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The use of light in cancer immunotherapy

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Chapter 7

Summary and General Discussion

The use of light in cancer immunotherapy

Decades of research have established the field of oncoimmunology and resulted in the new paradigm stating that tumors co-exist with the immune system throughout their life (1,2). Avoidance of immune clearance is now recognized as a major hallmark of cancer (3). As mutations may not only provide tumor cells their unique proliferative capacities but also introduce non-self characteristics, the immune system manages to eliminate many early developing tumors. This means that cancer cells that managed to progress to clinically apparent tumors either were not sufficiently recognizable by the immune system, or managed to actively suppress and evade immune attack. Cancer immunotherapy aims to facilitate immune attack by inducing new immune responses and/or boosting the efficacy of existing immune responses. Most forms of cancer immunotherapy are focused on T cells (4), as these are the most prominent immune cells capable of tumor cell killing based on their ability to recognize mutated self-proteins processed and presented in MHC molecules. Unfortunately, their efficacy in doing so can be heavily influenced by other immune and non-immune cells (5,6). While impressive clinical responses have been observed in some patients after cancer immunotherapy, many patients do not respond to therapy, which has raised the need to better understand immune evasion mechanisms and to develop superior (combination) therapies (7–9). This thesis shows several examples of how these two needs can be directly or indirectly addressed. Light plays a central role in all these examples, either as a diagnostic tool for live in vivo visualization of relevant cells and molecules during cancer immunotherapy, or therapeutically as an external energy source that triggers the ablation of tumors.

Immunogenic tumor ablation

Advanced cancer generally responds worse to therapy than early lesions. Advanced tumors are not only be bigger in size, but also have a longer evolutionary history of immune pressure and therefore had more time to develop immune suppression mechanisms. From this perspective, tumor ablation therapies that cause immunogenic cell death are attractive treatment options for advanced cancer, as they both reduce tumor burden and may induce or enhance anti-tumor immune responses. In comparison to other tumor mass-reducing protocols such as chemotherapy and radiotherapy, Photodynamic Therapy (PDT) is known for its high target-specific toxicity and limited side effects, and gaining recognition among surgeons for a tissue recovery with minimal scarring, when compared to surgery (10). PDT involves the administration of an inert photosensitizer which is then to be activated by exposure to light, using laser fibers or lamps as a light source. PDT

in clinical practice is therefore mostly applied to tumor types that are relatively accessible for the light source, such as skin lesions and tumors in the gastrointestinal and reproductive tract. However, many more cancer types can potentially be treated by PDT using interstitial fibers in a surgery setting. In this dissertation, we described two combination protocols of PDT and immunotherapy in preclinical models of advanced cancer. In chapter 2, combined treatment of tumor-bearing mice with PDT and therapeutic SLP vaccination could eradicate local (PDT-treated) and distant tumors, where either monotherapy showed limited efficacy. PDT induced and enhanced tumor antigen-specific CD8 T cell responses, which were necessary for the therapeutic effect (11). Chapter 3 described combination therapy of PDT and CTLA-4 immune checkpoint blockade in two highly mutated tumor models, and also showed superior efficacy of combination treatment in mice bearing two established tumors, fully dependent on the presence of CD8 T cells. Both studies emphasize the immunogenicity of tumor ablation by PDT, thereby functioning as an in situ vaccination, and suggest that this feature makes it an ideal combination partner for immunotherapy of large tumors.

The potential immunogenicity of PDT has been described before, most prominently by the group of Michael Hamblin, who urged collaborations between PDT experts and tumor immunologists in the search for combination treatments with immunotherapy (12). We believe that the combination regimens investigated in this thesis are good candidates for clinical application, as they combine PDT with clinically applied immunotherapeutics (7,13,14). Moreover, the two different combination protocols correspond to two different classes of human cancer. Tumors induced by oncogenic viruses can express known viral T cell antigens that are widely shared among patients, which allows antigen-specific immunotherapy. Our findings in chapter 2 indicate that if tumor antigens are known, combination treatment of PDT and SLP vaccination can provide a highly tumor-specific treatment. The enhanced clearance of distant secondary tumors suggests that spontaneous metastases, known or unknown, will also be attacked. Nonetheless, the majority of cancers arise not from oncoviruses but from familial or acquired mutations, in which case patients display a (mostly) unique tumor-antigenic profile. It is technically feasible to identify mutated T cell epitopes in every individual patient and produce personalized therapeutic peptide vaccines (15,16). However, alternative treatment strategies may be more appropriate, especially in those cancer types with a high mutational burden, which has been suggested to correlate to a higher neo-antigen load (17). The combination protocol described in chapter 3, using CTLA-4 immune checkpoint blockade, can be widely applied regardless of the tumor antigen makeup. The disadvantage of applying potent non-specific immunostimulatory treatments is the inevitable occurrence of off-target toxicity (18). Previous work in our group has

addressed this issue by supplying CTLA-4 blockers locally, strongly reducing toxicity while therapeutic efficacy is maintained (19).

Alternative ablation therapies have been shown to mediate comparable immunogenic effects as we describe here for PDT, and several preclinical studies have shown successful combinations with immune checkpoint blockade (20–23). Although several photosensitizers have been clinically approved, PDT has much fewer applications as a standard-of-care compared to other ablative therapies such as chemotherapy and radiotherapy. One might therefore argue that it is attractive to opt for combinations of chemotherapy or radiotherapy with immunotherapies to accelerate clinical translation. However, systemic immune stimulation by checkpoint inhibitors, as it is currently commonly applied, often already cause substantial side-effects. It may therefore be preferred to avoid increasing the therapeutic burden much further, as would happen considerably by radiotherapy and especially chemotherapy. From this perspective, the highly focused nature of PDT favors combinations of immunotherapy with PDT, which as a monotherapy is associated with only mild side-effects such as painful skin at the target site during treatment (24). Moreover, there are no indications that PDT treatment itself can be mutagenic, as is the case for both chemotherapy and radiotherapy. We believe that our studies have provided proof of concept of PDT-immunotherapy combinations, but additional preclinical and clinical studies are required to assess its broader clinical applicability.

Visualizing vaccines by optical imaging

Optical imaging of fluorescence or bioluminescence is a widely used technique to study biological or experimentally-induced processes in vitro and in vivo. Excellent technologies have been developed to visualize biomolecules, track cells, quantify bioactivities and study kinetics of physiological processes in vivo. Fluorescent dyes are usually coupled to the biomolecules of interest prior to administration, while bioluminescence is a feature of luciferase enzymes for which the coding gene can be introduced into the genome in order to visualize the cells of interest. Fluorescent dyes and bioluminescent enzymes therefore each have their own advantages and disadvantages and are applied accordingly (25). Compared to optical imaging, alternative techniques to visualize injected molecules offer some advantages, such as excellent spatial resolution in the case of MRI, and absolute quantification using nuclear imaging (26–28). Nonetheless, fluorescence imaging is often preferred and applied as it is non-invasive, fast and cost-effective (29,30). In particular, near-infrared (NIR) fluorescent dyes are preferred because light of NIR wavelength has superior tissue penetration compared to visible light, allowing the detection of signals from

deeper layers of the body or sample. Chapter 4 describes a feasibility study of SLP vaccine tracking using a conjugated NIR fluorescent dye. By live optical imaging, we visualized this NIR-SLP vaccine at the injection site and in vaccine-draining lymph nodes. The applicability of this vaccine imaging platform was assessed by formulating the NIR-SLP in several different pharmacological formulations, whose expected distinct kinetics were adequately identified by NIR fluorescence imaging. Similarly, chapter 5 describes the tracking of NIR-labeled protein vaccines encapsulated in biodegradable nanoparticles (NP), which were labeled which a different NIR dye to allow independent visualization of vaccine and carrier. Fluorescence imaging showed that encapsulated protein is retained longer at the injection site and accumulated more gradually in the draining lymph nodes, when compared to soluble protein in saline. NPs and protein had overlapping kinetics, suggesting that the vaccine is transported while still being encapsulated, rather than having been released at the injection site. Both studies showed that NIR-labeling of the vaccine does not affect its ability to achieve its ultimate functional purpose, the activation of CD8 T cells. These studies illustrate that NIR-labeling allows live vaccine visualization and quantification, which can be used as tool to address scientific research questions on vaccine functionality as the systemic localization and quantity of the vaccine may be determinants of its eventual therapeutic efficacy (31–33). Further studies using other NIR-labeled SLPs are required to determine whether our findings using a model SLP of chicken ovalbumin are generally applicable, or were instead partly or fully influenced by the specific characteristics of the chosen peptide (34). The safety and applicability of our NIR dye has been shown in a clinical study using a clinically applied and NIR-conjugated SLP vaccine, which could be visualized after administration and did not cause adverse effects. The experimental approach of vaccine delivery by nanoparticle carriers as applied in chapter 5 was chosen based on several potential advantages over soluble vaccine administration. For instance, non-adjuvanted soluble antigen is weakly immunogenic, which can be improved by carrier systems that can deliver antigen vaccines to antigen-presenting cells, and may also protect the protein from undesired degradation (35,36). Additional opportunities are co-encapsulation of adjuvants with the antigen and attaching targeting molecules on the surface (37,38). Moreover, unlike the widely used oil-in-water emulsions as alternative sustained-release formulations, nanoparticles can be labeled and imaged as we have described. To show the concept of dual color NP-vaccine imaging we made us of the model protein chicken ovalbumin for practical reasons regarding NIR labeling and encapsulation. As previous work in our group has shown that SLP vaccines are therapeutically superior to protein vaccines, it would be of interest to study NIR-labeled peptide vaccines encapsulated in nanoparticles and attempt to correlate kinetics to functionality (39).

Dual-color T cell imaging

Chapter 6 introduced the new T cell luciferase transgenic mouse termed TbiLuc, as a tool for bioluminescence imaging of T cell location and activation status using two separately controlled luciferases. T cells are involved in a wide variety of physiological and pathological conditions and are highly mobile due to their ability to differentially express homing ligands that determine their localization in the bloodstream or entry in peripheral lymphoid and non-lymphoid tissues (40,41). Therefore, there is substantial interest among researchers in being able to measure the presence of T cells throughout the body in time, which is conventionally solved by the laborious process of sacrificing experimental animals at different time points and analyzing T cell counts in different organs ex vivo. Our study is the first to address this issue by creating a T cell dual-luciferase transgenic mouse. Earlier attempts to apply bioluminescence imaging (BLI) for T cell tracking mostly used retroviral vectors carrying luciferase genes to transduce T cells, which requires T cell activation and is not very efficient (42–44). Several groups have solved this issue by creating T cell luciferase transgenic mice to supply a large pool of untouched luciferase-expressing T cells, but all used only one constitutively expressed luciferase (45–47). The basic concept of dual-luciferase T cell imaging was first shown by Na et al., who used an indirect approach of lentiviral transduction in bone marrow to create chimeric mice as a source of naïve T cells (48). We used a more straightforward approach to limit the constitutively expressed luciferase to T cells by coupling it to the human CD2 promoter, which in mice is specific to T cells (49). The two luciferases in the TbiLuc construct, termed CBG99 (click-beetle green) and PpyRE9 (a red mutant of the common firefly Photinus pyralis), both function with the common D-luciferin substrate. However, the considerably higher quantum yield of CBG99 proved problematic in simultaneous dual-color measurements in mice, as the emission of PpyRE9 could not be separated from the bulky CBG99 emission. Therefore, the novel cyclic substrate CycLuc1 was used (50,51), which produces a stronger PpyRE9 signal than D-luciferin, while the CBG99 signal was nearly absent. As simultaneous imaging using mixed substrates reduced the amount of signal, since substrates compete for the active sites of the luciferases, we used a sequential imaging setup and visualized T cell activation and expansion in an in vivo vaccination model. Although sequential imaging depicts the biological situation with a 3 hours difference due to the substrate washout time, which has to be taken into account when studying T cell processes with fast kinetics, the two relevant biological parameters of localization of T cells and their activation status can now be clearly distinguished. In conclusion, the TbiLuc transgenic mouse is a suitable model to visualize endogenous T cell responses, with possible applications in many fields such as vaccinology, cancer immunotherapy including T cell reinvigoration

by immune checkpoint blockade, auto-immune disease and transplantation. In addition, crossing TbiLuc to T cell receptor-transgenic mouse strains produces large numbers of light-producing cells of known antigen specificity, usually applied in adoptive T cell transfer models. This transgenic mouse model can be widely applied in immunology research, as T cells play a role in many physiological or pathological processes. One example is the field of cancer immunotherapy, due to the crucial role of T cells in the killing of malignant cells. The efficacy of cancer vaccines can be assessed by measuring T cell activation and expansion following vaccination. Similarly, reactivation of suppressed T cells in cancer models can be visualized upon treatment with immunomodulatory antibodies. Other applications lie in models of viral infection, transplantation and autoimmunity.

Concluding remarks

This manuscript has illustrated several applications of light in the field of cancer immunology and immunotherapy. The luciferase enzymes used in our studies are derived from animal species that naturally express them, which has sent researchers into the woods with a catching net searching for a new undiscovered enzyme. Visible light drives the physicochemical reaction underlying the therapeutic effect of PDT, whose potential was already observed by ancient civilizations. Given the vast amount of time that people have spent in the sun throughout human history, and nowadays would still like to, it is important to realize that light can have substantial effects on the human body. We should understand both its dangers and its benefits, and use this knowledge to our advantage.

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Appendices

Nederlandse Samenvatting

Dankwoord

Curriculum Vitae

List of publications