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Defining and studying B cell and T cell receptor interactions

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Abstract

B- and T-cell receptors (BCRs, Antibodies, and TCRs, or AIRs, adaptive immune receptors) are the means by which the adaptive immune system recognizes foreign and self-antigens, playing an integral part in host defense, as well as the emergence of autoimmunity. Importantly, the interaction between AIRs and their cognate antigens defies a simple key-in-lock paradigm and is instead a complex many-to-many mapping between an individual's massively diverse AIR repertoire, and a similarly diverse antigenic space. Understanding how adaptive immunity balances specificity with epitopic coverage is a key challenge for the field and terms such as broad specificity, cross-reactivity, and polyreactivity remain ill-defined and are used inconsistently. In this *Immunology Notes and Resources* article, a group of experimental, structural, and computational immunologists define commonly used terms associated with AIR binding, describe methodologies to study these binding modes, as well as highlight the implications of these different binding modes for therapeutic design.

Introduction

Adaptive immune receptors (AIRs) play a critical role in the generation of adaptive immune responses via interactions with their cognate antigens. The specific binding region of an AIR is known as the paratope, whereas the region of the antigen the paratope interacts with is known as the epitope. B cell receptors (BCRs) and their secreted counterparts, antibodies, recognize native three-dimensional epitopes, whereas T cell receptors (TCRs) bind to linear

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peptide fragments from larger proteins presented in complex with Major Histocompatibility Complexes (MHCs), also called Human Leukocyte Antigens (HLAs) in humans, or lipid and small molecule antigens presented by CD1 and MHC class I-related protein (MR1). AIRs are classically known for highly specific interactions with their cognate antigens. However, AIRs demonstrate multiple different binding modes that facilitate binding to both closely and distantly related antigens, as well as non-specific binding to unrelated antigens. While experimental and computational approaches to study AIR binding have considerably increased our understanding of their properties, it remains a challenge to clearly delineate the complexity of AIR binding into distinct phenomena. Here, we review current progress and remaining challenges in studying AIR binding and propose coherent definitions for important terms relating to specificity and cross-reactivity for both BCRs and TCRs, incorporating perspectives ranging from fundamental immunology to therapeutics and diagnostics design.

Genetic, Structural, and Binding features of AIRs

3D structure of BCRs and TCRs—The diversity, and thus antigen recognition breadth, of AIR repertoires is influenced by the statistics of variable (V), diversity (D), and joining (J) genes segment recombination (1). Specific germline genes (and alleles), as well as their frequencies, have been linked to neutralizing antibody classes (2–6), and autoantigen-specific binding in autoimmunity (7, 8). For BCRs, somatic hypermutation (SHM) of antibody genes add another layer of genetic complexities that can lead to distinct binding modes. Importantly, V(D)J gene usage shape the 3D structure of AIRs as well as the diversity of loops responsible for antigen recognition (9).

BCRs are transmembrane proteins located on the surface of B cells and are responsible for recognizing diverse antigens (10, 11). Structurally, a BCR can be subdivided into a membrane-bound immunoglobulin (mIg) and signaling domains (10) that are responsible for antigen-binding and B cell activation, respectively, resulting in the production of plasma cells, which secrete large quantities of secreted immunoglobulins (i.e., antibodies) (10). The classical IgG structure is composed of two identical sets of paired heavy and light chains, and the stem of the antibody, also known as the crystallizable fragment region (Fc), is responsible for interacting with other components of the immune system through Fc receptor binding (12, 13). The antigen-binding fragment (Fab) arms of the Y-shaped immunoglobulin structure are composed of heavy and light chains that can be subdivided into a constant (Fc) and a variable (Fv) domain (13, 14).

Genetically the variable region is comprised of recombined VDJ genes for the heavy chain and a recombined VJ genes for the light chain. Structurally, the variable fragment is comprised of six hypervariable loops, known as the complementarity determining region (CDR) loops, where the sequence and structural diversity of the antibody is concentrated (15). Together with the relative orientation of the heavy and the light chain (VH-VL) (16, 17), the CDR loops shape the antigen binding site, also known as the paratope (18). The CDR loops are separated by structurally conserved framework regions (FR), that display less sequence variability and are responsible for maintaining the immunoglobulin β-sheet structure to support antigen binding by the CDR loops, although the FR regions can

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contribute to antigen binding (19). Additionally, certain framework residues can influence the CDR loop conformations, thereby contributing indirectly to antigen recognition (20–22).

TCRs are transmembrane heterodimers consisting of disulfide-linked α and β chains, analogous to the heavy and light chain in antibodies, and the signal transducing invariant CD3 dimers (23). TCR signaling in canonical αβ TCRs is initiated by the receptor recognizing peptides (up to 24 residues long) bound to MHC proteins (pMHC) (24). Both α and β chains are involved in pMHC binding and can each be subdivided into a constant and variable domain (25). Similar to BCRs, each variable domain of the TCR is comprised of three CDR loops. However, the divergent antigen recognition needs of TCRs and BCRs have selected for structural differences in their recognition modes (26, 27). TCRs, with the exception of noncanonical T cell subsets such as Mucosal-Associated Invariant T cells and γδ T cells (28), have evolved to recognize small but highly diverse peptides in the context of pMHC presentation. Consequently, most TCRs bind pMHCs in a canonical binding mode using both α and β chains with a conserved docking polarity. While CDR1 and CDR2 loops predominantly interact with the MHC molecule, peptide recognition is primarily achieved by the CDR3 loops (reviewed in (29, 30)). While TCRs that bind with a non-canonical reverse docking polarity have been observed, recent studies suggest that canonical docking is essential for signaling and thus T cell activation (31).

In contrast, the binding modes of BCRs and antigens are highly diverse (reviewed in (32)), although each CDR loop still adopts characteristic canonical structures upon antigen binding, with the exception of the CDRH3 (15, 33). Unlike TCRs, BCR affinity can be improved by SHMs at positions that optimize the paratope, including positions that are not directly contacting antigen. For example, non-paratope SHMs can modulate the flexibility of CDR loops, which in turn can influence binding affinity (34, 35). Furthermore, such mutations can affect antigen binding affinity by modulating the interactions between the heavy and light variable domains (36–39).

Binding affinities and avidities—Antigen-binding affinity is defined as the strength of the molecular interaction between a single paratope and a single epitope. BCRs have bivalent binding potential, as a single BCR possesses two Fabs arms, each Fab possessing identical antigen-binding affinities. Furthermore, secreted antibodies can form structures with up to 12 identical Fab arms, such as for IgM isotype antibodies. Avidity is the combined binding affinity of the BCR, antibody, or TCR to multiple copies of an epitope, whether on the surface of the cell or in solution. As such, avidity is frequently assessed through the apparent affinity of multivalent AIRs to represent their physiologically relevant binding strengths more accurately.

Both the affinity and avidity of receptor binding play critical roles in cellular activation and differentiation, B cell selection, T cell tolerance, and antibody function. Avidity of BCRs and TCRs is achieved by the clustering of immune receptors within lipid rafts, which increases intracellular signaling (40, 41). The strength of signaling is dependent on both receptor affinity and avidity, which ultimately dictates cellular activation and differentiation. For example, B cells with low affinity BCRs are more likely to differentiate into germinal center B cells and memory B cells, whereas B cells with high affinity BCRs are more

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likely to differentiate into antibody secreting cells (42). In addition, T cells possessing TCRs with excessively high apparent affinity to self-antigens presented in the thymus during thymic selection are more likely to be deleted or differentiate into regulatory T cells (T_{reg}; reviewed in (43)). Antibody isotypes can compensate for lower affinity with higher avidity. For example, bivalent IgG isotype antibodies possess lower binding valency but often high affinity, owing in part to substantial SHM, whereas pentameric IgM isotype (the basal isotype of antibodies) antibodies often possess lower affinity and little-to-no SHM, but can still be potently neutralizing due to their potential for highly avid binding (44–48). Therefore, affinity and avidity play critical roles in B and T cell activation, as well as in antibody function.

Ultimately, whether a BCR or TCR binds its cognate antigen(s) and mediates effector functions is dependent upon its antigen binding affinity and avidity. The dissociation constant (K_D) between TCRs and pMHCs is typically in the micromolar (μM) range (29), with T cell responses reported for TCR-pMHC interactions with K_Ds as high as ~1 mM (49), whereas the K_D between BCRs/antibodies and antigens can often reach the nanomolar (nM) and picomolar (pM) range (50). The higher affinity of BCRs can at least be partly explained by the affinity maturation process via SHM, which is absent in mammalian TCRs. Moreover, high affinity (K_D < 100 nM) T cells clones are purged from TCR repertoires during thymic selection, presumably to limit the potential for autoreactivity (51). Although the monomeric affinities between AIRs and their cognate antigens have been well-studied, the physiological apparent affinities necessary for T cell and B cell activation remain ill-defined. For TCRs, this understanding may be improved by assays that assess TCR-pMHC interactions in their native membrane-bound context, as these measurements have been found to correlate better with T cell activation than kinetics measurements in solution (52).

Modes of AIR binding

Specificity for AIRs can be defined in relation to their antigen binding properties. However, owing to structural differences in the antigens bound by BCRs/antibodies and TCRs, these definitions should be tailored to each immune receptor class (Table 1).

Monospecificity—Monospecific binding of AIRs refers to the capacity of a receptor to only bind to a single epitope with a consistent binding interface and mode within the apparent affinity ranges described above. This definition implies that a monospecific receptor should not bind to mutated forms of the epitope but includes binding to variants of the same antigen provided that the epitope is unaltered. As such, monospecificity refers to the classic lock-and-key mode of AIR binding to antigen. However, true monospecificity according to this strict definition is exceedingly rare, as both antibodies and TCRs often recognize at least some point-mutated epitopes (53–60). In fact, it is believed that, due to the vast excess of potential pMHC antigens relative to the number of T cells in the naive repertoire, all T cells must recognize more than one pMHC to prevent gaps in immunosurveillance (61, 62).

More practically, monospecific AIRs may be defined as those with very limited breadth, where binding is lost upon comparatively minor mutation of the epitope relative to broadly

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specific AIRs (discussed below). Examples of monospecific antibodies according to this less stringent definition include those against the variable epitopes of coronavirus receptor-binding domain and influenza virus hemagglutinin (HA) head domain. Antibodies against these domains are functionally limited in their binding breadth against viral variants and on a molecular-level can be shown to lose binding affinity upon a relatively modest accumulation of mutations (53–58).

It should be noted that most monospecific AIRs are isolated following immunization, infection, or in diseased states, and screened only against antigens present in these states and their variants. As a result, receptors identified in this manner as monospecific may nevertheless bind to distinct unrelated antigens and may display features of cross-reactive and polyreactive receptors (described below) when assayed outside of those conditions.

Broad specificity—Broad specificity, also called broad reactivity, refers to AIRs that bind to multiple variants of a conserved epitope on polymorphic antigens (Fig. 1A). Broadly specific AIRs react with epitopes that are generally well conserved but have either minor residue or structural changes. As such, this definition includes binding to partially conserved epitopes on highly homologous but evolutionarily distinct proteins, including cross-species orthologs and proteins conserved between virus families. Unlike monospecific AIRs, broadly specific AIRs are able to retain physiologically relevant affinities for their epitopes in the presence of these changes.

Examples of broadly reactive antibodies include antibodies against the influenza virus HA stalk (stem) domain (63) and CD4 binding site of HIV (64). To accommodate binding to distinct variants of these partially conserved epitopes, broadly reactive antibodies often utilize multiple modes of recognition. For example, three mAbs that bind the HA stalk domain, PN-SIA28 (65), FI6v3 (66), and MEDI8852 (67), each exhibit multiple binding modes to recognize antigenic variants. In addition, broadly reactive antibodies often possess variable affinities for distinct variants of a partially conserved antigen. For example, broadly specific antibodies against HA antibodies often possess higher affinity for past influenza strains, relative to current circulating viruses, due to iterative rounds of affinity maturation against past strains (55, 57, 58).

For TCRs, this definition can be applied to TCRs that bind highly similar pMHC antigens, such as two length variants of a single peptide, or peptide variants that differ only in their MHC contacts, so long as the local structure of the peptide backbone and remaining side chains remains unaltered. Examples of broadly specific TCRs would therefore include MEL5, which binds the nonapeptide (AAGIGILTV) and decapeptide (EAAGIGILTV) of MART-1 presented on HLA-A*02:01 (68), as well as the MART-1 A27L heteroclitic decapeptide variant (ELAGIGILTV), which possesses enhanced binding to its presenting HLA allele (HLA-A*02:01) (69).

Cross-reactivity—We propose restricting the definition of cross-reactivity to recognition of a discrete motif that is shared in antigens that otherwise share little-to-no homology (Fig. 1B). This can include binding to structurally similar motifs in distinct antigens, including examples of molecular mimicry, wherein immune responses to infectious agents

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can potentiate autoimmunity due to cross-reactivity with unrelated autoantigens (70). One of the earliest and most clinically significant examples of this was the identification of antibodies and T cells that cross-reacted with group A streptococcal M protein and cardiac myosin (71, 72), which is implicated in the development of rheumatic fever. B and T cells recognized motifs shared between this bacterial antigen and self-antigen, including a repeated seven amino acid motif found in a variety of self-antigens including myosin, tropomyosin, and vimentin (73).

In addition, numerous anti-viral cross-reactive antibodies have been found to target glycan moieties on viral surface glycoproteins. One such antibody, 2G12, was initially discovered as an HIV antibody that binds to oligomannose cluster (74–76), but has also been shown to bind influenza HA with nM affinity (77). Similarly, a recent study has identified another glycan-directed antibody, mAb688, that can bind to at least four highly divergent viruses (hepatitis C virus [HCV], HIV, influenza, SARS-CoV-2) (78). The same study also discovered HIV/HCV cross-reactive antibodies that are independent of glycan for binding (78), implying a different structural motif shared across viruses. Moreover, antibodies against the HIV membrane proximal external region possess the capacity to cross-react with the viral membrane (79–81).

As noted above, pMHC cross-reactivity (also referred to as binding degeneracy) is an ingrained feature of TCRs owing to the vast diversity of potential peptide antigens that can be presented by MHC molecules (62). In addition, highly similar peptides may be frequently derived from highly dissimilar antigens due to shared statistical biases between proteomes (82), or molecular mimicry between foreign and self-antigens (61, 83). Cross-reactivity can also be applied to the binding of TCRs to highly distinct peptides presented by the same MHC allele, such as for the binding of 2C TCR to H-2K^b presenting DEV8 (EQYKFYSV) and SIY (SIYRYYGL) peptides, facilitated by a high degree of plasticity in the CDR loops upon binding (84, 85).

Furthermore, cross-reactivity is enhanced in high-affinity TCRs, often due to excess binding affinity to the MHC protein, independent of the bound peptide, resulting in a loss of peptide specificity (86). As such, TCR cross-reactivity has been a major impediment to the development of TCRs for adoptive therapy for cancer, wherein cross-reactivity can result in autoimmunity. The most notable example was the engineered affinity-enhanced TCR MAG-IC3 targeting an epitope from the cancer-testis antigen MAGE-A3 (EVDPIGHLY) presented by HLA-A*01:01, which led to the death of two patients in a clinical study (87). The likely source of this off-target toxicity was later traced back to cross-reactivity with a highly similar but unrelated peptide (ESDPIVAQY) derived from striated muscle-specific protein titin (88).

Notably, the true number of cross-reactive BCRs and TCRs, as well as the full extent of their cross-reactivity, is not known, as there is a near infinite number of antigen combinations to be analyzed. Therefore, the contributions of cross-reactivity to protection against infectious diseases and cancer as well as the initiation and exacerbation of inflammatory diseases is not well understood.

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Polyreactivity (BCR)—BCRs and antibodies that are capable of binding to many distinct antigens with diverse motifs are defined as polyreactive (Fig. 1C). Unlike broadly reactive or cross-reactive antibodies, polyreactive antibodies are predicted to have multiple antibody paratopes that interact with diverse epitopes found on different antigen classes (e.g., protein, lipid, glycan). Polyreactivity is also a phenomenon distinct from polyspecificity, which represents an extreme of cross-reactivity, with binding to many highly divergent antigens with meaningful affinities (89). Polyreactive antibodies generally exhibit weak interactions with many distinct motifs based largely on hydrophobicity and charge (reviewed in (90)). For example, polyreactivity to DNA is associated with hydrogen bonding surface patches on the antibody (91), and polyreactivity to negatively charged biological components, such as heparin and cell membranes, is associated with positively charged surface patches on the antibody (92).

Conformational variability of the binding site is required to accommodate distinct antigens. Therefore, polyreactive antibodies reveal a multitude of weakly populated conformations, each of which able to recognize different binding partners (56, 93–95). This view of the antigen binding site provides a direct link of polyreactivity with flexibility (94–96). On the other hand, specificity can be understood as a reduction of conformational states, which is accompanied by an increase in probability towards the conformation responsible for binding a specific antigen. Polyreactive antibodies tend to follow the conformational selection paradigm, as all conformations pre-exist in solution and the state probabilities shift upon binding to the respective antigens (97). Therefore, while monospecificity, broad specificity, cross-reactivity, and polyspecificity are primarily driven by the nature of the epitope, polyreactivity is primarily driven by inherent properties of the paratope.

Many polyreactive antibodies possess both highly specific binding interactions with cognate antigen(s) as well as polyreactivity as a secondary feature to augment their function. Notably, numerous broadly protective antibodies against influenza viruses and HIV possess polyreactivity, which is linked to improved antibody binding avidity and neutralizing capacity (56, 80, 98, 99). In some cases, polyreactive antibodies can have strong cross-reactive antibody binding to multiple distinct antigens, as well as possess lower affinity polyreactive binding to a variety of diverse antigens, such as highly mutated polyreactive anti-HIV antibodies identified during acute infection frequently bind gut microbiota (100–104).

Promiscuity (TCR)—The extent of TCR-pMHC cross-reactivity can be so substantial that TCR binding can be described as promiscuous, with cross-reactivity to vast numbers of unrelated peptide antigens from highly divergent antigens (Fig. 1D; reviewed in (105)). This highly degenerate or promiscuous binding occurs when the CDR loops of the TCR make few contacts with the peptide sidechains, and therefore the TCR tolerates many amino acid substitutions at the remaining peptide positions (62). The most well studied example is the autoimmune TCR 1E6, which was isolated from a patient with type 1 diabetes and recognizes a preproinsulin peptide (PPI_{15-24}) presented on HLA-A*02:01. This TCR was found to recognize over one million distinct decameric peptides in the context of this single HLA allele with up to 7 out of 10 peptide residues altered, due to TCR binding to only a small 3 amino acid motif in the center of the peptide (62, 106). In general, this highly

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degenerate binding is often associated with autoimmune TCRs, although all TCRs display a degree of cross-reactivity in their binding (107).

Resources and technologies to study AIR-antigen interactions

Numerous assays have been developed to understand AIR binding interactions. Here, we review traditional and emerging approaches to measure AIR binding breadth, with emerging technologies highlighted in Table 2.

Kinetics/Affinity measurements—To measure binding kinetics of BCRs and antibodies, surface plasmon resonance (SPR) and biolayer Interferometry (BLI) are typically used and are considered the gold-standard for calculation of accurate affinity measurements and kinetics. Similarly, the kinetics of TCR-pMHC interactions are frequently measured via SPR, with recent experimental advances allowing precise measurements of low-affinity interactions (49). However, these measurements require laborious reformatting of TCRs into soluble formats and do not incorporate the contributions of CD4 and CD8 coreceptor binding to avidity (108). In contrast, TCR avidity is often measured *in situ* and includes the contributions of coreceptor binding, either using fluorescently-labeled pMHC multimers (e.g., tetramers) or monomers, providing ‘structural avidity’ via coreceptor binding (108). In addition, kinetics measurements obtained by these ‘three-dimensional’ assay formats have been shown to correlate less accurately with T cell activation and functional responses than ‘two-dimensional’ assay formats in which both reagents are anchored into apposing membranes (reviewed in (109)).

Structural Biology—X-ray crystallography and cryo-electron microscopy (cryo-EM) are commonly used for structural characterizations of antibody-antigen complexes, although other techniques such as nuclear magnetic resonance (NMR) are also used. Negative stain EM can also analyze heterogeneous and polyclonal samples, which has led to development of EM-based polyclonal epitope mapping (EMPEM) (110, 111), a technique that has been applied to numerous infectious diseases, including influenza viruses, HIV, and coronaviruses (CoVs) (112–114). Cryo-EMPEM can be used to generate high-resolution antibody maps and was recently used to generate maps under 4Å of polyclonal sera against HIV envelope protein (113).

In contrast to antibody-antigen complex structures, TCR-pMHC complex structures have historically been characterized by X-ray crystallography instead of cryo-EM, owing in part to the relatively small size of TCR-pMHC complexes. While most structural characterizations of BCRs and TCRs focus on their soluble forms without the transmembrane domain, recent studies have reported the cryo-EM structures of full length BCR (10, 115, 116), full-length TCR-CD3 complex (23), and the membrane-bound TCR-CD3-pMHC complex (24). These structures have provided important mechanistic insights into BCR and TCR signaling.

Drawbacks of these methods include that protein samples for X-ray crystallography and cryo-EM often need to be at a high concentration that may not be physiologically realistic. In addition, X-ray crystallography and cryo-EM only have limited ability to capture structural dynamics.

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Molecular dynamics—The ability of AIRs to recognize and bind a variety of antigens is ultimately governed by dynamics, as their three-dimensional structures fluctuate constantly (18, 117). Therefore, structural and dynamic characterization of AIRs is important for understanding their biophysical properties and consequently antigen recognition (118). Molecular dynamics (MD) simulations can be used to characterize the conformational diversity of proteins by modeling conformational ensembles in solution (117). MD simulations capture the movements of atoms over time, following Newton's second law of motion. The resulting time-resolved motions can be used to reconstruct thermodynamics and kinetics of the captured conformational rearrangements, making it a powerful tool to elucidate biophysical properties of antigen recognition by antibodies (Fig. 2A) (97). Additionally, MD simulations allow the characterization of conformational consequences of point mutations that are otherwise hard to predict from single-static structures and models (20).

Machine Learning—Machine learning tasks focused on predicting AIR-antigen binding may take several forms such as classification of binders versus non-binders, prediction (or optimization) of affinity, and the prediction of paratope-epitope interactions. These problems may be formulated using sequence-only, structure-only, or hybrid approaches. Importantly, it was shown that combining sequence and structural information increases paratope-epitope interaction prediction accuracy (25, 119–122). Protein language models, which learn non-linear protein sequence similarity by training on million or even billions of sequences, have also shown a remarkable capacity to capture structure-based patterns from sequence-based data (123–125). However, we do not yet understand the underlying interaction rules of AIR-antigen binding, particularly the long-range sequence and structure-based patterns that are causally linked with binding. One promising approach to overcome data-limitations in machine-learning is to guide them through physics-based modeling of receptor-ligand binding energy landscapes. This technique can build upon the successes of statistical biophysics models of TCR-pMHC interactions (126–129), which have explained how thymic selection shapes the HIV-reactive T cell repertoire (127) and the breadth of TCRs binding a common pMHC (129). Ultimately, elucidating the rules of AIR-antigen binding may enable *in silico* screening of the binding behaviors discussed in this text (e.g., monospecificity, cross-reactivity, polyreactivity).

Structure prediction—Predicting protein structures directly from sequences has been one of the grand challenges in structural biology/computational chemistry (130). AlphaFold2 revolutionized the field by combining co-evolutionary information with machine learning to predict protein structures (131). However, predicting the structures of the variable domains of TCRs and antibodies due to their high diversity in length and structure, in particular the heavy chain CDR3 loop, still remains challenging (97, 132–134). Therefore, various antibody- and TCR- specific tools have been developed to advance structure prediction and consequently enhance the predictive power of the respective models (25, 135–137).

The performance of the available prediction tools has improved tremendously, resulting in structure models with high accuracy, reflected in low overall root-mean-square deviations relative to their X-ray crystal structures (25, 132, 135). However, special care has to be taken

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when basing experiments and conclusions on antibody models, as some of these structure models can suffer from physical inaccuracies, such as cis-amide bonds, D-amino acids or steric clashes (132). These issues can strongly deteriorate the quality of predictions that rely on accurate side-chain and backbone conformations such as antibody-antigen docking or surface property predictions (138, 139). Despite this, a recent study compared the predicted structure of a broadly neutralizing antibody binding to influenza virus HA to that generated via cryo-EM and found high accuracy in this prediction (140).

Broadly reactive and cross-reactive antibody binding—Testing the binding breadth of antibody responses is critical for understanding immunity against antigenically variable pathogens as well as antibodies against unknown antigens. Various assays have been developed to assess and define the cross-reactivity of antibodies, with various throughputs and resolutions. At low throughput, antibodies can be tested in tissue cross-reactivity assays, which rely on *ex vivo* immunohistochemistry to identify binding to sites not associated with an antibody's target of interest, or binding to its target of interest outside of the desired tissue (141). Importantly, these assays capture binding to the target of interest and potential off-targets in their natively expressed form, and therefore are often used to support first-in-human trials. However, they are incompatible with widely expressed targets and cannot resolve putative off-targets. In addition, a yeast-based platform has been recently described that allows for the enrichment and identification of putative off-targets in a library-based format (142).

An emerging technology in identifying BCR binding breadth is LIBRA-seq (Linking B cell Receptor to Antigen specificity through Sequencing), which uses antigen probes linked to unique DNA barcodes that are detected using barcoding microfluidic technologies, such as 10x Genomics (143). LIBRA-seq allows for the simultaneous analysis of the transcriptome, antibody gene usage, and specificity of individual B cells (Fig. 2B) and has been used to investigate binding breadth of antibodies targeting HIV, influenza viruses, and SARS-CoV-2 (143–145). Noteworthy, 10x Genomics has also generated a related product line known as BEAM (Barcode Enabled Antigen Mapping) that is able to link B cell specificity and T cell specificity to gene expression and V(D)J gene usage.

Another emerging technology for analyzing binding breadth and cross-reactivity is the use of PhIP-seq (Phage Immunoprecipitation sequencing) (146, 147). PhIP-seq utilizes phage display of proteomic scale peptide libraries, with peptides up to 90 amino acids in length. Using polyclonal or monoclonal antibodies for immunoprecipitation, antibody binding breadth, cross-reactivity, and potentially polyreactivity can be assessed. PhIP-seq has been applied to various diseases widely, including identifying antibody targets of autoreactive antibodies (148), identification of broadly reactive anti-coronavirus antibodies (149), and determining the seroprevalence of antibodies against human viruses (150). However, phage-displayed peptides represent linear epitopes and lack post-translational modifications, and therefore do not accurately mimic native epitopes for which antibodies were raised. Moreover, PhIP-seq has largely been applied to polyclonal antibodies, which makes it unclear if individual antibodies possess cross-reactivity/broad reactivity or if individual antibodies against antigens are present.

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Ig-Seq, also known as Ab-seq, combines proteomics and next-generation sequencing to connect polyclonal antibody specificities to monoclonal B cell clones that can be further tested for broad reactivity, cross reactivity, and polyreactivity (151–155). Polyclonal serum antibodies are subjected to mass spectrometry to determine the composition of clonotypes. In parallel, B cells are sequenced to generate a database of distinct clonotypes and for mAb production. B cells against a new trimer interface epitope of influenza HA (156) as well as antibodies against an egg-glycan (157) have been discovered using this technique. Ig-Seq elegantly allows for tracking of clonotypes overtime that can be linked to epitope specificity, binding breadth, function, and disease state (158, 159).

TCR cross-reactivity and promiscuity—Various platforms have been developed to assess TCR cross-reactivity and promiscuity. However, owing to the degenerate nature of TCR-pMHC interactions and the vast diversity of pMHCs, these assays must sample immense target spaces, utilize computational predictions, or both (60, 160, 161). Assessments of single amino acid variant (X-scan) libraries centered around a cognate peptide can provide insight into the key peptide contacts and the tolerance of the TCR to mutation at those positions (59). To assess TCR recognition more comprehensively, diverse (>1E8 variants) pMHC libraries can be expressed recombinantly in yeast- or phage-display systems, and motifs generated from these libraries can then similarly be used to predict TCR off-targets (61, 162). However, these systems cannot differentiate between activating and non-activating TCR-pMHC interactions (163). Therefore, mammalian display systems including SABR (164), MCR (165), T-Scan (166) have been developed to assess and define these interactions at high-throughput for both class I and II pMHCs.

A recently developed technique called RAPTR (Receptor–Antigen Pairing by Targeted Retroviruses) allows for high-throughput screening of TCR-pMHC interactions (167). RAPTR involves pseudotyping lentivirus with pMHC, which then can infect T cells with TCR that binds to the pMHC on the lentivirus, allowing a library-on-library screen (Fig. 2C). RAPTR has also been successfully applied to study BCR-antigen interactions (167).

Polyreactivity (BCR)—Analysis of polyreactive binding requires the use of diverse antigens. The most widely used and affordable assay is the polyreactive ELISA panel, which uses diverse antigens composed of distinct antigen types (56, 168, 169). Expanded panels frequently include HEp-2 binding to investigate self-reactive antibody binding to subcellular structures (58, 168). Moreover, soluble cell membrane and cytosolic protein extracts, baculovirus particles, and heat-shock proteins have also been used to determine polyreactive antibody binding (170–172). However, these approaches are limited to the antigens tested and therefore likely do not recapitulate the full antigen-binding landscape of polyreactive antibodies.

Limitations of AIR binding and therapeutic potential—The specificity of antibodies and TCRs is an important consideration for their development as therapeutics, as it shapes both their efficacy and safety in the clinic. Polyreactive antibodies have poor serum half-lives and biodistibutions, and may have increased propensities for immunogenicity, due to increased undesirable cell interactions, leading to higher internalization and clearance rates (89, 173). Separately, cross-reactivity can result in off-target toxicities, such as

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the emergence of capillary hemangiomas in patients treated with the anti-PD1 antibody camrelizumab due to cross-reactivity with pro-angiogenic receptors such as VEGFR2 (174). Similarly, off-target cross-reactivities can have disastrous consequences for TCRs. As noted above, clinical trials for a transgenic TCR T cell therapy utilizing an affinity matured TCR MAG-IC3 were halted due to severe cardiac toxicities (87). As such, it is of critical importance to both assess and understand the specificity of antibodies and TCRs to assess their capacity to be safely taken forward into the clinic.

Conclusions

We define commonly used terms to describe AIR binding modes, how these terms apply to discrete AIR classes (e.g., BCRs and antibodies versus TCRs), and methodologies to study these binding modes. While we intend to present consensus terminology for AIR binding modes, several outstanding questions remain relating to the physiologic roles of these binding modes on the generation of adaptive immune responses.

1. What are the physiologically relevant binding affinities for these various binding modes that are necessary for the activation and function of B and T cells?
2. What thresholds distinguish monospecificity from broad specificity, broad-specificity from cross-reactivity, cross-reactivity versus polyspecificity, polyspecificity from polyreactivity, and degeneracy from promiscuity?
3. What is the evolutionary significance of AIR binding modes? How does this relate to functional immune responses against foreign antigen and self-antigens?

While we do not put forward the answers to these questions here, we review emerging technologies that may shed light on these questions and highlight the importance for future studies to take into consideration how AIR repertoires, affinities, and binding modes shape protective and pathogenic immune responses.

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Abbreviations used in this article:

BCR	B cell receptor
TCR	T cell receptor
AIR	adaptive immune receptor
MHC	Major Histocompatibility Complex
HLA	Human Leukocyte Antigens

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MR1	MHC class I-related protein
V	variable gene
D	diversity gene
J	joining gene
SHM	somatic hypermutation
mIg	membrane-bound immunoglobulin
Fc	crystallizable fragment region
Fab	antigen-binding fragment
Fv	variable domain
CDR	complementarity determining region
VH	heavy chain
VL	light chain
FR	framework region
pMHC	peptide bound MHC
Tregs	regulatory T cells
K_D	dissociation constant
HA	hemagglutinin
SPR	surface plasmon resonance
BLI	biolayer interferometry
Cryo-EM	cryogenic electron microscopy
NMR	nuclear magnetic resonance
EMPEM	electron microscopy based polyclonal epitope mapping
CoV	coronaviruses
MD	molecular dynamics

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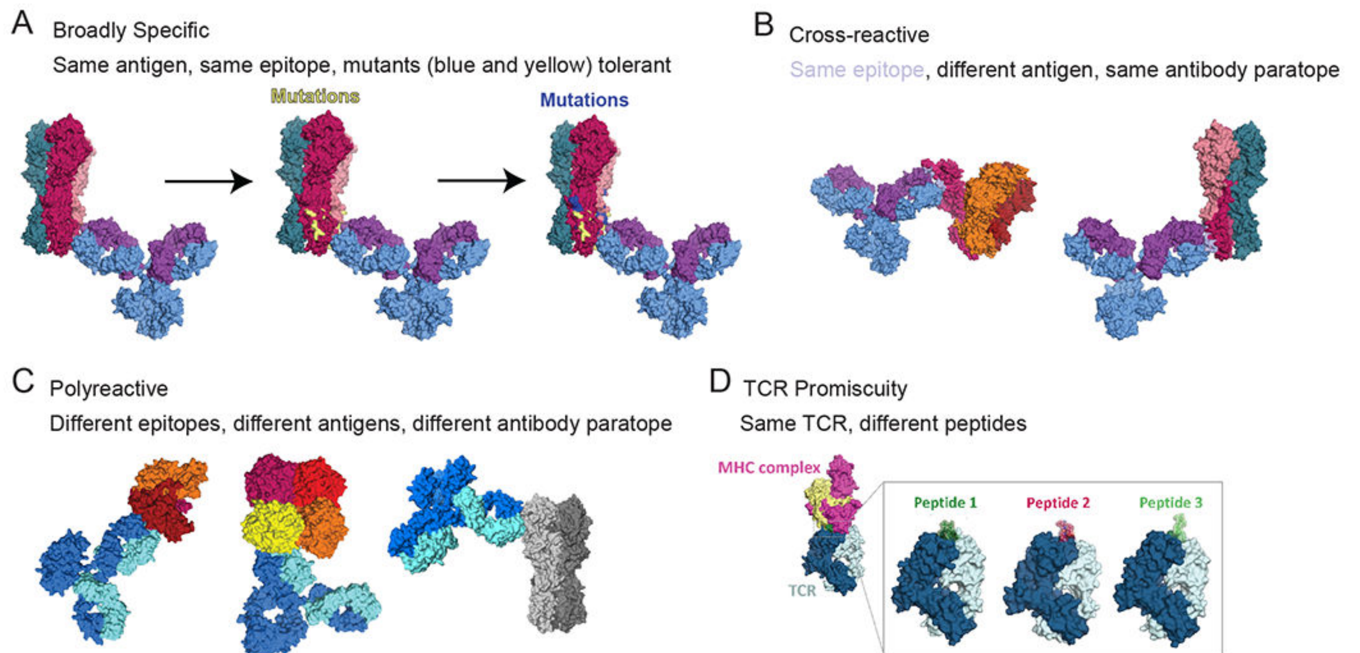

Figure 1:
AIR binding modes. (**A**) BCR/antibody broadly specific binding (PDB accession codes: 1IGY and 7T3D). (**B**) BCR/antibody cross-reactive binding (PDB accession codes: 1IGY, 5UTY and 7T3D). (**C**) BCR/antibody polyreactivity binding (PDB accession codes: 1IGY, 6DZL, 6Q23, 7T3D). (**D**) TCR promiscuous binding (PDB accession codes: 3QDG, 7N5C, 4MAY).

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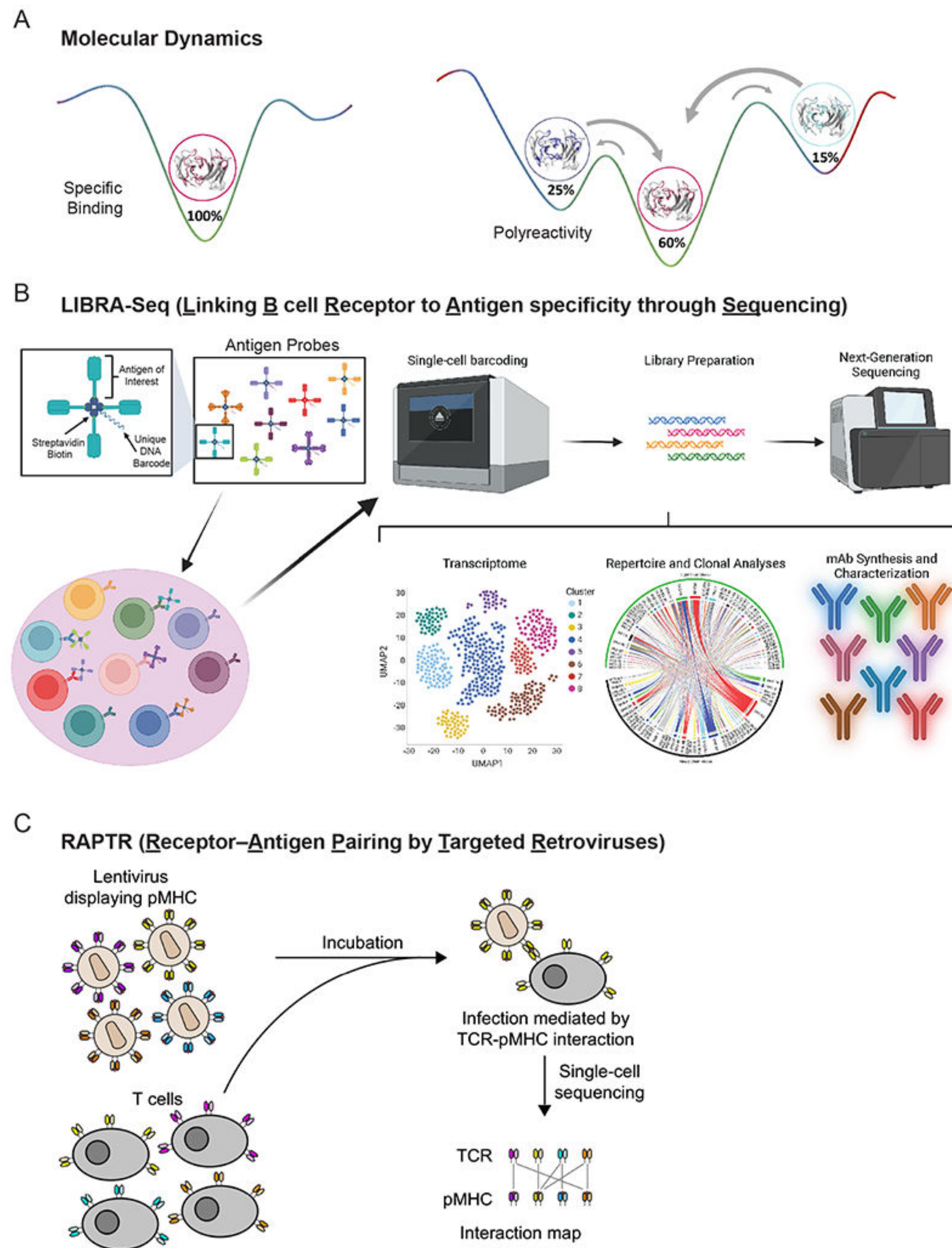

Figure 2:

Emerging technologies to study AIR binding modes. (**A**) Different antibody conformations as indicator of antigen-specific binding (left) and polyreactivity (right) using molecular dynamics. (**B**) Depiction of LIBRA-seq workflow and utility. Panel was made in part using Biorender.com. (**C**) Depiction of RAPTR and application to screening TCR:pMHC libraries.

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Table 1:

Binding mode definitions.

Term	Definition	Examples
Monospecificity	Monospecific binding of AIRs refers to their capacity to bind to a single epitope with a consistent binding interface and binding mode within the apparent affinity ranges for BCRs (K_D range ≈ pM-nM) and TCRs (K_D range ≈ μM-mM)	Antibodies targeting variable epitopes of SARS-CoV-2 RBD
Broad specificity	Broadly reactive specificities react with epitopes that are generally well conserved but have either minor residue changes or changes to the local structure	Broadly neutralizing antibodies against HIV or influenza viruses; TCRs cross-reactive with nonapeptide and decapeptide of MART-1
Cross-reactive	Recognition of a discrete motif that is shared in antigens that otherwise share little-to-no similarity	BCR and TCR binding to Streptococcal M protein and cardiac myosin; HIV and HCV binding antibodies
Polyreactive (BCR)	Binding to many distinct antigens with diverse motifs using multiple antibody paratopes that interact with diverse epitopes found on different antigen classes (e.g., protein, lipid, glycan)	Antibodies against influenza HA stalk domain; anti-HIV MPER antibodies
Promiscuity (TCR)	Binding to vast numbers of unrelated peptide antigens	TCR 1E6 binding to recognize over 1 million distinct decameric peptides in the context of HLA-A*02:01

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Table 2:

Emerging technologies to study AIR binding modes.

Approach	Readouts	References
Cryo-Electron Microscopy Polyclonal Epitope Mapping	Paratope:Epitope binding of polyclonal antibodies	(113)
Machine learning	AIR:Antigen binding; paratope:epitope prediction; affinity	(25, 119–125)
Molecular Dynamics and Structure Prediction	Confirmational AIR landscape predictions and probabilities; AIR:Antigen binding; paratope:epitope prediction	(25, 135–137, 140)
LIBRA-seq	Paired Gene expression, VDJ, and antigen binding; B cells only	(143)
Ig-Seq	Monoclonal serum specificities and clonal diversity	(151, 152, 156–159)
RAPTR	High-throughput screening of TCR-pMHC or BCR:Antigen interactions	(167)