IV. Limited impact of imatinib in a murine model of sclerodermatous chronic graft-versus-host disease

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Background
Sclerodermatous chronic Graft-versus-Host Disease (scl-cGvHD) is one of the most severe forms of cGvHD. PDGF and TGF-β play a significant role in the fibrosing process occurring in scl-cGvHD. This prompted us to assess the impact of the PDGF-r and c-Abl tyrosine kinase inhibitor imatinib on scl-cGvHD.

Methods
Balb/cJ mice were injected i.v. with 10.10^6 bone marrow cells and 70.10^6 spleen cells from B10.D2 donor mice after lethal irradiation at 7 Gy to induce scl-cGvHD. Mice were then given sterile water or imatinib by oral gavage at the dose of 150 mg/kg/day from day +7 after transplantation to the end of the experiment (day +52).

Results
Imatinib-treated mice had significantly lower levels of PDGF-r phosphorylation than control mice on day 29 after transplantation (P=0.033). However, the evolution of scl-cGvHD scores was similar between placebo and imatinib-treated mice.

Conclusions
Imatinib failed to ameliorate scl-cGvHD in a murine model of severe scl-cGvHD despite significant inhibition of PDGF-r. Future studies should assess the combination of imatinib with T-cell modulating agents.

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the main curative treatment for many hematological malignancies [359]. Its anti-tumor activity relies in large part on immune-mediated Graft-versus-Tumor effects (GvT effects) [360, 361]. However, donor immune cells contained in the graft can also attack healthy host tissues causing Graft-versus-Host Disease (GvHD)[362, 363]. GvHD can be divided into 2 syndromes, acute GvHD, historically defined as a GvHD reaction occurring within the first 100 days after allo-HSCT and chronic GvHD (cGvHD), that generally occurs beyond day +100 [364, 365]. While cGvHD has been associated with GvT effects [361, 366], it is also a major cause of morbidity/mortality in long-term transplant recipients [367].

Sclerodermatous cGvHD (scl-cGvHD) is one of the most severe forms of cGvHD and develops in approximately 20% of cGvHD patients [368]. Although scl-cGvHD shares common features with systemic fibrosis, the two syndromes differ both in terms of pathology (scl-cGvHD usually begins in the superficial layer of the skin and then extents to deeper layers of the skin while the opposite is generally true in systemic sclerosis), and in terms of clinical symptoms, with clinical features such as Raynaud's syndrome, pulmonary hypertension and cardiac dysfunction being frequently observed in patients with systemic sclerosis but infrequently in scl-cGvHD patients [369, 370].

The pathogenesis of cGvHD remains not fully understood. It is generally accepted that donor T cells are largely involved [362], since *in vitro* or *in vivo* T cell depletion of the graft are the most efficient methods for preventing cGvHD in humans [371, 372]. Further, data from the murine model of scl-cGvHD suggest that donor T cells involved in scl-cGvHD are primarilly $CD4^+$ T helper 2 (Th2) cells [373]. These Th2 cells secrete IL-4, IL-5, IL-10, IL-11 and IL-13 that stimulate other cells to release fibrosing factors such as IL-13, PDGF and TGF-β. These factors then induce fibrosis in the skin and other affected organs. Histocompatibility antigenic disparities between donor and recipient are also a risk factor for cGvHD (although to a lesser extent than for acute GvHD [374]), suggesting that cGvHD manisfestations are due to recognition of allogeneic antigens, such as major or minor histocompatibility antigens by donor T cells. Host thymus integrity could also play a role [152], although some studies failed to observe an association between thymic function and subsequent occurrence of cGvHD [156]. Finally, emerging data have also demonstrated an important role for Th1 and TH17 cells as well as for B cells in cGvHD pathogenesis [143, 200, 201, 375].

Imatinib (Glivec®; Novartis Pharmaceuticals) is a tyrosine kinase inhibitor developed as a competitive inhibitor of ATP for binding to BCR-ABL inducing

apoptosis of BCR-ABL dependent leukemic cells [376]. However, imatinib is not specific towards BCR-ABL and also targets other tyrosine kinases such as the stem cell factor c-kit, c-Abl (involved in transforming growth factor (TGF)-β signaling pathway), and platelet-derived growth factor receptor (PDGF-r) [376]. Given that the TGF-β and PDGF signaling pathways are largely involved in the fibrogenesis process in scl-cGvHD [373, 377], and given the ability of imatinib to inhibit T-cell proliferation *in vitro* [280], some clinical studies have assessed the impact of imatinib in patients with steroid-refractory cGvHD [302, 305, 306, 308, 378]. Unfortunately, these studies yielded conflicting results underlying the importance of re-assessing the impact of imatinib in scl-cGvHD in pre-clinical models. Here we investigated the impact of imatinib on scl-cGvHD in a classical scl-cGvHD murine model (B10.D2 (H-2^d) → BALB/cJ (H-2^d)) [373, 379].

Materials and methods

Mice and drugs

12 to 14 week-old B10.D2 (H-2^d, Jackson Laboratories, Bar Harbor, USA) and Balb/cJ (H-2^d, Jackson Laboratories) mice were used as donors and recipients, respectively, in a MHC-matched minor antigens disparate scl-cGvHD model. All mice were maintained in top-filtered cages in a standard animal facility and provided with sterilized food. Sterilized water supplemented with Baytril® 1% (Bayer HealthCare, Diegem, Belgium) was given from 3 days before transplantation to the end of the experiment (day +52). Water was changed every 2-3 days. All animal experiments were approved by the animal ethic committee of the University of Liege (local file number: 1438).

Imatinib (Glivec®) was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland). Imatinib was dissolved in sterile water and given by oral gavage at a dose of 150 mg/kg/day (50 mg/kg every morning + 100 mg/kg every evening).

Cell preparation for bone marrow transplantation

Spleen, femurs and tibias from B10.D2 mice were collected in sterile RPMI + FBS 10% + Penicilline/Streptomycine (P/S) 1%. After red blood cell lysis according to manufacturer's instructions (RBC lysis buffer; eBioscience, San Diego, USA), suspensions were passed through a 70 µm nylon filter and washed with PBS + FBS 3% + P/S 1%. Cells were then resuspended and mixed in pure PBS at a concentration of $70x10^6$ spleen cells and $10x10^6$ bone marrow cells/200 µL.

Bone marrow transplantation and scl-cGvHD

Balb/cJ recipient mice were transplanted as previously described by Jaffee and Claman [379]. Briefly, recipient Balb/cJ mice (H-2^d) were lethally irradiated with 7 Gy total body irradiation using a ^{137}Cs irradiator (GammaCell 40, Nordion, Ontario, Canada). Six hours later, recipients were injected with 200 µL of the

prepared cell suspension ($70x10^6$ spleen cells + $10x10^6$ bone marrow cells) from B10.D2 ($H\text{-}2^d$) mice. Recipient mice were monitored daily, while recipient body weights and GvHD scores (see next section) were recorded every 3 days. Mice were observed for a maximum of 52 days after transplantation. Imatinib treatment was started on day +7 post-transplant, was prepared in sterile water and was given orally at a dose of 150 mg/kg/day (50 mg/kg every morning + 100 mg/kg every evening, as previously reported [380]). Control mice received the same volume of sterile water. At the end of the experiment (day +52), mice were sacrificed. Spleen and bone marrow cells were collected in sterile RPMI + FBS 10% + P/S 1% while blood was collected in Microtainer® K2E tubes (BD Biosciences, San Diego, California, USA).

Assessment of scl-cGvHD

The severity of scl-cGvHD was assessed with a clinical scoring system as previously reported [45]. Briefly, ear-tagged animals were individually scored 3 times/week for five parameters: weight loss (1, loss between 10-20%; 2, loss > 20%), posture (1, kyphosis only at rest; 2, severe kyphosis when the animal moves), activity (1, moderate activity impairment; 2, no move unless stimulated), skin (1, erythema or scaling tail; 2, lesions on the body surface), and hair loss (1, loss > 1 cm^2; 2 loss > 2 cm^2). The clinical score was generated by summation of the five criteria scores (0 to 10/10). Animals reaching a score of 8/10 were sacrificed to avoid unnecessary pain according to our local ethic committee. Final scores for dead animals reaching the ethical limit score were carried forward for the remaining time points.

Skin biopsies and immunohistochemistry

On day +29 after transplantation, mice were anesthetized with an i.p. injection of a solution of xylasin/ketamin (10 mg/kg and 75 mg/kg, respectively). Skin samples of 0.5 cm^2 from the upper back were obtained and directly fixed in paraformaldehyde 10% before being paraffin-embedded 48 hours later and sectioned at 4-5 µm thickness. Paraffin-embedded samples were deparaffinized and blocked with a commercial protein block serum-free solution (Dako, Glostrup, Danemark). Skin sections were then incubated with one of the following antibodies at 4°C overnight: polyclonal rabbit anti-mouse phospho–c-Abl, polyclonal rabbit anti-mouse phospho-PDGFR (Abcam PLC, Cambridge, United Kingdom). Samples were finally quantified by using a scoring system for two criteria: the staining intensity and the area of staining. Each criterion received a score from 0 to 3 and multiplied with each other to obtain a global and unique score for each slide.

***In vivo* proliferation experiment**

Balb/cJ recipient mice were lethally (7 Gy Total body irradiation) irradiated and then administered with $10x10^6$ B10.D2 bone marrow cells on day 0. Fourteen

days later, spleens from B10.D2 donor mice were harvested and cells were isolated as previously described. Spleen cells were labeled with CFSE (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were then resuspended in PBS at a concentration of $70x10^6$ cells/200 µL. Recipient mice were injected i.v. with these cells and received imatinib or sterile water treatment for 5 days as described above. Mice were then sacrificed and bone marrow from femurs and tibias, blood, lymph nodes and spleen were collected. Harvested cells were then stained for CD4, CD8 and FoxP3 expression.

Flow cytometry

The reconstitution of the different T-cell sub-populations was evaluated by flow cytometry at the indicated time-points. Specifically, mice were bled via tail vein and blood was collected immediately in a BD Microtainer® K2E Tube (BD Biosciences, San Diego, USA). Blood volumes were measured and transferred into a FACS tube. Blood cells were washed with PBS + FBS 3% + P/S 1% and supernatants were then removed. Biotinilated antibody was added and incubated with cells for 30 minutes in the dark at 4°C. Cells were washed twice with PBS + FBS 3% + P/S 1%. Extracellular antibodies, including the streptavidin antibody, were added as a mix directly in tubes and incubated for 30 minutes at 4°C in the dark. Cells were washed twice with PBS + FBS 3% + P/S 1%. Cells were then fixed and permeabilized using the FoxP3 staining buffer set (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Cells were then incubated for 30 minutes at 4°C in the dark with intracellular antibodies and finally washed twice and resuspended with pure PBS in BD Trucount Tubes® to determine the absolute number of each lymphocyte population per µL of blood.

The following antibodies were used: anti-mouse CD3 V500 (clone: 500A2, BD Biosciences), CD4 eFluor 450 (clone: RM4-5, eBiosciences), CD8 Pe-Cy7 (clone: 53-6.7, eBiosciences), CD4 FITC (clone: GK 1.5, eBioscience), CD8 APC-Cy7 (clone: 53-6.7, eBioscience), CD62L Pe-Cy7 (clone: MEL-14, eBioscience), CD44 PerCP-Cy5.5 (clone: IM7, eBioscience) and anti-mouse FoxP3 APC (clone: FJK-16s, eBioscience) for T-cell subpoplations; anti-mouse B220 APC-eFluor 780 (clone: RA3-6B2, eBiosciences) for B lymphocytes; anti-mouse CD49b Biotin (clone: DX-5, eBiosciences) combined with a streptavidin PerCP-Cy5.5 was used for NK cells; and anti-human Ki-67 PE (BD Biosciences) was used to assay T-cell proliferation. Data were acquired on a FACS Canto II (Becton Dickinson) and were analyzed with FlowJo (Treestar Inc., San Carlos, CA).

Statistics

Statistical analyses were performed with the Graphpad® Prism 5.00 software (La Jolla, CA, US). The Mann-Whitney U test was used to compare GvHD scores

or flow cytometry data in the different experimental groups. Results were expressed as median±interquartile range with *p*-values considered as significant at ≤ 0.05.

RESULTS

Imatinib does not decrease *in vivo* proliferation of T-cell subsets

Given the primordial role of T cells in the pathogenesis of cGvHD, we first assessed the impact of imatinib on T-cell proliferation *in vivo*. Specifically, after 7 Gy TBI, Balb/cJ mice were transplanted with $10x10^6$ bone marrow cells from B10.D2 mice on day 0, followed by the injection of $70x10^6$ CFSE-labeled spleen cells on day +14. Treatment with sterile water or imatinib was started immediately after spleen cell injection. Mice were sacrificed 5 days later and spleen, lymph nodes, blood and bone marrow were collected and stained for flow cytometry experiments. As shown in ***Figure 1 (p.80)***, percentages of proliferation were comparable between control and imatinib mice for the different T-cell subsets in spleen, blood, bone marrow and lymph nodes. These data indicate that imatinib does not significantly inhibit *in vivo* proliferation of T cells in this lymphopenic host.

Imatinib does not affect cGvHD severity

Lethally irradiated Balb/cJ recipient mice were injected with $10x10^6$ bone marrow cells and $70x10^6$ spleen cells from B10.D2 donor mice. From day +7 after transplantation to the end of the experiments (day +52), mice received either sterile water or imatinib. Combined results of 3 consecutive experiments are reported. As shown in ***Figure 2 (p.81)***, imatinib did not impact cGvHD severity, since a comparable evolution of cGvHD scores was observed in the two groups of mice.

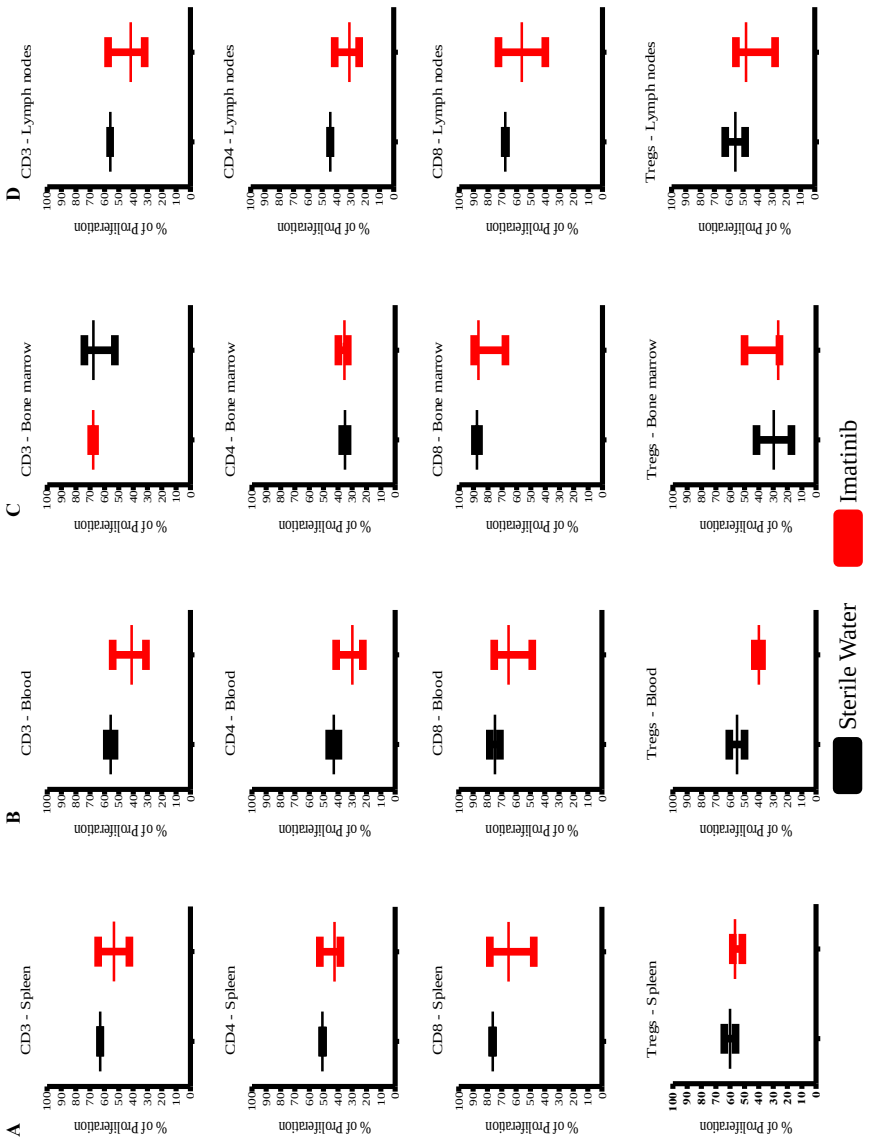

Figure 1: Imatinib does not decrease *in vivo* proliferation of T-cell subsets Balb/cJ mice were lethally irradiated and then injected i.v. with 10.10^6 bone marrow cells from B10.D2 mice on day 0 and 70.10^6 CFSE-labelled spleen cells on day +14. Mice were then treated with sterile water or imatinib by oral gavage at the dose of 150 mg/kg/day (50 mg in the morning and 100 mg in the evening) directly after spleen cell injection until sacrifice 5 days later. Proliferation was similar in the control (n=2) and imatinib groups (n=3) for CD3, CD4, CD8 and regulatory T cells in **(A)** spleen, **(B)** blood, **(C)** bone marrow and **(D)** lymph nodes. Results are expressed in median with interquartile range.

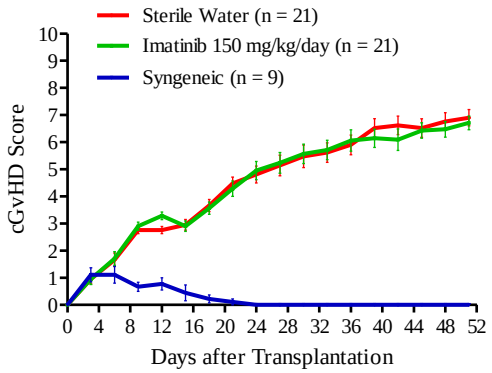

Figure 2: Imatinib does not affect cGvHD severity. Balb/cJ mice were injected i.v. with 10.10^6 bone marrow cells and 70.10^6 spleen cells from B10.D2 donor mice after lethal irradiation at 7 Gy. Mice were then given sterile water (n=21) or imatinib (n=21) by gavage at the dose of 150 mg/kg/day (50 mg in the morning and 100 mg in the evening) from day +7 post-transplant to the end of the experiment (day +52). Animals were individually scored 3 times/week for five parameters, as described earlier. Pooled GvHD scoring of three independent groups of mice given or not imatinib, showing no impact of the treatment on cGvHD scores (for syngeneic mice, n=9). Results are expressed with mean with SEM, unpaired T-test.

Imatinib decreases phosphorylation of PDGF-r and C-Abl

We next assessed whether PDGF-r and c-Abl signaling pathways were impacted by imatinib. Indeed, it has been previously reported that prevention of fibrosis by TKIs could be attributed at least in part to a direct inhibition of c-Abl and PDGF-r signaling pathways [298]. As shown in ***Figure 3 (p.82)***, there was a trend for lower levels of phosphorylated c-Abl on day +29 in imatinib-treated mice compared to control (*P*=0.1854) (***Figure 3A and B***). Further, imatinib-treated mice had significantly lower levels of PDGF-r phosphorylation than control mice (*P*=0.033) (***Figure 3C and D***). These data demonstrate that imatinib inhibits PDGF-r signaling *in vivo* in that murine model of scl-cGvHD.

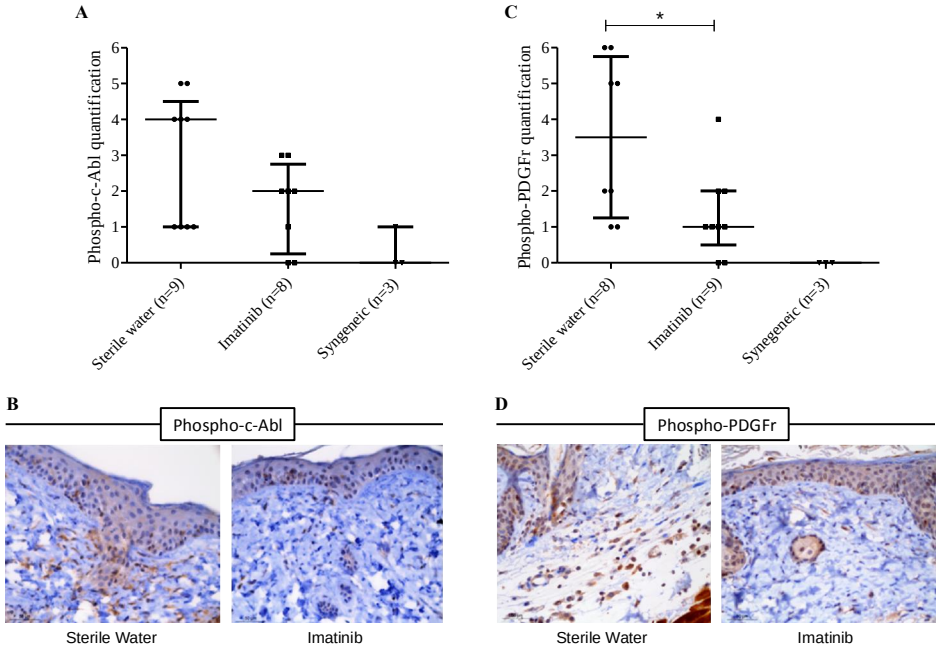

Figure 3: Imatinib decreases phosphorylation of PDGF-r and c-Abl. Skin samples from the upper back of each mice were harvested on day +29 post-transplant and directly fixed in formol 10% before being paraffin-embedded. Skin sections were then stained using anti-phospho PDGF-r and anti-phospho c-Abl to quantify phosphorylation levels of these receptors. Samples were quantified by multiplying the staining intensity (scored 0 to 3) by the stained area (scored 0 to 3). **(A-B)** Immuno-histological evaluation of the phosphorylation level of c-Abl (control: n=9, imatinib: n=8, syngeneic: n=3). **(C-D)** immune-histological evaluation of the phosphorylation level of the PDGF-receptor (control: n=8, imatinib: n=9, syngeneic: n=3). * = $P < 0.05$. Results are expressed in median with interquatile range, Mann-Whitney test.

Imatinib does not affect T-cell counts on day +21

We next assessed the impact of imatinib on T-cell subsets in scl-cGvHD mice 14 days after treatment initiation (D+21 post-transplantation).

a) Comparison between allogeneic and syngeneic graft

Figure 4 (p.84) shows that in compared to cGvHD control mice, animals given syngeneic grafts had higher absolute counts of $CD4^+$ T cells (P=0.001), naïve $CD4^+$ T cells (P=0.003), naïve $CD8^+$ T cells (P=0.03), and T_{regs} (P=0.002) in blood (***Figure 4***). We also compared the proliferation of the different T-cell subsets on day +21 by measuring their expression of Ki67. As shown in ***Figure 5 (p.85)***,

compared to control mice, mice given syngeneic grafts had higher proliferation of $CD4^+$ T cells (*P*=0.007), naïve $CD4^+$ T cells (*P*=0.002) and T_{regs} (*P*=0.006) but lower proliferation of $CD8^+$ T cells (*P*=NS).

b) Comparison between control and imatinib-treated cGvHD mice

As shown in ***Figure 4*****,** absolute counts of the various T-cell subsets were comparable in imatinib-treated and control mice. Finally, compared to control mice, imatinib-treated mice displayed higher proliferation of $CD4^+$ T cells (*P*=0.046), $CD8^+$ T cells (*P*=0.05), and T_{regs} (*P*=0.035) (***Figure 5***).

Imatinib does not affect T-cell counts on day +35

As for day +21, we also assessed the impact of imatinib on T-cell subsets in blood and on their proliferation on day +35 after transplantation (28 days after starting treatments).

a) Comparison between allogeneic and syngeneic graft

Figure 6 (p.86) shows that, compared to animals receiving allogeneic grafts, mice receiving syngeneic grafts had higher absolute counts of all T-cell subsets except for effector memory $CD8^+$ T cells that, on the contrary, were higher in allogeneic than in syngeneic mice (*P*=0.017 and *P*=0.036 respectively). Regarding analyses of proliferation assessed by the expression of Ki67, proliferation of $CD8^+$ T cells was lower in syngeneic mice than in allogeneic transplanted animals, treated or not with imatinib (*P*=0.0002 and *P*=0.001 respectively) (***Figure 7; p.87***). Finally, regarding central memory T-cell subsets, syngeneic recipients showed higher proliferation of $CD4^+$ T cells than allogeneic recipients, again treated or not with imatinib (*P*=0.011 and *P*=0.003 respectively) while proliferation of $CD8^+$ T cells was lower in syngeneic than in allogeneic recipients treated with imatinib (*P*=0.036).

b) Comparison between control and imatinib-treated cGvHD mice

As observed on day +21, absolute counts of T-cell subsets were also comparable whether animals received or not imatinib (***Figure 6***). Interestingly, proliferation of naive $CD4^+$ T cells was higher in imatinib-treated mice than in controls (*P*=0.03).

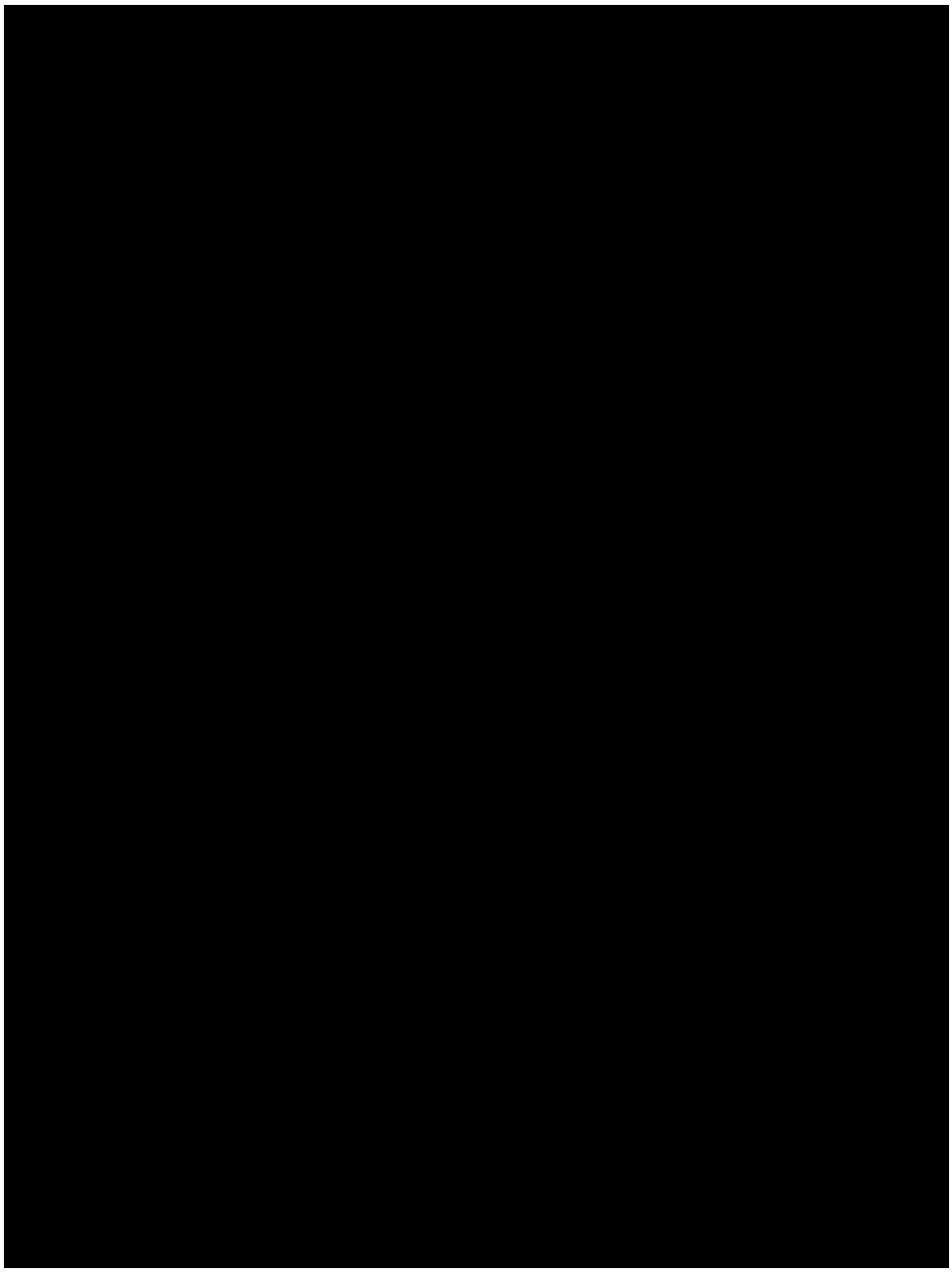

Figure 4: Imatinib does not affect T-cell (subset) counts on day +21. Blood samples from transplanted mice were collected at day +21 post-transplant to assess absolute numbers of T-cell subpopulations. Results indicate that imatinib does not affect absolute numbers of $CD4^+$ T cells, $CD8^+$ T cells or T_{regs}. * = P < 0.05; ** = P < 0.01; *** = P < 0.001. Control: n=12, imatinib: n=12, syngeneic: n=6. Results are expressed in median with interquartile range, Mann-Whitney test.

Figure 5: Imatinib increases T-cell proliferation at day +21. Blood samples were collected at day +21 post-transplant to assess T-cell (subpopulation) proliferation. The results indicate that the expression of Ki67 by $CD4^+$ T cells, $CD8^+$ T cells and T_{regs} was higher in imatinib-treated mice (n=12) than in controls (n=12), while no difference was observed for the other T-cell subsets. * = $P < 0.05$; ** = $P < 0.01$. Syngeneic mice: n=5. Results are expressed in median with interquartile range, Mann-Whitney test.

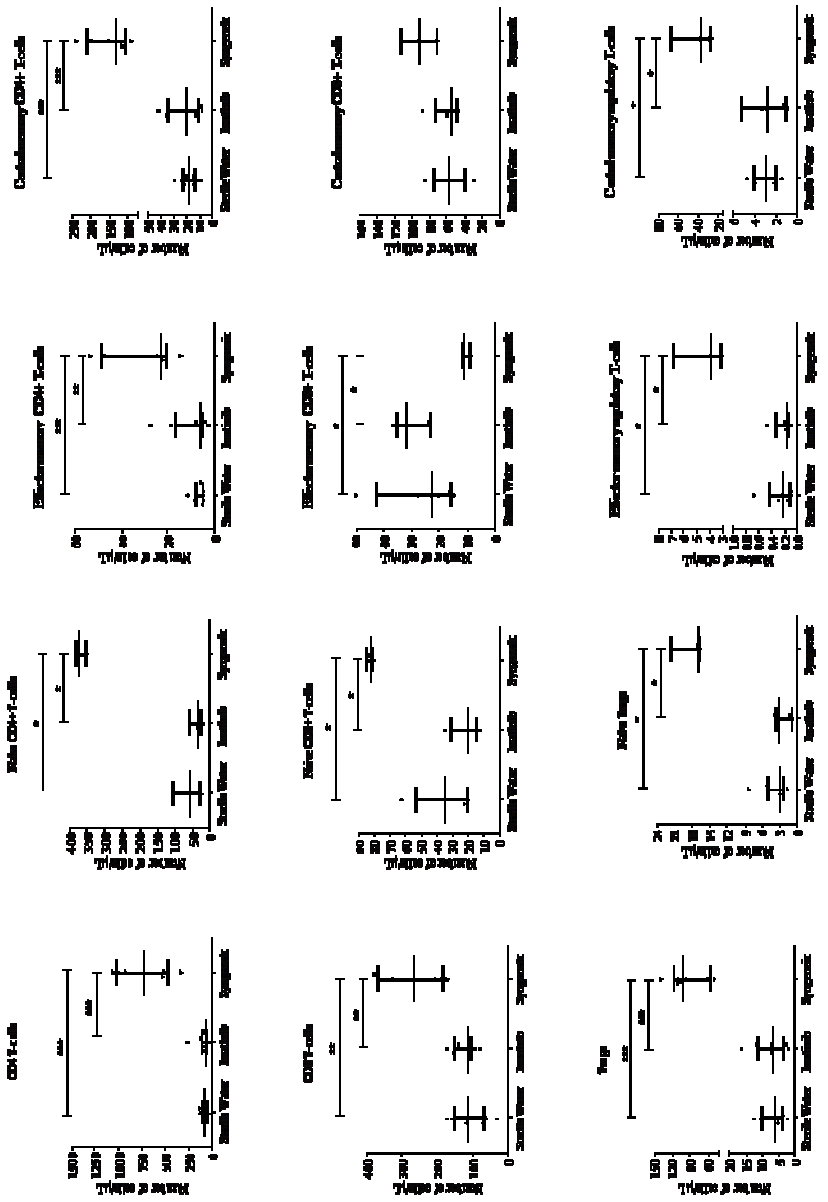

Figure 6: Imatinib does not affect T-cell (subset) counts on day +35. Blood samples were collected to at day +35 post-transplant to assess the impact of the treatment on absolute number of T-cell subpopulations. Imatinib treatment (n=10) did not affect the counts of T-cell subpopulations. However, counts of all T-cell subpopulations (except effector and central memory $CD8^+$ T cells) were higher in syngeneic recipients (n=6) than in scl-cGvHD mice. Control mice: n=9. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. Results are expressed in median with interquartile range, Mann-Whitney test.

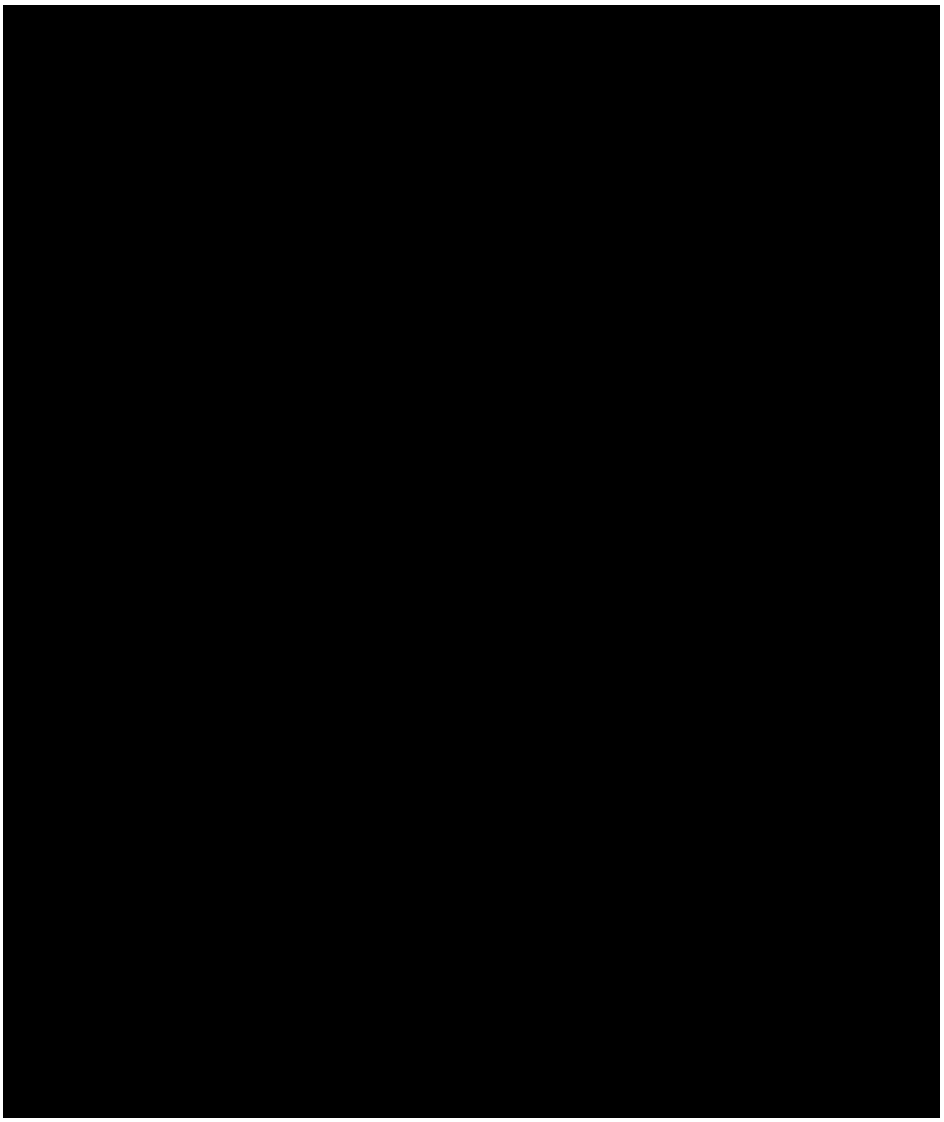

Figure 7: Imatinib does not affect T-cell proliferation at day +35. Blood samples were collected at day +35 post-transplant to assess T-cell proliferation. Proliferation of naive $CD4^+$ T cells was significantly higher in imatinib- (n=10) than in water-treated mice (n=8). Further, compared to scl-cGvHD mice, syngeneic mice (n=6) had a lower proliferation of $CD8^+$ T cells but a higher proliferation of naive and central memory $CD4^+$ T cells. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. Results are expressed in median with interquartile range, Mann-Whitney test.

DISCUSSION

PDGF and TGF-β cell signaling pathways play an important role in the fibrosing process occurring in scl-cGvHD. Imatinib, a well-demonstrated inhibitor of PDGF and TGF-β signaling pathways (by inhibiting PDGF-r and c-Abl tyrosine kinases), has been studied in patients with scl-cGvHD [302, 305, 306, 308, 378]. While some reports suggested a beneficial impact of imatinib in that setting [302, 306, 308, 378], this was not confirmed in other clinical studies [305]. This prompted us to investigate the impact of imatinib in a well-characterized murine model of scl-cGvHD in which PDGF-r, c-Abl and TGF-β have pivotal function in scl-cGvHD pathogenesis. Several observations were made.

First, *in vivo* administration of imatinib in that model was able to inhibit its targets. Indeed, phosphorylation levels of PDGF-r were significantly decreased in imatinib-treated compared to control mice, while a similar trend was observed for c-Abl. This is in concordance with the observations reported by *Zerr et al.* in a similar murine model of scl-cGvHD (with the exception that lower numbers of spleen cells were used in their study (2.10^6 versus 70.10^6 spleen cells/mice)) [298].

Secondly, imatinib administration failed to ameliorate scl-cGvHD. This is in contrast to what has been reported by *Zerr et al.* who observed that imatinib significantly decreased scl-cGvHD scores [298]. The reason for this apparent discrepancy is likely related to the lower numbers of spleen cells injected (35 times less) by *Zerr et al*., resulting in a less severe scl-cGvHD. Taken together, these observations might suggest that imatinib, when used as a single drug, might be mainly efficient in patients with moderate intensity cGvHD. These observations could be confirmed by a recent paper by de *Masson et al.*, in which they observed a limited efficacy of imatinib for patients affected by severe scl-cGvHD [308].

Thirdly, compared to mice transplanted with syngeneic grafts, we observed that allogeneic scl-cGvHD mice had higher counts of effector memory $CD8^+$ T cells but significantly lower counts of all other T-cell subsets. These data suggest that the scl-cGvHD process induces the expansion of effector memory $CD8^+$ T cells while impacting immune reconstitution probably in part by decreasing thymic neo-generation of T cells and increasing T-cell apoptosis as previously reported [381, 382]. Further, these data are consistent with recent observations by *Binsfeld et al.* in a murine model of graft-versus-myeloma effect consisting in the transplantation of BALB/cJ mice, previously injected with luciferase-transfected MOPC315.BM myeloma cells, with bone marrow cells and spleen cells from B10.D2 mice [45].

Fourthly, we observed that imatinib had only a limited impact on immune reconstitution in scl-cGvHD mice. This is in contrast with previously demonstrated *in vitro* inhibitory effects of imatinib on T-cell activation following TCR activation (through direct inhibition of LCK by imatinib) [280]. However, we demonstrated that imatinib does not decrease T-cell proliferation *in vivo* in a lymphopenic host. These data suggest that imatinib could be combined safely with T-cell modulating agents. Indeed, the lack of significant impact of imatinib in the current study contrasts with recent observations from our group in the same mouse model where T-cell modulation with either the mTor inhibitor rapamycin or the demethylating agent azacytidine were able to significantly ameliorate scl-cGvHD (Belle *et al.* and Fransolet *et al.*, submitted).

In conclusion, imatinib failed to alleviate scl-cGvHD in a severe murine model of scl-cGvHD despite significant inhibition of PDGF-r. Future studies should assess the combination of imatinib with T-cell modulating agents such as rapamycin or azacytidine.

AUTHORSHIP AND DISCLOSURES

LB, GF and FB designed the study. YB, GE and MH helped in the design of the study and in the interpretation of the results. LB, GF performed the experiments and analyzed the data. GE, MB and MH helped in the experiments. JS and PD performed the histopathological analyses. PD, LB and GF performed the skin biopsies. LB, GF and FB wrote the paper. All other authors edited and approved the manuscript.

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V. Mechanisms of sclerodermic chronic Graft-versus-Host Disease prevention by rapamycin

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Background
Sclerodermatous chronic Graft-versus-Host Disease (scl-cGvHD) is one of the most severe forms of cGvHD. Rapamycin (RAPA) has been shown to inhibit CD4 and CD8 conventional T cell (T conv) proliferation and differentiation without any inhibitory effect on regulatory T cells (T_{regs}). This prompted us to assess the impact of rapamycin on scl-cGvHD.

Methods
Balb/c mice were lethally irradiated and injected i.v. with 1.10^7 bone marrow cells and 7.10^7 spleen cells from B10.D2 mice. Balb/c recipient mice were then treated with rapamycin 1mg/kg every 24h from day +7 after transplantation until the end of the experiment (day +50).

Results
Rapamycin-treated mice had significantly lower cGvHD scores than vehicle-treated recipients from day +24 after transplantation. In comparison to control mice, rapamycin-treated mice had a lower T-cell proliferation, a lower infiltration of T cells in the lungs, a much higher proportion of naive T cells, and a higher proportion of T_{regs} expressing the CD103.

Conclusions
Rapamycin prevented experimental scl-cGvHD by different mechanisms including decreased T conv proliferation, decreased T-cell differentiation, decreased T-cell migration towards certain target organs and increased proportion of activated T_{regs}.

Manuscript *in preparation*

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative treatment for a wide range of hematological disorders [359]. When performed as treatment for malignant diseases, its anti-tumor activity relies in large part on immune-mediated graft-versus-tumor effects [360]. However, donor immune cells contained in the graft can also attack healthy host tissues causing Graft-versus-Host Disease (GvHD) [362]. GvHD can be divided into 2 syndromes, acute GvHD (historically defined as a GvH reaction occuring within the first 100 days after allo-HSCT) and chronic GvHD (cGvHD) that generally develops beyond day +100. In contrast to acute GvHD, which is primarilly an inflammatory process involving the skin, GI tract and/or the liver, cGvHD induces various clinical features resembling autoimmune and other immunologic disorders such as sicca syndrome, wasting syndrome, scleroderma, primary biliary cirrhosis, bronchiolitis obliterans and chronic immunodeficiency [128]. While cGvHD has been associated with graft-versus-tumor effects [361, 383], it is also associated with increased non-relapse mortality and is the first cause of mortality/morbidity in long-term transplant recipients [367]. This is particularly the case for sclerodermatous cGvHD (scl-cGvHD), which is one of the most severe forms of cGvHD and develops in approximately 20% of cGvHD patients [368].

Recent progresses have been made in our understanding of cGvHD pathogenesis [143, 362]. It is now well demonstrated that clinical manifestations of cGvHD in humans are mediated by a complex immune reaction involving donor conventional $CD4^+$ and $CD8^+$ T cells (T convs), donor regulatory T cells (T_{regs}), donor B cells, and donor regulatory B cells (Bregs) [143, 203, 384].

Rapamycin (RAPA) is a mTor inhibitor that acts several mechanisms of action. By inhibiting IL-2 signaling in T convs (but not in T_{regs} that depend primarrily on the STAT5 pathway for IL-2 signaling), it inhibits their proliferation and entry in cell cycle [385]. Further rapamycin inhibits $CD4^+$ T helper (Th) cell differentiation into Th1 and Th17 cells [386-388]. In contrast, rapamycin promotes T_{reg} by both favoring *de novo* generation of T_{reg} from naive $CD4^+$ T cells and by selectively expanding T_{reg}. Importantly, rapamycin also regulates $CD8^+$ T-cell trafficking by promoting expression of CD62L and CCR7 (favoring their migration into secondary lymphoid tissues) and favors the generation of memory $CD8^+$ T cell immunity [386-388].

In the current study, we assessed the impact of rapamycin in an experimental scl-cGvHD murine model with the aim of dissecting its mechanisms of action in that setting.

MATERIALS AND METHODS

Mice, drugs and cell lines

Twelve- to 14-week-old B10.D2 (H-2^d, Jackson Laboratories, Bar Harbor, USA) and BALB/cj (H-2^d, Jackson Laboratories) mice were used as donor and recipients, respectively, in an allogeneic bone marrow transplantation (BMT) model of scl-cGvHD. All mice were maintained in top-filtered cages in a standard animal facility and provided sterilized food. Sterilized water supplemented with Baytril® 1% (Bayer HealthCare, Diegem, Belgium) was given from 3 days before to the end of the experiment. All animal experiments were approved by the ethic committee from the University of Liège (File number: 1138). For *in vivo* experiments, 1 mg/kg Rapamycin (Rapamune, Pfizer, New York, USA), diluted in PBS (Lonza Verviers, Belgium) was administered i.p. every 24h from day +7 to the end of the experiment. For *in vitro* experiments, rapamycin powder was purchased from Sigma-Aldrich (St. Louis, USA) and stock solutions were prepared in DMSO at a concentration 1 mM. Further dilutions were performed in fully supplemented medium. NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza) supplemented with inactivated Fetal Bovine Serum (FBS) 10%, and Penicillin/Streptomycin (P/S) (Lonza) 1%.

MTT, apoptosis and cell cycle assays

NIH-3T3 cells were washed twice in PBS + P/S 1%, trypsinized and then resuspended in 10 mL of DMEM + FBS 10% + P/S 1% (complete media). A total of 57,600 (for MTT assay) or 180 000 (for apoptosis and cell cycle assay) cells/well were seeded in a 96 well-plate (final volume of 100 µL). Rapamycin was added 24 hours at a final concentration of 1000, 100, 10, 1, 0.1 or 0.01 nM. Cells were incubated for 48 hours, washed with PBS + P/S 1% and then collected. MTT assays were performed using the Cell Proliferation Kit I (MTT) according to manufacturer's instructions (Roche, Indianapolis, USA). Apoptosis assays were performed by flow cytometry using the FITC Annexin V Detection Kit I according to manufacturer's instructions (BD Biosciences). Cell cycle assays were performed by flow cytometry using the CycleTEST™ Plus DNA Reagent Kit (BD Biosciences) as previously reported [380]. The percentage of cells in the different phases of the cell cycle was determined with Modfit software (BD Biosciences) on at least 20,000 acquired events. The percentage of cells in cycle was calculated as follows: percentage = ((S + G_2/M cells)/Total cells) x 100.

Cell preparation for bone marrow transplantation

B10.D2 donor mice were sacrificed in agreement with the recommendation of our ethical committee. Spleen, femurs and tibias were collected in sterile RPMI (Lonza) + FBS 10% + P/S 1%. Bone marrow (BM) cells were flushed with RPMI + FBS 10% + P/S 1%. Spleens were crushed between two sterilized microscope slides. Cells were washed twice with PBS + FBS 3% + P/S 1% (staining buffer)

and treated with RBC lysis buffer (eBioscience, San Diego, USA) for 4 minutes at room temperature. Cells were passed through a 70 µm nylon filter mesh and washed twice with staining buffer. Cells were finally resuspended in PBS at a concentration of $70x10^6$ spleen cells/200 µL and $10x10^6$ BM cells/200 µL total volume.

Bone marrow transplantation and scl-cGvHD

Mice were transplanted as previously described [379]. Briefly, recipients BALB\c mice were lethally irradiated with 7 Gy total body irradiation. Six hours later, recipients were injected with $70x10^6$ spleen cells + $10x10^6$ BM cell from donor B10.D2 mice, mice from syngeneic groups received only BM cells. Survival was monitored daily and recipient body weights and GvHD scores were recorded every 2-3 days. The severity of scl-cGvHD was assessed with a clinical scoring system, by intergring the following 5 parameters: weight loss (grade 1, 10-20%; grade 2, > 20%), posture (1, kyphosis only at rest; 2, severe kyphosis when the animal moved), activity (1, moderate activity impairment; 2, no move unless stimulated), skin (1, erythema or scaling tail; 2, open lesion on the body surface). The final clinical score was generated by the summation of the five criteria scores as previously reported [45].

Histopathological analysis

Skin, liver and lungs were obtained and directly fixed in 10% formaldehyde for 48 hours before being paraffin-embedded and sectioned at 4-5 µM. Slides were then stained using an anti-CD3 antibody, following the manufacturer's instructions. Histological slides were then scanned with a 40x magnification using Hamamatsu Nanozoomer 2.0 HT (Hamamatsu Photonics, Geldern, Germany). Slides were examined by a blinded examinator (JS) using a scoring system consisting in an absolute count of CD3+ cells in three different hotspots for each slide. Total number of positive cells was then normalized in number of CD3+ cells per mm^2 for each sample.

Flow cytometry

Blood from tail vein was collected in BD Microtainer K2EDTA Tube (BD Biosciences, San Diego, USA). Spleen cells were obtained by crushing the spleen, and BM cells by flushing femurs and tibias with RPMI 1640 + 10% FBS. To obtain lungs and liver lymphocytes, heart was first perfused with 10 ml PBS, then lungs and liver were minced, digested for 1 h at 37°C in 1 mg/ml collagenase A (Roche) and 0.05 mg/ml DNaseI (Roche) in HBSS and lymphocytes were finally harvested by Ficoll-Paque (GE Healthcare, Freiburg, Germany) gradient centrifugation. Erythrocytes were depleted using RBC lysis buffer (eBioscience, Vienna, Austria) according to manufacturer's instructions. Cells were washed in staining buffer before processing for flow cytometry. The following antibodies, specific for mouse epitopes, were used: CD229.1-FITC

(30C7, BD), CD3-V500 (500A2, BD), CD4-eFluor450 (RM4-5), CD4-BV510 (RM4-5, SONY Biotechnology, Weybridge, UK), CD8-APCeFluor780 (53-6.7), CD8-PeCy7 (53-6.7), CD62L-PECy7 (MEL-14), CD62L-APCeFluor780 (MEL-14), CD44-FITC (IM7), CD44-APC (IM7), CXCR4-eFluor450 (2B11), CXCR3-PE (CXCR3-173), CCR3-AF647 (J073E5, SONY), CCR5-PerCPeFluor710 (HM-CCR5 (7A4)), B220-APCeFluor780 (RA3-6B2), CD49b-biotin (DX-5), CD103-BV510 (M290, BD), Bcl-2-eFluor450 (10C4), FoxP3-PE (FJK-16s), FoxP3-APC (FJK-16s) and the following antibodies, specific for human epitopes, were used: Ki-67-PE (B56, BD) and pSTAT5-APC (DY694, BD) (all from eBioscience unless indicated otherwise). Cells (1.5-2x10^6 cells/sample) were incubated with surface antibodies for 20 min at 4°C in the dark and washed twice with staining buffer. Intracellular staining for FoxP3 and Ki67 was performed by using the FoxP3 staining buffer set (eBioscience). For pSTAT5 staining, the PerFix EXPOSE reagent kit (Beckman Coulter, Fullerton, CA) was used following the manufacturer's instruction, as previously reported [389]. Absolute counts were obtained with BD Trucount Tubes (Becton-Dickinson). Data were acquired on a FACS Canto II flow cytometer (Becton Dickinson) and analyzed with the FlowJo software 10.0.7 (Tree Star Inc., Ashland, OR).

Serum cytokine levels

The concentration of mouse cytokines was determined in mice serum, after 4-fold dilution, using the Bio-Plex Pro Mouse Cytokine 23-plex Assay (Biorad Laboratories, Nazareth Eke, Belgium). The experiments were performed according to the manufacturer's recommendation, and results were acquired on Bio-Plex System and analyzed with Bio-Plex Manager Software 4.0 (Biorad Laboratories), as previously reported [390].

Statistical analyses

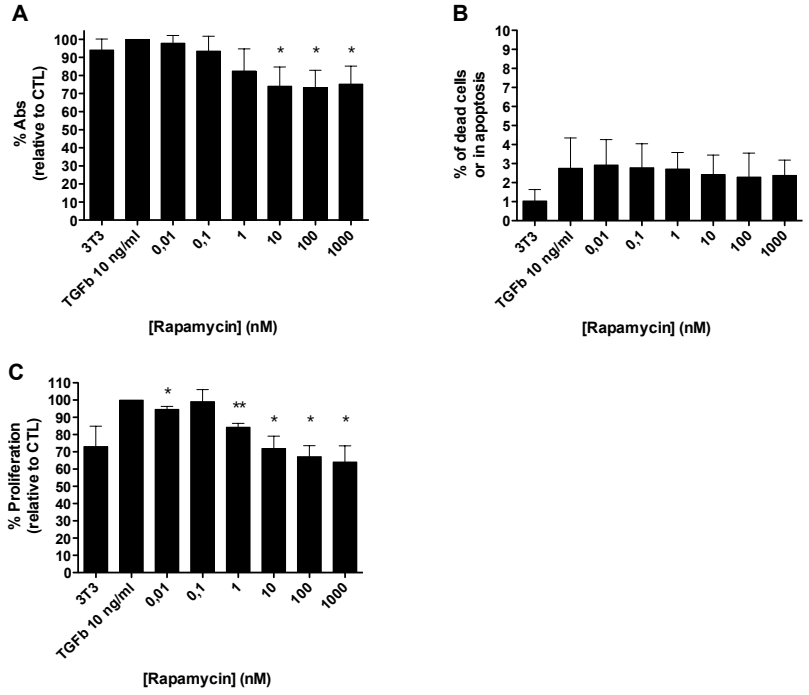
The Mann-Whitney test was used to compare flow-cytometry data, serum cytokine levels and immunohistopathological scores between different groups. Comparisons between GvHD score curves were made using unpaired Student's T test. *P* values < 0.05 were considered as statistically significant. Statistical analyses were carried out with Graphpad Prism 5.0 (Graphpad Software, San Diego, CA).

RESULTS

Rapamycin decreases proliferation of the fibroblastic cell line NIH-3T3

Since fibroblasts play a major role in scl-cGvHD, we first investigated the impact of rapamycin on the fibroblastic cell line NIH-3T3 *in vitro*. For these experiments, proliferation and collagen production by NIH-3T3 cells were induced with TGF-β 10 ng/mL for 48 hours in the presence or absence of various concentration of rapamycin. As showed in ***supplemental figure 1 (p.99)***, rapamycin decreased the proportion of cells in S and G2 phases at

concentrations ≥ 1 nM, and decreased metabolic activity (assessed with the MTT assay) at concentrations ≥ 10nM. In contrast, rapamycin had no impact on the percentage of cells in apoptosis.



Supplemental Figure 1. Rapamycin reduced collagen synthesis by TGF-β-stimulated fibroblasts *in vitro* by preventing cell-cycle entry and mitochondrial activity. (A): Rapamycin decreased proliferation of TGF-β stimulated fibroblasts. (B): Influence of a 48-hour pre-culture in the presence rapamycin on mitochondrial activity. (C): Apoptosis in filbroblast-cultured with rapamycin for 48h rapamycin. Results are expressed as mean ± SD. N ≥ 3, *p<0.05, **p<0.005 versus CTL, Student's paired t tests. CTL: control condition without rapamycin.

Rapamycin mitigates scl-cGvHD.

Next, we assessed the impact of rapamycin on scl-cGvHD. As showed in ***Figure 1 (p.100)***, rapamycin significantly reduced GvHD scores from day 24 to the end of the experiment demonstrating that rapamycin attenuated Scl-cGvHD severity.

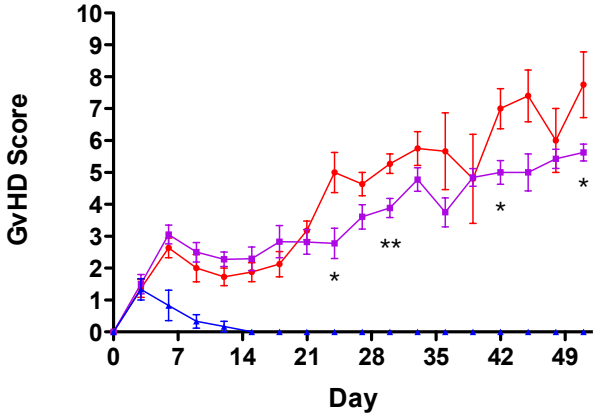


Figure 1. Rapamycin mitigates scl-cGvHD. Balb/cJ mice were lethally irradiated and injected i.v. with 1.10^7 bone marrow cells and 7.10^7 splenocytes from B10.D2 mice, 21 days before transplantation. Mice were then administered, or not, with 1 mg/kg of rapamycin (RAPA) i.p. every 24h from day 7 to 50.

Rapamycin reduces T-cell proliferation and increases their resistance to apoptosis

Because of the pivotal function of mTOR pathway in T-cell proliferation, we investigated whether rapamycin affected T-cell proliferation *in vivo*. As showed in ***figure 2A (p.101)***, a reduced expression of Ki67 was found in peripheral blood circulating $CD4^+$ and $CD8^+$ T cells at days +21 and +35 in rapamycin-treated mice (in comparison to control mice), although the differences reached statistical significances only for $CD4^+$ T cells.

To further investigate rapamycin impact on lymphocytes in lymphoid and target tissues, we sacrificed a cohort of animals at day +21 and analyzed T cells from spleen, BM, liver and lungs by flow cytometry. In agreement with observations made in the blood, we observed a lower frequency of $Ki67^+$ $CD3^+$ T cells in spleen, BM and lungs (but not in liver) of treated mice (***Figure 2B***).

In addition to its anti-proliferative effect, rapamycin was also reported to increase resistance to apoptosis [391]. Therefore we analyzed the Bcl-2 expression in $CD4^+$ and $CD8^+$ T cells in blood (***Figure 2C***) and in $CD3^+$ T cells in other tissues (***Figure 2D***) and found a higher Bcl-2 MFI in all organs of mice treated with rapamycin in comparison to control ones.

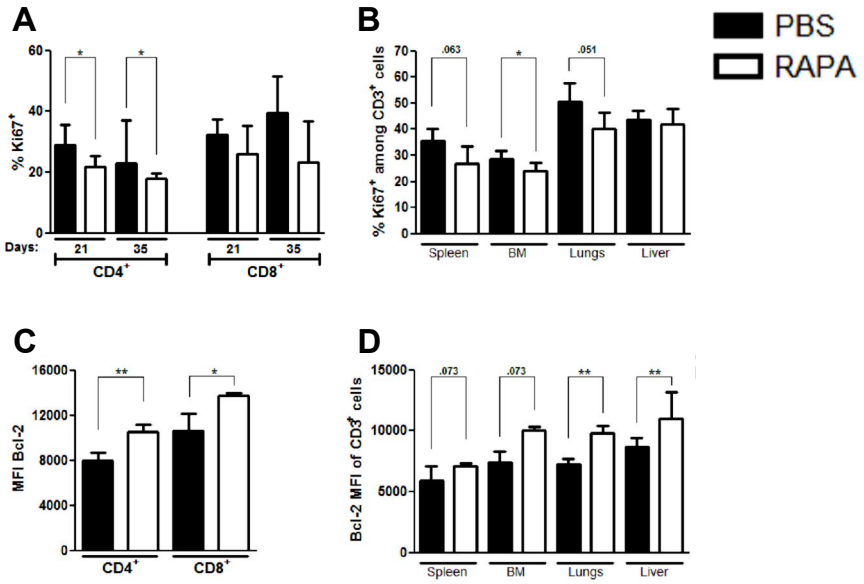


Figure 2. Rapamycin reduces T-cell proliferation and increases their resistance to apoptosis. Balb/cJ mice were lethally irradiated and injected i.v. with 1.10^7 bone marrow cells and 7.10^7 spleen cells from B10.D2 mice, 21 days before transplantation. Mice were then administered, or not, with 1 mg/kg of rapamycin i.p. every 24h from day 7 to 50. Flow cytometry analyses were performed on blood at days +21 and +35 (A) or on blood (C) and other organs at day +21 (D). Comparisons of (A) $Ki67^+$ cells within $CD4^+$ and $CD8^+$ T cells population, (B) $Ki67^+$ cells within $CD3^+$ cells population, (C) Bcl-2 expression (mean fluorescence intensity, MFI) of $CD4^+$ or $CD8^+$ T cells, (D) Bcl-2 expression (MFI) of $CD3^+$ T cells. Data show median values of 6-12 mice / condition in A and 6-7 mice / condition in B-C-D, with interquartile range (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$).

Rapamycin reduces T-cell trafficking to GvHD target organs

In light of the reduction of the proliferation and increase of resistance to apoptosis in rapamycin-treated mice, we assessed the impact of the drug on absolute T-cell counts. Higher counts of $CD4^+$ and $CD8^+$ T cells were found in the blood from treated animals when compared with control groups at days +21 and +35 (***Figure 3A-B; p.103***). Because we assumed that the absolute number of circulating T cells might also be related to their trafficking, we examined the expression of homing markers on T cells in the blood and peripheral tissues of mice treated or not with rapamycin. As showed in ***figure 3 C-F*** (spleen) and ***supplemental table 1*** (other tissues; ***p.104***), the expression of CCR3, CCR5, CXCR3 and CXCR4 was decreased by rapamycin at day +21 in the spleen and

other tissues (to a lesser extent), suggesting an impairment of T-cell trafficking in these animals.

The hypothesis of a lower homing of T cells in rapamycin-treated mice was assessed by blinded histological examination of T-cell infiltration in liver, lung and skin. In agreement with our flow cytometry observations, we observed significantly lower infiltrates of T cells in the lungs (P= 0.05) of rapamycin-treated mice, while there was a suggestion for a similar effect in the livers (P=0.14) but not in the skins (***Figure 3G***). In agreement with these observations and with the reduction of trafficking to non-lymphoid tissues, lower serum concentrations of the proinflammatory cytokines and chemokines IL-1α, MCP-1, MIP-1α and RANTES were observed in the blood of treated mice on day +21 after transplantation (***Figure 3 H***).

Rapamycin reduces T-cell differentiation

Because the expression of homing markers may correlate with T-cell differentiation, we compared the frequency of $CD4^+$ and $CD8^+$ T-cell subsets between treated and control groups in blood. As expected, a higher frequency of $CD44^-CD62L^+$ naive $CD4^+$ and $CD8^+$ T cells was found at days +21 and +35 in treated mice while dramatically decreased frequencies of $CD44^+CD62L^-$ effector memory T cells were observed (***Figures 4A-B***). Interestingly, rapamycin did not significantly impact the frequency of $CD44^+CD62L^+$ central memory T cells (data not showed).

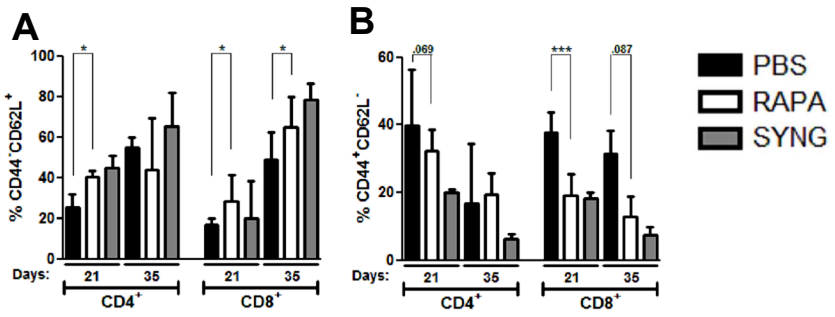


Figure 4. Rapamycin reduces T-cell differentiation. Balb/cJ mice were lethally irradiated and injected i.v. with 1.10^7 bone marrow cells and 7.10^7 spleen cells from B10.D2 mice, 21 days before transplantation. Mice were then administered, or not, with 1 mg/kg of rapamycin (RAPA) i.p. every 24h from day +7 to +50. Flow cytometry analyses were performed on blood at days +21 and +35. Comparisons of (A) $CD44^-CD62L^+$ cells frequency within $CD4^+$ or $CD8^+$ T-cell population and (B) $CD44^+CD62L^-$ cells frequency within $CD4^+$ or $CD8^+$ T-cell

population. Data show median values of 6-12 mice / condition, with interquartile range (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$).

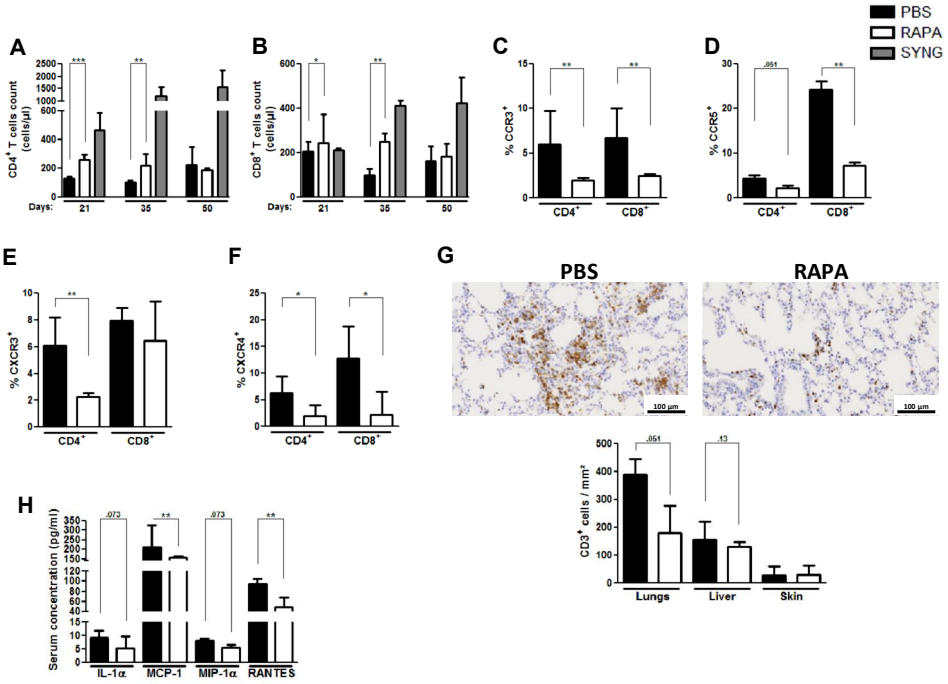


Figure 3. Rapamycin reduces T-cell trafficking to GvHD target organs. Balb/cJ mice were lethally irradiated and injected i.v. with 1.10^7 bone marrow cells and 7.10^7 splenocytes from B10.D2 mice, 21 days before transplantation. Mice were then administered, or not, with 1 mg/kg of rapamycin (RAPA) i.p. every 24h from day +7 to +50. Flow cytometry analyses were performed on blood at days +21, +35 and +50 (A-B) or on spleen at day +21 (C-D-E-F). Comparisons of (A) $CD4^+$ T cells and (B) $CD8^+$ T cells count, (C) frequency of $CCR3^+$ cells within $CD4^+$ or $CD8^+$ T cells population, (D) frequency of $CCR5^+$ cells within $CD4^+$ or $CD8^+$ T cells population, (E) frequency of $CXCR3^+$ cells within $CD4^+$ or $CD8^+$ T-cell population, (F) frequency of $CXCR4^+$ cells within $CD4^+$ or $CD8^+$ T cells population. (G) Histologic evaluation of $CD3^+$ cells infiltration in GvHD target organs. (H) Comparison of IL-1α, MCP-1, MIP-1α and RANTES serum concentrations at day +21 by Bio-plex. Data show median values of 6-12 mice / condition in A-B and 6-7 mice / condition in C-D-E-F-G-H, with interquartile range (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$).

Parameter	PBS		RAPA		*p*-value
	Median	IQR	Median	IQR	
Blood					
$CD4^+CCR3^+$	8.43	6.81-11.23	7.96	6.06-11	NS
$CD4^+CCR5^+$	11.34	8.49-15.5	8.12	5.16-13.10	NS
$CD4^+CXCR3^+$	22.25	17.43-29.98	20.2	15.5-23.7	NS
$CD4^+CXCR4^+$	17.20	9.57-27.7	15.1	9.46-19.7	NS
$CD8^+CCR3^+$	12.8	9.55-22.83	7.08	6.34-9.76	0.073
$CD8^+CCR5^+$	12.4	8.11-13.75	9.23	6.1-12.8	NS
$CD8^+CXCR3^+$	7.79	5.38-11.05	7.15	5.34-10.6	NS
$CD8^+CXCR4^+$	13.97	7.57-31.83	12.40	11.1-16.5	NS
Bone marrow					
$CD4^+CCR3^+$	4.09	1.8-6.19	2.58	1.26-3.29	NS
$CD4^+CCR5^+$	24.35	19.93-36.93	26.2	24.7-28.3	NS
$CD4^+CXCR3^+$	8.25	7.62-9.72	7.82	5.71-8.35	NS
$CD4^+CXCR4^+$	4.58	2.9-8.98	1.91	1.35-5.53	NS
$CD8^+CCR3^+$	6.5	5.7-12.55	7.11	5.05-13.9	NS
$CD8^+CCR5^+$	26.65	14.05-36.9	22.5	17.5-23.2	NS
$CD8^+CXCR3^+$	7.38	2.68-12.33	2.13	1.09-5.63	0.101
$CD8^+CXCR4^+$	14.1	7.21-18.48	3.77	2.65-5.96	0.0082
Lungs					
$CD4^+CCR3^+$	7.41	4.65-18.75	3.79	1.43-6.73	0.101
$CD4^+CCR5^+$	12	5.85-18.85	4.55	2.28-10.70	0.051
$CD4^+CXCR3^+$	6.28	4.08-8.28	3.36	2.46-4.18	0.035
$CD4^+CXCR4^+$	8.93	5.17-11.58	6.14	4.16-7.23	NS
$CD8^+CCR3^+$	5.8	3.38-9	2.5	1.92-3.47	0.014
$CD8^+CCR5^+$	11.05	6.25-15.40	5.52	3.77-11	0.101
$CD8^+CXCR3^+$	2.13	1.38-3.06	1.84	0.98-2.99	NS
$CD8^+CXCR4^+$	4.15	3.04-5.85	2.63	2.35-4.25	NS
Liver					
$CD4^+CCR3^+$	6.41	4.52-7.2	5.95	3.45-7	NS
$CD4^+CCR5^+$	4.9	3.6-6.04	4.76	2.44-7.6	NS
$CD4^+CXCR3^+$	12.65	9.83-17.45	7.97	6.19-10.2	0.014
$CD4^+CXCR4^+$	5.93	4.47-8.86	6.19	5.48-6.94	NS
$CD8^+CCR3^+$	0.86	0.32-2.42	2.05	0.34-5.89	NS
$CD8^+CCR5^+$	1.97	1.38-2.13	1.81	1.16-3.03	NS
$CD8^+CXCR3^+$	7.05	4.3-8.4	5.38	2.43-6.79	NS
$CD8^+CXCR4^+$	4.53	1.6-8.09	4.92	3.86-6.36	NS

Supplemental Table 1. Balb/cJ mice were lethally irradiated and injected i.v. with 1.10^7 bone marrow cells and 7.10^7 splenocytes from B10.D2 mice sensitized (severe model) or not (classic model) with 5.10^5 Balb/cJ splenocytes, 21 days before transplantation. Mice were then administered, or not, with 1 mg/kg of rapamycin (RAPA) i.p. every 24h from day +7 to +21. At day 21, mice were sacrificed and organs were harvested for flow cytometry analyses of CCR3, CCR5, CXCR3 and CXCR4 expression of $CD4^+$ and $CD8^+$ T cells. Data show median values of 6-7 mice / condition with interquartile range (IQR).

Impact of Rapamycin on regulatory T cells

Rapamycin was previously shown to promote T_{reg} *in vivo*. Consequently, we examined whether rapamycin affected T_{regs} in this experimental setting. Surprisingly, blood T_{reg} frequencies among $CD4^+$ T cells were significantly decreased by rapamycin at days +21 and +35 while absolute T_{reg} counts remained similar between rapamycin and PBS mice (***Figure 5A-B***). In line with these observations, the serum concentrations of IL-10, an immunoregulatory cytokine in part secreted by T_{reg}, were similar in rapamycin-treated and in PBS mice (***Figure 5C***). Similarly, in other tissues, T_{reg} frequency was not affected by the treatment excepted in lungs where it was increased by RAPA (***Figure 5D***).

We further investigated the impact of rapamycin on T_{reg} proliferation. As showed in ***figure 5E***, rapamycin increased T_{reg} Ki67 expression in blood. Since T_{reg} proliferation is mainly driven by STAT5 signaling following IL-2 binding to its receptor, we compared $_{phospho}$STAT5 level in T_{reg} from rapamycin and control mice. In concordance with the Ki67 expression, we observed higher $_{phospho}$STAT5 levels in T_{reg} from rapamycin-treated than in control mice in the classic model (***Figure 5F***). Accordingly, higher serum concentrations of IL-2 (p=0.18) were observed in rapamycin-treated mice (***Figure 5G***).

Because it was previously shown that $CD103^+$ T_{regs} are more suppressive and more prone to suppress cGvHD than total T_{reg} population [392], we investigated rapamycin impact on CD103 expression of T_{reg}. As showed in ***figure 5H***, rapamycin significantly increased T_{reg} expression of CD103.

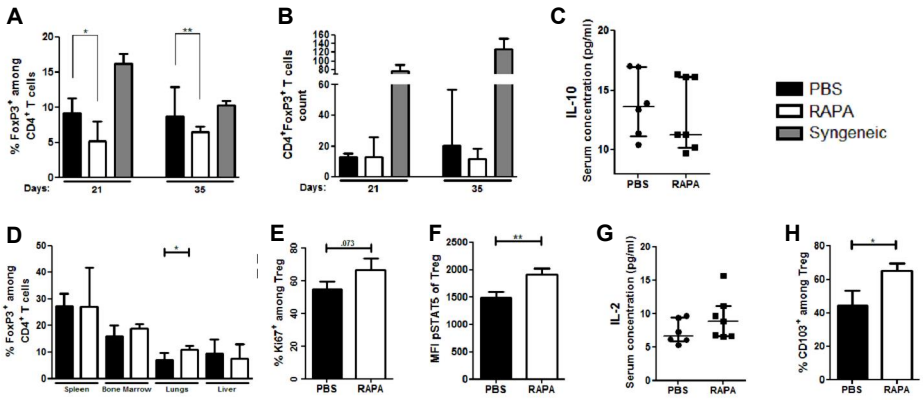


Figure 5. Rapamycin impact on regulatory T cells. Balb/cJ mice were lethally irradiated and injected i.v. with 1.10^7 bone marrow cells and 7.10^7 spleen cells from B10.D2 mice, 21 days before transplantation. Mice were then administered, or not, with 1 mg/kg of rapamycin (RAPA) i.p. every 24h from day +7 to +50. Flow cytometry analyses were performed on blood at days +21 and

+35 (A-B) or on spleen (E-F-H) and other organs at day +21 (C). (A) FACS comparison of FoxP3$^+$ cells frequency within CD4$^+$ T cells population, (B) FACS comparison of T_{reg} count (Trucount), (C) Bioplex comparison of IL-10 serum concentration at day +21, (D) FACS comparison of FoxP3$^+$ cells frequency within CD4$^+$ T cells population, (E) FACS comparison of Ki67$^+$ cells frequency within the T_{reg} population in blood at day +21, (F) FACS comparison of STAT5 phosphorylation level (MFI) of T_{reg}, (G) Bioplex comparison of IL-2 serum concentration at day +21, (H) FACS comparison of CD103$^+$ cells frequency within the T_{reg} population. Data show median values of 6-12 mice / condition in A-B and 6-8 mice / condition in C-D-E-F-G-H, with interquartile range (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$).

DISCUSSION

MTOR inhibitors such as rapamycin (sirolimus) or everolimus are current treatment options for GvHD prophylaxis and for patients with steroid-refractory cGvHD [393]. However, mechanisms of GvHD prevention/improvement by rapamycin have remained not fully understood. Here, we assessed the impact of rapamycin in a well-established model of scl-cGvHD. Several observations were made.

We first show that rapamycin ameliorated scl-cGvHD. These results are in concordance with previous reports in murine models as well as in a model of xenogeneic GvHD showing the ability of rapamycin given alone or in combination with low-dose IL-2 to ameliorate GvHD [385, 394].

This prompted us to look at possible mechanisms of GvHD prevention by rapamycin. We first observed that rapamycin decreased T-cell proliferation (assessed by Ki67 expression) *in vivo*. This is in concordance with prior observations showing that rapamycin decreased T-cell proliferation by several complex mechanisms including inhibition of IL-2 signaling in T convs, induction of non-protein coding RNA GAS5 [395] and through the deletion of autophagy-related genes [396].

Interestingly, the inhibition of T-cell proliferation by rapamycin did not translate to lower T-cell numbers *in vivo*. In the contrary, there was even higher numbers of T cells in the peripheral blood of rapamycin-treated mice. A first possible explanation for this apparent discrepancy could include a reduction of T-cell apoptosis by rapamycin. Indeed, we observed higher levels of BCL-2 expression by both CD4$^+$ and CD8$^+$ T cells in rapamycin-treated than in control mice. These observations are in accordance with previous studies that demonstrated that rapamycin increased the cellular concentration of BCL-2 and had anti-apoptotic effect in a B-cell lymphoma cell line and decreased T-cell apoptosis through autophagy [391, 397].

A second possible explanation might consist of decreased T-cell homing into non-lymphoid organs by rapamycin. Indeed, we observed lower infiltration by T cells in the lungs (and to a lesser extend the liver) of rapamycin-treated than in those from control mice while T-cell infiltration was similarly low in the skin from mice in the 2 groups. Mechanisms of decreased T-cell homing by rapamycin are probably multifactorial. First, we observed lower serum levels of MCP-1, MIP-1α and RANTES in the blood of rapamycin-treated mice, three cutaneous chemokines that play a pivotal function in scl-cGvHD in this model [211]. This is in line with a previous study showing that rapamycin decreases the secretion of these molecules by monocytes stimulated with LPS [398] and suggest that rapamycin does not regulate GvHD only through T-cell mediated mechanisms. Secondly, we observed lower expression of CCR3, CCR5, CXCR3 and CXCR4 by T cells from the spleen of rapamycin than of control mice on day +21 after transplantation. This is in accordance with prior studies showing that rapamycin reduced CCR5 expression by $CD4^+$ T cells and CXCR4 expression by gastric carcinoma cells [399, 400]. Thirdly, we observed a higher proportion of CD62L expressing T cells in rapamycin mice, in line with previous observations showing that rapamycin increases the expression of KLF2, which in turn prevent the downregulation of CD62L and CCR7, thus improving recirculation of T cells into secondary lymphoid tissues [401].

Besides inhibition of T-cell proliferation and T-cell homing to targeted organs, a third mechanisms of cGvHD prevention by rapamycin might consists into the inhibition of T-cell differentiation. Indeed, we observed a higher frequency of naïve T cells in rapamycin than in control mice, while the frequency of effector memory T cells was dramatically decreased by rapamycin. These observations are in concordance with the previously demonstrated inhibition of T-cell differentiation by rapamycin [387, 388, 402].

Finally we showed that rapamycin has mixed impact on T_{reg}. Indeed, rapamycin increased their frequency only in the lungs and even decreased their frequency in the peripheral blood. This was observed despite that T_{regs} from rapamycin-treated mice had higher Ki67 and pSTAT5 expression than control mice, suggesting better T_{reg} homeostasis in rapamycin-treated mice. This might indicate that rapamycin promoting effect on T_{regs} (extensively reported in literature) may be organ- and model-dependent. This hypothesis is supported by a study showing that rapamycin did not increase (and even reduced!) T_{reg} frequencies in lympho replete (WT) mice [342]. However, we also demonstrated higher expression of CD103 by T_{regs} from rapamycin-treated mice, suggesting that T_{regs} from rapamycin-treated mice might be more efficient at preventing cGvHD than those from control mice. Indeed, a prior study has

demonstrated that only $CD103^+$ T_{regs} were able to prevent cGvHD in another murine model of GvHD [392].

In summary, we observed that rapamycin prevented experimental scl-cGvHD by different mechanisms including decreasing T conv proliferation, decreasing T-cell differentiation, and perhaps by decreasing T-cell migration towards certain target organs and increasing the proportion of activated T_{regs}.

AUTORSHIP AND DISCLOSURES

LB, GF and GE performed the laboratory work and all statistical analyses for this study. JS and PD performed the pathological examinations. GE, LB and FB wrote the paper. All authors edited the mansucript. MH helped for bone marrow transplantation experiments. LB, Fba, SH-B and GE designed the study. JC and YB helped in the design of the study and in the interpretation of the results.

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