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CD19-targeting CAR T Cells for Treatment of B Cell Malignancies

From Bench to Bedside

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Abstract

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Immunotherapy for cancer is a young research field progressing at high speed. The first chimera of an antibody and a signaling chain was designed by Zelig Eshhar and was later further developed to enhance existing T cell therapy by combining a single-chain fragment of an antibody with the CD3 zeta chain of the TCR complex. T cells expressing these chimeric antigen receptors (CARs) could recognize and specifically kill tumor cells. However the T cells, lacked in persistence and tumor rejection did not occur. Thus, the CAR constructs have been improved by providing the T cell with costimulatory signals promoting activation. The focus of this thesis has been to evaluate second and third generation αCD19-CAR T cells for the treatment of B cell leukemia and lymphoma.

B cell tumors commonly upregulate anti-apoptotic proteins such as Bcl-2, which generates therapy resistance. In the first paper a second generation (2G) αCD19-CD28-CAR T cell was combined with the Bcl-2 family inhibitor ABT-737. ABT-737 sensitized tumor cells to CAR T cell therapy and may be an interesting clinical combination treatment. In paper II, the phenotype and function of a third generation (3G) αCD19-CD28-4-1BB-CAR T cell were evaluated. B cell-stimulated CAR T cells showed increased proliferation and an antigen-driven accumulation of CAR+ T cells. 3G CAR T cells had equal cytotoxic capacity, similar lineage, memory and exhaustion profile phenotype compared to 2G CARs. However, 3G CAR T cells proliferated better and had increased activation of intracellular signaling pathways compared to 2G CAR T cells. In paper III, αCD19-CD28-4-1BB-CAR T cells were used to stimulate immature dendritic cells leading to an upregulation of maturation markers on co-cultured dendritic cells. Hence, CAR T cells may not only directly kill the tumor cells, but may induce bystander immunity that indirectly aids tumor control. This thesis also include supplementary information about the development and implementation of protocols for GMP production of CAR T cell batches for a phase I/IIa clinical trial currently ongoing for patients with refractory B cell leukemia and lymphoma. So far, two patients have safely been treated on the lowest dose.

Keywords: chimeric antigen receptors, CAR T cells, T cell therapy, immunotherapy, CD19, Bcl-2 family inhibitors, B cell malignancies

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Till min familj

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Karlsson, H., Lindqvist, C., Fransson M., Paul-Wetterberg G., Nilsson B., Essand M., Nilsson K., Frisk P., Jernberg-Wiklund H., Loskog A. (2013) Combining CAR T cells and the Bcl-2 family apoptosis inhibitor ABT-737 for treating B-cell malignancy. *Cancer Gene Therapy*, 20(7):386-93.

II Karlsson H., Svensson E., Gigg C., Larsson R., Jarvius M., Olsson-Strömberg U., Savoldo B., Dotti G., Loskog A. CARs expressing CD28 and 4-1BB demonstrate increased intracellular signaling activity and proliferative capacity while maintaining a memory phenotype upon repeated antigen stimulation. *Submitted manuscript*

III Karlsson H., Gustafsson W., Olsson-Strömberg U., Savoldo B., Dotti G., Loskog A. CAR T cells induce DC maturation via a cell-cell contact-dependent process and enhance their ability to stimulate T cell responses. *Manuscript*

IV *Karlsson H., *Enblad G., Wikström K., Hållstrand C., Pettersson H., Blomberg P., Essand M., Amini R-M., Brenner M., Savoldo B., Dotti G., Hagberg H., Loskog A. Third generation CD28/4-1BB CAR T cells for refractory CD19+ B cell malignancy: Generation of a GMP protocol and evaluation of clinical grade batches. *Manuscript/Supplementary information*

*Authors contributed equally to the work

Contents

Abbreviations

1G	First generation
2G	Second generation
3G	Third generation
AIF	Apoptosis-inducing factor
ALL	Acute lymphocytic leukemia
AP-1	Activator protein 1
APAF-1	Apoptotic protease-activating factor-1
APC	Antigen-presenting cell
ATM	Ataxia Telangiectasia Mutated
Bad	Bcl-2-antagonist of cell death
Bak	Bcl-2 antagonist/killer
Bax	Bcl-associated X protein
Bcl-2	B cell lymphoma 2
Bcl-w	Bcl-2-like protein 2
Bcl-xL	B cell lymphoma extra large
Bid	BH3-interacting domain death agonist
BiTE	Bi-specific T-cell-engaging antibody
BCR	B cell receptor
BCR-Abl	Breakpoint cluster region/Abelson
Bim	Bcl2-interacting mediator of cell death
BMSC	Bone marrow stromal cell
CAIX	Carbonic Anhydrase IX
CAR	Chimeric antigen receptor
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
cFLIP	Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein
CLL	Chronic lymphocytic leukemia
CM	Central memory
CNS	Central nervous system
CR	Complete response
CRP	C-reactive protein
CRS	Cytokine release syndrome
CTL	Cytotoxic T lymphocyte

CREB	cAMP response element-binding protein
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CXCR	Chemokine (C-X-C motif) receptor
DAMP	Danger-associated molecular patterns
DAP10	DNAX activation protein 10
DC	Dendritic cell
DISC	Death-inducing signaling complex
DNA	Deoxyribonucelic acid
DLBCL	Diffuse large B cell lymphoma
EM	Effector memory
ERK	Extracellular-signal-regulated kinase
FADD	Fas-associated protein with death domain
GD2	Disialoganglioside
GDP	Guanosine-diphosphate
gp100	Glycoprotein 100
GMP	Good manufacturing practice
GTP	Guanosine-triphosphate
Grb-2	Growth factor receptor-bound protein 2
Her2	Human epidermal growth factor receptor 2
HLA	Human leukocyte antigen
IAP	Inhibitor of apoptosis protein
ICAM-1	Intercellular adhesion molecule 1
ICOS	Inducible T-cell costimulator
IFN	Interferon
Ig	Immunoglobulin
IGHV	Immunoglobulin heavy variable
IL	Interleukin
ITAM	Immune receptor tyrosine-based activation motif
LAT	Linker for activation of T cells
Lck	Lymphocyte-specific protein tyrosine kinase
LFA-1	Lymphocyte function-associated antigen 1
mab	Monoclonal antibody
MAGE	Melanoma-associated antigen
MAPK	Mitogen-activated protein kinase
MART-1	Melanoma antigen recognized by T cells 1
MCL	Mantle cell lymphoma
Mcl-1	Induced myeloid leukemia cell differentiation protein
MHC	Major histocompatibility complex
miRNA	Micro ribonucleic acid
MOMP	Mitochondrial outer membrane permeabilization
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa B
NK	Natural killer
NY-ESO	New York esophageal cancer

OR	Objective response
PAMP	Pathogen-associated molecular patterns
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
PI-3	Phosphatidylinositol-3
PIP2	Phosphatidylinositol biphosphate
PIP3	Phosphatidylinositol triphosphate
PKCθ	Protein kinase C theta
PLCγ	Phospholipase C gamma 1
PR	Partial response
Pre-B-ALL	Precursor B cell acute leukemia
PUMA	p53 upregulated modulator of apoptosis
Ras	Rat sarcoma
SAE	Serious adverse event
ScFv	Single-chain variable fragment
SCT	Stem cell transplantation
SLP-76	SH2 domain-containing leukocyte protein of 76kDa
Sos	Son of sevenless
SYK	Spleen tyrosine kinase
TARP	TCR gamma alternate reading frame protein
TBI	Total body irradiation
TCR	T cell receptor
TEL1	Telomere maintenance 1
TGFβ	Transforming growth factor beta
Th	T helper
TIL	Tumor-infiltrating lymphocyte
Tim-3	T cell immunoglobulin mucin-3
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory cell
V(D)J	Variable (diversity) joining
ZAP-70	Zeta chain associated protein of 70kDa

Summary of the thesis in Swedish

Populärvetenskaplig sammanfattning på svenska

Denna avhandling undersöker om immunterapi i form av CAR T celler är ett bra behandlingsalternativ för patienter med blodcancer.

En av tre personer drabbas någon gång under sitt liv av cancer. En grupp av cancer är hematologiska maligniteter som är en cancer i blodets celler. En person som drabbas av denna typ av cancer upplever ofta en trötthetskänsla, återkommande infektioner, feber, nattliga svettningar och viktnedgång. I dagsläget behandlas patienter med blodcancer framförallt med cytostatika. Cytostatika är en grupp av läkemedel som påverkar alla delande celler i kroppen vilket också medför omfattande biverkningar som exempelvis håravfall och mag-tarm besvär. För några patienter kan transplantation av stamceller från benmärgen hjälpa dem bekämpa cancern, men den typen av behandling är också associerad med stora risker och kan inte ges till alla patienter. I hopp om att finna en effektiv behandling med få biverkningar och potentiell bot har nya metoder så som immunterapi använts i hopp om att kunna använda kroppens eget immunförsvar för att bekämpa cancern.

T celler är en del av immunförsvaret och deras roll är att patrullera runt i kroppen och döda felaktiga celler exempelvis celler som infekterats av virus. De har ett mycket specifikt system för att känna igen de ”farliga” cellerna, en så kallad T cellsreceptor. Receptorn är ett protein som sitter på T cellens yta. När T cellen kommer i kontakt med en sjuk cell som receptorn känner igen så kommer T cellen att döda den sjuka cellen. Deras inbyggda förmåga att specifikt känna igen och döda ”farliga” celler kan användas för att på samma sätt döda tumörceller. Men för att dessa T celler ska kunna känna igen just tumörcellerna kan de behöva lite hjälp på traven. Därför har en chimär T cellsreceptor tagits fram. Den chimära T cellsreceptorn känner igen ett specifikt protein på tumörcellens yta. Behandlingen fungerar som följer: Patienten får lämna ett vanligt blodprov och ur detta blodprov renas T celler fram. Sedan förändras cellerna genetiskt med hjälp av ett virus som levererar den genetiska koden för den chimära T cellsreceptorn. Då cellen får den genetiska koden kan den börja tillverka den chimära T cellsreceptorn och receptorn sätter sig på T cellens yta. Sedan odlar man dessa T celler med tillväxtfaktorer för att de ska bli flera och ger sedan tillbaka cellerna till pati-

enten. Dessa nya T celler cirkulerar i kroppen tills de stöter på en tumörcell. Med hjälp av den chimära T cellsreceptorn kan de då känna igen tumörcellen och döda den.

I delarbetena i denna avhandling har olika typer av chimära T cells receptorer undersökts. Deras förmåga att döda tumörceller på labbet och i patienter har utvärderats. I första artikeln kombineras T cellerna med ett läkemedel (ABT-737) som hjälper till att göra tumörcellerna känsliga så att det blir lättare för T cellerna att döda dem. Det finns flera varianter av chimära T cellsreceptorer och i manus II har två olika varianter undersökts för att se om de skiljer sig åt i hur de fungerar, hur snabbt de delar på sig och blir flera, och hur effektivt de kan döda tumörceller. Deras förmåga att få andra celler i immunförsvaret att hjälpa till att döda tumörcellerna undersöks i manus III. I manus IV undersöks terapin i patienter med blodcancer. Bland annat har kvalitén på de T celler som producerats undersökts och hur patientens immunförsvar ser ut före och efter behandling.

Sammanfattningsvis, avhandlingen undersöker om immunterapi med så kallade CAR T celler kan användas som behandling av patienter med blodcancern B cellsleukemi och lymfom. Avhandlingen sträcker sig från studier av CAR T cellernas funktion i odlingsskålar ända in i kliniken där patienter behandlas med CAR T celler.

INTRODUCTION

INTRODUCTION

Introduction

Cancer is a large group of diseases with one common feature – uncontrolled growth of cells. It affects about one in three people in Sweden and is one of the leading causes of death in developed countries. Cancer immunotherapy is a relatively young field of research focusing on the potential of the immune system to fight tumors. Its recent success stories put cancer immunotherapy in the limelight and the journal Science gave cancer immunotherapy the prestigious award "the breakthrough of the year" in 2013. Cancer immunotherapy includes many different types of treatments, among them adoptive transfer of T cells. This thesis focus on the potential of engineered T cells to recognize and eradicate tumors of B cell origin.

The immune system

Our body is well protected by numerous cells working together to eliminate exogenous and endogenous dangers. The immune system functions as an intricate network, with surface-bound and soluble proteins providing cues. The immune system is commonly divided into an innate and an adaptive arm. During an infection, the innate immune system provides the initial response to danger via physical barriers (e.g. epithelial cells), soluble factors (e.g. complement and cytokines) and cells (phagocytic cells, natural killer (NK) cells, granulocytes and dendritic cells (DCs)). The primary purpose of the innate immune system is to provide a rapid non-specific response to any pathogen. This function is based on recognition of patterns common for groups of microbes, so called pathogen-associated molecular patterns (PAMPs). Also, molecules secreted by or expressed on stressed cells, so called danger-associated molecular patterns (DAMPs) can also trigger innate immune responses. The innate immune system also provides signals for the adaptive immune system.

The adaptive immune system is based on recognition of specific antigens in order to kill microbes, as well as infected or transformed cells. Both B and T cells recognize their targets via antigen-specific surface receptors. Recognition via the receptor leads to clonal expansion of the B or T cell and secretion of immune-activating cytokines. These processes create an immunological memory leading to faster immune response and pathogen eradication

upon reinfection. DCs function as an important link between the innate and the adaptive immune system. DCs capture antigens through ingestion of cells, debris and pathogens. Larger proteins are digested to peptides that are subsequently presented on major histocompatibility complex (MHC) on the cell surface. This allows for recognition of protein antigens by cluster of differentiation (CD) 4+ and CD8+ T cells. Binding of surface receptors recognizing PAMPs and DAMPs on DCs initiate maturation and the DCs become activated. Mature DCs promote robust activation of T cells. Cytokine-producing DCs will promote activation of other effector cells as well, such as NK cells and macrophages.[1]

Dendritic cells

There are several different types of DCs that all initially originate from hematopoietic stem cells. Roughly, some DCs are resident in the lymph nodes, other patrol peripheral tissues taking up antigens and migrate to lymph nodes upon activation, where both types present antigens to T cells. Steady-state DCs are immature and have a high capacity for taking up antigen and processing it for antigen presentation on MHC molecules. During maturation these features are ultimately downregulated in favor of improved antigen presentation due to increased MHC expression, for example.[2] DC maturation is induced by PAMPs and DAMPs as stated above but also by cytokines and costimulators such as CD40L.[3,4] Cells, debris and pathogens are taken up, processed and presented as peptides on MHC molecules on the DC surface. Extracellular proteins are presented on MHC class II and are presented to T helper (Th) cells while intracellular proteins are presented on MHC class I and presented to cytotoxic T lymphocytes (CTLs). However, DCs can also present extracellular proteins to CTLs through cross-presentation. The maturation of DCs comprises both phenotypical features, including increased expression of costimulators such as CD40, CD70, CD80, CD83, CD86, 4-1BBL and OX-40L, as well as functional abilities, such as secretion of cytokines. DCs can secrete both immunostimulatory cytokines such as tumor necrosis factor (TNF)α, interleukin (IL)-12, IL-6 and IL-1β, as well as immunosuppressive cytokines for instance IL-10 and transforming growth factor (TGF)-β, tilting the immune system towards an immunogenic or a tolerogenic response. Which cytokines that are secreted depend on environmental cues.[5] In absence of danger signals, tolerance is induced by the immature DCs. Functionally mature immunogenic DCs secrete IL-12 that activates NK cells, T cells and M1 macrophages, promoting Th1 responses connected to good anti-tumor activity.[1]

Cells of the adaptive immune system

T cells and B cells share ancestry, both differentiating from a common progenitor lymphocyte originating from a hematopoietic stem cell. While T cells mature in the thymus, and recognize antigens via the membrane-bound T cell receptor (TCR), B cells partially mature in the bone marrow, complete their maturation in secondary lymphoid organs and recognize antigens via mainly secreted antibodies. Hence, T cells are effective against intracellular pathogens and defect cells via recognition of protein peptides derived from those, while B cells are effective against soluble and membrane bound antigens that can be proteins, carbohydrates and lipids. In the following section I will focus on T cells.

T cell development

Precursor T cells leave the bone marrow and circulate in the blood to the thymus. In the thymus the progenitors of αβT cells start to express the TCR α- and β receptor as well as both coreceptors CD4 and CD8. The antigen recognizing α- and β-chain chains of the TCR undergo gene rearrangement to achieve a diverse immune repertoire. This so called V(D)J rearrangement is a recombination process where selection and joining of one variable (V), diversity (D) and joining (J) gene segment at each locus give rise to a TCR with discrete antigen specificity. Unsuccessful rearrangement will lead to apoptosis induction in the T cell. Subsequently, the TCR recognition specificity and affinity is scrutinized. Antigen-presenting cells (APCs) in the bone marrow present peptides on MHC molecules or human leukocyte antigen (HLA) in humans, to the immature T cells. T cells first undergo a positive selection where the T cells that bind to self-peptide/self-MHC with low but sufficient avidity receive survival signals and are thereby preserved for a subsequent negative selection. During the negative selection, T cells that bind with too high avidity to self-peptide/self-MHC will undergo apoptosis. During the positive selection, the T cell loose expression of either CD4+ or CD8+ based on the class of MHC that was recognized together with peptide during the process. CD8 is restricted to peptides presented on class I MHC while CD4 recognizes class II MHC.[1]

T cell activation

For a T cell to be able to recognize an antigen it needs to be processed and presented on a MHC molecule. T cell antigen recognition is then conferred via the α- and β-chains of the TCR. This will induce a signal via the CD3-TCR complex, often referred to as signal 1. However, for the T cell to become fully activated additional stimulatory signals are required or the cell will become anergic or tolerized. The second signal is provided via costimulation from APCs such as DCs, for example via CD80 and/or CD86 binding to CD28 on the T cell surface. The requirement of the second signal for acti-

vation is important in order to avoid responses to self-antigens. Costimulatory signals induce the transcription of anti-apoptotic B cell lymphoma extra large (Bcl-xL) and proliferation-stimulating cytokine IL-2 in the T cell. The third signal, provided in the form of cytokines secreted from DCs, promote differentiation into a specific effector phenotype. The α- and β-chains of the TCR dimerizes upon recognition of antigen presented on MHC. For the TCR to be able to signal it needs to form a complex with the ζ, ε, γ, δ chains of the CD3 complex.[6,7] The CD3 complex forms dimers; CD3γε, CD3δε and CD3ζζ that functions as separate signaling units coming together to confer signals induced by TCR antigen recognition. Signaling through the CD3 complex is mediated through the immune receptor tyrosine-based activation motif (ITAM).[6,7] When a T cell recognizes a peptide presented on MHC, an immunological synapse is formed. The function of the synapse is to stabilize the receptor complex and for already activated CTLs, enable T cell killing. The synapse includes the CD3/TCR complex, costimulatory CD4 or CD8, CD28 and LFA-1 on the T cell and a processed peptide presented on MHC II or MHC I, respectively, CD80/CD86 and intercellular adhesion molecule 1 (ICAM-1) on an APC. Other costimulatory receptors and ligands may be involved as well. CD4 or CD8 associates with the lymphocyte-specific protein tyrosine kinase (Lck). Formation of the immunological synapse brings Lck closer to the TCR/CD3 complex, enabling phosphorylation of the ITAMs of the ζ-chains leading to recruitment of two tyrosine kinases, ζ chain associated protein of 70kDa (ZAP-70) and spleen-tyrosine kinase (SYK). ZAP-70 phosphorylates linker for activation of T cells (LAT). Recruitment of LAT and SH2 domain containing leukocyte protein of 76kDa (SLP-76) leads to phosphorylation and activation of phospholipase-C gamma-1 (PLCγ), increasing the intracellular levels of calcium and activating transcription factors such as nuclear factor of activated T cells (NFAT). NFAT regulates, in complex with other transcription factors for instance activator protein 1 (AP-1), transcription of cytokines such as IL-2 which plays a crucial role in promoting proliferation and survival of the effector T cell.[6] Phosphatidylinositol-3 (PI-3) kinase is also recruited to the TCR complex, generating phosphatidylinositol triphosphate (PIP3) from membrane-bound phosphatidylinositol biphosphate (PIP2). PI-3 kinase is most efficiently activated downstream CD28. PIP3 provides a binding site on the inside of the plasma membrane for e.g. PLCγ and the IL-2-inducible T cell kinase (Itk). Once phosphorylated by ZAP-70, LAT serves as a docking site for growth factor receptor-bound protein 2 (Grb-2). Docking to LAT enables Grb-2 to recruit son of sevenless (Sos) that converts Ras-GDP to Ras-GTP. Ras-GTP triggers the mitogen-activated protein kinase (MAPK) cascade culminating in activation of extracellular-signal-regulated kinase (ERK) -1, 2 that in turn induces synthesis of AP-1 via transcription of c-fos. TCR signaling also activates the nuclear factor kappa B (NFκB) pathway, via PKCθ, leading to increased T cell survival through upregulation of the anti-

apoptotic protein Bcl-xL.[6,8,9] The transcription factor cAMP response element-binding protein (CREB), is also activated by phosphorylation by PKCθ and by calmodulin kinases activated by the increased intracellular calcium levels.[10]

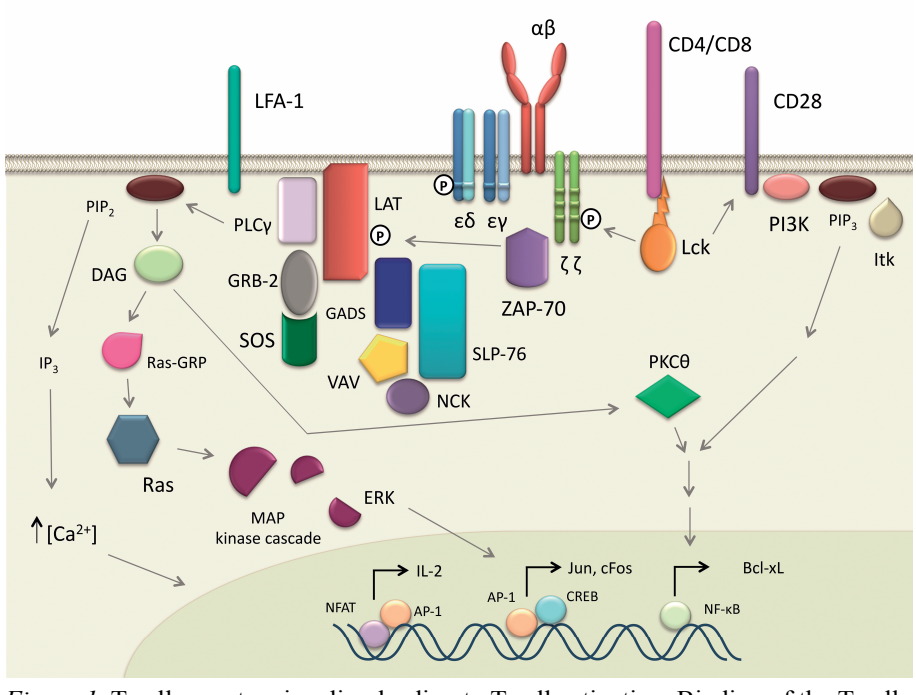

Figure 1. T cell receptor signaling leading to T cell activation. Binding of the T cell receptor and co-receptor CD4 or CD8 to antigen peptide loaded onto MHC, together with binding of CD28 to CD80/86 leads to Lck-mediated phosphorylation of the ITAMs of the CD3 molecules. ZAP-70 is recruited to the CD3 ζ chains and in turn phosphorylates LAT. LAT binds PLCγ, Grb-2 and Gads leading to recruitment of SLP-76 via Gads. LAT and SLP-76 forms the backbone of the complex organizing adapter molecules to form signaling complexes thereby activating several different pathways e.g. Ras-MAP kinase and Ca2+-mediated signaling pathways. Activation of transcription factors NFAT, NFκB and AP-1 leads to transcription of e.g. IL-2 and Bcl-xL, promoting survival and proliferation of the T cell.

Costimulatory and inhibitory molecules

T cells express several membrane-bound receptors that bind costimulatory molecules. Expression of some of these molecules is induced by TCR/CD3 engagement for instance members of the tumor necrosis factor receptor (TNFR) superfamily; 4-1BB (CD137) and OX-40 (CD134). Although 4-1BB and OX-40 are not dependent on CD28, CD28 can augment their expression on the T cell.[11] Costimulation via 4-1BB induces cytokine secretion, T cell expansion, upregulation of anti-apoptotic genes and prevents activation-induced cell death.[12] CD27 is expressed on naïve T cells and is initially upregulated upon T cell activation but expression decreases during expan-

sion and differentiation to effector cells. Costimulation via CD27 through ligation with CD70 leads to upregulation of Bcl-xL expression and promotes Th1 cell development. Interaction can also lead to secretion of soluble CD27. A transient expression of CD70 is induced on T cells upon TCR signaling and DCs through Toll-like receptor triggering.[13]

Also inhibitory molecules are upregulated upon T cell activation. Programmed cell death 1 (PD-1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) provide negative feed-back loops to avoid autoreactive responses and unnecessary tissue damage. Binding of PD-L1 to PD-1 leads to inhibitory signals via the PI3K and the Akt signaling pathway,[14] while CTLA-4 binding to CD80/CD86 leads to reduction of the T cell activating signals for instance through blockade of IL-2. Another recently identified T cell inhibitory receptor is the T cell immunoglobulin mucin-3 (Tim-3) which has been proposed to inhibit T helper (Th)1 responses. PD-1 expressed in conjugation with Tim-3 is thought to phenotypically indicate T cell exhaustion. Exhaustion is a state of decreased functionality, characterized by impaired killing, diminished cytokine production and decreased proliferation.[15]

T cell phenotype

As previously mentioned mature/post thymic T cells express CD4 or CD8 on their surface. This MHC restriction will also dictate the function these cells fill. CD4+ T cells are often referred to as T helper cells (Th) and T regulatory (Treg) cells, while CD8+ T cells are commonly called cytotoxic T cells (CTLs). Th cells are commonly divided into Th1, Th2 and Th17 cells based on their immunological functions for instance cytokine secretion profile. Th1 cells stimulate a proinflammatory response, involving secretion of type I cytokines like IFNγ, in turn activating APCs, stimulating a CD8+ T cell response.[16] Th1 cells can license DCs through interaction via CD40L/CD40. This in turn leads to upregulation of costimulatory molecules on the DC, and enhances IL-12 secretion. In response to antigen, Th2 CD4+ T cells secrete type II cytokines, e.g. IL-4, IL-5 and IL-13. Th2 cells can limit the activation of APCs and enhance humoral immunity as well as the influx of innate immune cells such as eosinophils and granulocytes.[17] Th17 cells secrete large amounts of IL-17 and potently induce inflammation. Th17 have been linked to autoimmunity and its role in cancer as pro- or anti-tumor is heavily debated.[18] Treg cells inhibit adaptive T cell responses against self-molecules and will thereby inhibit responses against self-tumor antigens as well. However, the inhibition is not antigen-specific per se. Tregs inhibit nearby immune cells through the secretion of the inhibitory cytokines IL-10, TGF-β or through cell-cell contact.[19] Tregs can be generated either in the thymus during development or are induced from naïve T cells in the periphery to control an ongoing immune response. CTLs also secrete cytokines. For example, upon antigen recognition CTLs instantly release IFNγ. The main function of

CTLs is like the name implies, to kill infected or malignantly transformed cells. Activated CTLs circulate the body until it come across a cell that is presenting the specific antigen recognized by the CTLs TCR on MHC I. CTLs can kill virus-infected cells and tumor cells through induction of apoptosis (see the section about T cell-induced apoptosis).

Naïve T cells have high expression of lymph node-homing molecules such as CD62L and CCR7. When naïve T cells respond to antigen they differentiate into effector cells, but a fraction of the naïve cells differentiate into antigen-specific memory T cells. Memory cells can be of central memory (CM) phenotype and of effector memory (EM) phenotype. CM T cells express high levels of CD62L and CCR7 while the EM T cells have low or no expression of these markers.[20] CM T cells are thought to reside in lymph nodes ready to respond to secondary pathogen challenge, while effector memory cells patrol the periphery.[21] Upon reinfection memory T cells can differentiate to effector T cells, possibly induced by pro-inflammatory cytokines secreted from local innate immune cells, such as IL-12, IL-18 and type I interferons.[22] Antigen experience affects the expression of CD45, CD45RA is found on naive T cells and CD45RO on antigen-experienced T cells, and can in turn be used as a marker for T cell memory phenotype.[23]

Bystander immunity

During immune responses, immune cells secrete cytokines and express different molecules on their surface in order to stimulate other immune cells to respond to the discovered danger. Local innate immune responses can attract leukocytes into the tissues resulting in activation of tissue APCs. These APCs secrete cytokines and express surface molecules that in turn can activate for instance T cells that are not specific for the antigen that initiated the innate immune response, thereby broadening the immune response to include cells that would not respond to the initial signals.[1]

As previously stated, immune cells respond to danger signals expressed by endogenous cells. But these signals are not exerted only through recognition of foreign intruders. Malignantly transformed cells can express defect proteins on the cell surface or in other ways provide cues for the immune system.

Tumor immunology

In 1957, Burnet and Thomas proposed the hypothesis of tumor surveillance.[24] As the term implies, immune cells would patrol the body, recognize aberrant endogenous cells and eradicate them before a tumor mass

had the possibility to form. Since then, the concept has been reevaluated proposing the process as a combination of host protective and tumor promoting functions of the immune system during tumor development and it has been termed cancer immunoediting.[25] Immunoediting moves through three phases: elimination, equilibrium and escape. During the first phase, cells and other components of the immune system recognize, target and eliminates tumor cells. How immune cells detect arising tumor cells is not clear. The transforming cell is possibly exerting danger signals, for instance secretion of type I IFNs that in turn alert DCs leading to priming of adaptive immune cells.[26] However, if the tumor cell can avoid detection by the immune system, equilibrium (the second phase) between tumor growth and elimination is reached. Tumor cells that express antigens derived from mutated cellular proteins or oncogenic viral proteins presented on MHC class I molecules can be detected and eliminated by CTLs. Tumor cells therefore evade the immune system by downregulation of the machinery for processing and presentation of proteins on MHC or downregulation of the MHC molecule itself. However, lack of MHC molecules makes the tumor cells prone to elimination by NK cells. This is circumvented by the tumor cells through expression of MHC molecule variants on their surface. Also, many tumors secrete immunosuppressive cytokines such as IL-10 and TGFβ leading to recruitment of Tregs and inhibition of DC function.[27] Tumor cells can also express ligands that can provide negative stimulation to infiltrating T cells, thereby inhibiting or reducing their cytotoxic function.[28,29] These are just a few examples of ways that tumors evade the immune system during the equilibrium phase that can go on for years. In the end, the evasion strategies will succeed and the tumor cells escape the immune system resulting in outgrowth of tumor lesions. However, cells of the adaptive immune system can be reactivated and/or reeducated to recognize the tumor.

T cell therapy

T cells have the potential to recognize tumor cells and kill them through the same mechanism as that they use to kill a virus-infected cell. This capacity can be utilized for treatment of cancer.

Tumor-infiltrating lymphocytes

T cells infiltrate tumor tissue and their presence is for most cancer types associated with a better prognosis.[26,30,31] Nevertheless, since most tumors form an immunosuppressive microenvironment, the majority of these infiltrating T cells become anergic and cannot muster sufficient anti-tumor response to induce tumor rejection.[32] In melanoma patients, it has been shown

that tumor-infiltrating lymphocytes (TILs) can be isolated, reactivated and expanded for reinfusion into the patients.

The first study to show responses in melanoma was conducted in 1988.[33] At this time IL-2 was under investigation as a monotherapy for melanoma and demonstrated higher response rates than available treatment at the time.[34] Due to the immunological responses seen in patients treated with IL-2 monotherapy and the likely IL-2-dependence after *ex vivo* culture, supportive IL-2 was given along with the infused TILs.[34] However, in the initial clinical trials, infused TILs failed to engraft and due to the low *in vivo* persistence, clinical effects where sparse and not durable.[33,35-37] Immunosuppressive cells such as Tregs,[38] and competition for endogenous cytokines[39] were considered an obstacle for *in vivo* engraftment. Therefore, lymphodepleting chemotherapy was given prior to TILs infusion to create a favorable environment for the transferred cells. The addition of pre-conditioning chemotherapy substantially increased the efficacy of TILs infusions.[40,41] With preclinical support of improved efficacy of treatment, total body irradiation (TBI) was added to the chemotherapy pre-conditioning prior to TILs infusion and was seen to increase the response rate even further in a group of patients with metastatic melanoma.[42] However, the trial was not randomized and the patient numbers were small. Still, a pooled analysis of these three sequential studies showed an objective response (OR) of 56% and complete responses in 22% of the patients[43] concurring with data from other groups reporting ~50% OR in their studies.[44,45]

However, with high anti-tumor effect comes high toxicity. At this point the adverse events of pre-conditioning alone could be severe. There have been reports of severe adverse events where the pre-conditioning could be the cause of death.[46] Also, treatment with systemic IL-2 is associated with high toxicity for the patient and can be life-threatening.[47] Autoimmune on-target effects have been seen for TILs with melanocyte-targeting specificity e.g. vitiligo[40,41], uveitis,[40-42] and hearing loss,[42] these side-effects were however transient and often linked to tumor regression.[41]

Treatments with TILs require pre-existing accessible tumor-reactive cells that can be isolated, reactivated and expanded *ex vivo*.[48] It is therefore not applicable to all types of cancers or patients. Success has so far been limited to melanoma[49] even though several solid cancers potentially could be treated with TILs.[50-52] However, clinical trials (phase II) recruiting patients with metastatic gastric, colorectal, pancreatic, hepatocellular cancer and metastatic cholangiocarcinoma (clinicaltrials.gov; NCT01174121) and cervical cancer, oropharyngeal, vaginal, anal and penile cancer (human papilloma-associated cancers) (clinicaltrials.gov; NCT01585428) are ongoing at NIH.

There are difficulties in accessing and isolating TILs, and especially expanding them to large numbers while maintaining cytotoxic capacity and avoiding exhaustion. Another drawback is the decreased and sometimes absent, MHC I expression on tumor cells which make the tumor invisible for T cell recognition and destruction. Another way of utilizing T cells in cancer immunotherapy is to gene engineer T cells to harbor specificity for a tumor antigen via cloned tumor-specific TCRs or chimeric antigen receptors.

TCR engineered T cells

The instability of the tumor accumulates defective proteins that are presented on MHC I. Hence, there are possibilities to find a tumor-restricted target using TCR engineered T cells. For MHC I positive tumors, such T cells are an interesting approach. T cells of unknown specificity can easily be isolated from peripheral blood of any cancer patient. With gene transfer, these T cells can be engineered to express a TCR of known specificity targeting any protein antigen, including intracellular antigens. However, to find a specific sequence that recognizes the tumor cells, a tumor-reactive clone is required from which the α- and β-chains of the TCR can be sequenced. Through transfer of the tumor-specific TCRαβ genes, T cells with tumor-specific recognition can be engineered. Recognition of antigen through the TCR is dependent also on recognition of the MHC molecule on which the peptide is presented and can therefore only be used in patients with matching HLA (matching between individual where the TCR has been isolated and the recipient). The most common type is HLA-A2 which is present in~50% of Caucasians and has therefore become the most common type of engineered TCRs.[53] HLA-A2-restricted TCRs have been engineered for MART-1,[54] gp100,[54,55] NY-ESO-1,[54,56] Her2,[57] CEA,[58] MAGE-A3,[59] TARP,[60] and the P53 suppressor gene[61] and through TCR transfer rendered a tumor-specific T cell clone. The first successful clinical trial utilized a MART-1 specific TCR cloned from TILs that had shown effect in a previous clinical trial[40] for melanoma.[54]

For antigens that are expressed on healthy tissue as well as the tumor, autoimmune responses are a factor to consider. As previously mentioned for TILs treatment of melanoma, TCR engineered T cells specific for MART-1 and gp100 causes melanocyte destruction with transient toxicity in skin, eyes and ears (skin rash, uveitis, hearing loss). The affinity of the receptor affects both clinical response and side-effects, clearly seen in two trials of MART-1 TCR. The initial clone had intermediate affinity for MART-1 and gave rise to OR in 2/15 patients and no autoimmunity was seen.[54] The clone was subsequently modified for increased affinity in order to increase efficacy of treatment and the new TCR induced partial responses (PR) in 6/20 patients, but all responding patients had autoimmune manifestations.[55] In a small trial

of metastatic colorectal carcinoma targeting CEA, all three patients had dose-limiting colitis.[58] Also, lethal cardiac toxicities[62] and lethal neurological toxicities[63] have been reported in two trials investigating TCRs targeting two different epitopes of MAGE-A3.

There are also additional aspects to consider when engineering a TCR. For signaling to occur in the T cell the TCR needs to associate with the CD3 complex, ending up with the transferred TCRs competing with the endogenous TCRs for CD3 complexes.[64] Therefore, high level expression of the transferred TCR as well as equal amounts of α- and β-chains is required for efficient signaling. Gene vector constructs enabling translation of equal amounts of α- and β-chains can be established through insertions of self-cleaving 2A peptides between the α- and the β-chain.[65] There is also a risk of the transferred α- and β-chains cross-pairing with endogenous TCR chains forming heterodimers with unknown specificity that potentially could lead to graft versus host disease. Much effort has been put into avoiding this, for instance; murinisation of the transferred chains to avoid cross-pairing[66] or incorporation of an additional cysteine residue leading to formation of a disulphide bond between the α- and β-chain.[67] Knockdown (using miRNA)[68,69] or knockout (using zink finger nucleases)[70] of endogenous TCRs on the T cells has also been used to avoid mispairing.

Additional considerations with TCR engineering are that the method is laborious and time-consuming, it requires HLA matching of patient and TCR clone and it needs proper presentation of the tumor-antigen on MHC that is commonly downregulated in tumor cells. To circumvent MHC recognition, T cells can be engineered with a receptor in which antigen recognition is due to an antibody domain instead of the α- and β-chain of the TCR.

Chimeric Antigen Receptor T cells

T cells of unknown specificity can easily be isolated from peripheral blood of any cancer patient. With gene transfer, these cells can be engineered to express a chimeric antigen receptor (CAR) directed against any surface-bound antigen. The first CAR, developed by Eshhar *et al* was constructed for the purpose of better understanding TCR signaling and antigen/TCR interactions.[71,72] Its potential as a T cell-based therapy was soon discovered and the constructs modified for the purpose. The first generation CAR consisted of a single-chain variable fragment of an antibody linked to a cytosolic signaling domain, for instance the ζ chain of the TCR-CD3 complex.[73]

These first generation CAR T cells showed specific killing of target cells and secreted IL-2 upon target-recognition *in vitro*.[73] However, when brought to the clinic the CAR T cells did not persist *in vivo* and their expansion was

limited.[74-76] The first signal for T cell activation was provided through recognition via the CAR, however tumor cells often lack expression of costimulatory molecules such as CD80 and CD86, and it is therefore likely that the CAR T cell did not receive the second signal required for T cell activation. To improve CAR T cell proliferation and *in vivo* persistence, a costimulatory molecule was added to the cytosolic part of the CAR construct. Several different molecules have been investigated, for instance CD28, 4-1BB (CD137) and OX-40 (CD134) and lately DNAX activation protein 10 (DAP10)[77] and inducible T-cell costimulator (ICOS).[78] Addition of the CD28 domain led to increased proliferation,[79-84] increased cytokine secretion upon antigen recognition[78,79,82,83] and resistance to Tregs and the immunosuppressive cytokines IL-10 and TGFβ.[81] The addition of CD28 also led to improved expansion and persistence *in vivo* both in preclinical studies[77] and in a clinical trial where the constructs were compared head to head.[85] Second generation CARs carrying 4-1BB showed increased cytokine secretion, enhanced *in vivo* persistence and an upregulation of anti-apoptotic genes.[86-88] In clinical studies, a 4-1BB CAR targeting CD19 has shown impressive results in patients with CLL[89,90] and ALL.[91] OX-40 in the CARs showed increased proliferation and cytokine secretion as well as a prolonged T cell survival.[78,92] The preferred costimulation for optimal response is still debated, even though several studies comparing different construct have been performed. Some have argued that costimulation with 4-1BB renders a stronger anti-tumor effect compared to CD28,[87,93] while others claimed them equal to the task.[88] In an attempt to further increase the efficacy of the CAR construct, third generation CARs were constructed including both CD28 and 4-1BB[87,88,94-96] or CD28 and OX-40.[92] In comparative investigations, Zhong *et al* found that a third generation CD28-4-1BB-CAR had an increased proliferation and cytokine release, as well as a superior *in vivo* persistence and anti-tumor response compared to first and second generation CARs. Also, signaling via PI3/Akt was augmented in these cells, giving rise to higher expression of anti-apoptotic Bcl-xL.[96] Comparing a first, second and third generation CAR, Tammana *et al* came to the conclusion that a second generation CAR including 4-1BB was superior to one with CD28 alone, while CD28 and 4-1BB in combination appeared to act in synergy and thereby raising the strongest anti-tumor response *in vivo*.[87] A third generation CAR combining CD28 and OX40, was showed by Pulè *et al* to secrete higher levels of cytokines and also induce a stronger activation of the NFκB pathway leading to increased levels of anti-apoptotic proteins compared to CD28 alone.[92] Some of the differences seen in these comparative studies can be attributed to differences in the constructs for instance the use of different transmembrane domains. The methods for gene transfer, T cell phenotype, culturing conditions as well as pre-conditioning and concomitant cytokine support at T cell infusion are just a few of the variables that can affect outcome.

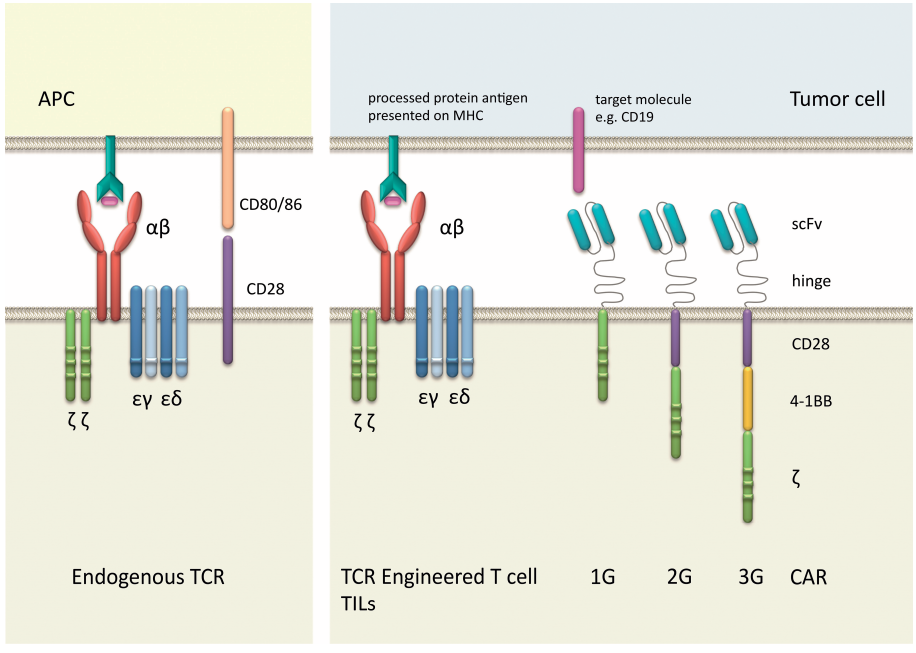

Figure 2. Overview of the structures of the endogenous T cell receptor (TCR)/CD3 complex and costimulatory molecules CD28 and CD80/86, TCR engineered T cells and tumor infiltrating lymphocytes (TILs) recognizing a specific antigen and chimeric antigen receptors (CARs) of the first (1G), second (2G) and third (3G) generation. CD28 and 4-1BB are examples of costimulatory molecules that can be used in the CAR constructs. MHC processing and presentation is required for recognition with the TCRs, while CARs can recognize antigen via direct binding.

Clinical studies

So far, CAR T cells have shown the strongest efficacy in treatment of B cell malignancies. There are currently 53 clinical trials of CAR T cells for cancer treatment registered at clincialtrials.gov. 39 are for treatment of B cell malignancies and 29 of those are targeting CD19. Clinical data has been reported from trials targeting several different antigens including hematological malignancies via CD19,[85,89-91,97-104] CD20[76,97] and LewisY,[105] metastatic renal carcinoma via CAIX,[106] neuroblastoma, sarcoma, melanoma via GD2,[92,107] neuroblastoma via CD171,[108] breast, lung, prostate, glioma via Her2,[109] and ovarian folate receptor-α[74] as well as malignant pleural mesothelioma, pancreatic, ovarian, and lung cancer via mesothelin.[110] The majority of these trials are investigating second generation CAR T cells. There is limited experience of third generation CAR T cells in the clinic. So far only two clinical trials have reported data of a third generation CAR. The first trial investigated a CAR directed against Her2, where recognition of Her2 on healthy tissue led to fatal outcome for one patient.[109] The second trial targeted CD20 in non-Hodgkins lymphoma and mantle cell lymphoma. However, modest effects were seen, possibly due to the lack of pre-conditioning treatment and

the use of a transient gene transfer system.[76] Currently, we have initiated a clinical trial treating CD19+ B cell lymphoma and leukemia with 3G CAR T cells as one of the first centers in Europe.

Clinical experience of αCD19 CAR T cells

Savoldo *et al* treated non-Hodgkins lymphoma patients with first generation or CD28-CAR targeting CD19. The trial gave crucial information of the requirement of costimulation, yet treatment did not lead to durable remissions, merely stable disease in two patients, possibly partly due to the lack of pre-conditioning.[85]

A second generation 4-1BB CAR showed impressive results in an initial study treating three patients with CLL.[89,90] Two of the three patients had a complete response while the third experienced a long-lasting partial response. The transferred T cells expanded up to 1000-fold and could still be detected in peripheral blood 180 days after infusion. Two patients with relapsed and refractory childhood pre-B-ALL were treated with the same 4-1BB CAR mentioned above. Complete remissions were observed in both patients, however one of the patients relapsed with a CD19 negative tumor approximately 2 months after treatment.[91] All patients were treated with pre-conditioning chemotherapy.

Kochenderfer *et al* initially reported a partial response in a patient with follicular lymphoma treated with a CD28-CAR.[98] Subsequently, patients with follicular lymphoma, CLL and splenic marginal zone lymphoma were treated resulting in one complete response, six partial responders and one patient with stabile disease.[99] Recently a trial from the same group comprising 15 patients with lymphoma reported 8 complete responders and 4 partial responders.[111] All patients were treated with aggressive regimens of pre-conditioning chemotherapy.

Brentjens *et al* evaluated CD28-CARs in chemotherapy-refractory CLL or relapsed B-ALL.[100] The patients were divided into two groups, of one receiving pre-conditioning. The responses were sparse. Two CLL patients that had received pre-conditioning had stable disease. Five additional patients with adult pre-B-ALL were treated with a CD28-CAR, all receiving pre-conditioning. All patients were minimal residual disease negative after T cell infusion. Four of the five patients received allogeneic stem cell transplantation 1-4 months after therapy, while the fifth patient had a relapse 90 days after treatment.[101] Subsequently, 16 patients with relapsed or refractory B-ALL were treated, 10 had complete remission of which 7 received allogeneic stem cell transplantation.[102]

With efficacy comes toxicity, and CAR T cell therapy is no exception. Most severe adverse events may relate to the pre-conditioning, but there are some reactions directly related to the CAR T cells.

CAR-related adverse events

In αCD19 CAR trials, B cell aplasia is an expected adverse event of efficient treatment due to expression of CD19 also on healthy B cells. The aplasia can be managed with intravenous infusions of immunoglobulins should the patient suffer from persistent infections. Tumor lysis syndrome has also been reported, generally related to large tumor load in the treated patient. Delayed appearance of adverse events, up to 50 days[90] following infusion can occur, and be related to the T cell dose and their proliferative capacity.

Cytokine release syndrome (CRS) is seen in nearly all responding patients in CAR trials. Initial symptoms are fever and features commonly seen in infections and can appear within days or weeks coinciding with peak T cell expansion. C-reactive protein (CRP) functions as a reliable surrogate marker. As IL-6 appears to be the central mediator of CRS, the αIL-6R monoclonal antibody (mab) tocilizumab has successfully been used to block severe CRS in patients.[91,112] Corticosteriods are also effective in blocking CRS but could potentially also negatively affect the T cell-induced anti-tumor response to a greater extent than tocilizumab.[102] It has also been suggested that the response to tocilizumab may be more rapid.[113] Macrophage activation syndrome is related to hemophagocytic lymphohistiocytosis[114,115] and has been reported in association with CRS in a αCD19 CAR T cell trial.[91] Macrophage activation syndrome could in part be driven by high levels of IL-6.[116] Further, recent αCD19 CAR trials have reported neurological side-effects[101,102,111] of unknown origin, possibly related to high levels of cytokines. Reports have included transient aphasia, confusion, facial nerve palsy, myoclonus,[111] delirium and seizure-like activity.[102] Interestingly, neurological toxicities and seizures have previously been reported in a trial treating patients with Blinatumomab, a CD3/CD19 bi-specific T-cell-engaging antibody (BiTE).[117] It has been suggested that the neurological side-effects are more prominent in CAR T cells with CD28[99,101,102,111] and that it potentially could be explained by the enhanced TNFα secretion associated with CD28 signaling.[118]

There have also been serious adverse events with fatal outcome reported. Morgan *et al* reported one death in a phase I clinical trial investigating a third generation αHer2 CAR. Her2 is overexpressed in tumor tissue but is also present on healthy tissues. The patient was infused with $1x10^{10}$ cells, which is by far exceeding the normal numbers used in other CAR trials ($1x10^{7}$ - $5x10^{8}$ cells/patient). Cause of death was most likely due to on-target off-tumor effect where the T cells recognized their target on lung tissue giv-

ing rise to a fatal pulmonary toxicity.[109] In a phase I αCD19 CAR treating CLL, one patient most likely had an undetected infection prior to treatment which was aggravated by the pre-conditioning chemotherapy, leading to fatal outcome.[46] In a αCD19-CD28-CAR study by Kochenderfer *et al*, one patient past away 18 days after T cell infusion due to influenza A pneumonia, nonbacterial thrombotic endocarditis and cerebral infarction.[99] In another study by Kochenderfer *et al* they recently reported that one lymphoma patient treated with αCD19-CD28-CAR died of an unknown cause 16 days after T cell infusion.[111] Overall, there have been few fatal outcomes considering the number of patients treated.

Engineering therapeutic effect and safety

There are many ideas on how to improve and modify CAR T cell therapy to achieve an optimal balance of anti-tumor effect, treatment availability and patient safety. Several targets for T cell therapy today are tumor-associated antigens that are expressed on both tumor cells as well as healthy tissues. To improve specificity and safety dual-targeting CARs have been constructed. Signaling through these constructs require presence of two different antigens on the target cell to enable CAR T cell killing, for instance the breast cancer antigens Her2 and mucin 1.[119] In a similar approach, another CAR was constructed with a ScFv specific for mesothelin linked to a CD3 zeta chain and *in trans* a CAR specific for folate receptor α linked to costimulatory CD28. Recognition of one target led to sparse T cell response while an encounter with a target cell expressing both antigens lead to a response similar to that of a regular second generation CAR.[120]

Tumor cells commonly lack costimulatory molecules and instead express immune inhibitory molecules on their surface. Switch receptors is a concept where for instance extracellular PD-1 is linked to cytoplasmic CD28, converting an inhibitory signal into a costimulatory one.[121]

Now when cell therapies begin to show durable responses in the clinic commercialization and streamlining to create an "off-the-shelf" product becomes the next natural step. A universal immune receptor platform could be one approach. A biotin-binding immune receptor platform is a receptor construct with signaling domains linked to an extracellular avidin providing a possibility to have a standard receptor construct that can be used to target a large variety of antigens. Target cells are labelled with biotin conjugated antigen specific antibodies or ScFv's and can thereby be recognized through avidin/biotin interaction.[122] Another is to develop allogenic T cells for CAR or TCR engineered therapy. Attempts to circumvent graft versus host disease include allogenic stimulation of the T cells while blocking CD28-mediated costimulation in order to induce tolerance,[123] inactivation of the TCRα con-

stant gene[124] or knockout of endogenous TCRs[70,125] and HLA[126] have been attempted.

For T cell therapy to be efficient in solid tumors migration to the tumor site is of the essence. Many tumors secrete different chemokines and immunosuppressive cytokines. Therefore, attempts to improve homing to and persistence in tumor tissue have been made. In a murine system for neuroblastoma αGD2 CARs showed increased anti-tumor effect when co-expressing chemokine receptor CCR2b.[127] Similarly, a mesothelin-directed CCR2b CAR increased migration towards CCL2, secreted by the tumor, and improved anti-tumor activity.[128] Also, αCD30 CAR co-expressing CCR4 displayed enhanced migration to CCL17-secreting Hodgkins lymphoma cells and increased anti-tumor efficacy.[129] Immunosuppressive cytokine TGFβ is commonly secreted from tumor cells. Improving T cell resistance to TGFβ has been addressed through dominant negative TGFβ receptors[130,131] and soluble TGFβ decoy receptors.[132] Also, reduced sensitivity to apoptosis can be conferred through siRNA silencing of FAS[133] or PD-1 ligands PD-L1 and PD-L2.[134]

A successful engraftment and expansion of transferred T cells is crucial for a strong immune response and tumor regression. However, a too powerful immune response can be fatal for the patient and therefore we need techniques to control it. As previously mentioned, tocilizumab can be used to modulate response-related symptoms as well as corticosteroids. But if the cells are attacking normal tissues it is desirable to knock-out the infused CAR T cells. For this purpose, suicide genes can be utilized. A suicide gene can be inserted into to the gene vehicle construct. There are several different methods investigated. For example, a herpes simplex virus-derived thymidine kinase gene that can be used in combination with administration of nucleoside analogs such as ganciclovir, leading to blocked DNA synthesis in the engineered cell. However, this technique functions only in proliferating cells and it has also been shown that incorporation of a viral gene renders the cells immunogenic, leading to a risk that the engineered cells are eliminated before it has an effect on the tumor.[135] Genes that induce apoptosis have been investigated as a potential alternative, among them inducible Fas,[136] inducible caspase 8[137] and inducible caspase 9.[138-141] Inducible caspase 9 is linked to small molecule that has high affinity for a non-toxic drug. When required the drug will be administrated to the patient leading to dimerization and thereby activation of caspase 9 and, hence, downstream apoptosis signaling in the engineered T cell. It has been shown to eliminate 90% of the engineered T cell population in a patient within 30 minutes.[138,141] The dimerization-inducing drugs tested so far have shown no adverse effects for patients, nor has the addition of a suicide gene for T cell treatment.[138,142]

Cell properties affecting engraftment in T cell therapies

There are some aspects of CAR T cell therapy that is relevant for all T cell therapies. How do we optimize the protocols to achieve engraftment of the transferred T cells and assure their *in vivo* persistence? Engraftment and *in vivo* expansion is crucial for *in vivo* persistence, which in turn correlates with cancer regression.[143] In the following sections, some of the major discussion topics in the field will be briefly reviewed.

T cell phenotype

In the beginning many groups focused on infusing CD8+ T cells because of their known cytotoxic function.[144] There was also a fear of transferring CD4+ Tregs and thereby supporting the immunosuppressive environment. However, TILs studies infusing CD4+ rich populations have shown positive effects on the patients,[145] and it is likely important that both CD4+ and CD8+ cells collaborate for successful treatment. The CD4+ cells can provide "helper" support for the transferred CD8+ cells, but also provide this support for endogenous T cells or NK cells broadening the immune response.[146]

In regards to maturation stage of the transferred T cells, the optimal phenotype is debated and so is the differentiation direction between the different stages of memory. A model of T cell differentiation in which cells proceed from naive cells to stem cell memory to central memory and thereafter effector memory has been suggested based on progressive phenotypic and functional T cell changes as well as the gene-expression pattern of progressive up- or downregulation according to the previously mentioned order.[147] In many early trials, effector and EM cells have been transferred and although these cells displayed strong tumor-reactive features in vitro they failed to induce tumor regression *in vivo*. One of the reasons may be due to the T cell phenotype and that a less differentiated cell would increase *in vivo* persistence. Some advocate that central memory cells are the preferred T cell phenotype, while others suggest naïve cells. In murine studies, EM cells were shown to lack persistence, whereas CM cells resulted in long-term memory response and differentiation into EM cells upon antigen-encounter[148,149] High number of CD8+CD27+ T cells has been associated with clinical response to TILs treatment[150] Genetically engineered T cells from CM cells persisted longer than effector cells from EM origin in a study in a primate model[151] Kalos *et al* reported that the persisting T cells in their patients treated with a 4-1BB-CAR were of CM phenotype.[89] Conversely, in a murine model, T cells of naive phenotype showed increased anti-tumor activity compared to cells of CM phenotype.[152] The presence of stem cell CM T cell has also been suggested. Gattinoni *et al* reported findings of a stem-like T memory cell expressing CD8+, CD45RA+, CCR7+, CD62L+, CD27+ and CD28+ with phenotypical features resembling naïve cells yet displaying traits of memory

cells.[147] Xu *et al* found a correlation between the frequency of CD8+, CD45RA+, CCR7+ cells within the infused CD19-targeting CAR T cells and their *in vivo* expansion in a trial treating lymphoma patients.[153] Naïve cells have larger replicative potential and longer telomeres than memory cells, due to their less differentiated phenotype.[154] Telomere length of transferred cells has been correlated with clinical response in melanoma patients treated with TILs.[42,155]

In vivo cytokine support

Expansion of T cells *in vivo* is crucial for engraftment of transferred cells. IL-2 effectively expands T cells in culture and *in vivo*. However, IL-2 can stimulate expansion of bystander Tregs.[156] Systemic treatment with IL-2 is also associated with severe adverse events and can only be administered to patients with a good general condition.[47] Due to the high toxicity of many cytokines, expression cassettes are incorporated into TCR or CAR constructs to provide local secretion *in vivo*. For example, cytokine cassettes have been tested for local administration of IL-2,[140] IL-12[157,158] and IL-15.[139,140]

Pre-conditioning

Pre-conditioning induces lymphopenia leading to homeostatic responses promoting proliferation of the transferred cells while restoring the size of the endogenous T cell pool. The depletion of cells also reduces the number of endogenous immunosuppressive cells such as Tregs and myeloid-derived suppressor cells.[39,159] In clinical trials of TILs pre-conditioning was required for durable responses. The early trials without pre-conditioning reported only modest or no responses to TILs treatment, while the response rate in these trials increased with increased intensity of pre-conditioning from low dose fludarabine and cyclophosphamide in combination, to increased dosage and time-span and subsequently addition of TBI.[43] A negative correlation between Treg (CD4+ Foxp3+ T cells) and clinical response was seen in these patients.[160] CAR T cell infusions without prior pre-conditioning showed transient effect but no durable responses.[85] In recent CAR studies where pre-conditioning was administered, durable responses have been seen.[90,91,102,111] In studies of B cell leukemia and lymphoma treated with CD19-targeting CAR T cells, chemotherapy pre-conditioning aims to reduce tumor burden and thereby reduce the risk of tumor lysis syndrome and cytokine release syndrome. Therefore, several different chemotherapy drugs have been utilized based on patient chemotherapy response. Heavy pre-conditioning treatment can have a strong positive effect on T cell engraftment and tumor elimination, yet pre-conditioning can cause severe side-effects.

We are currently investigating CAR T cells targeting CD19 that is expressed almost exclusively on B cells and is therefore an appropriate target for B cell malignancies.

Hematological malignancies

Hematological malignancies entail a large group of tumors originating from cells of the immune system, for example B or T cells. While hematological malignancies are cancers of the blood they are also cancers of the immune system. In a way infiltrating the system that should protects us against cancer. In this thesis I will focus on B cell malignancies. This is a very heterogeneous group of cancers that arise in different stages of the B cells development, from the early precursor B cell stage (e.g. pre B-ALL) to more mature B cells (e.g. CLL), and include both leukemias and lymphomas. Most B cell malignancies are highly dependent on their microenvironment for survival and propagation. In order to understand the underlying pathogenesis of B cell malignancies it is important to be familiar with B cell development.

B cell development

B cells and T cells originate from a common lymphoid progenitor cell. While T cells mature in the thymus, B cells partially mature in the bone marrow and complete their maturation in secondary lymphoid organs. The microenvironment in the bone marrow will allow for lymphoid progenitor cells to differentiate to pro-B-cells. For a B cell to be able to produce antibodies they must differentiate and undergo a rearrangement of the immunoglobulin genes. The rearrangement is a complex process where DNA double strand-breaks are induced and variable (V), diversity (D) and joining (J) gene segments are joined together to achieve the heavy and light chain of a functional antigen B cell receptor (BCR).[161] It is initiated at the pro-B-cell stage starting with rearrangement of the heavy chain. In the late pro-B-cell stage, the B cell also starts to express the BCR co-receptor CD19. During the pre-B cell stage the μ heavy chain is transcribed and subsequently synthesized in the cytoplasm and a small portion of these can associate with an invariant surrogate light chain, leading to low expression of a pre-BCR on the B cell surface. However, these receptors cannot recognize or respond to antigen. Next, a κ or λ light chain gene is rearranged, similarly to the heavy chain but only including V and J segments, producing a light chain that associates with the heavy chain to produce a complete IgM. The IgM is expressed together with an Igα (CD79a) and an Igβ (CD79b) functioning as an antigen receptor.[1,162] Immature B cells expressing a functional BCR exits the bone marrow. B cell maturation takes place in secondary lymphoid organs. In the germinal centers of lymphoid tissues antigen-activated B cells undergo clonal expansion,

class-switch recombination and the immunoglobulin heavy variable (IGHV) genes are modified through somatic hypermutation and high-affinity BCRs selected through affinity maturation.[163,164] These processes give rise to such high variability that the likelihood of finding two B cell clones with identical BCRs is virtually negligible. However, all these rearrangements require double-strand breaks, removal of intervening DNA and rejoining of the segments, giving rise to many opportunities for discrepancies to occur. Chromosomal translocations of Ig loci and a proto-oncogene are common in many B cell lymphomas, for example the BCL-2-IgH translocation seen in follicular lymphoma and are a result of mistakes during V(D)J recombination. Other lymphoma transforming translocations can occur during somatic hypermutation or in the IgH constant region during class-switching, events that also involve DNA strand breaks.[163]

In this thesis I will give a brief summary of four CD19+ B cell malignancies that has been part of my thesis work. They will be presented in the same order as the papers they appear in in this thesis, with an emphasis on CLL that has been the focus for the majority of the papers.

Precursor B cell acute lymphocytic leukemia

Diagnosis and incidence

Precursor B cell acute lymphocytic leukemia (Pre-B-ALL) is an aggressive disease that is more common in children than in adults. It is also the most common childhood malignancy, with about 70 new cases in Sweden each year. Peak incidence is at the age of 2-5 years. Patients are often tired, pale and have pain in the bones of their body, bruises that don't heal and are more susceptible to infections than normal. Since the transformation takes place in pre-B-cells in the bone marrow, diagnosis is confirmed with a bone marrow sample, showing an accumulation of CD19+ blasts crowding the healthy cells of the bone marrow. Epidemiologic studies indicate a potential role of infections in the etiology of ALL, as well as environmental factors such as exposure to ionizing and non-ionizing radiation.[165-167]

Treatment and prognosis

Age is a prognostic factor for pre-B-ALL. Children 1-9 years have a better outcome than infants and adolescent children. 85% of newly diagnosed children and young adults with pre-B-ALL are today cured with chemotherapy and supportive care.[168] However, the prognosis for patients with relapsed and chemotherapy-refractory pre-B-ALL is poor, especially for those relapsing after hematopoietic stem cell transplantation.[169] The majority of childhood ALLs harbor chromosomal alterations for instance hyperdiploidy, hypodiploidy or translocations e.g. TEL-AML1 and BCR-ABL. The TEL-AML1

fusion gene, present in 25% of pre-B-ALL patients is associated with a favorable outcome.[170] Translocation between chromosomes 9 and 22 (Philadelphia chromosome) occurs in approximately 5% of children with ALL. Infants (<6 months) are treated according to the international Interfant protocol. Current treatment protocol (NOPHO) in Sweden for childhood pre-B-ALL consists of induction, consolidation, late intensification phase and maintenance therapy (all in all 2 ½ years). Patients are divided in to risk groups based on genetic aberrations and lymphocyte count in peripheral blood before initial induction treatment. Consolidation treatment is decided based on response to induction treatment, genetic aberrations and CNS involvement (presence of leukemic cells in cerebrospinal fluid). The treatment consists of corticosteroids combined with different combination chemotherapy treatments of increasing intensity based on previously mentioned criteria. Philadelphia chromosome positive pre-B-ALL is historically associated with a very poor prognosis. However, the tyrosine kinase inhibitor imatinib is effective in this group of patients and its addition to standard chemotherapy treatment has greatly improved the event free survival of these patients.[171]

Chronic Lymphocytic Leukemia

Diagnosis and incidence

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia of the Western world.[172] Median age at diagnosis is ~70 years, but about 30% are younger than 65.[173] It affects twice as many men as women and approximately 500 individuals in Sweden every year.[174] The etiology is largely unknown. CLL is a very heterogeneous diagnosis, spanning from indolent forms not requiring treatment to aggressive disease with deadly outcome. Common symptoms are fatigue, frequent infections, fever, weight loss, night sweats, pain in the upper abdomen (spleen) and enlarged lymph nodes. CLL is often discovered in association with routine visits were patient blood samples are found to contain an irregular amount of lymphocytes. The diagnosis is based on lymphocytosis ($\geq 5.0 \times 10^9$/L) in peripheral blood containing mature B cells that express CD19, CD5, CD23, CD200 and display a low expression of surface immunoglobulins.[173] The cell of origin is debated and also the matter whether there is more than one cell giving rise to the genetically different B-CLL variants carrying mutated or unmutated IGHV. According to the two cell origin hypothesis, CD5+ B cells are suggested as the cells of origin, pre-germinal center cells giving rise to unmutated IGHV CLL and post-germinal center cells giving rise to the mutated IGHV CLL.[175,176] However, as a sole predecessor to both types of CLL, the marginal zone B cell is at this time considered to be the most likely candidate.[177]

Microenvironment in CLL

Many lymphomas are strongly dependent on their microenvironment, something that becomes apparent when attempting to culture the cells *in vitro*. Findings of deregulation of cell-cycle regulatory genes and defective apoptosis led the field to believe that CLL results from accumulation of cells rather than proliferation.[174] Circulating CLL cells are non-dividing, quiescent cells. However, a small portion of the CLL clone proliferates in proliferation centers or so called pseudofollicles in the lymph nodes.[178-180] Bone marrow stromal cells (BMSCs) functions as support for the CLL cells and can attract the CXCR4-expressing CLL cells via secretion of CCL12.[181,182] CD38 expression on the surface of the CLL cell allows for binding to CD31 on BMSCs leading to activation of ZAP-70 and downstream survival pathways.[182,183] CLL cells also interact with nurse-like cells present in the spleen and secondary lymphoid tissues, in a similar manner as with BMSCs, which protects them from spontaneous or drug-induced cell death.[182] BCR-signaling is activated through binding of autoantigens and environmental antigens giving rise to secretion of cytokines such as CCL3, CCL-17 and CCL-22 which in turn recruit T cells and monocytes.[181,184] Activated CD4+ CD40L+ T cells present in the proliferation centers,[183,185] can provide stimulation for the CLL cells via CD40L interactions and IL-4 secretion.[181] Interestingly, T cells in CLL patients display several dysfunctional features affecting their cytotoxic capacity, among them, inability to form a functional immunological synapse.[186] CLL has also been associated with autoimmune diseases and the CLL cells produce autoantibodies.[187,188]

Staging and prognosis

The prognosis for patients diagnosed with CLL varies greatly mirroring the heterogeneity of the disease. Clinical staging is based on Rai[189] or Binet[190] classification where the different stages are determined based on hemoglobin and platelet levels in peripheral blood, as well as palpable lymphadenopathy, hepatomegaly and splenomegaly.[173] Genetic characteristics of the CLL cell are currently the basis for prognosis evaluation. Mutation status of the heavy chain of the immunoglobin gene (IGHV) has been established as one of the most reliable prognostic markers in CLL, where a mutated IGHV is associated with a favorable prognosis. [175,176] However, there is an exception, the IGHV3-21 gene is associated with poor outcome, regardless of mutational status.[191] CD38 is commonly expressed in patients with unmutated IGHV genes and is associated with poor prognosis. High levels of ZAP-70 is often seen in unmutated CLL and might indicate a more aggressive disease.[174] In 80% of CLL patients chromosomal abnormalities can be found, of which deletion in chromosome 13q, the most common alteration in CLL, is associated with a favorable prognosis and an indolent disease course, whereas de-

letions in 11q or 17p are associated with a more aggressive disease and poor prognosis.[192] TP53 mutation in the remaining allele of 17p has been associated with drug resistance in CLL patients with 17p deletion.[193-195] TP53 mutation without 17p deletion is associated with a prognosis similar to that of 17p deletion patients.[173] Also, trisomy 12 affects 11-16% of CLL patients and these patients display a more intermediate disease course.[192] BCRs with high homology, so called stereotyped BCRs[196-199] have been found in patients with CLL and are present in more than 20% of CLL patients. [200,201] Today more than 100 subtypes are defined.

Treatment

Asymptomatic patients are kept under observation and treated upon progression or symptomatic disease. Approximately 1/3 of CLL patients never require treatment. CLL patients with symptomatic disease are primarily treated with combination chemotherapy of fludarabine and cyclophosphamide. Fludarabine refractory patients are treated with an addition of rituximab to previous chemotherapy treatment (FCR), bendamustine and rituximab in combination, alemtuzumab (αCD52 mab) or allogeneic stem cell transplantation. Patients that carry the 17p deletion/TP53 deletion are evaluated for allogeneic stem cell transplantation. Stem cell transplantation is only considered for younger patients with high risk genes or that experience early relapse. Patients with 17p deletion that are not eligible for stem cell transplantation are treated with alemtuzumab. Patients that are elderly and/or have comorbidities are treated with chlorambucil or bendamustine in order to reduce disease-related symptoms. Glucocorticoids are added to treatment for patients with autoimmune complications.[173]

The B-CLL cell dependency on microenvironmental cues has stirred interest in drugs modifying the CLL cells' interactions with its microenvironment. Among those is lenalidomide that has shown immunomodulating effects in CLL, for instance, repairing the dysfunctional synapse formation of T cells.[186] Clinical investigations has shown promising results, but treatment is associated with tumor lysis syndrome.[202] Also, several kinase inhibitors involved in BCR signaling e.g. ibrutinib, fostamatinib and idelalisib are currently under evaluation for treatment of CLL.[203]

Diffuse large B cell lymphoma

Diagnosis and incidence

Diffuse large B cell lymphoma (DLBCL) is the most common type of non-Hodgkin's lymphoma among adults and affects about 500 Swedes yearly. The median age of diagnosis is ~70 years. DLBCL comprise a heterogeneous group of lymphomas, although similar in morphology giving rise to a

wide variety of immunological characteristics and clinical manifestations and outcomes. Enlarged lymph nodes, pruritus, fever, night sweats and weight loss are common symptoms. Tumor cells express CD19 and CD20 and the proliferative fraction is high (30-95%). The most common forms of DLBCL, the germinal center-type and the activated B cell are considered to originate from germinal center or post-germinal center B cells.[163] Several autoimmune diseases increase the risk of developing B-cell lymphoma, for instance systemic lupus erythematosus[204], rheumatoid arthritis[204,205] and Sjögren's syndrome[206] have been associated with DLBCL.

Treatment and prognosis

Aberrant hypermutation of multiple oncogenes could play an important role in the pathogenesis of DLBCL.[207] For instance Bcl-2 overexpression has been reported in ~40-60% of DLBCL patients, and has been associated with a less favorable outcome.[208] MYC rearrangement is present in 5-10% of DLBCL patients and is associated with poor outcome[209,210] and in one trial, increased risk of CNS relapse.[210] Aggressive lymphomas are treated with Rituximab and combination chemotherapy (R-CHOP) which cures approximately 60-70% of the patients. Patients that relapse or are refractory to treatment receive salvage chemotherapy or high-dose chemotherapy followed by autologous stem cell transplantation.[211] However, in a recent retrospective study, the median overall survival for patients that progress after autologous stem-cell transplantation was <10 months.[212] Younger patients that respond to chemotherapy can receive allogeneic stem cell transplantation as an experimental treatment. About 1% of DLBCL patients have CNS involvement at diagnosis, and during the course of disease about 5% of patients are affected. These patients receive methotrexate and cytarabine as well as R-CHOP. Prophylactic treatment for CNS protection is also commonly used.[211]

Mantle cell lymphoma

Diagnosis and incidence

Mantle cell lymphoma (MCL) is classified as an aggressive lymphoma with a median survival of 3-5 years.[213] However, a small subset of patients has an indolent form that doesn't require treatment. Median age at diagnosis is about 65 years and the majority of patients are male (3:1). The incidence is low, approximately 6% of all non-Hodgkin's lymphomas. For the majority, the disease is spread, involving bone marrow, the gastrointestinal tract, lymph nodes and spleen.[163,214] MCL can also involve oropharyngeal and pulmonary sites and the skin. MCL share many features with CLL besides involvement of blood, bone marrow, spleen and lymph nodes. BCR signaling is important, stereotyped BCRs and both mutated and unmutated IGHV

occur also in MCL.[215] MCL cells are mature B cells defined as CD5+ CD23- CD200-.[214]

Treatment and prognosis

Blast morphology and Ki-67 are the most reliable prognostic markers.[214] 95% of MCL cases overexpress cyclin D1 promoting cell cycle entry. Inactivation of pro-apoptotic protein Bim has also been observed in MCL.[215] The most commonly mutated gene in MCL is the tumor suppressor gene ataxia telangiectasia mutated (ATM) that plays a crucial part in DNA repair, linking it to the rearrangements of the BCR. Loss or downregulation of ATM also affects apoptosis induction since ATM activates p53 upon DNA damage.[215] Nearly all cases are associated with BCL1-IgH translocation.[163] At present, there is no curative treatment available for MCL. High response is seen to first line chemotherapy. However, the majority of patients relapses and succumbs to their disease. Late relapses are common.[215] Patients are treated with different combination chemotherapy treatments where inclusion of rituximab improve prognosis.[216] Bendamustine has fewer side effects and is therefore recommended for older patients. Younger patients are treated with Rituximab and chemotherapy in combination followed by autologous stem cell transplantation. Bortezomib, temsirolimus, thalidomid and lenalidomid has shown promising results in MCL but is accompanied by severe adverse events. There are also some cases with localized disease that can be treated with radiation.[214]

B cell malignancies are a heterogenic group of tumors and despite the progresses in treatment for some of these tumors, there is still a large group of patients in need of new therapies. Tumor cells can become resistant to treatment. This can happen in many different ways. In hematological malignancies resistance is often conveyed through overexpression of anti-apoptotic proteins.

Apoptosis

Aberrant cells are constantly formed in the body due to the intricacy of the processes that control their function and replication. However, all cells have a contingency plan for dysfunction and are programmed to die if aberrations occur. Apoptosis is a programmed cell death and a way for the body to get rid of waste material in a secure way. It is also utilized by for instance T cells that kill their targets through induction of apoptosis. Several different stressors can trigger apoptosis. External stimuli originating from outside the cell lead to signaling via the extrinsic pathway, while signals inside the cell induce signaling trough the intrinsic pathway of apoptosis.

Intrinsic pathway of apoptosis

Stress stimuli such as DNA-damaging agents, heat or radiation can all trigger the intrinsic pathway of apoptosis. Intrinsic apoptosis signaling is mainly regulated by the B-cell lymphoma 2 (Bcl-2) family proteins. The Bcl-2 family consists of pro-apoptotic proteins e.g. Bax, Bid, Bak, Bim, Noxa, Puma and anti-apoptotic proteins e.g. Bcl-2, Bcl-xL, Bcl-w and Mcl-1. The pro-apoptotic proteins Bax, Bid and Bak are under normal conditions sequestered by anti-apoptotic proteins, for example Bcl-2 and Bcl-xL, preventing them from initiating apoptosis signaling. An apoptotic stimulus activates the transcriptional regulator p53 that in turn upregulates pro-apoptotic genes e.g. BAD, BAX, BID, BAK, APAF-1, FAS, TRAIL and downregulates anti-apoptotic genes such as BCL-2 and SURVIVIN. Also, p53 can directly activate Bax and Bad present in the cytosol.[217,218] The increased number of pro-apoptotic proteins will, due to their higher binding affinity, displace Bak and Bax from binding to the anti-apoptotic proteins Bcl-2 and Bcl-xL, thereby translocating them to the mitochondrial membrane.[219] Upon apoptotic stimulus Bax translocates to the mitochondria where it is inserted in the outer membrane. Bak, resident in the mitochondrial membrane, undergoes a conformational change facilitating dimerization with Bax giving rise to mitochondrial outer membrane permeabilization (MOMP). Due to the increased permeability of the mitochondrial membrane, the pro-apoptotic proteins cytochrome c, Smac, Omi, apoptosis-inducing factor and Endonuclease G are released into the cytoplasm.[218,220] In the cytoplasm, cytochrome c binds to and activates the scaffolding protein apoptotic protease-activating factor-1 (APAF-1), leading to formation of the apoptosome. The apoptosome functions as a platform for procaspase 9 dimerization and thereby its activation.[221] The active form, caspase 9, activates executioner caspases 3 and 7 through proteolytic cleavage.[217,221] AIF and endonuclease G induces apoptosis through translocation to the nucleus where they induce chromatin condensation, DNA damage and reactive oxygen species production. A control system as important as the apoptotic machinery needs to be tightly regulated. Several regulating molecules and feed-back loops can affect the course of action, enhancing or rescuing the cell from apoptosis, at different stages of induction. At an early stage, before apoptosome formation, the mitochondrial pathway is regulated by anti-apoptotic proteins of the Bcl-2 family. In this initial phase of apoptosis induction, Bcl-2, Bcl-xL and Mcl-1 all inhibit Bax/Bak dimerization. Bcl-2 sequesters Bax, Bad, Bim, while Bcl-xL blocks Bax, Bak, Bad, Bim and Bid, and Mcl-1 inhibits Bax and Bim. Pro-apoptotic proteins Bad and Noxa in turn block the anti-apoptotic proteins. Bad dimerizes with Bcl-xL and to a lesser extent with Bcl-2, while Noxa binds Mcl-1. In a later stage, after MOMP, apoptosis is regulated by members of the inhibitor of apoptosis family (IAP), including for instance X chromosome-linked IAP (XIAP), Survivin and Livin. XIAP can inhibit both initiator

(caspase 9) and executioner caspases (3, 7).[222,223] Smac and Omi, that are released from the mitochondria at MOMP inhibit XIAP, while Livin in turn inhibits Smac.[223] Survivin hinders the release of apoptosis-inducing factor from the mitochondria, inhibiting its pro-apoptotic function.

Extrinsic pathway of apoptosis

The extrinsic pathway is triggered via binding of ligand to Fas, tumor necrosis factor receptor (TNFR) or TNF-related apoptosis-inducing ligand receptor (TRAILR) on the cell surface. These so called death receptors share a similar signaling pattern. Binding of ligand (FasL, TNF or TRAIL, respectively) to the receptor results in receptor trimerizing and a conformational change of its cytosolic death domain, inducing an active state. This recruits the Fas-associated protein with death domain (FADD) to the cytosolic death domain of the receptor. FADD in turn binds procaspase 8, forming the death-inducing signaling complex (DISC) which leads to an autoactivation of procaspase 8.[224] Active caspase 8 can directly activate the executioner caspases 3 and 7 through proteolytic cleavage. The formation of DISC can be inhibited by cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (cFLIP) that through blocking activation of caspase 8 (also known as FLICE) regulates extrinsic apoptosis induction. Caspase 8 can also cleave Bid thereby producing its active form truncated Bid (tBid). This cross-talk between the intrinsic and the extrinsic pathway enhances the pro-apoptotic signaling in the cell. Upon activation, caspase 3 and 7 initiates disassembly of several structure-upholding proteins in the cell and degradation of DNA, giving rise to the typical appearance of an apoptotic cell; rounded with blebbing cell membrane and pyknotic nucleus. Surface exposure of phosphatidylserine and other phagocytic signals attracts phagocytes making sure that no debris is left behind.[217]

T cell-induced apoptosis

T cells kill their targets through induction of apoptosis, either via FasL-Fas interaction or through secretion of granzymes and perforin. As previously described, CD8+ T cells bind with the TCR and CD8 to the peptide/MHC complex on the target cell, leading to formation of an immunological synapse. Granzymes and perforin are subsequently released from the T cells into the formed synapse. Perforin is thought to facilitate the entry of granzymes into the target cell. Granzyme B is a serine protease that, once inside the target cell, induces apoptosis through truncating Bid, leading to MOMP, or by directly activating executioner caspases 3 and 7.[225,226] Granzyme A on the other hand induces apoptosis in a caspase-independent manner. There is much less known about how granzyme A induces apoptosis but current theories suggests that it induces translocation of the redox-sensitive SET-

complex from the ER to the nucleus where it inactivates proteins essential for DNA repair and releases nucleases to induce DNA-damage.[225,226]

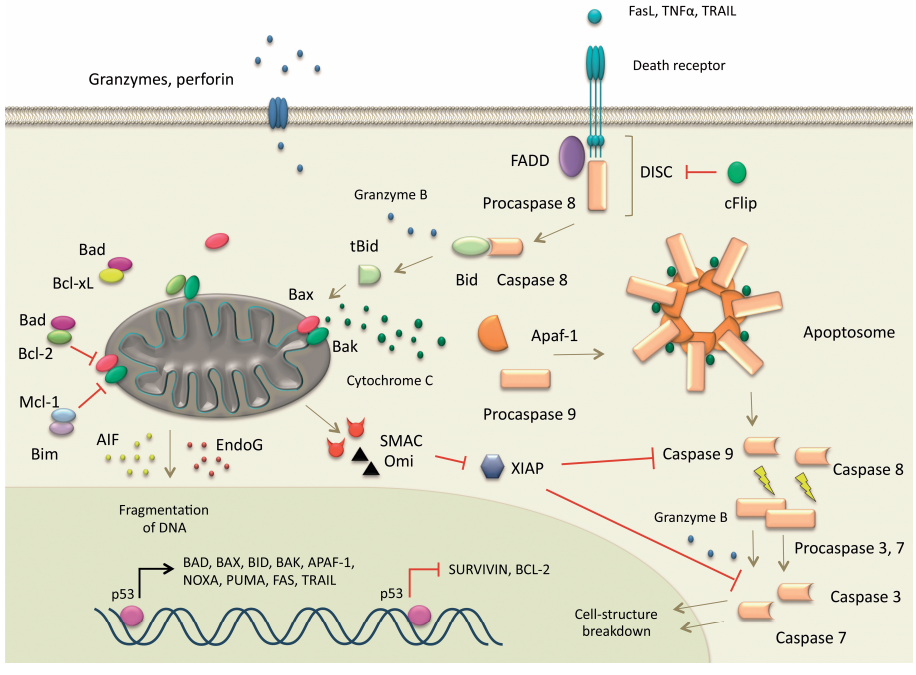

Figure 3. Apoptosis can be induced via the extrinsic pathway initiated via binding of ligand to death receptors leading to assembly of the death inducing signaling complex (DISC) activating caspase 8. Caspase 8 induces apoptosis by directly activates executioner caspase 3 and 7 or via Bid. The intrinsic pathway is triggered by e.g. DNA damage leading to p53-mediated upregulation of pro-apoptotic proteins giving rise to Bax/Bak dimerization in in the mitochondrial membrane inducing MOMP. As a result cytochrome c is released from the mitochondria leading to apoptosome formation and subsequent activation of caspase 9 and executioner caspases 3 and 7. T cells can induce apoptosis via death receptor interaction and via granzyme release. Granzyme B can activate Bid leading to MOMP and/or activate caspase 3 and 7 directly.

Apoptosis in malignant cells

In malignant cells, resisting cell death is considered a hallmark, commonly conveyed by a halted apoptosis machinery.[227] However, different types of tumors have different approaches to ensure their viability, for instance genetic mutations, epigenetic changes and overexpression of anti-apoptotic proteins. Mutations in p53 is a well-known genetic alteration that is present in the majority of human cancers, frequently associated with advanced disease and a poor prognosis.[228] In hematological malignancies, overexpression of anti-apoptotic proteins of the Bcl-2 family is common and is often associated

with chemoresistance and progressive disease. Pre-B-ALL, CLL, DLBCL and MCL tumor cells all overexpress Bcl-2 as well as other components of the apoptosis machinery.[208,229,230] For example, Mcl-1 is commonly overexpressed in CLL where the ratio Bax to Mcl-1/Bcl-2 correlates with progressive and/or resistant disease.[231] Genomic deletions in MCL cells results in Bcl-2, Mcl-1 and Bcl-xL upregulation, as well as Bim inactivation.[229]

IAPs are also overexpressed in numerous human cancers, often linked to poor prognosis and treatment failure.[223] Death receptor silencing resulting from receptor downregulation or through mutations in the caspase-binding death domain of the receptor has been seen to convey resistance to apoptosis induction via the extrinsic pathway.[228] Tumor cells can also evade apoptosis through release of decoy receptors that bind ligands of the death receptors, keeping them from inducing apoptosis via the extrinsic pathway.[224] Another way is through overexpression of cFLIP, thereby inhibiting DISC formation.[228] Activation of pro-survival pathways such as PI3K leading to activation of NFκB and inactivation of Bad and caspase 9 is also found in human tumors.

Targeting the apoptotic machinery

Molecules involved in apoptotic signaling are attractive targets for cancer therapy. Tumor cells are under apoptotic pressure, dodging the bullet through a vast repertoire of escape mechanisms (as discussed in previous section), but apoptosis can be reactivated in these cells. The apoptotic machinery can be targeted either through inhibition of anti-apoptotic molecules for instance via Bcl-2 family inhibitors and IAP inhibitors or by adding pro-apoptotic stimuli e.g. recombinant TRAIL. ABT-737 is a small molecule Bcl-2 family inhibitor that binds with high affinity to Bcl-2, Bcl-xL and Bcl-w, but not Mcl-1 or Bcl-2 related protein A1.[232,233] ABT-737 has shown high cytolytic effect in cell lines of hematopoietic origin.[233-235] In animal studies, ABT-737 induced regression of tumors, improved survival and resulted in a high rate of cures in the mice.[233] ABT-737 displaces Bim from the binding of Bcl-2, which through activation of Bax leads to MOMP, making circulating CLL cells that are dependent on Bcl-2 sensitive to ABT-737.[236] In an *in vitro* study mimicking lymph node microenvironment, cultured primary CLL cells concurrently upregulated Bcl-xL and Bcl-2-related protein A1 upon treatment, inducing a resistance to ABT-737 that could not be lifted with combination therapies.[237] Many important processes in the cell are subject to redundancy, leading to upregulation of one protein if another with the same function is inhibited. Resistance to ABT-737 has been correlated to high expression of Mcl-1 in several cancer cell lines.[234,238-241] However, High *et al* found no correlation between Mcl-1 expression and response to ABT-737 in *in vivo* ALL xenografts.[234] In primary CLL cells, the Mcl-1/Bcl-2 ratio has

been suggested to be predictive of ABT-737 response.[242] Also, ABT-737 has shown good results in pre-clinical studies when combined with Mcl-1 RNA-silencing[243] or platinum compounds[244] and synergy with more established therapies such as chemotherapy[224-226,238] and radiation.[233] The oral analogue of ABT-737, Navitoclax (ABT-263) has been investigated in phase I/II clinical trials treating both hematological malignancies[245] and solid tumors.[246] ABT-263 showed promising results in the clinic treating patients with CLL.[247] However, its main target Bcl-xL is required for platelet survival and its inhibition lead to severe dose-dependent thrombocytopenia, limiting the therapeutic potential of ABT-263.[246] Therefore, a derivative, ABT-199, targeting only Bcl-2 was generated.[248] Early clinical reports indicated that ABT-199 was effective and appeared to spare the platelets.[249,250] Recent reports from the phase I trial showed an 84% overall response rate of evaluable patients of which 23% had a complete response. In addition, fludarabine-refractory CLL patients and patients with 17p deletion responded well (89 and 82%).[251]

Targeting IAP to restore apoptosis in tumor cells has been attempted by several mechanisms of which small molecule IAP antagonists and antisense oligonucleotides has gather most attention.[223] Among these, molecules mimicking Smac has been shown to sensitize tumor cells to chemotherapy.[252] While testing different compounds antagonizing IAPs, Dougan *et al* found that besides inducing apoptosis, one of their compounds showed additional effects in the form of co-stimulation of T cells.[253]

Stimulation of the extrinsic pathway of apoptosis via recombinant human TRAIL has proved effective in phase I/II clinical trials.[254] However, TRAIL resistance has become an issue in many cancer types, including CLL. Both release of decoy receptors and high expression of Mcl-1 has been linked to TRAIL resistance.[224] Combination strategies to overcome resistance are therefore considered. Targeting both the intrinsic pathway with for instance Bcl-2 family inhibitors and the extrinsic pathway to simultaneous attack the tumor cell from several directions is an attractive strategy. As a matter of fact, death receptor ligand TRAIL has in combination with ABT-737 shown highly synergistic effects *in vitro* giving rise to upregulation of TRAIL receptor 2 expression, lifting ABT-737 resistance in these cells.[255] Similarly, T cells induce apoptosis through other mechanisms than the Bcl-2 family inhibitors and would therefore make an interesting combination therapy. In Paper I we investigated the combination of ABT-737 with a second generation αCD19-CD28-CAR.

Aims

The overall aim of the studies presented in this thesis was to improve CAR T cell therapy. Herein, pre-clinical and initial clinical data using αCD19-targeting CAR T cells for treatment of CD19+ B cell malignancies are presented. The specific aims for the four different studies are specified below.

Paper I

To investigate the apoptosis-inducing effect of second generation αCD19-CD28 CAR T cells in combination with the Bcl-2 family inhibitor ABT-737.

Paper II

To analyze a third generation αCD19-CD28-4-1BB CAR in aspects of:

- Cytotoxicity
- Proliferation
- Immunophenotypic features (memory, exhaustion)
- Cytokine secretion
- Intracellular signaling

Paper III

To evaluate the potential of third generation αCD19-CD28-4-1BB CAR T cells to induce bystander immunity through maturation of immature dendritic cells.

Paper IV/Supplementary information

To establish and evaluate a protocol for GMP production of third generation αCD19-CD28-4-1BB CAR T cells for clinical investigation.

To evaluate the produced patient batches for CAR expression and immunophenotypic features.

To evaluate the immunological status of the patients participating in the clinical trial before and after treatment.

Summary of papers

Paper I

2G CAR T cells (αCD19-CD28-CD3zeta), generated from blood samples from children with pre-B-ALL and healthy donors, were combined with ABT-737, a small molecule inhibitor targeting anti-apoptotic components in the intrinsic pathway of apoptosis. The oral analogue of ABT-737, ABT-263 (Navitoclax) is currently investigated in clinical trials. In our studies, CAR T cells as monotherapy induced apoptosis in five out of the five CD19+ cell lines tested. Three cell lines were also sensitive to ABT-737 as a monotherapy. Interestingly, when combining the two the apoptosis-inducing effect was significantly higher, also in less sensitive cell lines. Our data indicates that ABT-737 could be a potential drug for enhancement of CAR T cell therapy efficacy.

Paper II

2G CAR T cells (αCD19-CD28-CD3zeta) and 3G CAR T cells (αCD19-CD28-4-1BB-CD3zeta) generated from healthy donors and chronic lymphocytic leukemia (CLL) patients were cultured with IL-2, autologous B cells or the combination of the two. Repeated stimulation with B cells lead to an antigen-driven accumulation of CAR+ T cells and an increased proliferation. The CAR T cells were mainly CD8+ memory cells that maintained low expression levels of exhaustion markers throughout culture. 3G and 2G CAR T cells showed equal cytotoxic capacity, similar lineage, memory and exhaustion profile phenotype. However, 3G CAR T cells proliferated better and showed increased activation of intracellular signaling pathways compared to 2G CAR T cells. Interestingly, we also found differences in the kinase activity profile between the 3G and 2G CAR T cells post antigen stimulation. Several of these proteins are involved in the cell cycle, cell adhesion and exocytosis. Collectively, the enhanced properties of 3G CAR T cells could augment their anti-tumor effect *in vivo* compared to 2G CARs.

Paper III

Activated T cells express costimulatory molecules CD27, CD70 and CD40L that could potentially interact with their counterparts on dendritic cells (DCs). CD40L-CD40 interactions are known to induce upregulation of costimulatory molecules on DCs and thereby activating them and increasing their antigen-presenting abilities. 3G CAR T cells (αCD19-CD28-4-1BB-CD3zeta) were investigated for their potential to mature dendritic cells (DCs) as a model of bystander immunity induction. 3G CAR T cells were co-cultured with monocyte-derived immature DCs. After 48h co-culture, the DCs had increased expression of maturation markers (CD83, CD80, CD86) compared to before co-culture. These phenotypically mature DCs were subsequently stimulated with CMV peptide to investigate their potential to expand CMV+ T cells and thereby be proved functionally mature. Co-cultures were also performed in a transwell system where the cells share medium and can communicate via cytokines but cannot physically interact. In this system, no maturation of the DCs was seen, indicating that (in this setting) cell-cell contact is crucial for the maturation process since cytokines alone could not induce maturation. To conclude, CAR T cells could induce maturation of DCs and thereby broaden the immune response against tumors.

Paper IV

2G CAR T cells carrying costimulatory molecule 4-1BB has shown impressive results in clinical trials of leukemia patients. However, the same success has not been seen in lymphoma patients. 3G CARs has shown increased anti-tumor properties in preclinical studies and is now ready for clinical testing. We recently started a phase I/IIa clinical trial treating refractory lymphoma patients with 3G CAR T cells (αCD19-CD28-4-1BB-CD3zeta). The patients receive pre-conditioning chemotherapy prior to CAR T cell infusion. MTD will be determined through a dose-escalation with the first 6 patients receiving $2x10^7$, $1x10^8$ or $2x10^8$cells/m^2 (2 patients/dose), followed by 9 additional patients on the MTD. For this purpose, a GMP batch of a 3G αCD19-CD28-4-1BB-CD3zeta SFG retroviral vector was produced at Baylor College of Medicine. A protocol for GMP production of CAR T cells was developed and quality assured. The protocol has been implemented at Vecura, Huddinge and to this date 4 batches have been produced with a mean CAR expression of 54%. For 3 out of the 4 T cell batches, the expression of costimulatory markers, lineage (CD4, CD8) and memory phenotype has also been investigated. 2 batches have been used to treat patients in the ongoing clinical trial. Patient responses are currently under evaluation.

Conclusions

Paper I

ABT-737 sensitized tumor cells to apoptosis induction by CAR T cells, hence, combination therapy led to a significantly level of ongoing apoptosis compared to monotherapy with either treatment, also in cell lines less sensitive to ABT-737 monotherapy. Hence, our data indicates that ABT-737 or similar agents may be used in combination with CAR T cell therapy to enhance its efficacy.

Paper II

Repeated stimulation with B cells led to an antigen-driven accumulation of CAR+ T cells and an increased proliferation. 3G and 2G CAR T cells showed equal cytotoxic capacity, similar lineage, memory and exhaustion profile phenotype. However, 3G CAR T cells proliferated better and showed increased activation of intracellular signaling pathways compared to 2G CAR T cells. Collectively, the enhanced properties of 3G CAR T cells could potentially augment their anti-tumor effect *in vivo* compared to 2G CARs.

Paper III

3G CAR T cells could activate human dendritic cells through a cell-cell contact mechanism as shown by upregulation of maturation markers on co-cultured dendritic cells. Hence, CAR T cells may not only directly kill the tumor cells but may induce bystander immunity that indirectly aid tumor control.

Paper IV/Supplementary information

A protocol for CAR T cell GMP production was established. The clinical grade CAR T cell batches full-filled the release criteria and consisted of both effector and effector memory CAR T cells and we conclude that it is feasible to produce high numbers of clinical grade cells of high standard. Treatment of the first two patients enrolled at the lowest dose level was safe and at least one of them showed a clear response.

Future perspectives

In paper I, Bcl-2 family inhibitor ABT-737 was combined with CAR T cells resulting in increased treatment effect *in vitro*. However, navitoclax - the oral analogue of ABT-737 is associated with severe thrombocytopenia and therefore could investigations using the ABT-737 derivate ABT-199 be an interesting option. *In vivo* studies administrating ABT-199 as a pretreatment followed by CAR T cell infusion would be an interesting approach. However, first, its clinical safety as monotherapy needs to be established.

Paper II addresses an aspect that has not yet reached the limelight – signaling downstream CARs. Not much is known about how CARs actually works. Due to the signaling domain derived from the TCR/CD3 complex, signaling similar to the TCR is probable and supported by the few studies performed. Information about the signaling can provide important insights into how we should construct future CARs. CAR T targeting CD19 cells have shown promising/impressive results in clinical trials treating patients with B cell malignancies but with the current CARs pre-conditioning is still needed for efficacy. In lymphoma patients, aggressive pre-conditioning is required to reach similar effect as in leukemic patients with less pre-conditioning. This may be due to a strong local immunosuppression in the lymphoma lesions. Improvements in how we engineer the T cells could reduce the need for pre-conditioning. In our laboratory we have novel CARs that will be evaluated for resistance to immunosuppressive mechanisms.

CD19 as a target have many benefits. It is almost exclusively expressed on B cells, B cell aplasia is a relatively manageable "side-effect" and the majority of malignant B cells resides in the blood or tissues accessible to and commonly patrolled by T cells. Loss of CD19 expression due to tumor immunoediting, has only been reported in a few patients so far.[118] Targeting only one antigen will always lead to escape mutants lacking that particular antigen. In paper III we discuss the possibility of CAR T cells inducing bystander immunity. A broader immune response would reduce the risk of escape mutants and may also lead to a better immune profile in the tumor, hence, inducing a better milieu for the CAR T cell to function properly. The conclusions in this paper are considered in our novel CAR T cells that are yet to be evaluated.

An interesting approach would be to combine CAR T cells with immunomodulating drugs for instance lenalidomide that has been shown to restore the dysfunctional synapse formation present in CLL T cells, or combine CAR T cells with checkpoint blockade, for instance anti-PD-1 has been shown to potentiate T cell effect in preclinical studies.[256]

The clinical trial that is the basis of paper IV will hopefully lead us towards insights into the potential of 3G CAR T cells to induce bystander effects. So far, the best response rates in patients treated with CD19-targeting CAR T cells have been seen in patients with ALL.[116] It will be interesting to see if 3G CAR T cells can induce a stronger anti-tumor response in lymphoma patient than 2G CARs. The clinical trial will also enable correlation of immune markers in the patients to efficacy, to better understand how to measure tumor responses. Immune markers in patients prior to therapy may aid the selection of future patients to CAR T cell trials and finally, correlating the quality of GMP CAR T cell batches to responses may be crucial for us to understand what defines a good CAR T cell batch.

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