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

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# 6

## General Discussion

Local radiotherapy (RT) has been used for over a century to induce DNA damage, leading to cell cycle arrest and cell death in rapidly proliferating tumor cells. To improve RT efficacy, studies have primarily focused on improving radiosensitivity by modulating DNA damage and repair. The contribution of the immune system<sup>1</sup>, particularly CD8<sup>+</sup> T cells<sup>2</sup> to RT-induced tumor regression has long been neglected. In recent years, the concept has emerged that RT is potentially immunogenic, since dying cells can release danger-associated molecular patterns (DAMPs)<sup>3</sup> and tumor-associated antigens<sup>4</sup>. Such immunogenic cell death could lead to dendritic cell (DC) activation, causing tumor-specific T cell responses in the tumor-draining lymph node (TdLN)<sup>5,6</sup>. This idea has greatly raised interest in using RT as an *in situ* anti-cancer vaccine. This concept was reinforced by the discovery that tumor regression outside the field of RT, known as the abscopal response<sup>7</sup>, is T cell mediated<sup>8</sup>. Consequently, numerous clinical trials were initiated to combine RT with immunotherapy (IT) strategies, in order to enhance systemic anti-tumor immune responses<sup>9</sup>. However, despite these efforts, clinical successes are unsatisfactory, with overall response rates generally not exceeding 18%<sup>10</sup>. In part, disappointment arises from unrealistic expectations set by mouse studies using tumor models with highly immunogenic exogenous antigens<sup>5,6,11,12</sup>. Since endogenous tumor antigens are generally poorly immunogenic, these studies might overestimate the potential of RT to facilitate T cell priming to human cancer in the clinic. Additionally, while synergy between RT and IT in immune responsiveness is often claimed in mouse studies<sup>8,13-15</sup>, clinical reports indicate that systemic anti-tumor immunity primarily reflects the effects induced by IT alone and do not truly represent a combined effect of RT and IT<sup>16,17</sup>. For these reasons, the mechanisms that drive RT-induced T cell responses in the diversity of human cancers must be better understood. Only then can we rationally design the correct RT-IT combination strategies to synergistically increase systemic T-cell based immunity against cancer. This approach can be particularly useful for cancers that are not spontaneously immunogenic and are therefore in need of a “vaccination” approach<sup>18,19</sup>.

The work described in this thesis aimed to unravel and optimize important determinants for RT-induced T cell responses in poorly immunogenic tumors. To achieve this, I addressed the following questions, which will be explored in the subsequent discussion:

1. To what extent does RT induce T cell responses against cancer?
2. Which impediments and potential IT targets can be utilized to enhanced RT-induced T cell responses in poorly immunogenic cancers?
3. Can RT be exploited to overcome local immunosuppression in the tumor microenvironment (TME)?

## 1) What is the potential of RT to raise systemic T-cell immunity?

### RT responses are dictated by the tumor immune constellation

Traditionally, TMEs are classified as “immune-infiltrated”, “immune excluded” or “immune desert”, based on the varying levels of lymphocyte presence and localization<sup>20</sup>. However, these classifications primarily rely on the density of CD3<sup>+</sup> and/or CD8<sup>+</sup> cells on immunohistochemistry slides<sup>20</sup> and may not accurately predict tumor responsiveness to IT. Recently, multi-omics analysis of human pan-cancer tissues revealed distinct immune constellations among tumors of the same type<sup>21-23</sup>. These immune complexities significantly impact patient survival and response to IT<sup>21,22</sup>. Tumors containing high myeloid cell content and fibroblasts, along with low presence of lymphocytes, often show negative survival outcomes and treatment responses, as opposed to tumors containing high lymphocyte abundance, combined with elevated levels of DCs and interferon type I (IFN-I) signaling<sup>22</sup>. Interestingly, FOXP3<sup>+</sup> T regulatory cells (Tregs), generally considered immunosuppressive, are variably present among all immune contexts, but are typically associated with macrophage-enriched tumors<sup>23</sup>.

Tumor responses to RT can vary, despite equal radiosensitivity *in vitro*<sup>24</sup>. RT elicited systemic CD8<sup>+</sup> T cell-mediated anti-tumor immunity in mouse tumor models that were spontaneously T cell-infiltrated, but not in T cell-devoid tumors<sup>18,24</sup>. A recent patient study similarly identified that tumors enriched for IFN-I pathways, along with presence of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, activated NK cells and inflammatory macrophages are associated with enhanced tumor radiosensitivity<sup>25</sup>. Therefore, there appears to be a relationship between tumor immunogenicity and response to RT. In **chapter 2**, we analyzed records from The Cancer Genome Atlas (TCGA) using five pan-cancer immune phenotypes<sup>21</sup>. We observed reduced overall survival after RT in non-immunogenic cancers, described as “lymphocyte depleted”, in which the TME typically exhibits high myeloid-to-lymphocyte ratios<sup>21</sup>. We found that the transplantable TC-1 tumor model recapitulates human lymphocyte-depleted cancer and investigated the factors impeding the RT response (**chapter 2**)<sup>21</sup>. Despite expressing antigenic HPV16-derived E6 and E7 antigens, TC-1 tumors lack CD8<sup>+</sup> T cells and have a TME enriched with myeloid cell populations, as confirmed by single-cell RNAseq analysis<sup>26</sup>. This tumor model provides valuable insights into the requirements for tumor-specific T cell priming. Tumors like TC-1, which do not spontaneously provoke tumor-specific T cell responses, likely have not undergone T cell-mediated immune surveillance or pressure. As a result, they may remain susceptible to newly generated T cell responses upon RT and/or IT<sup>27</sup>. In agreement, the TC-1 tumor is highly responsive to CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses induced by vaccination (**chapter 5** and<sup>28,29</sup>). Moreover, the TC-1 tumor model effectively recapitulates (dys)functional T cell priming in the TdLN<sup>30</sup>. Additionally, we observed that TC-1 tumor growth triggers effector (e)Treg priming (**chapters 2, 3**) and attracts high levels of monocytes in the TdLN. These data illustrate the continuous interaction between the tumor and TdLN<sup>31</sup>, in this case resulting in the development of systemic immunosuppression.

In **chapter 2**, we discovered that RT of the TC-1 tumor caused priming of CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) in the TdLN, necessary to mediate tumor control. However, eTreg expansion, induced by the tumor and exacerbated by RT, limited CTL priming. This mechanism may also be relevant in human cancer, as evidenced by tumor-induced Treg expansion in the TdLN in patients with challenging-to-treat cancers like breast, colorectal, and hepatocellular carcinoma<sup>32</sup>. Our finding highlights the potential of RT to stimulate tumor-specific T cell priming in lymphocyte-depleted tumors and identifies simultaneous Treg priming as a key negative regulatory mechanism. Whether prediction of anti-tumor immunity can solely rely on T cell-to-Treg ratios found in the TME remains controversial, as it appears highly influenced by the cancer molecular subtype and disease stage<sup>23,33</sup>. Specifically, while the presence of Tregs is generally considered to negatively impact disease outcome, it may paradoxically be favorable for survival in head and neck, colorectal and esophageal cancer<sup>33</sup>. This suggests that the mere presence of Tregs may not be sufficient to accurately predict disease outcomes. A patient cohort study of invasive breast cancer indicated that the ratio of effector Tregs to T cells, which contained a population with a higher suppressive phenotype, rather than the total Treg to T cell ratio, was a better predictor of treatment responses<sup>34</sup>. Thus, instead of solely focusing on the entire Treg population, the functionality of the Tregs present in the TME should be considered to predict anti-tumor immune responses. Furthermore, recent RNA-seq data indicate that response to chemotherapy in Treg-enriched human cancers is associated with reduced suppressive Treg function, along with increased abundance of CD8<sup>+</sup> T cells and inflammatory M1-type macrophages<sup>35</sup>. Together, these findings highlight that the design of treatment strategies must encompass the broader tumor immune landscape, in addition to the Treg functional state.

## **The tumor-draining lymph node serves as a niche for pro- and anti-tumor immune responses**

Anti-tumor immunity primarily relies on CTL responses orchestrated in the TdLN<sup>36</sup>. Intravital imaging studies have revealed a two-step process for the effective priming of CTLs<sup>37-39</sup>, involving correct localization and activation of conventional (c)DCs in the TdLN. Initially, CD8<sup>+</sup> and CD4<sup>+</sup> T cells receive their first priming-step by encountering the cDC1 or cDC2 subset, respectively, in distinct areas of the LN. Subsequently, both T cell types interact with the same lymph-node resident or migratory cDC1s, enabling CD4<sup>+</sup> T cells to provide the necessary help signals for optimizing DC activation and CTL differentiation<sup>37,40</sup>. To achieve this, both cDC1s and cDC2s need appropriate activation and migration from the tumor to the TdLN<sup>41,42</sup>. However, cDC paucity<sup>43</sup>, inhibitory metabolic pathways<sup>44,45</sup>, and negative immunoregulatory mechanisms from the tumor<sup>46,47</sup> contribute to suboptimal DC activation, which extends to the TdLN and can lead to systemic immunosuppression. Additionally, tumors may enforce immunosuppression by causing

aberrant stromal remodeling and structuring in the LN, disrupting the normal dynamics required for lymphocyte interactions<sup>32</sup> and/or by altering the cellular composition of the LN. This involves attraction of immunosuppressive myeloid cells<sup>48</sup> and Tregs<sup>49</sup> (**chapter 2 and 3**), which could limit T cell functionality<sup>48,50</sup>. Notably, in the TdLN, tolerogenic cDCs may induce Treg priming and expansion<sup>51-53</sup>, which subsequently impair CTL differentiation by inhibiting cDC1 responses<sup>54</sup>. Aberrant immunity in the TdLN may also promote primary tumor progression and establish a microanatomic niche for metastases<sup>55,56</sup>, as it is often the first tissue infiltrated<sup>32</sup>.

While the role of the LN as orchestrator for adaptive immune responses has been well-established, its function in supporting tumor immunity and response to IT has only recently been recognized<sup>36,57,58</sup>. Studies in mice demonstrate that treatment efficacy of PD-1 inhibition is hindered when exit of T cells from the TdLN is blocked<sup>57,58</sup>. Additionally, direct administration of anti-PD-L1 to the TdLN alone has been shown to achieve tumor control<sup>57</sup>. Similar effects have been observed concerning CTLA-4 blockade<sup>59</sup>. Likewise, the TdLN plays a crucial role in generating RT-induced systemic immunity<sup>12</sup>, leading to both local (**chapter 2**) and abscopal<sup>11</sup> responses. Thus, effective anti-tumor immunity depends on the TdLN, despite potential immunosuppression. **Chapter 2** highlights the duality of this response in the context of RT, showing that RT can elicit CTL priming in the TdLN alongside Treg priming. These findings suggest that RT may support cDC1 activation by releasing DAMPs required for their migration to the TdLN. However, RT may also upregulate signals that hinder cDC1 recruitment to the TME<sup>60</sup>, potentially limiting the number of CTLs primed in the TdLN based on the initial cDC1 population present in the tumor. Conversely, since the tumor contains high levels of cDC2s, Treg priming induced by these cDC2 cells may dominate cDC1-induced CTL priming in the TdLN after RT. IT strategies now focus on restoring cDC1 function in the tumor by alleviating cDC scarcity, supporting antigen spillage, and providing activation mimicry<sup>43,61,62</sup>. However, whether these therapies are successful depends on the functional state of the TdLN<sup>63,64</sup> and the ability to overcome existing immunosuppression<sup>57,65</sup>.

In the clinic, the removal or irradiation of either the sentinel lymph node (the first lymph node receiving tumor drainage) or the entire LN basin is common to limit potential metastatic spread, as tumor involvement in these LNs are aberrant for survival<sup>66</sup>. However, the therapeutic benefit of this approach is controversial<sup>67</sup>. In view of the requirement for TdLNs to educate anti-tumor immune responses, treatment strategies should avoid removing or damaging lymph nodes when possible. In particular, in RT and RT-IT approaches, avoiding nodal irradiation is desirable, as the destruction of lymph nodes may compromise systemic anti-tumor immunity in the clinic<sup>63,64</sup>. Clinical studies should determine whether tumor-infiltrated LNs can still generate effective anti-tumor immune responses<sup>65</sup> and whether other LNs, beyond the sentinel LN, can support anti-tumor immunity. The stage of tumor development may also impact immunity in the TdLN, with advanced tumor burden being associated with enhanced systemic immunosuppression

and reduced responses to IT<sup>68</sup>. Therefore, preserving the TdLN may offer particular benefits for early-stage cancer, when tumor cells have not yet infiltrated in the TdLN and immunosuppression is relatively low. In **chapter 2**, we propose blockade of CD86, a costimulatory ligand expressed by cDCs and other myeloid cells, as a potential mechanism to alleviate immunosuppression in the TdLN, by disrupting Treg priming and supporting cDC1 activation (further discussed below). Particularly in advanced cancer, strategies that directly target suppressive immune responses in the TdLN may present an opportunity to salvage ineffective anti-tumor immunity.

## 2) Defining impediments and potential targets that prevent RT-induced systemic immune responses.

### The CD28 costimulatory axis dictates RT responses in Treg-rich tumor settings

#### 1) PD-1 and CTLA-4 blockade enable CD28 costimulation on (RT-induced) Tregs.

Clinically approved immune checkpoint blockades (ICB) of CTLA-4 and PD-(L)1 promote T cell responses by enabling cDC-mediated costimulation and aim to alleviate peripheral tolerance against the tumor. Specifically, PD-1 inhibits T cell costimulation by CD28<sup>69,70</sup>. CD28 promotes antigen-specific activation and clonal expansion of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells by various mechanisms<sup>71</sup>. Upon binding to its ligand PD-L1 or PD-L2 presented by cDCs, PD-1 recruits the SHP2 tyrosine phosphatase to its cytoplasmic tail and subsequently inhibits CD28 signaling<sup>70</sup>. CTLA-4, constitutively expressed on Tregs and upregulated by Tconvs following antigenic activation, can downregulate the CD28 ligands CD80 and CD86 on cDCs<sup>72</sup> and thereby attenuates CD28 costimulation of Tconvs<sup>71</sup>. Thus, both PD-1 and CTLA-4 prevent T cell responses by suppression of CD28 costimulation. CTLA-4 exerts its influence early during T cell priming, whereas PD-1 is upregulated following T cell activation<sup>73</sup> and each contribute to peripheral immune tolerance.

In **chapter 2**, we discovered that instead of promoting CTL responses, the blockade of CTLA-4 or PD-1 led to an increase in RT-induced eTreg expansion, which abrogated the therapeutic effects of RT. These findings highlight the limitations of current ICB approaches designed to enhance T cell responses. The majority of the costimulatory receptors are shared between both conventional T cells (Tconvs) and Tregs, which means that ICB might unintentionally activate suppressive Treg responses. Particularly, like Tconvs, Tregs require CD28 costimulation for clonal expansion<sup>74</sup> and may thus benefit from CTLA-4 and/or PD-1 blockade. These findings are crucial for clinical implementation of RT and ICB combinations<sup>9,10</sup>. Current “one-size-fits-all” approaches may inadvertently promote adverse treatment outcomes, especially when the patient’s unique immune parameters are not considered prior to treatment. For instance, the distinction between CTL and Treg responses following PD-1 blockade appears to be influenced by the balance between these cell populations in



the TME, along with the levels of PD-1 expression<sup>75</sup>. As a result, PD-1 blockade in cancers having high Treg-to-Tconv ratios was associated with significant tumor progression<sup>75,76</sup>. Likewise, the Treg-to-Tconv ratio also seems to underlie responses to CTLA-4 blockade<sup>77,78</sup>, and requires a setting in which CTLA-4 blockade favors Tconv over Treg priming. Therapeutic benefit to RT and CTLA-4 blockade has been described in mice<sup>13,15,79</sup> and in patients with metastatic non-small-cell lung cancer<sup>80</sup> and metastatic melanoma<sup>81</sup>. Combination of RT with CTLA-4 blockade in these settings likely enhanced CTL priming, based on the observed increase in TCR diversity among tumor-infiltrating T cells<sup>79,80</sup>. However, in T-cell devoid tumors, several factors may contribute to an unfavorable T cell-to-Treg ratio upon RT. This includes a higher cDC2 over cDC1 ratio in the TME (**chapter 2**), limited release of RT-induced DAMPs<sup>82</sup>, insufficient IFN-I responses<sup>15</sup>, and RT-induced suppressive factors hindering cDC1 maturation<sup>60,83</sup>. In such cases, CTLA-4 blockade may preferentially benefit CD28 costimulation of Tregs<sup>84</sup>, leading to their expansion and eTreg formation.

Considerable attention is directed towards improving efficacy of CTLA-4 blocking antibodies by incorporating the ability to deplete Tregs in the TME without affecting peripheral Tconvs, rather than only blocking ligand binding<sup>85,86</sup>. Moreover, due to its disruption of peripheral immune tolerance against the tumor, clinical application of CTLA-4 blockade is frequently associated with grade 3-4 severe adverse immune-related events, including conditions such as diarrhea, colitis, and severe skin rashes<sup>87</sup>. New approaches therefore aim to enhance CTL-to-Treg ratios, while limiting adverse immune-related toxicities. One strategy involves engineering antibodies that selectively bind to CTLA-4 in an acidic environment, such as the tumor site, but not in peripheral tissues, which is currently undergoing its first clinical trial<sup>88</sup>. Furthermore, bi-specific antibodies engineered to pair PD-1 blockade with a non-Treg specific IL-2-variant are promising, as they have shown to selectively engage tumor-specific T cell responses, without targeting Tregs<sup>89</sup>.

Collectively, our findings emphasize the importance of considering the Treg-to-Tconv ratio in the TME when contemplating RT and ICB combinations. Particularly, in tumors that provoke Treg responses, administration of PD-1 and/or CTLA-4 blockade may unintentionally enhance these responses by engaging CD28. Consequently, instead of promoting immune activation, ICBs, either alone or in combination with other treatments like RT, may inadvertently foster immune suppression. This, in part, provides insight into the lack of therapeutic responses observed in a subset of patients<sup>76,90,91</sup>.

## *2) The differential role of the CD28 costimulatory ligands CD80 and CD86 in supporting RT-induced CTL responses.*

The observed Treg response to CTLA-4 and PD-1 blockade in the TC-1 tumor model highlights that both Tconvs and Tregs benefit from CD28 costimulation mediated by its ligands CD80

and CD86, presented by cDCs. Although CD80 and CD86 diverge considerably regarding their sequence, biophysical characteristics, and cellular expression<sup>92,93</sup>, they are often considered to have similar immunological functions. In **chapter 2**, we discovered that upon RT, CD80 and CD86 differentially promote Tconv and Treg responses, respectively. This discovery presents CD86 blockade as a promising therapeutic approach to boost (RT-induced) anti-tumor Tconv responses and prevent systemic immunosuppression by Tregs. In our setting, CD86 blockade countered effector Treg expansion in the TdLN. Therefore, this approach may benefit patients with advanced disease, by potentially ameliorating systemic immunosuppression. Additionally, we observed that reducing the eTreg response by CD86 blockade facilitated RT-induced cDC1 activation and CTL priming against the tumor. This result suggests that the RT-induced eTreg response directly hinders CTL priming by inhibiting cDC1 activation in the TdLN, as has recently been described in another setting<sup>54</sup>. In the TME, Tregs rely on continuous interactions with cDCs to maintain their immunosuppressive properties, which depend on CD80 and CD86<sup>84,94</sup>. Thus, next to inhibiting the eTreg response in the TdLN, CD86 blockade likely also disrupts local immunosuppressive interactions in the TME. Importantly, we found that CD86 blockade does not affect the population of central (c)Tregs in the TdLN and non-TdLN. This finding has considerable implications for translation into the clinic, as CD86 blockade may present with fewer immune-related toxicities as compared to CTLA-4 blockade<sup>87</sup>. Specifically, because CTLA-4 is constitutively expressed on Tregs, its inhibition might inadvertently impact the entire Treg population, including those responsible for immune homeostasis. This concern could be avoided by preserving the cTreg subset.

Our discovery that CD86 is favored for CD28 costimulation of (RT-induced) Tregs can be attributed, in part, to its lower binding affinity towards CD28 and CTLA-4, unlike CD80<sup>95</sup>. Specifically, as Tregs express both CD28 and CTLA-4, CD86 and CD80 compete for facilitating CD28 costimulation. Since CD86 has a lower affinity for CTLA-4 than CD80, CD86 may remain available for mediating CD28 costimulation, bypassing the CTLA-4 restraint. In agreement, making CD80 available by CTLA-4 blockade allowed for CD80-mediated Treg responses<sup>96</sup>. While CD86 is constitutively expressed on cDCs, CD80 expression levels are strongly upregulated upon cDC maturation<sup>97</sup>. Moreover, recent research has demonstrated that CD80 forms cis-heterodimers with PD-L1, providing protection from CTLA-4-mediated downregulation while retaining its CD28 costimulatory capabilities<sup>98</sup>. These findings, together with its high affinity for CTLA-4, imply that CD80 requires tight regulation, that is apparently geared to support CD28 costimulation of Tconvs rather than Tregs, as we demonstrate. Considering the sequential expression of CD86 and CD80 on DCs<sup>71</sup>, optimal CD28 costimulation may require an initial stimulus initiated by CD86, followed by CD80. In **chapter 2**, we also observed that CTLs primed upon RT and CD86 blockade upregulated PD-1, which impaired RT-induced tumor control and survival. Thus, in presence of CD86 blockade, CD28 costimulation may still not be optimal for Tconv priming.

Recent studies indicate that the metabolic state of cDC2s dictates CD86-mediated Treg expansion<sup>99,100</sup>. Since tumors generally influence the cDC maturation state<sup>101,102</sup>, future treatment approaches should focus on mechanisms that alleviate these processes at a metabolic level to enhance cDC maturation and activation. For example, genetic ablation or inhibition of the prostaglandin receptors EP2 and EP4 may present an attractive strategy to promote cDC activation in the TME, warranting further investigation<sup>44</sup>.

Collectively, our findings in **chapter 2** highlight the role of CD28 costimulation in promoting both pro- and anti-tumor T cell responses, specifically in a Treg rich tumor setting. Particularly, blockade of CD86 emerges as a promising avenue for therapeutic intervention, especially in tumor settings featuring suppressive TdLNs, as it effectively dampens Treg responses both in the TdLN and the tumor. Importantly, since this approach preserved the cTreg population, the risk of unintended adverse effects may be reduced. Furthermore, considering the effects of RT in reducing tumor burden and its immunostimulatory potential observed in **chapter 2**, its combination with CD86 blockade offers a promising strategy, as this approach holds the potential to not only mitigate prevailing systemic suppression but also to facilitate Tconv responses in Treg-enriched cancers.

## Tregs as an impediment in cancer and anti-cancer therapies

As demonstrated in **chapter 2**, TC-1 tumor growth raises effector Treg responses that prevent anti-tumor immunity. In **chapter 3**, we closely examined these Tregs and found that TC-1 tumor development leads to a preferential accumulation of Helios<sup>+</sup> Tregs, that are likely thymus-derived<sup>103</sup>. These Tregs undergo initial effector differentiation before migration into the TME, where they adopt a more mature phenotype. These findings highlight the opportunity of the TC-1 tumor model to mechanistically study systemic Treg responses in an *in vivo* setting. Specifically, although the role of Tregs in driving metastatic seeding is becoming increasingly clear<sup>55,56</sup>, the exact mechanisms of initial Treg responses in the TdLN remains controversial. Since Tregs accumulate in the TdLN early during tumorigenesis<sup>104</sup> (**chapter 3**), uncovering these mechanisms could reveal potential avenues to counteract early immunosuppression (e.g., CD86 inhibition - **chapter 2**). Moreover, current approaches to restrain Tregs are limited by the availability of targetable molecules exclusively expressed by Tregs, and the difficulty to distinguish tumor-specific Tregs from healthy tissue to preserve homeostatic immune tolerance. Thus, identification of *in vivo* models that may recapitulate human processes are of high importance, as they may help to elucidate the mechanisms governing Treg responses in the tumor context.

Transcriptomic analysis of human Tregs obtained from blood, adjacent healthy tissue, and tumor tissue identified many similarities in the TCR repertoire and phenotype of healthy tissue- and tumor-resident Tregs<sup>105</sup>. This suggests that tumor-resident Tregs are likely specific for “self”

antigens expressed in healthy tissue rather than tumor-antigens. Similarly, in a mouse model of prostate cancer, tumor-resident Tregs were found to recognize normal prostate tissue-specific antigens, distinct from Tconv<sup>106</sup>. These Tregs originated from the thymus and subsequently accumulated in the prostate-dLN in an antigen-dependent manner, irrespective of tumor presence. This aligns with our understanding of tissue-specific Treg development in a homeostatic setting, which requires priming of Treg progenitor cells in the LN or spleen before migrating to nonlymphoid tissues<sup>107,108</sup>. Moreover, tumor-derived Tregs seem to follow similar adaptation trajectories as their counterparts in healthy tissue<sup>109</sup>, indicating a parallel developmental pattern. This observation is supported by the identification of a preserved conservative signature encoding a tissue-repair program in tissue-resident murine and human Tregs, which may be utilized by tumor-derived Tregs to support extracellular matrix re-organization and tumor growth<sup>105</sup>. Thus, given the potentially similar developmental trajectories of tumor-associated and homeostatic Tregs, the observed accumulation of Tregs in the TdLN (**chapter 2 and 3**;<sup>49,102</sup>) might stem from increased exposure to self-antigens due to proliferating and dying tumor cells. However, other factors may also influence Treg expansion in the TdLN, including the DC activation state<sup>53</sup> and the LN cytokine environment<sup>54,110</sup>, which requires further investigation.

In **chapter 3**, we observed that Tregs in the TME had enhanced expression of ICOS, CTLA-4, GITR, CCR8 and acquired expression of CXCR6 and CD39, compared to those found in the TdLN. These markers overlap with a mature (non-lymphoid tissue) phenotype<sup>109</sup>, suggesting that Tregs undergo further maturation in the tumor tissue following their initial priming in the TdLN. In a recent study, T cell trafficking between the TdLN and tumor revealed distinct phenotypic characteristics of newly arrived Tregs in the TME compared to those already present in the tumor<sup>111</sup>. Upon infiltration into the tumor, Tregs rapidly adopted a tumor-retained phenotype, characterized by the acquired expression of CD39 and LAG-3 and high expression of ICOS<sup>111</sup>. Since Tregs in homeostatic settings undergo tissue adaptation in non-lymphoid tissues<sup>107,109</sup>, it remains to be studied to what extent these findings truly reflect a tumor-restricted transformation. Nonetheless, studies in humans have demonstrated that tumor-resident Tregs exhibit phenotypes associated with enhanced activation and suppressor functions as compared to Tregs from healthy tissue<sup>112,113</sup>. This suggests the existence of environmental factors that may sustain the intra-tumoral Treg pool and impact their molecular reprogramming, such as local interactions with DCs<sup>84,94</sup> or macrophages<sup>114</sup>. Moreover, the presence of immunosuppressive metabolites, such as lactic acid, has also been implicated to affect intra-tumoral Tregs<sup>115,116</sup>.

Importantly, recent transcriptional characterization identified the chemokine receptor CCR8 as potential marker to differentiate tumor-derived Tregs from tissue-resident Tregs<sup>112,113,117</sup>. However, other studies have found that CCR8 could also be expressed on healthy tissue-resident Tregs<sup>105</sup>, raising concerns regarding unwanted elimination of these Tregs in the periphery upon CCR8-

targeting. Thus, a better understanding of the factors involved to differentiate between Tregs from the tissue or tumors is necessary to identify novel therapeutic targets in a more precise manner.

The factors driving Treg expansion upon RT (**chapter 2**) are currently unclear, as most mouse studies focus on stimulating CTL responses<sup>14,80,118,119</sup>. Next to pro-inflammatory responses, RT-induced tissue damage can also trigger inflammatory processes that may have counterproductive effects<sup>120</sup>, such as releasing the active form of TGF- $\beta$ , a potent suppressor of anti-tumor immune responses<sup>121</sup>. TGF- $\beta$  can suppress RT-induced DC activation in the TME<sup>122</sup> and may promote Treg expansion by converting CD4<sup>+</sup> Tconvs to Tregs<sup>123</sup>. In absence of TGF- $\beta$ , RT may release activin A, a TGF- $\beta$  superfamily member, which can also enhance Treg responses<sup>124</sup>. Additionally, RT may enhance levels of IL-33, a cytokine that promotes tissue protection<sup>125</sup>, in part by inducing Treg expansion<sup>126</sup>. However, the roles of TGF- $\beta$  and IL-33 in supporting RT-induced Tregs remain unclear, as their inhibition failed to reduce Treg expansion in irradiated tumor models<sup>127</sup>.

While RT has been shown to promote DC activation and migration to the TdLN<sup>24,82,119</sup>, the specific cues that determine Tconv versus Treg priming upon cDC encounter are not fully understood. Recent studies suggest that the metabolic rewiring of cDCs, along with the uptake of cell debris, plays a crucial role in determining their molecular differentiation state<sup>99,128</sup>. RT-induced metabolic alterations<sup>129</sup> and upregulation of CDKN1A, a protein that protects against RT-induced DNA damage, may potentially favor enhanced Treg priming in the TdLN<sup>130</sup>. Thus, further studies need to elucidate the metabolic and molecular pathways within cDCs that differentiate Tconv and Treg priming in the TdLN, particularly within the context of RT.

### 3) Overcoming local immunosuppression in the TME – The potential of RT and other strategies.

#### RT as a local immune modulator

Despite achieving sufficient tumor-specific CTL priming, therapeutic responses in the clinic can be impeded by local immunosuppression in the TME. In **chapter 4**, we describe a setting in which CTL priming in the TdLN was facilitated by a combined ICB approach, using a CD137 (4-1BB) agonist along with PD-1 inhibition. Addition of RT to this ICB combination enabled effective control of the primary tumor. However, it did not significantly contribute to systemic anti-tumor immune responses. This was evident by the similar outgrowth curves of a secondary non-irradiated tumor in the same mouse, treated with either ICB alone or RT combined with ICB. RNA-seq analysis indicated that RT did not induce CTL-intrinsic effects. Instead, it appeared to alleviate suppressive mechanisms imposed by the tumor, sensitizing it for CTL functionality. This effect was not observed in the non-irradiated tumor, despite high CTL infiltration, and low dose cisplatin was required to overrule immune suppression in the non-irradiated tumor. These findings emphasize

that despite raising systemic CTL responses, tumor-associated immune suppression may abrogate treatment benefit. Moreover, our results caution for the often reported “synergistic” systemic effects in clinical settings using RT in combination with ICB approaches<sup>81</sup>, since the contribution of RT to the systemic immune responses is often overestimated, especially in situations where CTL priming can be achieved with ICB treatment alone.

Specifically, CD137, a TNF receptor superfamily member, is upregulated on T cells upon antigen recognition and CD27 costimulation and plays a crucial role in mitigating cell death, promoting proliferation, facilitating memory formation, and reversing exhaustion<sup>131,132</sup>. While CD137 expression is primarily confined to B-, T- and NK cells, it is also detected on several myeloid-lineage cells, including cDCs<sup>132</sup>. In our setting, it is likely that agonistic CD137 antibody not only directly acts on T cells, but also potentially supports cDC activation (**chapter 4**). In this context, CD137 agonism alone is probably sufficient to facilitate CTL priming, which was further assisted by PD-1 blockade. This is reinforced by the increase of CD4<sup>+</sup> (FOXP3) Tconvs observed upon CD137 agonism, suggesting that anti-CD137 may contribute to enabling CD4<sup>+</sup> T cell help. For instance, CTLs primed in absence of sufficient CD4<sup>+</sup> T cell help lack important effector and memory functions, required to overcome negative regulation<sup>29,40,133</sup> and to facilitate anti-tumor immunity<sup>37</sup>. However, we found that enlargement of the tumor-specific CTL pool by therapeutic vaccination, which included an MHC-I restricted tumor-antigen alongside CD4<sup>+</sup> T cell helper epitopes, did not further improve control of the non-irradiated tumor when compared to our RT and combined ICB approach (**chapter 4**). Thus, CD137 agonism together with PD-1 inhibition likely adequately supports CTL priming, either by directly facilitating CD4<sup>+</sup> T cell help during priming by improving CD4<sup>+</sup> T cell responses and/or by mimicking the effects of CD4<sup>+</sup> T help on CTLs<sup>28</sup>. Consequently, the broad range of stimuli induced by CD137 agonism, together with PD-1 inhibition, may have outweighed the potential benefits of adding RT to foster systemic anti-tumor immune responses.

Our study emphasizes the importance of engaging every step in the cancer-immunity cycle to bolster therapeutic benefits, as CTL priming alone proved insufficient to overcome local immunosuppression in the TME. Moreover, despite comparable levels of CTL infiltration, the irradiated and non-irradiated tumor significantly differed in tumor control, suggesting a local impediment in CTL functionality. This constraint was unrelated to PD-1 signaling, nor was it attributed to the presence of neutrophils or tumor-associated macrophages (TAMs) (**chapter 4**). In such cases, conventional anti-tumor approaches, like RT and chemotherapy, may sensitize tumors to CTL responses. In the clinic, effective ICB responses are most prevalent in cases of limited tumor burden<sup>68</sup> and when T cells are present in the TME<sup>22</sup>. In more advanced tumor settings, irradiation to all metastatic tumor sites has been suggested<sup>134</sup>, as this may reduce tumor burden, while potentially enabling CTL priming within distinct TdLNs, situated in different anatomical locations. However, this approach is restricted to cancers presenting with identifiable

metastases on tissue sites capable of withstanding high dose RT. In settings characterized by diffuse disease spread or the presence of potential microscopic disease, other options should be considered. These encompass low-dose RT (below 2 Gy)<sup>135,136</sup> or administration of minimal doses chemotherapy, like cisplatin (**chapter 4**), both proven successful in facilitating local CTL responses. Thus, especially in settings with high tumor burden, conventional anti-tumor therapies may help to alleviate local immunosuppression, provided that they are applied in a rational manner.

## Autotaxin as a potential target to enable CTL-infiltration in the TME

Local CTL paucity is generally attributed to the absence of chemokine signals that guide and direct CTLs to the tumor vasculature and/or to physical obstructions, such as presence of cancer-associated fibroblasts and matrix metalloproteinases<sup>137</sup>. In **chapter 5**, we unveil a novel function for autotaxin (ATX), a lysophospholipase D secreted by tumor cells and other cells, in repelling CTLs from the TME. Specifically, ATX generates lysophosphatidic acid (LPA), the bioactive product, from extracellularly available lysophosphatidylcholine (LPC)<sup>138</sup>. The biological effect of ATX relies on six distinct G protein-coupled receptors (GPCRs) that LPA binds to, known as LPAR1-6. These LPARs play a significant role in various cellular responses, particularly in cell proliferation and migration<sup>138</sup>. LPAR1-3 are part of the “endothelial differentiation gene” (EDG) subfamily of GPCRs, while LPAR4-6 are more closely associated with the purinergic receptor family of GPCRs<sup>138</sup>. Involvement of LPAR1-3 are considered to enhance cellular responses, whereas LPAR4-6 are generally believed to counteract these responses by suppressing migration and invasion of diverse cell types<sup>139,140</sup>. In **chapter 5**, we demonstrate that ATX, secreted by human melanoma cells, impedes T cell migration through chaperoning and binding of LPA to LPAR6.

Although the pleiotropic role of ATX in stimulating tumor progression and metastases formation has been widely acknowledged<sup>141</sup>, its function in the context of tumor immunity has only recently been appreciated<sup>142</sup> and remains an active field of investigation. Specifically, the exact mechanisms by which LPA inhibits anti-tumor immunity remain obscure. For example, LPA may operate as a negative regulator of IFN type I production by cDCs in the TME<sup>143</sup>, or it may act directly on T cells by disrupting early TCR signaling<sup>144</sup>, preventing CTL-mediated tumor control. In the latter study, binding of LPA to LPAR5 significantly reduced the CTL functionality. In contrast, we observed that binding of LPA primarily repelled CTLs from the TME without adversely affecting their effector functions (**chapter 5**). This discrepancy could potentially be explained by the fact that T cells in the melanoma TME expressed various LPARs (**chapter 5**). Consequently, differences in LPA effects may arise from distinct molecular pathways triggered by specific activated LPA-receptors. The ultimate biological outcome is likely determined by the balance in LPA-receptors present on the T cell surface, emphasizing the need to further investigate the mechanisms underlying LPA-receptor expression. Regardless, these findings indicate that inhibition of ATX, or its mediator LPA, may act

as a potential target to enable CTL infiltration and functionality in the TME, while simultaneously mitigating its other tumor promoting effects, including the formation of tumor fibrosis<sup>145</sup>. The ATX inhibitor IOA-289, currently in clinical development (clinicaltrials.gov ID NCT05586516), has shown promise by enabling CD8<sup>+</sup> T cell infiltration, while simultaneously altering the chemokine milieu within the TME, improving tumor control<sup>146</sup>. However, this study does not address whether ATX inhibition directly recruits CD8<sup>+</sup> T cells from the periphery by alleviating its repellent effect, or if this recruitment is an indirect outcome influenced by other factors, such as reduced tumor fibrosis. Furthermore, whether the observed improved tumor control upon ATX inhibition is a direct consequence of enhanced CD8<sup>+</sup> T cell infiltration has not been functionally addressed. Nevertheless, ATX inhibitors offer a promising approach to potentially benefit current treatment strategies by overcoming local immunosuppression and inviting new T cells.

## Concluding remarks

The immune-modulating potential of RT to trigger systemic anti-tumor immune responses has garnered substantial interest in the past decade<sup>10,147</sup>. However, its translation to the clinic has been disappointing<sup>10,134</sup>, primarily due to the lack of insight into the requirements for synergy between RT and ICB approaches. The work presented in this thesis has elucidated important mechanisms that need to be considered when designing radio-immunotherapy strategies. Specifically, we provide evidence that *a priori* understanding of the effect of the tumor on the local and systemic immune response is required to rationally design treatments that benefit the vaccine potential of RT. These findings may provide a starting point for future clinical trials that aim to achieve combined responses between RT and ICB. Importantly, these trials should consider stratifying patients over immune archetype, rather than mere cancer subtype. Moreover, we propose that novel treatment strategies should extend beyond countering local suppression within the TME, as these should also focus on preserving the TdLNs and rectifying aberrant immune responses within these TdLNs. We provide evidence that combinations involving CTLA-4 and/or PD-1 blockade may inadvertently support Treg responses within Treg-enriched tumor settings, via CD28 costimulation. To address this, we suggest that CD86 blockade may alleviate CD28 costimulation to Tregs, which act both in the TdLN and tumor.

In summary, to achieve synergistic clinical responses, combined approaches involving RT requires a comprehensive understanding of how each therapeutic component contributes to optimizing anti-tumor immunity in a rational manner.



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