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ORIGINAL ARTICLE

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The effect of FTY 720 on engraftment in a model of spontaneous allograft acceptance

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Abstract Further development in organ transplantation requires the utilization of new immunosuppressive drugs that—in addition to being effective against rejection—do not block tolerance. We previously reported that FTY 720, a drug that alters lymphocyte trafficking, has marked anti-rejection properties. We now investigate how FTY 720 influences tolerance in a model of graft acceptance by donor-specific blood transfusion (DSBT). Two different transplant models—heart transplantation (Htx) and intestinal transplantation (Itx)—were studied. We performed orthotopic Itx and heterotopic Htx using fully mismatched inbred male RA ($RT1^P$) and PVG ($RT1^C$) rats as donors and recipients. Tolerance was induced by DSBT on pre-transplant day −12. To test the effect of FTY 720 on DSBT-induced tolerance, we administered FTY 720 orally prior to DSBT. Itx: control rats succumbed to rejection at 18 ± 4 days. DSBT alone prolonged survival to 101.9 ± 18 days ($P < 0.05$ vs untreated). Long-term survivors were tolerant (acceptance of secondary donor-specific Htx). Adjunction of FTY 720 prior to DSBT reduced survival to 55.9 ± 44.7 days ($P < 0.05$). However, long-term survivors still accepted secondary donor-specific Htx. Htx: control rats survived 9 ± 0.6 days. DSBT alone prolonged survival indefinitely (> 120 days) and induced tolerance (acceptance of secondary donor-specific Htx). Unlike in Itx, adjunction of FTY 720 prior to DSBT did not reduce Htx survival. Acceptance of secondary donor-specific Htx was not influenced by FTY 720. In Itx, FTY 720 counteracts the beneficial effect of pre-transplant DSBT and triggers acute rejection of primary, but not secondary grafts. In Htx, however, FTY 720 allows full development of tolerance. The mechanisms by which FTY 720 causes rejection in primary intestinal but not in heart grafts need to be elucidated.

Keywords FTY 720 · Tolerance · Rejection

Introduction

Despite availability of potent immunosuppressive reagents, rejection, acute and particularly chronic, remains a leading cause of graft loss after solid-organ allotransplantation. In addition to direct drug-related toxicity, long-term exposure to immunosuppression causes infection and malignancies [27]. Development of immunomodulatory strategies aimed at inducing hyporesponsiveness in vivo and at reducing the need for immunosuppression would therefore represent substantial progress in organ transplantation. That drugs active

against rejection can equally counteract development of tolerance—an immunologically active phenomenon—is an increasingly recognized observation [15]. In this context, it is of paramount importance that new drugs entering into the clinics be tested not only for their anti-rejection properties, but also for their effect on tolerance induction. Remarkably, many experimental studies analyze the effect of immunosuppressive drugs on rejection, but only few examine their impact on tolerance.

FTY 720, a new type of immunosuppressive agent, is a synthetic structural analog of myriocin, a metabolite of the ascomycete *Isaria sinclairii* [8]. Its chemical structure and mechanism of action are different from those of cyclosporin A (CsA), tacrolimus, and other current immunosuppressive drugs [1]. FTY 720 modifies lymphocyte trafficking through alteration of the expression or function of certain chemokine receptors [3]. This provokes a transient migration of lymphocytes from the peripheral blood to the secondary lymphoid organs, and causes, as a consequence, peripheral lymphopenia [5, 6, 28]. It has been shown that FTY 720 acts against acute rejection—not by affecting T-cell function, as classical immunosuppressive drugs such as CsA and tacrolimus do—but by blocking allograft infiltration by recipient lymphocytes, while preserving their function [4, 5, 6, 28].

We reported earlier that FTY 720 has marked anti-rejection effect, when used as induction or as rescue treatment after heart allotransplantation in rats [30, 31]. The aim of the present study is to analyze the effect of FTY 720 on tolerance in a model, in which grafts are accepted via donor-specific blood transfusion (DSBT) in an entirely immunosuppression-free environment. Because development of tolerance may vary with different organs, two separate organ transplant models, the highly immunogenic intestinal transplantation (Itx) model and the less immunogenic heart transplantation (Htx) model, were studied.

Materials and methods

Animals

Inbred male RA ($RT1^p$) and PVG ($RT1^c$) rats weighing 150 to 250 g were used as fully mismatched donors and recipients, respectively.

Model of intestinal and heart transplantation

Itx was performed according to the methods described by Monchik and Russell 19and Zhong et al. [32]. The entire donor small intestine, including the jejunum and ileum, was harvested in continuity with an abdominal aortic tube and the portal vein for vascular reconstruction. The recipient infra-renal aorta and vena cava were isolated. End-to-side anastomoses were performed, followed by removal of the native small intestine and reconstruction of intestinal continuity in an end-to-end fashion. Donor heart grafts were transplanted onto the infra-renal aorta and vena cava of recipients, by standard microsurgical techniques [21]. All transplanted rats that did not survive at least 5 days were considered as technical failures and were not entered into the present study. The technical failure rate was 15% after Itx and 3% after Htx.

Assessment of rejection and graft-versus-host disease

Following Itx, graft rejection was reflected by the animal's death. Rejection was characterized by a characteristic clinical course, including the development of a palpable abdominal mass, weakness, significant weight loss, and diarrhea. Development of acute and chronic rejection was further assessed by gross morphology and confirmed histologically. Clinical and histological signs of graft-versus-host disease (GVHD) were also looked for on the skin. Following Htx, the heartbeat was monitored daily by manual palpation through the abdominal wall. Rejection was defined as the time of complete cessation of heartbeat and was confirmed histologically.

Induction of tolerance by DSBT

By direct heart puncture, 1.5 ml of donor-specific RA blood was obtained and transfused into PVG recipients via the penile vein. In this specific RA-to-PVG rat combination, we previously reported that a single pre-transplant infusion with DSBT constantly induces tolerance [11, 13, 17].

Secondary Htx

In recipients of long-term surviving intestinal or heart grafts, we tested tolerance by performing secondary donor-specific (RA) and third-party (WKAH) Htx on post-transplant day 100. Htx was done in the right cervix, according to a previously described technique [17, 19].

Drugs

FTY 720 was generously provided by Novartis, Basel, Switzerland. It was diluted in distilled water and administered orally at a dose of 5 mg/kg per day for 3 days. It was administered on pre-transplant days −15, −14, and −13, e.g., immediately prior to DSBT administration on pre-transplant day −12.

Experimental design

The following experimental groups were studied in Itx and Htx, respectively (Table 1). In syngeneic controls (group 1), Itx and Htx were performed on RA rats. In allogeneic controls (group 2), RA grafts were transplanted into PVG recipients without any treatment. In the FTY 720-alone group (group 3), FTY 720 was given on pre-transplant days −15, −14, and −13 and allogenic transplantations were performed. In the DSBT-alone group (group 4), allogenic transplantations were performed 12 days after DSBT administration. In the FTY 720-plus-DSBT group (group 5), FTY 720 was given on pre-transplant days −15, −14, and −13, immediately prior to DSBT administration on pre-transplant day −12. Allogenic transplantations were subsequently performed. The recipients with long-term surviving primary grafts were killed on post-transplant day 120.

Table 1 Experimental groups

Group	Number		Administration
	Itx	Htx	
1. Syngeneic control	$n=6$	$n=6$	None
2. Allogeneic control	$n=6$	$n=6$	None
3. FTY°720 alone	$n=7$	$n=6$	FTY°720, 5°mg/kg per day on days −15, −14 and −13
4. DSBT alone	$n=7$	$n=6$	DSBT on day −12
5. FTY°720 + DSBT	$n=8$	$n=7$	FTY°720, 5°mg/kg per day on days −15, −14 and −13 DSBT on day −12

Anti-donor-specific antibodies

Anti-donor IgM and IgG were determined by flow cytometry on post-transplant day 120, with RA peripheral blood mononuclear cells (PBMCs) being used as targets. Four Htx and four Itx rats were examined. Aliquots of 0.5×10^6 PBMCs were cultured for 30 min at 4 °C with 100 µl of 1:10 diluted serum taken from recipient rats. After a secondary staining with fluorescein isothiocyanate (FITC)- conjugated anti-rat IgM (The Binding Site, UK) and anti-rat IgG (ST-AR 17, Serotec, Oxford, UK), the cells were examined by flow cytometry. Results were expressed as the relative mean channel fluorescence, calculated as the mean channel fluorescence of stained cells divided by the mean channel fluorescence of cells incubated with control serum and counterstained with FITC-conjugated anti-rat IgM or IgG antibodies [29].

Mixed lymphocyte cultures

Recipient PBMCs were isolated from heparinized blood by density gradient centrifugation over Percoll (Pharmacia, Uppsala, Sweden). To be used as stimulators, single-cell suspensions of splenocytes were prepared by the gentle compression of the minced spleen on nylon mesh. RPMI added with 10% fetal calf serum and 2-mercaptoethanol was used as culture medium. Cells were seeded in 96-well microtiter plates in replicates of four at a concentration of 5×10^5 cells per well. We added 5×10^5 cells per well of irradiated (3,000 rad) lymphocytes—used as stimulator—to the culture for 72 h, and determined ^{3}H-thymidine uptake, according to the standard technique [24].

Effect of FTY 720 on lymphocyte ratio and lymphocyte subpopulations

The lymphocyte ratio, (lymphocytes/leukocytes) ×100, and the percentage of lymphocyte subpopulations were analyzed in the peripheral blood, the spleen, and the mesenteric lymph nodes of three PVG recipients 1 day and 13 days after administration of a 3-day course of FTY 720 (5 mg/kg per day). The peripheral blood was hemolyzed. The spleen and the mesenteric lymph nodes were minced and passed through mesh. The cells were washed and suspended in phosphate-buffered saline (PBS). After being centrifuged, each specimen was incubated with the following monoclonal antibodies: PE-conjugated G4.18 (mouse IgG3, k, 22015B, Pharmingen, San Diego, Calif. USA) for CD3; PE-conjugated OX-35 (mouse IgG2a, k, 22025B, Pharmingen) for CD4; FITC-conjugated 341 (mouse IgG1, k, 22504D, Pharmingen) for CD8b; PE-conjugated OX-33 (mouse IgG1, k, 22175B, Pharmingen) for CD45RA (B-cell). We conducted two-color flow cytometric analysis using a standard FACScan (Becton Dickinson, N.J., USA).

Statistical analysis

Data were analyzed by unpaired Student's *t*-test, log-rank test, and Chi-square test. $P<5\%$ was considered as significant.

Results

Induction of tolerance by DSBT; in vivo discrepancy between Itx and Htx

Pre-transplant DSBT significantly prolonged graft survival after both Itx and Htx, compared with allogeneic controls (Tables 2 and 3). Allogeneic control Itx rats (group 2) survived for 18 ± 4 days, whereas DSBT-treated rats (group 4) survived for 101.9 ± 17.9 days ($P<0.05$). Htx in allogeneic control rats (group 2)

Table 2 Survivals of PVG recipients with Itx. In each group, individual graft survival, and mean survival time (*MST*) ± SD are shown

Group	Survival (days)	MST ± SD(days)	*P*
1. Syngeneic	> 120×6	> 120	
2. Allogeneic control	14.14.17.18.22.23	18.0 ± 3.8	
3. FTY°720 alone	17×4.18.20.21	18.1 ± 1.6	NS vs group 2
4. DSBT alone	78.88.89.98 > 120×3	101.9 ± 17.9	< 0.05 vs group 2
5. FTY°720 + DSBT	18.19.25.28.34.83 > 120×2	55.9 ± 44.7	< 0.05 vs group 4

Table 3 Survival of heart grafts in PVG recipients. In each group, individual graft survival and mean survival time (*MST*) ± SD are shown

Group	Survival (days)	MST ± SD(days)	*P*
1. Syngeneic	> 120×6	> 120	
2. Allogeneic control	8, 9×4, 10	9.0 ± 0.6	
3. FTY°720 alone	8, 9×3,10, 11	9.3 ± 1.0	NS vs group 2
4. DSBT alone	> 120×6	> 120	< 0.05 vs group 2
5. FTY°720 + DSBT	> 120×7	> 120	NS vs group 4

survived for 9 ± 0.6 days, whereas Htx in DSBT-treated rats (group 4) survived for more than 150 days ($P < 0.05$). However, DSBT allowed indefinite survival of only 43% of Itx recipients, whereas indefinite allograft survival was constantly achieved (100%) after Htx ($P < 0.05$). All recipients with long-term surviving intestinal or heart grafts (> 100 days) accepted secondary donor-specific—but not third-party—heart grafts (Table 4).

Table 4 Acceptance of second graft. We tested tolerance by performing a secondary donor-matched heart graft. In both Itx and Htx models, recipients with long-surviving grafts accepted donor-specific, but not third-party, heart grafts, independently of the adjunction of FTY°720 to DSBT. Thus, FTY°720does not influence acceptance of secondary grafts in both Itx and Htx model

Model	Treatment	Donor-specific graft (days)	Third-party graft (days)
Itx	DSBT alone	> 20×3	7×1
	FTY°720 + DSBT	> 20×2	7×1
Htx	DSBT alone	> 20×3	5×2
	FTY°720 + DSBT	> 20×3	8×1

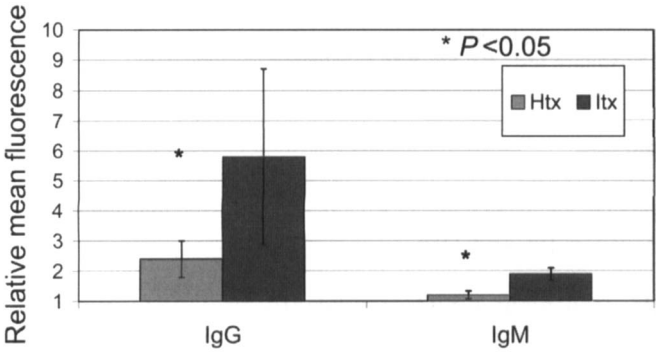

Fig. 1 IgG and IgM production in tolerant Htx and Itx recipients in group 4 (post transplant day 120). IgM to IgG class switch was observed in each model but a significantly larger amount of IgG was detected in Itx than in Htx ($P < 0.05$)

Anti-donor-specific IgM and IgG production and mixed lymphocyte cultures in tolerant animals at post-transplant day 120; in vitro discrepancy between Itx and Htx models

On post-transplant day 120, cellular and humoral responses against donor antigen were examined in tolerant animals of group 4. In the serum of tolerant Itx and Htx recipients, we detected larger amounts of anti-donor-specific IgG than IgM, suggesting development of a class switch from IgM to IgG (Fig. 1). The relative mean fluorescence was 5.4 ± 2.3 (IgG) vs1.9 ± 0.2 (IgM) in Itx ($P < 0.05$) and 2.4 ± 0.6 (IgG) vs 1.2 ± 0.1 (IgM) in Htx ($P < 0.005$). However, significantly higher levels of IgG were observed in Itx recipients than in Htx recipients (Fig. 1). The relative mean fluorescence was 5.4 ± 2.3 (Itx) vs 2.4 ± 0.6 (Htx); $P < 0.05$. As far as mixed lymphocyte cultures (MLCs) are concerned, tolerant Itx recipients displayed vigorous MLC responses against donor antigen, whereas immunological hyporesponsiveness was observed in tolerant Htx recipients (Table 5).

The effect of FTY 720 on the lymphocyte ratio and on lymphocyte subpopulations

Profound peripheral lymphopenia was induced by treatment with FTY 720: the lymphocyte ratio had dropped from 56.8 ± 10.4% to 14 ± 5.8% 1 day after treatment; $P < 0.0001$ (Fig. 2). Lymphocyte depletion was also observed in the spleen, but to a lesser extent than in the peripheral blood: the lymphocyte ratio in the spleen had dropped from 52.3 ± 1.2% to 41 ± 2% 1 day after treatment with FTY 720; $P < 0.005$ (Fig. 2). In the mesenteric lymph nodes, however, the percentage of lymphocytes did not vary significantly: 56.3 ± 10.3% before FTY 720 treatment vs 50.4 ± 3.2% 1 day after FTY 720 treatment; $P = 0.26$ (Fig. 2). FTY 720 affected not only T lymphocytes (including CD3, CD4 and

Table 5 Proliferation of peripheral blood lymphocytes from tolerant Itx and Htxrecipients (post transplant day 120). Tolerant Itx recipients showed vigorous MLC against donor antigen. On the other hand, immunological hypo-responsiveness was observed in tolerant Htx recipients

Subject	^{3}H-thymidine incorporation when cultured with		
	PVG stimulator mean (SD) cpm	RA stimulator mean (SD) cpm	WKAH stimulator mean (SD) cpm
Itx			
Animal 1.	972 (168)	47,420 (6,928)	34,537 (4,397)
Animal 2.	2,068 (291)	54,211 (5,579)	30,608 (726)
Animal 3.	2,405 (618)	33,154 (2,850)	18,638 (2,594)
Htx			
Animal 1.	1,921 (244)	2,733 (282)	9,136 (1,476)
Animal 2.	999 (272)	2,886 (168)	12,560 (2,672)
Animal 3.	4,168 (39)	10,699 (4,403)	25,719 (4,056)
Control			
Animal 1.	1,073 (222)	25,467 (11,001)	49,353 (8,520)
Animal 2.	2,455 (734)	41,821 (2,669)	46767 (3653)

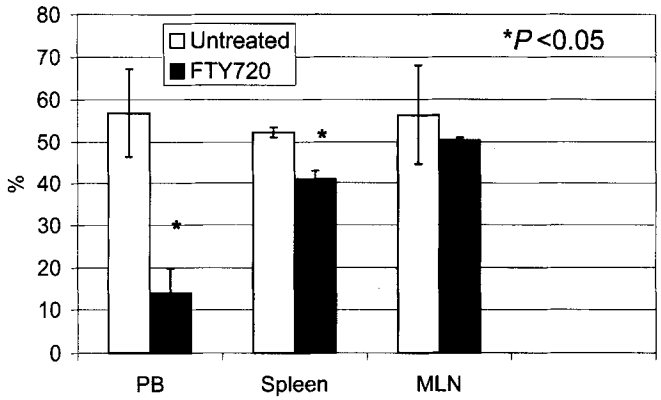

Fig. 2 Lymphocyte ratio in the peripheral blood (*PB*), spleen, and mesenteric lymph nodes (*MLN*) 1 day after FTY 720 administration. Significant lymphopenia is observed in the peripheral blood and the spleen ($P < 0.01$). More profound lymphopenia is observed in the peripheral blood than in the spleen. In the mesenteric lymph node, the lymphocyte ratio is unchanged

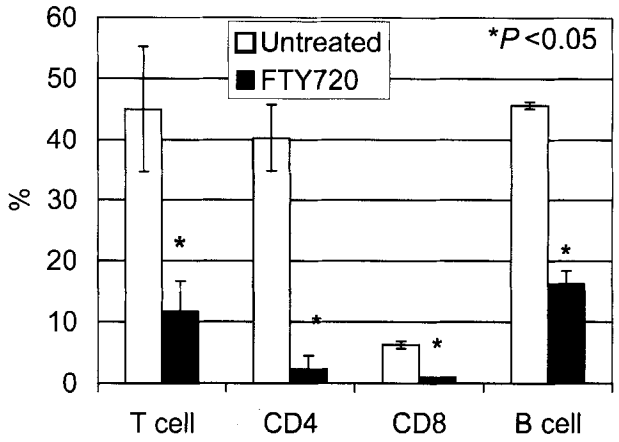

Fig. 3 Lymphocyte subpopulations in the peripheral blood (*PB*) 1 day after FTY 720 administration. FTY 720 significantly reduced not only T cells ($CD3^+$, $CD4^+$, $CD8^+$) but also B cells ($P < 0.01$)

CD8 positive cells), but also B lymphocytes. This phenomenon was particularly pronounced in the peripheral blood (Fig. 3). To determine whether the lymphopenia induced by FTY 720 is reversible, we examined the lymphocyte ratio in the peripheral blood 13 days after FTY 720 administration. By that time, lymphopenia in the peripheral blood had completely recovered and was not different from control values; $P = 0.21$.

Differential effect of FTY 720 on DSBT-induced tolerance after Itx and Htx

Itx

Tables 2 and 4 show the differential effect of FTY 720 on DSBT-induced tolerance after Itx.

Pre-transplant FTY 720 alone did not influence graft survival: recipients survived for 18.6 ± 2 days vs 18 ± 4 days in allogeneic controls [not significant (NS)]. With pre-transplant DSBT alone, all recipients survived for more than 2 months. As mentioned above, 43% of the rats (three of seven) survived indefinitely, whereas 57% of the rats (four of seven) succumbed between post-transplant days 78 and 98 after a typical clinical course of chronic rejection. Pathological findings confirmed that chronic rejection was the cause of death. Histological characteristics of chronic rejection included vasculopathy of the mesenteric artery, lymphoid depletion of the mesenteric lymph node and Peyer's patches and mucosal villous blunting. Histological features of acute rejection, such as lamina propria infiltration by mononuclear cells and endotheliitis, were absent. Finally, histological signs of GVHD were absent on histological examination of the skin.

In contrast, adjunction of FTY 720 prior to DSBT caused acute rejection in 62.5% of the rats (five of eight). Those rats succumbed within 40 days after transplantation. Acute rejection as cause of death was confirmed by histological examination, demonstrating the presence of massive mucosal necrosis with severe mononuclear cell infiltration and endotheliitis. Only 12.5% of rats (one of eight) succumbed to chronic rejection; 25% of rats (two of eight) survived indefinitely. The latter two animals accepted donor-specific heart grafts but rejected third-party grafts, similar to DSBT controls, the Itx rats (Table 4).

Htx

Tables 3 and 4 show the differential effect of FTY 720 on DSBT-induced tolerance after Htx.

Pre-transplant FTY 720 alone did not modify graft survival: recipients survived for 9.3 ± 1 days vs 9 ± 0.6 days in allogeneic controls (NS). With pre-transplant DSBT alone, all grafts survived indefinitely (> 120 days). In contrast to Itx, adjunction of FTY 720 prior to DSBT did not modify survival of primary grafts. In addition, and as in Itx, tolerized DSBT-treated recipients (with or without adjunction of FTY 720 prior to DSBT) accepted secondary donor-specific heart grafts but rejected third-party heart grafts (Table 4).

Discussion

Most experimental and clinical studies examining new immunosuppressive drugs usually focus on the anti-rejection properties of these compounds. Tolerance, like rejection, involves an active and specific response of alloreactive immune recipient cells towards donor

antigens. It is, therefore, possible that current immunosuppressive regimens—although highly effective in blocking rejection—negatively affect certain immune pathways that are important for tolerance induction [15]. We and others have previously reported that high-dose steroids and calcineurin inhibitors may block tolerance in certain experimental conditions [12, 15]. This may have important clinical implications, because induction of tolerance is no longer the 'holy grail', and trials of tolerance induction are on the verge of entering clinics. In addition, it is also becoming clear that allografts themselves induce a tolerogenic response that contributes actively to maintaining a state of tolerance [10]. It is thus crucial that future immunomodulatory/immunosuppressive protocols include drugs that do not block, but promote, tolerance.

FTY 720 is a new immunosuppressive agent that has recently received much attention because of its unique mechanism of action. Indeed, FTY 720 does not act as a classical immunosuppressor in that it does not alter T-cell function. Remarkably, T-cell activation upon encounter with donor antigen, cytokine production and proliferation capacity which are pivotal in rejection (but that can equally play a role in certain tolerogenic responses) is not modified by FTY 720 [4]. In concordance with other studies, we show in this study that FTY 720 simply acts by causing a profound lymphopenia that is reversible, since the lymphocyte count fully recovers within 2 weeks of treatment. Lymphopenia induced by FTY 720 has been shown to result from altered lymphocyte trafficking and temporary lymphocyte migration into the secondary lymphoid compartments [5, 28]. This is accompanied by reduced graft infiltration, and we and others have reported a marked anti-rejection effect of FTY 720 when it is used as an induction or as a rescue form of treatment [30, 31]. Absence of effect of FTY 720 on lymphocyte function led us to hypothesize that this new compound will not interfere with a series of lymphocyte-dependent mechanisms that may be instrumental in the development of tolerance. To our knowledge, no study that analyzes the potential impact of FTY 720 in models of tolerance is currently available.

We developed a model of tolerance induction in a completely immunosuppression-free environment [11, 13]. In the RA-to-PVG fully mismatched combination in rats, a single administration of DSBT is sufficient to induce long-term graft acceptance with no need for pharmacological immunosuppression. Long-term survivors are clinically tolerant, as assessed by acceptance of secondary donor-specific, but not third-party, heart grafts. In that model, 100% of heart grafts but only 43% of intestinal grafts were accepted, a difference that points to the higher immunogenicity of the bowel and the greater difficulty to promote engraftment of the intestine vs the heart. This model of graft acceptance in an immunosuppression-free environment is ideal for us to examine separately the effect of various immunosuppressive drugs on tolerance. For example, we showed earlier in that model that exposure to high doses of steroids prior to DSBT or at the time of transplantation blocks tolerance and triggers acute rejection of primary and secondary grafts [17]. We now use this model to study how FTY 720 influences a tolerogenic response in vivo.

We demonstrated that adjunction of FTY 720 counteracts the beneficial effect of pre-transplant DSBT and causes acute rejection in primary intestinal grafts. One explanation for the negative effect of FTY 720 on DSBT-induced acceptance of primary intestinal grafts is the following: FTY 720, by altering lymphocyte trafficking and temporarily sequestering lymphocytes into various secondary lymphoid compartments, may prevent certain donor/recipient lymphocyte interactions that are obligatory for tolerance to develop. This hypothesis, however, is unlikely, because lymphocyte localization to the peripheral lymph nodes was recently shown to be a prerequisite for tolerance induction [9, 26]. FTY 720, by promoting lymphocyte migration to the secondary lymphoid tissues, would promote, rather than inhibit, tolerance induction [9].

Alternatively, FTY 720 at the dose used in our study may have caused lymphocyte apoptosis and thus physical elimination of donor and/or recipient cells that are necessary for tolerance induction. That high doses of FTY 720—similar to the one used in our study (5 mg/kg per day)—cause apoptosis has been demonstrated in vitro and in vivo by others [20, 28]. In vitro exposure to high dose FTY 720 concentration (4×10^6) induces chromatin condensation, typical DNA fragmentation and formation of apoptotic bodies [28]. Lymphocyte apoptosis following administration of FTY 720 has been documented in vivo as well [20]. In the present study, we found a reduced lymphocyte population in the spleen, but no significant change and even a slight decrease in the lymphocyte population of the mesenteric lymph nodes, following exposure to FTY 720. This is in contrast with others, who found an increase in the lymphocyte population in the mesenteric lymph nodes following exposure to lower doses of FTY 720 [5], and represents indirect evidence that FTY 720, at the higher dose used in our study, may have caused some degree of lymphocyte deletion in the mesenteric lymph nodes. Finally, although low-dose FTY 720 does not alter lymphocyte function, it is not clear whether higher doses of FTY 720 do so, too.

In our study, the 'break of tolerance' by FTY 720 was partial and limited only to primary intestinal grafts. FTY 720 did not alter tolerance to secondary heart grafts in tolerant Itx recipients. In addition, FTY 720 did allow full development of tolerance to primary and secondary grafts after Htx. This stands in stark contrast

with earlier observations made by our group and others, when testing various immunosuppressive drugs, in particular steroids and calcineurin inhibitors [2, 7, 16, 17, 22]. Steroids were shown to trigger rejection of primary liver graft in a mouse model, where the liver is otherwise spontaneously accepted. It was hypothesized that steroids block a protective mechanism of activation-induced cell death (AICD) [2]. Remarkably, in a model identical to the one used in the present study, we showed that exposure to steroids prior to DSBT or at the time of Tx triggers acute rejection not only in primary grafts, but occasionally also in secondary grafts [17]. Break of tolerance to secondary grafts by steroids, but not by FTY 720, suggests that mechanisms involved in acceptance of secondary grafts, albeit affected by steroids, are resistant to FTY 720. We are now looking at the mechanisms of tolerance induction in our model, and in particular we are examining whether a phenomenon of AICD is in action.

Preliminary data indicate an early state of immune activation and, in particular, the presence of a predominantly Th1-type cytokine profile early post-transplantation in tolerant animals, an observation that is consistent with the theory of AICD (unshown data). Because FTY 720 does not act on T-cell function, activation, and proliferation, it would therefore not block tolerance in models in which AICD operates. Remarkably, the paradoxical break of tolerance by immunosuppression is not limited to steroids. Calcineurin inhibitors have been shown to block tolerance in certain models, an observation that could also be explained by the inhibitory action of calcineurin inhibitors on AICD, a phenomenon dependent upon interleukin-2 production [7, 16].

The deleterious effect of FTY 720 on acceptance of primary grafts was organ-specific. Indeed, FTY 720 failed to trigger acute rejection in primary heart grafts. One possibility is that tolerance obtained after Htx is more robust and thus more difficult to overcome than after Itx. Indeed, all Htx rats were easily tolerized, whereas only 43% of the DSBT-treated Itx animals became tolerant. Remarkably, and in contrast with tolerant Htx rats that displayed stable hypo-responsiveness, tolerant Itx rats had enhanced MLC responses, which may again point to a less profound and, therefore, more vulnerable state of tolerance after Itx than after Htx. Finally, tolerant Itx rats continued to produce large numbers of IgG and IgM antibodies against donor antigen, compared with tolerant Htx recipients. Complete suppression of immunoglobulin class switching from IgM to IgG has been found by Wasowska et al. to be a reliable surrogate of profound tolerance after DSBT. In contrast, persistence of a high anti-donor IgG production, as we saw in our Itx recipients, is a sign of an incomplete and more fragile form of tolerance [29].

A second explanation for the difference between Htx and Itx is the unique capacity of the intestine to induce GVHD and the fact that graft survival after Itx eventually depends upon an equilibrium between GVHD and rejection, a balance that may have been influenced by FTY 720 [23]. Although GVHD was found neither clinically nor histologically in our fully allogenic model, we cannot exclude that FTY 720 may have abolished a subclinical GVH response, thereby modifying that balance in favor of rejection [18, 23].

Finally, FTY 720 causes migration of lymphocytes to mesenteric lymph nodes and to Peyer's patches. Therefore, we cannot rule out the possibility that FTY 720 may have accelerated rejection by causing migration of recipient lymphocytes towards the Peyer's patches of the intestinal graft, thereby sensitizing rather than tolerizing the animals. For that reason, it has even been suggested that FTY 720 may not be an appropriate immunosuppressor in Itx (Volker Brinkman, Novartis, Basel, personal communication).

A single DSBT induced tolerance in non-immunosuppressed rats, an observation that has not been made in humans. Therefore, the question arises as to the clinical relevance of our findings and, in particular, whether the break of tolerance by immunosuppression observed here in a particular animal model also applies clinically. There is an increasing number of studies on steroid-free immunosuppression, particularly after liver transplantation. In those studies, the incidence of rejection is extraordinarily low, suggesting that active mechanisms of tolerance induction can be blocked by non-specific immunosuppression [14]. Preliminary studies indicate that regulatory/suppressor cells are involved in tolerance induction in our DSBT model (unshown data). Remarkably, these cells also seem to operate clinically in maintaining hypo-responsiveness in patients with stable allograft function [25].

In summary, we have studied the effect of FTY 720 in a DSBT tolerance model, where heart and intestinal allografts are accepted with no need for pharmalogical immunosuppression. In this model, we demonstrate that FTY 720 accelerates rejection of primary intestinal grafts. However, the survival of secondary grafts is not influenced by FTY 720, and this stands in high contrast to our previous observations that exposure to steroids (in an identical model) causes severe acute rejection, not only in primary, but also in secondary, donor-specific grafts. In addition, FTY 720 did not affect the tolerogenic potential of DSBT after Htx, either in primary or in secondary heart grafts. FTY 720 may emerge as an interesting compound to include in protocols of tolerance induction after heart, but not intestine, transplantation. Organ-specific mechanisms accounting for the deleterious effect of FTY 720 on acceptance of primary intestinal, but not heart, allografts, exact further investigation.

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