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*Research Article*

# TCPro: an In Silico Risk Assessment Tool for Biotherapeutic Protein Immunogenicity

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***Abstract.*** Most immune responses to biotherapeutic proteins involve the development of anti-drug antibodies (ADAs). New drugs must undergo immunogenicity assessments to identify potential risks at early stages in the drug development process. This immune response is T cell-dependent. *Ex vivo* assays that monitor T cell proliferation often are used to assess immunogenicity risk. Such assays can be expensive and time-consuming to carry out. Furthermore, T cell proliferation requires presentation of the immunogenic epitope by major histocompatibility complex class II (MHCII) proteins on antigen-presenting cells. The MHC proteins are the most diverse in the human genome. Thus, obtaining cells from subjects that reflect the distribution of the different MHCII proteins in the human population can be challenging. The allelic frequencies of MHCII proteins differ among subpopulations, and understanding the potential immunogenicity risks would thus require generation of datasets for specific subpopulations involving complex subject recruitment. We developed TCPro, a computational tool that predicts the temporal dynamics of T cell counts in common *ex vivo* assays for drug immunogenicity. Using TCPro, we can test virtual pools of subjects based on MHCII frequencies and estimate immunogenicity risks for different populations. It also provides rapid and inexpensive initial screens for new biotherapeutics and can be used to determine the potential immunogenicity risk of new sequences introduced while bioengineering proteins. We validated TCPro using an experimental immunogenicity dataset, making predictions on the population-based immunogenicity risk of 15 protein-based biotherapeutics. Immunogenicity rankings generated using TCPro are consistent with the reported clinical experience with these therapeutics.

**KEY WORDS:** anti-drug antibodies (ADA); computational approaches; immunogenicity; protein-based therapeutics.

## INTRODUCTION

Biotherapeutic proteins are used to treat a wide variety of diseases, ranging from the rare (e.g., hemophilia) to the common (e.g., diabetes and cancer). An important safety and efficacy concern with biotherapeutic proteins is the risk of immunogenicity. For some biotherapeutics, the prevalence of anti-drug antibodies (ADAs) in the patient population can be as high as 87% (1). ADAs sometimes affect the biological function of the biotherapeutic, and these are called neutralizing ADAs (nADAs) or neutralizing antibodies (NABs). However, even antibodies that do not directly affect the function of the proteins (sometimes referred to as binding antibodies) can enhance, or impede, the clearance of the therapeutic, and thus affect a drug's pharmacokinetics (PK) and/or the pharmacodynamics (PD) (2–4). In rare cases, immune responses can be severe, resulting in hypersensitivity reactions and even death (5–7). The economic costs of immunogenicity to patients and the healthcare system are high. In the clinic, immunogenicity may require dose escalations (8), alternative treatments (9), frequent and expensive testing, hospitalizations (10,11), and other events. During drug development, immune responses can result in drug disapprovals (3,12,13). The immunogenicity-related risk of failure reduces industry incentive to develop products that address unmet medical needs of patients with rare diseases (12,14). The decisions are complex and require an understanding of the severity of the immune response in the fraction of the patient population likely to be affected, as well as alternative options available to the physician (15).

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Non-clinical immunogenicity assessments are very useful. They can be used to select candidate drugs in early stages of development (14,16). In the clinic, such assays can help to identify individuals and sub-populations at greater risk of developing ADAs (17). It has been scientifically established that T-helper cells (Th; $CD4^+$ T cells) responding to therapeutic-protein epitopes are necessary to developing clinically meaningful ADA responses. These epitopes are presented to Th cells by human leukocyte antigen (HLA) molecules (major histocompatibility complex class II (MHC-II)) on the surface of antigen-presenting cells (APCs). Consequently, current strategies for determining immunogenicity risk mainly rely on measurement of immunological events that lead to the proliferation of antigen-specific T cells (16). In *ex vivo* systems, measurement of antigen-mediated proliferation of T cells is possible. Moreover, this measurement is the culmination of both antigen presentation by MHCII molecules and engagement of the peptide-MHC-II complex with antigen-specific T cell receptors (18,19). ADA responses are ultimately the product of (a) the generation of the relevant peptide-HLA complex, (b) antigen presentation to and activation of cognate T cells, (c) presentation and interaction of antigen-specific B cells with T-helper cells. This leads to the differentiation of naïve B cells to the antibody secreting plasma cells, as well as memory B cells. The measurements of these assays reflect the recall/memory of T cells when they respond to previously exposed therapeutics.

Given that T-helper cells have been shown to be necessary for eliciting sustained high-titer ADAs (20), assays that measure antigen-mediated T cell proliferation play an important role in the non-clinical risk assessment of immunogenicity (21,22). These assays are expensive and time-consuming, and their success is highly dependent on the quality of cells (16). In addition, T cell epitopes are MHC-restricted (23). The same foreign neo-sequence will engage differently with different MHCII variants. As MHC variants are diverse in the human genome, obtaining samples from a sufficient number of donors to represent the population vis-à-vis MHCII coverage can be challenging (24). Computational models for T cell responses following exposure to a neo-antigen are highly desirable to address an unmet need of non-clinical immunogenicity risk assessment. Such models could provide a cost-effective initial screening step for neo-sequences during the development of therapeutic proteins. Moreover, the model(s) could also be used to supplement information obtained from *ex vivo* assays by generating and simulating a much larger virtual donor cohort. The virtual donor cohort can thus include many more MHCII variants and align the frequencies of the individual MHCII variants to those found in a specific population (or sub-population) of interest.

In this paper, we present TCPro (the T cell proliferator), a computational tool simulating *ex vivo* peptide-induced T cell activation assays (time course thymidine incorporation and IL-2 ELISpot). TCPro employs non-linear ordinary differential equations (ODEs) to model the *in vivo* dynamics of T cell response to antigenic peptides. TCPro incorporates distinct features that allows emulating the antigen-presenting dendritic cell (DC) and $CD4^+$ T cell population dynamics and assay uncertainties (e.g., initial immune cell counts) in a typical *ex vivo* T cell assay. It uses in silico peptide-MHCII binding affinity predictions (25) and approximate cell numbers in assay wells to estimate antigen-induced T cell stimulation and, in turn, immunogenicity risk. We validated TCPro's cohort-level predictions against a dataset of *ex vivo* assays on 13 drugs with varying levels of immunogenicity. For a separate set of drugs with known frequencies of ADAs, we also analyzed impact of geographical regions and individual variations of MHCIIs in drug immunogenicity. Taken together, these results demonstrate the utility of TCPro in clinically relevant risk assessments of biotherapeutics.

## RESULTS

### Rationale and Description of the Model

To quantify potential immunogenicity of a protein-based drug, TCPro emulates *ex vivo* T cell assays (cytokine ELISpot and [$^3$H]-Thymidine incorporation) that are used to measure drug-dependent T cell activation. TCPro predicts whether a protein-based drug can trigger a response from $CD4^+$ cells of a human subject using three inputs: (1) amino acid sequence of the drug, (2) ratio of drug-specific $CD4^+$ cells in the subject's $CD4^+$ cell pool ($F_P$), and (3) HLA-DRB1 alleles of the individual (Fig. 1a). Based on the drug sequence, drug-HLA complex binding affinity is calculated using NetMHCIIpan version 3.2 (25). Initial drug sample concentration in the assay wells is assigned the same amount as used in the *ex vivo* assays (see "Materials and Methods" for details).

Next, using a set of non-linear ODEs, we model the time courses of the DCs and $CD4^+$ cells in artificial *ex vivo* assay wells. For each virtual human subject, we randomly assigned, within scientifically reported ranges (Tables S2 and S3 in S1 Text), an initial dendritic cell maturation signal concentration ($MS_0$) and initial counts of dendritic cells and naive $CD4^+$ cells in the peripheral blood mononuclear cell (PBMC) sample of the subject. TCPro simulates the scenario in the absence of the drugs as baselines. For the scenario with the drug present (drug simulations), the ODEs take $F_P$, $K_D$, and the drug concentration parameters as additional inputs. For each subject, an $F_P$ value is randomly sampled from a population distribution for $F_p$.

Each model simulation generates numbers of proliferated and activated (secreting IL-2) $CD4^+$ cells in an assay well as functions of time. The actual *ex vivo* experiments are often conducted as triplicate and sextuplicate wells. Therefore, for proliferation tests, three simulations, each for 5 to 8 days, are performed to mimic triplicate wells in the *ex vivo* assay. For the IL-2 cytokine secretion assay, six simulations, each for 8 days, are performed to mimic sextuplicate wells in the assay. For each simulated well, the model randomly assigns a PBMC density within the range of $4–6 \times 10^6$ PBMC per mL sample to match the experimental conditions. Hence, the numbers of predicted proliferating (or cytokine secreting) cells vary among simulated wells of a test subject. We compared the distributions of the cell counts generated from the drug simulations with those from the corresponding baseline (no drug) simulations. For the proliferation tests, if the distribution of cell counts in the drug simulations is significantly higher (ratio of the mean cell counts > 1.9 and $p$ value < 0.05) than that of the baseline in at least one of the measurement days (e.g., day 5), the drug is considered immunogenic for this individual, based on the proliferation test. A similar comparison is conducted for the IL-2 secretion test. Finally, if the subject is predicted to respond to the drug both in

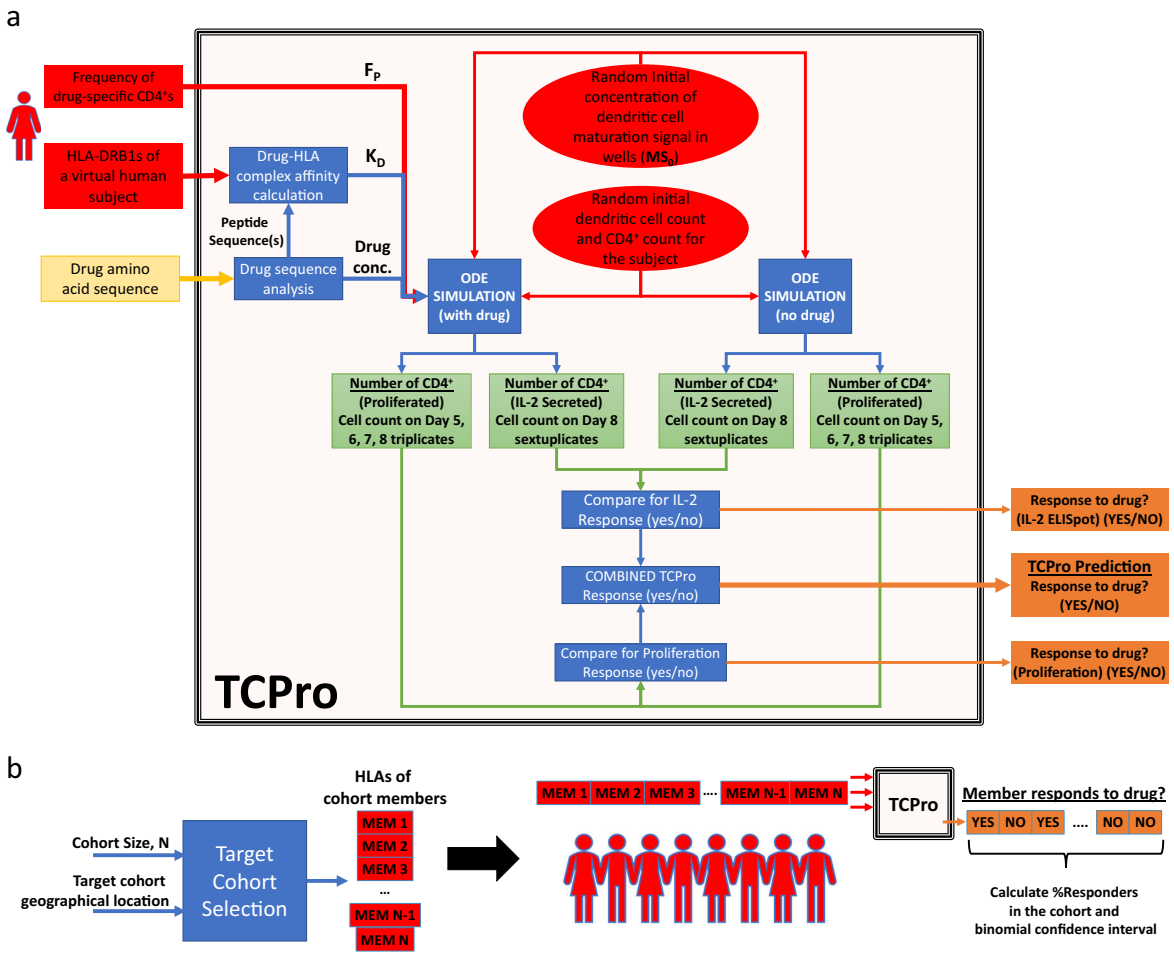


**Fig. 1.** TCPro workflow to predict the potential immunogenicity risk of a protein-based biotherapeutic on a population. As inputs, TCPro uses a set of HLA alleles of a target study cohort, the peptide sequence of the biotherapeutic and the ratio of the $CD4^+$ T cells in human blood that are drug-specific. **a** The schematic for individual-level simulation workflow for the drug immunogenicity prediction. Using a mathematical model based on ordinary differential equations, TCPro simulates the temporal dynamics of a cohort member's $CD4^+$ T cells in artificial *ex vivo* assay wells in the presence and absence (baseline) of the biotherapeutic. By comparing the final counts of drug-activated T cells to the baseline/no-drug response (see "Materials and Methods"), the model generates binary (yes/no) immunogenicity prediction for each individual. **b** For the population-level drug immunogenicity prediction, a target cohort with known HLA alleles is required as model input. The allelic distribution may vary by ethnicity. TCPro predicts the percentage of the cohort members responding to a given drug based on cohort HLAs

the IL-2 and the proliferation tests, TCPro labels the drug immunogenic for the subject based on combined assay results (see "Materials and Methods" for details).

To simulate the immunogenicity risk of a drug at the population level, a target cohort (e.g., the World or North American population) and cohort size *N* (Fig. 1b) must be selected as model inputs. Then, TCPro obtains a set of HLA-DRB1 molecules for each cohort member based on known distribution of HLA alleles of the cohort and predicts the percentage of the cohort members whose $CD4^+$ cells would respond to the studied drug. TCPro runs 100 iterations of this simulation to obtain statistical estimates on the predicted %Responders.

## Comparison of TCPro Prediction with Experimental Blood Clotting Factor VII (FVIIa) Dataset

T cell activation measured in *ex vivo* assays is associated with *in vivo* immune responses (26). In an *ex vivo* $CD4^+$ T cell assay, the blood donor cohort is selected based on the MHCII haplotypes that represent a population of interest (the world population is used as the default). Next, the blood samples are depleted of $CD8^+$ T cells and the remaining cells are treated with drugs in assay wells for 5 to 8 days. As a part of validation of TCPro, we used a set of experimental data containing *ex vivo* $CD4^+$ T cell assay results for 10 short peptide fragment samples (PEP A to J) from mutated human factor VIIa protein and three control samples (humanized anti-A33 antibody, exenatide, and keyhole limpet hemocyanin) administered on PBMC samples of a cohort of healthy donors. Immunogenicity of the samples was measured with two types of assays, namely the [$^3$H]-Thymidine incorporation-based proliferation assay and IL-2 secretion-based ELISpot assay. These two assays measure orthogonal markers for the response of a $CD4^+$ cell to a drug sample. The radioactive thymidine incorporation assay has greater accuracy, while ELISpot assay has higher sensitivity. In the laboratory, the drug-triggered T cell response is typically evaluated based on the combined results of the two assays.

We evaluated the immunogenicity prediction performance of TCPro at the cohort population level by comparing TCPro's predictions of percent responders with experimental data described above. We compared the results of TCPro independently with the results from proliferation (Fig. S6 a and b in S1 Text) and the ELISpot (Fig. S7 a and b in S1 Text) assays as well as the combined results from the two assay types (Fig. 2a and b). Comparing with combined results from the two assays, the overall mean absolute percent error (MAPE) of the TCPro-predicted median cohort responses is 4.2%. The discrepancy between the prediction of TCPro and two individual assays is greater. MAPE of the TCPro-predicted median cohort responses is 8.9% when comparing with the results from proliferation assay alone and 11.7% when comparing with the results from the ELISpot assay alone. We used only the combined ELISpot/proliferation test when presenting drug immunogenicity risk in the following sections.

### Analysis of Correlation of TCPro Predictions with Preclinical and Clinical Measurements of Immunogenicity

Percent drug immunogenicity predictions of *ex vivo* T cell response assays typically correlate well with the percentage of patients who develop antibodies against the drugs in clinical studies. To analyze that correlation for TCPro's immunogenicity predictions, we first obtained the HLA DRB1 alleles of 1272 healthy donors from the multinational 1000 Genomes study dataset (27) (S2 Dataset) which served as our virtual patient cohort. Next, we searched drugs with available information on their respective population frequencies of drug-specific CD4$^+$ cells (S3 Dataset) and amino acid sequences. We found 15 drugs satisfying that criteria. Eight of the drugs (adalimumab, etanercept, infliximab, ixekizumab, natalizumab, rituximab, secukinumab, ustekinumab) are known to directly alter immune cell behavior. The remaining seven (A33, bevacizumab, erythropoietin, exenatide, kogenate, trastuzumab, vatreptacog-α) are non-immunomodulatory. Last, for the set of drugs, we obtained published percent *ex vivo* experimental assay data and clinical immunogenicity data from clinical drug trials and observational studies and compared those with the TCPro's predictions for the world cohort ($N = 1272$) (Table I). There are many factors affecting quantification of antibodies developed against a drug *in vivo*, such as the assay method, route of administration, and patient disease status. However, we found that TCPro predictions correlated with the *ex vivo* assay and clinical immunogenicity outcomes. Also, TCPro in general predicted a lower percent of cohort immunogenicity for our set of immunomodulatory drugs, when compared with the non-immunomodulatory drugs.

### Predictions of TCPro Are Most Sensitive to the Frequency of Drug-Specific CD4$^+$ Cells

Next, to better understand the effects of different parameters on the immunogenicity predictions of TCPro, we ran a parameter sensitivity analysis in silico for PEP D as an example. We run the simulations with the lower and upper bond values of model parameters (Table S3 in S1 Text) and then calculated how much that variation would affect the predicted percent cohort response. We observed that the

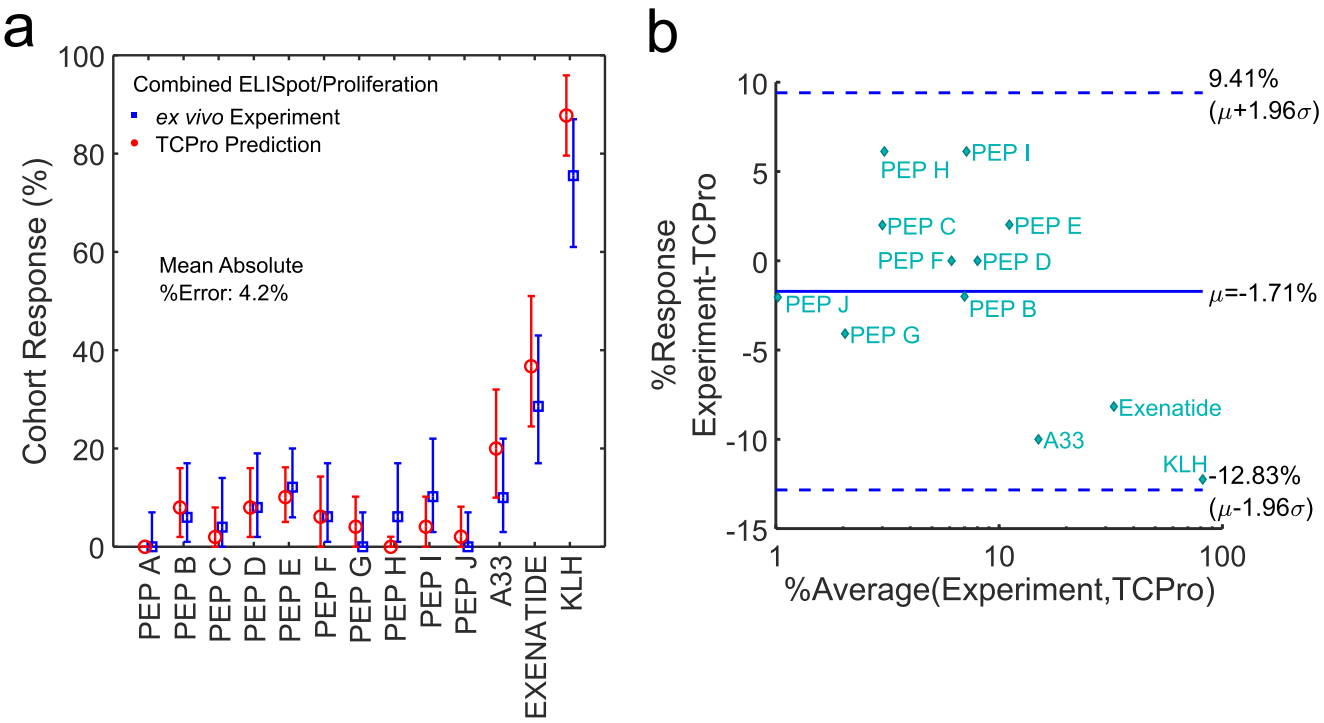


**Fig. 2.** **a** Using TCPro, we simulated a total of 2,340,000 assay wells for the set of drug-donor pairs with variable assay conditions (such as random initial numbers of immune cells in the wells and maturation signal concentration). The median cohort responses (circles for TCPro and squares for proliferation and ELISpot assays combined) and 95% binomial confidence intervals (lines) are shown for each peptide sample tested. **b** Bland-Altman plot is used to show the difference in median percent responders between experimental observation from ELISpot/Proliferation assay combined (blue squares in a) and the predictions from TCPro predictions (red circles in **a**). Normal distribution 95% confidence interval bounds are shown with blue dashed lines

**Table I.** Comparison of preclinical (*ex vivo* T cell activation assays) and clinical (ADA) drug immunogenicity along with TCPro predictions on the World ($N = 1272$) cohort from (27). When available, only the percent clinical immunogenicity data for moderate- or high-titer patients or persistent ADA+ patients are shown on the %Clinical immunogenicity data column

| Drug | Target(s) | % Immunogenicity | | Clinical trial details | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | TCPro (95% CI) | *Ex vivo* assays (95% CI)[REF.] | Clinical (95% CI) *N*: cohort size | Route | Assay method | Cohort geography | Cohort disease | Ref. |
| A33 | GPA33 | 19.97 (17.77, 22.17) | 10 (2, 18)[a] | 16.67 (7.41, 27.78), $N = 54$ | IV | SPR | USA | Metastatic CRC | (29,30) |
| | | | 18 (8, 30) (28) | 33.33 (8.33, 58.33), N = 12 | | | Australia | CRC | (31) |
| Bevacizumab | VEGF | 0 | 10 (2, 18) (1) | 0, $N = 25$ | IV | ELISA | USA | AST | (33) |
| | | | 30 (0, 60)[b] (32) | 10.53 (3.51, 19.3), $N = 57$ | | ECLIA | UK, Germany | M, healthy | (34) |
| Erythropoietin | EPO Receptor | 0 | 4.17 (0, 12.5) (35) | 1.20 (0.24, 2.40), $N = 416$ | SC | RIP | Multinational | CKD | (37) |
| | | | 44.44 (11.11, 77.78)[b] (36) | 1.37 (0.63, 2.11), $N = 946$ | IV | ELISA | South Korea | | (38) |
| Exenatide | GLP receptor | 35.30 (32.71, 37.89) | 28.57 (16.33, 40.82)[a] | 48.80 (47.05, 50.54), $N = 3154$ | SC | ELISA | Multinational | T2D | (39,40) |
| Kogenate | Factor IX, X, vWF | 15.88 (13.92, 17.92) | 100[b] (41) | 24.03 (20.65, 27.42), $N = 620$ | IV | Bethesda/Nijmegen | Multinational | PUHA | (42) |
| Trastuzumab | Her2/Neu | 0 | 4 (0, 10) (1) | 6.78 (4.07, 9.83), $N = 295$ | SC | ELISA | Multinational | F, BC | (43) |
| | | | 10 (0, 30)[b] (32) | 3.39 (1.36, 5.42), N = 295 | IV | | | F, BC | |
| | | | | 0.47 (0, 1.42), $N = 211$ | | | | F, BC metastatic | (44) |
| | | | | 0, $N = 398$ | | | | F, BC | (45) |
| Vatreptacog-α | Factor VIII, X | 32.55 (30.16, 35.1) | < 10 (14) | 11.11 (4.17, 19.44), $N = 72$ | IV | RIA | Multinational | M, PTHA/B | (3) |
| Adalimumab | TNFα | 6.53 (5.19, 7.94) | 4–56 (46) | 47.69 (35.38, 60.00), $N = 65$ | SC | ELISA | Spain | PPs | (48) |
| | | | 7.69 (0, 19.23)[c] (19) | 33.33 (17.95, 48.72), $N = 39$ | SC | ELISA | Italy | RA | (49) |
| | | | 22 (12, 34) (1) | | | | | | |
| | | | 56.25 (31.25, 81.25)[b] (47) | | | | | | |
| Etanercept | TNF | 0 | 7.14 (0, 21.43)[b] (32) | 0, $N = 47$ | SC | ELISA | Spain | PPs | (48) |
| | | | 8 (2, 16) (28) | 31.2 (11.02, 58.66), $N = 16$ | RA | ELISA | Colombia | RA | (50) |
| | | | | 11.5 (3.45, 20.69), $N = 58$ | SC | ELISA | Italy | RA | (49) |
| Infliximab | TNFα | 0.08 (0, 0.24) | 3.85 (0, 11.54)[c] (19) | 25.53 (17.02, 34.04), $N = 94$ | IV | Bridging ELISA | Spain | SA | (51) |
| | | | 7–18 (46) | 10.3 (0, 21.88), $N = 32$ | IV | ELISA | Italy | RA | (49) |
| | | | 14 (6, 20) (1) | 32.94 (23.53, 43.53), $N = 85$ | IV | Bridging ELISA | Spain | RA | (52) |
| | | | 20 (10, 32) (28) | 36.80 (28.80, 45.60), $N = 125$ | IV | ELISA | Belgium | CD | (53) |
| | | | 21.43 (0, 42.86)[b] (32) | 51.38 (45.52, 57.24), $N = 290$ | IV | ECLIA | Multinational | RA | (54) |
| | | | | 1.01 (0.0, 2.51), $N = 199$ | IV | ELISA | Multinational | CD | (55) |
| Ixekizumab | IL-17A | 12.66 (10.85, 14.45) | 56.25 (31.25, 81.25)[b] (47) | 7.24 (4.83, 9.92), $N = 373$ | SC | ELISA | Multinational | PPs | (56) |
| Natalizumab | α4 integrin | 7.00 (5.66, 8.49) | 2–10 (46) | 7.03 (3.78, 10.81), $N = 185$ | IV | ELISA | Multinational | CD | (57) |
| | | | 26.92 (11.54, 46.15)[c] (19) | 1.35 (0.68, 2.14), $N = 888$ | IV | Bridging ELISA | Sweden | MS | (58) |
| Rituximab | CD20 | 5.43 (4.25, 6.76) | 4–18 (46) | 10.59 (9.43, 11.79), $N = 2578$ | IV | ELISA | Multinational | RA | (59) |
| | | | 10 (2, 18) (1) | 0, $N = 37$ | IV | ELISA | USA | NHL | (60) |
| | | | 19.23 (3.85, 34.62)[c] (19) | | | | | | |
| Secukinumab | IL-17A | 0 | 6 (0, 14) (28) | 0.39 (0.18, 0.63), $N = 2842$ | SC | ECLIA | Multinational | PPs | (61) |
| | | | 6.25 (0, 18.75)[b] (47) | 0.5 (0.23, 0.78), $N = 2577$ | SC | Bridging ELISA | Multinational | PA, AS | (62) |
| Ustekinumab | IL-12/IL-23 | 5.43 (4.25, 6.76) | 6 (0, 14) (28) | 0.61 (0.17, 1.13), $N = 1154$ | IV | ECLIA | Multinational | CD | (63) |

*CKD* chronic kidney disease, *CRC* colorectal cancer, *BC* breast cancer, *RA* rheumatoid arthritis, *JIA* juvenile idiopathic arthritis, *PA* psoriatic arthritis, *AS* ankylosing spondylitis, *SA* spondyloarthritis, *CD* Crohn's disease, *PPs* plaque psoriasis, *UC* ulcerative colitis, *MS* multiple sclerosis, *NHL* non-Hodgkin's lymphoma, *CLL* chronic lymphocytic leukemia, *AST* advanced solid tumors, *PUHA* previously untreated hemophilia A patients, *PTHA/B* previously untreated hemophilia A/B patients, *T2D* type II diabetes, *M* male, *F* female, *SC* subcutaneous, *IV* Intravenous, *RIP* radioimmunoprecipitation, *SPR* surface plasmon resonance, *ELISA* enzyme-linked immunosorbent assay, *ECLIA* electrochemiluminescence assay, *RIA* radioimmunoassay

[a] Data from the current study

[b] In this assay, T cells were repeatedly stimulated over 3 weeks

[c] Donor is a responder if PBMC responded in either ELISpot or T cell proliferation

frequency of drug specific T cells shows a significant positive correlation with the predicted immune response (Fig. 3). The other significant parameters are the mean concentration of maturation signal in the wells ($\mu_{MS0}$), the rates of death ($\delta_{NT}$), and differentiation ($\beta_{NT}$) of the $CD4^+$ cells. The variation of the rest of the model parameters within their respective upper and lower limits did not have a meaningful impact on the predicted percent cohort response obtained. Similar trends are also observed in separate simulations for proliferation and ELISpot assays (Figs. S6c and S7c of S1 Text).

### Although Individual Immune Responses Vary Widely, Differences in Percent Responders at the Population Level Are Not Significant Among Geographically Sampled Cohorts

To study the potential variability of immunogenicity among different subpopulations, we used three geographical subpopulations (Finnish, Yoruba, and Japanese) in the 1000 Genomes dataset (27) whose HLA-DRB1 alleles are reported in S2 Dataset. For each drug shown previously in Table I, we performed TCPro simulations on the geographical cohorts and found limited variability among predicted percent responses against a drug from the retrieved cohorts (Fig. 4a for non-immunomodulatory drugs and Fig. 5a for immunomodulatory drugs). In addition, TCPro also allows for the analysis of individual-level drug immunogenicity risk based on T cell response, and for some of the drugs, we observed a substantial variation in immunogenicity risk among individuals (Figs. 4b and 5b). For example, with all else remaining equal, for an individual with HLA-DRB1 alleles *0101 and *1401, TCPro predicts a risk of exenatide immunogenicity five times higher than that for someone with homozygous *0302 alleles.

## DISCUSSION

*Ex vivo* immunogenicity experiments, although not required for every new biologic, are useful in assessing immunogenicity risk and are routinely used during the development of biopharmaceuticals. Here, we presented an in silico tool that can predict potential population and individual-level immunogenicity risks of biotherapeutics by simulating the *ex vivo* proliferation of $CD4^+$ T cells cultured with biopharmaceutical molecules. The predictions are performed through simulations of a mathematical immune response model informed by *ex vivo* parameter values obtained from literature. The results indicated that our predictions of drug immunogenicity on a select cohort compared well (4.2% mean absolute percent error) with the predictions of $CD8^+$-depleted PBMC-based immune response assays on 13 drug samples, including clinical and reproducibility benchmark protein molecules (humanized anti-A33 antibody, Exenatide and KLH). In addition, we analyzed the variation in immunogenicity of a collection of drugs on geographically-selected virtual human subject cohorts, concluding that the immunogenicity variation among such cohorts is not significant, even though a large variation of immune responses may be seen among individuals with different HLA types.

In practice, multiple factors can cause a large variability among the measured anti-biotherapeutic antibody development in the clinic and a deviation from *ex vivo* immunogenicity predictions. These factors include assay method, ADA cut-point criteria, drug dosage, treatment length and frequency, administration route, patient genetics and disease type or combined immunomodulatory therapy. Overall, TCPro immunogenicity predictions tend to correlate well with the clinically observed drug immunogenicities and levels of immunogenicity predicted by *ex vivo* assays with some exceptions. Vatreptacog-α is a recombinant Factor VIIa analog with three amino acid substitutions for which TCPro predicted a much higher population immunogenicity (33%) than *ex vivo* and *in vivo* tests. Only for vatreptacog-α in our set of drugs, we could not have access to a population distribution of frequencies of full-length drug-specific precursor T cells ($F_p$). Therefore, we opted to estimate an $F_P$ for vatreptacog by summing up the $F_p$ values of vatreptacog's two mutually exclusive non-wild-type short peptides obtained through *ex vivo* assay study in our laboratory. However, summation of the individual peptide $F_p$ values might not be a good estimate of full-length vatreptacog's $F_p$. Lamberth *et al.* (14) pointed that when the full-length vatreptacog protein was assayed using *ex vivo* assays, less than 10% of the donors responded. As the level of therapeutic protein immunogenicity tends to increase with the frequency of T cells specific for that protein, a low immunogenicity predicted for full-length vatreptacog by Lamberth and others suggests that the actual $F_p$ might be at a much lower value than the one we used ($F_P = 1.22$ $CD4^+$ per million) to arrive at our TCPro immunogenicity prediction. Also, TCPro's immunogenicity predictions for Kogenate and Erythropoietin are significantly lower than their *ex vivo* assay counterparts presumably because in those assays donor cells were treated multiple times over a 3-week span potentially boosting the immune cell memory response. Erythropoietin aggregation might also be another source of the high immunogenicity level observed by Delluc *et al.* (36). Nevertheless, in this version of TCPro, we have not accounted for the impact of drug aggregation on immunogenicity and TCPro's immunogenicity prediction for Erythropoietin better matches with that of an *ex vivo* assay of non-aggregated Erythropoietin (35). Last, there is a wide range of *ex vivo* and clinical immunogenicity demonstrated in the literature for the tumor necrosis factor (TNF) blockers Adalimumab, Etanercept and Infliximab and in general TCPro and *ex vivo* assays predicted lower immunogenicity. High levels of clinical immunogenicity might be explained by the repeated administrations of TNF blockers triggering immune memory response in patients. Also, TCPro response predictions are based on both the proliferation and cytokine secretion markers which may explain why they are lower than some of the *ex vivo* predictions that report donor responses only based on one marker type.

In its current state, TCPro is a generic in silico T cell activation assessment tool and an important missing feature of it is the drug-specific set of activity mechanisms that may initiate and sustain target mediated alteration of T cell activity in the wells. For example, there are many different interaction pathways involved in the activity of the immunomodulatory protein-based biotherapeutics. Therefore, to capture a more accurate picture of such large-scale interplay for all drugs *ex vivo*, TCPro should expand to include cytokine network model of all available immune cell types (and their

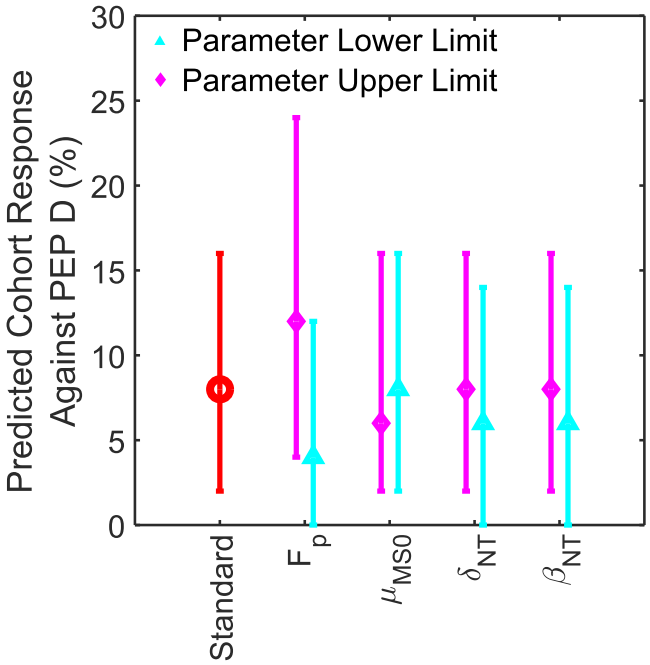


**Fig. 3.** The impact of model parameters on the TCPro prediction using the combined ELISpot/Proliferation assay method. PEP D (the standard, red line) is used as an example (see Fig. 2a) which shows the range of predictions by TCPro when inputs of all model parameters are the standard inputs for these parameters (Table S3 of S1 Text). We performed independent simulations for each parameter using the upper (purple) and lower (cyan) parameter bounds. Using $F_p$ as an example, the purple line represents the range of predictions by TCPro when the upper bound value is selected as model input for $F_p$, while the cyan line represents the range of prediction by TCPro when the lower bound value is selected. Under these two scenarios, inputs of all other model parameters are set to their standard input parameter values

subtypes) in the PBMC along with their relevant surface receptors. This remains as a very significant potential development direction for TCPro.

Apart from the missing hard-coded drug-specific activity mechanisms, our *ex vivo* assay modeling approach has several other limitations worth discussing. First, in silico peptide-MHC binding affinity predictors currently work only with canonical amino acids. TCPro cannot at this time assess the risk of immunogenicity for the "non-natural amino acids, nucleic acids, or post-translational modifications" on protein biotherapeutics using in silico tools (64). For example, the immunogenicity of KLH is partly due to its glycosylation (65). In addition, TCPro's success is partly contingent upon the performance of the method used to predict peptide-MHCII affinity. However, even though we used NetMHCIIpan in this work, TCPro is flexible and can make predictions with alternative affinity data. Second, experimental conditions, such as the amount of nutrients and cell debris in the assay wells, change with time; in our mathematical model, we are assuming that these factors do not have a direct effect on the immune response and rate parameters are time-independent. Third, our tool does not consider the proliferation or activation of B cells, monocytes, and natural killer cells naturally present in typical PBMC-based T cell activation assays; these cells are assumed to be inert in TCPro. Consequently, our model is reminiscent of the irradiated PBMC:T cell coculture proliferation assay demonstrated recently by Schultz *et al.* (19). Fourth, currently TCPro cannot explain fully the practical scenario of immune response to recall antigen administration since we do not yet model the activation of B cells nor do we model repeat biotherapeutic injections into the wells. Moreover, even though the monocytes and B cells can present antigens to T cells, TCPro assumes dendritic cells as the sole antigen-presenting cell species in the assays. This may be a valid assumption for the naïve $CD4^+$ cell subspecies as they strictly depend on DC for activation (66). Conversely, memory $CD4^+$ subspecies can be activated by the resting B cells as well (67). Even though B cells are not as efficient in antigen presentation as DCs (68), the B cell count in PBMCs is roughly ten times of the DC count which supports the inclusion of B cells in our mathematical model. The B cells, if included, would have increased the overall antigen presentation to T cells in TCPro and in turn increase the number of activated and proliferated T cells and immunogenicity responses predicted. On the other hand, it is difficult to predict the impact of incorporating monocyte activity on TCPro's predictions since they can both suppress and activate $CD4^+$ T cells (69).

Our mathematical model accounts for all $CD4^+$ cells, albeit in a generic manner. The memory T cells have a lower antigenic activation threshold than naïve cells; however, the memory cell count in the PBMC is expected to be much lower than the naïve T subspecies especially when it comes to biotherapeutics (as opposed to toxins and viral/bacterial surface proteins). Based on that observation and the lack of *a priori* knowledge of individual $CD4^+$ cell subspecies counts, TCPro does not make any distinction between the naïve and memory $CD4^+$ T cells. As a result, we assume that all $CD4^+$ subspecies ($CD45RA^+CD45RO^-$, memory and others) have the same potency of activation and proliferation when a biotherapeutic is encountered.

The endotoxin (maturation signal) amount in the assay wells is a key element in our model. Endotoxin typically is present in equipment and some cell cultures (70,71). We expect its amount to be somewhere between the maximum allowed limit in biotherapeutics and the LAL assay sensitivity lower limit for each well simulated. A high endotoxin content in a well decreases the *ex vivo* immune response assay sensitivity by increasing the number of mature APCs in the simulated well, and thereby the number of activated $CD4^+$ cells in the absence of a biopharmaceutical. Similarly, TCPro would yield better immunogenicity predictions if it is informed by an ideal experimental scenario in which each well's endotoxin content is measured in the beginning of the experiments. Nevertheless, it was shown that lymphocytes in PBMC samples can also promote toll-like receptor (TLR)-independent DC maturation (72,73) suggesting an alternative DC maturation pathway which we did not directly include in our model.

In addition, we did not consider in our model the potential effects of competing peptides on the p-MHCII complex formation. However, the relatively high sample concentration in assays may overcome competitive effects. Based on a published source (74), there are two to four million proteins per 1 fL of cell volume. This means that a single dendritic cell contains about a billion protein molecules. The concentrations used in T cell activation

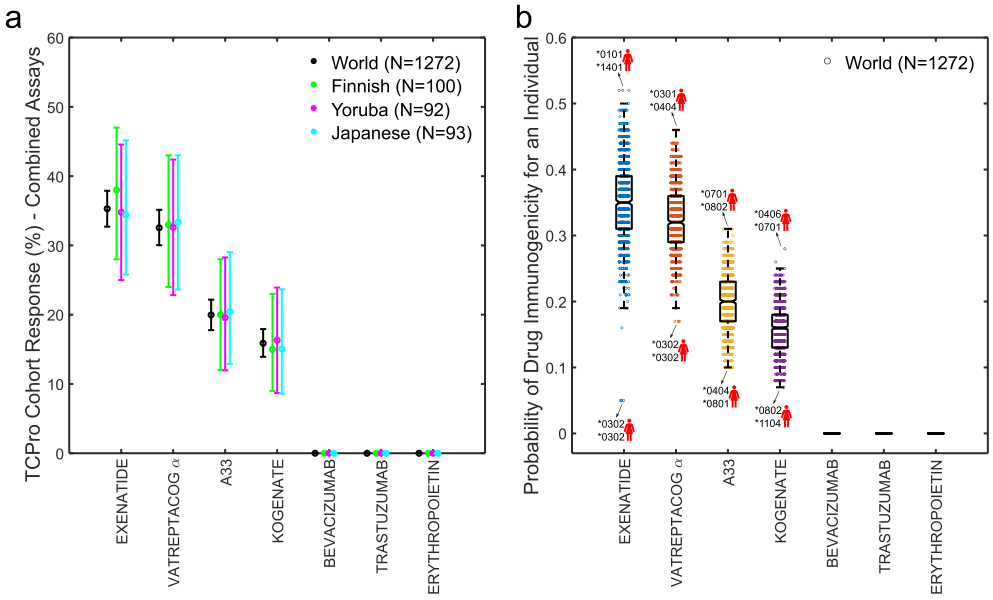


**Fig. 4.** Geographical and within-subject variations of immunogenicity in non-immunomodulatory drugs. **a** Analysis of the effect of geographical differences on TCPro-predicted drug immunogenicity using ELISpot/Proliferation combined assay predictions for a selection of non-immunomodulatory drugs. The medians (circles) and 95% binomial confidence intervals (error bar) for the cohort response are presented. Even though there are differences in levels of immunogenicity among the different drugs, we did not observe significant differences in a drug's predicted immunogenicity among different geographical cohorts drawn from the 1000 Genomes dataset. **b** TCPro immunogenicity predictions for each individual in the general World population shown in **a**. Each circle represents the probability of an individual responding to a drug. Probabilities are calculated by averaging 100 TCPro-simulated Bernoulli experiments (Member responded? Yes/No). The bottom and top edges of the box indicate the 25th and 75th percentiles, and the whiskers cover ~ 99% of the data points. HLA-DRB1 alleles of the cohort members with the lowest and highest immune response probabilities are shown in brackets

assays are on the order of 1 micromolar which results in ~ $10^{15}$ protein molecules per well. Considering that a single well contains a million cells, there are on average about a billion protein molecules available in a well of each cell. Since this number is roughly equal to the total number of proteins in a cell, the biotherapeutic-specific peptides will outcompete peptides from endogenous proteins in the cell. This assumption would not hold if lower sample concentrations are used in the assays, and peptide-peptide competition would then become important. In such a case, binding

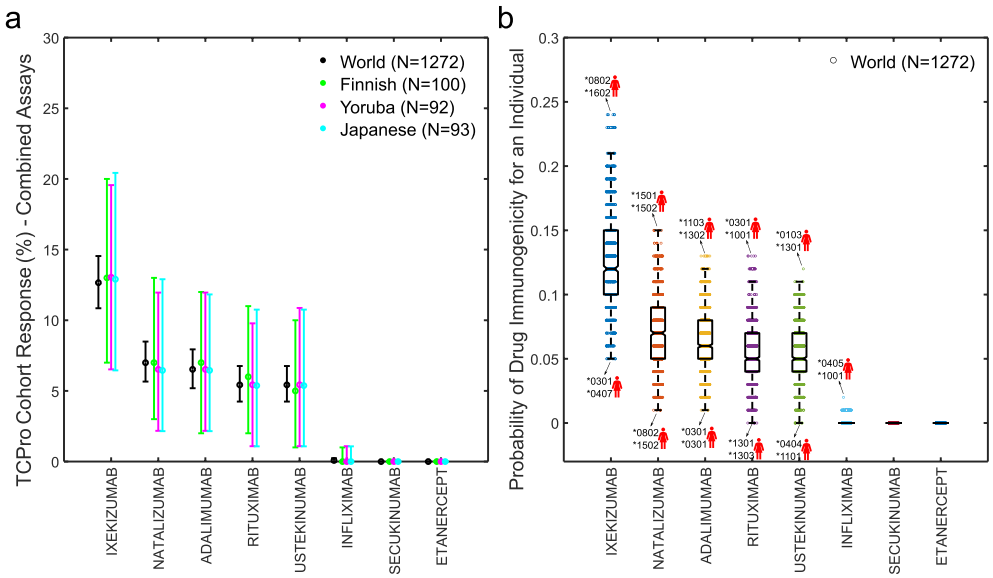


**Fig. 5.** Geographical and genetic variations among immunomodulatory drugs. **a** Analysis of the effect of geographical differences on TCPro-predicted drug immunogenicity. **b** Individual-level view of TCPro immunogenicity predictions for the World cohort shown in **a** for the immunomodulatory drugs

affinity %Rank information available from (25) can be used to model competition; or alternatively, separate differential equations for competing peptides can supplement (as described in (75)) the model.

Last, donor age, sex, and race might play a role in the baseline numbers of immune cells (76). Therefore, when using TCPro for making immunogenicity predictions on such specific populations, the initial cell number distributions must be modified accordingly. Similarly, we understand that *a priori* knowledge of epitope-specific precursor $CD4^+$ frequency is not very easy to obtain, and at this point, we suggest that a range (typically 0.1–1 per million $CD4^+$ per epitope per drug) could be scanned when running simulations with TCPro to obtain immunogenicity risk assessments.

Even though T cell activation is not a sufficient indicator of whether a biopharmaceutical will trigger antibody production, it is the necessary step in triggering the B cell response. Therefore, immunogenicity assays based on T cells are helpful in the drug development process and regulatory decision-making, especially in identifying biotherapeutics with a higher probability of being safe. TCPro is a quick immunogenicity risk characterization tool which can simulate immune responses in large and diverse populations. Equivalent *ex vivo* assays for comparable sample sizes would be cost prohibitive. Nevertheless, we do not expect TCPro to eliminate the need for performing *ex vivo* assays. Rather, our tool could be used as a preliminary tool for screening new drugs. TCPro users can extrapolate the immunogenicity risk quantified by their own *ex vivo* assays to larger populations or to populations with genetic, gender, or age makeups that are different than the ones used in their assays to obtain better assessment of confidence intervals of their immunogenicity predictions. We speculate that TCPro could become a more robust tool in the future contingent upon the introduction of drug specific activity mechanisms to the model, such as those of the immunomodulatory drugs acting specifically (or indirectly) on $CD4^+$ T cells. In addition, TCPro needs a population distribution of drug-specific precursor $CD4^+$ frequencies ($F_p$ in our model) as input which is generally obtained from *ex vivo* assays. Therefore, another obstacle in the road for TCPro to becoming a more independent and robust tool is the acquisition of drug $F_p$ values from an alternative source, presumably by means of computational predictions.

We foresee another utility of TCPro in simulation of immunogenicity arising in combination therapy scenarios and analyze correlations in immunogenicity. For example, the clinical immunogenicity rate of nivolumab is 11%; however, when combined with ipilimumab, that rate was observed to increase to 38% (77). TCPro also can be applicable in T cell epitope deimmunization studies (78). In the future, as *ex vivo* B cell activation assays become common, TCPro can be modified or extended to make predictions of B cell activation using the methodology laid out here. Finally, our model can be used as a flexible platform for assay development and optimization, because it allows temporal modification and analysis of cell numbers, possible endotoxin content of wells, alternative affinity prediction methods, and sample concentrations to mechanistically understand the interplay between various factors of immunogenicity.

## MATERIALS AND METHODS

### The Sequence of *Ex vivo* Events in Immune Response Against a Drug Is Modeled with a Non-linear ODE Model

We computationally modeled and simulated the sequence of immune response events against a drug sample in a typical *ex vivo* $CD8^+$-cell depleted PBMC:T cell time series proliferation assay and ELISpot interleukin-2 (IL-2) secretion assay using the Chen model (75,79) as our starting point. For a graphical summary of the model, see Fig. S1. First, immature DCs in the assay wells differentiated into mature dendritic cells due to the maturation signal ($MS_0$) presence in the wells (Eqs. 1–3 in S1 Text). Existence of the mature dendritic cells can cause spontaneous activation of the $CD4^+$ cells in the absence of antigen molecules in the wells. Also, mature DC can endocytose drug molecules where the molecules are trafficked to the endosomes and processed into shorter peptide fragments. There, the drug peptides bind the MHCII molecules. Using an in silico prediction tool for the peptide-MHC Class II molecule affinity (25), we modeled the drug fragment binding and presentation by the dendritic cells (Eqs. 5–10 in S1 Text) to the T cells. Drug-specific T cells recognize the DC-presented peptides and differentiate into activated T cells, secreting cytokines and proliferating strongly (Eqs. 11–17 in S1 Text). The initial number of drug-specific T cells are calculated by multiplying the frequency of precursor cells and randomly selected number of T cells of a human subject whose cells are assayed with the experiment.

### Model Inputs and Parameters

Most of the TCPro parameters came from the literature (Table S3 in S1 Text). One of the parameters we modeled is the concentration of the dendritic cell maturation signal, $MS_0$. To characterize $MS_0$, we hypothesized that the residual endotoxin present in assay wells causes spontaneous maturation of DCs, which triggers a small baseline T cell proliferation that depends on the number of mature DCs. Using that assumption, we statistically modeled $MS_0$ in the wells by fitting the distribution of the cytokine spot-forming cells in the medium-only IL-2 ELISpot assays (S1 Text). Next, for each virtual subject an $MS_0$ was randomly selected from the distribution. Another modeled input parameter is drug-specific precursor $CD4^+$ cells. To estimate $F_p$ for PEP A-J, Exenatide, A33 and KLH, we used our experimental data and assumed that the drug-specific T cells followed a Poisson distribution (Eq. 21 in S1 Text and S1 Dataset). In the simulations of the rest of the drugs, we randomly sampled $F_p$ from the published distributions as shown in S3 Dataset (32,36,41).

Additional input to TCPro is the pair of HLA-DRB1 alleles. For our TCPro validation simulations (Fig. 2), we obtained HLA allele information of each PBMC donor from the *ex vivo* experiment itself, using only those HLA-DRB1 alleles in our peptide-MHCII affinity predictions. The HLA allele frequency distribution of the donor set corresponded to the general frequency of HLAs in the world population (S1 Dataset). HLAs of the World, Finnish, Yoruba, and Japanese cohorts were obtained from the 1000 Genomes study as shown in S2 Dataset (27). Additionally, in the simulations, the initial concentration of the peptides (PEP A-J) in the assay wells was set at 5.0 μM, and all other protein drugs in this

work had initial concentrations of 0.3 μM, to mimic the concentrations used in our *ex vivo* experiments.

### TCPro Simulations

Using MATLAB R2017b, we solved the set of ordinary differential equations governing the *ex vivo* system with selected parameters and initial conditions (Tables S1 and S3 in S1 Text). We calculated the number of proliferated $CD4^+$ cells in a brief period of incubation time as a proxy for the standard radioactive [$^3$H]-Thymidine incorporation experiments after days 5 to 8. Similarly, we used the activated T cell counts throughout the incubation period (8 days) as a proxy for the spots per well (SPW) measurement in a typical IL-2 ELISpot experiment. Cultures treated with an immunogenic protein are expected to have a bigger population of activated/proliferated $CD4^+$ T cells than those cultured with only medium (baseline). For each donor, we defined T cell stimulation indexes by comparing the cell numbers in cultures with drugs to those of the ones grown with media only (Eqs. 18–20 in S1 Text). Next, by analyzing the stimulation indexes, we decided if a donor responded to a specific sample. Based on a donor cohort, we finally arrived at the percentage of donors whose $CD4^+$ cells responded to the tested samples (Fig. 1a). We simulated the T cell response assays for each donor and for each protein sample, repeating $N = 100$ times. During each repetition, each virtual cohort member was assigned randomly selected but unique immune cell type percent distributions, drug-specific precursor $CD4^+$ frequency and an $MS_0$ value. Also, the total number of cells within each well was randomly selected based on the experimental uncertainty. Finally, individual-level probability of drug immunogenicity was calculated as the number of successful Bernoulli trials for each cohort member response to the drug, divided by $N$. The full source MATLAB code of TCPro is available upon request.

### Parameter Sensitivity Analysis

We performed parameter sensitivity analysis by running TCPro, fixing one parameter at a time to its lowest or highest limit while keeping the rest of the parameters as shown in Table S3 in S1 Text (the standard set). The percent cohort response predictions were recorded. Some of the parameters do not have upper or lower limits ($\boldsymbol{\beta_{pM}}$, $\boldsymbol{\delta_{NT}}$, $\mathbf{K_{pM,N}}$, and $F_p$), and for those parameters we assigned artificial limits (50% of the original value for the lower limit and 200% for the upper limit).

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Investigation: ONY, ZES.
Methodology: ONY, ZES, JRM, MAT, HY.
Project administration: HY, ZES, MAT.
Resources: HY, ZES.
Software: ONY.
Supervision: HY, ZES, MAT.
Validation: ONY.
Visualization: ONY.
Writing—original draft: ONY, ZES, JRM.
Writing—review and editing: ONY, ZES, JRM, MAT, HY.

## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Disclaimer** This article reflects the views of the authors and should not be construed to represent FDA's views or policies.

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