INCREASED SEMI-ALLOGENEIC SKIN GRAFT SURVIVAL AFTER FTY720 TREATMENT

AUMENTO DA SOBREVIDA DO ENXERTO DE PELE SEMI-ALOGÊNICO APÓS TRATAMENTO COM FTY720

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ABSTRACT

FTY720 is a new compound which increases allograft survival in animal models through not fully elucidated mechanisms. **Purpose:** We investigated the effects of the FTY720 administration in semi-allogeneic skin graft survival in an animal model and its associated mechanisms. **Methods:** Both transplanted, Non-treated (Tx) and Treated (Tx+FTY720) groups were daily observed to determine the semi-allogeneic skin graft survival. In another set of experiments both groups were assessed in different time points for lymphocyte numbers and activation markers in blood and spleen. In a third experiment, mice recipients that accepted skin semi-allogeneic graft had spleen, blood, or axillary lymph node cells harvested and adoptively transferred to naïve C57BL/6 mice followed after 24 hours by a F1 skin semi-allogeneic graft transplantation. **Results:** Skin semi-allogeneic graft survival increased significantly in Treated Group (21.3±10.1 days) when compared to the Non-treated Group (12.9±0.4 days) ($p=0.002$). Five days after transplantation, Treated Group presented significantly lower amount of lymphocyte in spleen and blood (44.7±11.6x10^6 and 38.2±14.8%) than Non-treated mice (77.5±19.5x10^6 and 70.0±12.3%), respectively ($p<0.005$). MHC II expression in splenocytes was 26.3±5.6% in Treated, and 28.0±11.0% in Non-treated Group ($p=0.3$) whereas such marker presented a significant lower expression in blood: 5.6±4.1% versus 28.9±8.0% ($p<0.005$), respectively. Graft infiltrating cells were significantly higher in Non-treated than in Treated mice ($p<0.005$). Adoptive transfer caused no improvement in skin semi-allogeneic graft survival. **Conclusion:** The FTY720 treatment successfully improved mice skin semi-allogeneic graft survival, impairing antigen presentation and reducing graft cell infiltration. Functional tolerance after FTY720 administration was not observed.

Keywords: Immunosuppression, Leukocytes, Transplantation Immunology, Flow Cytometry

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INTRODUCTION

The major goal in transplantation is to develop clinically applicable protocols to induce alloantigen-specific tolerance which could be associated to the immunosuppressive drugs.

FTY720 is a synthetic compound analog to Isaria sinclaiiri extract successfully used in experimental autoimmune diseases, tumor growth and metastasis, transplantation and ischemia-reperfusion injury.[1-6] Moreover, FTY720 long-term administration (21 days) in mice has not been associated to toxic effects.[7, 8]

FTY720 shares the same receptor of sphingosine-1 phosphate (S1P) that regulates several biological functions of many cell types from proliferation and survival to migration and secretion.[9-11]

The S1P receptors $S1P_1$ and $S1P_4$ are expressed in T cells, B cells, and thymocytes at late developmental stages. S1P binding to $S1P_1$ causes the regulation of lymphocyte trafficking and distribution in lymphoid tissues.[12] The FTY720 binding to S1P receptors causes changing in the lymphocyte trafficking, redirecting these cells to the lymph nodes (LN) and Peyer's patches (PP), preventing their migration to inflammatory sites.[13]

In animal models of heart and skin transplantation, the FTY720 administration increased the allograft survival which was associated to decreased graft cell infiltration.[14, 15]

Further to the potential to avoid acute rejection by affecting the migration of leukocytes to the allograft (inflammatory site), FTY720 has another features that makes itself a strong candidate to prevent immune-mediated situations. In animal models, therapeutic doses of FTY720 do not impair the T cell activation, proliferation and immunological memory, suggesting that the drug may protect organ grafts without inducing generalized immunosuppression. Furthermore, FTY720 at higher than 5 mg doses has been shown to be well tolerated in renal transplant recipients, and does not appear to be associated to nephrotoxicity and side effects on the central nervous system and blood lipids induced by classical immunosuppressants. FTY720 was generally well tolerated along the 12-month study. Initial experience with FTY720 in phase I and previous phase II studies have shown the agent to be efficacious and well tolerated in renal transplant patients.[16] Another study found that FTY720 associated to FK506 in a murine skin allograft model caused fewer changes in kidney structure, and blood glucose levels were lower than in the FK506 monotherapy.[17] These evidences suggest a potential successful application of long-term FTY720 therapy in organ transplants survival.

It has been suggested that S1P and FTY20 can also act on T-regulatory cell. The profile of the S1P-receptor expression is similar for $CD4^+$ and $CD8^+$ T-cell subsets and for $CD4^+CD25^+$ regulatory T cells.[18, 19] *In vitro* concentrations of S1P between 10 and 100 nM protected T cells from apoptosis by inhibiting caspases and supported the effective function of both cytotoxic T lymphocytes and $CD4^+CD25^+$ regulatory T cells by the increased generation of IL-10 and expression of cytotoxic T-lymphocyte antigen 4.[20] Moreover, it has been shown that mice treated along 3 days (0.1-1.0mg/kg/day) with FTY720 presented an increasing population of $CD4^+CD25^+$ T cells both in blood and spleen.[21]

Considering our previous findings showing that FTY720 administration as a monotherapy or associated to CsA for 21 days improved the skin allograft survival in a fully mismatched mice strain combination [22], and that FTY720 is associated to the redirection of lymphocytes from blood to secondary lymphoid organs, thereby preventing their migration to the inflammatory site, it was our purpose to investigate whether FTY720 could cause any additional increase in skin graft survival when using semi-allogeneic mice strain combination. Also, we assessed the possible associated mechanisms and the development of functional tolerance.

METHODS

Animals

Eight to 10-week-old male F1 (C57BL/6 x BALB/c, donor) and C57BL/6 (H-2^b, recipient) mice were used (bred in a local colony) for the skin transplantation model. They received a standard mice diet and water *ad libitum*. Animals were cared for in accordance to the Principles of the Laboratory Animal Care (NIH publication No 86-23, reviewed in 1985) and regulations of the Brazilian Committee on Animal Experimentation (COBEA).

FTY720 (Novartis, Basel, Switzerland, 1mg/kg/day diluted in sterile distilled water) was administered daily by gavage starting 3 days before the skin semi-allogeneic graft transplantation and along a 21 day period (skin survival group).

Skin grafting

Donors and recipients were anesthetized with an intraperitoneal injection of Xylazine (Agribrands, Brazil) and Ketamine (Vetbrands, Brazil) diluted in 10 mL of sterile PBS (phosphate buffered saline solution-OXOID LTD Hampshire England). The skin tail of the F1 donor mice was removed, placed in cold saline, segmented in 1x1 cm^2 pieces and used to replace the previously removed back skin from the C57BL/6 recipient mice. The skin graft was fixed with 4-0 sutures at each corner, and animals were kept in individual cages for daily observation. In a set of experiments, all mice were daily observed in order to establish the rejection time (<90% of skin necrosis).

Time point evaluation

In another set of experiments, mice were used 5 days after transplantation (Treated and Non-treated Groups) for immunology assessment, taking into account that the immune response peak is believed to occur 5 days after transplantation in allograft experimental models.[23] Also, in other two groups (FTY720 Controls 1 and 2) C57BL/6 mice were not submitted to skin semi-allogeneic graft transplantation, but they received daily FTY720 along 9 or 21 days, followed by assessment in order to investigate the changing caused by the drug in the absence of semi-allogeneic stimuli. Still, there was a Control Group, with non-transplanted and non-treated mice assessed at the same time of the Non-treated and Treated Groups (5 days after skin semi-allogeneic graft transplantation.)

In the previously determined periods after the skin transplantation, mice were anesthetized as described before and placed on a temperature controlled surgical table (Braile Biomédica, Brazil) to perform the blood collection, spleen and ALN harvest. Blood samples were used for a smear test, whereas spleen and ALN cell numbers were determined. Blood and spleen cells were also phenotyped using flow cytometry.

Adoptive transfer

We assessed if the FTY720 administration was associated to the development of a functional regulatory cell population. To address such hypothesis in a separate experiment, we harvested cells from blood, spleen or axillary lymph nodes (ALN) of C57BL/6 recipient mice treated along a 14 day period with FTY720, transplanted with F1 donor mice skin and which did not rejected the graft up to the 10^{th} post transplant day. Harvested cells ($5x10^6$ splenocytes, 0.5 mL of blood, or $1x10^6$ cells from ALN) were adoptively transferred to the naïve C57BL/6 mice through the penile vein, and 24 hours later, F1 skin semi-allogeneic graft transplant was performed in C57BL/6 animals.

Blood smear test

A drop of blood (10μL) collected from the vena cava was deposited on a histological blade, and using the extender blade, the material was pressed in a 45º at constant speed dragging the drop of blood up to the end of the blade. Next, the material was covered by a glass slide. For the cellular staining the Panotico kit was used (Laborclin, Parana, Brazil) and the leucocytes were identified and counted using a microscope.

Lymphocyte counting

Upon the examination of the amount of cells in the spleen and ALN, single cell suspensions were prepared by pressing these tissues through a 400 µm sterile nylon mesh. Single cell suspension from spleen was submitted to 1 minute washing of distilled water aiming to cause hemolysis. Cells from spleen and ALN were counted into a Neubauer chamber with the aid of a microscope.

Flow cytometry

$1x10^6$ cells single cell suspension from spleen and blood were incubated with rat anti-mouse (BD Biosciences Pharmingen) CD54 PE (ICAM-1) or I-A^b PE (MHC class II) for surface marker staining. Cells from spleen were submitted to FACS buffer washing (PBS/2% FCS), whereas blood cells were submitted to FACS lysine solution and washed with FACS buffer. Both spleen and blood cells were evaluated in FACScalibur Flow Cytometer (BD Biosciences) using Cell Sorter software. Cell populations were classified as to the size (forward scatter) and complexity (side scatter) with a gate set in the lymphocyte population. At least 10000 cells were assessed.

Histology

Skin sections were stained with Hematoxylin and Eosin (H&E). Possible structure changes and cell infiltration were evaluated by a pathologist blinded to treatment arm. The infiltration score was defined as 0 (absence), 1 (mild), 2 (moderate), and 3 (intense).

Statistical analysis

Data are shown as mean ± standard deviation. The Kaplan-Meier analysis was used to assess the skin semi-allogeneic graft survival. The analysis of variance (ANOVA) followed by the Tukey post-test were performed to assess other parameters, except for cell infiltration in transplanted skin, where the Mood Median Test was used. The level of statistical significance was defined as p-value <0.05.

RESULTS

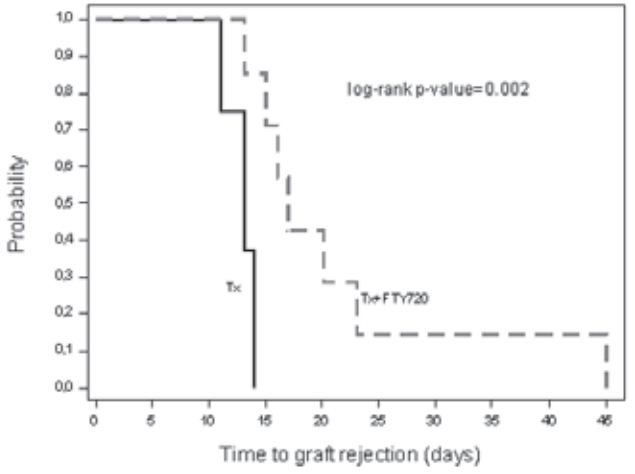
Increased skin semi-allogeneic survival

Figure 1 shows that FTY720 administered along 21 days in the Tx+FTY720 Group was associated to a significant increase (p=0.002) in skin semi-allogeneic graft survival when compared to the Tx Group. One of the Tx+FTY720 recipients rejected the transplanted skin 45 days after the transplant. Also, the FTY720 therapy delayed the first rejection signs, as shown in the macroscopic view of the transplanted skin in Figure 2.

Assessment of the amount of cells in the Axillary Lymph Nodes (ALN), Spleen and Blood of C57BL/6 recipients mice

It is commonly accepted that FTY720 promotes an increase in the allograft survival mainly by changing the migration and homing of lymphocytes via sphingosine 1-phosphate receptors and causing lymphopenia. The investigation of the amount of cells in different sites showed that there was a decrease of ALN in lymphocytes in all groups when compared to the Control Group. There was no difference in the amount of lymphocytes of ALN when comparing the Tx and Tx+FTY720 Groups. However, FTY720 administered for 9 or 21 days in the absence of skin transplantation caused a significant decrease in the amount of lymphocytes of ALN compared to the transplanted groups (p=0.007). The skin transplantation increased the amount of lymphocytes in the spleen, and the administration of FTY720 in the presence or absence of semi-allogeneic graft caused a significant decrease in that amount (p<0.005). The treated Group presented a significant decrease of lymphocytes in the blood compared to the Non-treated Group (p<0.005). However, the lowest percentage of lymphocytes was noted after 21 days in treated and non-transplanted mice (FTY720 Control Group 2). On the other hand, there was a significant increase of neutrophils in the blood of mice treated with FTY720 (p<0.005). These data are summarized on Table 1.

Figure 1. *F1 (C57BL/6 x BALB/c) skin allograft survival in C57BL/6 recipient in absence of treatment (Tx) and treated with FTY720 (Tx+FTY720).*



	Groups	
	Tx	**Tx+FTY720**
MST±SD	12.9±0.4 (n=8)	21.3±10.1 (n=7)

Figure 2. *Allograft rejection process in different time points (days) after skin allograft transplantation in one representative mice of each group. [A-C] Tx and [D-F] Tx+FTY720 groups showing the percentage of skin necrosis.*

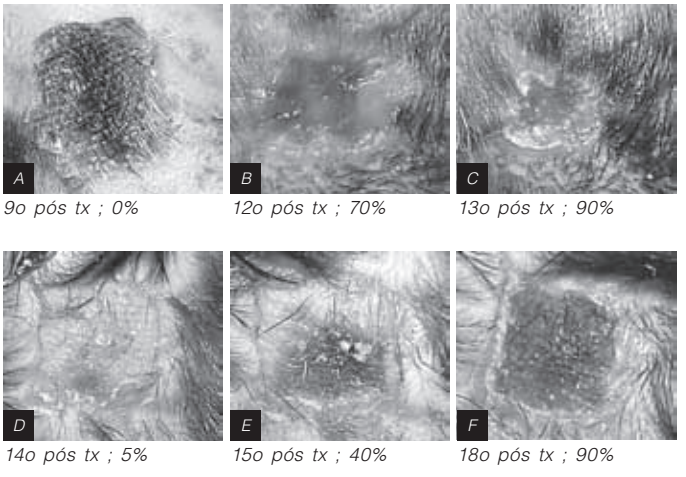


Table 1. *Leukocyte numbers observed in different compartments (ALN, spleen and blood) of C57BL/6 recipient mice and at different time points.*

Groups	ALN	Spleen	Blood	
	Lymphocyte (x106)	Lymphocyte (x106)	Lymphocyte (%)	Neutrophil (%)
Control	4.2±1.0[a]	28.5±3.2[a]	62.2±11.1[bc]	34.2±10.2[a]
Tx	2.5±0.9[c]	77.5±19.5[b]	70.0±12.3[c]	27.3±12.0[a]
Tx+FTY720	2.4±0.5[c]	44.7±11.6[a]	38.2±14.8[ab]	55.8±14.4[b]
FTY720 (1)	1.1±0.3[b]	48.6±3.6[a]	49.6±4.6[b]	56.2±11.3[b]
FTY720 (2)	0.8±0.1[b]	44.0±14.7[a]	25.2±6.6[a]	68.8±7.7[b]
p-value	[axbxc] p=0.007	[axb] p<0.005	[axbxc]p<0.005	[axb]p<0.005

Control group – *non-transplanted and non-treated mice evaluated 5 days after skin semiallogeneic graft translplantation.*
Non-treated group (Tx) – *transplanted and non-treated mice evaluated 5 days after skin semi-allogeneic graft transplantation.*
Treated group (Tx+FTY720) – *transplanted mice and treated during 9 days with FTY720 and evaluated 5 days after skin semi-allogeneic graft transplantation.*
FTY720 Control group (1) – *non-transplanted mice treated during 9 days with FTY720 followed by evaluation.*
FTY720 Control group (2) – *non-transplanted mice treated during 21 days with FTY720 followed by evaluation.*
Lymphocytes in ALN and spleen are expressed in x106 numbers. Lymphocyte and neutrophil numbers in blood are expressed in percentage.

Table 2. *MHC class II and ICAM-1 cell expression in spleen and blood, in C57BL/6 recipient mice at different time points.*

Groups	Spleen		Blood	
	MHC II (%)	ICAM-1 (%)	MHC II (%)	ICAM-1 (%)
Control	21.6±6.8	58.6±11.4	31.2±8.7[a]	86.4±3.9[a]
Tx	28.0±11.0	52.5±9.6	28.9±8.0[a]	56.9±22.2[ab]
Tx FTY720	26.3±5.6	54.1±5.7	5.6±4.1[c]	50.6±28.7[b]
FTY720 (1)	33.3±8.6	51.3±14.3	18.3±4.2[b]	45.8±10.6[b]
FTY720 (2)	30.2±9.5	46.4±12.8	16.9±3.0[b]	33.1±4.7[b]
p-value	p=0.3	p=0.52	[axbxc] p<0.005	[axb] p<0.005

Control group – *non-transplanted and non-treated mice evaluated 5 days after skin semiallogeneic graft translplantation.*
Non-treated group (Tx) – *transplanted and non-treated mice evaluated 5 days after skin semi-allogeneic graft transplantation.*
Treated group (Tx+FTY720) – *transplanted mice and treated during 9 days with FTY720 and evaluated 5 days after skin semi-allogeneic graft transplantation.*
FTY720 control group (1) – *non-transplanted mice and treated during 9 days with FTY720 followed by evaluation.*
FTY720 control group (2) – *non-transplanted mice and treated during 21 days with FTY720 followed by evaluation.*
MHC class II and ICAM-1 numbers are expressed in percentage.

Assessment of MHC class II and ICAM-1 cell expression in the Spleen and Blood of C57BL/6 recipient mice

Skin semi-allogeneic transplantation both in Treated and Non-treated Groups was associated to an increase in the MHC II molecules of splenocytes when compared to the Control Group (with no statistical significance). Upon the comparison of the Treated and Non-treated Groups to each other, the FTY720 treatment caused a slight but yet non-significant decrease in the expression of such molecule. Interestingly, FTY720 administered in non-transplanted animals caused an increase in the expression of MHC II in splenocytes (p=0.3). In the blood, FTY720 caused a significant decrease in the MHC II expression mainly among the Treated Group when comparing the Control and Non-treated Groups (p<0.005). The ICAM-1 expression had a significant decrease in the three groups treated with FTY720 (Tx+FTY720 and FTY720 Control Groups 1 e 2), in the blood compared to the Control and Non-treated Groups (p<0.005) (Table 2).

Reduced skin semi-allogeneic graft cell infiltration

Figures 3 and 4 show that leukocyte infiltration was significantly higher in Non-treated Group compared to the Treated Group (p<0.005).

Evaluation of Adoptive Transfer

Taking into account the results of the skin semi-allogeneic graft survival, amount of lymphocytes, cell phenotype, and graft infiltrating cells from FTY720 treated mice, we assessed whether the FTY720 could induce the development of regulatory or suppressor cells. C57BL/6 recipients with no signs of rejection of the F1 donor skin up to the 10th day had their blood (0.5mL), spleen (5x10^6 cells) or ALN (1x10^6) cells adoptively transferred (AT Group) to C57BL/6 naïve mice, who received 24 hours later the transplant of the F1 donor skin. Skin rejection in C57BL/6 adoptively transferred (blood, spleen, or ALN) mice was not statistically

Figure 3. *Leukocyte infiltration score (LIS) observed five days after F1 (C57BL/6 x BALB/c) skin allograft transplantation to C57BL/6 mice. Tx versus Tx+FTY720 p<0.005*

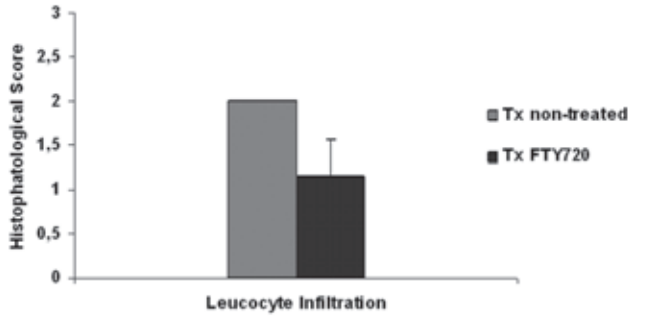
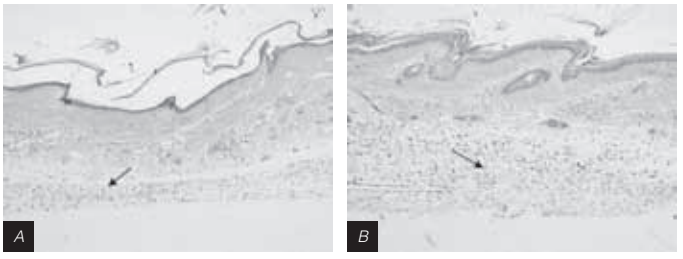


Figure 4. *Histology of skin allograft transplantation, H&E stain. A. One representative mice from Tx+FTY720 Group; B. One representative mice from Tx Group, both evaluated 5 days after transplantation. Arrow: leukocyte infiltration. Leukocyte infiltration score (LIS): 0 – absence; 1 – mild; 2 – moderate; 3 – intense.*



different from transplanted and non-adoptively transferred animals (Tx+FTY720 and Tx Groups), but there was a slight increase in the semi-allogeneic graft survival of mice receiving ALN adoptively transferred cells (p=0.12) (Figure 5). It has to be emphasized that the adoptively transferred cells were not associated to the accelerated rejection of the skin semi-allogeneic graft.

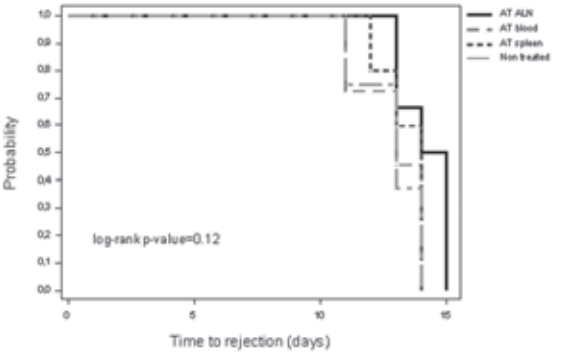
DISCUSSION

Allotransplantation stimulates cells from the immune system in promoting the proliferation, in changing the expression of the markers to the membrane activation, in inducing the secretion of growth factors, and migration to the graft site.[24,25] In agreement with these findings, it was observed in our model the increase in the amount of lymphocyte both in spleen and blood 5 days after the skin semi-allogeneic graft transplantation. It was also found a higher MHC II molecule expression in splenocytes and the skin histology showed infiltration of leukocytes. The mean skin survival time was 12.9 days.

FTY720 is a new synthetic compound that interacting with S1P (sphingosine 1-phosphate) is immunosuppressive in several T-cell-dependent assays.[26] Although the effects of the FTY720 in the S1P regulation of the lymphocyte traffic that accounts for its highly selective and relatively safe immunosuppressive profile, this is not yet fully elucidated.

We have shown previously that FTY720 increases mice skin allograft survival in a fully mismatched strain combination (BALB/c – C57BL/6).[22] In the present study, the FTY720 potential in preventing

Figure 5. *Skin allograft survival in C57BL/6 recipient mice non-treated or adoptively transferred (AT) with spleen cells (5x106), blood (0.5ml), or axillary lymph node cells (1x106).*



MST±SD (days)	Groups				
	Tx non-treated	AT (blood)	AT (spleen)	AT(ALN)	p-value
	12.9±0.4 (n=8)	13.1±1.2 (n=8)	13.4±0.9 (n=5)	14.2±0.2 (n=6)	0.12

rejection was assessed using a semi-allogeneic strain combination (F1 – C57BL/6 x BALB/c) as donors and C57BL/6 mice (H-2^b) as recipients. FTY720 (1mg/kg/day) administered along 21 days promoted a significant increase in the skin semi-allogeneic graft survival (21.3±10.1 days on Treated Group versus 12.9±0.4 on Non-treated mice).

FTY720 administration was associated to a lower amount of lymphocyte both in spleen and blood, but not in ALN, when mice were assessed five days after the skin allograft transplantation (after a nine days administration of FTY720). Morris et al. showed that FTY720 daily administered to C57BL/6 mice caused a sustained decrease in the lymphocytes of blood and spleen, whereas the accumulation of these cells in ALN was transient.[27] Furthermore, Chiba et al. observed that in a murine model of skin transplantation with a single oral FTY720 administration, the amount of peripheral lymph nodes (PLN) lymphocytes reached its highest peak 12h after the FTY720 administration and thus returned to the controlling level around 24 hours.[13] In a specific tolerogenic treatment (αCD2 plus αCD3, DST plus αCD40L), it was observed that 10, 20, and 30 days after heart transplantation there is an increase in total mononuclear cells in the lymph nodes (LN) from tolerant mice, whereas a decrease of these cells was observed in rejecting mice in LN.[28] These findings are in agreement with ours, and suggest that FTY720 could be associated to the development of tolerance. Yet, in contrast to the altered distribution of lymphocytes in FTY720-treated rats, L-selectin-deficient mice were reported to show 70% to 90% reduction in the amount of PLN lymphocytes and 30% to 55% increase in spleen cellularity. Accordingly, if most of the circulating mature lymphocytes rapidly are sequestered in PLN, mesenteric LN, and PP the systemic immune responses would be markedly suppressed. Based on these aspects, the sequestration of circulating mature lymphocytes is presumably a major mechanism of the immunosuppressive activity of FTY720.[13] However, more experiments need to be conducted in order to better elucidate this aspect of the FTY720 mechanism of action.

Mice treated with FTY720 along 9 days but not submitted to the skin transplantation (FTY720 Control Group 1) also presented a decrease

in lymphocytes from the spleen and blood. Moreover, a very important decrease in lymphocytes was observed in the blood of non-transplanted mice treated with FTY720 for 21 days (FTY720 Control Group 2) confirming the drug's most common effect (lymphopenia). However, the absence of skin transplantation was associated to a significant decrease in the lymphocytes from ALN both at 9 and 21 days of the FTY720 treatment. This result suggests that the drug affected in a different way non-activated and activated cells from the graft draining site (ALN). In agreement, Hofmann et al.[29] showed that FTY720 has different effects in naïve, effector and memory cells.

The MHC II expression is associated to the antigen presentation[30], and in the absence of treatment it was observed an increase of this molecule in splenocytes but not in blood 5 days after the skin semi-allogeneic graft transplantation, compared to the Control Group. In transplanted mice, the treatment with FTY720 caused a slight decrease in the MHC II expression in splenocytes, whereas the decrease was very dramatic in the blood lymphocytes, suggesting that FTY720 also acts through the impairment of the alloantigen presentation. In non-transplanted mice, FTY720 caused an increase in the MHC II of splenocytes, whereas a significant decrease of such molecule was observed in blood.

The ICAM-1 expression has been associated to the cell activation and migration.[31] In our experiments, it was observed a similar pattern of the ICAM-1 expression in splenocytes upon the comparison of groups 5 days after transplantation. However, it was observed a significant decrease of ICAM-1 expression in blood 5 days after transplantation both in Non-treated and Treated Groups, suggesting a migration of lymphocytes from blood to other sites. A more dramatic decrease of ICAM-1 in blood was observed in mice non-submitted to the transplantation and treated with FTY720 for 21 days (FTY720 Control 2) in agreement with our findings of highly decreased amount of lymphocyte in blood of those mice. It has been already described that FTY720 causes lymphopenia by changing the lymphocyte migration to peripheral lymphoid organs.[32] As to the possible populations expressing this markers, Chiba et al observed that no clear changes were caused in the amount of the red blood cells, thymocytes (CD41, CD81 or CD41CD81 subpopulation) or bone marrow cells 12 h after administration of FTY720 in rats with skin allograft transplantation. So, the authors suggested that changes in the lymphocyte distribution induced by FTY720 appears to be specific in mature lymphocytes, but there is lack of selectivity for T cells, B cells, or T cell subsets.[13]

The landmark for acute rejection is the development of inflammatory process in the graft[33], which is mediated by the increased traffic of leukocytes associated to the immune cells proliferation.[34] In our model, it was observed a graft cell infiltration either in Non-treated and in FTY720 Treated mice. However, 5 days after transplantation, the leukocyte infiltration score in skin semi-allogeneic graft was significantly lower in mice treated with FTY720. Our results are in agreement with data from Yanagawa et al.[14] showing that FTY720 caused a significant decrease in the semi-allogeneic graft cell infiltration in a rat model. Wang et al.[19] also showed that FTY720 was associated to less mononuclear infiltrating cell in experimental cardiac allograft. Using monoclonal antibodies to induce tolerance, Ochando et al.[28] found that tolerant mice presented a decrease in the amount of mononuclear cells infiltrated in the graft when compared to rejecting animals. Moreover, in tolerant mice there were an increased amount of $CD4^+CD25^+$ cells in grafts.

The major goal in transplantation is to develop clinically applicable protocols to induce alloantigen-specific tolerance which could be associated to the immunosuppressive drugs. Sawicka et al. showed that FTY720 administration caused an increase in the $CD4^+CD25^+$ T cells in blood and spleen, and the adoptive transfer from these cells to the naïve mice caused their accumulation in blood and spleen, but not in the lymph nodes. Moreover, those cells inhibited the infiltration of eosinophils and lymphocytes when transferred into OVA-sensitized C57BL/6 mice 24 hours before the allergen second exposure.[19] In order to evaluate whether the FTY720 administration caused the development of functional tolerance, we adoptively transferred cells from non-rejecting C57BL/6 recipients (transplanted with F1 skin and treated for 14 days with FTY720) to naïve C57BL/6 mice. Twenty-four hours later, the F1 skin was transplanted in adoptively transferred C57BL/6 mice and the skin semi-allogeneic graft survival determination was followed. It was not observed a significant increase in the semi-allogeneic graft survival due to AT cells in blood, spleen or ALN. However, ALN transferred cells caused a slight increase in the skin semi-allogeneic graft survival. Ochando et al.[28] observed that CD4+CD25+ T cells in LN and graft of tolerant mice (treated with αCD2 plus αCD3, DST plus αCD40L) increased from day 10, attaining very high levels 100 days after transplantation. In our model, cells from non-rejecting mice were adoptively transferred to naïve mice 10 days after transplantation without causing increased graft survival. It can be discussed that at this point, the development of the regulatory T cells in our model was not enough to transfer operational tolerance.

CONCLUSION

FTY720 has different effects on naïve and effector cells, and in a model using semi-allogeneic mice strain combination this drug significantly increased the skin graft survival mainly through lymphopenia, impairment of antigen presentation and decrease in the graft infiltrating cells. It was not possible to show the development of functional tolerance in this model.

RESUMO

FTY720 é um novo composto que aumenta sobrevida de aloenxertos através de mecanismos ainda pouco elucidados. **Objetivo:** Investigar o efeito da administração de FTY720 na sobrevida do enxerto de pele semi-alogênico em modelo animal e seus mecanismos associados. **Métodos:** Ambos os grupos transplantados, Não-tratados (Tx) e Tratados (Tx+FTY720) foram avaliados diariamente para determinar a sobrevida do enxerto. Os grupos foram ainda avaliados em diferentes momentos quanto à contagem linfocitária e ativação de marcadores celulares no sangue e baço. Em experimento paralelo, camundongos que aceitaram o enxerto semi-alogênico tiveram as células do baço, sangue ou linfonodos axilares coletados e transferidos para camundongos C57BL/6 naïve, que receberam enxerto semi-alogênico de F1 24 horas pós-transferência celular. **Resultados:** A sobrevida do enxerto semi-alogênico aumentou significativamente no Grupo Tx+FTY720 (21.3±10.1 dias), quando comparado ao Tx (12.9±0.4 dias) (p=0.002). Cinco dias pós-transplante, o Grupo Tratado apresentou diminuição significativa do número de linfócitos no baço e sangue ($44.7±11.6x10^6$

e 38.2±14.8%) em relação aos camundongos Não-tratados (77.5±19.5x10^6 e 70.0±12.3%) ($p<0.005$). A expressão de MHC II nos esplenócitos foi de 26.3±5.6% em Tx+FTY720 e 28.0±11.0% em Tx ($p=0.3$), enquanto no sangue, esse marcador apresentou significativa diminuição da expressão: 5.6±4.1% contra 28.9±8.0% ($p<0.005$), respectivamente. A infiltração celular no enxerto semi-alogênico foi significativamente maior em Tx em relação ao Tx+FTY720 ($p<0.005$). A transferência celular adotiva não causou aumento da sobrevida do enxerto semi-alogênico. **Conclusão:** O tratamento com FTY720 aumentou a sobrevida do enxerto de pele semi-alogênico, impedindo a apresentação de antígeno e reduzindo infiltração celular no enxerto. Não foi observada tolerância funcional após administração de FTY720.

Descritores: Imunossupressão, Leucócitos, Imunologia de Transplantes, Citometria de Fluxo

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