

Monitoring mRNA vaccine antigen expression in vivo using PET/CT

Corresponding Author: Dr Mark Sellmyer

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The study presents a novel approach for noninvasive, longitudinal monitoring of mRNA vaccine protein products using a positron emission tomography (PET) reporter gene system, "Vax-Tag," based on *Escherichia coli* dihydrofolate reductase (eDHFR). Overall, the manuscript presents a significant advancement in the field of mRNA vaccine research and development, offering a novel tool for noninvasive, longitudinal monitoring of protein expression. The findings have important implications for both preclinical studies and potential clinical applications. Below are detailed reviewer comments: Major modification comments:

1. The focus of the article should emphasize the tracking of antigen expression distribution. The current study places too much emphasis on the induction of immune response, which is not significantly different from already published articles and seems to deviate from the main topic.
2. The reviewer expects that simultaneous tracking of both mRNA and its expressed protein could provide greater academic value.
3. The authors mentioned that the S2P-eDHFR group did not produce antibodies against eDHFR, which is a very interesting finding. However, this was not explained in results and discussion sections. Please discuss this.
4. Line 176-178, "On day 1, mice vaccinated with S2P-eDHFR demonstrated a 5-fold increase in uptake of [18F]-FTMP in the ipsilateral popliteal LN and a 2-fold increased uptake in the injected muscle compared with mice vaccinated with eLNPs (Fig. 5B-C).", The mRNA expression in the ipsilateral popliteal lymph node is much higher than that in the injected muscle. However, following intramuscular administration of mRNA via LNP carrier, mRNA expression is predominantly localized in the muscle and liver by in vivo imaging of the bioluminescence of luciferase [PMID: 38557908, 37896985]. It may be a good discussion point to suggest supplementary experiments to verify the differences in results produced by the two methods.
5. Line 140 states that "...is a nonimmunogenic Vax-Tag in vivo". To further substantiate this claim, I would suggest adding a subset of animals that exclusively express eDHFR protein to determine whether it indeed induces an immune response. And line 209. "No immune response is detected against eDHFR in an NHP", additional experiments are needed to verify whether there is a cellular immune response against eDHFR in NHP.

Minor modification comments:

1. Line 467, 470, and 476. "50x10⁶ cells/mL, 25x10⁶ cells/mL, and 50x10⁶ cells/mL" should be revised to read: "5.0x10⁷ cells/mL, 2.5x10⁷ cells/mL, and 5.0x10⁷ cells/mL".
3. Line 496. A solitary rhesus monkey with N=1 is an insufficient sample size, which may undermine statistical significance, given that the test results lack robustness.
4. Line 714. Fig. 2B, please provide the original WB image with Maker.
5. Line 714. Please provide the figure legend for Fig. 2D, including the number of repetitions and standard deviation values, to enable accurate interpretation.
6. Line 754, Fig. 3. Statistical analysis is required to assess the significance of any differences observed among the various groups. Please provide the corresponding statistical tests and p-values to support the interpretation.
7. Figure 2A, why did not include a flexibility linker in the design of S2P-eDHFR mRNA.
8. Check the format of the references.
9. Please provide animal experiment ethics number.

Reviewer #2

(Remarks to the Author)

TO AUTHORS

The authors developed what they term "Vax-Tag", which is essentially a reporter gene fusion onto a protein intended to be expressed for vaccination, here in the context of SARS-CoV-2. The encoding happens at the mRNA level, hence does not only monitor mRNA (like reported in <https://www.nature.com/articles/s41551-019-0378-3>) but in fact the translated protein species as the relevant immunogenic molecule. They use the exogenous eDHFR reporter gene, an E. Coli-derived protein, in conjunction with its specific F-18-radiotracer (trimethoprim (TMP)). This reporter gene/radiotracer pair has been developed by the lead author over many years and has been applied in several different settings before. The eDHFR/TMP pair is a good choice due to excellent contrast but eDHFR as a foreign protein is potentially concerning as expected to be immunogenic on its own. The authors have previously published work showing comparably little immunogenicity towards eDHFR and speculated that this could be due to a potentially acquired tolerance towards this protein stemming from commensal microorganisms expressing similar dihydrofolate reductases. The authors performed vaccination experimentation in mice as well as one non-human primate and performed PET/CT imaging to identify immunization sites and draining lymph nodes containing cells that express the reporter-antigen. Sadly, this manuscript is not up to the quality standard expected from a high-impact publication such as Nature Communications with quite a few shortcomings.

The introduction could give a better overview of reporter gene imaging in mRNA vaccine development. The authors focused on SARS but the principle is much wider applicable, hence several publications dealing with other vaccines but using mRNA reporters have been left out despite them being examples for reporter gene methodology use in this context.

Results/Figures:

-Fig.1 is not really needed and resembles more a graphical abstract. It should be relegated from the main part of the manuscript.

-Fig.2: The MFI of S2P+ve cell subpopulation in S2P-eDHFR seems around 3-fold lower than in S2P; this is bigger in 2C than in 2D, whereby in 2D there are no error bars (suggesting N=1?!?). Moreover, 2B has only N=2. Experiments should be carried out with at least 3 biological repeats (for all non-NHP aspects).

-One obvious conclusion, namely that the S2P-eDHFR fusion appears significantly lower expressed compared to S2P is not followed up, neither experimentally nor in the discussion – this could have important consequences for immunization/antigen-presence and may be critical for wider development of the eDHFR reporter methodology as envisaged by the authors.

-The authors should also evaluate relative S2P vs. S2P-eDHFR expression in a set of different cell populations that are relevant to in vivo vaccination under realistic scenarios; and then perform relative expression analysis to quantify the impact of the eDHFR fusion across such a cell line panel.

-How exactly is the S2P mRNA fused to the eDHFR mRNA, what is the exact linker?

-Immunoblots in Fig.2B appear saturated and lack molecular weight standards; controls with unmodified S2P are missing.

Fig.3:

-Fig.3A-C: Authors should also demonstrate the immunization with the unmodified S2P and show whether the likely lower antigen expression achieved (as follows from Fig.2) has an impact on immunization and what the extent of this impact is on total produced antibodies; corresponding comparative figure panels should be shown. Ideally it would also be commendable to show this for LNPs carrying eDHFR alone (or a plasma membrane-tethered variant; e.g. CAAX or CAGgap-modified).

-Fig.3D: These T-cell experiments are interesting but again, do not reveal differences compared to S2P only immunization. Moreover, statistical analysis is required for each condition and the overall subject number of N=5 does not seem to be right in all cases; e.g. CD8+INFG+Boost panel appears as if there is N=7, while in the CD8+INFG+Prime panel N is only 5, could authors check that the N-number is the same across all panels? It would also be useful to indicate (e.g. via different symbols) which data points stem from the same subject. In the CD8+INFG+Prime panel, it seems %age for peptides B, E, F, and G may be larger for S2P-eDHFR vs eLNP (statistical analysis will tell).

Fig.4:

-In the corresponding methods it is not clear how many CFSE(lo)/(hi) splenocytes were ultimately administered per mouse?

-How did authors ensure that the antigen loading was comparable between CFSE(hiS2P) and CFSE(hiEDHFR)? What levels were achieved?

-Which peptide pools were used for generating CFSE(hiS2P) and CFSE(hiEDHFR)? This is not clear from methods and as described and displayed must be different compared to e.g. eDHFR peptides used for Fig.3.

-Fig.4B/C: the way the data are shown is not very helpful due to the "3D stacking". This should be made clearer by showing the data as in Fig.2C such that every histogram is clearly visible and not overlapping with others. When using color, meaning of color and consistency, at least within the same figure, would be very helpful for the onlooker and the authors should change this.

Fig.5:

-The authors should show the data in 5B using the same hue/grayscale overlays (alongside PET-only hue images) as in 5E; larger images should be shown.

-Marking of the immunization site on the animals is needed.

-Non-immunized control mice to clarify which tissues take up TMP are missing.

-Scale bars lack units in 5B and 5E.

-It is totally unclear which VOI precisely was analyzed for the Muscle data in 5C/D/F, and what were the selection criteria (authors elude to some thresholding in Methods but do not explain what/how it was done).

-It is good practice in such nuclear medicine experiments to have (sub-)cohorts that are sacrificed and their tissues harvested for ex vivo radioactivity analysis. That also enables histology/flow cytometry and all ex vivo data can then support

claims stemming from in vivo imaging. The authors should repeat this experiment and add such data (e.g. popliteal ex vivo lymph node analysis, demonstrating in lymph nodes that S2P-eDHFR expressing cells are present, adding ex vivo biodistribution data, demonstrating reach of antigen-expressing cells at immunization sites matching analysis in vivo volumes etc.). During lymph node harvesting and to identify lymphatics as guides it may be beneficial to employ dyes such as isosulfane blue or similar.

-5E please add Day-3 images as well as eLNP only mice; vice versa, please add S2P-only vaccinated animals to 5B/C/D.

-Radioactivity units should be in SI system (Bq, not Ci).

-What was the specific molar activity of the administered radiotracer?

-What was the vehicle for the radiotracer, saline?

-Why was 2h uptake time chosen?

-Were the mice receiving the radiotracer under anesthesia, then woke up, and subsequently re-anesthetized for imaging? Or were they under anesthesia for the whole 2h? What was the isoflurane carrier (air? Oxygen?) and what was its flow rate?

-How was thresholding done for PET image analysis?

-How were boundaries of 'tissues of interest' drawn? What criteria were used?

Fig.6:

This reviewer appreciates the ethical concerns, experimental complexities, and cost connected with NHP experimentation. However, N=1 is too anecdotal/little a number while absolutely vital controls also must be considered. I suggest performing the experiment with N=2x NHP per cohort and the following cohorts: S2P-eDHFR, S2P, and eLNP (if analysis of the first NHP show similar behavior as in mice, i.e. no response, N=1 for eLNP is sufficient). It is good to have the TMP imaging data from the NHP vaccinated with *Clostridium difficile* already.

-Images in S3 are too small/resolution to appreciate relevant features. In S4, it is somewhat surprising that the immunization site (L Deltoid) did not show signals on day-1 imaging.

-Why were days 1, 8, and then after a very long pause day 99 chosen? It would appear much more reasonable to chose a closer time point, e.g. day 16-24.

-What applies for image visualization for mice in Fig.5B/E is also valid for all NHP imaged (see above).

-It is a missed opportunity in the current NHP that the authors chose to not perform ex vivo experimentation as in 3D for the mouse model and/or mixed lymphocyte reactions to assess the NHP T cells' ability to respond to antigen. This should be added to future experiments in NHP is possible through withdrawing blood samples at different time points for this purpose.

General comments:

-P-values should be as numbers rather than just categories/asterisks; best to add instead of the asterisks into each figure panel

-Flow cytometry: Please show the full gating strategies used. This should include the position of positive and negative controls and essential information, such as the percentage of cells in the region or gate or event count, and statistics as appropriate. Axis legends should include the marker (e.g. antigen) and the fluorochrome/dye used. For more information how to prepare flow cytometry data analysis for publication please see the "Guidelines for the use of flow cytometry and cell sorting in immunological studies" (<https://onlinelibrary.wiley.com/doi/epdf/10.1002/eji.202170126>). This is relevant for all flow cytometry graphs in this manuscript.

-Animals: Methodology reporting should adhere to the ARRIVE 2.0 guidelines (as a bare minimum the ARRIVE 10 but better including the full recommended set; please see here for guidance: <https://arriveguidelines.org/arrive-guidelines>). This enables readers and reviewers to scrutinize the research adequately, evaluate its methodological rigor, and reproduce the methods or findings.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The author has solved almost all my concerns through revisions, but there are still some details that need to be added or explained.

1. Fig 6 c, the selected areas of day 35 and day1/day7 are inconsistent. Day35 obviously has no ribs and its shape is inconsistent with the other two pictures.

2. Discussion, lines 255-256, "As previously mentioned, this may be due to improved surface trafficking afforded by the canonical furin cleavage site present in S2P.", this does not explain that the S2P positivity rate of dendritic cells from the LN of S2Pf-eDHFR vaccinated mice is lower than that of S2P vaccinated mice, please check carefully.

3. Lines 380-383, "mRNA-LNPs were characterized for their hydrodynamic size, and polydispersity index (PDI), using dynamic light scattering (DLS), and the encapsulation efficiency and concentration measured using the RiboGreen RNA assay (Invitrogen, R11490)", the author does not seem to describe the characterization information of the prepared vaccine, including particle size, PDI, etc. In fact, the particle size of the vaccine has a certain impact on the immune effect. Different particle sizes may affect the spatial distribution of the vaccine at the injection site.

4. In line 385, "HEK293T cells transfected with 25 µg of S2P or S2Pf-eDHFR mRNA...", HEK293T cells were transfected with 25 µg mRNA. How was such a high dose determined? And please describe the transfection method.

Reviewer #3

(Remarks to the Author)

I have been requested to provide feedback on the revisions made in response to Reviewer #2's concerns. Reviewer #2 raised a total of 34 points, all of which were valid. This reviewer conducted a thorough analysis of the manuscript while maintaining focus on the overall context. The authors have also done an outstanding job addressing each of the 34 points raised by Reviewer #2. As a result, I believe the revised manuscript now meets the high-quality standards expected of a prestigious journal like Nature Communications.

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February 12, 2025
Nature Communications

We thank the reviewers for the opportunity to submit a revised and improved manuscript entitled, “**Monitoring mRNA vaccine antigen expression in vivo using PET/CT**” to *Nature Communications*. Our revisions are described below and nearly all the requested changes have been made. Below is our point-by point response and here is a summary of the key changes to the manuscript:

- Repetition of in vitro experiments including western blot and flow cytometry as well as expression of S2P and S2P^{Δf}-eDHFR in a cell line relevant to in vivo vaccination.
- Characterization of the immunogenicity of S2P^{Δf}-eDHFR compared with S2P in mice. We have also included a cohort of mice vaccinated against eDHFR alone to these studies.
- Ex vivo analysis including an [¹⁸F]FP-TMP biodistribution experiment and flow cytometry of lymphocytes isolated from mouse lymph nodes following vaccination with S2P, S2P^{Δf}-eDHFR and eDHFR.
- Statistical analysis has been performed for all figure panels and we have added a biostatistician as an author for biostatistical review.
- Repeat imaging of S2P^{Δf}-eDHFR in a second NHP on days 1, 7, and 35 and characterization of cellular immune response against S2P and eDHFR.

Reviewer #1

The study presents a novel approach for noninvasive, longitudinal monitoring of mRNA vaccine protein products using a positron emission tomography (PET) reporter gene system, “Vax-Tag,” based on *Escherichia coli* dihydrofolate reductase (eDHFR). Overall, the manuscript presents a significant advancement in the field of mRNA vaccine research and development, offering a novel tool for noninvasive, longitudinal monitoring of protein expression. The findings have important implications for both preclinical studies and potential clinical applications. Below are detailed reviewer comments:

We appreciate these positive comments. Note that we removed the “Vax-Tag” moniker as eDHFR has other applications as a PET reporter gene outside of vaccine research.

Major modification comments:

1. The focus of the article should emphasize the tracking of antigen expression distribution. The current study places too much emphasis on the induction of immune response, which is not significantly different from already published articles and seems to deviate from the main topic.

We substantially emphasized the imaging in the prose and rearranged the figures to have imaging come before immunogenicity (mouse → NHP) for a more logical flow.

The immunogenicity experiments performed in this study look at induction of immune response against eDHFR when expressed in vivo, which is significantly different from previous work we have done. The prior work analyzing the immunogenicity of eDHFR (Lee et al, *Molecular Therapy*, 2023) was in human ex vivo cultures exposed to peptides only, which is less stringent than immunization with eDHFR in immunocompetent animals. These experimental results are important to report because we are introducing a bacterial protein into mammalian species.

While the examination of S2P immunogenicity may not be significantly different from published work, it was important for us to explore given the relatively lower expression of S2P^{Δf}-eDHFR, as was a point raised by several of these revision comments.

2.The reviewer expects that simultaneous tracking of both mRNA and its expressed protein could provide greater academic value.

While we appreciate the value of simultaneous tracking of the mRNA and its expressed protein, this is out of scope for our study. There are already techniques that exist for tracking of the mRNA transcript (Lindsay et al, *Nature Biomedical Engineering*, 2019) and the LNP itself (Wong et al, *Nuclear Medicine and Biology*, 2017). Our approach is tailored to build upon these techniques to look at protein expression itself. We believe that tracking of the protein is most important given that it is the bioactive species. That being said, we do envision future studies where we compare LNP specific labels (e.g. Cu64) or radiolabeled mRNA complimentary sequences for dual monitoring, as the reviewer is suggesting.

3.The authors mentioned that the S2P-eDHFR group did not produce antibodies against eDHFR, which is a very interesting finding. However, this was not be explained in results and discussion sections. Please discuss this.

We added more discussion in body of the manuscript. Please see line 165, 210 (results) and 298-302 (discussion).

4.Line 176-178, "On day 1, mice vaccinated with S2P-eDHFR demonstrated a 5-fold increase in uptake of [18F]FP-TMP in the ipsilateral popliteal LN and a 2-fold increased uptake in the injected muscle compared with mice vaccinated with eLNPs (Fig. 5B-C).", The mRNA expression in the ipsilateral popliteal lymph node is much higher than that in the injected muscle. However, following intramuscular administration of mRNA via LNP carrier, mRNA expression is predominantly localized in the muscle and liver by in vivo imaging of the bioluminescence of luciferase [PMID: 38557908, 37896985]. It may be a good discussion point to suggest supplementary experiments to verify the differences in results produced by the two methods.

In the publication by Du et al (PMID: 38557908), figure 1F shows BLI images of luciferase expression in mice over a 48-hour time course following delivery with either LNP or LPP. It is true that luciferase expression is present in the liver of mice following intramuscular administration of mRNA via LNP carrier. However, this result is predominant at the earlier timepoints of 6 - 12 hours whereby at 24 hours, liver expression only remains in one mouse. The result is similar in Zhang et al (PMID: 37896985) figure 3B whereby liver signal decreases from 6 to 24 hours. In our experiments, the day 1 PET imaging timepoint (24 hours) is after peak liver expression, and the concentration of S2P^{Δf}-eDHFR may be too low to see signal above background. It is also important to consider the inherent differences in imaging techniques. BLI can capture very low amounts of enzyme expression and is capable of imaging very few transfected cells through catalytic signal amplification. eDHFR PET requires accumulation of the radiotracer to sufficient levels in transfected cells by non-covalent binding of eDHFR.

In the context of muscle expression, it is important to note that in vivo BLI has poor spatial resolution when compared with PET/CT imaging. In BLI, light emitted from luciferase-expressing tissues that are close together may overlap and make it difficult to distinguish the source. In mice, the popliteal lymph node lies between the hamstring muscles. Therefore, BLI signal coming from the hindlimb of mice in these two publications is likely a combination of muscle and LN signal, whereas we can discriminate between the muscle and LN in terms of expression of the vaccine using PET/CT.

All of our in vivo imaging data was confirmed with an ex vivo [¹⁸F]FP-TMP biodistribution study we added (**Supplementary Fig. 3**).

5. Line 140 states that "...is a nonimmunogenic Vax-Tag in vivo". To further substantiate this claim, I would suggest adding a subset of animals that exclusively express eDHFR protein to determine whether it indeed induces an immune response. And line 209. "No immune response is detected against eDHFR in an NHP",

additional experiments are needed to verify whether there is a cellular immune response against eDHFR in NHP.

We have repeated the ELISA and T cell polyfunctionality experiment with mice vaccinated with eDHFR exclusively and we do not see an antibody response or an immune response against the combined peptides (**Figs. 3-4, Supplementary Fig. 9**).

Additionally, we have vaccinated a second NHP with S2P^{Δf}-eDHFR and performed an ELISPOT to look at cellular immunity against both eDHFR and spike (**Fig. 7**). No cellular immune response against eDHFR was detected in either NHP.

Minor modification comments:

6. Line 467, 470, and 476. “50x106 cells/mL, 25x106 cells/mL, and 50x106 cells/mL” should be revised to read: “5.0x107 cells/mL, 2.5x107 cells/mL, and 5.0x107 cells/mL”.

Done.

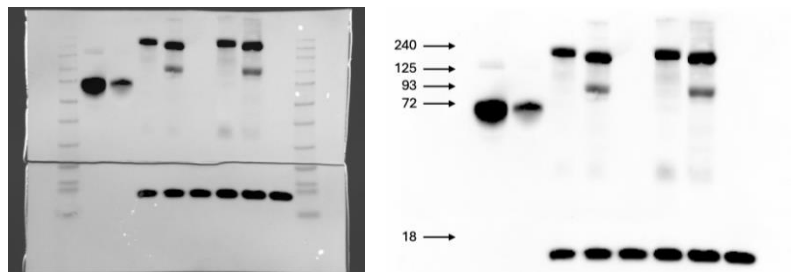
7. Line 496. A solitary rhesus monkey with N=1 is an insufficient sample size, which may undermine statistical significance, given that the test results lack robustness.

We have vaccinated an additional NHP with S2P^{Δf}-eDHFR and performed PET imaging on day 1, 7, and 35. The imaging data is consistent with our previous results in which there is high signal in the ipsilateral axillary lymph nodes on days 1 and 7 which decreases to background by day 35 (**Fig. 6**). We have also repeated immunogenicity experiments to include both NHPs (**Fig. 7**).

8. Line 714. Fig. 2B, please provide the original WB image with Maker.

Please note the Western blot has moved to **Figure 1c**. We have also provided all WB and repeats in the Source Data File using Image Lab (Bio Rad).

Lane 1: 100 ng S2 protein + 40 µg BSA
Lane 2: 25 ng S2 protein + 40 µg BSA
Lane 3: 40 µg S2P^{Δf}-eDHFR HEK293T lysate
Lane 4: 40 µg S2P HEK293T lysate
Lane 5: 40 µg HEK293T WT lysate
Lane 6: 80 µg S2P^{Δf}-eDHFR HEK293T lysate
Lane 7: 80 µg S2P HEK293T lysate
Lane 8: 80 µg HEK293T WT lysate



9. Line 714. Please provide the figure legend for Fig. 2D, including the number of repetitions and standard deviation values, to enable accurate interpretation.

We have added error bars to the flow experiments. Error bars are representative of 3 technical repeats within the same experiment, but the experiment has been repeated 3 times and the data is consistent. Please note this figure has moved to **Figure 1d**.

10. Line 754, Fig.3. Statistical analysis is required to assess the significance of any differences observed among the various groups. Please provide the corresponding statistical tests and p-values to support the interpretation.

We have run statistical analysis for the T cell polyfunctionality experiments to determine any difference in immune response detected against the various eDHFR peptides or pooled spike peptides. Details on the statistical test are included in the figure captions and p-values are displayed on the graphs.

No significant difference in immune response was determined between eLNP and S2P^{Δf}-eDHFR mice against any of the eDHFR peptides (A-G) (**Fig. 4a-c, Supplementary Fig. 8**). Similarly, no significant response was detected against the combined pool of eDHFR peptides in the eDHFR and S2P^{Δf}-eDHFR vaccinated mice in our follow up experiment (**Fig. 4d-e, Supplementary Fig. 9**).

11. Figure 2A, why did not include a flexibility linker in the design of S2P-eDHFR mRNA.

We have previously shown that eDHFR is functional as a direct fusion protein (Sellmyer et al, *Molecular Therapy*, 2020; Lee et al, *Molecular Therapy*, 2023; Etersque et al, *Nature Communications*, 2023) and as S2P is already a large protein we did not want to introduce a linker region unnecessarily.

12. Check the format of the references.

Done.

13. Please provide animal experiment ethics number.

Done.

Reviewer #2

The authors developed what they term “Vax-Tag”, which is essentially a reporter gene fusion onto a protein intended to be expressed for vaccination, here in the context of SARS-CoV-2. The encoding happens at the mRNA level, hence does not only monitor mRNA (like reported in <https://www.nature.com/articles/s41551-019-0378-3>) but in fact the translated protein species as the relevant immunogenic molecule. They use the exogenous eDHFR reporter gene, an E. Coli-derived protein, in conjunction with its specific F-18-radiotracer (trimethoprim (TMP)). This reporter gene/radiotracer pair has been developed by the lead author over many years and has been applied in several different settings before. The eDHFR/TMP pair is a good choice due to excellent contrast but eDHFR as a foreign protein is potentially concerning as expected to be immunogenic on its own. The authors have previously published work showing comparably little immunogenicity towards eDHFR and speculated that this could be due to a potentially acquired tolerance towards this protein stemming from commensal microorganisms expressing similar dihydrofolate reductases. The authors performed vaccination experimentation in mice as well as one non-human primate and performed PET/CT imaging to identify immunization sites and draining lymph nodes containing cells that express the reporter-antigen. Sadly, this manuscript is not up to the quality standard expected from a high-impact publication such as Nature Communications with quite a few shortcomings.

The introduction could give a better overview of reporter gene imaging in mRNA vaccine development. The authors focused on SARS but the principle is much wider applicable, hence several publications dealing with other vaccines but using mRNA reporters have been left out despite them being examples for reporter gene methodology use in this context.

We have added two additional mRNA reporter gene imaging techniques to the introduction (Skourti et. al., *Frontiers in Immunology*, 2023; Gurung et. al., *Science Translational Medicine*, 2024) in line 80-83 that we believe helps give two precedents in the field.

Results/Figures:

Figure 1

1. Fig.1 is not really needed and resembles more a graphical abstract. It should be relegated from the main part of the manuscript.

We have removed most of the figure and combined it with the data in figure 2. We chose to keep the schematics of our mRNA-LNP and transfection into antigen presenting cells with addition of [¹⁸F]FP-TMP to illustrate our system.

Figure 2

2. The MFI of S2P+ve cell subpopulation in S2P-eDHFR seems around 3-fold lower than in S2P; this is bigger in 2C than in 2D, whereby in 2D there are no error bars (suggesting N=1!?!). Moreover, 2B has only N=2. Experiments should be carried out with at least 3 biological repeats (for all non-NHP aspects).

We have added in error bars and completed biological repeats (N = 3). Please see the next comment (reviewer 2, comment 3) for a response to the lower MFI of S2P^{Δf}-eDHFR positive cells.

3. One obvious conclusion, namely that the S2P-eDHFR fusion appears significantly lower expressed compared to S2P is not followed up, neither experimentally nor in the discussion – this could have important consequences for immunization/antigen-presence and may be critical for wider development of the eDHFR reporter methodology as envisaged by the authors.

We appreciate this point and acknowledge the important implications of the notably lower expression of S2P^{Δf}-eDHFR versus S2P alone. We have included experiments to follow up on this finding (**Fig. 1d, Supplementary Fig. 5**) and have added more prose in lines 125-126, 246-250, 333-340.

In short, the canonical furin cleavage site was removed from our S2P^{Δf}-eDHFR construct to keep the eDHFR tag fused to S2P (note we have changed the naming convention of the construct for added clarity). We hypothesize that the furin cleavage site present in S2P may improve surface trafficking of the protein versus S2P^{Δf}-eDHFR. Both ^Δfurin and endogenous spike proteins have been used in the context of COVID-19 vaccines.

As demonstrated in the western blot, a second band is detected in the S2P lane around 90 kDa (**Fig. 1c**) corresponding to the glycosylated form of the S2 cleavage product whereas no cleavage product is observed in S2P^{Δf}-eDHFR. This effect is also demonstrated in Peacock et al, *Nature Microbiology*, 2021 and Prompetchara et al, *Nature Communications*, 2023.

Importantly, while there may be some inherent differences in expression kinetics between native and eDHFR fusion proteins, the immune response detected against spike in mice vaccinated with S2P or S2P^{Δf}-eDHFR is comparable, indicating that eDHFR fusion does not impact bioactivity. In future applications, researchers should empirically test the stability and immune responses elicited from native and eDHFR-fused proteins of interest (commented on in the discussion line 337-340).

4. The authors should also evaluate relative S2P vs. S2P-eDHFR expression in a set of different cell populations that are relevant to in vivo vaccination under realistic scenarios; and then perform relative expression analysis to quantify the impact of the eDHFR fusion across such a cell line panel.

We have repeated the transfection of S2P and S2P^{Δf}-eDHFR at molar concentrations followed by flow cytometry in the DC2.4 cell line which is an immortalized murine dendritic cell line (**Fig 1d, Supplementary Fig. 1**). We have also looked at expression in vaccinated mouse lymph nodes using flow cytometry (**Supplementary Fig. 5**). The data remains the same in which the positivity of S2P cells is higher than S2P^{Δf}-eDHFR cells.

5. How exactly is the S2P mRNA fused to the eDHFR mRNA, what is the exact linker?

There is no linker, rather a direct fusion is used in which eDHFR is fused to the C-terminal end of the S2P sequence. Please see the above comment (reviewer 1, comment 11) for further explanation as to why no linker was used.

6. Immunoblots in Fig.2B appear saturated and lack molecular weight standards; controls with unmodified S2P are missing.

Molecular weight markers and the recombinant SARS-CoV-2 spike S2 protein (SinoBiological, 40590-V08B) which was used to produce the SARS-CoV-2 spike S2 antibody used in all experiments (SinoBiological, 40590-MM05) were added to the western blot.

Figure 3

7. Fig.3A-C: Authors should also demonstrate the immunization with the unmodified S2P and show whether the likely lower antigen expression achieved (as follows from Fig.2) has an impact on immunization and what the extent of this impact is on total produced antibodies; corresponding comparative figure panels should be shown. Ideally it would also be commendable to show this for LNPs carrying eDHFR alone (or a plasma membrane-tethered variant; e.g. CAAX or CAGgap-modified).

We have completed this experiment looking at humoral and cellular immune responses against spike and eDHFR in eDHFR, S2P^{Δf}-eDHFR, and S2P vaccinated mice (**Figs. 3-4, Supplementary Fig. 9**). No impact on S2P immunogenicity and no immune response against eDHFR is detected.

8. Fig.3D: These T-cell experiments are interesting but again, do not reveal differences compared to S2P only immunization. Moreover, statistical analysis is required for each condition and the overall subject number of N=5 does not seem to be right in all cases; e.g. CD8+INFg+Boost panel appears as if there is N=7, while in the CD8+INFg+Prime panel N is only 5, could authors check that the N-number is the same across all panels? It would also be useful to indicate (e.g. via different symbols) which data points stem from the same subject. In the CD8+INFg+Prime panel, it seems %age for peptides B, E, F, and G may be larger for S2P-eDHFR vs eLNP (statistical analysis will tell).

Please see above comments (reviewer 1, comment 10; reviewer 2, comment 7). Statistical analysis was performed for all T cell polyfunctionality experiments in mice. Because many data points overlap, we did not indicate individual mice by different symbols, but we have made the entire data set available in the Source Data File.

For the initial T cell polyfunctionality experiment, sub-cohorts of mice were taken down at prime (N = 5) and boost (N = 5 or 7). This has been updated in the figure captions.

Figure 4

9. In the corresponding methods it is not clear how many CFSE(lo)/(hi) splenocytes were ultimately administered per mouse?

Line 475 – a total of 4e6 splenocytes were administered per mouse, 2e6 CFSE^{lo} and 2e6 CFSE^{hi}.

10. How did authors ensure that the antigen loading was comparable between CFSE(hiS2P) and CFSE(hiEDHFR)? What levels were achieved?

The same amount of each peptide was added to each sample under the same conditions. We cannot quantify the antigen loading efficiency which is based on the intrinsic binding of MHC class molecules to the peptides. There is no supporting data for antigen loading provided in the method development or other papers using this type of experiment (Haymaker et al, *Journal of Visualized Experiments*, 2017; Stark et al, *Methods in Molecular Biology*, 2020; Ramos da Silva et al, *Science Translational Medicine*, 2023).

11. Which peptide pools were used for generating CFSE(hiS2P) and CFSE(hiEDHFR)? This is not clear from methods and as described and displayed must be different compared to e.g. eDHFR peptides used for Fig.3.

We have updated the methods section to describe this more clearly. In the first T cell polyfunctionality experiment (eLNP versus S2P^{Δf}-eDHFR), T cells were exposed to individual eDHFR peptides (A-G) or all of the spike peptides pooled into one reaction. In the follow up T cell polyfunctionality experiment (eDHFR, S2P^{Δf}-eDHFR, S2P) all peptides were pooled together for both spike and eDHFR (all spike peptides in one reaction, all eDHFR peptides in the other). This method was also used in the in vivo cytotoxicity experiment. Regardless of whether the peptides were pooled or not, each peptide was maintained at the same concentration (2.5 µg/mL).

12. Fig.4B/C: the way the data are shown is not very helpful due to the “3D stacking”. This should be made clearer by showing the data as in Fig.2C such that every histogram is clearly visible and not overlapping with others. When using color, meaning of color and consistency, at least within the same figure, would be very helpful for the onlooker and the authors should change this.

The histograms have been updated and unstacked. Additionally, the figure has been color coordinated to assist the reader with interpretation of the results.

Figure 5

13. The authors should show the data in 5B using the same hue/grayscale overlays (alongside PET-only hue images) as in 5E; larger images should be shown.

It is critical to show whole animal MIP images to show the reader where else the radioactivity has accumulated at a particular time point, as opposed to a single slice. Black and white look up table (LUT) images are how we interpret clinical PET scans at our institution with hot metal overlays for PET/CT images. Black and white LUTs also allow more discrimination and linear relationship appreciation of activity, but would not work with overlaid CT images. For the experiment in 2B, supplementary figure 2 shows the isolated CT and PET/CT overlay images.

14. Marking of the immunization site on the animals is needed.

This is a great suggestion. We have added it.

15. Non-immunized control mice to clarify which tissues take up TMP are missing.

We chose to use the eLNP as our negative control for imaging experiments because it most closely simulates vaccination with S2P^{Δf}-eDHFR, just without the genetic component. The background uptake of [¹⁸F]FP-TMP observed in these mice, namely the gall bladder, intestines, bladder, and bone is consistent with several other imaging experiments we've done including in the development of [¹⁸F]FP-TMP in which naïve Balb/c mice were injected with [¹⁸F]FP-TMP and the biodistribution was assessed at 15, 60, and 120 minutes (Sellmyer et al, *PNAS*, 2017).

16. Scale bars lack units in 5B and 5E.

Units have been added.

17. It is totally unclear which VOI precisely was analyzed for the Muscle data in 5C/D/F, and what were the selection criteria (authors elude to some thresholding in Methods but do not explain what/how it was done).

ROIs were drawn on the CT images and representative muscle and lymph node ROIs for the mouse image analysis have been added to the supplemental (**Supplementary Fig. 2**). We did not use more complex thresholding or SUV_{peak} type of ROI analysis. Since the location of the eDHFR expression could be heterogenous within the tissue (be it lymph node or muscle), we chose to quantify the data using a standard PET and clinical measure, SUV_{max} .

18. It is good practice in such nuclear medicine experiments to have (sub-)cohorts that are sacrificed and their tissues harvested for ex vivo radioactivity analysis. That also enables histology/flow cytometry and all ex vivo data can then support claims stemming from in vivo imaging. The authors should repeat this experiment and add such data (e.g. popliteal ex vivo lymph node analysis, demonstrating in lymph nodes that S2P-eDHFR expressing cells are present, adding ex vivo biodistribution data, demonstrating reach of antigen-expressing cells at immunization sites matching analysis in vivo volumes etc.). During lymph node harvesting and to identify lymphatics as guides it may be beneficial to employ dyes such as isosulfane blue or similar.

We appreciate this reviewer's comment and agree with the importance of providing ex vivo analysis. We have performed an ex vivo biodistribution experiment with [^{18}F]FP-TMP (**Supplementary Fig. 3**) and flow cytometry on the lymphocytes harvested from mouse LNs following vaccination with eDHFR, S2P Δ eDHFR, or S2P (**Supplementary Figs. 4-5**). The data supports our in vivo imaging findings, namely expression of S2P Δ eDHFR in the ipsilateral LNs and injected muscle.

In the biodistribution experiment, [^{18}F]FP-TMP uptake was observed predominantly in the ipsilateral LNs and the injected muscle in the S2P Δ eDHFR group (**Supplementary Fig. 3**). The expression of eDHFR as a standalone protein may be too low to capture using [^{18}F]FP-TMP. Please see line 151-152 and 322-330 for further discussion of this point.

For flow cytometry, we used fluorescent markers against CD3, CD19, and NK1.1 to dump T cells, B cells, and NK cells, respectively, from our analysis. We then analyzed expression of S2P and eDHFR in the dendritic cells using a fluorescent marker against CD11c and staining with an Alexa Fluor 488 conjugated spike S2 antibody and TMP-JF646 (a fluorescent conjugate of TMP). TMP-JF646 staining confirmed expression of eDHFR in the S2P Δ eDHFR and eDHFR vaccinated mice, and lower expression is observed in the eDHFR cohort consistent with our biodistribution data (**Supplementary Fig. 4**). Similarly, expression of S2P in the S2P and S2P Δ eDHFR vaccinated mice was detected. Of note, we were not able to detect S2P expression at the tested antibody dilution in the first iteration of this experiment. Therefore, we optimized for S2P staining by using a higher dilution of S2 antibody and incubated for a longer period in the follow up experiment. Consistent with our in vitro data, S2P appears to express higher than S2P Δ eDHFR (**Supplementary Fig. 5**).

19. 5E please add Day-3 images as well as eLNP only mice; vice versa, please add S2P-only vaccinated animals to 5B/C/D.

The data shown in 5B-D is from a separate animal experiment from the data in 5E-F. In 5B-D, no S2P vaccinated mice were imaged. In this experiment, mice were imaged on day 1 and 3 to determine the optimal imaging timepoint for S2P Δ eDHFR. From this data, we determined that day 1 was peak expression and so we performed a follow up imaging experiment with mice vaccinated with either S2P Δ eDHFR or S2P alone and imaged on day 1 only and this data is shown in 5E-F. Please note that this data has moved to Figure 2. We have experimental timelines for clarity.

20. Radioactivity units should be in SI system (Bq, not Ci).

Done.

21. What was the specific molar activity of the administered radiotracer?

The specific activity is 19442 +/- 12277 Ci/mmol or 719354 +/- 454249 GBq/mmol (line 412).

22. What was the vehicle for the radiotracer, saline?

Yes, saline.

23. Why was 2h uptake time chosen?

The 2 hour uptake time was found to be optimal in the [^{18}F]FP-TMP/eDHFR development papers (Sellmyer et al, *PNAS*, 2017). This allows maximum washout of radiotracer from background tissues. In our NHP imaging experiments, we performed a dynamic scan over a 120 minute period and found the data to be consistent between 60 and 120 minutes.

24. Were the mice receiving the radiotracer under anesthesia, then woke up, and subsequently re-anesthetized for imaging? Or were they under anesthesia for the whole 2h? What was the isoflurane carrier (air? Oxygen?) and what was its flow rate?

The mice received the radiotracer while awake. Mice were anesthetized ~10 minutes prior to imaging and throughout the imaging time course. 2% isoflurane in oxygen at a flow rate of ~0.8 L/min was used.

25. How was thresholding done for PET image analysis?

No thresholding was done. Please see comments on SUV above (reviewer 2, comment 17).

26. How were boundaries of 'tissues of interest' drawn? What criteria were used?

Regions of interest were drawn on the CT images and confirmed on the PET/CT overlay images with two investigators.

Figure 6

27. This reviewer appreciates the ethical concerns, experimental complexities, and cost connected with NHP experimentation. However, N=1 is too anecdotal/little a number while absolutely vital controls also must be considered. I suggest performing the experiment with N=2x NHP per cohort and the following cohorts: S2P-eDHFR, S2P, and eLNP (if analysis of the first NHP show similar behavior as in mice, i.e. no response, N=1 for eLNP is sufficient). It is good to have the TMP imaging data from the NHP vaccinated with *Clostridium difficile* already.

We appreciate the suggestion and have repeated S2P $^{\Delta\text{f}}$ -eDHFR imaging in a second NHP. The second NHP followed the same vaccination schedule with vaccination on day 0 and boost on day 15. Imaging timepoints were adjusted to days 1, 7, and 35 to address the below comment. Blood was also sampled at day 7 and 35 for immunogenicity analysis. Overall, the imaging data remains consistent between the 2 NHPs. We see [^{18}F]FP-TMP notably in the injected muscle on day 1 and in the ipsilateral axillary lymph nodes on days 1 and 7. All tissues normalized to background levels by the day 35 timepoint (**Fig. 6, Supplementary Fig. 11**). We do not have funding nor scanner/NHP access to perform imaging on all the reviewer suggested groups.

28. Images in S3 are too small/resolution to appreciate relevant features. In S4, it is somewhat surprising that the immunization site (L Deltoid) did not show signals on day-1 imaging.

We increased image size for all NHP MIP images. NHPs were vaccinated in R deltoid. We have added immunization site markers to all imaging figures. We did observe high [¹⁸F]FP-TMP signal in the injected muscle in NHP 1 but less so in NHP 2. Several factors may be contributing to this observation including the use of a younger NHP, the use of recently produced mRNA-LNPs, variability in the location and technique of the immunization, as well as variability in the way that muscle cells are transfected following immunization between test subjects. Please see line 280-282 for further discussion of this point.

29. Why were days 1, 8, and then after a very long pause day 99 chosen? It would appear much more reasonable to choose a closer time point, e.g. day 16-24.

Thanks for the suggestion. We have adjusted the imaging timepoints in our repeat S2P^Δ-eDHFR NHP imaging study to address this comment. Based on this new data, we can see signal decrease to background by day 35.

30. What applies for image visualization for mice in Fig.5B/E is also valid for all NHP imaged (see above).

See above comment (reviewer 2, comment 13), noting that we are showing the full animal MIP image.

31. It is a missed opportunity in the current NHP that the authors chose to not perform ex vivo experimentation as in 3D for the mouse model and/or mixed lymphocyte reactions to assess the NHP T cells' ability to respond to antigen. This should be added to future experiments in NHP is possible through withdrawing blood samples at different time points for this purpose.

We performed an ELISPOT for IFN- γ and IL-2 to look at cellular immune responses in NHPs using N = 2 vaccinated with S2P^Δ-eDHFR and 2 naïve NHPs (Fig. 7).

General comments:

32. P-values should be as numbers rather than just categories/asterisks; best to add instead of the asterisks into each figure panel

The asterisks have been updated to show p-values.

33. Flow cytometry: Please show the full gating strategies used. This should include the position of positive and negative controls and essential information, such as the percentage of cells in the region or gate or event count, and statistics as appropriate. Axis legends should include the marker (e.g. antigen) and the fluorochrome/dye used. For more information how to prepare flow cytometry data analysis for publication please see the "Guidelines for the use of flow cytometry and cell sorting in immunological studies" (<https://onlinelibrary.wiley.com/doi/epdf/10.1002/eji.202170126>). This is relevant for all flow cytometry graphs in this manuscript.

Gating strategies for all flow cytometry experiments have been added to the supplemental.

34. Animals: Methodology reporting should adhere to the ARRIVE 2.0 guidelines (as a bare minimum the ARRIVE 10 but better including the full recommended set; please see here for guidance: <https://arriveguidelines.org/arrive-guidelines>). This enables readers and reviewers to scrutinize the research adequately, evaluate its methodological rigor, and reproduce the methods or findings.

We have ensured ARRIVE 10 reporting.

We thank you for the opportunity to resubmit this manuscript, and look forward for your response.

Here is a point-by-point response to each reviewer's comments for a revised manuscript, entitled "**Monitoring mRNA vaccine antigen expression in vivo using PET/CT**" to *Nature Communications*. Our revisions are described below and nearly all the requested changes have been made. Here is a summary of the key changes to the manuscript:

- We have added two authors, Hooda Said and Quy Cao, since initial submission. A related manuscript file with all authors consent to this change has been included in the submission.
- We have updated the day 35 axial slice to be consistent with day 1 and 7 and added LNP physiochemical data to the manuscript.
- All editorial comments have been incorporated and completed.
- We are supplying 3 Supplemental Movies of the rotating Maximum Intensity Projection (MIP) of the PET images for S2P^{Δf}-eDHFR vaccinated NHP 1 on day 1, 7, and 35. Please see below the reviewer comments for the movie legends. References have been added to the manuscript.

Reviewer #1

The author has solved almost all my concerns through revisions, but there are still some details that need to be added or explained.

1. Fig 6 c, the selected areas of day 35 and day1/day7 are inconsistent. Day35 obviously has no ribs and its shape is inconsistent with the other two pictures.

Thank you for this comment. We have updated the axial slice for day 35 to match day 1 and 7.

2. Discussion, lines 255-256, "As previously mentioned, this may be due to improved surface trafficking afforded by the canonical furin cleavage site present in S2P.", this does not explain that the S2P positivity rate of dendritic cells from the LN of S2P^{Δf}-eDHFR vaccinated mice is lower than that of S2P vaccinated mice, please check carefully.

We hypothesize that the furin site which is a canonical cleavage site present in the spike protein may improve surface trafficking of S2P versus S2P^{Δf}-eDHFR. This is why the S2P positivity rate of dendritic cells from the LN of S2P^{Δf}-eDHFR vaccinated mice is lower than that of S2P vaccinated mice.

3. Lines 380-383, "mRNA-LNPs were characterized for their hydrodynamic size, and polydispersity index (PDI), using dynamic light scattering (DLS), and the encapsulation efficiency and concentration measured using the RiboGreen RNA assay (Invitrogen, R11490)", the author does not seem to describe the characterization information of the prepared vaccine, including particle size, PDI, etc. In fact, the particle size of the vaccine has a certain impact on the immune effect. Different particle sizes may affect the spatial distribution of the vaccine at the injection site.

The physio-chem data for the LNPs used in these experiments was added to the manuscript. The hydrodynamic diameter of these mRNA-LNPs was approximately 71-78 nm with a PDI ranging from 0.05-0.13 of and an encapsulation efficiency of ~97-98%.

4. In line 385, "HEK293T cells transfected with 25 µg of S2P or S2P^{Δf}-eDHFR mRNA...", HEK293T cells were transfected with 25 µg mRNA. How was such a high dose determined? And please describe the transfection method.

This was determined to be the optimal amount of mRNA to transfect the cells with to achieve sufficient signal via Western blot given the relative sensitivity of the Anti-spike antibody that we used in our assays. We had tried previously using 5µg of mRNA and did not detect bands in S2P^{Δf}-eDHFR transfected cells. We have updated the methods section to specify that the mRNA was transfected with Lipofectamine MessengerMax (Invitrogen, LMRNA) according to manufacturer protocol.

Reviewer #3

I have been requested to provide feedback on the revisions made in response to Reviewer #2's concerns. Reviewer #2 raised a total of 34 points, all of which were valid. This reviewer conducted a thorough analysis of the manuscript while maintaining focus on the overall context. The authors have also done an outstanding job addressing each of the 34 points raised by Reviewer #2. As a result, I believe the revised manuscript now meets the high-quality standards expected of a prestigious journal like Nature Communications.

We appreciate this reviewer's recognition of our comprehensive effort to address each of the 34 points raised by reviewer #2 and acknowledgement that the revised manuscript meets the high-quality standards of Nature Communications.

Supplementary Movie captions:

1. Whole body maximum intensity projection of [¹⁸F]FP-TMP uptake in S2P^{Δf}-eDHFR vaccinated NHP 1 at 120 minutes post radiotracer injection on day 1. Movie is scaled from an SUV_{max} of 0-10 and represented on a rainbow color scale in which purple is minimum and red is maximum uptake.
2. Whole body maximum intensity projection of [¹⁸F]FP-TMP uptake in S2P^{Δf}-eDHFR vaccinated NHP 1 at 120 minutes post radiotracer injection on day 7. Movie is scaled from an SUV_{max} of 0-10 and represented on a rainbow color scale in which purple is minimum and red is maximum uptake.
3. Whole body maximum intensity projection of [¹⁸F]FP-TMP uptake in S2P^{Δf}-eDHFR vaccinated NHP 1 at 120 minutes post radiotracer injection on day 35. Movie is scaled from an SUV_{max} of 0-10 and represented on a rainbow color scale in which purple is minimum and red is maximum uptake.

Thank you for the opportunity to resubmit this manuscript, and we look forward to publication.