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REVIEW

Transfer of tolerance to heart and kidney allografts in the rat model

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Summary

Long-term allograft acceptance can be induced in the rat using a variety of maneuvers. One of the cardinal features of some models of tolerance is that once the tolerance state has been established, it can be perpetuated to naive recipients by the adoptive transfer of donor-specific regulatory cells. Such adoptive transfer studies have also addressed the capacity of T-cell subpopulations and non-T cells to transfer tolerance. However, tolerance cannot be transferred in all models. The underlying reasons for this are unclear with some studies pointing towards dose-dependent aspects and timing of expansion of T regulatory cells following tolerance transfer. Further exploration of this phenomenon will help us to understand better the mechanisms upon which allograft tolerance is based, and will provide new perspectives for further experimental studies.

Despite the introduction of new and potent immunosuppressive agents and the better control of acute rejection, transplant patients still require nonspecific immunosuppressive therapy that is associated with major side effects (drug toxicity, infections and malignancies) [1]. Thus, achieving tolerance in the clinic remains a major objective in transplantation. Since the experiments by Medawar *et al.* [2] in the 1950s, tolerance, which is defined as ''*the acceptance of a transplanted organ without indefinite immunosuppression*'', has been a major goal in organ transplantation [3]. In the context of solid organ transplants, two types of tolerance can be distinguished: central and peripheral tolerance. Central tolerance includes central thymic deletion, which requires the establishment of a chimeric immune system such that recipient T cells are educated by recipient (or donor) thymic tissue to donor antigens (Ags), resulting in the deletion of potentially alloreactive T cells. Peripheral tolerance can be achieved via a multitude of mechanisms including anergy (functional inactivity of T cells), ignorance (absence of reactivity to the donor alloantigen), peripheral deletion of alloreactive T cells by apoptosis and suppression of T-cell activity (by T regulatory cells) [4]. However, what actually differentiates central tolerance from peripheral tolerance is still unclear. In animal models, the phenomenon of allograft tolerance is classically characterized by donor specificity (tolerant recipients accept a secondary donor-specific allograft but reject third-party allografts [5,6]), as well as by the absence of chronic rejection (based on the histological analysis of grafts surviving long-term [6,7]). Finally, in many instances, once induced, peripheral tolerance can be maintained and perpetuated into naive recipients by regulatory cells, a phenomenon termed ''infectious tolerance'' [8]. Infectious tolerance implies the ability to transfer tolerance to unmanipulated recipients over multiple generations by cell transfer. In the infectious tolerance phenomenon, regulatory properties of cells from tolerant recipients can be transferred to a naive cell population, converting them into regulatory cells. Tolerance to a given alloantigen is spread to other alloantigens present on the same target cells. Originally described in the mouse [8], infectious tolerance was first described in

the rat by Kupiec-Weglinski's group who demonstrated that CD4-targeted therapy leads to permanent acceptance of cardiac allografts in presensitized rat recipients, and that donor-specific tolerance can be transferred by spleen cells in an infectious-type fashion [9].Our current knowledge of the mechanisms that enable the transfer of tolerance to an allograft is based on *in vivo* experiments in animal transplantation models, where, once a tolerant state is established, it can be perpetuated by the adoptive transfer of cells to secondary naive recipients over multiple generations.

In this paper, focusing on the rat model, we will review how tolerance transfer may be influenced by the tolerance induction protocols and highlight the different organ compartments and cell subtypes that are able to transfer this tolerance state.

We would like to add, that at some points was necessary to mention the results obtained in the mouse model. Experimental work in the mouse model has advantages compared with the rat model (for example, the availability of transgenic and ''knockout'' mice). Also, studies in mice are made easier by a greater availability of experimental tools such as monoclonal antibodies. However, here we focus on the rat model and will only refer to murine models when the data provided in these models provide a better explanation for some of the phenomena identified in the rat.

Differential capacity of immune compartments to transfer tolerance

The spleen is the most frequently used compartment to test the adoptive transfer of tolerance in experimental models. One of the pioneering experiments where spleen allografts were used to induce tolerance and where spleen cells were used to transfer tolerance to heart allografts was performed by Stepkowski *et al.* [10] Regardless of the protocol used for tolerance induction and the type of organ transplanted, in various strain combinations, splenocytes have been identified as robust and reliable cells for tolerance transfer. In the Lewis (LEW).1W to LEW.1A congenic major histocompatibility complex (MHC) incompatible rat strain combination [11], adoptive transfer demonstrated the presence of potent donor-specific regulatory cells in splenocytes. In this model, tolerance induction to an MHC-mismatched heart allograft ($RT1^u$ to $RT1^a$) could be achieved by treating recipients with a deoxyspergualine (DSG) analogue, LF15-0195, for 20 days following transplantation, with the tolerated grafts showing no signs of chronic rejection [12]. Tolerance is uniformly transferred with spleen cells from LF15-0195-treated recipients 100 days after transplantation. In the same strain combination, heart allograft tolerance could be induced by donor-specific blood transfusion to the recipient, 14 and 7 days before transplantation and tolerance could be transferred with 50×10^6 splenocytes to a secondary rat recipient [13]. Finally, again in the LEW.1W to LEW.1A strain combination, tolerance to heart allografts could be induced by treatment of recipients with AdCD40Ig or AdCD40Ig and anti-inducible costimulation (ICOS) [14]. In the latter model, adoptive transfer of 50×10^6 splenocytes was again sufficient to induce significant prolongation of allograft survival [14]. Splenocytes have been shown to transfer tolerance in numerous other models and in different rat strain combinations. In the LEW to dark A6OUT1 (DA) heart allograft model, where recipients were treated with anti-rat lymphocyte serum and intrathymic injection of LEW donor antigens, 50×10^6 spleen cells from tolerant DA rats conferred unresponsiveness to donor alloantigen *in vivo* and transferred tolerance to secondary untreated rat recipients [15]. In the same strain combination, but this time in donor specific transfusion (DST)-induced tolerance, adoptive transfer was achieved using 100×10^6 spleen cells [16]. In the inverse rat strain combination (DA to LEW), treatment of Lewis recipients of a DA cardiac allograft with a combination of AdCTLA4-Ig and anti-ICOS antibody induced tolerance, which was transferable with 50×10^6 spleen cells [17]. Thus, the spleen provides an abundant source of cells that are able to transfer tolerance. Nevertheless, there are several models of tolerance that are not associated with the capacity of splenocytes to transfer long-term graft prolongation to secondary hosts (*see below*).

In addition to the spleen, other compartments of the immune system (e.g. lymph nodes) have been reported to contain cells that are able to transfer tolerance. Indeed, in the RA to PVG[18] and LEW to DA [16] rat strain combinations, heart allograft tolerance was shown to develop after one-step DST priming with blood 12 days before transplantation [18] or splenocytes 7 days [16] before transplantation. In both the models, donor-specific tolerance was successfully transferred to secondary recipients using 100×10^6 lymph node cells or 100×10^6 splenocytes. Contrasting with these data, Zhai *et al.* [19] reported that, in the $LBNF_1$ to LEW model of heart allograft tolerance, only splenocytes successfully transferred tolerance (100×10^6), whereas the same number of lymph node cells failed to do so [19]. This difference may reflect the different methods of primary tolerance induction since Zhai *et al.* exposed the recipient rat to a skin graft 1 week before heart transplantation under the cover of 10 injections of anti-CD4 mAbs from the day of skin grafting to 3 weeks after heart transplantation. Further explanations of these discordant results can be found in a mouse model of heart allotransplantation. Lakkis *et al.* [20] showed that in

alymphoplastic (*aly/aly*) mice, which lack lymph nodes and Peyer's patches, rejection of cardiac allografts was considerably delayed in comparison with the wild-type and heterozygous (*aly/+*) recipients, which had normal secondary lymphoid organs. Splenectomized *aly/aly* mice accepted their cardiac allografts indefinitely [20]. These findings indicate that the fully vascularized allogeneic organ transplants do not induce a productive alloimmune response in the absence of secondary lymphoid tissue. In the context of tolerance transfer, lack of secondary lymphoid organs also abrogates generation of cells with regulatory capacity, which is crucial for tolerance transfer [20].

One could ask whether primary and secondary lymphoid organs are equally important in the induction and maintenance of tolerance. Some experiments have addressed the importance of primary and secondary lymphoid organs in tolerance development and transfer. Kitade *et al.* [18] examined the effect of thymectomy or splenectomy on graft survival and on the generation of Tregs in DST-treated rats. These authors showed that the thymus and spleen are required for the generation of Tregs but not for their expansion18. In agreement with this observation, Onodera *et al.* [21] showed that thymectomy prevents induction but not maintenance of infectious tolerance in CD4 mAb-treated rat recipients pre-sensitized with donor skin grafts. Chiffoleau *et al.* [12] documented results conflicting with the previously mentioned studies. In their experimental model, where tolerance was induced with the DSG analogue LF15-0195, the thymus was required neither to induce allograft tolerance nor to induce and expand regulatory cells in the periphery capable of transferring tolerance [12]. These results imply that the thymus is critical for generation of regulatory T suppressor cells, whenever recipients are prechallenged with donor antigens. In such a situation, one can hypothesize that recipient T cells are educated by recipient thymic tissue to donor Ags, resulting in the deletion of potentially alloreactive T cells.

Regarding the importance of different compartments in tolerance induction, followed by adoptive transfer, it is important to mention the presence of the graft itself. Kataoka *et al.* [16] showed that without the presence of a heart allograft, DST alone was ineffective in generating the regulatory cells capable of transferring tolerance.

Investigations have also been undertaken to determine whether the blood or graft infiltrating cells (GIC) have a role in tolerance transfer. The first evidence that blood can also transfer tolerance came from the studies by Bektas *et al.* [22]. In a LEW.1W to LEW.1A heart graft model, using two-step DST tolerance induction, these authors showed that 1 ml of full blood transfers tolerance. However, in the same strain combination, using the same protocol, Lair *et al.* showed that peripheral blood mononuclear cells (PBMC) are less efficient in transferring tolerance than spleen cells. In the latter study, adoptive transfer of 100×10^6 splenocytes from DST-treated recipients indefinitely prolonged graft survival in all recipients whereas $20–40 \times 10^6$ PBMC from the same animals induced long-term graft survival in only 50% of secondary recipients [13], although higher doses of PBMC were not tested.

Another cell population studied for their ability to transfer tolerance is GIC. Zhou *et al.* reported that, after oral administration of donor splenocytes, 70×10^6 renal allograft GIC (harvested 5 days after transplantation) could adoptively transfer tolerance to a naive animal in a BN to LEW strain combination [23]. Another indication that GIC are powerful in transferring tolerance was provided by Kataoka *et al.* [16] who reported that, in the LEW to DA strain and 60 days after transplantation and DST induction (7 days prior to the heart transplantation), $0.3–30 \times 10^6$ GIC transferred tolerance in 40% of secondary recipients. In the same experiment, 30×10^6 splenocytes from long-term surviving recipients were necessary to transfer tolerance, suggesting that GIC were 10–100 times more effective in transferring tolerance [16]. In discordance with this observation, 5×10^6 GIC from two-step DST-induced heart allograft tolerance in the LEW.1W to LEW.1A strain combination could not transfer tolerance [24].

Why is irradiation prior to tolerance transfer important?

In numerous studies where the capacity of different organ compartments or T-cell subpopulations to transfer tolerance was tested, sub-lethal whole body irradiation (3–5 Gy) of secondary recipients appeared to be a necessary experimental step. An explanation for this lies in a fact that, whenever T cells participate in tolerance transfer, preservation of the homeostasis of T-cell numbers is critical. The immune system tends to maintain its structure and function by establishing dynamic equilibriums controlled by multiple regulatory mechanisms. These mechanisms participate in the homeostatic control of T-cell numbers and population distribution [25,26]. If a population of regulatory T cells is introduced into a secondary recipient, the T cells expand to reach steady state numbers. In other words, different sub-populations of lymphocytes are in ''competition to occupy specific niches''. Irradiation of the secondary recipient prior to cell transfer would ensure that certain ''niches'' are empty and that there is no competition for them. In a situation such as this, the regulatory properties of cells from tolerant recipients can be transferred to a naive cell population, thus converting them into regulatory cells without competition with host cells.

However, in the model described by Degauque *et al.* [6] (induction using anti-donor MHC class II serum), an irradiation step was not necessary for successful tolerance transfer. In this model, T cells alone were not sufficient to transfer a state of dominant tolerance, but required the presence of $CD103^+$ DC which, in concert with T cells from tolerant recipients, educated naive host T cells [6] without competition for ''specific niches''. Bektas *et al.* [22] (using two-step DST tolerance induction) also reported tolerance transfer through full blood without exposing the secondary recipients to irradiation. Tolerance may thus also function through the education of naive host T cells and not through their expansion after irradiation.

Adoptive transfer identifies different T-cell subtypes as key players in tolerance transfer

$CD4^+$ T cells are powerful in transferring tolerance. Various types of cells have been described to transfer tolerance to naive syngeneic hosts when injected at the time of transplantation. The most studied populations are $CD4^+$ and $CD8^+$ T cells. Sometimes, even within the same strain combination, different tolerance induction protocols provide contradictory results as regards the capacity of certain cell sub-populations to transfer tolerance.

In the PVG to DA strain combination, following tolerance induction by anti-CD4 mAb therapy, 100 days post-transplantation, tolerance to heart transplants can be transferred with 20×10^6 $CD4^+$ T cells, whereas the same number of $CD8^+$ T cells is ineffective [5]. In the RA to PVG rat strain combination, where heart allograft tolerance develops after one-step DST priming with blood 12 days prior to transplantation [18], Kitade *et al.* showed that $CD4^+$ cells are more powerful than $CD8^+$ cells in transferring tolerance to a heart allograft. Similarly, regulatory cells develop after DST-induced acceptance of a LEW heart transplanted into a DA rat and Kataoka *et al.* identified $CD4^+$ cells that uniformly transferred tolerance, as regulatory cells. However, in this study, at day 60, the same number of $CD8^+$ cells (10×10^6) showed suppressive activity and transferred tolerance in 62% of grafts [16]. Heart transplantation performed in the same strain combination, where tolerance was induced using DST with splenocytes, again showed that 10×10^6 of $CD4^+$ cells fully transferred tolerance, whereas 10×10^6 of $CD8^+$ T cells transferred tolerance with limited capacity [16]. Finally, in the LEW to DA combination, both 10×10^6 of $CD4^+$ or $CD8^+$ T cells transfer tolerance to heart allografts. In this study, and in general, both $CD4^+$ and $CD8^+$ populations appeared to have regulatory activities, although the $CD4^+$ population played the dominant role [27].

Liu *et al.* reported that in their model of heart allotransplantation, where tolerance was induced in ACI recipients by multiple transfusions of UVB-irradiated blood from Lewis heart donors, $CD8^+$ T cells from tolerant ACI rats expressed FOXP3 and 25×10^6 of $CD8^+$ T cells transferred tolerance to naive secondary hosts and induced the up-regulation of the paired immunoglobulin-like receptor-B in Lewis dendritic cells and heart endothelial cells [28]. Similarly, Zhou *et al.* reported the generation of $CD8^+$ regulatory GIC in a renal allograft model after oral administration of donor splenocytes in the BN to LEW strain combination and showed that the $CD8^+$ GICs could adoptively transfer allograft tolerance to a naive recipient [23]. Finally, in a heart allograft model of AdCD40Ig-induced tolerance, Guillonneau *et al.* showed that adoptive transfer of 2.5×10^6 $CD8^+$ $CD45RC^{low}$ T cells resulted in indefinite allograft survival, whereas transfer of the same number of $CD8^+CD45RC^{high}$ T cells failed to inhibit early acute rejection [29].

Suppressive activity: $CD4^+CD25^+$ T cells, $CD4^+CD25^-$ T cells, or both?

Among the $CD4^+$ T cell populations, particular attention has been paid to the $CD4^+CD25^+$ regulatory T-cell subset. In a rat model of DST-induced graft survival prolongation (the RA to PVG rat strain combination), Pirenne *et al.* published a detailed description of induced Treg cells [18]. They found that both $CD4^+CD25^+$ (25×10^6) and $CD4^+CD25^-$ T cells (25×10^6) had the ability to transfer tolerance [18].

Studies performed by our own group in the LEW.1W to LEW.1A combination showed that 5×10^6 of $CD4^+CD25^+$ T cells (of spleen and thymus origin) from animals treated with a DSG derivative were highly efficient in transferring tolerance, whereas the same number of $CD4^+CD25^-$ cells only partially transferred tolerance [12]. In contrast, in the same strain combination, when the tolerance was induced by a DST protocol, tolerance transfer with 50×10^6 $CD25^-$ spleen cells was successful [13], whereas 4×10^6 of $CD25^+$ T cells from tolerant animals were unable to prolong graft survival following transfer to a naive host. This suggests that, even in the same genetic background and the same strain combination, different mechanisms operate when different tolerance induction protocols are applied. These data also show that CD25 may not be a stable marker for regulatory T cells in the periphery [30]. In support of this conclusion, Gavin *et al.* demonstrated that during the homeostatic process, $CD4^+CD25^+$ T cells that had divided more than five times no longer expressed the CD25 marker but remained highly potent in terms of their suppressive capacity [31].

On the subject of $CD4^+$ cells, interesting results have been obtained concerning the $CD4^+CD45RC^+$ and $CD4^+CD45RC^-$ populations. Kitade *et al.* shed new light on the phenotype of Tregs by showing that, *in vivo*, transfer of tolerance was not associated with the CD25 marker. Whereas only 10×10^6 $CD4^+CD45RC^-$ cells adoptively transferred tolerance, the same number of $CD4^+CD45RC^+$ cells were unable to do so [18]. One conflicting report with these data comes from Zhai *et al.*, who reported that $CD4^+CD45RC^+$ cells (and not $CD4^+CD45RC^-$) were hyporesponsive to alloantigen and were able to suppress normal T cell function in coculture assays [19], although nothing was reported on their *in vivo* properties.

T cell and non-T cell cooperation in the transfer of tolerance

Several studies have provided evidence that both T cells and non-T cells are required for the successful transfer of tolerance. In the heart allograft model, where tolerance was induced using AdCD40Ig, subtraction of the T-cell fraction from splenocytes resulted in heart allograft rejection. Moreover, we previously showed that in the LEW.1W to LEW.1A strain combination, kidney allograft tolerance can be induced using anti-donor MHC class II serum. In this model, tolerance was also transferred in an ''infectious'' manner over several generations using 80×10^6 spleen cells [6]. Moreover, splenocytes depleted of T cells or $CD103^+$ dendritic cells were no longer able to transfer tolerance. These data indicate that, in this model, transfer of tolerance requires the presence of both T and non-T cells. In the same strain combination in a model of DST-induced heart allograft tolerance, Lair *et al.* showed that 20×10^6 purified blood T cells had no effect in adoptive transfer [24]. In contrast, the adoptive transfer of 20×10^6 blood non-T cells from DST-treated recipients induced an indefinite graft survival in 40% of secondary recipients. The models mentioned above, including the different rat strain combinations, tolerance induction protocols and cell subtypes used to transfer tolerance efficiently, are outlined in Table 1.

Immune tolerance mechanisms involved in Treg development

Several studies have shown the contribution of central and peripheral immune tolerance mechanisms in the development of Treg capable of transferring tolerance. Kataoka *et al.* demonstrated that both LEW DST and a LEW heart allograft were necessary to generate regulatory lymphocytes in DA recipients. The adoptive transfer of cells from DA rats receiving only LEW DST, but no heart transplant, did not lead to LEW heart graft acceptance in irradiated naive recipients [16]. In the rat model, there is another piece of evidence showing that continuous presence of alloantigen is a critical factor in the development and maintenance of nonresponsiveness to donor antigens. Onodera *et al.* showed that normal LEW rats rejected LBNF1 hearts despite the hearts having been parked for 100 days in CD4 mAb-conditioned LEW hosts. The authors concluded that this tolerant state does not result from ''graft adaptation,'' and regulatory T cells were exposed to continuous stimulation by the donor antigens. Their results from both graft-free and adoptive transfer studies demonstrate that effective memory for suppression in the infectious tolerance pathway depends upon

Table 1. Overview for the tolerance transfer experiments in rat model.

Group	Strain combination	Graft type	Tolerance induction protocol	Cell subtype that is able to transfer tolerance	Long-term transfer success
Hall	PVG → DA	Heart	Anti-CD4 mAb	$CD4^+$ 20×10^6	6/6
Kataoka	LEW → DA	Heart	DST spleen (D-7)	$CD4^+$ 10×10^6	3/3
Kataoka	LEW → DA	Heart	DST spleen (D-7)	$CD8^+$ 10×10^6	5/8
Kataoka	LEW → DA	Heart	Spontaneous acceptance of donor liver	$CD4^+$ 10×10^6	4/5
Kataoka	LEW → DA	Heart	Spontaneous acceptance of donor liver	$CD8^+$ 10×10^6	4/7
Kitade	RA → PVG	Heart	DST blood (D-12)	$CD4^+$ 25×10^6	5/5
Kitade	RA → PVG	Heart	DST blood (D-12)	$CD4^+CD25^+$ 25×10^6	5/5
Kitade	RA → PVG	Heart	DST blood (D-12)	$CD4^+CD25^-$ 25×10^6	5/5
Chiffoleau	LEW.1W → LEW.1A	Heart	LF15-0195	$CD4^+CD25^+$ 5×10^6	4/4
Degauque	LEW.1W → LEW.1A	Heart	DST blood (D-7, D-14)	$CD25^-$ 50×10^6	4/9
Kitade	RA → PVG	Heart	DST blood (D-12)	$CD4^+CD45RC^-$ 10×10^6	6/6
Liu	LEW → ACI	Heart	DST (UVB-irradiated blood, D-21, D-14, D-7)	$CD8^+$ 20×10^6	3/5
Guillonnoeau	LEW.1W → LEW.1A	Heart	AdCD40-Ig	$CD8^+CD45RC^{low}$ 2.5×10^6	4/4
Lair	LEW.1W → LEW.1A	Heart	DST blood (D-7, D-14)	PBMC non-T cells 20×10^6	4/10

Summary of strain combination, graft type, tolerance induction protocols, and cell subtypes used in adoptive transfer experiments.

persistent donor-specific alloantigen stimulation and wanes about 3 weeks after allograft removal [21].

The thymus and spleen are also thought to be important in Treg generation. Kitade *et al.* showed that the thymus and spleen are required for the generation of DSBT-Tregs but not for their expansion. Thymectomy or splenectomy, when performed 4 weeks before DST, abrogated heart allograft tolerance. However, the same procedures performed on the day of transplantation did not influence tolerance [18]. The same conclusion that thymectomy prevents induction but not maintenance of regulatory T cells capable of transferring tolerance was also documented in Onodera *et al.*'s experiments [21]. Overall, the results of these studies suggest that both central and peripheral mechanisms of tolerance are involved in allograft acceptance and transfer of tolerance.

Timing of Treg expansion

Information about the timing of Treg expansion in the spleen and other compartments (including the graft itself) comes from Kitade *et al.* [18]. Using the RA rat strain as a heart donor and the PVG strain as a recipient in the context of a DST protocol, these authors showed that Tregs transferring tolerance are present in the spleen and lymph nodes in tolerant rats as soon as day 5 after transplantation. At 2 and 4 weeks post-transplantation, Tregs expanded and were present in all compartments tested: not only in the spleen and lymph nodes but also in the thymus and the graft itself [18]. When comparing the efficiency of 100×10^6 spleen cells and 100×10^6 lymph node cells to transfer tolerance 5 and 14 days after transplantation, they showed that 14 days post-transplantation, spleen and lymph node cells transferred tolerance in 100% of cases, whereas Tregs harvested on day 5 transferred tolerance in only approximately 40% of the cases. Moreover, a reduced number of splenocytes (10 and 25×10^6), which failed to transfer tolerance when taken from tolerant rats at day 5, fully transferred tolerance at day 14. Four weeks post-transplantation, Tregs were detected in all compartments analyzed and transferred tolerance with great success: 100% for $10–100 \times 10^6$ splenocytes, 100% for 100×10^6 lymph node cells, approximately 85% for 100×10^6 thymocytes, and approximately 80% for 10×10^6 GICs [18]. These data suggest a progressive expansion of regulatory cells during the development phase of tolerance and their maintenance thereafter. Other data from rat models support these conclusions. Liver transplantation from LEW to DA rats results in spontaneous acceptance. To detect the presence of cells regulating graft rejection, Kataoka *et al.* performed heart transplantation after adoptive spleen cell transfer from DA recipients who spontaneously accepted LEW hepatic grafts [27]. LEW cardiac allografts were rejected when splenocytes were adoptively transferred from DA rats bearing LEW livers for only 30 days. On the other hand, splenocytes from DA rats bearing LEW livers for >60 days completely transferred tolerance and all LEW cardiac allografts were accepted. This result again indicates that time was necessary for the expansion of regulatory cells that are able to transfer tolerance [27].

Transfer of tolerance is dose-dependent

The dose-dependent aspect of tolerance transfer has been documented in previously mentioned models of spontaneous acceptance of hepatic grafts. Splenocytes (100×10^6) from DA tolerant recipients (bearing LEW livers for >60 days) transferred tolerance with 100% of success compared with a 70% success rate for 50×10^6 splenocytes [27]. In the same strain combination, using different protocols for tolerance induction, Kataoka *et al.* provided another example that transfer of tolerance is dose-dependent [16]. In the LEW to DA heart allograft model, tolerance was induced by priming with donor splenocytes 7 days before transplantation. In this study, $30–100 \times 10^6$ splenocytes transferred tolerance to all recipients, but 1×10^6 and 10×10^6 splenocytes were insufficient to prevent acute rejection of the cardiac transplant [16].

Is it possible to extrapolate across models?

One of the questions arising at this point is whether it is possible to extrapolate results obtained in rodents to large animal models, or even more, to apply rodent tolerance induction protocols to nonhuman primate models (NHPs). Establishing successful protocols in old world monkeys, such as the rhesus macaque or baboon, would offer the possibility of moving towards transplantation tolerance in the clinic. On the whole, nearly all tolerogenic strategies that are successful in rodents have proven less effective in NHPs [32]. This can be explained by the homogeneity of rodent models – uniform age, environmental exposure and, most importantly, genetic background [32]. The advantage of performing experimental studies in rodents is that many rat and mouse inbred strains are available, which is not the case with large animals or monkeys. Moreover, tolerance transfer is always performed in the same strain combination, meaning that the cells responsible for transferring tolerance, even in the new recipient, face the same antigens and thus successfully transfer tolerance.

Another huge problem that exists between small animals and NHPs is environmental exposure to different pathogens and the development of heterologous immunologic memory. Memory cells present a clear threat to

antigens; they respond vigorously and in certain situations can exert cross-reaction to alloantigen [33]. This may explain failure to transfer tolerance in some circumstances. Finally, in large animals and NHPs, most tolerance induction protocols include calcineurin inhibitors that may inhibit the development and activation of Tregs [34] that have often been identified as key mediators of tolerance transfer in rodents.

Occasionally, some authors have been able to transfer tolerance with anergic T cells generated *ex vivo* in rhesus monkeys [35] or have been able to prolong graft survival by transferring peripheral blood lymphocytes from tolerant recipients in miniature swine [36]. Nevertheless, in this review, we wanted to focus on tolerance transfer in rat models and for this reason, the results obtained in mice, guinea pigs and large animals have been only mentioned briefly. Overall, translation of tolerance strategies from animals to humans has become increasingly difficult [32].

Conclusion

Tregs are present very early after transplantation in primary and secondary lymphoid organs. This was documented in numerous studies where different organ compartments were used in adoptive transfer experiments. In this way, Tregs can block the initiation of the alloimmune response and participate in the induction of tolerance. Tolerance transfer can also be successfully achieved with GICs, suggesting that Tregs can directly and locally protect the transplanted tissues. Different cell subpopulations have proven their capacity in tolerance transfer. The results that are sometimes contradictory, as mentioned by Pirenne, can be explained by mechanisms that may depend upon the strain combination and the species used [18]. Finally, both dose and time components can be critical factors in the transfer of tolerance.

Future immunosuppressive strategies should include agents that enable expansion of Tregs while preventing innate and adaptive immunity mechanisms and, in this context, experiments of tolerance transfer may help us to understand better the biology of Tregs. Clinical observations have shown that transplantation tolerance may be lost over time and Treg presence or absence could be used as a useful biomarker in the prognosis of graft survival. It will be very interesting to make correlations between Tregs, proven for their capacity to transfer tolerance, and their possible role in prediction of graft outcome in numerous rat models.

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VJ and DL wrote the paper. JPS and SB supervised the study.

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