The research described in this thesis has a focus on a unique T cell subset termed regulatory T cells (Treg). Tregs are immunoregulatory T cells that play an important role in immunological tolerance [1]. Treg development requires expression of the transcription factor FoxP[2], and there is extensive evidence that these cells can act in autoimmunity, transplantation and cancer [3]. Tregs can derive from the thymus (tTregs), but can also be induced in the periphery (pTregs) or in vitro (iTregs) [4]. They can be identified by a combination of markers, namely CD3, CD4, CD25, FoxP3, the absence of CD127, and furthermore by markers like CTLA-4 and Helios [5].

In Chapter 2 we explored whether factors that are known to promote the induction and survival of Tregs can also be used for the acquisition of an immunosuppressive phenotype by other immunoregulatory cell populations, such as CD1d-restricted invariant NKT (iNKT). iNKT cells represent a conserved immune cell subset able to produce pro- as well as anti-inflammatory cytokines upon stimulation, and it is this balance that determines the outcome of developing immune responses [6-8]. In a previous publication it was shown that freshly isolated iNKT cells can express FoxP3 upon exposure to TGF-ß and can acquire immunosuppressive properties upon culture with rapamycin [9]. In our studies, we investigated the effect of the suppressive cytokines IL-10 and TGF-ß alone or in combination with the mTOR inhibitor rapamycin. We found that IL-10 rather than TGF-ß induced FoxP3 expression, however, rapamycin was required for nuclear localization of FoxP3, which was otherwise localized in the cytoplasm. Furthermore, we showed that only iNKT cells with nuclear localization of FoxP3 were suppressive (iNKTregs). These iNKTregs could be beneficial when applying iNKT cells for immune-mediated inflammatory diseases. iNKTregs could also exert beneficial effects during hematopoietic stem cell transplantation (HSCT). As graft-versus-host disease (GVHD) is a serious complication of HSCT, and iNKT cell numbers can predict the incidence of GVHD [10], mTOR inhibitors could be of benefit in the treatment or prevention of GVHD.

The mTOR pathway is frequently deregulated in cancer and mTOR inhibitors are used in the treatment of several solid tumors including mRCC [11]. However, the clinical antitumor effect might be improved when the detrimental effect of mTOR inhibitors on the reported induction and expansion of Tregs can be counteracted [12–15]. Therefore, we analyzed the effect of several novel inhibitors of the PI3K/ mTOR pathway in Chapter 3. Our data indicate that inhibition of PI3K (i.e. upstream from mTOR) by BKM120 (Buparlisib) does not result in the proliferation advantage of Tregs over Tconv as is commonly observed when mTOR is directly inhibited by rapamycin [12–15]. In contrast, dual PI3K/mTOR inhibition by BEZ235 (Dactolisib) and single mTOR inhibition (rapamycin and everolimus) did. These data illustrate the presence of a complex signaling cascade in Tregs. While Tconv are dependent on the PI3K/ mTOR pathway for proliferation, Tregs are able to progress through the cell cycle via JAK/ STAT5 signaling by expression of the protein PIM 2, resulting in a selective growth advantage in the presence of rapamycin [16]. Although this might explain how Tregs are able to proliferate in
the presence of an mTOR inhibitor, it could also indicate a selective depletion of Tconv, since Tconv proliferation will be inhibited by mTOR inhibition (see also figure 1 of the introduction). The PI3K protein might have a more crucial role in this signaling cascade, since our data showed similar expansion rates for Tregs and Tconv in the presence of BKM120. However, future studies are needed to further unravel these mechanisms, knowledge of which may benefit the design of more effective cancer treatments.

It is known that Treg numbers increase in patients treated with the mTOR inhibitor rapamycin [17], and these results were recently confirmed for the mTOR inhibitor everolimus [18]. An increase in immunosuppressive Tregs may be beneficial in preventing transplant rejection and the control of autoimmune responses, but can be considered detrimental when aiming for effective anticancer immunity. Indeed, increased Treg numbers are associated with poor survival [19–21] and strategies that are able to prevent the mTOR mediated expansion of Tregs in cancer patients could therefore result in a more effective anticancer therapy response. To study this in more detail, we initiated a clinical trial in which everolimus, which at that time in cancer patients could therefore result in a more effective anticancer therapy response. To study this in more detail, we initiated a clinical trial in which everolimus, which at that time in cancer patients could therefore result in a more effective anticancer therapy response. To study this in more detail, we initiated a clinical trial in which everolimus, which at that time in cancer patients could therefore result in a more effective anticancer therapy response. To study this in more detail, we initiated a clinical trial in which everolimus, which at that time in cancer patients could therefore result in a more effective anticancer therapy response. To study this in more detail, we initiated a clinical trial in which everolimus, which at that time in cancer patients could therefore result in a more effective anticancer therapy response.

In vitro suppression assays with purified Tregs from these patients showed that their suppressive capacities were retained. In addition to the effects on Tregs, a significant increase in mMDSC, a significant decrease in immunoregulatory NK cells, classical CD141+ (cDC1) and CD1c+ (cDC2) DC subsets, as well as a decrease in the activation status of pDC and cDC1 was observed. These data indicate that the immunological effects of everolimus affect multiple immune cell subsets and altogether tip the balance in favor of immunosuppression, which underscores the requirement of combination treatment with agents able to negate immune suppression and boost T cell immunity. In Chapter 6 the results of the clinical trial in which everolimus was combined with several dosages and
schedules of metronomic oral CTX are described. These data indicate that a selective and significant Treg depletion in peripheral blood can be achieved when patients with mRCC are treated with the standard once daily oral dose of 10 mg everolimus in combination with 50 mg CTX once daily continuously. Both Treg percentages and absolute Treg numbers decreased after four weeks of this combination treatment and since the primary objective of this trial was to determine the recommended dose and schedule for metronomic CTX which, when combined with everolimus, resulted in optimal and selective Treg depletion, this dose was selected for the phase 2 part of the trial. Several adverse events (AE) were recorded, and the most common side effects were fatigue, anorexia, rash, cough, mucositis, nausea, anemia, and hypercholesterolemia. The overall incidence of these AEs was more or less comparable to that observed in the RECORD-1 trial, the phase 3 trial which led to market approval of everolimus.

Results of the extensive immunomonitoring that was performed in the phase 1 study are described in Chapter 7. The combination of 50 mg CTX once daily continuously with the standard dose of 10 mg everolimus once daily, which was selected for the phase 2 part of the study, not only resulted in depletion of Tregs, but also led to a reversal of the immunosuppressive effects in other immune cell subsets that were observed with everolimus treatment alone (see figure 2 of the introduction). These positive effects of the combination of everolimus and CTX on the immune response were hypothesized to contribute to improved survival, and therefore this combination was evaluated in the phase II part of the clinical trial, as reported in Chapter 8. Although the results of the phase 2 part were comparable to those observed in the phase 1 part of the trial, the trial as abrogated at the predefined interim-analysis after the inclusion of 24 patients, since the PFS was not improved from 50% to 70% at 4 months. Despite the termination of the phase 2 part of the trial, we did obtain relevant information from the comprehensive immunomonitoring data that may be taken into account in the design of future immunotherapeutic approaches, for instance in guiding which subsets to analyze to create a biomarker profile. The field of tumor immunobiology has been rapidly evolving over the past decade. Besides T cell-based cancer immunotherapy approaches, NK cells have been more intensely explored. As tumor cells are able to downregulate MHC class I molecules, cytotoxic T cells are no longer able to act, whereas NK cells still can [25]. One interesting finding in the here described trial was that the combination treatment of CTX and everolimus led to changes in the NK cell population. While four weeks of single everolimus treatment led to stable immunoregulatory NK cell percentages and increased cytotoxic NK cell percentages, addition of CTX reduced the effects on the cytotoxic subset, with even a significantly lower cytotoxic NK cell percentage in cohort 4 and 6, possibly contributing to the failure in obtaining clinical efficacy. Several drugs like antimetabolic agents, plant alkaloids and alkylating agents can lead to enhanced NK cell function [26], and one could envision that addition of one of these therapeutics to the here explored combination strategy could counteract the negative effects on the NK cell population. However, an important disadvantage of this approach might be the likely appearance of toxicity.
Furthermore, new therapies have been shown to be successful for several tumor types. Checkpoint inhibitors like ipilimumab (anti-CTLA-4), nivolumab and pembrolizumab (both anti-PD-1) are now registered for the treatment of several tumor types of cancer and recently nivolumab was registered for mRCC [27]. Unfortunately, it is still hard to predict which patients will benefit from these checkpoint inhibitors. Although expression of PD-L1 in the tumor is associated with better responses, patients with low PD-L1 expression in the tumor can still benefit from anti-PD-1 treatment regimens (28).

Recently the combination of nivolumab and ipilimumab was investigated for patients with mRCC, and especially patients with an intermediate to poor risk mRCC responded better to the combination treatment compared to sunitinib [29], leading to the FDA and EMA approval of the combination treatment for this patient category. Addition of ipilimumab might deplete Tregs, potentially contributing to the added value of the combination strategy. Unfortunately, most of the large clinical trials that led to the registration of checkpoint inhibitors lacked extensive immunomonitoring data. Taking into account that many immunological cell subsets express CTLA-4 and/or PD-1/PD-L1, immunomonitoring could help in the detection of biomarkers or create a biomarker profile for patients with a good response to immune checkpoint blockade therapy. Furthermore, the immunomodulatory effects of the treatment with CTX as described in this thesis, may be of additional value when combined with checkpoint inhibitors, simultaneously or in a sequential manner. As CTX resulted in reduced MDSC percentages, combination of checkpoint inhibitors with metronomic CTX could be of special interest taking into account the suppressive role of MDSCs and their possible role in the failure of checkpoint inhibitors [30].

The data described in this dissertation contribute to the understanding of the role of Tregs in cancer and possible mechanisms to counter the balance from an immunosuppressive to a more pro-inflammatory microenvironment.

**References**

Summarizing discussion and future perspectives

18.


