Development of novel anti-mouse CD19 single-chain variable-fragments and establishment of an immunocompetent B-cell malignancy mouse model for the investigation of CAR-T cell therapies

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Declaration

I, Francesco Nannini confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Abstract
The use of chimeric antigen receptor (CAR) modified T cells targeting human CD19 has demonstrated great success in treating relapsed/refractory (r/r) Diffuse Large B Cell Lymphoma (DLBCL) and B cell Acute Lymphoblastic Leukaemia (B-ALL). However, the injection of CD19-directed CAR T-cells can cause severe side effects, such as cytokine release syndrome (CRS), neurotoxicity, and tumour lysis syndrome. Moreover, around 50% of patients still relapse after CAR T-cell therapy.

The preclinical development of anti-CD19 CAR therapies has been primarily carried out in immunodeficient mice, limiting the possibility of understanding the interaction with the host immune cells and the “on-target, off-tumour” effects of the therapy.

The aim of this thesis was to develop an immunocompetent mouse model recapitulating the therapeutic and toxic effects reported in human CAR-T trials. The challenges in identifying the ideal setting for tumour and CAR T-cells engraftment has proven total body irradiation (TBI) effective for the establishment of tumour and engraftment of second-generation anti-mouse CD19 CAR T-cells.

Through this project, I have developed a model that demonstrates both efficacy and toxicity related to tumour burden and different doses of engineered CAR T-cells. The observation of elevated cytokines, such as IL-6, interferon-γ (IFN-γ), and MCP-1 in animals with the most severe side effects is similar to what is reported in human CD19 CAR-T trials. Moreover, B cell aplasia and persistence of CAR T-cells confirms the successful engraftment and function of anti-mouse CD19 CAR T-cells.

In addition to the available anti-mouse CD19 binding domain (1D3), I have isolated through hybridoma technology four additional antibodies with improved thermal stability and one antibody binding to a separate epitope compared to the reference clone 1D3. Biophysical characterization revealed a similar kinetic profile to the reference clone 1D3, which resulted in equivalent in vitro CAR T-cells performance.

In summary, this work successfully developed an immunocompetent mouse model for systemic B cell malignancy, which can be used to study the effects of anti-mouse CD19 CAR T-cells and explore improved CAR T-cell structures. Ultimately, the novel anti-mCD19 scFv developed through this project could be useful to construct and test alternative CAR architectures for in vivo applications.
**Impact statement**

DLBCL is the most common subtype of non-Hodgkin lymphomas, constituting up to 40% of cases worldwide. Although most commonly observed in older patients, DLBCL can affect any age group, including children. While 75% of B-ALL cases typically occur in children, its prognosis in adults is extremely poor. CAR T-cells have changed the prognosis of B cells malignancies, delivering complete remission in both adult and paediatric patients. However, despite these therapeutic improvements, a significant proportion of patients relapse or become refractory to CAR T-cell therapy. Extensive research to improve the outcome of these patients is currently ongoing, including work to better understand the interactions of the different components of the CAR structure (i.e. binding motif) in scenarios such as immunocompetent mouse models that are more relevant to the human disease.

The work presented in this thesis establishes an immunocompetent mouse model for testing CAR T-cell therapies in the context of B cell malignancy. The model was able to recapitulate most of the anti-tumour effects and the toxic side effects reported in clinical trials. The full mouse structures constructed and utilized for targeting mouse CD19 provide a robust methodology for testing cross-talk between the engineered CAR T-cells and the endogenous immune cells. Through the use of hybridoma technology, novel anti-mouse CD19 scFv have been isolated and characterized biophysically and in CAR format.

As the nature of the work is focused on a mouse homologue of human therapeutics, the results are of great value in two ways: (i) the established model represents a robust platform to test existing and improved CAR T-cell design, not only targeting CD19, but with the possibility to expand to other TAAs, either for CAR T-cells alone or in combination with other therapeutic agents; (ii) the newly generated anti-mouse CD19 binders greatly contribute to the number of available scFvs targeting mouse CD19. In CAR-T format, these binders are as efficacious as the currently existing reference clone, making them suitable for testing alternative CAR structures.

Together, the novel binders and the immunocompetent mouse model could be suitable for testing many immunotherapeutic approaches, including signal mixing CAR architectures, antibody-drug conjugates, and bispecific-T cell engagers (BiTEs) contributing to improving the understanding of the mechanism of action of these therapies.
Acknowledgments

I would like to start by saying thank you to my primary supervisor Martin Pule for the opportunity he has given me to embark in this PhD and not least his priceless support. Thank you to my secondary supervisor Sergio Quezada for allowing me to work under his animal licence and expertise. A grateful thank you to Claire Roddie for the continuous support and sympathy throughout my final years. Thank you to Brian Philips for the training he provided me during my first approaches with mice. A special thank you to Kerry Chester, Shimobi Onuoha and Mathieu Ferrari for all the knowledge they shared with me and for believing in my skills. Thank you, Samir Agrawal, for believing in a young scientist that had just landed in UK.

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The most important thank you goes to my family for giving me the opportunity to pursue my education. Particularly my mom for being there for me and the encouragements she unconditionally always provided. Thank you to the stars that have been watching me from above and for always warming me up from the inside. Not least, but vital thank you to my wonderful wife Arianna whose understanding, listening and love have been the never-ending fuel throughout all these years.

"Nothing is lost, nothing is created, everything is transformed."

Antoine-Laurent de Lavoisier
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<tbody>
<tr>
<td>ACT</td>
<td>Adoptive cell therapy</td>
</tr>
<tr>
<td>B-ALL</td>
<td>B cell acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>BCMA</td>
<td>B cell maturation antigen</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminescent imaging</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CABI</td>
<td>Centre for Advanced Biomedical Imaging</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric antigen receptor</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytokine bead array</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukaemia</td>
</tr>
<tr>
<td>CRS</td>
<td>Cytokine release syndrome</td>
</tr>
<tr>
<td>DLBCL</td>
<td>Diffuse-large B cell lymphoma</td>
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<tr>
<td>DPT</td>
<td>Day post tumour</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>E:T ratio</td>
<td>Effectors to targets ratio</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal-bovine serum</td>
</tr>
<tr>
<td>Fc</td>
<td>Immunoglobulin constant fragment</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HC</td>
<td>Heavy chain</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte antigen</td>
</tr>
<tr>
<td>HLH</td>
<td>Hemophagocytic lymphohistiocytosis</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish-peroxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cells</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMGT</td>
<td>International immunogenetics information system</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
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<tr>
<td>KLC</td>
<td>Kappa light chain</td>
</tr>
<tr>
<td>LC</td>
<td>Light chain</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAS</td>
<td>Macrophage activation syndrome</td>
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<tr>
<td>mCD19</td>
<td>Mouse CD19</td>
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<tr>
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<td>Monocyte chemoattractant protein-1</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MDSCs</td>
<td>Myeloid-derived suppressor cells</td>
</tr>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
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<td>Macrophage inflammatory protein 1b</td>
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<td>MMLV</td>
<td>Moloney Murine Leukaemia Virus</td>
</tr>
<tr>
<td>Nano-DSF</td>
<td>Nano-differential scanning fluorimetry</td>
</tr>
<tr>
<td>ORF</td>
<td>Open-reading frame</td>
</tr>
<tr>
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<td>Overlap-extension PCR</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDX</td>
<td>Patient-derived xenograft</td>
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<td>PIL</td>
<td>Personal individual licence</td>
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<td>p-MHC</td>
<td>Peptide-major histocompatibility complex (MHC) complex</td>
</tr>
<tr>
<td>PPL</td>
<td>Project licence</td>
</tr>
<tr>
<td>r/r</td>
<td>Relapsed or refractory</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>scFv</td>
<td>Single-chain variable fragment</td>
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<tr>
<td>SMART</td>
<td>Switching mechanism at the 5' end of the RNA transcript</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface-plasmon resonance</td>
</tr>
<tr>
<td>T2A</td>
<td>T2A ribosomal skip element</td>
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<tr>
<td>TAA</td>
<td>Tumour associated antigen</td>
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<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>$T_h$</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumour infiltrating lymphocytes</td>
</tr>
<tr>
<td>TME</td>
<td>Tumour microenvironment</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumours necrosis factor-alpha</td>
</tr>
<tr>
<td>xGVHD</td>
<td>Xenograft-graft versus host disease</td>
</tr>
</tbody>
</table>
1. Introduction

1.1. T cell immunotherapy

1.1.1. Genetic engineering of primary T-cells

Throughout millions of years of evolution, organisms have developed systems to prevent and protect them from external infective challenges. This immune response is divided into innate and adaptive response\(^1\). The innate system functions as the first, non-specific line of defence against non-self pathogens and comprises physical barriers such as the skin and immune cells such as natural killer (NK) and macrophages\(^2\). The innate response rapidly prevents the spread and movement of foreign pathogens throughout the body\(^3\). On the other hand, the adaptive response delivers targeted immunological defence tailored to specific organisms and provides immunological memory to prevent re-infection in the future\(^4\). Adaptive immune responses, divided into humoral and cellular, are delivered by B- and T lymphocytes. B cells are the primary mediators of humoral immune response and control the production of antigen-specific antibodies. They target and neutralise freely circulating pathogens, causing lysis or phagocytosis\(^5\). T cells are the key player in the cellular immune response\(^6\). Cellular immunity occurs inside infected cells, with the pathogen's antigens expressed on the cell surface or on an antigen-presenting cell (APC). These processed proteins/peptides are presented associated with the Major Histocompatibility Complex (MHC), and these specific epitopes are recognized by specific T cell receptor (TCR) structures expressed on the surface of T cells, triggering downstream events such as the lysis of the infected cells\(^7,8\) (Figure 1-1). The mechanisms and processes involved in TCR recognition of antigens has been extensively investigated\(^9,10\). Briefly, the TCR expressed on both CD4+ helper T cells and CD8+ cytotoxic T cells binds to the antigen to form a structure called the MHC complex on the APC’s surface. The formation of this complex triggers an initial antigen-specific activation of the T cells or “signal 1”. Both helper T cells and cytotoxic T cells require several secondary signals to become activated and able to effectively respond to the threat\(^11,12\). This “signal 2” is mediated by cytokines or co-stimulatory molecules such as B7.1 (CD80) and B7.2 (CD86) present on the surface of APCs\(^13\). Receiving only signal 1 without co-stimulation results in T cell unresponsiveness or anergy, a mechanism known as peripheral tolerance\(^14\).
Figure 1-1. Schematic of lytic activation of cytotoxic T-cells. CD8 + T cells recognise infected cells via their T cell receptor (TCR). The TCR binds complexes of peptides and MHC class I molecules at the surface of infected cells. Following CD8 + T cell recognition of the infected cell, the T cell effector mechanisms are triggered. The downstream effect can be lytic (killing of the infected cell) and/or non-lytic (secretion of cytokines such as IFN-γ and TNF-α, which recruits other immune cells population to fight the challenge).

The primary role of T cells is to recognise foreign antigens and reject/prevent infection. The T cell response is designed not to recognise self-antigens to prevent the damaging phenomenon of autoimmunity. For this reason, many researchers did not support the hypothesis of anti-cancer immunity as the majority of cancer antigens arise from self-antigens to which the adaptive immune system should have been ‘tolerised’. Robert Schreiber spent his career pushing back on this paradigm, arguing that the immune system can recognise tumour cells but that ‘cancer’ is a state in which the immune system has lost control. Cancer immunoeediting is composed of three phases: elimination, equilibrium, and escape. Seminal work by Scheiber and colleagues has built the foundations for the field of cancer immunotherapy\textsuperscript{15,16}.

Cancer immunotherapy relies on the concept of the body’s own immune system recognising and targeting cancer. It can be subdivided into checkpoint blockade and adoptive cell therapy. The significant advances identified in the field of cancer immunotherapy led to its nomination by \textit{Science} magazines as the 2013 scientific breakthrough for the promising anti-tumour efficacy shown in human clinical trials\textsuperscript{17}.
Loads of patients with advanced tumours have benefited from cancer immunotherapy, with many achieving complete remission. Adoptive cell therapy (ACT) has long been used to treat various types of cancers. Originally, unmanipulated cell therapy for cancer was applied in haemat-oncology with pioneering work in allogeneic bone marrow transplantation and donor lymphocyte infusions for relapsed and refractory leukaemias. Subsequent work by the Rosenberg group at the National Cancer Institute (NCI) led to the development of tumour infiltrating lymphocytes (TIL) therapy for immunogenic tumours such as melanoma\textsuperscript{18–20}.

Studies using autologous TILs in patients with advanced melanoma led to significant clinical responses in a proportion of cases\textsuperscript{21,22}. After decades of research, the adoptive transfer of TILs is one of the most important tools for the treatment of melanoma and several other tumours\textsuperscript{23}. However, the many obstacles associated with the use of TILs limit the anti-tumour capacity of TIL-based immunotherapy. ACT of TILs works by direct recognition of tumour-associated antigen (TAA) in the form of major histocompatibility complex (MHC)–peptide complexes (p-MHC)\textsuperscript{24}. Because TAAs are also expressed on self-tissues, immunotolerance can occur when using TILs exposed to p-MHCs derived from TAAs, resulting in anergic T cells\textsuperscript{25}. In addition, there are several mechanisms by which tumours cells can evade an effective immune response, such as downregulation of MHC molecules and autoantigens leading to immunosuppression and weak immunogenicity\textsuperscript{26}. Moreover, TILs from a significant proportion of patients cannot be obtained or sufficiently expanded prior to the infusion. However, the logistical challenge of isolating and expanding TILs from patient tumours and the lack of Phase III studies demonstrating clear efficacy have limited the widespread application of TILs in clinical practice. Furthermore, for some tumours, the lack of suitable target antigens makes the use of unmanipulated TILs/other adoptive cell therapies less compelling. In these cases, an attractive concept is engineering tumour antigen specificity into T cell products to redirect them to recognize tumours, providing an alternative approach to developing T cell therapies\textsuperscript{27}.

Two main engineered T cell strategies have been developed: one using T cells engineered with a synthetic TCR that recognizes human leukocyte antigen (HLA)-restricted TAA peptides\textsuperscript{28} (TCR-T cells) and the other utilising an antibody recognition domain linked to a TCR signalling molecule, to form a chimeric antigen receptor (CAR)\textsuperscript{29} (Figure 1-2).
TCR-T cells are generated by engineering patient T cells with TCR genes conferring known specificity to TAAs\textsuperscript{30}. Preclinical mouse models demonstrated excellent anti-tumour efficacy for metastatic melanoma\textsuperscript{31}, and synovial sarcoma\textsuperscript{32} and TCR-T cells have been shown to mediate tumour-regression in clinical trials, demonstrating that the specificity of T cells can be redirected against a specific TAA to mediate immunity to cancer\textsuperscript{33,34}.

However, TCR-T cell immunotherapy is MHC-restricted, limiting the use of these approaches to a subset of HLA-matched patients. In contrast, CAR structures recognize surface antigens on target cells via the antibody fragment in the extracellular portion of the chimeric receptor, thus eliciting cytotoxicity in an MHC-independent manner, broadening the application of genetically engineered T cells to virtually any surface TAA\textsuperscript{29,35}. The anti-tumour efficacy of CAR-engineered T cells has been largely

\textbf{Figure 1-2. Schematic of the T cell receptor (TCR) and chimeric antigen receptor (CAR) structures.} The TCR is a multisubunit antigen recognition complex in which the TCR\textalpha{} and TCR\textbeta{} chains bind peptides in the context of major histocompatibility complex (MHC) molecules and the signal is propagated through its associated molecules CD3\textgreek{z}, CD3\textepsilon{}, CD3\textgreek{y}, and CD3\textdelta{}. The CAR structure includes an extracellular domain derived from the fusion of the variable domains of an antibody to form a single chain variable fragment (scFv). The antigen recognition domain is tethered to the membrane by a flexible spacer domain fused to the cytoplasmatic tail of the CD3\textgreek{z} signalling molecule.
demonstrated\textsuperscript{36,37}, and it is clear that T cell-cancer immunotherapy is a promising treatment for haematological and solid tumours\textsuperscript{38}.

1.2 CAR T-cells for cancer immunotherapy

The advent of CAR T-cell immunotherapy has significantly changed clinical practice in haematological oncology with large data-sets available for lymphoma, leukaemia, myeloma, and other haematological malignancies\textsuperscript{39,40}. Early CAR-T studies provided evidence of feasibility for CAR T-cell production but lacked clinical efficacy\textsuperscript{41}. These studies were carried out with what is now known as first-generation construct design with the antigen-binding domain, typically a single-chain variable fragment (scFv), linked to CD3 zeta or FcR\(\gamma\) chain as a signalling domain. Activation through the CD3 zeta chain or FcR\(\gamma\) chain was not sufficient for a productive immune response\textsuperscript{42}. These suboptimal results rapidly led to the development of second-generation CARs, combining activatory and co-stimulatory signalling domains, and characterized by improved cytokine secretion, T-cell expansion upon repeated antigen exposure, and T-cell persistence\textsuperscript{43}. The most frequently used co-stimulatory molecules have been the signalling domains of CD28 or 4-1BB, although others have also been studied\textsuperscript{44,45}. The majority of clinical trials performed so far have used second-generation CARs\textsuperscript{46}. Third-generation CARs have two co-stimulatory domains to enhance the expansion further\textsuperscript{47}. However, direct comparisons with second-generation CARs have not yet been conducted in the clinical setting.
Finally, “armored” CAR T-cells have been the latest significant upgrade to the CAR architecture. An armored CAR vector includes a second gene, encoding a protein that either provides the resulting T cell with a survival or cytotoxicity advantage or modulates the tumour microenvironment\textsuperscript{48}. Examples of such proteins include the proinflammatory cytokine interleukin-12 (IL-12)\textsuperscript{49} and immunostimulatory molecules 4-1BB or CD40L\textsuperscript{50} (Figure 1-2). Early clinical trials utilizing “armored” CAR have demonstrated the safety and efficacy of this approach. A clear advantage over the other CAR structure is yet to be proven and is currently under clinical investigations\textsuperscript{51,52}.

![Figure 1-3. Schematic representation of the different generations of CAR T-cells. First generation of CAR T-cells contains single signalling module CD3ζ, while second and third generations contain one or two additional co-stimulatory (i.e. CD28 and 4-1BB) domains to enhance the survival as well as proliferation and persistence of activated CAR T-cells. The 4th generation CAR T-cells are additionally genetically modified with an inducible cytokine gene cassette (i.e. NFAT) to immediately secrete cytokines to boost CAR T-cell activation in a challenging tumour microenvironment.](image)

The adoptive transfer of T cells expressing engineered CAR receptors has shown great promise in humans. To date, the most striking success has been achieved by targeting CD19 on B cell malignancies resulting in the FDA approval of two CD19 CAR T-cell products for relapsed or refractory (r/r) B cell acute lymphoblastic leukaemia (B-ALL) in young adult patients and diffuse-large-B cell lymphoma (DLBCL) in adult patients\textsuperscript{37,41}.  

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Currently, many CARs for other non-B cell targets, including solid tumours, are under investigation both in preclinical and clinical trials\(^{38,53}\). CAR-associated toxicity is commonly described in the optimal scenario of specific activation of CAR T-cells via their target expressed on tumour tissue. This ‘on-target on-tumour’ toxicity is due to excessive T cell activation and is characterized by a T\(\text{h}1\)-dominated cytokine release syndrome (CRS) with the production of high levels of inflammatory cytokines such as interferon-\(\gamma\) (IFN-\(\gamma\)), IL-6, and IL-10\(^{36,54,55}\). The severity of the CAR T-cell-associated CRS correlates with tumour burden. Further, CAR-associated neurological complications ranging in severity from mild to life-threatening have also been reported across different clinical studies\(^{37,56,57}\). An additional risk from CAR therapy is where the target is also expressed on the normal tissue, potentially leading to an ‘on-target off-tumour’ toxicity and damage to healthy tissue\(^{58,59}\). Some of these safety concerns listed can be addressed effectively in the clinic, but others have not been anticipated with potentially fatal consequences.

1.2.1 CD19 as a tumour associated antigen

The initial clinical trials using first-generation CAR designs in patients with various cancers were disappointing due to lack of expansion and lack of clinical impact. However, in 2011, second-generation CAR T-cells targeting CD19 and encoding costimulatory domains emerged as the lead example of successful engineered-T cell therapies for cancer\(^{60}\). Several landscapes make CD19 a nearly ideal target. It is high-level expressed on B cell malignancies, it is vital for normal B cell development in humans, and it is not expressed outside the B cell lineage\(^{61}\). Patients successfully treated with CD19-CARs often develop profound B cell aplasia, but it has been largely managed clinically by replacement therapy with intravenous immunoglobulin, similarly to individuals with genetic deficiencies in B cells due to CD19 mutations\(^{62}\). Early results from CAR T cell studies investigating other targets indicated that the CD19 off-tumour cross-reactions could also be observed when targeting TAAs on different cell lineages\(^{63,64}\).

The use of anti-CD19 CAR T-cell therapy has changed the prognosis of patients with r/r DLCBL. In this setting, two CAR-T products have been approved by both the American Food and Drug Administration (FDA) and the European Medicines Agency (EMA): axicabtagene ciloleucel (Axi-Cel), approved in 2017 on the basis of the ZUMA-1 trial\(^{65}\) and tisagenlecleucel (Tisa-Cel), initially approved in the USA for
r/r paediatric or young adult B-ALL in 2017 and subsequently approved for adults with r/r DLBCL in early 2018 based on the results of the JULIET trial. Frederick Locke and colleagues presented the longest reported follow-up so far from a large, multicentre study of anti-CD19 CAR T-cell therapy. They showed that the safety profile of Axi-Cel and the durability of the responses remained unchanged with an additional year of follow-up, with some patients still in remission at 2 years following CAR-T infusion. These findings build on an earlier single-institution study by Kochenderfer and colleagues in which the same anti-CD19 CAR construct was used in r/r DLBCL, and four out of the five patients with complete responses achieved long-term remission. Comparisons across anti-CD19 CAR trials in terms of activity and toxicity are precluded by the differences in the CAR T-cell constructs (CD28 costimulatory domain for Axi-Cel, 4-1BB for tisagenlecleucel) as well as from differences in clinical trial designs. However, what is remarkable across anti-CD19 CAR T-cell trials is the consistent durability of the response, the absence of late or unexpected gene-therapy-related events, and the unique but manageable toxicities (i.e., cytokine release syndrome and neurotoxicity). The striking results of these clinical trials targeting CD19 have been instructive for understanding the limitations of this new modality and the challenges to improve CAR T-cell therapy function and safety. The outcomes of the pivotal and current CAR T-cell clinical trials targeting CD19 in B cell malignancies are summarized in Table 1-1.

<table>
<thead>
<tr>
<th>Clinical trial</th>
<th>Costimulatory domain</th>
<th>Clinical outcome</th>
<th>Toxicity (≥grade 3)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT02435849 (ELIANA)</td>
<td>4-1BB</td>
<td>60% CR</td>
<td>48% CRS 13% ICANS</td>
<td>Maude et al.,2018</td>
</tr>
<tr>
<td>NCT02445248 (JULIET)</td>
<td>4-1BB</td>
<td>40% CR 12% PR</td>
<td>22% CRS 12% ICANS</td>
<td>Schuster et al.,2018</td>
</tr>
<tr>
<td>NCT02348216 (ZUMA-1)</td>
<td>CD28</td>
<td>58% CR</td>
<td>11% CRS 32% ICANS</td>
<td>Locke et al.,2019</td>
</tr>
<tr>
<td>NCT02443831 (CARPALL)</td>
<td>4-1BB</td>
<td>86% CR</td>
<td>0% CRS 7% ICANS</td>
<td>Ghorashian et al.,2019</td>
</tr>
<tr>
<td>NCT02601313 (ZUMA-2)</td>
<td>CD28</td>
<td>67% CR 27% PR</td>
<td>15% CRS 31% ICANS</td>
<td>Wang et al.,2020</td>
</tr>
<tr>
<td>NCT02631044 (TRANSCEND NHL-001)</td>
<td>4-1BB</td>
<td>53% CR 20% PR</td>
<td>2% CRS 10% ICANS</td>
<td>Abramson et al.,2020</td>
</tr>
<tr>
<td>NCT03105336 (ZUMA-5)</td>
<td>CD28</td>
<td>76% CR</td>
<td>7% CRS 19% ICANS</td>
<td>Jacobson et al.,2020</td>
</tr>
</tbody>
</table>
1.2.2 Risks and challenges with CD19-targeted CAR T-cell therapy

B cell tumours are ideal targets for CAR T-cells because they express lineage-specific molecules such as CD19, CD20, and CD22 that are not expressed on other tissues. As described in section 1.2.1, anti-CD19 CAR T-cells have proven to be highly effective for treating B cell malignancies \(^{40}\). The risks and challenges are linked to (1) toxicities and (2) resistance to therapy/relapse.

**Toxicity:** various CAR T-cell-mediated toxicities, such as tumour lysis syndrome \(^ {60,79} \), cytokine release syndrome \(^ {56,60} \), neurotoxicity \(^ {80} \), and on-target off-tumour toxicity \(^ {58,81} \) have emerged, some with devastating consequences. After infusion of CD19 CAR T-cells, unique toxicities such as CRS \(^ {69} \) and neurotoxicity \(^ {82} \) can arise. These continue to be the subject of intense research to assist in defining effective prevention and treatment strategies. The onset of CRS coincides with activation and proliferation of CAR T-cells *in vivo* and typically occurs within the first few days after T cell infusion \(^ {83} \). CRS is characterized by a range of symptoms from mild in the majority of patients to severe with high fevers, hypotension, and capillary leak syndrome in some. After administration of CAR T-cells, serum analysis has shown elevated cytokines including IFN-\( \gamma \), IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein 1b (MIP-1b); as well as acute-phase proteins such as C-reactive protein and ferritin, indicative of systemic inflammatory response \(^ {84,85} \).

Risk factors associated with CRS include high tumour burden, presence of higher numbers of CD19-positive cells in the bone marrow, and a higher CAR T-cell dose \(^ {86,87} \). CRS is currently treated by the administration of a monoclonal antibody blocking the IL-6 receptor (Tocilizumab), other immune-modulating agents such as dexamethasone, and Anakirina inhibit cytokine production by activated CAR T and other immune cells utilized \(^ {55,88} \).

<table>
<thead>
<tr>
<th>Trial ID</th>
<th>CAR Domain</th>
<th>Response</th>
<th>Toxicity</th>
<th>Authors, Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT02926833 (ZUMA-6)</td>
<td>CD28</td>
<td>46% CR</td>
<td>4% CRS</td>
<td>Jacobson et al., 2019 (^ {76} )</td>
</tr>
<tr>
<td>NCT03761056 (ZUMA-12)</td>
<td>CD28</td>
<td>75% CR</td>
<td>20% CRS</td>
<td>Neelapu et al., 2020 (^ {77} )</td>
</tr>
<tr>
<td>NCT02935257 (ALLCAR19)</td>
<td>4-1BB</td>
<td>85% CR</td>
<td>0% CRS</td>
<td>Roddie et al., 2021 (^ {78} )</td>
</tr>
</tbody>
</table>

*Table 1-1. Clinical outcomes of the main and current anti-CD19 CAR T-cell trials. Summary of the clinical response and toxicity events greater or equal to grade 3. CR: complete remission, PR: partial remission, CRS: cytokine release syndrome, ICANS: immune effector cell-associated neurotoxicity syndrome.*
Although CRS is an expected toxicity, unforeseen neurologic complications (mild to life-threatening) have been specifically reported for CD19 and B cell maturation antigen (BCMA) CAR T-cell therapy. Symptoms include headache, delirium, aphasia, seizures, and loss of consciousness, in parallel with or after CRS and usually resolve over time. The main risk factors for neurotoxicity include high tumour burden or CAR T-cell dose, CRS, and pre-existing neurologic conditions. Neurotoxicity is associated with endothelial activation, disseminated intravascular coagulation, and increased blood-brain barrier permeability. The presence of fever and high serum IL-6 and MCP-1 concentrations identifies patients at high risk of subsequent severe neurotoxicity within the first 36 hours after CAR T-cell infusion.

The expected elimination of normal B cells is an ‘on-target’ side effect of targeting CD19. Transient or sustained loss of normal B cells is clinically well-tolerated, and B cells recover eventually in most patients when the number of CAR T cells decreases.

The design of vectors with suicide genes or inbuilt mechanisms to accelerate the elimination of potentially dangerous CAR T-cells is desirable. Examples include co-expression of a truncated format of the epidermal growth factor receptor (EGFRt), downstream of a T2A ribosomal skip element. In practice, EGFRt can be targeted by cetuximab in the event of CAR-associated toxicity, triggering CAR T-cell elimination. In preclinical models, cetuximab administration eliminated CD19 CAR T-cells and restored B cell numbers without leukaemia relapse. This approach could be applied to patients that achieve durable remissions but have persistent B cell aplasia.

**Resistance to therapy/relapse:** Disease relapse following anti-CD19 CAR T-cell therapy can occur in up to 50% of patients with B-ALL by 12 months after infusion, in two major patterns: early relapse of antigen-positive leukaemia or later relapse usually associated with antigen loss. Antigen-positive relapses typically occur within the first few months after successful induction of remission, often associated with limited CAR T-cell persistence and/or transient B cell aplasia, which suggests a loss of activity of the CAR T-cell product. Determinants of CAR T-cell persistence remain to be defined. In addition to inherent T cell quality (which might be patient-dependent and manufacturing-dependent) and initial T cell phenotype (including the ratio of CD4+/CD8+ T cells), they might include the co-stimulatory domain built into each unique CAR construct. Preclinical and clinical reports indeed indicate that CD28 co-stimulatory domain-containing CARs tend to persist less well than those containing a
4-1BB co-stimulatory domain.$^{37,100,101}$ Further observations in patients with a notable expansion and/or persistence of the CAR T-cells suggest a role for targeted genomic integration of the CAR construct to enhance persistence. Clonal expansion observed in a patient with CLL revealed that TET2 gene disruption results in CAR T-cell biology alterations, leading to enhanced potency and a central memory phenotype.$^{102}$ Similarly, specific integration of the CAR gene into the TCRα constant (TRAC) locus of the T cell genome using CRISPR–Cas9 editing results in better anti-tumour responses than those observed with conventionally transduced CAR T-cells in preclinical ALL model.$^{103}$ Strategies to improve persistence independently of CAR T-cell design and manufacturing are currently being under testing in the clinic; for example, combining CAR T-cell therapy with immune-checkpoint inhibitors or other immunomodulatory therapies provides a synergetic approach to optimize the durability of clinical responses. Evidence for increased PD-1 expression in CAR T-cells during the time from infusion to peak expansion has been demonstrated in clinical samples, and preclinical data support a role of PD-1–PD-L1 blockade in improving the effectiveness of CAR T-cell therapy.$^{104-106}$

Target antigen modulation is one of the most evident mechanisms of disease relapse following successful remission induction using CAR T-cells and accounts for approximately 9–25% of relapse cases in B-ALL.$^{37,41,96}$ It has also been described with other targeted immunotherapeutic approaches, such as bispecific T cell-engager (BiTE) antibody constructs or monoclonal antibodies.$^{107,108}$ This antigenic escape mechanism is similar to the acquired defects in antigen presentation or antigen loss observed with TCR-T cell-based therapies.$^{36}$ Sotillo and colleagues demonstrated that mutations affecting the CD19 gene and CD19 splicing variants lacking the CAR-recognized epitope are strongly enriched compared to samples analysed before treatment. In particular, exon 2 of CD19 was frequently spliced out, leading to the disappearance of the CD19 epitope that is recognized by the fmc63-based antigen-binding moiety of the CAR structure.$^{109}$ This study suggests that in some patients, the CD19 protein is still present, but it is truncated, lacking the epitope necessary to trigger CAR recognition and activation.$^{110}$ Established mechanisms leading to loss of CD19 expression also include aberrant trafficking of the protein to the cell surface, and the additive effect of prior targeted immunotherapies might further increase the complexity of immune evasion after CAR T-cell therapy.$^{111}$ After administering the BiTE Blinitumumab, a loss of response was associated with the emergence of CD19 negative relapses. As most
of these agents target similar epitopes of the CD19 molecule, the use of such agents might render CAR T-cell less effective or less persistent\textsuperscript{107}. Notably, prior administration of Blinatumumab was an exclusion criterion in the pivotal trial for tisagenlecleucel in patients with ALL\textsuperscript{70}. CD19 escape is not a form of resistance in patients with chronic lymphocytic leukaemia (CLL); failure of CLL therapy is likely due to lack of CAR T-cells proliferation after infusion\textsuperscript{112}. Independent of treatment-related antigen loss or modulation, inherent tumour heterogeneity also has a role in predisposing the emergence of an antigen-negative clone. CD19 has been considered ubiquitously expressed on all pre-B cell ALL clones, with the development of antigen-negative subclones upon CD19-targeted treatment. However, a more detailed analysis of pre-therapy CD19 expression is necessary, as rare patients have malignant cells with CD19 negativity or partial expression at diagnosis with data indicating the possibility that the malignant B cell progenitors are CD19\textsuperscript{-}, particularly in patients with BCR–ABL1 ALL\textsuperscript{113,114}. A different mechanism for CD19-negative relapse is called myeloid “lineage switch.” This phenomenon has been observed in the preclinical and clinical setting, with the emergence of myeloid subtypes (CD19-negative) following CD19-directed immunotherapy in ALL\textsuperscript{115,116}. Altogether, optimization of CAR T-cells for patients with B cell malignancies is the current frontier for research in this field of immunotherapy. However, many obstacles need to be overcome, and close attention to novel CAR constructs' design will be informative in identifying further barriers. To progress the field, representative preclinical models would be of enormous value, allowing investigators to test hypotheses around novel CAR's safety and efficacy.

**1.3 Pre-clinical models for CD19-targeted CAR T-cells**

The majority of preclinical studies used to investigate CAR T-cell function to date have focused on verifying the specificity and potency of CAR T-cells' antitumor activity \textit{in vitro} and \textit{in vivo}\textsuperscript{117,118}. The measurement of the specificity is usually performed early during CAR T-cell constructions and relies on the intrinsic specificity of the utilized scFv. Hence, specificity is typically assessed \textit{in vitro}, target-positive and -negative cells are tested in parallel, and the benefit of CARs is seen as fold increase above background. Non-specific activity determines the levels of the background, resulting in overall decreased potency again target-positive cells. Such assays prove CAR function and support translation to the animal model system\textsuperscript{119}.  

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For clinical translation, regulatory authorities generally require information regarding the specific agent to be used in the clinical trial. In the case of CAR T-cells, this means that testing in mouse models represents the only possible route to assess the actual clinical agent.

1.3.1 Xenograft models

In the past half-century, significant progress has been made in developing therapies for human cancers due to the contribution of more clinically relevant animal models. Nevertheless, the translation of these discoveries from mouse models to human trials has been hampered by the genotypic and phenotypic differences between humans and mice that rarely reflect the human disease scenario. The establishment of human cells and tissues into laboratory animals (xenograft model) will induce rejection of human tissues in a host animal with an intact immune system\textsuperscript{120}. From the first athymic mouse model\textsuperscript{121} in cancer research to the latest highly immunodeficient strains, the xenografting of human cells in a mouse model has been an invaluable tool to investigate and potentially improve the treatments of several human cancer\textsuperscript{122}. Particularly valuable in this area is the genetically modified strain of severe-combined immunodeficiency (NOD-SCID) mice that facilitates the engraftment of human PBMCs and hematopoietic stem cells (HSCs)\textsuperscript{123,124}. Although NOD-SCID mice are more efficient for human PBMC and HSC engraftment, residual innate mouse immune populations, including NK cells, limit other human cell and tissue engraftment\textsuperscript{125}. However, the success of human T-cell engraftment in most immune-compromised mice is limited since residual elements of the mouse immune system challenge the engrafting of human T cells\textsuperscript{126}. However, several studies have shown excellent human CAR T-cell engraftment in Nude, NOD/SCID, or SCID/Beige mice\textsuperscript{127} and more recently, in the highly immune-compromised NSG mouse (NOD/SCID IL-2Rγ\textsuperscript{-/-}). Further efforts had been made to promote xenograft engraftment efficiency by introducing other genetic deficiencies into mouse models. Targeted mutations at the interleukin-2 receptor (IL-2R) γ chain locus (IL2rg\textsuperscript{-/-}) of NOD-SCID mice (NSG) resulted in the complete absence of NK cells significantly improving the engraftment of human cells\textsuperscript{128,129}. Currently, most xenograft mouse models of CAR-T therapy use the NSG mouse strain\textsuperscript{130}. In this setting, xenograft models have the advantage of permitting the study of human CAR T-cells against human cancer cells, although this is essentially carried...
out in a vacuum, as interactions with other immune cells or healthy human tissues are missing\textsuperscript{131}. Nonetheless, xenograft mice serve as a valuable model for testing CAR-T efficacy and for validating proof-of-concept studies.

Barret and colleagues first described the use of NSG mice for xenogeneic transfer of a CD19\textsuperscript{+} human tumour cell line followed by infusion of human CAR T-cells. Using this model, they demonstrated that anti-CD19-directed CAR T cells were able to kill the CD19\textsuperscript{+} leukaemic B cells resulting in prolonged survival\textsuperscript{132}. This observation and subsequent research led the clinical team at the same institution to attempt the nascent therapy in their first human patient\textsuperscript{36}.

It is important to emphasize that human cancer cell line xenograft models lack a functional human immune system and other human tissues, which may modulate the anti-cancer activity and toxicity of CAR T-cells \textit{in vivo}.

Despite the advantages of the system, there are also several challenging problems. The approach of transferring human PBMCs into NSG mice only allows short-term experiments because the animals develop the xenograft-graft versus-host disease (xGVHD) within 4–5 weeks\textsuperscript{126}. The occurrence of xGVHD is associated with transduced cell persistence, and this is affected by the cytokine conditions used to culture the cells before injection into mice. Consequently, this limits the ability to investigate the long-term effects of CAR T cells in this model\textsuperscript{133}. In addition, the majority of these studies involve CAR T-cells targeting human antigens whose expression is restricted to the human tumour cells used to challenge the mice. Thus, targeting an antigen that may be expressed on the normal, healthy tissue cannot be evaluated. Nevertheless, these models were essential to establish the first proof of concept, and the majority of CAR T studies still rely on these xenograft mouse models for primary evaluation of efficacy.

Nevertheless, there are several significant challenges associated with existing immunodeficient models. Xenograft-graft versus host disease (xGVHD) is associated with transferred human T cells and limits the ability to investigate CAR T-cells' long-term effects in these models\textsuperscript{134,135}. Further, most of these models are designed to test CAR T-cells targeting human antigens where expression is restricted to the human tumour cells used for the model. Thus, an exploration of 'on target, off tumour' toxicity and the impact on the normal, healthy tissue cannot be evaluated.

An additional, more relevant, humanized mouse model has been used for the evaluation of CAR T-cells efficacy. The patient-derived xenograft (PDX) model consists of
injecting a primary tumour biopsy from a patient instead of human cell lines. In this setting, the tumour cells and the tumour microenvironment (e.g. immune cells) are present *in vivo*. Therefore, PDX models are increasingly used for personalized anti-cancer T cell therapies and are potentially more relevant than xenografted cancer cell line models for translation into the clinic.

As early as 2010, a patient’s tumour and autologous T cells were engrafted in NSG mice to show that the patient’s T cells can function as CAR T-cells. Another study showed the efficacy of CAR T-cells in PDX mice for independent hepatocarcinoma grafts from three different patient’s tumours. In two PDX mice, CAR T-cell therapy eradicated the tumours, while one was resistant and showed upregulation of immune checkpoint inhibitor molecules. These findings might suggest that a combinatorial CAR-T and immune checkpoint inhibitor treatment could be recommended for this particular patient.

PDX models allowed the assessment, to some extent, of human immune responses to primary cancer cells, which is relevant for clinical translation. These PDX models still have their limitations since a normal functional human immune system is not present in these humanized mice, and they cannot fully predict what might happen in cancer patients. That is why very recently, a more complex humanized mouse model was developed. Jin et al. developed a mouse model for B-ALL, in which cancer and immune cells are autologous. They produced two humanized mice using human CD34+ progenitor cells from the same donor. Firstly, they engrafted one NSG recipient mouse with CD34+ cells and fetal thymus to generate a humanized mouse with human immunocompetence since T-cell are educated on human thymic tissue. In parallel, they generated a second mouse engrafted with fetal CD34+ cells from the same human donor transduced with a B-ALL relevant oncogene. These latter mice developed human B-ALL autologous to the blood system of the first mice generated. They then engrafted these autologous B-ALL cells into the immune-competent humanized mice to create a valid B-ALL model. To test CAR T-cells in this model, they isolated T cells from the humanized mice, which they modified with an anti-CD19-CAR and subsequently reinfused into the B-ALL human-like mouse model.

This model has unique characteristics that can better mimic normal human T cell development and thymic selection processes while avoiding the risks of xGVHD. One major problem with this model is that the reconstitution of human myeloid cells in CD34+ HSC-transplanted mice is much lower than that under normal conditions, and
the maturation of these cells is hindered because of the lack of several human cytokines, including stem cell factor, GM-CSF, and M-CSF\textsuperscript{139}. Certain types of myeloid cells, such as macrophages, can be activated by CAR T-cells and play important roles in systemic immune responses. Hence it is important for toxicity modelling that the myeloid compartment is represented\textsuperscript{140}.

Though complex in the generation, these models are much more relevant for the evaluation of human CAR T-cell efficacy, resistance, and toxicity and have been used to mimic CRS caused by CD19-specific CAR T-cell therapy\textsuperscript{141,142}. However, while presenting many advantages, these humanised mouse models are not identical to a human host. Importantly, humanized mice cannot develop all characteristics of a human immune system. In particular, they lack innate immune cells due to the absence of human cytokine signalling and exhibit poor lymph node development\textsuperscript{139}. It has been shown that certain types of myeloid cells, such as macrophages, can be activated by CAR T-cells and play essential roles in systemic immune responses\textsuperscript{143–145}. A specific limitation in evaluating CAR-T therapy using this humanised mouse model may be in toxicity studies, as IL-6 was below the detectable levels and human cytokine-transgenic SGM3 mice producing human stem-cell factors (SCF), GM-CSF, and IL-3 might correct the defect\textsuperscript{138}. This rather convoluted approach would require the engraftment of human CD34+ cells into triple transgenic NSG mice\textsuperscript{141}. Along with potential long-term xGvHD, this approach results in extended experimental timelines with high costs, especially for large-size experiments. Moreover, non-hematopoietic tissues are of mouse origin, which may alter the anti-tumour responses.

1.3.2 Transgenic models

Less commonly, immunocompetent transgenic mice have been employed in CAR-T studies to determine treatment safety better. As most TAAs are not expressed only on tumours but also at lower or similar levels on healthy tissues, transgenic mice are useful for observing unexpected side effects of TAA-specific immunotherapies such as CAR-T treatment. Transgenic mice typically have a mouse TAA-knockout and human TAA knock-in and can be bred such that TAA expression patterns and levels are similar to those found in humans\textsuperscript{146,147}. Transgenic models utilize mouse T cells and have an intact immune system, like syngeneic models, but allow the study of human TAA-specific CAR constructs, like xenograft models. In the setting of anti-CD19 CAR therapy, two groups have performed a study to evaluate the effects of preconditioning in a transgenic
immunocompetent host in an attempt to recapitulate the life-threatening complications of this therapy, including CRS and neurological adverse events\textsuperscript{148,149}. Creating transgenic immunocompetent mouse strains for human TAAs is a laborious task that has been little utilized for anti-CD19 CAR therapy to date, but they have the potential to provide information that neither syngeneic nor xenograft models can give, as transgenic models can potentially reveal off-tumour toxicity specific to human TAAs and induce systemic immune responses elicited by mouse immune cells as reported by the work of Pennell and colleagues\textsuperscript{149}.

1.3.3 Syngeneic models

Syngeneic or immunocompetent allograft models use CAR T-cells, tumours, and target antigens that are all mouse-derived. The main advantage of this model is that the mice are immunocompetent, allowing for the observation of CAR T-cells within the context of a functional immune system\textsuperscript{150}. Additionally, syngeneic models can reveal on-target off-tumour toxicities, as healthy mouse tissue can express the target antigen at low levels, mirroring the expression patterns in human patients\textsuperscript{151,152}. However, the syngeneic model has drawbacks, as mouse biology does not always accurately recapitulate human biology. For example, mouse immune systems differ from the human one, and syngeneic models have mainly been unable to mimic CRS and do not allow the \textit{ex vivo} evaluation of the optimal human CAR T-cell. For example, an early study of anti-CD19 CARs used a syngeneic mouse model to demonstrate that a single infusion of CAR T-cells resulted in the long-term elimination of lymphoma and was more effective than using the anti-CD19 monoclonal antibody for antibody-derived cell cytotoxicity\textsuperscript{153}. Preconditioning via sub-lethal irradiation of the mice was critical to the efficacy of the treatment. They explored different CAR constructs and showed that a mutant CD3\(\zeta\) domain resulted in better persistence than wild-type CD3\(\zeta\). This model was helpful in detecting B cell aplasia as potential toxicity after CAR-T injection (also seen in patients) as mice were lymphoma-free, but healthy B cells were also undetectable. Another study confirmed that anti-CD19 CAR-T therapy eradicated both leukaemia and healthy B cells in a syngeneic mouse model and that CAR-T persistence and efficacy were enhanced with cyclophosphamide preconditioning\textsuperscript{154}. A CD19 CAR-T study published a few years later was able to determine that toxicities were dependent both on the CAR T-cell dose and on the presence of co-stimulatory domains in the CAR construct. The same group, showed that first-generation CARs (without co-stimulatory
domains) killed lymphoma cells but did not persist or cause adverse side effects\textsuperscript{155}. In contrast, second-generation CARs with CD28 co-stimulatory domains induced B cell aplasia and chronic toxicity accompanied by an increase in CD11b+Gr-1+myeloid-derived suppressor cells (MDSCs), which play a role in immunosuppression in the tumour microenvironment (TME). Elevated serum IFN-γ and tumours necrosis factor-alpha (TNF-α) levels upon treatment with second-generation CAR T-cells indicated possible CRS\textsuperscript{156}. This particular model used BALB/c mice, but other syngeneic models used C3H or C7BL/6 mice and did not show toxicities\textsuperscript{157,158}, suggesting that, while these models are important in evaluating CAR safety, side effects may vary between mouse strains. Nonetheless, syngeneic models are important in elucidating cytokine-induced changes in the TME and involvement of host immunity after CAR-T administration, and they can be used to focus on the effects of CAR T-cells on the TME instead of just on the direct lysis of tumours cells. They also permit the study of long-term persistence and safety of different CAR constructs in a host with a functional immune system, to more closely reflect the human-disease scenario.

Table 1-2 summarizes the main mouse models of B cell malignancies adopted to recapitulate CRS’s acute effects and the limitation they have encountered in modelling a human-like toxicity profile.

<table>
<thead>
<tr>
<th>Mouse Model</th>
<th>Description of the model</th>
<th>Limitation in modelling CRS</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humanized PDX</td>
<td>Systemic engraftment of human B-ALL in matched CD34+ FLC-humanized NSG mice</td>
<td>Poor reconstitution of human myeloid cells, which led to lack of IL-1 and IL-6 and consequent CRS</td>
<td>Jin et al., 2018\textsuperscript{138}</td>
</tr>
<tr>
<td>Humanized and transgenic</td>
<td>Systemic engraftment of human ALL-CM in HSC humanized SGM3 mice</td>
<td>Technically challenging model in triple transgenic mice. The lack of human cytokine signals and the non-haematopoietic mouse tissues might alter the extent of CRS</td>
<td>Norelli et al., 2018\textsuperscript{141}</td>
</tr>
<tr>
<td>Transgenic</td>
<td>Generation of hemizygous human CD19 transgenic B6 mice</td>
<td>The human CD19 expression levels reduced due to the hemizygotic approach that alters CAR-T cells activity</td>
<td>Pennel et al., 2018\textsuperscript{149}</td>
</tr>
<tr>
<td>Syngeneic</td>
<td>Systemic engraftment of mouse A20 B-cell lymphoma cells in Balb/c mice</td>
<td>The strong precondition regimen before infusion of CAR-T cells resulted in a lack of the acute effects of CRS</td>
<td>Cheadle et al., 2010,2014\textsuperscript{155,156}</td>
</tr>
</tbody>
</table>
Evolution of CARs

Chimeric antigen receptors (CARs) are engineered receptors that can graft the desired specificity onto an immune effector cell (T cell). CARs include three parts: an extracellular antigen recognition domain comprising a single-chain variable fragment (scFv) derived from an antibody), a transmembrane domain, and an intracellular T cell activation domain incorporating CD3ζ. CAR T-cell therapy is designed to redirect patient/donor T cells to target and destroy tumour cells. Since the initial development phase in 1989, CAR T-cells have been refined to include four generations of improved structures, but it is unlikely that the current formats of CAR design will enable T cells to overcome the various obstacles presented by both tumour cells and the hostile tumour microenvironment. Altogether, these successive generations of CAR T-cell therapy have generated a great deal of enthusiasm in cancer treatment leading to extensive research in the mechanism and improvements of CAR T-cells structures.

1.4.1 Structure and signalling

Chimeric antigen receptor (CAR) T cells have shown unparalleled therapeutic potential in treating refractory haematological malignancies. In contrast, solid tumours pose a much more significant challenge to CAR T cell therapy, which has yet to be overcome.

As this innovative therapeutic modality evolves, increasing effort is being invested in determining the optimal structure and assets of CARs to facilitate the transition from empirical to rational design and testing of CAR T-cells. The current CARs structure consists of an ectodomain, transmembrane domain, and endodomain. The ectodomain is derived from membrane protein outside the cytoplasm and exposed to the extracellular space. The ectodomain, in this case, consists of a signal peptide, an antigen recognition region, and spacer moiety. The role of a signal peptide is to direct the nascent protein into the endoplasmic reticulum. The antigen-binding properties of a CAR are defined by the antigen recognition domain, which usually consists of a single-chain variable fragment (scFv) derived from an antibody.

Table 1-2. Characteristics and limitations of anti-CD19 CAR T-cell mouse models. The table includes pre-clinical mouse models employed to recapitulate the effects of CAR T-cell to reflect the human scenario of B cell malignancies and the challenges and limitations in modelling CRS.
chain variable fragment (scFv). The feasibility of generating an scFv through linking the variable heavy (VH) and variable light (VL) chains of a monoclonal antibody by a short linker was first demonstrated in 1988, and scFvs normally retain the specificity and affinity of the original antibody. Different linker peptides have been effectively utilized for fusing the VH and VL chains. The majority of linkers used in CAR T cells encompass a polypeptide variation based on glycine (Gly), and serine (Ser) repeats. For instance, the (Gly<sub>4</sub>Ser)<sub>3</sub>–linker consists of three repeats of the pentapeptide Gly–Gly–Gly–Gly–Ser. The incorporation of these residues in the linker provides flexibility and minimizes the risk of interfering with the proper folding and function of the connected variable chains. Some necessary requirements for linker length may be conditional to the scFv molecule in use, as studies suggested that too short linkers may favour the formation of scFv aggregates. In the setting of scFv-based CAR T cells, this could lead to tonic signalling by antigen-independent CAR clustering. It is commonly accepted that the optimal linker length is within 15–20 amino acids, and many CAR constructs have utilized either (Gly<sub>4</sub>Ser)<sub>3</sub> or (Gly<sub>4</sub>Ser)<sub>4</sub> linkers. An important determinant of CAR functionality is the affinity of the scFv for its target antigen. The scFv used for CAR T cells structures usually has an affinity several orders of magnitude more than the natural TCR on T cells. In the native T-cell, TCR signalling is mediated through TCR in conjunction with the co-activatory receptors CD4, CD8, and the CD3 chains γ, δ, and ε, which contribute to the activation signal threshold. This is not the case for CAR signalling, which is independent of CD4 and CD8. The optimal antigen-affinity for CAR T cells has been shown to vary based on many factors, such as the costimulatory and spacer domains as well as the antigen density on the target cells and the CAR expression levels on T cells. Several groups suggest that there is a lower limit to CAR affinity, below which sufficient antigen-binding does not occur, leading to suboptimal CAR T cell activation. By contrast, it has been observed that an increase in CAR affinity does not improve CAR T cell function for high-density antigen-expressing cells, suggesting that there is also an upper limit to scFv affinity. The fine-tuning of CAR affinity allows the possibility to mitigate on-target off-tumour effects associated with a low-antigen expression on healthy tissue while retaining the effector function to eradicate antigen-overexpressing malignant cells. A key to this rationale comes from the work of Liu and colleagues. They generated different CAR variants carrying a range of affinities for human epidermal...
growth factor receptor 2 (HER2/neu). Low-affinity CAR T cells demonstrated effective anti-tumour activity both in vitro and in a xenograft mouse model while sparing healthy tissues expressing normal levels of HER2/neu\textsuperscript{177}. This reduced activation to normal cells is especially relevant considering a prescient case report in which a patient died some hours following CAR T cell infusion due to CAR recognition of lung epithelial cells expressing physiological levels of HER2/neu\textsuperscript{58}. This reduced activation to normal cells is especially relevant considering a prescient case report in which a patient died some hours following CAR T cell infusion due to CAR recognition of lung epithelial cells expressing physiological levels of HER2/neu\textsuperscript{58}. In addition to CAR affinity, the function is also affected by the location of the recognized epitope on the antigen\textsuperscript{178}. For example, CAR T-cells expressing an scFv that recognized a membrane-proximal epitope of CD22 had superior activity to CAR T-cells that recognized a membrane-distal epitope\textsuperscript{179,180}. In theory, an scFv-based CAR T cell can be redirected towards any antigen, knowing the amino acidic sequence of the antibody with the desired properties. However, Long and colleagues have demonstrated that there may be distinct properties to some scFv that limit their use in CAR T cell applications. Comparing CARs directed against either CD19 or GD2, using fmc63 and 14g2a scFv-clones, respectively, they identified that the framework regions of the 14g2a-scFv induced antigen-independent clustering of the anti-GD2-CAR, resulting in tonic CAR activation. This promoted exhaustion during in vitro culture and limited this CAR's ability to function in a xenograft mouse model. The authors attempted to salvage the anti-GD2 CAR by replacing the framework regions of 14g2a scFv for those of the anti-CD19-scFv (fmc63), but this resulted in abrogated CAR expression, showing that the framework regions may not always be exchangeable between constructs\textsuperscript{167}. Another factor that remains mostly unsolved is that many clinical CAR trials have used scFvs derived from murine antibodies, thus increasing the risk of an immune response against the CAR T cells, which could cause toxicity and potentially limit CAR T cells persistence\textsuperscript{59}. This issue may be in part resolved by humanizing murine scFvs or by generating the scFvs from human antibody sequences\textsuperscript{181,182}. However, the chimeric nature of these receptors means that even sequences derived exclusively from human proteins can potentially cause an immune response due to the creation of unnatural peptide sequences at the junction sites of the CAR domains. The mechanism is via either cell-mediated immunity or by eliciting the production of anti-idiotype antibodies\textsuperscript{183}. The spacer motif connects the scFv to the transmembrane domain and can also profoundly affect CAR-T-cell function by creating differences in the length and flexibility of the final CAR structure. The majority of CAR constructs have been
designed with either immunoglobulin G (IgG)-based spacers or portions of CD8α and CD28 extracellular domains. The optimal spacer length has been the subject of several studies and appears to be dependent both on the antigen and the position of the targeted epitope, showing that CAR T cells are more potently activated with an epitope that resides closer to the target-cell membrane\textsuperscript{179,184}. This observation led to the rationale that the length of the spacer may position the CAR T cell and the target cell at an optimal distance to more efficiently exclude the intracellular phosphatases during the synapse formation, which are responsible for the attenuation of CAR signalling\textsuperscript{185}. It has been observed that short spacer for CAR structures targeting CD19, CEA, interleukin-13 receptor alpha-2 (IL13Rα2), and membrane distal epitopes within the receptor tyrosine kinase-like orphan receptor 1 (ROR1) induce a more potent activation than CARs with longer spacer domains\textsuperscript{173,186,187}. In contrast, where the targeted epitope is relatively inaccessible, longer spacers are required to overcome steric hindrances and allow binding of the scFv to the antigen. Various groups have shown the necessity of longer spacers when targeting inaccessible or membrane-proximal epitopes in antigens such as ROR1, mucin 1 (MUC1), and neural cell adhesion molecule (NCAM)\textsuperscript{186,188}. Optimal spacer length may vary according to the targeted epitope and requires design adjustments and optimization when targeting novel TAA.

A distinct problem of using human IgG-derived spacers is their ability to retain binding to Fc-receptor (FcγR)-expressing cells through the heavy chain constant 2 (CH2) domain, causing off-target activation of CAR T cells\textsuperscript{189}. Moreover, this interaction activates human monocytes and NK cells \textit{in vitro}, which could have a detrimental biological effect in patients\textsuperscript{190}. These effects were also observed for IgG1 and IgG4-based hinges. However, several groups have re-designed their spacers to abrogate binding to FcγR by removing the CH2 domain or introducing specific mutations essential to FcγR-binding, thus avoiding the negative impact of using IgG-based spacers\textsuperscript{191,192}.

At present, we do not know the mechanisms underlying the above observational differences in scFv/spacers combinations. The dataset is too small for any general predictive rules or algorithms to have emerged as to which spacer will work best with which CAR-targeted antigen. Therefore, empiric testing of scFv/spacer domain combinations is necessary to determine optimal CAR structures, although this will likely change as more data and validation studies become available.
Between the hinge and the signalling endodomains lies the transmembrane domain. This is usually derived from CD3ζ, CD4, CD8, or CD28\textsuperscript{193,194}. In the same way as the spacer domain, the transmembrane was initially regarded as an inert structural link between the ecto- and endodomains of the CAR. It is now accepted that the transmembrane domain can influence the function of CAR-T-cells. It has been shown that first-generation CD19-specific CARs containing CD3ζ transmembrane domains are less stable on the cell surface over time in comparison to 2\textsuperscript{nd} generation CD19-specific CAR T-cells with CD28 transmembrane domains\textsuperscript{195}. Current practice is broadly to use transmembrane domains in conjunction with their respective spacer domains or intracellular costimulatory domains\textsuperscript{196,197}.

The endodomain is the functional portion of the chimeric receptor, and the most common domain used is CD3ζ relying on the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs)\textsuperscript{198}. Upon antigen recognition, CAR endodomains transmit activation and costimulatory signals to T cells. While most current CAR endodomains contain an activation domain derived from CD3ζ, other ITAM-containing domains have also been investigated, including the domain of the Fc receptor for IgE-\gamma, but proved to be less effective\textsuperscript{199}.

The addition of a costimulatory domain has been demonstrated to slightly improve the in vitro cytotoxic function of 2\textsuperscript{nd} generation CAR T cells. However, the secretion of cytokines, CAR T cell proliferation, and overall anti-tumour effects were significantly enhanced compared to first-generation CAR T cells\textsuperscript{47,200}. The most widely used costimulatory domains are derived from CD28 and 4-1BB, both of which have been extensively characterized and compared. CD28 was shown to promote rapid effector functions of T cells at the preclinical stage but provided limited in vivo persistence compared to CARs incorporating a 4-1BB costimulatory endodomain\textsuperscript{201}. On the other hand, CAR T cells containing 4-1BB displayed slower tumour eradication in a leukaemia mouse model but accumulated over time, resulting in comparable anti-tumour efficacy to CAR T cells with a CD28 endodomain\textsuperscript{202}.

Building on these preclinical reports, clinical trials testing second-generation CD28 or 4-1BB CARs have similarly shown different results in respect of the in vivo persistence of the engineered T cells. CD28-based anti-CD19 CAR T cells have shown potent anti-tumour activity for only a limited time. Therefore, it has been suggested as an effective bridge to transplant where the remission induced by the CAR T cell therapy is followed by HSC transplantation\textsuperscript{54,203}. 2\textsuperscript{nd} generation CAR T cells containing 4-1BB as
endodomain have shown similarly potent anti-tumour efficacy, but these have been reported to persist for several years in humans, conferring long-term immunological memory\textsuperscript{70}. However, it is important to remember that cross-study comparisons can be error-prone due to potential confounding factors outside the CAR structure, such as trial design, product manufacturing, and preconditioning regimens used.

On the other hand, costimulatory domains other than CD28 and 4-1BB have been mainly characterized in the preclinical setting. A CD27-based 2\textsuperscript{nd} generation CAR showed similar anti-tumour efficacy in a mouse model of ovarian cancer to CD28 or 41BB-based CAR T cells\textsuperscript{45}. Similarly, Guedan and colleagues have reported that incorporation of the ICOS costimulatory domain allows T\textsubscript{H}1–T\textsubscript{H}17 polarization in CAR T cells showing superior \textit{in vivo} persistence compared to the more traditional 2\textsuperscript{nd} generation endodomains\textsuperscript{204,205}. The clinical benefits of these alternative costimulatory endodomains over the well-established CD28 and 4-1BB is yet to be proven.

Third generation (3\textsuperscript{rd}) CAR designs incorporate multiple signalling domains, such CD28-41BB or CD28-OX40, to augment potency, cytokine production, and killing ability\textsuperscript{206,207}. Some groups have shown superior function of 3\textsuperscript{rd} generation CAR T cells both \textit{in vitro} and \textit{in vivo} compared to standard 2\textsuperscript{nd} generation structures. In contrast, other groups have observed no differences, and in some cases, 3\textsuperscript{rd} CAR T cells are claimed to perform worse than their 2\textsuperscript{nd} generation counterparts\textsuperscript{44,169,208}. The reasons for these observed differences remain unclear, and the variability in reporting likely reflects that \textit{in vitro} measurements of CAR T cell functionality do not always predict CAR T cell activity \textit{in vivo}. Indeed, excessive CAR signalling may be detrimental to T cell function, in line with reports of tonic signalling impairing CAR T cell performance\textsuperscript{103,209}. In the clinical setting, 3\textsuperscript{rd} generation CAR T cells were used to treat lymphoma and colon cancer without any demonstrable benefit in efficacy over 2\textsuperscript{nd} generation structures, but with more severe and adverse events observed\textsuperscript{58,193}. The generation of intracellular structures bearing two costimulatory domains within one receptor increases the complexity of CAR design, and it remains to be seen whether 3\textsuperscript{rd} generation CAR T cells will confer an improved clinical benefit compared to predecessor designs.
1.4.2 Limitations of current CAR-T and future CAR designs to improve potency and to minimise toxicity

Building up from the unprecedented successes in early phase trials of anti-CD19 CAR T cell for the treatment of r/r CD19-positive B cell malignancies, in which many patients have achieved long-term remission, the FDA approved two distinct anti-CD19 CAR T cell products for the treatment of both ALL and DLBCL, revolutionising the field of cancer immunotherapy. Currently, with more than 100 trials of CAR T cells ongoing worldwide, comparable remission rates have also been demonstrated to be possible even by targeting antigens other than CD19. As more patients are treated and longer follow-up data become available, it has become apparent that approximately 30–50% of patients who have achieved tumour-remission with anti-CD19 CAR T cells will relapse within 1 year following treatment. Tumour-relapses are not unique to agents targeting CD19; the initial clinical experience with other CAR targets, such as CD22, has pointed out that relapse will be a common and recurrent challenge. Loss or modulation of the target antigen and/or a lack of CAR T cell persistence, as well as product manufacturing failures, are among the more commonly cited barriers to effective CAR T cell therapy. Furthermore, similar successes have not yet been achieved in diseases beyond B cell leukaemia and lymphoma.

As CAR T cells become more widely used, understanding the limitations of this type of therapy and overcoming these obstacles will be crucial to harnessing the full potential of this highly effective treatment modality. Several groups have been investigating CAR design to enable T cells to overcome the diverse obstacles presented by both tumour cells and the hostile tumour microenvironment. Antigen loss and tumour escape maybe prevented by simultaneously targeting more than one TAA on the same cell, which can be achieved via the expression of multiple CARs with different specificities. Similarly, bispecific CARs (named tandem-CAR) have been generated, incorporating two separate antigen-binding domains within one receptor. Another strategy for targeting multiple antigens involves further modification of CAR T cells to produce bispecific T cell engagers molecules (BiTEs). These secreted proteins typically consist of two scFvs connected by a flexible linker, one scFv specific to CD3 and the other binding to a TAA; therefore, generating a physical link between the T cell and the cancer cell. Researchers have demonstrated BiTE-secreting CAR T cells to be effective in overcoming heterogeneity in tumour
antigen expression and circumventing antigen escape in preclinical models of leukaemia and solid tumours\textsuperscript{218,219}.

An alternative approach to generating T cell responses against multiple TAAs is to create CAR T cells that can also elicit an endogenous immune response. These agents are currently termed as “armored” CAR T cells, incorporating an immunomodulatory agent that can engage and modulate other cells of the host’s immune system\textsuperscript{220}. CAR T cells modified to express the proinflammatory molecule CD40 ligand (CD40L) provide one example\textsuperscript{221}. As well as having enhanced intrinsic functionality owing to CD40L costimulation, these cells can activate professional antigen-presenting cells and increasing the immunogenicity of tumour cells via engagement of CD40, thereby promoting epitope spreading and elimination of tumour cells by endogenous, unmodified T cells\textsuperscript{222}. Regardless of the number of TAAs targeted, antigen escape is always a concern with CAR T cell therapy, and thus recruitment of endogenous immune cells might be necessary to propagate the anti-tumour immune response.

Augmenting CAR T cells to secrete stimulatory cytokines that drive the proliferation, survival, and anti-tumour activity of T cells and change the immunosuppressive environment of solid tumours is an area of active investigation. Indeed, these 4\textsuperscript{th} generation CAR T cells, engineered to constitutively or conditionally secrete cytokines such as IL-12, IL-15 and IL-18, have shown promising results in reshaping the immunosuppressive TME to allow more effective immune responses\textsuperscript{50,223,224}.

In addition, dominant-negative receptors have been used to neutralize immunosuppressive ligands and chimeric switch receptors that can convert inhibitory signals into T cell stimulatory signals\textsuperscript{104,225,226}.

In parallel to the augmented potency of the therapy, safety concerns regarding the toxicity of CAR T cells have prompted the development of “off-switches” in the form of suicide genes that can provide a way to deactivate CAR T cells whether and when CRS or on-target off-tumour toxicities become unmanageable. Numerous approaches have been developed, some based on co-expression of the CAR and human cell-surface antigens for which FDA-approved therapeutic antibodies are available, including CD20, which is targetable with rituximab\textsuperscript{227}, or the extracellular and transmembrane domains of EGFR, which can be targeted with cetuximab\textsuperscript{94}. Antibody-mediated suicide has been shown to control the toxicity of engineered CAR T-cells in preclinical and clinical studies\textsuperscript{51}, but concerns exist around the limitations of this approach, such as the
on-target toxicity from the antibody used as well as the (limited) biodistribution of the drug, might limit their utility and further development.

An alternative and rapid onset safety switch has been developed using an apoptosis-triggering fusion protein. Here, conditional dimerization and activation of the fusion protein are triggered by systemic administration of an otherwise biologically inert small-molecule\textsuperscript{228}.

More recently, it has been demonstrated that the tyrosine kinase inhibitor dasatinib, an FDA-approved treatment of Philadelphia chromosome-positive chronic myeloid leukaemia and ALL, interferes with CAR activity. The way it does so is by inhibiting the lymphocyte-specific protein tyrosine kinase (LCK), responsible for the phosphorylation of the intracellular chains of the CD3 and ζ-chains of the TCR complex thereby disrupting signalling downstream of the activated CD3ζ domain\textsuperscript{229}.

An innovative approach developed to achieve greater tumour selectivity is the use of synthetic Notch receptors. These receptors have been engineered to recognize one TAA. Following TAA binding, the Nuclear transcription factor-κB (NF-kB) is cleaved from the intracellular domain inducing the expression of another transgene, such as a conventional CAR structure, specific for a second TAA. Thus, synthetic Notch receptor CAR T cells can exploit effector functions only against targets expressing both antigens concurrently\textsuperscript{230,231}. These synthetic receptors can also provide T cells with the conditional ability to secrete cytokines, express death receptor ligands, and differentiate into distinct T cell subsets only in the presence of targeted antigens\textsuperscript{232,233}.

A versatile approach to generate specific activation of T cells is represented by the split and programmable CAR system (SUPRA-CAR)\textsuperscript{234}. Using leucine zippers motifs, this platform allows the generation of zip-CAR T cells that can be redirected towards TAA by using zip-fused scFvs. The interaction between the zip motifs on the CAR and scFv assembles the structure into a full CAR able to eradicate antigen-expressing tumour cells. By exchanging the zip-scFv specificity, CAR T cells are redirected against multiple TAA without the requirement to reengineer T cells. The extent of the SUPRA-CAR T cell activity can also be regulated via zip-scFv concentration or by tuning the scFv- or zip-affinity for their cognate antigens. Multiple zip-CARs can be utilized to differentially control the activation of different T cell populations\textsuperscript{235}. Altogether, these and other technologies can provide a means to successfully target heterogeneous solid tumours in humans without increasing the off-tumour toxicities.
1.5 Binder discovery

Monoclonal antibodies (mAbs) are produced by B cells and specifically target antigens. The hybridoma technique introduced by Köhler and Milstein in 1975 has made it possible to obtain pure mAbs in large amounts, greatly enhancing basic research and their potential applications in the clinic. In parallel, scientific and technological advances have enabled the successful translation of mAbs to treat human diseases. Worldwide, more than 500 therapeutic mAbs have been studied in clinical trials by commercial companies, and numerous therapeutic mAbs have been approved for use in humans and are currently on the market, including many for the treatment of cancer.

In the last 10 years, CAR T cells have emerged as a form of directed immunotherapy based on antibody-antigen specificity. The chimeric nature of CAR structures enables the targeting of surface molecules in their native conformation. Unlike TCR targeting, engagement CAR engagement is independent of antigen processing and peptide presentation on MHC molecules. Full-length antibodies can be broken down into their corresponding Fab fragment and Fc domains, responsible for the interaction with FcRs. The Fab portion of the antibody, without the Fc domains, can be used to generate scFvs, composed of the variable heavy and variable light domains linked using a short flexible peptide linker. Once in the scFv or Fab format, different therapeutic modalities can be tested, such as BiTEs and CARs. These engineered fragments have been developed as therapeutics independently, and many are approved or under clinical investigation.

A major challenge in the CAR field is that it is currently necessary to test all the available scFv variants as no general rules are helping the CAR-binding domain design. Ligand-binding domain parameters such as affinity, avidity, and antigen epitope location have been shown to contribute to CAR T-cells functionality majorly. The affinity has been extensively modulated for a variety of scFvs to reduce the potential of “on-target, off-tumour” toxicity. Rational decrease of the affinity has been shown to be able to discriminate between high and low levels of target expression, sparing normal healthy tissues expressing low antigen levels. Despite the increase in specificity when targeting high-antigen expressing cancer cells, a reduction in affinity can also result in diminished cytotoxicity and cytokine production as reported for the anti-ROR1 scFv R12 and the anti-GD2 CAR based on the low-affinity scFv 14G2a. In contrast, recent clinical data of an anti-CD19 CAR harbouring a low-
affinity scFv with a fast off-rate, reported longer cell persistence and improved cytotoxic abilities\textsuperscript{72}, confirming the importance of the affinity in the antibody fragment selection. However, this type of measurement reflects only the monovalent behaviour of the scFv; in both CAR- T cells and native T cells, the strength of the interactions is given by multiple receptor-ligand interactions due to the formation of multiple immune-synapse at the T cells-target interface\textsuperscript{244,245}. This parameter is called avidity, and it is influenced by CAR expression levels, ligand densities on target cells, and affinity of individual ligand-binding domains. Assessment of avidity requires evaluation of ligand-binding domains in the context of CAR T-cells. Drent and colleagues have reported that of 120 affinity-modulated scFv clones against CD38, some did not show detectable binding when using standard affinity measurement techniques, but they did show binding when tested against ligand-coated beads. Specific binder interaction was furtherly confirmed by CAR T-cells killing, indicating avidity as a contributor to CAR T-cells activation\textsuperscript{246}. In addition, excessive tonic signalling, in an antigen-independent manner, can cause early exhaustion of T cells. The propensity of some scFv to aggregate plays a role in regulating CAR T-cell activity. A study utilising the anti-GD2 scFv 14G2a has proposed the framework regions of the scFv as responsible for CAR surface aggregation resulting in tonic signalling and exhaustion. They have shown that replacing the framework regions of anti-CD19 FMC63 CAR with the framework regions of anti-GD2 14G2a scFv resulted in increased exhaustion\textsuperscript{167}. The propensity of a scFv to aggregate is a consequence of protein misfolding caused by the low folding stability of the VH or VL domain. Moreover, scFv linkers can sterically constrain the VH-VL interaction, resulting in oligomerization\textsuperscript{247}. High CAR expression levels can facilitate these non-specific interactions enhancing the potential of aggregation on the cell surface\textsuperscript{248}. Lastly, the spatial location of epitope binding could have a bigger impact on CAR activity than variation in binding kinetics\textsuperscript{180,249}. The modular nature of the CAR’s structure allows for targeting difficult epitopes, including large and bulky cell surface receptors. CARs based on an scFv targeting the membrane-proximal region of the mesothelin (MSLN) molecule showed increased cytotoxicity and cytokine secretion \textit{in vitro} and \textit{in vivo} compared to a membrane-distal epitope targeting CAR. The group attributed the increase in functional responses to the rigid structure of the membrane-proximal region that enabled better signal transduction\textsuperscript{250}. Moreover, the authors explained that the membrane-distal region of MSLN functionally interacts with proteins such as CA125 (MUC16), which might compete or impede CAR binding. This suggests
that not only steric hindrance but also structural and functional characteristics of the targeted epitope need to be considered in the design of CARs. It has been shown that epitope location is also important for modulating the immune synapse distance, which regulates effective kinetic segregation of phosphatases and T cell degranulation. A CAR targeting the membrane-distal epitope of CD22 was found to have weaker signalling, lower cytotoxic efficiency, and sub-optimal degranulation compared to a CAR harbouring an scFv to a membrane-proximal epitope. Although the immune synapse distance can be easily tuned by utilising different spacer domains, it remains important to consider epitope location and functional and structural limits imposed by the nature of the target.

There are two main methods that allow the generation and isolation of mAb or mAb’s fragments for a specific target molecule: generating antibodies by immunizing animals or using naive antibody libraries selected through display platforms as phage display.

1.5.1 Hybridoma technology

The first and traditional screening method uses immunocompetent animals in the process of generating monoclonal antibodies. The antigen of interest, either in the form of purified protein or genetic material, is injected into the animal, typically mice and rats. This elicits an immune response, then isolation of B cells from either blood or other lymphoid tissues and fusion to a specific myeloma cell partner providing immortalization of the B cell in a hybridoma cell. Once a hybridoma has been generated, the secreted antibodies can be screened for specificity and isolated for further development. In this way, mAbs against almost any antigen can be created, but their therapeutic application is limited by both the incidence of a harmful immune response against the murine mAb and the lack of sufficient effector function for murine mAbs. Recent advancements in genome editing, such as the replacement of endogenous mouse antibody gene with that of human origin, have revolutionised the field. This technology has generated strains of humanised mice that, upon antigen challenge, the B cells from these mice will produce human antibodies. Though the diversity of the antibodies generated is limited to that of the human donor, this technology eliminates the arduous process humanization and affinity modulation of mouse-derived antibodies. However, when considering in vivo antibody’s generation methods, it is important to acknowledge that affinity maturation can be achieved in the animal by boost strategies. In addition, if in vivo strategies cannot achieve the production of
CDRs with the desirable qualities, it is always possible to perform in vitro affinity tuning to alter the strength of scFv binding to the target antigen. A seminal study by Liu and colleagues demonstrated that lower affinity CARs could induce greater target cell death than higher affinity CARs. Critically, these lower affinity CARs demonstrated a greater capacity to distinguish between overexpressed and physiological levels of antigen expression compared to higher affinity CAR T cells\(^{177}\). In summary, the use of in vivo platform to generate the antibody with the desired properties allows for processes such as affinity tuning to occur more rapidly than ex vivo engineering, nonetheless, the pool of potential binders may be narrowed as a result of the deletion of reactive CDR binders in tolerance if the target antigen has a high homology to the animal being used to generate the antibodies. Alternatively, the use of in vitro screening platforms such as phage display allows to identify a larger pool of diverse CDRs, however this larger pool of CDRs requires greater efforts for the isolation of binders to a given target.

1.5.2 Phage display

In vitro antibody-selections methods have been largely used as an efficient way of discovering high-quality reagents with wide use in biological, diagnostic, and therapeutic applications\(^{261-263}\). Nowadays, improved antibody-libraries provide a lot of alternatives and powerful ways to recognize the most interesting candidates by combining the generation of billions of components with fast screening and selection procedure\(^{264,265}\). One of the most widely used library methods is based on the use of filamentous phages, which seems to play an increasingly important role in the future of antibody discovery\(^{266,267}\). The phage display approach is based on the ground-breaking work of George P. Smith on filamentous E. coli phage M13 and the fusion of peptides to the phage envelope proteins, which allows the phenotypic in vitro selection of the corresponding peptide encoding gene fragment packaged in the same phage particle\(^{268}\). The selection process was called “panning” because of the resemblance to the method used to find gold\(^{269,270}\). All major phage display systems were based on antibody-pIII fusion proteins, but they differed in the antibody gene expression cassette location. The integration of the antibody-pIII encoding gene into the phage genome was not as successful as the more flexible phagemid system, which uncouples antibody-pIII expression from the expression of the phage proteins and from phage replication\(^{271,272}\). The phagemid bears a bacterial origin of replication and antibiotic selection, in addition
to the antibody-pIII expression cassette working as a normal plasmid in the absence of the rest of the phage proteins provided by the helper phage\textsuperscript{273}. This antibody surface display and expression system allow the switch between oligovalent and monovalent antibody displays by using different helper phages, such as using hyperphage for oligoclonal display\textsuperscript{274}. The starting point in the phage display technique is library construction which includes a huge collection ($>10^{10}$) of antibody genes encoding antibodies with unknown properties. Their design is critical to success. The generation of diversity in phage display libraries is achieved via immune or nonimmune sources. Nonimmune libraries such as naïve phage libraries tend to be large, often in the range of $10^9$–$10^{11}$ independent clones\textsuperscript{275,276}. These libraries, in theory, facilitate the isolation of antibodies specific to any antigenic epitope. However, in practice, maintaining diversity is challenging and can lead to the isolation of low affinity antibodies that will require further, time consuming, \textit{in vitro} engineering\textsuperscript{277}. Immune libraries, in comparison can be smaller, with high affinity antibody isolation possible from libraries as small as $1 \times 10^5$ unique clones\textsuperscript{278,279}. Immunisation of a host species leads to affinity maturation by the immune system of the recipient and expansion of antigen-binding B cells. RNA isolated from tissue sources rich in these B cells are used for library generation, increasing the likelihood that antibody presenting phage molecules in the library will confer the required binding specificity. Several species have served as the source of variable region diversity for antibody phage libraries. Typically, v-regions have been isolated from sources including mouse, rabbit, and human\textsuperscript{280–282}. In any display method, the selection antigen and method play an important role in the successful isolation of antibodies. A pure antigen is often required to avoid isolation of non-target specific antibodies. The generation of pure protein can often be a limiting factor, particularly for challenging protein targets. Correct display of the target antigen can also impact the selection of binders with the desired properties and specificity\textsuperscript{272}. Direct conjugation of proteins to a solid support via passive or covalent immobilization is rapid and simple; however, this method can occlude desired epitopes and present proteins in non-natural conformations. Solution-based selection strategies overcome this to some extent by allowing phage to bind to the biotinylated antigen in solution prior to the capture of the complex on streptavidin-coated magnetic beads\textsuperscript{283,284}. Nonetheless, this step does not remove the need for highly pure biotinylated reagents for panning. In addition, the irreversibility of the streptavidin-biotin interaction requires elution conditions that can reduce the recovery of infective phage particles for
subsequent enrichment steps\textsuperscript{285}. Several methods have been developed to avoid this, including engineered cleavage tags within the scFv coding sequence or the use of disulphide linked biotin moieties to allow elution using reducing agents such as DTT. Our lab has optimized a simple selection strategy that uses the StreptagII – StrepTactin system\textsuperscript{286} to directly capture protein from cell culture supernatant to a solid support. Strep-tagII is recombinantly fused and expressed with the antigen, mimicking biotin, while StrepTactin (a streptavidin analogue) enables high avidity stringent selection while also facilitating mild and efficient recovery with an inexpensive biotin solution. Screening for monoclonal antibodies can be performed either by antibody phage using enzyme-linked immunosorbent assay (ELISA) or by soluble expression of antibody fragments in \textit{E. coli} followed by immunoassays, such as flow cytometry\textsuperscript{287–289}. The isolated antibody fragment can be directly used or converted into other antibody formats for downstream development, e.g., CAR, IgG, and antibody-drug conjugates.

**Hypotheses and aims of the project**

Despite the great promise of anti-CD19 CAR T-cells for the treatment of B cell malignancies, concerns remain regarding its safety. CAR design and, in particular, the binder’s biophysical properties have been demonstrated to be critical for the activity and persistence CAR T-cells. Minimising toxicity and severe CRS, neurotoxicity, and persistent cytopenia is of paramount importance to the field, particularly given the populations of patients we are aiming to treat are often elderly with numerous co-morbidities and limited physiological reserve.

One of the significant limitations the field faces is that current \textit{in vivo} models poorly recapitulate the effects of CAR activity in humans. Models such as that devised by Norelli et al. have been able to demonstrate the crucial role of monocytes in triggering CRS, similarly to what has been reported in clinical trials. Although it is a very elegant humanized mouse model, it is not identical to a human host due to the absence of human cytokine signals, and the mouse origin of non-hematopoietic tissues might contribute to an altered CAR T-cells activity. In addition, the complex approach employed to develop this model makes it rather difficult for basic-research studies.

Given the above challenges, the overreaching aim of this PhD project is to develop a fully mouse immunocompetent model system to dissect the biology of responses/lack of response and toxicity of CD19 CAR T-cells. Further, the project aims to generate a
range of novel mouse anti-CD19 scFvs to investigate the impact of biophysical properties on CAR T-cell function both in vitro and in vivo. Specifically, my objectives are:

- To generate a fully mouse system of B cell malignancy
- To generate and test the activity of anti-CD19 mouse CAR T-cells both in vitro and in vivo
- To interrogate the impact of anti-CD19 mouse CAR T-cells on the endogenous immune compartments and cytokine response
- To develop novel anti-mouse CD19 scFv and to study their biophysical properties
- To compare the novel anti-mouse CD19 scFv as mouse CAR T-cells
2. Methodologies

2.1. Molecular biology

2.1.1. Molecular Cloning

2.1.1.1. PCR amplification and splicing by overlap extension PCR

Transgenes were created in a flexible manner by hybridisation of two or more DNA fragments using overlap extension PCR (OE-PCR) protocol. The DNA fragment were amplified using specific primers in the primary PCRs reaction from either parental plasmids or as de-novo gene fragments (gBlocks IDT DNA). PCR reactions were carried out as per manufacturer’s protocol using Phusion polymerase (NEB). The recombinant transgenes assembled by OE-PCR were generated to have the N-terminus containing a 5′restriction site (typically NCol site) and the C-terminus containing the 3′restriction site (typically MluI site) to facilitate the downstream cloning in the appropriate vector backbone. The DNA fragments generated by PCR were commonly run for 35 cycles of amplification. General cycling conditions for Phusion polymerase were the following: melting temperature: 98°C for 120s, annealing temperature 65°C for 30s and extension temperature 72°C. The duration of the extension was defined for each amplicon length generated; approximately 30 seconds for every 1000bp of DNA). Following PCR amplification, the DNA products were separated by gel electrophoresis and purified using QIAquick PCR purification kit (QIAGEN). Following PCR clean-up (Qiagen QIAquick clean-up kit), PCR digestion was performed using appropriate restriction enzymes and the insert was subcloned into appropriately digested destination plasmid.

2.1.1.2. Restriction endonuclease digestion

All the restriction enzymes used throughout the project were supplied by NEB with the recommended buffers, enzymatic reactions were set up as per manufacturer’s protocol. Two types of enzymatic digestions were performed: screening digestion inspecting the generated transgenes or cloning digestion were larger amounts of DNA were used to obtain the final expression plasmid. Screening digestion was performed using between 200-300ng of DNA and incubated for 1 hour at 37°C, while larger scale digests of up to 5µg of DNA were incubated for 3 hours. Digested products were purified by gel electrophoresis, as described earlier. In the event of buffer or temperature-incompatible
enzymes, sequential digests were carried out with a DNA purification step in between the two digestions and enzymes were inactivated at the end of each incubation (typically at 65°C)

2.1.1.3. DNA ligation and bacterial transformation
DNA ligations were performed using Quick Ligation™ kit (NEB). A 1:3 molecular ratio of vector: insert was used to increase the success of correct integration. Typically, 7.5µl of insert DNA was mixed with 1.5µl of vector DNA in a 20µl final reaction volume with 10µl Quick Ligase buffer, and 1µl Quick Ligase. Samples were incubated at room temperature (RT) for 5 minutes and immediately placed on ice to stop the reaction. The ligation mix was directly used to transform high efficiency chemically competent bacteria *E.Coli* (NEB, C2987H), 2µl of ligated DNA were incubated on ice for 5 minutes with 25µl of bacteria, followed by 42°C heat-shock for 35 seconds to allow the DNA to enter the cells. Transformed *E.Coli* bacteria were spread on an agar-dish containing the Luria-Bertani (LB) nutrients and the appropriate antibiotic for selection. The LB-agar plates containing the transformed bacteria were incubated at 37°C and allowed to grow overnight.

2.1.1.4. Small-scale DNA preparation
Small amount of purified DNA was generated for screening purposes (miniprep) and prepared as follows. From the LB-agar plates containing the transformed bacteria, single colonies were picked and used to start a 4ml liquid culture in LB media supplemented with 100µg/ml carbenicillin (or other antibiotics) and incubated with shaking at 220rpm overnight. Plasmid DNA was extracted from the bacterial culture using the Qiagen Miniprep kit (QIAGEN) following the manufacturer’s protocol. In the final step, DNA was eluted in 30µl of deionized H₂O (dH₂O). Products were validated by screening restriction digestion and DNA sequence confirmed by Sanger sequencing (Genewiz).

2.1.1.5. Large scale DNA preparation
Generation of DNA for *in vitro* and/or *in vivo* experiments required greater amounts of DNA. Large scale DNA midiprep (Macherey-Nagel) was performed as follows in accordance to the manufacturer’s protocol: 50ml of terrific broth (TB) were seeded with
100μg/ml of the appropriate antibiotic and 500μl of the small-scale bacterial culture and cultured overnight in a 37°C shaking incubator at 220rpm. After final step of DNA precipitation, the pellet was reconstituted overnight in 200μl of dH2O in the fridge. The purified DNA was then verified by multiple screening digestions to confirm the unique restriction pattern in comparison with the parental vector backbone.

2.1.1.6. DNA screening by gel electrophoresis

In order to separate DNA fragments of different sizes, typically a 1.2% (m/v) agarose gel was casted. 1.2g agarose were dissolved in 100ml Tris/Borate/EDTA (TBE) solution by heating in a microwave for 2 minutes. Upon cooling 5µl SYBR™ Safe Gel Stain (Thermo-Scientific) was added to facilitate DNA visualisation under ultraviolet light or blue light if the DNA was required for downstream applications. DNA samples were pre-mixed with loading buffer at a ratio of 5:1 (v/v) (Bioline) and ran at 150V alongside an appropriate DNA ladder, typically Hyperladder 1Kb (Bioline) for DNA size-reference.

2.1.1.7. DNA extraction from agarose gel

Following separation of the fragments by DNA electrophoresis, the bands visualised under blue light to prevent UV-mediated mutagenesis were excised from the gel using a sharp scalpel. The cut band was then followed by DNA extraction using Qiagen QIAquick gel extraction kit (QIAGEN), according to manufacturer’s instructions and DNA eluted in 30µl of dH2O.

2.1.1.8. Quantification and validation of DNA

DNA concentration of plasmid DNA was assessed using a nanodrop ND-1000 spectrophotometer. All the new generated plasmids were sent for sequencing at the small-scale preparation stage to confirm the DNA sequence (Genewiz). The electropherograms were analysed and the sequences aligned with the reference map using SnapGene® software. The main primers used for sequencing SFG plasmids were forward (MP406, ttacacagtcctgctgaccacc) and reverse (MP785, caagcggcttcggccagtaac) for SFGmR plasmids: MP406 as forward primer and MP8980 as reverse primer (gctaagtaacatctgtggcaatattg). The pVAC2 plasmids generated for the rat vaccination
were sequenced with forward (MP13901, cgcctcgcttgagtcgag) and reverse (MP13902, atcgaagtagagaagatgcaagcatg).

2.1.1.9. Plasmids

The SFG gammaretroviral vector (Addgene ID:22493) was used as vector backbone for the majority of the constructs generated. This plasmid was derived from the Molony murine leukaemia virus with the open-reading frame (ORF) located at the site of the deleted viral envelope gene. Multicistronic transgene expression was achieved by using either an internal ribosome entry site (IRES) or an in-frame foot-and-mouth-like 2A peptide for ribosomal skipping. SFGmR backbone is an improved version of SFG plasmids with an addition of the scaffold/matrix attachment region (S/MAR) to enhance gene expression stability of the viral insert. This vector backbone was used for the generation of all the constructs involved in mouse experiments. pVAC2 plasmids were used as genetic vaccination vector for the delivery of mouse CD19 transgene without intracellular signalling. This plasmid is optimized for transient high expression under a pCMV promoter. DNA sequences were obtained from the Dr Martin Pulé pMol plasmid archive or de-novo synthetized as gBlocks (IDT DNA) using uniprot.org as database reference for the DNA and protein sequences.

2.1.2. Solving hybridoma with 5’RACE PCR protocol

The overview of the protocol for solving hybridoma by 5’-Rapid amplification of cDNA ends (RACE) is summarised in figure 2-1.
Figure 2-I. Schematic of 5’RACE. Total mRNA is reverse transcribe into cDNA using oligo dT primers specific to the poly-A tail of the mRNA. The generated cDNA is poly-C tailed at the 3’ end using terminal deoxynucleotidyl transferase (TdT). An anchor primer annealing (NNNGGG) in combination with an constant region-specific primer (IgG or kappa) allows the amplification of variable chain. Subsequent nested PCR using inner primers is used to obtain an adequate amount of PCR product for direct cloning into a TOPO-TA vector for the determination on the DNA sequence.

2.1.2.1. RNA extraction and quality control
Typically, RNA was extracted from 5 x10^6 cells using RNAeasy™ kit (QIAGEN) and eluted in 40µl of nuclease-free dH2O (Sigma-Aldrich). RNA quantification and quality were assessed with BioAnalyzer or Tapestation instruments (Agilent) and samples were prepared according to the manufacturer protocol. The RNA integrity number (RIN) obtained was from the machines used to estimate the RNA quality for each sample, with great RNA integrity being with RIN>8.0.

2.1.2.2. cDNA conversion and 3’-polyC tailing
The cDNA reaction was performed using 7µg of RNA according to the QuantiTect® Reverse Transcription kit (QIAGEN) instructions. The success of the reaction and potential genomic contamination was assessed by PCR amplification of the housekeeping gene GAPDH followed by DNA electrophoresis. Following cDNA production, 3’ polyC tailing was performed by incubation of the cDNA with terminal transferase (TdT) and dCTPs, as follows: 10µL of cDNA was incubated at 95°C for 1 minute then immediately chilled on ice; 6µl of dH2O, 2µL TdT buffer,1µL dCTPs
(10mM) and 1µL TdT were added to the cDNA and the mixture incubated at 37°C for 30mins, followed by 75°C for 30mins. The poly-C tailed cDNA was subsequently purified with PCR clean-up kit (QIAGEN) and used as a template for the nested PCR.

2.1.2.3. Nested PCR for the identification of heavy and light chain sequences

The heavy and light chains were separately first amplified using outer primers; a 5′ forward specific for the poly-C tail forward primer with (MP12837, aeggtgcaaaacctcctcecaaatcggg) and a gene-specific reverse primers for either rat IgG2a (MP15164, tcctgggctaccacggggtac) or rat Ig kappa (MP16588, gatacacgactgagctcacgcgt) were used for first PCR amplification. 50µL reaction mix was containing 47µL Platinum PCR SuperMix high fidelity kit (Invitrogen), 1 µL polyC-cDNA, 1µL forward primer (10mM), 1µL reverse primer (10mM). This was run on the thermocycler using the following conditions: 1) 94°C 2mins; 2) 94°C for 30sec; 3) 56°C for 30sec; 4) 68°C for 30sec; 6) 68°C for 10mins and repeated for 25 cycles. The PCR products were subsequently cleaned up and used as template for the subsequent inner PCR reactions. The inner PCRs, also term ‘nested’, were performed separately for the heavy and light chains, a forward primer (MP12838, aeggtgcaaaacctcctcecaaatcggg) and specific inner primers for rat IgG2a (MP15163 gtcaccatggagaactttgttggagacagt) and rat Ig kappa (MP16587 ggaagatrgatacagttggtgcagcatc) were used to set up a 50µL PCR reaction with the same settings as above. Following DNA amplification, the PCR products were run on an agarose gel and the resulting smear from 400-600bp for each chain were cut out and extracted from the gel. The purified DNA bands were then subcloned into TOPO-TA vector for subsequent colony screening.

2.1.2.4. TOPO-TA cloning and colonies screening

The TOPO-TA cloning kit (Invitrogen) was used to facilitate the screening of the hybridoma amplicons generated through 5’RACE PCR. The TOPO-TA vector is supplied in a linearized format possessing a single 3’-thymidine (T) overhang with the topoisomerase I enzyme proprietary linked to the vector. Using Taq polymerase-containing products that possess a non-template-dependent terminal transferase activity, generated PCR amplicons will incorporate a 3’-terminal deoxyadenosine (A) functioning as an overhang for the direct cloning into the vector. The obtained ligation
mix was directly transformed into *E. Coli* bacteria and plated onto carbenicillin-selective agar plates pre-coated with the chromogenic substrate for β-galactosidase (X-gal). The chromogenic assay of blue/white colonies facilitates the isolation of the colonies incorporating the insert. White colonies were produced by the lack of galactosidase-mediated cleavage of the X-gal blue substrate due to the disruption of the galactosidase gene by introducing the DNA insert. Individually picked white colonies were grown overnight in 4ml of LB in the presence of 100µg/ml of carbenicillin for further analysis. The plasmid DNA was extracted from the bacteria and purified prior to Sanger sequencing for the identification of the HC and LC sequences.

2.1.2.5. SMART-5′RACE protocol

The SMART (switching mechanism at the 5′ end of the RNA transcript) strategy relies on the intrinsic properties of Moloney murine leukaemia virus (MMLV) reverse transcriptase and the use of a unique template switching oligonucleotide (TS oligo). During first-strand synthesis, upon reaching the 5′ end of the RNA template, the terminal transferase activity of the MMLV reverse transcriptase adds a few additional nucleotides (mostly deoxycytidine) to the 3′ end of the newly synthesized cDNA strand. These bases function as a TS oligo-anchoring site. Upon base pairing between the TS oligo and the appended deoxycytidine stretch, the reverse transcriptase switches template strands from cellular RNA to the TS oligo and continues replication to the 5′ end of the TS oligo. By doing so, the resulting cDNA contains the complete 5′ end of the transcript, and universal sequences of choice are added to the reverse transcription product. The simple version of a TS oligo is a DNA oligo sequence that carries 3 riboguanosines (rGrGrG) at its 3′ end. The complementarity between these consecutive rG bases and the 3′ dC extension of the cDNA molecule empowers the subsequent template switching (Figure 2-2). Following successful template switch and cDNA generation, the reaction was purified by ethanol precipitation, adding the following reagents into each 50µl sample. Bioline pink dye 1.5µl, 3M NaAc 5µl ice-cold 100% EtOH 125 µl. The mixture was then incubated at -20°C for 10 min and pelleted by spinning at >13,000rpm for 20 mins at 4°C. The pink pellet was washed twice with 250 µl 70% EtOH and spun at >13,000 rpm for 5 min at 4°C after each wash. cDNA was finally dissolved in 20µl of nuclease-free dH20.
The first round of PCR is carried out using a mixture of tail-specific forward primers (MP30734, cgacgtggactatcatgaacgcatactggtgatacaacgcagatg and MP30735, cgacgtggactatcatgaacgcatactggtgatacaacgcagca) at 2µM and 10µM respectively and a gene-specific reverse primer. For ratIgG, MP30737– ccaagatgcaggacagctgg- and ratIg kappa, MP30739-ctggctcaacgaggggtgtgcg- were used as gene-specific primers.

If the PCR gave a clean band, the fragments were directly sent for Sanger sequencing, otherwise cloned into a TOPO-TA vector and DNA excreted from individual colonies. In the case of faint or too many non-specific bands, a second round of PCR was carried out using inner primers: forward (MP30736, gactatcatgaacgcatactggtgatacaacgcagatg) and reverse (ratIgG MP30738 cgctggacaggtccaggtcagtcg and ratIg kappa MP30740 gtctttgtgctgtctgatcagtaac) and the DNA purified prior sequencing.
2.1.2.6. Analysis of the antibody sequences using IMGT server and subcloning as single-chain variable fragments

The International imMunoGeneTics information system (IMGT) is a high-quality and specialized database for immunoglobulins germline sequences. This is globally used as a reference for the sequence, genome, and structure of the loci involved in the expression of antibody’s chains from a wide variety of species, including mice, rats, camels, and humans\(^2\). The DNA sequence obtained through sequencing of the antibody’s variable chains was used as input for the IMGT V-quest query. This algorithm allows the alignment of unknown antibody DNA sequence to the most similar germline variable chain to identify the family and mutations of a rearranged antibody. After alignment, the software is able to identify HC and LC sequences based on the homology with the germline sequences of the desired species. In addition, it will also provide extensive annotations on the structures and evolution of the complementarity-determining regions (CDRs) of each variable chain. Upon identifying productive HC and LC, these were cloned as scFv format, generating a chimeric mouse IgG2a scFv-Fc antibody and as full structure chimeric mouse IgG2a/kappa antibody. The sequences were inserted into SFG backbones using OE-PCR for expression in mammalian cells to assess the antigen-specific binding.

2.1.3. Phage display

2.1.3.1. Genetic vaccination and validation of the immunization

Three *Wistar* rats were immunized with the target mouse CD19 (accession number: UniProtKB - P25918). Genetic vaccinations were carried out using DNA-coated gold nanoparticles at Aldevron, GmBH. Briefly, the gene coding for mouse CD19 was cloned into a pVAC2 expression plasmid, and 3mg of endotoxins-free plasmid were
shipped for the rat’s vaccination. A GeneGun™ system was used to deliver the coated gold nanoparticles intramuscularly. Twenty days later, the serum was received and tested for specific binding. Both pre- and post-vaccination sera were tested against target antigen-expressing SupT1 cells and non-transduced negative control SupT1s as well as the naturally expressing mouse cell line A20. Specific binding was assessed by flow cytometry as per general protocol and stained with a secondary anti-rat IgG antibody labelled with APC. Secondary antibody alone was acting as negative control and a commercial antibody for mouse CD19 as positive control.

2.1.3.2. Primary PCRs for the amplification of rat’s-variable heavy and light chains

The primary amplification of the two variable chains was performed with oligonucleotides designed on the *Rattus Norvegicus* germline sequences of heavy variable chain (VH) and kappa light chain (VK) genes; primers were designed to have the annealing portion with a melting temperature around 60°C. A PCR master mix was prepared using High Fidelity Phusion polymerase and buffer (NEB) in a 50µl reaction with 1µl of a 25nM working dilution of each primer and 1µl of the cDNA pool. An individual reaction for each forward primer containing 1µl of an equimolar pooled mix of the reverse primers was performed. The samples were heated at 98°C for 2 min, followed by 35 cycles of 98°C for 30 sec, 60°C for 40 sec, and 72°C for 40 sec with a 10 min final extension at 72°C. Each PCR product at approximately 400bp was purified from the agarose gel.

2.1.3.3. Assembly of the scFv library and sub-cloning onto phagemid vector

Assembly of the scFvs fragments was carried out using OE-PCR as described in the previous section. The approximately ~800bp PCR product was purified and 1µg of DNA digested with SfiI and NolI restriction enzymes (NEB) for the cloning into the phagemid vector pHEN1. Samples were ligated overnight using T4 ligase (NEB), as shown below. 500ng of digested vector were ligated to 267ng of the polyclonal insert for a 1:3 vector:insert ratio. The reaction was heat-inactivated for 20 minutes at 80°C and purified prior the electroporation in *E.Coli*.
2.1.3.4. **Electroporation in *E.Coli* and library size estimation**

The generated ligation mixture of plasmid DNA was cleaned-up by ethanol precipitation and re-suspended in 50 µl of dH2O and electroporated into *TG1 E.Coli* bacteria (MicroPulser™, Bio-Rad). The transformed bacteria were recovered in 2xTY medium (16g Tryptone, 10g Yeast Extract, 5g NaCl, Sigma-Aldrich) at 37°C C shaking at 225 rpm for 1h before spreading on a Bio-Assay Dish (Thermo-Scientific) containing 200ml of 2xTY-agar, 100µg/ml ampicillin and 1% glucose (w/v). Following overnight incubation, the dish was harvested, and the library re-suspended in 10ml of 2xTY and 15% glycerol (v/v) for storage in 500µl aliquots at -80°C. The successful generation and estimation of the library size were performed by counting the colonies plated on serially diluted 2xTY-agar/Amp/Gluc Petri-dishes. A number of single colonies were grown in liquid culture and the plasmid DNA was screened by colony PCR using M13 primers that generated a ~1000bp DNA in the presence of the full-length scFv insert (~800bp). The library glycerol stock was then used to inoculate the starting culture of the phage production for the first biopanning round.

2.1.3.5. **Strep-Tactin® beads coating**

The phage display biopanning rounds are traditionally performed using StrepTactin™ type II (IBA) magnetic beads coated with the target of interest. Mouse CD19 was genetically engineered to be fused with a mouse IgG Fc and a Strep-tag™ II (WSHPQFEKGGGSGGSGSAWSHPQFEK) and cloned as a secreted protein into a SFG expression plasmid. HEK293T cells cultured in a complete DMEM medium (Gibco) were transfected with 12.5µg plasmid DNA using GeneJuice® transfection reagent (MerckMillipore). After 48h, the cell supernatant was harvested and 0.2 µm filtered prior to the coating of magnetic beads. 5µl of beads suspension were incubated with 1.5ml of filtered cell supernatant for 1h at RT shaking. Supernatant from non-transfected 293T cells was used to coat magnetic beads as negative control during phage selection. The beads were magnetized and washed three times with PBS. The presence of mCD19-mIgGFc-Strep-tag II fusion protein on the beads was assessed by flow cytometry of a representative aliquot of NT-supernatant coated beads and mCD19-coated beads stained with anti-mCD19 or anti-mIgG polyclonal antibody. Previously generated phage libraries (human CD160 and human CD21) were used as a positive control of successful beads coating.
2.1.3.6. Phage production

To produce phage-scFv particles for the biopanning steps, a 2xTY/Amp/Gluc starting culture (OD600nm: 0.1) was inoculated with the library glycerol stock and grown to OD600nm: 0.4-0.5, before infection with 100µl of M13KO17 helper phage (NEB) followed by incubation for 40 min at 37°C without shaking. The bacteria were then centrifuged and re-suspended in 100ml 2xTY containing 100 µg/mL ampicillin, 50 µg/mL kanamycin without glucose overnight at 30°C shaking at 225rpm.

The next day, the culture was centrifuged at 3300 x g for 20 minutes at 4°C to remove bacterial cells, 18 ml of ice-cold 20% PEG-6000/2.5M NaCl was added to the phage-containing supernatant, and the solution was incubated 4°C for 1h. After centrifugation at 3300 x g for 20 minutes at 4°C, the supernatant was discarded, and the phage-containing pellet was re-suspended in 1.5 ml of dH2O. The solution was then centrifuged at 12,000 rpm in a microfuge for 5 minutes to pellet any remaining bacterial cells, and the supernatant was transferred into a clean 1.7 ml tube. 0.25ml of ice-cold 20% PEG-6000/2.5M NaCl added, the solution was incubated at 4°C for 1h then centrifuged at 12,000 rpm for 10 minutes; the supernatant was discarded and the final phage pellet was re-suspended in 1ml of dH2O. To assess the titre of phage particles, 10µl were kept aside for E.coli infection, and the remaining solution was divided into two ~500µl aliquots which were blocked with 1ml of PBS, 4%BSA (w/v) for 1h at RT and subsequently used for the rounds of biopanning.

2.1.3.7. Cell-based biopanning

The instability of mCD19 in a soluble format made the conventional beads-based strategy not feasible. An alternative approach that allows retaining the correct display of the target antigen is based on the use of whole mammalian cells expressing the target of interest. Other colleagues have successfully reported the use of this strategy to select phage display libraries. A20 cells were used as a natural source of mCD19 to select the phage library against, and a previously generated A20 KO for mCD19 was used as enrichment control through the rounds of selection. To increase the chance of specific enrichment, a round of negative selection on A20 KO cells was carried out after the first two rounds of positive selection on normal A20 cells. Two libraries were carried out in parallel with or without the round of negative selection step. Three
positive rounds of selection or two positives, one negative, and positive, were performed for the two parallel rounds of panning. 20 million target cells were blocked with 1ml of PBS, 4%BSA (w/v) for 1h at RT followed by incubation with blocked phage at RT in a sterile 4ml FACS tube with 200rpm for 2h. The phage-cells complex was washed with 3ml of PBS, 4%BSA (w/v) by centrifugation of cells at 400 x g for 5min and supernatant decanted for a total of 5 washes. Phages were eluted from cells with a solution of trypsin-EDTA for 5 minutes. Eluted phages were used to infect E.Coli to generate the glycerol stock for the next round of selection.

2.1.3.8. Enrichment and binding assessment

Individual colonies plated on 2xTY-agar/Amp/Gluc from the glycerol stock were grown in 4ml of 2xTY/Amp/Gluc liquid culture and the plasmid purified with the Qiagen miniprep kit. Approximately 300ng of extracted DNA from each colony was screened on agarose gel after SfiI and NotI restriction digestion. Expression of soluble scFv from positive bacteria colonies was obtained by re-suspending the polyclonal glycerol stocks from each round of biopanning or single colonies into 1ml of 2xTY, 100 µg/ml ampicillin and 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cultured at 30°C for further 16h shaking at 225rpm. After centrifugation at 3300 x g for 10min, 200µl of the induced bacteria supernatant was used as primary antibody for staining $10^5$ mCD19 positive supT1 cells followed by 1 µl of anti c-myc-DyLight549. Cells were acquired on a BD Fortessa x20 II flow cytometer. Anti mCD19 antibody was used as positive control and either induced bacteria supernatant from the previous round of biopanning or 2xTY medium as a negative control for the staining.

2.2. Protein work

2.2.1. Protein purification and validation

2.2.1.1. Protein A purification using AKTA system

ScFv-IgG2a cloned in SFG vectors were expressed by transient transfection in CHO cells following the 25ml small-scale production protocol from manufacturer (Gibco) using 20µg of purified DNA. Supernatants were harvest 8 days later and used for protein purification. Supernatant was span down at >3000 x g for 10 min and filtered through an 0.2µm filter prior to the purification. Pre-packed 1ml protein A columns
(GE healthcare) have been used for affinity purification, relying on the m1G2a-Fc of the fusion proteins. The column was drop-to-drop plug into an AKTA start system, and the purification controlled remotely with UNICORN software. In-house 20mM sodium phosphate pH 7.4 was used as a binding buffer, and 0.1M sodium citrate pH 3.0 was used as elution buffer. Each supernatant was run through the column and washed 20 times before fractionation into ten 0.5ml aliquots of eluted protein. pH was immediately neutralized with 120ul of Tris-HCL pH8.0 (Gibco). Absorbance at 280nm from the system was used to identify the fraction containing the protein, usually the 6th and the 7th fraction. The protein-containing fractions were dialyzed overnight in 10KDa cut-off dialysis-cassettes (Thermo-Fisher) 1:1000 in PBS.

2.2.1.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used technique that allows the separation of proteins based on their molecular weight. SDS is a detergent in the sample buffer where, along with boiling, and a reducing agent (normally DTT or B-ME to break down protein-protein disulphide bonds), it disrupts the tertiary structure of proteins. This brings the folded proteins down to linear molecules. Moreover, SDS coats the proteins with a uniform negative charge, masking the intrinsic charges. SDS binds uniformly to linear proteins meaning that the charge of the protein is now approximately proportional to its molecular weight. The PAGE of SDS-labelled proteins consists of a 2-dimensional protein separation in a polyacrylamide gel matrix to produce different pores sizes, giving various separating conditions. Samples were prepared as follows:5 µL of sample loading buffer,2µL of reducing buffer (not included if gel was to be run under non-reducing conditions), made up to 20µLfinal volume with the sample. The sample was then boiled at 95°C for 5 minutes. 15µL of each sample was run on a premade 4-12% SDS-PAGE gel (NuPAGE™ 4 to 12%, Bis-Tris, Thermo-Scientific) at 200V, 500mA for 45 minutes on constant voltage mode. 10µL of pre-stained protein ladder (Novex) was run as a size marker. Following the run, gels were either used for Coomassie staining or Western blotting.
2.2.1.3. Coomassie staining

Following SDS-PAGE, the gel was transferred to a staining tray, covered with Coomassie R-250 stain solution (Sigma-Aldrich), and stained for 60 minutes with gentle agitation on a rotating plate shaker. The staining solution was decanted, and the gel was destained with Coomassie destain solution (dH2O, methanol, and acetic acid in a ratio of 50/40/10 (v/v/v)) with gentle agitation. Further destain solution was changed as required until the protein bands became clearly visible over the blue background, typically overnight. Finally, the protein gel image was acquired using QuantStudio imager (GE Lifesciences).

2.2.1.4. Western blot

Following protein separation, the gel was transferred to a PVDF membrane using a wet transfer protocol. The gel containing the protein was assembled with layers of sponges and filter papers to make a conductive sandwich. All layers were pre-soaked in transfer buffer and air-bubbles removed. Proteins were transferred from the gel to the membrane by applying a constant electrical current of 10V for 1h. Following the transfer, the membrane was blocked with a solution of PBS with 4% BSA (Sigma-Aldrich) overnight at 4°C. The appropriate primary antibody was used for the staining in PBS with 4% BSA for 1 hour with gentle rotation. The membrane was then washed 3 times with PBS 0.1% Tween20 (Sigma-Aldrich) for 10 minutes each time. If secondary staining was required, this was performed as per primary staining. The final antibody is used for the detection as it can be supplied as a Horseradish-peroxidase (HRP)-conjugate antibody. The enzyme allows the detection of positive binding through the chemiluminescence reaction triggered by the addition of the appropriate substrate. Following washing, the sample was developed using ECL substrate (ThermoScientific). After 5 minutes of incubation, the substrate was gently removed by blotting on an absorbent paper. The proteins were then visualized using when the blot is sensitized on photographic X-ray film, which darkens in response to the emitted light, and the signal intensity is determined by the number of HRP molecules reacting with the substrate and the length of the exposure. Typically for 10-600 seconds.
2.2.2. Biophysical characterization

2.2.2.1. Melting temperature analysis

Melting temperature analysis was used to assess protein stability and was performed using nano-differential scanning fluorimetry (NanoDSF) (Prometheus NT.48, NanoTemper Technologies GmbH, Munich, Germany). It uses tryptophan or tyrosine intrinsic fluorescence to monitor protein unfolding. Both the fluorescence intensity and the fluorescence maximum depend on the surroundings of the two residues. Therefore, the ratio of the fluorescence intensities at 350nm and 330nm wavelengths is suitable to detect changes in the protein structure. The emission of fluorescence at 330nm and 350nm were measured with the temperature changes from 20 to 95°C, at a rate of increase of 1°C every minute. The fluorescence curves obtained were used to determine the differentials ratios of the two emissions to determine transitions in the protein’s state.

2.2.2.2. Kinetics study using Biacore SPR technology

The binding kinetic of the selected scFvs were studied at 25°C by surface-plasmon resonance (SPR) technology using either a Biacore™ X100 or T200 machines. Supernatant from 293T cells transfected with different anti mCD19 scFv -mIG2aFc was harvested at 48h and immobilized on a CM5 sensor chip using a mouse antibody capture kit (GE, healthcare). The purified mouse CD19 analyte (Sino Biological) was dialysed against the experimental running buffer HBS-P+ (GE, healthcare) prior to use. All steps were performed at a flow rate of 30μL/min, and the analyte in the X100 system was injected in a single-cycle kinetic mode at five different concentrations (3.7nM, 11.11nM, 33.33nM, 100nM and 300nM). While in the T200 system, the analyte was injected in a multi-cycle modality where each concentration of analyte is independently run through cycles of surface-regeneration. In both systems, the binding data were double-referenced using the interspots (representing unmodified chip surface) and the in-line buffer blank (“0 nM mCD19” injection) to count for non-specific interactions. The double-referenced sensorgrams were locally fit using the 1:1 Langmuir binding model for the extrapolation of Ka (on rate), Kd (off rate), and KD (affinity) (BiaEvaluation software Version 3.0).
2.2.2.3. Epitope competition using Biacore SPR technology
The assay was performed with Biacore 8K instrument (located at Autolus Therapeutics plc) using HBS-P+ as the running and dilution buffer (GE Healthcare). The built-in BIAevaluation software version (GE Healthcare) was used for data processing. The anti-mouse CD19 reference clone 1D3 was covalently coated on the gold surface of CM5 series S chip using amine coupling kit (GE Healthcare). Soluble recombinant His-tagged mCD19 (Sino Biological) was injected at saturating concentration (600nM). To determine binding competition, a second anti-mCD19 antibody was injected over the flow cells. Binding competition for the second antibody was determined by normalization of same antibody pair, to adjust for dissociation, and then compared with the Rmax obtained in the absence of the second antibody.

2.3. Tissue culture

2.3.1. Propagation of mammalian cells

2.3.1.1. Propagation of adherent cell lines
The adherent cell line HEK293T was routinely maintained in complete IMDM media (Iscove's Modified Dulbecco's Media) supplemented with 10% fetal-bovine serum (FBS) in 175cm² tissue culture-treated flasks (Thermo-Scientific) and incubated at 37°C in a 5% CO₂ atmosphere. 80%-confluent healthy cells were passaged twice a week by replating them at a 1:10 dilution. Briefly, supernatant was removed and cells incubated for 5 minutes at 37°C with 7ml trypsin/EDTA (Sigma-Aldrich). Cells were harvested by washing the flask with 13ml of complete IMDM, and after spinning at 400 x g for 5 minutes, cells were resuspended in fresh, complete media in a 175cm² flask.

2.3.1.2. Propagation of non-adherent cell lines
Non-adherent human cell lines were routinely cultured in complete RPMI-1640, (Roswell Park Memorial Institute Media, Sigma-Aldrich) with 10% FBS. SupT1 and Raji cell lines were maintained in a 75cm² flasks in 37°C incubators with 5% CO₂. Cells were passaged when the culture density was greater than 4x10⁶ cells/ml and split 1:10 twice a week with fresh complete RPMI

2.3.1.3. Propagation of Expi-CHO™ cells
The ExpiCHO ™ Expression System is designed to allow high-density transfection
of suspension ExpiCHO-S cells in a defined, serum-free medium. The system has been adapted to serum-free, high-density suspension culture using the manufacturer recommendation (Gibco). Cells were allowed to grow until a minimum density of $4 \times 10^6$ – $6 \times 10^6$ viable cells/mL at the time of subculturing. Cells were routinely maintained in 125ml vented Erlenmeyer shake flask (Corning) in 25ml of ExpiCHO™ Expression Medium (Gibco). Cells were incubated in an orbital shaker incubator (120rpm) at 37°C with ≥80% relative humidity and 8% CO₂.

2.3.1.4. Propagation of hybridoma cell lines
The rat monoclonal antibody (clone 1D3) against the mouse B cell antigen CD19 was purchased from the ATCC® collection (HB-305™). Cells were cultured in RPMI-1640 supplemented with 10% FBS, 10 mM HEPES, and 1.0 mM sodium pyruvate supplemented with 0.05 mM 2-mercaptoethanol in a 5% CO₂ incubator at 37°C. Hybridoma cells obtained from Aldevron™ after vaccination of three Wistar rats with mouse CD19 were cultured in DMEM (Gibco® Life Technologies) with 10% FCS supplemented with HFCS (Roche) and 1x Pen-Strep (Gibco®) as per manufacturer instructions.

2.3.1.5. Propagation of mouse cells
The BALB/c mouse B cell lymphoma cell line A20 (ATCC® TIB-208™) were cultured in RPMI-1640 supplemented with 10% Hi-FBS with 0.05 mM 2-mercaptoethanol and maintained at $1 \times 10^5$ and $1 \times 10^6$ cells/mL in a 5% CO₂ incubator at 37°C. A fresh spleen from a donor wild-type (WT) balc/e mouse was used to obtain fresh mouse lymphocytes. The whole spleen was manually homogenized and strained through a 0.70 μm filter in 5 ml of ACK lysis solution (Gibco® Life Technologies). After 5 min in ACK buffer, cells are washed with 25 ml Phosphate Buffer Saline (PBS, Sigma-Aldrich) and resuspended in complete media prior to activation.

2.3.1.6. Single-cell cloning by limiting dilution
For applications where a highly stable cell line was required, single-cell cloning was performed to identify a stable clone. Single-cell isolation was achieved by limiting dilution. Cells were diluted to a concentration of 1 cells/ml in appropriate culture media...
using a multi-step dilution to ensure that less than 100µl was never transferred. Cells were aliquoted in flat-bottom 96-well plate in 200µl fractions and cultured for 1-3 until a clear population had been established, which were subsequently screened for the expression of specific antibodies in the supernatant and expanded upon positive result.

2.3.1.7. Cryopreservation and recovery of cell lines
For the purpose of long-term storage and as a disaster-recovery backup source, all the generated cell lines were required cryopreservation. Cells were harvested whilst in the optimal exponential-growth phase. Following centrifugation, cells were resuspended at 5x10^6 cells/ml (10x10^6 cells/ml for Expi-CHO cells) in cryopreservation medium (90%FBS with 10% dimethyl-sulfoxide (DMSO)) and dispensed into appropriate cryovials (Corning) into 500ml aliquots. Cryovials were then placed into a “MrFrosty” freezing-device containing an isopropanol bath to slow down freezing to 1°C/min when transferred to a -80°C freezer. The following day, cryovials were then moved to a liquid nitrogen tank for long-term storage. As the cryoprotectant DMSO is highly toxic to cells, and it is imperative to minimise DMSO exposure during the thawing procedure. The cryovials were rapidly thawed in a 37°C water bath with agitation until no lumps of ice were visible. Once thawed, cells were immediately washed in 25ml of pre-warmed media, followed by centrifugation at 400x g for 5 minutes. Cells were then resuspended in a small volume of complete media (3ml) in a T25cm² flask and transferred to a 37°C incubator.

2.3.2. Transient transfection of 293T cells
2.0 x 10^6 293T cells were plated into 10cm-dishes (Corning) in a 10ml cell culture 24 hours prior to transfection. The following day optimal Cellular density was assessed by microscopic inspection, approximately 40-50% confluent dish. The transfection mixture was prepared with 30µl of GeneJuice (Merck) and 470µl of plain RPMI for each individual transfection. The mixture was incubated for 5 minutes prior to the addition to the DNA. 12.5µg of plasmid DNA was used and incubated at room temperature for 15 minutes with the transfection mixture. Afterward, the transfection was performed dropwise over each 293T cells dish.
2.3.3. Transient transfection of Expi-CHO cells for protein production

For optimal transfection of high-density suspension Expi-CHO cultures, the manufacturer protocol for ExpiFectamine ™ CHO transfection (Gibco) is specifically formulated to enable transfection without the need to change or add media to maximize protein concentration before purification. The standard protocol for transfection requires a total of $1.5 \times 10^8$ live CHO cells in exponential-phase of growth cultured in 25ml of ExpiCHO Expression Medium (Gibco) in a 125ml vented Erlenmeyer shake flask (Corning) and maintained in an orbital shaker incubator (120rpm) at $37°C$ with $\geq80\%$ relative humidity and $8\%$ CO$_2$. On the day of transfection, the procedure was carried out as per manufacturing protocol (Gibco). 20µg of sterile DNA were used for protein production, and supernatant harvested 8 days post-transfection.

2.3.4. Retroviral work

2.3.4.1. Retrovirus production in 293T cells

The delivery of the transgene of interest to permanently integrate into the host’s genome was achieved by using a non-replicant competent virus. A gammaretroviral packaging system expressed by co-transfection of 293T cells with an SFG vector containing the transgene, an envelope helper-plasmid encoding RD114, and the vector containing the packaging and integration signals, pEQ-Pam3-E encoding the genes Moloney Murine Leukaemia Virus (MMLV) and gag-pol genes. $2.0 \times 10^6$ 293T cells were plated into 10cm dishes as per transient transfection protocol. The triple transfections were performed by mixing the required plasmids’ DNA at an established ratio for each plasmid for a total of 12.5µg of DNA: 3/8 gagpol, 2/8 RD114, and 3/8 transgene vector. The supernatant was harvested at 48 hours and stored at 4°C for immediate use or aliquoted prior to “snap-freeze” in an ethanol bath to destroy every remaining cell and release all the viral particles. The supernatant was then stored at -80°C until use.

2.3.4.2. Retrovirus production in Phoenix-ECO packaging cells

The Phoenix-Ecotropic (ECO) cell line expresses the ecotropic envelope protein and is a second-generation retrovirus producer cell line for the generation of helper-free virus. Phoenix-ECO cells are a derivation of 293T cells that have been transformed with Gag-pol and hygromycin as the co-selectable marker while the envelope proteins were
inserted with diphtheria resistance as the co-selectable marker. 2.0x10^6 Phoenix-ECO cells were plated in 10ml of complete media into 10cm tissue culture treated dishes 24hours before transfection. Cellular confluence was assessed by microscopic examination to ensure optimal density, and the transfection protocol was carried out using the same procedure of standard 293T cells. 2.6µg of pCL-Eco49 were used as packaging plasmid combined with 4.7µg of the transgene vector (SFG or SFGmR). 24 post-transfection media was replaced with 5ml of complete RPMI-1640 with 10% FBS, and the following day supernatant was harvested prior to “snap-freezing” in an ethanol bath and storage at -80°C. Higher transduction efficiency was obtained using only 48 hours-harvested supernatants.

2.3.4.3. Retronectin coating preparation of tissue culture plates
RetroNectin (TakaraBio) has been reported to colocalise virion particles and cells, resulting in increased transduction efficiency for suspension cells299. RetroNectin plates were prepared a minimum of 24 hours before viral transduction by coating each well of a non-tissue culture 4-well plate with 500µl of PBS containing 4µl of RetroNectin. These plates were sealed with parafilm and stored at 4°C with the RetroNectin solution, reused a maximum of two repeats by direct transfer to fresh plates and stored as above until required.

2.3.4.4. Retroviral transduction of suspension human cell lines
Cells were harvested, counted, and resuspended at a concentration of 6x10^5 cells/ml. 500µl of this cell suspension was transferred to each well of the RetroNectin coated plate 1.5ml of retroviral supernatant was added to each well, with cells spin-transduced at 1000 x g for 40 minutes before returning to the incubator for 2 days before moving to a tissue-culture treated 24-well plate for expansion before assessing transduction efficiency by flow cytometry.

2.3.4.5. Retroviral transduction of suspension mouse cells
Mouse cells (primary cells or A20 cell line) were harvested, counted, and resuspended at a concentration of 2x10^6 cells/ml directly in neat virus obtained in Phoenix-ECO cells. A RetroNectin coated plate was blocked with 2ml of PBS 2% BSA (w/v) for 30
minutes and washed twice times with PBS prior to the addition of the cells. Plates were then spin at 800 x g for 90 minutes at 32°C and returned to the incubator overnight. The following each well was filled with 1.25ml of complete RPMI-1640 (10%FBS, 0.1mM β-mercaptoethanol, 10mM HEPES (Sigma-Aldrich)) and 100U/ml of IL-2 in case of primary mouse cells. Two days later, transduction efficiency was assessed by flow cytometry and cells were plated in a tissue-culture treated 24-well plate for expansion or in the case of primary cells used for in vivo experiments.

2.4. Flow cytometry

Flow cytometry experiments were performed using BD LSR Fortessa (BD Biosciences) or Cytoflex (Beckman Coulter) instruments. The information of all the antibodies and concentrations used throughout the project are summarized in Table 2-1.

<table>
<thead>
<tr>
<th>Application</th>
<th>Antigen</th>
<th>Fluorophore</th>
<th>Clone</th>
<th>Manufacturer</th>
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<td></td>
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<td>BV785</td>
<td>2C11</td>
<td>Biolegend</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>CD19</td>
<td>BV421</td>
<td>1D3</td>
<td>BD Biosciences</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>THY1.1</td>
<td>APC</td>
<td>H1551</td>
<td>eBioscience</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>CD34</td>
<td>FITC</td>
<td>RAM34</td>
<td>Biolegend</td>
<td>1:100</td>
</tr>
</tbody>
</table>

Table 2-1. Specifications of the antibody clones used. All the antigens for in vitro and in vivo staining refer to a mouse antigen. Stainings were performed in 100μl volume and the different required antibodies were pre-mixed together to ensure minimal pipetting errors.
2.4.1. General staining protocol

Approximately $2.5 \times 10^5$ cells were typically used for antibody staining. Live cells were counted (Neubauer chamber) and washed in phosphate-buffered saline (PBS) before adding the specific antibodies. Cells were spun down at 400xg for 5 minutes, and the wash solution was decanted. Then, cells were resuspended in 100µl of PBS containing the appropriate dilution of antibodies and live/death staining. After 30 minutes of incubation at room temperature, cells were washed and resuspended in 200µl PBS and placed on ice until flow cytometry acquisition. In some experiments, different or unknown numbers of cells were stained, and the details are indicated within each experimental section. Where multiple staining steps were required (i.e., unconjugated primary Ab and a fluorescent secondary antibody), samples were washed with PBS between individual staining steps. Isotype and/or non-transduced controls were included as a control to establish an appropriate comparison and gating. Mouse-derived cells were stained in the presence of mouse Fc-blocking receptors (Milteni), and in the case of primary mouse cells, these were pre-treated with ACK lysis buffer (Gibco) to remove red cells, washed with PBS, and resuspended in the staining mixture.

2.4.2. Staining for intracellular antigens

Intracellular staining of proteins requires cells to be fixed and permeabilised prior to staining. Stainings were all performed in a V-bottom 96-well plate. Following extracellular staining as above, cells were fixed and permeabilised by incubation of cell pellet in 100µl of cytofix/cytoperm solution (BD) for 10mins. After removing the fixative solution by centrifuging the cells at 800xg for 3 minutes, cells were washed with 100µl of wash solution (BD, Perm/Wash), then incubated with the appropriate antibodies for 20mins in Perm/Wash solution. Afterward, cells were washed twice by centrifuging at 800xg for 3 minutes in 100µl of perm/wash solution. Finally, cells were resuspended in 100µl of PBS and kept in the dark on ice until flow cytometry acquisition.

2.4.3. Compensation

Due to the nature of flow cytometry experiments, a multi-fluorophore staining carries the risk of spillover of the fluorophores’ emission into adjacent detection channels for similar emission-wavelength (i.e., APC and APC-Cy7). In order to control for this,
compensation may be required to ensure subtraction of the spillover emission from the appropriate channels. Compensation was undertaken using single-stained compensation beads (UltraComp beads, eBioscience). Beads are formulated to contain positive (capture rodent antibodies) and negative (do not react with antibody) populations. The compensation matrix for each experiment was calculated automatically using FACS Diva software (BD) and applied to all the samples during acquisition.

2.4.4. Normalisation using Counting Beads
CountBright™ (Invitrogen™) were vortexed for 1 minute before use, and one drop of beads was added in each staining condition. The use of counting beads allows the enumeration of cells by normalization of the number of cells acquired for every bead: 

\[
\text{[(cell's events / total bead's events) * average bead's events across all samples tested].}
\]

2.4.5. Flow cytometry-based sorting
Virally transduced cells were expanded to obtain at least 2x10⁶ live cells prior to cell sorting. Cells were stained in PBS with the appropriate antibody as per general protocol and resuspended in PBS/1% FBS. FACS-AriaIII (BD) was operated by UCL-Cancer Institute Core Flow Service and sorted cells collected into sterile tubes containing 100% filtered-FBS. Cells were subsequently recovered by PBS washing and resuspended in a small volume of complete media supplemented with 100µg/ml of broad-spectrum antibiotic Normocin (Sigma-Aldrich) in a 24-well plate until recovery cell growth.

2.4.6. Cytokine bead array
Due to the small sample available during mouse experiments, the use of a multiplex platform to simultaneously detect diverse cytokines was necessary. Cytokine bead array assays (CBA) uses fluorescence–encoded beads suitable for the analysis of various cytokines. In this project, 13 cytokines were simultaneously detected in mice’s plasmas using the LEGENDplex™ mouse inflammation panel kit (Biolegend). This panel allows simultaneous quantification of IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β, IFN-γ, TNF-α, and GM-CSF. Most cytokines in this panel are produced by innate immune cells, linking the innate and adaptive immunity and/or
bystander cells. The assay was carried out as per manufacturer instructions, and each sample was run in duplicate using 25µl of a 1:3 diluted plasma sample.

2.5. In vitro assays

2.5.1. Mouse lymphocytes activation and transduction
Freshly obtained lymphocytes from a balb/c mouse spleen were counted and resuspended at 1x10^6 cells/ml in RPMI-1640 with 10% FCS, 0.05 mM 2-mercaptoethanol, 10 mM HEPES supplemented with 1ng/ml IL-7 and 2µg/ml Concanavalin A (Sigma C5275) for lymphocytes activation (day 1). The following day cells were harvested, and the transduction protocol was carried out using retrovirus produced in Phoenix-ECO cells as described above. On day 5, cells were harvested to assess transduction efficiency by flow cytometry and used for in vivo experiments or alternatively transferred in a T75cm^2 flask at 2x10^6 cells/ml in complete media with IL-2 100 U/ml and cells used two days later for in vitro assays.

2.5.2. Cytotoxicity assay
Activated and CAR-transduced mouse lymphocytes were co-cultured together with target cells in complete RPMI-1640 media for 48h. Target and effector cells were resuspended at 1x10^5 cells/ml and combined with target cells at the desired effector to target ratio (E:T), keeping constant 5x10^4 target cells. Each condition was plated in triplicate. Cells were then spun at 400g for 5min, and the supernatant was transferred to a fresh plate and stored at -20°C for cytokine quantification. The cells were stained with 1:1000 viability dye (e780, Biolegend), target cells were stained for the marker gene (indicated within the text), and counting beads were added as described in the previous section. The 96-well plate was run through automated flow cytometry (CytoFlex, Beckman-Coulter) and analysed using FlowJo software (BD). The numbers of residual cells were normalized against the same number of counting beads, and the average of each replicate was compared to non-transduced lymphocytes to estimate the percentage of the specific killing of the target cells.
2.5.3. Cytokine release assay

Mouse IFN-γ and IL-2 were both quantified using the ELISA MAX™ kits (Biolegend, 431804, and 430104, respectively). Supernatants were stored at -20°C until required, thawed at RT, and used at 1:10 and 1:20 dilution respectively in assay-diluent buffer (Biolegend) to ensure adequate overlap with the standards provided in the quantification kit. The standard curve was generated by the optical density (OD) of the serially diluted standard proteins of known concentrations. Quantification was obtained from the interpolation of unknown samples with the standard curve to calculate the concentrations of the target proteins. The ideal assumption is that the best fit for the standard curve will be a line that passes as close as possible to all data points from the standard curve. All the ELISA data were fitted using linear regression equations, including only the points falling in the linearity range of the assay, resulting in $R^2 > 0.98$ as an estimate of the “goodness” of the fit [$y = mx + b$].

2.6. In vivo work

2.6.1. Experimental animals

Wild-type balb/c mice were purchased from Charles River (UK) and kept in IVC cages at Cruciform BSU (UCL) in the first two experiments, while the last two were performed in Centre of Advanced Biomedical Imaging (CABI) animal facility to access the use of both irradiator and IVIS Spectrum machines. Mice were allowed a week of acclimatisation before any procedure. The experiments were carried out under the project licence PPL12570 (70/9032), protocol 3, with moderate severity. The operator (PIL: I79BD4993) was adequately trained for all the procedures performed and supervised during i.v injections.

2.6.2. Intravenous injection and tail bleeding

Two main types of injections have been used thought the experiments. Intra-peritoneal injection (i.p) and intra-tail vein (i.v). The procedures were performed using 1mL syringe (BD Falcon) and 16Ø gauge needles (BD, Falcon). I.p injections have been used to deliver a maximum of 200µl volume of lymphodepleting chemotherapy (150 mg/kg cyclophosphamide or 50mg/kg Busulfan, Sigma-Aldrich) or substrate for bioluminescence imaging (luciferin 1mg/ml). Either tumour or CAR T-cells have been
injected i.v through the tail vein of the mice in a maximum volume of 200 µl of sterile-filtered PBS. Bleeding has performed by a small cut with a scalpel on the tail vein and the drops harvested in heparin-coated tubes (SARSTEDT, 16.443) for a maximum of 100 µl per mouse every 10 days. Blood was directly lysed with ACK lysis buffer (Gibco® Life Technologies), washed with PBS, and used for flow cytometry analysis or to separate the plasma for cytokines quantification.

2.6.3. Bioluminescence imaging of mice
Tumour engraftment and progression were monitored using tumour cells expressing Firefly luciferase (MP24905.SFG.Fluc_5xRed.Thy1.1) for in vivo tracking by bioluminescent imaging (BLI). The mice were anesthetized using Isoflurane (Pyramidal) and received 200µl of luciferin substrate by i.p injection. After 10 minutes, mice were imaged as many as five at a time in an IVIS Spectrum machine (Perkin Elmer). The images were subsequently analysed using the software LivingImage 3.0 (Perkin Elmer) to extrapolate the images and the radiance of each mouse. The data were plotted and analysed using GraphPad 9.2

2.6.4. Monitoring and euthanasia of experimental animals
Mice were monitored daily for evidence of poor health conditions, including a lack of grooming, hunched posture, and piloerection. Animals were weighed daily when possible and the temperature was monitored daily for signs of fever using a digital rectal probe (Bioss). Animals were culled if found not to be in satisfactory condition and according to protocol 3 of PPL 12570. At the time of the sacrifice, mice were euthanised according to schedule 1 method, which includes raising the concentration of C02 followed by cervical dislocation.

2.6.5. Tissues harvest and processing
2.6.5.1. Spleen
The spleen was removed and transferred to sterile PBS prior to cellular harvest. The spleen was macerated with gentle pressure at the top of 70µm cell strainer before red cell lysis with 5ml of ACK lysis buffer for 5mins. Lysis was neutralized and washed by addition of 25ml of PBS before filtration through a 45µm cell strainer to remove cell
clumps. Cells were subsequently centrifuged and resuspended in a PBS solution for downstream use.

2.6.5.2. Bone marrow

Both femur and tibia were removed from the left flank and transferred to chilled PBS pending cellular harvest. The ends of the femur were removed by scissors, and the contents of the bone were obtained by centrifugation at 1000xg in a homemade funnel inserted in a microfuge tube. The whole bone marrow samples were then resuspended into 50µl of ACK lysis buffer and incubated at room temperature for 5 minutes. Red cell lysis was then neutralized and washed with 250µl of PBS and spun at 400xg for 5 minutes. Cells were resuspended in 100µl and transferred in a V-bottom 96-well plate for downstream flow cytometry staining.

2.6.5.3. Whole blood

Whole blood was obtained from tail bleeding of each mouse or cardiac puncture after euthanasia and collected in heparin-coated capillary tubes. Approximately 100µl were collected and processed to obtain plasma for cytokine quantification. Samples were spun directly in the heparin tubes at 1200xg for 15 minutes in a pre-chilled centrifuge at 4°C. The plasma was collected and stored at -80°C until used for the cytokines’ quantification. The remaining cell pellet was lysated in 50µl of ACK buffer for 5 minutes and washed with 250µl PBS prior to flow cytometry staining.

2.6.5.4. Flow cytometry gating strategy for mouse tissues

Single-cell suspensions from mouse tissue were run on BD LSR Fortessa using the antibodies and described in table 2-1. Stained cells were initially gated to obtain single events by a 45° line in the FSC-H vs FSC-A. Viability dye-negative cells were subsequently gated to separately obtain CD45+ and CD11b+ cells. The CD45+ lymphocytes were further gated to obtain CD3+ T cells and non-tumour CD19 positive cells. Of the CD3+ cells, the marker gene mCD34 was used to derive the percentage of CAR T-cells, while the marker gene mTHY1.1 was used to determine the percentage of tumour cells within the CD19 positive gate.
2.6.6. Statistics

All statistical analyses were performed in GraphPad Prism v9.2. Unless otherwise stated, data were expressed as mean ± SEM. Statistical analyses for both in vitro and in vivo assays were undertaken by 2-way ANOVA with donor matching and Turkey post-test correction for multiple comparisons or 2-tailed student t-test, as indicated in figure legends. Statistical significance was indicated as follows in the figures:

****p<0.0001; ***p<0.001; **p<0.005; *p<0.05; and NS, non-significant (p>0.05).

3. Isolation and binding characterization of the anti-mouse CD19 clone 1D3 and its testing as mouse chimeric antigen receptor structure

3.1. Introduction

Tumour antigen recognition and engagement by the CARs results in activation of CAR T-cells, cytolysis of tumour cells, and release of cytokines. The cytokines subsequently promote the rapid expansion of CAR T-cells, followed by their memory differentiation. Although there has been a general understanding of the mechanism of action for CAR T-cells, the impact of structural key determinants influencing the rate and extent of CAR T-cell activity remains poorly understood. In addition, the interactions regulating CAR T cell activity and tumour regression in vivo are not fully recapitulated. For instance, it has been shown the importance of the scFv-affinity for its cognate target. Typically, scFvs used for CAR T cells structures have an affinity several orders of magnitude greater than the natural TCR. This has led several groups to elucidate the role of the binding domain in CAR structures, but the conclusion remains that empirical testing of scFv domains combinations is necessary to determine optimal CAR structures. Moreover, whether tumour regression is primarily the result of direct CAR T cell killing or whether the engineered T cells act by activating the host immune cells at the tumour site is another key question to be addressed. In 2010, two groups utilized an anti-mouse CD19 CAR to prove the efficacy of a 1st generation and 2nd generation (CD28ζ) CAR structure in two different mouse models of B cell tumours. Both the groups have designed their binding domain based on the only available anti-mouse CD19 monoclonal antibody clone (1D3), and similarly, they have shown tumour regression with prolonged B cell aplasia. Later the same group showed that 1D3-based CD28ζ CAR in BALB/c mice could result in rapid
toxicity potentially caused by elevated Th$_1$ cytokine levels (i.e., IFN-$\gamma$, TNF, and IL-2). Moreover, they have shown long-term expansion of CAR T-cell associated with prolonged production of Th$_2$ cytokines and have seen no differences based on preconditioning used or dosage, thus replicating some of the toxicity observed in humans. On the other hand, other studies employing the same anti-mouse CD19 CAR have observed no toxicity in different models of B cell tumours$^{154,95,49}$. Altogether, these results highlight the importance of the syngeneic model to study the effects of CAR T-cells in an intact immune system and show the limitation in understanding the CAR activity mechanism. In summary, CAR T cell killing dynamics have been only extensively analysed in vitro, limiting our understanding of the quantitative and qualitative requirements for optimal CAR T cell activity in vivo.

3.2. Aims

The work presented in this chapter aimed to generate and characterize the binding affinity of the anti-mouse CD19 scFv fragment 1D3 and its testing as 2$^{nd}$ generation mouse CAR structures.

3.3. Results

3.3.1. Isolation of the antibody sequence of the anti-mouse CD19 clone 1D3 from monoclonal hybridoma cells

The monoclonal antibody clone 1D3 against mouse CD19 is deposited as rat hybridoma on the ATCC cell bank, while the DNA sequence of the variable heavy and light chains is not available in the literature. The 1D3 hybridoma clone was obtained by fusing NS-1 myeloma cells with spleen cells obtained from Lewis rats immunized with transfected K562 cells expressing the extracellular domain of mouse CD19 and is an IgG2a, kappa rat isotype.

To determine the DNA sequence of the two-variable antibody ‘chains, the total RNA was extracted from three different aliquots of expanded 1D3 hybridoma cells. The quality and concentration of RNA were determined by capillary electrophoresis prior to performing the RT reaction for the conversion into cDNA (Figure 3-1A). An RNA integrity number (RIN) greater than 8 is considered good quality RNA, with 100-300ng/µl being the optimal concentration range for the subsequent RT reaction. The aliquot of RNA obtained from $1 \times 10^6$ hybridoma cells was used converted into cDNA.
and genomic DNA contamination from the RNA extraction was assessed by amplification of the housekeeping gene GAPDH. The presence of only a 600bp product showed no genomic DNA contamination by the lack of a bigger PCR product (900bp) containing the genomic intronic sequences (Figure 3-1B).

The 5’RACE protocol was performed as described in the method section, after the addition of the poly-C tail at the 5’ end of the cDNA. This was used for the Nested PCR to separately amplify the heavy and kappa-light chains using outer primers mapping on the 5’ poly-C tail and gene-specific at the 3’. The purified PCR products were then used as a template for the subsequent amplification using inner primers. An initial annealing temperature of 65°C and 1µl template were used to amplify the two chains, resulting in only the LC being successfully amplified. A second attempt using 61 annealing temperatures and two different amounts of template (1µl and 3µl) effectively amplified the HC as well (Figure 3-2A). After amplification of the HC and LC, the PCR products were cloned into a TOPO-TA vector and used to transform bacteria. Insert-positive white colonies were individually picked, and the DNA extracted for DNA sequencing. Approximately twelve colonies per chain were sequenced, and the HC and LC were aligned to the IMGT germline reference database. This confirmed the legitimacy of the sequences as rearranged Rattus Norvegicus variable chains. The genes were further aligned within each other to determine the functional consensus sequence of the two variable chains and to exclude potential point mutations that occurred during PCR amplification (Figure 3-2B).

**Figure 3-1.** RNA extraction and cDNA conversion from 1D3 hybridoma cells. [A] RNA quality and concentration assessment using the BioAnalyzer capillary electrophoresis. [B] PCR amplification of rat GAPDH gene at three different concentrations of cDNA to determine genomic DNA contamination.
The HC and LC sequences of the 1D3 clone obtained from the 5’RACE PCR were cloned into two antibody formats in an SFG gammaretroviral expression vector. A chimeric full human antibody was generated by cloning the HC and LC separately fused with human IgG1 and human kappa constant regions. Instead, a single-chain variable fragment format was generated by fusing together the two chains spaced by three repeats of GGGS linker in-frame with the mIgG2a-Fc portion. The mouse kappa-light chain signal peptide was used for the correct and high-efficiency secretion of the antibody fragment in the cell’s media (Figure 3-3).

Figure 3-2. Nested PCR and sequence alignment of the 1D3 heavy and light chains. [A] Agarose gel of the PCR products obtained from the second amplification of the Nested PCR. On the left the successful amplification of the 600bp product of the LC and on the right the amplification of the HC gene at 61°C annealing temperature using 1µl and 3 µl of template respectively. [B] Determination of the consensus sequence of the HC and LC sequence alignment.
Figure 3- 3. Schematic representation of the plasmid’s expression cassette. Three different plasmids were generated using the HC and LC of the 1D3 clone. These were cloned separately in-frame with human IgG1 and kappa constant-regions respectively to generate the chimeric full human mAb using the native human signal peptide. The two chains were fused together and spaced with 3XGGGS linker for the generation of the scFv-mIgG2a-Fc format. eGFP or eBFP were used as marker genes for transfections.

The generated plasmids were expressed in HEK293T cells, and specific binding on mouse CD19 was assessed by flow cytometry. 293T cells were transiently transfected with a plasmid encoding for mCD19 with defective endodomain to abrogate signal transduction and co-expressed with human CD34 as a marker gene. Control of binding and transfection was determined by staining 293T NT and mCD19 positive cells using the commercial anti-mouse CD19 antibody and anti-human CD34 for the presence of the marker gene (Figure 3-4A).
The supernatant containing either the human 1D3 mAb or the 1D3scFv-mIgG2a-Fc formats was stained using a fluorophore-conjugated secondary antibody specific for the constant region of the antibody tested. Both formats generated for the HC and LC of 1D3 clone, specifically stained mouse CD19 positive cells and not NT 293T cells (Figure 3-4B-C).

3.3.2. Kinetic characterization of 1D3 clone

The measurement of binder kinetics was performed using surface plasmon resonance (SPR) technology on a BiacoreX100 system. This instrument allows measurement in real-time of interactions occurring between two molecules to determine the strength of the binding, as association and dissociation rate for the calculation of the affinity. A ligand, typically an antibody, is immobilized on the gold surface of a Biacore chip, the analyte of interest (antigen) flows over the surface at a series of different concentrations. The instrument measures the angle and the intensity of the reflected

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Figure 3- 4. Binding test of 1D3 clone on 293T cells transfected to express mouse CD19. [A] Transfection efficiency assessment by flow cytometry of 293T cells expressing mCD19. Cells were double stained with amCD19-FITC and QBEND10-APC for the marker gene. [B] Supernatant containing 1D3 either as full antibody fused with humanFc (H+L-HuFc) or [C] as scFv fused with mouseFc (scFv-muFc) was used to stain transfected 293T cells and binding was detected with secondary antibodies anti-humanFc-V405 and anti-mouseFc-PE respectively.
light generated from the changes in the association and dissociation between ligand and analyte over time (Figure 3-5A).

The known instability of mouse CD19 protein when expressed in soluble format outside the cells requires the implementation of an alternative strategy to successfully characterize the 1D3 binding kinetic for mCD19. A soluble format of mCD19 protein fused with 6 histidines (HisTag) was immobilized on the surface of an NTA-cm5 Biacore chip directly from crude 293T supernatant. These allowed enhanced protein capture without the complications of protein loss during purification or storage observed with other approaches.
The Fab portion of chimeric human 1D3 mAb was used as an analyte at a known concentration to determine the kinetic profile of the clone (Figure 3-5B). The endodomain-truncated mouse CD19 protein was expressed in SFG vector in frame with the HisTag and with eBFP as a marker gene for transfection (Figure 3-6A). This was expressed in 293T cells and used fresh to coat the surface of an NTA biacore chip for the capture of histagged-proteins (Figure 3-6B). A purified version of the chimeric human 1D3 mAb was digested with papain to obtain just the Fab fragment. This process allowed the study of the kinetics, minimizing the avidity effect of a tetravalent full mAb and of background/non-specific interaction with the NTA chip. The successfully digested Fab fragment was confirmed on an SDS-page gel stained with Coomassie blue and showed no evidence of the Fc-domain of the mAb in the Fab lane (Figure 3-6C). The digested Fab fragment was then quantified using a BSA standard curve prior to the Biacore run (Figure 3-6D). Of the two flow channels available on the BiacoreX100, one was required as a reference channel, and the second one was used to capture mCD19 from transfected 293T cell supernatant. The capture levels between the various cycles of the machine run confirmed the rapid decay of the
protein in the supernatant. Over the course of 6 total cycles for a total of 3.5 hours, the capture levels decreased by approximately 10% (Figure 3-7A). Purified 1D3 Fab was used as the analyte in a sequentially injected series of 5 1:3 dilutions from 3.7nM to 300nM. The data were double reference-subtracted for the reference flow cell, and the blank runs and the processed curves were locally fitted to a 1:1 Langmuir binding model. The kinetic values measurement identified a high-affinity clone in the low-nanomolar range with a slow on and off rate (Figure 3-7B).

**Figure 3- 7. Kinetic measurement of 1D3 clone using BiacoreX100.** [A] Capture levels in response unit (RU) for mouse CD19 ligand protein on the surface of NTA chip over the course of the single cycle kinetic run. In green the start-up cycle of the machine and in red the capture levels for the sample runs. [B] 1D3 Fab was injected as analyte at 5 different concentration (3.70 nM, 11.11 nM, 33.33, nM, 100 nM and 300 nM). Double-reference subtracted sensorgram was fitted with the 1:1 Langmuir binding model showing a high affinity binder in the nanomolar range of KD.
3.3.3. *In vitro testing as second-generation mouse CAR*

The characterized anti-mouse CD19 clone 1D3 scFv was used to construct a full mouse 2\textsuperscript{nd} generation CAR structure to be used in the development of a B cell-malignancy immunocompetent mouse model. The design of CAR architectures is constantly refined, with three generations of CARs in which modulation of the endodomains is the focus for many research groups worldwide. In addition, fourth-generation CARs are also designed for constitutive secretion of cytokines to enhance cytotoxic and proliferation functions of CAR T-cells.

The most widely used endodomains adopted in pre-clinical and clinical settings are CD28 and 4-1BB fused with the CD8 stalk and transmembrane regions to create a 2\textsuperscript{nd} generation CAR structure. In our system, the mouse version of these two co-stimulatory domains fused with anti-mouse CD19 scFv (1D3) has been used to create a fully mouse second-generation CAR which also includes mouse CD8 stalk and transmembrane domains, and mouse CD3 zeta(ζ) (Figure 3-8A). In our lab, a 2\textsuperscript{nd} generation CD28ζ mouse CAR against EGFRvIII was already deposited in our plasmids archive and extensively characterized in a glioma mouse model.

The above plasmid backbone (SFGmR) is a variation of our standard gammaretroviral SFG plasmids with the addition of a scaffold/matrix-attachment region (S/MAR) known to improve long-term stability for the expression of the transgene. Along with the CAR structure, the plasmid backbone included a truncated version of mouse CD34 as a marker gene, in-frame with the T2A self-cleavage peptide for the expression as bicistronic transcript (Figure 3-8B).
To test the functionality of 1D3 in our CAR structure, I engineered mouse spleen-derived lymphocytes to express the construct, and these effector cells were challenged against mouse CD19 positive cell lines for the assessment of specific cytotoxic abilities and cytokine release potential.

While the CD28ζ architecture for the mouse CAR structure had already proved functional *in vitro* and *in vivo*, the corresponding 4-1BB version of this 2nd generation CAR hadn’t been generated and tested.

Although it’s outside of the primary goal of this project, a direct comparison of two different co-stimulatory domains as mouse CAR structure hasn’t been reported yet.
In order to be able to compare the two different co-stimulatory, the mouse signalling portion of 4-1BB domain was synthesized as Gblock™ (NM_001077509.1) and replaced mCD28 in the structure described above.

The functionality of the generated 4-1BBζ CAR was initially compared to the same CAR structure harbouring a known functional scFv (4G7) specific for the human version of CD19. These two CARs were compared against the mouse B cell line A20 modified to express our in-house marker gene (RQR8) and against Raji cells naturally expressing hCD19 and modified to express mCD19 (Figure 3-9A). Mouse lymphocytes obtained from a whole spleen were engineered to express the two CARs, and the transduction efficiency was assessed by flow cytometry. Live mouse lymphocytes cells gated on CD3+ cells and stained for the mCD34 marker gene demonstrated a high level of transduction, 74% and 67% for 1D3- and 4G7-CARs, respectively (Figure 3-9B).
The CAR-engineered effector cells and the target cells were used for a co-culture cytotoxic assay to determine the killing abilities and cytokine release potential of the mouse 4-1BBζ structure. Cells were plated at two different effectors to targets (E:T) ratio (1:1 and 1:2), and after 48h, cells were stained for the flow-cytometry-based killing assay and the supernatant used to assess the IFN-γ production as a measurement of T cell activation and cytokine release. Live cells gated were stained for the mouse effector cells using an anti-CD2 mAb, and the negative target cells were gated separately for the estimate of the number of residual cells, each condition was performed in triplicate (n=3) and normalized using counting beads, and the percentage of the live target was obtained by comparison with the NT-effector cells conditions (Figure 3-10).

Figure 3-9. Mouse CD19 positive target cell lines and CAR expression. [A] The mouse cell line A20 naturally expressing mouse CD19 were engineered to also include expression of a marker gene (RQR8), while the human cell line Raji was modified to express mouse CD19. These sorted target cell lines were stained for surface expression of mouse CD19 and compared against isotype control or non-transduced cells (NT) in both A20 and Raji cells respectively. [B] CAR transduction efficiency of mouse splenocytes, the expression of the intracellular marker gene (mCD34) was used to detect the level of expression of each CAR compared to NT cells.
The 1D3 CAR demonstrated good cytotoxic ability against A20 and Raji mCD19 cells at both 1:1 (p=0.001285 and p=0.006662) and 1:2 E:T ratios (p=0.008236 and p=0.001533) with an average percentage of live target cells of 11.9%, 30.9%, 15.5% and 70.9% respectively compared to 63.1%, 88.5%, 72.9% and 86.8% using the 4G7-based CAR. Raji cells are hard to kill, as reflected by 4G7 CAR being only able to modestly kill these target cells with 88.4% and 93.1% Raji NT cells left at 1:1 and 1:2, respectively. The 1D3 CAR version performed better against Raji cells at both the E:T ratio with 30.9% and 70.9% target cells left. No background toxicity was observed on mCD19 negative Raji cells (Figure 3-11A). Excellent cytokine release measured as IFN-γ production was observed for both 1D3 and 4G7 CARs in the presence of target-positive cells. 1D3 CAR produced significantly more cytokine compared to 4G7 against A20 and Raji mCD19 cells at both the 1:1 and 1:2 ratios with averages of 5055 pg/ml, 77988.3 pg/ml, 6699.8 pg/ml and 63373.8 pg/ml respectively using the 1D3-based CAR compared to 1899.9 pg/ml, 48139.6 pg/ml, 1591.9 pg/ml and 46956.1 pg/ml with the 4G7-based CAR (p= 0.001472, p= 0.003808 and p= 0.000124, p= 0.005157 respectively). Although no killing was observed, 4G7 CAR showed high levels of IFN-γ production.
γ release against Raji NT at both E:T ratios tested (p= 0.003459 and p= 0.000049) with averages of 52850.8 pg/ml and 52540.8 pg/ml at 1:1 and 1:2 E:T ratios respectively compared to 1613.6 pg/ml and 741 pg/ml using the 1D3-based CAR. This confirmed T cell activation in the absence of target positive cells and no significant difference in the background was observed for both 1D3 and 4G7 CARs in the absence of target cells (Figure 3-11B).
The significant difference in cytokine levels observed in the A20 and Raji target cell lines was probably due to the greater antigen density of the mCD19- engineered Raji cells compared to A20 cells, naturally expressing the antigen. In addition, the engineered Raji mCD19 expressed higher levels compared to human CD19 antigens, which might have contributed to the differences observed between 1D3 and 4G7 CARs. The generated 4-BBζ CAR using 1D3 scFv to target mouse CD19 has shown specific killing abilities with superior performance to the human counterpart 4G7 scFv when used in mouse architecture and mouse cells.

The next step was to compare the two mouse costimulatory domains CD28 and 4-1BB, to identify any major differences in killing performance prior to in vivo experimentation. Further cytotoxicity and cytokine release assays were performed using A20 cells expressing a balb/c mouse congenic marker gene (THY1.1.). This mitigates for the potential immunogenicity of using human-derived proteins as marker genes for in vitro and in vivo experiments as previously used. Moreover, these cells were further engineered using the CRISPR/Cas9 system to abrogate the surface expression of mCD19 and to be used as negative control for specific killing (A20 KO). As Raji are hard to kill, Supt1 cells instead represent an alternative target cell line to be used in this type of cytotoxic assay. Supt1 cells were virally transduced to overexpress mCD19 resulting in higher target density on the cell surface as demonstrated by the one-log difference in the MFI by flow cytometry staining (Figure 3-12A). Mouse spleen-derived lymphocytes were virally engineered to express the CD28ζ and 4-1BBζ CAR constructs with comparable transduction efficiency on CD3+ T cells (72.7% and 65.7% respectively) as determined by mCD34 marker gene expression (figure 3-12B).
The engineered mouse effecter cells and the target cells were used for a direct comparison between the two costimulatory endodomains, and the cytotoxic assay was performed at four E:T ratios from 1:2 to 1:16 to increase the challenge for the CAR T-cell of eliminating the target cells. IFN-γ was assessed in the supernatant after 48h as measurement of T cell activation and cytokine release. Live cells gated were stained for

**Figure 3-12. Mouse CD19 positive target cell lines and CAR expression.** [A] A20 cells were engineered for in vivo purposes to express an engineered version of the firefly luciferase (5xFluc) alongside the mouse congenic marker gene THY1.1. A20 cells negative for mCD19 (KO) were generated by CRISPR/Cas9 and the expression compared with the anti-mCD19 isotype control. The human cell line Supt1 was also engineered to express high level of mCD19 with human CD34 as marker gene. [B] CAR transduction efficiency for both m4-1BBζ and CD28ζ-engineered mouse splenocytes, mCD3+ T cells were gated from live cells and mCD34 marker gene expression levels were compared as histograms to NT cells.
the target cells discriminating from effector by staining mCD20 on A20 cells, and hCD34 was the marker gene on supT1 mCD19+ cells (QBEND10) and were used for the estimate of the number of residual cells, each condition was performed in triplicate and normalized using counting beads, and the percentage of the live targets was obtained by comparison with the NT-effector cells conditions (Figure 3-13).

Both the CARs tested have shown great cytotoxicity against both A20 and supT1 mCD19 cells at all the E:T ratios tested with a more marked reduction in killing at higher concentrations of supT1 mCD19 cells compared to A20 WT cells. Notably, supT1 NT cells were non-specifically killed (‘background’) with both CARs tested, probably as a consequence of an allogenic reaction between human and mouse cells. No background killing was observed against A20 cells KO for mCD19 (A20 KO) and no significant differences were observed in cytotoxicity between CD28 and 4-1BB costimulatory domains with averages of live supT1 mCD19 target cells of 9.9%, 38.4%, 79.6% and 132% for the CD28 CAR, while 4-1BB CAR resulted in 21.7%, 36.6%, 83.7% and 109% of A20 WT target cells at 1:2, 1:4, 1:8 and 1:16 E:T ratios.
respectively. Both the endodomains performed better at low E:T ratios against supt1 cells with loss of cytotoxicity at 1:8 and 1:16. To a lesser extent, a similar trend was observed against A20 cells without complete loss of killing at higher E:T ratios (Figure 3-14A). The cytokine release was measured as IFN-$\gamma$ and IL-2 production over the 48h co-culture with the target cells. High levels of IFN-$\gamma$ were produced with both the costimulatory domains, with CD28 releasing significantly more cytokine compared to 4-1BB at 1:4 and 1:8 ratio of supt1 mCD19 cells ($p=0.006008$ and $p=0.001299$) with averages of $18244.8 \text{ pg/ml}$ and $16784.6 \text{ pg/ml}$ respectively compared to $9062.5 \text{ pg/ml}$ and $3272.6 \text{ pg/ml}$ using 4-1BB endodomain. High levels of IFN-$\gamma$ were also produced at high E:T ratios on A20 WT cells ($p=0.006973$, $p=0.011282$ and $p=0.018566$) with averages of $18105.9 \text{ pg/ml}$, $16797.6 \text{ pg/ml}$ and $16364.2 \text{ pg/ml}$ at 1:2, 1:4 and 1:8 E:T ratios respectively using CD28 endodomain compared to $9013.2 \text{ pg/ml}$, $6696.1 \text{ pg/ml}$ and $4101.4 \text{ pg/ml}$ using 4-1BB endodomain respectively. No significant difference was observed in IFN-$\gamma$ production on target negative cells (Figure 3-14B). On the other hand, 4-1BB consistently released significantly more IL-2 than CD28, both against supT1 mCD19 at 1:2, 1:4 and 1:16 E:T ratios ($p=0.002949$, $p=0.005546$ and $p=0.001898$) with average IL-2 of $7238.6 \text{ pg/ml}$, $5919.6 \text{ pg/ml}$ and $2929.8 \text{ pg/ml}$ respectively compared to $1687.9 \text{ pg/ml}$, $1953.5 \text{ pg/ml}$ and $950.2 \text{ pg/ml}$ using CD28 endodomain. A similar trend was also observed at the same ratios on A20 WT cells with averages of $4882.4 \text{ pg/ml}$, $3145.5 \text{ pg/ml}$ and $2377.9 \text{ pg/ml}$ using 4-1BB-based CAR at 1:2, 1:4 and 1:16 E:T ratios respectively compared to 31.4 $\text{ pg/ml}$, 85.1 $\text{ pg/ml}$ and 505 $\text{ pg/ml}$ using CD28-based CAR T-cells ($p=0.002340$, $p=0.004263$ and $p=0.018395$) (Figure 3-14C). Unlike IFN-$\gamma$, there was a significantly more background signal against target-negative cells for 4-1BB endodomain compared to CD28 (supT1 NT, p= 0.008219 and A20 KO, p=0.001258, p=0.008340 and p=0.023330) with an average of $545.3 \text{ pg/ml}$ against 1:2 supT1 NT for 4-1BB endodomain compared to 18.1 $\text{ pg/ml}$ for CD28 endodomain and averages of $2628.1 \text{ pg/ml}$, $744.4 \text{ pg/ml}$ and $236.5 \text{ pg/ml}$ at 1:2, 1:4 and 1:8 respectively for 4-1BB endodomain against A20 KO cells compared to 18.9 $\text{ pg/ml}$, 19.4 $\text{ pg/ml}$ and 26.4 $\text{ pg/ml}$ respectively using CD28 endodomain. This is in line with clinical trial data in which 4-1BB exhibits more antigen-independent tonic-signalling compared to CD28.
3.4. Discussion

In this chapter, I have demonstrated the production and in vitro testing of full anti-mouse CD19 CARs. After solving the 1D3 hybridoma, I cloned anti-mouse CD19 in various antibody formats and demonstrated its specificity and high affinity for the target. The binder was then utilized to construct two second-generation CARs harbouring two different co-stimulatory domains (CD28 and 4-1BB). I successfully transduced mouse T cells with specific cytokine-release and in vitro killing of both engineered human cell lines and mouse B cell tumours.

The data presented in this chapter are in line with previous reports of the in vitro performance of the 1D3 scFv as anti-mouse CD19 mouse CAR. I have additionally characterised the cytotoxic ability of mouse 4-1BB endomain, demonstrating superior activity compared to 4G7 scFv in the same mouse CAR architecture. To date, the comparison between mouse CD28 and 4-1BB endomains remains unexplored. In a challenging cytotoxicity assay, I have shown that they possess similar killing abilities with significantly different cytokine release profiles. Higher levels of interferon-γ were produced CD28 endomain, while 4-1BB demonstrated higher levels of IL-2 but increased antigen-independent release in line with the data using human cells.

Despite their overall potent activation, neither of the endomains entirely eradicated mouse B cell tumour, and overall, they have shown poor performance at higher effector to target ratios, unlike in human constructs/cells. This could suggest that 1D3 scFv does not make a great CAR when compared to anti-human CD19 CARs. Also, the different biology of human and mouse cells might affect the activity of these cells explaining the discrepancies in killing performance.
In vivo studied discussed in chapter 4 were set up to provide a platform for further testing of anti-mouse CD19 CARs to recapitulate the efficacy and toxicity observed in human trials.

For the purpose of modelling CRS in vivo, I have decided to take forward the mouse CD28-based CAR structure. Both mouse CD28 and 4-1BB endodomains have demonstrated similar CAR T-cell activity; nonetheless, the use of mouse 4-1BB in mouse CAR structures have been little reported in the literature. The work of Li and colleagues have attempted to understand the biological differences between mouse CD28 and 4-1BB, highlighting different intracellular signalling of mouse 4-1BB compared to the human corresponding. They have shown that the inclusion of mouse 4-1BB endodomain produced a less efficacious CAR T-cell product compared to mouse CD28 which is not in line with the human clinical scenario. By swapping mouse 4-1BB with human 4-1BB in a full mouse CAR structure, the group was able to improve the persistence and activity of the human 4-1BB-based mouse CAR consistent with the observations in human data. In addition, it has been shown that human 4-1BB binds TRAF1-3 while mouse 4-1BB recruits TRAF1-2 confirming the different biology between the human and mouse endodomain. Further assays such as western blot or RNA sequencing could have helped better understand the differences in the signalling profile between mouse CD28 and 4-1BB endodomains, for example looking at downstream phosphorylation events such as ERK1/2 and transcription factors molecules like NF-kb and NFAT.

As shown in table 1-1, the majority of the human trials have reported a higher incidence of CRS using CD28-based CAR T-cells. It is also widely described in humans that CD28 endodomain produces higher levels of IFN-γ and TNF-α compared to 4-1BB, which contributes to a greater extent of CRS. Altogether, I have selected mouse CD28 for in vivo work as it appeared more relevant in driving CRS demonstrated both in this chapter and confirmed by other groups.
3.5. Conclusions
- I obtained the sequence of the anti-mouse CD19 clone 1D3 after solving hybridoma by 5’ RACE

- Different recombinant 1D3 antibody-formats maintained specificity for mouse CD19 positive cells

- Binding kinetics performed using SPR technology (Biacore) demonstrated a high affinity of the 1D3 clone for mouse CD19.

- Normal mouse T cells transduced mouse 1D3 CAR showed interferon-γ release specific and lysis of CD19 positive cells superior to 4G7 scFv.

- Mouse CD28 and 4-1BB endodomain demonstrated similar killing of mouse CD19 positive cells

- Interferon-γ was significantly higher with mouse CD28 endodomain, while mouse 4-1BB produced significantly more IL-2

- Mouse CAR T cells carrying 4-1BB endodomain showed significant IL-2 production in the absence of mouse CD19 target at low effector to target ratio compared to mouse CD28 CAR-T.

4. Development of an immunocompetent B cell tumour mouse model and in vivo performance of anti-mouse CD19 CAR T cells

4.1 Introduction
Chimeric antigen receptor (CAR) T cells represent a potentially curative strategy for B cell malignancies. In recent clinical trials, CAR T cells targeting the CD19 molecule have demonstrated remarkable activity in the treatment of B cell leukaemia and Diffuse-large B cell lymphomas (DLBCL)\textsuperscript{41,311}. These benefits, however, require patients to endure and survive the almost invariable short-term toxicity of CRS and,
less commonly, neurotoxicity\textsuperscript{312,313}. CRS occurring after CD19 CAR T cells infusion is frequently followed, within days, by neurotoxicity, and its severity correlates with the number of CAR T-cells infused, tumour burden, and the severity of the cytokine storm\textsuperscript{90}. CAR-T-associated CRS is thought to result from the activation of myeloid cells by highly activated T cells, and although antibodies to the IL-6 receptor (tocilizumab) can ameliorate CRS, they do not prevent or treat neurotoxicity\textsuperscript{314}. The majority of the studies evaluating the antitumor activities of CAR T-cells use both \textit{in vitro} cell culture-based assays and \textit{in vivo} tumour-bearing mouse models. \textit{In vitro} assays are used to assess certain aspects or functions, such as cytokine secretion, deregulation, and cytotoxicity, as well as proliferation upon target cell stimulation. These assays can indicate the recognition capability of CAR but are not as helpful for evaluating the efficacy and predicting the patient's response. The use of the immunodeficient NSG mouse has enabled the evaluation of human CAR T-cells against human cancers, and they have been extensively used as a platform for the development of CAR T-cells for novel TAAs antigens and disease types. Despite the widespread of xenograft mouse models, these have substantial limitations. Their compromised immune system does not allow the modelling of the crosstalk between CAR T-cells and the host immune cells. In addition, the native TCR expressed by human T cells can recognize mouse antigens, leading to xGVHD. Because of this xeno-activation, the growth kinetics and tissue distribution of infused CAR T-cells in these mice may not be able to simulate that of human conditions, and the lethality of xGVHD can interfere in the evaluation of the efficacy of the therapy. More recently, two groups have been able to recapitulate and prevent CRS in humanized mouse models, confirming the clinical finding that IL-6 is released from myeloid cells and is an important contributor to CRS\textsuperscript{141,315}. In addition, the authors have shown that pre-emptive use of IL-6- and IL-1-receptor antagonists inhibit macrophage functions and can prevent CRS without impairing tumour remission. They have also confirmed the clinical observation that the degree of tumour burden and T cell expansion influence the onset and severity of CRS\textsuperscript{316}. Although complex in their generation, these humanized models are more relevant for the evaluation of human CAR T-cell efficacy, resistance, and toxicity. Alternatively, a syngeneic mouse model for the investigation of CAR T-cell therapy has been rarely employed and is discussed in more detail in the general introduction. These models offer the advantage of being able to recapitulate and study the effects of CAR T-cells in their natural context of crosstalk with an intact
immune system. Also, these models are useful to investigate on-target off-tumour effects for the evaluation of the safety profile of the immunotherapeutic without the occurrence of 


\textsuperscript{150,151}. Two main mouse B cell tumours models have been used to investigate 1\textsuperscript{st} and 2\textsuperscript{nd} generation anti-mCD19 CAR therapy. The E\textsubscript{\textmu}-ALL01 cell line derived from a lymphoma of E\textsubscript{\textmu}-myc C57BL/6 mouse, has been used to model systemic models B cell tumours\textsuperscript{95,154}. Following a week of tumour growth, mice received pre-conditioning, either cyclophosphamide or total body irradiation (TBI), the day prior to the injection of 3x10\textsuperscript{6} CAR T-cells. These models were successfully able to recapitulate and revert the B cell aplasia associated with CD19 CAR therapy; nonetheless, they did not report any sign of CRS-related toxicity. The A20 mouse cell line has been alternatively used in a syngeneic model of B cell tumour. This B cell line is derived from a spontaneous lymphoma in an old BALB/c mouse\textsuperscript{317}. Gilham’s group have utilized this cell line to establish a systemic model of B cell malignancy. Tumour engrafted with cyclophosphamide preconditioning took up to two months to establish, followed by further preconditioning with either cyclophosphamide or TBI the day prior to the administration of up to 4x10\textsuperscript{6} CAR T cells\textsuperscript{155,156}. Elevated levels of IFN-\gamma and TNF-\alpha accompanied with an increase in MDSCs, resulted in rapid weight loss and acute toxicity. Although they did not find a statistical increase in IL-6, the toxicities have indicated possible CRS similar to the one observed in the clinical experience. In summary, syngeneic models can be important in evaluating and predicting CAR safety and side effects with the possibility of studying the changes induced by crosstalk with a functional host immune system.

4.2 Aims

The chapter aims to establish a syngeneic immunocompetent mouse model of B cell malignancy as a system to improve the understanding of the effects of CD19 CAR T-cell therapy.

4.3 Results

4.3.1 Comparison of two lymphodepleting regimens for the systemic engraftment of A20 tumour cells

The first report in the literature of the A20 syngeneic balb/c mouse model included a pre-conditioning regimen of 150 mg/kg of Cyclophosphamide the day before the injection of 5x10\textsuperscript{5} A20 tumour cells. After thirteen days, the mice were irradiated with 6Gy total body irradiation (TBI) and the day after were injected with autologous CAR
T-cells\textsuperscript{155}. A first attempt at replicating the above tumour engraftment condition failed to produce a detectable and consistent bioluminescent signal (BLI) in most of the mice, and upon sacrifice, I could not detect the presence of tumour cells by flow cytometry. The presence of a tumour \textit{in vivo} was monitored using A20 cells engineered to expresses Firefly luciferase (FLuc+) and the mouse congenic marker THY1.1 (aka CD90.1) for flow cytometry. Transduced cells were sorted by flow cytometry for high expression of the marker gene, and light emission was tested \textit{in vitro} using a plate reader for the photons count. The A20 cell line stably expresses high levels of the marker gene, and it has 3 logs more light emission than the unmodified cells (\(p<0.0001\)) (Figure 4-1).
Figure 4-1. Generation of an A20 reporter tumour cell line for in vivo tracking. [A] Schematic of the SFGmR retroviral plasmid for the co-expression of the red-Firefly luciferase (5xRed_Fluc) for in vivo tracking and mTHY1.1 as flow cytometry marker gene. [B] Flow cytometry assessment of stably transduced and sorted A20 cells, gated on live single cells and stained for the expression of mTHY1.1 as marker gene compared to NT A20 cells. [C] Bioluminescence (BLI) emission of the engineered cells in the presence of luciferin substrate and expressed as relative light in comparison to NT cells. The difference in the emission of light between A20 NT and A20 THY1.1 was analysed using unpaired t-test (****p<0.0001).
The light emission and the high expression of the marker gene facilitate the monitoring of tumour engraftment and separation from the normal B cells of the mouse. In order to identify the best tumour engraftment conditions, two different pre-conditioning drugs (150mg/kg of Cyclophosphamide and 50mg/kg of Busulfan) and three different tumour cell doses were tested (Figure 4-2).

After injection of the modified A20 tumour cells (day 0), mice were monitored daily for signs of pain and/or stress, and BLI images were taken as early as 5 days after tumour injection. On the day of the euthanasia, the spleens and bone marrow from the lower legs were harvested and processed for flow cytometry staining. Mice receiving three different dosages of tumour cells preconditioned with Busulfan, consistently showed little tumour engraftment by BLI with complete disappearance in all mice by day 26. On the other hand, mice pre-conditioned with cyclophosphamide had more consistent engraftment, especially in the group that received 3x10^6 tumour cells. The engraftment was already visible in the bone marrow after 8 days, and the mice required culling between day 15 and day 22. Interestingly, in mice that received the highest tumour-dose (5x10^6), after an initial spike of BLI signal, the tumour did not persist beyond day 15 (Figure 4-3A). The measurement of radiance from each mouse confirmed poor engraftment in the Busulfan pre-conditioned mice, with BLI signal gradually disappearing over time, while the mice that received cyclophosphamide and 3x10^6 tumour cells had a constant increase in tumour growth and required take down by day 22 (Figure 4-3B). On the day of the sacrifice, cells obtained from the spleen and...
bone marrow were analysed by flow cytometry for the presence of CD19+, THY1.1 double-positive A20 tumour cells and were found in the bone marrow of mice receiving 3x10⁶ tumour cells and cyclophosphamide-preconditioning. Although the sample size utilized for establishing the best A20 engraftment conditions was small, 150mg/kg of Cyclophosphamide followed by tail injection of 3x10⁶ tumour cells resulted in tumour-related deaths with systemic disease present in the animal’s bone-marrow as described in the literature.
**Figure 4-3. In vivo tracking of tumour engraftment.** [A] BLI emission throughout the 26 days of the experiment for each individual mouse (N=2) from the two cohorts receiving different doses of tumour cells. Light is expressed as a pseudo-colours luminescence scale. [B] The BLI signal was plotted as mean radiance (p/sec/cm²/sr) for each of the cohort studied and the differences were analysed using 2-way ANOVA with Turkey correction to account for multiple comparison (**p<0.001, *p<0.05). [C] Representative flow cytometry plot of mouse bone marrow cells evaluating the presence of tumour cells (mTHY1.1+) within the CD19+ B cells.
4.3.2 Comparison of mouse CD28 and 4-1BB costimulatory domains in 1D3-based second-generation CAR structure

The cyclophosphamide model described in 4.3.1 was used to investigate the efficacy of the anti-mouse CD19 CARs. The structures contained 1D3 scFv as a binding domain spaced by CD8 stalk and transmembrane regions and fused with the intracellular domain of either mouse CD28 or 4-1BB. In the work of Cheadle et al., they injected a 1st or 2nd generation CAR against mCD19 in mice pre-conditioned with 6Gy TBI before the administration of the cells\textsuperscript{155,156}. Due to the logistics of the animal house I was working in, I did not have access to an irradiator and an IVIS spectrum for the BLI images at the same time, which made it difficult to replicate the paper’s experimental conditions. However, the requirement of lympho-depleting pre-conditioning is necessary for the engraftment and success of CAR T-cells. To test our CARs in this model settings, I decided to pre-condition the mice with another dose of cyclophosphamide the day before the injection of the cells (Figure 4-4).

![Timeline of the in vivo experiment to compare CD28ζ and 41BBζ in the A20 tumour model.](image)

Figure 4- 4. Timeline of the in vivo experiment to compare CD28ζ and 41BBζ in the A20 tumour model. 3x10\(^6\) A20 cells were engrafted in 9 balb/c mice and pre-conditioned with cyclophosphamide before the injection of 4x10\(^6\) CAR modified lymphocytes from a donor mouse. 3 mice each received 1D3 with either CD28 or 4-1BB as endodomain and compared with mice receiving saline only. Tumour was monitored by BLI and mice were monitored for side effect. At the end of the experiment tumour presence and CAR T-cell engraftment were representing the readouts for this model.

Activated lymphocytes from the spleens of two healthy balb/c mice were transduced with either CD28ζ CAR or 4-1BBζ CAR. The transduction efficiency was estimated by flow cytometry staining of the marker gene mCD34 showing 72% and 66% positive for the two CARs, respectively (Figure 4-5).
CAR T-cells were administered at a high dose (4x10^6 CAR-positive cells) to increase the chance of tumour clearance but also to potentially highlight toxic effects of the therapy (i.e., CRS) that can be studied further. The tumour was monitored by BLI and engraftment was visible at day 7, consistent with the previous experiment data, with BLI signal coming from the bone marrow in 8 out of 9 mice. In accordance to Cheadle’s group model, on day 11, mice received either CAR T-cells or a saline solution and were monitored for side effects and tumour reduction. The experiment was terminated early on day 18, because of tumour reduction in 2/3 mice that received saline solution. The primary endpoint of seeing tumour reduction was not possible due to the lack of the control group (Figure 4-6A). This was the result of using chemotherapy as pre-conditioning before CAR T-cells injection; A20 cells are probably sensitive to cyclophosphamide which alone caused the complete regression of the tumour in the saline group. Interestingly, the group that received 4-1BBζ CAR did not control the tumour as well as CD28ζ. In humans, 4-1BB is known to contribute to a more delayed effect, and long-term sustained activity compared to CD28 endodomain (Figure 4-6B).
Figure 4-6. BLI monitoring of tumour progression and CAR T-cell efficacy. [A] BLI emission during the course of the experiment, each individual mouse from the three different cohorts (N=3) was imaged from a ventral position. At day 11 post-tumour injection 4x10^6 CAR-T cells were administered following pre-conditioning the day before. [B] BLI emission reported as radiance (p/sec/cm2/sr) from each experimental cohort and expressed in logarithmic scale. Differences across the three cohorts were analysed using 2-way ANOVA with Turkey correction for multiple comparison.
Flow cytometry analysis of bone marrow and spleen extracted at the day of the sacrifice confirmed the presence of both CD4+ and CD8+ CAR T-cells in the bone marrow of only one mouse in the CD28ζ group. Tumour cells were gated as CD19+THY1.1+ double-positive cells, while CAR T cells were stained for their marker gene (mCD34) in CD4 and CD8 T cells (Figure 4-7).

**Figure 4-7.** Flow cytometry analysis of cells from spleen and bone marrow. Flow cytometry plots from one mouse of the CD28ζ cohort as representative gating strategy for all the cohorts tested. Cells extracted from spleens [A] and bone marrow [B] were gated for single and live cells. The presence of tumour cells was identified from CD45+, CD19+ cells and distinguished from normal B cell as THY1.1+. CAR T-cells were gated from CD45+,CD19- cells and subgated in CD4 and CD8 T cell. From these subsets of cells the expression of mCD34 was used to identify the presence of CAR-T cells.
The spleen was negative in all the mice for both CAR T-cells and A20 cells (Figure 4-8A). Interestingly, 4-11Bζ-receiving mice had a significantly higher percentage of CD4 T cells (p= 0.040853) while CD28ζ had more CD8 T cells (p= 0.004274). The presence of tumour cells was detected only in the bone marrow of a mouse in the 4-1BBζ group, while it was absent in all the other mice (Figure 4-8B). Altogether, this model has demonstrated the unsuitability of cyclophosphamide as preconditioning regimen before the administration of CAR T-cells. A20 cells have been shown to be sensitive to cyclophosphamide. In addition, the 4-1BBζ cohort has shown little effect in controlling tumour growth.

Figure 4-8. Summary of flow cytometry staining in the spleen and bone marrow at the day of the sacrifice. Cells obtained from the spleen [A] and bone marrow [B] from each mouse and cell subpopulations were shown as percentage of their parental population (CD45+ for non-tumour CD19 as percentage of CD45+, CD4 and CD8 as percentage of CD19-, CD4 CAR and CD8 CAR as percentage of CD4 and CD8 respectively, tumour CD19 as percentage of non-tumour CD19). Each data point represents an individual mouse from the three differently treated groups at the day of the sacrifice.
takedown (day+18 post-tumour injection). Each biological replicate (N=3) was plotted individually and analysed using unpaired-t-test for the comparison between 4-1BBζ and CD28ζ CAR. ***p<0.001, **p<0.005, *p<0.05.

4.3.3 Tumour engraftment using total body irradiation (TBI)

The model described in 4.3.2 was limited by the lack of consistent and detectable tumour cells in the bone marrow. This raised concerns for the future study of tumour kinetic in terms of eradication and CAR T-cells characterization. Engraftment in the marrow is described in the literature and is a known niche for the A20 cells.\textsuperscript{156} Moreover, the sensitivity demonstrated by A20 cells to cyclophosphamide limits its use as a preconditioning regimen for the engraftment of CAR T-cells. Availability of a different animal facility (Centre for Advanced Biomedical Imaging, CABI, UCL) allowed the use of both TBI for the tumour engraftment and BLI imaging for monitoring. In the attempt to move forward and recapitulate the work of Cheadle and colleagues\textsuperscript{155}, I shifted towards TBI preconditioning to improve tumour engraftment. In order to evaluate the optimal dose of radiation, I tested two cohorts of 5 mice each, receiving sublethal doses of 3 or 6 Grey (Gy) TBI followed by the injection of 5x10^5 tumour cells (A20 THY1.1+ / Fluc+) 6 hours later, similar to the settings used by Cheadle and colleagues for the engraftments of CAR T-cells\textsuperscript{156} (Figure 4-9).

![Figure 4-9](image)

**Figure 4-9. Timeline of the in vivo experiment to compare two doses of total body irradiation (TBI) for the establishment of systemic A20 cells tumour.** Tumour pre-conditioning was administered as TBI at 3Gy and 6Gy in two different cohorts of 5 mice each. 5x10^5 A20 tumour cells were injected i.v. on the same day. Tumour growth was monitored throughout the experiment by BLI imaging, mice were sacrificed at any sign of pain. Finally, spleen and bone marrow were extracted to look at the presence of tumour cells.

The BLI imaging of the tumour showed a positive signal as early as day 7 and constantly progressed over time. The signal was clearly identifiable in the bone marrow of all the mice showing engraftment on both flanks of the group that received the higher dose of
radiations. The upper body of the mice, probably the lungs and the upper bone marrow (anterior legs and sternum), was also showing a strong signal (Figure 4-10A). Mice were monitored daily and were culled at day 20 when they started showing signs of weight loss and distress. The radiance from individual mice showed an exponential and constant progression with moderately aggressive and high burden tumour growth before mice required culling (Figure 4-10B).
On day 20, mice were euthanised, the cells from spleens and bone marrows were extracted and used for flow cytometry analysis looking for the presence of A20 tumour cells (CD19+, THY1.1+) (Figure 4-11). The effects of TBI on the immune system are known in mice to last around 7-10 days, with B cells ontogeny occurring earlier than T cells during HSCs development\textsuperscript{319}. Flow cytometry analysis showed that the majority of the cells were CD45+ lymphocytes with around 40% of T cells and 60% of B cells, confirming the recovery of the mice’s immune system in both groups.
Figure 4-11. Flow cytometry analysis of cells from spleen and bone marrow. Representative FACS plot of the gating strategy in spleen and bone marrow cells. Cells were gated first on viability dye, live cells were selected for CD45+, CD11b- lymphocytes. The proportion of T and B cells were differentially gated with CD3 and CD19, respectively. The percentage of tumour positive cells were obtained from the total number of CD19+ B cells using the marker gene THY1.1.
Bone marrow is normally expected to be less populated by T cells compared to peripheral blood, and in this experiment, indeed, the vast majority of BM cells observed were CD19+ B cells and CD45- cells, with an average of 10% CD3+ cells only present in the 3Gy group. CD19+ and THY1.1 double-positive A20 cells were identified in the bone marrow of nearly all the mice. Only the group that received 3Gy had two mice with very few or no tumour cells detected, while in the 6Gy group, all the CD19+ cells in the bone marrow were tumour cells and present in all the mice (Figure 4-12). This suggested that a 6Gy TBI, followed by the engraftment of A20 cells, would provide consistent tumour engraftment with detectable tumour cells in the mice bone marrow only.

![Flow cytometry staining of the spleen and bone marrow cells at the day of the sacrifice.](image)

**Figure 4-12. Flow cytometry staining of the spleen and bone marrow cells at the day of the sacrifice.** Percentage of CD3+ T cells and non-tumour CD19+ B cells were obtained from CD45+ cells in the spleen and bone marrow of the two cohorts receiving 3Gy and 6Gy TBI respectively. Tumour cells were obtained from the parental non-tumour CD19+ population as mTHY1.1 double positive cells. Each biological replicate was plotted individually (N=5) and the differences in the percentage of cells between 3Gy and 6Gy TBI were analysed using unpaired-t-test.

### 4.3.4 Identification of a therapeutic window for the administration of CAR T-cells

The tumour engraftment conditions given by the use of 6Gy TBI and subsequent injection of 5x10⁵ A20 cells have shown consistent and uniform engraftment in the previous experiment 4.3.3. Mice engrafted with these settings had a detectable tumour in the bone marrow already at day 7, with BLI emission increasing and disseminating in the animal over time. On day 20, the tumour burden had raised exponentially, and mice required culling. The administration of CAR T-cells for therapeutic purposes has been first investigated to assess at which day post-tumour injection these cells would
have been more effective at eradicating the established tumour. The long-lasting effect of TBI on the immune system was thought to be sufficient to allow engraftment of the cells without further lympho-depleting treatment prior to CAR T-cell injection. 12 mice were engrafted with A20 tumour and received a dose of $4 \times 10^6$ CAR-positive cells or saline at either day 10 or day 14 post-tumour injection (N=4). Mice were monitored for tumour by BLI imaging, and weight was recorded every two days. To evaluate the potential engraftment of CAR T-cells, after 12 days, a small amount of blood was taken from the tail-vein for flow cytometry staining (Figure 4-13).

Two spleens from healthy balb/c mice were extracted and processed to generate a lymphocyte suspension for transduction with 1D3-CD28ζ CAR. Two separate batches of spleens were independently transduced to generate CAR T-cells for the administration at days 10 and 14 post-tumour injections, respectively, and high-level expression of the CAR T-cell marker gene (mCD34) indicates excellent transduction (86.1% and 89.9%) for both time points (Figure 4-14).
At the time of CAR T-cells injection (days 10 and 14), the tumour was well established in almost all the mice except two (cohort CAR-T D+14). One mouse was completely excluded from this group because the tumour never engrafted, while a second mouse in the same group showed minimal BLI emission with abnormal tumour localisation, and this led to a rapid health decline after CAR T-cell administration, requiring culling (Figure 4-15).

**Figure 4-14. Transduction efficiency of mouse splenocytes.** Representative flow cytometry gating for the evaluation of CAR T-cells expression prior the injection into mice. Live cells were gated for CD3+ T cells and the expression of marker gene mCD34 compared to non-transduced cells (NT). Two separate batches of CAR T-cells were prepared for the in vivo administration either at day +10 or +14 post tumour injection.
The group that received saline progressed as expected to rapid tumour expansion with weight loss and sickness that required culling at days 17 and 24 (Figure 4-16A). Almost all the mice in the group that received CAR T-cells manifested a weight loss with different degrees of severity from gradual to a more sudden loss, resulting in the endpoint for these mice. Mice that received the therapy on day 14 did not control the tumour's progression and needed culling at days 24 and 35. Of the group that received CAR T-cells at day 10, almost all the mice (3/4) showed a decrease in BLI emission accompanied by weight loss over the following 15 days (day 25) (Figure 4-16B). Sudden weight loss and general sickness were observed in 2/4 mice after CAR

**Figure 4-15. Tumour progression monitoring.** BLI images throughout the course of the mouse experiment. Images were taken from a ventral position and light emission was expressed in pseudo-colours as radiance (p/sec/cm²/sr). DPT: day post tumour
administration; one was found dead on day 17, while the other two started to regain weight in the following days (Figure 4-16C).

![A] Survival

![B] Bioluminescence

![C] Weight

**Figure 4-16. Survival and mice monitoring.** [A] Survival curves for the three differently treated cohorts (saline only, administration of CAR-T cells at day 10 (D+10) and day 14 (D+14) post tumour injection. Survival curves were compared using the Log-rank (Mantel-Cox) test (N=4, N=3 for CAR at day 14). [B] The BLI emission for each cohort was reported as mean radiance (p/sec/cm²/sr) and expressed in logarithmic scale [C] Weigh monitoring in each cohort expressed as mean of the body weight in grams (g) for each cohort. Differences in BLI and weight were analysed using unpaired t-test comparing the means of each cohort (****p<0.0001, ***p<0.001, **p<0.005, *p<0.05).

The tumour signal in these two animals decreased to almost eradication at around day 35 with a stable condition and health recovery in the following weeks. The experiment was terminated at day 77 post-tumour injection and after being free from the tumour for over 40 days. Blood was collected from mice in all the groups after 12 days from
CAR T-cell administration to assess the presence of these cells in the peripheral blood. The group that received a saline injection was bled in parallel at day 12 as a control for the staining and cell populations. Live cells were gated for CD45+ lymphocytes to investigate T cell and B cell, and the proportion of CAR T-cells and A20 tumour, respectively (Figure 4-17A). The vast majority of the cells in the blood of the treated mice were CD3+ T cells, and normal B cells were detected in the saline group only, highlighting the efficacy of CAR T-cells at eliminating CD19+ cells. The group that received the therapy on day 10 had remarkable levels of CAR T-cells in all three mice with up to 10% positive cells in the CD3+ population. On the other hand, in the group which received CAR on day 14, only one mouse out of the two had detectable CAR T-cells in the blood (Figure 4-17B).

These results support published data regarding the relationship between the depth of the B cell aplasia and the response to the therapy. The remarkable engraftment of CAR T-cells in some mice might explain the weight loss and stress observed in the first two weeks from the therapy administration. On the day of the takedown, mouse cells from the spleen and bone marrow were stained for the presence of tumour and CAR T-cells and analysed by flow cytometry. Bone marrows of mice that did not receive the therapy were almost entirely populated by A20 tumour cells with very few CD3+ T cells, while the spleens had a more evenly and normally distributed proportion of T and B cells with an average of 55% and 40%, respectively. On the other hand, almost all the mice that received CAR T-cells, independently of the time when they received them, had nearly complete lack of CD19+ B cells both in the bone marrow and the spleen with a high percentage of CD3+ infiltrated in the bone marrow and circulating in the peripheral blood. Tumour was identified in 2/3 bone marrows of the mice that received CAR-positive cells at day 14, while it was not found in the other treated group. CAR T-cells were detectable in the bone marrow of all the mice with an average of around 15% positive cells in both the groups treated; CAR+ cells were also detected in the spleen with averages of 4% and 6% for CAR at day 10 and 14, respectively (Figure 4-17C).
Figure 4-17. Flow cytometry analysis of cells from spleen and bone marrow. [A] Representative flow cytometry plots and gating strategy for cells obtained from spleens and bone marrow of each mouse. Viability dye negative and single cells were gated for CD45+, then CD3+ T cells and CD19+ B cells were gated separately. CAR-T cells were obtained by the expression of the marker gene (mCD34+) from the parental CD3+ T cells, while tumour CD19 cells expressing mTHY1.1+ were gated from non-tumour CD19 B cells. [B] Flow cytometry analysis of cells obtained from tail bleeding after 12 days from CAR-T cells administration (N=2 for saline and CAR at day 14, N=3 for CAR at day 10). [C] Flow cytometry analysis of the spleen and bone marrow cells subpopulation extracted at the day of the animals’ takedown (N=4 for saline, N=3 for CAR at day 10 and 14). The parental populations were obtained as a percentage of CD45+ cells for CD3 and non-tumour CD19, while the parental cells of CAR and tumour CD19 were CD3+ and non-tumour CD19 respectively. The mean of the percentages were compared for each group using unpaired t-test (***p<0.005, *p<0.05).
Interestingly, a high percentage of CAR+ cells (~10%) remained in the bone marrow of the surviving two mice (CAR at day 10 group) long after tumour eradication, explaining the B cell aplasia and sustained tumour remission observed. CAR T-cells were effective in 50% of the mice that received them on day 10 after tumour injection, while no effect on tumour progression was observed in the group that received the cells 4 days later, highlighting the importance of the right time for the administration. Identifying CAR T-cells in the BM of all the mice reflected their good engraftment in the context of CD19+ target cells.

In the CAR at day 10 group, sudden weight loss and requirement for an early cull in 2 of 4 animals where CAR T-cell expansion was observed, suggesting that CAR-T toxicity rather than tumour progression may have been responsible for these deaths.

The group receiving CAR-T on day 14 post-tumour injection failed to fully control tumour growth. Interestingly, the CAR T-cells administered at day 14 resulted in a persistent and active response as shown by the elimination of non-tumour B cells comparable to the CAR administration at day 10 but did not fully clear tumour B cells from the bone marrow. These could have been due to (I) the higher tumour burden present on the day of the CAR T cells administration and (II) the lack of the benefits from the initial lymphodepleting TBI. After 14 days from the TBI, the animals reconstituted most of the immune system, which contributed to less effective therapy.

The presence of a higher amount of normal B cells and a less favourable environment for the engraftment of CAR T-cells might have contributed to the decreased activity of the CAR administered at day 14 compared to day 10.

In summary, this experiment shows that CAR T-cell engrafted and eradicating CD19+ tumours, especially when administered at day 10 post tumour. The long CAR-T persistence and the toxic effects (weight loss, and high levels of CAR T-cells in the bone marrow) might be a consequence of cytokine release syndrome (CRS), from the combination of high burden CD19+ disease in some mice and a high CAR T-cell dose. However, these results open the possibility to characterize and study the toxic and long-term effects of anti CD19 CAR therapy.

4.3.5 CAR T-cells dose de-escalation for toxicity/efficacy assessment

Experiment 4.3.4 demonstrates that the optimal therapeutic window for administration of the CAR T cells is at day 10 post-tumour injection. This model suggests that animals
can achieve long-term remission but with significant side effects that triggered the requirement for a cull in these animals. It is possible that the high dose of CAR T-cells administered, although in line with the dosage used in a similar model\textsuperscript{154,156}, could have contributed to this phenomenon. The observations from this experiment have concluded that the conditions established for this B cell tumour mouse model can be utilized to investigate both efficacy and toxicity of CAR therapy along with the crosstalk with an intact host immune system.

In order to better understand the side effects observed, a dose de-escalation model was used to investigate the number of administered CAR T cells as a determinant of the magnitude of toxic and anti-tumour effects. Three different doses of CAR T cells (4x$10^6$, 2x$10^6$ and 1x$10^6$ cells), based on 50\% of the highest dose tested in experiment 4.3.4, were injected in cohorts of 4 mice each at day 10 days following A20 tumour injection. A cohort receiving the highest dose (4x$10^6$) of non-engineered T cells (NT) has been used as an experimental control.

The primary endpoint was represented by the survival of tumour-free mice. In addition, the characterization of the potential side effects, such as CRS, was evaluated. Along with BLI imaging and body weight, the internal body temperature was recorded daily. As reported in humans, a spike in temperature following CAR T cell administration can indicates ongoing CRS, and the degree/duration of the fever can correlate with the severity of the cytokine storm. Measurement of inflammatory cytokines was performed on peripheral blood, and the timing was decided based on the increase in body temperature. As per the project-licence, the collection of up to 100µl of blood was allowed every 10 days. On the day of collection, the plasma was frozen down and used later for cytokine beads array (CBA) (Figure 4-18).
Three spleens from healthy balb/c mice were utilized to generate enough CAR T-cells to administer the three different doses tested (4x10^6, 2x10^6, and 1x10^6 cells). The lymphocytes extracted from spleens were gammaretroviral transduced with the 1D3-CD28ζ CAR construct and expression on CD3+ was evaluated by flow cytometry. Comparison with matching NT cells demonstrated that 73.6% of T cells successfully expressed the CAR construct (Figure 4-19).
All four cohorts of mice showed homogeneous tumour engraftment consistent with previous experiments. On the day of CAR administration (day 10), the A20 tumour cells had established a predominantly bone marrow niche, and some animals had a more disseminated tumour, also in the upper arms and sternum. Mice were euthanised if any sign of pain or discomfort arose, with tumour-free survivors culled after 74 days from the beginning of the experiment (Figure 4-20).

The cohort receiving 1x10⁶ CAR T cells performed best with 50% (2/4) overall survival (OS) at day 74, followed by 25% (1/4) OS in the cohort receiving the 4x10⁶ dose (median 60- and 55-days, respectively). The statistical difference was observed in comparison to both NT-receiving mice and the 2x10⁶ CAR T cells dose with a median of 20- and 18-day survival post-tumour injection (Figure 4-21A). In both the NT and 2x10⁶ groups, the BLI emission increased exponentially over time with all the animals.
culled by day 24 post-tumour injection. Interestingly, in the groups that received CAR T cells, poor animal health and weight loss, required early cull at day 17 and this may be related to CAR T toxicity. (Figure 4-21B).

The increase in side effects has also been confirmed by the spike in temperature observed at 3-, 7-, and 10-days post-CAR-T. Comparison with the cohort receiving NT cells demonstrated a statistically significant difference in temperature at day 2 for the group receiving 1x10^6 CAR T-cells (p=0.007119) with an average of 36.18 °C compared to NT 35.48 °C and at day 3 for the 4x10^6 CAR T-cells dose and (p=0.03966) with an average of 37.2 °C compared to NT 36.8 °C. On day 7, a more marked spike in temperature was observed for the 4x10^6 and 2x10^6 doses (p=0.003273 and p=0.001654) with averages of 37.63 °C and 37.4 °C respectively compared to NT 36.58 °C; at day 9 for the 2x10^6 CAR-T cells dose, the temperature was 36.7 °C and 35.67 °C for the NT-treated group (p= 0.00092), while the other two CAR-T treated groups (4x10^6 and 1x10^6 cells) demonstrated a statistically significant difference in temperature at day 10 from the group receiving NT cells, with average values of 37.08 °C, 37.2 °C and 35.97 °C respectively (p=0.013480 and p=0.001683) (Figure 4-21C). Weight confirmed the ongoing illness in some animals, and loss of ≥10% of body weight automatically
triggered the endpoint for those animals, while tumour-free mice kept gaining weight as a sign of healthy recovery (Figure 4-21D).

**Figure 4-21. Survival and mice monitoring.** [A] Kaplein-Meyer survival curve of the three differently treated mouse cohorts (4x10⁶, 2x10⁶ and 1x10⁶ CAR T-cells) and control group (NT) (N=4). Survival curves were compared using the Log-rank (Mantel-Cox) test. [B] BLI emission plotted as radiance (p/sec/cm²/sr), data were expressed as mean from each cohort and reported in logarithmic scale. Monitoring of the body temperature [C] and total body weight [D] in each cohort. The means of the different measurements were compared for each group using unpaired t-test. (**p<0.001, *p<0.005, *p<0.05).
As a spike of temperature was observed already at day 2-3 and the limitation in the performing repeated successive bleeding, mice were bled at this timepoint despite the later discovery of statistically significant temperature’ spikes. CBA analysis on the plasma of these animals highlighted inflammation with elevated inflammatory cytokines like TNF-α, IL-1, IL-10 and IL-23 in all the cohorts and no difference in any of the 13 cytokines tested. This was probably due to the inflammation caused by the tumour growth that masked the standalone effects of CAR T-cells. Interestingly, two mice in the cohorts requiring early takedown (4x10^6 and 2x10^6 CAR T-cells) had high levels of IFN-γ (42 pg/ml and 405.7 pg/ml respectively), only one mouse in the 4x10^6 and 2x10^6 cohorts had detectable levels of IFN-γ. IL-6 was also elevated in the 4x10^6 receiving group with an average of 82.4 pg/ml compared to NT 11.9 pg/ml. This may be consistent with a CRS type syndrome, akin to that reported in humans and might suggest a similar toxicity profile in these animals (Figure 4-22A). The small amount of blood material collected did not allow the simultaneous assessment of cell populations by flow cytometry. This was performed on a second bleeding 10 days later (13 days post CARs), and of the survivors, all showed excellent CAR-T engraftment with no detectable B cells (Figure 4-22B). In the 4x10^6 and 2x10^6 doses an average of 18.2% and 13.6% of CAR T-cells were detected, confirming their sustained engraftment in parallel to the return of the normal T and CD11b+ cells. At this stage of the model, we would expect to see a contraction in the percentage of circulating CAR T-cells; the lack of B cells confirmed that the engineered T cells are functionally active that also explains the CAR T-cells persistence as B cells are attempting to return to homeostasis. This scenario can be studied and investigated in a syngeneic immunocompetent model, but not in xenograft models where the immune cells don’t reconstitute adequately, and xGVHD can cause the termination of the experiment.
On the day of takedown, cell suspension from spleens and bone marrows were stained for flow cytometry analysis. Cells were gated on live CD45+ lymphocytes, looking at CAR T cells (mCD34+) and tumour cells (mTHY1.1+) in comparison to NT-treated mice (Figure 4-23A). Flow cytometry analysis showed good levels of CAR T-cell...
engraftment in the spleen, especially in the groups receiving 4x10^6 and 2x10^6 dosage with an average of 9.2% and 7.5%, respectively, but no significant differences between the doses tested. The percentage of monocyte in the spleen was significantly decreased in the group receiving 2x10^6 CAR T-cells with an average of 6.6% compared to 30.6% in the NT group (p=0.019751), but not compared to the averaged in the 4x10^6 and 1x10^6 (40.1% and 12.1% respectively).

In all the mice receiving CAR-T, the absence of B cell was a confirming feature of the efficacy of the therapy in which both the 4x10^6 and 2x10^6 cohorts showed significant differences with an average of 2.35% and 0.53%, respectively, compared to 21.6% in the NT-receiving cohort (p=0.031342 and p=0.002314).

In line with the reduction in the numbers of B cells, the percentage of CD3+ T cells was significantly higher in the cohorts receiving 4x10^6 and 2x10^6 CAR T-cells, 80.2% and 83.6% (p=0.041321 and p=0.023377), compared to NT (61.6%), but not the 1x10^6 dose (72.5%) (Figure 4-23B). A similar finding was also observed in the bone marrow with the addition of a greater level of CAR T-cells detected, and non-tumour B cells detected only in the NT-receiving group (9.4%), where tumour B cells were predominantly detected (88.3%). CD3+ T cells were detected with averages of up to 40% in all cohorts, but no statistical differences were observed compared to NT cells. CAR T-cells have been consistently detected in the bone marrow with averages of 27.1%, 39.1% and 16.4% in the 4x10^6, 2x10^6 and 1x10^6 cohorts, respectively. The different numbers of cell populations reflected the scenario of the spleen with absent monocytes in the cohort receiving 2x10^6 CAR T-cells and elevated T cells accompanied with a decreased number of B cells in all the cohorts. However, no significant difference was highlighted due to the majority of B cells present in the bone marrow were tumour B cells (88.3%, 49.9%, 36.9% and 46.5% in the NT, 4x10^6, 2x10^6 and 1x10^6 cohorts, respectively).

Notably, in the cohorts experiencing the worst toxicity (2x10^6), the significant reduction in the numbers of CD11b+ monocytes observed in the spleen was also observed in the bone marrow with an average of 1.5% compared to 24.4% in the NT-treated cohort (not significant). While in the 4x10^6 and 1x10^6 cohorts CD11b+, the percentage of CD11b+ was increased with averages of 41.4% and 47.3%, respectively (Figure 4-23C).

The marked reduction in the monocyte population combined with detectable tumour cells and a high number of CAR T cells might suggest that the toxic effects observed arose from the battle of the treatments against the tumour.
Figure 4-23. Flow cytometry analysis of cells from spleen and bone marrow at the day of takedown.

[A] Representative FACS plot of the gating strategy in spleen and bone marrow cells. Cells were gated first on viability dye negative and single cells were gated for CD45+, then CD3+ T cells and CD19+ B cells were gated separately as percentage of the CD45 parental population. CAR-T cells were obtained by the expression of the marker gene (mCD34+) from the parental CD3+ T cells, while tumour CD19 cells expressing mTHY1.1+ were gated from non-tumour CD19 B cells. CD11b cells were obtained as percentage of live single cells. Flow cytometry analysis of the percentage of cells obtained in the spleen [B] and bone marrow [C] at the day of the takedown for each mouse (N=4). The means from the different cell’s populations were compared for each cohort using unpaired t-test (**p<0.05, *p<0.05).
The CBA analysis showed differences among the treated groups for the 13 inflammatory cytokines analysed (Figure 4-24A). Consistent with human trials, elevated IFN-γ and IL-6 (non-significant) were predominantly observed in the cohort receiving 2x10^6 CAR T-cells, which experienced the worst side effects (sudden weight loss and poor animal health) and mice required immediate cull. An average of 136.7 pg/ml of IFN-γ and 1987.7 pg/ml of IL-6 were detected in the 2x10^6 cohort compared to 4.2 pg/ml and 21.4 pg/ml respectively in the NT cohort. Interestingly significantly higher levels of MCP-1 (p<0.0001) were also found in the 2x10^6 cohort with an average of 98.4 pg/ml and 21.9 pg/ml in the NT-receiving mice. These changes in the cytokine levels could suggest that CRS was ongoing in the 2x10^6 cohort. Cheadle and colleagues reported in a syngeneic model elevated IFN-γ, but not other CRS-like cytokines or any acute event similarly to other syngeneic B cell tumour models. On the other hand, elevated IFN-γ, IL-6, MCP-1 and IL-10 have been described in humanized models that reported acute side effects as a reflection of CRS. In addition, the elevated levels of MCP-1 in the 2x10^6 cohort were accompanied by a decrease in the percentage of CD11b+ monocytes both in the spleen and in the bone marrow, confirming the disruption in the homeostasis of the immune systems caused by CRS toxicity. However, the presence of a chemoattractant protein for monocytes might have caused these cells to migrate to a different organ, such as lymph nodes that were not included in the readouts. In the cohorts receiving 4x10^6 and 1x10^6, CRS-like toxicity was less clearly highlighted in the cytokine levels despite the increase in body temperature and the loss of weight. The 4x10^6 might have been a too high dose that limited the rate of engraftment and expansion of the CAR T-cells, while 1x10^6 had acute side effects as shown by the two early deaths, but the lower dose compared to the 2x10^6 might have mitigated the extent of CRS. Higher levels of IL-10 (p=0.0229) were also observed in the group with the two long-term survivors (1x10^6) with an average of 1106 pg/ml compared to 325.7 pg/ml in the NT cohort, in line with the known delayed onset of the immunosuppressive effect of this cytokine (Figure 4-24B).
Figure 4-24. Cytokine measurement at the day of sacrifice. [A] Cytokine bead array measurement performed on plasma obtained from the blood at day of take down for each individual mouse (N=4, N=3 for 1M CART) from the 4 different cohorts. The Legendplex™ inflammation panel included 13 cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β, IFN-γ, TNF-α, and GM-CSF) with a range of limit of detection between 3-12pg/ml. [B] Highlight of the most significant cytokines differences obtained with CBA analysis. The means of the different cytokines were compared for each group using unpaired t-test (****p<0.0001, ***p<0.001, **p<0.005, *p<0.05).  

In summary, of the three CAR T cells doses tested (4x10^6, 2x10^6 and 1x10^6 cells), not many differences were observed in the readouts performed. The 2x10^6 had the most CAR-related side effects, and 1x10^6 performed the best with 50% tumour-free survival. Although the 1D3-CD28ζ anti-mouse CD19 CAR was able to eradicate the tumour, the outcome also included human-like side effects with moderate control of tumour-growth. Compared to the corresponding human scenario, the high dose of CAR T-cell used for a mouse might have contributed to the small differences observed in the treated groups. While the experiment was performed only once, the outcomes have outlined the opportunity to study long-term effects of CAR T-cells and their interaction with the rest of the immune system and not least the potential side effects rising from CRS and in vivo expansion of CAR T-cells.  

4.3.6 CAR T-cells dose de-escalation for optimal CAR performance  
The three different doses of anti-mouse CD19 CAR T-cells administered in the previous dose de-escalation experiment have not shown major differences in therapy outcomes. In combination with a high tumour burden, the high dose of cells used might have exacerbated the side effects associated with CAR T-cells. While 2/4 and 1/4 of the mice that received 1x10^6 and 4x10^6 dose respectively achieved long-term tumour-free survival, the rest of the animals required earlier takedown. Although difficult to discern the tumour-growth from the CAR T-cells side effects, the increase in temperature in all the treated cohorts was commonly observed between day 9 and 10 and the high level of CAR T-cells engraftment accompanied with elevated serum cytokines in some animals have suggested possible CRS. Especially the cohort that received the 2x10^6 CAR T cells required early culling, and in parallel, two mice 1x10^6 cohort experienced similar acute early toxicity events. In addition, the significantly decreased percentage of monocytes and the presence of MCP-1 in the cohorts experiencing the worst toxicities (2x10^6) might indicate cytopenia as an additional side effect of excessive CAR T-cell activation. I concluded that the 2x10^6 and 1x10^6 doses
showed a CRS-like toxicity. In particular, the lower dose of 1x10^6 provided a 50% rate of beneficial versus side effects that could be useful to mimic a human-like scenario where severe CRS occurs in between 30-50% of anti-CD19 CD28ζ CAR T-cells. This prompted me to further investigate this relevant scenario optimizing the conditions to replicate and study CRS in the 1x10^6 CAR T-cell dose. In addition, to replicate the optimal conditions of CRS observed in the 1x10^6, a further dose de-escalation model was employed to better understand the performance of the 1D3-based CAR T-cells in a more challenging scenario. Starting from the most cost-effective CAR T-cell dose in terms of efficacy versus toxicity observed in the previous experiment (1x10^6), two further reduced doses were tested (1x10^5 and 1x10^4 CAR T cells). These ‘stress’ conditions would challenge the engineered T cells, but resemble more accurately the number of cells received by a patient (from 1x10^6 cells/kg). This experiment will allow us to further understand the potential limitations of the 1D3-based structure, and it will help to distinguish between tumour- and CAR–associated toxicities. The model has been carried out as described previously, with a survival endpoint with mice monitored throughout for tumour eradication and side effects (Figure 4-25).

Figure 4-25. Timeline of the A20 mouse model for the assessment of the minimal therapeutic dose of 1D3-CD28ζ CAR. Four cohorts of 4 mice each had been engrafted with 5x10^5 A20-FLuc+ A20 tumour cells to evaluate the lowest CAR T-cell dose to achieve tumour eradication with minimal toxic side effects. Tumour engrafted with TBI was monitored by BLI imaging and 10 days later each cohort received three different doses of 1D3-CD28ζ CAR T-cells (1x10^6, 1x10^5 and 1x10^4 cells) and the control cohort received 1x10^6 NT cells. Mice were monitored for weight loss, increase in body temperature and tumour growth. Blood was sampled 10 days after CAR-T cell administration measurement of CAR T-cell engraftment and cytokines levels in the plasma. Survival was the end point at which mice were sacrificed for the assessment of the presence of tumour cells, degree of CAR T-cells engraftment and cytokine levels.
Two spleens from healthy balb/c mice were utilized to generate enough CAR T-cells to administer three different doses ($10^6$, $10^5$, and $10^4$ CAR T-cells). The lymphocytes extracted from spleens were transduced with the 1D3-CD28ζ CAR construct, and the expression of the marker gene mCD34 was evaluated on CD3 positive T cells by flow cytometry. Comparing matching non-transduced cells (NT) have shown that 65.7% of T cells expressed the CAR construct (Figure 4-26).

![Flow cytometry images](image.png)

**Figure 4-26. Transduction efficiency of mouse splenocytes.** Representative flow cytometry gating for the assessment of CAR T-cells expression for in vivo administration. Live single-cells were gated for CD3+ T cells and the expression of the marker gene mCD34 in the CD28ζ CAR construct compared to non-transduced cells (NT).

Five mice per cohort received the three different doses of CAR T-cells ($1x10^6$, $1x10^5$ and $1x10^4$), and one control cohort received $1x10^6$ NT cells. On day 10 post-tumour injection, bone marrow tumour engraftment was observed in most of the mice when the CAR was administered, but at a lower intensity than previously observed. This might have been a consequence of a recent new ion-chamber and sources installation fitting in the SARPP irradiator. The lack of tumour engraftment was later observed in one mouse of the control group, and overall a lower tumour burden was established compared to previous experiments (Figure 4-27).
Nonetheless, the highest dose of CAR T cells tested (1x10^6) had a 100% OS after 50 days from tumour engraftment without evident side effects. On the other hand, most of the mice receiving the other two doses of CAR T cells did not manage to control the tumour, resulting in a small difference in the OS compared to the NT-receiving cohort (Figure 4-28A). By day 25, all the mice of the NT cohort required takedown, while 2 mice from the 1x10^5 and 1x10^4 cohort appeared to moderately control the tumour growth (Figure 4-28B). The body temperature did not show differences in the various cohort (Figure 4-28C), as well as the weight monitoring did not highlight any sudden weight loss except for one mouse in the 1x10^5 cohort, which triggered the endpoint for that mouse, but overall not acute side effects have reported (Figure 4-28D).

Figure 4-27. BLI imaging of representative timepoints throughout the experiment. Luminescence was represented as radiance (p/sec/cm²/sr) for each mouse and images exposed uniformly for visual comparison. Mice were euthanised at the onset of any signs of pain and/or stress and experiment concluded after 50 days post-tumour injection.
Figure 4-28. Survival and mice monitoring. [A] Kaplein-Meyer survival curve of the three differently treated mouse cohorts (N=5) (1x10⁶, 1x10⁵ and 1x10⁴ CAR T-cells) and control group receiving 1x10⁶ non-transduced cells (NT). Survival curves were compared using the Log-rank (Mantel-Cox) test. [B] BLI emission plotted as radiance (p/sec/cm²/sr), data were expressed as a mean for each cohort and reported in logarithmic scale. Monitoring of the body temperature [C] and total body weight [D] in each cohort. The means of the different measurements were compared for each group using unpaired t-test. (***p<0.005, *p<0.05).
Tail-vein bleeding was performed on day 10 after CAR T cells injection. The difference shown in the previous experiment for the $1 \times 10^6$ dose highlighted a good time point to assess cytokine levels. The absence of a spike in temperature was confirmed by the lack of differences observed in plasma cytokines levels in all the cohorts tested. IL-27 was significantly higher in the cohort receiving $1 \times 10^6$ CAR T-cells ($p= 0.035192$) compared to the NT cohort with averages of 1299 pg/ml and 269.8 pg/ml, respectively (Figure 4-29A). The increase in this pleiotropic cytokine, along with non-significant high levels of other pro-inflammatory (i.e., IFN-$\gamma$, average range 211.2–312.3 pg/ml) and immunosuppressive cytokines (IL-10, average range 1021–1890 pg/ml), might be an indication of an active polarization towards a Th1 response as a result of CAR T-cell administration. Flow cytometry analysis of the cell populations revealed the presence of CAR T cells only in the group receiving $1 \times 10^6$ and $1 \times 10^5$ doses with an average of 2.5% and 1.8%, respectively, with a statistical difference between the $1 \times 10^6$ and $1 \times 10^4$ cohorts (0.26% CAR T-cells) ($p=0.036768$). The overall low levels of CAR T cells engraftment were also reflected by the percentage of non-tumour CD19 positive cells still present in the peripheral blood compared to NT-treated mice (10.44%, 9.8%, 8.4% and 14.3% in the NT, $1 \times 10^6$, $1 \times 10^5$ and $1 \times 10^4$ respectively). No differences were observed in any of the cell populations analysed with all comparable amounts of CD11b+ cells (average range 31.3-48.6%) and CD3+ cells (average range 62.7-64.4%) (Figure 4-29B). In addition, the absolute count of each cell population confirmed the above difference. An average of 82.7 CAR T-cells was identified in the peripheral blood of the $1 \times 10^6$ dose compared to 23.6 in the $1 \times 10^5$ cohort and 1.2 CAR T-cells in the $1 \times 10^4$ cohort ($p=0.010538$). The comparable absolute count of the CD45+ lymphocytes (average range 454.7-1087 cells), CD11b+ cells (average range 638.2-1558 cells), CD3+ T cells (average range 289.3-697.3 cells) and non-tumour B cells (average range 40.3-177 cells) confirmed the lack of differences in the immune cells’ populations with
no signs of cytopenia and no tumour cells were detected in peripheral blood (Figure 4-29C).
On the day of the takedown, the spleen and the bone marrow were analysed by flow cytometry for the presence of tumour and CAR T-cells. Live cells were gated for CD3 and CD19 positive T and B cells, looking at the expression of the CAR T-cell marker gene (mCD34) and tumour (mTH1.1.) (Figure 4-30A). Good CAR T-cell engraftment was observed in spleens of the cohort receiving 1x10⁶ (6.3%), significantly different from the 0.29% detected in the 1x10⁴ cohort (p=0.003478). Similarly, the mice in the lower dose group (1x10⁵) had good CAR-T engraftment (6.1%), although these mice eventually required takedown as a consequence of tumour growth and weight loss. As expected, the presence of CAR T-cells was accompanied by the absence of detectable non-tumour CD19 positive cells with averages of 24.3%, 13.5%, 9% and 12.3% in the NT, 1x10⁶, 1x10⁵ and 1x10⁴ cohorts, respectively. In the cohort with long-term tumour-free survivors (1x10⁶), a statistically significant reduction in the percentage of CD11b positive cells (10.7%) was observed both compared to the NT and 1x10⁵ cohorts (p=0.005798 and p=0.001694) with averages of 40.2% and 43.8% respectively. Interestingly a high proportion of tumour cells were detected systemically in some mice of the NT (20%) 1x10⁵ (39.4%) and 1x10⁴ (47.7%) cohorts. This dissemination of the tumour outside the bone marrow has never been observed in previous models in this chapter and is not reported in the literature (Figure 4-30B). Similar to the spleen, in the bone marrow, high levels of CAR T-cells were detected in both 1x10⁶ and 1x10⁵ doses (22.6% and 20.8%, respectively), with a significant difference between the highest and the lowest doses (2.2% CAR T-cells) (1x10⁶ and 1x10⁴, p=0.000058). The B cell aplasia was more evident than in the spleen. In the bone marrow non-tumour CD19 positive cells were present at 18.5%, 17.6%, 1.1% and 34.5% in the NT, 1x10⁶, 1x10⁵ and 1x10⁴ cohorts, respectively. A statistically significant higher percentage of CD3 T cells were
identified in the cohort receiving 1x10^6 compared to the NT cohort (p=0.044741) with averages of 26.5% and 7.5% respectively. The complete absence of tumour cells in the 1x10^6 cohort was clear when compared against the NT and 1x10^5 groups (p= 0.011321 and p<0.000001) where tumour CD19 positive cells were detected with an average of 70.2% and 88.7%, respectively, while two mice receiving the 1x10^4 dose failed to show the presence of tumour cells in the bone marrow (average 43.3%). Interestingly, one mouse from the cohort with 100% tumour-clearance (1x10^6) had high levels of non-malignant CD19+ cells in concomitance to the presence of CAR T-cells (Figure 4-30C). The absolute cell count confirmed the statistical decrease observed in the spleen for the CD11b+ cells in the 1x10^6 compared to the NT-treated groups with average absolute cell counts of 3537 and 16402 cells, respectively (p=0.000639). Similarly, a statistical increase in the absolute number of CD3+ cells was observed in the cohorts receiving 1x10^6 compared to both the NT and the 1x10^5 cohorts (p=0.032062 and p=0.020257) with average counts of 16032, 5133 and 4377 cells, respectively. In addition, the number of CAR T-cells was statistically higher in the 1x10^6 (954.7 cells) compared to the cohort receiving 1x10^4 CAR T-cells (30.6 cells) (p=0.003556), but not different from the 1x10^5 cohort (465 cells), confirming an overall greater T cells expansion in the cohort receiving the highest CAR T-cells dose (Figure 4-31A). Likewise, in the bone marrow, more CD3+ T cells were present in the 1x10^6 cohort than the NT and 1x10^4 (p=0.043743 and p=0.043187) with average cell counts of 14195, 1583 and 1531, respectively. No other differences in cell count were significant; notably, CAR T- cells were detected in the 1x10^6 and 1x10^5 with average cell numbers of 3071 and 1002 cells, respectively, but there was no suggestion of cytopenia compared to the observations in experiment 4.3.5 (Figure 4-31B).
Figure 4-30. Flow cytometry analysis of cells from spleen and bone marrow. [A] Representative FACS plot of the gating strategy in spleen and bone marrow cells (N=4, N=5 for 10⁶ CART only). Cells were gated first on viability dye negative and single cells were gated for CD45+, then CD3+ T cells and CD19+ B cells were gated separately as percentage of the CD45 parental population. CAR-T cells were obtained by the expression of the marker gene (mCD34+) from the parental CD3+ T cells, while tumour CD19 cells expressing mTHY1.1+ were gated from non-tumour CD19 B cells. CD11b cells were obtained as percentage of live single cells. Flow cytometry analysis of the cells obtained the percentage of each cell population in the spleen [B] and bone marrow [C] at the day of the takedown for each individual mouse. The means of the different measurements were compared for each group using unpaired t-test (***p<0.001, **p<0.005, *p<0.05).

Figure 4-31. Absolute cell count. Counting beads were used to determine the absolute cell count in the spleen [A] and bone marrow [B] using 1000 bead’s events for the normalization. The means of the
different measurements were compared for each group using unpaired t-test (***p<0.001, **p<0.005, *p<0.05).

The analysis of the cytokines production on the day of the takedown confirmed the lack of the CAR T-cells-related side effects observed in the previous mouse model. There was an overall general inflammation, as demonstrated by the evenly high levels of IL-1α, IL-1β, IFN-γ, and IL-10 (Figure 4-32A). A moderate increase in TNF-alpha was observed only between the $1 \times 10^6$ and $1 \times 10^4$ cohorts ($p=0.0428$) with an average of 15.5 pg/ml and 6 pg/ml respectively, while IL-6 was significantly higher in the $1 \times 10^6$ cohort (223.6 pg/ml) compared to the $1 \times 10^5$ (26.4 pg/ml) and $1 \times 10^4$ (29.2 pg/ml) CAR T-cells doses ($p=0.0083$ and $p=0.0098$), but not compared to the NT-receiving cohort (96 pg/ml) (Figure 4-32B). A relevant difference in the cytokine profile of the long-term survivors of this model compared to the previous attempt might have been the length of time the mice remained free from the tumour. The earlier termination of this cohort had been a consequence of the building access restrictions at the time of the experiment.

In summary, unlike what was reported in the previous model, the highest dose of CAR T-cells tested ($1 \times 10^6$) resulted in the absence of side effects but complete tumour eradication. The presence of elevated IL-6 but no other relevant cytokines denoted the great CAR T-cells activation and prevented excessive activation of the engineered T cells. The two lower doses of CAR T-cells tested failed to control the tumour, although a good degree of engineered T cells was detected in the $1 \times 10^5$ cohort. The presence of B cell aplasia and increased T cell expansion have suggested that the $1 \times 10^5$ had a functional CAR T-cell response that progressively faded over tumour progression. Interestingly, the use of this lower dose would be more appropriate compared to the ratio of CAR T-cells per kilogram used in humans, and the data presented might suggest that the $1 \times 10^5$ could have worked with a more potent CAR T-cell response. An important difference that might have contributed to a lower CAR T-cells activation and related side effects was the lower tumour burden present on the day of the injection of the CAR T-cells compared to the previous model ($10^5$ vs. $10^6$). In addition, the aberrant tumour growth in some of the mice that required culling and the overall lower tumour burden observed might have been a consequence of an uneven dosage of radiation before the engraftment of the tumour. Our radiation core facility had recently changed the ion chamber in the TBI machine, and although performance was assessed after the adjustments work, not much optimization was carried out. The empirical calculation of the application of 6Gy with the new ion chamber resulted in the application of the dose
for 64sec, rather than 30sec with the previous settings. This might have contributed to
the variation of the observed, and mice were more likely to wake up during the
administration of the radiations failing to deliver the optimal conditions for studying
the toxic effects of CAR T-cell therapy in this model.

Figure 4-32. Cytokine measurement at the day of sacrifice. [A] Cytokine bead array
measurement performed on plasma obtained from the blood at day of take down for each
individual mouse of the four differently treated cohort (N=5). The Legendplex™
inflammation panel included 13 cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-
23, IL-27, MCP-1, IFN-β, IFN-γ, TNF-α, and GM-CSF). [B] Highlight of the most
significant cytokines differences obtained with CBA analysis. The mean of every cytokine
was compared for each cohort using unpaired t-test (**p<0.001, *p<0.005, *p<0.05).

4.4 Discussion

The data presented in this chapter demonstrated the successful establishment of a
syngeneic immunocompetent model of systemic B cell malignancy. I have
demonstrated the importance of effective pre-conditioning regimens for successful engraftment of the A20 tumour cells. Several groups have already proved the requirement of pre-conditioning for successful CAR T-cells engraftment in similar immunocompetent models, and it was mainly achieved through administration of total body irradiation (TBI) of the mice\textsuperscript{154,155}. I have shown that the A20 tumour cells were sensitive to cyclophosphamide, resulting in the impossibility of using this agent for the engraftment of CAR T-cells. Here I demonstrated that the application of TBI was sufficient for establishing a systemic A20 tumour and I demonstrated that the long-lasting effects of the radiations were sufficient to allow the engraftment of CAR T-cells. A previous report of a similar model using A20 cells administered a high dose of radiations prior CAR T-cell administration\textsuperscript{156}. Cheadle et al. recapitulated some CRS features (elevated IFN-gamma and TNF-alpha) and reported anomalous granuloma formation and disruption of the liver architecture, which have not been described in humans. Numerous groups have demonstrated the importance of having an immune system that can control the effects of CAR T-cells, and excessive pre-conditioning might alter the therapy's response, especially when employed in a mouse model\textsuperscript{127,141,315}.

In the model presented in this chapter I have demonstrated successful activity of the mouse anti-CD19 1D3-CD28ζ CAR, as demonstrated by the tumour eradication and B cell aplasia. Like the human scenario, the side effects reported in our model have increased with higher tumour burden and higher CAR T-cell dose\textsuperscript{54,92,321}. The observed increase in IFN-γ, IL-6, and IL-10 accompanied with weight loss and temperature rise was a possible indication of CRS, and the decreased number of monocytes in combination with elevated MCP-1 were in line with the reports of the importance of the monocytic compartments in controlling the effects of CAR T-cells\textsuperscript{141,315,322}. I have also demonstrated that the mouse 1D3-CD28ζ CAR had a functional response at previously unreported dosage, which might be relevant as a more challenging and accurate scenario for testing CAR T-cells. In addition, the comparison with standard anti-human CD19 CARs harbouring optimized scFvs might not be possible as 1D3 might not make a CAR as potent as the human ones.

In summary, the model presented here was able to recapitulate most of the human scenario with a degree of variability due to tumour burden and CAR T-cell dosage that
correlates with clinical trial observations. The CRS-like scenario, the cytopenic consequence of excessive CAR T-cells activation, and the ability to study long-term effects make this model adequate to characterise the effects of diverse anti-mCD19 therapy. Finally, the recapitulated important contribution of the on-target off-tumour in CAR T-cells activation supports the use of this model for studying the effects of targeting alternative mouse TAAs or alternative CAR architectures.

4.5 Conclusions
- Demonstrated that either Cyclophosphamide or Busulfan were insufficient for the establishment of a systemic A20 tumour model

- A20 tumour cells were sensitive to Cyclophosphamide, and this is not suitable as pre-conditioning before administration of CAR T-cells

- TBI using 6Gy have demonstrated to be suitable for the establishment of a systemic A20 tumour

- The effects of the TBI were sufficient to allow the engraftment of the 1D3-CD28ζ CAR when cells were administered 10 days post-tumour injection.

- Increased IFN-γ, IL-6, and IL-10 have been reported in the mouse model using a high dose of CAR T-cells

- I have shown that a sustained decrease in monocytes and elevated MCP-1 were associated with increased side effects

- The CRS observed in the previous model (4.3.5) was not replicated in this attempt. The difference in the preconditioning settings compared to the previous model have led to a 10 fold less tumour burden on the day of CAR T-cells injection. In humans, the side effects related to CAR T-cells are known to be related to tumour burden and to the therapeutic dose administered. The difference in tumour burden might
have certainly caused the discrepancy observed between this model and the one previously described. In addition, the detection of the tumour in the bone marrow both by BLI and flow cytometry confirmed the different degree of tumour dissemination compared to the previous mouse model that has certainly impacted the activity and expansion of CAR T-cells in the bone marrow resulting in less CAR T-cell infiltration and hence less CRS events. Further work will be needed to confirm the CRS-like toxicity observed in the previous model. More consistent delivery of the TBI preconditioning regimen before the administration of tumour cells would definitely benefit the reproducibility of the model. In addition, increasing the number of animals per cohort would help minimize the variations within each cohort.

- I have demonstrated that the 1D3-CD28ζ CAR have been able to achieve tumour eradication and long-term B cell aplasia at a high dose but failed in controlling the tumour at a more mouse-relevant dose (1x10^5)

5. Generation and characterization of novel anti-mouse CD19 antibody fragments

5.1. Introduction

The use of monoclonal antibodies as therapeutics requires optimizing several of their key attributes to achieve the best therapeutic performance. These include binding affinity and specificity, folding stability, solubility, pharmacokinetics, effector functions, and compatibility with the attachment of additional antibody domains (bispecific antibodies) and cytotoxic drugs (antibody–drug conjugates). Addressing these is a great effort, requiring reliable methods and powerful in vitro screening platforms. The binding domain of a CAR is usually derived from an antibody. However, provided that it exhibits specificity and high affinity for the target, it is not usually studied in depth. Historically, the mode of activation of CARs has been compared to TCR-MHC interaction during T-cell activation, in which the affinity of the TCR to its MHC-bound peptide ligand is tightly correlated with the efficiency of cellular activation. Efficient T cell activation requires serial triggering of a TCR that is generally of low affinity (K_d: 1 - 100 μM). This is relatively weak compared
with the reported affinities for the protein-protein interactions of the CAR binding domains currently in use\textsuperscript{173,177}.

More recently, a comparison of the contribution of scFv affinity on CAR performance has shown that high-affinity CARs exhibit less discrimination between target cells with high or low target expression levels, increasing the potential for normal tissue toxicity at low antigen levels. In some cases of low antigen expression tumours, an increased affinity results in augmented T-cell function in response to the tumour target. However, when antigen density is high on target cells, there seems to be no benefit in an increased scFv affinity\textsuperscript{172,175,177,325}. To date, 4 different anti-CD19 clones have been taken forward into clinical trials (SJ25C1, FMC63, CAT, and 4G7). The kinetic properties are reported for 3 of these scFvs and are summarized in table 5-1. Differences in affinity were mainly determined by the off-rates, leading to a significantly quicker dissociation from its target in the CAT scFv compared to FMC63 and 4G7. The faster off rate of CAT-CAR was shown to cause higher cytotoxicity and proliferative responses compared to the other CD19-CARs\textsuperscript{305}.

Evidence supports both $K_d$ and $K_{off}$ as determinants of T-cell function, where a lower affinity and a faster $K_{off}$ seem to contribute to an enhanced serial triggering of the CAR as a mechanism to improve functional avidity against targets with low antigen expression levels\textsuperscript{305,326}. In addition to scFv affinity, other crucial factors that might contribute to optimal CAR activity are expression level, location of the targeted epitope, length of the spacer, and the signalling domain. Therefore, a better understanding of the role of the antigen-binding domain would undoubtedly improve the design of future CAR structures.

<table>
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<th>Off-rate $K_d$ (1/s)</th>
<th>Dissociation constant $K_D$ (nM)</th>
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</tr>
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<td>CAT</td>
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<td>7.07E-02</td>
<td>116</td>
</tr>
</tbody>
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\textit{Table 5-1. Kinetic of anti-human CD19 clones.}

5.2. Aims

The project aimed to (I) generate a set of novel anti-mouse CD19 scFv and (II) characterize their biophysical properties for downstream integration into CAR structure to be tested in the A20 immunocompetent mouse model.
5.3. Results

5.3.1. Isolation of anti-mouse monoclonal antibodies from rat hybridoma cells

The anti-mouse scFv clone 1D3 discussed in chapter 3 has been shown to be specific and functional as CAR structures both in vitro and in vivo. The studied binding affinity have been demonstrated to be approximately 35nM with 10-fold difference in the on- and off-rates compared to the human CD19 binders. In order to model the impact of alternative binding kinetic on CAR-T function, I developed a series of novel anti-CD19 scFvs by generating hybridoma cells from rats immunized with mouse CD19, from which I could isolate the variable chain fragments for assembly as scFvs. A non-signalling version of mouse CD19 was cloned into a pVAC2 vector under the control of EF1α promoter and SC40 enhancer for high transgene expression (Figure 5-1A). The homology between rat and mouse CD19 shows a very high degree of similarity in the extracellular portion of the molecule (88%) (Figure 5-1B,C). The correct expression of the transgene was assessed in transiently transfected 293T cells, and flow cytometry shows high expression of mCD19 in the transfected cells (Figure 5-1D). Three Wistar rats were genetically vaccinated to express a non-signalling version of mouse CD19 on the cell surface.
The rat vaccination and hybridoma fusion were outsourced to Aldevron™, where sera-converted-rat B cells were fused with Y20 myeloma cells partner. Successful sera-conversion was evaluated in-house using the rat serum before and 20 days after

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**Figure 5-1. Generation of truncated-mouse CD19 protein for rat vaccination.** [A] Schematic of pVAC2 plasmid for the expression of tr-mCD19. The plasmid contains a CpG-free bacterial promoter (EM7) and a CpG-free Zeocin-resistance gene (Sh-ΔCpG). The EF1α gene promoter and its polyadenylation signal (EF1pAN) combined with SV40 enhancer drive expression of the transgene in mammalian cells. [B] Alignment of rat and mouse CD19-extracellular domain and [C] their relative percentage of homology. [D] pVAC2 expression in transiently transfected 293T cells. Cells were stained for flow cytometry with anti-mCD19 antibody using non-transfected cells (NT) as a negative control.
vaccination to stain mCD19 positive cells. All three rats have demonstrated effective sera-conversion specific for mouse CD19 positive cells (Figure 5-2).

Rats were euthanized, and B cells fused with the myeloma partner to generate ten 96-well plates containing oligoclonal hybridoma cells. The supernatant from these wells was tested for the ability to bind mouse CD19 positive Supt1 cells specifically. Due to the initial small amount of supernatant available (~100µl), the binding was compared to cells stained with only the secondary polyclonal antibody for rat IgG. The final bleed obtained from the vaccinated rats was used as a positive control for specific staining of

**Figure 5-2. Genetic vaccination of mCD19 in rats.** [A] The mCD19 negative cells (SupT1 NT) and mCD19 positive cells (A20, SupT1 mCD19) were used as negative and positive controls respectively and stained with the commercial anti-mCD19 antibody or a secondary anti-rat IgG. [B] Staining of rat’s serum obtained before and after genetic vaccination with mCD19. The serum from three different Wistar rats was used for flow cytometry staining of A20, SupT1 NT and Supt1 mCD19 respectively from bottom to top in the same order as panel A. The detection of binding was assessed using an APC-conjugated secondary anti-rat IgG antibody.
mCD19 positive and negative Supt1 cells (Figure 5-3). The median fluorescence intensity (MFI) was used to discriminate between positive and negative clones: moreover, negative clones had a very similar MFI to the secondary antibody alone, making them a further internal control.

In the 10 different hybridoma plates, the vast majority of the wells screened were negative, but a number of wells showed a clear increase in MFI. An average of 7 positive clones per plate were identified (range 2 to 14) in the 10 different (Figure 5-4).
The corresponding cells plate were thawed and expanded in larger volumes, and an aliquot was frozen prior to single-cell cloning. A density of 1 cell per well was obtained by serial 1:10 dilution from $10^5$ live hybridoma cells. Four plates were produced for each of the expanded positive clones and left to expand further until visible colonies. (Figure 5-5).

After limiting dilution, the supernatant from successfully expanded wells was used to assess that specific binding for mCD19 was retained. Six oligoclonal wells successfully grown as single clones and specifically bind mCD19 positive cells. An aliquot of the monoclonal hybridoma cells was banked before RNA extraction to solve the antibodies’ variable chains (Figure 5-6).

**Figure 5- 4. Binding test of rat hybridoma supernatant.** Analysis of the median fluorescence intensity (MFI) from the staining of 10x 96-well plate of hybridoma supernatant. Positive binding was detected with a polyclonal anti-rat IgG secondary antibody. The dotted line represents the background fluorescence of the staining on antigen negative cells.

**Figure 5- 5. Limiting dilutions of positive hybridoma clones.** [A] Schematic illustration of the limiting dilutions strategy used to obtain single cell clones from each of the positive oligoclonal hybridoma well. Oligoclonal hybridoma cells were expanded in a 6 well plate prior single cell dilution into 4x 96 well plates. Upon growth of the single clones, these were furtherly expanded in a 24-well plate prior to cell recovery for the isolation of the monoclonal antibody. [B] Brightfield image (20x) of growing hybridoma cells before and after single cell cloning.
The expected number of potential clones was 70 in total; however, the requirement for repeated manipulation of these fragile cells dramatically reduced clonal recovery. Moreover, the limiting dilution step combined with some non-specific clones has further diminished the total number of clones obtained from the hybridoma campaign (Table 5-2).

Figure 5-6. Binding test of the expanded single cell hybridoma clones. Flow cytometry staining using supernatant from the expanded single-cell hybridoma clone. The supernatant was used to stain both SupT1 NT and SupT1 mCD19 cells and the binding was detected with a secondary anti-rat IgG-APC antibody.
Table 5-2. Summary of the mCD19 hybridoma campaign. Screening and expansion rate of the positive hybridoma clones from each of the 10 initial oligoclonal plates. At least two passages of expansion for each positive well were required to obtain an adequate number of cells for the limiting dilution and final staining confirmation and the RNA extraction for the downstream variable-chains solving. Few successfully expanded clones exhibited non-specific binding assessed by flow cytometry on SupT1 NT and SupT1 mCD19 cells.

The rat heavy and kappa light chains for each of the six cloned isolated were obtained using a SMART 5’RACE protocol, as described in the methods section. The total mRNA obtained from the hybridoma cells was universally tagged to ease the subsequent PCR reaction using gene-specific primers. A 700bp PCR band were amplified for each of the clones, and the sequences were obtained through Sanger sequencing; when more than one single cell clone was available it was used to retrieve the antibody sequence. (Hyb#1_A6 and Hyb#3D3= 3 clones, Hyb#1_B6= 8 clones, Hyb#2_C11, Hyb#4_G4 and Hyb#6_G9= 1 clone) (Figure 5-7).
After RNA extraction, the cDNA was universally tailed at the 5’ end using SMART protocol. This was used as template for the amplification of the HC and LC using gene-specific primers. Each PCR was run on an agarose gel, and the correct-size band (~700bp) was extracted for DNA sequencing. Each of the sequences obtained was confirmed to be rat’s rearranged HC and KLC sequences and originated from four different families of HC (family 1, 2, 5 and 8) carrying unique CDR3s, both in the HC and in the KLC (Figure 5-8A). Phylogenetic analysis of the whole scFv sequence confirmed the presence of distinct sequences with minimal degrees of similarity (Figure 5-8B). The scFv sequences were then cloned into SFG plasmids for expression of both scFv-mIgG2aFc and chimeric mouse IgG2a/Kappa antibody formats. Only 4 out of six of the antibodies generated demonstrated specific binding of mCD19 positive supt1 cells. The 1D3 clone was used in both formats as positive control, and the four novel positive clones showed equivalent binding to 1D3 in both formats (Figure 5-8C).
Figure 5-8. Analysis of the variable-chains sequences from the monoclonal hybridoma clones. [A] Families identification of the HC and LC for the six different clones and their unique CDR3 of the HC. [B] Phylogenetic analysis of the whole scFv sequence of each clone. (0=completely different; 1=completely identical). [C] Flow cytometry staining using cell supernatant containing the anti-mCD19 clones either as scFv-mlgG2a-Fc or full mouse IgG2a antibody formats. Binding was detected with a secondary anti-mlgG antibody and secondary alone was used as negative control. 1D3 clone was used as control to stain both SupT1 NT and SupT1 mCD19 cells.
5.3.2. Phage display library generation and selection

To increase the chance of developing a diverse set of scFv with unique biophysical characteristics, a greater number of unique scFvs were required. From the hybridoma campaign, 4 functional and diverse clones were obtained. However, further antibody generation through the use of immune libraries and phage display to isolate specific scFv fragments can be performed. Our lab has successfully developed a combinatorial approach of rat vaccination and phage display to increase scFv diversity and stringency to obtain a high affinity-set of scFvs.

Phage display involves the expression of a library of interest on the surface of filamentous phages, in this case, an immune library from the mCD19 rat vaccination. Phages are then selected through the biopanning process against the target of interest, usually anchored on a solid support. After extensive washing, phages are eluted for the infection of *E.Coli* and amplification of the selected library before the next round of biopanning (Figure 5-9A).

Our traditional method employs StrepTagII tagged-antigen immobilized on streptactin-beads for the capture (and washing) of specific phages. These are then recovered from the beads with an excess of biotin to displace the target from the beads (Figure 5-9B). The requirement for a successful biopanning process is to have the target antigen stably displayed on the surface of the beads.

Mouse (and human) CD19 antigens are unstable as a secreted protein. This makes them difficult to use to reliably coat streptactin beads for standard biopanning (Figure 5-9C). To overcome this, Kerry Chester’s lab (and other groups) have successfully used phages selected on mammalian cells expressing the target antigen. A negative selection of target negative cells is strongly recommended to decrease the non-specific background.

In this project, I generated an A20 mouse cell line, knock-out (KO) for mCD19 through the use of CRISPR/Cas9 system, which was used as negative selection control, and the wild-type (WT) A20 cell naturally expressing the antigen for the positive selection. As explained in detail in the methods section, after washes, phages were eluted using a trypsin-EDTA solution for 5 minutes and used to infect *E.Coli* TG1 cells.
The cell suspension from the lymph nodes and the spleen that were not fused for hybridoma generation were used for mRNA extraction. This was extracted from several $5 \times 10^6$ cells aliquots and the quality of each RNA sample was assessed prior to cDNA

**Figure 5-9. Phage display method to discover anti-mCD19 scFvs.** [A] Schematic representation of the phage display process. [B] In-house biopanning strategy to select phage libraries against soluble antigens captured on magnetic beads. [C] Assessment of the coating of Streptactin™ magnetic beads with secreted truncated-mCD19, two alternative antigens were used as positive control of successful beads-coating (CD160 and CD21) and beads NT as negative control. Binding on the secreted proteins on the beads was detected either by using a commercial antibody or anti-mIgG for the staining of the mIgG2a-Fc spacer.
conversion. All the samples extracted from the lymph-nodes were not suitable for downstream applications, while the vast majority of the RNA obtained from spleen aliquots had RNA Integrity Number (RIN) greater than 7.9, hence were good quality for the subsequent reverse-transcription reaction (Figure 5-10A). RNA from three different aliquots (S1 1, S2 1, and S2 2) was pooled together for the cDNA conversion and this used as a template for the amplification of the HC and KLC.

Our set of primers for the amplification is designed on the IMGT database for *Rattus Norvegicus*, which lists 232 germline HV sequences with 13 families and 164 kappa LV sequences in 21 families. Both the forward and reverse primers are made of two parts: the annealing sequences to each specific gene family and a primer tail of a 21 to 33 bp DNA sequence. The 400bp PCR product obtained in the primary amplification was extracted from the agarose gel, and the pool of the amplified products was inspected for the correct size using capillary electrophoresis (Figure 5-10B, C). Almost all the KLC primers successfully amplified the variable genes (35 out of 39), while 18 out of 29 showed specific DNA amplification (Figure 5-10D).
Figure 5-10. Amplification of rat variable chains for the generation of rat’s immune library. [A] Capillary electrophoresis concentration and quality assessment of the RNA extracted from lymph nodes (LN) and spleen (S) of mCD19-vaccinated rats. RNA extracted from different frozen aliquots of homogenised tissues and quality expressed as RNA Integrity Number (RIN). [B] Representative gel image of the primary PCR amplification of the HC and LC with an expected 400bp band. [C] Purity assessment of the extracted primary product by capillary electrophoresis. [D] Summary of the successful primers (green) used to amplify the HC and LC.
The primer tail at the 5’ end of the VH gene and the 3’ end of the VL gene contained the annealing sites for the nested PCR outer primers and SfiI/NotI restriction sites for cloning into the phagemid vector (pHEN1<sup>296</sup>). The tails at the 3’ end of the VH and the 5’ end of the VL included the serine-glycine linker sequence (3xSGGGS) as overlapping regions. The primary PCR amplification of VH and VL chains incorporated the outer tail regions. Overlap extension PCR created a single amplicon encoding an scFv in the VH-VL orientation separated by a serine-glycine linker sequence. Due to the presence of a short PCR product (600bp), the correct DNA size (800bp) was serially extracted and purified from an agarose gel to increase the purity of the correct insert. As confirmed by capillary electrophoresis, after the first gel extraction, the shorter product was still present in the sample (Figure 5-11A), but after the second run of extraction and purification, the short band was almost undetectable with only the presence of fragments greater than 800bp (Figure 5-11B).
The extracted full-length scFv DNA band was purified and ligated into the phagemid vector for electroporation into TG1 E.Coli cells. The successful incorporation of the insert was assessed by DNA sequencing of a small number of individual bacterial colonies. The IMGT alignment analysis has demonstrated that of the 30 individually picked colonies, 29 contains a productive HC with only one carrying a stop codon in the framework region 1, while 28 had a productive KLC with two colonies containing a stop codon both in framework region 4. The circos plots show the pairing between the variable chains (HV and KV) genes and their associated joining region (J), highlighting great diversity for both the chains with all unique clones. As reported in the literature for rat’s germline genes usage\textsuperscript{195}, most of the HV clones were coming from family 5 followed by family 2 and 6, mainly associated with the J2 region. On the
other hand, the KV did not highlight any major difference in the gene usage with equally distributed variable genes paired equally with J5 and J1 followed by J2. The overall association of the variable genes for each scFv (HV-KV) reflects the non-redundancy and good diversity of the library’s clones (Figure 5-12).

**Figure 5-12. Analysis of the diversity of the rat anti-mCD19 phage library.** Circos representation of the pairing between the variable chains of the HC (HV) and LC (KV) with their corresponding joining region (J) as well as the pairing of each scFv clone (HV-KV).

The library size estimate was calculated by serial dilution of the library’s bacterial stock and plated on antibiotic-selective 2TY plates. From the manual count and considering...
the 10% non-productive colonies from the sequencing analysis, the library size was approximately $10^8$ diverse clones (Figure 5-13).

Our traditional biopanning method could not have been applied due to the instability of the mCD19 antigen when expressed as a soluble protein. Hence, I decided to employ a cell-based selection strategy for the correct display of the target molecule. For this purpose, A20 mouse cells naturally expressed mCD19 were used for positive selection. Due to the high background of non-specific binding, a negative selection step using A20 cells KO for mCD19 was performed to try to remove non-specific phage particles recovering the negative fraction from the wash steps. After two initials positive biopanning rounds, the library was furtherly selected on A20 WT with or without the negative step in the middle. The specific enrichment for mCD19 was estimated by colony count of the phage-infected *E.Coli* after each round of biopanning and compared with the library selected on mCD19 negative A20 cells. No enrichment was observed.
in the number of colonies between A20 KO and A20 WT throughout the whole biopanning process (Table 5-3).

<table>
<thead>
<tr>
<th>Phages</th>
<th>Titration</th>
<th>A20_KO</th>
<th>A20 WT</th>
</tr>
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<tbody>
<tr>
<td>P1</td>
<td>$10^{13}$</td>
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<td>P2</td>
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</tr>
<tr>
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</tr>
<tr>
<td>PPNP</td>
<td>$10^{10}$</td>
<td>$10^7$</td>
<td>$10^7$</td>
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*Table 5-3. Summary of the number of colonies obtained throughout the biopanning processes. This includes a positive round of selection against A20 WT (P) and negative selection against A20_KO cells (N). The number of phages corresponds to the colonies count of TG1 infected cells.*

In order to assess the specific enrichment for mCD19 after the phage display selection, the phage particles containing the scFvs were induced to secrete these as soluble Myc-tagged fragments. The supernatant from each of the rounds was used to stain Supt1 mCD19 positive cells detected by flow cytometry using an anti-Myc tag mAb. The unselected library (P0) and the secondary antibody were used as negative controls and a separate successful library (CD160) was used as both negative and positive controls for the induction of scFv expression from phage particles. Of the various rounds of biopanning for mCD19 none of these has shown any specific enrichment for the target antigen (Figure 5-14A). A small number of individual colonies were also picked and induced to express the scFv for a further binding assessment by flow cytometry, confirming that none of the colonies was able to bind mCD19 (Figure 5-14B). The DNA sequencing analysis showed that although all the scFv were functional, these seem to have gone towards monoclonal convergence with several repeated clones and, overall, very little diversity among all the sequences (Figure 5-14C).
**Figure 5-14. Analysis of the enrichment of the phage library for anti-mCD19 scFv.**

[A] Phage-infected E.Coli supernatant from each round of selection was used to stain SupT1 cells and a secondary anti-Myc antibody was used to detect binding. Commercial anti-mCD19 antibody and supernatant from a separate successful phage display library (CD160) were used as controls. [B] MFI of the staining using supernatant from twelve individually picked colonies, controls to stain SupT1 cells include commercial anti-mCD19 antibody and secondary anti-Myc antibody only. [C] Matrix of the alignment of the DNA sequence for each of the twelve individual colonies. From green to red represents the heatmap colours based on the percentage of homology.
5.3.3. Biophysical characterization of the novel anti-mouse CD19 scFvs

A total of six mAbs were obtained from the hybridoma-screening campaign, from which four of these (H2C11, H3D3, H4G4, H6G9) were confirmed to specifically bind mCD19 antigen both as full mouse antibody and scFv format. A layer of initial characterization for the discrimination of newly discovered antibodies includes the study of the kinetic, thermal stability, aggregation profile, and epitope competition to identify differences in the binding location. For comparison, the biophysical properties of the new scFv were assessed alongside the already available anti-mCD19 clone 1D3.

The first step for characterizing antibodies was to produce and purify the scFv fused with a mIgG2a-Fc expressed in SFG vectors. CHO cells transfected with these plasmids were used as producer cell lines to express the four antibodies plus 1D3 cloned in the same format as the other scFvs. The supernatants from small-scale transfected CHO cells were run through the AKTA™ start system and purified using a protein A pre-packed column with a high affinity for mIgG2a-Fc. The protocol for purification was run according to the manufacturer's instruction, and the column-bound scFvs were washed with 20 column-volumes before the fractionated elution. The whole process was monitored through the built-in UV-detector allowing real-time inspection of the various steps of the purification for the presence of the protein-peak (Figure 5-15A). The purified proteins were run on SDS-page gel to assess the purity of the proteins, and these were quantified using a BCA assay with BSA as a standard curve. All the proteins were successfully purified with no presence of residual albumin or other contaminant proteins. In reducing conditions, scFvs-Fc proteins were expected to run at 50KDa with only one single band present. Most of the proteins also contained small fragments at around 25KDa, which might have represented a breaking point between the scFv and its mIgG2a-Fc, potentially indicating lesser stable proteins. The quantification performed on the total protein demonstrated good amounts of the purified proteins with a concentration range between 0.4-0.8mg/ml (Figure 5-15B).
The kinetic characterization was assessed by SPR technology using BiacoreT200 system. The purified scFvs were captured on a protein A-coated CM5 chip, and commercial mCD19-His tag was injected as the analyte at different concentrations in a multi-cycle kinetic run. A range from 3.7nM to 600nM of analyte was injected in six independent cycles for each scFv, and the resulting sensorgrams were double

**Figure 5-15.** Protein purification of anti-mCD19 scFv-mIgG2a-Fc clones. [A] Overview of the purification performed on the AKTA start system. The process is monitored throughout all the steps using the UV (A280nm) detector. A protein A-pre-packed column are equilibrated with 10 column volumes (CV) of binding buffer, after sample application unbound proteins are washed-off with 20CV of binding buffer and the finally the proteins is eluted into ten different fraction using acidic sodium citrate (pH 3.0). Protein peak for scFv-Fc was consistently present in fraction 6 and 7. [B] SDS-page run and Coomassie staining of the purified scFv-Fc proteins. The expected size in reducing condition was ~50KDa. BSA standard curve was used for the quantification of each purified clone and concentration determine using BCA assay.
referenced subtracted for the reference flow-channel and the buffer-blank (0nM mCD19). The binding data were then locally fit with the 1:1 Langmuir binding model, and all the clones showed a perfect fit within the instrument's limit of detection (Figure 5-16A). The extrapolated values of association (Ka) and dissociation rate (Kd) were used to calculate the affinity (KD) for each scFv. All were high affinity with binding kinetics in the nanomolar range and Chi² value less than 1% of the global R_max, confirming the good fit of the binding model (Figure 5-16B). I identified a slow on and off-rate for almost all the scFvs with values very similar to 1D3 clone, H3D3 was the only clone with slower Ka and Kd compared to the rest of the clones (Figure 5-16C).
The melting temperature analysis was carried out as a measurement of the stability of each scFv. The thermal stability of a protein is typically described by the 'melting temperature' or 'Tm,' at which 50% of the protein population is unfolded, corresponding to the midpoint of the transition from folded to unfolded. This analysis was performed using a dedicated label-free machine, which uses nano differential scanning fluorimetry (Nano-DSF) to determine protein stability by employing intrinsic tryptophan or tyrosine fluorescence. Both the fluorescence intensity and the fluorescence maximum strongly depend on the close surroundings of these two residues. Upon unfolding, the ratio of the fluorescence intensities at 350 nm and 330 nm is suitable to detect any changes in protein structure (Figure 5-17A). The melting analysis for anti-mCD19 scFvs revealed different stability profiles with a wide range of temperatures from less than 50°C to 60 °C (Figure 5-17B). The Tm values of the inflection point 1 corresponding to the scFv-unfolding transition confirmed the stability observed in the SDS-page run with 1D3 and H3D3 being the least stable while H4G4 and H6G9 being the most stable clones. The inflection point 2, corresponding to the mIgG2a-unfolding transition, confirmed the validity of the run with all similar values for all the scFvs as expected for a disulphide-bond interaction (Figure 5-17C).
Figure 5-17. Melting temperature analysis of the anti-mCD19 scFv-mIgG2a-Fc clones. [A] Nano-differential scanning fluorimetry (DSF) analysis. First derivative of the ratio of the absorbance at 330nm and 350nm for each of the anti-mCD19 clone. Ramping of 1oC/min from room-temperature (25oC), the first peak represents the scFv transition from folded to unfolded while the second common peak represents the breaking of the disulphide-bonded Fc-domain. [B] Comparison of the melting temperature (Tm) profile of each scFv. [C] Summary of the Tm values obtained for the scFv (point 1) and the Fc (point 2) transitions.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Inflection Point 1</th>
<th>Inflection Point 2</th>
</tr>
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<tbody>
<tr>
<td>1D3</td>
<td>48.3°C</td>
<td>77.9°C</td>
</tr>
<tr>
<td>H2C11</td>
<td>53.4°C</td>
<td>77.0°C</td>
</tr>
<tr>
<td>H3D3</td>
<td>49.7°C</td>
<td>76.2°C</td>
</tr>
<tr>
<td>H4G4</td>
<td>60.5°C</td>
<td>75.8°C</td>
</tr>
<tr>
<td>H6G9</td>
<td>59.6°C</td>
<td>78.3°C</td>
</tr>
</tbody>
</table>
A further layer of characterization was represented by studying the competitive binding of each scFv against the reference clone 1D3. The competition assay was performed using Biacore 8K that allows the parallel run of up to seven scFvs, with the eight being the reference flow-channel. The experiment was set up to covalently coat the surface of CM5 chip with the reference clone 1D3. A saturating amount of mCD19 analyte (1µM) was then injected for binding on the 1D3 clone. Binding competition for the second antibody was determined by normalisation of the same antibody pair to adjust for dissociation and then compared with the Rmax obtained with the second antibody to determine an increase in non-competing antibodies (Figure 5-18A). An anti His-Tag antibody was used as a positive control for the binding on a non-competing location at the C-terminus of mCD19. Increased response units (RUs) were observed for both H3D3 and H4G4. Comparison with the competition profile of 1D3 against itself, H2C11 and H6G9 confirmed the Rmax increase of H3D3 and H4G4. While the latter increased in Rmax greater than the His-Tag, H3D3 had a lower increase in RUs close to the instrument limitation (1 RU) (Figure 5-18B). This suggested that H4G4 binds on a more distal epitope than 1D3, while H3D3 might bind to a partially overlapping epitope. I concluded that of the 4 novel anti-mouse CD19 antibodies, 2 had an overlapping epitope with 1D3, one partially overlapping, and one separate (Figure 5-18C).
5.3.4 Evaluation of the cytotoxic activity of the new anti-mouse CD19 scFvs

The characterized four novel anti-mouse CD19 scFvs were cloned into the same CAR architecture as the 1D3-based CAR showed in chapter 3 (Figure 3-8) to compare their cytotoxic activity as 2nd generation CD28ζ CAR structures.

Three different balb/c spleens were processed to obtain a single-cell suspension before viral transduction. The expression of the CAR marker gene (mCD34) was assessed by flow cytometry on live CD3 positive cells and compared to non-transduced T cells (NT). All constructs demonstrated high level of expression of the marker gene (between 86.2% and 89.2%), comparable to 1D3-CAR (Figure 5-19). CAR-engineered T cells
were then used in a cytotoxicity assay against 4 different targets cells (SupT1 and A20 cells), either mCD19 positive or negative cells, as previously shown in chapter 3 (Figure 3-12A). 5x10^4 effector cells were plated at 4 different E:T ratios (1:2, 1:4, 1:8 and 1:16) to determine killing abilities and cytokine release of the novel mCD19 scFvs in comparison to the reference 1D3 CAR. After 48h, cells were stained for the flow-

Figure 5-20. Representative cytotoxic assay gating strategy. Flow cytometry-based assay for the evaluation of the absolute number of target cells after 48h co-culture with anti-mCD19 CARs. Single cells gated from the FSC-H against FSC-A and subsequently gated for live cells only. T cells were discriminated from SupT1 target cells by their surface expression of mouse CD3 and CD45, while A20 target cells were identified by the expression of the marker gene (mTHY1.1). An equal number of counting beads was included in every sample to ensure accurate normalization of the absolute count of the number of residual target cells.
cytometry based killing assay. Single live cells were gated for A20 target cells based on the expression of the marker gene (mTHY1.1), while SupT1 targets were gated based on their lack of expression of mCD3 and mCD45. The residual number of target cells was normalized using counting beads and the percentage of live target was obtained by comparison with the NT-effector cells conditions. (Figure 5-20).

This assay has shown poorer overall cytotoxicity compared to that outlined in chapter 3. CAR-T cells have been able to modestly kill SupT1 mCD19 cells with an average of residual cells at a 1:2 E:T ratio of between 66.1% to 81.2%. Similarly, on A20 cells, the residual cells at a 1:2 ratio were between 61.6% to 80.4%. Nonetheless, all the CARs tested demonstrated cytotoxicity comparable to the 1D3-based CAR. No background killing was observed on antigen-negative cells compared to the non-transduced cells (Figure 5-21).

Figure 5-21. Cytotoxicity assay. Splenocytes from three wildtype balb/c mice’s spleens were processed and transduced (n=3) with retrovirus to express the five anti-mCD19 2nd generation CARs fused. Mouse T cells were incubated with target positive (SupT1 mCD19 and A20) and negative (SupT1 NT and A20 KO) cells at four different target ratios (1:2, 1:4, 1:8 and 1:16). After 48h co-culture, the percentage of residual target cells was obtained by normalization with the target cells of matched NT-T cells conditions. The different degree of cytotoxicity was compared with the reference control CAR (1D3-CD28ζ) using 2-way ANOVA with Turkey correction to account for multiple comparisons.
The 48h-harvested supernatant was used to assess IFN-γ and IL-2 production as a measurement of T-cell activation and cytokine release. Unlike the cytotoxicity assay, significant differences were observed with IFN-γ secretion in comparison to the reference 1D3 CAR. On target positive cells (Supt1 mCD19 and A20), all CARs demonstrated IFN-γ secretion, with H4G4 secreting significantly more (p<0.0001 and p=0.0025) than 1D3, while H2C11 and H6G9 produced significantly less IFN-γ at 1:2 (p<0.0001) and 1:4 (H2C11, p=0.0491) E:T ratios compared to 1D3. Similarly, H2C11 released significantly less IFN-γ than 1D3 CAR at 1:2 E:T ratio (p<0.0001).

On the other hand, there was not much IL-2 production overall. H2C11 produced significantly less IL-2 against mCD19 positive Supt1 cells at 1:2 E:T ratio (p=0.0439) than 1D3. H6G9 CAR demonstrated significant non-specific production of both IFN-γ and IL-2 at 1:2 and 1:4 ratios on antigen positive and negative cells with a very modest increase in the presence of mCD19. This might be a consequence of spontaneous protein aggregation of the scFv H6G9 or non-specific binder that results in ligand-independent activation167.

Despite improvements in scFv thermal stability and the addition of a further binding epitope, this campaign did not generate reagents with a significant enhancement of the in vitro cytotoxicity activity of current anti-mouse CD19 CARs. Notably, H2C11 showed significantly reduced cytokines while H6G9 produced significant background T cell activation, independent of target antigen. This latter event was unexpected and not highlighted by biophysical characterization. Overall, the similar kinetic profile of the novel scFvs compared to the reference 1D3 were a good predictor of the performance of each new CAR, as these demonstrated to have comparable activity to the reference CAR.
Figure 5-22. Cytokine release assay. The supernatant harvested after 48h of cytotoxicity assay (n=3) was used in an ELISA assay for the quantification of mouse IFN-gamma [A] and IL-2 [B]. The amount in pg/ml each condition was obtained by interpolation of each experimental condition with the standard curves with $R^2 \geq 0.99$. The differences in the amount of cytokines produced were compared with the reference control CAR (1D3-CD28ζ) using 2-way ANOVA with Turkey correction for multiple comparisons ($****p<0.0001$, **$p<0.005$, *$p<0.05$).
5.4 Discussion

This chapter presented the successful generation of novel high-affinity anti-mouse CD19 antibodies and their biophysical characterization. I have employed two different approaches (hybridoma and phage display) to obtain a set of antibodies with diverse properties. From the hybridoma campaign, I have obtained four unique mAb specific for mouse CD19. The high loss of potential hybridoma clones was a mixture of difficulties in culturing and recovering these cells. The intrinsic fragility of the hybridoma platform combined with continuous cell culturing and inexperience in handling these types of cells were known factors that affected the success of the campaign. The engineering of human CD19 has highlighted the instability of this protein when expressed in a soluble format, and attempt to improve its stability (i.e., fusion with Fc domains) have only marginally extended its half-life. Due to the structural homology between human and mouse CD19 I concluded that the hybridoma approach would have had higher chances of obtaining antibodies as no soluble protein was involved. Indeed, I have confirmed through the use of phage display that mouse CD19 was indeed unstable for conventional biopanning strategies and required the use of whole cells in the attempt to enrich for specific antibodies. The assemble phage display library showed productive sequences and great diversity but failed to be positively enriched after multiple rounds of biopanning. The analysis of the obtained sequences confirmed the aberrant phage-enrichment as the majority of the clones were identical and overall skewed towards this non-specific sequence. The use of whole cells have greatly affected the phage display selection process that combined with the “stickiness” of phage particles are a known source of non-specific background.

The characterisation of the four new anti-mouse CD19 antibodies' biophysical properties revealed a very similar high-affinity kinetics to the reference clone (1D3). One antibody clone showed a much slower off-rate than 1D3 compensated by an almost ten-fold difference in the on-rate, resulting in the same nanomolar affinity. The on-rates of the novel antibodies are more than 10 fold lower than the human gold standard CD19 binders (FMC63 and CAT) but comparable to 4G7, and also the off-rates fall in the same magnitude of 4G7 and FMC63 with overall lower affinity values. The lack of significant differences in the novel anti-mouse CD19 antibodies with the reference clone 1D3 might have been a consequence of the rats’ immunization process. The high
homology of CD19 in mice and rats could have resulted from the central immune tolerance to exclude higher affinity antibodies that might have caused a severe immune reaction in rats. The use of rabbits would have circumvented this issue, but no hybridoma fusion services were reasonably available. The thermal folding analysis highlighted a great improvement in the stability profile of the new clones with up to twelve degrees difference in the melting temperature. Low thermal stability is an indication of potential self-aggregation that results in the occlusion of the binding pocket, and in the context of CARs structure, it has been demonstrated to reduce the CAR surface expression and can induce antigen-independent signalling and, therefore non-specific T cell activation\textsuperscript{248,334}. The competition assay has interestingly revealed two additional epitopes for the binding of the antibodies. The modest increase in the response unit might suggest that the epitope of H3D3 might partially overlap with 1D3, while H4G4 binds to a separate portion of mouse CD19. Currently, no crystal structure is available for mouse CD19. The structural homology with human CD19 predicts a similar folding to the unique pattern of human CD19. Rather than a tandem of c-type immunoglobulin folds predicted from the amino acid sequence, the extracellular domain of CD19 exhibits an elongated $\beta$-sandwich formed by the two immunoglobulin domains\textsuperscript{335}. Fine-epitope mapping of the human CD19 binders revealed that most clones (FMC63, 4G7, and CAT) have distinct but overlapping epitopes located in the loops of exons 3 and 4\textsuperscript{72,336}. The immunodominance of one epitope in the human CD19 protein suggested a similar effect on mouse CD19, and indeed two antibodies shared a completely overlapping epitope and one partial-overlapping. The identification of a separate binding epitope for one of the antibodies is a feature that has not been described before in both mouse and human CD19. In the context of CAR-T cells, the location of the binding epitope has been demonstrated to be crucial for optimal T cell activation and cytotoxicity\textsuperscript{334,337}. Unlike the targeting of other antigens (i.e., CD22 and mesothelin), all the human CD19 CARs share the same binding spot and no major differences have been observed in their overall performance\textsuperscript{118,250,338}. Moreover, it has been shown that the occurrence of CD19 negative relapse is a consequence of a loss of the binding epitope\textsuperscript{99}. The alternative splicing reported in exon 2-5 and the immune pressure exerted by the therapy has been shown to be the main drivers of CD19 negative relapses with consequent non-applicability of CD19-targeting therapies\textsuperscript{36,109,110,339}. Although the mechanism of tumour escape by antigen-negative relapses has not been fully elucidated yet, the targeting of a different epitope might help elucidate the
emergence of CD19 negative relapses as well as the different performances of anti-CD19 CAR-T cells.

In summary, in this chapter, I have generated and characterised novel rat antibodies specific for mouse CD19. The high affinity of these clones was comparable to the reference clone. They exhibited higher thermal stability and binding to two additional epitopes on mouse CD19. The biophysical properties had suggested that these novel scFv would make a CAR with similar performances to 1D3, and indeed these have demonstrated comparable in vitro CAR cytotoxicity to the reference 1D3. Notably, the cytokine release assays have shown that H6G9 induces non-specific T cell activation, while H2C11 underperformed compared to 1D3 CAR. As these events were not related to their biophysical properties, it will be interesting to elucidate the cause of their different behaviour. Further studies could also investigate the contribution of the significant enhancement in stability and the additional binding epitopes to their in vivo performance.

5.5 Conclusions
- I have generated a signalling-deficient version of mouse CD19 and successfully expressed it in pVAC2 plasmid
- Rats immunized with the pVAC2 plasmid demonstrated good serum-conversion specific for mouse CD19
- The hybridomas generated from rats splenocytes were able to produce specific antibodies against mouse CD19
- After limiting dilutions and cloning of the positive hybridoma clones, four of them have demonstrated specific binding, both in full antibodies and scFv formats
- A phage display library assembled from the rats’ lymphocytes and selected against whole cells expressing mouse CD19 resulted in unsuccessful isolation of specific antibodies
The novel anti-mouse CD19 antibodies were successfully produced in CHO cells and purified for biophysical characterization.

Biacore technology was employed to study the kinetics of the antibodies highlighting all high-affinity antibodies comparable to the reference clone 1D3.

Melting temperature analysis revealed high thermal stability for all the identified clones, greater than the reference clone 1D3.

Competition assays with the reference clone 1D3 have demonstrated two overlapping epitopes, one partially overlapping and one separate.

CAR cytotoxicity assay has demonstrated that all the novel anti-mouse CD19 scFv possess comparable activity to the reference 1D3-based CAR.

H2C11 produced significantly less IFN-γ and IL-2 at low effectors to targets ratios and H6G9 significantly secreted high levels of these cytokines in the absence of antigen-positive cells.

6. General discussion and future work

Chimeric antigen receptor T-cells have demonstrated significant efficacy in treating relapsed or refractory (r/r) B cell malignancies. The most striking success has been achieved in this setting by targeting CD19 on B cell cancers that resulted in the FDA approval of two CD19 CAR T-cells products for r/r B cell acute lymphoblastic leukaemia (B-ALL) in young adult patients and diffuse-large-B cell lymphoma (DLBCL) in adult patients. Although anti-CD19 CAR T-cells have proven to be highly effective for treating B cell malignancies, risks and challenges arise coincidentally with the therapeutic benefits and remain related to toxicities and resistance to therapy associated with the occurrence of tumour relapses. Although some of these toxicity concern had been predicted and successfully managed in the clinic, others with potentially fatal consequences have not been foreseen. After infusion of CD19 CAR T-cells, various CAR T-cell-mediated toxicities, such as tumour lysis syndrome, cytokine release syndrome (CRS), neurotoxicity, and on-target off-
tumour toxicity\textsuperscript{58,81} can arise and remain subject of intense research. Although CRS was expected toxicity, unanticipated neurologic complications have been specifically reported for CD19 and B cell maturation antigen (BCMA) CAR T-cell therapy\textsuperscript{57,82}. One of the major concerns of CD19-directed therapy is the occurrence of disease relapse in which early events are normally associated with CD19 positive disease, while later episodes present a more challenging scenario where antigen modulation results in CD19 loss from the tumour cells. Lack of adequate persistence of CAR T-cells has been shown to be the main determinant for early relapses. The initial quality of the T cell product and the choice of endodomain (CD28 vs 4-1BB) have been demonstrated to play a crucial role in long-term persistence of the therapy. On the other hand, the mechanism involved in the loss of CD19 have been associate with the intrinsic plasticity and heterogeneity of tumours. Splicing events in the extracellular portion of CD19 have been shown to results in the loss of the binding epitope targeted by the most common anti-CD19 paratopes (i.e. FMC63 and CAT). The coexistence of CD19 negative B cell clones withing the initial diagnosis have emerged as a rare event inherent to the heterogeneity of the tumour and independent from the CD19-directed therapy. The optimization of CAR T-cells for the treatment of B cell malignancies is current at the frontline of research in this field of immunotherapy, but many obstacles still remained to be overcome and close attention to the design of improved CAR structures will be informative in identifying further barriers. In addition to this field, more representative pre-clinical models would be of huge value and would allow investigators to test hypotheses focused on the safety and efficacy of novel CARs.

The majority of preclinical studies used to investigate CAR T-cell function to date have been mainly employing severe-immunodeficient mice (NSG) in which human CAR T-cells can be directly tested in a xenograft tumour model. These models have been demonstrated as an invaluable tool for the early stage validation of anti-CD19 preclinical development. However, the xenograft model has great limitations when employed to study the effects of immunotherapy. The approach of transferring human PBMCs into NSG mice only allows short-term experiments because the animals develop xenograft-graft-versus-host disease (xGvHD) within 4–5 weeks\textsuperscript{126}. Most of the xenograft studies involve CAR T-cells targeting human antigens whose expression is restricted to the human tumour cells used to challenge the mice. Thus, targeting of the antigen, which may be expressed on normal, healthy tissue, cannot be evaluated. It is also important to emphasize that xenograft models lack a functional human immune
system and other human tissues, which may modulate the anti-cancer activity and toxicity of CAR T-cells \textit{in vivo}. Nevertheless, these models were essential to establish the first proof of concept, and most CAR T studies still rely on these xenograft mouse models for primary efficacy evaluation. The use of more disease-relevant models, like syngeneic immunocompetent mouse models, has been little employed for testing CAR T-cells. Advantages of these types of models include the observation of CAR T-cells within the context of a functional immune system\textsuperscript{150}. Additionally, syngeneic models can reveal on-target off-tumour toxicities, as healthy mouse tissue can express the target antigen at low levels, mirroring the expression patterns in human patients\textsuperscript{151,152}. However, mouse immune systems differ from that in humans, and syngeneic models have so far been unable to mimic CRS, and side effects may vary between mouse strains. Nonetheless, syngeneic models can be important in elucidating cytokine-induced changes in the tumor-microenvironment (TME) and the host immune system's involvement in modulating the effects of the CAR T-cell therapy in a more human-like disease scenario.

In this thesis, I have attempted to generate an immunocompetent syngeneic model to recapitulate the side effects associated with anti-CD19 CAR T-cells. I have generated 2\textsuperscript{nd} generation full mouse CAR T-cells backbones specific for mouse CD19 aiming at recapitulating the benefits and toxicities of CD19-directed cell therapy. Using the publicly available anti-mouse CD19 hybridoma (1D3), I constructed CD28 and 4-1BB-based CAR structures. As to date, the comparison between mouse CD28 and 4-1BB endodomains remains unexplored. In a challenging cytotoxicity assay, I have shown that they possess similar killing abilities with significantly different cytokine release profiles. Higher levels of interferon-\(\gamma\) were produced CD28 endodomain, while 4-1BB demonstrated higher levels of IL-2 but increased antigen-independent release in line with the data using human cells\textsuperscript{167,195}. I demonstrated that mouse CD28 was associated with higher levels of interferon-gamma \textit{in vitro} compared to 4-1BB, supporting the use of CD28 for the investigation of the acute side effects caused associated with CRS in an immunocompetent model of systemic B cell tumour. Despite their overall potent activation, neither of the endodomains fully eradicated mouse B cell tumour, and overall, they have shown poor performance at higher effector to target ratios, unlike the reported human equals\textsuperscript{303–305}. This could suggest that 1D3-based CAR does not make potent therapeutics when compared to the performance of anti-human CD19 CARs. I have established an immunocompetent mouse model of systemic B cell tumour that
was able to recapitulate most of the features of CD19-directed CAR therapy. This mouse model has been shown to adequately mimic the activity of the anti-CD19 CD28ζ CAR, as demonstrated by the tumour eradication and sustained B cell aplasia. Like in the human scenario, our model's side effects were greater, the higher the tumour burden and the CAR T-cell dose\textsuperscript{54,92,321}. The observed increase IFN-γ, IL-6, and IL-10 accompanied with weight loss and temperature rise was a possible indication of CRS, while the decreased number of monocytes in combination with elevated MCP-1 suggested more severe toxicity potentially related to cytopenia and hemophagocytic lymphohistiocytosis/macrophage activation syndrome (HLH/MAS). Similar to reports of CD19 clinical trials in which MCP-1 was significantly elevated in CRS grade \( \geq 3 \) as a result of the great CAR-T cells expansion\textsuperscript{145,316}. In the model where limited amounts of CAR T-cells were injected, a functional response was observed at previously unreported dosage, which might be relevant for modelling a more challenging and accurate scenario to test the performance of CAR T-cells. The downstream mechanisms balancing the cost-benefits of this therapy are of great interest in designing a finely tuned CAR structure to deliver the best therapeutic effects with minimal toxicities.

The model presented in this thesis attempted at recapitulating both the curative effect and the toxicity associated with the infusion of anti-CD19 CAR T-cell therapy in the context of systemic B cell malignancy. Specifically, modelling CRS was the main purpose of the mouse model. In summary, the model investigated here has highlighted acute side effects after the administration of CAR T-cells. The toxicity has been demonstrated to show CRS-like features (i.e., increase in inflammatory cytokines and sudden weight loss) associated with a great expansion of the CAR T-cells and a significant decrease in the CD11b positive monocytes with the most severe toxic events. The attempt at confirming the observed CRS resulted in the absence of such side effects but resulted in complete tumour clearance. The different settings of tumour engraftment for this latest model resulted in a significant reduction in the overall tumour burden in all the cohorts that impacted the extent of CAR T-cell activation and so the insurgence of toxicity. In conclusion, the model has been shown to adequately mimic the effects of the anti-CD19 CAR therapy reported in human trials, where 30-50% of patients treated with CD28-based CAR T-cells developed CRS. The robustness of the model in terms of consistently reproducing CRS remains to be improved. The TBI preconditioning regimen for the engraftment of the tumour cells is essential for the reproducibility of the model settings; in order to minimize the variations, a greater
number of mice could be employed (i.e., 8 mice per cohort) to achieve a uniform tumour burden before the injection of CAR T-cells. In addition, the precise timing of bleeding for the assessment of cytokine production after the infusion of CAR T-cells is essential to highlight the cytokine changes during CRS. In order to improve the chance to hit the right time point, a time course model in which different cohorts are sacrificed at different days from the injection of CAR T-cells could help elucidate the best timing for assessing cytokine production. Finally, in the models presented here, only cells derived from peripheral blood, spleen and bone marrow have been evaluated. The model would benefit from the inclusion of other tissues such as the lymph nodes. Also, the analysis of cerebrospinal fluid (CSF) and brain could highlight additional sites for tumour and CAR T-cells infiltration that might help elucidate the consequences of CRS toxicity and might also emphasize neurological toxicity and CAR infiltration in the brain, which has not been reported in any of the current syngeneic models for anti-CD19 CAR therapy.

As more novel CAR T-cell architectures are emerging to improve the therapeutical benefits of these cells, a deeper understanding of each CAR-component's functional role is necessary for the rational design of improved CAR therapies. The contribution of the binding-moiety (scFv) in the performance of CAR structures has been the subject of intense research. Parameters of the ligand-binding domain, such as affinity, avidity, and antigen epitope location, greatly contribute to CAR T-cells functionality. Comparisons of the contribution of the scFv affinity in CARs activity have shown that high-affinity CARs exhibit less discrimination between target cells with high or low target expression levels, reducing the potential for normal tissue toxicity expressing low antigen levels. Rational decrease of the affinity is able to discriminate between high and low levels of target expression, sparing normal healthy tissues expressing low antigen levels\textsuperscript{172,175,177}. Although the increase in specificity when targeting cancer cells expressing high levels of the antigen, a reduction in affinity can also result in diminished cytotoxicity and cytokine production. Recent clinical data of an anti-CD19 CAR harbouring a low-affinity scFv with a fast off-rate reported longer cell persistence and improved cytotoxic abilities\textsuperscript{72}. Both in CAR T-cells and native T cells, the strength of the interactions is given by multiple receptor-ligand interactions that allow the formation of multiple immune-synapse at the T cells-target interface\textsuperscript{244,245}. The resulting avidity has been demonstrated to be a further contributor to CAR T-cells activation\textsuperscript{246}. A detrimental effect on the activity of CAR T-cells has been linked to the
propensity of some scFv to spontaneously aggregate, resulting in antigen-independent activation with consequent tonic-signalling that can lead to early exhaustion of T cells. Although the modular nature of the CAR’s structure allows for targeting difficult epitopes, including large and bulky cell surface receptors, the spatial location of the binding epitope has been shown to have a bigger impact on CAR performance than binding kinetics alone. A CAR targeting a membrane-distal epitope produces a weaker signalling, lower cytotoxicity, and sub-optimal degranulation than a CAR specific to a membrane-proximal epitope\(^ {179}\). The immune synapse distance can be easily tuned by utilising different spacer domains, but it remains essential to consider epitope location together with functional and structural limits imposed by the biology of the target. Through the use of hybridoma technology I generated a diverse and novel set of functional anti-mouse CD19 scFvs with \textit{in vitro} activity compared to the reference 1D3 clone with one underperforming scFv and one delivering non-specific T cell activation. It would be interesting to explore the impact of the improved biophysical properties in the performance of CAR-T alongside toxicity in the A20 immunocompetent mouse model.

\textbf{6.1 Directions for further investigation}

The kinetics profile studied for the novel four anti-mouse CD19 scFv identified high-affinity binders that did not harbour the same biophysical properties of the anti-human CD19 binders. The unique off-rate identified in CAT19 scFv is 2-logs faster than all the others\(^ {305}\). Moreover, the on-rate is 2-log slower than the two main anti-human CD19 (FMC63 and CAT), making a direct comparison impossible. The previously unreported identification of a further non-competing epitope present on mouse CD19 makes these binders suitable for exploring the impact of unique localization on the binding on CD19. Although \textit{in vitro} data have failed to show differences in the CAR activity of the newly generated scFvs and the reference clone. As CD19 possesses a unique protein conformation, it has been hypothesized that epitope location might not significantly impact CAR T-cells performance, unlike the reports for CD22 and MSLN\(^ {118,250}\). Moreover, the improved thermal stability for the generated scFvs might have a significant impact on the activity of the CAR T-cells. As the stability increases, the tendency to produce antigen-independent tonic signalling decreases\(^ {247,248}\). This could turn the T cell product into a less exhausted phenotype able to deliver a better surface expression and a more persistent activity with minimal
chances of off-target, off-tumour effects. These effects would be observable only in the context of *in vivo* models in which the minimal differences of the scFvs’ biophysical characteristics might have a significant effect on the long-term efficacy and toxicity of the CAR product.

In patients with B cell leukaemia, the in vivo expansion and antitumor activity of co-infused CD28- and 4-1BB-engineered CAR T-cells have reported different expansion rates as expected from the two costimulatory domains. Cheng et al. demonstrated the feasibility and benefits from both co-stimulatory signals\(^{340}\). Although, they used the same CAR architecture targeting the same epitope delivering third generation-like signalling without using the appropriate structure. Traditionally a third-generation CAR is constructed in a unique cassette with CD28 and 41BB fused in-*cis* and expressed on the cell surface\(^{207}\). Third-generation CARs have demonstrated beneficial effects in a preclinical model in terms of long-term persistence and tumour clearance\(^{44}\). These benefits in humans are still unexplored, with only one trial reporting little improvements in the therapeutical outcome\(^{58,193}\). Modular assembly of multiple CAR structures can mix different binding moieties to increase the targetable antigen without the expression of multiple CAR structures\(^{230,235}\). This versatile approach has shown interesting results *in vitro* and NSG mouse models as it aims at minimizing the chance of antigen escape. An exciting and unexplored application of targeting two different epitopes on the same molecule might include delivering multiple costimulatory domains in-*trans*, allowing for mixing two or more different signals without increasing the intracellular size of the CAR structures. This is important as increased distance of CD3ζ from the cell surface results in sub-optimal activation of the ITAM motifs and indeed less potent CARs. Moreover, in addition to two costimulatory domains, the structure illustrated in figure 6-1 is suitable for mixing two or more different costimulatory signals without bulking up the individual CAR structure. The benefits of using alternative costimulatory domains such as OX40 and ICOS have already been demonstrated to provide effective stimulation either alone or in the *-cis* 3\(^{rd}\) generation CAR structures\(^{200,187,196}\). Finally, the implementation of in-*trans* signal mixing with simultaneous targeting of two different portions of the same molecule might diminish antigen-negative escape chances. Safety concerns of more potent CAR structures, like third generations, remain the biggest challenge to their clinical application. The generated immunocompetent model of B cell tumour will be perfectly suitable for characterizing the potency and
toxicity of the illustrated novel architecture compared to the current available anti-mouse CD19 2\textsuperscript{nd} generation CARs.

6.2 Final conclusions

In this thesis, I have developed a novel immunocompetent model of systemic B cell malignancy. This model can recapitulate the main effects of the CD19-directed CAR T-cell therapy described in humans. The variability in the outcome of the mouse CD19 model is a possible reflection of the natural biological variability that characterises this type of T cell therapy in humans. I have constructed and tested in vivo an anti-mouse CD19 2\textsuperscript{nd} generation CAR that summarizes the acute side effects of CRS with increased temperature, weight loss and elevated cytokines. The sustained B cell aplasia and long-term engraftment of CAR T-cells have confirmed the excellent activation of the engineered T cells in this model, making it suitable for long-term studies without the occurrence of xGVHD. Moreover, the expansion of CAR T-cells observed in the model

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{signal_mixing_structure.png}
\caption{Schematic of the signal mixing structure. Traditionally, a 3\textsuperscript{rd} generation CARs delivers two costimulatory signals in -cis. As two scFvs bind on two separate epitopes of the target molecule, these novel structures would deliver multiple signals in -trans. This approach would allow mixing multiple costimulatory signals without excessively increasing the distance from the membrane. In addition, it could potentially include more than two costimulatory molecules.}
\end{figure}
led to a drastic reduction in the monocyte compartment in the animals with more severe side effects, highlighting another potential complication of the therapy also observed in humans, such as cytopenia and HLH/MAS. This would require further investigation and could be of interest to help characterise more severe toxicities arising from the immune system failing to control the activity of CAR T-cells. Although not investigated in this thesis, the insurgence of neurological side effects could be a further line of investigation that would advance the understanding of this life-threatening complication. In addition, I have generated a novel set of anti-mouse CD19 binders with similar kinetics but improved stability and bind to a different location on the mouse CD19 molecule. However, the biophysical properties suggested that these novel scFvs would make a CAR with similar performances to the reference mouse CD19 CAR, as shown by their in vitro performance. Further studies will help to elucidate the role of the significant enhancement in stability and the additional binding epitopes in the design of improved anti-CD19 CAR T-cells. Collectively, this work emphasizes the importance of using an adequate mouse model that can mimic the human disease scenario to better understand the key players as determinants of optimal CAR function with minimal toxicity. I believe that this mouse model and the generated binders will be of great use as a platform to test alternative and improved CAR’ designs in the context of B cell malignancies that can be expanded further than CD19-directed cell therapy.

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