The use of light in cancer immunotherapy

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Chapter 1

General Introduction

The use of light in cancer immunotherapy

The development of treatment options for advanced cancer forms a major challenge in medical oncology. The breakthrough of immunotherapy for cancer has introduced promising new options, but nonetheless only a minority of cancer patients show clinical benefit. This situation has inspired two avenues of research to find solutions to this problem: mechanistic studies to decipher the working mechanisms of immunotherapies and to investigate why many patients do not respond, and translational studies developing combination treatments to achieve clinical benefit in situations where immunotherapy alone is not sufficient. This thesis explores both these avenues by investigating applications of visible light in immunotherapy of cancer. The first aim of this thesis is to develop optical imaging platforms for visualization of immune cells and immunotherapies, which can shed light on the immunological events after administration of immunotherapy. The second aim is to develop novel therapies combining light-based tumor destruction and different types of immunotherapies. The following paragraphs will discuss how the immune system can recognize and attack tumors, how immunotherapy aims to boost immune attack of tumors, and how light-based technologies can be applied in this context.

The immune system

The immune system comprises a set of cells and molecules that forms a defense system against disease, and can be subdivided into an innate and an adaptive immune system. The innate immune system is the only immune system in plants and insects, and provides an immediate but non-specific layer of defense. Jawed vertebrates, including humans and most animals used in biomedical research, have additionally developed an adaptive immune system characterized by slower but target-specific effector mechanisms, which moreover can establish memory to protect against future challenges with the same pathogen (1). Despite their distinct evolutionary origins, the innate and adaptive immune system collaborate in both the formation and the regulation of immune responses. To ensure robust immune defense while avoiding auto-immune disease, the adaptive immune system is trained to recognize its targets based on the distinction between self and non-self, distinguishing the body's own tissue from invading pathogens. It has now become clear that cancer cells can also be recognized by the adaptive immune system, as mutations in cancer cells cause deviation from 'self', rendering them susceptible to immune attack. The following paragraphs will discuss how cancer cells are recognized and attacked by the adaptive immune system.

T cells

T cells, also called T lymphocytes, form the cellular effector arm of the adaptive immune system. T cells are small lymphoid cells that are named after the thymus, a lymphoid organ that trains developing T cells to distinguish foreign elements from the body's own healthy tissue in order to avoid auto-immunity. Target-specificity, a core principle of the adaptive immune system, is mediated by the T cell receptor (TCR) complex on the cell membrane of T cells that specifically recognizes a specific peptide antigen in the context of MHC molecules on the surface of target cells. T cells acquire their TCR by gene rearrangement processes in the thymus, and are then exposed to positive and negative selection procedures that assure the deletion of T cells expressing a TCR that either has insufficient affinity for MHC to serve as functional T cells, or binds so strongly to MHC molecules presenting self-peptides that auto-reactivity may occur. Traditionally, two T cell subsets are distinguished based on the expression of either the CD4 or the CD8 co-receptor as part of the TCR complex, which are known as CD4 T cells and CD8 T cells, respectively (2, 3). Naïve T cells express a TCR recognizing a specific peptide-MHC (pMHC) complex, but cannot exert their effector functions until they are properly activated. T cell activation is mediated by the same mechanisms as T cell target recognition,

involving TCR recognition of the specific pMHC complex, but only when this pMHC complex is presented by professional antigen-presenting cells (APCs) (4, 5). Dendritic cells (DCs) are innate immune cells that are the most efficient professional APCs capable of activating T cells. DCs can engulf extracellular material and present epitopes in MHC class II molecules to CD4 T cells, which do not directly engage extracellular pathogens but aid the effector mechanisms of other immune cells, including macrophages and antibody-producing B cells, mostly by cytokines or cell-cell interactions. Because of their importance in helping other immune cells, CD4 T cells are also called T-helper (T_H) cells. Several classes of CD4 T cells exist, including T_H1, T_H2, T_H17 and the immunosuppressive subset of regulatory T cells (Tregs), which are characterized by the expression of distinct transcription factors, membrane markers and cytokines and are involved in shaping several different types of immune responses. CD8 T cells on the other hand recognize epitope in MHC class I molecules, which are loaded with peptides derived from intracellular antigens. Importantly, DCs are able to cross antigens from the endocytosis pathway to the MHC I pathway in a process called cross-presentation, allowing the activation of CD8 T cells specific for extracellular antigens engulfed by DCs (6). DCs present various extracellular and intracellular receptors that sense the tissue for signs of infection (or more generally, danger) and only in that case present co-stimulatory molecules on their membrane. Co-stimulation is crucial for proper T cell activation, forming an additional layer of security against autoimmunity besides the deletion of auto-reactive T cells during thymic selection, the presence of Tregs and the expression of suppressive co-inhibitory molecules such as CTLA-4 and PD-1. This means that a naïve T cell can only be activated by a DC that has sensed danger and has (cross-)presented non-self epitopes in the correct MHC class. These strict requirements of T cell activation are necessary to guard the body from unrestrained T cell responses that may lead to auto-immune disease.

Immune recognition of cancer

So far, the immune system has been described as a defense mechanisms against pathogens, which throughout our evolutionary history have indeed posed a major threat to our survival from early age on. In contrast, cancer is a disease that typically becomes clinically apparent and relevant at higher age, suggesting that cancer has played no role in the evolution of the immune system (7). However, research in the last decades has confirmed century-old observations that the immune system is nonetheless capable of recognizing and attacking cancer cells (8, 9). Tumors arise from normal cells of the body in which genes regulating proliferation and survival have become dysfunctional by mutations, leading to unrestrained proliferation.

Fortunately, mutations do not only drive tumorigenesis but also facilitate immune recognition, as mutated genes may give rise to new T cell epitopes (neo-epitopes) in formerly self-proteins (10-12). As all nucleated cells of the body continuously present peptides from intracellular proteins in MHC class I, mutations in cancer cells may thus lead to recognition and attack by CD8 T cells. The aforementioned process of antigen cross-presentation by DCs is required for successful activation of tumor antigen-specific CD8 T cells, since healthy DCs themselves do not contain the required intracellular mutated self-proteins for the classical MHC class I pathway. Instead, DCs can take up cellular material from dying tumor cells and cross-present tumor antigens to the MHC class I pathway, allowing the activation of tumor antigen-specific CD8 T cells (13). It is now generally established that T cell immunity is the primary immune effector system against tumors. Cancers with a higher mutation rate, particularly those induced by exogenous mutagenic factors such as sunlight (melanoma) and tobacco smoke (lung and bladder cancer), have been shown to contain more T cell neo-epitopes and are indeed best recognized and infiltrated by T cells (10). However, tumors still manage to escape initial recognition and clearance by T cells and develop into clinically apparent cancer. Two prominent mechanisms of immune evasion by tumors are down-regulation of tumor-antigen presentation and suppression of T cell functionality by maintaining an immunosuppressive tumor microenvironment (14-16). The hypothesis of immune-surveillance and immune-editing tells the co-evolutionary story of the shared history of tumors and the immune system: newly formed malignant cells are most often immediately recognized by the surveilling immune system based on their non-self features, whereas the few variants that possess or acquire evasion mechanisms are able to escape immune attack and continue to grow and reshape the tumor. The success of modern cancer immunotherapies is based on the induction and/or enhancement of T cell responses against the tumor (17).

Cancer immunotherapy

Tumor elimination by T cell immunity is especially challenging in the case of advanced cancer, in which tumors have successfully evaded immune clearance by preventing the induction or the functionality of T cell responses. Cancer immunotherapy comprises various different strategies to increase the number and the effector function of tumor-specific T cells, as these have the exclusive ability to recognize intracellular mutations in malignant cells. Prominent forms of cancer immunotherapy include the administration of exogenous tumor antigen (vaccination) and the blockade of immunosuppressive molecules or activation of immune-stimulatory molecules by administration of immunomodulatory antibodies.

Therapeutic vaccination against cancer involves the administration of tumor epitopes in the form of protein or peptide antigens, or of DCs pre-loaded with such antigens (18). Antigen vaccines are typically administered together with adjuvants to deliver danger signals to the DC, resulting in the expression of co-stimulatory molecules and ensuring proper T cell activation. Alternative methods of vaccine administration include antigen encapsulation into biodegradable nanoparticles, which protect the antigen from premature degradation and may also enhance delivery to DCs with the optional co-delivery of DC-activating signals (19-22). To restrict MHC presentation to professional APCs only, the concept of synthetic long peptide (SLP) vaccines was designed (23-26). SLP vaccines contain extra amino acid sequences flanking the T cell epitope, rendering them too large to be directly bound by MHC molecules. Instead, only DCs can take up the SLP and (cross-) present it into MHC class I and II molecules. It was shown that SLP vaccination is most efficient when both CD8 and CD4 T cell epitopes are included in the vaccine (24). Moreover, SLP vaccines lead to better antigen uptake, processing and presentation than full protein vaccines (27). An SLP vaccine consisting of a set of overlapping peptides covering the E6 and E7 oncoproteins of human papillomavirus 16 (HPV16) has been successfully applied in patients with HPV16-induced pre-malignant lesions, but it was not clinically effective against advanced HVP16-induced cancer (28-30). Improved efficacy of SLP vaccination has been shown by combination with conventional cancer therapies and by conjugating Toll-like receptor (TLR) ligands to the peptide (31, 32). Importantly, therapeutic peptide vaccination is not limited to cancer types involving widely shared antigens as in the case of HPV-induced cancer, as shown by recent studies targeting neo-epitopes with individually designed peptide vaccines (33-37).

Unlike SLP vaccination, immunomodulatory antibodies (IMAbs) in cancer immunotherapy boost anti-tumor T cell immunity in a non-antigen-specific manner. IMAbs are directed against molecules that regulate T cell activation and/or effector function, and may be agonistic or blocking antibodies depending on the role of the targeted molecule in the immune response (38, 39). All currently FDA-approved IMAbs are blocking antibodies targeting the immune checkpoint molecules CTLA-4 and the receptor-ligand pair PD-1 and PD-L1. CTLA-4 is expressed on T cells and may regulate both T cell priming and effector function, and aid the suppressive function of a CD4 T cell subset called regulatory T cells (Treg) (40-42). Impressive results in metastatic melanoma patients treated with CTLA-4 blocking antibody established the prominent position of immune checkpoint blockade as a form of cancer immunotherapy (43). PD-1 is an inhibitory receptor expressed on activated T cells which upon ligation by PD-L1 induces T cell apoptosis (44, 45). PD-L1 can be expressed on various cell types including cancer cells and tumor-infiltrating myeloid

immune cells (46). Blockade of the PD-1/PD-L1 axis by antibodies was clinically effective in a range of cancer types including melanoma and non-small cell lung cancer (NSCLC) (47-49). Combinations of PD-1 and CTLA-4 blocking antibodies were shown to further improve clinical responses, supporting the hypothesis that tumors may evade single IMAb treatment by applying alternative immunosuppressive molecules (50-53). Agonistic IMAbs are currently in clinical trial following promising pre-clinical results targeting the DC-activating molecule CD40 or T cell co-stimulatory molecules such as CD137 (4-1BB), OX40, ICOS and CD27 (54, 55).

Optical imaging

Optical imaging has a wide range of applications in biomedical research, all comprising the measurement of optical signals from cells, tissues or living animals. Live *in vivo* optical imaging is of particular interest as a non-invasive strategy to follow physiological or experimentally-induced processes in time within an individual experimental animal. The source of the optical signals can be fluorescent molecules which emit light after being excited by an external light source (fluorescence imaging, FLI), or luciferase enzymes which produce light as a product of a chemical reaction converting an administered substrate (bioluminescence imaging, BLI) **(Figure 1)**. These two forms of optical imaging each have their advantages and disadvantages (56). For instance, FLI allows the administration of fluorescent dyes into living animals, either as such or conjugated to experimental reagents, after which the fate of the administered molecules can be tracked in real-time. Fluorescent molecules do not intrinsically produce photons, but need to be excited by an external light source, and then absorb the energy of the incoming photons and subsequently emit photons of a slightly lower energy (i.e. higher wavelength), which form the actual signal of fluorescence imaging. Since the source of optical signals in whole-body imaging may be relatively deep, photons may be absorbed by the tissue they have to pass during excitation and emission. As photons with higher wavelength are less likely to be absorbed, the most commonly used fluorescent dyes for *in vivo* FLI are near-infrared (NIR) dyes whose wavelength lies slightly above the human visible spectrum. In BLI, photons are produced intrinsically by luciferases, which therefore do not need external energy sources for excitation (57, 58). The most commonly used luciferases have been isolated from animals including the firefly (*Photinus pyralis*), which also produce the substrate to fuel the light-producing reaction. Instead, biomedical BLI systems require the introduction of the luciferase gene into cells or animals by transfection or transgenesis, and the administration of substrate prior to imaging. Besides the extra technical effort, this gives the advantage of placing luciferase gene expression under the control of a

promoter of interest, enabling protein-specific and cell type-specific analysis by BLI. The enzymatic reaction of luciferases requires ATP and oxygen, thus the context of a living cell, allowing the use of BLI for cell viability assays. Moreover, luciferases have a relatively photon quantum yield compared to fluorescent dyes, allowing the detection of low amounts of cells. However, the higher quantum yield of luciferases is counteracted by the fact that luciferases emit light within the human visible spectrum, which is more prone to absorption by tissue than NIR fluorescent dyes. The choice between FLI and BLI will therefore depend on the characteristics of the experimental model in which they are to be applied.

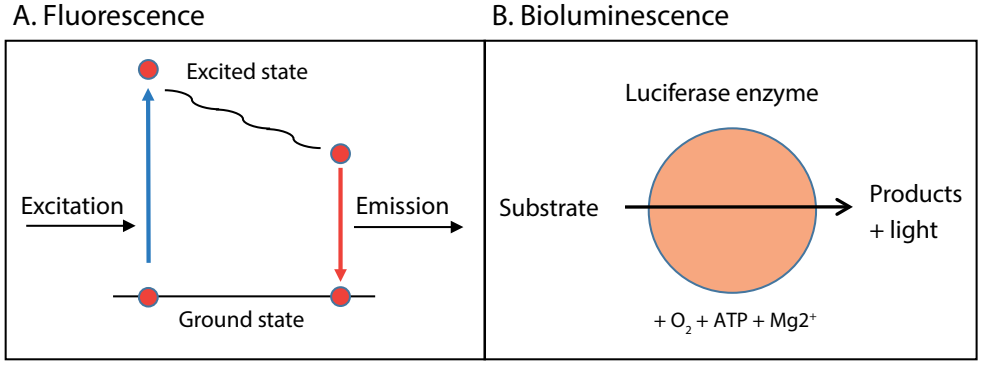

Figure 1. Optical imaging of fluorescent or bioluminescent molecules. (A) Fluorescence is the result of excitation of a fluorescent molecule, causing its electrons to reach the higher-energy excited state, after which they return to ground state, releasing the energy by the emission of light (emission) that can be measured by fluorescence imaging (FLI). Many fluorescent molecules exist in nature, but they do not produce light without a light source to excite them. **(B)** Bioluminescence is an enzymatic reaction of luciferase enzymes, fuelled by cellular ATP and co-factors, producing an oxidized product and visible light, which can be measured by bioluminescence imaging (BLI). Several animals produce luciferase enzymes that, as luciferase reactions are independent of external energy sources, can truly glow in the dark.

Photodynamic therapy

Besides measuring optical signals from biological samples, light can also be used to induce changes in cells and tissues. It is commonly known that exposure to light can directly influence the human body, such as skin pigmentation induced by the UV waves of sunlight, and regulation of the circadian rhythm by light exposure to the eyes. The ancient Indian and Chinese civilizations had already discovered that the application of certain plant extracts to the skin caused dramatic reactions to the skin following exposure to sunlight (59). In the early 20th century, the molecular basis of this 'photodynamic effect' was established. The photosensitive molecules could be isolated, but did not have any obvious effect on a protozoa culture unless exposed to a dose of light that by itself was also harmless. Moreover, oxygen was shown to be required for the photodynamic effect, and the mediators of the effect were extremely short-lived. Although the potential medical applications were realized

at the time, it took over 60 years until a cohort of patients with various types of cancer was treated with Photodynamic Therapy (PDT) showing generally positive results (60). Since then, several photosensitizers have been approved for a range of diseases including both cancer and benign skin conditions. In PDT of cancer, a photosensitizer is administered systemically or applied to the tumor topically, typically followed by a pause of several hours to allow photosensitizer distribution throughout the tumor, before the tumor is exposed to light. The light exposure excites the photosensitizer, which reacts with available oxygen to form the oxygen radicals that are the mediators of the cytotoxic effect of PDT (61). The resulting cancer cell death will alleviate tumor burden, but may also provide the tumor antigen and danger signals required to induce a tumor-specific T cell response (62, 63) **(Figure 2)**. This motivates combination therapies of PDT and immunotherapy to enable successful treatment of advanced tumors for which monotherapies are insufficient.

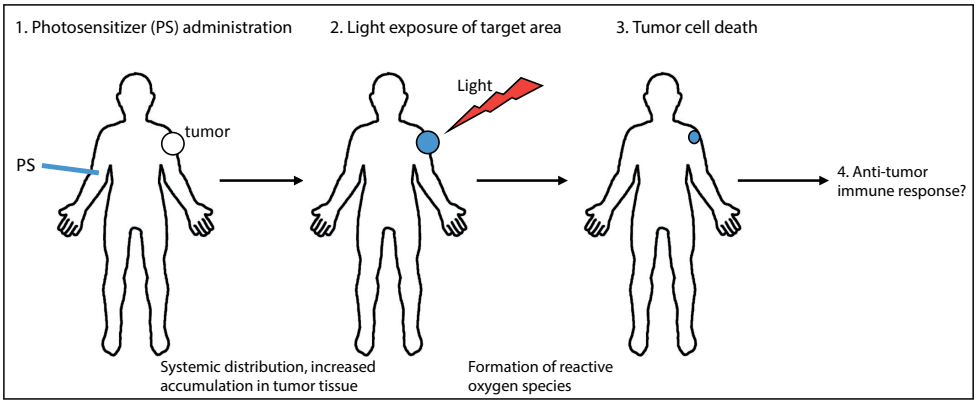

Figure 2. Photodynamic therapy of cancer involves several steps. Typical Photodynamic Therapy protocols involve the following steps. Step 1: systemic administration of a photosensitizer (PS), when then distributes through the body. Tumor cells may take up higher PS levels due to increased expression of lipid receptors on the membrane. Step 2: the PS is selectively activated in the tumor by exposing the tumor to visible light, which excites the PS and results in the formation of reactive oxygen species. Step 3: immediate and local damage to the plasma membrane and organelle membranes leads to tumor cell death. Step 4: massive tumor cell death may lead to the exposure of tumor (-associated) antigens and pro-inflammatory molecules to the immune system, which can induce and/or enhance anti-tumor immune responses.

Outline of this thesis

This thesis shows several different ways of using light in cancer immunotherapy. In **chapter 2**, we investigate combination therapy of PDT and therapeutic SLP vaccination in two aggressive mouse tumor models using an experimental setup in which neither monotherapy is able to eradicate the tumor. Besides following tumor outgrowth as the primary outcome parameter, we analyze the ability of single and combined therapy to induce CD8 T cell responses and the effect on distant identical

tumors. In **chapter 3**, we assess the efficacy of PDT in highly mutated tumor models that express several neo-epitopes that may be recognized by the immune system. We test whether T cells are involved in the effect of PDT and whether distant tumors are also affected. The effect of addition of CTLA-4 blocking antibody is investigated as a potent combination strategy without the need to know the neo-epitope profile of the individual tumor. In **chapter 4**, we test the feasibility of SLP vaccine tracking after vaccination by live *in vivo* fluorescence imaging using peptides labeled with a NIR fluorescent dye. We test whether NIR fluorescent dyes allow long-term vaccine visualization of the vaccination site and the vaccine-draining lymph nodes, and quantify the fluorescence signals at these sites to gather information on vaccine kinetics. In **chapter 5,** we use a similar approach to follow a model protein encapsulated in nanoparticles as a biodegradable delivery system for vaccines. Two fluorescent dyes are applied to independently visualize the nanoparticle carriers and the encapsulated protein vaccine, and the ability of encapsulated protein versus soluble administration to induce vaccine-specific CD8 T cell activation is assessed. In **chapter 6**, we show a T cell luciferase transgenic mouse that allows live *in vivo* visualization of T cells by bioluminescence imaging. We developed a dual-luciferase system where one luciferase is expressed constitutively and exclusively in T cells to report on the location of all T cells, while another luciferase is only expressed upon T cell activation to visualize T cell responses. Finally, **chapter 7** provides a general summary and discussion of the results reported in this thesis.

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