

1 Synergistic activity of IL-2 mutein with tolerogenic ImmTOR nanoparticles leads to massive expansion of
2 antigen-specific Tregs and protection against autoimmune disease

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22 **Abstract**

23 Low dose IL-2 therapy and IL-2 molecules engineered to be selective for the high affinity IL-2 receptor
24 have been shown to expand Tregs in vivo, and, in the case of low dose IL-2 therapy, has demonstrated
25 promising therapeutic benefit in autoimmune diseases. One of the potential limitations of IL-2 therapy
26 is the nonselective expansion of pre-existing Treg populations rather than induction of antigen-specific
27 Tregs, as well as potential activation of effector cells. We have recently developed biodegradable
28 nanoparticles encapsulating rapamycin, called ImmTOR, to induce selective immune tolerance to co-
29 administered antigens, such as immunogenic biologic drugs. Unlike Treg-selective IL-2 therapy, ImmTOR
30 alone does not increase total Treg numbers. However, here we demonstrate that the combination of
31 ImmTOR and an engineered Treg-selective IL-2 variant (termed IL-2 mutein) increases the number and
32 durability of total Tregs, as well as inducing a profound synergistic increase in antigen-specific Treg when
33 combined with a target antigen. We demonstrate that the combination of ImmTOR and an IL-2 mutein
34 leads to durable inhibition of antibody responses to co-administered AAV gene therapy capsid, even at
35 sub-optimal doses of ImmTOR, and provides protection in autoimmune models of type 1 diabetes and
36 primary biliary cholangitis. ImmTOR also showed the potential to increase the therapeutic window of
37 engineered IL-2 molecules by mitigating effector T cell expansion typically observed at higher doses of
38 IL-2 and preventing exacerbation of disease in a model of graft-versus-host-disease. At the same time,
39 engineered IL-2 molecules showed potential for dose-sparing of ImmTOR. Overall, these results establish
40 that the combination of ImmTOR and an IL-2 mutein show synergistic benefit on both safety and efficacy
41 to provide durable antigen-specific immune tolerance to mitigate drug immunogenicity and to treat
42 autoimmune diseases.

43

44 Introduction

45 The autoimmune disease field is experiencing a resurgence in the development of regulatory T cell
46 (Treg)-focused therapies to counterbalance autoreactive effector T cells in autoimmune disease¹. Two
47 divergent strategies that have emerged focus on either non-selectively expanding total Tregs or inducing
48 antigen-specific Tregs^{2,3}. Strategies to expand total Tregs frequently employ interleukin-2 (IL-2), a
49 critical Treg growth and survival factor^{4,5}. Tregs constitutively express a high affinity trimeric IL-2
50 receptor, comprised of the α (CD25), β (CD122) and γ (CD132) subunits (IL-2R $\alpha\beta\gamma$, $K_D \sim 100$ pM); whereas,
51 effector T cells and NK cells constitutively express an intermediate affinity heterodimeric IL-2 receptor,
52 comprised of only the β (CD122) and γ (CD132) subunits (IL-2R $\beta\gamma$, $K_D \sim 10$ nM). Low doses of IL-2 have
53 been used clinically to selectively target the high affinity IL-2R $\alpha\beta\gamma$ on Tregs⁶⁻⁹, and the difference in the
54 affinity of IL-2 for the two receptors has also been exploited by investigators to engineer IL-2 for the
55 treatment of either autoimmune diseases or oncology^{10,11}. Various engineered forms of IL-2, including
56 IL-2 muteins¹²⁻¹⁶, pegylated IL-2^{17,18}, IL-2-antibody complexes and fusion proteins¹⁹⁻²³, and CD25-IL-2
57 fusion proteins²⁴, have been designed to selectively engage the high affinity IL-2 receptor, leading to
58 biased expansion of Tregs in vivo. Large numbers of expanded and activated Tregs can provide
59 'bystander suppression' to inhibit immune responses in an antigen-independent manner, through the
60 production of immunosuppressive cytokines, sequestration of IL-2 from effector T cells, generation of
61 extracellular adenosine, and/or through Treg-mediated trogocytosis of co-stimulatory molecules
62 expressed by antigen-presenting cells²⁵⁻²⁷. Early phase clinical trials of low dose IL-2 have demonstrated
63 promising results in treatment of systemic lupus erythematosus (SLE), graft-versus-host disease (GVHD),
64 and other autoimmune conditions^{6-8,28}.

65 Despite early successes with low dose IL-2 therapies, antigen-specific immune tolerance has been a
66 long-standing goal for immunotherapy of autoimmune diseases, which are currently treated by systemic
67 immunosuppressive or immunomodulatory drugs. Indeed, preclinical studies suggest that antigen-
68 specific Tregs play a dominant role in preventing autoimmunity²⁹ and are more effective than total Tregs
69 in mitigating autoimmune disease³⁰. The ratio of Treg:effector T cells is thought to be an important
70 factor in immune homeostasis^{31,32}. Thus, the generation of large numbers of antigen-specific Tregs may
71 be necessary to offset the large pool of pre-existing autoreactive effector T cells in the context of
72 autoimmune disease. Autologous T cells can be engineered ex vivo to express specific T cell receptors
73 (TCRs) in order to generate large numbers of antigen-specific Tregs; however, this strategy has the
74 drawback of complex and costly manufacturing of personalized cell therapies. An alternative strategy is
75 the in vivo induction of antigen-specific Tregs. Early clinical trials of antigen-specific immune tolerance
76 strategies have shown encouraging results³³⁻³⁵.

77 We have developed biodegradable tolerogenic nanoparticles, termed ImmTOR, which encapsulate
78 rapamycin, a macrolide inhibitor of the mTOR pathway^{36,37}. ImmTOR nanoparticles selectively
79 biodistribute to the spleen and liver following intravenous injection, and they induce a tolerogenic
80 phenotype in antigen-presenting cells that endocytose the nanoparticles³⁷⁻³⁹. ImmTOR has been shown
81 to induce immune tolerance to a variety of co-administered antigens, as demonstrated by: 1) induction
82 of antigen-specific Tregs; 2) the ability to transfer tolerance from treated mice to naïve recipients; 3) the
83 ability to withstand subsequent challenge with the antigen alone; and 4) the ability to maintain
84 immunological responses to unrelated antigens³⁶. Therapeutic administration of ImmTOR + antigen has
85 been shown to inhibit disease relapse in a relapsing-remitting model of experimental autoimmune
86 encephalomyelitis (EAE)^{38,40}. In addition, ImmTOR enables repeated dosing of highly immunogenic

87 microbial therapeutic proteins, such as a fungal-derived uricase enzyme (pegadricase), a bacterial-
88 derived immunotoxin, and viral-derived gene therapy vectors^{33, 37, 41}. ImmTOR dosed in combination with
89 pegadricase has been shown to inhibit the formation of anti-drug antibodies in humans³³ and is being
90 evaluated in Phase 3 clinical trials for the treatment of chronic refractory gout (NCT04513366 and
91 NCT04596540).

92 Here we describe an in vivo strategy to leverage the benefits of polyclonal Treg expansion as well as
93 induction and expansion of antigen-specific Tregs using a combination of a Treg-selective IL-2 mutein
94 and ImmTOR co-administered with a target antigen. This combination therapy approach produces a
95 massive and synergistic increase in antigen-specific Tregs in vivo. When administered in combination
96 with an adeno-associated virus (AAV) gene therapy vector, treatment with ImmTOR and an IL-2 mutein
97 led to profound synergistic inhibition of anti-AAV antibodies, even at sub-therapeutic doses of ImmTOR.
98 Similarly, combining ImmTOR + IL-2 mutein treatment with a nanoparticle-encapsulated autoantigen
99 protected non-obese diabetic (NOD) mice from the development of Type 1 diabetes (T1D) and was
100 efficacious in a mouse model of primary biliary cholangitis (PBC). In addition, ImmTOR + IL-2 mutein
101 treatment increased the therapeutic window of engineered IL-2 by restraining effector cell activation
102 and preventing disease exacerbation in a model of GVHD. Taken together, these results suggest that the
103 combination of ImmTOR with a Treg-selective IL-2 molecule could be a modular and effective strategy to
104 promote specific immune tolerance to a variety of target antigens.

105

106 **Results**

107 **Combination of Treg-selective IL-2 + ImmTOR leads to biased Treg expansion**

108 To investigate the effects of combining ImmTOR and a Treg-selective IL-2 on nonselective expansion of
109 total Tregs, we employed a mouse IL-2 mutein fused to an Fc domain for extended in vivo half-life, as
110 described by Gavin and colleagues (known as Fc.IL2m)^{15, 42}. Mice treated with a single dose of Fc.IL2m
111 alone showed a dramatic increase in total splenic Tregs, with a peak at 4 days and levels declining back
112 to baseline by day 14 (Figure 1A, Suppl Fig 1), as previously described¹³. As expected, mice treated with
113 ImmTOR alone showed little or no increase in total Tregs (Figure 1A, B) compared to naïve mice.
114 However, the addition of ImmTOR amplified Treg expansion in response to Fc.IL2m. The Treg response
115 to ImmTOR+Fc.IL2m lagged behind that observed with Fc.IL2m alone, with levels peaking approximately
116 7 days after treatment (Figure 1A, B). Moreover, Treg levels remained significantly increased at 14 days
117 after treatment with ImmTOR+Fc.IL2m compared to both naïve mice and mice treated with Fc.IL2m
118 alone. The absolute number of Tregs and the percent Tregs of total CD4⁺ T cells showed dose-
119 dependent increases (up to 13-fold) following treatment with ImmTOR+Fc.IL2m compared to naïve mice
120 (Figure 1C), and striking increases were observed in the number and percentage of Ki67⁺ proliferating
121 Tregs and Helios⁺ stable Tregs (Figure 1C, Suppl Figure 2A). Whole animal imaging of mice expressing
122 red fluorescence protein (RFP) under control of the Foxp3 promoter provided in situ confirmation of
123 increased Foxp3 expression when ImmTOR was co-administered with Fc.IL2m (Figure 1D). Notably,
124 treatment with the Treg-selective Fc.IL2m led to increases in CD8⁺ cytolytic T cells (CTL), effector CD4⁺ T
125 cells (Teff), and natural killer (NK) cells at high doses of 18 and 27 µg (Figure 1E). In contrast, the
126 addition of ImmTOR to Fc.IL2m suppressed the expansion of these immune effector cell subsets. The
127 increased numbers of Tregs combined with suppression of effector cells resulted in substantially
128 increased Treg:effector cell ratios in animals treated with ImmTOR + Fc.IL2m compared to mice treated

129 with Fc.IL2m alone or naïve mice (Figure 1F). Similar effects of ImmTOR+Fc.IL2m treatment were
130 observed in the liver, although the degree of Treg expansion was lower than that observed in the spleen
131 (Suppl Figure 2B).

132 We observed that circulating Fc.IL2m showed slower clearance in animals treated with ImmTOR+Fc.IL2m
133 versus those treated with Fc.IL2m alone (Suppl Figure 3A). The clearance of Fc.IL2m correlated with the
134 kinetics of IL-2R α (CD25) expression, which was delayed in the ImmTOR+Fc.IL2m treated group (Suppl
135 Figure 3B), consistent with the delayed peak of Foxp3⁺ Treg (Figure 1A). Circulating levels of Fc.IL2m
136 were ~10-fold higher at Day 4 in animals treated with ImmTOR+Fc.IL2m compared to those that
137 received only Fc.IL2m. Treatment with ImmTOR+Fc.IL2m led to greater demethylation of the Foxp3 and
138 EOS genes compared to treatment with Fc.IL2m alone, although the demethylation pattern of the Helios
139 gene was similar in animals treated with ImmTOR+Fc.IL2m and those that received Fc.IL2m only (Suppl
140 Figure 3C). ImmTOR alone showed no significant effects on methylation patterns.

141 As Fc.IL2m is a mouse IL-2-derived mutein, we sought to evaluate the synergy of ImmTOR with a human
142 IL-2-derived molecule. To this end, we made use of an engineered Treg-selective human IL-2
143 immunocytokine, denoted F5111 IC, which is comprised of the human anti-IL-2 antibody F5111²⁰
144 covalently tethered to human IL-2²². F5111 IC has been shown to potently and selectively stimulate the
145 high affinity IL-2R resulting in robust in vitro activation and in vivo expansion of Tregs²². We evaluated
146 the activity of F5111 IC and ImmTOR in immunodeficient NOD SCID gamma mice 2-3 weeks after
147 engraftment with human peripheral blood mononuclear cells (HuPBMC). The HuPBMC mice, which are
148 prone to develop GVHD, showed marked expansion of Treg, CD8⁺ T cells, and NK cells after F5111 IC
149 treatment (Figure 2A). Although the mice did not show signs of GVHD at the time of treatment, the
150 expansion of effector cells may reflect sub-clinical inflammation of HuPBMC in response to host mouse
151 antigens. Importantly, the addition of ImmTOR to F5111 IC treatment enabled expansion of Treg but
152 inhibited the expansion of CD8⁺ T cells and NK cells (Figure 2A). We next evaluated the effects of F5111
153 IC and ImmTOR in a model of GVHD in which host mice were irradiated prior to transfer of HuPBMC,
154 which accelerates disease course. F5111 IC alone exacerbated disease, leading to increased mortality,
155 while the combination of ImmTOR + F5111 IC prolonged survival and improved disease scores (Figure 2B
156 and Suppl Figure 4). Treatment with ImmTOR alone was similarly efficacious, but the combination with
157 F5111 IC showed a trend to better durability of response.

158 We next assessed the effects of F5111 IC in a non-disease setting using engineered knock-in mice
159 expressing human IL-2R $\alpha\beta$, which can form functional IL-2 receptors with endogenously expressed
160 mouse IL-2R γ . F5111 IC induced robust Treg expansion in the engineered human IL-2R $\alpha\beta$ mice without
161 substantial expansion of CD8⁺ T cells (Figure 2C). The addition of ImmTOR to F5111 IC resulted in a
162 corresponding synergistic expansion of Tregs, similar to that observed in wild-type mice treated with
163 ImmTOR+Fc.IL2m. Collectively, the results of our mouse and human Treg expansion studies demonstrate
164 that combination treatment with ImmTOR and Treg-selective IL-2 molecules induces selective
165 promotion of Treg proliferation, synergistically enhancing the activity of the Treg-selective IL-2
166 molecules alone while restraining effector cell activation.

167 **ImmTOR+Fc.IL2m treatment ameliorates autoimmune hepatitis**

168 The activity of the ImmTOR+Fc.IL2m was evaluated in a model of autoimmune hepatitis induced by
169 systemic administration of the concanavalin A, a lectin that causes polyclonal lymphocyte activation and

170 hepatic infiltration of activated immune cells. Previous studies have shown that Treg depletion with
171 anti-IL-2R α antibodies exacerbated disease while adoptive transfer of Treg ameliorated disease⁴³. Both
172 ImmTOR and Fc.IL2m monotherapies inhibited infiltration of activated effector T cells, and the
173 combination treatment led to a further reduction in T cell infiltrates (Figure 3A). Similar, though more
174 modest, reductions were observed in activated NK cells; whereas reductions in activated NKT cells,
175 neutrophils, and macrophages were primarily mediated by ImmTOR (Suppl Figure 5). Both ImmTOR
176 and Fc.IL2m reduced production of serum interferon- γ (IFN- γ) and, to a lesser extent, of CXCL1
177 chemokine (Figure 3B). Combination treatment further reduced the levels of both IFN- γ and CXCL1,
178 whereas reductions in IL-6 were primarily mediated by ImmTOR. ImmTOR+Fc.IL2m administration also
179 induced increased production of FGF21, a hepatoprotective stress-response growth factor (Figure 3C).
180 Overall, this model illustrates the therapeutic potential for ImmTOR+Fc.IL2m combination therapy in
181 autoimmune hepatitis.

182 **ImmTOR+Fc.IL2m leads to synergistic induction and expansion of antigen-specific Treg**

183 ImmTOR has been shown to induce antigen-specific Treg to co-administered antigens^{36,37}. We therefore
184 sought to test whether ImmTOR+Fc.IL2m could enhance the induction of antigen-specific Treg when co-
185 administered with antigen. In these experiments, we utilized ovalbumin (OVA) as the target antigen
186 following adoptive transfer of OVA-specific OT-II CD4⁺ T cells. As expected, ImmTOR+OVA did not
187 expand pre-existing host Treg but induced OT-II Foxp3⁺ Treg (Figure 4A). In contrast, Fc.IL2m+OVA
188 expanded host Tregs but did not have a significant effect on OT-II Tregs. The triple combination of
189 ImmTOR+Fc.IL2m+OVA led to a profound synergistic expansion of OVA-specific OT-II Tregs, while also
190 increasing total host Treg compared to Fc.IL2m+OVA or ImmTOR+OVA treatment alone.
191 ImmTOR+Fc.IL2m without OVA had only a modest effect on OT-II Tregs, consistent with previous
192 findings that the target antigen must be co-administered with ImmTOR to induce antigen-specific
193 Tregs³⁷. Increasing amounts of OVA led to a dose-dependent increase in OT-II Treg in response to
194 ImmTOR+Fc.IL2m, plateauing at approximately 100 μ g OVA (Figure 4B). As anticipated, there was no
195 significant effect of increasing amounts of OVA on the host Treg response.

196 Free OVA is expected to biodistribute widely, whereas ImmTOR has been shown to biodistribute
197 selectively to the spleen and liver³⁷⁻³⁹. Thus, only a small proportion of the total free OVA dose is
198 expected to co-localize with the splenic and hepatic antigen-presenting cells that endocytose ImmTOR.
199 We therefore examined whether formulating ovalbumin in nanoparticles would improve the efficiency
200 of OT-II Treg expansion by increasing co-localization with ImmTOR. Nanoparticles encapsulating 0.05 μ g
201 OVA (NP-OVA) enabled significantly more OT-II Treg induction and expansion in response to
202 ImmTOR+Fc.IL2m treatment than did free OVA at 100-fold higher doses (Figure 4C). Maximal Treg
203 expansion was observed at 0.5 μ g nanoencapsulated OVA, with further increase of NP-OVA providing no
204 additional Treg elevation. Taken together, these OT-II mouse studies established that ImmTOR+Fc.IL2m
205 treatment leads to robust antigen-specific Treg expansion when co-administered with the target
206 antigen, particularly with nanoencapsulated antigen.

207 **ImmTOR+Fc.IL2m treatment synergistically inhibits anti-AAV antibody responses**

208 Combination ImmTOR+Fc.IL2m treatment was assessed for the ability to inhibit antibody responses to
209 an adeno-associated virus (AAV) gene therapy vector. Mice immunized with two doses of AAV8 on Day
210 0 and Day 56 developed rapid and robust anti-AAV immunoglobulin G (IgG) antibody responses (Figure

211 5). As previously reported^{44, 45}, ImmTOR was shown to inhibit anti-AAV antibody responses when co-
212 administered with AAV vectors at a dose of 200 µg; however, some animals exhibited late breakthrough
213 of anti-AAV antibodies at Day 91 (Figure 5). Suboptimal doses of 50 or 100 µg ImmTOR resulted in
214 earlier breakthrough of antibody responses. Similarly, Fc.IL2m co-administered with AAV on Days 0 and
215 56 showed weak modulation of AAV immunogenicity, with antibodies detected as early as 12 days after
216 immunization. In contrast, ImmTOR+Fc.IL2m combination treatment resulted in complete inhibition of
217 anti-AAV antibodies, even at sub-optimal doses of ImmTOR. Anti-AAV IgG levels were significantly lower
218 in animals treated with ImmTOR+Fc.IL2m compared to those treated with ImmTOR alone from day 33
219 onwards (Suppl Table ST1). These results indicate that combining ImmTOR with Fc.IL2m provides
220 synergistic benefit for the efficacy and durability of AAV immunogenicity mitigation.

221 Similar synergistic effects of ImmTOR+Fc.IL2m were observed using a high vector dose of 5E13 vg/kg
222 AAV8, showing control of anti-AAV IgG development for more than 4 months after AAV inoculation
223 (Suppl Fig 6A). Immune phenotyping of splenocytes also reflected the synergistic effect of ImmTOR and
224 Fc.IL2m. There were trends towards increased numbers of total splenic CD8⁺ T cells, CD4⁺ effector T
225 cells, and B cell plasmablasts four days after administration of a high vector dose of AAV alone (Suppl Fig
226 6B). Robust expansion of total Tregs was observed at Day 4 when Fc.IL2m was co-administered with
227 AAV; however, this was also accompanied by substantial expansion of CD8⁺ T cells and plasmablasts.
228 The combination of ImmTOR with Fc.IL2m co-administered with high dose AAV induced robust
229 expansion of total Tregs at Days 4 and 7 while inhibiting effector T cell expansion, resulting in
230 significantly elevated Treg:CTL ratios (Suppl Fig 6B, Suppl. Table ST2).

231 **Efficacy of ImmTOR+Fc.IL2m in autoimmune disease is enhanced by co-administration of** 232 **nanoencapsulated antigen**

233 We next evaluated the ability of ImmTOR + Fc.IL2m to prevent type 1 diabetes in the NOD mouse model.
234 NOD mice were administered 4 monthly treatments starting at 8 weeks of age (Figure 6A). ImmTOR and
235 ImmTOR+Fc.IL2m were administered in the absence or presence of nanoparticle-entrapped hybrid
236 diabetes peptide 6.9⁴⁶ (NP-HIP6.9). Whereas 7 out of 10 mice in the control group progressed to
237 diabetes by week 30, all treated groups showed evidence of disease protection (Figure 6A and 6B). The
238 cohort treated with ImmTOR+Fc.IL2m combined with NP-HIP6.9 was the only group that did not have a
239 single diabetic mouse by week 33. Notably, even in the absence of co-delivered antigen, the
240 ImmTOR+Fc.IL2m combination protected 9 out of 10 mice from diabetes at week 33.

241 We also assessed the activity of ImmTOR and Fc.IL2m in NOD.C3C4 mice, which spontaneously develop
242 an autoimmune disease of the liver which closely resembles primary biliary cholangitis (PBC)^{47, 48}. The
243 primary T cell epitope has been mapped to a peptide in the inner lipoyl domain of the E2 component of
244 the pyruvate dehydrogenase complexes (PDC-E2-ILD)⁴⁷. Mice were treated with three monthly doses of
245 ImmTOR, ImmTOR+Fc.IL2m or ImmTOR+Fc.IL2m combined with nanoencapsulated PDC-E2-ILD antigen
246 (NP-PDC-E2-ILD) (Figure 7A). Treatment with ImmTOR+Fc.IL2m significantly reduced bile duct epithelial
247 degeneration, biliary hyperplasia and liver inflammation (Figure 7B). Co-administration of NP-PDC-E2-
248 ILD provided additional benefit. Liver histology showed striking biliary pathology, with marked peri-
249 biliary mononuclear cell infiltrates, biliary hypercellularity and ductular ectasia in both female (Figure
250 7C-F) and male mice (Figure 7 G-J). Treatment with ImmTOR (Figure 7D and 7H), ImmTOR+Fc.IL2m
251 (Figure 7E and 7I), and ImmTOR+Fc.IL2m+NP-PDC-E2-ILD (Figure 7F and 7J), showed progressive
252 improvement of all histologic features, with the triple therapy showing only minimal residual disease

253 pathology. Collectively, these AAV immunogenicity and T1D and PBC autoimmune disease models
254 highlight the therapeutic promise of combining ImmTOR with a Treg-selective IL-2 molecule, particularly
255 in the context of antigen co-administration.

256

257 **Discussion**

258 We describe here profound synergistic activity between ImmTOR nanoparticles carrying rapamycin and
259 engineered IL-2 molecules that selectively activate Tregs to expand the number and durability of total
260 Treg population, as well as in inducing and expanding antigen-specific Tregs in the presence of a co-
261 administered target antigen. This combination therapy leverages the large body of preclinical and
262 clinical work showing that Treg-directed IL-2 therapy can be used to selectively expand pre-existing
263 Tregs in vivo, particularly memory and activated Tregs, for the treatment of a wide range of
264 autoimmune diseases,^{4, 5, 49} while adding the ability to induce autoantigen-specific Tregs, which have
265 been shown to be more effective than Tregs that are not disease-specific in animal models of
266 autoimmune disorders³⁰. While expansion of total Tregs has shown benefit in the treatment of
267 autoimmune diseases in animals and in humans⁶⁻⁹, the ability to induce Tregs of novel antigen specificity
268 may have additional benefits in autoimmune diseases driven by antigens for which natural thymic-
269 derived Tregs are unlikely to exist. These include neoantigens created by post-translational modification
270 of self-antigens, such as hybrid antigens in the case of type 1 diabetes and citrullinated antigens in the
271 case of rheumatoid arthritis, as well as diseases driven by environmental antigens, such as
272 transglutaminase-modified gluten proteins in the case of celiac disease^{32, 46, 50-54}. In addition, our
273 technology offers the possibility of inducing of antigen-specific immune tolerance to foreign therapeutic
274 proteins, such as microbial enzymes or viral gene therapy vectors, which would be desirable to mitigate
275 immunogenicity that can compromise the safety and/or efficacy of these treatments⁵⁵⁻⁵⁷.

276 Tregs are highly responsive to in vivo therapy with low dose IL-2 or engineered Treg-selective IL-2.
277 Curiously, purified Treg do not proliferate in response to IL-2 in vitro⁵⁸, suggesting that other signals,
278 such as endogenous antigens presented by antigen-presenting cells and/or co-stimulation, may be
279 required for Tregs to respond to IL-2. Although Tregs are primed to respond to IL-2 in vivo, they do not
280 respond to in vivo treatment with rapamycin-loaded nanoparticles (ImmTOR) alone (Figure 4A).
281 Rapamycin has been used to expand Tregs ex vivo, but the culture conditions require either the addition
282 of exogenous IL-2 or polyclonal activation of total T cells, which are a potential source of IL-2^{59, 60}. We
283 have demonstrated that in vivo administration of ImmTOR with a target antigen promotes induction of
284 antigen-specific Treg but does not impact total Treg abundance^{37, 38, 40} (Figure 4A). The number of
285 antigen-specific Tregs induced by ImmTOR is limited, perhaps because production of endogenous IL-2 is
286 limited to antigen-specific effector cells responding to the same antigen. We hypothesized that the
287 number and durability of antigen-specific Tregs could be further enhanced by the addition of exogenous
288 Treg-selective IL-2. Our in vivo results are consistent with in vitro studies showing that the addition of
289 rapamycin increased Treg abundance in T cells treated with IL-2 or activated with anti-CD3 and anti-
290 CD28 antibodies⁵⁹. Mechanistic studies indicate that the addition of rapamycin to IL-2 stimulated T cells
291 is due to increased frequency of Foxp3 expression rather than selective proliferation of Foxp3⁺ cells⁵⁹.

292 Interestingly, a single administration of Fc.IL2m with ovalbumin showed little or no specific expansion of
293 adoptively transferred OVA-specific T cells (Figure 4A). Recently, Gavin and colleagues demonstrated
294 that multiple cycles of treatment with an Fc.IL2m combined with OVA in the form of a dendritic cell-

295 targeted anti-DEC205-OVA fusion protein were required to induce and expand OVA-specific OT-II
296 Tregs⁴². Each cycle of treatment consisted of initially expanding total Tregs, including adoptively
297 transferred OT-II cells, using an IL-2 mutein alone, followed 2-4 days later with anti-DEC205-OVA. The
298 rationale for this approach was to provide a selective survival strategy for the OT-II Tregs by activating
299 them with antigen at the peak of total Treg expansion. A single cycle of IL-2 mutein followed by
300 DEC205-OVA treatment showed no significant increase in OT-II Tregs 6 days after initiation of treatment;
301 however, three weekly cycles of treatment increased OT-II Tregs from a baseline of ~3% to 18%. These
302 results are consistent with our results showing that the same engineered IL-2 mutein showed little or no
303 expansion of antigen-specific OT-II T cells after a single dose. In contrast, a single treatment of ImmTOR
304 + IL-2 mutein + OVA increased OT-II Tregs from a baseline of ~3% to ~45% by 7 days after treatment
305 (Figure 4A). Daniel et al. showed that everolimus, a second generation rapalogue could also expand
306 adoptively transferred antigen-specific Tregs when combined with the high affinity IL-2R-biased anti-IL-2
307 antibody JES6-1 complexed with IL-2 + antigen⁶¹. In this study, daily dosing of everolimus at 100µg/day
308 for 14 days, a regimen that is typically used to mediate chronic immune suppression to prevent graft
309 rejection, was required for Treg expansion. In contrast, our results show that a single dose of 100 µg
310 ImmTOR was sufficient to induce antigen-specific Treg expansion when combined with an IL-2 mutein.
311 Our results therefore suggest that combination treatment with ImmTOR and Treg-biased IL-2 molecules
312 may allow for more efficient expansion of antigen-specific Treg compared to previous strategies.

313 Combination therapies are warranted for complex and serious diseases, but these approaches are often
314 limited by additive or synergistic toxicity. ImmTOR co-administration with Treg-selective IL-2 may
315 represent a rare exception in which combination therapy is less toxic than the individual components.
316 The dose limiting toxicity of ImmTOR in human clinical trials has been stomatitis, a common rapamycin-
317 associated side-effect³³. Evaluation of the combination of ImmTOR with an IL-2 mutein showed
318 synergistic activity in preventing antibody responses to an AAV gene therapy vector, even at
319 subtherapeutic doses of ImmTOR (Figure 5), suggesting that the addition of an IL-2 mutein could allow
320 for dose-sparing of ImmTOR. Conversely, the primary concern of IL-2-based therapies is the activation
321 and expansion of effector cells, including CD4⁺ and CD8⁺ effector T cells, as well as NK cells⁶².
322 Rapamycin, the active component of ImmTOR, is known to inhibit effector cell proliferation, while being
323 permissive for Treg proliferation, and we observed that the addition of ImmTOR to high-dose IL-2
324 mutein therapy mitigated the expansion of effector cells in healthy mice (Figure 1E). Similarly, the
325 combination of ImmTOR with an IL-2 mutein mitigated effector cell expansion following administration
326 of high vector doses of AAV, which can cause hepatic inflammation⁵⁷ (Suppl Figure 6B). One potential
327 concern for Treg-selective IL-2 therapies is that in settings of inflammatory disease, activated effector T
328 cells can transiently express IL-2R α , leading to the formation of the high affinity IL-2R $\alpha\beta\gamma$ ^{10,11}. Indeed,
329 following adoptive transfer of human PBMC into immunodeficient mice, a setting which can lead to
330 GVHD, administration of the Treg-selective IL-2 fusion protein F5111 IC alone led to exaggerated
331 expansion of effector T cells (Figure 2A). However, this expansion was prevented by the addition of
332 ImmTOR. The increased expansion of effector cells observed in HuPBMC mice treated with F5111 IC
333 alone correlated with exacerbation of disease in a HuPBMC GVHD model. Notably the addition of
334 ImmTOR to F5111 IC significantly increased survival in this model (Figure 2B). Disease exacerbation has
335 also been reported for the IL-2/JES6-1 anti-IL-2 antibody complex in a mouse model of inflammatory
336 arthritis induced by infection with chikungunya virus⁶³. Administration of IL-2/JES6-1 during active
337 infection increased both Tregs and effector T cells resulting in disease worsening, while prophylactic
338 administration in healthy mice prevented subsequent disease. Thus the activity of engineered IL-2

339 molecules may be dose-limited due to its effects on effector cells in settings of inflammation. The
340 addition of ImmTOR increases the therapeutic window of Treg-selective IL-2 by restraining effector cell
341 activation while synergistically increasing Tregs. Another potential concern related to engineered IL-2
342 molecules is their potential for immunogenicity. ImmTOR has been shown to inhibit immunogenicity of a
343 variety of co-administered biologic therapies and could thus counteract potential anti-drug antibody
344 responses³⁷. Caution is warranted, as the combination of rapamycin with low dose IL-2 was reported to
345 induce transient impairment of β cell function in a small clinical trial conducted in patients with Type 1
346 diabetes⁶⁴. The authors speculated that the toxicity was related to IL-2, as β cell impairment was
347 observed in patients that did not receive the full course of rapamycin and was most significant in the
348 first month of therapy, concordant with IL-2 treatment. Our use of an engineered Treg-selective IL-2
349 molecules with long circulating half-life combined with ImmTOR could help mitigate potential toxicities
350 associated with low dose cytokine administration.

351 The combination of ImmTOR with engineered Treg-selective IL-2 molecules showed potent synergistic
352 activity in inhibiting antibody responses against an AAV gene therapy vector (Figure 5, Suppl Figure S5A).
353 Currently, AAV gene therapies are limited to a single systemic administration due to development of
354 high neutralizing antibody titers⁵⁷. Even low titers of neutralizing antibodies may block transduction;
355 thus, potent and durable inhibition of anti-capsid antibodies would be required to enable vector re-
356 administration. ImmTOR+Fc.IL2m combination therapy also showed potent activity in preventing type 1
357 diabetes in NOD mice (Figure 6). In this model, ImmTOR+Fc.IL2m combined with a nanoencapsulated
358 chromogranin A-insulin hybrid peptide provided the longest disease-free duration of activity, although
359 ImmTOR and ImmTOR+Fc.IL2m administered in the absence of exogenous antigen also provided strong
360 protection. Similarly, ImmTOR+Fc.IL2m showed significant activity in a mouse model of PBC, with
361 marked reduction of peri-biliary mononuclear cell infiltrates, biliary hypercellularity and ductular ectasia
362 (Figure 7). The addition of nanoencapsulated PDC-E2-ILD antigen further improved activity of
363 ImmTOR+Fc.IL2m. Taken together, these results reinforce the importance of driving antigen-specific
364 tolerogenic responses provided by co-administration of nanoencapsulated antigens. However, the
365 efficacy of ImmTOR+Treg-selective IL-2 alone in models of both T1D and PBC suggests the possibility
366 that this combination may be able to induce tolerogenic immune responses to endogenously expressed
367 autoantigens in the context of autoimmune disease. In summary, our work demonstrates that
368 combining ImmTOR with engineered Treg-selective IL-2 molecules provides a promising approach to
369 mitigate pathogenic autoimmunity by leveraging both bystander suppression through expansion of total
370 Tregs as well as inducing and expanding antigen-specific Tregs.

371

372 **Materials and Methods**

373 **ImmTOR, other nanoparticles and IL-2 mutein molecules**

374 Rapamycin containing nanoparticles (ImmTOR) were manufactured as described earlier^{34,35}. ImmTOR
375 doses were based on rapamycin content ranging from 50 to 300 μ g per mouse. Rapamycin (sirolimus)
376 was manufactured by Concord Biotech (Ahmedabad, India). Antigen-containing nanoparticles (NP) were
377 prepared using a water/oil/water (W/O/W) double-emulsion solvent evaporation method as
378 described³⁵. Briefly, PLGA (50:50) and pegylated polylactic acid (PLA-PEG) were dissolved in
379 dichloromethane to form the oil phase. An aqueous solution of antigen (chicken ovalbumin or OVA
380 protein, hybrid insulin peptide HIP6.9 (LQTLALNAARDP), or PDC-E2-ILD (amino acids 213-314)⁶⁵ was then

381 added to the oil phase and emulsified by sonication (Branson Digital Sonifier 250A). Following
382 emulsification of the antigen solution into the oil phase, a double emulsion was created by adding an
383 aqueous solution of polyvinyl alcohol and sonicating a second time. The double emulsion was added to a
384 beaker containing PBS and stirred at RT for 4 h to allow the dichloromethane to evaporate. The resulting
385 NPs were washed twice by centrifuging at $75,600 \times g$ for 50 min at 4 °C followed by resuspension of the
386 pellet in PBS. Concentration of extracted antigens was measured by HPLC. Dynamic Light Scattering
387 (DLS) analysis of particle size and PDI was performed using a Malvern Zetasizer Nano-ZS ZEN 3600. All
388 the nanoparticles loaded with antigens exhibited a particle size distribution ranging between 140-155
389 nm with a low polydispersity index (<0.15). Recombinant PDC-E2-ILD was manufactured by Genscript
390 (Piscataway, NJ) using its proprietary E. coli expression system. Mouse IL2 mutein (Fc.IL2m) was
391 constructed based on the sequence Fc.Mut24 published by Khoryati et al.¹³ The protein was
392 manufactured by Genscript, using its proprietary CHO mammalian expression system. F5111 IC was
393 produced as previously described²⁰.

394

395 **Viruses**

396 AAV8-SEAP was manufactured by SAB Tech (Philadelphia, PA, USA) using their proprietary helper
397 plasmid and AAV8AAP plasmids, and the plasmid containing the gene of interest. The plasmids were
398 transfected into adherent human embryonic kidney (HEK) 293 cells and harvested 72 hours after
399 transfection by cell lysis. The clarified supernatant from the harvest was purified by CsCl₂ gradient, and
400 the AAV8-containing fraction was collected. The viral vector band was assayed by SDS–polyacrylamide
401 gel electrophoresis (PAGE) gel and silver stain to determine a viral titer, which was then confirmed by
402 qPCR using ITR-specific primers.

403

404 **Mice**

405 Immunologically naïve, female C57BL/6 mice aged 36-52 days (or 17-18g) were purchased from Charles
406 River Laboratories (Wilmington, MA). Similarly aged B6.Cg-Tg(TcraTcrb)425Cbn/J mice (also known as
407 OT-II mice), expressing a T cell receptor (TCR) specific for chicken ovalbumin 323-339 peptide
408 (OVA323-339) in the context of I-Ab resulting in OVA-specific CD4⁺ T cells were purchased from Jackson
409 Laboratories (Bar Harbor, ME). Non-obese diabetic (NOD) NOD/ShiLtJ strain and FoxP3-IRES-mRFP
410 (C57BL/6-Foxp3tm1Flv/J) mice co-expressing expressing the Foxp3 (forkhead box P3) gene with
411 monomeric red fluorescent protein (mRFP) were also purchased from Jackson Laboratories. Human Tg-
412 IL-2/IL-2R α /IL-2R β mice carrying knock-out mutations for IL-2, and IL-2 receptor alpha and beta chains
413 and expressing their human homologues were purchased from Biocytogen (Wakefield, MA). NCG mice
414 (NOD-*Prkdc*26^{emCd52}*Il2rg*^{em26Cd22}/NjuCrl) carrying a mutation in *Sirpa* and knockouts of *Prkdc* and *Il2rg*
415 genes and thus lacking functional/mature T, B, and NK cells, along with reduced macrophage and
416 dendritic cell function were purchased from Charles River Laboratories. NCG mice were reconstituted
417 with human PBMC from one of three different donors used in separate studies per manufacturer's
418 instructions. A similar strain, NSG (NOD.Cg-*Prkdc*scid *Il2rg*tm1Wjl/SzJ), carrying mutations in *Prkdc* and
419 null allele of the *Il2rg* (*Il2rg*null) lacking functional/mature T, B, and NK cells was purchased from
420 Jackson Labs, and used in GVHD studies. NOD.c3c4 (NOD.B-(D3Mit93-D3Mit124)(D4Mit114-
421 D4Mit142)/1112Mrk) mice, known to spontaneously develop autoimmune liver disease and specifically,
422 primary biliary cholangitis (PBC)^{62, 63} were purchased from Jackson Labs and then bred in-house. To
423 minimize the potential effects of stress, mice were acclimated to the Animal Care Facility at Selecta for
424 at least three days prior to injection. All the experiments were conducted in strict compliance with NIH
425 Guide for the Care and Use of Laboratory Animals and other federal, state and local regulations and
426 were approved by Selecta's IACUC.

427

428 **Animal Injections**

429 Mice were injected (i.v., tail vein or retro-orbital plexus) with ImmTOR nanoparticles in the effective
430 range of 50-300 µg per mouse, or with NP-encapsulated protein or peptide antigens in the effective
431 range of 0.05-5 µg per mouse, or with Fc.IL2m (i.p. or i.v., retro-orbital plexus) or F5111-IC (i.v.) in the
432 effective range of 6.25-18.75 µg per mouse. Free ovalbumin (OVA) was administered i.v. in the effective
433 range of 20-500 µg per mouse. AAV8-SEAP vector injected i.v. at specified doses. NOD (type 1 diabetes
434 model) and NOD.c3c4 (PBC model) mice were treated with individual therapeutic components or their
435 combinations as described in Figure Legends three or four times total at 28-day intervals.

436

437 **GVHD model**

438 NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ; Jackson Laboratory #005557) were irradiated with 1 Gy
439 from an X-ray irradiator source and then reconstituted with 1×10^7 human PBMC. The next day, mice
440 were treated with a single dose of phosphate buffer saline (vehicle), ImmTOR (100 µg), F5111 IC (9 µg),
441 or their combination. Animals were assessed for disease activity 3 times per week. Each animal was
442 assessed for weight loss, posture, activity, fur texture, skin integrity, and paleness on a 2 grade scale as
443 indicated below. Animals losing more than 20% weight or moribund were removed from the study.

444

Criteria	Grade 0	Grade 1	Grade 2
Weight Loss	<10%	from $\geq 10\%$ to $\leq 20\%$	>20%
Posture	Normal	Hunching noted at rest	Severe hunching impairs movement
Activity	Normal	Mild to moderately decreased	Stationary unless stimulated
Fur Texture	Normal	Mild to moderate ruffling	Severe ruffling/poor grooming
Skin Integrity	Normal	Scaling of paws/tail	Obvious areas of denuded skin
Paleness	Normal	Slight paleness	Severe paleness

445

446 **T1D model**

447 NOD mice were enrolled in the study at 6-7 weeks of age with the first treatment at week 8. They were
448 monitored weekly using standard glucometer strips, and mice showing glucose levels >250 mg/dL on 2/3
449 successive measurements were considered diabetic and those scoring >500 mg/dL twice or >600 mg/dL
450 once were terminated. All animals in the study were terminated at 35 weeks.

451

452 **PBC model**

453 NOD.c3c4 mice were enrolled in the study at 8 weeks and treated for the first time at 10 weeks of age.
454 After termination at 24 weeks, livers were fixed, embedded in paraffin, sectioned, stained with
455 hematoxylin and eosin and then slide images were taken. The resulting slides were evaluated by a
456 certified veterinary pathologist, and microscopic findings were scored as follows: Grade 0 (normal):
457 finding not present, Grade 1 (minimal): a focal, subtle, or trivial change, Grade 2 (mild): an easily
458 identifiable change of limited severity and/or distribution, Grade 3 (moderate): an obvious change with
459 normal tissue remaining, Grade 4 (marked): an extensive change that obliterates much of the normal
460 tissue, Grade 5 (severe): a maximal change.

461

462 **Flow Cytometry (murine liver and spleen cell populations)**

463 Immediately after euthanizing mice (at 1-14 days after initial treatment), livers and spleens were
464 harvested and rendered into single cell suspensions. Livers were processed via collagenase 4
465 (Worthington, Lakewood, NJ) enzymatic digest according to manufacturer's recommended protocol.
466 Spleens were processed via mechanical passage through a 70 µm nylon mesh (ThermoFisher, Waltham,
467 MA). Next, a red blood cell lysis step was performed for both liver and spleen suspensions for 5 min at

468 room temperature in 150 mM NH₄Cl, 10 mM KHCO₃, 10 μM Na₂-EDTA; washed in PBS, 2% bovine serum;
469 then filtered again with a 70 μm nylon mesh. Cells were incubated 20 min on ice with anti-CD16/32 (Fc-
470 block, clone 93, BioLegend, San Diego, CA) then stained with the following antibodies directed toward
471 cell surface receptors: CD3e-BV421 (BioLegend, clone 145-2C11), CD4-PerCP-Cy5.5 (BioLegend, clone
472 RM4-5), CD8a-BV510 (BioLegend, clone 53-6.7), CD25-PE-CF594 (BD, clone PC61), NK1.1-AF700 (BD,
473 clone PK136), CD122-APC (BioLegend, clone TM-B1), and CD132-PE (BioLegend, clone TUGm2).
474 Adoptively transferred human PBMC were stained with the following antibodies: CD4-PerCP-Cy5.5
475 (BioLegend, clone SK3), CD8a-APC-Cy7 (BioLegend, clone RPA-T8), CD56-BV421 BioLegend clone HCD56),
476 CD3e-BV-510 (BioLegend, clone UCHT-1) and CD26-PE-CF594 (BD clone M-A251). After cell surface
477 labeling cells were then fixed and permeabilized according to manufacturer's recommended protocol
478 using a FoxP3 Transcription Kit (eBioscience, San Diego, CA). The targeted intracellular markers were
479 stained with FoxP3-PE (InVitrogen, Waltham, MA), clone FJK-16s, Ki67-A647 (BioLegend, clone 11F6) and
480 Helios-PE-Cy7 (BioLegend, clone 22F6). Analysis was performed via FACSymphony A3 Cell Analyzer (BD
481 Biosciences) with subsequent data analysis using FlowJo software (TreeStar, Ashland, OR).

482

483 **Whole animal imaging**

484 FoxP3-IRES-mRFP mice were used to measure in vivo FoxP3 expression after treatment with Fc.IL2m
485 alone or combined with ImmTOR. At various time-points post treatment, images were acquired of the
486 left side dorsal aspect with the AMI Imaging System charge-coupled-device camera and analyzed with
487 the Aura 4.0.7 software package (Spectral Instruments Imaging, Tucson, AZ). A region of interest (ROI)
488 was manually selected based on signal intensity. The area of the ROI was kept constant, and the
489 intensity was recorded as maximum [photons s⁻¹ x cm⁻² x sr⁻¹ (steradian)] within a ROI.

490

491 **Methylation Analysis**

492 Murine CD3⁺, CD4⁺CD3⁺ and CD4⁺CD25⁺ cells were isolated from splenocytes seven days post treatment
493 via immunomagnetic bead selection (Miltenyi, Gaithersburg, MD) using either negative selection of
494 untouched CD4⁺ T cells or positive selection of CD4⁺CD25⁺ T cells (both from Miltenyi). After careful
495 supernatant removal, accurate cell counting (Countess, ThermoFisher), cell pellets were then snap
496 frozen in liquid nitrogen, then stored on dry ice. Samples were then sent to EpigenDx (Hopkinton, MA)
497 for subsequent targeted NextGen bisulfite sequencing panel analysis using their in-house FoxP3
498 methylation panel N4V1P15 analysis.

499

500 **Serum cytokines and FGF21 determination**

501 Serum cytokine concentrations were determined using Meso Scale Discovery (MSD) U-PLEX 10-Assay
502 SECTOR™ Plates, Linkers, and corresponding capture and detection antibody pairs. Plates were read
503 using electrochemiluminescence detection on an MESO® QuickPlex SQ 120, with Discovery Workbench
504 software (version 4.0.13) for analysis (MSD®, Gaithersburg, MD). Assays were performed according to
505 manufacturer's instructions, and without alterations to the recommended standard curve dilutions.
506 Serum FGF21 concentration was determined by ELISA using the mouse/rat FGF21 commercial kit from
507 R&D Systems (Minneapolis, MN). Serum samples were run at a 1:10 dilution.

508

509 **Concanavalin A Challenge Model**

510 Concanavalin A (Con A) induced liver toxicity model was employed essentially as earlier described³⁶.
511 Briefly, mice were injected (i.v., r.o.) Con A at 12 mg/kg and then terminally bled at 6 or 12 hours post-
512 challenge with cytokine levels in serum determined by MSD as described above and liver tissues
513 collected simultaneously for single-cell suspension analysis by flow cytometry as described above or for
514 hematoxylin-eosin staining followed by microscopic evaluation.

515

516 **Enzyme-linked immunosorbent assay for IgG against AAV8**

517 The 96-well plates were coated overnight with AAV8, washed, and blocked on the following day,
518 followed by sample incubation (1:40 diluted serum). Plates were then washed, and the presence
519 of IgG was detected using anti-mouse IgG-specific horseradish peroxidase (HRP; 1:1500; Jackson
520 ImmunoResearch, West Grove, PA, USA). The presence of rabbit anti-mouse IgG-specific HRP was
521 visualized using trimethylboron substrate and measured using absorbance at 450 nm with a reference
522 wavelength of 570 nm. The optical density (OD) observed is proportional to the amount of
523 anti-AAV8 IgG antibody in a sample and was reported.

524

525 **Statistical Analysis**

526 Statistical analyses were performed using GraphPad Prism 9.4.1. To compare the mouse experimental
527 groups pairwise either multiple t test (for several time-points) or Mann-Whitney two-tailed test (for a
528 single time-point; individual comparison of two groups presented within the same graph) were used.
529 Significance is shown for each figure legend (* – $p < 0.05$, ** – $p < 0.01$; *** – $p < 0.001$; **** – $p <$
530 0.0001 ; not significant – $p > 0.05$). All data for individual experimental groups is presented as mean \pm SD
531 (error bars).

532

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535

536 **Author contributions**

537 T.K.K. conceived the idea for the combination therapy, T.K.K. and P.O.I. designed the research and wrote
538 the manuscript, M.F., A.M., G.R., and C.R. conducted the biological studies, T.C., M.F., G.R., and C.R,
539 analyzed samples, N.N. and L.D. formulated the antigen nanoparticles, F.F. analyzed the nanoparticles,
540 N.L. produced the protein products, D.VD and J.B.S. provided F5111 IC, P.G.T. analyzed the histology
541 slides, T.K.K., P.O.I., S.L., T.C., M.F., G.R., and C.R, analyzed the data, T.K.K., P.O.I., D.VD, and J.B.S. edited
542 the manuscript.

543

544 **Competing interests**

545 a

546

547

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687

688

689 Figure Legends

690 **Figure 1. Expansion of splenic Tregs by ImmTOR and IL-2 mutein.** **A.** Dynamics of Treg induction by
691 ImmTOR, Treg-biased IL-2 mutein (Fc.IL2m) or the combination thereof. Groups of mice (n= 3-7 mice per
692 timepoint) were treated as described, and spleens were harvested at times indicated, processed to single-
693 cell suspension, stained, and analyzed for CD3⁺CD4⁺CD25⁺FoxP3⁺ Treg abundance by flow cytometry. This
694 graph is a summary of four independent experiments. Error bars indicate mean +/- standard deviation
695 (SD). **B.** Representative graph of a 7-day timepoint shown in **A.** This graph is a summary of 2 independent
696 experiments (n=7 per group). Error bars indicate mean +/- SD. **C.** Dose-dependence of Treg induction by
697 Fc.IL2m alone or combined with ImmTOR. Groups of mice (n=4 per cohort) were treated with ascending
698 doses of 9, 18, or 27 µg of Fc.IL2m, alone or combined with 100 µg ImmTOR. Seven days after treatment,
699 mice were sacrificed and harvested spleens were then evaluated for total number of Tregs, proliferating
700 (Ki-67⁺) Tregs, and Helio⁺ stable Tregs by flow cytometry. Error bars indicate mean +/- SD. **D.** Whole animal
701 fluorescence imaging of transgenic mice expressing mRFP under control of the Foxp3 promoter. Mice
702 were treated with either Fc.IL2m alone (top row) or with Fc.IL2m+ImmTOR (bottom row) and analyzed 7
703 days after treatment. **E.** Effector cell populations induced by ascending doses of Fc.IL2m alone or in
704 combination with ImmTOR, as described in **C.** Total numbers of CD8⁺ cytolytic T lymphocytes (CTL;
705 CD3⁺CD8⁺), CD4⁺ T effector (Teff; CD3⁺CD4⁺CD25⁻), and NK (CD3⁻NK1.1⁺) cells are shown. Error bars
706 indicate mean +/- SD. **F.** Ratios of total number of Tregs relative to CTL, Teff, and NK cells after treatment
707 with ascending doses of Fc.IL2m alone or in combination with ImmTOR, as described in **C.** Ratios of the
708 value for each experimental group vs untreated control are indicated above the bars in **C, E** and **F.**
709 Representative graphs from one of two studies with similar results are shown. Error bars indicate mean
710 +/- SD. Statistical significance: * p < 0.05, ** p < 0.01, *** p < 0.001.

711 **Figure 2. Induction of Tregs by ImmTOR and human IL-2/anti-IL-2 antibody fusion protein in humanized**
712 **mice.** Mice were treated with F5111 IC (18.75 µg) alone or combined with ImmTOR (100 µg), and
713 splenocytes were harvested at 7 days post treatment and analyzed by flow cytometry. **A.** Human PBMC-
714 engrafted NSG (huPBMC) mice were treated at 1.5-3 weeks after PBMC engraftment. Treg
715 (CD3⁺CD4⁺CD25⁺FoxP3⁺), CTL (CD3⁺CD8⁺), and NK cell (CD3⁻CD56⁺) populations are presented as absolute
716 cell numbers, percent Tregs out of total T cells, and relative ratios of Treg:effector cells. A summary of 3
717 experiments using different PBMC donors is shown (n=10 per group). Error bars indicate mean +/- SD. **B.**
718 ImmTOR mitigates disease exacerbation by F5111 IC and prolongs survival in a HuPBMC model of GVHD.
719 NSG mice were irradiated and then reconstituted with 1x10⁷ human PBMC. The next day, mice were
720 treated with a single dose of saline, ImmTOR (100 µg), F5111 IC (9 µg), or the combination. Control
721 animals were irradiated but did not receive HuPBMC. **C.** Transgenic mice expressing human IL-2, IL-2Rα
722 and IL-2Rβ mice were treated as described (5 animals/group) or left untreated (3 animals/group). Treg,
723 Helios⁺ stable Treg, CTL, and NK total and proliferating cell populations are shown as fractions, absolute
724 cell numbers, or relative ratios. A representative experiment of 2 independent studies that resulted in a
725 similar outcome is shown. Error bars indicate mean +/- SD. Ratios of the value for each experimental
726 group vs untreated control are indicated above the bars in **A** and **C.** Statistical significance: * p < 0.05, **
727 p < 0.01, *** p < 0.001.

728 **Figure 3. Efficacy of ImmTOR + Treg-biased IL-2 mutein in a concanavalin A-induced model of**
729 **autoimmune hepatitis.** **A.** Female C57BL/6 mice (n=5 per cohort) were either left untreated or treated
730 with ImmTOR (200 µg) and Fc.IL2m (9 µg) individually or in combination. Mice were challenged 4 days
731 later with 12 mg/kg of concanavalin A (Con A) or left unchallenged (no Con A). At 12 hours after Con A

732 challenge, serum was drawn for cytokine analysis and livers were harvested and hepatic T cells were
733 assessed by flow cytometry. **A.** Activated (CD69⁺) and highly activated (CD69^{high}) T cells (CD3⁺) and CTL
734 (CD3⁺CD8⁺) are shown either as fractions of total or as absolute cell numbers. A representative experiment
735 of 4 studies that resulted in similar outcomes is shown. Error bars indicate mean +/- SD. **B.** Mice were
736 treated as in **A.** Serum IFN- γ , IL-6, and KC/GRO levels at 12 hours after Con A challenge (summary of 2
737 independent experiments, n=8-10 per group). Error bars indicate mean +/- SD. **C.** FGF21 serum levels prior
738 to and after Con A challenge. A representative experiment of 4 studies that resulted in similar outcomes
739 is shown (n=4 per group). Error bars indicate mean +/- SD. Statistical significance: * p < 0.05, ** p < 0.01,
740 *** p < 0.001, **** p < 0.0001.

741 **Figure 4. Induction of OVA-specific Tregs by combination of ImmTOR, IL-2 mutein, and ovalbumin.** OVA-
742 specific OT-II T cells from CD45.2⁺ donors were adoptively transferred into CD45.1⁺ C57BL/6 recipient mice
743 (n=3 per group) and 24 hours later treated with different combinations of ImmTOR (100 μ g), Fc.IL2m (9
744 μ g), and either free ovalbumin (OVA, 5-500 μ g) or nanoparticle-encapsulated OVA (NP-OVA, 0.05-5 μ g) or
745 left untreated. At 7 days after treatment, spleens were harvested and analyzed by flow cytometry. **A.** Total
746 splenic and OVA-specific Tregs induced by combinations of ImmTOR, Fc.IL2m, and free OVA (n=3/group).
747 Error bars indicate mean +/- SD. **B.** Antigen dose-dependent induction of OVA-specific donor Tregs by the
748 combination of ImmTOR, Fc.IL2m, and free OVA. The concurrent expansion of recipient Tregs was
749 unaffected by OVA. Total numbers of CD45.1⁺ (recipient) and CD45.2⁺ (OT-II donor) Tregs are shown (n=3
750 per group). Error bars indicate mean +/- SD. **C.** Antigen dose-dependent induction of OVA-specific donor
751 or recipient Tregs by the combination of ImmTOR, Fc.IL2m, and either free OVA or NP-OVA. Total number
752 of CD45.1⁺ (recipient) and CD45.2⁺ (OT-II donor) Tregs and the fractions of total cells are shown (n=6-7
753 per group). Error bars indicate mean +/- SD. Statistical significance: ** p < 0.01.

754 **Figure 5. Mitigation of antibody response to AAV vector by combination treatment with ImmTOR and**
755 **IL-2 mutein.** C57BL/6 mice (5 per group) were injected with AAV8 on Day 0 (2.7E12 vg/kg) and Day 56
756 (5.0E12 vg/kg) either alone or in combination with ImmTOR (50-200 μ g) and/or Fc.IL2m (9 μ g). Animals
757 were bled at the indicated timepoints indicated, and serum was analyzed for the presence of anti-AAV8
758 IgG antibody by ELISA. Timing of the second AAV8 administration is shown by arrows. Error bars indicate
759 mean +/- SD. Statistical analyses are shown in Supplementary Table ST1.

760 **Figure 6. Diabetes prevention by combination treatment with ImmTOR, IL-2 mutein, and nanoparticle-**
761 **encapsulated hybrid insulin peptide 6.9 (NP-HIP6.9).** Female NOD mice (n=10 per group) were left
762 untreated or treated with ImmTOR (100 μ g) only or ImmTOR and Fc.IL2m (9 μ g), in the absence or
763 presence of NP-encapsulated hybrid insulin peptide 6.9 (NP-HIP6.9, 1 μ g) starting at week 8 of age (4
764 treatments at 4-week intervals, shown as arrows). Mice were monitored up to 35 weeks of age. Blood
765 glucose was measured weekly, and mice scoring >250 mg/dL on 2/3 successive measurements were
766 considered diabetic and those scoring >500 mg/dL twice or >600 mg/dL once were terminated. Fractions
767 of diabetic mice (**A**) and individual mouse blood glucose levels (**B**) are shown with statistical significance
768 indicated. Statistical significance: ** p < 0.01.

769 **Figure 7. Efficacy of ImmTOR + IL-2 mutein with and without nanoparticle-encapsulated PDC-E2 antigen**
770 **in a mouse model of primary biliary cholangitis.** **A.** Schedule of treatment of NOD.c3c4 mice. Mice were
771 treated with three monthly doses as indicated starting at 10 weeks of age. **B.** Disease scores. Paraffin-
772 embedded liver sections were stained with hematoxylin & eosin of NOD.c3c4 mice and analyzed by an
773 independent veterinary pathologist. Bile duct degeneration, biliary hypertrophy, and liver inflammation

774 were graded on a 5-point severity scale as described in Materials and Methods. **C-J**. Histology.
775 Representative histology sections from female (**C-F**) and male (**G-J**) mice are shown at 5x magnification.
776 **C**. Untreated female. Vast majority of bile ducts show marked ectasia, some with intraluminal
777 accumulations of neutrophils (arrow), and dense biliary and intrahepatic mononuclear inflammatory
778 infiltrate (*). **D**. ImmTOR-treated female. Marked biliary mononuclear cell inflammation (*) occasionally
779 forming follicles (arrowhead) and multifocal duct ectasia (arrow). **E**. ImmTOR-IL-treated female. Biliary
780 mononuclear cell inflammation with small foci of peri-biliary hypercellularity (*) and multifocal duct
781 ectasia (arrow). **F**. ImmTOR-IL plus NP/PDC-E2.ILD-treated female. Mild biliary mononuclear cell
782 inflammation with small foci of peri-biliary hypercellularity (*) and mild biliary hyperplasia or bile duct
783 ectasia (arrow). **G**. Untreated male. Marked biliary mononuclear cell inflammation with densely cellular
784 accumulations surrounding bile ducts (*), with follicle formation (arrowhead), moderate bile duct
785 hyperplasia and bile duct ectasia (arrow). **H**. ImmTOR-treated male. Biliary mononuclear cell inflammation
786 with few foci of peri-biliary hypercellularity (*) and multifocal duct ectasia (arrow). **I**. ImmTOR-IL-treated
787 male. Biliary mononuclear cell inflammation with small foci of peri-biliary hypercellularity (*) and
788 multifocal duct ectasia (arrow). **J**. ImmTOR-IL plus NP/PDC-E2.ILD-treated male. Minimal biliary
789 mononuclear cell inflammation, rare foci of peri-biliary hypercellularity and, minimal biliary hyperplasia
790 or bile duct ectasia (arrow).

791 **Suppl. Fig. 1. CD3, CD4, CD25 and FoxP3 gating strategies.** Representative dot plots are shown for
792 phenotyping of untreated mice and mice treated with ImmTOR, Fc.IL2m (IL-2 mut), or ImmTOR+Fc.IL2m
793 combination therapy.

794 **Suppl. Fig. 2. Treg induction by Fc.IL2m +/- ImmTOR in spleen and liver.** **A**. Dose-dependence of Treg
795 induction by Fc.IL2m alone or combined with ImmTOR. Groups of mice (n=4 per cohort) were treated and
796 splenic Tregs were analyzed as described in Figure 1C. Fractions of Tregs out of total T helpers and of
797 proliferating (Ki67⁺) and Helios⁺ stable Tregs out of total Tregs are shown. Error bars indicate mean +/- SD.
798 **B**. Dynamics of hepatic Treg induction by combination treatment with ImmTOR and IL-2 mutein. Groups
799 of mice (n=3-7 per cohort per time-point) were treated with ImmTOR, Fc.IL2m or the combination thereof.
800 Livers were harvested on Day 4, 7, or 14, as indicated, processed to single-cell suspension, stained, and
801 analyzed by flow cytometry. Graphs represent summaries of 4 independent experiments. Total Treg
802 numbers and ratio of Treg-to-CD8⁺ T cells are shown. Error bars indicate mean +/- SD. Statistical
803 significance: * p < 0.05, ** p < 0.01, *** p < 0.001.

804 **Suppl. Fig. 3. Treg responses to Fc.IL2m +/- ImmTOR.** **A, B**. Dynamics of circulating Fc.IL2m (**A**) and CD4⁺
805 T cell IL-2R α (CD25) expression (**B**) after treatment with ImmTOR, Fc.IL2m, or the combination thereof.
806 Groups of mice (n=3-6 per group for each timepoint) were treated with Fc.IL2m and/or ImmTOR at the
807 indicated doses. The graphs represent summaries of 3 independent experiments. Error bars indicate
808 mean +/- SD. **C**. Demethylation of Treg-specific genes after treatment with ImmTOR, Fc.IL2m or their
809 combination. Groups of mice (n=9 per group) were treated with Fc.IL2m and/or ImmTOR at the doses
810 indicated, CD4⁺ T cells were isolated after 7 days, and status of methylation within FoxP3, EOS, and Helios
811 genes was assessed within multiple CpG islands as indicated. The graphs represent summaries of 2
812 independent experiments. Statistical significance: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

813 **Suppl. Fig. 4. ImmTOR improves GVHD disease scores.** NSG mice were irradiated, reconstituted with
814 HuPBMC and treated as described in Figure 2B legend. Disease activity index (DAI) was assessed three
815 times per week, as described in Materials and Methods.

816 **Suppl. Fig. 5. Effect of ImmTOR and IL-2 mutein on activation of hepatic NK, NKT, neutrophils, and**
 817 **macrophages after treatment with concanavalin** Female C57BL/6 mice (n=5 per cohort) were either left
 818 untreated or treated with ImmTOR (200 µg) and Fc.IL2m (9 µg) individually or in combination. Mice were
 819 challenged 4 days later with 12 mg/kg of concanavalin A (Con A), as described in Figure 3. At 12 hours
 820 after Con A challenge, serum was drawn for cytokine quantification and livers were harvested and hepatic
 821 T cells were analyzed by flow cytometry. (A) Fractions or total cell numbers of activated (CD69⁺ or CD69^{high})
 822 NK (CD3⁻NK1.1⁺), NKT (CD3⁺NK1.1⁺) cells, neutrophils (CD11b⁺GR-1⁺), and macrophages (F4/80⁺) are
 823 shown. (B) Serum concentrations of IFN-γ, IL-6 and CXCL1. (C) Serum concentrations of FGF21. Error bars
 824 indicate mean +/- SD. Statistical significance: ** p < 0.01.

825 **Suppl. Figure 6. Mitigation of antibody response to high AAV vector dose by combination treatment**
 826 **with ImmTOR and IL-2 mutein.** (A) C57BL/6 mice (n=6 per cohort) were treated with a single high vector
 827 dose of 5E13 vg/kg on Day 0 with or without ImmTOR (200 µg) and/or Fc.IL2m (9 µg). Groups treated
 828 with Fc.IL2m received additional Fc.IL2m doses on Days 28, 56, and 84. Anti-AAV8 IgG antibodies were
 829 assessed via serum blood draw on various days as indicated. (B) Spleens were harvested at the timepoints
 830 shown and processed into single-cell suspensions that were analyzed by flow cytometry. Populations are
 831 presented as fractions, absolute cell numbers, and relative ratios. Graphs are the summary of 2 identical
 832 studies with similar results. Error bars indicate mean +/- SD. Statistical analyses shown in Supplementary
 833 Table ST2.

834 **Supplementary Table ST1.** Comparison of anti-AAV IgG levels in mice treated with ImmTOR alone (50-200
 835 µg) vs. animals treated with ImmTOR (50-200 µg) combined with Fc.IL2m (9 µg) as described in legend to
 836 Figure 5. Mean values ± SD are shown for all time-points and statistical significance indicated (unpaired t-
 837 test; ns – not significant).

	Day 12	Day 19	Day 33	Day 47	Day 61	Day 75	Day 91	Day 104	Day 117
ImmTOR	0.08 ± 0.02	0.10 ± 0.11	0.40 ± 0.57	0.42 ± 0.58	0.48 ± 0.54	0.59 ± 0.67	0.80 ± 0.56	0.94 ± 0.57	0.91 ± 0.54
ImmTOR + Fc.IL2m	0.07 ± 0.02	0.07 ± 0.01	0.08 ± 0.02	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.03	0.06 ± 0.02	0.07 ± 0.02	0.08 ± 0.02
P value	ns	ns	0.039	0.036	0.0061	0.0065	<0.0001	<0.0001	<0.0001

838
 839 **Supplementary Table ST2.** Statistical significance of differences between all the splenocyte cell
 840 populations (fractions, absolute numbers and their ratios) shown in Supplementary Fig. 6B. The values
 841 for the group of mice treated with AAV and ImmTOR and Fc.IL2m combination vs. all other experimental
 842 groups at all time-points is shown. * p < 0.05, ** p < 0.01, ns – not significant.

	AAV			AAV + ImmTOR			AAV + IL-2mut		
	Day 1	Day 4	Day 7	Day 1	Day 4	Day 7	Day 1	Day 4	Day 7
Treg fraction (% of CD4 ⁺)	**	**	**	**	**	**	ns	ns	**
CTL, total	ns	ns	ns	ns	ns	**	ns	**	*
Treg:CTL ratio	**	**	**	**	**	**	ns	ns	**
Plasmablasts (% of CD19 ⁺)	ns	**	*	ns	ns	**	*	**	*
Plasmablasts, total	ns	*	ns	ns	ns	**	*	**	ns
T effectors, total	*	**	ns	ns	ns	*	ns	**	*

Figure 1

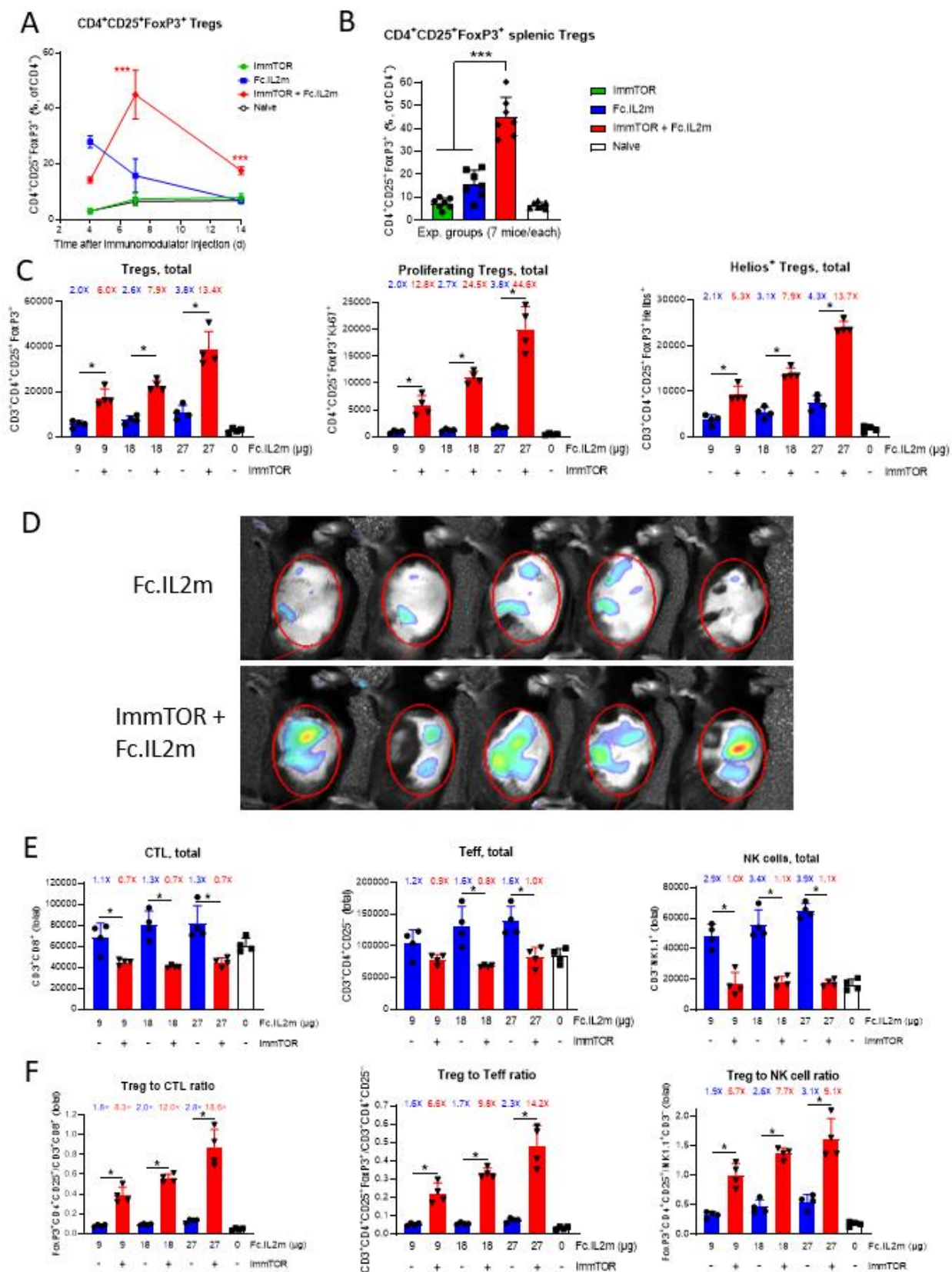


Figure 2

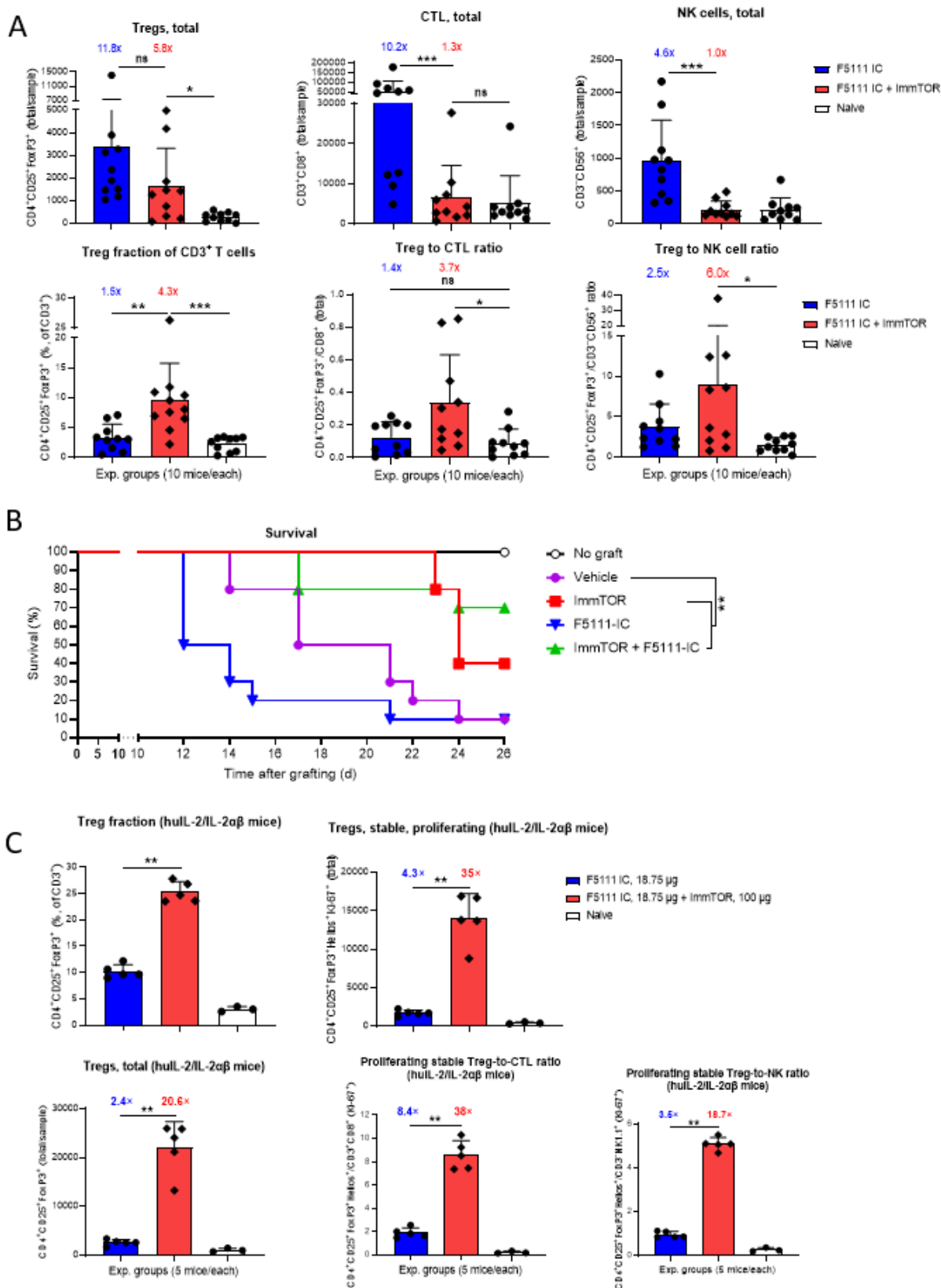


Figure 3

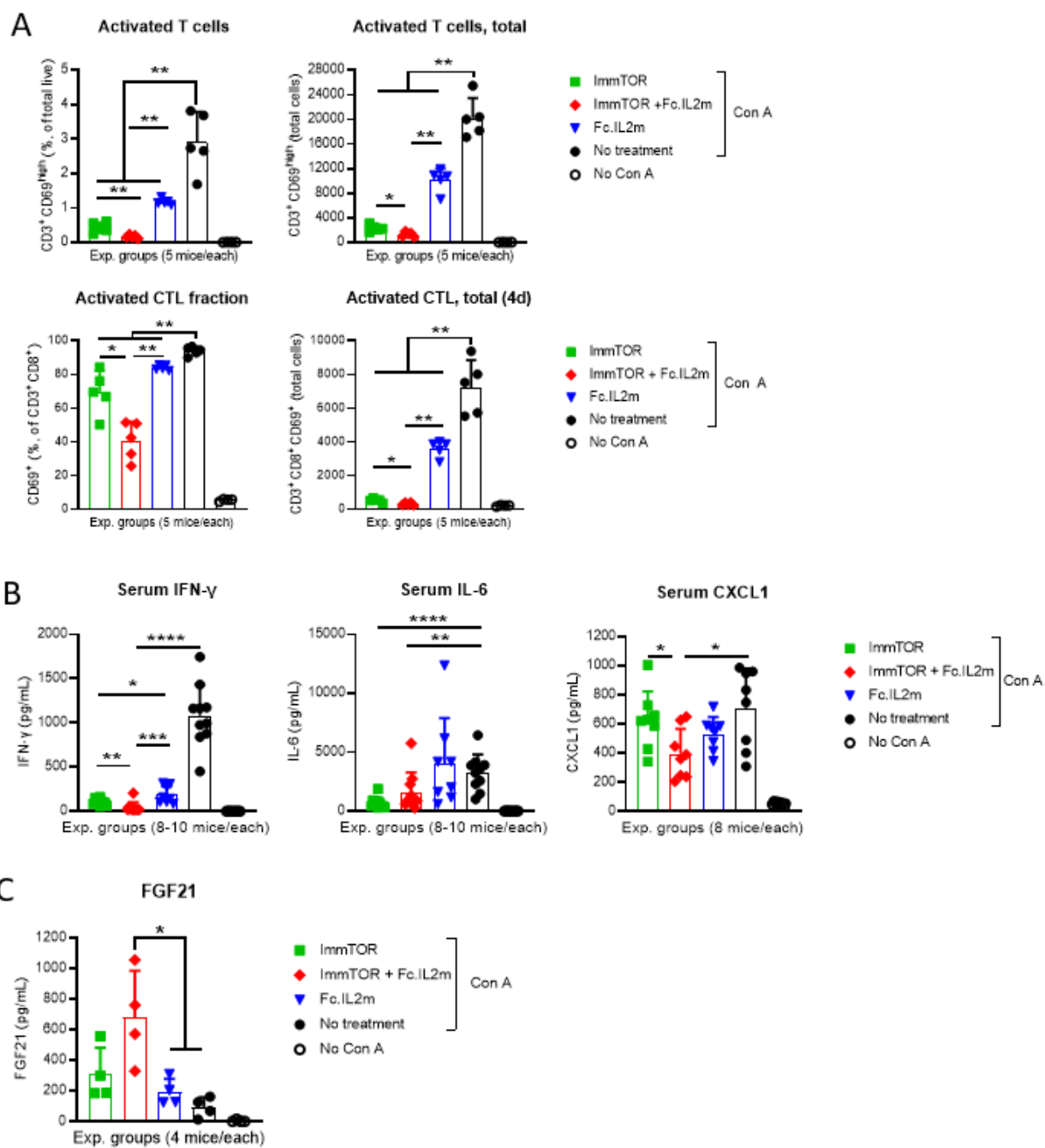


Figure 4

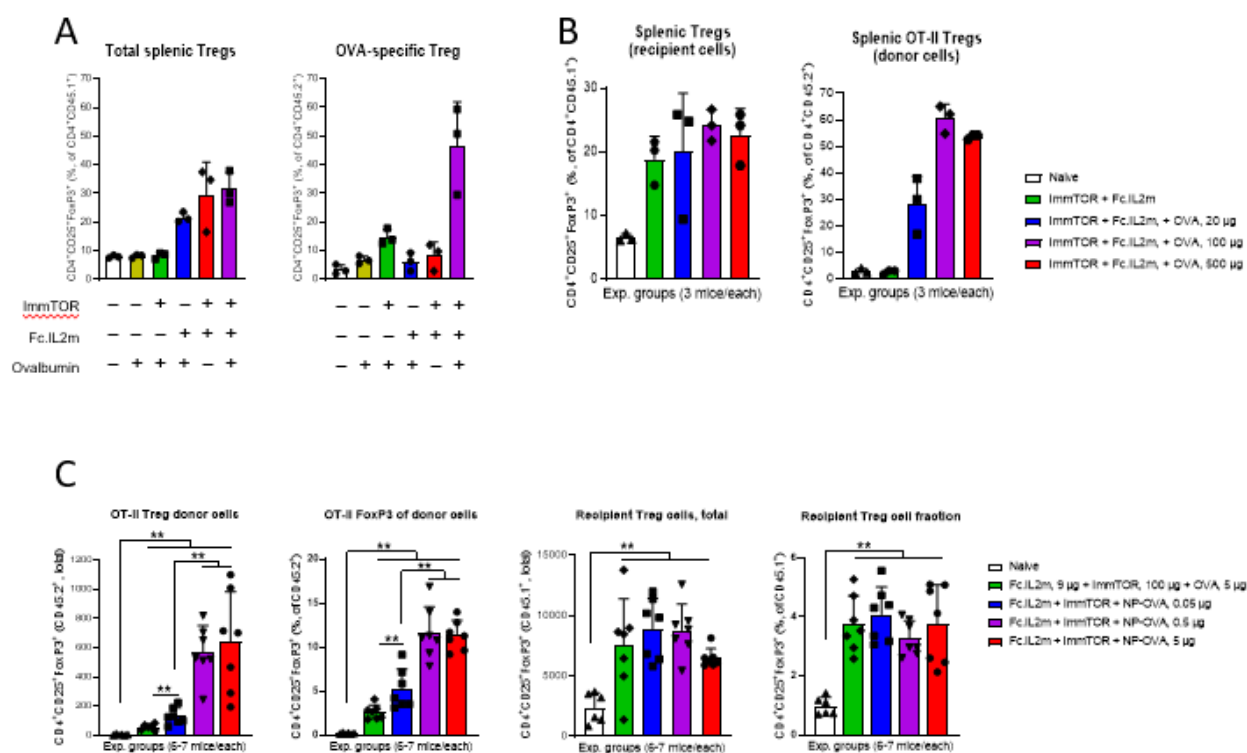


Figure 5

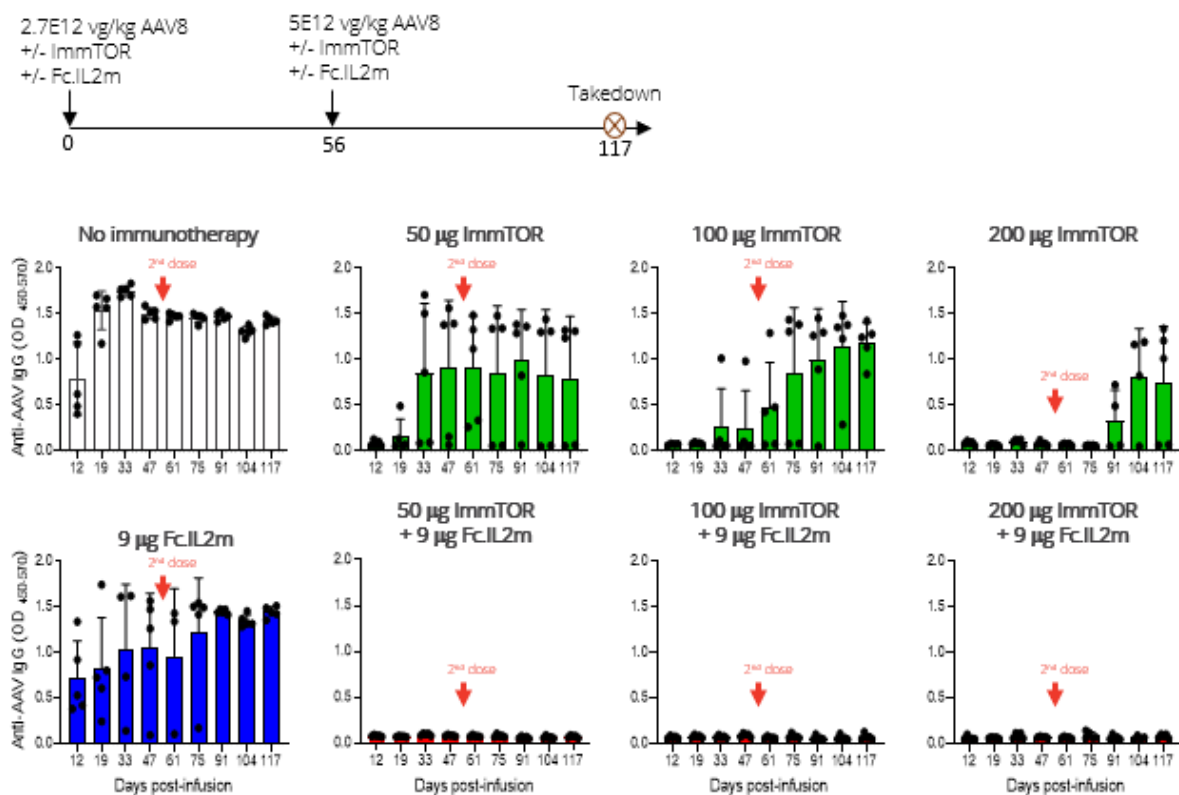


Figure 6

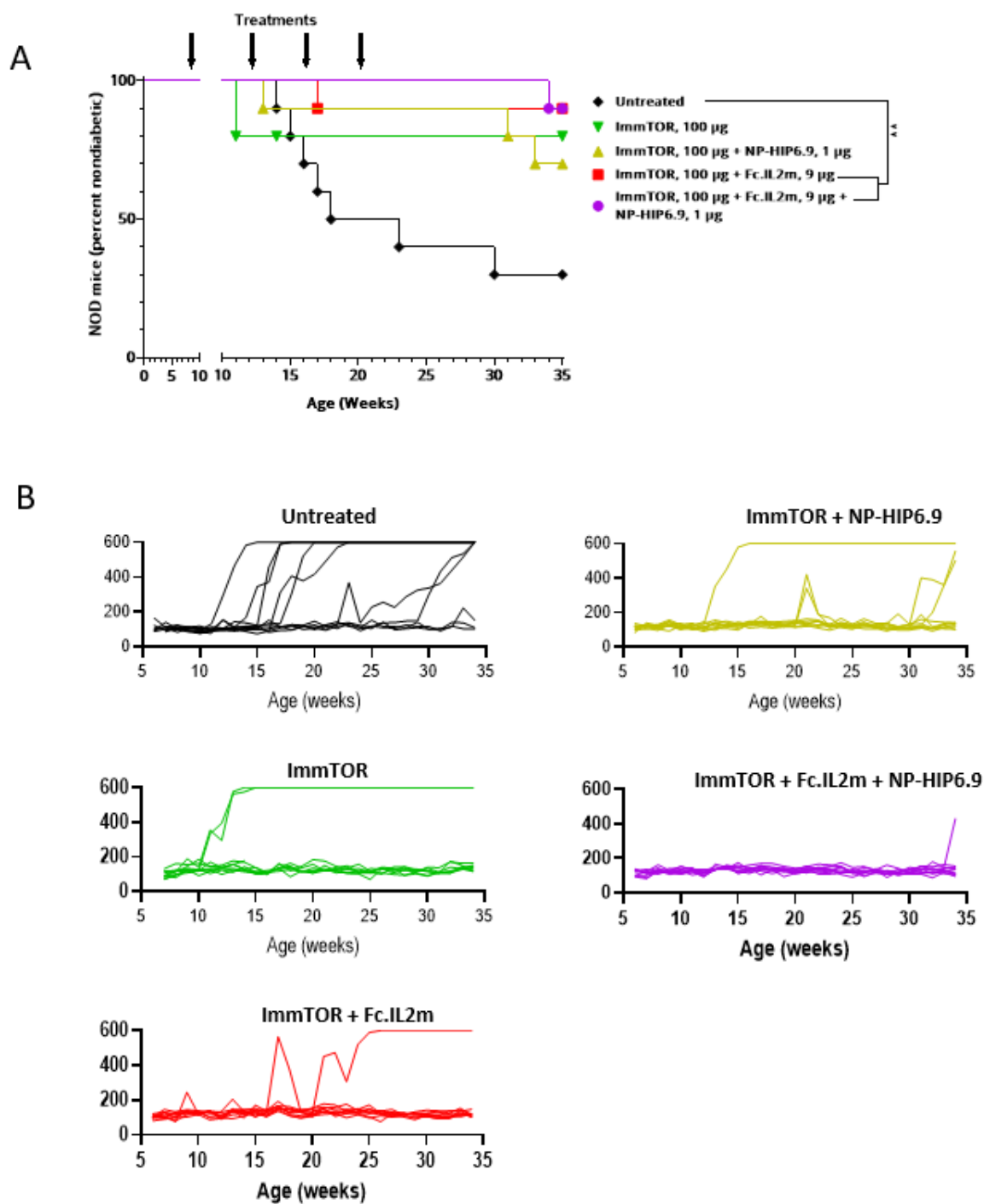
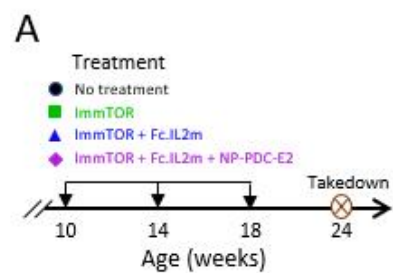
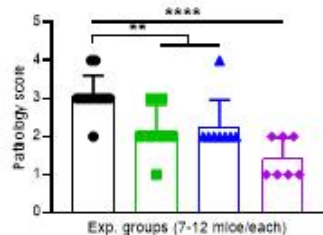


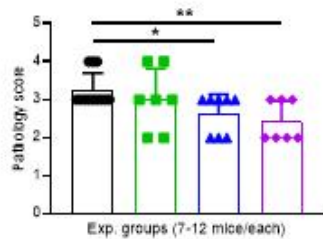
Figure 7



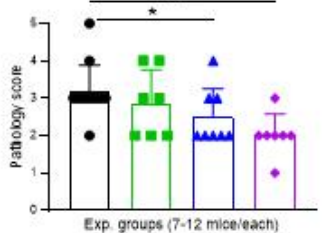
B Bile duct epithelium degeneration



Biliary hyperplasia/hypertrophy

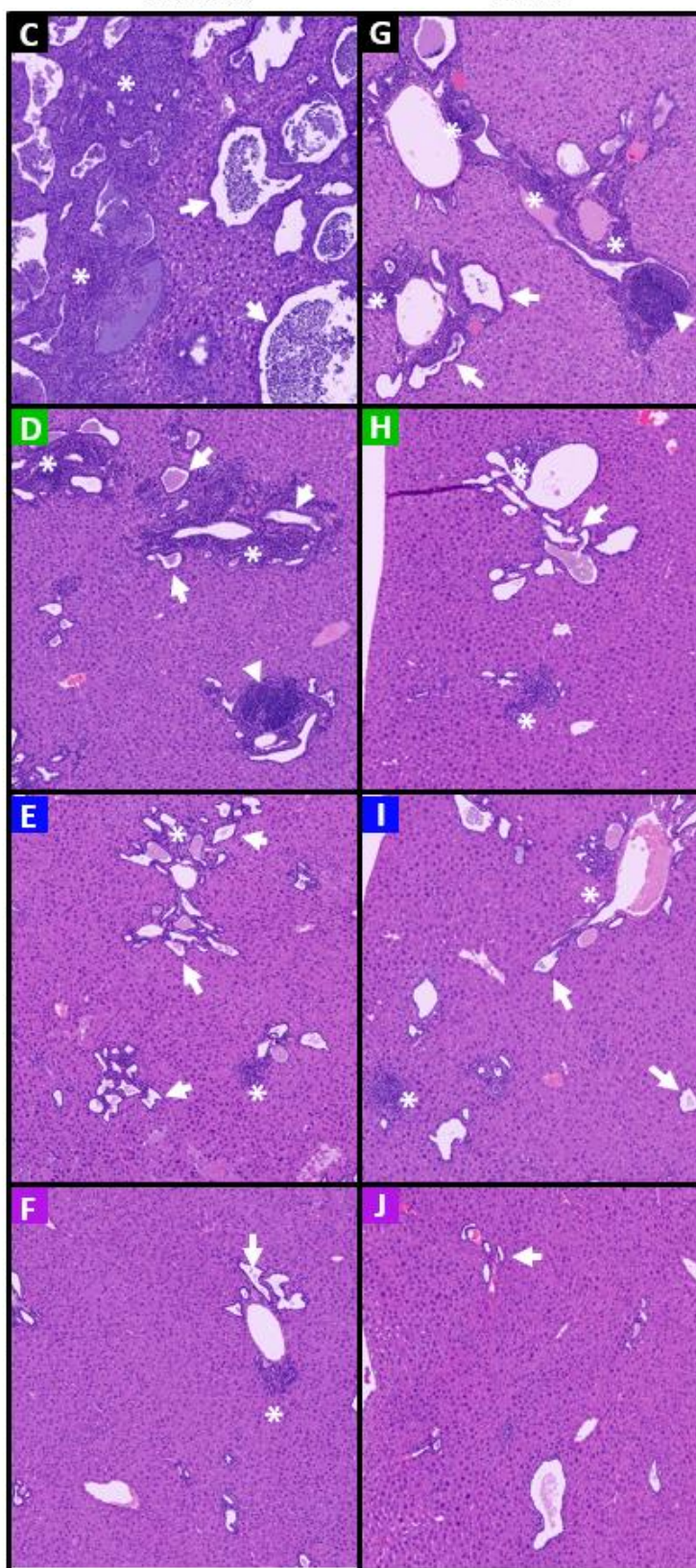


Liver Inflammation (MC, biliary)

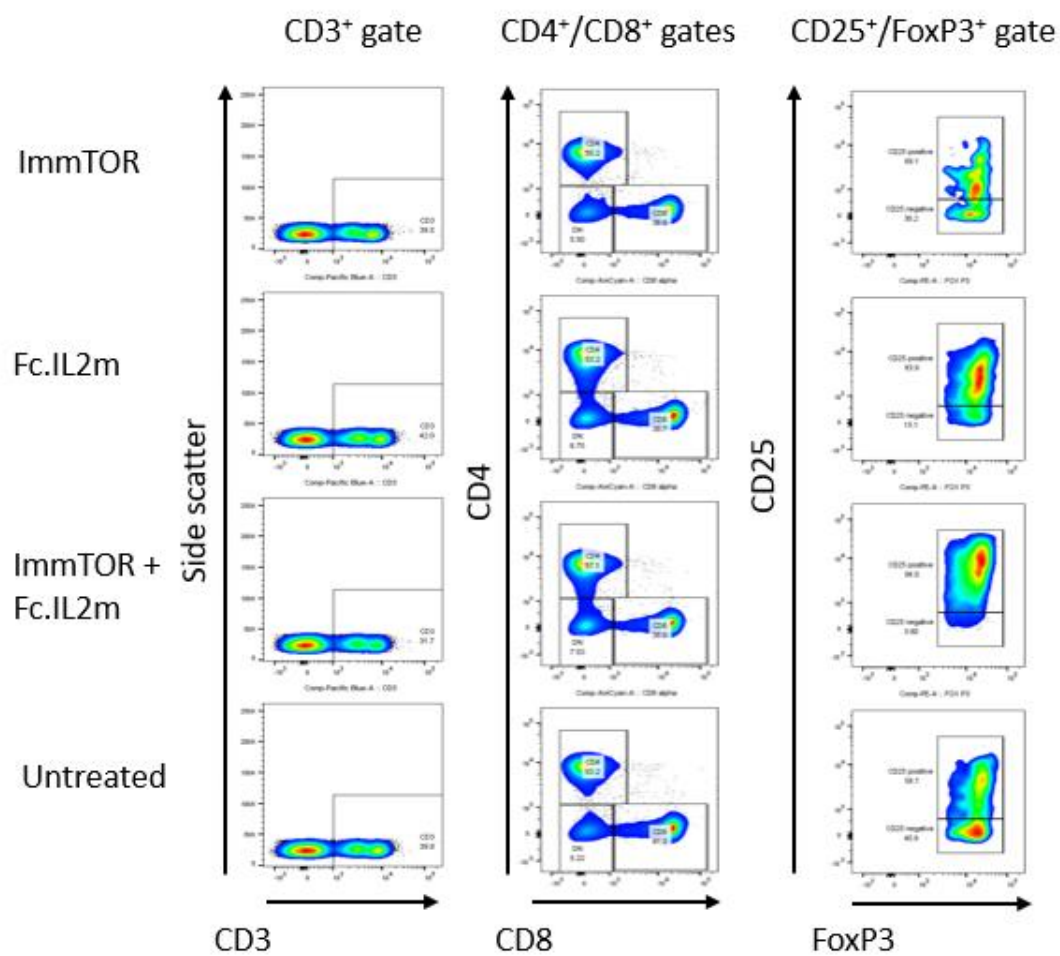


Female

Male

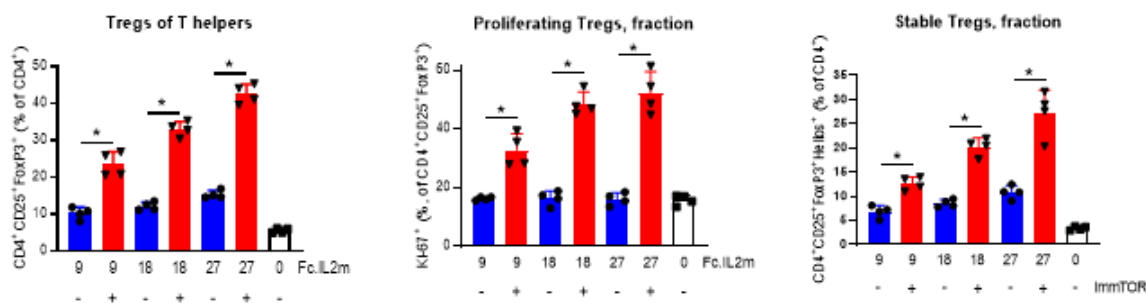


Supplemental Figure 1

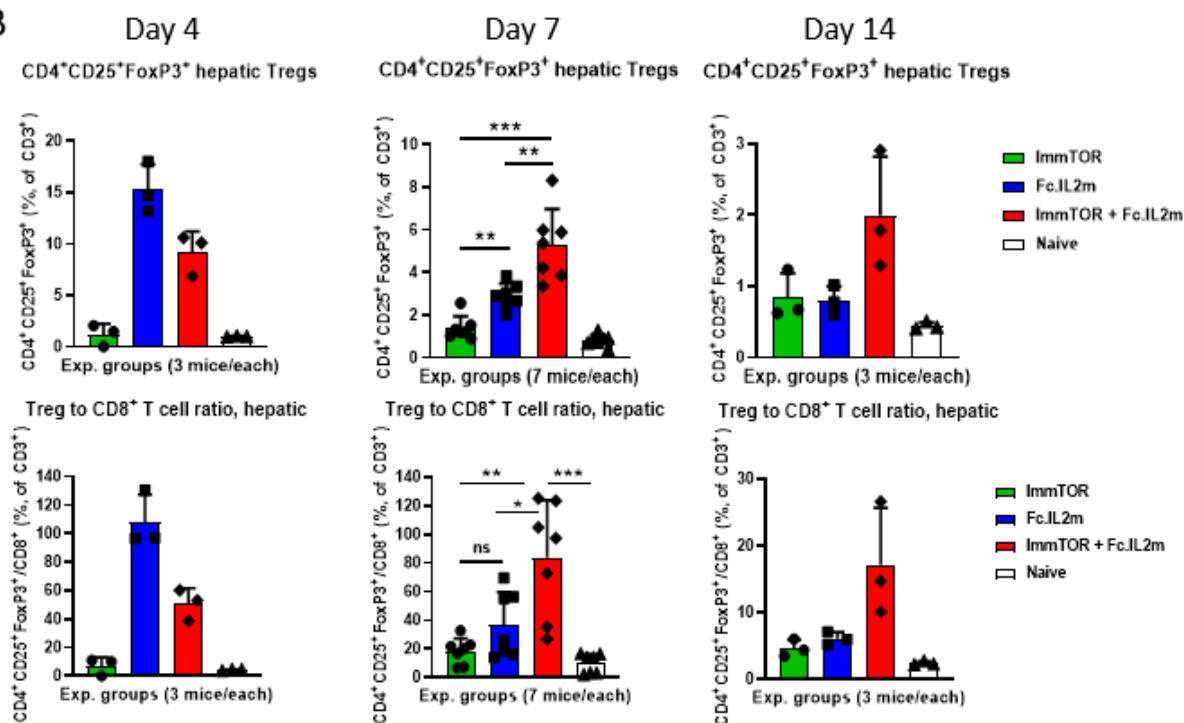


Supplementary Figure 2

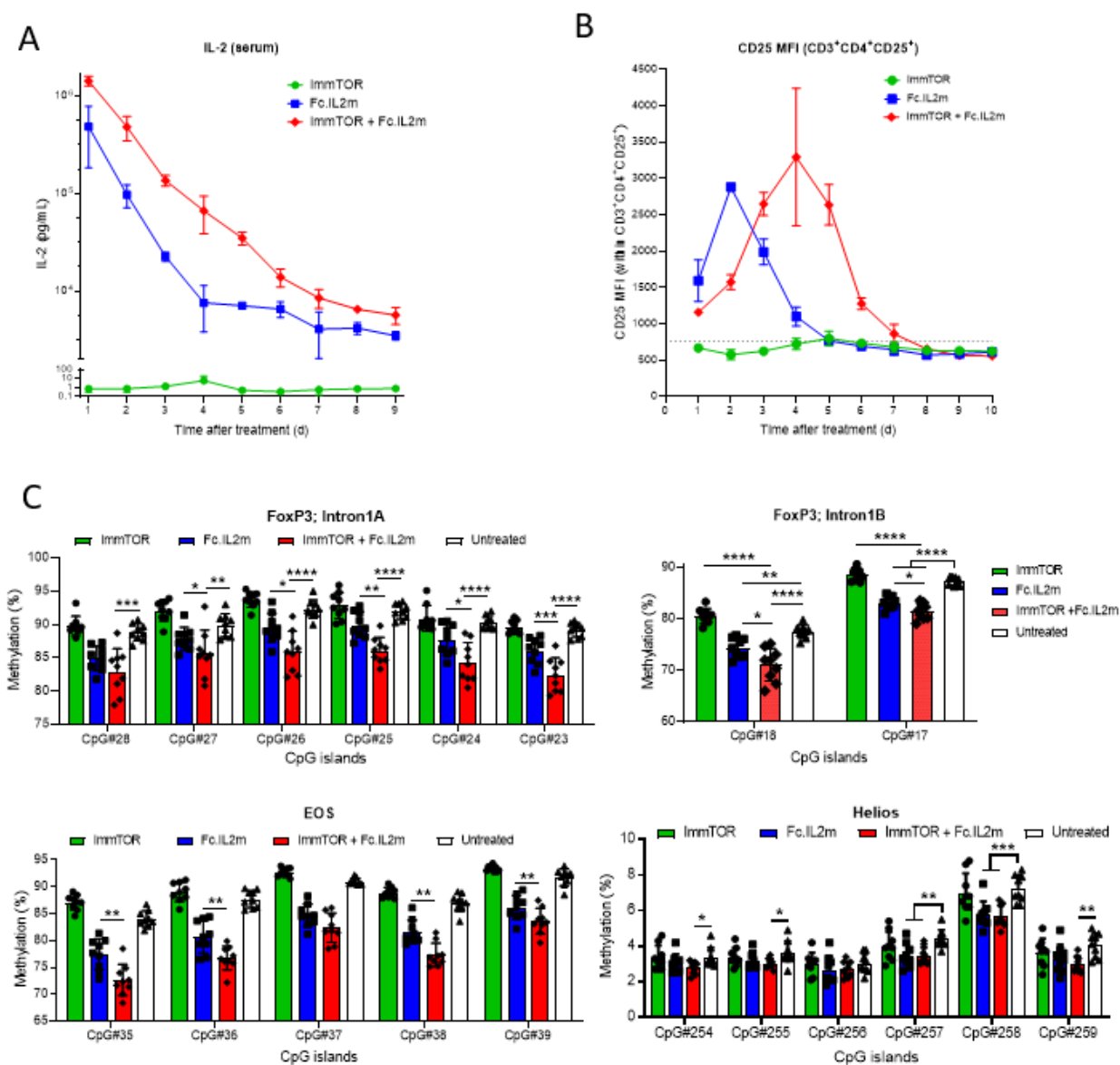
A



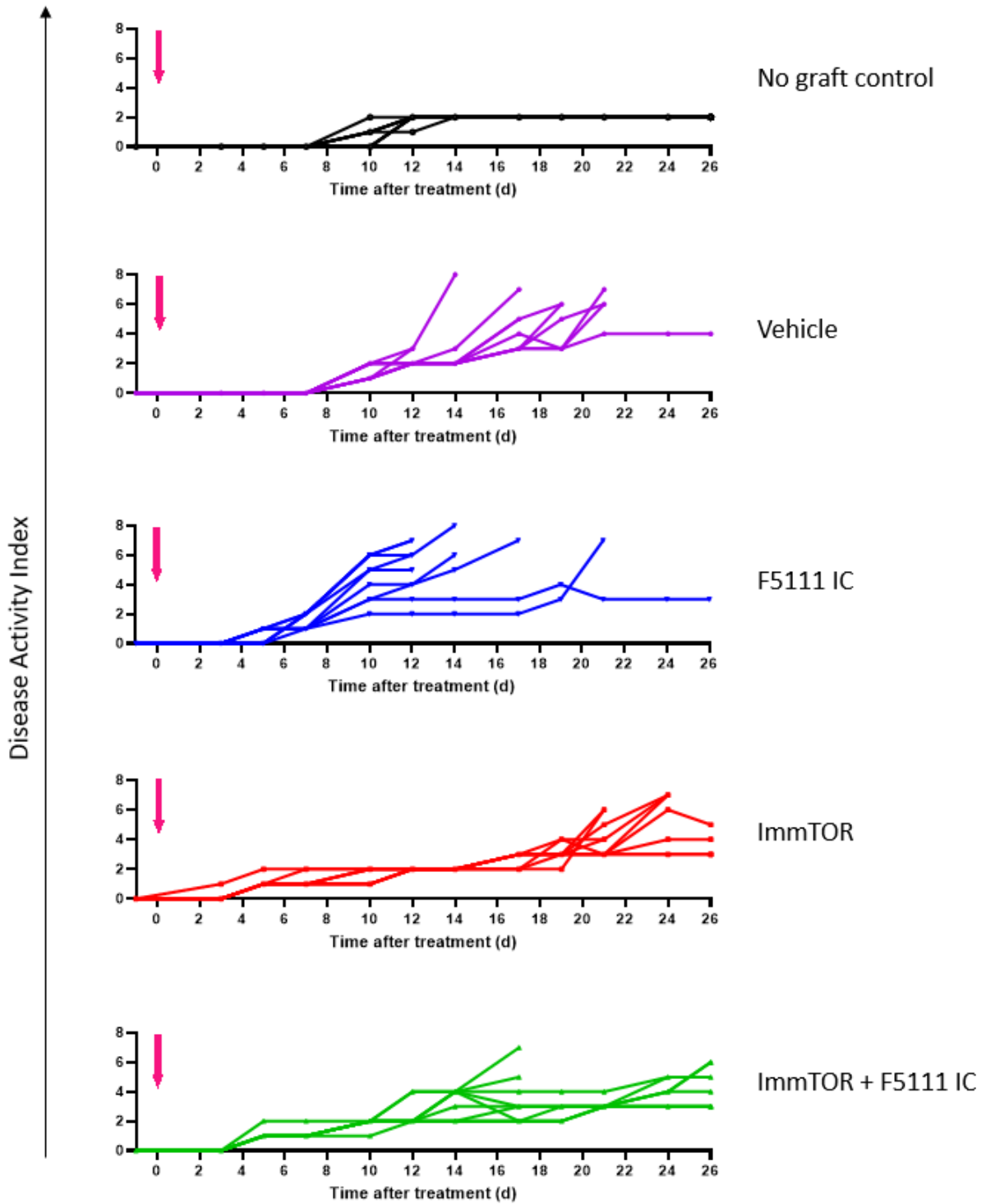
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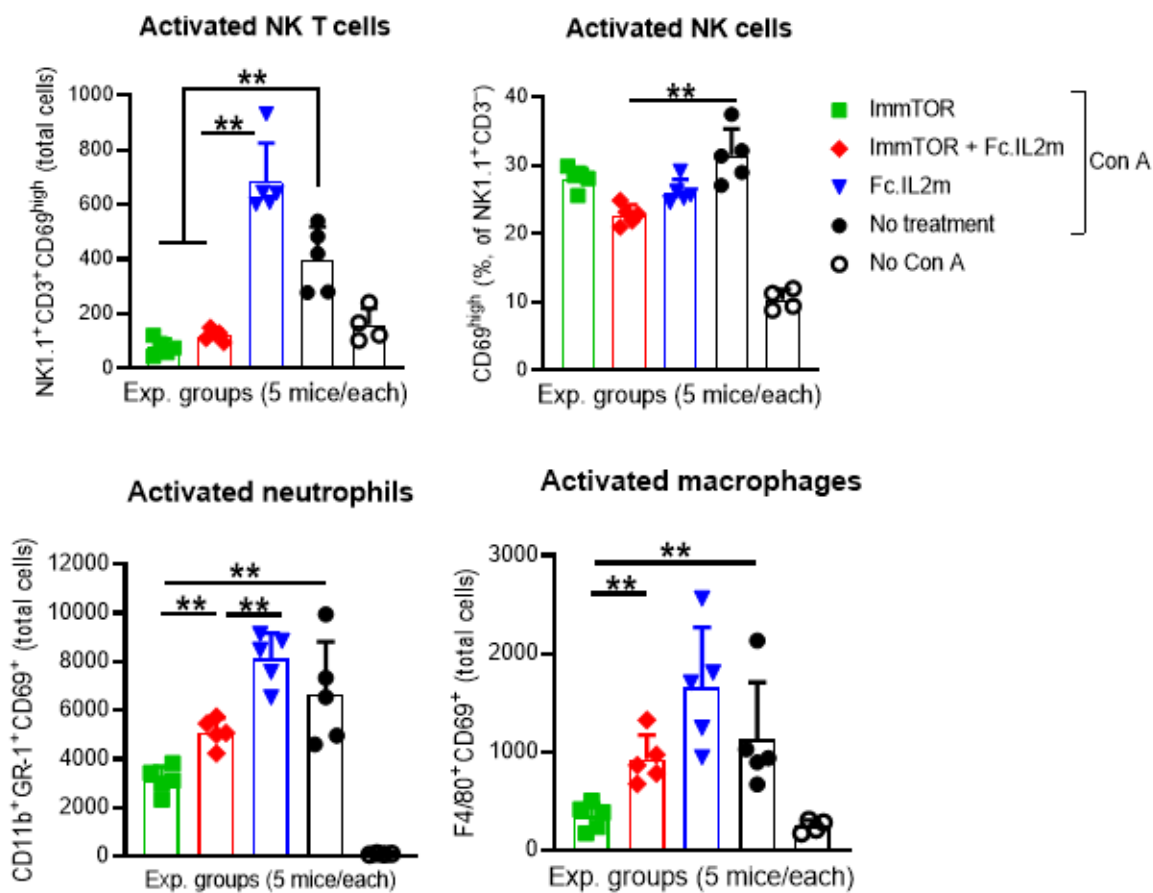
Supplemental Figure 3



Supplemental Figure 4

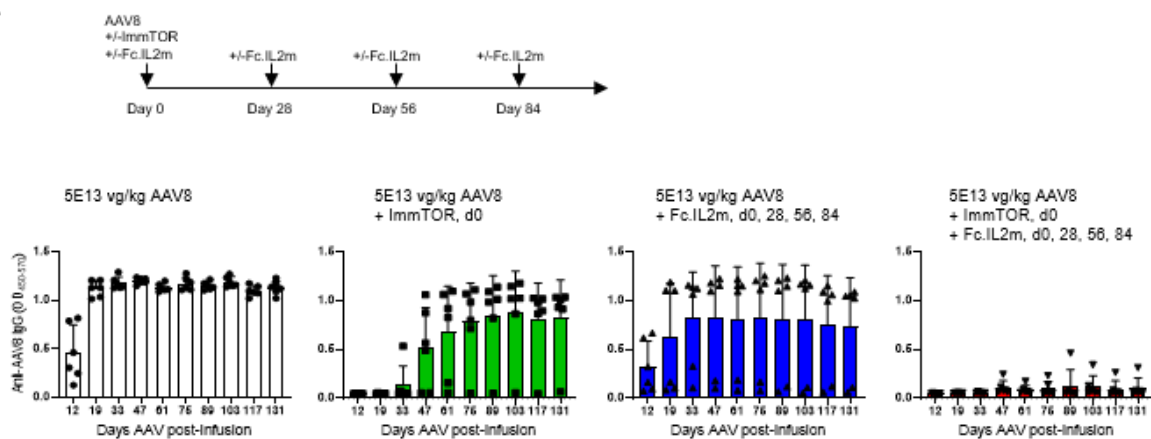


Supplemental Figure 5



Supplemental Figure 6

A



B

