

First application of dPCR for Lentiviral Vector Copy Number and Post-infusion monitoring of TCR-Engineered Regulatory T Cells

Cheng Rui Peh

cheng.peh@monash.edu

Monash University

Zhanrong cui

Monash University

Marine Besnard

Monash University

Khai Lee Loh

Monash University

Hanim Halim

Monash University

Janet Chang

Monash University

Tu Nguyen-Dumont

Monash University

Joshua Daniel Ooi

Monash University

Yi Tian Ting

Monash University

Article

Keywords: TCR-engineered regulatory T cells (TCR-Tregs), regulatory T cells (Tregs), lentiviral transduction, digital PCR (dPCR), quantitative PCR (qPCR), vector copy number (VCN), biodistribution, in vivo cell tracking

Posted Date: August 26th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10544631/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Additional Declarations: Competing interest reported. J.D.O. is a co-founder of Resseptor Therapeutics and has patents to protect the use of TCR-Tregs for specific autoimmune diseases.

Abstract

Regulatory T cell (Treg) therapies are safe but only had modest clinical benefit, and most programs still rely on polyclonal products and qPCR-based assays that limit both potency and quantitative precision. Antigen-specific TCR-engineered Tregs (TCR-Tregs) offer a more targeted alternative, yet their manufacturing properties and in vivo behaviour remain poorly defined. Here, we develop and validate a digital PCR (dPCR) assay for lentiviral vector copy number (VCN) quantification in human TCR-Tregs and matched CD4⁺ conventional T cells (Tconvs), and use it to link product characterisation with in vivo monitoring. At matched MOIs, Tregs acquired higher VCNs than Tconvs (0.75 vs 0.53 at MOI 1 and 1.50 vs 0.86 at MOI 5) and higher frequencies and MFI of GFP⁺ cells, indicating more efficient lentiviral integration and stronger transgene expression in Tregs, while modest change in GFP⁺ frequency with increasing MOI suggests that higher VCN does not boost transgene expression. In humanised NSG mice, dPCR enabled quantification of TCR-Tregs transgene in blood over 60 days and organs at day 30, revealing dose-dependent but non-linear expansion and organ-specific accumulation. These findings identify TCR-Tregs as favourable substrates for lentiviral engineering and establish dPCR as a unified, scalable tool for manufacturing control and post-infusion tracking.

Introduction

Digital PCR (dPCR) has been used for vector copy number assessment and biodistribution in other gene and cell therapies, but, to our knowledge, has not previously been applied to TCR-engineered regulatory T cells in an integrated manufacturing and in vivo tracking framework. Therapies using Tregs are emerging as a promising strategy to re-establish immune tolerance in autoimmune and inflammatory diseases. To date, approximately 80% of clinical trials have relied on polyclonal Treg products, which consistently prove safe and well tolerated but show, at best, modest and often transient clinical benefit¹. Studies have generated proof-of-concept data that TCR-Tregs can achieve more potent disease control than polyclonal Tregs both in vitro and in vivo²⁻⁴. For example, in lupus nephritis, Cheong and colleagues showed that Smith autoantigen-specific TCR-Tregs not only more potently suppressed Smith-specific inflammatory responses than mock-transduced polyclonal Tregs, but also markedly reduced renal pathology in a humanized mouse model, lowering mesangial proliferation from ~59–62% to ~14% of glomeruli and tubulointerstitial injury from ~50% to ~10% of the cortical area⁴. These data position TCR-Tregs as a compelling next generation of cell therapies for autoimmunity, but they also heighten the need for rigorous analytical frameworks that can quantify vector dose, product composition, and in vivo behaviour with sufficient precision for clinical translation.

Lentiviral vectors remain the principal platform for stable TCR expression in primary human T cells. In this setting, vector copy number (VCN) is a central quantitative determinant of both potency and insertional risk for advanced therapy medicinal products. Most Treg and broader T cell programs still measure VCN by quantitative PCR (qPCR). This method depends on external standard curves and shows limited precision and sensitivity at low copy numbers, which constrains its utility for longitudinal comparisons across batches and time points⁵. Digital PCR (dPCR) provides absolute, standard-curve-independent quantification and generally offers higher precision and sensitivity than qPCR, particularly in the low-copy range. These properties make dPCR an attractive platform for both manufacturing control and long-term tracking of gene-modified cells. In parallel, deuterium-labelling studies have defined population-level survival kinetics of infused Tregs in vivo, but isotopic approaches cannot distinguish engineered from unmodified clones or resolve the expansion and tissue distribution of specific TCR-Treg products⁶.

In this study, we develop and validate an in-house dPCR assay suite for lentiviral VCN quantification of TCR-engineered CD4⁺ Tregs and matched CD4⁺ Tconvs. Under standardised transduction conditions, we show that Tregs acquire significantly higher VCNs than Tconvs while maintaining comparable transgene expression via a linked GFP reporter, revealing a previously underappreciated, lineage-specific advantage that can be exploited to generate potent TCR-Treg products at lower vector inputs. We further demonstrate that our dPCR-based VCN measurements correlate closely with a commercial qPCR assay (Spearman $r = 0.83$), indicating comparable robustness, while providing higher precision and standard-curve-independent quantification that is particularly advantageous for longitudinal and cross-site product characterisation. Finally, leveraging a humanized NSG lupus nephritis model, we extend dPCR beyond manufacturing analytics to quantify the in vivo expansion and organ biodistribution of engineered TCR-Tregs across blood, spleen, liver, lung, and kidney, yielding tissue-level data that complement and extend earlier deuterium-labelling studies. The procedural roadmap provided here integrates dPCR-based VCN control with clone-specific in vivo tracking in TCR-Tregs, extending recently established analytical standards for engineered T-cell products and offering a template for IND-enabling workflows, including vector lot release, manufacturing quality control, and regulated biodistribution studies.

Results

Validation of In-house dPCR VCN Assay

The analytical specificity of the dPCR VCN assay primers was first assessed by conventional PCR. Human genomic DNA (without lentiviral integration) yielded a single 260 bp FoxP3 (Forkhead box P3) amplicon, whereas untransduced Jurkat 76 (J76) cells showed the same single band and transduced J76 cells showed two bands corresponding to FoxP3 (260 bp) and WPRE (Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element, 110 bp); no non-specific products or primer dimers were detected in any reactions, including no-template controls (Fig. 2B). Similar results were obtained with mock-transduced and transduced Tregs, with only the FoxP3 band in mock-transduced cells and both FoxP3 and WPRE bands in transduced cells, and Sanger sequencing confirmed that these amplicons matched their intended target sequences.

We then evaluated the duplex dPCR assay on the Absolute Q[™] instrument using transduced Tregs. Clear separation of positive and negative partitions was observed for both targets, with FAM fluorescence reporting WPRE and HEX fluorescence reporting FoxP3, and thresholds set between approximately 9–11 × 10³ and 0.9–1.1 × 10³ fluorescence units, respectively, allowing reliable classification of positive and negative reactions. To define the optimal input range, we next titrated the amount of genomic DNA per reaction: between approximately 395 and 39,467 cells per reaction the assay yielded consistent VCN estimates of 4.35–4.47 copies per genome, whereas at 39 cells per reaction the measured VCN dropped to 0.04, indicating loss of accuracy at very low input and establishing ~400 cells per reaction as a practical lower limit for reliable quantification. Finally, a VCN standard curve (Fig. 2D) generated with FoxP3

gene blocks spiked with WPRE gene blocks over a range of 0.5–10 copies per genome in three independent runs demonstrated excellent linearity ($R^2 = 0.9997$), supporting the reproducibility and repeatability of the in-house dPCR VCN assay.

qPCR vs dPCR

To assess concordance between our in-house dPCR VCN assay and a commercial qPCR assay, we compared VCN values with those obtained using the LentiX™ Provirus Quantitation Kit (Takara Bio Inc, Shiga, Japan) across 60 samples from five donors transduced at varying MOIs. Spearman's rank correlation showed a strong positive association between the two methods ($r = 0.83$; Fig. 2F). However, qPCR consistently yielded higher VCN estimates than dPCR (Fig. E), and this systematic bias was significant by Wilcoxon matched-pairs signed rank test ($p < 0.0001$), with qPCR values on average 2.25-fold higher than dPCR (mean fold-difference $\pm$ SD: 2.25 ± 1.43).

VCN Incorporation and GFP Expression Higher in Tregs than Tconvs

VCN measurements by qPCR and dPCR showed consistently higher VCN in Tregs than in Tconv cells across the MOI range tested (Fig. 3A). At MOI 1, Tregs had a mean VCN of 0.752 compared with 0.528 in Tconvs, and at MOI 5 the mean VCN in Tregs increased to 1.504, almost twice that of Tconvs (0.860). Increasing MOI led to higher VCN in both populations.

GFP, expressed from the TCR vector as a reporter of transgene expression, was quantified by flow cytometry as the percentage of GFP⁺ cells among single, live CD4⁺ cells (Fig. 3B-C). GFP expression was higher in Tregs than in Tconvs, with 4.15% versus 2.14% GFP⁺ cells at MOI 1 and 4.82% versus 2.16% at MOI 5; at MOI 5 this difference was statistically significant by two-way ANOVA ($p < 0.05$). Median fluorescence intensity (MFI) of GFP⁺ cells was also greater in Tregs than in Tconvs, indicating higher per-cell transgene expression in the Treg lineage. In contrast, increasing VCN did not result in higher GFP⁺ frequencies, indicating that additional vector copies did not further enhance detectable transgene expression at the population level.

dPCR Can Monitor and Detect TCR-Treg In Vivo

dPCR was used to quantify WPRE copies as a measure of TCR-Treg persistence in peripheral blood over 60 days in the SLE mouse model. In the medium-dose group (1×10^5 cells per mouse, $N = 6$), mean WPRE copies per 100 ng gDNA were 7.4 on day 1, 5.0 on day 8, 60.9 on day 30, and 9.67 on day 60. In the high-dose group (1×10^6 cells per mouse, $N = 6$), means were 12.3, 2.27, 38.0, and 3.8 copies per 100 ng gDNA on days 1, 8, 30, and 60, respectively (Fig. 3D). At days 1 and 60 in the medium-dose group, samples were only available from 5 of 6 mice, and data shown therefore reflect $N = 5$. Nonetheless, TCR-Tregs remained detectable for at least 60 days in both dose groups with a pronounced peak at day 30.

In a separate biodistribution cohort, TCR-Tregs generated from three patients were infused intravenously at low- (1×10^4 cells per mouse), medium- (1×10^5 cells per mouse), or high-dose (1×10^6 cells per mouse), and organs were collected 8 weeks post-infusion for WPRE analysis. WPRE copies per 100 ng gDNA were detectable in kidney, liver, lung, and spleen across all dose groups, with the highest median copy numbers in spleen for the low-dose group (19.3 copies per 100 ng gDNA) and in lung for the medium-dose group (22.9 copies per 100 ng gDNA), and with a peak median of 17.3 copies per 100 ng gDNA in spleen in the high-dose group (Fig. 3E). Overall, these data indicate that TCR-Tregs not only persist in the circulation but also distribute to lymphoid organs and selected non-lymphoid tissues within the first month after transfer.

Discussion

Regulatory T cell therapies represent a transformative opportunity for antigen-specific immune regulation in autoimmune disease, yet clinical translation has been hampered by the modest efficacy of polyclonal products and analytical challenges inherent to manufacturing engineered cell therapeutics. We developed and validated a dPCR assay for lentiviral vector copy number quantification in TCR-engineered Tregs, revealing three key findings: Tregs acquire significantly higher VCNs than conventional CD4⁺ T cells under identical transduction conditions; dPCR offers superior precision versus commercial qPCR while correlating strongly (Spearman $r = 0.83$); and dPCR-based WPRE quantification enables longitudinal tracking and organ-specific biodistribution analysis of TCR-Tregs in vivo.

Tregs consistently incorporated 1.4- to 1.7-fold higher VCNs than Tconvs at equivalent MOIs (MOI 5: Treg VCN 1.504 vs. Tconv 0.860) and together with their respective GFP⁺ frequencies (4.82% vs. 2.16%, $p < 0.05$), suggest a lineage-specific advantage of Tregs for lentiviral integration and transgene expression under matched transduction conditions. Recent CAR-T studies show VCN < 5 correlates with lower transgene expression (median 12% vs. 65% for VCN > 5 , $p < 0.0001$) and that higher VCN numbers related to higher functional avidity, although FDA recommends VCN < 5 to minimize insertional mutagenesis⁷. This Treg-specific advantage could reduce viral vector inputs, a critical cost driver, while maintaining target transgene dosing^{5,8}. Whether this reflects distinct cell-cycle kinetics, chromatin accessibility at Treg-specific loci such as demethylated *Foxp3* or enhanced survival of multiply transduced clones warrants genome-wide integration-site mapping⁹⁻¹¹.

Our assay's strong correlation with qPCR (Spearman $r = 0.83$) yet systematic 2.25-fold qPCR overestimation mirrors prior CAR-T comparisons showing ~1.4-fold bias^{12,13}. dPCR exhibited superior linearity ($R^2 = 0.9997$) across 400–8,000 cells/reaction, with accuracy degrading below ~400 cells (VCN dropping from 4.35–4.47 to 0.04 at 39 cells/reaction). Comparative studies show dPCR achieves tighter gene correlation ($R^2 = 0.99$ vs. 0.78 for qPCR) despite 2-3 logs narrower in dynamic range^{14,15}. For manufacturing release criteria requiring precise VCN determination, dPCR's absolute quantification without standard-curve dependency positions it as superior to qPCR^{13,16,17}. dPCR provided value in this study beyond in vitro VCN quantification by establishing a single analytical framework for both manufacturing control and post-infusion monitoring. The same principle used to quantify lentiviral VCN in vitro was extended through WPRE measurement to track engineered TCR-Tregs longitudinally in blood and tissues, thereby linking product attributes to in vivo behaviour. This

strategy parallels emerging practice in oncology, where dPCR is increasingly used to monitor CAR-T expansion with higher sensitivity than flow cytometry or qPCR and to quantify low-abundance liquid-biopsy biomarkers for disease monitoring^{18,19}. These precedents support adopting dPCR in TCR-Treg programs not only for product release analytics, but also for mechanistic and translational monitoring after infusion.

TCR-Tregs persisted up to 60 days with average day-30 expansion peaks in medium-dose (60.9 WPRE copies/100 ng gDNA) and high-dose (38.0 copies/100 ng gDNA) groups. Unlike deuterium labelling which cannot distinguish expansion from persistence, dPCR can distinguish engineered from endogenous cells, quantifies absolute numbers, and detects true expansion⁶. Organ biodistribution showed highest median WPRE in spleen (low-dose; 19.3 copies/100 ng gDNA and high-dose; 17.3 copies/100ng gDNA) and lung (medium-dose: 22.9 copies/100 ng gDNA), with TCR-Tregs detectable in both lymphoid (spleen) and non-lymphoid organs (lung, liver, kidney). The non-linear relationship between infused cell dose and day-30 WPRE accumulation indicates that medium doses (1×10^5) yielded higher TCR-Treg numbers than either lower (1×10^4) or higher (1×10^6) doses (60.9 vs. 38.0 WPRE copies/100 ng gDNA at day 30). One possible explanation, consistent with published IL-2-dependent Treg expansion models, is a biphasic regime in which low doses are limited by expansion time, medium doses achieve a favourable balance between starting cell numbers and local IL-2 availability, and high doses encounter relative IL-2 scarcity as the larger infused Treg population consumes available cytokine; however, this remains a hypothesis in our system because IL-2 was not directly measured²⁰⁻²². Tissue-specific differences in IL-2 production where higher production in lymphoid organs due to activated T cell populations could further explain organ-specific accumulation patterns²³. Human lymphoid-derived Tregs show ~60% Ki67+ proliferation vs. ~20% in blood, supporting lymphoid organs as primary sites of Treg expansion²⁴. Lower kidney WPRE despite lupus nephritis pathology may reflect limited local IL-2 availability in inflamed non-lymphoid tissues, transient tissue residency (~3 weeks), rapid turnover due to low Bcl-2 expression, or poor homing, with Tregs navigating complex macrophage populations undergoing M1/M2 transitions potentially limiting accumulation^{20,25-28}.

This study has several limitations that are important to consider when interpreting the data and planning next steps. First, the WPRE based dPCR assay cannot identify lentiviral integration sites, and VCN measurements reflect population averages rather than clonal copy number distributions. This means the assay cannot distinguish a uniformly transduced population from one in which a few highly transduced clones carry many integrations near genes of concern, so it informs overall vector load but not clonal architecture or insertional safety. Second, all in vivo experiments were performed in NSG mice, which lack key immune compartments and differ from humans in cytokine milieu and tissue physiology. As a result, the observed persistence, expansion, and homing patterns should be viewed as proof of concept rather than direct predictors of human pharmacokinetics or efficacy. Third, the sampling schedule (days 1, 8, 30, and 60) provides only a coarse view of TCR Treg kinetics: it confirms the presence of an expansion peak but cannot resolve the precise onset, duration, or shape of expansion and contraction phases, limiting the ability to build detailed pharmacokinetic models or optimize dosing intervals. Finally, our organ panel excluded lymph nodes, bone marrow, and mucosal tissues, which are key sites for Treg priming, long term survival, and tissue specialization; consequently, the biodistribution data likely underestimate total body persistence and may miss relevant reservoirs or homing patterns in unsampled niches.

Critical future work will require genome wide integration site sequencing to determine whether Treg specific chromatin features, such as demethylated *Foxp3* and distinct accessibility landscapes, create preferential integration patterns that could account for the higher VCN observed in Tregs. This is not only mechanistically relevant but also essential from a safety perspective, as CAR T studies have identified clonal expansions driven by integrations near genes such as TET2 and CBL, underscoring the regulatory importance of understanding where vectors insert^{29,30}. Whether higher VCN in Tregs translates into increased insertional risk or instead reflects a distinct, potentially safer integration profile remains a key question for clinical translation. Pairing integration site analysis with single cell RNA sequencing would also allow understanding the correlations between integration patterns and tissue homing, survival, and suppressive function to be defined at the clonal level, enabling rational engineering strategies to optimize TCR Treg potency and durability while satisfying safety expectations for advanced therapy products.

This study has identified a previously unappreciated, lineage-specific advantage of human Tregs in lentiviral engineering, showing that they acquire higher VCNs than matched Tconvs under identical conditions while maintaining comparable transgene expression. Building on this, we establish what is, to our knowledge, the first integrated dPCR framework tailored to TCR-engineered Tregs, comprising a validated duplex assay that delivers precise VCN quantification and enables clone-specific in vivo tracking, thereby directly linking manufacturing attributes to post-infusion expansion and biodistribution. Together, these findings position TCR-Tregs as both biologically advantaged and analytically tractable cell therapy candidates, and provide a practical template for future integration-site and clonal safety studies needed to support clinical translation of TCR-Treg products.

Materials and Methods:

Isolation of PBMCs From Blood:

Peripheral blood was obtained from HLA-typed HLA-DRB1*15:01–positive donors, including both patients with systemic lupus erythematosus (SLE) and healthy controls. SLE patients were adults who fulfilled American College of Rheumatology classification criteria, had anti-Smith autoantibodies, and were HLA-DR15 positive. Whole blood or buffy coat samples from patients and healthy controls were processed within 24 hours of collection. PBMCs were isolated by density-gradient centrifugation using Lymphoprep and SepMate tubes (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's instructions.

All experiments in this study used TCR-Tregs generated from healthy donor PBMCs, except for the biodistribution analysis, which was performed retrospectively on organs from earlier efficacy studies in the humanised lupus nephritis model, where mice had received TCR-Tregs derived from SLE patient samples.

TCR engineering of CD4+ Cells:

TCR-Treg production was done according to previously established protocol unless otherwise stated ⁴. In brief, T cell isolation was done from previously stored frozen PBMCs collected under ethics approval from Monash University Human Research Ethics Committee (approval ID: MUHREC41416). Tregs (CD4+CD25+CD127low) and Tconvs (CD4+CD25-CD127high) were isolated using EasySep™ Human CD4 Positive Selection Kit II and Human CD4+CD127lowCD25+ Regulatory T Cell Isolation Kit (STEMCELL technologies, Vancouver, Canada). 3 to 7 days after cell isolation, Tregs and Tconvs were transduced at multiplicity of infection 1, 3 and 5.

Flow Cytometry of TCR-engineered CD4+ Cells:

In vitro-expanded Tregs and Tconvs were first stained using LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Invitrogen™, Massachusetts, USA), followed by anti-human antibodies; CD4 PB (clone RPA-T4, BD). Post-staining, cells were analysed on the Cytex® Aurora. Further quantification analysis was performed on FlowJo 10.9.0 (Fig. 1B).

DNA Extraction:

Cell aliquots day 14 post-transduction were washed in phosphate-buffered saline, spun down and its pellet kept at -20°C prior to DNA extraction. Samples were extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), according to manufacturer's protocol. Extracted DNA samples were then measured for its concentration and quality using the Nanodrop. Samples were first stored at 4°C prior to VCN analysis then to -20°C for long-term storage.

Conventional PCR Validation of VCN Primers:

To check the specificity of primers designed (Table 1), they were tested on DNA extracted from PBMCs of healthy human controls, SM2-TCR-transduced Jurkat 76 (SM2-J76) and J76 using conventional PCR. In brief, PCR master mix used GoTaq® Green Master Mix (Promega, Wisconsin, USA), with final primer concentrations of 1µM. The master mix was then aliquoted into 0.2mL PCR tubes, prior to the addition DNA samples. Samples were then loaded onto a thermocycler and ran according to the following cycling conditions: an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 74°C for 30 seconds. A final extension step at 74°C for 5 minutes was performed, and the reactions were held at 8°C indefinitely. Post-PCR samples were resolved using gel electrophoresis (135 V, 50 mins) on 1% agarose gels containing 1X GelRed® Nucleic Acid Gel Stain (Jomar Life Research, Victoria, Australia).

Remaining samples were then processed using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) prior to Sanger sequencing at Micromon Genomics (Monash University, Australia) for further analysis, to ensure amplified sequence was correct.

In-house dPCR VCN Assay:

To measure VCN in transduced cells, an in-house dPCR assay was designed to amplify the WPRE region of 3rd generation LVV (vector genome) and the FoxP3 region of humans (reference target) (Table 1). As Foxp3 is present on the X chromosome, depending on biological gender, the calculation for VCN is as follow:

$$\text{Vector Copy Number} = \frac{\text{Copies}/\mu\text{L of LV vector genome}}{\text{Copies}/\mu\text{L of reference target}} \times 2 \text{ (Male) or } 4 \text{ (Female)}$$

DNA extracted from transduced cells were tested on the Absolute Q™ (Applied Biosystems™, Massachusetts, USA). On the Absolute Q™, samples were set-up on the manufacturer's MAP16 plate kit. Cycling conditions and assay set-up were according to the manufacturer's protocol. DNA template input was between 20ng – 150ng.

VCN Standard Curve:

Foxp3 (ChrX_GRCh38.p14_49252469-49252810) and WPRE gene blocks (Table 2) were synthesized by IDT (Integrated DNA Technologies, Singapore). Each gene block was diluted to a working concentration equivalent to 450 copies of Foxp3 per reaction, and reactions were spiked with 225–4500 copies of WPRE per reaction, to obtain VCN of 0.5 to 10. Samples were run alongside a no-template control (NTC) on three independent occasions. Standard curves were generated from these data and analysed using Pearson correlation.

dPCR Detection of TCR-Tregs In Vivo:

TCR-Tregs were evaluated both longitudinally in blood and at endpoint in tissues. Humanised NSG mice (NOD.Cg-Prkdc^{scid} H2-Ab1^{em1Mw} H2-K1^{tm1Bpe} H2-D1^{tm1Bpe} Il2rgtm^{1Wjl}/SzJ, Jackson Laboratories strain 025216) with lupus nephritis were generated using a previously described model⁴. Mice were assigned randomly to groups and their sex were not a consideration in the study design. In the persistence study, mice were monitored for TCR-Treg engraftment by submandibular blood collection over 60 days. In a separate biodistribution experiment, spleen, kidney, liver and lung were harvested post-euthanasia via CO₂ asphyxiation at day 30 for assessment of TCR-Treg localisation. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and analysed together with NTC using our in-house dPCR VCN assay.

qPCR VCN Assay:

DNA extracted were tested for their VCN using the QuantStudio™ 7 Flex Real-Time PCR system (Applied Biosystems™, Massachusetts, USA) Samples were prepared using Lenti-X™ Provirus Quantitation Kit (Takara Bio, California, USA), following manufacturer's instructions.

Ethics:

All procedures were performed in accordance with the Declaration of Helsinki. All donors provided written informed consent. Ethics was obtained from Monash Health hospital human research ethic committee (HREC-RES-22-0000-192A) for SLE patients and from Monash University for healthy control whole bloods and buffy coats (MUHREC41416). Animal ethics was obtained from the animal ethics committee at Monash Medical Centre (MMCB/2023/24). All methods were carried out in accordance with the relevant guidelines and regulations.

Statistical Analysis:

Data distributions were first assessed for normality prior to statistical analyses using GraphPad Prism version 10.2.0. VCN and GFP expression data from in vitro transduction experiments (Tregs vs Tconvs across MOIs) are presented as mean (SD) and were analysed using two-way ANOVA with cell type and MOI as factors; where appropriate, post hoc comparisons were performed with correction for multiple testing, and $p < 0.05$ was considered significant. For method comparison between dPCR and qPCR VCN measurements, paired data points (matched by donor, cell type, and MOI) were analysed using the Wilcoxon signed-rank test, and correlation between platforms was assessed by Spearman's rank correlation coefficient. Longitudinal WPRE copy numbers in peripheral blood and organ-level biodistribution data are presented as mean (SD) or as individual values with mean indicated; these descriptive statistics were used to visualise dose- and time-dependent trends, and no formal hypothesis testing was applied beyond the analyses specified above. All data shown represent biological replicates, except for the standard curve. Standard curve was generated from gBlock DNA and run in triplicate on three independent assay runs to assess technical reproducibility.

Declarations

Competing Interests

J.D.O. is a co-founder of Resseptor Therapeutics and has patents to protect the use of TCR-Tregs for specific autoimmune diseases.

Declaration of Interests:

J.D.O. is a co-founder of Resseptor Therapeutics and has patents to protect the use of TCR-Tregs for specific autoimmune diseases.

Funding:

This research was supported by grants from the Australia National Health and Medical Research Council (NHMRC) Investigator grant awarded to J.D.O..

Author Contribution

C.R.P., J.D.O., Y.T.T. and T.N.D. designed the research study. J.C. provided the samples. C.R.P., Z.C., K.L.L. and J.C. carried out experiments. C.R.P, M.B. and T.N.D. analysed the data. C.R.P. wrote the manuscript. All authors edited and approved the manuscript. J.D.O., Y.T.T. and T.N.D. supervised the research.

Acknowledgement

The authors would like to thank the Biochemistry Imaging Facility, Irene Hatzinsiriou, for her technical assistance. We would also like to thank the Thermofisher team in Melbourne, Australia, specifically Hashmath Hussain for providing his expertise and technical support.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article.

References

1. Rodrigues, K. B., Eggenhuizen, P. J., Bacchetta, R. & Good, Z. Regulatory T cell therapies: from patient data to biological insights. *Front. Immunol.* **16**, 1675114. 10.3389/fimmu.2025.1675114 (2025).
2. Lim, Z. F. S. et al. Gene-Engineered Ro60-Specific Regulatory T Cells Suppress Autoreactive Inflammatory Responses in Sjögren's Disease. *Available at SSRN 5586124*.
3. Eggenhuizen, P. J. et al. Engineered antigen-specific T regulatory cells suppress autoreactivity to the anti-glomerular basement membrane disease antigen. *Kidney International* 107, 751–756, doi:10.1016/j.kint.2025.01.005 (2025).
4. Ooi, J. et al. Smith-specific regulatory T cells halt the progression of lupus nephritis. (2023).

5. Murphy, L. A. et al. Digital polymerase chain reaction strategies for accurate and precise detection of vector copy number in chimeric antigen receptor T-cell products. *Cytotherapy* **25**, 94–102. 10.1016/j.jcyt.2022.09.004 (2023).
6. Bluestone, J. A. et al. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Science translational medicine* **7**, (2015). 315ra189-315ra189.
7. Norberg, S. M. et al. Impact of vector copy number on critical quality attributes of retrovirally engineered T cells. *Mol. Ther.* **34**, 1954–1962. 10.1016/j.ymthe.2025.12.048 (2026).
8. Stoops, J., Morton, T., Powell, J., Pace, A. L. & Bluestone, J. A. Treg cell therapy manufacturability: current state of the art, challenges and new opportunities. *Front. Immunol.* **16**, 1604483. 10.3389/fimmu.2025.1604483 (2025).
9. Guiraud, V. et al. Longitudinal analysis of lentiviral and retroviral chimeric antigen receptors' integration sites reveals distinct clonal evolutionary patterns. *Br. J. Haematol.* **206**, 1173–1177. 10.1111/bjh.20020 (2025).
10. Yang, F. et al. Differential regulation of Treg stability in human naïve and effector Treg subsets by TGFβ-signaling via ARKADIA-SKI axis. *Front. Immunol.* **16**, 1636434. 10.3389/fimmu.2025.1636434 (2025).
11. Shao, L., Zheng, Y., Somerville, R. P., Stroncek, D. F. & Jin, P. New insights on potency assays from recent advances and discoveries in CAR T-cell therapy. *Front. Immunol.* 16–2025. 10.3389/fimmu.2025.1597888 (2025).
12. Parcelier, A. et al. VCN Analysis Using Droplet Digital PCR Method in Hematopoietic Stem Cells and T Lymphocytes after Lentiviral Transduction: Optimization and Limitations. *Re:GEN Open* **2**, regen.0034, (2021). 10.1089/regen.2021.0034 (2022).
13. Lu, A. et al. Application of droplet digital PCR for the detection of vector copy number in clinical CAR/TCR T cell products. *J. Translational Med.* **18**, 191. 10.1186/s12967-020-02358-0 (2020).
14. Sojka, L. Enhanced Precision and Robustness in CAR-T Manufacturing: A Comparative Study of qPCR and dPCR. *Cytotherapy* **27**, S203. <https://doi.org/10.1016/j.jcyt.2025.03.413> (2025).
15. Peh, C. R. et al. Quantitation of Mycoplasma genitalium using droplet digital PCR. *Lett. Appl. Microbiol.* **76**, ovad064. 10.1093/lambio/ovad064 (2023).
16. Murphy, L. A., Mariani, R., Kohler, M. E., Fry, T. J. & Winters, A. C. Detection of Vector Copy Number in Bicistronic CD19xCD22 CAR T Cell Products with Digital PCR. *Blood* **138**, 4001–4001. 10.1182/blood-2021-153889 (2021).
17. Ma, J., Meyer, S., Olweus, J., Jin, C. & Yu, D. An adjusted droplet digital PCR assay for quantification of vector copy number in CAR-T cell and TCR-T cell products. *Immuno-Oncology Technol.* **25**, 101031. <https://doi.org/10.1016/j.iotech.2024.101031> (2025).
18. Galli, E. et al. Unlocking Predictive Power: Quantitative Assessment of CAR-T Expansion with Digital Droplet Polymerase Chain Reaction (ddPCR). *Int. J. Mol. Sci.* **25** 10.3390/ijms25052673 (2024).
19. Honoré, N., Galot, R., van Marcke, C., Limaye, N. & Machiels, J. P. Liquid Biopsy to Detect Minimal Residual Disease: Methodology and Impact. *Cancers (Basel)*. **13**. 10.3390/cancers13215364 (2021).
20. Kurt, A. S. et al. IL-2 availability regulates the tissue specific phenotype of murine intra-hepatic Tregs. *Front. Immunol.* 13–2022. 10.3389/fimmu.2022.1040031 (2022).
21. Harris, F., Berdugo, Y. A. & Tree, T. IL-2-based approaches to Treg enhancement. *Clin. Exp. Immunol.* **211**, 149–163. 10.1093/cei/uxac105 (2023).
22. Amit, I. et al. Negative feedback expansion of tregs caused by endogenous IL-2 limits the activity of IL-2-based therapies. *J. Cancer Immunol.* **5**, 29–39 (2023).
23. Owen, D. L. et al. Identification of Cellular Sources of IL-2 Needed for Regulatory T Cell Development and Homeostasis. *J. Immunol.* **200**, 3926–3933. 10.4049/jimmunol.1800097 (2018).
24. Attridge, K. & Walker, L. S. Homeostasis and function of regulatory T cells (Tregs) in vivo: lessons from TCR-transgenic Tregs. *Immunol. Rev.* **259**, 23–39. 10.1111/imr.12165 (2014).
25. Rothstein, D. M. & Camirand, G. New insights into the mechanisms of Treg function. *Curr. Opin. Organ. Transpl.* **20**, 376–384. 10.1097/mot.0000000000000212 (2015).
26. Qu, Z. et al. Tissue-resident macrophages and renal diseases: landscapes and treatment directions. *Frontiers in Immunology* Volume 16– (2025). 10.3389/fimmu.2025.1548053 (2025).
27. Sharma, R. & Kinsey, G. R. Regulatory T cells in acute and chronic kidney diseases. *Am. J. Physiol. Ren. Physiol.* **314**, F679–f698. 10.1152/ajprenal.00236.2017 (2018).
28. Burton, O. T. et al. The tissue-resident regulatory T cell pool is shaped by transient multi-tissue migration and a conserved residency program. *Immunity* **57**, 1586–1602e1510. 10.1016/j.immuni.2024.05.023 (2024).
29. Shah, N. N. et al. Clonal expansion of CAR T cells harboring lentivector integration in the CBL gene following anti-CD22 CAR T-cell therapy. *Blood Adv.* **3**, 2317–2322. 10.1182/bloodadvances.2019000219 (2019).
30. Fraietta, J. A. et al. Disruption of TET2 promotes the therapeutic efficacy of CD19-targeted T cells. *Nature* **558**, 307–312. 10.1038/s41586-018-0178-z (2018).

Tables

Table 1. Primer and probe sequence for in-house dPCR VCN assay

Amplicon	Primer/Probe	5' à 3'
FoxP3	Forward	CTTTATGCCAACCCAACC
	Reverse	GTTTCAGTTCAGAGACAGTCG
	Probe	HEX/CCTAGCGCCACACCTAACCC/BHQ1
WPRE	Forward	GCTTCCCGTATGGCTTTCA
	Reverse	TGCGTCAGCAAACACAGTG
	Probe	FAM/TTATGAGGAGTTGTGGCCCGTTGT/BHQ1

Table 2. Gene block for VCN standard curve

Gene Block	Sequence (5' to 3')
Foxp3	GCTAACCCCATCTCTGCACCTTGCCCTAAACGTACCCAGCTCTTACTCTAACTCCTTCCCAGCCTTTATGCCAACCCAACCCCATCTCCTTCCCTGGCCC
WPRE	TACGCTGCTTTAATGCCTTTGTATCATGCTATTGCTTCCCGTATGGCTTTCATTTTCTCCTCCTTGATAAATCCTGGTTGCTGTCTTTATGAGGAGTTGTG

Figures

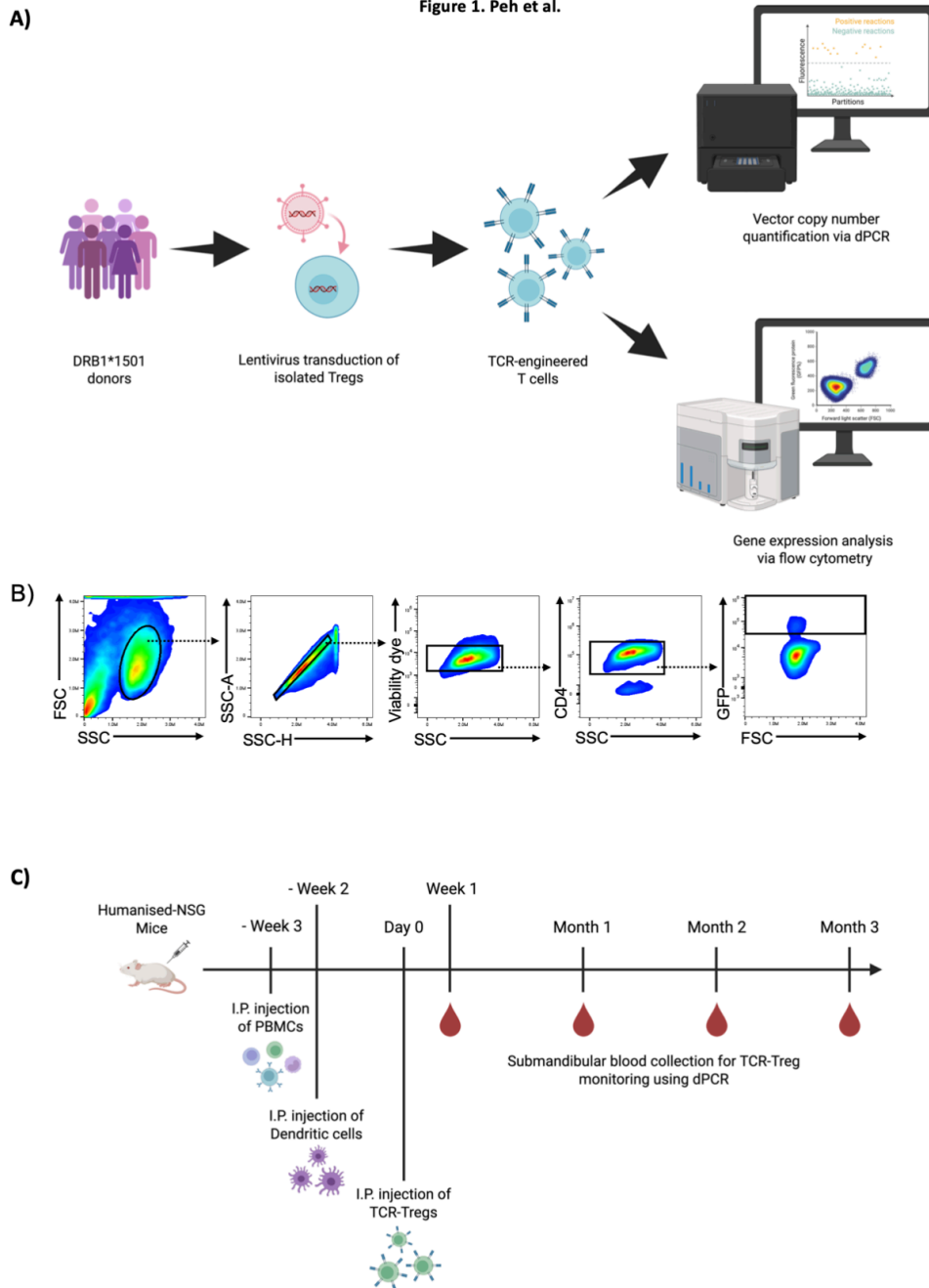


Figure 1

Experimental workflow and analysis. (A) CD4⁺CD25⁺CD127^{low} regulatory T cells and CD4⁺CD25⁺CD127^{high} conventional T cells were isolated from HLA-DRB1*15:01⁺ healthy donor peripheral blood by magnetic bead separation. Cells were transduced at multiplicities of infection (MOI) 1, 3, and 5 with a lentiviral vector encoding the TCR transgene and a GFP reporter and then expanded in vitro. Vector copy number (VCN) was quantified by digital PCR (dPCR) on genomic DNA and gene expression was measured by GFP reporter using flow cytometry. (B) Representative flow cytometry gating strategy showing sequential gating on lymphocytes, singlets, live CD4⁺ T cells, and GFP⁺ events used to quantify TCR-T cell transduction efficiency. (C) Previously established humanised NSG systemic lupus erythematosus (SLE) model - mice engrafted with human cells receive intraperitoneal injections of PBMCs, dendritic cells, and TCR-Tregs. Peripheral TCR-Treg abundance was monitored longitudinally by dPCR on genomic DNA extracted from submandibular blood collected at defined timepoints.

Figure 2. Peh et al.

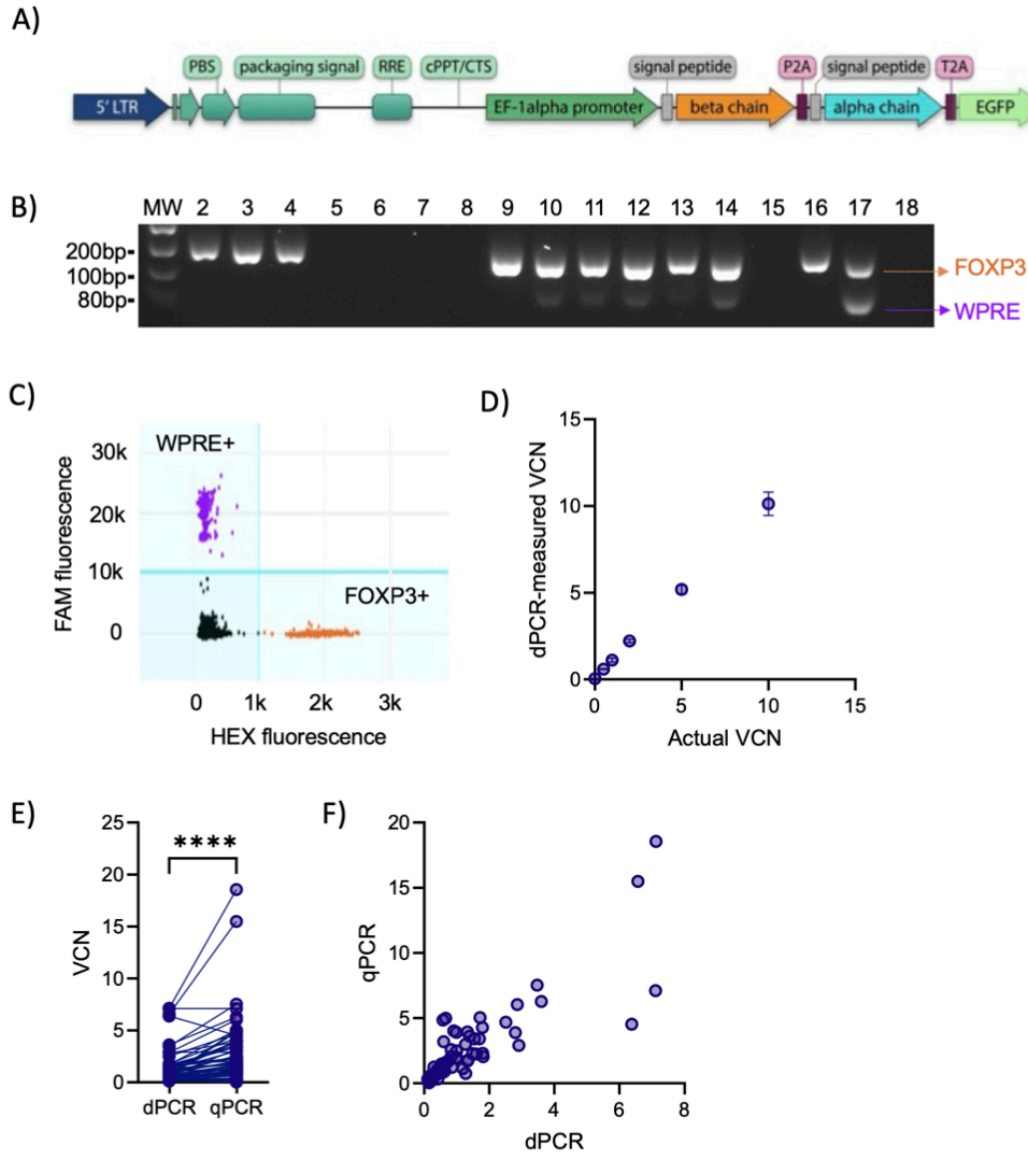


Figure 2

Validation of the in-house dPCR VCN assay. (A) Schematic of the TCR transgene insert. (B) 1% agarose gel (130 V, 50 min) showing dPCR VCN assay products from genomic DNA derived from human PBMCs (lanes 2–4), polyclonal Tregs (lane 8), TCR-Tregs (lanes 10–14), Jurkat-76 cells (lane 16), and TCR-transduced Jurkat-76 cells (lane 17); WPRE amplicons represent the transgene and Foxp3 amplicons the reference gene. (C) Representative three-dimensional scatter plot from the dPCR VCN assay illustrating separation of positive and negative reactions on the Applied Biosystems QuantStudio Absolute Q™ Digital PCR System. (D) Standard curve ($N = 3$) generated using Foxp3 gBlock DNA spiked with WPRE gBlock DNA to model samples with different VCNs ($R^2 = 0.9997$). (E-F) Comparison of dPCR and qPCR for VCN quantification in Treg and Tconv cells ($n = 5$). Data points were paired based on cell source, cell type, and MOI. (E) Statistical analysis of 60 data points using the Wilcoxon signed-rank test showed **** $p < 0.0001$. (F) Correlation between dPCR and qPCR VCN values, with Spearman's rank correlation coefficient $r = 0.8318$.

Figure 3. Peh et al.

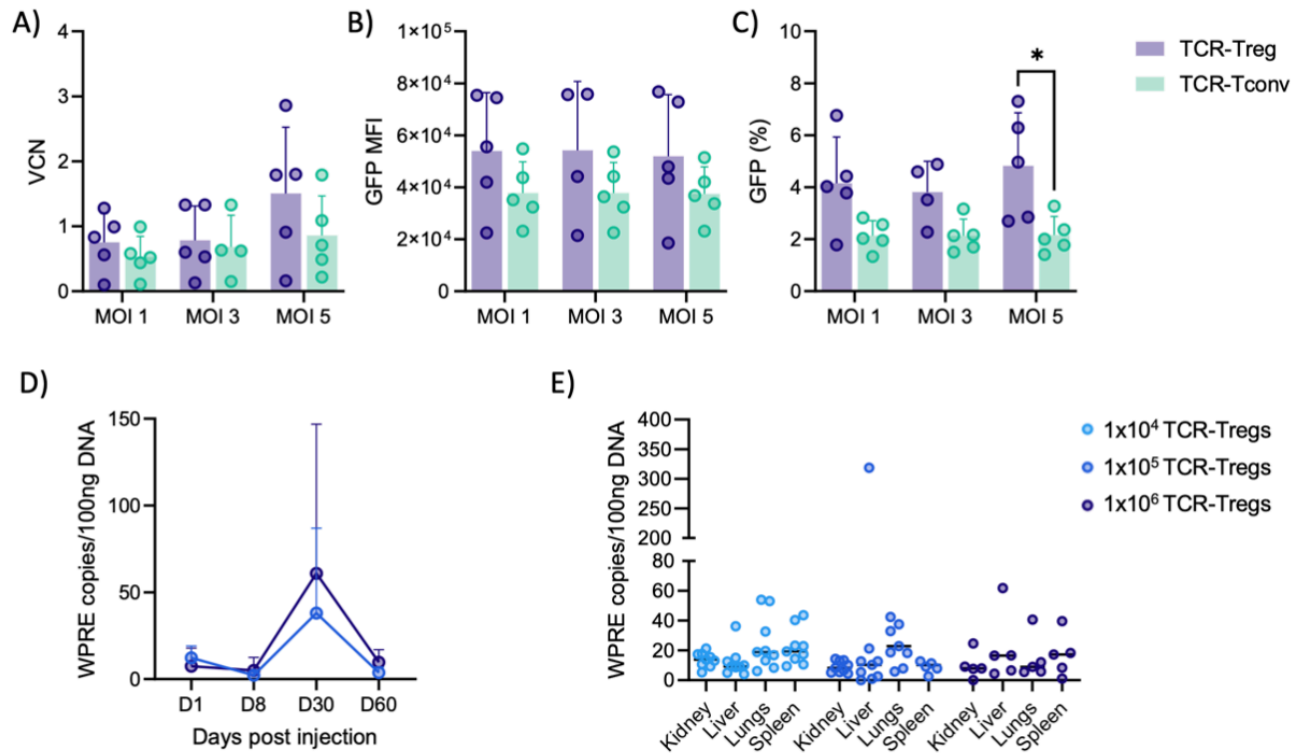


Figure 3

In vitro transduction characteristics and in vivo tracking of TCR-Tregs. (A) Vector copy number (VCN) in Treg and Tconv cells transduced at multiplicities of infection (MOI) 1, 3, and 5 with lentiviral vectors encoding the TCR transgene; cells were derived from healthy controls ($n = 5$) and VCN was quantified using the in-house dPCR assay, with data shown as mean (SD). (B,C) GFP expression in Treg and Tconv cells transduced at MOI 1, 3, and 5, measured by flow cytometry and presented as mean (SD, $n = 5$ healthy donors); a two-way ANOVA was used to test the effects of MOI and cell type, $*p = 0.0107$. (D) In vivo monitoring of TCR-Tregs in SLE-diseased humanised NSG mice. Mice were infused with TCR-Tregs and followed for 60 days, with cheek-bleed blood collected on days 1, 8, 30, and 60 for dPCR-based quantification of WPRE copies as a surrogate of circulating TCR-Treg abundance; data are shown as mean (SD) with at least 5 mice sample per time point. (E) Biodistribution of TCR-Tregs in SLE-diseased humanised NSG mice. Animals received TCR-Tregs at three doses (low, 1×10^4 ; medium, 1×10^5 ; high, 1×10^6 cells), and organs were harvested 8 weeks post-infusion; genomic DNA was extracted and WPRE copy number was quantified by dPCR as a measure of TCR-Treg abundance, with each point representing an individual mouse and horizontal lines indicating the mean.