Below please find our detailed point-by-point response to the reviewer comments. The reviewer comments are **in blue** and our responses are **in black**.

Re: JVI00500-19 (Single-cell virus sequencing of influenza infections that trigger innate immunity)  
  
Dear Dr. Jesse D Bloom:  
  
Below you will find the comments of the reviewers. As you will see, both reviewers were very enthusiastic about the work, and both had suggestions for text changes that would improve an already strong manuscript.    
  
*[Detailed information about the re-submission process has been removed from the reviewer response letter to avoid clutter.]*  
  
Thank you for submitting your paper to JVI.  
  
Sincerely,  
Julie Pfeiffer  
Editor, Journal of Virology

Thank you for the careful and rapid review of our manuscript. We were pleased that the reviewers liked the work, and appreciate their helpful suggestions. Below we detail how we have revised the manuscript to incorporate these suggestions.  
  
Reviewer comments:  
  
Reviewer #1 (Comments for the Author):  
  
This manuscript aims to quantify the contribution of influenza virus genetic variation to the observed heterogeneity of virus infection outcome. The experiments first enriched for interferon activated cells (which are rare in normal infections), then infected with well-characterized viral stocks, and finally characterized the full genotypes and transcriptomes of all viral genes present within single cells. This transcriptomic data is used to derive the viral transcriptional burden on cells and to quantify innate immune activation (IFN, ISG) on a per-cell basis. The genotype data provides information on mutations, deletions, and other changes in the viral genome(s) that infected those same cells. The results from these two data sets show that a majority of cells infected by viruses with mutations are IFN+ compared to cells infected by wild type viruses, but this difference was not statistically significant. Further analysis shows statistically significant associations with IFN+ cells and specific mutations, particularly in segments PB1 and NS1. Some of these mutations were engineered into viruses and experiments showed that 5/8 of these mutations increased the percentage of IFN+ cells relative to wildtype, with the greatest effect mutation yielding ~17% IFN+ cells.    
  
This paper presents leading edge methods to investigate the outcomes of viral infections at the single cell level. A major strength of the text is the explanation of the methods, which briefly and very clearly explains the overall methodology with plenty of supplementary information. The presentation of results and data is clear, with increasing levels of detail in the multiple supplements. The data in Figure 4 certainly represent a milestone in the influenza virus field, made all the more impactful by the exquisite presentation. Furthermore, there are followup experiments looking at the mechanisms by which specific viral variants (NS1 and PB1 mutants) stimulate IFN induction. These experiments show in detail that IFN+ can occur directly through mutations in NS1 (which is an IFN antagonist) when viral expression is high or indirectly through changes in polymerase function.    
  
This manuscript could be improved by contextualizing the statistical significance and magnitude of the observed effects with regard to the role of viral genetic variation in the heterogeneity of innate immune activation. In a few sections, some words could be interpreted by readers as an overstatement of the support of the data for this topic. We present specific suggestions (see Major Comment 1) to improve this aspect of the text. The abstract could clarify the background of interferon in the context of influenza infection for readers not familiar with the subject. Finally, a summary figure tracking the overall results (see Major Comment 1) might benefit the reader.  
  
Overall, we find this manuscript highly stimulating and useful for the innovation in the methods. We anticipate some aspects of this paper (esp. Figure 4) will become textbook knowledge in influenza virology. This paper is an exciting contribution to the influenza field, and indeed the broader virology community, which is currently characterizing viral infection dynamics in increasing detail.

We thank the reviewer for the nice summary of the study and its context.  
  
Major comments:  
  
1. One of our major points of our discussion reviewing this paper centered around contextualizing the statistical significance and magnitude of the observed effects with regard to the role of viral genetic heterogeneity in innate immune activation. Particularly given that the activation of IFN is so rare to begin with and that the data seem to support modest effects that are not always statistically significant. We have some suggestions to this effect.  
  
First, some of the language in the discussion and abstract should be qualified to benefit the reader's understanding (e.g. the word "crucial" in Line 374).

This is a good point. We have revised line 374 and several other places in the manuscript to make clear that viral genetic defects do not fully explain the heterogeneity and immune induction, and only some of the genetic defects are validated to actually increase immune induction.

Second, the data in Figure 3G can be used to provide some context showing that viral infection indeed increases the proportion of IFN+ cells, relative to uninfected cells. This result can also be highlighted with a statistical test of these proportions and the discussion could expand on how cell state could influence spontaneous IFN activation.

We have performed a Fisher’s exact test to confirm that the frequency of IFN positivity is much higher among infected than uninfected cells. As the reviewer suspected, the difference is highly significant (P < 10-5), consistent with our interpretation to the data. The results of this test have been added to the text. We have also elaborated on how the rare uninfected but IFN+ cells could have activated IFN due to a non-viral (cellular ligand) as is known to sometimes occur, and have added several citations describing the phenomenon.

Third, it may be helpful to conduct a post-hoc power test of data in Fig 5B to assess whether there was enough power to detect a significant difference between these proportions (0.184 and 0.306) given the sample size imposed by the single cell methodology.

We agree with the reviewer’s sentiment that the reason that the different in Fig 5B was not significant is probably because we had a modest sample size: we strongly suspect the difference would be significant with more cells. Our reasons include the fact that our validation experiments indeed show that some viral defects increase IFN induction, and that the trend *is* significant for certain sub-categories of mutations (as the reviewer nicely describes in his/her summary at the beginning of the review).

However, we have declined to perform a post-hoc power test. The reason is that such tests are considered a statistically suspect way to support the claim that things would have been significant with a larger sample. Specifically, see the following three blog posts for arguments against post-hoc power analyses:

* <https://dirnagl.com/2014/07/14/why-post-hoc-power-calculation-does-not-help/>
* <http://daniellakens.blogspot.com/2014/12/observed-power-and-what-to-do-if-your.html>
* <https://statmodeling.stat.columbia.edu/2018/09/24/dont-calculate-post-hoc-power-using-observed-estimate-effect-size/>

Fourth, it may be worth placing more emphasis on the fact that although the stated NS1 and PB1 mutations were well known to activate immunity, this is (if we are not mistaken) the first characterization at the single cell level. Finally, the discussion should place more emphasis on the fact that although IFN activation is rare, it could affect the course of the entire infection when founding infection size is small.

These are excellent suggestions, and we have modified the Discussion accordingly.

First, we now note that our study is the first to directly show that defects such stochastic absence of NS or mutations to NS or PB1 contribute to IFN induction in single cells.

Second, we have added text to the end of the Discussion about how rare IFN activation could infect the entire course of infection when the founding population is small—in fact, we used almost the exact words suggested by the reviewer, since these very nicely emphasize the point!

Additionally, it would be very useful to have a single figure that summarizes the major results addressing the central question of the paper. This figure could summarize the infected/uninfected proportions, immune activation/genotypes, and specific mutations that impact IFN, thus tracking the broad outlines of the full experiment. Readers may get lost in the rich data and helpful figures, which could dilute the main message of the text.

Thanks for this suggestion; we have seriously considered and discussed adding such a schematic “summary” figure of the results. However, we have ultimately decided against it. The reason is that (as the reviewer describes in his/her summary above), only some of the trends are statistically significant and so we validate the points through a combination of statistics and targeted validation experiments on single mutants. In addition, our data are clearly not sufficient to fully explain why some cells activate interferon. We have therefore been unable to create a summary figure that we aren’t worried is an “over-simplification.” We agree such figure would be nice for a review on the topic, but we can’t craft one that we feel strikes the right balance between accuracy and simplicity for the actual research paper itself.

2. We find Figure 4 a very interesting and effective figure. It is an accomplishment in both experimental methods and data visualization. I anticipate routine use of this figure in the classroom and we expect it will become textbook knowledge in virology.

Thank you for the kind words about this figure; we also think it is very interesting!  
  
3. There are a couple of terms used throughout the paper that could be substituted or clarified to benefit the reader. Viral infection outcomes is used throughout, but it is not always clear what this term refers to. In the context of the paper, the two measured infection outcomes we found were mRNA levels and immune state. If this is the case, "expression and immune state" is only one more word and provides flexibility to refer to one or the other.  

This is an excellent suggestion. We have revised the text in numerous places to use more precise terminology to refer to expression of viral genes or innate-immune activation rather than the vague term “infection outcome.”

The second term is the reference to mutations, deletions, and other modifications from "wild-type" as defects. While this is certainly commonplace in genetics and is convenient shorthand, it does have a connotation of being uncommon and negative. As Figure 4 so exquisitely shows (among many, many other studies), this heterogeneity is probably the norm for influenza viruses. As Brooke (2014) and many others have advocated, as a field we perhaps should be moving towards investigating and recognizing this heterogeneity as a standard part of influenza virus biology.

This is a good suggestion. We have completely eliminated use of the word “wild type” except when we are referring specifically to viral stocks generated in the validation experiment from genes that either match the unmutated (wild type) WSN strain or contain a specific mutant. We think this provides a good balance between avoiding the connotation that mutations are always rare and bad, while also maintaining clarity in specific cases where we have generated defined point mutants for validation experiments.

4. We find it very interesting that the coinfection rate is estimated to be 63% given a rough, "effective" MOI ~ 0.241 (290/1200). While the text explained this could be due to methodological biases, it may be worth indicating if there were any relationships between coinfection and expression or immune activation.

This is an excellent and important suggestion, and Reviewer 2 also suggested something similar. We have added a new Figure S5 that shows the frequency of IFN induction among known co-infected and other cells. There is ***not*** any tendency of co-infection to increase the rate of IFN induction. Therefore, we suspect that either infection is truly non-Poisson (for instance, maybe bigger cells provide a larger “target” for the virus and are more likely to be infected), or co-infection increases the chance that the cell produces enough viral mRNA to meet our detection threshold. However, since we can’t directly test these hypotheses with our data, we have simply slightly elaborated the discussion of this point along with adding Figure S5.   
  
Minor Comments:  
  
Line 18 - This line could be confusing to some readers if they do not have the background (stated well in the Introduction line 43) that influenza virus is very good at preventing IFN induction.

This line should be clearer now as one of the preceding sentences in the abstract has been clearly revised to state that infected cells “… only occasionally activated innate immunity.”

Line 47 - For the introduction, this statement could be written with more accessible language, paralleling the wording in the discussion (Lines 415-422), which is very understandable.

We have revised this wording.

Line 51 - This paragraph could perhaps more appropriately start by discussing NS1's well known role in suppressing IFN, setting a baseline for what the expectation should be. This could better contextualize the results for readers not familiar with influenza virus immunology.

This is a good suggestion: to provide background on how influenza has evolved to evade the innate-immune system in order to better explain why we expect mutations might reduce the efficacy of this evasion. We have added text to the paragraph that explains viral immune-evasion strategies, although we have kept it broader than just focusing on NS1 as other viral proteins are also implicated in immune evasion and activation.

Line 84 - To benefit the reader, the text could indicate (as done in Fig1 Supp4) that the Sendai infection was done at high MOI to validate the IFN activation of cells. As written may not be clear if text is still referring to Steuerman data.

We have ensured that there is no reference to the Steuerman data so that this is not confusing.

Line 109 - May want to spell out IFN negative, instead of IFN-, some readers found it easy to miss the - sign

We have switched the line in question to read “IFN-negative” rather than “IFN-“.

Line 209 - This is a really nice summary of the criteria used to call mutations and a frank assessment of the limitations of the approach

Thanks, we felt it was important to explain the mutation-calling clearly and be upfront about its limitations.   
  
Line 227 - A very nice figure, with a wealth of information, indeed

Thanks, we spent a great deal of time on this figure so are glad that the information was appreciated!  
  
Line 243 - A "favorable outcome for the virus" could be interpreted as increased viral replication, which doesn't necessarily follow from the proportion of viral mRNA expressed in the cell.

This is a good point, we have revised the text to be explicit and say: “high viral gene expression and immune evasion.”

Line 256 - "Also" is confusing in this context. Delete if retains intended meaning, otherwise should be rephrased

We have rephrased the relevant text.

Line 415 - This paragraph is a really good discussion of the importance of IFN+ cells in light of the fact that they are very rare in influenza infection.

Thanks.

Line 522 - This section is a very detailed description of the methods. Highly useful for the community.

Thanks, we hope that by making detailed methods available it will help others extend and apply our approach.  
  
Line 677 - The computational analyses are helpfully described and the full pipeline is open and available in GitHub and explained in several Jupyter Notebooks

Thanks. The computational analysis was a major part of this study, so we wanted to ensure that others can understand what we did and reproduce it if they want.  
  
Reviewer #2 (Comments for the Author):  
  
This is an excellent manuscript describing flu infection and IFN innate immunity at the single cell level. Following flu infection, the authors use cell sorting to enrich their sample in IFN+ cells and perform a transcriptomic analysis coupled with flu sequencing of individual cells. Their results confirm two previous findings: that IFN activation is highly variable among cells and that most flu viral particles carry mutations, including missing segments. Furthermore, the authors study the association between viral mutations and IFN responses at the single-cell level. Their data show that certain mutations in the viral genome make IFN activation more likely, but that this association is highly noisy, such that some cells infected with non-mutated viruses do activate IFN whereas some cells infected with mutated viruses (including mutations in the viral IFN antagonist NS) do not activate IFN. I have no major criticisms and congratulate the authors for their careful experimental design and data analysis. Below are some questions and comments that, in my opinion, are worth discussing.

Thank you for the nice summary of the paper, and the helpful suggestions. Below we describe how we have responded to each of them.  
  
The infection was made such that 25% of the cells were flu positive. This is a relatively high MOI considering that the authors wanted to study individual virus-cell pairs. This complicates mutation calling and data interpretation. Could the authors provide a justification for their MOI choice?

We have added text that answers this question: *“this moderately low MOI reasonably balances our desire to limit the number of co-infections with the cost of performing transcriptomics on uninfected cells.”* As that sentence explains, we were trying to balance keeping a relatively low MOI with the fact that single-cell RNA-seq is expensive on a per-cell basis, so having lots of uninfected cells becomes very costly.

As to whether our MOI was “relatively low” or “relatively high,” this is in the eye of the beholder. We note that the MOI is calculated in terms of infections that lead to “detectable viral transcription” (we are careful to note this in the text), not by plaque assay or TCID50. The fully infectious titers measured by one of those two assays would be lower as some particles that can undergo transcription are not fully infectious. We agree with the reviewer that 25% of flu positive cells is much higher than say 1% to 10% (which we might have chosen if we weren’t worried about cost). But other single-cell RNA-seq experiments on virus infected cells have used massively higher MOIs: for instance, Wang et al (*bioRxiv*, 2018) used at MOI of 5, and Zanini et al (*eLife*, 2018) used a MOI of 10. So compared to these studies, having only 25% of cells is infected is still relatively low.

The problems associated with using a relatively high MOI were worsened by the fact that multiply-infected cells turned out to be more frequent that expected. Specifically, if 25% of the cells were infected, the Poisson-expected fraction of infected cells receiving multiple viral particles was ca. 13.7%. Yet, the authors estimated that this percentage actually rose of 63% in their experiments. Possible reasons for this large deviation are briefly discussed in the results section: (1) that the MACS for selecting IFN+ cells indirectly selected for multiply infected cells; (2) that multiply infected cells were more likely to be identified as flu+; (3) that infection was actually not Poisson. My impression is that the data could actually help the authors discriminate between these alternative explanations. 1. Did cells carrying both synonymous variants expressed higher IFN and ISG levels than those carrying only one variant? 2. Did cells carrying both synonymous variants showed higher flu mRNA levels than those carrying only one variant? I understand that ca. half of the cells carrying only one variant were expected to be multiply infected, but since this fraction was 100% for cells carrying both variants, some differences between these two groups may still be detected if hypotheses 1 or 2 are correct. Concerning hypothesis 3, there are at least two possibilities the authors could discuss. 3a: that a large fraction of cells are not susceptible to infection for some reason; 3b: that a fraction of viral particles are aggregated. 

This is an important point, and Reviewer #1 had a similar comment (major comment 4 of Reviewer #1). As described in the response to that comment, we have ruled out explanation 1 using the data shown in the new FIG S5: co-infected cells are *not* more likely to be IFN+ in our experiment.

We think the explanation is therefore a combination of the explanations (2) and (3). Consistent with the reviewer’s suggestion, the amount of viral mRNA is slightly higher in co-infected cells (21% for known co-infected versus 18% for single-barcode infected), although we cannot directly say if this fully explains the non-Poisson behavior because we cannot quantify the effect that co-infection has on cells below our detection threshold. We also suspect that explanation (3) contributes. In some cases, it could be as simple as some cells being larger than others or growing partially on top of others, and so being more likely to be infected. Recent papers by the reviewer himself (Combe et al, 2015) and the editor of this submission (Aguilera et al, *mBio*, 2017) nicely discusses phenomenon similar to the last two explanation in the context of other viruses. Therefore, in addition to adding the new FIG S5 described at the beginning of the response to this point, we have also added some more discussion of possible explanations that cites these papers.

There appears to be no statistical association or even a positive correlation between IFN activation and viral yield (measured as % mRNAs belonging to the virus). Hence, these data seem to support the view that autocrine responses typically fail to prevent viral spread and that the efficacy of innate immunity against flu depends mainly on paracrine signaling. Could the authors elaborate on this point? 

This is a great point. We suspect that the reason is simply that IFN induction is too late to have an autocrine effect. We have added a sentence that discusses this point: *“Overall, the lack of reduced viral gene expression in IFN+ cells suggests that autocrine IFN signaling typically occurs too late to suppress viral transcription, and the well-known inhibitory effect of IFN against influenza depends mainly on paracrine signaling.”*

Rafael Sanjuán   
  
EDITORIAL NOTE: JVI policy only allows movies and large datasets (e.g., spreadsheets, large phylogenetic trees, etc.) as supplemental information. Please move figures that don't meet this criteria into the main body of the manuscript.

We contacted the editor requesting to retain our supplementary figures because the analysis of our high-dimensional data requires many accessory figures. The editor approved this request, forwarding the following e-mail from the Editor in Chief, Rozanne Sandri-Goldin:

Hi Diane and Julie,

Yes we can make an exception. I agree that the 13 supplemental figures are integral to the work and I would not want readers to have to find this information on a university website. The files on GitHub are large data sets and these must be made available for access on public data bases. The supplemental figures are not large data sets but are important summations of the work in the large data sets. The study involves single cell RNA-seq and generates massive amounts of data that requires the analysis in the body of the work and the supplemental data.

Roz

Note that her e-mail did request that we make the supplemental files available on a public database as well as on GitHub. We have therefore added them to DataDryad (<https://doi.org/10.5061/dryad.nh053c6>) where they will shortly become publicly available, and added a link to this DOI in the legends to the supplemental files.