

1 **SIMON, an automated machine learning system reveals immune signatures of**
2 **influenza vaccine responses**

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18

19 **Abstract (175):** Machine learning holds considerable promise for understanding complex
20 biological processes such as vaccine responses. Capturing interindividual variability is essential to
21 increase the statistical power necessary for building more accurate predictive models. However,
22 available approaches have difficulty coping with incomplete datasets which is often the case when
23 combining studies. Additionally, there are hundreds of algorithms available and no simple way to
24 find the optimal one. Here, we developed Sequential Iterative Modelling “OverNight” or SIMON,
25 an automated machine learning system that compares results from 128 different algorithms and is
26 particularly suitable for datasets containing many missing values. We applied SIMON to data from
27 five clinical studies of seasonal influenza vaccination. The results reveal previously unrecognized
28 CD4⁺ and CD8⁺ T cell subsets strongly associated with a robust antibody response to influenza
29 antigens. These results demonstrate that SIMON can greatly speed up the choice of analysis
30 modalities. Hence, it is a highly useful approach for data-driven hypothesis generation from
31 disparate clinical datasets. Our strategy could be used to gain biological insight from ever-
32 expanding heterogeneous datasets that are publicly available.

33 **[Main Text: 7,756]**

34 **Introduction**

35 The immune system is comprised of multiple cell types that work together to develop an
36 effective response to a given pathogen. However, which of these myriad cell types are important
37 in a particular response is not well understood. The increasingly common systems immunology
38 approach measures gene expression and different cells and molecules in the immune system during
39 an infection or vaccination and uses computational methods to discern which components are most
40 important (1-6). These studies have the practical goal of determining what makes one vaccine
41 formulation better than another or how individuals vary. In addition, it may suggest a mechanistic

42 understanding of how an effective immune response is achieved. To accomplish this, an accurate
43 modeling of the complex processes that lead to a successful outcome is crucial.

44 Over the past few years, many systems studies of influenza vaccination responses in human
45 beings have been analyzed computationally, but the results have not been consistent (2, 3, 7-10).
46 One reason for these inconsistent results are the relatively small sample sizes. Another is that
47 studies focus on only one biological aspect, for example molecular correlates of protection by
48 using transcriptome data (11). However, a more robust approach to understanding how a vaccine
49 works would involve analyzing multiple parameters from many individuals across different
50 populations to more accurately capture biological variability. Furthermore, this would increase the
51 statistical power, ultimately leading to the generation of classification and regression models with
52 more robust performance metrics. While the number of studies and the amount of data are
53 expanding dramatically, analyzing diverse samples across clinical studies remains challenging
54 (12). This is particularly true for data from flow and mass cytometry where the number of markers
55 analyzed can vary tremendously (13).

56 In this study, we develop an approach that optimizes a machine learning workflow through
57 a Sequential Iterative Modeling “OverNight” (SIMON). SIMON is specifically tailored for clinical
58 data containing inconsistent features with many missing values. SIMON utilizes multi-set
59 intersections to successfully feed such data into an automated machine learning process with
60 minimal sample losses. Our approach runs hundreds of different machine learning algorithms to
61 find the ones which fit any given data distribution, and this maximizes predictive accuracy and
62 other performance measurements. We used SIMON to analyze data from the Stanford Human
63 Immune Monitoring Center (HIMC) collected from five separate clinical studies of seasonal
64 influenza vaccination, obtained over eight years, with various platforms and expanding

65 parameters. This enabled a systems-level identification of features that correlate with protective
66 immunity to influenza. In the resulting models, we identified several previously unknown immune
67 cell subsets that correlated with a successful influenza vaccination outcome, as defined by antibody
68 responses. The impact of our findings is twofold. First, the study offers a new tool that can increase
69 the accuracy of predictions from heterogeneous biological datasets. Second, it provides new targets
70 for the development of the next-generation of influenza vaccines.

71 **Results**

72 *Subhead 1. Preprocessing of data collected across different clinical studies*

73 To test robustness of our approach, we used data from the Stanford HIMC. This data included
74 187 nominally healthy individuals between 8 and 40 years of age undergoing an annual influenza
75 vaccination recruited over eight consecutive seasons, from 2007 to 2014, and five clinical studies
76 (**Fig. 1A**). Blood samples were acquired before vaccination and on day 28 after vaccination. Over
77 3,800 parameters were measured at baseline. This included 102 blood-derived immune cell subsets
78 analyzed by mass cytometry (**Fig. S1, Table S1**). It also included the signaling capacity of over
79 30 immune cells subsets stimulated with seven conditions, which were evaluated by measuring the
80 phosphorylation of nine proteins (**Table S2**). Additionally, up to 50 serum analytes were evaluated
81 using Luminex bead arrays (**Table S3**). On day 28 after vaccination, the serum titer of
82 haemagglutinin-specific antibodies against all vaccine strains was determined using the
83 hemagglutination inhibition assay (HAI), which is the best-defined correlate of influenza
84 immunity induced by this vaccine (14). The HAI antibody titers were calculated as the fold change
85 between the HAI titer at day 28 relative to the baseline titer. High and low responders were
86 determined using metrics defined by the US Centers for Disease Control to evaluate influenza
87 vaccine efficacy: seroconversion and seroprotection (15). Individuals were considered to be high

88 responders if they had a protective HAI antibody titer to all vaccine strains (HAI antibody titer >
89 40) and if they seroconverted (geomean HAI titer > 4).

90 Out of 187 analyzed donors, 64 were identified as high responders and 123 as low responders (**Fig.**
91 **1B**). Overall, there were no major differences in the age, gender, or study year between the high
92 and low responders (**Fig. S2**). The only exception was that a higher proportion of adolescents were
93 high responders, which is in line with published data(16) (**Fig. S2B**).

94 *Subhead 2. Dealing with missing values using intersection function*

95 A major problem when using data across clinical studies and years is the lack of overlap
96 between the features measured. Indeed, even though the data comes from a single facility, in many
97 years there was an increase in the number of parameters measured, especially in the transition from
98 FACS analysis (12-14 parameters) to mass cytometry (25-34 parameters). Since all assays were
99 not performed across all studies and years (**Fig. S3**), our initial dataset had many missing values.
100 The dataset contained 187 rows/donors and 3,284 columns/features, yielding a total of 614,108 values.
101 However, 572,081 values were missing, resulting in high data sparsity. That is, the percentage of
102 missing values in the dataset was 93.2% (**Fig. S4**). Such high data sparsity, which is commonly
103 encountered in the clinical data, does not allow for straightforward statistical analysis. Therefore, we
104 had to reduce the number of missing values. Researchers and data scientists deal with missing values
105 either by deletion or by imputation of missing data (17). However, analysis of the missing data
106 distribution revealed that when all studies were combined, the dataset had missing values in every
107 column and every row and many of the columns and rows had sparsity of 90% (**Table S4**).
108 Therefore, if we deleted either rows or columns, this would result in data with zero subjects. This
109 approach was unsuitable. Additionally, effective imputation was strongly limited by the small
110 number of cases that could be used as prior knowledge. Overall, we concluded that the high number

111 of columns and rows with missing values made it impossible to use the whole dataset for further
112 analysis.

113 Since this could be a very useful dataset for predictive modeling of influenza vaccine
114 responses, we explored alternative ways to reduce the number of missing values. To ensure that
115 interpretation of the initial dataset was preserved and so as not to introduce bias, we selected
116 feature subsets from the original dataset without transformation by identification of the overlap
117 (i.e. intersection) between multiple donors. We hypothesized that by using intersection, we could
118 identify features shared across donors. Such a process could generate feature subsets that span an
119 entire initial dataset. Additionally, it was expected that reducing the number of features would
120 improve the performance of the model, such as was shown for random initial subset selection (18).

121 In the first step of SIMON, we implemented an algorithm, *mulset*, to identify features shared
122 across donors and generate datasets containing all possible combinations of features and donors
123 across the entire initial dataset (**Fig. 2**). While strategies to find an intersection among large number
124 of sets have been reported(19), detecting intersections in a dataset with 614,108 datapoints would
125 be challenging. The *mulset* was inspired by an approach commonly used in computer science to
126 accelerate detection of duplicated records across large databases (20). By using the intersect
127 function, we identified shared features between donors. These were converted to a unique shared
128 feature identifier using the hash function. This process allowed the rapid identification of donors
129 with shared features and the generation of datasets that can be used in further analysis. The *mulset*
130 algorithm calculated overlapping features between all donors, resulting in 34 datasets with
131 different numbers of donors and features (**Table S5**). After applying the *mulset* algorithm, the
132 dimensionality of the data was significantly reduced, since all generated datasets had a maximum
133 of 300 shared features. Eleven of the generated datasets had a higher number of donors than

134 features, with a maximum number of 143 donors that shared 49 features (**Table S5, dataset 8**).
135 Overall, the first step in the SIMON produced more restricted datasets with higher data quality and
136 reduced the number of features, making possible to continue the data analysis.

137 *Subhead 3. Automating the machine learning process and feature selection*

138 The next step, following data preprocessing, was to apply machine learning algorithms to
139 extract patterns and knowledge from each of the 34 datasets. To select relevant features, we based
140 our approach on the method for feature selection proposed by Kohavi and John (21). In the original
141 approach, termed wrapper, feature subsets were selected using two families of algorithms: the
142 decision trees and the Naïve-Bayes (21). In this study, we build upon this approach by adding
143 ensemble algorithms (of which Random Forest was previously shown to be suitable for feature
144 selection (22)) and other dimensionality-reduction algorithms, such as discriminant analysis. It is
145 widely recognized that a best algorithm for all datasets does not exist (23). Currently, choosing an
146 appropriate algorithm is done through a trial-and-error approach, with only a few algorithms tested.
147 To identify optimal algorithms more quickly and efficiently across a broad spectrum of
148 possibilities, we implemented an automated machine learning process in SIMON.

149 SIMON is described briefly in **Fig. 3**. The feature subset selection was performed by testing
150 multiple algorithms without any prior knowledge and user-defined parameters on each of the 34
151 datasets in a sequential and iterative manner. First, each dataset was split into 75% training and
152 25% test sets, preserving balanced distribution of high and low responders. The training set was
153 used for model training and feature selection. The accuracy of the feature selection was determined
154 using a 10-fold cross-validation, which was shown to out-perform other resampling techniques for
155 model selection (24). The test set was used for evaluating model performance on independent data
156 not used in the model training. In general, it is most efficient to train the model on the entire dataset.

157 However, in our case, it was important to have an independent test set to evaluate and then compare
158 performance of the many models we expected to obtain. Additionally, evaluating model
159 performance using only cross-validation is not sufficient to conclude that model can be applied to
160 other datasets. There could be a problem with overfitting, such as when a model does not generalize
161 well to unseen data. Second, a fully automated process of model training utilizing 128 machine
162 learning algorithms was done initially on the training set and repeated for each dataset. **Table S6**
163 provides a list of all machine learning classification algorithms used. Each model was evaluated
164 by calculating the performance parameters using the confusion matrix on the training and test sets.
165 A confusion matrix calculates false positive and negatives, as well as true positive and negatives.
166 This allows for more detailed analysis than accuracy, which only gives information about the
167 proportion of correct classifications, and therefore can lead to misleading results (25). In SIMON,
168 for each model we calculated the proportion of actual positive cases that were correctly identified
169 (i.e. sensitivity) and the proportion of correctly identified actual negative cases (i.e. specificity).
170 All performance parameters were saved in the MySQL database. Finally, to compare the models
171 and discover which performed best, we calculated an Area Under the ROC Curve (AUROC). This
172 is a widely-used measure of quality for the classification of models, especially in biology (26). A
173 random classifier that cannot distinguish between two groups has AUROC of 0.5, while AUROC
174 for a perfect classifier that separates two groups without any overlap is equal to 1.0 (27). Therefore,
175 the training and test AUROC are reported throughout the text and models are compared using that
176 metric of performance.

177 To test the feasibility of SIMON, we ran more than 2,400 machine learning analyses on 34
178 datasets. SIMON built models for 19 datasets, with an average of 54 models built per dataset
179 (**Table S7**). None of the 128 machine learning algorithms tested were able to build a model for 15

180 of the datasets. This indicates that those have poor data quality and distributions. Therefore, they
181 were discarded from further analysis. With the remaining 19 datasets, models were built with the
182 training AUROC values ranging from a minimum of 0.08 to a maximum of 0.92 (**Table S7**).
183 Overall, the automated machine learning process improved the performance of the models in all
184 19 datasets, with a gain of performance ranging from 30 to 91% (**Table S7**). This indicates that
185 SIMON facilitates the identification of optimal algorithms, which ultimately increases the
186 performance of models.

187 *Subhead 4. Performance estimation and model selection*

188 Before model comparison, other performance parameters were calculated, in addition to
189 AUROC, and were used to filter out poorly performing models with the goal of facilitating further
190 exploratory analysis. To remove random classifiers, all models with $AUROC \leq 0.5$ on both training
191 and test sets were discarded. Furthermore, all models in which specificity and sensitivity of both
192 training and test sets were < 0.5 (i.e. models with higher proportion of false positive and negative
193 values) were also removed. This restriction discarded models in which the classifier achieved high
194 performance, as indicated by a high AUROC, at the cost of a high false positive or negative rate
195 (28, 29). After applying these filters, many models were removed, decreasing the average number
196 of models per dataset to three (**Table S8**). Additionally, eight datasets were discarded. This
197 filtering step was essential to remove models which would otherwise be falsely evaluated as high
198 performing, such as those built using dataset 205, for which a high AUROC of 0.92 was obtained
199 at the expense of low specificity (0.06) (**Table S7**).

200 To compare models within one dataset and discover which performs best, the random
201 number seed was set before training with each algorithm. This ensured that each algorithm trained
202 the model on the same data partitions and repeats. Further, it allowed for comparison of models

203 using AUROC. In general, AUROC values between 0.9-1 are considered excellent, 0.8-0.9 good,
204 0.7-0.8 fair and values between 0.6-0.7 are considered as having poor discriminative ability (30).
205 In SIMON, models trained on six datasets were built with fair discriminative ability (max. train
206 AUROC between 0.7-0.8) (**Table S8**). To avoid overfitting, we additionally evaluated the
207 performance of each model on the test set, which was not used for building the model. In this case,
208 models trained on the three datasets were built with a fair discriminative ability (**Table S9**,
209 **datasets 5, 13, and 171**). One dataset (**Table S9, dataset 36**) was built with a good discriminative
210 ability (max. test AUROC 0.86), which could be generalized to an independent set. It should be
211 noted that maximum AUROC values did not necessarily come from the same model (e.g.
212 maximum train AUROC might come from model 1, while maximum test AUROC from model 2).
213 To account for that, we add another filter to remove all models with poor discriminative ability,
214 that is all models in which the train and test AUROC were less than 0.7. By applying this
215 restriction, we were left with only two datasets (datasets 13 and 36). These were used for further
216 analysis and feature selection. The model build on dataset 36, with the shrinkage discriminant
217 analysis, out-performed the other four models as evaluated by comparison of train AUROC (**Fig.**
218 **S5A, Table S10**). A model was built with train AUROC of 0.78 and it performed well on an
219 independent test set (test AUROC 0.86). The model build on dataset 13 with the Naïve- Bayes
220 performed better than the other model built for the same dataset (train AUROC 0.75, test AUROC
221 0.7) (**Fig. S5B, Table S11**).

222 Overall, SIMON facilitated exploratory analysis and discovery of models with good discriminative
223 performance by integrating the filtering steps and evaluating comprehensive model performance.

224 *Subhead 5. Identification of all-relevant cellular predictors using SIMON*

225 After selection of the best-performing models, we focused on feature selection. Our goal was
226 to use SIMON to identify all-relevant features to deepen our knowledge about the process that
227 drives antibody generation in response to influenza vaccination. To solve this problem, classifiers
228 were used in SIMON to rank features based on their contribution to the model. Features were
229 ranked depending on the variable importance score calculated for each model (31). The score
230 ranges from 0 to 100. Features with variable importance score of 0 are not important for the
231 classification model and can be removed from training the model.

232 First, we focused on the model built on dataset 13 with 61 donors (**Table S12**). Out of 76
233 features, 64 had measurable variable importance score and 15 features had variable importance
234 score above 50 (**Fig. 4A, Table S13**). The top-ranked feature that highly contributed to this model
235 was CD4⁺ T cells with the CD127⁻CD25^{hi} phenotype (described as regulatory T cells or Tregs(32))
236 that expressed CD161 and CD45RA markers (**Table S13, rank 1**). The frequency of Tregs with
237 CD161⁻CD45RA⁺ phenotype was shown to be significantly greater among the high responders
238 (**Fig. 4B**, FDR < 0.01). To further explain features that contributed to this model, we performed
239 correlation analysis. Correlation analysis revealed that Tregs with CD161⁻CD45RA⁺ phenotype
240 had a significant positive correlation with the top-ranked feature, CD161⁺CD45RA⁺ Tregs
241 (Pearson's $r = 0.54$, $p < 0.0001$ after multiple comparison adjustment using the B-H correction)
242 (**Fig. S6**). Additionally, CD161⁺CD45RA⁺ Tregs had a weak, but significant positive correlation
243 with CD161⁺ CD4⁺ T cells (Pearson's $r = 0.08$, $p = 0.001$ after multiple comparison adjustment
244 using the B-H correction), which had high variable importance score (**Table S13, rank 9**). Such
245 correlation indicated that these subsets might describe similar family of CD4⁺ T cells contributing
246 to the generation of antibody responses after influenza vaccination. Indeed, a recent study suggests

247 that expression of CD161 marks a distinct family of human T cells with a distinct lineage and with
248 innate-like capabilities (33).

249 To experimentally validate results from this model, we analyzed the phenotype and
250 functionality of immune cells before and after vaccination in the independent samples from 14
251 individuals (7 high and 7 low responders) (**Table S14**). We found that after stimulation with the
252 influenza peptides, CD161⁺ CD4⁺ T cells from high, but not low responders, produced TNF α in
253 the samples prior to vaccination (**Fig. 4C**). This indicated that CD161⁺ CD4⁺ T cells from high
254 responders had a pool of pre-existing influenza-specific T cells. Additionally, after vaccination,
255 the frequency of CD161⁺ CD4⁺ T cells with a CCR6⁺ CXCR3⁻ (Th17) phenotype in high
256 responders increased significantly (**Fig. 4D**).

257 The second most important feature in this model was CXCR5⁺ CD8⁺ T cells (also known as
258 follicular cytotoxic T cells) (34-36) with a CCR6⁺ CXCR3⁻ (Tc17) phenotype (**Table S13, rank**
259 **2**). Frequencies of CXCR5⁺ CD8⁺ T cells with Tc17 were significantly increased among the high
260 responders (**Fig. 4B**, FDR < 0.01). Additionally, frequencies of CXCR5⁺ CD8⁺ T cells with a
261 CCR6⁻CXCR3⁻ (Tc2) phenotype were also increased in the same group (**Fig. 4B**, FDR < 0.01).
262 CXCR5⁺ CD8⁺ T cells with Tc2 phenotype were also identified as important in this model (**Table**
263 **S13, rank 7**) and had a significant positive correlation with Tc17 CXCR5⁺ CD8⁺ T cells (Pearson's
264 $r = 0.66$, $p < 0.0001$ after multiple comparison adjustment using the B-H correction) (**Fig. S6**).
265 However, analysis of the experimental data showed no significant participation of CXCR5⁺ CD8⁺
266 T cells in vaccine-induced responses, even though in a few of the high responders there was an
267 increase of CXCR5⁺ CD8⁺ T cells with a Tc2 and Tc17 phenotype (**Fig. 4D**).

268 The results obtained in this model were confirmed using an R package, Boruta, that
269 implements a novel feature selection algorithm for identifying all relevant features (22). CD127⁻

270 CD25^{hi} CD4⁺ T cells with the CD161 expression and CXCR5⁺ CD8⁺ T cells with Tc2 or Tc17
271 phenotype were identified as important ($p < 0.05$, after multiple comparison adjustment using the
272 Bonferroni method), confirming findings obtained by SIMON (**Fig. S7A**).

273 Second, we explored the features selected in the better performing model built on dataset 36,
274 comprising of 40 donors (**Table S15**). Out of 103 features, 88 had measurable variable importance
275 scores ranging from 5 to 100 (**Table S16**). Of those, 17 features had a variable importance score
276 above 50 (**Fig. 4E**), indicating a strong contribution for this classification model. Interestingly, the
277 effector memory (EM) CD4⁺ T cells, previously reported to correlate with antibody responses to
278 influenza vaccine (37), were ranked in 5th place in our model. Moreover, B cells with memory
279 phenotype, including a subset of IgD⁺ CD27⁺ memory B cells identified in previous studies (3, 8,
280 38), contributed to our model (**Table S16**). Obtaining results supported by other studies gave us
281 confidence in further analysis of our classification model. Importantly the top four features
282 identified have not previously been implicated as playing a major role in antibody responses to
283 influenza vaccination, or indeed any antibody response. These included CD8⁺ T cells with
284 expression of CD27 or CD85j markers, and CD8⁺ T cells with varying degree of expression of
285 CCR7 and CD45RA markers, described as naïve, effector or terminally differentiated effector
286 (TEMRA) and memory subsets (39). Analysis of the data particularly indicated that
287 effector/TEMRA CD8⁺ T cells increased significantly among high responders (**Fig. 4F**, FDR <
288 0.01). In contrast, low responders had significantly higher frequency of early CD27⁺/CD28⁺ CD8⁺
289 T cells and naïve CD8⁺ T cells (**Fig. 4F**, FDR < 0.01). Moreover, the effector/TEMRA CD8⁺ T
290 cells were confirmed to contribute to this model by Boruta ($p < 0.05$, after multiple comparison
291 adjustment using the Bonferroni method) (**Fig. S7B**).

292 The top four features that contributed the most to this model, were CD8⁺ T cells in early or
293 late effector or memory states, indicating they might all be contributing to the influenza response
294 through the same underlying mechanism. Indeed, correlation analysis showed that the top ranked
295 subset, CD27⁺ CD8⁺ T cells, had a significant correlation coefficient with other subsets (naïve
296 CD8⁺ T cells $r = 0.80$, CD28⁺ CD8⁺ T cells $r = 0.85$, CD85j⁺ CD8⁺ T cells $r = -0.69$,
297 effector/TEMRA CD8⁺ T cells $r = -0.61$ and EM CD8⁺ T cells $r = -0.71$, $p < 0.0001$ after multiple
298 comparison adjustment using the B-H correction) (**Fig. S8**). Additionally, a specific subset of
299 CD8⁺ T cells expressing NK-cell-related receptor CD85j was identified as the TEMRA subset
300 (40), while the expression of CD27 or CD28 was indicative of the subsets of T cells with a naïve
301 or early differentiation phenotype (41).

302 In the analysis of the independent samples, EM CD8⁺ T cells from high responders produced
303 IL17A after influenza peptide stimulation, demonstrating that this population contained influenza-
304 specific T cells (**Fig. 4G**). Furthermore, the frequency of EM CD8⁺ T cells with a Tc17 phenotype
305 was significantly increased only in high responders after vaccination (**Fig. 4H**). Additionally, the
306 frequency of EM CD4⁺ T cells with Th17 phenotype was also increased in the same group of high
307 responders after vaccination (**Fig. 4H**).

308 In summary, SIMON allowed us to identify both known and novel immune cell subsets that
309 correlate with a robust antibody response to seasonal influenza vaccines. Particularly surprising
310 were the number of different CD8⁺ T cell subsets, which are not typically thought of as playing
311 any role in promoting robust antibody responses. We confirmed that IL17A producing EM CD8⁺
312 T cells, which contained a pool of pre-existing influenza T cells, were elevated in the high versus
313 low responders with independent samples.

314 **Discussion**

315 In this study, we developed a novel computational approach, SIMON, for the analysis of
316 heterogenous data collected across years and from heterogenous datasets. SIMON increases the
317 overall accuracy of predictive models by utilizing an automated machine learning process and
318 feature selection. Using the results obtained by SIMON, we identified previously unrecognized
319 CD4⁺ and CD8⁺ T cell subsets associated with robust antibody responses to seasonal influenza
320 vaccines.

321 The accuracy of the machine learning models presented in this work was improved in two
322 stages. First, to interrogate the entire dataset across different clinical studies, we integrated into
323 SIMON an algorithm, *mulset*, which generates datasets using multi-set intersections. This is
324 particularly suitable for data with many missing values. In our case, due to the high sparsity of
325 initial dataset, this step was essential for the further analysis. In general, clinical datasets are often
326 faced with the same problem, namely, that many features are measured on a small number of
327 donors. Due to the rapid advance of immune monitoring technology, many more parameters in our
328 studies were measured in the later years than earlier. The same situation might arise when
329 combining data collected in different facilities. An alternative approach might be the imputation
330 of the missing values, but this would likely introduce bias. Moreover, the major limitation of
331 effective imputation is the number of cases that could be used as prior knowledge. The sparsity of
332 our initial dataset was too high for effective imputation. By using intersections, SIMON selects
333 feature subsets by preserving the interpretation of the initial dataset and without introduction of a
334 bias. Overall, an automated feature intersection process increases statistical power by accounting
335 for variability among different individuals. Potentially, it could be applied across clinical studies.
336 Additionally, by reducing the number of features, this process improves the performance of
337 models. This will be particularly important for the application of SIMON on larger publicly

338 available datasets such as those stored in Gene Expression Omnibus repository (42) or ImmPort
339 (43).

340 Second, finding the machine learning algorithm most suitable for specific data distribution
341 allows for a better understanding of the data and provides much higher accuracy. The current state-
342 of-the-art in building predictive models is to test several machine learning algorithms to find the
343 optimal one. However, a single algorithm that fits all datasets doesn't exist. If an algorithm
344 performs well on a certain dataset, it doesn't necessarily translate well to another dataset (even if
345 it pertains to a closely related problem) (23). The overall accuracy of the predictive models depends
346 on rigorous algorithm selection. With so many machine learning algorithms available, choosing
347 the optimal one is a time-consuming task, often performed in a limited way (only dozens of
348 algorithms are tested). Recent work has shown that automated machine learning can identify
349 optimal algorithms more quickly and efficiently (44-46). Open competitions and crowdsourcing
350 (e.g. www.kaggle.com), in which many groups contribute machine learning algorithms to build
351 models for the same datasets, increases the accuracy and predictive performance of the models
352 (47). By developing an automated machine learning process in SIMON, we can quickly identify
353 the most appropriate machine learning algorithm (of the 128 tested) for any given dataset.
354 Additionally, SIMON offers an alternative perspective on the application of algorithms that might
355 never be used due to lack of expertise or knowledge necessary for their implementation. These
356 features of SIMON also allow biologists with domain knowledge but who are not computationally
357 adept to find the most effective tools with which to analyze their data.

358 In this study, we demonstrate the utility of SIMON and its automated machine learning
359 processes to discover the principal features that correlate with high versus low influenza vaccine
360 responders. We found it to be essential for identifying the best-performing models and extracting

361 the most important features that contribute to those models. Performance of each model built in
362 SIMON was automatically evaluated on both training and left-out test sets using well-known
363 measures, such as AUROC, specificity, and sensitivity. This ensured that the model was not
364 overfitted and that it could generalize to unseen data. Both models were selected by stringent
365 restrictions in the exploratory analysis and were built with AUROC scores between 0.7 - 0.8.
366 Nevertheless, since the goal of the study was to identify features that discriminate between high
367 and low responders in a high-throughput manner, these models were built using the algorithms
368 without any user-defined parameters. Therefore, each model could be fine-tuned, and its predictive
369 performance might be increased. This could be of interest for researchers interested in building
370 predictive models to identify features for use in diagnostic tests. In the future, we plan to improve
371 SIMON by implementing an automated tuning process for each model.

372 This study demonstrated the advantage of SIMON over the conventional approach, in which
373 one machine learning program is chosen by successfully identifying the immune signature driving
374 influenza immunity. Some of our findings, such as the importance of effector memory CD4⁺ T
375 cells and subsets of memory B cells, had been identified in previous studies (2, 8, 9) serving to
376 validate our approach. Additionally, SIMON has identified previously unappreciated T cell subsets
377 that discriminate between high and low responders. It is well known that T cells, in contrast to
378 antibodies produced by cells of B lineage, have the ability to provide durable and cross-protective
379 immunity by targeting internal conserved viral epitopes (48, 49). Therefore, the CD4⁺ and CD8⁺
380 T cell subsets identified in this study could be useful targets for the development of broadly
381 protective influenza vaccines. Influenza-specific CD4⁺ T cells have already been shown to be
382 important for the generation of influenza immunity (50, 51). This was confirmed in the current
383 study by showing that high responders had a pre-existing pool of influenza-specific CD4⁺ T cells

384 expressing CD161. Additionally, we found that CD8⁺ T cells with an effector/TEMRA, EM and
385 Tc17 phenotype and CXCR5 expression correlated with improved vaccine responses. These
386 subsets are particularly interesting candidates and it will be of considerable interest to understand
387 how they contribute to more robust antibody responses. CXCR5⁺ CD8⁺ T cells are enriched in the
388 B cell follicles of germinal centers (35, 52) and they can promote B cell survival and antibody
389 generation (36). CD8⁺ T cells with a Tc17 phenotype have been detected in the lungs of mice
390 challenged with influenza A virus (53). Using independent samples from donors that weren't
391 included in the building and testing of our model, we found that CD8⁺ T cells from high responders
392 contained influenza-specific cells with the ability to produce IL17A in response to peptide
393 stimulation. In a mouse model, IL17A has been shown to be important for the generation of the
394 antibody responses necessary to clear an influenza virus infection (54). This apparent role of
395 IL17A in the modulation of antibody responses and proper functioning of germinal centers has
396 only recently been described (55). Interestingly, CD161⁺ CD45RA⁺ Tregs, the other subset we
397 identified, have also been described as memory cells with the ability to produce IL17A (56).
398 Therefore, both cell types may provide IL17A.

399 Here, we demonstrate that a combination of systems biology tools, advances in the field of
400 machine learning, and experimental investigation, provides a new and more efficient way to gain
401 biological insight from complex datasets, despite high sparsity.

402 **Materials and Methods**

403 **Subjects, sample and data collection**

404 All clinical studies were approved by the Stanford Institutional Review Board and performed
405 in accordance with guidelines on human cell research. Peripheral blood samples were obtained at
406 the Clinical and Translational Research Unit at Stanford University after written informed

407 consent/assent was obtained from participants. Samples were processed and cryopreserved by the
408 Stanford HIMC BioBank according to the standard operating protocols (57). All materials and data
409 were analyzed anonymously.

410 In this study, we used data from one hundred and eighty-seven healthy donors that were
411 enrolled in influenza vaccine studies at the Stanford-LPCH Vaccine Program from the 2007 to
412 2014. This included the following studies: SLVP015 (NCT01827462, NIAID ImmPort accession
413 number SDY212, data analysis described in (58)), SLVP017 (NCT02133781, NCT03020498,
414 NCT03020537), SLVP018 (NCT01987349, NCT03022396, NCT03022422, NCT03022435,
415 NCT3023176, data analysis published in (59)), SLVP021 (NCT02141581), SLVP028
416 (NCT03088904) and SLVP029 (NCT03028974). Individuals were selected for this study based on
417 the following criteria: (1) age range from 8-40 years, (2) received inactivated influenza vaccine
418 (IIV, Fluzone, intramuscularly), (3) only data from the first visit (some donors came in consecutive
419 years), (4) HAI titer measured and (5) information about gender and age available.
420 Exclusion/inclusion criteria, samples that were acquired with timepoints and analyses performed
421 are described in the study record details at website repository for clinical studies
422 (www.ClinicalTrials.gov) using provided identifiers. All the protocols for sample analysis such as
423 immunophenotyping and determination of signaling responses to stimulation using flow or mass
424 cytometry, HAI titer determination and determination of cytokines/chemokines in samples using
425 Luminex assay are available online (57). Additionally, immunophenotyping using mass cytometry
426 was published in Leipold and Maecker (60). Phosphoflow assay using flow cytometry (for studies
427 SLVP15, SLVP18 and SLVP21 from 2007 to 2011), was described in (58, 59) or for mass
428 cytometry (for study SLVP21 in 2013) (61). Luminex assay was described in (58, 59). The HAI

429 assay was performed on sera from day 0 and day 28 using a well-established method (62) and was
430 described before (2, 58).

431 All data used were analyzed and processed at the HIMC, as previously described (63) and
432 uploaded to the Stanford Data Miner (64). Briefly, data from both Luminex assays were
433 normalized at the plate level to mitigate batch and plate effects. The two median fluorescence
434 intensity (MFI) values for each sample for each analyte were averaged, and then log-base 2
435 transformed. Z-scores $((\text{value} - \text{mean}) / \text{standard deviation})$ were computed, with means and
436 standard deviations computed for each analyte for each plate. Thus, units of measurement were
437 $Z_{\log 2}$ for serum Luminex. For phospho-flow data acquired on flow cytometer a fold change value
438 was computed as the stimulated readout divided by the unstimulated readout (e.g. 90th percentile
439 of MFI of CD4+ pSTAT5 IFN α stimulated / 90th percentile of CD4+ pSTAT5 unstimulated cells),
440 while for data acquired using mass cytometry a fold change was calculated by subtracting the
441 arcsinh (intensity) between stimulated and unstimulated (arcsinh stim – arcsinh unstim). For
442 immunophenotyping using mass cytometer units of measurement were percentage of parent
443 population.

444 **Aggregation of data and generation of feature subsets**

445 The preprocessed data from Stanford influenza datasets were obtained from HIMC Stanford
446 Data Miner (64). This included total of 177 csv files, which were automatically imported to the
447 MySQL database to facilitate further analysis. Datasets were merged using shared variables, such
448 as Donor ID, Study ID, gender, age, race, Donor visit ID, Visit year, Experimental data (connected
449 to Donor visit ID), Assay, Name and value of the measured analyte. Data harmonization across
450 different clinical studies was accomplished by introduction of feature termed Visit internal ID,
451 which allowed us to discriminate between different visits of the unique donor in different years.

452 We standardized names of the vaccines, for example TIV and IIV3 were named Inactivated
453 influenza vaccine. Finally, we calculated the vaccine outcome parameter using HAI antibody titers.
454 High responders were determined as individuals that have HAI antibody titer for all vaccine strains
455 above 40 and geometric mean (GeoMean) HAI fold change >4 . The fold change is calculated as:
456 GeoMean HAI antibody titer for all vaccine strains on day 28 / GeoMean HAI antibody titer for
457 all vaccine strains on day 0. To facilitate analysis, vaccine outcome was expressed as binary value:
458 high responders were given value of 1, while low responders value zero.

459 The aggregated dataset contained 187 donors and 3,284 features, yielding a total of 614,108
460 datapoints. The dataset had 93.2% of missing values (572,081 missing values). To deal with
461 missing values, in the first step of the SIMON we implemented a novel algorithm, *mulset* that
462 allows for faster generation of datasets with all possible combinations of features and donors across
463 initial dataset. To efficiently compute shared features and find quickly similarity between donors,
464 *mulset* algorithm generated unique feature identifier for each donor. Then, intersection between
465 the identifiers was used to identify shared variables. The identified, shared variables are then
466 converted to unique shared features identifiers using hash function. Finally, data was exported
467 from the database according to the shared features. In total, *mulset* generated 45 different datasets.
468 To avoid proceeding with machine learning process using datasets with misleading results, we
469 removed datasets with less than 5 features and less than 15 donors. After applying that restriction,
470 11 datasets were deleted, and final analysis was performed on 34 datasets.

471 **Overview of SIMON - Sequential Iterative Modeling “Overnight”**

472 To identify baseline immune predictors that can discriminate between high and low
473 responders following influenza vaccination, we applied sequential, iterative modeling “overnight”,
474 shortly SIMON. The SIMON allows for dataset generation, feature subset selection, classification,

475 evaluation of the classification performance and determination of feature importance in the
476 selected models. The SIMON was implemented in R programming language (65) (**Data file S1**).
477 First in SIMON we automated the process of dataset generation using *mulset* algorithm as
478 described above. Next, each dataset was partitioned into 75% training and 25% test set with
479 balanced class distribution of high and low responders using the function *createDataPartition*
480 from the Caret package (31). To prevent evaluation of small test sets that would lead to misleading
481 performance parameters, datasets with less than 10 donors in test sets were discarded. Next, the
482 model training using 128 machine learning algorithms suitable for classification training (**Table**
483 **S6**) was initiated for each train dataset. Test sets were hold-out for evaluation of model
484 performance on unseen datasets. This step was crucial to prevent overfitting. All algorithms were
485 processed in an automated way through the Caret library (31). Each model was evaluated using
486 10-fold cross-validation (24) repeated 3-times. Additionally, performance of each model was
487 evaluated on the test set which was held out before model training by calculating performance
488 from a confusion matrix using available R package (66). Furthermore, contribution of each feature
489 to the trained model was evaluated and variable importance score is calculated as described (31).
490 All prediction metrics and performance variables are stored in the MySQL database for the final
491 exploratory analysis. Detailed description of the overall processes is as follows.

492 *Model training and performance evaluation.* For each dataset, model training was performed
493 on train set using 128 machine learning algorithms (**Table S6**). All algorithms were implemented
494 without any user-defined parameters and with the default tuning parameters, as described in the
495 Caret library (31). For each model we defined type of resampling. In this study 10-fold cross-
496 validation repeated three times was used. Each model was then evaluated by calculating
497 performance measures using the confusion matrix. Confusion matrix or contingency table is used

498 to evaluate the performance of a classification model on a set of data for which the true values are
499 known. The confusion matrix has four categories (see illustration below).

		Actual	
		Positive (High responder)	Negative (Low responder)
Predicted	Positive (High responder)	True positives (TP)	False negatives (FN)
	Negative (Low responder)	False positives (FP)	True negatives (TN)

500 True positives (TP) are cases in which classification model predicted they are high responders and
501 indeed those cases were high responders, while true negatives (TN) correspond to cases correctly
502 labeled as low responders. Finally, false negatives (FN) and false positives (FP) refer to low
503 responders or high responders that were incorrectly labeled. From a confusion matrix, to evaluate
504 classification models we calculated following performance measures. Accuracy, a measure how
505 often the classifier is correct was calculated as $(TP+TN)/\text{total number of observations}$. Specificity,
506 the proportion of actual negative cases (low responders) that were correctly identified was
507 calculated as $TN/(FP+TN)$, while sensitivity (also known as recall or true positive rate), the
508 proportion of actual positive cases (high responders) correctly labeled was calculated as
509 $TP/(TP+FN)$. To summarize the performance of classification models over all possible thresholds,
510 we generated the Receiver Operating Characteristic (ROC) curve by plotting the sensitivity (y-
511 axis) and the false positive rate (the proportion of low responders misclassified as high responders),
512 which was calculated as 1-specificity (x-axis). Finally, we calculated the area under the ROC curve
513 (AUROC) using an R package (66) and used this measure to summarize the performance of the
514 models. AUROC has values between zero and one, and higher values indicate better performance.
515 Value of 0.5 indicates a random classifier, and this was used as a cutoff to remove classifiers that
516 could not distinguish between high and low responders better than by random chance. In this study,

517 10-fold cross validation was applied three times, the AUROC was calculated for each repeated
518 iteration, and the average AUROC (and other measures) are reported as an overall quantitative
519 estimate of classification performance. Additionally, before model training, same seed for random
520 number generator was applied (*set.seed* 1234). This resulted in the uniformity where for each
521 model same resamples were used for performance evaluation. From this, we compared models and
522 evaluated which model was performing better in terms of AUROC values by comparing
523 performance of the resampling distributions using functions described in the Caret (31).

524 *Independent evaluation of the trained model.* The performance of each model was
525 additionally evaluated on the test set which was held out before training the model (25% of the
526 dataset). The performance on the test set was evaluated exactly as described for the train set above.
527 Confusion matrix was built and all the performance measures, including the AUROC, as for train
528 set were computed. Test AUROC was used to select models, in addition to train AUROC.

529 *Variable importance score.* Contribution of each feature to the model i.e. variable
530 importance score was calculated using the Caret library (31). Briefly, evaluation of the variable
531 importance was calculated directly from the model specific metrics and the variable importance
532 scores were scaled to have a maximum value of 100. Since in SIMON we utilized many different
533 algorithms, the contribution of each feature to the model was estimated using the methods
534 appropriate for each algorithm, as described in R packages (see reference list for the **Table S6**).

535 **Feature selection using Boruta algorithm**

536 To evaluate the all-relevant features for the selected top-performing models built on datasets
537 13 and 36, we used an R package Boruta (22). Boruta algorithm performs as a wrapper algorithm
538 around Random Forest (22). The method is suitable for selection of all-relevant features, and this
539 is accomplished by comparing original features' importance with importance achievable at random

540 (estimated using permuted copies of the original features, called shadow features). In each
541 iteration, Boruta removes irrelevant features and evaluates the performance of the model. Finally,
542 analysis is finished either when all features are confirmed or rejected or when Boruta reaches a
543 specified limit of runs. Boruta was performed using following parameters: maximal number of
544 importance source runs, *maxRuns* at 1000, *pValue* confidence level 0.05, a multiple comparisons
545 adjustment using Bonferroni method was applied (*mcAdj* set to TRUE), feature importance was
546 obtained using Random Ferns (function *getImpFerns*) and to ensure reproducibility of the results
547 we set the seed for the random number generator (*set.seed* 1337). Tentative features were also
548 included returned in the Boruta results (*withTentative* argument was set to TRUE).

549 **Peptide stimulation and intracellular cytokine staining using mass cytometry**

550 Thawed PBMC were rested in X-VIVO™ 15 medium (Lonza) supplemented with 10% FCS
551 and human serum AB (Sigma) for 2 days at 10^7 cells/ml in 24-well plate following “RESTORE”
552 protocol (67, 68). For stimulation assay, 5×10^6 PBMC were seeded in 96-well V-bottom plates
553 (10^6 PBMC/well) and stimulated overnight (12-16h) with the influenza overlapping peptide pool.
554 Influenza peptide pool contained 483 peptides (20mers with 11aa overlap, Sigma Aldrich)
555 spanning the entire influenza proteome from the influenza strain A/California/07/2009 (dissolved
556 in DMSO at 20mg/ml, working concentration 0.2ug/ml/peptide) and 24 peptides with HLA-
557 A*0201-specificity (9-10mers, Sigma Aldrich) generated against influenza proteins
558 (hemagglutinin, nucleocapsid protein, matrix protein 1, nonstructural protein 1 and 2) from the
559 influenza strain A/California/07/2009 using prediction software NetCTL-1.2 (69) (dissolved in
560 water or PBS/DMSO at 20mg/ml, working concentration 2ug/ml/peptide) (**Table S18**). In both
561 assay unstimulated sample was prepared in which only medium without peptides containing 0.5%
562 DMSO was added. Protein transport inhibitor cocktail (eBioscience/Thermo Fisher) and antibody

563 against CD107a were added at the beginning of the assay. After peptide stimulations, PBMC were
564 washed with the CyFACS buffer (PBS supplemented with 2% BSA, 2 mM EDTA, and 0.1% sodium
565 azide) and stained with surface antibody cocktail (**Table S17**), filtered through 0.1µm spin filter
566 with 20µL/sample of Fc block (ThermoFisher) for 30min at 4°C. Cells were then washed with
567 CyFACS buffer and incubated for 5min at RT in 1xPBS (Lonza) with 1/1000 diluted cisplatin
568 (Fluidigm). Cells were then incubated for 1h at RT (or left at 4°C overnight) in the Iridium-
569 intercalator solution in fixation and permeabilization buffer (BD Cytotfix/Cytoperm™, BD
570 Biosciences). Then cells were washed with 1x permeabilization buffer (BD Perm/Wash™, BD
571 Biosciences) and stained for 30min at RT with intracellular antibody cocktail diluted in 1x
572 permeabilization buffer (**Table S17**). Cells were fixed with BD Cytotfix/Cytoperm™ and left
573 overnight until analysis, or immediately used for mass cytometry. Immediately before starting the
574 analysis, cells were washed in CyFACS buffer, then PBS and finally with MiliQ water. Prior to
575 data acquisition, cells were resuspended in MilliQ water containing 1/10 diluted normalization
576 beads (EQ Four Element Calibration Beads, Fluidigm) to the concentration of less than 8×10^5
577 cells/ml to achieve an acquisition rate of 400 events/s on the CyTOF Helios mass cytometer
578 (Fluidigm). In each sample 1-1.5 million cells were acquired. After acquisition, data were
579 normalized with the reference EQ passport P13H2302 (70) and further data analysis was
580 performed using FlowJo v10.

581 **Statistical Analysis**

582 All the statistical parameters (sample size, statistical tests, and statistical significance) are
583 reported in the Figures and Figure Legends. Significance of differences in frequencies of the
584 immune cell subsets between high and low responders in the datasets was calculated using the
585 Significance analysis of microarrays (SAM) (71) at false discover rate (FDR) < 1%. Mass

586 cytometry data between two groups after peptide stimulation were analyzed using the one-way
587 ANOVA Kruskal-Wallis test followed by Dunn's multiple comparison test, while paired samples
588 within groups were compared with two-tailed Wilcoxon matched-pairs signed rank test.
589 Additionally, pairwise t-test with the Benjamini-Hochberg (B-H) correction for multiple testing
590 adjustment with 0.95 confidence level was used to evaluate changes in the cell frequencies after
591 vaccination within groups. Pearson's correlation coefficient was used to evaluate the correlations
592 between features from the top-performing models. The Corrplot package in R was used to calculate
593 correlation coefficients, statistics and for visualization of the correlation matrix (72). P-values were
594 adjusted for multiple comparisons by using the Benjamini-Hochberg correction (73). Statistical
595 analyses were performed with GraphPad PRISM 7.04 (Graph Pad Software) or in R, and p values
596 above 0.05 were considered not significant.

597 **Code and data availability**

598 The source code of the *mulset* algorithm is available from <https://github.com/LogIN-/mulset>.
599 The *mulset* is available as an R package in CRAN, a repository of open-source software. Pseudo-
600 code for SIMON is available as **Data file S1**. All data used in SIMON analysis are available from
601 the Stanford Data Miner (www.datamt.net). Mass cytometry fcs files related to Figure 4
602 (<https://zenodo.org/record/1328286>) are available on a research data repository Zenodo
603 maintained by OpenAIRE and CERN (www.zenodo.org).

604 **Supplementary Materials**

605 Fig. S1. Distribution of high and low responders included in the initial dataset based on gender,
606 CMV status and study year

607 Fig. S2. Assays performed across different clinical studies and study years.

- 608 Fig. S3. Staining profiles and gating scheme of immune cell subsets analyzed using mass
609 cytometry.
- 610 Fig. S4. Visualization of the initial dataset in the context of missing values.
- 611 Fig. S5. Performance evaluation of models build on datasets 13 and 36 after applying restriction
612 filters.
- 613 Fig. S6. Heatmap of the correlation coefficients calculated between features from the dataset 13.
- 614 Fig. S7. Importance of features determined by Boruta.
- 615 Fig. S8. Heatmap of the correlation coefficients calculated between features from the dataset 36.
- 616 Table S1. List of 102 analyzed immune cell subsets showing gating strategy.
- 617 Table S2. Immune cell subsets and phosphorylation of proteins identified using phosphorylated
618 cytometry.
- 619 Table S3. Cytokines, chemokines and growth factors analyzed by Luminex.
- 620 Table S4. Sparsity calculated by column.
- 621 Table S5. 34 datasets generated using intersections.
- 622 Table S6. List of machine learning algorithms implemented in SIMON.
- 623 Table S7. List of all models built and their minimal and maximal AUROC values.
- 624 Table S8. List of all models with minimal and maximal AUROC values after applying
625 performance restriction filters.
- 626 Table S9. List of models with maximal train and test AUROC.
- 627 Table S10. All models built on dataset 36 after restriction filters applied.

628 Table S11. All models built on dataset 13 after restriction filters applied.

629 Table S12. Characteristics of individuals with high and low response to influenza vaccination
630 selected in the dataset 13.

631 Table S13. List of features and their variable importance score in dataset 13.

632 Table S14. Characteristics of individuals with high and low response to influenza vaccination
633 used for experimental validations.

634 Table S15. Characteristics of individuals with high and low response to influenza vaccination
635 selected in the dataset 36.

636 Table S16. List of features and their variable importance score in dataset 36.

637 Table S17. Antibody panel for ICS mass cytometry.

638 Table S18. List of peptides in the influenza peptide pool.

639 Data file S1. Pseudocode for SIMON.

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876
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892 HIMC, analyzed the data and revised the manuscript. C.L.D. was responsible for regulatory
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897 CRAN, a repository of open-source software. Pseudo-code for SIMON is available as **Data file**
898 **S1**. All data used in SIMON analysis are available from the Stanford Data Miner
899 (www.datamt.net). Mass cytometry fcs files related to Figure 4

900 (<https://zenodo.org/record/1328286>) are available on a research data repository Zenodo
901 maintained by OpenAIRE and CERN (www.zenodo.org).

902

903 **Figure legends**

904 **Fig. 1. Study design.** (A) One hundred and eighty-seven healthy donors (8-40 years of age) were
905 recruited across eight consecutive influenza seasons. Data acquired at the baseline (day 0)
906 included phenotypical and functional state (phosphorylated proteins) of immune cells
907 analyzed using flow or mass cytometry and serum analysis using Luminex assay. Individuals
908 were labelled as high or low responders, depending on the HAI antibody titers determined
909 on day 28 after vaccination. (B) HAI antibody responses to influenza vaccine strains in high
910 (H, red) and low (L, grey) responders across years. Numbers below x-axis indicate the
911 number of donors in each group. HAI responses are shown as geometric mean titer (GMT)
912 calculated as a fold change between day 0 and day 28 after vaccination for all vaccine strains.
913 Violin plots show distribution of individuals. The line shows the median. Seroconversion is
914 defined as 4-fold increase in HAI titer for all vaccine strains (denoted by a grey line).

915

916 **Fig. 2. Automated feature subset generation using multi-set intersect function.** Schematic
917 example showing the initial dataset with four features and four donors. Missing values are
918 indicated by white circles. Missing values are present in such a way that either removal of
919 donors or features would result in no data for analysis. **(A)** Using a multi-set intersect
920 function, the *mulset* algorithm, identified shared feature sets between donors. First, for each
921 donor, the algorithm determined the unique feature ID. Second, using the intersect function,
922 it identified shared features, which were then converted to shared features ID using hash
923 functions. Finally, the *mulset* algorithm searched the database and identified donors with
924 shared feature sets. **(B)** *Mulset* generated ten distinct datasets with defined feature and donor
925 numbers, as indicated.
926

927 **Fig. 3. Automated feature selection and machine learning process integrated in SIMON.**

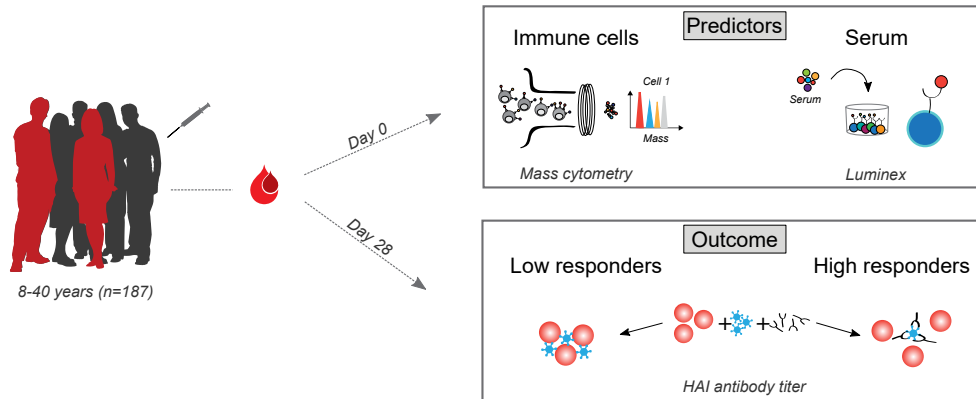
928 Before building a model, raw data were processed (cleaned, corrected, normalized, etc.)
929 using extract-transform-load (ETL) operations and the database was built. In the second step,
930 new features were created from the existing data, GMT of the HAI response was calculated,
931 and individuals were labelled as high or low responders. Third, datasets were generated using
932 multi-set intersection function. Each dataset was then used for model training in a fully
933 automated machine learning process, implemented in SIMON. Briefly, before training
934 started, each dataset was partitioned into training and test sets, which were excluded from
935 the model-building phase. Finally, in the exploratory analysis, each model was evaluated
936 based on its performance, and features were selected based on the importance score.

937

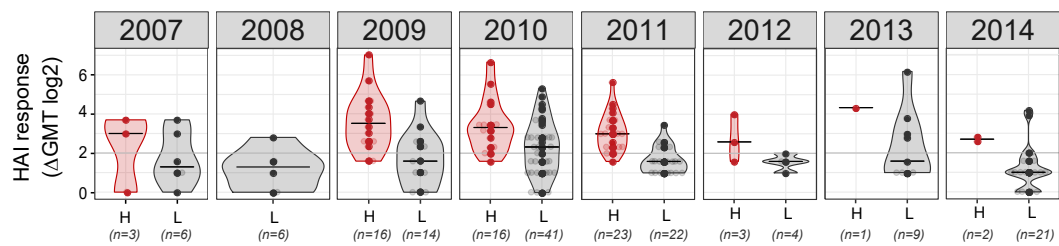
938 **Fig. 4. SIMON identifies cellular signature associated with the successful generation of**
939 **influenza immunity after vaccination. (A)** Features with variable importance score above
940 50 from the model built on dataset 13 are shown. **(B)** Raw data confirmed by SAM analysis
941 to be significantly changed in the donors from dataset 13, indicating frequency of cells (as a
942 percentage of the parent population). **(C)** Representative plot showing TNF α intracellular
943 staining of CD161⁺ CD4⁺ T cells in the unstimulated (-) or influenza peptide pool (+)
944 stimulated PBMC from high responder obtained before vaccination. Graph on the right
945 shows the frequency of TNF α ⁺ CD161⁺ CD4⁺ T cells from high responders (red circles) and
946 low responders (grey circles) in the samples before vaccination. Individual donors are
947 connected with lines. **(D)** Violin plots show distribution of frequency of CD161⁺ CD4⁺ T
948 cells and CXCR5⁺ CD8⁺ T with Tc2 and Tc17 phenotype in the PBMC samples derived
949 from high (red, n = 7) and low responders (grey, n = 7) analyzed before vaccination (-) and
950 on day 28 after vaccination (+). **(E)** Variable importance score of features selected in the
951 model built on dataset 36 with score above 50. **(F)** Significant immune cell subsets selected
952 by SAM analysis shown as raw data corresponding to donors from dataset 36, indicating
953 frequency of cells (as percentage of parent population). **(G)** Representative plot showing
954 IL17A intracellular staining of EM CD8⁺ T cells in the unstimulated (-) or influenza peptide
955 pool stimulated (+) PBMC from high responders, obtained after vaccination. The graph on
956 the right shows the frequency of IL17A⁺ EM CD8⁺ T cells from high (red circles) and low
957 (grey circles) responders in the samples after vaccination. **(H)** Violin plots show distribution
958 of frequency of CD4⁺ and CD8⁺ T cells, with indicated phenotypes analyzed in the PBMC
959 samples derived from high (red, n = 7) and low responders (grey, n = 7) before (-) and on
960 day 28 after (+) vaccination. Graphs shown in **(C, D, G and H)** represent combined data

961 from seven independent experiments. Violin plots show distribution of individuals. These
962 are represented by red circles for high responders and grey circles for low responders. The
963 line indicates the median. Statistical analysis between high and low responders was
964 performed with one-way ANOVA Kruskal-Wallis test followed by Dunn's multiple
965 comparison test. Analysis within groups before and after vaccination was calculated using
966 two-tailed Wilcoxon matched-pairs signed rank test. Significance in SAM analysis was
967 considered at $FDR < 0.01$. ns - not significant, * $p < 0.05$, ** $p < 0.01$.

A



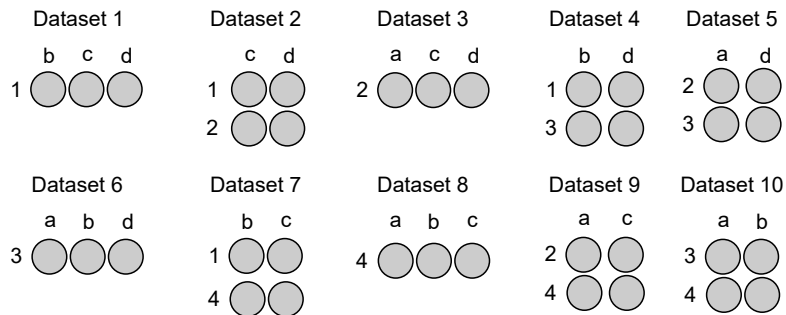
B

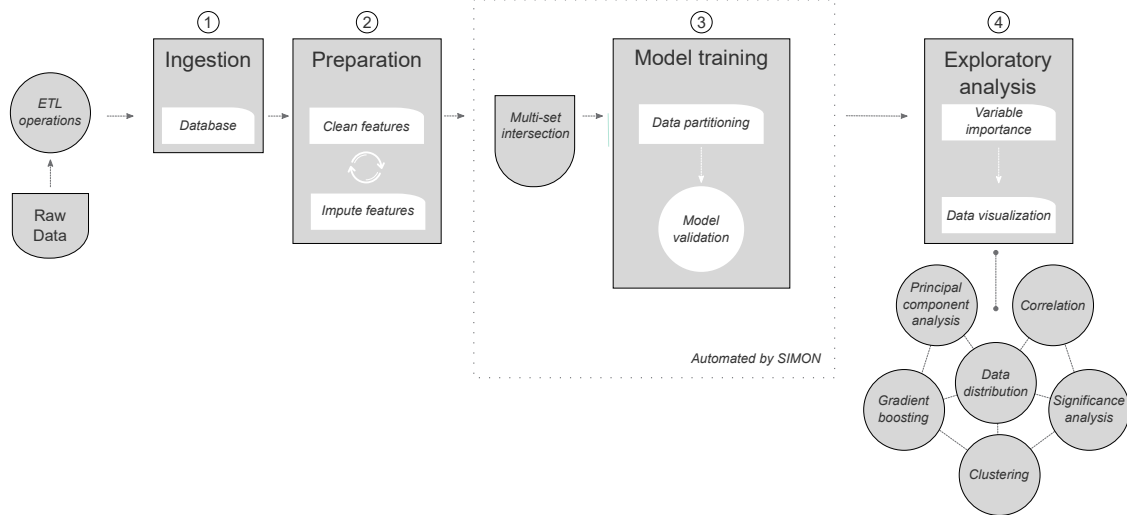




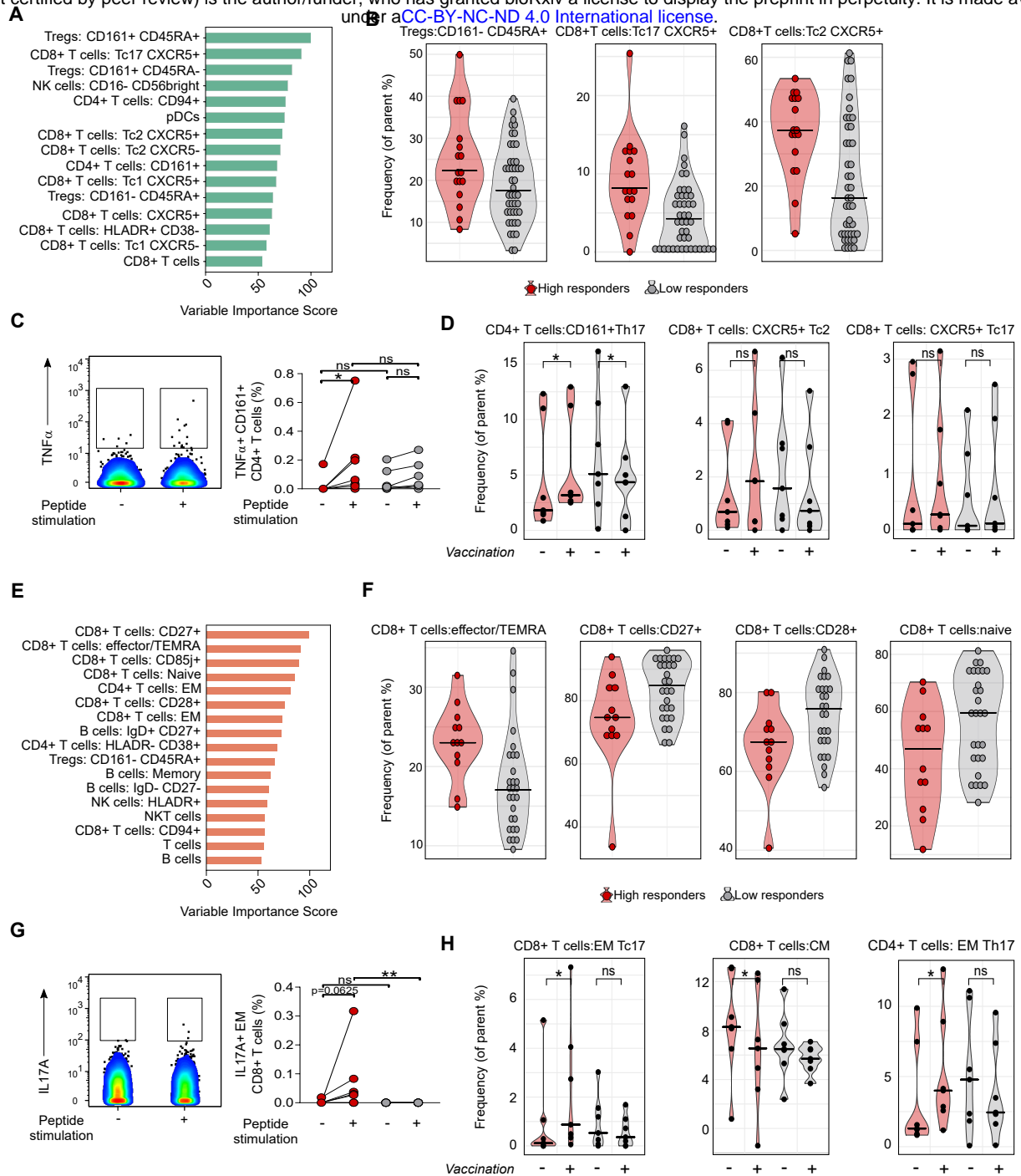
B

Generated datasets using multi-set intersect function





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Figure 3.



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Figure 4.