Division of Organ Replacement and Xenotransplantation Surgery
Kagoshima University
Graduate School of Medical and dental Science

Transplantation Tolerance: Allo-recognition and Regulatory T Cells

YUSUKE TOMITA

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Chapter

Title.

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Chapter 1: Overall Introduction

1-1. Overall aim

Overall aim of this thesis is better to understand mechanism of immunotolerace. More specifically, this thesis focuses on mechanisms of transplant peripheral tolerance.

1-2. Current obstacles in organ transplantation

Organ transplantation is currently the only definitive therapeutic method for end stage organ failure. Because of newly developed immunosuppresion regimens [1-4] as well as technical improvements over the past 20 years, both patient and graft survivals of allogeneic organs has markedly improved. Graft survival of allogeneic kidneys at 3 years exceed 85% [4, 5]. However, there are two major obstacles in current organ transplantation which are 1) organ shortage, and 2) life-long immunosuppressive therapy.

In the field of kidney transplantation (KTx), current statistics of end-stage renal disease (ESRD) patients in Japan indicate that while approximately 300,000 patients were under maintenance dialysis, KTx was performed on only 1600 patients each year. KTx, not dialysis, is the preferred treatment of choice for patients with ESRD. Because of the benefit of higher patient survival rate in KTx as opposed to survival rates on dialysis [6, 7], KTx is becoming an option even for patients with many risk factors In Japan, living KTx has been the most popular modality of organ procurement, and although the number of donation after brain death donors has been increasing since the organ transplant law was revised in 2010, there is not nearly enough to accommodate the current organ shortage. The number of recipients waiting for a transplant is

increasing. The average waiting time for dialysis patients to receive kidney grafts in Japan is 13 years as opposed to the 5 years in most countries. Recent efforts worldwide seek to expand the donor, including utilization of kidneys from expanded criteria deceased donors after circulatory death [8-10], dual kidney transplants [11, 12], revised graft perfusion methods for preventing re-perfusion injury [13, 14], new immunosuppressive regimens [15] and xeno-transplantation [16-18].

Currently, it is necessary for organ recipients to take life-long immunosuppression for preventing acute and/or chronic rejection following allogeneic transplantation [19]. Although development of new immunosuppressive drugs have dramatically improved the outcomes of transplantation during the last decade [4, 20], these drugs are not only a burden of the organ itself, but also associated with significant adverse effects, including cancers, infections, cardiovascular diseases and metabolic syndromes [21, 22]. These events are now one of the most frequent causes of graft loss in patient undergoing organ transplantation, only behind chronic rejection and death with a functioning graft.

1-3. Transplantation Tolerance

Tolerance of allo-graft is defined as a state in which there is a maintained immunological balance between donor and recipient without any drugs. First phenomenon of immunologic tolerance was discovered by Ray Owen in 1945. Placental fusion of blood exchange resulted in 50:50 mixed chimerism between dizygotic cattle twins [23, 24]. This phenomenon and subsequent follow-up studies yielded important clues about the mechanisms of successful tolerance induction in organ transplantation. Tolerance is classified into central (primary lymphoid organ; bone marrow and thymus) or peripheral (secondary lymphoid organ; spleen and lymph nodes) by major

location to promote tolerance. In addition, tolerance is classified by immunologic mechanisms such as deletion, anergy, ignorance and suppression.

1-4. Immunoregulatory mechanisms in transplantation tolerance

The nuclear transcription factor Foxp3 defines a special lineage of thymus-derived regulatory CD4 T cells and has an important role in the development of immune regulation [25, 26]. These cells and their counterparts induced in the peripheral lymphoid organs include both self- and allo-reactive T cells. The induced regulatory T (iTreg) cells are responsible for suppression of immune responses in transplantation.

Some previous mouse studies have showed that pre-transplant regulation may achieve long-term graft survival of vascularized cardiac allo-grafts, but not conventional non-vascularized skin grafts [27, 28]. However, when bi-directional regulation between donor and recipient was continued for more than 3 months regulation appeared to be required on both sides, not only towards donor allo-antigens but also towards recipient allo-antigens (Burlingham JW, not published). In addition, pre-transplant regulation on the donor side (toward the recipient), as well as the recipient side (toward the donor) promotes long-term graft survival of a kidney allo-graft in a depletional protocol [29]. I am seeking use of bi-directional regulation as a key component of a tolerance strategy in transplantation. Ultimately, I wish to apply this same strategy to achieve clinical transplantation tolerance (Details in Chapter 2).

Chapter 2: Induction of Regulatory T Cells for Transplantation Tolerance

2-1. Summary

Recently, cell-based therapies are the main strategy for induction of transplant tolerance in the United States and Japan. However, the mechanism of tolerance development is still unknown. In order to investigate the number of donor-specific Treg cells required for a powerful immunosuppressive impact, I focused on the amplification mechanism of iTreg cells for tolerance induction and maintenance. Transplant surgeons and immunologists are faced with a similar amplification problem when it comes to the "passenger" T lymphocytes within allo-grafts. These conventional donor CD4 T cells are also known to have large effects on graft rejection despite their very small numbers. In the case of graft-resident Treg cells, it is more difficult to explain the impact of relatively few Treg cells. The influence of pre-existing donor anti-recipient regulation status in living related kidney transplantation in lymphodepleted hosts are greatly amplified in their regulatory effect [29]. Whether the graft-resident Treg cells can involve amplification to the induction of tolerance has not yet been definitively answered.

2-2. Regulatory T cells and Cytokines

The main mechanisms of tolerance developed that clonal deletion/depletion of allo-specific clones, anergy and development of Treg cells, which elicit its effect mainly via anti-inflammatory cytokines (mainly TGFβ, IL10 and IL35) [30, 31]. Treg cells can be classified as either thymic-derived Treg (tTreg) cells or peripheral inducible (iTreg) cells. They are characterized by the expression of the transcription factor Foxp3 and CD25 (alpha chain of the IL2 receptor) [32]. While the exact function of Foxp3 itself is not fully understood, it is thought to downregulate the

nuclear factor of activated T cells [33]. In addition, other T cell subsets like conventional "bystander" T cells can produce regulatory cytokines without expressing those specific markers of Treg cells. Those cells are called Tr1 cells (TGFβ producing cells) and play a role in homeostatic produce of IL10 and TGFβ by effector T cells. It has been reported that iTreg cells and Tr1 differentiation can be induced by microchimerism under specific conditions [34, 35]. The soluble factors IL10 and TGFβ have reported significant interest in the Treg cells literature as the primary cytokines by which negative inhibition is mediated [36, 37]. Recently, IL35 has reported as a potent immunosuppressive cytokine of the IL12 family [33, 38]. This IL35 is a heterodimer formed by Epstein-Barr virus-induced gene 3 (Ebi3) and p35 (IL12α chain), that seems to be preferentially produced by Foxp3 positive Treg cells [2], but also by other leukocytes subsets, including regulatory B cells [39] and dendritic cells [40]. IL35 is one of the key molecules of immune regulation and infectious tolerance, which suppress the proliferation of effector T cells, while expanding non-Foxp3 regulatory T and B cells.

2-3. Intragraft resident Treg cells [30]

Given that Regulatory T cells are known to mediate both tolerance induction and tolerance maintenance in the model, investigators hypothesized that adoptive transfer of recipient derived Regulatory T cells (both peripherally and from within the graft) could lead to stable tolerance in a naive recipient [22, 24]. While adoptive transfer of leukapheresed Treg cells alone did not lead to tolerance induction, transplantation of the tolerated kidney (with or without peripheral Treg cells infusion) did lead to stable tolerance in the naive recipient [22]. These data suggested that the intragraft resident regulatory components, widely thought to be $CD4^+CD25^+Foxp3^+$ Treg cells, were capable of overcoming the intrinsic allo-reactive responses from the naive recipient [41-44].

In this way, adoptive transfer of intragraft resident Treg cells is thought to be capable of tolerance induction [24]. While important mechanistically, this model itself has little direct applicability to the clinic. However, these data strongly support the notion that tolerance is mediated by immunoregulatory cells and that, were these cells clinically available, transplantation tolerance might be readily achieved. There are questions surrounding this cell population. For example, it is unclear what percentage of intragraft resident cells are antigen specific, in contrast to tTreg cells and iTreg cells. If, for example, intragraft resident Treg cells are enriched with donor-specific Treg cells, these mechanisms by which this occurs might be exploited and extrapolated to the clinic.

CD40L (CD154)/CD40 is one of the key costimulatory mechanisms required for T cell activation. CD40L monoclonal antibody has used as a blocker of this costimulation pathway. After the clinical failure of CD40L blockade in humans and non-human primates (NHP), the interest in the CD40L/CD40 axis has reemerged due to promising results with CD40 blockade. In mice, donor-specific transfusion (DST) plus anti-CD40L blockade is a standard and successful protocol to induce donor-specific transplant tolerance, involving apoptosis, acquisition of regulatory cells, and suppression of proliferation of effector cells [45, 46]. Abbas and colleagues [47] have shown that there can be many resident T cells in transplanted organs and tissues, including both pro-inflammatory memory T cells and memory Treg cells. On day 30–40 after resolution of an inflammatory response in the skin, activated T cells, which had migrated from central lymphoid tissue, were maintained in the target tissue, thus developing "Treg memory" to that tissue. Mechanistically, it is thought that anti-CD40L leads to rapid changes in lymph node architecture and to the migration of Treg cells and effector T cells through high-endothelial venules [48]. While capable of tolerance induction, the kinetics of peripheral allo-specific regulatory T memory cells into tissues (other than the lymphoid tissue) are unknown. Whether Treg cells induce tolerance

directly or by virtue of facilitating other cell populations is unclear. Indeed, recently groups have reported that plasmacytoid dendritic cells are capable of facilitating hematopoietic cell engraftment. Below, I will address several addition cell populations, which may induce tolerance; however, it remains unclear if their function is by virtue of facilitation or by direct tolerogenic effects [49].

Chapter 3: Kinetics of alloantigen-specific regulatory CD4 T cell development and tissue distribution after donor specific transfusion and costimulatory blockade

3-1. Summary

The influence of donor-side regulation towards recipient antigens on graft outcome is poorly understood. Because this influence might be due, in part, to the accumulation of tissue-resident memory T cells in the donor organ, I used a standard murine tolerization model (donor specific transfusion, DST, plus CD40L blockade) to determine the kinetics of development and peripheralization of allo-specific regulatory T cell in lymphoid tissues and liver, a secondary lymphoid organ used in transplantation. I found that DST and CD40L blockade leads to a progressive and sustained T regulatory allo-specific response. The cytokines IL10, TGFβ and IL35 all contributed to the regulatory phenomenon as determined by trans-vivo delayed hypersensitivity (tv-DTH) assay. Unexpectedly, an early and transient self-specific regulatory response was found as well. Using double reporter mice (Foxp3-YFP, Ebi3-TdTomRed), I found an increase in Foxp3+CD25+ Tregs paralleling the regulatory response. The Ebi3+ CD4 T cells (IL35-producing) were mainly classic Tregs (Foxp3+CD25+), whereas TGFβ+ CD4 T cells are mostly Foxp3-negative, suggesting two different CD4 T regulatory cell subsets. Liver-resident TGFβ+ CD4 T cells appeared more rapidly than Ebi3-producing T cells, while at later time-points the Ebi3 response predominated both in lymphoid tissues and liver. The timing of the appearance of donor organ resident Treg subsets should be considered in experiments testing the role of bi-directional regulation in transplant tolerance.

3-2. Hypothesis and Specific Aims

I hypothesized that Tregs developed in response to DST and MR-1 will exhibit allo-antigen specificity, appear to other tissues besides the lymph nodes and spleen, where they produce immunosuppressive cytokines in response to specific tolerogen, and thereby mediate allo-specific linked suppression of DTH, a pre-condition for bi-directional regulation after transplant of that organ [29].

In this study, I aimed to determine the characteristics of the peripheral Tregs induced by DST and co-stimulator blockade. I was particularly interested in the kinetics of their development, and their migration into secondary lymphoid (transplantable) tissues, where they could potentially serve as facilitators of a robust tolerance via bi-directional regulation.

3-3. Introduction

The adaptive allo-immune response of the host direct, semi-direct, and indirect allo-recognition by T cells, and allo-specific B cells is thought to be responsible for rejection of organ transplants [50-52]. Several therapeutic treatments are needed in order to control the immune response and prolong graft survival. Nevertheless, in current clinical practice most of these treatments generate global immune suppression and as consequence increase the risk of severe and even life-threatening opportunistic infections. Furthermore, most of these drugs have significant side effects affecting the cardiovascular, endocrine and hematologic systems[53, 54].

In contrast, the best possible outcome after transplantation would be the development of graft-specific tolerance, in such a way that the immune system becomes unresponsive to graft-derived antigens without the need of immunosuppressive drugs [55]. A still suboptimal but desirable condition is the development of donor-specific regulation in the recipient immune system,

which is not sufficient to achieve complete tolerance, but is associated with longer graft survival and increased likelihood of successful withdrawal of immunosuppressive drugs [56, 57]. Notwithstanding, the induction of allo-specific tolerance or regulation is still challenging, partially because some physiologic aspects of regulation development, particularly on the donor side, are still unknown [55].

In order to analyze the role of donor-side regulation toward recipient antigens, I chose to study a mouse model, including lymphoid tissues and a secondary lymphoid organ typically used in vascularized organ transplantation, where donor specific transfusion (DST) and anti-CD40L monoclonal antibody are a well-known protocol to induce allo-specific tolerance. Such treatment, when applied to a future transplant donor, could give us insights as to the type of tissue-resident lymphocytes that give rise to bi-directional regulation in organ transplantation. It is known that DST and anti-CD40L treatment can induce Foxp3+ Treg cells, and IL10 as well as and TGFβ secretion by allo-specific Tregs [36, 58]. Far less is known about the induction of IL35-secreting T cells. Meanwhile, the allo-reactive effector T cell clones are depleted, anergized or shifted to regulatory functions by the CD40L blockade during the allo-response induced by DST, whereas other T cell clones are minimally affected [1, 29].

One way that host-based regulation approaches may fail to achieve long-term tolerance is the absence of a regulatory response on donor-side. For example, the highest level of pre-transplant regulation, both in Rhesus macaques and in human, was directed to non-inherited maternal antigen (NIMA) [59]. Yet analysis of >10,000 live-related kidney transplants in the U.S has shown that transplants from a maternal kidney donor fare worse than any other type of 1 HLA haplotype-mismatched graft within a family [47]. In contrast, grafts from a sibling kidney transplant donor fared much better if the mismatched HLA haplotype was the NIMA, as compared to the non-

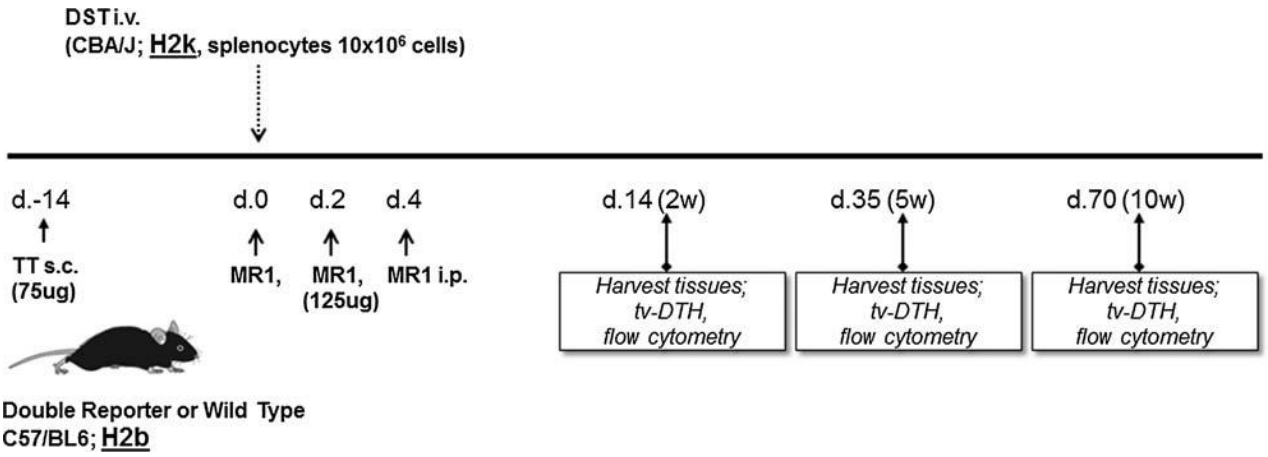
inherited paternal, or NIPA, mismatched siblings [60]. One possible explanation for this, so-called "NIMA paradox", is that the sibling donor's immune response to the host is also a NIMA response, i.e. conditioned by microchimerism and development in the same mother as the host. The maternal donor's response to the offspring as host is a memory response to inherited paternal antigens of her child and thus may be a sensitized response, subverting tolerance induction [45]. Analysis of graft outcomes in a group of 18, 1 HLA haplotype-mismatched donor-recipient pairs enrolled in a depletional protocol showed poor graft survival in cases where the recipient strongly regulated to donor antigens prior to transplant, while the donor did not regulate to the recipient. In contrast, excellent long-term outcome was seen in cases where both donor and recipient regulated to each other's antigen prior to transplantation [29].

IL35, a member of the IL12 family of cytokines, is a heterodimeric cytokine comprising the p35 (α-chain) shared by IL12 and Epstein-Barr virus-induced gene 3 (Ebi3) (β-chain) shared by IL27. The IL35 receptor is composed of gp130, the receptor for IL6 and the IL12 receptor β2. IL35 was first reported by Vignali et al. in 2007 [38], and subsequently has shown to play a critical role in the regulation of various immune responses. It is produced by Foxp3 positive T cells, and causes suppression of the proliferation of Th1 and Th17 effector T cells, and expansion of non-Foxp3 regulatory T- and B- cells. Besides these, activated dendritic cells, macrophages, endothelial cells, and smooth muscle cells can also express IL35. IL35 has also been identified as a novel cytokine in infectious tolerance, because it can convert CD4+ Foxp3 negative T cells into a non-conventional Treg, called iTr35, which produces IL35, but does not express Foxp3, IL10, or TGFβ [2].

3-4. Study design

Figure 1 shows the experimental timeline, including the time points when tetanus/diphtheria toxoid (TT/DT) immunization, DST and MR-1 treatment, euthanized for tv-DTH and flow cytometry assays were performed. I immunized wild-type and double reporter B6 mice with 70 µg TT/DT pediatric vaccine by subcutaneous injection in their inguinal pouch 2 weeks before initiation the tolerance protocol. I tolerized those mice with 1.0 x 10^7 CBA spleen cells (200µL) by retro orbital intravenous injection (DST) on day 0 and MR-1 (Bio X Cell) by intraperitoneal injection of 125 µg/dose on day 0, 2, and 4. In this model the CBA haplotype (H2k) is considered the "donor". Mice were euthanized on day.14, 35, and 70, spleen and lymph nodes (inguinal, axially, and surficial cervical) were harvested. Tv-DTH was performed using splenocytes, whereas flow cytometry was performed with lymph nodes, splenocytes and liver cells.

Figure 1. Study design. All wild type B6 and B6 Double Reporter (YFP-Foxp3/TdTomRed-Ebi3) mice were immunized s.c. with 70 µg tetanus toxoid/diphtheria toxoid (TT/DT) pediatric vaccine by subcutaneous injection in their inguinal pouch 2 weeks before initiation of the tolerance protocol. Then, these mice were tolerized with 1.0 x 107 CBA spleen cells by retro orbital intravenous injection (donor-specific transfusion or DST) on day 0, and anti-CD40L IgG (MR-1) by intraperitoneal injection of 125 µg dose on day 0, 2, and 4. Mice were euthanized on day 14, 35, and 70. Spleen, lymph nodes (inguinal, axial, and cervical), and liver were harvested for tv-DTH and flow cytometry assay.



3-5. DST + CD40L blockade induced a transient regulatory response to self-antigens but persistent allo-specific immunoregulation

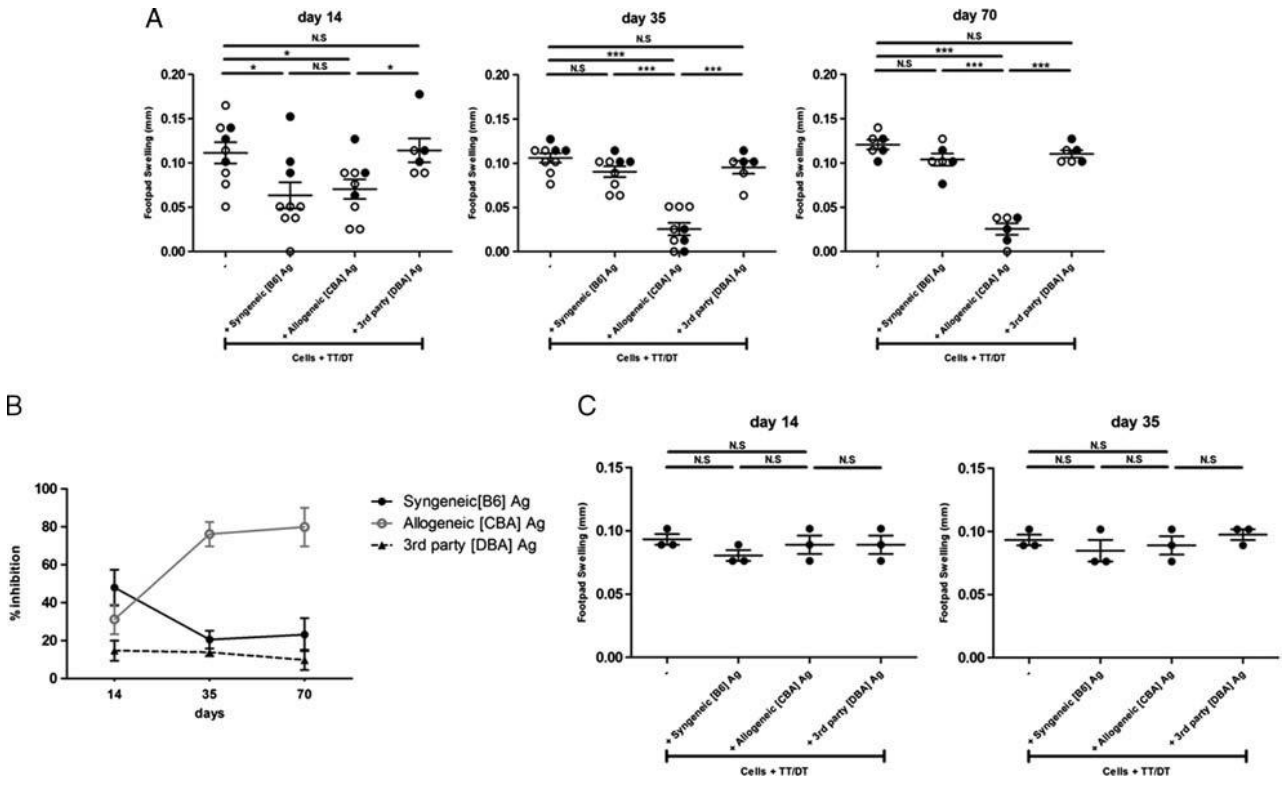
First, I determined the time course of allo-specific regulation as a measure of the immune-regulatory response. The timeline of treatment of the mice and harvest of spleen, lymph nodes and liver is shown in Figure 1. The functionality of memory allo-specific Tregs was analyzed at various times after the DST and anti-CD40L blockade treatment using the trans-vivo delayed type hypersensitivity (tv-DTH) linked suppression assay. Figure 2 shows one view of how linked-suppression of tv-DTH may occur in this assay. Treg cells recognizing an allo-peptide, or a self-peptide may be activated to release IL10, TGFβ or IL35, suppressing the Tetanus Toxiod/Diphtheria Toxoid (TT/DT)-specific recall response.

I found that DST and anti-CD40L blockade induced a sustained allo-specific regulation toward CBA antigen (H2k). This regulatory effect was already evident on day 14 after treatment, and persisted for up to 70 days after treatment ($P<0.001$ on day 35 and $P<0.001$ on day 70, respectively) (Figure 3A). Also, the magnitude of the allo-specific regulation increased between day 14 and 35 (Figure 3A, 3B). As expected, I found no regulation toward third party antigens (DBA/2; H2d). Unexpectedly, I observed linked suppression induced by self antigens (B6; H2b) on day 14 ($p\leq0.05$). This regulation occurred only at this early time point and disappeared by day 35 (P=N.S., % linked suppression with B6 antigen vs. TT alone). On day 35 and 70 neither self H2b nor third party H2d antigens elicited linked suppression of tv-DTH, suggesting that self-reactive T regulatory cells developed quickly, but subsided quickly in response to tolerization treatment (Figure 3B).

To determine if the DST was required or if MR-1 alone was sufficient to cause linked-suppression, similar experiments were performed on mice that received just MR-1 injections

(Figure 3C). In this case, no linked-suppression was seen, either on day 14 or day 35, consistent with the known mechanism of action for costimulatory blockade treatments, in which specific antigen challenge, and subsequent T cell-APC engagement are required to achieve the tolerogenic effect.

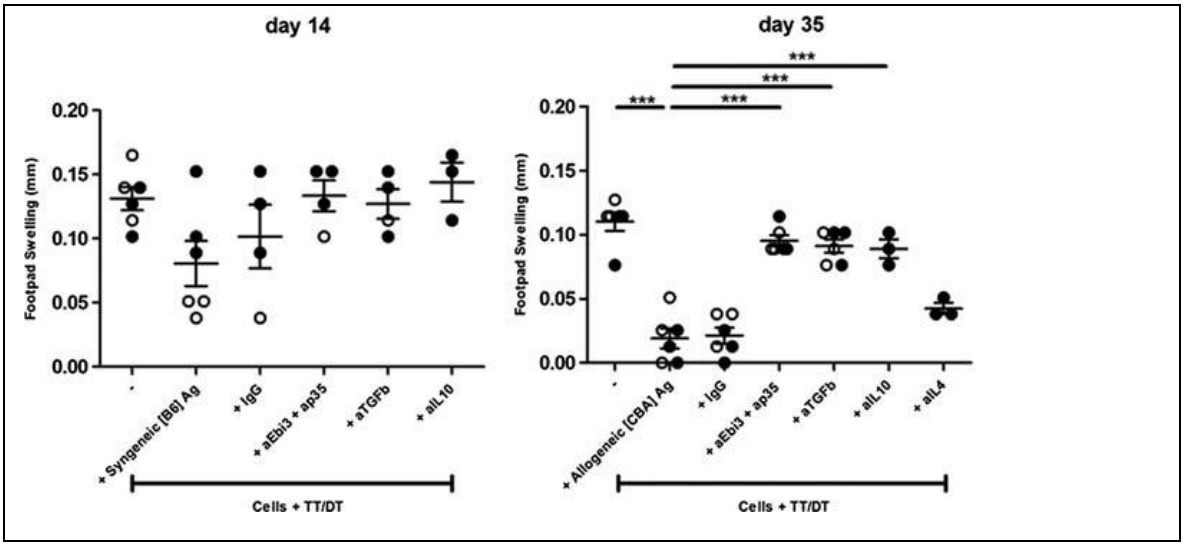
Figure 3. Time course of regulation response after DST and MR-1 tolerization. (A) The tv-DTH linked suppression response to syngeneic- [B6], allogeneic- [CBA], and 3rd party- [DBA/2] antigen preps on day 14, 35, and 70 after the DST and MR-1 treatment. Splenocytes from tolerized B6 mice at each time-point were tested in tv-DTH assay. The TT/DT response represents the positive control. Responses to TT/DT recall antigen (25 μg) were shown with each test antigen (20 μg) and analyzed by Student's t-test (* P≤0.05; *** P<0.001). Data were presented as mean with SEM from 6-9 mice in each group (Open symbols; wild type B6 mice, Close symbols; Foxp3/YFP, TdTomRed /Ebi3 reporter B6 mice). (B) Timeline of the linked suppression. Here is plotted in the Y axis the "% of inhibition", i.e: percentage of decrease in the TT response in response to the co-injection of each antigen [self B6, donor CBA, and 3rd party DBA/2]. While self plus allo-specific regulation was observed on day 14 after DST and MR-1 treatment, allo-specific regulation had developed in the central lymphoid tissue by day 35. There was no regulation at any time toward 3rd party-Ag. Self-regulation was transient at early time-points, whereas allo-specific regulation increased overtime, reaching a plateau on day 70. (C) Isolated impact of CD40L blockade. The footpad swellings to TT/DT challenge were not inhibited by any of the 3 antigens on day 14 and 35. Data were presented as mean with SEM from 3 mice in each group and analyzed by Student's t-test.



3-6. Immune-suppressive cytokines are required for DST + CD40L blockade induced regulation

Following the same experimental protocol, the tv-DTH assay was used to determine the regulatory cytokines that mediate linked-suppression, in this case, splenocytes from mice treated with DST and MR-1 were transferred into footpads of naïve syngeneic B6 mice and co-injected with TT/DT recall antigens, alloantigens, and blocking antibodies: a) anti-IL10, b) anti-TGFβ, or c) anti-Ebi3+anti-IL12p35 (anti-IL35). On day 35, all 3 blocking antibodies uncovered the allo-response, abrogating the regulatory phenomenon, suggesting that all 3 cytokines are involved in the allo-specific regulation developed after DST and MR-1 treatment (Figure 4). The co-injection of IgG isotype control did not elicit a significant effect, while αIL4 had only a marginal impact in linked-suppression. This profile was similar on day 35 and 70 after DST and MR-1 treatment ($P<0.001$ for all 3, IL10, TGFβ, and IL35 anti-cytokines vs. IgG control on day 35 and on day 70) (day 70 data not shown). These results indicate that the allo-specific regulatory phenomenon in the spleen, is mediated by IL10, TGFβ and IL35. In contrast, self-specific responses were uncovered only in half of the mice by IL10, TGFβ, and IL35 neutralization on day 14. There was no statistically significant abrogation of self-induced linked suppression, although a trend was observed (p=0.06, p=0.09, and p =0.06, respectively).

Figure 4. Abrogation of linked suppression by co-injection of blocking antibodies. Self- B6 regulation responses were variable, as measured by linked suppression of recall tv-DTH response on day 14. No significant differences in recovery of TT/DT response from linked suppression by self (B6) antigens were observed on day 14 with IL10, TGFbeta or IL35 neutralization, although a trend (p=0.06, p=0.09, and p=0.06, respectively) was observed. However, allo (CBA) -specific linked suppression responses on day 35 were clearly reversed by the IL10, TGFbeta or IL35 cytokine-neutralizing antibodies (all $p<0.001$). The co-injection of anti-IL4 antibody did not elicit a significant LS reversal effect relative to IgG isotype control. Data were presented as mean with SEM from 3-6 mice in each group (Open symbols, wild type B6 mice; closed symbols, Foxp3/YFP, TdTomRed /Ebi3 reporter B6 mice), and analyzed by Student's t-test (*** $p<0.001$).



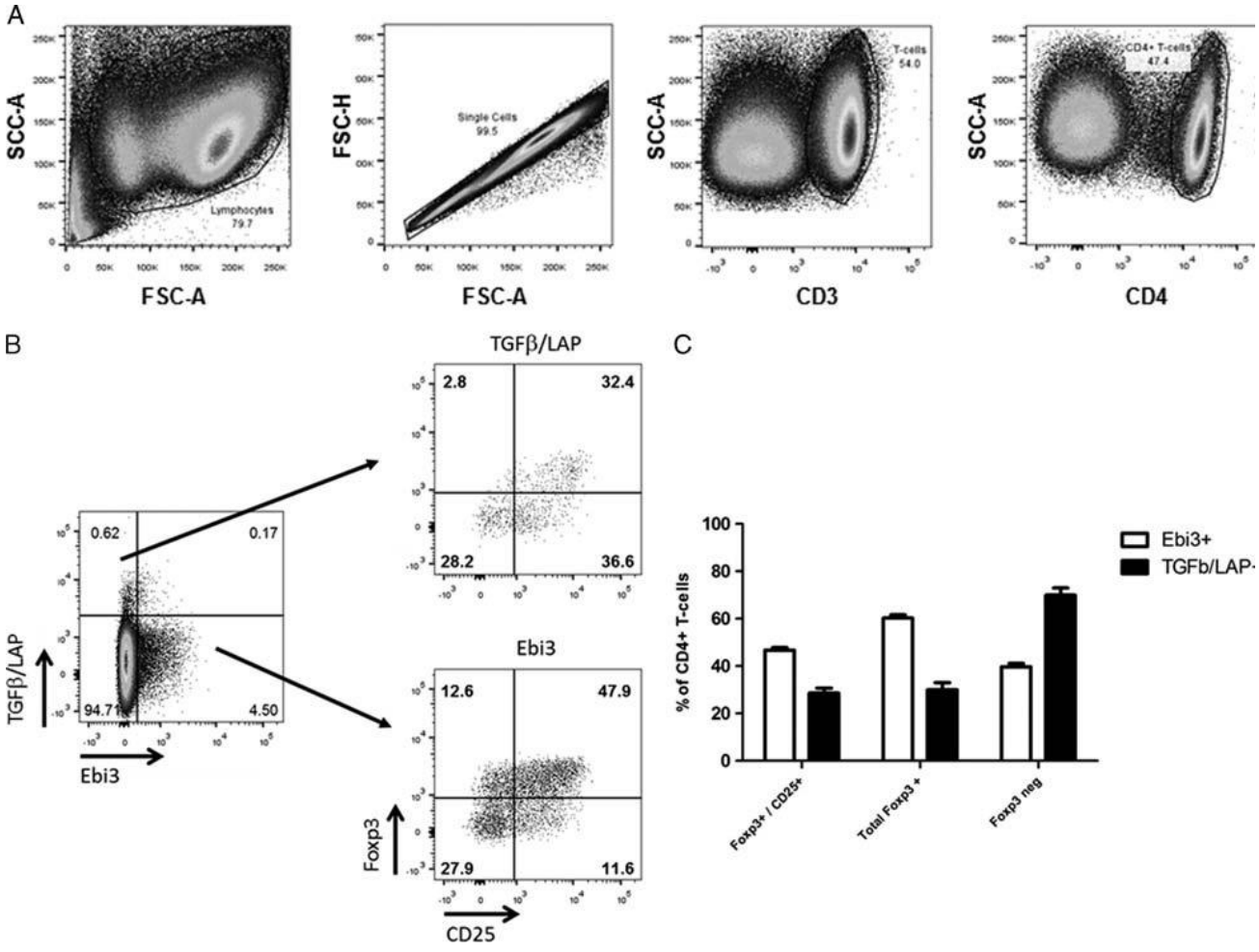
3-7. Ebi3-producer and TGFβ-producer regulatory T cells belong to different subsets

The mice used in the first set of experiments were double reporters (Foxp3/YFP and Ebi3/TdTomRed). The expression of the TdTomRed reporter indicates an active transcription of the Ebi3 gene. Ebi3 transcription and surface TGFβ/LAP expression in CD4 T cells of tolerized mice were analyzed with flow cytometry to determine the phenotype of the CD4 T cells producing 2 of the cytokines involved in the allo-regulation (Figure 5A, 5B).

Analyzing lymph nodes harvested on day 35 after treatment, I confirmed that approximately 50% of T cells actively transcribing Ebi3 (TdTomRed) were Foxp3+CD25+ Tregs. I also found that CD4 T cells which actively transcribed Ebi3, and the ones expressing surface TGFβ/LAP were different cells (Figure 5B, 5C). One third of TGFβ+ CD4 T cells showed the typical phenotype of Tregs (Foxp3+CD25+), but the majority were Foxp3neg, and within those TGFβ+Foxp3neg cells, only half were CD25+. No significant double positive Ebi3/TGFβ proportion was found either in lymph nodes or spleen on day 14; on day 35, this proportion was only slightly increased. Therefore, I conclude that the induced IL35-producing and TGFβ-

producing CD4 T cells belong to mostly non-overlapping subsets.

Figure 5. Immunophenotype of TGF Figure 5. TGFb and IL35 cytokine-producer CD4 T cells. (A) Gating strategy: live cells gated on FSC-A/SSC-A → singlets based on deviation from the linear function in FSC-A/FSC-H → T cells on CD3/SSC-A → CD4 T cells on CD4/SSC-A. Foxp3+ and Internal Ebi3+ cells are those lymphocytes expressing the YFP and the TdTomRed reporter (under YFP and Ebi3 promoter). (B) Cytokine profile of gating CD4 T cells in lymph nodes, which were harvested on day 35 after DST and MR-1 treatment, were analyzed by flow cytometry. Approximately 60% of the lymphocytes actively producing Ebi3 (TdTomRed) were Foxp3+ (classic Tregs). Approximately 65% of TGFβ+ cells are Foxp3 negative. The population of Ebi3/TGFβ double positives is around 0.2%. (C) Summary; phenotype of CD4 T lymphocytes producing Ebi3/IL-35 or TGFβ cytokines. IL35-producing and TGFβ-producing cells were mostly non-overlapping subsets differing in phenotype and cytokine profile. Data were from 7 mice in each group.



3-8. Peripheralization of regulatory T cells

To determine the dynamics of regulatory cells development and homing in peripheral tissues, lymph nodes, spleen and liver were harvested from naïve animals, and from mice treated with DST and MR-1, at days 14 and 35.

The Foxp3+CD25+ cells showed an increase over time in primary lymphoid tissues (spleen and lymph nodes) and liver ($p<0.01$ in lymphoid tissue and $p<0.001$ in liver on day 35, respectively) (Figure 6B, 7B). However, in liver, the proportion and the absolute number of Foxp3+CD25+ CD4 T cells were lower than that of lymphoid tissues (compare Figure 7B with 6B). The IL35-producing cells (Ebi3/TdTomRed+) had a frequency and an absolute number that significantly increased over time after tolerization treatment in lymphoid tissues and liver, with a similar pattern ($p<0.001$) in lymphoid tissue and in liver on day 35, respectively (Figure 6D, 7D). The trend of these IL-35+ cells was consistent with that of the Foxp3+CD25+ conventional Tregs. Interestingly, the kinetics of these cells was different from the TGFβ producer CD4 T cells. TGFβ+ CD4 T cells had a similar baseline in spleen and lymph nodes. The proportion and the absolute number of those cells increased at day 35 after DST and MR-1 treatment in a similar magnitude in both lymphoid tissues (Figure 6E). Remarkably, TGFβ-producer cells were nearly absent in the liver at day 0, but its proportion and the absolute number increased significantly on day 14 after treatment ($p<0.01$ and $p\leq0.05$, respectively), with further increase at day 35 ($p<0.001$ and $p<0.01$, respectively) (Figure 7E). This suggests a migratory process of the regulatory T cells into specific peripheral niches. It is interesting that after tolerization treatment the proportion of TGFβ+ cells amongst CD4 lymphocytes was significantly higher in the liver as compared with lymphoid tissues. These results indicate that IL35-producing and TGFβ-producing cells may have different

migratory kinetics in lymphoid tissues and liver.

Figure 6. Time course of increase in CD4 regulatory Tcells in tolerized double reporter B6 mice—lymph nodes (A) Gated CD4 T cells in lymph nodes, which were harvested on day 0 (untreated), 14, and 35 after DST and MR-1 treatment, were analyzed for Foxp3+ (reporter) and CD25+ by flow cytometry. (B) Comparison of the frequency and the total number of Foxp3+CD25+ cells in spleen and lymph nodes cells on day 0, 14, and 35 after the tolerized treatment. Total cell number means the absolute Foxp3+CD25+ CD4 T cells recovered from the same spleen and lymph nodes. (C) Gated CD4 T cells in lymph nodes over time after DST and MR-1 treatment were analyzed for Ebi3+ (reporter) and TGF□/LAP+ by flow cytometry. (D, E) Comparison of the frequency and the total number of Ebi3+ (D) and TGF□/LAP+ (E) cells in lymphoid tissues on day 0, 14, and 35 after the tolerized treatment. Data were presented as mean from 4-7 mice in each group and analyzed by Student's t-test (* P≤0.05; **P<0.01; *** P<0.001, Close symbols; spleen, Open symbols; lymph nodes).

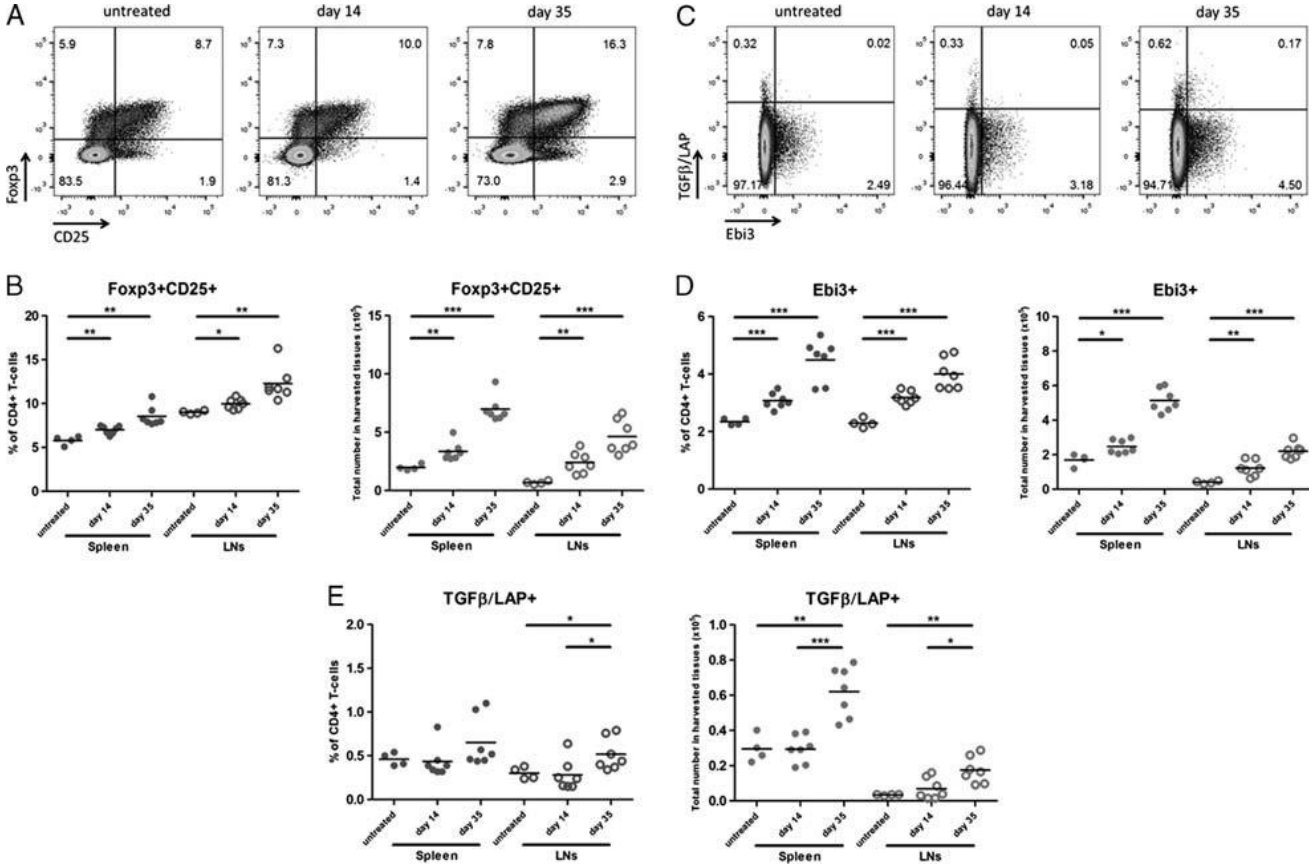
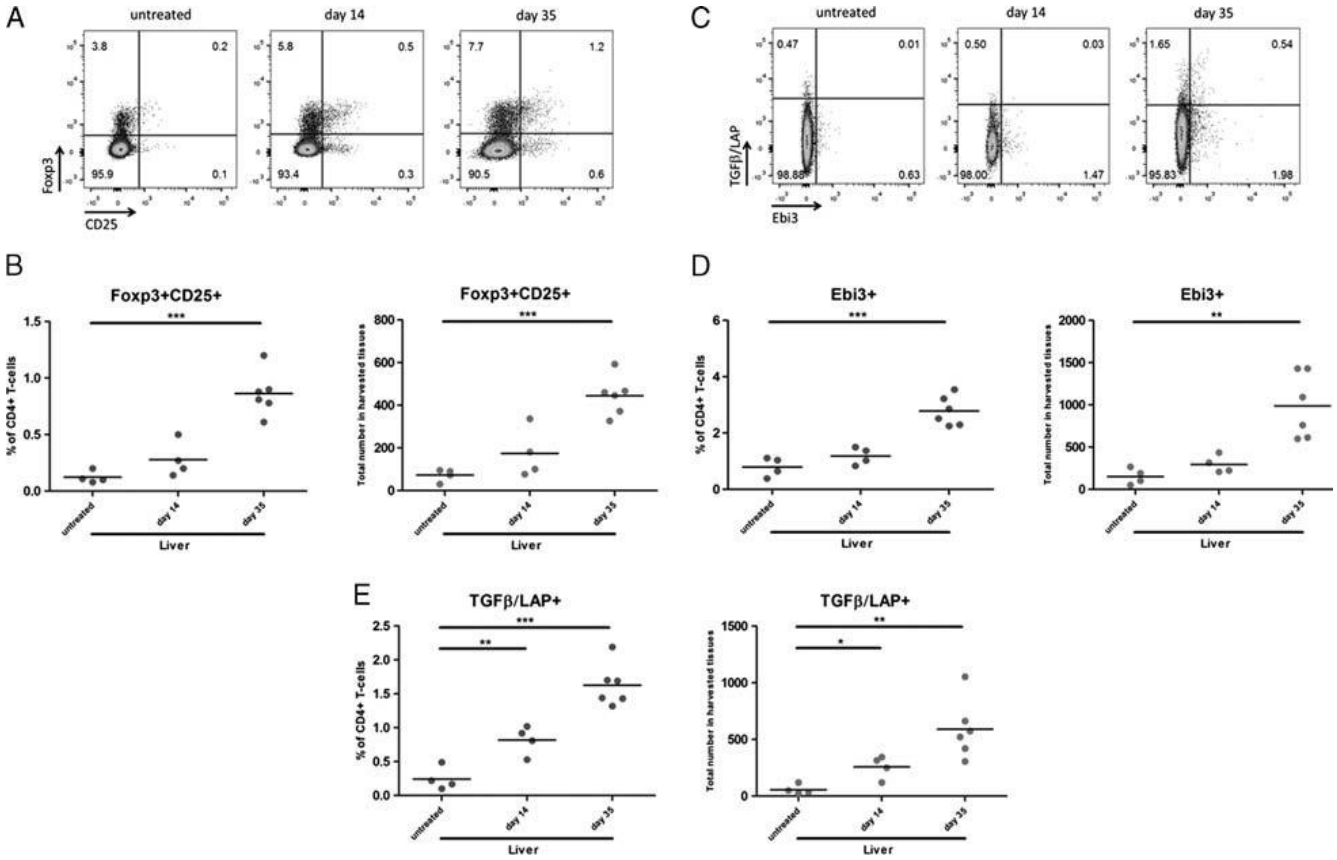


Figure 7; Time course of increase in CD4 regulatory Tcells in tolerized double reporter B6 mice—liver (A) Gated CD4 T cells in liver, which were harvested on day 0 (untreated), 14, and 35 after DST and MR-1 treatment, were analyzed for Foxp3+ (reporter) and CD25+ by flow cytometry. (B) Comparison of the frequency and the total number of Foxp3+CD25+ cells in liver on day 0, 14, and 35 after the tolerized treatment. (C) Gated CD4 T cells in liver over time after DST and MR-1 treatment were analyzed for Ebi3+ (reporter) and TGF□/LAP+ by flow cytometry. (D, E) Comparison of the frequency and the total number of Ebi3+ (D) and TGF□/LAP+ (E) cells in liver on day 0, 14, and 35 after the tolerized treatment. Data were presented as mean from 4-6 mice in each group and analyzed by Student's t-test (**p<0.01; *** p<0.001).

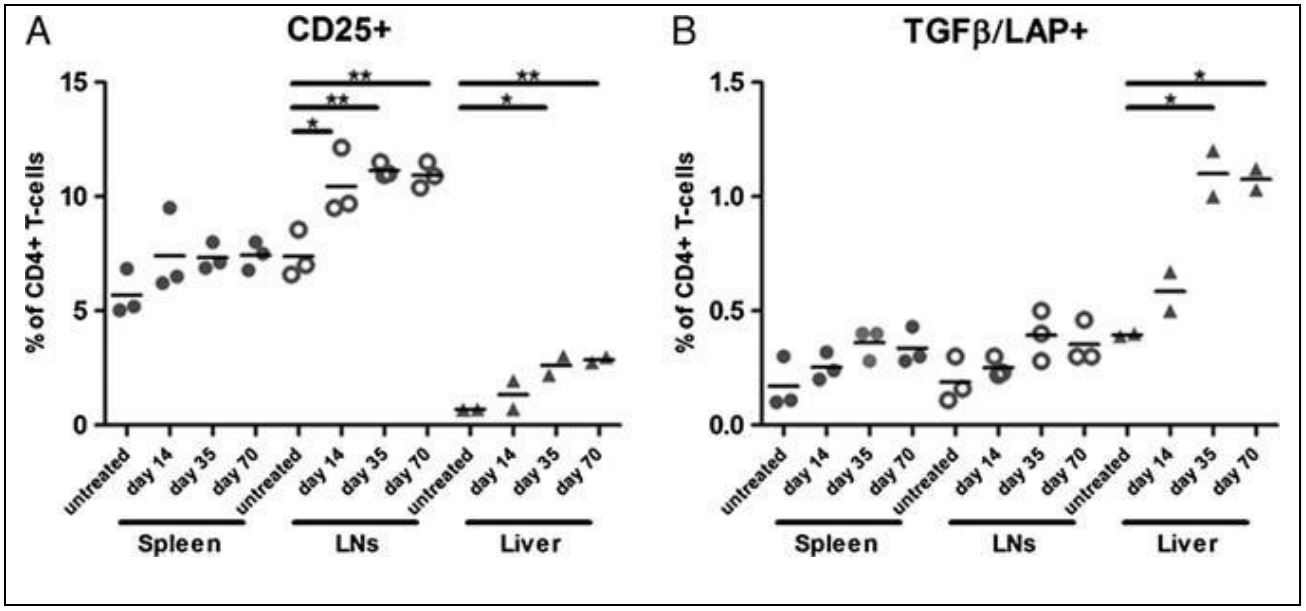


3-9. Regulatory T cells in tolerized wild-type B6 mice

To rule out the possibility that the dual-marker B6 mice had unusual Treg response kinetics, similar experiments were performed to assess the kinetics of the regulatory CD4 T cell development in wild-type B6 mice. The spleen, lymph nodes, and liver were harvested from wild-type B6 mice treated with DST and MR-1 at days 14, 35, and 70, and immunostained for CD3, CD4, CD25 and TGFβ/LAP.

As with the double reporter mice, DST and MR-1 treatment induced an increased frequency of CD4+CD25+ cells in lymphoid tissues (Figure 8A). Trends, but no significant differences between non-treated mice and tolerized mice were seen in spleen (p=0.2 on day 14, p=0.07 on day 35, and p=0.06 on day 70, respectively). However, there were significant differences in lymph nodes ($p\leq0.05$ on day 14, $p<0.001$ on day 35, and $p<0.001$ on day 70, respectively). In liver, CD25+ cells had a very low baseline frequency on day 14 (1.0% of CD4 T cells). These cells increased until day 35 after tolerization, but the proportion was lower than that of lymphoid tissues ($p\leq0.05$ on day 35, and $p\leq0.05$ on day 70, respectively). This trend was consistent with that of the YFP-labeled Foxp3+ sub-population in double reporter mice (Figure 6B, 7B). Interestingly, the proportion of TGFβ+ cells in CD4 T cells of wild-type B6 mice was lower than in the double reporter mice (compare Figure 6E and 7E), however the kinetics of appearance of these cells was similar.

Figure 8; Organ distribution patterns in spleen, lymph nodes, and liver of freshly harvested regulatory CD4 T lymphocytes of tolerized wild-type B6 mice. (A) Gated CD4 T cells in lymph nodes, which were harvested on day 0 (untreated), 14, 35, and 70 after DST and MR-1 treatment, were analyzed for CD25+ and TGF/LAP+ by flow cytometry. Comparison of the frequency of CD4+CD25+ cells in spleen, lymph nodes, and liver over time after the treatment. (B) Comparison of the frequency of CD4+TGF□/LAP+ cells in lymphoid and secondary lymphoid tissues on day 0, 14, 35, and 70 after the tolerized treatment. Data were presented as mean from 2-3 mice in each group and analyzed by Student's t-test (* $p\leq0.05$; ** $p<0.01$, Close symbols; spleen, Open symbols; lymph nodes, and Triangle symbols; liver).



3-10. Discussion

Pre-transplant regulation on the donor side (toward the recipient), as well as the recipient side (toward the donor), led to long-term graft survival of a kidney allo-graft in a depletional protocol [29]. Donor derived hematopoietic cells are transferred to the host in organ transplantation, creating a risk of graft versus host disease [61]. whereas stable microchimerism in the recipient was associated with improved long-term graft outcome [7, 62, 63]. Conlon et al [64]. showed that host CD4 T cells trafficking into germinal centers (GC) provided help for activated B cells to produce IgG allo-antibody via the indirect allorecognition pathway, whereas donor CD4 T cells via direct recognition pathway were able to activate host B cells to get them into GC, but were unable to induce class-switched allo-antibody. In rat heart transplantation, Ko et al. reported that selective depletion of donor leukocytes by antibody 2 weeks after engraftment had no effect upon long-term allograft function. However, passenger leukocyte depletion at the time of transplant was associated with development of severe chronic allograft vasculopathy [65]. Josien et al. have also showed that the presence of interstitial allo-specific dendritic cells in heart graft can elicit tolerance

in CD4 T cells from rats after DST treatment [46]. These results suggested that donor derived leukocytes in graft might play a role in either undermining, or facilitating successful engraftment both within the local environment of the transplant, and by their impact on distal immune sites.

Our study showed data about the progression of tolerance after DST and anti-CD40L monoclonal antibody treatment. Importantly, while self & allo-specific regulation was observed 2 weeks after tolerization, allo-specific regulation took ~5 weeks to develop in the lymphoid tissue (Figure 3B). In regards to this point, concerning the timing of graft placement in relation to the development of tolerance, I found that 5 weeks after the tolerization treatment was sufficient for allo-specific regulation to manifest itself in both the lymphoid tissue and liver. Rosenblum et al. [66] reported that there were many tissue resident T cells in transplanted organs, including inflammatory memory T cells and Tregs. On day 30-40 after resolution of an inflammatory response, activated T cells which had appeared from central lymphoid tissue were maintained in the target organ, thus developing "Treg memory" to that organ. This time frame corresponds to the kinetics of development of allo-specific linked suppression responses. Several studies have demonstrated that DST and anti-CD40L monoclonal antibody treatment induced tolerance, as evidenced by long-term tissue or solid organ allograft survival in mice [46, 50-52]. In these studies the transplantation was performed early after treatment (usually <7 days). In fact, in some cases DST was not performed, but anti-CD40L monoclonal antibody was injected the same day of transplantation, in such a way that the graft itself provided the allogeneic challenge needed for the costimulatory blockade to work [28, 29, 53]. Honey et al [53]. reported that anti-CD40L monoclonal antibody induced Ag-specific tolerance and linked suppression in CD4 T cells, but not CD8 T cells, suggesting that additional therapies to control aggressive CD8+ T cells may augment the tolerance state. Primarily vascularized skin allografts behaved similarly to heart allografts,

whereas conventional skin grafts were not prolonged by anti-CD40L, suggesting that rapid escape of "passenger leukocytes" provides the DST-like stimulus for tolerance induction [54].

Treg cell development in response to alloantigen stimulation by DST appears to rely on host antigen presenting cells, or the indirect pathway of allo-recognition, as early studies showed a requirement for MHC antigen sharing between host and donor in order for graft prolongation by F_1 DST alone [67]. Fully allogeneic DST did not prolong heart allograft survival. Co-stimulation blockade with anti-CD40L monoclonal antibody permits tolerization with a fully allogeneic DST. However, few studies have addressed the kinetics of allo-specific Treg induction on the key indirect allo-recognition pathway. Burrell and Bromberg [38] showed an early phase burst of proliferation and Foxp3-conversion of transferred allo-specific "indirect pathway" T cells in the first 72 hours after transfusion of BALB/c cells into B6 mice treated with anti-CD40L monoclonal antibody. This iTreg induction occurred mainly in the lymph nodes. Follow-up studies by the same group found that rapid matrix remodeling of the lymphoid vessels occurring within hours after DST and anti-CD40L monoclonal antibody treatment was essential for the early burst of T cell proliferation resulting in allo-specific iTreg, whereas by day 7 transfer of the same TcR transgenic T cell resulted in anergy and apoptosis [48]. This suggests a 2-phase model of naïve conventional T to Foxp3+ Treg conversion, followed by a phase in which tolerance may be based on allo-specific T cell anergy. Our studies have identified yet a third phase: the evolution of a robust, donor-specific immunoregulatory response, involving both Foxp3+ and Foxp3neg CD4 T cells.

The proportion of regulatory Foxp3+, TGFβ+, and Ebi3-producing CD4 T cells increased in lymphoid and peripheral tissues over time. The flow cytometry results corroborated the functional analysis of the regulatory function, i.e: the increase in regulatory CD4 T cells paralleled the increase of allo-specific linked suppression in tv-DTH assay. I found that the allo-specific

regulatory phenomenon in the spleen on day 35 was mediated by IL10, TGFβ and IL35. It is important to note that, in previous studies of mouse [68] and human [69] the regulation detected in the linked suppression tv-DTH assay was reversed by anti-Ebi3 and by anti-p35 tested separately, whereas anti-p28, the subunit of IL27 associated with Ebi3, had no effect. This suggests that the effects I am seeing are indeed due to the action of IL35. In a previous study by Maynard et al [70], it was found using a IL10 and Foxp3 dual reporter mouse, that leukocytes distributed to lung and liver were enriched in Foxp3+IL10neg Tregs, whereas the gut-homing Tregs included both Foxp3+IL10+ and Foxp3neg IL10+ subsets. Thus the tissue-specificity of different types of Tregs makes it important to consider the organ that is being transplanted, as far as bi-directional regulation is concerned. Interestingly the TGFβ-producing and IL35-producing cells were different in Foxp3 phenotype (Figure 5B, 5C). This was compatible with previous studies showing that, within the CD4 T cells, IL35 was secreted by classic Foxp3+ Tregs but not by Th0 or activated T effector cells [38]. Induction of allo-specific IL35 producing Tregs is implied by the abolition of tv-DTH linked suppression. Because this induction occurs after allogeneic challenge, the Ag-specific Tregs were most likely a peripheral (or inducible) type. Nevertheless, the possibility that DST-derived migratory dendritic cells can influence thymic Treg development cannot be entirely ruled out. In contrast to the Ebi3-producing Tregs, the majority of cells producing TGFβ were Foxp3neg (Figure 5C), as previously reported [71, 72]. The precise identity of the TGFβ+ CD4 T cells has yet to be determined--they might be either Tr1 cells or, alternatively, “Th3” CD4 T effector cells [73, 74] that synthesize the cytokine as part of their effector program.

I also found that TGFβ-producing and IL35-producing cells both appeared in the liver, but with different kinetics. Remarkably, TGFβ-producing cells appeared into peripheral tissues (liver) significantly faster than did IL35-producing cells. TGFβ-producing cells appeared in liver at day

14 after treatment, whereas IL35-producing CD4 T cells reached a maximum at day 35 (Figure 7D vs. 7E). Also, the fold-increase compared with baseline status was higher for TGFβ-producing CD4 T cells.

Bi-directional regulation implies a pre-transplant, induced unresponsiveness of the recipient immune system to donor antigens, and vice versa. The beneficial effects of this condition have been described in leukodepleted post-transplant kidney patients [29]. Regarding the application of our data to the study of bi-directional regulation in primarily vascularized transplant models, the results suggest that mice to be used as transplant donors should receive tolerization treatment at least 35 days before transplantation in order to allow regulatory cells to home to the organ.

3-11 Materials and Methods

3-11-1. Mice

Mice were bred and maintained at University of Wisconsin-Madison Hospital animal facilities according to specific pathogen free conditions. All animal care and handling were performed under institutional guidelines. C57 BL/6 (B6) [H2b], CBA/J (CBA) [H2k], and DBA/2 [H2d] were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA). In addition, it was used a specific strain of double reporter transgenic mice expressed YFP under the Foxp3 promoter and Td-Tomato-Red (TdTomRed) under the Ebi3 promoter (C57BL/6 background). Those mice were provided by Dr. Vignali (University of Pittsburgh).[46] Homozygous males and females were used to stablish breeding pairs and maintain the colony in a BSL-2 facility (Medical Science Center, University of Wisconsin-Madison). Reporter expression was confirmed by flow cytometry. I chose to use tolerance induction by DST and anti-CD40L monoclonal antibody (MR-1), as a means to

analyze our hypothesis.

3-11-2. Tissues processing

Spleen tissue was mechanically separated with micro-forceps and 100 μm nylon strainer. After washing on PBS, red blood cells and platelets were lysated with ACK buffer and then washed. After cell counting splenocytes were resuspended in desired volume. Lymph nodes were just mechanically separated with micro-forceps and 100 μm cell strainer, and then washed in PBS. To generate single-cell suspensions from liver tissues, it was mechanically separated with micro-forceps and a 100 μm cell strainer. Then, suspension was incubated with collagenase IV (2 mg/mL) and DNAse I (0.2 mg/mL) at 37°C for 60 minutes. After washing with PBS, cells were mononuclear cells-enriched using Percoll buffer (35%). Red blood cells were lysated with ACK buffer, and finally the cell suspension washed with PBS.

3-11-3. Tv-DTH assay

I used the mouse-to-mouse DTH transfer assay protocol described previously.[29, 75, 76] Briefly, 1.0 x10^7 splenocytes of B6, CBA, or DBA/2 were washed twice in sterile PBS, and resuspended in 0.1mL PBS containing 10 μg/mL PMSF (Sigma-Aldrich). The cells were then sonicated using a VR50 sonicator fitted with a 2-mm probe (Sonics). The disrupted cells were centrifuged for 30 min at 14,000 g at 4°C to remove debris. The protein content of the supernatant was determined using a micro BCA Protein Assay kit (Pierce). A total of 20 μg of protein was used for each injection in the DTH assays and referred to as either B6 [syngeneic/self], CBA [allogeneic/donor], or DBA/2 [3rd party] Ag. At the time points indicated in Figure 1, I euthanized and harvested a spleen, recovered splenocytes, and performed a tv-DTH assay. Specifically, I

injected 1.0 x 10^7 splenocytes from treated mice into syngeneic naïve mice footpads plus either: PBS [negative control], TT/DT recall antigen (25 μg) [positive control], test antigen (20 μg) [B6, CBA, or DBA/2 cell lysate], or a combination of TT/DT and test Ag [for linked suppression determination]. It is essential to notice that spleen cells DST is more efficient than bone marrow cells DST in regulation induction [72]. Before injection and after 24 hours, I used a dial-thickness gauge to measure footpad thickness; change in thickness, after subtraction of PBS value [net swelling] was expressed in units of 1.0 x 10^{-4} inches. To determine the extent of "linked suppression" I used the following formula:

$$\%Inhibition = \left(1 - \frac{(TT\ plus\ antigen)response}{TT\ response}\right)x100$$

The linked suppression was determined by comparing the footpad swelling response to TT/DT recall antigen alone, with the response to the combination of TT/DT and test antigen. The results were expressed as % inhibition of the recall response. When the % inhibition value with syngeneic Ag was = or > the % inhibition value with allogeneic-Ag, I considered the regulation in the mouse to be entirely non-specific. When the % inhibition value with syngeneic Ag was < the % inhibition value with allogeneic-Ag, I considered this to be evidence of allo-specific regulation. All values represent the observed tv-DTH response for the respective group minus the PBS control. Coinjected blocking antibodies were: αTGFβ (BD Pharmigen) (10 μg), αIL10 (R&D Systems) (10 μg), a combination of αp35 (R&D Systems) + αEbi3 (provided by Dr. Vignali, University of Pittsburgh) (1 μg of each), and αIL4 (BD Bioscience) (10 μg).

A scheme of how this assay might detect linked suppression caused by self- or allogeneic-Ag, is shown in Figure 2. The functionality of memory allo-specific Tregs was analyzed using tv-DTH assay. In this "single APC" model, suppression of the footpad swelling to TT/DT is "linked" when antigen sonicate is co-injected, because both the effecter T-cell recognizing TT/DT peptide,

and Tregs recognizing either self- or allo-peptides are seeing their p/MHC ligand on the same APC. If the APC is a dendritic cell, there is the possibility of thrombospondin- and IDO-mediated "trans" suppression effects [29]. The APC-Tregs interactions also result in production of regulatory cytokines IL10, TGFβ, or IL35.

3-11-4. Flow cytometry assay

Fluorochrome-labeled monoclonal anti-mouse antibodies were used at proper concentrations according to vendor technical sheets and/or titration experiments in our lab. Cells from spleen and lymph nodes were harvested and stained with CD3 (145-2C11), CD4 (RM4-5), CD25 (PC61), TGFβ/LAP (TW7-16B4) for 30 min at 4°C in FACS buffer. Depending on the panel, the following fluorochromes were used: eFlour450, BV421, APC, Alexa700, APC-eFlour780. Antibodies were purchased either from eBioscience, BD Bioscience or Biolegend. Fc blocking was made with anti-CD16/32 Ab. FITC (or equivalents) and PE (or equivalents) where avoided because they overlap the emission range of YFP and TdTomRed, respectively. YFP has an excitation peak at 514 nm and the emission peak is 527 nm (capture by 488B standard channel in flow cytometry). TdTomRed has an excitation peak of 557 nm and emission peak of 592 nm (captured by 561E standard channel). Flow cytometric analysis was performed on a BD FACS LSRII bench-top analyzer (BD Bioscience, San Jose, CA) and analyzed using FlowJo version 10 (Tree Star, Ashland, OR).

3-11-5. Statistical Analysis

Statistical analyses were performed by Graphpad Prism (GraphPad Software v5.01, San Diego, CA). The p values were calculated using Student t-tests and with 95% confidence interval

set as the measure of statistical significance (*P≤0.05, **P<0.01, ***P<0.001).

3-12. Abbreviations

DST: donor specific transfusion

tv-DTH: trans-vivo delayed type hypersensitivity

NIMA: non-inherited maternal antigen

NIPA: non-inherited paternal antigen

TT/DT: Tetanus Toxiod/Diphtheria Toxoid

GC: germinal centers

IL: interleukin

TGFβ: transforming growth factor beta

Ebi3: Epstein-Barr virus-induced gene 3

Foxp3: forkhead box p 3

Chapter 4: Conclusions

The results of our present study found new insights, which involved in a development of allo-specific tolerance. The mechanism of allo-specific tolerance was associated with not only the specific adaptive T cell response, but also the specific antigen presenting cells in the regulation of the immune responses.

I confirmed the existence of regulatory cytokines, IL35, TGFβ and IL10 in CD4 T cells in both lymphoid tissue and transplantable organ. Those cells can be either peripheral CD4 Treg cells ($Foxp3^{+}CD25^{+}$), which are mainly producers of IL35 and IL10, or Tr1 ($Foxp3^{neg}$) which essentially release TGFβ. The $IL35^{+}$ and $TGF\beta^{+}$ CD4 T cells in tolerance belong in other subset of classic Treg cells. I hypothesize that those cells are bystander CD4 T cells turned to a regulatory function in certain tissues via indirect response.

Finally, our study on induction therapy with donor specific transfusion (DST) plus CD40L co-stimulatory blockade was able to induce long-term allo-specific tolerance without further allogeneic challenge or co-stimulation boost. I also found that IL35-producing Treg cells were critical in development of regulation after allo-specific tolerization, but they homed peripheral tissues on day 35 after tolerization. The fact might be important factor to stablish an optimal timing between tolerance induction and transplantation. Remarkably, our results showed indirect evidence that IL35-producing Treg cells could release tolerogenic signals via exosomes-like nano vesicles to bystander CD4 T cells. This mechanism will play a critical role in the regulatory response. These findings provide valuable insights into future clinical trial of transplant tolerance.

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