# Anti-cancer vaccination – Immunomonitoring of a clinical phase I/II study in prostate cancer patients vaccinated with a RhoC-derived synthetic peptide

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**Chapter 3I** Immunomonitoring – technical aspects

**Summary**

Vaccination with peptides derived from tumor-associated or -specific antigens is a form of immunotherapy that specifically induces or reactivates T cell immune response to fight cancer. The aim of the present work was to assess the immune responses in prostate cancer (PCa) patients upon peptide vaccination in a clinical phase I/II study. PCa patients were repeatedly vaccinated with a RhoC-derived synthetic long peptide (SLP, 20 amino acids), which potentially activates CD8 and CD4 cells, emulsified in Montanide ISA-51. An immune response was detected in 86% of the patients after vaccination, which lasts at least ten months after the last vaccination. Vaccine-specific T cells were mainly poly-functional CD4 cells of the effector memory T cell phenotype. In total, three promiscuously presented human leucocyte antigen (HLA)-class II peptides were identified. No exhausted T cell phenotype was observed after multiple vaccinations. For one patient, a vaccine-directed CD8 cell response restricted by HLA-B*27:05 was detected in addition to the CD4 cell response. In conclusion, the RhoC-derived peptide is immunogenic and induces a long-lasting immune response in the majority of patients.

The second part of the thesis deals with technical aspects that are important for the presented clinical study. First, the common methods for the monitoring of clinical studies are described in a review, besides a newly established method for the identification of antigen-specific CD8 T cells. Methods that are described in this review are used for the identification of antigen-specific CD8 and CD4 cells using SLPs, which protocol optimization is described afterwards. We showed that the addition of Poly-ICLC (Hiltonol®), a toll-like receptor 3 agonist, together with an increased SLP concentration, lead to an optimized identification of peptide-specific cells. The optimized protocol could be used for the monitoring of clinical studies, as well as for the identification of epitopes, for example.

**Zusammenfassung**

Die Vakzinierung mit Peptiden, die von tumorassoziierten oder -spezifischen Antigenen entstammen, welche eine Art der Immuntherapie darstellt, verfolgt das Ziel, eine spezifische, gegen den Tumor gerichtete Immunantwort zu induzieren, oder eine vorhandene Immunantwort zu verstärken. Das Ziel der Arbeit war, zu untersuchen, ob solch eine Aktivierung des Immunsystems im Rahmen einer klinischen Phase I/II Peptid-Vakzinierungs-Studie bei Prostatakarzinom (PCa)-Patienten vorliegt. Die Patienten wurden wiederholt mit einem der RhoC Zielstruktur entstammenden synthetischen langen Peptid (SLP, 20 Aminosäuren), welches potentiell CD8 und CD4 Zellen aktivieren kann, zusammen mit Montanide ISA-51 vakziniert. Eine Vakzine-spezifische Immunantwort konnte in 86% der Patienten nachgewiesen werden, welche mindestens noch zehn Monate nach der letzten Vakzinierung nachweisbar war. Die Vakzin-spezifischen Zellen waren überwiegend multifunktionale CD4 Zellen, die einen Effektor-Gedächtnis-T-Zellen Phänotypen aufwiesen. Insgesamt konnten drei promiskuitiv präsentierte humane Leukozytenantigen (HLA)-Klasse II Peptide nachgewiesen werden. Die aktivierten CD4 Zellen zeigten auch nach wiederholten Vakzinierungen keinen Phänotypen der Exhaustion. Eine zusätzliche Vakzin-spezifische CD8 Zell Antwort, die durch HLA-B*27:05 restringiert war, konnte bei einem Patienten nachgewiesen werden. Die Untersuchung zeigt, dass das RhoC-entstammende Vakzin-Peptid immunogen ist und eine langanhaltende Immunantwort in einer Vielzahl von Patienten hervorruft.

Der zweite Teil der Arbeit befasst sich mit den technischen Aspekten die zur Durchführung der hier präsentierten klinischen Studie wichtig sind. Zuerst werden in einem *Review* die gebräuchlichsten Methoden zum Nachweis von Antigen-spezifischen T Zellen in der experimentellen Immuntherapie, neben einer neu etablierten Methode zur Identifizierung von Antigen-spezifischen CD8 Zellen, erläutert. Methoden, die mitunter beschrieben werden, werden in der Folge für den Nachweis von Antigen-spezifischen CD8 und CD4 Zellen mit Hilfe von SLPs eingesetzt, dessen Protokolloptimierung danach beschrieben wird. Wir zeigen, dass der Einsatz von Poly-ICLC (Hiltonol®), ein Toll-ähnlicher Rezeptor 3 Agonist, sowie eine erhöhte SLP Konzentration führte zu einem verbesserten Nachweis von Peptid-spezifischen Zellen. Das optimierte Protokoll findet sein Einsatzgebiet nicht nur im *Monitoring* von klinischen Studien, sondern zum Beispiel auch in der Identifizierung von Epitopen.

# Chapter 1I Introduction into prostate cancer, basic immunology, and anti-cancer immunotherapy with focus on prostate cancer

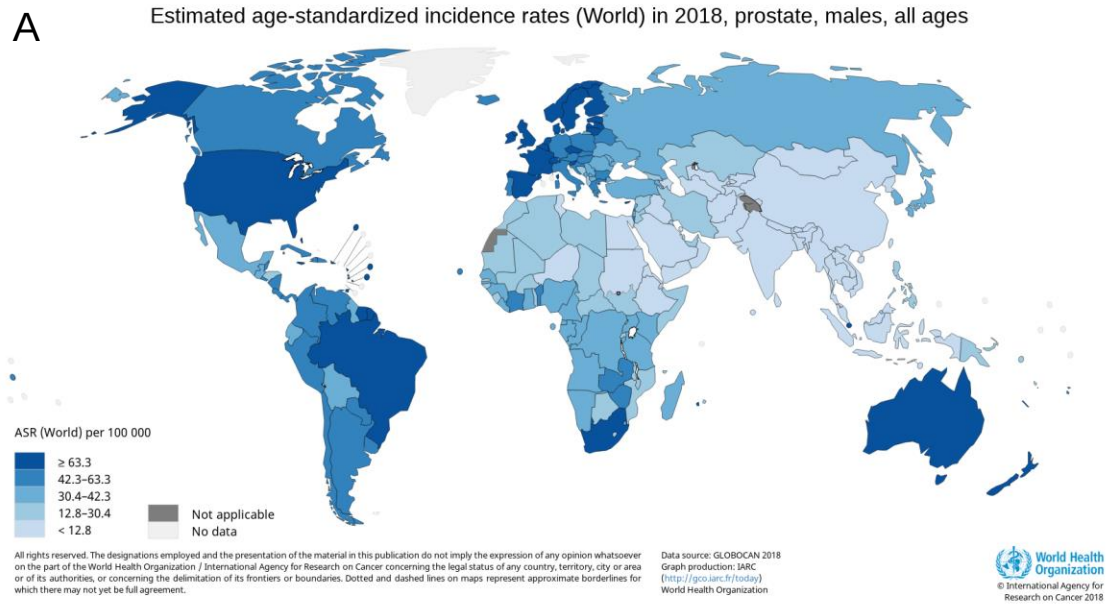
## 1.1 Prostate cancer

Function of the healthy prostate

The prostate gland is located below the bladder and is part of the male reproductive system. Naturally, it secretes prostatic fluid which is part of the ejaculate. The fluid contains different factors that contribute to the ejaculation process and quality of the semen, and therewith to male fertility. One of these factors is the prostate-specific antigen (PSA), a family member of the kallikrein-related peptidases, specifically secreted by epithelial cells of the prostate to thinner the semen and allow its release [1].

Epidemiology

The first case of prostate cancer (PCa) was published by John Adams, a surgeon from the London Hospital in 1853 [2]. It was initially described as “a very rare disease” but, nearly 170 years later, PCa is now the most common cancer in men in Germany and Denmark and the second most common cancer worldwide [3–6]. The GLOBOCAN 2018 database allows the comparison of the estimated incidence and mortality rates for 36 different cancer types, including PCa, in 185 different countries [7]. The estimated age-standardized incidence (A) and mortality (B) rates for PCa worldwide are shown in Figure 1.



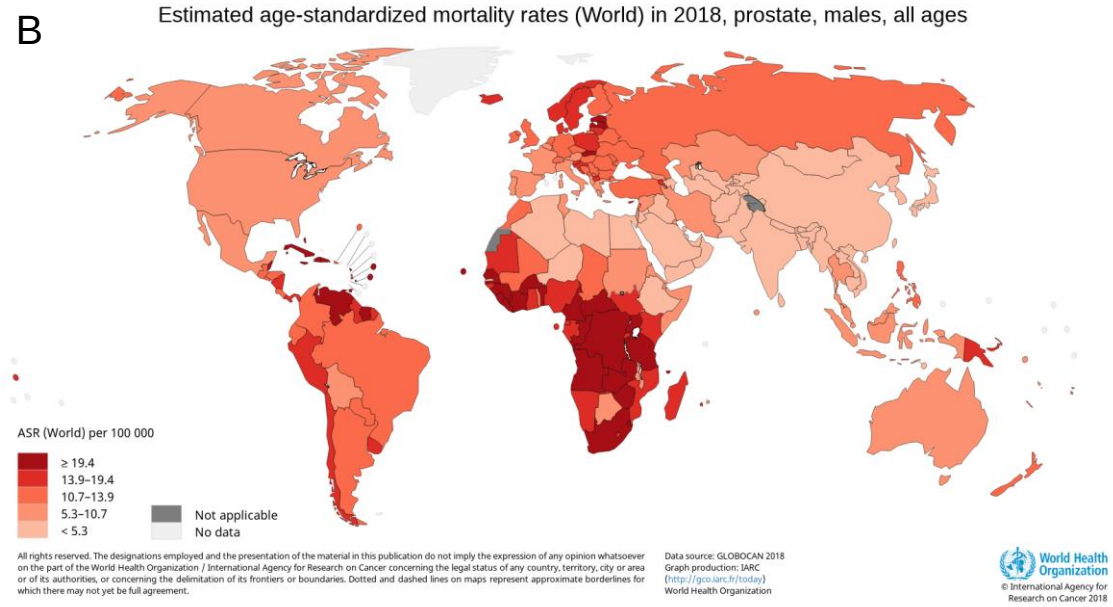



Figure 1: Worldwide estimated age-standardized incidence (A) and mortality (B) rates for prostate cancer among men of all ages. Created with [4].

The incidence rate varies a lot across the world but is highest for regions with a very high human development index and a high income [4,8]. Oceania, North America, and Europe show the highest incidence rates with 79.1, 73.7, and 62.1 cases per 100,000 individuals, respectively, whereas Africa and Asia show the lowest (26.6 and 11.4 per 100,000 individuals, respectively). The high incidence rates in developed countries are most likely attributable to the increased PSA testing [9,10], since PSA in the blood serum was introduced as biomarker for PCa in the late 1980s´ [11,12]. PSA will be discussed in details in section 1.1.1. The mortality rate varies to less extent than the incidence rate (48-fold range; Barbados 48.0 per 100,000 to Nepal 0.8 per 100,000 individuals) with highest for regions in Africa, Latin America, and the Caribbean [4].

The relative 5- and 10-year survival rates for PCa are 89% and 88% in Germany, respectively [5]. Assessment of the exact survival rates for PCa is nearly impossible as many factors contribute to the course of disease and different numbers are published. Clearly shown is that the survival rate dramatically drops when distant metastases occur. The difference in survival rate from the local disease to disease with distant metastases is reported from the American Cancer Society from nearly 100% to 31% for the 5-year survival rate [13] or from 89% to 6% for the 15-year survival rate in a population-based study performed in Sweden [14].

Etiology

The underlying mechanism of PCa initiation and progression is complex involving many different steps [15,16]. Different risk factors are associated with PCa formation. The risk for PCa has been shown to rise with age. Whereas men of age 35 have a risk of 0.1% to suffer from PCa in the next ten years, men of age 75 have a 5% risk [5]. Among others, ethnicity, nutrition, hormone levels, and shift work are risk factors for PCa [17,18], as well as the family history which is an important factor for PCa initiation [19]. The risk for PCa correlates with the degree of kinship, with a higher risk if a brother is affected versus the father [20,21]. In addition, PCa risk has been shown to increase with tumor stage of the brother, the onset age of the relative, and a stronger family history, e.g. if two brothers are affected compared to only one brother [22–24]. Interestingly, there is also a relation between the family history of breast cancer and an increased risk for PCa [21,24,25].

Adenocarcinoma of the prostate gland

Tumors of the prostate are classified by the World Health Organization (WHO) into epithelial, neuroendocrine, mesenchymal, hematolymphoid, and miscellaneous main tumor types, with various subtypes [26]. Beside other PCa tumor types like sarcomas (about 0.7% of PCa) [27] or the aggressive small cell carcinoma (0.5-2% of PCa) [28], the adenocarcinoma is the most frequent (>95%) PCa tumor type [29,30]. The clinical anti-cancer vaccine study which is later presented (Section 2) focused on PCa patients with adenocarcinoma.

### 1.1.1 Diagnosis and staging

The statutory medical checkup program for PCa in Germany is offered to men above age 45. It includes the physical examination of the genitals, the palpate of the lymph nodes, and the digital rectal examination (DRE) of the prostate, but does not cover the PSA test as an early-detection system [5]. If there is evidence for PCa, the DRE, PSA value, transrectal ultrasound (TRUS) guided biopsy and possible the multiparametric magnetic resonance imaging (mpMRI) are considered for primary diagnosis [31–33]. Is a man diagnosed with PCa, the so-called tumor staging takes place. The four main PCa stages are shown in Figure 2.

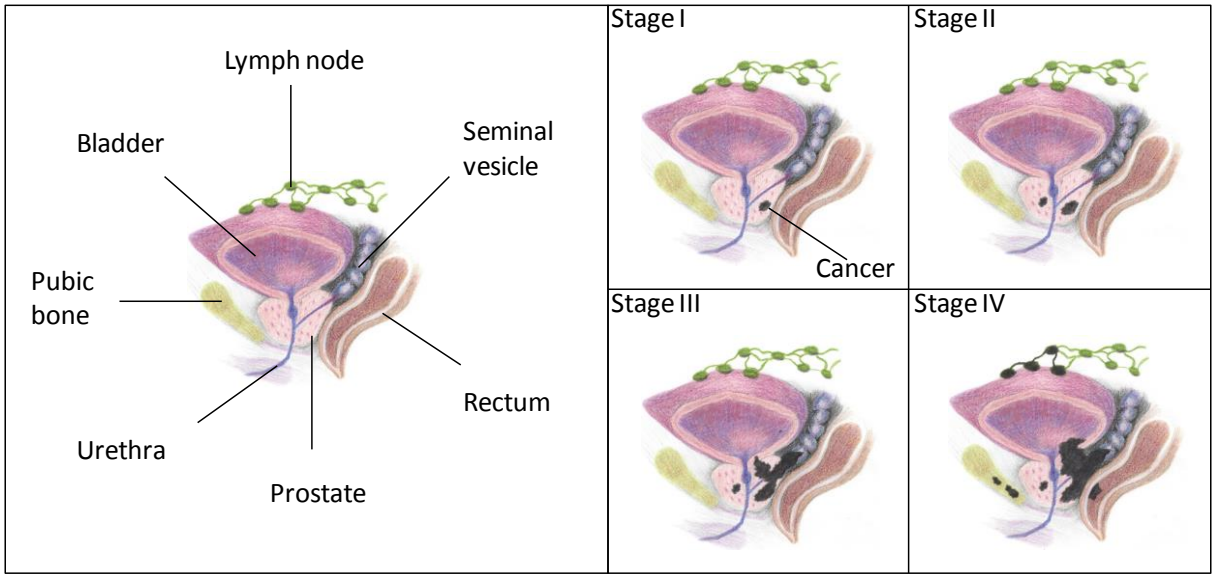



Figure 2: Main stages of prostate cancer. Shown are the healthy prostate and nearby organs (left panel) and the four main stages (I-IV) of prostate cancer (right panels). Graphics: T. Schuhmacher.

In stages I and II, the tumor foci is found locally either in one or both lobes of the prostate, whereas in stage III, the tumor starts to extend to nearby organs, like the seminal vesicle. Stage IV PCa describes the stage when the tumor invades further nearby organs like the rectum or bladder and possibly starts to spread to distant organs. Beside those four main stages, PCa is divided into further sub-stages [31,34]. The whole staging system is based on three main classifications: i) the Tumor Node Metastasis (TNM) classification, ii) the grade groups based on the Gleason score, and iii) the classification into risk groups.

Prostate specific antigen

PSA in men is predominantly and abundantly expressed by epithelial cells of the prostate [1,35,36]. It is considered as an organ- and not a PCa-specific marker, and levels can also be elevated due to non-cancerous causes [37–39]. The impact of the PSA-test as early-biomarker is controversially discussed and the benefit for patients is not clearly demonstrated [40,41]. Studies indicate that approx. 23% to 44% of the PSA positive tested PCa cases are over-diagnosed, meaning that the PCa would not have been diagnosed during the patient's lifetime without PSA testing [42,43]. Depending on the PSA-cut-off value, false-positive PSA test results (PSA value is elevated, but not due to PCa) occur for 4-19% of the screened individuals [40]. An over-diagnosis or false-positive PSA testing are accompanied by overtreatment and/or further unnecessary diagnosis options, which could cause wide-ranging complications for the patient [40]. Likewise, more high-grade PCa and metastatic

disease are observed at diagnosis, which is presumed to the low acceptance of the early PSA detection [44,45]. These discrepancies clearly show the need for more specific biomarkers to identify PCa at early stage. A promising candidate is the prostate cancer gene 3 (PCA3) [46,47], which has been shown to be 66-fold increased in PCa compared to benign tissue [48]. The PCA3 mRNA is measured in the urine of patients after DRE and normalized to the PSA mRNA result in the same sample [49]. The test was approved by the Food and Drug Administration (FDA) in 2012 [50] and is also available to the German/Danish patients [51]. Nevertheless, more sensitive biomarkers like molecular biomarkers are currently under investigation [52].

Although the PSA-test as early screening marker for PCa is controversially discussed, it is performed if requested by the patient and it is still a diagnostic marker [31,33]. The early-detection by single DRE is insufficient as its sensitivity and positive predicted value is low [33,53]. The PSA measurement is more sensitive than the DRE in detecting PCa and the combination of both methods could even improve the sensitivity and is recommended for early diagnosis [33,54–56]. Various forms of PSA exist in the blood and are measured to predict PCa, but will be not discussed further [57]. Usually, the total PSA value is measured in the blood serum (measurement depends on the machine calibration method) [33]. A biopsy is considered if the PSA value is ≥ 4 ng/ml [33]. Two kinetics of PSA are clinically determined: the PSA velocity (PSAV) and the PSA doubling time (PSADT). The PSAV addresses the absolute PSA per year, whereas the PSADT measures the exponential increase over time [31]. The calculation of PSADT and PSAV results in a higher predictive value than PSA alone, but is controversially discussed for untreated PCa patients [58–60]. The PSA value is used for the grouping into risk groups and the most important factor to screen for tumor recurrence (see also “risk groups” and 1.1.2.2).

Tumor Node Metastasis classification

The TNM classification describes the extent of primary tumor based on the DRE (T), the extent to nearby lymph nodes (N), and the distance spread to other sites (M). For the TNM classification, the current version of the UICC TNM classification should be used [31,33].

Table 1: TNM classification [31].

| | |
|---|---|
| T – Primary tumor (stage based on DRE only) | |
| TX | Primary tumor cannot be assessed |
| T0 | No evidence of primary tumor |
| T1 | Clinically inapparent tumor that is not palpable |
| T1a | Tumor incidental histological finding in 5% or less of tissue resected |
| T1b | Tumor incidental histological finding in more than 5% or less of tissue resected |
| T1c | Tumor identified by needel biopsy (e.g. because of elevated PSA) |
| T2 | Tumor that is palpable and confident within the prostate |
| T2a | Tumor involves one half of the lobes or less |
| T2b | Tumor involves more than half of one lobe, but not both lobes |
| T2c | Tumor involves both lobes |
| T3 | Tumor extends through the prostate capsula |
| T3a | Extracapsular extension (unilateral or bilateral) |
| T3b | Tumor invades seminal vesicle(s) |
| T4 | Tumor is fixed or invades adjescenct structures other than seminal vesicle: external sphinter, rectum, levator mucles, and/or pelvic wall |
| N – Regional (pelvic) lymph nodes | |
| NX | Regional lymph nodes cannot be assessed |
| N0 | No regional lymph node metastasis |
| N1 | Regional lymph node metastasis |
| M – Distance metastasis | |
| M0 | No distance metastasis |
| M1 | Distance metastasis |
| M1a | Non-regional lymph nodes(s) |
| M1b | Bone(s) |
| M1c | Other site(s) |

Gleason score and grade groups

The Gleason score is dominantly used as grading system for PCa. Multiple distinct tumor foci are typically observed at time of diagnosis in this highly heterogeneous disease [61]. Depending on the tumor size, at least eight (~30 cubic centimeter (cc)) or ten - twelve (>30 cc) systematic biopsies are recommended [31,61], followed by histological examination of the prostate biopsies. The most common and second most common glandular patterns are separately graded in accordance with a well-defined grading schematic. The grade groups range from one to five, with five describing the most advanced stage of PCa. The sum of the two numbers for the most and second most common patterns results in the Gleason score, which ranges from two to ten [62–65]. The International Society of Urological Pathology (ISUP) introduced a modified grading system for PCa in 2014 [62], which was accepted by the WHO in 2016 [63]. Histological examples of Gleason patterns, scores, and associated grade groups are shown in Figure 3.

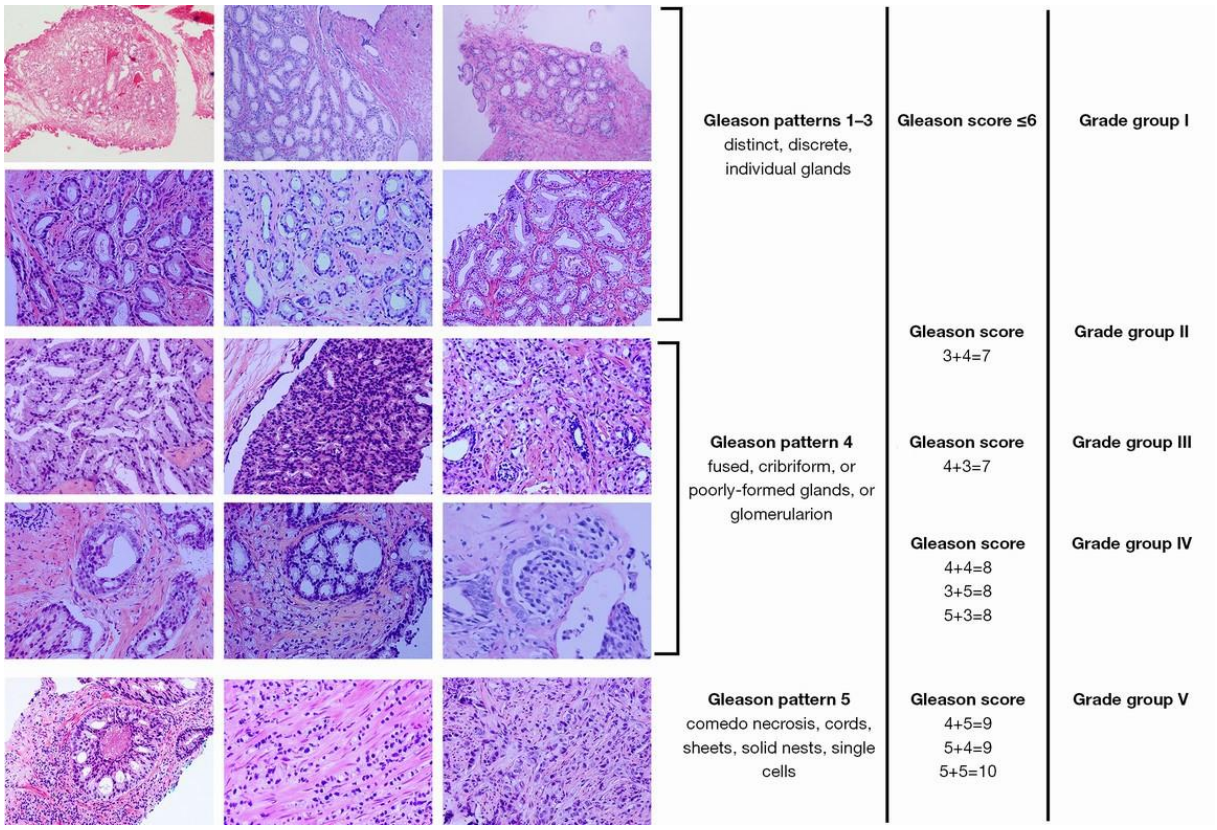



Figure 3: Gleason grading system and corresponding grade group system. Shown are typical hematoxylin and eosin stainings (100x-200x) of prostate adenocarcinoma tissues corresponding to the Gleason pattern, Gleason score, and grade group. Used with permission from AME Publishing Company [66].

## Risk groups

The localized PCa either shows a very mild course of disease without any symptoms for the patient, or a very aggressive form. It is further classified according to the probability of relapse after local therapy based on the PSA-value, Gleason score, and the T-category of the TNM classification of the primary tumor, which is summarized in Table 2 [33].

Table 2: Risk groups of prostate cancer based on the PSA-level, Gleason score, and T-category [33].

| Risk group | PSA [ng/ml] | Gleason score | T-category |
|---|---|---|---|
| Low-risk | ≤ 10 | 6 | T1c, T2a |
| Intermediate-risk | > 10-20 | 7 | T2b |
| High-risk | > 20 | ≥ 8 | T2c |

## 1.1.2 Standard therapies and recurrence

Different therapy options are available. Which therapy strategy is used depends on different factors, like the patient's age or expected lifespan at diagnosis. Guidelines for therapy management are available [33,67].

### 1.1.2.1 Main therapies for localized prostate cancer

90% of the PCa are diagnosed at a local stage [68]. The main therapy options are the active surveillance, watchful waiting, radical prostatectomy (RP), and radiotherapy.

Active surveillance and watchful waiting

Both wait-and-see approaches are used to prevent overtreatment of the patients. Whereas the active surveillance follows a strict time plan for follow-up and is based on a curative approach, the watchful waiting strategy is a palliative symptom guided therapy for frail patients. Watchful waiting is considered if the cancer-independent life span expectation of the patient is less than ten years. Active surveillance is considered for patients with low-risk PCa [33,67]. Metastasis occurrence and cancer progression are shown to be higher for monitored patients compared to patients undergoing RP or radiotherapy [69,70].

Radical prostatectomy

RP is a primary therapy method offered to patients with a localized PCa of all risk-groups. Following methods of surgery are used for RP: retropubic, perineal, laparoscopic, and robotic-assisted laparoscopic. Due to the missing randomized control trials, there is no clear preference for the surgery method regarding the oncologic outcome [33]. If the estimated risk for a pelvic lymph node metastasis is higher than 5%, a nodal removal is considered in addition to RP [67]. Men with low-risk PCa and a high expected life span (mean age 65) benefit from RP [71], as well as men with age ≤ 55 (98,8% 10-year survival rate) [72]. Nevertheless, the quality of life can be worsened by RP as urinary and sexual function problems could occur [73]. High-risk PCa patients may also benefit from RP, although the biochemical recurrence (BCR)-free survival (described in section 1.1.2.2) has been shown to depend on the number of simultaneously occurring risk factors like Gleason score, baseline PSA, or seminal vesicle invasion [74].

Radiotherapy

External beam radiation therapy is also offered to patients with localized PCa of all risk groups like RP. Dose escalation studies show an impact of 74 – 80 Gy on the BCR and are used for treatment [75–77]. Radiotherapy is offered for low-risk patients without androgen deprivation therapy (ADT, discussed in details in section 1.1.2.3), for intermediate-risk patients with short-term ADT (4-6 months), and for high-risk patients with long-term ADT (2-3 years) [67]. The ProtecT trial did not show any significant difference between the RP and radiotherapy group after ten years follow up. Of note, the additional ADT treatment was inconsistent between the two groups [69].

### 1.1.2.2 PSA relapse after first-line treatment of localized prostate cancer

BCR describes the PSA increase after first-line curative treatment and is used for the follow-up of patients. About 30% of patients treated in first-line with RP develop a BCR within ten years post-surgery [78,79]. BCR is defined by at least two successive measured PSA values: above >0.2 ng/ml after RP or >2 ng/ml after radiotherapy [33,80,81]. A single PSA value of ≥ 0.4 ng/ml after RP was shown to be a threshold for subsequent rise in the PSA and a strong predictor for future metastasis [82]. The PSADT is also an important factor after RP, as the metastasis-free survival is increased when PSADT is ≥10 months [83]. After PSA recurrence, the treatment options are limited. Main standard options are salvage radiotherapy after RP (min 66 Gy) or salvage RP after radiotherapy [33,80]. In case of local recurrence, the salvage radiotherapy after RP could improve the cancer-specific survival compared to no therapy after BCR [84]. The addition of hormone therapy, although no standard treatment for PSA recurrence, to salvage radiotherapy, delays BCR and clinical progression, and improves overall survival rate [85,86]. Nevertheless, both treatments can have severe side effects of grade 2 or higher involving e.g. the gastrointestinal and genitourinary tracts, as well as the cardiovascular system, or have been associated with hot flush and sweating [85–87].

### 1.1.2.3 Treatment options for metastasized prostate cancer

Metastasized PCa can be observed either at primary diagnosis or occur after localized treatment and remains mostly incurable. Treatment options are hormone therapy and chemotherapy, which will be discussed shortly next, as well as the castration-resistant PCa (CRPCa).

Hormone therapy and chemotherapy

Already in 1941, the critical effect of androgens on the proliferation of PCa cells was described by Charles Huggins [88,89]. The ADT therapy in such hormone-sensitive tumors aims to reduce tumor burden by reducing the androgen level either by suppressing the androgen secretion or inhibiting the action of circulating androgens. Therefore, different therapeutic approaches are considered in PCa, including surgical castration, anti-androgens, or inhibitors which suppress androgen synthesis [31]. ADT is today an accepted initial treatment for the hormone-sensitive metastasized PCa [33]. The addition of the cytostatic agent Docetaxel to ADT further improves the median overall survival by 13.6 -17 months (total median overall survival 57.6 months). Nevertheless, patients receiving ADT and/or chemotherapy show severe side effects of grade 3 or higher, like fatigue, allergic reactions, neutropenia, cardiac or nervous system disorders [90,91]. Median time to develop CRPCa was shown to be 11.7 months and 20.2 months for single ADT or ADT plus Docetaxel, respectively [90].

Castration-resistant prostate cancer

The mechanism behind the development of CRPCa includes e.g. androgen receptor-dependent mechanisms, defects in DNA damage repair, and the involvement of cancer stem cells (CSCs, discussed in more detail in the next section 1.1.3) [92,93]. CRPCa shows a low serum testosterone level (<50 ng/dL or 1.7 nmol/L) and either biochemical and/or radiological progression [31]. More than 80% of patients with CRPCa will develop metastasis (mCRPCa) [94]. Therapy options at this stage are all limited, and cure is not expected for those patients, whether metastatic or not [33].

### 1.1.3 Micrometastases – The cause of prostate cancer recurrence?

The multi-step mechanism of metastasis formation termed as “invasion-metastasis cascade” involves the local invasion of the primary tumor, intravasation and survival in the circulation, arrest at distant organ sites and extravasation, initial survival in the microenvironment, micrometastasis (MM) formation, and metastatic colonization [95]. PCa recurrence, as already described beforehand, is associated with a rise of PSA serum level. PCa cells, which already disseminated into the bloodstream and/or other organs could be the cause for the renewed increase of blood PSA. Indeed, circulating prostate cells or MM are found in the blood, bone marrow, or pelvic lymph nodes [96–98]. Circulating PCa cells are associated with BCR [99–101], as well as

pelvic lymph node MM. However, MM in the pelvic lymph nodes do not explain all cases of BCR [97,102,103] indicating that also other organs are affected by MM infiltration. Since PCa has been shown to metastasize commonly to the pelvic lymph nodes and bones, but also to distance lymph nodes, bones, liver, lung, and brain [104,105], these sites could also exhibit MM.

CSCs are a small subset of self-renewing cells which are able to resemble the tumor from which they were derived [106–108] and might contribute to tumor recurrence and metastasis formation. A small fraction (0,1%) of self-renewal CSCs are found in human PCa tumors [108]. Data sets for human breast cancer cells and PCa indicate a stem-like transcriptional state for metastatic samples and a shared gene signature between metastatic cells and primary tumor [109,110], supporting that circulating tumor cells can consist of CSCs.

Detecting circulating tumor cells or MM can be used for tumor staging and therewith for therapy decision. Nevertheless, PCa cells and also MM are difficult to detect, as they are of small size. It has been shown that MM are easily missed by routine pathologists [97]. With new techniques, like the positron emission tomography (PET) of $^{68}$Ga-labelled prostate-specific membrane antigen (PSMA), a transmembrane protein overexpressed in PCa like PSA, the detection of small-volume metastases is more likely. However, the utility of the method is shown in the context of secondary staging after primary therapy followed by BCR. Per lesion it shows a sensitivity and specificity of 80% and 97%, respectively, but the detection of metastases depends on the PSA value and on the PSMA expression itself. For example, with a pre-PET PSA of <0.2 ng/ml, 42% of the PSMA PETs were positive among BCR patients [111].

In summary, circulating tumor cells and MM can lead to disease progression and should be eliminated, ideally before the establishment of metastatic disease. One promising possibility for the specific elimination of cancerous cells is to activate the patients´ own immune system to fight such cells.

## 1.2 Cancer immunotherapy

Cancer immunotherapy is not a new concept. Already 2600 before Christ, the Egyptian physician Imhotep treated cancer or as he called it “swellings” by inducing infections at the tumor site [112]. The first systematic study using the immune system to fight cancer is most likely attributable to the work of William B. Coley in the late 19$^{th}$ century. He treated more than 1000 cancer patients, mainly patients with inoperable soft tissue and bone sarcomas, with bacteria or bacterial products, later known as Coley´s toxins, in order to activate the immune system, and achieved excellent results as the patients´ tumors disappeared [113]. In 1909, Paul Ehrlich hypothesized the presence of a “safeguard” in the organism to prevent frequently occurring carcinomas [114]. The theory was later supported by L. Thomas and M. Burnet, who formulated it into the immune surveillance theory stating that newly arising tumors are recognized as “non-self” by the immune system and are eliminated before clinical establishment [115–117]. Approximately at the same time, the first cancer vaccine study was published in 1959 by the Graham couple. Gynecologic cancer patients with “unfavorable prognosis” were vaccinated with Freund’s adjuvant and tumor lysate and survival, clinical outcome, and “complications” were reported [118,119]. Since then, many efforts have been made to understand the mechanism of tumor development, the role of the immune system therein, and to harness the immune system as possible tool to treat cancer. Dunn and Schreiber published the concept of cancer immunoediting, which describes three phases: elimination, equilibrium, escape, as shown in Figure 4. The first phase of elimination is equal to the immune surveillance theory, whereby tumor cells get killed after recognition by the immune system. In the best case, the healthy state is restored. If tumor cells survive the immune attack, for example by the outgrowth of poorly immunogenic cancer cell clones, the second phase, the dynamic equilibrium, is starting, which might occur for many years. Tumor escape is mediated by several mechanisms aiming at avoiding immune recognition. For example, due to the selective pressure of the immune system in the equilibrium, new tumor cell variants can occur by e.g. genetic or epigenetic changes, which may result in resistance and survival of the immune attack. These immunologically “invisible” cells escape the immune control, start to proliferate in an uncontrolled manner, and give rise to a clinically malignant disease [120,121].

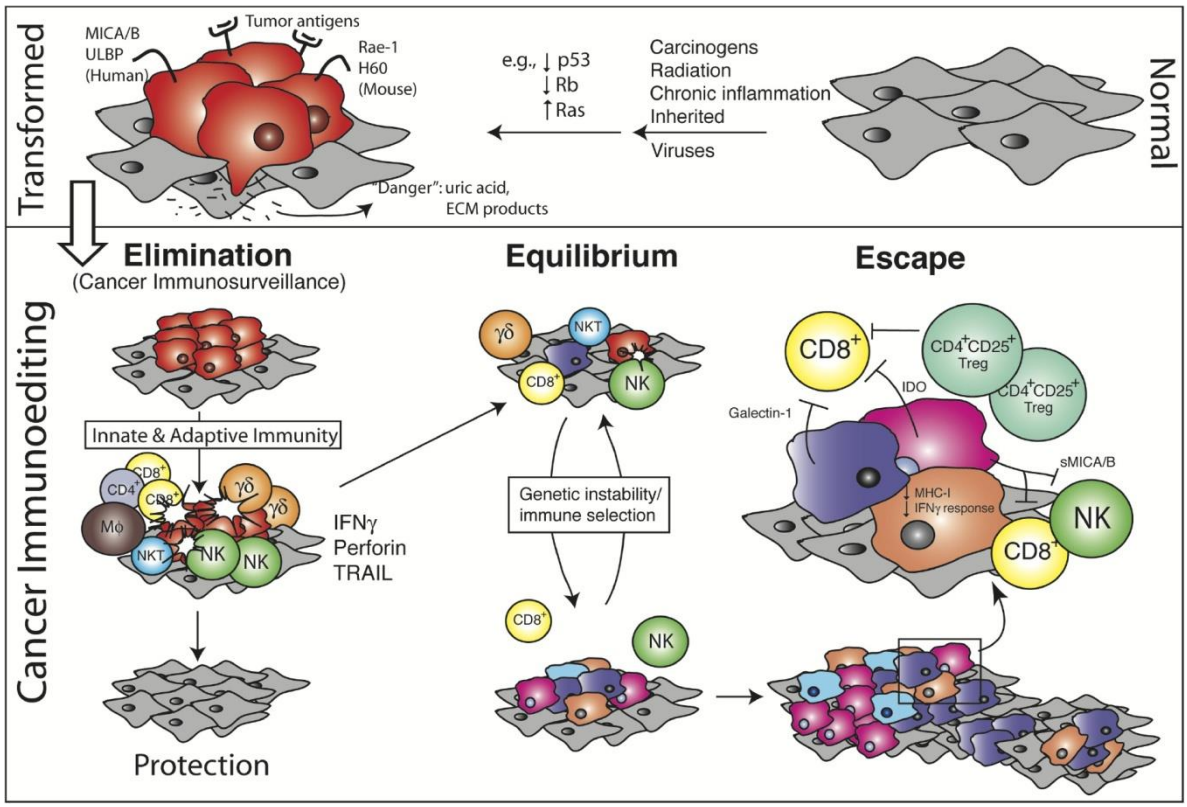



Figure 4: The concept of cancer immunoediting. When healthy cells (grey) undergo transformation and get malignant (red; top panel), they may express distinct tumor-specific markers, which can get recognized by immune cells as "danger" signal. In the first phase, the activated innate and adaptive immune cells will fight cancerous cells and may eradicate them. If this is not successful, tumor cells enter the second phase, the equilibrium. Here, the immune cells and cancer cells exist side by side. Due to selective immune pressure, new tumor variants might occur, evade the immune system, and give rise to a clinically malignant disease in the escape phase. Re-used with permission from Elsevier [120].

The escape from immune elimination was later added in the second version of "Hallmarks of cancer", first proposed in 2000 [122,123]. In the 20th century, major breakthroughs in cancer immunotherapy were achieved by using cellular therapies [124–126] or by targeting regulatory elements in the so-called checkpoint blockade immunotherapy (CBI) [127–129], which will be discussed in more detail in section 1.2.2. Although we learned a lot, there are still a number of open questions in the field of immunotherapy. Further research will help to understand the interplay between the immune system and cancer in more detail and allow the development of novel clinically effective immune therapies.

### 1.2.1 Cell war – The immune system in the battle against cancer

We have learned the basic knowledge about the immune system and its interplay between the innate and adaptive immune system from studies of infectious diseases,

where pathogens like bacteria or viruses invade the human body. This is also the fundament for immunological tumor research; immune cells fight not only pathogens but also tumor cells and invade solid tumors. There is strong evidence that the composition of tumor invading immune cells is important for the clinical outcome of the patient, as immune cells can not only fight cancerous cells but also support tumor growth. Important here are macrophages, natural killer (NK) cells, dendritic cells (DCs), B cells, and T cells, including cytotoxic T lymphocytes (CTLs), T helper (Th) cells (Th1, Th2, Th17) and T regulatory cells (Tregs) [130]. Myeloid-derived suppressor cells (MDSCs), which are supportive of tumor growth, also impact the clinical outcome for patients, however, are not in the focus of this thesis.

#### 1.2.1.1 The immune system – a highly complex cellular network simplified to basic knowledge

In this section, I will briefly discuss basic knowledge about the immune system upon initial pathogen infection, with focus on the above-mentioned cells and especially on T cells, as these cells are the main players of the later presented vaccine study (manuscript chapter 2).

Activation of the innate immune system and antigen presentation

The immune system consists of two arms, the innate and the adaptive immunities. Whereas cells of the innate immunity act in first line and very fast on a broad range of pathogens, are cells of the adaptive immune system more specific and act delayed. Macrophages and DCs are cells of the innate immune system and categorized as professional antigen-presenting cells (APCs). They are primarily found at places where pathogens could invade the organism, like the skin and the mucosa of solid organs. They recognize pathogens via interaction of pathogen-associated molecular pattern (PAMPs) e.g. lipopolysaccharides, dsRNA, CpG DNA, or danger-associated molecular patterns (DAMPs) produced by necrotic/stressed cells with so-called pattern-recognition receptors (PPRs), like Toll-like receptors (TLRs). The antigen uptake by APCs is mediated by different ways like the receptor-mediated phagocytosis or by macropinocytosis of soluble factors. After pathogen recognition and uptake, immune cells of the innate arm get activated and promote first local inflammation to reduce or prevent further spreading of the pathogen. For example, macrophages secrete cytokines like interleukin (IL) -1β, tumor-necrosis factor (TNF), and IL-12 to promote e.g. the permeabilization of the vascular endothelium and

facilitate entry of other immune cells and their activation. They further produce toxic products like nitric oxide (NO) to eliminate the engulfed pathogens in the phagolysosomes. If the infection cannot be cleared by cells of the innate immunity, cells of the adaptive immunity get activated and recruited. To this effect, pathogen-derived peptides are presented on the cell surface of the APCs to activate the cells of the adaptive immune system. APCs process extracellular antigens from e.g. invading bacteria in vesicles by proteases, which are activated upon pH reduction in the vesicle. The resulting 9-25 amino acids (aa) long peptides are loaded onto human leukocyte antigen (HLA, the human version of the major histocompatibility complex)-class II molecules and get transported on the cell surface. Antigens that are synthesized intracellularly upon e.g. viral infection are processed by the proteasome, transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP), loaded onto HLA-class I molecules, and transported to the cell surface. These peptides are generally 8-12 aa long. Beside these two classical ways of peptide processing and presentation mainly DCs are also able to process exogenous antigens either by the vacuolar or endosome-to-cytosolic way to present them on HLA class I molecules, a process called cross-presentation [131]. Each individual carries three major polymorphic genes for the two kinds of HLA molecules (HLA-A, -B, -C and HLA-DP, -DQ, -DR, respectively) among other minor classes, and each gene is expressed in at least two allelic variants. Peptides bind within the HLA-binding groove on defined positions with aa anchor motifs specific to an HLA-allotypic product. The sequence anchor motifs are well described for HLA-class I ligands but are less well defined for HLA-class II peptides. HLA-class II molecules are classically expressed by APCs, whereas HLA-class I molecules are expressed by all nucleated cells of the body.

Dendritic cells - The link between the innate and adaptive immunity

DCs are important for linking the innate and adaptive immunity. At least two different types of DCs are distinguished: plasmacytoid and classical myeloid DCs. Plasmacytoid DCs secret type I interferon (IFN), mostly upon viral infection, whereas classical DCs are directly involved in the activation of naïve T cells. Naïve T cells are immature T cells, which did not encounter their specific antigen yet, which is necessary for mediating T cell effector function. After antigen uptake, DCs get activated, mature and migrate to lymphoid organs (e.g. lymph nodes), where they present the antigen bound to the HLA molecule to naïve T cells for T cell priming.

The steps of T cell priming

T cells are cells of the adaptive immune system. They recognize specifically their cognate antigen-derived epitope presented on HLA-class I or -class II molecules by the T cell receptor (TCR). The TCR is expressed on the cell surface of T cells, and in most cases, is comprised of the α/β chain. Each T cell expresses a TCR specific to one antigen. The high diversity is generated by the V(D)J gene rearrangements that occur at both chain loci during the thymic T cell maturation. T cells are divided into two main populations: cluster of differentiation (CD) 8 T cells and CD4 T cells, expressing the co-receptor CD8 or CD4, respectively. CD8 T cells recognize peptides bound to HLA-class I molecules whereas CD4 T cells recognize peptides bound to HLA-class II molecules. Naïve T cells differentiate in the bone marrow, undergo selection in the thymus, and patrol through the blood and peripheral lymphoid organs in search of their cognate antigen presented by APCs. After a T cell enters the lymphoid organ, it binds temporarily on all APCs by the interaction of the adhesion molecules CD2, lymphocyte function-associated antigen (LFA)-1, intracellular adhesion molecule (ICAM)-3 on the T cell surface with their receptors CD56, ICAM-1, ICAM-2, CD209 on the APC cell surface. Although the adhesion is weak and only temporary, it is enough time to check for antigens. If the T cell recognizes its specific peptide:HLA complex, rapid signaling through the TCR triggers the conformation change of LFA-1. This leads to an affinity increase to ICAM-1 and ICAM-2 expressed by the APC and stabilizes the T cell-APC complex. The supramolecular activation cluster (SMAC) is built, which is stable for hours [132,133]. For the differentiation and proliferation of naïve T cells three signals are required. Signal one requires the binding of the TCR with its co-receptors CD4 or CD8 to the peptide:HLA complex. The interaction of the constitutively expressed co-stimulatory molecule CD28 on the cell surface of the T cell to CD80 or CD86 on the surface of APCs represents signal two, and provides the signal for survival and proliferation. This leads to the synthesis of IL-2 and the α-chain of the IL-2 receptor. After activation, other co-stimulatory molecules like ICOS or co-inhibitory molecules like cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are expressed, all being important for the processes that shape the immune response. The third signal is the secretion of cytokines (polarization factors) by DCs, which lead to the differentiation into various T cell effector types.

T cell differentiation

CD8 T cells differentiate into CD8 CTLs responsible for cell killing upon the recognition of their cognate antigen:HLA complex after e.g. viral infections. The induction of apoptosis in the target cell is primarily mediated by the release of cytotoxic granula that contain perforin and granzymes. This is mediated by exocytosis, whereby the lysosomal membrane of the granula is fusing to the CTLs membrane, detectable by the expression of LAMP-1 (CD107a), LAMP-2 (CD107b), and CD69 on the granula-delimiting outer membrane [134]. The expression of CD107a on the plasmatic membrane is often used to assess the cytotoxic potential of cells by flow cytometry [135]. CTLs are also known to secrete IFN-γ and TNF upon their activation to mediate further immune responses. CTLs also mediate apoptosis by the FAS–FAS ligand interaction [136,137], however, this mechanism is predominantly used by effector CD4 T cells [138].

CD4 T cells differentiate into various effector T cell subpopulations and include among others Th1, Th2, Th17, and Tregs. The polarization factors are IFN-γ and IL-12 (Th1), IL-4 and transforming growth factor (TGF)-β (Th2), IL-6 (Th17), and TGF-β and IL-2 (Tregs) [139]. Each subpopulation has its effector function and cytokine profile. In brief, Th1 cells secrete cytokines IFN-γ, IL-2, and TNF and are important in the immunity against extracellular pathogens like bacteria. They mediate help to macrophages to kill phagocytized bacteria more efficiently and stimulate the production of antibodies against extracellular pathogens by B cells, the third population of APCs that I will discuss later. Th2 cells secrete IL-4, IL-5, and IL-13, and mediate preferentially the activation of B cells to provide immunity against parasites. Th17 cells are defined by the production of IL-17 (IL-17A/F) but can switch from the Th17 to Treg or Th1 cytokine profile under certain conditions. Th17 cells are involved in many inflammatory and autoimmune disorders, as well as in the clearance of extracellular infections [139]. Fully differentiated T effector cells migrate to the inflammation site and support the immune response. After antigen clearance, most effector cells die up to a small proportion of long-living memory T cells which mediate a long-lasting immune response. After antigen rechallenge, memory T cells mediate rapid effector function. Tregs are a small cell population (approx. 5% of the blood circulating CD4 T cells) which is important to maintain immune homeostasis by e.g. downregulating effector cells after infection and to prevent autoimmune reactions. They suppress the immune system by different mechanisms like IL-2

deprivation, or by the secretion of inhibitory cytokines like TGF-β. They are mostly identified by the lineage markers forkhead box protein P3 (FoxP3) and CD25 [140]. CD127, the α-subunit of the IL-7 receptor, is inversely expressed with FoxP3 and is used for further identification of the cells [141]. In addition, it is shown that naïve and activated Tregs do not express CD154 [142,143].

Different T cell differentiation models are proposed [144]. For example, Sallusto et al. showed that the T lymphocyte differentiation state can be assessed by the expression of the homing receptor C-C chemokine receptor 7 (CCR7) and CD45RA, as shown in Figure 5 [145].

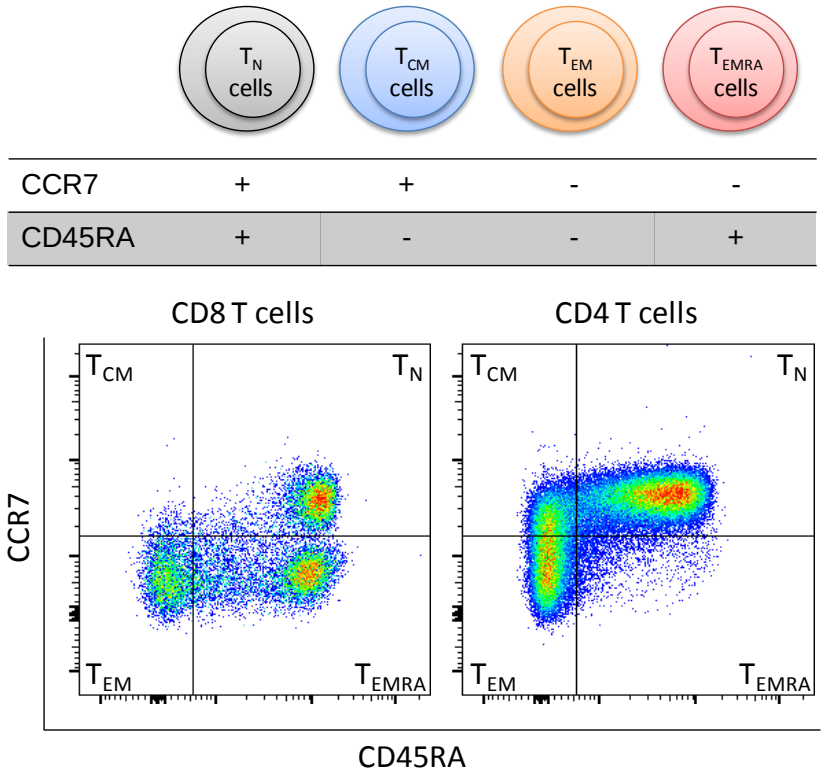



Figure 5**:** Phenotype of T cells based on CCR7 and CD45RA expression. $T_N$: naïve T cells, $T_{CM}$: central memory T cells; $T_{EM}$: effector memory T cells; $T_{EMRA}$: T effector memory T cells re-expressing CD45RA.

In the publication, they propose a stepwise differentiation model of T cells. Naïve T cells ($T_N$) are $CCR7^+CD45RA^+$. After recognition of their cognate antigen, they differentiate into central memory T cells ($T_{CM}$, $CCR7^+CD45RA^-$) and further into effector memory T cells ($T_{EM}$, $CCR7^-CD45RA^-$), which persist in the human body. $T_{CM}$ are memory T cells without immediate effector function, whereas $T_{EM}$ display immediate effector function and express IFN-γ and perforin. In the CD8 T cell

population, a subset of T effector memory T cells re-expressing CD45RA ($T_{EMRA}$) was detected, which was described to correspond to the already reported $CD27^-$ terminally differentiated effector T cell population. This population has been shown to predominantly express perforin and also IFN-γ, but to a lesser extent than the $T_{EM}$ [145]. Other *in vitro* studies examining viral-specific T cells also support this stepwise differentiation, showing furthermore a higher proliferation potential for $CCR7^+$ cells, whereas $CCR7^-$ cells show higher functional capacity [146]. CD4 $T_{EMRA}$ cells are also found in donors with a history of dengue virus and cytomegalovirus (CMV) where they are associated with viral control [147,148]. The four differentiation stages can be phenotypically further distinguished by the expression of CD28 and CD27 [149]. In addition, the CD4 $T_{EMRA}$ population can be discriminated by the G protein-coupled receptor 56 (GPR56), wherein $GPR56^+$ $T_{EMRA}$ have shown a distinct cytotoxic feature, a high clonal expansion capacity, and include the majority of virus-specific cells within the CD4 $T_{EMRA}$ population [150].

T cell exhaustion

The term T cell exhaustion was defined after the recognition that virus-specific CD8 T cells lose their ability to mediate cytotoxic activity, proliferation capacity, and show impaired maintenance of T cell memory upon chronic antigen-exposure. The distinct populations show changes, including the up-regulation of inhibitory receptors like program cell death protein 1 (PD-1), T cell immunoglobulin and mucin domain-containing protein 3 (Tim-3) or lymphocyte-activation gene 3 (LAG-3) on CD4 and CD8 T cells [151]. To assess the exhaustion phenotype, it is recommended to use several markers, since each marker alone could be also expressed upon activation. Apetoh et al. for example proposed guidelines to precisely define the CD8 T cell phenotypes in cancer, based on their functional state [152].

B cells – the second type of adaptive immune cells

B cells are also APCs and mediate humoral immunity. B cells recognize antigens specifically with their B cell receptor. (Surface) immunoglobulin diversity is also mediated by V(D)J gene rearrangement as for the TCR. In contrast to T cells, B cells recognize their antigen unbound, in soluble form. After recognition, B cells internalize the antigen into vesicles where it gets processed and later presented on the cell surface on HLA-class II molecules. CD4 Th cells that are specific for the same antigen recognize the peptide:HLA complex on the B cells and start expressing

CD154, which binds to CD40 on the B cells. The additional secretion of cytokines by the CD4 Th cells induces the antibody isotype switch. The activation of B cells leads to the proliferation and differentiation into antibody-producing plasma cells and memory B cells. B cells that recognize so-called thymus-dependent antigens get activated by the antigen but require a second signal: the help of CD4 T cells. Thymus-independent antigens, like bacterial polysaccharides, induce a humoral immunity without the help of CD4 T cells. The secreted antibodies lead to the neutralization, opsonization, and activation of the complement system. B cells do not typically cross-present exogenous antigen on HLA-class I molecules like DCs, but they are able to do so [153–155].

NK cells

NK cells are cells of the innate immunity. They mediate direct cell death of virus infected cells and stressed cells, but can also partner with B cells as they are able to recognize and kill antibody-opsonized cells. As these cells are not in the focus of this thesis, the following description of NK cell function is very brief: various viruses can block specifically the HLA-class I expression to prevent the presentation of viral antigens. The absence of HLA-class I expression is recognized by NK cells, which then induce cell killing (the missing self-recognition). This is regulated by killer-cell lectin-like receptors (KLR) and killer-cell immunoglobulin-like receptors (KIR). Furthermore, NK cells recognize stressed cells by the expression of e.g. MIC-A and MIC-B with their natural cytotoxicity receptor (NCR) and eliminate antibody-opsonized cells by antibody-dependent cell-mediated cytotoxicity (ADCC) by Fc-receptors like CD16 (FcγRIII).

#### 1.2.1.2 The interplay between the immune system and cancer

All mentioned immune cells contribute to anti-cancer immunity. Circumstances observed during (chronic) pathogen-induced inflammation are also happening during cancer development, like the T cell exhaustion [151]. The important steps for the initiation of T cell immunity against cancer are simplified in the so-called cancer-immunity cycle depicted in Figure 6.

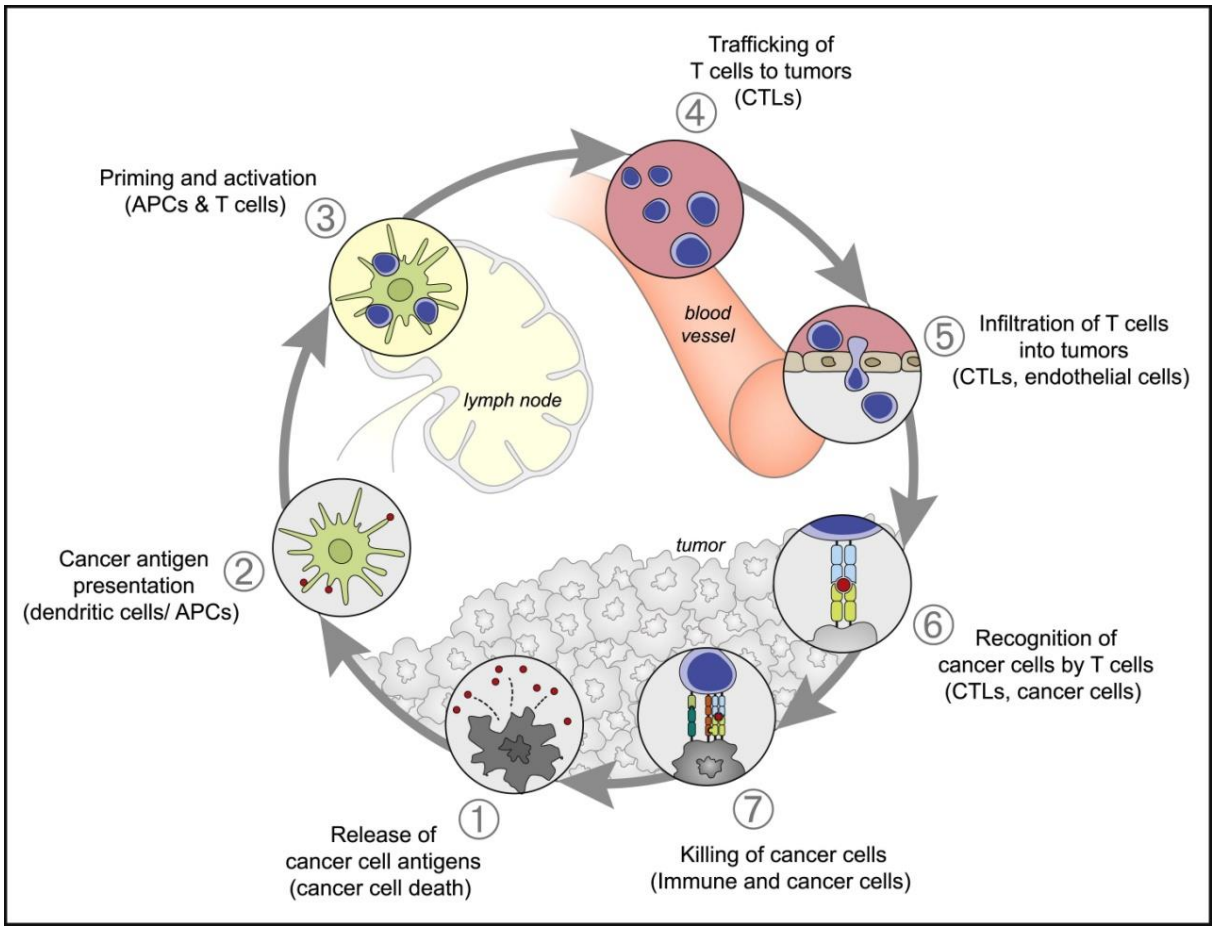



Figure 6: The cancer-immunity cycle. In the first step, antigens are released by the cancer cells by i.e. cancer cell death. APCs, like DCs, take up the antigen, get activated (step 2) and migrate to the draining lymph node, where they present the antigen to T cells (step 3). The primed T cells differentiate and proliferate, then travel throughout the bloodstream (step 4) to the tumor site, where they infiltrate the tumor (step 5). At the tumor site, they recognize cancer cells, which present their antigens on HLA-molecules (step 6), and eliminate these cells (step 7). The dying cancer cell releases cancer-related antigens during the dying process and the cycle starts again with step 1. Reused with permission from Elsevier [156].

Each step of the cycle is regulated by different stimulatory factors, like TNF-alpha or tumor necrosis factor receptor superfamily member 4 (OX-40), to support the immune response and likewise inhibitory factors, to slow down or stop the immune response. Negative regulators of the immune cells are for example cytokines like IL-10, or co-inhibitory molecules like PD-1, CTLA-4, or LAG-3 [156].

An inflammation at the tumor site is not always supportive of tumor elimination. It can also contribute to several hallmarks of cancer by the supply of factors that limit cell death, sustain proliferation, induce angiogenesis or contribute to the invasion and metastasis formation [123]. For example, macrophages within the tumor microenvironment, so-called tumor-associated macrophages (TAMs) can either fight cancerous cells or be supportive of tumor growth. M1 macrophages sense tumor-

associated antigens and induce an anti-tumor response [157]. They can either directly kill the tumor cells by NO production, or activate further cells like Th1 cells. M2 macrophages compose the second axis of TAMs, important for repair mechanisms in e.g. wound healing; they are often found in tumors and support tumor-growth. These cells are shown to induce a Th2 response [158,159]. In PCa for example, the infiltration of macrophages is associated with a positive prognosis, as long as they are not of the M2 type, whereas the infiltration of cytotoxic CD8 T cells does not affect the prognosis [160]. Here, the influence of CD4 T cells on prognosis is not mentioned, but these cells are known to be important to mediate e.g. help to CD8 T cells. Moreover, they are shown to promote anti-tumor immunity by different mechanisms like the direct killing of cancerous cells by the expression of granzymes and perforin, or by partnering with M1 macrophages and NK cells [161–163]. Since CD4 T cells can be specifically activated and mediate direct and indirect anti-tumor immunity, they are of high interest in anti-cancer immunotherapy. In the next section, the different strategies of immunotherapies for PCa are discussed based on current clinical approaches.

### 1.2.2 Immunotherapy in prostate cancer

Various forms of immunotherapy approaches, like CBI, adoptive cells transfer, and anti-cancer vaccines, aim to shift the escape and equilibrium phase to the elimination phase. T cells play a major role in all these approaches, as these cells can specifically fight tumor cells. Inflamed tumors with a high infiltration of immune cells, so-called “hot tumors”, are associated with a good response to immunotherapy especially after CBI (see also 1.2.2.1). They are characterized by a pre-existing immune response with e.g. high numbers of functional APCs, enriched Th1-type chemokines, and high numbers of functional T cells [164,165]. However, PCa is considered as “cold tumor”, associated with a low immune cell infiltration, mediated for example by an abnormal HLA-class I expression (e.g. down-regulation or selective loss) [166,167], a low mutational burden [168], as well as a suppressive tumor microenvironment (TME) [169,170]. This allows the tumor to escape from the immune system and makes immunotherapy challenging.

Two FDA-approved immunotherapy approaches are available to treat metastatic PCa: the cell-based anti-cancer vaccine PROVENGE (Sipuleucel-T; Sip-T) [171] and Keytruda® (Pembrolizumab) as CBI [172], both are currently not approved by the

European Medicine Agency (EMA). Nevertheless, approx. 13% (n=157) of the "recruiting" or "active, not recruiting" interventional clinical trials (n=1236) for PCa are immunotherapy approaches, including adoptive T cell transfer (ACT), anti-cancer vaccines, and antibody-based approaches, mostly in clinical phases I and II (Figure 7, snapshot 28.02.2020).

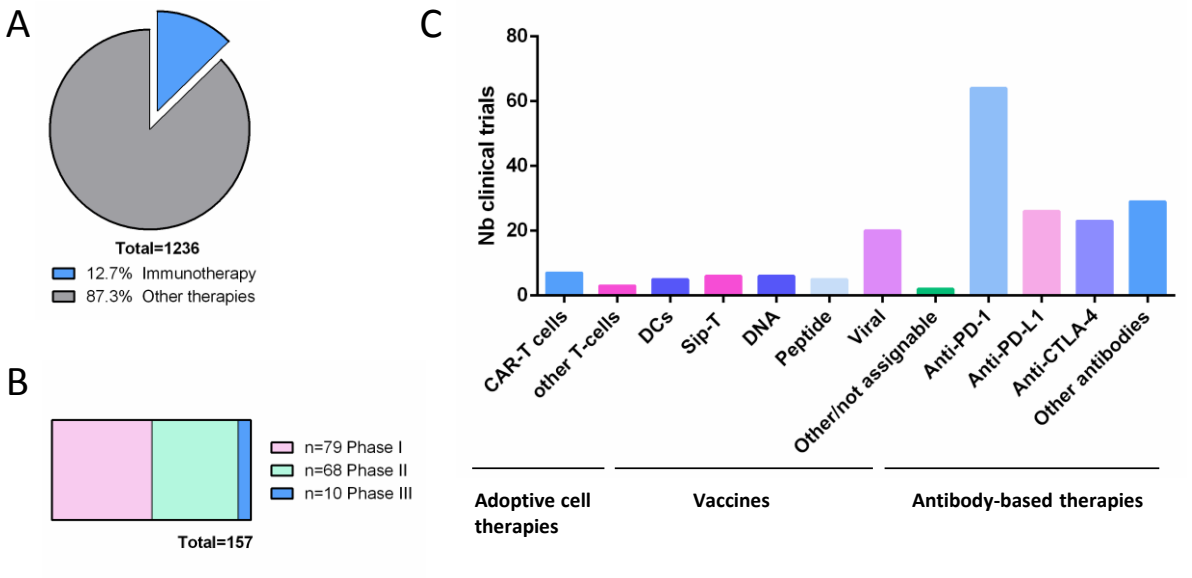



Figure 7: Snapshot (28.02.2020) of ongoing clinical trials for PCa with focus on immunotherapy. The data was processed with ClinicalTrials.gov [173]. Following basic criteria were applied as standard: "prostate cancer", "recruiting", "active, not recruiting", "interventional (clinical trial)", and "male". Following additional terms were applied for further individual search: "immune therapy", "CAR", "checkpoint", "PD1", "PDL1", "CTLA4", "Nivolumab", Pembrolizumab", Ipilimumab" "vaccine", "DNA vaccine", "cell vaccine", "DC vaccine", Sipuleucel-T", "ProstVac", "virus", "lymphocytes", "adoptive cell transfer", "engineered cells", and "immune stimulation". (A) After review of the results (exclusion of trials that contain the search item only as analysis marker; identification of duplicate hints, and combination therapies) unique ongoing immunotherapy (blue) and other approaches (grey; not reviewed) in PCa. (B) The associated clinical trial phases for the immunotherapy approaches in phases I to III (phase I consists of early phase I, phase I, and phase I/II stages; phase II includes also phase II/III) are shown. (C) Each clinical trial is categorized according to the approach, if applicable. Of note, some trials appear several times in the sections in case of a combinational therapy approach, e.g. PD-1 plus CTLA-4 therapy appears once in section PD-1 and once in section CTLA-4.

The available immunotherapy strategies either approved or under investigation will be elaborated in the next sections, with focus on anti-cancer vaccination. All PCa clinical trials mentioned later were identified by the approach described in the legend of Figure 7.

### 1.2.2.1 Antibody-based immunotherapy

For the treatment of PCa, 118 trials examine antibodies either in mono- or combination therapy. Sixty-four trials examine CBI therapy in PCa targeting PD-1, 26

trials target PD-L1, 23 trials target CTLA-4, and another 29 trials test antibodies against other checkpoint molecules or other antibody formats like bi-specific antibodies (Figure 7).

Targeting CBI molecules like PD-1, PD-L1, or CTLA-4 was groundbreaking in the treatment of solid tumors in recent years, especially in melanoma [127,128] or non-small cell lung cancer [174,175]. Preliminary work was made by James P. Allison and Tasuku Honjo, who were awarded with the Nobel Prize in 2018. The idea is to remove the “break” from the (exhausted) immune system, specifically effector T cells, by blocking the interaction of inhibitory molecules, or to enhance T cell activation by targeting co-stimulatory molecules like OX-40 or 4-1BB [176].

The effectiveness of immune CBI blockade is associated with several factors like the mutational burden [177,178], which correlates with neoantigen load [179], the HLA-class I genotype [180], or with a pre-existing immune response, which is suppressed by the expression of CBI molecules [181–183]. These are exactly the conditions which are, at least in part, most likely not present in the “cold tumor” PCa (median mutational burden PCa: ~1 somatic mutation per megabase; reference melanoma: 10 somatic mutations per megabase [168]). This could explain why CBI is poorly impacting the clinical course of PCa patients [127,184]. For PCa, it was indicated that the efficiency of CBI depends on the primary tumor and on the tumor stage. For example, PD-L1 is rarely expressed in primary PCa, but increasingly expressed in mCRPCa [185]. The phase II keynote-199 study examined the efficacy of Pembrolizumab (anti-PD-1 antibody) in cohorts of mCRPCa with and without PD-L1 expression [186], as the antibody previously showed an anti-cancer effect [187]. An objective response was detected in both patient cohorts, although the response rate (5% vs. 3%) and median overall survival rate (9.5 vs. 7,9 months) was slightly higher for mCRPCa patients with PD-L1 expression than without [186]. Nevertheless, the observed objective response rate is low compared to CBI of high mutational tumors like melanoma, non-small lung cancer or renal cell cancer [127,128,174]. In 2017, Pembrolizumab (Keytruda®) was approved by the FDA for solid tumors which are microsatellite instability-high or present a mismatch repair deficiency, as the response rate to this CBI was high regardless of the primary site [172,188,189]. Studies suggest that about 2-12% of PCa patients have the microsatellite instability-high/mismatch repair deficient phenotype [190,191]. For these patients,

Pembrolizumab could be a new therapy option. However, as most CBI monotherapies result in no or only a small clinical improvement for PCa patients, most ongoing studies favor the combination with e.g. a second CBI antibody (e.g. NCT03061539), with cell-based approaches (e.g. NCT01804465) or with DNA vaccination (e.g. NCT02499835). Only eleven clinical trials exclusively examine a CBI antibody in monotherapy. Nevertheless, CBI could have serious grade 3-4 side effects including fatigue, diarrhea, or colitis detected in 14-26% of the patients in monotherapy [127,184]. This could be even worse for combination therapy of CBIs, as shown for Nivolumab (anti-PD1) plus Ipilimumab (anti-CTLA-4) in melanoma for example [192]. First results of the CheckMate 650 study (NCT02985957) combining exactly these two antibodies for the treatment of mCRPCa demonstrate anti-tumor activity. Due to toxicity, only 40-50% of the patients were able to receive the planned four treatment cycles [193]. Further monoclonal antibodies are under clinical investigation for the treatment of PCa that target checkpoint molecules like OX-40 (NCT03217747) and B7-H3 (NCT02923180), or other molecules like CD38 (NCT04157517), VEGF (NCT00942331), or PSMA (NCT03724747).

Bi-specific antibodies like the in Tübingen developed PSMAxCD3 antibody CC-1 (NCT04104607) or the AMG160 (NCT03792841) are also investigated. The AMG160 is a fully-humanized, half-life extended BiTE (bi-specific T-cell engager antibodies), comprised of two single-chain variable fragments fused to an Fc domain. It is designed to target the tumor-associated antigen PSMA on PCa cells and CD3 on T cells simultaneously. The idea is to bring the activated T cells in close proximity to the cancerous cells to allow effector-mediated cell death [194].

#### 1.2.2.2 Adoptive cell transfer

Adoptive T cell transfer strategies are defined as cell-based immunotherapies. For this approach, T cells from the patients´ blood or tumor-infiltrating lymphocytes (TILs) from the tumor are taken, expanded *in vitro*, and are later infused back at high number. T cells can also be genetically modified before expansion, with CAR T cells or TCR engineered T cells [195].

Already in 1988, it became clear that autologous TILs can be used for anti-cancer treatment [196]. Cells were *in vitro* expanded and infused back to the patient together with IL-2. At the time of my clinical trial analysis (Figure 7), TILs from PCa were not

under investigation for anti-cancer treatment. However, Younger et al. demonstrated that the expansion of functional TILs from PCa with high tumor burden (> ~ 15 $mm^3$) is, at least in some cases, possible. The isolated TILs expressed PD-1, LAG-3, TIM-3, and CD28 and showed in general similarities to TILs isolated from melanoma or non-small cell lung cancer. Nevertheless, these results should be considered cautiously as only eight patients were examined [197]. Although PCa is considered as being a cold tumor, the relationship between immune cell infiltration seen for some PCa cases and the clinical outcome is not clear yet. BCR is associated with a high TIL infiltration [198,199], a high CD3 T cell infiltrate [200], especially of CD8 [201,202] and Treg cells [203], whereas also with a very low infiltration of CD3 cells [200].

CAR-T cells, in contrast to TILs, are designed to recognize and kill tumor cells independently of HLA molecules, which could help to overcome the tumor-escape mechanism of HLA-class I loss, seen for many tumors [204], also for PCa [205]. The CAR is a synthetic product composed of a TCR signaling unit (transmembrane and intracellular domain containing the immunoreceptor tyrosine-based activation motif (ITAM)) fused by a linker to an antibody variable domain recognizing the antigen of choice in the absence of an HLA-class II molecule, which is transfected into T cells after isolation [206,207]. Five generations of CAR are meanwhile available or under investigation; all have the same core structure, coupled to various additions in the intracellular signaling domain from one to the other generation, like co-stimulatory molecules, proteins e.g. inducible or constitutively expressed cytokines, or cytokine receptors [207]. Both CD8 and CD4 CAR-T cells are tested in animal models, as well as in the human setting, and have demonstrated a high cytotoxic potential [208–210]. Note that CD4 CAR-T cells were shown to be superior to CD8 CAR-T cells especially for long term immunity in mice [209] and with these CD4 CAR-T cells tumor regression (glioblastoma) was observed in a case report [210]. In a meta-analysis of 42 blood cancer and 18 solid cancer studies, CAR-T cell therapy showed clinical relevance for hematological diseases, although with severe side effects like the cytokine-release syndrome or neurotoxicities, but were less effective in solid tumors in mice [211]. One dose-escalation phase I study with CAR-T cells against PSMA in PCa reports partial clinical responses in 2/5 patients [212]. At the moment, seven clinical trials investigate the efficacy of CAR-T cell therapy in PCa (NCT03873805/ anti-prostate stem cell antigen (PSCA); NCT03089203/ anti-PSMA; NCT04227275/ anti-PSMA; NCT04053062/ anti-PSMA; NCT01140373/ anti-PSMA; NCT03013712/

anti-epithelial cell adhesion molecule (EpCAM); and NCT02744287/anti-BPX-601 (anti-PSCA).

Interestingly, one ongoing clinical phase I study (NCT03970382) is testing autologous gene-engineered CD4 and CD8 NeoTCR-T cells for a personalized approach in PCa. These cells are modified to express one autologous TCR that targets one neoepitope presented exclusively on the HLA of the patient´s tumor.

### 1.2.2.3 Anti-cancer vaccines – peptide vaccination in focus

Therapeutic anti-cancer vaccines are designed to induce or boost antigen-specific T cells against malignant cells. The underlying idea is to use the road of immune activation we learned from invading pathogens (see Figure 8; [213]**)** and from vaccinations against infectious diseases. Since the first melanoma patients who were treated with a vaccine containing a HLA-A*03 restricted MAGE-derived peptide in the mid 20$^{th}$ century [214], anti-cancer vaccines showed mostly convincing preclinical data [215,216], but often disappointing clinical results [217]. Open questions that remain are what is the best administration route for the vaccine (e.g. NCT03412786), the optimal vaccine scheduling (e.g. NCT02293707), or whether the anti-cancer vaccine might be more efficient in combination with another therapy (e.g. NCT00583752). Current efforts are made to improve the weakness of anti-cancer vaccines, with multiple studies addressing: I) appropriate tumor antigens, II) vaccine formulation, III) adjuvants, and IV) delivery vehicles [218]

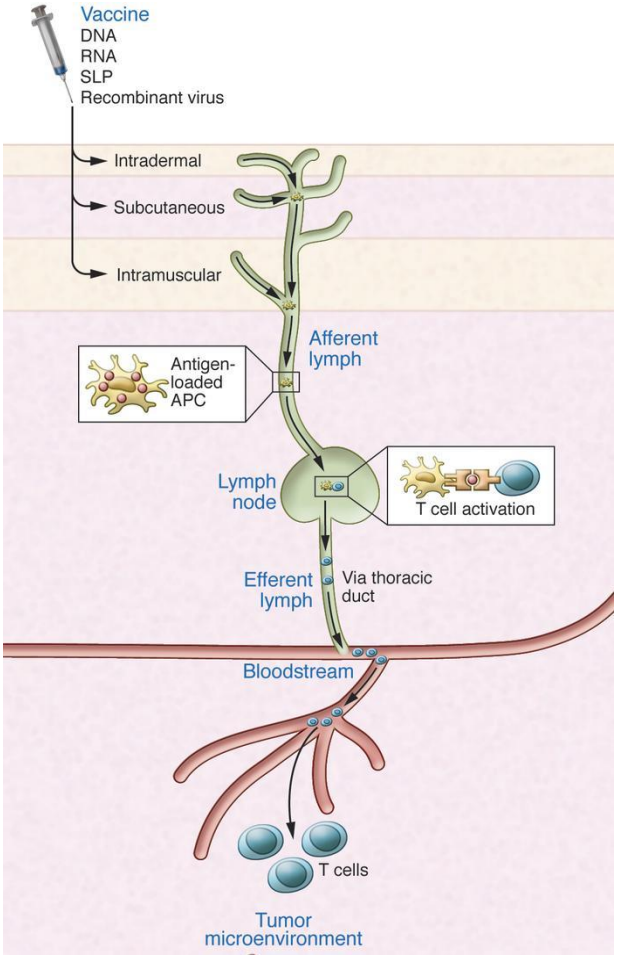



Figure 8: Therapeutic anti-cancer vaccines – mode of action. After vaccine application, skin resident APCs engulf the antigen, get loaded, and travel to the afferent lymph node where T cell priming takes place. Activated T cells travel to the tumor site, were they possible have to overcome an immunosuppressive milieu. Adapted from [213].

to induce a clinically relevant immune response, which at best will result in a long-lasting disease-free state for the patients.

Antigens targeted by anti-cancer vaccines

Two types of antigens are targeted by anti-cancer vaccines, either tumor-specific antigens (TSAs) or tumor-associated antigens (TAAs). Whereas TSAs are exclusively expressed by the tumor cells, like oncogenic virus-derived antigens or mutation-derived neo-antigens, TAAs are overexpressed or preferentially expressed on i) malignant cells or ii) tissue-specific cells. Further, TAAs include proteins that are involved in tissue differentiation and cancer-testis antigens [218]. TAAs are self-antigens for which high-affinity T cells are usually eliminated during T cell development to ensure self-tolerance [219]. Indeed, TAAs have been found to be expressed in the thymus of mice and humans, although with different expression levels [219,220]. Thus, anti-cancer vaccines that target TAAs need to overcome tolerance and stimulate lower affinity T cells to promote *in vivo* effector function [221]. TSAs like oncogenic viral antigens or products of somatic mutations are per se foreign to the immune system. These antigens are considered as being highly immunogenic as they lack central tolerance and can be recognized by high-affinity T cells. However, not all mutations are immunogenic and clinically relevant. Next-generation sequencing combined to high-throughput immunogenicity screenings or recently developed mass spectrometry workflows identifying naturally presented neo-epitopes are the methods of choice to identify those immunogenic and probably clinically relevant neo-epitopes [222]. Neo-epitopes differ between patients, as they arise from somatic mutations and are preferentially used for personalized-therapeutic vaccines, with recent clinical success [223,224]. Personalized neo-epitope vaccine approaches for PCa are not generally suitable due to the low mutational burden. Table 3 shows a selection of TAAs often targeted in PCa with the corresponding antigen category, clinical trial reference, and immunotherapy strategy.

Table 3: Selection of TAAs often targeted in prostate cancer.

| Antigen | Category | Immunotherapy approach + trial reference |
|---|---|---|
| PSA | Tissue differentiation antigen | Peptide vaccination [225,226], [227], [228], [229], [230], [231] |
| PSMA | Tissue differentiation antigen | Peptide vaccination [232], [233], [228], [229], [230] |
| PAP | Tissue differentiation antigen | Peptide vaccination [228], [229], [230], [231] |
| PSCA | Overexpressed tumor antigen | Peptide vaccination [229] |
| TARP | Overexpressed tumor antigen | Peptide vaccination [234] |
| NY-ESO-1 | Cancer-testis antigen | Protein vaccination [235] |
| hTERT | Overexpressed tumor antigen | Autologous DC vaccination (mRNA transfected) [236] |
| HER-2/neu | Overexpressed tumor antigen | Peptide vaccination [237], [229] |
| Survivin | Overexpressed tumor antigen | Peptide vaccination [238] |
| RhoC | Overexpressed tumor antigen | Peptide vaccination [chapter 2, present thesis] |

PSA: prostate specific antigen, PSMA: prostate-specific membrane antigen, PAP: prostatic acid phosphatase, PSCA: prostate stem cell antigen, TARP: T cell receptor gamma alternate reading frame protein, NY-ESO-1: New York esophageal cell carcinoma-1, hTERT: human telomerase reverse transcriptase, Her-2/neu: human epidermal growth factor receptor 2, RhoC: Ras homolog gene family member C

Vaccine formulation

Anti-cancer vaccines are composed of different formulations: cell-, viral vector-, nucleic acid- (DNA/mRNA), protein-, or peptide-based, all currently under investigation in clinical studies for PCa (see Figure 7C). The two major categories exploit either the whole antigen complexity (whole tumor vaccines) or follow an antigen-specific approach (selection of defined antigens) [218].

Cell-based vaccines are represented in both categories. For example, the whole antigen-complexity of a tumor is used by the vaccination with autologous modified whole tumor cells or allogeneic tumor cell lines [239,240], tumor cell lysate [241] also pre-loaded onto DCs [242], or with tumor-mRNA-transfected DCs [243]. This vaccine formulation does not require the identification of a “suitable” antigen, as the vaccine consists of either all patient-individual antigens or shared antigens if autologous or allogeneic tumor materials are used. The available characterized and/or uncharacterized antigens are then presented by APCs to T cells inducing immune responses to several antigens possibly bypassing the phenomenon of antigen loss. However, uncharacterized antigens could be also expressed by healthy cells, leading to an unwanted immune response. The preparation of autologous whole cancer vaccines presupposes the accessibility of the tumor for a biopsy, the presence of suitable antigens, and the absence of immunosuppression. Allogeneic vaccines lead

to allogeneic and tumor-specific immune reactions and do not require a biopsy (i.e. from established cell lines), however, the vaccine could be rejected or lead to massive production of cytokines for example [244,245]. In the tumor-vaccination history, a major breakthrough was made by the first FDA approval of a therapeutic anti-cancer vaccine in 2010. The approved autologous cellular Sip-T (PROVENGE) vaccination targets PAP-expressing cells in mCRPCa [171]. The clinical phase III trial showed a slight improvement of 4.1 months of the overall survival for patients receiving the therapy as compared to the placebo control group, with mostly mild to moderate (grade 1/2) adverse events. However, Sip-T therapy had no impact on the progression free survival [126]. In addition, the therapy is time consuming, costly, and varies in doses for single individuals and between patients [171]. Sip-T was also approved by the EMA in 2013, but withdrawn in 2015 [246].

Antigen-defined formulations contain only antigen/s (TAAs or TSAs) which have the potential to support anti-tumor immunity. The antigen/s need to be identified and are in best case validated (e.g. validation of expression on healthy tissue [247]) to reduce unspecific immune responses against healthy tissue.

Nucleic acid vaccines like RNA and DNA vaccines deliver genetic information for tumor antigens and are safe and easy to manufacture. Besides the delivery of the antigens, they can also trigger immune responses via interaction with nucleic acid sensing TLRs [248,249]. The different properties of the DNA/RNA vaccines are nicely compared in the review of Jahanafrooz et al. [250]. Six clinical trials are currently investigating nucleic acid vaccines for PCa in mono- or combination therapy (NCT04090528, NCT03532217, NCT02411786, NCT02499835, NCT03600350, NCT01341652).

Another formulation is the viral vector-based vaccine which is composed of recombinant viral vectors like poxviruses, adenoviruses, or measle viruses [251]. One of the most prominent examples in PCa is the PROSTVAC vaccination composed of PROSTVAC-V (vaccinia virus, rilimogene galvacirepvec) and ROSTVAC-F (fowlpox virus, rilimogene glafolivec), two live poxviral-based vectors containing the genes encoding for PSA and for the costimulatory molecules B7.1, ICAM-1, and LFA-3. In a clinical phase III trial for patients with asymptomatic or minimally symptomatic mCRPCa, PROSTVAC was well-tolerated and safe, however, it had no impact on overall survival and was stopped afterwards [252]. Clinical trials examining the effect

of PROSTVAC in other PCa conditions or in combination with other immunological therapies are still ongoing (e.g. NCT02506114, NCT02649439).

The most important formulation for the present study is the anti-cancer vaccination using peptides. These vaccines are easily produced under good manufacturing practice (GMP) and have shown an excellent safety profile [253], see also clinical trials listed in Table 3. The research on peptide-vaccination first concentrated on the induction of an immune response mediated by cytotoxic CD8 T cells, as it was believed that these are the therapeutically important cell type to reject tumors [254]. However, the induced immunity was often not long-lasting [255]. The potency of CD4 T cells to kill tumors *in vivo* was later demonstrated in a mouse model [256]. Moreover, since CD4 T cells are essential for CD8 T cell activation, expansion, and memory formation [257–259], peptide-vaccines nowadays are mostly designed to activate both CD4 and CD8 T cells. For that, either a mixture of characterized HLA-class I and -class II epitopes (synthetic peptides of 8-12 aa for HLA-class I and 13-18 aa for HLA-class II presentation) [260], or (overlapping) synthetic long peptides (SLPs; 15-35aa) [261] are administered to the patient. The peptide-vaccination using exact peptides requires the knowledge of the patients HLA alleles and the identification of the presented epitope/s, as the peptides directly bind to the HLA and do not need further *in vivo* processing. To select the vaccine peptides, the reverse immunological approach can be applied, whereby computational programs like SYFPEITHI [262] or netMHC [263] are used to predict the binding capacity of a certain HLA-class I peptide to a HLA-molecule based on known peptide binding motifs. The immunogenicity of selected potential binders has to be then further evaluated in cell-based assays. For most HLA-class I allelic products, the prediction tools work very well, as the binding motifs of HLA-class I ligands are well defined. The state of the art tool for predicting peptides binding to HLA-class II molecules is the netMHCIIpan 3.0 server [264]. Predicting binders to HLA-class II molecules is challenging due to the promiscuous binding of HLA-class II peptides, the lack of very precise binding motifs, and the possible influence of peptide flanking regions [265]. If tumor material is available for analysis, naturally presented HLA-class I and –class II ligands can be identified by mass spectrometry [266]. This approach has the advantage that the analyzed peptides naturally exist and do not need any binding predictions. The HLA Ligand Atlas, which is available online [267], lists naturally-presented HLA-class I and -class II epitopes which were found on a variety of benign

tissues. This platform could help to select suitable peptides for anti-cancer vaccination, by excluding peptides frequently presented on benign tissues in order to prevent unwanted immune responses against healthy organs. The identification of peptide binding motifs, the reverse immunology approach, and the MS identification of naturally presented peptides have been combined at the Department of Immunology in Tübingen for many years and have led to the design of several completed or ongoing vaccine-peptide based studies in cooperation with several departments at the University hospital of Tübingen (UKT) [238,260,268,269] and NCT02802943 (ongoing). For vaccinations with SLPs, patient individual HLA allotype does not necessarily have to be known, as the peptides are processed *in vivo*. SLPs can potentially contain HLA-class I and -class II epitopes to activate both CD8 and CD4 T cells simultaneously. They were shown to be superior to whole proteins, as they are more rapidly and efficiently processed by mouse and human DCs resulting in the activation CD4 T cells and, importantly, also of CD8 T cells by cross-presentation [270]. However, it cannot be excluded that some of the SLPs are trimmed by serum proteases and loaded exogenously onto the HLA-molecules [271]. SLP vaccine approaches often use a complete set of overlapping SLPs that cover the entire protein sequence [272].

At the moment, five clinical trials examine peptide vaccinations in PCa (see Table 4).

Table 4: Current peptide-vaccine clinical trials in prostate cancer (search procedure: see Fig.7)

| Disease | Phase | Antigen | Application | Adjuvant | Treatment condition | Ref. |
|---|---|---|---|---|---|---|
| Hormone-sensitive metastatic PCa | I | B-cell lymphoma extra large protein (Bcl-xl) | i.p. (10 patients)<br>i.m. (10 patients) | CAF09b (3 components: cationic liposomes, synthetic double-stranded RNA, Poly(I:C)) | Non-randomized; single treatment<br>Six vaccination in total per patient | NCT03412786 |
| Hormone-sensitive metastatic PCa | I/II | UV1: human telomerase reverse transcriptase catalytic subunit (hTERT) | i.d. | GM-CSF | Single-group assignment; dose-escalation study<br>Plus androgen blockade (GnRH-agonist and bicalutamide) | NCT01784913 and [273] |
| PSA recurrent Hormone-naïve and hormone independent PCa | IA/IIB | PSA | i.d. | IL-2 + GM-CSF | Single-group assignment, two-stage study<br>Phase IA: six vaccinations in total per patient of PSA/IL-2/GM-CSF<br>Phase IIB: same course as IA + six more vaccinations alternating between IL-2 alone and PSA/IL-2/GM-CSF | NCT02058680 |
| Progressive castration-resistant PCa | II | GX301 (4 peptides: TERT (540-548), hTERT (611-626), hTERT (672-686) hTERT (766-780)) | i.d. | Montanide ISA-51 + imiquimod | Randomized; three experimental regimes:<br>1) 8 vaccinations in 63 days<br>2) 4 vaccinations in 63 days<br>3) 2 vaccinations in 63 days | NCT02293707 |
| Adenocarcinoma with BCR within 3 years after RP or definitive radiation therapy | II | RV001V (RhoC) | s.c. | Montanide ISA-51 | Randomized; placebo-controlled; double-blind<br>12 vaccinations in total per patient either with Montanide ISA-51 + RV001V or only Montanide ISA-51 | NCT04114825 [274] |

i.p.: intraperitoneal; i.m.: intramuscular, i.d.: intradermal, s.c.: subcutan

In these clinical studies (Table 4) different antigens, vaccine application sites, adjuvants, or vaccine schedules are examined. Mostly patients with advanced PCa are included, although anti-cancer vaccines might be more effective in early stages [275] when the immune system is not suppressed by the TME. The active phase II clinical study targeting the Ras homolog gene family member C (RhoC; RV001 vaccination) was designed based on the results of the completed phase I/II study (NCT03199872) which is presented in chapter 2. Here, the vaccine is administrated subcutaneously (s.c). which is often used for peptide-vaccination in combination with Montanide [223,261,275].

Adjuvants and delivery vehicles

The term “adjuvant” comes from the Latin word “adiuvāre”, which means “help” or “support” [276]. Components used as adjuvants for therapeutic anti-cancer vaccines should ideally support the antigen uptake by APCs and induce full APC activation to promote CTL and/or Th1 immune response [253]. Delivery vehicles protect the vaccine components from degradation and should ensure a progressive delivery to the APCs [218]. A clear separation between an adjuvant and a delivery vehicle is not always possible. For example, Montanide ISA-51 (Montanide), a water-in-oil emulsion (incomplete Freund's adjuvant), is frequently given s.c. as adjuvant and delivery vehicle, and has an acceptable safety profile [277]. Montanide forms a depot which protects antigens from degradation and allows slow antigen release at the immunization site [277,278]. However, T cell sequestration and dysfunctionality after peptide vaccination with high-dose Montanide were observed in mice [279]. In patients, repeated peptide vaccinations with Montanide induced lymphoid-like structures, however, infiltrated cells were dysfunctional [280,281]. Other currently used adjuvants are e.g. GM-CSF, TLR ligands like CpG or XS15, and Poly-ICLC [253,282]. This addition of such adjuvants may reduce T cell sequestration and/or dysfunctionality, also in combination with Montanide [282]. Other delivery systems used for anti-cancer vaccines are e.g. nanoparticles [283], liposomes [284], and virosomes [285].

Although we have already gained a lot of knowledge since the first anti-cancer experiments were performed, a long and difficult road is still in front of us to develop clinically effective peptide-vaccinations for malignancies like PCa, which up to now remains eventually still incurable.

## 1.3 Aim of the thesis

The aim of the thesis was the assessment of the immunological response for the first-in men, clinical phase I/II study with the title “RV001V, a RhoC Anticancer Vaccine, Against Metastasis From Solid Tumours” (NCT03199872).

In this clinical study, a Rho-C derived SLP (20 aa) with the potential of simultaneously stimulating CD8 and CD4 T cells was used for the vaccination. The first step of the immunological assessment was to measure the immunological response against the vaccine-peptide before, during, and up to 13 months post-vaccination using Enzyme-Linked ImmunoSpot Assay (ELISpot). The responding cell population was later identified by intracellular cytokine staining assay (ICS). In a second step, if a vaccine-specific response was detected, a further in-deep analysis of the epitope/s, the presenting HLA allelic products, as well as the phenotypic analysis of the responding cells was performed using the flow cytometry technique. The results of the immunological assessment are shown in section 2 of the manuscript.

Our lab is specialized in the immunomonitoring of T cells after peptide vaccination using peptides of exact length. For the assessment of antigen-specific cells, several methods are routinely used to obtain complementary information on T cell quality and quantity. The specificity, advantages, and limitations of these common T cell monitoring methods, as well as the description of the ICAM-staining method that our laboratory recently introduced, are described in the focused review in section 3 part I.

For the monitoring of the presented clinical study, we wanted to use the vaccine peptide itself in order to readout simultaneously CD8 and CD4 T cell responses. As all methods available for immunogenicity measurement in the laboratory have been established and validated using short peptides, it was necessary to test and possibly optimize the protocols for the *in vitro* pre-sensitization of T cells, the ELISpot, and ICS. As model epitopes, known HLA-class I and –class II virus-derived peptides were elongated to 20mers according to the virus aa sequence. The immunological response against the exact and the elongated peptides was then assessed in healthy donors using ICS and ELISpot *ex vivo* and or after *in vitro* T cell pre-sensitization. The manuscript in section 3 part II describes this protocol optimization process.

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# Chapter 2I Immunomonitoring of a clinical phase I/II study in prostate cancer patients

# 2 Introduction: Targeting RhoC – an anti-cancer vaccine for solid tumors

The presented clinical study is a phase I/II study which examines as primary endpoint the safety and treatment-related side effects upon a SLP vaccination. The secondary endpoint examines the vaccine-related immune response. Patients who were diagnosed with an adenocarcinoma of the prostate gland and had undergone RP were included in the study. The RV001 vaccination is meant to fill the gap between the first-line therapy like RP or radiation therapy and the possibly needed second-line therapy if BCR, tumor recurrence or metastasis are observed (see Figure 9).

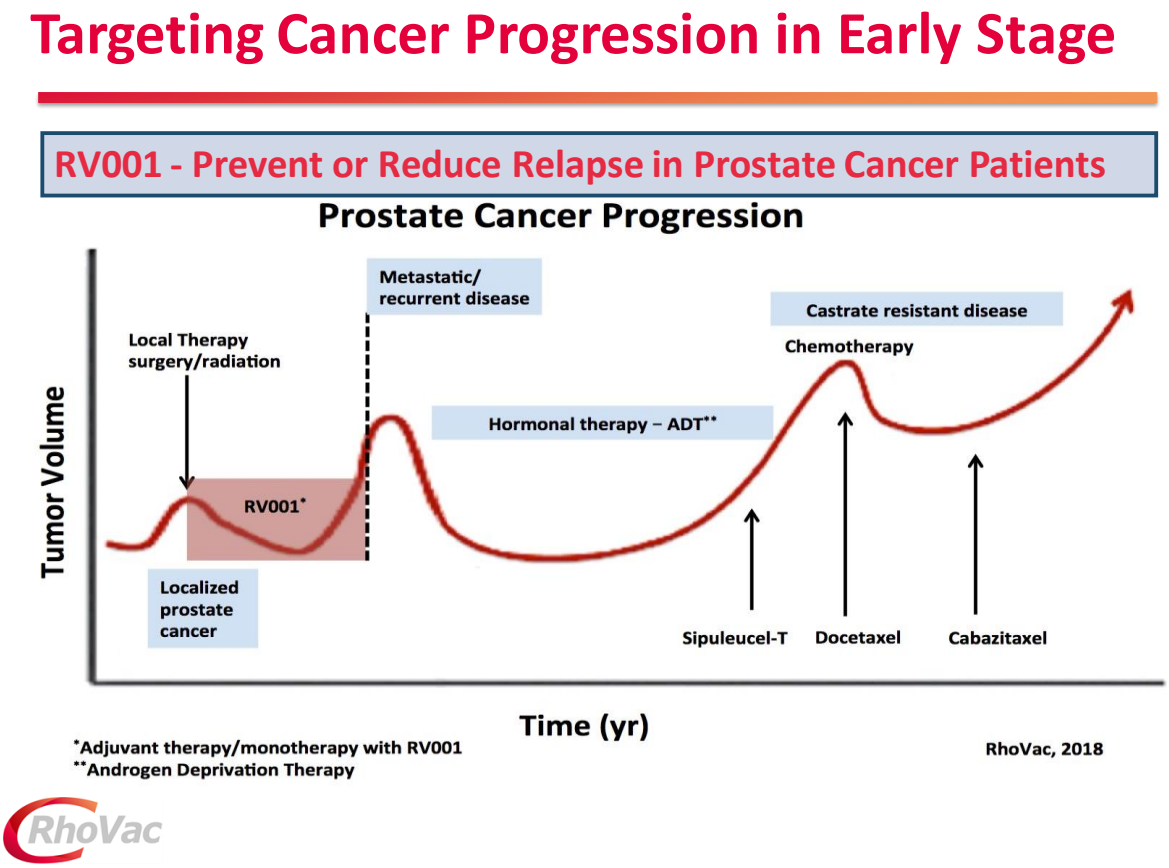


Figure 9: Placement of the RV001 synthetic long peptide vaccine therapy into the disease course over time. With permission from RhoVac AB.

The elimination of disease recurrence would improve the life of PCa patients immensely and prevent the need for additional aggressive treatments. The anti-cancer vaccine targets RhoC.

RhoC – in health and cancer

RhoC is a small GTPase, which is a so-called molecular switch cycling between its inactive GDP-bound and active GTP-bound conformations. It is closely related to other family members RhoA and RhoB (Rho subfamily), which both share 85% of sequence homology with RhoC [1]. The greater aa variability between the sequences is found in the carboxyl part (C-terminus; Figure 10A). This part is essential for their localization, due to post-translational modification, leading to the location of RhoB preferentially in endosomes, whereas RhoA and RhoB are located in the cytosol.


Figure 10: Amino acid (aa) sequence alignment. (A) Complete aa sequence alignment between the three GTPase family members RhoA (P61586), RhoB (P62745), and RhoC (P08134) in human. (B) C-terminal aa sequence (blue boxes) alignment between RhoA (P61586), RhoB (P62745), RhoC (P08134) and the peptide sequence of RV001. (*) - fully conserved residue; (:) – amino acid with strongly similarity (scoring > 0.5 in the Gonnet PAM 250 matrix); (.) – aa with weak similar properties (scoring ≤ 0.5 in the Gonnet PAM 250 matrix). Analyzed with Uniport – Align [4].

The Rho family members were identified regulators of the cytoskeleton [2], and although they share a high homology, they have different functions reviewed in [3]. They are also shown to have different functions in cancer [5]. Predominantly RhoC is involved in tumor invasion and metastasis formation in several solid tumor types like breast cancer or PCa [5-8] and is potentially useful as a clinical biomarker. RhoC is highly expressed in cancerous cells compared to healthy tissue and is shown to correlate with cancer progression [7,8]. Furthermore, studies report that RhoC is an

important factor for cancer stem cell formation, which is thought to be an essential step to metastasis and formation of new tumors in many cancer types [9,10].

Targeting RhoC by immunotherapy could lead to the elimination of advanced tumor cells and metastases and therewith reduce or even prevent BCR, as well as second-line therapy in PCa. Wenandy et al. identified a RhoC-derived 10 amino acid (aa) long HLA-A*03 restricted epitope (R**A**GLQVRKNK), using a modified version of the peptide (R**L**GLQVRKNK), which is spontaneously recognized by CD8 T cells of melanoma patients. The RhoC-specific T cells also showed a cytotoxic activity against various cancer cell types [11]. All these observations led to the idea that RhoC could be an adequate target for anti-cancer vaccination. The identified A*03-presented epitope is included in the C-terminal part of RhoC. An elongated version of this peptide could include further HLA-class I as well as -class II epitopes, activating both CD8 and CD4 T cells, respectively. However, the specific targeting of RhoC (and not RhoA and RhoB) allows only the elongation of the C-terminal aa. Hence, the SLP with the sequence ATRAGLQVRKNKRRRGCPIL (Figure 10B) was chosen as optimal vaccine sequence.

Clinical study protocol

The SLP vaccination targeting RhoC is applied after first-line therapy. The idea is that the first-line therapy reduces or even eliminates the primary tumor to create a more favorable immune cell to malignant cell ratio for immunotherapy. In a second step, the vaccine is applied, which primes and/or boosts RhoC-specific T cells to eliminate leftover primary tumor cells and metastases.

In the single arm, open label, clinical phase I/II study, the induction of an immune response was examined, beside vaccine safety and tolerance. The vaccine itself is composed of the above described RhoC-derived SLP RV001 (0.1 mg) emulsified in the adjuvant and delivery vehicle Montanide ISA-51 (1 ml). Twenty-two patients received in total 11 vaccinations s.c.: the first six every two weeks and the following boost vaccinations every four weeks (approx. 30 weeks of treatment). The vaccine was given alternating between the left and right arms because of the slow degradation of the depot. The complete immunological analysis, as well as the drug safety and PSA observations (examined by the clinic cooperation partner) are described in the following manuscript which is currently under revision for the *Journal of ImmunoTherapy of Cancer*.

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# Vaccination targeting RhoC induces long-lasting immune responses in prostate cancer patients: results from a phase I/II clinical trial

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The author of the present thesis planned, performed, and analyzed all immunological experiments. The author was strongly involved in the interpretation of the final data and in writing the manuscript.

## 2.1.1 Abstract

**Background:** Peptide-based vaccination is a rational option for immunotherapy of prostate cancer. In this first-in-man phase I/II study we assessed the safety, tolerability, and immunological impact of a synthetic long peptide vaccine targeting RhoC in prostate cancer patients. RhoC is a small GTPase overexpressed in advanced solid cancers, metastases, and cancer stem cells.

**Methods:** Twenty-two patients who had previously undergone radical prostatectomy received subcutaneous injections of 0.1 mg of a single RhoC-derived 20mer peptide emulsified in Montanide ISA-51 every two weeks for the first six times, then five times every four weeks for a total treatment time of 30 weeks. The drug safety and vaccine-specific immune responses were assessed during treatment and thereafter within a 13-month follow-up period. Serum level of prostate-specific antigen (PSA) was measured up to 26 months post-vaccination.

R**esults:** Most patients (18 of 21 evaluable) developed a strong CD4 T cell response against the vaccine, which lasted at least ten months following the last vaccination. Three promiscuously-presented HLA-class II epitopes were identified. Vaccine-specific CD4 T cells were polyfunctional and effector memory T cells that stably expressed PD-1 (CD279) and OX-40 (CD134), but not LAG-3 (CD223). One CD8 T cell response was detected in addition. The vaccine was well tolerated and no treatment-related adverse events of grade ≥3 were observed.

**Conclusion:** Targeting of RhoC induced a potent and long-lasting T cell immunity in the majority of the patients. The study demonstrates an excellent safety and tolerability profile. Vaccination against RhoC could potentially delay or prevent tumor recurrence and metastasis formation.

**Trial registration number:** NCT03199872

## 2.1.2 Background

Therapeutic anti-tumor vaccination may provide a safe and long-lasting immunotherapy treatment option for cancer patients. Many trials are ongoing worldwide, with most recent developments favoring a patient-individual approach [1-3]. It is acknowledged that vaccines should better be administered at an early stage of disease when the immune system of cancer patients is not yet suppressed. For advanced patients, vaccines could also be applied in combination with e.g. surgery, chemotherapy, or checkpoint inhibitor therapy [1,2]. In addition, cancer vaccines

should not only be designed for induction of cytotoxic T lymphocytes (CTLs), but also of effector CD4 T cells. CD4 T helper cells are crucial for CD8 T cell activation and expansion, as well as for the generation and maintenance of CD8 T cell memory [4-6]. They also display a range of anti-tumoral effects, such as secretion of TNF and IFN-γ [7,8], activation of macrophages or natural killer cells, and direct cytotoxicity, which together might be more powerful than the sole tumor killing by CTLs [9,10].

To stimulate both CD4 and CD8 T cells, vaccines containing either a mix of known HLA-class I and -class II epitopes [3,11] or (overlapping) synthetic long peptides (SLPs; 15-35aa) [1,12] can be used. SLPs have been shown to be rapidly and more efficiently processed compared to the whole protein, and to activate CD4 T cells, but also CD8 T cells by cross-presentation [13]. Since peptide processing takes place *in vivo*, prior knowledge of the precise T cell epitopes contained in the long peptides is not absolutely required, and such vaccines are generally applied to all patients, regardless of their HLA allotype.

The Ras homolog gene family member C (RhoC) belongs to the Rho GTPase family which comprises RhoA, RhoB, and RhoC (85% sequence homology), all involved in the regulation of cytoskeleton organization [14]. RhoC was shown to be an important player in tumor cell motility, invasion, and metastasis formation [15,16]. Since RhoC has a limited expression in normal cells but is highly expressed on advanced cancer cells and metastases [14,17], it could represents a suitable target for anti-cancer vaccination. Immunohistochemical analyses of tumor samples from prostate cancer (PCa) patients showed an increase in RhoC expression with advanced tumor stages and a strong correlation with the metastatic status. In addition, patients with RhoC expression have a significantly reduced overall-survival rate, indicating that RhoC could be used as a prognostic marker in PCa [18]. Furthermore, reports have demonstrated RhoC expression in cancer stem cells [19,20], which are also found in PCa [21]. In localized PCa the presence of micrometastases has been associated with biochemical recurrence (BCR) after first-line treatment by radical prostatectomy [22]. Targeting RhoC-expressing cancer cells and/or (micro) metastases by vaccination may therefore improve the clinical course of PCa patients and delay or prevent the onset of second-line therapies such as hormonal deprivation and/or chemotherapy. The immunogenicity of RhoC has been documented by our previous study, where CD8 T cells specific for a RhoC-derived 10mer anchor-modified peptide

were found in the blood of melanoma patients. Cloned T cells could specifically kill HLA-A*03 and RhoC expressing tumor cell lines *in vitro* [23].
In this clinical phase I/II study, we report the safety and immunogenicity of a 20mer SLP vaccine specifically targeting the RhoC protein in PCa patients.

## 2.1.3 Methods

**Study design and patients**

The study was an open-label, phase I/II trial. Patients previously treated with RP were identified, informed, and followed at Copenhagen Prostate Cancer Center, Department of Urology, University of Copenhagen, Rigshospitalet. Vaccinations were administered at Zelo Phase I Unit, DanTrials ApS, Copenhagen, Denmark. The trial was approved by the local ethical committee (H-1604701) and the European Union regulatory authorities (EuDRACT number: 2016-004189-24), and was conducted according to the Helsinki declaration. All patients gave informed consent. Prior to study entry, patients underwent screening procedures including a full physical examination, and in case of BCR, a metastatic workup with computer tomography and bone-scans. For inclusion and exclusion criteria, see online supplementary table S1. The primary endpoint of the study was the evaluation of the safety and tolerability of the vaccine. Treatment-emergent adverse events (TEAE) were analysed in accordance with the common terminology criteria for adverse events (CTCAE), version 4.03. The secondary endpoint was the investigation of the immunological responses against the vaccine.
Twenty-four patients were screened and 22 included in the study (table 1). Median time for surgery to study entry was 1.2 years (range: 0.3-12.8). Patients were vaccinated subcutaneously alternating between the right and left upper arms with the vaccine consisting of 0.1 mg SLP RV001 (20aa C-terminal sequence of the RhoC protein: ATRAGLQVRKNKRRRGCPIL; the 16 last amino-acid sequence is found only in RhoC, but not in RhoA/B, and is thus RhoC-specific) emulsified in Montanide ISA-51 (1 ml). Patients were vaccinated six times every two weeks, then five times every four weeks, resulting in a treatment duration of approximately 30 weeks (11 vaccinations in total). HLA-class I and II typing was performed before vaccination (visit 2, Tissue Typing Laboratory, Rigshospitalet, Copenhagen). For *in vitro* immunomonitoring, blood was taken before vaccination (visit 2), and after the $4^{th}$, $6^{th}$, and $11^{th}$ vaccinations (visits 6, 8, and 13). Follow up samples were obtained every

third month up to 13 months post-vaccination (visits 14 to 17). The study started in March 2017 and was completed in March 2019. Serum PSA levels were measured by routine clinical testing at every patient visit (median number of PSA measurements from study entry: 15 (range 13-17)). PSA doubling time was calculated using the MSK calculator available online [24]. In men with measurable PSA at study entry, doubling time was calculated from the first measurable PSA until study entry, and from study entry to end of follow-up. Median follow-up time for serum PSA was 2.5 years (range 2.4-2.7 years). Of note, Patient 015 withdrew from treatment after seven injections, but completed all visits for blood collection, except visit 13. Study design is shown in figure 1 and patient´s characteristics, PSA levels, and HLA-typing results are shown in online supplementary tables S2/S3.

Table 1: Main patient´s characteristics

| Parameter | Information |
|---|---|
| **No. of patients included** | 22 |
| **Median age (range)** | 66 (54-77) years |
| **PSA at baseline** | N = 20 ≤ 0,1 µg/L; N = 2 > 0,1 µg/L |
| **EOCG status at baseline** | N =22 status 0 |
| **Pathological Gleason Scores** | N |
| **6** | 3 |
| **7 (3+4)** | 10 |
| **7 (4+3)** | 6 |
| **8** | 1 |
| **9 (4+5)** | 2 |
| **pT category** | N |
| **pT2N0/Nx, R-** | 10 |
| **pT2N0/Nx, R+** | 5 |
| **pT3a/b N0/Nx, R-** | 1 |
| **pT3a/b N0/Nx, R+** | 5 |
| **pT3a N1, R+** | 1 |
| **All vaccinations performed** | N = 21 (Patient 015 received seven injections in total) |
| **Completed immunomonitoring** | N = 21 (Patient 002 excluded) |

T= tumor, N= nodes, R- and R+ = negative and positive margins

The following sections are prepared in accordance with the Minimal Information about T cell Assays (MIATA) guidelines.

## Cell samples

### Isolation of peripheral blood mononuclear cells (PBMCs)

Blood samples (100 ml, heparinized) were processed within 8 h after blood drawing (DanTrials ApS). PBMC isolation was performed according to a standard, pre-established protocol, using pre-filled 50 ml Leucosep$^{TM}$ tubes (Greiner Bio-One).

Cells were counted with trypan blue and Tuerks solution (both Sigma-Aldrich). Six to 13 x $10^6$ cells per cryovial (Nunc™, Sigma-Aldrich) were frozen in 1 ml heat-inactivated (hi) FBS (ThermoFisher) containing 10% dimethylsulfoxide (DMSO, Sigma-Aldrich). Cells were stored in freezing containers (Nalgene® Mr. Frosty, Sigma-Aldrich) at -70°C for 24-72 h and transferred to liquid nitrogen (-196°C). PBMCs were shipped on temperature-controlled dry ice to the Department of Immunology, Tübingen for immunological analyses. Cells were stored again at -196°C for 3-12 months before testing. PBMC samples from Patient 001 visit 2 and Patient 015 visit 13 were not available.

## Immunological assessments

### *In vitro* stimulation of antigen-specific T cells

PBMCs were thawed in IMDM (Gibco) containing 2.5% hi human serum (HS, Sigma-Aldrich), penicillin 100 units/ml and streptomycin 0.1 mg/ml (P/S, Sigma-Aldrich), and 50 µM β-mercaptoethanol (β-ME, Merck). After one washing step with serum-free medium (SFM, IMDM, P/S, 50 µM β-ME), cells were counted with trypan blue (Merck). The median live-cell yield after thawing was 63.5%. No cut-off was applied for further *in vitro* culture. On day zero, 1.0-3.5 x $10^6$ or 3.5-6.5 x $10^6$ PBMCs/well were seeded in T cell medium (TCM, IMDM with 10% hi HS, 1 x P/S and 50 µM β-ME) in a 48-well or 24-well plate, respectively (Cellstar®, Greiner bio-one), and further cultured at 37°C and 7.5% $CO_2$. On day one, cells were stimulated with 10 µg/ml RV001 peptide (purity ≥90%, PolyPeptide Laboratories France SAS) dissolved in 100% deionized water (dd$H_2O$; LiChrosolv, Merck) plus 20 µg/ml Poly-ICLC (Hiltonol®, Oncovir). Human interleukin (IL)-2 (R&D Systems) was added to the culture at 2 ng/ml on days three, five, seven, and nine. On day 12, cells were harvested, counted (median live-cell yield: 76,8%) and tested with enzyme-linked immune spot assay (ELISpot) and intracellular cytokine staining (ICS) assay.

### ELISpot

The Interferon (IFN)-γ ELISpot protocol has been described [25]. If not otherwise stated, 0.2 x $10^6$ cells were cultured per well in the presence of 50 µg/ml RV001 peptide for 26 h at 37°C and 7.5% $CO_2$ (triplicates). dd$H_2O$ (6 wells) and phytohemagglutinin-L (PHA-L, 10 µg/ml, Sigma-Aldrich, 1 well) were used as negative and positive controls, respectively. Spots were counted with the ImmunoSpot series 6 ultra-V analyzer (CTL Europe GmbH) according to a standard

protocol. Altogether, samples from n=21 patients were immunologically evaluated (results from Patient 002 were excluded because of inconsistent spot counts). For wells with counts above 2000 spots or stated as TNTC ("too numerous to count"), a count cut-off was set to 2000 spots. RV001-specific spot counts are defined as the mean spot counts in the RV001 stimulated wells minus the mean spot counts in the $ddH_2O$ wells. All spot counts are given in online supplementary table S5.

**Multiparameter flow cytometry**

PBMCs were analyzed by intracellular cytokine staining (ICS) either *ex vivo* or after culture. Thawed cells were rested in TCM for 4-6 h prior to the ICS. Pre-cultured cells were directly examined on day 12 or harvested from the ELISpot plate. Between 0.5 and 2 x $10^6$ cells per well (96-well plate) were stimulated with 50 µg/ml RV001 or 10 µg/ml of single overlapping 15mers (RV001-derived: ATRAGLQVRKNKRRR ($ATR_{15}$), AGLQVRKNKRRRGCP ($AGL_{15}$), LQVRKNKRRRGCPIL ($LQV_{15}$), all from JPT Peptide Technologies, ≥90% purity). $ddH_2O$ and Staphylococcus enterotoxin B (10 µg/ml, SEB, Sigma-Aldrich) were added as negative and positive controls, respectively. The CD107a antibody (Ab) was added together with the stimulus, protein transport inhibitors Brefeldin A (10 µg/ml, Sigma-Aldrich) and Golgi Stop (BD) 1 h thereafter. After 12 h at 37°C and 7.5% $CO_2$, cells were stained (Ab Panel 1, online supplementary table S4). For *ex vivo* analysis of PD-1, OX-40, and LAG-3 expression (Ab Panel 2, online supplementary table S4), fluorescence minus one controls were performed. After staining (for protocol see online supplementary table S4), cells were acquired on the same day on a LSRFortessa$^{TM}$ SORP (BD) equipped with the DIVA Software (Version 6). The analysis was performed with FlowJo (Version 10.6.1), gating strategies are shown in online supplementary figure S1. All results were audited. Frequencies of RV001-specific T cells are defined as: % marker positive cells in the RV001-stimulated sample minus % of marker-positive cells in the $ddH_2O$ sample.

**Identification of RV001-presenting allelic products**

Potential RV001-derived HLA-class I and -class II epitopes were tested by using HLA-matched human lymphoblastic cell lines (LCLs) as peptide-presenting cells. C1R and its HLA-B*27:05 transfectant (C1R-B*27) were cultured in RPMI 1640 supplemented with 10% hi FBS, P/S, 50 µM β-ME and G418 (Sigma-Aldrich) at 1 mg/ml. MGAR and H0301 were cultured in RPMI 1640 20% hi FBS, P/S, 50 µM β-

ME (for HLA-typing results, see online supplementary table S3). Per condition, 8 x $10^6$ LCL cells were loaded with 50 µg/ml RV001, or20 µg/ml RV001-derived single 15mer peptides ($ATR_{15}$, $AGL_{15}$), or with the respective amount of $ddH_2O$ in 1.5 ml of IMDM supplemented with 5% hi HS, P/S and 50 µM β-ME. After 7 h at 37°C, 7.5% $CO_2$, cells were washed three times. PBMCs stimulated with RV001 plus Hiltonol® for 12 days were either incubated with the pre-loaded HLA-matched LCLs at a 2:1 effector to target ratio, with 50 µg/ml RV001, or with 10 µg/ml of each 15mer peptide ($ATR_{15}$, $AGL_{15}$, $LQV_{15}$). $ddH_2O$ and 10 µg/ml SEB were used as controls. After 12 h, ICS was performed.

**Immunological response definition**

For each visit, a T cell response was defined by the DFR(2x) permutation resampling method [26]. A patient was defined as an immunological responder if at least two out of three analyzed times were tested positive in the ELISpot. If T cell reactivity against RV001 was already detected at baseline level, the patient was considered as a responder if response was boosted during vaccination (specific spot counts after vaccination ≥ 2 x specific spot counts at baseline). If less than three vaccination samples were evaluable (n=1), T cell reactivity detected at one time was enough to consider the patient as an immunological responder. Patients were grouped according to the sum of RV001-specific mean spot numbers for visits 6+8+13: strong- (n=7; ≥2500 spots), intermediate- (n=7; ≥1500-2500 spots), and weak/non- (n=7 including 3 non responders; 0-1500 spots) responders.

For the ICS, T cell reactivity was assessed within CD4 and CD8 T cell subsets. First, each activation marker (n=5) was stated as positive if the percentage of positive cells was ≥ three-fold for the peptide-stimulated cells compared to the $ddH_2O$ stimulated cell (and, ≥20 marker-positive cells were counted). Second, at least three out of five markers must be positive.

**Statistical comparisons**

Statistical analyses were performed with GraphPad Prism 6 (version 6.01). The Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilliefors corrected P value was used to check Gaussian distribution. One-way and two-way ANOVA were performed for single- and multiple-group comparisons, respectively. Correction for multiple comparisons was performed. Statistical differences were considered as significant for

P ≤ 0.05 (*), P ≤ 0.01 (**), and P ≤ 0.001 (***). Statistical test and number of patients included are given for each experiment.

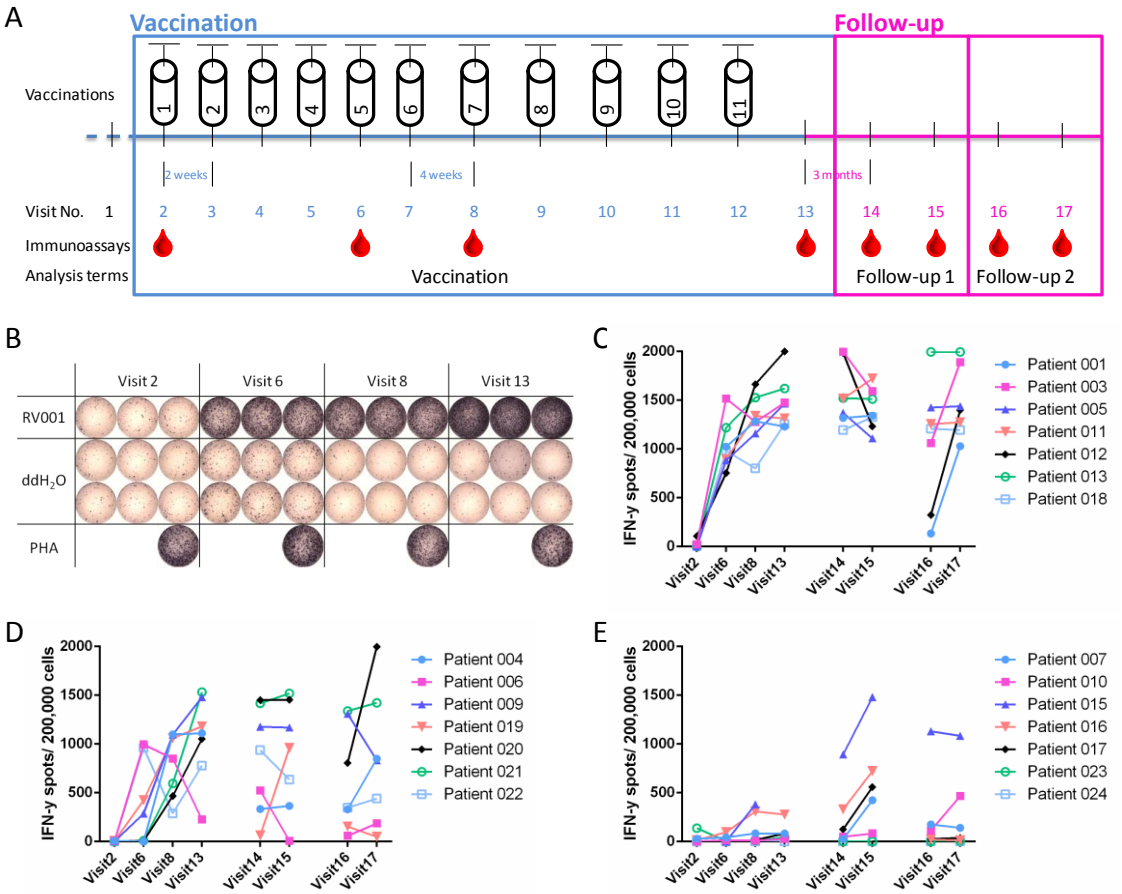
**General Lab Operation**

All experiments were performed with standard reagents and according to laboratory-standard protocols for culture, assays, and analysis. Protocols for ELISpot and ICS have been validated and the performance of the working group is regularly controlled by participation in external proficiency panels (CIP/CIMT, and Immudex).

## 2.1.4 Results

**Vaccination against RhoC induces potent and long-lasting RV001-specific T cell responses**

The immunological response against the RV001 vaccine was assessed on PBMC samples obtained during the vaccination phase and at all follow-up times for 21/22 evaluable patients (figure 1A). An exemplary IFN-γ ELISpot result (Patient 011) is shown in figure 1B. Patients were grouped according to the strength of their T cell response into strong- (figure 1C), intermediate- (figure 1D), and weak/non-responders (figure 1E). In most cases, vaccine-reactive T cells were detected after four vaccinations (visit 6). In strong-responders, T-cell frequencies reached a plateau at visit 13 (after 11 vaccinations), which lasted for 13 months post-vaccination. Weak-responder patients (Patients: 007, 015, 016, 017) show a maximal response mainly at visit 15, seven months post-vaccination. Specific mean spot counts per patient and visit are displayed in figure 1F. In total, 18/21 (86%) of the patients mounted a T-cell response during vaccination and 19/21 (90%) patients during the follow-up (light green). One spontaneous RV001-specific response was detected in Patient 012 PBMCs (visit 2), which was boosted by vaccination (approx. 20 fold) and lasted until the last follow-up visit (figure 1F). Interestingly, Patient 010, classified as non-responder during vaccination, developed a statistically significant response against the RV001 peptide at visit 15, which increased further at visits 16 and 17 (1.4 and 4 fold increase, respectively). Only one RV001-specific response was lost at the last follow-up visit (Patient 007). The high response rate among patients with various MHC allelic products indicates a broad immunogenicity of the vaccine. In addition, T cells were mostly detectable for at least ten months (visit 16) post-vaccination, suggesting the induction of a stable immunological memory.




F

| | Vaccination | | | | Follow-up 1 | | Follow-up 2 | |
|---|---|---|---|---|---|---|---|---|
| | Visit 2 | Visit 6 | Visit 8 | Visit 13 | Visit 14 | Visit 15 | Visit 16 | Visit 17 |
| 001 | na | 1025 | 1285 | 1230 | 1320 | 1342 | 134 | 1029 |
| 003 | 25 | 1518 | 1277 | 1477 | 1998 | 1593 | 1061 | 1891 |
| 004 | 7 | 0 | 1099 | 1110 | 335 | 365 | 332 | 850 |
| 005 | 2 | 883 | 1159 | 1467 | 1371 | 1109 | 1426 | 1440 |
| 006 | 5 | 996 | 850 | 225 | 524 | 8 | 60 | 186 |
| 007 | 30 | 42 | 85 | 82 | 26 | 424 | 175 | 140 |
| 009 | 2 | 287 | 1097 | 1481 | 1177 | 1169 | 1309 | 832 |
| 010 | 0 | 15 | 10 | 28 | 49 | 83 | 115 | 465 |
| 011 | 2 | 895 | 1339 | 1314 | 1516 | 1722 | 1256 | 1274 |
| 012 | 108 | 754 | 1664 | 1993 | 1986 | 1232 | 324 | 1400 |
| 013 | 0 | 1219 | 1528 | 1621 | 1523 | 1512 | 1995 | 1996 |
| 015 | 0 | 0 | 380 | na | 893 | 1481 | 1131 | 1084 |
| 016 | 14 | 96 | 309 | 278 | 331 | 723 | 23 | 13 |
| 017 | 2 | 4 | 16 | 85 | 127 | 559 | 28 | 37 |
| 018 | 7 | 981 | 802 | 1257 | 1195 | 1328 | 1207 | 1196 |
| 019 | 6 | 424 | 1060 | 1181 | 62 | 959 | 155 | 49 |
| 020 | 0 | 7 | 468 | 1053 | 1451 | 1454 | 805 | 1998 |
| 021 | 3 | 9 | 595 | 1533 | 1418 | 1520 | 1339 | 1422 |
| 022 | 3 | 963 | 289 | 779 | 938 | 636 | 347 | 440 |
| 023 | 138 | 6 | 11 | 44 | 0 | 0 | 0 | 0 |
| 024 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| Number of responder (%) | 1/20 (5%) | 12/21 (57%) | 18/21 (86%) | 17/20 (85%) | 18/21 (86%) | 19/21 (90%) | 19/21 (90%) | 18/21 (86%) |

**Figure 1: RV001-specific T cells are induced after RhoC vaccination. (A) Vaccination and monitoring schedule.** Patients were vaccinated 11 times. For immunoassays, blood was taken pre-vaccination, three times during the vaccination phase (vaccination) and four times post-vaccination (follow-up 1 and follow-up 2) (blood drops). PBMCs were pre-stimulated with the RV001 and expanded for 12 days before IFN-γ ELISpot testing (0.2 x 106 cells/well, except for Patient 21 visits 2-13, and Patient 012 visits 16+17: 0.17 x 106 cells/well). (B) Exemplary result of an ELISpot (Patient 011). ddH2O and PHA were used as negative and positive control, respectively. (C-E) RV001-specific mean spot counts per analyzed time and normalized to 0.2 x 106 cells/well. Three independent ELISpot experiments were performed (indicated by the gaps). The sums of RV001-specific mean spot numbers (V6+8+13) are shown for strong- (C; n=7; ≥2500 spots), intermediate- (D; n=7; ≥1500-2500 spots), and weak/non- (E; n=7; 0-1500 spots) responders. (F) RV001-specific mean spot counts per patient and visit normalized to 0.2 x 106 cells/well. Light green indicates a statistical significance according to the DFR(2x) permutation test. n=21 patients. na: cells not available.

**Vaccine-specific T cells are mainly polyfunctional CD4 T cells**

Multifunctional flow cytometry analysis was performed next to identify RV001-specific T cells in vaccine responders (n=18). Cells from one visit during vaccination were re-stimulated with the RV001 peptide for 12 h and tested in ICS. A representative example for Patient 011 visit 13 is shown in online supplementary figure S1A. Seventeen out of 18 patients (94%) showed a CD4 T cell response against the RV001 (figure 2A). Cells from Patient 007, classified as a weak-responder by ELISpot, did not reach the positivity threshold in ICS. CD4 T cells of strong-responder patients expressed more activation markers (mean sum of CD107a, CD154, IL-2, TNF, and/or IFN-γ: 21,7%, 95% CI 8,3 to 35,1) upon RV001 re-stimulation than CD4 T cells of intermediate-responders (mean sum: 9,0%, 95% CI 3,2 to 14,7) and significantly more as CD4 T cells of weak-responders (mean sum: 2,4%, 95% CI -3,8 to 9,0) (figure 2B). This supports our previous classification of patients in the three groups according to the ELISpot results. To assess multifunctionality, boolean gating was performed: 81% of the RV001-specific CD4 T cells expressed at least two activation markers and of these, almost half (43%) at least three markers simultaneously. When comparing the three patient groups, especially strong-responders showed a significantly higher frequency of RV001-specific T cells expressing the two-three marker combinations (figure 2C). Among all patients together, the three most frequent subsets of RV001-specific CD4 were those expressing CD154 and TNF only (mean: 2,8%, 95% CI 1.3 to 4.3), CD154+TNF+CD107a (mean: 2,5%, 95% CI 0.8 to 4.3), or CD154+TNF+CD107a+IFN-γ (mean 1,1%, 95% CI 0.1 to 2.1). On average, 0,49% (95% CI 0.19 to 0.80) of the RV001-specific cells within the CD4 T cell population express all five markers simultaneously (figure 2D).

PBMCs obtained from n=10 patients (including the non-responder Patient 007) after the vaccination was completed (follow-up 1 visits 14 and 15) were also examined. Nine out of ten patients still showed a CD4 T cell response against the RV001, while Patient 007 was still non-responder (not shown). In addition to a CD4 T cell response, Patient 004 showed at both visits also a CD8 T cell response against the RV001 peptide (figure 2E). In summary, this multiparametric analysis shows that RV001-specific T cells are mainly polyfunctional CD4 effectors, and identifies in addition the presence of (at least) one HLA-class I-presented epitope within the vaccine-peptide sequence.

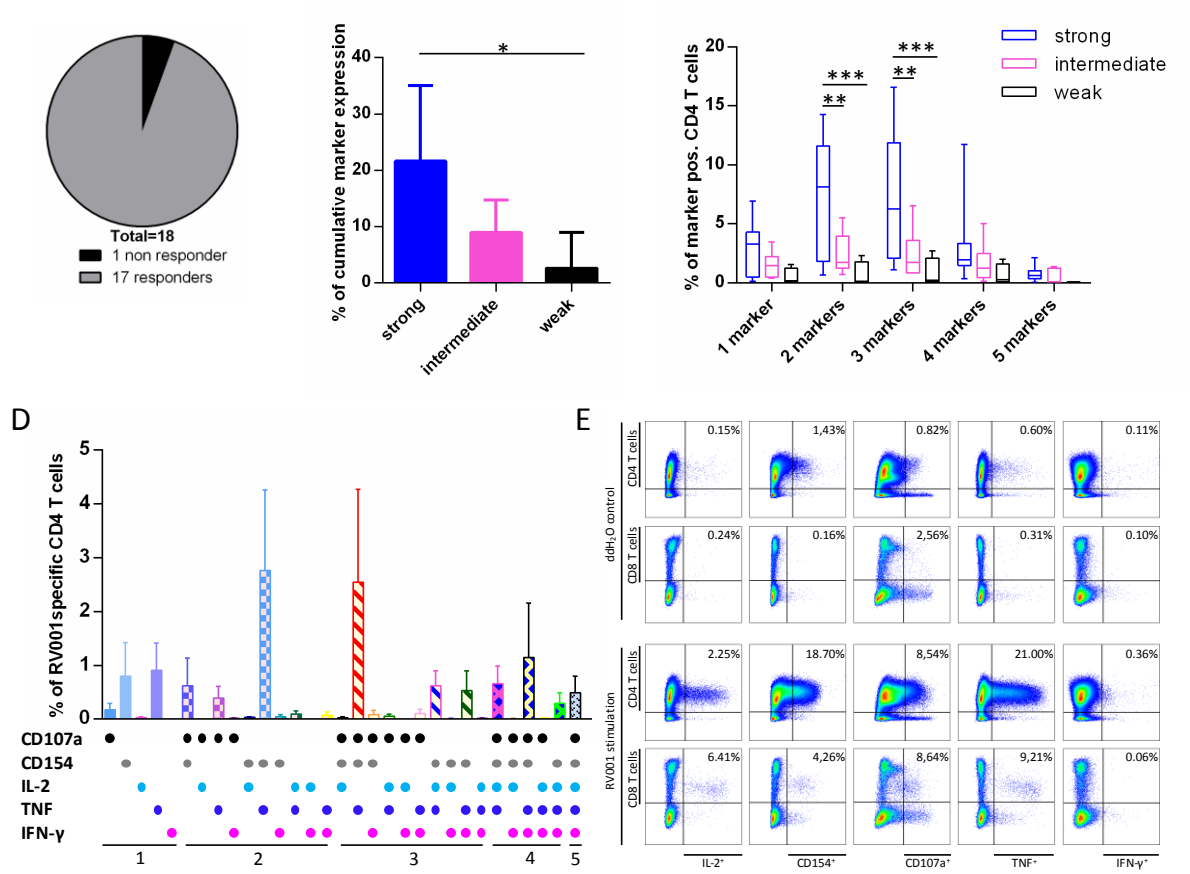



**Figure 2: RV001-responding cells are multifunctional**. $ddH_2O$ stimulated cells harvested from the ELISpot were re-stimulated with RV001 for 12 h. Expression of CD107a, CD154, IL-2, TNF, and IFN-γ was examined by ICS on live CD4 and CD8 lymphocytes and the % of RV001-specific cells calculated for each of the 5 markers within CD4 or CD8 cell subsets. (A) Overview of CD4 T cell responses during vaccination (n=18 patients). (B) Mean +95% CI of cumulative marker expression on RV001-specific CD4 T cells for strong- (n=7), intermediate- (n=7) and weak- (n=4) responders. Kruskal-Wallis test with Dunn´s post-test. (C) Min to max percentages of RV001-specific CD4 T cells expressing one to five markers simultaneously, classified per strong- (n=7), intermediate- (n=7), and weak (n=4) responders. Median values are indicated. Two-way ANOVA with Tukey´s post-test. (D) Mean +95% CI of RV001 specific CD4 T cells expressing each of the five activation markers or combinations thereof (all patients, n=18). (E) 12 day-cultured PBMCs from Patient 004 at visit 14 were re-stimulated with $ddH_2O$ (upper dot plot panel) or the RV001 (lower dot plot panel). The activation marker expression was examined on living CD4 (upper rows) and CD8 (lower rows) lymphocytes. Percentages of marker+ cells within CD4 or CD8 cells are given. P ≤ 0,05, **P ≤ 0,01, ***P ≤ 0,001. Responder groups are defined based on the ELISpot results.

## Vaccination against RhoC induces memory CD4 T cells

A long-lasting anti-tumor immune response is mediated by T-cell memory formation. To address the phenotype of RV001-specific T cells, we examined the differentiation status (CD45RA/CCR7), as well as the expression of OX-40 (activation marker), PD-1 (activation/exhaustion marker), and LAG-3 (exhaustion marker) by *ex vivo* multiparametric flow cytometry. Gating strategy for Patient 018 is available online supplementary figure S1B. Patients with a strong or intermediate IFN-γ response in the ELISpot were selected. RV001-specific T cells were identified by their TNF

expression after stimulation (12 h). This short culture-step does not modify the expression of the selected markers on T cells (data not shown). A representative overlay of $CD4^{+}TNF^{+}$ cells (black) on all CD45RA/CCR7 gated CD4 T cells (grey) is shown for Patient 009 in figure 3A. RV001-specific T cells were mostly effector memory T cells ($CD45RA^{-}CCR7^{-}$) and detectable already after the $4^{th}$ vaccination at visit 6. The response peaked between visits 6 and 13 for all 3 patients tested (see also online supplementary figure S2). PD-1 and to a lesser extent OX-40 (but not LAG-3, data not shown) were expressed on RV001-specific cells. However, median fluorescence intensities did not appear to increase in the course of the vaccination as compared to those of the whole CD4 subset, suggesting that vaccine-specific cells did not especially differentiate towards an exhausted phenotype upon repeated vaccination (figure 3B).

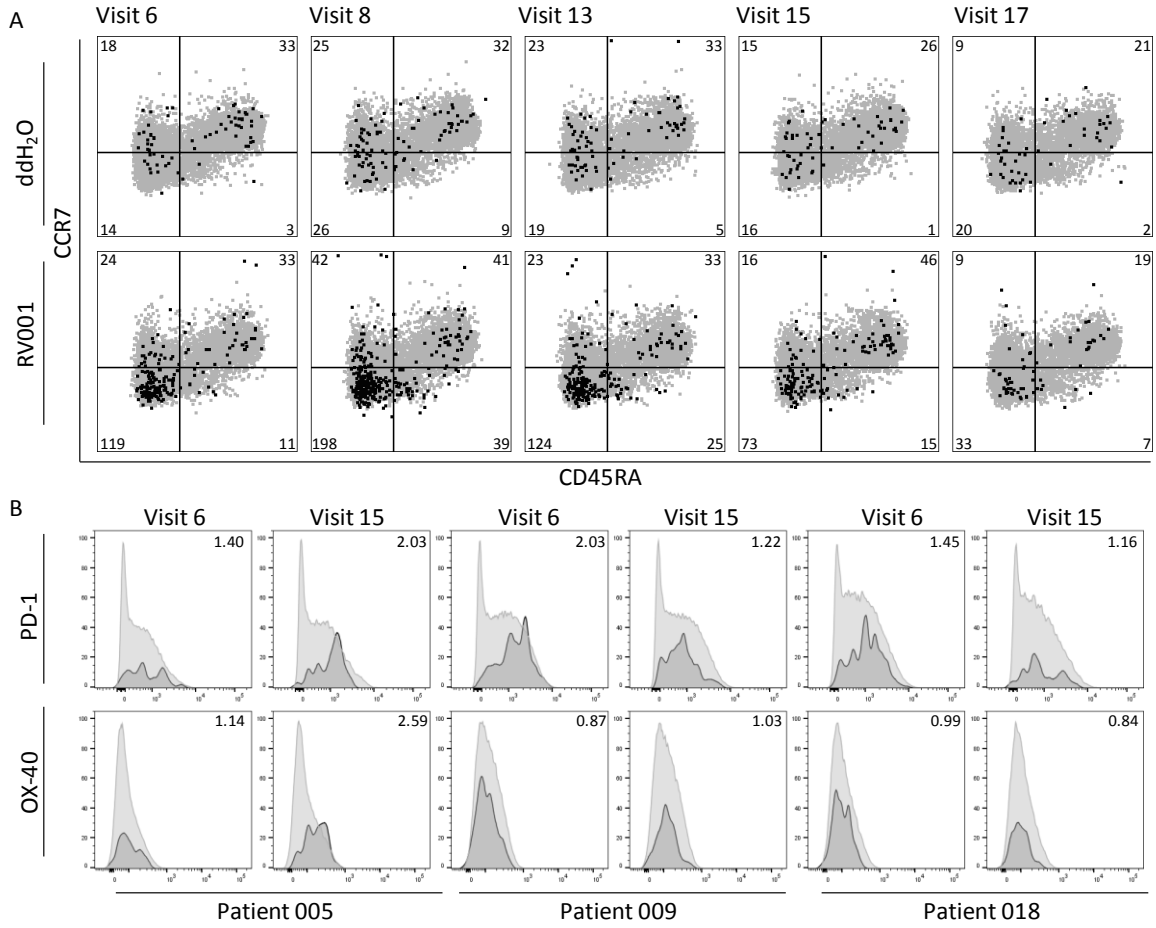



**Figure 3: RV001-specific T cells are effector memory T cells stably expressing PD-1 and OX-40 over time.** PBMCs from Patient 005 (visit 6 to visit 15) and Patients 009 and 018 (visit 6 to visit 17) were thawed, rested, and stimulated either with RV001 or $ddH_2O$ for 12 h. Live RV001-specific CD4 lymphocytes were identified by TNF expression and examined for the expression of CD45RA, CCR7, PD-1, OX-40, and LAG-3. (A) Exemplary results (Patient 009): $CD4^+TNF^+$ cells (black) are overlaid on the whole CD4 cell population (grey). Numbers indicate $CD4^+TNF^+$ cell counts. (B) Expression profile of PD-1(upper row) and OX-40 (lower row) for n=3 patients at visit 6 and visit 15. Numbers indicate median fluorescence intensity (MFI) ratios of the two receptors between $CD4^+TNF^+CD45RA^-CCR7^-$ cells (dark grey) and $CD4^+TNF^-CD45RA^-CCR7^-$ cells (light grey). Histograms show event counts normalized to mode.

## Several epitopes are recognized by T cells within the RV001 sequence

The 20 amino-acid long RV001 peptide might serve as a CD4 T cell epitope itself, but might also contain several shorter CD4 T cell epitopes. To identify such sequences, we expanded RV001-specific T cells from selected PBMC samples (n=7, two follow up visits each). After 12 days, cells were re-stimulated with RV001, or with single RV001-derived 15mer peptides ($ATR_{15}$, $AGL_{15}$, $LQV_{15}$; 15aa overlap) for 12 h, followed by ICS staining. Representative results for CD4 T cells of n=3 patients from visit 16 samples are shown in figure 4A and in online supplementary figure S3. We found that all three peptides could be recognized, albeit at various rates. Peptide

$AGL_{15}$ was recognized by cells from all three patients, peptide $LQV_{15}$ by cells from Patients 001 and 003, and $ATR_{15}$ by cells from Patient 019 only.

Next, we used two human LCLs MGAR and H0301 to identify the presenting HLA-class II allele/s for Patients 001, 003, and 019. According to the four-digit HLA-typing, the HLA-DRB1*13:02 allele was expressed by Patient 001, Patient 003, and by H0301, whereas HLA-DRB1*15:01, -DQB1*06:02, -DPB1*04:01 alleles were shared between Patient 019 and MGAR (online supplementary table S3). RV001 pre-stimulated PBMCs were mixed with pre-loaded (RV001, $ATR_{15}$, or $AGL_{15}$) LCLs for 12 h. Both 15mers were recognized by CD4 cells of Patients 001 and 003. For Patient 019, only a response against RV001 was detected, while co-incubation with $ATR_{15}$ or $AGL_{15}$ pre-loaded LCLs led to an increase in IFN-γ, CD154, and IL-2, but did not reach the pre-defined positivity threshold (figure 4B). Based on these findings, we concluded that several epitopes derived from the RV001 sequence are presented by HLA-DRB1*13:02, and possibly by DRB1*15:01, and/or -DQB1*06:02, and/or -DPB1*04:01, three alleles co-expressed in 80% of the HLA-DRB1*15:01$^+$ patients in the cohort.

The next step was to identify the presenting allele of the RV001-derived HLA-class I epitope that was recognized by CD8 T cells from Patient 004 (HLA-A*02/A*30$^+$, -B*18/B*27$^+$), as we had observed (figure 2E). PBMCs from Patient 004 visit 15 were pre-stimulated with RV001, and incubated with either the RV001 or the RV001 pre-loaded C1R or C1R-HLA-B*27 LCLs. A CD8 T cell response (CD107a, CD154, TNF, and IL-2 expression) was observed upon stimulation with the RV001 peptide or with the RV001 pre-loaded HLA-B*27 LCL, but not with the non-transfected LCL (figure 4C). These findings clearly show that a RV001-derived sequence is presented to CD8 cells by the HLA-B*27:05 allele.

In summary, the RV001 peptide contains at least three different HLA-class II peptides promiscuously presented on various HLA-class II allelic products, as well as one HLA-class I class I peptide presented by the HLA-B*27:05 allele.

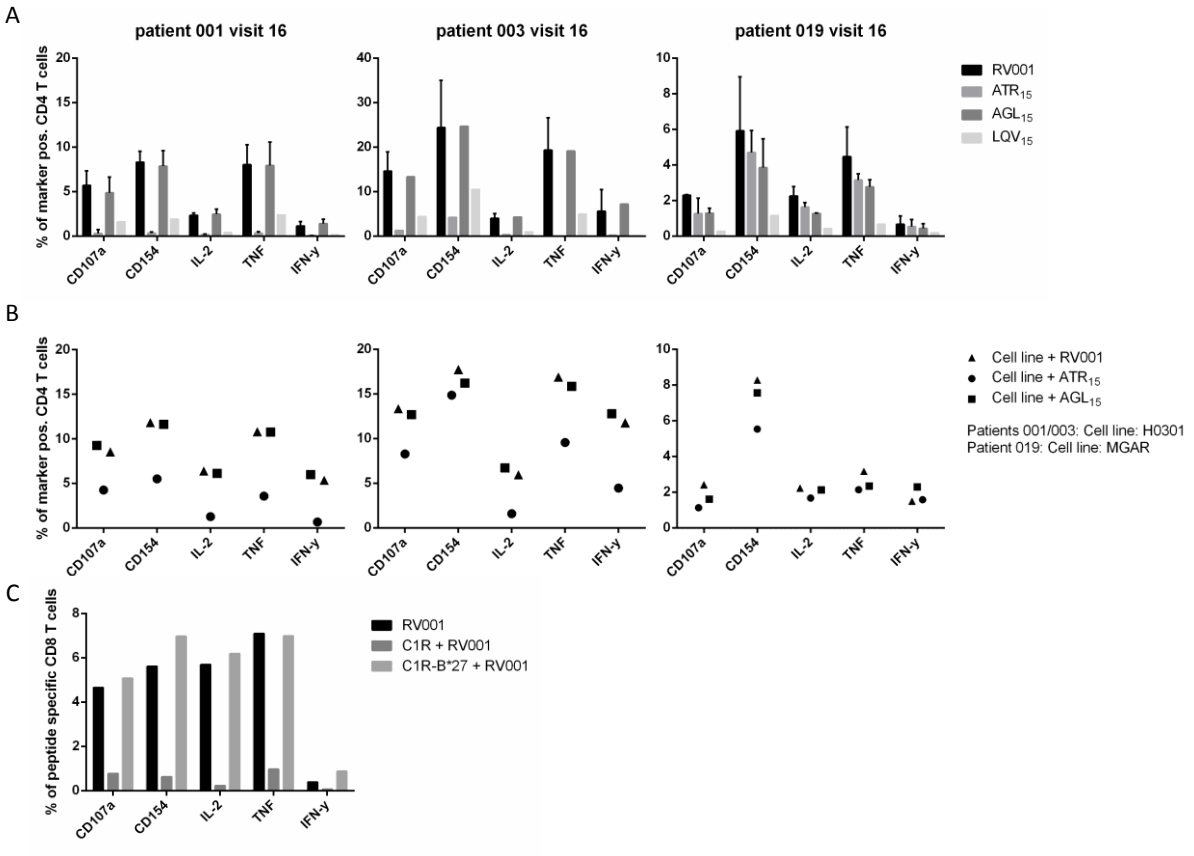



**Figure 4: The RV001 sequence comprises several promiscuous HLA-class II epitopes and one HLA-B*27:05 restricted epitope.** The expression of CD107a, CD154, IL-2, TNF, and IFN-γ was examined on live CD4 or CD8 cells. (A) Cells were re-stimulated with RV001 or with RV001-derived 15mer peptide ($ATR_{15}$, $AGL_{15}$, $LQV_{15}$) for 12 h. Shown are the percentage or mean +SD percentage (n=2 repeated measurements) of peptide-specific CD4 cells expressing each activation marker for three patients at visit 16. (B) LCLs were pre-loaded with RV001, $ATR_{15,}$ or $AGL_{15}$ and incubated with HLA-matched patient cells in at 1:2 ratio for 12 h. Shown are the specific percentages of CD4 cells expressing the indicated activation markers. (C) Cells were re-stimulated either with the RV001 peptide alone or with RV001-preloaded C1R or C1R-HLA-B*27:05 cells for 12 h in the ICS. Shown are the percentages of specific marker expression on CD8 cells.

## Vaccine safety

No adverse event led to discontinuation of treatment in any patient. Most frequent treatment-related events were fatigue and injection site reactions of grades 1 or 2. All patients experienced at least 1 treatment-emergent adverse event (TEAE) of injection site reaction that was considered related to the RV001 vaccine. Four patients (18%) experienced at least 1 grade 2 TEAE of injection site reaction. One patient had a TEAE of fatigue (grade 1) that was probably related to the RV001 vaccine, and one patient a TEAE of hot flush (grade 1), also probably related to the RV001 vaccine. No treatment-related side effect of grade 3 or higher occurred (table 2).

Table 2: Treatment-related side effects

| Side effects | Possible treatment-related*, Patients (%) | Probable treatment-related** Patients (%) | All treatment-related events Patients (%) |
|---|---|---|---|
| **Fatigue** | 2 (9.1) Grade 1 | 1 (4.5) Grade 1 | 3 (13.6) |
| **Injection site reactions** | 2 (9.1) Grade 1 | 18 (81.8) Grade 1<br>4 (18.2) Grade 2 | 22 (100) |
| **Viral upper respiratory tract infection** | 1 (4.5) Grade 1 | | 1 (4.5) |
| **Headache** | 1 (4.5) Grade 1 | | 1 (4.5) |
| **Paraesthesia** | 1 (4.5) Grade 1 | | 1 (4.5) |
| **Hot flush** | 1 (4.5) Grade 1 | 1 (4.5) Grade 1 | 2 (9.1) |
| **Total number of patients*** ** | 6 (27.3) | 22 (100) | 22 (100) |

N=22 patients; */** clinical events, including laboratory test abnormality, with a reasonable time sequence to administration of the vaccine, but which can also be explained by concurrent disease or other drugs or chemicals (*) or which can unlikely be attributed to concurrent disease or other drugs or chemicals (**). *** indicates the total number of patients reporting at least one event.

## 2.1.5 Discussion

Recent cancer vaccine studies targeting the tumor mutanome have demonstrated a high rate of immunological responses as well as encouraging clinical courses [1,2]. Such studies are *per se* individualized, since they target mutations that mostly occur in individual patients. Other vaccine strategies that are suitable for tumors with low level of mutational events focus on non-mutated, tumor-specific or tumor-associated antigens [3,11,27,28]. Prostate tumors generally harbor only few mutations, and respond poorly to checkpoint Ab therapy. Clinical studies have demonstrated that vaccination against prostate-associated or overexpressed antigens is safe, immunogenic and can impact favorably clinical course. Most of these trials were conducted at advanced stages of the disease (castration-resistant metastatic PCa), but few at earlier times, e.g. at biochemical relapse [27-30]. Immune intervention at BCR, when tumor load is limited and immunosuppression absent or limited might lead to more favorable clinical courses. In this context, targeting of antigens associated with cancer stem cells and metastases, like the RhoC protein, could improve tumor control.

The SLP vaccination against RhoC was safe for all patients over the complete treatment. Side effects were predominantly injection site reactions and fatigue (grade

1/2). These events are most likely related to the adjuvant and carrier Montanide ISA-51 [31] and did not necessitate specific medical intervention.

Immunogenicity of the vaccine was assessed in n=21 patients. Most of these (94%) had developed T cells specific for the RhoC peptide after the $4^{th}$ vaccination. We frequently observed high IFN-γ ELISpot counts (>1000 spots/ 200.000 cells), indicating excellent immunogenicity of the vaccine *in vivo* and/or a robust *in-vitro* proliferation capacity. T cell responses were overall stable and were detected almost one year after the last vaccination. This long-lasting functional immune response indicates the establishment of an immunological memory, which is essential for immunosurveillance of recurrent tumors and/or metastases. We found that anti-vaccine T cells belonged predominantly to the effector memory CD4 subset and were polyfunctional cells (> 80% of RV001 specific CD4 T cells expressed at least two of the activation markers/cytokines tested). They often expressed the cell-surface degranulation marker CD107a, suggesting that at least a fraction of those were cytotoxic effectors. Moreover, *ex vivo* phenotyping strongly (PD-1, LAG-3) suggests that RV001-specific cells did not shift towards an exhausted phenotype after multiple vaccine applications. We did not directly examine $CD4^+CD25^+FoxP3^+$ T regulatory cells (Tregs), but the fact that activated Tregs express no or very little CD154 [32] strongly suggests that essentially T helper cells were induced by the vaccine. Although the vaccine contained a single 20mer peptide, we could identify three HLA-class II 15mer RV001-derived epitopes. The high response rate in our patient cohort indicates that these, and possibly further as yet unidentified class-II epitopes, are presented promiscuously on several MHC-class II alleles (including DRB1*13:02). Hence, the RhoC vaccine can be applied broadly, independently of the patient's HLA allotype. In addition, a CD8 response restricted by HLA-B*27:05 could be observed in one patient. The exact epitope is under characterization, and further $HLA\text{-}B27^+$ patients will be assessed. Interestingly, RV001-specific CD8 T cells in that patient were polyfunctional, with high level of TNF and/or CD107a, IL-2, CD154. $CD154^+$ CD8 T cells (also called CD8 helper cells) have been shown to support their own expansion and differentiation and to activate dendritic cells to promote anti-tumor immunity [33]. Although the RhoC 20mer contains an embadded HLA-A*03 binding peptide, we did not observe any CD8 T cell response (one single time point tested) in the two patients carrying the HLA-A*03 allele [23].

CD4 T cells are now widely recognized as key players in anti-tumor immunity. Their role in dendritic cell and CD8 T activation, and in memory formation are well described [6,34,35]. CD4 T cells were also shown to kill HLA-class II positive tumors via granzyme/perforin release or Fas/Fas-L interaction [9,36], and there is also pre-clinical evidence that CD4 T cells can reject tumors better than CD8 T cells [10]. We could not test the HLA-class II expression of PCa tissues in our cohort, but HLA-class II expression has been detected on primary prostate cancer cells and on PCa cell lines, or could be induced by IFN-γ [37,38]. Indirect tumor killing by CD4 T cells can also be mediated by IFN-γ and TNF [7,8], as well as by the recruitment of nitric oxide producing macrophages within the tumor [39]. Altogether, our phenotyping and functional data indicate that the profile of vaccine-induced, RV001-specific CD4 T cells is in line with that of anti-tumor effectors.

Vaccine-based studies in mice and patients have started to unravel the contribution of CD4 T cells in tumor control [11,40,41]. High rate of polyfunctional CD4 cells, together with CD8 T cells, were detected in melanoma patients vaccinated with a personalized, neoantigen-based SLP vaccine containing up to 20 long mutated peptides. Four out of six patients had no recurrence 25 months post-vaccination, while the two patients with tumor recurrence achieved tumor regression when treated with an anti-PD1 Ab [1]. Case reports document tumor regressions after adoptive transfer of anti-tumor CD4 cells [42,43]. In mouse models, there is also evidence that CD4 chimeric antigen receptor (CAR) T cells are more potent than CD8 CAR T cells, as they can kill tumors, and in addition, exhibit long-lasting effector function [44,45].

Although clinical response was not a primary endpoint of this phase I/II study, patients were monitored for PSA serum levels and tumor progression. PSA doubling time is regarded as a surrogate parameter for PCa progression, and PSA doubling time can predict the risk of mortality in men with localized PCa [46]. Three patients progressed biochemically during follow-up (Patients 006, 015, 018). Two of these patients (Patient 006: followed for 24 months -12 PSA measurements-; Patient 018: followed for 52 months -9 PSA measurements-) had BCR at study entry. Patient 006 had a PSA increase from 0.5 to 1.1 µg/L 29 months following study entry, and Patient 018 from 1.1 to 1.5 µg/ml 24 months following study entry. Patient 015, who received only seven vaccinations, and presented with a pT2c R+ Gleason score 7 (4+3) PCa, developed BCR with a PSA doubling time of 1.2 years and final PSA level of 0.28 µg/L 26 months after vaccination end. When comparing the pre-study PSA doubling

time to that on study, we observed an increase from 1.3 to 2.1 years for Patient 006, and from 1.95 to 3.8 years for Patient 018. Patient 015 and 006 developed BCR 13 months post-vaccination (visit 17), however, PSA level in Patient 006 declined the last months on follow-up, and from a clinical perspective, the patient was considered as biochemically stable. No patient developed clinical signs of recurrence. Although these observations are encouraging, the finding of an increase in doubling time following vaccination shall be interpreted with caution, as PSA kinetics are influenced by the period over which the PSA values are measured (limited time span in our study) and the number of tests drawn [47]. It is estimated that approximately one in four patients undergoing RP will eventually experience BCR. The risk of BCR varies according to preoperative PSA and histopathological findings [48,49]. The individual risk of BCR the first year following enrolment in this study ranged from 2 to 19% and did not change substantially in the period of follow-up [50].

In summary, vaccination against RhoC is a well-tolerated treatment option which induces a long-lasting immune response in the large majority of patients. Vaccine-specific cells are polyfunctional and equipped for an anti-tumor response. A correlation between the induction of immune responses to RhoC upon vaccination and clinical outcome is at this stage premature, but will be examined in a recruiting double-blind, placebo-controlled, phase II trial for PCa patients in BCR (NCT04114825). RhoC vaccination to impair tumor spreading might also synergize with many tumor vaccines (such as those targeting patient-individual tumor antigens) or other therapies, and be a valuable approach for prostate cancer and for further tumor entities.

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## 2.1.7 Supplement Chapter 2I: Vaccination targeting RhoC induces long-lasting immune responses in prostate cancer patients: results from a phase I/II clinical trial

Supplementary table S1: Inclusion and exclusion criteria of the open label clinical phase I/II study

| Inclusion criteria | Exclusion Criteria |
|---|---|
| ≥ 18 years | Patient is a candidate for relevant therapies that are the current standard of care for their cancer disease |
| Patients prostatectomised (PT) due to histologically verified adenocarcinoma of the prostate gland who currently are not being treated, or expected within the next 8 months to be treated, with any anti-cancer treatment. Patients may or may not have measurable PSA. | Patient has been treated with Androgen Deprivation Therapy (ADT) or expected to receive such treatment within the next 8 months from enrolment. |
| Able to understand the study procedures and willing to provide informed consent. | Concurrent chemotherapy or radiotherapy within 12 weeks of 1st vaccination, or expected to receive such treatment within the next 8 months from enrolment. |
| Able and willing to comply with study requirements and complete all visits. | Patients have undergone major surgery or have had major bleeding within the last month prior to the first vaccination. |
| Using adequate contraceptive measures.<br>All non-vasectomized patients must use condoms during the study and for one month after the last vaccination with RV001V, or have a female partner who either has been post-menopausal for more than one year or is using a highly effective method of contraception (i.e., a method with less than 1% failure rate). | Patients with brain or leptomeningeal metastasis. |
| Eastern Cooperative Oncology Group (EOCG) status 0 or 1. | Prior treatment with any therapeutic anti-cancer vaccine(s). |
| Recovered/stabilized at grade ≤2 from all toxicities related to prior treatment(s) in accordance with Common Terminology Criteria for Adverse Events (CTCAE) | History of second malignancy (except for adequately managed basal cell carcinoma and squamous cell carcinoma of the skin). |
| Laboratory values obtained ≤30 days prior to the first vaccination, and more than 3 weeks after potential chemotherapy<br>• Haemoglobin ≥5.6 mmol/L<br>• Absolute granulocyte count ≥1.5 x $10^9$ /L<br>• Platelets ≥100 x $10^9$ /L<br>• Total bilirubin ≤1.5 x upper limit of normal (ULN)<br>• Creatinine ≤1.5 x ULN<br>• Alanine aminotransferase/aspartate aminotransferase/ alkaline phosphatase ≤2.5 | Patients in need of or treated the last 30 days before the first vaccination with systemic steroids or other immune suppressive therapy. Use of inhaled steroids, nasal sprays, and topical creams for small body areas is allowed. |

| | |
|---|---|
| xULN | |
| | History of alcohol or substance abuse within the last 5 years. |
| | History of acquired immune deficiency syndrome or positive serological test for human immunodeficiency virus infection. |
| | History of acquired immune deficiency syndrome or positive serological test for human immunodeficiency virus infection. |
| | History of viral hepatitis B as determined by positive antibody immunoglobulin M (IgM) to core antigen for hepatitis B or positive for hepatitis B surface antigen, or viral hepatitis C as determined by positive antibody for hepatitis C. |
| | Participation in any investigational trial or use of any investigational drug(s) within 30 days prior to inclusion in this trial. |
| | Any known serious infections, e.g. tuberculosis |
| | History of significant autoimmune disease such as: Inflammatory bowel disease, Systemic lupus erythematosus, Ankylosing spondylitis, Scleroderma, Multiple sclerosis. |
| | Severe medical conditions, such as but not limited to severe asthma/chronic obstructive pulmonary disease (COPD), New York Heart Association (NYHA) grading 3 or above, poorly regulated insulin-dependent diabetes, any significant organ damage as judged by the Investigator. |
| | Other medications or conditions that in the Investigator's opinion would contraindicate study participation for safety reasons or interfere with the interpretation of study results. |
| | History of drug allergies or known allergy/hypersensitivity to Montanide ISA 51, or intolerance to subcutaneous injection. |

Supplementary table S2: Patient´s characteristics

| Patient | Age | Weight [kg] | | PSA level [µg/L] | | | |
|---|---|---|---|---|---|---|---|
| | | Baseline (visit 1) | 13 months post-vaccination (visit 17) | Baseline (visit 1) | 4 weeks post-vaccination (visit 13) | 13 months post-vaccination (visit 17) | Last PSA measurement (months** from visit 13) |
| **001** | 64 | 91,5 | 83 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (24,0) |
| **002** | 74 | 93 | 92 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (23,6) |
| **003** | 69 | 87 | 87 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (24,0) |
| **004** | 76 | 100 | 100 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (22,4) |
| **005** | 65 | 104 | 108 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (21,2) |
| **006** | 71 | 112 | 112 | 0,50 | 0,60 | 0,80 | 1,0 (22,5) |
| **007** | 62 | 100 | 101 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (23,2) |
| **009** | 65 | 88,5 | 87 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (22,2) |
| **010** | 68 | 79 | 89 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (19,9) |
| **011** | 68 | 110,5 | 101 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (22,2) |
| **012** | 74 | 82 | 80 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (22,4) |
| **013** | 65 | 93 | 90 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (21,9) |
| **015*** | 73 | 95,5 | 93 | ≤ 0,10 | ≤ 0,10 | 0,20 | 0,28 (25,4) |
| **016** | 69 | 98,5 | 93 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (21,5) |
| **017** | 71 | 75 | 75 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (21,5) |
| **018** | 77 | 90 | 96 | 1,10 | 1,10 | 1,30 | 1,30 (21,7) |
| **019** | 70 | 72,5 | 73 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (21,8) |
| **020** | 55 | 110 | 109 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (21,5) |
| **021** | 72 | 96,5 | 97 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (20,6) |
| **022** | 54 | 83,5 | 91 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10(20,9) |
| **023** | 60 | 126 | 131 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (20,5) |
| **024** | 66 | 84,5 | 81 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 | ≤ 0,10 (21) |

*Patient received only seven vaccinations instead of 11.
**Assumption 30 days per months

Supplementary table S3: HLA-typing of individual patients and cell lines

| PATIENT/ CELL LINE | HLA-A (1) | HLA-A (2) | HLA-B (1) | HLA-B (2) | HLA-C (1) | HLA-C (2) | HLA-DRB1 (1) | HLA-DRB1 (2) | HLA-DQB1 (1) | HLA-DQB1 (2) | HLA-DPB1 (1) | HLA-DPB1 (2) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **001** | 01:01 | 02:01 | 08:01 | 40:01 | 03:04P | 07:01P | 03:01 | 13:02 | 02:01 | 06:04 | 01:01 | 04:02 |
| **002** | 29:01 | 68:01 | 07:05 | 50:01 | 06:02 | 15:05 | 07:01P | 10:01 | 02:02P | 05:01 | 02:01 | 13:01 |
| **003** | 01:01 | 11:01 | 08:01 | 40:01 | 03:04P | 07:01P | 03:01 | 13:02 | 02:01 | 06:04 | 04:01 | 04:01 |
| **004** | 02:01 | 30:02 | 18:01 | 27:05 | 01:02 | 05:01 | 01:01 | 03:01 | 02:01 | 05:01 | 04:01 | 04:01 |
| **005** | 02:01 | na | 07:02 | 51:01 | 02:02 | 07:02 | 04:04 | 11:01 | 03:01 | 03:02P | 04:01 | 16:01 |
| **006** | 02:01 | na | 08:01 | 44:02 | 05:01 | 07:01 | 01:01 | 14:54 | 05:01 | 05:03P | 03:01P | 04:01P |
| **007** | 01:01 | 68:02 | 08:01 | 14:02 | 07:01 | 08:02 | 03:01 | 13:03 | 02:01P | 03:01P | 01:01P | 02:01P |
| **009** | 11:01 | na | 35:01 | na | 04:01 | na | 01:03 | 14:54 | 05:01P | 05:03P | 04:01P | 04:02P |
| **010** | 02:01 | na | 08:01 | 15:01 | 03:03 | 07:01 | 03:01 | 04:01 | 02:01 | 03:02 | 01:01 | 04:01 |
| **011** | 01:01 | 32:01 | 08:01 | 15:01 | 03:03 | 07:01 | 07:01P | 07:01P | 02:01P | 02:01P | 04:01 | 17:01 |
| **012** | 02:01 | 68:01 | 40:01 | 44:02 | 03:04 | 07:04 | 11:01 | 12:01P | 03:01 | 03:01 | 09:01 | 03:01P |
| **013** | 02:01 | 68:01 | 27:05 | 44:02 | 02:02 | 07:04 | 11:01 | 15:01 | 03:02 | 06:02 | 03:01P | 04.01P |
| **015** | 02:01 | na | 07:02 | 40:01 | 03:04 | 07:02 | 04:04 | 15:01 | 03:02 | 06:02 | 03:01 | 05:01 |
| **016** | 24:02P | 25:01 | 39:06 | 44:02 | 05:01 | 07:02 | 04:01 | 08:01P | 03:02 | 04:02 | 02:01 | 04:01 |
| **017** | 02:01 | 32:01 | 40:01 | na | 03:04 | na | 04:08 | 15:01 | 03:01 | 06:02 | 02:01 | 04:01 |
| **018** | 01:01 | 02:01 | 40:01 | 51:01 | 03:04 | 15:02 | 04:04 | 13:02 | 03:02P | 06:04P | 04:01P | 222:01 |
| **019** | 03:01 | 24:02P | 07:02 | 35:03 | 07:02 | 12:03 | 15:01 | 15:01 | 06:02 | 06:02 | 04:01 | 04:01 |
| **020** | 02:01 | 32:01 | 07:02 | 40:02 | 02:02 | 07:02 | 15:01 | 16:02 | 05:02 | 06:02 | 04:01 | 04:01 |
| **021** | 02:01 | 03:01 | 15:39 | 18:01 | 04:01 | 07:01 | 11:02 | 11:04 | 03:01 | 03:01 | 04:02 | 10:01 |
| **022** | 11:01 | 31:01 | 27:05 | 44:02 | 02:02 | 05:01 | 04:01 | 12:01P | 03:01 | 03:02P | 03:01P | 04:02P |
| **023** | 02:01 | 32:01 | 15:01 | 15:07 | 03:03 | na | 04:04 | 04:04 | 03:02 | 03:02 | 02:01 | 04:01 |
| **024** | 02:01 | na | 15:01 | 51:01 | 03:04 | 15:02 | 01:01 | 04:04 | 03:02 | 05:01 | 04:01 | 04:01 |
| **MGAR** | 26:01 | 26:01 | 08:01 | 08:01 | 07:01 | 07:01 | 15:01 | 15:01 | 06:02 | 26:02 | 04:01 | 04:01 |
| **H0301** | 03:01 | 03:01 | 14:02 | 14:02 | 08:02 | 08:02 | 13:02 | 13:02 | 06:09 | 06:09 | 05:01 | 05:01 |

P: ambiguities; na: not available

Supplementary table S4: Monoclonal antibody panels for flow cytometry analysis

| | | Marker | Fluorophore | Clone | Manufacturer | Cat. No. |
|---|---|---|---|---|---|---|
| **Panel 1** | Extracellular staining | LD | Zombie Aqua | - | Biolegend | 423102 |
| | | CD4 | APC-Cy7 | RPA-T4 | BD | 557871 |
| | | CD8 | BV605 | RPA-T8 | Biolegend | 301040 |
| | | CD107a | FITC | H4A3 | BD | 555800 |
| | Intracellular staining | TNF | Pacific Blue | MAB11 | Biolegend | 502920 |
| | | IFN-γ | PE-Cy7 | 4S.B3 | BD | 557844 |
| | | IL-2 | PE | MQ1-17H12 | BD | 554566 |
| | | CD154 | APC | 24-31 | Biolegend | 310810 |
| **Panel 2** | Extracellular staining | LD | Zombie Aqua | - | Biolegend | 423102 |
| | | CD4 | APC-Cy7 | RPA-T4 | BD | 557871 |
| | | CD8 | AF700 | SK1 | Biolegend | 344723 |
| | | CD107a | FITC | H4A3 | BD | 555800 |
| | | CD45RA | BV711 | HI100 | Biolegend | 304138 |
| | | CCR7 | BV605 | G043H7 | Biolegend | 353224 |
| | | LAG-3 | BV650 | 1C3C65 | Biolegend | 369316 |
| | | OX-40 | APC | Ber-ACT35 | Biolegend | 350008 |
| | | PD-1 | BV421 | EH12.1 | BD | 565935 |
| | Intracellular staining | TNF | PE | MAB11 | Biolegend | 502908 |

Note: All fluorophore-coupled antibodies were pre-titrated.

Protocol: For staining, cells were washed in FACS-buffer (PBS without Ca/Mg (Lonza), 0,02% NaN3, 2 mM EDTA (both Sigma-Aldrich) and 2% hi FBS (Capricorn Scientific) and stained extracellularly for 20 min at 4°C. After fixation and permeabilization for 20 min at 4°C (Cytofix/Cytoperm, BD), cells were washed with permeabilization buffer (PBS 1X, 0,02% NaN3, 0,5% BSA and 0,1% Saponin (Sigma-Aldrich)) and stained intracellularly for 20 min at 4°C. Cells were finally washed with permeabilization buffer and resuspended in FACS-buffer before acquisition.

Supplementary table S5: IFN-γ ELISpot raw data spot counts per analysis time and patient

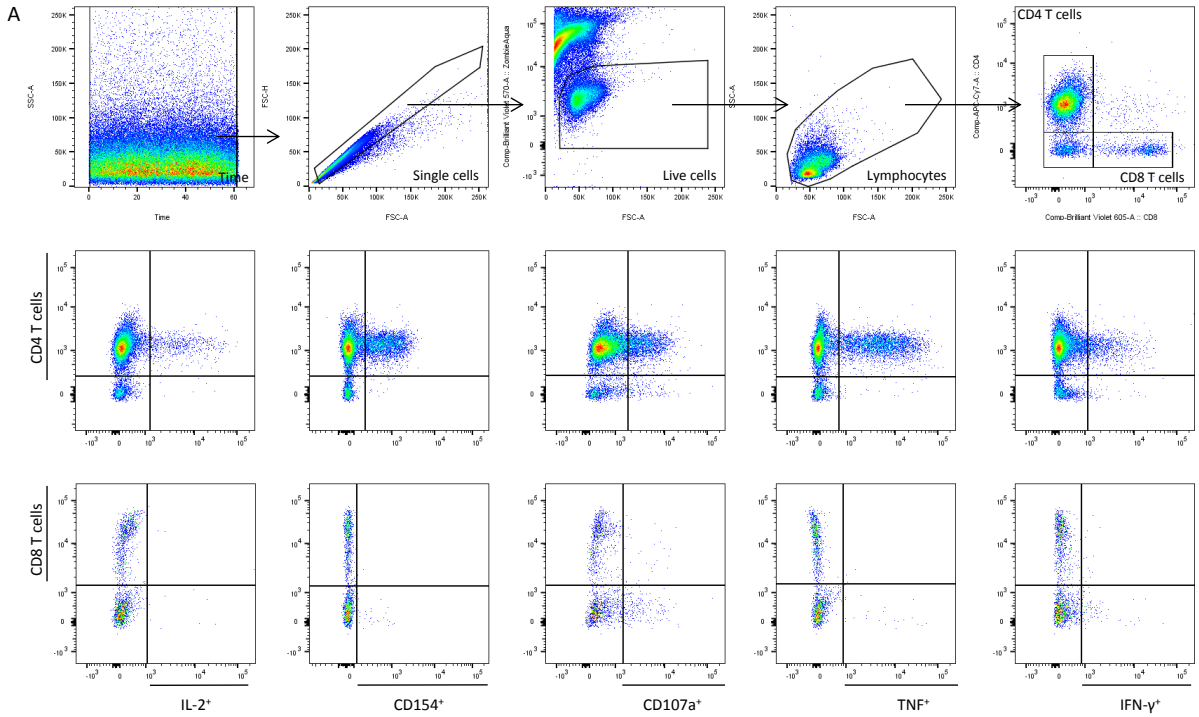
| Patient | Visit | Antigen | R1 | R2 | R3 | R4 | R5 | R6 | Patient | Visit | Antigen | R1 | R2 | R3 | R4 | R5 | R6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 001 | | RV001 | na | na | na | | | | 006 | | RV001 | 8 | 16 | 18 | | | |
| | Visit 2 | neg | na | na | na | na | na | na | | Visit 2 | neg | 5 | 14 | 17 | 5 | 4 | 9 |
| | | RV001 | 1203 | 853 | 1148 | | | | | | RV001 | 975 | 956 | 1102 | | | |
| | Visit 6 | neg | 44 | 71 | 55 | 29 | 28 | 32 | | Visit 6 | neg | 12 | 15 | 12 | 15 | 15 | 20 |
| | | RV001 | 1334 | 1292 | 1232 | | | | | | RV001 | 1026 | 807 | 882 | | | |
| | Visit 8 | neg | 0 | 2 | 0 | 4 | 2 | 1 | | Visit 8 | neg | 54 | 56 | 68 | 67 | 39 | 45 |
| | | RV001 | 1252 | 1276 | 1163 | | | | | | RV001 | 352 | 261 | 296 | | | |
| | Visit 13 | neg | 0 | 0 | 0 | 0 | 0 | 0 | | Visit 13 | neg | 69 | 217 | 47 | 49 | 42 | 42 |
| | | RV001 | 1260 | 1371 | 1328 | | | | | | RV001 | 438 | 499 | 653 | | | |
| | Visit 14 | neg | 0 | 0 | 0 | 0 | 0 | 0 | | Visit 14 | neg | 9 | 8 | 7 | 6 | 4 | 2 |
| | | RV001 | 1348 | 1341 | 1342 | | | | | | RV001 | 11 | 4 | 12 | | | |
| | Visit 15 | neg | 6 | 0 | 1 | 2 | 0 | 0 | | Visit 15 | neg | 1 | 1 | 1 | 0 | 2 | 3 |
| | | RV001 | 140 | 128 | 135 | | | | | | RV001 | 55 | 81 | 58 | | | |
| | Visit 16 | neg | 0 | 0 | 0 | 0 | 0 | 0 | | Visit 16 | neg | 5 | 6 | 6 | 5 | 3 | 4 |
| | | RV001 | 1044 | 1031 | 1013 | | | | | | RV001 | 220 | 213 | 226 | | | |
| | Visit 17 | neg | 0 | 0 | 0 | 0 | 0 | 0 | | Visit 17 | neg | 29 | 47 | 37 | 31 | 32 | 28 |
| 003 | | RV001 | 189 | 118 | 171 | | | | 007 | | RV001 | 59 | 69 | 67 | | | |
| | Visit 2 | neg | 137 | 115 | 145 | 137 | 131 | 142 | | Visit 2 | neg | 41 | 43 | 27 | 40 | 27 | 33 |
| | | RV001 | 1527 | 1560 | 1522 | | | | | | RV001 | 127 | 106 | 132 | | | |
| | Visit 6 | neg | 20 | 16 | 20 | 18 | 18 | 20 | | Visit 6 | neg | 71 | 77 | 69 | 96 | 107 | 59 |
| | | RV001 | 1345 | 1423 | 1399 | | | | | | RV001 | 98 | 98 | 93 | | | |
| | Visit 8 | neg | 96 | 149 | 108 | 118 | 99 | 100 | | Visit 8 | neg | 13 | 8 | 16 | 8 | 11 | 15 |
| | | RV001 | 1477 | 1416 | 1627 | | | | | | RV001 | 100 | 94 | 97 | | | |
| | Visit 13 | neg | 22 | 33 | 40 | 32 | 19 | 35 | | Visit 13 | neg | 14 | 23 | 14 | 17 | 5 | 16 |
| | | RV001 | 2000 | 2000 | 2000 | | | | | | RV001 | 27 | 33 | 37 | | | |
| | Visit 14 | neg | 3 | 2 | 4 | 3 | 2 | 0 | | Visit 14 | neg | 6 | 5 | 6 | 3 | 12 | 5 |
| | | RV001 | 1571 | 1665 | 1605 | | | | | | RV001 | 415 | 500 | 433 | | | |
| | Visit 15 | neg | 23 | 25 | 25 | 14 | 15 | 20 | | Visit 15 | neg | 21 | 33 | 27 | 24 | 22 | 28 |
| | | RV001 | 1080 | 1064 | 1040 | | | | | | RV001 | 188 | 216 | 208 | | | |
| | Visit 16 | neg | 0 | 0 | 0 | 0 | 0 | 0 | | Visit 16 | neg | 36 | 21 | 27 | 33 | 34 | 23 |
| | | RV001 | 2000 | 2000 | 2000 | | | | | | RV001 | 307 | 312 | 269 | | | |
| | Visit 17 | neg | 138 | 106 | 127 | 82 | 99 | 104 | | Visit 17 | neg | 155 | 131 | 159 | 179 | 158 | 154 |
| 004 | | RV001 | 54 | 11 | 22 | | | | 009 | | RV001 | 13 | 1 | 9 | | | |
| | Visit 2 | neg | 11 | 18 | 7 | 12 | 40 | 43 | | Visit 2 | neg | 3 | 1 | 7 | 7 | 9 | 7 |
| | | RV001 | 105 | 93 | 117 | | | | | | RV001 | 335 | 399 | 152 | | | |
| | Visit 6 | neg | 391 | 39 | 433 | 40 | 56 | 28 | | Visit 6 | neg | 5 | 12 | 8 | 8 | 6 | 12 |
| | | RV001 | 1133 | 1088 | 1104 | | | | | | RV001 | 1208 | 1065 | 1081 | | | |
| | Visit 8 | neg | 28 | 4 | 9 | 9 | 5 | 3 | | Visit 8 | neg | 27 | 26 | 19 | 16 | 17 | 19 |
| | | RV001 | 1194 | 1091 | 1140 | | | | | | RV001 | 1567 | 1592 | 1538 | | | |
| | Visit 13 | neg | 28 | 49 | 25 | 22 | 24 | 41 | | Visit 13 | neg | 46 | 183 | 141 | 53 | 43 | 43 |
| | | RV001 | 375 | 400 | 306 | | | | | | RV001 | 1094 | 1162 | 1279 | | | |
| | Visit 14 | neg | 33 | 35 | 21 | 19 | 20 | 27 | | Visit 14 | neg | 4 | 1 | 1 | 0 | 0 | 4 |
| | | RV001 | 382 | 379 | 343 | | | | | | RV001 | 1258 | 1206 | 1046 | | | |
| | Visit 15 | neg | 5 | 4 | 4 | 0 | 2 | 2 | | Visit 15 | neg | 1 | 0 | 0 | 1 | 1 | 1 |
| | | RV001 | 449 | 225 | 326 | | | | | | RV001 | 1324 | 1347 | 1263 | | | |
| | Visit 16 | neg | 6 | 0 | 0 | 1 | 2 | 2 | | Visit 16 | neg | 6 | 2 | 5 | 3 | 1 | 0 |
| | | RV001 | 883 | 884 | 807 | | | | | | RV001 | 805 | 827 | 874 | | | |
| | Visit 17 | neg | 13 | 6 | 9 | 5 | 10 | 6 | | Visit 17 | neg | 2 | 1 | 3 | 4 | 3 | 5 |
| 005 | | RV001 | 8 | 3 | 3 | | | | 010 | | RV001 | 31 | 19 | 17 | | | |
| | Visit 2 | neg | 4 | 2 | 4 | 3 | 4 | 2 | | Visit 2 | neg | 23 | 22 | 25 | 34 | 19 | 24 |
| | | RV001 | 894 | 836 | 962 | | | | | | RV001 | 82 | 84 | 96 | | | |
| | Visit 6 | neg | 17 | 13 | 5 | 26 | 11 | 16 | | Visit 6 | neg | 74 | 65 | 58 | 85 | 76 | 77 |
| | | RV001 | 991 | 1002 | 1516 | | | | | | RV001 | 71 | 61 | 96 | | | |
| | Visit 8 | neg | 12 | 7 | 9 | 9 | 15 | 13 | | Visit 8 | neg | 74 | 72 | 73 | 52 | 79 | 45 |
| | | RV001 | 1495 | 1439 | 1482 | | | | | | RV001 | 53 | 59 | 38 | | | |
| | Visit 13 | neg | 1 | 5 | 3 | 5 | 4 | 11 | | Visit 13 | neg | 26 | 35 | 21 | 9 | 24 | 15 |
| | | RV001 | 1408 | 1388 | 1317 | | | | | | RV001 | 170 | 130 | 128 | | | |
| | Visit 14 | neg | 0 | 0 | 0 | 0 | 0 | 0 | | Visit 14 | neg | 134 | 101 | 90 | 76 | 82 | 81 |
| | | RV001 | 1104 | 1100 | 1125 | | | | | | RV001 | 98 | 100 | 103 | | | |
| | Visit 15 | neg | 0 | 0 | 2 | 2 | 0 | 0 | | Visit 15 | neg | 17 | 16 | 17 | 14 | 20 | 22 |
| | | RV001 | 1444 | 1413 | 1424 | | | | | | RV001 | 101 | 143 | 112 | | | |
| | Visit 16 | neg | 0 | 1 | 1 | 3 | 2 | 2 | | Visit 16 | neg | 2 | 7 | 5 | 5 | 2 | 2 |
| | | RV001 | 1456 | 1432 | 1436 | | | | | | RV001 | 496 | 457 | 450 | | | |
| | Visit 17 | neg | 0 | 3 | 3 | 2 | 1 | 2 | | Visit 17 | neg | 5 | 2 | 3 | 4 | 1 | 0 |

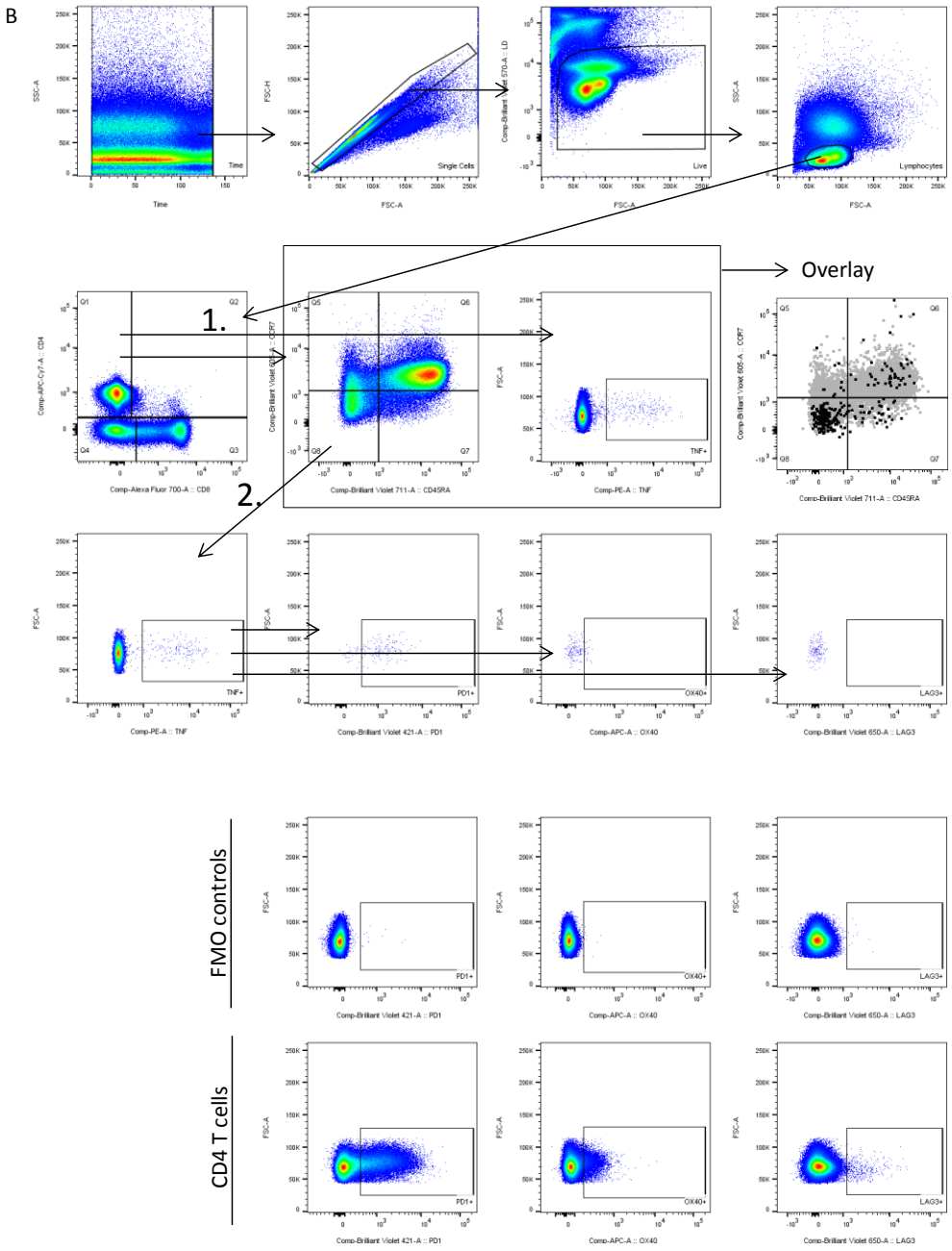
| Patient | Visit | Antigen | R1 | R2 | R3 | R4 | R5 | R6 | Patient | Visit | Antigen | R1 | R2 | R3 | R4 | R5 | R6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 011 | | RV001 | 25 | 40 | 47 | | | | 016 | | RV001 | 33 | 31 | 39 | | | |
| | Visit 2 | neg | 34 | 28 | 37 | 39 | 34 | 39 | | Visit 2 | neg | 11 | 18 | 31 | 20 | 21 | 21 |
| | | RV001 | 955 | 1028 | 1016 | | | | | | RV001 | 133 | 113 | 111 | | | |
| | Visit 6 | neg | 89 | 98 | 112 | 127 | 101 | 102 | | Visit 6 | neg | 15 | 23 | 22 | 21 | 20 | 36 |
| | | RV001 | 1357 | 1342 | 1374 | | | | | | RV001 | 408 | 371 | 328 | | | |
| | Visit 8 | neg | 24 | 13 | 16 | 15 | 20 | 22 | | Visit 8 | neg | 58 | 57 | 38 | 79 | 76 | 55 |
| | | RV001 | 1325 | 1368 | 1313 | | | | | | RV001 | 257 | 269 | 346 | | | |
| | Visit 13 | neg | 22 | 46 | 20 | 9 | 14 | 18 | | Visit 13 | neg | 22 | 15 | 9 | 9 | 16 | 8 |
| | | RV001 | 1551 | 1539 | 1556 | | | | | | RV001 | 361 | 436 | 374 | | | |
| | Visit 14 | neg | 32 | 33 | 48 | 21 | 27 | 37 | | Visit 14 | neg | 53 | 68 | 52 | 78 | 50 | 54 |
| | | RV001 | 1737 | 1724 | 1736 | | | | | | RV001 | 673 | 739 | 761 | | | |
| | Visit 15 | neg | 10 | 16 | 11 | 6 | 10 | 8 | | Visit 15 | neg | 3 | 2 | 0 | 1 | 1 | 2 |
| | | RV001 | 1279 | 1228 | 1272 | | | | | | RV001 | 24 | 35 | 19 | | | |
| | Visit 16 | neg | 6 | 7 | 5 | 2 | 1 | 3 | | Visit 16 | neg | 3 | 4 | 2 | 5 | 3 | 2 |
| | | RV001 | 1244 | 1229 | 1360 | | | | | | RV001 | 13 | 12 | 15 | | | |
| | Visit 17 | neg | 2 | 5 | 5 | 4 | 2 | 3 | | Visit 17 | neg | 2 | 0 | 0 | 0 | 0 | 1 |
| 012 | | RV001 | 132 | 113 | 97 | | | | 017 | | RV001 | 3 | 2 | 5 | | | |
| | Visit 2 | neg | 7 | 8 | 6 | 7 | 5 | 6 | | Visit 2 | neg | 3 | 2 | 1 | 1 | 1 | 3 |
| | | RV001 | 715 | 699 | 875 | | | | | | RV001 | 8 | 6 | 14 | | | |
| | Visit 6 | neg | 11 | 8 | 11 | 9 | 6 | 7 | | Visit 6 | neg | 8 | 4 | 2 | 2 | 13 | 3 |
| | | RV001 | 1674 | 1676 | 1680 | | | | | | RV001 | 27 | 14 | 17 | | | |
| | Visit 8 | neg | 4 | 14 | 26 | 19 | 5 | 11 | | Visit 8 | neg | 3 | 4 | 1 | 6 | 2 | 2 |
| | | RV001 | 2000 | 2000 | 2000 | | | | | | RV001 | 113 | 88 | 57 | | | |
| | Visit 13 | neg | 7 | 11 | 2 | 12 | 6 | 7 | | Visit 13 | neg | 0 | 0 | 0 | 3 | 3 | 1 |
| | | RV001 | 2000 | 2000 | 2000 | | | | | | RV001 | 146 | 134 | 159 | | | |
| | Visit 14 | neg | 14 | 15 | 13 | 20 | 12 | 12 | | Visit 14 | neg | 19 | 28 | 18 | 18 | 19 | 17 |
| | | RV001 | 1167 | 1214 | 1315 | | | | | | RV001 | 603 | 545 | 562 | | | |
| | Visit 15 | neg | 1 | 0 | 0 | 0 | 0 | 1 | | Visit 15 | neg | 22 | 14 | 15 | 5 | 6 | 6 |
| | | RV001 | 222 | 283 | 323 | | | | | | RV001 | 23 | 29 | 35 | | | |
| | Visit 16 | neg | 2 | 0 | 1 | 2 | 0 | 1 | | Visit 16 | neg | 0 | 0 | 1 | 2 | 2 | 0 |
| | | RV001 | 1228 | 1166 | 1176 | | | | | | RV001 | 38 | 36 | 45 | | | |
| | Visit 17 | neg | 0 | 0 | 0 | 0 | 1 | 0 | | Visit 17 | neg | 4 | 5 | 2 | 3 | 1 | 2 |
| 013 | | RV001 | 9 | 13 | 8 | | | | 018 | | RV001 | 19 | 39 | 27 | | | |
| | Visit 2 | neg | 13 | 9 | 14 | 12 | 14 | 7 | | Visit 2 | neg | 28 | 17 | 20 | 18 | 20 | 26 |
| | | RV001 | 1226 | 1218 | 1243 | | | | | | RV001 | 1061 | 1091 | 1096 | | | |
| | Visit 6 | neg | 13 | 12 | 8 | 11 | 7 | 7 | | Visit 6 | neg | 86 | 124 | 106 | 107 | 83 | 103 |
| | | RV001 | 1534 | 1543 | 1532 | | | | | | RV001 | 832 | 829 | 799 | | | |
| | Visit 8 | neg | 14 | 6 | 5 | 9 | 8 | 7 | | Visit 8 | neg | 16 | 17 | 19 | 21 | 13 | 21 |
| | | RV001 | 1639 | 1635 | 1618 | | | | | | RV001 | 1272 | 1295 | 1308 | | | |
| | Visit 13 | neg | 17 | 4 | 6 | 11 | 12 | 7 | | Visit 13 | neg | 36 | 34 | 25 | 54 | 24 | 34 |
| | | RV001 | 1505 | 1502 | 1603 | | | | | | RV001 | 1154 | 1187 | 1255 | | | |
| | Visit 14 | neg | 14 | 15 | 23 | 2 | 16 | 11 | | Visit 14 | neg | 2 | 4 | 4 | 5 | 5 | 5 |
| | | RV001 | 1583 | 1519 | 1469 | | | | | | RV001 | 1363 | 1353 | 1308 | | | |
| | Visit 15 | neg | 16 | 14 | 17 | 8 | 7 | 8 | | Visit 15 | neg | 8 | 17 | 22 | 9 | 11 | 11 |
| | | RV001 | 2000 | 2000 | 2000 | | | | | | RV001 | 1242 | 1215 | 1272 | | | |
| | Visit 16 | neg | 6 | 5 | 5 | 3 | 4 | 6 | | Visit 16 | neg | 36 | 45 | 41 | 35 | 29 | 32 |
| | | RV001 | 2000 | 2000 | 2000 | | | | | | RV001 | 1221 | 1203 | 1206 | | | |
| | Visit 17 | neg | 3 | 7 | 2 | 6 | 4 | 1 | | Visit 17 | neg | 17 | 19 | 14 | 12 | 16 | 8 |
| 015 | | RV001 | 4 | 2 | 3 | | | | 019 | | RV001 | 13 | 17 | 10 | | | |
| | Visit 2 | neg | 4 | 1 | 4 | 2 | 6 | 1 | | Visit 2 | neg | 11 | 4 | 1 | 3 | 7 | 16 |
| | | RV001 | 8 | 3 | 7 | | | | | | RV001 | 374 | 510 | 399 | | | |
| | Visit 6 | neg | 52 | 2 | 1 | 1 | 0 | 4 | | Visit 6 | neg | 3 | 3 | 3 | 4 | 4 | 3 |
| | | RV001 | 427 | 440 | 362 | | | | | | RV001 | 1324 | 1029 | 990 | | | |
| | Visit 8 | neg | 49 | 24 | 23 | 24 | 31 | 26 | | Visit 8 | neg | 6 | 12 | 8 | 7 | 2 | 291 |
| | | RV001 | na | na | na | | | | | | RV001 | 1199 | 1114 | 1246 | | | |
| | Visit 13 | neg | na | na | na | na | na | na | | Visit 13 | neg | 6 | 3 | 7 | 0 | 10 | 6 |
| | | RV001 | 962 | 873 | 864 | | | | | | RV001 | 52 | 53 | 81 | | | |
| | Visit 14 | neg | 11 | 4 | 15 | 4 | 4 | 4 | | Visit 14 | neg | 0 | 0 | 0 | 0 | 1 | 0 |
| | | RV001 | 1381 | 1413 | 1657 | | | | | | RV001 | 940 | 939 | 999 | | | |
| | Visit 15 | neg | 4 | 2 | 2 | 1 | 4 | 1 | | Visit 15 | neg | 0 | 1 | 1 | 0 | 0 | 0 |
| | | RV001 | 1121 | 1164 | 1121 | | | | | | RV001 | 123 | 251 | 90 | | | |
| | Visit 16 | neg | 5 | 1 | 4 | 8 | 5 | 4 | | Visit 16 | neg | 0 | 0 | 0 | 0 | 0 | 0 |
| | | RV001 | 1126 | 1178 | 1073 | | | | | | RV001 | 78 | 26 | 44 | | | |
| | Visit 17 | neg | 45 | 33 | 24 | 59 | 36 | 55 | | Visit 17 | neg | 0 | 0 | 0 | 0 | 0 | 0 |

| Patient | Visit | Antigen | R1 | R2 | R3 | R4 | R5 | R6 |
|---|---|---|---|---|---|---|---|---|
| 020 | | RV001 | 72 | 25 | 48 | | | |
| | Visit 2 | neg | 29 | 42 | 71 | 68 | 67 | 53 |
| | | RV001 | 167 | 117 | 166 | | | |
| | Visit 6 | neg | 93 | 126 | 171 | 171 | 172 | 125 |
| | | RV001 | 465 | 575 | 557 | | | |
| | Visit 8 | neg | 107 | 62 | 86 | 24 | 49 | 58 |
| | | RV001 | 1194 | 1115 | 1176 | | | |
| | Visit 13 | neg | 132 | 102 | 141 | 80 | 82 | 115 |
| | | RV001 | 1391 | 1423 | 1538 | | | |
| | Visit 14 | neg | 0 | 0 | 0 | 0 | 0 | 1 |
| | | RV001 | 1551 | 1369 | 1445 | | | |
| | Visit 15 | neg | 1 | 1 | 2 | 1 | 1 | 2 |
| | | RV001 | 826 | 804 | 788 | | | |
| | Visit 16 | neg | 3 | 1 | 0 | 1 | 0 | 1 |
| | | RV001 | 2000 | 2000 | 2000 | | | |
| | Visit 17 | neg | 2 | 1 | 0 | 4 | 3 | 1 |
| 021 | | RV001 | 3 | 3 | 4 | | | |
| | Visit 2 | neg | 0 | 1 | 1 | 1 | 1 | 1 |
| | | RV001 | 17 | 24 | 17 | | | |
| | Visit 6 | neg | 12 | 12 | 7 | 17 | 12 | 12 |
| | | RV001 | 488 | 539 | 496 | | | |
| | Visit 8 | neg | 3 | 1 | 2 | 0 | 1 | 2 |
| | | RV001 | 1292 | 1298 | 1321 | | | |
| | Visit 13 | neg | 0 | 0 | 1 | 1 | 2 | 0 |
| | | RV001 | 1425 | 1442 | 1397 | | | |
| | Visit 14 | neg | 5 | 1 | 6 | 3 | 6 | 2 |
| | | RV001 | 1465 | 1602 | 1497 | | | |
| | Visit 15 | neg | 2 | 0 | 1 | 2 | 2 | 3 |
| | | RV001 | 1321 | 1325 | 1377 | | | |
| | Visit 16 | neg | 2 | 2 | 2 | 3 | 2 | 2 |
| | | RV001 | 1428 | 1414 | 1435 | | | |
| | Visit 17 | neg | 2 | 4 | 5 | 2 | 4 | 3 |
| 022 | | RV001 | 15 | 14 | 4 | | | |
| | Visit 2 | neg | 9 | 2 | 8 | 12 | 9 | 7 |
| | | RV001 | 961 | 966 | 1017 | | | |
| | Visit 6 | neg | 18 | 14 | 14 | 27 | 18 | 21 |
| | | RV001 | 335 | 334 | 320 | | | |
| | Visit 8 | neg | 42 | 57 | 44 | 35 | 28 | 41 |
| | | RV001 | 945 | 878 | 805 | | | |
| | Visit 13 | neg | 83 | 100 | 107 | 112 | 106 | 76 |
| | | RV001 | 946 | 925 | 963 | | | |
| | Visit 14 | neg | 7 | 16 | 3 | 8 | 3 | 6 |
| | | RV001 | 623 | 642 | 649 | | | |
| | Visit 15 | neg | 3 | 5 | 0 | 0 | 1 | 3 |
| | | RV001 | 424 | 406 | 461 | | | |
| | Visit 16 | neg | 77 | 96 | 106 | 66 | 63 | 94 |
| | | RV001 | 480 | 421 | 432 | | | |
| | Visit 17 | neg | 3 | 6 | 5 | 4 | 2 | 5 |
| 023 | | RV001 | 358 | 433 | 475 | | | |
| | Visit 2 | neg | 293 | 288 | 314 | 218 | 267 | 323 |
| | | RV001 | 16 | 19 | 16 | | | |
| | Visit 6 | neg | 13 | 14 | 8 | 11 | 12 | 9 |
| | | RV001 | 39 | 35 | 26 | | | |
| | Visit 8 | neg | 26 | 28 | 21 | 17 | 23 | 21 |
| | | RV001 | 287 | 160 | 209 | | | |
| | Visit 13 | neg | 222 | 228 | 191 | 149 | 122 | 137 |
| | | RV001 | 3 | 1 | 0 | | | |
| | Visit 14 | neg | 0 | 2 | 1 | 1 | 2 | 1 |
| | | RV001 | 39 | 38 | 51 | | | |
| | Visit 15 | neg | 44 | 64 | 51 | 76 | 59 | 49 |
| | | RV001 | 24 | 28 | 44 | | | |
| | Visit 16 | neg | 20 | 25 | 38 | 39 | 48 | 27 |
| | | RV001 | 29 | 24 | 38 | | | |
| | Visit 17 | neg | 26 | 16 | 33 | 21 | 22 | 19 |

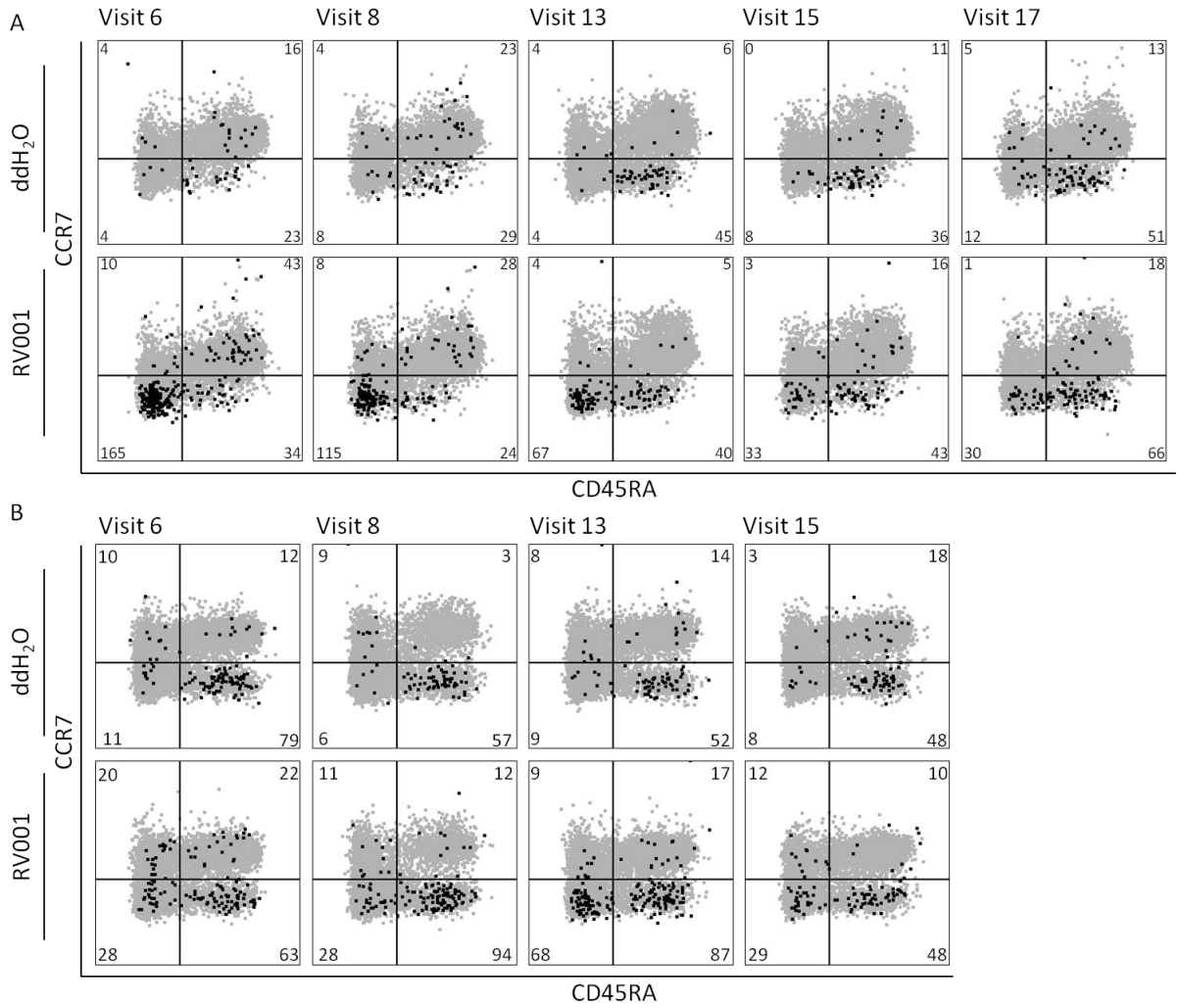
| Patient | Visit | Antigen | R1 | R2 | R3 | R4 | R5 | R6 |
|---|---|---|---|---|---|---|---|---|
| 024 | | RV001 | 0 | 0 | 0 | | | |
| | Visit 2 | neg | 0 | 0 | 1 | 0 | 0 | 0 |
| | | RV001 | 21 | 20 | 24 | | | |
| | Visit 6 | neg | 24 | 16 | 20 | 13 | 34 | 19 |
| | | RV001 | 5 | 5 | 6 | | | |
| | Visit 8 | neg | 7 | 5 | 13 | 5 | 8 | 14 |
| | | RV001 | 3 | 2 | 8 | | | |
| | Visit 13 | neg | 5 | 4 | 5 | 1 | 3 | 2 |
| | | RV001 | 1 | 2 | 3 | | | |
| | Visit 14 | neg | 2 | 0 | 1 | 2 | 4 | 3 |
| | | RV001 | 9 | 3 | 4 | | | |
| | Visit 15 | neg | 6 | 5 | 7 | 5 | 16 | 5 |
| | | RV001 | 2 | 3 | 5 | | | |
| | Visit 16 | neg | 5 | 4 | 2 | 9 | 6 | 2 |
| | | RV001 | 9 | 3 | 5 | | | |
| | Visit 17 | neg | 11 | 11 | 8 | 9 | 9 | 8 |

R: Replicate; neg = negative control ($ddH_2O$); na= not available. For all patients and tests, 0.2 x $10^6$ cells were platted/ well, except for Patient 021 visit 2 to visit 13 and Patient 012 visits 16 and 17, here 0.17 x $10^6$ cells were platted/ well. Patient 002 dropped out of the immunological analysis due to inconsistent results (data not shown). Visit 2 for Patient 001 and visit 13 for Patient 015 were not tested as no PBMC samples were available. Spots counts above 2000/well or TNTC (too numerous to count) are set to 2000 and marked in red. For details on the ELISpot, see Material and Methods.


A
Time
Single cells
Live cells
Lymphocytes
CD4 T cells
CD8 T cells
CD4 T cells
CD8 T cells
IL-2+
CD154+
CD107a+
TNF+
IFN-γ+

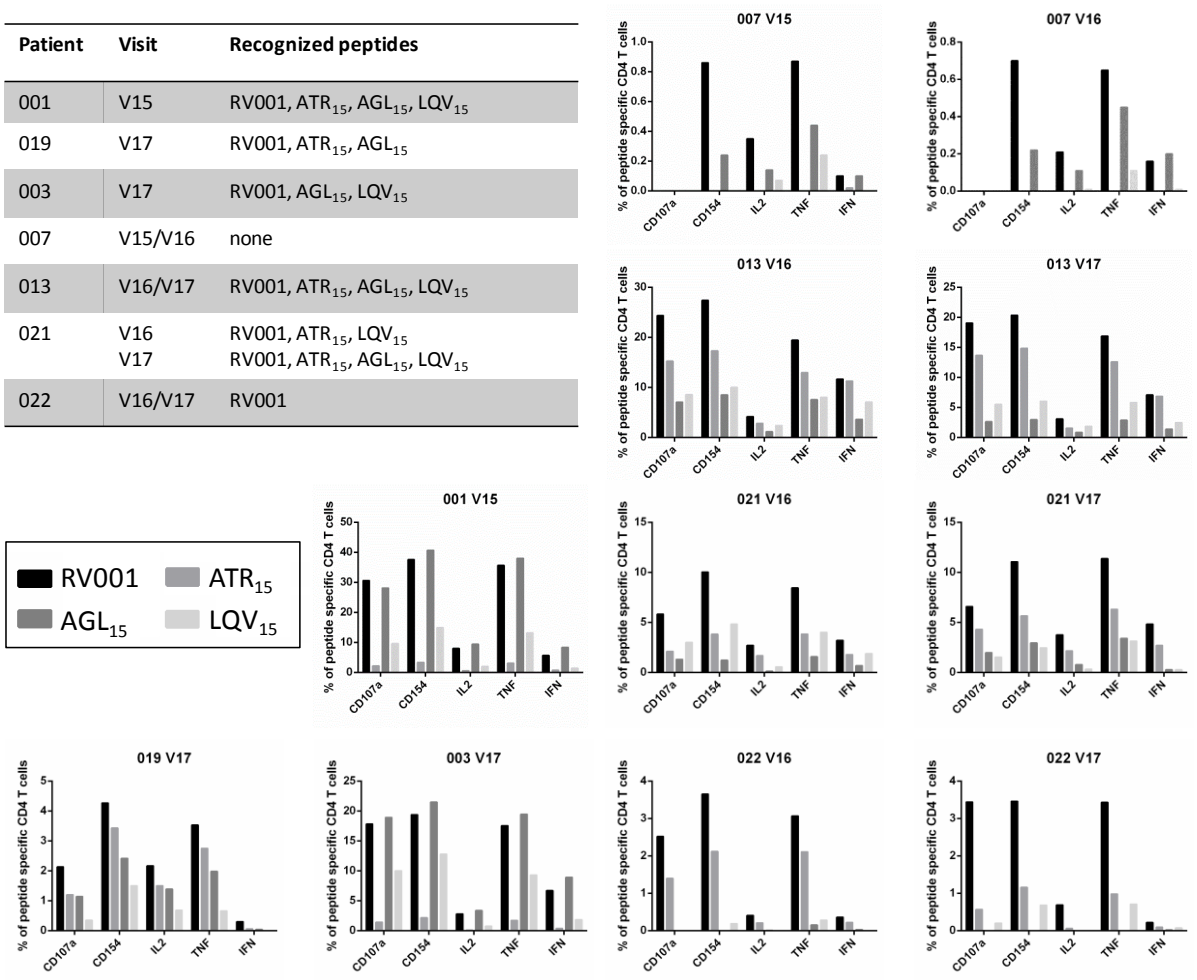



**Supplementary figure S1: Gating strategies. A: Gating strategy for the ICS analysis to identify RV001 specific T cell populations (Patient 011).** Upper row, left to right: sample flow over time, duplet exclusion (FSC-A/FSC-H), gating on live cells (FSC-A/Zombie Aqua dye), lymphocytes (FSC-A/SSC-A), and CD4/CD8 dot plot. CD4 T cells (middle row) and CD8 T cells (lower row) were analyzed for their expression of IL-2, CD154, CD107a, TNF and IFN-γ (from left to right). B: Gating strategy for the *ex vivo* flow analysis of RV001 specific CD4 T cells (Patient 018). Upper row, left to right: sample flow over time, duplet exclusion (FSC-A/FSC-H), gating on live cells (FSC-A/Zombie Aqua dye), lymphocytes (FSC-A/SSC-A). Gating 1: CD4 cells were examined for CD45RA/CCR7, as well as for TNF expression after RV001 peptide stimulation for 12 hr. The overlay of the TNF+ cells (black) onto the CD45RA/CCR7 dot plot (grey) shows that most of the RV001-specific CD4 cells were effector memory (CD45RA-CCR7-). Gating 2: The PD-1, OX-40, and LAG-3 expressions were further examined in the TNF+, CD45RA-CCR7- cells. Lowest 2 rows: As controls for the expression of PD-1, OX-40, and LAG-3, fluorescence minus one controls (FMOs) were used. FMO gates were set on the whole CD4 T cell population (shown on the dot plots) and copied to the CD4+CD45RA-CCR7-TNF+ (RV001-specific) cell population.




**Supplementary figure S2: Additional results of the e*x vivo* analysis of RV001 specific CD4 T cells (see also Fig. 3).** PBMCs from Patient 018 visit 6 to visit 18 (A) and Patient 005 visit 6 to visit 15 (B) were incubated with the RV001 peptide (lower row) or $ddH_2O$ (upper row) for 12 h. $TNF^+$ CD4 T cells were identified (black) and overlaid on the whole CD4 T cell population (grey). Numbers indicate the number the $CD4^+TNF^+$ cell counts in each quadrant. For gating, see Figure S1A.

| Patient | Visit | Recognized peptides |
|---|---|---|
| 001 | V15 | RV001, $ATR_{15}$, $AGL_{15}$, $LQV_{15}$ |
| 019 | V17 | RV001, $ATR_{15}$, $AGL_{15}$ |
| 003 | V17 | RV001, $AGL_{15}$, $LQV_{15}$ |
| 007 | V15/V16 | none |
| 013 | V16/V17 | RV001, $ATR_{15}$, $AGL_{15}$, $LQV_{15}$ |
| 021 | V16<br>V17 | RV001, $ATR_{15}$, $LQV_{15}$<br>RV001, $ATR_{15}$, $AGL_{15}$, $LQV_{15}$ |
| 022 | V16/V17 | RV001 |




**Supplementary figure S3: Additional ICS results for the identification of RV001-derived HLA-class II epitopes (see also Fig 4).** Table: tested patients and visits, as well as the peptides recognized by patients´ CD4 T cells (see Material and Methods). Bars indicate percentages of peptide-specific CD4 T cells expressing CD107a, CD154, IL-2, TNF, IFN-γ (IFN) when re-stimulated for 12 h with the peptides RV001 (black), $ATR_{15}$ (middle grey), $AGL_{15}$ (dark grey), $LQV_{15}$ (light grey). For gating, see Figure S1B.

# Chapter 3I Immunomonitoring – technical aspects

## Part I: Immunomonitoring: common methods

*Focused review: Adhering to adhesion: assessing integrin conformation to monitor T cells*

## Part II: Immunomonitoring: assay optimization

*Manuscript in preparation for submission: Optimization of a protocol for the simultaneous identification of functional antigen-specific $CD8^+$ and $CD4^+$ cells after in-vitro antigen stimulation using synthetic long peptides and Poly-ICLC*

# 3 Introduction: Immunomonitoring – technical aspects

Nearly daily new immune therapies or combinations of therapies are developed and tested in clinical studies. It is important to evaluate the efficacy of such immune therapies and best, to identify early biomarkers of the therapy. For the immunological assessment of T cells, the induction of an immune response has to be measured by e.g. the upregulation of receptors, secretion of cytokines, or the induction of cell proliferation. Nowadays, we know that not only the induction of an immune response is important for the immunotherapy, but also the phenotype of rare antigen-specific cells, the cellular interplay, or the tumor microenvironment. Therewith, it is not surprising that high-dimensional and high-throughput methods are developed to assess the heterogeneity of samples. One high-dimensional method is, for example, the cytometry by time of flight (CyTOF) single-cell analysis. It is a combination of flow cytometry and mass spectrometry using isotopes conjugated to antibodies, overcoming the limitation of fluorochrome spectral overlap which is a well known limitation in conventional flow cytometry. With this method, the assessment of 40 or even more markers simultaneously has become possible, allowing extended research on the tumor microenvironment and immune cell phenotypes, resulting in a comprehensive immunomonitoring [1-3]. The assessment of many markers at the same time is, however, still challenging for the data analysis [4]. Another method for the high-throughput single cell assessment of (rare) antigen-specific T cells uses DNA-barcoded MHC-class I multimers [5-7]. Besides the large-scale detection of antigen-specific T cells, this method allows the investigation of the T cell receptor (TCR) recognition pattern (TCR “fingerprint”) by measuring the affinities of TCRs to libraries of barcoded peptide-MHC multimers [8]. Such high-dimensional and high-throughput methods are not yet available for all laboratories as they are mostly cost- and time-intensive. They are also poorly adapted to routine measurements, hence, more suitable for basic research or discovery projects than for the monitoring of clinical trials.

Common methods that are frequently used to assess antigen-specific T cell responses with patient material are the Enzyme-linked Immunosorbent Assay (ELISpot), as well as the flow cytometry-based methods of fluorescence-labeled HLA-multimer staining, and the intracellular cytokine assay (ICS). All immunological assays used for a clinical immunomonitoring should be robust over a long period of

time (sometimes years), specific, as antigen-specific T cells are rare, and ideally require only a low amount of patient material. As the immunomonitoring of antigen-specific T cells takes several steps and deals with living material it is failure-prone. Detailed protocols that have to be strictly followed are of high priority to reduce technical failures and variability due to different operators. Furthermore, they allow the comparison of acquired data between patients and over time. It is important to validate the protocols to assess the specificity, reliability, and robustness of the assays [9]. The field of immunomonitoring has made a lot of efforts to improve the assays and implement guidelines to increase the comparability of data between different groups or centers, often based on harmonized proficiency panels [10-15]. Every protocol has critical points that should be strictly followed but often these are not obvious when papers get published. The Minimal Information About T cell Assays (MIATA) project was initiated in 2009 to implement a framework that contains all the information needed to understand the generated data and allow easier reproduction [16]. The focused review presented next (section 3, part 1) gives insights about the common T cell assays (ICS, ELISpot, HLA-multimer) used for immunomonitoring and besides explains the ICAM assay (assessment of functional CD8 T cells by immediate structural change of ICAM), which we have recently established in collaboration with Dr. Dimitrov et al. In particular, Table 1 in the focused review summarizes the common methods for T cell immunomonitoring and their main characteristics, advantages, and limitations.

The ELISpot and ICS were used to assess functional antigen-specific T cells in the clinical study presented in section 2. To overcome the problem of assay-related detection limit, rare antigen-specific cells can be expanded over several days using an antigen pre-sensitization step during which the synthetic peptide(s) and repeated addition of interleukin-2 are added to the culture. Such an *in-vitro* amplification is regularly performed in our laboratory (our established protocol foresees a culture during 12 days), coupled to the subsequent analysis of the expanded antigen-specific T cells (readout analysis). We are specialized in the monitoring of immune responses using synthetic short peptides (8-12 aa long for HLA-class I and 13-18 aa long for HLA-class II peptides), however, for the immunomonitoring of new projects like the RhoC vaccine study (section 2), we wanted to establish a protocol that allows the simultaneous readout of CD8 and CD4 T cells responses using SLPs. There was evidence that the identification of antigen-specific cells using SLPs may need an

optimized protocol, including a high peptide concentration and additional stimuli [17]. In the work presented in section 3, part 2, we tested and optimized our established protocols for the identification of antigen-specific cells using SLPs. The findings and the optimized protocol are presented as a pre-manuscript which is currently under preparation and will be submitted to a peer-reviewed journal as a methodological contribution.

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# Adhering to adhesion: assessing integrin conformation to monitor T cells

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The author of this thesis contributed to the focused review with writing the part of the "common methods for assessing antigen-specific T cells and their function" including parts: "The ELISpot: simple but refined", "FCM: single-cell, multi-parametric, and versatile", "Peptide-MHC multimer staining", "The intra-cellular cytokine staining" and Table 1: Main characteristics of immunological T cell assays.

## 3.1 Part I: Focused review: Adhering to adhesion: assessing integrin conformation to monitor T cells

### 3.1.1 Abstract

Monitoring T cells is of major importance for the development of immunotherapies. Recent sophisticated assays can address particular aspects of the anti-tumor T cell repertoire or support very large-scale immune screening for biomarker discovery. Robust methods for the routine assessment of the quantity and quality of antigen-specific T cells remain however essential. This review discusses selected methods that are commonly used for T cell monitoring and summarizes the advantages and limitations of these assays. We also present a new functional assay, which specifically detects activated $\beta_2$-integrins within a very short time following $CD8^+$ T cell stimulation. Because of its unique and favourable characteristics, this assay could be useful for implementation into our T cell monitoring toolbox.

### 3.1.2 The importance of T cell monitoring

T cells are key actors in many cancer immunotherapy approaches. With the increasing development of checkpoint blockade antibodies, adoptive transfer therapies, and new-generation cancer vaccines, the assessment of immune cell subsets has become indispensable. Monitoring of patient (T) cells delivers information on the mechanisms of action, persistence of transferred effector cells, and possibly on therapy resistance. In the context of vaccine development, it establishes immunogenicity of antigens and efficacy of adjuvants, and guides the choice of immune modulators and therapy combinations. It has also the potential to reveal early biomarkers of clinical efficacy [1].

Recent developments in genomics and in profiling of (single cell) TCR clonotypes [2,3] now allow browsing the full T cell repertoire from very few starting material. Coupled to methods for enriching selected antigen-specificities, they could soon deliver precious information on anti-tumor T cell response dynamics in cancer patients. These sophisticated, extremely high throughput approaches, are until now reserved to a few expert teams and associated to specific challenges [5]. Hence, straightforward T cell immunomonitoring methods that can be relatively easily implemented in daily-laboratory practice remain crucial tools for clinical development.

In the following paragraphs, we discuss those aspects of the most popular assays that we believe should be considered as basics in the context of clinical T cell immunomonitoring. We also describe a new method that we have recently developed, and which relies on a so far unexploited early event of T cell activation, i.e. the conformational and valency change of membrane-bound $\beta_2$-integrins.

### 3.1.3 Common methods for assessing antigen-specific T cells and their function

Antigen-specific T cells can be identified by phenotypic and/or functional hallmarks. In most settings, functional assessment requires an *in vitro* cell (e.g. whole blood or peripheral blood mononuclear cells i.e. PBMCs) re-stimulation phase in the presence of the relevant antigen(s) to be tested. Read-out can be then performed by measuring the upregulation of activation factors, the proliferation, the production of cytokines, and cytotoxic attributes such as degranulation or perforin/granzyme amounts.

For the monitoring of clinical studies, immune tests should be robust, able to detect low-frequency T cells from a limited amount of material, and amenable to a high number of samples. In addition, methods and instrumentation need to be stable over longer periods of time, possibly years, in order to allow a comparison of results obtained at various time points during therapy/follow up and from different patients enrolled in the trial. A number of methods are available for measuring T cell antigen specificity and function. Since there is no gold standard, they are employed according to the specific need and local know-how of the different immunomonitoring laboratories. The most widely used assays are the Enzyme-Linked Immunospot (ELISpot) and the flow cytometry-based methods that include peptide MHC (pMHC) multimer staining and intracellular cytokine staining (ICS). These tests deliver complementary information on the quantity and quality of the T cells and should be carefully chosen during the preparation phase of a study. The main characteristics, advantages, and limitations of these assays, are discussed below and summarized in Table 1.

**Table 1:** Main characteristics of immunological T cell assays

| Assay | Detection limit* | Stimulation time | Development time** | Parameter measured | Advantages | Limitations |
|---|---|---|---|---|---|---|
| **IFN-γELISpot** | 0.004 – 0.007% of PBMCs | 24-40 h | 4 h | • Secreted factor (e.g. cytokine) | • High throughput and robust<br>• Functional test<br>• Single cell level<br>• No protein transport inhibitor required | • Mostly monoparametric<br>• No information on the effector cell subsets<br>• Upper limit of detection (limited by the total number of spots that can be counted by the ELISpot reader) |
| **pMHC Multimer Staining (FCM)** | 0.01% of $CD8^+$ cells (in combinatorial down to 0.001%) | Not required | 1 h | • TCR specificity (extracellular) | • Single cell, multi-parametric measurement<br>• Live cell sorting possible<br>• Independent of function<br>• Very low background and detection limit | • No test of functionality<br>• Information on epitope and HLA restriction required<br>• Reagents must be produced for each specificity<br>• Less common for $CD4^+$ cells |
| **ICS (FCM)** | 0.01-0.04 % of $CD4^+$ or $CD8^+$ cells | 6-12 h | 2 h | • Soluble factors (intracellular)<br>• Activation markers (extra- and intra-cellular) | • Single cell, multi-parametric, functional measurement<br>• Discrimination between cell populations<br>• Pre-knowledge on the exact epitopes not required | • Depends on the kinetics of cytokine production<br>• Protein transport inhibition required, limiting the duration of the assay<br>• No live cell-sorting possible |
| **mICAM-1 staining (FCM)** | 0.01-0.04 % of $CD8^+$ cells | Minutes | 1 h | • $\beta_2$-integrin activation (extracellular) | • Single cell, multi-parametric, functional measurement<br>• Very early and quick read out<br>• Single reagent for any specificity (pre-knowledge on the exact epitopes not required)<br>• Live cell sorting possible | • No info on end-function<br>• Method is new and needs to gather more information (e.g. optimal storage conditions of mICAM-1 reagent to be established, application for clinical trial monitoring)<br>• Development needed for $CD4^+$ T cells |

* Indicative lower limit of detections may vary depending on cell types (whole blood, *ex vivo* PBMCs, cells after culture) and stimulation/staining conditions (medium, mAb & fluorochromes)

* Approximate times are given. Development times include all experimental steps but not the final analysis. For pMHC and mICAM-1multimer staining, extracellular staining with mAbs is included. For ICS, extracellular & intracellular staining steps, as well as permeabilization/fixation, are included.

The ELISpot: simple but refined

The ELISpot method was first described more than 30 years ago [6]. It is a relatively high throughput method that can be used for measuring a variety of secreted factors, provided that two monoclonal antibodies recognizing different epitopes of the targeted molecule (soluble analyte) are available. Interferon-γ (IFN-γ) is mostly used for assessing antigen-specific T cells, as this cytokine is produced in substantial quantity by both activated $CD4^+$ and $CD8^+$ T cells.

Briefly, suitable membrane-bottomed 96 well plates are coated with a monoclonal antibody (mAb) recognizing the analyte of interest e.g. IFN-γ. Cells are then added to the well and stimulated with the antigen (in general, short epitopes and long (>20 amino acids), overlapping peptides are used). After cell removal, a second biotinylated anti-IFN-γ mAb is added, followed by a streptavidin-coupled enzyme (e.g. alkaline phosphatase or horseradish peroxidase). Each activated and IFN-γ-secreting cell will give a colored spot after final incubation with a suitable precipitating substrate. The exact number of spots can be counted with an ELISpot reader and the frequency of antigen-specific cells calculated. Size of the spots, which gives information on the quantity and kinetics of cytokine production, is more rarely analyzed. The ELISpot assay is of high sensitivity, specificity, and accuracy due to the two antibodies recognizing different epitopes of the same analyte and to the signal amplification provided by the biotin-streptavidin interaction [7]. The technique can reach a detection limit of approximately 4 to 7 spots per 100.000 PBMCs (0.004 to 0.007%) in experienced laboratories [8,9], whereas the upper limit of quantification depends on the number of spots that can be discriminated by the ELISpot reader (typically between 1000 and 1500 spots/well). In most cases, cells are stimulated for 24 to 40 h, allowing for detection of late cytokines [10]. The duration of the stimulation is actually limited by the number of cells in the wells and the medium consumption. Although measurement of 2 to 3 parameters is possible, the assay is still mainly used as a mono-parametric test. Overall, ELISpot is a robust and sensitive method, but does not allow the identification of cytokine-secreting cell populations unless these are purified beforehand; this is rarely done with limited patient material.

The ELISpot method has been widely discussed and improved over the years, and very helpful guidelines and protocols are available [7,9,11]. As it is the case for any other assay including living cells, a number of parameters such as the number of

cells tested, the culture medium, the antigen concentration and format, the background reactivity and the incubation times can affect the final results. Many of these parameters have been identified by international harmonization efforts [12-14]. The analysis (i.e. the ELISpot reader parameters) should also be thoroughly performed [15]. Hence, each laboratory should establish and optimize the assay for its own in-house conditions, define optimal quantification and linearity ranges, and implement measures for controlling performance between operators and over time.

*FCM: single cell, multiparametric and versatile*

Apart from the ELISpot, other popular methods used for conventional T cell monitoring are based on flow cytometry (FCM). FCM is the prototype of a multi-parameter, single cell assessment method which allows the simultaneous phenotypic and functional characterization of various cell subsets contained in a cell mixture, for example PBMCs.

Automated single cell flow analysis was first mentioned in 1934 and further developed by Wallace Coulter in the 1950s. The first fluorescence-based commercial device, a “pulse cytophotometer”, and cell sorters, became available in the late 1960s. FCM has considerably improved since then, with major developments in the technology itself, as well as in the reagents and fluorochromes that are available. FCM remains an indispensable state of the art technique in basic research and in clinical development. Simultaneous measurement of more than 8 parameters is daily practice in many laboratories. Still, for rigorous and meaningful testing, and especially if many parameters are combined, it is absolutely essential to invest efforts in establishing and optimizing antibody panels and in controlling cytometer performance over time [16]. A number of specialized articles and books have already been published by leading experts in FCM [17-19] and specific tools are also available, such as tutorials on the websites of academic institutions or antibody manufacturers. Similarly to the ELISpot assay, harmonization initiatives have helped to increase performance and comparability of the results obtained at different centers [14,20-22]. Attention should be given not only to the experiments themselves, but also to their analysis. Flow gating strategies are not standardized and contribute substantially to inter-laboratory variation [23,24]. As FCM complexity is steadily increasing, such efforts should be sustained in the future.

Peptide-MHC multimer staining

The introduction of pMHC fluorescent multimers more than twenty year ago was a groundbreaking innovation which has boosted many aspects of T cell research, especially the characterization of low frequency antigen-specific T cells [25]. pMHC multimers bind to antigen-specific T cells due to the interaction of pMHC complexes with TCRs. The affinity of one pMHC molecule for its cognate TCR is generally low and not sufficiently stable to stain antigen specific cells. To bypass this problem, pMHC monomers (produced by *in vitro* refolding of biotinylated recombinant MHC chains in the presence of the peptide of interest) can be multimerized by taking advantage of the strong interaction between biotin and streptavidin (described in [26]). Various formats of pMHC multimers are available, from tetramers to more elaborate constructs containing 10 or more pMHC monomers [25,27]. Multimers are in principle very stable, but low affinity peptides might dissociate over time. Degradation can be prevented either by adding free peptide to the reagent, or by freezing multimers in the presence of glycerol, which will ensure stability of the reagents for at least 6 months [28]. pMHC class I tetramers can be produced in-house and are by far the most common multimers used to stain $CD8^+$ T cells. pMHC class II tetramers are more difficult to produce and remain rarely used for assessment of antigen-specific $CD4^+$ T cells.

The assay itself has a high specificity (< 0.002% in our hands for common virus-specific $CD8^+$ T cells) and a detection limit down to approx. 0.01% of $CD8^+$ T cells, allowing the examination of rare cell populations [9,29]. Optimizations, including combinatorial staining (usage of the same tetramers labelled with two different fluorochromes), can greatly improve the detection limit of the assay, increasing the chance to detect (tumor) antigen-specific T cells in *ex vivo* blood or PBMCs [30].

In combination with mAb that characterize T cell subsets, pMHC multimers are perfect reagents to identify antigen-specific cells of interest in a cell sample, without functional assessment. This can be an advantage, as all cells specific for a certain antigen will be detected, irrespective of their function. The problem with such “structural information” is that the cells detected may be anergic or dysfunctional and as such will probably not be efficient effectors. A well-known example in the virology field is the accumulation of Cytomegalovirus (CMV)-specific $CD8^+$ T cells in the

elderly; these cells can be detected by pMHC staining but are essentially dysfunctional [31].

The intracellular cytokine assay

The ICS assay presents the advantage of delivering comprehensive information on the functional profile of the T cell subsets of interest [32]. Upregulation of early functional markers can be detected, such as CD107a (degranulation, essentially for $CD8^+$ CTLs) or CD154 (CD40L, preferentially expressed on activated $CD4^+$ T cells and detected intracellularly, unless a CD40 mAb is added) [33,34]. This can be combined with the detection of intracellular cytokines. T cells that produce several cytokines at the same time, so-called polyfunctional T cells, have been associated with protection after vaccination and with favorable clinical outcome in various pathogen-related conditions [35]. A correlation with anti-tumor protection, however, has still to be determined. Nevertheless, polyfunctional T cells not only produce several cytokines which could reflect advanced effector function, but these cytokines, particularly IFN-γ are also produced in enhanced amounts at the single cell level [35].

ICS is mainly used when the exact epitopes and/or the MHC-restriction are not identified (e.g. when using overlapping (long) peptides for T cell screening), and for assessment of $CD4^+$ T cell responses [36,37]. It is an elaborate assay, and each step should be carried out carefully in order to deliver optimal results. Cell treatment (thawing, antigen stimulation, staining), mAb combinations, and analysis, need to be optimized in each laboratory. For the identification of low T cell responses in particular, it is important to keep the background cytokine/marker production in the unstimulated control condition as low as possible. This background varies between cytokines and is generally enhanced when cells have been cultured, but is optimally in the range of approx. 0.01%-0.04% (within $CD4^+$ /$CD8^+$ subsets), hence greater than that of pMHC multimers. Standardized protocols are available [38,39] and parameters important for performance have been identified in inter-laboratory testing exercises [21-23].

There are two intrinsic limitations to the ICS assay. First, the duration of the antigen stimulation is restricted. To enable intracellular staining of accumulated cytokines, cells are treated with protein transport inhibitors. Such inhibitors are toxic and should generally not be added for more than 12 h [38,33]. This time frame needs to be accommodated to the kinetics of production for the various cytokines that are to be

detected [10]. To circumvent this problem, one possibility is to first add the stimulus, and several hours later the inhibitors [40]. Second, the detection of intracellular structures requires the permeabilization and fixation of the cells. As a consequence, the cells cannot be used for live cell sorting and/or recovered for further *in vitro* culture. Finally, it is important to note that the combination of pMHC multimer staining and ICS is not possible, since antigenic stimulation triggers the rapid downregulation of the TCR, precluding multimer binding on cytokine$^+$ T cells.

### 3.1.4 The mICAM-1 assay: immediate structural changes indicate T cell function

The execution of CD8$^+$ T-cell effector responses requires strong adhesion to target cells (e.g. cancer cells), formation of the efficient immunological synapse and finally, killing of the target cells [41,42]. Adhesion is mediated mainly by activation of $\beta_2$-integrins, such as LFA-1 (heterodimer CD11a/CD18), which are expressed at high levels on circulating antigen-experienced T lymphocytes [43], but are maintained in an inactive state [44]. Following binding of the TCR to its specific antigen presented on target cell MHC molecules, integrin activation occurs within seconds by means of a process known as “inside-out” signalling. This leads both to an affinity increase and to clustering of membrane-bound integrins [45,46]. Because the integrins do not need to be synthesized *de novo*, this signaled adhesion response is very fast and allows binding to their ligands ICAM-1 (i.e. CD54), formation of the immunological synapse, a polarized release of secretory vesicles including cytokines, chemokines and lytic factors, and thereby effective cell killing.

As discussed above, different methods are being used for assessing antigen-specific T cells and the choice of one or several of these for routine application in a particular laboratory will depend on the information sought for, and often on the experience and the technical environment of the team. If the exact antigens are known, in particular for CD8$^+$ T cells, read-out with pMHC multimers will allow a very robust assessment of low frequency T cells, irrespective of their functionality. On the one hand, it means that functionally defective cells could be detected [31] but on the other hand, if effector cells do produce TNF, but not IFN-γ, they could be missed by IFN-γ ELISpot, but prove detectable with appropriate pMHC multimers, as we recently observed [37]. We have now introduced a new assay which identifies antigen-specific CD8$^+$ T cells by

specifically detecting activated integrin molecules with fluorescent ICAM-1 [47]. The principle of this assay is depicted in Figure 1, and relies on the interaction of activated LFA-1 with its ligand ICAM-1, which occurs rapidly during T cell activation. The affinity of activated LFA-1 for monomeric ICAM-1 (Kd = 0.5 µM) [48] is within the affinity range of the TCR for a monomeric pMHC (Kd = 0.1 – 400µM) [49], and weaker than the nanomolar affinity of an antibody for its antigen. In addition, the interaction LFA-1/ICAM-1 lasts a few seconds ($t1/2 = \ln 2/k_{diss}$ = ~ 7s) [48], and is in the same range as that of TCR/pMHC (0.5 to approx. 30s) [49,50]. Therefore, to stably detect the activated integrins, pre-assembled multimeric ICAM-1 (mICAM-1) with higher avidity had to be used. These multimers can be produced by pre-incubating recombinant ICAM-1-Fc molecules with fluorescent polyclonal anti-Fc antibodies, and used in FCM [51]. After carefully optimizing the multimer production and the staining conditions, we showed that the method is suitable for the detection of antigen-specific $CD8^+$ T cells against a range of antigens (e.g. CMV, HIV, EBV, Flu, YFV) and for various cell preparations (whole blood, fresh and frozen/thawed PBMCs, *in vitro* expanded T cells) [47]. We also used the assay to detect tumor antigen-specific $CD8^+$ T cells from prostate carcinoma patients who had received a multipeptide vaccine; hence, mICAM-1 binding can also be used to measure tumor antigen-specific T cells [47]. Compared with previous methods for assessing functional antigen-specific $CD8^+$ T-cells, our assay detected changes in the avidity of surface integrins rather than *de novo* production of (intracellular) proteins. This produces clear benefits, including the short activation time (typically only a few minutes when using short peptides, i.e. exact epitopes, as stimuli, and slightly longer - approx. 30 min - when using overlapping 15mers), and the simplicity of the staining procedure. The assessment of integrin activation can be combined with other staining reagents to derive detailed information about antigen-specific T cells, such as pMHC multimers, as well as surface and intracellular markers. The short stimulation time would not allow a significant change in the expression of these factors, which is the case for the long incubation time required to detect cytokines. Hence, the assay is likely to nearly reflect the *in vivo* situation. Significantly, we showed that i) while the two assays correlate very tightly, only a fraction of pMHC-tetramer positive cells also bind mICAM multimers after antigen-specific stimulation, ii) mICAM-1 staining highly correlated with cytokine production (IFN-γ and TNF) and CD107a upregulation, iii) mICAM-1 binding correlates very well with perforin and granzyme B expression, and iv) $CD8^+$ T cells that bind

mICAM-1 after antigen-stimulation can be found in both the effector and memory subsets. Based on these observations, we concluded that activated integrins represent a very early marker that identifies functional (very likely cytotoxic) $CD8^+$ T cells. mICAM-1 staining could be used not only for detection of antigen-specific cells, but also to address the effects of certain substances, or (immune) cell subsets, on T cell function. For example, we recently used the assay for assessing the impact of $G\alpha_s$-coupled receptor agonists and sleep on T cell function [52]. In addition, one attractive asset of the mICAM assay is that it preserves cell viability and cytokine production, allowing fast and easy isolation of functional cells [47].

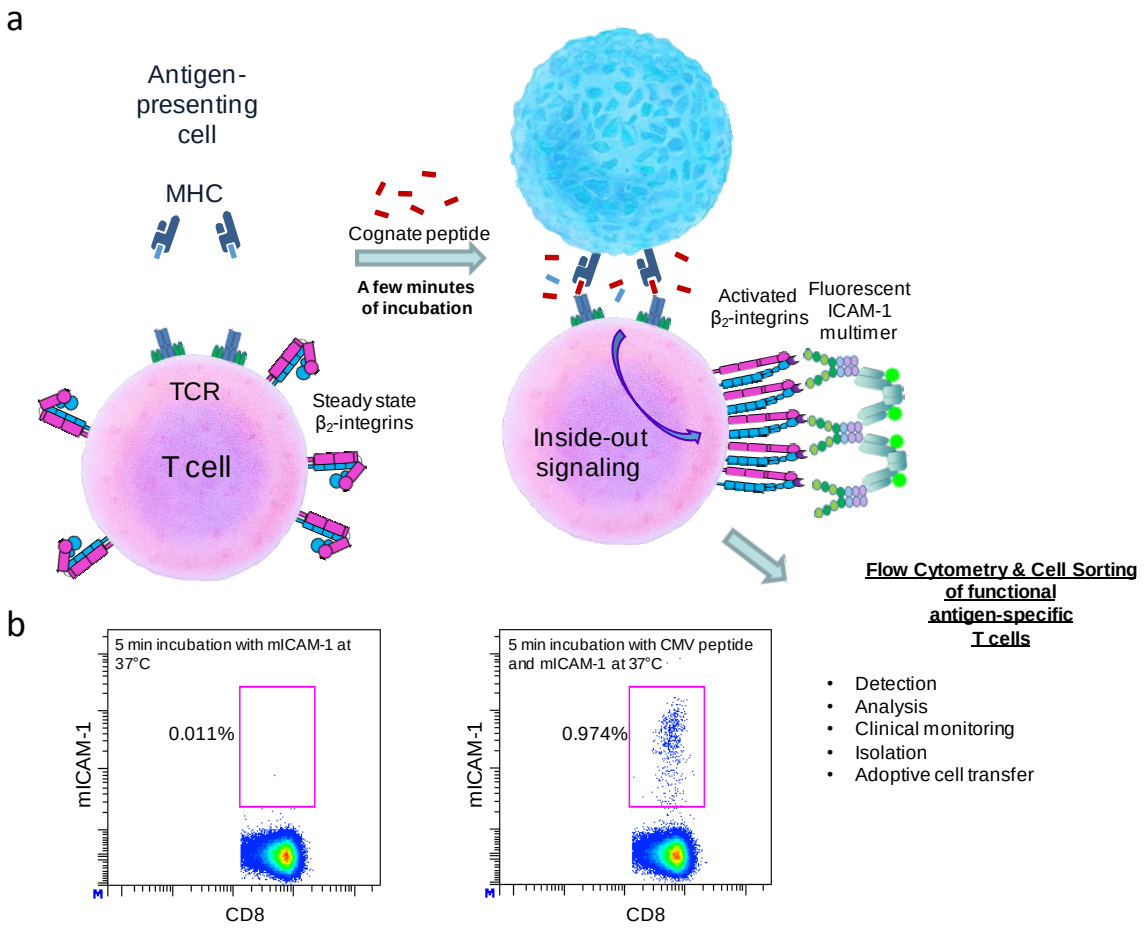


**Figure 1: Assessment of adhesion as a T cell monitoring tool.** a) Principle of the mICAM-1 assay: Following T-cell receptor-mediated stimulation, integrin activation occurs within seconds through a process known as "inside-out" signaling which leads to an affinity increase and a clustering of membrane-bound integrins. Fluorescent intercellular adhesion molecule (ICAM)-1 multimers bind specifically to activated $\beta_2$-integrins and can be used in flow cytometry for fast monitoring and isolation of antigen-specific T cells. b) Example of mICAM-1 (1.56 µg/ml) staining after 5 min activation of the blood of an HLA-A2+ CMV seropositive healthy donor in the absence (left) or presence (right) of the synthetic peptide NLVPMVATV (pp65-derived, HLA-A2 binding immunodominant epitope of CMV) at 4 µg/ml. Cells were stained with mICAM-1 PE, CD8 BV605, and CD3 BV510; dot-plots are gated on $CD3^+CD8^+$ lymphocytes.

The main characteristics of the mICAM assay are compared to those of established methods in Table 1. The background staining, i.e., the staining in the unstimulated control condition, is approx. 0.01%-0.04% in our hands, hence comparable to that of the ICS assay. Some individuals show an increased background staining, particularly when using frozen/thawed cells, but the overall signal-to-noise ratio can be optimized. The mICAM-1 reagent is stable for months when kept at 4°C, however, the background staining slightly increases when stored for more than a month under this condition. This can be prevented by freezing the multimers at -80°C (all unpublished data). For $CD8^+$ T cells, the combination of pMHC multimers and mICAM-1 staining is perfect for a fast, high-sensitivity assessment of total and functional numbers of antigen-specific T cells of interest.

### 3.1.5 Conclusion and perspectives

There is more than ever a major interest in the assessment of immune cells, and in particular T cells, in cancer immunotherapies and in pathogen-driven diseases. A number of assays are available to monitor antigen-specific T cells. Since none of these assays alone is able to capture the entire range of T cell properties and functions, the best option is probably to combine two complementary tests, especially when monitoring clinical studies. Assessment of conformation changes in adhesion molecules on T cells can be specifically detected with ICAM-1 multimers and exploited for rapid identification of functional T cells. The method could be useful for monitoring T cell immunity in health and disease, after vaccination, or during various immunotherapies. Because it preserves cell viability and functionality, it might also evolve as a precious tool to isolate highly functional $CD8^+$ T-lymphocytes for further gene expression or protein analysis, as well as for adoptive transfer strategies. Presumably, mICAM-$1^+$ antigen-specific $CD8^+$ T-cells with their strong functional capacity ensure protective immunity and thus can be used as correlates of protection. This, however, still needs to be evaluated. In the next step, we are planning to validate the assay and to implement it as an exploratory monitoring tool in the context of an upcoming multipeptide-based vaccination trial for glioma patients.

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# Optimization of a protocol for the simultaneous identification of functional antigen-specific $CD8^+$ and $CD4^+$ T cells after in-vitro antigen stimulation using synthetic long peptides and Poly-ICLC

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Manuscript in preparation

The author of the present thesis planned and analyzed all experiments and performed the experiments shown in Figures 1-4, S1, S3, and S4. The author was strongly involved in the interpretation of the final data and in writing of the manuscript.

## 3.2 Part II: Manuscript: Optimization of a protocol for the simultaneous identification of functional antigen-specific CD8+ and CD4+ cells after in-vitro antigen stimulation using synthetic long peptides and Poly-ICLC

### 3.2.1 Abstract

Synthetic long peptides (SLPs) are the format often used for the screening of antigen-specific $CD8^+$ and $CD4^+$ cell responses, especially when the source material for analysis is limited. Here, we present an *in vitro* protocol which allows the amplification of antigen-specific cells and the subsequent functional analysis of both T cell types using SLPs. Known viral-derived epitopes were elongated to 20mer SLPs on the N-, C-, and both termini for HLA-class I binders or on the N- and C termini for HLA-class II binders. The strength of $CD8^+$ T cell activation was dependent on the elongation site of the SLP. With peptide-stimulation only, $CD4^+$ T cell responses were completely lost in 22% of the tests performed *ex vivo*. The addition of a TLR agonist (Poly-ICLC) and an increased SLP concentration for T cell pre-sensitization in a 12 day *in vitro* culture, as well as an increased SLP concentration for the read-out of functional antigen-specific cells in the ELISpot and ICS, improved the detection of $CD8^+$ and $CD4^+$ T cell reactivities using SLPs.

### 3.2.2 Introduction

The identification of antigen-derived T cell epitopes is essential to basic and clinical immunology, such as the monitoring of immunotherapy or the mapping of virus- or tumor-derived antigens. To assess functional antigen-specific T cells, methods like the intracellular cytokine staining (ICS) assay or the interferon- (IFN-) γ Enzyme Linked ImmunoSpot Assay (ELISpot) are widely used. Such tests can be performed *ex vivo* on whole blood or peripheral blood mononuclear cells (PBMCs). However, since memory antigen-primed $CD8^+$ and $CD4^+$ T cells are generally present at very low frequencies in the blood, a pre-culture is often performed before testing to increase the frequency of the cells of interest and overcome the detection limit of these assays. During this pre-sensitization step, isolated PBMCs from patients or healthy volunteers are stimulated by the antigen of choice at the beginning of the culture and expanded by the addition of interleukin- (IL-) 2. The culture is typically continued for seven to twelve days before cell testing [1]. In our hands, the expansion

rate of antigen-specific cells is donor- and antigen-dependent and varies between 10-1000 fold. Synthetic peptides represent the most accessible and convenient format for stimulating T cells *in vitro*. Since $CD8^+$ and $CD4^+$ T cells recognize short HLA-epitopes (classically 8-12 amino acids (aa) for HLA-class I and 13-18 aa for HLA-class II presented peptides), such “minimal” short peptides can be used when the epitopes and/or the HLA-restriction are known or should be identified. This is for example the case for the monitoring of anti-cancer vaccines containing a mixture of HLA-class I and -class II binding peptides [2-4]. When the exact peptide is not known, (overlapping) synthetic long peptide (SLPs; > 15 aa) can be used in e.g. large-scale screening studies to assess the immunogenicity of pathogen-derived proteins [5], as they are of a suitable format to limit the size of peptide pools. Long peptides are used for vaccination studies, as they potentially contain HLA-class I and -class II peptides for the stimulation of both $CD8^+$ and $CD4^+$ T cells [6,7]. SLPs of 15-20 aa are considered to be of optimal length for the recognition of $CD4^+$ T cells, however, in most cases, they stimulate $CD8^+$ T cells less effectively than their short epitope counterpart, although this can be at least in part compensated by an increasing peptide concentration [8-11]. Hence, one method is to use SLPs for the monitoring of antigen-specific $CD4^+$ T cells, together with predicted short peptides or overlapping short peptide pools for the identification of $CD8^+$ T cells responses [6,12]. The simultaneous identification of both $CD8^+$ and $CD4^+$ antigen-specific T cells using only a single format is therefore imperfect.

In 2012, Singh et al. published a protocol that allows the *ex vivo* detection of low-frequency antigen-specific $CD4^+$ and $CD8^+$ T cells using SLPs of 30 aa[13]. Autologous adherent monocytes were stimulated with GM-CSF, peptide-pulsed, and used as antigen-presenting cells (APCs) to stimulate the non-adherent PBMC fraction. A higher peptide concentration (50 µg/ml) and the addition of Poly-IC, a Toll-like receptor (TLR) 3 agonist, together with IFN-α (Roferon-A) led to an improvement in the detection of T cell reactivities, in the range of 50-90% of the responses detected when using the short peptide. Although the authors were able to detect low frequencies of antigen-specific T cells *ex* vivo with this protocol, this might be not sufficient as often an amplification step is required before the functional analysis [6,12].

Here we present an improved protocol for both the pre-sensitization and the testing of antigen-specific T cells in bulk (no monocyte isolation is needed) PBMCs using SLPs. For the optimization of the method, we compared $CD8^+$ and $CD4^+$ T cell responses by ICS and ELISpot against known viral-derived HLA-class I and -class II epitopic peptides and their elongated (N-, C-, or N- and C-terminal) 20 aa long peptide versions. We show that not only $CD8^+$ T cell responses, but also $CD4^+$ T cell responses can be sub-optimally detected with SLPs. The addition of Poly-ICLC on day 1 and an increase in the SLP peptide concentration for the pre-stimulation and for the readout are sufficient to detect $CD8^+$ and $CD4^+$ responses at an acceptable level. This protocol is optimized for the simultaneous testing of both $CD4^+$ and $CD8^+$ cells using 20 aa long SLPs. It should be especially suitable when the number of starting PBMCs is limited, or the number of peptides to be tested is substantial, e.g. for the monitoring of patient-derived samples.

## 3.2.3 Material and Methods

PBMC isolation, cell freezing, and thawing

Buffy coats, mononuclear blood cell concentrates, or leukaphereses from healthy volunteers were obtained from the Center for Clinical Transfusion Medicine Tübingen). Participants gave informed consent and the study was approved by the ethical review committee of the University of Tübingen, project 713/2018BO2. PBMCs were isolated within 8 h-24 h after blood drawing. The blood products were diluted 1:4 (buffy coats, leukaphereses) or 1:8 (mononuclear blood cell concentrates) with PBS (homemade, 10x Lonza) and PBMCs were isolated using density centrifugation (Biocoll, Merck). PBMCs were washed twice with PBS and up to 30 x $10^6$ cells were resuspended in 1 ml freezing medium (heat-inactivated: h.i. fetal bovine serum (FBS, Capricorn Scientific) with 10% dimethylsulfoxide (DMSO, Sigma-Aldrich). Cyrovials (Nunc™, Sigma-Aldrich) were placed into a cryo container (Nalgene® Mr. Frosty, Sigma-Aldrich), stored up to four days at -80°C, and transferred to liquid nitrogen (-196°C). For all experiments, cells were thawed using IMDM (Gibco) supplemented with 2.5% h.i. human male AB serum (Sigma-Aldrich), 100 U/ml Penicillin/ 0.1 mg/ml Streptomycin (Pen/Strep; Sigma-Aldrich), and 50 µm β-Mercaptoethanol (β-ME; Merck).

Synthetic peptides

Known short HLA-class I (HLA-A*02) and HLA-class II (HLA-DR) restricted T cell epitopes were synthesized in-house using an automated peptide synthesis (ABI 433A, Applied Biosystems). Purity was assessed by reverse-phase liquid chromatography (e296, Waters). Each of these peptides was elongated to a 20mer SLP according to the viral protein sequence, at the N-, C-, and N and C- termini for HLA-class I, or at N and C- termini for HLA-class II peptides. All SLPs were ordered from JPT Peptide Technologies (Berlin, Germany). Lyophilized peptides were diluted in sterile $dH_2O$ containing 10% DMSO. Peptide origin, sequence, elongation-site, code, and purity are shown in Table 1.

Table 1: Virus-derived peptides

| | | Peptide length (aa) | Sequence | Elongation site | Code | Purity >50% | Purity >95% |
|---|---|---|---|---|---|---|---|
| HLA-class I | CMV pp65 strain AD169 Uniprot P06725 | 9 | **NLVPMVATV** | | CMV | - | >95 |
| | | 20 | GILAR**NLVPMVATV**QGQNLK | N + C | N-CMV-C | 89 | 98 |
| | | 20 | WPPWQAGILAR**NLVPMVATV** | N | N-CMV | 70 | 96 |
| | | 20 | **NLVPMVATV**QGQNLKYQEFF | C | CMV-C | 67 | 98 |
| | Matrix Influenza strain A/Puerto Rico/8/1934 H1N1 Uniprot P03485 | 9 | **GILGFVFTL** | - | INF | - | 100 |
| | | 20 | SPLTK**GILGFVFTL**TVPSER | N + C | N-INF-C | 78 | 90 |
| | | 20 | KTRPILSPLTK**GILGFVFTL** | N | N-INF | 66 | 98 |
| | | 20 | **GILGFVFTL**TVPSERGLQRR | C | INF-C | 63 | 98 |
| | EBV BRFL1 strain B95-8 Uniport P03209 | 9 | **YVLDHLIVV** | - | BRFL1 | 86 | - |
| | | 20 | PIVMR**YVLDHLIVV**TDRFFI | N + C | N-BRFL1-C | 53 | 98 |
| | | 20 | ACSIACPIVMR**YVLDHLIVV** | N | N-BRFL1 | 83 | 95 |
| | | 20 | **YVLDHLIVV**TDRFFIQAPSN | C | BRFL1-C | 52 | 96 |
| HLA-class II | CMV pp65 strain AD169 Uniprot P06725-1 | 15 | **YQEFFWDANDIYRIF** | - | CMV | - | 95 |
| | | 20 | NLK**YQEFFWDANDIYRIF**AE | N + C | N-CMV-C | 74. | 98 |
| | EBV EBNA2 strain B95-8 Uniprot P12978 | 15 | **PRSPTVFYNIPPMPL** | - | EBNA2 | 81 | - |
| | | 20 | SPE**PRSPTVFYNIPPMPL**PP | N + C | N-EBNA2-C | 60 | 99 |
| | EBV EBNA1 strain B95-8 Uniprot P03211 | 14 | **KTSLYNLRRGTALA** | - | EBNA1 | 72 | - |
| | | 20 | GGS**KTSLYNLRRGTALA**IPQ | N + C | N-EBNA1-C | 89 | 96 |
| CMV: cytomegalovirus; EBV: Epstein-Barr-Virus | | | | | | | |

12 day in vitro stimulation of PBMCs

For in vitro amplification of virus-specific cells, 1.0-3.5 x 106 or 3.6-6.5 x 106 PBMCs/ well were seeded in T cell medium (TCM; IMDM supplemented with 100 U/ml Pen/ 0.1 mg/ml Strep, 50 µM β-ME, and 10% h.i. human AB serum) in a 48- or 24-well plate, respectively (Cellstar®, Greiner bio-one) and cultured overnight at 37°C, 7.5%

$CO_2$. On day 1, cells were stimulated with 1, 5, 10, or 50 µg/ml peptides as indicated in the respective figure. Additionally, cells were supplemented with single addition of Poly-ICLC (Poly-IC stabilized with Poly-L-lysine and carboxymethyl cellulose [14], Hiltonol®; Oncovir; 20 µg/ml), GM-CSF (0.8 U/µl), the combination of both (either single or multiple additions) Poly-ICLC and GM-CSF (P+G, same concentration as for the single addition), or were left in media alone (standard condition) [2]. On days 3, 5, 7, and 9, 2 ng/ml recombinant human IL-2 (R&D Systems) was added to the culture. Cells were splitted 1:2 on day 5, 7, or 9 before the addition of IL-2 when the cell layer was > 70% confluent. On day 12, cells were harvested, counted manually using the Neubauer chamber and 0.1% trypan blue (Sigma Aldrich), and further analyzed for their functionality either with ICS or ELISpot. Of note, for the ELISpot analysis, the *in vitro* culture was performed in two independent replicates for each condition.

Intracellular cytokine staining assay (ICS)

PBMCs were either thawed approximately 8 h prior to the *ex vivo* ICS analysis (as described above), resuspended in TCM containing 1 µg/ml DNase I (Sigma-Aldrich) and rested at 37°C, 7.5% $CO_2$ until use, or directly tested on day 12 after the *in vitro* culture. 0.5-2 x $10^6$ cells/ well were seeded in a 96-well round-bottom plate (Cellstar®, Greiner bio-one) and stimulated with either 10 µg/ml short peptides or with 10 or 50 µg/ml elongated SLPs as indicated. DMSO (10% in $dH_2O$) and Staphylococcus enterotoxin B (SEB; 10 µg/ml Sigma-Aldrich) were used as negative and positive controls, respectively. The protein transport inhibitors Brefeldin A (10 µg/ml, Sigma-Aldrich) and Golgi Stop (BD) were added and cells were incubated for 12 h at 37°C, 7.5% $CO_2$. For the staining, cells were washed in FACS buffer (PBS without Ca/Mg (Lonza), 0,02% $NaN_3$ (Roth), 2 mM EDTA (both Sigma-Aldrich) and 2% h.i. FBS, and stained extracellularly for 20 min at 4°C. Cells were fixed and permeabilized (Cytofix/Cytoperm, BD) for 20 min at 4°C. After one washing step with permeabilization buffer (PBS 1X, 0,02% $NaN_3$, 0,5% BSA and 0,1% saponin (Sigma-Aldrich), cells were stained intracellularly for 20 min at 4°C. All antibodies were pre-titrated and are shown in Table 2. The cells were acquired on a LSRFortessa™ SORP (BD) using the DIVA software (Version 6). The data analysis was performed with the FlowJo software (Version 10.6.1). An exemplary gating strategy is shown in Supplementary Figure S1 for the *ex vivo* ICS. For the ICS analysis after the 12 day *in*

*vitro* culture, the lymphocyte (FSC-A/SSC-A) gate was enlarged. All results were manually audited.

Table 2: Monoclonal antibody panel for ICS

| | Marker | Fluorochrome | Clone | Manufacturer | Cat. No. |
|---|---|---|---|---|---|
| Extracellular staining | Live/Dead | Zombie Aqua | - | BioLegend | 423102 |
| | CD4 | APC-Cy7 | RPA-T4 | BD | 557871 |
| | CD8 | BV605 | RPA-T8 | BioLegend | 301040 |
| Intracellular staining | TNF | Pacific Blue | MAB11 | BioLegend | 502920 |
| | IFN-γ | FITC | B27 | BD | 554700 |

Flow cytometry staining of immune cell subsets

After the 12 day *in vitro* cultivation, 0.5 Mio. cells per test were used for the staining of immune cell subsets. FC receptors were blocked by adding 10 µl FC block (BD, final concentration 0.25 mg/ml) for 10 min at RT. Without a washing step, cells were subsequently stained with pre-titrated antibodies (Table 3) for 20 min, 4°C for T cell, B cell, NK cell, monocyte, and dendritic cell identification. A representative gating strategy is shown in Supplementary Figure S2. Cells were washed with FACS buffer and acquired on the LSRFortessa$^{TM}$ SORP (BD) using the DIVA software (Version 6). The data analysis was performed with the FlowJo software (Version 10.6.1).

Table 3: Monoclonal antibody panel for the detection of immune cell subsets

| | Marker | Fluorochrome | Clone | Manufacturer | Cat. No. |
|---|---|---|---|---|---|
| Extracellular staining | CD16 | BV786 | B73.1 | BioLegend | 360734 |
| | HLA-DR | BV711 | G46-6 | BD | 563696 |
| | CD14 | BV650 | M5E2 | BioLegend | 301835 |
| | Live/Dead | Zombie Aqua | - | BioLegend | 423102 |
| | CD8 | PE-Cy7 | SFCI21Thy2D3 | BeckmanCoulter | 737661 |
| | CD3 | PE-Cy5.5 | SK7 | eBioscience | 35-0036-42 |
| | CD56 | PE | B159 | BD | 555516 |
| | CD4 | APC-Cy7 | RPA-T4 | BD | 557871 |
| | CD19 | AF700 | HIB19 | BD | 561031 |
| | CD11c | APC | 3.9 | BioLegend | 301613 |

Enzyme-linked immunospot assay

The IFN-γ ELISpot assay is described in detail elsewhere [2]. The ELISpot analysis was performed in technical duplicates. 50.000 cells per well were seeded for the

viral-derived peptide stimulation and 300.000 cells per well for the background assessment (10% DMSO, negative control). Phytohemagglutinin-L (PHA, 10 µg/ml, Sigma-Aldrich) was used as positive control. Spots were counted with the ImmunoSpot series 6 ultra-V analyzer (CTL Europe GmbH) according to the laboratory standard protocol. No cut-off was set for the spot count.

Statistics

The GraphPad Prism 6 (version 6.01) software was used for statistical analysis. Normal distribution was checked by the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilliefors correction. For comparison of two parameters, the Mann-Whitney test was used. For single-parametric multi-comparison, a one-way ANOVA was performed. Statistical differences were considered as significant for $p \leq 0.05$ (*) or $p < 0.01$ (**). Information on the statistical test and number of data points tested are stated in the legend for each experiment.

## 3.2.4 Results

### *Ex vivo* detection of viral-specific T cells using SLPs alone is suboptimal

To assess whether $CD8^+$ and $CD4^+$ T cell reactivities can readily be detected when using long peptides instead of short epitopes, we used known HLA-class I and -class II viral-derived epitopes from the cytomegalovirus (CMV), Epstein-Barr-Virus (EBV), and Influenza virus (INF) as model antigens. Short epitopic peptides were elongated to 20aa SLPs according to the viral sequences. either at the N-, C-, or both N+C termini for HLA-class I peptides, or at the N+C termini for HLA-class II peptides. In a first step, we assessed the *ex vivo* immune response ($TNF^+IFN^+$ cytokine response in ICS) of $CD8^+$ or $CD4^+$ cells against the short peptides and the respective elongated SLPs in PBMCs of healthy donors (n=3 SLPs or n=1 SLP per specificity for elongated HLA-class I or -class II peptides, respectively; each tested in n=3 healthy donors (HD)). All donors had been pre-screened for the presence of viral-specific cells at various frequencies (0.02 – 8.80% peptide-specific cells within the $CD8^+$ or $CD4^+$ cell subset; tetramer staining/ICS; data not shown). SLPs were used at two purity grades (>90% and >50%). A representative gating strategy and examples of the flow cytometry results for HD6 and HD4 are shown in the Supplementary Figure S1A and B.

Antigen-specific $CD8^+$ cells (Figure 1A) and $CD4^+$ cells (Figure 1B) could be detected after the stimulation with the short peptides (set to 100%) in almost all cases (except

HD1 response against BRFL1). We also detected a response against most SLPs, however, the reactivity was often reduced compared to that against the corresponding short peptides, and occasionally, completely lost (example frequencies of peptide-specific cells: Figure 1B: HD4/HD6: EBNA1: 0.090%/0.013% N-EBNA1-C 0.00%/0.00%). The loss of response against the EBV-derived HLA-class II restricted SLP N-EBNA1-C in 2/3 donors was especially unexpected, since elongation was only two and three amino acids at each N- or -C terminus. We further observed that HLA-class I epitopes elongated in N-terminal mostly induced cytokine levels comparable to that of the matched short peptide, whereas the presence of a C-extension led to a decreased recognition of approximately 40% in mean in for 2/3 peptides tested (BRFL1 and INF, but not CMV). There was no statistically significant difference between the cytokine levels induced by the SLPs of the two purities (paired student´s t-test; data not shown); therefore we used only the peptides with the higher purity for all following experiments. These results clearly show that monitoring of $CD8^+$ or $CD4^+$ T cells with elongated (i.e. 20mer) peptides needs further improvement to decrease the chance of missing T cell responses.

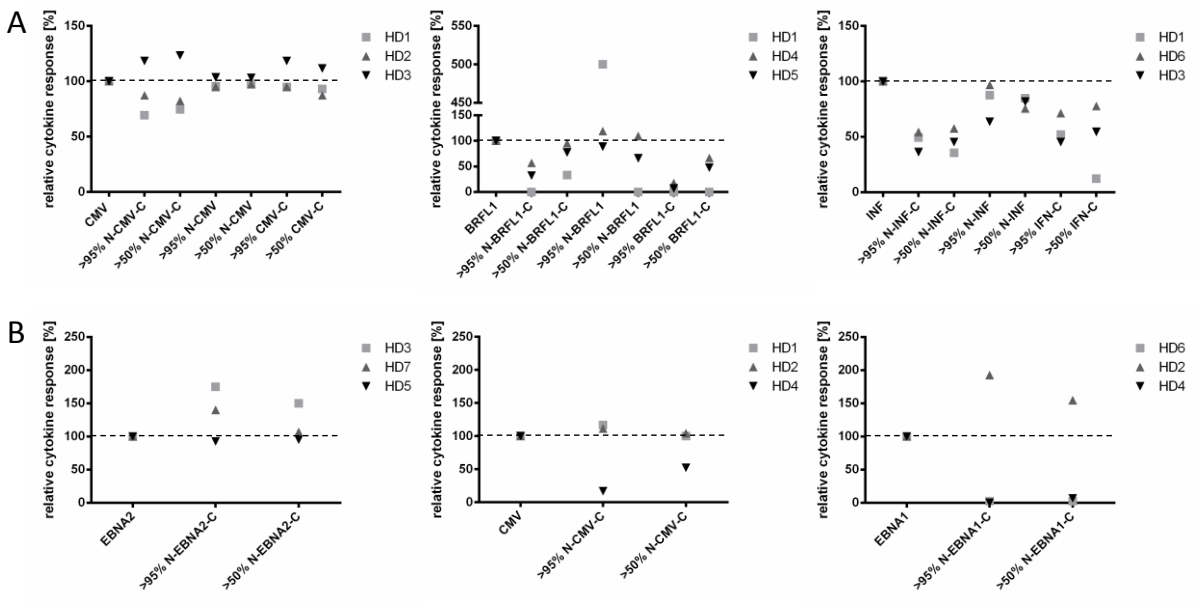


**Figure 1: *Ex vivo* detection of virus-specific T cell responses using synthetic long peptides is impaired in some cases.** PBMCs from healthy donors (HD, n=7, n=3 per virus protein) were stimulated with 10 µg/ml HLA-class I and -class II viral-derived epitopes from CMV, EBV (BRFL1/EBNA1/EBNA2) and Influenza virus (INF) of short length, and with their respective SLP version (20mers, N-peptide source-C: N- and C-terminal elongated; N-P: N-terminal elongated; P-C: C-terminal elongated) for 12 h. Two purities (>50% and >90%) of the SLPs were used. Single, live $CD8^+$ and $CD4^+$ lymphocytes were analyzed in ICS for their production of IFN-γ and TNF. Shown are the percentages of peptide-specific (background cytokine production in the negative control was subtracted) $CD8^+$ (A) and $CD4^+$ (B) cells that produce both cytokines ($IFN\text{-}\gamma^+TNF^+$). The anti-SLP cytokine response is depicted related to the response to the short peptides (range 0.012-2.22 % of $IFN^+TNF^+$ cells within the $CD4^+$ or $CD8^+$ subsets (numbers background subtracted), which is normalized to 100%.

## The addition of Poly-ICLC and GM-CSF improves the detection of viral-specific T cells with SLPs after 12 days *in vitro* stimulation

Many laboratories, including ourselves, use a short T cell presensitisation step (synthetic peptides + IL-2) to increase the frequency of antigen-specific cells in order to overcome the detection limit of functional assays like the ELISpot or ICS [1,2]. It was shown that a high peptide concentration loaded onto monocytes, together with the addition of GM-CSF and a TLR-3 ligand is favorable for the detection of in particular $CD8^+$ cells [13]. Therefore, we tested the addition of GM-CSF and Poly-ICLC (P+G) in our pre-sensitization culture We used cells from donor HD4, who had shown a response against the short HLA-class II EBNA1 peptide, but no response against the elongated N-EBNA1-C SLP in the *ex vivo* testing (Figure 1B). We stimulated HD4 PBMCs with 5 µg/ml of the EBNA1 or N-EBNA1-C peptides on day 1 without further addition (our standard setting) or in the presence of Poly-ICLC and GM-CSF (P+G). P+G was either added once on day 1 or on several days of the *in*

*vitro* culture (days 1, 3, 5, 7, and 9). On day 12, cells were re-stimulated with the same peptide as on day 1 and analyzed by ICS. For the restimulation, we used our standard concentration of 10 µg/ml for the short peptide, whereas we increased the peptide concentration of the SLP to 50 µg/ml [13]. Without the addition of P+G, $CD4^+$ T cells produced cytokines after restimulation with the SLP, however, the response was approx. 70-fold lower as compared to that obtained against the matched short peptide (approx. 6.3 % to 0.09% of the $CD4^+$ T cells, Figure 2A-B). With addition of P+G, we observed a 11-fold (approx. 1% of the $CD4^+$ cells) increase in cytokine-producing cells after SLP stimulation compared to the condition without P+G. No difference in the frequency of cytokine-producing cells was observed between the single or multiple additions of P+G. Although this T cell response was still approx. 6-fold lower as compared to that against the short peptide, cells were now readily detectable (Figure 2B). Hence, the addition of P+G can improve the detection of antigen-specific cells against SLPs.

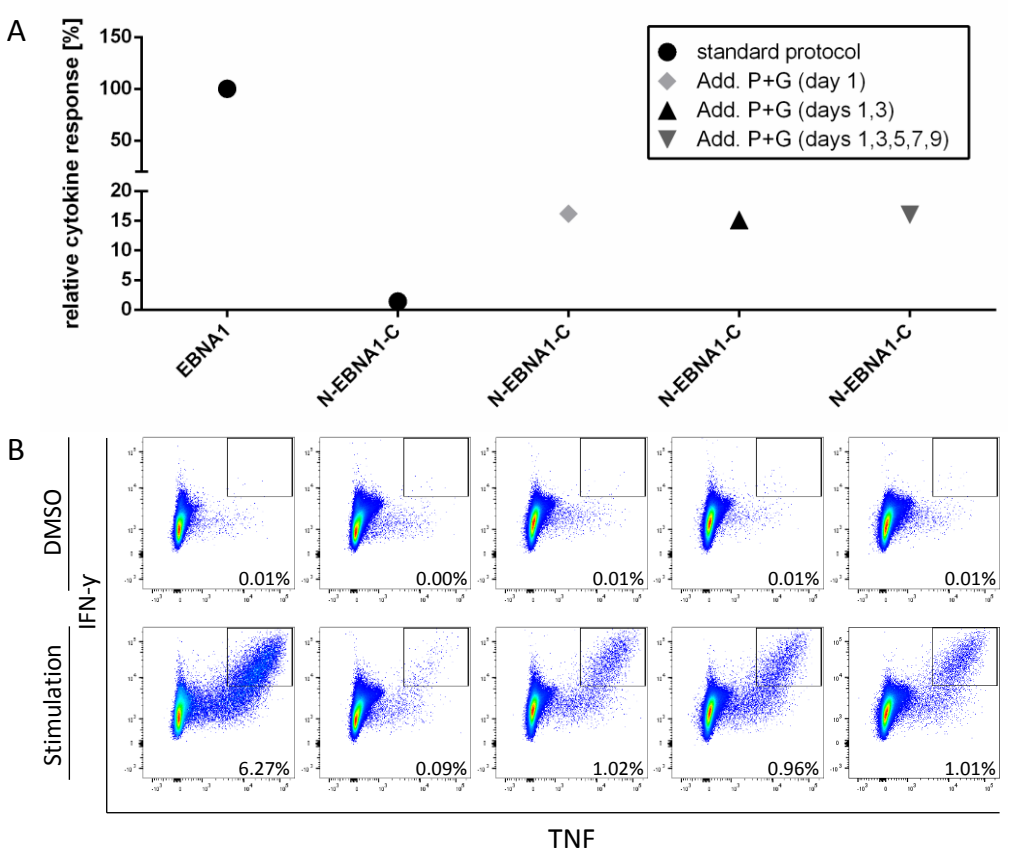


**Figure 2: The addition of Poly-ICLC and GM-CSF can improve the detection of viral-specific T cells after *in vitro* stimulation using synthetic long peptides (SLPs, 20mers).** PBMCs from healthy donor 4 (HD4) were stimulated with the short HLA-class II peptide from EBV EBNA1 (EBNA1) or its N+C-terminal SLP version (N-EBNA1-C, 20mer), both with a concentration of 5 µg/ml on day 1 without or with the additions of 20 µg Poly-lCLC and 0,8 U/µl GM-CSF (P+G). P+G was added once or repeatdlyduring the 12 day stimulation. On day 12, cells were re-stimulated with the same peptide as on day 1 for ICS analysis (10 µg/ml short peptide; 50 µg/ml SLP). Single cells, live $CD4^+$ lymphocytes were analyzed for their specific cytokine production (IFN-$\gamma^+$TNF$^+$ cells). (A) specific cytokine response (percentage of IFN-$\gamma^+$TNF$^+$CD4$^+$ cells after subtracting the background signal of the negative control (DMSO)) after SLP stimulation without (standard protocol, black dot) or with the addition of P+G on day 1 (light grey diamond), days 1 and 3 (black triangle), or days 1,3,5,7, and 9 (inverted dark grey triangle) relative to the cytokine response to the matched short peptide (standard protocol; black dot). (B) The respective flow cytometry dot plots are shown for each condition, either for the negative control (DMSO; upper panel), or after peptide stimulation (lower panel). Percentages indicate the frequencies of IFN-γ and TNF double positive events within the $CD4^+$ cell population.

## The single addition of Poly-ICLC is superior to the combination of Poly-ICLC and GM-CSF for T cell pre-sensitization with SLPs

To identify the minimum essential compound(s) that need to be added to the pre-sensitization step of the 12 day *in vitro* culture, we stimulated PBMCs with different peptides alone, with the combination of P+G, or with the single compounds Poly-ICLC or GM-CSF on day 1. IFN-γ ELISpot and ICS were performed on day 12 to identify functional antigen-specific cells. For the ELISpot, we seeded 300.000 cells/

well for the unstimulated control (Figure 3A; graph shows spot counts normalized to 50.000 cells), as this is the usual cell number that we use for PBMC monitoring, e.g. after anti-cancer peptide vaccination. For the cells stimulated with peptides, we seeded 50.000 cells/ well to reach a spot count within the quantification range of the ELISpot reader (Figure 3B). PBMCs of n=2 HD (HD5, HD8) were stimulated, each with one HLA-class I (N-INF (HD5), N-CMV (HD8)) and one HLA-class II (N-EBNA2-C (HD5), N-CMV-C (HD8)) SLP on day 1 and day 12 of the *in vitro* culture. The highest background spot count was observed for the combination of P+G (median 49.6) followed by the condition without addition (median 25.3), the single addition of Poly-ICLC (median 20.1), whereas the single addition of GM-CSF demonstrated a very low background spot number (median 5.6) (Figure 3A). The spot count for the stimulated cells was the highest for the condition with Poly-ICLC (median 861.3), followed by the conditions with P+G (median 727.1), without addition (573.9), and GM-CSF alone (median 84.01) (Figure 3B). For the ICS, we stimulated cells from n=2 HD with HLA-class I SLPs variants (N-INF, INF-C, N-INF-C (Figure 3C-E); EBV, N-EBV, EBV-C, N-EBV-C (not shown)), or from n=2 donors with one HLA-class II short peptide or the matched SLP variant (CMV, N-CMV-C (Figure 3F) or EBV, N-EBNA1-C (not shown)) on day 1. On day 12, cells were re-stimulated with the same SLPs as on day 1 or with the respective short peptide version. The response was the highest after SLP plus Poly-ICLC stimulation in 3/4 tests shown in Figure 3 C-F (HLA-class I and class II peptides). Altogether, higher frequencies were detected in 5/8 tests conditions with the single addition of Poly-ICLC, followed by the condition without any addition (2/8), and the single addition of GM-CSF (1/8). The background cytokine production was overall low, with no marked differences between conditions (data not shown). Taken together, the single addition of Poly-ICLC was sufficient and superior to the other conditions.

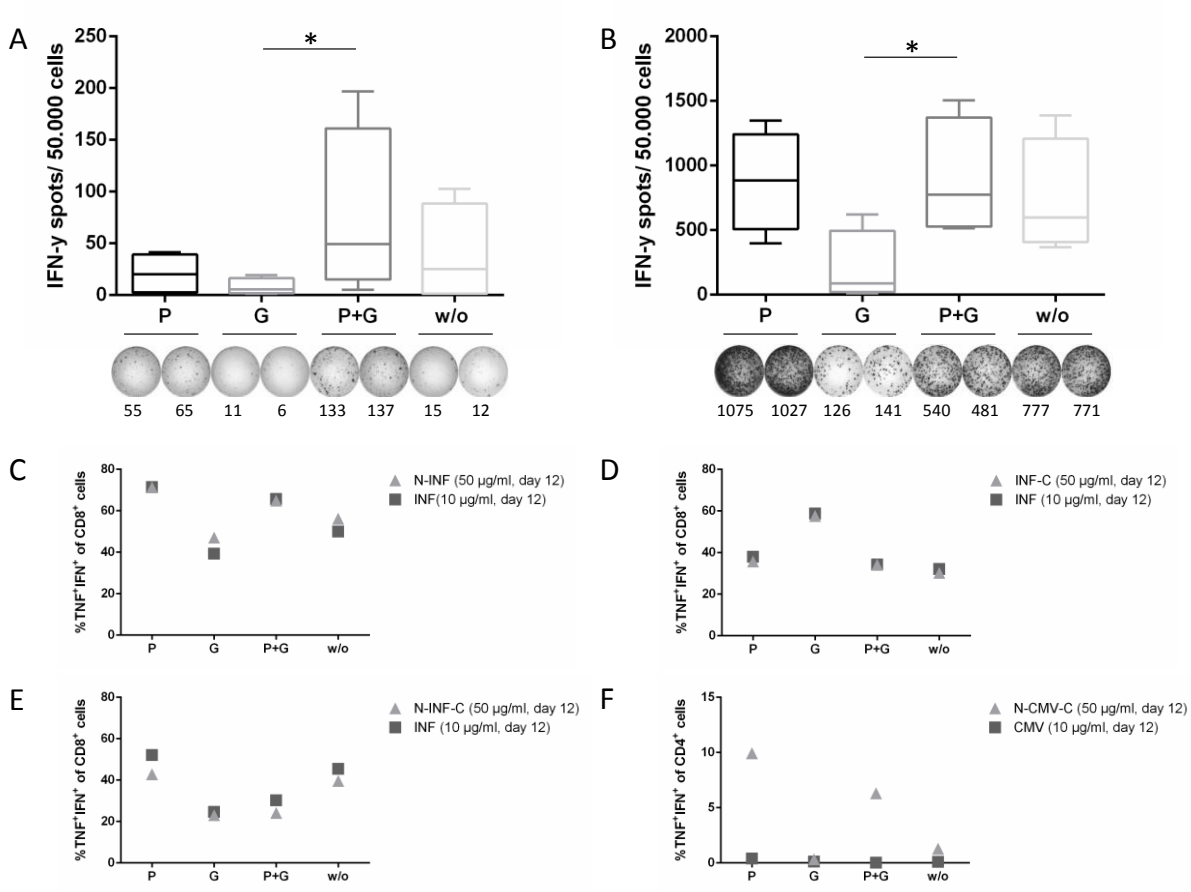


**Figure 3: The single addition of Poly-ICLC is superior to the combination of Poly-ICLC and GM-CSF for the detection of viral-specific T cells using SLPs (20mers).** Selected HD PBMCs were stimulated with viral-derived HLA-class I and HLA-class II SLPs (10 µg/ml, 20mers, N- and/or -C-terminal elongations are indicated) on day 1. In addition to the peptide stimulation on day 1, Poly-ICLC (20 µg/ml, P), GM-CSF (0.8 µg/ml, G), or the combination of P+G (same concentration as for the single addition) were added. Control stimulation was performed with peptide only (w/o). (A+B) Cells were re-stimulated with the respective SLP on day 12. IFN-γ ELISpot analysis of the background spot counts (A, negative control; 300.000 cells/well seeded, normalized to 50.000 cells) or spot counts after SLP stimulation (B; 50.000 cells/well seeded) derived from CMV (N-CMV-C) and EBV EBNA2 (N-EBNA2-C) (HLA-class II restricted peptides) or from CMV (N-CMV) and Influenza virus (N-INF) (HLA-class I restricted peptides). In total n=2 HDs. ELISpot analysis was performed in duplicates. Shown are the box and whisker plots of the mean IFN-γ spot counts per 50.000 cells. Median values are indicated. Statistical analysis: Friedman test with post-test Dunn´s multiple comparison. Representative ELISpot wells and spot counts (technical replicates) of PBMCs from HD5 for the negative control or the SLP N-INF stimulation are shwon. (C-F) ICS results. Cells were re-stimulated with 50 µg/ml of the respective SLP or 10 µg/ml short peptide on day 12. Shown are the specific frequencies (background subtracted) of cytokine ($IFN\gamma^+TNF^+$) $CD8^+$ (HD1; C-E) or $CD4^+$ (HD4, G) cells. Absolute frequencies (background subtracted) with short peptide on day 1 and day 12: 27,7% $IFN\gamma^+TNF^+CD8^+$ (INF); 4.70% $IFN\gamma^+TNF^+CD4^+$ (CMV).

**Optimal peptide concentration finding for simultaneous read-out of $CD8^+$ and $CD4^+$ T cells with SLPs**

The next step was to identify the optimal SLP concentration in addition to Poly-ICLC to read out cytokine-producing $CD8^+$ and $CD4^+$ cells. For this, we stimulated PBMCs from in total n=4 HDs with SLPs from n=2 viral sources each (EBV, CMV, and INF) resulting in altogether n=5 test conditions for HLA-class I peptides and n=2 test conditions for HLA-class II peptides. We tested different SLP concentrations on day 1 and day 12 (1, 10, 50 µg/ml). In all cases, Poly-ICLC was added on day 1. Matched short peptides were used as controls (standard concentrations) for the stimulation on day 1 (pre-sensitization) and the read-out on day 12 (1 µg/ml and 10 µg/ml, respectively). Representative results are shown in Figure 4A-C for the stimulation of $CD8^+$ cells with n=3 influenza virus (INF) -derived SLP variants and in Figure 4D for the stimulation of $CD4^+$ cells with n=1 HLA-class II EBV-derived SLP. The results for the HLA-class I N-CMV and CMV-C SLPs, as well as for the HLA-class II EBV N-EBNA-2-C peptide are shown in Supplementary Figure S3. In all cases, a response against the SLPs could be detected. Taken all experiments for HLA-class I SLPs into account, highest responses against the SLPs were detected in 3/5 test conditions when cells were stimulated with 10 µg/ml SLP on day 1 and 50 µg/ml on day 12. For the HLA-class II CMV-derived peptide, the highest response against the SLP was detected for 1 µg/ml (day 1) and 10 or 50 µg/ml on day 12, which was detected in 2/2 test conditions. Overall, optimal $CD8^+$ and $CD4^+$ T cell responses against SLPs reached between 25% and 204% of that obtained against the short epitopes. While optimal conditions might differ for $CD8^+$ or $CD4^+$ cells, 10 µg/ml SLP on day 1 and 50 µg/ml SLP on day 12 appeared to be the best condition for detection of T cell responses in both subsets simultaneously.

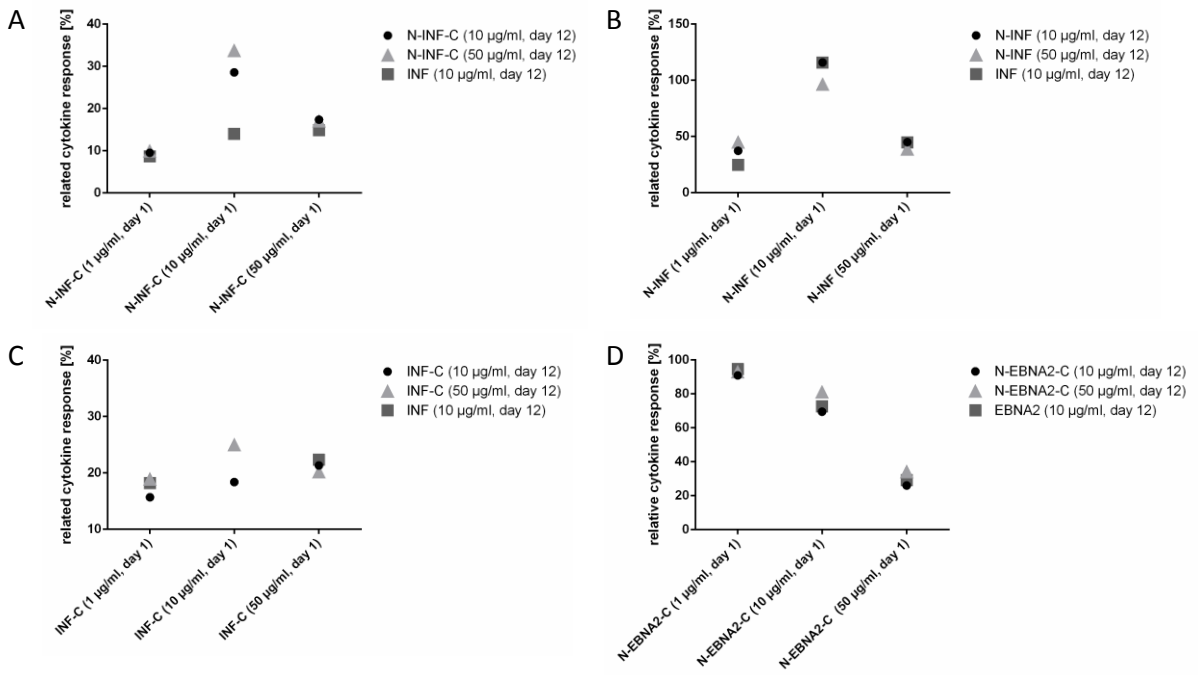


**Figure 4: Identification of the optimal SLP concentration for the readout of functional viral-specific CD8$^+$ and CD4$^+$ cells after *in vitro* expansion.** PBMCs from healthy donors were cultured with IFN- or EBV- derived short peptides or matched SLPs (20mers, N- and/or -C-terminal elongations are indicated) for 12 days. On day 1, cells were stimulated with 1 µg/ml (HLA-class I) or 5 µg/ml (HLA-class II) short peptide (standard concentrations) or with 1, 10, or 50 µg/ml of the SLP plus 20 µg/ml Poly-ICLC (x-axis). On day 12, cells were re-stimulated in the ICS with 10 µg/ml short peptide or with 10 µg/ml or 50 µg/ml of the respective SLP (graph legends). Single live CD8$^+$ or CD4$^+$ lymphocytes were analyzed for their specific IFN-γ and TNF expression (percentage of IFN-γ$^+$TNF$^+$ cells; background of the negative control was subtracted). Shown are the percentages of double positive cytokine CD8$^+$ cells (A-C, HD9) or CD4 cells (D, HD7) relative to the cytokine response detected with the standard protocol (short peptide on day 1 (1/5 µg/ml) and on day 12 (10 µg/ml). Absolute specific frequencies: 11.9% IFN-γ$^+$TNF$^+$ CD8$^+$ (INF); 16.3% IFN-γ$^+$TNF$^+$ CD4$^+$ (EBV EBNA2).

## The addition of Poly-ICLC on day 1 of the *in vitro* culture increases the frequencies of B cells on day 12

Finally, and based on our results that Poly-ICLC addition supports the recognition of SLPs, we asked whether this TLR3 ligand has an impact on the cell composition on day 12 of the *in* vitro culture. We analyzed different cell subsets (CD8$^+$ and CD4$^+$ T cells, NK cells, monocyte subsets (classical, non-classical, intermediate), dendritic cells (DCs), and B cells) by flow cytometry, with or without addition of Poly-ICLC on day 1 of the *in vitro* culture. We observed a significant increase in the frequency of B cells on day 12 when Poly-ICLC was added to the culture (Figure 5A; median 0.6% (w/o) vs. 1.3% (P) B cells within leukocytes). Although not significant, a slight increase in the frequency of HLA-DR positive B cells (median 95.1% vs. 99.3% HLA-DR$^+$ B cells w/o or with PolyICLC, respectively) as well as in the HLA-DR MFI of on B cells (median MFI 18945 (w/o) vs. 30931(P); Figure 5B) on day 12 were visible.

These results suggest that B cells are the APCs responsible for the improved SLP processing and presentation during the pre-sensitization step.

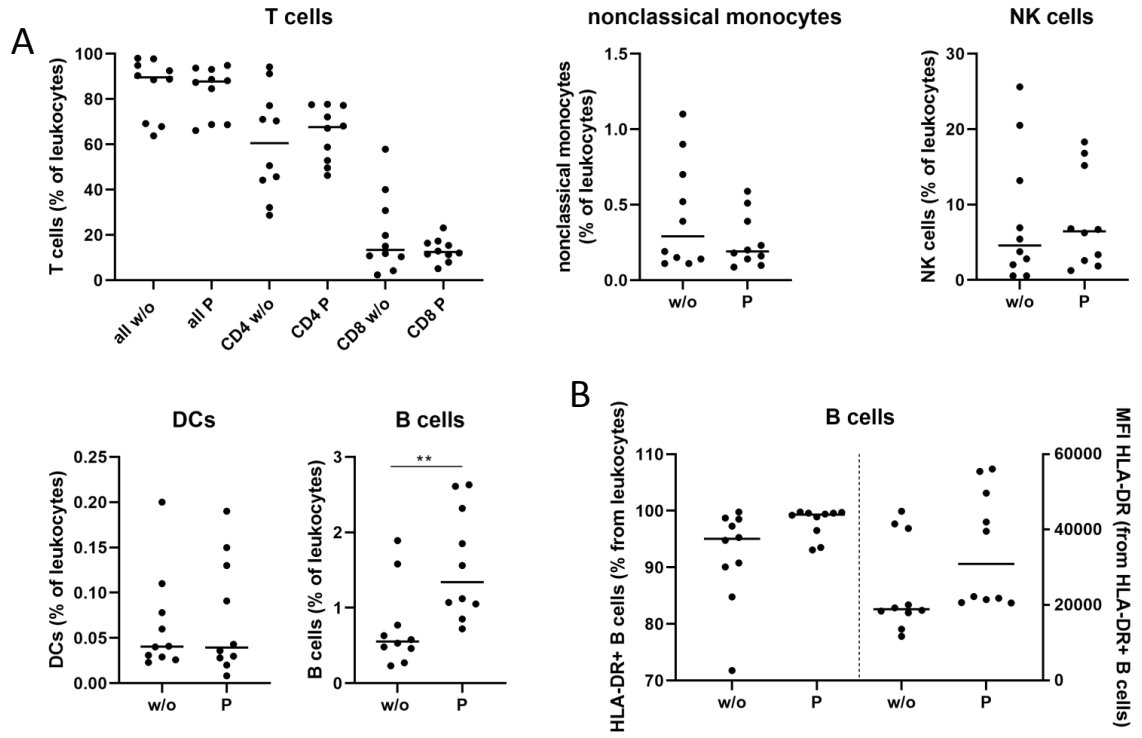


**Figure 5: Increased frequencies of B cells are detected upon addition of Poly-ICLC during T-cell presensitization.** PBMCs from 5 HDs were stimulated with peptide with (P) or without (w/o) 20 µg Poly-ICLC on day 1 of the *in vitro* culture. On day 12, cells were harvested and 500.000 cells stained to identify immune cell subsets. (A) Dot plot graphs show the frequency of live T cells ($CD3^+CD56^-$), $CD4^+$ T cells ($CD3^+CD56^-CD4^+$), $CD8^+$ T cells ($CD3^+CD56^-CD8^+$), B cells ($CD3^-CD56^-CD14^-CD19^+$), NK cells ($CD3^-CD56^+$), nonclassical monocytes ($CD3^-CD56^-CD14^-CD16^+$, or DCs ($CD3^-CD56^-CD19^-CD14^-CD11c^+$). (B) HLA-DR expression within B cells (left Y-axis) and corresponding HLA-DR median flurorescence (MFI) (right Y-axis) Bars indicate medians. One dot indicates one tested condition per donor. Statistical analysis: Mann-Whitney test. ** $p<0.01$.

## 3.2.5 Discussion

The identification and quantification of antigen-specific T cells is pivotal for many T cell based immunotherapies and epitope mapping studies. In many instances, T cells of interest are rare, and their robust and sensitive *in vitro* measurement remains a technical challenge. Therefore, approaches are being taken in order to increase the frequency of these rare cells *in vitro, such as* the *in vitro* pre-sensitization of cell cultures with synthetic peptides.

Here, we present an optimized protocol for the pre-sensitization and functional read-out of antigen-specific $CD8^+$ and $CD4^+$ cells using long synthetic peptides. As model antigens, we used described HLA-class I and -class II viral-derived short epitopes

that were elongated to 20 aa at the N-, C-, or N+C- termini. Frequencies of responding T cells were assessed by multiparameter ICS and by IFN-γ ELISpot. First, we asked whether SLPs are suitable for assessing of $CD8^+$ and $CD4^+$ responses in an *ex vivo* setting. For most specificities, effector cells could indeed be detected when using the short epitopic peptides and the respective SLPs. However, there were notable exceptions, both for HLA-class I as for HLA-class II binders. For $CD8^+$ T cell reactivities, N-terminus elongated peptides showed comparable results to the short peptide versions, whereas the response was decreased up to 93% if epitopes were elongated on the C-terminus (C- or N+C SLPs) if a response was detected against the short peptide. Our results are in accordance with previous observations that the capacity of recalling $CD8^+$ T cell responses depends on the elongation site (N, C, N+C) of the SLP [15]. However, it is not clear if the processing of SLPs is proteasome-dependent or if the peptides get trimmed by proteases, either extracellularly or in endosomal or lysosomal compartments [15,16]. To test proteasome-dependency in our system, we incubated the cells with the proteasome inhibitor Epoxomycin (5 µM) 30 min prior to SLP addition (n=1 SLP (CMV-C); n=2 HD). No effect on the activation of $CD8^+$ antigen-specific cells was observed (data not shown). This suggests a proteasome-independent peptide processing, possibly occurring through extracellular peptidases. For $CD4^+$ T cell, the complete loss of reactivities against SLPs in some donors was somehow unexpected, since HLA-class II molecules generally accommodate ligands of 8-25 residues; as an example, various length variants of the same core sequence are often identified in peptidomics studies [17,18].

We next aimed at establishing a suitable protocol for expansion and read-out of both HLA class-I and -class II restricted T cell responses based on the following considerations: 1) SLPs are efficiently processed and cross-presented by human monocyte-derived DCs for CD8 T cell presentation [16], and 2) An increased SLP concentration and the addition of GM-CSF and Poly-IC to monocytes used as APCs improved the T cell response *ex vivo* [13]. We therefore tested whether the addition of GM-CSF, which is routinely used to differentiate monocytes into DCs [13], and Poly-ICLC could improve the expansion of antigen-specific T cell when using SLPs instead of exact short epitopes. In our settings in the ICS and ELISpot, the addition of Poly-ICLC alone was found to be the best condition.

Notably, no changes in the frequencies of $CD8^+$ and $CD4^+$ cells were observed after the *in vitro* stimulation period in relation to the different additions (Supplementary Figure S4). Hence, the addition of Poly-ICLC, which has been shown to activate an innate immune response *in vivo* [19], improves also the immune response against SLPs *in vitro*.

To understand the reason for the increased frequency of antigen-specific cells in the presence of Poly-ICLC, we analyzed the immune cell subset composition after in vitro pre-sensitization (day 12 of the culture). We observed a significant increase of 46% in the frequency of B cells, together with an enhanced median fluorescence intensity of HLA-DR on these cells. The percentages of the other APC subsets tested, like monocytes and DCs, were not affected in the presence of Poly-ICLC. Although it has been shown that SLPs are not only presented by DCs, but also by B cells [16,20], blood human B cells generally do not express TLR3 [21], and do not respond to Poly-IC by proliferation or differentiation [22], indicating that they are likely not directly stimulated by Poly-ICLC. This suggests that indirect activation by TLR3 expressing PBMCs (T cells, myeloid DCs, and NK cells [23]) may happen during the pre-sensitization.

Although we show that SLPs can be used both for the pre-sensitization and at the read-out step, T cell responses were in some cases reduced compared to that obtained against the short epitopic peptide. Hence, T cell frequencies might be underestimated with long peptides, and, if a more exact quantification is required, the experiments should be repeated using short epitopes. We also tested whether it is possible to use the SLPs for the cell culture stimulation on day 1 and the short epitope for the restimulation on day 12. For HLA-class I restricted peptides this was possible in all cases. For HLA-class II restricted peptides in some cases the response was completely gone, but this phenomenon was donor-dependent. This might indicate that the SLP is processed in another short peptide with possibly a higher affinity dependent on the HLA type of the donor, which does not match the known short peptide or the 20mer binds directly [24]. For these donors, this did not exclude a detected response against the known short peptide when the short peptide was used on day 1 and day 12 of the *in vitro* culture.

Earlier reports propose to use SLPs at a higher concentration than short peptides [8, 11, 13,15]. As SLPs possibly contain both HLA-class I and -II epitopes, and in case of

limited PBMC material, a simultaneous read-out of both $CD8^+$ and $CD4^+$ responses will be preferred. To examine which SLP concentration is the best to use on day 1 in addition to Poly-ICLC and on day 12 of the *in vitro* culture to readout $CD8^+$ and $CD4^+$ antigen-specific T cell responses, we tested different concentrations. The optimal condition appeared to be the use of 10 µg/ml SLP on day 1 and 50 µg/ml SLP on day 12, although it is the optimal concentration for the readout of single $CD8^+$ T cell responses but not for the readout of $CD4^+$ T cells responses. For the readout of $CD4^+$ T cells the optimal concentration of 1 µg/ml SLP on day 1 and 10/50 µg/ml SLP had the highest efficacy to stimulate the cells. However, the loss in response was less strong for $CD4^+$ T cells (up to 25%) when the optimal condition for the readout of both cell types was used instead of the best concentration for single readout as for $CD8^+$ T cells when 1 µg/ml SLP on day1 and 10/50 µg/ml on day 12 (up to 72%) was used instead of the best concentration for single readout.

Finally, we have already applied the conditions for simultaneous $CD8^+$ and $CD4^+$ T cell assessment (10 µg/ml SLP on day 1 and 50 µg/ml SLP to readout) to monitor tumor antigen-specific T cells in vaccinated cancer patients. In a recent study, prostate carcinoma patients were vaccinated with a single 20aa long SLP emulsified in Montanide. The i*n vitro* monitoring demonstrated that 86% of the patients did respond to the vaccine by developing anti-vaccine $CD4^+$ T cells, and one simultaneous $CD8^+$ cell response was also detected (manuscript submitted). With the same protocol, we also verified that PBMC T cells from a patient vaccinated with a survivin derived class II epitope (15mer) could be detected with the cognate elongated SLP (20aa). The frequencies of specific $CD4^+$ cells were similar using the short and the long peptides (data not shown). Hence, the new improved protocol is not only suitable for the read-out of viral-specific responses but also for that of anti-cancer responses.

Taken together, we have established an improved pre-sensitization assay for the detection of low frequency antigen-specific $CD8^+$ and $CD4^+$ T cells in ICS and ELISpot using SLPs. Our protocol should be useful for both epitope mapping of e.g. pathogens and for the immune monitoring of T-cell based immunotherapies, especially anti-cancer vaccines.

## 3.2.6 References


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## 3.2.7 Supplementary Chapter 3I: Optimization of a protocol for the simultaneous identification of functional antigen-specific $CD8^+$ and $CD4^+$ T cells after in-vitro antigen stimulation using synthetic long peptides and Poly-ICLC

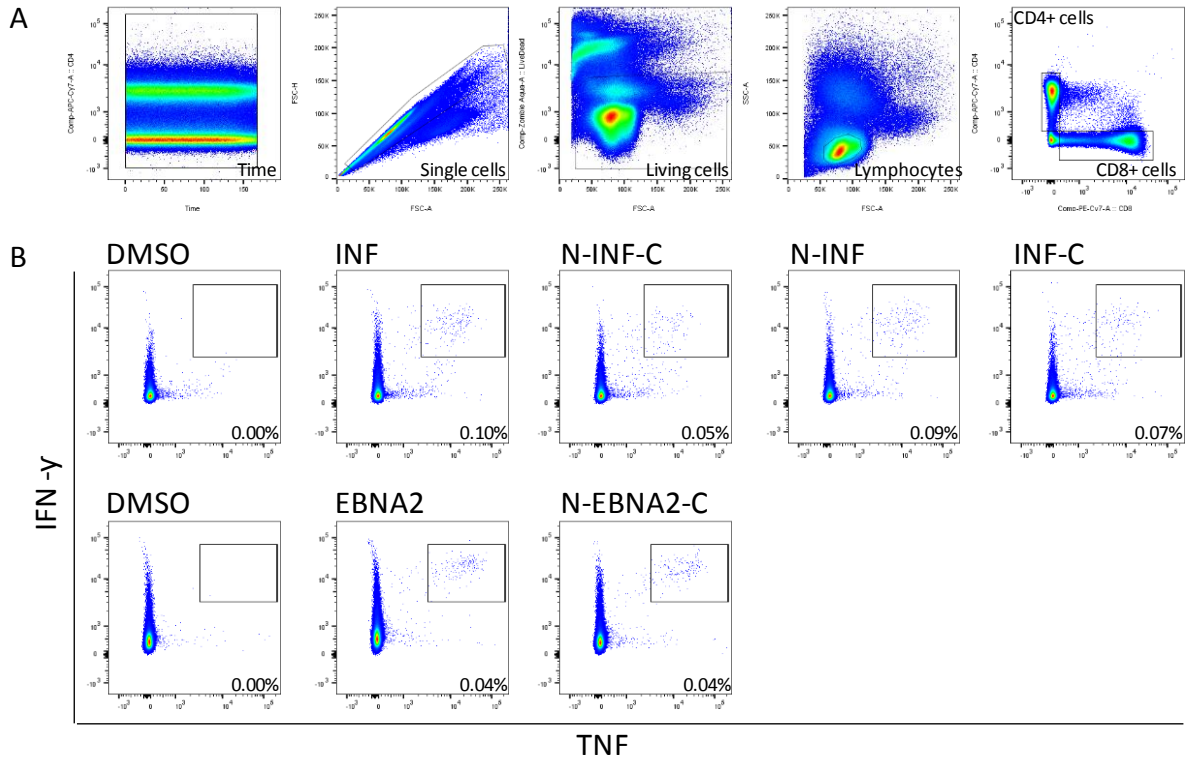


**Figure S1: Gating strategy and exemplary results for the *ex vivo* ICS**. (A) Gating strategy from left to right: Time, single cells, living cells, and lymphocytes, which were further gated for their CD8 or CD4 coreceptor expression. (B) Exemplary results for the IFN-γ and TNF coexpression of $CD8^+$ cells (upper row; Donor 6 (D6)) and $CD4^+$ cells (lower row, D5) after stimulation with DMSO (10% in water; negative control) or viral-derived peptides from the Influenza (INF) and EBV (EBNA2) viruses of short and elongated length (20mers, N-peptide source-C: N- and C-terminal elongated; N- peptide source: N-terminal elongated; peptide source-C: C-terminal elongated). Percentages indicate IFN-$\gamma^+$ $TNF^+CD4^+/CD8^+$ cells within the $CD4^+/CD8^+$ cell population.

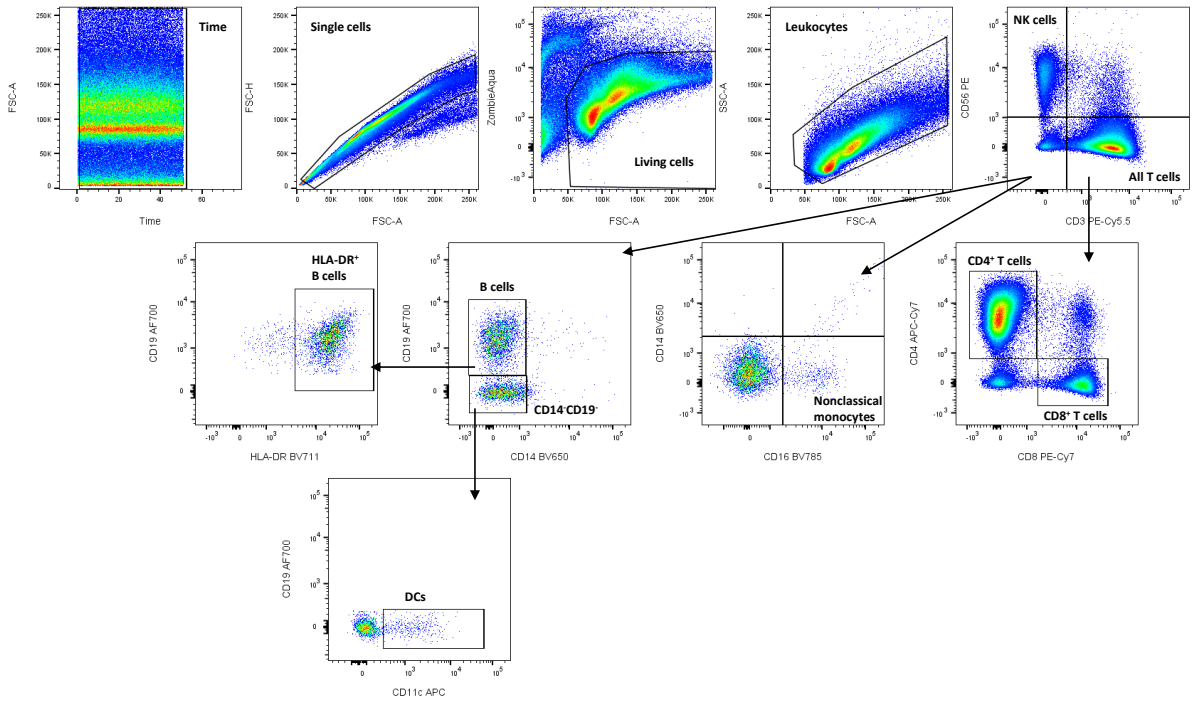


**Figure S2: Gating strategy for the identification of immune cell populations.** Gating strategy from left to right: shown are gates for time, single cells, living cells, and leukocytes. Leukocytes were further gated for NK cells ($CD3^-CD56^+$) and T cells ($CD3^+CD56^-$). T cells were subsequently gated for $CD4^+$ T cells ($CD3^+CD56^-CD4^+$) and $CD8^+$ T cells ($CD3^+CD56^-CD8^+$). $CD56^-$ and $CD3^-$ cells were gated for non-classical monocytes ($CD3^-CD56^-CD14^-CD16^{+)}$ and B cells ($CD3^-CD56^-CD14^-CD19^+$). $CD14^-$ and $CD19^-$ cells were analyzed for DCs ($CD3^-CD56^-CD19^-CD14^-CD11c^+$). The HLA-DR expression was analyzed for B cells.

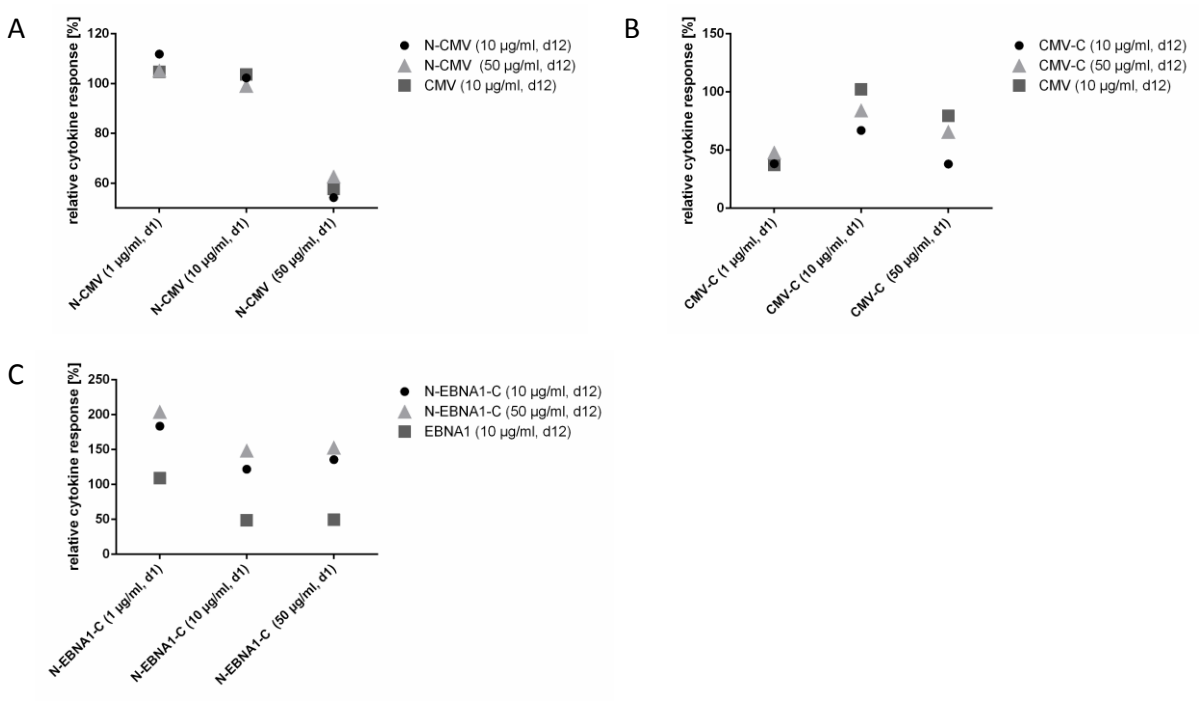


**Figure S3: Identification of the optimal SLP concentration for the read out of functional viral-specific CD8$^+$ and CD4$^+$ cells after *in vitro* expansion.** PBMCs from healthy donors were cultured with CMV- or EBV-derived short peptides or matched SLPs (20mers, N- and/or -C-terminal elongations are indicated) for 12 days. On day 1, cells were stimulated with 1 µg/ml (HLA-class I) or 5 µg/ml (HLA-class II) short peptides (standard concentrations) or with 1, 10, or 50 µg/ml of the SLP plus the addition of 20 µg/ml Poly-ICLC (x-axis). On day 12, cells were re-stimulated with 10 µg/ml of the short peptide or with 10 or 50 µg/ml of the respective SLP before ICS analysis (legend). Single live CD8$^+$ or CD4$^+$ lymphocytes were analyzed for their specific IFN-γ and TNF expression (percentage of IFN-γ$^+$TNF$^+$ cells; background of the negative control was subtracted). Shown are the percentages of double positive cytokine CD8$^+$ cells (A/B, healthy donor (HD2) or CD4$^+$ cells (C, HD10) relative to the cytokine response detected by the standard protocol (cells stimulated with the short peptide on day 1 (1/5 µg/ml) and on day 12 (10 µg/ml)). Absolute frequencies were: 58.7% IFN-γ$^+$TNF$^+$ CD8$^+$ (INF); 6.4% IFN-γ$^+$TNF$^+$ CD4$^+$ (EBV EBNA2).

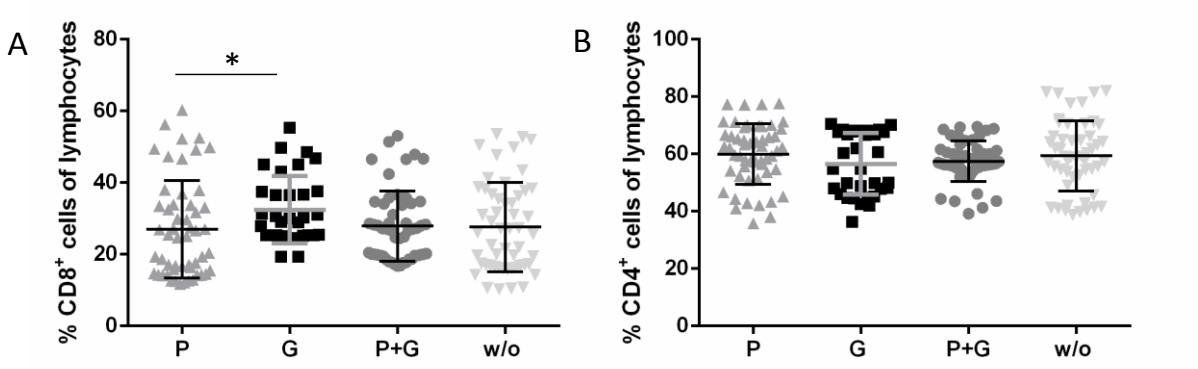


**Figure S4: The single addition of Hiltonol during T cell expansion does not alter the CD8 and CD4 cell frequencies.** On day 1 of the 12 day in vitro culture, PBMCs were stimulated with known viral-derived peptides of short length or with their respective SLP version (HLA-class I restricted epitopes derived from EBV BRFL1 and Influenza virus: N- and C-terminal, N-terminal, and C-terminal elongated; HLA-class II restricted epitopes derived from EBV EBNA1 and CMV: N- and C-terminal). In addition to the peptide stimulation, Poly-ICLC (20 µg/ml, P), GM-CSF (0.8 µg/ml, G), or the combination of P+G (same concentration as for the single addition) or no addition (w/o) was tested. On day 12, cells were re-stimulated with 10 µg/ml of the short peptide, with 50 µg/ml of the respective elongated version, or left unstimulated (10% DMSO control) and an ICS was performed. Shown are the frequencies of CD4 and CD8 cells within living lymphocytes. n=7 donors (except GM-CSF alone: n=4 donors), n=2 tests per donor and condition (short peptide) and n=3 tests per donor and condition (SLP). Statistical analysis: Kruskal-Wallis test with Dunn´s multiple comparison post-test. * $p \leq 0.05$.

# 4 General discussion and perspectives

In the last decades, immunotherapy made major breakthroughs with CBI [1-3], as well as cellular therapies [4-6]. The FDA has already approved two immunotherapies for PCa: the cell-based anti-cancer vaccine PROVENGE (Sipuleucel-T, Sip-T, Dendreon) [7] and the CBI Keytruda® (Pembrolizumab, anti-PD1) [8-10]. In 2010, Sip-T was approved for asymptomatic or minimal symptomatic metastatic castration-resistant PCa (mCRPCa) [7]. Although Sip-T is often stated as a “major breakthrough” for immunotherapy, as it was the first therapeutic anti-cancer vaccine ever approved by the FDA, it is of low acceptance and only 1 in 10 mCRPCa patients have been treated in the US between 2010 and 2016 [11]. This might be explained by: I) the absence of the “usual” response indicators as neither the time to progression (detected by computer tomography) nor the PSA level was significantly affected by the treatment, despite a modest increase in overall survival [6,7]; II) the vaccine itself is not clearly defined as it varies in the cell composition and dose not only between patients but also between the three doses for one patient [6]; III) the therapy is very expensive (approximately $100,000 in total) and stated as not cost-effective [11,12]. CBI therapy with Pembrolizumab (Keytruda®) was approved by the FDA in 2017 for the treatment of unresectable or metastatic solid tumors with characteristics of microsatellite instability with high or mismatch repair deficient [8-10]. For the first time, the FDA approved a cancer therapy, which was not based on the tumor origin e.g. for colon or lung cancer, but based on a specific genetic feature (biomarker) [10]. About 2-12% of PCa patients are tested positive for microsatellite instability biomarker and might profit from this therapy [13,14]. Indeed, a shrinking of several metastases (>30%) and a PSA level decrease of 63% was already reported for this therapy in early 2020, in a case report of a 58-year old man suffering from mCRPCa [15].

Both therapies are approved for metastatic PCa which represents an already advanced tumor state, and as all other therapies for metastatic PCa, they have no curative intention. For most PCa patients (90%), the cancer is diagnosed at local stage [16]. About 30% of those who are then treated with RP show BCR within ten years post-surgery [17,18], as detected by the recurring rise in PSA. MM and/or CSCs which have been already disseminated from the primary tumor before therapy might be the cause [19-21]. Eliminating those cells could potentially reduce or

prevent BCR, tumor recurrence, and/or metastasis formation. A therapy that achieves this goal would improve the patient's quality of life immensely, as it might prevent second-line treatments which can have severe side effects [6,9,22-26] and best, cure PCa.

One strategy to eliminate malignant cells specifically is the anti-cancer vaccination. Such immunotherapy has the advantage of being well tolerated by the patients and more importantly, of inducing long term tumor control by immune memory. Furthermore, peptides for vaccination can be easily manufactured under GMP conditions. In my opinion, anti-cancer vaccination for the treatment of in particular PCa patients in addition to the first-line treatment, should be superior compared to the treatments with CBI or CAR-T cells. CBI treatment might be a good option for the treatment of high mutational metastatic PCa, as described above, but is not appropriate for a "cold tumor" like PCa, as there is most likely no suppressed immune response in the tumor present of which the "brake" can be removed. CAR-T cells in contrast to peptide-vaccination require a more time consuming multistep manufacturing process as they have to be extracted from the leukapheresis of the patient and genetically engineered. In addition to their relevance in hematological diseases, they also showed severe side effects [27]. Nevertheless, anti-cancer peptide vaccines can be improved by several steps. The most suitable improvements and perspectives for the RV001 vaccination are discussed next.

The clinical phase I/II study (NCT03199872; "RV001V, a RhoC Anticancer Vaccine, Against Metastasis From Solid Tumours") presented here (section 2) is a SLP (20mer)-based vaccination, which specifically targets the small GTPase RhoC in PCa patients. RhoC is involved in tumor invasion and metastasis formation [28-30] and is highly expressed on high-grade localized carcinoma and high-grade metastatic carcinoma of the prostate [31]. The expression of RhoC is not limited to the prostate, as it is also overexpressed in other tumors e.g. breast cancer [30] or adenocarcinoma of the pancreas [32]. Hence, if successful, this vaccine should be applicable in more tumor entities where RhoC expression might serve as a prognostic biomarker for the therapy [30-32]. The patients in the RV001 study have been prostatectomized due to verified adenocarcinoma of the prostate gland. They were subcutaneously vaccinated in total eleven times with the SLP RV001 emulsified in Montanide ISA-51 (Montanide). The vaccination site was alternating between the left and right arms.

This might be superior to the single site injection for the induction of immune responses, and more vaccine sites might be even more favorable, as shown in a mouse model [33].

The aim of this thesis was the examination of the immunological response against the SLP vaccine, which was the secondary endpoint of the clinical study. For the immune monitoring of the clinical study as well as for upcoming projects, we wanted to establish a protocol that allows the detection of antigen-specific CD8 and CD4 cells with SLPs. SLPs are known to potentially activate CD8 and CD4 T cells and the simultaneous *in vitro* readout of both populations is preferable, as patient material is often limited. For the immunomonitoring of clinical studies, we usually perform a 12 day *in vitro* amplification of the rare vaccine-specific cells with subsequent functional assessment in the ICS and ELISpot. These laboratory standard methods are described in the focused review (section 3, part 1) and are well established in the team using short peptides. To test the possibility to use these established methods also for the readout of antigen-specific cells with long peptides, we used known virus-derived HLA-class I and -class II peptides, which were elongated to 20mers on the N-, C-, and both termini or only on both termini, respectively. With our standard *ex* vivo ICS protocol, we observed, that the efficacy of CD8 T cell immune response detection depends on the elongation site (N- vs C-term), as also seen by others [34]. However, and more importantly, the response against one HLA-class II viral SLP was completely lost in 2/3 healthy donors, and detection could only slightly be improved by our standard 12 day *in vitro* stimulation. The loss of response against HLA-class II SLPs was not expected, as the elongation of the short epitope was maximal 6 aa. This observation is especially important, as overlapping SLP peptide pools are often used for e.g. the assessment of antigen-specific CD4 T cells upon SLP vaccination [35,36] and for epitope mapping studies, as SLPs are believed to be appropriate for the readout of antigen-specific CD4 T cells [37]. Our results demonstrate the need for optimization of the established standard protocol to read out simultaneously CD8 and CD4 antigen-specific cells. With the addition of Poly-ICLC on day 1 and a higher SLP concentration on day 1 and day 12 of the 12 day *in vitro* culture, it was possible to readout antigen-specific CD8 and CD4 T cells in the ICS and ELISpot using SLPs (section 3, part 2).

Using the new optimized protocol, we observed a long-lasting immune response (up to ten months post-vaccination) in the majority of the immunological evaluable patients (18 out of 21 patients) of the RV001 clinical study. Anti-vaccine cells were found to be mainly polyfunctional effector memory CD4 T cells. This immune response was induced in 17 out of 21 patients after vaccination. We could identify three 15 aa long promiscuously-presented HLA-class II epitopes and no exhaustion phenotype of the vaccine-specific CD4 T cells was observed. A CD8 T cell response against the vaccine peptide was observed in one patient; this response was restricted by the HLA-B27:05, the exact peptide sequence recognized by these CD8 T cells is under investigation. Taken together, these results indicate an activation of the immune system upon vaccination in 86% of patients. These are very encouraging results, however, we did not directly examine the possibility yet that the vaccine-specific CD4 T cells can kill tumor cells and/or the clinical efficacy in this early stage clinical study. CD4 T cells can kill HLA-class II positive tumor cells directly or indirectly via e.g. the partnering with other cells like macrophages or NK cells [38,39]. There is also evidence that CD4 T cells can be more effective in tumor rejection than CD8 T cells *in vivo* [38]. The detected vaccine-specific polyfunctional CD4 T cells in the presented clinical study are mainly of the T effector phenotype ($CD154^+$) [40,41] with cytotoxic potential ($CD107a^+$, IFN–$\gamma^+$, and/or $TNF^+$), which strongly suggest that these cells are at least able to mediate an anti-tumor response [42-44]. Such polyfunctional cells were also detected after personalized neoantigen vaccination with SLPs after which 4 out of 6 patients had no recurrence 25 months post-vaccination [35]. Besides the choice of the antigen category (neo- vs tumor-associated antigen), the clinical study of Ott et al. [35] used the TLR3 ligand Poly-ICLC (Hiltonol®) as adjuvant. In our clinical study, Montanide alone was used, which is described as delivery vehicle and adjuvant, but the addition of Hiltonol is shown to improve the vaccine immunogenicity for CD8 and CD4 T cells [45]. The addition of Poly-ICLC to the Montanide vaccine might therefore also improve the immunogenicity of the RV001 vaccination. The comparison of the arms Poly-ICLC/Montanide/RV001 vs. Montanide/RV001 vs. placebo control would allow the identification of possible differences in the T cell immune response, and, for patients in BCR or tumor progression, it might give information on the link between the immune response and the clinical outcome of the PCa patients. One striking observation we made is that only one patient developed a vaccine-specific CD8 T cell

response in addition to the CD4 response. This might be due to the testing procedure, as only one visit time during the vaccination was tested in the ICS. Alternatively, CD8 T cells might get sequestered and deleted in the granuloma caused by Montanide, as it has been seen in mice [46]. However, in the same study, the sequestration was observed for immunization with short peptides (9 aa) rather than long peptides (20 aa). CD8 T cells sequestration at the injection site could be potentially prevented by the addition of an adjuvant, e.g. the TLR1/2 agonist XS15 which has recently been developed in the Department [47]. Another possibility might be that no HLA-class I epitope is present in the RV001 sequence. However, Wenandy et al. already identified an HLA-A*03 epitope in the RV001 sequence [48]. We also identified possible HLA-class I binders in the RV001 sequence using common prediction tools (SYFPEITHI [49] and netMHC [50]); using a T cell priming approach with artificial APCs, n=3 epitopes were recognized by T cells of healthy volunteers (data not shown) [51]. Future immunomonitoring steps are for example the examination of the cytotoxic activity of the CD4 T cells, the investigation of RhoC and HLA-class II expression on PCa cells of the vaccinated patients, an in depth analysis of the already detected CD8 T cells response and of further CD8 T cell responses, or the examination of extracellular vesicles (EV). For example, EVs were shown to be involved in the cell-cell communication between a tumor and its environment, and are potentially involved in the mediation of bone metastases in PCa [52].

In summary, a suitable protocol for the identification of antigen-specific cells using SLPs was established. This protocol was used for the monitoring of the first in man vaccine targeting RhoC, but it could also be applied for basic or clinical research with SLPs, for example for epitope mapping studies. The results of the clinical study were very encouraging, as an excellent safety profile meets a high rate of immunological responses against the vaccine peptide. Based on these findings, a second phase clinical trial was initiated (NCT04114825; currently recruiting). This double-blind, placebo-controlled study will examine the effect of the vaccine on the clinical outcome in PCa patients in BCR. In my opinion, the anti-cancer peptide-vaccination is a safe tool to treat malignancies, and with a suitable vaccine format and/or in combination with the right synergistic therapies, it will make its way into the clinic as standard therapy to improve life for tumor patients like PCa patients.

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# 5 Abbreviations

| | |
|---|---|
| aa | Amino acids |
| Ab | Antibody |
| ACT | Adoptive cell transfer |
| ADT | Androgen deprivation therapy |
| APCs | Antigen-presenting cells |
| BCR | Biochemical recurrence |
| CAR | Chimeric antigen receptor |
| CBI | Checkpoint blockade immunotherapy |
| cc | Cubic centimeter |
| CCR7 | C-C chemokine receptor 7 |
| CD | Cluster of differentiation |
| CRPCa | Castration-resistant prostate cancer |
| CSCs | Cancer stem cells |
| CTCAE | Common terminology criteria for adverse events |
| CTLs | Cytotoxic T cells |
| CTLA-4 | Cytotoxic T-lymphocyte-associated protein 4 |
| DCs | Dendritic cells |
| DRE | Digital rectal examination |
| EBV | Epstein-Barr virus |
| EMA | European Medicine Agency |
| FCM | Flow cytometry |
| FDA | Food and Drug Administration |
| Flu | Influenza Virus |
| FoxP3 | Forkhead box protein P3 |
| GPR56 | G protein-coupled receptor 56 |
| HLA | Human leukocyte antigen |
| ICAM | Intracellular adhesion molecule |
| IFN | Interferon |
| IL | Interleukin |
| INF | Influenza virus |
| LAG-3 | Lymphocyte-activation gene 3 |
| LFA | Lymphocyte function-associated antigen |
| mCRPCa | Metastatic castration resistant prostate cancer |
| MM | Micrometastasis |
| NK cells | Natural killer cells |
| NO | Nitric oxide |
| OX-40 | Tumor necrosis factor receptor superfamily member 4 |
| PBMCs | Peripheral blood mononuclear cells |
| PCa | Prostate cancer |
| PCA3 | Prostate cancer gene 3 |
| PD-1 | Program cell death protein 1 |
| PET | Positron emission tomography |
| pMHC | peptide major histocompatibility complex |
| PSA | Prostate-specific antigen |
| PSADT | PSA doubling time |
| PSAV | PSA velocity |
| PSCA | Prostate stem cell antigen |
| PSMA | Prostate-specific membrane antigen |
| RhoC | Ras homolog gene family member C |

| | |
|---|---|
| RP | Radical prostatectomy |
| Sip-T | Sipuleucel-T |
| SLPs | Synthetic long peptides |
| TAAs | Tumor-associated antigens |
| TAMs | Tumor-associated macrophages |
| TCR | T cell receptor |
| TEAE | Treatment-emergent adverse events |
| TGF | Transforming growth factor |
| Th cells | T helper cells |
| TILs | Tumor-infiltrating lymphocytes |
| TIM-3 | T cell immunoglobulin and mucin domain-containing protein 3 |
| TLR | Toll-like receptor |
| TNF | Tumor-necrosis factor |
| TNM | Tumor Node Metastasis |
| TME | Tumor microenvironment |
| T regs | T regulatory cells |
| TSAs | Tumor-specific antigens |
| WHO | World Health Organization |
| YFV | Yellow fever virus |

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# 7 Publications and posters

Publications in revisions

Juliane Schuhmacher, Sonja Heidu, Torben Balchen, Camilla Schmeltz, Jesper Sonne, Jonas Schweiker, Hans-Georg Rammensee, Per Thor Straten, Martin Andreas Røder, Klaus Brasso, Cécile Gouttefangeas. Vaccination targeting RhoC induces long-lasting immune responses in prostate cancer patients: results from a phase I/II clinical trial. Submitted to: *Journal of ImmunoTherapy of Cancer, 2020*

Publications

Tatjana Bilich, Annika Nelde, Jens Bauer, Simon Walz, Malte Roerden, Helmut R. Salih, Katja Weisel, Britta Besemer, Ana Marcu, Maren Lübke, Juliane Schuhmacher, Hans-Georg Rammensee, Stefan Stevanović, Juliane S. Walz. Mass spectrometry-based identification of a BCMA-derived T-cell epitope for antigen-specific immunotherapy of multiple myeloma. *Blood Cancer J. 2020 Feb 28;10(2):24. doi: 10.1038/s41408-020-0288-3.*

Julia Hahn, Manina Günter, Juliane Schuhmacher, Kristin Bieber, Simone Pöschel, Schütz Monika, Britta Engelhardt, Henrick Oster, Christian Sina, Tanja Lange, Stella E. Autenrieth. Sleep enhances numbers and function of monocytes and improves bacterial infection outcome in mice. *Brain Behav Immun. 2020 Jan 3. pii: S0889-1591(19)30708-1. doi: 10.1016/j.bbi.2020.01.001*

Cécile Gouttefangeas, Juliane Schuhmacher, Stoyan Dimitrov. Adhering to adhesion: Assessing integrin conformation to monitor T cells. *Cancer Immunol Immunother. 2019 Nov;68(11):1855-1863. doi: 10.1007/s00262-019-02365-1.*

Daniel Weishäupl, Juliane Schneider, Barbara Peixoto Pinheiro, Corinna Ruess, Sandra Maria Dold, Felix von Zweydorf, Christian Johannes Gloeckner, Jana Schmidt, Olaf Riess and Thorsten Schmidt. Physiological and pathophysiological characteristics of ataxin-3 isoforms. *Journal of Biological Chemistry, 2018 jbc.RA118.005801; doi: 10.1074/jbc.RA118.005801*

Posters

16th annual Cancer Immunotherapy Meeting (CIMT), Mainz, Germany

**Poster presentation**: An improved in vitro assay for monitoring peptide-specific T cell responses using synthetic 20mer peptides

17th annual Cancer Immunotherapy Meeting (CIMT), Mainz, Germany

**Poster price:** RhoC vaccination induces a robust and long-lasting immune response in a clinical phase I/II prostate cancer trial

34th Congress of the International Society for Advancement of Cytometry, Vancouver

Canada

**Poster presentation:** Activated integrins identify functional antigen-specific CD8+ T-cells within minutes after antigen stimulation

# 8 Curriculum Vitae

## Personal Details

**Juliane Schuhmacher, née Schneider**

Date of birth: 26.02.1988

## Graduation

| | |
|---|---|
| Since 10/2016 | Interfaculty Institute for Cell Biology (IFIZ), Tübingen, Department of Immunology, research group: PD Dr. Cécile Gouttefangeas |
| | Scholarship holder IRTG SFB685 Immunotherapy 10/2016 – 07/2017 |

## Education

| | |
|---|---|
| 09/2014 – 09/2016 | **Eberhard Karls University of Tübingen**<br>Study program: Molecular Cell Biology and Immunology (MCBI)<br>Degree: M.Sc. |
| | 02/2016 – 09/2016 **Master thesis**<br>Med. Klinik Innere II, Laboratory for dendritic cells, Research group: Stella Autenrieth, Tübingen |
| | Title: The Impact of Sleep on Different Immune Cells in the Lamina Propria and Lung in Mice |
| 09/2010 – 09/2014 | **University of Esslingen**<br>Study program: Biotechnology<br>Degree: B.Sc. |
| | 03/2013 – 08/2013 **Practical semester**<br>Fraunhofer Stuttgart, Institute for Interfacial Engineering and Biotechnology, Department of molecular cell technology |
| | Research group: Dr. Anke Burger-Kentischer<br>04/2014 – 07/2014 **Bachelor thesis** |
| | Synovo GmbH, Tübingen |
| | Title: Optimization of a Cell Based Assay for Testing Anti-Cancer Compounds which Target Macrophages |
| 2009 – 2010 | **Berufskolleg zum Erwerb der Fachhochschulreife**<br>Ludwigsburg |
| 2005 – 2009 | **Education as a certified nursery school teacher**<br>Mathilde-Planck-Schule, Ludwigsburg |
| 1998 – 2005 | **Middle school** |