Immunosequencing identifies signatures of cytomegalovirus exposure history and HLA-mediated effects on the T cell repertoire

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An individual's T cell repertoire dynamically encodes their pathogen exposure history. To determine whether pathogen exposure signatures can be identified by documenting public T cell receptors (TCRs), we profiled the T cell repertoire of 666 subjects with known cytomegalovirus (CMV) serostatus by immunosequencing. We developed a statistical classification framework that could diagnose CMV status from the resulting catalog of TCR β sequences with high specificity and sensitivity in both the original cohort and a validation cohort of 120 different subjects. We also confirmed that three of the identified CMV-associated TCR β molecules bind CMV *in vitro*, and, moreover, we used this approach to accurately predict the *HLA-A* and *HLA-B* alleles of most subjects in the first cohort. As all memory T cell responses are encoded in the common format of somatic TCR recombination, our approach could potentially be generalized to a wide variety of disease states, as well as other immunological phenotypes, as a highly parallelizable diagnostic strategy.

The ability of the adaptive immune system to adequately address an infection relies on the presence of T cells that generate $\alpha\beta$ -heterodimeric antigen-specific TCRs through V(D)J recombination. TCR specificity is mediated by primary sequence diversity: each mature TCRB gene is randomly rearranged at its complementarydetermining region 3 (CDR3) by combining noncontiguous variable (V), diversity (D), and joining (J) region gene segments of the germline locus. Nucleotide deletions and template-independent insertions at the V_{β} - D_{β} and D_{β} - J_{β} junctions further add to the diversity of the encoded receptors, resulting in highly diverse TCR β CDR3 regions^{1,2}. The TCRa chain is generated in a similar process and combines with the TCR β chain to form a TCR that binds its cognate antigen in the context of specific cell surface major histocompatibility complex (MHC) class I proteins. These are encoded by the highly polymorphic human leukocyte antigen (HLA) loci HLA-A, HLA-B, and HLA-C, and a TCR's antigen specificity is therefore further modulated by HLA context. Upon antigen recognition, activated T cells proliferate by clonal expansion and some become part of the memory compartment, where they can reside for many years as clonal populations of cells with identical TCR rearrangements by virtue of their descent from a common naive T cell³⁻⁵.

Healthy adults express approximately 10^7 unique TCR β chains on their ~ 10^{12} circulating T cells, which are drawn from a much larger pool of possible rearrangements⁵. Observing the same TCR β chain independently in two individuals is thousands of times more common

than would be expected if all rearrangements were equally likely⁶. It is expected that many TCR β sequences (especially those with few or no junctional insertions) are present in the naive T cell repertoires of most humans at any given time and the corresponding T cells will proliferate upon exposure to their target antigen in the proper MHC context⁷. Public T cell responses, in which a particular antigen is targeted by the same TCR sequence in multiple individuals, result when the space of potential high-avidity TCRB chains that could bind to a particular antigen-MHC complex includes one or more TCR β chains that also have a high likelihood of existing in the naive repertoire at any given time^{7,8}. TCR β sequences associated with a public T cell response to a particular antigen will only be intermittently observed in the naive compartment of subjects who have not been exposed to that antigen. However, T cells carrying these TCRB sequences undergo clonal expansion upon antigen encounter, which increases the probability that these sequences will be detected in the repertoire of exposed subjects, thus providing the basis for characterizing immunological memory across different individuals.

Despite historical limitations on sequencing depth and the limited size of investigational cohorts, there are many examples of public T cell responses to infectious diseases, such as CMV, Epstein–Barr virus (EBV), *Clostridium tetani*, parvovirus, herpes simplex virus (HSV), HIV, and influenza, as well as in malignancies and autoimmunity^{7,8}. Typically, these public T cell responses have been studied in the context of single antigens in a single HLA context by isolation

Received 15 September 2016; accepted 28 February 2017; published online 3 April 2017; doi:10.1038/ng.3822

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of specific antigen-bound T cells followed by low-throughput sequencing of the variable regions of the TCR β (and, in some cases, the TCR α) chains.

With the goal of identifying statistically significant associations between sets of TCR β sequences and phenotypes of interest, we immunosequenced the variable region of the TCR β chain to generate sizable libraries of public TCR β chains. By measuring the presence or absence of individual TCR β sequences in a large investigational cohort and statistically assessing their concordance with phenotypes of interest, we demonstrate that CMV serostatus and the presence of particular *HLA-A* and *HLA-B* alleles can be predicted with high confidence solely on the basis of the TCR β repertoire data generated from peripheral blood. We believe that our approach has the potential to be applied as a diagnostic strategy for a range of immune-related phenotypes.

RESULTS

Data acquisition

We immunosequenced the rearranged CDR3 TCRB region in a cohort of 666 healthy bone marrow donors (cohort 1), generating a total of 89,840,865 unique TCR β sequences, which are defined in this study as a unique combination of a V gene, a CDR3 amino acid sequence, and a J gene. This level of similarity does imply an identical TCRβ protein sequence, although, owing to HLA restriction and the potential to pair with different TCR α chains in different T cells, it does not guarantee that two such receptors will have identical antigen specificities. We observed an average of 192,515 (±80,630 s.d.) unique TCRβ sequences per subject; the level of variation observed between subjects is in line with the natural variation of T cell levels in the peripheral blood of healthy adults^{9,10}. For our proof-of-principle study, we selected CMV, a chronic virus that has been extensively studied as a model system for public T cell responses^{7,8,11} and which infects 30–90% of adults¹², thus providing high statistical power. We performed CMV serotyping for 641 subjects: 352 subjects were CMV negative (CMV⁻) and 289 were CMV positive (CMV⁺). For validation purposes, we also immunosequenced the CDR3 region of TCR β in an independent cohort of 120 subjects (cohort 2), which resulted in an average of 202,918 (±108,603) unique TCR β sequences per subject, and we performed CMV serotyping for all subjects in this cohort. Finally, we performed HLA typing for 626 subjects in cohort 1 to obtain HLA-A and HLA-B major allele calls. Demographic data for both cohorts are summarized in Table 1 and Supplementary Table 1.

Identification of CMV-associated TCRs

To determine whether CMV-associated TCR β sequences could be identified by their differential incidence among CMV⁺ and CMV⁻ subjects, we followed the experimental and analytical procedure outlined in **Figure 1**. After immunosequencing peripheral blood samples, we analyzed each unique TCR β chain identified for the 641 subjects in cohort 1 with known CMV serostatus for statistically significant enrichment among CMV⁺ subjects in comparison to CMV⁻ subjects. At a significance threshold of $P < 1 \times 10^{-4}$ (established as optimal via a cross-validation procedure) and a false discovery rate (FDR) of 0.14 (estimated by permutation of CMV status), we identified 164 CMV-associated TCR β chains (**Supplementary Table 2**) that displayed increased incidence among CMV⁺ subjects in cohort 1 in comparison to CMV⁻ subjects (**Fig. 2a**). The statistical approach used to identify these CMV-associated TCR β chains is described in detail in the Online Methods.

Next, we investigated the HLA restriction of each of these 164 CMV-associated TCR β sequences. As TCR-antigen binding occurs in the context of MHC proteins, which are encoded by the highly

Table 1	Cohort	demographics
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Sex, age, ancestry, known CMV status, and known HLA status are indicated, when available, for the 666 subjects in cohort 1 and the 120 subjects in cohort 2.

polymorphic HLA loci, the affinity of a given TCR for a given antigen is modulated by the HLA type of the subject². Hence, for each CMVassociated TCR β and each *HLA-A* and *HLA-B* allele identified for the subjects in cohort 1, we performed a Fisher's exact test¹³ to assess the significance of the enrichment of that TCR β among subjects positive for that HLA allele. At a significance threshold of $P < 1 \times 10^{-4}$, 45 of the 164 CMV-associated TCR β chains identified in this study were associated with at least one *HLA-A* or *HLA-B* allele (**Supplementary Table 2**). Several TCRs were significantly associated with both an *HLA-A* and an *HLA-B* allele, and no TCRs were associated with more than one *HLA-A* or *HLA-B* allele.

Several studies have identified TCRs that recognize CMV antigens through low-throughput, *in vitro* methods—mainly tetramer sorting of T cells with particular CMV epitopes followed by sequencing of a portion of the TCR β and/or TCR α locus—and report both private and public TCR sequences. **Supplementary Table 3** includes a list of 1,054 TCR β sequences, 917 of which are unique^{14–47}, that have been reported in the literature as being able to recognize CMV antigens (termed 'CMV reactive') from 34 such publications. Most of these TCR β sequences were identified on the basis of their reactivity to a small subset of welldefined epitopes from the CMV pp65 or E11 proteins, although a few of the studies report TCR β sequences that recognize collections of overlapping peptides from these and other CMV proteins. Many of these publications include HLA restriction information.

We first compared the list of 917 CMV-reactive TCR β sequences to the cumulative list of 89,840,865 unique TCR β sequences observed



Figure 1 Experimental and analytical overview. (a) For cohort 1, we obtained peripheral blood samples from 641 subjects with known CMV serostatus and the immunosequenced variable region of the TCR β locus. We then identified CMV-associated TCR β s, demonstrated that CMV⁺ subjects have more CMV-associated TCR β s in their repertoires than CMV⁻ subjects, and used a leave-one-out cross-validation approach to assess the diagnostic potential of screening for public TCR β s. (b) For cohort 2, we obtained peripheral blood samples from 120 subjects and immunosequenced the variable region of the TCR β locus in the same manner. Next, using the model resulting from analysis of the first cohort, we inferred CMV serostatus for all subjects and compared these results to the experimentally determined CMV status a posteriori, thus validating the diagnostic accuracy of the method. TPR, true positive rate; FPR, false positive rate.

in the repertoires of the subjects in cohort 1: 488 of 917 (53.2%) sequences were observed in our cohort of 666 subjects. For each of the 488 CMV-reactive TCR β sequences observed in our data set, we checked whether it had also been identified in this study as CMV associated (**Supplementary Table 2**) and identified 9 matches: 6 of these were significantly HLA restricted ($P < 1 \times 10^{-4}$) in our cohort, and in all 6 cases the HLA restriction we observed was consistent with previous reports (**Supplementary Table 4**). However, the vast majority of the CMV-reactive TCR β sequences displayed similar incidence among CMV⁺ and CMV⁻ subjects (**Fig. 2a**).

As the CMV-associated TCR β sequences identified in this study had to fall within a narrow range of incidence to occur frequently in CMV⁺ subjects but rarely in CMV⁻ subjects, thus disqualifying most truly public TCR β sequences from being identified by this method (**Fig. 2b**), we also performed a more thorough analysis to investigate



Figure 2 Identification of CMV-associated TCR β s. (a) Scatterplot showing the incidence of each TCR β sequence among CMV⁺ and CMV⁻ subjects in cohort 1. The number of TCR β s in each position is represented by both the transparency and size of the spot, with opacity and area mapped to the logarithm of frequency. CMV-associated TCR β sequences identified in this study are represented by green dots. All other TCR β s are represented by green dots. All other TCR β s are shown in gray. (b) Stacked bar chart depicting the distribution of the incidence of TCR β sequences in subjects in cohort 1 (the number of subjects in whom each TCR β is found). Previously reported CMV-reactive TCR β s is found). Previously reported CMV-reactive TCR β s identified in this study occupy a narrower range. Colors correspond to those defined in **a**.

the overlap with the 488 CMV-reactive TCR β sequences observed in our data set. In brief, we used a Mann-Whitney U test to determine whether these TCR β sequences were significantly more abundant in CMV⁺ subjects than in CMV⁻ subjects, restricting the analysis by HLA type for CMV-reactive TCR β sequences with reported HLA restriction. For each TCR β sequence, the fractional abundance in a subject was computed as the number of templates counted for that TCR β divided by the total number of template TCR β molecules in the sample. We then compared the list of fractional abundances in CMV⁺ subjects to that in CMV⁻ subjects. This analysis combines both presence or absence (as, by definition, the abundance metric must be larger than zero), the relative abundance of the TCR β in CMV⁻ and CMV⁺ subjects (a total of 372 such TCRβ sequences were observed in at least one CMV⁺ and at least one CMV⁻ subject), and HLA restriction (as the lists of CMV⁺ and CMV⁻ subjects that contained a specific TCR β sequence in their repertoire were restricted to those who possessed the HLA allele that restricted that CMV-reactive TCR β in their genome). We identified six CMV-reactive TCRβ sequences that were significantly enriched in abundance among CMV⁺ subjects at a *P*-value threshold of 1×10^{-4} . Five of these six TCR β sequences were present in both CMV⁺ and CMV⁻ subjects with the given HLA restriction, and four of them were also present in our list of CMV-associated TCR β sequences that were identified solely by the presence/absence criterion. Three of the six were reported as restricted to HLA-A2, and the other three were restricted to HLA-B7. By extension, five of our nine matches among CMV-associated TCRB sequences were identified by the incidence approach but not by the HLA-aware abundance comparison. In conclusion, the approach based on the presence or absence of a TCR β sequence in our data set is sufficient to recapitulate or even improve the results of an HLA- and abundance-aware analysis, and most CMV-reactive TCR β sequences previously reported in the literature are not more likely to be observed in-or be more abundant in-CMV⁺ subjects in our cohort.

Inference of CMV serostatus from public TCR β sequences

To control for differences in repertoire sampling depth, we plotted the number of CMV-associated TCR β sequences found in each of the 641 CMV-serotyped subjects in cohort 1 versus the total number of



Figure 3 The incidence of CMV-associated TCR β s is diagnostic of CMV serostatus. (a) Scatterplot comparing the distribution of the number of CMV-associated TCR β s to the total number of unique TCR β s sampled for CMV⁺ (orange) and CMV⁻ (blue) subjects in cohort 1. (b) ROC curves showing the classification performance of a classifier trained on data from cohort 1 and tested on all the data from cohort 1 (dotted line), by cross-validation in cohort 1 (dashed line), or by independent validation in cohort 2 (solid line). The black circle on each line corresponds to the maximum a posteriori (MAP) decision threshold. The inset depicts the MAP classification results for cohort 2 with the confusion matrix showing a sensitivity of 0.90 and a specificity of 0.89.

unique TCR β sequences observed in that subject (Fig. 3a). Although there was a clear separation between CMV⁺ and CMV⁻ subjects, the number of CMV-associated TCR β sequences increased with increased sampling depth for both CMV⁺ and CMV⁻ subjects, as expected. We hypothesized that the presence of many CMV-associated TCR β sequences in a subject would be diagnostic of CMV seropositivity. To test this hypothesis, we constructed a generative binary classifier that infers CMV serostatus from the number of CMV-associated TCR β sequences. The general framework for inferring subject-level phenotypes from immunosequencing data is described in the Online Methods. We trained this classifier on the CMV-serotyped subjects from cohort 1, tuning the P-value threshold for finding CMV-associated TCRB sequences while simultaneously estimating the performance of our classifier using exhaustive 'leave-one-out' cross-validation. The performance of this classifier, as measured by the area under the receiver operating characteristic curve (AUROC; Fig. 3a), was 0.99 for all data (dotted line) and 0.93 for the cross-validation data set (dashed line). Although the first model was overfitted, the robust performance observed in the cross-validation approach is encouraging.

To fully validate our method for inference of an individual's CMV serostatus, we collected peripheral blood samples from an independent set of 120 subjects who were not used to train the classifier (cohort 2), and we performed immunosequencing and CMV serotyping as described (**Table 1** and **Fig. 1b**). We observed very strong discrimination performance for our classifier on this validation cohort, as indicated by an AUROC value of 0.94 (**Fig. 3b**, solid line). This value is no less accurate than the one obtained from the cross-validation approach described above, suggesting that the cross-validation scheme was a fair estimate of model accuracy. The application of a maximum a posteriori decision threshold for CMV serostatus classification in cohort 2 resulted in a sensitivity of 0.90, a specificity of 0.88, and a diagnostic odds ratio of 70. Taken together, these results demonstrate the excellent diagnostic power of our approach.

In vitro confirmation of CMV antigen specificity

In order to confirm that the approach described above can identify TCR β sequences from TCRs that recognize CMV antigens, we

performed a MIRA (multiplexed identification of TCR antigen specificity) assay on a healthy HLA-A2⁺ adult^{29,48}. We screened approximately 200 million peripheral blood mononuclear cells (PBMCs) to identify TCR β sequences specific for any of 38 characterized HLA-A2-restricted peptide antigens, including CMV pp65 peptide NLVPMVATV (amino acids 495–503) and 37 unrelated epitopes (**Supplementary Table 5**).

Briefly, we found 1,840 antigen-reactive TCR β sequences in total, of which 69 (3.75%) were specific for CMV peptide CMV-pp65₍₄₉₅₋₅₀₃₎ and 1,771 were specific for the other non-CMV-derived epitopes (**Supplementary Table 6**). Of these 1,840 TCR β sequences and 69 CMV-specific TCR β sequences, 652 and 32, respectively, were observed in any of the subjects comprising cohort 1 in this study—consistent with the hypothesis that most TCRs are private.

When comparing these results with the list of 164 CMV-associated TCR β sequences generated by our association study in cohort 1, we found three TCR β sequences in common with the MIRA assay results; all three were specific for CMV in the MIRA experiment (3/3 overlapping TCR β sequences specific for CMV, while 69/1,840 were CMV specific overall: $P = 5.3 \times 10^{-5}$ by binomial test). Of these three, two (CASSLAPGATNEKLFF and CASASANYGYTF) were associated with HLA-A2 in both our study and previous studies^{32,36,41-43}. Thus, of the four CMV-associated TCR β chains that were confidently assigned to HLA-A2 in our study (**Supplementary Table 2**), half were seen in an experimental results derived from a single individual's T cells (**Supplementary Table 6**).

Supplementary Figure 1 shows the prevalence of each of the 652 MIRA TCR β sequences found in any subject from cohort 1 within CMV^+ and CMV^- individuals. Of the 32 CMV-reactive TCR\beta sequences also seen in cohort 1, some of which had high prevalence in the data, most appeared equally in CMV⁺ and CMV⁻ subjects. In addition to this incidence analysis, Supplementary Table 5 includes the results of a Mann-Whitney U test (with Bonferroni correction for 32 hypotheses) assessing clonal abundance in CMV⁺ and CMV⁻ subjects. Aside from the three TCR β sequences already identified as CMV associated by incidence alone, this analysis identified one additional TCR β that was more abundant in CMV⁺ subjects (CASSSANYGYTF). In conclusion, using this antigen-TCR matching assay, we have demonstrated that (i) several of the TCRβ sequences we identified as CMV associated using an association study do react to CMV epitopes in vitro and (ii) most TCR β sequences we identified as reacting to CMV epitopes in vitro do not appear to be more prevalent or more abundant in CMV-seropositive individuals in an epidemiological sense.

Inference of HLA type from public TCRs

As described above, we determined that our approach can find public TCR β sequences that have strong statistical association with CMV serostatus and that many of these TCR β sequences were restricted to particular HLA types in cohort 1. Thus, we hypothesized that, because HLA type plays a strong role in shaping the T cell repertoire⁴⁹, we should be able to find public TCR β sequences associated with particular HLA alleles using a similar framework. For each *HLA-A* and *HLA-B* allele present in cohort 1, we performed an association analysis similar to that used for CMV status determination, using *HLA-A* and *HLA-B* allele presence as the Boolean phenotype of interest. Notably, the analyst who conducted this study was not made aware of the HLA status of the subjects until after the analysis was completed.

Both positive and negative thymic selection based on interactions between TCRs and MHC proteins are known to affect T cell fate and, consequently, the presence of specific T cells in an adult human's peripheral blood^{49,50}. We therefore employed a two-tailed Fisher's



Figure 4 Identification of HLA-allele-associated TCR β s. For all *HLA-A* (top) and *HLA-B* (bottom) alleles observed in cohort 1, the graphs show the frequency of each allele (left), the number of allele-associated TCR β s identified (middle; the direction of the association is indicated as follows: light gray, positive association; dark gray, negative association), and the classification accuracy based on cross-validation (right; measured using *F*₁ score).

exact test to identify TCR β sequences that were either positively associated with specific HLA alleles (i.e., enriched in subjects carrying that HLA allele) or negatively associated with specific HLA alleles (i.e., suppressed in subjects carrying that HLA allele). This analysis resulted in the identification of 15,601 HLA-allele-associated TCR β sequences for the 61 *HLA-A* and *HLA-B* alleles observed in cohort 1, with all but 87 having positive associations in our data (**Fig. 4**, left and middle).

Next, for each allele, we performed exhaustive leave-one-out cross-validation to assess the ability of our classification framework to successfully infer the presence of HLA alleles by using a model that performs allele-associated TCR β identification and classifier training for each subject, including all of the subjects other than the one under consideration. This approach is described in detail in the

Online Methods. Classification was highly sensitive and specific for the more common alleles, with accuracy (measured by the F_1 score) diminishing with decreasing allele frequency (**Fig. 4**, right).

Finally, we inferred an HLA type for each subject, consisting of the set of HLA alleles whose individual classification model suggested that the allele was present in that subject, without explicitly enforcing homo- or heterozygosity at each locus. **Supplementary Table 2** includes the resulting HLA type inferences: of 626 HLA-typed subjects in cohort 1, 332 subjects were assigned cross-validated *HLA-A* allele inferences that exactly matched their known *HLA-A* alleles, 234 subjects were assigned exactly matched *HLA-B* allele inferences, and 138 subjects had both exactly matched *HLA-A* and *HLA-B* allele inferences.

These results demonstrate, in principle, the feasibility of HLA typing by immunosequencing. HLA typing was achieved for most HLA alleles in the data set, including those observed at lower frequencies. The inference of rare HLA types would require the acquisition of more data so that those alleles and their associated TCR β sequences would be better represented in the data set. Also, despite our initial hypothesis that the TCR repertoire would be shaped by both positive and negative thymic selection, we observed very few TCR β sequences negatively associated with HLA alleles. Although this observation warrants further study, it could suggest that very few TCR β sequences recombine frequently and are reliably deleted by negative selection. The relative contributions of positive and negative selection to the shaping of the TCR repertoire remain unclear. For example, the contribution of positive and negative intrathymic selection to the peripheral T cell repertoire might depend on the avidity of thymocyte interactions with selecting endogenous peptide-MHC (pMHC) complexes and their structural relationships with pMHC encountered in the periphery⁵¹, and it has been shown that expression of the MHCrestricting allele has, at most, a mild impact on T cell frequencies at the level of thymic selection, has barely an effect on homeostatic periphery expansion, and varies for different antigens⁵².

DISCUSSION

Using a very large set of immunosequencing data from over 650 healthy subjects, we performed a high-throughput screen for public $\text{TCR}\beta$ sequences whose presence is associated with CMV serostatus or with particular HLA-A and HLA-B alleles. First, we identified a set of 164 TCRB sequences that could be used to correctly predict the CMV serostatus of subjects in the training data set (by using a leave-one-out cross-validation strategy) and, more importantly, of subjects from an unrelated cohort, with very high specificity and sensitivity. For a small set of these TCR β sequences, we confirmed their specificity for CMV-pp65(495-503) in vitro, thus showing that, despite being blind to the biochemistry of actual TCR-MHC-antigen interactions, our statistical association method can find TCR β sequences that bind CMV epitopes. We believe that this approach can be generalized to identify exposure to other pathogens, as they should imprint a similar signature in the TCR repertoire of exposed individuals. Future experiments will extend this approach to other pathogens and to vaccination. Only a few of the CMV-reactive TCRB sequences previously reported in the literature were either seen more often in CMV⁺ subjects or were more abundant in CMV⁺ subjects than in CMV⁻ subjects in our cohort. This result is not unexpected: first, it has been established that a large majority of T cell responses are private rather than public, so it is not surprising that only half of the previously reported CMV-reactive TCRB sequences were observed in our study⁴³. Second, for a TCR β to be part of our list of CMVassociated clones, it must rearrange frequently enough to constitute a public response to CMV but not so frequently that it appears in the TCRβ repertoires of many naive subjects, such that truly public TCRβ sequences present in most repertoires at high frequency would not have been identified by our study. Third, the TCR β must belong to a TCR that does not react to any common stimulus other than CMV. T cell cross-reactivity is an essential characteristic of antigen recognition^{53,54}, with some estimates proposing that a single T cell can recognize up to a million different pMHC complexes⁵⁵, and thus it is possible that some of the previously reported TCR β sequences could be cross-reactive with epitopes from other pathogens or other proteins. For example, CMV-reactive clones have been shown to recognize peptides derived from human HLA proteins in the context of allotransplant^{56–58}. Finally, as we only profiled TCR β , the apparent lack of overlap with previously reported TCRs could represent commonly rearranged TCR β sequences that have a broad array of antigen specificities in different HLA contexts or that are paired with different TCR α chains, resulting in a different antigen specificity.

By extending our approach to HLA typing, we also showed that, for HLA alleles present at a high enough frequency in the training data set, we could correctly predict the HLA-A and HLA-B alleles of the majority of individuals in cohort 1. As more TCR sequencing data across thousands of subjects are accumulated and compiled, we expect that our approach will be useful in HLA typing all but the rarest of alleles. Therefore, low-resolution HLA typing may soon be an additional benefit of any immunosequencing experiment, achieved simply by consulting a database of known allele-associated TCR sequences.

Our study proves that particular immunological phenotypes (in particular, CMV infection and HLA type) qualitatively mold the T cell repertoire, leaving an imprint that can be read using immunosequencing. Because high-throughput sequencing of TCRs captures all T cell responses equally and because hosts store immunological memory in this common format regardless of the stimulus, we believe that reading T cell memory by looking for known public responses will be a viable strategy for simultaneously diagnosing a wide range of immunological conditions using a single peripheral blood sample and a simple, unified assay. While the initial effort of collecting and analyzing a large training cohort for each phenotype of interest may be substantial, our diagnostic approach has the unique and useful property that, following routine immunosequencing and once such a database of associated TCRs is available, the incremental effort required to test a sample for an additional phenotype is negligible. We believe that further exploration of this approach is warranted, both to refine our methodology and to include other applications, such as other chronic infections and autoimmune conditions, and to refine HLA inference. One caveat of our approach is that it will also detect TCRB sequences specific for any condition that is highly correlated with CMV seropositivity. The use of this approach for the diagnosis of multiple infections simultaneously in the context of correlated seropositivity will require additional experiments and new methodologies.

In summary, using an *ab initio* approach and a very large data set with high power, we demonstrated that TCR sequencing can provide a sensitive and specific diagnostic. We expect that, once a sufficient number of TCR association studies have been completed to enable a highly multiplexed assay, immunosequencing will become a costcompetitive alternative to current diagnostic methods.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

The authors would like to thank M. Chung and other technical staff in the Adaptive Biotechnologies immunosequencing laboratory for their work on this project, S. House for helping compile the list of CMV-reactive TCRB sequences from the literature, and C. Linkem and K. Boland for assistance with sample tagging for the immuneACCESS project. This work was funded in part by an award from the W.M. Keck Foundation Medical Research Program to H.S.R. and C.S.C.

AUTHOR CONTRIBUTIONS

J.G. and J.A.H. obtained the DNA samples and determined the CMV status and HLA type of the subjects. R.O.E., C.S.C., M.R., and H.S.R. conceived and designed the experiments. M.R. generated the sequence data. R.O.E., W.S.D., M.V., and C.D. analyzed the results. R.O.E. and W.S.D. performed the statistical analyses. M.V. and C.D. performed the literature searches of CMV-specific TCRs. J.K.H., E.J.O., and M.K. performed and analyzed in vitro confirmation experiments. R.O.E., W.S.D., M.V., M.K., and H.S.R. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Experimental cohort and study approval. For cohort 1, human peripheral blood samples were obtained from the Fred Hutchinson Cancer Research Center Research Cell Bank biorepository of healthy bone marrow donors. Donors underwent routine HLA typing and CMV serostatus testing at the time the samples were taken. For cohort 2, human peripheral blood samples were taken from healthy volunteers under a protocol to examine past and present exposure to infectious agents. In both cases, protocols were approved and supervised by the Fred Hutchinson Cancer Research Center Institutional Review Board, following written informed consent.

Immunosequencing. Genomic DNA was extracted from peripheral blood samples using the Qiagen DNeasy Blood Extraction kit (Qiagen). The CDR3 region of rearranged TCR β genes, defined according to IMGT⁵⁹, was amplified and sequenced using previously described protocols^{5,60}. Briefly, a multiplexed PCR method that uses a mixture of 60 forward primers specific to TCR V $_{\beta}$ gene segments and 13 reverse primers specific to TCR J $_{\beta}$ gene segments was employed. An average of 2.5 μ g of input DNA was used for each sample (range, 0.5–3.5 μ g). Reads of 87 bp were obtained using the Illumina HiSeq system. Raw HiSeq sequence data were preprocessed to remove errors in the primary sequence of each read and to compress the data. To remove both PCR and sequencing errors, a nearest-neighbor algorithm was used to collapse the data into unique sequences by merging closely related sequences.

Identification of phenotype-associated TCRs and inference of phenotype status. We developed a statistical learning framework for the identification of TCRs associated with particular subject phenotypes, as well as for the inference of phenotype status in novel subjects. In this study, the phenotypes analyzed were CMV status and presence of *HLA-A* and *HLA-B* alleles.

In brief, we performed an association analysis to identify a set of TCR β s that had significantly increased incidence among phenotype-positive subjects. We then defined a subject's phenotype burden as the number of these phenotype-associated TCR β s that were found among all unique TCR β s immunosequenced from that subject. Phenotype burden was modeled using a beta binomial likelihood and separately trained on phenotype-negative and phenotype-negative subjects (modeling a common beta prior for all phenotype-negative subjects and a common beta prior for all phenotype-positive subjects. The probability that a novel subject was phenotype positive or negative was taken as the posterior probability using the trained likelihoods and priors estimated from phenotype prevalence in the training data. Phenotype status inference was taken as the maximum a posteriori estimate (i.e., the phenotype status that maximizes this posterior probability). Each of these steps is described in more detail below.

Immunosequencing of a peripheral blood sample from each subject in both cohorts yielded a list of unique TCR β CDR3 sequences (cohort 1 mean = 192,515 ± 80,630, cohort 2 mean = 202,918 ± 108,603) identified by a V gene, a J gene, and the amino acid sequence of the CDR3. Our learning problem in its raw form consists of Boolean phenotype and TCR presence data (**Supplementary Fig. 2a**).

For each of *N* subjects, the phenotype is indicated as present (1) or absent (0), and each of the *M* TCR β s is indicated as present or absent in each subject. The training cohort in this study consisted of *N* = 666 subjects, from which *M* = 89,840,865 unique TCR β s were identified. We wished to define a binary classifier, train on these data, and infer the classes (phenotype statuses) of novel subjects from TCR β immunosequencing data.

Owing to the stochastic nature of the V(D)J recombination process, the set of possible TCR β s is extremely large, and any TCR β repertoire (i.e., each immunosequencing sample) very sparsely occupies this space. Also, many TCR β s from a novel subject are expected to be novel with respect to the *M* unique TCR β s identified in any training cohort. An important complication is that the binding affinity of a given TCR for a given peptide antigen is modulated across individuals by HLA type. Therefore, the features relevant for discrimination of phenotype status will segregate according to latent HLA variables. We introduce a feature selection and dimensionality reduction approach that attempts to accommodate the idiosyncrasies of immunosequencing data with a minimum of model complexity.

Identification of phenotype-associated TCRs. For each of the *M* features (TCR β s) present in the training data with a subject incidence of at least two, we assess the significance of association with class assignment (phenotype status) by performing Fisher's exact test¹³ on a 2 × 2 contingency table, counting the number of subjects in each class according to the presence and absence of the TCR β in question (**Supplementary Fig. 2b**). Defining a rejection region by setting a maximum *P* value, we identify a set of phenotype-associated TCR β s. **Figure 2a** shows the incidence of all TCR β s from cohort 1 among CMV⁺ and CMV⁻ subjects and highlights significantly CMV-associated TCRs.

To identify CMV-associated TCR β s, we performed a one-tailed Fisher's exact test to identify TCR β s enriched in samples from CMV⁺ subjects, presumably owing to their specificity for CMV antigen and clonal expansion following activation. To identify HLA-allele-associated TCR β s, we used a twotailed Fisher's exact test. Thymic positive selection is expected to favor specific TCR β s that promote MHC binding given an HLA context. Therefore, these TCR β s are enriched in allele-positive subjects. Conversely, thymic negative selection is expected to censor specific TCR β s that lead to excess TCR–MHC affinity in the same HLA context. These TCR β s are expected to be suppressed in allele-positive subjects.

Given a *P*-value threshold, the false discovery rate (FDR) among phenotypeassociated TCR β s may be determined by permutation of class labels (shuffling the second column in **Supplementary Fig. 2a**) according to the method of Storey and Tibshirani⁶¹. Let r_0 denote the number of rejected null hypotheses (phenotype-associated TCR β s) in the unpermuted data. Suppose we perform *b* random permutations of the class labels and, for each permutation *i*, we perform significance tests for all TCR β s and find r_i rejected null hypotheses at the same significance threshold set for unpermuted data. Assuming that most null hypotheses are true (i.e., most TCR β s are not associated with the phenotype under study), the FDR may be approximated as follows.

FDR
$$\approx \frac{1}{b} \sum_{i=1}^{b} \frac{r_i}{r_0}$$

To determine FDR among CMV-associated TCR β s at various significance thresholds, *b* = 100 permutations were performed (**Supplementary Fig. 3**).

Quantification of phenotype burden on the TCR repertoire. Having identified a catalog of phenotype-associated TCR β s as described above, we can quantify the phenotype's burden on a subject's TCR β repertoire by comparing this catalog to the list of TCR β s observed in the subject. Suppose immunosequencing data from subject *i* resulted in n_i unique TCR β s. Instead of a multidimensional feature representation that considers the incidence of each phenotype-associated TCR β in this subject, we simply count the number k_i of unique TCR β s out of the total n_i that are in our catalog of phenotype-associated TCR β s. This transformation reduces feature space dimensionality to 2 and has a simple interpretation as a measure of how much of the TCR β repertoire is devoted to the phenotype. We refer to this as 'phenotype burden'. **Figure 3a** depicts the distribution in this reduced feature space for CMV⁺ and CMV⁻ subjects, which shows strong discrimination.

In the case of disease-exposure phenotypes, each of the phenotype-associated TCR β s is likely HLA restricted and power to identify phenotype association will vary with HLA allele frequency among subjects in the training cohort. Therefore, this dimensionality reduction eliminates the segregation of subjects into discriminative subspaces according to HLA type in exchange for variation in univariate discrimination power according to HLA type.

Modeling of phenotype burden. We approach the learning problem on the phenotype burden data by observing that the reduced feature vector may be interpreted as a binomial pair of k_i successes in n_i trials (as long as k_i is much less than the total number of phenotype-associated TCR β s, avoiding saturation). Instead of employing a standard discriminative binary classifier on the k_i/n_i ratio, we preserve sampling depth information by constructing a generative model for k_i conditioned on n_i and class assignment. Let $c_i \in \{0,1\}$ denote the class assignment of subject *i*. The probability that any unique TCR β in the repertoire of subject *i* is associated with the phenotype is modeled as a binomial proportion p_i . We suppose that the p_i values are independently and identically beta-distributed random variables within each class as follows.

$$p_i \sim \text{Beta}(\alpha_{c_i}, \beta_{c_i})$$

With conditioning on class assignment, we thus have the beta binomial distribution for subject i

$$p(k_i|n_i,c_i) = \binom{n_i}{k_i} \frac{B(k_i + \alpha_{c_i}, n_i - k_i + \beta_{c_i})}{B(\alpha_{c_i}, \beta_{c_i})}$$

where B(\cdot , \cdot) denotes the beta function. The parameters { α_0, β_0 } and { α_1, β_1 } parameterize the beta-distributed prior for phenotype-negative and phenotype-positive subjects, respectively. These may be determined by maximizing the joint likelihood over all subjects.

$$p(k|n,c) = \prod_{i=0}^{N} p(k_i|n_i,c_i) = \left(\prod_{i:c_i=0}^{N} p(k_i|n_i,0)\right) \left(\prod_{i:c_i=1}^{N} p(k_i|n_i,1)\right)$$

Forming the log likelihood, dropping terms dependent only on the data, and exploiting parameter separability, we maximize

$$\ell_l(\alpha,\beta) = -N_l \log B(\alpha,\beta) + \sum_{i:c_i=l} \log B(k_i + \alpha, n_i - k_i + \beta), \ l = 0,1$$

where N_l denotes the number of subjects with class l. These objective functions have gradients

$$\begin{split} \frac{\partial \ell_l}{\partial \alpha} &= -N_l \left(\psi \left(\alpha \right) - \psi \left(\alpha + \beta \right) \right) + \sum_{i:c_i = l} \left(\psi \left(k_i + \alpha \right) - \psi \left(n_i + k_i + \alpha + \beta \right) \right) \frac{\partial \ell_l}{\partial \beta} \\ &= -N_l \left(\psi \left(\beta \right) - \psi \left(\alpha + \beta \right) \right) \\ &+ \sum_{i:c_i = l} \left(\psi \left(n_i - k_i + \beta \right) - \psi \left(n_i + k_i + \alpha + \beta \right) \right) \end{split}$$

where $\psi(.)$ denotes the digamma function. Standard numerical gradient ascent methods were used to determine the class-wise beta priors.

$$\{\alpha_l, \beta_l\} = \underset{\{\alpha, \beta\} \in \mathbb{R}^2_+}{\operatorname{argmax}} \ell_l(\alpha, \beta), \ l = 0, 1$$

Laplace smoothing of the most deeply sampled subject in each class (largest n_i) was used to regularize the likelihood. Densities for estimated priors from the CMV data are shown in **Supplementary Figure 4**.

Having determined these likelihood parameters from a joint model for all training subjects, we now consider a novel subject with the phenotype burden k', n'. Approximating class priors from Laplace-regularized class counts in the training data, the posterior probability of each class assignment for the novel subject is

$$p(c'=x|n',k') = \binom{n'}{k'} \frac{B(k'+\alpha_x,n'-k'+\beta_x)}{B(\alpha_x,\beta_x)} \frac{N_x+1}{N+2}, \ x = 0,1$$

We form the log-posterior odds ratio for class assignment as

$$F(k',n') = \log p(c'=1|k',n') - \log p(c'=0|k',n')$$

$$= \log(N_1 + 1) - \log(N_0 + 1) + \log B(\alpha_0, \beta_0)$$

- log B(\alpha_1, \beta_1) + log B(k' + \alpha_1, n' - k' + \beta_1)
- log B(k' + \alpha_0, n' - k' + \beta_0).

A decision function is defined by a threshold θ on this quantity.

$$\hat{c}(k',n',\theta) = \begin{cases} 0, F(k',n') \le \theta\\ 1, F(k',n') > \theta \end{cases}$$

The maximum a posteriori (MAP) classification corresponds to $\theta = 0$.

$$\hat{c}(k',n',0) = \underset{x \in \{0,1\}}{\operatorname{argmax}} p(c'=x|n',k')$$

With the classifier now defined, we may address a model selection question. In identifying phenotype-associated TCR β s, we must apply a *P*-value threshold. This threshold is a hyperparameter for the classifier. To select an optimal *P*-value threshold, we perform exhaustive leave-one-out cross-validation in the training data over a range of *P*-value thresholds and assess the cross-entropy loss at each *P* value used. For each subject held out, we recompute phenotype-associated TCR β s at the given threshold (feature selection) and fit likelihood parameters ({ α_0 , β_0 } and { α_1 , β_1 }) using only the remaining subjects. We then use this classifier to estimate the class probabilities for the subject held out. Iterating this process over all subjects gives the cross-validated class probabilities for each subject in the training set.

Let $q_i(\phi)$ denote the probability that $c_i = 1$ under the classifier built with subject *i* held out from feature selection and training, and using a *P*-value threshold of ϕ for identifying phenotype-associated TCR β s. The average crossentropy loss over all subjects using *P*-value threshold ϕ is defined as

$$L(\phi) = -\frac{1}{N} \sum_{i=1}^{N} \left[c_i \log q_i(\phi) + (1 - c_i) \log(1 - q_i(\phi)) \right]$$

By computing this loss function over a discrete set of *P* values ϕ , we may approximate a minimum.

$$\hat{\phi} = \underset{\phi \in \mathcal{O}}{\operatorname{argmin}} L(\phi)$$

Cross-entropy loss is a preferable metric to classification error, as it utilizes the probabilistic information provided by the generative model rather than the decision function per se. Minimizing $L(\phi)$ is tantamount to maximizing a joint likelihood of the class vector c, where the marginal probability for each subject i is computed from a model where subject i was held out. In modeling CMV data, a logarithmic grid of P values was used, indicating $\hat{\phi} = 10^{-4}$ as approximately optimal (**Supplementary Fig. 3**). Cross-entropy loss on this grid was also computed using a single model that was trained on all data. In this case, $L(\phi)$ monotonically decays with increasing ϕ , highlighting the importance of cross-validation to avoid overfitting to spuriously phenotypespecific TCR β s.

Assessing classification performance. To validate this learning framework, we acquired a second cohort of CMV-typed subjects as a testing cohort. To assess the performance of the method in classifying the presence of HLA alleles, leave-one-out cross-validation was performed. For each HLA allele, we held out each subject and built classifiers using the remaining subjects. HLAallele-associated clones may be both positively and negatively associated. As the vast majority of clones were positively associated, we excluded these clones in our dimensionality reduction. Our model could be augmented from beta binomial to Dirichlet multinomial to separately count the incidence of TCR β s negatively and positively associated with HLA alleles, but little improvement in discrimination is to be expected, as negative associations were relatively rare. In this case, $\hat{\phi} = 10^{-4}$ (from optimization on CMV data) was used as a fixed parameter, rather than optimized separately for each allele. This was necessary to avoid bias created by performing cross-validation for both model selection and model evaluation. Fixing this parameter is expected to provide a conservative assessment of classification performance, as the wide variation in HLA allele frequency entails variation in optimal P-value thresholds across alleles (which could be determined with more expensive nested cross-validation for both model selection and model evaluation). To call the HLA type of each subject, results from the classification on each allele were aggregated, with zygosity at each locus not explicitly enforced. Accuracy for these crossvalidated HLA-type inferences is described in the Results section.

Overlap with the literature. We compiled an exhaustive list of CMV-reactive TCR β s from 34 publications. Most of the published CDR3s conformed to the standard nomenclature (i.e., they extended from a cysteine to a phenylalanine, or C–F), but some did not. For those that were longer, we trimmed to the C–F fragment before comparing to the list of TCR β s identified in this study; those that were shorter or incomplete were used as published. In our overlap analysis, a match consisted of either the published TCR β or the TCR β identified in this study constituting a substring of the corresponding sequence.

MIRA assays. Peptide-based MIRA was performed as previously described²⁹. Briefly, approximately 200 million PBMCs from a single CMV⁺ HLA-A2⁺ donor were divided into 8 aliquots and incubated with different pools of 19 dextramers (Immudex) out of a total of 38 available dextramers. These included one dextramer with a CMV-derived epitope and 37 dextramers with non-CMV-derived peptides as a negative control to show the specificity of our assay. Dextramer-positive and dextramer-negative CD8⁺ T cells were sorted and immunosequenced as described above. Antigen-specific TCR β sequences were identified on the basis of the following criteria: first, we selected TCR β sequences that were significantly enriched in the positive sorted population in a subset (3, 4 or 5) of the 8 initial PBMC aliquots on the basis of a binomial model of differential abundance⁶². Next, we examined observed occupancy by using a maximum-likelihood framework to discern the best possible antigen address given the clone enrichment patterns. Significantly associated clones were then identified using a likelihood-ratio test comparing the best occupancy hypothesis to a null model of no enrichment across all aliquots. $TCR\beta$ sequences responding to CMV-pp65₄₉₅₋₅₀₃ using MIRA were then compared to associated sequences identified using population-based sequencing.

Statistics. CMV-associated TCR β s were identified using a one-tailed Fisher's exact test¹³. For each TCR β in the training cohort that was found in more than one subject, we formed a 2 × 2 contingency table counting the number of subjects in the training cohort according to CMV serostatus and presence of the TCR β in question. HLA-allele-associated TCR β s were found similarly by using allele presence and presence of the TCR β in question to generate a 2 × 2 contingency table. A two-tailed test was used to detect both positively and negatively associated TCR β s (corresponding to HLA modulation of positive

and negative thymic selection, respectively). FDR was computed by permutation, and a *P*-value threshold of 1×10^{-4} (FDR = 0.14) was selected by cross-validation.

Enrichment of pp65-specific TCRs among overlaps between the MIRA assay results and our catalog of CMV-associated TCR β s was calculated as follows. We assumed that the population frequency of pp65-specific TCR β s among all TCR β s identified by the MIRA experiment was 3.75% (empirically, using 69 pp65-specific TCR β s out of 1,840 MIRA TCR β s overall). Noting that three of three hits to our CMV-associated TCR catalog were pp65 specific in the MIRA experiment, we performed a one-tailed binomial test assessing the hypothesis that more than two of three trials were successes given a frequency of 3.75%. This led to a *P* value for enrichment of 5.3 × 10⁻⁵.

Data availability. All immunosequencing data underlying this study are freely available from https://doi.org/10.21417/B7001Z and can also be analyzed and downloaded from the Adaptive Biotechnologies immuneACCESS site at https://clients.adaptivebiotech.com/pub/Emerson-2017-NatGen.

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