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Improvement of adoptive T-cell therapy for Cancer

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Abstract

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Cancer immunotherapy has recently made remarkable clinical progress. Adoptive transfer of T-cells engineered with a chimeric antigen receptor (CAR) against CD19 has been successful in treatment of B-cell leukemia. Patient's T-cells are isolated, activated, transduced with a vector encoding the CAR molecule and then expanded before being transferred back to the patient. However some obstacles restrict its success in solid tumors. This thesis explores different aspects to improve CAR T-cells therapy of cancer.

Ex vivo expanded T-cells are usually sensitive to the harsh tumor microenvironment after reinfusion. We developed a novel expansion method for T-cells, named AEP, by using irradiated and preactivated allo-sensitized allogeneic lymphocytes (ASALs) and allogeneic mature dendritic cells (DCs). AEP-expanded T-cells exhibited better survival and cytotoxic efficacy under oxidative and immunosuppressive stress, compared to T-cells expanded with established procedures.

Integrating retro/lentivirus (RV/LV) used for CAR expressions randomly integrate in the T-cell genome and has the potential risk of causing insertional mutagenesis. We developed a non-integrating lentiviral (NILV) vector containing a scaffold matrix attachment region (S/MAR) element (NILV-S/MAR) for T-cells transduction. NILV-S/MAR-engineered CAR T-cells display similar cytotoxicity to LV-engineered CAR T-cells with undetectable level of insertional event, which makes them safer than CAR T-cells used in the clinic today.

CD19-CAR T-cells have so far been successful for B-cell leukemia but less successful for B-cell lymphomas, which present semi-solid structure with an immunosuppressive microenvironment. We have developed CAR T-cells armed with *H. pylori*neutrophil-activating protein (HP-NAP). HP-NAP is a major virulence factor and plays important role in T-helper type 1 (Th1) polarizing. NAP-CAR T-cells showed the ability to mature DCs, attract innate immune cells and increase secretion of Th1 cytokines and chemokines, which presumably leads to better CAR T-cell therapy for B-cell lymphoma.

Allogeneic-DCs (alloDCs) were used to further alter tumor microenvironment. The premise relies on initiation of an allo-reactive immune response for cytokine and chemokines secretion, as well as stimulation of T-cell response by bringing in tumor-associated antigen. We demonstrated that alloDCs promote migration and activation of immune cells and prolong the survival of tumor-bearing mice by attracting T-cells to tumors and reverse the immune suppressive tumor microenvironment.

Keywords: CAR T-cell therapy; AEP expansion protocol; scaffold matrix attachment region; non-integrating lentivirus; H. pylori Neutrophil-activating protein; allogeneic DCs

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Doctors are men who prescribe medicines of which they know little, to cure diseases of which they know less, in human beings of whom they know nothing.

----Voltaire

医者，为其无所悉之人，疗其不甚解之疾，施其稍许识之药。

---伏尔泰

To my dear family
致亲爱的家人

List of Papers

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

I **Jin C**, Yu D, Hillerdal V, Wallgren AC, Karlsson-Parra A[#], Essand M[#]*. Allogeneic lymphocyte-licensed DCs expand T-cells with improved anti-tumor activity and resistance to oxidative stress and immunosuppressive factors. **Molecular Therapy - Methods and Clinical Development**, 2014, 1:14001.

II **Jin, C**, Fotaki G‡, Ramachandran M‡, Nilsson B, Essand M[#]*, Yu D[#]*. Safe engineering of CAR T cells for adoptive cell therapy of cancer using long-term episomal gene transfer. **EMBO Mol Med,** 2016, 8: 702-71.

III **Jin C**[¶], Jing M[¶], Ramachandran M, Yu D, Essand M*. CAR T-cells armed with secreting H. pylori Neutrophil-activating Protein with improved therapeutic effects on tumors. **Manuscript.**

IV Fotaki G[¶], **Jin C**[¶], Ramachandran M, Kerzeli I, Karlsson-Parra A, Yu D[#]*, Essand M[#]*. Tumor antigen-loaded allogeneic dendritic cells augment therapeutic effect of adoptively transferred T-cells by altering tumor immune-microenvironment. **Submitted Manuscript.**

¶ Shared first authorship
‡ Shared second authorship
# Shared senior authorship
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Other papers by the author

I **Jin C**[¶], Yu D[¶], Cancer M, Nilsson B; Leja J, Essand M. Tat-PTD-modified oncolytic adenovirus driven by the SCG3 promoter and ASH1 enhancer for neuroblastoma therapy. **Hum Gene Ther**, 2013, 24:766-775.

II Yu D, **Jin C**, Ramachandran M, Xu J, Nilsson B, Korsgren O, Le Blanc K, Uhrbom L, Forsberg-Nilsson K, Westermark B, Adamson R, Maitland N, Fan X, Essand M. Adenovirus serotype 5 vectors with Tat-PTD modified hexon and serotype 35 fiber show greatly enhanced transduction capacity of primary cell cultures. **PLoS ONE** 2013, Jan 25, 8(1):e54952.

III Yu D, **Jin C**, Leja J, Majdalani N, Nilsson B, Eriksson F, Essand M. Adenovirus modified with a cell penetrating peptide exhibits therapeutic effect in experimental neuroblastoma and neuroendocrine tumors. **J Virol** 2011, 85: 13114-13123.

IV Ramachandran M, **Jin C**, Yu D. Eriksson F, Essand, M. Vector-encoded Helicobacter pylori neutrophil-activating protein promotes maturation of dendritic cells with Th1 polarization and improved migration. **J Immunol**, 2014, 193(5): p. 2287-96.

¶, Shared first authorship

Contents

Abbreviations

aAPC	Artifical antigen presenting cells
ASAL	Allo-sensitized allogeneic lymphocytes
alloDC	Allogeneic DCs
APC	Antigen presenting cells
BCR	B cell receptor
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CLIP	Class II associated invariant peptide
CMV	Cytomegalovirus
CRT	Surface-exposed calreticulin
CTLA	cytotoxic T-lymphocyte-associated protein
CTLs	Cytotoxic T-lymphocytes
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
EGFR	Epidermal growth factor receptor
EGFRvIII	EGFR variant III
ER	Endoplasmic reticulum
FAP	Fibroblast activation protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
HEV	High endothelia venules
HIV-1	Human immunodeficiency virus 1
HLA	Human leukocyte antigen
HMGB1	High mobility group protein B1
HP-NPA	Helicobacter pylori neutrophil activating protein
HSV-TK	Herpes simplex virus-thymidine kinase
ICAM	Intercellular adhesion molecule
iCARs	Inhibitory CARs
iCasp9	Inducible caspase-9
ICD	Immunogenic cell death
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN	Interferon
Ii	Invariant chain
IL	Interleukin
imDCs	Immature dendritic cells
ITAM	Immunoreceptor tyrosine-based activation motif
IVIg	Intravenous infusion of immunoglobulin

LFA	lymphocyte function-associated antigen
LTR	Long terminal repeats
LV	Lentivirus
mDCs	Mature DCs
MDSC	myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MLR	Mixed lymphocytes reaction
MM	Multiple myeloma
MoMLV	Moloney murine leukemia virus
MPO	Myeloperoxidase
NAP	Neutrophil activating protein
NIH	National Institutes of Health
NILV	Non-integrating lentiviral vectors
NK cell	Natural killer cells
PBMCs	Peripheral blood mononuclear cells
PD	Programmed cell death
REP	Rapid expansion protocol
ROS	Reactive oxygen species
RSV	Rous sarcoma virus
RV	Retrovirus
S/MAR	Scaffold matrix attachment region
SAF-A	Scaffold attachment factor protein
scFv	Single chain variable fragment
TAA	Tumor-associated antigen
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TfH	Follicular T helper cells
TGF	Transforming growth factor
Th cells	Helper T-lymphocytes
TIL	Tumor infiltrating lymphocytes
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T-cells
VSV-G	Vesicular stomatitis virus G glycoprotein

The human immune system

1. Overview of the human immune system

The immune system is a defense system that protects the host against infection and disease by identifying and eliminating foreign invaders [1]. It recognizes a wide range of pathogens from viruses to parasitic worms, and has the capability of distinguishing infected from uninfected cells.

The immune system contains two groups of organs or tissues: the primary lymphoid organs and the secondary lymphoid organs [2]. The primary lymphoid organs are the thymus and the bone marrow, which provide a microenvironment for generation and early selection of lymphocytes from immature progenitor cells. The secondary lymphoid organs include spleen, lymph nodes and lymphoid follicles in tonsils, Peyer's patches, adenoids and skin. The secondary lymphoid organs are the sites which provide conditions for survival and function of mature naive lymphocytes to interact with antigens effectively [2].

The human immune system is a complex system, which can be divided into innate and adaptive immunity. The innate immune system comprises physical and chemical barriers that contain epithelial surfaces, phagocytic cells and blood proteins (complement system and cytokines). It also includes innate leukocytes such as natural killer (NK) cells, mast cells, and phagocytic cells (e.g. monocytes, dendritic cells (DCs), macrophages and neutrophils). The innate immune system is regarded as the first line of protection against intruders by rapid activation, recognition and eradication of pathogens.

In contrast to the fast and "unspecific" innate immune system, the adaptive immune response has specificity for distinct molecules and provides the immune system with the ability to recognize and remember specific pathogens. The memory function of adaptive immunity enables more vigorous responses to repeated exposures to the same microbe. The adaptive immune system includes humoral immunity and cell-mediated immunity. In humoral immunity, B-cells interact with a specific antigen via their B-cell receptor (BCR), which leads to proliferation and differentiation to antibody-secreting plasma cells. Antibodies play a crucial role in humoral response by binding and neutralizing antigens. An antigen coated with antibodies can be eliminated in different ways, such as cross-linking of several antigens which forms clusters that are subsequently ingested by phagocytic cells or cleared

by activating the complement systems [2]. Cell-mediated immunity involves activation of cluster of differentiation (CD)4^+ helper T-lymphocytes (Th cells) and $CD8^+$ cytotoxic T-lymphocytes (CTLs) in an antigen-specific manner [2]. T-cells can not recognize antigen directly. Antigens need to be processed into small peptides and presented on major histocompatibility complex (MHC) molecules by antigen presenting cells (APCs). This process is called antigen processing and presentation [2]. T-cells are activated through specific T-cell receptor (TCR) binding to the MHC/peptide complex. The MHC can be divided into two major classes: class I and class II MHC molecules. $CD8^+$ T-cells recognize antigens presented by class I molecules and $CD4^+$ T-cells recognize antigens presented by class II molecules [2]. MHC I is expressed by all nucleated cells, while MHC II is only expressed on APCs. After antigen recognition, T-cells process to proliferation and differentiate into effector cells and kill target or secrete cytokines depending on phenotype. T-cells and B-cells can also differentiate into memory phenotype and be activated rapidly upon re-exposed to the same antigens, which lead a fast immune response. The innate and adaptive immune system work tightly with each other in eradication of invaders, and among this, DC has proved to have a unique ability to bridge the innate and adaptive immune systems.

2. Dendritic Cells

DCs have powerful key functions in the immune system which was first identified by Steinman and Cohn [3]. They usually reside in the peripheral tissues in an immature state and capture antigens upon infection. They are then activated through Toll-like receptors (TLRs) and cytokine receptors, and migrate to the secondary lymphoid organs via chemokine receptor signals such as CCR7. Upon activation, the DC matures and up-regulates MHC I and MHC II expression and also upregulates co-stimulatory molecules, such as CD86, CD80, CD40 and CD70 [4] on its surface to assist T-cell activation. In lymphoid organs, DCs may provide T and B lymphocytes with pathogen-related information from the affected tissue and thereby activate suitable antigen-specific immune responses [5].

DCs not only provide an important link between innate and adaptive immunity. They also have a key role in the polarization of adaptive immune responses and in that way contribute to the selection of the most efficient mechanisms against a particular pathogen. Cytokines secreted from DCs are the major regulators to drive DC maturation and polarization, such as interleukin (IL)-12.

Monocyte-derived DCs can be derived from peripheral blood mononuclear cells (PBMCs) *in vitro*. Monocytes cultured in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4 differentiate

to immature dendritic cells (imDCs). Subsequent treatment with ligands of TLRs and with cytokines can further initiate maturation of DCs. Cell-surface receptors such as CD80 (B7.1), CD86 (B7.2) that act as co-stimulatory molecules during T-cell activation process are upregulated during the maturation process.

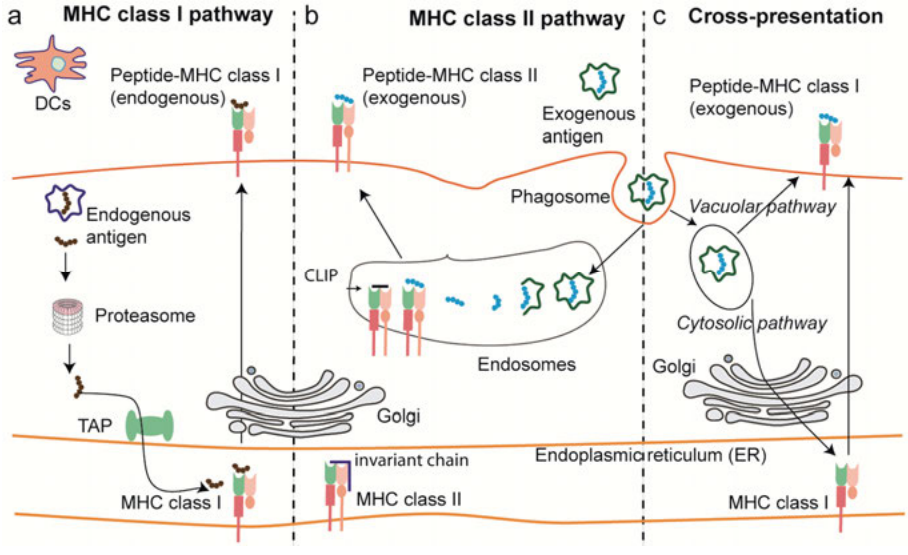


Figure 1. **Antigen-presentation pathways in dendritic cells. a)** MHC class I molecules present peptides that are derived from endogenous proteins and that are degraded in the cytosol by the proteasome. Peptides bind to MHC class I molecule at the endoplasmic reticulum (ER) and the MHCI/peptide complex is transferred to the cell membrane. **b**) MHC class II molecules bind to invariable chain in the ER and then transfer to endosomes. The invariable chain is cleaved into CLIP and replaced by exogenous peptide that are internalized by phagocytosis and digested by endosomes. The MHCII/peptide complex is transferred to the cell membrane. **c)** Cross-presentation of exogenous peptides on MHCI. Peptides from phagocytosed exogenous antigens are presented by MHC class I molecule by either the cytosolic or vacuolar pathways.

2.1 MHC class I pathway

MHC class I, a member of immunoglobulin superfamily, is expressed on all nucleated cells, can present peptides from endogenous antigens, including intracellular, cell membrane-associated and secreted antigens. The MHC class I molecule consists of an alpha chain with three domains and a single domain beta chain (β2 microglobulin). In humans, MHC class I is encoded by three major genes: HLA (Human leukocyte antigen)-A, HLA-B and HLA-C, with a polymorphism enabling them to bind to a variety of antigen peptides. All endogenous proteins are digested in the proteasomes into short

peptides of around 8-9 amino acids in length [6]. The digested peptides are transferred to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) protein. There, they are combined with MHC class I molecule to form a complex which is passing through Golgi vesicles and expressed on the cell surface for presentation to T-cells [7] **(Figure 1a)**.

During T-cell development in the thymus, self-reactive T-cells undergo selection and is deleted. However, when cells are infected with intracellular pathogens, such as virus or intracellular bacteria, the cellular machinery of the MHC class I pathway can present their antigenic peptides to $CD8^+$ CTLs, which becomes activated and lyse infected cells. In addition, mutated peptides from cancer cells are loaded on MHC I and can become targets for CTLs.

2.2 MHC class II pathway

MHC class II is only expressed on APCs, such as DCs, B-cells, macrophages and monocytes. It is a heterodimer, which consists of two homogenous chains, the α and β chain. Both chains are encoded by polymorphic MHC class II genes. The antigen-binding groove of MHC class II is open at both ends, so the antigen peptides presented by MHC class II are longer then peptides presented by MHC class I, typically 15-24 amino acids long.

The function of MHC class II is presenting peptides derived from exogenous proteins. MHC class II molecules are resident to the ER where they are associated with an invariant chain (Ii), in order to prevent binding of endogenous peptides. Peptides derived from exogenous antigen are processed by phagocytosis internalization and degraded in endosomes, where they can meet MHC class II-Ii complexes. When MHC class II-Ii complex reaches endosomes, Ii is cleaved into class II associated invariant peptide (CLIP), which is released from the MHC II molecule. CLIP is located in the groove of MHC class II until it is replaced by the exogenous antigenic peptide, facilitated by the HLA-DM (one of chaperone protein) **(Figure 1b)**.

$CD4^+$ T-cells is activated by TCR binding to MHC class II-peptide complex on the APCs. Subsequently, activated $CD4^+$ T-cells start to secrete cytokines for aiding $CD8^+$ T-cells in eradication of infectious cells or tumor cells. $CD4^+$ T-cells can also help B-cells.

2.3 Cross-presentation pathway

It is well understood how MHC class I presents endogenous antigen, but it has been found that MHC class I molecules can also present exogenous antigens, at least by DC [8]. There are several proposed mechanisms for cross-presentation [9, 10]. The cytosolic and vacuolar pathways are the two major ones [10]. The cytosolic pathway uses phagocytosed antigens transported into the cytosol that are processed by proteasomes and imported to the MHC

class I antigen loading pathway [11]. The vacuolar pathway is not via proteasome but by lysosomal degradation **(Figure 1c)**. Cross-presentation enables $CD8^+$ T-cells to also recognize exogenous peptides presented on MHC I molecules and is essential for priming T-cells against both viral and tumor-associated antigens (TAAs).

3. T-cells

3.1 T-cell maturation, activation and differentiation

Progenitor T-cells originate from hematopoietic stem cells from the bone marrow. They undergo a maturation process in the thymus before they are released into the peripheral tissues where they can get activated. T-cell maturation involves rearrangements of germ-line TCR genes where random genetic recombination events occur in the early stage of TCR production. This random recombination events generates a diverse repertoire of TCRs to match different antigens [2]. T-cells undergo two selection processes to generate functionally distinct subpopulations of mature T-cells. Positive selection is responsible for the creation of a self-MHC-restricted repertoire of T-cells and the negative selection is extremely important in generating a primary T-cell repertoire that are not self-responding [2].

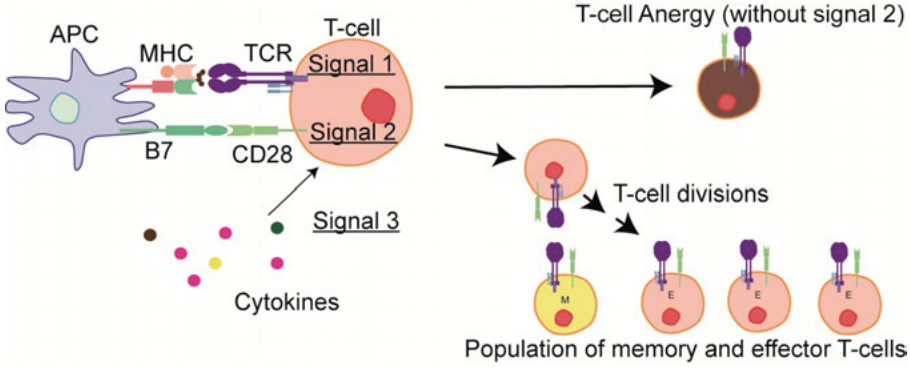


Figure 2. **T-cell activation**. Activation of T-cells is dependent on signal 1, 2 and 3. Signal 1 is the specific interaction between the TCR and the MHC/peptide complex. Signal 2 is co-stimulation, most commonly through CD28, and without signal 2 T-cells go into an anergic state. Signal 3 is cytokines binding to cytokine receptors. The cytokine profile directs the type of T cell response. Properly activated T-cells undergo proliferation and differentiation to generate both memory and effector T-cells.

T-cells circulate in the blood as resting cells in the G0 stage of the cell cycle and migrate through the high endothelia venules (HEV) mediated by chemokines, mainly CCL19 and CCL21 that are expressed in the HEV lumen [12]. Naïve T-cells, which have not yet encountered an antigen and express CCR7, stay in the secondary lymphoid tissues. When the TCR of a naïve T-cell encounters an antigenic peptide/MHC complex on an APC, they get activated. The interaction between a T-cell and an APC is initiated with integrin, such as lymphocyte function-associated antigen-1 (LFA-1) and CD2 on the T-cells and ICAM-1 and CD58 on APCs [13]. This interaction is weak and short-lived, followed by the TCR-MHC/peptide interaction, which is stronger and closer. CD8/CD4 binding to MHC class I/II, respectively also strengthen the TCR-MHC/peptide complex. In addition, co-stimulatory molecule interaction is also involved in the complex. Those interactions form the immunological synapse, which is a dynamics interaction and important for T-cell activation.

Upon interaction with APCs, the T-cell gets activated. The activation depends on three signals form the APC **(Figure 2)**. The **first signal** is generated by the interaction of the antigenic peptide-loaded MHC complex with the TCR-CD3 complex, and the **second signal** is provided mainly by interaction between CD28 on the T-cell and the members of the B7 family on the APC [2]. The role of co-stimulatory molecules (**Signal 2**) is important for optimal regulation of immune response conferred by T-cells, by controlling the initiation, expansion, survival and generation of memory T-cells. In fact, without signal 2, T-cells will go to a state of non-responsiveness referred to as anergy. Beside the CD28/B7 interaction, there are several other molecules involved in signal 2, such as CD40L/CD40, 4-1BB/4-1BB ligand, OX-40/OX40L and CD27/CD70. Mature DCs also deliver cytokines, which act as the **third signal,** for T-cells activation. After activation, T-cells are triggered to enter the G1 phase of the cell cycle, which results in the generation of a large number of effector T-cells and some memory T-cells. Effector T-cells are capable of cytokine secretion, help ability and cytotoxic killing activity. Memory T-cells are long-lived, quiescent cells that can quickly react to a subsequent challenge with the same antigen to generate a secondary response. This process is called priming response [14].

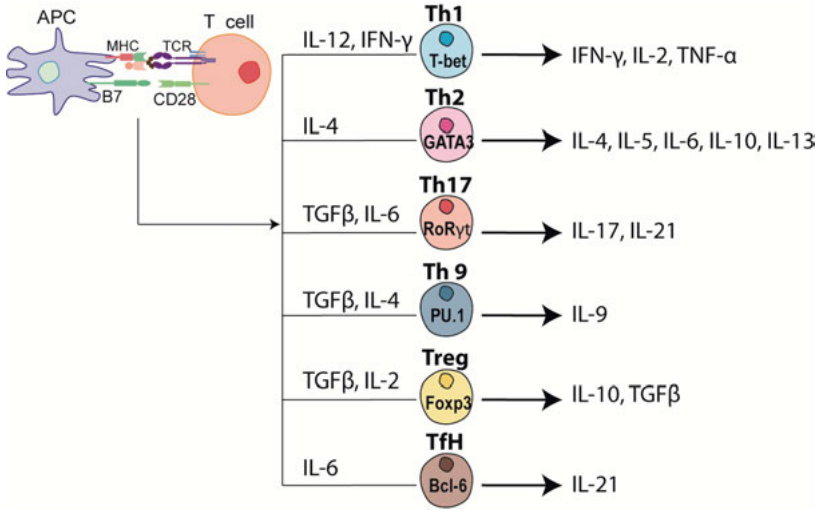


***Figure 3*. Helper T-cell differentiation.** Different cytokines and transcription factors control Th cell differentiation into different subsets. Th1 cells are controlled by IL-12, IFN-γ and transcription factor T-bet. Th1 cells are major in producing IFN-γ, IL-2 and TNF-α. Th2 cells are mainly mediated by IL-4 and transcription factor GATA3 and release IL-4, IL-5, IL-6, IL-10 and IL-13. Th17 cells are regulated by TGFβ, IL-6 and transcript factor RoRγt, producing IL-17 and IL-21. Th9 cells are controlled by TGFβ, IL-4 and PU.1, secreting IL-9. Tregs are regulated by TGFβ, IL-2 and Foxp3, and release IL-10 and TGFβ. TfH cells are mainly mediated by IL-6 and Bcl-6 and produce IL-21

DCs are the major type of APCs that aid in T-cell activation. Mature DCs (mDCs) also deliver a **third signal**, which is a polarization signal toward Th1, Th2, Th17, Th9, regulatory T-cells (Treg) or follicular T helper cells (TfH) immunity **(Figure 3)**. The process of T-cell differentiation is tightly regulated by cytokines and transcription factors [15]. Th1 response is mediated by T-bet, which is induced upon IL-12 and interferon (IFN)-γ stimulation. Th2 response is mainly regulated by GATA3 under stimulation of IL-4 and it is important in humoral immunity. Th17 response is under control of RoRγt, transforming growth factor beta (TGFβ) and IL-6. Th17 cells are involved in extracellular pathogens immunity and in inflammatory autoimmune disease. Recent evidence suggests that Th17 cells are also involved in tumor immunity [16]. The antitumor ability of Th17 cells may be due to recruitment of effector T-cells, NK cells and mDCs into the tumor microenvironment and tumor draining lymph nodes [16]. In addition, they can secret serval effector cytokines, such as IL-2, IL-17, GM-CSF, IFN-γ and tumor necrosis factor alpha (TNF)-α in the tumor microenvironment [16]. Th9 response is mediated by PU.1, TGFβ and IL-4. Th9, as a new player in adaptive immunity, contributes to immunopathology in allergy and autoimmunity [17]. Differentiation of Tregs is stimulated by TGFβ and IL-2 and also by

the transcription factor FoxP3. They mediate a suppressive effect in the tumor microenvironment. TfH cells are facilitated by IL-6 and Bcl-6, which is mainly helping B-cell maturation in the lymph node. T-cell differentiation is plastic in response to different cytokines and transcription factors.

In terms of tumor cell killing, a strong ability to induce CTL-mediated tumor killing is desired for Th1 polarization [18]. DCs have to be helped by $CD4^+$ T-cells, a process known as licensing or conditioning, in order to induce long-lived memory $CD8^+$ T-cells [19, 20]. The interaction between CD40L (CD154) on $CD4^+$ T-cells and CD40 on DCs is instrumental for this licensing process [21]. $CD4^+$ T-cell help enhances the expression of co-stimulatory molecules on DCs and induces secretion of bioactive IL-12 [22], which is central for induction of Th1 immunity.

3.2 T-cell function and T-cell inhibition

After activation, T-cells differentiate into different subsets and effector cells migrate to the sites of inflammation. Th cells ($CD4^+$) produce cytokines, such as IL-2, to provide survival signal for T cell functions and help CTL in their killing function. Once $CD8^+$ T-cells are activated, they are initiated to produce cytokines, such as IFN-γ and TNF-α. IFN-γ has multiple functions in immune response. It can up-regulate MHC expression on DCs, aid CTLs and is involved in Th1 immune response. However, more evidence suggests that programmed cell death (PD)-L1 expressed on hematopoietic cells and tumor cells is upregulated by IFN-γ secreted from NK cells and activated T-cells, which in turn interact with PD-1 to inhibit TCR-mediated effector function [23]. This function of IFN-γ helps tumor to escape from immune surveillance. Once CTLs form an immunological synapse, they begin to secrete granules towards the presynaptic membrane and release perforin and granzymes into synaptic cleft [24]. Perforin forms transmembrane pores that enable the diffusion of granzymes into the target cell cytosol, followed by initiating apoptosis in the target cells [24]. During granzymes release, T-cells upregulate CD107a on the surface to protect themselves from perforins. Therefore, CD107a is a marker for degranulation of T-cells and also for NK cells [25].

Immune responses are carefully regulated by inhibition function of immune cells, which can protect the immune system from over-activation when the pathogen is eliminated. T-cells activation can be inhibited by regulatory T-cells and inhibitory molecule interactions.

Regulatory T-cells, as suppressor T-cells, modulate the immune system by inhibitation of effector T-cell response, maintenance of tolerance to self-antigens and elimination of autoimmune responses. Tregs are a subpopulation of T-cells where most of them express CD4 together with high expression of CD25 (IL-2Rα), low expression of CD127 (IL-7Rα) and expression of the transcription factor FoxP3. Tregs suppress effector function of T-cells

in different ways. They can directly bind to effector T-cells and release perforin and granzymes to lyse effector T-cells [26]. MHC class II on the Tregs can bind to LAG3 on the effector T-cells that inhibit function of effector T-cells [27]. Cytokines, such as IL-10 and TGF-β released from Tregs are another import regulator in suppression of immune responses. IL-10 suppresses effector T-cell proliferation, migration and cytokine production and also inhibits antigen presentation by DCs through downregulation of MHC molecules [28]. TGF-β inhibits T-cell proliferation by blockage of cell cycle and IL-2 production from T-cells [29]. Tregs also inhibit DCs to block T-cell function by impairment of the maturation and antigen presentation.

Apart from expression of co-stimulatory molecules on T-cells after activation, they also express inhibitory molecules. Effector T-cells express higher levels of inhibitory molecules than naïve T-cells. Two important inhibitory molecules are cytotoxic T-lymphocyte-associated protein (CTLA)-4 and PD-1 that binding to CD86/CD80 and PDL1 respectively. CTLA-4 has higher affinity to interact with CD86/CD80, competing out CD28 binding to CD86/CD80, and delivers strong inhibitory signal to T-cells, which leads to dephosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) domains in CD3ζ chain resulting in blocking of activation-related gene expression of T-cells [30]. PD-1 binding to PD-L1 inhibits T-cell function through different pathways: inhibition of CD3ζ chain phosphorylation to suppress T-cell activation; downregulation of Ras and Bcl-xL to affect proliferation and cell survival respectively; dephosphorylation of P13K/Akt kinase for down-regulation of cytokine and cell survival related gene expression [31].

3.3 Allogenic T-cells response

Self-tolerant T-cells are generated after maturation process and released from thymus. Therefore, T-cells play a central role in rejection response to allogeneic tissue and a high frequency, around 1-10%, of T-cells can response to another individual's MHC [32]. The term "allorecoginition" describes the recognition of foreign tissue by the host. It can be divided into direct allorecoginition and indirect allorecognition [33].

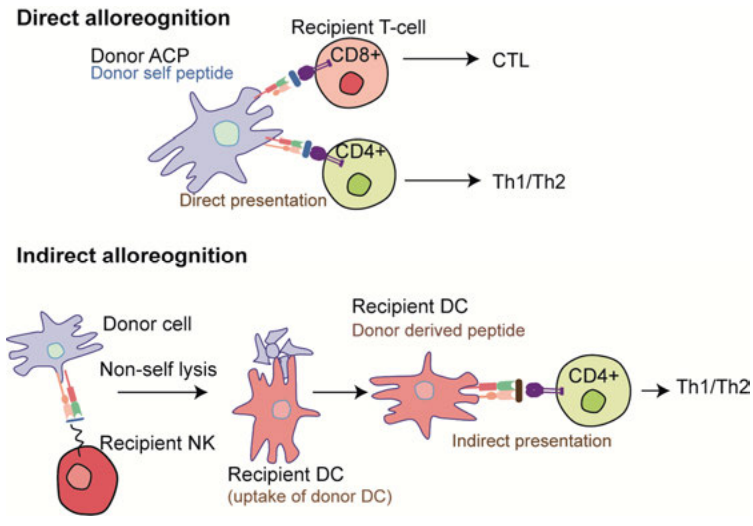


Figure 4. **Direct and indirect allorecoginition.** Allogenic T-cells recognize non-self cells in two different ways referred to as direct and indirect allorecognition. Both $CD4^+$ and $CD8^+$ allogenic T-cells can recognize non-self MHC and peptides directly, and differentiate into Th1/Th2 or CTL, in term of direct allorecoginition. Recipient DCs process and present non-self peptides from killed donor cells and stimulate mostly $CD4^+$ T-cell response, in term of indirect allorecoginition.

During direct allorecognition, the alloresponsive T-cells can directly recognize polymorphic allogeneic MHC. Recognition of foreign MHC on APC can prime both $CD4^+$ and $CD8^+$ host T-cells **(Figure 4)**. It has been reported that direct allorecognition is preferred for Th1 priming [34]. Regarding molecular mechanisms of allorecoginition, there are two theories proposed, "high determinant density" and "multiple binary complex". The "high determinant density" theory states that alloresponse may be firstly directed against residues on the non-self MHC and the bound peptide is of secondary importance, whereas if self and non-self MHC residues are quite closer, the alloresponse may turn to recognize endogenous peptides that are presented on non-self MHC, which is the case for the "multiple binary complex" theory [33].

During indirect allorecognition alloantigens can be processed as exogenous antigens and presented by host self-MHC on APC and be recognized by host T-cells **(Figure 4)**. $CD4^+$ T-cells dominate in indirect allorecognition. This response is slower than direct recognition but it is a long-term response to engraft tissue [33]. $CD4^+$ alloresponsive T-cells also help B-cells in antibody response to alloresponse through indirect recognition.

There is standard *in vitro* based assay based on allogenic T-cells response, which is the mixed lymphocytes reaction (MLR). It occurs between two or more allogenic lymphocytes population. After a few days of *ex vivo* culture, lymphocytes proliferate in the response to the un-matched MHC and generate an enriched cytokine environment [35].

Immune cells versus cancer cells

Cancers consists of a heterogenic malignant cells which have accumulated a variety of genetic mutations and loss of normal cellular regulatory processes [36]. These mutations result in the expression as tumor-associated neoantigens, which can be distinguished from proteins in normal cells and thereby be targeted by T-cells. The immune system has the capability to target tumor cells and kill them **(Figure 5)**.

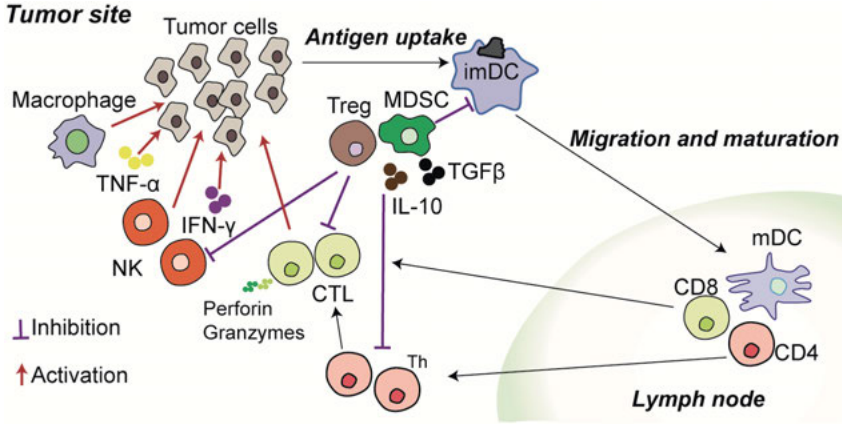


Figure 5. **Overview of immune cell killing of tumor cells in the harsh tumor microenvironment.** Tumor-associated antigens released from dying tumor cells can be taken up by resident imDCs, leading to maturation and migration of the DCs into draining lymph nodes. In the lymph nodes, the mature DCs prime naïve T-cells and tumor antigen-specific T-cells can get activated and migrate back to the tumor site, where $CD8^+$ T-cells turn into CTL and kill tumors cells by releasing perforin and granzymes. $CD4^+$ T-cells turn into T helper cells that aid CTL by releasing cytokines such as IFN-γ and TNF-α. Except from T-cells, innate cells such as NK cells and M1 macrophages are also involved in tumor cell killings. However, the tumor microenvironment often develops immune resistance to hamper effector immune cell function. This comes from accumulation of myeloid-derived suppressor cells (MDSCs) and Tregs together with high levels of IL-10 and TGFβ. Immune suppression can also be mediated by M2 macrophages, fibroblasts and tumor cells themselves.

Tumor-derived chemokine, cytokines and cell-death signals attract NK cells and they are activated by ligands expressed by tumor cells and by pro-inflammatory cytokines secreted from macrophages and DCs. Since NK

cells recognition of tumor cells is not MHC restricted and act on cells without MHC expression, they are crucial in tumor cell recognition and killing [37]. Activated NK cells unspecifically kill tumor cells and the lysed tumor cells serve as antigenic material and can be taken up by resident imDCs. NK cells also secrete IFN-γ and TNF-α during this process, which are important for DC activation and maturation. Mature DCs leave the tumor site after activation and migrate to draining lymph nodes or tertiary lymphoid organs sometimes formed in tumors where they present the antigenic messages to naïve T-cells, which include both $CD4^+$ and $CD8^+$ T-cells. On the one hand, $CD4^+$ Th cells can be activated and then produce cytokines that help proliferation and activation to tumor antigen-specific CTLs. On the other hand, the effector $CD8^+$ T-cells are also activated by DCs and can leave the lymph node and migrate to the tumor site and lyse tumor cells by releasing perforin and granzymes.

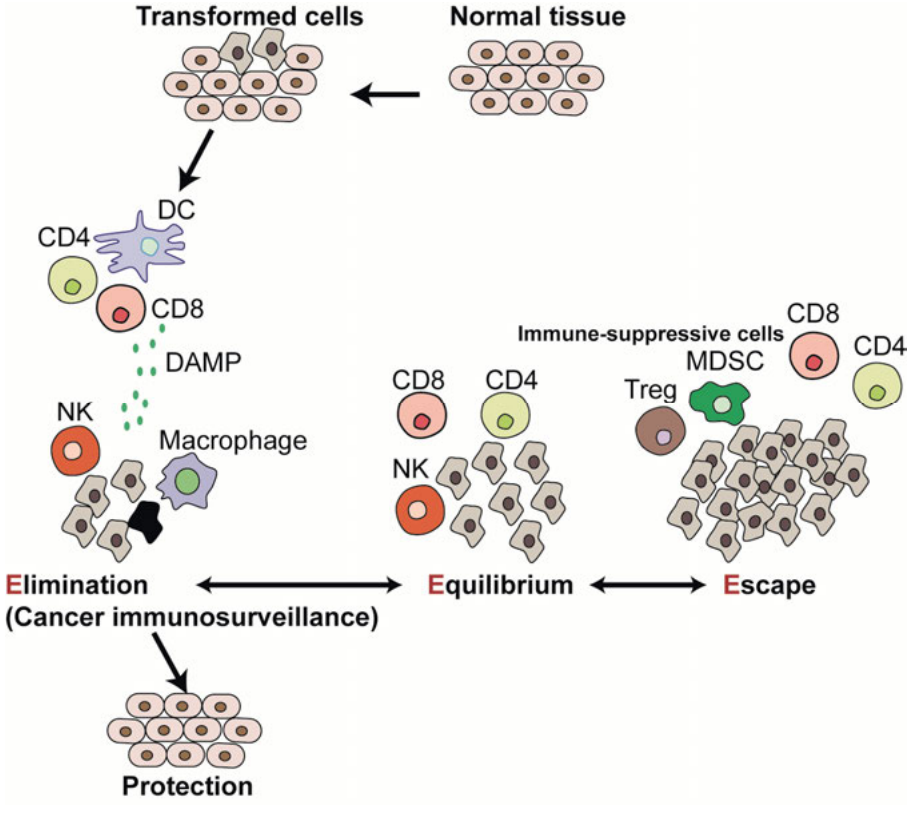


Figure 6. **The cancer immunoediting "3E model".** Elimination, equilibrium and escape. In the elimination phase immune cells work to remove newly transformed cells. The host is protected from cancer formation by the immune system. In cases where the elimination phase is inefficient the equilibrium phase occurs where immune cells keep tumor cells in check but tumor cells try to avoid the attack from immune cells. If avoidance is efficient the escape phase follows where tumor cells with major genetic and epigenetic changes start to grow and escape from immune cell recognition.

It has been understood for a long time that the immune system can clear existing tumor cells, but it is not clearly understood why tumors can reappear after clearance. After years of investigation several hypotheses were coined, one of them is called "cancer immunoediting". The cancer immunoediting hypothesis states three phases: **elimination**, **equilibrium** and **escape**, also known as the "3E" model [38] **(Figure 6)**. In the elimination phase, immune cells perform their normal functions as described above to kill newly transformed malignant cells. If all malignant cells were destroyed, the host would be protected. However, this is usually not the case and tumor cells go to a dynamic equilibrium phase with immune cells. In this phase, immune cells try to keep the tumor cells in check but the tumor cells go through several gene mutations to reduce the immunogenicity and escape recognition by immune cells. When the cancer cells successfully find the new way to escape

the immune system, they start to grow out of control. At this stage, the immune system do not have the capacity control the tumor cells anymore and this phase is called the escape phase**.**

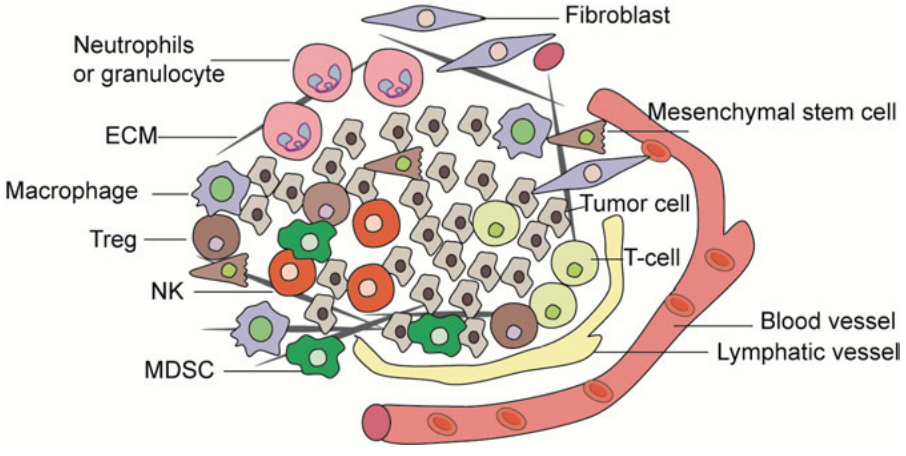


Figure 7. **Schematic representation of the tumor microenvironment.** Solid tumors consist of malignant cells and several populations of non-malignant cells including fibroblasts, mesenchymal cells and various immune cells, together with extracellular matrix and inflammatory mediators.

In the escape stage, tumor cells appear to be able to evade the immune system via several mechanisms. Firstly, tumor cells frequently down-regulate MHC molecules, which are necessary for efficient antigen presentation by malignant cells to T-cells and they provide poor co-stimulatory signals pushing T-cells to a state called clonal anergy [39]. Furthermore, structure of tumors also provides immunosuppressive microenvironment. Besides malignant cells, most solid tumors also consist of several population of non-malignant cells: fibroblasts, mesenchymal cells and various immune cells, together with extracellular matrix and inflammatory mediators **(Figure 7)** [40]. In many cases the malignant cells are in minority within a tumor [41]. The endothelial cells in the tumor bed can also act as a barrier by blocking effector T-cells from entering while letting regulatory T-cells through [42]. Importantly, suppressive cells also help tumor cells to evade the immune system, particularly $CD4^{+}CD25^{+}FoxP3^{+}$ T regulatory cells, tumor-associated macrophages and MDSCs. They impede T-cell function through the production of immunosuppressive molecules, such as arginase, nitric oxide synthase, TGF-β and IL-10. Tumor-derived factors, such as indoleamine-pyrrole 2,3-dioxygenase (IDO), create immunosuppressive tumor microenvironment by recruiting and activating MDSCs [43].

Cancer immunotherapy

1. Adoptive T-cells transfer

Adoptive immunotherapy is a treatment strategy where T-cells, isolated from a cancer patient, are activated and expanded *ex vivo* and re-infused into the host with the purpose of killing tumor cells [44, 45] **(Figure 8)**. The first report of adoptive T-cells transfer was from the Surgery Branch of the National Institutes of Health (NIH) in 1988, which used tumor infiltrating lymphocyte (TIL) from metastatic melanoma patient [46].

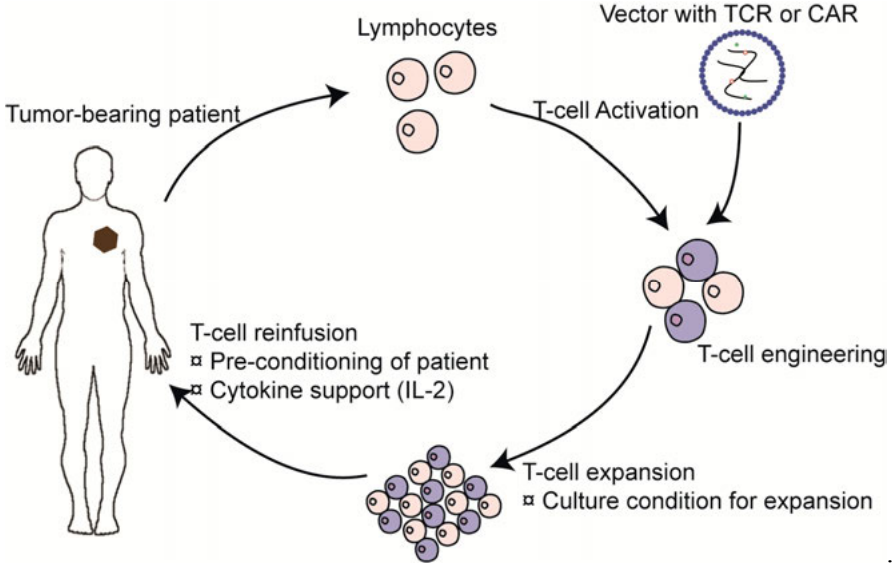


***Figure 8*. An illustration of adoptive T-cell transfer immunotherapy for cancer**. T-cells are either derived from the patient's tumor (TILs) or from patient's blood in which case they are genetically modified *ex vivo*, as illustrated by a vector delivering a TCR or CAR. Isolated T-cells are cultured under sterile conditions in media supplied with serum and IL-2 or other cytokines. After preconditioning of the patient with lymphodepletive therapy the expanded T-cells are transferred back into the patient.

Results from early TIL trails for malignant melanoma were encouraging but if was not until autologous TIL were combined with high-dose IL-2 and a pre-conditioning by cyclophosphamide and fludarabine that major responses were observed [44]. The results were further improved by combining the cyclophosphamide/fludarabine lymphodepletion with total body irradiation as precondition before TIL therapy [47]. The purpose of the precondition is to remove cytokines and leave room for infused T-cells proliferation [48]. Tregs can also be removed by lymphodepletion which is important to enhance the capacity of the infused T-cells [49]. TILs need to be isolated from tumors, followed by selection of highly reactive T-cell clones by IFN-γ assay and expansion *ex vivo*. It is not always possible to get good quality TILs. Therefore, if the target antigen is known blood-derived patient T-cells can be isolated and genetically modified *ex vivo* to express a new TCR or a chimeric antigen receptor (CAR) to redirect T-cell specificity towards tumor cells.

2. T cell engineering

2.1 TCR engineering of T-cells

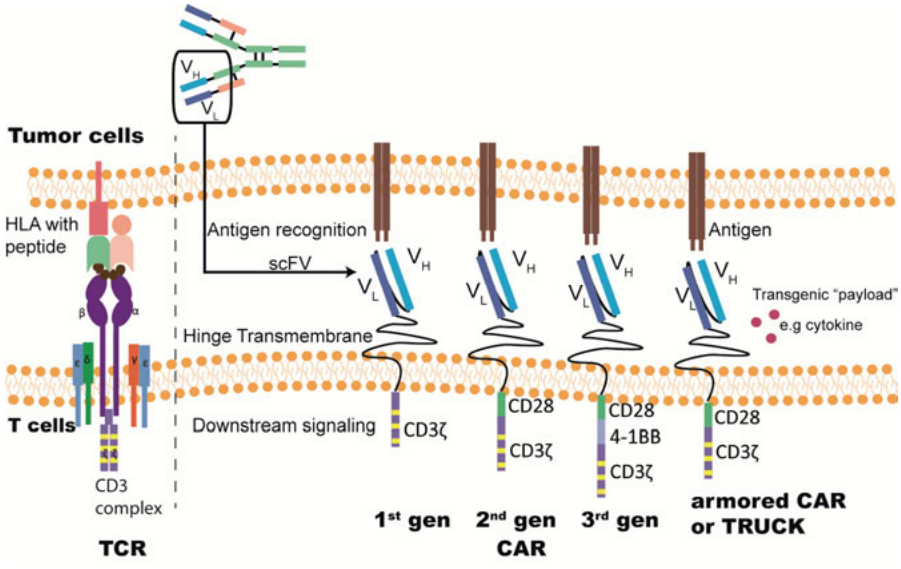


Figure 9. **Schematic representation of a TCR and CAR molecule**. The T-cell receptor (TCR) consists of an α- and β-chain complex for recognition of MHC/peptide complex on target cells and a CD3 complex including the ζ-chain for intracellular signaling upon target recognition. A chimeric antigen receptor (CAR) has an extracellular single-chain fragment from an antibody for recognition of an antigen on the surface of target cells and various intracellular signaling moieties. The first generation CARs have the CD3ζ intracellular signaling domain. The second generation CARs has in addition to CD3ζ an intracellular signaling domain derived from co-stimulatory molecule, e.g. the CD28 or 4-1BB. So called third generation CARs has CD3ζ and two costimulatory domains in tandem. Even more advanced CARs express a transgenic "payload" upon target cell recognition, such as secretion of IL12 and are sometimes being reffered to as armored CAR T-cells or TRUCK T-cells.

Approximately 95% of all T-cells contain an alpha and beta TCR chain and are referred to as αβ T-cells **(Figure 9)**, whereas about 5% of T-cells contain a gamma and delta TCR chain and are referred to as γδ T-cells. TCR genes for transfer are usually isolated from a high affinity αβ T-cell clone defined for a specific target antigen. Highly reactive T-cell clones are chosen because they can usually recognize and lyse target tumor cells efficiently. Methods to isolate high affinity TCR genes include the use of transgenic mice containing human HLA to generate TCRs against human antigen [50] or phage display technologies [51]. The main advantage of using TCRs to engineer T-cells is that their functions and signaling pathways are well understood. It is also the natural way by which T-cells recognize peptides from both intracellular and extracellular pathogens [52, 53]. However, antigen

recognition by TCRs is HLA-type restricted and TCRs cannot recognize non-protein tumor antigens, which can be disadvantageous when it comes to TCR use in T cell engineering.

Table 1. *Recent clinical trial using TCR engineered T-cells. Adapted from [54]and with some recent clinical studies.*

Antigen Target	Cancer	Year	Number of Patients	Response and commons
MART-1	Melanoma	2006	15	1 PR [55]
		2009	20	6 OR [56]
		2014	13	Not durable, combined with DC vaccine [57]
gp100	Melanoma	2009	16	3 OR [56]
CEA	Colorectal Cancer	2011	3	1 PR [58]
NY-ESO-1	Melanoma	2015	20	11 OR (4 CR) No "on-target/off-tumor" toxicity were observed [59]
	Synovial cell sarcoma	2015	18	11 OR No "on-target/off-tumor" toxicity were observed [59]
MAGE-3	Melanoma	2013	9	1 PR neurological toxicity were observed [60]

MART, melanoma antigen recognized by T-cells; CR, complete response; PR, partial response; OR, objective response; CEA, Carcinoembryonic antigen; MAGE, melanoma antigen.

Recent and ongoing clinical trials with TCR gene-modified T-cells are listed in **Table 2**. The first clinical study using TCR gene-modified T-cells targeted the melanocyte-specific MART1 antigen for treatment of refractory malignant melanoma. The anti-MART1 TCR transgene was cloned from a melanoma patient's TILs [55, 61]. Another example is an anti-CEA TCR used to treat metastatic colorectal cancer. For this study the anti-CEA TCR genes were cloned from an HLA-A2-transgenic mouse [58]. A TCR-target gp100 was also developed and used in clinical trial to treat melanoma [56]. Recently, a TCR targeting NY-ESO-1 was used in a clinical study to treat melanoma and synovial cell carcinoma [59, 62]. Although all clinical trials have some good outcome, "on-targt/off-tumor" effects have been oberved with TCRs targeting MART-1, gp100 and CEA [50, 56, 57]. NY-ESO-1 in particular was reported as a safe and effective target for treatment of melanoma with adoptive T-cells. In addition, one patient died due to "off-target" activity of anti-MAGE-A3 TCR-modifed T-cells, a TCR which also targeted MAGE-A12 and resulted in severe neurotoxicity [60].

2.2 CAR engineering of T-cells

CARs are generally designed by fusing a single chain variable fragment (scFv) from a monoclonal antibody as the antigen-recognizing extracellular domain with T-cell signaling domains to achieve downstream signaling upon

target recognition **(Figure 9)** [63, 64]. The **first generation of CARs** has only one intracellular signaling domain, usually the CD3ζ chain signaling domain from the TCR complex, which stimulates cytokine (IL-2 and IFN-γ) release upon T-cell activation. However, only CD3ζ signaling does not provide the necessary co-stimulatory signals for T-cell activation. **The second generation of CARs** has an additional T-cell intracellular signaling domain, which could influence T-cell effector functions like persistence, proliferation, trafficking, memory phenotype and cytokine production. The most widely used costimulatory domains are derived from CD28, followed by 4-1BB and thereafter ICOS, OX40 or CD27. **The third generation of CARs** have two co-stimulatory domains to further enhance the effector functions of CAR T-cells. The choice of cytoplasmic signaling domains is important to achieve optimal CAR activity. The most commonly used third generation CAR signaling domains include CD28/OX40 and CD28/4-1BB in addition to CD3ζ activation domain. In order to further improve CAR T-cell therapy and to shape the tumor microenviroment, **armored CAR T-cells** were devoloped to express a transgenic "payload" **(Figure 9)** [65]. Armoued CAR T-cells, sometimes also referred to as TRUCK T-cells release inducible IL-12 which and can enhance T-cell activaiton and Th1 polarization and attract innate immune cells to eradicate CAR antigen-negative cancer cells [65, 66].

The spacer region is the non-antigen binding extracellular domain of a CAR molecule. The most commonly used spacer regions are derived from the IgG (IgG1 or IgG4) Fc CH2-CH3 domain, the CD28 or CD8α hinge domain. The spacer region of the CAR is very important for optimal CAR function as it gives flexibility and length to allow proper dimerization of scFvs, thus improving its stability. Optimal spacer length is required for good effector-target interaction and a strong intracellular signaling [67, 68]. **The transmembrane region** is the least studied regions of the CAR molecule, which the most commonly used is derived from CD3ζ chain or CD28 molecule [69]. Early studies involving mutated CD3ζ chain transmembrane domains indicated that it is involved in stabilizing CAR interaction with other cell surface co-stimulatory molecules like endogenous TCR [69, 70]. This may have implication in CAR T-cell activation and function; however more detailed investigations are needed to confirm this. The transmembrane region can also determine the stability of CAR expression on the cell surface e.g. a first generation CAR with CD3ζ transmembrane region is less stably expressed on the cell surface than a first generation CAR with CD28 transmembrane region [71].

CARs containing **scFv extracellular domains** retain the specificity and affinity of a scFv antibody. Compared to TCRs, a major advantage of having scFv extracellular domain is that it bypasses the need for antigen presentation by MHC-I on tumor cells, as antibodies directly bind to cell surface proteins. This is advantageous in tumors that downregulate MHC-I to escape immune recognition. In addition, the CARs can also recognize non-protein

surface molecules such as carbohydrates and glycolipids. However, this limits the group of targets to cell surface-expressed antigens. The major challenge in designing a scFv is the choice of antigen that is exclusively expressed on tumor cells and the abundance of these antigens on the cell surface [69]. CARs can face competitive inhibition by soluble antigens that are released from tumors.

Table 2. *Recent clinical trials using CAR engineered T-cells. Adapted from [54]and with some recent clinical studies.*

Antigen target	Cancer	CAR construction	Year	Number of patients	Response and commons
CD19	Relapsed ALL	CD19CAR-41BB-CD3ζ (CTL019)	2014	30	27 CR Sustained remission of up to 2 years [72]
	Relapsed B-ALL	CD19CAR-CD28-CD3ζ	2014	16	14 CR [73]
	Diffusion large B-cell lymphoma	CD19CAR-CD28-CD3ζ	2015	9	4 CR [74]
	Relapsed CLL	CD19CAR-41BB-CD3ζ (CTL019)	2015	14	4 CR and 4 PR [75]
	Lymphoma and ALL	CD19CAR-CD28-41BB-CD3ζ (3G CAR)	2016	11	6 CR [76] (Sweden)
CD20	Relapsed or refractory NHL	CD20CAR-41BB-CD3ζ	2016	11	6 CR and 3 PR [77]
GD2	High risk neuroblastoma	GD2CAR- CD3ζ	2011	19	3 CR [78]
Her2	Her2+ sarcoma		2015	19	4 stable disease for12 weeks to 14 months

B-ALL, B-cell acute lymphoblastic leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin's lymphoma; CR, complete response; PR, partial response.

From 1994 to 2014, 101 clinical trials with CAR T-cells for treatment of cancer were performed in the US [79]. An increasing number of trails is currently being conducted in other regions, such as Europe, China and Australia. Recently published clinical trials using CAR T-cells are listed in **Table 3**. CAR T-cell therapy has demonstrated remarkable success when using anti-CD19 CAR T-cells for treating many different types of malignant B cells, including B-CLL, B-ALL and DLBCL [72-74]. Apart from being effective in treating patients, many patients also exhibit cytokine release syndrome, which is an associated symptoms together with CAR T-cell infusion which is mostly attributed to IFN-γ and IL-6 release. The related symptoms are fevers, kidney failure, cardiac dysfunction, hypoxemia and neurological toxicity. But in most cases, the side effect can be managed by immunosuppressive medicine, such as steroids or cytokine blockage antibodies (IL-6 blockage). The "on-target/off-tumor" toxicity is also observed in anti-CD19 CAR T-cells clinical trial, which is B-cell aplasia. However, it can be man-

aged clinically with monthly intravenous infusion of immunoglobulins (IVIg) [69].

Table 3. *Novel targets for CAR T-cells therapy to treat haematological malignancies. Adapted from [80]and with some recent clinical studies.*

Target	Cancer	CAR structure	Reference
CD22	FL, NHL, DLBCL, B-ALL	CD22CAR-CD28-CD3ζ	NCT02315612
ROR1	CLL, SLL	ROR1CAR-41BB-CD3ζ	NCT02194374
Igκ	CLL, low-grade B-cell malignancies	Igκ CAR-CD28-CD3ζ	NCT00881920
CD30	HL, NHL	CD30CAR-CD28-CD3ζ	NCT01316146
CD123	AML	CD123CAR-CD28-CD3ζ	NCT02159495
CD33	AML	CD33CAR-41BB-CD3ζ	NCT01864902
LeY	AML	LeYCAR-CD28-CD3ζ	NCT01716364
BCMA	MM	BCMACAR-41BB-CD3ζ	NCT02215967
CD138	MM	CD138CAR-41BB-CD3ζ	NCT01886976

FL, follicular lymphoma; NHL, non-Hodgkin's lymphoma; DLBCL, diffuse large B-cell lymphoma; B-ALL, B cell acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; SLL, small lymphocytic lymphoma; HL, Hodgkin's lymphoma; AML, acute myeloid leukemia; MM, multiple myeloma. ROR1, inactive tyrosine-protein kinase transmembrane receptor ROR1; BCMA, B-cell maturation antigen.

CD19 CAR T-cells used for B-cell malignancies have been intensively studied in the clinic, but also other targets of CAR T-cells have been studied both in pre-clinical and clinical studies for hematological malignancies, listed in **Table 4**. The expression patterns of CD22 and CD20 are similar to CD19 and can therefore also be utilized [77, 81]. Targeting ROR1 (highly expressed in tumorigenic B-cells) is another attractive strategy to reduce the possibility of "on-target/off-tumor" toxicity [82]. Low-grade lymphoma and B-CLL express monoclonal immunoglobulins carrying either the κ or λ light chain. It has been found that antibodies containing either κ or λ light chain have no functional differences. Therefore, CAR T-cells targeting κ light chain on malignant B-cells leave functional normal B-cells expressing λ light chain unharmed and vice verse, which minimize adverse effects [83]. Other potential targets for CAR T-cells to treat multiple myeloma (MM) are BCMA and CD138 [84, 85]. BCMA is a cell-surface receptor with restricted expression on plasma cells and CD138 is a type 1 integral membrane heparin sulfate proteoglycan expressed on both plasma cells and multiple myeloma cells. CD123, as a new target, is the IL-3 receptor subunit-α, with expression on malignant myeloid cells, as well as on endothelial cells and monocytes [86]. CD33 is another molecule expressed on malignant myeloid cells, but it is also expressed on some T-cells and hematopoietic stem cells [87]. LeY is expressed on a proportion of AML cells but also on gastrointestinal and pancreatic cells [88]. Therefore, those new targets for hematological malignancies still need to be carefully studied in the clinic in terms of

toxicity. However, novel CAR targets are required for the effective treatment of patients with hematological malignancies that do not express CD19.

CD19 CAR T-cells mediate long-term remissions of B-cell cancers. However, treatment of solid tumors have not yet seen much success with CAR T-cells. One reason is that solid tumor are composed of a complex and dynamic mass composed of several different cell types. Recent clinical trials by using GD2 CAR T-cells in neuroblastoma showed encouraging results [78]. However, there are several obstacles inhibiting CAR T-cell function, such as the immunosuppressive tumor microenvironment. Most solid tumors have a stromal compartment that support tumor growth and contributes to a suppressive microenvironment. Therefore, to direct CAR T-cells to stromal cells could be a potent new antitumor target. Fibroblast activation protein (FAP) is expressed on cancer-associated fibroblasts. Pre-clinical studies of using FAP-specific CAR T-cells have exhibited encouraging results in a mouse model [89] and a clinical trial for patients with malignant pleural mesothelioma is ongoing (NCT01722149).

In addition, the antigen targets in most of CAR T-cell trials are shared by tumors and normal tissues. Given the potential of "on-target/off-tumor" toxicity of CAR T-cells, the safety of CAR T-cell therapy for solid tumor needs to be carefully studied in pre-clinical and clinical trials. Pre-clinical studies trying to target solid tumor antigens with CAR T-cells are listed in **Table 5.** CAR T-cells targeting mesothelin and MUC16 are currently in clinical trials with a slow dose escalation/titration [79].

Table 4. *CAR targets for the treatment of solid malignancies. Adapted from [80] and with some recent clinical studies.*

Target	Cancer	Clinical trial
Mesothelin	mesothelioma, pancreatic and ovarian cancer [90]	NCT01583686
MUC1	Adenocarcinoma [91]	
MUC16	ovarian cancer [92] (with IL-12 secretion)	NCT00466531,
CAIX	kidney cancer [92]	
HER2	Sarcoma	NCT00902044
GD2	neuroblastom [78]	NCT01953900
CD171	neuroblastoma [93]	NCT02311621
CEA	adenocarcinoma liver metastases [94]	NCT02146466
PSMA	prostate cancer [95]	NCT00664196
IL-13Rα	glioma [96]	NCT02208362
Glypican-3	glioma [96]	NCT02395250
EGFRvIII	glioblastoma [97]	NCT02209376

MUC1, Mucin 1 cell surface associated; CAIX, carbonic anhydrase IX; HER2, human epidermal growth factor receptor 2; GD2, disialoganglioside 2; CEA, Carcinoembryonic antigen; PSMA, prostate-specific membrane antigen; EGFR, Epidermal growth factor receptor

One solution to overcoming the "on-target/off-tumor" toxicities is to target unique tumor antigens. The ideal antigen expressed on tumors would be result from oncogene mutation that contributes to malignant phenotype and cancer cell survival, such as hot-spot mutation in genes *like KARS, NRAS, ALK, PI3K* and *BRAF* [98]. One example of target-shared mutation in solid tumor is anti-EGFR variant III (EGFRvIII), a mutated truncated version of the epidermal growth factor receptor (EGFR). It has been showed that EGFRvIII is expressed in around 30% of patients with glioblastoma multiform. Therefore, pre-clinical and ongoing clinical are using specific anti-EGFRvIII CAR T-cells to treat glioma [97, 99]. Promising data have been shown in mouse model, but clinical activity still needs to be verified.

Another solution to minimize undesired "on-target/off-tumor" toxicities is to use CAR T-cells engineered with a safety mechanism. T-cells can be modified to carry "suicide" genes that can be turned when side effect happened during CAR T-cells therapy. Such genes include herpes simplex virus-thymidine kinase (HSV-TK) which sensitize the CAR T-cells to the antiviral medication ganciclovir [100], inducible caspase-9 (iCasp9) system which sensitize the CAR T-cells to a dimerized drug [101] and truncated EGFR which sensitize the CAR T-cells to the EGFR-specific monoclonal antibody cetuximab [102]. Safety of CAR T-cell can be regulated by the duration of receptor expression. Expression of CAR molecules on T-cells by RNA electroporation is time-limited, which can reduce long-term toxicity [103]. T-cells can also be co-transduced by two separate CARs targeting two separate

antigens, where one CAR has an activation signal and the other CAR has a co-stimulatory signal. This example has been demonstrated for CAR T-cells targeting two prostate cancer-associated antigens: PSCA and PSMA [104]. Another way is to deliver CARs with an inhibitory signaling domain, so called inhibitory CARs (iCARs), which target normal tissue dominant antigens. Inhibitory signaling domain can be derived from co-inhibitory molecular such as CTLA-4 and PD-1 [105]. "On-switch" CARs have also been developed, in which the antigen-binding and intercellular singling domains are separated into two parts [106]. Only in the presence of a small-molecule dimerizer, can the two components assemble and deliver an activation signal to T-cells [106]. Recently, a new "on-switch" CARs were designed by using synthetic Notch receptors to regulate CAR expression in order to reduce adverse effects. The Notch receptor is transmembrane protein that, when engaging an extracellular ligand, intramembrane proteolysis is induced leading to activation of a transcriptional regulator of the Notch intracellular domain. CAR T-cells can be engineered with a synthetic Notch receptor system, in which the extracellular synthetic CAR-Notch receptor can recognize antigen A and an active intercellular transcription regulator to initiate a second CAR directed toward an antigen B. When both antigens are expressed, the CAR-Notch T-cells will become active and kill the target cell [107].

2.3 Vectors for T cell engineering

Genetic engineering of T-cells for T-cell therapy is achieved by delivering genes for a TCR or CAR to T-cells using a gene delivery vector. The success of such gene delivery depends on efficient, stable and specific expression of the transgene in the modified T-cells. Most commonly used gene delivery vectors are virus-derived vectors, *e.g.,* gamma-retrovirus vector derived from Moloney murine leukemia virus (MoMLV) or lentivirus vector derived from human immunodeficiency virus 1 (HIV-1), both of which belong the *retroviridae* family of viruses.

The *retroviridae* virus family have positive-sense single-stranded RNA diploid genome which stably integrates a double-stranded cDNA copy of their pro-viral genome into the host cell genome by use of reverse transcription and integration [108]. These characteristics make them ideal gene delivery vectors to genetically modify target cells, leading to stable, and long-term transgene expression. Viral vectors derived from the *retroviridae* family are nowadays the most widely used delivery systems [109]. Safety levels of lentiviral vectors have been increasingly improved. This has been achieved by eliminating non-essential virulence genes and dividing the essential genes into separate expression plasmids. The infectious virus vector, carrying the gene of interest is produced by co-transfection of the plasmid encoding the gene of interest (vector expression cassette) together with the plasmids encoding essential genes (packaging expression cassettes) and the

plasmid encoding the envelope glycoprotein (envelope expression cassette). This results in the release of virus-like particles carrying a defective genome capable of infecting cells and integrating the transgene into the host cell genome but lack replication ability [110]. One of the most widely used envelope glycoproteins to pseudotype lenti-vectors is the vesicular stomatitis virus G glycoprotein (VSV-G). Pseudotyping with this pantropic envelope leads to a wide species and cell type tropism, which broadens their application in research and gene therapy.

Lentiviral vectors have been steadily improved over time to increase both their efficiency and biosafety. The most frequently used lenti-vectors today is the so-called third generation vectors with self-inactivating capabilities. The third generation lenti-vectors are safer as they are tat-independent [111], which is achieved through the replacement of the HIV-1 U3 region by a strong constitutive promoter, such as human early-intermediate cytomegalovirus (CMV) or Rous sarcoma virus (RSV) promoter [111]. The self-inactivating capability is achieved by removing a large part of the 3' U3 region containing transcriptional enhancers. Thus, after reverse transcription and integration, the enhancer at the 3' long terminal repeats (LTR) is not functionally duplicated at the provirus 5' end. In this way, the defective LTR does not act as a promoter and only the internal expression cassette in the integrated provirus is transcriptionally active. The elimination of all these virus elements theoretically reduces potential recombination with other retroviruses or retrovirus-like elements that could reconstitute an infectious HIV-like retrovirus.

However, there are still safety concerns when using retro or lentiviral vectors that need to be addressed like the generation of replication-competent viruses during production that could lead to a pathological viral infection. In addition, since retro and lentiviral vector integrate the transgene randomly into the host genome, it creates the possibility to activate a neighboring proto-oncogene or disrupt a suppressor gene, and thereby causing insertional mutagenesis, also referred to as vector-driven genotoxicity. These safety concerns have proven deleterious in several clinical studies when hematopoietic stem cells have been engineered e.g. insertional mutagenesis-driven clonal dominance or oncogenesis was observed in clinical gene therapy trials for X-linked severe combined immune deficiency [112], chronic granulomatous disease [113] and Wiskott-Aldrich syndrome [114]. This has not yet been observed when engineering T-cells.

Non-integrating lentiviral vectors (NILVs) have been developed to reduce the insertional mutagenesis-induced genotoxicity, by eliminating the integrase function of LV vectors [115]. Cells transduced with a NILV have double-stranded circularized DNA molecules accumulated inside the cell nucleus [116, 117]. These episomal circular DNA can facilitate transgene expression. However, the episomal DNA is diluted upon cell division, which

means that T-cell engineered by this method will not retain the TCR or CAR expression long enough to achieve a therapeutic effect.

Compared with viral vectors, plasmid DNAs has several advantages like no limitation on transgene size, limited biosafety issues and they are not subjected to sequence disruption. It is much cheaper to produce a clinical grade DNA plasmid than a viral vector. Furthermore, large amounts of plasmid vectors can be produced in a short period of time and are highly stable for long time storage. However, the use of plasmid DNA-based gene transfer in the clinics has been limited to vaccine strategies and *ex vivo* genetic modification of non-transformed human tissue /cells for therapeutic purposes [118, 119]. In terms of adoptive T-cell immunotherapy, T-cells can be electroporated to deliver a DNA plasmid carrying TCR or CAR [120]. However, the main disadvantage that limits its use is that plasmids lack the ability to replicate and when cells are dividing the plasmid will be lost from the daughter cells. To overcome this problem plasmids with transposon/transposase system that can promote integration of the gene into the genome to get long-term expression have been constructed [121], such as *sleeping beauty* system and *piggBac* system. However, also these systems can lead to insertional mutagenesis.

Another approach is to deliver *in vitro*-transcribed mRNA to T-cells by electroporation. Differently from a plasmid that needs to reach the cell nucleus the mRNA is active in the cytoplasm. However, mRNA delivered transgenes does, just like DNA plasmids, do not result in permanent genetic modification of T-cells. David et. al. used this approach to express CD19 CAR in T-cells for adoptive immunotherapy [122]. In this study, T-cells were electroporated with an optimized *in vitro* transcribed mRNA encoding a CAR against CD19. These mRNA modified CAR T-cells were then tested *in vitro* and *in vivo* for efficacy and it was observed that T-cells expressing an anti-CD19 CAR introduced by electroporation with optimized mRNA were potent and specific killers of $CD19^+$ target cells [122].

3. T-cell expansion *ex vivo*

In order to generate large amount of T-cells in a short period of time *ex vivo*, a robust T-cell expansion strategy is needed (**Table 5**). It often involves IL-2 and an agonistic stimulator of CD3 and CD28. By immobilizing anti-CD3 and anti-CD28 antibodies on beads to simultaneously deliver signal-1 and costimulatory signal-2, T-cell proliferation can be increased without provoking anergy or early apoptosis [123]. However, $CD4^+$ T-cells respond strongly to anti-CD3/CD28 bead stimulation compared to $CD8^+$ T-cells and can lead to an underrepresentation of $CD8^+$ T-cells in the CAR T-cell product. Another method used in the clinic is the rapid expansion protocol (REP) [124]. REP means that T-cells undergo a rapid expansion in culture media supplied

with anti-CD3 antibody (OKT3), exogenous IL-2, and irradiated allogeneic peripheral blood "feeder" cells usually from 3 different donors. The feeder cells promote T-cell expansion through expression of activating FcγRI (CD64) on monocytes that immobilize OKT3 and enhance its interaction with CD3 on T-cells. Feeder cells also promote a MLR where the T-cells expand and proliferate due to the allogeneic reaction [125]. Yet, another expansion methods used in laboratories is modified artificial APCs (aAPCs) to support T-cells proliferation [126]. The aAPCs can be modified in different ways to support T-cells function, such as expressing ligands for T-cells receptor (CD28, 4-1BB) [126], CAR ligands [127] and expression membrane-bound IL-15, IL-21 [128].

Table 5. *Ex vivo expansion used in clinical trials. Adapted from [129].*

Method	Expansion fold	Commons
Anti-CD3/CD28 beads+IL-2	49-385 fold	T-cells expansion from cancer patients [130, 131]
OKT3+IL-2+irradiated feeder cells		TCR engineered T-cells for melanoma [56]
		Anti-CD19 CAR engineered T-cells [132]
Artificial APC	150 fold	aAPC expression 4-1BB and CD28 [126]
Artificial APC	40000 fold	aAPC expression CD137 lig-and,CD86 CD64 and membrane-bound IL-15 IL-21 [128]

Most expansion protocols can achieve hundred to thousand-fold expansion folds during culture period ranging from 10 days to 6 weeks. In terms of expansion protocol, the optimal expansion time is unknown, but it seems that longer expansion may generate more terminally differentiated T-cells. Preclinical data suggests that the less differentiated phenotype the T-cells have the better anti-tumor efficacy *in vivo* [133].

For adoptive T-cell immunotherapy to be successful for cancer, some factors need to be considered. T-cells must be activated *in vivo* and expand to numbers capable of causing significant tumor destruction. Moreover, anti-tumor T-cells must survive long enough to completely eradicate all tumors cells. $CD8^+$ T-cells obtained *ex vivo* after expansion with the above-mentioned protocols have potent anti-tumor activity *in vitro*, but have been shown to be less effective in triggering tumor regressions *in vivo* [133]. In addition, effectors T-cells are repeatedly restimulated *ex vivo* to attain the number needed to have a significant effect in the clinical setting [134, 135]. However, repeated restimulation makes these cells exhausted and more sensitive to apoptosis and hostile factors in the tumor microenvironment such as reactive oxygen species (ROS) and immunosuppressive cytokines.

Methods

Detail of methods can be found in each paper. However, two of the most import components used in this study is listed below.

1. S/MAR element

As previously discussed, the ideal vector for genetic delivery must deliver genes with expression at a relevant therapeutic level without compromising the viability of target cells at either cellular or genetic level. One of the main limitations preventing generation of a clinically suitable plasmid DNA-vector is the transient expression of transgene. Additionally, in the case of retro or lentivirus-based vectors the risk of insertional mutagenesis (genotoxicity) caused by semi-random genomic integration of these vectors [136, 137].

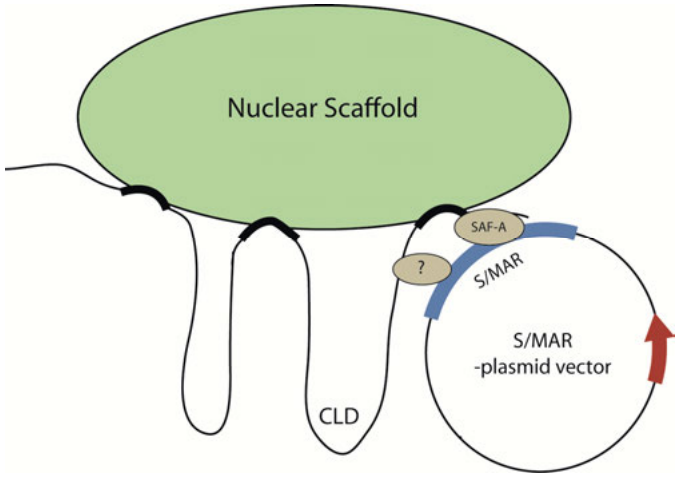


Figure 10. **The function of S/MAR elements**. S/MARs are sequences in the DNA, which work as an anchor to the chromatin. It consists as an inactive and active form. When the gene needs to be duplicated or transcribed, S/MARs are initiated by the association of scaffold attachment factor A (SAF-A) and are changed from an inactive to active form. S/MARs attach to nuclear matrix to assist gene duplication or transcription. When it is terminated, active S/MARs change back to an inactive form and disassociate from the matrix.

Scaffold matrix attachment region (S/MAR) elements are sequences in the DNA of eukaryotic chromosomes where the nuclear matrix attaches. They mediate structural organization of the chromatin within the nucleus [138]. S/MAR sequences are typically 300-500 bp and have a high AT content, which could be responsible for double helix destabilization and for the formation of secondary DNA structures [139, 140]. However, there is no S/MAR consensus sequence [139]. The S/MAR sequence binds scaffold attachment factor protein A (SAF-A) and provides mitotic stability by attaching the DNA to the nuclear matrix for the segregation of DNA into progeny cells **(Figure 10)** [141]. If the S/MAR sequence is included in a plasmid, it can persist as an extrachromosomal entity in the host cell. Human IFNβ-S/MAR-based vectors (the pEPI vector system) have been developed as non-viral plasmid vectors for persistent replication, maintenance and transgene expression [142]. The vector function is dependent on the S/MAR sequence downstream of the transgene and it is maintained at a low copy number (5-10 per cell). Continuous transcription of the transgene was observed in mammalian cell lines [142-144].

Therefore, we engineered CAR T-cells by using an S/MAR element included in non-integration lentivirus for the purpose of obtaining safe engineering of CAR T-cells.

2. *H. pylori* Neutrophil-activating Protein

Helicobacter pylori (*H. pylori*) neutrophil activating protein (HP-NAP) is a dodecameric protein (150-kDa) that acts as a virulence factor in *H. pylori* bacterial infection [145]. It is made of 12 monomeric subunits and each unit is around 17kDa and contains four α-helices [146]. The surface of the HP-NAP protein is highly positively charged and maybe has capacity of interact with and active human leukocytes [146].

HP-NAP plays a critical role in migration of neutrophils to inflamed tissue during *H. pylori* infection. HP-NAP promotes strong neutrophil and monocyte binding to endothelium and extravasation by upregulating surface expression of β2 integrin [145, 147]. It also activates neutrophils to produce ROS and myeloperoxidase (MPO) [145]. HP-NAP also stimulates secretion of other pro-inflammatory cytokines such as TNF-α and IL-8 [148, 149], which in turn induce adhesion molecules expression like V-CAM, I-CAM and secretion of CXCL8 by endothelial cells. In addition, HP-NAP can induce neutrophil secretion of macrophage inflammatory protein 1 alpha (MIP-1α) and MIP-1β (also called CCL4) [147]. These cytokines and chemokines attract chemotaxis of neutrophils to the site of inflammation.

HP-NAP is a TLR-2 agonist and it is chemotactic for neutrophils, monocytes and can mature DCs both *in vitro* and *in vivo*. It can stimulate secretion of IL-12 and IL-23, which are Th-1 polarizing cytokines [148, 150]. HP-

NAP stimulates monocytes to differentiate and mature to DCs by upregulating expression of HLA-DR, CD80 and CD86 [150, 151]. It also has a pivotal immunoregulatory function in aiding cytotoxic T-cell and NK cell activation. In addition, HP-NAP can interact directly with T-cells. It has been reported that HP-NAP can induce T-cells to secrete high level of IFN-γ and low level of IL-4, also suggesting a Th1 polarizing response [148]. This is coincident with reports that *H.pylori* infected humans indicate a strong Th1 polarizing response [148].

In terms of clinical application, HP-NAP also has a wide range of application. Firstly, it is used as a diagnostic marker for *H. pylori* infection and it is also associated with certain clinical outcomes of *H. pylori* infection. Secondly, HP-NAP can be a good target for vaccine development against *H. pylori.* It also has been reported that HP-NAP can be used as an adjuvant to stimulate immune responses against weak immunogen, such as used in immunoglobulin λ-chain in mice [152]. Thirdly, HP-NAP can be used in cancer immunotherapy. For example, recombinant HP-NAP has been used for bladder cancer [153] and a measles virus encoded HP-NP has been used for breast cancer [152], in both cases did it exhibit anti-cancer properties by inducing Th1 immune response in mice. It has also gained interest as an immunomodulating therapeutic agent for cancer therapy, either as recombinant protein or as transgene encoded by oncolytic adenoviruses [149, 154]. Based on those properties of HP-NAP, we engineered CAR T-cells with HP-NAP to improve T-cell therapy.

Present Investigations

Paper I

Aims: CAR T-cell therapy of cancer is a treatment strategy where T-cells are isolated a patient's blood, activated, engineered, and expanded *ex vivo* before being re-infused to the patient. The most commonly used T-cell expansion methods are the "rapid expansion protocol" (REP), which utilizes OKT-3, interleukin (IL)-2 and irradiated allogeneic feeder cells or anti-CD3/CD28 antibody beads. However, REP-expanded or bead-expanded T-cells are sensitive to the harsh tumor microenvironment and often short-lived after reinfusion.

Main findings: Here we demonstrate that when irradiated and preactivated allo-sensitized allogeneic lymphocytes (ASALs) are used as helper cells to license OKT3-armed allogeneic mature dendritic cells (DCs), together they expand target T-cells of high quality. The ASAL/DC combination yields an enriched Th-1 polarizing cytokine environment (IFN-γ, IL-12, IL-2) and optimal co-stimulatory signals for T-cell stimulation. AEP expanded engineered T-cells showed better survival and cytotoxic efficacy under oxidative stress and immunosuppressive environment, as well as superior proliferative response during tumor cell killing compared to the REP-protocol. Our result yields a robust *ex vivo* method to expand T-cells with improved quality for adoptive T cell immunotherapy of cancer.

Paper II

Aims: Chimeric antigen receptor (CAR) T cell therapy is a new successful treatment for refractory B cell leukemia. Success is relying on long-term CAR transgene expression in T-cells caused by gamma retrovirus (RV) or lentivirus (LV) integration in the T cell genome for long-term CAR transgene expression. However, uncontrolled RV/LV integration in host cell genomes has the potential risk of causing insertional mutagenesis.

Main findings: Herein, we describe a novel episomal and long-term cell engineering method using non-integrating lentiviral (NILV) vector containing a scaffold matrix attachment region (S/MAR) element, for either overexpression of transgenes or down-regulation silencing of target genes. The insertional events of this vector into the genome of host cells are below detection level. CD19 CAR T-cells engineered with a NILV-S/MAR vector

have similar levels of CAR expression as T-cells engineered with an integrating LV vector, even after numerous rounds of cell division. NILV-S/MAR-engineered CD19 CAR T-cells exhibited similar cytotoxic capacity upon CD19 expressing target cells recognition as LV-engineered T-cells and are as effective in controlling tumor growth *in vivo*. We propose that NILV-S/MAR vectors are superior to current vector options as they enable long-term transgene expression without the risk of insertional mutagenesis and genotoxicity.

Paper III

Aims: Chimeric antigen receptor (CAR)-engineered T-cells, targeting the pan B-cell marker CD19, have made remarkable progress in clinical studies for both acute and chronic B-cell leukemia. Success has also been observed for B-cell lymphomas although less so than for leukemia. The reason may be that the semi-solid structure of lymphomas leading to penetration obstacles and an immune suppression microenvironment within these tumors.

Main findings: *H. pylori* Neutrophil-activating Protein (HP-NAP) has been proven as a major virulence factor important for *H. pylori* infection and plays a crucial role in *H. pylori* induced inflammation. HP-NAP promotes DCs maturation and promotes Th1 polarization by inducing neutrophils, monocytes and DCs to secret IL-12. Therefore, we applied inducible HP-NAP in CAR T-cell therapy in our paper to convert the suppressive tumor microenvironment into a pro-inflammatory environment by inducing maturation of DCs, attracting innate immune cells and increasing secretion of Th1-type cytokines and chemokines, which support CAR T-cell therapy in lymphoma tumor both *in vitro* and *in vivo*

Paper IV

Aims: The tumor microenvironment has an important role in immune evasion and tumor progression and is strongly correlated with disease outcome. Cancer vaccines aim at initiating immune-activating responses in an immune-suppressed environment by linking innate and adaptive immunity in a coordinated attack against the tumor. Dendritic cells (DCs) can bridge innate and adaptive immune response and DC-based cancer therapies have therefore been developed. In most such therapeutic cancer vaccine trials, autologous DCs have been produced and modified *ex vivo* to present tumor-associated antigens (TAA) directly to T-cells.

Main findings: We propose to use allogeneic DCs (alloDCs), not as antigen presenters but as an adjuvant to initiate an allo-reactive immune response. AlloDCs can be prepared from blood monocytes of unrelated donors to secret cytokines associated with Th-1 responses and through adenovirus transduction express TAAs. The alloDCs will be attacked by the host im-

mune system and endogenous DCs can engulf material from the attacked cells, mature, migrate and initiate T-cell responses against the TAA. We have examined the effectiveness of alloDCs to promote migration and activation of immune cells both *in vitro* and *in vivo*, as well their therapeutic efficacy in a melanoma mouse model, where they are transduced to express human gp-100 as a model TAA. In combination with pMel-1 T-cells transfer, alloDCs prolong the survival of tumor-bearing mice by inducing T-cell persistence in the tumor.

Summary of the thesis in Chinese (论文中文简介)

人体的免疫系统就像给一个时刻准备战斗的部队一样，监控着人体免受外源“敌人”的侵扰。比如小到病毒，细菌，大到寄生虫等各类病原体和有害物质。免疫系统还有一个重要的工作就是把这些物质和自身的健康细胞组织区别开来。病原体的进化很迅速，它们可以快速的变化调整，来躲避“军队”的侦测和攻击。当然，人体作为高级生物也很聪明，为了能够在这场与病原体的对抗中胜利，人体也进化出了多种识别和消灭病原体的机制。人体的免疫系统由多种蛋白质，细胞，器官和组织所组成，它们之间相互作用，精密调控，共同构成了一个精细的动态网络。

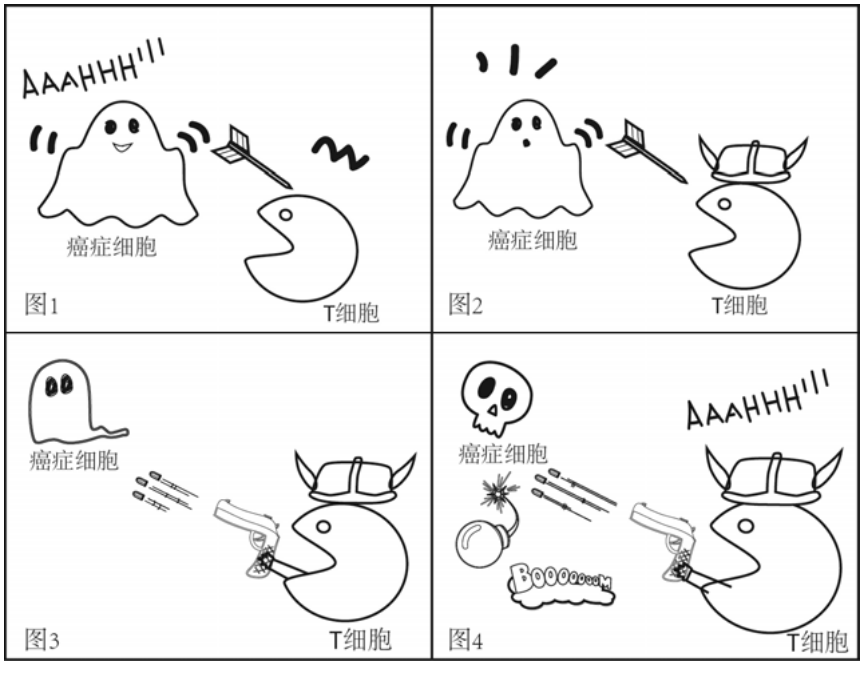
病原体攻击人体的时候，人体免疫系统的第一层障碍被称作“表层屏障”，比如皮肤，皮肤和呼吸道的分泌物，唾液，眼泪中的酶，以及在泌尿生殖系统和消化道中的共生的肠道菌。表层屏障作为先头部队，可以在抵抗的过程中发挥作用。但是一旦敌人成功的越过了先头部队，成功突破表层屏障进入人体内部，它们接下来就会遭遇到先天性免疫系统的识别和攻击。**先天性免疫**系统通常是通过“模式识别受体”与病原体识别后被触发。“模式识别受体”是先天免疫的关键部分，它能识别不同病原体中的保守部分，也可以识别发出警告信号的受损伤的细胞。这只后续部队的攻击是非特异的，一旦有敌人入侵的信号，它们会冲入战场，扫荡一番。受病原体侵扰的细胞就会被清理干净，这就是先天性免疫系统对抗病原体的方式，能够非常快速的反应，对广泛的病原体入侵做出反应，但不能对某一个病原体维持持久的战队力。组成这只有战斗力的主要成员有补体系统和细胞屏障。补体系统包含了超过 20 种不同的蛋白质，它们本身就是蛋白酶，与微生物结合之后，它们的蛋白酶活性被激活，快速增加，形成一个级联反应。这些分子的沉积会破坏病菌的细胞膜，达到杀菌的目的。细胞屏障是由独立的细胞方式来工作，“特种兵”作战模式。这些特种兵包括了有吞噬细胞（巨噬细胞，中性粒细胞和树突状细胞）、肥大细胞，嗜酸性细胞、嗜碱性细胞以及自然杀伤细胞。它们一接触攻击较大的病原体或者吞噬并杀死较小的微生物的方式，来识别消灭敌人。其中树突状细胞在“部队”中很重要，它可以吞噬了病原体，传递给 T 细胞（适应性免疫系统中的关键细胞之一），它承担了先天性免疫和适应性免疫之间的联络员。

适应性免疫系统，可以提供更强的免疫应答和免疫记忆，使得每个病原体都可以通过特征性的抗原表位而被免疫系统“记住”。适应性免疫系统接受到联络员提供的抗原信息，识别出“非自身”抗原，然后逐一击破。它是在“战斗”中具有高度的特异性“抗原特异性”，抗原的特异性使得应答的产生更有针对性。这种针对性的应答还可以通过体内的免疫记忆细胞来实现的。如果病原体再一次入侵机体，这些特异性的记忆细胞能够迅速地消灭这一病原体。适

应性免疫系统中的细胞被称为淋巴细胞，属于特殊类型的白细胞。B 细胞和 T 细胞是主要的淋巴细胞，由骨髓中的造血干细胞分化而来。B 细胞主要参与体液免疫，而 T 细胞则参与细胞免疫。B 细胞和 T 细胞都携带能够识别特定靶标的受体分子。B 细胞抗原特异性受体则是位于 B 细胞表面的抗体，负责识别整个病原体，每一种 B 细胞的细胞系都表达各不相同的抗体，因此，B 细胞抗原受体的集合代表了人体能够产生的所有抗体。T 细胞负责识别“非自身”靶标，如病原体，但需要抗原（病原体上的一些小片段）经处理并由一种被称为主要组织相容性复合体（MHC）的“自身”受体传递之后才能实现。T 细胞主要有两类：杀伤型 T 细胞和辅助型 T 细胞。

肿瘤在医学上是指细胞的异常病变，是身体的细胞有不受控制的增生而形成肿块，其可以分为良性肿瘤和恶性肿瘤。恶性肿瘤，增生的细胞还可以扩散到其他部位增生。正常细胞通过细胞增殖和细胞凋亡来达到平衡，但是肿瘤细胞与正常细胞相比，有基因、结构、功能和代谢的异常。但是肿瘤细胞来源于病变的“自身”细胞，它可以通过多种机制逃避人体免疫“部队”的检测和监控。

这几年免疫细胞治疗开始取得突破的进展，我的主要研究方向是 T 细胞的免疫治疗。免疫治疗就是通过增强机体本身的免疫系统来消除或抑制肿瘤。T 细胞治疗的基本思路是，从病人中提出来 T 细胞，在体外进行“再教育”，给它一个更强大，针对肿瘤更特异的“武器”（CAR），并且大量增殖，已得到一个数目庞大的队伍，然后回输给病人，在体内发挥效果。我的博士主要工作就是通过不同的方面来是增强 T 细胞治疗癌症的效果，并且提高治疗的安全性。



在这里我用了一个简单的四格漫画描述我的博士论文的主要研究内容的主要内容。T 细胞虽然具有杀伤性，但是面对强大并且狡猾的敌人“癌症细胞”还是处在下风。在 T 细胞的治疗过程中，T 细胞在肿瘤内还是很脆弱，敌人释放的环境会让 T 细胞容易死亡（图 1）。为了能让治疗型的 T 细胞在面对癌症细胞时有更好的保护，在体外增殖的时候，T 细胞经过特殊的增殖方式，拥有了坚硬的“盔甲”，可以抵抗肿瘤微环境的进攻 （图 2）。为了进一步增强 T 细胞的攻击性，赋予了它一个更特异，更安全的武器，其发挥的功能能极大的杀伤癌症细胞 （图 3）。肿瘤环境非常复杂，除了肿瘤细胞之外，还有很多支持肿瘤生长的细胞，可以看做敌人的后援部队。于是仅仅用 T 细胞杀伤是不够，还要充分的调动整个免疫系统（图 4）。通过同时加入树突状细胞，作为一个重要的武器，改变肿瘤的环境，更好的支持 T 细胞的杀伤。

总之，我的博士研究内容就是希望通过优化 T 细胞治疗的各个环节，增强 T 细胞治疗的效果。其中，体外特殊的增殖方法，即将运用在瑞典的 T 细胞临床试验中。也期望我所研究的内容能更多的用在病人身上，以达到治疗的效果。

致：景天 &我的家人

Future Perspective

1. The AEP T-cell expansion protocol into the clinic

For adoptive T-cell transfer, it is clearly important to produce T-cells *ex vivo* that are able to further proliferate *in vivo* after transfer, as this has been shown to translate to better antitumor efficacy. It has been reported that T-cells with a less-differentiated phenotype expanded in the presence of IL-7 or IL-15 have a better proliferation capacity *in vivo* [155]. Expansion using IL-7 and IL-15 can generate more memory phenotype T-cells from naïve precursors under GMP-compliant conditions [155]. In addition, long T-cell culture periods consume more reagents, especially human serum used currently in most centers, which is also expensive. Therefore, expansion of T-cells in serum-free medium needs to be considered in the future [156].

In order to evaluate the AEP expansion protocol in the clinic, we have evaluated it on T-cells from lymphoma patients. We found that CAR T-cells produced from the blood of lymphoma patients are as efficient as CAR T-cells produced from the blood of healthy volunteers. We are currently transferring the protocol to a GMP setting at Vecura (Clinical research center, Karolinska University Hospital). A phase I clinical trial with CD19 CAR T-cells is currently finishing (PI Gunilla Enblad) and the plan is to include our new expansion protocol in the follow up study.

In addition, we are currently testing the AEP T-cell expansion protocol in serum-free medium and also culturing T-cells in the presence of IL-7 and IL-15 instead of IL-2. Preliminary data suggests that T-cells have more central memory phenotype compare to culture supplied with only IL-2, which is in accordance with previous studies [155].

2. NAP CAR T-cell therapy for solid tumor

TRUCK T-cells (CAR T cells with inducible cytokine secretion) are modified to express pro-inflammatory cytokines, such as IL-12, upon encountering target cells that can regulate the suppressive tumor microenvironment in solid tumor in order to protect the infused CAR T-cell. CAR(IL12) T-cells have been proven to enhance antitumor efficacy and increase anti-suppressive function in the tumor microenvironment. Since the CAR(IL12) T-cells has been shown encouraging results in preclinical studies, an upcom-

ing trail is performed by using CAR(IL12) T-cells to target MUC-16 that over expressed on about 70% of all ovarian cancers [92].

In our study, we are using HP-NAP that is expressed and secreted upon CAR antigen recognition. The results showed that HP-NAP secretion from CAR T-cells worked as immunomodulatory agents to promote DCs maturation, to play important role in Th1 polarizing and to attract neutrophils, monocytes and host DCs. The *in vitro* results displayed high cytotoxicity in present DCs. We will continue the studies with *in vivo* models and furthermore confirm our concept in a solid tumor model (neuroblastoma) by using anti-GD2 CAR together with NAP secretion *in vivo*. We will also compare NP-NAP with IL-12 as transgenic payload in CAR T-cells.

3. Combination therapy for T-cell therapy

Since the immune response to tumors is dynamic it is in many cases difficult to obtain benefits from a single immunological treatment. According to evaluation of multiple components in the tumor microenvironment, tumors can be distinguished an immunogenic (hot) and non-immunogenic (cold). An immunogenic (hot) tumor microenvironment consists of infiltrating T-cells, proinflammatory cytokines, and PD-L1 expression, while a non-immunogenic (cold) tumor microenvironment lacks these components. Also, when T-cells are activated, they express PD-1 or CTLA-4 and are susceptible to blocking signals from tumor cells and other cells in the tumor microenvironment. The inhibitory interaction can be blocked by monoclonal antibodies against PD-1 and CTLA-4 and this approach has shown encouraging results in some malignancies, such as non-small cell lung and melanoma cancer [157, 158]. However, the success of this approach clearly needs pre-existing tumor-specific T-cells [159] and tumors need to be immunogenic.

Indeed, monoclonal antibody therapy is a good strategy to aid T-cell therapy in the clinical. Therefore, we are investigating combination of CAR(NAP) T-cells with anti-PD-1 antibody in solid tumors. Effects from NAP are expected to reverse immunosuppressive tumor environment resulting in better environment for T-cells cytotoxicity *in vivo* and the anti-PD-1 antibody to protect the CAR(NAP) T-cells from inhibitory signals. We also proposed to use alloDC transduced with adenovirus as a strong adjuvant to switch the tumor microenvironment. We do see that alloDCs treated tumors display more effector immune cells and less suppressive immune cells infiltration. Therefore, a combination of alloDCs, CAR(NAP) T-cells and anti-PD-1 antibody therapy may be beneficial in treating non-immunogenic tumor.

In terms of non-immunogenic tumors, the combination therapy may need to consider how to create an “immunogenic” tumor microenvironment. It is a clear indication in preclinical models that certain cytostatic drugs can induce

immunogenic cell death (ICD) that releases immunomodulatory “find-me” and “eat-me” signals and increase effective antigen presentation and T-cell activation. There is emerging evidences that oncolytic virus lysis of tumor cells also can induce ICD in tumors, which are mainly mediated by damage-associated molecular patterns (DAMPs) including surface-exposed calreticulin (CRT), secreted ATP and released high mobility group protein B1 (HMGB1) [160]. Therefore we are aiming to use oncolytic virus as ICD inducers to enhance treatment effects of non-immunogenic tumors. Preliminary data demonstrate that oncolytic adenovirus, Semliki forest virus and vaccine virus can induce ICD in different cancer cell lines and this can promote DC maturation. We will continue to study if combination therapy using oncolytic virus with CAR T-cells and/or anti-PD1 antibodies to further enhances antitumor immunity.

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