nature portfolio

Peer Review File

Antiviral CD4+ T and myeloid cell responses to influenza vaccines are attenuated in older adults

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors performed a cohort study on samples derived from young and older adults to assess differences in cellular responses to influenza vaccination aiming at elucidating factors resulting in the remaining high risk for severe influenza-related illnesses in older adults. To this end, they performed a comprehensive immune characterization. While humoral responses to the vaccination were not hampered in the older adults, they found profound differences in the T cell compartment with fewer influenza-specific CD4+ T cell responses and reduced antiviral T cell immunity. Thus, the authors reasoned that age-dependent differences in the transcriptional programming of myeloid and T cells confer reduced anti-viral and vaccination-induced responses.
The manuscript is well written, all methods are described concisely and the authors explain clearly how they came to their conclusions. Related age-dependent changes have been described, but the presented data set is novel and presents an additional aspect of age-dependent immune changes. The presented findings are interesting to all scientists working on improving vaccinations for the elderly and other high-risk groups.
However, there are questions that should be addressed in order to improve the manuscript and round up the narrative:

1. It is striking that older adults had a much stronger response (higher fold increase) against H1N1 as compared to younger adults. This is opposite of the expected. Do you have an explanation for this? The same is true for the B Victoria strain. For both strains the younger adults show almost no response. This should be discussed.
2. 65 years and older is usually the age that individuals are termed as elderly. Why did you choose to call them older adults instead?
3. It might be interesting to have a look at vaccine responders vs. non-responders within the group of the elderly adults. Previous publications have shown that such a closer look might reveal hidden patterns of immune senescence.
4. If I understand the gating strategy correctly, you gated all Th populations from the CD4+ memory population. What was your rational for this? Did you also check these populations within all CD4+ T cells?
5. Within the group of elderly adults, samples from individuals vaccinated with three different types of vaccines were included. Have you checked for differences between the different vaccines that the elderly individuals received? Especially whether the individuals who received the MF59 adjuvanted vaccine are responsible for outliers in the elderly groups observed for some of the shown data sets? It is important to check for this as adjuvanted vaccine-induced responses can differ drastically from non-adjuvanted ones.
6. Do you have any additional information on the health status of the participants? Or on previous vaccinations? This is an important point that should also be included in the discussion. The theory of original antigenic sin and its implication for following influenza encounters and its possible implication for the observed effects should be discussed. What is the median proportion of individuals getting vaccinated against influenza each year? Is it likely that the participants got vaccinated the previous years? Or is it more likely that they were vaccinated inconsistently? Do elderly individuals usually get influenza vaccination more regularly as compared to younger adults?
7. For me it is not clear, what the mass cytometry data was used for! Can you please make sure throughout the manuscript what data is derived from flow and what from mass cytometry? Why were both methods used? What was the incentive behind this? There are a lot of markers in the mass cytometry antibody table that are not described in the manuscript. Can you please elaborate on that?
8. Line 205: You state that similar differences can be observed between young and older adults. Can this be quantified? From simply looking at the color patterns, I would not completely agree that there are similar changes but rather age-specific differences.

9. Line 211: According to figure 4C, older adults have higher frequencies of Th2 cells as compared to younger adults with and without stimulation. Please correct this statement or explain. Additionally, there is no statistically significant difference between CM CD4+ T cells before and after stimulation according to Fig. 4C and Th2 cells appear to be lower after stimulation, please clarify.
10. Line 268: No significant changes between unstimulated and stimulated samples from young adults are shown for CD14 monocytes in the respective figure. Please comment/ correct.
11. Line 313: In the figure SIGLEC14 is shown. Please explain/ correct.
12. Line 358: None of the current influenza vaccines contain alum as adjuvans. Please correct.
13. Line 360: As most vaccines are given at a younger age and influenza vaccines do not usually contain alum, in my opinion this would not explain this shift. Can you maybe elaborate a little more how you come to this conclusion?
14. Line 392: Is there respective information on the health status of the participants that might help to put this into context?
15. 469: What data set was produced using plasma samples?
16. Figure 6: For C and E it would be helpful to add the cell population explanations to the respective axes in order to easier understand what is shown. And also add a label showing which graphs show data from the younger and which from the older adults.

Reviewer #2

(Remarks to the Author)
Jared M. Oakes et al.,
Antiviral CD4+ T and myeloid cell responses to influenza vaccines are attenuated in older adults.

The authors reported the combined in vitro and ex vivo assays with human samples to investigate B, CD4+ T, and myeloid cell responses to influenza vaccine antigens in younger and older adults. Due to the limitations mentioned in the manuscripts authors very well explained the key points.

A lot of experiment work was performed nicely for the manuscript, which is commendable. Overall, the manuscript is written with very abundant experimental details. Nonetheless, the quality of some types of data certainly has little room for improvement but due the limitations authors adhere the present scenario and performed the study.

Questions and queries:
1. Was the sample size statistically powered to detect differences in T-cell and myeloid responses? If yes what basis calculation done?
2. A participants did not receive the same influenza vaccine formulation (standard-dose, high-dose, adjuvanted)?, how might this affect outcomes?
3. Has authors compared same number of participants data for HAI titers and functional AIM assay? If yes, is there any changes?
4. Missing the numbers participants in the figures legends ? Kindly include.
5. What is the mechanistic link between attenuated interferon signatures and impaired T-helper responses?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In my opinion, the manuscript has improved substantially and now meets the standards for publication. I therefore recommend that it be accepted for publication.

Reviewer #2

(Remarks to the Author)
The authors reported the combined in vitro and ex vivo assays with human samples to investigate B, CD4+ T, and myeloid cell responses to influenza vaccine antigens in younger and older adults. Due to the limitations mentioned in the manuscripts authors very well explained the key points. A lot of experiment work was performed nicely for the manuscript, which is commendable. Overall, the manuscript is written with very abundant experimental details.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors performed a cohort study on samples derived from young and older adults to assess differences in cellular responses to influenza vaccination aiming at elucidating factors resulting in the remaining high risk for severe influenza-related illnesses in older adults. To this end, they performed a comprehensive immune characterization. While humoral responses to the vaccination were not hampered in the older adults, they found profound differences in the T cell compartment with fewer influenza-specific CD4+ T cell responses and reduced antiviral T cell immunity. Thus, the authors reasoned that age-dependent differences in the transcriptional programming of myeloid and T cells confer reduced anti-viral and vaccination-induced responses.

The manuscript is well written, all methods are described concisely and the authors explain clearly how they came to their conclusions. Related age-dependent changes have been described, but the presented data set is novel and presents an additional aspect of age-dependent immune changes. The presented findings are interesting to all scientists working on improving vaccinations for the elderly and other high-risk groups.

However, there are questions that should be addressed in order to improve the manuscript and round up the narrative:

1. It is striking that older adults had a much stronger response (higher fold increase) against H1N1 as compared to younger adults. This is opposite of the expected. Do you have an explanation for this? The same is true for the B Victoria strain. For both strains the younger adults show almost no response. This should be discussed.

We expected similar antibody responses based on data from improved vaccines for people over 65. The younger adults in this cohort are repeat influenza vaccinees, and had higher baseline pre-vaccination titers, which explains the reduced fold change in HAI titers post-vaccination.

In response: We have discussed this in lines 125-129 and 566-569.

2. 65 years and older is usually the age that individuals are termed as elderly. Why did you choose to call them older adults instead?

We used the term older adult since it has been used more often in recent publications, and is the recommended terminology according to the current NIH style guide (https://stagetestdomain3.nih.gov/nih-style-guide/age#:~:text=the%20elderly,these%20terms%2C%20ask%20for%20specifics).

3. It might be interesting to have a look at vaccine responders vs. non-responders within the group of the elderly adults. Previous publications have shown that such a closer look might reveal hidden patterns of immune senescence.

We agree and acknowledge that investigating vaccine responders vs non-responders would be very interesting and likely provide additional findings. However, the majority of our individuals would be classified as “responders” by their antibody responses, and that is the current definition of a vaccine responder. As we note later, we attribute this to studying a very healthy

group of individuals. We did not further divide the adults over 65 due to low statistical power and our confidence in any findings from such a small number of individuals.

In response: We highlight the need for further longitudinal studies in older adults with and without frailty and co-morbidities in lines 666-668.

4. If I understand the gating strategy correctly, you gated all Th populations from the CD4+ memory population. What was your rational for this? Did you also check these populations within all CD4+ T cells?

The reviewer is correct that the different Th subsets parent population is memory CD4+ and not all CD4+ T cells. We excluded naïve CD4+ T cells, also sometimes termed Th0, because they have not differentiated into a specific T helper subset.

5. Within the group of elderly adults, samples from individuals vaccinated with three different types of vaccines were included. Have you checked for differences between the different vaccines that the elderly individuals received? Especially whether the individuals who received the MF59 adjuvanted vaccine are responsible for outliers in the elderly groups observed for some of the shown data sets? It is important to check for this as adjuvanted vaccine-induced responses can differ drastically from non-adjuvanted ones.

We investigated whether the adults over 65 who received the adjuvanted vaccine were outliers; they were not. While it is possible that there may be differences between the current vaccines currently approved for older adults, we did not split the group into three groups due to low statistical power and confidence in any findings.

6. Do you have any additional information on the health status of the participants? Or on previous vaccinations? This is an important point that should also be included in the discussion. The theory of original antigenic sin and its implication for following influenza encounters and its possible implication for the observed effects should be discussed. What is the median proportion of individuals getting vaccinated against influenza each year? Is it likely that the participants got vaccinated the previous years? Or is it more likely that they were vaccinated inconsistently? Do elderly individuals usually get influenza vaccination more regularly as compared to younger adults?

This was a population in generally good health. As a condition of study participation, all older adults were living independently. We excluded individuals who were on chronic immune suppression or who had been on corticosteroids for more than 2 consecutive weeks in the 2 months prior to vaccination. Of these individuals, one had controlled type II diabetes, and two met criteria for obesity (BMI $\geq$ 30).

This was also a highly vaccinated population. Of the 15 older adults, all received influenza vaccination the prior year (the 2018-2019 season); nine individuals received HD vaccine, but the dose of vaccine was not documented in the other 6 individuals. 31 of the 32 younger adults received influenza vaccination the prior year.

In response: We added these study participant details to the study cohort description in lines 118-130.

7. For me it is not clear, what the mass cytometry data was used for! Can you please make sure throughout the manuscript what data is derived from flow and what from mass cytometry? Why

were both methods used? What was the incentive behind this? There are a lot of markers in the mass cytometry antibody table that are not described in the manuscript. Can you please elaborate on that?

The mass cytometry was used for high dimensional phenotyping. At the time of the analysis we hypothesized that older adults would have higher frequencies of senescent cells that might be associated with poor humoral or cellular immune responses. However, we found in our cohort that CD4+ senescent cell frequencies were not significantly different between groups (discussed in lines 572-574). Furthermore, both groups generated robust humoral responses to vaccination. However, despite the overall CD4+ phenotypic similarities between groups and comparable serologic responses, older adults had reduced cellular responses to vaccination.

We provide a full list of markers in supplemental table 2. The additional markers were used to identify other cell subsets that yielded insignificant results for this study. Additionally, the mass cytometry panel used in this study was used in prior studies for general immune cell phenotyping.

In response: We reference supplemental data 2 in the results section where we describe the phenotypic analysis (Lines 373-403) and added to the discussion of immune senescence (Lines 577-582)

8. Line 205: You state that similar differences can be observed between young and older adults. Can this be quantified? From simply looking at the color patterns, I would not completely agree that there are similar changes but rather age-specific differences.

This method uses a nearest neighbor search that quantifies the fold change based on an alpha value of 0.1. We agree that there are small areas of difference between the two age groups; however, this type of analysis can be driven by outliers, so we used a more robust metric (sccomp) in Fig 4C and S5D.

In response: We have amended the text in lines 424-428.

9. Line 211: According to figure 4C, older adults have higher frequencies of Th2 cells as compared to younger adults with and without stimulation. Please correct this statement or explain. Additionally, there is no statistically significant difference between CM CD4+ T cells before and after stimulation according to Fig. 4C and Th2 cells appear to be lower after stimulation, please clarify.

We thank the reviewer for the in-depth investigation of our study, and apologize for this error on our part.

In response: We have amended the text in lines 428-433 to match the figure.

10. Line 268: No significant changes between unstimulated and stimulated samples from young adults are shown for CD14 monocytes in the respective figure. Please comment/ correct.

We apologize for the incorrect statement in the text.

In response: We have removed the incorrect text in line 486-489.

11. Line 313: In the figure SIGLEC14 is shown. Please explain/ correct.

We apologize and appreciate the reviewer's detailed review.

In response: We have edited the text in line 532 to remedy this error.

12. Line 358: None of the current influenza vaccines contain alum as adjuvans. Please correct.

and,,,

13. Line 360: As most vaccines are given at a younger age and influenza vaccines do not usually contain alum, in my opinion this would not explain this shift. Can you maybe elaborate a little more how you come to this conclusion?

We agree with the reviewer. Influenza vaccines do not contain alum adjuvant, and most vaccines (including many with alum) are given at a younger age. We originally referenced literature suggesting that a lifetime of exposure to other vaccines containing alum could potentially explain a shift in T cell responses to Th2 phenotype. However, it is more plausible our findings are a consequence of aging itself rather than from cumulative vaccine exposure.

In response: We have deleted this section and revised the discussion in lines 585-592.

14. Line 392: Is there respective information on the health status of the participants that might help to put this into context?

All the adults in this study were generally healthy. As mentioned above, all 65 and older individuals were living independently and ambulatory, and had few aging-associated co-morbidities. we hypothesize that the proinflammatory monocytes present in people over 65 with and without stimulation are likely due to inflammaging in these individuals.

In response: We added detail to the health status of participants in lines 119-134. We discussed this in lines 622-626.

15. 469: What data set was produced using plasma samples?

The plasma samples were used to collect remaining cells not isolated with Ficoll to form a pellet for downstream HLA and KIR typing (Methods lines 238-240).

16. Figure 6: For C and E it would be helpful to add the cell population explanations to the respective axes in order to easier understand what is shown. And also add a label showing which graphs show data from the younger and which from the older adults.

We thank the reviewer for this helpful comment for data visualization and have amended the figure as requested.

Reviewer #2 (Remarks to the Author):

Jared M. Oakes et al.,

Antiviral CD4+ T and myeloid cell responses to influenza vaccines are attenuated in older adults.

The authors reported the combined in vitro and ex vivo assays with human samples to investigate B, CD4+ T, and myeloid cell responses to influenza vaccine antigens in younger and older adults. Due to the limitations mentioned in the manuscripts authors very well explained the key points.

A lot of experiment work was performed nicely for the manuscript, which is commendable. Overall, the manuscript is written with very abundant experimental details. Nonetheless, the quality of some types of data certainly has little room for improvement but due the limitations authors adhere the present scenario and performed the study.

Questions and queries:

1. Was the sample size statistically powered to detect differences in T-cell and myeloid responses? If yes what basis calculation done?

This was designed as an exploratory study and not specifically powered to find differences in antibody or cellular responses between groups. We wouldn't have been able to power our study to detect differences in T cell and myeloid transcriptional responses, since no official thresholds exist for differences between transcriptional profiles at the single cell level. However, in past studies we were able to detect differences in serologic responses between groups of older adults who received high dose vs. standard dose vaccine (Pilkinton et. al. Vaccine 2018) and differences in CD4+ cellular responses among solid organ transplant recipients after SARS CoV-2 vaccination using the same flow cytometry assay (antigen-induced marker assay; Yanis et. al. Transplant Inf. Dis 2021).

2. A participants did not receive the same influenza vaccine formulation (standard-dose, high-dose, adjuvanted)?, how might this affect outcomes?

All younger adults received the same standard dose vaccine formulation. While older adults received different formulations, each of these newer formulations has been shown to enhance antibody and/or cellular immune responses in older individuals and are the current standard of care. Our group has demonstrated improved antibody responses to high dose vaccines in adults 65 and older (Pilkinton et. al. Vaccine 2017 PMID 27919633). All three of these vaccines have been shown to enhance both cellular and antibody immune responses compared to standard dose vaccines in older adults (Li et. al. NPJ vaccines 2021 PMID 41166714). These more immunogenic vaccines are not currently licensed for individuals younger than 65. Despite all older adults in our study receiving a version of these enhanced formulations, and the majority receiving the high dose vaccine, we still found older adults had lower cellular responses compared to younger adults. This strengthens our overall conclusions.

In response: We address this point in lines 564-575.

3. Has authors compared same number of participants data for HAI titers and functional AIM assay? If yes, is there any changes?

And…

4. Missing the numbers participants in the figures legends ? Kindly include

Yes, the reviewer is correct. We evaluated HAI titers and AIM assays on all individuals.

In response: The number of individuals for each figure has been added in each legend.

5. What is the mechanistic link between attenuated interferon signatures and impaired T-helper responses?

In response: We added the following text to the discussion “Stimulation of interferon genes in innate immune cells shapes the differentiation when interacting with T cells (PMID 23332752 & 23178119). ISG expression has also been shown to regulate the effector functions of CD4+ T cells for antiviral immunity (PMID 35091453 & 25790790).”, in lines 629-631