nature portfolio

Peer Review File

Innate immune responses against mRNA vaccine promote cellular immunity through IFN-β at the injection site

Editorial Note: Parts of this peer review file have been redacted as indicated to maintain the confidentiality unpublished data.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

mRNA-based vaccines are composed of lipid nanoparticles enclosing mRNA. Both components are capable of generating immune responses, but the contribution from each part is poorly understood. Considering the rapid development of mRNA vaccines for preventing various diseases, it is crucial to dissect, at the cellular resolution, the mechanisms underlying the immune response. In the publication, Kim et al. aim to address this challenge and establish a workflow to uncover the early immunological response elicited by mRNA vaccine components at the site of injection.

Using an SARS-CoV-2 S-protein-based mRNA vaccine, they performed two types of injections in mouse muscle – one injection containing empty lipid nanoparticles (LNP) and another with lipid nanoparticles plus mRNA. The researchers performed and analyzed single-cell RNA-seq to uncover the immune response at two different time points: 2 hours and 40 hours post-injection. A PBS injection was also performed as a control. Altogether, the dataset encompasses 5 data points and 2 conditions, along with one control, generating 11 different single-cell RNA datasets.

The authors report the transcriptome of 83,000 cells (line 102) that have been analyzed using a conventional scRNA-seq data analysis workflow, and it is branded as an "injection site atlas." Briefly, the authors revealed the induction of a specific population of fibroblasts containing a high proportion of the spike protein and the mobilization of dendritic cells (DC). The publication addresses a major challenge in better characterizing mRNA vaccines, and the experiments are well-conducted. However, I have major comments on the experimental design of the single-cell experiments and the analysis of the results.

Major comments

1. I expected 11 different conditions to be reported, but it appears that only 10 have been acquired. The absence of Empty LNP at 40 hours post-injection is noticeable. Could the authors provide an explanation for this omission?

2. The number of replicates is crucial information and should be clearly stated for each condition in the single-cell RNA-seq experiments. The authors should demonstrate mice-to-mice variability in supplementary data. The number of replicates needs to be directly added to Figure 1A. If only one mouse has been acquired at a specific time point, or mice have been pooled without addressing variability, additional experiments should be conducted.

3. Figures 1d and 1f should display an integrated UMAP with all conditions, but the boost injections are missing in the panels. In panel 1f, could the author also provide the boost condition?

4. Cellular proportion changes between conditions are presented in Panel 1g using bars. Uncovering statistically different changes in cellular populations requires appropriate tools such as scCODA (PMID: 34824236; Büttner et al. Nat Comm). Otherwise, the analysis may be obscured by confounding factors. The number of cells per condition should not exceed a few hundred (between 500 and 1000 cells), ensuring proportional changes are rigorously assessed with proper statistical tools.

5. To understand how cells are affected by different treatments, the authors performed a PCA analysis with all cell types. While the approach is interesting, using Milo (PMID: 34594043) might be more powerful and better suited to the analysis.

6. In Figure 2c, I am having difficulty understanding why the points are linked together.

7. It appears that the analysis of the boost response has not been done. Could the authors elaborate on this aspect of the story?

Minor comments

- Line 80 and 102: why the number of analysed cells differ between these two lines?

- Gene name nomenclature should be correctly written in italic

Reviewer #2 (Remarks to the Author):

In this study, Kim et al investigated innate immune signaling following SARS-CoV-2 mRNA vaccination using single-cell transcriptome analysis. They identified injection site fibroblasts are enriched with spike mRNA which led to the induction of IFN-b from these cells. They also found that mRNA-LNP triggered migratory (m)-DCs to produce IFN-b at the injection sites and the draining lymph nodes. Furthermore, IFN-b production from these cells enhanced antigen-specific cellular responses. These findings are interesting to the field of mRNA vaccinology. There are several concerns to be addressed:

1.In Fig. 6 and Extended data Fig. 10, the authors showed that IFN-b production at the injection sites enhances antigen-specific cellular immune responses using IFN-g ELISOPT analysis. In the discussion (lines 304-315), the authors further postulated the potential role of mDC- ISGs in promoting CD8 T cell responses- this appear to be overdrawn as it is not supported by ELISPOT data. Whether and how IFN-b- producing fibroblasts and mDCs promote CD4+ and CD8+ T cell responses following vaccination is not clear in this study. Thus, additional data such as IFN-g staining of CD4+ and CD8+ T cell subsets in the vaccinated mice will strengthen this study.

2.Fig. 4g showed ELISA data of sera IFN-b levels at 16h, is there a statistical difference between LNP+mRNA group and LNP or PBS group? The levels of IFN-b in LNP+mRNA is very low, though detectable. Have the authors measured IFN-b levels in the injection site muscle tissue lysates?

3.Fig. 3b: There are no error bars. Are these data representative of one sample per group?

4.Extended data Fig. 8, missing figure legends (PBS, LNP, LNP+mRNA) for all regulons except Nfkb1 and Nfkb2.

Reviewer #3 (Remarks to the Author):

Kim et al perform a detailed analysis in mice of the innate response at the site of injection, in mice immunized with an mRNA-LNP vaccine. Their analysis demonstrates that the LNP component of the vaccine stimulates a pro inflammatory response, whilst the mRNA component stimulates a type 1 IFN response especially in fibroblasts at the injection site, which also harbor the mRNA molecules contained in the vaccine. They further demonstrate that supplementation of IFN-beta with a LNP stimulates a type 1 IFN signature and enhanced CD8+ T cell response, whilst blockade of IFN-beta in mice immunized with LNP-mRNA impairs the induction of antigen specific CD8+ T cells, in line with previous reports.

Taken together these results provide new insights into the mechanisms by which vaccination with mRNA-LNP stimulates innate response at the site of immunization and the impact of this on the ensuing T cell response. The study is well performed and will be of wide interest to the readers of Nature Communications. However the following points need to be addressed. In general many of the figures need to be better described with clearer legends and annotations. For example:

1. Fig 1f: It is not clear what the colored dots in the UMAPs represent.
2. Fig 2e: Not clear what this is meant to show.
3. Please label ALL supplementary figures with the figure numbers
4. Supp Fig 3b: It is not clear what the colored dots in the UMAPs represent.

Response to Reviewers

We thank the reviewers for their valuable feedback. We are grateful that all three reviewers recognized the merit of our study on the early immunological response to mRNA vaccination. Following the reviewers' constructive suggestions, we have conducted additional experiments and analyses to address the questions and concerns they raised. Thanks to this feedback, we believe our manuscript has been strengthened with more robust analysis and additional experimental evidence. Please find the detailed comment-by-comment responses below.

Reviewer #1

The authors report the transcriptome of 83,000 cells (line 102) that have been analyzed using a conventional scRNA-seq data analysis workflow, and it is branded as an "injection site atlas." Briefly, the authors revealed the induction of a specific population of fibroblasts containing a high proportion of the spike protein and the mobilization of dendritic cells (DC). The publication addresses a major challenge in better characterizing mRNA vaccines, and the experiments are well-conducted. However, I have major comments on the experimental design of the single-cell experiments and the analysis of the results.

1. I expected 11 different conditions to be reported, but it appears that only 10 have been acquired. The absence of Empty LNP at 40 hours post-injection is noticeable. Could the authors provide an explanation for this omission?

We appreciate the reviewer's comment. Our study focuses on the early immune responses at the injection site, including the main entry cell types and their gene expression changes within one day after the vaccine injection. We generated 2hr, 16hr, and 40hr LNP-mRNA post-injection (p.i.) samples to assess the dynamics of early immune responses, which confirmed the highest spike-mRNA detection rate at 2hrs (**Fig. 3a**) and the highest number of DEGs and migratory DC responses peaking at 16 hrs (**Fig. 2a**). This reassured that our 2hr / 16hr time point selection is adequate to investigate the early uptake and responses, consistent with previous reports[1,2]. The generation of single-cell transcriptome data of empty LNP injection conditions was confined to these two main time points, limited by budgetary considerations. Following the suggestions from the reviewer's comments #1-4 and #1-5, we revised our analysis by focusing on the cellular alterations shown in 2 and 16 hours p.i. samples.

We have updated our figures and manuscript (**Fig. 1f-i** and **line 104-109** in the revised manuscript).

2. The number of replicates is crucial information and should be clearly stated for each condition in the single-cell RNA-seq experiments. The authors should demonstrate mice-to-mice variability in supplementary data. The number of replicates needs to be directly added to Figure 1A. If only one mouse has been acquired at a specific time point, or mice have been pooled without addressing variability, additional experiments should be conducted.

We fully agree with Reviewer #1's concerns regarding the necessity of controlling variability to ensure robust conclusions. Our research design was strategically devised to first identify biological differences using single-cell transcriptome data in a comprehensive and unbiased manner. Subsequently, we validated these findings using orthogonal experimental methods, including RNA in situ hybridization, ELISPOT, FACS, and ELISA, a choice driven by the budgetary considerations inherent in single-cell analysis. Additionally, we explored the hypothesis that the first and second injections might elicit comparable innate immune responses, thus serving as natural replicates in assessing the early immune response to mRNA vaccines. Supporting this hypothesis, we observed minimal differences in transcriptomic responses and cell type compositions between the prime and boost injections (**Extended Data Figs. 3a and b** in the revised manuscript). Furthermore, the major axes of transcriptomic responses were consistent (**Extended Data Figs. 3c and d** in the revised manuscript).

By considering samples from the same time point and injection type as replicates, we were able to re-analyze our data with more powerful analytical tools such as scCODA, which was suggested by the reviewer's comments #1-4 and #1-5. The renewed analysis clearly revealed infiltration of neutrophils and monocytes accompanied by LNP injection, particularly after 16 hours from the injection (**Fig. 1f,g** in the revised manuscript), and global wave of transcriptomic shifts across various cell types in the injection site (**Fig. 1h,i** in the revised manuscript). Also, to strengthen the findings of this study, we have conducted additional experiments such as ELISA and flow cytometric analysis, which clearly demonstrated mRNA-specific induction of IFN-β (**Fig. 4g** in the revised manuscript) and their contributions to the formation of cytotoxic CD8 T cell responses (**Fig. 6k-n** in the revised manuscript).

To further ensure the reproducibility of our key findings from single-cell transcriptome data, **we have analyzed an independent set of single-cell transcriptome analysis from our on-going project**, which aims to

analyze differences among various ionizable lipid components. We named this dataset the "lipid test." In this study, we tested various ionizable lipid components such as SM-102[3] (CAS No. 2089251-47-6), C12-200[4] (CAS No. 1220890-25-4), in addition to ALC-0315[5] used in the current study and control phosphate buffered saline (PBS) injections. The latter two conditions are identical to the 16hr post injection experiments in the current study, with the same experimental and bioinformatic pipelines used for the analysis.

To evaluate whether the key findings from our study is conserved in these replicate conditions and other conditions where different chemical moieties were used for LNPs, we concatenated the "lipid test" dataset with the dataset used in the current study. From the integrated single-cell transcriptome dataset we successfully identified major cell types (**Fig. 1d,e** and **Appendix Fig. 1a**), and also found prominent immune cell infiltration including monocytes and neutrophils (**Fig. 1g** in the revised manuscript and **Appendix Fig. 1b**). Notably, we found that all of the LNP+mRNA vaccine samples, despite their differences in ionizable lipid components, overlap with each other on the global single-cell transcriptome landscape, and show distinct patterns from PBS or LNP injected samples (**Appendix Fig. 1c**).

One of the major findings in this study is that the mRNA vaccine-induced injection site transcriptional responses can be decomposed into two major axes: PC1 response in stromal cells that features upregulation of inflammatory cytokines, and interferon responsive PC2 response, which is prominent only in LNP+mRNA injections, highlighted in migratory dendritic cells (**Fig. 2b-d** in the revised manuscript). We conducted differential gene expression analysis on each of the cell types in the "lipid test" dataset and projected it onto the PC space using the feature matrix discovered in this study. We discovered that both the PC1 and PC2 responses are robustly observed in all of the LNP+mRNA injection samples, regardless of the chemical moiety in tested ionizable lipid components (**Appendix Fig. 1d,e**). We also found that PC1 responses are found in the stromal cell population, and the PC2 responses are prominent in the migratory dendritic cell (DC) population (**Appendix Fig. 1f**). Furthermore, we discovered that stromal PC1 responses are robustly found in all of the LNP-injected samples, unlike the PBS-injected sample, and the PC2 responses featured in migratory DC population are detected only in the LNP+mRNA injected samples, in accordance with the findings from our study (**Appendix Fig. 1g**).

Next, we investigated transcriptional changes in the fibroblast population. Integration of the fibroblast population from the "lipid test" dataset with the current dataset successfully reconstituted fibroblast subclusters originally identified in our study (**Fig. 3e,f** and **Appendix Fig. 1h,i**). By comparing subcluster compositions across different injection conditions, we found that the injection site fibroblasts in all of the LNP-injected samples showed

significant transcriptional shifts towards Cxcl5^{+} fibroblasts, regardless of the ionizable lipid components (**Appendix Fig. 1j-l**). We also analyzed transcriptional diversities in the migratory DC population (**Appendix Fig. 1m,n**), which revealed LNP+mRNA-specific induction of interferon-responsive migratory DCs (mDC_ISG), across all of the ionizable lipid variants (**Appendix Fig. 1o-q**), as described in this study (**Fig. 5g**).

Overall, we have evaluated the earliest responses at the mRNA vaccine injection site with various ionizable lipid components. Notably, we discovered that either the LNP-induced transcriptional reactions, such as increase in tissue myeloid infiltration and induction of damage-associated fibroblasts[6] (Fib_Cxcl5), or the mRNA-specific responses at the migratory DC (mDC_ISG) are robustly found in all of the tested mRNA vaccine moieties. Since our analysis included major ionizable lipid components widely used in commercial Covid-19 vaccines (ALC-0315 for Bnt162b2 (Comirnaty) from Pfizer and SM-102 for mRNA-1273 (Spikevax) from Moderna[3,5,7]), we believe that the key findings from our study could be translated into a generalized principle of mRNA vaccine adjuvanticity. As we are preparing an independent manuscript using the "lipid test" dataset, we would like to present this only to the reviewers for now, as a further support of the reproducibility of our single-cell analysis.

[redacted]

3. Figures 1d and 1f should display an integrated UMAP with all conditions, but the boost injections are missing in the panels. In panel 1f, could the author also provide the boost condition?

We appreciate this critical comment. Regarding the differences between the primary and boost shots of the vaccine, we have updated our analysis (**Extended Data Fig. 3a-d** in the revised manuscript), and as the reviewer pointed out, we have included layouts showing the overall distribution patterns of primary and boost shot on a single-cell transcriptome landscape (**Extended Data Fig. 3a** in the revised manuscript).

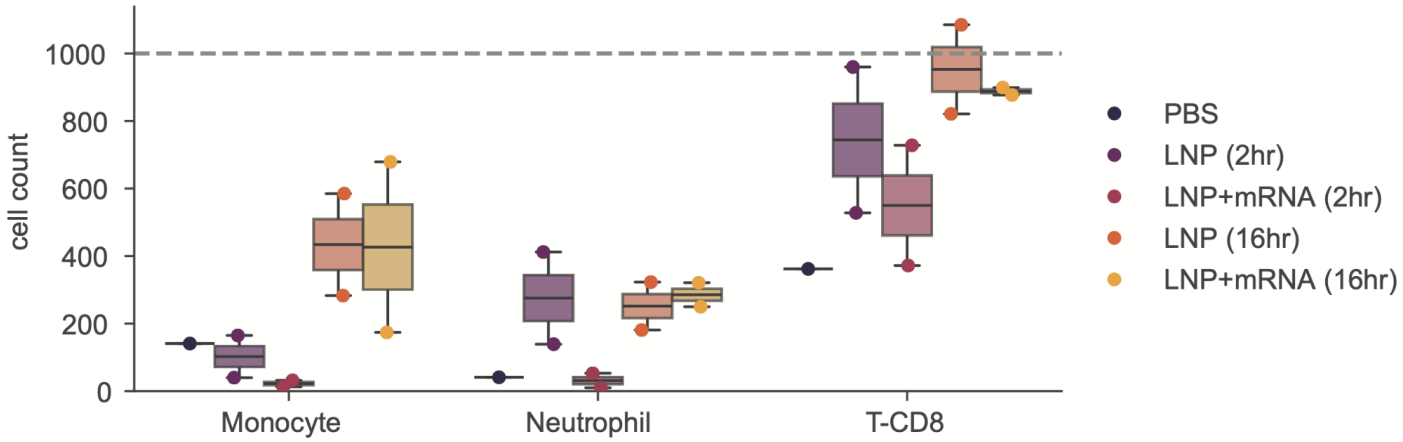
We have updated our figures and manuscript (**Extended Data Fig. 3a** and **line 123-125** in the revised manuscript).

4. Cellular proportion changes between conditions are presented in Panel 1g using bars. Uncovering statistically different changes in cellular populations requires appropriate tools such as **scCODA** (PMID: 34824236; Büttner et al. Nat Comm). Otherwise, the analysis may be obscured by confounding factors. The number of cells per condition should not exceed a few hundred (between 500 and 1000 cells), ensuring proportional changes are rigorously assessed with proper statistical tools.

We are thankful for this helpful and practical comment. Using the Bayesian statistical model the reviewer suggested[8], we systematically compared cell type compositions of vaccine injection and the control samples, which revealed an increase of monocyte, neutrophil and CD8 T cells at the injection site, at least 16 hours after the

injection (**Fig. 1g** and line in the revised manuscript). Also, as the reviewer recommended and the original article of the model suggests[8], we have further checked that the number of cells per condition mostly not exceeded a few hundred cells (**Appendix Fig. 2**).

We have updated our figures and manuscript (**Fig. 1g, Extended Data Fig. 3b** and **line 105-107** in the revised manuscript).



[Appendix Fig. 2] Number of cells from each condition. Raw, un-normalized cell counts from various treatment conditions are displayed on the box plot.

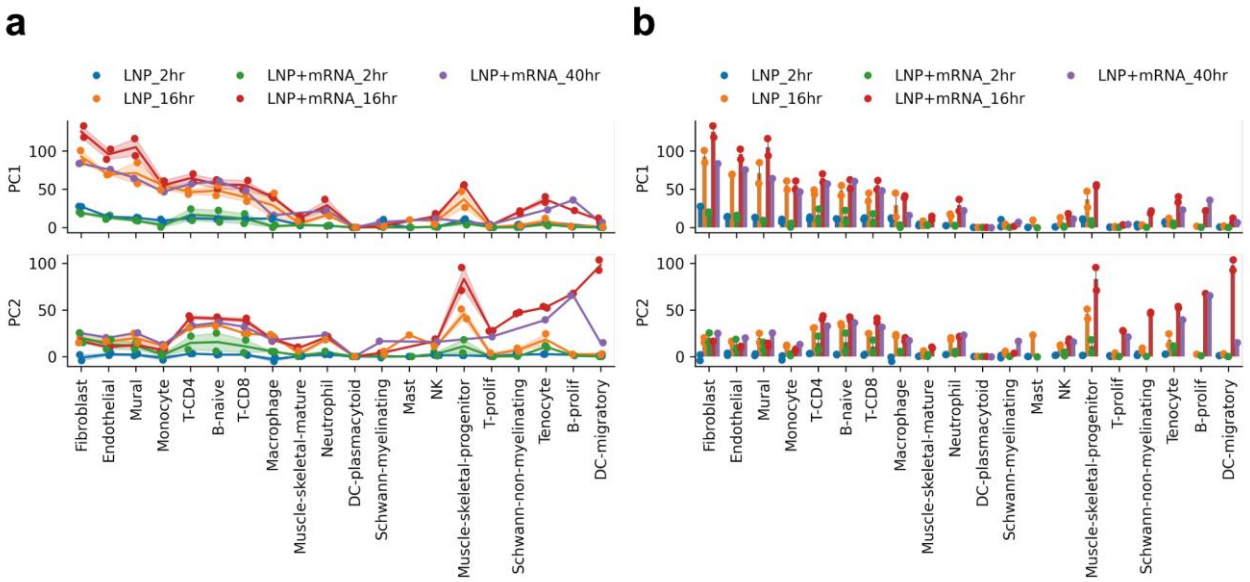
5. To understand how cells are affected by different treatments, the authors performed a PCA analysis with all cell types. While the approach is interesting, using **Milo** (PMID: 34594043) might be more powerful and better suited to the analysis.

We appreciate this highly insightful comment. As the reviewer suggested, we have conducted differential abundance testing on the LNP or LNP+mRNA injected samples using Milo[9], to reveal cell communities highly affected by the injections (**Fig. 1h** in the revised manuscript). The abundance test revealed widespread transcriptional shifts across various cell types, including T cells, B cells, endothelial cells and fibroblasts, both in the LNP and LNP+mRNA injections (**Fig. 1h,i** in the revised manuscript). We also conducted differential abundance testing according to the number of shots given, which yielded no significant differences (**Extended Data Fig. 3c** in the

revised manuscript), thus supporting our finding that the differences between the primary and boost shot at this early time point are not significant.

We have updated our figures and manuscript (**Fig. 1h,i**, **Extended Data Fig. 3c**, and **line 107-109** in the revised manuscript).

6. In Figure 2c, I am having difficulty understanding why the points are linked together.



[Appendix Fig. 3] Comparison of display methods regarding differential PC responses. a,b, PC1 and PC2 projections of cell type DEG vectors from various injection conditions are displayed in (**a**) line plots and (**b**) bar plots.

We are thankful for the thorough review. In this figure, we wanted to (1) display which cell types are mainly responsible for the PC1 and PC2 responses in DEG vectors, and (2) which responses are more affected by the different conditions, especially focusing on the empty LNP vs LNP+mRNA conditions. The cell types are ordered in x-axis based on their contributions in PC1 and PC2 responses, with left-located cell types (such as Fibroblast, Endothelial, etc.) showing strong PC1 response whereas right-located cell types like migratory dendritic cell population accounting for the PC2 response. Because of this feature, we considered the linked dot plots, which results in better visualization of the decoupling PC2 responses of LNP and LNP+mRNA injections at DC-migratory population compared to the bar plot representation (orange and red lines in **Appendix Fig. 3a**; orange and red bars in **Appendix Fig. 3b**)

7. It appears that the analysis of the boost response has not been done. Could the authors elaborate on this aspect of the story?

We appreciate this insightful comment. First, as the reviewer suggested, we displayed overall distribution patterns of primary and boost shot on a single-cell transcriptome landscape (**Extended Data Fig. 3a** in the revised manuscript). Next, we compared the cell type compositions between the primary and boost shot, and scCODA[8] revealed potential increase in CD4 T cells and proliferating B cells in the boost shot samples of LNP and LNP+mRNA injections, respectively (**Extended Data Fig. 3b** in the revised manuscript). Nonetheless, another differential analysis, Milo[9], revealed no consistent alterations in cell communities between the primary and boost conditions (**Extended Data Fig. 3c** in the revised manuscript). Finally, we investigated the vectors of differential gene expressions using the PC weight matrices used in this study (**Fig. 2b,c**), which revealed no substantial variations among the primary and boost shots, compared to the differences originating from the shot compositions (**Extended Data Fig. 3d** in the revised manuscript). Overall, primary and boost shots have no substantial differences in terms of very early responses (less than 16 hours p.i.) at the injection site. We believe that earliest responses at the injection sites are dominated by the vaccine adjuvanticity rather than antigen-specific reactions, as demonstrated by the strong immunogenic responses elicited by LNP injections without mRNAs, and it gives potential explanation for why the boost shot shows no significant boosting in the early phase of innate immune reactions.

We have updated our figures and manuscript (**Extended Data Fig. 3a-d** and **line 123-125** in the revised manuscript).

Minor comments

- Line 80 and 102: why the number of analysed cells differ between these two lines?

We apologize for confusing descriptions. The cell counts in line 80 (from the original manuscript) originally meant to represent the whole amount of single-cell transcriptome data analyzed in this study, including injection site (muscle) samples, which have 83,094 cells, and draining lymph node samples, which includes 8,507 single-cell profiles. The cell counts in the line 102 only counts for the single-cell profiles from the injection site samples. To prevent confusions, we have updated our manuscript.

We have updated our manuscript (**line 80-81** in the revised manuscript)

- Gene name nomenclature should be correctly written in italic

We apologize for incorrect labels.

We have updated our figures in the revised manuscript (**Fig. 1e**, **Fig. 2e,f**, **Fig. 3f**, **Fig.4b,f**, **Fig. 5d**, **Extended Data Fig. 5c,d**, and **Extended Data Fig. 6d** in the revised manuscript).

Reviewer #2

In this study, Kim et al investigated innate immune signaling following SARS-CoV-2 mRNA vaccination using single-cell transcriptome analysis. They identified injection site fibroblasts are enriched with spike mRNA which led to the induction of IFN-b from these cells. They also found that mRNA-LNP triggered migratory (m)-DCs to produce IFN-b at the injection sites and the draining lymph nodes. Furthermore, IFN-b production from these cells enhanced antigen-specific cellular responses. These findings are interesting to the field of mRNA vaccinology. There are several concerns to be addressed:

1.In Fig. 6 and Extended data Fig. 10, the authors showed that IFN-b production at the injection sites enhances antigen-specific cellular immune responses using IFN-g ELISOPT analysis. In the discussion (lines 304-315), the authors further postulated the potential role of mDC- ISGs in promoting CD8 T cell responses- this appear to be overdrawn as it is not supported by ELISPOT data. Whether and how IFN-b- producing fibroblasts and mDCs promote CD4+ and CD8+ T cell responses following vaccination is not clear in this study. Thus, additional data such as IFN-g staining of CD4+ and CD8+ T cell subsets in the vaccinated mice will strengthen this study.

We appreciate this highly insightful comment. As the reviewer suggested, we have conducted IFN-γ staining of T cell subsets in the vaccinated mice. We acquired spleen cells from the vaccinated mice, 2 weeks after the boost shot injection. T cells were stimulated with peptides of spike protein, against which mice were immunized, and the IFN-γ expression of T cells were evaluated with flow cytometry. First, we found a strong induction of CD8 T cell responses against spike proteins in the mRNA vaccine (LNP+mRNA) injected mice (**Fig. 6k** in the revised manuscript). Notably, neutralization of IFN-β at the injection site, which is achieved by co-administration of anti-IFN-β along the vaccine injection, has significantly impaired the amount of spike-specific CD8 T cell responses (**Fig. 6k** in the revised manuscript). We have further examined spike-specific T cell responses against spike proteins in the subunit vaccine immunization strategy, in which LNP was used as an adjuvant. Interestingly, we discovered that co-administration of IFN-β has substantially boosted antigen-specific CD8 T cell responses in the subunit vaccine strategy (**Fig. 6l** in the revised manuscript). For all of the vaccine strategies tested, the antigen-specific T cell responses in the CD8-negative T cell population were shown to be minimal (**Fig. 6k,l**). Previous report on the mRNA vaccine immunization in mice model[1] has reported that ~30% of CD8 T cells and ~1% of the CD4 T cells in spleen were antigen-specific, 3 weeks

after the boost shot. Overall, these results highly support the major finding of our study, which emphasizes the role of injection site IFN-β in the formation of robust antigen-specific CD8 T cell responses.

We have updated our figures and manuscript (**Fig. 6k,l** and **line 279-283** in the revised manuscript).

2. Fig. 4g showed ELISA data of sera IFN-b levels at 16h, is there a statistical difference between LNP+mRNA group and LNP or PBS group? The levels of IFN-b in LNP+mRNA is very low, though detectable. Have the authors measured IFN-b levels in the injection site muscle tissue lysates?

We appreciate this critical comment. To address the issue, we have measured IFN-β concentration in both the injection site and blood sera. Since the transcriptional induction of IFN-β occurred at the earliest phase of vaccine injection (**Fig. 4a,b**), we have collected samples from the earlier time point (4 hours after injections; previously samples were collected 16 hours after injection). Notably, the measured serum IFN-β levels were substantially higher in the 4 hour p.i. samples (~100pg/mL per sample), compared to 16 hours p.i. samples (~2pg/mL per sample) (**Fig. 4g** in the original and revised manuscript). We also measured IFN-β levels at the muscle tissue lysates, and discovered that only the LNP+mRNA injections gave significant increase in the muscle IFN-β levels (**Fig. 4g** in the revised manuscript).

We have updated our figures and manuscript (**Fig. 4g** and **line 212-215** in the revised manuscript).

3.Fig. 3b: There are no error bars. Are these data representative of one sample per group?

We apologize for the incorrect way of data visualization. We have multiple samples per group, and we have revised our figures with error bars.

We have updated our figure (**Fig. 3b** in the revised manuscript).

4.Extended data Fig. 8, missing figure legends (PBS, LNP, LNP+mRNA) for all regulons except Nfkb1 and Nfkb2.

We apologize for the label omissions.

We have updated our figure (**Extended Data Fig. 8d-k** in the revised manuscript)

Reviewer #3

Taken together these results provide new insights into the mechanisms by which vaccination with mRNA-LNP stimulates innate response at the site of immunization and the impact of this on the ensuing T cell response. The study is well performed and will be of wide interest to the readers of Nature Communications. However the following points need to be addressed. In general many of the figures need to be better described with clearer legends and annotations. For example:

1. Fig 1f: It is not clear what the colored dots in the UMAPs represent.

We sincerely apologize for the label omissions. Dot colors represent the origin of the cells on the integrated single-cell transcriptome data landscape.

We have updated our figure with color labels (**Fig. 1f** in the revised manuscript)

2. Fig 2e: Not clear what this is meant to show.

We apologize for the insufficient descriptions. Each dot represents a differential expressed genes (DEG) vector, which consists of log-fold changes of genes compared to the PBS injected samples. X and Y coordinates of each DEG vector represent the PC1 and PC2 projection of the DEG vectors. Although we have provided some biological interpretation of PC1 and PC2 axes (**Fig. 2d** and **Extended Data Fig. 4a-c**), we believe that the projections of the well-known inflammatory cytokine genes (**Fig. 2e** in the revised manuscript) and interferon-responsive genes (**Fig. 2f** in the revised manuscript) could provide more intuitive explanations for PC1 and PC2 axes. For instance, we found that the upregulation of *Il6* and *Tnf*, well-known inflammatory cytokine genes, is prominent on the high-PC1 low-PC2 dots (on the right bottom side in **Fig. 2e**), whereas the upregulation of *Ifit3* and *Oasl1*, typical interferon-responsive genes, is highly noticeable on the low-PC1 high-PC2 spots (on the upper left corner in **Fig. 2f**) .

We have updated our figure with labels on the colorbar (**Fig. 2e,f** in the revised manuscript)

3. Please label ALL supplementary figures with the figure numbers

We apologize for the omissions.

We have updated our figures with figure numbers (**Extended Data Fig 1-10** in the revised manuscript)

4. Supp Fig 3b: It is not clear what the colored dots in the UMAPs represent.

We apologize for the omissions of the labels. The dot colors represent the sample origin of the cells on the integrated single-cell transcriptome data landscape.

We have updated our figure with color labels (**Extended Data Fig. 3a** in the revised manuscript)

References

1. Li, C. *et al.* Mechanisms of innate and adaptive immunity to the Pfizer-BioNTech BNT162b2 vaccine. *Nat. Immunol.* **23**, 543–555 (2022).
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7. Polack, F. P. *et al.* Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *N. Engl. J. Med.* **383**, 2603–2615 (2020).
8. Büttner, M., Ostner, J., Müller, C. L., Theis, F. J. & Schubert, B. scCODA is a Bayesian model for compositional single-cell data analysis. *Nat. Commun.* **12**, 6876 (2021).
9. Dann, E., Henderson, N. C., Teichmann, S. A., Morgan, M. D. & Marioni, J. C. Differential abundance testing on single-cell data using k-nearest neighbor graphs. *Nat. Biotechnol.* **40**, 245–253 (2022).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Altogether, the authors have addressed my comments. The study is extensive and provides an overview of the early response to vaccines at 2 and 16 hr post vaccination. The study is overall well conducted and the authors have provided unpublished data in the rebuttal letter showing the reproducibility of the data.

Reviewer #2 (Remarks to the Author):

The authors have addressed all my prior concerns. I recommend acceptance of the manuscript.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily addressed my comments.