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In Vivo Expansion of Antigen-Specific Regulatory T Cells through Staggered Fc.IL-2 Mutein Dosing and Antigen-Specific Immunotherapy

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ABSTRACT

In mice, Ag administration in the absence of adjuvant typically elicits tolerogenic immune responses through the deletion or inactivation of conventional CD4 T cells and the formation or expansion of regulatory CD4 T cells (Treg). Although these “Ag-specific immunotherapy” (ASI) approaches are currently under clinical development to treat autoinflammatory conditions, efficacy and safety may be variable and unpredictable because of the diverse activation states of immune cells in subjects with autoimmune and allergic diseases. To reliably induce Ag-specific tolerance in patients, novel methods to control T cell responses during ASI are needed, and strategies that permanently increase Treg frequencies among Ag-specific CD4 T cells may provide long-lasting immunosuppression between treatments. In this study, we present an approach to durably increase the frequency of Ag-specific Treg in mice by administering ASI when Treg numbers are transiently increased with individual doses of a half-life-extended Treg-selective IL-2 mutein. Repeated weekly cycles of IL-2 mutein doses (day 0) followed by ASI (day 3) resulted in a 3- to 5-fold enrichment in Treg among Ag-responsive CD4 T cells. Expanded Ag-specific Treg persisted for more than 3 wk following treatment cessation, as well as through an inflammatory T cell response to an Ag-expressing virus. Combining Treg enrichment with ASI has the potential to durably treat autoimmune disease or allergy by increasing the Treg/conventional CD4 T cell ratio among autoantigen- or allergen-specific T cells. *ImmunoHorizons*, 2021, 5: 782–791.

INTRODUCTION

Immune homeostasis is mediated in part through a tug-of-war between pro- and anti-inflammatory T cells, in which populations of self-antigen- and foreign-Ag-reactive T cells are chronically restrained by immunosuppressive regulatory CD4 T cell (Treg). In autoimmunity, self-reactive conventional CD4 T cell (Tconv) overpower Treg, resulting in detrimental attack of self-tissues, and one approach to restore balance is to boost the quantity and

quality of Treg relative to Tconv (1). Based on extensive preclinical work, enhancing Treg in autoimmune patients is currently being tested through two main approaches: 1) stimulation of Treg growth and function via therapeutic administration of rIL-2 or IL-2 variants (muteins) (2, 3), and 2) adoptive transfer of in vitro-expanded patient-derived Treg (4, 5). Although reports of clinical efficacy in autoimmune diseases are forthcoming for IL-2 muteins and adoptive Treg therapy, robust and consistent reversal of autoimmunity with low-dose IL-2 has not yet been

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Abbreviations used in this article: ASI, Ag-specific immunotherapy; DC, dendritic cell; DEC-OVA, anti-DEC205–OVA fusion protein; Fc.IL2m, Fc-fused IL-2 mutein no. 27 containing IL-2 mutations M33D and V106D; FVIII, factor VIII; LN, lymph node; pTreg, Treg from peripheral Tconv; Tconv, conventional CD4 T cell; Treg, regulatory CD4 T cell; tTreg, thymically derived Treg; VSV-OVA, vesicular stomatitis virus–encoding OVA.

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demonstrated (6–9). If these approaches ultimately fail to reach clinical trial end points, poor efficacy may result from 1) weak Treg agonism and/or poor Treg-selectivity with low-dose IL-2 or IL-2 muteins, 2) induction of IL-2R nonresponsiveness following extended IL-2 or IL-2 mutein treatment, 3) poor persistence of adoptively transferred Treg, and/or 4) low representation of Treg specific for disease-associated Ags within enhanced Treg populations. Regarding this last limitation, preclinical experiments in which Treg specificity is engineered with transgenic TCRs or chimeric Ag receptors revealed that, unlike polyclonal Treg, Ag-directed Treg were highly effective in halting and reversing autoimmunity (10–13). Although methods for adoptive transfer of Treg with engineered specificity are currently under clinical development (14), pharmacological approaches to enhance endogenous Ag-specific Treg are potentially more scalable and warrant further investigation.

In a wide range of mouse models of autoimmunity, allergy, and transplant rejection, Treg enrichment with IL-2/Ab complexes (IL-2c) (15) prior to or during Ag challenge consistently suppressed disease and pathology (16–22), raising the possibility that Ag priming in the presence of elevated Treg frequencies will durably alter the balance between Ag-specific Treg and pathogenic Tconv in patients. An example of combined Treg enrichment and Ag-specific immunotherapy (ASI) in mice is the complete suppression of factor VIII (FVIII) Ab formation in FVIII-deficient mice treated with a combination of IL-2c and FVIII protein (23, 24). In this setting, FVIII-specific Treg were elicited and, upon isolation and transfer, could establish tolerance in untreated mice. These studies also demonstrated that Treg enrichment before FVIII challenge was more effective than concomitant treatment (24). Leveraging the same system, we recently reported that combining a Treg-selective Fc-fused IL-2 mutein with FVIII gene therapy also established durable suppression of FVIII Ab formation (25, 26).

To bridge these findings to clinical trial design, more research is needed to find the optimal dosing strategies that enforce tolerogenic responses to ASI and increase the relative abundance of Ag-specific Treg, and our goal in this work was to define a specific dosing regimen that permanently augmented this ratio. Because Ag-stimulated Tconv should upregulate CD25 and consequently increase Fc.IL-2 mutein-responsiveness, we considered the timing of ASI following Fc.IL-2 mutein treatment to be an important parameter to optimize to ensure that residual Fc.IL-2 mutein would not act on ASI-stimulated CD25⁺ Tconv. Our characterization of Fc.IL-2 muteins suggested that the time point of peak Treg enrichment was a likely indicator of the moment when the Fc.IL-2 mutein fell below biologically active levels (26); thus, we reasoned that ASI at time points centered around peak Treg enrichment should reveal the best strategy. With an optimal dosing regimen selected, we then explored the durability of Ag-specific Treg enrichment following treatment cessation and through acute viral infection.

MATERIALS AND METHODS

Mice

Female 5- to 6-wk-old C57BL/6J (Stock No: 000664) and B6.SJL-Ptprc^a Pepc^b/BoyJ (Stock No: 002014) (CD45.1) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Female OT-II mice were bred in house, and maintained in specific pathogen-free conditions. All experiments involved female mice at the age of 8–13 wk. Experiments were executed under an animal study proposal approved by the Animal Care and Use Committee of Benaroya Research Institute (Seattle, WA).

Adoptive transfer

Cell suspensions were generated from pooled inguinal, mesenteric, brachial, axillary, and superficial cervical lymph nodes (LNs) isolated from female OT-II mice. Two million LNs cells in 50 μ l of PBS were injected retro-orbitally into anesthetized B6.SJL mice. Anesthesia was induced by 1–4% isoflurane.

Dosing regimens of Fc-fused IL-2 mutein no. 27 containing IL-2 mutations M33D and V106D and anti-DEC205-OVA fusion protein

The Fc.IL-2 molecules were expressed in HEK293 cells and were purified from supernatants by protein A chromatography (Olympic Protein Technologies, Seattle, WA). For testing the pharmacodynamics of Treg enrichment, C57BL/6J mice ($n = 5$) received a single 3- μ g i.p. dose of Fc-fused IL-2 mutein no. 27 containing IL-2 mutations M33D and V106D (Fc.IL2m), and flow cytometry was performed with saphenous vein blood samples obtained on days 0, 3, 4, 5, and 7.

Four days after the adoptive transfer of OT-II LN cells, recipient B6.SJL mice were injected i.p. with 3 μ g of Fc.IL2m or PBS, after which mice received 0.5 μ g of anti-DEC205-OVA or PBS on day 2, 3, or 4 after Fc.IL2m treatment. Mice received one or three weekly Fc.IL2m/anti-DEC205-OVA fusion protein (DEC-OVA) treatment cycles. After the last treatment cycle, mice were euthanized, and LNs and spleens were harvested for immunophenotyping.

Because of the large size of these experiments, not all treatment groups were included in each replica experiment. Figures represent the aggregate of all replica experiments, and the number of individual experiments performed is noted in the figure legends.

Vesicular stomatitis virus-encoding OVA infection

Following three Fc.IL2m/DEC-OVA treatment cycles (D22), B6.SJL hosts were infected intranasally with vesicular stomatitis virus-encoding OVA (VSV-OVA) at a dose of 10⁴ PFU in 50 μ l of sterile PBS. For this procedure, mice were anesthetized with 1–4% isoflurane. Infected mice were housed in an Animal Biosafety Level 2 room.

Enrichment of OT-II donor T cells

Pooled spleen and LN cells were isolated from B6.SJL-recipient mice and RBCs were lysed with Life Technologies ACK lysing

buffer at room temperature for 5 min. Washed cell suspensions were counted (Invitrogen Countess II Automated Cell Counter) and a small aliquot was retained for immunophenotyping, and remaining cells were pelleted and resuspended in 45 μ l of MACS buffer per 10^7 cells. Anti-CD45.2-PE (5 μ l per 10^7 cells) was added to each sample and incubated for 30 min at 4°C. Cells were washed and resuspended in 90 μ l of MACS buffer per 10^7 cells, and were incubated with anti-PE microbeads (Miltenyi, Auburn, CA) at 20 μ l per 10^7 cells for 10 min at 4°C. After washing and resuspension in 500 μ l of MACS buffer per 10^8 total cell numbers, cells were applied to LS MACS columns in the magnetic column holder (Miltenyi, Auburn, CA). Columns were washed three times with 3 ml of MACS buffer, removed from the magnetic separator, and placed on 15-ml collection tubes. MACS buffer (5 ml) was added to each column and cells were flushed out by firmly pushing the plunger into the column. Column-bound cells were counted and stained for flow cytometric analysis.

Flow cytometry and statistics

For surface staining, cells were incubated at 4°C for 30 min in staining buffer (0.5% BSA in PBS) with the following conjugated Abs (from BioLegend unless otherwise specified): anti-CD3 (145-2C11; BD Biosciences), anti-CD4 (RM4-5), anti-CD8a (53-6.7), anti-CD25 (PC6.1), NK1.1 (PK136), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-CD45.2 (104), anti-CD45.1 (A20), TCR-V β 5.1/5.2 (MR9-4), and TCR-V α 2 (B20.1). All samples were also stained for dead cells with Zombie Aqua Kits from BioLegend according to the manufacture instructions. Cells were washed after surface staining and permeabilized for 30 min with eBioscience Fix/Perm buffer at 4°C. In PermWash staining buffer, cells were stained for 30 min at 4°C with anti-Foxp3, (FJK-16S; eBioscience), anti-Ki67 (B56, BD), and anti-Helios (22F6; Invitrogen). To allow for OT-II T cell enumeration, the entire column-bound fraction was analyzed by flow cytometry. Data were acquired on LSRII Flow Cytometer (BD Bioscience), and analyzed using FlowJo software (TreeStar) (Supplemental Fig. 1). Only mice yielding >50 detectable donor OT-II cells were included in the analyses and figures. Student *t* tests were used to compare population pairs: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, and ******p* < 0.00001.

RESULTS

Expansion of Ag-specific Treg following repeated cycles of ASI during the peak of Fc.IL2m-mediated Treg enrichment

To track the frequency of Ag-specific Treg in mice with a polyclonal T cell repertoire, we used congenically marked B6.SJL mice that received 2×10^6 LN cells from OT-II TCR transgenic mice, which, owing to endogenous TCR expression, contain OVA-reactive Treg at a frequency of ~3–6% of CD4⁺ T cells (27, 28). In this system, the frequency of OT-II T cells in recipient mice was only ~10-fold higher than the typical frequency of naive CD4 T cells specific for a single peptide Ag (29), necessitating donor cell enrichment by magnetic bead selection

to enumerate and phenotype rare OT-II T cells after the treatment regimen (30). For the ASI modality, we employed the DEC-OVA, which targets OVA to DEC205-expressing dendritic cells (DCs) (31). We selected DEC-OVA as our ASI because previous reports have shown that Ag delivered to DEC205⁺ DCs can stimulate both proliferation of pre-existing thymically derived Treg (tTreg) and formation of new Treg from peripheral Tconv (pTreg), potentially increasing the likelihood of achieving Ag-specific Treg enrichment with our protocol (32–34). We selected a 0.5- μ g dose of DEC-OVA because this dose should be processed and presented by MHC class II on DC within 3 h and stimulate OT-II cells for up to 2–3 d following a single injection (31). In our evaluation of Fc.mutins, Fc.Mut27 (IL-2 mutations M33D, V106D) and the well-characterized Fc.Mut24 (N103R, V106D) (26) possessed very similar CD25-dependency, in vivo half-life, and Treg-selectivity (Supplemental Fig. 2); and Fc.Mut27 (hereafter referred to as Fc.IL2m) was used for this work.

Following a single injection of 3 μ g of Fc.IL2m, Treg in blood increased 3-fold on day 3, returning to baseline by day 7 (Fig. 1A). Because Fc.IL2m treatment of OT-II-recipient mice should increase the frequency of both OT-II and host (B6.SJL) Treg, subsequent immunization with DEC-OVA during peak Treg enrichment should further stimulate OT-II Treg growth and survival and confer a numerical advantage over other Treg following their return to baseline frequency on day 7. OT-II Tconv responses to DEC-OVA in the presence of expanded OT-II and host Treg may also enable or enhance their conversion into pTreg through processes akin to infection tolerance (35). Thus, we set out to determine the optimal day to administer DEC-OVA following each weekly Fc.IL2m dose. DEC-OVA dosing was evaluated shortly before, during, and after peak Treg frequencies, or day 2, 3, and 4 after each Fc.IL2m dose. Three treatment cycles were performed, as we postulated that repeated Fc.IL2m/DEC-OVA treatment would elicit more robust OT-II Treg enrichment. The treatment regimen was initiated 4 d following OT-II cell transfer, and on day 22, CD45.2⁺ donor cells were column-enriched from splenocyte and LN cell suspensions and OT-II Treg and Tconv were enumerated by flow cytometry (Fig. 1B).

Consistent with our initial hypothesis, we found that the Fc.IL2m/ASI combination treatment was most effective at enriching OT-II Treg when DEC-OVA was administered on day 2 or 3 after each Fc.mutinin dose (Fig. 1C). In contrast, treatment with DEC-OVA alone modestly increased the frequency of OT-II Treg, and treatment with Fc.IL2m alone had no lasting effect. The durable nature of Treg enrichment with the Fc.IL2m/DEC-OVA combination treatment regimen was specific to OT-II cells, as the frequency of Treg among total host CD4 T cells returned to baseline at day 22 (Fig. 1D). Importantly, the combination treatment did not alter the frequency of OT-II Tconv among total CD4 T cells, and a significant negative correlation between the percent of Treg among OT-II CD4 T cells and overall OT-II Tconv frequency was not observed, indicating that OT-II Treg enrichment resulted from OT-II Treg expansion rather than OT-II Tconv contraction

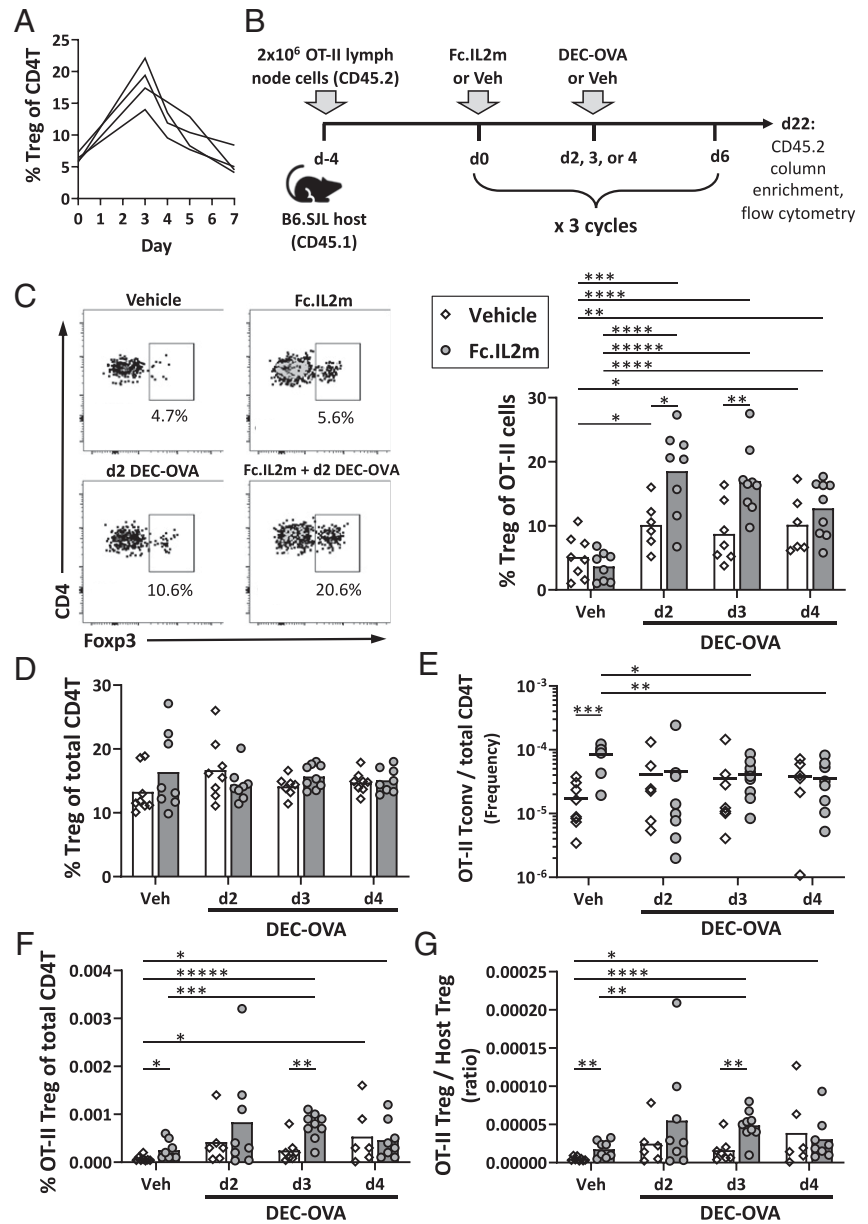


FIGURE 1. Repeated cycles of ASI during peak Fc.IL2m-mediated Treg enrichment results in sustained expansion of Ag-specific Treg.

(A) A single 3- μ g dose of Fc.IL2m results in maximal Treg enrichment in peripheral blood on day 3, which returns to baseline on day 7. (B) Schematic of exploratory Fc.IL2m/ASI treatment regimen in B6.SJL-recipient mice harboring OT-II CD4 T cells. (C) Representative flow cytometry plots and aggregate data showing the percentage of Treg among CD45.2 OT-II CD4 T cells. (D) Percent Treg among total CD4 T cells in recipient mice. (E) Frequency of OT-II Tconv (CD4⁺ Foxp3⁻) cells among total (donor + recipient) CD4 T cells. (F) Percentage of OT-II Treg among total CD4 T cells. (G) Ratio of OT-II Treg to host Treg. Open diamonds, vehicle (PBS); filled circles, Fc.IL2m. Data represent the aggregate of four individual experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, ***** p < 0.00001.

(Fig. 1E, Supplemental Fig. 3). The frequency of donor OT-II cells was highly variable, possibly due to variability in engraftment efficiency (Fig. 1E), and in this experiment—but not in subsequent experiments—we observed an unexpected increase in OT-II engraftment with Fc.IL2m treatment alone (Fig. 1E–G). Relative to the percentage of Treg among OT-II T cells (Fig. 1C), variability in OT-II frequency resulted in high variability in calculations of OT-II Treg among total CD4 T cells and the ratio of OT-II to host Treg (Fig. 1F, 1G). Although the combination treatment was also found to increase OT-II Treg among host cells—particularly for the day 3 ASI (Fig. 1F, 1G)—the percentage of Treg among OT-II CD4 T cells (Fig. 1C) was arguably the most relevant readout for this approach as it conveyed the ratio of suppressor to effector T cells among cells responding to a particular Ag. Thus, the combination of Treg enrichment and ASI

imprinted an elevated Treg frequency among Ag-specific CD4 T cells. For subsequent experiments, we chose to administer DEC-OVA on day 3 following Fc.IL2m or vehicle dosing, as this time point resulted in the most significant increase in OT-II Treg frequency relative to Fc.IL2m treatment alone.

Ag-specific Treg enrichment requires multiple Fc.IL2m/ASI cycles and persists following treatment cessation

For our protocol to serve as an effective Treg vaccine, it was important to characterize the durability of Ag-specific Treg enrichment following treatment cessation. To explore this, we performed a similar set of experiments in which OT-II-recipient mice were treated with weekly DEC-OVA alone, or DEC-OVA 3 d after individual Fc.IL2m doses. Following three treatment

cycles, some mice were evaluated at the completion of the third cycle, whereas others were left untreated for an additional 22 d. Furthermore, to determine whether three cycles were indeed necessary to observe any OT-II Treg enrichment, we included a third set of mice that received only one treatment cycle (Fig. 2A).

In comparing one and three weekly cycles of Fc.IL2m/DEC-OVA, Fc.IL2m, or DEC-OVA alone, or vehicle-control groups, we found no evidence of enrichment among mice receiving only one cycle, whereas three cycles of the combination treatment again elicited more than 3-fold OT-II Treg enrichment (Fig. 2B). Notably, this expansion persisted for an additional 3 wk following treatment cessation (Fig. 2B). As seen in our previous experiment, the frequency of OT-II Tconv did not differ between groups (Fig. 2C), and the frequency of OT-II Treg increased relative to total host CD4 and host Treg cells (Fig. 2D, 2E). Thus, the Fc.IL2m/ASI combination protocol was capable of establishing a durable increase in Ag-specific Treg.

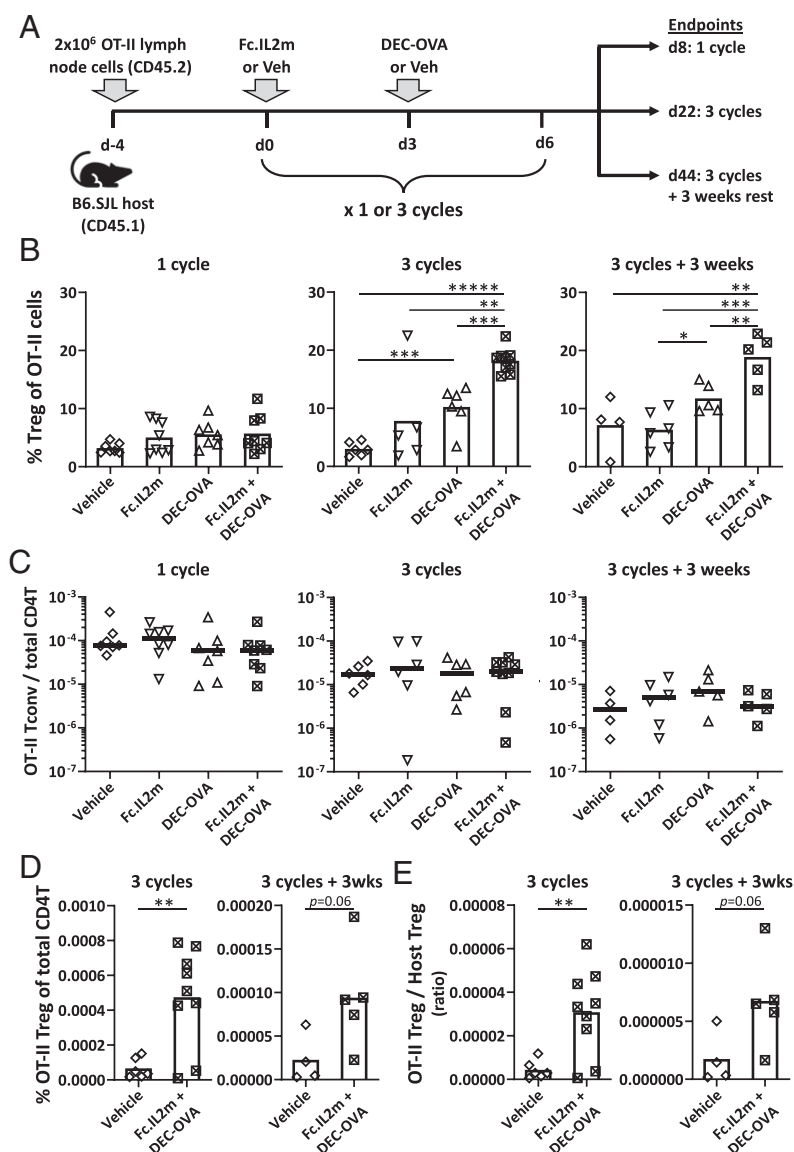
Regarding Treg phenotype following three treatment cycles, expression of transcription factor Helios was similar among the different treatment groups, and was slightly (albeit, insignificantly) lower in OT-II Treg relative to host Treg (Fig. 3A, 3B). CD25 and Ki67 expression was also similar among treatment groups, with a slight increase in Ki67⁺ cells in the Fc.IL2m/ASI combination group, potentially reflecting increased proliferation in response to both stimuli. Relative to host Treg, overall CD25 levels were slightly higher in OT-II Treg regardless of treatment group, a phenotype that may have resulted from stimuli present in the niche populated by transferred OT-II Treg (Fig. 3).

Expanded Ag-specific Treg persist through an acute antiviral immune response directed against the tolerizing Ag

We next asked whether the Fc.IL2m/DEC-OVA-exposed OT-II population would retain its high Treg frequency following

FIGURE 2. More than one Fc.IL2m/ASI cycle is necessary for Ag-specific Treg enrichment, which persists for at least 3 wk following treatment cessation.

(A) Schematic of comparison of one and three weekly treatment cycles, and three cycles followed by 3 wk without treatment. (B) Percentage of Treg among OT-II CD4 T cells in different treatment groups. (C) Frequency of OT-II Tconv among total (donor + recipient) CD4 T cells. (D) Percentage of OT-II Treg among total CD4 T cells. (E) Ratio of OT-II Treg to host Treg. Data represent the aggregate of two individual experiments. $p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.



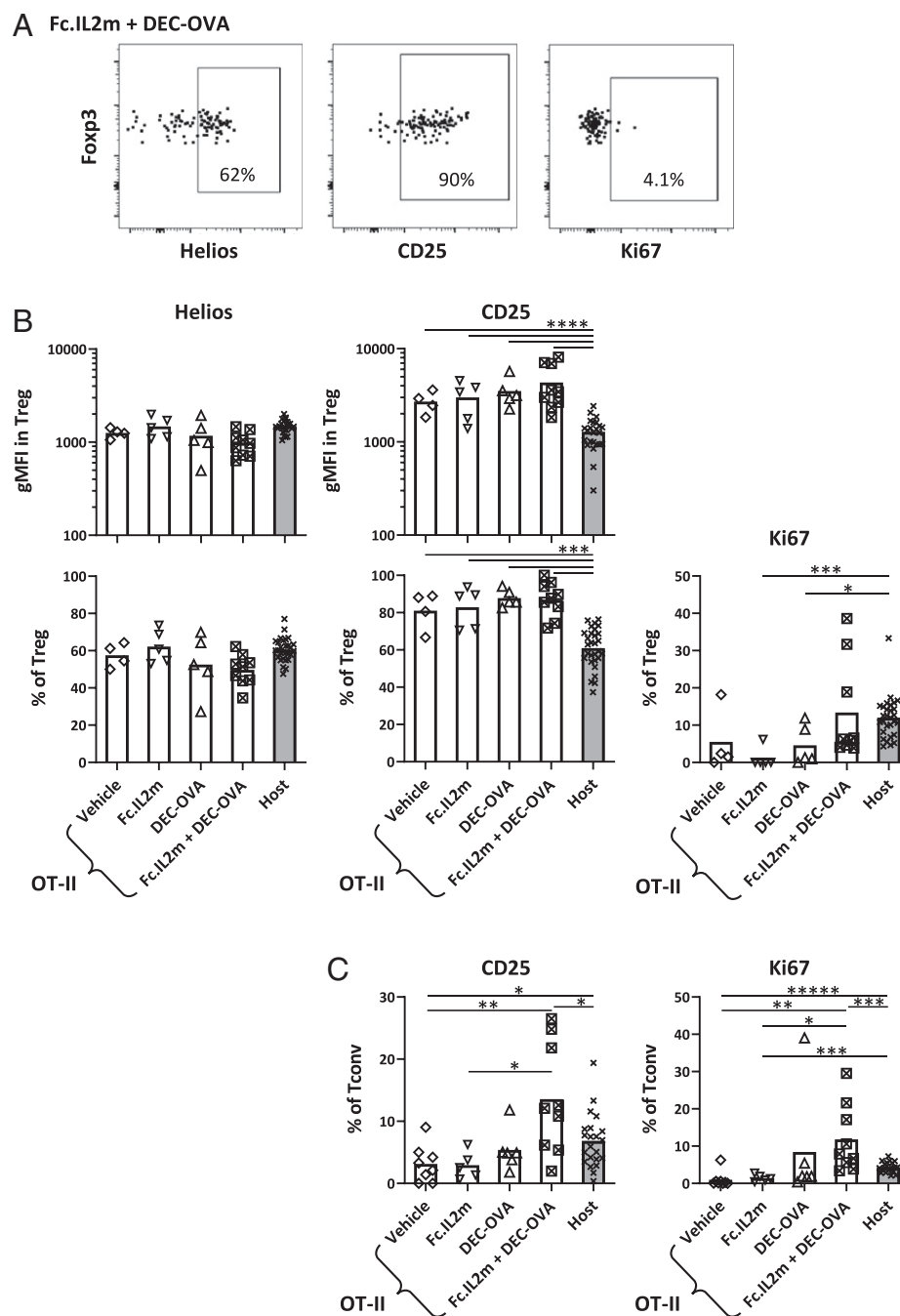


FIGURE 3. Phenotypes of OT-II and host Treg and Tconv following three treatment cycles (Fig. 2A, day 22).

(A) Examples of gate placement for OT-II Treg. The same gates were applied for other populations. (B) Helios and CD25 geometric MFI (top) or percentage of Helios⁺, CD25⁺, or Ki67⁺ cells (bottom) for OT-II Treg of the three treatment groups and host Treg for all treatment groups combined. Flow cytometry data containing more than 10 Treg cells were included in the analysis. (C) Percentage of CD25⁺ or Ki67⁺ among OT-II Tconv in the three treatment groups and host Tconv for all treatment groups combined. All host T cell data were derived from the pooled spleen + LN cell suspension prior to column enrichment of CD45.2⁺ T cells. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, ******p* < 0.00001.

Ag stimulation in a proinflammatory context, and whether the enriched OT-II Treg would suppress the OT-II Tconv response to the same challenge. Following OT-II transfer and three weekly cycles of the different treatment groups, we intranasally infected half of the mice in each group with VSV-OVA (day 22) and analyzed column-enriched OT-II cells 1 wk postinfection (day 29) (Fig. 4A).

Following intranasal VSV-OVA infection, OT-II cells adopted a CD44⁺ CD62L⁺ effector T cell phenotype, indicating robust T cell activation (Fig. 4B). Notably, OT-II Treg frequency markedly declined in the infected PBS and Fc.IL2m groups, yet remained high in the infected DEC-OVA-treated groups. Consequently, a 4.5-fold OT-II Treg enrichment observed for the uninfected combination treatment group

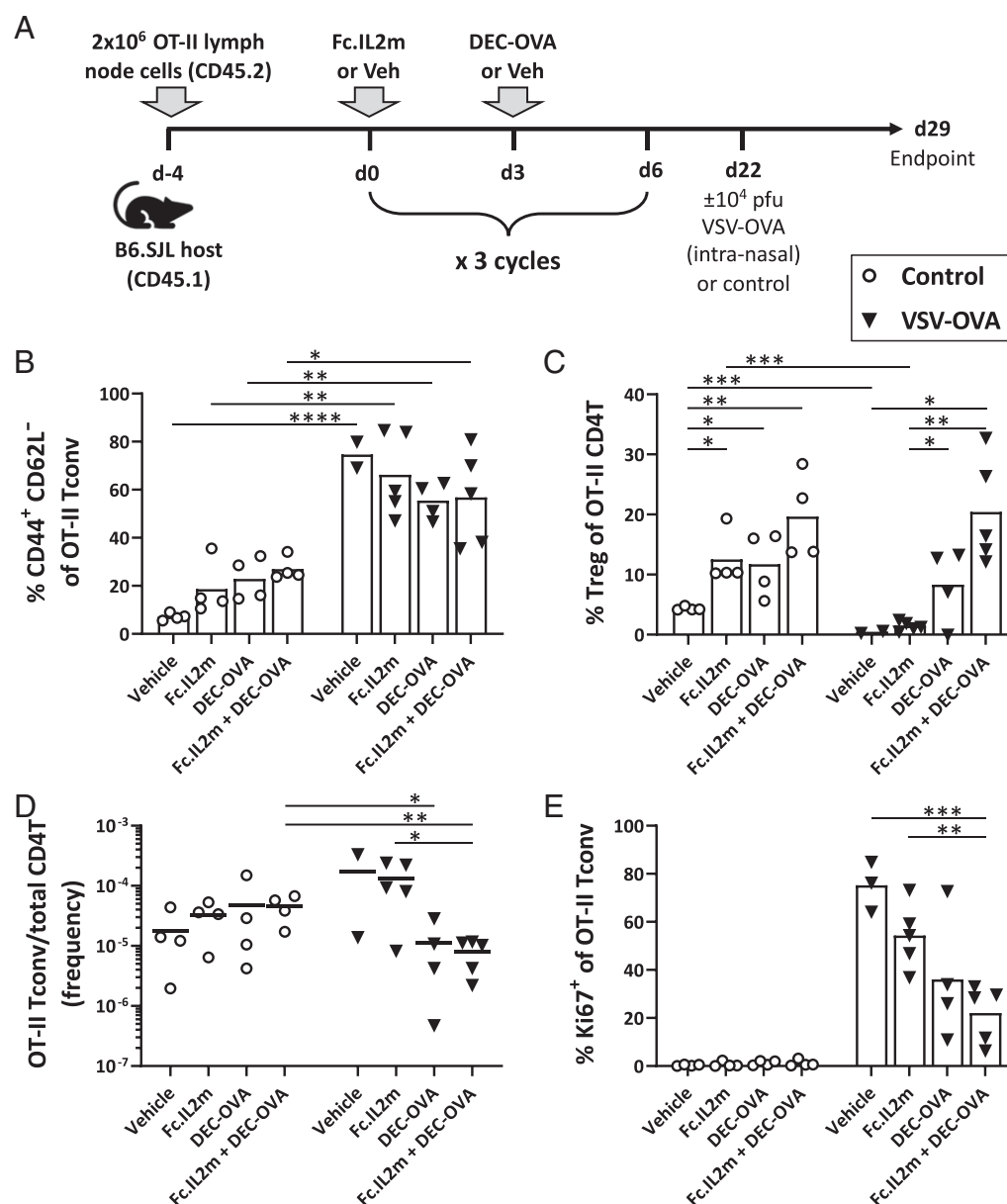


FIGURE 4. OT-II Treg enrichment persists through acute VSV-OVA infection.

(A) Schematic of experimental plan, in which OT-II mice received three treatment cycles followed by intranasal VSV-OVA infection. (B) Percentage of CD44⁺ CD62L⁻ OT-II Tconv. (C) Percentage of Treg among OT-II CD4 T cells. (D) Frequency of OT-II Tconv among total (donor + recipient) CD4 T cells. (E) Percentage of Ki67⁺ cells among OT-II Tconv. Data represent the aggregate of two individual experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

transitioned to a 14-fold increase following VSV-OVA infection (relative to vehicle groups) (Fig. 4C). Among the uninfected animals, we also observed a small increase in effector OT-II Tconv following DEC-OVA or combination treatment, consistent with the expected DEC-OVA stimulation of OT-II cells (31); however, this response coincided with the large enrichment of OT-II Treg in the combination group (Fig. 4B, 4C; uninfected). As in previous experiments, no significant change in OT-II Tconv frequency was observed among the groups, except for modest increases following VSV-OVA infection in the vehicle and Fc.IL2m groups and modest decreases in the DEC-OVA and combination treatment groups, with the most significant difference being a 5-fold decrease in the combination group following infection (Fig. 4D). Consistent with this

observation, VSV-OVA-induced Ki67 in OT-II Tconv was reduced in DEC-OVA-treated mice, a decline that was significant in mice cotreated with Fc.IL2m (Fig. 4E). VSV-OVA infection also induced proliferative responses in OT-II Treg, which were partially attenuated by prior DEC-OVA exposure (Supplemental Fig. 4). This finding suggests that previous DEC-OVA exposure rendered the OT-II cells resistant to infection-induced expansion, either due to cell intrinsic processes such as anergy or due to dominant suppression mediated by the enriched OT-II Treg. Thus, the Fc.IL-2 mutein/ASI treatment protocol is capable of durably expanding Ag-specific Treg that persist through inflammatory processes and may suppress Tconv reactive to the same Ag during acute viral infection.

DISCUSSION

The goal of ASI is to re-establish immune tolerance in autoimmune patients by delivering self-antigens or allergens to tolerate rather than activate B and T lymphocytes, and, as such, is considered a tolerizing vaccination strategy (36–39). In laboratory mice, which harbor a uniquely quiescent immune system, tolerogenic vaccination is effective when Ag is simply delivered without additional response-modifying agents (40). These tolerizing approaches include vaccination with 1) free peptide epitopes, 2) nanoparticles bearing peptide, whole Ag, or MHC-peptide complexes, 3) Ag bound to whole cells or RBCs, and 4) Ag coupled to Abs that target DCs—as we have employed in this study (31, 36, 39–41). In humans, and perhaps more so in autoimmune or allergic patients, chronic immune homeostasis exists in a more activated and variable state than that of laboratory mice, making it difficult to predict how an individual will respond to Ag stimulation of proinflammatory lymphocytes in the absence of immunomodulators. Consequently, multiple groups are exploring immunosuppressive adjuvants to enhance tolerogenic responses. These approaches are largely focused on specific molecular entities, such as inhibitors of immunostimulatory pathways (e.g., abatacept or rapamycin), and agonists of inhibitory pathways (rTGF- β or IL-10) (38, 41). It may be difficult, however, to determine which pathway will be most active in particular patient groups and whether manipulating one pathway will elicit compensatory mechanisms that diminish efficacy.

In this work, we have taken a cellular approach—Fc.IL-2 mutein-mediated Treg enrichment—to enforce tolerogenic responses to ASI. Treg employ multiple adaptive suppressive pathways to restrain different types of inflammation, increasing the likelihood that Treg enrichment will reliably control responses to ASI in different diseases and patient populations (6). For our experimental system, we used mice that had received OT-II TCR transgenic T cells, which contain a low proportion of OVA-responsive Treg. Transferred OT-II T cells were present at frequencies of 10^{-5} to 10^{-4} , retained their baseline percentage of Treg, and could be recovered and enumerated by magnetic bead selection and flow cytometry.

Consistent with our hypothesis that ASI administration at the peak of Treg enrichment would be most effective, we found that DEC-OVA dosing at day 2 and day 3 after Fc.IL2m treatment were roughly equivalent at maintaining higher OT-II Treg frequencies, but day 4 ASI was less effective. Interestingly, one cycle was not sufficient to increase OT-II Treg, whereas three cycles resulted in a significant 3- to 4-fold OT-II Treg enrichment. Thus, Treg accumulation may have resulted from an incremental step-wise process in which each cycle preferentially activated proliferative and survival pathways in OT-II Treg, and it is interesting to speculate that additional cycles would further increase OT-II Treg frequencies. Importantly, OT-II Treg enrichment persisted for at least 3 wk following treatment cessation and persisted through a strong, acute inflammatory response to VSV-OVA infection. In the vehicle

and Fc.IL2m-alone groups, VSV-OVA infection led to a marked decline in OT-II Treg, a finding that is consistent with previous research on the fate of naturally occurring virus Ag-responsive Treg following infection (42). In contrast, OT-II Treg percentages remained similar following VSV-OVA infection in DEC-OVA groups with the highest frequency being in mice that also received Fc.IL2m. Thus, although Fc.IL-2 mutein treatment alone can promote Treg expansion, TCR stimulation of expanded Treg is capable of improving their competitive fitness in a subsequent antiviral response. This study demonstrates the durability of the Fc.IL-2 mutein/ASI approach and suggests that a relative loss of Treg among CD4 T cells promoting inflammatory processes could be reversed by this protocol.

A shortcoming of our study is that the source of the expanded OT-II Treg was not characterized as growth of previously existing tTreg or newly generated pTreg. It should be noted that mice treated with anti-DEC205-fused Ag and other subimmunogenic methods of Ag delivery can develop pTreg that coexpress Helios, a transcription factor that is also expressed in tTreg and that enforces Treg phenotypic stability (32–34, 43–46). Although we cannot rule out that some Treg expansion resulted from de novo pTreg formation, we are unaware of reports of pTreg development from OT-II Tconv (e.g., from RAG-deficient OT-II T cells) following OVA administration other than oral delivery (47), and it has been postulated that OT-II Tconv have a poor intrinsic capacity for pTreg conversion (48). Other phenotypes that may shed light on the precursor/product relationship are high neuropilin-1 expression and an unmethylated Treg-specific demethylated region; however, a strict relationship between these markers and pTreg/tTreg delineation has been called into question (44, 49–51). Furthermore, high Helios expression was recently considered to be a better, albeit not perfect, tTreg marker (52). Although the origin of our expanded OT-II Treg is clearly of interest, we prioritized characterizing their stability, as this functional characteristic more directly bears on how they may behave in an inflammatory/autoimmune environment in patients. The maintenance of expanded OT-II Treg following treatment cessation and through an acute VSV-OVA infection, and the similarity in their Helios and CD25 expression with host Treg [important for Treg stability (45, 53) and a marker of Foxp3 function (54), respectively], suggest the expanded Treg should possess characteristics of tTreg (55). Defining the transcriptome and epigenome of expanded Ag-specific Treg is warranted to further understand their phenotypes and functional potential.

It is important that future evaluation of this approach explore different dosing strategies and efficacy in suppressing autoimmunity, not only to determine if durable immunosuppression can be achieved with a few treatment cycles, but also to explore whether the degree of Ag-specific Treg enrichment is different when the responding cells are already Ag-experienced. Answering this latter question in our model by treating after VSV-OVA infection may be challenging as we observed a nearly complete loss of OT-II Treg during the VSV-OVA response in our vehicle-control group. For future protocol

characterization in mice, higher Fc.IL-2 mutein doses and longer treatment cycles should be tested, such as 10 μ g of Fc.Mut24 (26) with 10–14 d cycles. Furthermore, this strategy could be leveraged to increase endogenous Ag-specific Treg enumerated with MHC class II tetramers (56), and efficacy could be evaluated in spontaneous autoimmune models such as (NZB $\times$ NZW)F1 lupus with the Hsp70 autoantigen (57) or NOD T1D with pancreatic islet Ags. In patients, similar approaches could be attempted with half-life-extended IL-2 muteins and ASI modalities currently in clinical development, where Ag-specific Treg enrichment could also be monitored with existing MHC tetramer technologies (42). In humans, treatment time points should be adjusted based on the pharmacodynamics of Treg enrichment and the pharmacokinetics of the IL-2-based therapeutic to ensure that ASI is administered after the IL-2 therapeutic falls below levels that will stimulate ASI-induced CD25⁺ Tconv. Furthermore, cycle duration should be extended based on ASI pharmacodynamics to increase the likelihood that ASI-induced CD25 on Tconv declines before subsequent IL-2 therapy doses. Indeed, stimulating Fc.IL-2 mutein-expanded Treg with ASI may be necessary to fully realize their potential to establish lasting tolerance and may obviate a requirement for sustained Treg enrichment through potentially deleterious chronic IL-2 mutein treatment. Furthermore, because ASIs carry the risk of activating rather than tolerizing pathogenic T cells in patients, adding IL-2 mutein expansion to existing ASI strategies may increase their safety and efficacy.

DISCLOSURES

M.A.G. was employed by Amgen, Inc., but does not own Amgen stock. He is currently employed by Omeros Corp., where his work is unrelated to the subject matter of this manuscript. The other authors have no financial conflicts of interest.

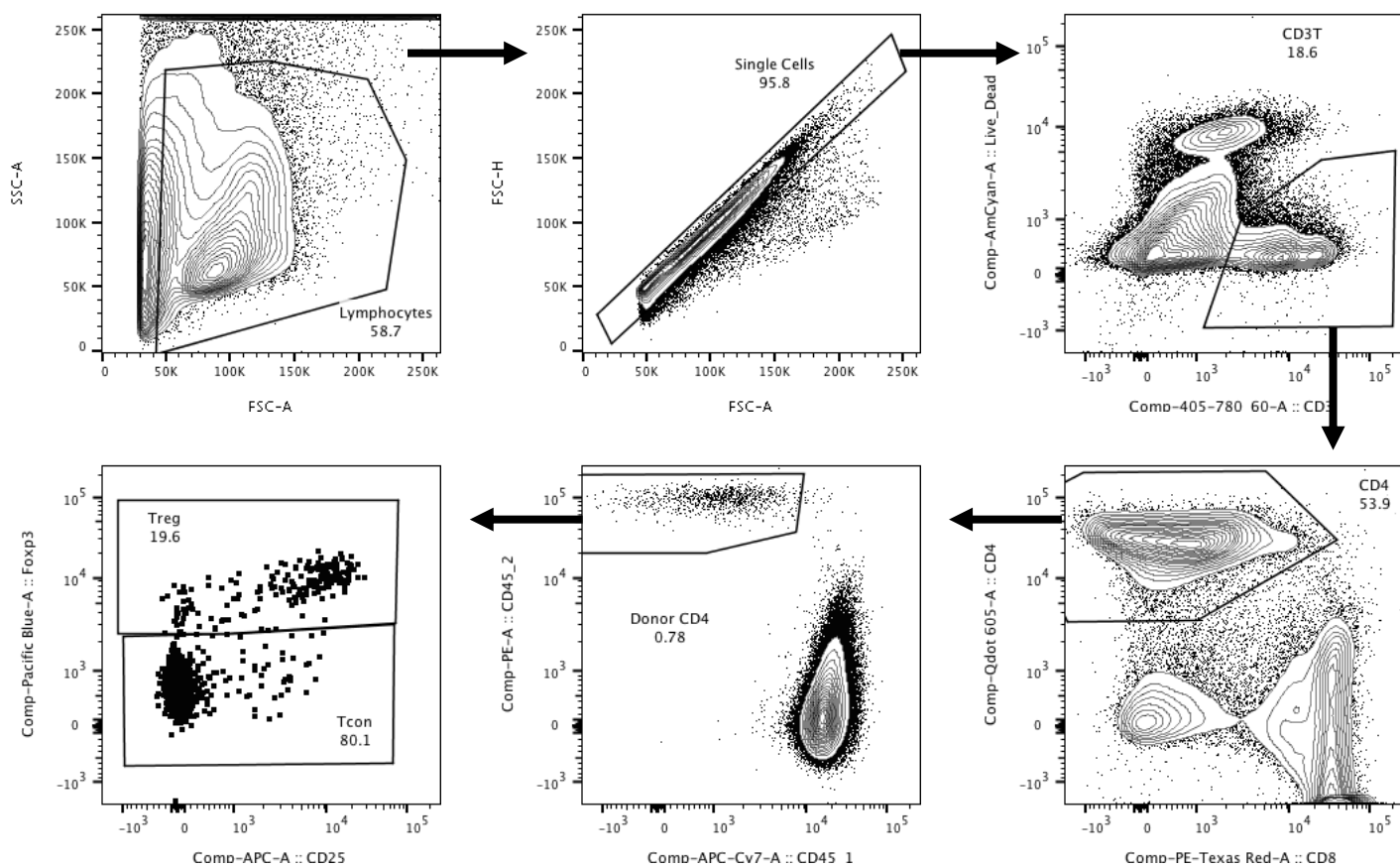
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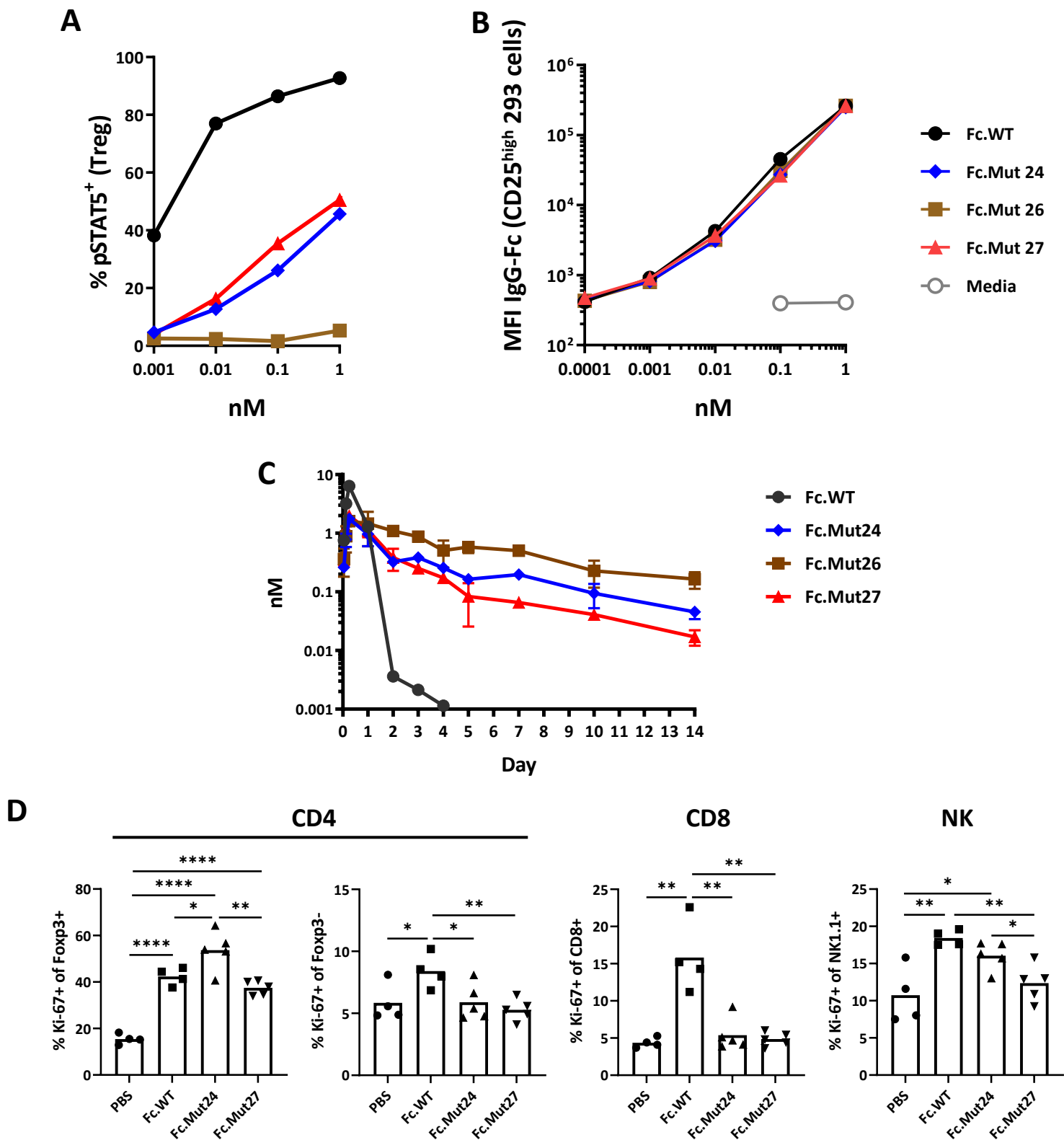
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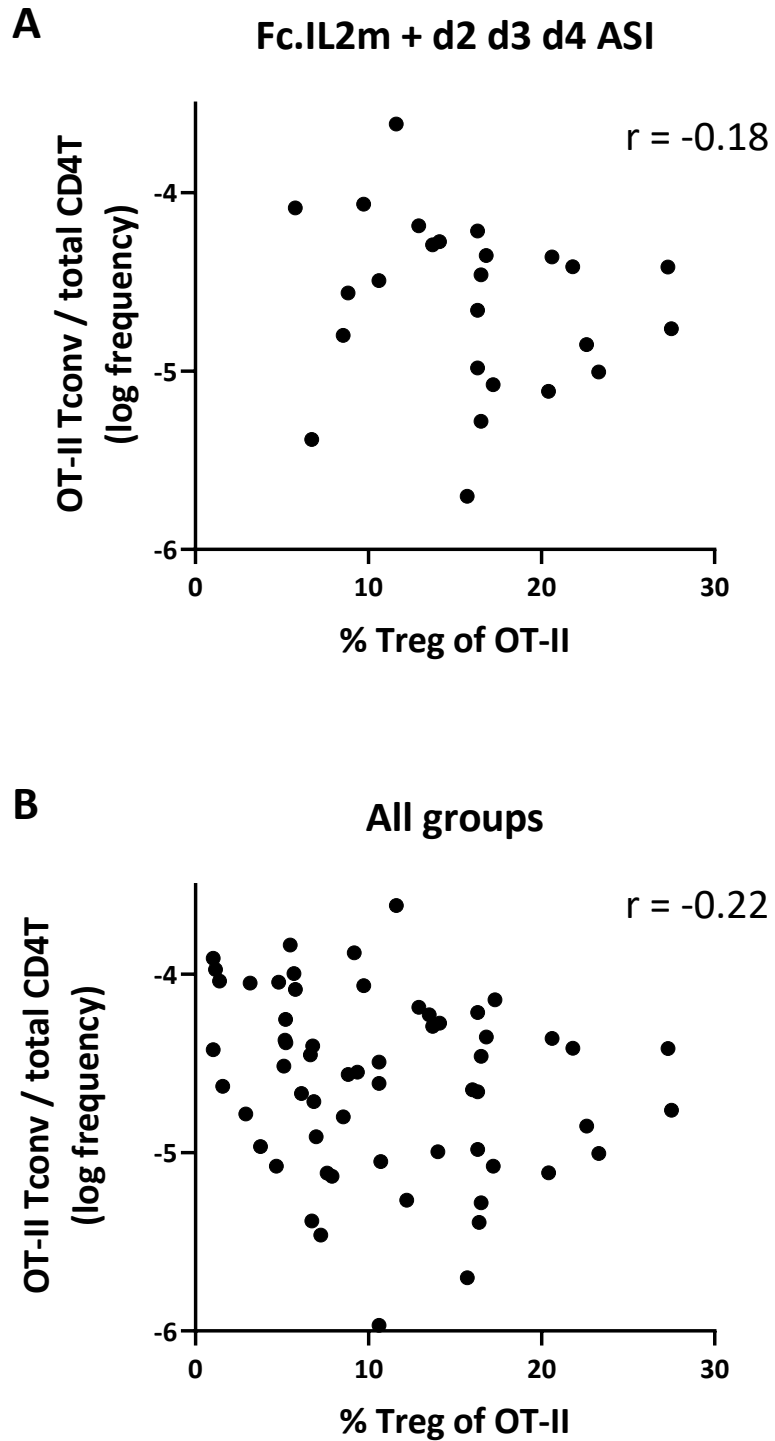


Supplemental Fig. 1. Gating strategy.



Supplemental Fig. 2. Fc.Mut24 and Fc.Mut27 possess similar potency, CD25 affinity, pharmacokinetics, and Treg-selectivity.

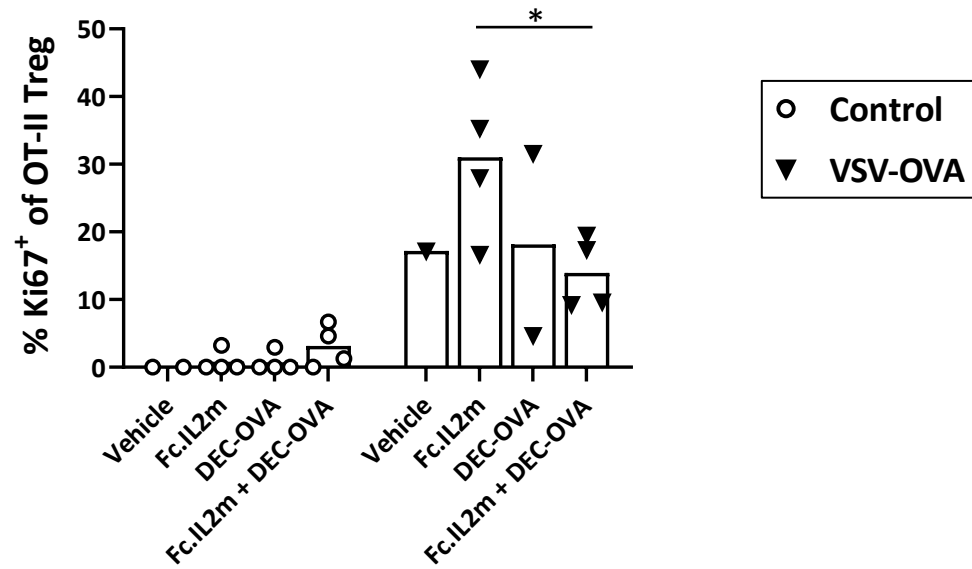
(A) B6 splenocytes were stimulated with the indicated concentrations of Fc.IL-2 muteins. The percentage of pSTAT5⁺ cells among Foxp3⁺ Treg is shown. **(B)** CD25-expressing HEK 293T/17 cells were coated with titrated Fc.IL-2 proteins for 15 minutes at room temperature, then washed and stained with fluorescently labeled anti-mouse Fc and anti-CD25, as described in (23). Median fluorescence intensity of anti-Fc in gated CD25^{high} cells is shown. **(C)** B6 mice received a single 4 μ g subcutaneous dose of the indicated Fc.IL-2 fusion protein, and serum levels of Fc.IL-2 muteins were determined by ELISA. Data are means $\pm$ SD; n = 2 to 3 mice per time point, as described in (25). **(D)** B6 mice received a single 3 μ g intraperitoneal dose of the indicated Fc.IL-2 fusion protein, and on day 3 inguinal LN cells were analyzed by flow cytometry. The % Ki67⁺ cells of the indicated populations is shown.



Supplemental Fig. 3. Increases in the % Treg of OT-II CD4 T cells does not correlate with a loss of OT-II Tconv.

(A) All mice that received Fc.IL2m and DEC-OVA ASI on days 2, 3, and 4.

(B) All mice from all groups.



Supplemental Fig. 4. Ki67 induction in OT-II Treg following VSV-OVA infection.

Data is shown for samples containing >10 Treg cells.