

# **Programmable mRNA Therapeutics for Controlled Epigenomic Modulation of Single and Multiplexed Gene Expression in Diverse Diseases**

Corresponding Author: Dr Charles O'Donnell

**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)

Major comments

Gene regulation using epigenetic editing is considered an innovative approach to overcome various diseases. However, an important issue for successful application to diseases is how to programmatically control the expression of target genes. In this study, Charles et al. presented a novel approach named " Epigenetic Controller (EC) " to control gene expression through epigenetic changes. They showed that, both in vitro and in vivo, ECs modulate the expression levels of one or more genes with a single dose, and in vivo the effects persist for weeks to months. In addition, EC could regulate multiple gene clusters such as CXCL1-8, indicating inflammatory diseases. The results imply that EC may be applicable not only to inflammatory diseases but also to genetic disorders and many sporadic intractable diseases. The methodology is well designed and described. This paper is also new and influential not only in the field of inflammation but also in the field of medicine in general, where patients are waiting for new therapeutic interventions.

Minor comments

The effectiveness of EC in targeting different levels of gene expression, as demonstrated in the paper, is certainly clear. However, in order to regulate multiple gene clusters, it should be taken into account that the effects will not only affect the gene of interest, but also other genes of interest. In this regard, it would be desirable to show directly how non-target genes are regulated by bisulfate analysis. If there is no direct evidence, at least the possibility that ECs regulate non-target genes should be carefully discussed in the Discussion section, even if the regulation of non-target genes may not have a strong effect on the phenotype.

In the Introduction, the authors state that ECs target three types of non-coding regulatory elements: transcription promoters, cis-regulatory-elements (cREs), and DNA loop anchors. To aid the reader's understanding, a schematic and detailed description of how EC works could be added to the figure and its legend. In vivo studies have not clearly described side effects from treatment. It would be desirable to at least indicate or discuss whether side effects such as weight loss and tissue damage are observed with treatment.

Please discuss the importance of selecting or developing a delivery system that is appropriate for clinical applications, citing several reviews that compare their delivery methods to the methods they used in the present study.

Reviewer #3

(Remarks to the Author)

Review of O'Donnell et al.,

Title: Programmable mRNA Therapeutics for Controlled Epigenomic Modulation of Single and Multiplexed Gene Expression in Diverse Diseases

In their manuscript O'Donnell et al. propose a platform for precision epigenomic therapies utilizing mRNA. The rationale

behind therapeutic use of epigenomic controllers is that they allow pre-transcriptional control of many genes at once. This is an interesting approach which potentially allows to treat diseases driven by multiple genes. The use of mRNA to produce epigenomic controllers allows rapid and flexible development of a platform programmable to target different gene clusters. This approach is certainly interesting and opens a new avenue in utilizing mRNA therapeutics, however in my opinion, the data presented in this manuscript lacks evidence to support claims about precision and safety of this platform.

Most of the results presented in this manuscript refer to experiments performed on different cell lines. When in vitro studies are crucial for initial testing and better understanding of specific mechanisms, it is problematic to claim “controlled epigenomic modulation of gene expression in diverse diseases” based primarily on cell culture studies.

#### Major points

1. In figure 2A-C authors analyze transcription inhibition and methylation after targeting different genes with different effectors in different cell lines. To analyze precision of this system it would be important to analyze possible off target inhibition/methylation in these cells. One of the most challenging issues with mRNA therapeutics is targeted in vivo delivery. The system proposed in this manuscript is designed to affect expression of many genes at once increasing a risk of possible off target effect. Therefore, for safety reasons targeted delivery is crucial in this case. How do authors propose to address this issue?
2. In figure 2D authors show long term inhibition of PCSK9 protein expression in serum and its gene promoter methylation in liver after single injection of epigenomic controllers. How do authors explain prolonged effect when mRNA/protein half-life is much shorter than observed ~200 days effect?
3. Section describing preparation of LNP for in vitro transfection lacks a list of specific lipids used. Different LNP formulations may have very distinct transfection efficiency, hence for reproducibility of these results it is crucial to specify which lipids were used. Similarly, for the LNP preparation for in vivo use; in this case authors specified using MC3 lipid, but it lacks description of other lipids used in the mix. Authors described RNA to lipid ratio to be 2:1, when typically, it is 3:1. Hence to make sure, was it indeed 2:1 or perhaps it is a typo?
4. In figure 3B, authors observed the most efficient inhibition across CXCL1,2,3,8 genes when using CXCL-EC2 treatment. However, in panel 3C this treatment was not used in human donor neutrophils. The combination of CXCL-EC1-2 showed modest inhibition of CXCL1 and 2 genes, but robust CXCL1 and 2 protein downregulation. Could authors explain this discrepancy? Additionally, there are no statistics on the protein expression bar (3C, right graph), however, I assume these differences are significant?
5. The study performed in mouse model of asthma lacks information of how mRNA-LNP was delivered and which cells/organs were targeted? As mentioned before, targeted delivery of mRNA therapeutics remains the main challenge. In this study authors aim to epigenetically silence CXCL1-8 gene cluster with “precision targeting of NF-kB signaling”. NF-kB is a critical signaling pathway involved in innate and adaptive immunity. While it would be beneficial to be able to specifically silence it in various disease settings, its overall activity is crucial for organism's homeostasis. Taking this into account, I find it puzzling that in figure 5 authors analyzed only the presence of three immune cell types in bronchoalveolar lavage fluid of treated mice without showing overall impact of this treatment on these mice.

#### Minor points:

1. Most figures/ figure legends lack information regarding n and number of experiments performed.
2. Some figures are small and difficult to read eg. Suppl. Fig 2.
3. In figure 2A, graph on the right contains various columns labeled MQ1 or KRAB – please specify what is difference between these columns.
4. In methods section describing mRNA production, please specify if you are using modified or unmodified mRNA.
5. In figure 5 please specify what ‘DEX’ is referring to.

#### Reviewer #4

##### (Remarks to the Author)

The authors have shown that CXCL1-8 can be co-repressed by delivery of a construct that methylates the promoters of this set of chemokines by repressing the NF-kB promoter/enhancer element. This is an innovative approach that the authors show can significantly reduce chemokine levels induced by IL-1 or LPS in vitro and in vivo. The approach also resulted in a reduction in neutrophil chemotaxis. This is important since suppressing only one of these chemokines is not sufficient to inhibit CXCR1/CXCR2 mediated chemotaxis.

The study is technically valid and appropriately controlled.

A major issue with inhibition of NF- $\kappa$ B-mediated regulation of chemokine expression through delivery of nanoparticles carrying the CRISPR construct that methylates promoters is that it will need to be delivered to specific cell types to be effective. It has been previously that systemic delivery of an NF- $\kappa$ B inhibitor or inhibition of NF- $\kappa$ B specifically in myeloid cells stimulates tumor growth in part by shifting the tumor infiltrating macrophages to an M2-like phenotype. The study described in this report did not examine the effect of the totality of cells effected by delivery of the nanoparticles that methylate the promoters of these CXCL chemokine. It is absolutely essential that other cell types be examined to have a full picture of the pleiotropic effects of these transcriptome modulators.

Version 1:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

Review of O'Donnell et al., 2nd round.

Title: Programmable mRNA Therapeutics for Controlled Epigenomic Modulation of Single and Multiplexed Gene Expression in Diverse Diseases

I would like to start by thanking the authors for addressing the remarks from the first round and for correcting the issues mentioned in the minor points. I would be very interested in reading the publication on the use of these epigenomic controllers in cancer. Is it already available on PubMed?

Regarding the response to major point 1, thank you for addressing the issue of the specificity of your epigenomic controllers. I am interested in knowing which tissues were analyzed by whole-genome methylation sequencing. Additionally, I only see data for Pcsk9-EC, not CXCL-EC. I believe it is important to analyze each of the designed clusters in vivo, as they are intended for therapeutic use.

In my original remark, I was not only concerned with whether these controllers affect off-target genes but also with whether they might affect on-target genes in tissues or cells where they are not intended to act, which could potentially cause serious side effects. For this reason, I respectfully disagree with the authors' opinion that the discussion about the delivery vehicles is outside the scope of this study. I believe it is a crucial aspect, which I elaborate on further in my reply to point 5.

Thank you for clarifying points 2, 3, and 4.

Regarding the response to point 5, thank you for performing experiments to demonstrate targeted lung delivery. In Supplemental Figure 4, the authors showed cell tropism data indicating that the cells expressing tdTomato are airway epithelial cells and also macrophages in the lungs. The image suggests that macrophages predominantly express tdTomato, but since no quantification is provided, I cannot draw a final conclusion. This result suggests that, in the context of a therapeutic experiment using the same LNPs, lung macrophages will receive CXCL-EC, which is designed to inhibit NF- $\kappa$ B signaling. This could potentially cause serious side effects, as the authors mentioned themselves: "[...] dangers of pan- and/or systemic NF- $\kappa$ B inhibition, which has been previously reported to stimulate tumor growth in some cancer models by promoting an M2-like phenotype in tumor-infiltrating macrophages." How do the authors propose to address this concern?

Reviewer #4

(Remarks to the Author)

For the most part the reviewers have addressed my comments--but I would still like to know how the phenotype of lung macrophages was affected by the EC treatment.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

They authors have adequately addressed my comments in the revised version of the manuscript. Therefore, I have no further comments.

Reviewer #4

(Remarks to the Author)

The authors have done a good job of addressing comments except for point 5 raised by the other reviewer. It does appear that the LNP delivery of CXCL1-8 EC is targeting both macrophages and lung epithelial cells since both express tomato. I

think additional work on specificity of delivery to lung epithelial cells and not myeloid cells is clearly needed.

Also, there is an important question to be addressed that I did not bring up in the initial review: It is interesting that the data in figure 5 show nearly equal inhibition of neutrophils and B cells (58% versus 51%) and 41% of the T cell migration into the BALF post LPS challenge was inhibited by the CXCL EC. One would not think that B and T cells were that dependent on CXCR2 mediated migration. CXCR2 expression is very low if detectable in T cells (Idorn M et al *Oncolmunology*, 2018; Liu et al, *Eur J Immunol*, 2020; Jin et al, *Nat Commun*, 2019)) and the Human Protein Atlas shows that only lung and skin B cells express a bit of CXCR2; Could something more be occurring than inhibition of CXCL1-8 or is there an indirect effect such that inhibition of neutrophil CXCR2 leads to reduction in other factors that recruit B and T cells or are other chemokines also altered by the CXCL ECs? It would be important to show that the lung epithelial cells receiving delivery of the LNP - CXCL1-8 EC only show a reduction in CXCL1-8 expression without effecting other T and B cell recruiting chemokines.

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## **Manuscript NCOMMS-23-55459-T point-by-point response**

### **Reviewer #1 (Remarks to the Author):**

#### Major comments

Gene regulation using epigenetic editing is considered an innovative approach to overcome various diseases. However, an important issue for successful application to diseases is how to programmatically control the expression of target genes. In this study, Charles et al. presented a novel approach named " Epigenetic Controller (EC) " to control gene expression through epigenetic changes. They showed that, both in vitro and in vivo, ECs modulate the expression levels of one or more genes with a single dose, and in vivo the effects persist for weeks to months. In addition, EC could regulate multiple gene clusters such as CXCL1-8, indicating inflammatory diseases. The results imply that EC may be applicable not only to inflammatory diseases but also to genetic disorders and many sporadic intractable diseases. The methodology is well designed and described. This paper is also new and influential not only in the field of inflammation but also in the field of medicine in general, where patients are waiting for new therapeutic interventions.

**Response:** We appreciate the reviewer's comprehensive summary and share in enthusiasm on the impact of this approach to a wide range of therapeutic settings.

#### Minor comments

The effectiveness of EC in targeting different levels of gene expression, as demonstrated in the paper, is certainly clear. However, in order to regulate multiple gene clusters, it should be taken into account that the effects will not only affect the gene of interest, but also other genes of interest. In this regard, it would be desirable to show directly how non-target genes are regulated by bisulfate analysis. If there is no direct evidence, at least the possibility that ECs regulate non-target genes should be carefully discussed in the Discussion section, even if the regulation of non-target genes may not have a strong effect on the phenotype.

**Response:** We appreciate the reviewer's questions about the off-target effects from ECs as genomic specificity may be influenced by a number of factors. By design each EC targets only a single epigenomic regulatory DNA element, which in turn modulate a single gene or an intended set of multiple genes. For example, a promoter targeting EC would only directly engage the regulatory chromatin controlling a single gene, targetable cREs that control multiple genes are also identifiable by modeling and experimental data, as is shown in this manuscript. However, to determine whether an EC potentially engaged a non-targeted genomic site, multiple lines of evidence must be integrated. Since natural downstream "on-target" effects can include the same epigenetic change an EC imparts, a genome-wide assessment of differential epigenetic change must be combined with an

assessment of genome-wide transcriptomic change, and interpreted according to directionality and local genomic DNA and regulatory context.

In our revised manuscript, we have analyzed in vivo tissue samples from the Pcsk9-EC study to perform both whole genome methylation sequencing and RNA-seq across time. With such data, potential direct EC effects could be indicated by an observation of concomitant change in methylation and gene transcription, both with directionality consistent with EC effect, at the same genomic locus. Such loci can then be prioritized for additional interrogation to rule out potential downstream “on-target” effects. When comparing all Pcsk9-EC treated animals to PBS-treated controls, modeling timepoint data as a covariate for increased power, we identified only 2 other genic regions outside Pcsk9 that showed an increase in DNA methylation, with only the Pcsk9 gene showing both differential methylation and change in gene expression (Tables 1 and 2). We address these data in the text of the results section on page 6 and in the discussion on page 12. We believe that the genomic specificity of ECs as demonstrated by these data reinforce one of the unique advantages of ECs as potential drugs as compared to other modalities with lower selectivity.

In the Introduction, the authors state that ECs target three types of non-coding regulatory elements: transcription promoters, cis-regulatory-elements (cREs), and DNA loop anchors. To aid the reader's understanding, a schematic and detailed description of how EC works could be added to the figure and its legend.

**Response:** In our revised manuscript, we have added clarity to the legend of Figure 1:

“Gene transcription is controlled by elements such as promoters, cREs, and anchors within Insulated Genomic Domains. A systematic approach based on a library of epigenomic controllers (ECs) allows precision control of gene programs through modulation of epigenetic state. Briefly, epigenomic effectors, chosen from an in-house library, are fused to customized DNA binding domains to form ECs. These ECs target specific elements that control transcription and can be applied to induce a tuned transcriptional response of one or multiple IGD genes.”

In vivo studies have not clearly described side effects from treatment. It would be desirable to at least indicate or discuss whether side effects such as weight loss and tissue damage are observed with treatment.

**Response:** Since mice are not a suitable model for nonclinical toxicology evaluation, we did not examine these animals for toxicity. However, we do appreciate the reviewer’s concerns about potential in vivo side effects from treatment, and we did not observe any overt toxicities in tissues from these animals. We have included text and a supplemental figure to indicate this for the Pcsk9-EC in vivo study on page 6: “Pcsk9-EC was well tolerated without any significant weight loss observed compared to PBS-treated control mice (Supplemental Figure 2), and Pcsk9-EC-treated mice did not exhibit any overt liver tissue damage.” We have also included text and Supplemental Figure 5 to indicate these

observations for the Cxcl-EC2 in vivo study on page 11: “We did not observe any differences in weight in the EC treated group relative to Dexamethasone or LPS controls.” Moreover, we observed an EC-related protective effect from LPS-induced lung damage upon evaluation of lung tissue pathology. We have included this data as text on page 11 and example images in Figure 5B.

Please discuss the importance of selecting or developing a delivery system that is appropriate for clinical applications, citing several reviews that compare their delivery methods to the methods they used in the present study.

**Response:** The reviewer highlights an important part of the drug development process. However, this manuscript is focused on the development of the epigenomic controller platform, and a comprehensive discussion of delivery vehicles falls outside of the scope of this study. Given the importance of delivery, we have included more details about the lipid formulations used in this study, both in vivo and in vitro, in the methods section. On page 10 and in Supplemental Figure 4 of our revised manuscript, we have also included biodistribution data of the LNP used in the MM-Cxcl-EC in vivo study. This LNP arose from an internal screen of commercial and proprietary lipid mixes, and, as shown in the data in Supplemental Figure 4, it yields highly specific delivery to the lung. On page 12 of our revised manuscript, we have also added to our discussion on the appropriateness of LNPs for the delivery of ECs:

“This coupling between genomic and cell-type specificity highlights the importance of a delivery system that is appropriate for clinical applications. LNPs are well suited for epigenomic mRNA therapeutics. First, this pairing enables decoupled PK/PD, conferring durable gene control with only transient potential drug product side effects. Second, it allows redosability to target variable therapeutically beneficial dosing regimes. Further, the maturity of LNP technology and CMC production knowledge, and its well characterized safety profiles, allows for straightforward clinical development paths. While outside of the scope of this study, larger scale formulation screens and associated biodistribution studies remain critical for clinical development of ECs.”

### Reviewer #3 (Remarks to the Author):

Review of O'Donnell et al.,

Title: Programmable mRNA Therapeutics for Controlled Epigenomic Modulation of Single and Multiplexed Gene Expression in Diverse Diseases

In their manuscript O'Donnell et al. propose a platform for precision epigenomic therapies utilizing mRNA. The rationale behind therapeutic use of epigenomic controllers is that they allow pre-transcriptional control of many genes at once. This is an interesting approach which potentially allows to treat diseases driven by multiple genes. The use of mRNA to produce epigenomic controllers allows rapid and flexible development of a platform programmable to target different gene clusters. This approach is certainly interesting and opens a new avenue in utilizing mRNA therapeutics, however in my opinion, the data presented in this manuscript lacks evidence to support claims about precision and safety of this platform.

Most of the results presented in this manuscript refer to experiments performed on different cell lines. When in vitro studies are crucial for initial testing and better understanding of specific mechanisms, it is problematic to claim "controlled epigenomic modulation of gene expression in diverse diseases" based primarily on cell culture studies.

**Response:** We are grateful for the reviewer's appreciation of the breadth of applicability of this approach and recognize that this manuscript is not comprehensive of how Epigenomic Controllers can be used to address all disease processes. We believe the title is appropriate for this manuscript, as we have demonstrated platform translatability in two in vivo settings. First, a CXCL1-8 targeting EC was applied to a common murine model of acute lung injury, demonstrating favorable functional readouts. Second, a liver-focused PCSK9 study demonstrated serum reduction of this hypercholesterolemia factor. Moreover, this platform has been applied directly to the treatment of cancer, with a full description of the pre-clinical development of an EC treating hepatocellular carcinoma accepted for publication in Nature Communication (NCOMMS-23-45655) and a Phase I/II clinical trial (NCT05497453). With the ability to control the levels of one or multiple genes pre-transcriptionally, this approach has and continues to be applied to a broadening number of clinical indications.

#### Major points

1. In figure 2A-C authors analyze transcription inhibition and methylation after targeting different genes with different effectors in different cell lines. To analyze precision of this system it would be important to analyze possible off target inhibition/methylation in these cells. One of the most challenging issues with mRNA therapeutics is targeted in vivo delivery. The system proposed in this manuscript is designed to affect expression of many genes at once increasing a

risk of possible off target effect. Therefore, for safety reasons targeted delivery is crucial in this case. How do authors propose to address this issue?

**Response:** We appreciate the reviewer's questions about off-target effects from ECs as genomic specificity may be influenced by a number of factors. By design each EC targets only a single epigenomic regulatory DNA element, which in turn modulate a single gene or an intended set of multiple genes. For example, a promoter targeting EC would only directly engage the regulatory chromatin controlling a single gene, targetable cREs that control multiple genes are also identifiable by modeling and experimental data, as is shown in this manuscript. However, to determine whether an EC potentially engaged a non-targeted genomic site, multiple lines of evidence must be integrated. Since natural downstream "on-target" effects can include the same epigenetic change an EC imparts, a genome-wide assessment of differential epigenetic change must be combined with an assessment of genome-wide transcriptomic change, and interpreted according to directionality and local genomic DNA and regulatory context.

In our revised manuscript, we have analyzed in vivo tissue samples from the Pcsk9-EC study to perform both whole genome methylation sequencing and RNA-seq across time. With such data, potential direct EC effects could be indicated by an observation of concomitant change in methylation and gene transcription, both with directionality consistent with EC effect, at the same genomic locus. Such loci can then be prioritized for additional interrogation to rule out potential downstream "on-target" effects. When comparing all Pcsk9-EC treated animals to PBS-treated controls, modeling timepoint data as a covariate for increased power, we identified only 2 other genic regions outside Pcsk9 that showed an increase in DNA methylation, with only the Pcsk9 gene showing both differential methylation and change in gene expression (Tables 1 and 2). We address these data in the text of the results section on page 6 and in the discussion on page 12. We believe that the genomic specificity of ECs as demonstrated by these data reinforce one of the unique advantages of ECs as potential drugs as compared to other modalities with lower selectivity.

This manuscript is focused on the development of the epigenomic controller platform, and a comprehensive discussion of delivery vehicles falls outside of the scope of this study. However, we do agree with the reviewer on the importance of delivery, and so we have added to our discussion on the appropriateness of LNPs for the delivery of ECs on page 12 of our revised manuscript.

2. In figure 2D authors show long term inhibition of PCSK9 protein expression in serum and its gene promoter methylation in liver after single injection of epigenomic controllers. How do authors explain prolonged effect when mRNA/protein half-life is much shorter than observed ~200 days effect?

**Response:** We thank the reviewer for pointing out a potentially confusing aspect of EC-mediated therapy. We have added language to help emphasize the distinction and

advantage of ECs in the results section on page 5. This includes highlighting and adding citations on "hit-and-run" kinetics, where the deposition of epigenetic marks persist and continue to modulate gene expression after any LNP-encapsulated mRNA therapeutic would have degraded.

3. Section describing preparation of LNP for in vitro transfection lacks a list of specific lipids used. Different LNP formulations may have very distinct transfection efficiency, hence for reproducibility of these results it is crucial to specify which lipids were used. Similarly, for the LNP preparation for in vivo use; in this case authors specified using MC3 lipid, but it lacks description of other lipids used in the mix. Authors described RNA to lipid ratio to be 2:1, when typically, it is 3:1. Hence to make sure, was it indeed 2:1 or perhaps it is a typo?

**Response:** We thank the reviewer for pointing out a potentially confusing description of the approach. A 2:1 ratio has been developed as a successful process parameter at scales necessary for in vivo studies. Similarly, a 3:1 flow rate ratio has been developed as a success process parameter in an orders of magnitude smaller Spark-based in vitro formulation. Accordingly, we have updated the Methods sections on Preparation of LNPs for in vitro transfection (page 14) and Preparation of LNPs for in vivo dosing of mice (page 15) with a more detailed description.

4. In figure 3B, authors observed the most efficient inhibition across CXCL1,2,3,8 genes when using CXCL-EC2 treatment. However, in panel 3C this treatment was not used in human donor neutrophils. The combination of CXCL-EC1-2 showed modest inhibition of CXCL1 and 2 genes, but robust CXCL1 and 2 protein downregulation. Could authors explain this discrepancy? Additionally, there are no statistics on the protein expression bar (3C, right graph), however, I assume these differences are significant?

**Response:** We thank the reviewer for identifying potential confusion in these panels, along with the clerical omission. We have updated these figures and legends for clarity. Figure 3B highlights the contributions of individual and combined ECs in cell line data. Notably, CXCL-EC1 highly inhibits CXCL8, but with compensatory CXCL1/2/3 up-regulation. CXCL-EC2 induces broad down-regulation across CXCL1/2/3/8, while the combined CXCL-EC demonstrates additive effects. Protein level assessment has been plotted by pg/ml to highlight the difference in levels between CXCL1/2/3/8 upon stimulation, where less activated genes exhibit lower relative levels of inhibition. Figure 3C demonstrates the effects of CXCL-EC in disease-relevant stimulated primary asthma lung fibroblasts.

5. The study performed in mouse model of asthma lacks information of how mRNA-LNP was delivered and which cells/organs were targeted? As mentioned before, targeted delivery of

mRNA therapeutics remains the main challenge. In this study authors aim to epigenetically silence CXCL1-8 gene cluster with “precision targeting of NF-kB signaling”. NF-kB is a critical signaling pathway involved in innate and adaptive immunity. While it would be beneficial to be able to specifically silence it in various disease settings, its overall activity is crucial for organism’s homeostasis. Taking this into account, I find it puzzling that in figure 5 authors analyzed only the presence of three immune cell types in bronchoalveolar lavage fluid of treated mice without showing overall impact of this treatment on these mice.

**Response:** We thank the reviewer (as well as Reviewer #4) for identifying a potentially confusing aspect of the study. In our revised manuscript, we have included more details about the LNP screening performed to identify a lung-specific delivery vehicle (Page 10 and Figure 5). For clarity, our ECs do not systemically inhibit NF-kB signaling, nor do they inhibit the full spectrum of NF-kB downstream targets. Instead, our ECs inhibit gene expression of the Cxcl gene cluster by targeting NF-kB-related regulatory elements found on DNA within that locus. We have added clarifying language on page 11: “Importantly, by selectively inhibiting NF-kB activation of the Cxcl cluster specifically in the lung, we avoid the dangers of pan- and/or systemic NF-kB inhibition, which has been previously reported to stimulate tumor growth in some cancer models by promoting an M2-like phenotype in tumor infiltrating macrophages.”

Minor points:

1. Most figures/ figure legends lack information regarding n and number of experiments performed.

**Response:** We thank the reviewer for pointing out this oversight. Our revised manuscript includes more details about sample sizes and statistics for each figure

2. Some figures are small and difficult to read eg. Suppl. Fig 2.

**Response:** Our revised manuscript contains larger/higher resolution images to ensure reviewability, especially Suppl. Fig 2.

3. In figure 2A, graph on the right contains various columns labeled MQ1 or KRAB – please specify what is difference between these columns.

**Response:** We thank the reviewer for identifying this potentially confusing labeling. Our revised manuscript identifies each column as a separate EC and grouped by MQ1 or KRAB effectors.

4. In the methods section describing mRNA production, please specify if you are using modified or unmodified mRNA.

**Response:** We have clarified the revised manuscript in the methods section on page 14 that “unmodified” NTPs are used in the IVT reaction.

5. In figure 5 please specify what 'DEX' is referring to.

**Response:** In the revised manuscript, we have clarified in the text and in the figure legend that DEX refers to a control treatment: "Dexamethasone, a synthetic glucocorticoid used for the treatment of inflammatory disorders."

**Reviewer #4 (Remarks to the Author):**

The authors have shown that CXCL1-8 can be co-repressed by delivery of a construct that methylates the promoters of this set of chemokines by repressing the NF-kB promoter/enhancer element. This is an innovative approach that the authors show can significantly reduce chemokine levels induced by IL-1 or LPS in vitro and in vivo. The approach also resulted in a reduction in neutrophil chemotaxis. This is important since suppressing only one of these chemokines is not sufficient to inhibit CXCR1/CXCR2 mediated chemotaxis.

The study is technically valid and appropriately controlled.

A major issue with inhibition of NF-kB-mediated regulation of chemokine expression through delivery of nanoparticles carrying the CRISPR construct that methylates promoters is that it will need to be delivered to specific cell types to be effective. It has been previously shown that systemic delivery of an NF-kB inhibitor or inhibition of NF-kB specifically in myeloid cells stimulates tumor growth in part by shifting the tumor infiltrating macrophages to an M2-like phenotype. The study described in this report did not examine the effect of the totality of cells effected by delivery of the nanoparticles that methylate the promoters of these CXCL chemokines. It is absolutely essential that other cell types be examined to have a full picture of the pleiotropic effects of these transcriptome modulators.

**Response:** We thank the reviewer (as well as Reviewer #3) for identifying a potentially confusing aspect of the study. In our revised manuscript, we have included more details about the LNP screening performed to identify a lung-specific delivery vehicle (Page 10 and Figure 5). For clarity, our ECs do not systemically inhibit NF-kB signaling, nor do they inhibit the full spectrum of NF-kB downstream targets. Instead, our ECs inhibit gene expression of the Cxcl gene cluster by targeting NF-kB-related regulatory elements found on DNA within that locus. We have added a clarifying sentence on page 11: “Importantly, by selectively inhibiting NF-kB activation of the Cxcl cluster specifically in the lung, we avoid the dangers of pan- and/or systemic NF-kB inhibition, which has been previously reported to stimulate tumor growth in some cancer models by promoting an M2-like phenotype in tumor infiltrating macrophages.”

## REVIEWER COMMENTS

**Reviewer #3 (Remarks to the Author):**

***Review of O'Donnell et al., 2nd round.***

***Title: Programmable mRNA Therapeutics for Controlled Epigenomic Modulation of Single and Multiplexed Gene Expression in Diverse Diseases***

***I would like to start by thanking the authors for addressing the remarks from the first round and for correcting the issues mentioned in the minor points. I would be very interested in reading the publication on the use of these epigenomic controllers in cancer. Is it already available on PubMed?***

The use of Epigenomic Controllers in cancer is under study in the clinical trial numbered NCT05497453. Preclinical data on this approach has been published in Senapedis W., et al. "Targeted transcriptional downregulation of MYC using epigenomic controllers demonstrates antitumor activity in hepatocellular carcinoma models." Nature Communications (2024), PMID: 39285180 (<https://doi.org/10.1038/s41467-024-52202-y>). We have included this citation on page 2 of the revised manuscript.

***Regarding the response to major point 1, thank you for addressing the issue of the specificity of your epigenomic controllers. I am interested in knowing which tissues were analyzed by whole-genome methylation sequencing. Additionally, I only see data for Pcsk9-EC, not CXCL-EC. I believe it is important to analyze each of the designed clusters in vivo, as they are intended for therapeutic use.***

Whole-genome methylation sequencing was performed on genomic DNA isolated from murine whole liver tissue. In our revised manuscript we further clarify this on page 6.

The specificity analysis was performed on the murine controller Pcsk9-EC (using an LNP) to characterize representative molecular targeting of an EC in vivo, directly assaying liver tissue under physiological conditions over the course of 6 months. This serves as an example of the level of specific targeting achievable using this modality, with a complete analysis performed using multiple genome-wide data sets on pages 6 and 7.

This publication evaluates the effects and the potential of the CXCL-EC drug substance; tested using a tool LNP. As such, this tool composition would not be a therapeutic product. While a representative off-target profile can be shown using a murine Pcs9-EC targeting the endogenous murine Pcsk9 gene in vivo, such a similar experiment would not be possible for the human targeting CXCL-EC in preclinical settings. Even surrogate models likely result in less translatable information. For example, the murine immune system can function differently, and the Cxcl locus does not conserve the same exact genes, which could not be targeted by CXCL-EC, and required

the development of a different design of guide-based tool EC surrogates for validation, MM-Cxcl-EC<sub>1</sub> and MM-Cxcl-EC<sub>2</sub>. Moreover, these treatments use tool LNP formulations rather than a therapeutic grade product.

***In my original remark, I was not only concerned with whether these controllers affect off-target genes but also with whether they might affect on-target genes in tissues or cells where they are not intended to act, which could potentially cause serious side effects. For this reason, I respectfully disagree with the authors' opinion that the discussion about delivery vehicles is outside the scope of this study. I believe it is a crucial aspect, which I elaborate on further in my reply to point 5.***

We appreciate the reviewer's perspective on the importance of LNP tropism and agree that a thoroughly validated LNP would be critical for clinical development of a drug product. Much like other drug modalities, ECs impart their on-target effect to any cell type they are delivered to. Albeit, some EC designs can mitigate off-tissue effects, such as by specifically targeting a cRE that is only active during NF- $\kappa$ B signaling in certain cell types, as CXCL-EC does. (In cell types without an active cRE, there may be no impact on endogenous gene regulation.)

However, the focus of this manuscript is to demonstrate the on-target effects of the LNP payload and translated protein(s) that perform epigenomic control – modular payloads with biological capabilities that could be applied across an entire platform of indications. In vitro and in vivo studies performed in this manuscript utilize a tool LNP. Clinical LNPs remain the focus of active development with several commercial products that have been well-characterized and deployed in clinical trials (Pardi N, et al. J. Control Release 2015; Thi, TTH, et al. Vaccines 2021). We have revised the manuscript to emphasize this focus on drug substance and not clinical drug product in several areas, including changes to the Introduction (p. 3), Results (p. 10), and Discussion (p. 12) sections.

***Thank you for clarifying points 2, 3, and 4.***

***Regarding the response to point 5, thank you for performing experiments to demonstrate targeted lung delivery. In Supplemental Figure 4, the authors showed cell tropism data indicating that the cells expressing tdTomato are airway epithelial cells and also macrophages in the lungs. The image suggests that macrophages predominantly express tdTomato, but since no quantification is provided, I cannot draw a final conclusion. This result suggests that, in the context of a therapeutic experiment using the same LNPs, lung macrophages will receive CXCL-EC, which is designed to inhibit NF- $\kappa$ B signaling. This could potentially cause serious side effects, as the authors mentioned themselves: "[...] dangers of pan- and/or systemic NF- $\kappa$ B inhibition, which has been previously reported to stimulate tumor growth in some cancer models by promoting an M2-like phenotype in tumor-infiltrating***

***macrophages.” How do the authors propose to address this concern?***

In vivo studies in this manuscript are intended to establish proof of concept of the drug substance engaging the designed target in the intended manner using tool LNPs. Off-tissue delivery assessment and biodistribution studies of a clinical LNP would be important steps for specific in-human studies but are beyond the scope of this platform demonstration. For additional clarity, page 11 has been updated to state that CXCL-EC acts by “selectively inhibiting the Cxcl cluster from responding to NF-kB signaling in lung, while not interfering with other essential NF-kB-related processes across the genome, we avoid the dangers of agents with pan- and/or systemic NF-kB inhibition,...”. Therefore, this non-systemic approach, which only targets NF-kB activation of the Cxcl locus, may avoid the M2-like phenotype as previously referenced.

The tissue imaging analysis presented in the manuscript evaluated the overall expression of tdTomato protein in lung tissue after LNP treatment. A qualitative assessment of the sections based on cell morphology suggests high expression of reporter protein in macrophages and airway epithelium (Supplemental Figure 4). The intended design of CXCL-EC is to inhibit overstimulation of the Cxcl locus, and not broad NF-kB signaling, in cell types for which Cxcl locus overstimulation is pathogenic.

Given this targeting approach for Cxcl-EC, it would even be advantageous to transfect immune cells in the lung, as this would further minimize the inflammatory signaling propagated by the Cxcl cluster.

**Reviewer #4 (Remarks to the Author):**

***For the most part the reviewers have addressed my comments - but I would still like to know how the phenotype of lung macrophages was affected by the EC treatment.***

In the referenced in vivo study, we were only able to identify alveolar macrophages in naïve mice, which is in line with publications that describe LPS stimulation as capable of inducing necrosis of these macrophages (Dagvadorj J, et al. Immunity. 2016). As previously seen and confirmed in our study, macrophage frequency decreases to minimal levels in this LPS disease model, which limits any assessment of EC treatment in this population.

**Reviewer #4 (Remarks to the Author)**

***The authors have done a good job of addressing comments except for point 5 raised by the other reviewer. It does appear that the LNP delivery of CXCL1-8 EC is targeting both macrophages and lung epithelial cells since both express tomato. I think additional work on specificity of delivery to lung epithelial cells and not myeloid cells is clearly needed.***

As we previously responded to Reviewer 3, we agree that thoroughly validating the delivery of a final drug product is essential for human trials. However, the development of clinical-grade LNPs remains extensive and we firmly believe that such a study remains out of the scope of this manuscript. We reiterate that this manuscript focuses on the mechanism and capabilities of an mRNA drug substance that can impart specific epigenetic control over gene transcription. In our most recent revision of the manuscript, we have emphasized this concept in the text by clarifying the in vivo studies were performed utilizing tool LNPs, allowing characterization of the precise molecular effects of Epigenetic Controllers, which could be modularly applied across a platform of indications.

***Also, there is an important question to be addressed that I did not bring up in the initial review: It is interesting that the data in figure 5 show nearly equal inhibition of neutrophils and B cells (58% versus 51%) and 41% of the T cell migration into the BALF post LPS challenge was inhibited by the CXCL EC. One would not think that B and T cells were that dependent on CXCR2 mediated migration. CXCR2 expression is very low if detectable in T cells (Idorn M et al Oncol Immunology, 2018; Liu et al, Eur J Immunol, 2020; Jin et al, Nat Commun, 2019)) and the Human Protein Atlas shows that only lung and skin B cells express a bit of CXCR2; Could something more be occurring than inhibition of CXCL1-8 or is there an indirect effect such that inhibition of neutrophil CXCR2 leads to reduction in other factors that recruit B and T cells or are other chemokines also altered by the CXCL ECs? It would be important to show that the lung epithelial cells receiving delivery of the LNP - CXCL1-8 EC only show a reduction in CXCL1-8 expression without effecting other T and B cell recruiting chemokines.***

Immune response in vivo is complex with substantial paracrine signaling. Activated neutrophils will secrete other cytokines that can trigger a broader cascade of immune cells, which can explain the results seen in Figure 8. For example, CXC chemokines such as Cxcl9/10 (Cxcr3 on B- and T-cells) and Cxcl13 (Cxcr5 on B- and T-cells) are expressed by neutrophils (Tecchio C, et al. “Neutrophil-Derived Cytokines: Facts Beyond Expression.” *Frontiers in Immunology* (2014). PMID: 25374568; Lo LW, et al. “Marginal Zone B Cells Assist With Neutrophil Accumulation to Fight Against Systemic *Staphylococcus aureus* Infection.” *Frontiers in Immunology* (2021). PMID: 34040603).

In our revised manuscript, we acknowledge this potential in the results section: “Although B-cells and T-cells commonly lack Cxcr2 expression and would be thought to be unaffected by perturbation of Cxcl1 signaling, we also observed concomitant inhibition of their recruitment into BALF (Figure 8A). Since activated neutrophils are known to secrete other chemokines, we show here that selective inhibition of a key cytokine may have the potential to disrupt paracrine feedback loops to drive broad impact to the immune response in vivo.”