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Karolinska Institutet, Stockholm, Sweden

CRIPTO-1: POTENTIAL TARGET FOR CANCER IMMUNOTHERAPY?!

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Cripto-1: Potential target for cancer immunotherapy?!

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“If people never did silly things,
nothing intelligent would ever get done.”

(Ludwig Wittgenstein)

ABSTRACT

Cancer immunotherapy refers to the stimulation of the immune system to eliminate cancer cells and can be applied by several different means. One form of cancer immunotherapy is therapeutic vaccination with the goal to generate *de novo*, and boost existing tumor antigen specific T cells. A crucial step in the development of a cancer vaccine is antigen selection. Ideally, an antigen should be uniquely expressed by tumor cells with low or no expression in healthy tissue. Also, the antigen should also be immunogenic and expressed on the cell surface, to be targetable both by cellular and humoral immunity. Cripto-1 (Cr-1) is a membrane bound oncofetal glycoprotein expressed in human cancer. Furthermore, Cr-1 is involved in epithelial-mesenchymal transition and found to be expressed by cancer stem cells (CSC). Thus, Cr-1 could represent a potential candidate for cancer immunotherapy. Circulating Cr-1 has also been found at elevated levels in patients with cancer and has been proposed as a biomarker in patients with non-small cell lung cancer. However, T cell reactivity against Cr-1 has not yet been reported in humans. We studied if vaccination against Cr-1 would result in protective anti-tumor immunity in models of melanoma and breast cancer. Furthermore, we explored the potential of soluble Cr-1 as a prognostic biomarker for patients with advanced melanoma and evaluated peripheral T cell reactivity to Cr-1 in these patients.

We found that Cr-1 DNA vaccination elicits a Cr-1 specific cellular and humoral immune response in C57Bl/6 and BALB/c mice respectively. The induced Cr-1 directed immune response was protective in murine melanoma and mammary carcinoma models. Prophylactic vaccination particularly reduced metastatic burden and partially prevented tumor formation from CSC. In patients with advanced melanoma we showed that low serum levels of soluble Cr-1 after surgery correlated with longer survival. Additionally, we detected Cr-1 reactive T cells in peripheral blood of patients with advanced melanoma and the presence of Cr-1 reactive T cells before surgery correlated with improved survival.

In summary, vaccination against Cr-1 elicits antigen-specific protective immune responses against melanoma and breast cancer. Furthermore, soluble Cr-1 protein and T cell reactivity against Cr-1 can serve as biomarkers in patients with advanced melanoma. Altogether, our observations demonstrate that Cr-1 is a potential target for cancer immunotherapy.

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- I. Ligtenberg MA, **Witt K**, Galvez-Cancino F, Sette A, Lundqvist A, Lladser A, Kiessling R. Cripto-1 vaccination elicits protective immunity against metastatic melanoma.
Oncoimmunology, 2016 Jan 8; 5(5):e1128613

- II. **Witt K***, Ligtenberg MA*, Conti L, Lanzardo S, Ruiu R, Wallmann T, Tufvesson-Stiller, Chambers BJ, Rolny C, Lladser A, Lundqvist A, Cavallo F, Kiessling R. Cripto-1 plasmid DNA vaccination targets metastasis and cancer stem cells in murine mammary carcinoma.
Cancer Immunol Res, 2018 Nov; 6(11): 1417-1425

- III. **Witt K**, Björk Gunnarsdottir F, Rao D, Houlder E, Wolodarski M, Sette A, Kiessling R, Lundqvist A, Pico de Coana Y. Peripheral T cell reactivity against Cripto-1 correlates with overall survival in patients with advanced melanoma.
Manuscript

* Equal contribution

RELATED PUBLICATIONS

not included in this thesis

- I. Ligtenberg MA, Mougiakakos D, Mukhopadhyay M, **Witt K**, Ladser A, Chmiellewski M, Riet T, Abken H, Kiessling R. Coexpressed catalase protects chimeric antigen receptor-redirected T cells as well as bystander cells from oxidative stress-induced loss of anti-tumor activity.
J Immunol, 2016 Jan 15;196(2):759-66

- II. Mao Y*, van Hoef V*, Zhang X, Wennerberg E, Lorent J, **Witt K**, Masvidal L, Liang S, Murray S, Larsson O, Kiessling R, Lundqvist A. Il-15 activates mTOR and primes stress-activated gene expression leading to prolonged antitumor capacity of NK cells.
Blood, 2016 Sep 15;128(11): 1475-89

- III. Sarhan D, Leijonhufvud C, Murray S, **Witt K**, Seitz C, Wallerius M, Xie H, Ullén A, Harmenberg U, Lidbrink E, Rolny C, Andersson J, Lundqvist A. Zoledronic acid inhibits NFAT and IL-2 signaling pathways in regulatory T cells and diminishes their suppressive function in patients with metastatic cancer.
OncImmunology, 2017 Jun 14;6(8):e1338238

* Equal contribution

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LIST OF ABBREVIATIONS

ACT	adoptive cell therapy
ADCC	antibody-dependent cellular cytotoxicity
APC	antigen presenting cells
CEA	carcinoembryonic antigen
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
Cr-1	Cripto-1
CSC	cancer stem cell
DAMP	damage-associated molecular pattern
DC	dendritic cell
dMMR	mismatch repair deficiency
DNA	deoxyribonucleic acid
Dvl3	dishevelled-3
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
ESCC	esophageal squamous cell carcinoma
FZD7	frizzled 7
FDA	Food and Drug administration
GITR	glucocorticoid-induced TNFR-related protein
GM-CSF	granulocyte-macrophage colony stimulating factor
gp100	glycoprotein 100
GPI	glycosylphosphatidylinositol
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HER2	human epidermal growth factor receptor 2
HNSCC	head and neck squamous cell cancer
HPV	human papilloma virus
IDO	indoleamine 2,3-dioxygenase
IFN	interferon

i.v.	intravenous
LAC	lung adenocarcinoma
LAK	lymphokine activated killer
MAGE-1	melanoma-associated antigen 1
MART-1	melanoma antigen recognized by T cells 1
MHC	major histocompatibility complex
MMTV	mouse mammary tumor virus
MSI	microsatellite instability
MUC-1	mucin-1
NSCLC	non-small cell lung cancer
OS	overall survival
OX40	tumor necrosis factor receptor superfamily member 4
PAP	prostatic acid phosphatase
PD-1	programmed cell death-1
PD-L1	programmed death-ligand 1
PD-L2	programmed death-ligand 2
PFS	progression-free survival
PR	progesterone receptor
RCC	renal cell carcinoma
RNA	ribonucleic acid
s.c.	subcutaneous
TAA	tumor associated antigens
TCR	T cell receptor
TNF	tumor necrosis factor
TLR	toll-like receptor

1 INTRODUCTION

1.1 CANCER

The term cancer describes a heterogeneous group of diseases. Cancers are characterized by uncontrolled growth, cell division, and the ability to disseminate to distant locations. What all cancers have in common are characteristics required for cell transformation and malignant progressions. In 2000, Hanahan and Weinberg proposed six hallmarks of cancer shared among all tumor types: 1) sustained proliferation, 2) evading growth suppressors, 3) replicative immortality, 4) induction of angiogenesis, 5) resisting cell death, and 6) activation of invasion and metastasis. (1) It is essential for tumors to acquire all 6 traits in order to progress, however it appears to be irrelevant, in which order they occur. (1)

It is generally accepted that tumor development is a multistep process of genetic and epigenetic alterations through which normal cells turn into malignant cell formations called cancer. Somatic mutations occur frequently in cells and accumulate over time. A small number of genes have been identified as oncogenes, which are often found mutated in cancer. (2, 3) Oncogenes are able to promote cell transformation through disabling or evading stringent intrinsic cellular control pathways for proliferation and division. Throughout tumorigenesis, cancer cells undergo a positive selective pressure for cell clones with beneficial mutations for survival and proliferation. (4, 5)

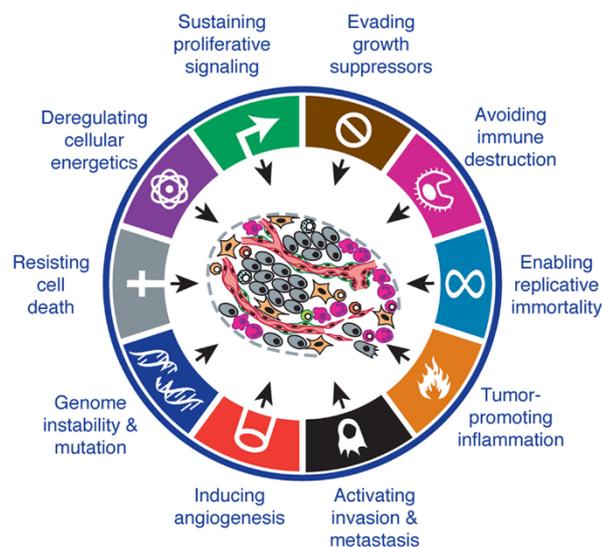


Figure 1: Hallmarks of cancer (adapted from Hanahan and Weinberg, 2011) (6)

The traditional six hallmarks reflect the one-sided view researchers have had on cancer for decades. While cancer research has primarily been focused on just the tumor cells themselves, it has become apparent there is a crosstalk between tumor cells, tissue stroma and

immune cells. The introduction of 4 additional hallmarks is the result of research seeing cancer as a complex and heterogeneous cell system. (6) The addition of the two hallmarks ‘tumor promoting inflammation’ and ‘avoiding immune destruction’ acknowledge the significance of the close crosstalk between tumor cells and the immune system (Figure 1). (6)

1.1.1 Epithelial-mesenchymal transition

Metastases are the most life-threatening for all cancer patients. Although immunotherapy has prolonged overall survival (OS) for patients with metastatic disease, long time responses are only observed in a very small fraction of patients. (7-9)

There is extensive evidence that epithelial-mesenchymal transition (EMT) plays a major role in the metastatic process. (10, 11) The expression of EMT associated proteins in cancer has been linked to tumor progression. (10, 12, 13) EMT is a fundamental cellular process during embryogenesis and highly conserved among different species. (14) It is defined by phenotypical and molecular changes including loss of cell adhesion, decreased proliferation and increased cell motility. (10, 15, 16) Snail, Twist and ZEB are key transcription factors in the EMT signaling network. (10) EMT can be initiated by various mechanisms including TGF β , TNF α , and hypoxia. (17-20) Loss of e-cadherin, through nuclear re-localization of β -catenin, activates the expression of Snail and transcription factors related to proliferation, migration and invasion. (21, 22) Wnt signaling enhances EMT by preventing the degradation of β -catenin in the nucleus. (23) Decreased cell adhesion, through loss of e-cadherin, was found to promote metastasis. (24) After migration of tumor cells from the primary tumor, mesenchymal-epithelial transition is required to establish metastatic colonies. (25)

Tumor cells that undergo EMT have been shown to escape immune recognition through various mechanisms, including cytoskeletal changes, expression of PD-L1, and changes in antigen repertoire, among others. (26-28)

1.1.2 Cancer stem cells

Cancer stem cells (CSCs) are subpopulation of cancer cells with characteristics similar to normal stem cells. (29, 30) They have self-renewal capacity and tumor initiating potential. (29, 30) CSCs have been identified and isolated from a range of solid tumors including breast, colon and pancreatic cancer. (31-36) Known stem cell markers ALDH, CD133 and CD44, are used to identify CSC within tumors. (37) In epithelial tumors, dedifferentiation of tumor cells through genetic or epigenetic alterations can give rise to CSCs. (30, 38) There is evidence for a tight link between EMT and stem cell phenotype in cancer. (39, 40) The EMT process can induce a CSC phenotype through shared pathways like notch, Wnt, and hedgehog, which control stem cell renewal and maintenance in normal stem cells. (41-43) CD44 is a target of β -catenin. Induction of EMT in breast cancer cells resulted in expression of CSCs markers. (44, 45) Because CSCs often have an EMT phenotype, they have been proposed as a source for metastasis. (46)

CSCs are involved in tumor progression and are resistant to chemotherapy and radiotherapy. (47-51) Several mechanisms have been identified that are the basis for the increased therapeutic resistance compared to non-stem cell like tumor cells. (52-55) Due to treatment resistance and intrinsic tumorigenicity, CSCs are a potential cause for relapse. (56)

1.1.3 Melanoma

Melanoma is a malignant transformation of melanocytes and the most aggressive skin cancer. The most common form is cutaneous melanoma that arises from the basal layer of the epidermis. Exposure to ultraviolet radiation is the main risk factor to develop melanoma, especially exposure at young age. (57, 58) The incidence rates of melanoma have been rising strongly in recent years. (59, 60) Cutaneous melanoma has a high mutational burden. (61) The most common recurrent mutations are in the proto-oncogenes BRAF and NRAS, and the tumor suppressor genes CDKN2A and PTEN. (62) The classic melanoma staging is based on the adverse prognostic factors of Clark's level of invasion and Breslow's tumor thickness, complemented with ulceration status and mitotic rate. (63-66) The 5-year OS rates, before the introduction of targeted therapies, range from 91% at stage I, through 62% and 57% at stage II and III to only 25% at stage IV. (67) The primary treatment option for melanoma is surgery. For metastatic melanoma, treatment with BRAF-inhibitors, anti-CTLA-4-antibodies, MEK-inhibitors or anti-PD-1-antibodies is indicated.

1.1.4 Breast cancer

A quarter of all cancer diagnoses in women are breast cancer, which makes it the most common malignancy of this sex worldwide. (68) It develops most commonly (95%) in the ductal and lobular tissue of the breast. The primary treatment strategy is surgery, but neo-adjuvant and adjuvant chemo- and radiotherapy is needed in more advanced disease. The intrinsic molecular patterns, based on gene expression analysis, subdivide breast cancers into 4 subtypes. The 'luminal A' subtype is estrogen receptor (ER) and/or progesterone receptor (PR) positive and human Epidermal growth factor Receptor 2 (HER2) negative, and usually only requires endocrine treatment. The 'luminal B', 'HER2-enriched' and 'triple-negative' (negative for ER, PR and HER2) subtypes require chemotherapy and endocrine therapy (depending on receptor expression) and trastuzumab treatment (depending on HER2 expression). (69) Together with the size and grade of the tumor, lymph node status and distant metastasis, these parameters are used to determine prognosis and optimal treatment. (70) The prognosis for 'triple-negative' subtype is particularly bad, most likely due to the lack of targeted therapy and instead higher tendency to metastasize. (71, 72) The 3-year OS by stage at the time of diagnosis is 99% for stage I, 94% for stage II, 76% for stage III and 42% for stage IV disease. (73)

1.2 TUMOR IMMUNOLOGY

1.2.1 Immunosurveillance

The concept of tumor surveillance by the immune system is not new. Already in 1909, Paul Ehrlich hypothesized that the immune system recognizes and eliminates neoplastic cells, preventing tumor formation. (74) The first experimental evidence for immune surveillance of tumor growth was obtained from serial transplantation experiments in inbred mice with methylcholanthrene induced tumors. Inoculated tumor regressed in a fraction of mice and tumor development was prevented following subsequent inoculations. (75, 76) Later studies proposed that the presence of tumor specific antigens is involved in tumor rejection and the term immunosurveillance was introduced. (77-80) A great number of murine studies, reviewed by Dunn et al., show that both innate and adaptive immunity are involved in tumor control. (81)

In addition to these findings in animal studies, evidence for immune surveillance in humans was collected. In retrospective studies, patients on systemic immune suppression were found to have increased incidence rates of non-viral induced cancer. (82-86) Presence of tumor infiltrating T cells and NK cells positively correlates with good prognosis in various types of cancer. (87-95) In parallel, tumor reactive T cells and antibodies were identified. (96-98) It has become evident that the immune system closely interacts with tumors throughout various stages of tumor progression. Dunn et al. redefined this interaction as immune editing and implemented the 3 E's of immunoediting: Elimination, Equilibrium, and Escape, defining three distinct phases. The immunological features of the three phases are shortly presented below and have been discussed by Dunn et al. and updated by Mittal et al. (Figure 2). (81, 99, 100)

Elimination

The elimination phase stands at the beginning of tumorigenesis. During cell transformation, danger signals are expressed on the cell surface, activating the innate immune response. This immune activation is further enhanced through secretion of IFN γ and IL-2 resulting in production of chemokines and recruitment of other immune cells. Tumor cells are killed by innate immune cells and tumor associated antigens (TAAs) are released. The TAAs can then be phagocytized and presented by locally activated DCs. Tumor specific T cells can then be activated by DCs in the draining lymph node and migrate to the tumor. Activated T cells eliminate tumor cells and enhance the tumor directed immune response through secretion of pro-inflammatory cytokines. If elimination of tumor cells is unsuccessful, progression to equilibrium will slowly occur. (81, 100)

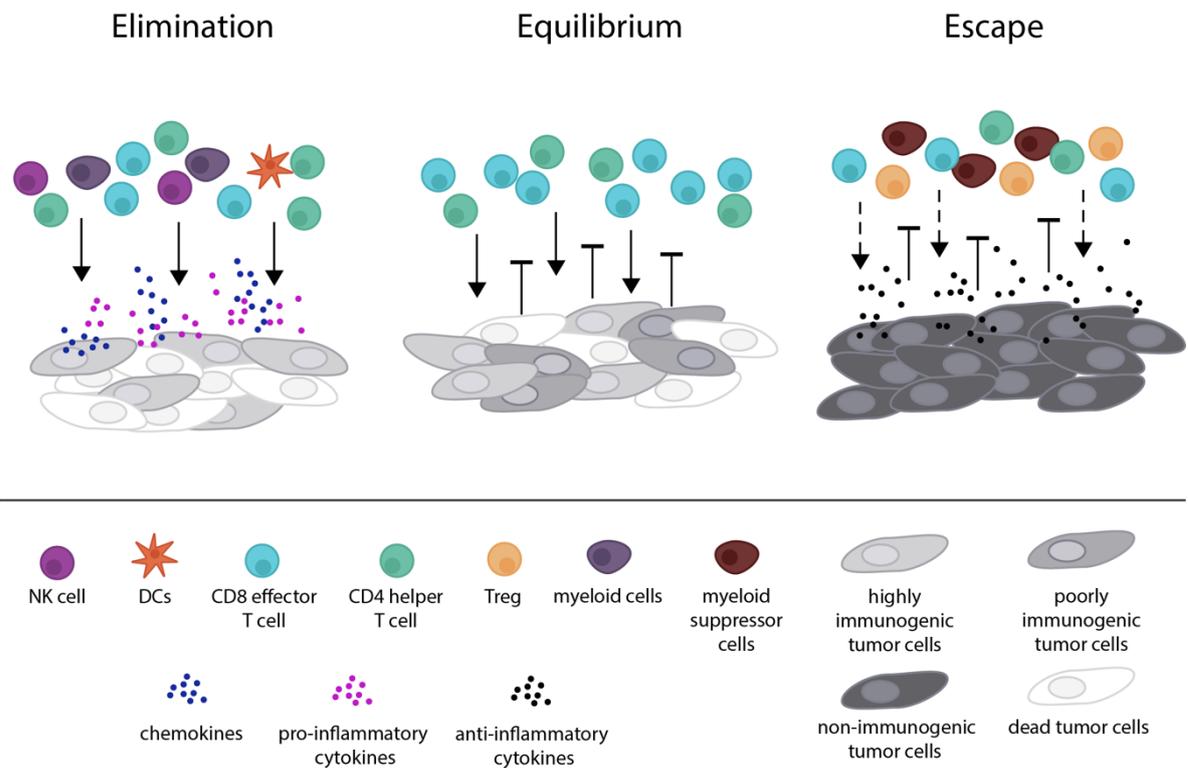


Figure 2: The three E's of cancer immunoediting (adapted from Dunn et al., 2004) (81)

Equilibrium

Through mutagenesis, tumor cells can acquire features increasing their resistance to elimination by the immune system. Even though transformed cells are still eliminated by the innate and adaptive immune response, it is no longer possible to eradicate them completely. This balance of proliferation and division on one side, and elimination on the other side is called equilibrium. While this phase can last for a long period of time, the tumor cells are under constant selective pressure by the local immune response. Immunogenicity will gradually decrease and immune suppressive mechanisms will slowly increase, ultimately leading to escape of the tumor. (81, 100)

Escape

This final phase is reached when tumor cells are no longer recognized by the immune system due to various mechanisms, including resistance to apoptosis, down regulation of MHC class I, and defects in antigen processing among others. It is known that tumors in this phase actively interfere with the immune response through secretion of anti-inflammatory cytokines and promotion of immune regulatory cells. (81, 100)

1.2.2 Cancer immunotherapy

Cancer immunotherapy is the stimulation of the immune system to eliminate cancer cells. This can be achieved through a number of different strategies, activating, modulating and enhancing the cancer patient's immune system. Here we will discuss three approaches in depth: cytokines, adoptive cell therapy and immune checkpoint blockade.

Cytokines

Recombinant cytokines were among the first immunotherapeutic agents tested in patients with advanced solid tumors. Efficacy of type I interferons was evaluated both in hematological and solid tumors. (101) Even though responses in patients with solid tumors were limited, IFN α was approved for melanoma.

IL-2 has been identified as a T cell growth factor and enabled long term in vitro culture of lymphocytes. While only a small fraction of patients with metastatic cancer responded to high dose IL-2, responses were long lasting. (102, 103) High dose IL-2 was most effective in patients with metastatic melanoma and renal cell carcinoma (RCC) and later FDA approved. (102, 103) Cytokine therapies can come with great toxicities due to unspecific stimulation of the immune system and therefore limit the overall efficacy. Therefore, IL-2 was later given in lower doses in combination with other immune therapies to enhance their efficacy. (104, 105) Although *ex vivo* IL-2 expanded lymphokine activated killer (LAK) cells had anti-tumor efficacy in mouse models, adoptive transfer of LAK cells together with high dose IL-2 in patients with advanced tumors did not have any synergistic effect. (106-108)

Adoptive cell therapy

In 1988, the first report was published on adoptively transferred *ex vivo* expanded tumor infiltrating lymphocytes (TILs) supported by short course of IL-2 in patients with metastatic melanoma. (109) In a follow up trial, objective clinical response rates were reported in one third of the patients, but these were not long lasting. (110) Combination of adoptive TIL transfer with prior lymphodepletion increased response rates and induced durable response. (111-113) While adoptive cell therapy (ACT) has been a great success in melanoma treatment, it is still in early phases for other solid tumor types. Particularly the culture and retrieval of TILs have been bottlenecks for the application of this therapy in other metastatic tumors. The efforts in this field have been adequately reviewed by Foppen et al. (114)

Other therapeutic strategies have been explored to overcome the limitations of TIL therapy. Genetic engineering of peripheral blood T cells to recognize tumors is an alternative approach. T cells transduced with T cell receptors (TCRs) recognizing known tumor antigens have been tested in clinical trials. Partial clinical responses have been reported but were accompanied by severe autoimmune responses and unexpected neurotoxicity. (115-117) An additional disadvantage is the MHC restriction of the TCR. Only a subset of patients, with matching HLA phenotype, is eligible to receive TCR transduced T cells.

Chimeric antigen receptors (CARs) are fusion proteins consisting of intracellular TCR signaling components and an extracellular single-chain variable fragment of an antibody. The advantage of CAR transduced T cells is that they are not restricted by epitope presentation on MHC molecules. However, the fact that target antigens need to be expressed on the cell surface is a major hurdle for CAR T cell therapy. CAR T cells against various antigens have been tested in clinical trials.

The most promising results were obtained with CD19 targeted CARs in hematological malignancies. (118) Adverse events include B cell aplasia, as CD19 is a B cell lineage marker, and neurotoxicities. (119) Recently CD19 CAR T cells got approved by the FDA for treatment of relapsed and refractory acute lymphoblastic leukemia and non-Hodgkin lymphoma.

Immune checkpoint inhibition

The discovery and characterization of inhibitory receptors on T cells enabled the development of T cell targeted immunotherapies. Several monoclonal antibodies targeting two distinct inhibitory signaling pathways, CTLA-4 and the PD-1/PD-L1 axis, in T cells have been approved for treatment of advanced tumors.

T cell priming and activation is dependent on both, stimulation via the TCR and through co-stimulatory signaling via CD28. CTLA-4 is an inhibitory receptor on T cells and a homolog to CD28, a co-stimulatory receptor important during T cell activation and priming. (120) Strong stimulatory signals result in the upregulation of CTLA-4 and consequently T cell anergy. (120, 121) In contrast to CTLA-4, the inhibitory receptor PD-1 is expressed on antigen experienced T cells, often referred to as exhausted. (122) PD-1 is often expressed on tumor-infiltrating T cells. (123) The ligand PD-L2 is mainly expressed on antigen presenting cells (APCs) and monocytes, while the expression of PD-L1 can also be induced on tumor cells and stromal cells in an inflammatory environment. (124, 125) Similar to CTLA-4 signaling, PD-1 signaling inhibits T cell activity and is important for maintenance of peripheral tolerance. (126)

In preclinical studies, both blockade of CTLA-4 and PD-1 resulted in rejection of tumors and prolonged survival. (127, 128) Blockade of CTLA-4 in patients with advanced melanoma induced durable responses and significantly prolonged OS. (7, 129-131) The development of a CTLA-4 blocking antibodies was a great success and induced previously unseen durable responses in a small fraction of patients with metastatic melanoma. In 2011, CTLA-4 blockade was approved by the FDA in advanced malignant melanoma. However, severe toxicities were reported in all clinical trials. It was shown that CTLA-4 inhibition broadens the peripheral T cell repertoire and potentially releases auto-reactive T cells causing autoimmune reactions. (132) In contrast to CTLA-4 blockade, inhibition of PD-1 resulted in higher response rates and longer survival. (9, 133, 134) In addition, the treatment was accompanied with less severe side effects. Response to PD-1 blockade was reported in several cancer types including melanoma, non-small cell lung cancer (NSCLC), and RCC.

(135, 136) Several PD-1 and PD-L1 blocking antibodies have received clinical approval. Despite the great success of checkpoint inhibition for treatment of advanced tumors, mechanisms of resistance, both intrinsic and acquired, have been revealed. (137) Failure to respond to therapy is categorized as either primary or acquired resistance. Primary resistances to anti-PD-1/-L1 therapy are associated with low tumor PD-L1 expression and low degree of T cell infiltration. Response-rates are also lower in tumors that harbor low levels of somatic mutations. Furthermore, recent studies show that the diversity and composition of the gut microbiome influences the responses to anti-PD-1 therapy. (138, 139)

Recent results show that patients that develop acquired resistance to anti-PD-1 therapy have a higher frequency of T cells that have gained the expression of alternative immunological checkpoints including; TIM-3, LAG-3, VISTA. (140) This has encouraged the launch of an overwhelming number of clinical trials to combine anti-PD-1 therapy with other forms of immunotherapy as well as conventional therapies. Secondly, analyses of tumors from patients that progressed on anti-PD-1 treatments have revealed that defective interferon-signaling and antigen presentation can occur. (141) Current research focuses on understanding resistance mechanisms, ultimately aiming to develop new therapeutic strategies to overcome resistance to treatment.

1.2.3 Cancer-Immunity Circle

The cancer-immunity circle describes the key steps essential for the generation of an anti-tumor immune response and successful elimination of tumor cells by immune cells. (142) The cycle includes 7 individual steps represented in Figure 3.

Antigen presentation by professional APCs and the priming of CD4⁺ and CD8⁺ T cells occurs in the draining lymph node. An inflammatory environment and immune stimulatory signals are essential to overcome tolerance at this stage. Once T cells are primed and activated they have to migrate to and infiltrate into the tumor. In the tumor microenvironment, the primed and activated T cells have to encounter their tumor antigens presented on the tumor cells, in order for them to kill the tumor cells. Dying tumor cells are then releasing tumor-associated antigens that can be phagocytized by APCs and again presented to T cells in the lymph node. The cycle of anti-tumor stimulation is endless and allows for constant generation of tumor specific T cells. However, we know that tumors are able to evade immune recognition. (81, 99) Immune escape of tumor cells can occur at each step and mechanisms of immune evasion at each step of the cancer-immunity cycle have been reviewed by Chen and Mellman. (142) The development of multifaceted immune therapeutic strategies is essential to overcome tumor escape mechanisms. It is crucial to identify these immune evasion mechanisms within a tumor to combine immunotherapies successfully.

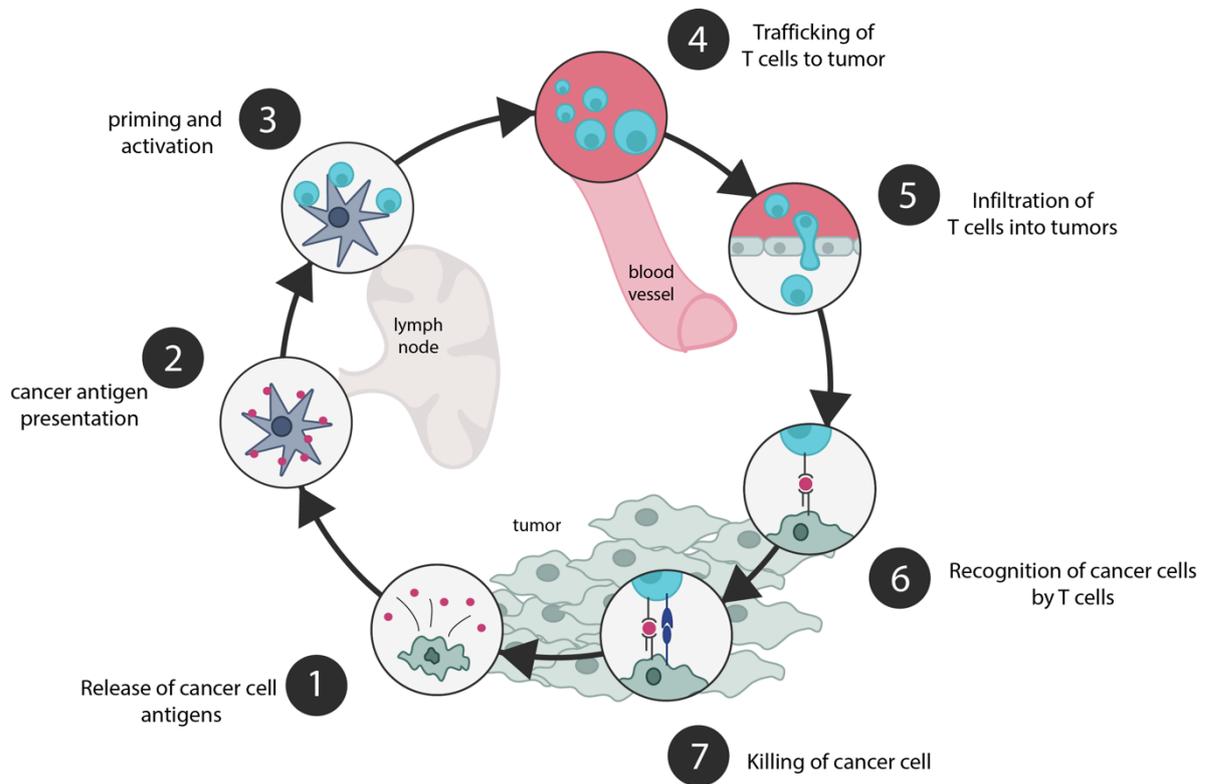


Figure 3: Cancer-immunity circle (adapted from Chen and Mellman, 2013) (142)

1.2.4 Tumor immune profiles

Cancer is caused by genetic alterations and while mutations can change protein functions, they are also reflected in the epitopes presented on MHC class I and II on tumor cells. Neoantigens may be recognized by tumor reactive T cells as they are foreign and tumor specific. The presentation of neoantigens on MHC class I and II was first described in murine tumor models. (143, 144) Even though only a fraction of mutated antigens is immunogenic, a protective tumor directed, neoantigen specific, T cell response was induced in mice. (145-148) Neoantigens and neoantigen-reactive T cells have been identified in human tumors. (149) Two recent clinical trials evaluated the therapeutic potential of neoantigens. (150, 151) Patients with advanced melanoma were immunized with RNA or peptides corresponding to patient derived neoantigens. Clinical responses were observed in both studies and neoantigen-reactive T cells were found in the blood post vaccination. Even though the mutational load strongly differs between tumor types and the mutanome (repertoire of mutations) is unique for each patient, it has become apparent that patients with high mutational load respond better to PD-1/PD-L1 blockade. (61, 152-155) This discovery has led to the approval of anti-PD-1 for tumors with microsatellite instability (MSI) or mismatch repair deficiency (dMMR). (156-160) MSI is caused by dMMR and results in high mutation burden in cancer.

Response to PD-1/PD-L1 blockade is highly dependent on a pre-existing anti-tumor immune response and neoantigens appear to contribute strongly to this. (161) Nevertheless, approximately one third of patients have long lasting responses after PD-1/PD-L1 blockade,

independently of the tumor mutational load. (162) The tumor immune profiles proposed by Chen and Mellman may explain the differences in response to PD-1/PD-L1 blockade within one tumor type. (163) It has become evident, that tumors exhibit heterogeneous immune phenotypes. Immune profiling of tumors by IHC revealed three distinct patterns correlating to response to PD-1/PD-L1 blockade. (161, 164, 165) Localization of immune subsets within the tumor is an important criterion for treatment response. Solid tumors consist of the parenchyma, containing the transformed cells, and the surrounding stroma. The identified profiles are: immune-inflamed, immune-excluded and immune desert (Figure 4). (163) These immune phenotypes are not determined by the tumor type, but rather by genetic and environmental factors.

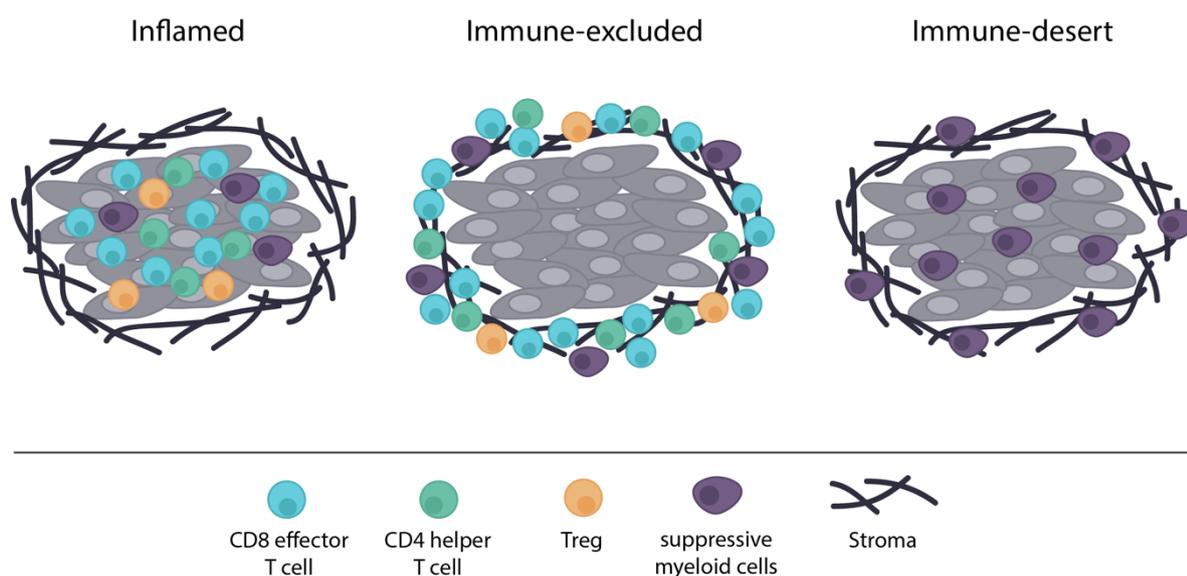


Figure 4: Tumor immune phenotypes (adapted from Chen and Mellman, 2017) (163)

Inflamed

The immune-inflamed phenotype is characterized by an abundance of CD8 and CD4 T cells in the parenchyma. Immune regulatory cells like Tregs and suppressive myeloid cells are present as well. (161, 166-173) Expression of PD-L1 is often high on the immune infiltrating cells. (161, 170, 171, 174, 175) The parenchyma is enriched for inflammatory cytokines and chemokines. (161, 170, 171, 173) This profile suggests a pre-existing tumor directed immune response with recognition of the tumor by T cells. Based on the characteristics of the profile, tumors belonging to this profile are suitable candidates for treatment with PD-1/PD-L1 blockade. Indeed, several studies have shown that the majority of patients responding to PD-1/PD-L1 blockade have inflamed tumors. (161, 168, 171, 176) However, response to this treatment is not guaranteed, as many other factors in the local environment may affect the activation of tumor reactive T cells.

Immune-excluded

In contrast to inflamed tumors, immune-excluded tumors lack immune cell infiltration. CD8 and CD4 T cells as well as myeloid cells are present in the tumor, but localized in the stroma surrounding the parenchyma. (161, 165, 177, 178) The cause of this typical lack of infiltration into the tumor parenchyma has been explained in different ways. (177-179) While checkpoint inhibition still activates the tumor reactive T cells, they'll be unable to infiltrate and leave the stroma. Clinical responses are consequently rarely seen. (161) Anti-VEGF antibodies in combination with checkpoint inhibition are able to increase T cell infiltration into tumors, through increase of chemokines in circulation and upregulation of adhesion molecules on tumor vasculature. (180-182)

Immune-desert

Tumors with immune desert phenotype lack T cells completely in the parenchyma. Myeloid cells can be present in the tumor. (161, 164, 165, 167) It appears that tumors with this phenotype completely lack a tumor-directed immune response. (163) Tumors with this phenotype seldom respond to PD-1/PD-L1 inhibition. (161)

Treatment regimens for this tumor profile must aim to both elicit a tumor directed immune response and ensure antigen presentation by tumor cells. Until today, patients with tumors of immune-desert phenotype have very poor prognosis and respond poorly to immune therapy. Therapeutic vaccines could fill this gap, able to elicit *de novo* immune responses directed against a great number of possible antigens including neoantigens. (183)

1.3 CANCER VACCINES

One possibility to elicit a tumor directed immune response is by using vaccines. Vaccines are potent generators of novel, and enhancers of pre-existing, immune responses and have been highly effective in preventing infectious diseases. In cancer treatment, however, vaccines have not yet been successful. In contrast to infectious diseases, tumors are in the majority of cases not caused by foreign infectious agents. Hurdles for cancer vaccines are immune suppression by the tumors themselves or the cells within the tumor microenvironment. (184-186) The decrease of vaccination efficacy with tumor progression is illustrated in Figure 5.

Cancer vaccines can be divided into prophylactic and therapeutic vaccines. Prophylactic vaccines are of interest when cancer development should be prevented. Unlike therapeutic vaccines, prophylactic vaccines are administered in healthy individuals. Two prophylactic cancer vaccines targeting the viruses HBV and HPV, known to cause liver cancer and cervical, have been FDA approved. Both HBV and HPV vaccination prevents latent infection of the host with the virus. Incidence rates of HCC decreased strongly upon approval of HBV vaccination. (187) The first HPV vaccination was only approved in 2006, but a decreased incidence of cervical carcinomas has already been reported. (188)

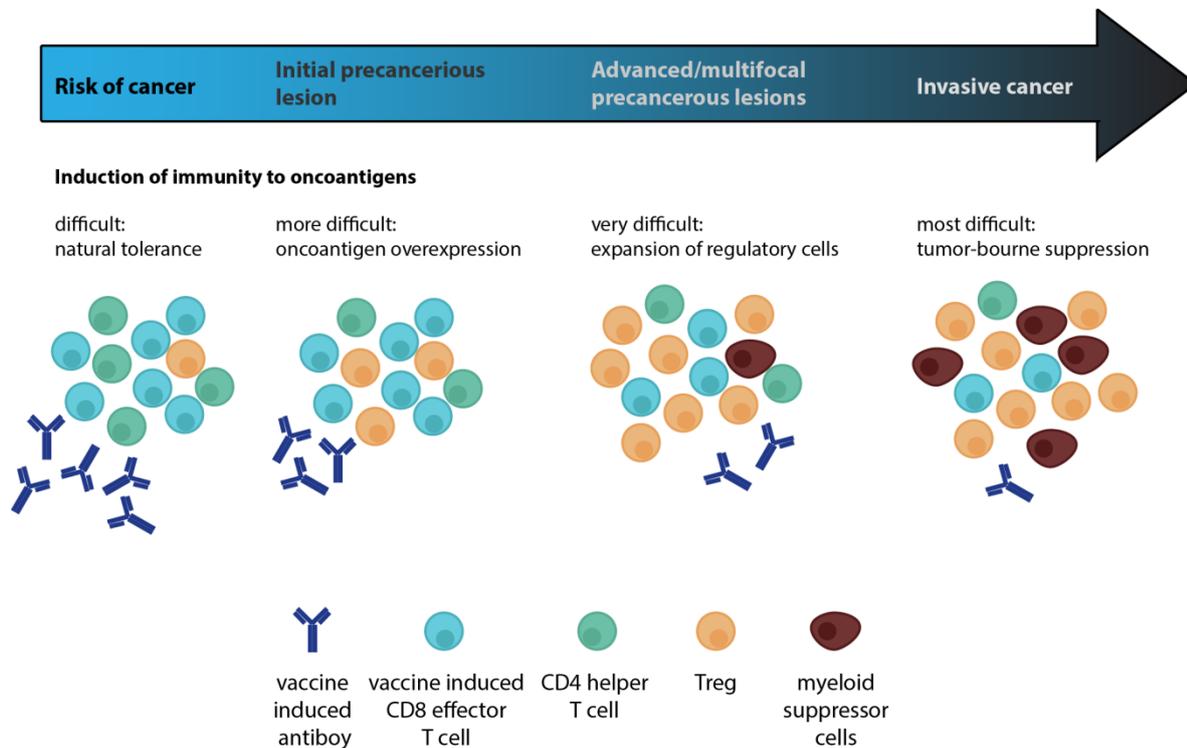


Figure 5: Impact of tumor burden on vaccination efficacy (adapted from Lollini et al. 2006) (189)

The idea of using vaccines in cancer therapy is not novel. Already in 1891, William Coley made the observation that some patients, who got bacterial infections after surgery, showed spontaneous tumor regressions. Based on his observations, he treated inoperable sarcoma patients by intratumoral injection of *Streptococcus pyogenes* and *Serratia marcescens*, now known as the Coley toxin, to stimulate their immune system. (190, 191) However, his work was highly controversial at that time. In bladder cancer, intravesical instillation of the BCG vaccines is standard adjuvant treatment. (192-194) The BCG vaccine contains live attenuated *Bacillus Calmette-Guérin* and acts on the same principles as the Coley toxin.

From a therapeutic cancer vaccine perspective neither the BCG vaccine, nor the Coley toxin, are considered specific vaccines. Both act as immune stimulants, but don't aim to generate a tumor specific immune response. It has been a great effort to identify tumor specific vaccine targets and platforms that elicit strong tumor directed immune responses. Therapeutic cancer vaccines are commonly categorized based on their formulation into cell based vaccines, protein/peptide vaccines, and genetic vaccines. Each formulation has its own advantages and disadvantages (Table 1).

Table 1: Advantages and disadvantages of vaccine formulations

	Advantages	Disadvantages
cell based vaccines (autologous, allogeneic and DC vaccines)	<ul style="list-style-type: none"> • Vaccination with full antigen spectrum if loaded with tumor lysate • Tumor antigens can be unknown • No HLA restriction[†] • Personalized • DCs are potent APCs 	<ul style="list-style-type: none"> • Work intensive • Autologous tumor cell vaccines require large amounts of primary tumor • Monitoring of immune response is challenging since antigens are unknown[†]
peptide/protein vaccines	<ul style="list-style-type: none"> • Off the shelf • More cost effective than cell based vaccines 	<ul style="list-style-type: none"> • Immunogenic epitope needs to be present within peptide sequence (potential HLA restriction) • Not immunogenic by themselves
genetic vaccines	<ul style="list-style-type: none"> • Cost efficient production • Easy manufacturing process • Can be immunogenic by themselves • Antigen can be unknown 	<ul style="list-style-type: none"> • Risk of anti-virus immune response when viral vectors are used for packaging

[†]except for DCs loaded with HLA-restricted peptides

1.3.1 Cell based vaccines

Tumor cell vaccines (autologous and allogeneic) and DC vaccines belong to the cell based vaccines. For autologous tumor vaccines, patient derived tumor cells are irradiated and inoculated into the same patient usually together with adjuvants. Autologous tumor cell vaccines have been among the first cancer vaccines to be tested in a range of different tumor types. (195-201) The great advantage of autologous tumor cell vaccines is that they include the whole spectrum of antigens expressed in the tumor piece that was used for vaccine preparation. There is no need to identify immunogenic peptides. However, these vaccines are highly personalized and require large amounts of resectable tumor tissue. (202)

Allogeneic tumor cell vaccines are similar to autologous cancer vaccines but differ in the source of antigen. Several established tumor cell lines, with known antigen expression are used for vaccination. This allows for standardization and off-the-shelf vaccines. (202)

A disadvantage is of course that the antigen profile of the vaccine might not fit optimally to the patients' tumor antigen profile. (203) Although, allogeneic tumor cell vaccines are more

immunogenic and have been tested in different tumor types including melanoma, prostate, and lung cancer. (204-209)

Dendritic cells (DCs) are professional APCs and potent elicitors of anti-tumor immune responses. For vaccination, autologous DCs are generated *ex vivo* from precursor cells derived from the patient's blood. Before re-infusion, they are loaded with tumor antigens and matured. DCs can be loaded with a great range of material, including tumor lysates, tumor cell lines, proteins and peptides. (210) It has become apparent that DC subsets vary in their ability to elicit strong immune responses. A lot of focus has been put on identifying methods to generate potent DCs for vaccination. (211, 212) Potent DCs need to express co-stimulatory molecules (CD40, CD80, CD86) and secrete cytokines to enhance T cell response. (213) Sipuleucel-T is an FDA approved DC vaccine in prostate cancer. Monocytes are loaded with a fusion protein of prostate tumor antigen PAP and GM-CSF. While the first clinical trials only showed minor anti-tumor efficacy, new studies are conducted in advanced prostate cancer for combinations with endocrine therapy and chemotherapy. (8, 214)

1.3.2 Peptide vaccines

Peptide vaccines have a clear advantage over the cell based vaccines, as they are universal. Due to the low immunogenicity of proteins and peptides, potent vaccines should include immune stimulatory adjuvants. One limitation of peptide vaccines is their MHC class I restriction. The peptides used for vaccination contain epitopes recognized by CTLs. Peptide vaccines can only elicit an immune response, if the epitopes within a peptide can be presented on the patient's MHC class I molecules. Consequently, the number of patients benefiting from peptide vaccines is limited to those expressing epitope binding MHC class I molecules. Over the past decades a number of TAAs with CTL epitopes have been described, i.e. gp100, MAGE-1, MART-1, CEA, MUC-1 and PAP. (215-220)

One of the first peptides that was used as a vaccine antigen was gp100. gp100 contains a HLA-A2 specific peptide between amino acid 209-217. Reactivity towards this epitope was confirmed *in vitro* for HLA-A2+ melanoma patients. (221) In phase II clinical trials, gp100 vaccination were tested together with high does IL-2 treatment in advanced melanoma patients. Vaccination elicited CTL responses in patients with advanced melanoma, but did not have clinical benefit. (222) In a follow up trial, the vaccine was injected together with incomplete Freud's adjuvant and high does IL-2 treatment. The adjuvant increased the response rate and prolonged progression free survival (PFS). (223) Clinical responses to vaccines can be increased by targeting multiple antigens. Efficacy of a multi-peptide vaccination in HNSCC was evaluated in a phase II clinical trial. Vaccination prolonged OS from 3.5 to 4.9 months. More interestingly, patients with CTL reactivity to multiple peptides had better clinical responses than patients with single reactivity. (224)

The results of these trials show that peptide vaccines are able to induce antigen specific CTL responses. Adjuvants and the inclusion of multiple peptides can boost the vaccine efficacy and result in clinical benefit.

1.3.3 Genetic vaccines

In genetic vaccines, antigens are encoded on RNA fragments or DNA plasmids. They can be administered naked or packaged in viral vectors. One advantage of DNA and RNA vaccines is that multiple antigens can be delivered in one immunization, allowing for broader and stronger immunizations. (225) Viral vectors are highly immunogenic and efficient delivery systems for antigens. However, sequential administration of the same viral vector also induces a vector directed immune response. As a consequence, the virus particles are removed from the host before host cells can be infected and present the antigens, resulting in a decrease of vaccine potency. (226) In contrast to viral based vaccines, nucleic acid vaccines can be administered over a long period without loss of potency.

1.4 DNA VACCINES

DNA vaccines encode the antigen and are delivered intradermal or intramuscular. They induce an antigen specific immune response through a direct transfection of APCs and indirect APC activation via the transfection of somatic cells. Injection of DNA bears the risk of genome integration. To reduce the risk of integration to the minimum, the DNA plasmid should not contain a mammalian origin of replication and homology with the human genomic sequence should be avoided. (227, 228)

At the injection site, the plasmid is taken up by keratinocytes and myocytes and localizes into the nucleus. The antigen will then be expressed by the transfected cells, processed and peptides will be presented on MHC class I and could directly stimulate antigen specific cytotoxic T cells, if present in the local environment. Crucial for a strong immune response, is the antigen uptake by APCs through phagocytosis of transfected cells. Antigen loaded APCs migrate to the lymph node, where they will present the antigen on MHC class II and MHC class I, through cross presentation, to CD4 and CD8 T cell, respectively. CD4 T cells can further induce antigen specific B cell responses. (225) Tissue resident APCs can also directly be transfected and activate CD4 and CD8 T cells. (229) DNA vaccines allow for encoding full length protein or long peptide, increasing the possibility of CD4 epitopes being presented on MHC class II. Activation of CD4 T cells is a crucial step in generating an antigen specific humoral response. (Figure 6)

DNA plasmids are often of bacterial origin and therefore immunogenic by themselves. They activate toll-like receptors (TLRs) and damage-associated molecular patterns (DAMPs), resulting in type I interferon (IFN) responses and pro-inflammatory cytokine responses. (225,

230-233) Vaccination results in a local inflammation augmenting the antigen directed immune response. Activation of NF κ B and type I IFN signaling have been identified as essential for successful DNA vaccination. (233-235)

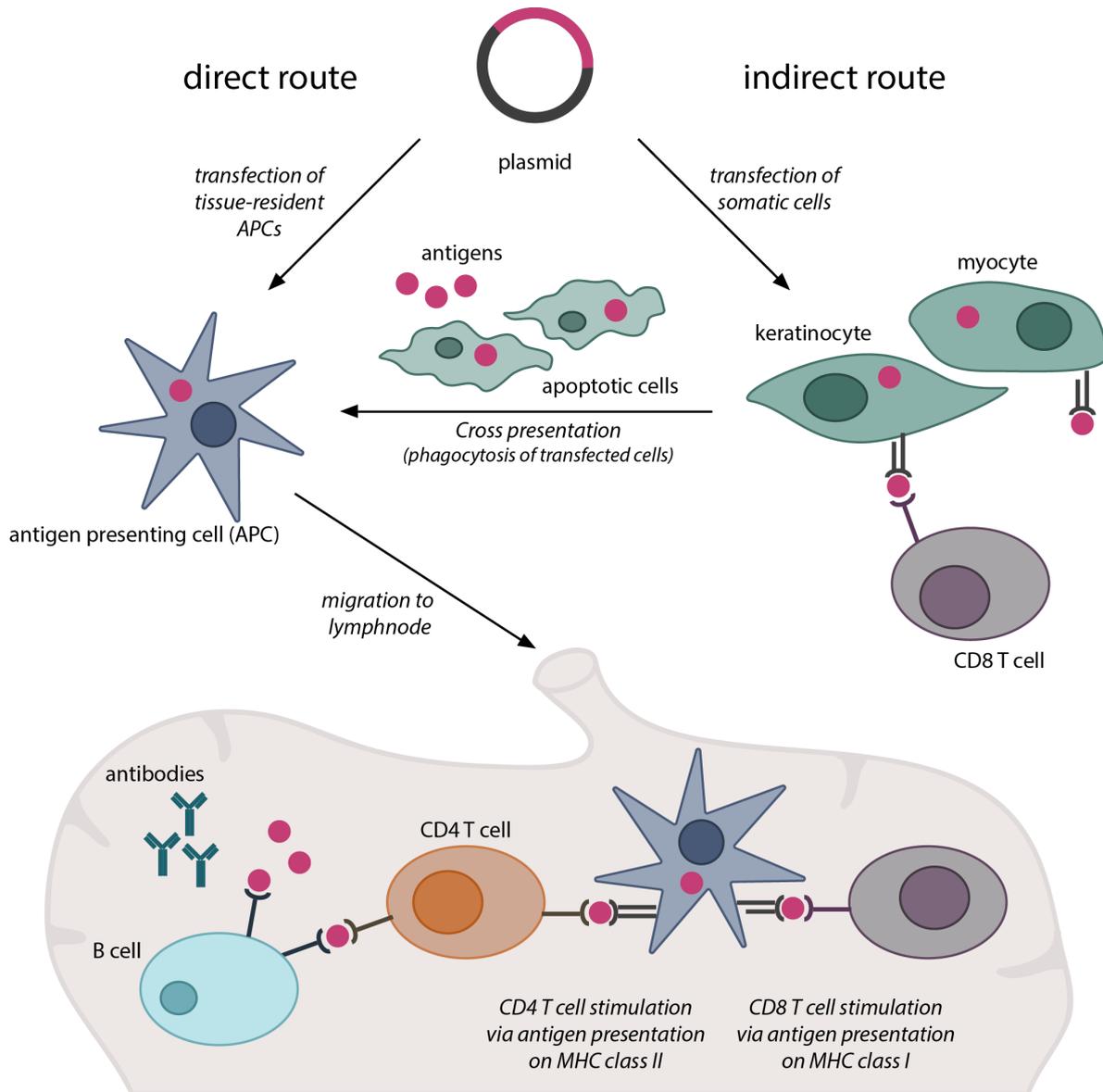


Figure 6: Mechanisms of action for DNA vaccination

1.4.1 Enhance immunogenicity of DNA vaccines

For DNA plasmids to elicit an immune response, the DNA plasmid is required to be taken up by the cells and transported into the nucleus. Electroporation can be used to increase the transfection rate *in vivo*. The electric field forms nanopores in the cell membrane and forms an electrical gradient within the cells. (236, 237) The negatively charged DNA moves through the electric field and enters the cells. (236) Electroporation increases the transfection efficacy by increasing the number of infected cells and co-transfecting cells with several

plasmids. (238, 239) In addition, immunogenicity of DNA vaccines was reported to be increased after electroporation, through local tissue damage. Inflammatory cytokines are secreted at the electroporation site and APC recruited. (240, 241) Finally, electroporation DNA vaccination was shown to increase antigen specific immune response when compared to DNA vaccination by itself. (242-244) Despite all these benefits, it requires a lot of optimization to set up electroporation protocols for optimal antigen expression and immune response, while avoiding greater cell damage. (245)

Once the DNA plasmid is in the cell, optimal antigen expression is key for successful DNA vaccination. DNA plasmids are not replicated in the cells and therefore the antigen should be expressed under a highly efficient promoter. The CMV promoter is one of the most active promoters and was found superior to other promoters. (246) Antigen expression is affected by mRNA stability and translation efficacy. It is known that mRNA with great levels of AU in the sequence has decreased stability. (247) Consequently, are the encoded proteins less abundant. A second bottleneck in mRNA translation is the amount of tRNAs. tRNAs are the link between the mRNA and amino acid. Each amino acid binds to 2 or more tRNAs. Low abundance of some tRNAs can affect protein expression. Increased immunogenicity of vaccination was reported when the antigen sequence was modified from low to high abundant tRNAs. (248-250)

Even though DNA vaccines are immunogenic by themselves, a number of strategies have been evaluated to enhance antigen specific immune responses. Co-injection of TLR and DNA sensor agonists has been shown to enhance anti-tumor immunity in murine tumor models. (251-253) Enhancing immune stimulation through fusion of microbial peptides with antigens has been tested in mouse models. (254, 255) In a phase I clinical trial in colon cancer, safety of a CEA-tetanus toxoid CD4 epitope was investigated. (256) No anti-tumor efficacies were evaluated. Safety of co-injection of GM-CSF and IL-2 together with DNA vaccination was examined in a trial in metastatic breast cancer. (257) Vaccination induced long-term humoral and cellular responses, but clinical benefit was not evaluated. One clinical trial that reported clinical benefit was conducted in patients with cervical intraepithelial neoplasia. The vaccine targeted the viral proteins E6 and E7 of HPV-16 and 18. Patients receiving the vaccine in this double blinded placebo trial had increased rates of local infection and regression. (258)

1.5 THERAPEUTIC CANCER VACCINES IN MELANOMA AND BREAST CANCER

Efficacy of therapeutic cancer vaccines has been evaluated in both melanoma and breast cancer. Several vaccine trials in advanced melanoma have been performed at the NCI Surgery Branch, mostly peptide vaccines targeting melanoma antigens. Despite these great efforts, the objective response for all trials together was only 2.6 %. (259) In 2011, results from a trial combining gp100 peptide vaccination with IL-2 in patients with advanced

melanoma reported prolonged PFS. (223) Combination of gp100 peptide vaccination with CTLA-4 blockade did not have any synergistic effect. (7) In 2015, FDA approved T-VEC, an oncolytic virus producing GM-CSF, for treatment of patients with advanced non-resectable melanoma. The phase III clinical trial that led to the approval of T-VEC, durable responses were observed in 16% of the patients and only 25% in the control arm. (260) In 2017, results from two vaccine trials targeting neoantigens reported clinical responses in patients with advanced disease. (150, 151)

Significantly less vaccine trials have been performed in breast cancer. Similar to the studies in melanoma were peptide vaccine most commonly tested, targeting HER2 or MUC-1 among others. The majority of the vaccines has been tested in disease free patients. Although antigen specific immune responses were elicited, only minor clinical benefit was observed. (261-267)

1.6 CHALLENGES IN CANCER VACCINE DEVELOPMENT

The field has faced three major challenges, (i) the systemic immune suppression in cancer patients, (ii) the lack of suitable antigens, (iii) monitoring of efficacy. When analyzing cancer vaccine trials retrospectively these challenges are likely the reason why the trials failed.

Monitoring of vaccine efficacy

Clinical trials in cancer have classically been evaluated based on ‘hard’ criteria. Treatment response in the clinical trials was assessed and determined based on the RECIST criteria, evaluating tumor shrinkage. (268) Tumor shrinkage as response criterion has been appropriate for therapeutic agents directly targeting tumor cells. However, with the development of immunomodulatory drugs and cancer vaccines, these criteria have been found to be less appropriate. Additional evaluation criteria for objective response have been suggested including patient survival, progression free survival, and stable disease. (259, 269, 270) Based on the development in the field of cancer immunotherapy the RECIST criteria have been updated in 2009. (271)

Immune suppression

Vaccination trials are often performed in late stage patients. Systemic immune suppression dampens the vaccine efficacy and limits the elicited immune response, while the local tumor microenvironment inhibits the anti-tumor directed immune response within the tumor. The MUC1 peptide vaccination was tested in both early and late stage colon cancer patients, which allows us to draw conclusions on the impact of advanced tumors on anti-tumor immunity. (272) While vaccination in patients with adenocarcinoma elicited minor MUC1 specific responses, the vaccination in a subset of patients with advanced adenoma induced strong humoral responses. (272, 273) Response to vaccination was inversely correlated to circulating myeloid derived suppressor cells, an indicator of systemic and local immune suppression in cancer. (273) A phase I DNA vaccination trial in melanoma targeting both

TRP2 and gp100 found that vaccination induced a stronger immune response in patients without tumor present at the time of vaccination than in patients with tumor burden. (274)

Patient selection and adjustments to these conditions in the vaccination setting can be the solution for this problem.

Antigen selection

Cancer vaccine antigens are typically categorized based on their biological origin in the following categories: 1) Oncoproteins, 2) Oncofetal, 3) Tissue differentiation, 4) Viral, 5) Stem cell/EMT antigens, and 6) Others. (183) A comprehensive list of human tumor antigens, reported to be recognized by T cells, was published by Renkvist et al in 2000 and was updated by Novellino et al. in 2004. (275, 276)

Antigen selection is a critical step in the development of cancer vaccines. An antigen needs to fulfill a number of requirements in order to be a suitable target for vaccination. Vaccine antigens need to be exclusively expressed on tumor tissue with low or no expression in healthy tissue. The antigen should also be immunogenic and expressed on the cell surface, to be targetable both by cellular and humoral immunity. For cancer vaccine antigens, it is also of interest if the protein is involved in key mechanisms of cancer progression. Targeting of these proteins, may not eradicate tumors completely, but at least halt progression. Proteins associated with the EMT process and cancer stem cells, have therefore been under investigation as suitable targets for cancer vaccines. Two phase I vaccine trials with vaccines targeting T-box transcription factor Branchury, a driver of EMT, have shown safety of the vaccines and presence of Branchury specific T cells after vaccination. (277-279) Prophylactic vaccination with irradiated induced pluripotent stem cells controlled tumor growth in murine mammary carcinoma and melanoma models. (280) No clinical trials targeting stem cell specific antigens have been performed yet.

Identification of suitable targets is a hurdle within the cancer vaccine field. An algorithm to streamline antigen identification has been proposed and tested. (281) The algorithm will allow for identification of candidate targets based on functional and immunogenic criteria, including expression level, specificity, number of antigenic epitopes and cellular location. (281)

Combination therapy

Selection of the study cohort is an essential step in clinical trial design and has a strong impact on clinical outcome. Many vaccine trials have been performed in late stage patients. We know today that patients with advanced carcinomas are often systemically immune suppressed. In several clinical trials, it was found that large tumor burden decreases the anti-tumor efficacy of vaccines. (272-274, 282)

Combination of cancer vaccines with other treatments can potentially enhance efficacy and clinical benefit. Classical cancer treatments like chemotherapy and irradiation have been

found to have additional immune stimulatory effects besides their anti-tumor activity. (283, 284) Some forms of chemotherapy and in particular at low dose, has been shown to synergize with cancer vaccines in clinical trials. (207, 285, 286) The impact of chemotherapy and radiotherapy on cancer vaccines in preclinical and clinical trials has been discussed by Hodge et al. and Andersen et al. (287, 288) Cancer vaccines have also been tested in combination with checkpoint inhibition. PD-1 inhibition resulted in complete responses in two stage IV melanoma patients that relapsed after personal neoantigen vaccination. (150) The T-VEC vaccine has been combined with both anti-CTLA-4 and anti-PD-1 inhibition and tested for efficacy in patients with advanced melanoma. Both trials reported increased objective response rate for the combination of T-VEC with immune checkpoint inhibition over checkpoint blockade alone. (289, 290)

Cancer vaccines are often combined with GM-CSF, a potent immune stimulator through activation and maturation of DCs. (291) GVAX is a GM-CSF secreting tumor cell based vaccine. (209, 292, 293) It has been tested in several cancer types including melanoma, prostate and, pancreatic cancer. (294-296) Combination of GVAX in advanced pancreatic cancer with CTLA-4 inhibition was found to increase survival over CTLA-4 inhibition alone. (297) A vaccine consisting of mutant ras peptides given in combination with GM-CSF elicited clinical responses in patients with advanced pancreatic cancer, which correlated with the induced antigen immune response. (298) However, another trial showed that addition of IL-2 to the vaccine regiment diminished the effect of vaccination with mutant ras peptides and GM-CSF alone. (299) The results show, that multi-combination trials with cancer vaccines can be inferior to single combinations.

Combinations of cancer vaccines with other immunomodulatory agents targeting IDO, OX40 and GITR have been tested in pre-clinical tumor models and show promising results. (300-303)

1.7 CRIPTO-1

Cripto-1 (Cr-1) is a glycoprotein and was first cloned in 1989. (304) It is a membrane bound protein, anchored to the cell membrane with GPI, but can also be shed when the anchor is cleaved by GPI-phospholipase D. (305, 306) Cr-1 belongs to the EGF-CF protein family. (307) Members of this family are essential during early embryogenesis, implicated to be involved in the regulation of germ layer formation and the orientation of anterior posterior axis. (308, 309) In adult tissue, Cr-1 is expressed in low levels. (310, 311)

Lacking an intracellular domain, Cr-1 is a co-receptor in the activation of intracellular signaling by Nodal and other TGF β family member, Glypican-1, and Wnt family members. (312-315) During embryogenesis these signaling pathways activate cell proliferation, survival, migration and differentiation (Figure 7). (312, 313)

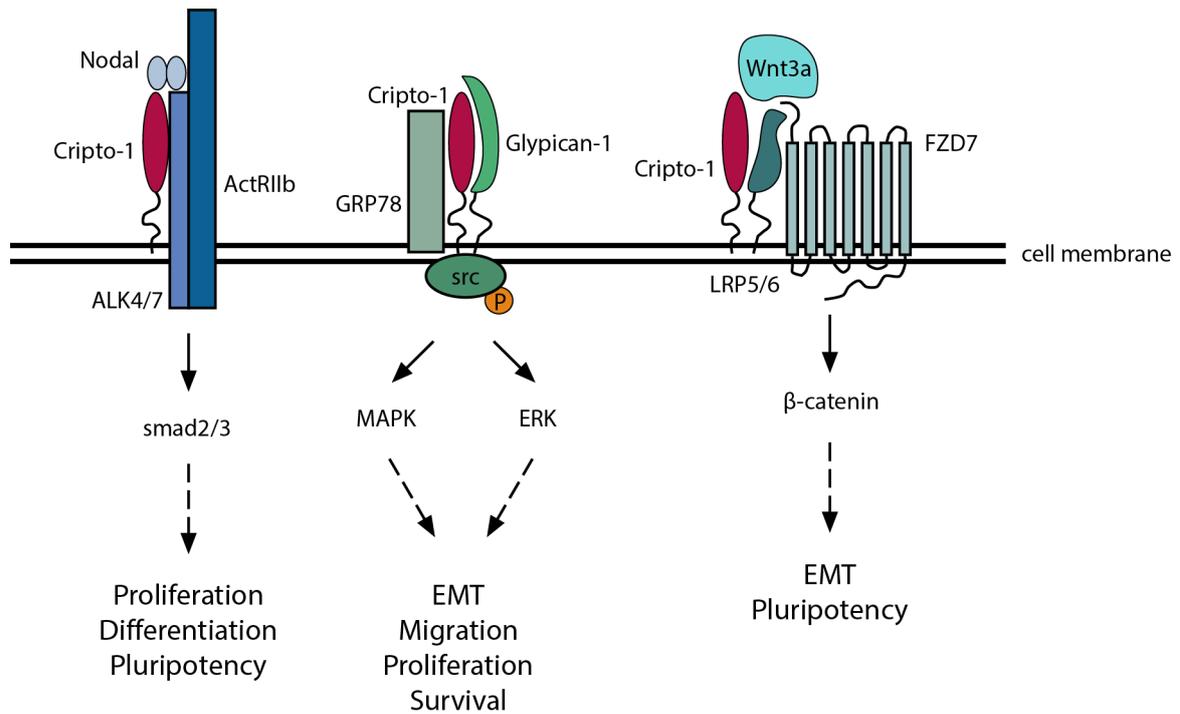


Figure 7: Cripto-1 signaling pathways (Adapted from Bianco et al., 2004) (312)

1.7.1 Role of Cripto-1 in cancer

It is known that there are many parallels between embryogenesis and tumor development. Conserved signaling pathways for cell proliferation, plasticity and motility during embryogenesis are often reactivated in cancer and critical for tumor progression. (316) Similar to other embryogenic proteins found to be re-expressed in cancer, Cr-1 expression has been identified using qPCR and IHC in a great number of human tumors. (317-319) An overview is presented in Table 2.

In particular, high expression of Cr-1 was correlated with worse prognosis in breast cancer, HCC, and glioblastoma. (320-323) A study in bladder cancer described not only the correlation of high Cr-1 expression with shorter OS and PFS, but also a positive correlation between Cr-1 expression and tumor grade. (324) While a study in a cohort of ESCC patients did not evaluate survival, they did find a correlation between Cr-1 expression and tumor grade. (325) A survival study in lung adenocarcinoma correlated high expression of Cr-1 within the tumor, together with high serum levels of the lung cancer tumor marker CEA, with poor prognosis in lung adenocarcinoma. Cr-1 was found to correlate better with shorter survival than CEA serum levels did. (326) In a study on gastric cancer, Cr-1 expression was evaluated together with e-cadherin, a protein which is associated with epithelial cells and lost during EMT. Patients with low expression of e-cadherin and high expression of Cr-1 had the worst prognosis in this cohort. (327)

Table 2: Summary of Cripto-1 expression in human tumors

Tumor type	Subtype	Method of detection	Reference
Breast cancer		IHC, ICC	(320, 328, 329)
Bladder cancer		IHC	(330)
Brain tumors	Glioblastoma	mRNA, IHC	(322, 323)
Cervical cancer		IHC	(331)
Colorectal cancer		IHC	(332-334)
Endometrial cancer		IHC	(335)
Gall bladder cancer		IHC	(336)
Gastric cancer		mRNA, IHC	(327, 337)
Hepatocellular carcinoma		IHC	(321)
Lung cancer	NSCLC, LAC	IHC, mRNA	(326, 338, 339)
Melanoma	cutaneous & uveal	IHC	(340, 341)
Pancreatic cancer		IHC	(342, 343)
Ovarian cancer		IHC	(344-346)

Cr-1 can also be shed from the extracellular surface. In a number of different studies, Cr-1 protein was detected in the sera of patients, while only low levels were found in healthy controls. (347-349) In breast cancer, colon cancer, and HCC, levels of soluble Cr-1 (sCr-1) were not correlated with stage of disease. (347, 349) Interestingly, sCr-1 levels have been associated with worse prognosis and shorter survival in patients with NSCLC and glioblastoma multiforme. (323, 350, 351)

Until today, it remains unclear why Cr-1 is linked to poor prognosis and tumor stage in some tumor types while not in others. The fact that Cr-1 is found to be present in a large number of different tumor types, suggests that the function of Cr-1 within tumors is independent of the tumor type and rather specific for certain phenotypes of tumors. In conclusion, several studies have investigated Cr-1 in different tumors and cancer cell lines, allowing us to draw

preliminary conclusions on the functions of Cr-1 in cancer. Expression of Cr-1 was in particular linked to EMT and the presence of cells with stem cell phenotype within tumors.

1.7.2 Cripto-1 and epithelial-mesenchymal transition

Cr-1 has been implicated to play a crucial role in the EMT process during the germ layer formation facilitating signaling of TGF- β and Wnt family members. (352) Wnt/ β -catenin signaling is known to be involved in EMT and cell plasticity both in embryogenesis and cancer. (353) Knockout of Cr-1 causes embryonic lethality at day 7.5, unable to form mesoderm and endoderm. (354) Crosstalk of Cr-1 with Wnt in the EMT process has been shown during mouse embryonic development. Wnt \neg and β -catenin \neg results in similar phenotype as for Cr-1 knockout. (355) Knockout of Wnt3 in particular, resulted in reduced expression of Cr-1 implicating a regulation of Cr-1 expression downstream of Wnt3a signaling. (355, 356) The role of Cr-1 in EMT in cancer has been studied in the Cr-1 overexpressing MMTV mammary carcinoma model. (357) Overexpression of Cr-1 induced EMT-like morphological changes and resulted in increased migration and invasion resulting in development of hyperplasia and adenocarcinoma in the MMTV model. Within these tumors, an increase in mesenchymal proteins was detected, while proteins associated with epithelial cells were decreased. (357-359) In addition, studies in melanoma cell lines have shown that Cr-1 enhanced cell motility *in vitro* via Nodal and src signaling. (340)

1.7.3 Cripto-1 and cancer stem cells

It has become evident, that EMT and stem cell phenotype are tightly linked cellular programs, both in stem cell and in epithelial cell populations. (45, 360, 361) Cr-1 itself has been found to link EMT to stem cell phenotype in cells. Cr-1_{hi} expressing cells within the NTERA2/DC EC cell line displayed an EMT phenotype and enhanced capacity to form spheroids *in vitro*, when compared to the Cr-1_{low} subpopulation. (362)

Cr-1 can also regulate stem cell phenotype. It was shown that Cr-1 is critical for the maintenance of pluripotency in human end mouse embryonic stem cells. (363) In a number of recent studies, the role of Cr-1 in CSCs has been explored. In colorectal cancer, Cr-1 has been found to regulate the CSC compartment. (364) For CSC like cells in esophageal squamous cell carcinoma, Cr-1 is a functional marker correlated to patient outcome. (365) While Cr-1 is known to be linked to poor prognosis, the mechanism was still unclear. (321) In a recent study, the role of Cr-1 in CSC within HCC was further explored. It was found that Cr-1 enhances Wnt signaling by stabilizing Dvl3, resulting in strong activation of β -catenin. (366) In addition, it has become clear that Cr-1 is part of the stem cell gene network. Cr-1 is a direct target of the transcription factors Nanog and Oct4. (367-369) This regulation has been confirmed in human embryonal carcinoma cells. (362) Within stem cells, Cr-1 mediated Nodal signaling induced transcription of Nanog, creating a positive feedback loop. (367, 368)

It remains to be shown, that this feedback loop is involved in maintenance of stem cell phenotypes in tumor cells. Expression of Cr-1 has been described in CSCs in both melanoma and in colorectal cancer. (364, 370)

Based on our knowledge of Cr-1 within a great signaling network regulating both cell proliferation and EMT, we suspect Cr-1 to be involved in tumor development and progression. The association of Cr-1 with EMT processes may explain why Cr-1 is associated with poor prognosis. CSCs and cells with stem cell phenotype have been shown to be resistant to classical cancer treatments, including chemo-and radiotherapy. (371) The expression of Cr-1 in especially in these subsets of tumor cells, implicates the importance of Cr-1 in treatment resistance and relapse.

Taken together, these findings propose that Cr-1 is an interesting candidate for cancer immunotherapy with the potential to control hallmarks of aggressive tumors. Thus both distant spread, by blocking EMT, and relapse post treatment, by targeting CSCs, may be reduced.

1.7.4 Potential of targeting Cripto-1 in cancer

Due to its important biological function in cancer, Cr-1 is not a novel target in cancer therapy. A number of different therapeutic strategies have been evaluated for efficacy in murine tumor models.

Cr-1 antisense oligonucleotides bind Cr-1 mRNA and inhibit the translation. Antisense oligonucleotides to Cr-1 have suppressed growth of human breast, ovarian and colon cancer. (372-374) A follow up study in colon xenograft models showed that injection of multiple antisense oligonucleotides targeting growth factors including Cr-1 inhibited tumor growth. (374)

In addition to antisense oligonucleotides, Cr-1 binding therapeutically active antibodies have been developed and tested in vitro as well as in xenograft models. Antibodies targeting different epitopes of the Cr-1 protein have been developed. By blocking the assembly of Cr-1 in receptor complexes, downstream signaling was inhibited and tumor growth controlled. (375-378) One Cr-1 specific antibody conjugated to the cytotoxic component DM4 has been tested in a Phase I clinical trial (NCT00674947). The results of this study have not been published yet. Apart from this antibody, no other Cr-1 targeting treatment has been tested in the clinic so far.

2 AIMS OF THE THESIS

The thesis has evolved around the tumor associated antigen Cr-1. Based on the previous studies on the presence and function of Cr-1 in human tumors, we identified Cr-1 as a valuable target for aggressive cancers. In contrast to previous studies, we have investigated this protein in cancer from an immunological point of view. In study I and II we evaluated the immunogenicity of Cr-1 upon DNA vaccination with Cr-1 in two mouse strains. Study III is a translational study in which we assessed the presence of sCr-1 protein and Cr-1 specific T cells in the blood of human advanced melanoma. Taken together, the findings presented in this thesis highlight the relevance of Cr-1 as a protein of interest in cancer immunotherapy.

3 RESULTS & DISCUSSION

3.1 STUDY I

Cripto-1 vaccination elicits protective immunity against metastatic melanoma

Background

Checkpoint inhibitors targeting CTLA-4 or the PD-1/PD-L1 axis have induced long lasting responses, in particular in melanoma. (131, 379) However, it has become evident that large numbers of patients with metastatic disease will not response or relapse. (7, 162, 380, 381) One reason why patients are resistant to treatment with checkpoint inhibitors is the lack of tumor reactive T cells. This has been shown to be caused by a general absence of tumor antigens or insufficient antigen presentation. (382) Vaccination is a therapeutic strategy to generate *de novo*, and boost existing tumor antigen specific T cells. One bottle neck in cancer vaccine development has been the lack of suitable targets. The oncofetal protein Cr-1 is part of cellular signaling pathways during embryogenesis inducing EMT, migration and invasion. (383) These processes constitute hallmarks of aggressive cancers. (1) The expression of Cr-1 was reported in a large number of human cancers. (384) Based on its biological function and tumor specific expression we identified Cr-1 as a suitable target for vaccination in cancer therapy.

Aim of the study

The intention of this study is to assess if murine Cr-1 (mCr-1) is an immunogenic antigen and if mCr-1 DNA vaccination can elicit a mCr-1 directed immune response. In addition, we address the question, if the elicited immune response is anti-tumor directed and protective in a murine melanoma model.

Results

We assessed the immunogenicity of mCr-1 through identification of potential CD8⁺ T cell epitopes. Using an overlapping peptide library (15-mer), covering the full length mCr-1 protein, and *in silico* epitope prediction tools, we identified three candidate peptides (9-mers). All candidate peptides stabilized H2-K^b on RMA-s cells.

Mice were vaccinated at week 8 and 10 by intradermal injection of full length mCr-1 encoding DNA plasmid (pmCr-1), or empty plasmid as control (pVAX-1), followed by electroporation of the injection site. Immunization-induced immune responses were evaluated 14 days after the second injection. T cell activation within splenocytes from pmCr-1 and pVAX-1 immunized mice were evaluated *in vitro*. Only peptide mCr-1₁₆₋₂₅ strongly activated cytotoxic T cells from pmCr-1. This finding was confirmed in an *in vivo* cytotoxicity assay.

Based on these findings we hypothesized that Cr-1 vaccination elicits Cr-1 peptide recognizing cytotoxic T cells in C57Bl/6 mice. If Cr-1 is indeed a relevant tumor antigen in cancer, we proposed that Cr-1 vaccination can control tumor growth. We chose to test this hypothesis in the syngeneic murine melanoma model B16F10. The B16F10 cell line gives rise to aggressive tumors *in vivo* and is widely used to study solid tumors, when injected s.c., and metastases, when injected i.v.. Cr-1 expression in the B16F10 cells was confirmed by western blot. After prophylactic vaccination, mice were challenged with s.c. injected B16F10 cells. We observed reduced tumor sizes in the pmCr-1 vaccinated group compared to the control group. Immunization prior to i.v. challenge with B16F10 resulted in great reduction of lung tumors in the pmCr-1 group.

Significance

In this study, we were able to confirm the potency of targeting Cr-1 in cancer therapy and extend this knowledge by exploring a vaccine based therapeutic approach.

Cripto-1 has been identified and tested in other pre-clinical studies as a target in cancer therapy. (372-378) These strategies aimed to inhibit the signaling function of Cr-1 or its expression and were reported to inhibit tumor growth. One disadvantage of the suggested treatment strategies is that they would need to be administered continuously or for a long time in order to maintain tumor control. We have identified Cr-1 as a suitable target for vaccination. It is a cell surface expressed protein, overexpressed in tumors in comparison to somatic cells, and is associated with hallmarks of advanced tumors. (310) As the platform for antigen delivery we chose DNA vaccination followed by electroporation. DNA vaccines are not only easy and cost efficient in the manufacturing process, we also had previous experience from working with DNA vaccines. (234, 251, 257) Breaking tolerance against the endogenous protein Cr-1 was critical in our study. Indeed, we found that prime boost DNA vaccination followed by electroporation elicited Cr-1 specific CTLs and protection against challenge with tumor cells. The potency of homologous prime-boost DNA vaccination has been previously shown in other studies. (234, 385) However, it has been shown that heterologous vaccination regimens combining DNA vaccination with protein or viral based vaccination are more potent. (225) We considered combining DNA and protein vaccination but attempts to produce recombinant Cr-1 protein were unsuccessful and commercially available protein too expensive. We can only speculate that a heterologous prime boost vaccination, could have elicited a stronger Cr-1 directed immune response resulted in greater protection. In murine tumor models, internal tolerance could be overcome by xenogeneic DNA vaccination. (386, 387) However, clinical benefit of syngeneic over xenogeneic immunization was not observed in clinical trials. (388-392). Due to the lack of clinical relevance, we did not further explore the beneficial effects of xenogeneic vaccination in our study. We know from previous studies that adjuvants can enhance immunogenicity of DNA vaccines. (251-253) In this study we found it sufficient to perform electroporation in combination with DNA vaccination to elicit a Cr-1 directed immune response. We hypothesize that inclusion of additional agonists in the vaccine formulation would further

increase immunogenicity of the vaccine and a potential therapeutic benefit should be evaluated in additional studies.

Monitoring of immune response post vaccination is a crucial step in the evaluation of immunogenicity. It is therefore essential to identify immunogenic peptides that allow for monitoring. Overlapping peptide libraries, like we used in our study, are commonly used to identify MHC binding epitopes. (393, 394) Using *in silico* prediction we identified potential immunogenic epitopes with the highest binding affinity to H2-K^b within the hits of the first screen. However, it was not the epitope with the highest binding to H2-K^b. In line with our findings, a study comparing immunogenicity of high and low MHC binding peptides found that low affinity peptides were more potent in generating CTLs with longer avidity. (395) Therefore, binding affinity might not be a fully reliable marker for immunogenicity of epitopes. Stability of the epitope MHC class I epitope was identified as more predictive for CTL specific immunogenicity than epitope binding affinity. (396)

To investigate if Cr-1 vaccination is protective we chose the murine melanoma model B16F10. The B16 and B16F10 murine melanoma models are widely used syngeneic melanoma models, especially in vaccine studies. (397) Despite the fact that they express known melanoma antigens, they don't fully resemble human melanoma. (398) The B16F10 cell line has greater metastatic potential than the parental B16 cell line and endogenously expresses Cr-1. (399) The growth speed of both the s.c. and i.v. injected B16F10 cells in C57Bl/6 mice only allowed us to do prophylactic vaccination. The i.v. injection of B16F10 cells is often used as a metastasis model. (400) Nevertheless, it rather reflects the formation of independent pulmonary nodules, and is not a suitable model to study metastasis. While we observed great reduction of pulmonary foci in immunized mice, our results are only an indication that Cr-1 vaccination can target the metastatic process.

Taken together, we showed that Cr-1 DNA vaccination elicits an immune response in C57Bl/6 mice. We further identified an immunodominant epitope and demonstrated that prophylactic vaccination was protective in the B16F10 murine melanoma model.

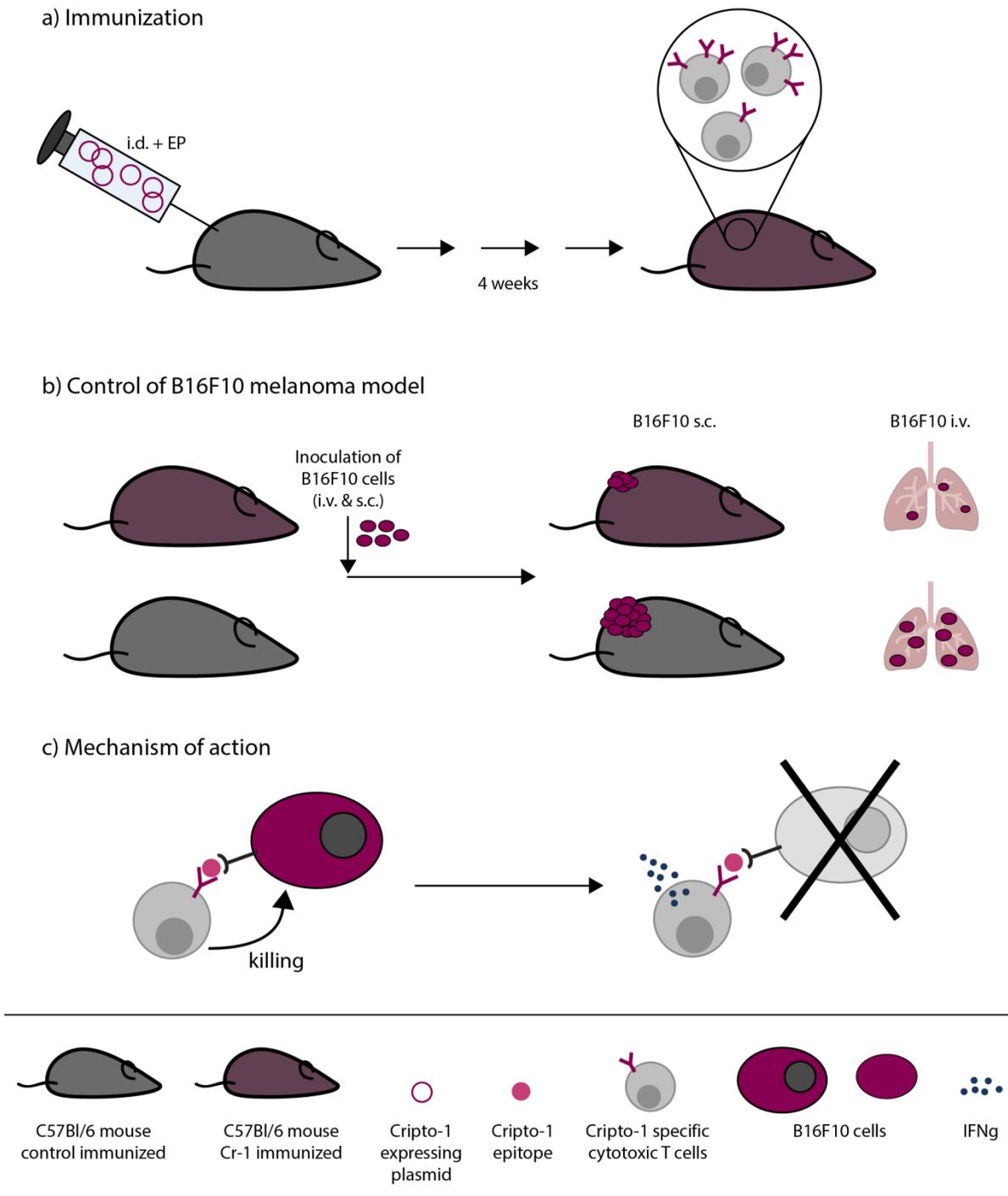


Figure 8: Summary of study I

a) Immunization of naïve C57Bl/6 mice with pmCr-1 elicits cytotoxic T cells. b) Control of i.v. and s.c. inoculated B16F10 cells in immunized mice. c) Vaccine induced CTLs recognize Cr-1 expressing tumor cells.

3.2 STUDY II

Cripto-1 plasmid DNA vaccination targets metastasis and cancer stem cells in murine mammary carcinoma

Background

Based on the findings in study I we propose that Cr-1 is a suitable antigen for cancer vaccines. A number of studies have investigated the function of Cr-1 during embryogenesis and in cancer. It was shown that Cr-1 is involved in Wnt signaling, regulating EMT during both embryogenesis and cancer. (401, 402) Recently Cr-1 expression was associated with metastasis in colorectal cancer. (403) These findings support the relevance of Cr-1 within the metastatic process. However, it remains unclear if metastasizing cells express Cr-1 and if Cr-1 is expressed in metastases.

Targeting Cr-1 is also of interest because of its association with cancer stem cells. Cr-1 is part of a signaling network, including Nanog, Oct-4, in stem cell maintenance. (310, 367, 369, 404) Cr-1 expression was shown in melanoma derived cells with stem cell phenotype. (405) A subset of Cr-1_{high} expressing cells within the embryogenic cancer cell line were reported to have a stem cell phenotype and increased capacity to form spheroids *in vitro*. (306) Further studies are required to understand the role and function of Cr-1 in cancer stem cells.

Presence of EMT and cancer stem cells is a feature of aggressive, often treatment resistant, tumors. (1, 38) The fact that Cr-1 expression in tumors is linked to both these phenotypes, suggests that Cr-1 vaccination may be suitable for treatment of aggressive metastatic tumors.

Aim of the study

The purpose of this study is to elucidate the full potential of Cr-1 vaccination. We investigate if Cr-1 immunization targets and controls spontaneous tumor metastasis in mice. Further we explore the effect of Cr-1 vaccination on cancer stem cells.

Results

We chose the murine 4T1 mammary carcinoma model to study the effect of Cr-1 vaccination on metastasis and the primary tumor in parallel. The 4T1 model is a commonly used model to study the metastatic process because the primary tumors metastasize within 2 weeks after inoculation. (406) In contrast to the B16F10 cell line used in study I, Cr-1 expression was weak in the 4T1 cell line. Therefore, we overexpressed Cr-1 in the 4T1 cell line (4T1mCr-1). Naïve BALB/c mice were immunized against Cr-1 using the same protocol as in study I. 4T1mCr-1 cells were orthotopically injected in both tumor growth and metastatic burden were evaluated. As reported in study I, pmCr-1 vaccination slowed down growth of the primary tumor but was unable to eradicate tumors. The metastatic burden in the lung was

strongly reduced. We decided to confirm our findings in the clinically relevant spontaneous mammary carcinoma model, BALB-neuT. BALB-neuT mice were vaccinated at week 10 and 12. Mice do not exhibit palpable tumors, but show dysplasia in the mammary fat pad at the time point of vaccination. Despite the lack of Cr-1 expression in the primary tumors, metastasis to the lungs was strongly reduced in this model. Our data confirms that Cr-1 vaccination has the ability to specifically control metastasis.

It was left to confirm what type of immune response Cr-1 vaccination induced in BALB/c mice. We were unable to identify Cr-1 reactive cytotoxic T cells in pmCr-1 immunized mice. On the other hand, we detected Cr-1 binding antibodies in the sera of vaccinated mice. Antibodies have been shown to have anti-tumor activity via activation of NK cells, a process known as antibody-dependent cellular cytotoxicity (ADCC). (385, 407) We therefore hypothesized that ADCC could be a mechanism of tumor control after Cr-1 vaccination. In mice, only antibodies of subtype mIgG2a, and to a lesser extent, IgG2b can mediate ADCC. The majority of Cr-1 binding antibodies in the sera of pmCr-1 vaccinated mice belonged to these two subtypes. *In vitro* cytotoxicity assays with NK cells as effector cells in the presence of sera of pmCr-1 vaccinated mice confirmed our hypothesis that Cr-1 binding antibodies facilitate targeting of Cr-1 expressing tumor cells by NK cells.

To evaluate the effects of Cr-1 vaccination on cancer stem cells, we confirmed the expression of Cr-1 *in vitro* cultured cancer stem cells TUBO P3. In the next step, TUBO P3 cells were s.c. injected in immunized mice. Cr-1 vaccination was partially successful in controlling CSCs *in vivo*. Prophylactic pmCr-1 immunization prevented tumor formation by TUBO P3 cells in one third of the mice and reduced tumor growth in another third.

Significance

In this study we confirmed the findings from study I on the protective effect of Cr-1 vaccination on tumor growth and extended the knowledge regarding the potential benefit of vaccination targeting metastasis and CSCs.

A limitation of the first study was that we used a transplantable model to evaluate the effect of Cr-1 vaccination on tumor growth and that we used a model that is not ideally suitable to study metastasis. For this second study, we therefore chose the 4T1 mammary carcinoma model to study the effect of vaccination on metastasis in a more relevant setting. Upon injection of 4T1 cells into the mammary fat pad of BALB/c mice, primary tumors are established that metastasize to distant organs. (408) Orthotopic transplantable mouse models are of greater clinical relevance than transplantable models as they partially mimic the morphology and growth of natural tumors (409, 410). We were able to confirm that prophylactic Cr-1 vaccination also has a protective effect in the BALB/c strain and particularly decreased metastasis in the lung. A limitation to the relevance of our results is that we overexpressed mCr-1 in the 4T1 cell line due to very low endogenous expression. We therefore confirmed our findings in the BALB-neuT mammary carcinoma model. This model has the great advantage of being a genetically engineered spontaneously developing tumor

model, overexpressing rat HER2 under the MMTV promoter. (411, 412) Oncogene-induced hyperplasia is developed around week 4 and progresses until palpable tumors occur around week 20. The primary tumors also metastasize to the lungs. (413) Tumor development in the BALB-neuT model is similar to human breast cancer development. (414, 415) The findings from both models together let us conclude that Cr-1 vaccination specifically targets metastasis. The effect of Cr-1 vaccination also appeared to be robust, since the two animal models were run at independent laboratories.

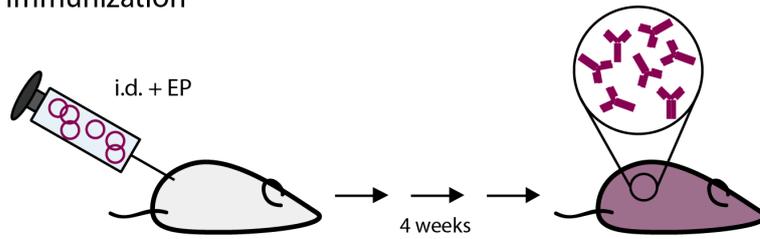
A hurdle in both study I and II was the lack of tools to detect Cr-1. Cr-1 vaccination reduced metastasis in the BALB-neuT mice despite Cr-1 being undetectable in the primary tumors by western blot. This raised the question whether Cr-1 is only expressed on a subset of cells undergoing EMT in the primary tumor, on circulating tumor cells or on the metastases. Evaluation of Cr-1 expression in the lung metastases by qPCR was a not an ideal method. We detected Cr-1 mRNA in the lung tissue, but were unable to confirm that the expression is in the metastasis. We tested several commercially available human Cr-1 antibodies, but none of them was suitable for detecting murine Cr-1 by either immunofluorescence or flow cytometry.

In contrast to our findings in the C57Bl/6 mouse strain, vaccination was protective through induction of Cr-1 specific IgG2a and IgG2b antibodies in the BALB/c mouse strain. We suggest that this discrepancy is caused by the differences between the genetic backgrounds of these two mouse strains. A recent study identified that BALB/c mice are predisposed to a greater IgG2a response post vaccination. (416) Additional studies in mice have shown that tumor specific IgG2a antibodies are sufficient for tumor control in the BALB-neuT mouse model. (417-420) The activation of T helper cells was essential in this protection. (421)

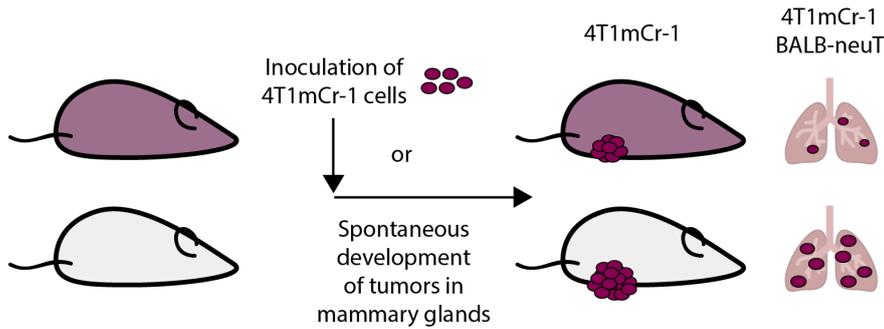
In addition to the validation of the humoral response, we screened for Cr-1 reactive T cells. Splenocytes from vaccinated mice were simulated with peptides from the overlapping peptide library and IFN γ production was evaluated by flow cytometry. The flow cytometry based screening is less common than the ELISA or ELISPOT based analysis of the IFN γ response, but has been done in other studies. (422, 423) We were unable to detect Cr-1 reactive T cells in our screening. We could speculate that Cr-1 does not contain a dominant immunogenic epitope or that our assay was not sensitive enough to detect low frequency clones. It remains to be shown if long time *ex vivo* stimulation of splenocytes with the Cr-1 peptide library could increase the frequency of Cr-1 reactive T cells allowing for detection. The detection of antigen specific T cells by IFN γ production is a sensitive and a commonly used method to detect antigen specific T cells post vaccination.

In summary, we were able to confirm that Cr-1 DNA vaccination elicits a protective immune response in another mouse strain and three different mammary carcinoma models. In addition, we showed the potency of Cr-1 vaccination to target metastasis and CSCs.

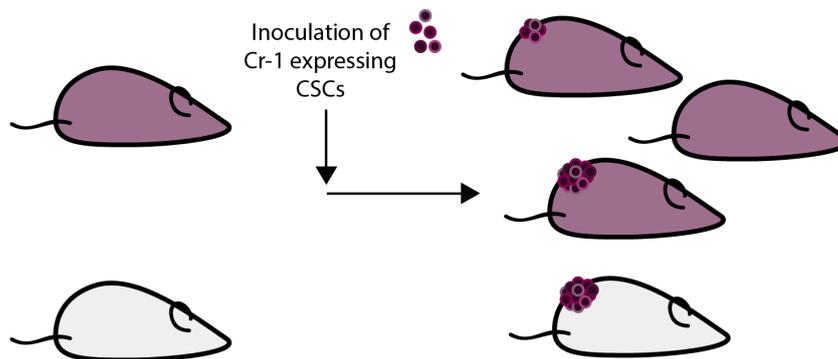
a) Immunization



b) Control of mammary carcinoma models



c) Partial control of tumor establishment from CSCs



d) Mechanism of action

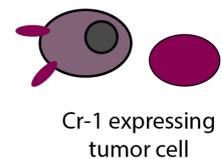
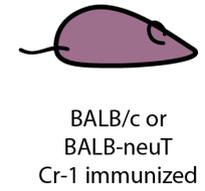
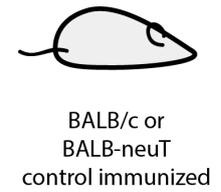
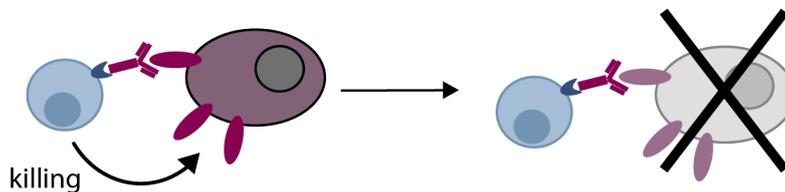


Figure 9: Summary of study II

a) Immunization of naïve BALB/c mice with pmCr-1 elicits a humoral response. b) Control of primary tumor and metastasis in orthotopically inoculated 4T1mCr-1 model. Control of metastasis in BALB-neuT mice, which spontaneously develop mammary tumors. c) Partial control of tumor formation from breast cancer stem cells. d) NK cell mediated ADCC of Cr-1 expressing tumor cells.

3.3 STUDY III

Peripheral T cell reactivity against Cripto-1 correlates with overall survival in patients with advanced melanoma

Background

The expression of Cr-1 has already been shown, including, lung cancer, breast cancer and gastric cancer. (383) It is known that Cr-1 can be shed and it was reported to be present in the sera of patients with breast cancer, colon cancer, NSCLC, hepatocellular carcinoma and glioblastoma. (323, 347-349) However, the biological function of sCr-1 remains unclear. It has become evident that high levels of sCr-1 are related to poor prognosis in NSCLC and are a biomarker for prognosis (350, 424). The evidence for the presence of Cr-1 in melanoma is still limited. Cr-1 expression was described in stem cell like cells isolated from an aggressive melanoma cell line and a small number of primary human melanoma cell lines. (340, 405) It was also detected by IHC in a small study on uveal melanoma.

In study I and II we showed that murine Cr-1 vaccination induced Cr-1 specific immune responses in mice. While CTL epitopes have been described for a number of tumor associated antigens, including cancer testis antigens NYESO-1 and MAGE-A, immunogenicity of human Cr-1 remains to be shown. (425, 426)

Aim of the study

The aim of this study is to identify the relevance and correlation with survival of soluble Cr-1 (sCr-1) and T cell reactivity to Cr-1 in patients with advanced melanoma.

Results

Forty-one patients with stage IIIb, IIIc and IV melanoma, scheduled for surgical removal of a melanoma lesion, were included in our patient cohort. Two blood samples were collected; one before and one after surgery, with a median time of 34 days between the blood draws.

Based on previous reports, we evaluated Cr-1 protein concentrations in the serum, both pre- and post-surgery. sCr-1 was present in more than 80% of stage IIIc and IV patients, but only in 50% of stage IIIb patients before surgery. Surgery did not influence the levels of sCr-1 found in the patient sera. However, high levels of circulating Cr-1 protein (>587.4 pg/ml) after surgery correlated with worse survival.

We next aimed to identify Cr-1 reactive T cells in the patient peripheral blood PBMCs. PBMCs were stimulated for 12 days with the human Cr-1 15-mer peptide library, based on the protocol to detect tumor reactive T cells in PBMCs established by Weide et al. (427) Using this assay, Cr-1 reactive CD4⁺ and CD8⁺ T cells were detected in 60% of the patients before surgery, mainly in stage IIIb patients. The presence of Cr-1 reactive CD4⁺ and CD8⁺ T cells in circulation correlated with better OS and longer PFS in this cohort. Surgical removal

of melanoma lesions increased T cell reactivity to Cr-1, in particularly that of CD8⁺ T cells. T cell reactivity post-surgery was not correlated with prognosis.

Deeper analysis of the cytokine profile of Cr-1 reactive CD4⁺ and CD8⁺ T cells revealed that mainly pro-inflammatory cytokines (IFN γ , TNF α , IL-2) were produced in response to Cr-1 peptide stimulation. In line with the increase in total T cell reactivity, we observed an increase in multi-cytokine responses in patients after surgery. Interestingly, specific cytokine responses in Cr-1 reactive CD4⁺ T cells were more predictive for OS than those in CD8⁺ T cells.

Significance

In this study, we explored the intrinsic immunity against Cr-1 in melanoma patients to potentially identify sCr-1 or T cell reactivity to Cr-1 as a novel biomarker in advanced melanoma.

In line with studies on other cancer types, we were able to confirm the presence of sCr-1 in the sera of advanced melanoma patients. (323, 347-349) Cripto-1 expression has been described before in samples from early and late stage melanoma. (340) However, we did not yet confirm expression of Cr-1 in the tumor samples from our patient cohort.

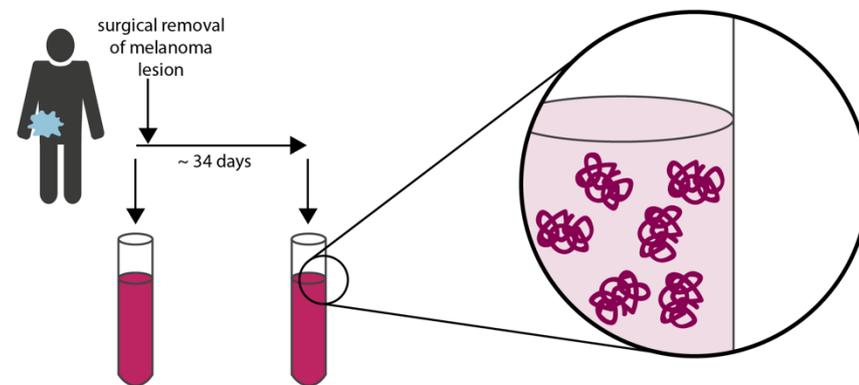
High concentrations of sCr-1 in post-surgical patient sera were associated with shorter survival, although we did not observe a significant decrease of sCr-1 protein in the serum of patients after surgery. One possible explanation could be that serum Cr-1 levels did not yet normalize within our follow-up period. Studies on other serum biomarkers IGF-1, CEA and CA19-9 have found that normalization occurs between one and three months after surgical removal of the tumor. (428, 429) A drawback of our serum analysis is that we did not include serum from healthy donors yet. The detected serum levels in our patient cohort and the mathematically determined cut-off point for low and high Cr-1 levels are in line with the findings in other studies. (323, 347-349) However, inclusion of sera from healthy donors will deepen our knowledge on the biological and clinical relevance of our findings.

For the first time, we were able to Cr-1 specific T cells in cancer patients. Circulating T cells that are reactive to known melanoma antigens and neoantigens have previously been identified in melanoma patients. (430-432) Using a long-term T cell stimulation assay, we detected Cr-1 reactive T cells in the peripheral blood of patients with advanced melanoma. We used a human Cr-1 overlapping peptide library to detect Cr-1 reactive T cells, which allowed us to evaluate responses in all patients, independent of their HLA subtype. However, this brings a disadvantage, since 15 amino acid long peptides do not optimally bind to MHC class I. We followed and established 12-day stimulation protocol. (427) Due to low amounts of frozen PBMCs, we stimulated our T cell cultures with peptides alone, while a more optimal setting would have been to include peptide-loaded autologous PBMC. This has shown to significantly increase the frequency of peptide reactive T cells. (433) Because of the long pre-stimulation and the low frequency of cytokine producing T cells after re-stimulation

with the peptide library, we decided against a quantitative analysis. This is a drawback of the study, as we cannot correlate response intensity to outcome. However, there are indications that T cell reactivity is associated with longer survival. In order to confirm that Cr-1 reactive T cells were primed by tumor derived antigens and are not an artifact, Cr-1 T cell reactivity should be evaluated in healthy donor PBMC.

In summary we confirmed the presence of soluble Cr-1 in the sera of patients with advanced melanoma and its correlation with survival. Surgical removal of tumor mass increased the reactivity of circulating CD8⁺ and CD4⁺ T cells to Cr-1. Our data suggest that T cell cytokine responses to Cr-1 correlate with survival as well.

a) Serum analysis



b) Stimulation of peripheral Cr-1 specific T cells

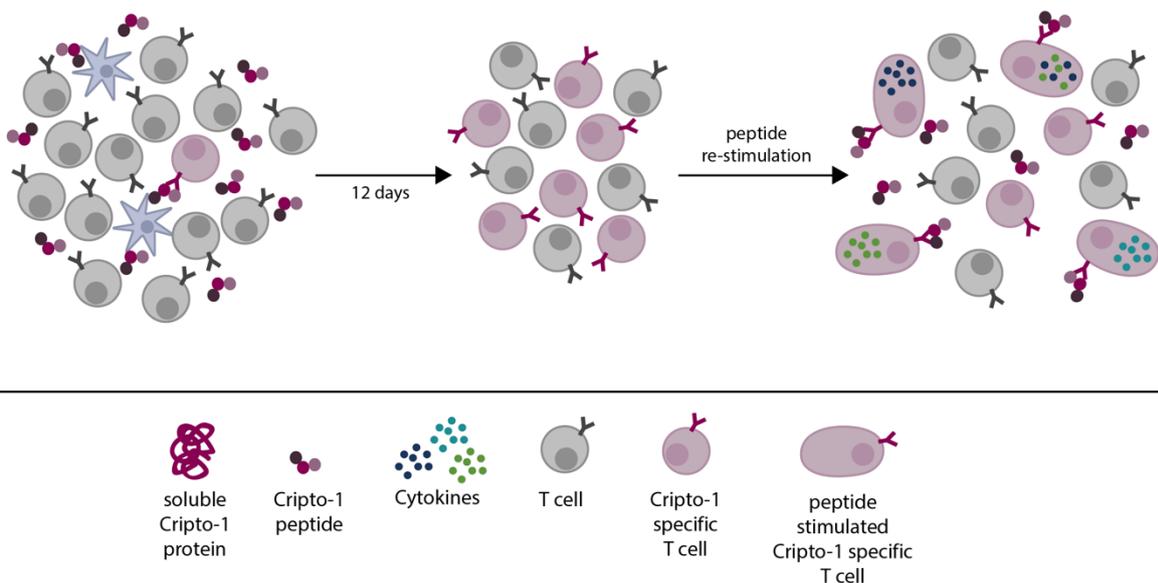


Figure 10: Summary of study III

a) Two blood samples were collected from all patients, one before and one after surgical removal of melanoma lesion. Soluble Cr-1 in serum was measured by ELISA. b) PBMCs are stimulated with Cr-1 peptides for 12 days prior to re-stimulation and in vitro detection of Cr-1 reactive T cells.

4 CONCLUSION

With the clinical approval of the first checkpoint inhibitor in melanoma treatment in 2011 and the Nobel Prize award in 2018 for the discovery of using the inhibition of immune checkpoints in cancer therapy, immunotherapy got into the focus of scientist, clinicians, and the general public.

Increasingly more research is being conducted within the field of tumor immunology, broadening our knowledge and deepening our understanding for the interaction between the tumor and the immune system. This valuable knowledge enables us to develop novel immunotherapeutic strategies, and even more importantly, allows us to identify the right patient groups. Cancer vaccines can be of great benefit to patients who lack tumor specific T cells and will not respond to the blockade of the PD-1/PD-L1 axis. Vaccination with TAAs can increase the diversity of tumor reactive T cells and enhance reactivity against self-antigens.

In this thesis, we have demonstrated that Cr-1 is a target for cancer immunotherapy. In study I and II we showed that a vaccine based treatment approach elicited Cr-1 specific immune responses in two mouse strains. Moreover, it was protective in murine melanoma and murine mammary carcinoma models. We further showed that Cr-1 vaccination specifically reduced metastasis and partially prevented tumor formation from CSCs. In study III we investigated the potential of Cr-1 as a biomarker in advanced melanoma. We found that both low levels of sCr-1 in the sera and the presence of circulating Cr-1 reactive T cells correlated with better survival in our cohort. Altogether, we identified Cr-1 as a potential biomarker and interesting target for cancer immunotherapy.

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“Life is about the people you meet”

(unknown)

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