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Finland

IN VITRO CELL EXPANSION AND CTLA4
IN ADVANCED T-CELL THERAPIES

Tanja Kaartinen

ACADEMIC DISSERTATION

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Life begins at the end of your comfort zone.

- Neale Donald Walsch
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:


The publications are referred to in the text by their roman numerals (I-III). The original publications are reproduced with permission of their copyright holders.

In addition, some unpublished data are presented.
ABSTRACT

T-cell function is a promising therapeutic target and remedy in modern medicine. Various ways of modifying T-cell response are under development with a view to treating cancer, autoimmune diseases, and transplantation-related complications. T-cell function can be steered by altering target recognition or cosignaling receptors as well as by inducing immunological memory or regulatory T cells (Tregs). Unwanted immune responses can be curtailed by administering Tregs and, perhaps, long-lasting immunological tolerance can be induced. Cytotoxic T cells can be directed against cancer cells. Considerable T-cell numbers are required for clinical efficacy. Therefore, *in vitro* cell expansion is often necessary and cultures are commonly supplemented with interleukin (IL)-2. As T-cell activation, proliferation, effector differentiation, and the development of memory are inherently coupled to each other, excessive stimulation during expansion may lead to exhaustion. Hence, cells with weaker therapeutic potency may be produced.

In this thesis, various methods of T-cell activation and *in vitro* cell expansion were evaluated particularly in the context of personalized medicine and cell therapy.

Good therapeutic response to T-cell therapy in cancer depends in part on the survival of T cells and T-cell memory. The present study demonstrated that the proportion of memory T cells could be increased by limiting the length of *in vitro* T-cell expansion and by reducing the amount of IL-2.

This study further showed that as a result of *in vitro* expansion Tregs expressed higher levels of the Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) cosignaling receptor. CTLA4 is a central molecule for the Treg-mediated inhibition. The level of CTLA4 expression in Tregs correlated with higher inhibitory function of the cells. Apparently, high CTLA4 receptor expression after cell expansion was in part a result of changes in the alternative splicing of CTLA4 messenger RNA (mRNA). It was also found that the splicing preferences and the expression levels of CTLA4 mRNAs were associated with genetic variation in the T-cell cosignaling receptor gene region.

This thesis provides new knowledge that can be applied in the evaluation of individual variation in T-cell immunity and the production of therapeutic T cells. The T-cell expansion method that was developed here is directly applicable in T-cell manufacturing, and the findings may have substantial clinical relevance.
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>ATMP</td>
<td>advanced therapy medicinal product</td>
</tr>
<tr>
<td>CAR</td>
<td>chimeric antigen receptor</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein diacetate N-succinimidyl ester</td>
</tr>
<tr>
<td>CHAI</td>
<td>CTLA4 haploinsufficiency with autoimmune infiltration</td>
</tr>
<tr>
<td>CLL</td>
<td>chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CRS</td>
<td>cytokine release syndrome</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T lymphocyte-associated antigen 4</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>eQTL</td>
<td>expression Quantitative Trait Locus</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>flCTLA4</td>
<td>full-length CTLA4</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box p3</td>
</tr>
<tr>
<td>GMP</td>
<td>good manufacturing practice</td>
</tr>
<tr>
<td>GVHD</td>
<td>graft-versus-host disease</td>
</tr>
<tr>
<td>HAMA</td>
<td>human anti-mouse antibody</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>HSV-TK</td>
<td>herpes simplex I virus thymidine kinase</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible costimulator</td>
</tr>
<tr>
<td>IDO</td>
<td>indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>liCTLA4</td>
<td>ligand-independent CTLA4</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MACS</td>
<td>magnetic cell sorting</td>
</tr>
<tr>
<td>MART-1</td>
<td>Melanoma antigen recognized by T-cells 1</td>
</tr>
<tr>
<td>MNC</td>
<td>mononuclear cells</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NHL</td>
<td>non-Hodgkin lymphoma</td>
</tr>
<tr>
<td>PD-1</td>
<td>programmed death-1</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>REP</td>
<td>rapid expansion protocol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>scFv</td>
<td>single-chain variable fragment</td>
</tr>
<tr>
<td>sCTLA4</td>
<td>soluble CTLA4</td>
</tr>
<tr>
<td>SSP</td>
<td>sequence-specific primers</td>
</tr>
<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
</tr>
<tr>
<td>TCM</td>
<td>central memory T cell</td>
</tr>
<tr>
<td>T Eff</td>
<td>effector T cell</td>
</tr>
<tr>
<td>TEM</td>
<td>effector memory T cell</td>
</tr>
<tr>
<td>T M</td>
<td>memory T cell</td>
</tr>
<tr>
<td>T SCM</td>
<td>T memory stem cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>helper T cell</td>
</tr>
<tr>
<td>TIL</td>
<td>tumor-infiltrating lymphocyte</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>Tr1</td>
<td>T regulatory type 1 cell</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
</tbody>
</table>
1 REVIEW OF THE LITERATURE

1.1 T-CELL FEATURES

T cells are a subpopulation of lymphocytes, white blood cells, that are able to recognize specific structures presented by human leukocyte antigen (HLA) molecules via the unique T-cell receptor (TCR) found on their surface. T cells have three major roles in the immune system (Figure 1). Together with antigen-presenting cells (APCs), helper T cells initiate the adaptive arm of the immune reaction. The cytotoxic subpopulation of T cells monitors the body for infected and transformed cells. Finally, regulatory T cells are able to suppress the immune response and generate peripheral immunological tolerance. Thus, T cells are indispensable in terms of health (Janeway et al. 2001).

Because of the potency of T cells, their functions present both a target and remedy to modern, advanced medicine. The improvement of T-cell response is pursued in the treatment of infections and cancer to better combat these diseases. On the other hand, efforts are made to attenuate the undesired T-cell reactions in organ and cell transplantation or autoimmune diseases to maintain tissue health. In medicine, many features of the T-cell biology could be exploited.

1.1.1 ANTIGEN RECOGNITION

T-cell progenitors originate from bone marrow hematopoietic stem cells (HSC) and mature to functional T cells in the thymus. During maturation, each T cell gains its unique receptor for the recognition of antigens (Ag), the structures capable of inducing an immune response. The random rearrangement of TCR genes provides each T cell a unique TCR with distinct specificity for antigens. Before the T cells can leave the thymus, they are quality controlled. First, positive selection ensures that the newly generated TCRs can bind sufficiently their counter-receptors presenting the Ags, i.e. HLA molecules. Then, negative selection results in the deletion of T cells with TCR that recognizes self-Ags, antigenic peptides from the own body, thereby preventing them from eliciting an autoimmune response.

Mature T cells circulate in the bloodstream and enter into lymphoid tissues. The first signal required for T-cell activation is delivered when a specific Ag, which can be recognized by a particular naïve T cell, is presented by HLA molecules of an APC. But additional signals are required for proper immune activation (Janeway et al. 2001).
1.1.2 COSIGNALING RECEPTOR CD28 AND CTLA4, ICOS, AND PD-1

In addition to the Ag-specific signal mediated by TCR and HLA interaction, a simultaneous second signal, i.e. cosignal, is needed. Its purpose is to indicate danger. Dendritic cells (DCs), which belong to APCs, upregulate CD80 (B7-1) and CD86 (B7-2) cosignaling receptor ligands upon final maturation following an intake of antigens and migration from tissues to the lymph nodes (Table 1). Also, macrophages and B cells, the other APC types, start expressing these ligands as a response to immune activation. On T cells, engagement of CD28 by CD80 or CD86 provides the essential cosignal for naïve T cells. Engagement induces intracellular signaling leading to transcriptional changes followed by cellular activation (Janeway et al. 2001).
Table 1  A simplified summary of B7/CD28 cosignaling family molecules. For complete review for receptor-ligand interactions and functions, see (Carreno and Collins 2002) and (Chen and Flies 2013).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Expression on T cells</th>
<th>Function</th>
<th>Ligand(s)</th>
<th>Ligand Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD28</td>
<td>constitutive</td>
<td>activating</td>
<td>CD80,</td>
<td>DCs, B cells, macrophages (induced, CD86 constitutively at low level);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD86</td>
<td>CD80 on T cells (induced)</td>
</tr>
<tr>
<td>CTLA4</td>
<td>induced</td>
<td>inhibitory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sCTLA4</td>
<td>induced?</td>
<td>inhibitory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICOS</td>
<td>induced</td>
<td>activating</td>
<td>B7-H2</td>
<td>DCs, B cells, macrophages (constitutive), non-hematopoietic cells (induced)</td>
</tr>
<tr>
<td>PD-1</td>
<td>induced</td>
<td>inhibitory</td>
<td>PD-L1, PD-L2</td>
<td>DCs, B cells, macrophages (induced), non-hematopoietic cells (constitutive and induced), PD-L1 on T cells (induced)</td>
</tr>
</tbody>
</table>

Several families of T-cell cosignaling receptors have been identified (Chen and Flies 2013). CD28 belongs to the B7/CD28 family. After the activation of T cells by Ag and CD28 signaling, the expression of other family members, such as Cytotoxic T lymphocyte-associated antigen 4 (CTLA4), Inducible costimulator (ICOS), and programmed death-1 (PD-1), is induced (Carreno and Collins 2002, Keir and Sharpe 2005, Bour-Jordan et al. 2011). They all are close homologs of each other, and the genes encoding them are located close together. The significance of B7 family cosignaling receptors for the T-cell response is indicated by detrimental and even fatal outcomes upon elimination of their function in animal models. In humans, there is substantial evidence that autoimmune susceptibility is associated with genetic variation in the T-cell cosignaling receptor genes (Gough et al. 2005, Bour-Jordan et al. 2011). The specific genes or mechanisms causing this are not known. Since polymorphisms in these genes, for the most part, do not change amino acids in the receptor proteins, the association could derive from differences in gene expression or splicing (Knight 2005).

The balance between various signals and the cytokine environment determines the functional outcome of the Ag recognition. ICOS further enhances T-cell activation. It modifies the cytokine secretion by the T cell and may influence the subsequent B-cell response. In contrast, CTLA4 and PD-1 counteract the functions of CD28 and ICOS. Both T-cell proliferation and cytokine response are inhibited by PD-1 engagement (Carreno and Collins 2002).

The requirement for CD28 signaling, as well as the expression and function of the other cosignaling molecules and their ligands, are context
dependent. The launching of the effector functions in peripheral tissues, e.g. at the site of infection, and the activation of memory T cells (T_M) in lymph nodes or tissues (described in the following sections), are less dependent on CD28 signaling. While CTLA4 probably is pivotal in the regulation of T-cell activation in lymphoid tissues, PD-1 and ICOS play a more major role in the termination and modification of the response in the periphery (Keir and Sharpe 2005, Bour-Jordan et al. 2011). Besides, PD-1 has a role in the thymic T-cell maturation process. Last, the induced expression of CD80 on activated T cells can add further complexity to the cosignaling network. Engagement of T cell-expressed CD80 by PD-L1 or CTLA4 leads to inhibition of T-cell growth and effector functions (Chen and Flies 2013).

1.1.2.1 CTLA4 and its soluble isoform

The mechanisms behind CTLA4-mediated inhibition are more complex than the sole, direct signal-induced alteration in transcription (Bour-Jordan et al. 2011, Walker 2013). First, CTLA4 was found to increase the T-cell activation threshold (the number of engaged TCRs needed), decrease interleukin (IL)-2 secretion, and to arrest cell cycle (Krummel and Allison 1996, Blair et al. 1998). Negative signaling inside the T cell by CTLA4 was the obvious mechanistic explanation (Rudd and Schneider 2003). Next, CTLA4 was found to share its ligands with CD28 and to have a higher affinity for them. Therefore, CD28 has to compete for ligands, which results in diminished activation signaling. Finally, a ligand-independent function, where CTLA4 in the immunological synapse interrupts TCR signaling, has also been proposed (Bour-Jordan et al. 2011).

Regulating the expression of T-cell cosignaling receptors and their ligands is central to defining their specific functions. The cell surface expression of CTLA4 is under particularly tight regulation by restricted trafficking and rapid internalization. The translocalization of CTLA4 to and from the cell surface is a prerequisite for its precise function. Therefore, the CTLA4 receptor mainly has an intracellular localization (Valk et al. 2008, Tai et al. 2012).

Functional mechanisms and significance of soluble CTLA4 (sCTLA4), the secreted CTLA4 isoform (Magistrelli et al. 1999, Oaks et al. 2000), are not well known. sCTLA4 arises from alternative splicing of the CTLA4 transcript and lacks the transmembrane domain. As the ligand-binding part is retained, sCTLA4 is also able to bind CD80 and CD86. It has been demonstrated to have an inhibitory function (Oaks et al. 2000, Huurman et al. 2007, Simone et al. 2009, Ward et al. 2013). Many reports have shown sCTLA4 to be present in the serum of autoimmune patients (Oaks and Hallett 2000, Mayans et al. 2007, Simone et al. 2009, Cao et al. 2012) and one report in the serum of leukemia patients (Simone et al. 2012). However, the results have been suspected to be a misinterpretation due to unspecific and inappropriate test methods. Antibodies recognizing the immunoglobulin (Ig)-like domain
of the CTLA4 receptor were utilized in immunoassays that were poorly controlled and apparently, instead of sCTLA4, Ig domains of different serum proteins were detected (Tector et al. 2009, Esposito et al. 2014). sCTLA4 is expressed at a lower level compared to the full-length CTLA4 receptor (Ueda et al. 2003, Perez-Garcia et al. 2013). Its expression is either induced (Perez-Garcia et al. 2013) or repressed (Magistrelli et al. 1999, Oaks et al. 2000) upon T-cell activation, depending on the strength of stimulation (Ward et al. 2013).

CTLA4 also plays a prominent role in regulatory T cells (Tregs, (Sakaguchi et al. 2009)), which is discussed later in this thesis. Current knowledge has led to the realization that CTLA4 has a dual role in T-cell immunity (Bour-Jordan et al. 2011): i) as a T cell-intrinsic regulator in conventional effector T cells and ii) as an extrinsic regulator when expressed on Tregs.

Expression of the inhibitory T-cell cosignaling receptors, such as CTLA4 and PD-1, which are also called checkpoint molecules, is often induced on T cells that have entered into a tumor. This way the tumor avoids being destroyed by T cells. Ipilimumab (Ward et al. 2014, Clifton et al. 2015), a biological medicine targeting CTLA4 entered European markets in 2011 (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/002213/WC500109302.pdf). It is a CTLA4 specific antibody (Ab) that is used for the treatment of melanoma. Together with other cancer immunotherapies, anti-CTLA4 Abs are considered a breakthrough in the field (Couzin-Frankel 2013). It binds to CTLA4 and blocks its function. As a consequence, T-cell inhibition is abrogated providing stronger T-cell immunity against tumor cells. Another mechanism that might operate with anti-CTLA4 monoclonal antibodies (mAbs) is related to Tregs. The immunosuppressive microenvironment inside tumor tissue induces not only inhibitory molecules but also recruits immunosuppressive cells, such as Tregs, to the site. The binding of anti-CTLA4 mAb to Tregs allows their elimination by Ab-dependent cellular cytotoxicity (Clifton et al. 2015). The outcome is the same: enhanced T-cell immunity against the tumor.

Another kind of CTLA4-based medicine, abatacept (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Summary_for_the_public/human/000701/WC500048936.pdf), is used in autoimmune disorders, e.g. in rheumatoid arthritis (Ward et al. 2014). It is a soluble fusion protein of CTLA4 and Ig (CTLA4-Ig). It binds to CD80 and CD86 on the surface of APCs, thereby preventing APCs from offering costimulatory signals for T cells via CD28. The resulting T-cell inhibition ameliorates the autoimmune inflammation.
1.1.3 CLONAL EXPANSION AND EFFECTOR FUNCTIONS

The number of naïve T cells that initially recognize a given antigenic structure can be low and insufficient for the elimination of the target. Therefore, the activated T cell self-replicates multiple times in order to generate a clone of T cells with an identical TCR and specificity against the activating antigen. IL-2 delivers this signal required for sustained and efficient T-cell proliferation, i.e. clonal expansion.

The expression of IL-2 and CD25, the α-chain of the heterotrimeric high-affinity IL-2 receptor, is directly regulated by CD28 signaling (Bour-Jordan et al. 2011). The expression of IL-2 and CD25 is induced within hours following T-cell activation. Activated T cells are the principal producers of this essential cytokine, hence making IL-2 an autocrine T-cell growth factor.

IL-2 mediates its functions predominantly through the high-affinity IL-2 receptor. The trimeric receptor is formed by the non-signaling IL-2 receptor α-chain, the IL-2 receptor β-chain and the common cytokine receptor γ-chain. The binding of IL-2 to CD25 induces a conformational change which increases the IL-2 binding affinity for the β-chain. The β- and γ-chains, the signaling components of the receptor, combine with the initial IL-2-CD25 complex. After the cytokine engagement, the cytokine-receptor complex is internalized and degraded, except the CD25 subunit which is recycled back to the cell surface. A heterodimeric receptor consisting of the β- and γ-chains is also capable of binding IL-2 but only with intermediate affinity. In the presence of high IL-2 levels, CD8+ naive and memory T cells can be stimulated by the dimeric IL-2 receptor as well. Interestingly, the CD25 subunit, expressed on the surface of a DC, can upon binding of IL-2 provide trans-presentation of the cytokine for the dimeric IL-2 receptor on the T cell (Boyman and Sprent 2012).

After clonal expansion, activated T cells leave the lymph nodes and migrate into the inflamed tissues. There they launch their effector functions following a re-encounter with their specific target (Figure 1). APCs that are located in the tissues present Ags on their HLA class I and II molecules. Also, all nucleated cells of the body express HLA class I molecules and can therefore directly present intracellular Ags to CD8+ cytotoxic T cells. Depending on the T-cell subtype, T cells can either destroy the target carrying the specific structure, e.g. virus-infected cell (CD8+ T cells), or activate an army of other immune cells to fight against the intruder (CD4+ helper T cells). The Ab production of B cells, cytotoxic function of CD8+ T cells, further presentation of foreign antigens by APCs, and the activation of macrophages to ingest and eliminate microbes are dependent on CD4+ T cells. Their effector functions are mediated via cytokines and cell surface receptors (e.g. interferon (IFN)γ and CD40L, respectively).

Recombinant IL-2, aldesleukin, is used in the treatment of melanoma and renal cell carcinoma (Boyman and Sprent 2012). The goal is to promote T-cell and natural killer cell activation and proliferation in order to eliminate tumor cells. A severe side effect, a vascular leak syndrome, can be induced
when the medicine engages to the high-affinity IL-2 receptor on endothelial cells.

1.1.4 LIMITING THE T-CELL RESPONSE
Mechanisms for the restriction and termination of the active T-cell response are needed to avoid excessive reactions. Peripheral tolerance restrains also self-reactive T cells that have eluded thymic negative selection. Both inhibitory cosignaling receptors and regulatory T cells play a role here.

Tregs inhibit the functioning of conventional T cells, and several Treg types are known. CD4+CD25+Foxp3+ (Forkhead box p3) Tregs (Sakaguchi et al. 2007) represent a constant population in blood and are indispensable for the generation of self-tolerance (Sakaguchi et al. 1995). A total of 2-10% of CD4+ T cells are Foxp3+ Tregs (Seddiki et al. 2006, Liu et al. 2006). Regulatory T cells are discussed in more detail in the latter parts of this thesis.

Upon the termination of an immune response, apoptosis limits the number of responding T cells and hence the overall response activity during the T-cell contraction phase. Fas (also named CD95) -mediated activation-induced cell death, which can be triggered by persistent Ag stimulation together with IL-2 signaling, causes effector T cells to die in a controlled manner. The survival of effector T cells is also limited by reduced survival signaling offered by cytokines. The levels of cytokines and their receptors on T cells are strictly regulated to maintain T-cell homeostasis: the secretion of IL-2 is ceased by the end of the response and the receptor for IL-7, the most important homeostatic cytokine in resting state, is not expressed by effector T cells (Rochman et al. 2009, Boyman and Sprent 2012).

1.1.5 T-CELL MEMORY
A small share of activated T cells avoids apoptosis and remains in the body as memory T cells. They are in a resting state but provide rapid protection against recurrent infection by the same pathogen. Due to immunological memory, a resistance develops and upon secondary exposure the pathogen can often be destroyed before any symptoms occur.

Memory T cells are further divided into subsets according to their most distinctive functions. The cells in the subsets are, however, heterogeneous in expression of many cell surface proteins, and particular functions, like secretion of certain cytokines, are often overlapping between memory subsets. The most primitive subset, T memory stem cells (TSCM), represents a subpopulation with self-renewal ability and superior proliferation capacity upon stimulation (Gattinoni et al. 2011). TSCM are multipotent; they act as progenitors for central memory (TCM) and effector memory T cells (TEM), which offer more rapid functional potency. TSCM and TCM home to lymphoid tissues where they can be stimulated by their specific Ags if those are
presented by APCs. T_{CM} represent 'reactive memory' (Sallusto et al. 2004): they provide efficient proliferation, produce mainly IL-2 and smaller amounts of effector cytokines, and differentiate into effector T cells. Based on gene expression data and functional studies, the differences between T_{SCM} and T_{CM} appear small and mainly quantitative, the main divergence being the outstanding competence of T_{SCM} to proliferate. Notably, also T_{CM} are capable to self-renew (Busch et al. 2016). T_{EM} provide 'protective memory': they home into inflamed tissues where they are activated by encountering with Ags and subsequently launch their immediate effector functions. In comparison to T_{CM}, T_{EM} secrete more effector cytokines, like IFN\(_\gamma\) and tumor necrosis factor (TNF), instead of IL-2 (Farber et al. 2014).

The process for memory T-cell generation is not known but a progressive model is surmised. In that model, each less-differentiated T-cell subset acts as a precursor for the next one (naïve \(>\) T_{SCM} \(>\) T_{CM} \(>\) T_{EM} \(>\) T_{Eff}, (Farber et al. 2014)).

The efficacy of vaccination is based on immunological memory. The Ags administered via vaccination cause at most a mild immune response that still allows the generation of memory T and B cells. They patrol the body and can offer even life-long immunity.

### 1.2 ADOPTIVE T-CELL THERAPY

Cell therapy can be regarded as a next step in the development of medical treatments that so far have included traditional pharmaceuticals (small molecules), biopharmaceuticals (therapeutic proteins e.g. antibody), and medical devices (Mason et al. 2011). Cells used as medicine differ fundamentally from molecule-based medicines because they are living and thus able to react to, and be modified by, their changing surroundings (Salmikangas et al. 2015). Their long-term effects are difficult, even impossible, to test thoroughly in animal models and are thus not yet fully established. Therefore there must be a balance between the risks and benefits for the patient as well as a comparison to other treatment options. Today, cell therapy is predominantly used for severe diseases with an unmet medical need. Reflecting the unknown long-term safety profile, early stage clinical trials are conducted in target patients instead of healthy volunteers and thus, although primarily intended to show safety, also early signals for efficacy are sought.

Adoptive T-cell therapy is defined as the administration of \textit{ex vivo} processed T cells (Figure 2). It provides new treatment options for refractory or advanced cancer and infections (Maus et al. 2014) and for controlling immune tolerance (Riley et al. 2009). T cells are highly target-specific compared to current standard pharmaceuticals e.g. cytostatic drugs or immunosuppressants, and thus may cause fewer side effects. On the other hand, the extremely complex cellular interactions may lead to unexpected
adverse effects. Intravenous infusion is the usual route for cell administration but also local delivery near to the site of action can be used.

Figure 2  An illustration of the concept for adoptive T-cell therapy using gene-modified chimeric antigen receptor (CAR) T cells as an example. Depending on the T-cell product, particular steps can be omitted or modified. Figure from (Barrett et al. 2014b).

In this literature review, therapies utilizing only T cells of adaptive immunity are discussed, with innate T lymphocytes, such as natural killer T cells and γδ T cells, excluded.

1.2.1 REGULATORY T CELLS
Inappropriate immune reactions are encountered in allogeneic cell and organ transplantations as well as in autoimmune diseases. Tissue damage is caused particularly by T cells that specifically attack harmless targets either on transplanted allogeneic organs or cells (rejection), on tissues of the allogeneic stem cell recipient (attacked by the T cells in the graft, graft-versus-host disease, GVHD), or on body’s own tissues (autoimmunity, e.g. destruction of the insulin producing cells in type 1 diabetes, T1D). Current treatment options, such as life-long immunosuppressive medication and other immunomodulatory agents (e.g. thymoglobulin), are not specific but induce a general decline in immunity increasing the risk for infections and cancer. In addition, despite insulin replacement therapy, a major proportion of T1D patients suffer from serious secondary complications. Severe GVHD represents an unmet clinical need that may lead to death. In these situations,
regulatory T cells may offer a way toward long-lasting immunological tolerance.

1.2.1.1 Clinical experience: safety and efficacy

During the last seven years, about a dozen clinical trials have been conducted to test the safety and potency of Tregs for clinical therapy (Trzonkowski et al. 2015). General immune suppression, similar to current immunosuppressive regimens using traditional pharmaceuticals, and the possible induction of immune activation due to effector T cell impurities in the Treg product or unstable Treg identity, have been identified as potential concerns. Administering Tregs seems safe according to the results from patients with GVHD (90 patients in six centers, (Trzonkowski et al. 2009, Edinger and Hoffmann 2011, Brunstein et al. 2013, Martelli et al. 2014, Bacchetta et al. 2014, Theil et al. 2015)), autoimmunity (46 patients in three centers, T1D (Marek-Trzonkowska et al. 2014, Bluestone et al. 2015) and Crohn’s disease (Desreumaux et al. 2012)), and in liver transplantation (10 patients in a single study, (van der Net et al. 2016)). Though a malignant disease was developed in two GVHD patients (Theil et al. 2015) the causal connection to Tregs or to the immunosuppressants commonly in use is unclear. However, regarding general immune suppression, it is too early to draw conclusions about long-term safety, since the follow-up period for most patients has been at most one year (Trzonkowski et al. 2015), except for one study with the follow-up of seven years (Bacchetta et al. 2014). In many trials, the persistence of Tregs has been poor, thus not necessarily revealing the whole picture of the long-term safety.

Tregs have been used as both the treatment and prophylaxis for GVHD (Trzonkowski et al. 2015). The prophylactic approach, where Tregs are administered either at the time of HSC transplantation (Brunstein et al. 2011, Brunstein et al. 2013, Martelli et al. 2014) or a few months after (Edinger and Hoffmann 2011, Bacchetta et al. 2014), seems most promising. The rationale behind the procedure is two-part: i) immune suppression induced by Tregs directly hinders alloreactive GVHD-causing T cells, and ii) faster immune reconstitution due to tapering of immunosuppressive medication strengthens protection against infections and enhances the desired graft-versus-leukemia effect, thus also lowering the risk for relapse.

In autoimmune disorders, adoptive Tregs have shown early potency in patients with Crohn’s disease (Desreumaux et al. 2012) and T1D (Marek-Trzonkowska et al. 2014). Expectations are high since, in preclinical animal models, existing autoimmune diseases have not only been suppressed but reversed (Tang et al. 2004).

In summary, clinical data for adoptive Treg therapy is scarce and study protocols so variable, that the definitive proof of efficacy has yet to materialize.
1.2.1.2 Mechanisms of action

Regulatory T cell may be defined as a T cell exerting inhibitory function. However, two cell types are used in the clinic (Trzonkowski et al. 2015), CD4+CD25+Foxp3+ Tregs (Sakaguchi et al. 2007) and T regulatory type 1 cells (Tr1, (Vignali 2008)).

Foxp3+ Tregs differentiate into Tregs during maturation in the thymus. Alternatively, these cells are induced from conventional CD4+ T cells in response to TCR stimulation combined with Transforming growth factor β signaling (Th3, (Vignali 2008)). The transcription factor Foxp3 is imperative for the Treg generation in the thymus and their functional activity (Hori et al. 2003, Fontenot et al. 2003, Khattri et al. 2003).

Tr1 cells are conventional peripheral CD4+ T cells (non-Tregs) that are induced by IL-10 and have a suppressive function. With IL-10 secretion as their main mechanism, they typically do not express Foxp3 (Vignali 2008).

The main difference between thymic and peripheral Tregs (both Foxp3+and Tr1) is the source of Ags that their TCRs recognize: self-Ags for thymus derived Tregs and foreign Ags for peripherally derived Tregs. Suppressive function is not confined to the recognized Ag but, similarly to conventional T cells, Tregs need activating signals to execute their functions. Therefore the choice of polyclonal or Ag-specific Tregs may be an important factor determining clinical efficacy (Trzonkowski et al. 2015, van der Net et al. 2016).

Foxp3+ Tregs employ multiple immunosuppressive mechanisms, both cell contact dependent and mediated by soluble factors (Figure 3). Effector T cells are either directly inhibited by Tregs or indirectly via APCs. The particular mechanism(s) in use may depend on the context (anatomical location or disease), the Treg subtype, and the target cell characteristics. They may also be deployed sequentially (Vignali 2008). However, the CTLA4-related mechanisms seem to be shared by all Foxp3+ Tregs (Sakaguchi et al. 2009, Walker 2013).
1.2.1.3 CTLa4 in Treg function

The deletion of CTLa4 function leads to uncontrolled, detrimental T-cell proliferation in mice (Waterhouse et al. 1995). Effector T cells expressing CTLa4 were found to be inhibited by CTLa4 ligation (Walunas et al. 1994), revealing its cell-intrinsic role in the regulation of T-cell homeostasis (Bour-Jordan et al. 2011). CTLa4 was defined as a negative cosignaling receptor for T-cell activation. Due to the strong genetic association (Ueda et al. 2003, Haimila et al. 2004), the CTLa4 gene region is considered a general autoimmune susceptibility region (Gough et al. 2005). Autoimmune-related functional and numerical Treg impairment (Dejaco et al. 2006) and the constitutive, Foxp3-controlled CTLa4 expression in Tregs (Miyara et al. 2009, Sakaguchi et al. 2009) led to the understanding of the key role CTLa4 plays in Tregs (Walker 2013). The phenotype of Treg-specific CTLa4 deletion that is characterized by a broad immune dysregulation is similar to Foxp3-defective mice and humans (Brunkow et al. 2001, Bennett et al. 2001) and points to the essential function of CTLa4 in sustaining self-tolerance (Wing et al. 2008).

The cell-extrinsic CTLa4 function means that immune-controlled T cells do not have to express CTLa4 themselves (Bour-Jordan et al. 2011). Several
detailed mechanisms for CTLA4-mediated inhibition in Tregs have been proposed (Figure 4, (Sakaguchi et al. 2009)). First, CTLA4 can outcompete the activating CD28 for the cosignaling receptor ligands, CD80 and CD86. This mechanism may still mainly work in cell-intrinsic fashion on effector T cells. Second, in order to abolish the activation of T cells, CTLA4 on Tregs directly removes these ligands from the surface of APCs by trans-endocytosis (Qureshi et al. 2011). Third, CTLA4-provoked events in the APC induce indoleamine 2,3-dioxygenase (IDO), a tryptophan-depleting enzyme that engenders immune restraint (Fallarino et al. 2003, Grohmann et al. 2003, Cribbs et al. 2014). The depletion of the essential amino acid tryptophan and the action of its proapoptotic metabolites, kynurenines, mediate the inhibition.

The sCTLA4, an alternatively spliced isoform (Magistrelli et al. 1999), also has the ability to engage with CD80 and CD86 (Oaks et al. 2000). In a diabetogenic mouse model, the expression of CD86 on the APC surface was downregulated by Treg-secreted sCTLA4 (Gerold et al. 2011). The inhibitory function of mouse and human Tregs is diminished by specific elimination of sCTLA4 and in murine models, this leads to autoimmunity and reduced tumor control (Gerold et al. 2011, Ward et al. 2013).

Figure 4  CTLA4-mediated inhibitory functions and IL-2-related effects of Foxp3+ Tregs.
Figure from (Sakaguchi et al. 2009).

1.2.1.4 IL-2 and Tregs

Shimon Sakaguchi originally revealed the existence of Foxp3+ Tregs by demonstrating that constitutively CD25-positive CD4+ T cells are indispensable for the generation of normal self-tolerance (Sakaguchi et al. 1995). Only later, Foxp3 was found to be a better Treg marker, although still
not exclusive (Hori et al. 2003). CD25, the IL-2 receptor α-chain, is one of the three IL-2 receptor components, which together form the high-affinity IL-2 receptor. The α-chain provides high-affinity binding for the receptor upon cytokine engagement. The other receptor chains, the IL-2 receptor β-chain and the common cytokine receptor γ-chain, which are expressed by Tregs as well, are responsible for the signal transduction (Boyman and Sprent 2012). In Tregs, the transcription of CD25 and the repressed expression for IL-2 are directly controlled by Foxp3 (Figure 4, (Sakaguchi et al. 2009)). Signaling through the IL-2 receptor is crucial for the survival of Tregs and a deficiency of IL-2 or CD25 disrupts self-tolerance (Malek and Bayer 2004). Because of their high dependency on IL-2, Tregs act as IL-2 sinks. Therefore, also CD25 contributes to the T-cell suppression by limiting IL-2 availability. Besides, signaling through the IL-2 receptor boosted Treg function via STAT5 activation (Chinen et al. 2016).

Interestingly, Tregs simultaneously represent both an anergic (no/low IL-2 production in response to Ag) and in terms of conventional T cells, an activated phenotype (e.g. expression of CD25, CTLA4, and Foxp3 and repressed expression of CD127, the IL-7 receptor). Epigenetic control by demethylation of the Foxp3 gene plays a key role in the stable and constitutive expression of these downstream genes (Floess et al. 2007).

1.2.1.5 Treg production

Blood has normally been used as starting material for Treg generation, except for one study that utilized cord blood derived Tregs (Brunstein et al. 2011). The donor of the starting material depends on the treatment indication. The patient’s own autologous blood has been used in the cases of autoimmunity and organ transplantation while blood from the original donor for the HSC transplantation has been used in a GVHD setting. Tregs that have been tested in patients can be roughly categorized into four groups based on their method of production (Trzonkowski et al. 2015):

i. fresh polyclonal Foxp3+ Tregs that are administered directly after enrichment without further in vitro expansion,
ii. expanded polyclonal Foxp3+ Tregs,
iii. alloantigen-specific Foxp3+ Tregs, and
iv. polyclonal or Ag-specific Tr1 cells (Table 2).

Clinical data from the trials using alloantigen-specific Foxp3+ Tregs (iii) have yet to be published.

In general, methods for the production of polyclonal Tregs are simpler than for Ag-specific Treg cells (Tang and Bluestone 2013, Putnam et al. 2013, Trzonkowski et al. 2015, van der Net et al. 2016). Also, the Ag-specific protocol carries the risk for cellular impurities, in forms of allogeneic APCs, with potentially harmful effects. Often at least two rounds of activation are needed for sufficient Treg expansion (Putnam et al. 2009, van der Net et al.
The total processing time for the expanded products varies from two up to eight weeks. The anticipated Treg cell numbers needed for treatment might be higher in the polyclonal setting due to the weaker activation stimulus provided \textit{in vivo} (Tang and Bluestone 2013). The numbers of fresh Tregs directly enriched from blood (Edinger and Hoffmann 2011, Martelli et al. 2014) are considered insufficient for effective clinical therapy for most applications (Riley et al. 2009, Edinger and Hoffmann 2011, Tang and Bluestone 2013).

### Table 2 Technical steps for different Treg production methods.

<table>
<thead>
<tr>
<th>Technical step</th>
<th>fresh Tregs</th>
<th>polyclonal Tregs</th>
<th>alloAg-specific Tregs</th>
<th>Tr1</th>
</tr>
</thead>
<tbody>
<tr>
<td>starting population</td>
<td>CD4⁺CD25⁺</td>
<td>↓</td>
<td>MNC</td>
<td></td>
</tr>
</tbody>
</table>
| → methods for selection | • CD8⁺ elimination → CD25⁺ enrichment (MACS)  
 |                       | • CD4⁺CD25⁺CD127⁻/low (FACS)  |           |     |
| activation           | CD3/CD28 beads | allo APCs  | APC/feeder* & IL-10 ± Ag # |     |
| expansion            | CD3/CD28 beads |                 |                       |     |

* autologous or allogeneic depending on the setting  
^ not always used for alloAg-specific Tregs  
# if Ag-specific Tregs are produced (Desreumaux et al. 2012)  
FACS = fluorescence-activated cell sorting, MACS = magnetic cell sorting, MNC = mononuclear cells.

#### 1.2.2 ANTIGEN-SPECIFIC EFFECTOR T CELLS

The TCR repertoire of mature T cells has a theoretical potential to recognize all imaginable peptide fragments that are not of normal self-origin, naturally including pathogenic peptides as well. Thus, T-cell reactivity against some transformed self-derived proteins exists as well, enabling tumor cell immunosurveillance (Dunn et al. 2002).

In addition to pathogen-specific T cells and tumor-infiltrating lymphocytes (TILs) discussed in detail below, the generation of tumor Ag specific T-cell clones derived from blood represents a less frequently used approach (Hunder et al. 2008).
PATHOGEN-SPECIFIC T CELLS

After hematopoietic stem cell transplantation, slow immune reconstitution leaves the patient susceptible to normally harmless infections. These infections are one of the main causes of transplant-related mortality. Current anti-viral medicines have a limited applicability and efficacy in this severely ill and specific patient population. Patients may suffer viral and fungal infections, and T cells specific for these pathogen types could be used for therapy. In the course of 20 years, Epstein-Barr virus (EBV), cytomegalovirus (CMV), and adenovirus have been the most frequent targets (Saglio et al. 2014). While Aspergillus-specific T cells would fulfil an unmet clinical need (Papadopoulou et al. 2016), only one clinical study has been published so far (Perruccio et al. 2005).

The original donor for the HSC transplantation is used as the source for the pathogen-specific T cells. A more recent approach is to use third-party donors allowing banking of virus-specific T-cell products. In this setting, GVHD avoidance requires either careful matching for the tissue types or strict selection for the virus-specific T-cell lines. Previous exposure to the specific pathogen is required for the donor to be eligible. Blood-derived cells are either activated with pathogenic peptides, with responding cells selected based on their IFNγ secretion, or alternatively they may be selected directly through the binding of their TCR to tetramers mimicking HLA-Ag peptide complexes. The processing takes one day, making the T-cell products rapidly available (Saglio et al. 2014).

Protective immunity against Aspergillus is mediated through CD4+ Th1-type T cells and their cytokines (IFNγ and TNF). In contrast, for the most part, CD8+ T cells are pursued for viral infections owing to their direct cytotoxic functions against infected cells.

Worldwide, hundreds of patients in phase I trials have received pathogen-specific T cells after allogeneic HSC transplantation either as prophylaxis or for treatment. Occurrence of GVHD in CMV-targeting therapy is a potential concern and wider clinical benefit still remains uncertain. EBV therapy is safe and evidence for its clinical efficacy is strong (Saglio et al. 2014).

A novel endeavor is to use autologous virus-specific T-cells against malignancies that carry viral Ags but the efficacy of this concept has not yet been proven in the clinic (Schuessler et al. 2014).

TUMOR-INFILTRATING LYMPHOCYTES

The hypothesis that immune system could inhibit or prevent tumor development was presented in 1909 by Paul Ehrlich and then by Burnet and Thomas in 1957 through the concept of lymphocyte tumor immunosurveillance (Dunn et al. 2002). Indeed, the number of lymphocytes that naturally infiltrate into the tumor tissue in melanoma and several other types of solid cancer correlates with longer patient survival (Dunn et al.
2002). Thus, at least some of the infiltrated lymphocytes are expected to specifically recognize and destroy tumor cells.

The first clinical study of adoptively transferred TILs was published in 1988, reporting a significant but brief treatment response (Rosenberg et al. 1988). Since then patient preconditioning and IL-2 administration for the induction of a long-term response have been conceived as parts of TIL therapy. Early-stage TIL trials in metastatic melanoma, in which altogether over 300 patients were treated, showed reproducible response rates between 20 to 72%, with a mean of ~50% (Besser et al. 2015). TILs are often used as salvage therapy when the disease is refractory to other treatments. Considering the patients’ clinical status, and the up to 10 year disease-free survival times seen in some of the patients (Rosenberg et al. 2011), the efficacy is impressive. Administration of TILs as such seems safe but severe toxicities, induced by the lymphodepletive preconditioning and high-dose IL-2 administration after TILs, can be expected. On-target but off-tumor autoimmunity occurs as a direct adverse effect of TILs when the TILs target tumor-associated, but otherwise normal self-Ags, such as the differentiation Ag Melanoma antigen recognized by T-cells 1 (MART-1, (Dudley et al. 2002)). The autoimmunity detected against melanocytes in eyes, ears and skin has, however, been reported to be transient.

The clinical success of TILs has been confined to melanoma, although the treatment of other solid cancers has been actively explored for two decades (Besser et al. 2015). In melanoma, the three key determinants for successful TIL therapy are fulfilled: safe access to the patient’s tumor tissue, active T-cell infiltration into the tumor, and high mutation rate in the tumor cells (Lawrence et al. 2013). Mutations increase tumor immunogenicity by providing novel, tumor-specific antigenic peptides, i.e. neoantigens. A novel approach is to utilize the viral specificity of TILs against virus-induced cancers, such as papilloma-associated malignancies (Stevanovic et al. 2015).

Generation of TILs for adoptive therapy starts with a tumor biopsy. Tissue fragments are cultured in vitro until cells that had infiltrated into the tumor outgrow from the tumor cell mass. Either standard or so-called ‘young’ TILs can be generated (Tran et al. 2008). ‘Young’ TILs are cultured at this stage for 10-18 days, and all separate lymphocyte cultures with different Ag-specificities are pooled. The standard TIL protocol takes additional 10-18 days during which expansion and selection of tumor-reactive clones is performed. Finally, both TIL types are further expanded for two weeks by a rapid expansion protocol (REP). In summary, TILs are autologous, heterogeneous lymphocytes of intratumoral origin that are expanded in vitro for five to seven weeks (Besser et al. 2015).

The direct killing activity of CD8⁺ T cells due to the TCR-specific recognition of tumor Ags is the apparent functional mechanism of TILs. However, the depletion of CD4⁺ T cells from the TIL products may weaken therapeutic potency (Dudley et al. 2013). Furthermore, a patient with advanced gastrointestinal carcinoma was treated solely with CD4⁺ TILs...
specifically recognizing a neoantigenic mutation in the receptor tyrosine-protein kinase ERBB2 interacting protein (ERBB2IP, (Tran et al. 2014)). Transferred CD4+ cells produced Th1 cytokines, IFNγ, TNF, and IL-2, and expressed cytotoxic activity, leading to long-lasting tumor regression.

1.2.3 GENE-MODIFIED T CELLS

One restriction of the TIL approach is the lack of tumor immunogenicity. First, most tumor-associated Ags are normal self-Ags. T cells with TCRs that strongly recognize self-Ags are deleted in the thymus during T-cell maturation. Thus, many tumors do not bear immunogenic proteins. Second, tumor cells gain advantage by impairing intracellular Ag processing and/or HLA I presentation, thus avoiding T-cell recognition. Genetic instability in rapidly dividing tumor cells offers an opportunity for such development. Also the immunosuppressive tumor microenvironment limits Ag presentation. Third, these immunosuppressive soluble factors and cells inhibit the maturation and function of APCs (Kaufman and Disis 2004). As a result, in many tumor biopsies effective TILs are difficult to find.

Genetic engineering of cells provides tools for synthetic reconstruction of T cells with desired Ag-specificity. The gene fragments or constructs can be introduced into cells with several methods (Maus et al. 2014): with chromosome-integrating viral vectors (gammaretroviral, lentiviral), with transposons for permanent gene transfer, or with non-integrating methods for transient expression of the gene (adenoviral vectors, RNA transfection). A risk for the induction of malignant transformation through replication-competent virus and insertional mutagenesis is among the major safety concerns linked to therapies using genetic modifications. This risk has been addressed by modern vector technology with the split-genome design, which, by deleting required elements from the vectors, eliminates the potential for replication, and with self-inactivating vectors enabling the vector genome activation (Bear et al. 2012, Schambach et al. 2013).

T-CELL RECEPTOR (TCR) –ENGINEERED T CELLS

The molecular characterization of tumor-recognizing TCRs on TILs and the advances in gene therapy allowed in vitro engineering of therapeutic cells. Any particular TCR with a known specificity can be inserted into T cells derived from the patient’s blood, regardless of the T cells’ original specificities. This genetic engineering process redirects naturally existing T cells against a new, desired target. Consequently, availability of tumor-specific T cells, a critical aspect in the TIL therapies, could be achieved. Thus, TCR-engineered T cells, also called TCR-modified T cells, are being developed against various malignant disorders (Ikeda 2016).

A MART-1 specific TCR identified in TILs from a melanoma patient was used in the first TCR-engineered clinical study (Morgan et al. 2006). The
TCR sequence was incorporated into T cells of metastatic melanoma patients using a gammaretroviral vector. With a response rate of 13%, only modest clinical efficacy was attained and no adverse reactions were detected. The clinical response rate was increased to 30% in another set of patients using a high-affinity TCR for the same Ag (Johnson et al. 2009). This time, autoimmunity that was similar to but more severe than with MART-1 targeting TILs was seen.

Naturally existing TCRs against tumor-associated self-Ags (as in many TILs) commonly bind their targets with only low to intermediate affinity. To achieve better clinical outcomes with TCR-engineered T cells, their affinities have been frequently enhanced either through genetic modification of the original TCR sequence or by immunizing mice (i.e. using mouse-derived TCR sequence). However, use of such affinity-enhanced TCRs significantly increases the risk for adverse effects, since such reactivity has never existed in humans (Ikeda 2016).

Although the goal is to broaden the applicability of adoptive T-cell therapy to other cancer types, in the clinical trials using TCR-engineered T cells >60% (n=88) of patients have suffered from melanoma (Ikeda 2016). Among the twelve early-stage clinical trials, hematologic, breast, colorectal, esophageal, and synovial cell cancers have been the other targets (Maus et al. 2014, Ikeda 2016). Results from these trials show response rates between 11% and 80% (mean ~40%). Very encouraging results (80% response rate, 70% with complete response) were obtained in tumor antigen NY-ESO-1 -targeted myeloma study conducted along with autologous HSC transplantation (Rapoport et al. 2015). The expected response rate with the standard care utilizing autologous HSC transplantation alone is less than 40%. The use of TCR-engineered cells was safe, and GVHD encountered in patients was shown to be caused by the standard autologous HSC transplantation.

Severe adverse effects including deaths have occurred with TCR-engineered T-cell therapies. In contrast to TIL treatment, these side effects have arisen directly from the adoptively transferred T cells – and their TCR specificity. Affinity-enhanced TCR recognizing melanoma-associated antigen MAGE-3 caused cardiac toxicity and two patients died (Linette et al. 2013). Engineered T cells cross-reacted with a peptide derived from an unrelated titin protein that is expressed in the cardiac muscle (Linette et al. 2013, Cameron et al. 2013). This off-target reaction was induced by a titin-derived peptide with limited sequence similarity to MAGE-3 (5 of 9 amino acids). Two more deaths occurred using another type of affinity-enhanced MAGE-3 reactive T cells (Morgan et al. 2013). Neurological toxicity was caused by on-target but off-tumor T-cell reactivity due to unforeseen MAGE expression in the brain.

TCR cross-reactivity appears completely physiologically in nature (Mason 1998). It may cause potential off-target risks in T-cell therapies. Normal tissue expression of targeted Ags may cause on-target off-tumor risks. The
development of TCR-engineered T cells requires extensive, complicated, and laborious preclinical testing for the prediction of concomitant side effects (Linette et al. 2013, Cameron et al. 2013, Morgan et al. 2013, Ikeda 2016). Unfortunately, preclinical animal models are not useful (Maus et al. 2014). Thus, several types of safety switches have been developed and introduced into the engineered cells in order to eliminate rapidly the transferred cells if severe or uncontrolled adverse reactions occur (Jackson et al. 2016). Even so, careful anticipation of the risks is a prerequisite when novel TCR-engineered T-cell types are clinically tested.

A shortcoming of TCR-engineered T-cell therapy is its HLA-restricted nature. When T cells recognize their specific antigenic peptide through TCR binding, they simultaneously bind to the HLA molecule presenting the peptide. This binding to the HLA molecule is also TCR specific, meaning that a particular T cell can only recognize the specific Ag in combination with a particular HLA type. Therefore, the HLA type recognized by the TCR to be used needs to be known - and the recipient of the cells must carry this HLA type. Fortunately, extensive HLA matching covering also other HLA loci, required in, for example, allogeneic cell or organ transfer, is not needed. Most of the TCRs utilized recognize HLA-A2 molecules, which are found in ~50% of Caucasians (Ikeda 2016).

In protocols for the production of TCR-engineered T cells, peripheral blood is used as starting material (usually obtained by apheresis), T cells are activated with an anti-CD3 antibody that binds to the endogenous TCR complex, and viral vectors are used for the permanent insertion of the additional TCR gene into the chromosomal DNA. In vitro culture of the cells takes 7 to 10 days. One center has used an additional 14 day REP protocol for increasing the cell yield (e.g. (Morgan et al. 2013)).
**Table 3** Comparison of TILs, TCR-engineered T cells, and CAR T cells.

<table>
<thead>
<tr>
<th></th>
<th>TILs</th>
<th>TCR-engineered T cells</th>
<th>CAR T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>source for starting material</td>
<td>tumor biopsy</td>
<td>blood</td>
<td>blood</td>
</tr>
<tr>
<td>differentiation status and number of cells prior expansion</td>
<td>Ag-primed, exhausted? few</td>
<td>mixed, adequate?</td>
<td>mixed, adequate?</td>
</tr>
<tr>
<td>genetic modification</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>restrictions of target Ag</td>
<td>natural tumor-Ags</td>
<td>intracellular, secreted, cell surface Ags</td>
<td>cell surface Ags</td>
</tr>
<tr>
<td>requirement for natural Ag presentation</td>
<td>needed</td>
<td>needed, HLA-restriction limits patient eligibility</td>
<td>independent</td>
</tr>
<tr>
<td>safety in regard to target specificity</td>
<td>least risky</td>
<td>most risky</td>
<td>small risk</td>
</tr>
</tbody>
</table>

**CHIMERIC ANTIGEN RECEPTOR T CELLS**

The main obstacle for overcoming cancer with immunotherapies is the immunosuppressive microenvironment created by the tumor. As a result, antigen presentation is inhibited (Kaufman and Disis 2004, Munn and Bronte 2016). Both TILs and TCR-engineered T cells are dependent on Ag presentation carried out either directly by tumor cells or APCs (Table 3).

Gene technology also allowed the utilization of non-T-cell derived Ag-binding receptors in T cells. Usually Ag-specificity is achieved by exploiting antibodies but also protein ligands can be used (Brown et al. 2015, Jackson et al. 2016). In contrast to TCRs recognizing processed linear peptides, B cells secrete antibodies that specifically recognize unprocessed, three-dimensional structures. T cells can be activated through antibody-based Ag recognition by connecting antibody segments with the CD3ζ domain from the TCR complex, a signaling domain that normally delivers the signal into the T cell ((Gross et al. 1989), Figure 5). Thus, T cells can be specifically activated by cell surface molecules without Ag processing and presentation or HLA restrictions. The requirement for Ag presentation for adoptive T-cell therapies is thus abrogated. These synthetic receptors were first named T-bodies and later chimeric antigen receptors (CARs).
3.1.2.3.1 Safety

Although cancer is the obvious target for CAR T-cell therapy, persistent infections could be treated with it as well. In fact, first clinical CAR T cell trial was against human immunodeficiency virus (HIV) infection (Mitsuyasu et al. 2000, Deeks et al. 2002). CARs in these trials did not use antibody-based recognition; instead the natural HIV envelope receptor CD4 was used as the extracellular, Ag-binding part. It was connected to the CD3ζ domain and the idea was to eliminate HIV envelope expressing, i.e. HIV infected T cells. Only a modest effect on viremia was observed. Importantly, long-term safety of gene-modified T-cell therapy was demonstrated in these trials (Scholler et al. 2012). During a follow-up period of 11 years in 43 patients, retroviral transgene integration into the genome did not lead to transformation or clonal expansion and did not favor integration near genes controlling cell growth. This data reveals that cell transformation due to genome modification induced insertional mutagenesis is not shared by all cell therapies involving gene modification. Susceptibility for transformation can depend on the transgene or cell type, with HSCs possibly presenting a higher risk (Newrzela et al. 2008, Schambach et al. 2013).
CARs are cellular synthetic receptors that are not found in nature. Two potential safety risks regarding the receptor structure may arise: The CAR itself can be recognized as foreign by the body’s natural immune cells (immunity against transferred CAR T cells) or the CAR, similarly to the affinity-enhanced TCR in TCR-engineered T cells, can recognize unintended structures (off-target immunity i.e. toxicity). However, compared to TCRs the target specificity of the antibodies is high and cross-reactivity less likely (Harris and Kranz 2016). So far, off-target reactivity has not been reported.

The antibody segments in CARs are often derived from mouse Abs. Usually both heavy and light chain single-chain variable fragments (scFv) from the murine antibody are used (Dotti et al. 2014). Mouse-derived CARs, as well as TCRs, are able to induce the production of human anti-mouse antibodies (HAMA, (Lamers et al. 2006, Till et al. 2008, Davis et al. 2010)). These IgG class antibodies have not caused harm in patients. Instead, repeat dosing of mesothelin-targeted CAR T cells induced IgE HAMA generation and caused anaphylaxis and death in one case (Maus et al. 2013). On-target off-tumor reactions were anticipated due to mesothelin expression on normal tissues and therefore transient CAR expression was used as a safety procedure. Repeated dosing of CAR T cells was employed in order to prolong the treatment time. Mesothelin targeting was shown to be safe but repeated dosing of cells bearing mouse-derived components is now considered to be a potential safety concern. Murine-derived scFv domains in CARs can be humanized, which may also improve clinical efficacy hindered by HAMAs (Lamers et al. 2006, Davis et al. 2010) or cell-mediated immunity (Lamers et al. 2006, Turtle et al. 2016a, Turtle et al. 2016b).

Since only a few tumor Ags are selectively expressed on malignant cells, on-target off-tumor toxicity is a main concern when developing CARs against new targets. Although CARs do not harness CD3 receptor complex signaling in its entirety, they are effective molecules requiring only ~50 target molecules per target cell to react (Maus et al. 2014). TCRs can respond against only 1-10 target Ags. Therefore, a very low level of Ag expression in normal tissues can be sufficient for the response activation: Carbonic anhydrase IX expression in the biliary tract triggered CAR-induced hepatitis in patients treated for renal cell carcinoma (Lamers et al. 2006) and ERBB2 expression on lung epithelial cells led to the death of a patient treated for advanced colon cancer (Morgan et al. 2010). ERBB2 (also known as HER2) targeted immunotherapy using exactly the same regulatory-approved monoclonal antibody, trastuzumab, as well as utilizing CAR T cells with another scFv (Ahmed et al. 2015) has been safe. The most likely explanation for the unfortunate death was the huge cell dose given (10^{10}, typical adult CAR T-cell dosing <5x10^{8} corresponding to <5x10^{6}/kg, (Jackson et al. 2016, Turtle et al. 2016c)), which resulted in massive CAR T-cell infiltration into the lungs in 15 minutes.

In summary, the complexity of CAR T-cell therapy may carry risks and clinical application must be approached with caution.
1.2.3.2 Clinical efficacy
Clinical efficacy with first generation (1G) CARs, which only carried the CD3ζ signaling domain, was modest. Mimicking nature, the addition of a signaling domain from the CD28 T-cell cosignaling receptor into the CAR improved the efficiency (Figure 5). These second generation (2G) CAR T cells expand better in vivo after transfusion and exhibit longer persistence (Savoldo et al. 2011). CARs with signaling domains from other cosignaling receptors, such as 41BB, OX40, and ICOS, have also been developed. ICOS signaling in CAR T cells favored a Th17 type response (Guedan et al. 2014). Furthermore, third generation (3G) CARs containing the CD3ζ domain and two cosignaling domains, most often CD28 and 41BB, are used. Comparisons between different cosignaling domains in 2G CARs (Milone et al. 2009, Frigault et al. 2015, Kawalekar et al. 2016) or between 2G and 3G CARs (Milone et al. 2009, Karlsson et al. 2015) have shown some functional differences but their impact on clinical performance is not known.

Data for clinical efficacy of CAR T cells in solid cancers is still limited although dozens of trials are under way (n=60, www.clinicaltrials.gov). The results that are available have evoked cautious optimism (Jackson et al. 2016): GD2-CAR for neuroblastoma (Louis et al. 2011), PSMA-CAR for prostate cancer, mesothelin-CAR for malignant pleural mesothelioma (Maus et al. 2013, Beatty et al. 2014), CEA-CAR for adenocarcinoma liver metastases, HER2-CAR for glioblastoma (Ahmed et al. 2015), and IL-13-CAR for glioma (Brown et al. 2015). Strategies for increasing the clinical efficacy include the local administration of CAR T cells, bigger cell doses, and the breaking down of the immunosuppressive tumor microenvironment.

1.2.3.3 Clinical use of CD19-targeted CAR T cells
Hematologic malignancies offer a feasible target for novel immunotherapies for two reasons. First, the tumor-induced immunosuppressive microenvironment is not as big an obstacle as in solid tumors. Second, hematologic cell types have lineage specific surface markers and thus the on-target off-tumor toxicities are more predictable. Indeed, the biggest breakthrough in the field of advanced cell therapy has been the CD19-targeted CAR T cells against refractory B-cell malignancies. Phase I and II trials conducted in different centers consistently produce astonishing complete response rates between 51 to 93% in acute lymphoblastic leukemia (ALL, altogether 165 patients in four centers, (Davila et al. 2014, Maude et al. 2014, Lee et al. 2015, Turtle et al. 2016a, Turtle et al. 2016c)) along with encouraging responses in chronic lymphocytic leukemia (CLL, altogether 31 patients in four centers with overall response rates up to 89%, (Brentjens et al. 2011, Kochenderfer et al. 2012, Turtle et al. 2016c)) and non-Hodgkin lymphoma (NHL, altogether 69 patients in four centers with overall response rates up to 68%, (Savoldo et al. 2011, Kochenderfer et al. 2015, Turtle et al.
A multicenter phase II trial for pediatric ALL is ongoing and shows consistent clinical results (Maude et al. 2016). Currently, there are 121 ongoing CAR T-cell trials for lymphoma or leukemia worldwide (www.clinicaltrial.gov).

Adverse effects for CD19-targeted CAR T cells can be significant but in the great majority of cases manageable (Turtle et al. 2016c). The expected on-target off-tumor consequence is the depletion of normal B cells. This B cell aplasia can be long-term depending on the persistence of transferred CAR T cells. It predisposes the patient to infections through the lack of antibodies but can be managed with monthly Ig infusions. Two other toxic effects, cytokine release syndrome (CRS) and neurological toxicity, are encountered in all clinical trials that show efficacy and may be related to the tumor burden. CRS and neurotoxicity are also experienced with blinatumomab, a bispecific anti-CD3–anti-CD19 mAb that activates endogenous T cells while simultaneously binding to target B cells. Blinatumomab can also be used for leukemia. CRS and neurologic effects are usually transient but may require intensive care. The pathophysiology of neurotoxicity is not understood. On the other hand, CRS is induced by cytokines, particularly IL-6, which are released in response to immunological danger signals originating from damaged tumor cells after CAR T-cell attack. These toxicities can be treated with an anti-IL6R mAb, tocilizumab. Corticosteroids, which inhibit CAR T cell function as well, are used in the most severe cases. Still, two patients treated for lymphoma have died of severe CRS and neurotoxicity (Turtle et al. 2016b). In this trial, cell dose at $2 \times 10^6$/kg was determined as the maximum dose tolerated, with $20 \times 10^6$/kg being excessively toxic. In CD19-targeted CAR therapy, only one more contingently CAR T-cell-related but undecipherable death has occurred (Kochenderfer et al. 2015).

Hope of a permanent cure for formerly refractory cancer with CAR T cells has arisen from the long-lasting remission attained in some of the patients treated against CD19+ malignancies. In one clinical center, the rate of disease-free survival at 1 year among the complete responders in ALL was 44% (Maude et al. 2014, Turtle et al. 2016c). Two thirds of the relapses were due to CD19- escape mutants. However, about dozen patients with ALL, CLL, and NHL have now remained in remission for several years (Maude et al. 2014, Turtle et al. 2016b, Turtle et al. 2016c) indicating that a cure may be within reach.

**1.2.3.4 CAR T-cell generation**

CAR T cell production follows the steps that are also used for TCR-engineered T cells: blood T cells activated with anti-CD3 Ab are transduced, usually, with a viral vector carrying the CAR sequence and expanded for one to two weeks. Again, the direct mechanism for tumor cell elimination is achieved by CD8+ T cell cytotoxicity, even though a mix of both CD4+ and CD8+ T cells is generated.
A major drawback hampering the wider use of CAR T-cell therapy results from the normal antibody function: only structures that are exposed outside the cell membrane can be targeted. Unfortunately, many of the useful tumor Ags are intracellular. A novel approach to circumvent the need for cell surface expression is to use scFvs derived from antibodies that recognize tumor Ag-derived peptides upon HLA presentation (Liu et al. 2016). However, Ag presentation by tumor cells is needed and thus one of the greatest advantages of CARs is lost.

Genetic engineering has introduced further options for improving T-cell therapies and several discoveries are currently being tested in the clinic as well (reviewed in (Jackson et al. 2016)). Both efficacy and safety are in focus, and CAR T cells bearing, for example, additional synthetic receptors for chemokines to improve homing, or engineered to produce cytokines that activate bystander immunity (IL-12) are being developed, in addition to safety switches. Dual-specific CAR T cells are tested for increased specificity and to decrease the risk for escape mutants.

The factors that determine clinical efficacy can depend on the treatment protocol or the T-cell product and once known could be applied more comprehensively.

1.3 **IN VITRO T-CELL EXPANSION**

T cell numbers that can safely be collected from tumor biopsies or blood are considered insufficient for clinical efficacy, with pathogen-specific T cells being a prominent exception. Multiplication of the cell number, i.e. expansion, is thus a common part of most adoptive T-cell modalities. *In vitro* T-cell expansion can also provide other benefits, such as improving the purity of desired cell type or functionality due to cell activation. Conversely, in case of nonphysiologic cell manipulation, expansion evokes safety issues.

T-cell biology is the foundation for all T-cell expansion technologies, with cell culturing techniques providing the framework. *In vivo*, T-cell proliferation takes place during an immune reaction. Tregs, too, proliferate upon immune response but later in order to limit the activation (Tang and Bluestone 2013). Therefore, T-cell expansion *in vitro*, for both conventional and regulatory T cells, is based on T-cell activation. All three physiological signaling events, TCR-CD3 mediated Ag-recognition (first signal), cosignal through CD28 (second signal), and the IL-2 -delivered signal (third signal), are typically included in the clonal expansion-based *in vitro* proliferation.

The technical solutions are either based on soluble anti-CD3 antibodies with or without anti-CD28 or the use of these antibodies immobilized on beads (Maus et al. 2014). Formerly, APCs were used but, in addition to the improved control and safety, CD28-specific Abs bypass the possible interference of CTLA4 engagement that would result in T-cell inhibition. However, cord blood Tregs were recently expanded with artificial APCs, a
Review of the literature

transduced cell line providing the CD3 signal via anti-CD3 Ab and the cosignal via CD86 (McKenna et al. 2017). Cellular expansion was more efficient compared to beads exploiting solely CD28 binding. Perhaps cells were providing stronger CD28 stimulus, which is known to be required for in vitro expansion of Tregs (Hombach et al. 2007). APCs are also utilized in the production of Ag-specific Tregs in order to enrich the desired T-cell clones (Putnam et al. 2013). In case of the oligo-clonal alloreactive Tregs, no substitute for APCs exists. However, if the specific autoantigen driving the autoimmune disorder was known, APC-based methods could be replaced either with HLA-tetramer systems (Neudorfer et al. 2007) or even with genetic engineering (Noyan et al. 2016). Traditionally, in the REP phase of TIL production, soluble anti-CD3 antibody together with autologous feeder cells are used (Dudley and Rosenberg 2003). Recently, anti-CD3/CD28 beads have been introduced (Baldan et al. 2015).

As mentioned before, there has not been a firm consensus on the exploitation of CD28 in effector T-cell expansion. Several facts in basic biology point to benefits received through cosignaling. Naïve T cells, the most abundant subset in methods starting from blood, cannot be activated without CD28. The lack of this non-redundant cosignal drives them to anergy (Schwartz 1990) or can lead to Treg differentiation (Wraith 2016). Also, the full activation of Ag-experienced T cells benefits from CD28 signaling. Finally, CD28 stimulation maintains telomere length (Weng et al. 1996). Combined, these advantageous effects produce higher cell numbers for treatment, increase potency, and prolong performance time.

The anergic state of Treg cells complicates their expansion. In addition to intense CD28 signaling, high IL-2 concentration helps to break the anergy (Hoffmann et al. 2004, Hombach et al. 2007, Putnam et al. 2009). In addition, IL-2 is needed for the survival of Tregs and the maintenance of their function (Malek and Bayer 2004, Nelson 2004).

IL-2 is called the T-cell growth factor because of its central function in clonal expansion. Thus, IL-2 supplementation is used extensively for in vitro T-cell expansion. However, ample proliferation eventually leads to lower potency owing to expression of inhibitory molecules (e.g. CTLA4, PD-1), cell exhaustion, or senescence (Shen et al. 2007). Continuous activating signals may cause activation-induced cell death. Furthermore, IL-2 itself drives T cells toward effector differentiation (D'Souza and Lefrancois 2003, Yu et al. 2003), with both IL-2 signaling strength and duration affecting CD8+ memory (Boyman and Sprent 2012). Excessive effector T-cell content at the expense of memory cells is an undesired outcome in T-cell products intended for treatment of cancer. Clinical results have indicated that T-cell persistence is needed (Robbins et al. 2004, Maude et al. 2014) and that it depends at least in part on immunological memory (Powell et al. 2005, Kalos et al. 2011, Louis et al. 2011). A growing body of evidence from preclinical animal models supports the utilization of less-differentiated early memory T cells with greater proliferation capacity and survival after infusion (T_{SCM}, T_{CM}), instead
of cells that bear the apparent cytotoxic function (T_{EM}, T_{Eff}, (Gattinoni et al. 2005, Gattinoni et al. 2011, Barrett et al. 2014a, Sommermeyer et al. 2016)).

Because T-cell proliferation and effector/memory differentiation are inherently coupled upon clonal expansion (Crompton et al. 2014), novel methods founded on homeostatic proliferation are currently being tested (Gattinoni et al. 2005, Cieri et al. 2013, Xu et al. 2014, Gomez-Eerland et al. 2014, Gargett and Brown 2015, Singh et al. 2016). In vivo, homeostatic proliferation affects naïve and memory T cells in lymphopenic conditions where the level of lymphocytes is reduced and is responsible for the life-long low-level replication of these cells (Jameson 2002, Rochman et al. 2009). In these situations, the proliferative signal is mainly delivered by IL-7, but IL-15 is used in vitro to enhance the effect. Expression of the IL-7 receptor, CD127, is confined to naïve and memory T cells, thus being nearly absent on activated T cells (Rochman et al. 2009) and Tregs (Seddiki et al. 2006, Liu et al. 2006). Proliferation in response to IL-7 causes the cell to retain its initial differentiation phenotype. Regarding the homeostasis of naïve cells, weak TCR signaling in the form of self-peptide recognition and sense of T-cell ‘space’ (lack of T cells) is required in addition to cytokines. Experiences of using these cytokines for in vitro T-cell expansion instead of IL-2 are mostly restricted to CD8\(^+\) T cells (Cieri et al. 2013, Xu et al. 2014, Gomez-Eerland et al. 2014, Gargett and Brown 2015, Sabatino et al. 2016).

Cell composition in the starting material has a critical impact on the cell subset composition of the final product. Cancer treatment with chemotherapy alters the differentiation state of blood T cells towards effector phenotypes (Singh et al. 2016, Sommermeyer et al. 2016). The presence of naïve or T_{SCM} cells is essential for the generation of T cells that are capable of persisting in vivo and to provide improved clinical performance (Singh et al. 2016). In vitro expansion of less-differentiated T cells is, therefore, also approached by selecting more primitive starting populations for the expansion (Sommermeyer et al. 2016) or by interrupting the effector differentiation using signaling inhibitors (Sabatino et al. 2016).

All forms of in vitro T-cell expansion alter the characteristics of the cells. Therefore, both cell quality and safety need to be controlled. Uncontrolled proliferation could lead to genetic instability and malignant transformation. Also, expansion can influence the cellular plasticity compromising the cell identity and function.

1.4 CELL THERAPY REGULATION AND CURRENT STATUS IN FINLAND

Authority regulations apply to all tissue and cell therapeutics, as well as to blood products for transfusion medicine. Two ex vivo processing categories, minimal vs. substantial manipulation of the cells, classify T cells respectively, either as tissue products (Tissue Act 101/2001 on the Medical Use of Human
Organs and Tissues) or as advanced therapy medicinal products (ATMP, Article 17 of Regulation (EC) No 1394/2007). In practice, only pathogen-specific T cells that are produced with the reversible HLA-tetramer binding method (Neudorfer et al. 2007) and Tregs isolated utilizing the same technique and used without further expansion are regarded as tissue products. In the European Union, ATMPs need marketing authorization from the European Medicine Agency through a clinical trial demonstrating the safety and efficacy. Conducting a clinical trial also requires regulatory approval. As an option for individualized treatment of individual patients with ATMPs, a national production authorization, so-called hospital exemption, can be requested from the competent national authority controlling the quality and safety (in Finland from Fimea; Määräys 5/2014 19.12.2014 Dnro 002646/00.01.00/2014).

ATMPs are medicines, and the production must follow good manufacturing practice (GMP). GMP is a comprehensive quality system implemented to ensure that the patient is not placed in risk, and it covers all aspects of drug manufacturing (European Guide to Good Manufacturing Practice, Eudralex, http://ec.europa.eu/health/documents/eudralex/vol-4_en). To meet current GMP requirements, cell production needs to be conducted in clean rooms or in closed systems for ensuring sterility, with traceability that covers the starting material (tissue, blood) and raw materials used in the production method (e.g. serum or other growth factors) as well as the final product. Quality control, release testing, and quality assurance need to be in place. Methods and instruments for the production and quality control must be validated, every procedure instructed, and all personnel appropriately trained. Documentation for each relevant step during and related to the manufacturing process (e.g. training of cleaning personnel) is mandatory.

Overall, eight ATMPs have been approved in the European Union (http://www.ema.europa.eu/docs/en_GB/document_library/Committee_meeting_report/2017/01/WC500219367.pdf); the first, ChondroCelect® (TiGenix), for cartilage repair in 2009. By 2016, 14 Finnish patients have received this autologous cellular medicine (reference Finnish Red Cross Blood Service). Three approved medicines have been withdrawn from the market for business reasons (ChondroCelect®, MACI® and Provenge®).

Zalmoxis® (MolMedSpA), the only T cell-based product with marketing authorization was approved in 2016. It can be used to improve engraftment and immune reconstitution in HSC transplantation. These donor-derived T cells are genetically modified by retroviral transduction to express two transgenes, a truncated form of the human Low-affinity nerve growth factor receptor LNGFR and the herpes simplex I virus thymidine kinase (HSV-TK Mut2). T-cell treatment can lead to acute GVHD, which can be abolished by a HSV-TK Mut2 -activated pharmaceutical that kills the modified T cells causing the adverse effect. Zalmoxis® increased the patient one-year overall survival rate from 37% to 49%.
In Finland, three advanced cell therapy trials were recorded before 2016 (www.fimea.fi/valvonta/kliiniset_laaketutkimukset/tilastotietoa_kliinisista_laaketutkimuksista), one in 2011 and two in 2015. For comparison, more than 700 clinical trials were registered globally in the field of cell and gene therapy and regenerative medicine in 2016 (http://alliancerm.org/page/clinical-trials-products). Tregs, TILs, virus-specific T cells, and CAR T cells are studied in European clinical trials (n=3, 41, 9, and 11, respectively, www.clinicaltrials.gov), however not yet in Finland. Instead, the Finnish Red Cross Blood Service is allowed by the special permission by the competent authority to import virus-specific T cells from Germany. An urgent cell production order for individual Finnish HSC transplantation patients is coordinated through the Finnish Stem Cell Registry. This special procedure has been conducted for eight severely ill patients between years 2014 and 2017.

Two cell therapy products are produced under the national production authorization (hospital exemption) in Finland. A tissue-engineered product consisting of autologous adipose stem cells is used for the reconstruction of large bone defects (BioMediTech, (Wolff et al. 2013)). Refractory GVHD can be treated with immunosuppressive allogeneic mesenchymal stromal cells (Finnish Red Cross Blood Service, (Salmenniemi et al. 2016)).
2 AIMS OF THE STUDY

The studies of the present thesis aimed at creating sufficient expert knowledge for the development of T-cell-based cellular therapies at the Finnish Red Cross Blood Service. An objective was to develop therapeutically meaningful ways to expand and activate T cells and to evaluate their functionality. Also, characterization of genetic variation regulating activation of T cells can be used for the development of personalized medicine.

Specific aims for each publication were:

I to reveal how different in vitro cell expansion conditions affect the composition and therapeutic potency of T-cell products. In addition, this knowledge is used to develop an effective T-cell expansion protocol that can be applied to the clinical production of CAR T cells.

II to determine the relevance of the CTLA4 receptor and its soluble isoform to the potency of Tregs in inhibiting the T-cell response. Both freshly isolated and in vitro expanded Tregs were studied.

III to evaluate whether genetic polymorphisms in T-cell cosignaling receptor genes affect the expression of CTLA4.
3 MATERIALS AND METHODS

3.1 ETHICS AND HUMAN MATERIAL

This study was approved by the institutional review boards of the Finnish Red Cross Blood Service (I-III) and the Hospital District of Helsinki and Uusimaa (I and III). Informed consent was obtained from the involved participants when applicable (I and III). Human T cells (I) and Tregs (II) were expanded in vitro, starting from buffy coats that were processed from the donated blood of healthy blood donors (I and II). Also, whole blood samples from two leukemia patients were used for T-cell expansion (I). Study subjects, who voluntarily participated in the functional genetic studies (III) were healthy adults.

3.2 METHODS

The methods that were used in the studies are described in the original publications (I-III) or, regarding the additional unpublished data, are briefly described here. All the methods are listed in Table 4. T cell phenotyping was based on antigens and their expression patterns that are shown in Table 5. The gene polymorphisms that were genotyped in study III are presented in Table 6.
### Materials and methods

#### Table 4

*The methods used in the thesis.*

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Method</th>
<th>Publication</th>
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<tbody>
<tr>
<td>Cell isolation</td>
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<tr>
<td>- MNC</td>
<td>- density-gradient centrifugation</td>
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</tr>
<tr>
<td>- T cells (CD3⁺)</td>
<td>- positive selection with magnetic beads</td>
<td>I</td>
</tr>
<tr>
<td>- Treg, Th</td>
<td>- FACS sorting</td>
<td>II</td>
</tr>
<tr>
<td>- CD4⁺ T cells</td>
<td>- negative selection with RosetteSep</td>
<td>III</td>
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<tr>
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<td>I</td>
</tr>
<tr>
<td>- primary Treg</td>
<td></td>
<td>II</td>
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<td>Genetic modification</td>
<td>lentiviral transduction</td>
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<td>Phenotyping/Ag detection</td>
<td>flow cytometry</td>
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<tr>
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<tr>
<td>- T-cell activation</td>
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<td>- Ag specific activation via CAR</td>
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<td>Analysis of mRNA</td>
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<tr>
<td>- RFLP</td>
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<td></td>
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<tr>
<td>- SSP (allele specific)</td>
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</table>

# Co-cultures of different cell types
* Unpublished data

CFSE = carboxyfluorescein diacetate N-succinimidyl ester, MNC = mononuclear cells, RFLP = restriction fragment length polymorphism, SSP = sequence-specific primers. qRT-PCR = quantitative reverse transcriptase-polymerase chain reaction.

### CTLA4 BLOCKING EXPERIMENTS

To study the functional significance of CTLA4 in Tregs before (denoted as fresh Tregs herein) and after expansion, a modification of the immunosuppression test measuring responder cell proliferation (II) was used. CFSE-stained MNCs were stimulated for 5 days using a soluble anti-CD3 antibody (clone Hit3a from BioLegend, USA) at 0.5 μg/mL to induce a
suboptimal proliferative response (~50% of the responder cells proliferating at the end of the assay). Proliferation was measured with flow cytometry. MNCs were co-cultured with Tregs using different cell ratios (1:1, 1:0.5, and 1:0.2 responders to Tregs). A blocking anti-CTLA4 antibody (clone BNI3 from BD Pharmingen, USA) was used at 10 μg/mL. Suppressive function was compared to positive proliferation controls that also included anti-CTLA4 antibody (MNC + anti-CD3 Ab + anti-CTLA4 Ab). Same amount of isotype matching control antibody with irrelevant specificity (mouse IgG2a, clone eBM2a from eBioscience, USA) alone inhibited the proliferation of responder cells. This inhibition was even stronger than Treg-mediated inhibition. However, the anti-CTLA4 antibody did not have a similar effect on responder cells but rather slightly increased their proliferation. Also, in certain co-cultures of responder and Treg cells the presence of anti-CTLA4 antibody did not alter the responder cell proliferation (Table 8). Based on this data, the effect of the isotype control was considered unspecific and irrelevant regarding CTLA4 blocking experiments, and it was not used for data analysis. The effect of CTLA4 blocking was compared to identical co-cultures without any antibody. Expanded Tregs were tested in flat-bottom culture wells. For fresh Tregs U-bottom wells, which allow cell contact also for small cell numbers and thus more uniform testing conditions, were used.

**Table 5**  
*Antigens and their expression patterns used for phenotyping. Sequential differences in expression between T-cell differentiation subtypes are shown using underlining. TSCM, TSCM-like, and TCM cells represent less-differentiated, early memory T cells.*

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Publication</th>
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<tbody>
<tr>
<td>T cells</td>
<td>CD3+</td>
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<td>cytotoxic T cells</td>
<td>CD3+ CD8+</td>
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<tr>
<td>helper T cells</td>
<td>CD3+/CD14- CD4+</td>
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<td>TSCM-like</td>
<td>CD95+ CD45RO+ CD45RA+ CD27+</td>
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<tr>
<td>TCM</td>
<td>CD95+ CD45RO+ CD45RA- CD27+</td>
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<tr>
<td>TEM</td>
<td>CD95+ CD45RO+ CD45RA- CD27-</td>
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<tr>
<td>TEff</td>
<td>CD95+ CD45RO- CD45RA+ CD27-</td>
</tr>
<tr>
<td>Tregs additional markers</td>
<td>CD4+ CD25+ CD127 low/- Foxp3, CTLA4</td>
</tr>
</tbody>
</table>
Materials and methods

Table 6  Genotyped T-cell cosignaling receptor gene polymorphisms (III).

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>reference cluster ID*</th>
<th>Genotyping method</th>
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<tbody>
<tr>
<td>CTLA4-318C/T</td>
<td>rs5742909</td>
<td>RFLP</td>
</tr>
<tr>
<td>CTLA4+49A/G</td>
<td>rs231775</td>
<td>RFLP</td>
</tr>
<tr>
<td>CT60G/A</td>
<td>rs3087243</td>
<td>RFLP</td>
</tr>
<tr>
<td>ICOS IVS1+173T/C</td>
<td>rs10932029</td>
<td>SNaPshot</td>
</tr>
<tr>
<td>ICOS c.602A/C</td>
<td>rs10183087</td>
<td>RFLP</td>
</tr>
<tr>
<td>ICOS c.1624C/T</td>
<td>rs10932037</td>
<td>SNaPshot</td>
</tr>
<tr>
<td>ICOS c.2373G/C</td>
<td>rs4675379</td>
<td>SSP</td>
</tr>
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3.3 STATISTICAL ANALYSES

The differences between T-cell expansion conditions were analyzed with Mann-Whitney test (I), between fresh and in vitro expanded Tregs using paired t-test (II), and between genotypes by Kruskal-Wallis test (III). Correlations were analyzed using Spearman’s correlation (II). The dependence of cytotoxicity on effector to target cell ratios and IL-2 levels was tested using a linear modelling analysis (I). Differences were considered statistically significant when two-sided P values were <0.05. Analyses were performed using GraphPad Prism (version 6.07, I and II), R for the linear modelling analysis (version 3.3.1, I), StatsDirect (version 2.7.7, II), or SPSS software (version 14.0, III).
4 RESULTS

The results presented here are a summary focusing on the most significant findings in each study with respect to the context and topic of the thesis. More detailed results are reported in the original publications.

4.1 T cells cultured in a low IL-2 concentration are functionally potent with early memory phenotype (I)

Efficacy of T-cell therapy for cancer and infections, including the CAR T cells, can be improved using cell products containing less-differentiated early memory T cells. To this end, systematic studies were conducted on the effects of IL-2, “the T-cell growth factor”, and the in vitro expansion time on the balance between T-cell numbers and cell composition. Blood-derived T cells (11 donors: 9 healthy and 2 leukemia patients) were expanded using anti-CD3/CD28 beads and supplemented with varying concentrations of IL-2 ranging from 0 to 300 IU/mL. The cell phenotype was analyzed on the tenth and twentieth days of the expansion. The growth kinetics was monitored until day twenty.

Figure 6  Memory T-cell subsets after 10-day in vitro expansion under varying IL-2 conditions (0 IU/mL n=5, 5 IU/mL n=6, 20 IU/mL n=11, 100 and 300 IU/mL n=10). Means and 95% confidence intervals are depicted. Transparent data points represent patient samples and colored the samples from the healthy donors. Mann-Whitney test was used to calculate statistical significance (*P < 0.05).
Results

After ten days of expansion, cultures supplemented with a lower concentration of IL-2 had a higher proportion of less-differentiated TCM and, on the other hand, a lower relative amount of effector T cells (Figure 6). The overall content of early memory T cells (TSCM, TSCM-like, and TCM) was high but decreased along with the increasing levels of IL-2: the cultures without exogenous IL-2 had an average of 88% of early memory T cells and the cultures supplemented with 300 IU/mL IL-2 still had 61% (P=0.03). Naïve T cells were not detected after expansion. Between 20 to 300 IU/mL IL-2, the memory T-cell generation from the leukemic donors was in a similar way dependent on the exogenous IL-2 as from the healthy donors (Figure 6). The two patient samples were not studied in the two lowest IL-2 concentrations. Patient material yielded products with smaller proportions of early memory T cells and higher levels of more differentiated T cells (TEM and TEff). At day 20, the proportion of early memory T cells had decreased to about a half of the day 10 levels (I, Figure 5).

Half of the expansions were entirely deficient in TSCMs at the 10-day time point (Figure 6, 55% TSCM negative, n=11). All concurrent cultures from the same cell material were negative for TSCMs indicating that the lack was not IL-2 dependent. Further expansion until day 20 induced TSCM in those cultures that previously were negative but decreased TSCM levels in other cultures (n=6, Figure 7a). Poor expansion during the first week of in vitro expansion

Figure 7

a) Change in TSCM during in vitro T-cell expansion (n=6). Memory status was phenotyped on days ten and twenty of the expansion. Representative data from expansions with 20 IU/mL IL-2 is shown. b) Slower proliferation until the 6th day of the in vitro expansion was linked to the lack of TSCM on day 10 (n=6 for d10 TSCM negative and n=5 for d10 TSCM positive cultures, P=0.03). Expansion kinetics was studied in expansions with 20 IU/mL IL-2. Mann-Whitney test was used to calculate statistical significance between expansions that were either positive or negative for TSCM on day 10. The transparent symbols represent expansions that were negative for TSCM on day ten.
was associated with the lack of TSCMs on day 10 (n=11, Figure 7b). A three-fold difference in the cumulative fold expansion before the sixth expansion day was detected, corresponding to 4- and 13-fold mean expansion for expansions lacking TSCMs or positive for TSCMs, respectively, on day 10 (using 20 IU/mL IL-2, P=0.03).

The T-cell cultures consisted of CD4+ and CD8+ T cells. A longer expansion time yielded products with a prevalence of CD8+ cells: there was a 2- to 4-fold decrease in CD4+ cells from day 10 to day 20 (e.g. from 43% to 18% using 20 IU/mL IL-2, d10 n=11 and d20 n=6, P=0.03; I, Figure 6).

As expected, when less exogenous IL-2 was used for the expansion, the resulting cell numbers were lower (Table 7; I, Figure 4). 100 IU/mL IL-2, the current standard used in CAR T-cell production, as well as 300 IU/mL IL-2 produced exponential proliferation kinetics beyond twenty days. The cell expansion was modest with low IL-2 supplementation (~10-fold at day 10 with 0-5 IU/mL).

In vitro functionality of the expanded T cells was studied after 10-day expansion using CAR T cells as an example. All CD19-targeted CAR T-cell expansion products had multiple functions against their targets, CD19+ leukemia cell lines, regardless of which IL-2 concentration was used for expansion (n=3; I, Figure 7C-F). The T cells proliferated, secreted IL-2 and IFNγ, and showed potent cytotoxicity in response to exposure to target cells. When higher IL-2 levels were used during T-cell expansion, the killing activity increased (n=4, 0 vs. 100 IU/mL IL-2 P<0.0001). This increase is in agreement with the results showing increased effector T-cell generation.

### Table 7

<table>
<thead>
<tr>
<th>T-cell expansion kinetics under different IL-2 conditions. n = 5-12 depending on the conditions.</th>
</tr>
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<tbody>
<tr>
<td>cumulative fold expansion on day 10 (mean (min-max))</td>
</tr>
<tr>
<td>No IL-2</td>
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</tr>
<tr>
<td>20 IU/mL IL-2</td>
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<tr>
<td>100 IU/mL IL-2</td>
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<td>300 IU/mL IL-2</td>
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4.2 Treg expansion decreases alternative CTLA4 splicing and yields more potent CTLA4 receptor bearing cells (II)

CTLA4 is significant for the function of regulatory T cells but the relevance of its soluble isoform, sCTLA4, is not clear. Expression of CTLA4 isoforms was studied before and during in vitro Treg expansion, along with the immunosuppressive function of the cells. Current reagents cannot distinguish between the CTLA4 receptor and sCTLA4 proteins. Also, the CTLA4 receptor mainly has an intracellular localization due to its tightly controlled surface expression. Therefore the mRNA levels of CTLA4 receptor and sCTLA4 were measured. Tregs were isolated from the blood of healthy donors with FACS sorting (denoted as fresh Tregs herein). Some of the cells were directly analyzed, while the remainder were expanded using anti-CD3/CD28 beads with a very high concentration of IL-2 (1 000 IU/mL). High supplemental IL-2 was used to break the physiological Treg anergy. Expanded Tregs were analyzed after a 2-week expansion.

Figure 8  
a) mRNA levels of the soluble and full-length CTLA4 isoforms (sCTLA4 and fCTLA4, respectively) in fresh and Tregs expanded for 14 days. fCTLA4 encodes the CTLA4 receptor, sCTLA4 being its differently spliced variant. Mean ± standard deviations are shown, n=7. A paired t-test was used to calculate statistical significance (*P=0.0002). b) Expansion kinetics of Tregs that were expanded using anti-CD3/CD28 beads with 1 000 IU/mL IL-2, n=7. Figures from (Kaartinen et al. 2016).
In vitro expansion decreased the expression of sCTLA4 (Figure 8a). However, shortly after the CD3/CD28-mediated activation, at the three-hour time point, the sCTLA4 expression was in fact induced in comparison to unstimulated cells (II, Figure 4). In contrast, the mRNA level for the CTLA4 receptor, full-length CTLA4 (flCTLA4), increased as a result of expansion (P=0.01). An 11-fold mean increase in the intracellular, total CTLA4 protein content was achieved with the 2-week expansion of Tregs (range 5-17, n=7; II, Figure 1A). The data includes both CTLA4 receptor and sCTLA4. The expansion kinetics of Tregs was biphasic (Figure 8b): the cell proliferation was modest during the first week (on average eightfold) but subsequent re-stimulation provided a 210-fold mean expansion (range 50-350, n=7).

The immunosuppressive potential of fresh and expanded Tregs was compared in a T-cell proliferation assay (Table 8). Expanded Tregs were to some extent more immunosuppressive than Tregs that were tested directly after isolation (with 1:1 responder cell to Treg ratio, P=0.27). The strength of the T-cell activation manifested either as a suboptimal or optimal proliferative response (25-60% and 75-100% of responder cells proliferating after 4-day co-culture) did not influence the Treg-mediated inhibition (II, Figure 2).

Table 8

<table>
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<tr>
<th>suppression %</th>
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<tr>
<td><strong>responders:Tregs</strong></td>
</tr>
<tr>
<td>1:1</td>
</tr>
<tr>
<td>blocking:</td>
</tr>
<tr>
<td>1:1</td>
</tr>
<tr>
<td>1:0.5</td>
</tr>
<tr>
<td>1:0.2</td>
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</table>

Table 8: Immunosuppressive potential of fresh and expanded Tregs. The inhibition of responder cell proliferation was tested on suboptimal response (25-60% of responder cells proliferating) after 4 day co-culture (n=3, except n=7 for fresh Tregs). Immobilized anti-CD3 antibody (clone UCHT1) was used for the responder cell activation. The effect of the total CTLA4 blocking (both CTLA4 receptor and sCTLA4 together) was studied in 5-day cultures (n=3). In the blocking studies, a soluble anti-CD3 antibody (clone Hit3a) was used. Studies were conducted in U-bottom culture wells, except the blocking studies with expanded cells which were done in flat-bottom wells. Percentages for Treg suppression were calculated comparing the co-cultures with positive proliferation controls (similar cultures including only stimulated responder cells). Detected differences were tested by non-parametric tests (Mann-Whitney or Wilcoxon matched-pairs signed rank test) and were not statistically significant. Mean ± standard deviations are shown.
Results

The CTLA4 blocking (both CTLA4 receptor and sCTLA4) slightly inhibited the immunosuppressive function of fresh Tregs but only with high responder cell to Treg ratios (1:0.5 and 1:0.2, Table 8). This weak blocking effect did not reach statistical significance (P=0.25). With these cell ratios, fresh Tregs were equally potent as suppressors as the expanded cells. However, the testing conditions were not totally free from experimental differences (U- vs. flat-bottom culture wells were used respectively for fresh or expanded cells), thus leaving the significance of this data open. The correlation between the mRNA levels of CTLA4 isoforms and the level of Treg-mediated suppression points to a role for flCTLA4 in Treg immunosuppressive function (r = 0.93, n=7 including 5 fresh and 2 expanded cell samples, P=0.007; II, Figure 5). Hence, CTLA4 mRNA levels may be utilized to predict the functional potency of Treg products. sCTLA4 expression was negatively correlated with the inhibitory function (r = -0.57, P=0.2). The inhibiting function of expanded Tregs was not affected by CTLA4 blocking under testing conditions slightly different to those used for fresh cells (U- vs. flat-bottom culture wells were used respectively for fresh or expanded cells, Table 8).

4.3 Genetic variation in T-cell cosignaling receptor genes regulates the mRNA expression of CTLA4 in addition to alternative splicing (III)

CTLA4 is a key regulator of T-cell response against tumors, mediating Treg function, and restraining autoimmunity. We studied how genetic variation in the CTLA4 gene region on chromosome 2, containing genes for CD28, CTLA4, ICOS, and PD-1, changes the expression of the full-length CTLA4 receptor and its secreted sCTLA4 isoform. mRNA levels were measured in blood CD4+ T cells, including CD4+ Tregs, of 17 healthy volunteers before and during anti-CD3 and CD80 induced stimulation.

Depending on a genetic polymorphism in the ICOS gene (IVS1+173T/C, rs10932029), both CTLA4 isoforms were expressed differently upon T-cell activation (Figure 9). Homozygous carriers of the T allele showed on average a two-fold increase in the expression of both flCTLA4 and sCTLA4 shortly after T-cell activation. Without activation no difference was seen (data not shown). Regardless of genetic variation, T-cell activation mostly induced the expression of both CTLA4 splicing isoforms (Figure 9).
Figure 9  T-cell activation-induced change in the mRNA expression of CTLA4 isoforms (sCTLA4 in (A) and flCTLA4 in (B)) in 17 healthy volunteers carrying either the TT or TC genotype in the IVS1+173 polymorphism (rs10932029) in ICOS gene. Means for each genotypic group are shown in the left panel where at 3h time point in A) n=7 for TT and n=8 for TC genotype and at 6h time point in B) TT n=6 and TC n=5. Statistical significance was analyzed using the Kruskal-Wallis test. Figures from (Kaartinen et al. 2007).
Results

In resting cells, the alternative splicing of CTLA4 varied according to another genetic variation located near the CTLA4 gene (CT60G/A, rs3087243). The mean sCTLA4 level was higher, 0.06, in 7 GG homozygotes compared to 0.11 in 10 persons carrying the A allele (9 GA + 1 AA, P=0.01, Figure 10). This genetic effect was only revealed in non-activated cells. Conflicting reports of the functional role of CT60G/A polymorphism has been published. Our data contradicts the reports by Atabani and Mayans (Atabani et al. 2005, Mayans et al. 2007) and supports the work by Ueda.

Figure 10 The mRNA expression level of the soluble CTLA4 isoform (sCTLA4) in resting CD4+ T cells of 17 healthy volunteers (n=7 for CT60 GG and n=10 for other CT60 genotypes (9 GA and 1 AA)). CT60 (rs3087243), an intragenic polymorphism, is located between the genes encoding CTLA4 and ICOS. Means for each group are shown. Statistical significance was analyzed using the Kruskal-Wallis test. Figure from (Kaartinen et al. 2007).
5 DISCUSSION

5.1 *In vitro* cell expansion in adoptive T-cell therapies (I & II)

The effect of IL-2 on memory T-cell differentiation
In this study, a simplified and lean *in vitro* expansion protocol was developed for the production of T cells with potent effector functions (I). By lowering the amount of supplemental IL-2 during the *in vitro* expansion, a higher proportion of less-differentiated memory T cells was generated. Cell products that have a substantial amount of early memory T cells are expected to perform clinically better due to improved *in vivo* survival, as denoted in cancer therapy (Robbins et al. 2004, Maude et al. 2014).

The expansion method presented in this thesis is directly applicable for the production of CAR T cells and TCR-engineered T cells. Today, standard methods for CAR and TCR-engineered T-cell production utilize 100 or more IU/mL of IL-2 (Mock et al. 2016), which in this study generated a more unfavorable ratio between early memory T cells and effectors compared to T cells expanded without or with only a low level of supplemental IL-2. Therapeutic approaches based on tumor infiltrating T cells, TILs, would also benefit from the maintenance of cell longevity, as the starting material in itself represents Ag-primed and even exhausted cells. Current TIL protocols use as high a level of IL-2 as 3 000–6 000 IU/mL for several weeks (Donia et al. 2012). The *in vitro* expansion of TILs, however, has its special challenges as a result of the limited starting cell number.

Current methods for the *in vitro* expansion of Tregs commonly use 300 IU/mL (Putnam et al. 2013). In this study (II), Treg expansion was conducted under 1 000 IU/mL of IL-2. Studies addressing exhaustion or functional impairment of Tregs after excessive *in vitro* expansion have not been published. However, Tregs with a naïve-like phenotype have *in vitro* functionality that exceeds the potency of more effector-like Tregs (Hoffmann et al. 2006, Lamikanra et al. 2017). In the present study, Tregs were potent suppressors after expansion, therefore suggesting that a 2-week expansion using a very high level of IL-2 does not cause deterioration of Tregs derived from healthy donors.

Other novel approaches for the production of early memory T cell enriched products are based on IL-7 and IL-15 induced homeostatic T-cell proliferation (Gargett and Brown 2015), signaling inhibitors that interrupt effector cell differentiation (Sabatino et al. 2016), or selection of less-differentiated starting cell populations before expansion (Sommermeyer et al. 2016). The increase in early memory T cells using homeostatic cytokines instead of the commonly used IL-2 concentrations (100–300 IU/mL, (Cieri et
Discussion

al. 2013, Xu et al. 2014, Gargett and Brown 2015)) is similar in magnitude to the increment demonstrated here by limiting IL-2 supplementation (5-30%). In a recent study, the use of IL-7 and IL-15 yielded 10-20% more TSCMs compared to expansions without any cytokine supplementation (Singh et al. 2016). Singh et al. also revealed a simultaneous IL-7 and IL-15 induced decrease in TCM and, interestingly, an increase in TEff.

T-cell expansion in low IL-2 concentration also provides other benefits in addition to the favorable cell composition. First, compared to other approaches requiring complicated multistep processes, it offers a simple and cost-effective GMP-grade procedure for clinical use. Second, the safety profile of cells produced with methods that are based on clonal expansion is better known than with the more novel methods. The utilization of signaling inhibitors in conjunction with T-cell activation or homeostatic cytokines detached from the physiological homeostatic environment (e.g. signaling for ‘space’) may also modify other characteristics of the cells. Furthermore, robust IL-2 or IL-15 signaling with concurrent viral activation of oncogenes has been linked to insertional mutagenesis (Newrzela et al. 2011).

The appropriate clinical cell dose for T-cell therapies is not known and may depend on the cell phenotype in the product. Dose-escalation studies are needed to define the T-cell expansion conditions providing the best balance between cell subset composition and sufficient total cell number.

The generation of TSCMs and CD4+ T cells

The generation of TSCMs, a memory T-cell subset bearing superior proliferative capacity and ability to self-renew, was inconsistent over time and between expansions (I). TSCM generation and the expansion kinetics of the cultures were connected. The lack of TSCMs on day 10 was detected in cultures that displayed a slow T-cell proliferation during the first week of expansion. The subsequent intensive proliferation may be related to the presence of TSCMs later on day 20. Also, vice versa: when good proliferation was achieved during the first week of expansion, TSCMs were generated in cultures already before day 10 but then their proportion decreased by day 20. These results may imply that for product comparability, it is more important to follow the individual growth kinetics of the cells instead of limiting the production process to a beforehand defined length. This data illustrates the sensitivity of primary cell culturing and the challenges encountered in cell manufacturing.

Considering the proposed progressive model, where memory T-cell generation is a stepwise process from more primitive cells to cells with increasing effector functions (Farber et al. 2014), it was surprising to find (I) that cultures initially deficient for the most primitive subsets, naïve and TSCM, later contained TSCMs. Also, only naïve T cells have been demonstrated to give rise to the memory stem cell population during in vitro T-cell expansion.
(Singh et al. 2016). It is possible that those subsets were present at all times during expansion but only at low levels below the detection limit.

Most in vitro expansion protocols seem to be incapable of generating TSCMs (CD95+CD45RO-CD45RA+CD27+, (Cieri et al. 2013, Xu et al. 2014, Gomez-Eerland et al. 2014, Gargett and Brown 2015)). Finding out the factors inducing the generation of TSCMs in the present system (I) would be of great interest both scientifically and with regard to production of adoptive T-cell products. The TSCM formation was not dependent on the level of supplemental IL-2. Signals provided by IL-7, IL-15, and IL-21 (Gattinoni et al. 2011, Cieri et al. 2013, Sabatino et al. 2016) may be central to the generation of TSCMs and TSCM-like cells, a subset not detected in blood but which has been depicted in in vitro expansions (CD95+CD45RO-CD45RA+CD27+, Figure 6, (Cieri et al. 2013, Xu et al. 2014, Gomez-Eerland et al. 2014, Gargett and Brown 2015)). T cells are not known to produce IL-7 or IL-15 but IL-21 and IL-9 are secreted by activated CD4+ T cells (Rochman et al. 2009). IL-9 supports T-cell survival and is produced in the late phases of the T-cell response (Rochman et al. 2009, Parrot et al. 2016). Cytokine secretion was not measured in our expansion cultures. However, based on the importance of CD4+ cells for the T-cell memory formation (Janssen et al. 2003, Shedlock and Shen 2003) and correlation between the clinical T-cell persistence and the numbers of CD4+ T cells in cell products (Louis et al. 2011), we hypothesize that the generation of TSCMs is dependent on CD4+ T-cell derived factors produced after these cells’ peak proliferation during immune activation.

Assuming that in vitro generated early memory T cells have a similar homing capacity to their physiological counterparts, they will not enter peripheral tissues. Rather, they will be activated if they meet their target antigens in lymphoid organs. This event depends on efficient Ag-presentation by APCs or the presence of target cells in lymphoid tissues. Thus, the clinical success of early memory cell enriched T-cell products in cancer may be highlighted in hematological malignancies. Clinical data from trials treating patients with non-hematological tumors is pending (Gomez-Eerland et al. 2014, Gargett et al. 2016).


The functional potency of Tregs following in vitro expansion

Tregs were slightly better immunosuppressors after in vitro expansion than their fresh, non-expanded counterparts (II), in line with earlier reports obtained in humans and animal models (Hoffmann et al. 2004, Chai et al. 2008, Theil et al. 2015). The lower Treg content in the product is one
Explanation for the weaker performance by fresh cells. Cellular impurities result from the difficulties in dissociating Tregs from conventional T cells. Although different in function, Tregs are, in essence, similar to other T cells. They circulate in the body, constantly monitoring their environment, need activating signals to launch their functions, and have different phenotypes in resting and activated states. Considering this, it makes sense that T cells and Tregs exhibit the same surface markers and are therefore difficult to separate from each other in practice. Most clinical Treg methods use magnetic bead-based selection (CD4+CD25+) instead of FACS sorting (CD4+CD25+CD127−/low) due to much easier applicability in GMP. As a consequence, the purity of isolated, non-expanded Tregs can be lower (Seddiki et al. 2006, Liu et al. 2006) but during subsequent expansion, Tregs are enriched. However, the stringent FACS-based Treg selection used in our study points to other reasons than impurities behind the differences between expanded and fresh cells.

The studies reported here demonstrate that expanded Tregs expressed higher levels of CTLA4, which also predicted more potent Treg function. CTLA4 is known to play a key role in the immunosuppressive function of Tregs (Sakaguchi et al. 2009). However, blocking the function of CTLA4 only had a moderate effect on the Treg-mediated inhibition of fresh Tregs. After expansion, even the slight influence seen by CTLA4 blockade disappeared. These findings suggest that the Treg population obtained by sorting was using CTLA4-mediated suppressive mechanism. However, CTLA4 was not irreplaceable for their function. In vitro T-cell expansion is based on activation of the cells through TCR. This activation can also arm other immunosuppressive mechanisms in Tregs, like inhibitory cytokines, in addition to CTLA4. Versatile mechanisms would be more readily usable and could better compensate for each other.

In conclusion, in vitro expansion of Tregs is helpful, not only because it offers higher cell numbers for therapeutic use but also because the functional potency of Tregs is strengthened. The activating nature of T-cell expansion presumably upregulates a wide range of function-related molecules. These results provide support for the development of clinically feasible in vitro expansion methods for Tregs.

5.2 Role of the CTLA4 receptor and its soluble isoform in immunology (II & III)

CTLA4 isoforms in Tregs
Study (II) showed that the expression of RNA encoding for the CTLA4 receptor is linked to enhanced Treg immunosuppression. In contrast, the level of sCTLA4 showed a negative correlation. These results suggest, in line with other reports (Gerold et al. 2011, Ward et al. 2013), that the function of
CTLA4 receptor is more important than the function of sCTLA4 and is likely to be sufficient for the immunosuppressive function of Tregs, even without the sCTLA4 isoform.

Gerold et al. were able to segregate the function of sCTLA4 from the CTLA4 receptor by specific silencing of the soluble isoform in a T1D mouse model (Gerold et al. 2011). Tregs deficient for sCTLA4 could not inhibit the expression of CD86 on DCs as efficiently as cells expressing sCTLA4. The elimination of sCTLA4 impaired the in vivo function of Tregs, but the functional in vitro effect was only seen with high responder cell to Treg ratios (≥ 1:0.2). Interpretation of the studies conducted in mice is complicated by the presence of a third CTLA4 isoform lacking the extracellular domain, ligand-independent CTLA4 (liCTLA4), which is absent in humans. The importance of sCTLA4 was only seen when higher amounts of liCTLA4 were simultaneously produced. sCTLA4 elimination delayed the onset of autoimmune disease, but a direct comparison to the CTLA4 receptor deletion was not made. The complete loss of CTLA4 gene in mouse Tregs has spontaneous, fatal consequences (Wing et al. 2008).

Ward et al. addressed the function of sCTLA4 in human Tregs (Ward et al. 2013). First, using an sCTLA4-specific novel antibody, they demonstrated human Tregs to be prominent producers of secreted sCTLA4 protein. Specific sCTLA4 blocking reduced the inhibitory function of Tregs, which was stronger with higher responder to Treg ratios (1:0.5 and 1:0.1) in contrast to 1:1 cultures that provide more cell contact. Unfortunately, no comparison to CTLA4 receptor blocking was conducted.

Together, these studies imply that sCTLA4 functions as an inhibitory molecule in Tregs. The function of sCTLA4 seems to matter only when cell contact between Tregs and their target cells is scarce, suggesting a secondary role compared to the CTLA4 receptor. However, inside the body, in the natural physiological context, the availability of contact between Tregs and their targets is not known.

**sCTLA4 in immune response**

When sCTLA4 was discovered, it was thought to be present mainly in resting T cells and its expression to be repressed by T-cell activation (Magistrelli et al. 1999, Oaks et al. 2000). Our results show that upon the induction of Treg cell expansion by T-cell activating factors (CD3/CD28 beads, II) and TCR-complex mediated activation of CD4+ T cells (III), the sCTLA4 expression is in fact upregulated. Other reports confirm these findings (Perez-Garcia et al. 2013, Ward et al. 2013). Only exceptionally strong T-cell stimulation leads to the downregulation of sCTLA4 (Ward et al. 2013). The possibility that sCTLA4 production is suppressed due to strong but incomplete activation conducted without CD28 cosignal has not been studied. Even so, the idea that in resting state sCTLA4 would function as a regulator of immune activation or T-cell homeostasis is not a plausible explanation due to the
activation-related expression profile. Compared to the receptor, a similar but possibly distinct role in activated T cells is suggested for sCTLA4 by the concurrent expression and the recently accepted inhibiting function (Oaks et al. 2000, Huurman et al. 2007, Simone et al. 2009, Ward et al. 2013).

The downregulation of CD28 ligands on the surface of APCs is the only known mechanism for sCTLA4 (Huurman et al. 2007, Gerold et al. 2011). Another potential mechanism is the induction of IDO via CD80/CD86 signaling. Other mechanisms used by the CTLA4 receptor, like trans-endocytosis of CD28 ligands from the surface of APCs or direct inhibitory signaling in T cells themselves, cannot be employed by a soluble factor. However, the expression of CD80 is induced on activated T cells (Chen and Flies 2013). Therefore, one possibility is that sCTLA4 binds its ligands on the surface of other T cells - or even in an autocrine manner. On the other hand, by using soluble CTLA4 instead of the receptor, a T cell could avoid potential intrinsic inhibitory signals but still be able to inhibit other cells, a mechanism that would suit regulatory cells. As a secreted protein sCTLA4 at least has a wider functional range compared to the CTLA4 receptor, which functions in cell contact.

Interestingly, sCTLA4 is secreted by certain tumor cell lines (Ward et al. 2014). Furthermore, effector T cells or Tregs that infiltrate into the tumor could secrete sCTLA4 in a manner similar to the increased expression of checkpoint molecules (CTLA4, PD-1). In a mouse model of melanoma, specific targeting of sCTLA4 improved the tumor control as efficiently as the targeting of total CTLA4 (Ward et al. 2013). Therefore, an inhibitory role in the tumor microenvironment has also been proposed for sCTLA4.


In summary, the role and functional mechanisms for sCTLA4 are far from clear. However, it is evident that sCTLA4 plays a part in the regulation of immune response and is an attractive molecule in immune-related diseases, like autoimmune disorders or cancer.

5.3 Exploring the CTLA4-mediated functional effects of T-cell cosignaling receptor gene variation (III)

Upon T-cell activation, the expression of both CTLA4 isoforms was regulated in the same way by one polymorphism (IVS1+173T/C), but alternative splicing was affected by genetic variation in CT60G/A polymorphism in resting cells only. In our extended genetic analysis, two additional polymorphisms were associated with CTLA4 expression in a similar manner: upon stimulation one polymorphism (rs11571300) influenced the expression
of both isoforms but another was linked to altered sCTLA4 levels only (rs231755, (Haimila et al. 2009)). These results show that genetic variation affects CTLA4 expression differently in resting and activated T cells.

Substantial evidence indicates that genetic variation in the gene segment containing CD28, CTLA4, ICOS, and PD-1 genes is associated with susceptibility to many immune-related disorders, and this association is presumed to derive from differences in gene expression. To use functional genetics in personalized medicine, the effects must be validated and be concordant between studies. However, the numerous genetic disease association studies, even systematic whole genome screenings and those utilizing genetic tools such as expression Quantitative Trait Locus (eQTL) databases, as well as the functional genetic studies conducted to date, reflect the complexity of CD28 family cosignaling receptors at multiple levels. First, the complex and strong genetic linkage disequilibrium in the region obfuscates the identification of disease-linked genetic polymorphisms (Haimila et al. 2004). Second, the intricate regulation of CTLA4 protein expression in general in addition to the in part discrete genetic regulation of the splicing isoforms, and third, the complex functions of CTLA4, with both cell-intrinsic and extrinsic effects wrought via multiple mechanisms, make the design of functional studies difficult. Lastly, the fact that CTLA4 plays different roles in immunology depending when and where anatomically and on which type of cell it is expressed complicates the interpretation of the results. These levels of complexity are not separate from each other but intertwined.

As an indication of the current status of the functional CTLA4 genetics, it can be noted that there is no consensus on what the immune disorder-associated CTLA4 characteristics are. Some suggest that the genetic disease-predisposing phenotype is CTLA4 deficient (Kouki et al. 2000, Ligers et al. 2001, Anjos et al. 2002, Maurer et al. 2002, Howard et al. 2002) and others that CTLA4 is over-expressed (III, (Wang et al. 2002, Anjos et al. 2004, Haimila et al. 2009, Perez-Garcia et al. 2013). The third group of data points to the deficient expression of the sCTLA4 isoform (III, (Ueda et al. 2003, Atabani et al. 2005, Perez-Garcia et al. 2007, Haimila et al. 2009)).

Two recent publications demonstrated the clinical consequences of reduced CTLA4 expression (Kuehn et al. 2014, Schubert et al. 2014). Rare heterozygous mutations in the CTLA4 gene caused a severe disorder (CTLA4 haploinsufficiency with autoimmune infiltration, CHAI) with functionally impaired Tregs and disturbed T and B-cell homeostasis. In the affected families, several polymorphisms led to the same outcome, though not in all the family members carrying the mutation.

eQTL databases established during the past few years provide a powerful and systematic tool for analyzing the effects of gene variation on gene expression. Table 9 shows a list of genetic polymorphisms that were found to regulate the CTLA4 gene in blood according to the eQTL database of Westra et al. (Westra et al. 2013). If we want to proceed toward personalized
medicine by utilizing gene markers for CTLA4, we should first know the net effect of all these variants.

Considering the immune regulated nature of CTLA4 expression and our results demonstrating different functional effects in resting and activated T cells, the most relevant variants may be found only using stimulated samples. In line with this, the polymorphism influencing the CTLA4 expression in stimulated cells only (IVS1+173T/C, rs10932029) or the one identified in our extended study (rs11571300, (Haimila et al. 2009) are not found in the eQTL database (Table 9). Therefore, eQTL data that are based on in vitro stimulated samples, or patient material would better reflect the physiological context where the genes, and cells expressing them, function. Such databases are emerging (Ye et al. 2014, Peters et al. 2016, Li et al. 2016). We have also produced a small eQTL bank based on activated and non-activated T cells from ~50 blood donors (Saavalainen et al. unpublished).

The possibility that direct CTLA4 protein measurement in cells, and in Tregs particularly, can be applied in personalized medicine could be explored. It might be simpler to measure the functional outcome that apparently causes the immune disturbance than it is to reveal the very complex and possibly somewhat individual genetics behind it (Kuehn et al. 2014, Schubert et al. 2014). Potential scenarios for the utilization of the CTLA4-related personalized medicine in cell therapies can be envisioned. For example, virus-specific T-cell products derived from donors with a low expression of CTLA4 may be associated with a higher risk for unwanted T-cell activity, such as GVHD, and hence may not be suitable for all patients.

To conclude, elucidating the functional effects of genetic variation on CTLA4 is complex and the subsequent impacts on immunity are not yet known but are most likely highly context-dependent.

Table 9  Genetic variation nearby the CTLA4 gene (cis) affecting CTLA4 expression in blood cells according to the eQTL database of Westra et al. (Westra et al. 2013), http://genenetwork.nl/bloodeqtlbrowser/.
5.4 CONCLUSIONS

The aim of these studies was to test how in vitro cell expansion influences the characteristics of human regulatory T cells and T cells with potent effector functions, and to assess the significance of CTLA4 isoforms and the role of genetic variation in controlling CTLA4 expression.

The expansion of T cells under reduced cytokine conditions produced a higher percentage of early memory T cells, which likely perform better in adoptive T-cell therapy. Compared to other approaches to generate memory T-cell enriched products, the method described here is technically simpler and more cost-efficient, and its safety is better known. Also, this method is capable of generating TSCMs, the T cells with the highest survival potency. The functional potency of Tregs was associated with the RNA levels of the CTLA4 receptor and was slightly strengthened by the in vitro expansion. Therefore, the expansion of Tregs is beneficial in two ways. The number of Tregs in the blood, which is originally low, can be multiplied, and the cells simultaneously gain a better inhibitory capacity. Determining a good T-cell product for clinical use is, however, not straightforward. A balance between the cell phenotype or function and cell number is required, and these properties are often interdependent. Future studies addressing this balance are needed.

The soluble isoform, sCTLA4, seems secondary to its receptor counterpart, both in Treg function and in expression. Although the regulation of the isoforms was similar in response to T-cell activating signals, distinct features were seen in resting CD4+ T cells, in which genetic variation played a role, and on the other hand, in expanded Tregs compared to fresh Tregs. Further research is required to elucidate the role of sCTLA4 and the immunological impact of gene variation in the CTLA4 gene region.

In conclusion, the studies presented in this thesis provide new knowledge that can be utilized in the production of therapeutic T cells and broaden the knowledge base for potential CTLA4-based personalized medicine. As the T-cell expansion method described here, and the observations made using it, are directly applicable in T-cell manufacturing, these research results may also have substantial clinical significance.
The study described in this thesis was carried out at the Finnish Red Cross Blood Service in Helsinki during 2003-2007, 2008-2010, and 2014-2017. I am grateful to the Director of the Blood Service, Professor Martti Syrjälä, as well as to the other, present and former, directors of our organization, Dr Jarkko Ihalainen, adj.prof Kari Aranko, and Dr Jarmo Laine. I find it admirable that a non-profit institution responsible for providing all blood products for Finnish hospitals and patients still offers such strong encouragement to do research. I appreciate the chance I was given to focus on research instead of research grant applications, and I am proud that this thesis is the fruit of my paying job.

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