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Engineering Therapeutic Regulatory T Cells to Overexpress G Protein-Coupled Receptor 15 Improves Functional Fitness for In Vivo Gut Homing

The imbalance of proinflammatory and anti-inflammatory T cells in the gut lamina propria is a hallmark of inflammatory bowel diseases (IBDs) such as Crohn's disease (CD) and ulcerative colitis (UC). Thus, although regulatory T cells (Tregs) sufficiently counteract effector T cells under homeostatic conditions, they fail to control their proinflammatory behavior in the inflamed intestine, which contributes to the perpetuation and chronification of the disease.[1]

Current therapeutic approaches to IBDs predominantly aim to restrain the signaling, survival, or recruitment of proinflammatory immune cells but do not achieve long-standing remission in many cases. Hence, specifically strengthening regulatory processes seems a promising alternative strategy for improved treatment of IBDs.

We previously developed a protocol for the good manufacturing practice (GMP)-compliant production of ex vivo expanded Tregs (hereafter called investigational medicinal [IMP]-Tregs) for autologous cell-transfer therapy, which results in a cell product that is cluster of differentiation (CD)$25^{+}CD127^{-}$ and characterized by high expression of Foxp3, cytotoxic T-lymphocyte associated protein 4, and Helios, and very low or no secretion of interleukin 2, interferon-γ, and interleukin 17.[2,3]

We recently completed a phase I trial with these cells in UC. Clinically, early results are promising,[4] and mechanistically, we have demonstrated that IMP-Tregs exhibit improved immunosuppressive function. An indispensable prerequisite for these Tregs to counteract inflammation in the gut, however, is that they reach the mucosa in a process called gut homing. Although we previously were able to show that some of these cells show such migration to the gut in vivo,[3,5] only a part of the IMP-Tregs is equipped with surface molecules relevant for trafficking to the gut, which will probably limit their efficacy. In particular, we have observed that IMP-Tregs express only low levels of G protein-coupled receptor 15 (GPR15),[2] a surface receptor that has previously been described to drive T-cell homing to the large intestine[6,7] by enhancing $\alpha4\beta1$-dependent and $\alpha4\beta7$ integrin-dependent firm adhesion.[8]

Thus, we set out to explore strategies to improve the gut homing capacity of IMP-Tregs and chose the approach of engineering these cells to overexpress GPR15. To this end, we used a GMP-approved vector containing the complementary DNA encoding GPR15 to produce GMP-compliant GPR15 messenger (m)RNA and electroporated IMP-Tregs with this mRNA (Figure 1*A*).

To investigate the resulting expression of GPR15 on the cell surface of engineered IMP-Tregs, we analyzed IMP-Tregs after electroporation with GPR15 mRNA or after mock electroporation by flow cytometry (gating strategy and staining controls shown in Supplementary Figure 1*A–G*). We observed a robust increase in GPR15 expression up to 24 hours after electroporation, with peak levels reached 6 hours after electroporation (Supplementary Figure 1*H*). Focusing on this time point, we found that in all independent experiments, GPR15-engineered IMP-Tregs expressed clearly more GPR15 on their surface (Figure 1*B*), whereas the expression of other molecules, such as integrin $\alpha4\beta7$ or $\alpha4\beta1$, remained similar (Supplementary Figure 2*A*). Moreover, there was no difference in the expression of GPR15 on IMP-Tregs after mock electroporation or without electroporation, implying that electroporation per se had no effect on GPR15 expression (Supplementary Figure 2*B*). Furthermore, we only observed a minimal and not significant decrease of the viability of the engineered IMP-Tregs during the whole procedure (Supplementary Figure 2*C*).

To explore whether increased GPR15 expression was functionally relevant for gut homing, we compared the dynamic adhesion of engineered vs control IMP-Tregs treated with the GPR15 ligand (GPR15L) to mucosal addressin cell adhesion molecule 1 and vascular cell adhesion molecule 1 as the intestinal endothelial ligands for the integrins $\alpha4\beta7$ and $\alpha4\beta1$. Whereas GPR15L treatment alone increased dynamic adhesion to both molecules, this was even more the case in GPR15-engineered Tregs compared with mock-engineered Tregs (Figure 1*C*).

In a next step, after confirming that the highly homologous mouse GPR15L promotes the adhesion of GPR15-engineered IMP-Tregs to mucosal addressin cell adhesion molecule 1 similar to human GPR15L in adhesion assays (Supplementary Figure 2*D*), we took advantage of a humanized in vivo mouse model of intestinal cell trafficking[9] and injected labeled GPR15-engineered or mock-engineered IMP-Tregs into the ileocolic artery of $Rag1^{-/-}$ mice with dextran sulfate sodium colitis. Flow cytometry of lamina propria mononuclear cells from these mice showed substantially increased homing of GPR15-engineered IMP-Tregs after 1 and 3 hours (Figure 1*D* and Supplementary

[§]Authors share co-senior authorship.

Abbreviations used in this paper: **CD, Crohn's disease; GMP, good manufacturing practice; GPR15, G protein-coupled receptor 15; GPR15L, G protein-coupled receptor 15 ligand; IBDs, inflammatory bowel diseases; IMP-Tregs, investigational medicinal product-regulatory T cells; mRNA, messenger RNA; Tregs, regulatory T cells; UC, ulcerative colitis.**

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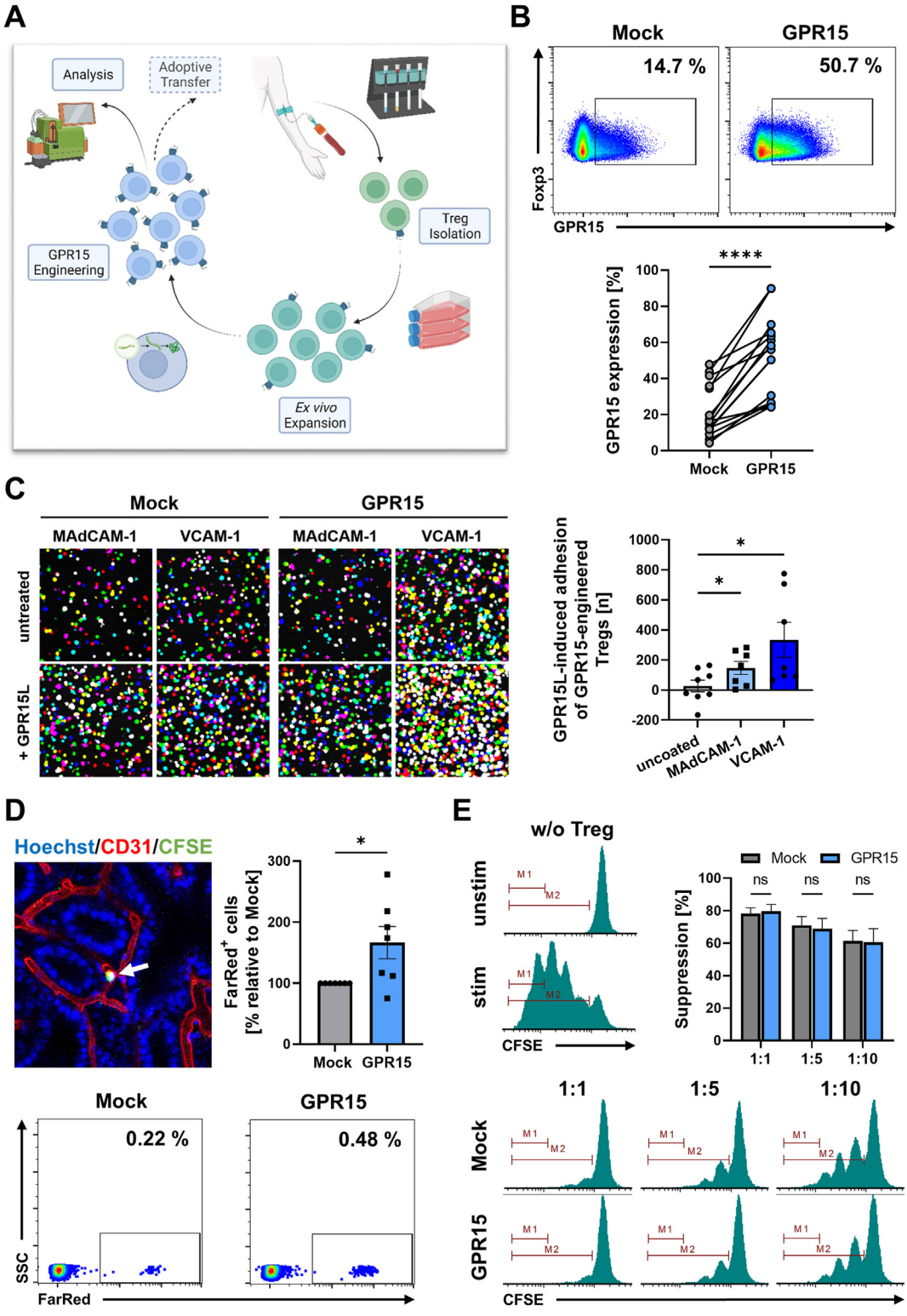
Figure 2*E*). Together, these data demonstrated that GPR15 overexpression results in improved functional fitness of IMP-Tregs for gut homing.

To exclude that such manipulation hampered the suppressive functions of the IMP-Tregs, we performed coculture experiments with IMP-Tregs and autologous CD25$^-$ T-responder cells and determined carboxyfluorescein succinimidyl ester dilution in the latter. Engineered IMP-Tregs, irrespective of whether they were GPR15-engineered or mock-engineered, substantially suppressed the proliferation of CD8$^+$ T cells at all 3 different tested Treg–to–T-responder cell ratios (Figure 1*E* and Supplementary Figure 2*F* and *G*).



Together, these data show that our approach is successful in overexpressing functional GPR15 on IMP-Tregs without decreasing their suppressive potential. This seems a promising way to improve the effectiveness of adoptive Treg cell therapy and might similarly be used to additionally overexpress further molecules relevant for gut homing.

In contrast to other strategies to engineer therapeutic cell products, the approach chosen leads to transient overexpression of GPR15. This seems sufficient, because IMP-Tregs are transferred within 1 hour after thawing in our clinically established protocol, and thus, overexpression for up to 24 hours leaves substantial time for engineered IMP-Tregs to circulate and finally home to the gut.[9,10] Moreover, the particular advantage of this concept is that the cells are not permanently altered on the genetic level, which clearly limits the potential of safety issues and regulatory hurdles for human application.

In conclusion, our data are the first to describe engineered GMP-compliant expanded Tregs with superior gut homing capacity and nominate them for future clinical studies in IBDs.

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Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://dx.doi.org/10.1053/j.gastro.2024.10.003.

Figure 1. Increased GPR15 expression and enhanced homing of IMP-Tregs after engineering with GPR15 mRNA. (*A*) Overview of the workflow: Tregs were isolated from human blood and ex vivo expanded according to our GMP-approved protocol. Afterwards, IMP-Tregs were electroporated with mRNA encoding GPR15 and analyzed by different in vitro and in vivo methods. *Dashed arrow*: Future perspective of adoptive autologous transfer to patients. Created with licensed BioRender.com. (*B*) Representative and quantitative flow cytometry of GPR15 expression on IMP-Tregs engineered with GPR15 mRNA or receiving mock treatment (N = 15; paired *t* test). (*C*) Dynamic adhesion assays of engineered IMP-Tregs in the presence or absence of GPR15L perfused through capillaries coated with coating buffer (uncoated), mucosal addressin cell adhesion molecule 1 (MAdCAM-1) or vascular cell adhesion molecule 1 (VCAM-1) as indicated. *Left panel*: Representative images merged from 7 differentially colored high-power fields from a representative dynamic adhesion assay (200× original magnification). *Right panel*: Pooled quantification of GPR15-dependent dynamic adhesion of GPR15-engineered IMP-Tregs (adhesion without GPR15L subtracted from adhesion with GPR15L treatment (N = 8; mixed-effects analysis). (*D*) Humanized in vivo mouse model of intestinal cell trafficking. *Upper left panel*: Representative intravital confocal microscopy image of a labeled IMP-Treg recruited to the inflamed colon of a *Rag1*$^{-/-}$ mouse. *Other panels*: Representative (*lower panels*) and quantitative (*upper right panel*) flow cytometry of GPR15-engineered or mock-engineered IMP-Tregs accumulated in the lamina propria of *Rag1*$^{-/-}$ mice after transfer to the ileocolic artery (N = 7; 1 outlier removed; 1-sample *t* test). (*E*) *Upper left panel*: Representative histograms of CD8^{+} T-cell proliferation in the absence (w/o) of engineered IMP-Tregs under unstimulated (unstim) and stimulated (stim) conditions as determined by flow cytometry. *Upper right panel*: Percentage suppression of all divided cells (M2) in the presence of engineered IMP-Tregs at the indicated Treg–to–T-responder cell ratios (N = 7; multiple paired *t* test). *Lower panel*: Representative histograms of CD8^{+} T-cell proliferation in the presence of engineered IMP-Tregs at Treg–to–T-responder cell ratios of 1:1, 1:5, and 1:10. CFSE, carboxyfluorescein succinimidyl ester. *$P < .05$, ****$P < .0001$.

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Conflicts of interest
These authors disclose the following: Markus F. Neurath has served as an advisor for Pentax, Giuliani, MSD, AbbVie, Janssen, Takeda, and Boehringer. Sebastian Zundler received speaker fees from Takeda, Roche, Galapagos, Ferring, Falk, Lilly, and Janssen. Markus F. Neurath and Sebastian Zundler received research support from Takeda, Shire (a part of Takeda), and Roche. Britta Siegmund has served as consultant for AbbVie, Abivax, Arena, BMS, Boehringer, CED Service GmbH, Celgene, CT Scout, Endpoint Health, Falk, Forga Software, Galapagos, Janssen, Lilly, Materia Prima, Pfizer, Takeda, Pharma Insight, and Predictimmune; speaker fees from AbbVie, BMS, CED Service GmbH, Falk, Ferring, Galapagos, Janssen, Lilly, Materia Prima, Takeda, and Pfizer, and grant support from Arena/Pfizer (served as representative of the Charité). The remaining authors disclose no conflicts.

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Data Availability
All data are available from the corresponding author upon reasonable request.

Supplementary Methods

Donors and Samples

This study was approved by the Friedrich-Alexander-Universität Erlangen-Nürnberg Ethics Committee (151_12B, 417_19), and all samples were obtained after informed written consent.

Messenger RNA Production

The complementary DNA sequence of human GPR15 (ENST00000284311.5) was synthesized and cloned into the pGEM4Z64A vector using Sal1 and Pac1 restriction sites (BioCat). GPR15 mRNA production was performed with the mMESSAGE mMACHINET7 Ultra Transcription Kit (Invitrogen) and the RNeasy Kit (Qiagen), according to the manufacturer's instructions.

Ex Vivo Regulatory T-Cell Expansion

Ex vivo expansion of human Tregs was performed according to a recently published GMP-compliant protocol.[e1] After 14 days of ex vivo expansion Tregs were electroporated with or without mRNA encoding GPR15 using GenePulser Xcell system (Bio-Rad) at 1 μg/1$\times$10^6 cells and 1.5$\times$10^7/100 μL OpitMEM (Gibco) with a square-wave protocol at 500 V for 4 ms. After 3 hours of incubation in medium at 37°C cells, were cryopreserved and stored in liquid nitrogen until use. For further analyses, Tregs were thawed, left to rest for 1 hour, and subsequently used for experiments. Viability was determined directly before and after electroporation as well as 1 hour after thawing.

Flow Cytometry

To analyze GPR15 and $\alpha 4\beta 7$ integrin expression, engineered Tregs were stained with Fixable Viability Dye efluor506 (Invitrogen) for 30 minutes at 4°C, washed once with phosphate-buffered saline, and stained with the following antibodies for 15 minutes at 4°C: CD25 (allophycocyanin/Cy7, A251, BioLegend), CD127 (allophycocyanin, A019D5, BioLegend), CD49d ($\alpha 4$; fluorescein isothiocyanate, MZ18-24A9, Miltenyi Biotec), integrin-$\beta 1$ (peridinin-chlorophyll-protein /Cy5.5, TS2/16, BioLegend), integrin $\beta 7$ (peridinin-chlorophyll-protein/Cy5.5, FIB27, BioLegend), and GPR15 (BV421, SA302A10, BioLegend). Cells were washed once with phosphate-buffered saline, fixed overnight at 4°C using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience), and intracellularly stained with an antibody against Foxp3 (phycoerythrin, 236A/E7, Invitrogen) or an isotype control (phycoerythrin, mouse IgG1 κ P3.6.2.8.1, eBioscience) for 30 minutes at 4°C. Subsequently, cells were analyzed on a MacsQuant10 (Miltenyi Biotec) instrument, and data were evaluated using FlowJo 10.8 Software (BD Bioscience).

In Vitro Dynamic Adhesion Assays

In vitro dynamic adhesion assays were performed as previously described.[e2,e3] Briefly, miniature borosilicate capillaries (Vitrocom) were coated with Fc chimera of recombinant human vascular cell adhesion molecule 1 (BioLegend) or recombinant human mucosal addressin cell adhesion molecule 1 (R&D Systems) at a final concentration of 5 μg/mL (in 150 mmol/L NaCl with 10 mmol/L HEPES) and incubated for 1 hour at 37°C. Plastic tubing was connected to the capillaries and inserted in a peristaltic pump (Baoding Shenchen Precision Pump Company). Engineered IMP-Tregs were resuspended in adhesion buffer (pH 7.4; 150 mmol/L NaCl, 10 mmol/L HEPES, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and 1 mmol/L $MnCl_2$) at a final concentration of 1.5$\times$10^6 cells/mL, incubated with or without 350 nmol/L human GPR15L or mouse GPR15L (both PeproTech), and perfused through the capillaries using the peristaltic pump. After 3 minutes, capillaries were rinsed to remove nonadherent cells and imaged using a SP8 confocal microscope (Leica). Seven images were randomly acquired per capillary, and the number of adhering cells was calculated using Fiji (National Institutes of Health).

Suppression Assays

Suppression assays were performed according to our GMP-compliant IMP lot-release protocol as described previously.[e1] In brief, autologous CD25-negative lymphocytes were labeled with carboxyfluorescein succinimidyl ester (Thermo Fisher Scientific) and cocultured with IMP-Tregs at defined ratios. Cocultures were stimulated with anti-CD3/-CD28 beads (Miltenyi Biotec) and incubated at 37°C for 3 days. Cells were harvested and stained with propidium iodide and anti-CD8 (BD Biosciences). Treg-mediated suppression was determined as the reduction of proliferation based on the percentage of divided CD8$^+$ cells compared with control samples without IMP-Tregs.

Humanized In Vivo Gut Homing Model

The in vivo gut homing model is described in detail elsewhere.[e4] In brief, a dextran sodium sulfate (1.5% in drinking water) colitis was induced in immunodeficient *B6.129S7-Rag1<tm1Mom>/J* (*Rag1*$^{-/-}$) mice for 7 days. After 1 week, engineered Tregs were thawed, stained with CellTrace FarRed (Thermo Fisher Scientific), and injected into the ileocolic artery of the mice. Between 3.3 and 7.7$\times$10^6 cells were injected, but the number of injected cells was identical for every pair of mock-engineered and GPR15-engineered IMP-Tregs. At 1 or 3 hours after injection, the mice were euthanized, and lamina propria mononuclear cells were isolated using a Lamina Propria dissociation kit (Miltenyi Biotec), followed by density gradient centrifugation with Percoll (GE Healthcare). Cells were fixed overnight at 4°C using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). The frequency of FarRed$^+$ cells was analyzed by flow cytometry using a MacsQuant10 instrument, and data evaluation was performed with FlowJo 10.8 Software (BD Bioscience).

Animal experimentation was approved by the Government of Lower Franconia.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism 8.3.0 (GraphPad Software), and results are shown as

individual values, scatter dot plots with mean and standard error of the mean, or bar graphs with mean and standard error of the mean. Outliers were identified with the ROUT test ($Q = 1\%$) and excluded from the analyses where indicated. To test for normal distribution, a Shapiro-Wilk test was performed. For data with normal distribution, statistical differences were tested using paired Student t tests when comparing 2 groups and 2-way analysis of variance when comparing 3 groups. For data without normal distribution, Wilcoxon's test was performed. For analyses with relative comparisons, 1-sample t tests and for dynamic adhesion assays with mucosal addressin cell adhesion molecule 1 or vascular cell adhesion molecule 1, a mixed-effects analysis was performed. An α-value of $P < .05$ was defined as statistically significant.

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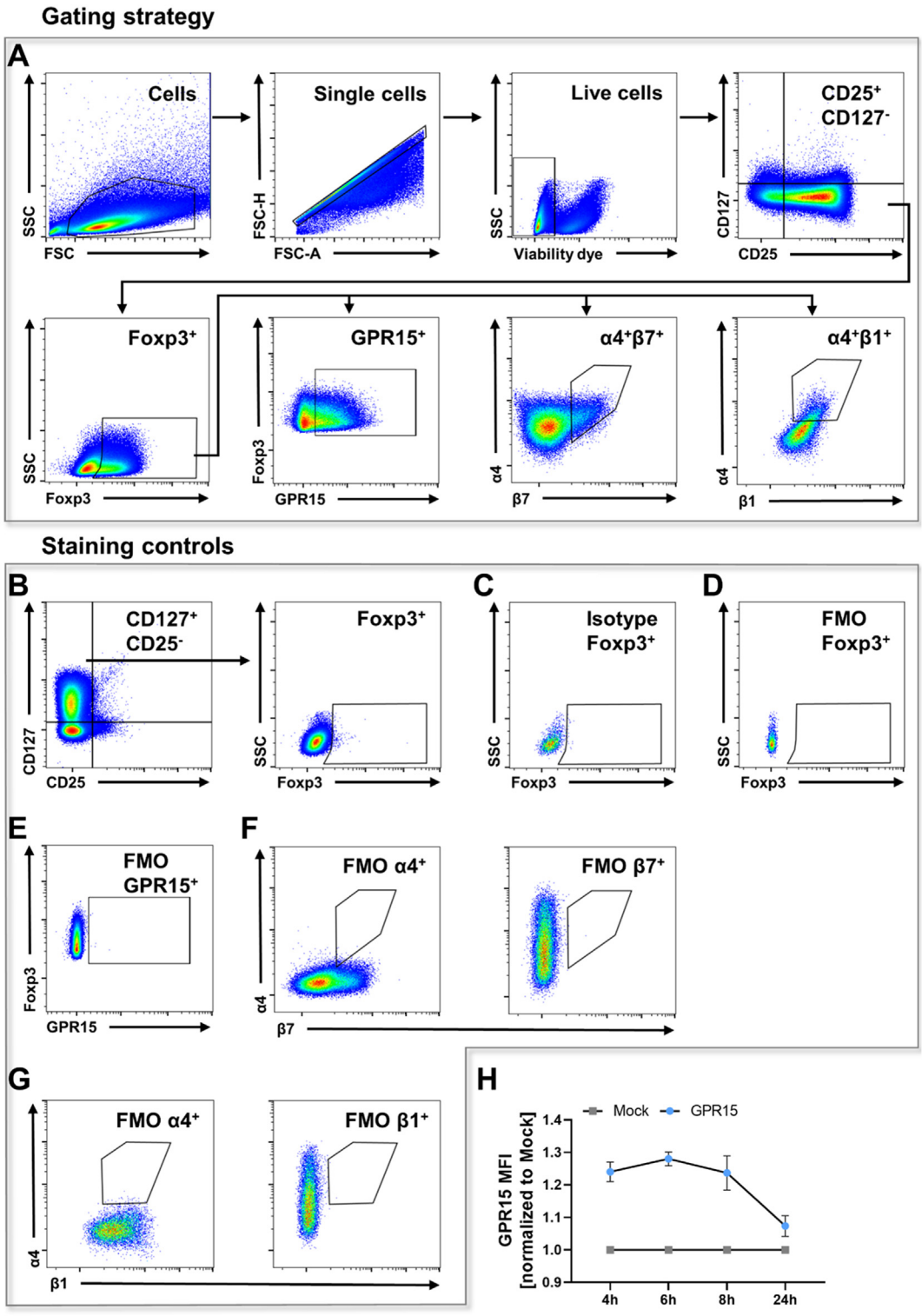
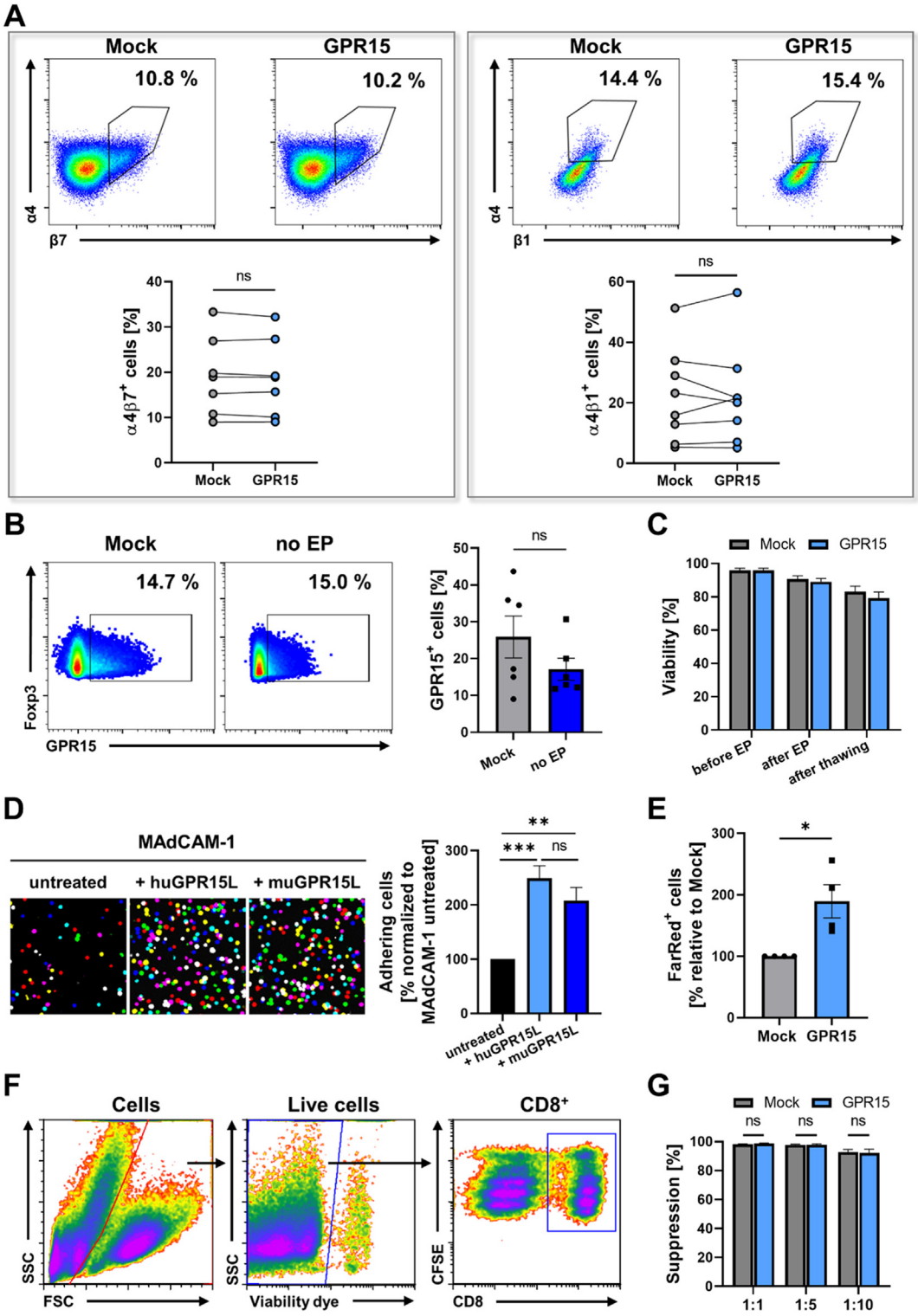


Supplementary Figure 1. (*A*) Gating strategy for the analysis of GPR15 and $\alpha 4\beta 7$ and $\alpha 4\beta 1$ integrin expression on engineered IMP-Tregs. To analyze GPR15 and $\alpha 4\beta 7$ integrin expression, living $CD25^{+}CD127^{-}Foxp3^{+}$ IMP-Tregs were selected. (*B–G*) Staining controls of flow cytometry data. Foxp3 staining on nonexpanded $CD127^{+}CD25^{-}$ cells from the (*B*) peripheral blood as well (*C*) as isotype and (*D*) fluorescence minus one (FMO) control for Foxp3 on $CD127^{-}CD25^{+}$ cells. (*E*) $GPR15^{+}$, (*F*) $\alpha 4\beta 7^{+}$, and (*G*) $\alpha 4\beta 1^{+}$ cells were defined using FMO controls. (*H*) Kinetics of GPR15 expression of engineered IMP-Tregs after electroporation normalized to mock control 4, 6, 8, and 12 hours after electroporation (N = 3). FSC-H, forward scatter height; SSC, side scatter.



Supplementary Figure 2. (*A*) Representative and quantitative flow cytometry of $\alpha4\beta7$ and $\alpha4\beta1$ expression on the surface of IMP-Tregs engineered with GPR15 mRNA or receiving mock treatment (N = 7–8; paired *t* test). (*B*) Representative and quantitative flow cytometry of GPR15 expression on the surface of IMP-Tregs receiving mock electroporation (EP) or no electroporation (N = 6; Wilcoxon's test). (*C*) Viability of IMP-Tregs engineered with GPR15 mRNA or receiving mock treatment before and after electroporation as well as after thawing, as determined by flow cytometry (N = 6). (*D*) Quantification of dynamic adhesion of GPR15-engineered IMP-Tregs perfused through capillaries coated with mucosal addressin cell adhesion molecule 1 (MAdCAM-1) in the presence or absence of human (hu)GPR15L or mouse (mu)GPR15L (N = 8; 1-way analysis of variance). (*E*) Humanized in vivo mouse model of intestinal cell trafficking. Quantitative flow cytometry of GPR15-engineered or mock-engineered IMP-Tregs accumulated in the lamina propria of *Rag1*$^{-/-}$ mice with dextran sodium sulfate colitis 3 hours after transfer to the ileocolic artery (N = 4; 1-sample *t* test). (*F*) Gating strategy for identifying CD8^{+} responder cells in proliferation suppression assays. (*G*) Percentage suppression in the first generation of divided cells (M1) in the presence of GPR15- or mock-engineered IMP-Tregs at the indicated Treg–to–T-responder cell ratios (N = 9; multiple paired *t*-test). FSC, forward scatter; SSC, side scatter. *$P < .05$, **$P < .01$, ***$P < .001$; ns, not significant.