Everyone works better together: rational improvements to radio- and immunotherapy combinations

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General Introduction & Scope of thesis

General introduction

The T cell response to cancer

$CD8^+$ and $CD4^+$ T cells can defend higher organisms against pathogens and cancer through the recognition of foreign ("non-self") molecules, called antigens. $CD8^+$ T cells are particularly effective in targeting tumors, because they can identify intracellular antigens presented by MHC Class I molecules that are expressed on all cell types, including all tumor variants. $CD8^+$ T cells differentiate into cytotoxic T lymphocytes (CTL) upon activation that can directly kill infected or cancer cells. In contrast, $CD4^+$ T cells recognize antigens presented in the context of MHC Class II, which is predominantly expressed on antigen-presenting immune cells (APCs). They can therefore directly recognize only certain cancers and are primarily involved in immunomodulatory functions. $CD8^+$ T cells are activated (primed) in secondary lymphoid organs and after clonal expansion and effector differentiation, go into the blood, from where they can reach and infiltrate infected or cancerous tissues. For successful cancer immunotherapy, a durable and self-sustaining anti-tumor $CD8^+$ T cell response is crucial. Efficient priming of effector T cells relies on the activity and migration of dendritic cells (DCs) from the tumor[1]. DCs encompass three different lineages, including plasmacytoid (p) DCs and conventional (c)DC type 1 (cDC1) and type 2 (cDC2). cDC1s and cDC2s are discerned in migratory and lymph node-resident subsets[2]. cDCs are specialized in engulfing infected cells and cellular debris, which they process and present as antigens via MHC Class I and II molecules to $CD8^+$ and $CD4^+$ T cells, respectively. cDCs need to be adequately activated to express specific costimulatory molecules and cytokines to induce (prime) $CD8^+$ and $CD4^+$ T cell responses. Activation of DCs in cancer depends on the release of danger-associated molecular patterns (DAMPs) from dying cancer cells. Additionally, cDCs require "help" or licensing from $CD4^+$ T cells through MHC Class II-mediated interaction to fully mature[3-5]. $CD4^+$ T cell help equips $CD8^+$ T cells with effector and memory functions required to overcome negative regulation[6,7]. Intravital imaging argues that priming of effective $CD8^+$ T cell immunity occurs in two steps: The initial priming step takes place separately for $CD8^+$ and $CD4^+$ T cells in distinct areas of lymphoid organs, involving cDC1s and cDC2s, respectively. Subsequently, $CD8^+$ and $CD4^+$ T cells interact with the same lymph node-resident or perhaps migratory cDC1, where $CD4^+$ T cells provide their help signals allowing for effector $CD8^+$ T cell differentiation to occur[5,8,9]. cDCs are crucial in determining whether a response by conventional $CD8^+$ and $CD4^+$ T cells (Tconv) occurs. Insufficient DC activation can lead to tolerogenic mechanisms, including the development of non-responsive (anergic) T cells or the priming of regulatory $CD4^+$ T cells (Tregs)[10]. Tregs promote immune tolerance by attenuating conventional T cell (Tconv) responses through inhibition of cDC activation and migration[11,12] or by suppression of effector T cell function[13].

Thus, a series of intricate and carefully orchestrated steps is required to mount an effective anti-tumor T cell response. Several bottlenecks commonly hinder this response, including the lack of recognizable non-self antigens, insufficient presence of T cells with tumor antigen-specific T

cell receptors (TCRs) in the patient's repertoire, inadequate abundance of cDCs in the tumor, or inadequate activation of cDCs, and effector T cell suppression in the tumor micro-environment (TME). Targeting of these bottlenecks has been the focus of current cancer immunotherapy strategies and has resulted in a surge of clinical trials in the past years[14,15]. However, only a small fraction of patients with solid tumors benefit from these treatments[16], emphasizing the requirement for a better understanding of the immunogenicity and immune responsiveness of cancers.

Interplay between tumors and the immune system

Recent advances in multi-omic analysis of human cancer have provided valuable insights into the relationship between tumors and the immune system and the impact of the immune response on patient survival[17,18]. These studies have revealed that tumors of the same type, e.g. non-small cell lung cancer (NSCLC) or colon cancer can present a wide range of immune constellations that can be favorable or unfavorable in terms of overall patient survival and response to immunotherapy. Generally, lymphocyte paucity, in combination with high levels of fibroblasts and/or myeloid cells, is typically associated with a higher mortality rate, whereas a high abundance of lymphocytes, often accompanied by elevated levels of DCs and robust IFN responses, is strongly linked to improved response to antibody-based immunotherapy and overall survival[17,18]. These "inflamed" tumors usually meet the required conditions for T cell priming in the TdLN, but suboptimal priming, combined with immunosuppression in the TME often impairs their activity, resulting in T cell exhaustion[19]. In such cases, antibody-based blockade of the inhibitory pathways imposed by PD-(L)1 or CTLA-4 can restore anti-tumor T cell responses[20,21]. In contrast, tumors lacking T cells generally experience immunosuppression or immune ignorance at an early stage in development, rendering them unresponsive to the same immunotherapy approaches. For these tumors, strategies should focus on inducing T cell priming rather than overcoming immunosuppression in the TME[22].

Tumor progression not only affects the local immune landscape but also triggers systemic immune alterations[23]. This leads to a state of general immunosuppression, typically due to the mobilization of immunosuppressive myeloid cells in the host, creating a favorable environment for tumor metastasis to distant organs[24]. The tumor-draining lymph nodes (TdLNs), acting as downstream lymph draining site for (tumor) tissues, are often the first affected sites. This results in significant immunosuppression that hinders effective tumor-specific T cell responses[25], presenting another hurdle for effective cancer immunotherapy.

Cancer immunotherapy

Immunotherapy strategies are designed to overcome barriers that impede effective anti-tumor T cell responses. Among these, therapeutic vaccination aims to expand the pool of tumor-reactive

T cells. This is achieved by incorporating either tumor-associated "self" antigens like cancer-testis antigens, or "non-self" antigens including virus-derived antigens and tumor-specific neoantigens[26]. However, clinical benefit of vaccination is limited by challenges in antigen identification (for both $CD8^+$ and $CD4^+$ T cells), inadequate MHC class I expression on tumors, local and systemic immunosuppression and tumor burden[26]. To overcome these hurdles, adoptive cell therapy (ACT) with autologous or allogeneic tumor-specific T cells was proposed as a promising strategy[27]. ACT involves infusion of highly active effector T cells, and recent advances in genetic engineering of tumor-specific TCRs and the development of chimeric antigen receptor (CAR)-T cell have further improved its effectiveness, particularly in hematopoietic malignancies. However, outcomes with ACT depend on the presence of defined tumor antigens, the availability of tumor-specific T cells with the appropriate functional capabilities and resistance to exhaustion, and have a risk of life-threatening toxic off-target effects[28].

Immunomodulatory antibodies, commonly known as immune checkpoint blockade (ICB), like anti-PD-(L)1 and anti-CTLA-4 alleviate suppression imposed on tumor-specific T cells existing within patients. This approach presents a potential solution to lack of defined tumor-specific antigens. The co-inhibitory receptors PD-1 and CTLA-4 attenuate CD28 costimulation[29,30], required to support T cell division, metabolism, and survival[31] through distinct mechanisms. PD-1 dampens CD28 costimulation by binding to its ligands PD-L1 or PD-L2, which are presented by cDCs. This interaction causes the recruitment of SHP2 tyrosine phosphatase to PD-1, which leads to CD28 dephosphorylation and subsequent signaling inhibition[32]. CTLA-4, constitutively expressed on Tregs and upregulated by Tconvs post activation, downregulates the CD28 ligands CD80 and CD86 from the cell surface of cDCs[30]. Thereby, it plays a crucial role in peripheral tolerance by establishing Treg-mediated inhibition of the priming of self-reactive T cells[33]. Notably, PD-(L)1 blockade can act in the TME, but also promotes T cell priming in the TdLN[34,35]. Impressive curative responses following CTLA-4 blockade were observed in melanoma[36], whereas PD-1 inhibition achieved significant successes in non-small-cell lung cancer[37], renal carcinoma[38], and head and neck squamous carcinoma[39]. Additionally, the combination of CTLA-4 and PD-1 blockade can be synergistic, as was first shown in melanoma[40] and has yielded considerable successes in other difficult-to-treat cancers, including mismatch-repair proficient colorectal cancer[41] and advanced esophageal cancer[42]. However, the mechanism underlying this synergistic response has not yet been fully elucidated[21,43]. Despite these successes, response rates for most solid tumors are disappointing[16], which can be attributed to the lack of pre-existing T cell responses in these tumors. Thus, especially for cancers displaying robust immunosuppression, immunotherapy approaches should aim to 1) evoke an effective, preferably endogenous, anti-tumor T cell response without the requirement for prior identification of tumor antigens, and 2) overcome prevailing immunosuppression. To achieve this, ICB may be combined with radiotherapy (RT)[44].

Radiotherapy

RT is a common cancer treatment given to over 50% of patients for curative or palliative purposes[45]. RT inflicts DNA damage and selectively targets tumor cells, owing to their high proliferation rate and frequent loss of DNA repair capabilities. This leads to either a permanent cell cycle arrest or tumor cell death[46]. In contrast to chemotherapy, RT is applied locally to the tumor field and thereby offers the advantage of reduced off-target toxicity to healthy tissues. Moreover, RT not only causes tumor cell death, but also induces local immunomodulatory effects. For example, RT targets endothelial-cellular junctions and enhances the expression of adhesion molecules, potentially resulting in improved permeability and enhanced infiltration of circulating immune cells into the TME[46]. Additionally, RT may expose immunogenic neoantigens by upregulating mutated gene expression[47,48] and it can potentially increase the expression of MHC Class I on tumor cells[49]. Furthermore, depending on the anatomical location, proliferative state, and level of differentiation, RT may also target other cellular components in the TME, including immunosuppressive cells[46]. Thus, in addition to reducing tumor size, RT can create a more immune-permissive environment, potentially reducing the likelihood of local tumor recurrence.

Radiotherapy and immunogenicity

RT has gained increasing interest for its potential effects on systemic anti-tumor immunity. In 1953, Robin Mole observed that local RT may result in anti-tumor effects outside the field of treatment, described as the abscopal effect[50]. The contribution of the immune system to this effect was not identified until 1979, when it was observed that an intact immune system was crucial for RT-induced tumor control[51]. Many years later, the abscopal effect to RT was shown to depend on the recruitment of effector T cells to the tumor[52]. However, the fact that RT could support T cell priming remained unknown for two decades until the groundbreaking study by Lugade, et al.[53], who demonstrated that local RT supports the differentiation of tumor-specific effector T cells in the TdLN. These studies not only emphasized the importance of T cells for achieving curative effects with RT, but also highlighted the potential of RT to generate new T cell responses.

Upon causing tumor cell death, RT may release cellular debris, including antigens and DAMPs required to achieve DC maturation and migration to the TdLN[54]. Specifically, RT-induced DNA damage activates the nucleic acid sensor cGAS in tumor cells, leading to the activation of the Stimulator of Interferon Genes (STING) pathway and subsequent production of type I interferons (IFN-I)[55,56]. Release of IFN-I is crucial to improve the activation and migration of DCs[57]. Thus, RT can essentially kickstart the cancer-immunity cycle to promote endogenous anti-tumor T cell responses[58]. In principle, this may be further supported by concomitant ICB, but the combination of RT and such immunotherapy thus far has not consistently achieved combined curative

responses[44]. This is likely primarily due to our limited understanding of the mechanisms by which RT affects immune responses in tumors with varying immune complexities. Specifically, spontaneously immunogenic tumors containing tumor-specific T cells in the TME prior to treatment may exhibit intratumoral T cell activation upon RT alone[59] or in combination with ICB[60]. This may lead to regression of the irradiated tumor without the requirement of a systemic T cell response. In contrast, tumors lacking tumor-specific T cells in the TME prior to treatment, require for regression the development of a RT-induced anti-tumor T cell response in the TdLN.

Various factors limit the effectiveness of RT in eliciting systemic anti-tumor T cell responses, including the lack of RT-induced release of antigens and DAMPs[61,62], insufficient recruitment of DCs[63] and immunosuppression in the TME[64]. Additionally, RT may cause the upregulation of inhibitory molecules, including PD-L1 and CD73[63,65,66], further contributing to immunosuppressive effects that could limit curative responses. Importantly, these factors are often overlooked or diluted in pre-clinical mouse studies, as these typically take advantage of tumor models that contain dominant, non-self antigens and therefore poorly reflect human cancers[53,67-69]. It is currently unclear to what extent RT can induce T cell priming in the human cancer setting, specifically in immunosuppressive tumors lacking a tumor-specific T cell infiltrate prior to treatment. Therefore, we need a comprehensive, mechanistic understanding of how RT affects the T cell response to poorly immunogenic tumors. This is essential to rationally combine RT with specific immunotherapeutic interventions, to achieve a synergistic effect on anti-tumor immunity.

Scope of the thesis

In this thesis, I used mouse tumor models of different immune complexities to define and optimize determinants for RT-induced T cell priming and subsequent anti-tumor immunity. First, I identify, in a tumor model resembling human lymphocyte-depleted cancer, impediments that prevent systemic RT-induced T cell responses and present interventions that overcome these impediments. Next, I describe work investigating the role of RT and other interventions to overcome local T cell suppression in the TME, using different tumor models.

In **chapter 2**, we examined the potential and challenges of using RT to generate novel T cell responses in a tumor model representing human lymphocyte-depleted cancer. We observed that low lymphocyte levels and high myeloid cell content negatively impact overall survival after RT in human tumors. We utilized the transplantable TC-1 tumor model to replicate these immune characteristics in mice. This tumor model is characterized by systemic immunosuppression, indicated by increased monocyte and Treg levels in the TdLN and tumor. In this model, RT promotes $CD8^+$ T cell priming in the TdLN, required for RT-induced tumor control. However, concurrent Treg priming, which is spontaneously induced by the tumor and further exacerbated by RT, hindered these responses. We

proved that in this setting, CTLA-4 and PD-1 blockade further enhanced RT-induced Treg responses and resulted in failed tumor control. Mechanistically, we identified CD28 costimulation, engaged upon CTLA-4 and PD-1 blockade, as the main driver of the RT-induced Treg response. We discovered that the CD28-ligand CD86 promoted this Treg response. Inhibition of CD86, but not CD80, prevented RT-induced Treg expansion, enhanced cDC1 activation and CTL priming, and together with anti-PD-1 led to improved RT-induced tumor control and overall survival.

This chapter presents compelling evidence for the potential of RT to induce a potentially tumor eradicating CTL response even in the presence of systemic Treg-based immunosuppression. Furthermore, it emphasizes the significance of considering the patient's tumor immune profile, particularly the Tconv/Treg ratio, when designing combination strategies involving RT. This is crucial, as conventional ICBs may inadvertently promote undesired Treg responses. In addition, we propose CD86 blockade as a promising potential therapeutic target to prevent (RT-induced) Treg responses in the TdLN and tumor.

The influence of Tregs as an impediment to (RT-induced) anti-tumor immunity has received much attention by earlier studies[64,70,71]. However, clinical targeting of Tregs has been impeded by the limited availability of targetable molecules exclusively expressed by Tregs, and the challenge of identifying targets that can discriminate tumor-specific Tregs from healthy tissue to preserve homeostatic immune tolerance. Thus, a comprehensive characterization of the development and origin of tumor-induced Treg responses is crucial to identify potential targets that can effectively differentiate them from healthy surrounding tissue. In **chapter 3**, we characterize the Treg response triggered by TC-1 tumor development and highlight potential future experiments necessary to better identify the factors to determine potential targets for Tregs.

In **chapter 4**, we study the potential of RT to promote $CD8^+$ T cell responses in the TME locally. Here, we investigated the use of RT as a strategy to overcome anti-PD-1 resistance in a transplantable breast carcinoma model. Combined infusion of inhibitory anti-PD-1 and agonistic anti-CD137 facilitated the priming of CTLs, while RT created a T-cell permissive TME. Additionally, the presence of an unirradiated second, contralateral tumor, further emphasized the contribution of RT in supporting local CTL activity, as CTL infiltration in these "metastatic" tumors was not enough to improve tumor control and survival. Rather, low-dose systemic cisplatin infusion created a permissive CTL environment in the non-irradiated lesion, leading to prolonged overall survival. This chapter proposes that even following sufficient CTL priming and infiltration, local immunosuppressive mechanisms within the TME may prevent CTL functionality, alongside PD-1 blockade. In such cases, strategies like RT and cisplatin can be used to overcome these impediments and restore CTL reactivity.

Classically, TMEs have been categorized into "immune desert"; lacking T cell infiltration, "immune excluded"; containing T cell restricted to the tumor border, and "immune infiltrated";

characterized by T cell infiltration[72]. However, the mechanisms that prevent immune infiltration are not completely understood. In **chapter 5**, we uncover a novel role for autotaxin (ATX), a lysophospholipase D secreted by tumor cells and other cells, in preventing CTL infiltration into the TME. Through production of lysophosphatidic acid (LPA), we show that ATX secreted by human melanoma cells prevents T cell migration, predominantly through binding to the G protein-coupled receptor 6 (LPAR6). Upon anti-cancer vaccination of tumor-bearing mice, enforced ATX overexpression in tumor cells did not interfere with the development of systemic T cell responses, but prevented CTL infiltration into the TME, resulting in abrogated tumor control and survival.

This chapter is fundamental in uncovering a potential mechanism employed by tumors to prevent T cell infiltration, even in the presence of optimal CTL responses. In addition, it offers a promising therapeutic opportunity, that may be combined with existing anti-tumor immune interventions for use in the clinic.

Importantly, the findings in **chapter 4** and **chapter 5** illustrate the significance of effectively engaging every step in the cancer-immunity cycle to establish a durable anti-tumor immune response. Specifically, despite the generation of robust tumor-specific CTL responses, either by using immunomodulatory antibodies or by vaccination, tumor control was not achieved unless local immune inhibition was alleviated.

Finally, in **chapter 6**, I discuss the concepts and clinical implications explored throughout this thesis considering the current literature.

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