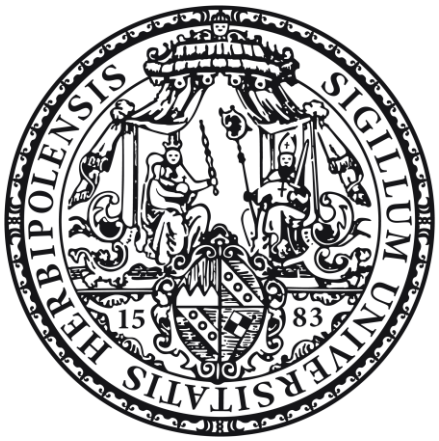

Differential requirement for CD28 co-stimulation on donor T cell subsets in mouse models of acute graft versus host disease and graft versus tumour effect

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Unterschiedlicher Einfluss der CD28 Kostimulation auf Donor-T-Zell-Populationen in Mausmodellen der akuten Graft-versus-Host Disease und des Graft-versus-Tumor Effekts

Doctoral thesis for a doctoral degree

at the Graduate School of Life Sciences,

Julius-Maximilians-Universität Würzburg,

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Würzburg, 2017

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1 Summary

Hematopoietic stem cell transplantation is a curative therapy for malignant diseases of the haematopoietic system. The patients first undergo chemotherapy or irradiation therapy which depletes the majority of tumour cells before they receive the transplant, consisting of haematopoietic stem cells and mature T cells from a healthy donor. The donor T cells kill malignant cells that have not been eliminated by the conditioning therapy (graft versus leukaemia effect, GvL), and, therefore, are crucially required to prevent relapse of the tumour. However, the donor T cells may also severely damage the patient's organs causing acute graft versus host disease (aGvHD). In mice, aGvHD can be prevented by interfering with the co-stimulatory CD28 signal on donor T cells. However, experimental models using conventional CD28 knockout mice as T cell donors or αCD28 antibodies have some disadvantages, i.e. impaired T cell development in the thymus of CD28 knockout mice and systemic CD28 blockade with αCD28 antibodies. Thus, it remains unclear how CD28 co-stimulation on different donor T cell subsets contributes to the GvL effect and aGvHD, respectively.

We developed mouse models of aGvHD and the GvL effect that allowed to selectively delete CD28 on certain donor T cell populations or on all donor T cells. $CD4^+$ conventional T cells (Tconv cells), regulatory T cells (Treg cells) or $CD8^+$ T cells were isolated from either Tamoxifen-inducible CD28 knockout (iCD28KO) mice or their wild type (wt) littermates. Allogeneic recipient mice were then transplanted with T cell depleted bone marrow cells and different combinations of iCD28KO and wt T cell subsets. Tamoxifen treatment of the recipients caused irreversible CD28 deletion on the iCD28KO donor T cell population. In order to study the GvL response, BCL-1 tumour cells were injected into the mice shortly before transfer of the T cells.

$CD4^+$ Tconv mediated aGvHD was efficiently inhibited when wt Treg cells were co-transplanted. In contrast, after selective CD28 deletion on donor Treg cells, the mice developed a late and lethal flare of aGvHD, i.e. late-onset aGvHD. This was associated with a decline in iCD28KO Treg cell numbers around day 20 after transplantation. CD28 ablation on either donor $CD4^+$ Tconv cells or $CD8^+$ T cells reduced but did not abrogate aGvHD. Moreover, iCD28KO and wt $CD8^+$ T cells were equally capable of killing allogeneic target cells *in vivo* and *in vitro*. Due to this sufficient anti-tumour activity of iCD28KO $CD8^+$ T cells, they had a therapeutic effect in our GvL model and

25% of the mice survived until the end of the experiment (day 120) without any sign of the malignant disease. Similarly, CD28 deletion on all donor T cells induced long-term survival. This was not the case when all donor T cells were isolated from wt donor mice. In contrast to the beneficial outcome after CD28 deletion on all donor T cells or only $CD8^+$ T cells, selective CD28 deletion on donor $CD4^+$ Tconv cells completely abrogated the GvL effect due to insufficient $CD4^+$ T cell help from iCD28KO $CD4^+$ Tconv cells.

This study demonstrates that therapeutic inhibition of the co-stimulatory CD28 signal in either all donor T cells or only in $CD8^+$ T cells might protect patients from aGvHD without increasing the risk of relapse of the underlying disease. Moreover, deletion of CD28 on donor Treg cells constitutes a mouse model of late-onset aGvHD which can be a useful tool in aGvHD research.

2 Zusammenfasung

Die hämatopoetische Stammzelltransplantation ist eine heilende Therapie für maligne Erkrankungen des blutbildenden Systems. Die Patienten müssen sich zuerst einer Chemotherapie oder einer Strahlentherapie unterziehen, welche den Großteil der Tumorzellen beseitigt, bevor sie das Transplantat erhalten. Dieses besteht aus hämatopoetischen Stammzellen und reifen T-Zellen eines gesunden Spenders. Die transplantierten T-Zellen töten die malignen Zellen, die zuvor durch die Chemo- bzw. Strahlentherapie nicht zerstört wurden (Graft versus Leukämie Effekt, GvL), und sind daher essenziell, um ein Rezidiv der Tumorerkrankung zu verhindern. Die T-Zellen des Spenders können aber auch die Organe des Patienten schwer schädigen und dadurch die akute Graft versus Host Disease (aGvHD) verursachen. In Mäusen kann die aGvHD verhindert werden, indem man das kostimulatorische Signal des CD28 Moleküls moduliert. Mausmodelle, in denen konventionelle CD28 Knock-out Mäuse als T-Zell-Donoren verwendet werden oder αCD28 Antikörper eingesetzt werden, haben einige Nachteile, wie zum Beispiel eine gestörte T-Zell Entwicklung in CD28 Knock-out Mäusen oder die systemische Blockade des CD28 Moleküls mit Antikörpern. Dadurch blieb bislang unklar, inwiefern CD28-Kostimulation auf verschiedenen T-Zell-Populationen zum GvL Effekt und zur aGvHD beiträgt.

Wir haben Mausmodelle der aGvHD und des GvL Effekts entwickelt, die ermöglichen, das CD28 Molekül entweder nur auf bestimmten Spender-T-Zell-Populationen oder auf allen Spender-T-Zellen zu deletieren. Hierfür wurden $CD4^+$ konventionelle T-Zellen (Tconv Zellen), regulatorische T Zellen (Treg Zellen) und $CD8^+$ T-Zellen von Tamoxifen-induzierbaren CD28 Knockout (iCD28KO) Mäusen bzw. deren wildtypischen (wt) Wurfgeschwistern isoliert. Den allogenen Empfängermäusen wurden dann T-Zell-depletierte Knochenmarkszellen und verschiedene Kombinationen aus iCD28KO und wt Spender-T-Zellen transplantiert. Die Behandlung der Empfängertiere mit Tamoxifen führte zu einer irreversiblen Deletion von CD28 auf den iCD28KO T-Zell-Populationen. Um den GvL Effekt zu untersuchen, wurden den Mäusen kurz vor dem T-Zell-Transfer BCL-1 Tumorzellen injiziert.

Die von den $CD4^+$ Tconv Zellen verursachte aGvHD konnte sehr gut kontrolliert werden, indem zusätzlich wt Treg Zellen transplantiert wurden. Im Gegensatz dazu entwickelten die Mäuse einen späten und tödlichen Schub der aGvHD, auch late-onset

aGvHD genannt, wenn die CD28 Expression auf den Treg Zellen des Spenders deletiert wurde. Dies ging mit einem Rückgang der iCD28KO Treg-Zellzahlen ca. 20 Tage nach Transplantation einher. Die Deletion von CD28 auf $CD4^+$ Tconv Zellen oder auf $CD8^+$ T-Zellen reduzierte die aGvHD, konnte diese aber nicht vollständig verhindern. Des Weiteren waren iCD28KO und wildtypische $CD8^+$ T-Zellen gleichermaßen in der Lage, allogene Zellen zu töten, *in vivo* wie auch *in vitro*. Aufgrund dieser hinreichenden Anti-Tumor-Antwort hatten iCD28KO $CD8^+$ T-Zellen einen therapeutischen Effekt in unserem GvL Modell und 25 % der Tiere überlebte bis zum Versuchsende (Tag 120) ohne Anzeichen des Tumors. Ein Langzeitüberleben der Tiere wurde auch beobachtet, wenn das CD28 Molekül auf allen Spender-T-Zellen fehlte. Dies war nicht der Fall, wenn alle Spender-T-Zellen von wt Mäusen isoliert wurden. Im Gegensatz zur CD28 Deletion auf entweder allen Spender-T-Zellen oder nur auf den $CD8^+$ T-Zellen, ging der GvL Effekt vollständig verloren, wenn CD28 nur auf den $CD4^+$ Tconv Zellen entfernt wurde, da diese dann keine ausreichende T-Zellhilfe mehr leisten konnten.

Die vorliegende Arbeit zeigt, dass eine therapeutische Blockade des kostimulatorischen CD28 Signals entweder in allen Spender T-Zellen oder nur in $CD8^+$ T-Zellen vor der aGvHD schützen könnte ohne gleichzeitig das Risiko eines Rezidivs zu erhöhen. Darüber hinaus steht mit der Deletion von CD28 auf Treg Zellen ein Mausmodell der late-onset aGvHD zur Verfügung, welches für die weitere Erforschung dieser Krankheit nützlich sein kann.

3 Introduction

Hematopoietic stem cell transplantation (HSCT) is a very efficient therapy for numerous diseases. Besides various genetic disorders, the main indication for this treatment are neoplastic malignancies of myeloid or lymphoid origin [1]. The therapeutic power of HSCT can be attributed to two mechanisms: 1) depletion of the patient's hematopoietic system by chemotherapy or irradiation and replacing it with healthy hematopoietic stem cells and 2) the anti-tumour activity of transferred allogeneic T cells. However, the donor-derived T cells can severely damage host tissue by their cytotoxic effector functions and, thereby, induce acute graft versus host disease (aGvHD) [reviewed in 2]. Besides infectious diseases and recurrence of the underlying malignancy, aGvHD is the major cause for post-transplant morbidity and mortality [reviewed in 3]. Clinically, aGvHD primarily involves the skin, liver and gastrointestinal tract and manifests as rash, jaundice and diarrhoea. To prevent aGvHD while at the same time allowing a sufficient anti-tumour response after HSCT, the transferred immune cells need to be tightly controlled. In addition to donor T cells, which belong to adaptive immunity, also cells of innate immunity are crucial for the pathophysiology of aGvHD and the GvL.

3.1 Innate Immunity

The immune system of mammals consists of numerous cell types and various humoral factors and involves different organs. It has evolved as a mechanism to defend the body against all kinds of diseases and can be divided into two branches, innate immunity and adaptive immunity. Innate immunity provides a first and immediate defence against pathogens and, for this purpose, uses different mechanisms of protection. Epithelial barriers provide a mechanical barrier that prevents invasion of microbes. In addition, soluble factors and specialised cells of mostly myeloid origin fight against incorporated pathogens. Phagocytes, including macrophages and neutrophils, can engulf and digest microbes whereas other cells, like eosinophils and mast cells, release pro-inflammatory mediators. In addition, natural killer (NK) cells, which belong to the innate lymphoid cells, can kill pathogens or infected cells [summarized in 4]. A common feature of the cells of innate immunity is their ability to sense pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) through pattern recognition receptors. Activation of these

receptors triggers the release of pro-inflammatory cytokines that can be sensed by cells of the adaptive immune system [reviewed in 5]. Dendritic cells (DCs) are professional antigen presenting cells (APCs). They build a bridge between innate and adaptive immunity as their main function is to present pathogen-derived antigens to T cells in the context of major histocompatibility complex (MHC) class I and class II molecules [reviewed in 6]. DCs and other cells of the innate immune system play a critical role in the development of aGvHD. The conditioning of patients by irradiation and chemotherapy causes the release of PAMPs and DAMPs and subsequent activation of innate immune cells. Mature donor APCs initiate and amplify the T cell-driven aGvHD by release of pro-inflammatory mediators [reviewed in 7].

3.2 Adaptive Immunity

In contrast to the innate immune system, the adaptive immune system mounts highly specific immune responsess against a particular pathogen and can further mediate long-lasting protection. The major cellular players of adaptive immunity are B cells and T cells. While B cells develop in the bone marrow, T cells mature in the thymus. Both cell types acquire their antigen specificity through random somatic recombination of receptor-encoding gene segments such that every mature T and B cell is equipped with a unique T cell or B cell receptor [summarized in 8]. Upon recognition of their cognate antigen, B cells differentiate into memory B cells and plasma cells, the latter producing antibodies which are mediators of the humoral immune response. While antibody production of B cells is involved in the pathophysiology of chronic GvHD, the major cellular effectors of acute GvHD are T cells [reviewed in 9]

3.2.1 T cell activation

T cell activation takes place in the secondary lymphoid organs, e.g. the spleen and the lymph nodes. Naïve T cells patrol through the body via the blood stream and lymph vessels and scan professional APCs in lymphoid organs for their cognate antigen. During an infection, professional APCs that have sensed and internalized the microbes also migrate to secondary lymphoid organs where they present antigenic peptides on MHC molecules. In more detail, phagocytosed proteins are processed and their peptides presented on MHC class II or, via a process called cross-presentation, on MHC class I molecules. In contrast, cytosolic antigens are predominantly presented on

MHC class I molecules but can also be processed and enter the pathway for presentation on MHC class II molecules [summarized in 10]. Of note, the T cell receptor (TCR) of $CD4^+$ T cells recognises peptides in the context of MHC class II molecules whereas $CD8^+$ T cells are restricted to MHC class I. In case the MHC-peptide complex binds to the TCR, an intracellular signalling cascade is initiated. Full activation of T cells, however, requires a co-stimulatory signal (see 3.2.2). Numerous co-stimulatory molecules have been identified that belong to either the immunoglobulin superfamily (e.g. CD28, ICOS) or the tumour necrosis factor receptor superfamily (e.g. 4-1BB, CD27 or Ox40) [reviewed in 11]. Of those molecules, CD28 is considered to be the major co-stimulatory receptor because it is, in contrast to e.g. ICOS, constitutively expressed on naïve T cells and involved in the initiation of the T cell signalling cascade [reviewed in 12]. The ligands for CD28 on antigen presenting cells are CD80 and CD86, which are both upregulated upon activation of the APC. CD86 is more abundantly expressed on APCs under steady state conditions and is induced more rapidly upon activation. This suggests that CD86 is the major ligand in initial T cell priming [reviewed in 13]. CD80 might, however, be a more potent ligand to CD28 during cognate T cell APC interaction as conformational changes in the CD28 molecule after TCR stimulation allow for bivalent interaction with CD80 [14]. Upon ligation with CD80 or CD86, CD28 is phosphorylated at the YMNM and PYAP motives of its intracellular tail. This recruits the adaptor protein growth factor receptor bound protein 2 (GRB2), protein kinase Cθ (PKCθ), lymphocyte cell-specific protein-tyrosine kinase (LCK), phosphoinositide 3-kinase (PI3K) and RAS guanyl nucleotide-releasing protein (RASGRP). PI3K and RASGRP enhance AKT signalling and thereby induce the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and nuclear factor of activated T cells (NFAT). This increases the expression of IL-2, the glucose transporter GLUT1 and the anti-apoptotic protein BCL-xL, thus, leading to cell proliferation and survival [15, reviewed in 11]. Nuclear translocation of NFAT, and subsequent IL-2 production, are further dependent on GRB2 and LCK, the latter also being part of the TCR signalling cascade [reviewed in 11].

CD80 and CD86 cannot only co-stimulate T cells through CD28 but also lead to co-inhibition through the Cytotoxic T-lymphocyte Antigen-4 (CTLA-4, CD152) receptor. CTLA-4 is upregulated on conventional T cells during activation and binds CD80/CD86 with higher affinity than CD28 [16]. It, therefore, outcompetes CD28 in binding to their

shared ligands and allows for effective control of T cell responses. Mice lacking CTLA-4 develop an autoimmune syndrome. This is not the case if additionally CD28 is deleted [17, 18].

3.2.2 Two Signal Hypothesis

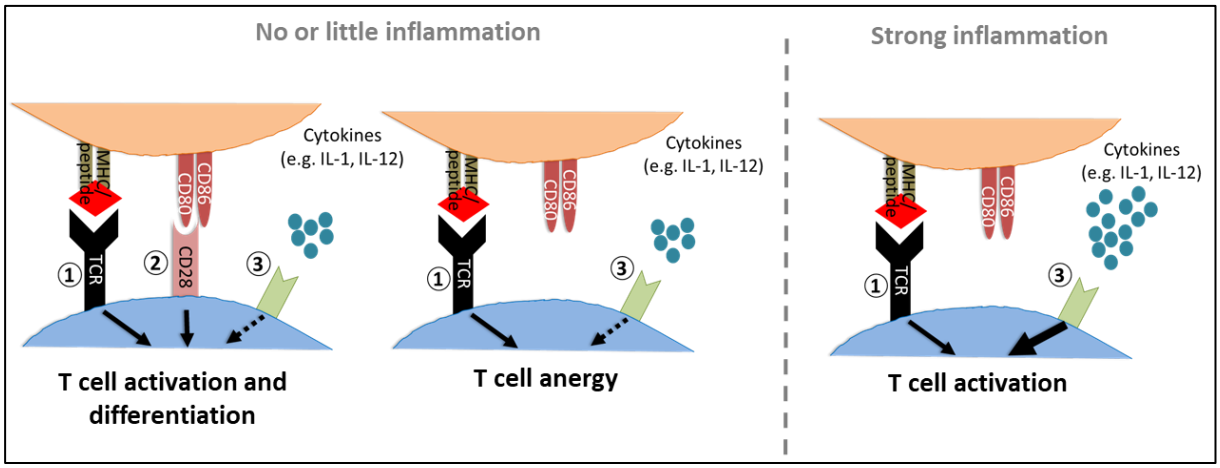

Figure 1: Three-Signal-Modell of T cell activation. Full T cell activation requires signalling through the TCR (Signal 1) and co-stimulation via CD28 (Signal 2). Cytokines (Signal 3) drive the differentiation of CD4⁺ T cells into different T helper subsets. In absence of CD28 stimulation, T cells become angergic. In highly inflammatory situations signal 3 can compensate for lack of CD28 stimulation.

TCR signalling in the absence of CD28 co-stimulation renders naïve T cells anergic [19, 20, reviewed in 21]. This mechanism of peripheral tolerance helps to avoid activation of self-reactive T cells and is called the two-signal-model of T cell activation (see Figure 1) [reviewed in 11]. The model, as we know it today, is based on the observations of Bretscher and Cohn in 1970 [22]. They were the first ones, describing that two signals are required to activate lymphocytes, whereby signal two is needed to interpret signal one. Remarkably, at the time when Bretscher and Cohn proposed their “theory of self-nonself discrimination”, the two distinct types of lymphocytes (T cells and B cells) were just about to be discovered. Lafferty and Cunningham and others further developed the model of Bretscher and Cohn [reviewed in 23]. Today, it is still a matter of debate if co-stimulation through CD28 integrates into the TCR signalling cascade in a qualitative or quantitative manner, or both [reviewed in 24, 25]. An argument for a mere quantitative contribution of CD28 in T cell activation came from a study showing that co-stimulation lowered the number of TCRs that need to be triggered in order to activate T cells [26]. Moreover, CD28 ligation was found to amplify the transcriptional response to TCR ligation rather than inducing a distinct set of genes [27, 28]. On the other hand, a qualitative role of CD28 in T cell activation might come

from its role in establishing first T cell-APC contacts. When compared to TCR/MHC-peptide interactions, the binding of CD28 to its ligands occurs with faster kinetics and might, thus, be important for initiating the APC -T cell contact [reviewed in 29]. This hypothesis is supported by a recent study, showing that CD28 enhances TCR signalling even in absence of its cytosolic domain [30].

Recently, the two-signal-hypothesis has been extended to a three-signal-model [reviewed in 31]. The third signal is derived from cytokines and is required for optimal effector functions of antigen-activated $CD4^+$ and $CD8^+$ T cells. The pro-inflammatory cytokines IL-12 and INFα/β, therefore, support clonal expansion as well as formation of long-lived memory cells during a $CD8^+$ T cell response [32]. For $CD4^+$ T cells, IL-1 is thought to enhance proliferation and differentiation and functions as “signal three” [33].

Despite the central role that CD28 plays in the priming of $CD4^+$ and $CD8^+$ T cells, it is clearly not essential for activating T lymphocytes. This is demonstrated by mice that constitutively lack CD28 molecules but are still capable of mounting immune responses against pathogens [34]. In fact, in highly inflammatory situations, CD28 co-stimulation becomes dispensable (see Figure 1) [35, 36]. There are a number of other signals that might have the potential to compensate for the lack of CD28 co-stimulation: the “signal three” cytokines IL-1, IL-12 and INFα/β, IL-2 [reviewed in 37] or co-stimulatory molecules like Ox40 and 4-1BB [38, 39].

3.2.3 T cell subsets

During thymic development, $CD4^+$ $CD8^+$ T cell precursors lose one of the two co-receptors CD4 or CD8 and become restricted to the $CD4^+$ T helper (Th) cells or the $CD8^+$ cytotoxic lymphocytes (CTL) lineage. CTLs, as their name indicates, are professional killers that can directly destroy infected or malignant cells. There are two different mechnisms by which CTLs induce apoptosis in their target cells. They release granules by exocytosis which contain Perforin and Granzyme B. Perforin is a protein that inserts into the cell membrane of the target cell and forms pores through which Granzyme B can enter. Granzyme B is a protease that cleaves pro-caspase 3 and induces apoptotic pathways [reviewed in 40]. Moreover, cytotoxic T cells express Fas ligand (FasL) on the cell surface which binds to Fas on the target cell. Thereupon,

cleavage of pro-caspase 8 activates downstream caspases and ultimately leads to apoptosis of the target cell [reviewed in 41].

$CD4^+$ T helper cells contribute to the clearance of an infection by providing help to CTLs, for instance by production of IL-2, by stimulating B cells to produce antibodies or by activating macrophages to destroy ingested microbes [summarized in 42]. Depending on the cytokine milieu in which T cell activation takes place, $CD4^+$ T cells can differentiate into different subsets of helper cells. Th1 cells produce Interferon γ (IFNγ) and are mediators of intracellular immunity whereas Th2 cells secrete IL-4 and provide protection against extracellular pathogens. Additionally, other subsets, like Th17 or Th9 cells that express distinct effector cytokines have been described [reviewed in 43]. In patients undergoing HSCT, high IFNγ cytokine levels correlate with a higher incidence to develop aGvHD indicating a major role of Th1 cells in disease pathology [44, 45] . In most *in vivo* models of aGvHD, and also the mouse model used in this study, it is known that aGvHD is mainly driven by Th1 cells [46, 47]. However, under certain circumstances also Th17 cells are able to mediate lethal aGvHD [reviewed in 48]. While $CD4^+$ T cells alone are sufficient to induce aGvHD, cooperation of $CD4^+$ and $CD8^+$ T cells is required for the GvL effect [49, 50]. Regulatory T cells also express the CD4 co-receptor but are functionally different from conventional $CD4^+$ T cells. In contrast to T helper cells, they do not contribute to but inhibit inflammatory processes and are crucial in terminating and regulating immune responses.

3.2.4 Regulatory T cells

Regulatory T cells (Treg cells) either develop in the thymus (thymic, natural Treg cells) or differentiate in the periphery (induced Treg cells) from naïve $CD4^+$ T cells [reviewed in 51]. In 2003, Foxp3 was found to be the master regulator of $CD4^+CD25^+$ Treg cells [52-54]. This discovery was based on previous observations in Scurfy mice [55] and IPEX patients who suffer from autoimmunity as a consequence of mutations in the Foxp3 gene. $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells exert their suppressive function through different mechanisms [reviewed in 51, 56, 57]: 1) They secrete the anti-inflammatory cytokines IL-10 and TGFβ. 2) They constitutively express the high affinity IL-2 receptor and thereby reduce the availability of this cytokine for conventional T cells. 3) They express Granzyme B and Perforin and act in a cell to cell manner 4) They constitutively express CTLA-4 and remove CD80 and CD86 from the cell surface of activated APCs

through a process called trans-endocytosis [58]. By this mechanism, Treg cells indirectly prevent CD28 co-stimulation of conventional T cells.

Treg cells themselves are highly dependent on CD28 signalling. This is illustrated by a 60% - 80% reduction of Treg cells in thymus and periphery of CD28-/- mice when compared to wild type mice [59-61]. Mechanistically, CD28 signalling is required for upregulating Foxp3 during Treg cell development [62]. In the periphery, CD28 is furthermore crucial for homeostatic Treg proliferation in response to self-antigens. This was demonstrated by a rapid decline in Treg cell numbers after induced CD28 deletion [59]. Moreover, CD28-depleted Treg cells also showed impaired effector functions [59].

3.3 Immunological tolerance

The concept of immunological tolerance was already proposed over 50 years ago by Medawar and Burnet even before MHC restriction and T cell subsets were known [63]. It describes the capability of the immune system to mount effective immune responses against pathogens and malignant cells but not against self-antigens. Tolerance of the adaptive immune system can either be generated in the thymus, central tolerance, or acquired in the periphery, peripheral tolerance [reviewed in 64]. Mechanisms of central tolerance are necessary because the T cell receptor repertoire is generated by random recombination events that also lead to self-reactive receptors. Central tolerance ensures that these potentially auto-reactive T cells are deleted in the thymus through a mechanism called negative selection. Positively selected, thus MHC-restricted single positive T cell precursors migrate to the thymic medulla. There, the transcription factor AIRE specifically expressed in medullary thymic epithelial cells (mTECs) drives the expression of tissue restricted antigens in these cells [65]. Like professional APCs, mTECS can present antigens on MHC class I and MHC class II molecules and provide co-stimulatory signals through CD80 [reviewed in 66]. Thymocytes bearing a TCR that recognises any of the presented self-antigens die by apoptosis. The role of CD28 during thymic selection is not yet fully understood. CD28-deficient mice show a 50 % increase in thymic cellularity indicating a role in negative selection [67-69]. However, as CD28 was reported to inhibit positive selection of thymocytes [70], this could also account for higher cell numbers in thymi of CD28 knock-out mice. In fact, negative selection of conventional $CD4^+$ and $CD8^+$ T cells in the thymus requires different co-stimulatory receptors and is not exclusively dependent on CD28 [71, 72].

Some thymocytes that bear auto-reactive TCRs are not negatively selected but instead become regulatory T cells. This is another mechanism of central tolerance. In contrast to maturation of conventional T cells, CD28 signalling is essential for the generation of thymus-derived regulatory cells. As described above (see 3.2.4), CD28 deletion leads to a drastic reduction in Treg cell frequencies in mice. Mechanistically, CD28 co-stimulation is necessary for the generation and survival of $CD4^+$ $Foxp3^-$ $CD25^+$ precursors rather than affecting the TCR repertoire of the Treg population or skewing $CD4^+$ T cell precursors towards a Tconv phenotype [73]. Under the influence of IL-2 and IL-15, $CD4^+$ $Foxp3^-$ $CD25^+$ thymocytes upregulate Foxp3 expression and become Treg cells [74] .

Eventually, and even though the mechanisms of central tolerance are very efficient, some potentially harmful, self-reactive T cells might leave the thymus. Therefore, peripheral tolerance is required to tightly control the immune system and prevent autoimmunity. Induced apoptosis in auto-reactive T cell clones is one mechanism of peripheral tolerance and requires interaction of Fas and FasL [reviewed in 75]. This explains to some extent why mice carrying a mutation in the Fas gene suffer from lymphoproliferative disorders [76]. In addition, Bim, the natural antagonist of Bcl-2, plays a critical role in the deletion of auto-reactive CD8 T cells [77].

Another way to control self-reactive T cells is to induce anergy. As discussed in chapter 3.2.2, CD28 co-stimulation is crucial for T cell activation. Immature $CD80^{low}$/$CD86^{intermediate}$ APCs that present self-peptides on MHC molecules induce TCR signalling without sufficient co-stimulation and render T cells unresponsive to further stimuli. Apart from that, the co-inhibitory receptors CTLA-4 and PD-1 are important players in peripheral homeostasis and mice, deficient for either one of the two receptors, develop autoimmunity [78, 79, reviewed in 80]. Despite these similarities, CTLA-4 and PD-1 exert their functions in controlling T cell activation via different mechanisms. CTLA-4 limits early T cell responses in lymphoid organs by removing CD80 and CD86 from the DC cell surface [58]. In contrast, PD-1 transduces a negative signal to previously activated T cells in the periphery. The differential expression patterns of their ligands might at least partially account for this difference. CD80 and CD86, the shared ligands for CD28 and CTLA-4, are mainly expressed by professional APCs, whereas the ligands for PD-1, PD-L1 and PD-L2, are on non-haematopoietic cells and APCs [reviewed in 81]. Taken together, in non-inflammatory

situations, APCs mediate co-inhibitory signals and render T cells tolerogenic rather than induce immune responses [reviewed in 82].

Another way how antigen presenting cells can act tolerogenic, is by generating induced Treg cells (iTreg). In the appropriate microenvironment, certain DC subsets can induce Foxp3 expression in $CD4^+$ T cells [83, 84]. In contrast to Treg differentiation in the thymus, iTregs cannot only be generated against auto-antigens but also against foreign antigens [85]. This expands the potential TCR repertoire of the Treg population and argues for distinct functions of thymus-derived natural Tregs (nTreg) and iTregs [reviewed in 86, 87]. In murine models of organ transplantation, both, nTreg and iTreg, prevent graft rejection [88-90]. Furthermore, Treg have been shown to inhibit aGvHD and induce long-term survival after allogeneic haematopoietic stem cell transplantation in mice [91-93]. The efficacy and applicability of adoptive Treg cell therapy to treat human aGvHD, however, is still a topic of investigation [94-96, reviewed in 97].

3.4 Alloresponses

The fact that T cells recognise antigens only when presented on MHC molecules links innate immunity to adaptive immunity and ensures that the adequate type of T cell response is mounted against a certain pathogen. However, in the unnatural situation of hematopoietic stem cell transplantation, MHC restriction is the basis of graft rejection and graft versus host disease. T cells normally recognise self MHC/ foreign peptide complexes but due to structural mimicry, they can also be activated by allogeneic MHC/peptide complexes. Still, there is a major difference between conventional T cells responses and allo-recognition: The frequency of alloreactive T cells is 100 -1,000 fold higher than the precursor frequency of T cells specific for any single self MHC/ peptide complex resulting in around 5-10% of alloreactive T cells in the periphery [98, reviewed in 99]. There is evidence that TCRs have an inherited affinity towards MHC and that positive selection for low-affinity interactions with self-MHC/peptide complexes in the thymus further enriches for T cells with cross-reactivity towards allogeneic MHC/peptide structures [100, reviewed in 101]. Moreover, it has been shown that T cells can recognise multiple peptide/MHC complexes, which could also explain the high frequency of alloreactive T cell clones within the T cell repertoire [reviewed in 99].

The molecular basis of alloreactivity lies in the high polymorphism of MHC molecules. The MHC complex is located on chromosome 6 in humans and chromosome 17 in

mice and contains over 200 genes. In humans, there are three different type I MHC genes (HLA –A, -B and –C) and three pairs of MHC class II α and β- chain genes (HLA-DP, HLA–DQ, HLA-DR) which are co-dominantly expressed [summarized in 102]. Each of these MHC molecules binds a different range of peptides, increasing the chance that pathogenic antigens will be presented by APCs. Additionally, MHC genes are highly polymorphic. Evolutionary, this is an advantage because it increases the probability that within a population at least some individuals can mount an adequate immune response against a new pathogen [reviewed in 103].

In mice, there are three MHC I genes (H-2K, H-2D and H-2L) and the two MHC II gene loci (I-A and I-E) [summarized in 10]. In inbred mouse strains, of course, MHC polymorphism is lost. Instead, each different strain expresses one certain haplotype of MHC genes. C57BL/6 mice, for instance, have the haplotype H-2b and have the genes $H\text{-}2K^{b}$, $H2\text{-}D^{b}$ and $I\text{-}A^{b}$ but do not have an I-E gene. In contrast, BALB/c mice have the haplotype H-2d and possess the genes $H\text{-}2K^{d}$, $H2\text{-}D^{d}$, $I\text{-}A^{d}$ as wells as $I\text{-}E^{d}$. Regarding mouse models of haematopoietic stem cell transplantation, this means that transfer of C57BL/6 bone marrow cells into BALB/c recipient mice leads to a full MHC mismatch. Additionally, the two different mouse strains will also differ in other polymorphic antigens, so called minor histocompatibility antigens (miHA). In this example major and minor mismatches activate the transplanted C57BL/6 T cells via the direct and indirect pathway of allorecognition, respectively, and lead to lethal graft versus host disease (Figure 2) [reviewed in 104]. However, in other MHC matched HSCT models, minor mismatches alone are sufficient to cause graft versus host disease, even though with less morbidity [reviewed in 104].

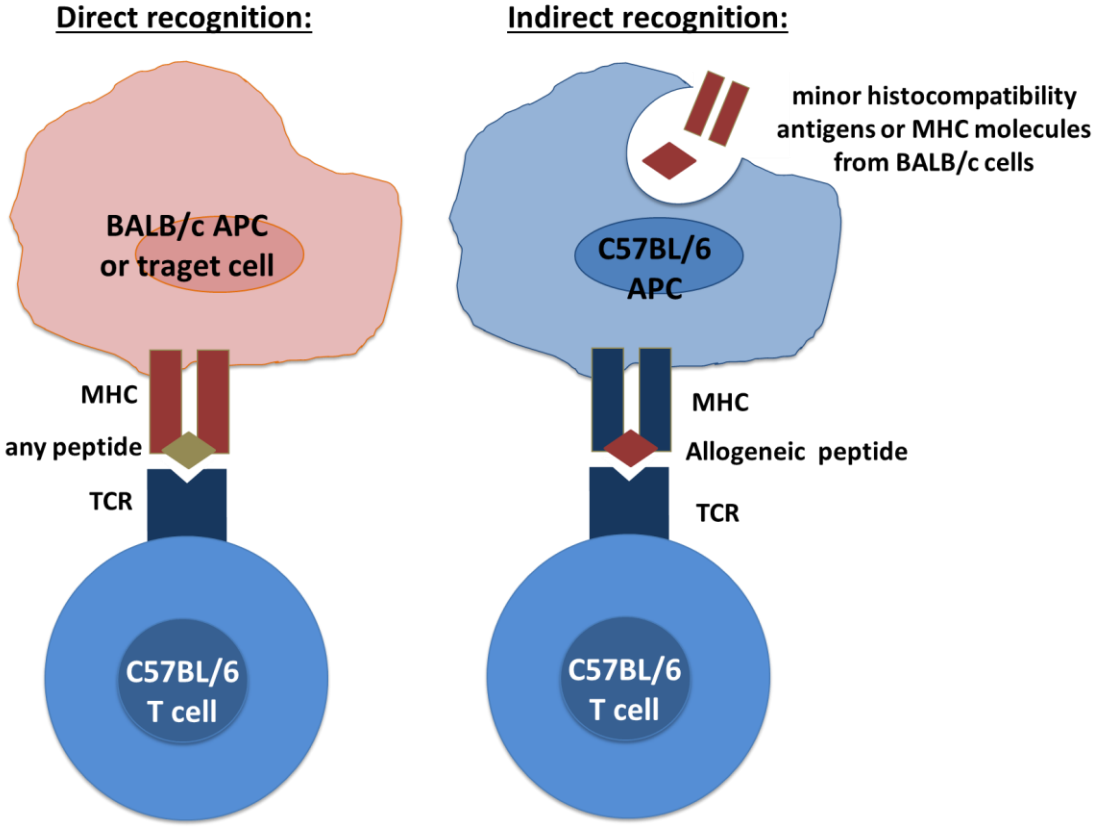

Figure 2: Direct and indirect recognition of allogeneic MHC molecules and minor histocompatibility antigens in the C57BL/6 →BALB/c transplantation model. C57BL/6 T cells recognise allogeneic MHC/peptide complexes on BALB/c APCs or target cells. The origin of the peptide bound to the MHC molecule is irrelevant in direct allo-recognition. Donor-derived (C57BL/6) APCs take up host (BALB/c) proteins and can activate donor T cells via the indirect pathway of allorecognition. The processed peptide presented on the MHC molecule needs to be either derived from allogeneic MHC molecules or minor histocompatibility antigens. Based on: [104, 105]

3.5 Haematopoietic stem cell transplantation

One of the pioneers in the field of haematopoietic stem cell transplantation was Edward Donnall Thomas who performed the first transfer of bone marrow cells between human individuals in 1957, which was before the HLA system was discovered [106]. Even though this first trial failed to cure the patients, 15 years later, E. Thomas performed the first successful HSCT and for this was awarded with the Nobel Prize in medicine in 1990 [107, reviewed in 108]. Moreover, HLA-matching allowed transplantations between unrelated donors and made this therapy available to a larger cohort of patients. Since then, improved conditioning strategies, the use of peripheral blood stem cells as well as optimized treatment and prophylaxis of opportunistic infections constantly increased the number of patients undergoing haematopoietic stem cell

transplantations over the last decades. In 2016, almost 16 000 allogeneic HSCTs were performed within Europe [109].

Despite all the improvements that have been achieved since the first transplantations, acute GvHD is still a major cause of morbidity and mortality after allogeneic HSCT. The incidence of aGvHD is highly dependent on the degree of mismatch and ranges between 35 % and 80 % [110, reviewed in 7] . Acute GvHD can be classified in four grades: mild (I), moderate (II), severe (III) and very severe (IV). The prognosis for severe aGvHD is very poor, with only 5 % (grade IV) to 25 % (grade III) surviving longer than five years [reviewed in 7].

3.6 Pathology of acute graft versus host disease

The pathology of acute graft versus host disease can be conceptualised into three phases (see Figure 3): 1) Activation of APCs, 2) Donor T cell activation and 3) Effector phase.

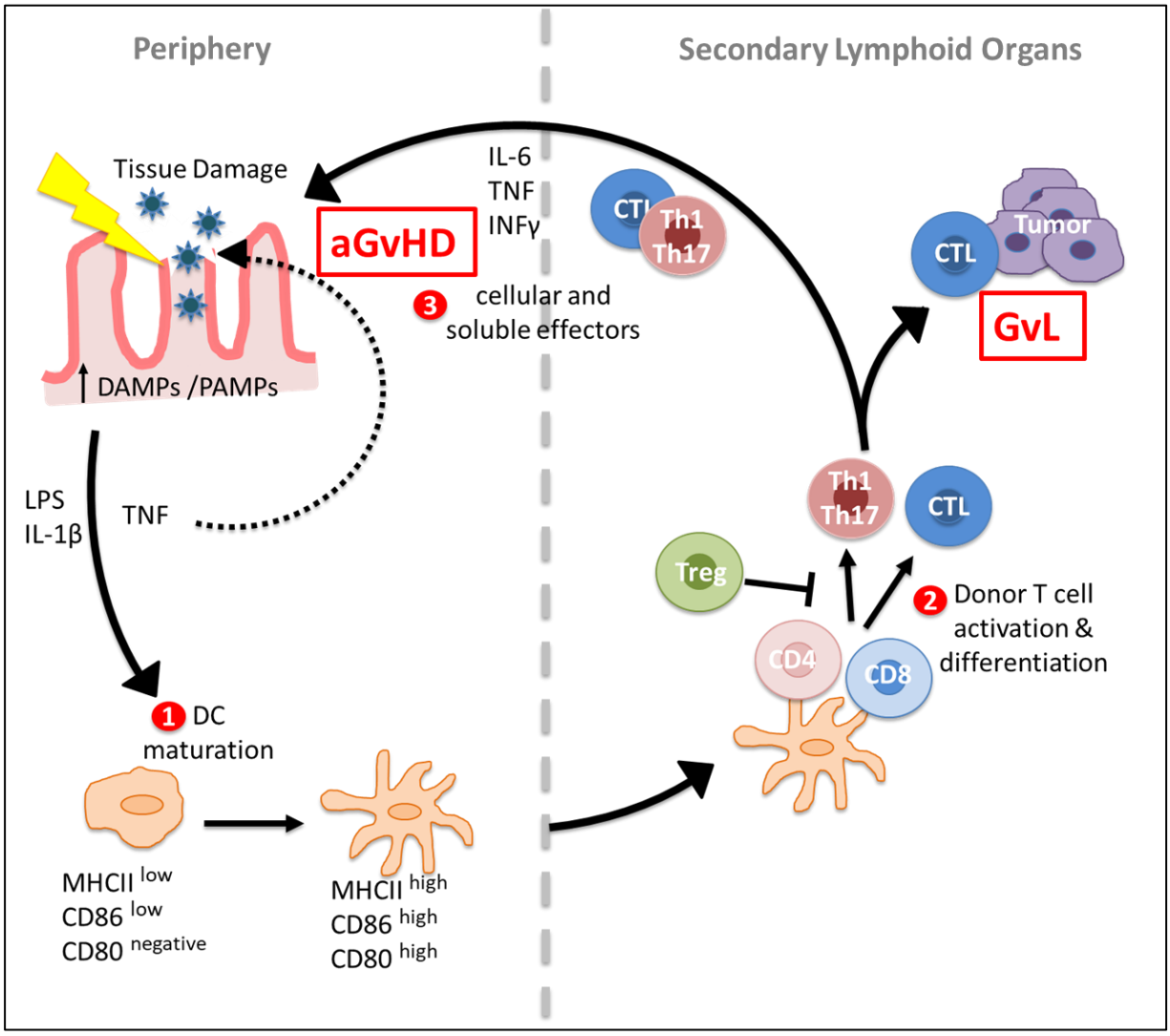

Figure 3: Schematic depiction of aGvHD and GvL pathology: Tissue damage, caused by the conditioning regime leads to release of pro-inflammatory mediators and DC maturation. In secondary lymphoid organs, T cells are activated and differentiate into effector cells which cause aGvHD either by direct cytotoxicity or by secreting proinflammatory cytokines. The GvL effect is predominantly mediated by CTLs.

1) Activation of APCs

The pathophysiology of aGvHD starts even before transfer of donor cells with the conditioning regime of the patient. The conditioning regimen that is applied before infusion of donor haematopoietic cells depends on individual patient-related factors and might be either myeloablative (high-dose), of reduced intensity or nonmyeloablative [reviewed in 111]. In humans, depletion of the myeloid compartment is achieved by either total body irradiation or chemotherapy, for instance with alkylating agents like cyclophosphamide or busulfan [reviewed in 111]. Most mouse models of aGvHD apply irradiation to deplete the haematopoietic system of the recipient [reviewed in 104]. The consequence is massive tissue destruction and the expression of DAMPs on damaged cells. Residual host APCs that have survived the conditioning can sense these danger signals, get activated and thereupon are capable of efficiently priming donor $CD4^+$ and $CD8^+$ T cells [reviewed in 112] . Disruption of the epithelial barrier in the gastrointestinal tract, in particular, enhances activation of the innate immune system as it allows the translocation of microbial products like lipopolysaccharide (LPS) into the circulation [reviewed in 7]. Host conventional DCs, but not host B cells, have been shown to be sufficient to initiate lethal aGvHD [113]. They might, however, not be essential in priming allogeneic T cells, as depletion of recipient cDCs, pDCs and B cells did not prevent aGvHD in an experimental model [114]. Instead, recipient non-haematopoietic antigen presenting cells or donor APCs might contribute to the initiation phase of aGvHD.

2) Donor T cell activation

Spleen, mesenteric lymph nodes and Peyer's patches are infiltrated by naive donor T cells as early as one day after T cell infusion [115]. There, alloreactive donor T cells are responding to host APCs by massive proliferation and differentiation into effector cells. The release of cytokines, mainly IL-2, TNF and IFNγ skews naïve T cells towards a Th1 immune response [47, reviewed in 7]. The crucial step of effector T cell differentiation can be suppressed by $CD4^+$ $CD25^+$ regulatory T cells. Donor Treg infusion has been very potent in inhibiting aGvHD in mice and is a promising therapeutic strategy in humans [91, 93, 94, 116-118, reviewed in 119].

3)The effector phase

The effector phase of aGvHD involves soluble and cellular effectors of innate and adaptive immunity [reviewed in 3]. IL-1, IL-6, IFNγ and especially TNF are key effector cytokines in aGvHD pathology. The latter is released by monocytes and macrophages upon LPS stimulation but also by primed donor T cells and can directly cause tissue damage, particularly in the gastro-intestinal tract [reviewed in 48, 120]. This creates a vicious circle in which TNF directly amplifies destruction of the mucosal barrier, further propagates the release of pro-inflammatory effectors and ultimately results in a "cytokine storm". The primary cellular effectors in aGvHD are donor T cells and NK cells. They can lyse target cells either by Fas-FasL interaction or through release of Perforin and Granzyme B. The Fas/FasL pathway might predominantly account for tissue damage in the liver, as hepatocytes express large amounts of Fas [reviewed in 7].

3.7 Interfering with CD28 signalling in GvHD

The important role of CD28 co-stimulation for T cell activation and its involvement in peripheral tolerance and Treg development renders it a potential target for aGvHD therapy. Until now, different technical approaches have helped to at least partially elucidate the role of CD28 signalling in aGvHD and the GvL effect.

3.7.1 Conventional $CD28^{-/-}$ mice

In 1993, mice that constitutively lack CD28 receptors were generated by inserting a neomycin cassette into exon 2 of the CD28 gene, thereby disrupting gene expression [121]. Using these mice as T cell donors in different mouse models of allogeneic HSCT brought first insights into the role of CD28 co-stimulation during aGvHD induction. CD28-deficient donor lymphocytes could induce lethal aGvHD in a major MHC mismatch model, which was, however, delayed when compared to wildtype lymphocytes [36]. Both, $CD4^+$ and $CD8^+$ T cells, contributed to the aGvHD pathology in absence of CD28 as shown by applying an MHC class II or MHC class I driven mouse model, respectively [122]. In addition, transfer of CD28-deficient T cells into unirradiated recipient mice revealed that co-stimulation of donor T cells is required for proliferation, IL-2 production and prevention of graft rejection. However, regarding the crucial role of CD28 during T cell maturation, constitutive deletion of the receptor might

alter the composition of the T cell compartment of the donor mice in these experiments, rendering their interpretation difficult.

3.7.2 Antibodies and antibody based approaches

Another approach to interfere with CD28 signalling is to use monoclonal antibodies (mAb), directed either against the CD28 molecule or its ligands CD80/CD86. A first study using αCD80 and αCD86 mAbs revealed that the combination of both antibodies is highly effective in preventing mice from $CD4^+$ and $CD8^+$ mediated aGvHD in an MHC disparate model [123]. In contrast, each antibody alone only resulted in partial protection from $CD4^+$ T cell mediated aGvHD [123]. This argues for distinct physiologic functions of CD80 and CD86 in activation of T cells. Targeting CD80/86, however, does not only inhibit T cell co-stimulation through CD28, but also disrupts T cell inhibition through CTLA-4, which shares the same ligands with CD28. Thus, a αCD28 mAb (clone 37.51) was superior to CTLA4-Ig treatment in a murine model of aGvHD [124]. However, the beneficial effect of this antibody turned out be mediated rather by selective depletion of allo-reactive T cells, than by blockade of CD28 signalling [125, 126]. Indeed, the clone 37.51 only partially impairs interaction of B7 molecules with CD28 but instead provides additional co-stimulatory signals to T cells *in vivo*, leading to an IFNγ dependent induction of apoptosis [125].

In order to investigate the effect of physiologic CD28 co-stimulation *in vivo*, Beyersdorf et al. used the αCD28 mAb clone E18 that attaches to CD28 near the binding site of CD80 and CD86. Therefore, administration of mAb E18 to healthy mice has similar effects as genetic deletion of CD28, e.g. a reduction in Treg cell frequencies among $CD4^+$ T cells [127]. Furthermore, application of mAb E18 in a major mismatch model of aGvHD resulted in reduced clinical pathology and enhanced survival of recipient mice [127]. However, the molecular mechanisms accounting for this effect are beyond just blocking CD28 co-stimulation. In contrast to the observed effects in a non-inflammatory situation, treatment with mAb E18 increased Treg cell frequencies in a highly inflammatory milieu. Accordingly, the beneficial effect of E18 in the aGvHD model was reduced when Treg cells were depleted from the transferred donor T cells [127]. The paradoxical effect that mAb E18 has on the Treg compartment in steady state versus inflammation is best explained as follows: During inflammation, allo-reactive Tconv cells are more dependent on CD28 co-simulation than Treg cells, such

that E18 treatment during aGvHD inhibits the expansion of Tconv cells to a greater extent than that of Treg cells [reviewed in 128].

In order to achieve a mere blockade of CD28 co-stimulation without confining effects of intact αCD28 mAbs, a monovalent αCD28 Fab-Fragment (FR104) was tested in non-human primates undergoing MHC mismatched hematopoietic stem cell transplantation after myeloablative conditioning. Blockade of CD28 co-stimulation in this model protected graft recipients from aGvHD and impaired $CD8^+$ T cell proliferation [129]. In addition, FR104 has been shown to inhibit allo-activation of T cells *in vitro* by enhancing CTLA-4 signalling while at the same time allowing for efficient immune responses against pathogens [130].

Besides conventional αCD28 antibodies, like the clones E18 and 37.51, another type of αCD28 antibody has been identified which is able to induce proliferation in T cells without simultaneous TCR stimulation [131, 132]. These superagonisitc αCD28 mAb clones share their capability to selectively target and expand Treg cells *in vivo* and *in vitro* [133-137]. Accordingly, pre-treatment of donor T cells either *in vivo* or *in vitro* with a superagonistic αCD28 mAb increased Treg frequencies within the transferred T cell population and protected recipient mice from lethal aGvHD while sparing the GvL effect [117, 138].

Taken together, the mode of action of different αCD28 mAb clones can vary from CD28 blockade to superagonistic simulation and their effects might be different in steady-state-situations and inflammatory situations like aGvHD. Monovalent antibody Fab fragments are, thus, a better approach to induce mere CD28 blockade during allogeneic T cell activation in aGvHD. However, they have the disadvantage to systemically inhibit CD28 signalling. This means that the effects seen with these agents cannot be assigned to different donor T cells populations.

3.7.3 Antibody-independent strategies

Antibody-based approaches have been very instructive in elucidating the role of CD28 co-stimulation during aGvHD induction. However, some disadvantages like inherent immunogenicity, in case that the antibody originates from a different species,

bioinstability or unwanted Fc-mediated signalling have led to the development of antibody-independent strategies to interfere with CD28 co-stimulation.

One of the first constructs used to interfere with CD28 co-stimulation was a fusion protein of CTLA-4 and the Fc part of IgG1 (CTLA4-Ig), the latter mainly enhancing the serum half-life of the protein [139]. CTLA4-Ig efficiently blocks CD80 and CD86 and, like αCD80+αCD86 mAb treatment, injection of CTLA4-Ig improved aGvHD pathology and induced long-term survival in otherwise lethal mouse models [140, 141]. CTLA4-Ig is now successfully used in the clinics to treat e.g. rheumatoid arthritis but has failed to show sufficient efficacy in both, a canine model of aGvHD and a model using humanised recipient mice [142, 143].

Recently, a chemically synthesised antagonistic molecule, a so-called peptoid, that specifically binds and blocks CD28 has been developed [144]. Blockade of CD28 co-stimulation with this peptoid delayed, but did not abrogate, lethal aGvHD in mice in a major mismatch model of HSCT [144]. However, like Fab fragments of αCD28 antibodies, general CD28 blockade by this peptoid does not allow to investigate the requirements for CD28 co-stimulation of different T cells populations to induce aGvHD.

Donor lymphocyte infusions very often are performed with the intention to prevent or treat a relapse of leukaemia in patients. In this case, high allo-reactivity by donor T cells through boosting CD28 signalling might be beneficial. In a very recent study a CTLA-4/CD28 chimera gene was introduced into donor T cells before transfer into allogeneic recipient mice [145]. The gene product, consisting of the extracellular part of CTLA-4 and the intracellular domain of CD28, has a similarly strong affinity to CD80/CD86 as CTLA-4, but transduces a co-stimulatory CD28 signal. T cells bearing the CTLA-4/CD28 chimeric protein mediated enhanced GVL effect but also caused more severe aGvHD [145].

3.8 Tamoxifen-inducible CD28 knock-out mice

Studying the role of CD28 on donor T cells during aGvHD has been limited by using conventional CD28 knock-out mice as T cells donors, as they have an altered T cell compartment [59-61]. Therefore an inducible CD28 knock-out (iCD28KO, B6.Thy1.1+/- ERCre +/- CD28 flox/-) mouse was generated, in which CD28 expression is ablated after the mice or their cells are exposed to Tamoxifen [146].

Tamoxifen is an estrogen receptor antagonist, initially developed to treat estrogen-sensitive breast cancer. As Tamoxifen does not bind to murine estrogen receptors, it does not interfere with hormone signalling in wild type mice, nevertheless, some cytotoxic side effects have been described [147]. In iCD28KO mice, Tamoxifen binds to the estrogen 2 receptor which is ubiquitously expressed under the Rosa26 promoter as a fusion protein with the Cre-recombinase (ERCre). As a consequence, the heat-shock protein 90 (HSP90) is released from the ERCre fusion protein, enabling the translocation of the enzyme to the nucleus (see Figure 4). In iCD28KO mice, the exons two and three of one CD28 allele are flanked by loxP sites ("floxed") and are excised by ErCre-mediated recombination. As the other CD28 allele is disrupted by a neomycin cassette, this results in an irreversible ablation of CD28 expression in iCD28KO mice or their cells upon treatment with Tamoxifen or its metabolite, 4OH-Tamoxifen, respectively [146, 59].

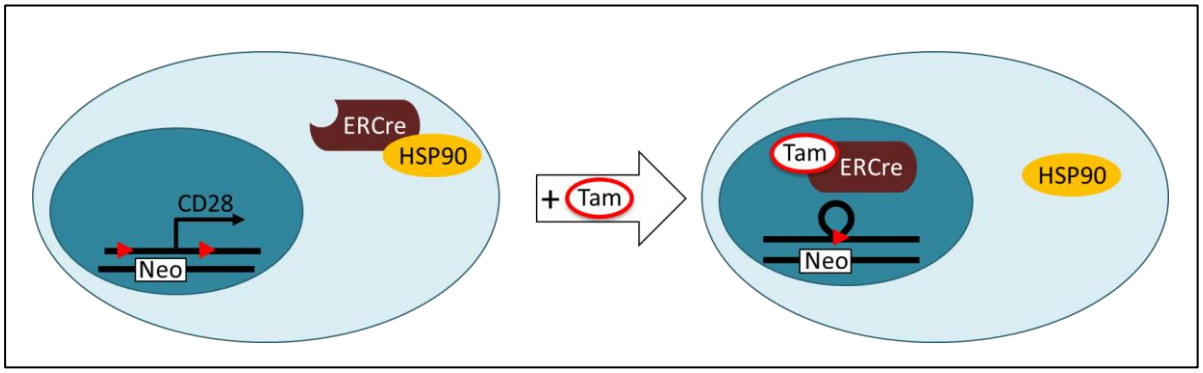

Figure 4: Tamoxifen-induced ablation of CD28 expression in iCD28KO cells. In iCD28KO cell, one CD28 allele is disrupted by a neomycin cassette. In the absence of Tamoxifen, HSP90 is bound to ERCre, sequesters it to the cytoplasm and allows transcription of the "floxed" CD28 allele. Binding of Tamoxifen to ERCre recombinase removes HSP90, resulting in nuclear translocation of the enzyme and excision of the loxP flanked CD28 gene sequence.

4 Aim of the study

Despite substantial research in the field, the precise mechanism of how CD28 signalling in different donor-derived lymphocyte populations contributes to aGvHD induction and the GvL effect is still not fully understood. As described in chapter 3.7, using $CD28^{-/-}$ mice as T cell donors or systemically blocking CD28 signalling post transplantation in mouse models of aGvHD, are approaches that face considerable limitations. Especially the controversial effects of different αCD28 mAb clones on Tconv and Treg cells in inflammatory and steady-state-like situations raises the need for a better model to study the role of co-stimulation on donor T cell subsets during aGvHD.

We have, therefore, developed a mouse model of aGvHD, where we can genetically delete the CD28 molecule on different donor T cell populations after they have been transferred into recipient mice. In this model, the donor T cells are derived from Tamoxifen-inducible CD28 knock-out (iCD28KO) mice. Unlike $CD28^{-/-}$ mice, iCD28KO mice undergo normal T cell development and loose CD28 expression only when treated with Tamoxifen. We transferred $CD4^+$ Tconv cells alone or together with $CD8^+$ T cells and/or Treg cells from iCD28KO mice or their wild type littermates into allogeneic hosts. Treatment of the T cell recipients with Tamoxifen induced CD28 deletion on the susceptible donor T cell populations.

By analysing the allo-responses of CD28-depleted donor T cells in this model we wanted to answer the following scientific questions:

1) Is CD28 expression on donor $CD4^+$ or $CD8^+$ T cells required for aGvHD induction?

2) Do Treg cells need CD28 to suppress aGvHD pathology?

3) How does CD28 deletion on $CD4^+$ Tconv cells, $CD8^+$ T cells or Treg cells impact the GvL effect?

5 Materials

5.1 Chemical and Biochemical Reagents

Chemical	Manufacturer
2-mercaptoethanol	Invitrogen/ Life technologies
4-OH Tamoxifen	Sigma-Aldrich/ Merck KGaA
Ammonium chloride (Na_4Cl)	Sigma-Aldrich/ Merck KGaA
Bovine Serum Albumin (BSA)	AppliChem GmbH
Calcium chloride	AppliChem GmbH
Citric acid	Merck KGaA
Disodium hydrogen phosphate (Na_2HPO_4)	Carl Roth GmbH & Co. KG
Entellan	Merck KGaA
Ethanol	AppliChem GmbH
Ficoll (Histopaque 1077)	Sigma-Aldrich/ Merck KGaA
Formaldehyd (37 %)	AppliChem GmbH
Fetal Calf Serum (FCS)	Gibco/Life technologies
L-glutamine	Carl Roth GmbH & Co. KG
Magnesium chloride ($MgCl_2$)	AppliChem GmbH
Magnesium Sulfate ($MgSO_4$)	Merck KGaA
Neomycin	Bela- pharm
Non-essential amino acids MEM	Gibco/ Life technologies
Normal rat serum	In-house
Penicillin	InfectoPharm Arzneimittel und Consilium GmbH
Polymyxin B	Sigma-Aldrich/ Merck KGaA
Potassium bicarbonate ($KHCO_3$)	AppliChem GmbH
Potassium chloride (KCl)	AppliChem GmbH
Potassium di hydrogen phosphate (KH_2PO_4)	Carl Roth GmbH & Co. KG
Proleukin ® (recombinant, human IL-2)	Novartis AG
Roti-Mount Fluor Care	Carl Roth GmbH & Co. KG
Sodium azide (NaN_3)	AppliChem GmbH

Sodium Carbonate (Na_2CO_3)	AppliChem GmbH
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG
Sodium Citrate ($Na_3C_6H_5O_7$)	AppliChem GmbH
Sodium hydroxide (NaOH)	Carl Roth GmbH & Co. KG
Sodium Pyruvate ($C_3H_3NaO_3$)	Sigma-Aldrich/ Merck KGaA
Streptomycin	AppliChem GmbH
Tamoxifen (40 mg pill)	Hexal AG
Tris ($C_4H_{11}NO_3$)	Merck KGaA
Tween 20	Sigma-Aldrich/ Merck KGaA
Xylene	AppliChem GmbH

5.2 Kits and cell staining dyes

Kit	**Manufacturer**
Mouse CD 4 T cell isolation kit	Affymetrix/Thermo Fisher
Mouse CD8 T cells isolation kit	Affymetrix/Thermo Fisher
Mouse Treg isolation kit	Miltenyi Biotec GmbH
anti-CD90.2 MircoBeads	Miltenyi Biotec GmbH
anti-Biotin MircoBeads	Miltenyi Biotec GmbH
LEGENDplex™ Assay for Cytoine detection	BioLegend, Inc.
Foxp3 Transcription Factor Fixation kit	eBioscience, Inc. /Thermo Fisher

Dye	**Manufacturer**
DAPI	Carl Roth GmbH & Co. KG
Eosin-G Solution 0.5 %	Carl Roth GmbH & Co. KG
Haematoxylin	Inst. for Pathology, University Würzburg
Viability Dye eFluor 780	Invitrogen/ Life technologies
Streptavidin PE	Becton Dickinson GmbH
Streptavidin PE Cy7	BioLegend, Inc.
CFSE	Molecular Probes/ Life technologies
Trypan Blue	AppliChem GmbH
Annexin V Cy5	Becton Dickinson GmbH

5.3 Antibodies:

Antigen-specific antibodies and Fab Fragments:

Specificity	Conjugate	Clone	Manufacturer
B220	FITC	RA3-6B2	BioLegend, Inc.
CD25	FITC / PE Cy7	PC61	BioLegend, Inc.
	Biotin	7D4	BioLegend, Inc.
CD28	APC	E18	BioLegend, Inc.
CD28 Fab	Unconjugated	E18	EXBIO Praha, a.s., in-house
CD3	PerCP Cy5.5	17A2	BioLegend, Inc.
	FITC	145-2C11	BioLegend, Inc.
CD4	PE / Biotin / BV 421	GK1.5	BioLegend, Inc.
	Pacific Blue	RM4-5	BioLegend, Inc.
	Alexa 700	RM4-5	Becton Dickinson GmbH
CD8	Biotin	53-6.7	Becton Dickinson GmbH
	Alexa 700	YTS156.7.7	BioLegend, Inc.
CD80	PE	16-10A1	Becton Dickinson GmbH
CD86	PE	GL1	Becton Dickinson GmbH
CD90.1	BV 510	OX-7	BioLegend, Inc.
CD90.2	Alexa Fluor 700	30-H12	BioLegend, Inc.
Fas	unconjugated	Jo2	Becton Dickinson GmbH
Foxp3	APC	3G3	Miltenyi Biotec GmbH
	Percp Cy5.5 / eFluor 450/ eFlour 660	FJK-16s	eBioscience, Inc
Granzyme B	PE / eFluor 450	NGZB	eBioscience, Inc.
H-2K^b	Biotin	AF6-88.5	Becton Dickinson GmbH
I-A/I-E	Alexa Fluor 700	M5/114.15.2	eBioscience, Inc.
I-A^b	Percp Cy5.5	AF6-120.1	BioLegend, Inc.
Ki-67	PE	B56	Becton Dickinson GmbH
BCL-1 idiotype	unconjugated	Mc10-6A5	gift from Prof. M.Glennie
Rat IgG	PE	F(ab')2 Fragment	DIANOVA Vertriebs-Gesellschaft mbH
T-bet	PerCP Cy5.5	eBio4B10	eBioscience, Inc.

Isotype Control antibodies:

Isotype	**Conjugate**	**Manufacturer**
Rat IgG2a$_{,κ}$	PE	Becton Dickinson GmbH
Mouse IgG2b$_{κ}$	APC	BioLegend, Inc.

5.4 Solutions, Buffers and Media

Solutions**:**

1.8 % NaCl:

18 g	NaCl
	ad 1 l ddH_2O

3.7 % Formaldehyde:

100 ml	Formaldehyde (37 %)
900 ml	PBS

BSS (Balanced Salt Solution I, II):

110 ml	BSSI
110 ml	BSSII
	ad 1 l ddH_2O

BSSI:

50 g	Glucose
3 g	KH_2PO_4
11.9 g	Na_2HPO_4
0.5 g	phenol red
	ad 5 l ddH_2O

BSSII:

9.25 g	$CaCl_2$
20 g	KCl
400 g	NaCl
10 g	$MgCl_2$
10 g	$MgSO_4$
	ad 5 l ddH_2O

BSS/ 0.1 % BSA:

110 ml	BSSI
110 ml	BSSII
2 g	BSA
	ad 1 l ddH_2O

Trypan Blue Solution:

0.04 % (w/v)	Trypan Blue
0.05 % (w/v)	NaN_3
	in PBS

Buffers:

Citrate Buffer:

0.18 µM	$C_6H_8O_7$
0.82 µM	Sodium Citrate (0.1 M)
	in ddH_2O, pH 6.0

FACS Buffer:

0.1 % (w/v)	BSA
0.02 % (w/v)	NaN_3
	in PBS

PBS (phosphate buffered saline):

138 mM	NaCl
2.7 mM	KCl
6.5 mM	Na_2HPO_4
1.5 mM	KH_2PO_4
0.9 mM	$CaCl_2$,
0.5 mM	$MgCl_2$
	in ddH_2O, pH 7.4

PBS-T:

500 µl	Tween 20
	ad 1 l PBS

TAC Buffer:

20 mM	Tris
0.83 % (w/v)	NH_4Cl
	in ddH_2O, pH 7.

Media:

Cell culture medium:

RPMI 1640 Medium (Gibco) supplemented with:

10 %	fetal calf serum (FCS)
1mM	Sodium Pyruvate
2 mM	L-glutamine
0.05 -2mM	Non-essential amino acids MEM
30 µM	2-Mercaptoethanol
100 µg/ml	Streptomycin
100 U/ml	Penicillin

5.5 Consumables

Consumable	Manufacturer
Cell culture flasks	Greiner Bio-One GmbH
Cell culture plates (96-, 48-, 24- and 12- well)	Greiner Bio-One GmbH
Cell strainer	Corning, Inc.
Cover slips	Paul Marienfeld GmbH & Co. KG
Dispenser tips	BRAND GmbH & Co. KG
Embedding cassettes	A. Hartenstein GmbH
Eppendorf tubes (1.5 ml and 5 ml)	Eppendorf AG
FACS bullets	A. Hartenstein GmbH
FACS tubes	Sarstedt AG & Co
LS and LD Columns	Miltenyi Biotec GmbH
Needles (20 gauge and 25 gauge)	B. Braun Melsungen AG
Object slide, SuperFrost Ultra plus	Menzel™, Thermo Fisher Scientific Inc.
Pipette tips (10 µl, 100µl, 1000µl)	A. Hartenstein GmbH
Pipette tips (300 µl)	BRAND GmbH & Co. KG
Serological pipettes	Sarstedt AG & Co
Syringes (1 ml)	B. Braun Melsungen AG
Syringes (20 ml)	Henke-Sass Wolf GmbH
Tissue culture dish	Greiner Bio-One GmbH
Tubes (15 ml and 50 ml)	Sarstedt AG & Co

5.6 Instruments

Instrument	Manufacturer
Analytical Balance	Mettler Toledo GmbH
Centrifuge (5415C)	Eppendorf AG
Centrifuge (Megafuge 1.0R)	Heraeus / Thermo Fisher Scientific Inc.
FACS LSR II	Becton, Dickinson and Company
Fluorescence microscope (DMi8) with DFC3000G camera	Leica Microsystems GmbH
Freezer (-80°C)	Thermo Fisher Scientific Inc.

Fridge	Candy Hoover Group S.r.l.
Haematocytometer (Neubauer)	A. Hartenstein GmbH
Heatblock	Eppendorf AG
Incubator (Heracell)	Thermo Fisher Scientific Inc.
Laminar flow hood (Herasafe)	Thermo Fisher Scientific Inc.
Light-optical microscope	Leica Microsystems GmbH
Magnetic cell separators	Miltenyi Biotec GmbH and Affymetrix
Microtome	Leica Microsystems GmbH
Multichannel pipette	Eppendorf AG
Paraffin embedding station	
pH meter	Hanna Instruments Deutschland GmbH
Pipette Controller	BRAND GmbH & Co. KG
Pipettes	Eppendorf AG
Stepper	Eppendorf AG
Ultrasound instrument	Electrosonic,Inc.
Vortex mixer	Scientific Industries, Inc.

5.7 Software

Program	**Application**
ImageJ 1.50i	Fluorescence microscopy image analysis
Microsoft Office - 365ProPlus for Windows - 2011 for Mac	Data management and manuscript preparation
Graphpad Prism v6	Statistical analysis and graphs
FlowJo v8.8.7	FACS analysis
EndNote X7	Reference Management
FACS Diva	FACS acquisition
LASX	Fluorescence microscopy image acquisition
LEGENDplex™ data analysis software	Analysis of LEGENDplex Cytokine Assays

6 Methods

6.1 Mice

BALB/c OlaHsd and C57BL/6J OlaHsd mice for aGvHD experiments were obtained from ENVIGO RMS GmbH (Venray, Netherlands). iCD28KO mice (B6. ERCre$^{+/-}$ CD28$^{flox/-}$ Thy1.1$^{+/-}$) and their wild type littermates (B6. ERCre$^{+/-}$ CD28$^{+/-}$ Thy1.1$^{+/-}$) have been previously generated and described by Gogishvili et al. [146]. The lineage was maintained by crossing B6.ERCre$^{+/+}$ CD28$^{-/-}$ mice that express the Cre recombinase, fused to the estrogen receptor, under the control of the Gt(ROSA)26Sor promoter with mice that carry one floxed CD28 allele (B6.Thy1.1$^{+/+}$ CD28$^{flox/+}$). iCD28KO, B6.CD28$^{-/-}$, B6.Thy1.1$^{+/+}$ mice and wild type BALB/c and C57BL/6J mice used for *in vitro* experiments were bred in the animal facility of the Institute for Virology and Immunobiology of the University of Würzburg.

6.2 Cell Isolation from lymph nodes, spleen, liver and bone marrow

Mice were either sacrificed by cervical dislocation or in CO_2 narcosis. In order to obtain single cell suspensions of lymph nodes, the cervical, inguinal, axillary, brachial and mesenteric lymph nodes were grinded through a 70 µm cell strainer. The cell suspension was centrifuged at 602 *g* and the cells were resuspended in a defined volume of BSS/BSA buffer for counting. Similarly, single cell suspensions from the spleen were prepared and, in addition, erythrocytes were lysed by resuspending the cell pellet on a vortex mixer in 3 ml ddH_2O before addition of 3 ml 1.8 % NaCl. This short hypo-osmotic shock disrupts erythrocytes but not nucleated cells. To obtain mononuclear cells from the liver, the organ was perfused with PBS and pressed through a 70 µm cell strainer. 3 ml of Ficoll buffer were placed in a 15 ml tube and were overlaid with the single cell suspension of the liver cells. After centrifugation at 1470 *g* at room temperature the mononuclear cells could then be isolated from the interphase. Bone marrow cells were obtained by flushing femora and tibiae with BSS/BSA buffer followed by erythrocyte lysis with TAC-Lysis buffer for 10 minutes at room temperature.

6.3 Cell separation with magnetic beads

6.3.1 T cell depletion of splenocytes and bone marrow cells

In principle, cell separation protocols were performed in BSS/BSA buffer according to the manufacturer's instructions. In order to deplete T cells from bone marrow cells or splenocytes, single cell suspensions ($1x10^8$ cells/ml) were incubated with normal mouse Ig (nmIg, 20 µg/ml) to block FC receptors before adding anti-CD90.2 MircoBeads (1:10) for 15 minutes at 4°C. CD90.2 positive, bead-bound cells, were then depleted by using magnetic cell separation columns.

6.3.2 Depletion of cell suspensions from $CD4^+$ or $CD8^+$ T cells

$CD4^+$ or $CD8^+$ T cells were depleted by first blocking cell suspensions (1×10^7 cells/ml for CD4 depletion, 1×10^8 cells/ml for CD8 depletion) with nmIg (20 µg/ml) before staining them with αCD4-Biotin antibody or αCD8-Biotin antibody (15 minutes, 4°C). Subsequently, cells were washed with BSS/BSA and incubated with anti-Biotin beads (15 minutes, 4°C). Bead-bound $CD4^+$ and $CD8^+$ cells were depleted on an LS or LD column, respectively.

6.3.3 Isolation of $CD4^+$ Tconv cells, $CD4^+$ $CD25^+$ Treg cells and $CD8^+$ T cells

$CD4^+$ or $CD8^+$ T cells were isolated from lymph node cells by negative depletion. $1x10^8$ cells/ml were placed into a sterile FACS tube, incubated with the respective biotinylated antibody cocktail (1:10) for 10 minutes at room temperature and then washed with BSS/BSA buffer. Subsequently, the cells were incubated with streptavidin-coated magnetic beads (1:10) for 5 minutes at room temperature before the FACS tube was placed into the magnet for another 5 minutes. The supernatant contained $CD4^+$ or $CD8^+$ T cells. When $CD4^+CD25^+$ conventional T cells (Tconv) were isolated, biotinylated anti-CD25 antibody (1:250, clone 7D4) was added to the antibody cocktail. For separation of $CD4^+CD25^+$ regulatory cells (Treg) and $CD4^+$ $CD25^-$ Tconv we used a Treg Isolation Kit. In brief, $CD4^+$ T cells were obtained from spleen or lymph node suspensions by negative selection. Subsequently, purified $CD4^+$ T cells were labelled with an anti-CD25 PE conjugated antibody, washed and incubated with anti-PE beads before loading them onto LS columns. $CD4^+$ $CD25^-$ Tconv were collected in the flow-through whereas $CD4^+CD25^+$ Treg could be eluated from the LS column.

6.4 CFSE labelling

For labelling of target cells with CFSE (Carboxyfluorescein succinimidyl ester), cells were washed three times with BSS, resuspended in BSS at a dilution of 1×10^7 cells/ml (BALB/c and C57BL/6 splenocytes) or 2×10^7 cells/ml (BCL-1 and T8-28 tumour cells) before incubation with CFSE (1:2000) for 5 minutes at room temperature. The labelling was stopped by addition of 45 ml BSS/BSA

6.5 Flow cytometry experiments

For flow cytometry analysis, up to 10^6 cells were placed in a 96 well V bottom plate, and unspecific binding of fluorochrome-labelled antibodies was blocked with αCD16/αCD32 antibodies (clone 2.4G2 supernatant, diluted 1:5 in FACS buffer) directed against FcγII/III receptors. Cell surface markers were then stained with the respective fluorochrome-conjugated antibodies for 15 min at RT and either directly used for flow cytometric analysis or washed with PBS and fixed with fixation buffer (30 minutes, RT) for subsequent intracellular staining. Antibodies directed against intracellular markers were diluted in permeabilization buffer and incubated with the cells for 45 minutes at room temperature. In case of indirect staining, the cells were washed three times before adding the secondary antibody. Fluorescently labelled cells were resuspended in 100 µl FACS buffer and analysed on a BD™ LSR II flow cytometer equipped with the FACS Diva software. For further analyses of the data, FlowJo software was used.

6.6 Bead-based cytokine analysis

TNF and IFNγ concentrations in the serum were assessed with a bead-based immunoassay according to manufacturer's instructions. In brief, anti-TNF and anti-IFNγ beads were incubated with diluted serum samples. Subsequently, the cytokines that were bound to the beads were detected with biotinylated anti-TNF and anti-INFγ antibodies. After adding a PE-conjugated streptavidin detection reagent, the assay was analysed by flow cytometry. Absolute TNF serum concentrations were calculated from the MFI of the PE signal with the LEGENDplex™ Data Analysis Software.

6.7 aGvHD mouse model

For all aGvHD experiments, male BALB/c mice at the age of 9 weeks were used as T cell recipients. In order to reduce the gut flora of the recipient mice, their drinking water was supplemented with Neomycin (250 mg/l) and Polymyxin B (0.5 mg/l), starting four days before transplantation and sustained until 27 days after transplantation. The myeloablative conditioning of the BALB/c mice was achieved by total body irradiation with a single dose of 8 Gy generated by a Faxitron X-ray source 24 h before cell transfer. T cell-depleted bone marrow cells were obtained from male C57BL/6 mice at the age of 8 weeks and iCD28KO mice, their wild type (wt) littermates or Thy1.1$^{+/+}$ mice were used as T cell donors. Recipient mice were intravenously injected with 1×10^7 TCD-BM cells alone or together with T cells in 200 µl of sterile PBS. In order to induce CD28 deletion on the transferred iCD28KO T cells, the recipient mice were fed 1.25 mg of Tamoxifen in 100 µl drinking water by oral gavage on four consecutive days, starting with the day of T cell transfer. The Tamoxifen solution was prepared from a 40 mg pill resolved in 3.2 ml of drinking water. In one experiment CD28 was deleted *in vivo* before transplantation by treating the donor mice with the same Tamoxifen preparation from day -4 to day -1 before T cell transfer. In some experiments, we tested the *in vivo* killing capacity of the donor T cells by transferring a mixture of 6×10^6 or 8×10^6 CFSE labelled target cells, consisting of BCL-1 lymphoma cells and TCD splenocytes from BALB/C mice and C57BL/6 mice in a 2:1:1 ratio, one day before analysis. When we analysed the GvL effect, 3×10^3 freshly thawed BCL-1 lymphoma cells were injected i.v. in 200 µl PBS 4 hours before transfer of bone marrow and T cells.

6.7.1 Tracking and analysis of alloreactive T cells or BCL-1 lymphoma cells by flow cytometry

Single cell suspensions of spleens, mesenteric lymph nodes and livers of recipient mice were counted using trypan blue exclusion. Flow cytometric analysis (see 6.5) was further used to assess the percentage of donor T cells within the organs and allowed to calculate absolute donor T cell numbers. Furthermore, phenotype and proliferation of donor T cells was analysed by staining for activation markers or analysing CFSE dilution, respectively. By indirectly staining the B cell receptor of BCL-1 cells with an anti-idiotype antibody (Mc10-6A5) and a PE-labelled anti-rat secondary antibody, we

could also calculate absolute BCL-1 cell numbers in the spleen. The absolute number of BCL-1 cells in the spleen was used to define the cause of euthanization of recipient mice in GvL survival experiments: if we detected more or than or equal to 10^7 BCL-1 cells in the spleen, we assumed that the high tumour burden was the reason why recipient mice reached the humane endpoint, whereas, if we found less than 10^7 BCL-1 cells in the spleen of recipient mice, aGvHD was supposed to be the cause of euthanization.

6.7.2 Clinical scoring of recipient mice

The clinical appearance of each mouse was monitored daily and recorded every other day by scoring the mice according to the scheme shown in Table 1. Mice that reached a cumulative score of 8 or higher were killed for humane reasons. In addition, mice with a score of 2 in the categories “Spontaneous activity”, “Abdomen” and “Breathing”, as well as a weight loss of over 30 % for longer than two days were euthanized.

	Score:		
Category:	0	1	2
1) Weight loss	< 10%	>10 %, < 25 %	> 25 %
2) Posture	normal	hunching at rest	severe hunching, impaired movement
3) Spontaneous activity	normal	mild to moderately decreased	stationary unless stimulated
4) Fur texture	normal	mild to moderate ruffling	severe ruffling/ poor grooming
5) Skin integrity	normal	scaling of paws/ tail	obvious areas of denuded skin
6) Conjunctivitis	absent	one eye only or mildly in both eyes or dull eyes	severe in both eyes or sunken bulbi
7) Licking or itching of inflamed skin	absent	< 1x/ min	> 1x/ min
8) Abdomen	normal	spleen palpable but not reaching right lower quadrant or ascites suspected	enlarged spleen extends to right lower quadrant or ascites highly likely
9) Stool	normal	loose stools/ swollen anal mucosa	strong diarrhoea/ sticky black stool
10) Breathing	normal	flatter than normal/ little use of accessory respiratory muscles	strained/ extensive use of accessory respiratory muscles

Table 1: Scoring scheme for evaluation of clinical signs of aGvHD and GvL (from [135])

6.7.3 Histological analysis of small and large bowel

To assess intestinal tissue damage, histological sections of small and large bowel were stained with haematoxylin and eosin. The organs were fixed in 3.7 % formaldehyde, sequentially dehydrated in ethanol and embedded in paraffin (performed at the Institute for Pathology of the University of Würzburg). 4 µm thick sections from small and large bowel were generated with a microtome. Tissue sections were deparaffinized, rehydrated in xylol and ethanol, and subsequently stained with 0.05 % Eosin (5 minutes, room temperature (RT)) and Haematoxylin (15 minutes, RT) followed by incubation in cold tap water (10 minutes) to induce blue colouring of nuclei. Sections were mounted with Entellan and a cumulative histological score for small and large bowel was determined according to the scoring scheme shown in Table 2.

Small bowel categories:	**Scores**					
lamina propria lymphocytic infiltrate	(0) normal	(0,5) focal and rare	(1) focal and mild	(2) diffuse and mild	(3) diffuse and moderate	(4) diffuse and severe
villous blunting						
luminal sloughing of cellular debris						
outright crypt destruction						
Large bowel categories:						
lamina propria lymphocytic infiltrate						
Mucosal ulceration						
outright crypt destruction						

Table 2: Histological scoring scheme for small and large bowel, adopted from [148, 149].

6.7.4 Immunohistochemical staining of gut resident Treg cells

For immunohistochemical staining of Treg cells, 4 µm paraffin sections were deparaffinized and boiled in citrate buffer for 30 minutes for antigen-retrieval. After blocking unspecific antigen binding with 10 % BSA/PBS, Tregs were stained with αFoxp3 eFluor660 antibody in 1% BSA/PBS overnight and the slides were mounted in Roti-Mount Fluor Care containing DAPI. Fluorescence microscopy of small and large bowel was performed on a Leica DMi8 microscope equipped with an HCXPL FLUORTAR L 40×/0.60 DRY objective and a DFC3000G camera. The LAS X software and Image J software were used for image acquisition and analysis, respectively. Foxp3 and DAPI staining were detected in the LED-405 channel and Y5 channel, whereas the RHOD channel was used to identify autofluorescent signals. For

quantitative analysis, the numbers of Foxp3 and DAPI positive cells were counted in 10 high power fields of both, the small and the large bowel.

6.8 Cellular assays and cell culture

6.8.1 Mixed lymphocyte reactions

One-way mixed lymphocyte reactions were carried out in a round-profile 96-well plate in 200 µl of RPMI medium in at least two technical replicates. 2×10^5 T cell depleted BALB/c splenocytes were co-cultured with 2×10^5 responder cells of C57BL/6 origin for four days at 37°C and with 5 % CO_2. Depending on the scientific question, either whole lymph node cells were used as responder cells or 1×10^5 $CD4^+CD25^-$ and 1×10^5 $CD8^+$ T cells, isolated from $CD28^{-/-}$ mice, C57BL/6 mice, iCD28KO mice or their wt littermates, were mixed. Where indicated, recombinant human IL-2 was added to the cultures at a concentration of either 10^{-7} M or 10^{-8} M. To block CD28 signalling in the cultures, 10 µg/ml of an αCD28 Fab Fragment (clone E18) was added. Alternatively, splenocytes and lymph node cells from iCD28KO mice or their wt littermates were incubated with 10 µM 4OH-Tamoxifen at 37°C for one hour before isolation of $CD4^+CD25^-$ or $CD8^+$ T cells.

6.8.2 Killing assay

Killing assays were performed with freshly thawed BCL-1 or T8-28 cells, both originally isolated from a BALB/c mouse, as target cells. 1×10^4 CFSE labelled BCL-1 or T8-28 cells were seeded per well (V-bottom 96-well plate) together with 1.3×10^3 - 1×10^5 effector cells of C57BL/6 origin in 200 µl of RPMI medium. The plates were spun down shortly (190 *g*) to ensure maximal cell contact and then incubated at 37°C and with 5% CO_2 for 4 hours. Effector cells were previously generated in a 4 day MLR culture. In some experiments, αCD28 Fab Fragment (clone E18) was added to block CD28. In order to exclude target cell lysis by $CD4^+$ T cells, the effector cells were either depleted from $CD4^+$ cells prior to the killing assay or $CD8^+$ T cell-depleted effector cells were used as a negative control. Addition of αFas antibody to the target cells served as a positive control for induction of apoptosis. Killing assays were analysed by flow cytometry after staining with Annexin V and Viability Dye. The percentage of specific lysis was calculated as follows:

$$\%\ specific\ lysis = \frac{\%\ dead\ cells\ - \%\ dead\ cells\ (neg.control, no\ effector\ cells)}{100 - \%\ dead\ cells\ (neg.control, no\ effector\ cells)} \times 100$$

6.9 Statistical analysis

Summary graphs and statistical testing was done with GraphPad Prism 6.0d. *p* values of less than 0.05 were considered as statistically significant (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

7 Results

7.1 Tamoxifen-induced CD28 deletion on donor $CD4^+$ T cells

7.1.1 CD28 deletion of donor Tconv after transplantation

To investigate the requirement for CD28 co-stimulation on $CD4^+$ donor Tconv cells for aGvHD induction, we established a new mouse model of HSCT, that allows to delete CD28 molecules exclusively on the donor Tconv cells after transplantation. In this model, Tconv cells were isolated from Tamoxifen-inducible CD28 knock-out (iCD28KO, B6.Thy1.1$^{+/-}$ ERCre$^{+/-}$ CD28$^{flox/-}$) mice or their wild type littermates (wt, B6.Thy1.1$^{+/-}$ ERCre$^{+/-}$ CD28$^{+/-}$) and mixed with T cell depleted bone marrow (TCD-BM) cells from C57BL/6 mice. Transferring this cell mix into irradiated BALB/c recipient mice provided a full MHC mismatched model of aGvHD. After transplanting the donor cells, we fed the BALB/c recipients with Tamoxifen for four consecutive days by oral gavage to induce CD28 deletion (Figure 5A). Donor T cells were identified in the spleen of recipient mice due to the expression of the congenic marker Thy1.1 (Figure 5B). Before transfer into the recipient mice (day 0), the CD28 expression on the wt and iCD28KO donor Tconv cells was similar (Figure 5C). On day 3 post transplantation, iCD28KO Tconv cells were almost completely CD28-deficient but also wt Tconv cells showed reduced CD28 expression as compared to day 0. The latter is most likely caused by a transient internalization of CD28 after binding to CD80/CD86 in the allogeneic environment. Accordingly, 7 days after transplantation, wt Tconv cells again expressed high amounts of CD28 on the surface. In contrast, iCD28KO Tconv cells remained CD28-deficient (Figure 5C).

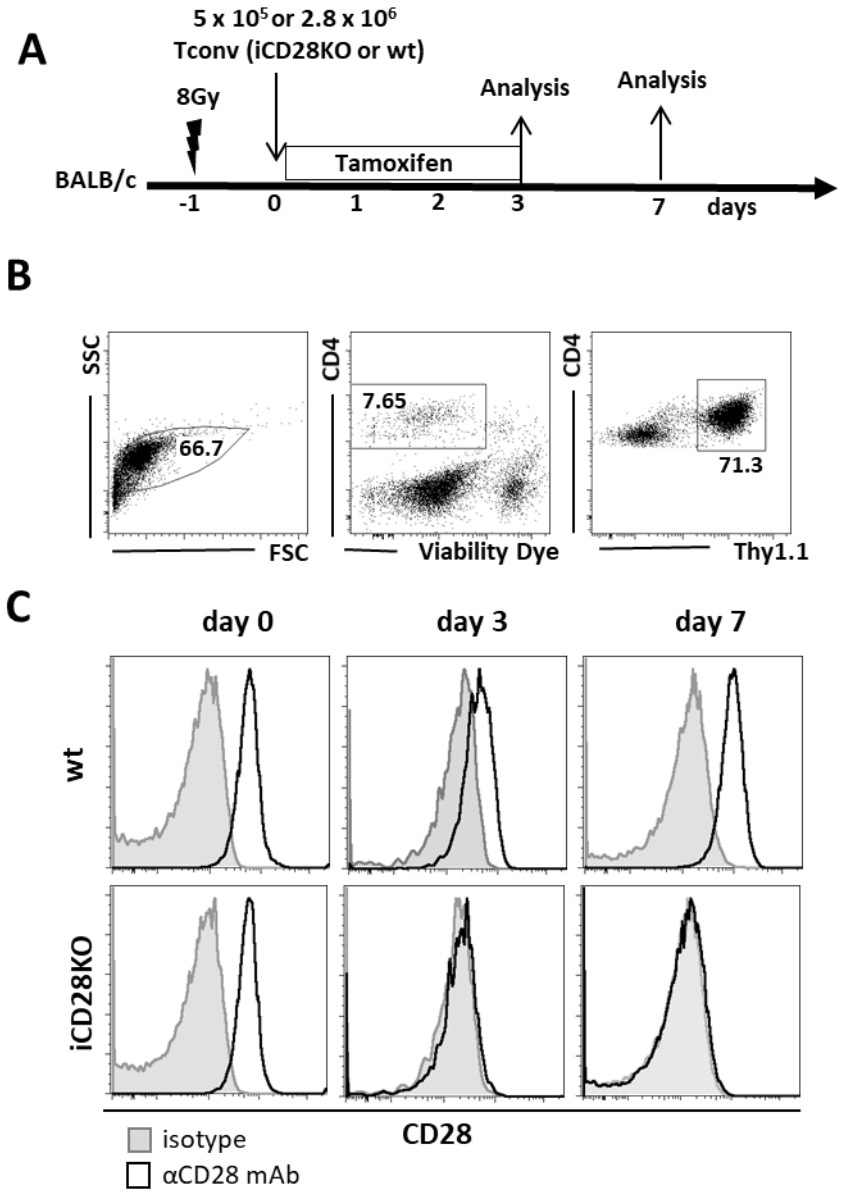

Figure 5: CD28 deletion on donor iCD28KO Tconv cells by Tamoxifen treatment of recipient mice. (A) Lethally irradiated BALB/c mice were transplanted with 10^7 TCD-BM cells and 2.8 x10^6 or 5 x10^5 Tconv cells of wt or iCD28KO mice. CD28 deletion on donor Tconv cells was induced by Tamoxifen treatment of the recipient mice. (B) Donor Tconv cells were identified in the spleen post transfer by expression of Thy1.1. (C) CD28 surface expression on donor T cells was assessed by FACS before (day0) or at day 3 and 7 after T cell transfer in the spleen of recipient mice. Published in [150].

7.1.2 CD28 deletion does not impair proliferation and expansion of donor CD4$^+$ Tconv

We next investigated if CD28 deletion on donor Tconv cells reduces their proliferation and expansion during aGvHD. Carboxyfluorescein succinimidyl ester (CFSE) is a cell staining dye that penetrates the cell membrane and binds to lysine residues of intracellular proteins and other amine sources [151, 152]. Upon cell division, each

daughter cell is left with half of the CFSE-labelled molecules. CFSE dilution within a cell population, thus, can be used to analyse cell proliferation. We applied this method to investigate the effect of CD28 deletion on the proliferation of Tconv cells after transfer into allogeneic recipients. CFSE dilution of donor wt and iCD28KO Tconv cells was similar at day 3 after transplantation (Figure 6A). Neither the percentage of dividing cells nor the average number of cell divisions by proliferating cells (proliferation index) differed between wt and iCD28KO cells (Figure 6A). Moreover, also 7 days after transplantation, the frequency of dividing Tconv cells was not reduced upon CD28 deletion as shown by staining for the proliferation marker Ki-67 (Figure 6B). Furthermore, we did not observe an upregulation of Foxp3 in the transferred Tconv cells, demonstrating that there was no induction of Treg cells (Figure 6B).

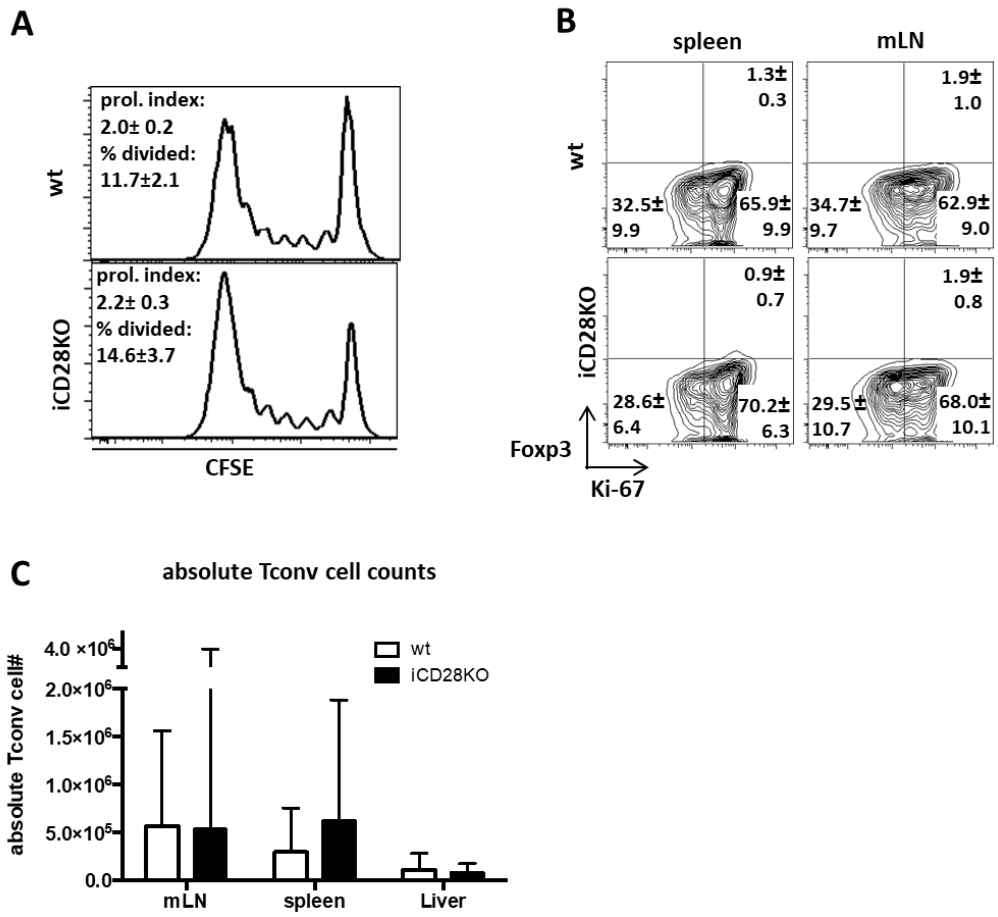

Figure 6: Proliferation and expansion of donor Tconv cells after CD28 deletion. 2.8 × 10^6 CFSE labelled (A) or 5 × 10^5 unlabelled (B-C) iCD28KO or wt Tconv cells were transferred together with TCD-BM into irradiated BALB/c recipient mice. (A) CFSE dilution of labelled donor Tconv cells was measured on day 3 after transplantation in the spleen of recipient mice. Mean + SD of the proliferation index and the percentage of divided cells are shown for three independent experiments (n=4 mice/group). (B) Ki-67 and Foxp3 expression and (C) absolute donor Tconv cell counts of unlabelled donor Tconv cells was assessed on day 7 after transplantation in the mesenteric lymph nodes (mLN), spleens and livers of recipient mice. Data are shown as mean percentages + SD (B) and median + range (C) of three independent experiments (n=8 mice/group). Published in [150].

Additionally, CD28 deletion also did not affect the recovery of donor Tconv cells from the spleens, mLNs and livers of the hosts (C). Taken together, these data show that iCD28KO and wt Tconv cells proliferated and expanded equally well when CD28 was deleted post transplantation.

7.1.3 $CD4^+$ T cell expansion and Treg cell frequencies are not affected by CD28 deletion neither before nor after T cell transfer

As shown in Figure 5, it took about three days before CD28 protein expression on donor $CD4^+$ Tconv cells was depleted by Tamoxifen treatment of the recipient mice. We wanted to exclude that initial CD28 expression on donor iCD28KO T cells during the first 3 days after transplantation might curtain possible effects on proliferation and expansion of donor T cells in our model. Therefore, we deleted CD28 on donor T cells before transplantation by feeding the donor iCD28KO mice or their wt littermates with Tamoxifen for 4 consecutive days, starting 4 days before transplantation (Figure 7A). Purified $CD4^+$ T cells were transferred together with TCD-BM cells into allogeneic BALB/c recipient mice. To test the effectivity of CD28 deletion, we analysed CD28 expression on $CD4^+$ T cells by flow cytometry either at the day of transfer or three days after transplantation (Figure 7B). CD28 surface expression on the iCD28KO but not on wt T cells was reduced by 70.2 % at the day of transplantation and by 94.1 % three days later. The residual CD28 surface expression on the day of transplantation, thus, was not due to insufficient genetic ablation but rather caused by a slow turn-over of CD28 protein in resting T cells.

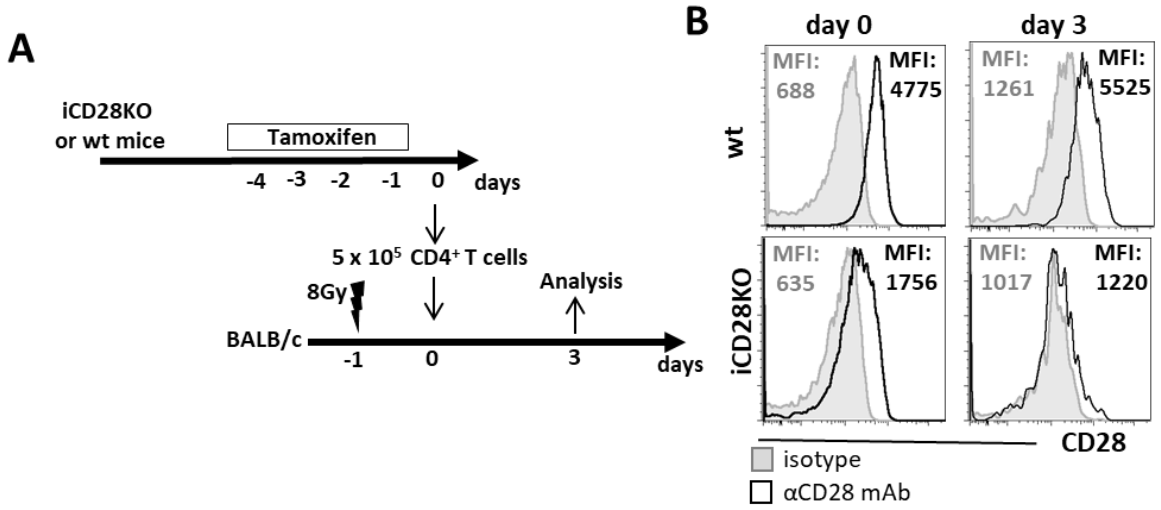

Figure 7: Tamoxifen treatment of the donor iCD28KO mice before transplantation depletes CD28 expression on transferred T cells. 10^7 TCD-BM cells were transferred together with 5×10^5 CD4⁺ T cells from iCD28KO mice or wt littermates into irradiated BALB/c recipient mice. (A) CD28 deletion was induced by treatment of the donor mice with Tamoxifen before transplantation. (B) CD28 surface expression on donor T cells in the spleen was assessed before transplantation (day 0) and three days after transfer by FACS staining and compared to an isotype control. Numbers indicate Median Fluorescence Intensity (MFI) of αCD28mAb staining or control. Published in [150].

We now had two models of CD28 deletion on donor T cells, deletion before and after transplantation, which we could compare in terms of T cell expansion and effects on the Treg cell compartment.

We transferred CD4⁺ T cells of wt or iCD28KO mice into BALB/c recipients and induced CD28 ablation before or after T cell transfer by either treating the donor mice (Figure 7A) or the recipient mice (Figure 5A), respectively. Neither the genetic background of the donor T cells (wt or iCD28KO) nor the timepoint of CD28 deletion with Tamoxifen (before or after transplantation) had an impact on the expansion of donor CD4⁺ T cells in the allogeneic host (Figure 8A and B). In addition, the frequencies of Foxp3⁺ CD25⁺ Treg cells within the donor CD4⁺ T cell population were similar in wt and iCD28KO donor CD4⁺ T cells after either treating the donor mice or the recipient mice with Tamoxifen (Figure 8A and B). Of note, during aGvHD development, Treg frequencies were generally decreased (Figure 8A and B). This has also been observed by others previously [153].

Furthermore, we analysed the phenotype of CD28-deleted Treg cells during aGvHD induction. CD25 expression on the Treg cells was increased on day 3 and 6 after transplantation, on both, wt and iCD28KO Treg cells, and independent of the Tamoxifen treatment schedule (Figure 8C). Thus, CD28 expression was dispensable

for activating donor Treg cells during the first days of aGvHD. Moreover, Foxp3 expression of the donor Treg cell population was not altered upon CD28 deletion and was similar when assessed before or 3 and 6 days after transplantation (Figure 8C). Taken together, our observations demonstrate, that during the first days of aGvHD induction, CD28 signalling has no effects on the expansion of donor CD4$^+$ T cells or on donor Treg cell frequencies.

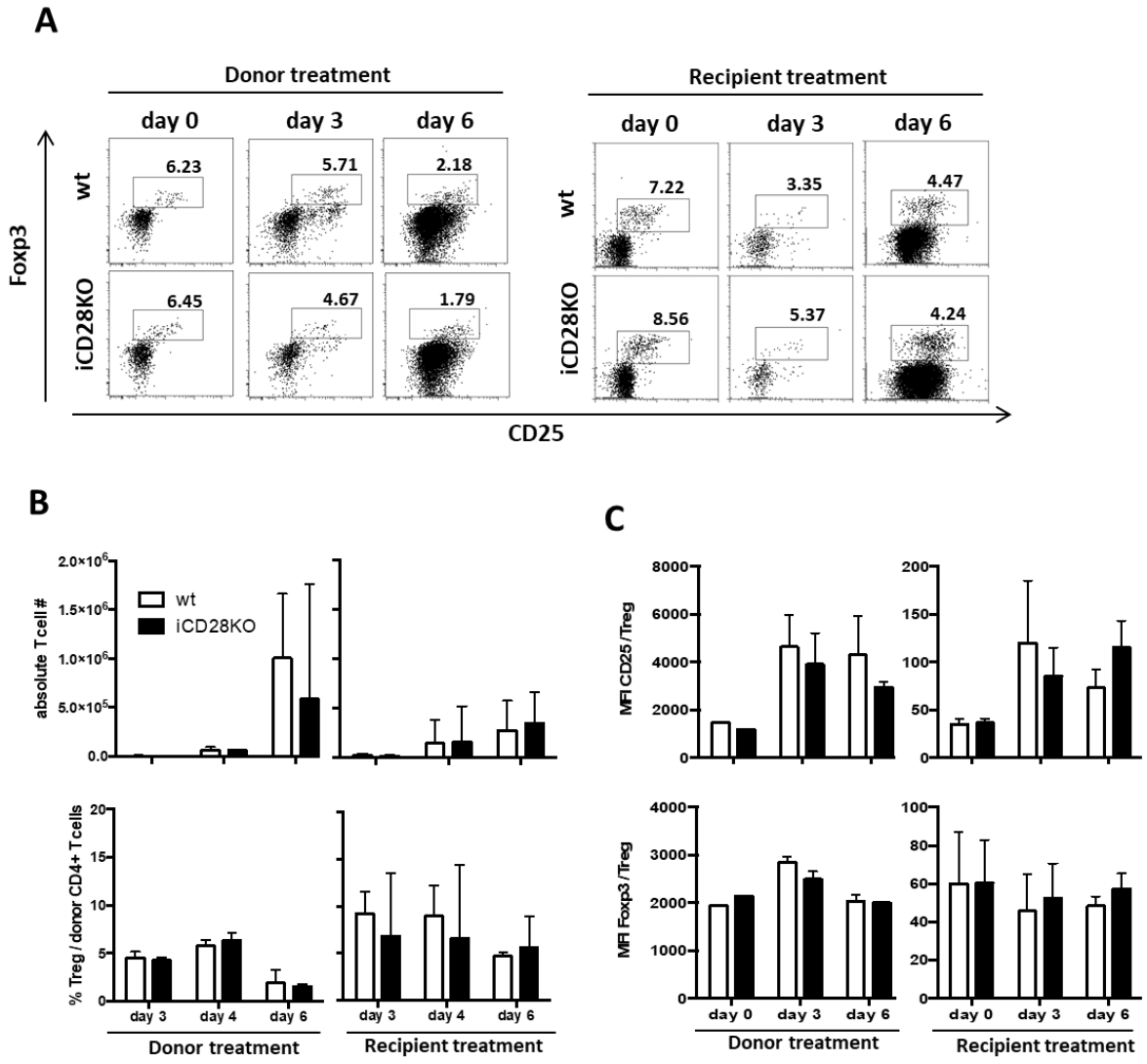

Figure 8: CD28 deletion on donor CD4$^+$ T cells before or after transplantation has no effect on allogeneic expansion of donor CD4$^+$ T cells or Treg cell frequencies and phenotype. BALB/c recipient mice were transplanted with 10^7 TCD-BM cells and 5×10^5 CD4$^+$ T cells from iCD28KO mice or wt littermates and CD28 deletion induced by Tamoxifen treatment of the donor or the recipient mice. (A) Foxp3$^+$ CD25$^+$ donor Treg cells were identified within donor CD4$^+$ T cells either on the day of transplantation (day 0) or in the spleen of recipient mice on day 3 and 6 after transplantation. (B) Absolute number and frequencies of Treg cells within the donor CD4$^+$ T cells. (C) Median fluorescence intensity (MFI) of CD25 and Foxp3 on donor Treg cells. n=3 mice/group, mean + SD. Published in [150].

Therapeutic blockade of CD28 in allogeneic HSCT patients would most likely be performed post transplantation. Therefore, we decided that for the following experiments, CD28 expression on donor T cells should be ablated after transfer by Tamoxifen treatment of the recipient mice.

7.1.4 CD28-deficient CD4$^+$ Tconv cells are able to induce lethal aGvHD

iCD28KO and wt CD4$^+$ Tconv cells proliferated and accumulated equally well in the allogeneic host. We next wanted to investigate if both were also similarly capable of inducing aGvHD. The pro-inflammatory cytokine TNF is a critical mediator of aGvHD pathology and produced by donor CD4$^+$ Tconv cells [reviewed in 154]. Seven days after transplantation of iCD28KO or wt CD4$^+$ Tconv cells, the concentration of TNF in the serum of iCD28KO CD4$^+$ Tconv recipients was lower as in recipients of wt CD4$^+$ Tconv cells (Figure 9A). In contrast, we could not observe a difference in the histopathological score of small and large bowel of wt and iCD28KO CD4$^+$ Tconv recipients, indicating that they were equally able to cause tissue damage in the intestine (Figure 9B). Still, CD28-deficient CD4$^+$ Tconv cells caused less clinical signs of aGvHD than wt CD4$^+$ Tconv cells, shown by a reduced clinical score of recipient mice on day 7 after transplantation (Figure 9C).

We next analysed whether CD28 deletion on CD4$^+$ Tconv cells also enhances the survival of the recipient mice. When we compared the clinical score of mice transplanted with wt or iCD28KO CD4$^+$ Tconv cells during a time period of 80 days, we observed a transient reduction in aGvHD symptoms upon CD28 deletion between day 7 and day 15 after transplantation (Figure 9D). However, this did not result in long-term survival of iCD28KO CD4$^+$ Tconv recipients, even though CD28 deletion increased the median survival from 8 (wt CD4$^+$ Tconv cell recipients) to 23 (iCD28KO CD4$^+$ Tconv cell recipients) days after transplantation (Figure 9E). *Post mortem* analysis of CD28 expression on donor T cells showed that lethality was indeed due to CD28-deleted iCD28KO CD4$^+$ Tconv cells and not mediated by an outgrowth of CD28 positive T cells within the donor T cell population (Figure 9F).

In summary, our data demonstrate that in this major mismatch model of aGvHD, CD28 deletion on donor CD4$^+$ Tconv cells only transiently protects from aGvHD but fails to mediate long-term survival.

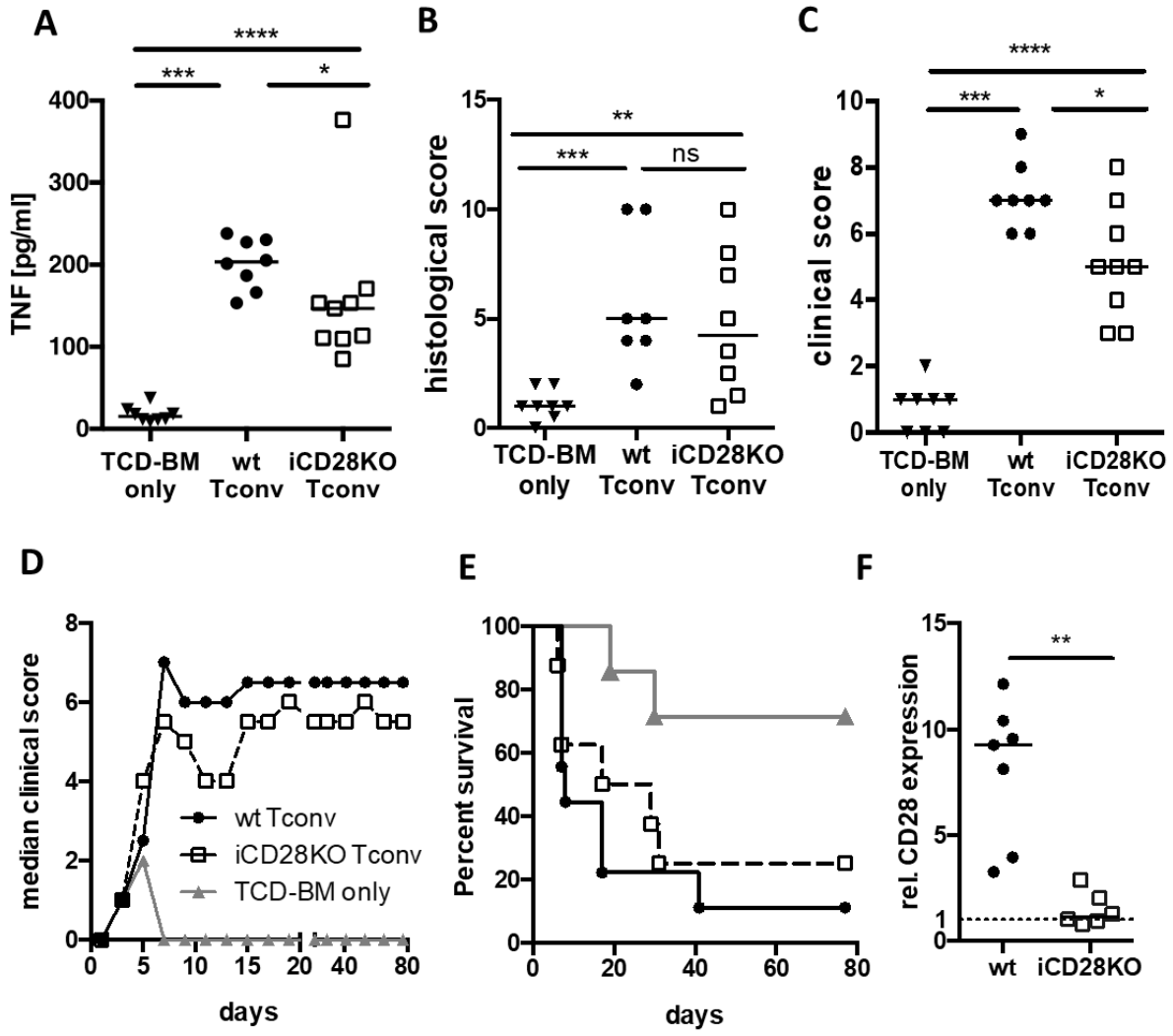

Figure 9: CD28 deletion on donor CD4⁺ Tconv cells does not protect from lethal aGvHD. BALB/c recipient mice were injected with TCD-BM and 5 × 10⁵ iCD28KO or wt CD4⁺ Tconv cells and subsequently treated with Tamoxifen. (A) TNF serum concentrations, (B) histological score of small and large bowel and (C) clinical score of recipient mice was assessed on day 7 after T cell transfer. Lines indicate the median of the data from three independent experiments; two-tailed, unpaired Mann–Whitney test. (D) Median clinical score, including final clinical score of mice that were euthanized before day 80, and (E) survival of recipient mice until day 80 after transplantation. (F) Post mortem analysis of CD28 expression on donor CD4⁺ Tconv cells. Relative CD28 expression: ratio of MFI of specific CD28 staining/MFI isotype control staining. (D-F) data of two independent experiments were pooled (n=8 mice/ group). Published in [150].

7.2 Effects of CD28 deletion on donor Treg cells

7.2.1 Donor Treg cell phenotype and expansion are not affected by CD28 deletion

As described in 3.3, regulatory T cells protect from aGvHD in different animal models and are currently tested in patients undergoing allogeneic HSCT. As CD28 is critical for Treg cell maturation and homeostasis (see 3.2.4), we wanted to know whether CD28 deletion on donor Treg cells affects their expansion or phenotype after transfer into allogeneic hosts. We transplanted BALB/c recipient mice with TCD bone marrow, $CD4^+$ Tconv cells from B6.Thy1.1$^{+/+}$ mice and Thy1.1$^{+/-}$ Treg cells from iCD28KO mice or wt littermates (Figure 10A). Upon Tamoxifen treatment of the recipient mice, CD28 was completely deleted on Thy1.1$^{+/-}$ iCD28KO Treg cells but not on wt Treg cells (Figure 10B). Selective CD28 deletion on donor Treg cells neither had an impact on the expression of CD25 or Foxp3 nor on the absolute number of Treg cells, recovered from spleens and mLNs of recipient mice (Figure 10C-E). This is in line with our previous observations, where we deleted CD28 on total $CD4^+$ donor T cells (Figure 8).

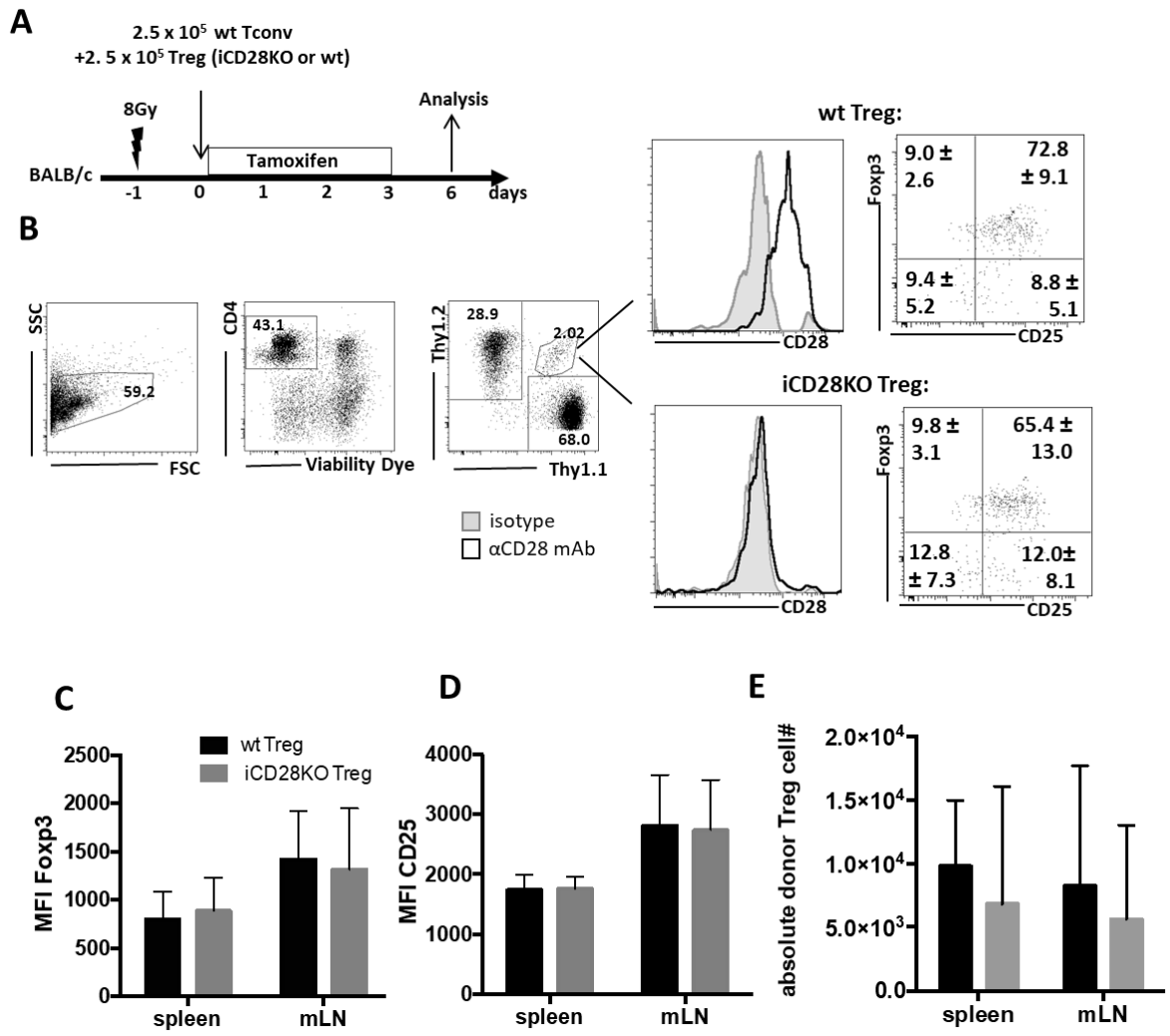

Figure 10: CD28 deletion on donor Treg cells has no impact on Treg cell recovery or phenotype. (A) Irradiated BALB/c mice received TCD-BM together with 2.5 × 10^5 Thy1.1+ $CD4^+$ Tconv and 2.5 × 10^5 Thy1.1+/Thy1.2+ Treg cells isolated from iCD28KO mice or wt littermates. CD28 deletion on donor Treg cells was induced after transplantation by Tamoxifen treatment of the recipients for four consecutive days. (B) On day 6 after transplantation, donor Treg cells were identified in the mesenteric lymph nodes (mLN) of hosts by expression of Thy1.1 and Thy1.2 and expression of Foxp3, CD25 and CD28 on donor Treg was analysed. Percentages of $CD25^+$ and/or $Foxp3^+$ cells are shown as mean+SD). Median fluorescence intensity of Foxp3 (C) and CD25 (D) staining of donor Treg cells and the absolute number of donor Treg cells (E) from spleens and mLNs of recipient mice are shown as mean+SD (C and D) and median+range (E). n= 7 mice / group, pooled data from three independent experiments. Published in [150].

7.2.2 CD28 costimulation is not required for suppressive function of Treg cells early after transplantation

As CD28 deletion did not alter the expansion and phenotype of Treg cells after transfer into allogeneic hosts, we investigated if CD28-deficient Treg cells were also able to inhibit $CD4^+$ Tconv cell mediated aGvHD. Both, iCD28KO and wt Treg cells, were equally able to suppress accumulation of donor $CD4^+$ Tconv cells in secondary lymphoid organs of the recipients (Figure 11A). Moreover, donor Treg cells reduced the concentration of TNF in the serum of the mice independent of CD28 expression as compared to mice that received only $CD4^+$ Tconv cells (Figure 11B). In addition, CD28-deficient Treg cells suppressed tissue damage in small and large bowel and were found in the gut in equal numbers as wt Treg cells (Figure 11C-E). Thus, CD28 deletion on donor Treg cells did not impair their capacity to prevent $CD4^+$ Tconv cell-mediated inflammation in the host.

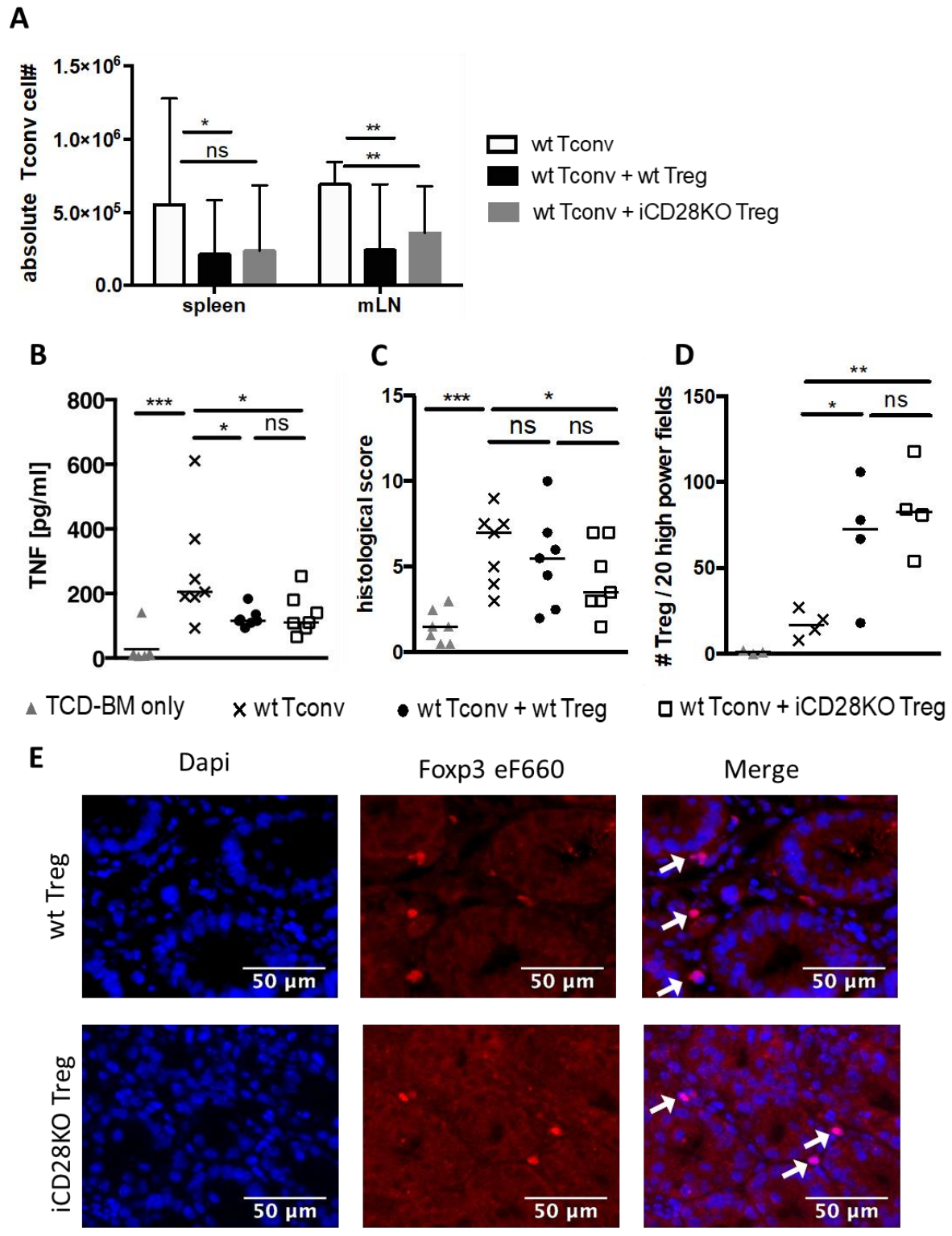

Figure 11: CD28-deficient Treg cells suppress $CD4^+$ Tconv cell mediated inflammation in vivo. *Irradiated BALB/c recipient mice were reconstituted with 2.5 × 10^5 $Thy1.1^{+/+}$ $CD4^+$ Tconv cells and 2.5 × 10^5 Treg cells from iCD28KO mice or wt littermates and analysed 6 days after transplantation. (A) Absolute $CD4^+$ Tconv cell numbers as recovered from spleen and mLN (median + range). (B) TNF serum concentration of recipient mice and (C) cumulative histopathological score of recipient mice, lines indicate the median score. (D) Paraffin sections of small and large bowel were stained with αFoxp3 antibody and DAPI and the number of $Foxp3^+$ Treg cells assessed in ten high power fields (200x magnification) of each organ. (E) Representative fluorescence microscopy images of small bowel. (A-C): n= 7 mice/ group of three independent experiments, unpaired Mann–Whitney test (comparisons between Treg recipients: two-tailed; all other comparisons: one-tailed). Published in [150].*

7.2.3 CD28-deficient Treg cells do not protect from lethal aGvHD

Our results demonstrated that CD28-deficient Treg cells efficiently inhibited donor CD4⁺ Tconv cells during the first week after transfer into the allogeneic hosts. We next wanted to investigate if they are further able to prevent aGvHD long-term.

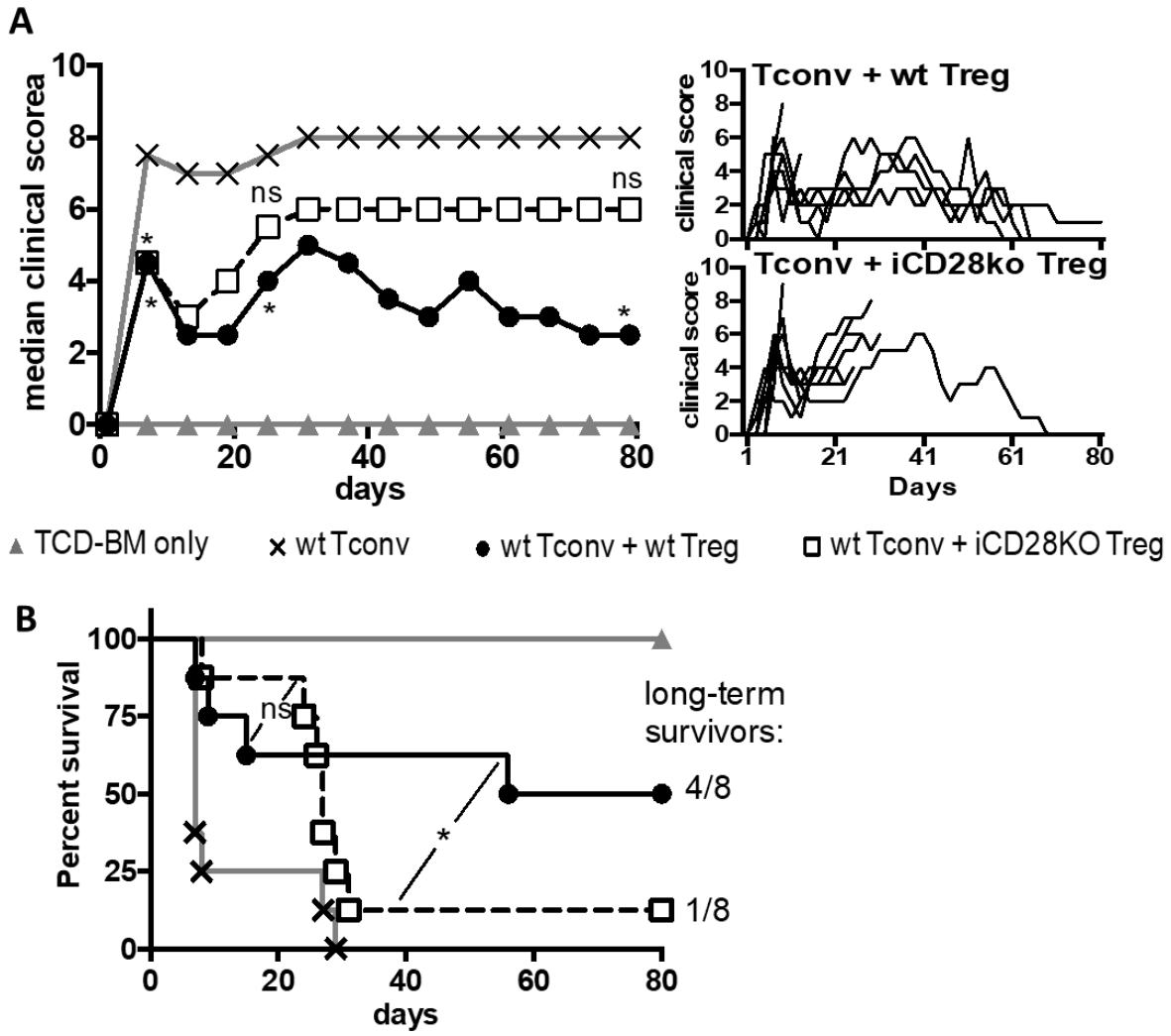

Figure 12: CD28-deficient Treg cells do not protect recipient mice long-term. 1 × 10⁷ TCD-BM cells and 1.25 × 10⁵ CD4⁺ Tconv cells were transferred into BALB/c recipients either alone or together with and 2.5 × 10⁵ Treg cells from iCD28KO donors or wt littermates. CD28 deletion was induced by Tamoxifen treatment of the hosts from day 0 to day 3. (A) Clinical scores of recipient mice until day 80. Left: Median clinical score including the final score of mice that had to be killed for humane reasons before day 80. P values refer to a one-tailed Mann–Whitney test between Tconv only and Tconv + Treg recipients on day 7, 25, and 80. Right: individual scores of Treg recipients, each line is representing one mouse. (B) Survival of recipient mice and ratio of long-term survivors in the Treg recipient groups. Mantel–Cox test was performed between the two Treg recipient groups until day 24 or from day 25 until the end of the experiment, n = 8 mice/group; pool of two independent experiments. Published in [150].

We transplanted BALB/c mice with wt CD4⁺ Tconv cells and Treg cells from iCD28KO mice or wt littermates and induced CD28 deletion on donor Treg cells by Tamoxifen

treatment of the hosts. Both, iCD28KO and wt Treg cells, reduced the clinical score of recipient mice when analysed on day 7, at the peak of aGvHD activity, as compared to mice that received $CD4^+$ Tconv cells alone (Figure 12A, left panel). Consequently, Treg cells could protect recipient mice from lethal aGvHD until about day 20 after transplantation, independent of CD28 expression (Figure 12B). However, only wt Treg cell recipients survived a second flare of aGvHD, whereas almost all iCD28KO Treg cell recipients had to be humanely killed due to the severity of aGvHD symptoms (Figure 12A, right panel and Figure 12B). Thus, even though CD28-depleted Treg cells could suppress aGvHD early after transplantation, they failed to protect the recipient mice long-term. Instead, iCD28KO Treg cell recipients developed a late and lethal flare of the disease. Similar disease courses are also seen in patients undergoing allogeneic HSCT and are classified as late-onset aGvHD [155].

7.2.4 Impaired survival of CD28-deleted Treg cells results in late-onset aGvHD

To elucidate why CD28-deficiency on donor Treg cells results in late-onset aGvHD, we repeated the experiment shown in Figure 12 but sacrificed the mice on day 19, which is just before iCD28KO Treg cell recipients had succumbed to aGvHD in the previous experiment.

In contrast to what we had observed on day 6 after transplantation, the frequency and number of Treg cells 19 days after transfer into the allogeneic recipients was drastically reduced upon CD28 deletion in the spleen, the mLN and the liver, one major target organ of aGvHD (Figure 13A and B). We further quantified the number of Treg cells in the intestine by fluorescence microscopy. CD28-depleted Treg cells were found less frequently in the small and large bowel as compared to CD28-sufficient Treg cells (Figure 13C and D).

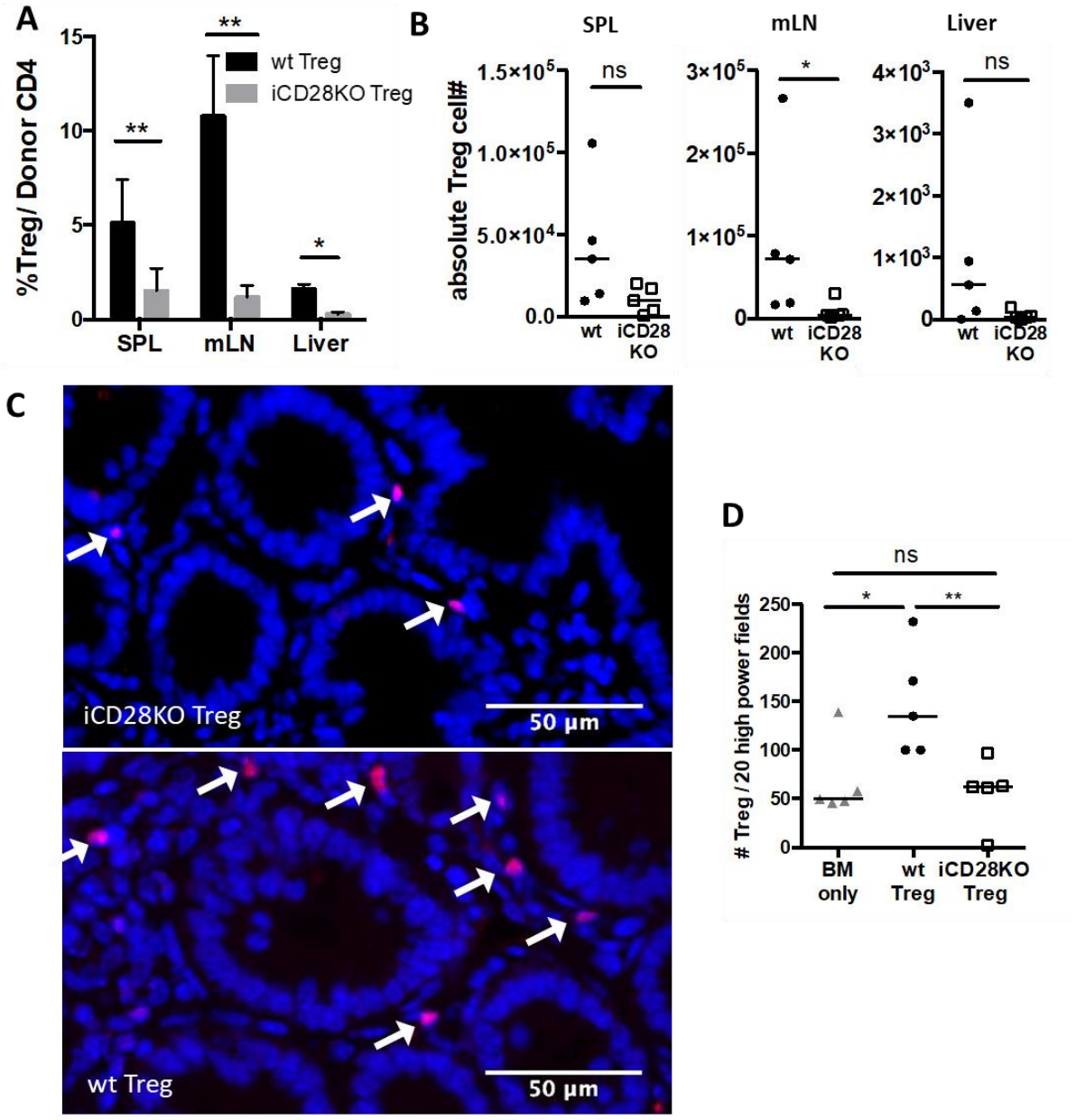

Figure 13: Impaired recovery of CD28-depleted donor Treg cells at day 19. BALB/c recipient mice were injected with 1 × 10^7 TCD-BM cells, 4 × 10^4 $CD4^+$ Tconv cells and 2.5 × 10^5 Treg cells from iCD28KO mice or wt littermates, fed with Tamoxifen from day 0 to day 3 after transplantation and analysed on day 19. (A) Frequency of Treg cells within the donor $CD4^+$ T cell population (median+ range) and (B) absolute Treg cell numbers, recovered from the spleens, mLNs and livers of recipient mice (lines indicate the median). (C) Representative images of paraffin sections from the small bowel stained with αFoxp3 and DAPI. (D) Quantification of $Foxp3^+$ Treg cells in 10 high power fields of small and large bowel each (200x magnification). (A-D): n= 4-5 mice/group, two-tailed unpaired Mann-Whitney test. Published in [150].

A migratory defect of iCD28KO Treg cells was unlikely to be the cause of lethal late-onset aGvHD in iCD28KO Treg recipients, as Treg numbers were reduced in both, the secondary lymphoid organs and the two target organs we looked at, liver and intestine. The recovery of iCD28KO Treg cells could, however, also be due to either impaired proliferation or enhanced apoptosis of iCD28KO Treg cells in comparison to wt Treg

cells. We used a viability dye to differentiate viable and dead Treg cells in the spleen and mesenteric lymph nodes of recipient mice and observed that the frequency of dead cells within the Treg cell gate was higher in recipients of iCD28KO Treg cells (Figure 14A and B). However, there was no difference in the expression of the proliferation marker Ki-67 between viable CD28-deficient and -sufficient Treg cells (Figure 14C). Taken together, our data demonstrate that iCD28KO Treg cells do not survive long-term in the allogeneic recipients and therefore cannot protect from a second flare of aGvHD.

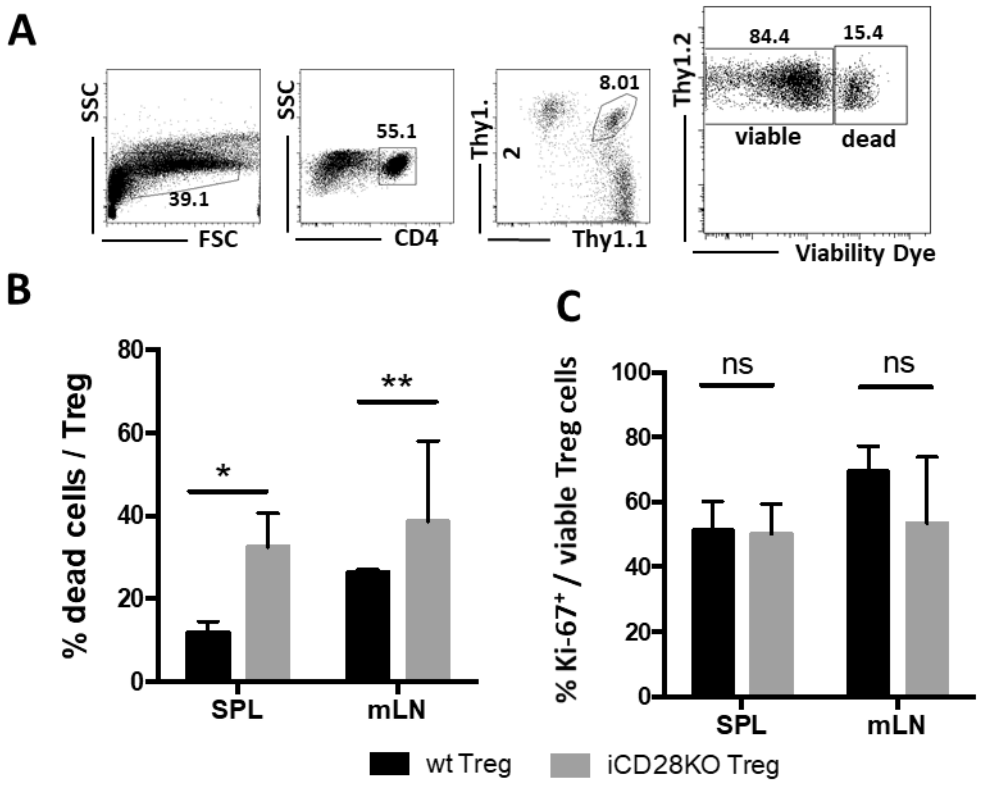

Figure 14: Impaired survival of CD28-depleted Treg cells in the allogeneic host. BALB/c recipients were reconstituted with TCD-BM, 4 × 10^4 $CD4^+$ Tconv cells and 2.5 × 10^5 Treg cells from iCD28KO mice or wt littermates before Tamoxifen treatment. (A) Representative dot plots showing the gating strategy to differentiate viable and dead Treg cells in secondary lymphoid organs at day 19 after transplantation. (B) Percentage of viability dye positive cells within the Treg gate. (C) Frequency of Ki-67^+ cells among viable Treg cells. (B-C): median + range, n= 4-5 mice/group, two-tailed unpaired Mann-Whitney test. Published in [150].

7.3 Effect of CD28 co-stimulation on CD8$^+$ T cell expansion and aGvHD

7.3.1 Reduced CD8$^+$ T cell expansion in the mLNs upon CD28 deletion

CD8$^+$ T cells are crucial to mediate the GvL effect, but also contribute to aGvHD or may even be sufficient to induce lethal aGvHD in MHCI-driven animal models [reviewed in 104].

To investigate the role of CD28 deletion in donor CD8$^+$ T cells during aGvHD, we transferred BALB/c recipient mice with TCD bone marrow, CD4$^+$ Tconv cells from wt mice and CD8$^+$ T cells from Thy1.1$^{+/-}$ iCD28KO mice or their wt littermates (Figure 15A). CD28 deletion was induced on donor CD8$^+$ T cells by Tamoxifen treatment of the recipient mice. Three and six days after transplantation, CD28 surface expression was assessed on the transferred CD8$^+$ T cells recovered from the spleen and mesenteric lymph nodes of recipient mice (Figure 15B and C). CD28 expression on wt CD8$^+$ T cells generally is lower when compared to CD4$^+$ T cells and was even further decreased after transfer into the allogeneic host (see Figure 5C and Figure 15C, day 3). Like in CD4$^+$ T cells, this was probably due to transient internalisation of CD28 upon ligand binding because CD28 expression on wt CD8$^+$ T cells was recovered 6 days after transplantation. In contrast, CD28 was completely deleted on iCD28KO CD8$^+$ T cells at day 6 and already slightly reduced at day 3 after transplantation when compared to wt CD8$^+$ T cells (Figure 15C).

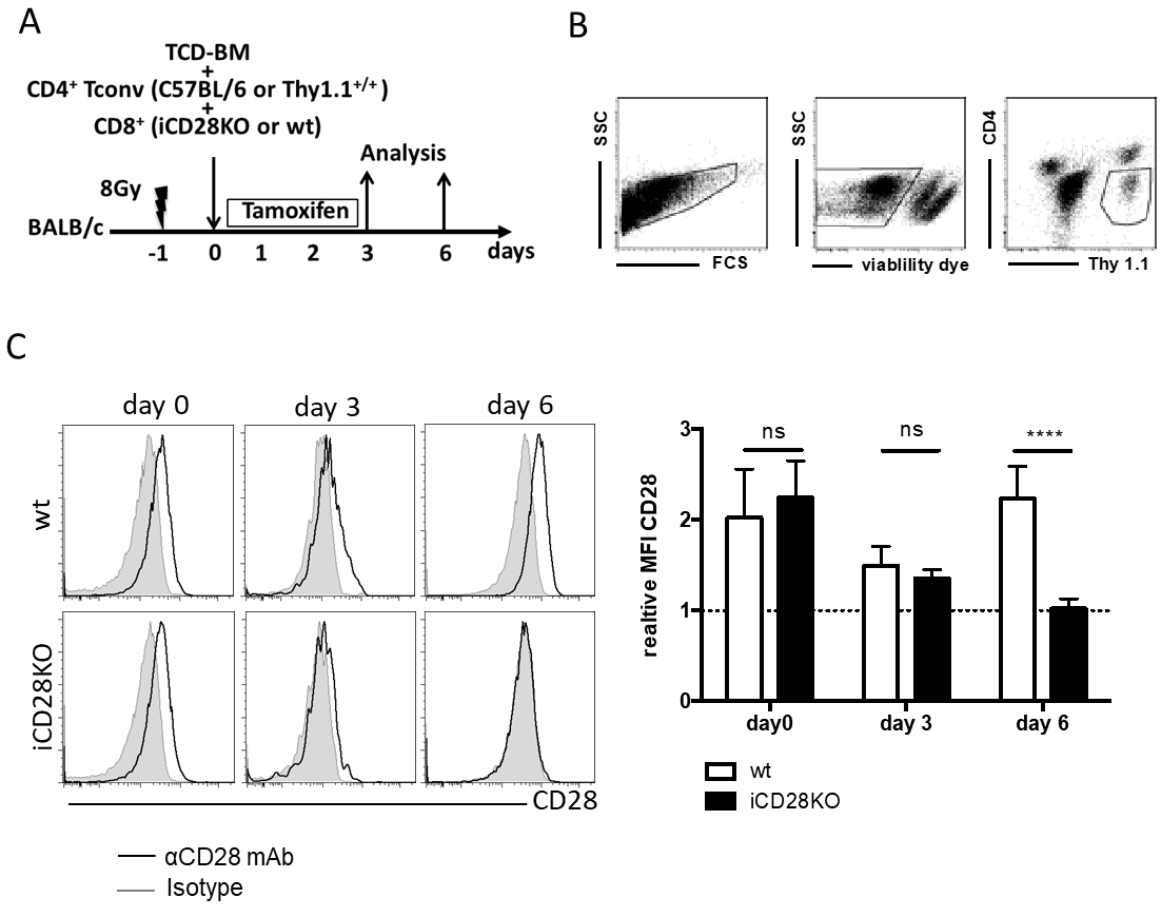

Figure 15: CD28 deletion on donor CD8⁺ Tcells. (A) BALB/c recipient mice were transferred with 1 × 10⁷ TCD-BM cells together with 1 × 10⁶ C57BL/6 (day 3 analysis) or 2.5 × 10⁵ Thy1.1⁺/⁺ (day 6 analysis) CD4⁺CD25⁻ Tconv cells and equal numbers of CD8⁺ T cells from iCD28KO mice or their wt littermates. Recipients were subsequently fed with Tamoxifen to induce CD28 deletion on donor CD8⁺ T cells. (B) FACS gating strategy to identify donor-derived CD8⁺ T cells in spleens (SPL) and mesenteric lymph nodes (mLN) of recipient mice on day 6 after transplantation. (C) CD28 expression on splenic iCD28KO or wt donor CD8⁺ T cells 3 and 6 days after transfer. Left panel: exemplary histograms of stainings with αCD28 mAb and isotype control antibody, right panel: quantification of CD28 expression, relative MFI= ratio of MFI of specific staining /MFI of isotype control staining; mean+SD; n= 3-6 mice / group in two independent experiments; two-tailed unpaired t-test.

After CD28 deletion, we found less CD8⁺ T cells in the mesenteric lymph nodes but not in the spleens of recipient mice (Figure 16A). Moreover, the frequencies of CD8⁺ T cells among all transferred donor T cells were reduced (Figure 16B). In contrast to what we had observed for Treg cells 19 days after transplantation (see Figure 14B), increased cell death of CD28-depleted donor CD8⁺ T cells did not account for the impaired recovery of iCD28KO CD8⁺ T cells in the mLNs of recipient mice (Figure 16C).

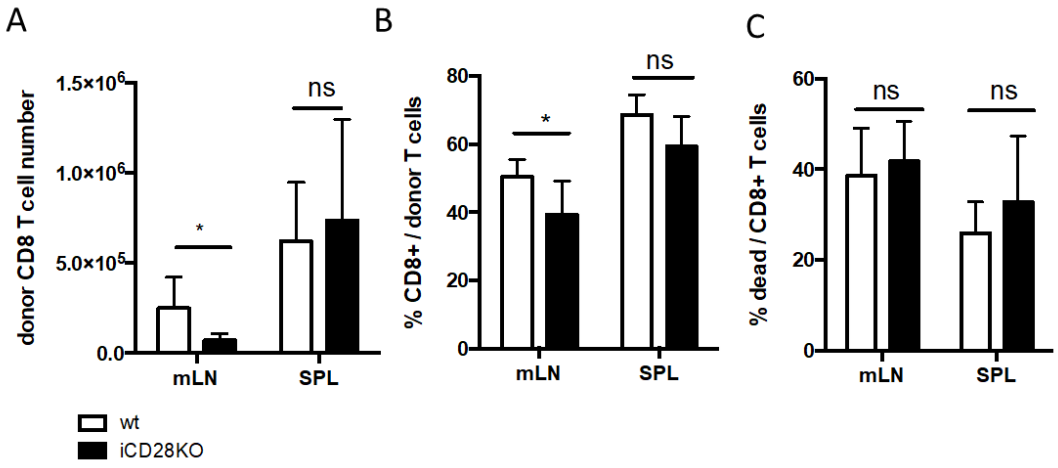

Figure 16: Impaired expansion of CD28-deficient CD8⁺ T cells in the mLN. Mice were transplanted as shown in Firgure 14 A. (A) Absolute numbers and (B) frequencies of donor CD8⁺ T cells in the mLN and SPL on day 6 after transplantation. (C) Frequency of viability dye positive (dead) cells within the donor CD8⁺ T cell population (gated as Thy1.1⁺ CD4⁻); (A) – (C): n=6 mice/group, two independent experiments, mean + SD, two-sided unpaired t-test.

We next investigated if CD28 co-stimulation is required for efficient proliferation of donor CD8⁺ T cells after allogeneic HSCT. We transferred wt CD4⁺ Tconv cells and CFSE-labelled CD8⁺ T cells from iCD28KO mice or their wt littermates into irradiated BALB/c mice. Subsequently, we treated the recipients with Tamoxifen, sacrificed them 3 days after transplantation and analysed the CFSE dilution of the transferred CD8⁺ T cells (see Figure 15A). Due to CD28 deletion, the proliferation of CD8⁺ T cells was slightly, but not significantly, reduced in the mLN and the peripheral lymph nodes (pLN). This was indicated by less CFSE^{low} cells as well as a lower division index (average number of cell divisions) of donor iCD28KO CD8⁺ T cells (Figure 17A). In contrast, CD28 deletion did not affect the proliferation of CD8⁺ T cells in the spleen (Figure 17A).

Of interest, the frequencies of host-derived BALB/c APCs in the mLNs and the pLNs were much higher as compared to the spleen (Figure 17B). Even though this did not result in an increased host APC/ T cell ratio in the lymph nodes compared to the spleen (Figure 17C), the donor derived MHCII⁺ cells in the spleen might function as spacers, impairing host-APC/ T cell contacts and limiting the effect of CD28 deletion on donor T cell activation and expansion (Figure 17D). Moreover, as CD28 expression on day 3 after transplantation is generally very low, the effect of Tamoxifen-induced CD28 depletion on donor CD8⁺ T cell proliferation is probably very limited at this time point. Taken together, our data indicate, that CD28 deletion on donor CD8⁺ T cells might

impair their proliferation in the mesenteric lymph node, but not in the spleen, where donor-derived APCs might impede T cell / host APC contacts.

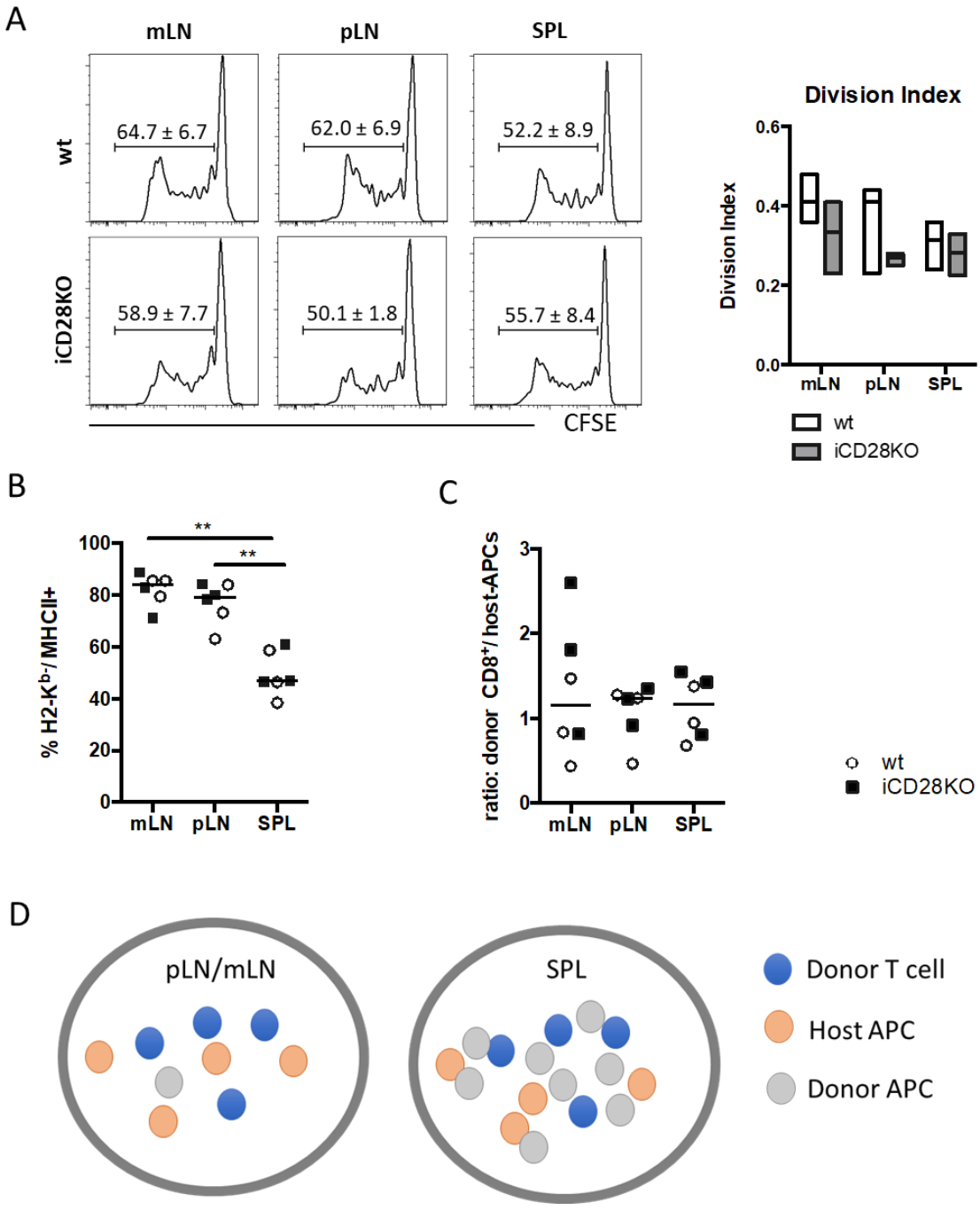

Figure 17: Slightly reduced proliferation of donor CD8⁺ T cells upon CD28 deletion. BALB/c recipient mice received 1 × 10⁷ TCD-BM cells, 1 × 10⁶ CD4⁺CD25⁻ Tconv cells from C57BL/6 mice and 1 × 10⁶ CFSE labelled CD8⁺ T cells from either iCD28KO mice or their wt littermates and were analysed three days later (see Figure 14A). (A) CFSE dilution among donor CD8⁺ T cells. Left panel: representative histograms of CFSE labelled CD8⁺ donor T cells, mean percentages ± SD of CFSE^{low} cells, right panel: division index and proliferation index, box shows the range and the median. (B-C) BALB/c APCs were identified in the mLN and SPL of recipient mice as H2-k^{b-} and MHCII⁺; two-sided Mann-Whitney test (B) Frequency of BALB/c APCs of all MHCII⁺ cells and (C) ratio of CD8⁺ donor T cells and BALB/c APCs; the lines indicate the grand median. (D) Schematic depiction of cellular composition in the lymph nodes and the spleen. (A)- (C): n= 3 mice/group.

In order to get a clear answer to the question whether CD28 co-stimulation is required for efficient CD8$^+$ T cell proliferation and expansion, we addressed this point in an MLR co-culture experiment *in vitro* (Figure 18A). Blocking CD28 co-stimulation on CD8$^+$ T cells with an αCD28 mAb Fab fragment reduced both, the frequency of proliferating CD8$^+$ T cells and the absolute number of CD8$^+$ T cells in the cultures (Figure 18B and C). Furthermore, we simulated the inflammatory situation during aGvHD by activating the APCs with LPS prior to the MLR cultures. LPS pre-treatment of the APCs reduced the effect of CD28 blockade on the proliferation of CD8$^+$ T cells, but not on CD8$^+$ T cell expansion (Figure 18B and C).

Taken together, we could show, that CD28 blockade on CD8$^+$ T cells reduced the proliferation and expansion of CD8$^+$ T cells in an *in vitro* co-culture with allogeneic APCs. As there were no syngeneic APCs present in the cultures, this rather mimicked the situation in the mLN than in the spleen during aGvHD (see Figure 17D). Thus, our *in vitro* data support our *in vivo* finding that in the lymph nodes of mice suffering from aGvHD, iCD28KO CD8$^+$ T cells do not proliferate as good as wt CD8$^+$ T cells.

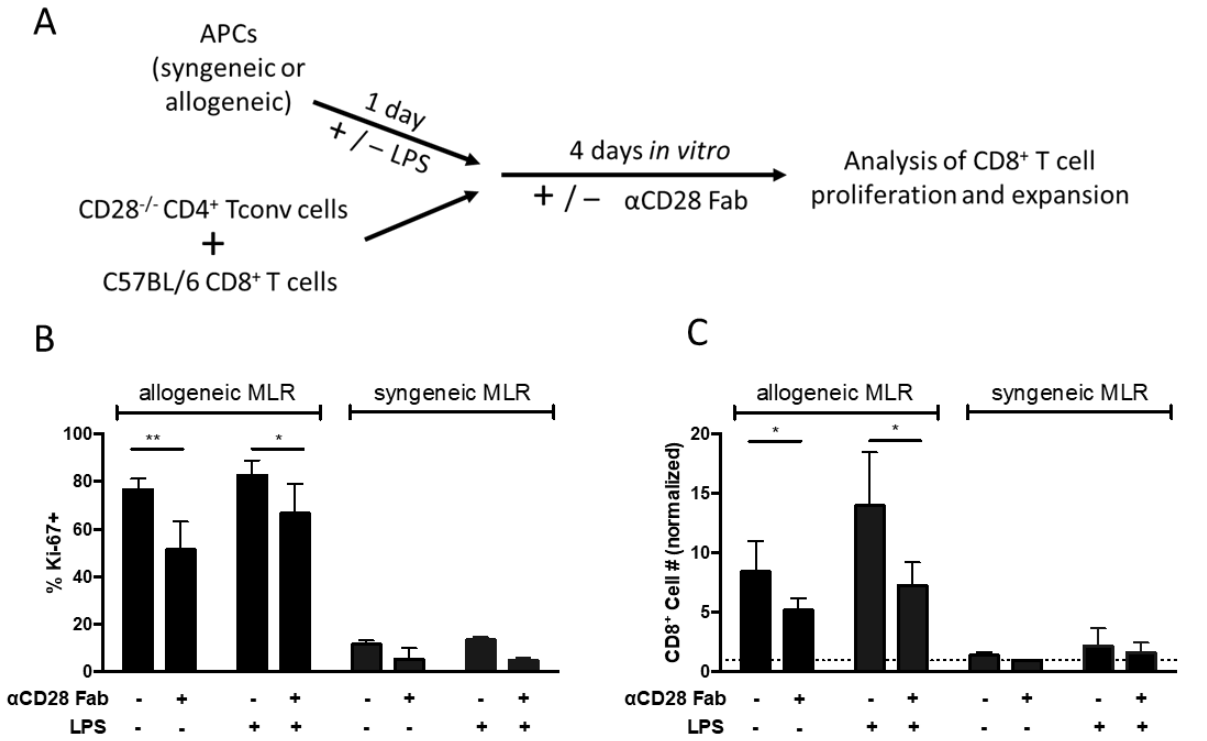

Figure 18: Reduced Proliferation of allo-reactive CD8$^+$ T cells upon CD28 blockade. (A) CD8$^+$ T cells from C57BL/6 mice were co-cultured with CD4$^+$CD25$^-$ T cells from CD28$^{-/-}$ mice and TCD splenocytes from BALB/c or C57BL/6 mice that were either pre-treated with LPS or untreated. CD28 co-stimulation of CD8$^+$ T cells in the mixed lymphocyte reactions (MLR) was blocked with αCD28 mAb Fab fragment. (B) Expression of Ki-67 and (C) CD8$^+$ T cell expansion were assessed after 4 days; n= 5, data were pooled of three independent experiments, two-tailed, paired t-test.

7.3.2 CD28 deletion on CD8$^+$ T cells has no effect on systemic cytokine release but reduces clinical signs of aGvHD

To investigate the effects of CD28 deletion on CD8$^+$ T cells on the clinical parameters of aGvHD, we induced aGvHD in BALB/c recipient mice as described in Figure 15A and analysed the recipients 6 days after transplantation. Both, INFγ and TNF were upregulated in the serum of T cell recipients when compared to mice that were transplanted with TCD BM cells alone. However, we could not observe a difference between recipients of iCD28KO and wt CD8$^+$ T cells (Figure 19A and B). Furthermore, TNF serum concentrations of mice transplanted with CD4$^+$ Tconv cells and CD8$^+$ T cells were two- to four-fold lower when compared to TNF concentrations of mice transplanted with equal numbers of only CD4$^+$ Tconv cells (Figure 9A and Figure 19B). TNF secretion during aGVHD directly potentiates tissue damage in the gut [reviewed in 154]. Consequently, also the histopathological score of small and large intestine was lower after transfer of CD4$^+$ and CD8$^+$ T cells as compared to transfer of only CD4$^+$ T cells (Figure 9B and Figure 19C). Moreover, we could not observe a difference in the intestinal tissue damage of iC28KO or wt CD8$^+$ T cell recipients (Figure 19C). Still, CD28 deletion on donor CD8$^+$ T cells reduced the clinical score of recipient mice, indicating that other target organs than the gut contributed to overall disease severity (Figure 19D). In summary, our data show that CD28 deletion on CD8$^+$ T cells caused less signs of aGvHD, which is, however, not due to reduced serum cytokine concentration or less intestinal tissue damage.

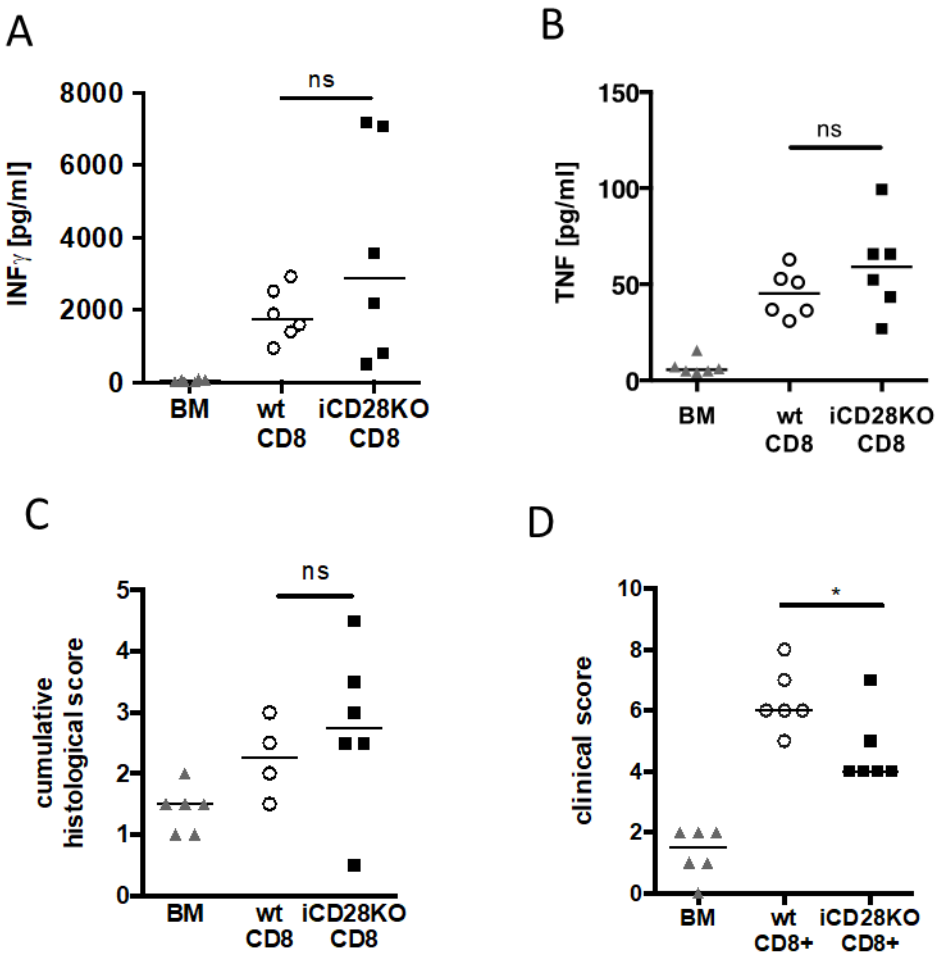

Figure 19: CD28 deletion on donor CD8⁺ T cells causes less clinical signs of aGvHD. Lethally irradiated BALB/c mice were reconstituted with 2.5 × 10⁵ Thy1.1⁺/⁺ CD4⁺ Tconv cells and 2.5 × 10⁵ CD8⁺ T cells from iCD28KO mice or their wt littermates, treated with Tamoxifen and analysed on day 6 after transplantation as shown in Figure 14A. (A) Concentration of IFNγ and (B) concentration of TNF in the serum of recipient mice. (C) cumulative histological score of small and large bowel of recipient mice and (D) clinical score. (A)-(D): n=4-6 mice/ group, two independent experiments; lines indicate medians, two-tailed unpaired Mann-Whitney test.

7.3.3 Killing of allogeneic target cells by CD8⁺ T cells is not reduced after CD28 deletion or blockade

The GvL effect relies on the capacity of CD8⁺ T cells to efficiently kill allogeneic target cells in a contact dependent manner. To investigate the contribution of CD28 to the cytotoxic effector function of allo-reactive CD8⁺ T cells, we transplanted BALB/c mice with wt CD4⁺ Tconv cells and CD8⁺ T cells from iCD28KO mice or wt littermates. One day before analysis, the mice were injected with CFSE-labelled target cells (Figure 20A). We assessed the cytotoxic capacity of the transferred CD8⁺ T cells by calculating the odds ratio of allogeneic target cells (BALB/c splenocytes or BCL-1 cells) and syngeneic target cells (C57BL/6 splenocytes) (Figure 20B). In both, recipients of wt and iCD28KO CD8⁺ T cells, the allogeneic target cells were eliminated equally well

and much more efficiently as compared to mice that were transplanted with TCD BM cells alone (Figure 20B). Because the number of $CD8^+$ T cells in the spleen was not affected by CD28 deletion (Figure 16A), we could assume that the ratio of effector and target cells was not altered due to CD28 deletion on CD8 T cells. Thus, our results demonstrate that CD28 expression on donor $CD8^+$ T cells was not required to kill allogeneic target cells *in vivo*.

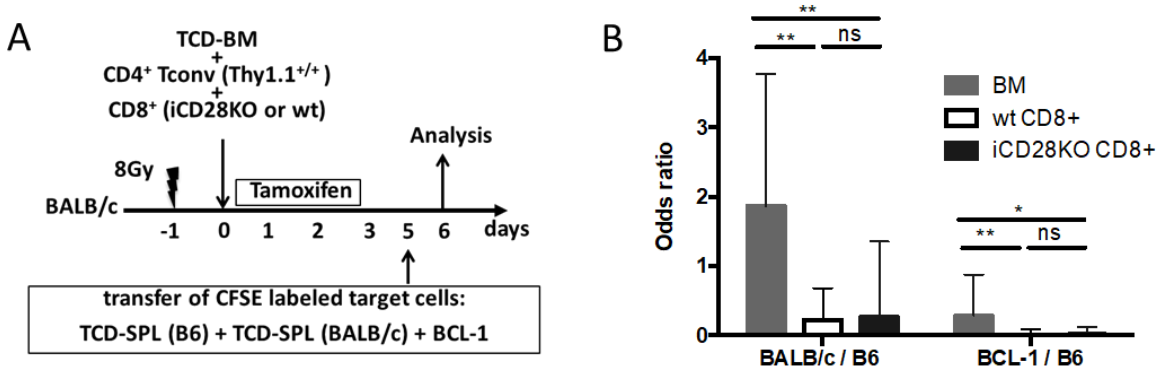

Figure 20: Killing of allogeneic target cells by $CD8^+$ T cells is independent of CD28 co-stimulation. BALB/c recipient mice were transplanted with Thy1.1$^{+/+}$CD4$^+$ Tconv cells and 2.5 × 10^5 iCD28KO or wt CD8$^+$ T cells and analysed on day 6 after transplantation as shown in Figure 14A. (A) In the same experiment, CFSE labelled BCL-1 lymphoma cells and TCD splenocytes from C57BL/6 and BALB/c mice were injected into the recipient mice one day before analysis (B) Odds ratio of labelled target cells in the spleen of recipient mice; median + range; unpaired Mann-Whitney test, comparisons between T cell recipients: two-tailed, all other comparisons: one-tailed; n=6 mice/group in two independent experiments;

BCL-1 is a B-cell lymphoma cell line that expresses CD80 and CD86 but not CD28. In contrast, T-cell lymphomas, like the T cell clone T8-28, express CD28 but not CD80 or CD86 (Figure 21A). We wanted to know if interaction of CD28 with CD80/86 is needed for efficient killing of allogeneic target cells by $CD8^+$ T cells. For that reason, wt $CD8^+$ T cells were activated in an MLR co-culture together with $CD28^{-/-}$ $CD4^+$ Tconv cells and allogeneic APCs, and then used as effector cells in a killing assay with BCL-1 or T8-28 cells as target cells (Figure 21B). $CD8^+$ T cells killed BCL-1 and T8-28 cells in a dose dependent manner and regardless of whether αCD28 Fab fragment had been added to the killing assay or not (Figure 21C). CD8-depleted effector cells had only a minimal killing capacity towards the target cells, demonstrating that the cytotoxicity of $CD4^+$ T cells could be neglected in this assay (Figure 21C). In addition, we observed that killing of BCL-1 cells solely relied on Fas-independent pathways whereas apoptosis of T8-28 cells could be induced by an αFas antibody and was further enhanced by Fas-independent mechanisms of effector cells (Figure 21C).

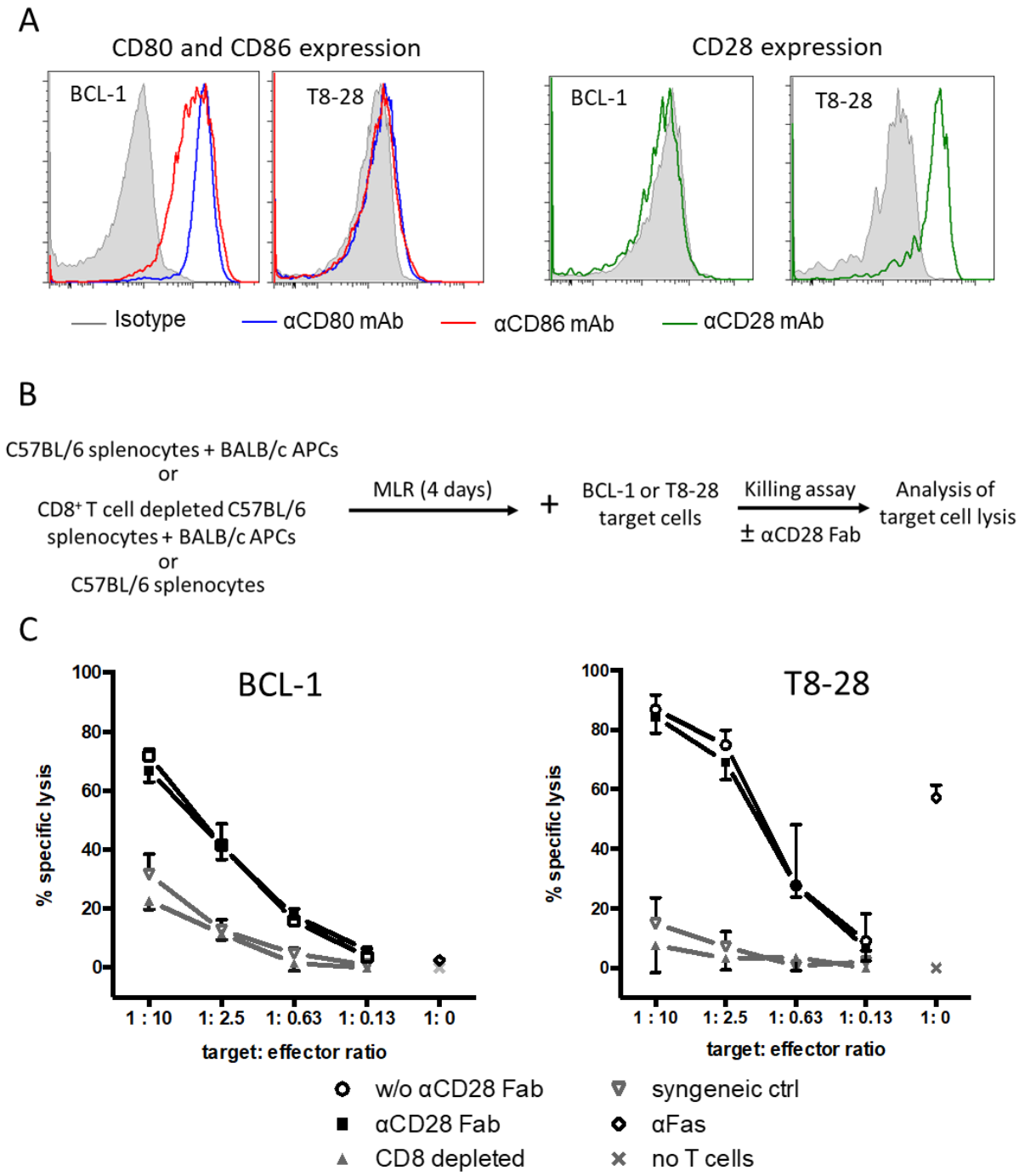

Figure 21: CD8⁺ T cells kill allogeneic target cells in vitro despite CD28 blockade. (A) Freshly thawed BCL-1 and T8-28 cells were stained with αCD80 mAb, αCD86 mAb and αCD28 mAb or isotype control antibodies (B) Total C57BL/6 lymph node cells or CD8⁺ T cell-depleted lymph node cells were activated for 4 days in an allogeneic MLR with TCD BALB/c splenocytes or cultured alone (syngeneic ctrl) in presence of 10^{-7} M IL-2. Four days later, cultured cells served as effector cells in a killing assay with BCL-1 and T8-28 cells as target cells. CD28 was blocked during the time of the killing assay with αCD28 mAb Fab fragment. (C) % specific lysis of target cells; n= 4, mean values and SD of three independent experiments.

7.4 Requirement of CD28 on CD4$^+$, CD8$^+$ and Treg cells for the GvL effect

7.4.1 CD28 deletion on CD4$^+$ Tconv cells but not CD8$^+$ T cells ablates the GvL effect

Having studied the effects of CD28 deletion on CD4$^+$ Tconv cells and CD8$^+$ T cells on aGvHD, we next wanted to investigate the role of CD28 co-stimulation on conventional donor T cells in a mouse model where the recipient mice could develop aGvHD and/or lymphoma.

BALB/c recipient mice were lethally irradiated and injected with BCL-1 lymphoma cells. Four hours later, we transferred CD4$^+$ Tconv cells, CD8$^+$ T cells and Treg cells from iCD28KO mice or their wt littermates in a 2:1:3 ratio into the recipient mice. We needed to transplant equal numbers of Treg cells and conventional T cells (CD4$^+$ and CD8$^+$) as otherwise the mice would prematurely die from aGvHD before we were able to analyse the GvL effect. CD28 deletion on donor iCD28KO T cells was again induced after transplantation by treating the recipients with Tamoxifen until day 3 after transplantation (Figure 22A). Each mouse that was killed for humane reasons in this survival study was analysed *post mortem* to identify the cause of euthanization. If we found more than 1×10^7 BCL-1 cells in the spleen of recipient mice, we assumed that the lymphoma burden and not aGvHD was the cause of death. This was the case for all mice that received only TCD-BM cells (grey symbols) and had to be killed around 30 days after transplantation due to splenomegaly, breathing difficulties or both (Figure 22B and C). In comparison, mice survived significantly better, when they were transferred with wt T cells and TCD BM cells (black symbols). While 75 % of the recipients in this group developed delayed but lethal lymphoma, 25 % of the recipients showed efficient anti-tumour activity but had to be killed due to severe aGvHD symptoms (Figure 22B and C).

The therapeutic effect of the donor T cells was ablated when CD28 was deleted on donor CD4$^+$ Tconv cells (red symbols) (Figure 22B). In this group, similarly to the BM control group, none of the recipients showed efficient anti-tumour activity, i.e. only one mouse survived until day 120 and had 1.6×10^6 lymphoma cells in the spleen upon analysis (Figure 22C).

In contrast to CD28 deletion on donor CD4$^+$ Tconv cells, selective CD28 deletion on donor CD8$^+$ T cells even enhanced the survival of the mice from 42 days (wt T cell

recipients, black symbols) to 62.5 (iCD28KO CD8$^+$ T cell recipients, green symbols) (Figure 22B). Moreover, CD28 deletion on donor CD8$^+$ T cells induced tumour-free long-term survival, which was not observed for recipients of CD28-sufficient T cells (Figure 22B). While the percentage of mice that succumbed to aGvHD was the same in recipients of wt T cells and recipients of iCD28KO CD8$^+$ T cells, less mice had to be killed because of the tumour burden (Figure 22C). This is in line with our previous observations *in vivo* and *in vitro* (see Figure 20 and Figure 21), where we could not see a diminished killing of allogeneic target cells by CD8$^+$ T cells upon CD28 deletion or blockade.

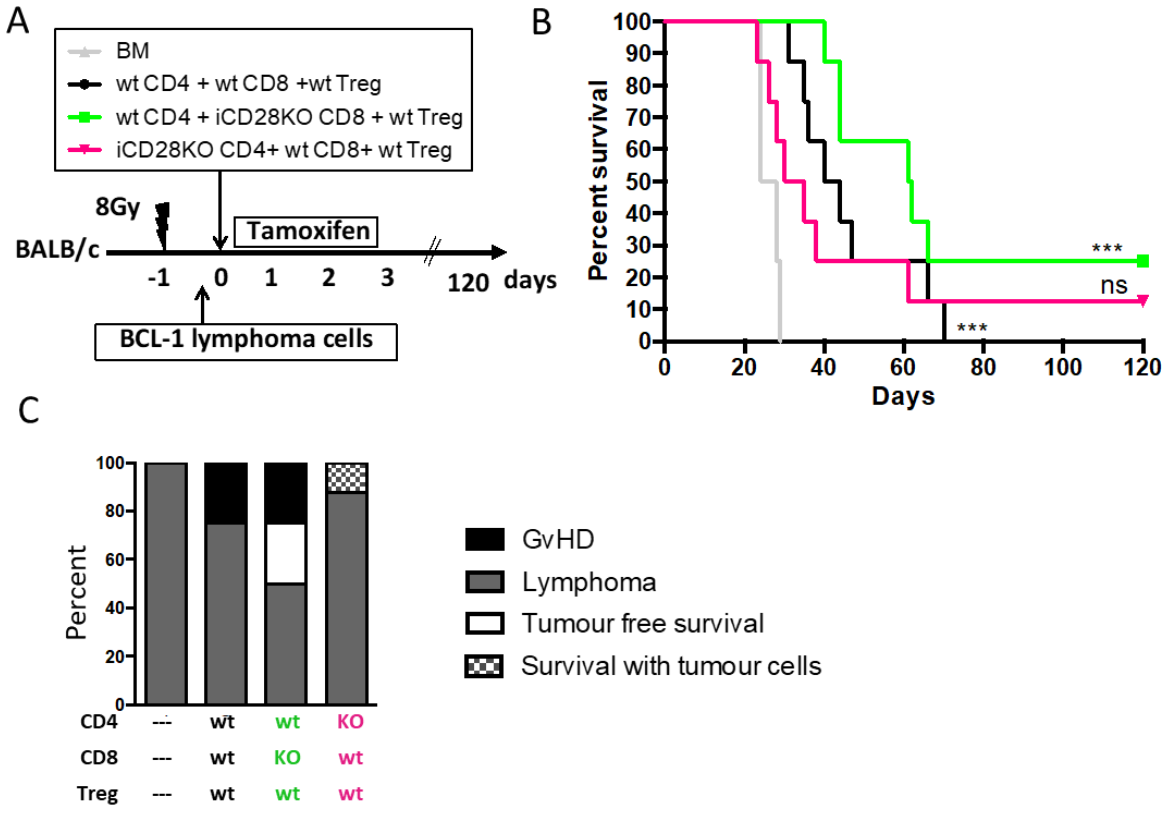

Figure 22: CD28 deletion on CD4$^+$ Tconv cells but not CD8$^+$ T cells impairs GvL effect in vivo. *(A) Lethally irradiated BALB/c mice were injected with BCL-1 lymphoma cells 4 hours before transfer of TCD BM cells with or without 8 × 10^4 CD4$^+$ Tconv cells, 4 × 10^4 CD8$^+$ T cells and 2.5 × 10^5 Treg cells from either iCD28KO mice or their wt littermates. (B) Percent survival and (C) cause of euthanization of recipient mice until day 120 after transplantation; n= 8 mice/ group, Mantel-Cox survival test between BM only recipients and T cell recipients.*

To elucidate why CD28 deletion on CD4$^+$ T cells but not CD8$^+$ T cells impaired the GvL activity, we analysed the frequency of CD8$^+$ and CD4$^+$ T cells among all donor T cells recovered from the spleen of the recipient mice *post mortem*. While CD8$^+$ T cell frequencies were not decreased upon CD28 deletion, the frequency of CD4$^+$ donor

Tconv cells was reduced when CD28 was selectively deleted on these cells (Figure 23A and B). This indicated that CD28 expression is required for the survival or memory cell differentiation of CD4$^+$ Tconv cells. Consequently, iCD28KO CD4$^+$ T cells probably provided only little T cell help to CD8$^+$ T cells, resulting in an insufficient anti-tumour response.

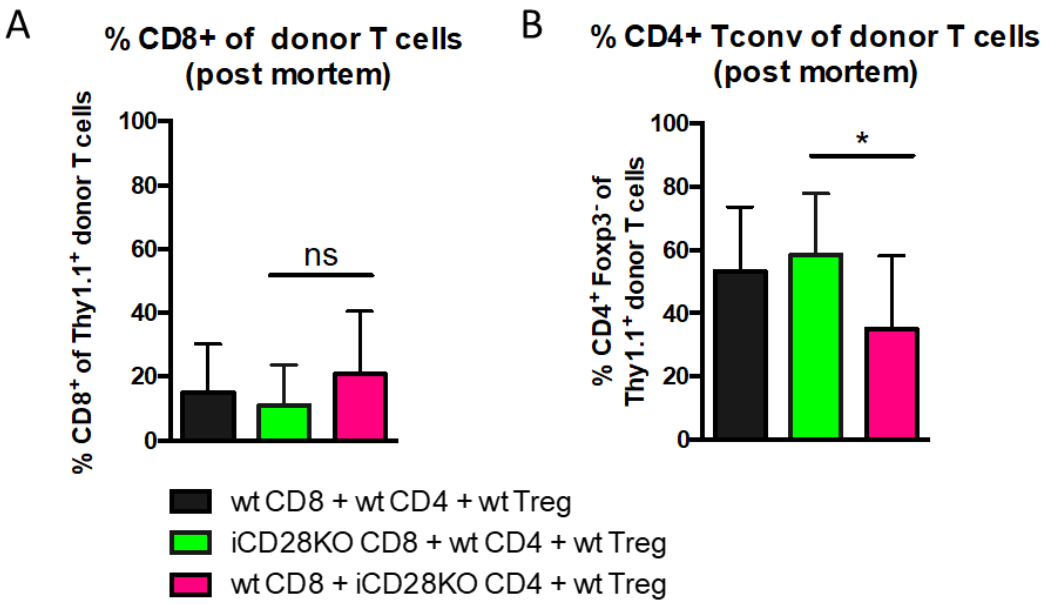

Figure 23: Reduced recovery of donor CD4$^+$ Tconv cells after CD28 deletion in vivo*. BALB/c recipient mice were injected with BCL-1 lymphoma cells before receiving CD4$^+$ Tconv cells, CD8$^+$ T cells and Treg cells from iCD28KO mice or wt littermates as described in Figure 21 A. (A) Frequency of CD8$^+$ and (B) CD4$^+$ T cells among the transferred Thy1.1$^+$ donor T cells, analysed post mortem until day 120 after transplantation; n= 6-8 mice/ group, data pooled of three independent experiments, two-sided unpaired t-test.*

7.4.2 CD28 expression on CD4$^+$ T cells is required for optimal helper function and anti-tumour activity

We next wanted to test if CD28 deletion on donor CD4$^+$ Tconv cells could, indeed, account for the impaired anti-tumour activity by CD8$^+$ T cells. Therefore, we analysed the effect of Tamoxifen-induced CD28 deletion on either CD4$^+$ Tconv cells or CD8$^+$ T cells when activated together in an MLR culture for four days (Figure 24A).

Due to a short incubation with Tamoxifen prior to the MLR cultures, the amount of CD28 protein on the cell surface was only depleted by around 46 % on iCD28KO CD4$^+$ Tconv cells (data not shown). Still, wt CD8$^+$ T cells that were activated together with iCD28KO CD4$^+$ Tconv cells expressed less T-bet and less Granzyme B than wt CD8$^+$ T cells, derived from MLRs with wt CD4$^+$ Tconv cells (Figure 24B). Consequently, there

were fewer T-bet$^+$ CD8$^+$ effector cells when they were co-cultured with iCD28KO CD4$^+$ Tconv cells. (Figure 24C). This impaired effector CD8$^+$ T cell differentiation could not be rescued by addition of 10^{-8} M IL-2 to the MLR cultures (Figure 24B and C).

In contrast to what we had observed when CD28 was selectively depleted on CD4$^+$ Tconv cells, the frequency of T-bet$^+$ CD8$^+$ effector T cells was not reduced, when iCD28KO CD8$^+$ T cells were co-cultured with wt CD4$^+$ Tconv cells (Figure 24B and C). This was not due to insufficient deletion of CD28, as CD28 expression on effector iCD28KO CD8$^+$ T cells was reduced by 94 % (data not shown).

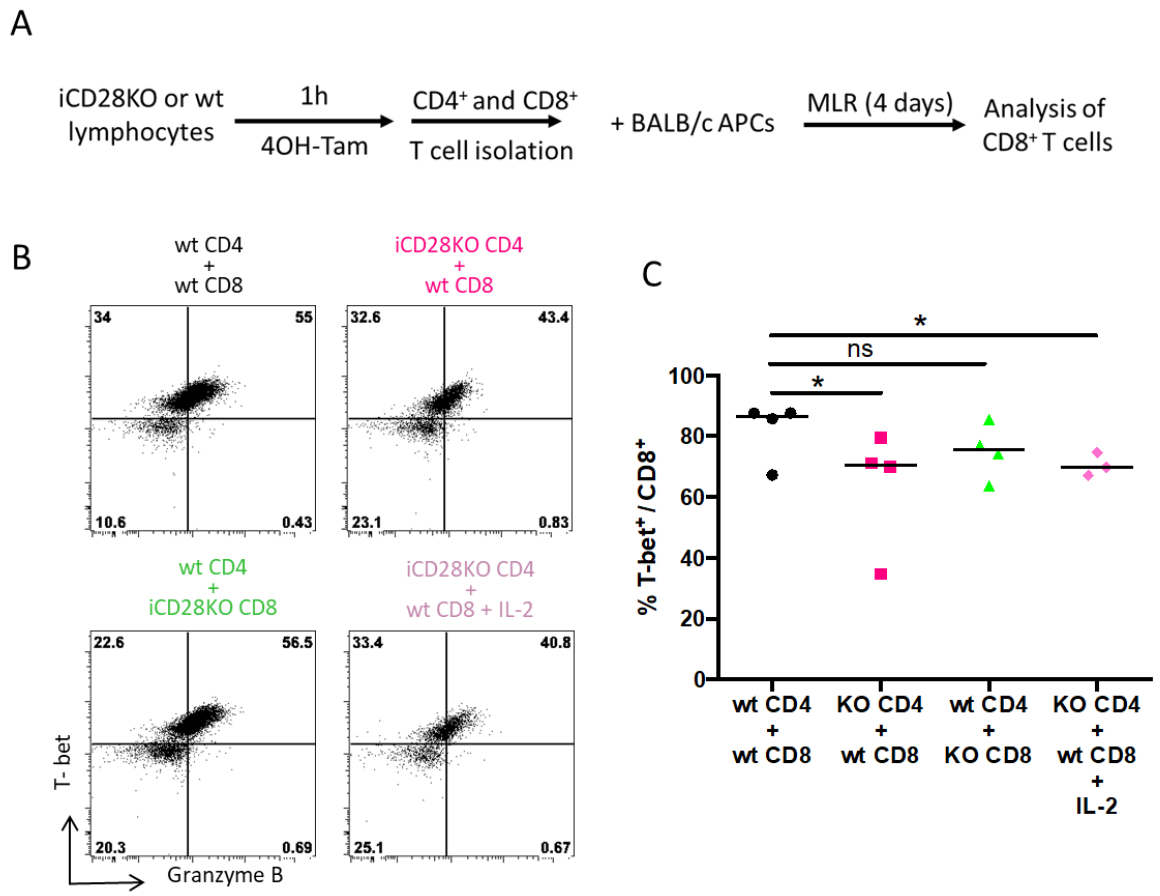

Figure 24: CD28 depletion on CD4$^+$ T cells impairs CD8$^+$ effector T cell differentiation. (A)CD4$^+$ Tconv and CD8$^+$ T cells from iCD28KO mice or their wt littermates were pre-treated with 4OH-Tamoxifen for 1 hour and then activated in mixed lymphocyte reactions with TCD BALB/c splenocytes for 4 days. Where indicated 10^{-8} M recombinant IL-2 was added to the cultures. (B) Exemplary dot blots of CD8$^+$ T cells showing Granzyme B and T-bet expression. (C) Frequency of T-bet$^+$ effector cells among CD8$^+$ T cells, n=4, two-sided paired t-test.

We next wanted to test the allo-reactivity of CD8$^+$ T cells that had previously been activated in the MLR cultures shown in Figure 24. For that reason, we depleted CD4$^+$ cells from MLR culture cells and assessed the capacity of the remaining cells, mainly

CD8⁺ T cells, to induce cell death in BCL-1 target cells (Figure 25A). iCD28KO CD8⁺ T cells showed a similar cytotoxicity towards BCL-1 cells as wt CD8⁺ T cells that had been co-cultured with wt CD4⁺ Tconv cells (Figure 25B). However, wt CD8⁺ T cells, that were activated in an MLR culture together with iCD28KO CD4⁺ Tconv cells, were less efficient in killing BCL-1 target cells as wt CD8⁺ T cells derived from MLR cultures with wt CD4⁺ Tconv cells (Figure 25B).

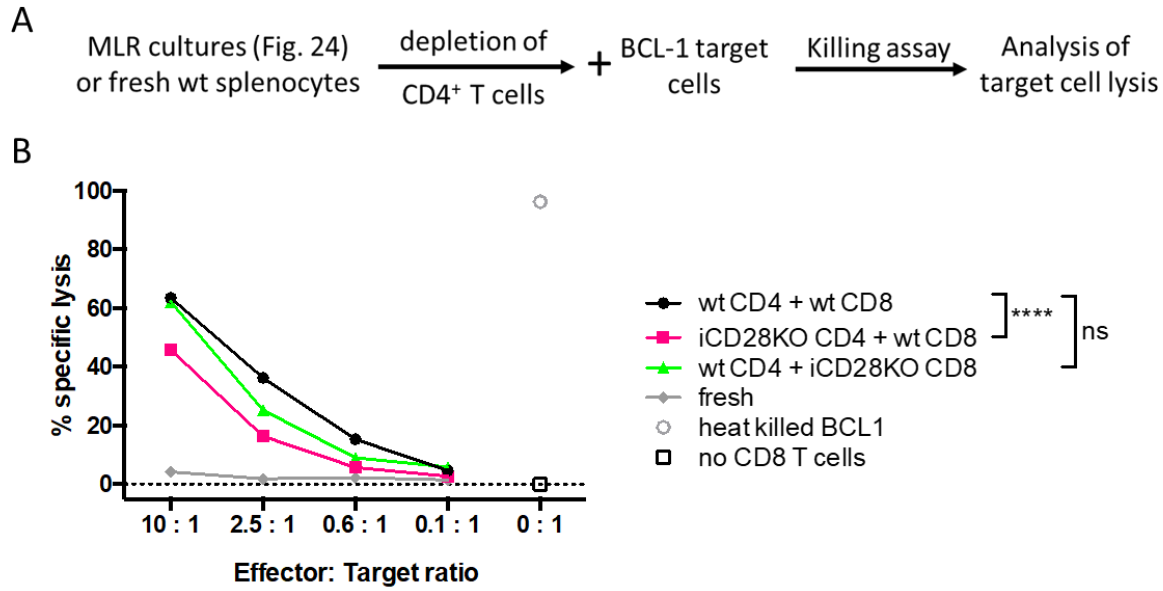

Figure 25: CD28 expression on CD4⁺ T cells enhances CD8⁺ T cell mediated killing of allogeneic target cells in vitro. *CD8⁺ T cells and CD4⁺ Tconv cells from wt or iCD28KO mice were activated in MLR cultures as described in Figure 23. (A) CD4⁺ T cell-depleted MLR cultures or, as control, freshly isolated CD4⁺ T cell-depleted splenocytes served as effector cells in a killing assay with BCL-1 lymphoma cells as target cells. (B) % specific lysis of target cells; two-way repeated measures ANOVA test, n=4, pooled data of three independent experiments.*

Taken together, these *in vitro* experiments recapitulate what we had observed *in vivo* and demonstrate that CD28 co-stimulation is not required for anti-tumour activity of CD8⁺ T cells. In contrast, CD4⁺ Tconv cells required CD28 signals to mediate efficient T cell help and to allow for optimal effector differentiation and -function of CD8⁺ T cells.

7.4.3 Late-onset aGvHD correlates with enhanced GvL effect.

We had previously observed that CD28 deletion on donor Treg cells causes a late and lethal flare of aGvHD, i.e. late-onset aGvHD (see Figure 12). In order to investigate if late-onset aGvHD is associated with enhanced anti-tumour activity, we deleted CD28 selectively on Treg cells in the same aGvHD/GvL experiments as described in 7.4.1 (see Figure 22 and Figure 26A). In line with our previous observations, recipients of

CD28-depleted Treg cells (blue symbols) developed a late flare of aGvHD which was associated with an enhanced anti-tumour effect as only 25 % of the recipients developed a lymphoma compared to 75 % of wt T cell recipients (black symbols) (Figure 26B and D). However, the severity of late-onset aGvHD required the euthanization of the mice around 30 days after transplantation (Figure 26 C). Consequently, transfer of wt $CD4^+$ Tconv cells and $CD8^+$ T cells together with iCD28KO Treg cells did not prolong the survival of the mice when compared to TCD-BM only recipients (grey symbols) (Figure 26D).

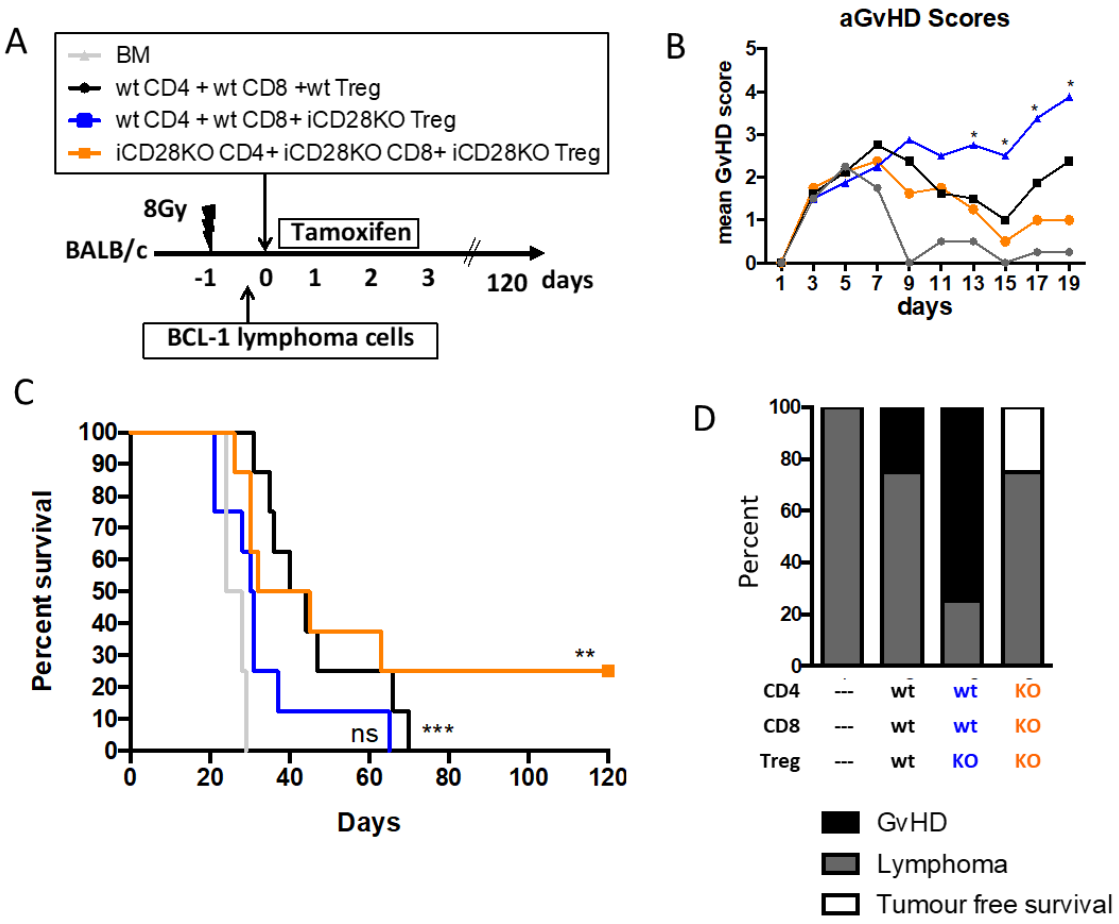

Figure 26: Late-onset aGvHD correlates with enhanced GvL effect. (A) One day after lethal irradiation, BALB/c recipient mice were first injected with BCL-1 lymphoma cells and 4 hours later with TCD BM cells alone or together with 8 × 10^4 $CD4^+$ Tconv cells, 4 × 10^4 $CD8^+$ T cells and 2.5 × 10^5 Treg cells from either iCD28KO mice or their wt littermates. (B) Mean aGvHD score until day 20 after transplantation; One-tailed Mann-Whitney test between wt T cell recipients (black) and iCD28KO Treg recipients (blue) on days 13, 15, 17 and 19. (C) Percent survival of recipient mice; Mantel-Cox survival test between BM only recipients and T cell recipients. (D) Cause of euthanization until day 120 after transplantation. (B-D): n= 8 mice/group.

In contrast to selective deletion on Treg cells, none of the recipients developed a lethal aGvHD when CD28 was deleted on all donor T cells (orange symbols) (Figure 26B

and D). This demonstrates that late-onset aGvHD required CD28 protein expression by $CD4^+$ Tconv cells and $CD8^+$ T cells. Moreover, the proportion of recipient mice developing a lethal lymphoma was similar in recipients of CD28-depleted T cells (orange symbols) and CD28-sufficient T cells (black symbols) (Figure 26D), showing that the GvL effect was not impaired when CD28 was deleted on all donor T cells. As 25 % of the mice that received CD28-deficient donor T cells survived until the end of the experiment without an outgrowth of BCL-1 cells, the prognosis of this experimental group was better that of mice, transplanted with wt T cells (Figure 26C and D).

All in all, our *in vivo* GvL experiments show that donor Treg cells and $CD4^+$ Tconv cells need to express CD28 to maintain the therapeutic effect of the T cell transplant. In contrast, CD28 deletion on all donor T cells induced long-term survival of recipient mice. The best therapeutic effect was achieved by selective CD28 deletion on donor $CD8^+$ T cells as this enhanced the median survival of recipient mice and induced tumour-free survival in 25 % of the mice.

8 Discussion

In the past, CD28 co-stimulation during aGvHD was studied by using blocking antibodies or conventional CD28 knockout mice as T cell donors [36, 122, 127]. The disadvantages of these models, i.e. impaired T cell development in $CD28^{-/-}$ mice and systemic CD28 blockade with antibodies, however, hampered the interpretation of these experiments. By using inducible CD28 knock-out mice as T cell donors, we were now able to investigate the role of CD28 co-stimulation on different donor T cell subsets without facing the limitations of previous mouse models of allogeneic HSCT. The effects of Tamoxifen-induced CD28 deletion on donor $CD4^+$ T conv cells, $CD8^+$ T cells and Treg cells on both, aGvHD and the GvL response, is summarised in Figure 27 and will be discussed in more detail in this chapter.

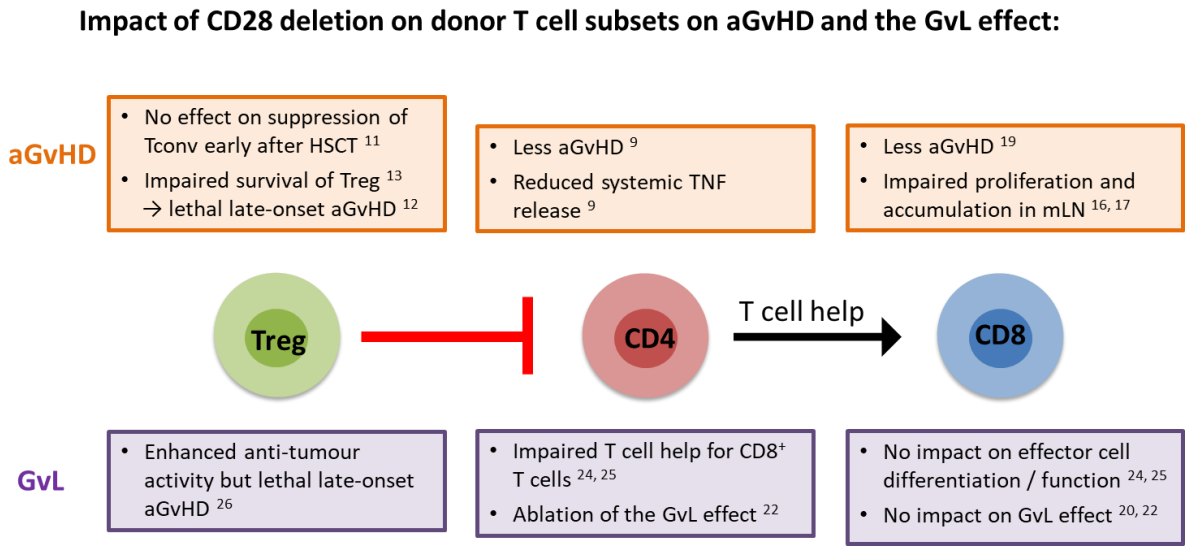

Figure 27: Impact of CD28 deletion on different donor T cell subsets on aGvHD and the GvL effect. The numbers in superscript refer to the numbers of the Figures, where each observation was shown in the results section. As we have not addressed the suppression of $CD8^+$ T cells by Treg cells, this is not depicted in the simplified scheme.

8.1 Less inflammation in recipients of iCD28KO $CD4^+$ Tconv cells

We first analysed the kinetics of CD28 deletion on donor iCD28KO $CD4^+$ Tconv cells upon tamoxifen treatment of the recipient mice and observed almost full ablation of CD28 expression on $CD4^+$ Tconv cells by day 3 after transplantation (Figure 5C). We also saw a decrease in CD28 expression in wt $CD4^+$ Tconv cells after transfer into the allogeneic recipients. This can be explained by internalisation of the receptor after binding to its ligands CD80/CD86 [156].

As iCD28KO donor T cells still express CD28 in the first 2-3 days after transplantation, it is possible that the first donor T cell / host APC contacts occur in the presence of CD28. To determine the impact of the initial CD28 expression on iCD28KO T cells, we deleted CD28 on donor $CD4^+$ T cells either before transfer into the recipients or after transplantation by feeding the donor mice or the recipient mice with Tamoxifen, respectively. Independent of the Tamoxifen treatment schedule, we saw that both, wt and iCD28KO $CD4^+$ T cells, were equally well expanding and that the Treg frequencies were comparable (Figure 8). For an efficient Th1 differentiation however, continuous CD28 expression on primed $CD4^+$ T cells is necessary [157]. Consequently, when we deleted CD28 on $CD4^+$ Tconv cells after transplantation, we observed less inflammation and less signs of aGvHD on day 7 after transplantation (Figure 9A-C).

Even though iCD28KO $CD4^+$ Tconv cells transiently reduced aGvHD symptoms, the mice still succumbed to lethal aGvHD (Figure 9D and E). This demonstrates that in the highly inflammatory and lymphopenic situation of aGvHD, lack of CD28 signalling can be at least partially compensated by “signal 3” stimuli, like pro-inflammatory cytokines or other co-stimulatory molecules [38, 39, reviewed in 37]. In other studies, using conventional CD28 knockout mice, the magnitude of aGvHD was modulated to varying degrees, depending on the exact model used: Comparably to our inducible knock out model, constitutive CD28 deficiency on donor T cells only delayed aGvHD after transfer into fully MHC-mismatched recipients ($H\text{-}2^b \rightarrow H\text{-}2^d$) [36]. In contrast, transplantation of $CD28^{-/-}$ $CD4^+$ T cells into MHC class II disparate F1 recipients (C57BL/6 → (B6xbm12)F1) induced long-term survival of recipient mice [122]. Therefore, it is likely that in a less inflammatory model of aGvHD, with lower levels of “signal 3” cytokines, the effect of inducible CD28 deletion would be higher.

8.2 CD28 deletion on donor Treg cells provides a model of late-onset aGvHD

We investigated the requirement of CD28 co-stimulation on donor Treg cells in aGvHD. In contrast to previous studies, where CD28 was blocked with an αCD28 Fab fragment or an intact αCD28 mAb (clone E18), we could not observe an increase in Treg frequencies after inducible CD28 deletion on total donor $CD4^+$ T cells, i.e. $CD4^+$ Tconv cells and Treg cells (Figure 8B) [127, 130, 158]. The reason why blockade of CD28 with αCD28 antibody (-fragments) but not genetic CD28 deletion increases Treg cell frequencies remains unclear. One could, however, speculate that the effect of the E18

antibody and even that of αCD28 Fab fragments is not only due to abrogation of endogenous CD28 stimulation.

Also, when we selectively deleted CD28 only on donor Treg cells, the number of iCD28KO and wt Treg was similar at day 6 after transplantation (Figure 10E). Consequently, CD28-deficient and CD28-sufficient Treg cells were equally able to suppress the accumulation of donor $CD4^+$ Tconv cells and reduced aGvHD scores as well as TNF serum concentrations until six days after aGvHD induction (Figure 11A and B, Figure 12). Thus, at the first peak of disease activity, CD28 expression on Treg cells was dispensable for their suppressor function. In fact, also in other inflammatory models, CD28-deficient Treg cells were able to, at least partially, control immune responses [159, 160]. The $Foxp3^{Cre}$ $CD28^{flox/flox}$ Treg cells, used in these experiments showed, however, deficits in migration to non-lymphoid tissues [160]. In contrast to that, we found equal numbers of wt and iCD28KO Treg cells in the intestine at day 6 after transplantation, indicating that the homing of Treg cells to the gut was not affected by CD28 deletion in our model (Figure 11D and E).

We further investigated the capability of iCD28KO Treg cells to prevent lethal aGvHD as it has been shown for wt Treg cells before [91, 94, 116, 117, 138, 161].

While CD28-sufficient Treg cells induced long-term survival, CD28-deficient Treg cells protected the mice only during the first 2 to 3 weeks after transplantation but could not control a second flare of disease around day 20 (Figure 12). We further found out that the cause of this lethal and late flare of aGvHD is the impaired survival of iCD28KO Treg cells in the allogeneic host (Figure 13). Thus, 3 weeks after allogeneic HSCT, CD28 expression was important for Treg survival, as it has been previously demonstrated for steady-state situations [59-61, 162].

Our observations lead us to the following hypothesis shown in Figure 28: The conditioning of the recipient mice leads to tissue damage which in turn causes the release of high amounts of pro-inflammatory mediators. Early after aGvHD induction, these pro-inflammatory mediators either directly compensate for the lack of CD28 co-stimulation on donor Treg cells or enhance the expression of other co-stimulatory molecules on APCs and/or Treg cells which replace the CD28 signal. However, at a later phase of the disease, when these mediators have subsided, CD28 is again important for Treg survival as it is in steady-state conditions.

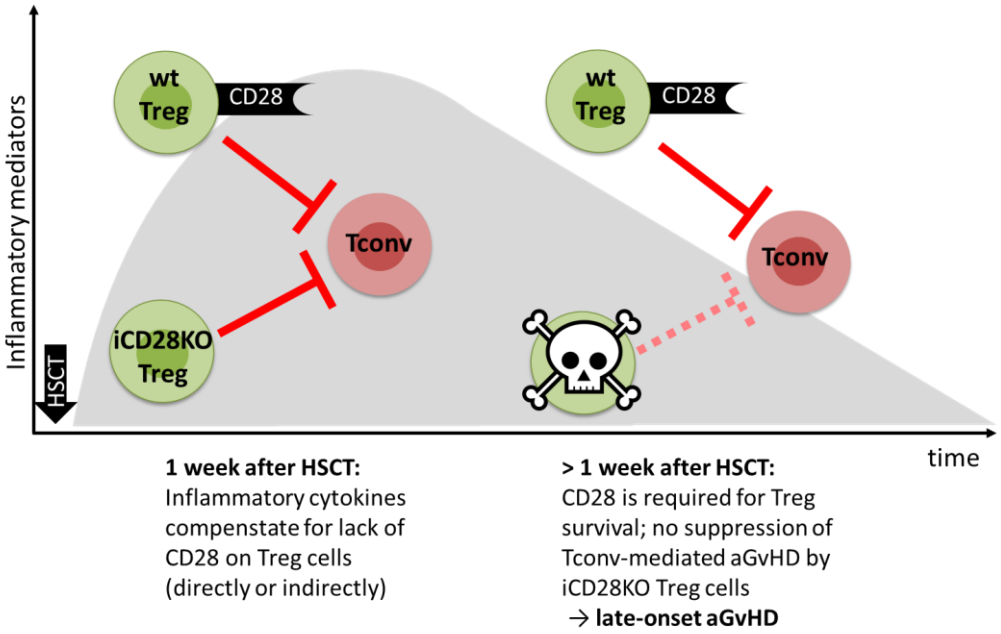

Figure 28:Pro-inflammatory mediators directly or indirectly compensate for lack of CD28 co-stimulation early after aGvHD induction.

About 10 % of aGvHD patients in the clinics suffer from a late form of aGvHD, also called late-onset aGvHD, which is similar to the disease course that we saw in iCD28KO Treg recipient mice [155, 163-165]. Thus, by transplanting iCD28KO Treg cells together with wt $CD4^+$ Tconv cells we generated a mouse model that mimics late-onset aGvHD in humans. It is not yet clear whether the pathophysiology of late-onset aGvHD and classical aGvHD differs. Due to the late timepoint of disease onset, it is however likely that inflammatory mediators resulting from the conditioning regimen of the patients only play a minor role in late-onset aGvHD. Instead, angiogenic factors might be involved which are elevated in both, late-onset and classical aGvHD [155]. Therefore, our new mouse model might be a useful tool to study the pathophysiology of late-onset aGvHD. In addition, the model allows to test the efficacy of experimental and standard therapies towards late-onset aGvHD.

8.3 CD28-deficient CD8$^+$ T cells cause less aGvHD but efficiently kill allogeneic target cells

We found that CD28-sufficient CD8$^+$ T cells proliferated more than CD28-deficient CD8$^+$ T cells *in vivo* and *in vitro* and, therefore, they were found in higher numbers in the lymph nodes of recipient mice (Figure 16A and B, Figure 17A, Figure 18). This is in line with a previous study, showing that naive TCR-transgenic iCD28KO CD8$^+$ T cells have an impaired clonal expansion upon stimulation with their cognate antigen *in vivo* [166].

Even though we found less iCD28KO CD8$^+$ T cells in the mLN, where mostly gut-homing of T cells is induced, the intestinal tissue damage of iCD28KO CD8$^+$ T cell recipients was comparable to that of wt CD8$^+$ T cell recipients but generally low when compared to experiments where only CD4$^+$ Tconv cells were transplanted (Figure 19C and Figure 9B). This is in line with lower TNF serum concentrations in recipients of CD4$^+$ and CD8$^+$ T cells compared to CD4$^+$ Tconv cell recipients (Figure 19B). Moreover, the TNF concentrations were not reduced upon CD28 deletion on CD8$^+$ T cells (Figure 19B). This is, however, not surprising because donor CD8$^+$ T cells, in contrast to CD4$^+$ Tconv cells, do not considerably contribute to systemic TNF release during aGvHD [167, reviewed in 48]. Thus, we found no evidence that CD28 expression on CD8$^+$ T cells enhances intestinal tissue damage. Still, the clinical score of iCD28KO CD8$^+$ T cell recipients was lower than that of wt CD8$^+$ T cell recipients (Figure 19D). This suggests that CD28-deficient CD8$^+$ T cells cause less pathology in other target organs. The liver tissue for example is prone to Fas-mediated apoptosis through T cells as it expresses high amounts of Fas [168-170]. Upon activation, CD4$^+$ and to a higher extend CD8$^+$ T cells upregulate Fas ligand (FasL) [169, 171]. Optimal FasL expression on T cells further requires CD28 co-stimulation [172]. Therefore, it might be that iCD28KO CD8$^+$ T cells are less cytotoxic towards liver tissue.

In contrast to the expansion of CD8$^+$ T cells in mesenteric lymph nodes, their effector function was not impaired upon CD28 deletion *in vivo* and *in vitro* (Figure 20 and Figure 21C). This has also been described by others previously [173, 166]. In the BCL-1 model we used for our experiments, the GvL effect relies on the Granzyme B and Perforin expression of donor T cells because BCL-1 cells were insensitive to Fas-mediated apoptosis (Figure 21C). We could, however, neither detect a difference in

Granzyme B nor Perforin expression upon CD28 deletion in $CD8^+$ T cells *in vivo* and *in vitro* (Figure 24 and data not shown). Others have shown, that blocking CD28 with the CTLA-4Ig molecule during aGvHD even increases the expression of these cytotoxic molecules, probably because it also abrogates CTLA-4-mediated T cell inhibition [174].

8.4 Selective deletion of CD28 on donor $CD8^+$ T cells provides a means to inhibit aGvHD but maintain the GvL effect

As discussed above (see 8.3), $CD8^+$ T cells need CD28 co-stimulation to optimally proliferate and expand in in the mesenteric lymph nodes, where T cells that are primed to home to aGvHD target tissues. Thus, we observed less signs of aGvHD in iCD28KO $CD8^+$ T cell recipients (Figure 19D).

The GvL effect is mainly mediated in the spleen, where the BCL-1 tumour cells predominantly grow. In contrast to the lymph nodes, wt and iCD28KO $CD8^+$ T cells proliferated equally well in the spleen (Figure 17). Thus, also the GvL effect was similar in recipients of CD28-sufficient and CD28-depleted $CD8^+$ T cells (Figure 22). To answer the question why CD28 deletion had no effect on $CD8^+$ T cell proliferation in the spleen, we analysed the cellular composition of the spleen and found an abundant accumulation of donor derived MHC class II^+ APCs (Figure 17B and D). Due to the early timepoint (day 3 after transplantation) the donor-derived MHC class II^+ cells could not be the progeny of transplanted stem cells but must have been contained in the T cell-depleted bone marrow inoculum. They might represent B cell progenitors, mature B cells or dendritic cells that are found in the bone marrow [reviewed in 175]. How these donor APCs affect CD28-driven proliferation of $CD8^+$ T cells in the spleen is not clear. On one hand, they might act as spacers, hampering the donor T cell/ host APC contacts and generally impairing proliferation in the spleen. Consequently, the effect that CD28 deletion could have on the expansion of the $CD8^+$ T cells is limited in this organ. On the other hand, the donor-derived APCs could secrete pro-inflammatory mediators that compensate for CD28 deficiency on donor $CD8^+$ T cells in the spleen.

Lack of CD28 co-stimulation has been shown to impair or even abrogate $CD8^+$ recall responses in different experimental models [166, 176-178]. While effector T cells predominantly use glycolysis for their energy supply, memory T cells rather depend on

oxidative phosphorylation [reviewed in 179]. Without CD28 co-stimulation during the initial priming, memory $CD8^+$ T cells have a reduced mitochondrial respiratory capacity and cannot mount a sufficient immune response upon re-stimulation [178]. This would suggest that in our aGvHD model, iCD28KO $CD8^+$ T cells would be less efficient in eliminating BCL-1 cells, that are the source of lethal lymphoma around 25 days after transplantation. This is, however, not the case. In contrast, selective CD28 deletion on donor $CD8^+$ T cells even enhanced the median survival of the recipient mice and less mice died due to the tumour burden (Figure 22). Therefore, it is possible that iCD28KO $CD8^+$ T cells receive enough CD28 signal during the first 1-2 days after transplantation to endow them with a latent mitochondrial capacity. Moreover, CD28 deficiency on $CD8^+$ T cells might be compensated by other factors, e.g. cytokines and other co-stimulatory molecules, as we have postulated for $CD4^+$ Tconv cells and Treg cells (see 8.2 and 8.3). Indeed, activated T cells transiently express 4-1BB, which has been shown to be particularly important during secondary immune responses and for expansion of memory $CD8^+$ T cells [180-182]. As BCL-1 cells express high amounts of 4-1BB ligand, it would be possible that they substantially provide co-stimulation, thus contributing to overcoming deficits in memory formation of CD28-deficient $CD8^+$ T cells [183].

8.5 CD28-deficient $CD4^+$ Tconv cells do not provide sufficient T cell help to $CD8^+$ T cells

$CD4^+$ Tconv cells play a crucial role in establishing a protective immune response against infections by providing T cell help to $CD8^+$ T cells [reviewed in 184]. $CD4^+$ T cell help is mediated through the release of IL-2 and the interaction of CD40L with CD40 on APCs. Continuous CD28 expression on $CD4^+$ Tconv cells is critical for their differentiation into T helper cells [157]. Our *in vitro* data show that only CD28-sufficient $CD4^+$ Tconv cells provide optimal T cell help to $CD8^+$ T cells, resulting in maximal expression of effector molecules like Granzyme B (Figure 24). We could not compensate this defective T cell help by iCD28KO $CD4^+$ Tconv cells through addition of external IL-2. Thus, either the dose of IL-2 (10^{-8} M) was suboptimal or CD40-CD40L interactions were predominantly mediating the CD28-dependent T cell help. Indeed, $CD4^+$ T cells express higher levels of CD40L after stimulation with αCD3 and αCD28

antibodies as compared to stimulation with αCD3 mAb alone [185, 186]. In turn, CD40 "licences" APCs to prime cytotoxic CD8⁺ T cells by inducing the expression of CD80 and CD86 on APCs [187, reviewed in 188]. Therefore, CD28 deletion on CD4⁺ T cells indirectly dampens the maturation of CD8⁺ effector T cells (Figure 29).

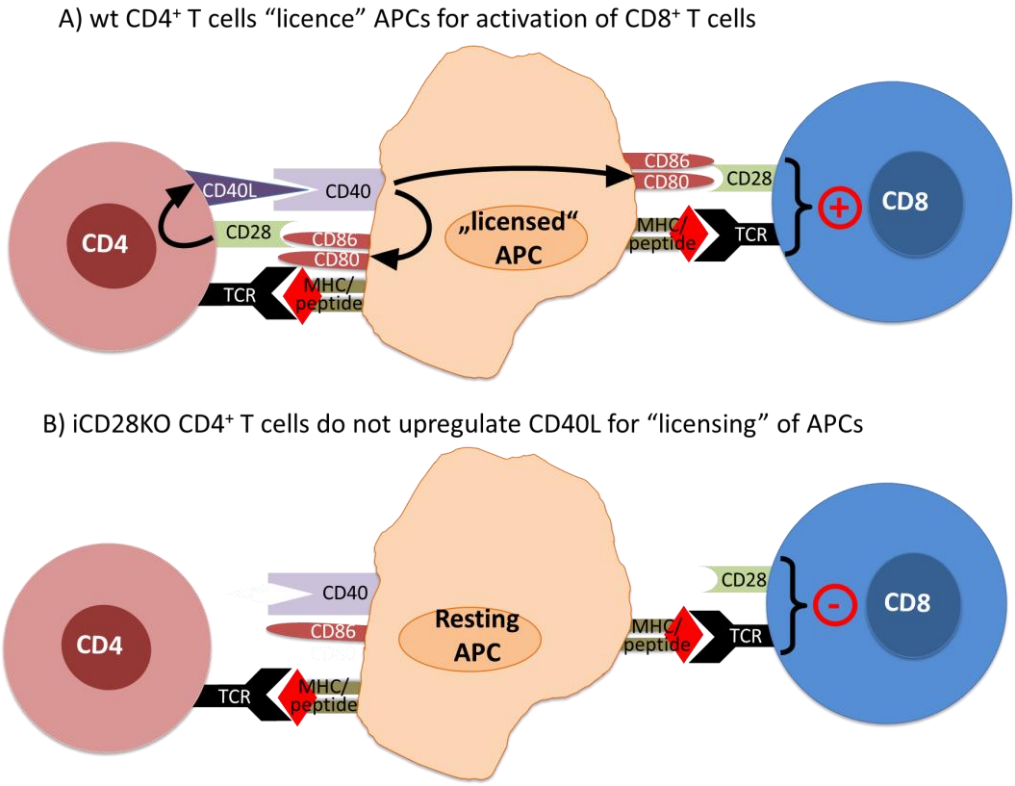

Figure 29: Licensing of APC by CD4⁺ T cells is CD28-dependent. A) CD28 signalling in CD4⁺ T cells induces the expression of CD40 ligand. CD40 signalling in APCs enhances the expression of CD86 and CD80 and licenses the APCs to activate CD8⁺ T cells. B) Impaired upregulation of CD40L in CD28-deficient CD4⁺ Tconv cells prevents licencing of APCs. Based on: [185-187]

Upon CD28 deletion on CD4⁺ donor Tconv cells *in vivo* we observed a loss of the anti-tumour response (Figure 22). This was accompanied by a reduction in CD4⁺ Tconv cell frequencies in the animals, analysed *ex vivo* between day 23 and 120 (Figure 23B). As we had not seen a decrease in CD4⁺ Tconv cell frequencies at day 7 after transplantation (Figure 6C), this suggests, that the initial expansion of CD4⁺ T cells in the inflammatory environment right after irradiation does not depend on CD28. In contrast, memory differentiation and long-term survival of CD4⁺ Tconv cells might require co-stimulation through CD28 and lack of CD28 signalling cannot be compensated by other co-stimulatory molecules, as it could be the case for 4-1BB in

$CD8^+$ T cells [182]. In line with our observations, delayed donor lymphocyte infusions need to contain $CD4^+$ T cells, in order to obtain a sufficient anti-tumour response [50]. Instead, $CD4^+$ T cells were dispensable for the $CD8^+$ T cell-mediated GvL effect when both cell types were transferred together directly after irradiation, when tissue damage results in systemic release of pro-inflammatory mediators [50]. Together with experiments in infectious disease models [189, 190], these observations suggest, that under very inflammatory conditions, when APCs are for example activated by Toll-like receptor signals, $CD8^+$ T cell responses can be independent of $CD4^+$ T cell help. In the GvL model we used, as well as in patients with relapsing leukaemia, the tumour again manifests itself several weeks after transplantation, thus, at a timepoint, when $CD4^+$ T cell help is relevant.

8.6 Late onset aGvHD interferes with the GvL effect

By selective deletion of CD28 on donor Treg cells we have found a means to induce a late flare of $CD4^+$ Tconv mediated aGvHD (Figure 12). We also observed a strong increase in the clinical GvHD score in our GvL model, when we transferred wt $CD8^+$ T cells together with wt $CD4^+$ Tconv cells and iCD28KO Treg cells, (Figure 26B). Even though this correlated, as expected, with a strong anti-tumour response, the mice did not survive better than bone marrow controls because they had to be sacrificed due to severe late-onset aGvHD (Figure 26C and D). Thus, CD28 expression on Treg cells is fundamental for their therapeutic potential in aGvHD patients.

Systemic blockade of CD28, for instance with the pegylated αCD28 Fab fragment FR104, might, however, be beneficial for patients [191]. In our mouse model, 25 % of the mice that had received CD28-deficient donor T cells survived without tumour cells in the spleen. This is in line with published data, where the ablation of CD28 co-stimulation with blocking αCD80/αCD86 antibodies diminished aGvHD but maintained the response against BCL-1 cells [192]. In addition, none of the recipients of CD28 depleted T cells had to be killed because of the severity of aGvHD symptoms in our model. This might provide a therapeutic window where we could further increase the number of transferred iCD28KO T cells without inducing too strong aGvHD. By that, we could eventually further enhance the survival of the recipient mice.

8.7 Clinical relevance and experimental therapeutic strategies

By Tamoxifen-inducible deletion of CD28 we could very precisely define how co-stimulation of different donor T cell subsets affects the GvL effect and aGvHD. As described above, CD28 deletion on all donor T cells seemed to be superior to transplantation of wt T cells because it reduced aGvHD severity and induced long-term survival in 25 % of the recipient mice. Recently, FR104, an antagonistic pegylated αCD28 Fab fragment has been tested in a first clinical trial [193]. FR104 was safe and well tolerated by healthy humans and was able to suppress aGvHD in primates and humanized mice [129, 143, 193]. Even though for safety reasons the doses given to the study participants (max. 1.5mg/kg body weight) were lower than those used in the animal models (5mg/kg body weight), FR104 had an immunosuppressive effect in humans and, therefore, might soon be evaluated in further clinical trials with patients after allogeneic HSCT [193]. Our data suggest, that FR104 treatment would be beneficial for these patients.

In contrast to our experiments, where we deleted CD28 only on donor T cells, systemic CD28 blockade also inhibits CD28 co-stimulation on host cells. One approach to avoid this would be to incubate the donor T cells with blocking agents before transfer into the patients. Recently, *in vitro* experiments have shown that human T cells can be tolerized towards allo-antigens when activated in an MLR culture in presence of FR104 and thereupon are less responsive in a secondary MLR without FR104 [130]. Moreover, in mice, T cells caused less aGvHD symptoms, when they had been tolerized with a murine αCD28 Fab molecule before transplantation [130]. Thus, ex vivo tolerization of donor T cells with FR104 might also be a promising approach in clinical applications.

Systemic CD28 blockade is technically more feasible than selective CD28 blockade on certain T cells subsets. However, our data suggest that CD28 deletion on $CD8^+$ T cells is the best strategy to control aGvHD but maintain the GvL effect. Bispecific antibodies could be a tool to translate this finding from basic research into a therapeutic approach. Bispecific antibodies recognise two distinct epitopes and can either block or activate their target molecules or just bind to them without eliciting a response [reviewed in 194]. The CD47/CD19 antibody for instance has experimentally been used to target B cell lymphomas. It binds to $CD19^+$ (malignant) B cells and blocks the CD47 molecule specifically on those cells but not on $CD19^-$ cells. Thereupon, CD47

can no longer interact with SIRPα on macrophages and other immune cells, which otherwise would protect the tumour cells from being phagocytosed [195]. Accordingly, a bispecific antibody, targeting CD8 and CD28 might be able to block CD28 co-stimulation on $CD8^+$ T cells only. One would, however, need to carefully choose the right αCD28 mAb clone, as some display intrinsic agonistic capacities *in vivo* (see 3.7.2).

All in all, this study contributed to a better understanding of how CD28 blockade can be used in clinical applications to treat and modulate aGvHD. Importantly, our data show that CD28 blockade either on all or only on $CD8^+$ T cells provides a means to inhibit aGvHD without ablating the GvL effect. We further demonstrate that the beneficial effect of Treg therapy is dependent on CD28 expression by the Treg cells.

9 Abbreviations

°C	Degree Celsius
µ	Micro
aGvHD	Acute graft versus host disease
APC	Antigen presenting cell
BM	Bone marrow
BSA	Bovine serum albumin
BSS	Buffered salt solution
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidylester
CTL	Cytotoxic lymphocyte
Ctrl	Control
DAMP	Danger-associated molecular pattern
DAPI	Diamidinphenylindol
DC	Dendritic cell
ddH_2O	Double-desalted water
et al.	Et alteri
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf (bovine) serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead-Box-Protein P3
FSC	Foreward scatter
g	Gram
GvL	Graft versus leukaemia
Gy	Gray
h	Hour(s)
HSCT	Haematopoietic stem cell transplantation
iCD28KO	Inducible CD28 knock-out
IFNγ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
l	Liter
LPS	Lipopolysaccharide

M	Molar
mAb	Monoclonal antibody
MFI	Median fluorescence intensity
mg	Milligram
MHC	Major histocompatibility complex
min	Minute(s)
ml	Millilitre
mLN	Mesenteric lymph nodes
MLR	Mixed lymphocyte reaction
ns	not significant
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered Saline
PE	Phycoerythrin
PerCp	Peridinin chlorophyll
pg	Picogram
pLN	Peripheral lymph nodes
RT	Room temperature
SD	Standard deviation
SPL	Spleen
SSC	Sideward scatter
TCD	T cell depleted
Tconv cell	Conventional ($CD4^+$ $CD25^-$) T cell
TCR	T cell receptor
Th cell	T helper cell
TNF	tumor necrosis factor
Treg cell	Regulatory ($CD4^+$ $CD25^+$ $Foxp3^+$) T cell
w/o	Without

10 References

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11 Appendix

11.1 Acknowledgements

Most of all, I would like to thank PD Dr. Niklas Beyersdorf for his excellent supervision during my time as a master student and PhD student in his lab. I could not have imagined having a better advisor and mentor for my first steps as a researcher. Thank you for your outstanding support, for sharing your immense knowledge and for giving me the opportunity to start my scientific career in your lab.

I wish to thank Prof. Dr. Thomas Kerkau, for his constant support, his precious scientific advice and for carefully reviewing my thesis. I also thank Prof. Dr. Jürgen Löffler for his valuable comments during our thesis committee meetings and Prof. Dr. Dandekar for taking over the chair of my thesis committee.

I want to thank my dear colleagues at the Institute for Virology and Immunobiology in Würzburg. My special thanks go to Dr. Claudia Hollmann for reviewing my thesis and for our great cooperation in all kinds of mouse issues. Thank you, Sandra Werner and Nelli Wolf for teaching me how to handle big experiments and for your fantastic technical support. In the same regard, my gratitude goes to Franziska Seifert, Elena Vogel, Christian Linden and Arthur Berg and the team of the animal facility. I further thank my lab mates Stefanie Haack, Teresa Wiese, Xin Ding, Daniela Langenhorst, Claudia Hahn and all past members of the lab for the good times we had together.

My gratitude goes to the José Carreras Leukämie-Stiftung and the Graduate School of Life Sciences for funding and for their constant support.

I also wish to thank those who made me forget about the science in the evening and on weekends: the people of the TG Höchberg, the SG Eltmann and the Uni Würzburg Judo Team and my good friends Anika, André, Steffen, Janine, Björn and Katja who was thoroughly reviewing my thesis. Thank you also Franzi and Simon for your long-lasting friendship.

I am sincerely grateful to my boyfriend Florian and my family for supporting me in every regard and for always encouraging me in my professional and private life.

11.2 Curriculum Vitae

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Practical experiences

Feb. 2014 - Dec. 2017	University of Würzburg, Institute for Virology and Immunobiology, Master thesis and PhD thesis in the group of PD Dr. Niklas Beyersdorf: "CD28 co-stimulation in a new mouse model of acute graft versus host disease"
Dec. 2013 - Feb. 2014	Fraunhofer Institute for Cell Therapy and Immunobiology, Leipzig, Internship in the group of Dr. Daniel-Christoph Wagner: "Dendritic cell subsets in a murine model of stroke disease"
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Education

Since 2015	University of Würzburg, PhD student of the Graduate School of Life Sciences
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11.3 Publications

Uri A, Werner S, Lühder F, Hünig T, Kerkau T, Beyersdorf N: Protection of mice from aGvHD requires CD28 co-stimulation of donor CD4+ Foxp3+ regulatory T cells. 2017, Front. Immunol. 8:721.

Pösel C, **Uri A**, Schulz I, Boltze J, Weise G, Wagner DC: Flow cytometric characterization of brain dendritic cell subsets after murine stroke. 2014, Exp Transl Stroke Med. 2014; 6: 11.

Langenhorst D, Haack S, Göb S, **Uri A**, Lühder F, Vanhove B, Hünig T, Beyersdorf N: CD28 costimulation of Th1 cells enhances cytokine release in vivo; submitted

11.4 Oral presentations

June 2017: Late-onset acute graft versus host disease is associated with enhanced graft versus tumour activity in a new mouse model; German Meeting of Immunoregulation; Berlin

May 2017: CD28-deficient Treg do not mediate long-term protection from aGvHD in mice; 12th Annual Meeting – Immunology Training Network Meeting of the Universities Erlangen, Tübingen and Würzburg; Kloster Schöntal

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July 2016: CD28 expression by donor CD4^{+} Foxp3^{+} regulatory T cells is required for long-term but not short-term suppression of acute graft versus host disease in mice; 11th Annual Meeting – Immunology Training Network Meeting of the Universities Erlangen, Tübingen and Würzburg; Obertrubach

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Sept. 2017: Annual Meeting of the German Society for Immunology; Erlangen

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Sept. 2015: European Congress of Immunology, Vienna, Austria

July 2015: 10th Annual Meeting – Immunology Training Network Meeting of the Universities Erlangen, Tübingen and Würzburg; Blaubeuren

11.6 Affidavit

I hereby confirm that my thesis entitled “Differential requirement for CD28 co-stimulation on donor T cell subsets in mouse models of acute graft versus host disease and graft versustumour effect“ is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Unterschiedlicher Einfluss der CD28 Kostimulation auf Donor-T-Zell-Populationen in Mausmodellen der akuten Graft-versus-Host Disease und des Graft-versus-Tumor Effekts“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum Unterschrift