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

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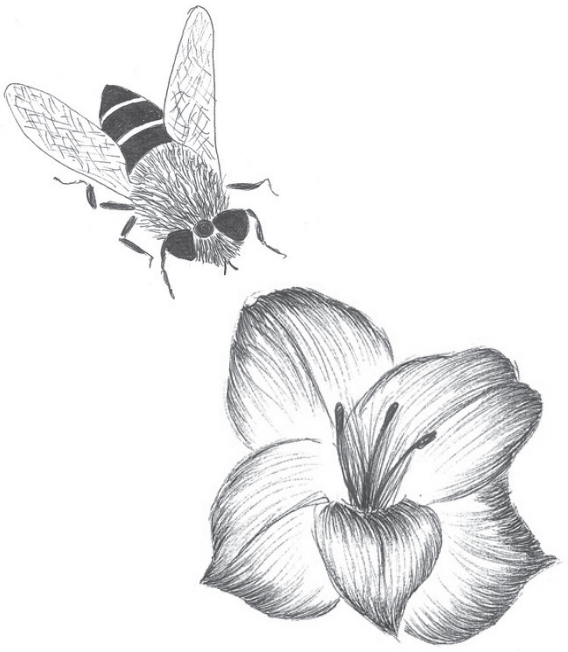

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Addenda

English Summary

In this thesis, I describe the role of T cells in eliminating cancer cells, alone or in combination with radiotherapy (RT), immunotherapy (IT) or conventional chemotherapy. Since therapeutic interventions have advanced to combat infectious diseases, cancer, along with cardiovascular diseases, has emerged as a prominent global health challenge in the 20th century. Although cancer is typically considered a single disease, it is now clear that it encompasses a multitude of diseases determined by the tissue of origin, the underlying genetic alterations, and the presence of non-tumorous cells, including immune cells. During tumor development and treatment, these cells are in continuous crosstalk with each other, giving rise to evolving cellular ecosystems.

Cancers are generally treated with conventional therapies designed to kill rapidly dividing tumor cells, like RT and chemotherapy. RT is a local treatment that aims to reduce tumor masses, without harming the surrounding healthy tissue. Therefore, RT generally has less severe side effects than chemotherapy, which is administered throughout the body to combat systemic cancer spread. However, RT efficacy is limited by intrinsic and acquired resistance of cancer cells.

While it was originally thought that RT primarily acts by causing tumor cell cycle arrest and death, it is now evident that it also has the capacity to activate immune cells, including T cells. The evolutionary role of CD8$^+$ and CD4$^+$ T cells is to protect us against infection by viruses and other micro-organisms. Particularly, CD8$^+$ T cells can mature into cytotoxic T lymphocytes (CTLs), which are highly effective in directly killing infected cells by recognizing pathogen-derived, foreign (“non-self”) molecules, called antigens. CD8$^+$ T cells are activated (primed) to expand and differentiate into CTLs in tissue draining lymph nodes (TdLN) in response to antigen recognition and specific costimulatory signals and cytokines delivered by conventional dendritic cells (cDCs). After priming, these CTLs exit the lymph node and migrate through the blood to the infected tissue. CD4$^+$ T cells are crucial to provide help for effective CTL and antibody responses. Besides combating infections, T cells can also employ the same mechanisms to target cancer cells.

Since tumors originate from the body’s own cells, they are not easily recognized as “foreign” and therefore do not typically raise a T cell response. RT can help to overcome this problem, since RT-induced tumor cell damage and death can cause the release of inflammatory molecules and antigens that can activate cDCs and tumor-specific T cell responses. However, tumors also develop immunoregulatory mechanisms that prevent or inhibit T cell responses. These inhibitory processes can act locally in the tumor microenvironment (TME) but can also involve the TdLN and may therefore become systemic. Not only can these processes interfere with the generation of new T cell responses, but they can also facilitate the spread of cancer beyond the primary tumor. One such mechanism used by the tumor is the recruitment and expansion of FOXP3$^+$ CD4$^+$

T regulatory (Tregs) cells. Tregs employ multiple mechanisms that suppress T cell activity, either directly or indirectly (i.e., via cDCs). Therefore, they play an essential role in preventing excessive and self-reactive immunity.

To enhance efficacy by overcoming immune suppression, clinical trials often combine RT with IT, particularly utilizing antibody-mediated immune checkpoint blockade (ICB) targeting inhibitory receptors such as PD-1 or CTLA-4. ICB aims to enhance anti-tumor T cell responses by either blocking of immune inhibitory receptors or engaging immune stimulatory molecules. The combined approach of RT and IT holds the potential to enhance the effectiveness of local RT and generate systemic anti-tumor T cell responses, which are crucial for preventing metastatic progression. However, achieving improved combined responses in the clinical setting remains challenging, primarily due to a lack in understanding of the mechanisms that drive RT-induced T cell responses across the diversity of human cancers. By improving our understanding of the factors required to initiate and support RT-induced T cell responses, we can enhance the clinical efficacy of RT. This may lead to a rational use of treatment strategies that can effectively overcome local or systemic immune suppression. This thesis contributes to a deeper understanding of how RT affects T cell responsiveness and provides guidelines for optimizing the combination of RT with IT.

In **chapter 2**, we analyzed publicly available records from The Cancer Genome Atlas (TCGA) and observed that RT was negatively associated with survival of patients with cancer types that have low lymphocyte- and high myeloid cell content. The transplantable TC-1 tumor model in mice replicated these immune characteristics and was therefore used to study the spontaneous and RT-induced T cell response. We found that TC-1 tumor development was associated with systemic immunosuppression, indicated by increased monocyte and Treg cell levels in the TdLN and tumor. RT to TC-1 tumors induced CTL priming in the TdLN. However, concurrent Treg priming, initiated by the tumor and further exacerbated by RT, inhibited overt CTL responses and CTL-based tumor rejection. We found, contrary to our expectations, that blockade with CTLA-4- or PD-1, further increased RT-induced Treg responses and did not promote tumor rejection. This is an important observation since similar effects could potentially occur in human tumors of this type. We elucidated that Treg expansion was caused by engagement of the costimulatory receptor CD28 on Treg cells. Therefore, this type of ICB may unintentionally support immunosuppressive Treg responses in tumor settings that favor Tregs over T cells. Our research further revealed that the CD28 ligands CD80 and CD86, expressed by cDCs, differentially promote T cell and Treg responses, respectively. Blocking CD86, but not CD80, attenuated RT-induced Treg responses and supported RT-induced CTL priming. Additional PD-1 blockade to this combination treatment improved RT-induced tumor control and overall survival. This study reveals the potential of RT to induce CTL responses even in the presence of Treg-mediated immunosuppression and highlights CD86 blockade as a promising therapeutic approach to increase CTL-based immunity in such tumors.

The influence of Tregs on preventing anti-tumor immunity has garnered significant attention in recent years. However, the exact mechanisms responsible for tumor-induced Treg priming in the TdLN remain unclear. Tregs can develop in the thymus (tTregs) in response to self-antigens, after which they migrate to the lymph nodes in an immature state to undergo initial Treg differentiation. Subsequently, these Tregs migrate to the tissue where they further adapt a tissue-resident phenotype. Alternatively, Tregs can emerge from the conversion of mature $CD4^+$ T cells in the periphery (pTregs) that are specific against non-self antigens.

In **chapter 3**, we characterize the Treg response initiated upon TC-1 tumor development in the TdLN and tumor. We reveal that TC-1 tumor growth preferentially drives expansion and effector differentiation of tTregs in the TdLN and their increased presence in the tumor, where they adapt a more mature phenotype. We propose future experiments that can elucidate the mechanisms governing tumor-induced Treg responses in the TdLN and the developmental trajectories of Tregs in healthy tissues and tumors. This information is essential for identifying novel therapeutic targets to prevent tumor-associated Treg responses without eliciting immune-related adverse effects.

Chapter 4 details the contribution of RT to systemic anti-tumor CTL responses within the context of combined ICB treatment, involving blockade of the inhibitory receptor PD-1 and stimulation of the costimulatory receptor CD137, in a transplantable breast carcinoma model. In this setting, the combined anti-PD-1 and anti-CD137 treatment promoted CTL priming. Contrarily, RT did not directly contribute to a systemic T cell response, but enabled CTL activity in the TME. Accordingly, rejection of a non-irradiated tumor implanted on the contralateral flank in the same mouse was not improved upon combined treatment with RT and ICB compared to ICB alone. This study highlights that RT can enable tumor rejection by overcoming local immunosuppression within the TME. Furthermore, we show that low-dose chemotherapy infusion, in the form of cisplatin, enabled CTL activity in the non-irradiated tumor, leading to improved overall survival. This chapter emphasizes that in settings where CTL priming can be achieved by ICB treatment alone, conventional treatments like RT and low dose chemotherapy, can help override local immunosuppression when applied in a rational manner, based on mechanistic insights.

In **chapter 5**, we uncover a novel role of autotaxin (ATX), a lysophospholipase D secreted by tumor cells and other cells, in repelling CTLs from the TME. Mechanistically, we reveal that lysophosphatidic acid (LPA), the bioactive product of ATX, hinders T cell migration primarily by binding to the G protein-coupled receptor 6 (LPAR6). We experimentally enforced ATX overexpression in TC-1 tumor cells and implanted these cells in mice. ATX production by the tumor cells did not disrupt the induction of a systemic CTL response by vaccination but impeded the infiltration of these CTLs into the TME, resulting in significantly reduced mouse survival. Given that ATX inhibitors are currently in development for clinical use, these findings reveal a promising

therapeutic opportunity for enhancing anti-tumor immunity. Together with our findings in **chapter 4**, this study emphasizes that treatments designed to facilitate CTL activity, such as conventional ICB approaches, may not succeed unless we have a comprehensive understanding of the specific CTL inhibitory mechanisms at play and address them carefully.

Finally, in **chapter 6**, I discuss the findings described in this thesis and provide context within the current literature. Additionally, I explore future perspectives regarding the valuable role of RT in enhancing the anti-tumor immunity, particularly within the diverse human tumor landscapes. Together, these findings contribute to a rational application of RT to establish durable anti-tumor immune responses against a range of cancer types.