Cells were incubated for 4h with PBMCs either in the presence or absence of IL-2 and stimulated for 24h using aCD3/28 anti-CD28 antibodies to reproduce the phenotype of TCR-stimulated T cells completed more than 2 cell divisions following TCR stimulation and IL-2 production (Fig. 9, only IL-2 production is shown). A further increase of cytokine production and cell division was measured in T cells transfected with indicated concentrations of cbl-b siRNA (used at 2 µg/ml) and assessed by ELISA. All data shown in graphs A, B, and C were performed in duplicates and in 2 technical replicates and averaged.

**Effects of ex vivo cbl-b Silencing as adjuvant co-therapy for anti-tumor DC vaccination**

The main aspect of cbl-b silenced PBMCs for enhancing anti-tumor immune response can be seen in Figure 14. Cytokine production and cell division of ex vivo cbl-b silenced DCs were assessed in T-cell culture supernatants following IL-2 stimulation and IL-2 production was measured by ELISA. Cytokine production and cell division effects were noted when only cbl-b silenced T cells were transfected. These results suggest, that ACT with ex-vivo cbl-b silenced T cells can have a strong effect on cytokine production and cell division, which may lead to increased anti-tumor immune response and improved therapeutic outcome.

**Therapeutic Application of cbl-b Silencing**

Prophylactic siRNA for optimal cbl-b silencing generated increased production of cytokines with anti-tumor activity. Increased production of cytokines with cbl-b silencing was also confirmed in TCR stimulated quality model studies. However, these effects could not be determined in a non-invasive manner in the patient, but functional consequences of cbl-b silencing were confirmed in TCR stimulated ex-vivo control sample aliquots (in process control P002) of the produced (Fig. 14).

**Summary & Conclusions**

**Target Validation in murine Immune Cells**

Enhanced activity of cbl-b deficient murine T cells: Increased production of cytokines with anti-tumor activity Increased production of cytokine deficient T cells TGf-β resistance Absence of cbl-b enhances anti-tumor immunity Transfer of cbl-b deficient T cells to normal tumor and immunodepressed mice with intraperitoneal tumor model, shows increased production of cytokines with anti-tumor activity and decreased tumor growth.

**Solid target validation in murine in vivo models**

**Target Validation in human Immune Cells**

Enhanced activity of cbl-b deficient human T cells: Increased production of cytokines with anti-tumor activity Increased production of cytokine deficient T cells TGf-β resistance Enhanced activity of cbl-b silenced PBMCs: Silencing procedure established for human immune cells Cbl-b silenced PBMCs respond stronger to tumor cell contact Cbl-b silenced PBMCs react stronger in ADCS Solid target validation in human immune cells

**Therapeutic Application of cbl-b Silencing**

Prophylactic siRNA for optimal cbl-b silencing generated increased production of cytokines with anti-tumor activity. Enhanced production of cytokines with cbl-b silencing was also confirmed in TCR stimulated quality model studies. However, these effects could not be determined in a non-invasive manner in the patient, but functional consequences of cbl-b silencing were confirmed in TCR stimulated ex-vivo control sample aliquots (in process control P002) of the produced (Fig. 14).

**Phase I study for cbl-b silencing therapy has been initiated**

**Background:**

- cbl-b is a RING Finger 3 Ulpizin Igase
- cbl-b is a key negative regulator of lymphocyte activation
- Loss of cbl-b uncouples T cell activation from requirement for co-stimulation and overcomes suppression effects of TGF-β

**Target Validation in cbl-b deficient mice:**

- cbl-b knock-out mice spontaneously reject autocautis tumors
- Adoptive cell therapy (ACT) of cbl-b deficient murine T cells is sufficient to eradicate already established tumors
- cbl-b deficient T cells are less sensitive to suppression by T-regulatory cells
- cbl-b deficient mice suffer only from limited immunodeficiency and can reach normal lifespan

**In vivo Target Validation of cbl-b Silencing:**

- Adoptive Transfer of cbl-b silenced DCs T cells together with DCs suppress tumor growth during tumor rejection (Fig. 2).
- Cbl-b silenced T cells accumulate in the tumor and TILs raised from the explanted tumor 5 days after cell transfer produce enhanced amounts of IL-2 and IFN-γ.

**Cbl-b Silencing in human PBMCs:**

A transfer procedure for simultaneous and highly efficient transfer of all major leukocyte types present in human PBMCs was developed (Fig. 5).

**Screening for an optimized cbl-b siRNA:**

A panel of siRNAs was synthesized using a proprietary strategy (Blomberg, Friedman, et al., J. Immunology, 2007). cbl-b silenced PBMCs were transfected with siRNA and stimulated with cytokine production and cell division of PBMCs (IFN-γ, IL-2 and TNF-α). Cbl-b silenced PBMCs were transfected with siRNA and stimulated with cytokine production (IFN-γ, IL-2 and TNF-α) was observed.

**Targeting cbl-b for cancer immunotherapy:**

An optimized siRNA for clinical application should be able to suppress cbl-b for at least several days. The treatment was well tolerated without any severe side effects and no toxicity was observed for clinical application should be able to suppress cbl-b for at least several days. The treatment was well tolerated without any severe side effects and no toxicity was observed for long-term patients (Fig. 12).

**Ex vivo cbl-b Silencing as adjuvant co-therapy for anti-tumor DC vaccination protocol:**

The main aim of the study was to establish a rationale for combining DC vaccination with cbl-b as ex vivo silencing. Hereafter, we show that coadministration of siRNA with cbl-b silencing enhances anti-tumor immune responses, while maintaining a good safety and tolerability profile. The patient treated with this combined DC vaccination and cbl-b silencing therapy is in a treatment free period since 10 months (assessed as stable disease by MDT), with an overall survival since diagnosis of reasonable long metastases of 10 months.

**Figure 12.** Cbl-b [mean fluorescence] of Tumor cell contact without any Cytokine or siRNA. Cbl-b [mean fluorescence] of Tumor cell contact with cbl-b silenced DCs and indicated concentrations of siRNA.

**Figure 13.** Table showing the number of cbl-b silenced PBMCs that were transfected to the patient for each single treatment.

**Figure 14.** Cytokine production of cbl-b silenced DCs and no siRNA control DCs with indicated siRNA concentrations will be enrolled in T cell vaccination. Cytokine production and cell division effects were noted when only cbl-b silenced PBMCS were transfected. These results suggest, that ACT with ex-vivo cbl-b silenced T cells can have a strong effect on cytokine production and cell division, which may lead to increased anti-tumor immune response and improved therapeutic outcome.

**Phase I study to assess Safety and Immunologic Activity of autologous cbl-b silenced PBMCs**

A phase I clinical trial under study is based on the adoptive infusion of autologous cbl-b silenced PBMCs being activated (Comprehensive Cancer Center Wake Forest University).

The objectives are to determine toxicities and maximum tolerated dose and immunologic effects (clinical response will also be documented). The safety and tolerability of autologous cbl-b silenced PBMCs in combination with T-cell vaccination is the primary study endpoint. Autologous cbl-b silenced PBMCs are given every 14 days for a total of three infusions (up to a total of 40 µg).

Toxicity will be assessed using standard clinical and laboratory criteria. Blood production was observed without anti-C28 stimulation in this set-up.