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Review

Genotype–phenotype landscapes for immune–pathogen coevolution

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Our immune systems constantly coevolve with the pathogens that challenge them, as pathogens adapt to evade our defense responses, with our immune repertoires shifting in turn. These coevolutionary dynamics take place across a vast and high-dimensional landscape of potential pathogen and immune receptor sequence variants. Mapping the relationship between these genotypes and the phenotypes that determine immune–pathogen interactions is crucial for understanding, predicting, and controlling disease. Here, we review recent developments applying high-throughput methods to create large libraries of immune receptor and pathogen protein sequence variants and measure relevant phenotypes. We describe several approaches that probe different regions of the high-dimensional sequence space and comment on how combinations of these methods may offer novel insight into immune–pathogen coevolution.

Highlights

The set of all possible pathogen and immune receptor sequences, along with the phenotypes that define their fitness and interactions, define a large and high-dimensional landscape across which immune–pathogen coevolution takes place.

Recent high-throughput phenotyping assays exploit next-generation sequencing to measure relevant phenotypes of large libraries of pathogen or immune receptor sequences, dramatically increasing our ability to characterize the genotype–phenotype maps underlying immune–pathogen coevolution.

Three broad classes of approaches have been used to explore these coevolutionary landscapes. First, random or saturating mutagenesis methods analyze all (or many) variants in the direct mutational vicinity of a small set of focal sequences. Second, combinatorial methods make it possible to analyze all (or many) possible combinations of some specific set of mutations. Finally, directed evolution methods explore how focal sequences adapt across successive rounds of mutagenesis and selection.

Significance

Understanding immune–pathogen coevolution is essential for predicting and ultimately controlling disease. This coevolution takes place over a complex landscape defined by the relevant pathogen and immune receptor sequences, along with the phenotypes that determine how they interact. Thus, it is important to survey the recent high-throughput empirical methods that have transformed our ability to characterize the properties of large and high-dimensional genotype–phenotype maps and their implications for our understanding of immune–pathogen coevolutionary dynamics.

Immune–pathogen coevolution

Our adaptive immune systems are engaged in a continuous coevolutionary struggle with the pathogens that attack us [1,2]. This immune–pathogen coevolution takes place across a range of spatial and temporal scales: evolution in individual infections is driven by interactions between pathogens and an individual host immune system, often over the course of days or weeks, while global pathogen evolution is driven by the collective immune responses of whole, often geographically dispersed populations across years or decades [3–6]. This pathogen evolution, in turn, drives shifts in both individual and population-wide host immune repertoires [7–10]. The dynamics of this coevolutionary process within individuals and across the population is key to understanding, predicting, and ultimately controlling disease.

Like any evolutionary process, two key components shape these dynamics: the relevant **genotype–phenotype maps** (see Glossary) (which define the landscapes on which coevolution takes place) and the **population genetic environment** (the selection pressures, population sizes, mutation rates, and other factors that determine how evolution navigates the landscape) (Box 1). While both components are important and incompletely understood, here we focus on recent work exploiting technological advances to characterize the former: the genotype–phenotype maps of immune and viral proteins that define the coevolutionary landscape. Exploring these landscapes is a daunting prospect, because the size of the relevant sequence spaces is so massive that we cannot possibly explore these spaces comprehensively. However, by probing landscape structure in ways that are guided by evolution, we can hope to understand essential features that drive the coevolutionary process.

Inferences from observational data

Scientists have tracked pathogen sequence evolution since the advent of Sanger sequencing nearly 50 years ago, with a particular focus on viruses such as influenza. In the past two decades, rapid advances in sequencing technology have led to a dramatic expansion in the scale and

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Box 1. Fitness landscapes

We can think of the map between genotype and fitness as a '**fitness landscape**', which we often visualize as an actual landscape (complete with mountains and valleys), where the 'height' represents fitness and horizontal distances are proportional to the similarity between genotypes (see Figure 3 in main text) [124]. Populations can then be visualized as evolving across these landscapes by acquiring mutations, generally following an upward trajectory to higher fitness, with their exact dynamics depending on the population genetic environment (e.g., the selection pressures, population size, mutation rate, etc.). Hence, given some knowledge of these processes, fitness landscapes can be used to predict future evolution. A key question is the extent to which landscapes are 'smooth' (with steadily uphill paths towards higher fitness) versus 'rugged' (with local optima that can create traps and dead ends). Because ruggedness arises from non-additive (i.e., epistatic) interactions between mutations, it is particularly important to characterize these interactions.

While this low-dimensional intuition is appealing, it is also important to remember that genotype space is in fact vast and extremely high-dimensional. Even for a short peptide of ~20 amino acids, there are more than 10^{26} potential sequence variants, with a complex web of mutational relationships. The typical structure of such high-dimensional landscapes can run counter to our low-dimensional intuition (e.g., in considering the likelihood of local peaks and dead ends). In addition, the implicit assumptions that fitness landscapes are static breaks down when considering coevolving entities such as pathogens and immune receptors. Instead, these coevolutionary landscapes can act more as 'seascapes', which constantly shift in response to the opposing selection pressure [125]. For example, a virus may acquire antibody escape mutations, temporarily improving its fitness and allowing it to move upward in the landscape, but this region of the landscape will then shift downwards as antibodies mature to recognize the mutant virus [125].

The dynamic, high-dimensional nature of immune–pathogen coevolutionary landscapes makes the relationship between genotype and fitness impractical to characterize comprehensively using either experimental or computational methods. Instead, there have been significant advances in understanding the underlying structure of these landscapes by mapping the relationship between sequence and individual components of fitness (e.g., host receptor affinity, antibody escape) and the relative importance of these components in various selection environments (e.g., individuals with distinct immune responses). While seemingly more complicated, this type of decomposition can prove useful because these components are governed by biophysical and population genetic rules, mitigating the need to exhaustively chart sequence space [126].

scope of these surveillance studies and we now have access to detailed databases containing thousands to millions of sequences of viral pathogens, such as influenza, Zika, Ebola, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and many others [4,11–13]. Massive databases of bacterial pathogen sequences are also widely available [14,15]. These resources offer a unique window into pathogen evolution at the nucleotide level and often allow researchers to identify novel variants and track their dynamics through space and time [16,17].

Observational studies of the sequence diversity of immune receptors within an individual also date back a few decades and numerous studies have used these datasets to characterize the immune response [18–23]. As with the tracking of pathogen evolution, recent advances in sequencing methods have led to a proliferation of more comprehensive analyses of the B or T cell repertoires within individuals [24,25] and of how these repertoires change over time (e.g., in response to vaccination) [23,26,27]. These studies shed light into the evolution of our immune receptor repertoires, for example, by classifying B cell receptor (BCR) sequences into clonal lineages [28,29] and using within-lineage diversity to infer aspects of the **affinity maturation** process [30–32]. Aspects of the overall statistics of repertoire sequences (e.g., the frequency distribution of different variants) can also be used to infer parameters of the **V(D)J recombination** process that generates naive BCR sequences [33] and the influence of a history of pathogen exposure and/or vaccination on shaping repertoire statistics [30,34,35].

However, while advances in sequencing have dramatically improved our ability to observe sequence evolution in both pathogens and immune receptor repertoires, it remains more difficult to connect this sequence evolution with relevant phenotypes. That is, which of the mutations that we observe in influenza virus or SARS-CoV-2 evolution might lead to immune escape? Which of the BCR sequences binds a particular antigen and how has this changed across a clonal lineage

during affinity maturation? Addressing these questions is needed to understand the significance of observed pathogen and immune evolution and for any attempt to predict future coevolution.

To some extent, we can infer the phenotypic effects of specific mutations based on observational data (Figure 1). When multiple time points are available, the **relative fitness** of a specific pathogen strain can be directly estimated from its growth [36]. However, while this has been successfully applied to SARS-CoV-2 [37,38], there are potential caveats related to population structure and sampling biases, which can lead to misleading estimates of strain frequencies over time. In addition, these approaches are often not viable for less densely sequenced viruses. This type of analysis is also extremely difficult in the context of antibodies: as affinity maturation occurs entirely within lymph nodes, only the end result of this process is usually sequenced, so we lack any direct measurements of antibody frequency changes over time. Even experiments sampling directly from lymph nodes (in mice [39,40] or in humans [41,42]) result in the disruption of the germinal center, stopping the possibility of assessing an evolutionary process.

When time course data are not available, sequence variation at a snapshot in time can still provide some insight into selection pressures. For example, analyses based on the frequency of mutations or on the statistics of inferred phylogenetic relationships between sampled sequences have been used to analyze antigenic selection in influenza virus infections (and hence to predict future viral evolution) [43–45], as well as antibody responses to vaccination [42]. These approaches essentially allow us to infer past increases in frequency of specific pathogen strains or antibody variants (or groups of strains/variants). Some of this work also combines phylogenetic data with phenotypic information (e.g., **cross-neutralization** data for influenza [46–48]), which

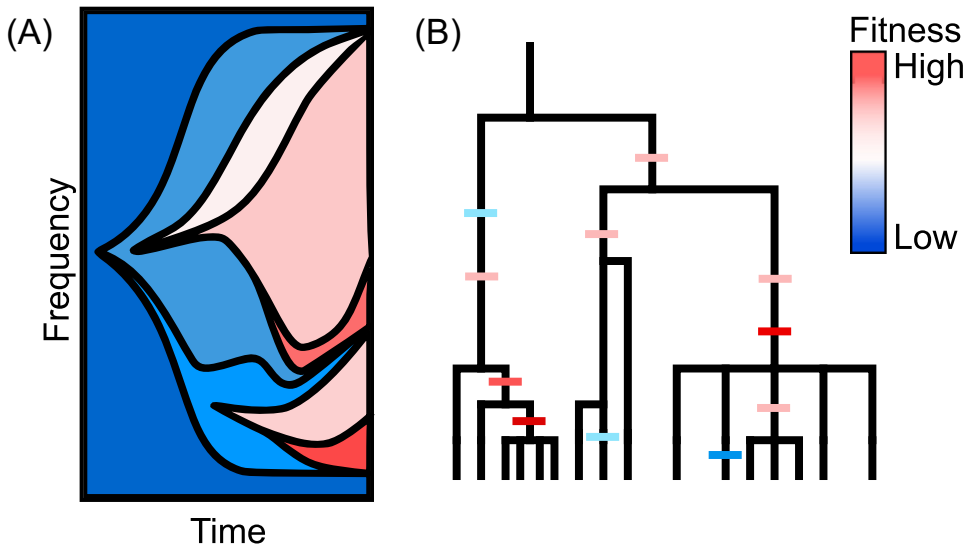

Figure 1. Inferring fitness from observational data. (A) **Muller diagram** showing changes in frequency of lineages within a population over time. Each color refers to a specific genotype with a given fitness [corresponding to the phylogeny in (B); trajectories illustrate the changes in frequency of each lineage through time]. These frequency changes can be used to infer fitness of each genotype (though as noted in the text, this may be confounded by population structure, sampling biases, and other factors). (B) For illustration purposes, a hypothetical reconstructed phylogeny from a population sample collected at a single timepoint is shown. The structure of this phylogeny can be used to infer the fitness effect of each mutation.

Glossary

Affinity maturation: process by which antibodies increase their affinity against pathogens through rounds of somatic hypermutation and selection.
Antigenic drift: gradual accumulation of mutations in a pathogen, leading to changes in effectiveness of an immune response.
Biased mutation profile: set of probabilities of mutation types that are biased relative to other somatic mutations.
Cross-neutralization: the ability of antibodies to neutralize related pathogen strains.
Deep mutational scanning (DMS): method to analyze the effects of genetic variation by systematically introducing mutations into genes and measuring their effects on the corresponding phenotype.
Directed evolution: method of evolving biomolecules towards desired properties through experimentally imposed cycles of mutagenesis and selection.
Epistasis: non-additive interactions between mutations.
Extant sequences: sequences present in nature.
Fitness landscape: map between genotype (nucleotide sequence) and fitness (often represented by a proxy phenotype such as binding affinity, expression, antigenicity, etc.).
Focal sequence background: region of the protein sequence that is not mutated with respect to the starting (focal) sequence.
Fragment-antigen binding domain (Fab): part of an antibody that binds antigens. Constituted by two domains (constant and variable) for both the heavy and light chains. Used in antibody display.
Genotype–phenotype maps: map between genotype and a specific phenotype.
Historical contingency: influence of initial mutations on the evolutionary accessibility of future mutations.
Kinetic exclusion assays: measure the fraction of ligand bound to a receptor over a short period of time to quantify ligand–receptor interactions.
Low-dimensional antigenic space: hypothetical low-dimensional representation of all antigens that would retain accurate distances between immunological properties of each strain.

can improve the accuracy of inferred selection pressures but requires careful curation of the phenotypic data [43,47]. By contrast, other studies have focused entirely on phylogenetic information [49]. However, irrespective of these details, these types of analyses require relatively large datasets and make assumptions regarding evolutionary dynamics [50]. In addition, it is particularly challenging to apply these approaches to antibody sequence diversity, largely because of the properties of affinity maturation: namely, antibodies evolve in a handful of generations [39], under strong selection pressures [30], and with a **biased mutation profile** [51]. These complications violate assumptions of many population genetic models, making it difficult to apply standard methods.

Connecting genotype to phenotype

The limitations of these purely observational methods highlight the need for direct experimental measurements that connect genotypic diversity to the phenotypes that drive immune–pathogen coevolution. There are numerous phenotypes that influence the fitness of pathogens and antibodies and it is impossible to measure them all. However, several key phenotypes (e.g., protein stability, binding affinity to relevant target proteins, and neutralization of pathogenic strains by sera) are thought to be major drivers of immune–pathogen coevolution [52–56]. While these traits do not encompass all the selection pressures relevant for coevolution, they are useful proxies for important aspects of this process.

One approach to characterize these phenotypes involves direct analysis of protein structures, such as in early work using the structure of the influenza hemagglutinin (HA) protein to pinpoint sites of potential importance for immune evasion [57,58]. However, protein structure alone is usually insufficient to predict the effect of specific mutations. This motivates the use of methods that directly measure phenotypes, which often rely on traditional biochemical and immunological assays. Neutralization assays, for example, measure the ability of monoclonal antibodies (or of polyclonal sera) to inhibit viral infection of host cells [59]. This is relevant for analyzing population-level viral evolution [37,46], though it is less informative about intra-host immune evolution because it does not reveal the underlying antibody sequence changes. Other approaches tend to focus on more specific phenotypes, such as binding between surface proteins and antibodies. These measurements are typically made using purified proteins, using various techniques such as **surface plasmon resonance**, **kinetic exclusion assays**, titration-based methods, and ELISA [60–63]. While these measurements may not be as straightforward to interpret as neutralization data, they often provide a more direct insight into the mechanistic basis of antibody–pathogen interactions and hence can be more useful for making predictions.

Numerous studies have used these experimental measurements in combination with epidemiological models to infer and predict immune–pathogen coevolutionary dynamics. For example, serum reactivity studies can be used to define immune-similarity metrics between antigens, in the hope of defining a relatively **low-dimensional antigenic space** (see, e.g., [46,64,65]); one can then model **viral escape dynamics** through this space (see, e.g., [66]). Other studies make simplifying assumptions about the broader coevolutionary landscape (e.g., assuming that the effects of viral and antibody variants on binding affinities are additive to predict the dynamics of HIV-1 escape from monoclonal antibodies [67]).

A key limitation of these traditional phenotypic measurements is throughput: individual experiments can only test one pathogen strain or antibody at a time and are both time-consuming and expensive. As a result, studies have focused on relatively small sets of pathogen or antibody sequences. For example, one can isolate the relatively small fraction of B cells that bind a specific

Muller diagram: graphical representation showing the frequency of all sequence variants within a population over time.
Population genetic environment: factors that determine how a population generates variation and navigates the fitness landscape.
Relative fitness: measure of fitness compared with other variants in the population; this determines relative changes in frequency over time.
Reverse genetics: set of methods that infer the function of a gene by engineering specific mutations and observing their effects on phenotype.
Sequence logo plots: graphical representation of the effects of individual mutations on a given phenotype. Refers to additive effects in a specific focal background and does not include potential epistatic effects.
Single-chain variable fragment (scFv): fusion protein of antibody heavy and light variable regions. Used in antibody display.
Surface plasmon resonance: label-free method to measure the concentration of ligands bound to a surface coated with a receptor and, hence, quantify ligand–receptor interactions.
Tite-Seq: high-throughput approach to estimate the binding affinities of tens of thousands of protein variants to a given ligand.
Training neural networks: statistical approach for inferring parameters of a neural network based on a given data set.
V(D)J recombination: gene recombination process that generates naive antibody and T cell receptor diversity.
Viral escape dynamics: evolutionary changes in virus structure leading to immune escape.

antigen from blood samples and characterize the binding of these sequences to that antigen [68–71]. However, this still constrains the analysis to one small part of the landscape that was explored by affinity maturation in one particular individual. While one can alternatively create and analyze other types of sequence variants, basic constraints on throughput will still limit analysis to extremely small corners of the genotype space. This limitation can be circumvented by making simplifying assumptions (e.g., additivity of mutational effects) that allow us to infer the phenotypes of unobserved genotypes. However, these inferences are likely to fail because of widespread **epistasis** across the large diversity of viral strains and antibody sequences (Box 1). The key problem is that even small changes in viral or antibody proteins can have dramatic and context-dependent effects on their interactions [72].

Exploring large and high-dimensional landscapes

To more comprehensively analyze the large and high-dimensional genotype–phenotype maps that are relevant for immune–pathogen coevolution, extensive efforts have been devoted in recent years to developing methods to dramatically increase the throughput of the phenotypic measurements described earlier. These approaches typically use next-generation sequencing to measure relative enrichments of pathogen or immune receptor sequences across some experimentally imposed selection pressure, making it possible to conduct phenotypic measurements for large libraries of sequences in parallel (Figure 2). For example, numerous studies measure the effect of large libraries of sequence variants of influenza HA [73–76], SARS-CoV-2 spike protein [77,78], or HIV-1 envelope [79] on the ability of the corresponding viruses to infect target cells. Similar methods have been used to characterize the interactions between a library of antibody variants and their cognate antigen [80,81]. Other studies have developed '**Tite-Seq**' methods to make precise quantitative measurements of the binding dissociation constants of large libraries of antibody sequences to specific antigen proteins [54,82,83] (or conversely of large libraries of pathogen proteins to specific antibodies and target proteins [55,84–86]). These can be undertaken by displaying the library of interest on the surface of yeast, sorting the yeast cells into

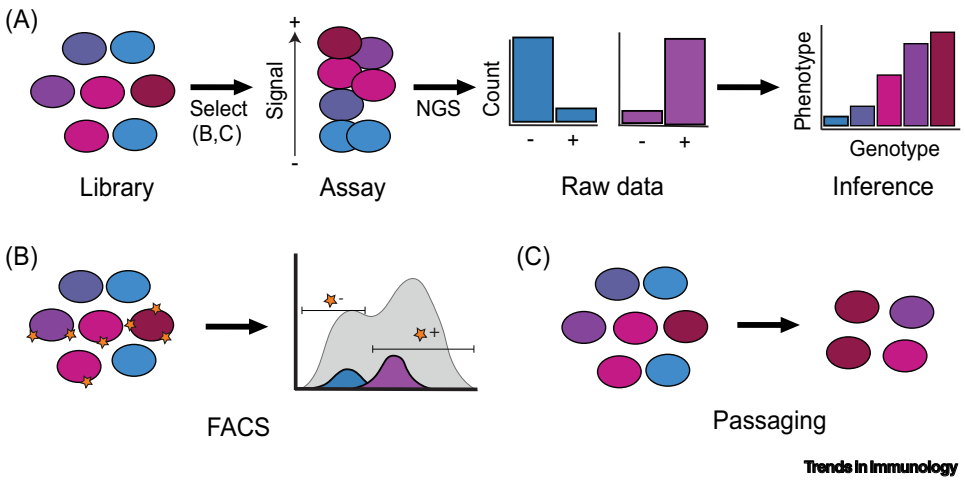

Figure 2. High-throughput phenotypic measurements. (A) Large libraries of protein sequence variants can be assayed for various phenotypes of interest by conducting some assay, grouping variants based on the result of this assay [e.g., selecting using flow cytometry based on binding to a fluorescently labeled antibody as in (B), or based on some other experimental selection pressure as in (C)] and sequencing to determine the relative enrichment of each variant within each group. (B) A fluorescence-activated cell sorting (FACS)-based method to measure phenotypes such as binding or protein expression, in which the protein of interest is labeled with fluorescent tags for FACS-based cell sorting, is shown. (C) A direct passaging method, in which viral variants are incubated with target cells and allowed to replicate, is shown. After some time, successfully replicating (i.e., infectious) variants are isolated [95,136,137]. Abbreviation: NGS, next-generation sequencing.

bins based on binding of the surface-expressed protein to fluorescently labeled target, and sequencing these bins. In principle, similar types of approaches could conceivably be used to dramatically increase the throughput of other types of phenotypic testing; for example, this might be done by using methods analogous to Tite-Seq to measure the ability of large libraries of antibody variants to neutralize a specific viral strain, or the ability of a specific antibody sequence to neutralize a large library of viral variants.

High-throughput approaches often (though not always) rely on simplified or idealized systems, for example, viruses may be represented by a single protein that is recombinantly produced and antibodies may be truncated to enable expression on display systems [e.g., a **single-chain variable fragment (scFv)**, or **fragment-antigen binding domain (Fab)**] (Box 2); this can make it possible to explore phenotypes across a much larger portion of sequence space. However, despite these increases in throughput, the potential sequence space relevant for even a small viral protein (or a relatively short variable region in an antibody sequence) is so vast that we can still only hope to sample a tiny fraction of possible variants. Consequently, phenotypic measurements must be restricted to a well-chosen and relevant subset of genotypes. The choice of these subsets must be guided both by the specific research questions addressed (e.g., sampling variants in a way that is ideally related to the natural evolutionary process) and by technical constraints (particularly on the methods used to construct large variant libraries). Next, we describe three broad categories of approaches to the construction of these libraries used to explore distinct parts of antibody and pathogen sequence spaces (Figure 3, Key figure).

Local exploration

In the short term, evolution always acts locally, by sampling genotypes in the immediate mutational neighborhood of **extant sequences**. This has motivated extensive efforts to explore the local mutational landscape around antibody or pathogen sequences of particular interest (Figure 3A). For instance, numerous studies have used the **deep mutational scanning (DMS)** approach [87,88] to assess the phenotypic effect of various mutations on a specific **focal sequence background**. In these studies, libraries containing variants with one to a few amino acid substitutions from the focal sequence are generated and subjected to a selection pressure

Box 2. Relevant expression and display technologies

Numerous advances in protein expression technology over the past several decades have helped enable high-throughput measurements of various phenotypes. These technologies often rely on culturing phage or microbes, which produce recombinant protein either in solution or displayed on cell surfaces. Screening phenotypes of soluble proteins typically requires individual measurements for each variant because there is no way to link specific variants with their sequence in a bulk assay. While these soluble protein screens can often be conducted in 96- or 384-well plate format, the need for individual measurements still limits throughput significantly. For this reason, high-throughput phenotyping measurements are often carried out using either a protein display strategy (where the variant sequence is encoded on a plasmid or in the genome) or by linking a variant-specific tag to each protein molecule (e.g., using an mRNA or a ribosome display system [127]).

Protein display technologies have been established in phage, prokaryotic, and eukaryotic systems and have been used to measure various protein phenotypes, especially binding affinity [128–131]. For example, phage display was first introduced in 1985 [132] and has become a mainstay in novel therapeutic development, especially for antibody treatments. The relatively small size of bacteriophages makes it possible to analyze enormous libraries (up to tens of millions of variants) in a single measurement [132]. Although yeast-display libraries are substantially smaller than those in phage, they enable protein expression with eukaryotic post-translational modifications, which can crucially influence viral protein and antibody receptor function [131]. Still, most yeast-display systems are constrained to individual protein domains, limiting their utility for examining phenotypes involving multiple domains or oligomers. Recently, there have been several advances in human cell display technologies (e.g., engineered T cell receptor display), enabling display of full-length, natively processed viral proteins and immune receptors [78,133]. Separately, **reverse genetics** approaches enable construction of viral libraries [134,135], where viral protein variants are expressed in virions containing the corresponding genetic information and 'fitness' can be easily measured by viral replication.

Key figure

Strategies for exploring sequence space

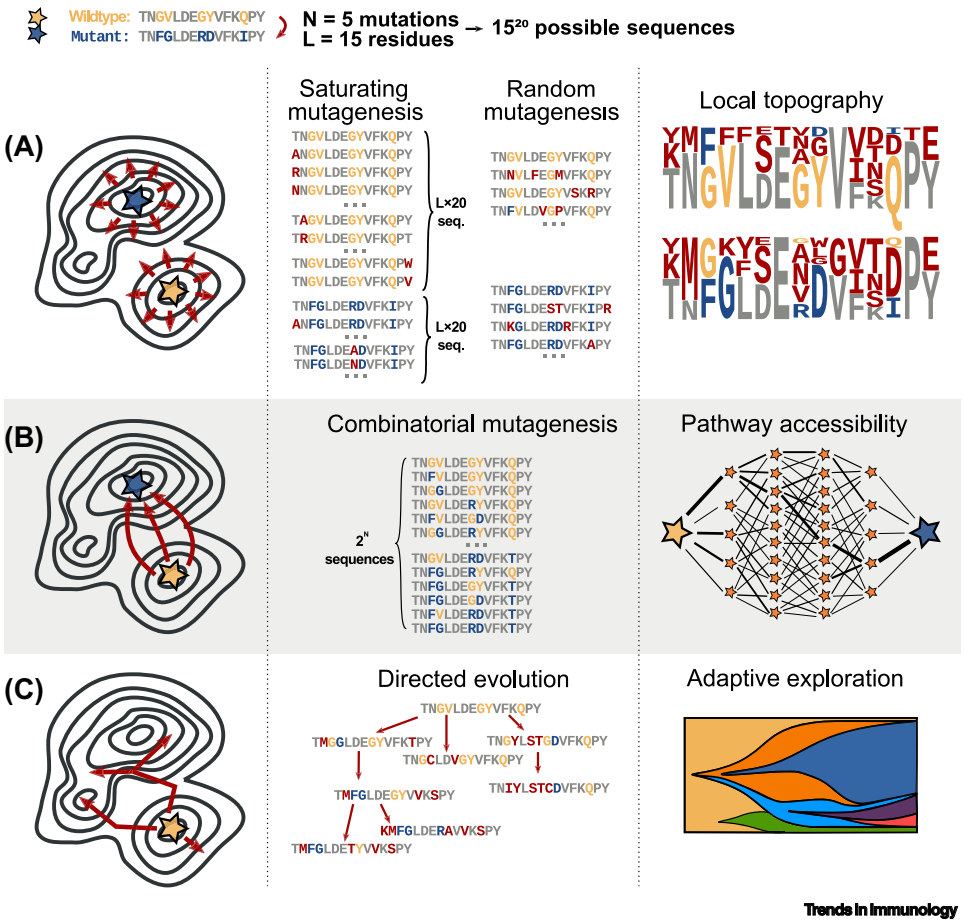

Figure 3. We show an example of a short 15-residue peptide (with genotype space consisting of $15^{20} = 3 \times 10^{23}$ total possible sequence variants), which has evolved from a wild-type sequence to a mutant with five mutations at the sites indicated in blue. Shown are several types of variant libraries that explore different subsets of the sequence space (left) and lead to different observables (right). (A) Local exploration. These methods focus on the local mutational landscape around a specific focal genotype (here the wild-type or mutant sequences) using saturating mutagenesis (i.e., constructing all genotypes one mutation away from the focal genotype, 20*L total variants) or random mutagenesis. **Sequence logo plots** (right) are commonly used to summarize the impact (in the focal genotype) of all possible mutations at each site on the phenotype. This approach surveys both negative and positive mutational effects but does not provide information on epistasis. (B) Combinatorial exploration. Here the library contains all possible combinations of N mutations separating the mutant from the wild-type sequence (2^N total variants); both positive and negative mutational effects as well as epistatic interactions can be analyzed. All possible trajectories from wild-type to mutant sequences are shown on the right, with lines indicating the probability of each based on an evolutionary model given the measured phenotypes corresponding to each genotype (right). These probabilities can be used to define 'pathway accessibility', which refers to the likelihood of each possible pathway given a particular evolutionary model of natural selection and genetic drift. (C) Random exploration with selection. These libraries are generated with multiple rounds of random mutagenesis and selection; this approach is suited primarily to studying mutations with positive effects. The resulting population dynamics are shown on a Muller diagram, which shows the changes in frequency over time of each sequence variant (right).

such as binding affinity [89–94]. Following selection, relative enrichment of each variant is measured (more recent studies can also measure absolute binding affinities; see [55,85,86]).

DMS studies have identified mutations relevant to immune escape and pathogen function in many different viral systems involving influenza [73,75,95], hepatitis C [96], HIV-1 [79], and numerous SARS-CoV-2 variants [55,86], among others. This work has obvious direct implications for short-term evolutionary dynamics. For example, a number of studies on influenza virus show that the HA sequence is highly tolerant to mutations, enabling rapid **antigenic drift** [73,92,95,97]. Similar work on the SARS-CoV-2 spike protein has identified three receptor-binding domain (RBD) sites with mutations that increase binding affinity to the host cell receptor ACE2, while other RBD patches are constrained and therefore might be desirable targets for potential therapeutic antibodies [55,86]. Later DMS studies have identified other key spike protein sites in which mutations are likely to lead to immune escape variants [85,98]. Although these studies have largely been focused on viral proteins, the same strategies have been applied to investigate libraries of antibody variants, for example, by scanning complementarity-determining regions of relevant antibodies for mutations that enhance their binding to various antigens [81,82,99,100].

While this body of work has provided a wealth of insight into immune–pathogen coevolution, these approaches also have key limitations. The central constraint is that comprehensive DMS libraries are expensive (and/or laborious) to create, so while we can use this approach to comprehensively explore local sequence space, we can do so only around a small number of focal sequences of interest. Random mutagenesis can be used to create libraries more cheaply (at the cost of not being comprehensive), but the number of focal sequences we can explore remains relatively small. Thus, these approaches are generally limited to analyzing the landscapes that are relevant for very short-term coevolutionary dynamics around a handful of strains of interest. Some studies attempt to extrapolate to larger genetic distances. For example, this can be done by estimating the combined effects of multiple mutations based on their individual mutation effects in a DMS study [101], or by **training neural networks** (e.g., using SARS-CoV-2 receptor binding motif mutagenesis data [102]). However, widespread epistasis unavoidably means that the accuracy of any type of extrapolation of this kind will often fall off rapidly with mutational distance [103]. This places fundamental limits on the extent to which we can use these approaches to predict evolutionary dynamics.

Combinatorial exploration

Given the widespread importance of epistasis in immune–pathogen coevolution, a natural alternative approach is to construct combinatorial libraries of antibody or pathogen protein variants involving various combinations of mutations at some set of sites (Figure 3B). This allows us to characterize phenotypes that are relevant for potential longer-term evolutionary trajectories involving these sites (with the tradeoff that we are limited to just these sites and thus cannot explore potential trajectories involving other mutations elsewhere in the sequence). For example, recent work used this approach to show that epistasis between several SARS-CoV-2 Omicron BA.1 spike protein RBD mutations strongly interacted to modulate ACE2 binding affinity [78,104,105].

Many combinatorial studies attempt to construct all possible combinations of specific sets of mutations to provide a comprehensive characterization of the corresponding high-dimensional landscapes. This makes it possible to measure the phenotypes of all possible evolutionary intermediates between some ancestral sequence and its evolved descendant. These studies have been used to study protein evolution in a variety of contexts (e.g., to characterize constraints on the evolution of beta-lactamase [106–108] or to measure the importance of epistasis in a

fluorescent protein [109]). Recent work by us and others applied these methods to study immune–pathogen interactions, for example, by constructing all possible combinations of mutations leading from germline antibody sequences to several broadly neutralizing anti-influenza virus antibodies [54,83], or from the ancestral Wuhan Hu-1 strain of the SARS-CoV-2 spike protein RBD to the Omicron BA.1 variant [84,110]. We then measured the binding affinities of antibodies to relevant antigens, as well as the binding affinities of spike protein variants to human ACE2 and monoclonal antibodies. The structure of the resulting landscapes has provided some insight into how these antibody and viral strains evolved (e.g., that CR-9114, an extremely broadly neutralizing antibody, likely evolved in response to a specific sequential antigen exposure history).

These combinatorial studies allow us to comprehensively analyze the high-dimensional landscape defined by all possible evolutionary trajectories between the ancestral and evolved sequence (i.e., all possible orders in which this specific set of mutations could have occurred [107,111]). However, this is inherently retrospective and does not provide any insight into alternative potential evolutionary trajectories involving mutations at other sites. This is not a fundamental limitation of the approach: in principle, it is possible to construct combinatorial libraries involving any set of mutations of interest. For example, in future work it may be useful to analyze 'chimeric' libraries corresponding to variants across a broader phylogenetic tree; this could provide insight into how initial mutations constrain future evolution ('**historical contingency**') [103,112]. Other studies might focus on libraries of mutations that were identified based on structural analyses. Another promising avenue for future work is to integrate combinatorial approaches with data from DMS and mutagenesis studies. For example, one can identify a set of key mutations from DMS studies in a specific focal background and then explore the combinatorial landscape defined by these variants. Alternatively, one can conduct a combinatorial analysis, use it to identify key intermediate genotypes along likely evolutionary trajectories, and then conduct DMS or mutagenesis around these focal genotypes.

Regardless of how we select sets of mutations, a key constraint on combinatorial exploration of genotype–phenotype maps is the exponential explosion of the size of the library as the number of mutations involved increases. While it is generally relatively inexpensive to construct large libraries using combinatorial assembly methods, the scale at which we can measure corresponding phenotypes (even with high-throughput methods) becomes limiting. For example, Tite-Seq-based binding affinity assays are constrained by experimental limitations on flow cytometry throughput and sequencing costs; this renders the measurement of phenotypes of more than a few hundred thousand variants challenging [82]. Thus, if we wish to analyze combinatorially complete landscapes, we can consider at most, sets of 16–18 mutations. Alternatively, it is possible to consider combinatorial landscapes of larger sets of mutations in a less comprehensive way, for example, by grouping mutations into subsets and looking at all possible combinations of these subsets, or by making incomplete libraries consisting of a random sampling of possible mutation combinations [113]. This random sampling approach may be particularly promising, as it does broadly survey many potential epistatic interactions and hence may provide a better basis for extrapolating landscapes by inferring phenotypes of unobserved genotypes.

Random exploration guided by selection

A third approach to the characterization of high-dimensional genotype–phenotype maps is to attempt to directly recreate the process by which evolution samples these landscapes: mutational exploration combined with natural selection (Figure 3C). This essentially involves conducting **directed evolution**, in which one alternates rounds of mutagenesis with artificial selection for some phenotype of interest [114,115]. For example, a recent study conducted directed evolution of the SARS-CoV-2 spike protein RBD, finding that rounds of mutagenesis and selection for

expression and ACE2 binding led the original Wuhan Hu-1 strain to accumulate mutations that were common across the natural phylogeny [116].

Directed evolution studies are particularly widespread in the context of antibody affinity maturation, where numerous studies have evolved antibody sequences towards high affinity for a variety of specific antigens [117–122]. However, these studies have been primarily used for optimization, with the goal of rapidly producing antibodies with desired binding properties [115,123]. In principle, however, we could also use directed evolution in a more exploratory way, to artificially produce large numbers of replicate evolutionary trajectories through a high-dimensional landscape and measure relevant phenotypes of each intermediate. Similar approaches should also make it possible to emulate and analyze coevolutionary trajectories by simultaneously selecting antibodies to bind pathogen proteins and pathogen proteins to evade binding by corresponding antibodies. The variants identified in these directed evolution studies might then form the basis for subsequent combinatorial studies and the final evolved sequences (as well as key intermediates) might serve as focal genotypes for DMS analysis. These are exciting areas for future work, which offer the potential for entirely novel ways to explore high-dimensional coevolutionary landscapes.

Concluding remarks

As with any high-dimensional genotype–phenotype map, the central problem in empirically characterizing immune–pathogen coevolutionary landscapes is the massive scale of sequence space. Regardless of how rapidly we improve experimental methods and throughput, we will never be able to comprehensively survey even a small fraction of all possible genotypes. A key challenge is therefore to determine which combinations of the approaches described earlier, along with other strategies that we may not yet have conceived, can provide the most power for extrapolation (see Outstanding questions). That is, what types of measurements (and which computational approaches) will best allow us to accurately infer the larger-scale structure of the coevolutionary landscape from inevitably limited data? There is reason to be optimistic that this is possible: evolution itself cannot and does not comprehensively explore sequence space. Instead, it samples and selects trajectories based on relatively limited information. Thus, if we can collect a similar sort of information, it should be possible to make at least general statistical predictions about how evolution will act. This has the potential to improve our ability to understand, and eventually even predict, the effectiveness of antibody affinity maturation in response to vaccination or pathogen exposure and the corresponding evolution of pathogen immune escape.

We also note that throughout this review, we have focused on methods to empirically characterize genotype–phenotype maps. An equally important task is the development of theoretical and computational methods to predict how evolution navigates these complex high-dimensional landscapes. These methods can help us to understand what aspects of the landscapes are most crucial in shaping coevolutionary trajectories, which in turn can then shape future experimental directions.

Outstanding questions

What variant library structure provides the most power to infer the phenotypes of unobserved genotypes? How does the choice of model used for this inference affect the accuracy of this extrapolation and how does this accuracy change as a function of genetic distance?

What aspects of landscape structure are most crucial for determining coevolutionary dynamics and what types of empirical approaches can be used to most efficiently characterize these aspects?

What phenotypes or combinations of phenotypes are most relevant for determining selection pressures in nature? How can we improve the design and throughput of empirical methods to measure more complex phenotypes that may be better proxies for these pressures?

What general themes can emerge from the study of landscapes across many different pathogen and immune receptor proteins and phenotypes? Are there common patterns in the statistical structure of genotype–phenotype maps across many systems and, if so, what general implications does this have for immune–pathogen coevolution?

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Declaration of interests

No interests are declared.

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