Blocking T-cell egress with FTY720 extends DNA vaccine expression but reduces immunogenicity

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Abstract
Optimal immunogenicity from nucleic acid vaccines requires a balance of antigen expression that effectively engages the host immune system without generating a cellular response that rapidly destroys cells producing the antigen and thereby limiting vaccine antigen expression. We investigated the role of the cellular response on the expression and antigenicity of DNA vaccines using a plasmid DNA construct expressing luciferase. Repeated intramuscular administration led to diminished luciferase expression, suggesting a role for immune-mediated clearance of expression. To investigate the role of cell trafficking, we used the sphingosine 1-phosphate receptor (S1PR) modulator, FTY720 (Fingolimod), which traps lymphocytes within the lymphoid tissues. When lymphocyte trafficking was blocked with FTY720, DNA transgene expression was maintained at a constant level for a significantly extended time period. Both continuous and staggered administration of FTY720 prolonged transgene expression. However, blocking lymphocyte egress during primary transgene administration did not result in an increase of transgene expression during secondary administration. Interestingly, there was a disconnect between transgene expression and immunogenicity, as increasing expression by this approach did not enhance the overall immune response. Furthermore, when FTY720 was administered alongside a DNA vaccine expressing the HIV gp140 envelope antigen, there was a significant reduction in both antigen-specific antibody and T-cell responses. This indicates that the developing antigen-specific cellular response clears DNA vaccine expression but requires access to the site of expression in order to develop an effective immune response.

KEYWORDS
immunity, immunotherapy, infection, vaccine, virus

Received: 4 May 2021 | Accepted: 24 September 2021
DOI: 10.1111/imm.13429

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INTRODUCTION

DNA vaccines are gene-based delivery modalities for the expression of vaccinating transgenes. They have promise for the future of vaccinology, particularly against infectious agents for which conventional forms of vaccination have not conveyed the necessary levels of immunity or are deemed too risky to employ. First described in the early 1990s, DNA vaccines are composed of a plasmid DNA backbone, into which a transgene that expresses a candidate vaccine antigen of interest is inserted [1–3]. Despite the promise, DNA vaccines have yet to translate into clinically approved prophylactics for disease prevention in humans, although a number of DNA vaccines have been approved for use in the veterinary field [4–8], and more recently, RNA-based vaccines have been approved for use in humans [9, 10].

One potential area for optimisation is to enhance and sustain vaccine antigen expression at the injection site. Unlike protein vaccines, following intramuscular delivery, DNA vaccines need to be expressed by host cells local to the immunisation site or by migrating APCs [11]. To achieve this, the administered DNA has to transit both the cell and nuclear membranes, and following this, the transfected cells need to express the delivered vaccine-encoded gene, in effect manufacturing the vaccine in situ. Finally, the expressed vaccine antigen then needs to be trafficked to secondary lymphoid structures such as the spleen or draining lymph nodes, where effective activation of antigen reactive T and B cells occurs to induce a highly specific and protective immune response. Therefore, enhanced and sustained expression of vaccines may lead to amplified levels of vaccine antigen production, potentially leading to significantly magnified immune responses.

The induction of an immune response to the DNA transgene may have an impact on the expression of the transgene antigen. This can occur at two levels, innate and adaptive immunity. DNA vaccines have an inherent immunostimulatory property and can induce an innate immune response while transiting to the nucleus and encountering intracellular pattern recognition receptor sensing molecules such as STING1 [12] or TBK1 [13] in the cytosol. The triggering of innate immunity is essential for promoting adaptive immunity to DNA vaccines. However, activating a type I interferon response may limit the expression of the foreign transgene through the activation of interferon-stimulated genes (ISGs) resulting in the subsequent downregulation of DNA transgene expression [14]. In contrast, the complete absence of interferon alpha signalling reduces the immunogenicity of a DNA vaccine [15]. Likewise, the adaptive immune response can have an impact on transgene expression, as T cells activated by the vaccine and specific to the target antigen will recognize cells expressing the transgene, leading to their clearance [16–18]. Again, this can have mixed outcomes, and the immune-mediated destruction of antigen-producing myocytes may add to immune reactivity of DNA vaccines by generating a localized inflammatory process [16].

Extending the duration of vaccine transgene expression may be beneficial, especially in the induction of antiviral antibody responses, for example against HIV-1. The development of vaccine-elicited neutralizing antibodies with the necessary breadth to protect against diverse strains of HIV has so far proven elusive. Analysis of infection induced antibodies has been used to gain insight for vaccine development. Analysis of VRC01-lineage antibodies (a clade of broadly neutralising antibody that targets the CD4 binding site) revealed a rapid rate of bnAb evolution to an early autologous gp120 molecule, which then slowed down over time. However, despite this decline in mutation rate, antibody evolution appears to continue in response to chronic persistence of antigen, thereby enabling continued somatic hypermutation [19]. These studies suggest that methods that can prolong antigen exposure to the immune system, potentially by recurring vaccination, slow release, persistence or combinations thereof within vaccinated individuals, might have the desired effect of promoting B-cell bnAb production. Therefore, mechanisms to prevent immune-mediated clearance of cells expressing vaccine-encoded transgenes, by primed T cells may help extend transgene expression. Especially since, recent evidence suggests that repeated exposure to antigens can lead to the establishment of long-lasting tissue resident T cells that could more rapidly eliminate transduced cells [20].

We hypothesized that varying the length of exposure to the expressed vaccine antigen may impact the immunogenicity. We used the FDA-approved drug fingolimod hydrogen chloride (FTY720), a known sphingosine-1-phosphate receptor (S1PR) modulator, as an adjunct treatment to DNA vaccination. FTY720 is currently licensed to treat multiple sclerosis and can trap T cells within secondary lymphoid tissues [21]. While FTY720 can alter the numbers of various lymphocyte populations in peripheral blood (e.g. B cells and NK cells), its effects are more pronounced on T cells [22–25]. S1PR expressed on the cell surface promotes the movement of T cells towards a gradient of S1P, which is found at higher concentrations in blood and lymph and at lower concentrations in tissues. During T-cell activation by cognate antigen, the surface expression of S1PR1 on T cells is inhibited for several days, preventing the activated T cells from sensing S1P and exiting secondary lymphoid tissues. We therefore used the FTY720 to down-modulate the expression of S1PR on T cells, thereby sequestering antigen-specific T cells away.
from plasmid DNA vaccine-transduced and antigen-expressing myocytes. The aim was to foster a period of treatment-prolonged antigen translation and potential immune priming. We evaluated the use of co-administered FTY720 with a DNA encoded luciferase reporter gene, and a plasmid construct that expresses a model HIV-1 vaccine antigen, CN54-gp140. We show that administration of FTY720 can have profound effects on extending the duration of DNA plasmid transgene expression while simultaneously affecting both antigen-specific humoral and cellular immune responses.

**MATERIALS AND METHODS**

**Plasmids, protein and peptides**

The Luciferase and HIV-1 vaccine constructs were developed and prepared using the Auxo-GTU® vector technology. The HIV-1 CN54-gp140 clade C/B gene (codon optimized for mammalian expression) and the Luciferase gene were inserted into the Auxo-GTU® vector backbone and DNA vaccine produced by FIT BIOTECH (Finland) as described previously [26]. Recombinant CN54-gp140 protein was purchased from Polymun Scientific. The gp140 stimulatory peptides, 15mers overlapping by 11, were devised, synthesized (Insight Biotechnology) and pooled together. The luciferase peptide (DYQGFQSMYTFVTSH) was custom synthesized (>80% purity) by ProImmune Ltd, while Luciferase protein from *Phobotimus pyralis* (98% purity) was purchased from Sigma-Aldrich.

**Mice, vaccinations and sampling**

Female BALB/c mice (Harlan), 6–8 weeks old, were placed into groups of *n* = 5 and housed in a fully acclimatized room. All animals were handled, and procedures performed in accordance with the terms of a project license granted under the UK Home Office Animals (Scientific Procedures) Act 1986. Food and water were supplied ad libitum. Mice received plasmid vaccinations via the intramuscular (IM) route using 20 µg of the plasmid. Immediately after IM injection of plasmid, the injection site was electroporated (EP) using electrodes and an ECM 830 square-wave electroporation system (BTX). Pulses consisted of 100 V of positive and negative polarity at a rate of 1 pulse/s, with each pulse lasting 50 ms. All repeat vaccinations, using the same vaccine construct, were delivered into the same quadriceps muscle. In experiments using two different plasmid constructs, the different DNAs were administered into the opposite quadriceps.

Tail bleeds were collected weekly without anticoagulant and centrifuged in a Heraeus Biofuge pico (Fisher) at 1000g for 20 min. The serum was harvested and transferred into fresh 0.5-ml micro-centrifuge tubes (Starlab) and stored at −20°C until antibody titres were determined by ELISA.

To modulate plasmid transgene expression, FTY720 (Selleckchem) was diluted in sterile PBS to a final concentration of 1 mg/kg and 100 µl administered to lightly anaesthetized mice via the intraperitoneal route. The FTY720 was administered 24 h prior to plasmid vaccination and then every 24 h thereafter.

For ELISpot splenocyte cultures, lymphocytes were isolated from single-cell suspensions from spleens of immunized and control mice. Briefly, mice were euthanized, and their spleens were removed aseptically and placed into individual 20-ml universal tubes (Greiner) containing 2 ml RPMI 1640 medium (Sigma Aldrich Ltd). The spleens were then placed into Petri dishes and single-cell suspensions made by disrupting the spleens. This was achieved by grinding the spleens through a 70 µm Nylon Cell Strainers (BD Falcon) using forceps. The cell suspensions were then centrifuged at 350 g for 10 min. The supernatants were decanted, and the pelleted cells resuspended in ACK lysis buffer for 5 min (Gibco). The cell suspensions were vortexed and centrifuged at 350 g for 10 min. The pelleted cells were decanted and resuspended in 1 ml RPMI 1640 medium. This step was repeated three times with the cells resuspended in complete RPMI and then filtered through a 100 µm Filcon unit (BD Biosciences). The cells were counted using a haemocytometer and trypan blue exclusion, with viable cell concentrations adjusted to 1 × 10⁶ cells/ml.

**Bioluminescence imaging**

Real-time bio-imaging of IM DNA vaccinations were performed using a multispectral Carestream In Vivo FX Pro system. Briefly, 20 µg GTU-Luc-GFP plasmid was administered IM with EP to anaesthetized BALB/c mice. Luciferase activity was measured 15 min after i.p. administration of D-luciferin (150 mg/kg; Promega). Bioluminescence images were acquired and analysed using Carestream software using 60-s exposures.

**Antibody ELISA**

A quantitative immunoglobulin ELISA protocol described previously [27, 28] was followed. Briefly, 5 µg/ml gp140-coated ELISA plates were blocked with 200 µl/well 1% BSA/0.05% PBST. After washing, samples were diluted and added to the plates for 1 h before washing
and addition of a 1:4000 dilution of anti-mouse IgG-HRP (Southern Biotech). Samples were developed using 3,3’,5,5’-teramethyl benzidine (TMB) and the reaction stopped using Stop solution (Insight Biotechnologies). The ELISA standard consisted of coating with anti-mouse Kappa (1:3200) and Lambda (1:3200) light chain (Serotec), blocking as before and then adding 50 µl of highly purified polyclonal mouse IgG (Southern Biotech) in a 1:5 dilution down the plate, starting at 200 ng/ml. Absorbance was read on a spectrophotometer (VersaMax – Molecular Devices) using SoftMax Pro GxP v5 software.

**IFN-γ ELISpot**

IFN-γ ELISpot assays (MABTECH) were carried out on splenocytes as per the manufacturer’s instructions and as described previously [27, 28]. Plates were blocked for 30 min using complete RPMI before addition of 100 µl of 1 x 10⁶ cells/ml. Next, either 5 µg/ml PHA (Sigma) stimulated cells were served as controls. To detect spots, biotinylated anti-IFN-γ antibody was added at 1 µg/ml for 2 h before washing and incubating with Streptavidin-HRP for 1 h. Plates were washed as before and 100 µl/well of TMB substrate added.

**Flow cytometry**

Isolated splenocytes (100 µl of 5 x 10⁶ cells/ml) were assessed for reactivity towards vaccine antigen using two pools containing 78 peptides of 15-mers overlapping by 11 aa of HIV-1 CN54gp140 Env (Insight Biotechnology). Cell samples were incubated for 1 h in 5 µg/ml Brefeldin A before being incubated for 5 h with 1 µl of each CD28 and CD49d monoclonal antibody, and 2 µg/ml of the relevant peptide pools. Samples were washed twice in FACs buffer (2.5% FCS in PBS) before staining with Zombie Aqua (BioLegend) and then surface staining for 1 h at 4°C with anti-CD3 (Clone 500A2, BD Biosciences), anti-CD4 (Clone GK1.5, BD Biosciences), anti-CD8 (Clone 53-6.7, eBioscience), anti-CD44 (clone IM7, BioLegend) and anti-CD62L (clone MEL-14, BioLegend) antibody. Samples were washed a further two times before fixing in 1.5% methanol-free paraformaldehyde (Polysciences) in PBS. Samples were analysed on a FACS Canto II instrument with FACS Diva software. Data analysis was performed with FlowJo (BD). Samples with gated CD3+ lymphocytes populations less than 5% were designated below the limit of detection.

**Statistical analysis**

Where two groups are being assessed, statistical analysis of the data was carried out using a Mann-Whitney non-parametric t test (unpaired) or Wilcoxon signed-rank test (paired) using GraphPad PRISM software. Alternatively, where multiple groups are being compared, a non-parametric Kruskal–Wallis test was used, followed by adjusting for multiple comparisons using Dunn’s test.

**RESULTS**

Sequential DNA administration results in reduced transgene expression

We used luciferase to investigate the impact of the immune response on transgene expression. We administered DNA expressing firefly luciferase intramuscularly with electroporation which we have previously shown to dramatically increase expression [26]. Luciferase expression was assessed using in vivo imaging at multiple timepoints after DNA transgene administration (Figure 1a). After the first DNA administration, luciferase expression was rapidly detectable in the muscle on Day 2 post-vaccination (9.8 x 10⁵ RLU ± 3.96 x 10⁵) (Figure 1b). Expression peaked at day 7 (3.9 x 10⁶ RLU ± 1.04 x 10⁶) after DNA administration and was detectable up to 21 days (1.1 x 10⁵ RLU ± 4.8 x 10³) after transgene delivery. At no point was luciferase expression detected by in vivo imaging in the region of the draining lymph nodes or spleen. The mice were subsequently rested for 3 weeks prior to re-administration of the same plasmid DNA construct expressing the identical luciferase transgene, with electroporation. Compared with luciferase expression levels detected after the first vaccination, transgene expression was considerably lower after the second administration, with a reduction in expression level recorded as early as Day 2 (3.3 x 10⁵ RLU ± 5.8 x 10⁵) and a statistically significant reduction in expression detected by Day 7 post-administration (2.4 x 10⁵ RLU ± 3.2 x 10⁴, p < 0.0005). By Day 2, there was a 57% (±14.9% SEM) reduction in vivo luciferase expression in the inoculated muscle. By Day 7 after the second administration, expression was 91% (±2.9% SEM) of the first administration (Figure 1c).

Collectively, this shows that upon administration of a
second, identical DNA construct, the peak expression levels are significantly lower in the muscles.

**FIGURE 1** Sequential DNA vaccination results in reduced transgene expression. Female BALB/c mice (n = 5/group) were vaccinated intramuscularly with electroporation using 20 µg AuxoGTU® plasmid (FIT BIOTECH) expressing the luciferase firefly transgene. Mice were vaccinated on day 0 with luciferase activity followed for 21 days post-vaccination with imaging occurring on days 0, 2, 5, 7, 16 and 21. The mice were then given a 3-week recovery period before the experiment was repeated (a). Vaccine transgene expression was verified as a function of photon emission (Relative Light Units, RLU) using a multispectral Carestream In Vivo FX Pro system (USA) (b). The percentage change in luminescence was calculated between the values obtained from the first vaccination and corresponding values of the second vaccination (c). Statistical analysis was performed using Wilcoxon signed-rank test. *p ≤ 0.05

**FTY720 alters DNA transgene expression kinetic**

To address the potential impact of the immune response on transgene expression levels after a second, identical vaccination, we used fingolimod (FTY720), an immunomodulatory drug which blocks the sphingosine-1-phosphate receptor, thereby trapping T cells in lymph nodes [29, 30]. We used this to assess the impact of reduced circulating T cells on luciferase transgene expression. BALB/c mice were administered with FTY720, 24 h before and then every day, for 21 days, after DNA vaccination with a luciferase transgene. In mice that did not receive FTY720 treatment, there was transient transgene expression that peaked in the first week and was cleared by 21 days post-vaccination (Figure 2a); there was no detectable luciferase expression in these control mice after day 24 of the study (Figure 2a). In line with our hypothesis, that circulating T cells might impact DNA transgene expression, 21 days of continuous administration of FTY720 after the primary vaccination (red line), enabled extended transgene expression for up to 50 days, after which luciferase expression was undetectable. Compared to the control group there was significantly more luciferase expression from day 12 (p < 0.05) to day 42 (p < 0.005) in the group where FTY720 was administered. The total luciferase expression was calculated as the area under the curve for each group and was 2.9-fold greater in the FTY720 treated group compared to non-FTY720-treated group (p < 0.005; Figure 2c). When the FTY720 treatment was halted at day 21, the RLU rapidly declined (Figure 2a).

Next, because we used FTY720 to prevent immune cells from clearing transfected myocytes during a vaccine prime, we evaluated if the initial exposure to FTY720 had either no effect or could even extend expression of transfected myocytes after a second homologous boost vaccination. This could signify a level of tolerance induction or development of an aberrant immune response. Additionally, we asked the question that if FTY720 was administered during the vaccine boost rather than during the prime, could it prevent immune-mediated clearance despite pre-existing immunity. To answer these questions, we switched the FTY720 treatment regimens after a 37-day washout. When the groups were switched around, the previously FTY720-treated group
had a similar response peak and kinetic to the group that had not been treated with FTY720 in the primary DNA administration. In both groups, there was detectable transgene expression, but it was significantly lower than the primary administration. Expression was cleared after 14 days in both groups, with a slightly faster clearance
kinetic in the group that did not receive FTY720 during the primary DNA administration (Figure 2a). These data show that a 21-day FTY720 treatment regimen did not have a negative impact on immune responses that clear transfected cells arising through DNA vaccine boosting. Moreover, it demonstrated that FTY720 was unable to prevent clearance of transfected myocytes in the context of pre-existing immunity. As circulating T cells in blood are effectively suppressed during FTY720 treatment, the presence of tissue resident immune cells could be behind the clearing of transfected myocytes.

Having seen that FTY720 administration can modulate transgene expression, we looked to see whether alternative drug regimes, comparing continuous to staggered administration, would also impact expression over multiple DNA administrations. For this schedule, mice were given three vaccinations, using a luciferase expressing DNA construct, at 3-week intervals. They either received no FTY720 (controls) or FTY720 every day (continuous) or the drug between days 7 and 21 after each of the first two immunisations (staggered), with a third DNA administration with no treatment (Figure 3a). There were repeated waves of luciferase expression followed by clearance after each of the three DNA administrations in the control (non-FTY720) group (Figure 3b). The duration of transgene antigen expression was reduced after each successive administration. Both the constant and staggered FTY720-treated groups had sustained luciferase expression to day 42. When the FTY720 was removed, the constant group had sustained expression out to day 58, whilst expression in the staggered group began to decline. We hypothesized that the altered dynamic was associated with transgene-specific T cells clearing cells expressing the luciferase. To investigate the effect of FTY720, we measured luciferase-specific IFN-γ expression by cells isolated from the spleens of mice. Mice that received no FTY720 had significantly more luciferase-specific T cells in response to stimulation with either peptide ($p < 0.001$, Figure 3c) or whole luciferase protein ($p < 0.001$, Figure 3d). This suggests that the altered transgene expression is associated with differential T-cell dynamics.

**Blocking lymphocyte trafficking reduces the immune response to DNA vaccination**

Having observed altered transgene expression after FTY720 administration, we wanted to evaluate the effect on the immune response. To do this, the mice used in Figure 3 were also vaccinated with an HIV gp140 expressing DNA construct in the opposite leg to the luciferase expressing plasmid and were left untreated or co-administered FTY720. We measured anti-gp140 antibodies in the blood at various time points after DNA vaccination. There was no antigen-specific antibody detectable at day 7 or 14 after the priming vaccination (Figure 4a). At day 28, there were antigen-specific antibodies detectable in all groups, though there was no difference in the antibody titre between treatment groups. At day 49 after the first immunisation, the untreated group had significantly more antibody than the constant group. We also investigated T-cell responses. There were significantly more IFN-γ-producing cells in response to gp140 peptide pools (Figure 4b) or total protein (Figure 4c) in the control FTY720 untreated mice than the groups that had been vaccinated and treated with FTY720.

We next investigated whether there was an impact on the memory phenotype of T cells in the spleens from vaccinated mice (Figure 3a). Expression of markers of central (TCM: CD62L+CD44−) or effector (TEM: CD62L−CD44+) memory was measured on splenic CD4+ or CD8+ T cells by flow cytometry. There was a significantly higher percentage of live CD4+ cells in the spleens of mice immunized in the absence of FTY720 (i.e. none group) than the constantly treated animals ($p < 0.005$, Figure 5a). Of the mice with viable CD4+ T cells, there was no observable difference in CD44+ CD62L+ (TCM) or total protein (Figure 4b) between FTY720 untreated mice than the constant treated group (Figure 5b). Similarly, no observable difference was seen in the CD44+ CD62L- (TEM) when gating on viable CD4 T cells (Figure 5c). Interestingly, although there was no significant difference in the viability of CD8+ T cells (Figure 5d), a slight reduction in TCM and an increase in TEM cells was detected, although the sample numbers are not suitably powered to detect a significant difference (Figure 5e,f). It should be stressed that we carried out these studies on splenic CD4 and CD8 T cells, and not peripheral T cells; therefore, it is impossible to determine if the FTY720 had any effect on circulating T cells. However, previous reports have shown FTY720 administration does impact peripheral T-cell counts [31, 32].

**DISCUSSION**

In this study, we investigated the interplay between T cells, DNA vaccine expression and immunogenicity. To prevent T-cell egress from secondary lymphoid tissues, we used the drug FTY720. We observed that transgene expression was significantly prolonged when T cells could not leave these immune structures. However, blocking T-cell trafficking also significantly reduced the immunogenicity of administered vaccines. In this study, we utilized luciferase to track expression, as we have previously observed it to be more sensitive than fluorescent proteins [33]. We observed
An ideal vaccine would induce a stable, long-lived immune memory, avoiding the need for subsequent booster administrations. One approach might be to mimic viral infections which can leave a durable, if not permanent change to the immune system. Vaccination with live-attenuated viruses (e.g., vaccinia, measles, mumps, rubella, varicella zoster virus) can elicit remarkably stable antibody responses \( t_{1/2} = 50–200 \text{ years} \) [36]. Though this is not the case for all viruses, some induce much shorter lived antibody responses enabling re-infection, for example respiratory syncytial virus [37, 38] and possibly coronaviruses [39]. One feature of viral infection and vaccination that has been linked to a durable immune response is the extended exposure to antigen. The use of adjuvants for protein vaccines that form a depot has been proposed as a way to mimic the extended antigen exposure [40]. By forming a depot, vaccine antigens can be released over prolonged periods of time, ensuring continued immune stimulation driving germinal centre formation, B-cell affinity maturation and neutralizing antibody production. Hence, mechanisms that could extend the half-life of vaccine antigens within the body, could be a promising avenue of investigation when it comes to designing more protective vaccines.

Genetic-based vaccines have tremendous potential as prophylactic treatments against endemic and newly emerging pathogens as they are safe, stable, easily scalable and rapid to generate. However, DNA vaccines have not yet lived up to the expectation in human clinical trials [41]. Unlike conventional vaccines, nucleic acid vaccines need to be expressed prior to inducing an immune response. For DNA vaccines, expression requires the vaccine transgene to cross both the cellular and nuclear membranes. Subsequent expression of the DNA vaccine antigen may only have a relatively short duration before being cleared by the activated immune system, with clearance likely occurring through the combined actions of cytotoxic T lymphocytes (CTL) and NK cells. Interestingly, previous studies evaluating immune-mediated elimination of transfected target cells, suggests that CD4 T cells, via CD4+ T-cell Fas/FasL myocyte signalling, may be central to DNA vaccine antigen clearance [16]. Hence, by sequestering CD4 T cells away from sites of vaccination or even eliminating them, we hypothesized it may be possible to prolong antigen expression and enhance immunogenicity of DNA vaccines. In a separate study, oral FTY720 in renal allograft recipients was shown to impact both circulating CD4 and CD8 T cells, making FTY720 an interesting candidate for preventing T cell-mediated elimination of transduced cells [31]. We did indeed observe an increase in protein expression; interestingly, this only occurred when the FTY720 was given during the primary transgene

**FIGURE 4** Constant and staggered administration regimens of Fingolimod modulates vaccine-elicited immune responses. Mice \( (n = 8/\text{group}) \) were immunized with plasmid DNA expressing gp140 with and without FTY720 as described in Figure 3. Anti-gp140-specific IgG was measured in mouse sera \( (n = 5/\text{group}) \) every 7 days \( (a) \). On Day 72, the mice were euthanized, and splenic tissues harvested for assessment of T-cell responses. IFN-\( \gamma \) ELISPOT assay results \( (n = 8/\text{group}) \) after stimulation with gp140 peptide \( (b) \) and gp140 protein \( (c) \) are shown. Statistical significance was assessed using a non-parametric Kruskal–Wallis test for comparisons of median values among the different groups, followed by multiple comparisons testing using Dunn’s test.

\*\( p \leq 0.05 \); **\( p \leq 0.005 \); ***\( p \leq 0.0005 \). SFU, spot forming units

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a significant reduction in transgene expression on the second administration, suggesting immune-mediated clearance, both in BALB/c and C57BL/6 strains of mice having known T-cell epitopes within luciferase [34]. We utilized the known immune response to luciferase in mice to track the interface of immunity and expression, but it is a caveat for other uses of bioluminescence, for example tracking tumours where an immune response to the luciferase may facilitate clearance [35].
administration. When FTY720 was given during the second transgene administration, expression levels were not changed. It is possible that this could have happened as a result of tissue resident memory T cells (TRM) accumulating at the injection site clearing transfected cells, a phenomenon we have previously seen for another DNA vaccine [42]. Although we have not evaluated the accumulation of TRM at the vaccine site in this manuscript, it nevertheless raises important clinical questions related to vaccine delivery. For instance, do tissue tropic immune cells, recruited to sites of vaccine-induced inflammation, ultimately interfere with DNA, RNA or even viral vector transgene expression? As TRM are known to persist, extending vaccine dosing schedules may not circumvent this problem if clearance is by this memory T-cell subset. Rather, it could be speculated that subsequent inoculations of homologous vaccine preparations in a separate tissue site may exhibit a therapeutic benefit, by separating transfected myocytes from TRM, and enabling greater transgene expression before clearance. Alternatively, using heterologous DNA or viral vector-prime and protein-boost regimens may avoid this issue. As we enter a new vaccine era, where gene-based vaccine delivery is now in use and likely to become more prevalent, studies deconvoluting anti-vector immunity and the immune-mediated clearance of transfected cells will need to be carried out to ensure optimal vaccine delivery and expression.

However, rather than seeing an immune potentiating effect of extended antigen expression following FTY720 administration, we saw a significant reduction in response. This was seen with both constant and staggered drug administration regimes. Taken together, the work presented herein, provides evidence that the S1P receptor modulator, FTY720, significantly impacts vaccine-elicited humoral and cellular immune responses. Using HIV gp140 as a model vaccine antigen, we observed significantly reduced CD4 T-cell numbers capable of responding to ex-vivo antigen re-stimulation and significantly reduced vaccine-elicited antibody titres in FTY720 treated animals. Moreover, we show that FTY720 impacts CD4 and CD8 TCM and TEM ratios and the overall viability of CD4 T cells. Overall, the data show that FTY720, particularly if given regularly, can negatively impact immune responses to vaccines.

**ACKNOWLEDGMENTS**

This work was funded by CUT’HIVAC under a European Commission FP7 award (grant number 241904). PM & RS were funded by the Department of Health & Social Care using UK Aid funding and is managed by the Engineering and Physical Sciences Research Council (EPSRC, grant no. EP/R013764/1; note that the views expressed in this publication are those of the authors and not necessarily those of the Department of Health and Social Care). We are also grateful to the Fondation Dormeur for provision of an equipment grant.

| Figure 5 | Fingolimod administration depletes CD4 T cells within secondary lymphoid organs. The splenic cell viability and different memory cell subsets were analysed in mice (n = 8/group) by flow cytometry. In some treatment groups, recovery and cell viability were low, and therefore only n = 3 are shown. Live CD4 (a) or CD8 (d) splenic T cells were measured. Cell populations were gated based on CD44 and CD62L expression to identify TCM (CD44+, CD62L−) and TEM (CD44+, CD62L−) populations for CD4 (b and c) and CD8 T (e and f) cells, respectively. Statistical significance was assessed using the Mann–Whitney U test. *p ≤ 0.05 |
Special thanks go to Andres Männik, Ioana Stanescu, Mart Ustav, and Fit Biotech (Finland) for the provision of the GTU-Luc-GFP and GTU-CN54-gp140 plasmids used in these studies. We also thank Dietmar Katinger (Poxvirus Scientific, Austria) for the provision of the CN54-gp140 antigen.

Schematics were created using BioRender software (BioRender.com).

**CONFLICT OF INTEREST**
The authors declare no conflicts of interest.

**AUTHOR CONTRIBUTIONS**
JM Conceptualisation, Investigation, writing – Original Draft; PM Conceptualisation, Investigation, Supervision, writing – review & editing; JP Investigation; KK Investigation, JT Writing – review & editing; RS Conceptualisation, Funding Acquisition, Supervision, Writing – review & editing. All authors read and approved the final manuscript.

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**How to cite this article:** Mann JFS, McKay PF, Klein K, Pankrac J, Tregoning JS, Shattock RJ. Blocking T-cell egress with FTY720 extends DNA vaccine expression but reduces immunogenicity. Immunology. 2022;165:301–311. [https://doi.org/10.1111/imm.13429](https://doi.org/10.1111/imm.13429)