Title: Regulatory T cell expansion by a highly CD25-dependent IL-2 mutein arrests ongoing autoimmunity

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Abstract

Interleukin-2 (IL-2) controls the function of regulatory T cells (Treg), and defects in the IL-2 pathway contribute to multiple autoimmune diseases. Although IL-2 therapy has been efficacious in certain inflammatory conditions, the capacity for IL-2 to also activate inflammatory effector responses highlights the need to develop IL-2-based therapeutics with improved Treg-specificity. We targeted conserved residues in IL-2 that interact with the IL-2-receptor, and identified an optimal IL-2 mutein with reduced potency, but enhanced Treg selectivity due to increased dependence on the CD25 IL-2-receptor subunit. IL-2 mutein treatment led to greater Treg enrichment and reduced agonism of effector cells across a wide dose range, and sustained resolution of spontaneous autoimmunity. Thus, IL-2 mutein treatment allows for selective Treg enrichment and targeted control of the autoimmune response.

One Sentence Summary

A CD25-dependent IL-2 mutein selectively expands regulatory T cells and provides safe and effective control of autoimmunity.

Main Text

Regulatory T cells (Tregs) maintain immune homeostasis and prevent autoimmunity (*1*, *2*). Tregs rely on interleukin 2 (IL-2) for their maintenance and function (*3*), and boosting IL-2 activity has been explored for treatment of autoimmunity. The IL-2 receptor (IL-2R) is expressed either as an intermediate-affinity dimer, composed of IL-2Rβ (CD122) and the common cytokine receptor gamma chain (CD132), or a high-affinity trimer that includes CD25, which has no signaling capacity and instead functions by binding IL-2 with high affinity, presenting it to the CD122/CD132 signaling complex (*4*). CD25 is constitutively expressed by most Foxp3$^+$ regulatory T cells (Tregs) (*5*–*7*), making them highly responsive to limiting amounts of IL-2. CD25 is also induced on conventional CD4$^+$ and CD8$^+$ T cells after TCR stimulation, and CD122/CD132 are highly expressed on memory CD8$^+$ T cells and NK cells (*8*). To avoid activation of these effector cells, IL-2 has been tested at the lowest biologically active doses in autoimmune patients (*9*). Nevertheless, balancing the efficacy and the safety of IL-2 therapy remains a major concern, and thus there is a pressing need for IL-2 based therapeutics with improved Treg-selectivity.

We hypothesized that reducing IL-2 binding to CD122 would increase CD25 dependency and improve Treg-selectivity in therapeutic settings. For this, we generated constructs for twenty-eight mouse IL-2 variants (IL-2 muteins, table S1), mutating conserved residues with known functional importance at the CD122 contact interface (*10*). To improve pharmacokinetics and tissue distribution, the IL-2 muteins were fused to a mutated IgG2a Fc domain with abolished effector function (hereafter referred to as Fc.WT and Fc.muteins). We expressed the constructs in HEK 293T/17 cells and screened the Fc.muteins for activity by measuring phosphorylation of signal transducer and activator of transcription 5 (STAT5) in Tregs. The Fc.muteins ranged from full activity comparable to wild-type Fc.IL-2 (Fc.WT) to complete functional abrogation (fig. S1).

Since weaker mutein activity (indicating diminished IL-2/CD122 binding) should translate into enhanced Treg-selectivity, we selected four mutein candidates exhibiting intermediate (Fc.Mut24 and Fc.Mut12) to weak (Fc.Mut25 and Fc.Mut26) activity for further evaluation. When titrated on freshly isolated splenocytes, Fc.Mut24, Fc.Mut12 and Fc.Mut25 showed reduced signaling activity on Treg cells that was highly CD25-dependent, but unlike Fc.WT did not stimulate $CD4^{+}Foxp3^{-}$ T cells, $CD8^{+}$ T cells or NK cells even at high doses (Fig. 1, A-C). We confirmed with CD25-expressing HEK cells that the Fc.muteins bound CD25 with the same affinity as Fc.WT (fig. S2), establishing that reduced signaling potency was due to impaired interaction with CD122 or CD122/CD132 heterodimers. To assess the Treg selectivity of the Fc.muteins under conditions more closely resembling inflamed tissues, we used splenocytes that were pre-activated with anti-CD3, which results in elevated CD25 expression and enhanced IL-2 responsiveness of both $CD4^{+}Foxp3^{-}$ and $CD8^{+}$ T cells. Again, we found that the Fc.muteins were more Treg-selective than Fc.WT across a wide dose range (fig. S3A), and that decreasing potency of the Fc.muteins correlated with enhanced dependence on CD25 for signaling (fig. S3B).

To extend these findings *in vivo*, we compared responses of IL-2 sensitive lymphocytes in the lymph nodes of B6 mice after treatment with Fc.WT, Fc.Mut12, Fc.Mut24 and Fc.Mut25. Interestingly, despite its weaker activity *in vitro*, Fc.Mut24 was even more effective than Fc.WT at expanding the Treg population *in vivo*, whereas Fc.Mut12 showed weaker activity and Fc.Mut25 had no effect (Fig. 1, D and E). Additionally, Fc.Mut24 showed the greatest degree of Treg expansion vs. effector $CD8^{+}$ T cells and NK cells (Fig. 1, F and G), and based on these results we selected Fc.Mut24 as the optimal candidate for Treg-enrichment based therapy.

We compared the kinetics of *in vivo* responses in Treg cells to Fc.WT and Fc.Mut24 by examining Tregs cells in the blood between 3-7 days following a single treatment. In Fc.WT-treated mice, the

percentage of Tregs in the blood peaked on day 3 and declined rapidly to near baseline by day 7. By contrast, the response to Fc.Mut24 peaked at day 4, then slowly declined but remained elevated compared to baseline for at least a week (Fig. 2, A and B). Furthermore, at all timepoints the ratios of Treg:$CD8^+$ cell and Treg:NK cell were higher in Fc.Mut24-treated mice than in Fc.WT-treated ones, indicating that Treg-selectivity is maintained throughout the response (Fig. 2, C and D). Thus, despite being a weaker agonist of IL-2R signaling *in vitro*, we observed more robust and sustained responses of Treg cells to Fc.Mut24 *in vivo*. This led us to hypothesize that due to its weaker activity, Fc.Mut24 should exhibit reduced receptor-mediated clearance, resulting in prolonged exposure to Treg cells. To test this, splenocytes pre-activated with anti-CD3/CD28 were coated with Fc.WT or Fc.Mut24 and cultured for up to 24h. We assessed surface retention of the IL-2 fusion protein at various time points by staining cells for CD25 and the Fc portion of cell-associated Fc.IL-2. Whereas the amount of Fc.WT on the surface of $CD4^+CD25^{hi}$ cells rapidly declined, surface retention of Fc.Mut24 was dramatically prolonged, and we detected substantial levels of Fc.Mut24 on the cell surface even after 24h (Fig. 2, E and F). Accordingly, the half-life of Fc.Mut24 *in vivo* was approximately three times longer than Fc.WT, and was closer to that of the functionally inactive mutein Fc.Mut26 (Fig. 2G). Thus, diminished consumption via IL-2R signaling results in extended pharmacokinetics of Fc.Mut24, leading to enhanced Treg enrichment *in vivo*.

The narrow dose-range that must be used to avoid effector cell activation is a barrier to the implementation of IL-2-based therapies in autoimmune diseases. To determine if the increased CD25-dependency of Fc.Mut24 would enable a wider-dose range while maintaining Treg-selectivity, we assessed Treg and effector lymphocyte activation in response to increasing doses of Fc.WT or Fc.Mut24. As indicated by induction of the proliferation marker Ki-67, both Fc.WT

and Fc.Mut24 activated Tregs in a dose-dependent manner. However, whereas Fc.WT also activated $CD4^+Foxp3^-$ T cells, $CD8^+$ T cells and NK cells, Fc.Mut24 showed only low activity in NK cells at the highest doses tested (Fig. 3A). Consequently, there was a robust dose-dependent increase in the frequency of Tregs compared to the effector populations in Fc.Mut24-treated animals, with Tregs reaching more than 50% of $CD4^+$ T cells at the highest dose. In contrast, the Treg response to Fc.WT plateaued at less than 30% Treg (Fig. 3, B to E). Stimulation of NK cells by Fc.Mut24 *in vivo* is curious given their lack of CD25 expression and the inability of Fc.Mut24 to stimulate NK cells *in vitro*. However, NK cells express extremely high levels of CD122, and trans-presentation of IL-2 by CD25-expressing cells to $CD122^{hi}$ cells has been reported (*11*). Thus, this NK response may represent the limit of Treg specificity that can be achieved by selectively targeting IL-2 to $CD25^+$ cells.

To determine if the Treg-selectivity of Fc.Mut24 is maintained at sites of ongoing autoimmunity, we treated 9-week old female non-obese diabetic (NOD) mice with a single 15μg dose of Fc.WT, Fc.Mut24, or PBS, and assessed responses of different lymphocyte populations in the spleen, pancreatic lymph node and pancreas. In the NOD model of type 1 diabetes (T1D), autoimmunity begins as early as 3 weeks of age (*12*), and is characterized by infiltration of $CD25^+$ effector T cells in the pancreas (*13*). Indeed, both $CD4^+Foxp3^-$ and $CD8^+$ T cells in the pancreas, but not the pancreatic lymph node, of NOD mice upregulated Ki-67 in response to high-dose Fc.Mut24 (fig. S4). However, despite this apparent loss of Treg-selectivity in an IL-2R-proximal response, we still observed dominant expansion of Tregs in all tissues of Fc.Mut24-treated NOD mice that was superior to that in Fc.WT-treated animals (Fig. 4, A to D).

In the NOD T1D model, low-dose IL-2 treatment led to significant disease protection, whereas, conversely, high-dose IL-2 treatment accelerated disease (*13*). Thus, this model should allow us

to determine if the improved Treg-selectivity and pharmacokinetics of Fc.Mut24 would translate to therapeutic efficacy across a wider dose-range and with reduced dosing frequency. Beginning at 10 weeks of age, we treated NOD mice with either a low (3μg) or high (15μg) dose of Fc.WT or Fc.Mut24 for a total of 5 doses over 18 weeks, and monitored blood glucose levels until 34 weeks of age. By 25 weeks, all PBS-treated control mice developed diabetes. In contrast, low-dose Fc.Mut24 treatment offered substantial disease protection that was comparable to that observed in Fc.WT-treated mice. However, whereas the efficacy of high-dose Fc.WT was somewhat reduced, high-dose Fc.Mut24 further enhanced protection against diabetes, with 75% of the mice remaining disease-free (Fig. 4E). Consistent with our *in vitro* and i*n vivo* Fc.mutein characterization, this finding indicates that Fc.Mut24 is immunosuppressive across a wide dose range compared to Fc.WT.

To further evaluate the therapeutic efficacy of Fc.Mut24, we compared Fc.WT and Fc.Mut24 in the NOD model using a dose escalation regimen in which mice were given a single low-dose (3μg) of Fc.WT or Fc.Mut24, followed 4 days later by a 12 week course of bi-weekly high-dose (15μg), a strategy that would still allow Treg to return to baseline frequencies between doses (Fig. 2). Indeed, consistent with previous studies, this high-dose/bi-weekly Fc.WT treatment significantly accelerated disease development compared to PBS-treated controls. In contrast, Fc.Mut24 conferred a significant and durable protection against diabetes lasting for at least 20 weeks following treatment cessation (Fig. 4F). Importantly, histological examination of the mice that were diabetes-free at the end of the study showed a dramatic reduction in the severity of insulitis in the Fc.Mut24-treated mice compared to PBS controls (Fig.4, G and H). Taken together, these results reveal the capacity for transient, periodic Treg enrichment with Fc.Mut24 to maintain

efficient and targeted control of autoimmunity, despite the presence of Fc.mutein-responsive pathogenic T cells at the site of autoimmune inflammation.

A recent clinical trial investigating the effects of low-dose IL-2 (LD IL-2) therapy across 11 autoimmune diseases has shown encouraging signs of efficacy using a dose of 1 MIU/injection in all patients (*14*). However, finding a "universal" dosage in autoimmunity is very challenging. In addition to the heterogeneity observed in the physiopathology of autoimmune diseases, some patients may also carry intrinsic alterations of the IL-2 pathway (*15*) that can limit the efficacy of the treatment, as in T1D (*16*). Our results demonstrate that increased CD25-dependency of Fc.muteins translates into an improved dynamic range of selective Treg enrichment and a greater flexibility in dosing strategies, without compromising efficacy or safety. Furthermore, due to prolonged immune modulation following individual doses, we found that chronic Treg enrichment was not required to provide lasting protection against predisposed autoimmunity. Recently, human IL-2 muteins have been described with similar Treg selective properties *in vitro* and in the clinic (*17–19*), and our findings provide a strong proof-of-concept for IL-2 mutein therapy. Indeed, through optimal and targeted control of the autoimmune response, this approach represents a more efficient and safer alternative to LD IL-2.

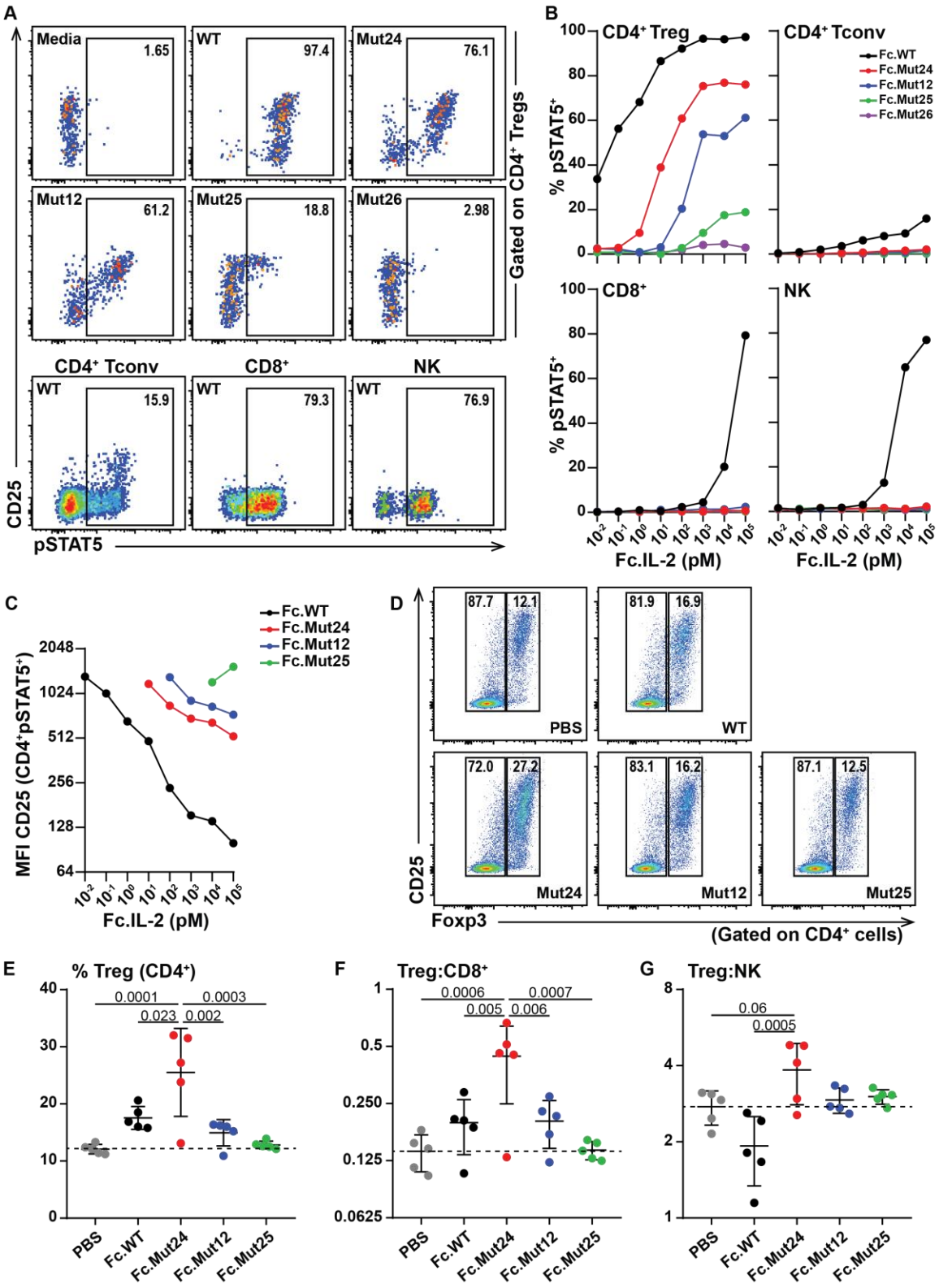
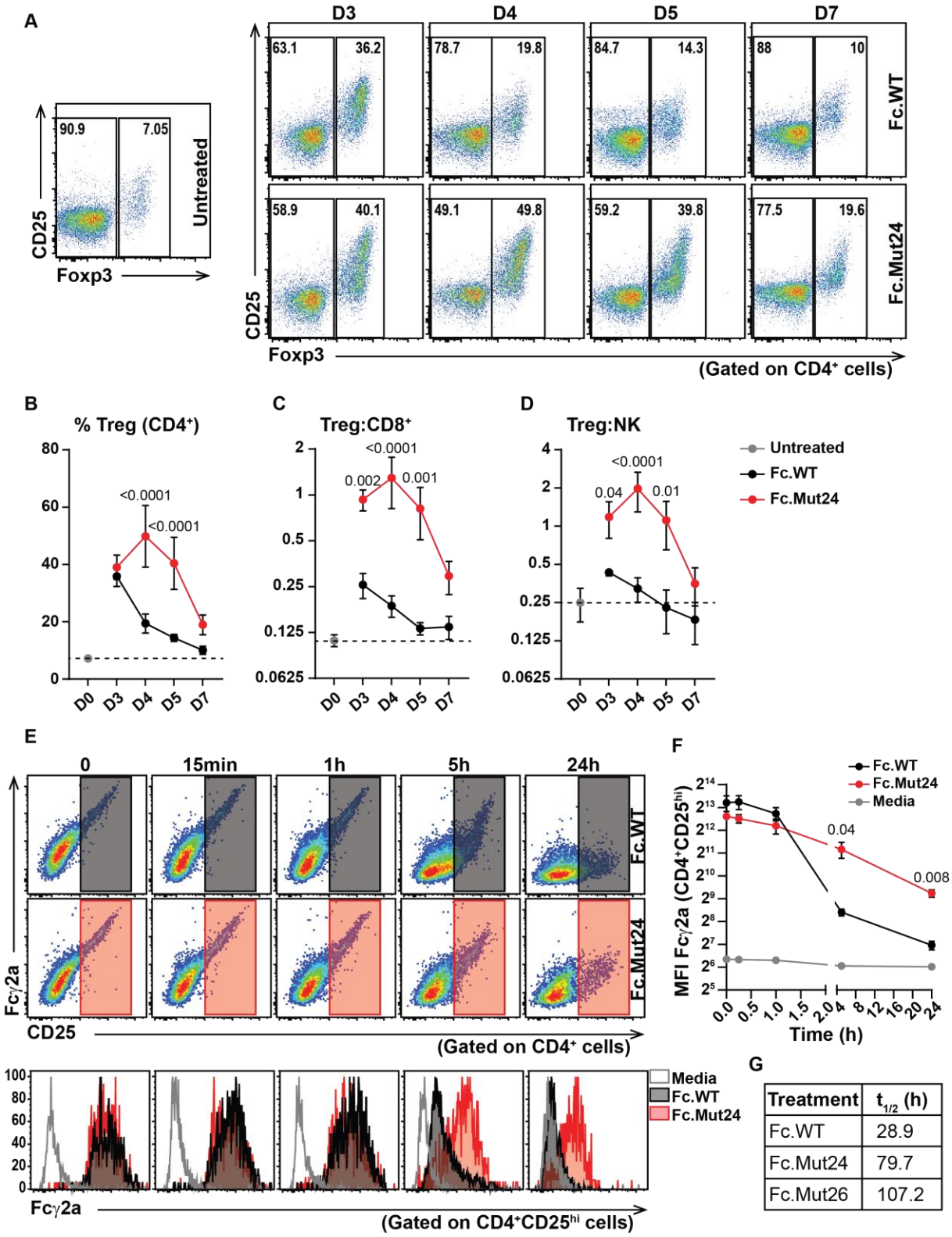


Fig. 1. Fc.muteins show a weak signaling potency but a high Treg selectivity. (**A-C**) B6 Splenocytes were stimulated with the indicated purified Fc.IL-2 fusion proteins. (**A**)

Representative flow cytometric analyses for the 100nM dose. (**B**) pSTAT5 dose-response curves for the indicated populations. (**C**) CD25 median fluorescence intensity on $CD4^+pSTAT5^+$ cells. Data are representative of at least two independent experiments. (**D-G**) B6 mice were treated with a single dose of PBS or the indicated Fc.IL-2 (8μg), and inguinal lymph nodes were harvested four days later. (**D**) Representative flow cytometric analyses of Treg frequency. (**E**) Frequencies of Treg and ratios of (**F**) Treg:$CD8^+$ and (**G**) Treg:NK are summarized. Data shown as mean ± SD, significance determined by one-way ANOVA followed by Tukey post-test. Representative of at least two independent experiments with n=5 mice/group.



Treatment	$t_{1/2}$ (h)
Fc.WT	28.9
Fc.Mut24	79.7
Fc.Mut26	107.2

Fig. 2. Sustained Treg enrichment *in vivo* and lower target mediated clearance of Fc.Mut24.

(**A-D**) B6 Mice were treated with Fc.WT or Fc.Mut24 (15µg) and blood was collected at different

times for analysis. (**A**) Representative analyses of Foxp3 and CD25 expression. (**B**) Treg frequencies and ratios of (**C**) Treg:$CD8^+$ and (**D**) Treg:NK are summarized. Data shown as mean ± SD, significance determined by two-way ANOVA followed by Tukey post-test. Representative of two independent experiments with n=4-5 mice/group. (**E-F**) Anti-CD3/CD28 activated B6 splenocytes were coated with Fc.WT or Fc.Mut24 (1nM), washed, and cultured for the indicated times. (**E**) Upper and middle: Representative staining for surface Fc.IL-2 and CD25 on $CD4^+$ cells. Bottom: Analysis of Fcγ2a staining on $CD4^+CD25^{hi}$ cells. (**F**) Analyses of median fluorescence intensity of Fcγ2a on $CD4^+CD25^{hi}$ cells. Data shown as mean ± SD, significance determined by repeated-measures two-Way ANOVA with the Geisser-Greenhouse correction followed by Tukey post-test. Data representative of three biological replicates. (**G**) *In vivo* half-life of the indicated Fc.IL-2 molecules.

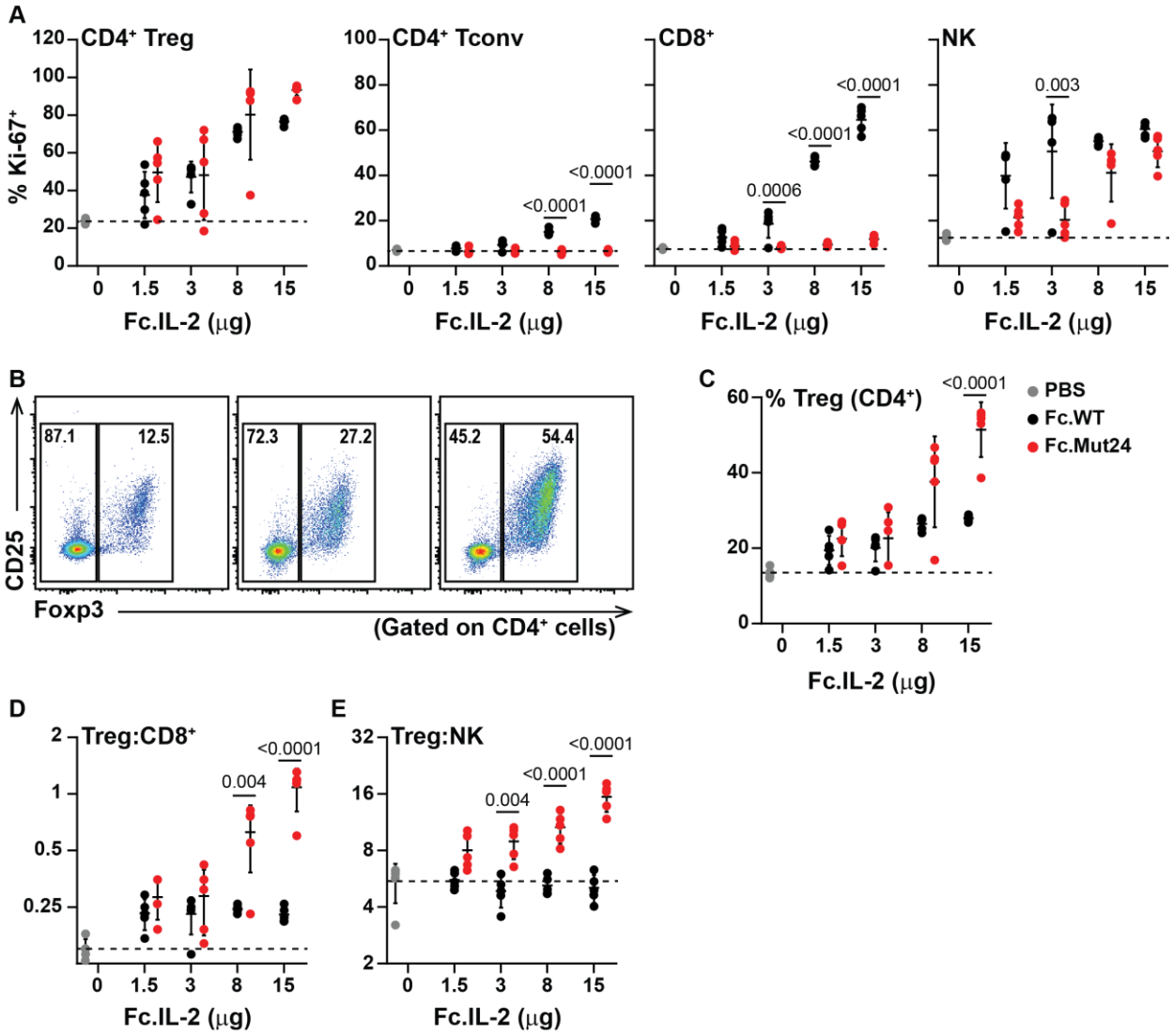


Fig. 3. Fc.Mut24 remains Treg-selective across a wide dose range *in vivo*. B6 Mice were treated with either PBS or Fc.IL-2 and inguinal lymph nodes were harvested four days later for analysis by flow cytometry. (**A**) Representative analyses of Foxp3 and CD25 expression for the 15μg group. (**B**) Treg frequencies, ratios of (**C**) Treg:CD8$^+$ and (**D**) Treg:NK, and (**E**) percentages of Ki-67$^+$ cells in the indicated populations are plotted. Data shown as mean ± SD, significance determined by two-way ANOVA followed by Tukey post-test. Representative of at least two independent experiments with n=5 mice/group.

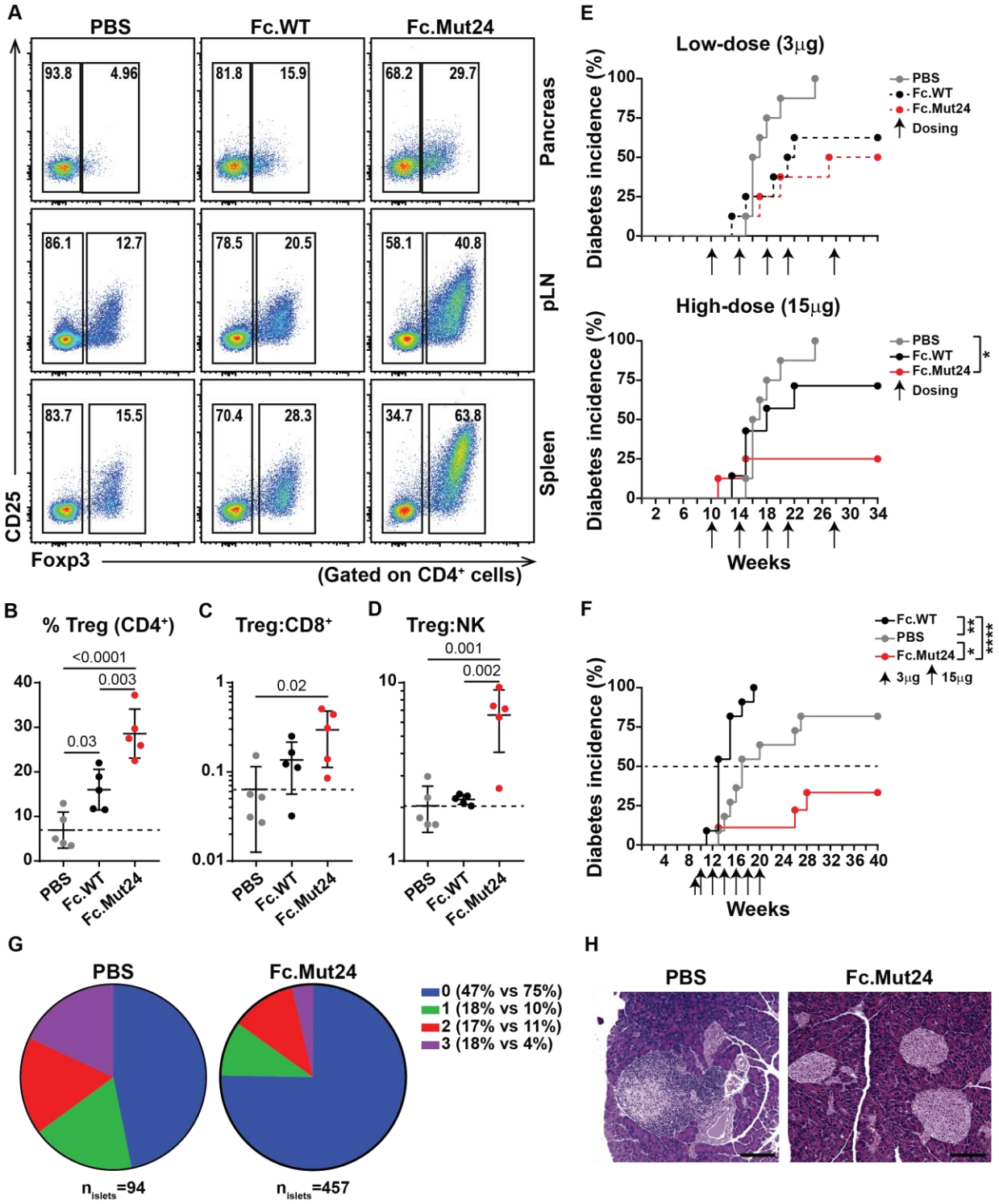


Fig. 4. Fc.Mut24 induces selective Treg enrichment and arrests ongoing autoimmunity in NOD mice. (**A-E**) Female NOD mice were treated with PBS or Fc.WT or Fc.Mut24 (15μg) and tissues were harvested four days later. (**A**) Representative analyses of Foxp3 and CD25 expression

in the indicated tissues. (**B**) Treg frequencies and ratios of (**C**) Treg:CD8$^+$ and (**D**) Treg:NK in the pancreas. Data shown as mean ± SD, significance determined by one-way ANOVA followed by Tukey post-test. Representative of two independent experiments with n=5 mice/group. (**E-F**) Diabetes incidence in female NOD mice treated with PBS, Fc.WT or Fc.Mut24. Arrows indicate time of dosing. Significance determined by Log-rank (Mantel-Cox) test, *p<0.05, **p<0.01, ****p<0.0001, n=7-8 in (**E**) and n= 9-11 in (**F**). (**G-H**) Histological analysis of insulitis severity in disease-free mice at the end of the experiment shown in (**F**). (**G**) Percentages of islets with no infiltration (0), peri-infiltration (1), <50% infiltration (2), and >50% infiltration (3). (**H**) Representative H&E staining of a grade 3 islet from a PBS-treated mouse and three grade 0 islets from an Fc.Mut24-treated mouse (scale bar 100μm).

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Competing interests: M.A.G. owns stock in Amgen, Inc.

Supplementary Materials

Materials and Methods

Figs. S1 to S4

Table S1