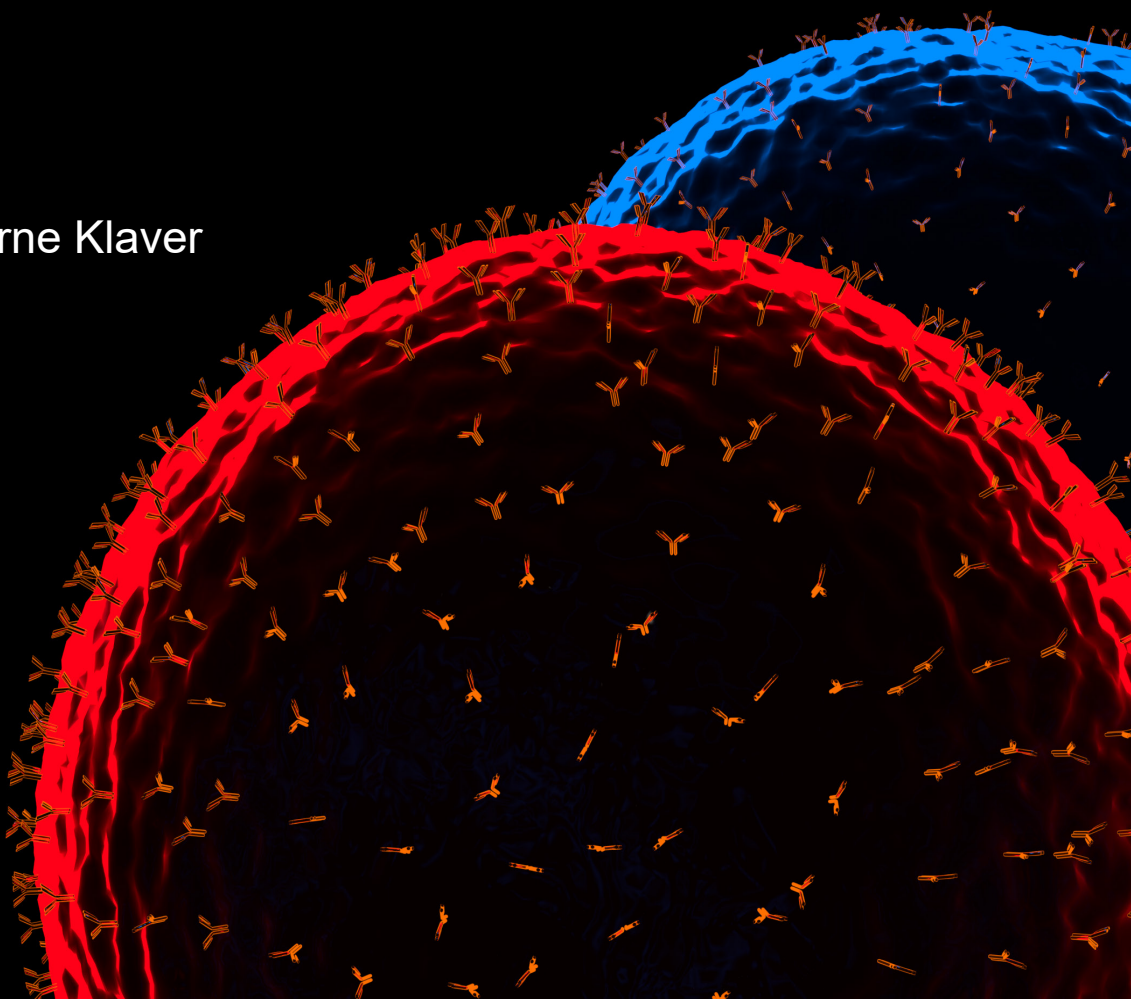
IMMUNE TREATMENTS OF SOLID TUMORS AND T CELL MONITORING

Yarne Klaver



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Immune Treatments of Solid Tumors and T Cell Monitoring

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Immuuntherapieën van solide tumoren en T cell monitoring

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Contents

Chapter 1

General introduction into cancer (immune)biology and immunotherapy

INTRODUCTION

Cancer is a disease that is the leading cause of death in the Netherlands for both men and women.[1] Cancer is a disease that involves abnormal and uncontrolled growth of cells that have the potential to invade and spread (metastasize) to other parts of the body. When cancer is detected early and has not yet metastasized to lymph nodes or other organs, it is curable by resection. Unfortunately, cancer is often detected in a stage where it already has metastasized. To date, in most cases metastasized cancer is not curable, and treatment of metastasized disease aims at preventing disease progression. The "traditional" treatment modalities in oncology are in addition to resection or debulking of tumors, radiotherapy and chemotherapy. However, one of the new promising therapeutic options that has emerged in the last decade is Immunotherapy.[2] Immunotherapy of cancer is a strategy that harnesses the body's immune system to combat tumors.

IMMUNE SYSTEM

The immune system protects us from extraneous agents and pathogens but plays also a role in the recognition and eradication of premalignant or malignant cells. The immune system can roughly be divided into two subsystems: innate- and adaptive immune system. These two subsystems are complementary and strongly interact with each other.

Innate immune system

The main compartments of the innate immune system are physical and chemical barriers (skin, mucous membranes, saliva, etc.), and the complement system. In addition, the innate immune system also harbours multiple immune cells like: basophils, dendritic cells, eosinophils, mast cells, macrophages, neutrophils, and natural killer cells. All these immune cells have their own specific role in the recognition and elimination of pathogens. In addition, macrophages and dendritic cells are important not only in immediate recognition of pathogens but also in killing, digestion and exposing ("presentation") of pathogen-derived antigen fragments (peptides), called "epitopes". Therefore, these cells are also known as antigen-presenting cells (APC).[3-5] The presented antigens can subsequently be recognized by T cells from the adaptive immune system. Lastly, the initial activation of innate immune cells at the site of inflammation can set off production of chemo- and cytokines by innate immune cells themselves and/or tumor cells, fibroblasts or endothelial cells. These chemo- and cytokines attract other immune cells to the site of inflammation.

Adaptive immune system

The adaptive immune system consists out of highly specialised cells, which are able to recognize unique antigens. The adaptive immune system roughly comprises (antibody producing) B cells, CD4+ helper T cells, and CD8+ cytotoxic T cells. After presentation of pathogenic antigens by APCs, B cells can recognize antigens directly via the B cell receptor (BCR), whereas T cells recognize antigens as peptides presented by major histocompatibility complex (MHC)

molecules via the T Cell Receptor (TCR). These receptors are unique and arise mainly from genetic recombination of the DNA encoded segments in individual T and B cells. This enables the formation of T and B lymphocytes that are able to distinguish many different antigens, including self-antigens that are presented by healthy tissues. It is therefore critical for lymphocytes to undergo selection during development from lymphocyte precursor to lymphocyte, in which process self-reactive lymphocytes are eliminated. Upon antigen recognition by the BCR or TCR, B or T cells become activated, rapidly expand and clear aberrant cells positive for the target antigen. After reduction of a certain antigen (for instance a pathogen), immunological memory cells are formed, which, upon a second encounter with the same pathogen, enable accelerated expansion of antigen-specific lymphocytes leading to fast clearance and protection against such a pathogen.

Aside from all the above effector immune cells, there are also suppressive immune cells that dampen immune responses. This is important, since keeping immune responses in-check prevents overt reactions and autoimmunity. Examples of suppressive immune cells include: Myeloid derived suppressor cells (MDSC) [6], M2-macrophages [7], and T regulatory cells (Tregs) [8]. The relevance of these cells is illustrated by the notions that mice that are depleted of Tregs [9], or humans who have immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome due to mutations in FOXP3 have severely increased autoimmunity. [10, 11]

ANTI-TUMOR IMMUNE RESPONSES AND ITS HISTORY

Tumors are quite heterogeneous, not only with respect to genetic characteristics of tumor cells themselves, but also with respect to presence and activation of stromal cells, such as fibroblasts, endothelial cells, and immune cells. Here, I would like to zoom in on the immune constituents of tumors. When present, generally the case in subsets of tumors of any histological origin, immune constituents comprise many different types of immune cells, as illustrated in Figures 1A-G. Rudolf Virchow, already in the 19th century, has described the presence of immune cells in tumors, and being ahead of his time pointed to a link between inflammation and cancer. Based on these and other observations hinting at the potential of the immune system as a mean to treat cancer, researchers in the 1950s demonstrated that it was possible to immunize animals and prevent tumor formation. In these experiments, tumors (induced by carcinogens) were excised from an animal and, following a short time culture, re-transplanted onto animals. Strikingly, animals were able to reject a second injection with tumor cells.[12-15] These experiments implied that there must be antigens on tumor cells that can be recognized by the immune system. Nowadays we know that CD8 T cells play a dominant role in recognizing and killing tumor cells. In fact, it has been shown that numbers and spatial distribution of CD8 T cells in cancer can strongly influence disease outcome. For example, a high frequency of tumor infiltrating lymphocytes (TILs), and in particular the CD8 T cells, correlates with an increased survival in melanoma, head and neck, breast, urothelial, ovarian, colorectal and lung cancer patients [16, 17]. Importantly, the seminal experiments of Virchow also demonstrated that tumors initially evade immune reactions. There are several

immune evasive mechanisms that delay or put on-hold anti-cancer immunity. One strategy that tumors exploit to evade CD8 T cell responses is to diminish or prevent antigen presentation and recognition; other. Other strategies may include: reduction in numbers of effector immune cells; enhanced numbers of immune suppressor cells; reduction of antigen expression and/or enhanced expression of immune and metabolic checkpoints.[18, 19]

CANCER IMMUNOTHERAPY

To treat cancer by using the patients own immune system, several approaches have already shown promise in clinical trials. The most effective strategies include: 1) antibodies targeting immune checkpoints (checkpoint therapy), 2) adoptive T cell therapy with ex vivo expanded TILs, and 3) adoptive T cell therapy with ex vivo genetically modified and expanded T cells. Also, several other therapeutic have been tested in clinical trials, like treatment with immune modulators (e.g., cytokines, such as Interleukin-2 (IL-2) or Interferon alpha); vaccination with tumor cell preparations, incl. tumor derived proteins and antigenic peptides alone or in combination with antigen presenting cells; or treatment with oncolytic viruses. Below, we will highlight vaccination therapy since this is one of the subjects in this thesis, and subsequently checkpoint therapy and adoptive T cell therapy.

Vaccination

Instead of adoptively transferring effector T cells, or use of checkpoint inhibiting antibody therapy to (re)activate T cells, T cells can also be primed and/or boosted ***in vivo*** by tumor vaccination therapy. This can be done by injecting (tumor-derived) proteins or peptides into the patient. These proteins or peptides are absorbed and processed by APCs and presented to T cells.[20] Alternatively, APCs can be generated ***ex vivo*** from isolated blood monocytes and loaded with tumor antigens (proteins or peptides) prior to injection into patients to activate patient T cells. The vaccine-activated T cells then specifically recognize and attack the tumor cells.[21]

Checkpoint inhibitors

A major breakthrough in the field of cancer immunotherapy was the discovery of immune checkpoints and the development of antibodies that bind to such inhibitory receptors (iRs) on T cells. By blocking the iRs (or their corresponding ligands on tumor cells) T cells are released from their inhibitory state and are enabled (again) to kill tumor cells. The first blocking antibodies that were evaluated clinically were ipilimumab, nivolumab and pembrolizumab, and targeted ‘Cytotoxic T Lymphocyte Associated protein 4’ (CTLA4) and ‘Programmed cell Death-1’ (PD1). Clinical trials using these checkpoint inhibitors have shown promising results, with durable responses in a significant proportion of patients, in particular in melanoma patients [22-24]. Checkpoint inhibitors are part of the standard treatment portfolio and now FDA-licensed as new therapeutic agents for the treatment of patients with metastatic melanoma, non-small cell lung cancer, renal cell carcinoma, Hodgkin lymphoma and bladder

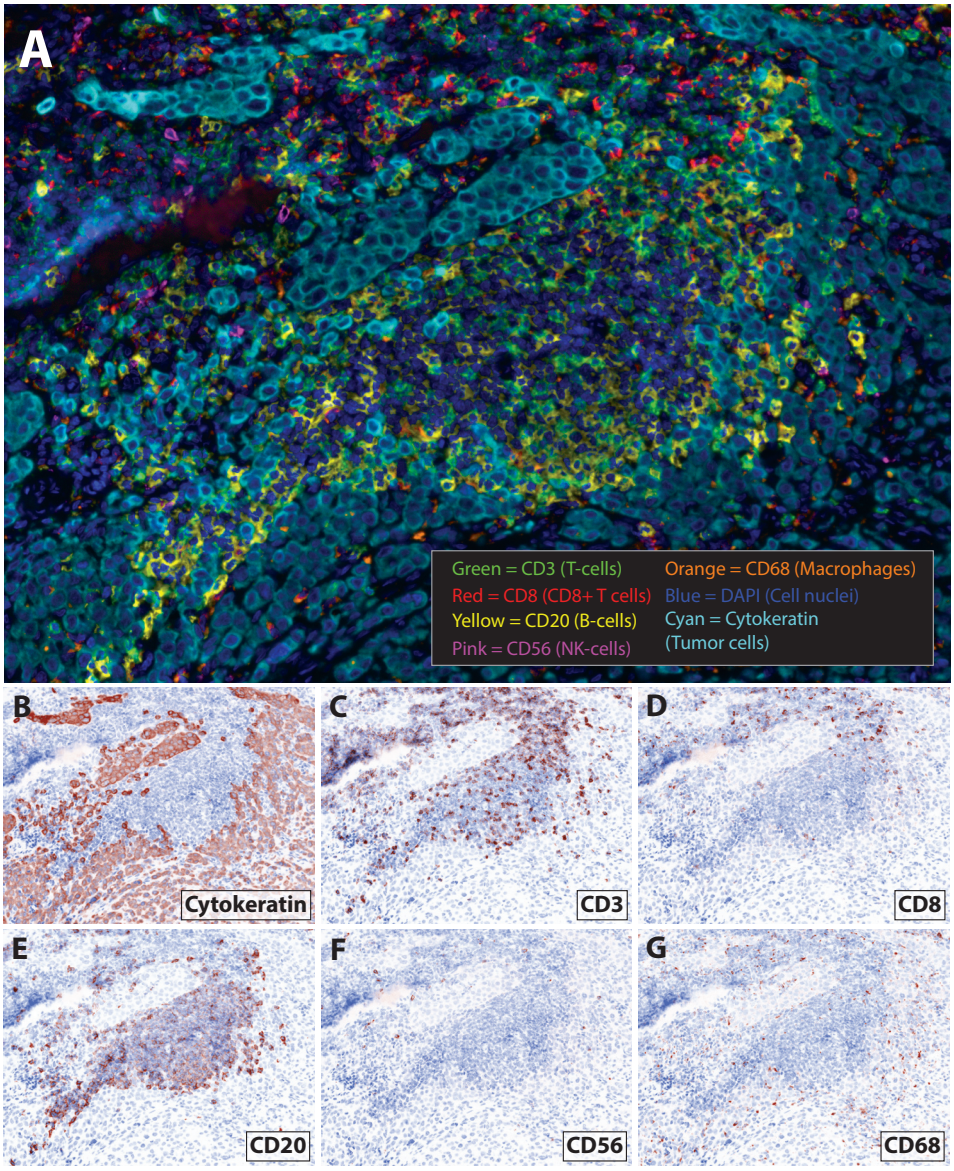

Figure 1: Intra-tumoral immune cells represent a diversity of different cell types.

1A: Multiplex staining of a lymph node metastasis of bladder cancer with antibodies directed to several Cluster of Differentiation (CD) markers that typically distinguish different immune cells. Four-micrometer thick formalin-fixed, paraffin-embedded (FFPE) whole tissue slides were probed using a tyramide signal amplification multiplexing technique and were exposed to the following antibodies and fluorophores: CD3 (Clone SP7; Sigma-Aldrich, St. Louis, United States), Opal 520; CD20 (Clone L26; SanBio, Uden, the Netherlands), Opal 620; CD8 (Clone C8/144B; SanBio), Opal 570; CD68 (Clone KP-1; SanBio), Opal 540; CD56 (Clone MRQ-42; SanBio), Opal 650; Cytokeratine (Clone AE1/AE3; Thermo Fisher, Waltham, United States), Opal 690; DAPI (PerkinElmer, Waltham, United States). All opal Dyes were also derived from PerkinElmer. Each multiplex stained slide was digitally processed by Vectra 3.0 InForm tissue finder software. **B-G)** bright field images of single markers as displayed in A produced with InForm tissue finder software. Figure was generated in laboratory of Tumor Immunology, Department of Medical Oncology, Erasmus MC.

cancer [25], but also for patients with tumor showing microsatellite instability, irrespective of tumor type.[26]

Adoptive T-cell therapy

The concept of adoptive T cell therapy is to transfer into patients autologous effector T cells that are able to find and destroy tumor cells. There are two major classes of adoptive T cell therapy.

Tumor Infiltrating Lymphocytes (TIL)

Rosenberg and colleagues explored the adoptive cell transfer (AT) of autologous T cells (derived from excised tumor tissues and rapidly expanded ***ex*** vivo) for the treatment of metastatic melanoma from 1988 onwards[27, 28], but it was only until 2002, when combining AT of TIL with prior lymphodepletion with fludarabine and cyclophosphamide that substantial clinical successes were obtained[29-31]. With the optimized protocol up to 60% clinical responses were obtained [29-31] . Figure 2A displays a graphical representation of AT with TILs. After these (single institution) successes with TIL therapy more studies in other centers followed, mainly focusing on the treatment of melanoma and with objective responses around 50% and complete responses around 20%.[32, 33] Also in renal cell carcinoma and ovarium cancer there are efforts made to use TIL therapy.[34] Key to the success of TIL therapy is the ability to isolate tumor reactive T cells from the tumor biopsy.

Receptor engineered T cells

As it is not always possible to obtain TILs from tumors, an alternative approach was taken, in which T cells from patients' blood are genetically engineered with immune receptors that endow these T cells with tumor specificity. The immune receptors can be antibody based, i.e., Chimeric Antigen Receptors (CAR) or T cell receptors (TCR) that specifically target an antigen of interest expressed by malignant cells. CARs are generally single-chain variable fragments (scFv) derived from monoclonal antibodies that are fused to a transmembrane and a signaling domain.[35] A graphical representation of AT with CAR/TCR engineered T cells is depicted in figure 2B. When introduced in T cells binding of the CAR with its antigen leads to T cell activation. Trials with first generation CARs showed limited success, but trials with second generation CARs have shown more promising results. Second generation CARs harbor an extra intracellular domain derived from co-stimulatory receptors, such as CD28 or 4-1BB (CD137). Specifically, adoptive T cell therapy with genetically modified second generation CD19 CAR T cells has shown impressive clinical results in hematological CD19 expressing B cell malignancies [36, 37]. Treatment of acute Lymphoblastic B-cell Leukemia (ALL) with CD19 CAR T cells resulted in complete responses between of 70 to 100%.[38] This recently resulted in the FDA approval of CD19 CAR T cell products for treatment of acute lymphocytic lymphoma (ALL), and certain types of non-Hodgkin lymphoma (NHL).[39]

The advantage of treatment of cancer with adoptive transfer of TCR transduced T cells is that

not only extracellular proteins, but also intracellular proteins can be targeted since intracellular proteins are also processed and presented as peptides by MHC molecules at the cell surface. The treatment with T cells expressing the NY-ESO1 TCR demonstrated clinical objective responses in 61% of the patients with synovial cell sarcoma and 55% of patients with melanoma (55%). This trial showed that T cell therapy with the NY-ESO1 TCR is an effective therapy for cancer patients are refractory to other treatments.[40] In solid tumors adoptive therapy with CAR- or TCR- engineered T cells in different tumor types do show clinical responses, but results are not yet as impressive nor long-lasting as with the CD19 CAR T cells.[32, 41] Of great importance in CAR and TCR T cell therapy is the choice of antigen that is targeted by the receptor. When the targeted antigen is expressed not only by the tumor, but also by healthy tissue, this could lead to severe (auto-immune like) toxicities.[32, 42] Also, due to the potency of T cells high levels of cytokines can appear in patients resulting in shock. This is called a cytokine release syndrome (CRS). Luckily, these cytokine storms are manageable and transient.[43]

FOCUS OF THIS THESIS: *IMMUNE MONITORING*

Despite the above-mentioned promise of immunotherapeutic approaches, a significant fraction of patients does not yet respond to these therapies. To date there is no tool available to select patients for immunotherapies as there is a lack of markers that robustly predict therapy outcome or provide sufficient understanding into the underlying mechanisms that define unsuccessful treatment or toxicity. It is of pivotal importance to obtain such markers, since this will lead to improved stratification of patients for immune therapy. Patient stratification can prevent unnecessary treatment and increase cost-effectiveness of these treatments. To discover predictive markers, extensive monitoring of immunological as well as clinical parameters within clinical trials is mandatory.

This thesis is split into two parts. In the first part, we have focused on immune monitoring tools in a CAR T cell phase-I trial to find parameters that correlate with in vivo behavior of administered T cells.

In the second part, we have monitored changes in T cells numbers, T cell clonality (number of different TCRs) and T cell phenotype in the blood compartment in patients receiving Dendritic Cell therapy in a phase-I trial. In addition, we have assessed the characteristics of intratumoral T cells in several soft tissue sarcoma (STS) subtypes in order to identify new parameters for immune monitoring in these tumor types.

PART 1: Immune monitoring in adoptive T cell therapy.

From 2003 until 2011, the laboratory of Tumor Immunology, Department of Medical Oncology, Erasmus Medical center has initiated and conducted a clinical phase I trial with CAR T cells targeting Carboxy-anhydrase IX (CAIX) to treat patients with clear cell renal cell carcinoma (RCC). Twelve patients with metastatic RCC expressing CAIX were treated with CAR T cells. In

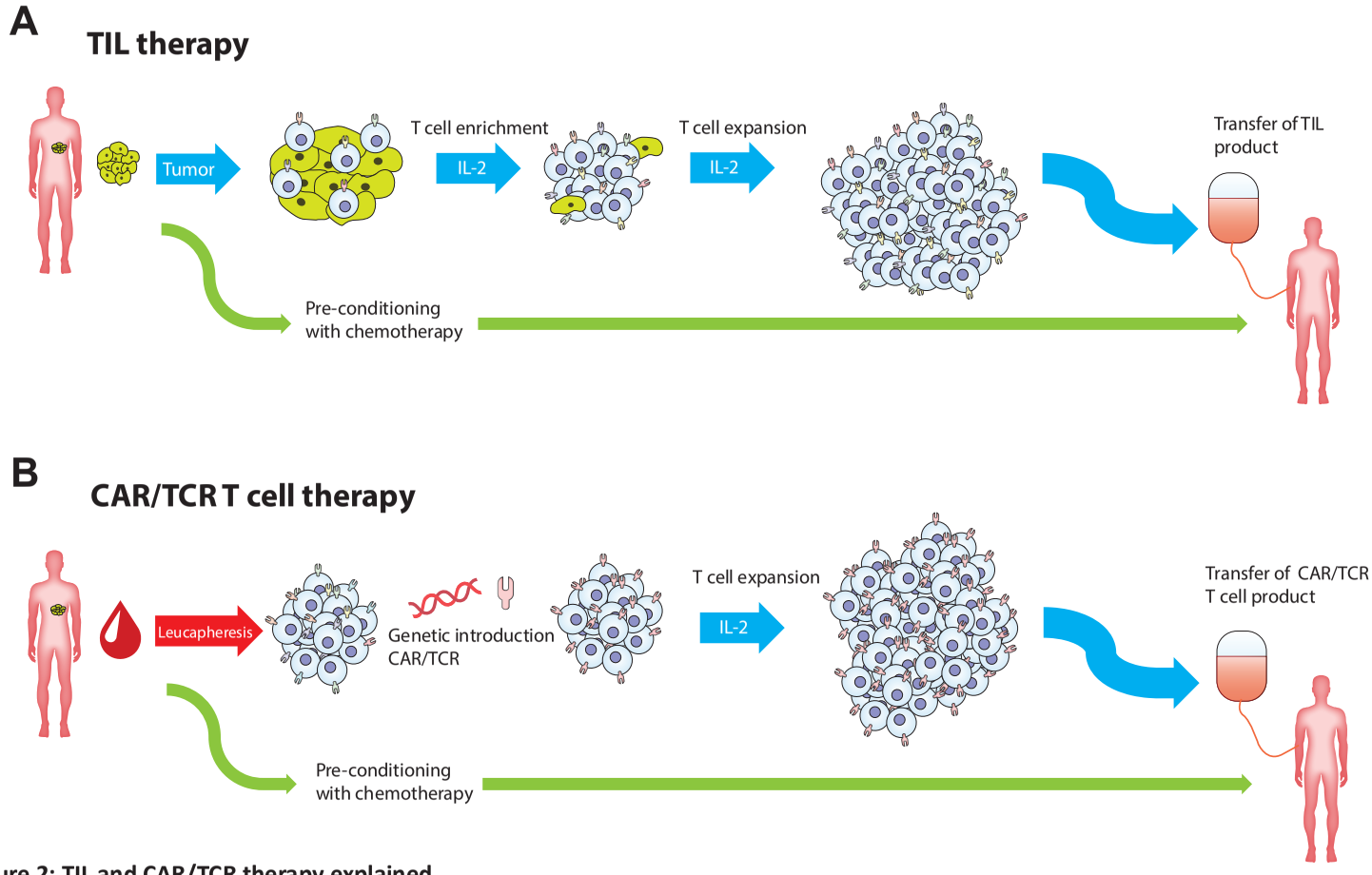

Figure 2: TIL and CAR/TCR therapy explained

A) Graphical representation of TIL therapy. Tumor is removed and processed into a single cell suspension. Subsequently TILs are enriched and expanded and transferred to patients following lymphodepleting chemotherapy. **B)** Graphical representation of CAR and TCR therapy. Patients undergo leukapheresis. Peripheral blood mononuclear cells are subsequently transduced with CAR or TCR-encoding genes and expanded with supportive cytokines. Then, the expanded CAR/TCR T cells are infused patients following lymphodepleting chemotherapy.

this trial no clinical responses were observed, and patients experienced transient toxicities due to CAIX expression in the bile ducts. This trial, the first of its kind in Europe, demonstrated that CAR T cells were highly potent *in vivo* as was reflected by the observed on-target/off-tumor toxicity**.** It remains not fully clear why no anti-tumor effects were seen against CAIX-expressing tumors. Importantly, in this trial, patients were extensively monitored by collecting and storing blood samples on multiple time points following start of treatment. This enabled in-depth of post infusion *in vivo* CAR T cell behavior and characteristics, which may explain the lack of anti-tumor effects. Along these lines, we have correlated gene-modified T cell numbers in blood with therapy effectiveness [36, 44-46], and we have looked into T cell features that predict peripheral persistence of the infused CAR T cells. In **Chapter 2,** we have studied cytokine profiles during ex vivo CAR T cell preparation, and identified cytokines are potential surrogate markers for CAR T-cell persistence in peripheral blood.

Next, we have extensively analysed the cellular composition of the infusion product in **Chapter 3**, in particular whether certain T cell characteristics, such as T cell maturation status in the CAR T cell infusion product relate to *in vivo* T cell expansion.

Chapter 4 provides an up-to-date and complete overview of patient characteristics, treatment schedules, CAIX CAR T cell characteristics, treatment observations and clinical observations of this phase I CAIX CAR T cell study. Subsequently, in **Chapter 5** we review current literature on adoptive T cell trials with a special focus on monitoring of the T cell product and patients pre-and post-treatment. Together these chapters give recommendations for design and monitoring strategies for future CAR/TCR T cell trials

PART 2: immune monitoring beyond adoptive T cell therapy.

The second part of this thesis also provides in-depth T cell monitoring, but in settings different from the above-mentioned adoptive T cell therapy.**Chapter 6** describes an extensive analysis of the immunological changes during the treatment of pleural mesothelioma patients treated with autologous monocyte derived dendritic cells that were loaded with mesothelioma tumor cell lysate. This phase-I trial demonstrated an impressive clinical response in one patient with almost 70% tumour size reduction, and in an additional 2 out of 9 patients stabilization of disease.[47] This chapter zooms in on changes in peripheral blood T cell phenotype and T cell clonality.

In **Chapter 7,** we have assessed T cell signatures of 5 different Soft Tissue Sarcoma (STS) subtypes, for which immune landscapes are less well described nor immune therapies are standardly considered. The aim of this study is to detect differences between STS subtypes to enable more rationalized design for (combinatorial) immunotherapies. Differences between STS subtypes in T cell numbers, phenotype, and clonality have been assessed and supplemented with assessments of immune gene analyses.

Finally, in **Chapter 8**, main results of chapters 2, 3, 6 and 7, and anticipated implications for T cell monitoring of cancers, and their consequences for future immune therapies are summarized and discussed.

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Chapter 2

Plasma IFN-γ and IL-6 levels correlate with peripheral T-cell numbers but not toxicity in RCC patients treated with CAR T-cells

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ABSTRACT

Autologous T-cells genetically modified to express a Chimeric Antigen Receptor (CAR) against carboxy-anhydrase-IX (CAIX) were administered to twelve patients with CAIX-positive metastatic renal cell carcinoma. Here, we questioned whether plasma cytokine levels following treatment or in vitro cytokine production from the T-cell infusion products could serve as predictors for peripheral T-cell persistence or in vivo T-cell activity. We demonstrated that CAR surface as well as gene expression are down-regulated following T-cell infusion, and that peripheral numbers of CAR T-cells are best captured by flow cytometry and not by qPCR. Numbers of CAR T-cells in blood correlated with plasma levels of IFN-γ and IL-6, but not with any of the other cytokines tested. Plasma IFN-γ or IL-6 levels did not correlate with liver enzyme values. Thus, out of 27 cytokines tested, IFN-γ and IL-6 levels in plasma are potential surrogate markers for CAR T-cell persistence in solid tumors.

INTRODUCTION

With the use of gene transfer technologies, T-cells can be genetically modified to stably express antibody molecules on their cell surface. The first generation of Chimeric Antigen Receptors (CARs) consisted of constructs in which an extracellular antibody-based recognition domain was combined with the intracellular signaling domain of the CD3-zeta (CD3ζ) or Fc(**ε**)RIγ chains into one single protein.[1] In the so-called second or third generation CARs, one or two additional intracellular co-signaling domains that are generally derived from the CD28 or 4-1BB co-stimulatory molecules, are added.[2, 3] The introduction of such an extra intracellular co-stimulatory domain increases the clinical effectivity of CAR T-cells, and coincided with enhanced in vivo persistence, and in vivo expansion of CAR T-cells.[2, 4-6] In recent years, CAR T-cell therapy has shown impressive clinical responses in hematological B-cell malignancies [7]. Also correlations between T-cell persistence and clinical effectivity have been described.[2, 4-6] In solid tumors, however, the number of clinical CAR T-cell studies has lagged behind with only a few clinical responses reported.[8, 9]

Obviously, in this emerging field there is a need for markers that provide information about early CAR T-cell persistence and in vivo activity.[7] With the increasing number of studies valuable data becomes available to perform such analysis.

One of the first CAR T-cell trials, performed at Erasmus MC Rotterdam, used a CAR:Fc(ε)RIγ directed to carboxy-anhydrase-IX (CAIX), an antigen that is over-expressed on Renal Cell Carcinoma (RCC). T cells were transduced with the CAIX CAR by the SFG γ-retroviral vector. [10] A total of twelve RCC patients were treated in this phase-I dose escalating trial with CAR T-cells and low dose IL-2 without prior chemotherapy in three separate cohorts (see materials and methods). Four out of eight patients in cohorts 1 and 2 experienced severe, but transient liver toxicities, which were most likely due to CAIX antigen expression on the surface of epithelial cells lining the bile ducts in the liver, and its recognition by the administered CAIX CAR T-cells. [11, 12] Another four patients in cohort 3 were pre-treated with CAIX monoclonal antibodies (mAb) to preferentially block CAIX in the liver but not in RCC lesions, a scheme that successfully prevented severe liver toxicity in these patients.[11, 12] Though we could demonstrate in vivo activity of CAIX CAR T-cells, as measured by the observed on-target liver toxicities, objective clinical responses were not seen.[11, 13] In the study described here, we measured the concentration of an extended set of cytokines in blood samples taken at multiple time points during treatment, as well as in culture supernatants from T-cell infusion products, and assessed whether or not cytokine values correlated with numbers of circulating T-cells that express CAR and/or liver toxicity. To the best of our knowledge, such information was not yet available for CAR T-cell treatments of a solid tumor.

MATERIALS AND METHODS

Patient treatment schedule and evaluation

Patients were diagnosed with clear cell RCC with progressive disease, not suitable for curative surgery, for whom no standard treatment existed, and with the primary tumor expressing CAIX.[12] Specific patient characteristics are described elsewhere.[11] Patients were treated after written informed consent, and treated according to three patient cohorts due to serious adverse events observed in the first patients treated.[11] In short, in cohort 1, it was aimed to assess toxicity and to establish the maximum tolerated dose (MTD) of CAIX CAR T-cells by an in-patient dose escalation scheme. Treatment consisted of intravenous administration of $2x10^7$ T-cells at day 1; $2x10^8$ T-cells at day 2; and $2x10^9$ T-cells at days 3-5 (treatment cycle 1) and days 17-19 (treatment cycle 2). Simultaneously patients received twice daily subcutaneous injections of IL-2, 5 x 10^5 IU/m^2 on day 1-10 and day 17-26. Because of liver toxicity, the schedule was changed in cohort 2. it was aimed to assess several dose levels starting at 1 x 10^8 CAR T-cells per infusion and extending to 2, 4, 8, 16, 20, 25, and 30 × 10^8 cells in subsequent dose levels, and applying a maximum of 10 T-cell infusions at days 1–5 and days 29–33 combined with IL-2, subcutaneously, 5 × 10^5 IU/m^2 twice daily at days 1–10 and days 29–38.

In cohort 3, patients were treated as in cohort 2, but received an extra intravenous infusion of 5 mg of the anti-CAIX mAb G250, 3 days before start of each series of CAR T-cell infusions, in order to block CAIX antigen in the liver and leaving accessible CAIX antigen at RCC tumor sites.[14-16] Patients from this latter cohort were not included in the analyses of in vivo parameters because of additional pre-treatment with anti-CAIX mAb, which has led to differences in T cell persistence between the first two cohorts versus the third cohorts.[11] For the analyses of infusion products, however, all patients and treatment cycles were included, as preparations of CAR T-cells were independent of patient cohorts and treatment cycles.

Preparation of CAIX CAR T-cell infusion products and their supernatants

Patient peripheral blood mononuclear cells (PBMCs) from leukapheresis were activated in a complete Mixed Medium (MixMed) [17] using 10 ng/mL CD3 mAb OKT3 (Janssen-Cilag Beerse, Belgium), without addition of exogenous IL-2. At days 2 and 3, T-cells were transduced with the CAIX CAR vector (batch #M4.50086; BioReliance, Sterling, UK) as described [10] in the presence of 100 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands). From day 4 onward, T-cells were expanded in complete MixMed supplemented with 360 IU/ml IL-2. Lymphocytes were counted every 2–3 days and adjusted to $0.5x10^6$ cells/ml by adding fresh culture medium and IL-2 until day 15. At culture days 2, 3, 4, 7, 9, 11 and 14 aliquots of culture supernatants were collected, cleared by centrifugation (10 min at 3000g) and stored at -70°C for retrospective analysis.

Blood samples

We obtained blood samples at regular intervals before, during, and after treatment for flow

cytometric analysis, isolation of PBMCs, genomic DNA, RNA and plasma was as described elsewhere. [13, 18] We aliquoted and cryopreserved PBMCs in liquid nitrogen, and stored genomic DNA, RNA and serum samples at -70°C.

Enumeration of transduced T-cells in infusion products and blood samples

Number of T-cells with membrane expression of CAIX CAR in cultures as well as blood was assessed by flow cytometry (FCM) using the anti-CAIX CAR idiotype mAb NUH82 [19] (kindly provided by Dr. E. Oosterwijk, Nijmegen, The Netherlands) (Limit of quantification: 0.01% CAIX-CAR+ cells within CD3+ cells).[18, 20] Gene expression of CAIX CAR was assessed by qRT-PCR. To this end, RNA was isolated from T cell cultures or blood samples using the ChargeSwitch Total RNA Cell Kit (Invitrogen, Carlsbad, CA, USA) and QIAamp® RNA Blood Minikit (Qiagen, Valencia, CA, USA), respectively, according to the manufacturer's guidelines. Complementary DNA (cDNA) synthesis was done using Reverse Transcriptase Superscript III (Invitrogen) under standard conditions. The quantitative real time PCR to detect CAIX CAR cDNA copies was performed as described previously.[18] CAIX CAR RNA levels in blood were only assessed in cohort 2 and 3. Additionally, genomic DNA was isolated from blood aliquots and T cell cultures using the QI Amp DNA mini kit (Qiagen, Hilden, Germany). The quantitative real time PCR to detect CAIX CAR DNA copies was again performed as described previously. [18]

Assessment of cytokine levels

The concentrations of IL-1β, IL-2ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, Eotaxin, FGF-Basic, G-CSF, GM-CSF, IFN-γ, IP-10 (CXCL10), MCP-1, MIP-1α, MIP-1β, PDGF-bb, RANTES, TNF-α, and VEGF in plasma samples and culture supernatants were determined using a commercially available 27-multiplex bead assay (BioRab Laboratories, Inc., Veenendaal, The Netherlands/Minneapolis, MN, USA) according to the manufacturers' instructions. For array specificities and lower limit of quantification levels, see Supplementary Table S1.

Statistical analysis

Cytokine levels in culture supernatant were normalized to the levels at day 2 (i.e., 40h after anti-CD3 mAb activation of PBMCs) and the cytokine levels in plasma were normalized to the baseline plasma levels at day 1. The Spearman correlation coefficient method was used to assess linear association between two continuous variables. P-values < 0.05 were considered significant. Differences between two categories with respect to paired continuous parameters were determined using an exact Wilcoxon rank sum test. Statistical analyses were performed with SPSS software (version 21) for Windows (IBM Corporation, Illinois, U.S.A.). Graphpad Prism v5.0 was used to prepare graphs and calculation of the Area Under the Curve (AUC) for cytokines and CAR T-cell numbers was performed using non-normalized data in linear X- and Y-axis plots.

RESULTS

Patients

In total, twelve RCC patients were treated with CAR T-cells. Specific patient characteristics are described elsewhere.[11] Four out of eight patients treated in the first two cohorts developed grades 3-4 liver toxicities and therefore three of them did not receive a second cycle of CAR T-cell infusions. Six out of eight patients developed anti-CAR cellular immune response, and 6 out of eight patients developed a humoral immune response. These responses became particularly prominent during and after treatment cycle two, compromising CAIX CAR T-cell numbers.[20] For this reason, we performed an analysis of circulating CAR T-cell numbers and cytokine levels during the first treatment cycle only(cohort 1: day 1 until 17, and cohort 2: day 1 until 29).

CAIX CAR T-cell numbers in patient blood peak between days 5- 8 after onset of treatment

CAIX CAR T-cells and CAIX CAR DNA copies were quantified in patient blood by FCM and qPCR, respectively (Fig. 1A,B) and were clearly detectable in all patients during the first cycle of CAR T-cell treatment. Since some of the patients received a second cycle of CAR T-cells at day 17, we only displayed the CAR T-cell numbers during the first 16 days. Patient 6 demonstrated very high levels of CAR DNA copies/µl at days 5, 10 and 16, which was in contrast to measurement by FCM. In addition, qPCR measurements of CAR DNA levels in patient 5 decreased between days 5 and 8 while the number of CAR T-cells as measured by FCM increased after day 5. Statistical analysis revealed a significant correlation between the number of CAIX CAR T-cells and CAIX CAR DNA copies (Spearman correlation; r=0.83; P=0.04) on day 5 only, but no significant correlation on any other day during the first treatment cycle.

CAR DNA copy numbers remain constant but do not reflect CAR expression by T-cells.

Using T-cell cultures, we observed a significant decline in CAIX CAR membrane expression (expressed as mean fluorescence intensity, MFI) from culture day 14 to 18 (i.e., treatment days 1 to 5). (Fig. 2A). Also, CAR RNA levels decreased during T cell culture, as presented for infusion products of day 1 versus 5 (Fig. 2B). Expectedly, CAR DNA levels remained constant (Fig. 2C). Analysis of patient blood after treatment showed a decline of the CAIX CAR RNA:DNA ratio (Fig. 2D), which extends the in vitro observation and suggests the existence of CAR DNA-containing T cells with a down-regulated expression of CAR. Of note, we calculated a relatively high RNA:DNA ratio for patient 6 at day 8 (Fig. 2D), because of an (unexpected relatively low CAR DNA level at day 8 when compared to day 5 and day 10 (Fig. 1B).

Kinetics of cytokine levels in patient plasma

We have analysed the levels of 27 cytokines in patient plasma at various time points during

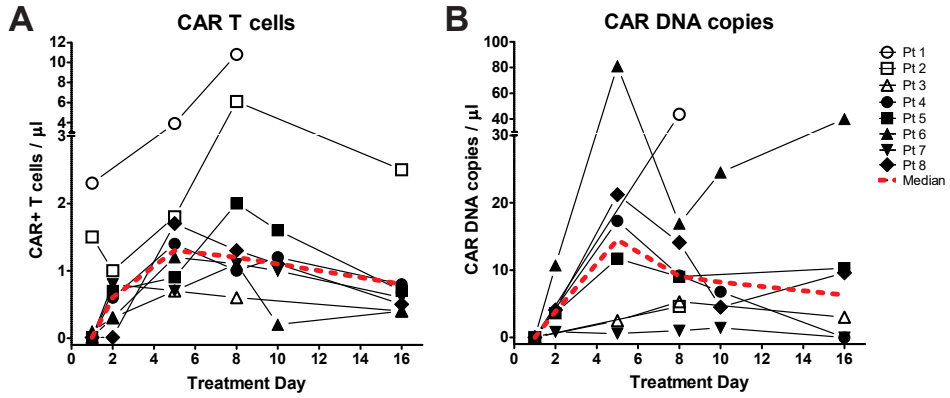

Figure 1: Kinetics of CAIX CAR T-cell numbers and CAIX CAR DNA copies in blood.

Patients were treated with CAIX CAR T-cells (days 1-3/5) and monitored for blood numbers of CAIX CAR T-cells by FCM **A)** and CAIX CAR DNA copy numbers by Q-PCR **B)** during and after treatment up to day 16 of treatment cycle 1. Results are expressed in numbers per microliter blood. Values for 8 individual patients treated in cohorts 1 and 2 and the median observation are shown.

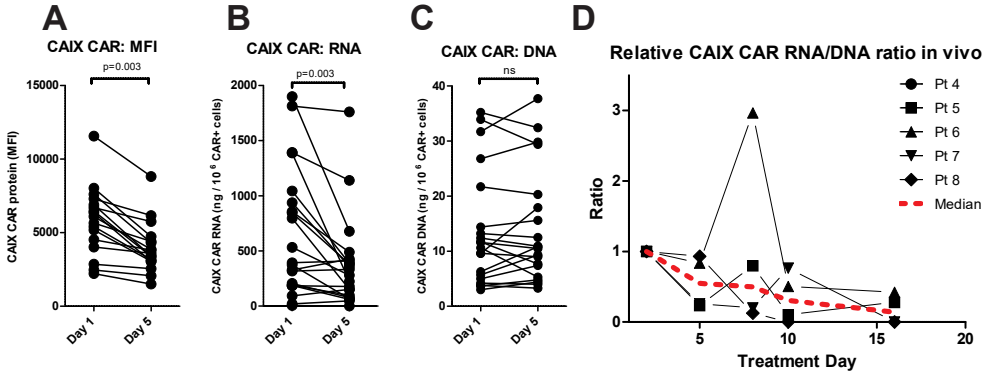

Figure 2: CAIX CAR expression decreases during T cell culture and after T cell infusion in patients.

Patient CAIX CAR T cell cultures at treatment days 1 and 5 (i.e., culture days 14 and 18) were analysed for **A)** CAIX CAR membrane expression by T cells, expressed as mean fluorescence intensity (MFI); **B)** CAIX CAR RNA levels, and **C)** CAR DNA levels, both expressed as ng per 10^6 CAR-expressing T cells. A-C: 19 paired samples; **D)** Patient blood samples after CAIX CAR T cell infusion were analysed for CAIX CAR RNA and DNA levels and data is presented as RNA:DNA ratio's relative to treatment day 2, i.e., 1 day after first single CAIX CAR T cell infusion; 5 patients from cohort 2 were included ratio of blood levels of CAIX CAR RNA over CAIX CAR DNA. Results are expressed relative to the value at treatment day 1 (day 1 ratio=1).

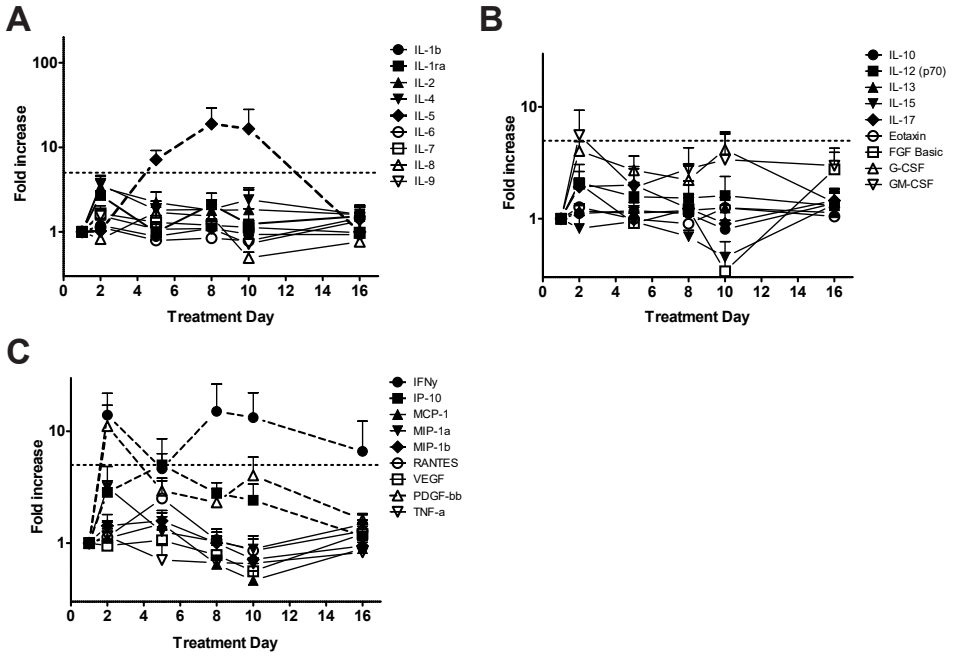

Figure 3: Kinetics of cytokines levels in patient plasma during CAIX CAR T-cell treatment.
Plasma samples were collected from 8 patients during and after CAIX CAR T-cell treatment up to day 16 and analysed for multiple cytokines using the Bio-Plex™ human cytokine 27-plex; for assay specificities see Supplemental Table S1. Patients presented with considerable variation in baseline (pre-treatment) cytokine levels, therefore we normalized cytokine levels relative to baseline for subsequent analysis. Cytokine levels relative to day 1 (pre-treatment) are presented in panels **A, B, C,** showing 9 cytokines each. Dashed lines represent cytokines demonstrating a > 5-fold increase on at least 1 time point and a > 2-fold increase at 2 or more time points after the first CAR T-cell infusion. Horizontal dotted lines indicate 5-fold increases relative to day 1.

treatment with CAR T-cells, see Supplemental Fig. S1. CAR T-cell therapy induced fluctuations in plasma levels of about half of the analysed cytokines. Most of these cytokines peaked at day 2 (after the first CAR T-cell infusion), and declined thereafter, whereas other cytokines either peaked at later time points (i.e., IL-5, IFN-γ, IP-10) or showed constant elevated levels, (i.e., G-CSF, GM-CSF, PDGF-bb). The mean levels of IL-5, IFN-γ, IP-10 and PDGF-bb showed an over 5-fold increase from baseline on at least 1 time point, and an over 2-fold increase from baseline at 2 or more time points after the first CAR T-cell infusion. The mean levels of the following cytokines showed an over 2-fold increase from baseline between days 2 to 16, i.e., IL-1ra, IL-2, IL-4, IL-12(p70), IL-17, G-CSF, FGF-Basic, G-CSF. (Fig. 3A-C).

Plasma levels of IFN-γ and IL-6 correlate to numbers of CAIX CAR T-cells in blood

In order to assess whether changes in plasma levels of cytokines were related to peripheral

persistence of CAR T-cells, we analysed the correlation between the absolute CAR T-cell numbers in blood and the plasma levels of individual cytokines. Since peak levels of either T-cell numbers or cytokines differed per patient between days 5 and 8, we used areas under the curve (AUC). AUCs were determined between the last day of T-cell infusion and the day at which CAR T cell numbers generally started to decline (day 5 and 8, respectively). Using this approach, we found strong correlations between CAIX CAR T-cell numbers as determined by FCM and IL-6 (Spearman correlation; r=0.86; P=0.01) as well as IFN-γ (Spearman correlation; r=0.73; P=0.04), but not for any of the other cytokines analysed (Fig. 4). No such correlation was found for CAIX CAR DNA copy numbers and cytokine levels.

IFN-γ and IL-6 are not constitutively produced by CAIX CAR T-cells

The different plasma cytokine patterns raised the question whether these might be the consequence of either constitutive or CAR-mediated production by the infused CAR T-cells. We previously showed that CAR T-cells upon CAIX specific interaction in vitro produced predominantly IFN-γ, and low levels of IL-5, TNF-α and IL-4.[13] To distinguish between constitutive and CAR-mediated cytokine production in this study, we assessed the cytokine production during the CAR T-cell culture period prior to T-cell infusion (Supplemental Fig. 2). In these cultures PBMCs were stimulated for 2 days with CD3-mAb (without exogenous IL-2), followed by CAR transduction and subsequent culture with IL-2 and without CAIX antigen until T-cell infusion. The CD3 mAb T-cell activation induced an initial burst in the production of many cytokines (day 2), after which we observed a clear decline in the accumulation of most of them, including IL-6 and IFN-γ (Fig. 5A,B; supplemental Fig. 2). These observations suggest that the elevated IL-6 and IFN-γ levels measured in plasma during and after therapy, are not constitutively produced by CAR T-cells nor that such production depends on IL-2. The latter observation is noteworthy since CAR T-cell treatment in patients was administered concomitantly by subcutaneous IL-2 injections up to day 10. Aside from IL-6 and IFN-γ, also IP-10 and PDGF-bb demonstrate similar patterns of production during CAR T-cell culture and in patient plasma.

Interestingly, a gradual increase of cytokines the levels during the 14 day culturing period was observed for IL-2 (added to the culture), IL-5 and IL-13, and to a lesser extent for IL-4, , IL-12(p70), FGF-basic, GM-CSF, MIP-1a, Rantes and VEGF (Fig. 5A). As previously reported[13], IL-5 production by (CAR) T-cells is mediated by IL-2; and indeed both the IL-5 levels in culture supernatants and in plasma clearly coincided with the application of exogenous IL-2. Accordingly, cessation of the in vivo IL-2 injections at day 10 was followed by a sharp decline in plasma IL-5 levels of the patients (day 16). From the cytokines that were constitutively produced by the cultured CAR T-cells at time of infusion, only IL-4 showed a weak raise in plasma levels at treatment day 2 (Fig. 3A). In addition, the day 2 elevated plasma levels of IL-1ra, G-CSF, GM-CSF, MIP-1a and TNF-a cannot be related to constitutive production by CAR T-cells. Collectively, our data support the notion that constitutively produced cytokines by CAR T-cells hardly affect plasma cytokine levels following CAR T-cell infusion.

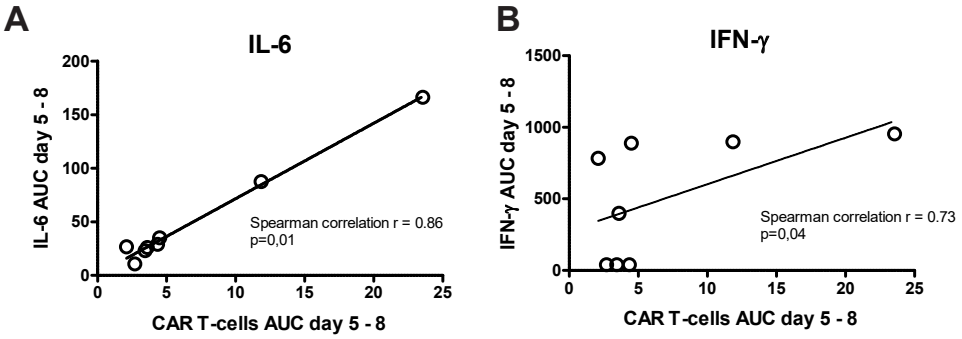

Figure 4: Plasma levels of IL-6 and INF-γ correlate with circulating CAIX CAR T-cell numbers

CAIX CAR T-cell numbers in blood, expressed as Area Under the Curve (AUC), between days 5 to 8 (i.e., during the 3 days following the last CAR T-cell infusion) for each individual patient were correlated with the absolute plasma levels of **A)** IL-6 and **B)** INF-γ, also expressed as AUC over same time period. Statistical significance was assessed by Spearman correlation analysis; p-value < 0.05 is considered significant.

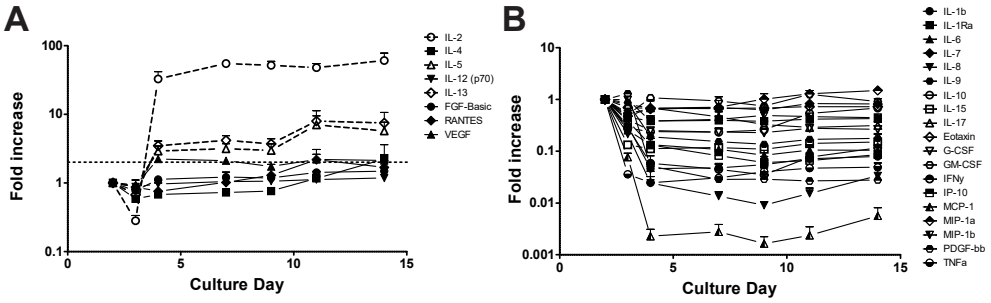

Figure 5: Kinetics of cytokine levels in supernatants of CAIX CAR T-cell infusion products

PBMCs were activated by soluble anti-CD3 mAb (days 0+1), transduced with the CAIX CAR (days 2+3) and subsequently expanded in an IL-2-supported culture up to day 16. Culture supernatants were collected every 2-3 days from day 2 onwards to day 16 and analysed for their cytokine levels using the cytokine 27-plex; for details see legend in Fig. 3. Kinetics of the absolute values (mean +/- standard error; n=5) per cytokine is shown in Supplemental Fig. S2. Cytokine levels in CAR T-cell culture supernatants relative to day 2 (post-activation) are presented in panels A, B; panel **A)** 8 cytokines with increasing levels. Dashed lines represent cytokines demonstrating an over 5-fold increase relative to day 2. Horizontal dotted line indicates a 2-fold increase relative to day 2; panel **B)** 19 cytokines with decreasing levels.

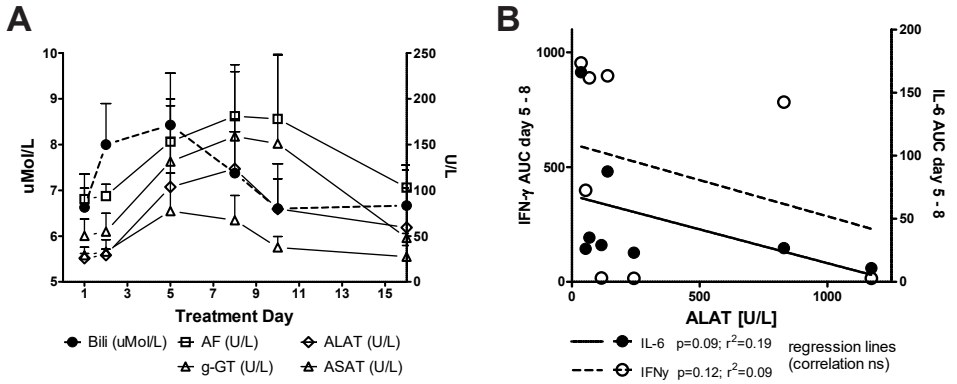

Figure 6: No significant correlation between plasma levels of cytokines and liver enzymes.
Blood samples from 8 patients during CAIX CAR T-cell treatment were and analysed for levels of liver enzymes and cytokines; panel **A)** presents levels of bilirubin (Bili), alkaline phosphatase (AP), gamma-glutamyl transpeptidase (γ-GT), aspartate transaminase (ASAT), and alanine transaminase (ALAT) (mean +/- standard error; n=8). Levels of liver enzymes were correlated with plasma cytokine levels; and correlations were found not to be significant; panel **B)** example of two non-significant correlations between the AUC of IL-6 and IFN-γ with the AUC of ALAT between days 5-8.

Plasma cytokine levels do not correlate with CAIX CAR T-cell activity in terms of liver toxicity

During treatment, all 8 patients showed slight to dramatic elevations of liver enzyme levels in blood, reaching Common Terminology Criteria for Adverse Events (CTCAE) grade 3-4 in 3 patients (patients 1, 3 and 8) during treatment cycle 1, highly likely due to recognition by CAR T cells of CAIX expressed on the bile duct epithelium. Blood levels of the liver enzymes bilirubin (Bili), alkaline phosphatase (AP), gamma-glutamyl transpeptidase (g-GT), aspartate transaminase (ASAT), and alanine transaminase (ALAT) during treatment are represented in Fig. 6A. The recognition of CAIX on the bile duct epithelium by the CAR T cells (so-called "on-target" toxicity) coincided with an inflammation reaction in the portal triangles around the bile ducts. The direct and indirect actions of the CAR T cells upon recognition of CAIX on bile duct epithelium, i.e., T cell effector functions (killing and cytokine release, a.o. IFN-γ) and induction of an inflammatory reaction respectively, lead to an increase of all liver enzymes. We used these liver enzyme elevations as a surrogate marker for CAR T cell activity (Supplementary Fig. 4). Although mean blood levels of AP, g-GT, ASAT, and ALAT displayed a similar kinetics as the numbers of CAR T-cells in blood (assessed with either FCM or Q-PCR) (Fig. 6A, and Fig. 1A-B), neither liver enzymes nor their derivative, the CTCAE toxicity grading, did significantly correlate with CAR T-cell numbers. Moreover, we could not demonstrate any correlation between plasma cytokine levels and liver enzyme levels or toxicity grading (Fig. 6B).

DISCUSSION

Here we provide an extensive analysis of cytokine plasma profiles in metastatic RCC patients treated with T-cells transduced with CAR:Fc(ε)RIγ specific for CAIX [11, 12], and assessed whether cytokine plasma levels relate to ***in vivo*** numbers and activity of CAR T-cells in terms of on-target liver toxicity.

CAIX CAR T-cells in patient blood were quantified by FCM for CAR protein and qPCR for CAR DNA. However, in contrast to a previous report [21], we did not find significant correlations between numbers of CAIX CAR T-cells and CAIX CAR DNA copies, except for the last treatment day (day 5). In addition to existing variation in CAR DNA copy numbers in T-cell preparations between individual patients [11], we would like to argue that CAR DNA levels do not directly reflect the level of CAR membrane expression and may in fact overestimate the number of T cells that are reactive to CAIX in vivo. Notably, a decrease in CAR surface expression was accompanied by a decrease in CAR mRNA expression in T cell infusion products as well in blood following T-cell treatment, possibly the consequence of decreased gene transcription in non-activated T-cells. In a previous study, using a similar γ-retroviral vector backbone, a decreased expression has also been observed for a TCR transgene, which was attributed to a waning LTR-driven gene transcription due to metabolic quiescence of gene-transduced T-cells.[22] This loss of transgene expression might be vector dependent as so far no such event has been described in other studies using different vector backbones.

Our observation that following T-cell infusion, CAR RNA:DNA ratio in blood decreased suggests that circulating CAR T-cells have not been activated in vivo. Yet, blood cytokine signature suggests in vivo CAR T cell activation. Therefore we anticipate that CAR T cells that have encountered CAIX target antigen (in tumor and liver) and have released cytokines will be located in the respective tissues.

Most CAR T-cell trials, especially in solid tumors, have monitored only a limited number of blood cytokines aside from IFN-γ and TNF-α. Some CD19 CAR T-cell trials reported data on multiple cytokines and showed similar plasma patterns as described in the current study, including a prominent increase of IFN-γ and IP-10 levels in the first days after a (single) CAR T-cell infusion, and subsequent normalization to baseline.[3, 4, 6, 21, 23] In the current study, IFN-γ increased >15 fold , though not as high as in a second generation CD19 CAR T-cell trial where a >1000 fold increase of IFN-γ levels was observed.[21] The prominent increase in plasma IFN-γ levels in the latter studies may be attributed to the extent of the disease burden in these hematological patients as well as the co-stimulatory format of the CAR used yielding higher activity. The increased levels of IP-10 and PDGF-bb may be the consequence of increased levels of IFN-γ since the latter cytokine is known to prime IP-10 and PDGF-bb production by amongst others, monocytes, endothelial cells and fibroblasts.[24, 25]

In our study, we also observed a clear increase in IL-5 plasma levels in vivo, which, based on our in vitro data, we link to IL-2-induced production of this cytokine. Aside from our own ob-

servations, others also described that IL-2 can induce production of IL-5 and IL-13 by T-cells.[26] Accordingly, in another CAR T-cell trial targeting Epidermal Growth Factor Receptor 2 (HER2) positive sarcoma without IL-2 administration, no changes in IL-5 or IL-13 plasma levels were observed.[27] In the reported CD19 CAR T-cell trials no IL-2 was co-administered to the patients, which may explain that no changes in IL-5 plasma levels were reported.[6] Thus, our current observation strongly suggests that the elevation in IL-5 plasma levels is rather an IL-2 induced effect than a demonstration of CAR T-cell activity upon CAIX-antigen recognition.[13]

In the recently reported CD19 CAR T-cell trials, plasma cytokine levels of GM-CSF, IL-2, IL-10, TNF-α, IFN-γ and in particular IL-6 were closely monitored because these cytokines were strongly elevated in patients with clinical symptoms of a cytokine storm.[2-4, 6, 21, 23, 28-32] In CD19 CAR T-cell studies, cytokine storms are a frequent observation and are probably mainly due to the extended tumor burden in these patients with a high load of accessible targets together with highly active CAR T-cells. In our CAIX CAR T-cell trial only one patient showed a high increase of IL-6 plasma levels. Interestingly, this particular patient received the highest CAIX CAR T-cell dose, but did not present with clinical symptoms of a cytokine storm.

All patients demonstrated peak numbers of CAR T-cells as well as peak levels of cytokines between the last day of infusion (day 5) and day 8, i.e., up to 3 days after the last T-cell infusion. A correlation was observed between levels of IL-6 and IFN-γ and number of CAR T-cells, suggesting that these cytokines represent a measure of ***in vivo*** CAR T-cell activity. In fact, plasma levels for these cytokines may reflect exposure of CAR T-cells to CAIX antigen since days 5-8 post infusion PBMCs produced significant levels of IFN-γ after ***ex vivo*** CAIX recognition.[13] From the kinetics of IL-6 and IFN-γ in CAR T-cell culture supernatant it can be concluded that it is unlikely that constitutive production or IL-2 is involved in phenomenon. In addition, no correlations were found between the absolute number of B-cells, CD4+, CD8+, or CD15+CD56+ cells in patient blood and plasma cytokines. Of note, no correlations were found between the CAIX CAR DNA copy numbers and plasma cytokine levels. This contributes to our earlier statement that CAR T-cells measured by FCM may give a better representation of the in vivo presence of functional CAR T-cells since CAR DNA measured by qPCR also detects CAR T-cells with down-modulated CAR expression and thereby not functional CAR expressing cells.

In addition to peripheral T-cell persistence, we attempted to correlate plasma cytokine levels to liver enzyme abnormalities as a measure of on-target effects, and assessed whether plasma levels of liver enzymes could serve as correlates for ***in vivo*** activity of CAIX CAR T-cells towards the CAIX antigen expressed on the bile duct epithelium.[11, 12] Despite apparently similar kinetics of some of the liver enzymes, CAR T-cell numbers and some cytokines, no statistically significant correlations were found between the levels of liver enzymes and the latter two parameters. This is probably due to the fact that T-cell numbers and cytokine levels in the periphery do not adequately reflect the presence and activity of CAR T-cells in the liver parenchyma and bile ducts.

In conclusion, in the current study we found a correlation between plasma levels of IL-6

and IFN-γ and peripheral numbers of CAIX CAR T-cells but not with liver toxicity. We argue that monitoring of CAR T cells in patients should preferably be performed by FCM using an anti-CAR antibody to specifically detect those T cells reactive towards target antigen. Even though the number of patients in our analysis was limited, and our analyses may be underpowered to detect differences with respect to other cytokines, we advocate the measurements of plasma levels of IFN-γ and IL-6 during T-cell therapy trials. We argue that these two cytokines serve as indicators for T-cell persistence. Further studies are warranted to establish whether IFN-γ and IL-6 are associated with anti-tumor activities of gene-modified T-cells.

ACKNOWLEDGEMENTS

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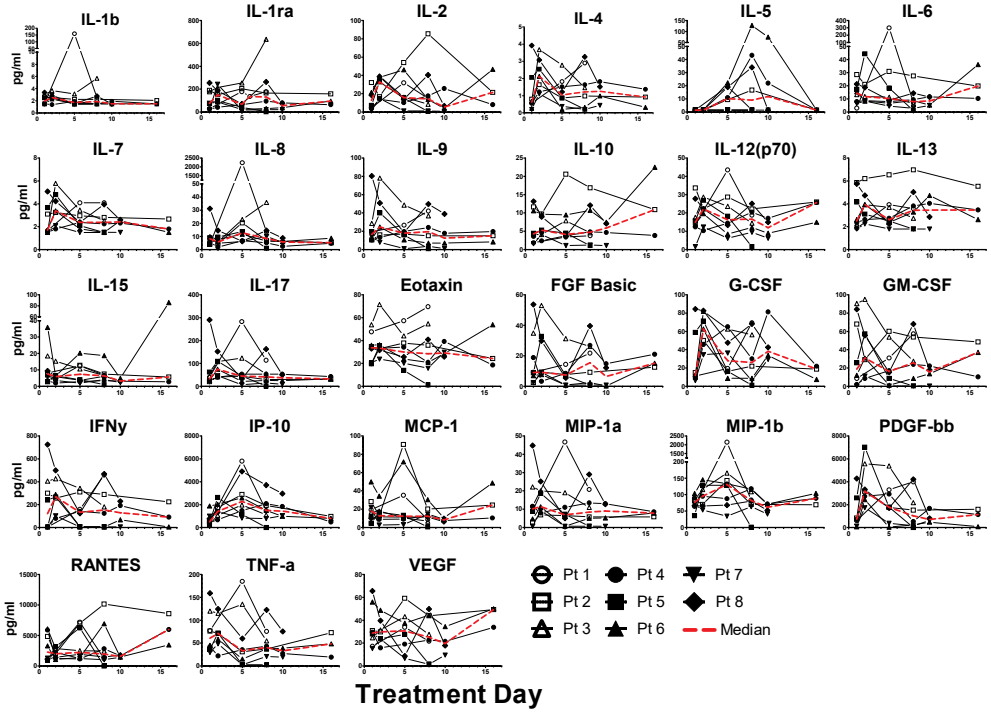
SUPPLEMENTARY DATA

	Lower limit of detection	Range standard curve (pg/mL)	
CYTOKINE	[PG/ML]*	MIN	MAX
Hu IL-1b	*3,1*	*3,2*	*52178*
Hu IL-1ra	*5,0*	*5,2*	*85727*
Hu IL-2	*1,3*	*1,3*	*21614*
Hu IL-4	*0,4*	*0,4*	*6303*
Hu IL-5	*3,1*	*3,1*	*50609*
Hu IL-6	*2,2*	*2,3*	*38185*
Hu IL-7	*3,1*	*3,1*	*50240*
Hu IL-8	*1,9*	*1,9*	*31062*
Hu IL-9	*2,0*	*2,1*	*34790*
Hu IL-10	*2,2*	*2,2*	*36166*
Hu IL-12(p70)	*3,2*	*3,3*	*53588*
Hu IL-13	*3,6*	*3,7*	*60505*
Hu IL-15)	*2,1*	*2,1*	*34157*
Hu IL-17	*2,7*	*3,1*	*50051*
Hu Eotaxin	*2,3*	*2,3*	*37440*
Hu FGF basic	*1,6*	*1,6*	*26755*
Hu G-CSF	*2,4*	*2,4*	*40031*
Hu GM-CSF	*0,9*	*1,2*	*19070*
Hu IFN-g	*5,0*	*3,2*	*52719*
Hu IP-10	*6,8*	*6,7*	*109911*
Hu MCP-1(MCAF)	*1,4*	*1,4*	*22566*
Hu MIP-1a	*1,5*	*1,4*	*22566*
Hu MIP-1b	*2,1*	*2,0*	*33283*
Hu PDGF-bb	*3,3*	*3,2*	*51933*
Hu RANTES	*2,2*	*2,2*	*35250*
Hu TNF-a	*5,6*	*5,8*	*95484*
Hu VEGF	*3,3*	*3,4*	*56237*

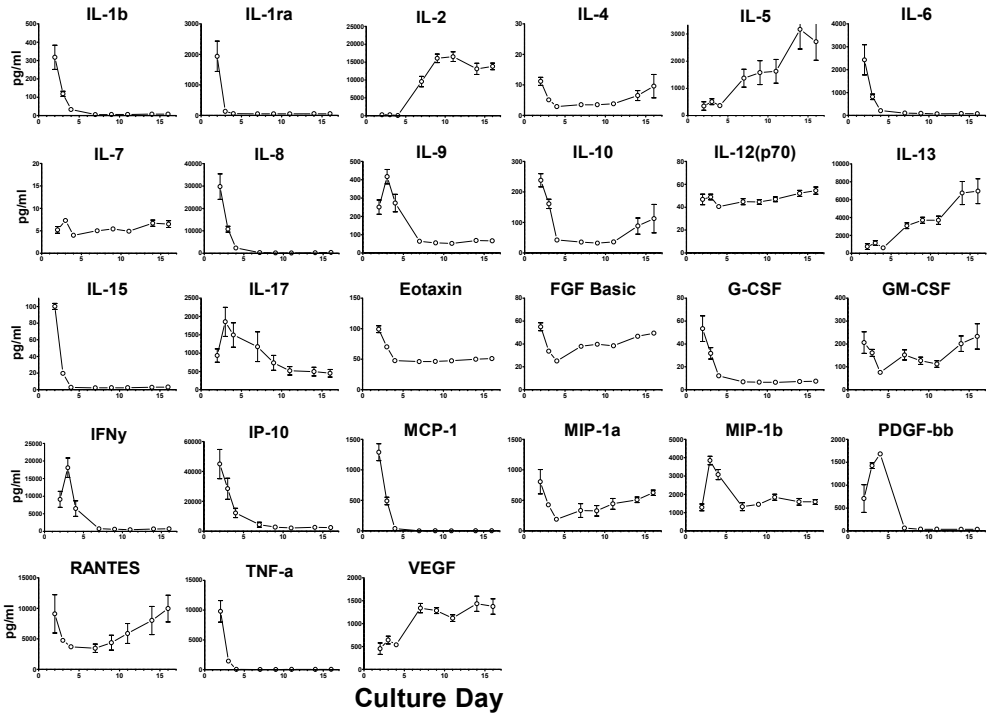
Supplementary Table S1 - Bio-plexTM human cytokine 27-plex bead array specificities

* L.O.D. is defined as the value calculate d from the standard curve at the point of 2 standard deviations above the mean background MFI (ten replicates).



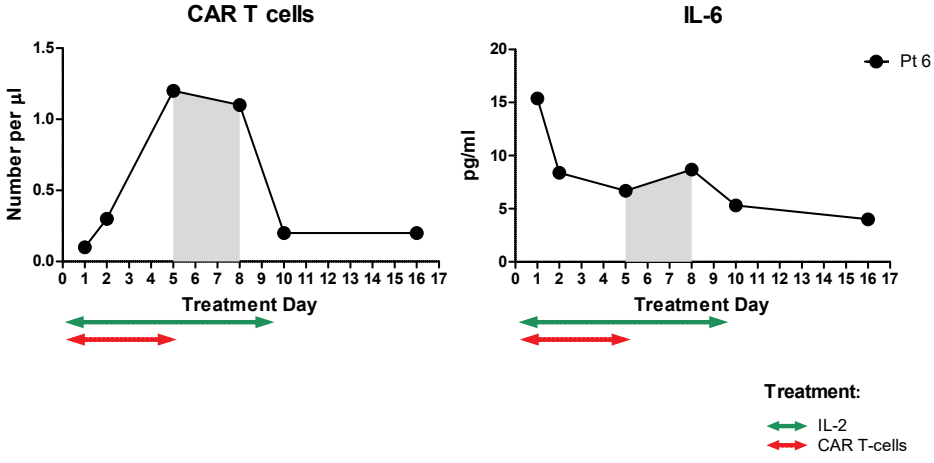
Supplemental Figure 1: Kinetics of cytokines levels in patient blood during CAIX CAR T-cell treatment.

Plasma samples were collected from 8 patients during and after CAIX CAR T-cell treatment up to day 16 of treatment cycle 1 and analyzed for multiple cytokines using the Bio-PlexTM human cytokine 27-plex; for assay specificities see Supplemental Table S1. Absolute values for individual patients and median observations are shown per cytokine and per time point



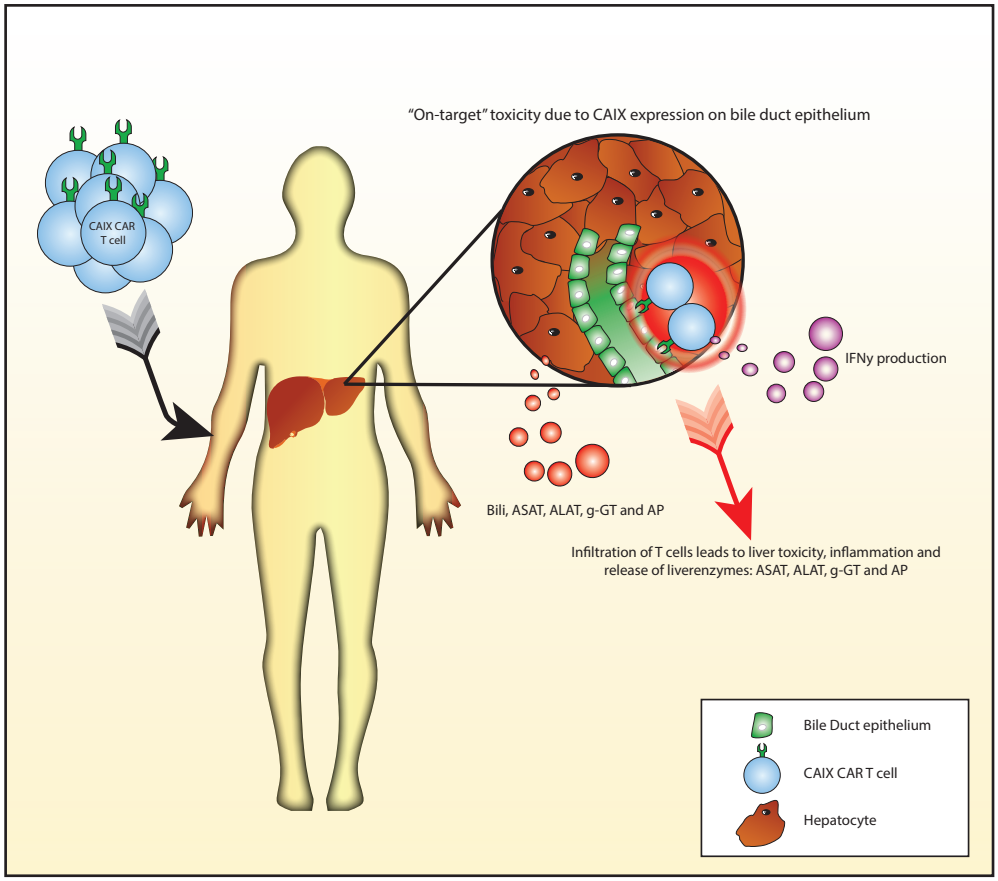
Supplemental Figure 2: Kinetics of cytokine levels in CAIX CAR T-cell culture supernatant.

PBMC were activated by soluble anti-CD3 mAb (days 0+1), transduced with the CAIX CAR (days 2+3) and subsequently expanded in an IL-2-supported culture up to day 16. Culture supernatants were collected every 2-3 days from day 2 onwards to day 16 and analyzed for their cytokine levels using the cytokine 27-plex; Absolute values (mean +/- standard error; n=5) are shown.



Supplemental Figure 3: AUC calculations of cytokine levels

Area under the curves (AUCs) were determined between the last day of T-cell infusion and the day at which CAR T cell numbers generally started to decline (day 5 and 8, respectively). Graphpad Prism v5.0 was used to prepare graphs and calculation of the Area Under the Curve (AUC) for cytokines and CAR T-cell numbers and was performed using non-normalized data in linear X- and Y-axis plots.



Supplemental Figure 4: On target toxicity as a surrogate marker of T cell activity.

On target toxicity due to recognition of CAIX on the bile duct epithelium by the CAIX CAR T cells. Upon recognition of CAIX on bile duct epithelium CAR T cells exert direct T cell effector functions, such as killing and cytokine release (amongst others, IFN-γ), and induced an inflammatory reaction. It is assumed that this sequence of actions has led to an increase of all liver enzymes. We used these liver enzyme elevations as a surrogate marker for CAR T cell activity.

Chapter 3

T cell maturation stage prior to and during GMP processing informs on CAR T cell expansion in patients.

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ABSTRACT

Autologous T cells were genetically modified to express a Chimeric Antigen Receptor (CAR) directed towards carboxy-anhydrase-IX (CAIX) and used to treat patients with CAIX-positive metastatic renal cell carcinoma. In the present study, we questioned whether the T cell maturation stage in the pre-infusion product affected CAIX CAR expression and function ***in vitro*** as well as ***in vivo*** CAR T cell numbers and expansion. During the 14 days expansion of CAR T cells prior to administration, we observed shifts from a predominant CD4 to a CD8 T cell phenotype, and from a significant fraction of naïve to central effector T cells. Surface expression of the CAR was equally distributed among different T cell subsets and T cell maturation stages. During T cell culture days 14 to 18 (which covered patient treatment days 1 to 5), T cells demonstrated a decline in CAR expression level per cell irrespective of T cell maturation stage, although the proportion of CAR positive T cells and CAR-mediated T cell effector functions remained similar for both CD4 and CD8 T cell populations. Notably, patients with a higher fraction of naïve CD8 T cells at baseline (prior to genetic modification) or central effector CD8 T cells at 2 weeks of CAR T cell culture demonstrated a higher fold expansion and absolute numbers of circulating CAR T cells at 1 month after start of therapy. We conclude that the T cell maturation stage prior to and during CAR T cell expansion culture is related to *in vivo* CAR T cell expansion.

INTRODUCTION

Despite clinical successes in B-cell malignancies, adoptive transfer of T cells genetically modified with chimeric antigen receptor (CARs) or T cell receptors (TCRs) to treat solid tumors is challenged by limited patient responses.[1] The efficacy of adoptive T cell therapy (in hematological malignancies) correlates with numbers and persistence of circulating modified T cells [2-5]. Building on this notion, several approaches have been explored to improve persistence of genetically modified T cells ***in vivo***. For example, the introduction in receptors of intracellular domains from the CD28 and/or CD137 co-stimulatory molecules has led to an increased CAR T cell persistence as well as expansion ***in vivo***, and consequently in clinical responses.[6, 7]

Furthermore, preclinical studies in mice and monkeys suggest that improved ***in vivo*** persistence and anti-tumor responses are obtained when T cells in early stages of differentiation (such as naïve or central memory cells) are used for genetic modification and treatment[8, 9]. In fact, the differentiation state of $CD8^+$ T cells appeared to be inversely related to their capacity to proliferate and persist.[10, 11] We have previously generated CAR T cells directed against carboxy-anhydrase-IX (CAIX) and treated patients with CAIX-positive metastatic renal cell carcinoma (mRCC).[12] Here, we assessed T cell maturation stage prior to and during CAR T cell expansion cultures, analyzed whether the T cell maturation stage affects CAR expression and function ***in vitro*** as well as the ***in vivo*** properties of CAR T cells, in particular expansion potential and absolute numbers of circulating CAR T cells.

MATERIALS AND METHODS

Patients and treatment

Patients, diagnosed with CAIX-positive mRCC and for whom no standard treatment was available, were included in this phase-I trial. Patients were treated in 3 cohorts, and aimed to assess toxicity and to establish the maximum tolerated dose of the number of CAR T cells. Treatment schedule was previously presented [13] and was, in brief: in cohort 1, treatment consisted of intravenous administration of $2x10^7$ T-cells at day 1, $2x10^8$ T-cells at day 2, and $2x10^9$ T-cells at days 3-5 (treatment cycle 1) and days 17-19 (treatment cycle 2). Simultaneously, patients received twice daily subcutaneous injections of IL-2 at 5 x 10^5 IU/m^2 on days 1-10 and days 17-26. Because of liver toxicity [13] this schedule was changed in cohort 2, to a classic 3x3 dose-escalating phase I schedule starting at 1 x 10^8 CAR T-cells per infusion and extending to 2, 4, 8, 16, 20, 25, and 30 × 10^8 cells at subsequent dose levels, and applying a maximum of 10 T-cell infusions at days 1–5 and days 29–33 combined with sc IL-2 at 5 × 10^5 IU/m^2 twice daily at days 1–10 and days 29–38. In cohort 3, patients were treated as in cohort 2, but received an extra iv infusion of 5 mg of the anti-CAIX mAb G250, 3 days before start of each series of CAR T-cell infusions, in order to block CAIX antigen in the liver and leaving accessible CAIX antigen at RCC tumor sites.[14-16] For the analyses of CAR T cell persistence, only patients treated in cohort 2 and 3 were as-

sessed until day 29, as from day 29 eight out of nine patients received a second treatment cycle of CAR T cells. Since patients in cohort 1 received varying numbers of CAR T cells, and 1 patient already received a second cycle of CAR T cells on days 17-19, cohort 1 was not assessed in the persistence analyses.

Patients did not receive lympho-depleting pretreatment. The clinical protocol and amendments were approved by governmental regulatory authorities (Central committee on research involving Human Subjects) as well as the Erasmus MC institutional medical ethical review board. The clinical protocol (DDHK97-29/P00.0040C) adheres to the declaration of Helsinki protocols. Patient characteristics are detailed elsewhere.[13]

T cell infusion product and post-treatment blood sampling

Patient peripheral blood mononuclear cells (PBMC) from leukapheresis (n=9) were activated at day 0 with soluble anti-CD3 mAb OKT3 (10ng/mL) without IL-2. At days 2 and 3 T cells were retrovirally transduced with the CAIX CAR in the presence of 100 IU/ml IL-2. From days 4 to 18 T cells were expanded in medium supplemented with 360 IU/ml IL-2. Patients were treated with 5 daily infusions of 'fresh' CAIX CAR T cells harvested from culture at days 14 to 18[13, 17]. We obtained blood samples at regular intervals before, during, and after treatment for direct flow cytometric (FCM) analysis and isolation and cryopreservation of PBMC in liquid nitrogen[18, 19]

Flow cytometry

CAIX CAR-positive T cells in cultures and blood samples were assessed by FCM using the anti-CAIX CAR idiotype mAb NuH82, as described in [18] and supplementary figure S1. The starting T cell product (PBMC from leukapheresis) and T cell cultures were analyzed for various lymphocyte subsets, a.o. CD4+ and CD8+ T cells and T cell maturation subsets using following markers: CD27, CD28, CD45RA, CD45RO, CD62L and CCR7. The starting T cell product (PBMC from leukapheresis) is referred to the "baseline" sample or measurement in the rest of this study. In addition, T cell maturation subsets were analyzed for CAR-expression (supplementary figure S1) and CAR-mediated effector function in response to CAIX+ RCC cell line (SKRC-17 MW1-clone4) by means of up-regulated expression of CD107. Information on antibodies and staining combinations used in multi-color FCM is specified in supplementary Table S1. Samples were measured on the FACS Canto, and analyzed with FCS express v. 4.07 software (De Novo software). Gating strategy to determine the T cell maturation stage is demonstrated in Supplementary Fig 1A+B.

Statistical analysis

Statistical analyses were performed with SPSS software (version 21) for Windows (IBM Corporation, Illinois, U.S.A.). Graphpad Prism v5.0 was used to prepare graphs.

RESULTS

T cell phenotype

Patient PBMC at baseline (from leukapheresis) were activated, transduced with the CAIX CAR and expanded. During the 14 days of expansion, the phenotype of the T cells shifted significantly. Although the extent varied per patient, T cell cultures reproducibly demonstrated a shift from a $CD4^+$ to a $CD8^+$ predominance compared to baseline (Figure 1A, supplementary Figure S2). During the 18 days culture period, individual markers expressed on $CD3^+$ T cells demonstrated a clear shift, especially during the first 14 days. (Supplementary Figure S3). When assessing CD8 T cell maturation according to the markers CD45RA, CD45RO, CD27 and CD28, we observed that during culture the most prevalent subset drastically shifted from T_{ES} at baseline to T_{EM} at day 14. (Figure 1B; see legend to Figure 1 for the definition of maturation stages: T_N (Naïve), T_{INT} (Intermediate between T_N and T_{CM}), T_{CM} (Central Memory), T_{EM} (Effector Memory), T_{CE} (Central Effector) and T_{ES} (End Stage) T cells). Following T cell culture, there was also a decrease in the fraction of T_N and an increase in the fraction of T_{CE}, yet the overall change was in favor of younger T cells (T_{CM} + T_{EM}) at the expense of further maturated T cells (T_{CE}+T_{ES}). $CD4^+$ T cells showed also a culture-dependent decrease in the fraction of T_N and an increase in the fraction of T_{EM}, yet the most prevalent subset at baseline, i.e., T_{CM}, remained unchanged (Figure 1C). Interestingly, in contrast with $CD8^+$ T cells cultured $CD4^+$ T cells harbored almost no T_{CE} cells. Additional analysis with different T cell maturation markers (CD45RA, CCR7 (CD197) and CD62L) showed high concordance with the maturation stages and kinetics as described above (Supplementary Figure S4).

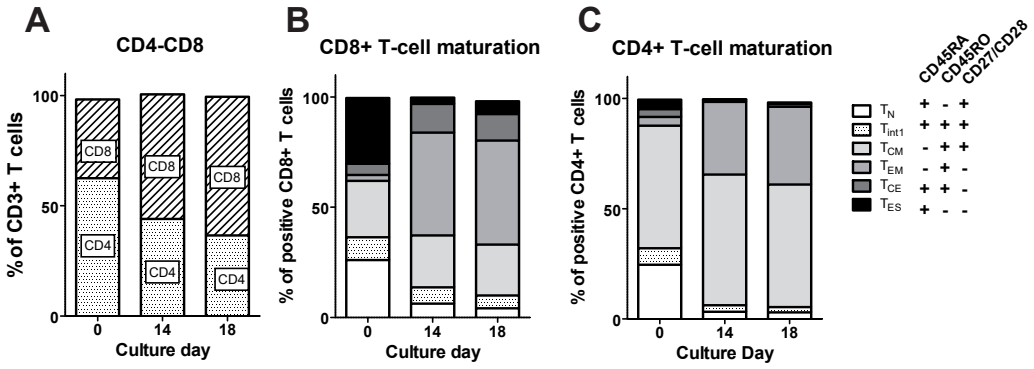

Figure 1: T cell phenotype and maturation during clinical T cell expansion cultures.

A) Proportions of CD4 and CD8 T cells at baseline (leukapheresis, day 0) and culture day 14. Data of individual patients are presented in supplementary Figure S1. Maturation stages of CD8+ **B)** and CD4+ **C)** T cells at baseline and culture day 14 and at day 18 defined according to the expression of CD45RA, CD45RO and CD27/CD28 as indicated in insert: Naïve, T_N: CD45RA+, CD45RO-, CD27/CD28+; Central Memory, T_{CM}: CD45RA-, CD45RO+, CD27/28+; Effector Memory, T_{EM}: CD45RA-, CD45RO+ or CD27/CD28-; Central Effector, T_{CE} : CD45RA+, CD45RO+, CD27/CD28-) and End Stage T_{ES}: CD45RA+, CD45RO-, CD27/CD28- T cells. A small fraction was CD45RA+, CD45RO+ and CD27/CD28+ and this population was defined as interphase (Int1) T cells. Data are presented as stacked bars of means of 9 patients.

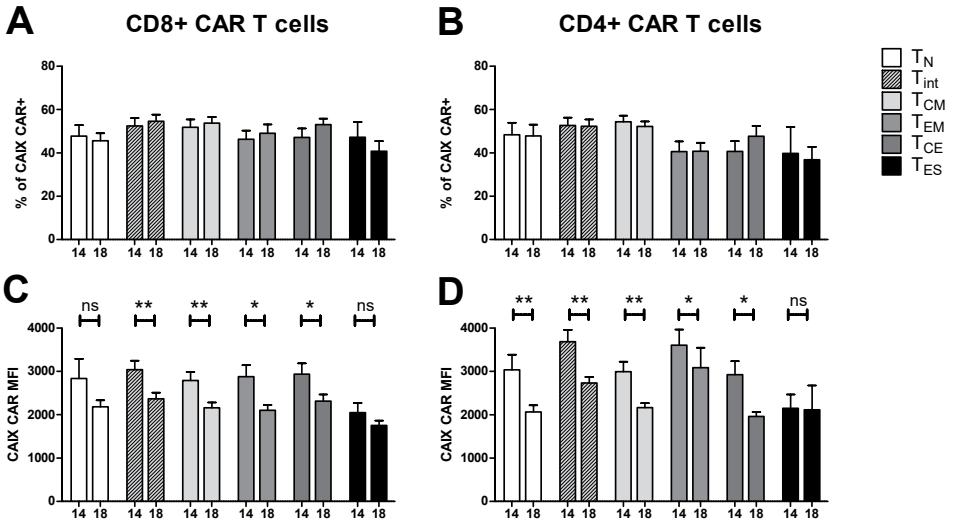

Figure 2: CAIX CAR expression of T cells in infusion products according to T cell maturation.
Proportions of CAIX CAR-positive CD8+ **(A)** and CD4+ **(B)** T cells and CAIX CAR expression levels (expressed as mean fluorescence intensity, MFI) on CD8+ **(C)** and CD4+ **(D)** T cells at culture days 14 and 18 according to T cell maturation. Differences between culture days 14 and 18 with respect to paired continuous parameters were determined using an exact Wilcoxon rank sum test. * $p < 0.05$; ** $p < 0.01$; ns: not significant. Bars represent mean + SEM from 16 independent clinical CAIX CAR T cell cultures for the treatment of 9 patients, of which 7 received infusions in two treatment cycles. See also legend to Figure 1.

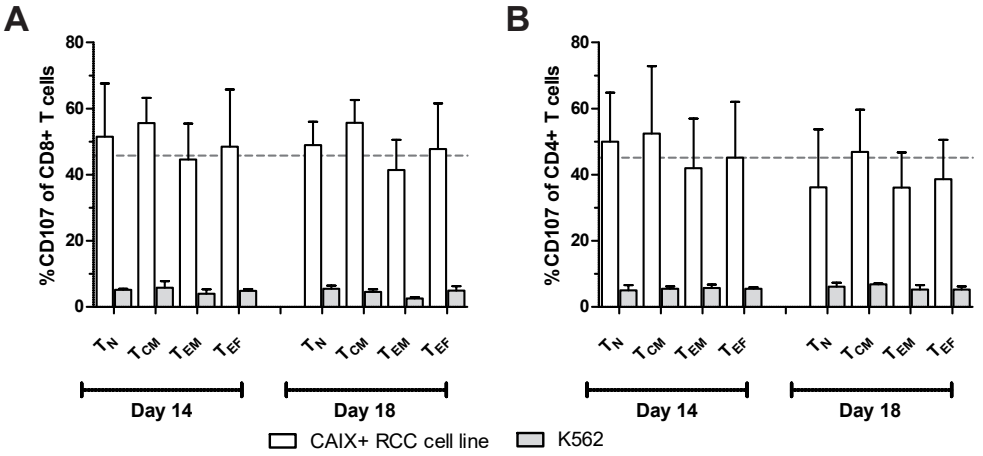

Figure 3: CAIX CAR-mediated function by T cell maturation subsets.
CAIX CAR T cell cultures at days 14 and 18 were assayed for CAIX CAR-mediated cytolytic (degranulation) potential. CAIX CAR T cells were co-cultured (2 hours) with the CAIX-positive RCC cell line (SKRC-17 MW1-clone4) or the CAIX-negative cell line K562 and subsequently analyzed by flow cytometry. Results are presented as proportions of CD107 positivity within differently matured CD8+ **A)** and CD4+ **B)** T cells. Dotted line represents the average proportion of CD107 positivity of CD8+ T cells after co-culture with the CAIX positive RCC cell line. T cell maturations subsets were defined by the markers CD45RA and CCR7 as follows: T_N: CD45RA+,CCR7+; T_{CM}: CD45RA-,CCR7+; T_{EM}: CD45RA-,CCR7-; T_{EF}: CD45RA+,CCR7-. Bars represent mean + SEM (n=3).

CAIX CAR expression in T cell maturation subsets

At culture day 14, the proportion of CAIX CAR-expressing cells was about equal for various T cell subpopulations, such as CD3 positive T cells in combination with CD4, CD8, CD56, CD57, TCRγδ, CD45RA, CD45RO, CD62L or CCR7 (supplementary Figure S5). The proportion of CAIX CAR-expressing cells within $CD8^+$ and $CD4^+$ T cells was stable during patient treatment (culture days 14-18), and appeared highest in the $T_{INT}{}^+ T_{CM}$ and lowest in the T_{ES} maturation stages (Figure 2A+B). When considering the CAR expression level per cell (MFI), we observed a significant decrease of CAR expression between days 14 to 18 for almost all maturation stages with exception of $CD8^+ T_N$, $CD8^+ T_{ES}$ cells, and $CD4^+ T_{ES}$ cells (Figure 2C+D). This observation is in extension to a previous report on a general loss of CAR expression during the last 5 days of the T cell culture.[20] Data at MFI level also reinforced the above observation that CAR expression was highest in the $T_{INT} + T_{CM}$ and lowest in the T_{ES} maturation stages (Figure 2C+D). Further, we analyzed the CAIX CAR-mediated function (degranulation) and found no significant differences in CD107-upregulation between the different maturation stages or T cell culture times following co-culture of CAR T cells with a CAIX-positive RCC cell line or CAIX-negative K562 cells. Thus, the relatively small decrease in CAR expression did not result in a measurable decrease in CAR-mediated function (Figure 3A+B).

T cell maturation correlates with in vivo CAR T cell expansion.

We analyzed whether phenotype of T cells at baseline and after culture correlated with numbers of CAR T cells in patient blood and expansion (fold increase) of these CAR T cells during 5 days after the last infusion. Kinetics of circulating CAR T cell numbers irrespective of maturation stage in patients have been reported elsewhere.[20] Here, we reveal a significant correlation between the fold increase in CAR T cell numbers in patients and the proportions of $CD8^+ T_N$ cells at baseline (Figure 4A) and $CD8^+ T_{CE}$ in the infusion product at culture day 14 (Figure 4B) and on day 18 ($r=0.683$, $p=0.042$ (data not shown). Patients with higher proportions of $CD8^+ T_N$ cells in the baseline PBMC and $CD8^+ T_{CE}$ in the infusion product, in general, had higher absolute numbers of circulating CAR T cells up to 29 days after the first infusion (Figure 4C+D). Our in vitro analyses considered both CD8+ and CD4+ T cells (Figures 1-3). Correlations between the relative occurrence of T cell maturation stages (prior to and during T cell processing) and numbers of T cells in post-treatment patient blood samples were assessed for both CD8+ and CD4+ T cells. Significant correlations, however, were only found for CD8+ T cells but not CD4+ T cells (data not shown). We found no correlations between proportion of CAR-positive T cells or CAR expression (MFI) in the infusion product and CAR T cell numbers or expansion in patients (data not shown).

DISCUSSION

Here we document T cell phenotypic changes during IL-2-supported CAIX CAR T cell cultures in preparation of a clinical trial to treat RCC patients. During the 2-week expansion period, T cells skewed from a CD4 to CD8 phenotype and the proportion of naïve CAR T cells (T_N:

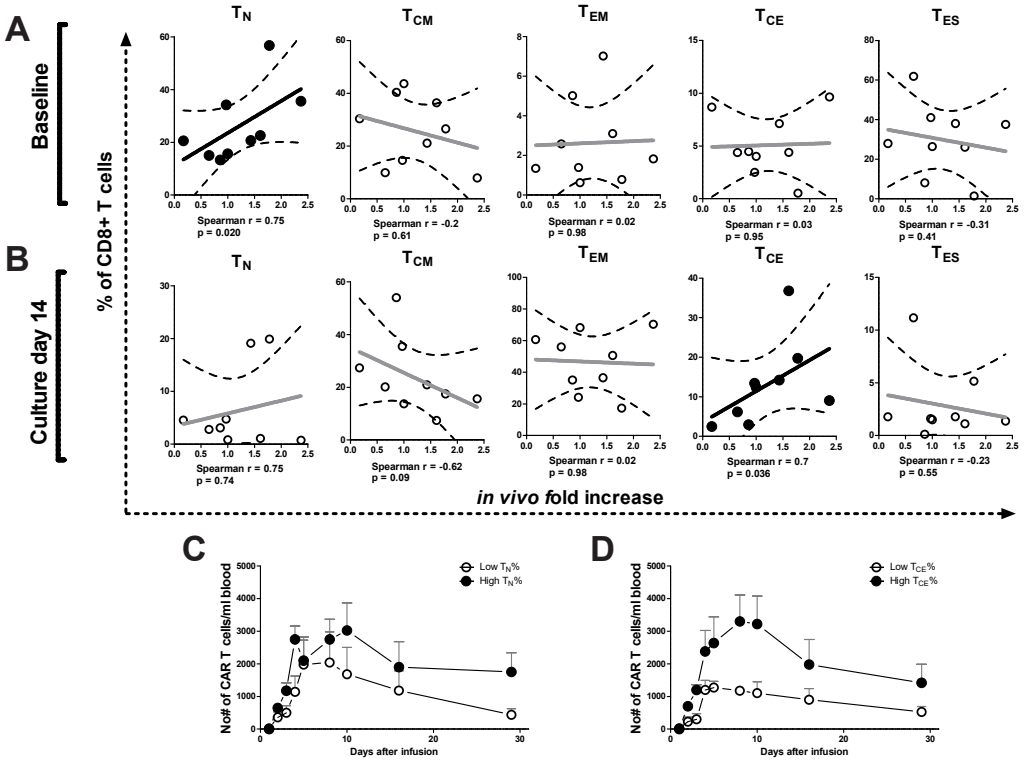

Figure 4: Correlations between T cell maturation (pre-infusion) and increase of CAR T cell numbers in patients.

Correlation plots of proportions of T_N, T_{CM}, T_{EM}, T_{CE}, and T_{ES} cells at baseline **A)** and culture day 14 **B)** versus increase of CAR T cell numbers in vivo during the first 5 days after the last T cell infusion of treatment cycle 1 (see refs [13, 20]). Dotted lines represent the 95% confidence bands of the best fitted line. The Spearman correlation coefficient method was used to assess linear association. P-values less than 0.05 were considered significant. Patients were divided into above or below median values (high and low, respectively) of T_N at baseline (day 0) **C)** or T_{CE} cells at culture day 14 **D)** and plotted for absolute numbers of circulating CAR T cells during treatment cycle 1 (days 1-29). See also legend to Figure 1 for further details.

$CD45RA^+$,$CD45RO^-$,$CD27/CD28^+$) strongly declined, while the proportions of T_{EM} ($CD45RA^-$, $CD45RO^+$, $CD27/CD28^-$) and T_{CE} ($CD45RA^+$, $CD45RO^+$, $CD27/CD28^-$) cells significantly increased. We noted a shift from T_N cells to more maturated stages as was described before for other transduced T cell products cultured with IL-2.[21] Interestingly, also the frequency of T_{ES} declined during the CAR T cell culture. We anticipate that the less maturated cells like T_{CM} and T_{EM} have proliferated faster, and thereby have overgrown the T_{ES} cells. We demonstrated a small decline in CAR expression level per cell during the 5-day CAR T cell culture covering the days of T cell administration (day 14-18) that was independent of T cell maturation stage and did not affect CAR-mediated function. We conclude that the 5 sequential and 'freshly' prepared clinical preparations of CAIX CAR T cells had about equal phenotypic and functional properties.

In adoptive CAR T cell treatment, circulating numbers, persistence and ***in vivo*** expansion potential of infused CAR T cells are currently the only parameters revealed to be associated with improved clinical outcome.[22] Most CAR T cell trials targeting CD19 in hematological malignancies show strong T cell expansion, mainly due to a high load and accessibility of target antigen, the nature of tumor cells (B cells being able to provide co-stimulation) and highly active (second generation) CAR T cells. Along these lines we sought to define pre-infusion characteristics that may predict the ***in vivo*** behavior of infused CAR T cells. Here, we showed that patients with more $CD8^+$ T_N cells in baseline PBMC (from leukapheresis) or more $CD8^+$ T_{CE} in the infusion product at culture day 14, showed a higher fold increase in numbers of CAR T cells ***in vivo***, resulting in higher blood levels of CAR T cells in these patients for at least 4 weeks. In fact, a correlation exists between characteristics of both baseline PBMC (proportion $CD8^+$ T_N) and infusion product (proportion $CD8^+$ T_{CE}) and numbers of circulation CAR T cells after treatment. Such findings bear clinical relevance, as younger T cells were shown to positively correlate with clinical effectiveness in adoptive T cell trials.[1, 23, 24] To our knowledge, our study is the first CAR T cell trials targeting a solid tumor, in which a correlation is demonstrated between pre-infusion and pre-expansion T cell characteristics and ***in vivo*** CAR T cell expansion potential. In only one other non-CD19 CAR trial, targeting neuroblastoma with GD2 CAR T cells, a correlation was described between ***in vivo*** CAR T cell persistence and the proportion of $CD4^+$ or T_{CM} cells in the infusion product.[24] Our observation is in line with the report that effector T cells derived from naive rather than memory T cell subsets possess superior features for adoptive immunotherapy, though no correlations were made with in vivo parameters.[25]

The observation that the proportion $CD8^+$ T_N in baseline PBMC determines the ***in vivo*** behavior of CAR T cells is intriguing and provides a means to manipulate leukapheresis products for a better clinical outcome, especially for patients with low proportions of T_N cells in PBMC. This could include enrichment for T_N cells before transduction or changing the culturing conditions by addition of cytokines such as IL-15 and IL-21 during expansion[21, 26]. These data may provide clues to adapt the ***in vitro*** T cell processing towards optimal T cell fitness and may enable to develop an improved protocol for adoptive T cell therapy**.**

FUNDING

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ACKNOWLEDGEMENTS

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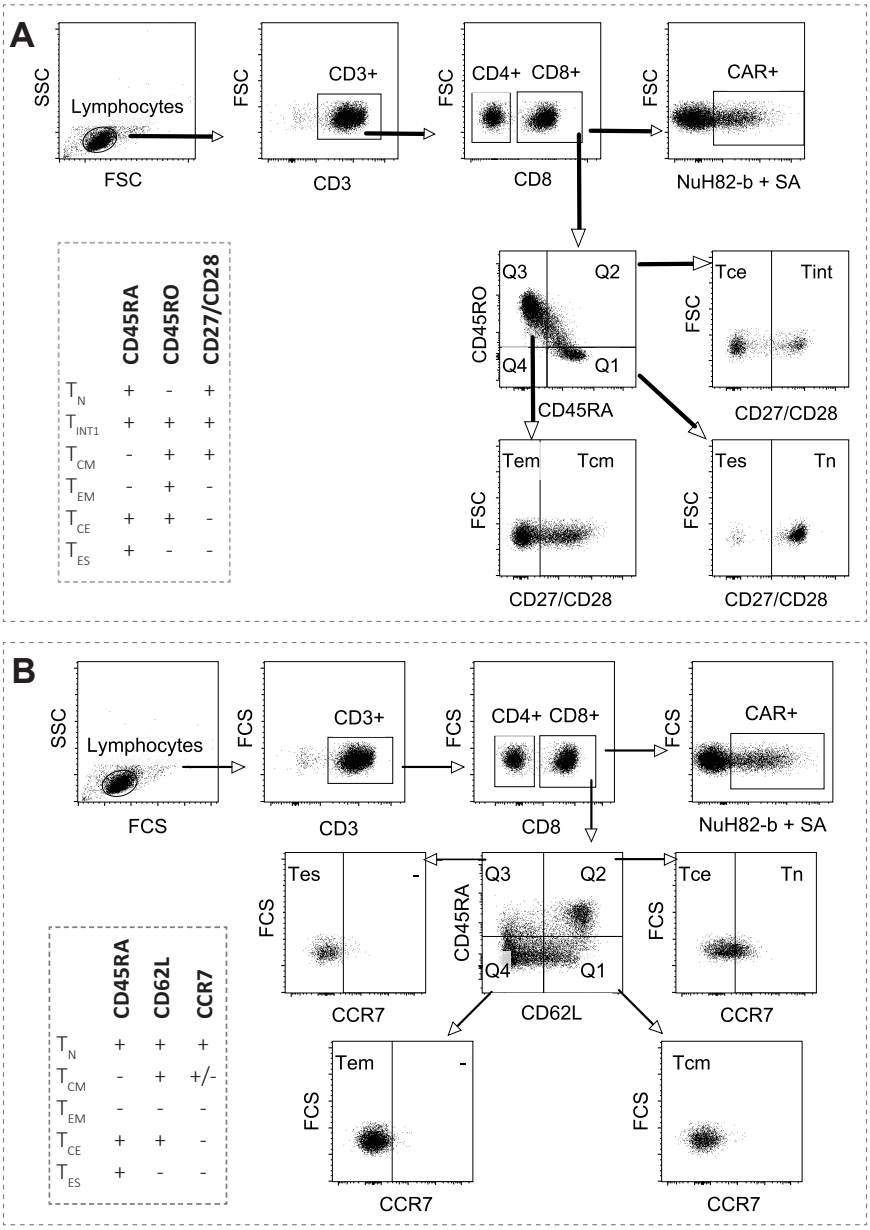
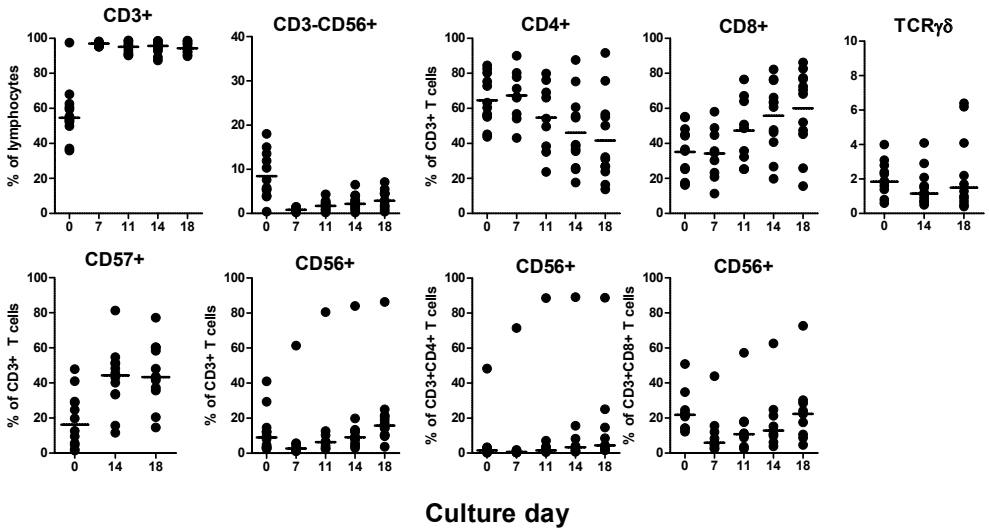
SUPPLEMENTARY DATA



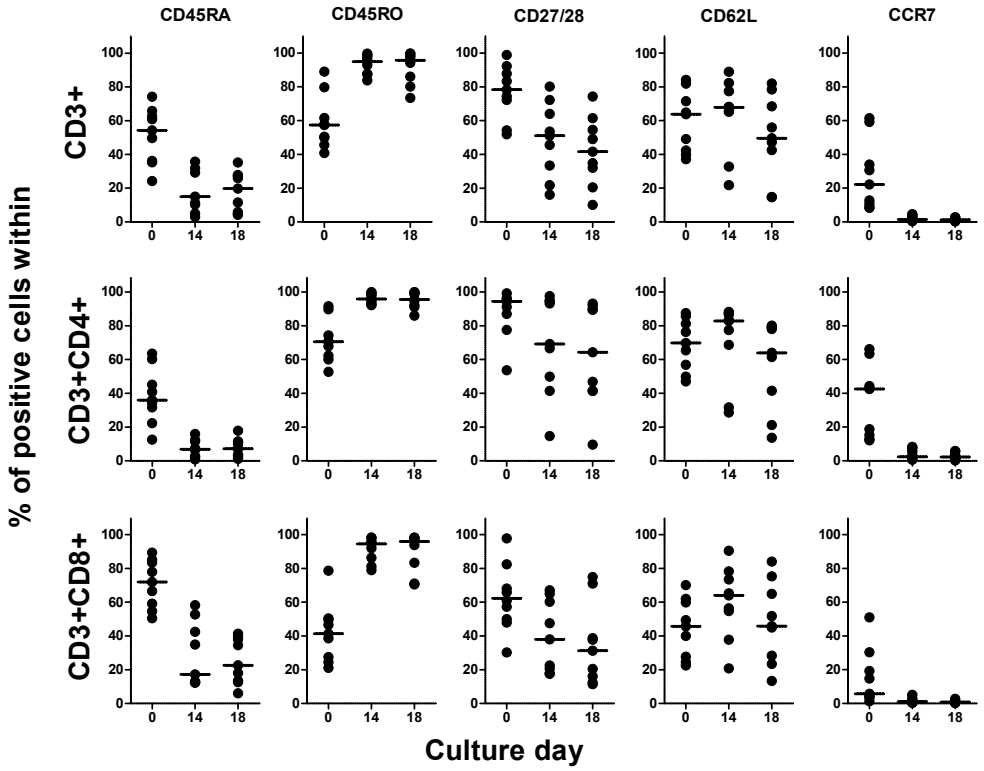
Supplementary figure S1: Gating strategy of T cell cultures for CAR expression and T cell maturation

Gating strategy for the CD45RA/CD45RO/CD27/CD28 maturation panel, and (B) the gating strategy for the CD45RA/CD62L/CCR7 maturation panel. Both panels also determined the CAIX CAR expression (upper right panels). The NuH82-biotin (b) + Streptavidin (SA) gate was set based on an "unstained" tube, in which the NuH82-biotin mAb was left out in the first staining step. A Boolean gating approach, was used to automatically generate the combinations of the maturation stage and CAIX CAR expression. Plots show a representative T cell culture at day 14 of the culture.



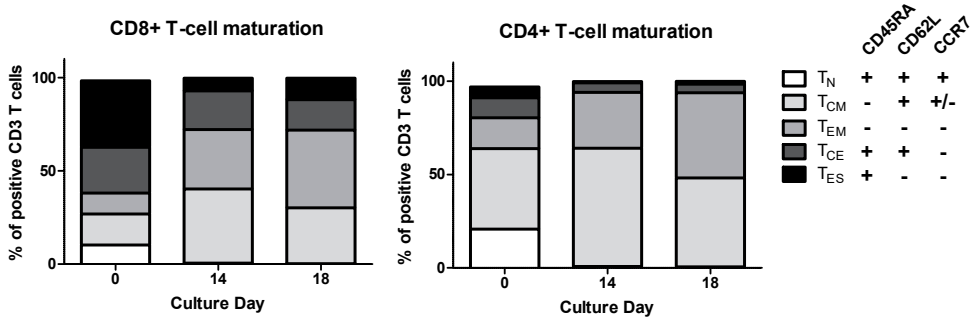
Supplementary figure S2: Kinetics of major lymphocyte subsets during CAIX CAR T-cell expansion cultures.

Patient PBMC from 9 patients were activated, transduced and cultured two-times as detailed in Materials and Methods section to generate therapeutic doses of CAIX CAR T-cells for treatment cycle 1 and treatment cycle 2; for both clinical cultures, non-transduced T-cells were cultured in parallel, resulting in 4 independent T-cell cultures per patient. FCM analysis of lymphocyte subsets revealed that phenotypic shifts were independent of transduction and reproducible for each patient, therefore mean values of the 4 individual cultures are presented (one dot per patient per time point). Day 14 and day 18, represent the infusion product on the first and last of the 5 daily infusions of CAIX CAR T-cells. Presented lymphocyte subsets: CD3+; CD3-CD56+ (NK); CD3+CD4+; CD3+CD8+; CD3+TCRgd+; CD3+CD57+; CD3+CD56+, and % CD56+ of CD3+CD4+, and % CD56+ of CDC3+CD8+. Horizontal lines represent the median values.



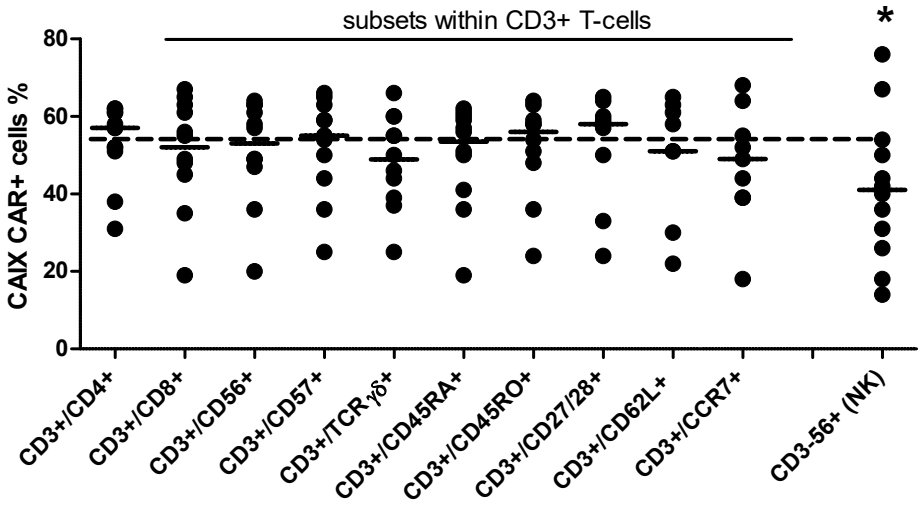
Supplementary figure S3: Kinetics of T-cell maturation markers expressed by different T-cell subsets during CAIX CAR T-cell expansion cultures.

Patient PBMC from 9 patients were propagated as detailed in the legend to Figure S2. FCM analysis of maturation markers CD45RA, CD45RA, CD27/CD28, CD62L and CCR7 on CD3+ T-cells and CD3+CD4+ and CD3+CD8+ T-cells was performed at baseline and culture days 14 and 18. The mean values of the 2 clinical CAIX CAR T-cell cultures are presented (one dot per patient /time point). Horizontal lines represent the median values.



Supplementary figure S4: T-cell maturation during clinical T-cell expansion cultures using CD45RA, CD62L and CCR7.

CAIX CAR T-cell cultures were generated as described. Proportions of T-cell maturation subsets within CD8+ (A) and CD4+ (B) T-cells were assessed at baseline (PBMC from leukapheresis, day 0), culture day 14, and culture day 18 according to the expression of CD45RA, CD62L and CCR7 as indicated the in insert: Naïve, TN: CD45RA+, CD62L+, CCR7+; Central Memory, TCM: CD45RA-, CD62L+, CCR7+/-; Effector Memory, TEM: CD45RA-, CD62L-, CCR7-; Central Effector, TCE: CD45RA+, CD62L+, CCR7-; and End Stage TES: CD45RA+, CD62L-, CCR7-; T-cells. Data are presented as stacked bars of means of 9 patients.



Supplementary figure S5: CAIX CAR is expressed homogenously among different T-cell subsets.

CAIX CAR T-cell cultures were generated as described. At culture day 14 proportions of CAIX CAR+ cells were assessed per lymphocyte subset by FCM. The mean values of CAIX CAR expression of the 2 individual CAIX CAR T-cell cultures are presented (one dot per patient). Lymphocyte subsets inlcuded: CD3+; CD3+CD4+; CD3+CD8+; CD3+CD56+; CD3+CD57+; CD3+TCRgd+; CD3+CD45RA+; CD3+CD45RO+; CD3+CD27/28+; CD3+CD62L+; CD3+CCR7+, and CD3-CD56+ (NK) cells. Graphs represent individual (dot) and median values (horizontal line). Differences from expression in CD3+ were tested using the paired Student t-test, * $p < 0.05$.

Chapter 4

Treatment of metastatic Renal Cell Carcinoma (mRCC) with CAIX CAR-engineered T-cells a complete study overview

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ABSTRACT

We studied safety and proof of concept of a phase I/II trial with Chimeric Antigen Receptor (CAR) T-cells in patients with metastatic renal cell carcinoma (mRCC). The CAR was based on the G250 mAb that recognized an epitope of carboxy-anhydrase-IX (CAIX). Twelve patients with CAIX+ mRCC were treated in three cohorts with a maximum of 10 daily infusions of $2x10^7$ to $2x10^9$ CAR T-cells. Circulating CAR T-cells were transiently detectable in all patients and maintained antigen-specific immune functions following their isolation post-treatment. Blood cytokine profiles mirrored CAR T-cell presence and in vivo activity. Unfortunately, patients developed anti-CAR T-cell antibodies and cellular immune responses. Moreover, CAR T-cell infusions induced liver enzyme disturbances reaching CTC grades 2 to 4, which necessitated cessation of treatment in 4 out of 8 patients (cohort 1+2). Examination of liver biopsies revealed T-cell infiltration around bile ducts and CAIX expression on bile duct epithelium, adding to the notion of on-target toxicity. No such toxicities were observed in four patients that were pretreated with G250 mAb (cohort 3). The study was stopped due to the advent of competing treatments before reaching therapeutic or maximum tolerated dose in cohort 3. No clinical responses have been recorded. Despite that, from this trial numerous recommendations for future trials and their immune monitoring could be formulated, such as choice of the target antigen, format and immunogenicity of receptor and how the latter relates to peripheral T cell persistence.

INTRODUCTION

Adoptive transfer of antigen-specific T-cells has shown therapeutic successes in the treatment of viral infections and tumors.[1-5] Treatment of patients with gene-engineered T-cells equipped with either chimeric antigen receptors (CARs) or T-cell receptors (TCRs) provides an attractive strategy to provide therapeutic immunity against malignancies. Despite some marked successes in hematological malignancies[6, 7], so far, gene-engineered T-cells failed to yield anti-tumor responses in a substantial number of patients in solid tumors.[8-12]

Improved insight into several aspects is urgently needed in order to fully explore the potential of gene-engineered T-cells as a treatment option for solid tumors. One of the main challenges in the field of T-cell engineering is, for example, receptor specificity. Engineered T-cells endowed with high-affinity receptors proved significantly toxic when tumor-associated antigens were targeted that were also expressed, even at low level, on normal tissue,[8, 11-15]so-called 'on-target' toxicity. Other important aspects include choice of receptor format, strategies to prolong T-cell persistence and to reduce immunogenicity and sensitization of the suppressive tumor micro milieu.[16]

We have designed a first generation CAR directed against carboxy-anhydrase-IX (CAIX) and treated patients with CAIX-expressing metastatic renal cell carcinoma (mRCC)[17]. In this paper we present a study overview and will summarize the clinical observations and immune monitoring performed on this clinical study including published and non-published data.

CAIX CAR T-CELLS

The first generation CAR was constructed from the single chain variable domain (scFv) of the murine monoclonal antibody (mAb) G250 and the intracellular part of the γ-chain from the Fc(ε)RI receptor present on mast cells.[18, 19] The G250 mAb recognizes an epitope on CAIX, which is frequently overexpressed on clear cell RCC.[20] Following retroviral introduction of the CAIX CAR into primary human T-cells, the CAR became surface expressed, which enabled T cells to recognize CAIX and to exert antigen-specific effector functions, such as cytokine production and killing of RCC cell lines.[21] Next, we established and validated a Good Manufacturing Procedure (GMP)-compliant protocol, based on T-cell activation with sCD3 mAb, retronectin-based transduction and IL-2 supported T-cell expansion to generate CAR T-cells in a closed culture system for patient treatment.[21-23]

PRE-TREATMENT OBSERVATIONS: ANALYSES OF INFUSION PRODUCTS

The transduction protocol highly efficiently transduced all lymphocyte subsets, including CD4+, CD8+, CD57+ and TCRγδ+ T-cells.[24] Detailed phenotypic analysis showed that the CAR T-cell cultures were skewed towards differentiated CD8+ T-cells (as defined by markers CD45RA, CD45RO, CD62L, CCR7 and CD28). Of note, during culture all differentiation markers shifted along the anticipated T-cell differentiation lines.[25] At culture day 14 the majority of

the T-cells were central memory (T_{CM}) and effector memory (T_{EM}) T-cells and the CAR expression was slightly higher on T_{CM} and T_{EM} when compared to the naive (T_N) and end stage (T_{ES}) T-cells.[24] Remarkably, all T-cell differentiation stages within both CD4+ and CD8+ T-cell subsets exerted similar levels of CAR specific CD107a mobilization, suggesting the CAR expression levels in all of these subsets was above a functional expression level and that in addition to CD8 CAR T cells also CD4 CAR T cells may exert effector T cell functions.[24]

Patients were treated with multiple T-cells infusions (see below) that were freshly prepared on culture days 14 to 18 in two treatment cycles. The CAR T-cell infusions of treatment day 1 (culture day 14) versus day 5 (culture day 18) had similar T-cell phenotypes and proportions of CAR expressing T-cells, yet the expression level (mean fluorescence intensity, MFI) of the CAR was lower at day 5. The latter observation was confirmed by decreased CAR mRNA levels at treatment day 5 versus day 1 while the CAR DNA levels were equal.[26],[27] Our observations are in line with those of Burns et al.[28], who further showed that the loss of transgene expression in human lymphocytes transduced with a similar (MFG) retroviral vector was LTR-driven, and subject to global cellular mechanisms.[28]

Further characteristics of the pre-infusion CAIX CAR T-cells are summarized elsewhere.[29] In short, T cell infusion products showed a median of 61% were CD8+ (range, 18–83%) and 53% (range, 24–65%) expressed the CAIX CAR. The CAR T-cells had incorporated a median of 2.6 copies of the CAR transgene in their DNA (range, 1.2–12.9). We reported a median CAIX-specific cytolytic activity of 107 $LU_{20}/10^6$ CAR T-cells (range, 18–372) and interferon-γ (IFN-γ) production of 29 ng/24 hours/10^6 CAR T-cells (range, 1–47). Specific IFN-γ production by T-cell from the therapeutic infusions was at least 20-fold higher than production of interleukin-5 (IL-5), tumor necrosis factor (TNF-)α, and IL-4.[29]

PATIENT TREATMENTS

Between March 2003 and December 2010, we treated 12 patients with CAIX-expressing metastatic RCC, not amendable for curative surgery, and for whom no standard treatment existed.[8] Specific patient characteristics are described elsewhere.[29]

Patients were not subjected to lymphodepleting preconditioning and were treated with two cycles of multiple intravenous (i.v.) infusions of CAR T-cells accompanied with subcutaneous (s.c.) IL-2 injections (2x/d IL-2, $5x10^5$ IU/m^2) during 10 days from the start of a treatment cycle. Patients were treated in three cohorts, e.a. featuring a treatment scheme that included adaptations in response to serious adverse events, see Figure 1.[29]

Cohort 1, the study was designed as a phase I/II study comprising of an in-patient dose escalation (i.e., day 1, $2x10^7$ T-cells; day 2, $2x10^8$ T-cells; day 3, $2x10^9$ T-cells), followed by a consolidation dose of $2x10^9$ T-cells at days 4-5 (treatment cycle 1) and $2x10^9$ T-cells at days 17-19 (treatment cycle 2) (Figure 1 – scheme 1). In this scheme, three patients were treated, two of which developed dose-limiting toxicities in terms of liver enzyme disturbances, hence the treatment protocol was amended.

The amended protocol was a conventional 3×3 phase I study, applying a maximum of up to 10 CAR T cell infusions at days 1–5 (treatment cycle 1) and days 29–33 (treatment cycle 2), at a start dose of 1×10^8 CAR T-cells per infusion and projected escalations to 2-, 4-, 8, 16-, 20, 25- and $30x10^8$ CAR T cells per infusion. The study comprised 2 steps, the first without 'protective measures' in order to assess the net maximum tolerable dose (MTD) of the CAIX CAR T cells with IL-2 support, and a second step including 'protective measures' being i.v. infusions of 5 mg of the anti-CAIX mAb G250 3 days before start of each treatment cycle. The pretreatment with G250 mAb was intended to block CAIX in liver and prevent elevation in liver enzyme values, while not blocking CAIX in RCC metastasis.[30-32] In step 2, we aimed at increasing the MTD of the CAIX CAR T cells and accomplish a clinical effective dose. In case dose limiting toxicities were recorded in 2 patients at a particular dose level in step 1, subsequent patients would be treated at the same dose level in step 2.

In cohort 2, 5 patients were treated according to step 1 of the conventional 3×3 phase I approach (Figure 1 – scheme 2). At the starting dose of 1×10^8 CAR T-cells per infusion (maximum cumulative dose of 1×10^9), again a dose-limiting toxicity (DLT) was observed with respect to liver enzyme values in the third patient after 10 infusions (cumulative dose 1×10^9 CAR T cells) and in the fifth patient after 3 infusions (cumulative dose 0.3×10^9 CAR T cells). Therefore, cohort 2 was closed without a proper assessment of a MTD of CAIX CAR T-cells.

In cohort 3, 4 patients were treated as in cohort 2, but with pre-treatment of an i.v. infusion of 5 mg of the anti-CAIX mAb G250 3 days before start of each T cell treatment cycle (Figure 1 – scheme 3). Three patients were treated at the starting (cumulative) dose of 1×10^9 CAR T-cells without toxicity and the CAR T-cell dose was increased to 2×10^9 CAR T-cells (cumulative) for the next 3 patients. Due to limited patient accrual because of competing treatments given the introduction of VEGFR-TKIs with proven activity in mRCC patients, the study was terminated after one patient in the second dose level of cohort 3.

CLINICAL OBSERVATIONS

No clinical responses were noted and the median overall survival was 9.5 months (range: 3–33 months) for patients treated in cohorts 1+2 (n = 8 patients), and 12.5 months (6–24 months) for cohort 3 (n = 4 patients).

Toxicities were restricted to elevations in blood levels of liver enzymes in 4 out of 8 patients treated in cohort 1 and 2, as described above. Liver biopsies taken from 3 of these patients revealed CAIX expression on bile duct epithelium, discrete cholangitis with inflamed portal triangles and infiltration of T-cells, including CAIX CAR T-cells.[8, 29] We concluded that the liver toxicity was probably due to the specific interaction of the CAR T-cells with CAIX expressed on the bile duct epithelium. Indeed, blocking of CAIX in the liver by G250 mAb infusion allowed treatment of the next 4 patients (cohort 3) at a (cumulative) dose of $1\text{-}2x10^9$ CAR T-cells without any toxicity. From one patient a peripheral metastasis was excised however no significant T-cell infiltrate was observed by immunohistochemistry.

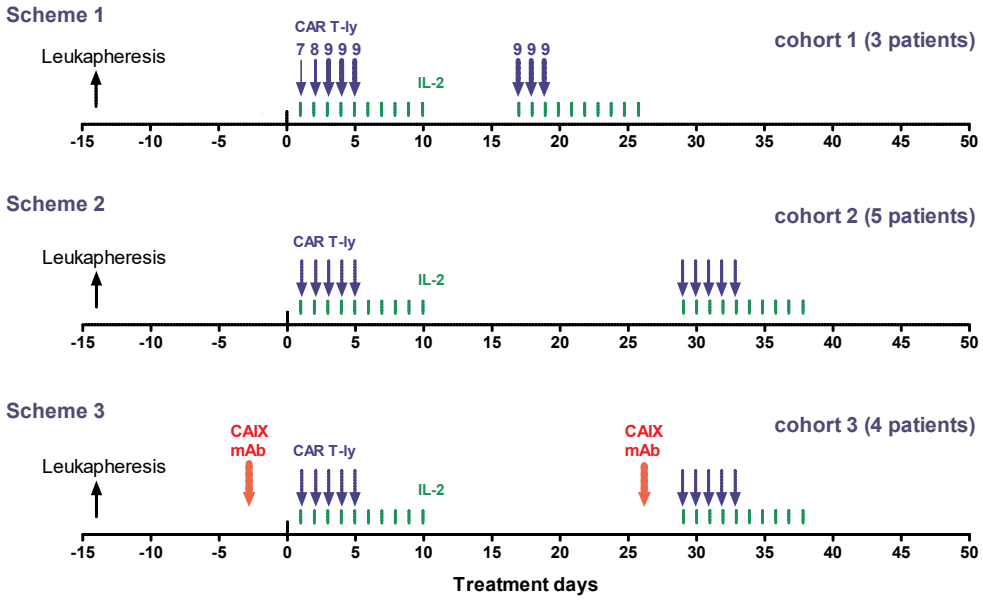

Figure 1: Treatment schemes

Treatment schemes, adapted from: Lamers, C.H., et al. Immune responses to transgene and retroviral vector in patients treated with ex vivo-engineered T cells. Blood 117, 72-82 (2011), ©the American Society of Hematology. ***In cohort 1 (scheme1),*** we treated patients with CAIX CAR T-cells in an in-patient dose escalation (i.e., day 1, $2x10^7$ T-cells; day 2, $2x10^8$ T-cells; day 3, $2x10^9$ T-cells), followed by a consolidation dose of $2x10^9$ T-cells at days 4-5 (treatment cycle 1) and $2x10^9$ T-cells at days 17-19 (treatment cycle 2), in combination with sub cutanous (s.c.) injections of $5x10^5$ IU/m^2 human recombinant interleukin-2 (IL-2, Chiron, Amsterdam), twice daily administered at days 1-10 and days 17-26. For each treatment cycle a new T cell culture was initiated from which fresh T cell infusions were prepared. Patients 1 and 3 developed liver enzyme disturbances reaching CTC grades 3-4 following 4 T-cell infusions, which necessitated cessation of treatment in patients 1 and 3, corticosteroid treatment in patient 1 and reduction of the maximal T-cell dose to 2 x 10^8 T-cells in patients 2 and 3[ref [8]]; ***In cohort 2 (scheme 2)***, we treated patients with CAR T-cells in a conventional phase I strategy with a maximum of 10 CAR T-cell infusions at days 1-5 and days 29-33 and staring at a CAR T cells dose of $1x10^8$ per infusion and projected dose escalations to 2-, 4-, 8, 16-, 20, 25- and $30x10^8$ CAR T cells per infusion. CAR T cell infusions were in combination with IL-2, s.c., $5x10^5$ IU/m2 twice daily administered at days 1-10 and days 29-38. ***In cohort 3 (scheme 3),*** we treated patients as in cohort 2, but applied a strategy to block CAIX CAR recognition of cognate antigen on normal liver tissue. To that end we included an extra i.v. infusion of 5 mg cG250 mAb (kindly provided by L. Old, LIRC New York), three days prior to start of each series of CAR T-cell infusions, which blocks CAIX in the liver and leaving accessible CAIX at RCC tumor sites[31, 32]. ***Of note***, in this clinical trial patients were not subjected to pre-treatment lymphodepletion conditioning

PATIENT MONITORING

CAR T-cells were quantified in blood by FCM using the anti-idiotype mAb NuH82. In addition, CAR DNA copies and CAR mRNA levels were quantified by qPCR and RT-qPCR, respectively. CAR T-cell, DNA and mRNA levels showed peak levels between treatment days 4 to 8 (for both cycles); peak levels were: CAR T-cells, median 2.7 cells/ul (range 0.8 – 10.0); CAR DNA levels; 0.070 fg/ul (0.018 – 0,566) which was equal to 10 transgene copies/ul (3 – 81); and CAR mRNA levels, 0.139 fg/ul (0.024 – 0.905). CAR T cells, DNA and mRNA levels gradually decreased between both treatment cycles, but levelled off more rapidly after the second treatment cycle, see Figure 2A-C. This observation may have been caused by an anti-CAIX CAR immune response (see below), which became prominent after the second treatment cycle.[33] This notion is supported by the observation that patients treated in cohort 3, receiving the protective G250 mAb infusions, had detectable G250 mAb blood levels up to treatment day 10, but developed less anti-CAIX CAR immunity[33], and showed longer persistence of the CAIX CAR T-cells (Figure 3A,B). Here we add additional data to our previous observation on the loss of CAR membrane expression following T cell administration to patients.[27] The ratio of the CAIX CAR mRNA : DNA levels in blood gradually declined after treatment cycle 1 and showed a steep decrease after treatment cycle 2 (Figure 1D). Decreased CAR gene and surface expression are indicative for limited in vivo lymphocyte activation.[28] In vivo expansion of the infused T-cells were only seen in treatment cycle 1 in just 3 out of 8 patients (cohort 2+3; Figure 2A).

Our observations on limited persistence of CAR T-cells concur with other reports on first generation CARs.[9, 34, 35] However, in case virus specific T-cells were gene-modified with a first generation CAR, these CAR T-cells persisted for months to years, likely due to repetitive viral re-stimulation.[36] Second generation CARs harbour intracellular co-signalling domains derived from molecules such as CD28 or 4-1BB. T-cells with second generation CARs showed improved in vivo expansion and prolonged persistence irrespective of lymphodepleting preconditioning.[37-40] These results have boosted the number of trials using second generation CD19 CAR T-cells to treat B-cell malignancies, which so far have shown impressive clinical results.[41, 42]

Blood cytokine levels were assessed using multiplex bead technologies, and revealed cytokine peak levels at day 5 (median; range, 5-8) in cycle 1 and at day 3 (range, 1-5) in cycle 2. Most prominent were elevations of IL-2, IL-5 and IFN-γ, potentially driven by IL-2 administration and in vivo activation of CAR T-cells.[29] Significant fluctuations were also recorded for: IL-1ra, IL-4, IL-12(p70), FGF-basic, G-CSF, GM-CSF, IP-10 and PDGF-bb. Analysis of cytokine levels after the last T-cell infusion revealed that IFN-γ and IL-6 levels correlated with numbers of peripheral CAR T-cells but not with liver enzyme toxicity scores.[26]

Post-treatment PBMC displayed CAIX-specific T-cell functions, both cytolysis and IFN-γ production.[29, 43] The CAIX-specific IFN-γ production peaked simultaneously (days 5-8) with the numbers of CAR T-cells in the circulation. Moreover, we showed that IFN-γ production by

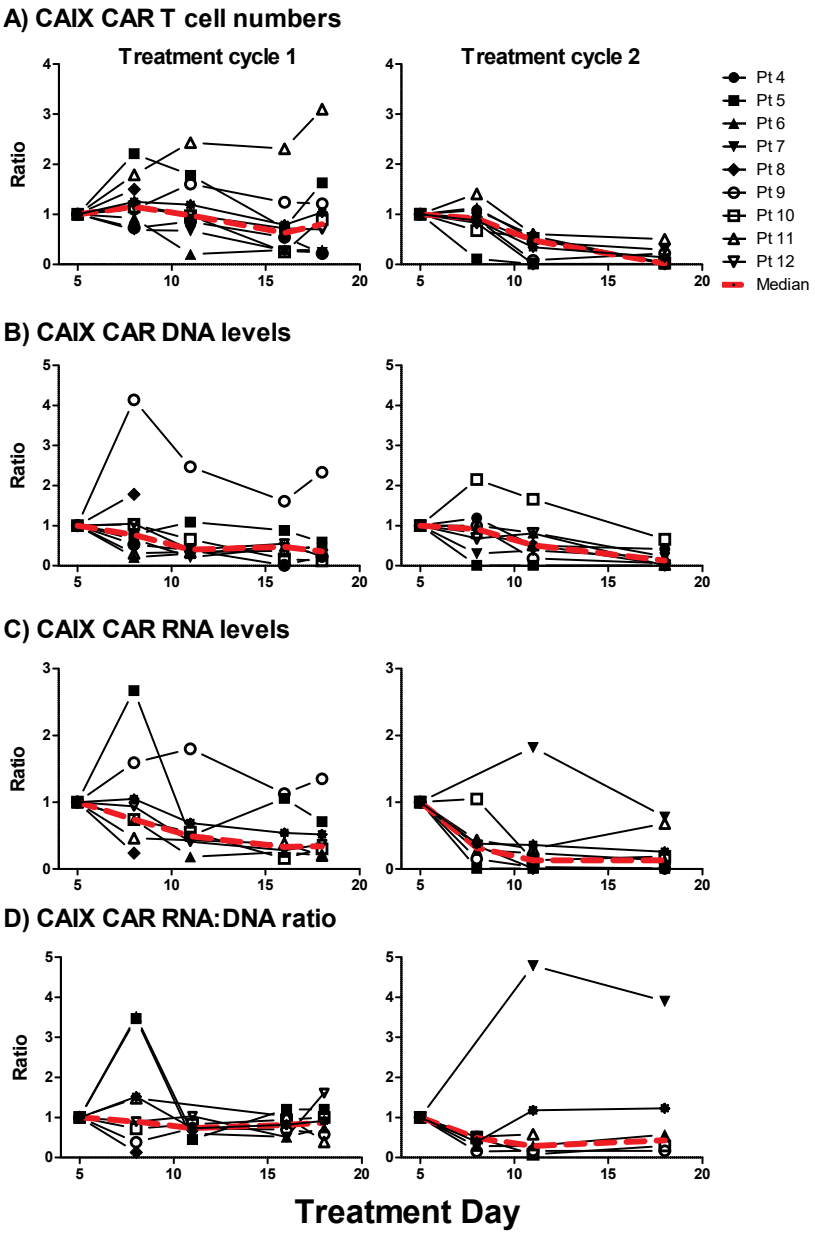

Figure 2: In vivo loss of CAIX CAR transgene expression

Patients were treated with CAIX CAR T-cells (days 1-5) and monitored for blood numbers of CAR T-cells by FCM **A)** and blood levels of CAR DNA **B)** and RNA **C)** by PCR and RT-PCR, respectively. Results are expressed relative to the value at treatment day 5 (day 5 ratio=1). **D)** relative CAR RNA over CAR DNA ratio. Values for 9 individual patients treated in cohorts 2 (filled symbols) and 3 (open symbols) and the median observation are shown.

post-infusion PBMC correlated with pre-infusion CAIX CAR T-cell IFN-γ production potency.[29] Thus, CAR T-cells maintain their transgene-specific immune functions in vivo.

IMMUNOGENICITY

The CAIX CAR is constructed from the variable parts (Fv) of the murine mAb G250. The humanized G250 mAb has been applied for treatment of mRCC in monotherapy or in combination with IL-2 or IFN-γ. In such therapies, patients received bi-weekly doses of 50mg G250 mAb with a human Fc portion for 12 weeks, without noticeable induction of anti-G250 mAb immune responses.[44]

However, patients treated with CAR T-cells developed distinct anti-CAIX CAR humoral immune responses in 7 out of 12 patients (not in 3 out of 4 patients treated in cohort 3) and cellular immune responses in 9 out of 10 evaluable patients.[33] Human anti-CAIX CAR antibodies were directed against the G250 mAb's idiotype and were able to neutralize CAR-mediated T-cell functions. Mapping of the anti-CAR cellular immune responses revealed reactivities against CDR2/3 and Vk FR3/4 domains. Remarkably, patients showed unique and single epitopes and none of the epitopes covered 'fusion' proteins that are part of the CAR. Of note, in this analysis we also detected immunity towards vector-encoded epitopes expressed by the CAR T-cells.[33] Thus, murine Fv domains in the context of a CAR and presented on T-cells can serve as strong immunogens[33, 45] when compared to the soluble mAb.[44] Only a few studies report on immunogenicity of CAR or TCR modified T-cells[34, 46], probably due to the commonly applied non-myoablative patient pretreatment.

SUMMARY

Infusion product observations

First-generation CAR genes showed homogeneous transduction efficiency and, once expressed, good antigen-specific function within different T-cell subsets/differentiation stages;[23, 24]

Infused patient CAR T-cells displayed predominantly a central memory (Tcm) and effector memory (Tem) phenotype;[24]

Clinical observations

- CAR T-cells did not significantly expand in vivo, nor persist > 4 weeks post infusion and showed gradually decreasing CAR gene and surface expression;[26, 27, 33]
- Blood cytokine profiles, in particular IFN-γ and IL-6, mirrored CAR T-cell presence and in vivo T-cell activity;[26, 29]
- CAR T-cells displayed antigen-specific functions;[43]
- Patients presented with dose limiting elevations of liver enzymes in blood highly likely as a consequence of specific recognition of CAIX on lining cells of the bile ducts

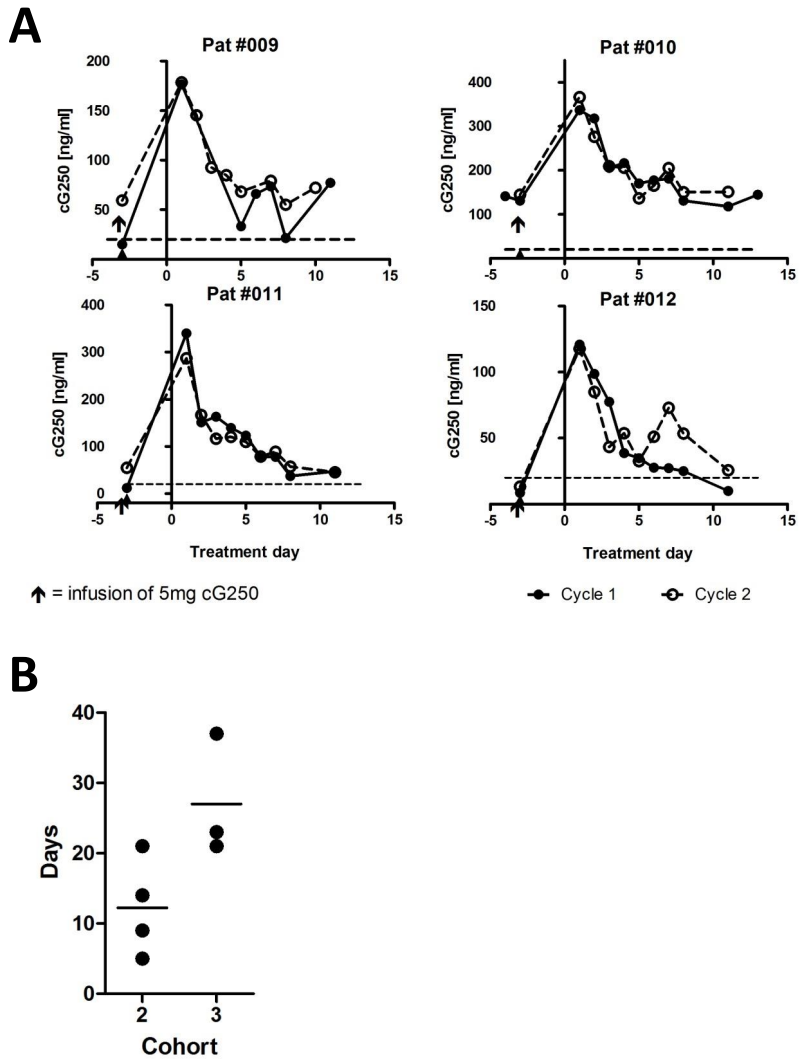

Figure 3: Patient pre-treatment with cG250 mAb infusions prolonged CAR T-cell persistence.

Patients in cohort 3 received i.v. 5mg of cG250 mAb, 3 days prior to T-cell infusion in both treatment cycles 1 and 2. Blood levels of cG250 mAb were assayed by sandwich ELISA using the anti-Id G250 mAb NuH82. **A)** Kinetics of cG250 blood levels during and after treatment cycle 1 and 2 for individual patients 9 to 12 (blood cG250 t1/2 values were pt 9: Cycle (C) 1, could not be determined; C2 (mean values) 2.3 days; pt 10, C1 2.9; C2 2.7 days; pt11 C1 1.7; C2 1.7 days; pt 12 C1 1.3, C2 1.5 days); **B)** CAR T-cell persistence is defined as last day of detectable FCM values relative to the start of treatment cycle 2 in patients receiving a cumulative dose of $1x10^9$ CAIX CAR T-cells (cohort 2: pt 4-7; cohort 3: pt 9-11; t-test, p value = 0.053). Of note, pt 12 received cumulative dose of $2x10^9$ CAIX CAR T-cells and developed anti-CAR antibodies from day 6 of treatment cycle II onwards[29], which interfered with the cG250 mAb detection assay.

by CAR T-cells;[8, 29]

- Blocking CAR by parental mAb (G250) infusion decreased liver enzyme values in blood;[29]
- CAR T-cells induced both humoral and cellular immune responses in patients directed against murine Fv domains, and which preceded loss of CAR T-cells;[33]

GENERAL REMARKS

- CAR T-cell treatment schedule, including 2 treatment cycles with repeated doses of freshly generated cells was logistically not convenient, and resulted in a reduced CAR expression in the latest T-cell infusions[26] and induction of anti-CAR immune responses;[33]
- Study was stopped due to competing therapies with TKIs prior to establishing a therapeutic dose. No clinical responses were recorded.
- CAIX CAR T-cells displayed strong on-target effects, however the presence of CAIX on liver tissue rendered this treatment not feasible to explore further.

RECOMMENDATIONS

We recognized as major limitations in presented first-generation CAIX CAR T-cells clinical study, i) the on target/off organ toxicity; ii) immunogenicity of the CAIX CAR receptor (supported by treatment scheme); and iii) lack of T-cell persistence and therapy efficiency.

In order to improve safety of receptor-engineered T-cell therapy, target antigens should be selected that are uniquely expressed by tumor cells and not on normal somatic tissues. For CARs it is quite a challenge to find such tumor specific targets, whereas for TCRs promising candidates have been identified within the groups of both Cancer Testis (CT) antigens and neo-antigens[47, 48], e.g. MAGE-C2[49, 50] and NY-ESO[51]. In case a self-antigen is targeted, the biological effect should also be taken into account and, where possible, preserved, e.g., the effect of B-cell aplasia following CD19 CAR T-cells treatment can be overcome by immunoglobulin infusions.[52] Targeting CT antigens might reveal unanticipated toxicities when applying affinity enhanced TCRs[12, 53, 54], emphasizing the need for thorough preclinical screening.[55]

In the vast majority of clinical studies, immunogenicity of the receptor has not been recognized as a possible limitation, most likely due to the applied non-myoablative preconditioning of patients in most studies, single T-cell infusions and a recent dominance of CD19 CAR T cell studies.[41, 42] Yet, our study clearly demonstrated the immunogenicity of xenogeneic protein sequences presented by T-cells.[45] Therefore, for construction of CARs and TCRs we advocate the use of human CDRs.

To date, it has been shown that T-cell persistence and therapy efficiency improves from modification of the receptor design, in particular, by including a co-stimulatory domain in the receptor.[41, 42] In addition, T-cells armoured with features that can adapt the immune-sup-

pressive tumor microenvironment, e.g., by receptor-mediated local production of cytokines, chemokines or scFvs also bear therapeutic potential.[16, 56-58]

T-cells with a 'young' phenotype demonstrate improved persistence and therapy outcome.[59, 60] Strategies to generate T-cells with a 'young' phenotype in vitro include, i) T-cell activation using CD3 and CD28 co-activation[61-63], ii) T cell culture with common-γ cytokine (IL-7, IL-15, IL-21) support in culture[62-65] and iii) (pre-)selection of T-cells subsets (e.g., CD62L selections).[66] These strategies are included in an upcoming TCR T cell adoptive therapy clinical trial at Erasmus MC to treat patients with MAGE-C2 positive tumors with co-stimulatory TCR T cells.[50, 63, 67]

There still remains a need for markers in adoptive T-cell therapy, whether related to pre-treatment infusion product or post-treatment blood measurements, that correlate with therapy effectiveness.[41] Extensive monitoring of these experimental studies is a prerequisite for obtaining a better understanding of the biology and mechanisms and will reveal tools and recommendations to improve the adoptive receptor-engineered T-cell approach.

ACKNOWLEDGEMENTS

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Chapter 5

Adoptive T-cell therapy
a need for standard immune monitoring

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ABSTRACT

Cancer immune therapy, in particular the use of checkpoint inhibitors and adoptive transfer of T cells has recently demonstrated significant clinical responses against several tumor types. Unfortunately, these therapies are frequently accompanied by severe toxicities, underscoring the need for markers that provide information on therapy response. Monitoring immune responses in the tumor micro-environment and peripheral blood prior to and during these therapies will provide better insight into the mechanisms underlying clinical activities, and will potentially enable the identification of such markers. In this review, we present an overview of adoptive T cell trials conducted with a special focus on immune monitoring, and argue that accurate monitoring of T cells is pivotal to further development of immune therapies to treat cancer.

INTRODUCTION

Immunotherapy is becoming part of the systemic treatment options for cancer patients. In particular immunomodulating agents, like the checkpoint blocking antibodies that block the negative regulators of T cell immunity. Recently, ipilimumab, that blocks CTLA-4 and nivolumab and lambrolizumab, that block PD-1, have drawn a lot of attention.[1-8] These agents applied as monotherapy have shown promising clinical results, in particular in patients with metastatic melanoma. The combination of ipilimumab and nivolumab resulted even in a higher objective response rate up to 53%, whereas adverse effects were comparable to those experienced with monotherapy.[9] Investigations in clinical application of these agents for other cancer types is rapidly expanding.

In addition to immunomodulating drugs, another promising immunotherapeutic approach is adoptive T cell therapy, an immunotherapeutic treatment that is based on the principle of transferring autologous and ex vivo expanded tumor-specific T cells to patients. In recent years, T cell therapy has yielded impressive clinical results in patients who had previously failed to standard treatments. Adoptively transferred T cells have the capacity to kill antigen-expressing tumor cells and thereby provide a direct cytolytic effector function. Therapy with tumor infiltrating lymphocytes (TILs) has shown initial objective responses in about 50% of the metastatic melanoma patients, and complete responses that ranged between 10 and 20%, including durable complete responses beyond 3 years. [10-13] Although these results are promising, the majority of patients are not eligible for TIL therapy, as not all patients have pre-existing tumor reactive T cells and in many cancers it is still difficult to identify and obtain tumor reactive lymphocytes. To overcome this limitation, the genetic introduction of chimeric antigen receptors (CARs) and T cell receptors (TCRs) into autologous T cells is considered an alternative, rendering T cell therapy available for a larger group of patients. CARs are single-chain variable fragments (scFv) derived from monoclonal antibodies that are fused to a transmembrane and a signaling domain. When introduced in T cells binding of the CAR with its antigen leads to T cell activation. Trials with first generation CARs showed limited success, but trials with second generation CARs have shown more promising results. Second generation CARs harbor an extra intracellular cosignaling domain derived from CD28 or 4-1BB (CD137). Recently in two clinical trials with second-generation CAR T cells, patients with B-cell acute lymphocytic leukemia (B-ALL) were treated with a CAR targeting CD19, and remarkable high complete response percentages of 90% were reported.[14-16] Also, several TCR gene-modified T cell trials have shown successes, although with variable response rates. T cells endowed with a TCR against NY-ESO1/HLA-A2 showed an objective response in about 50% of patients with metastatic melanoma, and in about 70% of patients with metastatic synovial cell sarcoma with no detectable toxicities.[17] Other TCR trials have shown anti-tumor responses that in some cases were accompanied by toxicities affecting tissues that expressed the same or a highly similar antigen as the one targeted by the introduced TCR transgene. See for a recent review.[18]

Trials with immunotherapeutic agents, like checkpoint inhibiting antibodies, have shown response patterns distinct from those of the traditional cytostatic/-lytic chemotherapeutic agents. These immunomodulating agents can show late responses with unconventional kinetics, immune related adverse events, and unusual late patterns of toxicity, but also have the ability to re-respond to therapy after progression.[19] In contrast, adoptively transferred T cells have the capacity to directly kill tumor cells, enabling fast tumor reduction, but also can initiate late adaptive responses by other compartments of the immune system. Yet, adoptive T-cell therapies also have shown unique clinical toxicities, either caused by overt reactivity of transferred T cells toward easily accessible and high numbers of (mostly hematological) tumor cells causing cytokine release syndrome (CRS), or by reactivity of transferred T cells toward healthy tissues expressing low levels of the target antigen or antigen that is highly similar to target antigen.[18, 20]

Although promising clinical results are seen in the T cell therapy trials described above, it is still hard to predict the efficacy and safety of treatment with T cells. The development of tools to identify patients who are likely to benefit from adoptive T cell therapy is needed. Immune markers that are related to the tumor microenvironment or peripheral blood could possibly give more detailed, and specific information about the patient's eligibility and, combined with the characteristics of the transferred T cells, might be predictive for effects of T cell therapy.

In 2011, the joint International Society for Biological Therapy of Cancer-Society for Immunotherapy of Cancer (iSBTc-SITC), US FDA and NCI Taskforce for the development of biomarkers in immune therapy trials stated: "good biomarkers offer prospect for focusing expensive and invasive therapies on the right populations, monitoring disease progression, therapeutic benefits as well as facilitate drug development" and formulated recommendations and guidelines in an attempt to standardize and harmonize immune monitoring assays.[21] We endorse these efforts, and emphasize that accurate immune monitoring is pivotal to our understanding and further development of T cell therapies, and other immunotherapeutic agents to treat cancer.

In this review, we provide an overview of TIL, CAR- and TCR T cell clinical trials with a focus on immune monitoring. Next, we list the different methodological efforts to monitor immune markers to better select eligible patients, and predict antitumor and adverse effects. Finally, we discuss the value of immune parameters and the feasibility of assessing such parameters in future cancer immune therapy trials. Donor lymphocyte infusion (DLI) following allogeneic stem cell transplantation or the use of intentionally mismatched killer cells without stem cell transplantation have shown clear clinical effectiveness, but are not part of the current review. [22-24]

CURRENT IMMUNE MONITORING IN ADOPTIVE T CELL THERAPY TRIALS

In most clinical studies, in addition to exploring the antitumor effects and toxicity of adop-

tively transferred T cells, attempts were made to identify markers associated with treatment outcome. These efforts particularly focused on peripheral persistence of postinfusion T cells as well as phenotype and function of preinfusion T cells.

TIL trials

The first TIL trial with impressive cancer regression and treatment response was described by Dudley et al. reporting a partial response in six of 13 melanoma patients that received nonmyeloablative lymphodepletion (NMA) before autologous TIL infusion.[25] Subsequently, they confirmed these results for a total of 35 patients, attempted to identify characteristics of the administered T cell treatment that could predict patient response, and reported that the infused T cell clones were no longer detectable in blood 2 weeks after infusion.[26] Robbins et al. showed that in these patients [25, 26] there was a significant correlation between tumor regression and the degree of the peripheral persistence of the transferred T cell clones. [27] In addition, a case study has been reported showing a very long persisting T cell clone in blood that expressed relatively high levels of the CD28 and CD27 co-stimulatory markers, indicating a beneficial feature of a putative slow differentiation towards an end stage effector T cells.[28]

Besser et al. studied the effect of shortly cultured TILs, designated “young TILs” (Y-TIL). Despite the small number of patients, the results of this study were promising, especially since preparation of Y-TILs was much less labor intensive.[29] In a Phase II trial, Besser et al. demonstrated a significant difference between the days of TIL culture prior to infusion of responding (about 13 days) and nonresponding (about 19 days) patients. There was no difference in CD28 or CD27 expression on TILs between the responding and nonresponding patients in this study, though the total number of infused CD8+ T cells was significantly higher in the responding group.[10] Dudley et al. used Y-TIL and additionally enriched the transferred cells for CD8+ T cells. The latter study demonstrated that the in vivo persistence of CD8+ T cells at 1 month after Y-TIL infusion highly correlated with response, and again demonstrated that a longer culture time of TIL was significantly more prevalent in the non-responder group.[30] Radvanyi et al. have looked at the expression of several co-inhibitory molecules before infusion on the administered TILs. They examined the expression of PD-1 (CD279), BTLA (CD272), LAG3 (CD223), and Tim-3 (CD366) on TILs in the infusion product. Notably, responding patients displayed significantly higher percentages of CD8+, BTLA+ T cells as well as more differentiated T-effector cells.[31]

In search for a discriminating marker for TIL functionality, most of the TIL-trials assessed IFN-γ production of the cultured T cells after stimulation with autologous tumor cells, allogeneic matched melanoma cell lines or tumor epitope peptides. Initially IFN-γ production was used as a selection criterion for the clinical use of the TIL since IFN-γ production may correlate with a higher response rate.[32] However, other studies could not find an association between a positive in vitro antitumor reactivity of the cultured T cells and clinical response. [29, 31] Ellebaek et al monitored the T cell functionality with intracellular staining of IFN-γ

and TNF-α in CD3+, CD8+ PBMCs after stimulation with autologous melanoma tumor cells, and demonstrated elevated percentages of cytokine-positive peripheral CD8+ T cells in one patient with a complete response.[12] Even though this patient was previously vaccinated with autologous DCs and the TIL product contained a high percentage of 'vaccine specific' T cells, the above results are indicative of increased numbers of tumor-specific CD8+ T cells following TIL treatment. Verdegaal et al, treated patients with peripheral blood-derived polyclonal tumor-specific T cells in combination with low-dose IFN-α , and analyzed cytokine profiles of infused T cells, and found predominantly Th-1 cytokine production (IFN-γ, IL-2, TNF-α) in responding patients, and predominantly Th-2 cytokine production (IL-4, IL-5, IL-10) in nonresponding patients. These data suggest that a predominant Th-1 cytokine profile is beneficial for clinical outcome although results were not statistically significant due to low patient number.[33]

CAR T cell trials

One of the first CAR T cell trials has been performed by our own group, in which a CAR was used against CAIX antigen that is overexpressed on renal cell carcinoma (RCC). Eight patients were treated with 1x109 CAR T cells and low dose IL-2 without prior chemotherapy. Four of them presented with severe, but transient liver toxicities, which were most likely due to CAIX antigen expression on the surface of epithelial cells lining the bile ducts in the liver (albeit at a low level).[34] Another four patients received a preinfusion of 5 mg of the humanized anti-CAIX mAb to block CAIX antigen expressed on normal tissues while leaving CAIX antigen on RCC tissue available for T cell treatment. Subsequently, these four patients were treated with 1x109 (n=3) or 2x109 (n=1) CAR T cells and low dose IL-2 without the occurrence of any toxicity.[34, 35] Transgene-specific immune functions of CAIX CAR T cells prior to infusions were maintained in vivo and ex vivo production of IFN-γ by post treatment PBMC correlated with IFN-γ production of pretreatment CAR T cells. The used T cell doses were low and did not yield clinical responses. CAR T cells did not persist in the circulation for more than 30 days following infusion due to an immune response directed against the CAR construct.[35-37] Also another CAR T cell trial in ovarian cancer patients showed a very short in vivo CAR T cell persistence, and in addition this study revealed a lack of localization of 111In-labeled CAR T cells to the tumor sites, except in one patient.[38] In contrast to the above two studies, Brenner et al. showed prolonged in vivo persistence of a CAR that was introduced in Epstein-Barr virus (EBV)-specific T cells and emphasized the importance of costimulatory molecules which were more present on these EBV specific T cells. They demonstrated that persistence of even low levels of CAR T cells at or beyond 6 weeks was associated with a significantly longer time to disease progression. Interestingly, they observed that CAR T cell in vivo expansion and persistence were highly concordant with the proportion of CD4+ T cells and central memory CD8+ T cells (CD45RO+ CD62L+) in the infused T cell product.[39, 40] Notably, Gattinoni et al have identified a human stem cell-like memory T cell, and likewise, Xu et al. demonstrated in a clinical setting that a subset of CD8 T cells that phenotypically resembled these "T-memory

stem cells" within the pool of CAR T cells correlated with in vivo T cell expansion.[41, 42]

The introduction of so-called second generation CARs, harboring an extra intracellular cosignaling domain derived from CD28 or 4-1BB, seems to increase clinical activity of transferred T cells as well as their in vivo expansion and persistence. The group of June used a CAR specific for CD19 with addition of a 4-1BB costimulatory domain coupled to CD3ζ, to treat three B-CLL patients and demonstrated an over 1000-fold in vivo expansion of the CAR T cells. These CAR T cells were able to traffic to bone marrow (BM) and continued to express functional CARs at high levels for at least 6 months post-treatment. All three patients in this study obtained an objective response, of which two were complete responders.[43] Prolonged T cell persistence and in vivo expansion were also reported for second generation CARs including a CD28 co-stimulatory domain.[44-47] Collectively, these results have boosted the number of trials using CD19 CAR T cells to treat B-cell malignancies, which so far have shown impressive clinical results.[48] The in vivo persistence and expansion of CAR T cells appear to be markers of therapy effectiveness. In addition, trafficking to and infiltration of sites of solid tumors by CAR T-cells is of importance, though so far such monitoring was only performed in a few trials. In addition to the suggested T cell differentiation[39, 41], one may anticipate a role for costimulatory or inhibitory T cell molecules with respect to T cell persistence and expansion. Although studies are limited, some trials have looked at PD-1 expression, but no clear correlations with outcome or CAR T cell persistence have been found.[15, 16, 43, 49, 50]

TCR T cell trials

Morgan et al. performed the first clinical TCR T cell trial and treated 17 melanoma patients with MART-1/HLA-A2-specifc T cells and obtained an objective response in two (12%) of them.[51] The ex vivo cultured TCR T cells displayed specific IFN-γ release prior to infusion, and TCR T cells persisted in the circulation for at least 2 months and maximally up to a year in the two responding patients. In a subsequent study, Johnson et al. used a higher affinity MART-1 TCR, and a TCR specific for the gp100/HLA-A2 in melanoma patients resulting in an increased (30%) objective response.[52] In the latter study, TCR T cells persisted at high levels in the blood of all patients 1 month after treatment and shifted toward a less differentiated T cell phenotype. PBMC obtained from responding patients showed a higher ex vivo antitumor reactivity than PBMC obtained from nonresponders. The fact that some nonresponding patients also had very high levels of circulating TCR T cells suggest that also using this approach, T cell persistence may be an important, yet not exclusive criterion related to tumor regression.

Choice of target antigen is critical to the success of T cell therapy trials, which has been reviewed elsewhere.[18] In a clinical trial in melanoma and synovial sarcoma patients, Robbins et al. used a TCR that targeted the cancer testis antigen NY-ESO-1/HLA-A2 and obtained significant objective responses in 55 and 61% of melanoma and synovial sarcoma respectively. [17, 53] In this study, tumor regression could not be correlated with specific function of pe-

ripheral TCR T cells as measured by NY-ESO-1 tetramer staining and IFN-γ Elispot; however, they found that responders had a significant higher total number of infused T cells.

METHODOLOGY OF IMMUNE MONITORING IN ADOPTIVE T CELL THERAPY TRIALS

To support comparison across studies and acquisition of reliable data, robust, standardized, and validated methods are highly needed. Here, an overview is provided of the most commonly used techniques for immune monitoring.

T cell persistence

In early studies using TIL clones or highly selected tumor-reactive T cells populations, TCR-Vb analysis provided insight in the in vivo fate of transferred T cells, see Table 1.[25, 54]. The polyclonal origin of therapeutic TILs may make this approach less applicable to monitor persistence of administered TILs. Recently, tools have become available to screen TIL cultures for their specificity toward a variety of tumor-associated or specific antigens. T cell specificities toward known epitopes of differentiation antigens, overexpressed antigens and Cancer Testis (CT) antigens can be screened by flow cytometry (FCM) and using peptide-MHC (pMHC) multimers, even for multiple epitopes in small volumes of the same sample [55, 56]. More recently also methods have been developed to identify epitopes of antigens that arise as a consequence of mutations in the cancer cell DNA, and T cells recognizing these so-called neo-epitopes (see below: Immune markers in tumor environment). Both neo-epitope-specific CD8+ T cells [57-59] and CD4+ T cells [60, 61] have been identified in TIL cultures from melanoma, cholangiocarcinoma, and ovarian cancer patients. Once single or multiple T cell specificities have been identified, one is able to in vivo track these T cells by means of FCM pMHC multimers or TCR-Vb analysis.[60, 62]

In clinical studies using gene (both CAR and TCR)-engineered T cells both quantitative polymerase chain reaction (qPCR) and FCM are applied to detect and quantify transferred T cells in blood. These two techniques, although routine methods in multiple laboratories, have to be set up and validated to specifically monitor the T cell population of interest. Quantitative PCR is based on primers and probes targeting and amplifying CAR or TCR-encoding DNA (either genomic DNA or copied DNA from RNA) and needs positive control DNA in order to obtain quantifiable results.[50, 63] Most CAR T cell studies listed in Table 2 apply qPCR for in vivo tracking of CAR T cells in blood and other compartments like bone marrow and tumor sites. FCM can be as sensitive in detecting CAR T cells in blood as qPCR [37, 63], provided specific mAbs are available, e.g., anti-idiotype (Id) mAbs that specifically bind the CAR variable binding region. [63, 64] Such anti-Id mAbs can be applied both on freshly drawn whole blood samples as well as cryopreserved PBMC. Using FCM to detect CAIX CAR T cells in patient blood, we were able to set a limit of quantification of 0.01% CAIX-CAR+ cells within the CD3+ T cells [37]. Advantage of FCM is the ability for simultaneous detection of multiple parameters on a single cell, which would allow to detect CARs (or TCRs) in combination with markers linked

to T cell differentiation, activation and exhaustion on these cells. [43, 65] So far, the number of adoptive TCR T cells studies is limited (Table 3), but to date both qPCR and FCM have also been applied for in vivo tracking of TCR T cells. In case of FCM, investigators have monitored T cells both with TCR-Vb mAbs specific for the introduced TCRb-chain and pMHC multimers, often combined with staining for T cell differentiation markers.[52, 66] In fact, peptide-MHC multimers have become one of the most widely used tools to measure Ag-specific T cells in humans and is based on commonly studied HLA alleles. However, van Buuren et al. showed that detection of Ag-specific T cells for a given peptide is affected by micropolymorphic differences between HLA subtypes and advise to consider this issue in pMHC-based personalized immunomonitoring.[67]

T cell effector function

T cell effector functions often monitored include cytolytic potential, cytokine release and proliferation upon antigen-specific encounter (see Tables 1-3). Such assays revealed that upon adoptive transfer, gene-modified T cells maintain their transgene-specific immune functions in vivo.[35, 36] Assays used for patient monitoring of these receptor-specific functions, generally are co-cultures of post-treatment PBMC with tumor target cells or peptide loaded antigen presenting and assessment of antigen-specific T cell functions, such as cytolysis by 51Cr release [35, 36, 40], CD107 degranulation [43, 68, 69] and more commonly by cytokine production. With respect to cytokines, IFN-γ and to a lesser extent TNF-α production have been analyzed in individual cells using Elispot [17, 52], intracellularly using FCM staining [12, 68, 69] or in culture supernatant using ELISA.[35] Assessment of cytokine concentrations in PBMC culture supernatant reflects the specific reactivity of immune cells within this cell population. Elispot allows quantifying the number of specific cytokine producing cells in the PBMC, whereas IC-FCM enables direct enumeration of cytokine producing cells within lymphocyte subsets in combination with other T cell markers.

Measurements in serum/plasma

Elevated cytokine blood levels, in particular of the most prominent Th-1 effector cytokine IFN-γ, are considered a measure of in vivo activity of transferred T cells.[36]. Cytokine blood levels are commonly assessed by ELISA or bead-based multiplex assays, with the last methodology allowing simultaneous detection of multiple soluble factors in a small test sample[43, 70]. Cytokine blood levels are also prominent markers for (severe) adverse events such as tumor lysis syndrome (TLS) and CRS including inflammatory markers such as Fractalkine, GMCSF, IL-6, TNF-α, IFN-γ, IL-2Ra, CRP and Ferritin.[14-16]

IMMUNE MARKERS & THEIR VALUE IN T CELL THERAPY TRIALS

As described above, monitoring efforts of most of the T-cell therapy trials focus on the characteristics of the transferred T cells and the in vivo T cell persistence, phenotype and function. In tumor immunology field there is a strong belief that monitoring of immune markers

in tumor environment and in peripheral blood are required for further fine-tuning of T cell approaches. In recent years, several studies performed in a wide spectrum of tumor types and treatments other than T cell therapy, have revealed several interesting immune markers in the tumor environment and peripheral blood that are potentially suitable as prognostic and or predictive markers in adoptive T-cell therapy.

Immune markers in tumor micro-environment

There is mounting evidence that in melanoma, colon-, ovarian-, and lung cancer, the presence of immune cells in the tumor, in particular CD8+ T cells, strongly correlates with clinical outcome.[71-74] Notably, Zhang et al. showed that in ovarian cancer patients, a survival advantage following chemotherapy was only observed in patients with TILs and not in patients without TILs.[74] Galon et al. reported that high numbers of CD45RO+, CD8+ T cell populations, both in the center and in the invasive margin of colo-rectal tumors, have a more favorable prognostic value when compared to conventional clinicopathological (TNM) criteria that are based on tumor burden (T), presence of cancer cells in regional lymph nodes (N) and presence of metastases (M).[75-78] Based on these results, authors advocate routine patient evaluation of presence and characteristics of TILs, among others numbers and ratio of CD4 and CD8 T cells, defined in a so-called “Immunoscore”.[79] In addition, the T-helper cell (Th) signature of the intra and peri-tumoral T cells mirrors their anti-tumor (often a Th-1 profile: IFN-γ, IL-12, TNF-α) or pro-tumor (often a Th-2 profile: like IL-4, IL-10 and TGF-β) feature, which might serve as a possible marker.[80]

In addition to immune markers related to cellular immunity also markers related to humoral immunity have been considered. In head and neck squamous cell carcinoma, not only CD8+ TILs in metastatic tumors but also peri-tumoral CD20+ B-cell infiltration[81] and high numbers of B-cells in lymph node metastases were associated with favorable outcome.[82] Moreover, in ovarian cancer the presence of both CD20+ B-lymphocytes and CD8+ T-lymphocytes correlated with favorable prognosis, compared with the presence of CD8+ T-lymphocytes alone.[83, 84] Schmidt et al. described the predictive value of immunoglobin G kappa chain (IGKC) gene expression toward response to anthracycline-based neoadjuvant chemotherapy in breast cancer. IGKC, primarily derived from tumor infiltrating plasma cells, was consistently associated with metastasis-free survival across different molecular subtypes in lymph node-negative breast cancer. In addition to breast carcinoma, IGKC gene expression was prognostic for overall survival in patients with non-small-cell-lung cancer (NSCLC) and colorectal cancer.[85]

When looking at immune suppressive immune cells, it has generally been considered that increased frequencies of CD4+, CD25Hi, FOXP3+ regulatory T cells (Tregs) in various types of tumors are correlated to poor patient prognosis.[86-88] Unexpectedly, however, some studies showed a better control of loco regional tumors in case of higher numbers of Tregs.[89, 90] So far, data on Tregs appear inconclusive, possibly due to Tregs being a cell type definition that has not been uniformly defined to serve as a proper immune marker in cancer to

date. The same holds for tumor associated myeloid derived suppressor cells (MDSC), which are also considered to have a negative prognostic value for survival in patients with cancer. In example, in patients with head and neck cancer, early myeloid lineage (CD34+) cells were found in tumor infiltrates, and their levels were associated with the suppression of T cells. [91] Only a few studies have focused on the characterization of human MDSC infiltrating tumor sites. Such efforts are generally hampered by the cellular heterogeneity in the myeloid compartment, the plasticity of MDSCs in response to the complex environment of tumors, as well as technical difficulties such as loss of CD15+ and CD30+ cellular subsets after processing of tumor samples for flow-cytometry or after cryopreservation.[92, 93]

In almost all adoptive T cell therapy trials, patients are pretreated with nonmyeloablative lymphodepletion (NMA) before administration of the T cells, which, at least in part, overcomes suppression by endogenous immune cells. Some trials intensified the chemotherapeutic pretreatment with total body irradiation. Although no significant differences were seen in overall response rate among the patient cohorts, there was an increased rate of complete responses associated with increasing dose of total body irradiation.[11, 13] This is suggestive for the fact that there are still immune inhibiting cells present after chemotherapeutic NMA that may limit the therapeutic efficacy of transferred T cells.

Aside from presence and diversity of immune cells in the tumor, also the prevalence and abundance of tumor specific antigens, in particular selected MAGE antigens or neo-antigens are thought to be important for the ability to induce an antitumor T cell response.[94, 95] Of note, melanoma the cancer type most prominently treated in TIL and TCR T cell therapy studies has the highest median prevalence of such somatic mutations. Identification of neoantigen epitopes recognized by CD8 T cells was realized by a combination of next generation sequencing techniques and algorithms that identify epitopes that are processed and presented by tumor cell MHC class I. Such neo-epitope specific CD8 T cells can be isolated using pMHC-sorting, and subsequently can be used to isolate the neo-epitope specific TCR. [57-59] Recently, MHC class II neo-epitopes were identified by using peptides that covered individual mutation derived neo-epitopes and that were loaded on B-cells to screen for CD4+ TILs. IFN-g producing cells were enriched and exposed to techniques to capture TCR genes. [61] Recently, Snyder et al. demonstrated a neo-antigen landscape that is specifically present in tumors that show a strong response to CTLA-4 blockade. This neo-antigen signature potentially enables the determination on forehand whether patients would benefit from CTLA-4 therapy or not, and thereby reduce unnecessary side-effects and costs of treatment.[96] Along these lines, the presence of T cells that recognize these neo-antigens could serve to predict therapy outcome in cancer (immune)therapy, especially in cancer treatment with checkpoint inhibitors or TILs.

Immune markers in peripheral blood

Blood levels of CD8+, CD28- effector T cells have shown to be significantly increased in both melanoma and hemato-oncological patients. [97-99] These early observations were con-

firmed by Tsukishiro et al. who demonstrated that in head and neck cancer patients frequencies of CD8+, CD45RO-, CD28+ T cells were low, whereas frequencies of memory CD45RO+, CD28+ T cells and effector CD8+, CD28- T cells were high when compared with normal healthy and age-matched controls.[100] Notably, after surgical resection, the frequency of CD8+, CD28- T cells normalized in head and neck cancer patients indicating that the presence of tumor influenced the numbers of these T cells in the blood. In addition, Czystowska et al. showed in patients with this type of tumor that the frequency of less differentiated CD8+, CCR7+ T cells in blood was much lower when compared to normal controls, and demonstrated that disease-free survival was significantly shorter in patients with less than 28% of circulating CD8+, CCR7+ T cells.[101] Interestingly, Almand et al. found in breast cancer patients a population of CD34+, CD33+, CD15- cells in blood, consisting for one third of immature macrophages and dendritic cells and two thirds of immature myeloid cells, and were able to reduce ex vivo T cell proliferation and T cell effector functions.[102]

Since NMA greatly depletes cells in the peripheral blood, the cell composition of this compartment prior to therapy has probably less influence on the efficacy of T cell therapy efficacy. However, progress in receptor design, such as co-stimulatory receptors [103] in combination with T cell preparation towards optimally fit T cells [104], may eliminate the need for NMA and IL-2 and may make treatments more simplified and less of a burden to patients. Such developments would also allow monitoring of immune cells in the peripheral blood prior to and during T cell treatment in search for parameters that are related to therapy outcome.

Immune markers to monitor safety of T cell therapy trials

Monitoring of immune markers can also possibly lead to the detection of parameters that are able to predict adverse effects of T-cell therapy. Tables 1-3 depict the immune related adverse effects of TIL, CAR and TCR T cell therapy trials. The adverse effects and toxicities related to NMA are not depicted in Tables 1-3, though in most trials NMA led to grade 3-4 hematological toxicities like neutropenia, thrombocytopenia, and lymphocytopenia. In some patients this could lead to opportunistic infections.[105] Also, the IL-2 which was administered in the majority of trials to support persistence and function of transferred T cells, led to transient adverse effects like chills, low grade fever, nausea and fatigue.

In the TIL trials in melanoma, mainly melanocyte destruction, or in some cases vitiligo and uveitis, was observed. In the clinical CAR and TCR T cell trials more severe adverse effects of T cell infusion have been demonstrated, generally because of “on-target” toxicity, where the infused T cells attack healthy tissues expressing low levels of the target antigen or a highly similar antigen.[34, 36, 52, 69, 70, 106] These toxicities most likely relate to the specificity and affinity of the receptor under study, and are expected to be addressed or even resolved by choosing receptors with tumor-specific specificities, such as selected MAGEs and neo-epitopes.

In addition to on-target toxicities, several CD19 CAR T cell trials also reported the so-called

"cytokine release syndrome" (CRS), which is characterized by a systemic inflammatory response, highly elevated cytokine levels and massive T cell activation and proliferation. Severe CRS can lead to life threatening conditions with vascular leakage, hypotension, respiratory and renal insufficiency, cytopenias, and coagulopathy.[15] It was shown that the plasma cytokine (IFN-γ) levels in CAR T cell-infused patients correlate with tumor burden of the patient[16] and that the blood levels of C-reactive protein (CRP) can serve as a reliable indicator for the severity of CRS.[15] Maude et al. showed that CAR T cell-infused patients who had severe CRS had significantly higher peak levels of IL-6, CRP, ferritin and soluble interleukin-2 receptor than patients with milder CRS. It is noteworthy that severe CRS symptoms can effectively be treated with the anti-IL-6 receptor antibody Tocilizumab.[14]

CONCLUSIONS AND FUTURE DIRECTIONS

In adoptive T cell therapy, the pre- and postinfusion characteristics of transferred T cells as well as other immune markers will provide valuable information toward the mechanisms that underlie clinical responses, and potentially enables the identification of prognostic and/or predictive markers.

So far, parameters that have been recognized to be relevant for effective and save T cell therapy are primarily related to the T cell product:

- For TILs, parameters that have shown significant differences between responding and nonresponding patients primarily include shorter culture times, higher absolute CD8+ T cell numbers and expression of CD8+ T cell memory markers (e.g., CD28, CD27), CD8 T cell co-inhibitory molecules (e.g., BTLA) and CD8 T cell Th1 cytokine production (IFN-γ, IL-2, TNF-α). In general, these parameters were also linked to longer in vivo persistence of postinfusion TILs.
- For CAR T cells, receptors including a costimulatory module (2nd generation receptor), a high proportion of CD4+ T cells and a high proportion of naive (CD45RA+, CCR7+) or central memory (CD45RO+, CD62L+) CD8+ T cells correlated with in vivo persistence and expansion of CAR T cells, which in turn seems to be indispensable for effective CAR therapy.
- In the relatively limited number of TCR T cell studies, no clear predictors of the T cell product have been assigned yet. Again, T cell persistence is considered an important although most likely not exclusive criterion related to tumor regression.

The increasing number of patients that are being treated in dozens of currently running adoptive T cell trials (see at: www.clinicaltrials.gov), extend the available data on the characteristics of pre- and post-infusion T cells, with special attention to T cell differentiation and costimulatory/inhibitory molecules and T cell functions. These extended data sets will better allow the finding of correlates between the properties of the T cells and therapy outcome.

In future immune monitoring of T cell therapy trials, we think pretreatment analysis of immune markers might be of great additional value. Various tumor-related and systemic im-

mune parameters have already been investigated for their prognostic and predictive value in cancer patients treated with conventional therapies. From such studies, the most significant tumor-related marker with prognostic value is the number of intra- and peritumoral CD8+ T cells, in addition peritumoral CD20+ B-cell and immunoglobin G kappa chain (IGKC) gene expression have been associated with favorable therapy outcome. More recently the assessment of the "neoantigen landscape" allowed response prediction to treatment with anti-CTLA-4 checkpoint inhibitors. These tumor-related markers bear great promise for identifying also those patients that are likely to benefit from adoptive T cell therapy. A first step could be implementation of the so-called "Immunoscore", which characterizes the immune cell infiltration in tumors, and may provide a novel manner to classify cancer. However, quantitative immunostainings, necessary to score immune cell infiltration, is complex and there are considerable protocol variations across laboratories, which underline an urgent requirement for assay harmonization. The worldwide Taskforce "Immunoscore" is trying to achieve this assay harmonization and implementation.[79]

So far, systemic immune parameters with prognostic value are scarce, for example, disease-free survival was significantly shorter in patients with less differentiated CD8+, CCR7+ T cells in head and neck cancer patients. Pretreatment analysis of local and systemic immune parameters has not yet been considered in adoptive T cell therapies. In addition, such analyses may be troubled by the fact that patients generally receive NMA prior to cell infusion. However, pretreatment immune status may correlate with the extent and quality of immunological reconstitution following NMA, and as such could be informative towards persistence and function of adoptively transferred T cells. Also, considering the impressive results in trials with checkpoint inhibitors, determination of coinhibitory and costimulatory molecule expression on (infused) T cells or peritumoral (immune) cells may provide a valuable addition to immune monitoring panels. Future developments to improve T cell therapy trials, such as newer costimulatory CARs or TCRs and optimized T cell preparation regimens, may eliminate the need for NMA and IL-2 and are expected to enlarge the window to monitor immune cells in peripheral blood prior to and during T-cell treatment.

Importantly, confirmation of proposed and identification of new immune markers relies on accurate banking of sufficient biosamples from preinfusion T cells as well as cells, serum/plasma, DNA and RNA samples from blood taken prior to and at regular intervals postinfusion. Such bio-banks will facilitate controlled and systematic analyses and allow multicenter (inter) national initiatives to obtain better powered studies. Sample preparation, storage and documentation should be performed according to international standards. Finally, immune monitoring assays require harmonization and standardization in order to improve comparison of different trials. To this end, we endorse the recommendations by the iSBTc-SITC/FDA/NCI Workshop on Immunotherapy Biomarkers, MIATA (Minimum Information About T cell Assays) guidelines, and other initiatives, which collectively highlighted the importance of standardization and harmonization of these monitoring protocols. [21, 107-113]

In conclusion, adoptive T cell therapy trials are extending rapidly. Analysis of local and peripheral immune markers, prior to and post-therapy, has only scarcely been part of the monitoring of these therapies. Integrated analysis of the therapeutic T cell product as well as local and systemic immune parameters should be part of contemporary immune monitoring and should guide development of future adoptive T cell therapies.

MALIGNANCY	NO. OF PATIENTS	OR (%)	PR	CR	IMMUNE RELATED ADVERSE EFFECTS	IMMUNE MONITORING OF PATIENT	IMMUNE MONITORING OF INFUSION PRODUCT	REF.
MELANOMA	15	0 (0)	0	0	nr	Recovery of immune cells after NMA	TIL Phenotype: CD4, CD8 TIL cytokine release: IFN-γ, IL-2	[54]
MELANOMA	35	18 (51)	15	3	melanocyte destruction (n=17), vitiligo (n=12), uveitis (n=3)	T cell persistence, MHC-I/II expression in recurrent tumor	TIL Phenotype: CD4, CD8 TIL cytokine release: IFN-γ TIL TCR expression	[25, 26]
MELANOMA	TIL: 12, Young TILS: 8	2 (17) 1 (13)	2 0	0 1	Vitiligo (n=1)	nr	TIL cytokine release: IFN-γ TIL culture time	[29]
MELANOMA	20	10 (50)	8	2	Vitiligo (n=1)	nr	TIL phenotype: CD4, CD8, CD27, CD28. TIL cytokine release: IFN-γ TIL culture time	[10]
MELANOMA	Cohort 1 (NMA, IL-2): 33 Cohort 2 (NMA + RTx, IL-2): 23	19 (58) 11 (48)	16 9	3 2	nr	PBMC phenotype: CD4. CD8	TIL phenotype: CD4, CD8	[30]
MELANOMA	Cohort 1 (NMA, IL-2): 43 Cohort 2 (NMA + RTx (2 Gy), IL-2): 25 Cohort 3 (NMA + RTx (12 Gy), IL-2): 25	21 (49) 13 (52) 18 (72)	16 8 8	5 5 10	Uveitis (n=1)	T cell persistence Cytokine plasma levels: IL-7, IL-15	TIL phenotype: CD4, CD8, CD27 TIL cytokine release: IFN-γ Telomere length	[11, 13]
MELANOMA	31	15 (48)	11	4	nr	nr	TIL phenotype: CD4, CD8 TIL cytokine release: IFN-γ TIL culture time	[114]
MELANOMA	6	2 (33)	0	2	nr	PBMC cytokine release: IFN-γ, TNF-α	TIL phenotype: CD4, CD8, CD27, CD45RO, CCR7, CD107a TIL cytokine release: IFN-γ, TNF-α	[12]
MELANOMA	31	15 (48)	13	2	nr	T cell persistence	TIL phenotype: CD4, CD8, CD27, CD28, CD62L, CD45RA, CD272, CD279, TIM3, CD223, CD270 TIL cytokine release: IFN-γ Telomere length	[31]
MELANOMA	57	23 (40)	18	5	nr	nr	Enumeration: CD8 T cells TIL phenotype: CD45RA, CD62L, CD27, CD28, CD69, CD57, CD25, PD-1, CTLA-4 TIL culture time	[115]
MELANOMA	Cohort 1: 34 (CD4 + CD8 TILs) Cohort 2: 35 (CD8 TILs)	22 (65) 11 (31)	10 4	12 7	nr	T cell persistence T cell phenotype: CD4, CD8	TIL phenotype: CD4, CD8 TIL cytokine release: IFN-γ	[32]

Table 1 overview of clinical TIL trials with an emphasis on immune monitoring.

Abbreviations: CR (Complete Response) According to RECIST criteria. IL (Interleukin), CTLA-4 (Cytotoxic T Lymphocyte associated protein 4), CTx (Chemotherapy), DC (Dendritic cell), IFN-α (Interferon-alpha), IFN-γ (Interferon-gamma), NMA (Nonmyeloablative Lymphodepletion), NR (None reportef), OR (Objective response), PBMC (Peripheral Blood Mononuclear cell), PR (Partial response), RTx (Radiotherapy), TNF-α (Tumor necrosis factor-alpha)

MALIGNANCY	ANTIGEN	ENDOO-MAIN(S)	PATIENTS	OR (%)	PR	CR	IMMUNE RELATED ADVERSE EFFECTS	IMMUNE MONITORING OF PATIENT	IMMUNE MONITORING OF INFUSION PRODUCT	REF.
RCC	CAIX	CAR:CD4-Fc(ε)R1γ	Cohort 1: CAR T cell (n=3) Cohort 2: Low dose CAR T cell (n=5) Cohort 3: CAIX mAb, low-dose Car T cells (n=4)	0 (0) 0 (0) 0 (0)	0 0 0	0 0 0	Cholangitis, grade 2-4 (liver) toxicities (n=3)	CAR T cell persistence; Cytokine blood levels: IFN-γ, TNF-α, IL-2, IL-4, IL-5, and IL-10; CAR T cell cytotoxicity: 51Chromium release assay; CAR T cell cytokine release: IFN-γ; CAR Immunogenicity: anti-CAR humoral and cellular immunity	CAR T cell phenotype: CD3, CD4, CD8 CAR T cell cytokine release: IFN-γ, TNF-α, IL-2, IL-4, IL-5, and IL-10 CAR T cell cytotoxicity: 51Chromium release assay	[34-36]
OVARIAN CANCER	FR	CAR:CD3ζ	8 6	0 (0) 0 (0)	0 0	0 0	-	CAR T cell persistence; CAR T cell imaging with Indium111 labelled T cells; CA-125 serum levels	CAR T cell cytokine release: IFN-γ, IL-10, IL-2. CAR T cell phenotype: CD4, CD8	[38]
NEURO-BLASTOMA	CD171 (L1-CAM)	CAR:CD3ζ	10	1 (10)	1	0	-	CAR T cell persistence	CAR T cell cytokine release: IFNγ, TNF-α IL-2, IL-4, IL-5, MCP-1, RANTES, SDF-1 CAR T cell phenotype: CD45RO, CD62L, CD27, CD28, CD56, CCR2, CCR5, CXCR4, PD-1, GranzymA, Perforin, HyTK, CER7; CAR T cell cyto-toxicity: 51Chromium release assay	[50]
NHL	CD20	CAR:CD3ζ	7	3 (43)	1	2	-	CAR T cell persistence	CAR T cell phenotype: CD2, CD3, CD4, CD5, CD8, CD11a, CD25, CD28, CD44, CD45RA, CD45RO, CD49d, CD56, CD62L, CD94, CD95, CD127, CD134, CD137, CD154, CD314, CCR5, CCR6, CCR7, CXCR3, CXCR4, CXCR5, TCRab; CAR T cell cytotoxicity: 51Chromium release assay	[116]

MALIGNANCY	ANTIGEN	ENDOO-MAIN(S)	PATIENTS	OR (%)	PR	CR	IMMUNE RELATED ADVERSE EFFECTS	IMMUNE MONITORING OF PATIENT	IMMUNE MONITORING OF INFUSION PRODUCT	REF.
NHL	CD19 or CD20	CAR:CD3ζ	4	0 (0)	0	0		CAR T cell persistence; CAR T cell cytotoxicity: 51Chromium release assay	CAR T cell phenotype: CD4, CD8 CAR T cell cytotoxicity: 51Chromium release assay	[117]
COLON	ERBB2	CAR:CD3ζ-CD28-CD137	1	0	0	0	Cytokine storm, death n=1	Plasma cytokines: IFN-γ, GM-CSF, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12	CAR T cell phenotype: CD3, CD4, CD8 CAR T cell cytokine release assay: IFN-γ	[106]
NEUROBLAS-TOMA	GD2	CAR:CD3ζ	19	4 (21)	1	3	-	CAR T cell persistence CAR T cell phenotype over time	CAR T cell phenotype: CD3, CD4, CD8, CD11a, CD11c, CD18, CD19, CD27, CD28, CD45RA, CD45RO, CD38, CD54, CD56, CD62L, CD106,. CD162, CCR2, CCR4, CCR5, CCR7, CXCR3, CXCR4, TCRab, TCRgd CAR T cell cytotoxicity: 51Chromium release assay	[39, 40]
ALL & CLL	CD19	CAR:CD28-CD3ζ	10	0 (0)	0	0	Death 48h after infu-sion, sepsis syndrome (n=1)	CAR T cell persistence Cytokine blood levels: IL-2, IL-7, IL-12, IL15, IFN-γ, TNF-α CAR T cell cytotoxicity: 51Chromi-um release assay Trafficking of CAR T cells post mortem	CAR T cell phenotype: CD4, CD8, CCR7, CD25, CD27, CD28, CD62L, FOXP3 CAR T cell cytotoxicity: 51Chromium release assay CAR T cell cytokine release: G-CSF, GM-GSF, IFNa, IFN-γ, IL-2, IL-3, IL-4, IL-5, IL-10, IL12, IL-13, IL-15, TNF-α, IL-17, MIP-1	[45, 118]

MALIGNANCY	ANTIGEN	ENDOO-MAIN(S)	PATIENTS	OR (%)	PR	CR	IMMUNE RELATED ADVERSE EFFECTS	IMMUNE MONITORING OF PATIENT	IMMUNE MONITORING OF INFUSION PRODUCT	REF.
CLL	CD19	CAR:CD137-CD3ζ	3	3 (100)	1	2	tumor lysis syndrome n=1, long term B-cell aplasia	CAR T cell persistence + expansion; CAR T cell trafficking to BM Cytokine levels (Plasma/BM): IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-1Ra, IL-2Ra, GM-CSF, G-CSF, MIP-1α, MIP-1β, MCP-1, CXCL9, CXCL10,. IFN-γ, IFN-α, TNF-α CAR T cell phenotype: CD4, CD8, CD25, CD27, CD28, CD62L,CD127, PD-1, CCR7 CAR T cell cytotoxicity: CD107a degranulation assay	CAR T cell phenotype: CD4, CD8, CD25, CD27, CD28, CD62L, CD127, PD-1, CCR7 CAR T cell cytotoxicity: CD107a degranulation assay	[43, 119]
NHL	CD19	CAR:CD3ζ / CAR:CD28-CD3ζ	6	0 (0)	0	0	-	CAR T cell Persistence + expansion	CAR T cell phenotype: CD3, CD4, CD8, CD28, CD45RA, CD45RO, CD62L, CCR7 CAR T cell cytotoxicity: 51Chromium release assay	[44]
NHL & CLL	CD19	CAR:CD28-CD3ζ	8	6 (75)	5	1	Long term B-cell aplasia (n=4)	CAR T cell persistence Cytokine blood levels: IFN-γ, TNF-α	CAR T cell phenotype: CD4, CD8, CD28, CD45RA, CD62L, CCR7 CAR T cell cytotoxicity: CD107a degranulation assay CAR-T cell cytokine release: IFN-γ, TNF-α, IL-2	[68]
NHL & CLL	CD19	CAR:CD28-CD3ζ	10	3 (30)	2	1	Tumor lysis syndrome (n=1)	CAR T cell persistence CAR T cell phenotype: CD4, CD8, CD27, CD28 CD45RA, CD57, PD-1, CCR7	CAR T cell phenotype: CD4, CD8, CD27, CD28, CD45RA, CD57, PD-1, CCR7 CAR T cell cytokine release: IFN-γ, TNF, IL-2 CAR T cell cytotoxicity: CD107a degranulation assay	[49]
AML	Lewis Y	CAR:CD28-CD3ζ	5	0 (0)	0	0	-	CAR T cell persistence CAR T cell imaging with Indium111 labelled T cells Cytokine blood levels: IFN-γ, IL-2, IL-4, IL-5, IL-10, TNF	CAR T cell phenotype: CD4, CD8 CAR T cell cytokine release: IFN-γ, IL-2	[46]

Table 2: Page 4 of 4

MALIGNANCY	ANTIGEN	ENDOO-MAIN(S)	PATIENTS	OR (%)	PR	CR	IMMUNE RELATED ADVERSE EFFECTS	IMMUNE MONITORING OF PATIENT	IMMUNE MONITORING OF INFUSION PRODUCT	REF.
DLBCL & CLL	CD19	CAR:CD28-CD3ζ	15	12 (80)	4	8	-	CAR T cell persistence CAR T cell phenotype: CD4, CD8, CD45RA, CCR7, PD-1 CAR T cell trafficking (fine needle aspiration LN) Cytokine blood levels: IFN-γ, TNF-α, IL-6	CAR T cell phenotype: CD4, CD8, CD45RA, CCR7, PD-1 CAR T cell cytokine release: IFN-γ, TNF, IL-2 CAR T cell cytotoxicity: CD107a degranulation assay	[120]
ALL	CD19	CAR:CD137-CD3ζ	30	27 (90)	0	27	Cytokine release syndrome(n=30)	CAR T cell persistence + expansion Inflammatory markers/cytokines in blood: IL-6, IFN-γ, CRP, Ferritin	nr	[14]
ALL	CD19	CAR:CD28-CD3ζ	16	14 (88)	0	14	Cytokine release syndrome (n=4)	CAR T cell persistence + expansion (BM/PB) Cytokine/inflammatory blood levels: Fractalkine, GMCSF, IL-5, IL-6, TNF-α, IFN-γ, IL2Ra. CRP	CAR T cell phenotype: CD3, CD4, CD8 CAR T cell cytotoxicity: 51Chromium release assay	[15, 16]
ALL	CD19	CAR:CD28-CD3ζ	21		0	14 (68)	Cytokine release syndrome (n=3)	CAR T cell persistence + expansion PB/CSF CAR T cell phenotype: CD4, CD8, CD45RO, CD95, CCR7 Max. copies of CAR per 100ng DNA Cytokine/inflammatory blood levels: GMCSF, IL-2, IL-6, IL-10, TNF-α, IFN-γ. CRP	CAR T cell phenotype: CD4, CD8, CD45RO, CD95, CCR7 CAR T-cell proliferation	[47]

Table 2 Overview of clinical CAR trials. Abbreviations:

ALL (acute lymphatic leukemia), AML (acute myeloid leukemia), BM (Bone Marrow), CAIX (carboxy-anhydrase-IX), CLL (Chronic Lymphatic Leukaemia), CR Complete Response), CRP (C-reactive protein), CSF Cerebral Spinal Fluid, CTx (chemotherapy), CXC4 (chemokine ligand 4), DLBCL (diffuse large B-cell lymphoma), G-CSF (granulocyte colony stimulating factor), GMCSF (granulocyte macrophage colony stimulating factor), HyTK (Hygromycin Phosphotransferase-Thymidine Kinase), HSCT (Hematopoietic stem cell transplantation), IFN-α (interferon-alpha), IFN-γ (interferon-gamma), IL (interleukin), mAb (monoclonal antibodies), MCP-1 (monocyte chemotactic protein 1), MIP-1 (macrophage inflammatory protein–1a), NHL (non hodgkin lymphoma), OR (objective response), PB (peripheral blood), PD-1 (programmed cell death protein 1), PR (partial response), RANTES (chemokine (C-C motif) ligand 5), RCC (renal cell carcinoma), RTK (receptor tyrosine kinase inhibitor), SDF-1 (stromal cell-derived factor 1), TNF-α (tumor necrosis factor-alpha)

MALIGNANCY	ANTIGEN	PATIENTS	OR (%)	PR	CR	IMMUNE RELATED ADVERSE EFFECTS	IMMUNE MONITORING OF PATIENT	IMMUNE MONITORING OF INFUSION PRODUCT	REF
MELANOMA	Mart -1/HLA-2	17	2 (12)	2	0	nr	TCR T cell persistence TCR T cell cytokine release: IFN-γ	TCR T cell cytokine release: IFN-γ	[51]
MELANOMA	Mart-1 /HLA-2(high avidity) gp100 /HLA-2	20 16	6 (30) 3 (19)	6 2	0 1	destruction of normal melanocytes (n=14), anterior uveitis (n=11), hearing loss (n=10) destruction of normal melanocytes (n=13), anterior uveitis (n=4), hearing loss (n=5)	TCR T cells persistence TCR T cell phenotype: CD4, CD8, CD45RA, CD27, CD28. TCR PBMC cell cytokine release: IFN-γ, IL-2 Cytokine blood levels: IFN-γ	TCR T cell cytokine release: IFN-γ, IL-2 TCR T cell phenotype: CD4, CD8, CD45RA, CD27, CD28. TCR	[52]
COLON	CEA/ HLA-A2	3	1 (33)	1	0	Severe inflammatory colitis (n=3)	TCR T cell persistence TCR T cell phenotype: CD4, CD8, CD45RA, CD45RO, CD27, CD28 Serum CEA	TCR T cell cytokine release: IFN-γ TCR T cell phenotype: CD4, CD8, CD45RA, CD45RO, CD27, CD28	[66]
MELANOMA & SYNOVIAL CELL SARC	NY-ESO-1/ HLA-A2	20 18	11 (55) 11 (61)	7 10	4 1	nr	TCR T cell persistence TCR T cell phenotype: CD4, CD8	TCR T cell cytokine release: IFN-γ	[17, 53]
MELANOMA & MULTIPLE MYELOMA	MAGE-A3(EVD)/ HLA-A1	2	0 (0)	0	0	Lethal cardiac toxicity (n=2)	TCR T cell persistence Cytokine blood levels; VEGF, IL-1β, IL-2, IL-4, IL-5, IL6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-2R, HGF, GM-CSF, MCP-1, IFN-γ, TNF-α	TCR T cell phenotype: CD3, CD4, CD8, CD45, CD62L	[70]
MELANOMA	MAGE-A3(KVA)/ HLA-A2	9	5 (56)	3	2	Changes in mental status n=3, coma and death n=2	TCR T cell persistence TCR T cell phenotype: CD4, CD8, CD45RO, CD62L, TCRβ TCR T cell cytokine release: IFN-γ, TNF-α	TCR T cell phenotype: CD4, CD8, CD45RO, CD62L, TCRβ TCR T cell cytokine release: IFN-γ TCR T cell cytotoxicity: CD107a degranulation assay	[69]

Table 3: Overview of clinical TCR T cell trials.

Abbreviations: Ab (antibody), CEA (carcinoembryonic antigen), CR (complete Response), CTx (chemotherapy), GMCSF (granulocyte macrophage colony stimulating factor), gp100 (glycoprotein 100), HGF (hepatocyte growth factor), HLA (human leukocyte antigen), IFN-α (interferon alpha), IFN-γ (interferon-gamma), IL (interleukin), MAGE-A3 (melanoma-associated antigen 3), MART-1 (melanoma antigen recognized by T cells 1), MCP-1 (monocyte chemotactic protein 1), NMA (nonmyeloablative lymphodepleting chemotherapy, nr (none reported), OR (objective response), PR (partial response), TNF-α (tumor necrosis factor-alpha, V

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Chapter 6

Autologous dendritic cell therapy in mesothelioma patients enhances frequencies of peripheral CD4 T cells expressing HLA-DR, PD-1 or ICOS

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ABSTRACT

Introduction: Malignant pleural mesothelioma (MPM) is a malignancy with a very poor prognosis for which new treatment options are urgently needed. We have previously shown that dendritic cell (DC) immunotherapy provides a clinically feasible treatment option. In the current study, we set out to assess the immunological changes induced by DC immunotherapy in peripheral blood of MPM patients.

Methods: Peripheral blood was collected from nine patients enrolled in a phase I dose escalation study, before and after treatment with DCs that were pulsed with an allogeneic tumor lysate preparation consisting of a mixture of five cultured mesothelioma cell lines. We used immune profiling by multiplex flow cytometry to characterize different populations of immune cells. In particular, we determined frequencies of T cell subsets that showed single and combinatorial expression of multiple markers that signify T cell activation, maturation and inhibition. Therapy-induced T cell reactivity was assessed in peptide/MHC multimer stainings using mesothelin as a prototypic target antigen with confirmed expression in the clinical tumor lysate preparation. T cell receptor (TCR) diversity was evaluated by TCRB geneβ PCR assays.

Results: We observed an increase in the numbers of B cells, CD4 and CD8 T cells, but not NK cells at 6 weeks post treatment. The increases in B and T lymphocytes were not accompanied by major changes in T cell reactivity towards mesothelin nor in TCRB diversity. Notably, we did observe enhanced proportions of CD4 T cells expressing HLA-DR, PD-1 (at 2 weeks after onset of treatment) and ICOS (6 weeks) and a CD8 T cell population expressing LAG3 (2 weeks).

Discussion: DC immunotherapy using allogeneic tumor lysate resulted in enhanced frequencies of B cells and T cells in blood. We did not detect a skewed antigen-reactivity of peripheral CD8 T cells. Interestingly, frequencies of CD4 T cells expressing activation markers and PD-1 were increased. These findings indicate a systemic activation of the adaptive immune response and may guide future immune monitoring studies of DC therapies.

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a solid tumor of the pleural lining that is strongly related to the exposure to asbestos (1). Overall survival is poor with a median survival of less than a year, and conventional therapies like chemotherapy and radiotherapy being able to improve survival only by a few months (2). Immunotherapy has evolved as an important new treatment modality in various kinds of cancer, with checkpoint inhibitors currently being FDA approved as first line therapy for several cancer types. Initial results of clinical studies for MPM with checkpoint inhibitors as second line treatment showed response rates of 9-25% (3).

MPM is characterized by a strong immunosuppressive component, with relatively low numbers of T cells infiltrating the tumor (1, 4). These low numbers of tumor-infiltrating T cells have prognostic value in MPM (5) and might explain the relatively low response rates to checkpoint inhibitors (1).

Furthermore, in MPM patients dendritic cells (DCs) have been shown to be reduced in numbers and in antigen-processing function compared to healthy controls, which negatively affected survival outcomes (6). The reduced functionality of DCs is thought to relate to low intra-tumoral T cell numbers. Along these lines, DC vaccination represents a promising therapeutic strategy.

Previously, we have developed a cellular therapy for MPM, consisting of autologous DCs pulsed with autologous tumor lysate with the intention to cover a broad range of tumor antigens (7). This vaccination strategy was shown to be safe with promising clinical outcomes (7, 8). However, the availability and quality of tumor material that could be obtained, limited the feasibility of the treatment with DCs loaded with autologous tumor lysate. To overcome this limitation, DC vaccination using allogeneic tumor lysate was developed and tested for safety and feasibility in a phase I clinical trial (9). Allogeneic tumor lysate derived from five ***in vitro*** cultured clinical-grade human mesothelioma cell lines was used to pulse autologous DCs and the resulting DC vaccine was administered to patients i.d. and i.v. once every two weeks for three cycles, with a booster vaccination at three and six months after the start of treatment. The study was set up as a dose escalation study with three cohorts of three patients, and each cohort received 10 million, 25 million or 50 million DCs per vaccination, respectively. By circumventing the immunosuppressive tumor immune environment and providing enhanced tumor antigen presentation with DC vaccination, impressive objective responses could be obtained, as exemplified by a tumor reduction of ~70% at 6 weeks post-treatment in one of the patients in this phase-I trial (9).

In the current study we aimed to characterize the immunological changes induced by DC immunotherapy in these nine MPM patients. For a better understanding of the immunological changes induced by DC immunotherapy we monitored peripheral blood, which is the preferred compartment for sequential sampling. We used extensive multiplex flow cytometry

with a focus on T cell activation and inhibitory markers and characterized T cell specificity using peptide-MHC multimers to obtain a detailed immune profile and immune dynamics following DC immunotherapy.

METHODS

Patients

The nine patients in this study participated in a first-in-human clinical trial as described by Aerts et al (9). In short, all patients had pathologically-proven MPM and were included in the study at least 6 weeks after their last chemotherapy treatment, or were treatment-naive if they had refused chemotherapy treatment. After inclusion in the study, patients received leukapheresis, which was used as a source of autologous DCs.

The DCs were prepared as described (9) and pulsed with a lysate, consisting of a mixture of five *in vitro* cultured mesothelioma cell lines. Patients received a total of 3 vaccinations every two weeks and blood samples were obtained at baseline and at week 2, 4, 6 and 8 following initial vaccination. Booster vaccinations were administered at 3 and 6 months (9). One third of the dose was administered intradermally (i.d.), and two thirds of the dose intravenously (i.v.). As this was a dose escalation study, patients 1-3 received 10 million DCs per vaccination, patients 4-6 received 25 million DCs per vaccination and patients 7-9 received 50 million DCs per vaccination. Patients 7 and 9 did not receive their second booster vaccination due to shortage of patient material. All other patients completed the full treatment scheme (Table S1 in Supplementary Material). For flow cytometry (FCM) analysis, cohort 1 was not included since the collected peripheral blood samples of patients in cohort 1 were immediately processed and stored. For cohort 2 and 3 the protocol was amended to enable absolute immune cell quantification.

Collection and processing of peripheral blood samples

Ethylene diamine tetra acetic acid (EDTA) anticoagulated peripheral blood was drawn from patients at baseline prior to the first vaccination (week 0), at 2 weeks after the first vaccination, i.e., prior to the second vaccination (week 2) and 2 weeks after the third vaccination at week 6 and analyzed within 6 hours by multiplex FCM. One ml of whole blood was used for multiplex FCM and from the remaining blood, peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll density gradient centrifugation, and were stored at-80 °C for further analyses.

Multiplex flow cytometric assessment of numbers and phenotype of immune cells

To enumerate immune cell populations, whole blood (100µl) was stained with the 'absolute numbers' panel (Table S2 in Supplementary Material) and incubated for 15 min at room temperature. Subsequently, 2 ml of lysis buffer (NH_4Cl: 8,26 mg/ml, $KHCO_3$: 1 mg/ml and EDTA:

37µg/ml) was added to the blood and incubated for 15 min at room temperature. Subsequently 100µl of Flow-Count Fluorospheres (Beckman Coulter Inc) was added and samples were measured on a BD LSRFortessa™ flow cytometer. A minimum of 10,000 CD45+ cells were measured to enable clear distinction of defined immune cell populations. Subsequently, data was analyzed with FlowJo version X (FlowJo, LCC) using the gating strategy as exemplified in Figure S1 in Supplementary Material. Values were expressed as cells per microliter. To determine the phenotype of T cells, the T cell maturation, activation, co-inhibitory and co-stimulatory receptors were analyzed on whole blood with different FCM panels (Table S2 in Supplementary Material). 100µl of whole blood was stained with each of the panels and incubated for 15 minutes at room temperature. Subsequently, 2 ml of lysis buffer was added to the blood and after an incubation of 15 minutes at room temperature, the cell suspensions were centrifuged at 450g for 5 minutes, washed and resuspended in buffered 0.1% paraformaldehyde (PFA). A minimum of 30.000 CD3+ cells were measured on the LSRFortessa™ flow cytometer to obtain clearly detectable immune populations. The FCM data were analyzed with FlowJo version X.

Determination of mesothelin-specific T cells

PBMCs were thawed and subsequently T cells were rapidly expanded with a feeder system as described elsewhere (10). After this rapid expansion protocol, T cells were co-cultured with artificial antigen presenting cells (aAPC) (11) loaded with mesothelin peptide A: SLLFLLFSL (mesothelin$_{20\text{-}28}$), or mesothelin peptide B: VLPLTVAEV (mesothelin$_{531\text{-}539}$)(Immudex, Copenhagen, Denmark)**.** The aAPC cell line is based on K562 cells, retrovirally transduced with CD80, CD83, and HLA-A2 for optimal antigen presentation and co-stimulation, and enables enrichment of antigen-reactive T cells with protocols optimized in our laboratory (12). 2.5 x 10^6 aAPC cells/ml were incubated at room temperature for five hours with 10µg/ml peptide and subsequently irradiated (120 Gy). Subsequently, these peptide-loaded aAPC cells were co-cultured with T cells (ratio 1:20) in T cell medium (RPMI Hepes [Lonza] supplemented with 10% human serum [Sanquin, Amsterdam, The Netherlands], 1% L-glutamine and 1% penicilline/streptomycine), 180 IU IL-2 per ml (Chiron, Amsterdam, The Netherlands), and 5 ng IL-15 per ml (Le-Perray-en-Yvelines, France). Mesothelin-driven T cell expansion was continued for 4 cycles, after which T cells were examined for their binding ability to corresponding mesothelin peptide-HLA-A2 complex multimers. In this study, we performed a maximum of 4 peptide-specific expansion cycles.

Dextramer A: PE-conjugated HLA-A2 dextramer with peptide SLLFLLFSL (mesothelin$_{20\text{-}28}$) and dextramer B: APC-conjugated HLA-A2 dextramer with peptide VLPLTVAEV (mesothelin$_{531\text{-}539}$) were both ordered from Immudex (Copenhagen, Denmark). Dextramer staining was performed according to manufacturer's protocol. Anti-CD3-BV711 (clone UCHT1) and anti-CD8-FITC (clone SK1) mAbs (both from BD Biosciences) were used together with dextramers for extracellular staining. 4',6-diamidino-2-phenylindole (DAPI) was used as viability dye. Samples were measured on an LSR-II flow cytometer (BD). Fluorescence-minus-one (FMO) con-

trols were used to enable gating and determine dextramer-positive populations.

GeneScan T cell clonality analysis

Cell pellets from PBMC samples were frozen and stored until further use. Genomic DNA was isolated using the AllPrep DNA / RNA Mini kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. T-cell receptor (TCR) β gene repertoire was measured using commercially available multiplex TCR Vβ-Jβ PCR assays (Invivoscribe, San Diego, CA, USA) as developed and approved by the BIOMED-2 / EuroClonality consortium (13). GeneScan fragment analysis was done on an ABI 3130 xl instrument (ThermoFisher Scientific) and data were analyzed using PeakScanner software. Data interpretation was based on GeneScan patterns of duplicate PCR results, largely following the EuroClonality guideline (14).

Statistical analyses

Statistical analyses and graphs were made with Graphpad Prism v5.0. For comparison of changes from baseline measurements, Wilcoxon signed-ranks test was used to test for significance between baseline measurements and other time points. The heatmap was made in R for windows version 3.4.1 using the package in "gplots" (https://cran.r-project.org/web/packages/gplots/). Clustering was performed with a complete agglomeration method, and distance matrix of all variables was computed with euclidean distance.

RESULTS

Numbers of B, CD4 and CD8 T lymphocytes in peripheral blood increase upon vaccination with DCs

We first assessed the absolute numbers of specific lymphocyte subsets (CD3+CD4+, CD3+CD8+, CD3-CD19+ and CD3-CD56+) from the six patients treated in the second and third cohort (dosage 25 million and 50 million DC per vaccination), using the gating strategy as shown in Figure S1 in Supplementary Material. Patients showed a significant increase at week 6 (after 3 vaccinations) compared to baseline in (median) numbers of $CD4^+$ T cells (increase from 522 to 634 cells/μl), $CD8^+$ T cells (from 256 to 304 cells/μl) and B cells (from 162 to 199 cells/μl), but not Natural Killer (NK) cells (Figure 1). Together, these results are indicative for a potentiation of adaptive immunity in peripheral blood after DC immunotherapy.

Mesothelin-specific T cells are detectable in peripheral blood of mesothelioma patients

Next, we investigated whether the increased numbers of T cells due to DC vaccination harbored vaccine-specific CD8 T cells. To identify vaccine-specific CD8 T cells, we selected mesothelin as a prototypic antigen based on the following lines of evidence. Firstly, RNA sequencing and western blot data obtained from the mesothelioma cell lines used to generate the lysate preparation validated mesothelin mRNA and protein expression. Secondly, immune

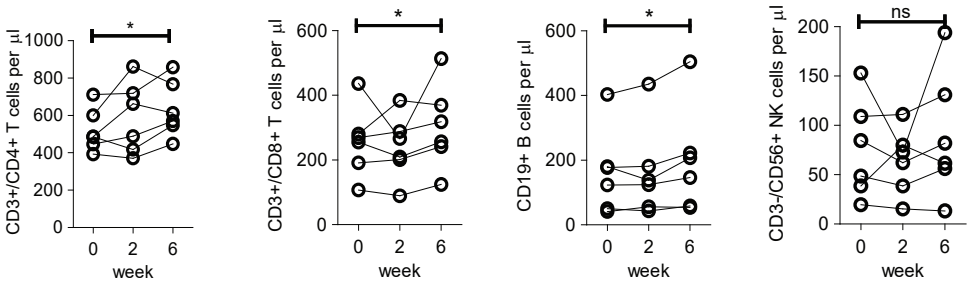

Figure 1: Absolute number of lymphocyte subsets in peripheral blood of patients before and after DC immunotherapy.

Quantification of absolute numbers of CD4 T cells (CD45+/CD3+/CD4+), CD8 T cells (CD45+/CD3+/CD8+), B cells (CD45+/CD3-/CD19+), and NK cells (CD45+/CD3-/CD56+) in peripheral blood of patients in cohort 2 and 3 on baseline prior to the first vaccination (week 0), 2 weeks after the first vaccination, i.e., prior to the second vaccination (week 2) and 2 weeks after the third vaccination (week 6). Differences between week 0 and week 6 with respect to paired continuous parameters were determined using the exact Wilcoxon signed rank test. * $p < 0.05$; ns: not significant.

histochemistry confirmed the expression of mesothelin in eight out of eight available patient biopsies. Lastly, mesothelin peptide/HLA-A2 complexes with reported immune reactivity (15, 16), (peptide A: SLLFLLFSL and peptide B: VLPLTVAEV) were bound by CD8 T cells derived from patient skin biopsies after challenge with DC vaccine (9), as summarized in Table S2 in Supplementary Material. To determine whether DC therapy would induce changes in the frequency of mesothelin-specific CD8 T cells, we measured the binding of two mesothelin-peptide/HLA-A2 multimers by CD8 T cells in pre- versus post-vaccination peripheral blood samples. To this end, T cell fractions were first propagated using four T cell culture cycles in the presence of mesothelin peptides (A and B). Figures 2A and 2B show flow cytometry plots for one of the two mesothelin epitopes (peptide B) in propagated CD8 T cell fractions from HLA-A2-positive patients (8 out of 9 patients). These analyses show that the frequency of mesothelin-specific CD8 T cells is variable among patients, and already pre-exists in 5 out of 8 HLA-A2 positive patients and does not change significantly upon treatment. Similar data were obtained for a second mesothelin epitope (peptide A; Figure S2 in Supplementary Material) and no correlation was observed between the frequencies of CD8 T cells specific for the two epitopes analyzed (data not shown).

In addition, we studied TCRB diversity of total T cells as a global measure of T cell reactivity via GeneScan TCRB PCR (Figure 2C). While some patients showed dominant peaks (e.g. patient 9), the GeneScan patterns were generally similar in pre-treatment and post-treatment PBMC fractions from the MPM patients, indicating that DC therapy did not induce clonal T cell expansion or selection. Collectively, these findings show that DC vaccination-specific T cells are detectable, but that this is not accompanied by an overall enhancement of frequencies in peripheral blood, nor obvious shifts in T cell TCRB repertoire.

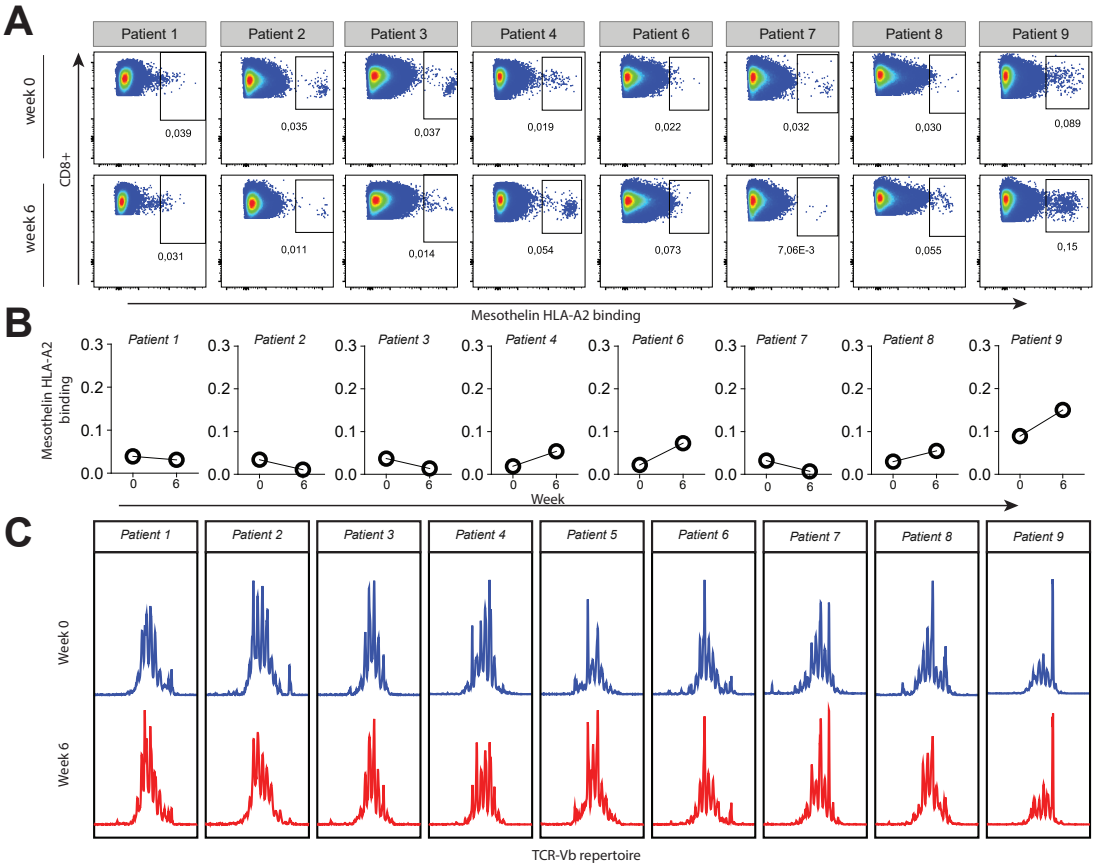

Figure 2: Mesothelin-specific CD8 T cells in HLA-A2 positive patients are measurable in pre- and post-vaccination samples.

A) Peripheral blood samples were collected at baseline (week 0) and 2 weeks after the third vaccination (week 6). CD8 T cells were propagated in four culture cycles with mesothelin-peptide-B loaded aAPC, after which mesothelin-peptide B/HLA-A2 dextramers were used to detect mesothelin-specific CD8 T cells. Gating was based on the negative controls, FMO staining and the non-CD8 T cell population. All HLA-A2 positive patients are shown; patient 5 was excluded (haplotype HLA-A3/HLA-A68). **B)** Values of gated dextramer-binding CD8 T cells - as shown in panel A - presented as proportions of total CD8 T cells. **C)** Relative frequencies of complementarity determining region-3 (CDR3) lengths in pre- and post-therapy total T cells from PBMC samples of all patients. The Y-axis represents relative frequency as assessed by fluorescence intensity, with the various CDR3 lengths on the X-axis. A polyclonal repertoire would follow a normal distribution.

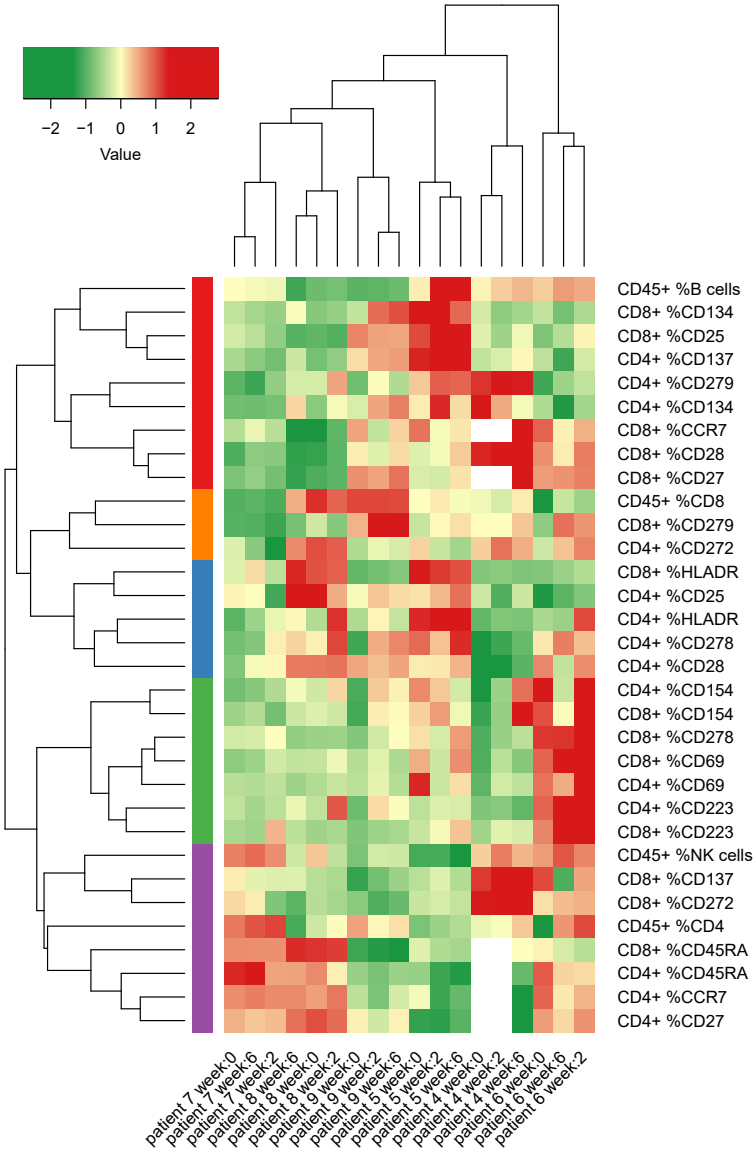

Figure 3: Heatmap of percentages of several different lymphocyte subsets and T cell surface markers in peripheral blood from patients in cohort 2 and 3 (MCV004 – MCV009).

Columns represent different patient samples at week 0, week 2 and week 6 after start of treatment. Rows represent the proportions of CD4 T cell, CD8 T cell and CD45 lymphocyte populations that express defined markers. Percentages were normalized according to mean values of all measurements and were clustered by non-hierarchical clustering.

Increased frequencies of PD-1, HLA-DR and ICOS positive CD4+ T cells after DC vaccination.

We then evaluated changes in frequencies of CD4 or CD8 T cells expressing surface activation, maturation or co-signaling markers and compiled a heatmap of all variables measured by FCM at pre- and post-therapy time points (Figure 3). Hierarchical clustering of samples showed clustering of the different time points per patient for the measured variables. This demonstrates that intra-individual differences over time are relatively small, compared to inter-individual differences in the immunological variables that were measured.

To further explore immunological parameters that were modulated by DC immunotherapy, we assessed the expression of the various surface markers at multiple time points. Figure 4A-D display the expression of T cell activation, co-inhibitory and co-stimulatory markers that were significantly altered either early after the start of treatment (2 weeks after the first vaccination) or later during treatment (2 weeks after the third vaccination). In particular, $CD4^+$ T cells in peripheral blood showed a significant gain of HLA-DR^+ and PD-1^+ T cells (Figure 4A, B) after the first vaccination. After 3 vaccinations, we detected a significant increase of CD278 (inducible T-cell co-stimulator; ICOS) positive $CD4^+$ T cells compared to baseline (Figure 4D). CD8 T cells did not show any significant increase of these markers, although $CD8^+$ T cells demonstrated a significant enrichment of $CD223^+$ (Lymphocyte activation gene-3; LAG-3) T cells (Figure 4C). No significant changes were observed in the following markers on both $CD4^+$ and $CD8^+$ T cells: CD25, CD69, CD272 (BTLA), CD137 (4-1BB), CD154 (CD40L), CD134 (OX40); (Figure S3 in Supplementary Material).

In summary, these findings show that following autologous DC therapy in mesothelioma patients the frequencies of circulating CD4 T cells expressing HLA-DR, PD-1 or ICOS, as well as CD8 T cells expressing the co-inhibitory receptor LAG3 are significantly increased.

DISCUSSION

Here we document on the effects of treatment of MPM patients with DC immunotherapy – autologous dendritic cells loaded with an allogeneic tumor lysate prepared from five human MPM cell lines – on blood immune composition. We observed an increase in absolute numbers of B cells, and $CD4^+$ and $CD8^+$ T cells in peripheral blood, suggesting induction of both a cellular and humoral immune response. Additionally, we found a significant increase of HLA-DR, PD-1, and ICOS-positive $CD4^+$ T cells, and an increase of LAG3-positive $CD8^+$ T cells after treatment with DC vaccination therapy.

The DC immunotherapy was designed to induce a broad immune response towards multiple tumor antigens present in the tumor lysates (9). To detect whether the increase in T cell numbers was accompanied by a gain of vaccine-specific T cells, we chose to monitor mesothelin-specific T cells in peripheral blood during treatment. Since mesothelin was determined to be present in the tumor lysate, and expressed on the tumor cells of the patients, it

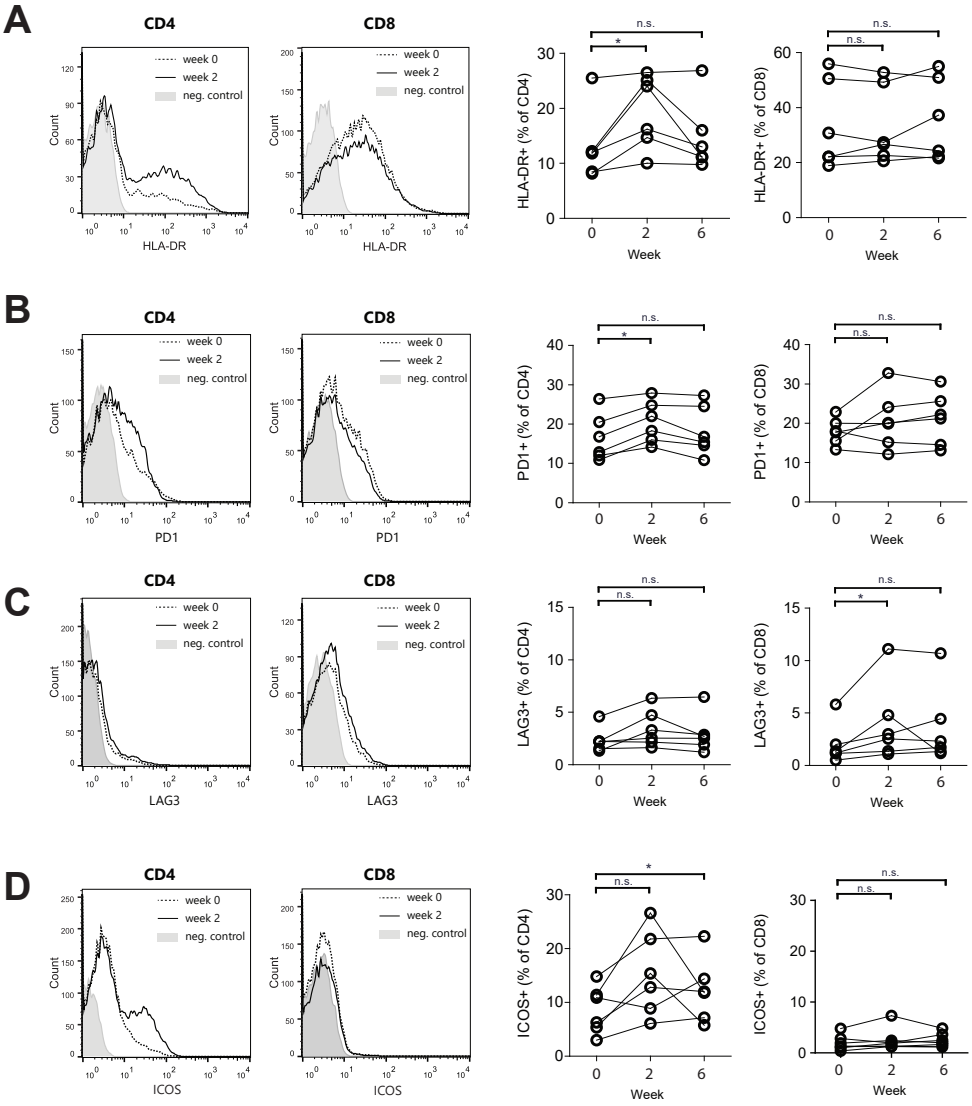

Figure 4: T cell activation and co-stimulatory molecule expression changes induced by DC immunotherapy.

Representative histograms of flow cytometry data (left) and quantification for patients 4 to 9 at week 0, week 2 and week 6 after start of treatment (right). **A)** Proportions of HLA-DR-positive CD4 and CD8 T cells. **B)** Proportions of PD-1-positive CD4 and CD8 T cells. **C)** Proportions of LAG3-positive CD4 and CD8 T cells and **D)** Proportions of ICOS-positive CD4 and CD8 T cells. * p<0.05- Wilcoxon paired signed-rank test, n.s. = not significant.

was regarded to be a relevant antigen in this setting. Interestingly, after mesothelin-derived peptide-driven ***in vitro*** T cell propagation, $CD8^+$ T cells that bind mesothelin peptide/HLA-A2 complexes could already be detected in baseline blood samples of the majority of patients, suggesting that a mesothelin-specific immune response was already present in these patients prior to therapy. However, DC vaccination did not induce changes in frequencies of the mesothelin-specific T cells in peripheral blood. Additionally, as a measure of T cell clonality, we analyzed the TCRB repertoire in pre- and post-treatment blood samples and confirmed that no major repertoire shifts occurred. Our combined findings of an increase in CD4+ and CD8+ T cells and non-skewed TCRB repertoire suggest that DC vaccination induces polyclonal T cell responses that still might be enriched in tumor-specific T cells. As we only have monitored specificity against one major model antigen (mesothelin) in this study, we might have missed an increase of tumor antigen-specific T cells with other specificities. Furthermore, our study did not address whether changes occurred in T cell reactivity in the local compartment. However, removal of tumor can be harmful in MPM due to a substantial risk of local tumor outgrowth at the intervention site (17), which precludes analysis of local tumor material for monitoring purposes.

Some other studies employing DC immunotherapy in different types of cancer have reported an increase in the frequency of tumor-specific T cells in peripheral blood after treatment, as determined by IFN-γ ELISPOT (18) or HLA-A2 MHC multimers (19). However, in these studies specific peptides were used to pulse DCs, which therefore have likely led to a more easily detectable clonal expansion of T cells. Palucka and colleagues found increased frequencies of MART-1-specific $CD8^+$ T cells after DC vaccination using allogeneic tumor lysate in 3 out of 21 patients, with detectable numbers of these T cells already present at baseline in 2 out of these 3 patients (20). In previous studies with DC vaccination in mesothelioma using autologous tumor material, we found significantly increased cytotoxicity against the tumor after treatment, showing induction of a tumor-specific response by DC immunotherapy (7). In the current study, no tumor material was collected for ethical reasons, but we anticipate a comparable induction of a tumor-specific immune response based on the clinical outcomes in our patient cohort (9). As shown here and by others, monitoring of specific T cells in peripheral blood upon immunotherapy with a broad antigen repertoire, is challenging due to the unknown antigen composition and low frequency of individual T cell clones, and requires either very immunogenic antigens like NY-ESO-1 or MART-1 (21, 22) or ***in vitro*** enrichment steps, which makes it less attractive for future use in clinical practice.

Next to T cell specificity, we investigated T cell phenotype, using extended multiplex flow cytometry. We demonstrated that treatment-related differences were most notable for $CD4^+$ T cells, with an increase in surface expression of ICOS, PD-1 and HLA-DR after treatment. This signifies T cell activation, because T cells upregulate HLA-DR (23), ICOS (24) and PD-1 (25) after (TCR) stimulation.

Interestingly, both HLA-DR^+ CD4 T cells and $ICOS^+$ CD4 T cells have been described to in-

crease after treatment with ipilimumab (anti-CTLA-4 monoclonal antibody) (26). Moreover, an increase of $ICOS^+$ CD4 T cells is suggested as a possible pharmacodynamic biomarker for response to this checkpoint inhibitor (27-29). Notably, the best responding patient (#5) in our study showed the highest numbers of $HLA\text{-}DR^+$ $CD4^+$ and $ICOS^+$ $CD4^+$ T cells. Our flow cytometry panels did not allow analysis of co-expression of ICOS and HLA-DR. Others have demonstrated that $ICOS^+$ T cells also expressed CD45RO (24), indicating that T cells bearing high levels of ICOS may be in an advanced maturation phase. This would be in concordance with the low proportions of CCR7 and CD27-positive T cells in our patient (#5), which are markers of less maturated T cells. In the current study patient numbers are too low to correlate the immune monitoring data with clinical outcome. Yet, our findings suggest that the $ICOS^+$ $CD4^+$ T cell subset may have value in the immune monitoring of future trials with DC immunotherapy. Furthermore, Fan and colleagues have described a functional role for ICOS in the anti-tumor immune response (30), providing a rationale for the combination of DC immunotherapy and engagement of ICOS-signaling in future treatments.

In conclusion, vaccination with a broad spectrum of antigens (as is the case with the allogeneic lysate used in the current study) in MPM patients, induced an increase in T cell and B cell numbers in peripheral blood. No evidence was found for a mono- or oligo-clonal T cell expansion, thus suggesting broad activation of the T lymphocytes after therapy. Additionally, changes in the frequencies of defined immune cell markers, in particular the increase of T cell activation markers in $CD4^+$ T cells, demonstrated treatment-associated changes that we would propose as parameters to be included in the monitoring of DC vaccination treatments. Future studies with larger patient groups should evaluate their relation with treatment efficacy.

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SUPPLEMENTARY DATA

Table S1: Patient characteristics*

							Skin responses to DCK, DC and lys †			Presence Msln-specific T cells in skin biopsy ‡	
Patient	*Pretreatment (chemo)*	*DC dose (per vaccination)*	*Number of vaccinations*	*Best response (RECIST)*	*HLA haplotype*	*Msln expression diagnostic biopsy#*	*DCK*	*DC*	*lys*	*Msln peptide A*	*Msln peptide B*
1	No	10 million	5	SD	A1/A2	++	+	-	-	-	-
2	No	10 million	5	SD	A2/A68	++	+	-	+	+	+
3	Yes	10 million	5	SD	A2/A2	++	+	-	-	-	-
4	Yes	25 million	5	PR	A1/A2	++	+	-	-	*ND*	*ND*
5	No	25 million	5	PR	A3/A68	++	+	+	-	*ND*	*ND*
6	Yes	25 million	5	SD	A1/A2	++	+	+	-	-	-
7	No	50 million	4	SD	A2/A24	*ND*	+	+	-	+	+
8	Yes	50 million	5	SD	A2/A24	++	+	+	-	+	+
9	Yes	50 million	4	SD	A2/A32	+	+	+	-	+	+

* Patient characteristics are further detailed Aerts et al (2017)

Abbreviations: DCK, DC's pulsed with lysate and KLH; DC, DC's pulsed with lysate only; lys, tumor lysate; Msln, mesothelin; ND, not determined; SD, stable disease; PR, partial response.

++ = >75% positive tumor cells; + = 25-75% positive tumor cells;

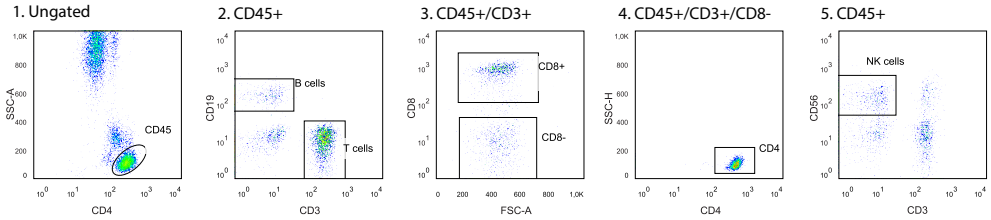
† positive (+) if induration > 2 mm 48 hours after injection.

‡ positive (+) when dextramer-binding CD8 T cell frequency ≥2x negative controls

Table S2: List of antibodies used for multiplex flow cytometry

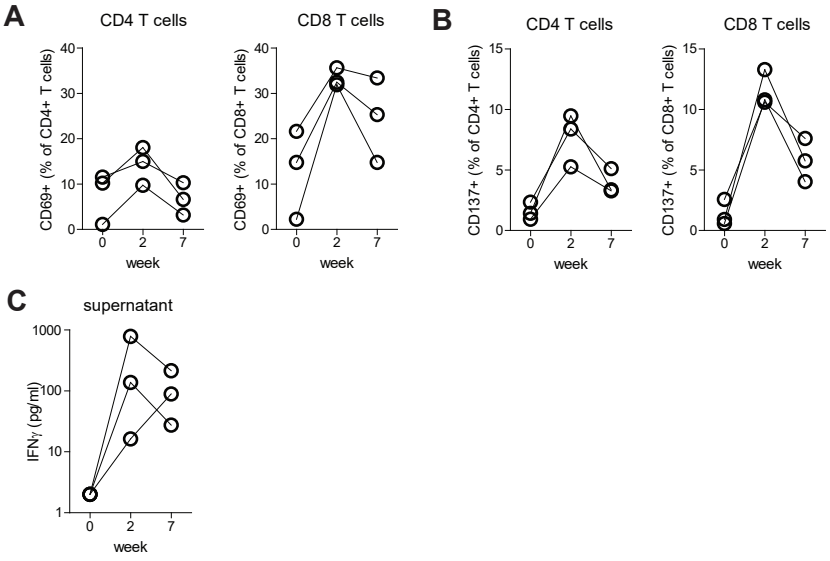
MARKER	LABEL	SUPPLIER	CAT.NO	CLONE	PANEL*
CD3	FITC	BD Biosciences	345764	SK7	1
CD3	Pacific Blue	BD Biosciences	558117	UCHT1	2,3,4,5
CD4	PE-Cy7	BD Biosciences	348809	SK3	1
CD4	BV510	BD Biosciences	560769	RPA-T4	2,3,4,5
CCR7	PE	R&D	FAB197P	150503	2
CD8	APC-Cy7	BD Biosciences	348813	SK1	1
CD8	PerCP	BD Biosciences	345774	SK1	2,3,4,5
CD19	APC	eBioscience	17-0199-42	HIB19	1
CD25	APC	BD Biosciences	340907	2A3	3
CD27	APC-Cy7	BD Biosciences	560222	M-T271	2
CD28	APC	BD Biosciences	559770	CD28.2	2
CD45	PerCP	BD Biosciences	345809	2D1	1
CD45RA	PE-Cy7	BD Biosciences	337186	L48	2
CD56	PE	Dako	R7251	C5,9	1
CD57	FITC	BD Biosciences	333169	HNK-1	2
CD69	PE-Cy7	BD Biosciences	335792	L78	3
CD127	PE	BD Biosciences	557938	hIL-7R-M21	3
CD134	FITC	eBioscience	11-1347-42	ACT35 (ACT-35)	5
CD137	PE	eBioscience	12-1379-42	4B4 (4B4-1)	5
CD154	APC-Cy7	Biolegend	310822	24-31	5
CD223	PE-Cy7	eBioscience	25-2239-42	3DS223H	4
CD272	PE	Biolegend	344506	MIH26	4
CD279	APC-Cy7	Biolegend	329922	EH12.2H7	4
CD278	PE-Cy7	eBioscience	25-9948-42	ISA-3	5
HLA-DR	APC-Cy7	BD Biosciences	335831	L243	3
TCRgd	FITC	BD Biosciences	347903	11F2	1

*Panel 1: Absolute numbers, Panel 2: Maturation, Panel 3: Activation/Tregs, Panel 4: Co-inhibitory markers, Panel 5: Co-stimulatory markers



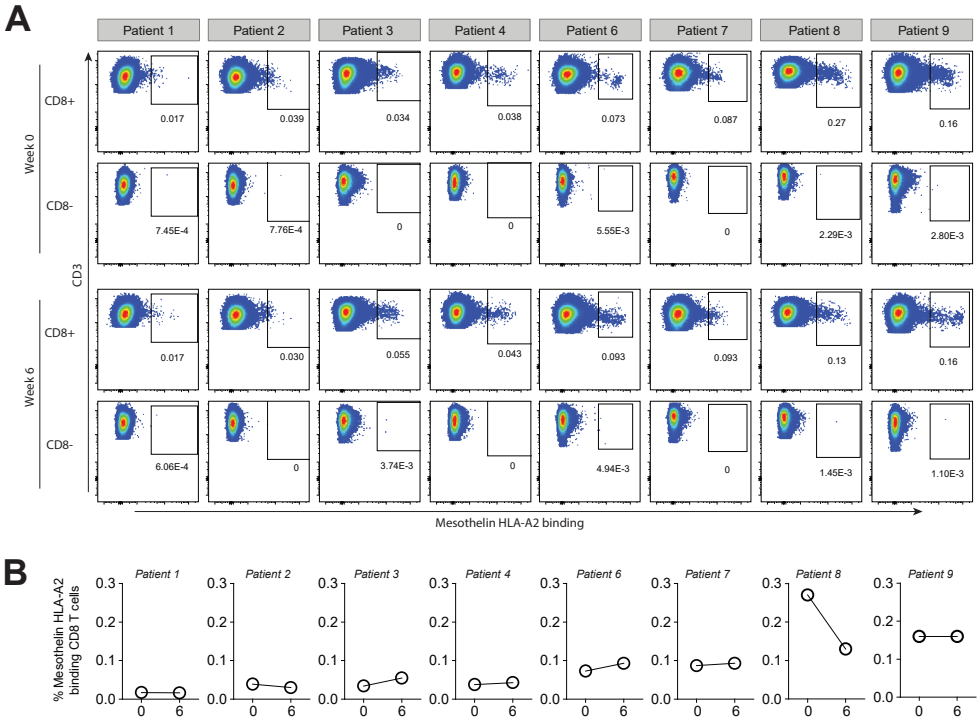
Supplementary figure S1: Example of gating strategy preceding the enumeration of immune cell populations.

Lymphocytes were defined as CD45+, SSC-A^{low} (plot1); from CD45+, SSC-A^{low} lymphocytes, T cells (CD3+CD19-) and B cells (CD19+CD3-) were defined (plot 2). T cells were further subdivided in CD8+ T cells, and CD4+ T cells (plot3 and 4). Within the CD45+, SSC-A^{low} lymphocytes, NK cells were defined as CD56+CD3- (plot 5).



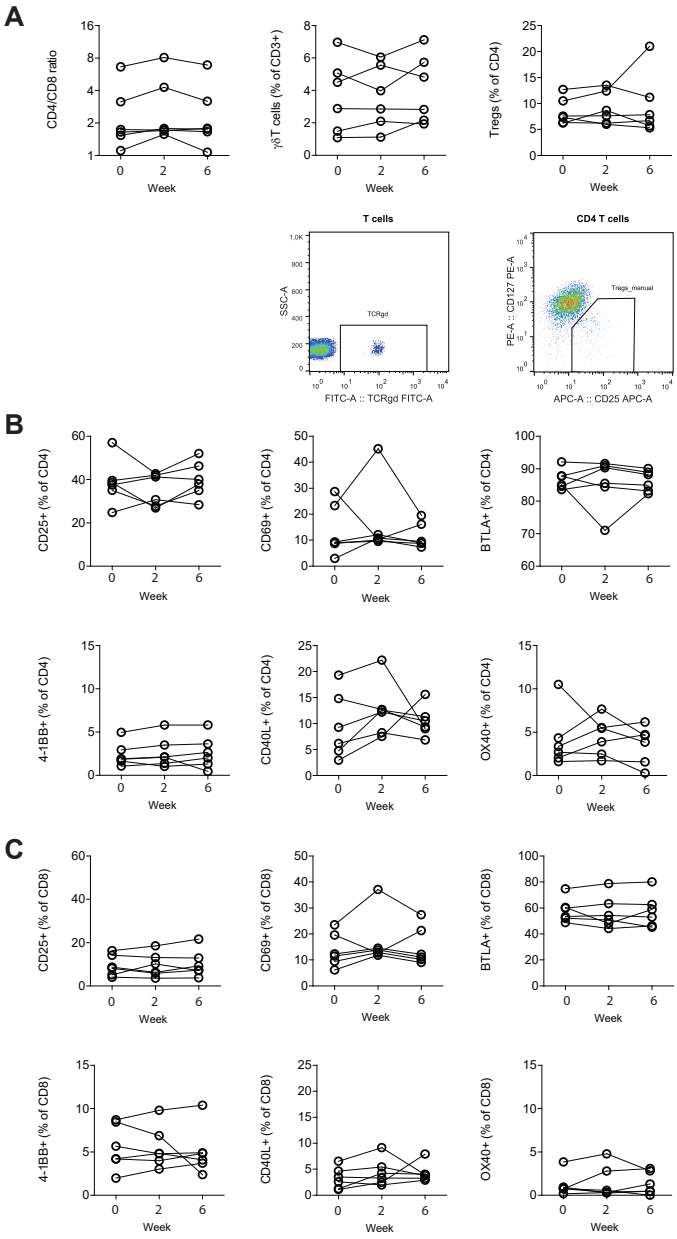
Supplementary figure S2: Activation of post-therapy T cells after co-culture with lysate-pulsed DCs.

Lysate-pulsed DCs were co-cultured for 24h with autologous PBMC obtained at week 0, 2 or 7. **A)** Proportions of CD4 and CD8 T cells expressing CD69, as determined by flow cytometry. **B)** Proportions of CD4 and CD8 T cells expressing CD137, as determined by flow cytometry. **C)** Concentration of IFNγ in the culture supernatant after 24h, as determined by ELISA.



Supplementary figure S3: Mesothelin peptide A-specific CD8 T cells in HLA-A2 positive patients.

A) Peripheral blood samples were collected at base line (week 0) and 2 weeks after the third vaccination (week 6). CD8 T cells were propagated in four culture cycles with mesothelin-peptide loaded aAPC after which mesothelin-peptide/HLA-A2 dextramers were used to detect mesothelin specific CD8 T cells. **B)** Values of gated tetramer positive CD8 T cells - as shown in panel A - presented as proportions of mesothelin-B HLA-A2-binding cells of total CD8 T cells. Gating was based on the negative controls: FMO staining and the non-CD8 T cell population. All HLA-A2 positive patients are shown; patient 5 was excluded (haplotype HLA-A3/HLA-A68).



Supplementary figure S4: Additional frequencies of T cell populations or ratios, as well as expression of cell surface markers, showing no change between week 0, 2 and 6.

A) CD4/CD8 T cell ratio, γδT cells and regulatory T cells (Treg) percentages (upper panels) and representative flow cytometry plots (lower panels; γδT cell and Treg). **B)** CD4 T cells positive for CD25, CD69, CD272 (BTLA), CD137 (4-1BB), CD154 (CD40L) and CD134 (OX40). **C)** CD8 T cells positive for CD25, CD69, CD272 (BTLA), CD137 (4-1BB), CD154 (CD40L) and CD134 (OX40)

Chapter 7

In contrast to other Soft tissue sarcomas, GIST shows enhanced numbers of immune check-point-negative CD8 T cells

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ABSTRACT

Introduction: Soft tissue sarcomas (STS) are a heterogeneous group of malignant tumors with over 50 subtypes, differing in tumor biology and clinical outcome. Local T cell immunity is often associated with tumor evolution and therapy response. Here, quantities and qualities of tumor-infiltrating lymphocytes (TIL), and T cell evasive mechanisms that occur in STS subtypes are investigated.

Methods: Liposarcoma (LPS), gastro-intestinal stromal tumors (GIST), leiomyosarcoma (LMS), myxofibrosarcoma (MFS) and pleomorphic sarcoma (PMS) were freshly obtained after surgery and dissociated into single cell suspensions. lipoma and melanoma were taken along as tumors representing a benign soft tissue tumor and immunogenic tumor, respectively. T cells were counted per mg tumor tissue, and phenotypically analyzed by flow cytometry, with emphasis on CD8 T cell maturation (assessed via CD45RA and CCR7) and T cell co-inhibition (PD1, LAG3, TIM3). Also, T cell numbers and localization were determined by quantitative in situ stainings. Next Generation Sequencing data from bulk tumor tissues were analyzed for TCR diversity, mutational load, relative frequencies of immune cell populations, and expression of immune-related genes.

Results: GIST, MFS and PMS were observed to have relatively high numbers of CD8+ TILs with highest fractions of differentiated CD8+ T cells in GIST and LPS. These CD8+ TILs generally expressed the checkpoints PD-1, LAG3 and TIM3, with highest co-expression in MFS and PMS but nearly absent co-expression in GIST. Interrogation of NGS data revealed that fractions of dominant T cell clones according to TCR-Vβ sequence were lowest in GIST and LPS, whereas mutational load was relatively low for all STS subtypes. Interestingly, in silico analyses revealed that in GIST, myeloid-derived cells are underrepresented, and the expression of co-stimulatory ligands, such as B7-2, ICOSL and 4-1BBL, is substantially lowered compared to other STS subtypes.

Conclusion: STS subtypes differ in quantity and quality of CD8+ TILs, making these subtypes amenable to different forms or combinations of immune therapy. In particular GIST seems to lack immunogenicity and may require additional T cell co-stimulation to enhance responsiveness to checkpoint inhibition.

INTRODUCTION

Soft Tissue Sarcomas (STS) are a group of heterogeneous tumors of mesenchymal origin with over 50 different subtypes that can originate from fat, muscle, nerves, fibrous, endothelial, or deep skin tissues. For most patients with non-metastatic STS standard care of treatment includes surgical resection with or without peri-operative (neo)adjuvant chemo- or radiotherapy. Dependent upon the disease stage and histological subtype, on average 25-50% of these patients develop recurrent and/or metastatic disease, and the median overall survival of metastasized STS after treatment by chemotherapy is only 10-15 months.[1, 2] Therefore there is an urgent need for novel and effective therapies for the treatment of advanced STS.[3] GIST is different from most STS subtypes, since 85-90% of tumors harbor a KIT tyrosine kinase, or platelet derived growth factor receptor α (*PDGFRA*) mutation resulting in great clinical responses to targeted (neo)adjuvant therapy with Imatinib.[4]

Immune therapies have clearly demonstrated therapeutic value in various tumor types. In STS, the clinical exploration of immunotherapeutic strategies has been limited. For example, interferons (α/β)[5, 6], interleukin-2 [7], and cancer vaccines have been tested and induced limited anti-tumor activity in a small fraction of STS patients showing stable disease but no radiological responses .[8] In another example, adoptive transfer of NY-ESO-1 TCR gene-modified T cells showed objective responses in 11 out of 18 (61%) patients and with 3- and 5-year survival rates of 38% and 14%, respectively. [9, 10] In addition, and despite pronounced clinical responses in several other tumor types, immune checkpoint inhibiting antibodies show inconclusive results in STS.[11-15] In fact, first studies revealed that some STS subtypes respond to treatment with anti-PD1 therapy[16], though others report no effect of anti-PD-1 therapy.[17] A recent phase 2 study treating 85 metastatic STS patients with nivolumab (anti-PD1, n=43) or nivolumab plus ipilimumab (anti-CTLA4, n=42) concluded that nivolumab monotherapy does not warrant further study in an unselected STS population given the limited efficacy. Yet, the nivolumab plus ipilimumab combination treatment arm showed promising efficacy in certain STS subtypes like undifferentiated pleomorphic sarcoma and leiomyosarcoma.[18]

It has become evident that the numbers and activation state of immune cells in tumors, in particular CD8-positive tumor-infiltrating lymphocytes (TIL), have predictive value with respect to the responsiveness to immune therapies.[19, 20] Local CD8 T cell immunity varies significantly between STS subtypes, which may explain the variable clinical effects of immunotherapies when treating these tumor types.[21, 22] Interestingly, local CD8 T cell immunity is strictly controlled [23], and can be captured by multiple parameters, such as the presence of CD8 cells, Major histocompatibility complex (MHC) molecules, suppressor cells, as well as immune and metabolic checkpoints.[24, 25] Present study, demonstrates a multi-parameter inventory of the immune microenvironment in general, and local CD8 T cell profile in particular of different STS subtypes with the intent to get more insight into the potential immunogenicity of the most common STS types. To this end, gastro-intestinal stro-

mal tumors (GIST), leiomyosarcoma (LMS), liposarcoma (LPS), myxofibrosarcoma (MFS) and pleomorphic sarcoma (PMS) are prospectively assessed for quantities and qualities of TILs, and T cell evasive mechanisms by the use in situ stainings and single cell flowcytometry, as well as in silico analyses of NGS data to assess mutational load, TCR diversity, frequencies of immune cells, and expression of immune-related genes.

MATERIALS AND METHODS:

Patients and samples

Patients with non-metastatic STS eligible for surgery at the Erasmus MC were included and covered the following subtypes: gastro-intestinal stromal tumor (GIST) untreated and Imatinib treated, leiomyosarcoma, liposarcoma, myxofibrosarcoma and pleomorphic sarcoma. Benign lipoma and melanoma were included as controls. Part of tumor resection specimen was formalin fixed and paraffin embedded (FFPE) for routine pathology to confirm STS subtype; and retrieved from the department of Pathology, Erasmus MC, for in situ stainings. The remainder of the tissue was processed into a single cell suspension to analyze numbers and phenotypes of intra-tumoral T cells. Patient characteristics are shown in Table 1. The study protocol has been approved by the institutional medical ethical review board (MEC-2012-436).

Tumor-derived single cell suspensions

Freshly obtained tumor material was dissociated on the day of acquisition or was stored overnight in complete medium (RPMI-1640 w/ Hepes [Lonza, Verviers, Belgium], supplemented with 6% human serum [Sanquin, Amsterdam, The Netherlands], 1% L-glutamine [Lonza) and 1% penicilline/streptomycine [Lonza]) at 4°C prior to dissociation. The tumor tissue was weighted, cut in small fragments of approximately 1 mm^3, placed in RPMI-1640 w/ Hepes supplemented with 1% penicilline/streptomycine with 0.1 mg/ml collagenase (Type A, Sigma-Aldrich, St. Louis, MO, USA) and 10 μg/ml DNAse (Roche), and mechanically dissociated using the gentleMACS™ Dissociator (program C and program h_tumor_01) (Milteyi, Bergisch Gladbach, Germany). Subsequently, tumor pieces were incubated for 60 min at 37°C and 5% CO_2 under continuous rotation, after which cells were passed through a 70μm strainer (Greiner bio-one, Kremsmünster, Austria) and washed once with complete medium.

Flow cytometry

Flow cytometry (FCM) panels were used to assess frequencies of T cell subsets according to expression of T cell maturation markers and T cell co-inhibitory receptors. For these panels, the following mAb-conjugates were used: CD3-Pacific Blue (clone UCHT1, BD Biosciences, San Jose, CA, US), CD4-BV785 (clone RPA-T4, Sony Biotechnology, San Jose, CA, US), CD8-BV605 (clone SK1, BD Biosciences), CD14-PerCP (clone MϕP9, BD Biosciences), CD272-APC (BTLA, clone MIH26, Sony Biotechnology), CD223-PE-Cy7 (LAG3, clone 3DS223H, eBioscience, San

Diego, CA, US), CD279-APC-Cy7 (PD1, clone EH122H7, Biolegend, San Diego, CA, US), CD366-FITC (TIM3, clone F38-2E2, eBioscience), CCR7-PE (clone 150503, R&D Systems, Germany) and CD45RA-BV510 (clone HI100, BD Biosciences). All antibodies fluorochrome combinations were selected and titrated to prevent spillover in the different fluorescence channels (data not shown). For stainings, 100 μl of single cell suspension was incubated with antibodies for 15 minutes at room temperature, washed with phosphate-buffered saline (PBS, Lonza), and re-suspended in 1% buffered Paraformaldehyde (PBS/PFA). FCM data acquisition was performed on a Celesta flow cytometer (BD Biosciences) equipped with FACSDiVa 8.xsoftware (BD Biosciences) following gating on single live cells (7-AAD negative; 7-AAD from BD Biosciences). Analysis was performed using FlowJo software (Ashland, Oregon, USA). Unstained controls were taken along for every sample and used to set thresholds for markers. T cell number per gram of tumor was calculated by measuring CD3+ T cell counts in 100 μl suspension (10% of total volume) divided by tumor weight.

Immunohistochemistry

FFPE tissues were cut into 4-μm sections and stained with anti-CD8 (clone: SP57, Ventana, Basel, Switserland) and anti-PD1 antibodies (clone: NATIOS, Cell Marque, St. Louis, MO, US). Stained slides were developed with DAB chromogen, counterstained with hematoxylin and mounted in Optisure Ventana coverslips. Whole tissue slides were scanned (magnification: 10x) using Vectra 3.0 (Perkin Elmer, Waltham, Massachusetts, USA). Regions of interest (12 in total: 6 at the tumor margin, and 6 at the tumor center; all evenly distributed) were selected for multispectral imaging (20x). To capture morphological heterogeneity, various subtype-specific tissue finding algorithms were generated using InForm tissue finder software (Perkin Elmer; Version 2.3) that enabled calculation of the number of CD8+ cells per mm^2 tumor tissue. Spatial distribution of CD8+ T cells was categorized according to: (1) ignorant tumors (with <10% of all nucleated cells being CD8+ T cells); (2) inflamed tumors (low, medium and high with 10-29, 30-60, >60% of cells being CD8+ T cells, respectively); and (3) excluded tumors (with >10% of cells being CD8+ T cells, and >90% of these CD8+ T cells at tumor border (see Figure 1C for representative IHC sections that reflect the different spatial distribution patterns and scores).[26] Spatial distribution of both CD8+ as well as PD1+ T cells was assessed by two authors (YK and RW) with high concordance (86,5%); and all non-concordant slides were discussed, and when not resolved, a 3rd author re-evaluated and categorized the slide.

In silico analyses

Relative frequencies of immune cell populations

Affymetrix U133A data were collected from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) entries GSE17743 and GSE71121. Number of patient tumor samples per STS subtype are displayed in Supplementary table 1. Raw .cel files were processed using RMA normalization (per entry), and subsequently uploaded and analyzed with

the CIBERSORT algorithm with permutations set at 100 (https://cibersort.stanford.edu/).[27] From the CIBERSORT output, only samples with a significant threshold ($p<0.05$) for deconvolution of the immune cell populations were selected.

Differential gene expression

RNAseq data were gathered from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) entry GSE71121. Number of patient tumor samples per STS subtype are displayed in Supplementary table 1. Raw FASTQ files were aligned with STAR [28] using reference genome GRCh38, and gene expression read counts were processed in R. Genes with read counts lower than 0.5 counts per million in at least 15 patient samples were excluded from analysis. The Limma package [29, 30] with Voom transformation (with trend) was used to estimate the mean-variance relationship of log-counts, and the Limma empirical Bayes analysis pipeline was used to compute differential expression of selected sets of immune-related genes among STS subtypes.

Mutational load

Whole Exome Sequencing (WES) data were obtained from The Cancer Genome Atlas (TCGA) and downloaded from the GDAC Firebrowse website as "level 4" data. Mutational load was determined by the sum of synonymous and non-synonymous mutations per Megabase DNA. Since GIST samples are not present in TCGA, publicly available raw WES data from the Sequence Research Archive (SRA, https://www.ncbi.nlm.nih.gov/sra), entry SRP042250 (FASTQ files, paired Tumor and Blood samples), were downloaded and subsequently analyzed by the TCGA DNAseq Variant calling pipeline to determine the mutational load (as described above). (https://docs.gdc.cancer.gov/Data/Bioinformatics_Pipelines/DNA_Seq_Variant_Calling_Pipeline/).

TCR clonality

MiXCR software (https://mixcr.readthedocs.io/en/latest/quickstart.html) was used to analyze T Cell Receptor (TCR)-Vβ diversity.[31] RNAseq FASTQ files (GSE71121) were used to determine number of different TCR-Vβ sequences.

Statistical analysis

Statistical tests were performed in R. Significant differences between continuous variables of two groups were tested with the Mann-Whitney-U test. Significant differences between continuous variables of 3 or more groups were tested with the Kruskall-Wallis test. Results were considered as significant when $p<0.05$.

Table 1: Patient characteristics.

Age (years)	*median*	*range*
	61,5	(25 - 86)
Primary tumor size (cm)	*median*	*range*
	9	(1,4 - 55)
Gender	*n=*	
Male	41	73,2%
Female	15	26,8%
Histology	*n=*	*distribution (%)*
Lipoma	6	10,2%
Melanoma	4	6,8%
GIST	9	15,3%
Leiomyosarcoma	7	11,9%
*Liposarcoma**	15	25,4%
Myxofibrosarcoma†	8	13,6%
Pleomorphic sarcoma‡	10	16,9%

*Well-differentiated n=8; Dedifferentiated n=3; Myxoid n=7; † 4 of 8 and ‡ 5 of 10 patients were pre-treated with radiotherapy

RESULTS

GIST, myxofibrosarcoma and pleomorphic sarcoma demonstrate high number and inflamed distribution of CD8+ T cells

Tumor specimen from STS subtypes were analyzed by flow cytometry (FCM) as well as ***in situ*** staining to assess quantity of intra-tumoral T cells. FCM analyses revealed considerable variation in numbers of CD8+ T cells per gram of tissue across STS subtypes, but these quantities were nonetheless significantly enhanced in GIST and myxofibrosarcoma when compared to liposarcoma, but not when compared to leiomyosarcoma and pleomorphic Sarcoma (Figure 1A). T cell enumeration in lipoma, considered benign and relatively non-immunogenic, and Melanoma, considered immunogenic, demonstrated very low (almost absent) and high numbers of T cells per gram of tissue, respectively, the latter comparable to T cell numbers observed in GIST and myxofibrosarcoma.

In situ quantification demonstrated similar heterogeneity in numbers per mm^2 of tissue

across STS subtypes, and again higher number of CD8+ T cells were observed in GIST and myxofibrosarcoma when compared to liposarcoma (Figure 1B).

Next, spatial distribution of CD8+ T cells within STS was assessed, which was categorized according to T cell ignorance; exclusion and different extents of inflammation (see for details, Materials and Methods). Representative in situ stainings of these different CD8 T cell distribution patterns are shown in Figure 1C; and frequencies of these patterns per STS subtypes are summarized in Figure 1C. About one-third of leiomyosarcoma and liposarcoma demonstrated the ignored phenotype (27 and 35%, respectively), whereas only about one-tenth of GIST, myxofibrosarcoma and pleomorphic sarcoma demonstrated lack of T cells (7, 7 and 13%, respectively). myxofibrosarcoma, pleomorphic sarcoma and GIST showed high percentages of inflamed tumors, where myxofibrosarcoma and pleomorphic sarcoma had more highly inflamed tumors compared to GIST (29, 20 and 7%, respectively). GIST had highest fraction of tumors (57 %) with medium inflamed spatial distribution of T cells. Melanoma tumors showed highest fraction (50%) of the CD8 T cell excluded pattern compared to all other STS subtypes, and lipoma only displayed an ignorant phenotype.

GIST and liposarcoma show no signs of T cell activation

The activation status of CD8+ TILs was measured via FCM analysis of the expression of T cell differentiation markers as well as co-inhibitory receptors. When assessing T cell differentiation, leiomyosarcoma, myxofibrosarcoma and pleomorphic sarcoma showed the highest fractions of T effector memory (T_{EM}) and T CD45RA+ effector memory (T_{EMRA}) cells (Figure 2A). In contrast, GIST showed the lowest fraction of T_{EM} cells within CD8+ TILs, being significantly different from myxofibrosarcoma and pleomorphic sarcoma (Figure 2B). Fractions of T_{EMRA} within CD8+ TILs were more or less reciprocally related to those of T_{EM}.

When assessing CD8+ TILs for the expression of inhibitory receptors, PD1 was abundantly expressed (Figure 3A). Interestingly, myxofibrosarcoma and pleomorphic sarcoma showed the highest fractions of PD1+ T cells, whereas GIST displayed the lowest fractions of PD1 positive T cells. Also, fractions of TIM3+ T cells, but not LAG3+ T cells, were higher in myxofibrosarcoma and pleomorphic sarcoma compared to GIST and liposarcoma. When looking into co-expression of PD1, LAG3 and TIM3, only a relatively small fraction of CD8+ TILs displayed all three inhibitory receptors (Figure 3B). We observed that TIM3 was mostly co-expressed with PD1, or with PD1 and LAG3, whereas LAG3 was mostly co-expressed with PD1. In fact, TIM3 or LAG3 were hardly co-expressed by PD1-negative CD8 TIL. Although patterns in IR (co-) expression were not specific for particular STS subtypes, it was notable that GIST demonstrated the lowest fraction of PD1+ LAG3+ TIM3+ CD8+ TILs. pleomorphic sarcoma had the highest fraction of PD1+ LAG3+ TIM3+ CD8+ TILs, being comparable to melanoma.

GIST and liposarcoma lack dominant T cell clones.

To assess whether numbers of intra-tumoral CD8 T cells and level of T cell activation in STS subtypes correspond to a changed TCR repertoire diversity and antigen load, publicly avail-

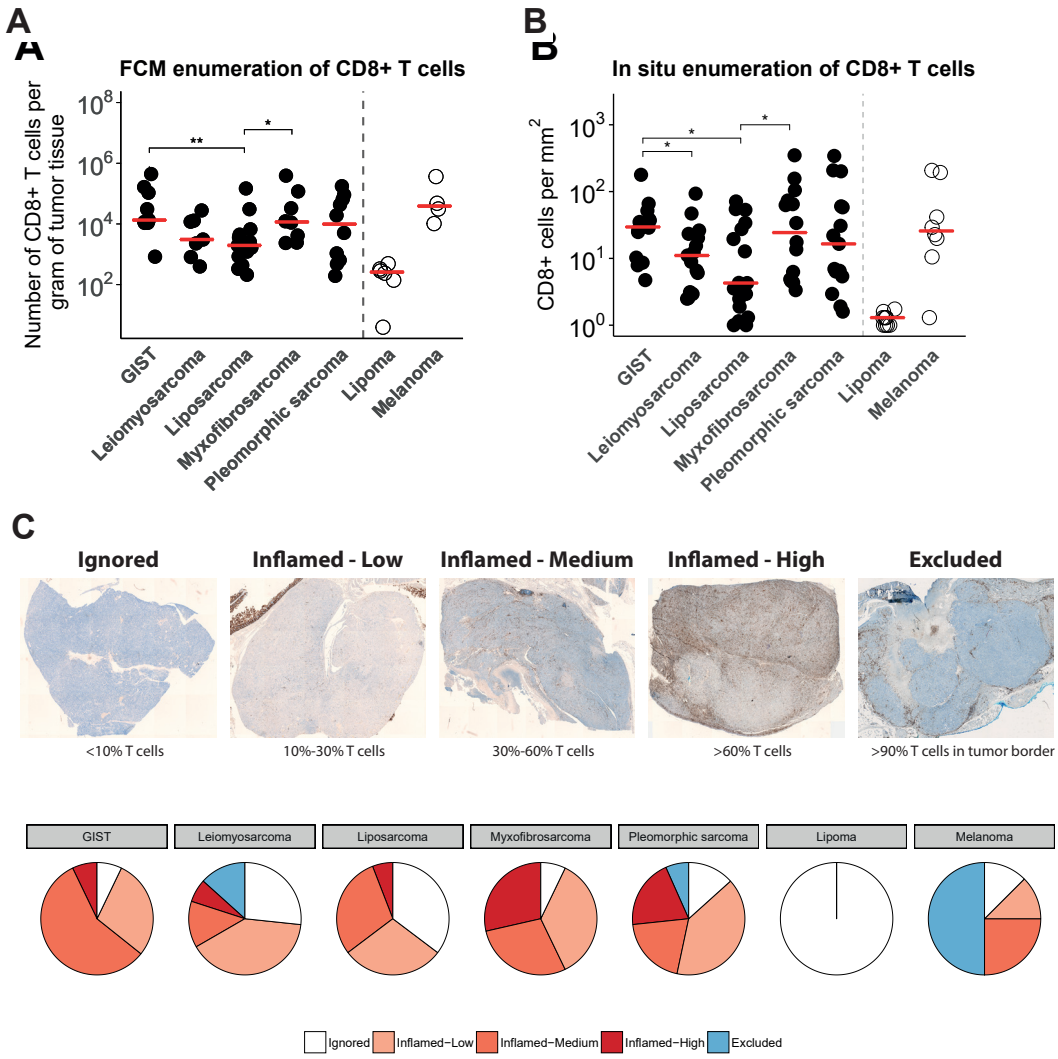

Figure 1: GIST and myxofibrosarcoma sarcoma have highest number and predominantly inflamed distribution of CD8+ T cells.

A) Absolute numbers of CD3+ T cells per gram of tumor tissue assessed by FCM after tissue dissociation of surgical specimen of GIST (n=9), leiomyosarcoma (n=7), liposarcoma (n=12), myxofibrosarcoma (n=8), pleomorphic sarcoma (n=9), lipoma (n=5) and Melanoma (n=4). Individual (symbols) and median (horizontal lines) observations are shown, Kruskal-Wallis test: p = 0,049. **B)** *In situ* quantification of CD8+ T cells per mm^2 in FFPE tissue sections of different STS subtypes (n= 15 specimen per STS subtype), Kruskal-Wallis test: p = 0,038. **C)** In situ stainings showing representative examples of different categories of spatial distribution of CD8+ T cells (bright field whole slide images, see materials and methods for details); lower panel: pie charts displaying the frequencies of the different categories of spatial T cell distributions for STS subtypes. *represents p-value <0.05 according to Mann-Whitney U test.

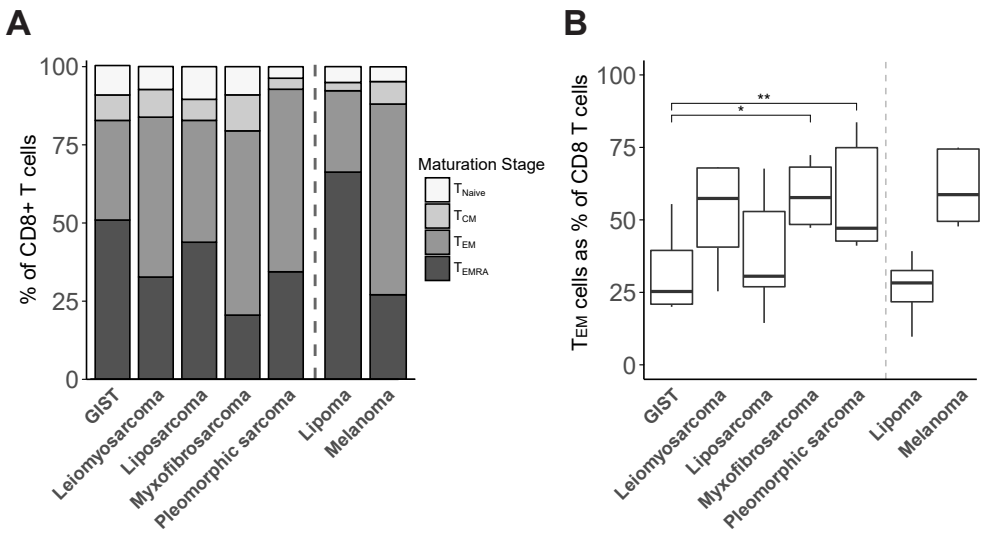

Figure 2: GIST shows low fraction of effector memory CD8+ T cells.

A) FCM analysis of maturation stages of CD8+ T cells in different STS subtypes based on expression of CD45RA and CCR7 using the following definitions: Naïve (CCR7+/CD45RA+, T_N), Central Memory (CCR7+/CD45RA-, T_{CM}), Effector Memory (CCR7-/CD45RA-, T_{EM}), and Effector Memory expressing CD45RA T cells (CCR7-/CD45RA+, T_{EMRA}). **B)** T_{EM} cells as percentage of CD8+ T cells. Included in analysis of (A) and (B): GIST (n=8), leiomyosarcoma (n=6), liposarcoma (n=10), myxofibrosarcoma (n=8), pleomorphic sarcoma (n=9), lipoma (n=3) and Melanoma (n=4); *represents p-value <0.05 according to Mann-Whitney U test.

able NGS data was interrogated. First, using the MiXCR tool [31], number of different TCR-Vβ sequences (a measure of different T cell clones) was determined, and showed a high level of variability across STS subtypes (Figure 4A), with some subtypes having <10 and others having >1000 different T cell clones. GIST showed a trend towards a higher number of T cell clones when compared to other STS subtypes, but when analyzing the frequency of the ten most dominant T cell clones, GIST and liposarcoma had the lowest fractions when compared to leiomyosarcoma, myxofibrosarcoma and pleomorphic sarcoma. Moreover, the most dominant T cell clone in GIST was present at a much lower frequency compared to the most dominant T cell clone in all other STS subtypes (Figure 4B).

Collectively, investigations of intra-tumoral CD8+ T cells in STS subtypes demonstrated that GIST, myxofibrosarcoma and pleomorphic sarcoma have relatively high numbers of such T cells. GIST and liposarcoma harbor low fractions of CD8+ T_{EM} cells, and low fractions of CD8+ T cells expressing PD1 or co-expressing PD1 and TIM3 when compared to myxofibrosarcoma and pleomorphic sarcoma. This low level of T cell activation in GIST and liposarcoma was accompanied by significant lower abundance of dominant T cell clones. These findings are schematically represented in Figure 8A.

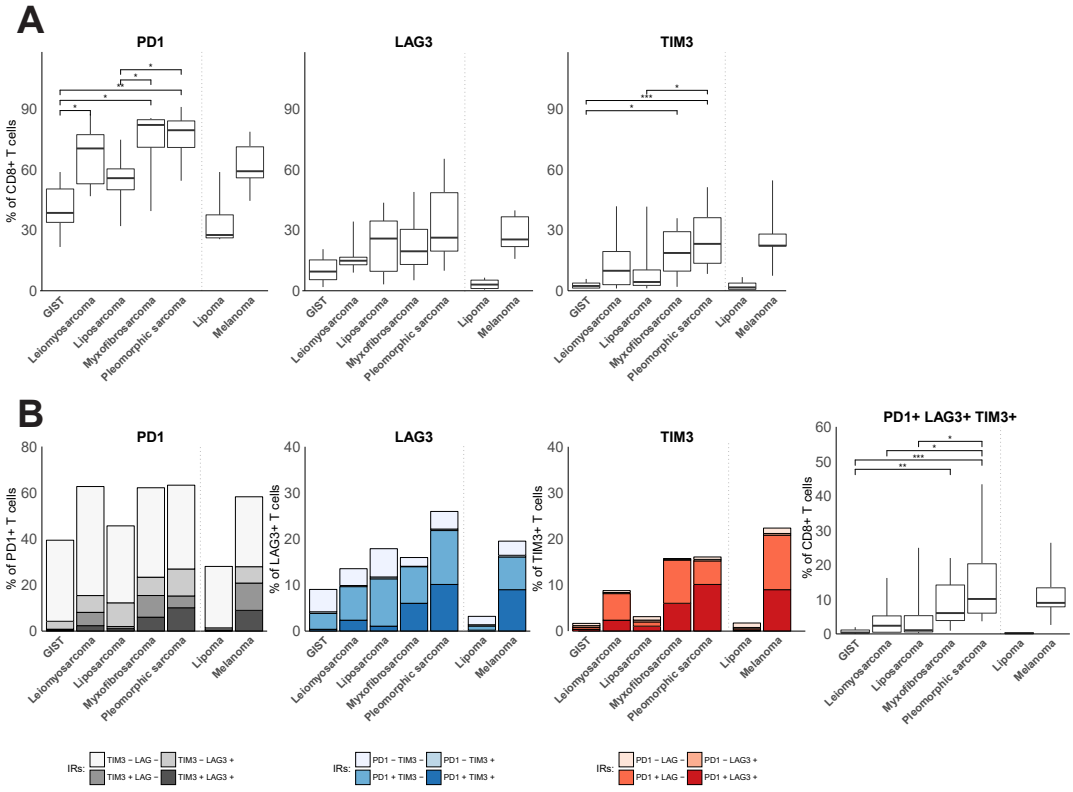

Figure 3: GIST and liposarcoma show decreased fractions of immune checkpoint-positive CD8 T cells.

A) FCM analysis of fractions of PD1, LAG3, and TIM3-expressing CD8+ T cells in different STS subtypes represented by box-and whisker plots. **B)** FCM analysis of fractions of CD8+ T cells co-expressing PD1, LAG3 and/or TIM3 in different STS subtypes. FCM analysis was only performed on samples with at least 200 CD8+ T cells in the staining tube and included: GIST (n=8), leiomyosarcoma (n=6), liposarcoma (n=10), myxofibrosarcoma (n=8), pleomorphic sarcoma (n=9), lipoma (n=3) and Melanoma (n=4); *represents p-value <0.05 according to Mann-Whitney U test.

GIST shows a non-differential mutational load nor antigen presentation

In addition to the analyses focusing on intra-tumoral CD8+ T cells, the immune micro-environment of STS subtypes was investigated. To this end, tumor mutational burden and expression of genes involved in antigen processing and presentation was analyzed with NGS data. Analysis of mutational load, as a surrogate marker for neo-antigens, demonstrated substantial differences among STS subtypes (Figure 5A). In general, the mutational load is low in STS, yet myxofibrosarcoma and pleomorphic sarcoma had the highest mutational load, which was significantly higher than that of leiomyosarcoma and liposarcoma. GIST did not significantly

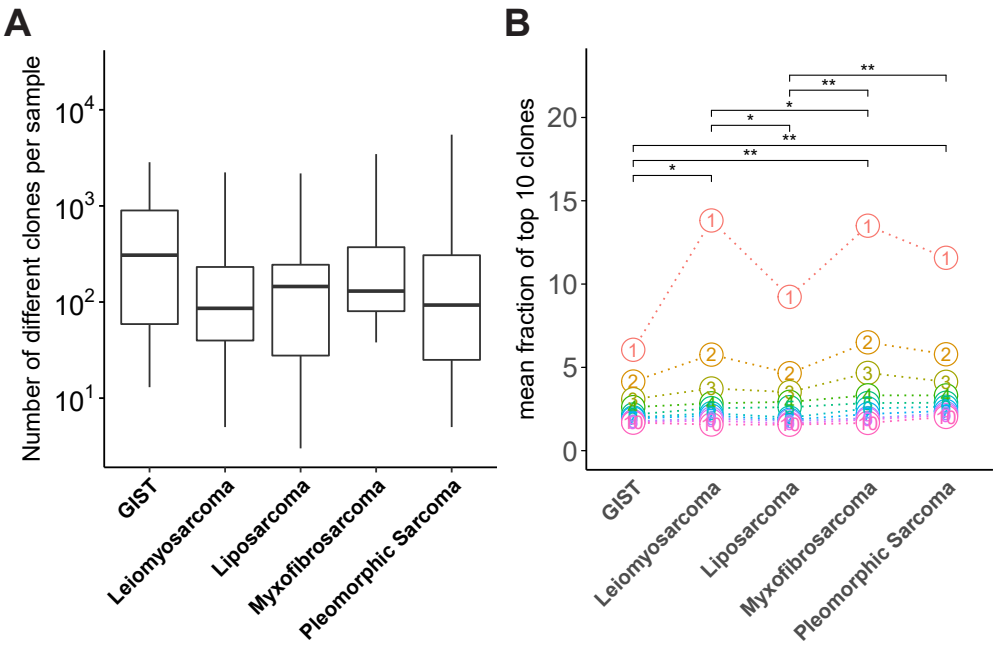

Figure 4: GIST and liposarcoma show no enrichment of TCR-Vb sequences.

A) Number of different TCR-Vβ sequences in STS subtypes determined by MiXCR RNAseq analysis of publicly available data (see materials and methods for details), and displayed as box and whisker plots. **B)** Mean fraction of the 10 most dominant T cell clones of all T cell clones with circles representing these T cell clones. Dots align the T cell clones according to dominance among the different STS subtypes.*represents p-value <0.05 according to Wilcoxon test.

differ in mutational load when compared to other STS subtypes, arguing that the lower dominance of T cell clones and lack of T cell activation in this STS subtype is not related to a lower level of neo-antigens.

Next, the expression of genes involved in antigen processing and presentation via MHC class I was analyzed (Figure 5B) and did not find significant differences between STS subtypes with respect to β2-microglobulin (B2M), HLA-A, HLA-B, HLA-C, and Transporter associated with antigen Processing 1 and 2 (TAP1 and 2). Also, the expression of genes involved in MHC class II (HLA-DMB, HLA-DPA1, HLA-DPB1, HLA-DQB1, and HLA–DRB1) did not differ among STS subtypes (Figure 5C). Although the expression of some single genes did differ between STS subtypes, the difference in expression was lower than 2-fold.

GIST shows differential presence of immune suppressor cells, and decreased expression of T cell co-stimulatory ligands.

Besides measures of immunogenicity (as described above), parameters of local T cell suppression, such as the presence of immune suppressor cells or T cell co-stimulatory or inhibitory ligands were examined. To determine the relative frequencies of various immune cell

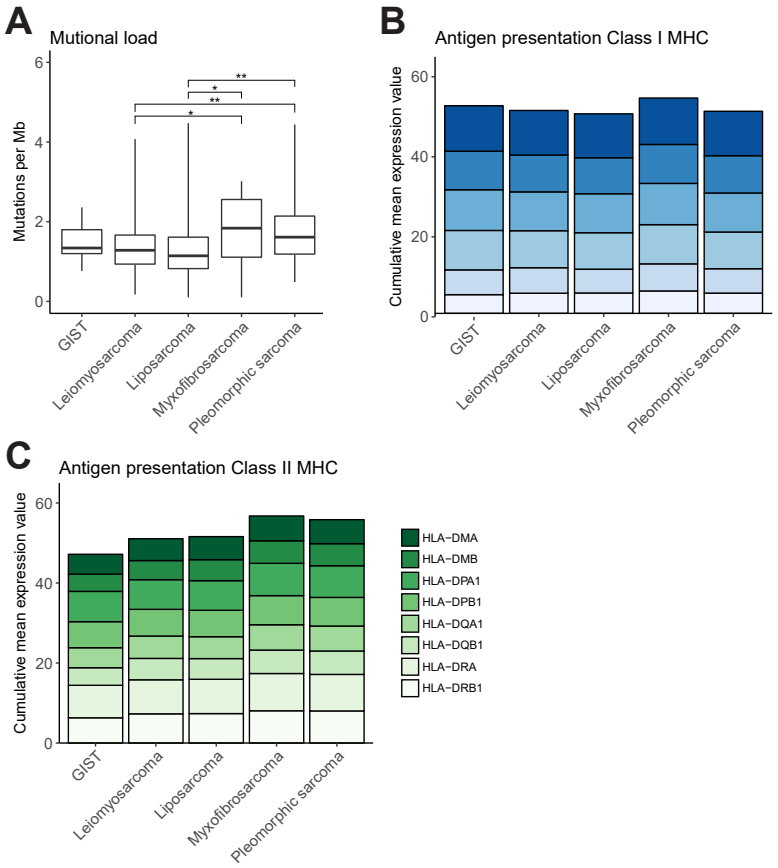

Figure 5: STS subtypes show differential mutational load but non-differential expression of genes involved in antigen presentation.

A) Mutational load in different STS subtypes defined as mutations per Mb according to publicly available NGS data, and displayed as box and whisker plots. **B)** Expression level of genes involved in MHC-class I antigen processing and presentation. **C)** Expression level of genes involved in MHC-class II antigen presentation *represents p-value <0.05 according to Mann-Whitney U test

types, gene expression data of 257 different STS tissues was analyzed with the CIBERSORT algorithm [27] (Figure 6). With respect to regulatory T cells (Tregs), pleomorphic sarcoma showed a significantly lower fraction of these suppressor T cells compared to GIST and leiomyosarcoma. The fraction of undifferentiated macrophages (M0), which may represent myeloid-derived suppressor cells (MDSC), was observed to be significantly lower (almost absent) in GIST compared to all other subtypes, and in leiomyosarcoma compared to liposarcoma and myxofibrosarcoma. The fraction of immune-suppressive M2 macrophages was observed to be higher in myxofibrosarcoma and pleomorphic sarcoma compared to all other STS subtypes, and GIST showed the lowest fraction of M2 macrophages among all STS subtypes.

Finally, differential gene expression of co-stimulatory and inhibitory ligands was assessed. We

observed that GIST shows a lowered expression of PDL2, but not PDL1 (Figure 7A). Ligands for LAG3, MHC class II molecules, were already assessed and these do not differ among STS subtypes (Figure 5B). The ligand for TIM3, Galectin-9, shows a lowered expression in leiomyosarcoma when compared to liposarcoma, myxofibrosarcoma and pleomorphic sarcoma. Notably, when analyzing T cell co-stimulatory ligands, CD86, but not CD80, shows a lowered expression in GIST when compared to all other STS subtypes, and in leiomyosarcoma compared to pleomorphic sarcoma and myxofibrosarcoma (Figure 7B). Also, the ICOS ligand and the 4-1BB ligand showed lowered expression in GIST, whereas the OX40 ligand did not differ significantly among STS subtypes.

Collectively, the in-silico analyses of the immune micro-environment of STS subtypes revealed that myxofibrosarcoma and pleomorphic sarcoma have a higher mutational burden compared to leiomyosarcoma and liposarcoma, but not GIST, and that genes involved in antigen processing and presentation were similarly expressed in all STS subtypes. Fractions of Tregs were higher and fractions of myeloid cells were lower in GIST compared to other STS subtypes. Expression level of the co-inhibitory ligand PDL2 was low in GIST and expression level of Galectin-9 was lowest in leiomyosarcoma and highest in myxofibrosarcoma and pleomorphic sarcoma. Expression levels of co-stimulatory ligands CD86, ICOSL, and 4-1BB ligand were all low in GIST compared to other STS subtypes. These findings are schematically represented in Figure 8B.

DISCUSSION

In the current study, we have analyzed the STS subtypes GIST, myxofibrosarcoma, liposarcoma, pleomorphic sarcoma and leiomyosarcoma for quantity and quality of CD8+ T cells as well as immune parameters of the tumor micro-environment using a combination of FCM, microscopy and in silico tools. A high concordance between FCM and ***in situ*** analysis for T cell quantity per STS subtype was seen, and it was shown that GIST, myxofibrosarcoma and pleomorphic sarcoma harbored the highest number of intra-tumoral T cells. Numbers of CD8+ T cells were comparable to those as seen in an immunogenic tumor type like melanoma. For pleomorphic sarcoma our data concerning T cell enumeration is supported by a recent study from Pollack and colleagues [32], but this study lacked GIST and myxofibrosarcoma. Also, quantity of TILS was assessed via CD3D gene expression. In another study, T cell quantity in GIST was compared to those of other STS subtypes, however, in this study T cell quantity was expressed as frequency of total CD45+ cells, not enumerated by absolute numbers, and spatial distribution was not assessed.[33] When addressing T cell localization within STS tumors, our microscopic analyses revealed that GIST, myxofibrosarcoma and pleomorphic sarcoma mostly showed a T cell-inflamed phenotype, featuring an even distribution of CD8+ T cells in tumor center as well as margin.

CD8+ T cell activation was assessed via FCM according to expression levels of T cell maturation markers as well as inhibitory receptors. We observed significantly lower fractions of ef-

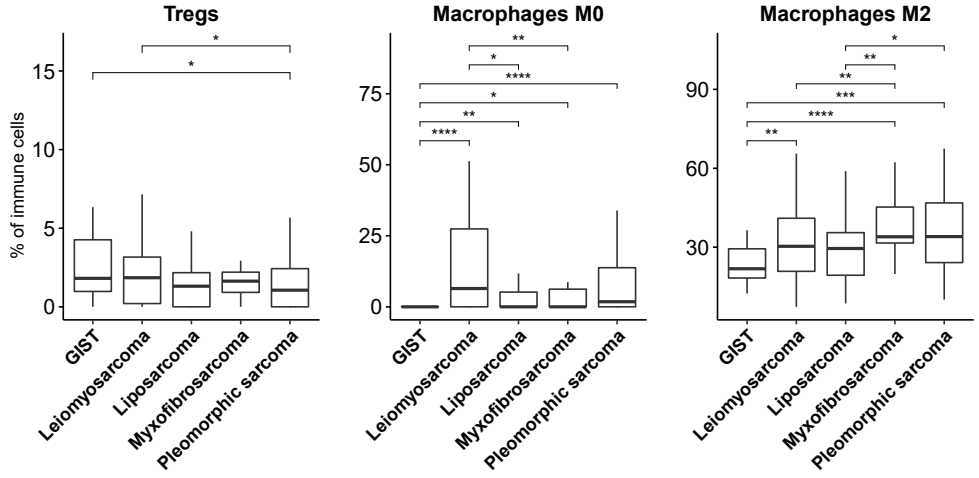

Figure 6: GIST shows decreased fractions of suppressive myeloid cells.

Fractions of immune cell subtypes in different STS subtypes as determined by CIBERSORT algorithm according to publicly available RNA data, and displayed as box and whisker plots. Number of analyzed tumors per STS subtype is described in materials and methods. *represents p-value <0.05 according to Mann-whitney U test.

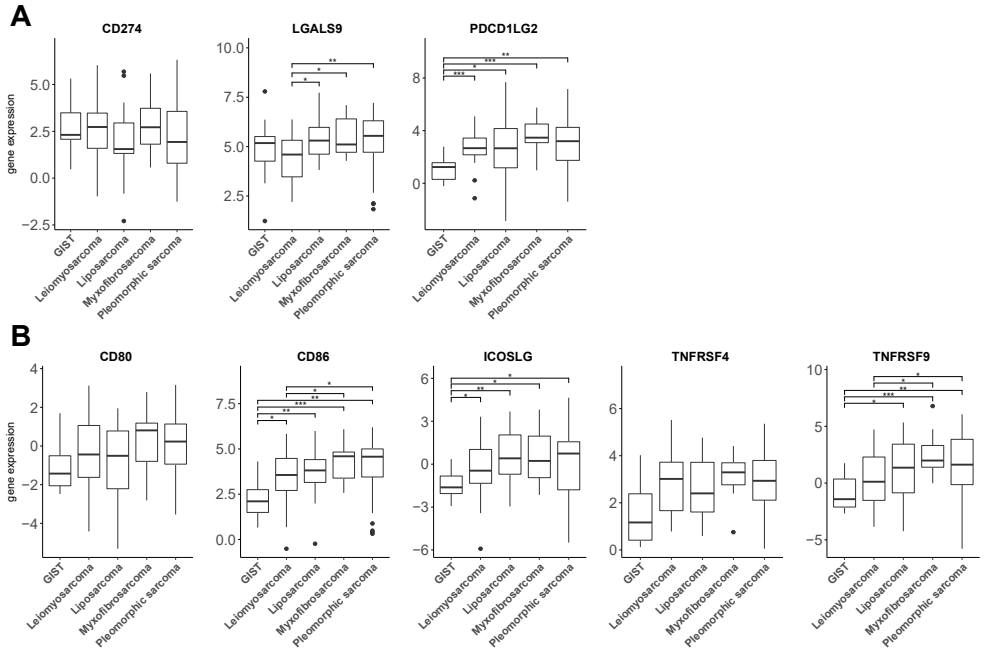

Figure 7: GIST shows decreased expression of PDL2, CD86, ICOSL and 4-1BBL genes.

A) RNA expression of IR ligands PDL1 (CD274), PDL2 (PDCD1LG2), and Galectin-9 (LGALS9) in different STS subtypes according to publicly available data, and displayed as box and whisker plots. **B)** RNA expression of co-stimulatory ligands CD80, CD86, ICOS ligand (ICOSLG), OX40 ligand (TNFRSF4), and 4-1BB ligand (TNFRSF9) in different STS subtypes according to publicly available data, and displayed as box and whisker plots. Number of analyzed tumors per STS subtype is described in materials and methods. *represents p-value <0.05 according to Mann-whitney U test.

fector memory CD8+ T cells (T_{EM}: CD45RA-, CCR7-) in GIST but not in myxofibrosarcoma nor in pleomorphic sarcoma, despite the fact that all three STS subtypes harbor equal numbers and similar spatial distributions of CD8+ T cells. In addition, GIST differed from myxofibrosarcoma and pleomorphic sarcoma by having the highest fraction of effector memory expressing CD45RA CD8+ T cells (T_{EMRA}: CCR7-/CD45RA+). The CD8+ T_{EMRA} cells are generally considered to be terminal effector cells, however, differentiation to this terminal effector phenotype is not necessarily the result of vigorous T cell receptor (TCR)-mediated stimulation, but can also be the result of excessive cytokine stimulation.[34, 35] Another recognized measure of T cell activation through TCR stimulation [36] is increased expression of inhibitory receptors (IRs). Again, GIST and also liposarcoma displayed lower fractions of PD1, LAG3 and especially TIM3-expressing CD8+ T cells when compared to myxofibrosarcoma and pleomorphic sarcoma. In fact, GIST demonstrated low numbers of PD1+LAG3+TIM3+CD8+ T cells, which are considered functionally compromised T cells [37], when compared to the latter two STS subtypes. These phenotypical analyses of CD8+ TILs argue that in GIST, despite high numbers of these T cells, there is limited TCR-driven T cell activation. For CD4+ T cells differences in maturation stages of CD4+ TIL were not as outspoken as for CD8+ TIL. Fractions of IR-expressing CD4+ T cells were lower compared to CD8+ T cells, especially for LAG3 and TIM3 the fractions of T cells were much lower and did not show any differences between STS subtypes (supplementary data).

To explore any differences in TCR-mediated stimulation across STS subtypes, we examined TCR diversity using in silico tools and publicly available NGS data. Analysis into the number of TCR-Vβ sequences demonstrated a high level of variation and revealed, when analyzing the 10 most abundant TCR-Vβ sequences, that GIST and liposarcoma had a significantly lower frequency of dominant T cell clones. This observation is particularly true for the single most dominant T cell clone, and is suggestive for a more polyclonal T cell repertoire in GIST and liposarcoma, and a more oligoclonal T cell repertoire in myxofibrosarcoma and pleomorphic sarcoma, the latter finding being in agreement with a report by Pollack and colleagues. With respect to parameters of immunogenicity, mutational burden as well as expression of genes involved in antigen processing and presentation were analyzed. Mutational load of tumors, often considered a surrogate marker for potentially mutated peptides that can be presented by MHC molecules (neo-antigens) on malignant cells, has been associated with higher response rates to checkpoint therapy and adoptive T cell therapy with TILs [38-40]. Interestingly, in leiomyosarcoma reduced expression of genes encoding neo-peptides was identified as a potential mediator of resistance to immune checkpoint therapy.[41] Although it has already been reported that STS subtypes differ with respect to genomic alterations, [22, 42, 43] only few studies have assessed mutational load in STS subtypes in relation to number and phenotype of CD8+ TILs. Along these lines, the TCGA database has recently been thoroughly interrogated for mutational load in STS subtypes, but did not include GIST.[22] In our analyses, we observed a generally low mutational load for all STS subtypes when compared to Melanoma and other tumor types[44], yet this load was different between the STS

subtypes showing significantly higher mutational load in myxofibrosarcoma and pleomorphic sarcoma compared to liposarcoma. Altered expression of genes involved in antigen processing and presentation have, besides mutational load, also been associated with resistance to checkpoint therapy [45]. We observed no differential gene expression of components of MHC class I and II presentation among different STS subtypes. Others did find differences in gene expression between antigen presentation molecules, however most of such differences were found between synovial sarcoma and other subtypes.[32]

Subsequently, parameters that may affect CD8+ T cell function, such as the presence of immune-suppressive cells and expression of co-signaling ligands were analyzed. The presence of Tregs, M0, and M2 macrophages in different STS subsets, did not show significantly higher fractions in GIST when compared to the other highly CD8+ T cell-rich myxofibrosarcoma and pleomorphic sarcoma. For the co-inhibitory ligands, GIST showed a lowered expression of PDL2, but not PDL1 when compared to other STS subtypes. Previous studies have shown longer median progression free- and overall survival in patients with head and neck squamous cell cancer treated with pembrolizumab, in case tumors were PDL2-positive but not PDL2-negative.[46] PDL2 expression is regulated by both interferon (IFN)β and γ, [47] and lower expression of PDL2 in GIST is suggestive for a lower interferon-I signature potentially reflecting the lower level of T cell activation found in this study. For the co-stimulatory ligands, clearly lowered expression for ligands of CD28 (CD86), ICOS (ICOSL) and 4-1BB (41BBL, TNFRSF9) was found. These ligands are generally expressed by innate immune cells, such as dendritic cells and macrophages, but also by tumor cells, and fulfill critical roles in T cell activation although the exact role in cancer immune therapy is not yet clarified [48]. CD86 expression has been reported to be significantly higher in pleomorphic compared to liposarcoma; however, GIST and myxofibrosarcoma were not included in this analysis.[32] To the best of our knowledge this is the first study that investigated the expression of a large series of co-signaling ligands in GIST and demonstrated a profoundly lowered level of expression of PDL2, CD86, ICOSL and 41BBL in this STS subtype. The underlying mechanism of why these ligands show a lower expression in GIST compared to other STS subtypes requires further studies. We would like to argue, however, that lowered expression of co-stimulatory ligands in GIST, but not lowered immunogenicity, may be at the basis for less CD8+ T cell activation as we discussed above. We also observed significantly lower fractions of CD137 positive CD8+ T cells in GIST compared to other STS subtypes (measured by FCM), but since we did not have this data for all samples data is not shown.

Our study has several limitations, such as: relatively small numbers of tumors; gene-expression analysis only included dedifferentiated liposarcoma while our FCM analysis included dedifferentiated/well-differentiated and myxoid liposarcoma; and some GIST tumors used in FCM analysis were pre-treated with Imatinib (n=5 out of 9), and some pleomorphic sarcomas (and myxofibrosarcomas were pretreated with radiotherapy with no significant differences between pre-treated and untreated tumors in any of the parameters analyzed (data not shown). Notwithstanding these shortcomings, this study is by our knowing the first study

with an in-depth analysis of STS subtype immunogenicity using a wide scale of different techniques.

In conclusion, our study revealed that quantity and quality of CD8+ T cells as well as parameters of the tumor immune micro-environments that affect numbers and function of CD8+ T cells are clearly different among STS subtypes (Figure 8). Building on these data, we speculate that myxofibrosarcoma and pleomorphic sarcoma are suitable candidates for checkpoint therapy. Since checkpoint therapy is primarily effective in inflamed tumors [49, 50], not all myxofibrosarcomas and pleomorphic sarcomas are expected to equally benefit from checkpoint therapy, providing a rationale to further stratify patient tumors according to immune parameters. For tumors that are less immunogenic (i.e., having a lowered mutational burden), other therapeutic options may be required (e.g. radiotherapy, chemotherapy) to sensitize tumors for checkpoint therapy or adoptive T cell therapy. With respect to GIST, we speculate that treatment with co-stimulatory agonistic antibodies may show efficacy.

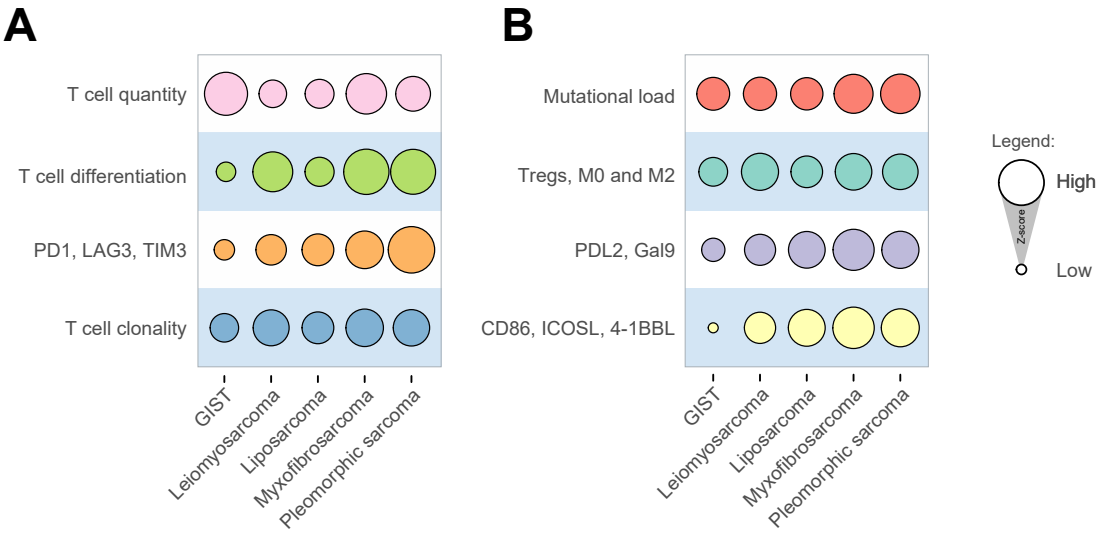

Figure 8: Overview of immune parameters of different STS subtypes.

Immune analyses of different STS subtypes are schematically summarized according to two sets of parameters, those that reflect intra-tumoral CD8 T cells **A)** as well as those that reflect the immune micro-environment **B)**. CD8 T cell-related parameters include: T cell quantity (i.e., number of T cells per gram as presented in Figure 1A); T cell differentiation (fraction of effector memory cells, Figure 2B); Co-inhibitory receptors (fraction of T cells co-expressing PD1, LAG3 and TIM3, Figure 3B) and T cell clonality (fraction of 10 most dominant clones, Figure 4B). Parameters related to immune micro-environment include: mutational load (mutations per Mb, Figure 5A); Inhibitory immune cells (fractions of regulatory T cells, and M0 and M2 macrophages, Figure 6); Co-inhibitory ligands (expression of PDL2 and Galectin9, Figure 7A); and Co stimulatory ligands (expression of CD86, ICOSL and 4-1BBL, Figure 7B). The listed parameters were utilized to compute Z-scores*. Circle sizes represent mean Z scores that are normalized (range:-0.82 to 0.83) for all parameters per STS subtype. *Z-scores were calculated as follows: (X- μ) / σ where X is the value of the parameter, μ is the population mean, σ is the standard deviation.

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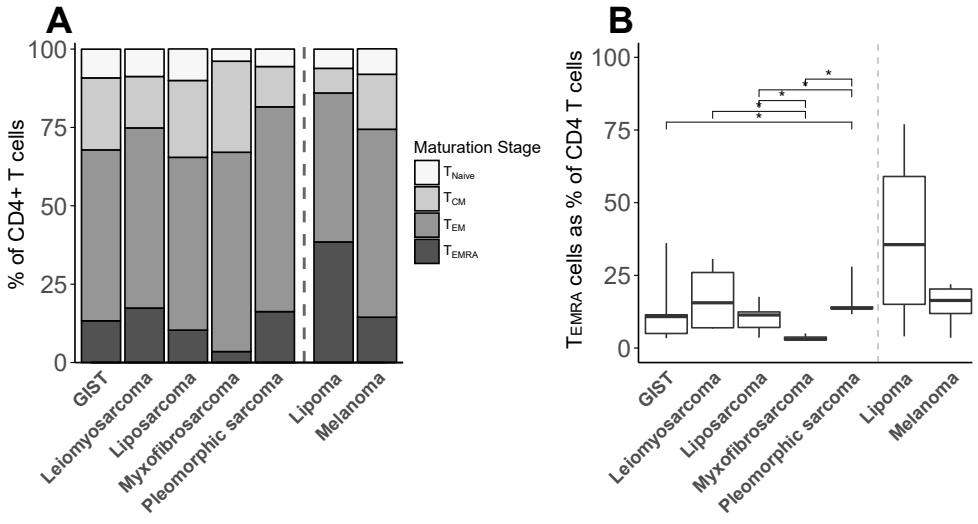
SUPPLEMENTARY DATA

	RNASEQ (GSE71121)	MICROARRAY (GSE71121)	DNASEQ (TCGA)
GIST	10	29[1]	9[2]
Leiomyosarcoma	36	89	98
Liposarcoma	18	45	56
Myxofibrosarcoma	15	43	22
Pleomorphic sarcoma	42	89	50

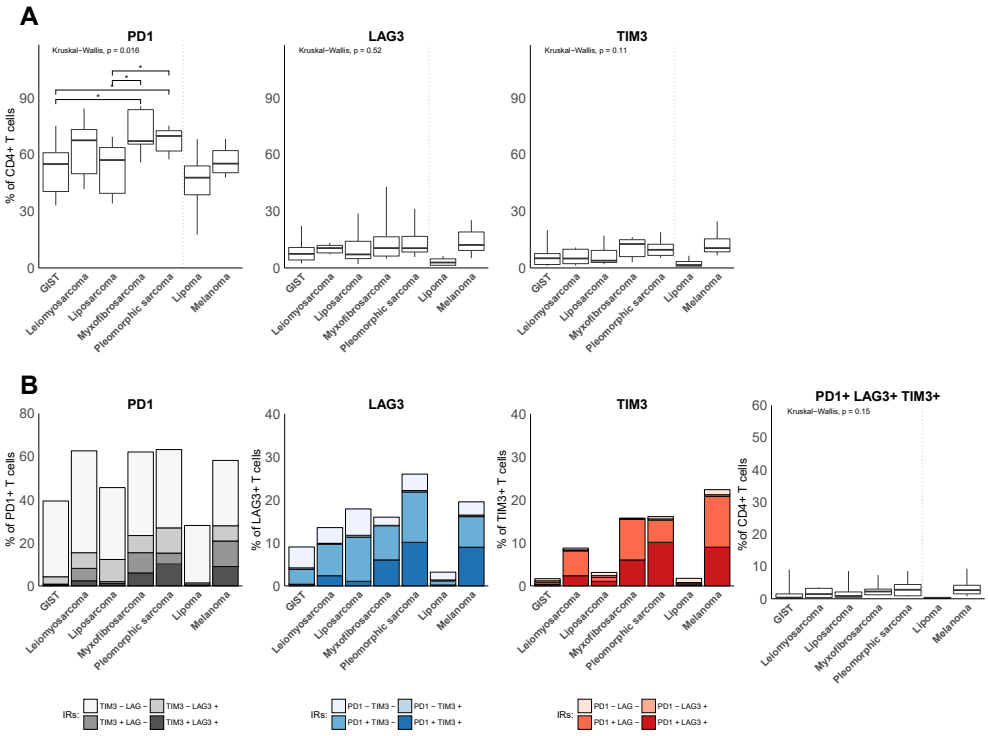
[1]GSE17743, [2]SRP042250

Supplementary Table 1: number of samples in different publicly available data sets.



Supplementary Figure S1: Differential fractions of matured CD4+ T cells in STS subtypes.

A) FCM analysis of maturation stages of CD4+ T cells in different STS subtypes based on expression of CD45RA and CCR7. See Figure 2 for definitions. **B)** T_{EMRA} cells as percentage of CD4+ T cells represented by box-and whisker plots. See figure 2 for patient numbers; *represents p-value <0.05 according to Mann-whitney U test.



Supplementary Figure S2: Inhibitory receptor (co-)expression on CD4+ TIL.

A) FCM analysis of fractions of PD1, LAG3, and TIM3-expressing CD4+ T cells in different STS subtypes. **B)** FCM analysis of fractions of CD4+ T cells co-expressing PD1, LAG3 and/or TIM3 in different STS subtypes. FCM analysis was only performed on samples with at least 200 CD4+ T cells in the staining tube. See figure 3 for patient numbers; *represents p-value <0.05 according to Mann-whitney U test.

Chapter 8

General Discussion

GENERAL DISCUSSION

The research presented in this thesis consists of studies on: (1) monitoring of immunological parameters during cellular therapy and (2) assessment of immune signatures in different tumor types. Accordingly, this final chapter is split up into two parts. Part 1 summaries our findings to define markers that give a better understanding of mechanisms that may underlie successful and safe adoptive T cell treatment. Part 2 of this thesis further focuses on monitoring of T cells in settings other than adoptive T cell treatment. With respect to part 2, we first monitored changes in peripheral T cell characteristics during Dendritic cell (DC) therapy of mesothelioma. Secondly, we assessed intratumoral T cell characteristics, and explored specific differences between soft tissue sarcoma (STS) subtypes.

PART 1: IMMUNE MONITORING IN CHIMERIC ANTIGEN RECEPTOR (CAR) T CELL THERAPY.

Adoptive transfer of T-cells has demonstrated significant clinical responses in several tumor types. Especially T cells genetically engineered to express CD19 specific CARs have shown impressive clinical responses in B cell malignancies. This recently resulted in the FDA approval of CD19 CAR T cell products for treatment of acute lymphocytic lymphoma (ALL), and certain types of non-Hodgkin lymphoma (NHL).[1] However, in solid tumor types, this approach is less well developed and translational as well as clinical studies are needed to get more insight into parameters that determine efficacy and toxicity from CAR T cell therapies. **Chapter 2 to 5** of this thesis describe findings from a phase-I clinical trial using a CAR:Fc(ε)RIγ directed to carboxy-anhydrase-IX (CAIX), an antigen that is over-expressed on Renal Cell Carcinoma (RCC). In this phase I dose escalation trial, patients with metastatic renal cell carcinoma were treated in five cycles with this first-generation CAR. A total of 12 patients were treated in three cohorts. Unfortunately, no objective clinical responses were observed, though in vivo activity of CAIX CAR T-cells was reflected by observed on-target liver toxicities.[2, 3] The last cohort of patients were pre-treated with CAIX (G250) antibodies to prevent on-target toxicity, and demonstrated significantly lower toxicities compared to the first two cohorts.[4] In this trial we performed extensive monitoring of pre-infusion T cell products and in vivo parameters to identify predictors of effective T cell therapy.

Quantification of CAR T cells in blood of patients

In **chapter 2** we have assessed the number of infused CAR T cells by two different assays: 1) flow cytometry (FCM), and 2) quantitative PCR in peripheral blood. Peripheral persistence of adoptively transferred T cells is the net result of proliferation, cell death and extravasation to peripheral tissues of transferred cells in vivo and has been correlated with response.[5-8] In the Rotterdam CAR T cell trial, CAR T-cells in patient blood were quantified by FCM for CAR protein and qPCR for CAR DNA and RNA. However, in contrast to a previous report [9], we found no significant correlations between numbers of CAIX CAR T-cells and CAIX CAR DNA copies. This is possibly due to limited number of patients, and a wide variation in CAR DNA

CHAPTERS 2 AND 3: MAJOR FINDINGS

- Peripheral numbers of CAR T-cells are best captured by flow cytometry and not by quantitative polymerase chain reaction.
- CAR surface as well as gene expression are down-regulated following T-cell infusion
- The presence of CAR T-cells in blood correlated with plasma levels of IFN-γ and IL-6
- During the 14 days in vitro expansion of CAR T cells prior to administration:
  - T cell phenotype shifted from predominantly CD4+ to a CD8+ T cells
  - T cell maturation shifted from naïve to predominantly central effector T cells
- High fraction of naïve CD8 T cells at baseline (prior to genetic modification) or central effector CD8 T cells at 2 weeks of CAR T cell culture correlated with fold expansion and absolute numbers of circulating CAR T cells in patients at 1 month after start of therapy.

copy numbers found in T-cell preparations of individual patients [2]. In addition, CAR DNA levels might not directly reflect the level of CAR protein expression and may in fact overestimate the number of T cells that are reactive to CAIX in vivo. In fact, CAR surface expression decreased in time in T cell infusion products as well in T cells derived from blood following T-cell treatment. This decrease in CAR surface expression was accompanied by a decrease in CAR mRNA expression, the latter potentially reflecting a decreasing gene transcription during the culture of non-reactivated T-cells. In previous studies, using a similar γ- retroviral vector backbone, a decreased surface expression has also been observed for a TCR transgene which has been attributed to a waning LTR-driven gene transcription due to metabolic quiescence of gene-transduced T-cells.[10] This loss of transgene expression might be vector dependent as so far no such event has been described in other studies using different vector backbones. Based on the findings in **chapter 2**, we recommend enumeration of CAR T cells using FCM even though this technique requires immediate processing of patient, good antibodies, and facilities with advanced expertise with respect to flow cytometers and cellular processing.

Markers that correlate to CAR T cell numbers and activity in blood of patients

Next in c**hapter 2**, we assessed the plasma cytokine levels during patient treatment with CAIX CAR T cells and correlated these to the peripheral CAR T cells number and measures of toxicity. Since cytokine levels are easy to assess, these would represent useful surrogate marker for T cell persistence. Interestingly, out of 27 cytokines tested, only IL-6 and IFN-γ correlated with CAR T cells numbers in vivo. In the CD19 CAR T-cell trials, plasma cytokine levels of GM-CSF, IL-2, IL-10, TNF-α, IFN-γ and in particular IL-6 were closely monitored because these cytokines were strongly elevated in patients with clinical symptoms of cytokine release syndrome. [5, 7-9, 11-17] In CD19 CAR T-cell studies, cytokine release syndromes may occur due to high accessibility of CD19-positive B cells (tumor cells as well as healthy B cells) together with the use of second generation CD19 CARs, including CD28 or 4-1BB (CD137) co-stimulatory domains. [18] In our 'first-generation' CAR T-cell trial directed towards CAIX-positive Renal Cell

Cancer, only one patient showed a high increase of IL-6 plasma levels. Interestingly, this particular patient received the highest CAIX CAR T-cell dose but did not present with the typical clinical symptoms of cytokine release syndrome. Along these lines, we have 'only' detected about a 15-fold increase of IFN-γ levels , whereas in the second generation CD19 CAR T-cell trials an over 1000 fold increase of IFN-γ levels were observed.[9]

We have used liver enzyme elevations as surrogate markers for CAR T cell activity since CAIX expressing bile ducts of patients were targeted by these T cells which led to liver toxicity. Although mean blood levels of Alkaline phosphatase (AP), Gamma-glutamyltransferase (GGT), Aspartate transaminase (ASAT) and Alanine transaminase (ALAT) displayed similar kinetics as the numbers of CAR T-cells in blood, neither liver enzymes nor CTCAE toxicity grading did significantly correlate with CAR T-cell numbers. Bearing in mind the small number of patients, we still argue that the above-mentioned IL-6 and IFN-γ serve well as indicators for peripheral persistence and in vivo activity of gene modified T cells.

T cell infusion characteristics affecting CAR T cell numbers and activity in vivo.

Aside from in-patient parameters, we were also interested whether specific characteristics of the T cell infusion product could affect peripheral T cell numbers. **In chapter 3** we assessed the T cell maturation stage prior to and during GMP processing and how these maturation stages correlate with CAR T cell numbers/expansion in patients. Preclinical studies in mice and monkeys suggest improved in vivo persistence and anti-tumor responses when T cells in early stages of differentiation (such as naïve or central memory cells) are used for genetic modification and treatment[19, 20]. In fact, the differentiation state of CD8+ T cells appeared to be inversely related to their capacity to proliferate and persist.[21, 22] Previously, we have described a shift from naïve T cells to more maturated stages during IL-2-containg cultures of transduced T cells.[23] In our CAIX CAR study, we showed that patients with more CD8+ naïve T cells at baseline (from leukapheresis) or more CD8+ T central effector cells at culture day 14 demonstrated a higher fold increase in numbers of CAR T cells in vivo, resulting in higher blood counts of CAR T cells in these patients for at least 4 weeks. These findings may bear clinical relevance as younger T cells have been shown to positively correlate with clinical effectiveness in CD19 CART cell trials.[24-27] In fact, the CAIX CAR T cell trial is the first CAR study targeting a solid tumor that reveals a correlation between pre-infusion and pre-expansion T cell characteristics and in vivo CAR T cell numbers. In only one other non-CD19 CAR trial, targeting neuroblastoma with GD2 CAR T cells, a correlation was described between in vivo CAR T cell persistence and the proportion of CD4+ or central memory T cells in the infusion product.[25] Our observations are in line with the notion that effector T cells derived from naive rather than memory T cell subsets possess superior features for adoptive immunotherapy [28], though in the latter study no correlations were made with in vivo parameters. Based on these findings we advocate for enhancing T cell persistence in vivo by increased fractions of CD8+ T naïve cells before transduction (e.g., by selection of the naïve

CD8+ T cell fraction) and/or the reduction of T cell maturation during the in vitro culture of gene-transduced T cells by using T cell co-stimulatory agents and cytokines IL15 and IL21 during T cell processing. [23]

Overall lessons from the Rotterdam CAR T cell trial

In **chapter 4** we give a thorough overview of the CAIX CAR T cell trial and the results that were collected in the past years, which enabled us to formulate the following recommendations for upcoming receptor (CAR/TCR) gene-modified T cell studies:

On target/off organ toxicity

The CAIX CAR T cell trial was the first trial using CAR-engineered T cells in Europe. This study demonstrated demonstrating ***in vivo*** activity of CAR T cells, unfortunately this was reflected by on target toxicity of CAR T cells attacking CAIX expressing bile duct epithelial cells, potentially masking anti-tumor efficacy of these CAR T cells. Also, in other trials with CAR T cells, severe toxicities have been described. [16] In recent years several tools have been established to better ensure T cell therapy safety. For example, target antigens should be carefully selected using in silico and laboratory techniques to ensure unique expression by tumor cells and not on normal somatic tissues.[29, 30]. In addition, screenings are available to detect binding of the CAR molecule to surface molecules that are expressed by healthy cells in the body. [31] Alternatively, receptor-engineered T cells can be co-transduced with a so-called death-switch, e.g., human caspase-9 that triggers apoptosis of T cells upon the treatment with small molecules.[32] Especially in early phase-I trials with CAR T cell the latter approach would, at early signs of toxicities, potentially limit development of (severe) adverse events.

Immunogenicity of CAR

In most clinical studies, immunogenicity of the CAR has not been measured nor recognized as a possible limitation of therapeutic efficacy, most likely due to the applied non- myeloablative preconditioning of patients in most studies, together with single T-cell infusions. Yet, our study clearly demonstrated the immunogenicity of xenogeneic protein sequences presented by T-cells.[33] Therefore, for the construction of CARs and TCRs, we advocate the use of fully human complementarity-determining regions (CDRs), and patient pre-treatment with lymphoablative preconditioning followed by infusion of only a single dose of CAR T cells.

Lack of T-cell persistence and therapy efficacy

In the CAIX CAR T cell trial, the CAIX expressing renal cell tumors did not show any shrinkage on radiological assessment. This is likely due to lack of CAR T cell persistence, and possibly the lack of sufficient accumulation and activation of CAR T cells within tumor tissue due to an immunosuppressive microenvironment. In our study, patients were not pre-treated with lymphoablative preconditioning, and were treated with a 1st generation CAR. In subsequent studies, the introduction of a CD28 or CD137 intracellular signalling domain into the CAR (2nd generation CARs) led to increased CAR T cell persistence and the first clinically relevant

responses. Also, patients participating in these studies were lympho-ablated, which is hypothesized to deplete inhibitory immune cell subsets like Myeloid-derived suppressor cell and T regulatory cells.

The best clinical results with CAR T-cells so far, have been achieved in haematological diseases treated with CD19 CARs. In these patients, the malignant, CD19 expressing B cells are present in blood and bone marrow and are easily accessible for CAR T cells. In contrast, to eradicate solid tumors is much more challenging. In order to sensitize the tumor micro-environment and thus support infiltration and action of the CAR T cells, several new strategies are now developed like CAR T cells that, upon activation, secrete factors like IL-12 [34-36], IL-18 [37] and HVEM. [38, 39] Future clinical trials are pending in order to see the effects of these new CARs. See Kunert et al, for an overview of gene-engineering strategies to enhance therapeutic efficacy of CAR T cells.[40]

Finally, in **chapter 5** we narrate extensively about immune monitoring performed in other clinical adoptive T cell trials. We provide an overview of clinical responses, and the immune monitoring performed in trials with TILs, and CAR/TCR gene modified T cells. This manuscript was published in 2015 and included studies until 2015. Interestingly, to date (2018) the topic, and issues pointed out in this chapter are still timely since good and robust predictors of efficacy and safety of treatment with T cells, and especially in solid tumors, are still lacking. [41] At this point, a total of 18 studies have published results about CAR T cell treatment in solid tumors. In these trials, no uniform efforts have been made to monitor immunological changes in patients and correlate these with clinical parameters. However, we emphasize that a better understanding of tumor immunity but also the immunological state of the patient, and analysis of the T cell infusion product are pivotal to identify predictors of clinical responses. In future clinical studies, we advocate that such parameters should be included in study protocols.

PART 2: IMMUNE MONITORING BEYOND T CELL THERAPY

The second part in this thesis describes the characterization of T cells in two different settings. First, we assessed immune cell changes in peripheral blood during dendritic cell (DC) therapy and second, we assessed the differences in immune profiles in different sarcoma tumor subtypes.

Dendritic Cell vaccination study in mesothelioma

Chapter 6 documents the immune effects of treatment of mesothelioma patients with autologous DCs loaded with an allogeneic tumor lysate. The clinical outcomes of this first-in-human phase-I clinical trial have been published previously and demonstrated promising clinical responses. In nine enrolled patients two partial responses were observed, of which one remarkable response of 70% tumor reduction in a treatment-naïve patient within six weeks from start of treatment and which lasted for two years.[42] In this phase I dose escalation

study, peripheral blood was collected before and after each of three treatments with DCs that were pulsed with a lysate prepared from a mixture of five mesothelioma cell lines. Using multiplex flow cytometry, we observed an increase in the peripheral numbers of B cells, CD4 and CD8 T cells, but not of NK cells at week 6 (i.e., 2 weeks after the last of 3 vaccinations). Next, we assessed whether vaccination would enrich for T cells recognizing tumor antigen. To this end, mesothelin, which was expressed by all the mesothelioma cell-lines that were used to load the dendritic cells, was used as a lead antigen. We were unable to demonstrate major changes in T cell reactivity towards mesothelin. Also, analysis of TCRβ diversity did not show shifts towards clonality, and do not suggest a T cell response directed against a single antigen as a result of DC therapy. Others demonstrated that vaccination of melanoma patients with gp-100 peptide loaded DCs induced a broad T cell response to a wide variety of (neo) antigens. Not only the number of neoantigen-specific T cells increased, but also the number of clonotypes per antigen increased, indicating a further diversification of the repertoire.[43] As shown in **chapter 6** and by others, monitoring of specific T cells in peripheral blood upon vaccination, is challenging due to the unknown antigen specificity and low frequency of individual T cell clones, and may be circumvented either by vaccination with very immunogenic antigens like NY-ESO-1 or MART-1 [44, 45] or ***in vitro*** enrichment of specific T cells, which may make it less attractive for future use in clinical practice.

Besides T cell clonality, we also assessed changes in frequencies of T cells positive for a variety of markers. Treatment-related changes were most notable for $CD4^+$ T cells, showing an increased frequency of cells positive for ICOS, PD-1 and HLA-DR after treatment. This is indicative for T cell activation, as T cells upregulate HLA-DR [46], ICOS [47]1BcUF1Q and PD-1 [48] after activation through TCR engagement. In the study described in **chapter 6** patient numbers are too low to correlate the immune related data with clinical outcome. Yet, our findings on the abovementioned activation markers may proof useful for future studies with larger patient groups to evaluate their relationship with treatment efficacy.

TILs and immune micro-environment in sarcoma subtypes

In **chapter 7**, we have generated a multi-parameter inventory of the immune microenvironment as well as CD8+ TILs of different STS subtypes. We aimed to thoroughly chart the immunogenicity of most common STS types, which in turn may provide valuable information about the STS subtype eligibility for immunotherapy. STS are a group of heterogeneous tumors of mesenchymal origin with over 50 different subtypes, for which the exploration of immunotherapeutic strategies has been limited. First studies show that some STS subtypes may respond to anti-PD1 Ab therapy [49, 50] as well as to adoptive T cell therapy.[51] A recent phase 2 study treating 85 metastasized STS patients with anti-PD-1 Ab nivolumab (n=43) or nivolumab plus anti-CTLA4 Ab Ipilimumab (n=42) concluded that nivolumab monotherapy does not warrant further study in an unselected STS population given the limited efficacy. Yet, the nivolumab plus ipilimumab combination treatment showed promising efficacy in certain STS subtypes like undifferentiated pleomorphic sarcoma and leiomyosarcoma.[52]

Since in other tumor types it is evident that numbers and activation state of immune cells in tumors, in particular CD8+ TILs, have predictive value with respect to the responsiveness to immune therapies [53, 54], we have extended these studies to different STS subtypes. Using Liposarcoma (LPS), Gastro-intestinal stromal tumors (GIST), Leiomyosarcoma (LMS), Myxofibrosarcoma (MFS) and pleomorphic sarcoma (PMS), we have measured quantities and qualities of TIL, and T cell evasive mechanisms via in situ stainings and single cell flow-cytometry, as well as DNA and RNA sequencing data analysis. Our data demonstrated clear differences between the different STS subtypes concerning intra-tumoral T cell numbers, T cell activation and maturation, and clonality. GIST, MFS and PMS appear to have the highest T cell numbers, while GIST have much lower fractions of activated and maturated T cells, and in GIST the TCR repertoire is more diverse compared to MFS and PMS (Figure 8, chapter 7). These data suggest that in GIST, only limited TCR-driven T cell activation/expansion occurs. To explain the differences found between the STS subtypes, we further analyzed the different STS subtypes for: antigen presentation/processing, mutational load, inhibitory cell subsets and inhibitory and co-stimulatory ligands. No differential gene expression was observed for molecules of both the MHC-I and MHC-II antigen presentation pathways. Mutational load was somewhat higher in MFS and PMS compared to LMS and LPS, however low compared to other tumor types like melanoma.[55] Inhibitory cell subsets like M2 macrophages were low in GIST and high in MFS and PMS. The expression of PD1L2 was low in GIST compared to all other STS subtypes and the TIM3 ligand Galectin-9 was lowest in LMS and highest in MFS and PMS. Most notably, expression of costimulatory ligands CD86, ICOSL, and 4-1BB ligand were all lowest in GIST, compared to other STS subtypes, and generally highest in MFS and PMS. In conclusion, quantity and quality of CD8+ T cells as well as parameters of the immune micro-environments are clearly different among STS subtypes. Building on these data, we speculate that MFS and PMS are suitable for checkpoint therapy. Since checkpoint therapy is primarily effective in inflamed tumors [56, 57], not all Myxo- and Pleomorphic sarcomas are expected to equally benefit from checkpoint therapy, providing a rationale to further stratify patient tumors according to immune parameters, such as density of PD1-positive CD8 T cells. For tumors that are less immunogenic (i.e., having a lowered mutational burden), other therapeutic options may be required (e.g. radiotherapy, chemotherapy) to sensitize tumors for checkpoint therapy or adoptive T cell therapy. With respect to GIST, we speculate that treatment with co-stimulatory agonistic antibodies may show efficacy.

Collectively, **part 2** of this thesis showed approaches of immune monitoring in two different settings namely: I) during DC therapy in peripheral blood to monitor response to DC vaccination, and II) in a pre-clinical setting, by monitoring the tumor microenvironment, in particular the intratumoral T cells, to find new rationales for (immuno)therapy.

In an era where there is more emphasis on personalized medicine, we advocate that new clinical study protocols should always include additional monitoring of the tumor microenvironment, and/or the peripheral immunity. Preferably before, during, and if possible after treatment. Especially sampling before and after treatment can give valuable information about

therapy induced changes which in turn could lead to rationales to combine therapies. With a variety of techniques like multiplex flow cytometry, multiplex in situ immunohistochemistry, and next generation DNA/RNA sequencing becoming more affordable and accessible nowadays, we would like to advocate in-depth analysis and (immuno)monitoring of both the tumor and peripheral blood should be a standard in every immunotherapeutic clinical trial.

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Appendices

English Summary

SUMMARY

Immune therapy has proven to be an important new treatment strategy in cancer. The "traditional" treatment modalities in oncology are in addition to resection or debulking of tumors, radiotherapy and chemotherapy. Immunotherapy of cancer is a strategy that harnesses the body's immune system to combat tumors. Despite the promise of immunotherapeutic approaches, a significant fraction of patients does not yet respond to these therapies. To date no markers are yet available to robustly predict therapy outcome or provide enough understanding into the underlying mechanisms that define unsuccessful treatment or toxicity. It is of pivotal importance to obtain such markers, since this will lead to improved stratification of patients for immune therapy. In addition, patient stratification can prevent unnecessary treatment and increase cost-effectiveness of these treatments.

Chapter 1 starts with a general introduction into immunology, cancer and subsequently describes different approaches how the immune system could be exploited to combat cancer. This chapter also describes the focus and structure of this thesis.

This thesis is split into two parts. In the first part (**chapters 2 to 5**), we have focused on immune monitoring tools that were used in a CAR T cell phase-I trial to find parameters that correlate with in vivo behavior of administered T cells. In the second part (**chapter 6 and 7**), we have monitored changes in T cells numbers, T cell clonality and T cell phenotype in the blood compartment in a phase I trial with Dendritic Cell therapy. Also, in the second part, we have assessed the characteristics of intra-tumoral T cells as well as the immune micro-environment in several soft tissue sarcoma (STS) subtypes in order to identify new immune monitoring.

PART 1: IMMUNE MONITORING IN CHIMERIC ANTIGEN RECEPTOR (CAR) T CELL THERAPY

Chapter 2 describes immune monitoring performed in a phase-I CAR trial treating patients with metastasized clear cell renal cell carcinoma (RCC). Twelve patients were treated with CAR T cells targeting Carboxy-anhydrase IX (CAIX). In this trial no clinical responses were observed, and patients experienced transient toxicities most likely due to low CAIX expression in bile ducts. In **chapter 2** the number of infused CAR T cells was assessed by two different quantitative assays using blood samples: 1) flow cytometry (FCM), and 2) qPCR. No significant correlations between numbers of CAIX CAR T-cells and CAIX CAR DNA copies were found. We concluded that CAR DNA levels do not directly reflect the level of CAR protein expression and may in fact overestimate the number of T cells that are reactive to CAIX in vivo. Subsequently, we have set out to find surrogate markers for T cell persistence. Notably, we found that plasma levels of IL-6 and IFN-γ correlated with CAR T cells numbers in vivo. Finally, in **chapter 2** we have assessed whether liver enzyme elevations can act as surrogate markers for in vivo CAR T cell activity. Unfortunately, neither liver enzymes nor CTCAE toxicity grading did significantly correlate with CAR T-cell numbers.

In chapter 3 we have assessed T cell maturation stages prior to and during transduction and expansion, and how these maturation stages correlate with CAR T cell numbers/expansion in patients. **Chapter 3** showed that patients with higher fractions of naïve CD8+ T cells at baseline (from leukapheresis) or central effector CD8+ T cells at culture day 14 demonstrated a higher fold increase in CAR T cells numbers in vivo, resulting in higher blood counts of CAR T cells in these patients for at least 4 weeks. Based on these findings we advocate to implement strategies to increase fractions of naïve CD8+ T cells before transduction (e.g., by selection of naïve CD8+ T cell fraction from patient blood) and/or reduce T cell maturation during in vitro culture of T cells (e.g. by using T cell co-stimulatory agents and cytokines IL-15 and IL-21).

Chapter 4 gives a thorough overview of the Rotterdam CAIX CAR T cell trial and the results that were collected in the past years, which enabled us to formulate recommendations for upcoming receptor (CAR/TCR) gene-modified T cell studies, particularly with respect to: on target/off organ toxicity, immunogenicity of CAR, and T-cell persistence and therapy efficacy.

Chapter 5 describes immune monitoring performed in other clinical adoptive T cell trials. An overview is provided of clinical responses, and the immune monitoring performed in trials with TILs, and CAR/TCR gene modified T cells. This chapter emphasizes that a better understanding of tumor immunity but also the immunological state of the patient, and analysis of the T cell infusion product are all pivotal to identify predictors of clinical responses. In future clinical studies, we advocate that such parameters should be included in study protocols.

PART 2: IMMUNE MONITORING BEYOND T CELL THERAPY

Chapter 6 documents the immune effects in mesothelioma patients treated with autologous DCs loaded with an allogeneic tumor lysate. In nine enrolled patients two partial responses were observed, of which one remarkable response of 70% tumor reduction in a treatment-naïve patient within six weeks from start of treatment and which lasted for two years. Patients in this trial demonstrated an increase in the peripheral numbers of B cells, CD4 and CD8 T cells, but not of NK cells after vaccination. Subsequent analysis of T cells specific for mesothelin, an antigen that is highly present in tumor lysate, as well as bulk T cells did not show shifts towards TCR clonality due to vaccination. This suggests that the T cell response is not directed against a single antigen as a result of DC therapy. Other treatment-related changes were most notable for CD4+ T cells, showing an increased frequency of cells positive for ICOS, PD-1 and/or HLA-DR after treatment, changes that are indicative for T cell activation. In the study described in **chapter 6** patient numbers are too low to correlate the immune related data with clinical outcome. Yet, our findings may proof useful for future studies with larger patient groups to evaluate their relationship with treatment efficacy.

Chapter 7 describes a multi-parameter inventory of the immune microenvironment as well as CD8+ TILs of different STS subtypes, thereby aiming to provide valuable information about subtype eligibility for immunotherapy. Data demonstrated clear differences between the different STS subtypes concerning intra-tumoral T cell numbers, T cell activation and matura-

tion, as well as T cell clonality. Gastro intestinal stromal tumors (GIST), myxofibrosarcoma and pleomorphic sarcoma have the highest T cell numbers, while GIST has much lower fractions of activated and maturated T cells, and in GIST the TCR repertoire is more diverse compared to myxofibrosarcoma and pleomorphic sarcoma. Further analyses of the STS tumors for: antigen presentation/processing, mutational load, inhibitory cell subsets and inhibitory and co-stimulatory ligands revealed no differential expression of genes involved in antigen presentation/processing. Mutational load was somewhat higher in myxofibrosarcoma and pleomorphic sarcoma compared to leiomyosarcoma and liposarcoma, however mutational load was overall low compared to other tumor types like melanoma. Frequencies of inhibitory cell subsets were low in GIST and high in myxofibrosarcoma and pleomorphic sarcoma. The expression level of inhibitory ligands was also low in GIST compared to all other STS subtypes, and highest in myxofibrosarcoma and pleomorphic sarcoma. Most notably, expression level of costimulatory ligands was lowest in GIST, and generally highest in myxofibrosarcoma and pleomorphic sarcoma. Building on the collection of these data, we speculate that myxofibrosarcoma and pleomorphic sarcoma are suitable for checkpoint therapy. With respect to GIST, we speculate that treatment with co-stimulatory agonistic antibodies may show efficacy.

Finally, in **Chapter 8** the main findings of this thesis are described and discussed. In an era where there is more emphasis on personalized cancer treatment, it is pivotal that new clinical study protocols include additional monitoring of the tumor micro-environment, and/or peripheral immunity. Preferably before, during, and if possible after treatment tumor and/or blood samples should be collected, which will provide valuable information about therapy-induced changes which in turn rationalize choices for new (combination) therapies. With a variety of techniques like multiplex flow cytometry, multiplex in situ immunohistochemistry, and next generation DNA/RNA sequencing becoming more and more affordable and accessible, we recommend in-depth immune monitoring of both tumor and peripheral blood becoming part of immunotherapeutic clinical trials.

Nederlandse Samenvatting

LEKENUITLEG EN NEDERLANDSE SAMENVATTING

In Nederland overlijden drie op de tien mensen aan de gevolgen van kanker. Kanker is daarmee de belangrijkste doodsoorzaak. Tot op heden is uitgezaaide (gemetastaseerde) kanker in de meeste gevallen niet te genezen en is de behandeling van gemetastaseerde ziekte gericht op het voorkomen van ziekteprogressie. De “traditionele” behandelingsstrategieën in de oncologie zijn chirurgie, radiotherapie en chemotherapie, maar in de laatste jaren is ook immuuntherapie sterk in opmars. Immuuntherapie is anders dan de traditionele behandelingen, omdat deze zich richt op het verbeteren of herstellen van de patiënt zijn/haar eigen afweer tegen de kanker, terwijl traditionele behandelingen zich richten op het direct doden maken van kankercellen. Een aantal immuuntherapieën hebben al indrukwekkende klinische resultaten laten zien, met een verbeterde overleving ten opzichte van chemo- of radiotherapie, en soms volledige genezing. Dit hoofdstuk geeft een korte introductie over het immuunsysteem, de interactie hiervan met oncologische ziektebeelden en hoe het immuunsysteem wordt gebruikt voor immuuntherapieën. Na deze introductie volgen de belangrijkste resultaten uit dit proefschrift.

HET IMMUUNSYSTEEM

Het immuunsysteem beschermt ons tegen ziekteverwekkers (pathogenen), zoals bacteriën, virussen en parasieten, maar speelt ook een rol bij het opsporen en uitroeien van ontspoorde (kwaadaardige) cellen. Het immuunsysteem kan grofweg worden verdeeld in twee subsystemen: 1) het aangeboren en 2) het verworven immuunsysteem. Deze twee subsystemen zijn complementair en zorgen tezamen voor een effectieve immuunrespons.

Aangeboren immuunsysteem

De belangrijkste onderdelen van het aangeboren immuunsysteem zijn de fysieke en chemische barrières (huid, slijmvliezen, speeksel) en het complementsysteem. Dit laatste systeem bestaat uit een reeks eiwitten die ervoor zorgen dat immuuncellen pathogenen makkelijker kunnen herkennen en vernietigen. Daarnaast bestaat het aangeboren immuunsysteem ook uit een reeks immuuncellen zoals: basofielen, dendritische cellen, eosinofielen, mestcellen, monocyten, macrofagen, neutrofielen en “natural-killer” cellen. Al deze cellen hebben hun eigen specifieke rol in de herkenning en eliminatie van pathogenen. Ook zijn deze immuuncellen, en dan met name de macrofagen en dendritische cellen belangrijk voor het herkennen, doden, verwerken en vervolgens presenteren van pathogeen-afkomstige eiwitfragmenten, ook wel “antigenen” genoemd. Macrofagen en dendritische cellen worden daarom ook wel antigeen-presenterende cellen genoemd. De gepresenteerde antigenen kunnen vervolgens worden herkend door cellen uit het adaptieve immuunsysteem, dat hiertegen een specifieke en sterke afweer ontwikkelt tegen dat pathogeen. Ten slotte produceren de geactiveerde cellen van het aangeboren immuunsysteem stoffen, chemo- en cytokines genoemd, die andere immuuncellen aantrekken, waardoor er op de plaats waar het pathogeen zit een ontsteking ontstaat.

Verworven immuunsysteem

Het verworven immuunsysteem bestaat uit zeer gespecialiseerde cellen die antigenen kunnen herkennen. Het adaptieve immuunsysteem omvat (antistof producerende) B-cellen, CD4+ helper T cellen en CD8+ celdodende (cytotoxische) T cellen. B-cellen herkennen antigenen direct via de B-celreceptor (BCR). T cellen hebben een T cel receptor (TCR) dat kleine antigeen fragmenten (peptiden) herkent die worden gepresenteerd door moleculen van het major histocompatibility complex (MHC) op het oppervlak van bijvoorbeeld macrofagen of dendritische cellen. De BCRs en TCRs zijn uniek en komen voort uit genetische recombinatie van de DNA-gecodeerde segmenten bij de aanmaak van de B- en T-cellen. Deze recombinatie vormt B- en T-lymfocyten die veel verschillende antigenen herkennen, waaronder antigenen die worden gepresenteerd door gezonde weefsels. Om dit te voorkómen ondergaan lymfocyten een selectie tijdens de uitgroei van stamcel naar lymfocyt, waarbij lymfocyten die in staat zijn gezonde weefsels te herkennen worden geëlimineerd. Wanneer de overgebleven B of T cellen een antigeen herkennen, worden deze geactiveerd en vermeerderen deze cellen zich snel)expansie). Vervolgens zullen deze cellen het pathogeen dat het antigeen tot expressie brengt aanvallen en elimineren. Na eliminatie van dat antigeen worden er immunologische geheugencellen gevormd. Hierdoor zullen bij een tweede infectie met hetzelfde pathogeen, de antigeen-specifieke B en T cellen sneller uitgroeien en het pathogeen sneller elimineren.

Naast de hierboven beschreven ‘reactieve’ immuuncellen zijn er ook immuuncellen die dergelijke reacties kunnen afremmen. Dit is belangrijk om een hyperactiviteit van het immuunsysteem te voorkomen en daarmee de ontwikkeling van auto-immuniteit. Er zijn verschillende van deze remmende immuuncellen, zoals “Myeloid derived suppressorcellen” (MDSC), “M2-macrofagen” en “T-regulatory cellen” (Tregs). Zij brengen moleculen tot expressie en produceren stoffen, cytokines, die een remmende werking hebben op de ‘reactieve’ immuuncellen.

Tumoren als verstoorde mini-organen

Tumoren bestaan niet alleen uit kankercellen maar ook uit cellen van bloedvaten, ondersteunende weefsels en verschillende immuuncellen. Al deze niet kankercellen samen in een tumor vormen de tumor micro-omgeving en zijn bepalend voor de eigenschappen van tumoren en daarmee ook hun gevoeligheid voor (immuun)therapie. Tegenwoordig weten we dat CD8+ cytotoxische T cellen erg belangrijk zijn in het herkennen en doden van tumorcellen. Het aantal en de locatie van deze immuuncellen in tumoren bepaalt de prognose van patiënten. Een hoge frequentie van tumor infiltrerende lymfocyten (TIL), en met name de CD8+ cytotoxische TILs, correleert bijvoorbeeld met een verhoogde overleving van patiënten met melanoom (huid)-, hoofd en nek-, borst-, urotheel-, eierstok-, colorectale en longkanker. Veel immuuntherapieën zijn er op gericht om deze T cellen te activeren, of om op een andere manier een antitumor respons te forceren.

Immuuntherapieën in kanker

Hierbij onderscheiden we therapieën met stoffen, die de immuunreactie van het lichaam tegen kanker(cellen) versterken, en daarnaast zijn er therapieën waarbij extra immuuncellen aan de patiënt worden toegediend (adoptieve cellulaire therapie).

Niet cellulaire immuuntherapieën

Bij niet cellulaire immuuntherapieën worden stoffen (bijv. cytokines, zoals Interleukine-2 (IL-2) of interferon-α aan de patiënt toegediend die de immuuncellen 'oppeppen'). Een ander voorbeeld is 'tumorvaccinatie', het toedienen van tumor-afkomstige antigenen en peptiden om een specifieke immuunreactie tegen de tumor op te wekken. Deze antigenen worden opgenomen door antigeen presenterende cellen die vervolgens T cellen activeren. Tot zover hebben deze strategieën echter slechts beperkte resultaten opgeleverd in de behandeling van kanker.

Een andere strategie waarmee in meerdere tumortypen indrukwekkende klinische resultaten worden behaald is de zogeheten 'immuun checkpoint' therapie. Immuun checkpoints zijn eiwitten op het oppervlak van immuuncellen die ervoor zorgen dat het immuunsysteem niet over-reageert. Een voorbeeld hiervan is de "programmed cell death-1 receptor" (PD1). Deze komt tot expressie op onder andere CD8+ T cellen, zodra deze T cellen worden geactiveerd. Als PD1 bindt met een ligand voor PD1 (PDL1 of PDL2) aanwezig op het oppervlak van een andere cel, zal PD1 een signaal afgeven waardoor de immuuncel geremd wordt. Sommige tumortypen maken hier slim gebruik van en hebben PDL1 op hun celoppervlak. Hierdoor worden geactiveerde T cellen geremd wanneer zij in contact komen met deze kankercellen. Immuun checkpoint therapie (in dit geval PD1) blokkeert deze interactie tussen PD1 en diens ligand. Hierdoor worden de immuuncellen niet meer geremd wanneer zij de tumorcel weer kunnen elimineren.

Cellulaire immuuntherapieën

Bij cellulaire immuuntherapie worden (in het laboratorium bewerkte) immuuncellen aan de patiënt toegediend. De twee belangrijkste cellulaire therapieën zijn het toedienen van dendritische cellen of T cellen.

Dendritische cel therapie

Bij dendritische cel therapie wordt een vaccin toegediend, bestaande uit dendritische cellen beladen met antigeen. Deze dendritische cellen (DCs) worden in het laboratorium gemaakt uit monocyten die worden verkregen uit het bloed van patiënten en verder worden opgekweekt in aanwezigheid van cytokinen en beladen met tumorantigenen (eiwitten of peptiden). Deze 'beladen' DCs worden vervolgens teruggegeven aan de patiënt om T cellen in de patiënt te activeren. De vaccin-specifiek geactiveerde T cellen kunnen de tumorcellen vervolgens herkennen en aanvallen.

In plaats van het ***in vivo*** (in de patiënt) activeren van T cellen door middel van checkpoint in-

hibitors of toegediende DCs, kunnen tumor-specifieke T cellen ook ***ex vivo*** worden vermeerderd of zelfs worden gegenereerd, zodat zij een specifiek tumor antigeen herkennen. Dit heet T celtherapie. In het kort zijn er twee belangrijke vormen van T cel therapieën:

T cel therapie met Tumor Infiltrerende Lymfocyten (TIL)

Bij TIL therapie worden er uit een tumor(biopt) T cellen (op)gekweekt en teruggegeven aan de patiënt (Figuur 1A). Deze TILs bevatten in meer of mindere mate tumor specifieke T cellen, die initieel 'geremd' zijn, maar eenmaal geïsoleerd van hun micro-omgeving weer functioneel zijn. De TILs worden vervolgens ***ex vivo*** vermeerderd tot grote aantallen en teruggegeven aan de patiënt. Met deze therapie zijn zeer indrukwekkende klinische resultaten behaald, vooral in patiënten met gemetastaseerd melanoom met klinische objectieve radiologische responsen rond de 50%, en in 20% van de gevallen zelfs volledige genezing van patiënten. Buiten de behandeling van patiënten met melanoom zijn de klinische resultaten van TIL therapie echter nog beperkt.

T cel therapie met Chimeric Antigen Receptor (CAR) of TCR T cellen

Omdat het niet altijd mogelijk is om TILs uit tumoren te verkrijgen, is er een alternatieve manier ontwikkelt om T cellen te gebruiken voor therapie. Hierbij worden T cellen uit het bloed van patiënten geïsoleerd en vervolgens in het laboratorium bewerkt (genetisch gemanipuleerd), waardoor zij een specifieke receptor tot expressie brengen en daarmee een antigeen op tumorcellen kunnen herkennen. Er worden twee typen receptoren onderscheiden: 1) receptoren gebaseerd op antistoffen, de zogeheten Chimeric Antigen Receptors (CAR), of 2) receptoren gebaseerd op T cel receptoren (TCR). Beide type receptoren kunnen specifiek binden aan een antigeen dat tot expressie gebracht wordt op kankercellen, de CAR aan membraan antigenen en de TCR aan een eiwit fragment (peptide) in het MHC complex, dat kunnen ook peptiden zijn van intra cellulaire eiwitten. Wanneer een CAR of TCR aan een antigeen bindt, zal de T cel geactiveerd worden en de (kanker)cel vernietigen.

CARs zijn afgeleid van het antigeen-bindend deel van een antistof en bevat een transmembraan, en een intracellulair domein, waardoor het verankerd is in de celmembraan en na binding aan antigeen een signaal de cel in kan sturen. Het principe van CAR/ TCR-T cel behandeling wordt weergegeven in figuur 1B. Studies met de 'eerste generatie' CARs lieten slechts beperkte klinische resultaten zien, maar studies met tweede generatie CARs hebben veelbelovende resultaten opgeleverd. Deze tweede generatie CARs hebben een extra ingebouwd signaalversterkings-domein dat is afgeleid van costimulatoire moleculen. Vooral in hematologische ziekten van B cellen, die het antigeen CD19 tot expressie brengen, hebben CARs gericht tegen het CD19 indrukwekkende klinische responsen laten zien. Bijvoorbeeld in Acute Lymfatische B-cel Leukemie (B-ALL) werd er complete genezing gezien in 70 tot 100% van de patiënten.

Een nadeel van CAR T cellen ten opzichte van TCR T cellen is echter dat alleen extracellulaire, en geen intracellulaire antigenen kunnen worden gekozen. Helaas zijn de klinische resultaten

van de CAR- en TCR- T celtherapie in solide tumoren nog niet zo indrukwekkend als voor de CD19 CAR in hematologische maligniteiten. Voor een effectieve CAR en TCR T celtherapie is de keuze van het antigeen waarop de receptor is gericht essentieel. Wanneer het antigeen namelijk niet alleen door de tumor maar ook door gezond weefsel tot expressie wordt gebracht kan dit leiden tot ernstige toxiciteit (auto-immuniteit).

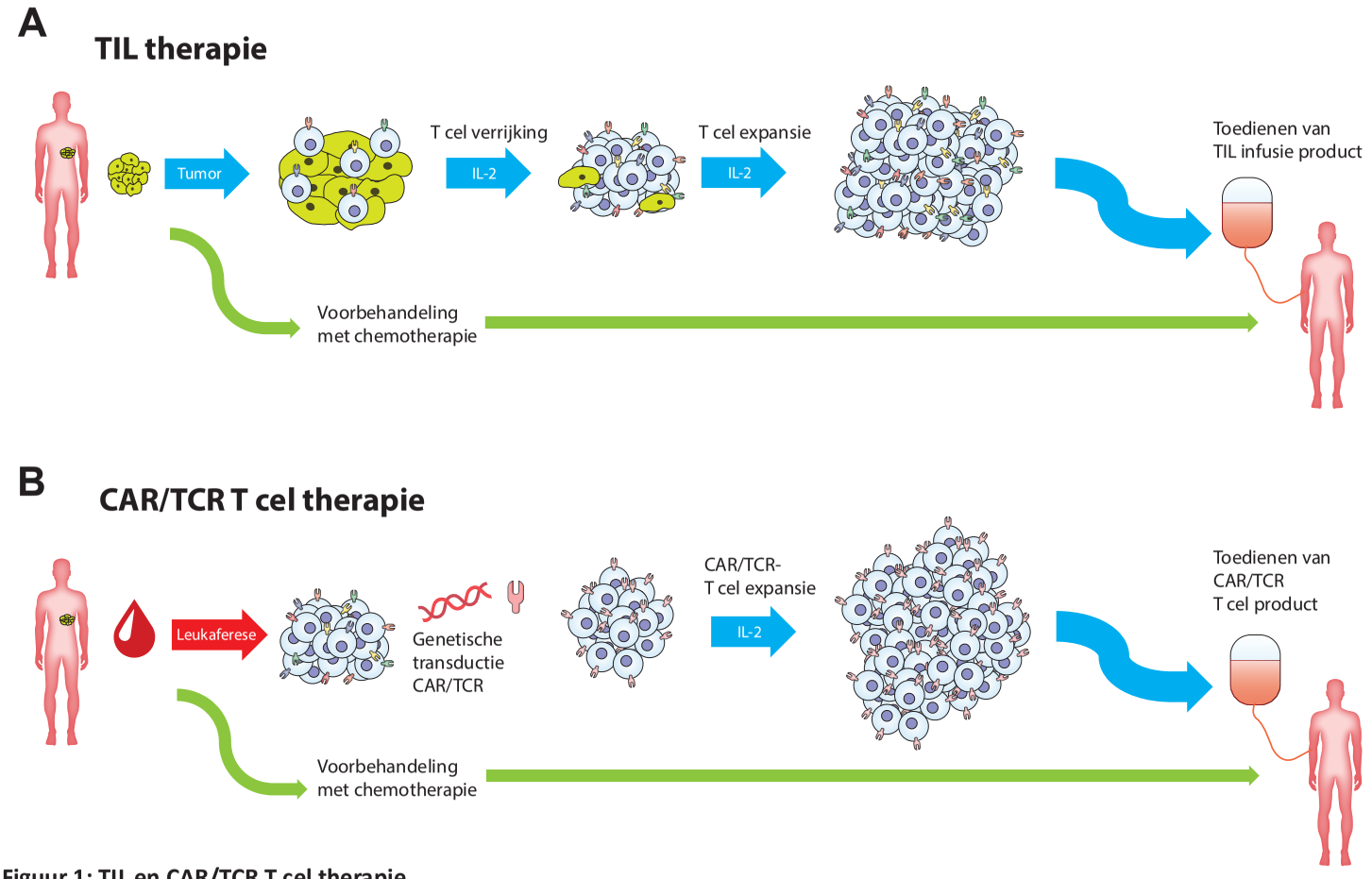
FOCUS VAN DIT PROEFSCHRIFT: IMMUUN MONITORING

Ondanks de hierboven beschreven veelbelovende ontwikkelingen reageert een aanzienlijk deel van de patiënten niet op immuuntherapie. Tot nu toe zijn er nog geen goede klinische of immunologische merkers (parameters) die aangeven of een patiënt mogelijk baat heeft of niet bij immuuntherapie. Ook zijn er geen goede merkers bekend die de onderliggende mechanismen van een succesvolle behandelingen, of juist het ontbreken daarvan, of het optreden van bijwerkingen voorspellen. Het is zeer belangrijk om dit soort merkers voorhanden te krijgen, omdat dit kan leiden tot betere selectie van patiënten en ook nieuwere inzichten die de therapie kunnen verbeteren. Verbeterede patiënt selectie is belangrijk omdat dit onnodige behandelingen voorkomt, kwaliteit van leven verbetert, en de kosten effectiviteit verhoogt. Om dit soort merkers te vinden is het uitgebreid volgen (monitoren) van immunologische en klinische parameters binnen patiënt studies essentieel.

Dit proefschrift is opgesplitst in twee delen. In het ***eerste deel*** hebben we ons gericht op verschillende immuun monitoring strategieën in een fase-I CAR T cel studie die een indicatie kunnen geven over de aantallen CAR T cellen die in het bloed van patiënten terug te vinden zijn, en daarmee voorspellend kunnen zijn voor de therapie uitkomst. In het ***tweede deel*** hebben we, ten eerste, in een fase-I DC vaccinatie studie gezocht naar merkers van een immuunrespons in patiënten tegen het vaccin. Ten tweede zijn de karakteristieken van T cellen in tumoren bestudeerd, waarbij we ons gericht hebben op weke delen tumoren (sarcomen), met als doel verschillende sarcoom subtypen uit elkaar te trekken wat betreft voorspelde gevoeligheid voor immuuntherapie.

Deel 1: T cel monitoring in CAR T cel therapie

Hoofdstuk 2 tot en met hoofdstuk 4 van dit proefschrift beschrijven de bevindingen van een fase-I klinische studie, waarin twaalf patiënten zijn behandeld met CAR T cel therapie. In deze studie werd gebruikt gemaakt van een CAR gericht tegen het carboxy-anhydrase-IX (CAIX) antigeen. Dit antigeen komt hoog tot expressie op het niercelcarcinoom. Helaas werden er geen klinische responsen gezien. Wel werd gezien dat CAR T cellen een sterke activiteit vertoonden aangezien patiënten levertoxiciteit ontwikkelden. Nader onderzoek toonde vervolgens aan dat het CAIX antigeen in lichte mate ook voorkomt op de cellen in de grote galwegen. Om deze toxiciteit tegen te gaan werden de laatste serie patiënten voorbehandeld met een CAIX bedekkend antistof. Dit resulteerde in aanmerkelijk minder toxiciteit vergeleken met de andere patiënten. Gedurende deze studie is er zeer uitgebreid onderzoek gedaan en zijn vele verschillende parameters gemeten. Zo zijn onder andere de eigenschappen van T cellen in



Figuur 1: TIL en CAR/TCR T cel therapie.

A) schematische weergave TIL therapie. **B)** Schematische weergave van CAR of TCR T cel therapie.

het infusie product onderzocht, maar ook zijn er verschillende in vivo parameters gemeten om te kijken welk van deze parameters mogelijk een effectieve T cel therapie zouden kunnen voorspellen.

Bepalen van het aantal CAIX CAR T cellen in bloed van patiënten

In **hoofdstuk 2** is bloedonderzoek beschreven gericht op 1) het aantal CAR T cellen gemeten met flow cytometrie (FCM) en spiegels van 2) CAR DNA en CAR RNA gemeten met moleculaire technieken. Het aantal CAR T cellen dat we meten is het netto resultaat van het aantal CAR T cellen dat is toegediend, het aantal dat na transfusie verloren gaat en het aantal dat door groei erbij komt. Meerdere studies hebben al laten zien dat er een verband bestaat tussen het aantal CAR/TCR T cellen dat na toediening aanwezig is in het bloed van de patiënt en de respons op therapie. Daarom is het belangrijk om dit aantal nauwkeurig te kunnen meten. Interessant is dat wij geen duidelijk verband vonden tussen de metingen van het aantal CAR T cellen met FCM en het aantal CAR DNA kopieën. Daarnaast zagen we een afname van de mate van CAR expressie op T cellen, mogelijk geassocieerd met vermindering van de algemene T cel activiteit.

Het meten van aantallen CAR T cellen in patiënten kan dus het beste gedaan worden door middel van FCM. Het enige nadeel van FCM is dat afgenomen bloedmonsters meteen opgewerkt moeten worden en dat instituten een flow cytometer moeten hebben of in staat moeten zijn om samples meteen in te vriezen.

Surrogaatmerkers voor CAR T cel aantallen

Tijdens de CAR T cel behandeling is bij de patiënten op meerdere tijdspunten bloed afgenomen, waarin cytokines zijn gemeten. In **hoofdstuk 2** is er gekeken of de concentratie van cytokines correleerden met het aantal CAR T cel in het bloed en met de geobserveerde bijwerkingen. Van verschillende cytokines die zijn gemeten, correleerden alleen interleukine-6 (IL-6) en Interferon-gamma (IFN-γ) met het aantal CAR T cellen (gemeten met FCM). Er zijn geen vergelijkbare studies bekend die de cytokine concentraties direct hebben gecorreleerd met het aantal T cellen. Wel zijn in enkele CAR T cel studies cytokine concentraties bepaald tijdens de therapie. In deze eerdere studies zijn vooral IFN-γ en IL-6 nauwgezet bestudeerd, omdat deze cytokines sterk verhoogd waren in patiënten met een zogeheten cytokine storm, waarbij zeer hoge cytokine concentraties waarneembaar waren na toediening van CAR T cellen. Cytokine stormen komen relatief vaak voor in patiënten met een grote hematologische tumormassa die worden behandeld met CD19 CAR T cellen. In de Rotterdamse CAIX CAR T cel studie werden er lang niet zulke hoge cytokine waarden gezien. Een belangrijk punt in deze studie is het kleine aantal patiënten, waardoor het moeilijk is in te schatten of de data ook naar een grotere groep mensen valt te extrapoleren. Desondanks zien wij duidelijk dat IL-6 en IFN-γ potentieel belangrijke parameters zijn die een reflectie geven van het aantal CAR T cellen, en mogelijk CAR T-celreactiviteit in patiënten.

Ex vivo CAR T cel eigenschappen voor CAR T cel aantal en activiteit in patiënt

Naast de parameters die gemeten kunnen worden in patiënten gedurende T cel therapie, wordt er in **hoofdstuk 3** gekeken naar de eigenschappen van de CAR T cellen in het infusieproduct. T cellen doorlopen verschillende maturatiestadia gedurende hun levensloop, die deels wordt beïnvloed door hun omgeving en de signalen die zij krijgen. In **hoofdstuk 3** hebben we gekeken of de T cel maturatiestatus, voor en gedurende het proces van transduceren en vermeerderen van T cellen, invloed had op de aantallen cellen in de patiënt. We vonden dat patiënten met relatief meer jonge CD8+ CAR T cellen in het uitgangsmateriaal (T cellen vóór het transduceren en vermeerderen), meer CAR T cellen in het bloed hadden dan patiënten met minder jonge T cellen. Ook als het CAR T cel product na kweek verrijkt was voor zogeheten 'effector T cellen' resulteerde dat in hogere CAR T cel aantallen in het bloed. De CAIX CAR T cel studie laat, als eerste CAR T cel studie in vaste tumoren, zien dat er een verband bestaat tussen T cel karakteristieken in het infusie product en aanwezigheid van CAR T cellen in de patiënt.

In **hoofdstuk 4** geven we een compleet overzicht van het beloop en de gepubliceerde resultaten van de CAIX CAR T cel studie en hebben de volgende aanbevelingen geformuleerd voor toekomstige CAR (TCR) T cel studies wat betreft de volgende aspecten:

1. On target/off organ toxiciteit

Om in de toekomst bijwerkingen van CAR T cellen te voorkomen, is het essentieel om antigenen te selecteren die alleen tot expressie komen op tumor en niet op de gezonde cellen. In de afgelopen jaren zijn er meerdere technieken ontwikkeld om T cel therapie veiliger te maken. Er zijn nu verschillende computer en laboratoriumtesten beschikbaar die kunnen aantonen of antigenen tot expressie komen op gezonde cellen in het lichaam, en of de CAR behalve het bekende antigeen ook andere antigenen herkent. Daarnaast kan er in CAR T cellen een 'noodrem' worden ingebouwd, een zogeheten 'kill-switch'. Indien nodig kunnen deze CAR T cellen snel worden verwijderd uit het lichaam.

2. Herkenning van de CAIX CAR door het eigen immuunsysteem

In de CAIX CAR T cel studie werd er een reactie gevonden van het immuunsysteem van de patiënten tegen de CAR, dat voor een deel uit vreemd (muizen) eiwit bestaat. In de meeste klinische CAR T cel studies wordt geen melding gemaakt van een immuunrespons tegen de gebruikte CAR. Dit komt hoogstwaarschijnlijk doordat in de meeste T cel studies vooraf het immuunsysteem van de patiënten sterk wordt afgezwakt door een vorm van chemotherapie. Daarnaast zijn in de nieuwe generatie CARs de vreemde eiwit sequenties geminimaliseerd of volledig afwezig. Verder wordt er nu in meeste CAR studies maar één gift CAR T cellen gegeven, terwijl in de CAIX CAR T cel studie patiënten maximaal 10 giften van CAR T cellen kregen toegediend.

3. Gebrek aan T cel persistentie en effectiviteit van de therapie.

In de CAIX CAR T cel studie werd gebruik gemaakt van een eerste generatie CAR. Ondanks het feit dat er wel degelijk CAR T cel activiteit werd gezien in de vorm van levertoxiciteit, werden er geen radiologische anti-tumor responsen gezien. Er zijn een aantal mogelijke oorzaken en oplossingen hiervoor:

- CAIX CAR T cellen persisteerden relatief kort in patiënten (**hoofdstuk 2**). De nieuwe tweede generatie CAR T cellen zijn echter aanmerkelijk langer detecteerbaar in patiënten.
- Immunosuppressieve eigenschappen van de tumor die T cel activiteit verminderen. Er zijn op dit moment meerdere nieuwe strategieën ontwikkeld om de tumor omgeving meer toegankelijk te maken voor T cellen. Toekomstige klinische trials moeten aantonen of dit ook daadwerkelijk leidt tot betere klinische responsen.

In **hoofdstuk 5** wordt een overzicht gepresenteerd van de immuunmonitoring die is gedaan in andere studies met klinische TIL en CAR/TCR T cel therapie. Dit hoofdstuk is gepubliceerd in 2015 en refereert alleen studies tot dat moment. Interessant is dat momenteel (2018) het onderwerp van immuunmonitoring en de belangrijkste punten in dit hoofdstuk nog steeds erg actueel zijn aangezien goede predictoren voor adoptieve T cel therapie nog steeds ontbreken. Met name voor solide tumoren is er nog weinig data (18 gepubliceerde studies), maar in deze studies is er maar beperkt gekeken naar de immunologische veranderingen in patiënten gedurende de therapie of de eigenschappen van het T cel product; iets dat wij juist willen aanraden om de kans op het vinden van gewenste predictoren te vergroten.

DEEL 2: T CEL MONITORING IN NIET- T CEL THERAPIE

Wat betreft T cel monitoring in niet-T cel therapieën, is als eerste gekeken in patiënten behandeld met DC vaccinatie therapie, en ten tweede is gekeken in onbehandelde sarcoom subtypen.

T cel monitoring in dendritische cel vaccinatie

Hoofdstuk 6 beschrijft een studie waarin getracht is een immuunreactie te meten in het bloed van patiënten met mesothelioom die zijn behandeld met DC vaccinatie. De patiënten in deze studie werden behandeld met autologe DCs die zijn opgekweekt uit monocyten en vervolgens zijn beladen met een lysaat gemaakt uit mesothelioom tumor cellijnen. De therapie werd goed verdragen en in één van de negen behandelde patiënten werd duidelijke een klinische respons gezien, met 70% tumorreductie 6 weken na start behandeling, en deze reductie hield 2 jaar aan.

In het bloed werd immuun activatie gemeten na toediening van het DC vaccin in de vorm van een toename van het aantal B cellen, en CD4 en CD8 T cellen. Vervolgens is onderzocht of er in het bloed van deze patiënten T cellen konden worden aangetoond gericht tegen

mesotheline, een veelvoorkomend antigeen in het tumorlysaat, en of deze T cellen in aantal toenamen door de DC therapie. Er werd echter geen verandering gezien in het aantal mesotheline-specifieke T cellen vóór en ná behandeling. Een effectieve T cel reactie kan leiden tot uitgroei van bepaalde T cel klonen, waardoor er een verschuiving kan optreden van een polyklonaal naar een oligoklonaal repertoire van TCRs. Analyse van de totale TCR diversiteit in het bloed toonde echter evenmin veranderingen vóór en ná behandeling. De bevindingen in **hoofdstuk 6** zijn suggestief voor een brede cellulaire immuunreactie. Wat betreft CD4+ T cellen in het bloed zagen we ná DC therapie een vermeerdering van de fractie T cellen positief voor activatie merkers. Het aantal patiënten in deze studie is te klein om deze data ook aan klinische uitkomsten te correleren. Desondanks zijn de gevonden activatiemerkers interessant om in toekomstige studies in grotere patiënt cohorten te analyseren, en te correleren met klinische parameters.

T cel en tumor monitoring in sarcomen

In **hoofdstuk 7** van dit proefschrift zijn de CD8 TILs en de eigenschappen van de micro- omgeving van verschillende sarcoom subtypes in kaart gebracht. Het doel van deze studie was om meer inzicht te krijgen in de aanwezigheid en staat van immuuncellen in de tumor en hoe de tumor micro-omgeving hier invloed op heeft. Sarcomen zijn een zeer diverse groep van tumoren, en de eerste studies met immuun checkpoint therapie en adoptieve T cel therapie hebben laten zien dat sommige sarcoom subtypes mogelijk gevoelig en sommige subtypes niet gevoelig zijn voor deze therapieën. Ons onderzoek geeft mogelijk inzicht in welke immuuntherapieën effectief kunnen zijn voor de verschillende sarcoom subtypes.

Hoofdstuk 7 beschrijft dat er duidelijke verschillen zijn in T cel aantallen, en hun activatie- en maturatie status, tussen vijf onderzochte sarcoom subtypen. Gastro-intestinale stromale tumor (GIST), myxofibrosarcoom, en pleomorf sarcoom hebben de hoogste aantallen T cellen in de tumor. Interessant is dat van deze drie subtypes, GIST een veel lagere fractie geactiveerde en mature cellen heeft, en een meer polyklonaal T cel repertoire. Deze waarnemingen zijn erg suggestief voor minder antigeen-gedreven reacties van T cellen in GIST. De sarcoom subtypen zijn verder onderzocht op aanwezigheid van (i) antigeen presenterende moleculen, (ii) het aantal DNA mutaties, (iii) remmende immuuncellen en (iv) remmende of stimulerende liganden. Er werden tussen de sarcoom subtypes geen verschillen gevonden in de expressie van antigeen presenterende moleculen. Wel werden in myxofibrosarcoom en pleomorf sarcoom een hoger aantal DNA mutaties gevonden ten opzichte van het leiomyosarcoom en liposarcoom (een aanwijzing voor verhoogde immunogeniciteit). GIST had relatief de laagste aantallen met immuunsuppresieve cellen. De aanwezigheid van de remmende moleculen was lager in GIST dan in alle andere sarcomen. Ook was de aanwezigheid van stimulerende moleculen het laagst in GIST, en was deze juist hoger in het myxofibrosarcoom en het pleomorf sarcoom. Op basis van deze bevindingen zouden het myxofibrosarcoom en pleomorf sarcoom het meest in aanmerking komen voor immuun checkpoint therapie. Sarcoom subtypes die minder immunogeen zijn zouden mogelijk eerst moeten worden voorbehandeld

(bijvoorbeeld met radio- of chemotherapie) om tumoren gevoeliger te maken voor checkpoint therapie. Daarentegen zou GIST mogelijk baat hebben bij een benadering waarin de immuun-stimulerende moleculen worden geactiveerd.

CONCLUSIE

Er worden de laatste jaren veel klinische studies uitgevoerd met (nieuwe) immuuntherapieën in verschillende tumorsoorten. Dit wordt vaak gedaan terwijl er tevoren onvoldoende informatie is verzameld over de immunologische kenmerken van de tumor die een patiënt heeft. Meerdere studies hebben ondertussen aangetoond dat de tumor micro-omgeving mede bepalend is voor het al dan niet aanslaan van immuuntherapie. Daarom is het essentieel om in nieuwe (klinische) studie protocollen op de juiste wijze de tumorkarakteristieken, maar ook de eigenschappen van immuuncellen in het bloed in kaart te brengen. Bij voorkeur vóór, tijdens en indien mogelijk ná behandeling. Deze informatie kan vervolgens weer leiden tot identificatie van predictoren voor therapie succes.

Met het pallet aan beschikbare technieken, zoals multiplex flowcytometrie, multiplex in situ immunohistochemie en Next Generation DNA/RNA-sequencing (die steeds betaalbaarder en toegankelijker worden) zou immunologische analyse van tumor en bloed standaard moeten worden opgenomen in klinisch kankeronderzoek.

About the author

Yarne Klaver was born in Zeist, the Netherlands on the 20th of April, 1989. He attended the Herman Jordan Lyceum where he graduated in 2007. Subsequently he started studying medicine at Maastricht University and obtained his Bachelor of Science degree in 2011. It was already during the first years of his bachelor when Yarne became fascinated by the complexities and intricacies of oncology and immunology. It was therefore that in his last year of medical school, he did his scientific internship at the Experimental Immunology department (Exim) at the AMC in the group of Prof. dr. Eric Eldering. His research (under supervision of dr. E. Slinger) aimed to determine the effects of different Toll-like receptors on cell viability and proliferation in chronic lymphocytic leukemia. After finishing this scientific internship Yarne graduated and obtained his Master of Science degree in 2014 at Maastricht University.

In 2014 he started working at the Tumor Immunology lab supervised by dr. Cor HJ Lamers (co-promotor), and dr. Reno Debets, at the Department of Medical Oncology, chaired by Prof. dr. Stefan Sleijfer (promotor) at the Erasmus Medical Centre in Rotterdam. Throughout his PhD study, Yarne's work revolved around immune monitoring in different cancer (immuno) therapies and analysis of the immune microenvironment in different cancers. Most of the results of his work are described in this thesis. From 2018 onwards, Yarne started working as an ANIOS in Internal Medicine at the Elisabeth TweeSteden Ziekenhuis (ETZ) hospital in Tilburg. In January 2019 he was allowed to start his residency in Internal Medicine at the ETZ in Tilburg under the supervision of dr. M. van Kasteren (ETZ) and dr. A. Zandbergen (Erasmus MC Rotterdam). In the future, he hopes to be able to bridge the results found in the laboratory to the clinic in order to improve patient care.

PHD - Portfolio

1. PhD training

	YEAR	ECTS
GENERAL ACADEMIC SKILLS		
Integrity course	*2016*	*0,3*
IN-DEPTH COURSES (E.G. RESEARCH SCHOOL, MEDICAL TRAINING)		
Molecular Immunology course	*2016*	*3*
POSTER PRESENTATIONS		
Dutch Society for Immunology, Noordwijkerhout	*2015*	*0,5*
Molecular Medicine Day, Rotterdam	*2015/2017*	*1*
CIMT meeting 10-12 May, Mainz, Germany	*2016*	*0,5*
CRI-CIMT-EATI-AACR meeting 6 – 9 sept. 2017, Mainz, Germany	*2017*	*0,5*
ORAL PRESENTATIONS		
Tumor Immunology Platform (TIP) Rotterdam EMC	*2015-2017*	*3*
Josephine Nefkens Institute Scientific Research Meeting	*2015-2017*	*3*
Dutch Tumor Immunology meeting Breukelen	*2015*	*1*
T cell consortium (TCC), Rotterdam	*2015-2017*	*3*
Medical oncology meeting	*2016-2017*	*0,6*
European conference of immunology	*2018*	*0,3*
CONFERENCES		
Dutch Society for Immunology:Kaatsheuvel	*2014*	*1*
Dutch Society for Immunology Noordwijkerhout	*2015*	*1*
Molecular Medicine Day, Rotterdam	*2015-2017*	*1*
Dutch Tumor Immunology meeting, Breukelen	*2015/2017*	*1*
CIMT meeting 10-12 May 2016, Mainz, Germany	*2016*	*1*
CIMT meeting 6-9 September 2017, Mainz, Germany	*2017*	*1*

SEMINARS AND WORKSHOPS		
Course: Basic and Translational oncology	*2015*	*1,8*
Course: Basic Introduction Course on SPSS	*2015*	*0,5*
Course: R	*2016*	*1*
Course: Survival analysis	*2017*	*0,5*

OTHER MEETINGS		
T cell consortium (TCC) meetings (~6x yearly), Rotterdam	*2015-2017*	*1*
JNI scientific research meetings (~36x yearly), Rotterdam	*2015-2017*	*4,5*
Tumor Immunology Platform (TIP) (~36x yearly), Rotterdam	*2015-2017*	*4,5*
Medical oncology meeting	*2016-2017*	*1*

2. Teaching activities

SUPERVISING PRACTICAL'S AND EXCURSIONS		
Minor Oncology (Medicine Erasmus MC)	*2015*	*0,5*
Junior med School	*2017*	*0,3*
Tutor Bachelor Medicine (Year 1)	*2017*	*1,5*

SUPERVISING THESES		
Maud Rijnders 2015-2016; MolMed Master student	*2015-2016*	*3*

	Total ECTS	*42,8*

A

List of Publications

» **Adoptive T-cell therapy: a need for standard immune monitoring.**
Klaver Y, Kunert A, Sleijfer S, Debets R, Lamers CH;
Published in: Immunotherapy. 2015;7(5):513-33. doi: 10.2217/imt.15.23. Review.

» **Treatment of metastatic renal cell carcinoma (mRCC) with CAIX CAR-engineered T-cells-a completed study overview.**
Lamers CH, ***Klaver Y***, Gratama JW, Sleijfer S, Debets R.
Published in: Biochem Soc Trans. 2016 Jun 15;44(3):951-9. doi: 10.1042/BST20160037. Review.

» **Plasma IFN-γ and IL-6 levels correlate with peripheral T-cell numbers but not toxicity in RCC patients treated with CAR T-cells.**
Klaver Y, van Steenbergen SC, Sleijfer S, Debets R, Lamers CH.
Published in: Clin Immunol. 2016 Jul 1;169:107-113. doi: 10.1016/j.clim.2016.06.014.

» **T Cell Maturation Stage Prior to and During GMP Processing Informs on CAR T Cell Expansion in Patients.**
Klaver Y, van Steenbergen SC, Sleijfer S, Debets R, Lamers CH.
Published in: Front Immunol. 2016 Dec 26;7:648. doi: 10.3389/fimmu.2016.00648.

» **The Systemic- Immune- Inflammation Index Independently Predicts Survival and Recurrence in Resectable Pancreatic Cancer and its Prognostic Value Depends on Bilirubin Levels: A Retrospective Multicenter Cohort Study.**
Aziz MH, Sideras K, Aziz NA, Mauff K, Haen R, Roos D, Saida L, Suker M, van der Harst E, Mieog JS, Bonsing BA, ***Klaver Y***, Koerkamp BG, van Eijck CH.
Published in: Ann Surg. 2018 Jan 12. doi: 10.1097/SLA.0000000000002660.

» **Autologous dendritic cell therapy in mesothelioma patients enhances frequencies of peripheral CD4 T cells expressing HLA-DR, PD-1 or ICOS**
Klaver Y, de Goeje PL, Lambers ME H., Langerak AW, Kunert A, Lamers CHJ, Aerts JGJV., Debets R and Hendriks RW
Published in: Front Immunol. 2018 Sep 7;9:2034. doi: 10.3389/fimmu.2018.02034.

Dankwoord

DANKWOORD

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Beste **Cor**, de eerste hoofdstukken van dit boekje waren niet mogelijk zonder al het werk dat jij en de mensen betrokken bij de *CAIX CAR trial* hebben verricht. Jij was dan ook voor de totstandkoming van deze hoofdstukken absoluut onmisbaar. Elke keer als we tijdens onze besprekingen met Reno tot het punt kwamen met de conclusie dat we bepaalde data miste, wist je wel weer een mapje of DVD’tje tevoorschijn te toveren met de benodigde data. We zijn zelfs in de krochten van de Daniël, in de stoffige archieven gedoken en uiteraard mét resultaat. Ook tussen de stapels papier op je bureau wist je feilloos papers te vinden die onze hypotheses konden ondersteunen en nog vaker ook weer onderuit konden halen. Dit heeft vooral in de laatste hoofdstukken erg geholpen. Misschien nog wel net zo belangrijk, ben je altijd een mijn “lab vader” geweest en heb je er in het begin voor gezorgd dat ik snel in het lab kon aarden. Zo nu een dan een geruststellende blik als de wanhoop blijkbaar weer van mijn gezicht droop, evenals de gesprekken die ik nodig had om soms iets te ventileren zal ik niet vergeten. Heel erg veel dank hiervoor.

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Dan zijn er binnen het Tumor Immunologie lab nog de mensen die het fundament van de groep vormen en ervoor zorgen dat het lab niet veranderd in een totale anarchie.

Ten eerste **Cor B.** Cor, jij bent binnen het lab een van de belangrijkste pijlers die ervoor zorgt dat het geen totale puinzooi wordt. Altijd sta je voor iedereen klaar en schiet je iedereen direct te hulp ook als dit betekent dat je zelf in de knoop komt met je andere taken. We hebben niet veel direct samengewerkt aan projecten, maar indirect hebben we heel veel werk verzet samen. Waar ik Pris had voor de voetbal, had ik jou voor álle andere onderwerpen waarover ik kon ouwehoeren en kon discussiëren. Tot mijn grote vreugd kon je je ook opwinden in deze discussies, waardoor dat soms een doel ***an sich*** werd voor mij. Het was een mooie symbiose. Dank voor de prettige samenwerking, de leuke pauzes, je duidelijke standpunten en het feit dat ik altijd op je kon terugvallen met dingen die speelden.

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