molecular systems biology

Apoptosis and other immune biomarkers predict influenza vaccine responsiveness

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 03 February 2013

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who accepted to evaluate the study. As you will see, the referees find the topic of your study of potential interest and are supportive. They raise however a series of concerns, which should be convincingly addressed in a revision of this study. The recommendations provided by the reviewers are very clear in this regard.

We would also kindly ask you to include the detailed baseline and post-vaccination measurements, including the parameters indicated in Table 1, as 'dataset' in Supplementary information, so that other can reproduce the machine-learning classifier and possibly build upon the dataset.

Please note that supplementary tables with more than 50 rows should be supplied as CSV, tab-delimited text or Excel files (see http://www.nature.com/msb/authors/index.html#a3.2.2.12). Thank you for submitting this paper to Molecular Systems Biology.

Referee reports:

Reviewer #1 (Remarks to the Author):

The manuscript by Furman et al provides a very valuable dataset of describing human immune parameters through several technologies. The strong relevance to vaccinology and medicine is a great strength of the paper. The computational approach in this manuscript is sound and disciplined. I respect that the authors were restrained in machine learning, not to over-interpret the data.

However there is an over interpretation of some of the biological validation data. Overall this is an excellent study that should be published as soon as possible.

In reviewing the paper, the following (largely minor) points were identified. All of these can be addressed by reworking the text, and will enhance the paper.

1. The title is an overstatement. Since the study is only focused on influenza vaccination, and the biomarkers are only in humans, the title should state: "Apoptosis as a biomarker of influenza vaccine responsiveness in humans."

2. Introduction, 2nd paragraph. It must be stated at the beginning previous work has used systems approaches to identify biomarkers or signatures that predict immunity to influenza vaccine (Nakaya et al, NI 2011).

3. Introduction: last paragraph. The authors state that the immune predictors found here are likely to be candidate predictors for other immune responses, and help define the signatures of "immune health." There is no data to support this claim, at present. Furthermore, "immune health" is a broad term - a biomarkers that predicts responsiveness to influenza vaccine may have little or no relevance in predicting responsiveness to another vaccine, or predisposition to asthma or diabetes and, as such, cannot be considered to be a biomarker of "immune health."

4. Figure 2: several previous studies have demonstrated higher pre GMT baseline titers in the elderly (e.g. J Infect Dis. 2010 Nov 1;202(9):1327-37). How do the authors explain their results?

5. Page 5 bottom: "We selected an HA region previously shown to contain epitopes for 5 neutralizing antibodies against an H5N1 influenza strain(13)." Why H5N1? The peptide array was designed for H5N1, but the vaccine was formulated for other strains. How well does the peptide array cover the vaccine strains and influenza virus in general?

6. Page 6, top: "As anticipated, there were significantly more GR within the subjects with low pre-GMT compared to those with high pre- GMT ($P < 0.05$ by t test) (Fig. 3A)." How do the authors define GR here? In the previous figure they define GR as subjects who achieved seroconversion (HAI titers at day 28 >4 fold relative to baseline, in 2 or more of the strains).
There may be a typo: do the authors mean that there were more GR in the subjects with high pre-GMT compared to those with low?

7. Figure 3A: what do the negative numbers on the y axis represent?

8. The classifier, elastic net, was supposedly applied all the way
since page 6, but only mentioned at the end of page 8. It will be very
helpful to summarize the computational strategy at the beginning.

9. The explanation of how the predictive model is formed and cross-validated be greatly expanded, and made, in general terms, accessible to the intended audience. For those who would like to dig deeper into the technical details, better referencing of sources would be beneficial. Specifically, there should be a better explanation of how the feature selection is done for each model. It appears that the most informative factors are selected out of the pool of available paramaters in an automated manner, to generate the model that best fits the data. How is this accomplished? Does the algorithm start with the entire set of parameters and then drills down by throwing out the parameters that do not improve the fit? Or is it seeded with a set of parameters, which is then expanded to include additional regressors that allow to achieve better fit?

10. The mouse experiments must be interpreted with caution. Fas is known to be highly expressed on many activated immune cells (e.g. Germinal center B cells), and the reduced antibody titers in Fas knockout mice cannot be taken as functional validation of the baseline expression of Fas being a negative correlate of antibody titers. It is important to highlight this as a major caveat in the interpretation

Minor points

1. Authors refer to 89 patients; however there are 91 listed in Table 1.
2. The core value of pre-GMT as the universal negative correlate of protection is undermined by the notion that pre-GMT is lower in the elderly population. Authors state (p.5) that pre-GMT is a stronger determinant of vaccine response in young patients.Therefore, it follows that predictive models that include pre-GMT or peptide reactivity factors that associate with pre-GMT have limited applicability to the elderly population. This calls for some discussion or clarification by the authors.
3. It is unclear what the number and width of hexagons on figures 3A and 6A symbolize.
4. Page 7: Authors state: "Thus, we identified two HA peptides that can distinguish individuals with a high versus low pre-vaccine HAI titer and to which high antibody levels are detrimental for the HAI activity in response to influenza vaccination." However, H1_23 has near-zero regression coefficient, and, therefore, does not contribute to the fit of the model (Fig. 4B). Which prompts me to re-iterate my question about how does the
feature selection works in this method.
5. Table S2 is absent from the manuscript, at least I cannot see it.
6. Table S1 should include information on whether the peptide reactivity was increased or decreased in elderly compared to young for each HA peptide.
7. "Regulator activity" panel in the online data should be explained; It would also be informative if the columns in clustering diagrams for gene modules be marked as "young" or"elderly".

Reviewer #2 (Remarks to the Author):

The authors have applied classification and regression techniques to a large dataset of covariates in a study of the humoral response to influenza vaccination. Their results provide novel insights into the correlates of responsiveness under the given circumstances, and perhaps beyond.

There are just a few minor criticisms that should be dealt with.

The main concern is that the authors repeatedly refer to "the antibody response" or "the HAI antibody response" and claim, for example, that they predict this response "with 84%" accuracy. The antibody response is an enormously complicated thing, and the authors clearly don't intend to claim to predict it in detail. What they are predicting, as far as I can tell, is the dichotomous classification according to antibody titers post-immunization. If this is correct, this crucial piece of information needs to be made much more prominent, and certainly made clear whenever the modeling of the response is described. For example, even in the methods, where the model for the logistic regression is described, the dependent variable is not mentioned.

Page 4: "The immune predictors found in this study are likely to be candidate predictors for other immune responses..." Why is that? I suggest the language be toned down to "may be candidate predictors..." or "may have value as predictors..."

Page 5, last complete paragraph: The fact that the older cohort had significantly less pre-GMT than the young cohort does not itself suggest that pre-GMT is a stronger determinant of the vaccine response in young than in older adults. An analysis of correlations would address this question.

Page 6, line 6: Why t-test? It's a test of proportions, right? Why not a contingency table?

Page 7: "none of this was age-related." None of what? Be specific.

Page 8, line 5-6: Again, unclear. What quantities are being examined here? (pre-existing antibodies is not a quantity), and what is meant by "associated with"?

Page 8, "We next tested ... baseline variation ...are indicative..." The variation would not be indicative but the values of these covariates might be. And then the verb would agree, too.

Beyond these details, I urge the authors to revise the paper for clarity. There are many results, and their relevance is not always made clear.

Reviewer #3 (Remarks to the Author):

This is an important paper which has used a "systems biology" approach to identify predictors of

good and poor response to the influenza vaccine in young and elderly humans. This represents an enormous body of work which, although not perfect, is a major contribution to the field.
Whole blood, serum, and PBMC (peripheral blood mononuclear cells) have been analyzed from 30 younger (20-30 years of age) and 61 older (61-greater than 89 years of age). Microarray analysis was done on whole blood. Fifty serum cytokines and chemokines, 15 immune cell subsets and the antibody response after vaccination with the trivalent influenza vaccine were analyzed as well as the cell signaling response to cytokines by phospho Flow (pFlow) for phosphorylated STAT proteins. The patient population was well characterized for conditions known to influence the immune response, e.g. CMV status, previous immunizations etc. (see Table 1). The introduction provides good justification for why this work is important as the elderly do not respond well to vaccines or infections. Thus the paper provides an excellent resource for a molecular systems analysis of the immune system in younger and elderly subjects and several biomarkers are revealed.

Although finally stated very briefly in the discussion, the rationale and justification for using peptide microarrays for detection of influenza-specific antibodies to linear epitopes should be given when they are introduced. This is a good, feasible method to quantify the antibody response but will miss the conformational (and glycosylated) determinants which antibodies naturally predominantly recognize. The fact that these authors WERE able to identify both negative and positive peptide predictors of the HAI response, many of which were age-associated, is a worthwhile/positive contribution of the paper.
The analysis is good in that it presents both aging as a variable and also when it is factored out - as aging is the major predictor of poor response.

Some points for possible correction/clarification:
Presentation of the data for the pre-GMT (geometric mean titer) and a potential hypothesis for higher values of this being associated with poorer sero conversion are not clear for Figure 2 or from the text. Figure 3 is better but young and elderly results were grouped together there - these points should be represented by different symbols. Figure 3B identifies 4 peptides for which pre-vaccine-reactive antibodies are significantly higher in pre-GMT high samples than in pre-GMT low. Presumably the hypothesis is that these are inhibiting a subsequent response (by binding and eliminating those epitopes?) but this is not stated and the significance and hypothesis for this is not clear.
Also are there cases where the positive response to peptides tracks with a good HAI? Fig 3B only showed peptides which track with pre GMT high but these track with a lower fold increase in response.
It is not clear how values in Fig 4B relate to those in Fig 3B. Were H3-5 and BH-14 also analyzed in Fig 4B? Only one, H3_8 seems consistent; it is higher in pre-GMT high (Fig 3B) and in Fig 4B shows a lower fold increase.
The data presented seem clear that apoptosis pathways (APO) are a positive predictor of good response whether aging is left in the model (model 1) or taken out (model 3). It is not as clear why the PROL (regulation of B cell proliferation) pathway is negatively correlated with survival as molecules in this pathway are necessary for plasmablast growth and survival which these authors have previously shown do correlate with good response. The argument for "beneficial self renewal" being necessary for a good response at this point (and in the references cited) seems more appropriate/substantiated for T cells than for B cells.
Data in Fig 6B are mixed for showing APO high is better represented in the 7 peptides predictive of HAI titer in response to vaccine : 4/7 of these are higher in APO high but 3/7 are lower in APO high. Therefore these data are difficult to interpret as supporting the hypothesis that APO high leads to more GR (good responders) via these responses. It is positive that Fig 6A does show that APO high have more GR (51% vs 9% GR in APO low).
On page 11 the key data for serum cytokines should be indicated (Fig 5B), although FasL was not a strong indicator and IL-12p40 just a little better.
Model 3, excluding age - identified a new set of features. Supl Fig 3 should be included in body/ regular Figures of paper. Very interesting data.
For Figures 7A and 7B the titers to vaccine were lower in FasL mutant mice but the conclusion/interpretation is flawed as FasL also affects T cell responses and it would not be surprising that for these reasons the titers are lower (not because of decreased apoptosis).
Discussion
It is clear that they have identified some HA linear epitope peptides for antibody reactivity for existence in the pre-vaccine repertoire but it is not clear how these would explain the negative effect

of baseline HAI titers (on sero conversion) - just give some ideas for this (e.g. do these block stimulation of B cells if these determinants are removed, but this is a little harder to see this for the antibody which sees the whole HA and not just linear determinants like T cells do). Their suggestion that inhibiting the reactivity of 2/4 peptides could improve the influenza vaccine response is not clear as these peptides change from year to year with different vaccines (?)
The discussion could include a better idea of which cells are thought to play a significant role in this as whole blood was analyzed. Are they thinking this is most valid for T cells? At this point it is also not clear what they would recommend to be used to identify poor responders (what molecule would be measured?) and what would the predictive values be (for poor response)?

In conclusion this is a very novel paper and shows association of apoptosis with robust antibody response as well as particular HA linear determinants for which antibody can be measured before vaccination and associates with poor response.

1st Revision - authors' response 01 March 2013

Thank you for sending us the reviewers' very positive comments. They raise many important issues regarding the association between pre-existing antibodies to the influenza vaccine as well as the response to the vaccine. They also comment on the possible implications of our findings and what we should or shouldn't say. All very constructive and helpful in refining and clarifying the interpretation of the data and we appreciate the work they have done. We address each point in our response as well as in the revised manuscript where appropriate.

Please find below our detailed response to the referees' comments after the editorial decision letter, as per the journal guidelines.

Point-by-point reponse

Reviewer #1 (Remarks to the Author):

The manuscript by Furman et al provides a very valuable dataset of describing human immune parameters through several technologies. The strong relevance to vaccinology and medicine is a great strength of the paper. The computational approach in this manuscript is sound and disciplined. I respect that the authors were restrained in machine learning, not to over-interpret the data. However there is an over interpretation of some of the biological validation data. Overall this is an excellent study that should be published as soon as possible.

R: We appreciate the reviewer's observations and comments.

In reviewing the paper, the following (largely minor) points were identified. All of these can be addressed by reworking the text, and will enhance the paper.

1. The title is an overstatement. Since the study is only focused on influenza vaccination, and the biomarkers are only in humans, the title should state: "Apoptosis as a biomarker of influenza vaccine responsiveness in humans."

R: We agree and have changed the title accordingly in the revised manuscript.

2. Introduction, 2nd paragraph. It must be stated at the beginning previous work has used systems approaches to identify biomarkers or signatures that predict immunity to influenza vaccine (Nakaya et al, NI 2011).

R: We thank the reviewer for pointing out this oversight. We now refer to the work of Nakaya et al. (Nat Immunol, 2011)[1] in the revised Introduction (Page 3, line 17): "This 'systems immunology' approach to vaccination has been pioneered in the studies of Sékaly(*8*) and Pulendran(*9*) in analyses

of yellow fever vaccine and more recently, Nakaya et al. in influenza vaccination who used gene expression data from post-vaccination samples to identify differences between responders and non-responders(*10*)."

3. Introduction: last paragraph. The authors state that the immune predictors found here are likely to be candidate predictors for other immune responses, and help define the signatures of "immune health." There is no data to support this claim, at present. Furthermore, "immune health" is a broad term - a biomarker that predicts responsiveness to influenza vaccine may have little or no relevance in predicting responsiveness to another vaccine, or predisposition to asthma or diabetes and, as such, cannot be considered to be a biomarker of "immune health."

R: We agree that immunological health is a broad term that likely has many aspects, but since very little is known, what better place to start than analyzing vaccine responses? This is certainly one measure of immunological health and we suspect that there will be some common features in the variables governing the responses to other vaccines. But we agree that we were stating this too strongly in the original text and so we have modified this in the revised version to state that (Page 4, line 20): "The immune predictors found here may also be important in the response to other vaccines and help to define metrics of immunological health(*13*)."

4. Figure 2: several previous studies have demonstrated higher pre GMT baseline titers in the elderly (e.g. J Infect Dis. 2010 Nov 1;202(9):1327-37). How do the authors explain their results?

R: There are conflicting results in the literature as to whether the young or the elderly have a higher pre-GMT[4-7]. An explanation for these discrepancies has been suggested by Sasaki et al. (2011)[8] who used plasmablast-derived polyclonal antibodies and found greater influenza-specific heterovariant reactivity in antibodies from older versus young vaccine recipients. This degree of cross-reactivity results in a higher dependency between pre-GMT and the response to vaccination, of elderly versus young subjects. This could have a number of implications for vaccinology and suggests differential strategies in young versus older based on the history of circulating strains. To clarify this we have added the following text (Page 15, line 9): "Several previous studies have compared the pre-GMT (baseline titers) to influenza in elderly versus young individuals with no general consensus (*45-48*). These discrepancies have been examined in detail by Sasaki et al. (2011)(*49*) who used plasmablast-derived polyclonal antibodies and found greater influenza-specific heterovariant reactivity of antibodies from older versus young vaccine recipients. Thus, the pre-GMT in the elderly is more dependent on their previous exposure history than that of young subjects. This is relevant for vaccinology as it suggests differential strategies in young versus older subjects based on the history of circulating strains."

5. Page 5 bottom: "We selected an HA region previously shown to contain epitopes for 5 neutralizing antibodies against an H5N1 influenza strain(13)." Why H5N1? The peptide array was designed for H5N1, but the vaccine was formulated for other strains.

R: As mentioned by the reviewer, the 2008-2009 TIV was composed of strains other than H5N1*. However, highly conserved sequences are characteristic for neutralization epitopes on HA across different influenza virus strains including those used here[9]. Furthermore, for peptide microarray construction we screened reactivity to each vaccine strain (H1N1, H3N2, B) for an HA region to which broadly neutralizing antibodies to H5N1 have been reported[10]. We have modified the main text on Page 6, line 14 as described in the next response (below).
*These are: A/South Dakota/06/2007 (A/Brisbane/59/2007(H1N1)-like virus), A/Uruguay/716/2007 (A/Brisbane/10/2007(H3N2)-like virus), and B/Florida/4/2006.

How well does the peptide array cover the vaccine strains and influenza virus in general?

R: Our peptide microarray technology is able to print 144 unique peptides per array (sample) in tripicate (432 total spots), which corresponds to a region of 237, 231 and 257 aminoacids for the H1N1, H3N2 and B strains. Thus, our peptide array covered ~40% of the HA protein sequence. In order to increase the likelihood of positive findings we focused on a region of a H5N1 protein to

which broadly neutralizing activity has been reported[10]. To observe aminoacid-level changes and fully cover the HA protein sequence for all 3 vaccine strains one would need to scale up to several hundreds of peptide spots. This could be achieved by using silicon-based peptide arrays as recently reported by Price et al. (2012)[11].
We have modified the text as follows (Page 6, line 13): "Our peptide microarray technology is able to cover ~40% of the HA protein sequence for each vaccine strain. Thus, to maximize the number of positive hits, we selected an HA region found in an H5N1 influenza strain containing epitopes for neutralizing antibodies against different influenza strains (broadly neutralizing antibodies)(*14*)."

6. Page 6, top: "As anticipated, there were significantly more GR within the subjects with low pre-GMT compared to those with high pre- GMT ($P < 0.05$ by t test) (Fig. 3A)." How do the authors define GR here? In the previous figure they define GR as subjects who achieved seroconversion (HAI titers at day 28 >4 fold relative to baseline, in 2 or more of the strains).
There may be a typo: do the authors mean that there were more GR in the subjects with high pre-GMT compared to those with low?

R: For all the analyses here, good responders (GR) were defined by whether the subject made a good response (4-fold or greater increase in) for two or more vaccine strains, as described in Methods and illustrated in Fig. 2A and B. We find more GR in the pre-GMT low compared to high, as many others have described, and this was our major motivation for the analysis comparing peptide reactivities at baseline with pre-GMT. There was no typo or a change in criteria.

7. Figure 3A: what do the negative numbers on the y-axis represent?

R: We apologize for the confusion. The y-axis of Figure 3A corresponds to standardized levels of pre-GMT (z-score). The figure label has been modified to "pre-GMT (z-score)".

8. The classifier, elastic net, was supposedly applied all the way
since page 6, but only mentioned at the end of page 8. It will be very
helpful to summarize the computational strategy at the beginning.
R: We thank the reviewer for this suggestion. We have now added the following text (on Page 7, line 7): "To do so, we used the 'elastic net'(*15*) with 5-fold cross-validation, a machine learning procedure that enables the discovery of relevant features from high-dimensional data and estimates the performance of the resulting prediction model, such that the most informative factors are selected out of the pool of available parameters in an automated manner, to generate the model with the lowest error. In 5-fold cross-validation, the samples are randomly partitioned into five sets and four sets are used to "train" the algorithm and blindly predict the outcome of the fifth set (test set). This process is repeated iteratively five times until all sets are tested. Then a cross-validated area under the ROC curve (cvAUROC) can be computed to assess the goodness of the model without the known problem of overfitting."

9. The explanation of how the predictive model is formed and cross-validated be greatly expanded, and made, in general terms, accessible to the intended audience.

R: Addressed above (point 8).

For those who would like to dig deeper into the technical details, better referencing of sources would be beneficial. Specifically, there should be a better explanation of how the feature selection is done for each model. It appears that the most informative factors are selected out of the pool of available parameters in an automated manner, to generate the model that best fits the data. How is this accomplished? Does the algorithm start with the entire set of parameters and then drills down by throwing out the parameters that do not improve the fit? Or is it seeded with a set of parameters, which is then expanded to include additional regressors that allow to achieve better fit?

R: This comment was partially addressed above (point 8). In addition, we have provided (revised Methods section, page 28, line 18) additional details of the methods and references as follows:
"*Cross-validation and feature selection for prediction of antibody response to vaccination*

An integral part of our training algorithm is a procedure for fitting a logistic regression model with *l1* and *l2* penalties, the elastic net penalty, a regularization algorithm that uses cyclical coordinate descent in a pathwise fashion as described(*15, 72*). The optimization cost can be stated as:

$$1/n \sum_{t=1}^{n} \log(1 + \exp(-y_t(\beta^T x_t + \alpha))) + \lambda \sum_{i=1}^{p} |\beta_i| + \kappa \sum_{i=1}^{p} \beta_i^2,$$

where *n* is the number of donors in the sample, *p* is the number of predictors, *x*t denotes a vector of predictor values for subject *t* and *yt* is observed outcome (poor versus good responder based on the seroconversion to 0-1 or 2-3 strains respectively)."

10. The mouse experiments must be interpreted with caution. Fas is known to be highly expressed on many activated immune cells (e.g. Germinal center B cells), and the reduced antibody titers in Fas knockout mice cannot be taken as functional validation of the baseline expression of Fas being a negative correlate of antibody titers. It is important to highlight this as a major caveat in the interpretation

R: We agree with the reviewer's comment and have modified the text accordingly. The Fas-deficient *lpr* mice have several defects in the T cell and B cell compartments and defective apoptosis via Fas, activation-induced, as well as the pathway involving FcγRII[14-16]. Though, conflicting results have been reported for the *in vivo* B cell response[17,18]. Our results are in agreement with those of Yakahashi et al. (2001) who demonstrated that these mice have defects in clonal selection and the establishment of the memory B cell repertoire[18]. The main text (Page 15, line 20) has been modified as follows: "It is known that the *lpr* mice have defects in T cell and B cell development. Thus our results might not be due to the apoptosis defect in these mice (although this is suggested by the apoptosis gene module) but these other factors. However, there is no agreement as to whether the GC formation and B cell responses are normal (*50, 51*). In particular our results argue against a previous study that reported that memory and antibody-forming cell populations appear to be normal in *lpr* mice(*51*). However, more recent studies have shown that these mice have defects in clonal selection and the establishment of the memory B cell repertoire(*50*)."

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Minor points

1. Authors refer to 89 patients; however there are 91 listed in Table 1.

R: We apologize for the confusion; only 89 of 91 subjects' serum specimen sets (pre and pos-vaccine) were complete for HAI testing. We have modified the text as follows (Page 5, line 3): "We carried out an influenza vaccine study in 91 young and older ambulatory subjects to better characterize immune parameters from peripheral blood that associate with vaccine responsiveness. 89 individuals' serum specimen sets (pre- and post-vaccine) were complete for the assays performed here."

2. The core value of pre-GMT as the universal negative correlate of protection is undermined by the notion that pre-GMT is lower in the elderly population. Authors state (p.5) that pre-GMT is a stronger determinant of vaccine response in young patients. Therefore, it follows that predictive models that include pre-GMT or peptide reactivity factors that associate with pre-GMT have limited applicability to the elderly population. This calls for some discussion or clarification by the authors.

R: We appreciate this observation as it helps us to clarify this point. We have conducted new analysis to address this (shown in Supplementary Fig. 1 of the revised Results section). The conclusion that pre-GMT is a stronger predictor of vaccine response in young individuals does not mean that it has no predictive value in the elderly. We have followed reviewer 2's suggestion (below) in: "Page 5, last complete paragraph: The fact that the older cohort had significantly less pre-GMT than the young cohort does not itself suggest that pre-GMT is a stronger determinant of the vaccine response in young than in older adults. An analysis of correlations would address this question" and have conducted new analysis for comparison of pre-GMT between GG and PR in

young and older separately (Supplementary Fig. 1 of revised Results section). While larger differences are observed in younger individuals, we observe significant differences in pre-vaccine HAI titer between GR and PR in older subjects (Supplementary Fig. 1 of revised Results section). Therefore, it is relevant to include pre-vaccine geometric mean titer (pre-GMT) in the prediction models for the older group. We refer to these new results in Page 6, line 2: "The older cohort had significantly less pre-GMT than the young cohort ($P = 0.0017$) (Fig. 2C), which suggests that pre-GMT is a stronger determinant of the vaccine response in young than in older adults. To address this in detail we compared the pre-GMT levels in PR versus GR in young or older individuals. Effectively, the differences between PR and GR were more pronounced for the young ($P = 0.005$) than for the older group ($P = 0.039$) (Supplementary Fig. 1)."

3. It is unclear what the number and width of hexagons on figures 3A and 6A symbolize.

R: The hexagons represent unique samples. To clarify this, we have incorporated in legend to Figure 3A: "Individuals were sorted by pre-GMT levels and divided into high or low pre-GMT (A) and reactivities against HA peptides were compared (B)" and for Fig. 6A: "Individuals were sorted and divided by expression levels of genes in the APO module (A) and reactivity to HA peptides was compared between individuals with high or low gene expression (B)"

4. Page 7: Authors state: "Thus, we identified two HA peptides that can distinguish individuals with a high versus low pre-vaccine HAI titer and to which high antibody levels are detrimental for the HAI activity in response to influenza vaccination." However, H1_23 has near-zero regression coefficient, and, therefore, does not contribute to the fit of the model (Fig. 4B). Which prompts me to re-iterate my question about how does the feature selection works in this method.

R: What we intended to say in Page 7, is that the reactivities against H1_23 and H3_8 were strongly associated with the levels of pre-GMT and computationally selected as predictors of vaccine response both in Model 1 (which included age) and Model 3 (where age is forced to be removed from the model), which suggested to us that these peptide predictors could be important for vaccine response. To make this more clear we have modified the text as follows (Page 8, line 2): "Thus, we identified two HA peptides that can distinguish individuals with a high versus low pre-vaccine HAI titer and to which high antibody levels are negatively associated with the HAI activity in response to influenza vaccination"
The reviewer's concern regarding the method used has been addressed in point 9 (Reviewer 1, major points).

5. Table S2 is absent from the manuscript, at least I cannot see it.

R: Table S2 was submitted as a separate file, we do not know why the reviewer might not have received it. It is now submitted following the journal guidelines for Supplementary Material.

6. Table S1 should include information on whether the peptide reactivity was increased or decreased in elderly compared to young for each HA peptide.

R: Thank you, we have updated Table S1 with the information suggested by the reviewer.

7. "Regulator activity" panel in the online data should be explained; It would also be informative if the columns in clustering diagrams for gene modules be marked as "young" or "elderly".

R: We thank the reviewer for this suggestion and have added labels (empty bars for young, black bars for older) in clustering diagrams for gene modules' "regulatory programs"; Supplementary Fig. 2 in the new version.

Reviewer #2 (Remarks to the Author):

The authors have applied classification and regression techniques to a large dataset of covariates in

a study of the humoral response to influenza vaccination. Their results provide novel insights into the correlates of responsiveness under the given circumstances, and perhaps beyond.

There are just a few minor criticisms that should be dealt with.

The main concern is that the authors repeatedly refer to "the antibody response" or "the HAI antibody response" and claim, for example, that they predict this response "with 84%" accuracy. The antibody response is an enormously complicated thing, and the authors clearly don't intend to claim to predict it in detail. What they are predicting, as far as I can tell, is the dichotomous classification according to antibody titers post-immunization. If this is correct, this crucial piece of information needs to be made much more prominent, and certainly made clear whenever the modeling of the response is described.

R: We agree with the reviewer that the antibody response implies an intricate process that we do not intend to predict as a whole. We employ the HAI assay because it is a direct measure of antibodies produced *in vivo* against viral HA proteins in response to infection or vaccination and and is generally accepted as the gold-standard for gauging the effectiveness influenza vaccines. We have incorporated the following text (Page 5, line 10): "We used the HAI assay because it is a direct measure of antibodies produced *in vivo* against viral HA proteins in response to infection or vaccination and is generally accepted as the gold-standard for gauging the effectiveness influenza vaccines."

For example, even in the methods, where the model for the logistic regression is described, the dependent variable is not mentioned.

R: We have incorporated additional details in response to Point 9 of reviewer 1 and updated the Method section, and the dependent variable is now mentioned as follows (Page 28, line 21

"The optimization cost can be stated as:

$$1/n \sum_{t=1}^{n} \log(1 + \exp(-y_t(\beta^T x_t + \alpha))) + \lambda \sum_{i=1}^{p} |\beta_i| + \kappa \sum_{i=1}^{p} \beta_i^2,$$

where *n* is the number of donors in the sample, *p* is the number of immune predictors, *x*t denotes a vector of predictor values for subject t and *y*t is observed outcome (poor versus good responder based on the seroconversion to 0-1 or 2-3 strains respectively)."

Page 4: "The immune predictors found in this study are likely to be candidate predictors for other immune responses..." Why is that? I suggest the language be toned down to "may be candidate predictors..." or "may have value as predictors..."

R: We agree with this. We have addressed this point above (point 3, Reviewer 1).

Page 5, last complete paragraph: The fact that the older cohort had significantly less pre-GMT than the young cohort does not itself suggest that pre-GMT is a stronger determinant of the vaccine response in young than in older adults. An analysis of correlations would address this question.

R: We thank the reviewer for this suggestion. This point has been addressed above (point 2 minor points, Reviewer 1).

Page 6, line 6: Why t-test? It's a test of proportions, right? Why not a contingency table?

R: We thank the reviewer and agree with this suggestion. We have conducted Fisher's exact test for data depicted in Fig. 3A as well as those shown in Fig. 6A.

Page 7: "none of this was age-related." None of what? Be specific.

R: We thank the reviewer for highlighting this typo and apologize for the confusion. We have modified the text in Page 7, line 21 as follows: "Of note, two of the peptide predictors identified in Model 1 (H1_23, NH2-FALSRGFGSGIINSNAPMD-COOH and H3_8, NH2 SSGTLEFNNESFNWTGVTQ-COOH) were significantly more prevalent in subjects with high pre-GMT (Fig. 3B) and neither of these peptide predictors were age-associated (see below)"

Page 8, line 5-6: Again, unclear. What quantities are being examined here? (pre-existing antibodies is not a quantity), and what is meant by "associated with"?

R: We appreciate this criticism and have modified the text in Page 8, line 22: "These findings demonstrate that the levels of some pre-existing antibodies targeting viral HA peptides correlate with pre-vaccine HAI titers…"

Page 8, "We next tested ... baseline variation ...are indicative..." The variation would not be indicative but the values of these covariates might be. And then the verb would agree, too.

R: We have modified the text as follows (Page 9, line 6): "We next tested the hypothesis that the baseline levels of immune parameters and subjects' characteristics are indicative of an ability to respond to vaccination"

Beyond these details, I urge the authors to revise the paper for clarity. There are many results, and their relevance is not always made clear.

R: Thank you, we have worked extensively on the revised manuscript to do so.

Reviewer #3 (Remarks to the Author):

This is an important paper which has used a "systems biology" approach to identify predictors of good and poor response to the influenza vaccine in young and elderly humans. This represents an enormous body of work which, although not perfect, is a major contribution to the field.
Whole blood, serum, and PBMC (peripheral blood mononuclear cells) have been analyzed from 30 younger (20-30 years of age) and 61 older (61-greater than 89 years of age). Microarray analysis was done on whole blood. Fifty serum cytokines and chemokines, 15 immune cell subsets and the antibody response after vaccination with the trivalent influenza vaccine were analyzed as well as the cell signaling response to cytokines by phospho Flow (pFlow) for phosphorylated STAT proteins. The patient population was well characterized for conditions known to influence the immune response, e.g. CMV status, previous immunizations etc. (see Table 1). The introduction provides good justification for why this work is important as the elderly do not respond well to vaccines or infections. Thus the paper provides an excellent resource for a molecular systems analysis of the immune system in younger and elderly subjects and several biomarkers are revealed.

Although finally stated very briefly in the discussion, the rationale and justification for using peptide microarrays for detection of influenza-specific antibodies to linear epitopes should be given when they are introduced. This is a good, feasible method to quantify the antibody response but will miss the conformational (and glycosylated) determinants which antibodies naturally predominantly recognize. The fact that these authors WERE able to identify both negative and positive peptide predictors of the HAI response, many of which were age-associated, is a worthwhile/positive contribution of the paper.
The analysis is good in that it presents both aging as a variable and also when it is factored out - as aging is the major predictor of poor response.

R: We are grateful of the reviewer's enthusiastic comments and have included the rationale for using peptide microarrays in our study in the Introduction section (Page 4 line, 9): "We characterized the reactivity of influenza-specific antibodies recognizing HA peptides from baseline serum samples with the aims of (1) investigate the HA linear epitope specificities in pre-vaccine antibodies, and (2) finding the minimal set of pre-existing antibodies that could potentially explain the negative effect of baseline HAI titers on the response to the influenza vaccine(*11, 12*)".

Some points for possible correction/clarification:
Presentation of the data for the pre-GMT (geometric mean titer) and a potential hypothesis for higher values of this being associated with poorer sero conversion are not clear for Figure 2 or from the text.

R: Despite the negative correlation between pre-vaccination and fold-increase in antibody titers has been repeatedly reported, there are limited data on the mechanism of how this happens. We agree that this is an important characteristic of the response to influenza and it is our motivation for including the analysis of peptide reactivity at baseline and pre-GMT. Work from He X-S et al. (2008)[22] has suggested that pre-existing flu-specific memory CD4 T cells that stimulate a B cell response could also activate (CD56dim)-NK cells that are able to inhibit antigen-presentation by dendritic cells, thereby suppressing the subsequent CD4 T cell help to B cells. However, to date, there are no conclusive results explaining how pre-existing antibodies actively suppress the subsequent antibody response to vaccination. To clarify this, we have modified the main text on Page 5, line 19: "An important feature of the immune response to influenza in humans is the presence of pre-existing HAI antibodies, the titer of which negatively correlates with responsiveness (fold increase) to the vaccine(*11, 12*). Pre-existing flu-specific memory CD4 T cells seem to play an important role by activating (CD56dim)-NK cells that are able to inhibit antigen-presentation by dendritic cells, thereby suppressing the subsequent CD4 T cell help to B cells(*11*)."

Figure 3 is better but young and elderly results were grouped together there - these points should be represented by different symbols.

R: We have updated Fig. 3A with distinct colors for young and elderly subjects.

Figure 3B identifies 4 peptides for which pre-vaccine-reactive antibodies are significantly higher in pre-GMT high samples than in pre-GMT low. Presumably the hypothesis is that these are inhibiting a subsequent response (by binding and eliminating those epitopes?) but this is not stated and the significance and hypothesis for this is not clear.

R: We thank the reviewer for highlighting this. We do not have an answer of how this happens yet; one possibility relates to pre-existing flu-specific memory CD4 T cells that stimulate a B cell response (as cited above from work of He X-S, et al. 2008). We have modified the following text on Page 14 line 15: "We found a set of four peptides, the reactivities of which correlated with the pre-vaccination HAI titer. In two of these, robust activity negatively correlated with the HAI response to influenza vaccination. Thus, our system can rapidly identify GR from PR based on their pre-vaccination antibody repertoire against HA protein regions with known neutralization activity(*14*), since conserved sequences are characteristic for neutralization epitopes on HA across different influenza virus strains(*44*). The negative effect of pre-existing antibodies on the response to vaccination has been suggested to be due to pre-existing flu-specific memory CD4 T cells that inhibit antigen-presentation by dendritic cells, thereby suppressing the subsequent CD4 T cell help to B cells(*11*). Therefore, it is possible that such set of memory CD4 T cells are able to maintain a background T-cell dependent antibody production represented by the reactivities against the linear epitopes we found here. Further studies are needed to clarify the mechanisms underlying these observations."

Also are there cases where the positive response to peptides tracks with a good HAI? Fig 3B only showed peptides which track with pre GMT high but these track with a lower fold increase in response.

R: This is a definitively interesting question to tackle, although, the focus of our study is on baseline (pre-existing) measurements and therefore, we did not analyze the actual response to peptide reactivity.

It is not clear how values in Fig 4B relate to those in Fig 3B. Were H3-5 and BH-14 also analyzed in Fig 4B? Only one, H3_8 seems consistent; it is higher in pre-GMT high (Fig 3B) and in Fig 4B shows a lower fold increase.

R: In Fig. 4B we show the results of cross-validation procedures where the most informative peptide predictors are selected out of the pool of available parameters in an automated fashion to generate the model with the best fit. Therefore, all peptide candidates were included for the analysis. We believe the reviewer may have missed H1_23, which together with H3_8 were indeed peptide predictors (with negative sign) identified by this method and significantly more prevalent in subjects with high pre-GMT (Fig. 3B). The fact that H3_5 and BH14 (also significantly more prevalent in subjects with high pre-GMT (Fig. 3B)) were not selected as predictors of vaccine response does not implies inconsistency of the findings, rather it indicates that these are less informative than each of the peptide predictors listed in Fig. 4C (both for Models 1 and 3).

The data presented seem clear that apoptosis pathways (APO) are a positive predictor of good response whether aging is left in the model (model 1) or taken out (model 3). It is not as clear why the PROL (regulation of B cell proliferation) pathway is negatively correlated with survival as molecules in this pathway are necessary for plasmablast growth and survival which these authors have previously shown do correlate with good response. The argument for "beneficial self renewal" being necessary for a good response at this point (and in the references cited) seems more appropriate/substantiated for T cells than for B cells.

R: While it seems logical that the up-regulation of genes involved in B cell proliferation and plasma cell differentiation would enhance the serological response, results from different studies point to a different conclusion. Heightened CD40 signaling essential for this process, disrupts germinal center formation[24] and direct B cells to extrafollicular plasma cells. Furthermore, in CD40L transgenic mice, the production of late-appearing high-affinity antibodies is severely down-regulated[25]. Therefore, it is appealing to hypothesize that augmented PROL expression (which includes CD40L) and the preferential generation of extrafollicullar plasma cells during the GC reaction results in the generation of antibodies that weakly bind to viral antigens. Of note, the CD320 gene also clustered with CD40L in module PROL. This gene has been shown to participate in GC differentiation by directing B cells to plasma cells[26] but is not clear what the consequences of up-regulation of this gene are.
We thank the reviewer for these comments and have modified the text in Page 17 line 20 as follows:
"Our study also identifies a link between cell survival and proliferation, and defective antibody responses. We found that the CD40L gene clustered in a module enriched for proliferation genes (module PROL), which is a negative indicator of the vaccine response. While molecules in gene module PROL (e.g. CD40L, CD320) are critical for plasmablast growth and survival, results from different studies suggest that elevated proliferation signals from these molecules have a detrimental effect. For example, heightened CD40 signaling causes B cells to shunt into an extrafollicular plasma cell fate and this prevents the generation of long-lived bone-marrow plasma cells. This has consequences for the B cell response including the premature termination of the humoral immunity and the disruption of GC formation *in vivo*(*64*). Therefore, it is appealing to hypothesize that weaker responses to the vaccine in individuals with augmented CD40L expression result from the preferential generation of extrafollicullar plasma cells that compromise the accumulation of somatic mutations in the GC B cells, dampening late-appearing high-affinity antibodies, as demonstrated in transgenic CD40L mice(*65*). An interesting finding in support of this hypothesis is the impairment in GC formation found in old mice with poor responses to vaccine(*66*). Further studies in humans could potentially address this.
Our results also suggest the presence of other genes in the PROL module with similar functions to that of CD40L, which could contribute to the diminished antibody responses. For example, the gene CD320 has been shown to participate in GC differentiation by directing B cells to mature into plasma cells(*67*)."

Data in Fig 6B are mixed for showing APO high is better represented in the 7 peptides predictive of HAI titer in response to vaccine : 4/7 of these are higher in APO high but 3/7 are lower in APO high. Therefore these data are difficult to interpret as supporting the hypothesis that APO high leads to more GR (good responders) via these responses. It is positive that Fig 6A does show that APO high have more GR (51% vs 9% GR in APO low).

R: We apologize for the confusion. Fig. 6B shows the significant results from comparing peptide reactivity in individuals APO low versus APO high. Effectively, 7 of 14 were automatically selected for prediction of the HAI response for Model 1 and Model 3 together (Fig. 4). The comparison the reviewer is suggesting must be done considering the contribution of each peptide predictor to the

resulting models 1 and 3 (regression coefficient, shown for Model 1 in Fig. 4B). Please note that by doing so, the results are no longer difficult to interpret; for example H1_1 is better represented in APO low (Fig. 6B) and is a negative predictor (Fig. 4B), H1_6 is better represented in APO high individuals and is a positive predictor, H3_21 is better represented in APO low and is a negative predictor, etc.

On page 11 the key data for serum cytokines should be indicated (Fig 5B), although FasL was not a strong indicator and IL-12p40 just a little better.

R: The data in now indicated in the main text (Page 12, line 6): "For serum cytokines, we found that soluble Fas ligand (sFasL) and IL-12p40 were negative predictors (Fig. 5B)."

Model 3, excluding age - identified a new set of features. Supl Fig 3 should be included in body/ regular Figures of paper. Very interesting data.

R: We thank the reviewer for his/her interest in our results and the suggestion. Fig. S3 is now included in the body of the paper as Fig. 7A and B.

For Figures 7A and 7B the titers to vaccine were lower in FasL mutant mice but the conclusion/interpretation is flawed as FasL also affects T cell responses and it would not be surprising that for these reasons the titers are lower (not because of decreased apoptosis).

R: We have addressed this above (reviewer 1, point 10):
Our response:
The Fas-deficient *lpr* mice have several defects in the T cell and B cell compartments and defective apoptosis via Fas, activation-induced, as well as the pathway involving FcγRII[14-16]. Though, conflicting results have been reported for the *in vivo* B cell response[17,18]. Our results are in agreement with those of Yakahashi et al. (2001) who demonstrated that these mice have defects in clonal selection and the establishment of the memory B cell repertoire[18]. The main text (Page 15, line 20) has been modified as follows: "It is known that the *lpr* mice have defects in T cell and B cell development. Thus our results might not be due to the apoptosis defect in these mice (although this is suggested by the apoptosis gene module) but these other factors. However, there is no agreement as to whether the GC formation and B cell responses are normal (*50, 51*). In particular our results argue against a previous study that reported that memory and antibody-forming cell populations appear to be normal in *lpr* mice(*51*). However, more recent studies have shown that these mice have defects in clonal selection and the establishment of the memory B cell repertoire(*50*)."

Discussion
It is clear that they have identified some HA linear epitope peptides for antibody reactivity for existence in the pre-vaccine repertoire but it is not clear how these would explain the negative effect of baseline HAI titers (on sero conversion) - just give some ideas for this (e.g. do these block stimulation of B cells if these determinants are removed, but this is a little harder to see this for the antibody which sees the whole HA and not just linear determinants like T cells do).

R: This has been addressed above (Reviewer 2). We have added the following text to the new version (Page 5, line 19): "An important feature of the immune response to influenza in humans is the presence of pre-existing HAI antibodies, the titer of which negatively correlates with responsiveness (fold increase) to the vaccine(*11, 12*). Pre-existing flu-specific memory CD4 T cells seem to play an important role by activating (CD56dim)-NK cells that are able to inhibit antigen-presentation by dendritic cells, thereby suppressing the subsequent CD4 T cell help to B cells(*11*)."

Their suggestion that inhibiting the reactivity of 2/4 peptides could improve the influenza vaccine response is not clear as these peptides change from year to year with different vaccines (?)

R: We thank the reviewer for this observation; have removed the statement and incorporated the following text on Page 14, line 16: "…In two of these, robust activity negatively correlated with the

HAI response to influenza vaccination. Thus, our system can rapidly identify GR from PR based on their pre-vaccination antibody repertoire against HA protein regions with known neutralization activity(*14*), since conserved sequences are characteristic for neutralization epitopes on HA across different influenza virus strains(*44*). The negative effect of pre-existing antibodies on the response to vaccination has been suggested to be due to pre-existing flu-specific memory CD4 T cells that inhibit antigen-presentation by dendritic cells, thereby suppressing the subsequent CD4 T cell help to B cells(*11*). Therefore, it is possible that such set of memory CD4 T cells are able to maintain a background T-cell dependent antibody production represented by the reactivities against the linear epitopes we found here. Further studies are needed to clarify the mechanisms underlying these observations."

The discussion could include a better idea of which cells are thought to play a significant role in this as whole blood was analyzed. Are they thinking this is most valid for T cells?

R: Our results suggest that T cells are highly represented in the immune signatures with respect to the association with apoptosis. Analysis of correlation between gene modules and cell phenotypes showed that the APO module is correlated with CD8 and CD4 EM cells (R = -0.27 and -0.24, respectively; $P < 0.001$). There is also great deal of evidence that the constant activation of clonal T cell populations may lead to increasing numbers of memory cells that fill the immunological space, compromising responses to newly encountered antigens[27]. Based on these observations and our results, we hypothesize an accumulation of apoptosis-resistant memory T cells, preferentially occurring in older subjects, which suppress the generation of an immune response against novel antigens.

At this point it is also not clear what they would recommend to be used to identify poor responders (what molecule would be measured?) and what would the predictive values be (for poor response)?

R: While this is one important goal of our studies, we cannot yet recommend with certainty which molecules to measure to identify good versus poor responders. This study is the first to address baseline parameters that can predict vaccine response, and consequently there are limited resources that we can use to compare our results with those of others, and find the key measurements that can be generalized across different human populations and to diverse vaccination strategies. However, further studies from our group including more subjects, and different populations as well as twin cohorts will be used to address this.

In conclusion this is a very novel paper and shows association of apoptosis with robust antibody response as well as particular HA linear determinants for which antibody can be measured before vaccination and associates with poor response.

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