Stable Immunosuppression Under Low-dose Tacrolimus Monotherapy is Dependent Upon Immunological Regulation

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vorgelegt von

Anja Nadine Kammler

aus

Straubing

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Prof. Dr. Edward K. Geissler

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Abstract

Allograft acceptance in solid organ transplantation might not be determined by mechanisms unique to the tolerant state but rather by the balance between the effector and regulatory immune response. In consequence, this quantitative view of tolerance implies the existence of marginal states, wherein regulatory responses are just insufficient to prevent rejection, or in which regulatory responses are just sufficient to prevent rejection but are readily disturbed. The presence of low-dose immunosuppression might be supportive in both scenarios and thus, allograft acceptance is promoted. This work aims to formally show that marginal states of allograft acceptance under low-dose immunosuppression exist and are dependent on regulation. Thus, a low-dose Tacrolimus monotherapy was combined with a weak regulation-inducing protocol in the fully mismatched BALB/c-to-C57BL/6 skin transplantation model.

Orally administered Tacrolimus in doses of 150 mg per kg food was therapeutic and prevented allograft rejection when administered before or at the time of transplantation. Tacrolimus at 75 mg/kg proved subtherapeutic when administered in monotherapy. The combination of costimulatory blockade with anti-CD154 antibody and a donor-specific transfusion (DST) led to moderate prolongation of allograft survival. The combination of anti-CD154 + DST and Tacrolimus at 75 mg/kg was not more effective than anti-CD154 + DST treatment alone, when Tacrolimus therapy was started 7 days prior to transplantation. However, when Tacrolimus was introduced seven days post transplantation, a remarkable synergism between the induction therapy and the low-dose immunosuppression could be observed and allograft survival was significantly enhanced. This finding was supported by *in vitro* T reg suppression assays, where effector T cell division is additionally suppressed in the presence of low doses of Tacrolimus.

In line with our hypothesis, it was further demonstrated that in the model of low-dose Tacrolimus in combination with weak-regulation induction, allograft acceptance can be broken. This was done by 1) withdrawal of immunosuppression, 2) enhancing the effector response and 3) disrupting the regulatory response. Thus, it was proven that stable immunosuppression in marginal states of allograft acceptance depends upon the balance of regulatory and effector responses.

The findings of this work have far-reaching implications for patient management, interpretation of immunomonitoring studies and clinical tolerance-induction studies.

Zusammenfassung

Die Akzeptanz eines Transplantats wird möglicherweise nicht durch einzigartige Toleranzmechanismen bestimmt, sondern vielmehr durch die Bilanz aus der Effektorantwort und der regulatorischen Immunantwort. Diese quantitative Ansicht der Toleranz umfasst auch die Existenz von Grenzfällen, wobei die regulatorische Immunantwort gerade nicht ausreicht, um die Transplantasabstoßung zu verhindern; oder wobei die regulatorische Immunantwort zwar gerade eben ausreicht, die Abstoßung zu verhindern, aber ohne weiteres gestört werden kann. Die Gegenwart von niedrig dosierter Immunsuppression kann in beiden Szenarien unterstützend wirken und dadurch möglicherweise die Akzeptanz des Transplantats vorantreiben. Mit dieser Arbeit soll formell gezeigt werden, dass diese Grenzfälle der Transplantatsakzeptanz unter Therapie mit niedrig dosierten Immunsuppressiva existieren und dass sie auf Regulation angewiesen sind. Dazu wurde eine niedrigdosierte Tacrolimus-Monotherapie mit einen schwach-regulationsinduzierendem Protokoll kombiniert und dies im BALB/c-auf-C57BL/6-Hauttransplantationsmodel mit vollständiger Gewebemerksmals-Inkompatibilität angewandt.

Oral verabreichtes Tacrolimus in Dosen zu 150 mg pro kg Futter hatte therapeutische Wirkung und verhinderte die Transplantatsabstoßung, wenn die Therapie vor, oder zum Zeitpunkt der Transplantation gestartet wurde. Als Monotherapeutikum hatte Tacrolimus in Dosen zu 75 mg pro kg Futter keine therapeutische Wirkung. Die Kombination aus Kostimulationsblockade mit dem anti-CD154 Antikörper und einer donorspezifischen Transfusion (DST) führte zu einer moderaten Verlängerung des Transplantatüberlebens. Die Kombination aus der Behandlung anti-CD154 + DST mit 75 mg/kg Tacrolimus zeigte sich nicht effektiver als die Behandlung mit anti-CD154 + DST alleine, wenn die Tacrolimustherapie sieben Tage vor der Transplantation gestartet wurde. Wenn hingegen die Tacrolimustherapie erst sieben Tage nach der Transplantation gestartet wurde, konnte ein bemerkenswerter Synergismus zwischen der regulationsinduzierenden Behandlung und der niedrig dosierten Immunsuppressionstherapie beobachtet werden, wobei das Transplantatüberleben signifikant verbessert wurde. Diese Erkenntnis wurde durch *in vitro* T reg Suppressionsassays bestätigt, wo eine zusätzliche Suppression der Effektor-T-Zellantwort in Anwesenheit von niedrig dosiertem Tacrolimus beobachtet wurde.

In Übereinstimmung mit unserer Hypothese konnten des Weiteren gezeigt werden, dass die Transplantatsakzeptanz im Modell der Kombination von niedrig dosiertem Tacrolimus mit einer schwachen Regulationsinduktion zerstört werden kann. Dies geschah durch: 1) Entzug der immunusppressiven Therapie, 2) Verstärkung der Effektorantwort oder 3) Abbruch der regulatorischen Immunantwort. Damit konnte gezeigt werden, dass die stabile

Immunsuppression in oben beschriebenen Grenzfällen der Transplantatsakzeptanz von der Bilanz aus der Effektorantwort und der regulatorischen Immunantwort abhängt.

Die Erkenntnisse, die im Rahmen dieser Arbeit gewonnen wurden, haben weitreichende Auswirkungen auf das Patientenmanagement, die Interpretation von Studien zur Definierung von Biomarkern und klinischen Studien zur Induktion von Toleranz gegenüber dem Transplantat.

1 Introduction

1.1 Transplantation – an overview

Almost 60 years ago, the first successful human kidney transplantation was performed in Boston by Joseph E. Murray and colleagues. This event was preceded by the work of half a century. Not only did the surgical techniques have to be established in order to transfer tissue or organs, but also unforeseen rejection of the grafts between different individuals had to be overcome. By transplanting between identical twins, Murray could bypass the latter problem. It was the work of several researchers that explained the rejection of grafts. Already in 1912, it was described by Georg Schöne that a second set skin transplant fails more rapidly than the original rejected one. James B. Murphy showed two years later that lymphoid cells were responsible for the destruction of (tumour -) grafts. The same conclusion was drawn by Leo Loeb 20 years later, based upon his rat skin transplant model [1]. Sir Peter B. Medawar, the “father of transplantation” [2], showed in the mid-1940s with controlled and precise experiments on rabbits that skin graft rejection was an immunologic reaction [3]. Snell and Gorer identified the major histocompatibility complex (MHC), the genetically encoded information responsible for the graft rejection [4]. Strategies to reduce the recipients’ immune response were developed in the following years in order to overcome rejection. The combination of refined operation techniques and immunosuppressive treatment opened the door for transplantation as a widely spread therapy for organ failure and dysfunction.

Transplantation is the only curative therapy for end-stage organ failure. This includes end-stage heart failure [5], end-stage renal disease [6], end-stage liver disease [7] and diabetes with end-stage renal failure [8]. By end of June 2013, over 10,000 patients in Germany alone were registered on the Eurotransplant waiting list for solid organ transplantation. During the first half of the year 2013, a total of 1,622 solid organ transplants have been performed in German transplant centres [9]. Despite being a widely-spread live-saving therapy, long-term transplant outcomes are not satisfactory and transplantation remains an experimental field.

1.2 Terms in transplantation

Transplantation in general is the transfer of cells, tissue or organs from a donor to a recipient. In autotransplantation, the donor himself is also the recipient; this for example may be the case in skin transplantation to treat burn. If the recipient is another individual than the donor, the term allotransplantation is used. Here we discriminate three different possibilities:

Syngeneic transplantation describes the transfer of cells, tissue or organs (then called syngraft or isograft) between genetically identical individuals, in humans, this only refers to transplantation between monozygotic twins. Allogeneic transplantation (of an allograft or homograft) is the transfer of cells, tissue or organs between genetically distinct members of the same species. Xenotransplantation is the term used for the transfer of a xenograft between members of two different species.

1.3 Basic concepts

1.3.1 T cell activation

1.3.1.1 TCR signalling

In the 1980's, the structure of the antigen receptor on T cells was characterised [10,11]. The αβ-T cell receptor (TCR) consists of an α and a β chain, that form a heterodimer. The αβ heterodimer forms a TCR complex with the noncovalently associated CD3 and ζ proteins upon binding MHC–peptide complexes [12] (Figure 1).

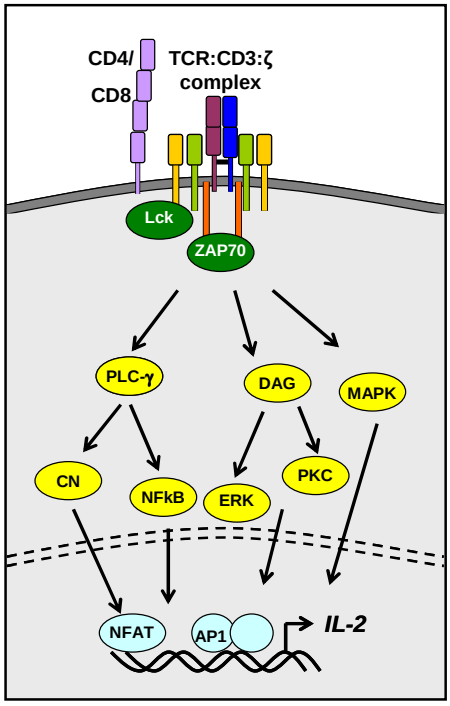

Figure 1: Activation of T cells, adapted from Janeway's Immunobiology [275]

The CD3 and ζ chains contain ITAMs (immunoglobulin receptor family tyrosine-based activation motif) that are essential for the intracellular signalling. The TCR complex clusters with a co-receptor (either CD4 or CD8), thus, the co-receptor associated protein tyrosine kinase Lck phosphorylates the ITAMs in the CD3 and ζ chains. This leads to binding and

activation of the intracellular protein tyrosine kinase ZAP-70, which phosphorylates several other cytoplasmic molecules, including LAT (Linker for Activation of T-Cells) and SLP-76 [13]. Thus, several signalling pathways are activated, such as MAP – kinase pathways, a PLCγ1-calcium - dependent pathway and a Diacylglycerol (DAG) – pathway. These pathways lead to activation of Extracellular-signal Regulated kinases (ERK) or Janus kinase (JAK), Calcineurin (CN) and enzyme protein kinase C (PKC), respectively. This leads to the activation of transcription factors such as NF-AT, NFκB or AP-1. These factors are responsible for the expression of genes required for proliferation, differentiation and effector functions of T cells [14,15].

1.3.1.2 Costimulation

For a functional T cell response, a second activation signal, besides TCR ligation, is necessary. This is transduced by so-called costimulatory molecules [15]. Costimulatory molecules can be grouped into the CD28/B7 family and the tumour necrosis factor (TNF) family. CD28 binds to the B7 molecules CD80 and CD86 on antigen – presenting cells (APCs) [16]. CD28 signalling pathways via phosphatidylinositol 3-kinase (PI3K) amplify TCR signalling pathways [15], and blocking the CD28 signalling whilst TCR signalling is present leads to anergy [17]. The inducible costimulatory molecule (ICOS) is a CD28 homolog that binds to B7h and seems to be important in effector cell differentiation [18]. The CD28/B7 family also includes negative costimulatory (i.e. coinhibitory) molecules such as CTLA and PD-L1. Both have been described as part of the suppressive mechanism of regulatory T cells [19,20]. Members of the TNF / TNFR – family of costimulatory molecules are, amongst others, CD40L (CD154), OX40, 4-1BB (CD137) and GITR. Since costimulatory molecules of the TNF – family are in general expressed upon activation, they may play a role in effector and memory responses rather than in naïve T cell responses [18]. Blockade of different costimulatory pathways in animal models of transplantation has been proven to be successful in prolongation of allograft survival [21]. Further, antibodies against costimulatory moleculed are in use or, considered to be, in the clinic.

1.3.2 T cell specification

The vast majority of T cells express the αβ-TCR. These cells comprise two lineages which are defined by their ability to bind distinct major histocompatibility complexes (MHC). They express either the MHC-class I – binding protein CD8 or the MHC-class II – binding protein CD4. With regard to their functional task within the immune response, they are also referred to as cytotoxic $CD8^+$ cells, and helper or regulatory $CD4^+$ cells [22]. A T cell that has not yet encountered antigen is called “naïve”; after activation, T cells proliferate and can differentiate into effector T cells. $CD8^+$ cells can differentiate into cytotoxic lymphocytes (CTL) which

mainly kill infected cells and contribute in allograft rejection. There are several distinct subsets of effector $CD4^+$ cells, mainly T_H1, T_H2 and T_H17 helper T cells and regulatory T cells (T reg). Differentiation into a distinct subset occurs in response to the present cytokine milieu and involves both transcriptional activation and epigenetic modification of target genes [23] (Figure 2). Each of these subsets has a special cytokine profile and expresses specific transcription factors [24].

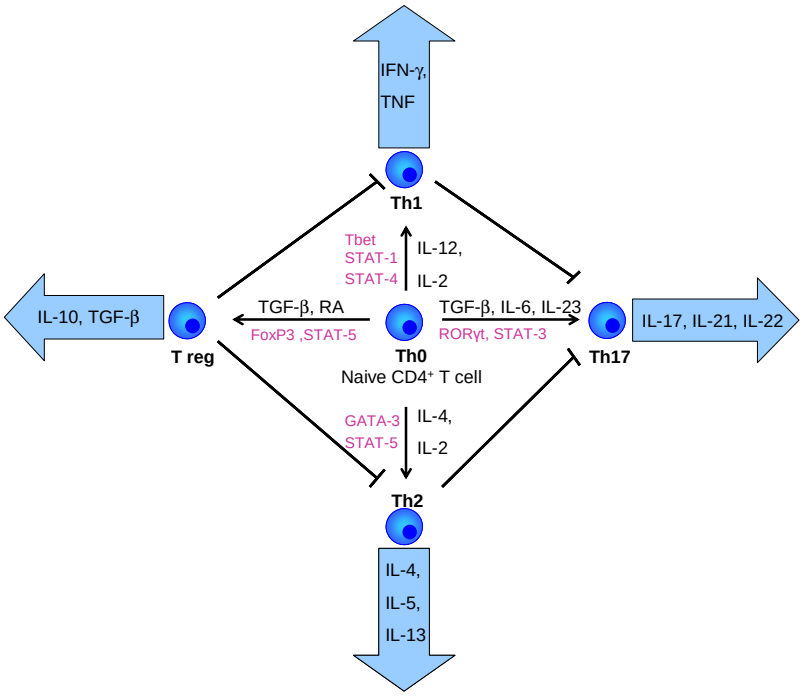

Figure 2 T cell polarisation

During the last years, there is increasing evidence that the differentiation into different T cell subsets is not terminal. In *in vitro* and *in vivo* studies, a T cell plasticity within and between the helper and regulatory cell subsets could be shown [23].

1.4 Allorecognition

The underlying genetic basis of graft rejection after allogeneic transplantation (or allotransplantation) was studied in the 1940s by Snell and Gorer. Using congenic inbred mouse strains, one region in the genome could be identified that was responsible for rejection of an allograft, the major histocompatibility locus, named H-2. Later it was found that the locus consists of multiple genes; therefore it was named *major histocompatibility complex* (MHC) [4]. The MHC in human was called *human leukocyte antigen* (HLA). The genes of the MHC code for the antigen-presenting MHC molecules, of which there are two classes: MHC class I and class II; respectively. There is also a third class of MHC genes

(MHC class III) that encode complement proteins or cytokines such as TNFα, but not all are involved in immune funtions. The MHC class I and II molecules serve the same process, which is antigen presentation to T cells. Without processed antigen being presented to them in MHC context (together with costimulation), T cells cannot be activated. MHC class I molecules present antigenic peptides of intracellular origin to $CD8^+$ T cells, whereas $CD4^+$ T cells recognize exogenous antigens presented on MHC class II molecules (Figure 3).

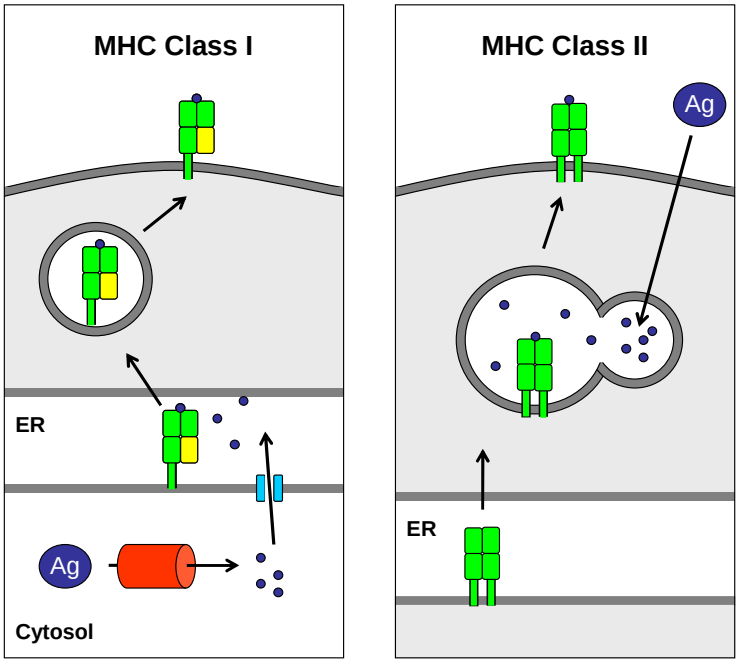

Figure 3 Major histocompatibility complex: antigen presentation, adapted from Janeway's Immunobiology [275]

Every nucleated cell type expresses MHC class I molecules, but the expression of MHC class II molecules is generally limited to antigen-presenting cells (APC), though it can be induced in other cells such as endothelial cells or fibroblasts [25].

The ability of individual organisms to discriminate self- from non-self-antigens is known as allorecognition. It describes the process of recipient cells recognizing foreign antigen presented on a MHC, as it inevitably happens in allo- and xenotransplantation. During development in the thymus, T cells undergo positive and negative selection. T cells that bind with too high affinity to self-MHC, or do not bind to self-MHC will be deleted. The T cell repertoire is then tolerant towards self-antigens, but recognizes non-self antigens. So far, three different pathways of allorecognition have been described: 1) direct, 2) indirect and 3) semidirect allorecognition.

1.4.1 Direct Allorecognition

The process of recipient T cells recognizing antigen presented via intact donor MHC on donor APC (here: dDC) is termed direct allorecognition (Figure 4).

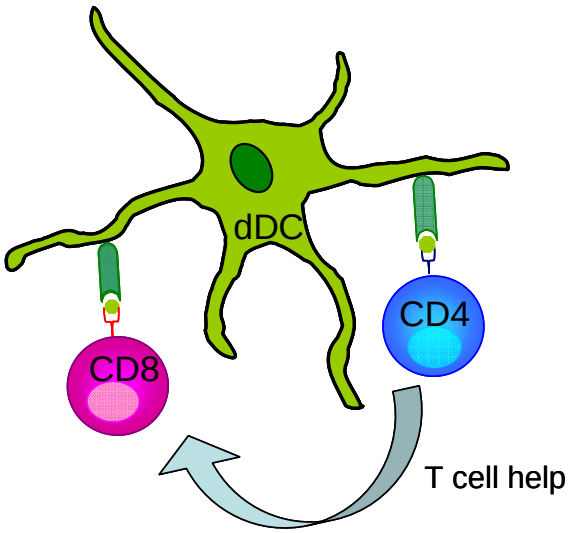

Figure 4 Direct allorecognition

Two theories have been brought up to explain the underlying mechanisms of the interaction between host T cell receptor (TCR) and donor MHC: the “high determinant density” model and the model of “multiple binary complexes” [26,27]. Briefly, the former theory holds it that the T cells can directly recognize the allogeneic MHC itself and not only peptides bound to the MHC [26-28]. In consequence, the density of ligands for alloreactive T cells is very high in opposition to the density of peptide-specific ligands. Therefore, T cells with low-affinity receptors are also able to respond to foreign MHC, which leads to the high incidence of alloreactivity observed. The second theory of “multiple binary complexes” suggests that the complex of a variety of bound peptides together with an allogeneic MHC is recognized by alloreactive T cells. Subsequently, a single MHC molecule can stimulate many different alloreactive T cells [29,30]. Both theories are not mutually exclusive and may account for the high incidence (up to 7%) of alloreactive T cells described in the literature [31]. It has been proposed that in the case of structurally different MHC molecules between donor and recipient, the alloreactivity is directed against the MHC itself (“high determinant density”), whereas the alloreactivity against the peptides in an allogeneic MHC complex (“multiple binary complexes”) may be predominant when the MHC molecules do not differ substantially [32].

Experimental proof of the participation of direct allorecognition in rejection has been given by depleting the graft of donor APC prior to transplantation. This leads to prolonged allograft survival [33], yet is eventually not sufficient to prevent rejection (see below). Since the intact

donor APC that must be present in the graft and host to elicit direct allorecognition, will be eliminated by the host's immune response, the contribution of this pathway is temporarily limited.

1.4.2 Indirect Allorecognition

T cells can also recognize donor histocompatibility antigen that is processed and presented by self-MHC (here: rDC) molecules; which is referred to as the indirect pathway of allorecognition (Figure 5).

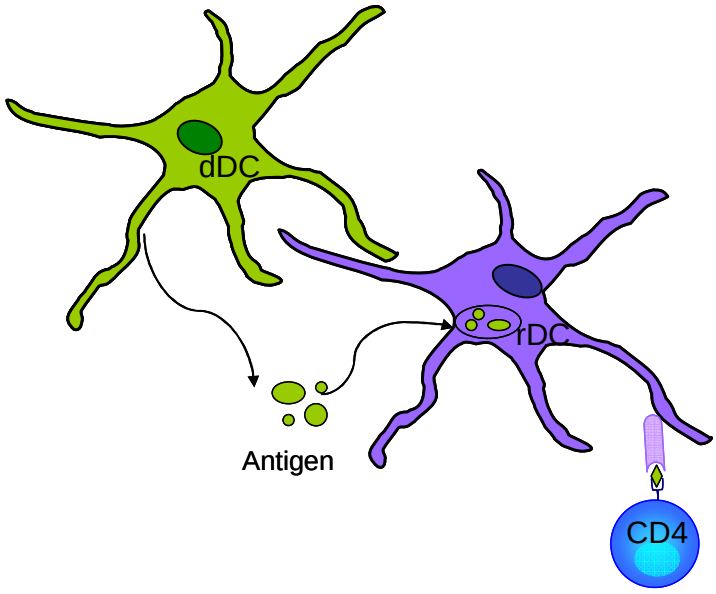

Figure 5 Indirect allorecognition

The processing of peptides derived from donor MHC molecules is a common event [34,35]. This occurs when apoptotic donor cells are taken up by host antigen-presenting cells. Additionally, the peptides can be shed from the surface of donor cells (here, a dDC is shown as an example). The existence of an indirect way of alloantigen presentation came into focus in a rat transplantation model. Here, after depletion of passenger donor APC in the graft, rejection did eventually occur [36]. The importance of this second pathway of allorecognition was demonstrated by Auchincloss et al. in a skin transplantation model. $CD8^+$- depleted mice without MHC class I molecules were able to reject a MHC class II deficient skin graft via a $CD4^+$ response. Since $CD4^+$ cells do not interact with MHC class I molecules (the remaining MHC class in the graft), the donor antigens must have been presented by host MHC class II [37].

This aforementioned data proves the sufficiency of an indirect allorecognition to cause graft rejection in the absence of direct allorecognition. Host dendritic cells are constantly trafficking

in the body and also in the graft itself, which expresses donor MHC molecules. Therefore, indirect allorecognition that can occur every time after transplantation may mount an immune response leading to rejection. Thus, the indirect pathway is probably the dominant way of allorecognition in the long term.

1.4.3 Semi-direct Allorecognition

Experimental data indicated that T cells with indirect allospecifity can amplify or suppress T cells with direct allospecifity [38-40]. This phenomenon has been first explained by a four-cell, unlinked, model: $CD4^+$ helper or suppressor T cells interact via the indirect pathway with recipient DC, whereas $CD8^+$ cells directly recognize donor cells. Work of several groups showed the ability of DC to acquire intact MHC molecules from other cells *in vitro*, which was then further investigated by Herrera *in vivo* [41]. A third pathway of allorecognition was described then, the semi-direct allorecognition (Figure 6).

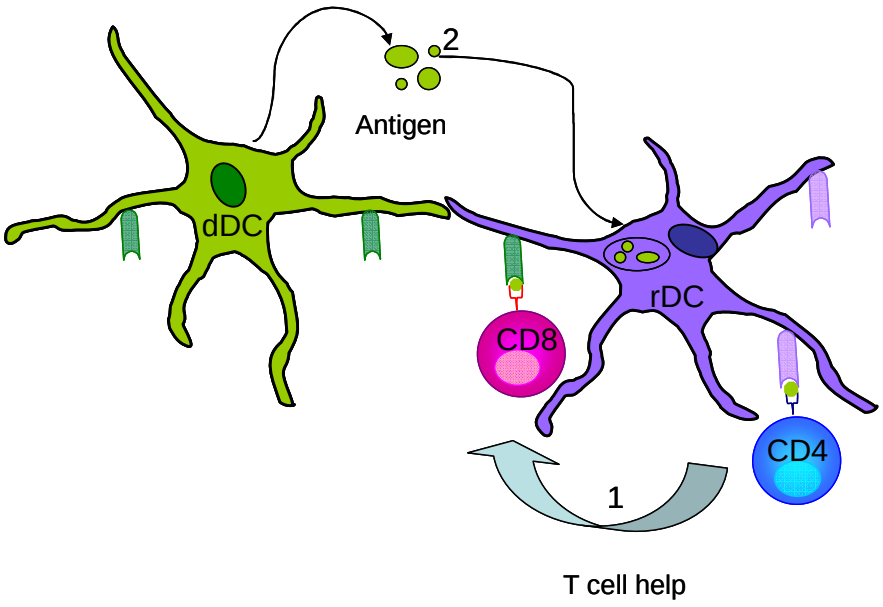

Figure 6 Semidirect allorecognition

Semi-direct allorecognition refers to direct pathway T cells recognizing intact, allogeneic MHC:peptide complexes that have been transferred from donor cells to recipients DC and are presented on their surface. Additionally, indirect pathway can recognize donor peptides that were internalised and are presented via the MHC class II on the same DC.

1.5 Rejection

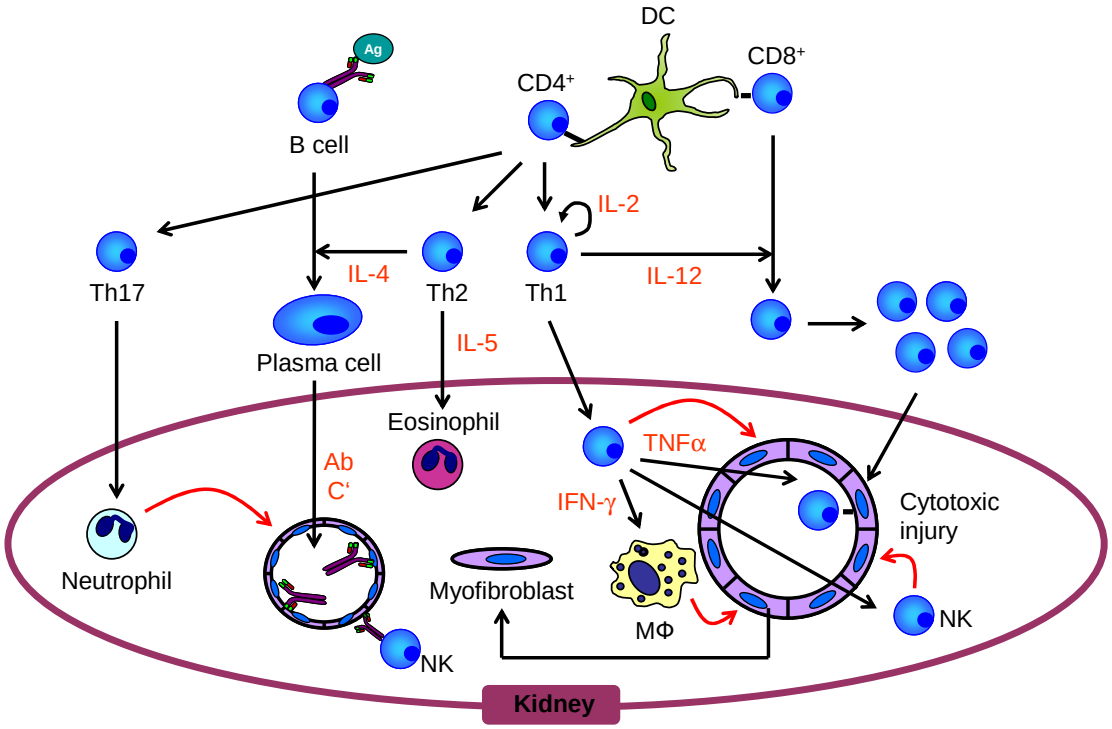

Figure 7 Rejection of an allograft

1.5.1 Hyperacute rejection

Hyperacute graft rejection occurs usually within minutes after transplantation, when preformed recipient antibodies bind to donor endothelial ABO or major histocompatibility (MHC) antigens. This can activate the complement cascade or mediate antibody-dependent cellular toxicity (ADCC), leading to damage of endothelial cells, culminating in intra-vascular thrombosis and tissue injury [42,43]. Hyperacute rejection inevitably leads to allograft loss, but occurs nowadays rarely due to pre-transplant bloodtyping and T cell crossmatch testing [44]. Hyperacute rejection also prevents interspecies transplantation (Xenotransplantation).

1.5.2 Acute rejection

Acute rejection occurs usually within days or weeks after transplantation. Even when the recipient receives immunosuppressive treatment, acute rejection episodes can occur repeatedly. Acute rejection can be cellularly (lymphocytes) or humorally (antibody) mediated. Every transplantation leads inevitably to tissue damage and thus to activation of the innate immune system. Innate immune cells such as neutrophils, macrophages and natural-killer cells (NK cells) express pattern recognition receptors (PRR) which recognize damage-associated molecular pattern molecules (DAMP) [45,46]. This antigen-independent

inflammatory response can promote further injury, e.g. caused by the production of tissue-damaging molecules such as reactive oxygen species (ROS) or nitric oxide (NO) [47]. The cells of the innate immune system produce cytokines, such as IFNγ, IL-6 or IL-12, leading to the activation of the adaptive immune system [48,49]. Work by Chalasani et al. indicated that the innate immune response towards the graft is necessary for an effective adaptive, antigen- dependent, immunity [50]. Dendritic cells (DCs) are considered to be the link between innate and adaptive immunity [51]. Immature DCs traffick through non-inflamed tissues, but exert immunogenic effects, when they receive a maturation signal. This can be provided by DAMPs following transplantation. Activated DCs migrate to the lymphoid tissue, presenting antigen to T cells, stimulating the adaptive antigen response [52]. There is also data demonstrating the influence of chemoattractants from neutrophils and macrophages on the optimal recruitment of T cells to the allograft [53].

Among the infiltrating cells in an acutely rejected allograft, T cells indeed build up the largest population [54,55]. Based mainly on experience from animal experiments, acute rejection is seen predominantly as a T cell mediated process. Athymic or neonatally thymectomized mice fail to reject MHC-mismatched skin grafts unless reconstituted with T cells from untreated syngeneic wildtype mice, after which the transplants will be rejected rapidly. T cells contribute to graft rejection in various ways after activation [56,57]. The release of proinflammatory cytokines (IL-1, IFNγ, TNF) triggers further graft infiltration by macrophages, monocytes, eosinophils and others, which promotes additional antigen-independent cytotoxicity. But also T cells, mostly MHC class I restricted $CD8^+$ T cells, can secrete cytotoxic molecules thus inducing apoptosis of the target cells. Cytotoxic T cells lyse target cells via two distinct mechanisms, the perforin/granzyme pathway or the Fas/FasL pathway [58]. It could be shown that $CD4^+$ T cells, but not $CD8^+$ T cells are essentially required to initiate rejection of an allograft [59]. $CD4^+$ T cells, mostly T_H1 helper cells, mediate delayed-type hypersensitivity responses (DTH) in an antigen-specific manner [60].

Activated $CD4^+$-T cells can also provide B cell help by cytokine production and expression of costimulatory molecules. B cells can act as APC for T cells, secrete inflammatory cytokines / chemokines and produce alloantibodies [61,62]. B cell infiltration was reported in acute rejection episodes of human kidney, liver and heart [61,63-65]. Studies on B cell-deficient µMT mice were performed, showing that B cells and antibodies are not required for acute rejection [66-68]. However, there is data indicating a significant role of B cells in acute rejection. Impaired indirect alloantigen presentation by B cells was followed by reduced antibody production and $CD4^+$-T cell division in murine cardiac transplantation [61]. Depletion of B cells using the anti-CD19 or anti-CD20 antibodies aggravated or ameliorated rejection

depending on the organ transplanted and the intensity of the rejection [69]. Further, antibodies produced by B cells can induce complement and therefore lysis of graft cells. If the Fc receptor on NK cells or macrophages recognizes antibodies, this can lead to antibody mediated cellular cytotoxicity (ADCC) mounting in apoptosis of the graft cells. The aforementioned effector mechanisms of cytotoxicity, DTH, Lysis and ADCC will then lead to a rapid rejection of the transplanted tissue.

1.5.3 Chronic Rejection

Chronic rejection refers to chronic allograft injury mediated by immunologic factors in contrast to other mechanisms such as drugs, ageing or infection. The chronically rejecting organ displays vasculopathy with distinct histologic injury depending on the type of solid organ [70,71], leading to diminished function and eventually loss of the organ. In general, chronic rejection is influenced by both alloantigen-dependent and alloantigen-independent mechanisms [70]. A rat kidney-retransplantation model established by Tullius et al. [72] demonstrated that early immunohistological changes in the chronic rejected allograft during the first 12 weeks are predominantly antigen-dependent and can be reversed by retransplantation back to the donor strain. In later retransplanted isografts, fibrotic injury continued to progress and even isografts did display immunohistological alterations similar to chronic rejection [73], indicating that late events in chronic rejection are antigen-independent.

Grafts undergoing chronic rejection display perivascular inflammation and fibrosis [74,75]. The pathognomonic lesion in chronic rejection is obliterative arteriopathy (OA), caused by fibrointimal hyperplasia [71]. The initial damage of endothelium and exposure of collagen causes repair mechanism involving fibrin and other clotting proteins and platelets [70,71,76]. Various factors such as platelet activating factors (PAF), platelet-derived growth factor (PDGF), tumour necrosis factor (TNF), leukotrienes and thromboxane are released, which can also lead to induced proliferation and migration of smooth muscle cells (SMC) [76-78]. The endothelial activation comes together with the upregulation of MHC II and adhesion molecules, supporting the infiltration of leukocytes [79-82]. The microscopic picture in the initial stage in rodents shows that monocytes/macrophages and T cells are predominant, but also eosinophils, plasma cells, DCs, B cells and mast cells were found [75,76,83-86]. T cells and macrophages build the arterial inflammatory response, with the lymphocytes attached to the intima and the macrophages permeating adventitia, media and intima of the vessel [71,79,86,87]. At this stage, cytokines such as tissue growth factor beta (TGF-β), interferon-γ (IFN γ), IL-1 and tumour necrosis factor (TNF) are expressed in the graft, as well as the chemoattractants RANTES (CCL5) and monocyte chemotactic protein-1 (MCP-1) [83,84,88]. This leads to further attraction of macrophages/monocytes and T cells, that in turn produce

more cytokines and chemoattractants. PDGF, released by endothelial cells, SMC, platelets and activated macrophages, and TNF, released predominantly by activated macrophages, stimulate the proliferation of SMC and their release of extracellular matrix proteins [76,89,90]. TGFβ activates extracellular matrix deposition and is expressed in grafts undergoing chronic rejection [70,91,92]. It has been demonstrated that TGFβ contributes to fibrosis, e.g. by upregulating connective tissue growth factor (CTGF) in fibroblasts and SMC, which has mitogenic effects on fibroblasts [93,94]. Häyry [95] communicates following hypothesis that is supported by many data including the above mentioned: SMC replicate in autocrine or paracrine response to the cytokines and growth factors. Extracellular matrix expressing metalloproteinases and proteolytic enzymes contribute to the migration of SMC to the intima, where they start remodelling the vascular wall. This leads to arterial narrowing and occlusion of small vessels, followed by damage of the surrounding tissue due to ischemia and fibrotic changes.

1.6 Regulatory immune cells in Transplantation

Whenever there is an activation of the immune system, i.e. an inflammatory process, there is also a regulatory response to control inflammation and thus prevent the host organism from damage. This is also true in transplantation. Indeed, the immune cell populations leading to rejection of an allograft also harbour regulatory cells that can suppress the effector response. These specialised cells either underwent selection processes for regulatory function or were driven into a regulatory phenotype on site (of the allograft). Other mechanisms to regulate the immune response are: ignorance, anergy and deletion [96]. Ignorance simply refers to T cells ignoring antigen, either because T cells cannot enter the sites where the antigens are expressed (immuno-priviledged sites) or because the antigen signal does not overcome the threshold to lead to a T cell response. T cells can also be rendered anergic, i.e. non-responding to further stimulation. This happens when the TCR is stimulated without adequate costimulation and signalling through alternative receptors occurs. Deletion of T cells does not only take place in the thymus (central deletion), but also in the periphery. Antigen-reactive T cells can be depleted by activation-induced cell death (AICD) upon restimulation of the TCR with signalling through other receptors such as Fas. This might occur in $CD8^+$ T cells that are activated without $CD4^+$ T cell – help [96,97].

In their ground-breaking publication in 1953, Billingham, Brent and Medawar already suggested that leukocytes able to suppress allospecific immune responses do exist [98]. Since then, different regulatory cell populations have been reported, such as regulatory T cells, B cells and macrophages, tolerogenic DCs and myeloid-derived suppressor cells

(MDSCs). The presence of these cells in a transplant recipient can promote acceptance of the allograft.

1.6.1 Regulatory T cells

Various T cell populations with regulatory function in transplantation have been discovered, including $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ regulatory T cells (Figure 8).

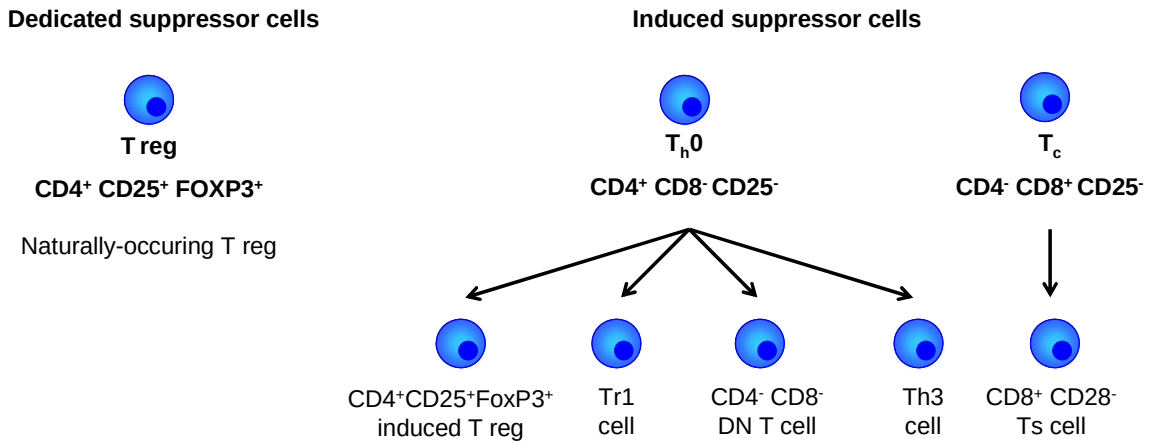

Figure 8 Regulatory T cell subsets

1.6.1.1 $CD4^+$ regulatory T cells

Naturally occurring, thymus-derived $CD4^+$ regulatory T cells (T regs) form a self-renewing, actively dividing and differentiated population and maintain tolerance in the periphery, mainly against self-antigens [97]. They are characterised as $CD4^+CD25^+FoxP3^+$ cells, as well as the population of induced T regs. In mice, FoxP3 is expressed exclusively on T cells and it is necessarily and sufficiently responsible for suppressive function in $CD4^+$ T regs [99-101]. In addition, T regs constitutively express high levels of the costimulatory molecule glucocorticoid-induced tumor necrosis factor receptor (GITR) [102]. GITR is described to enhance proliferation of both T regs and effector T cells [103]. Altogether, induction of GITR signalling has been shown to abrogate T reg suppression [104].

$FoxP3^+$ T regs highly express both PD-1 (programmed death receptor) and PD-L1 (programmed death receptor – ligand 1), a major coinhibitory receptor - ligand team [105]. PD-L1 was found to have a major role in the induction and maintenance of induced T regs, thus promoting tolerance [20]. Induced T regs are $CD4^+$ T cells that inducibly express FoxP3 and differentiate after encountering antigen in a tolerogenic microenvironment, thus are converted from potential effector T cells. Both naturally occurring and induced T regs can

respond to alloantigens in a graft-protecting way. Thymus-derived T reg might be predominantly present in the initial period after transplantation, later, induced T regs probably play a more important role [106,107].

T regs (thymus-derived and induced) suppress proliferation and / or activation of naïve and memory $CD4^+$ and $CD8^+$ effector T cells, B cells, and the function of NK cells, macrophages and DCs [108,109]. They can exert their effects via cell-contact dependent mechanisms. Via binding of the cytotoxic T lymphocyte antigen 4 (CTLA4), a receptor constitutively expressed on T regs, to the costimulatory molecules CD80 and CD86 on DCs, their activity can be inhibited [110]. Further, this can induce the production of the enzyme indoleamine 2,3-dioxygenase (IDO) by DCs, which, due to tryptophan deprivation, leads to attenuated T cell proliferation [108]. T regs themselves produce cytokines such as IL-10 and TGFβ. Interleukin-10 is an anti-inflammatory cytokine produced by many cells of the adaptive and innate immune system. In *in vivo* models of inflammatory bowel disease and transplantation, blockade or absence of IL-10 prevents T reg – mediated regulation [111,112]. TGFβ is a cytokine that is important for the development of induced T regs and in fact might be produced in part in an autocrine fashion [113]. It can be also expressed on the cell surface of activated T regs. TGFβ produced by T regs can inhibit the activation of effector T cells [114]. Another, more recently described, mechanism of suppression by T regs could be by IL-35 secretion [115]. However, the roles of these cytokines as suppressor mechanism is not completely clear, since in vitro data is often controversial [116]. Other mechanisms that have been described are cytotoxic activity of T regs via granzyme B and perforin [117] or apoptosis – inducement due to IL-2 depriviation [118].

In addition to T regs (thymus-derived and induced), other $CD4^+$ regulatory T cells have been described, such as T_R1 cells. These are distinct peripherally induced regulatory T cells that are negative for FoxP3 - expression, develop in response to IL-10 and can secrete IL-10 and TGFβ [119]. Further, TGFβ – producing T_H3 cells have been described [116].

1.6.1.2 $CD8^+$ regulatory T cells

$CD8^+CD28^-$ have been described in human kidney transplant patients after leukocyte depletion treatment. They use cell contact mechanisms to inhibit T cell activation via APCs and seem to be a distinct subset from a IL-10-producing $CD8^+$ T cell population [107]. The latter can be generated in vitro and can inhibit T cell responses through IL-10.

1.6.1.3 $CD4^-CD8^-$ regulatory T cells

Cells that express CD3 and the αβ-TCR, but neither CD4 nor CD8 (or the NK cell marker NK1.1) are so-called double-negative T cells. This immunosuppressive population has been first described in a mouse skin transplantation model, where graft survival could be enhanced. The suppressive mechanism shown was cell-contact - and Fas – dependent killing of $CD8^+$ cytotoxic T cells [120]. This cell population has also been described in animal models of diabetes and graft-versus-host-disease and could be isolated from human blood. In further experiments, double-negative regulatory T cells also had suppressive effects on $CD4^+$ T cells, B cells, and APCs [121].

1.6.2 Regulatory B cells

B cells in transplantation may have more than an antibody-producing role. Regulatory B cells secreting IL-10 have been described as immunosuppressive in models of autoimmunity such as experimental autoimmune encephalomyelitis (EAE), IBD, arthritis and diabetes [122]. Further, they were also detectable in human patients [123]. B regs have been described to induce populations of regulatory T cells in animal models of colitis and EAE [124]. CD40 and CD80/86 engagement is necessary for the establishment and/or function of B regs [125]. In a mouse transplantation model, hindered IL-10 production of regulatory B cells does not interfere with tolerance induction. It rather is suggested to be dependent on direct interaction between B cells and target cells [126]. In human kidney transplant patients after CD52 – (lymphocyte / monocyte) depletion, the repopulating B cells had B reg and transitional B cell phenotypes [127]. Transitional B cells are poor costimulators and thus may lead to T cell unresponsiveness [128]. Interestingly, the presence of naïve and transitional B cells after transplantation is associated with a positive outcome and a B cell gene signature was described in immunosuppressive-free patients with maintained graft function (operationally tolerant). Such B cell related gene markers were *Cd20*, *Ms4-a1* and *Fcrl1* [129,130].

1.6.3 Regulatory macrophages

Macrophages are activated quickly upon tissue damage as it occurs in transplantation, as already mentioned earlier. But macrophages do not only promote graft damage, they can also contribute in wound healing. Macrophages are often classified into two groups, the classically activated M1 – macrophages and the alternatively activated M2 – macrophages [131]. Further, regulatory macrophages have been described. Genome microarray studies on M regs induced *in vitro* from mouse and human monocytes show that these macrophages have a gene expression profile different from M1 and M2 polarised macrophages

[132](Hutchinson, unpublished data). Markers for these mouse M regs are typical macrophage – markers such as CD11b, F4/80, CD68 and CD14. Further these M regs express only intermediate levels of MHC II and low levels of costimulatory molecules CD80, CD86 and CD40. Further, they express PD-L1 and CD11c [132]. A variety of different stimuli has been shown to induce suppressive function of macrophages. Amongst these are M-CSF, IL-10, vitamin D, IFNγ, immune complexes and repetitive TLR stimulation, reviewed in [133]. Thus, no unique phenotype can be described for suppressor macrophages. IL-10 secretion may be one mode of action of regulatory macrophages [134]. Further, production of iNOS in mouse M regs or IDO in human M regs, has been described [132](Hutchinson, unpublished data). By these means, M regs may directly inhibit activation and proliferation of effector T cells. In addition, this leads to a microenvironment that can promote induction of regulatory T cells (Walter, unpublished data). M regs have already been used as cell therapy in kidney transplant patients [135], and interestingly, these two patients are maintained on additional immunosuppressive therapy at unexpectedly low doses.

1.6.4 Tolerogenic DCs

Mature dendritic cells can efficiently activate T cells and improve memory T cell responses. In steady state conditions, DC found in the peripheral lymphoid tissue are not fully mature. In order to achieve immunity, the antigen needs to be coadministered with a maturation stimulus [136]. If the antigen is delivered without a maturation signal, the immature DCs will engage T cells, but this lead to unresponsiveness [137]. It has also been shown that injection of *ex vivo* antigen.pulsed DC under the absence of maturation signals leads to downregulation of the immune response and induction of T regs [138]. Further, DCs might promote tolerance in response to tolerogenic signals such as IL-10 and TGFβ or to signals coming from T regs [109,139]. As already mentioned previously, IDO is one of the mechanism by which tolerogenic DCs can suppress T cell responses [139]. In addition, tolerogenic DCs inhibit T cells via IL-10 or heme oxygenase 1 (HO-1) [140,141]. Immature myeloid tolerogenic DCs can promote allograft acceptance in solid organ transplantations [142]. It has also been described that the population of plasmacytoid DCs (pDCs) which express more PD-L1 correlate with increased numbers of T regs in liver transplant patients [143]. This induction of T regs by pDCs has also been observed in animal models of transplantation [144].

1.6.5 Myeloid - derived suppressor cells (MDSCs)

MDSCs are a heterogeneous population of myeloid progenitor cells present in tissues during inflammation. They were first described in cancer patients, and now their

immunosuppressive function has been acknowledged in other diseases and transplantation [145]. Several subsets of MDSCs have been defined in both human and mouse. Common phenotypical markers of mouse MDSC subsets are expression of CD11b and Gr1 [146]. Activated T cells, stromal cells and, in cancer, tumour cells produce factors such as macrophage–colony stimulating factor (M-CSF), granulocyte-macrophage-CSF (GM-CSF), IL-6 or prostaglandins that regulate expansion and activation of MDSCs [146]. Upon activation, MDSCs can inhibit T and B cell responses by production of iNOS and arginase 1 [147,148]. Release of reactive oxygen species (ROS) is also part of the suppressive function of MDSCs [145]. In a murine skin transplantation model, MDSCs producing IL-10 and HO-1 did prolong allograft survival [149]. It has been shown that MDSCs can induce T regs [150] and in murine islet transplantation, this enhancement is mediated by expression of PD-L1 [151].

1.7 Immunosuppressive treatment in Transplantation

1.7.1 Overview

The first drugs successfully used to prevent acute rejection in transplantation between non-identical individuals were steroids (cortisone) and Azathioprine, a chemotherapy drug found to be effective in kidney transplantation in the early 1960s [152]. Azathioprine inhibits *de novo* purine synthesis and has an anti-proliferative effect on T and B lymphocytes [153]. The immunosuppressive therapy in transplantation could be expanded years later when the calcineurin-inhibitor Cyclosporine was introduced in the clinic in 1978 [154]. Thus, the one year survival time of an allograft increased dramatically [155]. In the following, more immunosuppressive drugs have been introduced into transplantation. In 1982, the type 2 isoform inosine 5'-monophosphate dehydrogenase (IMPDH) inhibitor mycophenolate mofetil (MMF) was developed. Studies showed that MMF is, in contrast to Azathioprine, more lymphocyte-specific and more effective in preventing graft rejection [156,157]. Thus, and because it is effective in combination with other immunosuppressants, MMF has largely replaced Azathioprine in the clinic [158].

In 1986, a new calcineurin-inhibitor (CNI) called Tacrolimus, was discovered and found to be more potent than Cyclosporine. The mechanisms of action of CNI will be discussed below. In 1989, the immunosuppressive properties of Rapamycin (Sirolimus), a microbial product with structural similarity to Tacrolimus, were further tested in transplantation models [159]. Rapamycin binds the same protein as Tacrolimus, but does not inhibit calcineurin. It acts on the mammalian target of Rapamycin (mTOR), thus leads to cell-cycle arrest in T cells [160].

In general, immunosuppressive therapy includes glucocorticoids and small-molecule immunosuppressive drugs as the drugs mentioned above. A third group are protein immunosuppressive drugs including fusion proteins such as CTLA-4-Ig, depleting antibodies and non-depleting antibodies [158].

Antibodies as induction therapy have been used since the early 1980's. Anti-Thymocyte globulin (ATG) and Campath-1H (Alemtuzumab) are widely used antibodies depleting T and B cells (and the latter to a lesser extend NK cells, monocytes and macrophages). Basiliximab is a non-depleting anti-IL2R antibody inhibiting lymphocyte proliferation. Further protein immunsuppressive drugs are developed (e.g. non-depleting CD40L antibodies ASKP1240 or 4D11) or in use (e.g. CTLA4-Ig) for blockade of the costimulatory CD40/CD40L or the CD28/CD80/CD86 pathways [21].

The common therapy protocol in transplantation includes an antibody such as Basiliximab with higher doses of CNI in the induction phase with an additional anti-proliferative drug (MMF) and tapered steroids. The maintenance phase then is based mostly on the CNI, with possible addition of MMF or Rapamycin to reduce CNI doses and toxicity [161,162]. Since CNIs as Cyclosporine and Tacrolimus are the basis of current standard immunosuppressive therapy, they will be described in more detail [162].

1.7.2 Calcineurin Inhibitors

1.7.2.1 Cyclosporine (CsA)

Cyclosporine is a fungal metabolite discovered in a screening program for immunosuppressive agents in 1972 [154]. Cyclosporine is a calcineurin-inhibitor that inhibits T cell proliferation and was introduced in the clinic by Sir Roy Calne six years later [163]. Since then, it has been used in transplantation and is in use until now. Its mechanism of action will be described below in context with another CNI.

1.7.2.2 Tacrolimus (FK-506)

Tacrolimus is a macrolide lactone ($C_{44}H_{69}NO_{12}$) that could first be isolated from *Streptomyces tsukubaensis* – cultures in Japan in the mid-1980's (Figure 9) [164].

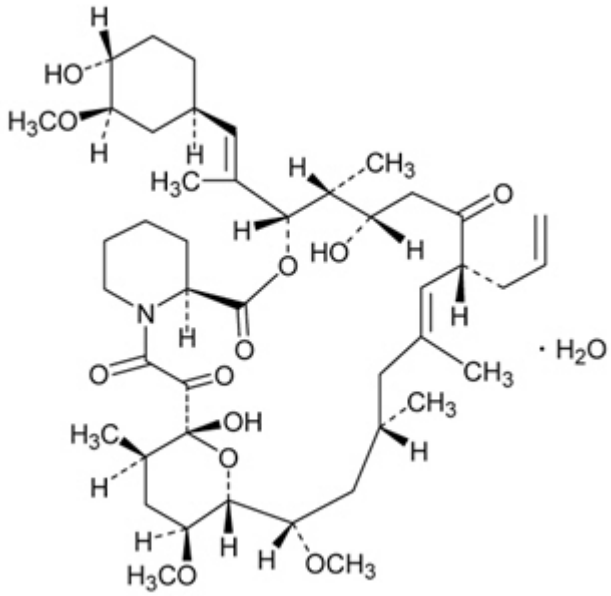

Figure 9 Chemical structure of Tacrolimus. Source: www.medlibrary.com

Various animal studies followed to further evaluate the immunosuppressive and anti-lymphocytic effects and soon, Tacrolimus was given to acutely rejecting transplant patients as "rescue" therapy. In 1990, a liver transplant study started, using Tacrolimus as first-line therapy [165]. Subsequently, Tacrolimus has been widely used in solid organ and bone marrow transplantation. The drug has been described as being up to 100-fold more potent in *in vitro* suppression assays than the CNI Cyclosporine [166]. Further, it was shown that Tacrolimus has suppressive effects on T cells without affecting myeloid cells at the same concentrations [167].

Activation of a T cell via engagement of the TCR results in activation of the calcium – calcineurin - NF-AT – pathway. Once Tacrolimus has entered the cell, it binds to the abundant FK506-binding protein FKBP-12, which is a cytosolic immunophilin. The FKBP-FK506 complex then competitively binds to calcineurin, a Ca^{2+} / calmodulin-dependent proteine phosphatase enzyme [168], and thus the calcium-dependent signal transduction pathway in T cells is interrupted (see Figure 10). Without Calcineurin, the cytosolic subunit of the nuclear factor of activated T cells (NF-ATc) will not be dephosporylated, thus the translocation to the nucleus is blocked. Therefore, NF-ATc cannot form a complex with the nuclear component of the nuclear factor of activated T cells (NF-ATn), which is necessary for promoter-binding of the IL-2 gene und subsequent production of IL-2 [169], a crucial cytokine for T cell activation. Also, further genes regulated through NF-AT are affected by calcineurin-

inhibitors, such as IL-4, IFNγ or Fas-ligand [170]. By inhibition of calcineurin with Tacrolimus, the activation, differentiation and proliferation of naïve and memory effector $CD4^+$ and $CD8^+$ T cells is effectively suppressed [171,172]. In its mode of action, Cyclosporine is similar to Tacrolimus. The correspondent immunophilin for Cyclosporine is Cyclophilin A, the formed complex can also bind calcineurin with the above described consequences. In comparator studies, evidence was found that Tacrolimus is superior to Cyclosporine treatment regarding acute rejection episodes and graft loss [173,174].

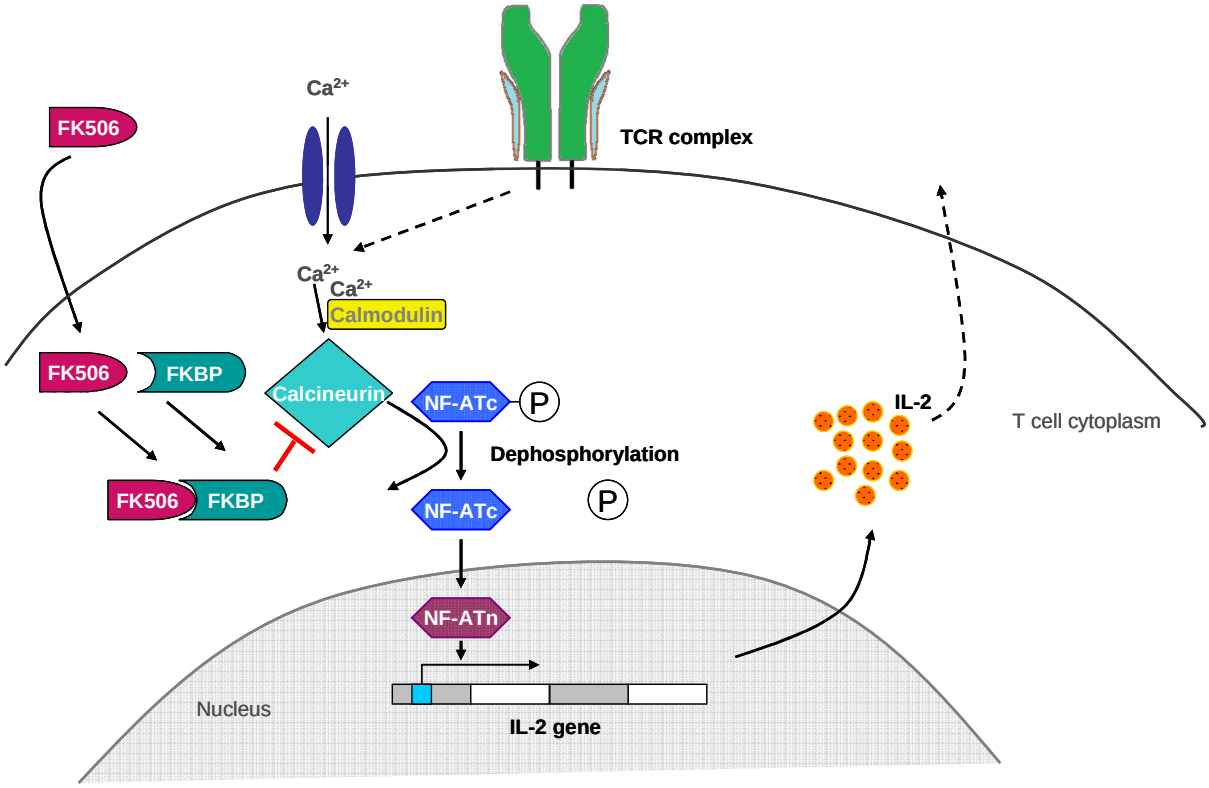

Figure 10 Effector mechanims of Tacrolimus

1.7.3 CNI toxicity – a trade off?

The short-term graft survival could be strikingly improved by the use of calcineurin inhibitors. Yet, the long-term outcome did not change much [175], due to further problems arising by numerous adverse drug-related effects. The toxic effects of both Tacrolimus and Cyclosporine are described similar: Nephrotoxicity and chronic kidney damage, neurotoxicity, disturbances of glucose metabolism and susceptibility to malignancy have been associated with both treatments [176,177]. Both MMF and Rapamycin in combination alone or together with either Tacrolimus or Cyclosporine were subject of various studies in order to spare / minimise the CNI doses. Late conversion from CNI-MMF treatment to a combination of MMF and Rapamycin did not improve renal function, in fact it was harmful to kidney transplant patients with already impaired renal function. An early conversion results only in an initial

better renal function. Additionally, also Rapamycin has adverse effects such as proteinuria, bone marrow suppression and, of note after an operative procedure, impaired wound healing [178]. In the Efficacy Limiting Toxicity Elimination (ELITE)-Symphony study [161], graft survival and acute rejection episodes with *de novo* Rapamycin in combination with MMF were worse than with the Tacrolimus-MMF treatment. Further, treatment with low-dose Tacrolimus (3-7 ng/ml) in combination with MMF had the best outcome (renal function and graft survival) compared to normal or low-dose Cyclosporine in combination with MMF [178]. Disregarding the low-dose use, the general toxicity profiles of Tacrolimus, Cyclosporine and Rapamycin were found to be retained, but by minimising the doses of CNI in stable renal transplant patients, impaired renal function can be improved [179,180].

1.7.4 Pharmacokinetics

Immunosuppressive drugs have variable pharmacokinetics in the individual patient. Thus, drug monitoring is important to achieve optimal efficient dosages to exert therapeutic effects with minimised side effects. A method used widely in the clinic is the measurement of trough levels (C_0), i.e. the concentration immediately before intake of a new dose of the administered drug.

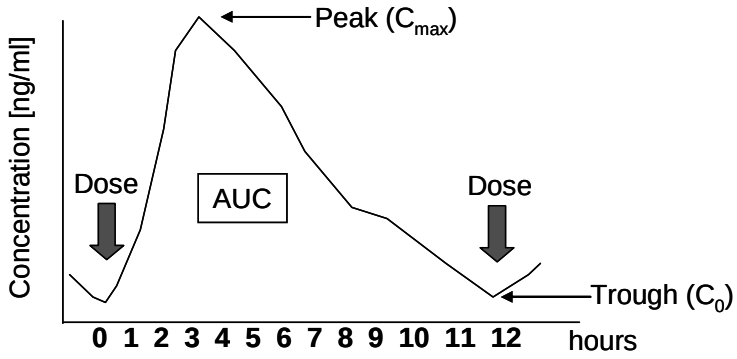

Figure 11 Concepts in drug monitoring (compare [[107,181])

After the intake, there is an initial absorption phase where drug levels then reach a peak (maximum concentration C_{max}) until the concentration then falls off to C_{min}. The total drug exposure between two doses is the area under the concentration-time curve (AUC). To determine the AUC, the drug concentration should be measured at several different time points to create a 12 hour pharmacokinetic profile [181]. This is not feasible in the clinic for every patient. Thus, measurement of Tacrolimus C_0 - trough levels, often reported as correlated with AUC [182-185], was recommended in 2009 by the KDIGO clinical practice guideline [186]. Nonetheless, there are studies reporting that other time points such as C_2 or C_4 correlate better with the AUC [182,183], though the relevance of this in regard to acute rejection episodes remains unclear. Important for the clinic is the fact that the differences in the maximum concentration C_{max} do not affect graft survival time, if the AUC stays the same

[181,187]. In fact, Tacrolimus C_{max} seems to correlate with the incidence of certain adverse effects [188,189]. So patients should receive Tacrolimus treatment that guarantees a certain AUC to avoid acute rejection, but does not lead to strong peak concentrations in order to reduce unnecessary adverse effects.

1.8 Tolerance - inducing strategies

1.8.1 Costimulatory blockade with anti-CD154 in animal models

1.8.1.1 Effects of anti-CD154

Treatment with anti-CD154 antibody to block the CD40/CD154 pathway of costimulation showed graft survival prolongation effects in various models. This has been reported in different species (mouse, rat, non-human primate) and organs (islet, skin, heart, or kidney) [190-195]. Yet, in a full mismatch murine skin transplant model, anti-CD154 therapy alone has been reported as non-sufficient to prolong allograft survival [195].

The mode of action of anti-CD154 blockade has been extensively examined in mouse solid organ transplant models. In a multiple minor mismatch model of murine skin transplantation, (B10.BR to CBA/Ca), anti-CD154 antibody induced antigen-specific tolerance in $CD4^+$ T cells. For this, however, the $CD8^+$ effector response had to be controlled, which was done by either thymectomy or depletion with an anti-$CD8^+$ antibody prior to transplantation. When anti-CD154 antibody was given alone, this resulted in significantly prolonged, yet not indefinite graft survival in this minor mismatch model, despite a delay in the $CD8^+$ cell-mediated rejection could be observed. It was further demonstrated that the induced CD4+ cells were capable of linked suppression, since donor $\times$ third party F1 grafts were accepted in mice tolerised with anti-CD154 and thymectomy [194]. The same group showed in further experiments that the tolerance achieved in their multiple minor mismatch model of anti-CD154 following $CD8^+$ depletion is infectious, i.e. imposed on naïve CD 4^+ T cells [196]. It was concluded that the impact of anti-CD154 antibody cannot be explained only by its ability to cause activation-induced cell death (AICD) of effector $CD4^+$ or $CD8^+$ T cells. Rather, anti-CD154 led to generation of a regulatory CD4+ T cell population that is responsible for the tolerance observed.

Ferrer et al. demonstrated that CD154 blockade caused late modest conversion of donor-reactive $FoxP3^-$ $CD4^+$ T cells into induced $FoxP3^+$ T regs in a transgenic mouse model. It was further shown that the injection of anti-CD154 could delay the expansion of donor-reactive $CD8^+$ T cells [197].

1.8.1.2 Combined treatment of anti-CD154 + DST

As it has been described by different groups, the administration of a donor-specific transfusion (DST) in combination with anti-CD154 treatment enhances the graft survival prolongation effect [198-201]. Yet, whilst in cardiac, islet, or minor mismatch skin transplant models the administration of a DST under costimulatory blockade is sufficient to induce permanent graft survival [200,202], in the B/c-to-B/6 full mismatch skin transplant model it is not. The Rossini group reports skin graft survival in this stringent model for ~50 days [195].

This group could show that only the combination of anti-CD154 and DST resulted in the rapid activation and subsequent deletion of alloreactive $CD8^+$ T cells in a transgenic mouse model [203]. These results were later confirmed by Ferrer et al. who additionally showed, as mentioned above, that anti-CD154 blockade induces $FoxP3^+$ T reg in the periphery. The increase in the frequency of graft-specific T regs was further enhanced by the combination of anti-CD154 + DST [197]. Further work of other groups on the effect of combined treatment of anti-CD154 and DST demonstrated that transgenic CD4+ T cells were not depleted, but this treatment induced hyporesponsiveness of the alloreactive CD4+ T cells [199,204]. Recent experiments in a B/c-to-B/6 skin transplant model indicated that administration of anti-CD154 + DST attenuates antigen-specific T cells responses by skewing CD8+ T cells towards short-lived effector cells [205].

1.8.2 Clinically applied strategies

Despite steady progress in the field of transplantation during the last decades, leading to reduction of acute rejection; transplant recipients still have a lower quality of life and a lower life expectancy compared to the general population. This is mainly due to maintenance immunosuppressive therapy [206]. Additionally, even with immunosuppressive treatment, long-term graft survival has not been improved substantially. In fact, the adverse effects of immunosuppression, especially Calcineurin-Inhibitors, contribute to this effect [207]. Therefore, much effort has been made to achieve immunosuppression withdrawal. How can this be done?

In the early 1990s, Thomas Starzl et al. reported five liver-transplanted patients that had stopped taking immunosuppressive drugs for 5 up to 13 years, but had normal liver functions [208]. This long-term drug-free acceptance of an allograft without signs of chronic rejection is described as operational tolerance in transplantation [209]. Additionally, the patient should be fully immunocompetent, and the tolerance should be antigen-specific. Further, the absence of a donor-specific response measurable by donor-specific antibodies (DSA) in transplanted

patients has been described [210]. In the clinic, the term operational tolerance is used, since it could not been proven that it is true tolerance. This is, as defined by animal experiments, a transferable and dominant regulation [211]. In the following, more patients, mostly due to noncompliance, have been found to accept their graft without immunosuppression. It has become an important goal to deliberately induce tolerance towards the allograft in transplant patients.

Tolerance induction through haematopoietic chimerism in pre-immune rodent models has been done for 60 years now [98]. Translation of numerous successful small animal protocols to large animals and humans came with various difficulties. So far, allograft tolerance in human renal transplantation could be induced only in combination with haematopoietic chimerism [212]. But this does not come easily, the preparative treatments are harsh and the therapy is not without side effects such as graft-versus-host-disease (GvHD). The risk of developing GvHD, a disease that can be fatal and life-threatening, in the context of transplantation may not be warranted.

Induction therapy, i.e. high intensity of immunosuppression at the time of transplantation; that is tapered in the following is the conventional approach to minimise immunosuppression, especially CNIs. These strategies, also including lymphodepletional antibodies and other biologics, did not lead to operational tolerance in patients, where immunosuppressive drugs could be withdrawn definitely. Yet, the doses could be reduced, albeit not to a level where the adverse effects would not occur [213].

Operational tolerance and thus withdrawal of all immunosuppression seems extremely difficult to achieve for all transplant patients. A more imminently achievable goal might be the induction of a recipient regulatory response that allows minimisation of maintenance immunosuppression. This can be described as partial (or “prope”) tolerance, a state of the immunological regulation of the recipient that is just insufficient to prevent rejection over a certain time. Here, by very low doses of immunosuppression, this state could be supported to promote long-term allograft survival, without raising adverse effects and toxicity [214].

1.9 Tolerance – a balance?

There are two general ways to explain tolerance in transplantation. One that might be called a qualitative account of transplant tolerance holds it that tolerance is a unique state that is entirely distinct from states of rejection or stable immunosuppression. There are several possible mechanisms discussed that can lead to this tolerance, e.g. the presence of antigen-specific regulatory T-cell populations in tolerant patients as opposed to patients that will undergo rejection. Another mechanism could be the total clonal deletion or complete anergy of effector cells in tolerant patients. It is thinkable that the allograft has become an immuno-priviledged site in tolerant patients; therefore effector cells do not have physical access to the graft. Another concept in transplantation immunology proposed by Stockinger et al. [215] holds it that in an organism there are several niches that can be populated by effector cells or regulatory cells. In consequence, in tolerant patients these niches would be predominantly filled by regulatory cells in contrast to the population by effector cells in rejecting patients.

In contrast to this “qualitative view”, tolerance and rejection might be seen as resulting from the quantitative balance between effector and regulatory response. Consequently, tolerance occurs if the regulatory response predominates whereas rejection will take place if the regulatory response is exceeded by effector cells. We can imagine that such a balance does not result in a black & white - picture, it rather suggests different degrees of stability. Further, this quantitative account implies the existence of marginal conditions, wherein neither regulation nor effector responses predominate. This could be the explanation for following situations: 1) The effector cells marginally exceed the regulatory cells, leading to a weak rejection that can be controlled by low doses of immunosuppression. 2) The regulatory response marginally exceeds the effector response, leading to an unstable state that can be supported in favour of regulation by low doses of immunosuppression.

2 Aim

The aim of this work was to give formal proof that marginal states of allograft acceptance do exist. As described above, the existence of marginal conditions, wherein neither regulatory nor effector responses predominate might explain certain observations in the clinic. Further, these states may influence immunosuppressive treatment of transplant patients and, eventually, the tolerance-inducing therapies. Yet, so far, no experimental model of such states exists. Therefore, a low-dose Tacrolimus monotherapy in C57BL/6 – mice and a weak tolerance-inducing protocol in the BALB/c-to-C57BL/6 skin transplantation model was established in this project. Both treatments were then combined to answer the question whether low-dose Tacrolimus therapy can support the allograft survival in mice treated with a weak tolerance-inducing protocol. Further, it was crucial to show that neither treatment alone did lead to comparable graft survival and that the disruption of either immunosuppression or regulation would lead to rejection of the allograft.

3 Materials and Methods

3.1 Materials

3.1.1 Instrumentation

Item	Manufacturer	Headquarters
Safety cabinet DIN 12950	Clean Air/Telstar	Woerden Netherlands
Centrifuge 5417C	Eppendorf	Hamburg, Germany
FACS Canto II	Becton, Dickinson & Company (BD)	Franklin Lakes, NJ, USA
LED cold-light source KL1500	Schott	Mainz, Germany
Light Cycler 480	Roche	Basel, Switzerland
Megafuge 1.0R	Heraeus Instruments	Hanau, Germany
Microscope M651	Leica	Wetzlar, Germany
Microscope SMZ168	Motic	Wetzlar, Germany
Mikrotom	SLEE	Mainz, Germany
NanoDrop 2000c	Thermo Fischer Scientific	Waltham, MA, USA
pipetus® pipetting controller	Hirschmann Laborgeräte	Eberstadt, Germany
Precision microplate reader	Molecular Devices	Sunnyvale, CA, USA
Steam cooker DG2438	Severin Elektrogeräte GmbH	Sundern, Germany
Thermal Pad Model	SHOR-LINE	Kansas City, KS, USA
Thermal cycler PTC-200	MJ Research Inc.	St.Bruno, QC, Canada
Zeiss Axio Observer Z1 microscope	Zeiss	Jena, Germany

3.1.2 Consumables

Item	Manufacturer	Headquarters
Capilliaries with Na- Hep. 9UL	Hirschmann Laborgeräte	Eberstadt, Germany
cell culture plates, different sizes	Corning	Corning, NY, USA
Cell Strainer 70 µm or 100 µm	BD Falcon	Franklin Lakes, NJ, USA
Cover slides	Carl Roth	Karlsruhe, Germany
EDTA Tubes/ Probengefäß 1,3 ml K3E	Sarstedt	Nürnbrecht, Germany
Embedding cassettes	Carl Roth	Karlsruhe, Germany
FACS Tubes for flow cytometry	Sarstedt	Nürnbrecht, Germany
Falcon tubes	Greiner Bio One	Frickenhausen, Germany
MACS columns (MS, LD, LS)	Miltenyi Biotec	Bergisch Gladbach, Germany
MACS Pre- Separation Filters 30 µm	Miltenyi Biotec	Bergisch Gladbach, Germany
Lightcycler 480 Multiwell Plate 96	Roche	Basel, Switzerland
Pipette tips 1ml	Corning	Corning, NY, USA
Pipette tips 250 µl	Sarstedt	Nürnbrecht, Germany
Pipette tips, with filter, different sizes	Biozym	Hessisch Oldendorf, Germany

Pipette tips10 µl	Starlab	Hamburg, Germany
Qia-shredder columns	Qiagen	Venlo, Netherlands
Reaction tube 2 ml + 0,5 ml	Eppendorf	Hamburg, Germany
Serological pipettes	Corning	Corning, NY, USA
Steriflip/ Stericup-Unit	Merck Millipore	Darmstadt, Germany
Super Frost Plus Microscope Slides	Thermo Fischer Scientific	Waltham, MA, USA

3.1.3 Operation consumables

Item	**Manufacturer**	**Headquarters**
Bepanthen ointment for eyes	B. Braun	Melsungen, Germany
Cannulaes, different sizes	Becton, Dickinson&Company (BD)	Franklin Lakes, NJ, USA
Depilation creme asid® med	ASID BONZ	Herrenberg, Germany
Feather disposable scalpell No.11	Feather Safety Razor Co., Ltd.	Osaka, Japan
Forceps BD331R and BD215R	Aesculap / B. Braun	Melsungen, Germany
Forceps Dumont #5	FST	Heidelberg, Germany
Gauze swabs	Hartmann	Heidenheim, Germany
Medical tape 3M™ Durapore™	3M	St. Paul, MN, USA
Mepitel™ wound contact layers	Mölnlycke Health Care	Gothenburg, Sweden
Needle holder FD241R	Aesculap / B. Braun	Melsungen, Germany
Scissors BC 110R	Aesculap / B. Braun	Melsungen, Germany
Suture thread 4-0 Prolene	Johnson & Johnson Medical GmbH	New Brunswick, NJ, USA
Suture thread 5-0 Sofsilk	Johnson & Johnson Medical GmbH	New Brunswick, NJ, USA
Suture thread 7-0 Ethilon	Johnson & Johnson Medical GmbH	New Brunswick, NJ, USA
Syringes, different sizes	Becton, Dickinson&Company (BD)	Franklin Lakes, NJ, USA

3.1.4 Reagents

Item	**Manufacturer**	**Headquarters**
2- Mercaptoethanol, 55 mM in DPBS	GIBCO / Life technologies	Carlsbad, CA, USA
2-Propanol	Merck	Darmstadt, Germany
7-AAD	Becton, Dickinson&Company (BD)	Franklin Lakes, NJ, USA
ACK Lysis Buffer	GIBCO / Life technologies	Carlsbad, CA, USA
Albumine Bovine Fraction Sol 7,5%	Sigma Aldrich	St. Louis, MO, USA
Anti- Biotin- Microbeads	Miltenyi Biotec	Bergisch Gladbach, Germany
Aquatex	Merck	Darmstadt, Germany
Atropinsulfat 0,5 mg/ml	B. Braun	Melsungen, Germany
Biocoll Separating Solution	Biochrom / Merck	Darmstadt, Germany
BSA Solution 7,5%	Sigma Aldrich	St. Louis, MO, USA
CD45.2- Biotin, mouse	Miltenyi Biotec	Bergisch Gladbach, Germany
Citrate buffer pH 6.0	Zytomed Systems	Berlin, Germany

DAB+ Substrate Chromogen System	DAKO	Hamburg, Germany
DEPC Treated water	VWR	Radnor, PA, USA
Detection Reagent 1	GIBCO / Life technologies	Carlsbad, CA, USA
Detection Reagent 2	GIBCO / Life technologies	Carlsbad, CA, USA
Diphtheria Toxin	Sigma Aldrich	St. Louis, MO, USA
DMSO	Sigma Aldrich	St. Louis, MO, USA
DNA Zap (Solution 1 + 2)	Ambion / Life technologies	Carlsbad, CA, USA
Dulbecco's PBS	Sigma Aldrich	St. Louis, MO, USA
ECL Plus WB Detection System	GIBCO / Life technologies	Carlsbad, CA, USA
EDTA solution 0,5 M, Ultra Pure	GIBCO / Life technologies	Carlsbad, CA, USA
Eosin Y solution	Sigma Aldrich	St. Louis, MO, USA
Ethanol	Merck	Darmstadt, Germany
FcR Blocking Reagent mouse	Miltenyi Biotec	Bergisch Gladbach, Germany
Fetal Calf Serum (FCS)	Sigma Aldrich	St. Louis, MO, USA
Fixable Viability Dye eF506	eBioscience	San Diego, CD, USA
Flourescent Mounting Medium	DAKO	Hamburg, Germany
Foxp3 Fixation/Permeabilization Concentrate	eBioscience	San Diego, CD, USA
Foxp3 Fixation/Permeabilization Diluent	eBioscience	San Diego, CD, USA
Foxp3 Perm Buffer 10x	eBioscience	San Diego, CD, USA
Glucose 5%	B.Braun	Melsungen, Germany
Glutamax 100x	GIBCO / Life technologies	Carlsbad, CA, USA
Goatserum	Sigma Aldrich	St. Louis, MO, USA
H_2O_2 solution, 30%	University Regensburg	Regensburg, Germany
Heparin Na 25000 I.E.	Rotexmedica	Amt Trittau, Germany
IC Fixation Buffer	eBioscience	San Diego, CD, USA
Isoflurane Baxter	Baxter	Deerfield, IL, USA
Ketamin	WDT	Garbsen, BRD
Liquid DAB+ Substrate Chromogen System	DAKO	Hamburg, Germany
Mayer's Hemalum solution	Carl Roth	Karlsruhe, Germany
MEM Non-essential Aminoacids100x	GIBCO / Life technologies	Carlsbad, CA, USA
NaCl 0,9%	B.Braun	Melsungen, Germany
Paraformaldehyd - solution (PFA)<5%	Pathology Deptartment UKR	Regensburg, Germany
Penicillin/Streptamycin	Invitrogen / Life technologies	Carlsbad, CA, USA
Periodic Acid solution	Merck	Darmstadt, Germany
Roti®-Histokit	Carl Roth	Karlsruhe, Germany
Roti®-Histol	Carl Roth	Karlsruhe, Germany
RPMI 1640 medium	GIBCO / Life technologies	Carlsbad, CA, USA
Schiff reagent	Merck	Darmstadt, Germany
SensiTek HRP	ScyTek Laboratories	Utah, UT, USA
Sodium Pyruvat 100 mM, 100x	GIBCO / Life technologies	Carlsbad, CA, USA
SuperScript® III First-Strand-Synthesis SuperMix	Invitrogen/ Life technologies	Carlsbad, CA, USA
Sybr® Green Dye	Invitrogen/ Life technologies	Carlsbad, CA, USA

Tacrolimus (FK-506), >99% purity	LC-Laboratories	Woburn, MA, USA
Tacrolimus (FK-506), >99% purity	biorbyt	Cambridge, UK
Tacrolimus (Prograf) 5mg/ml solution	Astellas	Chuo, Japan
Tacrolimus food produced by	SSNIFF	Soest, Germany
TMB Substrate Reagent Set	Sarstedt	Nürnbrecht, Germany
Trypan Blue Sol. 0,4%	Sigma Aldrich	St. Louis, MO, USA
Tween 20	Sigma Aldrich	St. Louis, MO, USA
Vybrant CFDA SE Cell Tracer Kit	Molecular Probes / Life technologies	Carlsbad, CA, USA
Xylazin	Bernburg AG	Bernburg, Germany

3.1.5 Kits

Item	**Manufacturer**	**Headquarters**
CD4+CD25+ Regulatory T Cell Isolation Kit, mouse	Miltenyi Biotec	Bergisch Gladbach, Germany
Epidermis dissociation kit, mouse	Miltenyi Biotec	Bergisch Gladbach, Germany
FlowCytomix Mouse Th1/Th2/Th17/Th22 13 plex kit	eBioscience	San Diego, CA, USA
IFNγ Quantikine ELISA kit, mouse	R&D Systems	Minneapolis, MN, USA
Pan T cell isolation kit II, mouse	Miltenyi Biotec	Bergisch Gladbach, Germany
RNeasy Plus mini kit	Qiagen	Venlo, Netherlands
T reg expansion kit, mouse	Miltenyi Biotec	Bergisch Gladbach, Germany

3.1.6 Antibodies

3.1.6.1 For injection

See 1.2.1.10, Application of antibodies

3.1.6.2 For Histology

Antibody	**Fluorochrome**	**Clone**	**Isotype**	**Manufacturer**
anti-mouse FoxP3	purified	FJK-16s	rat IgG2a, κ	eBioscience
rat IgG2a, κ	purified	eBR2a		eBioscience
goat-anti Rat IgG1 Fab2-B	Biotin			Santa Cruz

3.1.6.3 For FACS

Antibody	Fluorochrome	Clone	Isotype	Manufacturer
anti-mouse B220	V450	RA3-6B2	rat IgG2a, κ	BD biosciences
anti-mouse CD115	APC	AFS98	rat IgG2a, κ	eBioscience
anti-mouse CD11b	eF450	M1/70	rat IgG2b, κ	eBioscience
anti-mouse CD11b	V450	M1/70	rat IgG2b, κ	BD biosciences
anti-mouse CD11b	APC	M1/70	rat IgG2b, κ	eBioscience
anti-mouse CD11c	PE	HL3	aH IgG1	BD biosciences
anti-mouse CD11c	APC-eF780	N418	aH IgG	eBioscience
anti-mouse CD137	APC	17B5-1H1	hamster IgG2	Miltenyi Biotec
anti-mouse CD138	APC	281-2	rat IgG2a, κ	BD biosciences
anti-mouse CD19	FITC	1D3	rat IgG2a, κ	BD biosciences
anti-mouse CD19	AF647	1D3	rat IgG2a, κ	eBioscience
anti-mouse CD19	APC-H7	1D3	rat IgG2a, κ	BD biosciences
anti-mouse CD21/35	PE-Cy 7	8D9	rat IgG2a, λ	eBioscience
anti-mouse CD23	FITC	B3B4	rat IgG2a, κ	eBioscience
anti-mouse CD25	PE	PC61	rat IgG1, λ1	BD biosciences
anti-mouse CD25	APC	3C7	rat IgG2b, κ	BD biosciences
anti-mouse CD27	APC-eF780	LG.7F9	aH IgG	eBioscience
anti-mouse CD274	PE	MIH5	rat IgG2a, λ	eBioscience
anti-mouse CD279	FITC	J43	aH IgG	eBioscience
anti-mouse CD28	PE	37.51	sH IgG2, λ1	BD biosciences
anti-mouse CD314	PE	CX5	rat IgG1, κ	eBioscience
anti-mouse CD3e	PE-Cy 7	145-2C11	aH IgG	eBioscience
anti-mouse CD4	PerCP-Cy 5.5	RM4-5	rat IgG2a, κ	BD biosciences
anti-mouse CD4	PE-Cy 7	RM4-5	rat IgG2a, κ	BD biosciences
anti-mouse CD4	APC	GK1.5	rat IgG2b, κ	eBioscience
anti-mouse CD4	APC-H7	GK1.5	rat IgG2b, κ	eBioscience
anti-mouse CD44	FITC	IM7	rat IgG2b, κ	eBioscience
anti-mouse CD45.2	PerCP-Cy 5.5	104	mouse IgG2a, κ	BD biosciences
anti-mouse CD49b	V450	DX5	rat IgM, κ	BD biosciences
anti-mouse CD62L	APC	MEL-14	rat IgG2a, κ	eBioscience

anti-mouse CD8a	V450	53-6.7	rat IgG2a, κ	BD biosciences
anti-mouse CD90.2	FITC	53-2.1	rat IgG2a, κ	eBioscience
anti-mouse CD93	PE	AA4.1	rat IgG2b, κ	eBioscience
anti-mouse F4/80	PE-Cy 7	BM8	rat IgG2a, κ	eBioscience
anti-mouse FoxP3	FITC	FJK-16s	rat IgG2a, κ	eBioscience
anti-mouse FoxP3	APC	FJK-16s	rat IgG2a, κ	eBioscience
anti-mouse GR-1	PerCP-Cy 5.5	RB6-8C5	rat IgG2b, κ	eBioscience
anti-mouse IgG	FITC			eBioscience
anti-mouse IgM	APC	II/41	rat IgG2b, κ	eBioscience
anti-mouse Ly6C	FITC	AL-21	rat IgM, κ	BD biosciences
anti-mouse Ly6G	PE	1A8	rat IgG2a, κ	BD biosciences
anti-mouse MHC I (H-2K^{b})	FITC	AF6-88.5	rat IgG2a, κ	BD biosciences
anti-mouse MHC I (H-2K^{b})	PE-Cy 7	AF6-88.5	mouse IgG2a, κ	eBioscience
anti-mouse MHC I (H-2K^{d})	APC	SF1-1.1.1	mouse IgG2a, κ	eBioscience
anti-mouse MHC I (H-2K^{d})	eF450	SF1-1.1.1	mouse IgG2a, κ	eBioscience
anti-mouse MHC II	PE	M5/114.15.2	rat IgG2b, κ	eBioscience
anti-mouse NK1.1	PerCP-Cy 5.5	PK136	mouse IgG2a, κ	eBioscience
anti-mouse Siglec H	PerCP-eF710	440c	rat IgG2b, κ	eBioscience
anti-mouse TCRγδ	APC	GL-3	aH IgG	eBioscience

Isotype control	**Fluorochrome**	**Clone**	**Manufacturer**
rat IgG2a, κ	AF647		BD
hamster IgG2,κ	APC	B81-3	BD
rat IgG2a, κ	APC		eBioscience
rat IgG2a, κ	APC	eBR2a	eBioscience
rat IgG2b, κ	APC	A95-1	BD
aH IgG	FITC	eBIO299Arm	eBioscience
rat IgG2a, κ	FITC	eBR2a	eBioscience
rat IgG2b, κ	FITC	A95-1	BD
rat IgM, κ	FITC	R4-22	BD
aH IgG	PE	eBIO299Arm	eBioscience
rat IgG1, κ	PE		eBioscience
rat IgG1, λ1	PE	A110-1	BD
rat IgG2a, κ	PE		eBioscience

rat IgG2b, κ	PE		eBioscience
sH IgG2, λ1	PE		eBioscience
aH IgG1,κ	PE-Cy 7		BD
rat IgG2a, κ	PE-Cy 7		eBioscience
rat IgG2a, λ	PE-Cy 7	eBR2a	eBioscience
mouse IgG2a, κ	PerCP-Cy 5.5	G155-178	BD
rat IgG2a, κ	PerCP-Cy 5.5		BD
rat IgG2b, κ	PerCP-Cy 5.5		eBioscience

3.1.7 Buffers and solutions

MACS-Buffer:	0.5%	BSA
	2 mM	EDTA
		in PBS
		sterile filter, store cold

supplemented medium:	43 ml	RPMI
	5 ml	FCS
	0.5 ml	Penicillin/Streptamycin
	0.5 ml	Glutamax
	0.5 ml	Non-essential Amino Acids
	0.5 ml	Sodium Pyruvate
	0.1 ml	2- Mercaptoethanol
		sterile filter, store cold

T-TBS (10x):	24.2 g	TRIS base
	80 g	NaCl
		add ddH_2O to 1l
		adjust pH to 7.58
		dilute in ddH2O for 1x T-TBS

3.1.8 Primers

All primers were QuaniTect primers ordered from Qiagen.

3.1.8.1 Housekeeping genes

gene	a.k.a.	order no	name
Gapdh		QT01658692	glyceraldehyde-3-phosphate dehydrogenase
Hprt		QT00166768	hypoxanthine guanine phosphoribosyl transferase
Ppia		QT00247709	peptidylprolyl isomerase A
Rn18s		QT02448075	18S ribosomal RNA
Tbp		QT00198443	TATA-box binding protein

3.1.8.2 Genes of interest

gene	a.k.a.	order no	name
Ccr2		QT02276813	chemokine (C-C motif) receptor 2
Cd200		QT00145817	CD200 antigen
Cd79b		QT00243663	CD79B antigen
Col1a		QT00162204	collagen, type I, alpha 1
Cxcl10		QT00093436	chemokine (C-X-C motif) ligand 10
Ebi3		QT00155596	Epstein-Barr virus induced gene 3
Fcrl1		QT02249912	Fc receptor-like 1
Fcrlb		QT01539006	Fc receptor-like B
Foxp3		QT00138369	forkhead box P3
Grem1		QT01039983	gremlin 1
Gzmb		QT00114590	granzyme B
Hmmr		QT00127505	hyaluronan mediated motility receptor (RHAMM)
Hmox1		QT00159915	heme oxygenase (decycling) 1
Hs3st1		QT02257283	heparan sulfate (glucosamine) 3-O-sulfotransferase 1
Ido1		QT00103936	indoleamine 2,3-dioxygenase 1
Ifng		QT01038821	interferon gamma
Il13ra2		QT00176162	interleukin 13 receptor, alpha 2
Inos		QT00100275	nitric oxide synthase 2, inducible
Lag3		QT00113197	lymphocyte-activation gene 3
Man1a		QT00132034	mannosidase 1, alpha
Ms4a1	CD20	QT01058330	membrane-spanning 4-domains, subfamily A, member 1
Nav3		QT01050133	neuron navigator 3
Pdcd1	PD-1	QT00111111	programmed cell death 1
Pdcdlg1	PD-L1	QT00148617	Programmed cell death 1 ligand 1
Pdgfa		QT00197610	platelet derived growth factor, alpha
Pnoc		QT00102480	prepronociceptin

Sh2d1b1		QT01049195	SH2 domain protein 1B1
Sh2d1b2		QT00522221	SH2 domain protein 1B2
Slc8a1		QT01044862	solute carrier family 8, member 1
Tcaim	Gm1129	QT00281771	T cell activation inhibitor, mitochondrial
Tcl1		QT00103530	T cell lymphoma breakpoint 1
Tgfb1		QT00145250	transforming growth factor, beta 1
Tlr5		QT02328221	toll-like receptor 5
Tmem176b	TORID	QT00198037	transmembrane protein 176B
Tnfrsf4	OX40	QT00109151	tumor necrosis factor receptor superfamily, member 4
Trem1		QT00153979	triggering receptor expressed on myeloid cells 1
Trem2		QT00157969	triggering receptor expressed on myeloid cells 2

3.1.9 Software

Apart from the conventional software (e.g. Microsoft office) following software programs were used:

software	**application**
Axio Vision LE	Zeiss microscope software
BD FACSDiva 6.0	Flow cytometry data
Flow Jo 7.6.5	Flow cytometry data
Genecluster 3.0	Cluster analysis qPCR data
Gpower 3.1	Statistical planning
GraphPad Prism 4	Various graphs and statistics
Java Tree	Cluster analysis qPCR data
LightCycler® 480 Software, Version 1.5	qPCR data
Reference Manager 11	Compilation of references
SigmaPlot 11.0	Kaplan-Meier survival curves
SoftMax Pro	ELISA data generation

3.1.10 Mice

C57BL/6J, BALB/cAnNCrl, C3H and Rag1-/- (B6.129S7-Rag1tm1Mom/J) mice were purchased from Charles River or The Jackson Laboratory. FoxP3-GFP-DTR mice were bred in house and were provided by Prof. Dr. Stefan Fichtner-Feigl.

In general, male mice of 18-20 g (6-8 weeks of age) were used for experiments. Exceptions (for sex) are female C57BL/6J mice in the male-to-female minor antigen mismatch model and FoxP3-GFP-DTR mice, where male and female mice were used to increase group sizes. Here, treatment and control groups were mixed for sex to ensure comparability.

3.2 Methods

3.2.1 Methods involving mice

Animal experiments were approved by the local authorities (AZ: 54-2532.1-15/12 and 54-2532.1-06/13).

3.2.1.1 Treatment of mice

In general, mice from one cage were randomised for treatment in order to have accurately matched control groups. If this was not done for certain treatments, data from an historical control group was used as reference, which is indicated in the respective figure legend.

3.2.1.2 Skin-Transplantation

Donor mice were euthanized by CO_2 asphyxiation. Tail skin was removed with a ventral anto-posterior cut and placed in ice-cold sterile PBS for a maximum of 3 hrs. Tail skin from one donor was sufficient for 5 - 6 skin grafts. In some cases, the spleen was then removed to prepare a single cell suspension for the donor specific transfusion (DST).

Recipient mice were anaesthetised with a mixture of 3.6 mg Xylazine, 27.3 mg Ketamine in 1000 µl 0.9% NaCl at 40 µl per 10 gram bodyweight. Once in narcosis, the back of the recipient was shaved and depilated using depilatory cream. Mice were placed on a warming plate (37°C) to avoid cooling and the back skin was swabbed with medicinal Isopropanol. Then the upper layers of the skin were removed to obtain a square of ~1 cm in diameter, leaving the subcutis with the blood supplying vessels intact. Sterile 0.9% NaCl-solution (prewarmed to 37°C) was used to keep the tissue wet and elastic. From the donor tail, a size-matching full-thickness piece was trimmed and placed on the wound bed, carefully avoiding overlapping. The graft was fixed in all four edges using a single interrupted suture with a 7.0 suture thread and covered with sterile Mepitel® wound dressing and a piece of sterile gauze swab. Subsequently, the mice were wrapped in medical adhesive tape and kept warm until wake-up. The bandages were removed 7 days post-transplantation.

3.2.1.3 Graft monitoring

After transplantation, skin grafts were monitored for signs of rejection. This was initially done daily, then twice per week. In case of signs of rejection, grafts were monitored in shorter time intervals. Skin grafts were examined for thickening, signs of inflammation, haemorrhagic spots or scarring. Grafts with less than 20% viable, intact tissue were considered as rejected.

3.2.1.4 Donor specific transfusion

The donor spleen was meshed under sterile conditions (laminar flow cabinet) using a 100 µl nylon mesh and the plunger of a 2ml syringe. The cell-suspension was washed with sterile PBS and centrifuged (250g, 4°C, 5 min, standard). The supernatant was aspirated and for Erythrocyte-lysis, the pellet was resuspended with 3 ml sterile ACK buffer and then immediately centrifuged. After aspirating the supernatant, the cells were washed twice with 10 ml sterile PBS. The cell number was adjusted to 20 x 10^6 cells / ml, per ml 12.5 µl Heparin (60 U) were added to avoid cell coagulation. Recipient mice (prior to transplantation) were placed in a fixation chamber and the tail veins expanded by placing the tail in warm water (ca. 45°C). Using a 1 ml syringe with a $27^{3/4}$ gauge needle, 250 µl of the cell suspension (i.e. 5 x 10^6 cells) were injected i.v. in a lateral tail vein. Bleeding was stopped by compression with a sterile gauze swab. Immediately after releasing the mice from the fixation chamber, they received an i.p. injection of anti-CD154 antibody in 200 µl PBS. Mice were allowed to sit for at least 1h before proceeding to skin transplantation.

3.2.1.5 Retransplantation

3.2.1.5.1 Donor

On d50 post-transplantation, some mice with an intact graft were anaesthetised with a mixture of 3.6 mg Xylazine, 27.3 mg Ketamine in 1000 µl 0,9% NaCl at 40 µl per 10 gram bodyweight. After careful shaving of the surrounding area, the graft was cut out and trimmed before placing in ice-cold PBS for a maximum of 5 min. The donor was killed afterwards by cervical dislocation and organs and blood were removed for analysis.

3.2.1.5.2 Recipient

The recipients were prepared for skin transplantation as described above. The wound bed for the transplant was prepared regarding the exact size of the donor tissue. The intact graft was retransplanted without attached tissue of the first recipient. The following approach was done as described above.

3.2.1.6 Effector cells from sensitised mice

To obtain sensitised effector T cells against BALB/c – antigen, C57BL/6 – recipients received one BALB/c – transplant on d0. Seven days later, recipient mice were set on 75 mg/kg Tacrolimus – food. On d21, after completed rejection of the first allograft, recipients received a second BALB/c – transplant under continuation of the food. After successful rejection of the second allograft, mice were sacrificed on d37 and spleens were harvested. T cells were

sorted as described elsewhere (see below, Pan T cell isolation kit II). After sorting, cells were resuspended at 40 x 10^6 cells / ml with 12.5 µl Heparin (60 U), and 250 µl (i.e. 10 x 10^6 cells) were injected i.v. in a lateral tail vein.

3.2.1.7 Transfer of LN cells

Graft-draining lymph nodes (axilliary, inguinal) from allografted mice treated with anti-CD154 + DST +Tac-75 or Tac-100 were removed on d50. T cells were individually per mouse sorted as described elsewhere (see below, Pan T cell Isolation Kit II). After sorting, cells were resuspended at 4 x 10^6 cells / ml with 12.5 µl Heparin (60 U), and 250 µl (i.e. 1 x 10^6 cells) were injected i.v. in a lateral tail vein of a Rag1$^{-/-}$ mouse.

3.2.1.8 Splenectomy

For spleen removal, mice were anaesthetised as described before. Once in narcosis, mice were placed right-laterally on a 37°C warming plate and on the left side a small area below the ribcage was shaved and swabbed aseptically. A small incision in this hair-free area of 1 – 1.5 cm was made to access the abdomen. The spleen was exteriorised and placed carefully on a sterile gauze swab next to the incision. The splenic artery and veins were then ligated, cut, and the spleen removed. The peritoneum, abdominal muscle and skin are then sutured performing the single interrupted suture technique with a 4.0 Prolene suture thread.

3.2.1.9 Thymectomy

Thymectomised mice were ordered at Jackson Laboratory, USA. Mice were thymectomised at 4 - 6 weeks of age, when already a certain repertoire of mature T cells has been formed. Littermates of these mice were left untreated. After 1 week, mice were shipped to the animal housing facilities in Regensburg and after a further recreation time of 1 week, mice were transplanted.

3.2.1.10 Application of antibodies

Antibodies or Isotype controls were injected i.p. in sterile PBS in an end-volume of 200 µl / injection/ mouse. Doses were given as indicated in the table below. All antibodies and Isotype controls were ordered from BioXcell, MA, USA.

Antibody / Fusion protein	Clone	Dose	Days (relative to STx on d0)
anti-mouse CD134L	RM134L	0.5 mg/day	0, 2, 4, 8
anti-mouse CD154	MR-1	0.5 mg/day	0, 1, 3, 6
anti-mouse CD25	PC-61.5.3	1 mg/day	50, 53, 56, 59
anti-mouse GITR	DTA-1	1 mg/day	50, 53, 56, 59
anti-mouse IL10R	1B1.3A	0.5 mg/day	50, 52, 54, 56, 58, 60, 62, 64
anti-mouse PD-L1	10F.9G2	0.25-0.5 mg/day	50, 52, 54, 56, 58, 60, 62, 64
anti-mouse TGFβ	1D11.16.8	1 mg/day	50, 52, 54, 56, 58, 60, 62, 64
CTLA4-Ig (hum/hum)		0.5 mg/day	1, 3

Isotype control		Dose	
HRPN	rat IgG1	0.5-1 mg/day	as control for aIL10R / aCD25
LTF2	rat IgG2b	0.25-1 mg/day	as control for aGITR/ aPD-L1
MOPC21	mouse IgG1	1 mg/day	as control for aTGFβ

3.2.1.11 Application of Tacrolimus therapy

Tacrolimus was given at doses of 25 mg, 50 mg, 75 mg, 100 mg and 150 mg per kg food. For this, the required amounts of Tacrolimus (FK-506) were shipped to the food supplying company SSNIFF, where pellets including the desired dose were produced. Thus, the Tacrolimus-powder was incorporated into the pellets. To discriminate medicinal food from normal mouse food, the pellets were dyed with light green color. The mice were provided with Tacrolimus food ad libitum by members of our working group. Additionally to the food, a bolus dose of 1 mg/kg bodyweight of Tacrolimus (Prograf®) in 5% glucose solution was administered i.p. on the day of food change and two consecutive days. For this, the stock solution of 5 mg/ml Tacrolimus (Prograf®) was diluted 1:25 with the glucose solution (working solution: 200 µg/ml) and 5 µl/ g body weight were injected.

3.2.1.12 Application of Diphtheria toxin

Under sterile conditions, lyophilized Diphtheria toxin was reconstituted with 1 ml sterile PBS to achieve a stock solution of 1 mg/ml. For further dilution, a small aliquot was withdrawn from the vial using a syringe and transferred to a 1.5 ml test tube. The amount of substance was determined with a 200 µl pipette and subsequently diluted 1:10 with sterile PBS (e.g. for a 50 µl aliquot, 450 µl PBS were added). This 1:10 dilution was then transferred to a 50 ml Falcon tube and diluted 1:40 with sterile PBS to achieve a final concentration of 2.5 ng/µl. Mice were injected i.p. with 10 µl = 25 ng/ gram bodyweight (i.e. 25 µg/kg bodyweight) every other day for a total of six injections.

3.2.1.13 Toxicology

Measurements were performed by the Institute for Clinical Chemistry and Laboratory Medicine, University Hospital of Regensburg.

3.2.1.13.1 Tacrolimus

Serum-levels of Tacrolimus in mice were measured by LC-MS/MS in EDTA-blood. For this, mice were bled at indicated time points retro-bulbary under a brief Isoflurane-narcosis. Per analysis, 100 – 200 µl blood was taken.

3.2.1.13.2 Creatinine

Serum-levels of Creatinine were measured in the serum of heparinised blood by photometric analysis. Mice were bled retro-bulbary on d50 to collect 150 µl blood. Serum was obtained by Clinical Chemistry.

3.2.2 Molecular biology

3.2.2.1 RNA isolation

Single cell suspensions were pelleted and lysed by addition of 350 µL of RLT buffer supplemented with 1% (v/v) 99% 2-mercaptoethanol. For whole-organ qPCR analysis, whole organs were removed, wrapped in aluminium foil, snap-frozen in liquid N_2 and smashed (in the foil) between two metal blocks. The crushed organs were then lysed with 350 µl supplemented RA1 buffer and vortexed vigorously. Subsequently, the tissue samples were applied on a Qia-shredder column and centrifuged for 2 min at high speed (20,000 rcf). The cell lysates were stored at -80°C until RNA extraction. RNA was eluted in 30µL ddH_2O. RNA was isolated using the RNeasy Plus mini kit from Qiagen and the protocol “Purification of Total RNA from animal Cells“. Throughout the handling with cells, RNA, and cDNA contaminations with nucleases were avoided using RNaseZap® and nuclease free filter-tips.

3.2.2.2 cDNA synthesis

First-strand cDNA synthesis from total RNA samples was done using the SuperScript® III First-Strand-Synthesis SuperMix according to the manufacturer’s protocol.

The RNA was used in the highest concentration possible in the case of very low RNA yields due to limited numbers of cells, especially for skin samples. Thus, RNA concentration of the samples was measured with a NanoDrop spectrophotometer. Since 6 µl is the maximum

amount that can be used in the protocol, this volume was used for the sample with the lowest RNA concentration. RNA of the corresponding samples was diluted accordingly with RNAse / DNAse free H_2O.

For first-strand cDNA synthesis, the components (see below) were combined in a 0.2 ml reaction tube and incubated for 5 min in a thermal cycler at 65°C.

component	amount
up to 5 µg total RNA	max. 6 µl
50µM oligo(dT) primer	1 µl
Annealing Buffer	1 µl
RNAse / DNAse free water	fill to 8 µl

After incubation, the tubes were immediately placed on ice for 2 min and quickly spun down. Then, the following reagents were added to the tubes:

component	amount
2x First-Strand Reaction mix	10 µl
SuperScript III / RnaseOUT Enzyme Mix	2 µl

This was followed by short vortexing and spinning before samples were incubated for 50 min at 50°C in a thermal cycler. The reaction was terminated by a 5 min incubation step at 85°C. Tubes were then chilled on ice for 10 min and then stored at -20°C until further processing.

3.2.2.3 Quantitative real-time PCR

The quantitative polymerase chain reaction (qPCR) can be used to quantify a selected polynucleotide sequence by amplifying its concentration to a level at which an accurate detection can be made [200,201].

This level is the so-called crossing point (CP) which is defined as the number of PCR cycles necessary to detect the first reliable fluorescence signal from the dye Sybr Green added to the reaction. PCR amplifies the targeted nucleic acid in the sample and this amplification is considered to be exponentially in the most progressive phase. The fluorescent dye SYBR Green will bind to the minor groove of double-stranded DNA. The fluorescence is greatly enhanced upon DNA-binding [202].The resulting DNA-dye-complex absorbs blue light (λmax = 488 nm) and emits green light (λmax = 522 nm).

3.2.2.3.1 Quantitative real-time PCR setup

If necessary, cDNA was diluted 1 to 5 with nuclease-free water. Master mixes per primer for the target and the housekeeper genes listed above were prepared as a multiple of the reagent volumes shown below:

component	amount
10x Primer (Quantitect)	2.5 µl
QuantiTect SYBR Green PCR Kit	12.5 µl
cDNA	2 µl
ddH_2O	8 µl

Each cDNA sample was assayed in three technical replicates. 25 µl of the mastermix were transferred into real-time PCR 96-well plates.

The PCR was performed on a Roche Lightcycler 480 as follows: First, during a pre-incubation time of 15 min at 95°C, the FastStart Taq DNA polymerase is activated. Then, 40 amplification cycles follow (temperature targets see below).

Target °C	hold	step
94	15s	denaturation
55	30s	annealing
72	30s	elongation

At the end of the 40 amplification cycles amplicons were melted for 5 s at 95°C followed by an annealing step for 1 min at 65°C. Melting curve analysis was performed by stepwise heating-up of the sample until 97°C. The fluorescence signal decreases slowly until the melting temperature of the amplicon is reached. Then, a strong decline of the fluorescence signal will be detectable. The derivative function of the melting curve will display a peak at the melting point of the amplicon. If more than one peak is present, this indicates contaminating DNA or primer-dimers.

3.2.2.3.2 Analysis of qPCR data

Statistics, qPCR:

RT-PCR data was normalized against a set of 5 stable housekeeping genes (18S-rRNA, GAPDH, TBP, PPIA, Actb. For RT-PCR a nonparametric Mann-Whitney test was conducted on a per-gene basis for pairwise comparisons between the clinical groups. Gene-wise multiple testing adjustments were performed using the Holm-Bonferroni correction [203]. A statistical criterion for identification of differentially expressed genes adjusted $P < 0.05$.

Clustering analysis for the dLN qPCR data was done by Dr.Dr. J. Hutchinson (Department of Experimental surgery, University Hospital of Regensburg)

3.2.2.4 IFNγ -ELISA

The principle of the enzyme-linked immunosorbent assay (ELISA) method is the antibody-based antigen detection. A commercially available ELISA kit based on the sandwich ELISA principle was used to measure IFNγ-production. The kit was used following the manufacturer's instructions for cell culture supernatants. Test samples were analysed in duplicates. For analysis, a linear regression line was plotted using the mean values of the duplicates of the standard curve. On the basis of the equation of this regression line with the mean OD of the sample duplicates as x-value, the amount of IFNγ was calculated. OD values below the OD values of the highest dilution of the standard were not considered. Appropriate conduction of the ELISA was checked with the internal control of the kit, which was conducted every time.

3.2.3 Cell biological methods

3.2.3.1 Determination of cell numbers

Cells in single cell suspensions were counted using a Neubauer haemocytometer. For this, cells were diluted 1 to 10 (v/v) with trypan blue and 10 µL of the mixture were placed in the space between the haemocytometer and the cover slip. If dilution was required, cells were diluted 1 to 10 (v/v) with PBS prior to dilution with trypan blue. Cell concentrations in the sample were calculated according to Formula 1. The mean value of four independent areas containing 50 to 100 cells counted was calculated in order to minimise the counting error.

$$Concentration\ \ of\ \ cells = \frac{number\ \ of\ \ cells\ counted\ \ in\ one\ chamber}{volume\ of\ \ chamber} \times dilution\ \ factor$$

Formula 1 Calculation of cell numbers using Neubauer haemocytometer

3.2.3.2 Preparation of single cell suspension

3.2.3.2.1 Spleen and LN

The spleen and / or dLN (axilliary, inguinal) were removed and stored in cold PBS on ice for a maximum of 30 min. Then, organs were mashed using a 100 µm cell strainer and a 2 ml BD Discardit II syringe plunger, if required, under sterile conditions. Cells were then pelletised (5 min, 270 rcf, 4°C) and the supernatant (SN) discarded. If necessary,

Erythrocyte-lysis was performed using 3 ml ACK-Buffer, resuspended cells were immediately spun down and the SN aspirated. If Erythrocyte-lysis was not necessary, or after the lysis, cells were washed twice with 10 ml PBS and then used for further procedures.

3.2.3.3 MACS sorting

Sorting cells using the Miltenyi MACS-system was performed according to the manufacturer's manual. In detail, cell separation procedures are described below.

3.2.3.3.1 $CD4^+CD25^+$ T reg Kit (Miltenyi)

Single spleen cells were counted, pelletised and resuspended in 40 µl ice-cold MACS-Buffer per 10^7 cells. Then, 10 µl Biotin-Antibody Cocktail per 10^7 cells were added and cells were incubated for 10 min in the refrigerator at 4°C. Subsequently, 30 µl ice-cold MACS-Buffer, 20 µl anti-Biotin beads and 10 µl of CD25-PE antibody per 10^7 cells were added, followed by an incubation time of 15 min in the refrigerator at 4°C. Afterwards, cells were washed with 1 ml ice-cold MACS-Buffer per 10^7 cells, pelletised and the SN was discarded. The pellet was resuspended in 500 µl ice-cold MACS-Buffer. A MACS LD-column was placed in the MACS separator magnet and rinsed with 2 ml ice-cold MACS-Buffer, the flow-through was discarded. The labeled cells were then applied through a MACS-filter on the LD column, saving the flow-through (representing the $CD4^+$ cells) in a 15 ml Falcon tube. The column was washed twice with 2 ml ice-cold MACS-Buffer, saving the flow-through in the tube containing the $CD4^+$ cells. These were then centrifuged, the SN discarded and the cells resuspended in 90 µl ice-cold MACS-Buffer. Further, 10 µl Anti-PE- MicroBeads were added, then the cells were incubated for 15 min in the refrigerator at 4°C. After washing the cells with 1 ml ice-cold MACS-Buffer per 10^7 cells, the cells were pelletised and the SN was discarded. The pellet was resuspended in 500 µl ice-cold MACS-Buffer. A MACS MS-column was placed in the MACS separator magnet and rinsed with 500 µl ice-cold MACS-Buffer, the flow-through was discarded. The labeled cells were then applied on the MS column and magnetically sorted. The flow-through was saved in a 15 ml- Falcon, representing the $CD4^+CD25^-$ fraction. The column was washed thrice with 500 µl ice-cold MACS-Buffer, saving the flow-through in the $CD4^+CD25^-$ -tube. The column was placed on a fresh 15 ml Falcon tube. Then, 1 ml ice-cold MACS-Buffer was applied to flush the column with the provided plunger into the tube, which elutes the $CD4^+$ $CD25^+$ cells. To further increase the purity of the $CD4^+CD25^+$ -population, these cells were applied on a second MS-column and magnetically separated. The cell populations were kept in buffer on ice until further handling.

3.2.3.3.2 CD45.2 Sort

Single cell suspensions from spleen, dLN and skin were counted, pelletised and resuspended in 100 µl ice-cold MACS-Buffer per 10^7 cells. Additionally, 10 µl CD45.2 Biotin-antibody per 10^7 cells were pipetted to the cells, which were then incubated for 10 min in the refrigerator at 4°C. Then, cells were washed with 10 ml ice-cold MACS-Buffer, pelletised and the SN was discarded. Now, cells were resuspended in 80 µl ice-cold MACS-Buffer per 10^7 cells and 20 µl anti-Biotin beads per 10^7 cells were added, followed by an incubation time of 15 min in the refrigerator at 4°C. Afterwards, cells were washed with 10 ml ice-cold MACS-Buffer, pelletised and the SN was discarded. The pellet was resuspended in 500 µl ice-cold MACS-Buffer. Magnetic separation was performed with the autoMACS™ Pro Separator as indicated in the manufacturer's manual using the program "Possel-s" to collect the $CD45.2^+$ cell fraction. This program was chosen to achieve highest possible yield especially of the skin samples.

3.2.3.3.3 Pan T cell isolation kit II (Miltenyi)

Single spleen cells were counted, pelletised and resuspended in 40 µl ice-cold MACS-Buffer per 10^7 cells. Then, 10 µl Biotin-Antibody Cocktail per 10^7 cells were added and cells were incubated for 10 min in the refrigerator at 4°C. Further, 30 µl ice-cold MACS-Buffer and 20 µl anti-Biotin beads per 10^7 cells were added, followed by an incubation time of 15 min in the refrigerator at 4°C. Afterwards, cells were washed with 10 ml ice-cold MACS-Buffer, pelletised and the SN was discarded. The pellet was resuspended in 500 µl ice-cold MACS-Buffer. Magnetic separation was performed with the autoMACS™ Pro Separator as indicated in the manufacturer's manual using the program "Deplete" to collect the unlabelled T cell fraction containing $CD4^+$ and $CD8^+$ T cells .

After all sorts, purity FACS stains were performed.

3.2.3.4 Suppression Assay

After MACS-sorting, $CD4^+CD25^-$ cells were put into culture in flat-bottom 96-well plates at 1 x 10^5 cells per well together with aCD3/aCD28 coated beads in a total of 250 µl volume of supplemented cell media. For suppression assays, 1 x 10^5 $CD4^+CD25^+$ cells per well were added, the volume of beads was increased accordingly, the final volume of cells and reagents in media was maintained as 250 µl. Tacrolimus was added at final concentrations of 0.25, 0.5, 1, 1.5 and 2 ng/ml. Cells were incubated for exactly 48h at 37°C with 5% CO_2.

After incubation, the supernatants were harvested into 1.5 ml reaction tubes on ice and spun down at 300g to pellet cells. Then, the supernatant was transferred to a fresh 1.5 ml reaction tube and frozen at -20°C until further processing.

3.2.3.5 Suppression Assay – CFSE

Briefly, the setup for the culture was as described above.

However, MACS-sorting of the cells was modified: the $CD4^+$ cell fraction was stained with CFDA-SE and cells were rested overnight in supplemented medium in 6-well plates (max 7 x 10^6 cells / well). On the next day, $CD4^+$ cells were labelled with CD25-PE and then further processed as described above in the respective section.

After 48h-incubation at 37°C with 5% CO_2, the supernatants were harvested as described above. Further, the wells were rinsed with PBS to obtain the cultured T cells. These were pooled together with the pellet of the supernatant harvest and then stained for FACS analysis.

3.2.3.6 CFSE-labelling

Cells to be labelled with CFSE were adjusted with pre-warmed PBS (i.e. 37°C) to 10 x 10^6 cells / ml. The CFSE stock aliquots (0.5 mM) were diluted 1:125 with pre-warmed PBS to obtain a 4 µM CFSE-working solution. This was then mixed 1:1 with the cells, resulting in a final concentration of CFSE of 2 µM. The suspension was incubated for 15 min at 37°C in the water bath in the dark. The reaction was abrogated by adding FCS at 10% of the final volume. Cells were then spun down (300 g, 10 min, RT) and SN was discarded. The pellet was resuspended in supplemented medium, cells were plated in a 6-well-plate and recovered over night at 37°C in the incubator.

3.2.3.7 FACS staining

All flow cytometric analysis (fluorescence-activated cell sorting, FACS) were measured using BD Canto II. The used antibodies with the respective fluorescence conjugates are listed above. If not stated otherwise, all steps were done on ice. When sufficient material was available, 1 x 10^6 cells were dispensed into FACS tubes, otherwise as much cells as possible were used. After a wash with 1 ml PBS and a centrifugation step (300 g, 5 min, 4°C) the supernatants were either aspirated or discarded. This will be referred to as “wash step” in this work and can be performed with different buffers. In the following, the pellet was

resuspended in 1 ml PBS and 1 µl eF506 Viability Dye was added to the tubes, which were then vortexed and incubated in the dark on ice for 30 min. After that cells were washed twice with PBS and resuspended in 100 µl FACS buffer containing 10 µl mouse FcR blocking reagent and incubated on ice for 30 min. Master mixes of the antibodies desired for each staining were prepared according to the dilutions listed above. After another wash with FACS Buffer, the master mix (max. 70 µl) was added. The tubes were vortexed briefly and incubated on ice in the dark for 30 min. This was followed by two wash steps. If no intracellular staining was required, then 200 µl FACS Buffer were added to each tube and cells were stored at 4°C in the dark until analysis, but for not more than 3 h. In the case of FoxP3 – staining, cells were resuspended instead in 1 ml Fix/Perm solution and incubated for 30 min – max. 18 h in the refrigerator. Subsequently, cells were washed with 2 ml Perm Buffer. 90 µl Perm Buffer and 10 µl mouse FcR blocking reagent were added to the cells. After 15 min incubation, 10 µl of the FoxP3-antibody-working solution were pipetted per tube and cells were incubated for 30 min on ice. Cells were washed twice with Perm Buffer, then 200 µl FACS buffer were added and cells were stored in the refrigerator until analysis for a maximum of 24 h.

FACS-Panels used:

Laser detection	405		488				633	
Fluorochrome	V450 / eF450	V500	FITC	PE	PerCP Cy 5.5 / PerCPeF710	PE-Cy7	APC	APC-eF780 / APC-H7
T cell								
	CD8a	viability dye	CD44	CD28	CD4	CD3e	CD62L	CD27
T reg								
	CD8a	viability dye	FoxP3	CD25	CD4	CD3e	CD137	CD27
B cell								
	B220	viability dye	CD23	CD93	CD4	CD21/35	CD138	CD19
NK/NKT								
	CD49b	viability dye	CD19	CD314	NK1.1	CD3e	TCRgd	CD27
DC								
	CD8a	viability dye	Ly6C	MHC II	Siglec H	CD4	CD11b	CD11c
Macrophage/MDSC								
	CD11b	viability dye	Ly6C	Ly6G	GR-1	F4/80	CD115	CD11c
T reg II								
(in vitro, CFSE)	CD8a	viability dye	CFSE	CD25	CD4	CD3e	FoxP3	-

3.2.3.8 Crossmatch – FACS

To measure donor-specific antibodies (DSA) and unspecific antibodies (NDSA), serum from treated mice was incubated with donor splenocytes (BALB/c) and splenocytes from self (C57BL/6). First, 2.5 x 10^5 BALB/c splenocytes were incubated together with 2.5 x 10^5 C57BL/6 (B/6) splenocytes and 10% mouse FcR blocking reagent in 50 µl for 20 min on ice. Then, 50 µl serum - dilution was added per tube. Blank controls were treated accordingly, but no serum was added. For the standard curve, pooled sera from five sensitised mice were used. These have had rejected 2 consecutive BALB/c – skin grafts under 75 mg/kg Tacrolimus. The pooled sera were serially two-fold diluted in PBS, from a 1:10 dilution up to a 1:1280 dilution. As negative control, sera from naïve B/6 mice were used (NMS). The test sera from mice were used in a 1:40 dilution. Test-sera were done in duplicates, the standard-curve sera, NMS sera and blanks in triplicates. The serum was incubated for 90 min on ice together with the cells. This was followed by three wash steps with FACS Buffer (see above). Further, 10 µl mouse FcR blocking reagent was added to the cells and incubated for 10 min on ice. Without washing, 10 µl anti-mouse IgG-FITC antibody working solution was added and incubated for 60 min on ice. After one wash step, 10 µl each of following anti-mouse antibody working solutions were pipetted into the tubes: IgM–APC, CD3-PE, $H2K^d$-eF450 and $H2K^b$- PeCy7. Cells were incubated for 30 min on ice and then washed twice. Then 50 µl FACS buffer were added and samples were measured.

3.2.3.8.1 Principle and Analysis of the Crossmatch FACS

The flow cytometry crossmatch (FCXM) has been first decribed in 1983 [204] and has been used since then in clinics to measure anti-donor antibodies. The above described Crossmatch FACS follows the T cell FCXM in principle: Antibodies in the serum will bind to the splenocytes, the anti-mouse IgG antibody will bind to these antibodies (Figure 12).

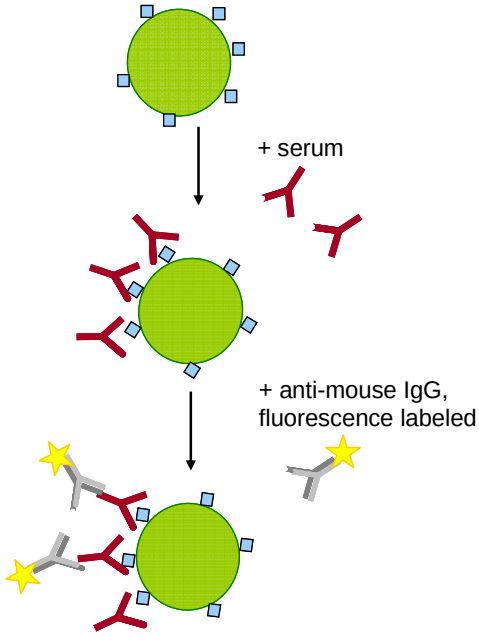

Figure 12 Principle of crossmatch FACS

For the analysis using the FlowJo 7.6.5 software, a lymphocyte gate was set in the forward-scatter / sideward-scatter plot. Using the signal for the CD3 and the $H2K^d$- antigen it was discriminated between B/c splenocytes (donor, $H2K^d$) or B/6 splenocytes (self, $H2K^b$). The PE-signal of the $H2K^d$ positive and $H2K^d$ negative population than shows the IgG response bound on B/c or B/6 cells. The mean fluorescence intensity (MFI) of the PE-signal was used for further analysis. A limit of detection was determined as mean MFI of the three NMS samples plus the threefold standard deviation (SD). Samples with a MFI below the limit of detection were considered as negative for IgG antibody. The same applies for the APC-signal of the IgM response.

3.2.4 Histology

3.2.4.1 Paraffin-embedded samples

Harvested skin grafts, kidneys or spleens were placed in embedding cassettes and put in 5% paraformaldehyde. Fixation of the tissue samples was done by Pathology Department, University Hospital of Regensburg. Finally, samples were stored in melted paraffin to ensure complete infiltration. For embedding, samples were placed in an embedding mold which was subsequently filled with melted paraffin. Skin samples were embedded standing upright. The paraffin blocks were cooled on a cooling plate and then stored at RT until sectioning.

Sectioning was performed on a microtome. For this, the block was first trimmed to obtain an optimal cutting surface. Then, 4 µm sections were cut and transferred to a 50°C water bath.

After unfolding, sections were mounted on Superfrost Plus microscope slides and dried for 24h at 37°C, before proceeding to immunohistochemistry and histology stains / reactions.

3.2.4.2 Haematoxylin & Eosin staining

To deparaffinise tissue samples, slides were placed in Roti®-Histol for 10 min, before rehydrating them in a graded alcohol series. Briefly, slides were placed for 10 min each in 100%, 96% and 70% EtOH followed by 10 min ddH_2O. Subsequently, sections were incubated for 7 min in Mayer's hemalum solution to stain nuclei and then washed for 15 min with lukewarm running tap water. To stain cytoplasm, slides were afterwards incubated for 3 min in Eosin Y solution. This was followed by short washes in ddH_2O and 70% EtOH, 90 sec in 96% EtOH and 150 sec in 100% EtOH. After a final incubation for at least 5 min in Roti®-Histol, sections were mounted with Roti®-Histokit and coverslips and air dried before analysis with the Zeiss Axio Observer.

3.2.4.3 PAS (Periodic-Acid-Schiff)-reaction

Deparaffinisation and initial rehydration were done as described above. Sections were then incubated for 8 min in periodic acid solution, washed in running tap water and rinsed in ddH_2O. This was followed by 15 min incubation with Schiff reagent to stain glycogen and polysaccharides, and a washing step for 5 min in running tap water. After rinsing in ddH_2O, sections were counterstained in Mayer's hemalum solution and washed with lukewarm running tap water for 3 min to stain nuclei. This was followed by short washes in ddH_2O and twice in 70% EtOH, 1 min in 96% EtOH and twice for 5 min in 100% EtOH. After a final incubation for 15 min in Roti®-Histol, sections were mounted with Roti®-Histokit and coverslips and air dried before analysis with the Zeiss Axio Observer.

3.2.4.4 Masson-Trichrome Staining

The Masson-Trichrome-Staining was performed in the Pathology Department, University Hospital of Regensburg.

3.2.4.5 FoxP3 – Staining

Deparaffinisation and initial rehydration of skin or spleen sections as staining control were done as described above. Then, sections were cooked for 20 min in citrate buffer in a steam cooker and then cooled down for 20 - 30 min at RT. This was followed by three washing steps in ddH_2O for each 5 min and a 10 min incubation step in 3% H_2O_2 solution. Again,

sections were washed three times in ddH_2O for each 5 min and then once in T-TBS for 5 min. Sections were then shortly drained and blocked with 5% goat serum in T-TBS for 1h at RT in a wet chamber. Afterwards, the primary antibody (FoxP3 or corresponding Isotype control) were added in a 1:50 dilution in blocking serum and incubated over night at 4°C in a wet chamber. On the consecutive day, sections were washed three times in T-TBS for 5 min. Then, the secondary antibody (Goat anti- rat IgG1 Fab2-B, Biotin Conjugated) was added in a 1:100 dilution in blocking serum and incubated for 1h at RT in a wet chamber. This was followed by three washing steps in T-TBS for each 5 min and an incubation step for 30 min at RT with HRP reagent. After three more washes in T-TBS for 5 min each, sections were stained for 90s with DAB reagent, reaction was stopped in ddH_2O. Counterstaining was done with Mayer's hemalum solution for 8 min and a washing step with lukewarm running tap water for 10 min to stain nuclei. Then sections were mounted with Aquatex and coverslips and air dried before analysis with the Zeiss Axio Observer.

3.2.5 Statistics:

Statistical tests were performed as indicated in the respective figure legend.
For statistical analysis of qPCR data, refer to section 1.2.2.3.2.

4 Results

4.1 Oral administration of Tacrolimus in mice

In later experiments, the effect of Tacrolimus on skin allograft survival in marginal states of allograft acceptance was examined. Therefore, a subtherapeutic Tacrolimus therapy had to be established. For this, it was necessary to keep mice over many weeks on controllable serum levels of Tacrolimus, therefore a reliable dosing protocol had to be developed. This can be achieved by different ways, such as oral administration, repeated i.p. injections, implanting osmotic pumps or subcutaneous drug pellets. Over the recent years, our group has been working successfully with the oral administration of drugs. Considering this and the minor stress of this application route, specially produced food supplemented with desired doses of Tacrolimus was given as the daily diet *ad libitum.* Thus, food containing 50 mg, 75 mg, 100 mg and 150 mg Tacrolimus per kilogram of food was fed to male C57BL/6 mice over a time span of 153 days and serum levels of Tacrolimus were measured. Since it was anticipated that additional bolus injections were necessary to quickly achieve measurable serum levels, mice received i.p. injections of Tacrolimus on three consecutive days starting on the day of drug food administration. The mean serum levels increased dose-dependently, mean Tacrolimus serum levels of 0.88 µg/l ± 0.2, 3.7 µg/l ± 1.4, 8.94 µg/l ± 5.42 and 22.04 µg/l ± 11.2, respectively, were achieved (Figure 13).

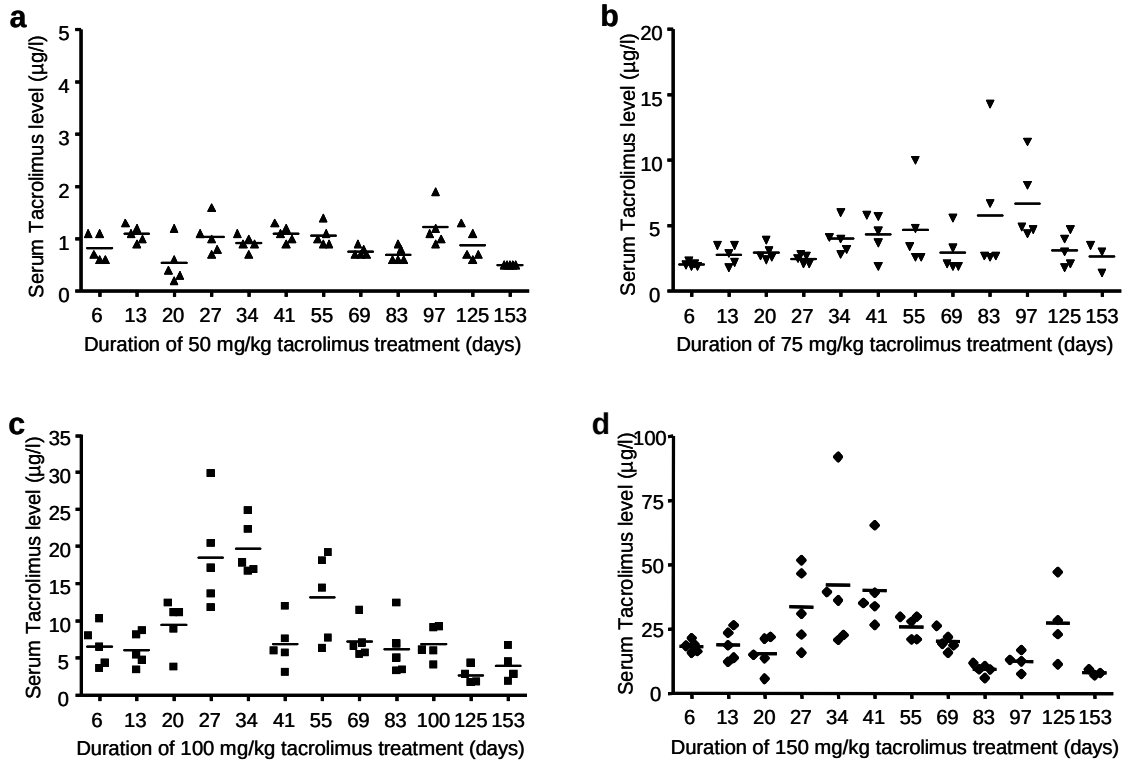

Figure 13: Relationship between oral Tacrolimus administration and serum levels in C57BL/6 mice. B/6 mice were set on food containing (a) 50 mg,(b) 75 mg,(c) 100 mg, or (d) 150 mg Tacrolimus per kilogram food. Mean serum levels of Tacrolimus are displayed in scatter plots.

Over a time span of 10 weeks, clearly titratable serum levels could be distinguished. For example, on d20 after induction, mice treated with 50 mg, 75 mg, 100 mg and 150 mg per kilogram food had serum levels of 0.5 µg/l ± 0.4, 2.9 µg/l ± 0.59, 9.5 µg/l ± 3.4 and 15.6 µg/l ± 6.6. However, there are fluctuations in the measured values throughout the groups, which led to an overlap in serum levels of mice fed with 75 mg/kg and 100 mg/kg Tacrolimus-food at the last four measurements starting on d83. These fluctuations might have occurred for several reasons, such as amount of ingested food shortly before bleeding or absorption of Tacrolimus in individual mice. Interestingly, the mean serum levels in mice receiving higher doses of 100 mg/kg or 150 mg/kg peaked early on d34 after induction of the drug therapy. This might correspond to toxicity effects of these doses. Taken together, it was shown that long-term treatment of mice with food at doses of 50 mg, 75 mg, 100 mg and 150 mg Tacrolimus per kilogram leads to titratable, dose-dependently increasing serum levels.

4.2 Toxic effects of Tacrolimus administration

Tacrolimus as immunosuppressive therapy is very effective in preventing acute rejection, yet it has many unwanted side effects, such as increased susceptibility to infection, malignancies, and nephrotoxicity [158]. Specifically, it was planned to investigate toxic effects of the established oral Tacrolimus administration. As a first read-out parameter, body weight of the mice treated with 50 mg, 75 mg, 100 mg and 150 mg Tacrolimus per kilogram food was determined (Figure 14). Whereas weight curves for mice fed with doses up to 100 mg/kg show a similar course, mice treated with 150 mg/kg Tacrolimus displayed reduced weight gain after the first month of treatment (150 mg/kg vs. 50 mg/kg: p = 0.023; 150 mg/kg vs. 75 mg/kg: p = 0.068; 150 mg/kg vs. 100 mg/kg: p = 0.038).

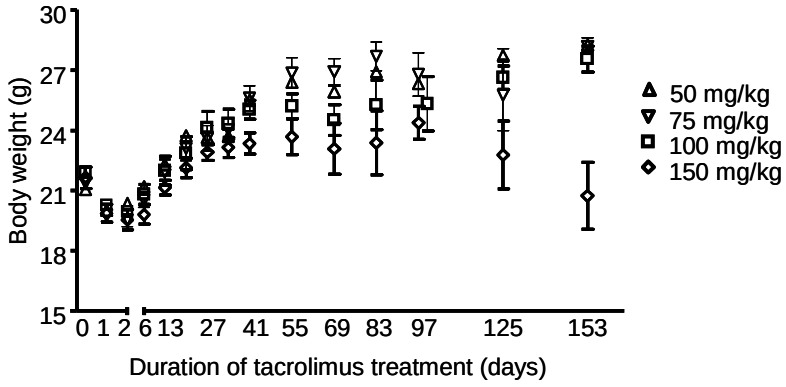

Figure 14: Weight curve in C57BL/6 mice during oral administration of Tacrolimus. Weight curves of mice from Figure13. Mean body weight is displayed with SD.

The aforementioned Tacrolimus nephrotoxicity has been reported to be dependent on the dosing regimen and occurs in 17%- 44% of kidney transplant patients [221,222]. Increased serum creatinine is a consequence of loss of renal function though nephrotoxicity in humans and mice [223,224]. Serum creatinine was measured after 50 days of treatment in untreated mice and sentinel mice treated with 75 mg/kg, 100 mg/kg or 150 mg/kg Tacrolimus food (Figure 15). A trend towards higher levels of serum creatinine in mice receiving higher doses of Tacrolimus indicates mild (100 mg/kg) or enhanced (150 mg/kg) nephrotoxicity compared to no changes in mice treated with 75 mg/kg Tacrolimus.

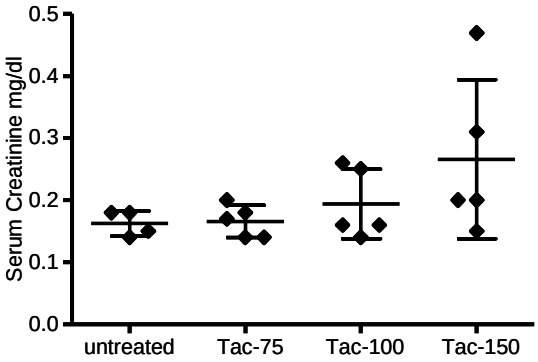

Figure 15: Serum Creatinine levels in sentinel mice. C57BL/6 mice were treated with oral Tacrolimus at 75, 100 and 150 mg/kg or left untreated. On d50, Serum Creatinine was measured. Scatter plots display mean and SD. The observed changes are statistically not significant (Student's T-Test, pairwise comparison with untreated group).

Chronic Calcineurin-Inhibitor (CNI) – induced nephrotoxicity is histopathologically characterised by hyaline arteriolopathy, interstitial fibrosis, and tubular atrophy, and occurring glomerular changes [224,225]. In order to examine CNI-induced nephrotoxicity, kidneys of mice treated with 50 mg, 75 mg, 100 mg and 150 mg Tacrolimus per kilogram food were removed after ~5 months (d150 –d153), H&E stained and histopathologically analysed. The analysis was done with the help of a pathologist, PD Dr. med. P. Rümmele (University Hospital of Regensburg). In none of the samples, regardless of the Tacrolimus treatment, CNI-typical damage was observed. However, in 3/4 kidneys from mice treated with 150 mg/kg Tacrolimus, tubular necrosis and inclusion bodies were observed (Figure 16).

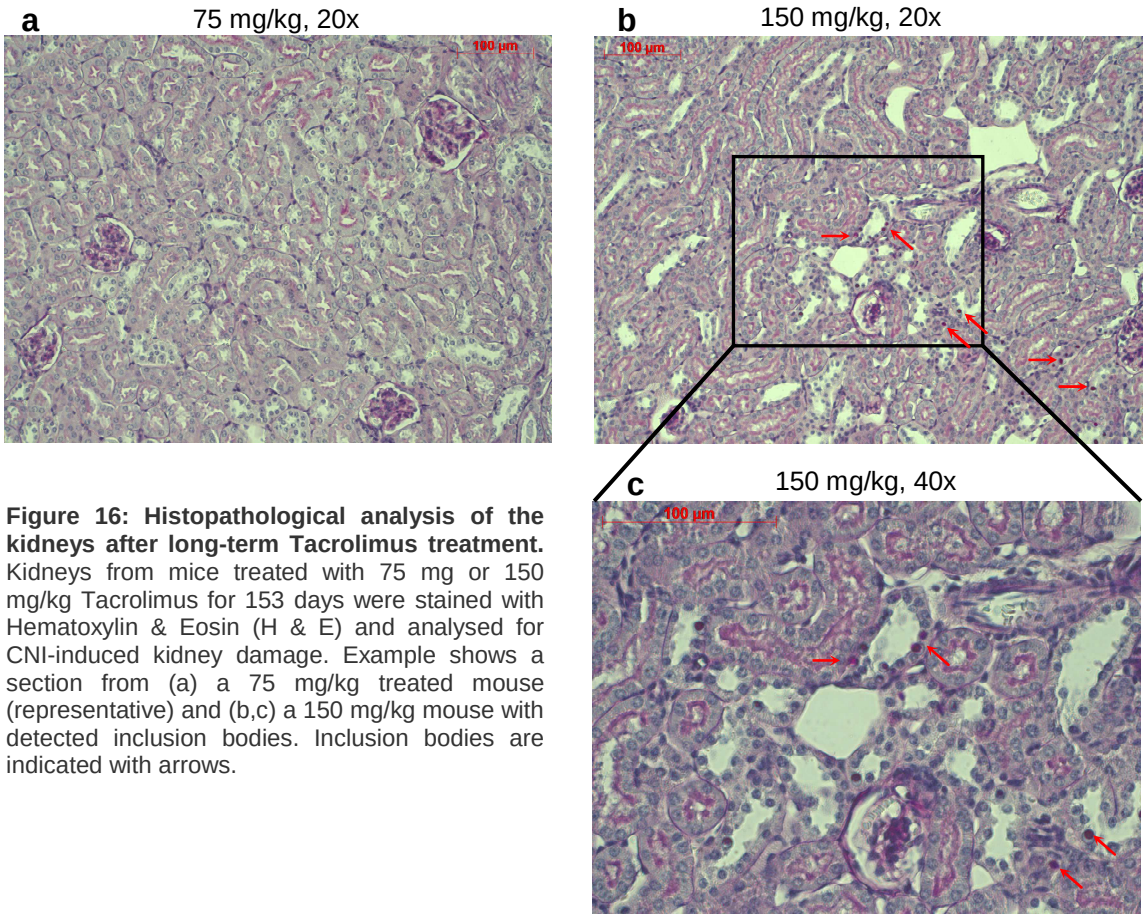

Figure 16: Histopathological analysis of the kidneys after long-term Tacrolimus treatment. Kidneys from mice treated with 75 mg or 150 mg/kg Tacrolimus for 153 days were stained with Hematoxylin & Eosin (H & E) and analysed for CNI-induced kidney damage. Example shows a section from (a) a 75 mg/kg treated mouse (representative) and (b,c) a 150 mg/kg mouse with detected inclusion bodies. Inclusion bodies are indicated with arrows.

Treatment with Tacrolimus in high doses has been reported as a risk factor for polyoma virus (BKV, JCV and SV-40) infection, a major complication after renal transplantation that can also occur in native kidneys. The presence of inclusion bodies and tubular injury morphologically defines BKV nephropathy [226]. It is possible that the mice might have been subjected to virus-infection due to the immunosuppression with high doses of Tacrolimus. Therefore, histological sections of the kidneys from the presumably infected mice were tested for polyoma virus (SV-40), Adenovirus and Cytomegalovirus (tests and analysis were performed by the Department of Pathology). None of the stainings gave positive (i.e. specific) results. Taken together, kidney damage is observed in mice treated with therapeutic doses of Tacrolimus (150 mg/kg), whilst mice receiving low doses of Tacrolimus (75 mg/kg) do not show any signs for kidney damage. Thus, the dose of 75 mg Tacrolimus per kg food does not seem to be nephrotoxic after long-term treatment.

4.3 Introducing Tacrolimus monotherapy into a skin transplantation model

An experimental model of marginal states of allograft acceptance must reflect the balance between effector and regulatory response where neither is predominant. Therefore, a model had to be established where neither the low-dose Tacrolimus monotherapy nor the weak regulation inducing therapy alone lead to allograft survival comparable to true tolerance. With the following experiments it was determined what doses of Tacrolimus in the BALB/c-to-C57BL/6- skin transplantation model did not prolong allograft survival, and were thus subtherapeutic. When Tacrolimus monotherapy was started seven days prior to transplantation, only doses of 100 and 150 mg/kg significantly prolonged allograft survival (Fig 17a: MST 100mg/kg or 150 mg/kg vs. Untreated: 13.1 ± 1.79 or 135 ± 0 days vs. 8.3 ± 0.42 days; $p = 0.003$ or $p < 0.001$). Tacrolimus food administered in doses of 25, 50, and 75 mg per kg food did not have any effect on the allograft survival (MST: 8.7 ± 0.33, 9.1 ± 0.52 and 8.3 ± 0.42, respectively). When Tacrolimus monotherapy was started on the day of transplantation, both 100 and 150 mg/kg doses prolonged allograft survival significantly compared to the untreated group (Fig 17b: MST: 12.2 ± 0.74 or 64 ± 4.14 days vs. 8.3 ± 0.42 days; $p = 0.003$ or $p = 0.001$). Administration of Tacrolimus starting seven days post-transplantation did not lead to significant prolongation of allograft survival in both doses of 75 and 150 mg/kg (Fig 17c: MST: 11 ± 0.78 or 9.6 ± 0.6 days vs. 8.3 ± 0.42 days).

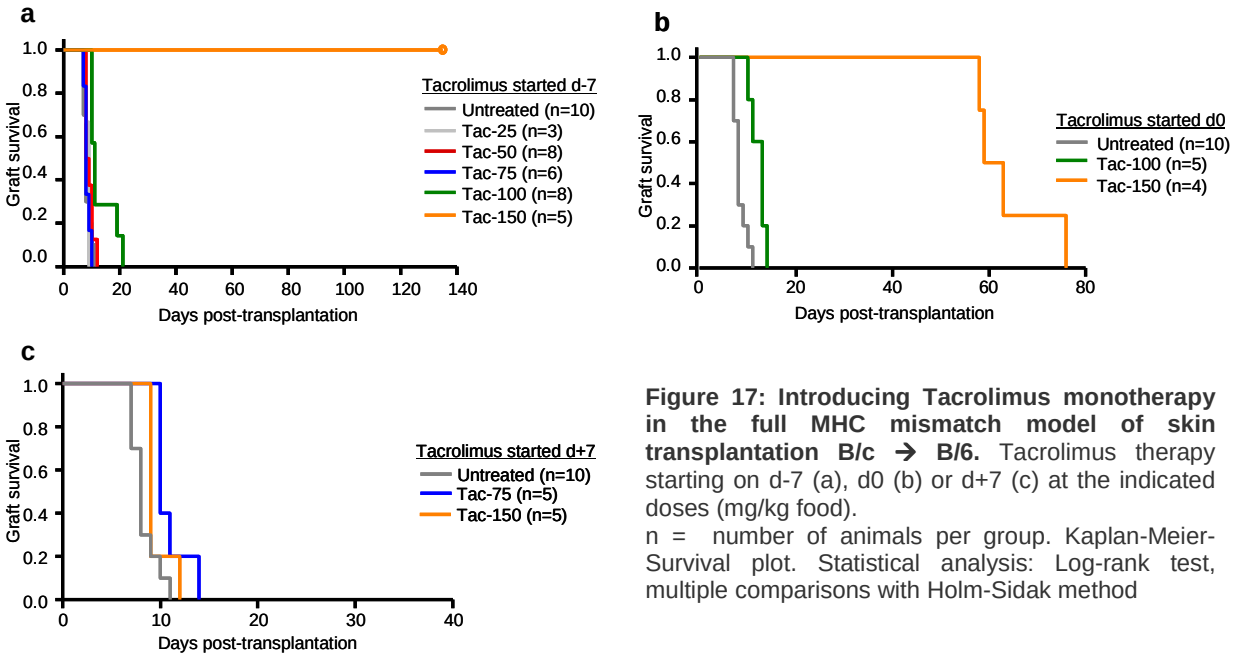

Figure 17: Introducing Tacrolimus monotherapy in the full MHC mismatch model of skin transplantation B/c → B/6. Tacrolimus therapy starting on d-7 (a), d0 (b) or d+7 (c) at the indicated doses (mg/kg food).
n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test, multiple comparisons with Holm-Sidak method

When given prior to or post transplantation, 75 mg/kg Tacrolimus had no observable effect, showing no significant prolongation of allograft survival in a BALB/c-to-C57BL/6 - skin

transplantation model. In contrast, 100 mg/kg doses led to a small, albeit significantly prolongation when administered prior to or at the time of transplantation. Started on d-7, Tacrolimus doses of 150 mg/kg had a pronounced significant effect on the allograft survival, therefore, this dose given at early time points is considered as therapeutic.

4.4 Defining a weak regulation inducing therapy

For the combination of a low-dose immunosuppression therapy with a weak regulation inducing therapy to establish a model of marginal states of allograft acceptance, it was necessary to define a weak regulation inducing therapy that alone would not lead to indefinite allograft survival comparable. Therefore, a model of strong regulation induction in the BALB/c-to-C57BL/6 – skin transplant combination was (B/c → B/6) established as a "standard". The group of Li described the use of triple costimulatory blockade to induce tolerance in a mouse skin transplant model [227], in which anti-CD154 and anti-OX40L antibodies and the fusion protein CTLA4-Ig were administered for one week following transplantation. With this treatment in the strain combination of DBA/2-to-C57BL/6 (DBA → B/6), Li et al. observed survival of 100% grafts over 100 days [227].

Since Tacrolimus monotherapy had been established in C57BL/6 mice, Li's protocol was applied in the B/c → B/6 – strain combination (MST vs. Untreated: 69.7 ± 11 vs. 8.3 ± 0.42 days; $p < 0.00001$; Figure 18). Yet, this significant effect did not match the graft survival described by Li et al. Allograft survival of a specific organ varies between different donor-recipient-strain combinations also in the stringent full MHC-mismatch model [228]. Hence, the triple costimulatory blockade was also used in a C57BL/6-to-BALB/c – strain combination (where BALB/c is the recipient, B/6 → B/c) and in the strain combination of DBA/2-to-C57BL/6 (DBA → B/6) that had been used in Li's experiment. Briefly, triple costimulatory blockade in these strain combinations had a similar effect than in the B/c → B/6 – model. Mean survival times of 57.2 ± 25 vs. 8.5 ± 4.6 days; $p < 0.001$ (B/6 → B/c) and 61.2 ± 25.4 vs. 11.8 ± 1 days; $p < 0.001$ (DBA → B/6) were achieved (Figure 18).

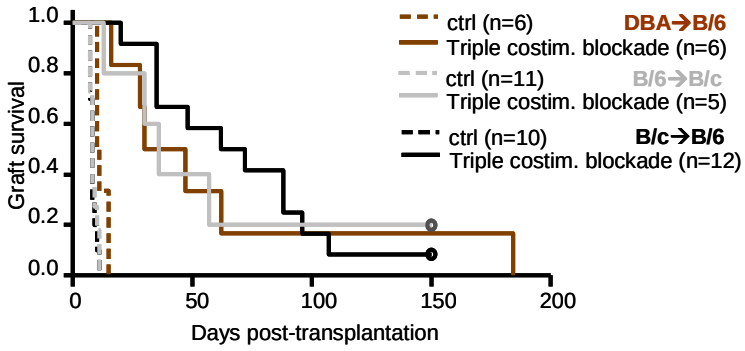

Figure 18: Triple costimulatory blockade in three strain combinations. Recipient mice were treated with anti-CD154, anti-OX40L and CTLA4-Ig (Triple costimulatory blockade) starting on the day of transplantation. ctrl: mice received PBS injections. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

Triple costimulatory blockade reproducibly prolongs allograft survival in a fully MHC-mismatched skin transplant model, yet, it does not lead to indefinite graft survival. Nonetheless, the B/c → B/6 – combination led to the most significant prolongation of graft survival. Thus, the mean survival time of skin graft in this combination will be used as the reference to define weak regulation-inducing protocols.

In order to achieve this less prolonged graft survival, mice were treated with anti-CD154 antibody alone or with anti-CD154 antibody in combination with a donor-specific transfusion (DST) of BALB/c-splenocytes. Treatment with only one component of the triple costimulatory blockade was considered to lead to a minor prolongation of the graft survival and in other transplantations models it has been shown to prolong allograft survival [194]. It is described in the literature that the combination of a costimulatory blockade with a DST enhances the prolongation effect [195]. As shown in Figure 19, the injections of anti-CD154 antibody on day 0, 1, 3 and 6 relative to skin transplantation lead to a MST of 21.8 ± 3.2 days, which is a significant prolongation of the allograft survival compared to untreated mice ($p < 0.001$). Treatment with the antibody in combination with a DST on the day of transplantation increases the graft survival time significantly (MST anti-CD154 + DST 40 ± 6; $p < 0.001$) and more profoundly than treatment with anti-CD154 alone ($p = 0.003$).

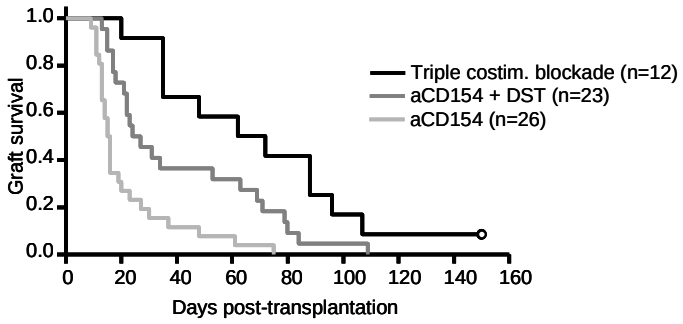

Figure 19: Treatment with anti-CD154 and DST is a weak regulation inducing protocol. Recipient mice were treated with anti-CD154 alone or in combination with DST, starting on the day of transplantation. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test, multiple comparisons with Holm-Sidak method

Yet, both treatment options did not result in the allograft survival prolongation achieved with the triple costimulatory blockade (anti-CD154 vs. triple costimulatory blockade: $p < 0.001$ and anti-CD154 + DST vs. triple costimulatory blockade: $p = 0.018$). Hence, both the treatment with anti-CD154 antibody alone or in combination with a DST is considered as weak regulation-inducing protocols.

4.5 Combination of low-dose Tacrolimus therapy with a weak regulation-inducing protocol

After both low-dose Tacrolimus therapy and weak regulation-inducing protocols were established, these treatments were combined to build a model of marginal conditions in allograft acceptance. First, Tacrolimus in doses of 50 mg/kg in the food was adjoined to induction therapy with anti-CD154 antibody. By giving the Tacrolimus 7 days before transplantation measureable drug levels at the time of the surgery could be ensured (Figure 13). When Tacrolimus therapy was introduced on d+7, interference of Tacrolimus with T reg induction should be avoided. Treatment with anti-CD154 has been shown to induce regulatory T cell responses [229] and it was assumed that this may play a role in this model. T regs are dependent on IL-2 and the expression of this cytokine is inhibited by Tacrolimus [170,230].

Mice received Tacrolimus therapy starting either 7 days before or 7 days after skin transplantation. The antibody doses were administered as usual on d0, 1, 3 and 6 respective to the day of transplantation (d0). As shown in Figure 20a, allograft survival was not significantly different in mice treated with anti-CD154 and Tacrolimus at 50 mg/kg starting on d -7 and in mice with antibody-treatment alone (MST anti-CD154 + TAC-50 on d-7 vs. anti-CD154 alone: 26.7 ± 6.7 vs. 21.8 ± 3.2 days; p = 0.81). The delayed introduction of Tacrolimus on d+7 had a significant further effect on prolongation of graft survival compared to antibody-treatment alone (MST anti-CD154 + TAC-50 on d+7 vs. anti-CD154 alone: 40.2 ± 6.9 vs. 21.8 ± 3.2 days; p = 0.012).

Further, the weak-regulation inducing protocol using anti-CD154 and DST was combined with Tacrolimus at 50 mg/kg on d-7 or d+7 relative to transplantation. Administration of Tacrolimus starting one week prior to transplantation did not have an additional graft survival prolonging effect (MST anti-CD154 + DST + TAC-50 on d-7 vs. anti-CD154 + DST: 35.7 ± 10 vs. 40 ± 6 days; p = 0.65, Figure 20b). In contrast, the late induction of Tacrolimus treatment starting seven days post transplantation did enhance graft survival compared to treatment with anti-CD154 and DST (MST anti-CD154 + TAC-50 on d+7 vs. anti-CD154 + DST: 64.5 ± 4.7 vs. 40 ± 6 days; p = 0.004).

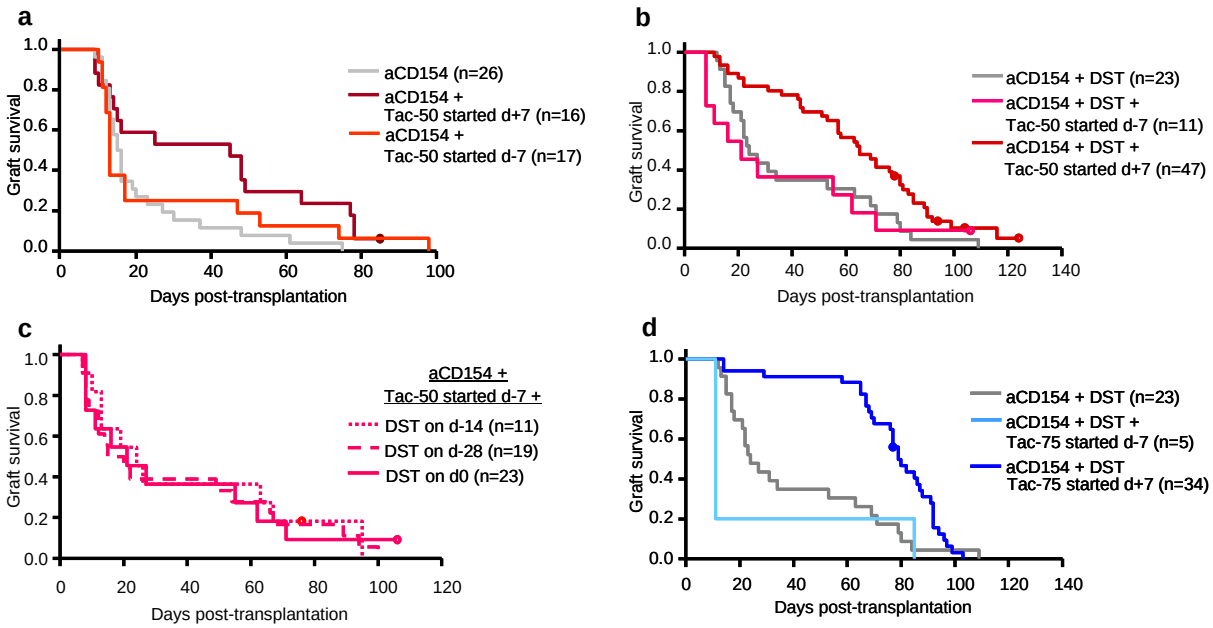

Figure 20: Low-dose Tacrolimus therapy combined with a weak regulation inducing protocol does significantly prolong allograft survival. Recipient mice were treated with anti-CD154 alone or in combination with DST; this was combined with Tacrolimus therapy starting on d-7 or d7. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test, multiple comparisons with Holm-Sidak method

Taking this difference in early and late Tacrolimus administration into account, it was presumed that the presence of immunosuppression at the time of treatment with anti-CD154 + DST does not have any additional beneficial effect on the induced regulation. Therefore, mice were treated with a DST together with a single dose of anti-CD154 either 28 or 14 days prior transplantation. Tacrolimus therapy was then induced on d -7 and mice were given the usual anti-CD154 treatment beginning on the day of transplantation. However, no effect on graft survival was observed when DST was advanced (MST anti-CD154 + DST (d-28) + TAC-50 on d-7 vs. anti-CD154 + DST (d-14) + TAC-50 on d-7 vs. anti-CD154 + DST + TAC-50: 39.3 ± 10.3 vs. 35.2 ± 7.6 days vs. 35.7 ± 10 days, respectively; p = 0.82 and 0.72, Figure 20c). Thus, we conclude that the effect of Tacrolimus enhancing the prolongation of allograft survival caused by anti-CD154 + DST only occurs when Tacrolimus is introduced after the transplantation.

The question arose whether Tacrolimus in higher doses would, in combination with the anti-CD154 + DST protocol, lead to further prolonged allograft survival. Therefore, Tacrolimus food at 75 mg/kg was given starting on d -7 or d +7, together with anti-CD154 + DST treatment on the day of transplantation. As expected, the early induction of Tacrolimus at 75 mg/kg did not have an effect on graft survival prolongation caused by anti-CD154 + DST treatment (MST anti-CD154 + TAC-75 on d-7 vs. anti-CD154 + DST: 25.8 ± 14.8 vs. 40 ± 6 days; p = 0.35, Figure 20d). When Tacrolimus at 75 mg/kg was administered starting one

week after transplantation, allograft survival was significantly prolonged (MST anti-CD154 + TAC-75 on d+7 vs. anti-CD154 + DST: 76.4 ± 3.7 vs. 40 ± 6 days; $p < 0.001$).

To summarise, Tacrolimus monotherapy combined with either anti-CD154 or anti-CD154 + DST significantly prolongs allograft survival, when the drug is administered 7 days after tolerance induction and transplantation. Graft survival is superior when regulation is induced by the combination of anti-CD154 treatment with a DST.

4.6 Dose-dependent effect of Tacrolimus and two modes of action

The weak regulation-inducing protocol based on anti-CD154 injections and DST was combined with subtherapeutic doses of Tacrolimus at 50, 75 and 100 mg/kg in the food, as shown in Figure 21. Thus, the allograft survival prolongation caused by anti-CD154 + DST treatment (MST 40 ± 6 days) was significantly increased in a dose-dependent effect to 64.5 ± 4.7 days with the 50 mg/kg treatment ($p = 0.035$) and to 76.4 ± 3.7 days with 75 mg/kg Tacrolimus food ($p < 0.001$). When Tacrolimus was administered at 100 mg/kg food, the mean survival time was 134 ± 5.5 days. At d150, the experiment was stopped with over 50% of thus treated mice still had an intact allograft with no visible signs of rejection.

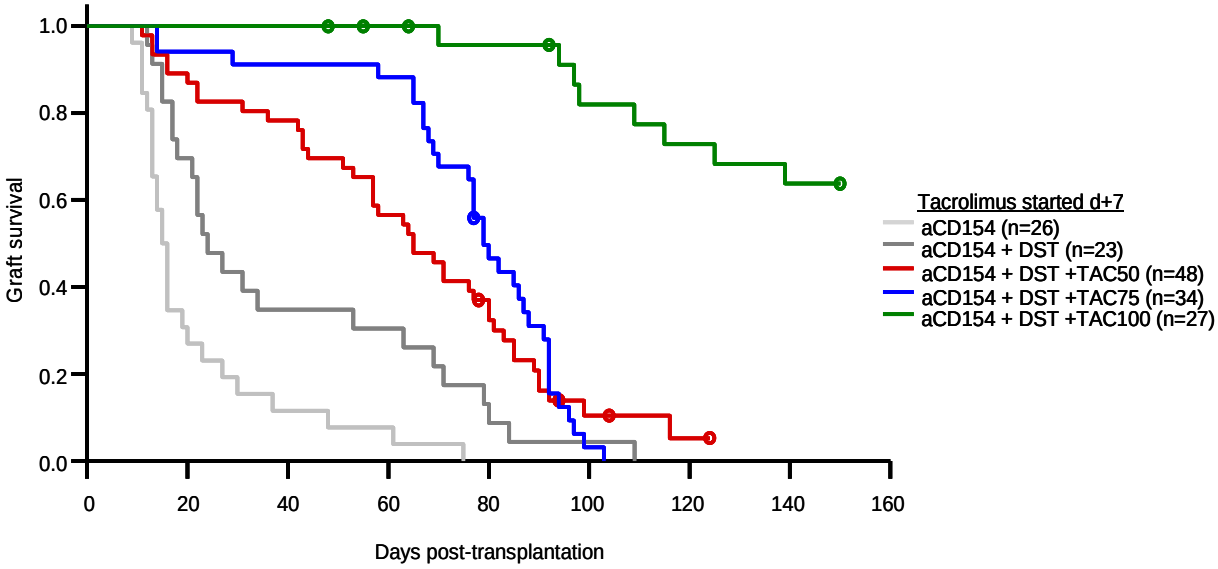

Figure 21: Dose-dependency. Recipient mice were treated with anti-CD154 alone or in combination with DST; this was combined with Tacrolimus therapy at doses of 50, 75 or 100 mg/kg food starting on d7. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test, multiple comparisons with Holm-Sidak method

The data obtained with different doses of Tacrolimus is depicted in a dose-response plot in Figure 22. Thus, the data is separated into four groups: 1) Tacrolimus monotherapy started one week prior to transplantation, 2) on the day of transplantation, 3) one week after transplantation and 4) Tacrolimus therapy in combination with tolerance induction by anti-

CD154 + DST. When started on the day of transplantation or one week prior, Tacrolimus monotherapy at 100 mg/kg leads to marginal prolongation of allograft survival, whereas doses of 150 mg/kg significantly prolonged graft survival (Figure 17). Interestingly, when Tacrolimus is administered on week post transplantation, synergism with the tolerance-inducing therapy can be observed. More precisely, by combining low-doses of Tacrolimus with the tolerance inducing therapy, the achieved graft survival was comparable to that obtained with therapeutic doses (150 mg/kg) Tacrolimus.

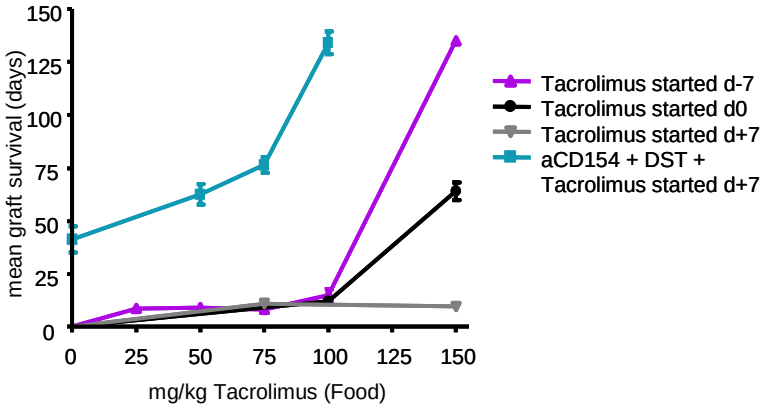

Figure 22: Synergism. Dose-response curves showing data obtained from mice treated with Tacrolimus in doses of 25, 50, 75, 100 or 150 mg/kg food at indicated time points, with or without combination with anti-CD154 + DST.

Tacrolimus in this model shows two modes of action: The allograft survival prolongation effect of high doses of Tacrolimus at 150 mg/kg decreases drastically between the introduction at one week prior to transplantation to introduction one week post transplantation (Figure 23). In contrast to this, Tacrolimus at low doses in combination with tolerance induction leads to graft survival prolongation when it is introduced at the time of transplantation or up to 7 days later (MST induction on d0 vs. d+3 vs. d+7: 74.3 ± 2.7 vs. 75.9 ± 1.7 days vs. 76.4 ± 3.7 days, respectively; p = 0.8 and 0.09).

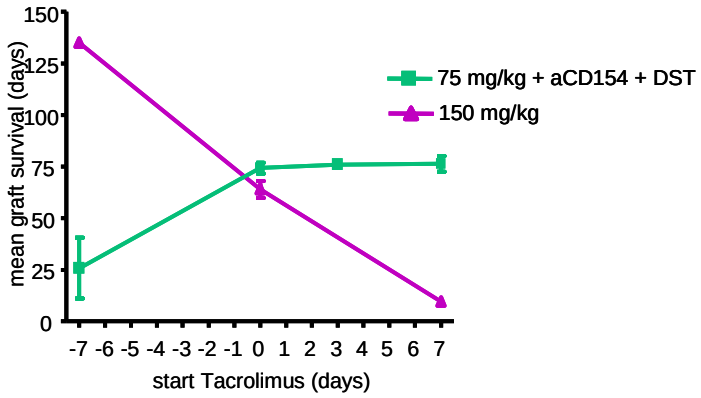

Figure 23: Tacrolimus has two modes of action. Immunosuppressive effect of Tacrolimus (treatment with Tacrolimus at 150 mg/kg food) and regulation supportive effect (treatment with anti-CD154 + DST and Tacrolimus at 75 mg/kg food). n = min. 4 per data point. Statistical analysis: Log-rank test

Taken together, when given at high doses and at early time points (preferably before transplantation), Tacrolimus is a general immunosuppressive drug. Surprisingly, Tacrolimus can also support regulation in this model, when it is given in low doses and at later time points (at the time of transplantation or up to one week later).

4.7 Tacrolimus in low-doses relatively enhances suppression by T regs

One possible explanation for enhanced regulation-dependent allograft survival under low-dose Tacrolimus treatment is that T regs are less susceptible to suppression by low-dose Tacrolimus than effector T cells. This hypothesis was tested using in vitro suppression assays, where $CD4^+CD25^-$ effector T cells (Teff) were cultured alone or 1:1 with $CD4^+CD25^+$ regulatory T cells (T reg) in the absence or presence of Tacrolimus at low doses (0.25 – 2 ng/ml). Suppression of effector T cells by regulatory T cells was detected by measuring the expression of the proinflammatory cytokine IFNγ by ELISA.

The addition of Tacrolimus alone led to dose-dependent suppression of the IFNγ expression (Figure 24). Addition of Tacrolimus to cocultures of Teffs and T regs led to significantly enhanced suppression of the IFNγ response compared to cocultured Teff and T reg without Tacrolimus (% of suppression: 0 ng/ml Tacrolimus vs. 0.25 ng/ml Tacrolimus: 50.8% ± 17.7 vs. 85.5% ± 8.7, $p = 0.0124$). At doses of 0.5 ng/ml Tacrolimus, the suppression effect was further increased (% of suppression: 0 ng/ml Tacrolimus vs. 0.5 ng/ml Tacrolimus: 50.8% ± 17.7 vs. 90.4% ± 4.5, $p = 0.0049$). When Tacrolimus doses were additionally increased, the IFNγ expression in the Teff-T reg-cocultures in some experiments was even suppressed towards the limit of detection.

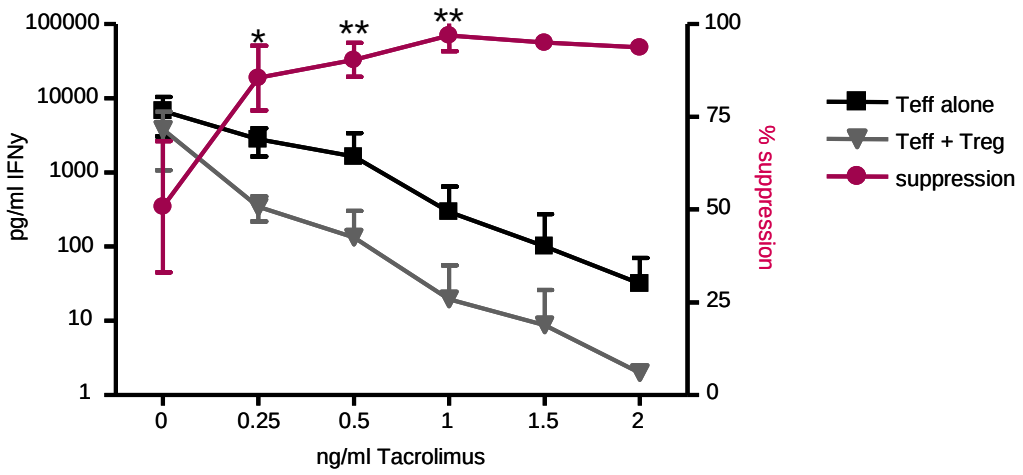

Figure 24: T reg Suppression assay. $CD4^+CD25^-$ effector cells (Teff) were cultured either alone or in a 1:1 ratio with $CD4^+CD25^+$ regulatory cells (T reg) under anti-CD3/anti-CD28 polyclonal stimulatory conditions and Tacrolimus addition as indicated. After 48h, supernatants were harvested and IFNγ was measured by ELISA. Mean and SD of 4 independent experiments are shown. The black and grey curve show the IFNγ production; the red line indicates percentage of suppression. Statistical analysis: pairwise comparison with 0 ng/ml Tacrolimus condition with Student's T-Test (* $p < 0.05$, ** $p < 0.01$)

These *in vitro* data show that Tacrolimus in low doses suppresses the IFNγ expression of effector T cells and disproportionately increases the suppressive effect of regulatory T cells in cocultures. If the low doses of Tacrolimus used in the *in vitro* assay did reflect the *in vivo* levels of Tacrolimus in the lymphoid tissue, the observed effect that low doses of Tacrolimus enhance suppression by T regs could account for the enhanced survival prolongation effect of subtherapeutic Tacrolimus observed in our *in vivo* model.

To facilitate readability, the treatment with anti-CD154 + DST + Tacrolimus at 75 mg/kg started on d+7 will be referred to as MD-75, where M stands for the anti-CD154 clone MR-1 and D for DST.

4.8 Allograft acceptance vs. chronic rejection

The previous experiments in mice treated with anti-CD154 + DST and Tac-75 (MD-75) indicated that regulation- and immunosuppression-dependent marginal states of allograft acceptance exist. However, despite a long-term acceptance of allografts in MD-75 mice, rejection eventually sets in at about 60 days (Figure 21). This late rejection raised the question whether the grafts were lost due to delayed acute rejection or chronic rejection. The features of acute and chronic rejection are not equal and can be distinguished by: 1) gross pathology, 2) histopathology and 3) molecular markers as detailed in the introduction. Thus, stable allografts from MD-75 mice at day 50 post transplantation were monitored for signs of acute or chronic rejection. As comparison for acute rejection, B/6 mice were transplanted with B/c allografts and received no further treatment. For chronic rejection control, a minor antigen (H-Y) mismatch model was used: B/6 female mice received a graft from B/6 male donors without additional treatment.

MD-75 allografts undergoing rejection resemble acutely rejecting allografts in their macroscopic appearance (Figure 25). The sudden onset of acute rejection in B/c-to-B/6 transplant recipients without further treatment (acute rejectors) on day 7 (± 1d) is mostly characterised by haemorrhagic, necrotic lesions in surrounding normal graft tissue with full hair growth. These necrotic lesions spread over the graft tissue until the peak of rejection on day 9 (± 1d), when the lesions often cover the complete graft tissue. In contrast to this, grafts in the chronic rejection model were rejected with much slower kinetics. Slowly progressing hair loss and scarring of the graft tissue were observed, whilst necrotic lesions did not occur.

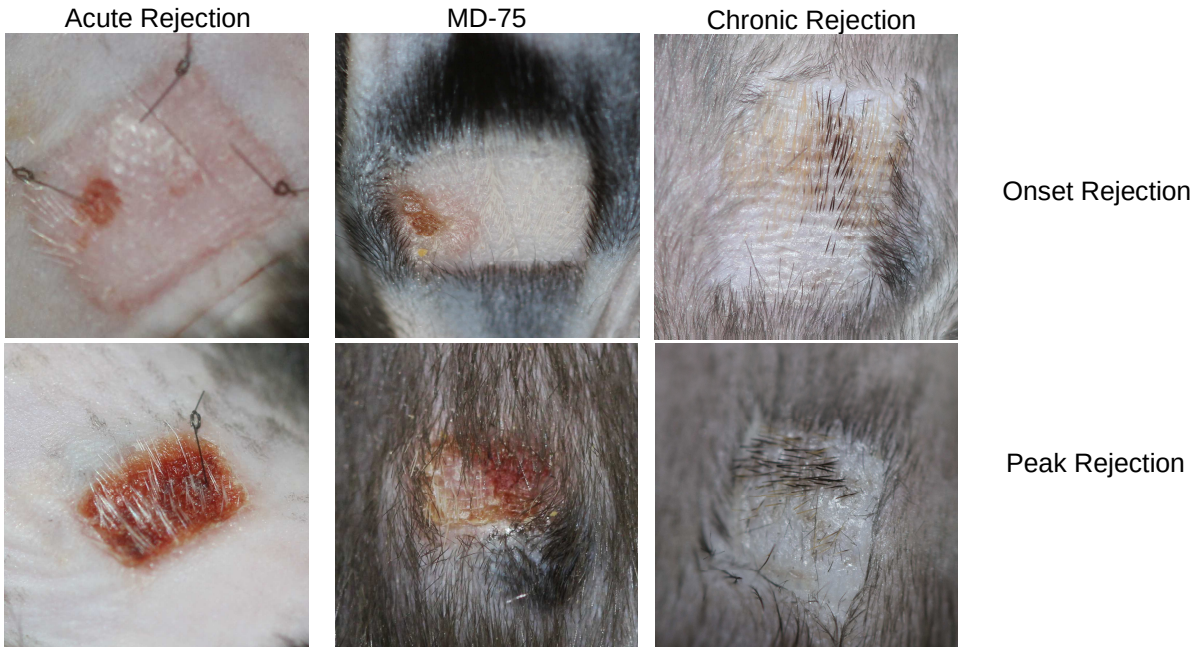

Figure 25: Macroscopic pathology of rejection. Both the onset (upper panel) and peak (lower panel) of rejection are shown. Left: Rejection in a B/c-to-B/6 skin transplant model without treatment (acute rejection). Middle: Rejection in MD-75 mice. Right: Rejection in minor-mismatch (male-to-female) model (chronic rejection). Photos are representative.

Further, the grafts were examined microscopically. Analysis of H&E stained samples was done with the help of a pathologist, PD Dr. med. P. Rümmele (University Hospital of Regensburg). Acutely rejecting grafts were harvested when necrotic changes were macroscopically manifest, but before haemorrhagic lesions covered the whole graft tissue. In all samples, thickening of the skin in total and the epidermis in particular due to massive infiltration of inflammatory cells (mostly plasma cells and lymphocytes) was obvious (Figure 26a). The skin structure was massively damaged with skin adnexal structures mostly destroyed. Polynuclear giant cells were detected; the inflammatory cell infiltrate caused apoptosis of keratinocytes, fibrinoid necrosis and partial epidermolysis.

Skin grafts from a minor antigen mismatch model (male-to-female) were harvested upon detection of hair loss and scarring approximately one month post transplantation. These grafts underwent chronic rejection as microscopically characterised by manifest sclerosis and fibrosis (Figure 26b). The skin graft was thickened and the skin adnexal structures were dissolving. A minor cell infiltrate was present in the epidermis, which overlaid a belt of sclerosis. Underneath the sclerotic zone, an infiltration of inflammatory cells was present. This stratification is pathognomic for chronic rejection.

Additionally, allografts from MD-75 mice on d50, when no signs of rejection were observed, were compared to syngeneic grafts (B/c to B/c) on d150 (Figure 26c + d). The syngeneic

grafts did not show any abnormalities except minimal infiltration of leukocytes in some samples, without any signs of fibrosis or destructive changes. MD-75 allografts on d50 displayed intact skin adnexal structures (hair follicles, sebaceous glands) with an intact epidermis. Neither apoptosis nor necrosis was found. Yet, infiltration of leukocytes occurred and was mostly perivascular with single cells in the epidermis. This infiltrate was not connected with destruction of tissue, but rather seemed non-aggressive. Minor fibrosis or sclerosis were occasionally detected, but not in all samples. No stratification was detected, contradicting chronic rejection.

To further address the initial question whether MD-75 mice undergo delayed acute or chronic rejection, a typical MD-75 skin graft was harvested during rejection for histology, when haemorrhagic spots were visible (Figure 26e). Microscopical analysis showed no fibrosis or sclerosis, but inflammatory destruction of rete pegs and adnexal structures. In the epidermis, inflammatory cells were detected, together with apoptosis and necrosis and beginning detachment of the epidermis. Thus, the features of an acute rejection process are manifest and the findings argue against chronic rejection taking place in MD-75 mice that reject after long-term acceptance.

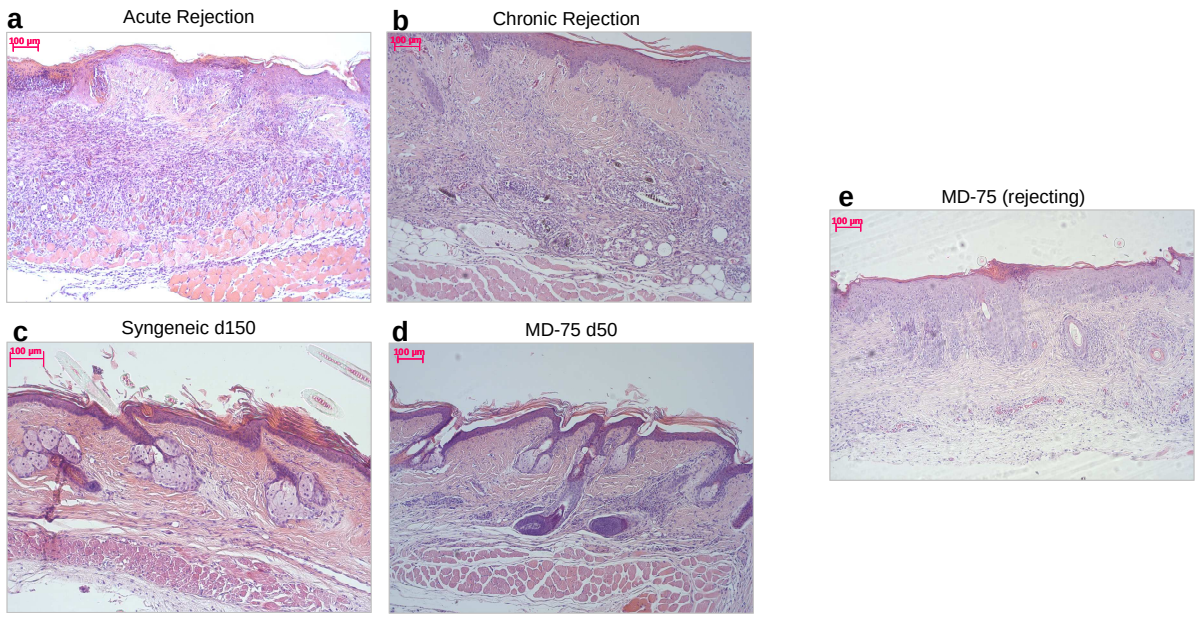

Figure 26: Microscopic pathology of rejection. (a) Rejection in a B/c-to-B/6 skin transplant model without treatment (acute rejection, d7). (b) Rejection in minor-missmatch (male-to-female) model (chronic rejection, d31). (c) Syngeneic (B/c to B/c) (d150). (d) MD-75 graft (d50). (e) Rejection in a MD-75 mouse (d61).

The possibility of chronic rejection was additionally examined by molecular RT-PCR analysis. TGFβ is a fibrogenic cytokine and its elevated expression has been described in chronically rejecting skin grafts [231]. The expression of TGFβ in allogeneic grafts of MD-75 mice on d50

without macroscopical signs of rejection was not significantly different from the expression in syngeneic MD-75 grafts. Moreover, the expression in both the syn- and allograft was significantly less than in chronic rejecting grafts (Figure 27: p = 0.016).

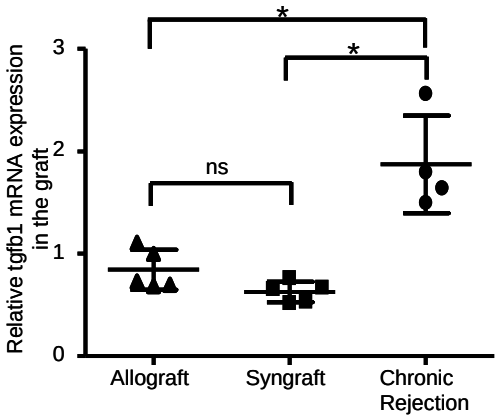

Figure 27: TGFβ- RT-PCR in skin grafts. Syn- and allografts from MD-75 mice were pooled on d50 post transplantation; skins from mice undergoing chronic rejection were pooled at the peak of rejection. RNA was isolated from CD45.2$^+$ leukocytes and levels of TGFβ were determined. n = 4 (chronic) or 5 pools per group, (at least 2 grafts / pool) . Scatter plots show the mean and standard deviation. Statistical analysis: Mann-Whitney-Test, two-tailed (*: $p < 0.05$ ns: $p > 0.05$).

To summarise, macroscopical, microscopical and molecular analyses indicate that rejection that takes place in MD-75 mice after long-term acceptance of the allograft is consistent with delayed acute rejection rather then chronic rejection.

4.9 Absence of donor-specific antibodies in MD-75 mice

In clinical studies, operationally tolerant patients that are drug-free and have stable allograft function [209] have been reported as having no or only low amounts of donor-specific antibodies (DSA) [129,210]. Presence of DSA in turn might be correlated with rejection [232]. The flow cytometry crossmatch (FCXM) has been first described in 1983 [220] and has been used since then in clinics to measure anti-donor antibodies. A modification of a T cell FCXM was developed to measure anti-donor-antibodies in mice. Briefly, serum from sensitised mice (pooled) or mice treated with anti-CD154 + DST + 75 mg/kg Tacrolimus food (MD-75) was incubated with donor and recipient splenocytes. After washing away excess sera, fluorescence-labeled antibodies were used to capture target IgG and IgM antibodies bound to the splenocyte cell surface. Thus, both donor-specific and self-specific antibodies can be detected.

For the analysis, first a lymphocyte gate was set in the forward-scatter / sideward-scatter plot (Figure 28, representative example). Using the fluorescence signal for the CD3 and the H2K^d antigen, it was discriminated between B/c splenocytes (donor, H2K^d) or B/6 splenocytes (self,

H2K^b). As shown in the histogram plot, IgG antibody from sensitised mouse serum has bound to H2K^d positive B/c cells, thus indicating DSA. As expected, no IgG antibodies can be detected in serum from naïve B/6 mice (NMS), neither with specific nor self-specific binding capacities.

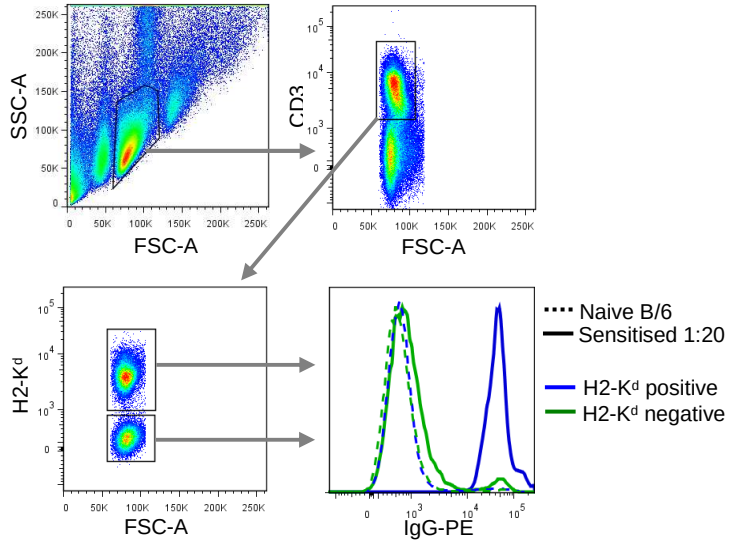

Figure 28: Crossmatch gating strategy. Gating strategy in one representative example is shown.

The serial two-fold dilution of the pooled sensitised mouse serum displays a dose-response curve for donor-specific IgG (Figure 29) and very low median fluorescence signals (MFI) for the H2K^b specific IgG response.

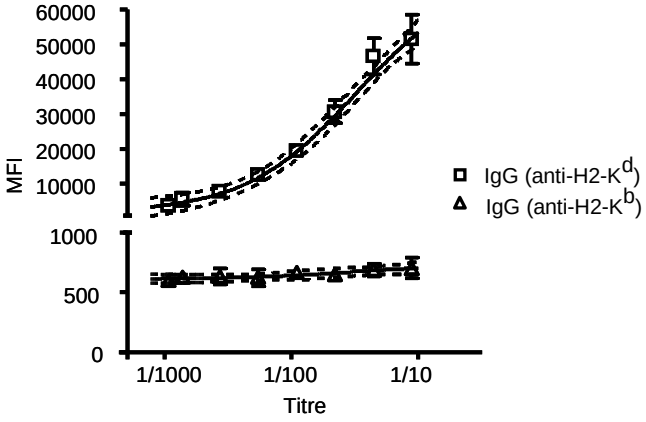

Figure 30: Titration curve. Anti-H2-K^d IgG and anti-H2-K^b IgG response from serial two-fold dilution of serum from a sensitised mouse pool. Data points expressed as mean of triplicate MFI is displayed with the standard deviation. Curve analysis was done as a sigmoidal dose-response fit of data with 95% confidence intervall.

A limit of detection was defined as mean MFI of the NMS sera plus the threefold standard deviation. Samples with a MFI below the limit of detection were considered as negative for IgG antibody.

To further characterise our model of weak regulation in combination with low-dose Tacrolimus therapy, levels of DSA were determined in mice bearing the allogeneic transplant for 50 days. Both the IgG response against donor and self of these mice lies beneath the limit of detection (Figure 30a). Further, serum from mice with a syngeneic B/6 transplant and treatment with anti-CD154 + DST + Tacrolimus at 75 mg/kg was analysed. The donor-specific IgG-response of both mice with allograft or syngraft was below the limit of detection (Figure 30b).

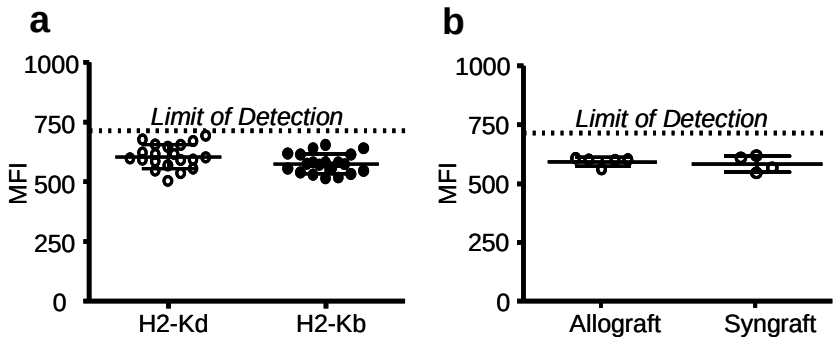

Figure 30: Analysis of donor-specific antibodies in transplanted mice. Limit of detection was defined as mean MFI of the NMS sera + 3x SD. Mean and standard deviation are shown in the scatter plots. (c) Anti-H2-K^d IgG and anti-H2-K^b IgG response from allografted mice treated with anti-CD154 + DST + Tac-75 (n = 20). (d) Anti-H2-K^d IgG response of allografted (n = 5) or syngrafted (n = 4) mice treated with anti-CD154 + DST + Tac-75.

The absence of DSA is evidence that the treated mice do not have donor reactivity. This indicates that on d50 after transplantation, the mice are not undergoing acute rejection and probably also not undergoing chronic rejection.

4.10 Genotypic characterisation of MD-75 mice with an allograft

4.10.1 Gene expression profile in skin grafts

In transplantation research, it is a focus to define biomarkers of tolerance, allowing identification of patients that are tolerant, or in line with the findings of this work, in marginal states of allograft acceptance. We have set up a panel of over 20 genes associated with states of tolerance or rejection (Section 3.1.8.2). The skin allografts of MD-75 mice were examined for the expression of these genes. Gene expression in MD-75 mice with a syngraft, mice treated with triple costimulatory blockade, mice undergoing acute and mice undergoing chronic rejection was measured as control.

Skin grafts from either syn- or allografted MD-75 mice or mice treated with triple costimulatory blockade and 75 mg/kg Tacrolimus food were removed on d50 post

transplantation. In addition, skin grafts from acutely rejecting (untreated after transplantation) and chronically rejecting (male-to-female minor mismatch model) were removed on the peak of rejection. qPCR analysis of whole graft tissue of single grafts was performed, examining for the expression of the genes from our panel. However, only six genes of the panel (pdcdlg1, tmem176b, gm1129, man1a, cxcl10 and hmox) were reliably detectable (Figure 31).

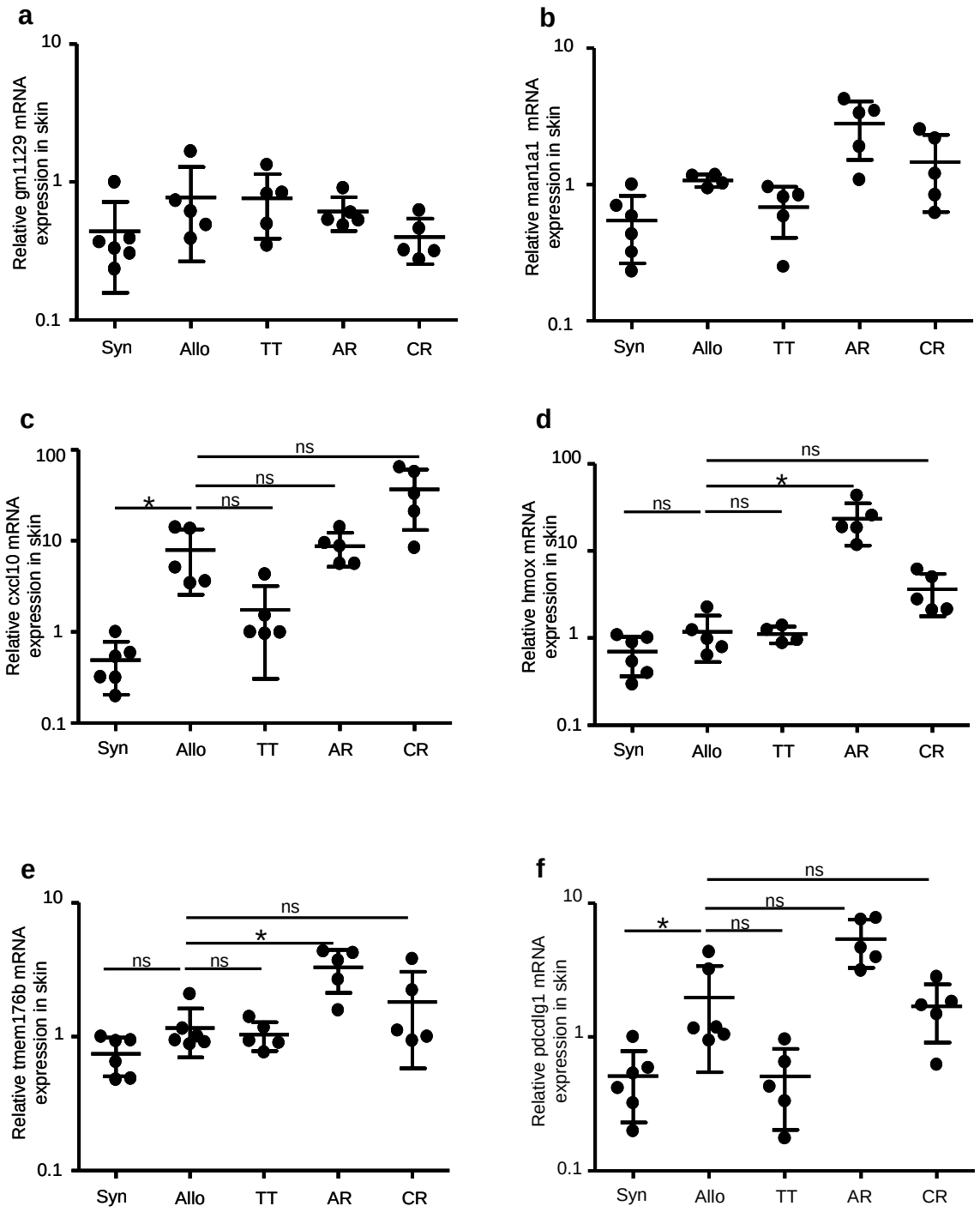

Figure 31: Gene profile of the skin graft. Groups: syn: MD-75 mice with syngraft. Allo: MD-75 mice with allograft. TT: mice treated with triple costimulatory blockade and 75 mg/kg Tacrolimus food. AR: Transplanted mice, untreated, acute rejection. CR: male-to-female minor mismatch transplantation, chronic rejection. qPCR analysis of whole single grafts on d50 (syn, allo, TT) or on the day of rejection (AR, CR). n = 4-6 per group. Scatter plots show mean with SD. Statistical analysis: pair-wise group comparison (Allo vs. Others) with Mann-Whitney-Test and Holm-Bonferroni correction for multiple analysis.

gm1129, the tolerance-associated gene (TOAG-1), which encodes TCAIM (Figure 31a), which has been described as highly expressed during graft acceptance in mice heart and rat kidney transplant models, was not significantly different expressed in grafts of allografted MD-75 mice [233].

Together with TOAG, also alpha-1, 2-mannosidase (man1a 1) was described in these models by the same group as being upregulated during induction therapy and graft acceptance and downregulated in the case of rejection. The expression of man1a 1 showed no significant differences in MD75 mice with an allograft compared to the control groups (Figure 31b).

In human transplant studies, high intragraft expression of chemokine CXCL10 (IP-10) has been reported in conjunction with both acute and chronic rejection [234,235]. The cxcl10-expression in MD-75 allografted mice was significantly higher than the expression in MD-75 mice with a syngraft ($p = 0.0043$, Figure 31c), but not significantly different to the three other control groups.

Heme-oxygenase 1 (hmox) is an enzyme, whose expression has been discussed controversially in the context of allograft acceptance and rejection [236]. In a mouse heart transplantation model using CD154 + DST to induce tolerance, intragraft expression of hmox was reported to have a protective effect, in other, rodent and human, models, hmox espression was upregulated during rejection [236,237]. In comparison to acutely rejecting allografts, hmox-expression was significantly reduced in MD-75 allografted mice ($p = 0.0079$), which in addition did not show significantly altered hmox expression compared to syngrafted MD-75 mice, triple costimulatory blockade treated mice or mice undergoing chronic rejection (Figure 31d).

Overexpression of tmem176b (tolerance-related and induced transcript, TORID) in the graft was first reported in a rat cardiac transplantation model [238]. Whilst the expression of tmem176b in MD-75 mice with an allograft was not significantly different from syngrafted MD-75 mice, triple costimulatory blockade treated mice or mice undergoing chronic rejection, it was significantly decreased in comparison to acutely rejecting allografts ($p = 0.0087$; Figure 31e).

The last gene that came up in the qPCR analysis of whole skin grafts was pdcdlg1 (PD-L1). As already mentioned, PD-L1 has a role in the regulation and inhibition of the T cell response. Interestingly, the expression of pdcdlg1 is significantly upregulated in MD-75 mice

with an allograft compared to MD-75 mice with a syngraft ($p = 0.0043$). The upregulation in comparison the mice treated with triple costimulatory blockade is not significant; the expression in chronically rejecting grafts is comparable. Expression of pdcdlg1 is higher in mice undergoing acute rejection, yet, this is not significantly different from MD-75 mice with an allograft (Figure 31f).

To summarise, none of the previously reported tolerance markers TOAG/TCAIM, TORID, alpha-1, 2-mannosidase or heme-oxygenase 1 was found to distinguish MD-75 mice with an allograft from neither MD-75 mice with a syngraft nor mice treated with triple costimulatory blockade. Yet, allografted MD-75 mice do express more CXCL-10, a chemoattractant for lymphocytes, and the inhibitory receptor ligand PD-L1. In conclusion, qPCR analysis of skin grafts of allograftes MD-75 mice shows a mixed picture. The expression of tolerance-related markers can be detected as well as markers for rejection, which is consisted with our hypothesis of marginal states and the balance between regulatory and effector responses.

4.10.2 Gene expression profile in draining LN

In order to obtain a more detailed gene expression profile of allograft acceptance in our model, the dLN were analysed for the expression of genes from our panel. Indeed, 22 of these genes associated with tolerance and rejection were reliably quantified by qPCR. In total, dLNs from 10 mice undergoing acute rejection, 10 mice undergoing chronic rejection, 5 mice treated with triple costimulatory blockade and 75mg/kg Tacrolimus were analysed together with dLNs from 6 syngrafted MD-75 mice and 13 allografted MD-75 mice (Figure 32). The data obtained from the qPCR is depicted as an unsupervised, hierachical cluster analysis. The acutely rejecting mice cluster all together, showing a consistent gene expression in the group. However, this is not the case for the other treatment groups. In general, single syngrafted MD-75 mice cluster next to single or grouped allografted MD-75 mice. Chronically rejecting mice cluster partly together or with triple costimulatory treated mice. The gene profile of the dLN of allografted MD-75 mice is heterogenous within the group. Half of the mice in this group cluster directly together, while the other MD-75 mice are spread and found together with syngrafted MD-75 mice, triple costimulatory treated mice and some chronically rejecting mice.

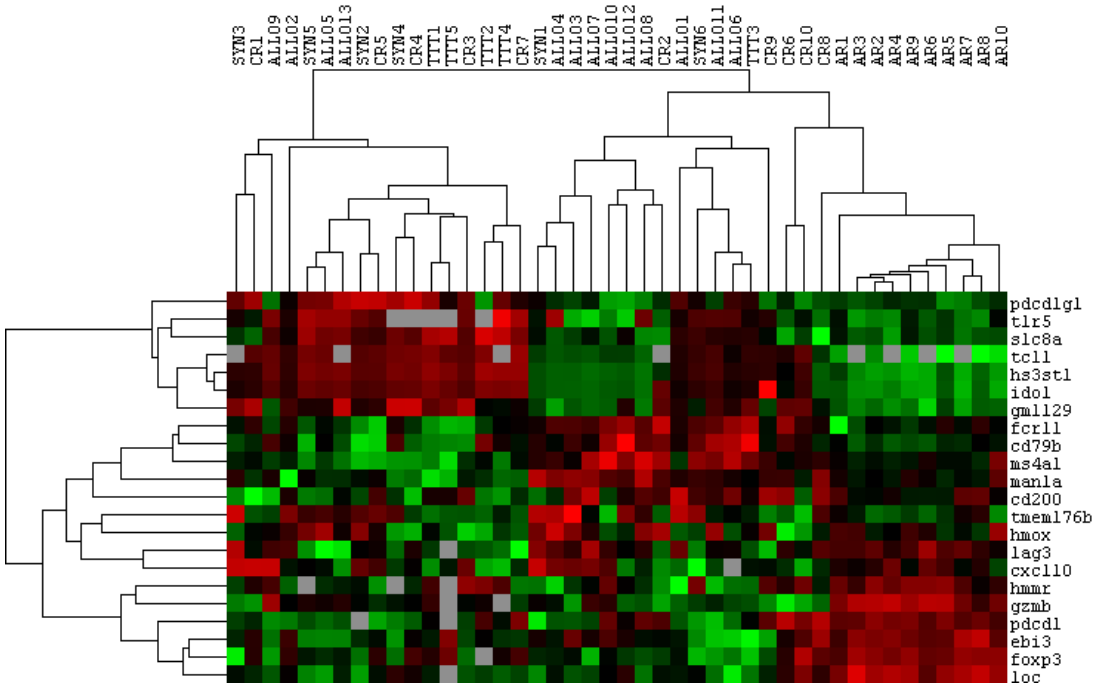

Figure 32: Gene profile of the dLN. syn: MD-75 mice with syngraft. Allo: MD-75 mice with allograft. TT: mice treated with triple costimulatory blockade and 75 mg/kg Tacrolimus food. AR: Transplanted mice, untreated, acute rejection. CR: male-to-female minor mismatch transplantation, chronic rejection. qPCR analysis of whole dLN (axilliary, inguinal) of single mice on d50 (syn, allo, TT) or on the day of rejection (AR, CR). Green shading indicates lower expression of a certain gene in comparison to the median of all samples, whereas red shading indicates higher expression. Grey boxes indicate missing data points.

In conclusion, a genetic profile unique to allografted MD-75 mice in the dLN could not be determined. This was also the case for syngrafted MD-75 mice, triple costimulatory treated mice and mice undergoing chronic rejection. Thus, the expression of the selected genes associated with states of tolerance or rejection in dLN cannot be used as a biomarker to determine the state of allograft acceptance in MD-75 mice.

4.11 A model of marginal states of allograft acceptance

So far, it could be shown that the addition of a low-dose Tacrolimus monotherapy to a weak regulation-inducing protocol synergistically prolongs allograft survival in allogeneic murine skin transplantation. It remains to be tested whether this experimental model is indeed reflecting marginal states of allograft acceptance, wherein neither the effector nor the regulatory response predominates. In these marginal states, a disturbance of either the regulation or the immunosuppression should tip the balance and lead to rejection of the graft. Thus, the same must apply for the experimental model of low-dose Tacrolimus and anti-CD154 + DST. The balance in marginal states should be disturbed by 1) withdrawing immunosuppression, 2) enhancing the effector response or 3) disrupting the regulatory response. Therefore, this was tested in the experimental model.

4.11.1 Withdrawal of Immunosuppression leads to allograft rejection

Unless stated otherwise, the experiments described below were in general performed with allografted mice treated with Tacrolimus food at 75 mg/kg (Tac-75) and anti-CD154 + DST, since the allograft survival was over 90% (i.e. 91.4%) on d50 post transplantation (i.e. d50).

Withdrawal of the immunosuppressive treatment was done first to determine the effect on allograft survival. Tacrolimus food was switched to normal diet on d50, this led to significantly accelerated rejection of the allograft than in the control group, where Tacrolimus food was continued (Figure 33: MST withdrawal vs. continuation: 61.4 ± 1.5 vs. 74.8 ± 4.9 days, p = 0.007).

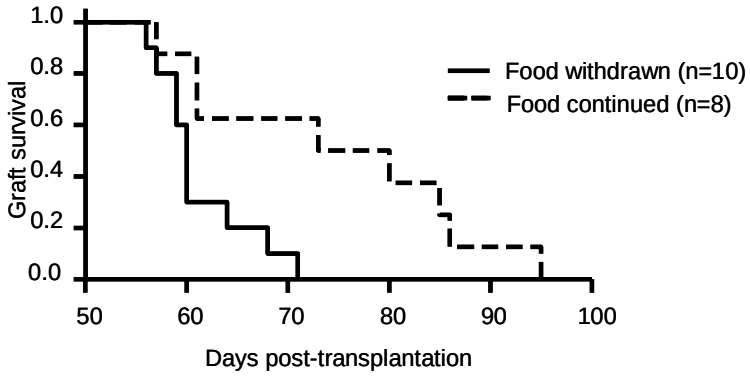

Figure 33: Withdrawal of immunosuppression. On d50 post transplantation, Tacrolimus diet was continued or withdrawn in MD-75 mice. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

4.11.2 Enhancing the effector response leads to allograft rejection

To enhance the effector response, MD-75 mice were treated on d50 with 10 x 10^6 $CD4^+$ and $CD8^+$T cells from sensitised B/6 mice that had previously rejected two B/c allografts under Tac-75. Control mice received PBS only. Importantly, no other treatment modification was done, i.e. Tacrolimus food was continued. The injection of effector T cells precipitated earlier rejection of the allograft in comparison to the control group (Figure 34: MST effector cell transfer vs. no transfer: 62.4 ± 2 vs. 76.8 ± 3.8 days, p = 0.007).

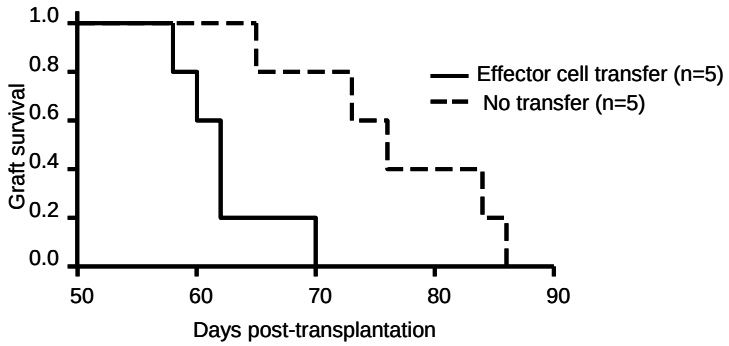

Figure 34: Effector cell transfer. On d50 post transplantation, MD-75 mice received i.v. 10 x 10^6 T cells from sensitised mice or PBS only . n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

The effector T cell transfer might be seen as a non-physiological challenge, so a second method to enhance the effector response was tested: A second skin transplant was applied to recipients on d50, which received either a skin graft from original donor (B/c) or from third party strain (C3H) (Figure 35). Mice that were challenged with a C3H graft rejected the original first graft (that is B/c origin) with similar kinetics than the MD-75 group that had not received any further treatment (MST challenge C3H vs. MD-75: 79.4 ± 4.4 vs. 82 ± 2.1 days, p = 0.81). However, mice that were challenged with a B/c graft rejected the first, original B/c graft significantly earlier than the MD-75 control group (MST challenge B/c vs. MD-75: 70.7 ± 1.8 vs. 82 ± 2.1 days, p = 0.0014).

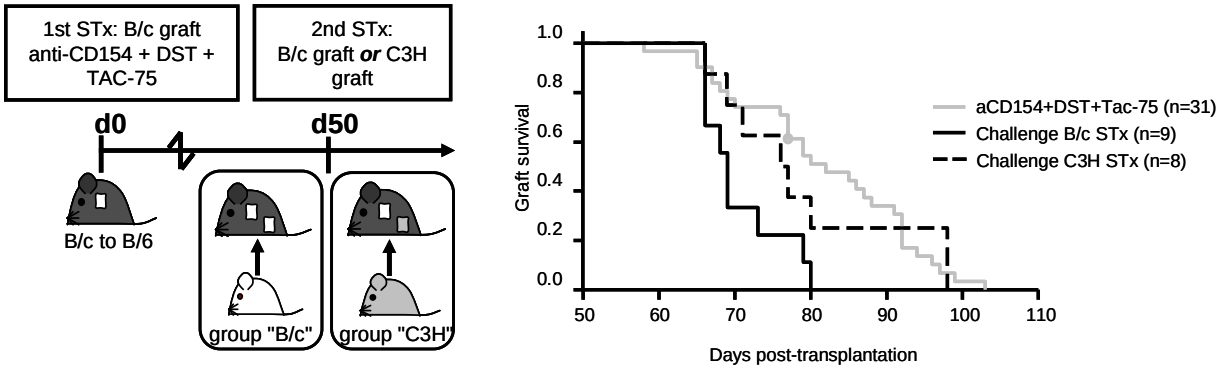

Figure 35: In vivo effector cell transfer. On d50 post transplantation, MD-75 mice received a second transplant that was either B/c (donor) or C3H (third party). Allograft survival of the first allograft (B/c) in both groups is shown together with allograft survival of MD-75 mice that received no further treatment (historical control group). n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test, multiple comparisons with Holm-Sidak method

In addition, mice that were treated with anti-CD154 + DST + Tacrolimus food at 100 mg/kg (in the following: MD-100) received i.v. injections of 10 x 10^6 effector T cells on d50. At this time point, graft survival was 100%. Treatment with effector cells precipitated premature graft rejection also in the MD-100 group compared to the untreated MD-100 group (Figure 36: MST effector cell transfer vs. no transfer: 78.3 ± 6.5 vs. 134.1 ± 5.5 days, p < 0.001).

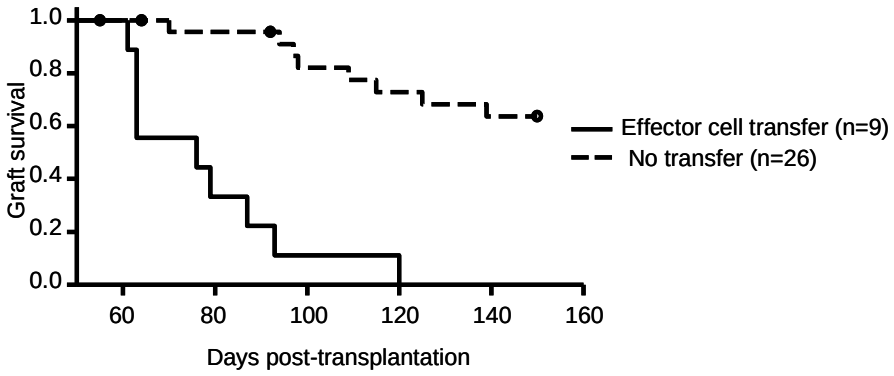

Figure 36: Effector cell transfer. On d50 post transplantation, MD-100 mice received i.v. 10 x 10^6 T cells from the sensitised mice. As control group, allograft survival of MD-100 mice without further treatment is shown (historical control group). n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

Taken together, these experiments show that enhancing the donor-specific effector response led to disturbance of marginal states and thus rejection of the allograft.

4.11.3 Disrupting regulation leads to allograft rejection

It follows from the above stated hypothesis that rejection caused by a disrupted regulatory response is another proof for having established an experimental model of marginal states. Therefore, MD-75 mice were treated on d50 post transplantation with antibodies against molecules that play a role in regulation in transplantation.

Treatment with anti-PD-L1 antibody caused early rejection of the allograft compared to treatment with the isotype control antibody (Figure 37: MST aPD-L1 vs. Isotype: 65.9 ± 2.6 vs. 76.4 ± 4.4 days, p = 0.045).

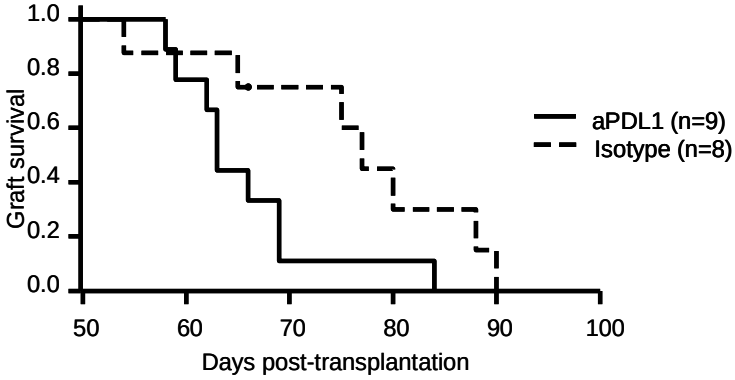

Figure 37: Anti-PD-L1 antibody Starting on d50 post transplantation MD-75 mice received 8 injections of anti-PD-L1 antibody or isotype control. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

Further, treatment with anti-GITR antibody led to significantly earlier rejection compared to the corresponding isotype control group (Figure 38: MST aGITR vs. Isotype: 69 ± 5.6 vs. 89.3 ± 6.6 days, p = 0.019).

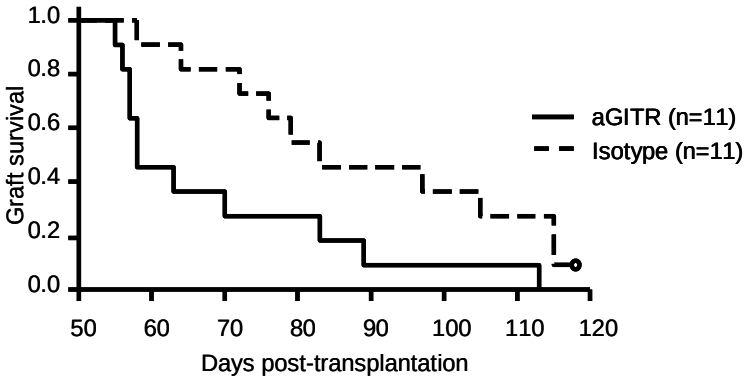

Figure 38: Anti-GITR antibody. Starting on d50 post transplantation MD-75 mice received 4 injections of anti-GITR antibody or isotype control. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

The importance of PD-L1 and GITR in the allograft acceptance in this model implies the involvement of regulatory T cells. T regs are defined as $CD4^+CD25^+FoxP3^+$ T cells and in mice, CD25 is a useful cell surface marker for T regs [109,239]. Therefore, it was planned to deplete T regs by an anti-CD25 antibody. This was done initially in wildtype C57BL/6 mice by administration of the depleting CD25 clone PC61 [240]. Flow cytometric analyses of spleen, LNs and peripheral blood showed significant reduction in relative numbers of $FoxP3^+$ T cells of 44.6%, 49.1% and 55.5%, respectively (Figure 39).

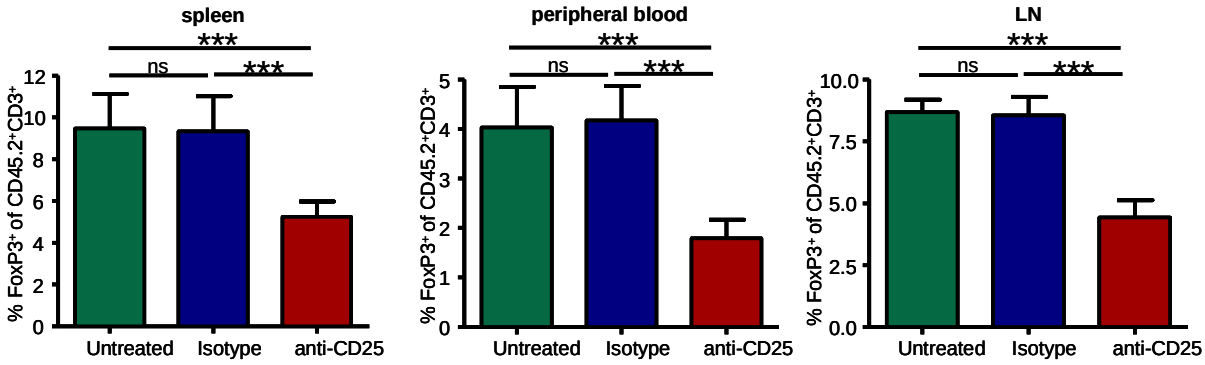

Figure 39: Depletion with anti-CD25. C57BL/6 mice were treated with 4 injections of anti-CD25 antibody (clone PC 61), isotype control or left untreated (animals per group: n = 6). Mean percentage of $FoxP3^+$ cells and standard deviation are shown. Statistical analysis: unpaired t-test, two-tailed (***: $p < 0.0005$, ns: $p > 0.05$).

When MD-75 mice were treated with the depleting anti-CD25 antibody, no effect on the allograft survival could be observed (Figure 40: MST anti-CD25 vs. Isotype: 92.5 ± 4.2 vs. 93.5 ± 8.5 days, $p = 0.41$). Thus, it was concluded that not only $CD4^+CD25^+$ T regs were depleted, but also $CD25^+$ activated effector T cells.

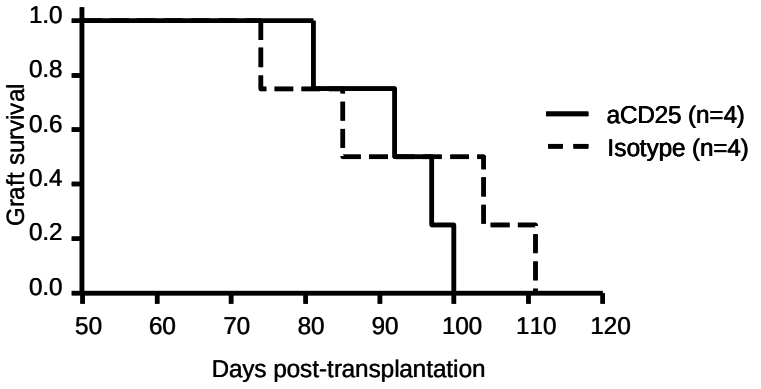

Figure 40: Anti-CD25 antibody. Starting on d50 post transplantation MD-75 mice received 4 injections of anti-CD25 antibody (clone PC61) or isotype control. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

A second approach to determine whether $FoxP3^+$ regulatory T cells are responsible for the regulation of the anti-donor response and thus allograft acceptance, was to use a genetically

modified mouse strain that allowed depletion of FoxP3$^+$ cells. Briefly, these mice were generated by Rudensky's group on a C57BL/6 background and carry the human diphtheria toxin receptor (DTR) fused to green fluorescent protein (GFP) sequences under the control of the FoxP3 promoter [241]. Thus, FoxP3$^+$ T reg (not FoxP3$^-$ T cells) express both GFP and the DTR. By administration of diphtheria toxin (DTx) to these Foxp3-GFP-DTR mice, a transient and very specific depletion of FoxP3$^+$ T reg can be achieved [241].

Foxp3-GFP-DTR mice were treated with anti-CD154 + DST and Tacrolimus food at 75 mg/kg to induce allograft acceptance. The allograft survival was significantly different in these mice compared to wildtype C57BL/6 mice that were treated with anti-CD154 + DST and Tacrolimus food at 75 mg/kg at the same time as control group (Figure 41: MST Foxp3-GFP-DTR vs. wildtype mice: 37.1 ± 10.8 vs. 75.5 ± 8.3 days, p = 0.027). These Foxp3-GFP-DTR mice had not received any DTx to deplete the FoxP3$^+$ T reg.

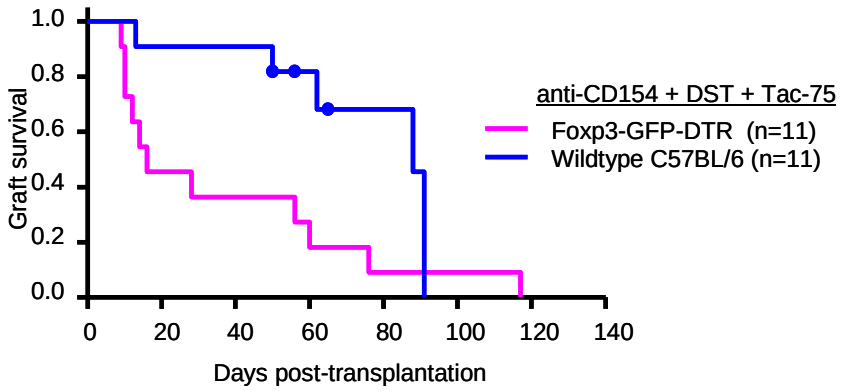

Figure 41: Treatment with anti-CD154 + DST + Tac-75 in FoxP3-GFP-DTR mice. Together with wildtype B/6 mice, FoxP3-GFP-DTR mice received a B/c transplant and were treated with anti-CD154 + DST + Tac-75 to induce allograft acceptance. No further treatment was done. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

This difference was an unexpected result since to our knowledge, no pathophysiological difference in these mice in any experiments has been observed. Measurement of Tacrolimus levels in the blood of non-transplanted Foxp3-GFP-DTR mice did not show different levels as would be expected in mice treated with 75 mg/kg Tacrolimus food (mean Tacrolimus level over 3 weeks after introduction: Foxp3-GFP-DTR vs. C57BL/6 sentinel mice: 2.2 µg/l ± 0.5 vs. 2.6 µg/l ± 0.5, p = 0.38). Thus, it was planned to induce allograft acceptance in our model by increasing the low-dose Tacrolimus in the Foxp3-GFP-DTR mice. Hence, they received anti-CD154 + DST and Tacrolimus in doses of 100 mg/kg. On d50 after transplantation, mice with an intact allograft (over 90%) were treated with i.p. injection of 25 ng/g bodyweight DTx or PBS as control. Injection of DTx did precipitate early rejection of the allograft in Foxp3-GFP-DTR mice in comparison with PBS-treated Foxp3-GFP-DTR mice (Figure 42: MST DTx injection vs. PBS injection: 58.8 ± 0.9 vs. 111 ± 4.7 days, p < 0.001).

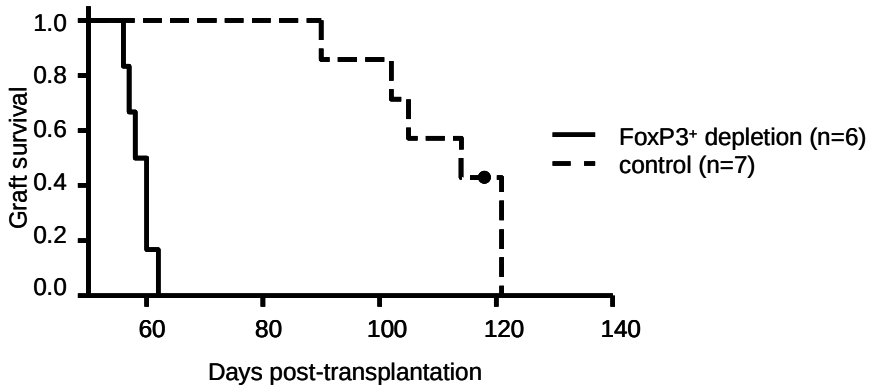

Figure 42: Treatment with anti-CD154 + DST + Tac-100 in FoxP3-GFP-DTR mice. FoxP3-GFP-DTR mice received a B/c transplant and were treated with anti-CD154 + DST + Tac-100 to induce allograft acceptance. On d50 post transplantation, mice randomisedly received i.p. injections of Diphtheria Toxin or PBS as control. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test, multiple comparisons with Holm-Sidak method

In contrast, in wildtype C57BL76 mice treated with anti-CD154 + DST + Tac-100, the injection of 25 ng/g bodyweight DTx did not precipitate rejection (data not shown). The allograft survival in Foxp3-GFP-DTR mice treated with PBS was not significantly different from wildtype C57BL76 mice treated with anti-CD154 + DST + Tac-100. Therefore, it was shown that the specific depletion of T regs led to disruption of regulation in marginal states and thus allograft rejection.

To further examine the importance of regulatory T cells in this model of allograft acceptance, their suppressive mechanisms were targeted. Injections with anti-IL10R antibody did not have an effect on allograft survival with reference to the isotype control treatment (Figure 43: MST aIL-10R vs. Isotype: 77.1 ± 2.4 vs. 78.8 ± 5.4 days, p = 0.6).

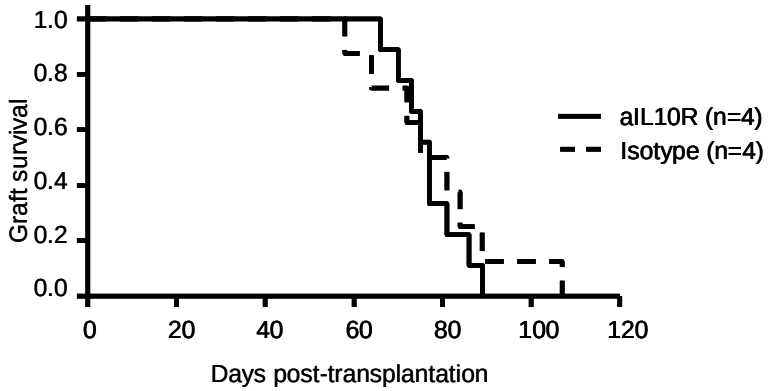

Figure 43: Anti-IL10R antibody. Starting on d50 post transplantation MD-75 mice received 4 injections of anti-IL10R antibody or isotype control. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

In contrast to this, injections of anti-TGFβ antibody, provoked rejection at significantly earlier time points than the Isotype control (Figure 44: MST aTGFβ vs. Isotype: 69 ± 1.4 vs. 81 ± 2.2 days, p = 0.002).

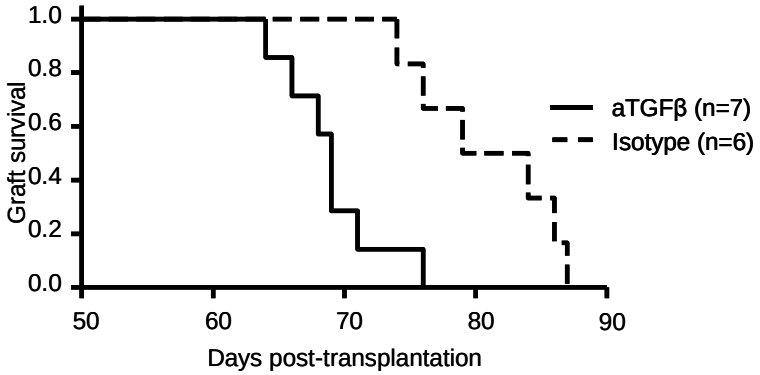

Figure 44: Anti-TGFβ antibody. Starting on d50 post transplantation MD-75 mice received 4 injections of anti-TGFβ antibody or isotype control. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

Therefore, regulation in marginal states of allograft acceptance in MD-75 mice is dependent on peripheral T reg producing TGFβ rather then on IL-10 producing natural T reg.

Taken together, the disruption of regulation in the experimental model by disturbing PD-L1, GITR and TGFβ pathways and by depleting FoxP3$^+$ T regs leads to rejection of the allograft. It was therefore demonstrated that in the model of low dose Tacrolimus in combination with weak regulation induction, allograft acceptance can be broken. This was done by 1) withdrawal of immunosuppression, 2) enhancing the effector response or 3) disrupting the regulatory response. Thus, we are convinced that we have established an experimental model of marginal states of allograft acceptance.

4.12 Location of regulatory and effector cell populations

4.12.1 Regulators and effectors in spleen and dLN

If our hypothesis is correct and there is a balance between regulatory and effector cells, then it should be possible to detect both regulatory and effector cells at the same anatomical sites. In MD-75 mice, the spleen was surgically removed 50 days post transplantation. Allograft survival in these animals was not affected significantly when compared to allograft survival of otherwise untreated MD-75 mice (Figure 45a: MST splenectomy d50 vs. MD-75: 77.3 ± 2.4 vs. 81.9 ± 2.1 days, p = 0.16). This raised the question whether spleen was even necessary for the induction of allograft acceptance in mice treated with low-dose Tacrolimus and anti-CD154 + DST induction. Thus, splenectomy was performed in C57BL/6 mice at d -7 pre-transplant, then these mice and naïve control mice were treated with anti-CD154 + DST and

Tacrolimus at 75 mg/kg. No significant difference was observed between the splenectomised and untreated groups (Figure 45b: MST splenectomy d-7 vs. MD-75: 68.6 ± 15.4 vs. 53 ± 13.2 days, p = 0.44). Hence, the spleen is dispensible for marginal states of skin allograft acceptance.

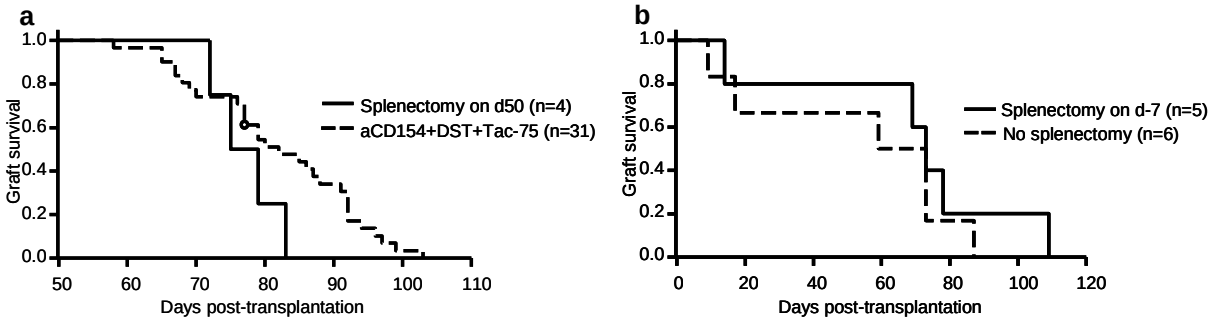

Figure 45: Splenectomy. (a) On d50 post transplantation MD-75 mice underwent splenectomy. As control group, allograft survival of MD-75 mice without further treatment is shown (historical control group). (b) Mice underwent splenectomy 7 days prior to transplantation and treatment with anti-CD154 + DST + Tac-75. Allograft acceptance was induced in non-surgically altered mice as control. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

The surprising result that the spleen was not necessary for the induction or the maintenance of allograft acceptance in the experimental model was further explored at a cellular and molecular level. Mice were treated with anti-CD154 + DST + Tac-75 and received either a C57BL/6 syngraft or a BALB/c allograft. On d50 post transplantation, leukocyte populations from spleen were analysed by flow cytometry. A panel of antibody-combinations was developed to analyse different leukocyte populations. Analyses of B cells, NK cells, DCs, MDSCs and macrophages did not display any significant differences between mice with a syngraft or mice with an allograft (Figure 46, gating strategy see Appendix).

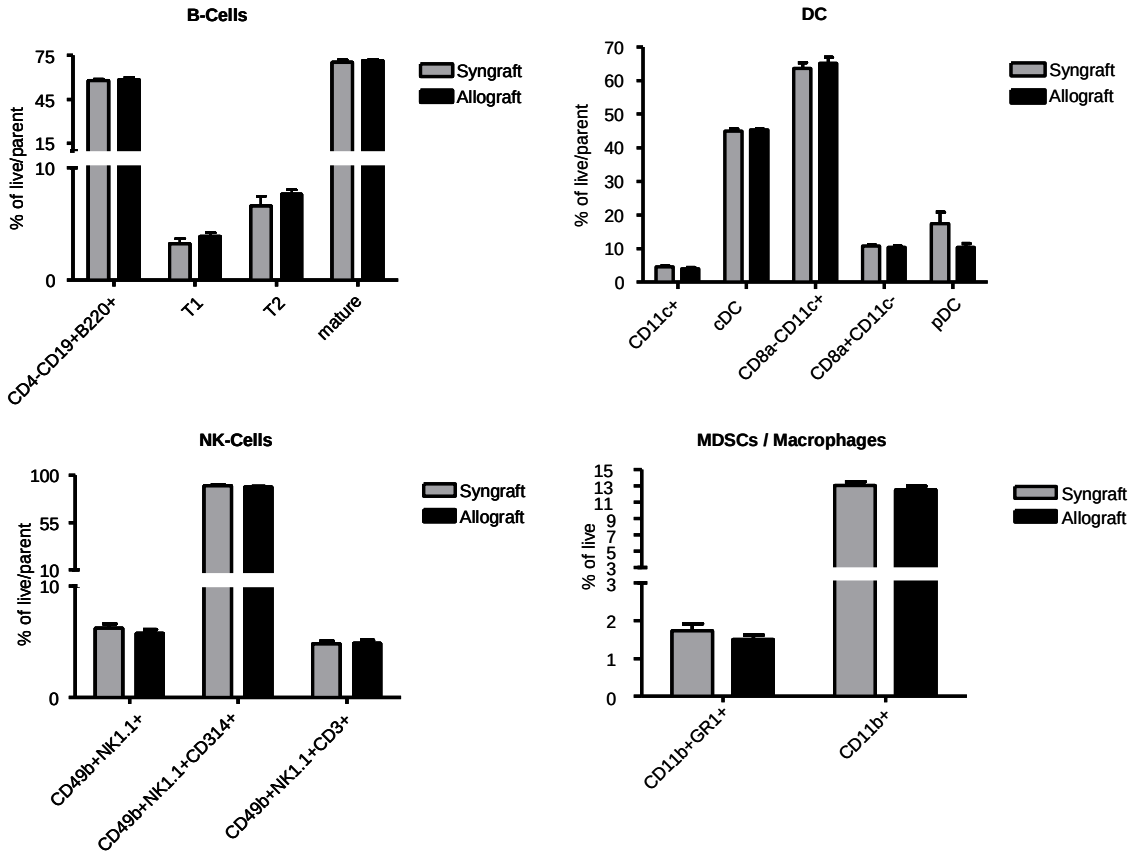

Figure 46: Panel analysis of spleen. Spleens from MD-75 mice with a syn- or an allograft were analysed on d50 post transplantation. Column plots show the mean and standard deviation. n = 9 per group. Statistical analysis: Mann-Whitney-Test, two-tailed.

The frequency of the $CD8^+$ population was decreased in allografted mice, but not significantly (Figure 47a 35.1% ± 1.2 vs. 38.2% ± 3.6, p = 0.07). The percentage of $CD4^+$ amongst $CD3^+$ T cells was significantly higher in spleens of mice bearing an allograft (55% ± 1.3 vs. 51.4% ± 4.3, p = 0.014). This finding indicates a possible shift in the effector or regulatory cell populations. Hence, subpopulations of $CD4^+$ T cells were analysed. No significant changes were detected in either central or effector memory T cells; nor in the percentage of naïve or mature T cells (data not shown). Further, no significant difference in the frequency of $CD25^+$ $FoxP3^+$ regulatory T cells or $CD25^+$ $FoxP3^-$ T cells was observed (Figure 47b: 1.4% ± 0.8 vs. 1.2% ± 0.8, p = 0.6 and 4% ± 2.9 vs. 3.6% ± 1.7, p = 0.73, respectively).

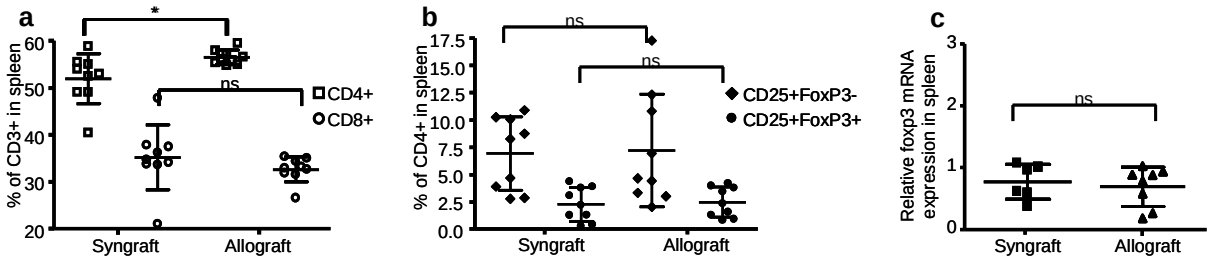

Figure 47: Analysis of spleen Spleens from MD-75 mice with a syn- or an allograft were analysed on d50 post transplantation. (a+b) n = 9 per group. (c) n = 6 (syngraft) or 8 (allograft). Scatter plots show the mean and standard deviation. Statistical analysis: Mann-Whitney-Test, two-tailed (*: $p < 0.05$, ns: $p > 0.05$).

In concordance with the FACS data, quantitative RT-PCR analysis of relative *foxp3* mRNA expression did not reveal any differences between mice with a syngraft and those with an allograft (Figure 47c: p = 0.49). In summary, no striking differences in the percentage of regulatory and effector cell populations were found in the spleens of mice with a syngraft and mice with an allograft. It was shown that the spleen is not necessary for the induction of allograft acceptance in our model and is even dispensable in the maintenance of allograft acceptance.

Therefore, the graft draining LN (inguinal and axilliary) were examined by FACS, focussing on the T cell populations. The frequencies of both $CD4^+$ and $CD8^+$ amongst the $CD3^+$ population were not different in mice with an allograft in comparison to mice with a syngraft (Figure 48a). Allografted mice seem to have slightly, yet not significantly, elevated percentages of $CD25^+$ $FoxP3^+$ cells in the draining LN (allo vs. syn: 7.6% ± 0.9 vs. 6.8% ± 0.8, p = 0.12; Figure 48b). The percentages of the $CD25^+$ $FoxP3^-$ cells were similar in both groups (Figure 48b: p = 0.69). Further, relative expression of foxp3 mRNA in whole dLN was comparable between mice with a syngraft and mice with an allograft (Figure 48c: p = 0.59).

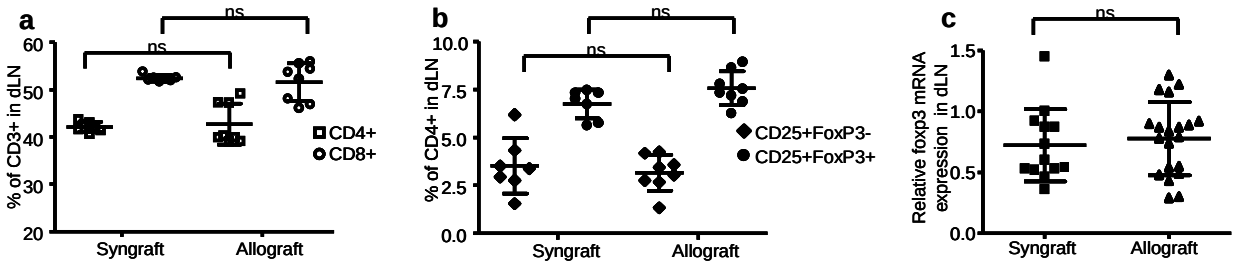

Figure 48: Analysis of dLNs Draining LNs from MD-75 mice with a syn- or an allograft were analysed on d50 post transplantation. (a+b) n = 7 (syngraft) or 8 (allograft). (c) n = 13 (syngraft) or 20 (allograft). Scatter plots show the mean and standard deviation. Statistical analysis: Mann-Whitney-Test, two-tailed (ns: p > 0.05).

Animal experiments, as first done in Waldmann's group [211], demonstrated that tolerance in transplantation is "infectious" and can be transferred by cell transfer. Thus, a cell transfer experiment was performed. Since cells from the spleen were not necessary for the maintenance of allograft acceptance in MD-75 mice, $1x10^6$ total $CD4^+$ and $CD8^+$ T cells from the draining LNs of MD-75 mice were transferred on d50 into Rag $1^{-/-}$ mice, which in general lack mature T and B lymphocytes [242]. Fourteen days after the cell transfer, mice underwent transplantation of both a B/c and a C3H skin allograft. The T cells originating from MD-75 mice will recognise B/c antigen as donor antigen and C3H antigen as third party antigen. As shown in Figure 49a, reconstituted Rag $1^{-/-}$ mice rejected C3H grafts significantly earlier than the B/c grafts (MST C3H vs. B/c: 13 ± 0.4 vs. 19.5 ± 1.1 days, p < 0.001). This experiment was also done with T cells from dLN of MD-100 mice and again, the C3H grafts

were rejected significantly earlier than the B/c grafts (Figure 49b: MST C3H vs. B/c: 15.5 ± 1.9 vs. 47.8 ± 10 days, $p < 0.001$).

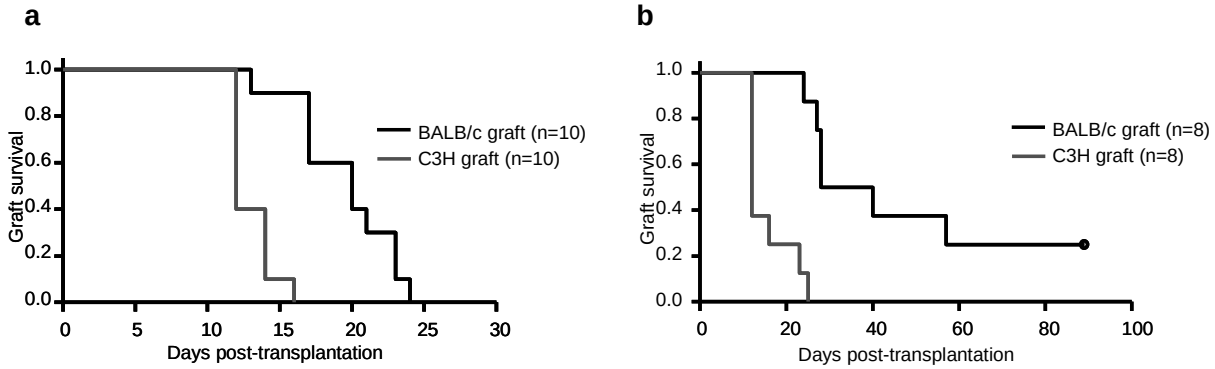

Figure 49: T cell transfer from dLNs. (a) On d50 post transplantation, 10 $Rag1^{-/-}$ mice received i.v. 1 x 10^6 T cells from MD-75 mice with an intact allograft on d50. (b) On d50 post transplantation, 8 $Rag1^{-/-}$ mice received i.v. 1 x 10^6 T cells from MD-100 mice with an intact allograft on d50. 14 days later, $Rag1^{-/-}$ mice received both a B/c (donor) or C3H (third party) transplant. n = number of grafts per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

Thus, it seems that the allograft acceptance generated in our model can be transferred and the donor-specific regulatory cells are at least in part located in the dLN. As control, Rag $1^{-/-}$ mice were reconstituted with T cells from the dLN from mice treated with either 75 mg/kg or 100 mg/kg Tacrolimus for 50 days and received B/c and C3H transplants 14 days later. These experiments were still ongoing by the time of submission of this thesis. Therefore, the results obtained and described above have to be interpreted carefully and it must be conceded that they can give only indices, not proof.

4.12.2 Regulators and effectors are also located in the graft

After examination of spleen and dLN, another possible location of regulatory and effector cell populations was analysed, namely the skin graft itself. Indeed, an experiment previously done to generate an in vivo effector response (Figure 35) offered more read-out information. As already described, MD-75 mice received a second transplant ("challenge") on d50 that was either a B/c or a C3H allograft. Figure 50 depicts allograft survival of both the first allograft, which is B/c in both groups, and of the second allograft, which is either B/c or C3H. The second allografts were rejected with similar kinetics, regardless whether they were donor (B/c) or third party (C3H) origin (MST second graft: B/c vs. C3H: 10.9 ± 1.6 vs. 13.3 ± 1.7 days, $p = 0.45$). As shown before, application of a second graft from donor precipitates rejection of the first graft (Figure 35). Interestingly, despite rejection of a second B/c graft located next to the first B/c graft (Figure 50), the latter was rejected significantly later (MST days post-transplantation: first B/c graft vs. B/c challenge graft: 10.9 ± 1.6 vs. 20.7 ± 1.8

days, p = 0.003, Figure 51). This hints at the presence of regulatory cells in the first allograft that suppressed the donor-specific effector cell enough to delay rejection.

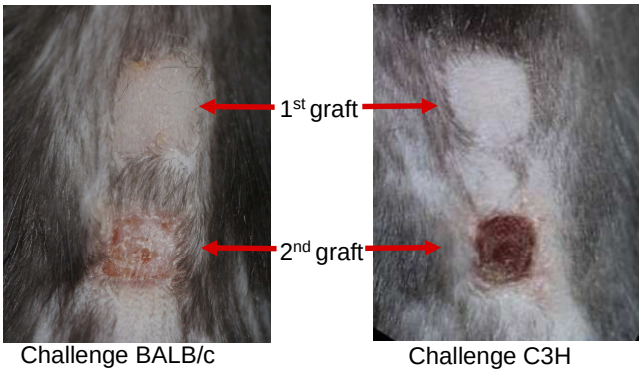

Figure 50: Challenge. On d50 post transplantation, MD-75 mice received a second transplant that was either B/c (donor) or C3H (third party). Allograft survival of first allograft (B/c) in both groups is compared with allograft survival of the second graft (B/c or C3H).

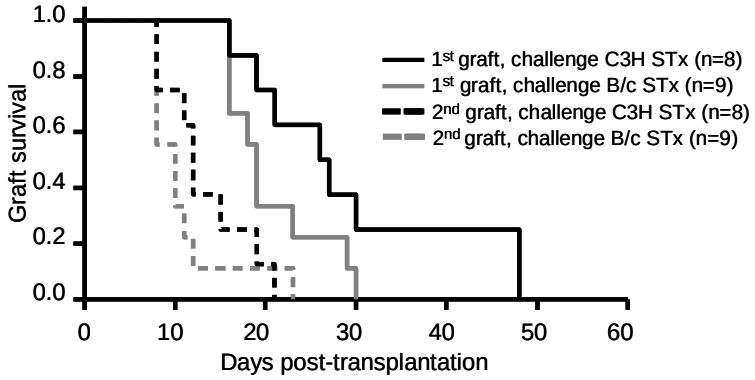

Figure 51: Challenge graph. On d50 post transplantation, MD-75 mice received a second transplant that was either B/c (donor) or C3H (third party). Allograft survival of first allograft (B/c) in both groups is compared with allograft survival of the second graft (B/c or C3H). n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test, multiple comparisons with Holm-Sidak method

To further examine regulatory and effector cells in the B/c grafts of B/6 mice treated with anti-CD154 + DST + Tac-75, grafts were explanted on d50 and retransplanted onto Rag1$^{-/-}$ mice. After 14d, leukocytes from dLNs of these Rag1$^{-/-}$ mice were analysed by flow cytometry (Figure 52). Both CD25^{+} FoxP3^{+} and CD25^{+} FoxP3^{-} CD4^{+} T cells were detected. Since Rag 1$^{-/-}$ mice lack T cells, these cells must have come out of the graft and originate from the B/6 recipient.

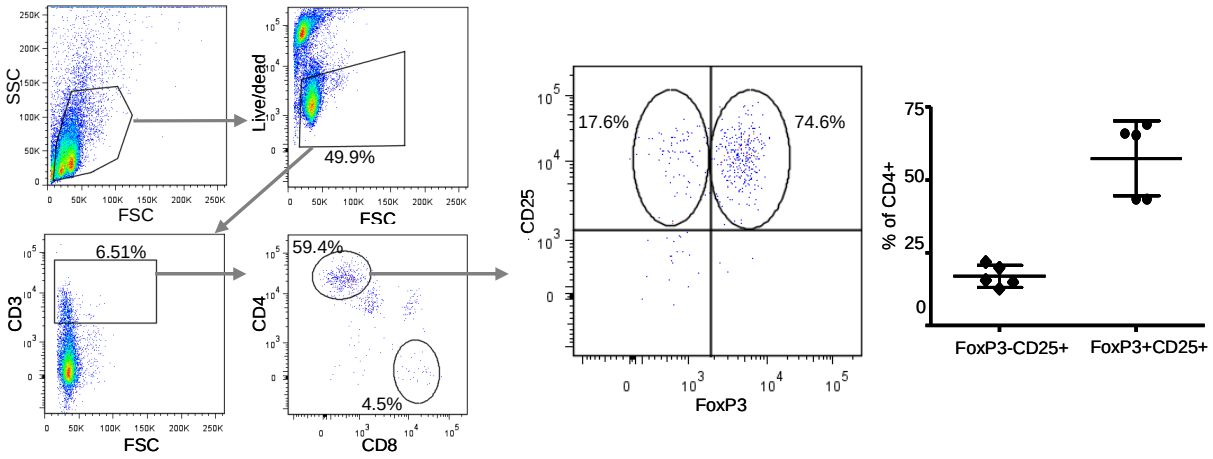

Figure 52: Effector and regulatory T cells transferred with graft. On d50 post transplantation, allografts from MD-75 mice were retransplanted onto Rag1$^{-/-}$ mice. 14 days later, dLNs from the Rag1$^{-/-}$ mice were analysed by flow cytometry. n = 5. Flow cytometry-plots from one representative sample is shown. Gates and arrows indicate the gating strategy; numbers in the plots indicate percentages of the parent population. The scatter plot shows percentages of FoxP3^{-} CD25^{+} FoxP3^{+} CD25^{+} of all five mice and the mean and SD.

In a transfer experiment depicted in Figure 53, grafts from MD-75 mice were retransplanted onto Rag1$^{-/-}$ mice. In addition, the recipients also received a second transplant from a naïve B/c donor on the same day.

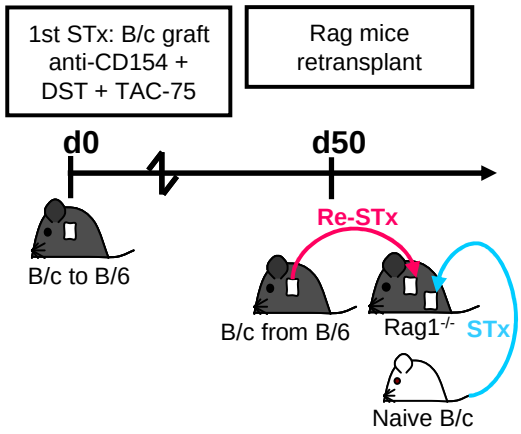

Figure 53: Experimental design retransplantation. On d50 post transplantation, allografts from MD-75 mice were retransplanted onto Rag1$^{-/-}$ mice. Additionally, these Rag1$^{-/-}$ mice received a skin graft from a naïve B/c donor.

It was planned to reconstitute the Rag1$^{-/-}$ mice 14 days later with T cells from a B/6 wildtype animal and to assess graft survival in order to test the hypothesis that the regulatory cells in the graft from a MD-75 mouse will prevent rejection from occurring as early as in naïve B/c graft. Surprisingly, before the reconstitution could be done, the Rag 1$^{-/-}$ mice rejected the graft from a MD-75 mouse, and also the graft from a naïve B/c donor. Three “naïve” grafts showing signs of rejection (haemorrhagic spots, hardening) were analysed by flow cytometry (Figure 54). In these B/c grafts, the majority of leukocytes (CD45.2) detected was negative for H2-K^{d} (BALB/c MHC I) and positive for H2-K^{b} (C57BL/6 MHC I). This population

contained $CD4^+$ T cells. The existence of $H2\text{-}K^b$ positive cells in the "naïve" grafts of B/c origin can be only explained by migration.

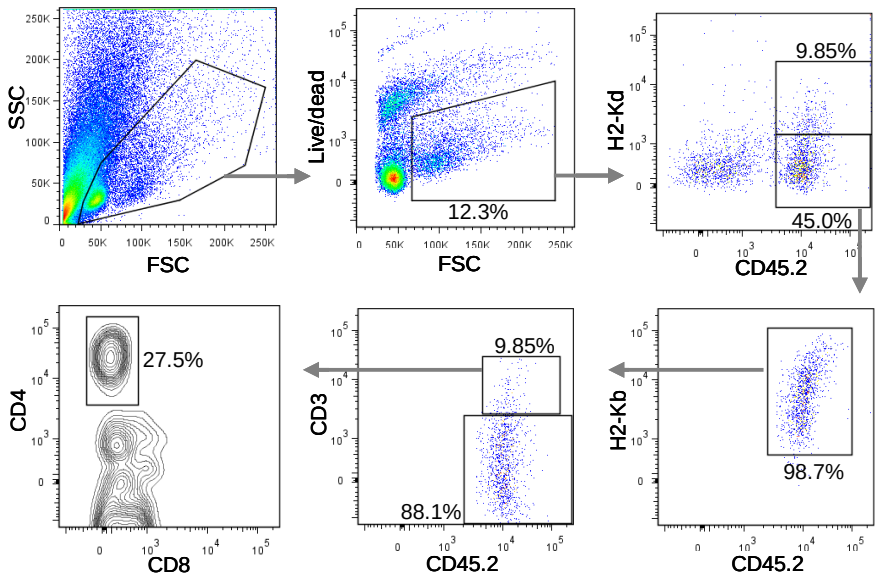

Figure 54: FACS analysis $Rag1^{-/-}$ with double transplants. One representative Flow cytometry plot shows the presence of T cells in the "naïve" graft undergoing rejection after the retransplanted graft had already been rejected. T cell were found in all 3 analysed grafts.

Taken together, these data indicate that effector cells from the C57BL/6 mouse receiving a B/c graft under anti-CD154 + DST + Tac-75 must have migrated into the graft (the "MD-75 graft"). Once the "MD-75 graft" was retransplanted onto a $Rag1^{-/-}$ mice together with a "naïve" B/c graft, these cells must have migrated to the "naïve" graft and then, they re-entered the "MD-75 graft" to cause allograft rejection.

To avoid the rejection of the allografts as observed above, another transfer experiment was designed as follows: One group of $Rag1^{-/-}$ mice received a retransplanted 50d - graft from a MD-75 mouse, whereas a second group of $Rag1^{-/-}$ mice received a "naïve" B/c graft from untreated donors (Figure 55).

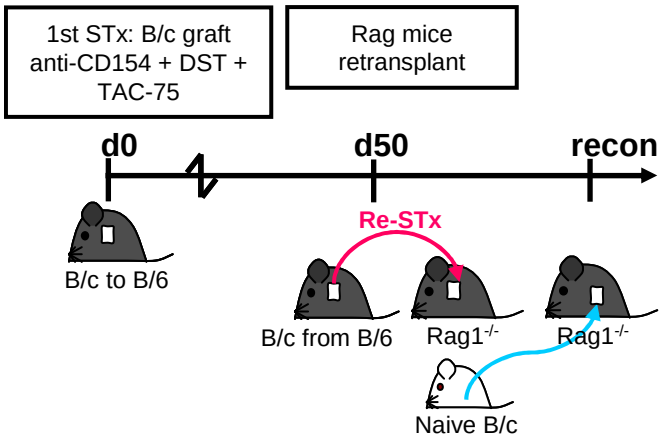

Figure 55: Experimental design retransplantation and reconstitution. On d50 post transplantation, allografts from MD-75 mice were retransplanted onto Rag1$^{-/-}$ mice. A second group of Rag1$^{-/-}$ mice received a skin graft from a naïve B/c donor. After 14 days, recipients were reconstituted with 1x10^6 T cells from untreated B/6 wildtype mice.

After 14 days, the recipients were reconstituted with 1x10^6 T cells from untreated B/6 wildtype mice. This precipitated allograft rejection in recipients with a "MD-75 graft" and in recipients with a "naïve" graft with similar kinetics (MST graft "MD-75" vs. "naïve": 14.4 ± 1.5 vs. 11.7 ± 1 days, p = 0.23; Figure 56). These results show that the regulation in the "MD-75 graft" alone is not strong enough to keep the balance and prevent rejection in the new setting, the Rag1$^{-/-}$ mouse.

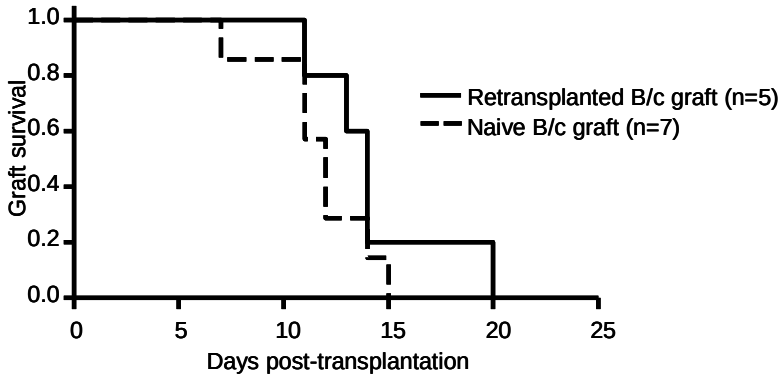

Figure 56: Transfer of regulation by retransplantation of the skin graft. On d50 post transplantation, allografts from MD-75 mice were retransplanted onto Rag1$^{-/-}$ mice. A second group of Rag1$^{-/-}$ mice received a skin graft from a naïve B/c donor. After 14 days, recipients were reconstituted with 1x10^6 T cells from untreated B/6 wildtype mice. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

Further, the presence of regulatory T cells was examined on the molecular level by qPCR analysis of foxp3 mRNA in the skin graft. For this, skin grafts of MD-75 mice were pooled (at least 3 grafts / group) and CD45.2$^+$ leukocytes were sorted. The control groups were mice that received a syngraft and the same treatment with anti-CD154 + DST + Tac-75. The foxp3 expression in all samples from mice with a syngraft is given as the fold expression of the maximum dilution of the reference allograft pool that could be detected using the manufacturer's instructions for the foxp3 primer. This was necessary, since the signal for

foxp3 came up only at 35 cycles or not at all, with no congruent results in the triplicate measurements. In contrast to this, expression of foxp3 mRNA could be detected reliably in all pooled samples from mice with an allograft (mean relative expression in syngrafted mice vs. allografted mice: 0.12 ± 0 vs. 1.6 ± 0.5, p = 0.002, Figure 57).

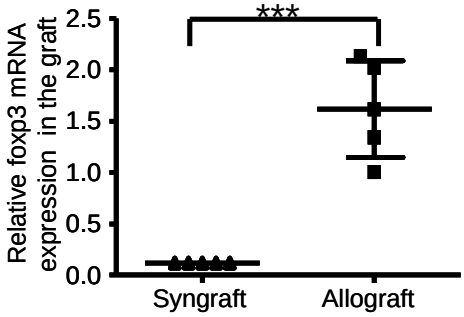

Figure 57: FoxP3- RT-PCR in skin grafts. Syn- and allografts from MD-75 mice were pooled on d50 post transplantation. RNA was isolated from CD45.2$^+$ leukocytes and levels of FoxP3 were determined. n = 5 pools per group, (at least 3 grafts / pool) . Scatter plots show the mean and standard deviation, FoxP3 expression in syngraft-pools was set according to the detection limit. Statistical analysis: One-sample T-Test, two-tailed (***: p < 0.005).

In addition, it was possible to detect FoxP3$^+$ cells in allografts of MD-75 on d50 or at the time point of rejection by immunohistochemical staining with anti-FoxP3 antibody (Figure 58). Judging from the phenotype of these cells (size, shape), they were lymphocytes.

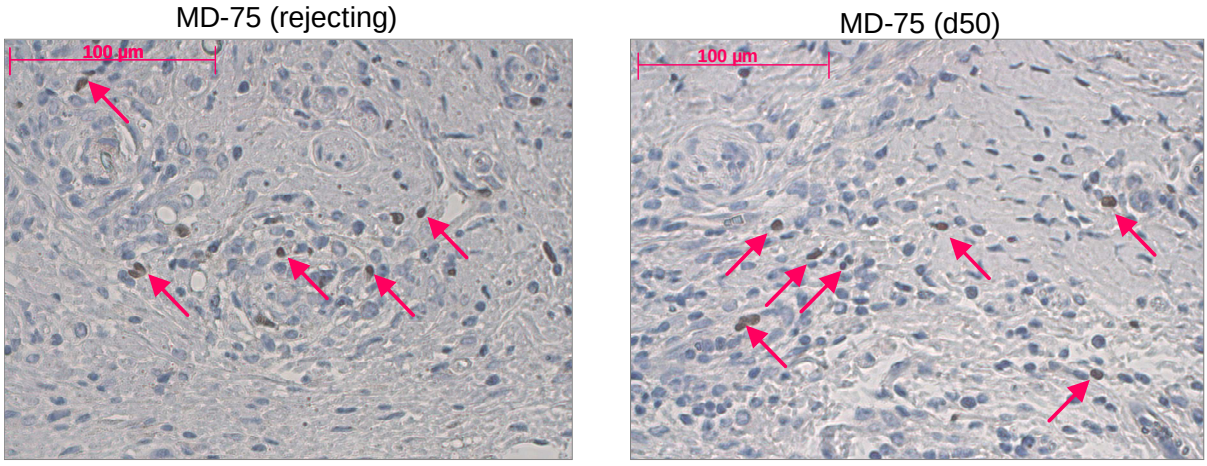

Figure 58: Immunohistochemical FoxP3 staining. FoxP3$^+$ cells were detected in skin sections of mice with a MD-75 allograft on d50 or a MD-75 allograft undergoing rejection od d61.

In summary, graft transfer experiments indicated the presence of both regulatory and effector T cell populations in the grafts of MD-75 mice. This was further confirmed by cellular and molecular analyses of FoxP3, indicating the presence of regulatory T cells. In addition, the presence of effector cell populations was indicated by transfer experiments fortified by flow cytometry data. These results fit the expectation that a balanced mixture of effector and regulatory cells in our model should be detectable at the same sites.

4.12.3 Analysis of myeloid cells in the graft

The skin grafts of MD-75 mice after d50 after syn- or allotransplantation were further analysed for cell populations with effector or regulatory function. No significant difference was observed in the MDSC compartment of allogeneic or syngeneic skin grafts (Figure 59: MST $CD11b^+Gr1^+$: syngraft vs. allograft: 2.5% ± 1% vs. 3.2% ± 1.2%, p = 0.19).

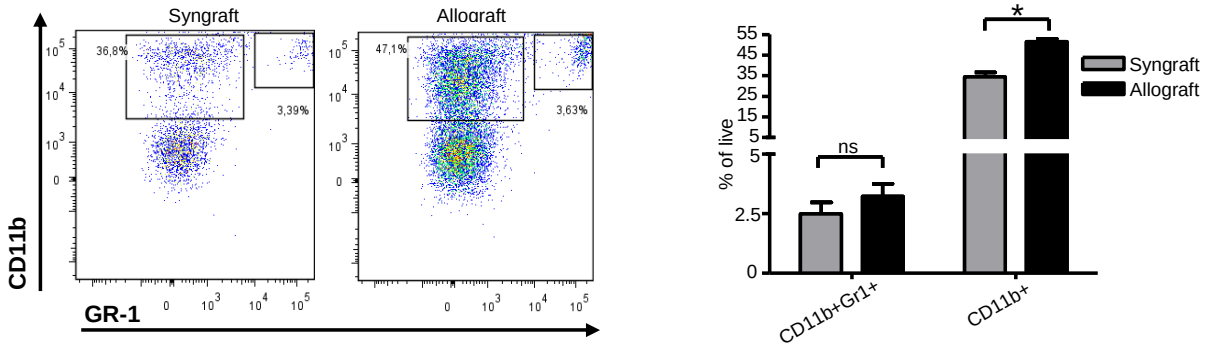

Figure 59: Myeloid cell analysis in skin grafts I. Syn- and allografts from MD-75 mice were analysed by Flow cytometry on d50 post transplantation. FACS plots showing gating information are representative examples. Column plots show the mean and standard deviation of $CD11b^+Gr1^+$ MDSCs and $CD11b^+$ macrophages. Statistical analysis: Mann-Whitney-Test, two-tailed (*: $p < 0.05$, ns: $p > 0.05$).

Yet, the percentage of $CD11b^+$ cells in allografts was significantly increased compared to the $CD11b^+$ population in syngeneic grafts (Figure 59: MST $CD11b^+$: syngraft vs. allograft: 34.5% ± 4.2% vs. 51.5% ± 3.1%, p = 0.02). Therefore, this population was examined further. Using the cell surface markers CD11b and CD11c, five distinct populations were gated in the skin grafts (Figure 60): 1) $CD11c^+$ $CD11b^{++}$, 2) $CD11c^-$ $CD11b^{++}$, 3) $CD11c^+$ $CD11b^+$, 4) $CD11c^-$ $CD11b^+$ and 5) $CD11c^+$ $CD11b^-$.

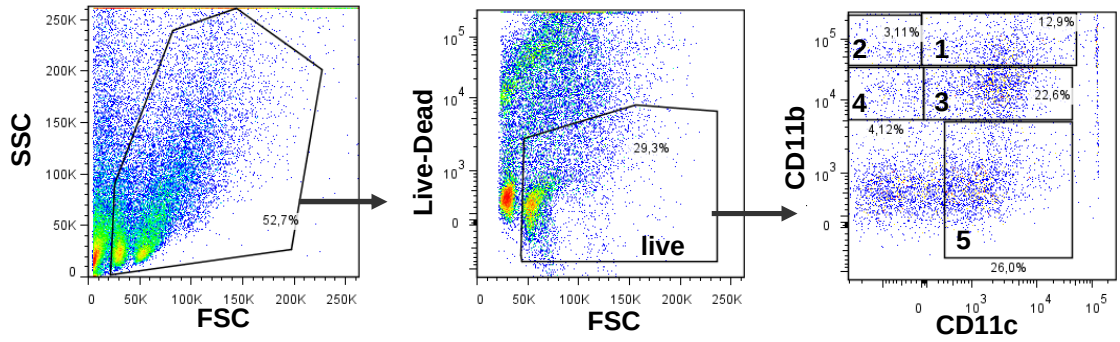

Figure 60: Myeloid cell analysis in skin grafts II. Syn- and allografts from MD-75 mice were analysed by Flow cytometry on d50 post transplantation. One representative plot is shown to demonstrate the gating strategy. .

The percentages of populations 1, 3 and 4 were significantly increased in allografts compared to syngrafts (Figure 61: $p = 0.005$, $p < 0.001$ and $p = 0.0004$, respectively). In contrast, the percentage of population 5 was significantly decreased in allografts ($p < 0.001$).

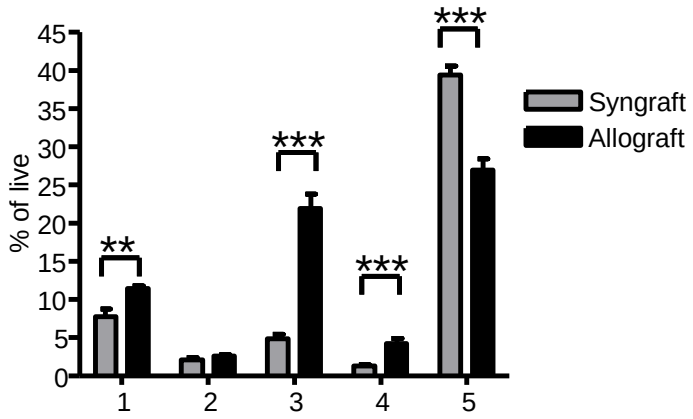

Figure 61: Myeloid cell analysis in skin grafts III. Skin grafts from MD-75 mice with a syn- or an allograft were analysed on d50 post transplantation. n = 12 per group. Column plots show the mean and standard deviation in population 1 – 5, gating strategy as described above. Statistical analysis: Mann-Whitney-Test, two-tailed (**: $p < 0.01$, ***: $p < 0.001$).

The populations in allografts were further characterised by flow cytometry. Population 1 expressed high levels of Dectin-1 and F4/80, a marker of mature macrophages, whilst the expression of both markers in population 3 was low or absent (Figure 62). Expression of MHC class II and the costimulatory molecules CD80 and CD86 was absent in population 3, indicating a non-immunogenic phenotype. In contrast, population 1 showed a heterogenic expression of MHC class II and intermediate levels of CD80 with absent CD86 expression, thus indicating partially matured antigen-presenting cells. The macrophage-restricted receptor Sialoadhesin (CD169) was expressed in both populations 1 and 3. Mononuclear phagocytes in the mouse dermis have been described as Dectin-1 positive cells also expressing the mannose receptor (CD206) and the macrophage galactose-/N-acetylgalactosamine-specificlectin (mMGL / CD301) [243]. Population 1 was negative for CD301 expression, as was population 3. Yet, in contrast to population 3, cells of population 1 did express the mannose receptor.

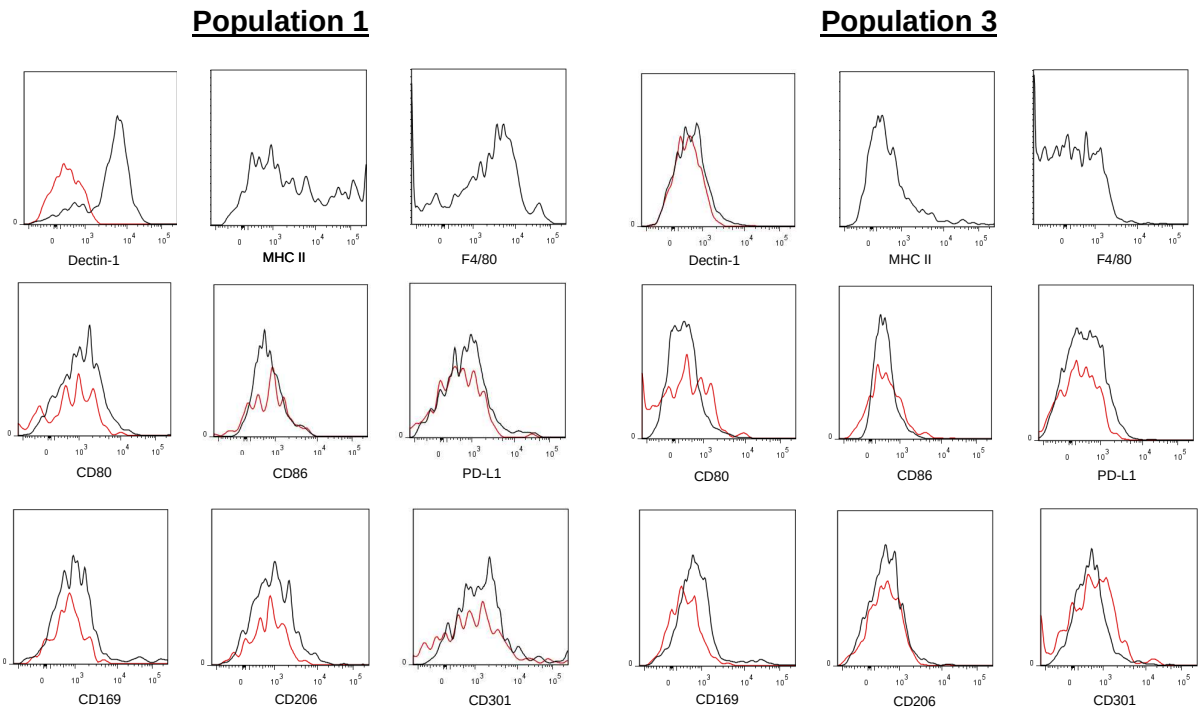

Figure 62: Expression profile of Population 1 and 3. Histogram plots, black lines represent specific signals; red traces represent isotype controls. Data are representative profiles of allografted mice.

The above described expression profiles are indicative of population 3 harbouring immature macrophages whilst population 1 is consistent with partially or completely matured macrophages. In allografts, the ratio of population 3 to population 1 is significantly enhanced compared to syngrafts (Figure 63, $p < 0.001$), skewing towards more immature macrophages in the graft.

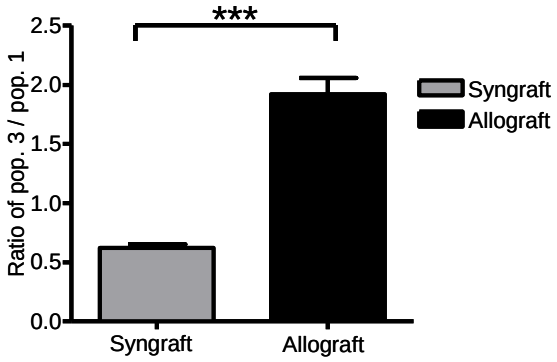

Figure 63: Ratio of Population 1 and 3. Mean and SD are displayed for . Data are representative profiles of allografted mice.

In addition, PD-L1 expression was detected in population 3 in contrast to population 1 (Figure 62).

Thus, non-immunogenic macrophages expressing PD-L1 might be a possible target for the anti-PD-L1 antibody treatment that precipitated rejection in MD-75 mice.

4.13 The balance tips

As indicated by previous data, allograft acceptance in MD-75 mice fails and grafts eventually undergo delayed acute rejection. In order to detect changes in the cellular compartment in MD-75 mice, effector and regulatory T cells were analysed further. As already mentioned and shown in Figure 21, on day 50 post transplantation, over 90% of MD-75 allografts are intact, but the grafts are rejected in the following weeks. Thus, effector and regulatory T cells in dLN from MD-75 mice on d50 were compared to dLN from MD-75 mice at a time point, where 50% of the grafts in this experimental group were rejected or rejecting (day 94). A significant increase in the percentage of $CD8^+$ T cells was observed comparing d50 mice with the "50% rejection" group (Figure 64a: d50 vs. "50% rejection": 51.3% ± 3.3% vs. 55.1% ± 3.2%, p = 0.015). The percentage of $CD4^+$ T cells in dLN of "50% rejection" mice with both intact and rejecting/rejected grafts was significantly decreased compared to d50 mice (d50 vs. "50% rejection": 43.3% ± 3.6% vs. 35.7% ± 2.1%, p = 0.002). The ratio of cytotoxic CD8+ T cells over CD4+ T cells was significantly increased in the "50% rejection" group compared to the d50 group (p = 0.002, Figure 64b).

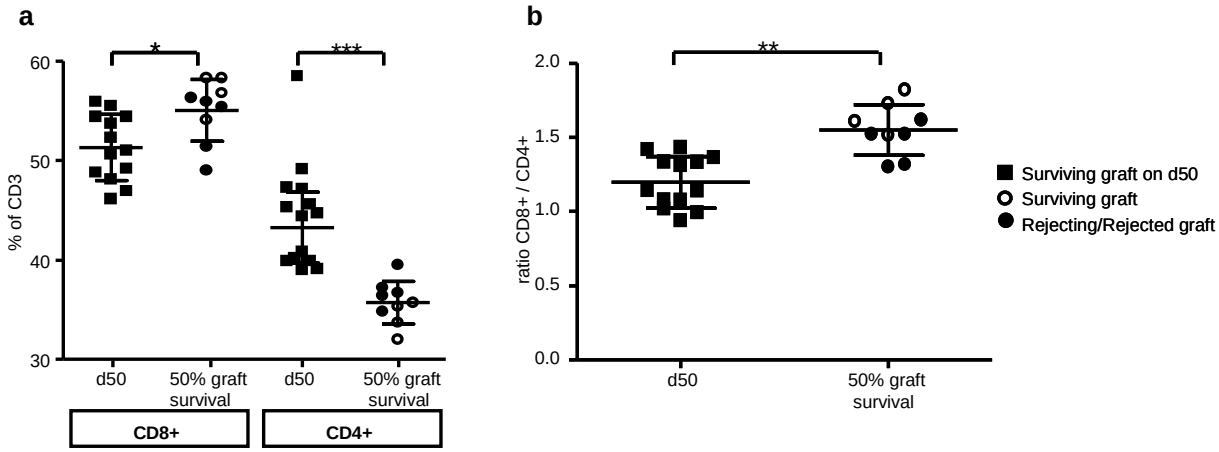

Figure 64: Analysis of $CD4^+$ and $CD8^+$ T cells in dLNs in MD-75 group with 50% rejection. Draining LNs from a experimental group of MD-75 mice with a syn- or an allograft were analysed when 50% of the mice had rejected the graft. Percentages of $CD4^+$ and $CD8^+$ of these mice were compared with data obtained from MD-75 on d50 with intact allografts. Scatter plots show the mean and standard deviation. Statistical analysis: Mann-Whitney-Test, two-tailed (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

In addition, the $CD4^+$ T cell compartment was analysed for the percentages of $CD25^+FoxP3^+$ T regs and $CD25^+FoxP3^-$ effector T cells. No significant changes were detected in the percentage of $CD25^+FoxP3^+$ amongst the $CD4^+$ population (Figure 65a: d50 vs. 50% graft survival group: 8% ± 1% vs. 7.3% ± 1.2%, p = 0.26). In contrast to this, the percentage of $CD25^+FoxP3^-$ amongst the $CD4^+$ population was significantly increased in the 50% graft survival group (d50 vs. 50% graft survival group: 3% ± 0.8% vs. 6.5% ± 1.4%, $p < 0.001$). The ratio of $CD4^+$ effector cells over $CD4^+$ regulatory was significantly increased in the "50% rejection" group compared to the d50 group ($p < 0.001$, Figure 65b).

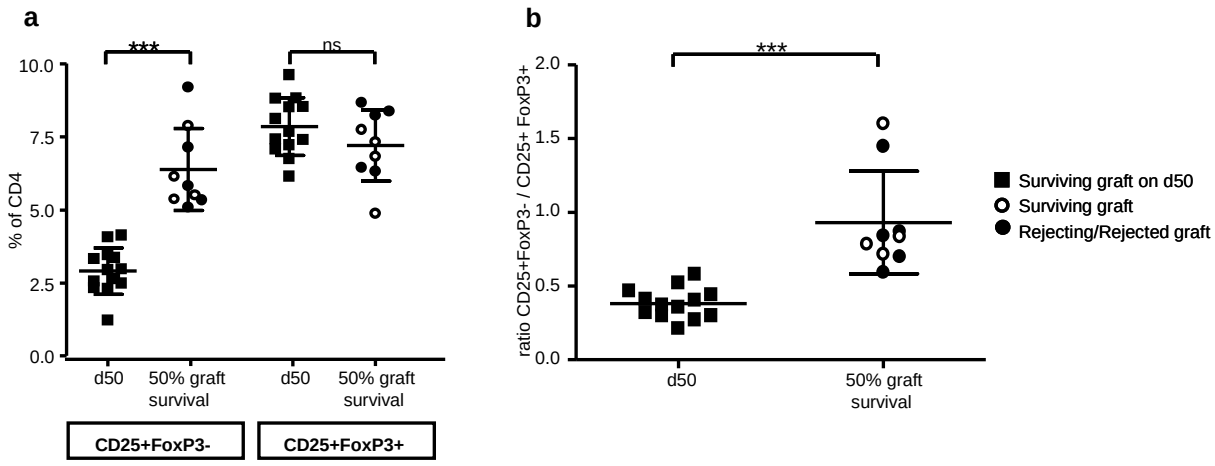

Figure 65: Analysis of dLNs in MD-75 group with 50% rejection. Draining LNs from a experimental group of MD-75 mice with a syn- or an allograft were analysed when 50% of the mice had rejected the graft. Percentages of $CD25^{+}FoxP3^{-}$ and $CD25^{+}FoxP3^{+}$ of these mice were compared with data obtained from MD-75 on d50 with intact allografts. Scatter plots show the mean and standard deviation. Statistical analysis: Mann-Whitney-Test, two-tailed (***: $p < 0.001$).

Thus, the population of $CD4^{+}$ effector T cells in the dLN increases over time and this increase is detectable in both mice undergoing rejection and mice with an intact allograft on d94. Nonetheless, it has to be mentioned that with these measurements, the whole polyclonal T cell pool is analysed and that therefore no conclusions can be made concerning the donor-antigen-specific populations of effector and regulatory T cells.

We then hypothesised that the late rejection that occurs in MD-75 mice is caused by donor-reactive T cells that emigrate from the thymus after the treatment with anti-CD154 + DST. Therefore, allograft acceptance was induced in C57BL/6 mice after they had been thymectomised at 5-6 weeks of age, together with their littermates that did not undergo thymectomy. Both groups were treated in conformity with the standard protocol of anti-CD154 + DST + Tac-75 and graft survival was monitored (Figure 66).

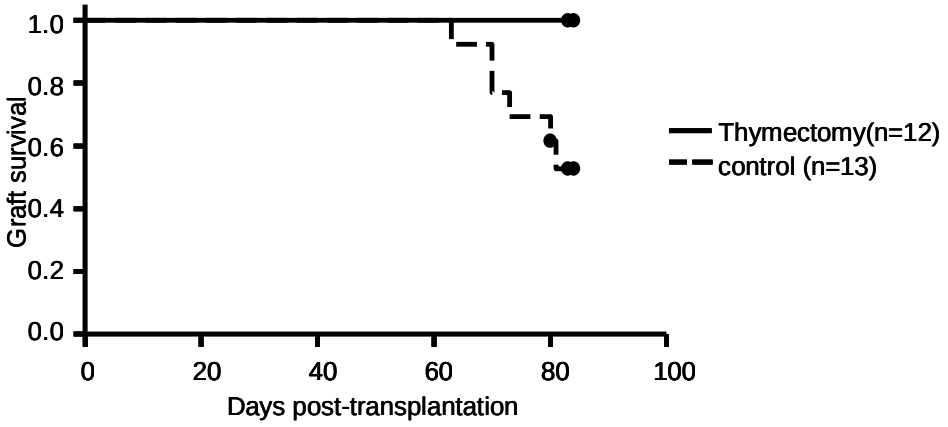

Figure 66: Thymectomy before induction therapy. Early thymectomised or untreated littermates were transplantated with a B/c graft and treated with anti-CD154 + DST + Tac-75 and graft survival was monitored. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

The “conventional” MD-75 mice rejected with similar kinetics as previously observed with MD-75 treatment (MST: 78.8 ± 2.1 days, 50% of the mice had rejected, p = 0.007). In contrast to this, none of thymectomised mice rejected the graft up to date (d84). Thus, we conclude that late rejection in MD-75 mice is caused, in part, by recent thymic emigrants.

4.14 Marginal states of allograft acceptance might be converted into operational tolerance

A logical consequence that follows from our hypothesis and the previous data is the desire to tip the balance of effector and regulatory cells in favour of regulation. By enhancing the regulatory response, it might be possible to withdraw immunosuppression completely and to achieve stable long-term allograft survival, i.e. operational tolerance. Therefore, MD-75 on d50 post transplantation received a second weak regulation induction (anti-CD154 + DST) and Tacrolimus food was withdrawn. In comparison to the allograft survival time of previous Tacrolimus withdrawal groups (Figure 33), mice receiving this boost therapy did reject the allograft significantly later (Figure 67: MST withdrawal vs. boost: 61.4 ± 1.5 vs. 87.5 ± 6.4 days, p < 0.001).

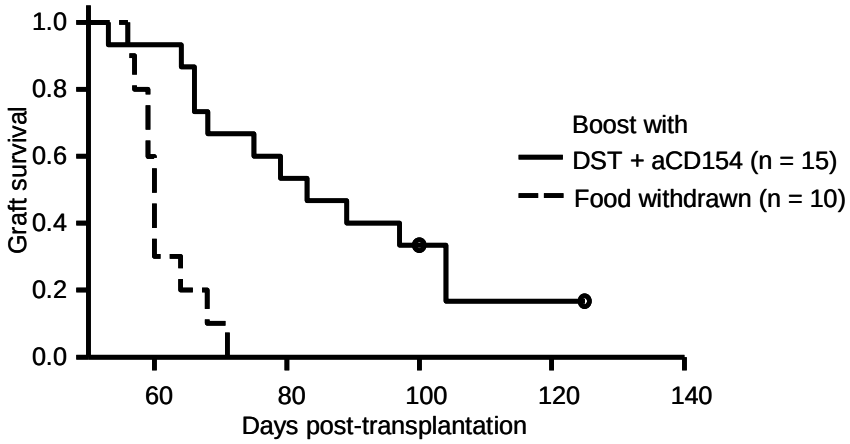

Figure 67: Boost of marginal states. Starting on d50 post transplantation MD-75 mice received a DST together with 4 injections of anti-CD154. At the same time, Tacrolimus food was switched to normal diet (withdrawal of immunosuppression) Allograft survival was compared to a historical control group, where Tacrolimus food was withdrawn from MD-75 mice on d50. n = number of animals per group. Kaplan-Meier-Survival plot. Statistical analysis: Log-rank test

These data indicate that it is feasible to achieve operational tolerance by boosting the regulatory response in marginal states, which is consistent with our hypothesis.

5 Discussion

Tolerance in transplantation can be seen as the qualitative result of the balance of the effector and regulatory response. This further implies the existence of marginal states of allograft acceptance. In such marginal states, neither the effector nor the regulatory response is predominating, which leads to an unstable condition of non-rejection. In these cases, low-dose immunosuppression might control a weak effector response or support a marginally exceeding regulatory response. A mouse skin transplant model of marginal states of allograft acceptance under low dose immunosuppression was established with this work. The existence of marginal states of allograft acceptance resulting from a balance between effector and regulatory response might have an influence on the development of new treatment strategies.

Allogeneic skin transplantation in mice is a widely used method to conduct research on graft survival and tolerance inducing strategies [3,195]. This is due to various reasons: skin transplantation is a very stringent model, involving a primary non-vascular graft that is highly immunogenic. We established a low-dose Tacrolimus monotherapy that is subtherapeutic and a weak-regulation inducing protocol, that does not lead to permanent graft acceptance in a murine skin transplant model. By combining both subtherapeutic treatments, an experimental model of marginal states of allograft acceptance under immunosuppression was built. The weak-regulation inducing protocol was established as treatment with a donor-specific transfusion (DST) of whole donor splenocytes in combination with a short course of anti-CD154 injections for costimulatory blockade or treatment with anti-CD154 alone.

Using this experimental model, we formally show that marginal states of allograft acceptance under low-dose immunosuppression are dependent upon immunological regulation. This regulation is supported by low doses of the CNI Tacrolimus, as shown *in vivo* and *in vitro*; and mediated by regulatory T cells. Our data demonstrate that the late rejection that occurs in marginal states of allograft acceptance is of an acute type and mediated by thymic emigrants. Importantly, we could show that the boost of regulation in marginal states and thus further prolongation of allograft survival is achievable.

5.1 Synergistic effect of Tacrolimus and regulation

Depletion of alloreactive $CD8^+$ T cells on the one side and the induction of regulatory T cells and hyporesponsiveness on the other side are not sufficient to induce tolerance with indefinite allograft survival in the full mismatch B/c-to-B/6 skin transplant model with anti-

CD154 + DST. Therefore, and because the induced allograft survival was significantly reduced in comparison to the tolerance inducing protocol of triple costimulatory blockade, we consider treatment with anti-CD154 + DST as a weak tolerance inducing strategy in the stringent model of B/c-to-B/6 skin transplantation.

The use of Tacrolimus monotherapy in the allogeneic skin transplant model showed that doses of 50, 75 and 100 mg per kg food had no or a small prolongation effect on allograft survival at the introduction time points tested. In contrast, doses of 150 mg/kg were found to be therapeutic when given before or at the time point of transplantation. Reflecting the clinical situation, therapeutic doses of Tacrolimus led to kidney damage, possibly due to viral infection. Yet, the inclusion bodies detected in mice treated with 150 mg/kg must still be identified, since histological staining for BK-virus, CMV and Adenovirus did not give positive results. Molecular analysis by virus-specific qPCR would be an approach to clarify the histopathological findings. Importantly, no or only mild kidney damage was observed after long-term treatment in mice under low-dose Tacrolimus (75 and 100 mg/kg, respectively), illustrating the benefit of CNI minimisation.

Regarding the compatibility of CNIs and tolerance induction with costimulatory blockade, conflicting data have been published. Some groups reported antagonistic effects of CNI treatment; when combined with costimulatory blockade, this resulted in the abrogation of tolerance [244-246]. In contrast, there is also data showing neither beneficial or adverse effects of combined treatment with CNI and costimulatory blockade [246-248]. The combination of costimulatory blockade with CNI could also enhance prolongation of allograft survival. This was demonstrated with the combination of CTLA4-Ig and suboptimal CsA in a rat cardiac model [249] as well as the combination of ICOS and suboptimal Tacrolimus in a rat liver transplant model [250].

Our data of Tacrolimus monotherapy and the data obtained when Tacrolimus at high (150 mg/kg) or low doses (75 mg/kg) was combined with the weak tolerance induction therapy show two modes of action of Tacrolimus. Interestingly, apart from the immunosuppressive feature, Tacrolimus also supports regulation in our allogeneic skin transplant model when given in low doses. Moreover, this synergistic effect was found to prolong allograft survival dose-dependently, comparing doses of 50, 75 and 100 mg/kg.

This finding is unexpected in the light of previously published data. Tacrolimus as a Calcineurin inhibitor prevents the dephosphorylation of NF-AT, thus blocking the transcription of the IL-2 gene and therefore IL-2 production. This results in suppression of the activation,

differentiation and proliferation of naïve and memory effector $CD4^+$ and $CD8^+$ T cells [171,172], preventing rejection of allografts. But the production of IL-2 (mainly by $CD4^+CD25^-$ T cells) is also necessary for the expansion and suppressive activity of $CD4^+CD25^+$ regulatory T cells [251,252]. In clinical studies, Tacrolimus levels were inversely correlated with T reg numbers [253] and patients under Tacrolimus treatment had lower numbers of circulating T regs than patients treated with Rapamycin [254]. Several studies, human or mouse, showed detrimental effects of Calcineurin inhibition on the generation and function of T regs [255-259]. On the basis of these data, it was concluded that CNI have a generally negative effect on regulatory T cells [260]. Yet, T regs from patients under CNI treatment were still capable of suppression in *ex vivo* cultures [257,261] and in lung transplant patients, the expansion of peripheral $CD4^+CD25^+$ T reg was not inhibited by CNI-based treatments [262].

We thus contemplated that T regs might be less susceptible to suppression by Tacrolimus in low doses than effector T cells. Therefore, we performed *in vitro* suppression assays in the presence of low doses of Tacrolimus to examine the *in vivo* observed regulation-supportive effect. Our *in vitro* data show an enhanced suppression of IFNγ expression in the presence of low doses of Tacrolimus in T reg suppression assays which might indicate a possible delayed impact on T reg function. It was described by Takada et al. that the distribution of Tacrolimus to the lymphatic circulation is extremely low (less than 0.2%) [263]. We can only speculate whether the Tacrolimus doses used *in vitro* (0.25 – 2 ng/ml) correspond effectively to the subtherapeutic Tacrolimus doses that further enhanced the allograft survival prolongation effect of anti-CD154 + DST in our *in vivo* model. Yet, under the assumption that they do, then the increased suppressive function of T regs is one probable reason for the beneficial effect of low-dose Tacrolimus on the weak tolerance inducing therapy in vivo.

5.2 Breaking marginal states by disrupting regulation

It follows from our hypothesis that in marginal states, a disturbance of either the regulation or the immunosuppression should tip the balance and lead to rejection of the graft. Our data show that by 1) withdrawing immunosuppression, 2) enhancing the effector response or 3) disrupting the regulatory response, allograft acceptance is abrogated.

We have shown with several approaches that regulation is the basis for allograft acceptance in our model of marginal states under low-dose immunosuppression. As mentioned above the presence of regulatory T cells in transplanted animals treated with anti-CD154 + DST has been demonstrated by various groups and together with our *in vitro* data, this was the reason

for us to disrupt T reg mediated regulation. Yet, whilst murine T regs express CD25, treatment with depleting anti-CD25 antibody could not break allograft acceptance. This is possibly due to the fact that anti-CD25 does not specifically deplete T regs, but also activated effector cells that express CD25 [264].

The transmembrane protein GITR is predominantly and highly expressed by T regs, yet its expression can be upregulated in activated $CD4^+$ and $CD8^+$ T cells. The suppression of $CD4^+CD25^-$ T cells by $CD4^+CD25^+$ T regs has been shown to be dependent on GITR expression on the T regs [104]. Neutralisation of GITR signalling by agonistic anti-GITR antibody abrogated suppression *in vitro* and, in line with our findings, *in vivo* in transplant models [104,265].

PD-L1 and PD-1 are highly expressed on $FoxP3^+$ T regs, but also on various other cell types [105]. PD-L1 blockade might thus be rather unspecific and not directly blocking regulatory T cells. Yet, it has been described in a cardiac transplant model that blockade of PD-L1 promotes expansion of $CD8^+$ and $CD4^+$ effector cells, decreased the number of $FoxP3^+$ T regs in the grafts and, as observed in our model, caused allograft rejection [266].

The depletion of regulatory $CD4^+$ T cells in FoxP3-DTR mice by DT is highly specific, since FoxP3 expression is generally restricted to suppressive T cells [267]. In FoxP3-GFP-DTR mice, in contrast to C57BL/6 wildtype mice, allograft acceptance could not be established by combining anti-CD154 + DST with Tacrolimus at 75 mg/kg. This finding was unexpected, since the FoxP3-GFP-DTR mice were backcrossed to C57BL/6 background and the serum levels of Tacrolimus were comparable to wildtype C57BL/6 mice. The allograft survival curve of FoxP3-GFP-DTR mice treated with anti-CD154 + DST and Tacrolimus at 75 mg/kg and without DT-injections was similar to C57BL/6 mice treated with anti-CD154 + DST alone. Thus, we suspected that higher doses of immunosuppression were necessary to induce marginal states of allograft acceptance. Indeed, when the anti-CD154 + DST treatment in FoxP3-GFP-DTR mice was combined with Tacrolimus at 100 mg/kg, long-term allograft survival as observed in wildtype mice was induced. Depletion of T regs by DT-injections did abrogate allograft survival demonstrating that allograft survival in our model is dependent upon regulation mediated by T regs.

We consider the fact that allograft acceptance in our model was abrogated by treatment with anti-TGFβ antibody as an indication for the involvement of induced T regs. The induction of T regs was described after treatment with anti-CD154 + DST [197]. TGFβ has not only been

shown as important for the induction of T regs but also as an inhibitor of effector T cell function [113,114].

In line with data from Waldmann et al., we hypothesised that the regulation in our model of marginal states is transferrable [268]. However, classical skin transfer experiments onto T cell depleted mice ($Rag1^{-/-}$) and subsequent reconstitution with T cells did not show transferrable regulation. Indeed, already before reconstitution, the balance between regulatory and effector response was skewed, presumably due to the loss of immunosuppression. Yet, our data from the dLN-cell transfer indicate that donor-specific regulatory T cells were present in MD-75 mice and could be transferred.

Altogether, our data show that allograft acceptance under low-dose immunosuppression is dependent upon regulation.

5.3 Collapse of marginal states

In our experimental model of marginal states of allograft acceptance under low-dose Tacrolimus therapy, the balance between regulatory and effector response is skewed towards regulation and thus, long-term graft survival is achieved. However, whilst over 90% of the mice had an intact graft on day 50, rejection eventually occurred in 97% before 100 days. This brings up the question, what causes the balance between effectors and regulators to tip towards rejection? Possible explanations may include: 1) (partial) loss of regulation, 2) increasing effector responses or 3) diminishing effect of immunosuppressive treatment.

Our data comparing MD-75 mice with an intact graft on day 50 with a group of MD-75 mice with 50% rejecting/rejected and 50% intact surviving allografts did not show significant differences in the number of total $FoxP3^+$ T regs in the dLNs. On the current evidence, it cannot be excluded that the number of allospecific $FoxP3^+$ T regs was decreased, since allospecificity was not assessed in our system. Thus, the loss of allospecific T regs and thus regulation remains a possibility that could account for rejection in our model of marginal states. This will be tested in future experiments using MHC dexamers.

Depletion of $CD8^+$ T cells by depleting CD8-antibody [194] or DST [195,203] has been reported as necessary for the induction of long-term allograft survival in the skin transplant model. But in the stringent B/c-to-B/6-skin transplant model, $CD8^+$ T cell depletion by combination of anti-CD154 + DST alone does not prevent rejection [195]. Also in our model of marginal states under low-dose immunosuppression, rejection occurs eventually. We

could show that thymectomy in adult mice prior to the induction therapy with anti-CD154 + DST + Tac-75 and allotransplantation significantly delayed rejection. This is in line with the findings of Rossini and colleagues, who have reported over 80% graft survival at d250 in thymectomised mice treated with anti-CD154 + DST without additional immunosuppression [198]. Thus our data are consistent with the conclusion that recent thymic $CD8^+$ emigrants are responsible for late rejection in this model. In the group of MD-75 mice with 50% rejecting/rejected and 50% intact surviving allografts, the percentage of $CD8^+$ T cells in the dLNs was significantly increased compared to MD-75 mice on d50. Together, these data indicate that recent $CD8^+$ thymic emigrants enhancing the effector response might precipitate allograft rejection in our model of marginal states. One possibility to further examine this hypothesis would be to deplete $CD8^+$ T cells on d50, which should lead to enhanced prolongation of the allograft survival.

Iwakoshi et al. demonstrated that decline of anti-CD154 antibody in a transgenic costimulatory/DST – model in combination with increased numbers of alloreactive $CD8^+$ T cells correlates with rejection. Specifically, they showed that anti-CD154 concentrations below 50 µg/ml could not prevent generation of T cell-responses in C57BL/6 mice and in transplanted transgenic CBA mice, this threshold level correlated with the initiation of rejection on d50 [269]. Yet, about 50% of those mice kept their graft for another 100 days. In the light of successful induction of indefinite allograft acceptance with anti-CD154, as mentioned above, it remains unclear whether the persistence of costimulatory blockade really is required for the maintenance of regulation and thus graft acceptance. We think it is possible that the decline of the anti-CD154 antibody means that $CD4^+$ T cells receive adequate costimulation and that these activated $CD4^+$ T cells provide critical help for $CD8^+$ effector T cell responses.

There is evidence for all three described mechanisms to contribute to the collapse of marginal states of allograft acceptance. Further work needs to be done to clarify the potential collaborative involvement to ultimately target these mechanisms in order to prevent late rejection in marginal states.

A question that remains is whether the rejection that occurs in mice treated with anti-CD154 + DST + low-dose Tacrolimus is purely antigen-specific. In our model of marginal states characterised by the balance between effector and regulatory response, one can imagine that minor inflammatory conditions might add up to the effector response and thus tipping the balance and causing rejection. Mechanisms that could account for non-alloreactive fortification of the effector pool are bystander activation and heterologous immunity.

Bystander activation of T cells describes the TCR-independent, non-antigen specific activation of T cell responses by cytokines during an antigen-specific immune response [270]. Heterologous immunity refers to the reactivation of memory T cells generated during an earlier immune response by a second, unrelated immunogenic stimulus [271]. Here, the TCR of memory cells against antigen A cross-reactively recognizes an epitope of antigen B that is shared with antigen A or structurally similar to epitopes from antigen A.

Bacterial infection with *Listeria monocytogenes* has been described to break allograft survival induced by anti-CD154 + DST in a murine cardiac transplant model due to IL-6 and IFNβ production [272]. In an allogeneic skin transplant model with anti-CD154 + DST treatment, infection with lymphocytic choriomeningitis virus abrogated allograft survival in contrast to syngraft survival [273]. However, the contribution of both bystander activation and heterologous immunity to the late rejection observed in our model remains speculative, since we did not perform any infection experiments to precipitate rejection. The animal husbandry during the experiments does not exclude the possibility of infections, as suggested by the finding of inclusion bodies in 150 mg/kg sentinel mice.

5.4 Boost of marginal states

We observed in our model that the balance between the effector and regulatory response tipped and skewed towards rejection. In order to boost the regulatory response, we treated mice after 50 days with a second round of induction therapy under withdrawal of Tacrolimus and thus achieved delayed allograft rejection. These data show that it is also possible to tip the balance towards stronger regulation and possibly eventually tolerance (i.e. allograft acceptance without immunosuppression). Data from a rat kidney transplant model shows that short-term low-dose CNI treatment in combination with the single administration of regulatory T cells could replace permanent immunosuppression by reducing circulating memory T cells [274]. The application of T reg or M reg in our skin transplant model is a promising approach to boost the regulation in marginal states to long-term allograft acceptance without immunosuppression, i.e. true tolerance.

The feasibility to boost the regulatory response in marginal states is of importance with regard to the clinical situation, where there might be already patients under immunosuppression with regulatory responses just not sufficient to prevent rejection. If this regulation could be enforced, then operational tolerance seems an achievable goal.

6 Conclusion & Perspectives

The objective of our project was to prove that marginal states of allograft acceptance exist. Therefore, we established a model combining weak regulation with low-dose Tacrolimus to achieve significantly prolonged allograft survival. Importantly, neither the low dose Tacrolimus therapy nor the weak regulation alone led to such a prolonged allograft survival. We proved our hypotheses that marginal states can be disrupted by withdrawing the immunosuppression, enhancing the effector response or disturbing the regulatory response, thus posing the following questions: What information do we gain from the experimental model of marginal states in regard to clinical situations? Are these marginal states under low-doses of CNI – immunosuppression desirable?

With caution, we think that marginal states under low-doses of CNI – immunosuppression are desirable in the clinical situation. For the deliberate induction, it must be ensured that the balance will not be skewed towards rejection. Therefore, the underlying immune responses have to be identified, monitored and antagonised if necessary. Additionally, it must be kept in mind that any safe reduction of immunosuppression is of great benefit to the patient, if thus the adverse effects can be minimised and chronic allograft damage can be delayed or avoided. Importantly, the achievement of marginal states of allograft acceptance may be a potentially meaningful result of tolerance-inducing therapies and seems more immediately achievable than full operational tolerance. With regard to clinical therapy, it might be beneficial to use inductive prime-boost strategies in combination with low doses of immunosuppression which then will be extended to post-operative tolerance-promoting therapies. The idea to boost regulation at a later time point would be a novel therapeutic approach in the field of transplantation.

The model system established in this thesis gives not only formal proof for the existence of marginal states of allograft acceptance, it further provides a foundation for: 1) determining biomarkers that reflect immunological regulation under immunosuppression, 2) understanding underlying immunological mechanisms of marginal states, 3) testing new therapeutic strategies that favour the induction of immune regulation, 4) understanding mechanisms of rejection in marginal states in order to antagonise them or 5) developing strategies to boost the regulatory response.

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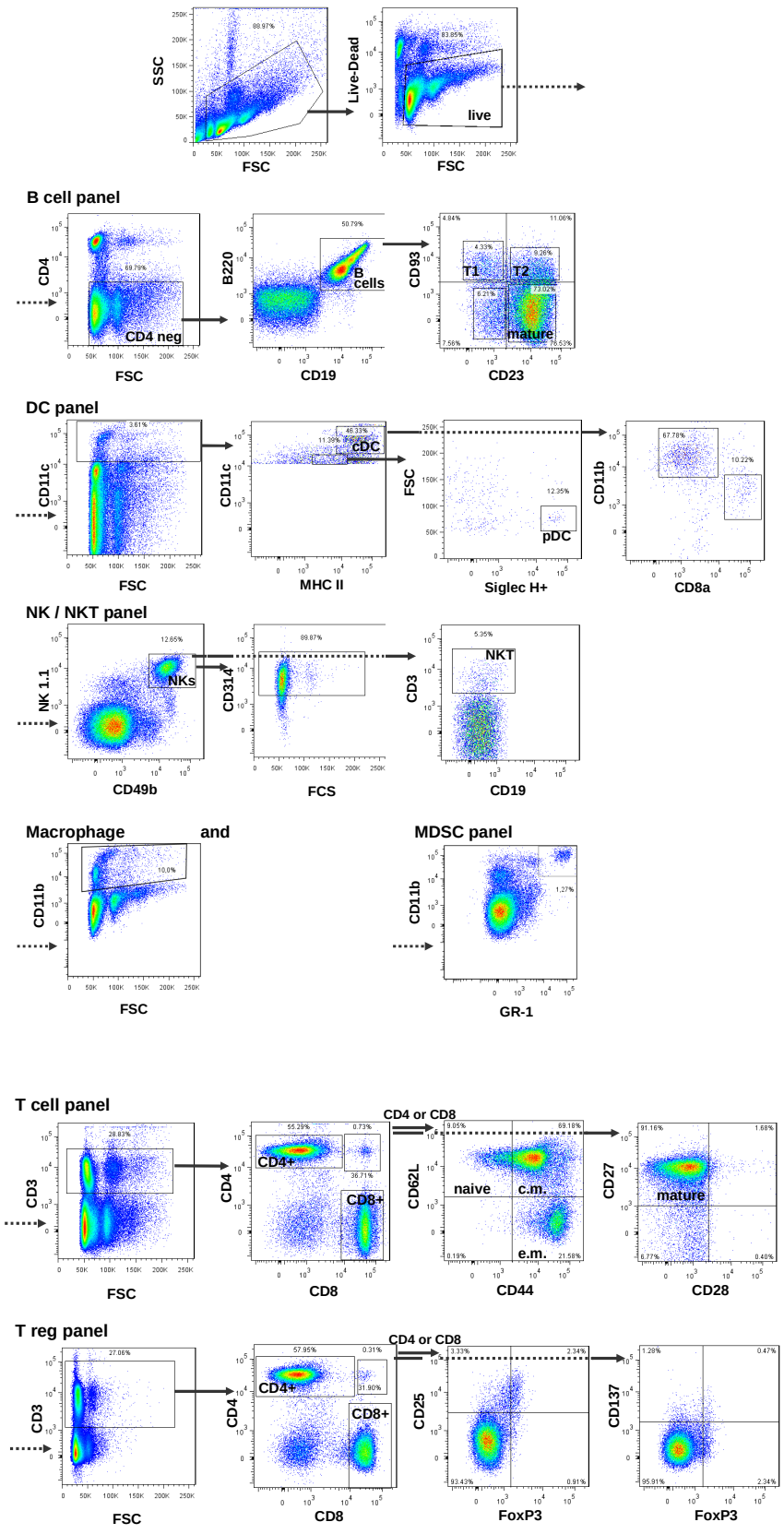
8 Appendix

8.1 List of Figures

8.2 Supplementary figure



Supplementary figure I: Panel Gating strategy

8.3 Abbreviations

°C	Degree Celsius
µ	Micro
µg	Microgram
µl	Microliter
ACK	Ammonium-Chloride-Potassium
ADCC	Antibody dependent cellular toxicity
Ag	Antigen
aH	Armenian Hamster
ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Antigen presenting cell
APC	Allophycocyanine
APC-Cy7	Allophycocyanine-cyanine 7
AUC	Area under the curve
BD	Becton, Dickinson and Company
BKV	BK-virus
BM	Bone marrow
BMDC	Bone marrow derived cell
BSA	Bovine serum albumine
c_0	Trough level
Ca^{2+}	Calcium
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CD40L	CD40 ligand
CFDA-SE	Carboxyfluorescein diacetate N-succinimidyl ester
CFSE	Carboxyfluorescein succinimidyl ester
c_{max}	Maximum concentration
c_{min}	Minimum concentration
CMV	Cytomegalovirus
CN	Calcineurin
d	Day
DAG	Diacylglycerol kinase
DC	Dendritic cell
dDC	Donor-DC
ddH_2O	Double-distilled water
dil.	Dilution
dLN	Draining LN
DN	Double negative
DNA	Desoxyribonucleic acid
DST	Donor-specific transfusion
DT	Diphtheria toxin
DTH	Delayed type hypersensitivity
DTR	Diphtheria toxin receptor
e.g.	Exempli gratia (for example)

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
et al.	Et aliae (and others)
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
Fc	Fragment crystallisable
FCS	Fetal calf serum
FcγRIIA/C	Fc-gamma-receptor II A/C
Fig.	Figure
FITC	Fluorescein isothiocyanate
FK-506	Tacrolimus
FKBP	FK506 binding protein
FoxP3	Forkhead box P3
g	Gram
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced TNFR family related gene
GM-CSF	Granulocyte / macrophage colony-stimulating factor
G-SCF	Granulocyte-colony stimulating factor
GVHD	Graft-versus-host disease
h	Hour
H&E	Haematoxilin & Eosin
HLA	Human leukocyte antigen
i.e.	Id est (that is)
i.p.	Intraperitoneally
i.v.	Intravenously
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-R	Interleukin-receptor
JAK	Janus kinase
JCV	JC-virus
k	Kilo
kg	Kilogram
l	Liter
Lck	Lymphocyte-specific protein tyrosine kinase
LD50	Lethal dose, 50%
LN	Lymph node
Ly6C	Lymphocyte antigen 6 C
Ly6G	Lymphocyte antigen 6 G
m	Milli
m	Meter
M	Molar
M reg	Regulatory Macrophage
MACS	Magnetic cell separation
MAPK	Mitogen-activated protein kinase
MD-100	Treatment: anti-CD154 + DST + 100 mg/kg Tacrolimus
MD-75	Treatment: anti-CD154 + DST + 75 mg/kg Tacrolimus
MDSC	Myeloid-derived suppressor cell
mg	Milligram

MHC	Major Histocompatibility Complex
min	Minute
ml	Milliliter
mRNA	Messenger ribonucleic acid
MST	Mean survival time
n	Number
n	Nano
ND	Not determined
NF-AT	Nuclear factor of activated T cells
NF-Atc	Nuclear factor of activated T cells, cytosolic
NF-Atn	Nuclear factor of activated T cells, nuclear
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B
ng	Nanogram
NK	Natural killer
ns	Not significant
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase-chain reaction
PD	Programmed death
PD-L1	Programmed death-ligand 1
PE	Phycoerythrin
PE-Cy 7	Phycoerythrin-cyanine 7
PerCP	Peridinin-chlorophyll-protein complex
PerCP-Cy5.5	Peridinin-chlorophyll-protein complex-cyanine 5.5
pg	Picogram
PI3K	Phosphatidylinositol 3-kinases
PKC	Proteine kinase C
PLCγ	Phospholipase C[gamma]
qPCR	Quantitative real-time PCR
QT	Quantitect
R&D	Reserach & diagnostics
RA	Retinoic acid
RAG	Recombination activation gene
rcf	Relative centrifugal force
rDC	Recipient-DC
RNA	Ribonucleic acid
ROS	Reactive oxgen species
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Real-time PCR
SD	Standard deviation
SEM	Standard error of the mean
sH	Syrian hamster
STAT	Signal transducers and activators of transcription
SV-40	Simian virus 40
T reg	Regulatory T cell
Tac	Tacrolimus
TBS	Tris-buffered saline

TCR	T cell receptor
TGFβ	Transforming growth factor β
Th	T helper
TNF	Tumour necrosis factor
Ts	Suppressor T cell
T-TBS	TBS supplemented with 0.5 % (v/v) Triton-X 100
USA	United States of America
v/v	Volume/volume
w/v	Weight/volume
ZAP70	Zeta-chain-associated protein kinase 70

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FIN