Sustained optogenetic expression in skeletal muscle is limited by host adaptive immunity, but preserved with targeted immunosuppression

Supplemental Material

An expanded description of the side effects of rapamycin/cyclosporin treatment:

Methods: Animal weight was recorded twice weekly throughout the study. 12 weeks following the initiation of immunosuppression, blood glucose was measured, and animals were placed in metabolic cages for 24 hours to assess urine output and chow and water intake.

Results: All animals displayed behavioural indicators of good health (normal activity, posture, gait, movement, and grooming). However, body weight and weight gain differed significantly between sexes and immunosuppression treatment groups (2-way RM-ANOVA, males: FigureS2A, $F(9,117) = 193.7$, $p < 0.0001$, females: FigureS2B, $F(9, 117) = 102.0$, $p < 0.0001$).

Across the 13-week study, rapamycin/cyclosporin-treated males did not gain weight (0 versus 13 weeks; Tukey, $p=0.13$), whereas all other animals, male and female, showed significant weight gain (Tukey: vehicle-treated males, $p=0.002$; vehicle-treated females, $p=0.009$; prednisolone-treated males, $p=0.01$; prednisolone-treated females, $p=0.002$; rapamycin/cyclosporin-treated females, $p=0.003$). In males, weight differences emerged early. After two weeks of treatment, rapamycin/cyclosporin-treated males were 18% lighter than vehicle-treated males (Tukey, $p<0.01$; FigureS2A), and by 13 weeks, they were 41% lighter (Tukey, $p<0.0001$). Rapamycin/cyclosporin-treated females were also lighter than their vehicle-treated counterparts, although differences were smaller and developed later (Tukey, $p<0.03$; FigureS2C). They were 16% lighter after 4 weeks (Tukey, $p=0.03$) and 23% lighter after 13 weeks (Tukey, $p=0.003$).

The body weight of prednisolone-treated males did not differ from either rapamycin/cyclosporin- or vehicle-treated males (Tukey, $p>0.05$). In females, prednisolone treatment resulted in consistently higher body weights compared to rapamycin/cyclosporin treatment, with prednisolone-treated females being 12-15% heavier from 6 weeks and onwards (Tukey, $p=0.01$ to $p=0.03$). The only exception was at week 8 (Tukey, $p>0.05$). By week 13, prednisolone-treated females were also 10% lighter than their vehicle-treated counterparts (Tukey, $p=0.05$).

After 12 weeks of immunosuppression, metabolic parameters including blood glucose, water consumption, urine output and chow consumption were measured in all animals. Only rapamycin/cyclosporin-treated males showed elevated blood glucose levels (FigureS2C), which were 3.4-fold higher than vehicle-treated males (Tukey, $p<0.0001$), and 2.9-fold higher than prednisolone-treated animals (Tukey, $p<0.0001$). These males also consumed more water (FigureS2D, Tukey: vs vehicle and prednisolone: $p<0.0001$) and produced more urine (FigureS2E, Tukey: vs vehicle and prednisolone, $p<0.0001$).

In contrast, blood glucose, water consumption and urine output did not differ between treatment groups in female animals (Tukey, $p>0.05$), and while chow consumption did not differ between groups in either sex (FigureS2F). Fixed and interaction statistics for all metabolic measures are outlined in Supplemental Material Table S2.

Supplemental figures:

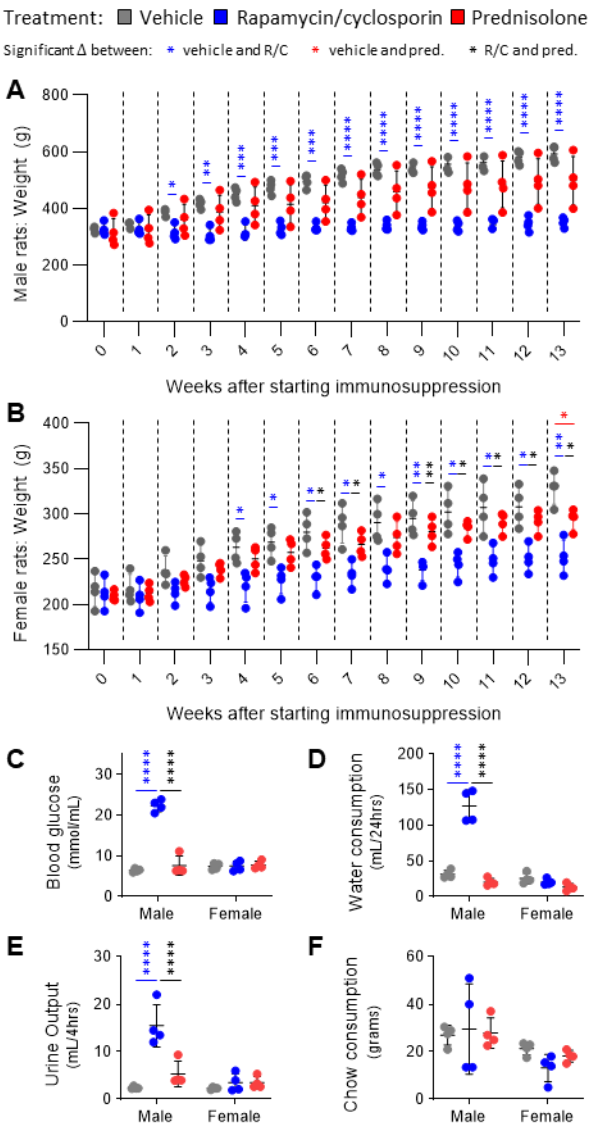

Figure S1 | Rapamycin and cyclosporin reduced growth rates in males and females, but altered metabolic parameters were specific to male animals. (A) Male rats treated with rapamycin/cyclosporin showed reduced weight gain starting two weeks after immunosuppression began (2-way RM-ANOVA, $F(9,117) = 193.7$, $p < 0.0001$). (B) Female rats treated with rapamycin/cyclosporin or prednisolone also showed reduced weight gain, but these effects emerged later—after 4 and 13 weeks of immunosuppression respectively (2-way RM-ANOVA, $F(9, 117) = 102.0$, $p < 0.0001$). (C-E) In male rats, rapamycin/cyclosporin treatment led to higher (C) blood glucose levels (2-way ANOVA, sex × treatment, $F(2, 18) = 86.8$, $p < 0.0001$), (D) water consumption (2-way ANOVA, sex × treatment, $F(1, 24) = 32.5$, $p < 0.0001$), and (E) urine output (2-way ANOVA, sex × treatment, $F(2, 18) = 20.0$, $p < 0.0001$) compared to rats treated with prednisolone or the vehicle control. None of these metrics differed between treatment groups in females. (F) Standard chow consumption did not differ between treatment groups in either sex (2-way ANOVA, sex × treatment, $F(2, 18) = 0.2$, $p = 0.8$).
Mean±SD, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05

Supplemental tables:

Table S1 | Additional statistics for light-evoked electromyography. Main and interaction effects from the linear mixed model (fixed effects: immunosuppression regimen [vehicle, prednisolone, rapamycin/cyclosporin], stimulation [unstimulated, stimulated] and endogenous EMG group [1 to 10]; random effect: animal ID). Pairwise comparisons are described in Figure 4.

Source of Variation	**F (DFn, DFd), P value**
Intercept	*F(1, 22) = 1926, p < 0.0001*
Immunosuppression	*F(2, 21) = 33, p < .0.0001*
Stimulation condition	*F(1, 334) = 246, p < 0.0001*
Endogenous EMG bin	*F(9, 344) = 285, p < 0.0001*
Immunosuppression × Stimulation condition	*F(2, 334) = 196, p < 0.0001*

Table S2 | Additional statistics for metabolic measures. Main and interaction effects (two-way ANOVA: sex × immunosuppression group [vehicle, rapamycin/cyclosporin, prednisolone]). Pairwise comparisons are described in Figure S2.

Source of Variation	**F (DFn, DFd), P value**
Blood glucose	
Sex	*F(1, 18) = 70.3, p < 0.0001*
Immunosuppression	*F(2, 18) = 86.8, p < 0.0001*
Sex × Immunosuppression	*F(2, 18) = 88.4, p < 0.0001*
Water consumption	
Sex	*F(1, 18) = 85.6, p < 0.0001*
Immunosuppression	*F(2, 18) = 64.5, p < 0.0001*
Sex × Immunosuppression	*F(2, 18) = 60.0, p < 0.0001*
Urine output	
Sex	*F(1, 18) = 24.1, p = 0.0001*
Immunosuppression	*F(2, 18) = 20.0, p < 0.0001*
Sex × Immunosuppression	*F(2, 18) = 15.3, p = 0.0001*
Chow consumption	
Sex	*F(1, 18) = 8.7, p = 0.009*
Immunosuppression	F(2, 18) = 0.2, p = 0.8
Sex × Immunosuppression	F(2, 18) = 0.8, p = 0.5