

# **Tumor metastasis – mechanisms and prevention**

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Dedicated to my family



# ABSTRACT

Metastatic spread of tumor cells accounts for the majority of cancer-related deaths. We have developed cancer vaccines based on the immunostimulatory adjuvant cholera toxin A1 (CTA1), including vaccines that deliver tumor antigens and CTA1 to cross-presenting type 1 conventional dendritic cells (cDC1). We also explored mechanisms of epithelial-mesenchymal transition (EMT), in which cancer cells acquire features that facilitate metastasis. **In paper I**, we used fusion vaccines that comprised of a cDC1-targeting anti-CD103 single-chain antibody (aCD103) and the CTA1 adjuvant fused with MHC class I and II-restricted epitopes from the model tumor cell antigen ovalbumin (OVA). The rationale was to deliver the antigen (OVA) and the adjuvant (CTA1) to highly efficient antigen-presenting cells (CD103-expressing cDC1) to achieve optimal induction of T cell-mediated immunity. This CTA1-I/II-aCD103 vaccine construct induced robust antigen-specific CD8<sup>+</sup> T cell responses along with a Th17-polarized CD4<sup>+</sup> T cell response and efficiently reduced primary growth and metastasis of B16F1-OVA melanoma in prophylactic and therapeutic settings. **Paper II** expanded these findings by demonstrating that CTA1-based cancer vaccines enhanced immune responses against the endogenous tumor-associated antigens TRP2 and Twist1 in mice. This led to significant protection against metastasis in B16F1 melanoma and 4T1 breast cancer models. **In paper III**, we explored the mechanisms underlying the induction of EMT, in which epithelial tumor cells acquire mesenchymal characteristics that facilitate metastatic spread. The results revealed that EMT, orchestrated by the induction of transcription factors such as SNAIL and SLUG, was triggered by reactive oxygen species (ROS), which mediate oxidative stress in the malignant microenvironment. The ROS-induced EMT led to metastasis of breast cancer *in vivo*, which could be mitigated through genetic and pharmacological inhibition of NOX2, a principal ROS-producing enzyme expressed by tumor-infiltrating myeloid cells. **In summary**, this thesis identified CTA1-based fusion vaccines that control the growth and dissemination of tumor cells and unveiled novel and potentially targetable mechanisms relevant to EMT-induced metastasis.

**Keywords:** cancer vaccines, adjuvant, metastasis, EMT, cDC1s, TAAs



# SAMMANFATTNING PÅ SVENSKA

Uppkomst av metastaser är den vanligaste dödsorsaken för patienter med solida tumörsjukdomar. Utöver konventionell cancerbehandling – kemoterapi, strålning och kirurgi – finns det få behandlingsstrategier som förhindrar metastasering. Detta avhandlingsarbete belyser strategier för att inducera immunreaktioner mot cancerceller genom att konstruera optimalt immunogena vacciner och identifierar mekanismer som bidrar till att metastaser uppkommer.

T-cellsförmedlade immunreaktioner mot cancerceller erfordrar att en typ av immunceller, dendritiska celler (DC), presenterar antigen för T-celler. En undergrupp av DC, konventionella DC typ 1 (cDC1), är höggradigt effektiva antigenpresenterande celler. Vi utvecklade därför rekombinanta fusionsvacciner i vilka tumörantigen kopplades dels till en adjuvans (koleratoxin subenhet A1, CTA1), som förstärker immunstimulering av vaccinet, dels till en antikropp (anti-CD103) som avsåg att effektivare leverera tumörantigen och adjuvans till cDC1. Vi tillverkade vacciner som avsåg att aktivera CD4<sup>+</sup> T-celler (hjälpceller) och sådana som även aktiverade CD8<sup>+</sup> (celldödande) T-celler.

I **arbete 1** använde vi tumörceller (melanom) som modifierats så att de uttrycker ovalbumin, vilket kan initiera T-cellsförmedlad immunreaktivitet. Dessa tumörceller implanterades subkutant till möss i studier av lokal tumörtillväxt eller injicerades venöst för att framkalla lungmetastaser. Vi fann att ett vaccin som bestod av både CTA1, anti-CD103 och antigen som avsåg att samtidigt aktivera T-celler av både CD4- och CD8-typ, gav upphov till robusta immunsvaret. Detta vaccin reducerade effektivt tumörtillväxt i möss och förhindrade lungmetastasering. Vaccinet gav ett varaktigt skydd mot

metastasering och var effektivt både när det gavs profylaktiskt och när det gavs till möss med etablerade melanomtumörer.

I **arbete 2** utvidgade vi dessa fynd genom att använda naturligt förekommande tumörantigener. Vi fann att ett vaccin som fusionerats med en dimer från *Staphylococcus aureus* protein A förbättrade immunsvaren mot de tumörassocierade antigenen TRP2 och Twist1 hos möss, och att vaccinkonstrukt som innehöll dessa antigen skyddade mot metastasering av melanom och bröstcancer.

*Epithelial to mesenchymal transition* (EMT) bidrar till uppkomst av metastaser från epiteliära tumörer, t ex bröstcancer, genom att tumörceller omvandlas till mesenkymala celler som lossnar från tumören och sprider sig via blod- eller lymfkärl. I **arbete 3** studerades hur s.k. oxidativ stress, som innebär överproduktion av reaktiva syremetaboliter (*reactive oxygen species*, ROS) i vävnader, påverkar EMT. Vi fann att transkriptionsfaktorer som medierar EMT, såsom SNAIL och SLUG, inducerades av ROS liksom att ROS-inducerad EMT gav upphov till tillväxt och metastasering av bröstcancer i möss. Dessa effekter kunde motverkas genom genetisk eller farmakologisk hämning av NOX2, ett ROS-producerande enzym som uttrycks av tumörinfiltrerande myeloiska celler.

Sammanfattningsvis kan detta avhandlingsarbete bidra till utveckling av mer effektiva tumörvacciner för att förhindra metastasering och till ökad förståelse för mekanismer av betydelse för uppkomst av metastaser.



# LIST OF PAPERS

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

- I. Arabpour M, **Paul S**, Grauers Wiktorin H, Kaya M, Kiffin R, Lycke N, Hellstrand K, Martner A. An adjuvant-containing cDC1-targeted recombinant fusion vaccine conveys strong protection against murine melanoma growth and metastasis. *Oncoimmunology*. 2022 Aug 24;11(1):2115618.
  
- II. **Paul S**, Kaya M, Johnsson O, Grauers Wiktorin H, Törnell A, Arabpour M, Hellstrand K, Martner A. Targeting murine metastatic cancers with cholera toxin A1-adjuvanted peptide vaccines. *Submitted*
  
- III. Kaya M, Johnsson O, **Paul S**, Issdisai N, Dongre A, Hellstrand K and Martner A. NRF2 activation by myeloid cell-derived oxidative stress induces SNAI-driven EMT and metastasis in breast cancer. *Manuscript*

Other papers not included in this thesis:

- I. Grauers Wiktorin H, Aydin E, Kiffin R, Vilhav C, Bourghardt Fagman J, Kaya M, **Paul S**, Westman B, Bratlie SO, Naredi P, Hellstrand K, Martner A. Impact of Surgery-Induced Myeloid-derived Suppressor Cells and the NOX2/ROS Axis on Postoperative Survival in Human Pancreatic Cancer. *Cancer Res Commun.* 2024 Apr 25;4(4):1135-1149.
  
- II. Nilsson MS, Komic H, Gustafsson J, Sheybani Z, **Paul S**, Rolfson O, Hellstrand K, Wennström L, Martner A, Thorén FB. Multiomic single-cell analysis identifies von Willebrand factor and TIM3-expressing BCR-ABL1<sup>+</sup> CML stem cells. bioRxiv 2023.09.14.557507



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# ABBREVIATIONS

ANN – Artificial neural networks

APC – Antigen-presenting cell

BA – Binding affinity

BCR – B cell receptor

BM – Bone marrow

cAMP – Cyclic adenosine monophosphate

CAR – Chimeric antigen receptor

CD spectroscopy – Circular dichroism spectroscopy

cDC – Classical dendritic cell

CT – Cholera toxin

CTA1 – Cholera toxin subunit 1A

CTL – Cytotoxic T cell

CTLA-4- Cytotoxic T lymphocyte associated protein-4

DAMP – Damage-associated molecular pattern

DC – Dendritic cell

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

ECM – Extracellular matrix

EMT – Epithelial to mesenchymal transition

ER – Endoplasmic reticulum

FDA- Food and drug administration

FDC – Follicular dendritic cell

FSC – Forward scatter

FT-IR – Fourier transform infrared

GBM – Glioblastoma

GC – Germinal center

GFP – Green fluorescent protein

GM-CSF – Granulocyte-macrophage colony-stimulating factor

H<sub>2</sub>O<sub>2</sub> – Hydrogen peroxide

HDC – Histamine dihydrochloride

HER2 – Human epithelial growth factor receptor 2

HLA- Human leukocyte antigen

HPLC – High performance liquid chromatography

HR – Hazard ratio

i.d. – intradermal

i.m. – intramuscular

i.n. – intranasal

i.p. – intraperitoneal

i.v. – intravenous

ICI – Immune checkpoint inhibitor

IFN – Interferon

IgG – Immunoglobulin G

IL – Interleukin

LNP – Lipid nanoparticle

MDSC – Myeloid-derived suppressor cell

MET – Mesenchymal to epithelial transition

MHC – Major histocompatibility complex

mRNA – Messenger RNA

NADPH – Nicotinamide adenine dinucleotide phosphate

NADPH oxidase – NOX

NK – Natural killer

NSCLC – Non-small cell lung cancer

OV – Oncolytic viruses

OVA-Ovalbumin

PAMP – Pathogen-associated molecular pattern

PBMC– Peripheral blood mononuclear cells

PBS – Phosphate-buffered saline

pDC – Plasmacytoid DC

PLC- Peptide loading complex

PRR – Pattern recognition receptor

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RT–qPCR – Real-time quantitative polymerase chain reaction

s.c. – Subcutaneous

ScFv – Single chain variable fragment

SSC – Side scatter

TAA – Tumor-associated antigen

TAM – Tumor-associated macrophage

TAP – Transporter associated with antigen processing

TCR – T cell receptor

TGF – Transforming growth factor

TIL – Tumor-infiltrating lymphocyte

TLR – Toll-like receptor

TME – Tumor microenvironment

TSA – Tumor-specific antigen

WBC – White blood cell

WGS – Whole genome sequencing



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# INTRODUCTION

## 1. CANCER

According to the World Health Organization (WHO) there were approximately 20 million new cases of cancer and 9.7 million cancer-related deaths worldwide in 2022 (1). Cancer is assumed to arise because of unfavorable changes in the deoxyribonucleic acid (DNA), which may be inherited (germline mutations) or acquired (somatic mutations). Mutations in the DNA occur frequently during normal cell division, but cells possess DNA repair systems that can identify and repair alterations. However, these systems are not foolproof, and failure of DNA repair mechanisms and accumulation of mutations may result in transformation of a healthy cell to a cancer cell. On a broader level, factors such as hormones, the status of the immune system, or environmental factors, may increase the frequency of genetic alterations and thus enhance the risk of cancer development. Environmental factors impacting DNA modifications may include physiological and xenobiotic sources, nutritional status, metabolic or oxidative conditions, exposure to toxins and therapeutics, infections and exposure to radiation (2). In addition to altering DNA sequence or structure, these factors may influence the expression of the genetic code (epigenetic modification), typically by methylation of DNA or by modifying histones (3). Both genetic and epigenetic modifications are involved in the emergence of malignancies (4). Epigenetic alterations can be inherited through cell division but may not always be stable (5).

The transformation of a healthy cell to a cancer cell may involve changes in gene expression related to *oncogenes*, which are mutated forms of normal genes (proto-oncogenes) that promote growth and division of healthy cells. When activated through mutations, oncogenes drive uncontrolled cell

proliferation. In many cancers, however, mutations instead occur in *tumor suppressor genes*. These genes normally act to maintain controlled cell growth and proliferation. When mutated or deleted, their inhibitory effect is reduced or eliminated, leading to unchecked cell growth (6). The phenotypic behavior attained during tumorigenic transformations typically results in uncontrolled proliferation, resistance towards controlled cell death, reduced responsiveness to growth-suppressive signals, dysfunctional energy metabolism and/or an instable genome. These aspects, along with enhanced inflammation and angiogenesis, are commonly referred to as the ‘hallmarks of cancer’ (4).

## 2. CANCER THERAPY

**2.1 Chemotherapy.** The historically most used and important cancer therapies, including chemotherapy, surgery and radiotherapy, can be traced far back in history. Modern chemotherapy originated from the use of nitrogen mustard during the 1940s. This drug was derived from chemical warfare agents used during the first world war and was initially used for treating lymphomas. Later, alkylating agents such as chlorambucil and cyclophosphamide were introduced (7, 8). Methotrexate became one of the first regimens to attain temporary remission of leukemia in children in the 1940s (9). Drugs such as 6-mercaptopurine and 6-thioquanine were widely utilized in leukemia in the 1950s (10, 11). 5-fluorouracil has been an established chemotherapeutic agent since the 1950s and is used to treat solid tumors such as colorectal and head and neck cancer (12). Corticosteroids has been incorporated into cancer treatment protocols since the 1950s to mitigate treatment-related side-effects (13).

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It was later discovered that different chemotherapeutic agents target tumor cells at different phases of the cell cycle, and that combination chemotherapy yielded improved therapeutic efficacy and also reduced resistance and toxicity. Based on their mechanism of action, chemotherapies are often classified as *alkylating agents*, which cause DNA strand breaks and cell death (14), *antimetabolites*, which deplete nucleotides or insert fraudulent nucleotides (15), and a broader class of *antineoplastic agents* (16).

Combinatory treatments using cytoxan, methotrexate and fluorouracil were applied to breast cancers and became standard of care for many years (17, 18). A combination of chemotherapies consisting of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), introduced in the 1970s, is still in use for patients with non-Hodgkin lymphoma (19-21).

**2.2 Targeted therapy.** The 1990s marked the introduction of targeted therapies, often originating from genome sequencing to identify abnormalities that are unique to cancer cells. A prototypic example is the identification of protein kinases only acting in cancer cells, leading to the development of cancer cell-specific or -selective protein kinase inhibitors (22, 23). Drugs against chronic myeloid leukemia (imatinib (24)), gastrointestinal stromal tumors (ripretinib (25)) and hepatocellular tumors (sorafenib (26)) were some of the first targeted therapies to gain approvals by Food and Drug Administration (FDA) (27). In addition, BRAF, which forms part of the mitogen-activated protein kinase signaling pathway that controls proliferation and survival, can be targeted in patients with advanced melanoma carrying mutated *BRAF*. BRAF inhibitors (including vemurafenib) may provide pronounced albeit often transient reduction of melanoma metastasis in humans (28).

**2.3 Surgery.** The advent of general anesthesia in the late 20<sup>th</sup> century allowed for multiple invasive forms of cancer surgery for the removal of, *e.g.*, abdominal, gynecologic, breast and prostate tumors (29, 30). In recent times, the benefit of surgery has been bolstered using chemotherapy in the pre-operative phase (neoadjuvant therapy) (31) or after surgery (adjuvant therapy) (32) and by the availability of improved imaging, which allows the removal of cancerous tissue not observed during surgery (33). More recent development in cancer surgery includes non-invasive procedures such as laparoscopic surgery (34), radiosurgery, and laser surgery (35).

**2.4 Radiotherapy.** Shortly after the discovery of X-rays in 1895, radiotherapy was established in cancer treatment, with significant developments following Marie Curie's discovery of radium in 1898 (36, 37). The affected area is exposed to a concentrated beam of ionizing radiation, usually delivered from a linear accelerator that produces high-energy X-rays or electrons. In the 1960s, improved radiation targeting was developed to concentrate radiation to the tumor lesion aiming to minimize impact on non-cancerous tissue, which was followed by several other improvements (38), including modulating doses of radiation to further spare healthy tissue (37).

Overall, the evolution of cancer treatments has translated into gradual but significant improvement of the survival of cancer patients over time, but there are still challenges to address. New treatment in the form of more precise targeted therapies and immunotherapies may overcome some of these challenges.

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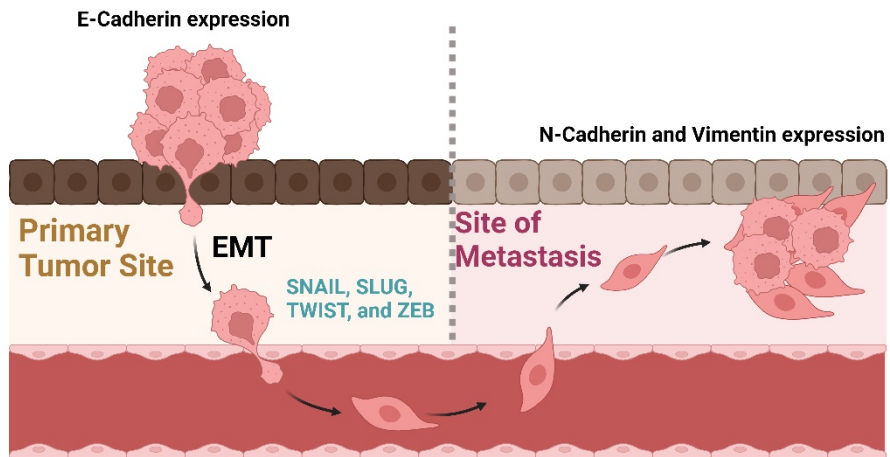
### 3. METASTASIS

Metastasis is defined as the establishment and growth of secondary tumors at sites distant from the primary tumor. A study from 2019 calculated that about two-thirds of cancer-related deaths from solid cancer were due to metastasis (39). The properties of the metastatic tumor and the site of metastasis have been suggested to depend on the primary seeding tumor cells, but the pathobiological mechanisms involved in metastasis remain incompletely understood, which limits the recognition of druggable targets. The understanding of the molecular mechanisms leading to metastasis is crucial to identify new interventions (40); yet there are surprisingly few available drugs that directly target metastasis (41).

**3.1 Epithelial to mesenchymal transition.** Cancer cells that metastasize leave the site of the primary tumor, circulate in the bloodstream or lymphatics and then need to cope with the high oxygen levels in the blood stream, escape immunosurveillance and finally adapt to and expand in a secondary site (42, 43). *Epithelial to mesenchymal transition* (EMT) is a process that is considered essential for epithelial malignant cells to metastasize. During EMT, epithelial cancer cells acquire features of mesenchymal cells, which allows for their detachment from the primary tumor to facilitate metastatic spread (Fig. 1). EMT comprises trans-differential mechanisms governed by reversible biochemical changes, including epigenetic changes (44).

A broad range of growth factors and signaling pathways regulate the mesenchymal transition (45, 46). Key players that mediate EMT include SNAIL, SLUG, TWIST and ZEB family proteins (4). These act as transcription factors to repress epithelial markers like E-cadherin while promoting mesenchymal traits such as N-cadherin and vimentin (47, 48). The net result is that epithelial cells lose their polarity and adhesion to the

epithelium, gaining migratory and invasive capabilities. EMT is essential in normal embryogenesis and wound healing, and the deregulated EMT in cancer is an example of how malignant cells utilize normal biological processes to promote metastasis (49).



*Figure 1. Epithelial-to-mesenchymal transition leading to metastasis*

The phenotypic fate for cells undergoing EMT is not a binary choice between epithelial or mesenchymal; instead, the cancer cells can acquire a broad spectrum of transitional phenotypes with invasive and metastatic characteristics. These intermediate EMT phenotypes have variable and occasionally increased potential to disseminate and trigger metastasis (50).

The malignant microenvironment may determine the initiation and evolution of EMT. Mesenchymal-like cells generally grow near endothelial and inflammatory cells. Inflammatory cells are recruited to the tumor microenvironment (TME) by chemokines produced by the tumor cells and

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promote angiogenesis and a vascularized inflammatory niche that contribute to a milieu favoring EMT. Furthermore, fibronectin alignment derived from cancer-associated fibroblasts may aid tumor cell migration (51). Additionally, extracellular matrix (ECM) pressure, metabolic stimuli and hypoxia may also facilitate EMT (52). While the process of EMT is essential for the initiation of metastasis, the reverse process of mesenchymal to epithelial transition (MET) is crucial for the subsequent establishment and growth of metastatic cells at distant sites (53).

## **4. THE IMMUNE SYSTEM**

**4.1 Innate immunity.** The body's first line of defense against pathogens and other threats is denoted as innate immunity. In addition to providing protection by multiple defense mechanisms, innate immune cells, particularly dendritic cells (DCs), play a role in initiating and activating adaptive immune responses. The innate immune system includes phagocytes, natural killer (NK) cells, and soluble factors such as complement. It generates immune responses with a broad range of antigen specificity. The innate immune system thus swiftly identifies and recognizes patterns with wide specificity, and it is generally not considered adaptive, meaning that innate defense mechanisms do not become stronger at re-exposure and cannot form memory (54). Despite being non-adaptive, innate immune mechanisms can effectively clear pathogens, infected cells and transformed cells (55). The rapid response of innate immunity is crucial, given the high replication rate of some microorganisms.

Serum proteins in the blood, such as complement, attach to the surface of microorganisms, flagging them as foreign and sometimes lysing them or coating them with proteins to make them more accessible to phagocytes for

engulfment and destruction by phagocytosis. This process, known as opsonization, enhances the efficiency of pathogen clearance. Phagocytes patrolling the blood for potential invaders use germline-encoded pattern recognition receptors (PRRs) on their cell membranes to identify foreign microorganisms. Unmethylated CpG motifs on bacterial DNA or other cell wall or membrane components of microorganisms can trigger a broad cascade of immune responses, including phagocytosis. Additionally, the secretion of cytokines, such as interleukins (IL) and tumor necrosis factors (TNF), by immune cells into the extracellular fluid results in the activation and infiltration of white blood cells (WBCs). These cytokines play a complex but crucial role in coordinating the innate and adaptive immune responses against pathogens (56, 57).

**4.2 Adaptive immunity.** The adaptive immune system, the operative second line of defense in vertebrates, employs specific and sophisticated mechanisms to protect against pathogens. Comprising cytotoxic and helper T cells as well as antibody-producing B cells, this system utilizes destructive responses to eliminate host-invading pathogens and infected or transformed cells. Due to the destructive nature of these responses, it is crucial that adaptive immune reactions are tightly regulated to prevent harm to the host. A fundamental aspect of the adaptive immune system is its ability to distinguish foreign entities from self. Failure in this distinction can result in destruction of normal tissue with ensuing autoimmune disease.

To prevent strong adaptive responses against self-antigens, adaptive immune cells undergo a process of central tolerance, where T cells and B cells recognizing self-antigens are eliminated through apoptosis. Additionally, the innate immune system aids in differentiating between antigens that should elicit strong adaptive responses and those that should be ignored or tolerated.

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Factors of microbial origin often activate innate immune cells to elicit strong adaptive immune responses and can be used as adjuvants to tailor immune responses to treatments such as vaccines. This activation not only triggers an innate immune response but also enables the adaptive immune system to respond to the administration of a foreign antigen. Another remarkable feature of the adaptive immune system is its ability to distinguish between similar antigens, such as proteins with slight variations in their amino acid compositions or optical isomers of a molecule, such as mutated peptides (58).

### 4.3 Myeloid cells

**4.3.1 Neutrophils.** Polymorphonuclear phagocytes, or neutrophils, are considered the first line of defense of cellular innate immunity. They constitute the largest number of leukocytes in circulation, with a production rate of approximately  $10^{11}$  cells per day in the bone marrow (BM). During homeostasis, neutrophils travel through the circulation, migrate into tissues in response to insults, and are removed by macrophages after performing their tasks (59). The mechanisms utilized by neutrophils to degrade invading pathogens include phagocytosis, intracellular degradation, release of granules, and the development of extracellular traps.

Recent studies have highlighted that the neutrophil repertoire extends beyond mere phagocytosis of invading pathogens. Neutrophils may thus influence inflammation by producing cytokines and inflammatory factors in response to various signals (60, 61). Transcriptional changes can alter neutrophil responses, and microenvironments can influence the functional phenotypes of neutrophils, promoting heterogeneity among these cells (62).

**4.3.2 Monocytes and macrophages.** Monocytes are BM-derived WBCs that respond to inflammation and infection (63). They circulate in the bloodstream

and can migrate into tissues where they differentiate into macrophages and dendritic cells. Thereby, monocytes contribute to phagocytosis of pathogens and apoptotic cells and in presenting antigens to T cells (64).

Macrophages are tissue-resident myeloid cells that differ in phenotype and function depending on the tissue in which they reside. Macrophages engulf and digest pathogens, dead cells and debris through phagocytosis. They are also sentinel cells that respond to danger signals via PRRs. This leads to production of cytokines, chemokines and lipid metabolites that are involved in recruitment of other immune cells, including monocytes, to the site of inflammation (63). Both monocytes and macrophages express an array of PRRs, including Toll-like receptors (TLRs), on the surface and intracellularly. These receptors bind to pathogen-associated molecular patterns (PAMPs) expressed on microbes and damage-associated molecular patterns (DAMPs) from injured cells, which dictates the resulting immune response (65). In the context of cancer, monocytes/macrophages can differentiate into tumor-associated macrophages (TAMs), which often promote tumor growth, metastasis and an immunosuppressive (TME). Factors present within the milieu may polarize the TAMs into M1-like cells, that are assumed to support anti-tumor immune responses, or M2-like cells, that suppress immune activity (66).

**4.3.3 Myeloid-derived suppressor cells.** The myeloid-derived suppressor cells (MDSCs) are pathologically activated monocytes or neutrophils with enhanced immunosuppressive features. They are classified into two main subsets based on their myeloid lineage: monocytic and granulocytic/polymorphonuclear MDSCs (M-MDSCs and PMN-MDSCs, respectively). Immune stimulation by pathogens, inflammation or cancer activates myeloid compartments, leading to sustained presence of myeloid-associated growth factors and inflammatory signals that trigger expansion and

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activation of MDSCs. During their development, myeloid cells expand and become conditioned in the spleen or BM and may subsequently be activated to differentiate into MDSCs in peripheral tissues (67). MDSCs are defined by their ability of inhibiting T and NK cell-mediated immune responses. Their immunosuppressive capacity largely stems from activation of the transcription factor STAT3. MDSC characteristically show upregulated ER stress along with enhanced expression of S100A8/A9, arginase 1 and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) enzyme of myeloid cells, NOX2 (68).

The M- and PMN-MDSCs have been suggested to confer immunosuppression by partly different mechanisms. Hence, suppression by PMN-MDSCs has been suggested to depend mainly on production of NOX2-derived reactive oxygen species (ROS), prostaglandin E<sub>2</sub>, arginase 1 and peroxynitrite, while M-MDSCs may be more dependent on production of nitric oxide, suppressive cytokines and expression of regulatory molecules such as PD-L1 (69). In cancer, the frequency of MDSCs is often elevated, both systemically and in the TME and premetastatic niches. MDSC-elicited immunosuppression may thus pave the way for escape of tumor cells to form metastases (70, 71). Recent studies imply that MDSCs may be aberrantly induced by anti-tumoral NK cells at tumor sites, thus suggesting a mechanism that drives activation of MDSCs to further allow tumor cells to escape immunity (72).

**4.3.4 Antigen-presenting cells: dendritic cells.** DCs are phagocytic immune cells with specialized properties of processing and presenting antigen on its surface that is detectable by other cell types to perform specialized functions. DCs are essential in adaptive immune responses and for the efficacy of therapeutics aiming at training the immune system against antigens to produce a combinatory innate and adaptive immune response.

DCs in humans and mice are broadly classified into classical DCs (cDCs) and plasmacytoid DCs (pDCs). These cells are formed from a common progenitor found in the BM called macrophage DC progenitor that can differentiate towards both macrophages and DCs. The cDC lineages are further subdivided into cDC1s and cDC2s (73). Genetic profiling of cDC1s shows their developmental associations with genes such as IRF8, Id2, Bcl6, Nfil3 and Batf3 (74-78). cDC2s comprise of two functionally different subsets represented transcriptionally by the genetic expression of Notch2 and KLF4 (79, 80). cDC1s can be genetically distinguished from cDC2s by the relatively higher expression of IRF4 and lower expression of IRF8/IRF8 (81). Transcription factor E2-2 is responsible for the development of pDCs (82).

cDC1s play a role in a mechanism denoted cross-presentation of antigens on major histocompatibility complex (MHC) class I to CD8<sup>+</sup> T cells, which is crucial for T cell-mediated antitumor and antiviral effects (83). cDC1s are primarily known to stimulate antigen-specific CD8<sup>+</sup> T cells and the Th1 subtype of T helper cells owing to the IL-12 expression observed in cDC1s (84). cDC2s are involved in control of infections, partly via their production of IL-23 in a Notch-2-dependent manner (79). Impaired development of cDC2s has been related to reduced priming of Th17 cells (81, 85, 86).

pDCs constitute a unique DC phenotype involved in the production of type I interferons (IFN-I), which are highly potent antiviral and immunomodulatory glycoproteins. Induction of this cell type has been observed in viral infections (87, 88) but also in autoimmune diseases and inflammatory conditions (89). pDCs may develop from lymphoid or myeloid precursors in the BM. Stimulation of TLR-7 and TLR-9 in the endosomes of pDCs results in secretion of high levels of IFN-I by these cells in response to ribonucleic acid (RNA) and DNA, respectively. While pDCs can present antigens to T cells,

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they are considered less efficient than cDCs. They do however promote Th1 differentiation by providing type IFN-I (88). The surface markers that define the phenotype of pDCs in humans are CD303 and CD304 along with ILT7 (90). Surface markers defining mouse pDCs are CD11c, Ly6C, Siglec-H, B220 and BST2 (91).

Another specialized DC subset is follicular DCs (FDCs) that support in the development of germinal centers (GC) and the formation of high affinity antibodies (92, 93). FDCs originate from stromal cell precursors and migrate to B cells follicles in secondary lymphoid tissues, where they interact with B cells (94, 95). FDCs produce CXCL13 that attracts CXCR5<sup>+</sup> B cells and T follicular helper cells to the follicles (96). They also promote B cell survival by production of B cell-activating factor (97, 98). One of the unique properties of FDCs is their capacity to retain immune complexes and antigens over extended periods, aiding in the formation of B cell memory (99). High expression of CR1 and CD21 on FDCs facilitates their capture and retention of immune complexes (93, 100).

## **4.4 Lymphoid cells**

**4.4.1 NK cells.** Natural killer (NK) cells are structurally large granular lymphocytes (101) with constitutive ('natural') cytotoxicity against foreign cells (102, 103). They form a part of innate immunity acting against infected and transformed cells. NK cells express germline-encoded activating and inhibitory receptors, which allows for their recognition of altered or stressed cells (104). Inhibitory receptors integrate signals from normal cells and prevent aberrant activation of NK cell killing.

When the balance is shifted away from inhibitory signals and towards stimulation of activating receptors, NK cells are instructed to kill. NK cells

mediate killing primarily by releasing granzyme and perforin, but also other effector mechanisms such as Fas ligand engagement may contribute. NK cells also produce IFN- $\gamma$  to activate other immune cells and can exert antibody-dependent cellular cytotoxicity (ADCC), recognizing and killing antibody-coated target cells. Therapeutic strategies involving NK cells are primarily established in cancer treatment, with ongoing research in other areas like diabetes and autoimmune diseases (105).

**4.4.2 B cells.** B cells lymphocytes within the adaptive immune system that produce antibodies that target pathogens or antigens. B cells have a unique B cell receptor (BCR) that is structured as a surface-bound antibody. Upon recognition of an antigen by the BCR, the antigen is taken up by the B cell and can be presented on MHC-II to T cells. If effector CD4<sup>+</sup> T cells recognize the specific antigen presented on MHC-II, they can provide T cell help and stimulate B cells to differentiate into antibody-producing plasma cells, or into memory B cells, which provide long-term immunity against that particular antigen (106, 107). Antibodies are part of the immunoglobulin (Ig) glycoprotein family and have a Y shaped structure with two heavy chains and two light chains, with variable regions at the tips responsible for antigen recognition and binding. Various subtypes of antibodies exist and the Fc fragments located at the base of the antibodies for certain subclasses can mediate interaction with Fc receptors expressed by multiple immune cell types (108, 109). Antibodies may act by neutralization, by providing opsonization and by activating ADCC (110).

**4.4.3 T cells.** Together with B cells, T cells serve as key players in adaptive immune responses. These lymphocytes develop in the thymus where they are subjected to positive and negative selection to ensure the evolution of functional T cells not prone to react to self-antigens to prevent autoimmunity.

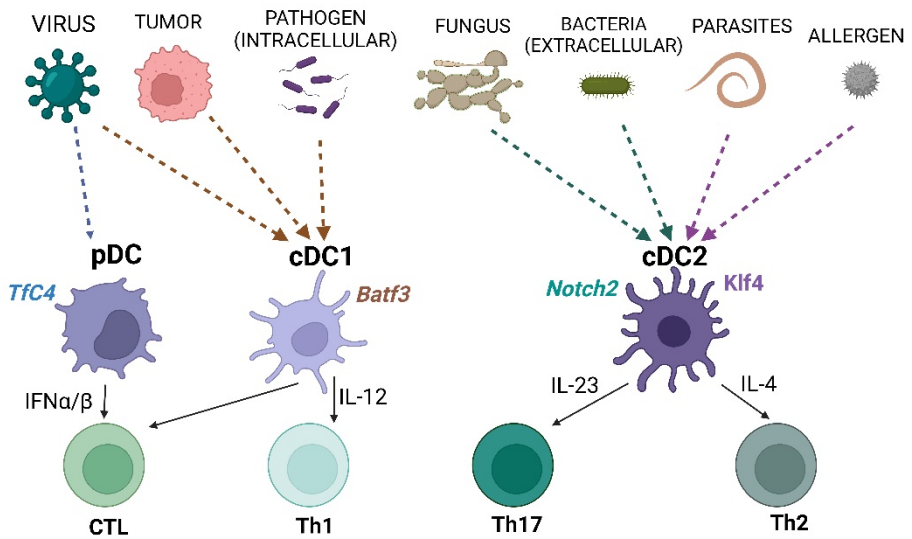
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T cells recognize peptide antigens presented in complex with MHC. Antigens are taken up by APCs and presented as a complex with MHC (or human leukocyte antigen (HLA)) class I or II which may be recognized by a T cell carrying a T cell receptor (TCR) specific for that MHC-antigen complex (111). TCRs are transmembrane receptors that are typically composed of an alpha and a beta chain with variable zones. These variations facilitate their recognition of different kinds of antigens (112, 113). Certain types of T cells carry specialized delta or gamma chains which facilitates recognition of specific types of antigen (114). The TCR recognizes peptide-MHC complexes. While CD3 transmits activation signals, CD8 and CD4 help with T cell activation by binding to MHC class I and II, respectively (112, 115).

T cells can be classified into CD8<sup>+</sup> cytotoxic T cells (CTL) and CD4<sup>+</sup> helper T cells. CD8<sup>+</sup>T cells are effector cells in the killing of cancer cells and virus-infected cells. The activation of naïve CD8<sup>+</sup> T cells is initiated when APCs such as DCs present a specific antigen on MHC I to T cells in a lymph node or in follicles, resulting in proliferation and differentiation of cytotoxic effector T cells. The activated CD8<sup>+</sup> T cells traffic into the circulation in search for cells exposing the specific antigen target on MHC I, which triggers the effector killing function of the T cell (112). CTLs secrete granzyme and perforin to kill target cells, and express FasL which can bind to the receptor Fas to stimulate caspases that eventually leads to apoptosis of target cells. Secretion of cytokines such as IFN- $\gamma$  by CTLs helps stimulate and activate other immune cells, thereby enhancing the overall immune response (116, 117) .

The other major subset of T cells is the T helper cells, also referred to as CD4<sup>+</sup> T cells due to their expression of the CD4 co-receptor. The TCR of CD4<sup>+</sup> T cells form complexes with antigens displayed on MHC II molecules of DCs instead of MHC I. When activated, helper T cells differentiate and polarize into

variants of cytokine-secreting effector T helper cells that modulate functions of other immune cells, including B cells to generate T cell-dependent antibody production (118, 119). Based on the signals that the CD4<sup>+</sup> T cells receive from the APC in form of co-stimulation and cytokine stimulation, the CD4<sup>+</sup> T cell polarize into different helper T cell subsets, such as Th1, Th2 and Th17 cells (120). Fig. 2 describes which subsets of APCs are responsible for priming a specific T cell subtype, via production of polarizing cytokines.



**Figure 2.** DC subtypes, activating stimuli and their influence on T cell priming

The Th1 cells produce IFN- $\gamma$  that stimulates antigen-presenting functions of APCs, the killing functions of macrophages and the production of IgG1 and IgG3 antibodies by B cells (120, 121). The IFN- $\gamma$ -induced stimulation of macrophages also generates a positive loop since it triggers IL-12 production by macrophages that enhances IFN- $\gamma$  production of the Th1 cells (122).

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Th2 cells are assumed to participate in defense against parasitic infections and in allergy. These cells produce IL-4, IL-5 and IL-13 which are involved in orchestrating recruitment of eosinophils and facilitating clearance of parasitic infections (123, 124). Th17 cells have an ascribed role in mucosal immune responses against extracellular invading pathogens such as bacteria and fungi. These cells produce IL-17 that is regarded as proinflammatory since it is involved in the recruitment of neutrophils. Th17 cells also produce IL-22 that protects and repairs epithelial tissues (125-127) .

T cells that are activated in the absence of co-stimulation, or thymic T cells that were selected based on weak recognition of self-antigens, may be polarized into a T cell phenotype with inhibitory effects on immune responses. These cells, regulatory or suppressor T cells (Tregs), secrete IL-10, transforming growth factor (TGF)- $\beta$  and IL-35 that dampen the activities of effector T cells and other immune cells. In addition, they express cytotoxic T lymphocyte-associated protein (CTLA)-4 that binds to B7 molecules on APCs and inhibit their ability to activate effector T cells. Tregs express the high affinity receptor for IL-2 and may thus consume IL-2 in the microenvironment to thereby limit access to this cytokine for effector T cells. Tregs are defined by their expression of the transcription factor FoxP3 and by low expression of CD127 along with high expression of CD25 and CTLA-4 (127, 128). Upon elimination of disease, most effector T cell populations die but a small population achieves a memory phenotype to be preserved for immune responses to recurrent infection (129).

#### **4.5 Antigen presentation**

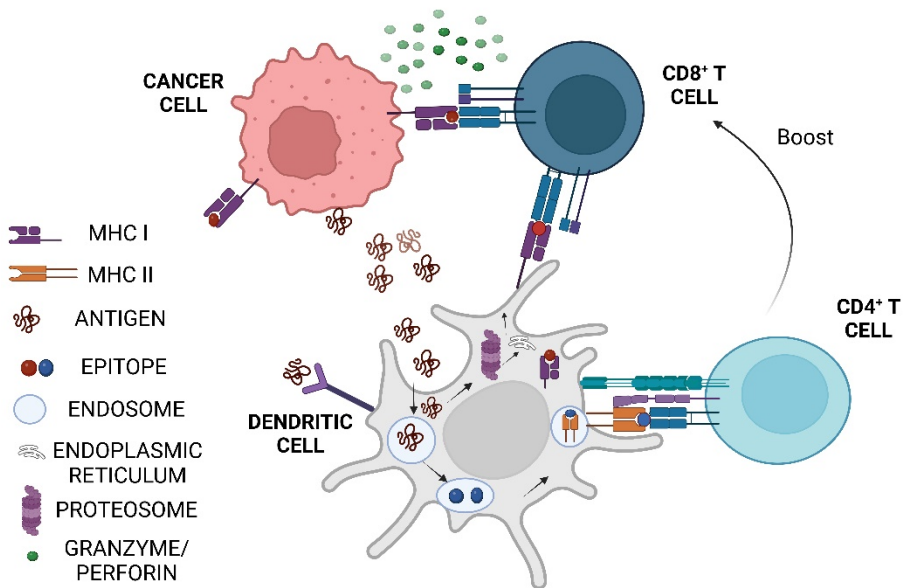
CD4<sup>+</sup> and CD8<sup>+</sup> T cells can only recognize their specific antigen when presented by an APC within a MHC molecule. This process is referred to as antigen presentation (130). MHCs are of two main types, MHC-I and MHC-

II, and are trans-membrane-based glycoproteins with the potential to bind to short peptides.

Expression of MHC-II is observed on multiple APCs including DCs, macrophages, thymic epithelial cells and B cells. Expression of MHC-II is induced by IFN- $\gamma$ . Antigens reach the endosomal or lysosomal compartments of APCs after processes such as endocytosis, phagocytosis, autophagy or micropinocytosis. Structurally, a MHC-II molecule consists of a heterodimer of  $\alpha$  and  $\beta$  transmembrane subunits. The proper transport of MHC-II molecules to antigen-presenting compartments depends on the invariant chain (I<sub>i</sub>), which guides their endocytic transportation through the Golgi apparatus (131, 132). It also prevents non-specific binding of peptides and proteins in the endoplasmic reticulum (ER) during the transport (133, 134). The signals facilitating this pathway include two dileucine motifs that are recognized by clathrin based adaptor proteins (135, 136). The invariant chain associated with the MHC-II complex is degraded by proteases (133), leaving a fragment, CLIP (class II-associated invariant chain peptides), that occupies the binding groove until antigenic peptides are loaded (137). An MHC II homolog, which in humans is known as HLA-DM and in mice denoted H2-M (138), facilitates the catalytic release of CLIP, allowing MHC-II to bind lysosomal peptides. HLA-DM also helps dissociate weakly bound peptides, enabling the selection of immunodominant peptide epitopes with high affinity (130, 139).

Structurally, MHC-I molecules comprise a glycosylated transmembrane heavy chain and a  $\beta_2$ -microglobulin ( $\beta_2m$ ) subunit. The heavy chain associates with  $\beta_2m$  dimer, which undergoes chaperone-mediated folding in the ER. Preparation for peptide binding occurs through assembly into the peptide loading complex (PLC). Priming of naive CD8<sup>+</sup> T cells often require presentation or cross-presentation, where exogenous antigens are presented on

MHC-I molecules. Proteins in the cytosol undergo proteasomal degradation into peptides and are transported into the ER by the ATP-dependent transporter associated with antigen processing (TAP), where they undergo further processing by aminopeptidases. ER aminopeptidases, such as ERAAP1 (ERAAP in mouse) and ERAAP2, trim peptides to 8-10 amino acid sequences, promoting their binding to MHC-I molecules (140). Peptide binding to MHC occurs within PLC (130, 141). An overview of the processing of antigens to be presented in the context of MHC-I and MHC-II is shown in Fig. 3.



**Figure 3.** Antigen processing and presentation on MHC class I and class II in the context of cancer

## 4.6 Tumor antigens recognized by T cells

**4.6.1 Tumor-specific antigens.** Tumor-specific antigens (TSAs) are derived from non-synonymous somatic or virus-induced mutations in cancer cells and are thus not present in healthy cells. Necrosis of tumor cells results in the release of TSAs. DCs may engulf, process and present the TSAs on MHC II to CD4<sup>+</sup> T cells or cross-present them on MHC I to CD8<sup>+</sup> T cells with resulting activation of anti-neoplastic T cells. The binding affinities of TSAs to MHCs are often higher than other forms of antigens (142). In addition, TSAs are often potent at eliciting strong immune responses, since T cells and B cells responding to these antigens are not negatively selected for during development. The absence of these antigens in healthy cells also reduces the risk of natural Tregs being present that otherwise may favor immune tolerance. TSAs are thus conceivable antigens for cancer vaccine formulations (143).

**4.6.2 Tumor-associated antigens.** Tumor-associated antigens (TAAs) are antigens that are expressed at elevated levels in cancer cells compared with healthy cells. Genetic amplifications and post-translationally acquired modifications contribute to the abnormal expression of TAAs in malignant cells (142). TAAs are broadly categorized into three types: overexpressed antigens, differentiation antigens and cancer/testis antigens. Overexpressed antigens are typically self-proteins that exhibit moderate expression in normal tissues but are significantly upregulated in cancer cells. Differentiation antigens are selectively expressed by specific cell types and the malignant cells from which they arise. Cancer/testis antigens are typically expressed only in the testis and various cancer tissues, making them potential targets for cancer vaccines due to their limited expression in normal cells (143).

TAAs may not be optimal therapeutic targets, as responsive T cells against certain TAAs may be lacking due to negative selection in the thymus.

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Alternatively, the presentation of TAAs may induce immune tolerance or autoimmune reactions (144). However, the use of immunostimulatory adjuvants may harness an immunogenic potential of certain TAAs while mitigating immune evasion.

#### **4.7 Melanoma antigens**

Melanoma arises from mutated melanocytes primarily located in the epidermis. Although melanoma accounts for only about 5% of skin cancers, it is responsible for majority of skin cancer-related mortality (145). While diagnosis and subsequent surgery at early and primary stages is often curative, patients at advanced and metastatic stages have reduced prospects for long-term survival (146). The introduction of checkpoint inhibitors has revolutionized the treatment of metastatic melanoma, providing cure for a significant fraction of patients (147). However, many patients either do not respond to available treatments, including checkpoint inhibitors, or develop resistance (148). Therefore, there is a critical need for treatments that improve response rates or resensitize patients to checkpoint inhibitors. Like other tumor antigens, melanoma-targetable antigens can arise from protein overexpression, mutations or genetic alterations, including gene fusions. Mutations and other genetic alterations may give rise to patient-specific neoantigens (149)). Additionally, TAA differentiation antigens that are normally expressed during the differentiation of melanocytes, are often overexpressed in melanoma cells including gp100 (150), tyrosinase-related proteins (TRP) 1 and 2 (151, 152) and melan-A (153). Studies have shown that tolerance to TRP2 can be overcome, and that vaccination or pulsing of APCs with this peptide allows CTL-dependent tumor clearance in cancer models, positioning TRP2 as a candidate for TAA-based cancer vaccines (154-156). Also, the TAA testis

antigens melanoma antigen gene (MAGE) family antigens and NY-ESO-1 are immunogenic proteins that are frequently overexpressed in melanoma (157).

#### **4.8 Breast cancer antigens**

Breast cancer is the leading cause of cancer-related mortality in women worldwide with more than 2 million cases/year. Although mortality in breast cancer has dropped significantly in the past decades, there were 680,000 deaths from breast cancer in 2020 (158). For early-stage cancer, surgery in combination with radiotherapy is the standard therapeutic option, followed by adjuvant systemic treatment if applicable. In case of metastatic stage of the disease, prolongation of survival and maintenance of quality of life remain the main goals (159). Previously described TAAs that are upregulated in melanoma are upregulated also among breast cancer cells (160). In addition, the basic helix-loop-helix TWIST-family of transcription factors are upregulated in multiple metastatic cancers, including breast cancer (161-163). The physiological function of the TWIST family of transcription factors is to act as embryonic regulators, but they also drive EMT alongside the SNAI-family of transcription factors that are also overexpressed in breast cancer (164-166)

#### **4.9 Model antigen – ovalbumin**

Ovalbumin (OVA) is a protein derived from chicken egg white, and thus is not expressed in non-transgenic mice (167). Therefore, tolerance mechanisms are not in play for OVA, which has been used as a model antigen in multiple research applications, including immunotherapeutic vaccines as it provides a convenient model to achieve strong immune stimulation (168). In this thesis, murine melanoma cells expressing OVA were employed to achieve immune-mediated clearance of melanoma cells *in vivo*, and OVA epitopes were

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generated in studies of the anti-tumor and immunostimulatory efficiency of melanoma vaccines.

## **5. REDOX REACTIONS IN TUMORS**

The TME is a complex and dynamic milieu composed of various cell types, ECM components, and soluble factors that collectively influence tumor progression, immune response, and therapeutic outcomes. Many cancers exhibit elevated levels of ROS (169), which are generated as byproducts of cellular metabolism and by inflammatory tumor-infiltrating myeloid cells. While excessive levels of ROS can damage cellular components, they also play a crucial role in modulating signaling pathways by altering redox-sensitive transcription factors and signaling proteins (170).

Metabolic reactions within mitochondria or peroxisomes serve as sources of ROS, which can modulate proliferation, apoptosis and metabolism (171-173). Additionally, members of the NOX family generate ROS by transferring electrons across membranes, catalyzing the conversion of oxygen to superoxide or hydrogen peroxide ( $H_2O_2$ ). Superoxide dismutases facilitate the reduction of superoxide to form  $H_2O_2$  (174).

ROS impacts various cancer-related processes, including proliferation, inflammatory responses, DNA integrity, metabolic reprogramming and apoptosis, all of which may influence tumor development and spread (169). ROS promote tumor progression through the suppression of adjacent immune cells (175). By producing ROS, MDSCs and TAMs may contribute to the immunosuppression in the tumor microenvironment. NOX2-derived ROS are thus potent inhibitors of NK cell function (176) and MDSC-derived ROS

inhibit TCR recognition of MHC-peptide complexes, resulting in impaired activation and proliferation of T cells (177-180). Additionally, the damaging effects of ROS on DNA, along with the upregulation of certain metabolic pathways, have been associated with metastasis in multiple cancer forms (181, 182).

All cells have defense systems to cope with elevated levels of ROS. Hence, upon oxidative stress, transcription factors such as NRF2 become activated (183). This transcription factor, which is a key regulator of the cellular antioxidative response, induces expression of an array of antioxidative enzymes that help protect cells, including tumor cells, from oxidative stress damage (184). Exposure of cancer cells to oxidative stress may thus lead to activation of NRF2 that may stimulate pathways that enhance cellular survival and resistance to oxidative cells, along with metabolic reprogramming, which may lead to tumor progression and metastasis (185). However, in some cases elevated levels of ROS in tumor cells have instead been associated with tumor cell death, especially in conjunction with ROS-inducing chemotherapy (186, 187). The NRF2 pathway in immune cells may, however, be activated in lymphocytes to maintain immune-mediated killing of tumor cells in adoptive cell therapy, including the transfer of engineered NK cells or T cells (188).

The NOX family of enzymes (NOX 1-5), responsible for catalyzing superoxide and H<sub>2</sub>O<sub>2</sub> formation, are differently expressed within cell types. Myeloid cells, including monocytes, granulocytes and macrophages, express high levels of NOX2 (189). The NOX2 complex consists of the membrane-bound units gp91<sup>phox</sup>, also known as CYBB, and p22<sup>phox</sup> (CYBA). It also contains several cytosolic subunits including 47<sup>phox</sup> (NCF1), p67<sup>phox</sup> (NCF2), and p40<sup>phox</sup> (NCF4) (190, 191). The activation of NOX2 is associated with the translocation of cytosolic subunits to the membrane in response to DAMPs,

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PAMPs, microbial peptides, cytokines and growth factors, where NOX2-derived ROS play a critical role in pathogen elimination (192). In DCs, ROS generated within phagolysosomes leads to alkalization by proton consumption, aiding in preservation of peptides presented to T cells (193-195).

## **6. IMMUNE TARGETING THERAPIES IN CANCER**

**6.1 Monoclonal antibodies.** Monoclonal antibodies have emerged as cancer therapy due to their ability to specifically target antigens expressed by cancer cells, growth factor receptors and immune check points. Rituximab is an engineered monoclonal antibody that targets CD20 and is used to eradicate B cells in non-Hodgkin's lymphoma and chronic lymphocytic leukemia (196). Another FDA-approved monoclonal antibody is trastuzumab that targets human epithelial growth factor receptor 2 (HER2) receptors. It is used in HER2-positive breast cancer, where it blocks signaling via this growth factor receptor (197). A third class of FDA-approved monoclonal antibodies are the immune check inhibitors (ICI), described in the next section.

**6.2 Immune checkpoint inhibitors.** ICIs represent a breakthrough in cancer therapy (198). ICIs may exert significant anti-tumor efficacy as monotherapy, including the complete and durable disappearance of metastatic tumors (199). In a more recent development, combination strategies employing ICIs alongside, *e.g.*, chemotherapy have markedly prolonged the survival of patients with several types of cancer with established metastasis (200, 201). The principal mechanism of ICI is the targeting of co-inhibitory receptors on the surface of T cells and/or cancer cells that negatively regulate immune reactions. This pathway of immunosuppression may be utilized by cancer and stroma cells to promote inactivation of cancer cell-reactive T cells.

Programmed cell death 1 (PD-1), programmed cell death ligand 1 (PD-L1) and cytotoxic T lymphocyte antigen 4 (CTLA-4) are three such receptors (202). Monoclonal antibodies targeting immune checkpoints typically bind and block co-inhibitory receptors, thereby restoring immune functionality (203). FDA-approved anti-PD1 based treatments for cancer include nivolumab, pembrolizumab and cemiplimab. FDA approved PD-L1-inhibitors include atezolimumab, durvalumab and avelumab (204). Examples of clinical indications of ICI therapy include non-small cell lung cancer (NSCLC) (205), metastatic melanoma (206), and metastatic cutaneous squamous cell carcinoma (207), but its efficacy is currently explored in many other cancer forms. With complete response rates ranging from 20-40% of treated patients (208), the success of these therapies depends on factors such as mutational burden of cancer cells, the TME including its infiltration by myeloid cells and T cells, ECM composition and expression levels of co-inhibitory receptors on T cells and tumor cells (203).

**6.3 T cell transfer therapy.** In T cell transfer therapy, also referred to as adoptive T cell therapy, patient T cells are amplified to improve cancer cell recognition and elimination. These therapies comprise two main modalities: chimeric antigen receptor (CAR) T cell therapy and tumor-infiltrating lymphocyte (TIL) therapy. In CAR T cell therapy, T cells isolated from the patient's peripheral blood are genetically engineered to express CARs that target specific tumor antigens. The currently approved indications for this therapy include B cell lymphoma and lymphoblastic leukemia (209). However, CAR-T cell therapy has not yet proven as successful in the treatment of solid tumors (210).

In TIL therapy, lymphocytes that naturally infiltrate the tumor are isolated, expanded and infused back into the patient in combination with the T cell-

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activating cytokine IL-2. Non-myeloablative lymphodepletion, to reduce the patient's existing immune cells, often precedes this therapy (211, 212).

**6.4 Oncolytic viruses.** Oncolytic viruses (OVs) have a natural or engineered tropism for selectively replicating in neoplastic cells, leading to the increased expression of pathogen and DAMPs. At the end of the replication process, the infected tumor cells die through oncolysis, resulting in the release of TAAs. In addition to eradicating the infected tumor cells, OVs may thus also provoke anti-neoplastic immune responses (213, 214). Many OVs are genetically engineered to carry immunomodulatory transgenes that induce the expression of immune-activating proteins within the TME, creating an immunoreactive (“hot”) TME. Examples include OVs designed to deliver granulocyte-macrophage colony-stimulating factor (GM-CSF) (215) or IL-2 and IL-12 to enhance immune infiltration into tumors (216). Talimogene laherparepvec (T-VEC, Imlygic) is an FDA-approved oncolytic herpesvirus that is indicated for the treatment of advanced melanoma (215). Combination strategies involving OVs and ICIs are currently being explored (217).

**6.5 Cancer vaccines.** Vaccines are routinely used in training the immune system to generate responses against infectious agents. In cancer immunotherapy their impact has thus far been less significant and continued research is essential to mitigate the current challenges. With the advent of immune checkpoint blockade there has been a shift in focus, but certain characteristics of cancer vaccines could make them an attractive therapeutic option in the future. For example, while modalities such as CAR-T cell therapy are effective at targeting known tumor antigens on the cell surface, cancer vaccines may offer a superior alternative by targeting a broader range of antigens, including those expressed intracellularly. Checkpoint blockade is assumed to be efficacious in tumors with an immunoreactive

microenvironment rich in T cells, whereas cancer vaccines can prime new T cells via APCs (218). The next section will focus on state-of-the-art advancements and challenges of research in this area.

## **7. CANCER VACCINES**

The development of cancer vaccines to elicit anti-neoplastic immunity has been in focus for the past 30 years (219). Despite extensive efforts, most cancer vaccine attempts have failed to generate measurable responses in larger clinical trials. This may be due to difficulties in inducing immunity against relevant cancer antigens, as well as the extensive immunosuppression observed in many patients. The successful introduction of immunotherapies targeting immunosuppression has resulted in renewed interest in exploring if cancer vaccines can promote anti-neoplastic immunity in combination with other immunotherapies, including those targeting immunosuppression. Additionally, there is room for innovative approaches of targeting specific compartments of the immune system to gain improved vaccine efficacy. Advances in the areas of improved antigen selection, development of effective vaccine formulations and effective combination therapies have modernized and rejuvenated the field.

The concept of a cancer vaccine is to train the immune system to identify and eliminate malignant cells, or viruses that cause cancer. Vaccines can either be administered as preventive or therapeutic treatment. The preventive vaccine approach is routinely used for vaccines designed to protect from virus infections with potential to transform normal cells into malignant cells. This concept relies on the vaccine generating an immunological memory that can be readily revoked upon encountering the virus, thereby providing protection.

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The various compartments of the immune system involved in vaccine-induced training include the APCs, helper and cytotoxic T cells, NK cells and tumor-resident myeloid cells. Spatiotemporal factors involved in the interaction of these compartments along with the local and systemic nature of immune suppression in cancer are major obstacles to the success of cancer vaccines (220, 221). However, even unsuccessful cancer vaccines have contributed to the understanding of antigen immunogenicity, preservation of trained cell populations and role of combination therapies.

Most therapeutic cancer vaccines that are currently in clinical development consist of a formulation of tumor antigens administered in combination with immunomodulatory factors, such as adjuvants or other costimulatory agents. Broadly, cancer vaccines can be divided into six major categories: tumor cell vaccines, DC vaccines, DNA and RNA vaccines, viral vaccines and peptide vaccines (219).

**7.1 Tumor cell vaccines.** One of the earliest therapeutic cancer vaccines, developed in early 1990s, utilized irradiated tumor cells to provide a source of multiple immunogenic antigens. These cells were irradiated to render them non-replicating, while preserving their ability to stimulate an immune response. The tumor cells may be genetically modified to produce GM-CSF, which enhances the recruitment and maturation of APCs, including DCs, to the vaccine site (222). Mature, antigen-bearing DCs then migrate to the lymph nodes, where they present antigens and evoke a T cell response (223, 224). This vaccine, GVAX, used both autologous and allogeneic cells in the development of personalized and off-the shelf vaccines. Although GVAX demonstrated promising clinical outcomes in early trials, the phase 3 VITAL-1 and VITAL-2 trial were discontinued in 2008 due to lack of efficacy in improving overall survival (225). Nevertheless, the therapy is being explored

in phase 1 and 2 trials in combination with ICI or chemotherapy (226).

**7.2 Dendritic cell vaccines.** While tumor cell-based vaccines depend on endogenous APCs to present antigens to T cells and initiate an immune response following vaccination, DC vaccines utilize differentiated *ex vivo* matured DCs that are reinfused to patients to initiate anti-tumor responses (227-229). In a typical DC vaccination scheme, monocytes are harvested from patients using leukapheresis and are differentiated *ex vivo* in the presence of cytokines, such as GM-CSF, to generate monocyte-derived DCs. These cells are then pulsed with tumor antigens and activated using, for example, cytokines and TLR agonists (230). Antigen-loaded and activated cells are reinfused to patients by either subcutaneous (s.c.) or intradermal (i.d.) routes. This approach of generating DC vaccines is, however, expensive and logistically challenging (231), and the success rate of DC vaccines in terms of objective reduction of tumor mass in clinical trials has rarely exceeded 15% (232). In addition to multiple unsuccessful phase 3 trials, however, Sipuleucel-T (Provenge) demonstrated sufficient efficiency in advanced prostate cancer to attain regulatory approval.

Currently, there are 108 listed clinical trials for DC or APC vaccines, out of which the vast majority (103) utilize DC as the APC. Almost one fourth of these trials are aimed at treating brain tumors with emphasis on glioblastoma (GBM). Among these trials, only 4% are in phase III. A DC-based vaccine is being investigated in a phase II trial to assess its effectiveness in preventing disease recurrence in patients with acute myeloid leukemia who are in remission after chemotherapy.

Multiple DC vaccines have succeeded in eliciting anti-tumoral T cell response but have failed to translate into a measurable or relevant clinical improvement.

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While the efficacy of DC vaccines is still being investigated in clinical trials, basic research is also ongoing aiming at understanding factors limiting the success of these vaccines. Factors affecting clinical translatability include inability to target or expand the highly potent cDC1s in an *ex vivo* setting, lack of stable antigen presentation, exhaustion or tolerance of tumor antigen-specific T cells, lack of sufficiently primed Th1-specific CD4<sup>+</sup> T cells and inability to activate T cell function in a suppressive TME (233, 234). While obtaining and expanding monocyte derived DCs (moDCs) is easier than cDC1, these cells do not possess adequate cross-presentation capacity to prime CD8<sup>+</sup> T cells (83, 235). Current research involves optimization of antigen presentation, increased focus on inclusion of CD4 epitopes in vaccine formulations, combinations with ICIs and culture and preservation of specialized DCs. In addition, advances in gene sequencing and the emergence of algorithms in the identification of immunodominant neoantigens have led to loading of DCs with patient-specific neoantigens via messenger RNA (mRNA) transfection (230, 236-238). Approximately one tenth of clinical trials with DC vaccines utilized neoantigen peptides. The efficiency of combination therapies has motivated 75% of current DC vaccine trials to be conducted as combination trials. Around 30% of the DC vaccines are combined with chemotherapy, most commonly temozolomide in cases of GBM. Some DC vaccines are used in combination with cytokines, such as GM-CSF, to enhance survival and function of the DC or co-administered with ICIs (219).

**7.3 Peptide-based vaccines.** Compared with the high manufacturing, production and logistical costs associated with DC vaccines, peptide vaccines are less expensive and have easier vaccination schemes. Peptides containing tumor antigens can be injected into the dermal or s.c. space to provide endogenous APCs with tumor antigens. Activation of the immune system with immunomodulating agents (adjuvants) is essential for the success of a peptide-

based vaccines as peptides alone have low immunogenicity. Adjuvants may cause maturation of APCs, often via stimulating PRR expressed by the cells. Additionally, peptide vaccines are often co-administered with cytokines such as GM-CSF that can promote the recruitment and maturation of DCs.

Early studies of peptide-based vaccines utilized short sequence peptides of 8-9 amino acids as MHC-I restricted minimal epitopes that could form peptide-MHC complexes to stimulate T cell activity without being processed by DCs. However, occasionally these short peptides could bind directly to MHC I even without uptake and cross-presentation. When this occurred and peptides were presented by non-APCs this could lead to T cell anergy (239). The field is thus moving towards synthetic long peptides which must be taken up and cross-presented by APCs.

Peptide vaccines are currently being evaluated in multiple phase III trials (240, 241). Out of various cancer vaccine trials about one third are peptide-vaccines for tumors in the brain, lung, breast and skin. Even though activation of APCs with adjuvants is of importance for the success of peptide-based vaccines, more than one third of these contained no reported adjuvant. In roughly half of the peptide-vaccine trials TAAs were utilized, out of which peptides from telomerase reverse transcriptase (hTERT) and HER2 were the most used antigens. In about 30% of the peptide-vaccine trials, patient-specific neoantigens were utilized. This development has become possible due to recent advances in sequencing of tumor material and availability of more accurate antigen-binding prediction algorithms and softwares (142, 242).

Only one peptide-vaccine has reported favorable phase III clinical trial results. This vaccine, Tedopi (OSE2101, OSE Immunotherapeutics), is composed of five TAAs and PADRE, a CD4 helper epitope. The vaccine was administered to patients with NSCLC who were previously non-responsive to ICI therapy.

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There was a significant increase in overall survival (hazard ratio 0.59,  $p=0.017$ ) in vaccinated patients, which has encouraged compassionate use in the European Union (243). The trial was disrupted during the COVID-19 pandemic and a confirmatory trial is awaited (219).

**7.4 DNA vaccines.** DNA vaccines are composed of plasmid DNA encoding tumor antigens. They are designed to target host cells, which take up the plasmid and express the encoded antigens. The administration of foreign genetic material in this manner can induce an immune response via activation of DNA PRR. However, while DNA vaccines can be inherently immunogenic, adjuvants are often administered in conjunction with DNA vaccines in clinical trials to enhance the immune response (244). Some plasmids are engineered to express not only tumor antigens, but also cytokines and chemokines, which can provide additional immune-stimulatory effects in the targeted microenvironment (244).

A major advantage of DNA vaccines is their stability, which simplifies storage and logistics, making them cost-effective compared to other types of vaccines. One challenge is the need for efficient intracellular delivery to facilitate DNA uptake and expression (245). Recent methods for delivery include intramuscular injection followed by electroporation. Electroporation transiently disrupts the lipid bilayer of the cell membrane, allowing the plasmid DNA to enter the cytosol. This technique has renewed interest in DNA vaccines as a clinically relevant therapy (246).

A recent review summarized ongoing trials with 20 DNA vaccines, in which one or multiple plasmids expressed combinations of antigens, adjuvants and/or cytokines. The DNA vaccines were administered via the intradermal or intramuscular routes, often followed by electroporation, which enhanced the uptake and immunogenicity 100 to 1000-fold (247). These vaccines targeted

breast cancer, anogenital cancer, prostate cancer and lung cancer. None of these trials have yet reached phase III. Most DNA vaccines were administered in combination with other therapies, such as ICI, chemotherapy or cytokines.

Notable vaccine candidates include WOKVAC, which encodes three TAAs (248, 249). Another example is INO-3112, which contains plasmids encoding the E6 and E7 antigens from human papilloma virus (HPV) types 16 and 18, alongside IL-12. This vaccine was co-administered with durvalumab (ICI) via intramuscular (i.m.) injection (250). A T cell response was observed in 60% of patients in a phase I trial (251).

**7.5 mRNA vaccines.** mRNA vaccines represent one of the most advanced techniques in the field of cancer vaccines by delivering antigens encoded by mRNA sequences. The tumor antigens are encoded in synthetic mRNA, which is often modified to enhance stability and translation efficiency. The mRNA may be encapsulated in lipid-nanoparticles (LNPs) to prevent degradation and to facilitate cellular uptake. The mRNA itself acts as an intrinsic adjuvant by stimulating PRR. Also, the lipid-based delivery vehicles may have adjuvant-like effects (252, 253). mRNA vaccines are typically delivered via i.m. or s.c. injection. The potential benefit of mRNA cancer vaccines is currently explored in multiple clinical trials (254, 255). This development is likely bolstered by the successful use of mRNA vaccines in SARS-CoV-19 infection.

Advancements in the development of algorithms and prediction software to identify immunogenic neoantigen epitopes, along with optimization of delivery systems, have resulted in renewed clinical interest in these vaccines (142, 256). Many mRNA vaccines utilize neoantigens while others focus on TAAs. Several phase II trials using RNA vaccines have been conducted for melanoma (255, 257) and head and neck squamous cell carcinoma (258).

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mRNA-4157 is a personalized cancer vaccine comprising up to 20 patient-specific neoantigen sequences encoded by mRNA encapsulated in LNPs. This vaccine, developed by Moderna, showed efficacy in improving recurrence free survival in melanoma (255) and other solid tumors (259) when co-administered with the ICI pembrolizumab. Another noteworthy mRNA vaccine encodes TAAs instead of neoantigens and is encapsulated in lipoflexes. The immunogenicity of this vaccine is further enhanced by addition of MHC I-trafficking domains and CD4-based epitopes (260, 261). These modifications aim to improve antigen translation, DC activation, MHC presentation. In a phase I trial, this vaccine elicited immune responses towards at least one antigen in 75% of patients (262).

**7.6 Viral vaccines.** Viral vaccines may involve the use of replication-incompetent virus or the delivery of genetic material resulting in antigen expression. These strategies can also be immunogenic, acting as natural adjuvants (263). To avoid neutralization of virus due to humoral responses, heterologous techniques are used which employ different viruses for priming and boosting immunity (264). While several studies are ongoing, the results of larger trials in cancer are still awaited (219).

## **8. ADJUVANTS**

Adjuvants aim at increasing vaccine immunogenicity when delivered alongside antigens. Molecules that serve as adjuvants includes natural extracts, particulates and small synthetic molecular compounds (265). Their mechanism of action was insufficiently understood until concepts emerged linking innate immune control to adaptive responses (266). Adjuvants may stimulate adaptive immune responses by activating innate immune cells via PRR signaling

pathways (267). Synthetically produced molecules and delivery particles aim to mimic the dimensional and spatial structure of pathogens to produce adjuvant-like effects (268).

Adjuvants typically generate co-stimulatory signals and enhance antigen-presentation by APCs. The co-stimulatory signals include upregulated expression of CD40, CD80 and CD86 on the surface of APCs along with production of inflammatory cytokines such as IL-6, IL-10, IL-12 and TNF- $\alpha$ . These factors are intended to enhance the ability of naïve T cells to engage in adaptive responses (269, 270). Based on the mechanism stimulated – either enhancing antigen presentation or enhancing co-stimulation – adjuvants are broadly categorized as antigen delivery systems or immunostimulants.

Immunostimulants act as danger signals that bind to PRRs on APCs, which leads to their maturation and render them in an activated state. Hence, the matured APCs have enhanced capacity to trigger adaptive immune responses against co-administrated antigens (271). Different types of stimulants may bind to different PRRs on APCs that results in a variable pattern of costimulatory signaling (272-274). Stimulation of PRRs can also result in enhanced recruitment of immune cells to the site of vaccination (275).

Delivery systems are molecules that encapsulate or interact with antigens for enhanced or targeted delivery to APCs (276). These delivery systems may also extend the duration for which the antigen is available in the microenvironment of the APCs by slow release, creation of immune niches and better preservation of the antigen within the cargo (277). Additionally, nano-emulsion molecules such as MF59, AS03 or hydrogels may promote sustained antigen release (274, 278).

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The slow release of antigens may also lead to production of higher affinity antibodies, as antigens presented with longer duration allows for B cells to conduct multiple rounds of affinity selection (279). Changing the composition or structure of the adjuvant may allow for a more sustained release of antigens. The size of the adjuvant and spatial structure also plays a crucial role, since the immune system is constructed to identify small particle antigens with repeated structural patterns that resemble the size and structure of pathogens. A repetitive pattern is also considered favorable for the aggregation of B cell receptors which can result in antibody production even without T cell help (280-282). Delivery systems may additionally provide a protective layer limiting antigen degradation. For example, naked mRNA molecules would be degraded by enzymes present in the serum and thus delivery via encapsulation in LNPs may be advantageous (283-285).

**8.1 Mechanisms of action of adjuvants.** The typical mechanism of action for adjuvants is to stimulate APCs via PRRs. In addition, targeting of receptors on DCs, such as CL (C-type lectin) or Fc receptors, may enhance uptake of antigens. The fusion of antigens to the CL receptor DEC-205-specific antibodies was found to enhance antigen presentation by DCs (286).

Adjuvants can improve antigen delivery to lymph nodes, which are the initial sites of activation of naïve immune cells. There are two broad mechanisms by which antigens enter the lymph nodes. Antigens encapsulated within delivery molecules may thus penetrate the gaps of the interstitial matrix of the afferent lymphatic system by passive diffusion, provided they have appropriate dimensions (5 to 100nm) and have surface attributes like hydrophobicity and net negative charge. This allows them to reach the lymph nodes (287). Transport and accumulation in the lymphatic system is also facilitated by hydrophilicity of the molecules (288).

Another mechanism, denoted albumin hitchhiking, transports vaccine antigens to the lymph node to trigger a 30-fold increase in T cell activation. In this process, antigens or other molecules are designed to bind to circulating endogenous albumin, which aids in transport to the lymph nodes (289-291).

Many cancer vaccine antigens need to be presented on MHC I molecules by APCs for CD8<sup>+</sup> T cells for efficacy. Exogenous antigens that are internalized by APCs into endosomes or lysosomes are typically presented on MHC II molecules to CD4<sup>+</sup> helper T cells. For the antigen to be cross-presented on MHC I, some of the antigenic peptides need to be transported to the cytosol, from where they can be further transported into ER to be loaded on MHC I (292, 293). Certain adjuvants may enhance cross-presentation by proton adoption within the acidic endosomal or lysosomal vesicles. This mechanism leads to inflow of chloride ions and fluids from the cytoplasm that can disrupt the endosome or lysosome and release antigens into the cytoplasm (294, 295).

Adjuvant molecules may also bind to membranes of endosomes or lysosomes to weaken them and thereby enhance antigen release into the cytoplasm (296). Incorporation of photosensitizing components in adjuvants is an emerging technology, which allows damage to endosomal membranes by light exposure. A study by Ji *et al.* demonstrated that this mechanism led to improved CD8<sup>+</sup> T cell-dependent immune reactivity (274, 297, 298).

**8.2 Adjuvants in cancer research.** Montanide ISA 720 and ISA-51 are water-in-oil based emulsions that serve as adjuvants due to their ability to trap soluble antigens in a depot at the site of injection, facilitating their slow release. These adjuvants are reported to enhance activation of CD8<sup>+</sup> and CD4<sup>+</sup> T cells when co-administered with long peptide epitopes of the oncoproteins E6 and E7 (299).

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TLR agonists are category of adjuvants that enhance vaccine efficacy through PRRs stimulation of innate immune cells (300). TLR agonists that are evaluated in clinical trials include polylysine, carboxymethylcellulose (Poly-ICLC, TLR3 agonist) (301), CpG oligodeoxynucleotide (CpG ODN, TLR9 agonist) (302), monophosphoryl lipid A (MPLA, TLR4 agonist) (303), imiquimod (TLR7 agonist) (304) and resiquimod (TLR7, TLR8 agonist)(305, 306).

Multiple emerging adjuvants have shown efficacy in preclinical models, but their clinical benefit is yet to be established. A combination of CD40 agonists and TLR agonists is a conceivable strategy, where CD40 agonists enhance antigen presentation and the TLR agonists enhance expression of co-stimulatory molecules, including CD40 (307, 308). DNA vaccines can directly activate stimulator of interferon gene protein (STING), a transmembrane protein found in the ER, that mediates strong IFN-I induction which enhances immunogenicity (309, 310). Unspecific targeting and toxicity are challenges that need to be overcome for several adjuvants (311). Immunostimulatory cytokines such as GM-CSF aid in DC maturation and antigen presentation and have shown efficacy in preclinical models, and immunostimulatory cytokines are included in several ongoing cancer vaccine trials (302, 306, 312, 313).

**8.3 CTA1.** The cholera-inducing enterotoxin from *Vibrio cholerae*, cholera toxin (CT), possesses strong immunomodulatory effects after binding to GM1 ganglioside receptors present in multiple cell types. Thus, components of CT have been evaluated as vaccines (against *Vibrio cholera*) or as mucosal adjuvants (against co-administrated infectious antigens) (314, 315). The potential impact of CT derivatives as cancer vaccine adjuvants is, however, less studied.

CT is composed of a pentamer of B subunits, which is responsible for GM1 binding and uptake, and a noncovalently linked toxic A subunit. The A1 subunit of CT is an ADP-ribosyl transferase that modifies the G protein (Gs) alpha subunit, locking it in its active form. This results in a continuous activation of adenylate cyclase and elevated levels of cyclic adenosine monophosphate (cAMP). High intracellular cAMP levels disrupt normal ion transport systems, resulting in a massive efflux of chloride ions and water from cells, causing the predominant symptoms of cholera (316, 317).

Additionally, CT is endowed with immunomodulatory properties, likely mediated by activation of APCs that also express GM1. Hence, CT activates the expression of costimulatory molecules and cytokines by APCs, which appears to be attributed to the ADP-ribosylating effect of CTA1 (318).

While the toxicity of CT precludes its *per se* usage as an adjuvant in human vaccines, CT derivatives with retained immunostimulatory effects but reduced toxicity, have been developed and show promising results. Lycke *et al.* developed such an adjuvant that is composed of the enzymatically active CTA1 subunit fused to a dimer from the D-domain of *Staphylococcus aureus* protein A (319). The efficiency and toxicity profile of this adjuvant, referred to as CTA1-DD, has been characterized in mice and primates (non-human) models, making it an adjuvant candidate for use in humans (319, 320).

CTA1-DD elicits strong T and B cell immune responses against co-administrated or conjugated antigens, both at the mucosa and systemically. The DD moiety of CTA1-DD promotes uptake by DCs (321, 322). Thereafter the ADP-ribosylating effect of the CTA1 subunit stimulates DC activation (322, 323), leading to enhanced T cell stimulation of presented antigens.

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In addition, CTA1-DD stimulates potent and long-lasting antibody responses against co-administrated antigens. This is thought to depend on the ability of the adjuvant to activate CD4<sup>+</sup> T cells, along with enhancing GC reactions. This in turn gives rise to the generation of long living plasma cells and B cells of memory phenotype (324-326). CTA1-DD has also been shown GC to directly interact with follicular dendritic cells (FDCs) through binding and stimulation of the complement receptors CR1 and CR2 via the DD subunit. This in turn affects organization of B cell follicles and reactions in the GC (327-329). Soluble factors and receptor ligands expressed by FDCs provide proliferative and differentiation signals to B cells resulting in their strong activation along with the selection of a memory phenotype with high affinity and long-lived plasma cells (328, 330-333).

Studies have demonstrated that the CTA1-DD-adjuvanted vaccines elicit cell-mediated as well as humoral immune responses that may provide protection against pathogens including human immunodeficiency virus, rotavirus and influenza virus (334-337). In these studies, antigens were either admixed or genetically fused to CTA1-DD (338, 339).

In a recent study, a novel CT variant was generated where CTA1 was coupled to a CD103 single chain variable fragment (ScFv) antibody instead of DD. While the CTA1-DD construct was taken up by cDC1 as well as by cDC2, the CTA1-aCD103 adjuvant was preferentially taken up by CD103<sup>+</sup> cDC1. Targeting of CD103<sup>+</sup> APCs through the inclusion of the ScFv anti-CD103 antibody entailed pronounced upregulation of antigen-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells (322).

cDC1s are considered to promote the development of Th1 cells and to facilitate cross-presentation of antigens to CD8<sup>+</sup> T cells. In contrast, cDC2s primarily support the priming of Th2 and Th17/follicular helper T cells (T<sub>fh</sub>) subsets

(322, 340, 341). This distinction aligns with the responses triggered by cDC1 and cDC2, respectively, following stimulation with adjuvants such as CpG, LPS, poly I:C (342, 343). However, the functional impact of the CTA1-enzyme in targeting cDC1 is noteworthy. The ADP ribosylation properties of CTA1 have thus been shown to facilitate the differentiation and priming of antigen specific Th17 cells. This finding contrasts with the traditional function of cDC1s and may highlight unique properties of the CTA1-based adjuvant (322).

## **9. IMMUNOINFORMATICS IN VACCINE DESIGN**

Immunoinformatics is an emerging field in which antigen epitope-based cancer vaccines are designed, aiming at enhancing immunogenicity and ensuring safer products (344). The general workflow of vaccine design involves the recognition of cancer-based antigens, investigation of protein and peptide composition and structural motifs, prediction and characterization of immunodominant T cell epitopes along with assessment of affinity scores defining interactive potential of the epitope. This computational methodology has the potential to map numerous biological entities *in silico*, thereby significantly reducing the time and cost associated with the vaccine development workflow, often referred to as ‘needle-to-needle’.

Whole exome sequencing (WES) of healthy DNA and matched tumor sample can aid in identification of peptides that possess specific non-synonymous mutations (345). Many algorithms allow for the screening of a very broad range of antigens to help in the selection of an antigen pool (346, 347). These programs also include affinity prediction for binding affinities (BA) between peptides and HLA molecules, affinity ratios between wild-type and mutated and peptide-HLA complex stability scores (347). RNA of whole-genome

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sequencing (WGS) is utilized to identify antigens from non-mutated genes, abnormally processed or translated RNA or genetic fusions (344).

Structural assessment of a protein is essential to understand the impact of the conformation on its interplay with immunity. Assessment of the physicochemical attributes of the protein such as molecular weight, stability, half-life and grand average of hydropathicity is done using the sequences of amino acids in the protein. Available bioinformatics tools have shown the potential to generate successful structural predictions with high immunogenicity (348). Some software utilizes auto cross covariance from the physicochemical properties to predict antigenicity (349).

T cell responses elicited by cancer vaccines depend on the TCR and HLA molecules. Whether a peptide can serve as a T cell epitope relies on multiple factors such as its length, structure and sequence. During prediction, a choice of epitope that provides higher coverage of HLA alleles allows its use in a higher proportion of patients. Prediction algorithms that follow the NNAlign\_MA model use data from BA and mass spectrometry of HLA-eluted ligands to make epitope predictions. Some commonly used programs that operate using this model are NetMHCpan and NetMHCIIpan (350). Mass spectrometry has proven helpful in presenting the sequence motifs which are presented by the various HLA molecules and are part of the immunopeptidome. The model provides BA proof and probability of a peptide having natural binding properties to MHC (351). MHCflurry 2.0 is another algorithm that utilizes BA and is also training using *in vitro* data from antigen presentation with peptide hits and negative controls. This system highlighted the significance of using proteasome cleaved peptides (352). Choice of prediction software can be made based on success of the various *in vitro* data included during software training. While some suggest that affinity-based

prediction may be superior, others trust elution-based training. Software such as Allergen FP 1.0 or ToxinPred uses length or peptide sequence data to predict allergenic or toxic peptides through comparison with data sets of known allergens or toxins (353, 354).

All predictions can help in making streamlined choices out of vast ranges of peptides, but experimental validation is needed. Prediction stage of vaccine development is followed by synthesis and purification stages. Two common methods of synthesis are soluble and solid phase synthesis. The characterization of the product is usually done using mass spectrometry along with Fourier Transform Infrared (FT-IR) spectroscopy to determine functional attributes (355). Circular dichroism (CD) spectroscopy is a useful tool to assess 2D and 3D structural properties (356). Commonly used techniques for protein purification include high performance liquid chromatography (HPLC), ion exchange chromatography or affinity chromatography (357, 358). In the current state scenario, a combination of high throughput technologies and software in genomics, proteomics and immunomics are necessary for personalized predictions followed by generation of preclinical proof of immunomodulation in experimental models.

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## AIM

The overarching aims of this thesis were to gain understanding of mechanisms influencing metastasis and to explore if stimulation of immunity using novel cancer vaccines may protect against metastasis.

**Paper I** aimed to develop a vaccine for murine melanoma by targeting a tumor cell antigen and an adjuvant, cholera toxin subunit A1 (CTA1) to a cross presenting population of antigen-presenting cells (cDC1).

**Paper II** aimed to determine how the mode of vaccine delivery and the composition of a cancer vaccine may affect metastasis and immunogenicity. This study also addressed the potential importance of the CTA1 adjuvant for enhancing tumor cell clearance by vaccines that incorporate endogenous tumor-associated antigens.

**Paper III** aimed to explore how myeloid cell-derived oxidative stress may influence epithelial-to-mesenchymal transition (EMT) and metastasis.

# METHODOLOGY

## 1. CELLULAR ASSAYS

**1.1 Migration and invasion assays.** Studies have shown that changes in cellular migration can provide insights into invasiveness, phenotypic alterations, and mechanisms related to malignant progression (359). In this thesis, migration and wound-healing assays were employed to assess how human and murine cancer cell lines respond to ROS.

For the migration assay, cells that migrated across a polycarbonate membrane in migration chambers were quantified. Changes in migratory behavior were evaluated under the influence of ROS. To investigate the role of myeloid cell-derived ROS for cancer cell migration, a chamber was used where monocytes were placed in the lower chamber while cancer cells were seeded in the upper chamber.

Additionally, two-dimensional migration was assessed using wound healing assays. A scratch was created in a cell monolayer and the rate of closure was monitored over time under different experimental conditions. Gene expression analysis was also performed on cells from these assays to further explore changes associated with ROS stimulation and inhibition.

## 2. IMMUNOINFORMATICS

The initial step to design a cancer vaccine targeting cancer cells is the identification of immunodominant peptides that can be successfully processed by DCs and presented to T cells in order to elicit an antigen specific immune response. To assess the immunodominant nature of peptides, their structural binding affinity to HLA or MHC haplotypes can be assessed using Artificial

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Neural Network (ANN)-based software such as PyMOL ([www.pymol.org](http://www.pymol.org)), MEMHC-2.0 (Minimal Epitope for maximum MHC Coverage, Version 2.0), and NetMHC-4.0/NetMHCIIpan-4.0 (DTU Health Tech).

The binding affinity analysis (BA) performed for the peptides-based antigens utilized in this thesis, using NetMHC-4.0/NetMHCIIpan-4.0 is demonstrated below. This ANN uses gapped sequence alignment with insertions and deletions to predict the patterns of binding between the peptides and MHC. The readout generates a predictive BA value in nanomolar with a rank of this BA relative to 400,000 other peptides of natural random origins.

Based on the thresholds set for ranking, the software predicts strong and weak binders but also the core part of the peptide that binds to the MHC. Table 1 shows the BA and ranking for the binding of MHC I (H-2-Kb) restricted ovalbumin peptide SIINFEKL (p257-267), melanoma antigen based TRP2 peptide SVYDFFVWL (p180-188) and MHC II (H-2-IAb) restricted OVA peptide ISQAVHAAHAEINEAGR (p323-339; denoted p323) to the MHC haplotypes present in the mouse strain C57BLK6/J. In a similar manner, table 2 show the BA of the Twist 1 peptides LKLAARYIDFLYQVL (p153-167), LYQVLQSDEL (p163-172), KIQTLKLAARYIDFL (p149-163), DKLSKIQTLKLAARY (p145-159), and SVWRMEGAWSMSASH (p 192-p206) to the MHC haplotypes of mice strain BALB/c (360). This evaluation on peptide BA for various MHC haplotypes was an important step to determine which peptides to include in our TAA based vaccines.

**Table 1: Peptide-MHC binding for MHC haplotypes of the C57BL6/J strain**

Sequence	MHC class (haplotype)	BA (nM)	%Rank	Core
SIINFEKL	I (H-2-Kb)	19.4	0.060	SIINFEKL
SVYDFVWL	I (H-2-Kb)	13.0	0.040	SVYDFVWL
ISQAVHAAHAEINEAGR	I (H-2-IAb)	662.78	0.57	VHAAHAEIN

**Table 2: Peptide-MHC binding for MHC haplotypes of BALB/c**

Sequence	MHC class (haplotype)	BA (nM)	%Rank	Core
LKLAARYIDFLYQVL	I (H-2-Kd)	3835.02	2.50	RYIDFLYQVL
	I (H-2-Ld)	4428.49	1.00	LKLAARYIDF
LYQVLQSDEL	I (H-2-Kd)	1489.87	1.00	LYQVLQSDEL
KIQTLKLAARYIDFL	I (H-2-Ld)	2495.93	0.60	LKLAARYIDF
	II (H-2-IAd)	311.39	3.92	TLKLAARYI
DKLSKIQTLKLAARY	II (H-2-IAd)	217.39	2.24	LSKIQTLKL
	II (H-2-IAd)	943.37	1.46	IQTLKLAAR
SVWRMEGAWSMSASH	II (H-2-IAd)	287.61	3.48	MEGAWSMSA

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### 3. VACCINE PREPARATION

**3.1 Production of CTA1-DD mutant.** The pUC19 vector was used as a template for site-directed mutagenesis and subcloning. To generate CTA1-pUC19, a 582 base-pair DNA fragment encoding the 194 amino acids of CTA1 was cloned into the vector (361). Insertion of the DD dimer was performed as described (362). The construct containing CTA1-DD in vector (320) was produced in *E. coli* using established protocols and purified through affinity chromatography on an IgG-Sepharose column (363). Each culture yielded 8-16 mg of fusion protein, with endotoxin levels maintained at <5 ng LPS/ $\mu$ g protein (323).

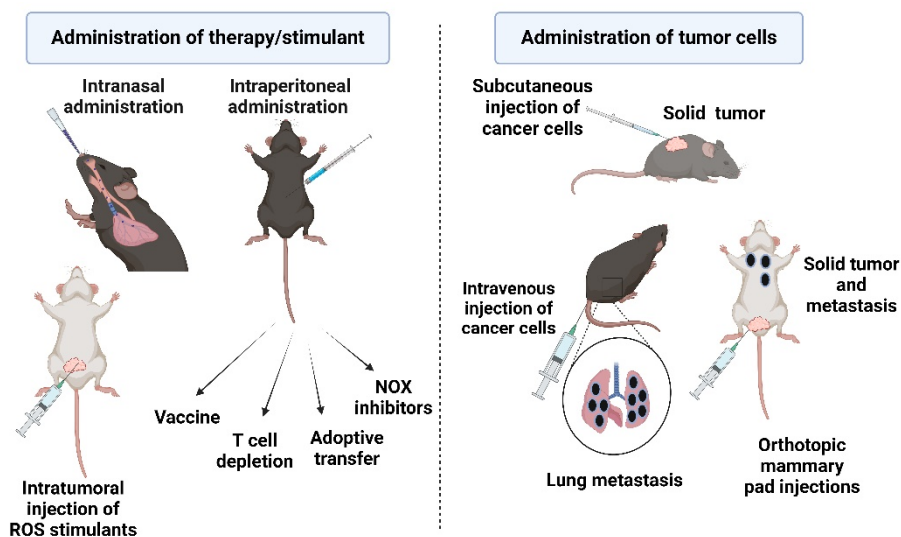
**3.2 Production of CTA1-peptide-aCD103 fusion construct.** To manufacture a CD103 scFv, variable regions from the heavy (VH) and light (VL) chains of the CD103 antibody were cloned and produced in M290 hybridoma. The required regions of the VL and VH chains were subjected to amplification using RT-PCR followed by sequencing. A sequence with a genetic composition of the 4 GGGGS linker was used to connect the amplified regions. The genetic sequence for CTA1 was fused to the N-terminal of the construct. The MHC I H-2-Kb restricted ovalbumin peptide SIINFEKL and MHC II I-Ab restricted peptide p323 were then fused to the construct (322). The end-product was subjected to purification using affinity chromatography and the endotoxin levels were maintained under 0.1 EU per mg of purified protein. Agmatine assay was used to confirm the enzymatic activity of the CTA1 (362).

**3.3 Preparation of vaccine mixtures.** Immunodominant peptides with sequences identified through immunoinformatics were purchased primarily

from Schafer-N, and diluted in 5% Dimethyl Sulfoxide (DMSO) in Phosphate Buffered Saline (PBS) and mixed with the required concentration of CTA1-DD.

## 4. MOUSE MODELS

A variety of therapeutic and cancer-inducing techniques were employed in the studies to explore the molecular mechanisms involved in metastasis and to assess the immunotherapeutic efficacy of cancer vaccines in clearing primary tumors and metastases. Figure 4 presents a schematic summary of the employed methods.



*Figure 4. Mouse models showing the administration of therapy or tumors*

**4.1 Intranasal and intraperitoneal vaccination.** For intranasal vaccinations (i.n.), mice were anesthetized using an isoflurane chamber and allowed to

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breathe in the vaccine formulation in 20  $\mu$ L of formulation solution. For intraperitoneal (i.p.) vaccination, mice were injected 100  $\mu$ L of vaccination formulation. Based on the vaccination scheme, mice usually received two or three vaccine doses prophylactically or therapeutically, either exclusively i.n. or via a combination of i.n. and i.p. administrations.

**4.2 Vaccination combined with depletion of T cell subsets.** To assess the role of CD8<sup>+</sup> or CD4<sup>+</sup> T cells in tumor clearance, these subsets of cells were depleted in vaccinated and control mice via i.p. injections of anti-mouse CD8 or CD4 monoclonal antibodies. The antibodies were administered once before tumor cell challenge and twice after tumor challenge.

**4.3 Adoptive T cells transfer.** To assess the functionality of T cells after vaccination, CD8<sup>+</sup> or CD4<sup>+</sup> T cells were isolated from single cell suspension of lymph nodes of vaccinated mice and adoptively transferred to naïve mice prior to challenge with tumor cells.

**4.4 Other therapies.** In the EMT study, tumor-bearing mice were not vaccinated, but treated with the NOX2 inhibitor histamine dihydrochloride (HDC) i.p. to investigate its effect on tumor growth. Additionally, some mice received intratumoral (i.t.) injections with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or with the NOX2 stimulating hexapeptide Trp-Lys-Tyr-Met-Val-d-Met (WKYMVM) followed by assessment of tumor growth and metastasis.

**4.5 Implantation of solid tumors.** In the melanoma model of solid tumors, mice were anesthetized using an isoflurane nasal tube, followed by subcutaneous (s.c.) injection of tumor cells in the flank, or in the case of breast cancer cells, orthotopically in the breast pad. Orthotopic injection of breast cancer cells also formed metastasis at advanced stages.

**4.6 Metastasis models.** Mice restrained in tubular chamber received intravenous (i.v.) tail vein injections of murine melanoma and breast carcinoma cell lines that metastasized to the lungs, as assessed two-three weeks post tumor cell challenge. The metastatic foci formed in the lungs by the black melanoma cells were enumerated macroscopically, while quantification of breast cancer cells, that were luciferase-tagged, was monitored using IVIS technology (Perkin Elmer). In the solid tumor models and in metastasis models, tissues such as lungs, spleen and tumor were isolated when mice were sacrificed, processed, and analyzed by down-stream applications such as flow cytometry, ELISpot, RT-PCR or Western blot.

## **5. FLOW CYTOMETRY**

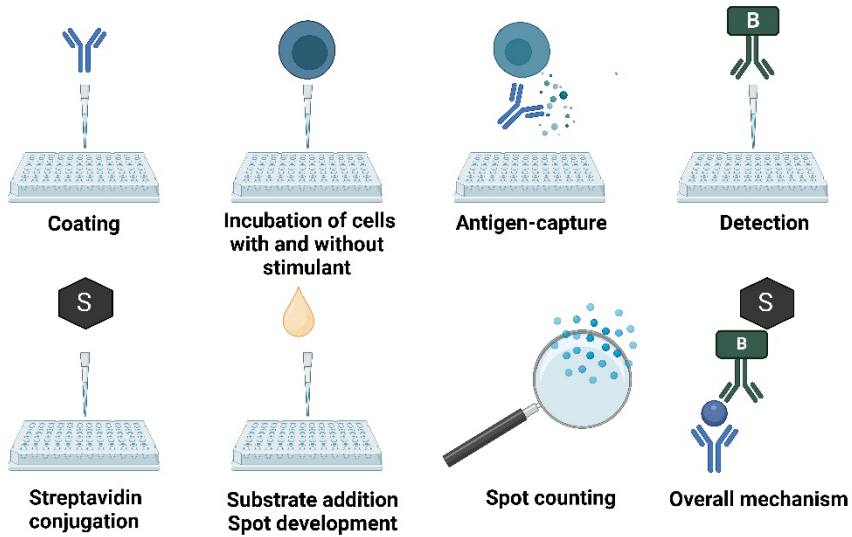
Flow cytometry analyzes single cells suspended in a buffered saline solution as they pass through one or more lasers. The relative structure of each cell is assessed by measuring the scattering of visible light in two directions: forward scatter (FSC), which indicates cell size, and side scatter (SSC), which reflects the granular nature or internal complexity of the cell. Graphs plotting FSC against SSC facilitates the identification of cell populations for further characterization based on additional biomarkers. Detailed characterization is achieved by staining cells with fluorescently labeled antibodies specific to the markers of interest (*e.g.*, CD8-PE), fluorescence-emitting dyes (*e.g.*, propidium iodide), or transfected fluorescent proteins such as green fluorescent protein (GFP) or luciferase. This allows for utilization of another key aspect of flow cytometry, the excitation of fluorophores using different lasers, followed by the detection of fluorescence emitted by the stained cells. Selected cell populations can also be sorted as bulk or single cells for expansion and culture of specific cells or clones (364).

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## 6. ELISpot AND INTRACELLULAR CYTOKINES

Enzyme-linked immunosorbent spot (ELISpot) detects the presence of cytokine-producing specific T cells. In this technique, solid surface pre-coated with antibodies are utilized. Cells to be tested for cytokine production are seeded on antibody-coated plates and stimulated with antigens. Cells that have previously encountered the same antigen are expected to become activated by the stimulation and release cytokines that bind to the pre-coated antibodies. The bound cytokines are subsequently detected by antibodies tagged with streptavidin or horseradish peroxidase (HRP) conjugates. These are then exposed to substrate solutions to develop visible spots, with each spot representing a cytokine producing cell. The complete mechanism of the assay is illustrated in Fig. 5.

This method has become standard for measuring antigen recall responses following vaccination (365). The assay quantifies the immune response generated by immune cells upon antigenic stimulation and also provides a functional measure of the cells' potency in cytokine production. We utilized this method to measure IFN- $\gamma$  and IL-17 production by CD8<sup>+</sup> and CD4<sup>+</sup> T cells isolated from the lungs and spleens of vaccinated mice and controls when stimulated with antigens corresponding to those included in the vaccine. Additionally, we detected double-positive cell populations using dual-color ELISpot assays.



*Figure 5. Schematic demonstrating antigen capture and detection principle of ELISpot*

In addition, T cell recall responses can be measured by restimulating cells with the specific antigens, followed by staining for T cell populations and intracellular cytokines along with detection by flow cytometry.

## 7. GENE AND PROTEIN EXPRESSION

*In vitro*-treated tumor cells, as well as tissue from murine tumors, were analyzed for gene expression and protein levels by real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot, respectively.

For gene expression analysis, mRNA isolated from cells or tissues was reverse transcribed into complementary DNA (cDNA), which was then amplified using PCR primers specific to the genes of interest. The use of fluorogenic primers enables real-time monitoring of the PCR reaction by detecting changes

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in fluorescent intensity through fluorescence resonance energy transfer (FRET). This method calculates the initial and relative concentrations of the transcript of interest in the experimental material, measured during the exponential phase of the reaction, compared to standards (366).

For protein expression measurements by Western blot, lysates were prepared from the relevant cells or tissues. In this technique, proteins are separated based on size by gel electrophoresis using sodium dodecyl sulfate (SDS) polyacrylamide gel, followed by immobilization on nitrocellulose or polyvinylidene fluoride (PVDF) membranes. Non-specific binding sites on the membrane are blocked, followed by probing with primary antibodies targeting specific proteins, and staining with fluorescent or chemiluminescent secondary antibodies to facilitate detection. Software tools are used to quantify protein levels through densitometric analysis of the bands visible on the membrane (367, 368).

## **8. BIOINFORMATICS**

Datasets from The Cancer Genome Atlas (TCGA) (369) were evaluated by biological mechanistic pathways using Gene Set Enrichment Analysis (GSEA). TCGA provides pre-characterized genomic, proteomic, and transcriptomic datasets across various cancer types, along with matched healthy samples. GSEA leverages predefined biological information regarding expression patterns and biochemical pathways to assess the positioning of gene sets within a ranked list, correlating them with differences in phenotypic classes. The program calculates an enrichment score that indicates the deviation from zero while traversing the list, identifying over-representation of genes: the run sum statistic increases if a gene is present and decreases if it is absent. The program's multiple analytical features contribute to its robustness and utility in analyzing molecular profiles (370).

In addition, data sets present in the Single Cell portal ([singlecell.broadinstitute.org](http://singlecell.broadinstitute.org)) and in the Kaplan–Meier plotter database ([kmplotter.com](http://kmplotter.com)) were analyzed to define the identity of cells expressing selected genes and the impact of gene expression on survival.

## 9. STATISTICAL ANALYSIS

Statistical analyses were conducted using GraphPad Prism (version 9 or updated). For comparisons between two groups, a two-tailed unpaired Student's *t*-test was employed. When comparing multiple groups (three or more), one-way ANOVA was utilized, followed by Tukey's or Sidak's multiple comparison tests for post hoc analysis. Survival analysis was performed using Kaplan-Meier curves, with comparisons between different groups assessed using the Log-rank (Mantel-Cox) test. Normality for parametric analyses was evaluated using the Shapiro-Wilk test, while the Mann-Whitney U test or Kruskal-Wallis test was applied for non-parametric comparisons. In some studies, the ROUT (Robust Regression and Outlier Removal) test was used to identify outliers. The number of biological replicates is indicated by 'n'. Two-sided p-values are represented as follows: \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ), \*\*\* ( $p < 0.001$ ), \*\*\*\* ( $p < 0.0001$ ).

All schematic figures are created using [biorender.com](http://biorender.com)

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# RESULTS AND DISCUSSION

## PAPERS I and II

One of the main challenges in developing effective cancer vaccines is identifying immunodominant peptide epitopes from cancer antigens that can trigger clinically significant T cell responses. A key step in selecting optimal peptide sequences from the antigen of interest is the usage of immunoinformatic software to evaluate binding affinities to the HLA or MHC molecules of the species in which the vaccine will be tested. The binding affinity predictions generated by this software can help identify peptides that are likely to elicit a stronger immune response, potentially sufficient to eliminate cells expressing the same antigen. However, multiple studies have highlighted that vaccines composed solely of antigens often fail to induce sufficiently robust immune responses due to self-tolerance mechanisms, ineffective targeting of DCs, and insufficient stimulation of innate immune pathways. Addressing these challenges requires the inclusion of immunomodulatory agents, such as adjuvants, to boost innate immune activation (371). Additionally, incorporating DC-targeting molecules could improve the delivery of antigens to DCs, which are highly efficient at antigen presentation, thereby enhancing CD8<sup>+</sup> and CD4<sup>+</sup> T cell-mediated immune responses.

In our studies, we initially used the immunodominant epitopes SIINFEKL and p323, derived from ovalbumin, as model antigens to develop an experimental system to evaluate the anti-metastatic immune responses elicited by these peptides in an OVA-expressing murine model of melanoma. These peptides have been widely used due to their optimal binding affinities to MHC molecules in mice, which correlate with the strength of the immune responses

they generate. To assess the importance of incorporating immunomodulatory agents and targeting molecules in vaccine formulations, we administered these antigens to mice either alone, admixed with the adjuvant CTA1-DD, or as chemically fused constructs comprising the CTA1 molecule, the selected antigens, and either the DD-moiety or a single-chain variable fragment (scFv) targeting the CD103<sup>+</sup> cDC1 dendritic cell population, known for its superior ability to cross-present antigens to CD8<sup>+</sup> T cells.

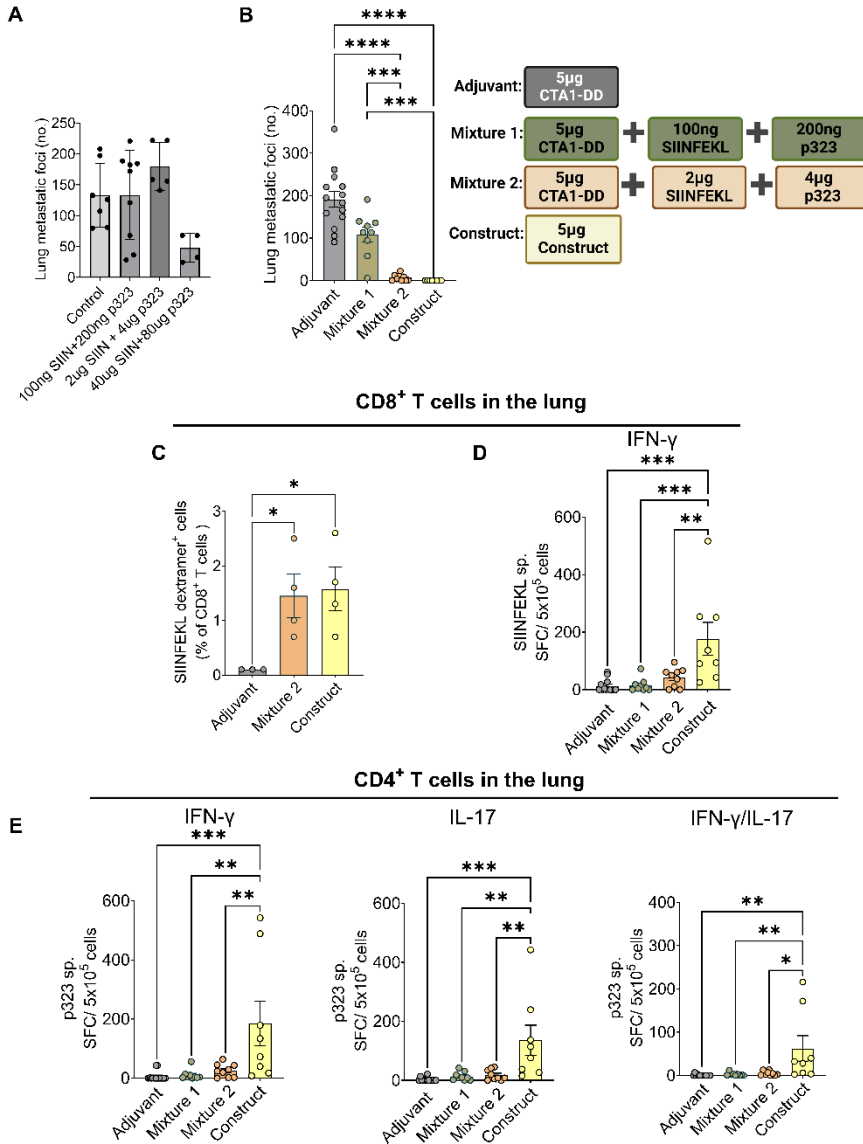
While CTA1-DD has been extensively studied as an adjuvant in the context of various infectious diseases (334-337), its potential as an adjuvant for cancer vaccines was previously not investigated. Our initial experiments involved administering OVA peptides (SIINFEKL and p323) to C57BL6/J mice, either alone, combined with the CTA1-DD adjuvant, or as chemically fused CTA1-constructs. The mice were later challenged with i.v. tail-vein injections of the OVA-expressing murine melanoma cell line B16F1-OVA, which metastasizes to the lungs. We assessed the reduction of lung metastatic foci along with the upregulation of SIINFEKL and p323-specific IFN- $\gamma$  and IL-17-producing functional T cells in the lungs as key immune response readouts (Fig. 6).

The goal of these experiments was to determine the role of the adjuvant in eliciting an anti-cancer immune response and to demonstrate the potential advantages of administering peptides chemically coupled to a fusion construct compared with a mixture of the same components. Our results indicated that a high dosage of OVA peptides (40  $\mu$ g SIINFEKL and 80  $\mu$ g p323) was required to generate a detectable immune response (Fig. 6A). From a clinical perspective, such high peptide levels would likely be difficult to achieve, underscoring the need for adjuvants to elicit a sufficiently strong immune response.

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Our study further showed that administering lower doses of antigens (2  $\mu\text{g}$  SIINFEKL and 4  $\mu\text{g}$  p323) significantly reduced lung metastasis when admixed with 5  $\mu\text{g}$  of CTA1-DD. However, complete metastasis clearance was only achieved when the peptides were conjugated to CTA1-DD. In the setting of conjugation, as low doses as 100 ng SIINFEKL and 200 ng p323 were sufficient, demonstrating the advantages of using a construct-based vaccine (Fig. 6B). These observations were further supported by post-vaccination ELISpot assays, which showed the greatest increase of SIINFEKL and p323-specific  $\text{IFN-}\gamma^+$  and/or  $\text{IL-17}^+$   $\text{CD8}^+$  and  $\text{CD4}^+$  T cells in the lungs of mice vaccinated with the constructs (Fig. 6 C-E). These results thus demonstrate that chemically fused constructs combining adjuvants, antigens, and targeting molecules may provide superior immune induction at lower antigen doses.

We also investigated if the specific DC population targeted by the vaccine may affect its efficacy. We utilized the CTA1-SIINFEKL-p323-DD construct (denoted as CTA1-I/II-DD) for broad DC targeting and compared it to the more specific targeting of migratory cDC1s achieved by the CTA1-SIINFEKL-p323-aCD103 construct (denoted as CTA1-I/II-aCD103). Both constructs conferred effective protection against metastasis when administered prophylactically followed by a challenge with B16F1-OVA cells (Fig. 7 A, B). However, the DD-based vaccine elicited a lower number of SIINFEKL tetramer<sup>+</sup> T cells compared to the aCD103-based vaccine (Fig. 7C). This observation highlighted the importance of targeting CD103-expressing cDC1s, which are highly efficient in cross-presenting antigens to  $\text{CD8}^+$  T cells.



**Figure 6. Metastasis protection and T cell activation by CTA1-based adjuvants when admixed or fused to antigens.** (A) Normalized mean number of B16F1-OVA lung metastasis ( $\pm$  s.e.m.) formed in PBS-treated C57BL/6 mice (control) or mice vaccinated with different doses of the SIINFEKL and p323 OVA-peptides. (B-E) Mice

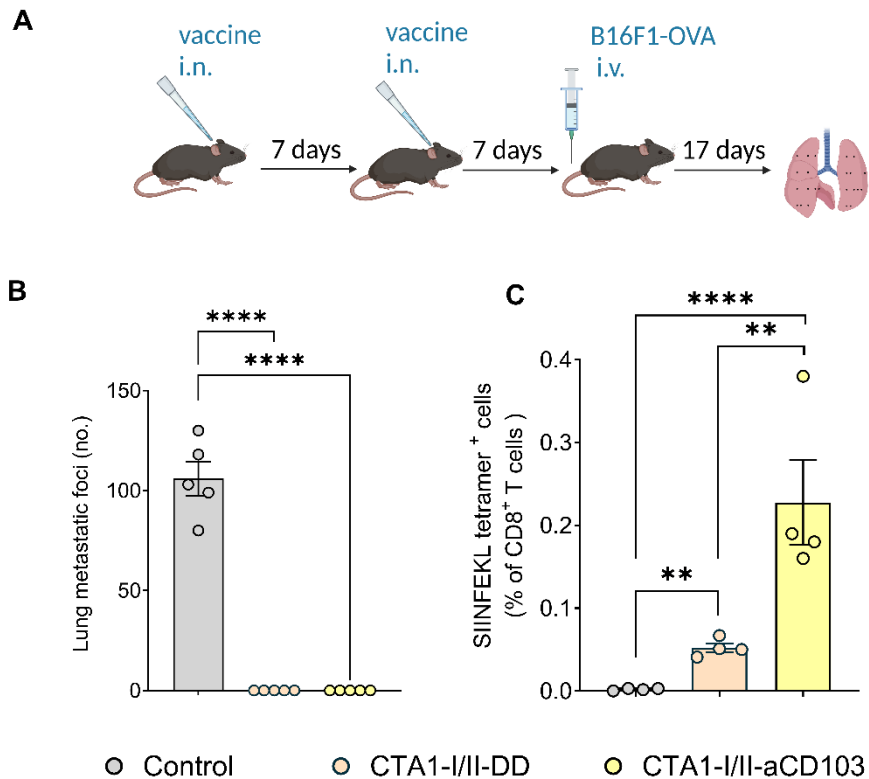
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were vaccinated twice with CTA1-DD adjuvant, CTA1-DD admixed with 100 ng SIINFEKL and 200 ng p323 (mixture 1), CTA1-DD admixed with 2  $\mu$ g SIINFEKL and 4  $\mu$ g p323 (mixture 2) or CTA1-I/II-based SIINFEKL/p323 fusion proteins (CTA1-I/II-aCD103 or CTA1-I-DD and CTA1-II-DD; construct), after which they were i.v. challenged with B16F1-OVA melanoma cells. (B) Normalized mean number of lung metastases ( $\pm$  s.e.m.). (C) Frequency of SIINFEKL dextramer<sup>+</sup> CD8<sup>+</sup> T cells in lungs. (D-E) Number of (D) SIINFEKL-specific IFN- $\gamma$ -producing CD8<sup>+</sup> T cell and (E) p323-specific IFN- $\gamma$ -producing and/or IL-17-producing CD4<sup>+</sup> T cells in lungs at the experimental endpoint as determined by ELISpot. Statistics by ANOVA followed by Sidak's multiple comparison test (n=3-14 mice per group).

Studies have shown that a subset of cDC2s can also express CD103, which can be distinguished from cDC1s by their expression of CD11b (372-374). cDC2s are primarily involved in presenting antigens to CD4<sup>+</sup> T cells and promoting the priming of Th2, Tfh, and Th17 cells (374). However, a previous study demonstrated that the efficacy of the aCD103-based vaccine construct in eliciting both CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses was compromised in cDC1-deficient Batf3<sup>-/-</sup> mice, but not in mice deficient in CD103<sup>+</sup> cDC2 (322). This thus indicates that while this construct may also target CD103<sup>+</sup> cDC2s, its activity largely depends on cDC1s.

In addition to understanding the role of the DC subtypes targeted by the vaccine construct, it was equally important to explore which activated T cell subsets generated an anti-metastatic immune response. C57BL6/J mice were prophylactically vaccinated with the CTA1-I/II-aCD103 construct, followed by the depletion of CD8<sup>+</sup> or CD4<sup>+</sup> T cell subsets and challenge with B16F1-OVA cells (Fig. 8A). CD8<sup>+</sup> T cell depletion allowed the formation of lung metastases after vaccination, while mice that underwent CD4<sup>+</sup> T cell depletion or control treatment were still completely protected from metastasis,

emphasizing the role of CD8<sup>+</sup> T cells in mediating the protective immune response (Fig. 8B).



**Figure 7. Comparison of a vaccine construct targeting pan DC populations to a construct targeting CD103<sup>+</sup> cDC1s** (A) Schematic of vaccination timeline (B) Mean number of lung metastases ( $\pm$  s.e.m.) post i.v. challenge with B16F1-OVA cells in mice receiving indicated vaccine constructs ( $n=5$ , statistics by ANOVA) (C) Flow cytometry plot showing the SIINFEKL tetramer<sup>+</sup> CD8<sup>+</sup> T cells in the lungs ( $n=4$ , statistics by ANOVA).

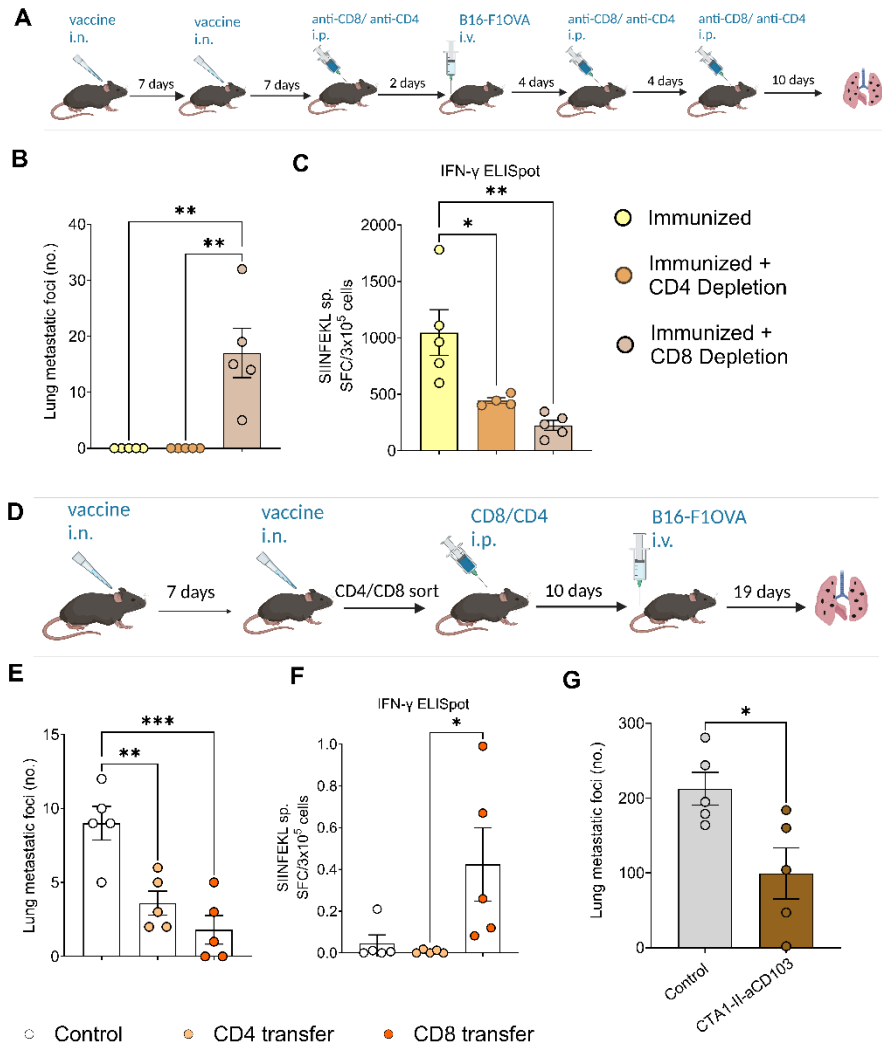
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Analysis of SIINFEKL-specific IFN- $\gamma$ -producing CD8<sup>+</sup> T cells in lungs at the experimental endpoint supported a significant reduction of these cells following treatment with anti-CD8 depleting antibodies. Interestingly, CD4-depleted mice also showed a notable reduction in IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells, suggesting that CD4<sup>+</sup> T cells provide support for an optimal activation and development of antigen-specific CD8<sup>+</sup> T cells (Fig. 8C).

To further examine the interdependence of these T cell subtypes, CD8<sup>+</sup> and CD4<sup>+</sup> T cells were isolated from the spleen and lymph nodes of vaccinated mice and thereafter adoptively transferred into naïve mice via i.p injection, followed by challenge with B16F1-OVA cells (Fig. 8D). While both CD4<sup>+</sup> and CD8<sup>+</sup> T cell transfers conferred protection against metastasis, only the CD8<sup>+</sup> T cell transfer entailed significant upregulation of SIINFEKL-specific IFN- $\gamma$ <sup>+</sup> cells. Nevertheless, the metastasis clearance observed in mice receiving CD4<sup>+</sup> T cells indicates that they provided some level of metastasis protection (Fig. 8E-F). In an infectious diseases setting, vaccines incorporating both class I and class II antigens have proven most effective upregulating T cell responses (375). Some studies have also demonstrated that a CD4<sup>+</sup> T cell response is operable (376). Our study shows that a vaccine construct containing only a class II antigen was able to elicit partial metastasis clearance (Fig 8G).

Understanding the roles and interdependence of CD8<sup>+</sup> and CD4<sup>+</sup> T cells is critical in vaccine development, as it highlights the importance of including antigens that can prime both T cell subtypes. Our findings support a role for CD4<sup>+</sup> T cells in boosting CD8<sup>+</sup> T cell vaccine responses, but also suggest a role for CD4<sup>+</sup> T cells in anti-tumor immunity beyond conferring CD8<sup>+</sup> T cells help to administrated antigens. While the detailed mechanism remains to be elucidated, it may relate to CD4<sup>+</sup> T cell-generated cytokines that create a more immune-responsive “hot” microenvironment, with enhanced influx of immune

cells and enhanced MHC expression along with more general T cell priming against various endogenous tumor antigen epitopes.



**Figure 8.** *CD8<sup>+</sup> T cells are necessary for the antimetastatic properties of CTA1-II-aCD103* (A-C) Mice were vaccinated prophylactically with CTA1-II-aCD103

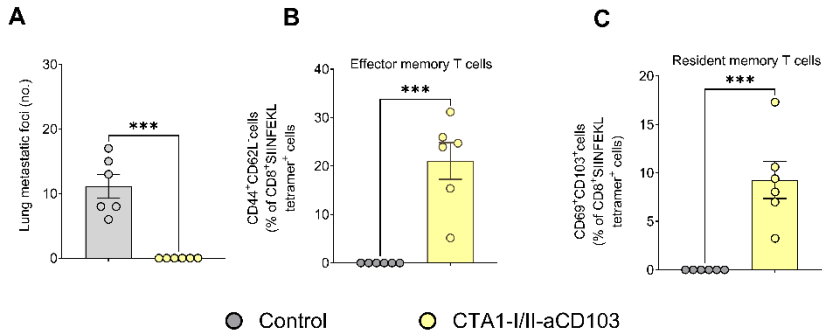
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followed by depletion of CD8<sup>+</sup> and CD4<sup>+</sup> T cell subsets before and after i.v. tail vein injection of B16F1-OVA cells. (A) Schematic timeline. (B) Mean count of lung melanoma metastases  $\pm$  s.e.m. formed in mice that received CTA1-I/II-aCD103 vaccine with or without CD8 and CD4 depletion. (C) SIINFEKL-specific IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells in the lungs of mice measured by ELISpot ( $n = 4-5$ , statistics by ANOVA). (D-F) T cell subsets from spleens and lymph nodes of vaccinated mice were i.p. adoptively transferred to naïve mice that were i.v. challenged with B16F1-OVA cells. (D) Schematic timeline. (E) Mean number of lung metastatic foci  $\pm$  s.e.m. formed in mice receiving control (PBS, white dots), CD4<sup>+</sup> T cells (yellow dots) or CD8<sup>+</sup> T cells (red dots). ( $n = 5$ , statistics by ANOVA). (F) SIINFEKL-specific IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells in the lungs of mice measured by ELISpot at the endpoint ( $n = 5$ , statistics by ANOVA). (G) Mice were prophylactically vaccinated twice with CTA1-p323-aCD103 (denoted as CTA1-II-aCD103) or PBS (control) followed by an i.v. challenge with B16-F1OVA. Plot shows mean number of lung metastatic foci  $\pm$  s.e.m., two week post challenge ( $n=5$ , statistics by unpaired Student's T test).

Following T cell activation, a small population of the primed T cells acquire epigenetic changes, allowing their long-term persistence. These memory cells respond more rapidly to their cognate antigens upon re-encounter (377). An ideal cancer vaccine should generate persistent antigen-specific memory T cells, which responds to future tumor recurrence also long after vaccination.

To test the longevity of the T cell responses induced by prophylactic administration of the CTA1-I/II-aCD103 vaccine, vaccinated mice were challenged with B16F1-OVA cells three months post-vaccination. The vaccine still conferred complete protection against metastasis (Fig. 9A). This was accompanied by sustained presence of SIINFEKL tetramer<sup>+</sup> and SIINFEKL-specific IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells in lungs of vaccinated mice post tumor cell inoculation. Flow cytometry analysis revealed that a large fraction of SIINFEKL-specific CD8<sup>+</sup> T cells exhibited an effector memory phenotype (CD8<sup>+</sup>CD44<sup>+</sup>CD62L<sup>-</sup>) (Fig. 9B) and a resident memory phenotype

(CD8<sup>+</sup>CD69<sup>+</sup>CD103<sup>+</sup>) (Fig. 9C). These resident memory T cell phenotypes are known to establish long-term residency in non-lymphoid tissues, such as the lungs (378).



**Figure 9. The CTA1-II-aCD103 vaccine confers long-term memory of antigen specific T cells.** Mice were prophylactically vaccinated two times with CTA1-II-aCD103 followed by a challenge with i.v. tail vein injections of B16F1-OVA murine melanoma cells, three months after the last vaccine dose (A) Count of lung metastatic foci (mean  $\pm$  s.e.m.) in controls and vaccinated mice (B) Flow cytometry analysis of CD8<sup>+</sup>CD44<sup>+</sup>CD62L<sup>-</sup> effector memory T cells out of the SIINFEKL specific CD8<sup>+</sup> T cells in lungs. (C) Flow cytometry analysis of CD8<sup>+</sup>CD69<sup>+</sup>CD103<sup>+</sup> resident memory T cells out of the SIINFEKL specific CD8<sup>+</sup> T cells in lungs (n=6, statistics by Student's *t* test)

While the choice of immunodominant antigens and immunomodulatory adjuvants is critical for defining the nature and magnitude of the immune response, an important albeit less studied factor is the route of vaccine administration. Studies have shown that the route of administration can affect the magnitude and nature of the immune response, since different APC populations are targeted. For instance, systemic administration might provide access to a greater number of APCs and prime a larger pool of naïve T cells (379). Intranasal delivery, on the other hand, targets mucosal APCs and likely

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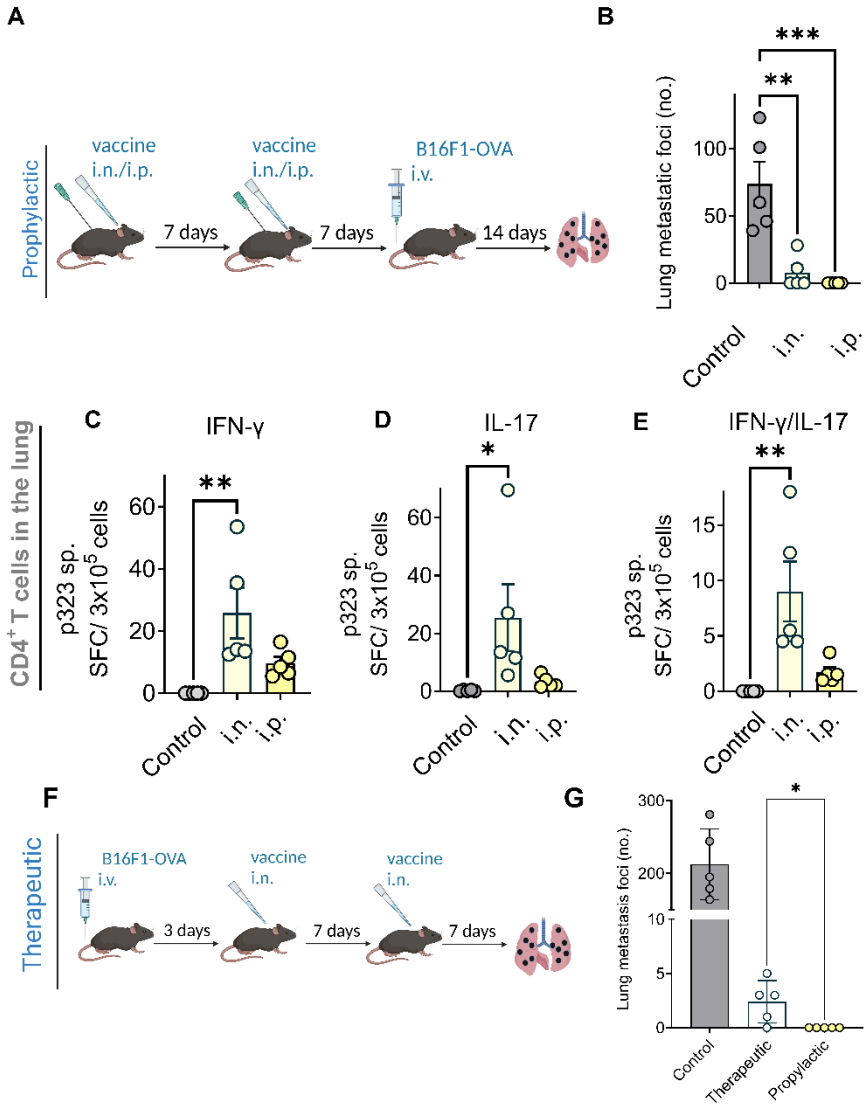
elicits more potent immune responses in the mucosa and systemically, while the systemic responses may be weaker, considering a reduced effect on mucosal immunity (380). Clinically, the route of administration may also influence toxicity and the ease of administration.

We compared local and systemic vaccination routes using i.n. and i.p. injections, followed by challenge with B16F1-OVA cells. Although there were no significant differences in metastasis clearance between the two routes (Fig. 10A,B), the local (i.n.) route was more effective at eliciting MHC II-restricted p323-specific IFN- $\gamma$ <sup>+</sup> and IL-17<sup>+</sup> CD4<sup>+</sup> T cells at the lung metastasis site (Fig. 10C-E), whereas the systemic route (i.p.) tended to produce more robust systemic CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses.

For a cancer vaccine to be clinically relevant, it should elicit an anti-tumor immune response also in the setting of an established tumor. We found that while our vaccine was more effective when administered prophylactically, it significantly reduced local tumor growth and metastasis also when administered in a therapeutic setting (Fig. 10 F- G).

As discussed, identifying appropriate immunodominant TAAs is likely crucial for the efficacy of cancer vaccines. It is also relevant to take into consideration that many overexpressed or differentiated TAAs are subject to immune tolerance mechanisms, which may limit their efficacy even if they theoretically should be able to induce a T cell response. We hypothesized that the use of the CTA1 adjuvants could enhance TAAs presentation to APCs, while also leveraging their immunodominant properties in vaccine formulations. Using our established system with CTA1-based mixtures and constructs, we prepared vaccine combinations that included the CTA1-DD adjuvant, combined with peptides from the melanoma-associated antigen TRP2, and from the breast cancer antigen Twist1. TRP2 is a differentiation TAA expressed in melanoma

(381, 382) while Twist 1 is an EMT transcription factor that is overexpressed in various metastatic cancers (383).



**Figure 10. Route of administration and therapeutic vaccination (A-E)** Mice were i.n. or i.p. vaccinated with CTA1-III-aCD103 followed by challenge with B16-F1-OVA

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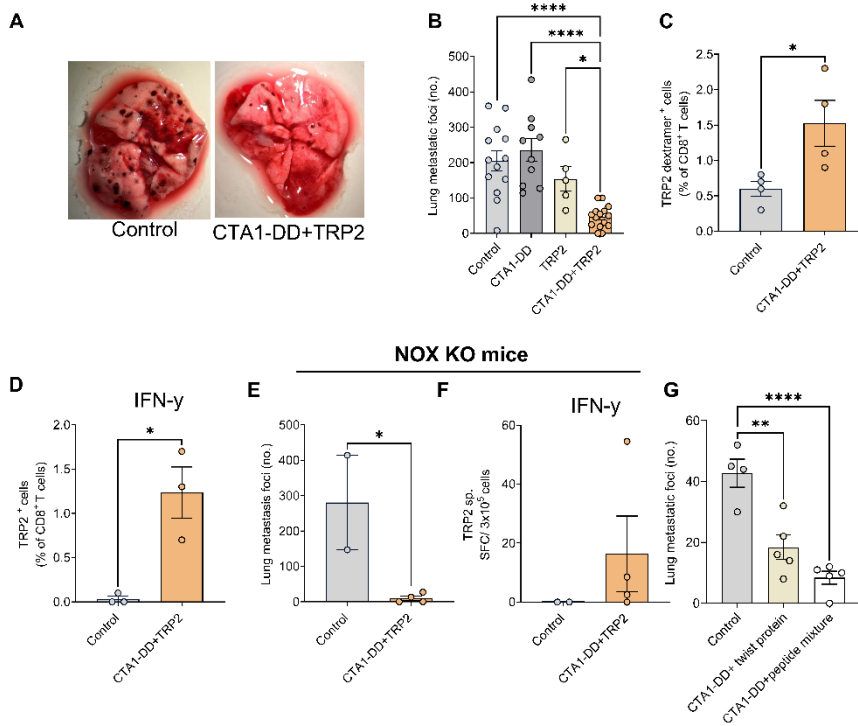
cells. (A) Schematic timeline. (B) Count of lung metastatic foci (mean  $\pm$  s.e.m.). (C-E) Number of p323 specific (C) IFN- $\gamma^+$ , (D) IL-17 or (E) IFN- $\gamma^+$ IL-17 $^+$  CD4 $^+$  T cells in lungs by ELISpot ( $n=5$ , statistics by ANOVA). (F-G) Mice were i.v. challenged with B16-F1OVA melanoma cells. They were either treated before (prophylactic) or three and 10 days later (therapeutic) with i.n. CTA1-I/II-aCD103 vaccine. (F) Schematic timeline of therapeutic vaccination. (G) Lung metastatic foci (mean  $\pm$  s.e.m.) in controls and mice vaccinated therapeutically and prophylactically ( $n=5$ , statistic by ANOVA)

In the ensuing experiments, C57BL6/J mice were prophylactically immunized with two doses of 100  $\mu$ g of the TRP2-derived SVYDFVWL peptide in combination with 5  $\mu$ g CTA1-DD via a combination of i.p. and i.n. routes for optimal protection. One week after the last vaccine dose, mice were challenged with B16F1-OVA cells via i.v. injection. Counts of macroscopic lung metastases and quantification of antigen-specific and functional T cells in lungs and spleens were performed two to three weeks post-challenge.

The SVYDFVWL peptide, when administered with the adjuvant but not alone, conferred significant protection against metastasis, highlighting the necessity of adjuvants in vaccine formulations (Fig. 11A-B). This combination also resulted in a significant accumulation of TRP2-dextramer $^+$  CD8 $^+$  T cells at the lung metastasis site (Fig. 11C), which was further validated using ELISpot. In addition, splenocytes from vaccinated mice, when stimulated with the TRP2 peptide sequence, showed a significant increase in intracellular IFN- $\gamma$ , a marker of functional antigen-specific CD8 $^+$  T cells, implying that also a systemic immune response was induced by the vaccine (Fig. 11D).

Since myeloid-derived ROS may suppress cytotoxic T cells in the tumor microenvironment, potentially hindering antigen-specific T cell responses (384, 385), we also vaccinated NOX2-KO mice with this antigen-adjuvant combination. Vaccinated NOX2-KO mice showed an almost complete

reduction in metastasis formation, along with induction of TRP2-specific CD8<sup>+</sup> T cells (Fig. 11E-F). These results suggest that NOX2-derived ROS may impede evolution of antigen-specific T cells and/or reduce their ability to eliminate metastasis.



**Figure 11. Endogenous TAAs confer protection against metastasis only in combination with CTA1-DD adjuvant.** (A-D) C57BLK6/J were immunized twice with PBS (control), CTA1-DD, TRP2 antigen only or TRP2 in combination with CTA1-DD, followed by i.v. challenge with B16F1-OVA cells. (A) Micrographs showing representative lungs. (B) Macroscopic count of lung metastatic foci (mean  $\pm$  s.e.m.)(n=5-15, statistics by ANOVA) (C) Flow cytometry analysis showing the frequency of TRP2 dextramer<sup>+</sup> CD8<sup>+</sup> T cells in the lungs (n=4, statistics by unpaired student's T test) (D) TRP2-specific IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T in spleen as measured by flow cytometry (n=3, statistics by unpaired student's T test) (E-F) NOX2-KO C57BL6/J

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*mice were vaccinated or not with CTA1-DD+TRP2 followed by B16F1-OVA challenge. (E) Lung metastatic count (mean  $\pm$  s.e.m.) in the lungs. (F) TRP2 specific IFN  $\gamma$ <sup>+</sup> CD8<sup>+</sup> T in the lung as measured by ELISpot (n=2-4, statistics by unpaired student's t test). (G) BALB/c mice were vaccinated twice with PBS (control), twist protein combined with CTA1-DD or a recombinant mixture of Twist1 peptides along with CTA1-DD, followed by i.v. inoculation of 4T1 murine breast cancer cells. Formed lung metastatic foci (mean  $\pm$  s.e.m.) in lungs are shown (n $\leq$ 5, statistics by ANOVA)*

We next assessed Twist1 as a vaccine antigen candidate in combination with the CTA1-DD adjuvant. By immunoinformatic analysis of peptide-binding affinity to BALB/c MHC haplotypes, five Twist1 peptides were identified. BALB/c mice were prophylactically vaccinated with either the intact Twist1 protein or a mixture of recombinant Twist1 peptides in combination with CTA1-DD. Mice were then challenged with 4T1 breast cancer cells via i.v. tail vein injection, followed by counts of lung metastases.

The intact Twist1 protein as well as the Twist1 peptide mixture conferred significant protection against 4T1 metastasis when these antigens were administered together with CTA1-DD (Fig. 11G). Additional experiments suggested that two of the Twist1 peptides, LKLAARYIDFLYQVL (MHC I) and SVWRMEGAWSMSASH (MHC II), were most important for protection against metastasis. These results imply that CTA1-DD may enhance metastasis-protective T cell responses against endogenous tumor antigens across tumor models and mouse strains.

Although chemically fused constructs of adjuvants, antigens, and targeting moieties provide superior immune regulation compared to simple mixtures between antigen and adjuvant, their design and production can be labor-intensive. Thus, to preclinically assess antigen immunodominance and adjuvant potency, a faster methodology is often needed. Our studies with

adjuvant-antigen mixtures offer an efficient way to test the immunodominance of antigens in murine models of metastatic cancer.

### **PAPER III**

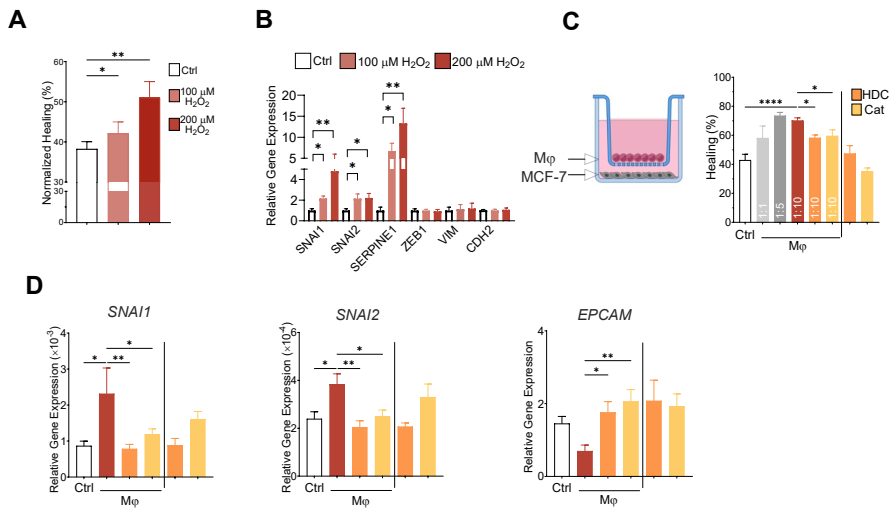
In paper III we explored mechanisms linking EMT and metastasis to an inflammatory tumor microenvironment. This study was based on the hypothesis that NOX2-derived ROS from myeloid cells might influence breast cancer cell EMT, ultimately leading to metastasis.

In initial experiments, H<sub>2</sub>O<sub>2</sub> was used as the source of ROS. Wound healing assays in the presence or absence of H<sub>2</sub>O<sub>2</sub> were conducted using the breast cancer cell line MCF-7 to assess effects on their migratory capacity. The addition of H<sub>2</sub>O<sub>2</sub> enhanced scratch closure in a dose-dependent manner, suggesting increased cell migration, potentially due to EMT induction (Fig. 12A). Further analysis using RT-qPCR revealed dose-dependent upregulation of EMT-associated genes such as *SNAIL1*, *SNAIL2*, and *SERPINE1* in H<sub>2</sub>O<sub>2</sub>-treated cells (Fig. 12B).

A main rationale of this study was to assess the impact of NOX2-derived ROS produced by myeloid cells on EMT gene expression. To test this, a co-culture system was established using transwells with MCF-7 cells cultured in the bottom well and myeloid CD14<sup>+</sup> monocytes isolated from healthy donor peripheral blood mononuclear cells (PBMCs) added to the top chamber. Addition of ROS-producing monocytes in the co-culture setting resulted in enhanced wound (scratch) healing of MCF-7 breast cancer cells, which was mitigated by the NOX2 inhibitor histamine dihydrochloride (HDC) or the ROS scavenger catalase (Fig. 12C). Several EMT markers were upregulated in breast cancer cells after exposure to NOX2-expressing monocytes, including *SNAIL1* and *SNAIL2*, while the epithelial marker *EPCAM* was downregulated in

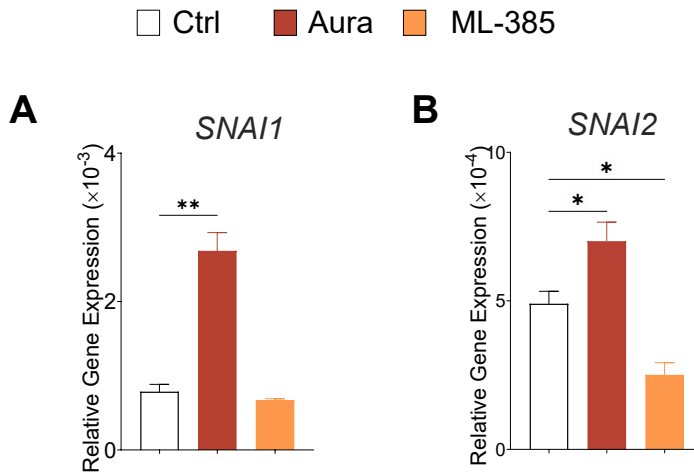
the presence of myeloid-derived ROS, effects that were reversed by HDC and catalase (Fig. 12D).

The study further explored which redox-dependent signaling pathways that might be driving the EMT-related gene expression. Reactome gene-set analysis of MCF-7 cells exposed to H<sub>2</sub>O<sub>2</sub> identified NRF2 as an induced pathway. In accordance, western blot analysis revealed increased NRF2



**Figure 12. ROS induces EMT.** (A-B) MCF-7 cells were treated or not with H<sub>2</sub>O<sub>2</sub>. (A) Healing of a scratch in plated MCF-7 cells at 48 hours (n=5, statistics by mixed-effects model with Holm-Šidák multiple comparison test). (B) Expression of EMT markers SNAI1, SNAI2, SERPINE1, ZEB1, VIM and CDH2 at 48 hours assessed by RT-qPCR (n=6, statistics by Kruskal-Wallis with Dunn's multiple comparison test). (C-D) CD14<sup>+</sup> monocytes (Mφ) isolated from healthy donor PBMCs were added to inserts placed for 2h on plated MCF-7 cells. Some wells were treated with 200 U/ml catalase (cat) or 200 μM HDC. (C) Healing of a scratch in plated MCF-7 cells was measured at 24 hours. (D) Expression of EMT markers at 24h (n=3-7, statistics by one-way ANOVA with Holm-Šidák multiple comparison test).

protein levels in MCF-7 cells co-cultured with monocytes, which were reduced upon HDC or catalase treatment. Analysis using the UCSC Genome Browser identified NRF2-binding motifs near the EMT-associated genes *SNAIL1* and *SNAI2*. Activation of NRF2 in MCF-7 cells with auranofin led to increased expression of *SNAIL1* and *SNAI2*, while NRF2 inhibition using ML-385 reduced *SNAI2* levels (Fig. 13A, B). Together, these results suggest that ROS-induced NRF2 activation was responsible for activation of the EMT-related transcription factors *SNAIL1* and *SNAI2*.



**Figure 13. ROS induced *SNAIL1* and *SNAI2* are dependent on NRF2 activation.** MCF-7 cells were treated with the NRF2 inducer Auranofin (Aura) or the NRF2 inhibitor ML-385. Expression of (A) *SNAIL1* and (B) *SNAI2* was assessed after 6 hours by RT-PCR. (n=3-4, statistics by one-way ANOVA with Holm-Šidák multiple comparison test)

To test the hypothesis that ROS stimulates EMT in an *in vivo* setting, we used an orthotopic model of breast cancer. Luciferase-tagged 4T1 murine breast cancer cells were injected into the mammary fat pad of BALB/c mice. Once tumors reached ~5 mm, they were injected intratumorally (i.t.) with either H<sub>2</sub>O<sub>2</sub>

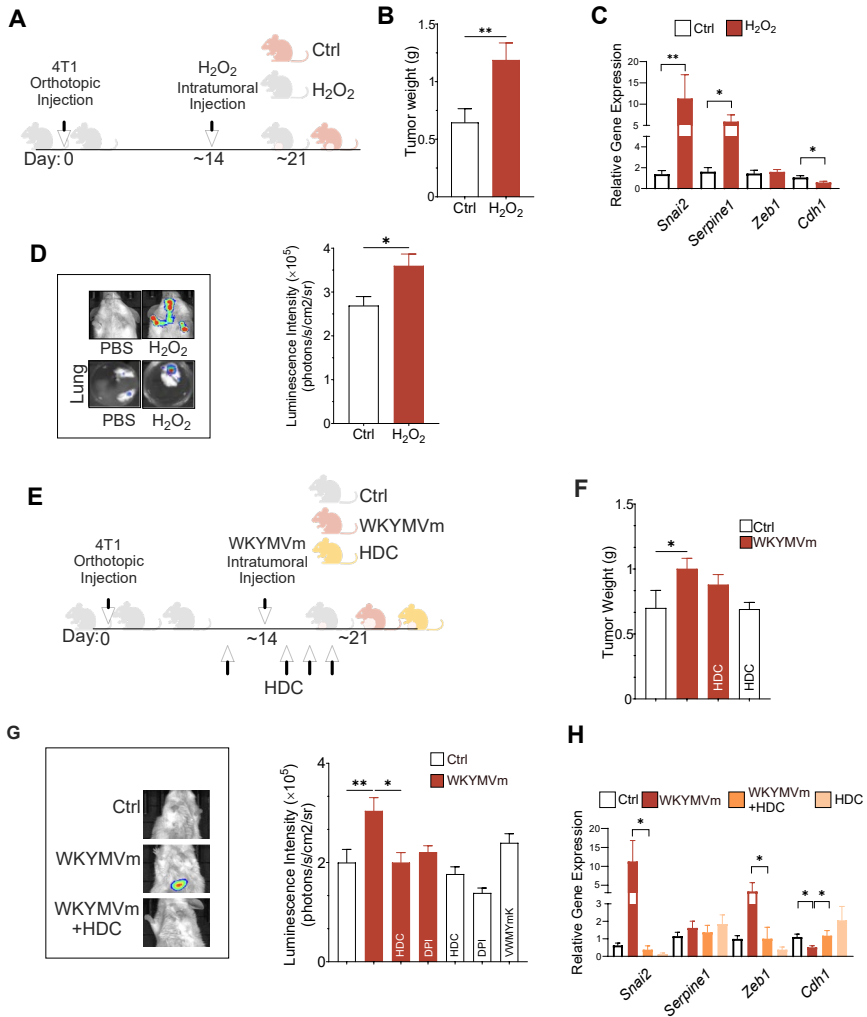
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or PBS. Tumor growth was measured, while metastasis was monitored via IVIS imaging. H<sub>2</sub>O<sub>2</sub> treatment significantly promoted primary tumor growth and metastasis, which correlated with upregulation of *Snai2* and downregulation of the epithelial marker *Cdh1* in the primary tumor, confirming EMT induction (Fig. 14A-D).

To determine if NOX2-derived ROS from myeloid cells might cause similar effects on tumor growth *in vivo*, the NOX2-stimulatory peptide WKYMVm was injected i.t. in orthotopic 4T1 tumors. This strategy aimed to enhance ROS production by tumor-infiltrating myeloid cells (Fig 14E). Administration of WKYMVm significantly enhanced primary tumor growth and metastasis, and these effects were reversed by treatment with the NOX2 inhibitor HDC (Fig.14F-G). The i.t. injection of WKYMVm also tended to enhance EMT marker expression while reducing expression of the epithelial marker *Cdh1*, effects that were reversed by HDC treatment (Fig. 14H).

Data mining of public databases showed correlations between expression of NOX2 subunits and EMT-related genes in breast cancer tumors, further supporting a link between NOX2 and EMT. Furthermore, analysis of publicly available breast cancer datasets showed that a high expression of the NOX2 subunit *NCF2*, and the EMT-related factors *SNAIL* and *SERPINE1* associated with poor metastasis-free survival.

In summary, results presented in paper III support that NOX2-derived ROS from myeloid cells promote EMT and metastasis in breast cancer. This induction appears to be at least in part mediated through the redox-sensitive transcription factors NRF2.



**Figure 14. Extracellular ROS enhances tumor growth and metastasis in an in-vivo setting of murine breast carcinoma.** (A-D) BALB/c received orthotopic inoculation of luciferase-tagged 4T1 murine breast cancer cells in the mammary fat pad followed by *i.t.* injections of PBS or H<sub>2</sub>O<sub>2</sub>. (A) Schematic timeline. (B) Tumor weight at endpoint ( $n=13-15$ , statistics by Mann-whitney test). (C) Expression of EMT markers Snai2, Serpine1, Zeb1 and Cdh1 in primary tumor measured by qRT-PCR ( $n = 8-14$ /group, Mann-Whitney test). (D) Bioluminescent imaging of metastasis ( $n=13-15$ , statistics by Mann-Whitney test). (E-H) BALB/c mice with orthotopically implanted 4T1-luc tumors,

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received i.t. injections of a NOX2 inducing WKYMVm peptide, a scrambled peptide (VWVMYmK) or PBS (control). Some mice were i.p. treated with HDC. (E) Schematic timeline. (F) Tumor weight at the endpoint (n=9-17, statistics by Kruskal-Wallis with Dunn's multiple comparison test). (G) Metastasis formation as measured by bioluminescence (n= 9-17, statistics by Kruskal-Wallis with Dunn's multiple comparison test). (H) Expression of EMT markers Snai2, Serpine1, Zeb1 and Chd1 in the primary tumor by RT-qPCR (n= 6-17, statistics by Kruskal-Wallis with Dunn's multiple comparison test)

## CONCLUDING REMARKS

The essence of this thesis can be summarized by a popular phrase ‘Hitting two targets with one arrow’. Researchers around the globe will likely agree that most of their work aims to address multi-faceted challenges. In the field of immunotherapy for advanced cancer, there is a need for the same ‘arrow’ to target and activate appropriate immune populations, while avoiding immunosuppression. A landmark study in ‘Nature cancer’ by Lin *et al.*, heralds cancer vaccines as the next frontier in immunotherapy (218). As discussed in earlier parts of this thesis, extensive pre-clinical and clinical evaluation of cancer vaccines has been conducted worldwide over the years, yet the full potential of this immunotherapeutic approach is yet to be harnessed.

One major limitation to the clinical success of cancer vaccines has been difficulties in identifying immunodominant epitopes capable of overcoming immunotolerance and eliciting robust tumor-specific immune responses. Another critical factor is the nature of the APCs that are targeted to present vaccine-antigens to T cells. A third factor to consider, is that the vaccine must overcome the immunosuppressive nature of the TME in a cancer context (386). Addressing these challenges with innovative strategies could position cancer vaccines as a promising future avenue for immunotherapy, offering a clear rationale and broad applicability across various cancer types and stages.

Advances in molecular biology and the increasing role of Artificial Neural Networks (ANN) in research are paving the way for cancer vaccines to become a viable form of personalized cancer therapy (387, 388). ANN-based immunoinformatics software now enables the prediction of effective antigens from individualized tumor genomic data. The use of lipid nanoparticle (LNP)-encapsulated mRNA-based vaccines also holds promise for improving target

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specificity (389, 390). However, it is essential not only to achieve these significant breakthroughs but also to carefully monitor the "baby steps" that lay a solid preclinical foundation.

These foundational steps include identifying immunodominant antigens, selecting potent adjuvants to elicit strong innate and adaptive immune responses, optimizing vaccine formulations and administration methods to enhance immune cell targeting, and reducing the immunosuppressive features of the TME. A significant portion of this thesis has been devoted to addressing these critical early-stage challenges.

In the first study (**Paper I**), we successfully employed an adjuvant, previously validated in mucosal vaccines against infectious diseases, within a cancer vaccine formulation for the first time. This formulation consisted of a fusion construct combining the CTA1-adjuvant, immunodominant epitopes from the model antigen OVA, and a single-chain variable fragment antibody targeting aCD103. The vaccine specifically targeted adjuvant and antigens to CD103<sup>+</sup> cDC1s, known for their superior cross-presentation abilities. The CTA1 adjuvant activated the DCs to efficiently present antigens, that promoted the expansion of OVA-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells with anti-tumor capacity. This study demonstrated the advantages of precise targeting in cancer vaccines and established a pilot model that can be adapted for various murine metastasis models using other immunodominant epitopes.

In the second study (**Paper II**), we explored the impact of vaccine formulation and administration routes on T cell-mediated immune responses. Our findings underscored the critical role of adjuvants in unlocking the immunodominant potential of endogenous tumor-associated antigens (TAAs), which are otherwise prone to immune tolerance mechanisms. Additionally, we observed that inhibiting reactive oxygen species (ROS) within the tumor

microenvironment (TME)—a known contributor to immune suppression—could enhance the efficacy of cancer vaccines.

The third study (**Paper III**) extended our understanding of the mechanistic pathways driving epithelial-mesenchymal transition (EMT). We demonstrated that myeloid-derived ROS induces the upregulation of EMT markers through an NRF2-dependent mechanism, linking ROS in the TME to EMT and, subsequently, metastasis. Notably, inhibiting ROS in the TME using histamine dihydrochloride (HDC) was able to counteract this process, revealing a promising therapeutic strategy for reducing metastasis. Furthermore, ROS inhibition within the TME could potentially complement cancer vaccines by preserving antigen-specific T cells while mitigating the immunosuppressive features of the TME.

Although further research is needed to fully elucidate the mechanisms of tumor metastasis and the role of cancer vaccines in prevention of metastasis formation, the studies presented in this thesis may offer promising preclinical models. They contribute to our understanding of inflammation for metastasis and highlight the potential of CTA1-adjuvanted, peptide-based cancer vaccines in targeting metastatic disease.

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