Beneficial Role of Rapamycin in Experimental Autoimmune Myositis

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Abstract

Introduction: We developed an experimental autoimmune myositis (EAM) mouse model of polymyositis where we outlined the role of regulatory T (Treg) cells. Rapamycin, this immunosuppressant drug used to prevent rejection in organ transplantation, is known to spare Treg. Our aim was to test the efficacy of rapamycin in vivo in this EAM model and to investigate the effects of the drug on different immune cell sub-populations.

Methods: EAM is induced by 3 injections of myosin emulsified in CFA. Mice received rapamycin during 25 days starting one day before myosin immunization (preventive treatment), or during 10 days following the last myosin immunization (curative treatment).

Results: Under preventive or curative treatment, an increase of muscle strength was observed with a parallel decrease of muscle inflammation, both being well correlated (R² = -0.645, p<0.0001). Rapamycin induced a general decrease in muscle of CD4 and CD8 T cells in lymphoid tissues, but spared B cells. Among T cells, the frequency of Treg was increased in rapamycin treated mice in draining lymph nodes (16.9±2.2% vs. 9.3±1.4%, p<0.001), which were mostly activated regulatory T cells (CD62LlowCD44high): 58.1±5.78% vs. 33.1±7%, treated vs. untreated, p<0.001). In rapamycin treated mice, inhibition of proliferation (Ki-67) is more important in effector T cells compared to Tregs cells (p<0.05). Furthermore, during preventive treatment, rapamycin increased the levels of KLF2 transcript in CD44low CD62Lhigh naive T cell and in CD62Llow CD44high activated T cell.

Conclusions: Rapamycin showed efficacy both as curative and preventive treatment in our murine model of experimental myositis, in which it induced an increase of muscle strength with a parallel decrease in muscle inflammation. Rapamycin administration was also associated with a decrease in the frequency of effector T cells, an increase in Tregs, and, when administered as preventive treatment, an upregulation of KLF2 in naive and activated T cells.

Introduction:

Idiopathic inflammatory myopathies are a heterogeneous group of different diseases, classified into four main categories: dermatomyositis, polymyositis which frequently overlap other connective tissue diseases, immune-mediated necrotizing myopathy, and sporadic inclusion body myositis [1–3]. Polymyositis presents endomyssial inflammatory cell infiltrates (rich in CD8+ T-cells), surrounding and invading non-necrotic muscle fibres, in parallel with a diffuse histocompatibility class I overexpression [4]. The treatment of polymyositis consists of corticosteroids frequently associated with other immunosuppressive drugs [5]. The obligatory and often severe side effects of these drugs, which have to be taken for several months or years, prompted us to propose alternative treatments, which were tested in experimental animal models.

We previously described the induction of experimental autoimmune myositis (EAM) in mice by immunization with partially-purified myosin [6]. This EAM model mimics closely polymyositis, showing muscle weakness and inflammation, and extensive CD8+ T cells and macrophages infiltrates. We further demonstrated that it was possible to induce the disease by adoptive transfer of unsorted lymph node cells or in vitro restimulated sorted CD4+ T cells in wild type mice. In this EAM model, we also demonstrated that regulatory T cells (Treg) can ameliorate the disease phenotype [6].

Tregs represent around 10% of CD4+ T cells. Tregs in mice and humans express the transcriptional factor FoxP3 encoding for the forkhead/winged-helix transcription factor scurfin [7–9]. Quantitative or qualitative deficits in the Treg compartment have been reported in many mouse models of autoimmune diseases and in humans [10–14].

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Rapamycin is a known potent immunomodulator with less side effects compared to other immunosuppressants (e.g. ciclosporin). Rapamycin has been used for a decade in transplant patients to prevent graft rejection [15]. In vivo, rapamycin has been described to increase the percentage of Treg [16–18].

The aim of this study was to test the efficacy of rapamycin in vivo in our EAM model and to investigate the effects of the drug on different immune cell sub-populations, with the ultimate goal of developing a new therapeutic approach for polymyositis.

**Methods**

**Mice**

Six to ten-week-old female BALB/c mice were purchased from Janvier Laboratories. All mice were kept under specific pathogen free conditions and manipulated according to European council directive 86/609/EEC. The study was approved by the Regional Ethical Committee 3 of Ile-de-France.

![Figure 1: Preventive administration of rapamycin permits to decrease severity of EAM. A: muscle strength (time to fall) improvement in rapamycin-treated mice. B: gastrocnemius, quadriceps, and triceps muscles inflammatory infiltrates evaluated by histological grading after hematoxylin-eosin staining as illustrated in C: the first two upper images represent a gastrocnemius section of an EAM mouse untreated with a histological score of 4 (HE, ×20). The middle picture also shows an invaded/tunnelized fiber (arrow, HE, ×40). The third image panel represents a gastrocnemius section of an EAM mouse treated with 3 mg/kg/day of rapamycin, displaying a histological score of 1 (HE, ×20). D: correlation between muscle strength (time to fall) and histological grade of inflammation.](https://www.plosone.org/article/funding-10.1371/journal.pone.0074450.g001)
Evaluation of Muscle Strength

Muscle strength was evaluated using an inverted screen test as described previously [6,19] one day before mice sacrifice. Briefly, a mouse was placed at the center of a wire mesh screen, which was inverted horizontally and the time to fall off the screen was recorded. All mice were evaluated independently by one investigator who was blinded to the immunization protocol.

Preparation of Myosin

Myosin was partially purified according to the method previously reported [6]. Briefly, A total of 30 ml of chilled 0.3 M/L KCl-0.15 M/L sodium phosphate buffer (pH 6.5) was added to 10 g of minced muscle tissue and kept on ice for 45 minutes. This homogenate was centrifuged at 15,000 rpm for 30 minutes at 4°C, and the supernatant collected. The filtrate was then diluted with 15 volumes of chilled Milli-Q-filtered (Millipore,
Figure 3. Effect of rapamycin on Treg cells. Representative dot plot of flow cytometry analysis of draining lymph nodes for percent of Treg (CD4+/CD25+:FoxP3+) in CD4+ (A) and in CD4+/CD44high (B) in controls and rapamycin (3 mg/kg/day) treated mice. Percent of Treg (CD4+/CD25+:FoxP3+) in CD4+ (A, right) and in CD4+/CD44high (B, right) in controls and rapamycin (3 mg/kg/day) treated mice. C: Histogram representative of the difference in activation of Treg (CD62Llow cell) and naïve Treg (CD62Lhigh) in controls and rapamycin (3 mg/kg/day) treated mice. D: Suppressive test. Horizontal lines indicate means. Suppressive activity of sorted CD4+/CD25+ T cells (Treg) from controls (white bars) and rapamycin (3 mg/kg/day, black bars) mice on the proliferation of autologous CD4+/CD25+ T cells (responders) stimulated with irradiated (15 Gy) splenocytes. Proliferation of responder cells (Teff) was measured by 3H-Tymidine incorporation (counts per minute, cpm). Results are indicated as percentage inhibition (±SD) compared to a max (ratio 1:0). Different Teff:Treg ratios were tested. E: Percent of Ki-67 positive cells (i.e. proliferative cells) in activated effector (aT) and activated regulatory (aTreg) T cells in controls and rapamycin (3 mg/kg/day) treated mice.

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Primer sequences
We looked at the expression of KLF2 in different cell populations using the following primers: forward 5'-AGCC-TATCTTGGCGTCCTTT-3', reverse 5'-CGCCTCGGGTT-CATTTC-3'. We normalized our results with the expression of hypoxanthine-guanine phosphoribosyl transferase (HGPT) in the same cells, using the following primers: forward 5'-CT-TCCTCCTAGACCGCTTT-3', reverse 5'-ACCTGGTTC-ATCATCGCTAA-3'.

Statistical Analysis
Data are presented as mean (±SD) for continuous variables and percentage for qualitative variables. Non-parametric Mann-Whitney test was used to compare variables. A p value < 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism version 4.0 and Instat version 3.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results
Preventive treatment with rapamycin results in a reduction of inflammatory infiltrates parallel to an increase in muscle strength
The first step in our study was to investigate preventive effects of rapamycin on EAM by testing two doses of the drug: 1 and 3 mg/kg/day.
Rapamycin was very well tolerated by all mice for the entire duration of the administration protocol, for instance no weight loss was observed (data not shown).
Compared with controls, a dose-dependent increase in muscle strength was observed (time to fall: controls 66.6 ± 4.6 seconds, Rapa 1 mg 138 ± 80 seconds, p = 0.05; Rapa 3 mg 325 ± 1001 seconds; p < 0.001 for control vs Rapa 3 mg, Figure 1A). This was accompanied with a parallel decrease in muscle inflammation attested by a lower histological score in EAM animals treated with rapamycin (controls, 3.55 ± 0.67, Rapa 1 mg, 1.85 ± 0.54, p < 0.001, Rapa 3 mg, 0.92 ± 0.69; p < 0.001 for control vs Rapa 3 mg, Figure 1B and Figure 1C). In the treated and control animals, clinical and pathological scores showed good correlation (R² = 0.645, p < 0.0001, Figure 1D).

Preventive treatment with rapamycin induced a general reduction in frequency of T cells

To explore the mechanisms involved in the improvement of muscle inflammation, we analysed lymphocyte population changes induced by rapamycin in different lymphoid organs. As shown in Figure 2, by comparison to controls, rapamycin induced a significant decrease in the absolute number of lymphocytes in every tested lymphoid organ (draining or non draining lymph nodes and spleen, Figure 2 A) with a dose effect. For instance, in popliteal draining lymph nodes, the number of total lymphocytes was 21.8 ± 6.9 ± 10⁶ in control mice compared to 11.5 ± 2.7 ± 10⁶ (p < 0.001) in Rapa 1 mg-treated animals.

The lymphopenia observed in rapamycin-treated mice was due to a decrease in CD4 and CD8 T cell populations, i.e. among CD3⁺ T cells, the decrease in number was equally partitioned between CD4⁺ and CD8⁺ T cells (Figure 2B), leading to a significant decrease in the overall T cell frequency (Figure 2C). Rapamycin had no effect on B cells whose absolute number remained stable (Figure 2B) and percent relative to T cells increased (Figure 2B) due to the reduction in T cell frequency. CD4⁺/CD8⁺ T cells ratio was not different in Rapa 1 and 3 mg/kg/day compared to the control group (figure 2B).

In the CD4⁺ T cell compartment, we observed an increase of pre-activated CD4⁺ T (CD69⁺) in popliteal draining lymph nodes

![Figure 5. Change in KLF2 pathway induced by rapamycin treatment.](image)

**Figure 5. Change in KLF2 pathway induced by rapamycin treatment.** A: Histogram plot of the shift of CCR7 expression in controls and rapamycin-treated (3 mg/kg/day) mice showing naive T cell subset (nT:CD62L⁺CD44⁻). B: Shift of CCR7 expression in controls and rapamycin-treated (3 mg/kg/day) mice in naive T cells (nT:CD62L⁺CD44⁻) an activated T cells (aT:CD62L⁻CD44⁻). C: Q-RT-PCR of KLF2 in naive T cells (nT:CD62L⁺CD44⁻), activated T cells (aT: CD62L⁻CD44⁻), naive regulatory T cells (nTreg:CD62L⁺CD44⁻), activated Treg cells (aTreg: CD62L⁻CD44⁻) in controls and rapamycin treated (3 mg/kg/day) mice.

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![Figure 6. Beneficial effect of rapamycin (3 mg/kg/day) in curative treatment of EAM.](image)

**Figure 6. Beneficial effect of rapamycin (3 mg/kg/day) in curative treatment of EAM.** Rapamycin (or water for control animals) was given orally for 10 days at a dose of 3 mg/kg. A: strength of mice evaluated by inverted screen test (time to fall in seconds). B: Gastrocnemius muscle inflammatory infiltrates evaluated by histological grading after haematoxylin-eosin staining. C: Percent of Treg cells (CD3⁺CD4⁺CD25⁺FoxP3⁺) in draining lymph nodes from controls and rapamycin-treated (3 mg/kg/day) mice.

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in rapamycin treated mice, 14.1±1.5% vs. 7.4±2.6% in controls mice (p<0.001) (figure 2D).

Despite CD4+ T cells decrease, rapamycin spares Tregs

Because changes were more marked in animals treated with rapamycin 3 mg/kg/day (Rapa 3 mg), we focused our efforts in comparing this group to control mice. An increased percent of Treg (defined as CD4+CD25+FoxP3+ T cells) was observed in the CD4+T cells compartment in Rapa 3 mg mice compared to controls (16.9±2.2% vs. 9.3±1.4%, p<0.001, Figure 3A) in draining lymph nodes. In the activated CD4+ T cells compartment (CD3+CD44+high), a higher proportion of Treg was observed in rapamycin treated mice (Figure 3B). In the Treg compartment, the percentage of activated Treg (CD62LlowCD44highTreg) was increased in rapamycin treated mice compared to control (58.1±5.78% vs. 33.1±7%, p<0.001, Figure 3C).

As rapamycin increased the relative frequency of Tregs, we tested the suppressive activity of this subset of T cells. To test Treg function, suppressor assays were performed, showing that regulatory functions of Treg were not modified by rapamycin treatment compared to control (37% vs. 39% of suppression at 2:1 suppressor:effector ratio, Figure 3D).

In the activated regulatory T cells (CD4+FoxP3+CD62Lhigh-T cells), we observed (Figure 3E) a decrease of Ki-67 cells (19±1.6 vs. 17±1.4, p<0.05) in rapamycin treated mice compared to control mice. We observed the same effect in effector T cells (CD4+FoxP3+CD62Lhigh-T cells, Figure 3E). Furthermore, inhibition of proliferation under rapamycin is more important in effector cells compared to Tregs cells (p<0.05). Because rapamycin treated mice have an increase percentage of Treg in draining lymph nodes, we wanted then to analyse Treg infiltration within muscles. Infiltrates were mainly composed by macrophages (CD11b), and CD8+ T cells (data not shown). Some CD4+ T cells were also observed (figure 4A), but Treg (CD4+FoxP3+ cells) remained sparse (figure 4A). Like for total CD4+ T cells in draining lymph nodes, there was a decrease in the absolute number of Tregs within the muscle following treatment with rapamycin even if the percentage seemed to remain stable. For 5 different mice in both groups (rapamycin treated and control), one representative cryosection was selected from the most inflamed muscle and FoxP3 and CD4 positive cells were counted. In control mice, on 5 representative sections, we counted 16 FoxP3 positive cells. In rapamycin treated mice (Figure 3E) a decrease of Ki-67 cells (19±1.6 vs. 17±1.4, p<0.05) in rapamycin treated mice compared to control (58.1±5.78% vs. 33.1±7%, p<0.001, Figure 3C).

Rapamycin modified the KLF2 pathway

An increase in the percent of Treg but also in the frequency of pre-activated CD69+ effector T cells (figure 2D) was observed in draining lymph node of Rapa 3 mg mice whereas muscular infiltrates decreased. Therefore, we hypothesized that a T cell trafficking modification under rapamycin treatment could represent one of the mechanisms of action of the drug.

KLF2 is a transcription factor which controls three important genes for lymphocyte homing: the integrins CCR7 and CD62L, involved in the entry of T cells in lymph nodes, and S1P1, involved in their exit [21]. mTOR is known to negatively regulate KLF2 [21]. We have then tested if rapamycin is able to modulate these proteins. After 25 days of treatment with rapamycin, an up-regulation of CCR7 was observed at the surface of CD44+CD62Lhigh naive T cell (897±126 mean fluorescent units [mfi] in control mice vs. 1352±414 mfi in rapamycin treated mice in draining lymph nodes, p<0.001) but also in CD62LlowCD44high activated T cells (920±168 mfi vs. 1243±343 mfi, p<0.05, Figure 5A and B). This result is in line with the observed increase level of KLF2 transcripts (Figure 5C) in CD44lowCD62Lhigh naive T cell (1.05±0.09 vs. 2.1±0.14, fold change over HPRT; p<0.05) and in CD62LlowCD44high activated T cell (0.44±0.08 vs. 1.02±0.18, fold change over HPRT; p<0.05). In naive regulatory T cells, we observed an increase of KLF2 (1.0±0.15 vs 2.0±0.19, p<0.05) but not in activated Tregs (Figure 5C).

Curative treatment with rapamycin allows for a reduction of inflammatory infiltrates, increase in Treg frequency, and a simultaneous increase in muscle strength

The final step of our study was to test the effect a curative treatment in mice with established EAM (i.e. once EAM is induced by myosin immunization). At the third immunization, all mice presented inflammatory infiltrates within muscle (Figure S2A). Compared to control mice, we observed an increase of muscle strength with rapamycin (time to fall: 64±43 seconds vs. 134±70 seconds, p<0.01, Figure 6A) and a decrease of muscle inflammatory infiltrates (3.5±0.6 vs. 2.2±0.5, p<0.001, Figure 6B). However, muscular infiltrate was higher in the curative treated mice than the preventive treated mice (2.2±0.5 vs. 0.92±0.69, p<0.001). In the treated and control animals, clinical and pathological scores showed good correlation [23] like during the preventive treatment. As in the preventive treatment, we observed an increase in Treg frequency in draining lymph nodes of rapamycin treated mice (11.9±1.92% of Tregs) compared to control mice (9.3±1.4% of Tregs; p<0.001, Figure 6C). However, curative treatment had no significant impact on the KLF2, CCR7 cell surface expression comparatively to preventive treatment (data not shown). As for the preventive treatment, we observed around 20% of Treg (FoxP3+) among the CD4+ cells within the muscle inflammatory infiltrates in treated and untreated mice (data not shown).

Discussion

In this study we showed that rapamycin decreases the severity of myositis (both clinical and pathological end points of the disease) in our model of EAM. This was achieved with both preventive and curative treatment. The amelioration of the disease phenotype was associated with a decrease in effector T cells (Teff) which was not compensated by an increase of proliferation of activated cells and a relative increase of Treg cells in draining lymph nodes. In addition, there is a change in the KLF2 pathway in preventive treatment.

A beneficial role of rapamycin in mice models of autoimmune diseases like experimental autoimmune encephalomyelitis (EAE) [17], auto-immune arthritis [22], or NOD mice (diabetic model) [23] have already been reported. Mechanism involved in disease improvement includes a lymphopenia [21] as we observed in our study. Unlike other publication [24], we observed only a modulation of T cells, but not of B cells. Such discrepancy may be explained by the fact that rapamycin inhibits mainly proliferating cell [6,21]. Rapamycin also induced an increase in the frequency of Treg cells in draining lymph nodes of EAM mice. Others have reported such observation in human and mice for other diseases but not for EAM and polymyositis [16–18,23].

The reason why anti-proliferative effect of rapamycin spare Treg may be explained by the fact that mTOR pathway is not involved in the entry of T cells in lymph nodes, and S1P1, involved in disease like experimental autoimmune encephalomyelitis (EAE) [21].
cells among Teff cells compared to Tregs. On the other hand, we did not observe an increase percentage of Treg in muscular compartment. This is in contrast with other models, in particular in EAE [17] in which Treg were strongly increased in the central nervous system and to a less extend in draining lymph nodes. Differences in immunisation protocols, in the site of inflammation (muscles vs. central nervous system), and in the treatment regimens, may induce different patterns of T cells proliferation and trafficking [25].

In our study, we also observed an increase in the expression of T cell homing marker KLF2 in rapamycin treated mice (in preventive treatment), KLF2 is a transcription factor which positively regulate the expression of the integrin CD62L, and the chemokine receptor CCR7 involved in the entry of lymphocytes in lymph nodes [26–29]. Notably KLF2−/− mice have decrease naive T cell and accumulation of T cells within tissues especially muscles because of an over expression of CCR-3 an CCR-5 [30].

Indeed, recent in vitro data showed that the KLF2 is down regulated by mTOR pathway and that rapamycin increased its expression [31]. For the first time, we show in vivo an increase of KLF2, expression in naive and effector T cells with the administration of rapamycin.

Thus together our data suggest that rapamycin improved EAM by inhibiting effector T cell responses, including autoreactive T cells proliferation (Ki-67+ cells), but also maintaining effector T cells in draining lymph nodes. Furthermore, by sparing Treg proliferation in draining lymph node, rapamycin permits to increase Treg/Teffector cells ratio which is crucial to improve Treg-mediated immunosuppression [24].

Curative treatment also permitted to decrease the severity of the myositis but less efficacious than the preventive treatment did. This discrepancy is probably due to the fact that in curative regimen muscular inflammation is already established whereas by definition in preventive settings treatments avoid muscular infiltrates and/or that the duration of treatment is longer in the preventive protocol.

Rapamycin has been extensively used as immunosuppressant in transplantation. In recent years, a growing interest in the sparing effect of rapamycin on Tregs [32] has promoted the initiation of several clinical trials in which the drug has been tested in autoimmune diseases like diabetes (Rapamune in Type 1 Diabetes, National Institute of Allergy and Infectious Diseases, number NCT00525889). To date, no cases were reported in which rapamycin was used to treat polymyositis, nevertheless, two cases of refractory dermatomyositis treated by rapamycin with good results have been published [33,34], suggesting that the drug could have a beneficial effect in patient affected by autoimmune myositis.

Conclusion
Those observations suggest that rapamycin may represent an effective new therapeutic approach in patients with polymyositis, allowing to reduce steroid administration. This approach may be particularly beneficial in this patient population, as a deficiency in Treg frequency has been reported in individuals affected by polymyositis [35].

Supporting Information

Figure S1 Description of preventive treatment (A) and curative treatment (B) by rapamycin in the EAM model. (TIFF)

Figure S2 Kinetic of histological score and correlation between muscle strength (time to fall) and histological grade. A: histological score of animals over time after 3 weekly immunizations against myosin at day 0, 7, and 14 (D0, D7, and D14, respectively). B: correlation between muscle strength (time to fall) and histological grade of inflammation after curative treatment of EAM. (TIFF)

Author Contributions
Conceived and designed the experiments: DK BS OB. Performed the experiments: NP YA. Analyzed the data: NP YA. Contributed reagents/materials/analysis tools: DK BS OB. Wrote the paper: NP YA.

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