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Development of a strategy and computational application to select candidate protein analogues with reduced HLA binding and immunogenicity

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Summary

Unwanted immune responses against protein therapeutics can reduce efficacy or lead to adverse reactions. T-cell responses are key in the development of such responses, and are directed against immunodominant regions within the protein sequence, often associated with binding to several allelic variants of HLA class II molecules (promiscuous binders). Herein, we report a novel computational strategy to predict 'de-immunized' peptides, based on previous studies of erythropoietin protein immunogenicity. This algorithm (or method) first predicts promiscuous binding regions within the target protein sequence and then identifies residue substitutions predicted to reduce HLA binding. Further, this method anticipates the effect of any given substitution on flanking peptides, thereby circumventing the creation of nascent HLA-binding regions. As a proof-of-principle, the algorithm was applied to Vatreptacog α, an engineered Factor VII molecule associated with unintended immunogenicity. The algorithm correctly predicted the two immunogenic peptides containing the engineered residues. As a further validation, we selected and evaluated the immunogenicity of seven substitutions predicted to simultaneously reduce HLA binding for both peptides, five control substitutions with no predicted reduction in HLA-binding capacity, and additional flanking region controls. *In vitro* immunogenicity was detected in 21·4% of the cultures of peptides predicted to have reduced HLA binding and 11·4% of the flanking regions, compared with 46% for the cultures of the peptides predicted to be immunogenic. This method has been implemented as an interactive application, freely available online at http://tools.iedb.org/deimmunization/.

Keywords: antigen/peptides/epitopes; bioinformatics; MHC/HLA; regulation/suppression; T cell.

Introduction

Native and engineered proteins are widely used as drugs to treat a variety of different ailments. One of the issues often encountered in their use is that proteins, whether of self or non-self origin, are often associated with some degree of potential immunogenicity. This immunogenicity, in turn, may be associated with decreased efficacy and/or potential safety issues. Accordingly, developing the capacity to predict potential immunogenicity, affording strategies to decrease the potential immunogenicity of protein drugs, has been the subject of intense investigation.[1–25] Several

Abbreviations: AUC, area under ROC; EPO, erythropoietin; FN, false negative; FP, false positive; HLA, human leucocyte antigen; IEDB, immune epitope database; IL-2, interleukin-2; MCC, Matthew's correlation coefficient; PBMC, peripheral blood mononuclear cells; ROC, receiver operating curve; TCR, T-cell receptor; TN, true negative; TP, true positive

groups, including De Groot and co-workers, have worked on this subject and produced valuable contributions.[26–29]

Several different variables affect protein immunogenicity, including their propensity to form aggregates, the degree of difference from self, uptake by antigen-presenting cells and, as it is well established that HLA class II binding is a necessary (although not sufficient) condition for T-cell immunogenicity, their content in terms of HLA-binding epitopes. CD4 T-cell responses are mediated by HLA class II molecules and are involved in responses to exogenously administered proteins. For this reason, HLA class II binding is a particularly relevant variable in the context of protein immunogenicity.[30]

Several methodologies are available to measure and predict HLA binding, as reviewed elsewhere.[31,32] Prominent among the various approaches is the direct measurement of peptide-binding capacity using purified HLA molecules in quantitative assays, and prediction of binding using matrix, neural network and machine-learning algorithms.[33] Relatively few studies have defined the thresholds of measured or predicted binding associated with immunogenicity for class I[34,35] or more relevant to the current study, for certain HLA class II alleles.[36–39]

In the clinical setting, the application of methodologies to predict and reduce immunogenicity based on HLA-binding requires the development and validation of strategies to address HLA heterogeneity. HLA class II molecules are encoded by four main loci, namely DRB1, DRB3/4/5, DQ and DP, and each locus is polymorphic. Thousands of different HLA variants exist, and this high degree of diversity is reflected in most human populations in general, and patient populations in particular.[40] In a series of different studies we showed that HLA molecules encoded by each of these loci often have a significant degree of overlap, both inter-locus and intra-locus, in terms of their peptide-binding repertoire. Indeed, unbiased clustering analysis showed that HLA class II can be grouped into several main supertypic binding specificities.[41–43] Peptide epitopes binding multiple HLA alleles (promiscuous epitopes) account for most responses in the general population.[44–48]

We have previously shown that focusing on a subset of 46 HLA class II molecules most represented in the general population worldwide allows us to account for about 90% of HLA variants expressed.[49] We further showed that a subset of 27 alleles also allows for covering a substantial majority of HLA molecules.[41] Further, a method based on calculating the median HLA binding for a set of seven prototype alleles (the '7-allele method') was shown to be effective in predicting immunogenicity on a population level.[50] Here we evaluate different methods to predict reduced immunogenicity, describe the implementation of a methodology to select among different modifications, and provide a freely available online tool to accomplish this goal.

Materials and methods

Training data set

The training data set was extracted from a previously published erythropoietin (EPO) de-immunization study.[51] In that study, 15mer peptides with a 10-residue overlap were generated from EPO and tested in five donors. Based on interleukin-2 (IL-2) ELISPOT assays and binding affinities to 15 selected alleles, two regions were identified as highly immunogenic. Different amino acid substitutions were applied to these regions and peptides with minimum immunogenicity were selected.

Accordingly, the EPO data set consists of two groups of peptides. The first set pertains to the identification of immunogenic regions in the protein, and includes 31 peptides (15mers) spanning the entire EPO sequence. Of these 31 peptides, four were immunogenic in $\geq 50\%$ of the donors. These four peptides span two regions, encompassing residues 91–120 (region 1) and 136–150 (region 2).

A second set of peptides pertains to the selection of non-immunogenic variants, where Tangri *et al.* probed the capacity of 12 analogues of region 1 (targeting positions 101–115) and nine analogues from region 2 (targeting position 16–150) to confer reduced immunogenicity.[51] The sequence and immunogenicity of each analogue can be found in Table 1.

Prediction approach selection

We applied Immune Epitope Database (IEDB) -recommended (consensus, see ref. 52) methods to predict HLA class II binding capacity for three different sets of alleles: (i) the same 15 alleles described in the EPO paper,[51] (ii) the seven-allele subset described by Paul *et al.*[50] and (iii) the 26 most common and/or representative alleles,[41] referred as 26-allele method throughout this manuscript. The seven-allele method predicts immunogenicity based on the median centile predicted binding of seven alleles representative of the binding motifs most commonly recognized in the general human population.

Performance evaluation parameters

Performance of the different prediction approaches was evaluated calculating Matthew's correlation coefficient (MCC) and the area under the receiver operating characteristics curve (AUC) and the following parameters:

TP, non-immunogenic peptide predicted as non-immunogenic
FP, immunogenic peptide predicted as non-immunogenic
TN, immunogenic peptide predicted as immunogenic
FN, non-immunogenic peptide predicted as immunogenic
Sensitivity, ratio of correct predictions in non-immunogenic peptides

Table 1. Antigenic regions from erythropoietin and their mutant analogues

	Peptide sequence	% Responding donors	Immunogenicity
Region 1			
Wild	GLRSLTTLLRALGAQ	100	+
Analogue 1	GARSLTTLLRALGAQ	75	+
Analogue 2	GERSLTTLLRALGAQ	100	+
Analogue 3	GGRSLTTLLRALGAQ	75	+
Analogue 4	GPRSLTTLLRALGAQ	0	−
Analogue 5	GSRSLTTLLRALGAQ	100	+
Analogue 6	GLDSLTTLLRALGAQ	50	+
Analogue 7	GLGSLTTLLRALGAQ	75	+
Analogue 8	GLRRLTTLLRALGAQ	50	+
Analogue 9	GLRSDTTLLRALGAQ	50	+
Analogue 10	GLRSLTDLLRALGAQ	25	−
Analogue 11	GGRSLTDLLRALGAQ	0	−
Analogue 12	GSRSLTDLLRALGAQ	25	−
Region 2			
Wild peptide	DTFRKLFRVYSNFLR	100	+
Analogue 1	DTFRKDFRVYSNFLR	25	−
Analogue 2	DTFRKLFDVYSNFLR	75	+
Analogue 3	DTFRKLFGVYSNFLR	100	+
Analogue 4	DTFRKLFRDYSNFLR	50	+
Analogue 5	DTFRKLFRGYSNFLR	50	+
Analogue 6	DTFRKLFRRYSNFLR	75	+
Analogue 7	DTFRKLFRVYDNFLR	0	−
Analogue 8	DTFRKLFRVYSDFLR	75	+
Analogue 9	DTFRKDFRVYDNFLR	0	−

Peptide sequences belonging to two different antigenic regions of wild-type erythropoietin protein are shown together with all the tested mutant analogues. For each sequence, the percentage of responding donors and corresponding immunogenicity (arbitrarily defined as $\geq 50\%$ of the donors responding) are shown. The data were published previously[51] and are shown here for reference purpose only.

Specificity, ratio of correct predictions in immunogenic peptides

Accuracy, ratio of correct predictions in immunogenic and non-immunogenic peptides.

$$\mathrm{MCC} = (\mathrm{TP} \times \mathrm{TN} - \mathrm{FN} \times \mathrm{FP}) / \sqrt{(\mathrm{TP} + \mathrm{FN}) \times (\mathrm{TP} + \mathrm{FP}) \times (\mathrm{TN} + \mathrm{FP}) \times (\mathrm{TN} + \mathrm{FN})}$$

Development of an online tool

Python scripts for an automated pipeline were divided into two parts, where the first part selects predicted promiscuous binding regions in a protein and the second part applies and evaluates the predicted effect of various amino acid substitutions on the selected region/peptide (s). The scripts were written and implemented using the DJANGO platform (https://www.djangoproject.com/).

Experimental validation

For experimental validation we selected the Recombinant factor VIIa (Vatreptacog α) protein, where three mutations were made in the wild-type Factor VII to increase its bioactivity.[18,19] We predicted promiscuous binding regions in the mutated and wild-type factor VII sequences. From the immunogenic region (positions 291–310), several variants with decreased immunogenicity were predicted. For experimental validation, we selected 35 peptides that included 14 analogues with predicted reduced binding, along with seven neighbouring and four wild-type peptides. As controls, 10 analogues predicted with no decrease in HLA binding were included.

In vitro immunogenicity assays

We used donations of peripheral blood mononuclear cells (PBMCs) from five healthy adults from San Diego, CA. All donors provided written informed consent for participation in the study. This study was performed with approvals from the Institutional Review Board at the La Jolla Institute for Allergy and Immunology (protocols; VD-101-0513 and VD-059-0813). Peptides were purchased from A&A (San Diego, CA) as crude material on a small (1 mg) scale and resuspended in DMSO at a final concentration of 40 mg/m.

The PBMCs were isolated by density gradient centrifugation, according to the manufacturer's instructions. Cells were cryopreserved in liquid nitrogen suspended in fetal bovine serum containing 10% (volume/volume) DMSO. Culturing of PBMCs for *in vitro* expansion was performed by incubating in RPMI-1640 (Omega Scientific, Tarzana, CA) supplemented with 5% human AB serum (Gemini Bioscience, Liverpool, UK), 1% GlutaMAX (Gibco, Grand Island, NY), and 1% penicillin/streptomycin (Omega Scientific) in 24-well plates at a density of 2×10^6 cells per well. Peptides were added to the cultures at a concentration of 50 μg/ml. Every 3–4 days, 10 U/ml IL-2 (eBioscience, San Diego, CA) in medium was added to the cultures.

After 14 days of culture with individual peptides, the cells were harvested and re-stimulated with the peptides at 5 μg/ml in an interferon-γ and IL-5 dual ELISPOT as previously described.[53] To be considered positive, a peptide response had to match three different criteria: elicit at least 320 spot-forming cells per 10^6 PBMCs, stimulation index ≥ 2 and $P \leq 0{\cdot}05$ by Student's *t*-test or by a Poisson distribution test.

Results

Equivalent performance of various prediction strategies on the two main immunogenic regions of EPO

A previous study evaluated the immunogenicity of EPO for human T cells *in vitro*, identifying two main immunogenic regions.[51] Modification of these regions was then shown to reduce immunogenicity of the whole protein.[51] In that study, it was further demonstrated how the two main immunogenic regions could be identified on the basis of measured promiscuous binding affinity to 15 common HLA molecules. Figure 1(a) shows the previously reported and published EPO immunogenicity. The EPO data from Tangri *et al.* were chosen as they are the only data set, to the best of our knowledge, in which analogues of the given protein designed for lower HLA binding have also been experimentally tested for immunogenicity.

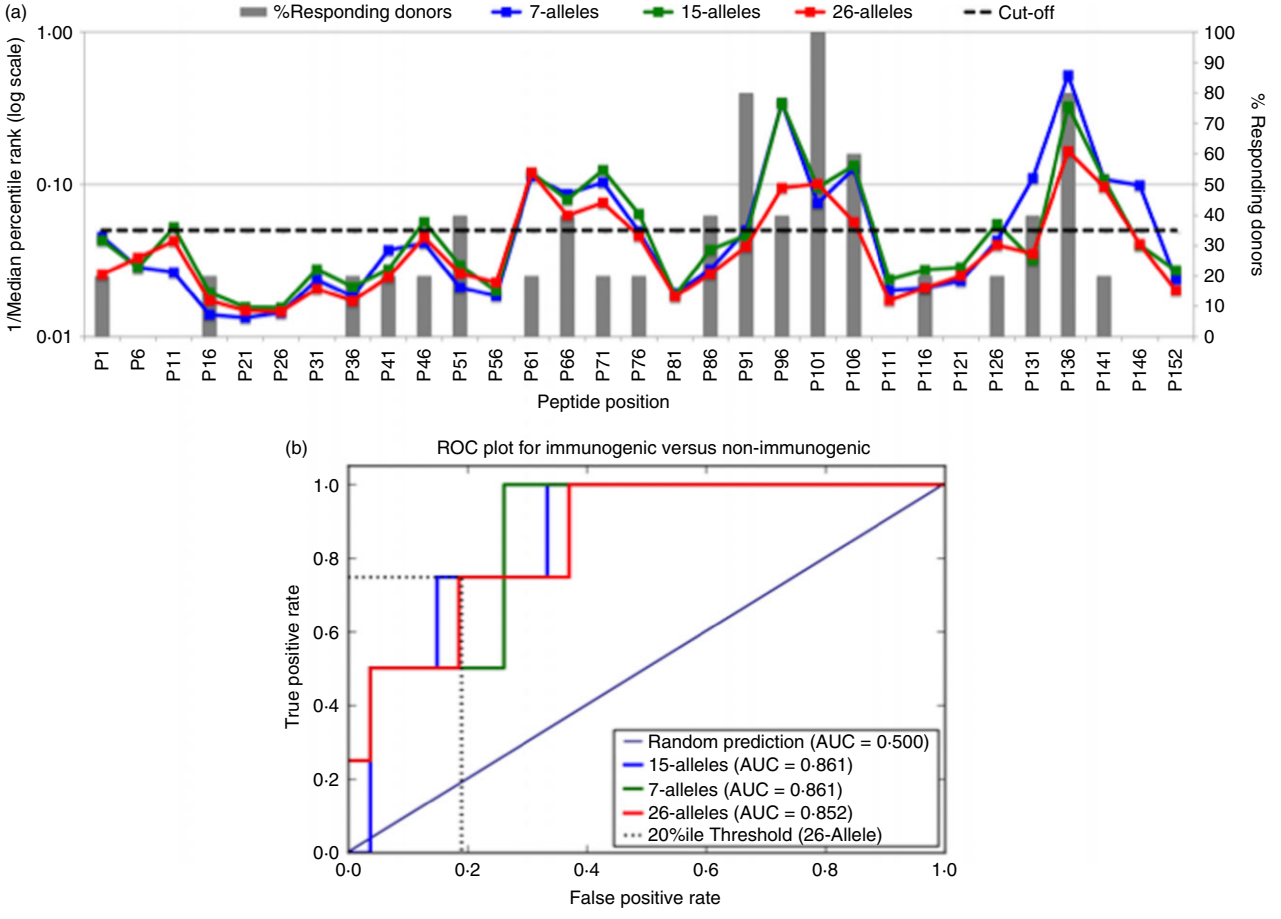

Figure 1. Comparison of erythropoietin (EPO) immunogenicity based on *in vitro* and *in silico* studies. (a) Prediction profiles of different *in silico* approaches to predict immunogenicity based on previous published experimental studies.[51] (b) ROC plots of the performances of median percentile rank for 7-allele, 15-allele and 26-allele methods. The dotted line shows the performance with chosen threshold (20%ile) for the 26-allele method. [Colour figure can be viewed at wileyonlinelibrary.com]

Herein, we wanted to evaluate several commonly available prediction methods for their capacity to identify these two main immunogenic regions. First, we used the 'IEDB-recommended' method[52] to predict the HLA-binding affinities of the various peptides for the same 15 alleles used in the EPO study above. Additionally, we evaluated the efficacy of predictions for a panel of 26 alleles most common in the general population,[41] and a recently described seven-allele subset (the '7-allele method'[50]), for identifying the protein regions that are bmost immunogenic at the population level, as described in the Materials and methods.

To visualize the results of the various methods, Fig. 1(a) shows the 1/median centile predicted values for the 15 alleles originally used, the 7-allele method, and for the 26-allele panel, each overlaid to the published immunogenicity values. The dotted line in the figure highlights the 1/median value of 0·05, which corresponds to a 20th centile median value, previously defined as a broad immunogenicity threshold for the general population.[37,38,45,50]

Receiver operating characteristics (ROC) plots provide a threshold-independent approach to compare the efficacy of different models. Based on the ROC curve, an AUC value can be calculated to express overall performance.[54] In this context, AUC values of 0·5 represent random performance, whereas a value of 1 is a perfect prediction. ROC plots and associated AUC values for the median centile for the 7-, 15- and 26-allele methods are provided in Fig. 1(b). From these results we concluded that the performance of the various methods is similar, with AUC values in the 0·852–0·861 range.

The nature of substitutions considered in de-immunization projects

Previous studies[46] have reported different strategies to generate amino acid substitutions for the purpose of reducing immunogenicity (for recent studies see refs 2,5,9,10,55–57). In several studies, alanine replacements have been used (see refs 12,25,56,58,59). The rationale for alanine replacement is that it is a relatively conservative or semi-conservative substitution for most amino acids, and as such is hypothesized to be less likely to interfere with the biological activity of the protein being considered. The potential drawback of using alanine is that the mild nature of the change might not impact HLA binding in a sufficiently decisive manner. An alternative approach, proposed by Tangri *et al.*,[51] was to introduce non-conservative substitutions as a targeted strategy to interfere with HLA binding. Here, we review the published data to gain additional insights into these issues.

Previous studies by Stern *et al.*[60] solved the X-ray crystal structure of the influenza HA 307–319 epitope and HLA DR1 complexed to a T-cell receptor (TCR). It was determined that the peptide residues in positions 1, 4, 6 and 9 of a 9mer core (corresponding to residues 309, 312, 316 and 319 of the peptide) were the primary HLA contact residues, while −1, 3 and 8 were the main TCR contact residues.

Independent studies determined the HLA-binding capacity of HA 307–319 analogues to DR1.[61] As summarized in Fig. 2, alanine substitutions only interfere with binding in position 1, but not any of the other three anchor positions (i.e. positions 4, 6 and 9). This is

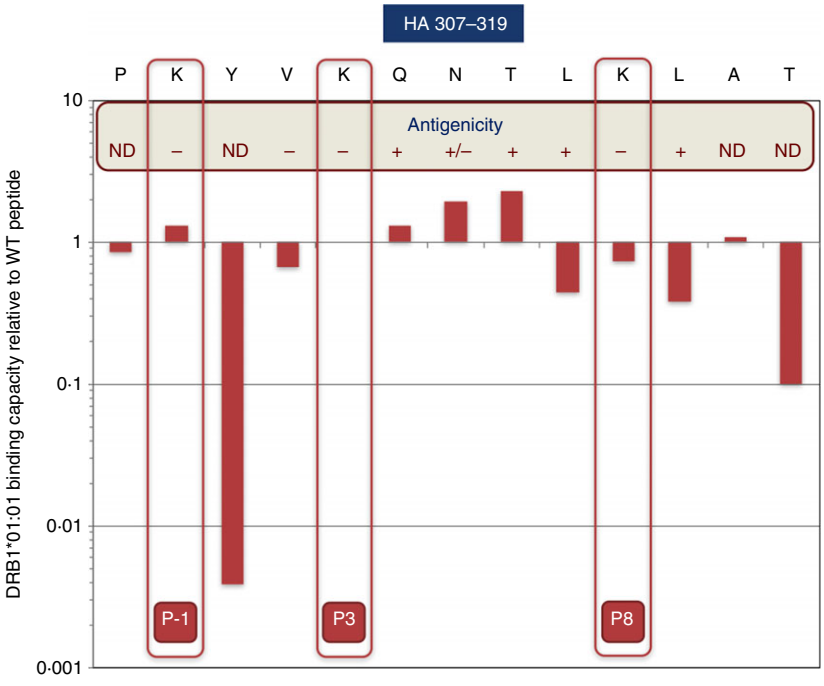

Figure 2. DRB1*01:01 antigenicity of alanine containing HA 307–319 analogues. The binding capacity for each amino acid residue after alanine mutation is shown in relation to the wild-type (WT) peptide. [Colour figure can be viewed at wileyonlinelibrary.com]

explained by the fact that alanine is a semi-conservative substitution for the native residues found in positions 4 (Q) and 9 (L), and is a conservative substitution for the T found in position 6 (an alanine substitution actually increases binding in this position). Conversely, by disrupting binding by more than 10-fold, non-conservative substitutions (K or E) correctly identify all four HLA contact residues.

At the level of T-cell recognition, the effect of alanine substitutions on most positions was investigated by Alexander *et al.*[62] The results, also summarized in Fig. 2, indicate that alanine substitutions abrogate T-cell recognition at all three main TCR contact residues. These findings were not limited to this particular HLA/epitope combination, as the same conclusions are reached by analysis of X-ray structures, binding and T-cell activation assays for the HA peptide in the context of DRB1*0401,[63,64] and DRB1*0701[65] and of MBP 85–99 epitope in the context of HLA DRB1*15:01.[66]

In conclusion, these results indicate that alanine substitutions are a feasible strategy to broadly interfere with TCR recognition. However, as HLA binding is retained in most instances, it is concerning that the analogue drug might elicit a new set of T cells with different TCR specificity. The data indicate that alanine substitutions are not a feasible strategy to broadly interfere with HLA binding, and non-conservative substitutions may be more suited to this purpose.

EPO analogues associated with reduced immunogenicity

The Tangri *et al.* study[51] described several single substitution replacements that were evaluated for their effect on immunogenicity. Here we tabulated these analogues (Table 1), recording their sequence and effect on immunogenicity. We define, for the purpose of our analysis, reduced immunogenicity as an instance where the response was reduced by more than 50% in 50% or more donors.

Evaluation of the 26-allele method to predict analogues associated with decreased immunogenicity

Next, we evaluated the capacity of the various methods described above to predict decreased immunogenicity in the Table 1 data set. For each method we recorded the number of true positives (TP), false positives (FP), false negatives (FN) and true negatives (TN). The MCC was also calculated for comparison between the different approaches tested. This measure is most appropriate when the data sets for validation are associated with binary outcomes (yes/no in terms of experimentally reduced immunogenicity and yes/no in terms of predictions). MCC can range from −1 to 1, where −1 indicates a perfect negative correlation, 0 means a random distribution and 1 indicates a perfect positive correlation. In general, MCC values of +0·70 or higher indicate a very strong positive relationship, +0·40 to +0·69 a strong positive relationship, +0·30 to +0·39 a moderate positive relationship, and values of +0·20 to +0·29 indicate a weak positive relationship.[67]

Preliminary analyses showed that the 26-allele method had a slightly better performance than the other methods. Accordingly, as criteria to predict decreased immunogenicity, in relative terms in comparison with the wild-type epitope, we considered the difference of the 26-allele median centile of a given analogue compared with the median for the wild-type sequence. The two EPO regions were evaluated separately or combined. Examining performance using different median value thresholds, a difference of 8·5 was found to be optimal. This cut-off was empirically defined. The data in Table 2 were given to evaluate the performance of our prediction model in both the immunogenic regions of EPO. The performance was evaluated in terms of sensitivity, specificity, accuracy and MCC, with MCC values of 0·5, 0·5 and 0·41 for Region 1, Region 2 and both regions combined. Based on these results we proceeded to implement an algorithm that, for any given analogue sequence, would automatically calculate the difference in the median of 26-allele method value between the wild-type potential epitope and the analogue in question, as described in more detail in the following sections.

A scheme to weigh the potential effect of introducing new epitopes in flanking regions

Any de-immunization strategy must account not only for decreasing the HLA-binding capacity of the targeted

Table 2. Performance of 26-allele methods for both regions of erythropoietin taken separately or together

Parameter	Region 1	Region 2	Both region together
TP	4	1	5
FP	4	0	4
TN	4	6	10
FN	0	2	2
Sensitivity	1·00	0·33	0·71
Specificity	0·50	1·00	0·71
Accuracy	0·67	0·78	0·71
MCC	0·50	0·50	0·41

Erythropoietin regions have been evaluated separately or combined to evaluate the decrease of immunogenicity predicted by the 26-allele method using different parameters (TP, true positive; FP, false positive; TN, true negative; FN, false negative; Sensitivity, Specificity, Accuracy and MCC, Matthew's correlation coefficient) as described in the Materials and methods section.

epitope, but also avoid the unintended creation of new epitopes in neighbouring sequences. In this context, amino acid substitutions in a 15mer peptide can also affect a maximum of two-neighbouring peptides in a protein sequence (as we are considering sets of sequential 15mer peptides overlapping by 10 residues). In some cases, namely when a potential epitope is at the C- or N-terminus of a protein, only one neighbouring 15mer is found, whereas in the case of an isolated peptide, no neighbouring 15mer is found.

To evaluate the potential impact of creating other immunogenic regions in neighbouring peptides, we have adopted a scheme that also scores neighbouring 15mers (Table 3). In this arbitrary schema, a lower score is associated with a more preferred analogue, specifically one predicted to have no effect on neighbouring peptides, or to confer reduced immunogenicity. The arbitrary score varies in range from 1 to 10, where the lowest score (i.e. 1) is given to an analogue where no neighbouring peptide is present (i.e. 15mer peptide subjected to de-immunization), as by definition, no neighbouring peptide can be affected by the substitution. The next lowest score (2) is given to the 'next-best' scenario, namely, only one neighbouring peptide is present and the analogues of such neighbouring peptides (either C- or N-terminal peptides) were also predicted to have reduced immunogenicity, based on a lower median score in comparison with the wild-type peptide. The next score would be 3, corresponding to the case in which both neighbouring peptides are present and are predicted to be associated with reduced immunogenicity. The next lowest score of 4 is given to analogues where, out of two neighbouring peptides, one is either absent or is predicted with reduced immunogenicity, while the other has no change in immunogenicity, and so on. The highest score is assigned to analogues where additional immunogenic sites are created in the neighbouring region. This schema (Table 3) is utilized to rank and evaluate the results of the de-immunization predictions in the predictive tool described in the following sections.

Table 3. Ranking strategy for scoring the potential de-immunized analogues of a given sequence with neighbouring peptides

Immunogenicity for Neighbouring peptide (1)	Immunogenicity for Neighbouring peptide (2)	Score
Absent	Absent	1
Absent	Reduced	2
Reduced	Reduced	3
Absent	Neutral	4
Reduced	Neutral	5
Neutral	Neutral	6
Absent	Increased	7
Reduced	Increased	8
Neutral	Increased	9
Increased	Increased	10

Predicted immunogenicity of neighbouring peptide in the two erythropoietin regions was evaluated and categorized as Absent (when no neighbour peptide is present), Neutral (when neighbour peptide median score is the same of the wild-type), Reduced (when neighbour peptide median score is lower than that of the wild-type) or Increased (when neighbour peptide median score is higher than that of the wild-type). A ranking score was assigned based on the combination of the two regions. Lower scores correspond to more preferred analogues.

Development of an interface for 26-allele method immunogenicity calculations

Overall, the de-immunization tool consists of a two-step process (Fig. 3): (i) identification of immunogenic region and (ii) evaluation of all analogues to identify those with decreased binding affinity and that do not affect neighbouring peptides. To facilitate implementing this process in the context of a generally usable protein de-immunization tool, we first developed a web application (Fig. 4) that accepts the sequence of proteins or peptides and automatically generates as output the specific regions that are predicted to be dominant epitopes at the population level. Median values of the predicted 26-allele centile are also reported.

Accordingly, in the input module, the protein sequence is entered, and the user can select a threshold/cut-off value for determining predicted immunogenic regions in that protein (Fig. 4a). A threshold value of 20% is used as the default, as it was previously defined as a broad immunogenicity threshold (derived *in vitro*) for the general population,[45] and also associated with optimal performance (in terms of MCC) in the case of data from the EPO study described above. However, the user can apply any threshold value that fits with their specific needs or context.

Our prediction strategy is based on fixed median values. We previously demonstrated that median values perform best in other immunogenicity predictions,[50,52] whereas the use of other parameters such as means or minima might bias the selection in favour of single 'outlier' values. Alternative methods in addition to medians and fixed values could be implemented in future versions of the tool.

After user input is submitted, an intermediate page (Fig. 4b) displays the potentially immunogenic regions predicted by the 26-allele method, automatically parsing sequences into 15mer peptides overlapping by 10 residues. This interactive page allows for selection of one or more of the various predicted peptides (15mers) for further analysis, which automatically redirects to a subsequent results page.

In the case of the EPO sequence example, this page at the default setting returns eight peptides, including the

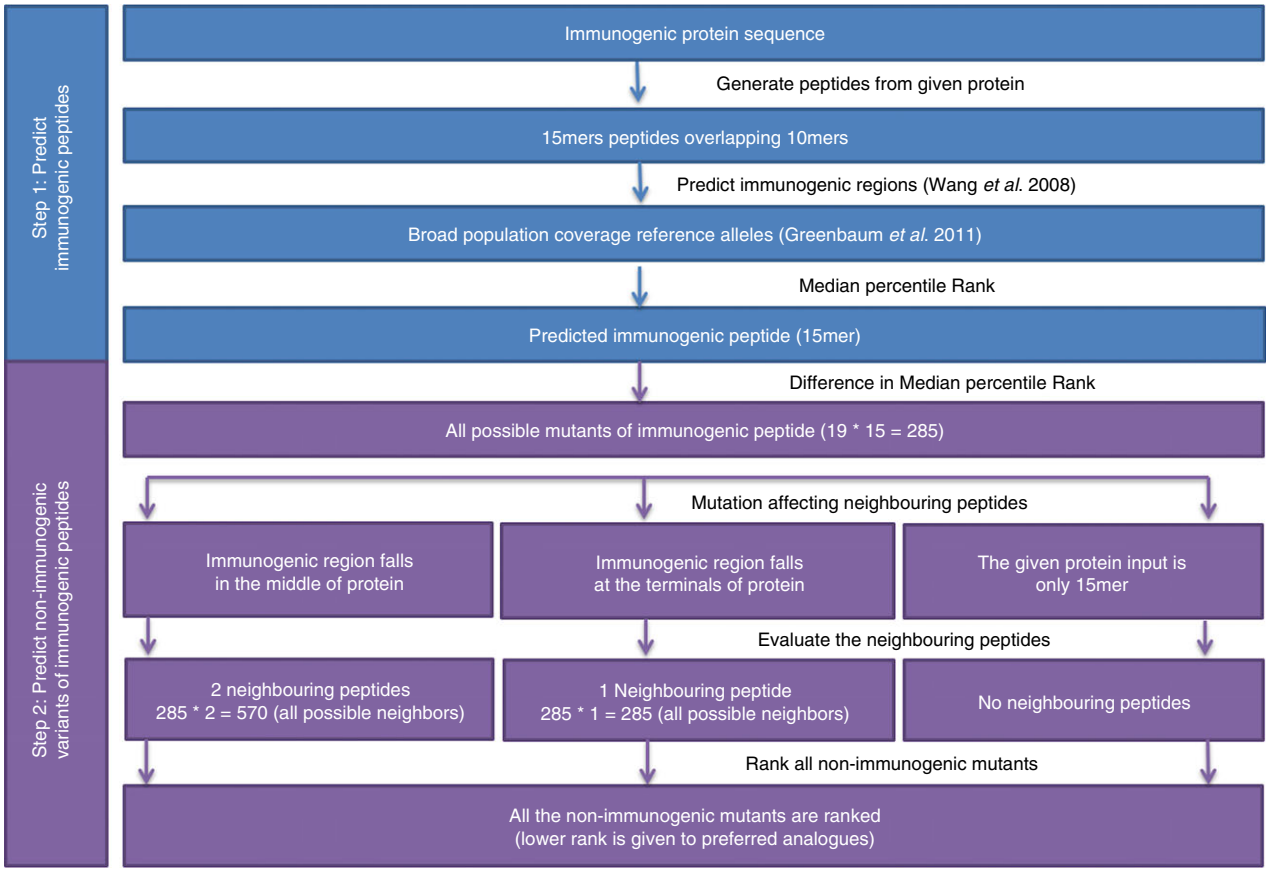

Figure 3. Schematic presentation of de-immunization tool. The de-immunization process is summarized in its two-step process. In the first step, the immunogenic regions are identified using the 26-allele method (median percentile rank), whereas in the second step all the possible variants of immunogenic peptides are screened to identify those with decreased (predicted) binding affinities, compared with the wild-type sequence. As a final step, all the lower binding mutants are then ranked according to their predicted effect on neighbouring peptides. [Colour figure can be viewed at wileyonlinelibrary.com]

two peptides (136–150 and 101–115) for which analogue substitutions were analysed experimentally in the Tangri study. Most of these eight are consecutive overlapping 15mers corresponding to three broad regions: 61–85, 96–120 and 136–155. Two of these regions correspond to the main immunogenic regions detected by the experimental analysis.

Output screen for the de-immunization tool

The algorithm automatically calculates the difference in median percentile rank for the 26-allele 'IEDB-recommended' method value between the wild-type potential epitope and each of the possible 285 analogues (15 positions × 19 possible substitutions). The results are summarized in tabular format, with the first row representing the wild-type peptide and the following rows representing the scores of the various analogues (Fig. 4c). For each analogue peptide sequence the median centile rank, as well as the difference in centile rank between wild-type and analogue, is shown. The output also shows the effect of the substitutions on neighbouring peptides (columns CN1, CN2, NN1, NN2). These columns represent the (one/two) neighbouring peptide(s) at the C and N termini, respectively (Fig. 4c). In the last column, a score that reflects the cumulative effect of the substitution on neighbouring peptides is also provided, where lower values are preferred for de-immunization. The summary table includes, by default, only analogues with a difference greater than 8·5, but the user can select and opt for different thresholds (more or less stringent) (Fig. 4c). The results are sorted according to the scheme shown in Table 3, and then by median difference for the 26-allele method.

As noted above, the tool operates in a two-step manner. The first step is to identify immunogenic peptides, which is relatively quick (less than 5 seconds for a 15mer). The second step is to evaluate all the possible variants of each 15mer, along with their flanking peptides (285 variants for a 15mer peptide). This step, in its present implementation, takes around 9 minutes (550·01 seconds to be exact). Considering the computational intensive nature of the calculation, we also offer to email the results to the user.

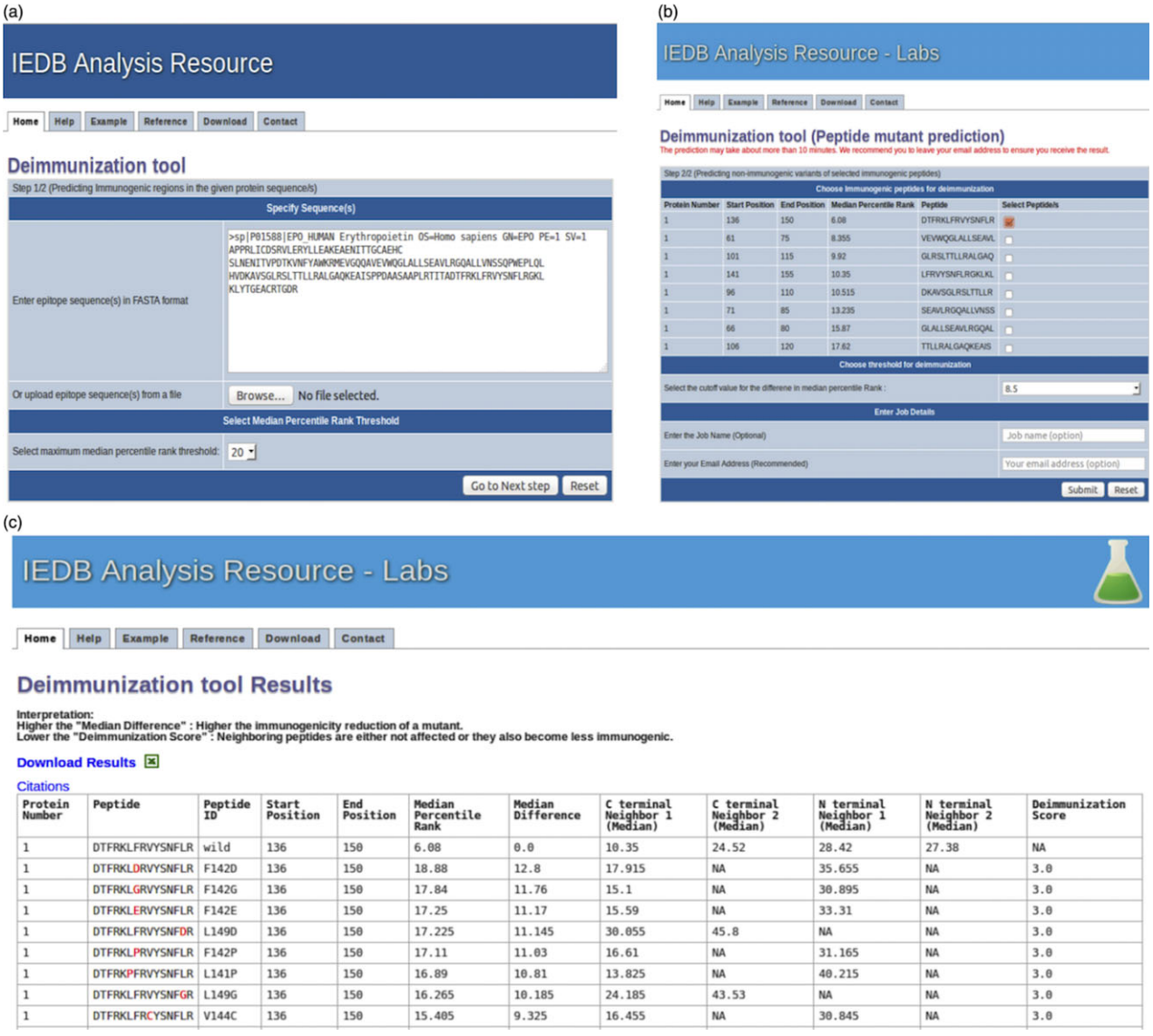

Figure 4. Web-tool interface for 26-allele method immunogenicity calculations. The web de-immunization tool interface is available in the IEDB Analysis Resource (http://tools-dev.internal.iedb.org/deimmunization/). The tool comprises an input page where sequence and parameter can be added or selected (a), an intermediate page displays the potentially immunogenic regions predicted (b) and a final page that summarizes the overall protein de-immunization results (c). [Colour figure can be viewed at wileyonlinelibrary.com]

Application of the de-immunization tool to select factor VII analogues with reduced immunogenicity

To illustrate the utility of the programme, we used the sequence of Vatreptacog α, an engineered factor VII molecule whose development was discontinued because of immunogenicity problems during human clinical testing.[68] In humans, the factor VII sequence is self, and hence largely non-immunogenic. However, the introduction of several mutations within the wild-type sequence to improve drug efficacy inadvertently rendered the drug immunogenic.[18,19]

When the Vatreptacog α sequence was run through the de-immunization tool, it was found that the 26-allele method predicted 10 peptides, corresponding to seven regions (as two contiguous peptides were predicted in the 196–215, 271–290 and 291–230 regions). Vatreptacog α was created with three mutations (V158D, E296V, M298Q) in the wild-type sequence. Out of these three mutations, two mutations were predicted to increase immunogenicity in the 291–310 region, spanning two of the predicted 15mers (position 291–305 and position 296–310) (Table 4).[69] The remaining six regions predicted to be immunogenic were identical to the wild-type factor VII sequence.

Accordingly, we have chosen the 291–310 region for experimental validation. This region, and specifically the

Table 4. Comparison of predicted peptides in wild-type and mutant Factor VII

		Wild-type factor VII		Mutant factor VII	
Start	End	Median	Sequence	Median	Sequence
166	180	10·91	WQVLLLVNGAQLCGG	10·91	WQVLLLVNGAQLCGG
181	195	11·54	TLINTIWVVSAAHCF	11·54	TLINTIWVVSAAHCF
196	210	12·39	DKIKNWRNLIAVLGE	12·39	DKIKNWRNLIAVLGE
201	215	15·18	WRNLIAVLGEHDLSE	15·18	WRNLIAVLGEHDLSE
241	255	9·595	HDIALLRLHQPVVLT	9·595	HDIALLRLHQPVVLT
271	285	15·325	RTLAFVRFSLVSGWG	15·325	RTLAFVRFSLVSGWG
276	290	17·55	VRFSLVSGWGQLLDR	17·55	VRFSLVSGWGQLLDR
291	**305**	**11·47**	**GATALELMVLNVPRL**	**6·965**	**GATALVLQVLNVPRL**
296	**310**	**15·615**	**ELMVLNVPRLMTQDC**	**15·75**	**VLQVLNVPRLMTQDC**
381	395	18·095	SQYIEWLQKLMRSEP	18·095	SQYIEWLQKLMRSEP

Start and end sequence positions of predicted wild-type and mutant analogues are shown together with their sequence, and their relative median score. Peptides shown in bold type (291–305 and 296–310) have different residues in wild-type and recombinant Factor VII and were therefore selected for de-immunization analysis.

nested 291–305 peptide, was ranked first within the 10 predicted peptides with a median value of 6·965. It must be noted, however, that not all predicted regions were immunogenic, probably because these other regions are 100% self, and so not recognized due to T-cell tolerance.

For further analyses, we selected two peptides (291–305 and 296–310) covering the predicted immunogenic region, and generated the list of analogues expected to be associated with reduced immunogenicity. In the case of peptide 291–305 we identified 32 different analogues that are predicted to be associated with reduced immunogenicity (Table 5A). Of those, 13 represented substitution of L297, one of L296, two of Q298, seven of L300, eight of L305 and one for R304. In the case of peptide 296–310, 45 substitutions were predicted to have reduced immunogenicity (see Supplementary material, Table S1). These substitutions correspond to seven different positions (V297, V299, L300, N301, V302, R304, L305).

Scrutinizing the two sets we observed that 25 (see Supplementary material, Table S2) different substitutions were predicted to have detrimental effect for both predicted epitopes, and also to have favourable profiles in terms of neighbouring peptides (scored as a '3'; see Table 3). These 25 substitutions would therefore be predicted as the most promising for further testing. Four positions (L297, L300, R304 and L305) accounted for all 25 substitutions.

Experimental validation of the de-immunization tool's predictions

To experimentally validate the output of the de-immunization tool, we next synthesized and tested in *in vitro* T-cell immunization assays a panel of peptide analogues based on the 291–305 and 296–310 wild-type epitopes, and including the two neighbouring peptides (one C-terminal, one N-terminal) (Table 5B). The panel included analogues predicted to be associated with decreased immunogenicity for both epitopes. The set of analogues encompassed seven substitutions for each epitope, made at four different positions (297, 300, 304 and 305). In addition, for each of those seven substitutions, we synthesized seven neighbouring peptides, as only one neighbouring peptide is affected by each common substitution from the two selected 291–305 and 296–310 peptides. Finally, as controls, 10 peptides – representing five substitutions, each in the two different frames (291–305 and 296–310) – predicted (based on increased median affinity) to be associated with no decrease in immunogenicity were included.

All peptides were tested for immunogenicity with human PBMCs from five normal donors using a 2-week *in vitro* re-stimulation protocol,[53] followed by ELISPOT assays measuring the number of cells secreting interferon-γ or IL-5 in response to a 20-hr stimulation with each specific peptide. Given the small number of donors and peptides tested, compiled data are shown in Fig. 5(a, b). The results for individual peptide data are provided in the Supplementary material (Table S3). In the case of control peptides, positive responses were detected in 46% of the cultures of those controls predicted to be immunogenic, but only in 21·4% of the decreased immunogenicity analogues and 11·4% of the flanking region controls (Fig. 5a). When response magnitude is considered, the corresponding (geometric mean) responses were 704, 445 and 360 spot-forming cells, respectively (Fig. 5b). One-tailed Mann–Whitney *U*-tests of control versus decreased immunogenicity analogues was associated with significant differences for both frequency and magnitude of response (Fig. 5a, b).

Table 5. List of mutant Factor VII peptides evaluated for immunogenicity. De-immunization analysis performed on peptide regions 291–305 of Vatreptacog α sequence. (A) List of sequences with predicted substitution to reduce immunogenicity in the peptide 291–305. Median values, differences with respect to the wild-type sequence, the effect on neighbour peptides (columns CN1, CN2, NN1, NN2) and relative score are shown. (B) Three groups of peptide analogues (immunogenic, non-immunogenic and neighbour peptides for the non-immunogenic) have been selected for further experimental validation

(A) Peptide	Sequence	Median	Difference	CN1	CN2	NN1	NN2	Scores
Wild	GATALVLQVLNVPRL	6·965	0	15·75	40·405	20·815	40·665	NA
L297P	GATALVPQVLNVPRL	29·03	22·065	29·435	NA	23·83	NA	3
L297G	GATALVGQVLNVPRL	25·1	18·135	27·95	NA	23·7	NA	3
L297D	GATALVDQVLNVPRL	24·91	17·945	27·255	NA	23·08	NA	3
L297C	GATALVCQVLNVPRL	24·585	17·62	25·09	NA	26·23	NA	3
L297E	GATALVEQVLNVPRL	24·11	17·145	27·09	NA	21·56	NA	3
Q298P	GATALVLPVLNVPRL	21·57	14·605	21·09	NA	21·055	NA	3
L300C	GATALVLQVCNVPRL	19·75	12·785	34·225	NA	22·085	NA	3
Q298G	GATALVLGVLNVPRL	19·33	12·365	19·14	NA	24·805	NA	3
L305D	GATALVLQVLNVPRD	18·515	11·55	37·665	65·34	NA	NA	3
L297R	GATALVRQVLNVPRL	18·29	11·325	25·43	NA	22·115	NA	3
L297H	GATALVHQVLNVPRL	18·155	11·19	24·295	NA	22·655	NA	3
L305E	GATALVLQVLNVPRE	18·115	11·15	35·605	63·515	NA	NA	3
L297Q	GATALVQQVLNVPRL	17·87	10·905	24·265	NA	22·45	NA	3
L305C	GATALVLQVLNVPRC	17·81	10·845	35·94	66·38	NA	NA	3
L305P	GATALVLQVLNVPRP	17·8	10·835	31·08	65·785	NA	NA	3
L297T	GATALVTQVLNVPRL	17·69	10·725	26·78	NA	23·695	NA	3
L297K	GATALVKQVLNVPRL	17·48	10·515	24·235	NA	21·955	NA	3
L300W	GATALVLQVWNVPRL	17·39	10·425	22·125	NA	21·93	NA	3
L297S	GATALVSQVLNVPRL	17·365	10·4	25·305	NA	21·695	NA	3
L300R	GATALVLQVRNVPRL	17·125	10·16	26·415	NA	21·92	NA	3
L305G	GATALVLQVLNVPRG	16·625	9·66	33·365	65·88	NA	NA	3
L305N	GATALVLQVLNVPRN	16·37	9·405	32·895	64·735	NA	NA	3
L300H	GATALVLQVHNVPRL	16·295	9·33	29·685	NA	21·9	NA	3
L300G	GATALVLQVGNVPRL	16·29	9·325	31·105	NA	22·51	NA	3
L297N	GATALVNQVLNVPRL	16·26	9·295	24·82	NA	22·45	NA	3
L297A	GATALVAQVLNVPRL	16·155	9·19	25·695	NA	21·335	NA	3
L300K	GATALVLQVKNVPRL	15·905	8·94	20·79	NA	21·935	NA	3
L305W	GATALVLQVLNVPRW	15·825	8·86	23·81	41·925	NA	NA	3
L305Q	GATALVLQVLNVPRQ	15·815	8·85	29·325	63·305	NA	NA	3
V296P	GATALPLQVLNVPRL	15·61	8·645	18·375	NA	24·135	NA	3
L300P	GATALVLQVPNVPRL	15·485	8·52	40·37	NA	21·165	NA	3
R304G	GATALVLQVLNVPGL	15·48	8·515	24·575	44·165	NA	NA	3

(B) Analogues	Start	End	Sequence	Median
Control analoges with no decrease in immunogenicity				
V299M	291	305	GATALVLQMLNVPRL	4·35
P303A	291	305	GATALVLQVLNVARL	4·625
A294F	291	305	GATFLVLQVLNVPRL	4·715
V299F	291	305	GATALVLQFLNVPRL	5·005
A294Y	291	305	GATYLVLQVLNVPRL	5·17
V299Y	296	310	VLQYLNVPRLMTQDC	6·435
V302S	296	310	VLQVLNSPRLMTQDC	7·73
Q298F	296	310	VLFVLNVPRLMTQDC	7·85
Q298Y	296	310	VLYVLNVPRLMTQDC	8·085
V302A	296	310	VLQVLNAPRLMTQDC	8·46
Analogues with predicted reduced immunogenicity				
L297P	291	305	GATALVPQVLNVPRL	29·03
	296	310	VPQVLNVPRLMTQDC	29·435

Table 5 *(Continued)*

(B) Analogues	Start	End	Sequence	Median
L297G	291	305	GATALVGQVLNVPRL	25·1
	296	310	VGQVLNVPRLMTQDC	27·95
L300G	291	305	GATALVLQVGNVPRL	16·29
	296	310	VLQVGNVPRLMTQDC	31·105
L300R	291	305	GATALVLQVRNVPRL	17·125
	296	310	VLQVRNVPRLMTQDC	26·415
R304G	291	305	GATALVLQVLNVPGL	15·48
	296	310	VLQVLNVPGLMTQDC	24·575
L305E	291	305	GATALVLQVLNVPRE	18·115
	296	310	VLQVLNVPREMTQDC	35·605
L305D	291	305	GATALVLQVLNVPRD	18·515
	296	310	VLQVLNVPRDMTQDC	37·665
Neighbouring peptides for non-immunogenic analogues				
L297P	286	300	QLLDRGATALVPQVL	23·83
L297G	286	300	QLLDRGATALVGQVL	23·7
L300G	286	300	QLLDRGATALVLQVG	22·51
L300R	286	300	QLLDRGATALVLQVR	21·92
R304G	301	315	NVPGLMTQDCLQQSR	44·165
L305E	301	315	NVPREMTQDCLQQSR	63·515
L305D	301	315	NVPRDMTQDCLQQSR	65·34

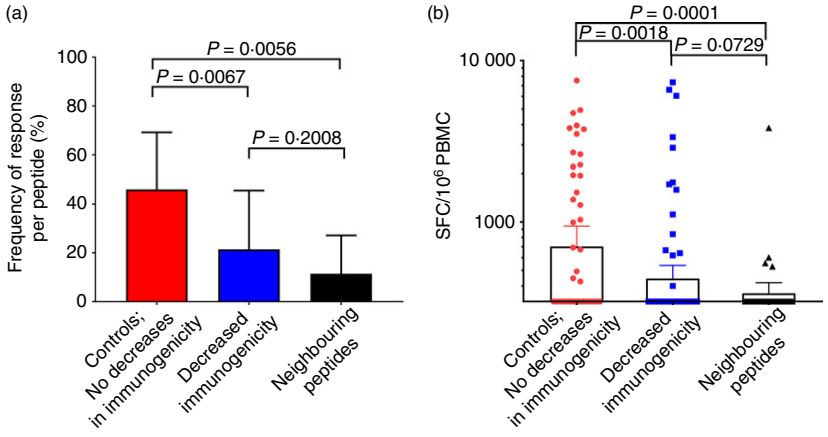

Figure 5. T-cell reactivity against peptides with increased or decreased immunogenicity. *In vitro* ELISPOT reactivity measured as the sum of interferon-γ (IFN-γ) and interleukin-5 (IL-5) producing cells after 2 weeks stimulation of peptides with no decreased immunogenicity (red), decreased immunogenicity (blue) or belonging to neighbouring peptides (black). (a) Frequency of responses per peptide analysed expressed as mean $\pm$ SD. (b) Magnitude of responses per peptide analysed expressed as geomean with 95% CI. Statistical analyses are performed using one-tailed Mann–Whitney U-test. [Colour figure can be viewed at wileyonlinelibrary.com]

Discussion

The present report represents, to the best of our knowledge, the first freely accessible method to automatically identify substitutions of potential value in terms of de-immunization of protein sequences, in general, and protein drugs in particular. The tool, available to the public through the IEDB's Analysis Resource (http://tools.iedb.org/deimmunization/), is designed to allow user flexibility, as different cut-offs can be selected, if the user wishes to deviate from the default settings.[70–72]

Protein immunogenicity is of serious concern in the context of protein immunotherapeutics.[7] T cells are key players in determining protein immunogenicity, directly through hypersensitivity reactions, and indirectly through providing the T-cell help required for immunoglobulin switch and generation of high-affinity antibody responses through affinity maturation. There is a clear appreciation

for the fact that T-cell immunogenicity is a multifactorial process. Main factors include protein availability and pharmacodynamics, protein uptake and processing from immune cells, binding to HLA molecules and recognition from a suitable repertoire of antigen-specific T cells.

Although each of these factors can be targeted and modified for the purpose of decreasing immunogenicity, our approach specifically targets HLA-binding capacity. As such, we emphasize that our method is potentially complementary and synergistic with methodologies aimed at modulating other variables. Our approach is based on the notion that HLA-binding affinity is a key requisite for T-cell immunogenicity. Several studies indicate that HLA binding is necessary (albeit not sufficient) for T-cell immunogenicity, and in that respect, if HLA-binding activity is decreased or abolished, a corresponding decrease in immunogenicity is to be expected.[6] The validation studies that we report herein, also in the context of previous studies using EPO as a model system, demonstrate that this is indeed the case.[51]

The purpose of this tool is to afford the capacity to predict immunogenicity at a population level, to include not only the DRB1 locus, but also accounting for DP and DQ loci as previously described in the 7-allele method.[50] The tool is not designed to predict immunogenicity of a specific allele, for which purpose a completely different strategy involving testing HLA-matched donors and using HLA-specific predictions would be recommended. To ensure broader HLA allele coverage, we selected the 26-allele method[41] instead of the 7-allele method, as they have shown equivalent performance. Importantly, we find that the best substitutions to significantly reduce HLA-binding capacity are in general non-conservative substitutions. This is in agreement with the bulk of immunochemical knowledge, which demonstrates that conservative substitutions are unlikely to significantly affect binding, even if introduced at key anchor positions. This has relevance in terms of the potential use of these substitutions, and additional experimental tests and/or structural simulations are required to select which of these substitutions is compatible with retaining protein function and drug potency.

Other de-immunization studies also based on introducing single amino acid substitutions into known epitopes have been reported in the literature (see, for example, refs 2,5,9,10,12,25,55–59, as cited above). In those studies, mild and conservative substitution strategies, such as 'alanine scans', were shown to be effective. These types of conservative substitutions are probably effective due to interference with TCR recognition (and have been routinely used to map TCR contact residues). At the same time, however, and as shown in Fig. 2, because conservative substitutions do not reliably and significantly interfere with MHC binding, they are subsequently vulnerable to the elicitation of a new set of T cells. By contrast, our approach takes into consideration non-conservative substitutions that are able to disrupt both HLA binding and TCR recognition.

At the same time, it is possible that whereas conservative substitution strategies may elicit a new wave of T cells that recognize a different set of TCR accessible residues on the same epitope region, they may have an important advantage of being less likely to perturb protein function. Further, it is also possible that the use of 'low profile' substitutions, such as alanine, might alleviate this problem, as it was shown that HLA class II restricted T cells often focus on large and charged amino acid side chains.

It is important to emphasize that modification of HLA-binding capacity does not address the central issue of T-cell tolerance. T cells are in general tolerant or non-reactive to self-protein sequences, and therefore any modification of self-protein sequences can lead to increases in immunogenicity because the molecule is perceived as non-self and promptly recognized by the immune scrutiny. In this light, protein modification of totally self-molecules should be used judiciously, and determinations of self/non-self status should be pursued in parallel.

This is a key feature of our approach as it provides an actionable strategy that can be implemented in the general population. Algorithms exist to predict binding capacity for several hundred different allelic variants expressed at different loci, but to search for substitutions simultaneously affecting such large numbers of alleles is not practical. Further, the impact of different substitutions must be envisioned at a global population level. Several previous studies in single-allele or genetically inbred animal models have provided evidence to support the concept that reduced binding can confer reduced immunogenicity. What is required, however, is the ability to 'jump' to human patient populations associated with a large degree of heterogeneity, in general, and at the level of HLA class II in particular. Previous studies have defined sets of alleles that are representative of HLA diversity on the one hand, and the extensive repertoire convergence existing at the level of peptide-binding specificity of HLA class II molecules on the other.[41] Our approach provides a strategy to identify candidate amino acid substitutions for decreased immunogenicity, globally applicable to heterogeneous populations.

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Disclosures

The authors declare no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Predicted substitutions in the peptide 296–310 for immunogenicity reduction.

Table S2 Predicted common substitutions to reduce immunogenicity with the highest score from both the peptides.

Table S3. Experimental validation of the deimmunization tool's predictions.

