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Next-generation sequencing and protein mass spectrometry for the comprehensive analysis of human cellular and serum antibody repertoires

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Recent developments of high-throughput technologies are enabling the molecular-level analysis and bioinformatic mining of antibody-mediated (humoral) immunity in humans at an unprecedented level. These approaches explore either the sequence space of B-cell receptor repertoires using next-generation deep sequencing (BCR-seq), or the amino acid identities of antibody in blood using protein mass spectrometry (Ig-seq), or both. Generalizable principles about the molecular composition of the protective humoral immune response are being defined, and as such, the field could supersede traditional methods for the development of diagnostics, vaccines, and antibody therapeutics. Three key challenges remain and have driven recent advances: (1) incorporation of innovative techniques for paired BCR-seq to ascertain the complete antibody variable-domain VH:VL clonotype, (2) integration of proteomic Ig-seq with BCR-seq to reveal how the serum antibody repertoire compares with the antibody repertoire encoded by circulating B cells, and (3) a demand to link antibody sequence data to functional meaning (binding and protection).

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Introduction

Since the landmark discovery of antibody (or immunoglobulin, Ig) in blood serum more than 100 years ago, we now know conclusively that serum is composed of a complex spectrum of distinct antibodies (is polyclonal) and is generated by individual B-cell clones through extraordinary modes of genetic recombination, diversification, and selection by antigen (*anti*body *gen*erator) according to rules outlined in the paradigmatic 'clonal selection theory'. Remarkably, however, there had been no way to identify, and determine the relative concentrations, of the monoclonal antibodies (mAbs) that comprise the serum polyclonal pool elicited in response to vaccination or natural infection, until recently [1••,4••,5]. Understanding the composition of the antigen-specific serum antibody *protein* repertoire, the properties (e.g., affinities, epitopes recognized) of the respective Ig, and finally, the relationship between circulating Ig and the presence of clonally expanded peripheral B cells is profoundly important for the comprehensive understanding of humoral antibody responses.

The current era of modern genomics and proteomics is providing extraordinary new tools for examining antibody repertoires. Next Generation Sequencing (NGS) allows millions of B cell receptor (BCR) sequences to be obtained in a single experiment, and NGS approaches to studying the human antibody repertoire [6] not only aim to aid in the discovery of elite antibodies potentially useful as therapeutics, but also to comprehensively catalogue the antibody sequences that are elicited during an adaptive immune response [7]. Previously a limitation with NGS, the ability to obtain the endogenous variable heavy and light chain (VH:VL) pairs within NGS datasets is now feasible [1••,8••,9,10]. This paired VH:VL sequencing represents a major breakthrough in BCR repertoire analysis, obviating the need for multiplexed screening to identify functionally paired VH and VL. NGS has also provided a stepping stone to the direct characterization of serum antibodies (Ig) using NGS database-driven high-resolution protein mass spectrometry [1••,2,3,4••], providing a direct means to comprehensive delineation of the antibody repertoire.

Two antibody repertoires: the cellular and the serological

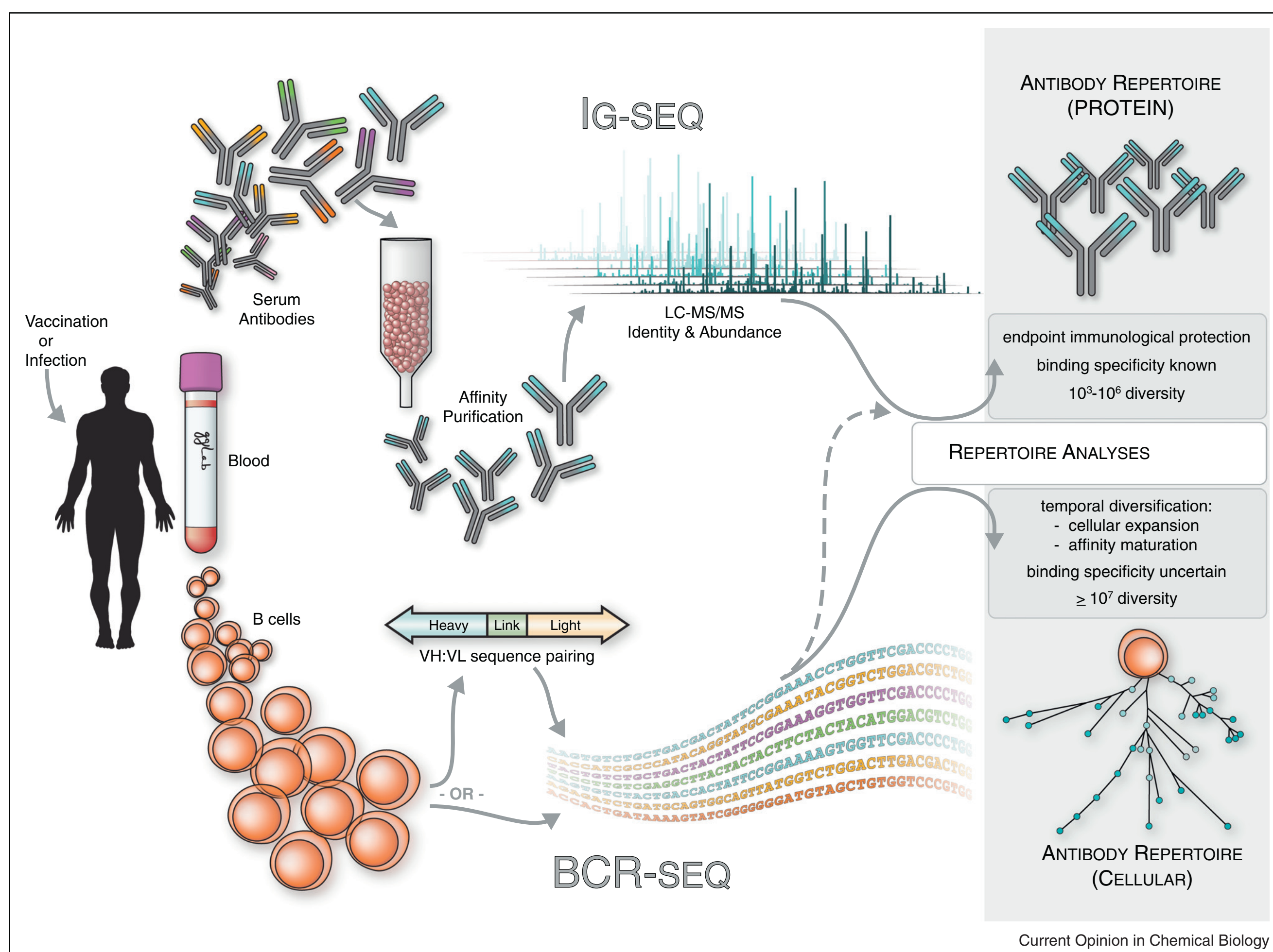
B cells, serum immunoglobulin, and persistence of the antibody repertoire

The mechanisms of antibody diversification and B-cell differentiation have been expertly reviewed elsewhere

[6,11,12] and are beyond the scope of this review. For purposes of introduction, it suffices to state that antibodies — the B cell antigen receptors (BCRs) — are composed of two heavy (H) and two light (L) polypeptide chains. Each chain consists of a constant (C) region and a variable region (VH or VL) which encodes the site of antigen binding. Antibody VH and VL domains each contain three juxtaposed spans of hypervariable sequence termed complementarity determining regions (CDRs), and their non-covalent association forms the antigen-binding site. CDR-H3 lies at the center of the antigen binding site, is the most diverse in terms of sequence, length and structures, and is typically a primary determinant of antibody specificity.

Whereas all newly formed B cells express antibody on their surface as BCR, and subsequently emigrate from their generative site in bone marrow to seed the periphery, it is only a small minority that might ultimately differentiate during the course of an immune response to become memory B cells (mBCs) and an even smaller fraction that secrete their BCR as soluble antibody. In this regard, we can conceive of the functional antibody repertoire as consisting of two major components: (1) the set of BCRs expressed on the surface of B lymphocytes, and (2) the collection of soluble Ig found in blood and secretions, produced predominantly (>95%) by terminally differentiated plasma cells in the bone marrow (BMPCs) [13] (Figure 1). Humoral immunity against pathogens can be

Figure 1



Analysis of human antibody repertoires from peripheral blood. The functional antibody repertoire consist of two major components: (bottom) the total set of BCRs expressed on the surface of peripheral blood B cells, and (top) the collection of soluble serum antibody circulating in the blood. The ability to compare and functionally characterize these two types of antibody repertoires provides a new paradigm in the study of the humoral response. This involves the isolation and proteomic analysis of affinity purified serum antibody (Ig-seq, top) in parallel with VH:VL pairing and/or NGS of peripheral B cell V gene repertoires (BCR-seq, bottom). The bioinformatic analyses of both the diversified cellular humoral immune response and the endpoint serological antibody response provides an avenue for effective antibody discovery, exhaustive antibody repertoire characterization, and an improved understanding of humoral immunity.

sustained for greater than a half century, requiring steady-state expression of serum antibodies by long-lived BMPCs [13]. However, bone marrow specimens are often impracticable to obtain in humans, and the vast majority of studies that examine the human antibody repertoire interrogate peripheral blood plasmablasts (PBs), or circulating mBCs, or else all peripheral blood mononuclear cells (PBMCs). Acutely activated PBs peak transiently in the blood 6–8 days after antigen exposure and can be highly enriched in B cells specific to the antigen (although this is not always the case) whereas the mBC repertoire represents a highly diverse record of an individual's immune history. Both of these peripheral B cell subsets have been examined in a large number of studies of the total and antigen-specific repertoire (reviewed extensively in Ref. [6]).

In summary, since antibody repertoires encompass two major components — the quiescent mBC cellular and the PC-secreted serological — both of which are generated during a primary response to antigen, and both of which persist for sustained yet indeterminate life spans, it is reasonable to ask what degree of overlap there is in the molecular composition of these compartments. If they are not congruent, as there is reason to think [12], then what might be the selective mechanisms that govern their differential recruitment, and what might be the consequences to protective humoral immunity?

Antibody repertoire metrics — the clonotype

Since B cells can potentially be triggered by antigen to proliferate, mutate, and expand to become a lineage of highly identical cells, it is a useful metric to enumerate antibody *clonotypes*. In the classical sense, a clonotype is defined by a unique B-cell specificity [14]. However, the earliest and still most common BCR-seq studies have focused on the H-chain CDR-H3 region — its length, peptide sequence, and IGHV and IGHJ gene usage patterns — to define VH clonotype dynamics. Molecular definitions of *clonotype* in this regard are typically the same germline IGHV and IGHJ by assignment, same CDR-H3 length, and ≥90% peptide sequence homology. A recent and significant innovation in BCR-seq is the development of methods to maintain the correct pairing of the VH and VL in the B-cell repertoire [8••,9,10]. This is achieved through single-cell sorting, VH:VL linkage PCR performed in an emulsion, and NGS. In regards to clonotyping methods, this technological advance will allow more accurate typing of the complete VH:VL antibody clonotype and can additionally take account of important features in VL domains.

Finally, recent advances in the integration of protein mass spectrometry and personalized VH:VL BCR-seq datasets (Ig-seq) allows high-resolution measurement of the actual antigen-specific Ig clonotypes present in serum [1••] (Figure 1). Since affinity chromatography is used to pull down clonally related serum antibodies of the same antigen specificity, the Ig-seq methodology allows all aspects of a clonotype (binding specificity plus molecular sequence) to be defined and validated.

Generalizable principles gleaned from normal antibody repertoires: determinism, size, and diversity

Natural variation within the primary repertoire

Because of the sheer size of antibody repertoires, estimates of breadth and diversity are limited by sampling and only recently have statistical approaches become feasible, with the human peripheral B cell repertoire size recently estimated to contain $\sim 10^6$–10^7 unique CDR-H3 [15]. However, the theoretical sequence space of the repertoire far exceeds the antibody diversity found within an individual at any one point in time, and shared antibody sequences are extremely uncommon [16–18]. Yet, at a broader level, the overall usage of the germline IGHV, IGHD, and IGHJ segments that encode the variable region has been shown to be unequally distributed, yet at a very consistent ratio among individuals, indicating a measured amount of determinism in the generation of primary antibody diversity [15,19]. It was subsequently shown that this determinism is due to genetic factors intrinsic to B cells [20–24].

Unlike the antigen-specific mBC repertoire, which is very diverse and, in the case of chronic or repeated infections, can comprise millions of distinct clones, the serological repertoire is orders of magnitude more restricted; this has been documented in mice infected with West Nile virus [25], and there is indirect evidence that these compartments also differ in humans [12]. Likewise, as described below, comparison of the antigen-specific VH gene repertoire of transient PB cells with the steady-state serum antibody repertoire (in other words, the serological memory) has revealed that a very small fraction of the VH clonotypes encoded by peripheral B cells are observed in the polyclonal serum response (<5% of the VH clonotypes in the peak response PB repertoire and <0.1% in the steady-state peripheral mBC repertoire) [1••].

The dramatic discordance between humoral immunity and the VH gene repertoire in antigen-stimulated peripheral B cells is not widely appreciated but it can be readily illustrated with a simple quantitative analysis of humoral immunity. For circulating antibodies to be physiologically relevant, they have to be present in serum at concentrations exceeding their equilibrium binding constant, K_D. Assuming an average K_D of IgGs to persistent antigen exposure or re-stimulation of 1–5 nM (or approx. 0.2–1.0 μg/ml) [26] and given that serum titers to pathogens rarely exceed 100 μg/ml it follows that the diversity of physiologically relevant antibodies present in the serological repertoire must be of the order of 10^2–10^3 (100 μg/ml divided by 0.2–1 μg/ml), or 3 or more orders of

magnitude smaller than the typical antigen-specific peripheral mBC repertoire. In regards to an *upper* bound, the total serological antibody repertoire could be as large as 10^6 distinct binding specificities (clonotypes) [27]. This argument assumes that (1) the lowest Ab concentration required for the elimination of antigen ~10 ng/ml, (2) there is 10 mg/ml total IgG in serum, and (3) each distinct antibody is present at threshold concentration. Even at 10^6, this still places the serological repertoire at least 10^1 smaller, perhaps 10^3 smaller, than the cellular repertoire by lower-bound BCR-seq estimates [15]. Proteomic analyses from our lab have yielded estimates of IgG clonotypic diversity of $\geq 10^4$ (Schätzle and Georgiou, unpublished observation). The discrepancy between the peripheral mBC (and also the antigen-specific peak response PB repertoire) and the serum antibody repertoire argue strongly that determination of the serological repertoire is critical for a comprehensive understanding of antibody-mediated protection mechanisms.

Generalizable principles gleaned from vaccine-induced antibody repertoires: dynamic cellular repertoires, steady-state serological repertoires, and convergence

BCR-seq of vaccine-specific VH antibody repertoires

Almost all vaccines confer immunity through the induction of antibodies in serum or in mucosal tissues [28]. Systematic analysis of the antibody-mediated humoral immune response to vaccination at high-throughput requires experimental distinction between the vaccine-specific and the total antibody repertoire in an individual. One approach to inferring antigen specificity is to examine the dynamics in the peripheral B cell repertoire in response to vaccination. Laserson *et al.* used BCR-seq in three individuals across 2 years, analyzing 38 blood samples before and after vaccination [19]. VH were selected for antibody characterization based upon increases in NGS clone frequency and found to be specific to the vaccine, albeit very few were of high affinity. Jackson *et al.* showed a direct correspondence between the number of clonally expanded peripheral B cell lineages at day 7 post-vaccination and an increase in serum titer [29••]. Jiang *et al.* similarly used BCR-seq dynamics before and after vaccination to identify expanded vaccine-specific antibody lineages within the peripheral B cell repertoire [30•]. They found that 11 of 16 confirmed influenza-specific VH sequences mapped to the expanded clonotypes. Further, lineage structure analysis revealed that elderly patients have fewer, more highly mutated IgG clonotypes as compared to younger patients. Wu *et al.* also detected higher IgG mutation in the elderly after influenza vaccination, as well as significantly longer CDR-H3 in the IgM and IgA lineages that were expanded at day 7 post-vaccination [31]. In a study on age-dependent responses to 23-valent pneumococcal polysaccharide vaccine (PPSV23) [32], BCR-seq spectratyping of IgM CDR-H3 demonstrated that the humoral response to PPSV23 was dominated by antibodies with short, hydrophilic CDR-H3s. The older group exhibited a noticeably attenuated response to vaccination at day 7, most likely reflective of reduced baseline (Day 0) IgM diversity and longer CDR-H3 lengths. Clearly, these studies and others [33–35] indicate the significance of antibody diversity and CDR-H3 characteristics to the humoral immune response and support the use of such metrics for studying vaccine efficacy. A refinement of these metrics should include a transition from VH-only BCR-seq to BCR-seq of complete VH:VL clonotypes, as well as a quantitative exploration of their absence or presence in the serological repertoire using Ig-seq.

BCR-seq of vaccine-specific paired VH:VL antibody repertoires

Other, more conventional methods for distinguishing antigen specificity include the labeling of antigen-specific mBCs or the isolation of bulk PBs using flow cytometry [36]. For a variety of viral infections and most immunizations, the appearance of vaccine-specific PBs is strikingly consistent, peaking at ~7 days post-vaccination or day 10 for primary vaccinations [37]. This 'plasmablast signature' and its predictive capacity for the *magnitude* of antibody production has been observed by several research groups [38–40]; how this might relate to the functional quality or exact molecular nature of the end-point serological antibody response is unknown and has yet to be comprehensively examined, but current investigations are tantalizing [1••,29••].

As but one example, DeKosky *et al.* isolated tetanus toxoid (TT) specific PBs on day 7 post-vaccination [8••] and, using emulsion linkage PCR and BCR-seq from 200 sorted cells, identified 86 TT-specific antibody VH:VL pairs in a single experiment. A significant improvement in throughput utilizes a newly-developed, low-cost single-cell emulsion-based technology which flows B cells through a nozzle to encapsulate individual B cells in lysis/PCR reaction droplets that contain magnetic beads for mRNA capture [41••]. Subsequent emulsion RT-PCR generated VH:VL amplicons are used for 2 × 300 Illumina MiSeq NGS. This method increases the B-cell VH:VL yields 100×, to $>2 \times 10^6$ B cells per experiment with demonstrated pairing precision >97%. This orders-of-magnitude increase in B-cell throughput and VH:VL sequencing depth allows millions of B-cells to be processed and interrogated in a single experiment by a single experimentalist. Thus, an advantage of this method is that it allows complete sequencing of *all* antigen-specific B-cell VH:VL pairs within a finite collected pool (e.g., day 7 PBs or total mBCs at 14 days post-booster vaccination [36]).

Serum antibody proteomics (Ig-seq) of vaccine-specific antibody repertoires

It is now also possible to identify affinity-purified serum antibodies using high-resolution proteomics [1••,2,3,4••]. The goal of serum antibody proteomics, or Ig-seq, is to

systematically identify the distinct antibodies present in a serum sample, as assayed using protein tandem mass spectrometry (Figure 1). The mass spectra data are interpreted in reference to a personalized BCR-seq database derived from the same donor. This process can be simplified to the quantitation and sequence determination of CDR-H3 peptides. The remaining sequence of each antibody can then be retrieved from the BCR-seq reference database. In a study of the immune response to tetanus toxoid (TT), Lavinder *et al.* exhaustively characterized the constituent serum antibodies elicited by a vaccine and discovered that the steady state anti-TT serum IgG repertoire is composed of a limited number of antibody clonotypes (80–100), with three clonotypes accounting for >40% of the response [1••]. Importantly, only a small fraction (<5%) of TT-specific, vaccine-responsive PB clonotypes at day 7 were found to encode antibodies that could be detected in the serological memory response 9 months post-vaccination. This suggests that only a minority of the antigen-specific, transient PBs give rise to long-lived BMPCs. This result is not altogether unexpected since huge variability in both the number, kinetics, and the antigen-specificity of transient PBs has been repeatedly observed for a variety of vaccination and natural infection contexts [42]. This is also in agreement with previous data demonstrating that only a fraction (5–10%) of responding PBs migrate to the bone marrow after vaccination [43]. These differences in the peak responding PBs and the effective levels of serum antibodies is a significant finding in that the antigen-specific repertoire in vaccinated humans is typically assessed by DNA sequencing of these responding PB clonotypes, a large number of which (>95% for both donors here) do not constitute the post-boost steady-state antigen-specific serum IgG repertoire.

Molecular convergence of antibody responses

Lastly, in the course of the TT study summarized above, we discovered a VH clonotype shared between two donors (a stereotype) and also with a third donor analyzed independently in a distinct laboratory [44], providing intriguing evidence for the existence of 'public' clones, or 'convergent immune signatures,' emerging after antigen challenge. BCR-seq of VH repertoires has discovered the emergence of stereotyped clones in other vaccinations, such as seasonal influenza H1N1 vaccination [29••] and Dengue viral infection [45•]. The convergent detection of stereotyped or 'public' serum clonotypes detected by BCR-seq and Ig-seq might correlate strongly with vaccine efficacy, seroconversion, or the production of neutralizing antibodies.

With certain antigens, convergence has been shown to be quite prevalent, producing VH clonotypes that are universally identified across individual antibody repertoires. Vollmers *et al.* developed a BCR-seq barcoding technique that uniquely identified each starting VH or VL transcript [16]. This allowed consensus-based filtering of sequencing error to allow an accurate measurement of the memory recall response to vaccination. It also provided an accurate measurement of antibody repertoire convergence, revealing that 25 of ~100,000 VH sequences were shared between vaccinated individuals. However, all of these were of low abundance and had very low amounts of mutation and short CDR-H3, indicating stochastic overlap within the naïve B cell repertoire. Jackson *et al.*, however, identified the molecular convergence of an antibody response to influenza H1N1 vaccination when comparing data with H1N1-vaccinated donors from two additional studies, revealing a stereotypic VH clonotype [29••]; although a striking result, it remains to be determined if this stereotyped rearrangement exists not merely as a VH-only but also as a VH:VL clonotype, and whether this clonotype exists in the serum antibody repertoire or can be correlated with seroconversion and viral neutralization. It is not entirely known how common stereotypic B cell responses are in vaccines. However, as detailed below, shared antibody sequences have enormous significance as potential biomarkers in both infectious disease and autoimmunity [46].

The antibody repertoire in the disease state

Infectious disease

Broadly neutralizing antibodies (bNAbs) directed against HIV and influenza viruses have been identified via the cloning of antibody V genes from peripheral B cells isolated from infected patients that displayed neutralizing serum titers [47–49]. Sequence analyses of bNAbs and homologous V genes of antigen-specific cells from the individual from which the bNAbs had been isolated, are providing insights on the evolution of broadly protective B cell immunity, on preferential usage of certain germline V genes, on somatic hypermutation patterns, and other features of neutralizing immune responses.

The significance of the information gained from these studies notwithstanding, it is not clear whether peripheral B cell-encoded bNAbs actually play a dominant role in the serological protection against infection *in vivo*. As discussed above, serological immunity is overwhelmingly contributed by BMPCs, which are often experimentally inaccessible in humans. Nonetheless, it should be appreciated that it is the cells of the BMPC compartment and not peripheral B cells, which actually secrete the Ig that maintains long-term serological memory. So far, it has not been ascertained whether bNAbs isolated from peripheral mBCs are actually present in the serum at all, let alone at physiologically relevant concentrations (i.e., above K_D) as is required in order for these antibodies to play a role in virus elimination and protection *in vivo*.

The most compelling examples of bNAb functionality are from convergent humoral responses that occur within chronic HIV infection. It is known that up to 25% of individuals with advanced HIV can develop bNAbs

against the virus. NGS is now being used to track antibody and viral co-evolution [50], and the identification of elite bnAbs is now central to the study of HIV. One of these bnAbs, VRC01, is specific to the CD4 binding site of gp120 and can cross-neutralize ~90% of HIV-1 isolates. Like many other identified HIV-specific antibodies, it shows striking amounts of affinity maturation (70 amino acid differences from germline). Wu *et al.* isolated VRC01-like antibodies from separate HIV-1 infected donors using FACS sorting against a CD4-binding site probe [51]. BCR-seq and phylogenetic analysis of both donors revealed similarities between the affinity maturation pathways for the VRC01-like antibodies and demonstrated how BCR-seq can be used to identify large clades of antibody sequences based upon selective criteria, such as V(D)J usage, amount of mutation, and sequence identity to known mAbs. Subsequent studies [52–55] further demonstrated the power of BCR-seq and phylogenetics as a general strategy for the de novo identification [53] and phylogenetic VH:VL pairing [54•] of VRC01-like and other bnAbs from HIV-1 infected donors.

Autoimmunity and cancer

Autoantibody repertoires likely contain a wealth of information both in regards to the early diagnosis of immunopathology, as well as providing an increased understanding of disease progression. Unfortunately, very little is known regarding these potentially significant sources of biomarkers. In a pair of recent studies [56,57] on patients with multiple sclerosis (MS), expanded B cell clonotypes in the periphery (peripheral blood in one study and cervical lymph nodes in the other) were overlapping with B cell sequences found in the CNS of the patient. It was shown that the founding members of these overlapping clonotypes were prevalent in the cervical lymph nodes and that overlapping members in the peripheral blood were primarily class-switched B cells. In addition to cross-tissue overlap, convergence among individuals has also been detected in the autoantibody response. Doorenspleet *et al.* used BCR-seq from peripheral blood and joint synovial fluid B cells in patients with early and established rheumatoid arthritis (RA), demonstrating potential convergence in the dominant B cell lineages within synovial fluid of early RA patients [58]. These dominant lineages heavily utilized the V segment IGHV4-34 and had significantly longer CDR-H3. A few recent studies have also used high-resolution mass spectrometry to proteomically identify molecular signatures in the autoreactive antibody response to the Ro/La ribonucleoprotein complex in patients with Sjögren's syndrome (SS) [59–61]. Although these studies only identified a handful of public (shared) antibody lineages and V gene mutations across SS patients, it highlights the great potential of serum antibody proteomics in autoimmune biomarker discovery.

Similarly, the antibody response to tumor-associated antigens can provide early diagnostic cues in detecting malignancy [62]. A series of studies have utilized BCR-seq, as well as serum antibody proteomics [63], to facilitate the early detection and monitoring of Non-Hodgkin's lymphoma [64], acute lymphoblastic leukemia [65,66], chronic lymphocytic leukemia [67], B lymphoblastic leukemia [68], and multiple myeloma [63,69]. Typically, these lymphocyte malignancies are detected and monitored via PCR specific to the malignant lineage(s), which requires patient-specific primers to examine values of minimal residual disease (MRD). BCR-seq circumvents patient-specific reagents and maintains or increases sensitivity for monitoring MRD [70]. Additionally, there is great potential for BCR-seq and Ig-seq in developing further metrics for diagnostic and prognostic applications in both autoimmunity and cancer.

Conclusions and future perspectives: integration of the cellular repertoire (BCR-seq) with the serological repertoire (Ig-seq)

Functional annotation of DNA sequences obtained in BCR-seq is an enormous challenge, and it has now become a common appeal in the field to link function with sequence [71,72]. Indirect approaches to link function with sequence might include experimental designs which allow all four IgG subclasses to be separately examined, rather than IgG *en masse*, which would link sequences with known differential features of antibody half-life, effector functions, or even altogether new clinical entities such as IgG4-related disease [73]; or, in certain cases, convergence or dynamics within BCR repertoires can often lead the discovery effort for antigen-specific clonotypes in response to vaccination or infection. However, this is not a generalizable strategy and such obvious levels of dynamics or determinism may be restricted to certain antigens or only evident in cases where the humoral response is robust or ongoing.

The recent development of paired VH:VL BCR-seq and Ig-seq represents a new paradigm for repertoire analysis and of antibody discovery in which functionality (binding) is directly linked to NGS of natively-paired antibody gene sequences. This ability to quickly link paired NGS data and antibody functionality is a key step forward in antibody discovery and repertoire analysis. Not only is this applicable to the vaccine-elicited antibody repertoire, but it also enables the link between antibody sequence and function to be ascertained in infectious disease, as well as autoimmunity and cancer. As a pair of relevant examples where such approaches are necessary, the recently identified auto-antigen, KIR4.1 [74], represents a potential target in the discovery of autoantibody profiles in MS. Or, in the case of vaccines which incorporate tumor-specific antigens, such as epidermal growth factor receptor variant III (EGFRvIII) glioblastoma peptide vaccine, wherein it has been shown that the elicitation of antibody titers can be correlated with an increase in overall survival rate in the treatment of glioblastoma brain tumors [75].

The discovery of functionally linked antibody sequences in such patients has obvious implications, whether as a means of identifying molecular biomarkers of disease or in the discovery and development of mAb therapeutics. In this last regard, these technologies could conceivably be deployed as emergency response platforms for mining true human mAbs from the convalescent whole blood of survivors recovered from emerging viral disease epidemics [76], or even in the production of mAbs as part of any general, global viral eradication initiative, such as that presently ongoing for poliovirus — the 'endgame.'

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. •• Lavinder JJ, Wine Y, Giesecke C, Ippolito GC, Horton AP, Lungu OI, Hoi KH, DeKosky BJ, Murrin EM, Wirth MM *et al.*: **Identification and characterization of the constituent human serum antibodies elicited by vaccination**. *Proc Natl Acad Sci U S A* 2014, **111**:2259-2264.

The first molecular-level analysis of the vaccine-elicited serum antibody repertoire, using a personalized BCR-seq gene database to interpret peptide spectra from high-resolution protein mass spectrometry of the serum antibodies (Ig-seq). Importantly, the majority of the Day 7 plasmablast clonotypes identified by BCR-seq could not detected by Ig-seq in the steady-state serological repertoire at nine months post-vaccination.

2. Wine Y, Boutz DR, Lavinder JJ, Miklos AE, Hughes RA, Hoi KH, Jung ST, Horton AP, Murrin EM, Ellington AD *et al.*: **Molecular deconvolution of the monoclonal antibodies that comprise the polyclonal serum response**. *Proc Natl Acad Sci U S A* 2013, **110**:2993-2998.

3. Boutz DR, Horton AP, Wine Y, Lavinder JJ, Georgiou G, Marcotte EM: **Proteomic identification of monoclonal antibodies from serum**. *Anal Chem* 2014, **86**:4758-4766.

4. •• Cheung WC, Beausoleil SA, Zhang XW, Sato S, Schieferl SM, Wieler JS, Beaudet JG, Ramenani RK, Popova L, Comb MJ *et al.*: **A proteomics approach for the identification and cloning of monoclonal antibodies from serum**. *Nat Biotechnol* 2012, **30**:447.

The first study to utilize high-resolution LC–MS/MS to identify constituent monoclonal serum antibodies. Using database-driven proteomic identification of both VH and VL-derived peptides from the sera of mice and rabbits, a total of 50 antigen-specific mAbs were identified by ELISA, with 19 of these confirmed by Western blot analysis.

5. Sato S, Beausoleil SA, Popova L, Beaudet JG, Ramenani RK, Zhang XW, Wieler JS, Schieferl SM, Cheung WC, Polakiewicz RD: **Proteomics-directed cloning of circulating antiviral human monoclonal antibodies**. *Nat Biotechnol* 2012, **30**:1039-1043.

6. Georgiou G, Ippolito GC, Beausang J, Busse CE, Wardemann H, Quake SR: **The promise and challenge of high-throughput sequencing of the antibody repertoire**. *Nat Biotechnol* 2014, **32**:158-168.

7. Mathonet P, Ullman CG: **The application of next generation sequencing to the understanding of antibody repertoires**. *Front Immunol* 2013, **4**:265.

8. •• DeKosky BJ, Ippolito GC, Deschner RP, Lavinder JJ, Wine Y, Rawlings BM, Varadarajan N, Giesecke C, Dorner T, Andrews SF *et al.*: **High-throughput sequencing of the paired human immunoglobulin heavy and light chain repertoire**. *Nat Biotechnol* 2013, **31**:166-169.

Once a major drawback for BCR-seq, the ability to retain the native pairing of the antibody heavy and light chain sequences is required to clone and express mAbs. Normally this is done by combinatorial screening of heavy chain/light chain (VH:VL) pairs for function. This study represents a major breakthrough by utilizing high-throughput linkage of native VH:VL, followed by BCR-seq. Greater than 5×10^4 single B cell VH:VL pairs can be identified in a single experiment.

9. Tan YC, Blum LK, Kongpachith S, Ju CH, Cai X, Lindstrom TM, Sokolove J, Robinson WH: **High-throughput sequencing of natively paired antibody chains provides evidence for original antigenic sin shaping the antibody response to influenza vaccination**. *Clin Immunol* 2014, **151**:55-65.

10. Lu DR, Tan YC, Kongpachith S, Cai XY, Stein EA, Lindstrom TM, Sokolove J, Robinson WH: **Identifying functional anti-*Staphylococcus aureus* antibodies by sequencing antibody repertoires of patient plasmablasts**. *Clin Immunol* 2014, **152**:77-89.

11. Rajewsky K: **Clonal selection and learning in the antibody system**. *Nature* 1996, **381**:751-758.

12. Tarlinton D, Good-Jacobson K: **Diversity among memory B cells: origin, consequences, and utility**. *Science* 2013, **341**:1205-1211.

13. Slifka MK, Ahmed R: **Long-term humoral immunity against viruses: revisiting the issue of plasma cell longevity**. *Trends Microbiol* 1996, **4**:394-400.

14. Klinman NR, Press JL: **Characterization of B-cell repertoire specific for 2,4-dinitrophenyl and 2,4,6-trinitrophenyl determinants in neonatal balb-c mice**. *J Exp Med* 1975, **141**:1133-1146.

15. Arnaout R, Lee W, Cahill P, Honan T, Sparrow T, Weiand M, Nusbaum C, Rajewsky K, Koralov SB: **High-resolution description of antibody heavy-chain repertoires in humans**. *PLoS ONE* 2011, **6**:e22365.

16. Vollmers C, Sit RV, Weinstein JA, Dekker CL, Quake SR: **Genetic measurement of memory B-cell recall using antibody repertoire sequencing**. *Proc Natl Acad Sci U S A* 2013, **110**:13463-13468.

17. Glanville J, Kuo TC, von Budingen HC, Guey L, Berka J, Sundar PD, Huerta G, Mehta GR, Oksenberg JR, Hauser SL *et al.*: **Naive antibody gene-segment frequencies are heritable and unaltered by chronic lymphocyte ablation**. *Proc Natl Acad Sci U S A* 2011, **108**:20066-20071.

18. Boyd SD, Marshall EL, Merker JD, Maniar JM, Zhang LN, Sahaf B, Jones CD, Simen BB, Hanczaruk B, Nguyen KD *et al.*: **Measurement and clinical monitoring of human lymphocyte clonality by massively parallel V-D-J pyrosequencing**. *Sci Transl Med* 2009, **1**:12ra23.

19. Laserson U, Vigneault F, Gadala-Maria D, Yaari G, Uduman M, Vander Heiden JA, Kelton W, Taek Jung S, Liu Y, Laserson J *et al.*: **High-resolution antibody dynamics of vaccine-induced immune responses**. *Proc Natl Acad Sci U S A* 2014, **111**:4928-4933.

20. Ippolito GC, Hoi KH, Reddy ST, Carroll SM, Ge X, Rogosch T, Zemlin M, Shultz LD, Ellington AD, VanDenBerg CL *et al.*: **Antibody repertoires in humanized NOD-SCID-IL2R gamma(null) mice and human B cells reveals human-like diversification and tolerance checkpoints in the mouse**. *PLoS ONE* 2012, **7**:e35497.

21. Larimore K, McCormick MW, Robins HS, Greenberg PD: **Shaping of human germline IgH repertoires revealed by deep sequencing**. *J Immunol* 2012, **189**:3221-3230.

22. Benichou J, Glanville J, Prak ETL, Azran R, Kuo TC, Pons J, Desmarais C, Tsaban L, Louzoun Y: **The restricted D-H gene reading frame usage in the expressed human antibody repertoire is selected based upon its amino acid content**. *J Immunol* 2013, **190**:5567-5577.

23. Ippolito GC, Schelonka RL, Zemlin M, Ivanov II, Kobayashi R, Zemlin C, Gartland GL, Nitschke L, Pelkonen J, Fujihashi K *et al.*: **Forced usage of positively charged amino acids in immunoglobulin CDR-H3 impairs B cell development and antibody production**. *J Exp Med* 2006, **203**:1567-1578.

24. Trad A, Tanasa RI, Lange H, Zemlin M, Schroeder HW Jr, Lemke H: **Clonal progression during the T cell-dependent B**

cell antibody response depends on the immunoglobulin DH gene segment repertoire. *Front Immunol* 2014, **5**:385.

25. Purtha WE, Tedder TF, Johnson S, Bhattacharya D, Diamond MS: **Memory B cells, but not long-lived plasma cells, possess antigen specificities for viral escape mutants**. *J Exp Med* 2011, **208**:2599-2606.

26. Foote J, Eisen HN: **Kinetic and affinity limits on antibodies produced during immune-responses**. *Proc Natl Acad Sci U S A* 1995, **92**:1254-1256.

27. Langman RE, Cohn M: **The E-T (elephant-tadpole) paradox necessitates the concept of a unit of B-cell function — the protecton**. *Mol Immunol* 1987, **24**:675-697.

28. Plotkin SA: **Correlates of vaccine-induced immunity**. *Clin Infect Dis* 2008, **47**:401-409.

29. •• Jackson KJL, Liu Y, Roskin KM, Glanville J, Hoh RA, Seo K, Marshall EL, Gurley TC, Moody MA, Haynes BF *et al.*: **Human responses to influenza vaccination show seroconversion signatures and convergent antibody rearrangements**. *Cell Host Microbe* 2014, **16**:105-114.

The humoral immune response to influenza produces a robust wave of peripheral blood plasmablasts that peak shortly after vaccination. Analysis of these plasmablast VH sequences revealed a striking correlation between increased polarization of B cell clonality and seroconversion. Significantly, this study also demonstrated a convergent influenza-specific heavy chain stereotype across three donors from independent studies, indicating that convergent signatures may be readily detected in antigen-specific antibody repertoires.

30. • Jiang N, He JK, Weinstein JA, Penland L, Sasaki S, He XS, Dekker CL, Zheng NY, Huang M, Sullivan M *et al.*: **Lineage structure of the human antibody repertoire in response to influenza vaccination**. *Sci Transl Med* 2013, **5**:171ra19.

BCR-seq of the PB repertoire elicited by influenza vaccination in young and elderly patients revealed striking differences in clonal diversity and somatic hypermutation. This has direct implications in known age-related differences in vaccine efficacy, highlighting the importance of key characteristics of the antibody repertoire in humoral protection.

31. Wu YC, Kipling D, Dunn-Walters DK: **Age-related changes in human peripheral blood IgH repertoire following vaccination**. *Front Immunol* 2012, **3**:193.

32. Ademokun A, Wu Y-C, Martin V, Mitra R, Sack U, Baxendale H, Kipling D, Dunn-Walters DK: **Vaccination-induced changes in human B-cell repertoire and pneumococcal IgM and IgA antibody at different ages**. *Aging Cell* 2011, **10**:922-930.

33. Gibson KL, Wu YC, Barnett Y, Duggan O, Vaughan R, Kondeatis E, Nilsson BO, Wikby A, Kipling D, Dunn-Walters DK: **B-cell diversity decreases in old age and is correlated with poor health status**. *Aging Cell* 2009, **8**:18-25.

34. Guerrettaz LM, Johnson SA, Cambier JC: **Acquired hematopoietic stem cell defects determine B-cell repertoire changes associated with aging**. *Proc Natl Acad Sci U S A* 2008, **105**:11898-11902.

35. Wang C, Liu Y, Xu LT, Jackson KJL, Roskin KM, Pham TD, Laserson J, Marshall EL, Seo K, Lee JY *et al.*: **Effects of aging, cytomegalovirus infection, and EBV infection on human B cell repertoires**. *J Immunol* 2014, **192**:603-611.

36. Frolich D, Giesecke C, Mei HE, Reiter K, Daridon C, Lipsky PE, Dorner T: **Secondary immunization generates clonally related antigen-specific plasma cells and memory B cells**. *J Immunol* 2010, **185**:3103-3110.

37. Giesecke C, Frolich D, Reiter K, Mei HE, Wirries I, Kuhly R, Killig M, Glatzer T, Stolzel K, Perka C *et al.*: **Tissue distribution and dependence of responsiveness of human antigen-specific memory B cells**. *J Immunol* 2014, **192**:3091-3100.

38. Obermoser G, Presnell S, Domico K, Xu H, Wang YY, Anguiano E, Thompson-Snipes L, Ranganathan R, Zeitner B, Bjork A *et al.*: **Systems scale interactive exploration reveals quantitative and qualitative differences in response to influenza and pneumococcal vaccines**. *Immunity* 2013, **38**:831-844.

39. Tsang JS, Schwartzberg PL, Kotliarov Y, Biancotto A, Xie Z, Germain RN, Wang E, Olnes MJ, Narayanan M, Golding H *et al.*: **Global analyses of human immune variation reveal baseline predictors of postvaccination responses**. *Cell* 2014, **157**:499-513.

40. Pulendran B: **Systems vaccinology: probing humanity's diverse immune systems with vaccines**. *Proc Natl Acad Sci U S A* 2014, **111**:12300-12306.

41. •• DeKosky BJ, Kojima T, Rodin A, Charab W, Ippolito GC, Ellington AD, Georgiou G: **Ultra-high throughput antibody VH:VL sequencing using a novel single-cell flow focusing technology**. *Nat Med* 2014. (in press).

DeKosky *et al.* report here the development of a low-cost single-cell emulsion-based technology for sequencing paired antibody VH:VL repertoires from $>2 \times 10^6$ B cells per experiment with >97% pairing precision. In three separate experiments using the B cells from human volunteers, this study generated 10 times more complete antibody sequences (both VH and VL information) than what has been deposited in Genbank over the last 25 years.

42. Fink K: **Origin and function of circulating plasmablasts during acute viral infections**. *Front Immunol* 2012, **3**:78.

43. Hofer T, Muehlinghaus G, Moser K, Yoshida THEM, Hebel K, Hauser A, Hoyer BEOL, Dorner T *et al.*: **Adaptation of humoral memory**. *Immunol Rev* 2006, **211**:295-302.

44. Franz B, May KF Jr, Dranoff G, Wucherpfennig K: **Ex vivo characterization and isolation of rare memory B cells with antigen tetramers**. *Blood* 2011, **118**:348-357.

45. • Parameswaran P, Liu Y, Roskin KM, Jackson KK, Dixit VP, Lee JY, Artiles KL, Zompi S, Vargas MJ, Simen BB *et al.*: **Convergent antibody signatures in human dengue**. *Cell Host Microbe* 2013, **13**:691-700.

This study provides the most striking example of convergence within the antibody repertoire. BCR-seq of the antibody repertoire in healthy and dengue-infected human donors revealed several convergent BCR clonotypes found only in acutely infected dengue patients. This convergence was not detected in post-recovery patients, indicating a limit of detection for such signatures within the highly diverse mBC repertoire at steady-state post-exposure.

46. Kodadek T: **Chemical tools to monitor and manipulate the adaptive immune system**. *Chem Biol* 2014, **21**:1066-1074.

47. Wrammert J, Smith K, Miller J, Langley WA, Kokko K, Larsen C, Zheng NY, Mays I, Garman L, Helms C *et al.*: **Rapid cloning of high-affinity human monoclonal antibodies against influenza virus**. *Nature* 2008, **453** 667-U610.

48. Kwong PD, Mascola JR: **Human antibodies that neutralize HIV-1: identification, structures, and B cell ontogenies**. *Immunity* 2012, **37**:412-425.

49. Corti D, Lanzavecchia A: **Broadly neutralizing antiviral antibodies**. *Annu Rev Immunol* 2013, **31**:705-742.

50. Liao HX, Lynch R, Zhou T, Gao F, Alam SM, Boyd SD, Fire AZ, Roskin KM, Schramm CA, Zhang Z *et al.*: **Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus**. *Nature* 2013, **496**:469-476.

51. Wu X, Zhou T, Zhu J, Zhang B, Georgiev I, Wang C, Chen X, Longo NS, Louder M, McKee K *et al.*: **Focused evolution of HIV-1 neutralizing antibodies revealed by structures and deep sequencing**. *Science* 2011, **333**:1593-1602.

52. Zhu J, O'Dell S, Ofek G, Pancera M, Wu X, Zhang B, Zhang Z, Mullikin JC, Simek M, Burton DR *et al.*: **Somatic populations of PGT135-137 HIV-1-neutralizing antibodies identified by 454 pyrosequencing and bioinformatics**. *Front Microbiol* 2012, **3**:315.

53. Zhu J, Wu X, Zhang B, McKee K, O'Dell S, Soto C, Zhou T, Casazza JP, Mullikin JC, Kwong PD *et al.*: **De novo identification of VRC01 class HIV-1-neutralizing antibodies by next-generation sequencing of B-cell transcripts**. *Proc Natl Acad Sci U S A* 2013, **110**:E4088-E4097.

54. • Zhu J, Ofek G, Yang YP, Zhang BS, Louder MK, Lu G, McKee K, Pancera M, Skinner J, Zhang ZH *et al.*: **Mining the antibodyome for HIV-1-neutralizing antibodies with next-generation sequencing and phylogenetic pairing of heavy/light chains**. *Proc Natl Acad Sci U S A* 2013, **110**:6470-6475.

Antibodies that are broadly cross-reactive to multiple HIV-1 isolates have been identified in a number of patients. These antibodies effectively

neutralize the virus and their discovery provides a potential template to understanding HIV-1 protection and vaccine efficacy. Here, the bNAb B cell lineages were examined by BCR-seq and a method for phylogenetic pairing of antibody heavy and light chains is presented. Significantly, matched phylogenetic pairs of new bNAb variants demonstrated low autoreactivity (as compared to mismatched), indicating these antibodies retain high specificity.

55. Gao F, Bonsignori M, Liao HX, Kumar A, Xia SM, Lu XZ, Cai FP, Hwang KK, Song HS, Zhou TQ *et al.*: **Cooperation of B cell lineages in induction of HIV-1-broadly neutralizing antibodies**. *Cell* 2014, **158**:481-491.

56. Stern JNH, Yaari G, Vander Heiden JA, Church G, Donahue WF, Hintzen RQ, Huttner AJ, Laman JD, Nagra RM, Nylander A *et al.*: **B cells populating the multiple sclerosis brain mature in the draining cervical lymph nodes**. *Sci Transl Med* 2014:6.

57. Palanichamy A, Apeltsin L, Kuo TC, Sirota M, Wang SZ, Pitts SJ, Sundar PD, Telman D, Zhao LZ, Derstine M *et al.*: **Immunoglobulin class-switched B cells form an active immune axis between CNS and periphery in multiple sclerosis**. *Sci Transl Med* 2014:6.

58. Doorenspleet ME, Klarenbeek PL, de Hair MJ, van Schaik BD, Esveldt RE, van Kampen AH, Gerlag DM, Musters A, Baas F, Tak PP *et al.*: **Rheumatoid arthritis synovial tissue harbours dominant B-cell and plasma-cell clones associated with autoreactivity**. *Ann Rheum Dis* 2014, **73**:756-762.

59. Thurgood LA, Arentz G, Lindop R, Jackson MW, Whyte AF, Colella AD, Chataway TK, Gordon TP: **An immunodominant La/SSB autoantibody proteome derives from public clonotypes**. *Clin Exp Immunol* 2013, **174**:237-244.

60. Lindop R, Arentz G, Chataway TK, Thurgood LA, Jackson MW, Reed JH, McCluskey J, Gordon TP: **Molecular signature of a public clonotypic autoantibody in primary Sjogren's syndrome: a forbidden clone in systemic autoimmunity**. *Arthritis Rheum* 2011, **63**:3477-3486.

61. Arentz G, Thurgood LA, Lindop R, Chataway TK, Gordon TP: **Secreted human Ro52 autoantibody proteomes express a restricted set of public clonotypes**. *J Autoimmun* 2012, **39**:466-470.

62. Murphy MA, O'Leary JJ, Cahill DJ: **Assessment of the humoral immune response to cancer**. *J Proteomics* 2012, **75**:4573-4579.

63. Barnidge DR, Tschumper RC, Theis JD, Snyder MR, Jelinek DF, Katzmann JA, Dispenzieri A, Murray DL: **Monitoring M-proteins in patients with multiple myeloma using heavy-chain variable region clonotypic peptides and LC–MS/MS**. *J Proteome Res* 2014, **13**:1905-1910.

64. He J, Wu J, Jiao Y, Wagner-Johnston N, Ambinder RF, Diaz LA Jr, Kinzler KW, Vogelstein B, Papadopoulos N: **IgH gene rearrangements as plasma biomarkers in Non-Hodgkin's lymphoma patients**. *Oncotarget* 2011, **2**:178-185.

65. Faham M, Zheng J, Moorhead M, Carlton VE, Stow P, Coustan-Smith E, Pui CH, Campana D: **Deep-sequencing approach for minimal residual disease detection in acute lymphoblastic leukemia**. *Blood* 2012, **120**:5173-5180.

66. Gawad C, Pepin F, Carlton VE, Klinger M, Logan AC, Miklos DB, Faham M, Dahl G, Lacayo N: **Massive evolution of the immunoglobulin heavy chain locus in children with B precursor acute lymphoblastic leukemia**. *Blood* 2012, **120**:4407-4417.

67. Logan AC, Gao H, Wang C, Sahaf B, Jones CD, Marshall EL, Buno I, Armstrong R, Fire AZ, Weinberg KI *et al.*: **High-throughput VDJ sequencing for quantification of minimal residual disease in chronic lymphocytic leukemia and immune reconstitution assessment**. *Proc Natl Acad Sci U S A* 2011, **108**:21194-21199.

68. Wu D, Emerson RO, Sherwood A, Loh ML, Angiolillo A, Howie B, Vogt J, Rieder M, Kirsch I, Carlson C *et al.*: **Detection of minimal residual disease in B lymphoblastic leukemia by high-throughput sequencing of IGH**. *Clin Cancer Res* 2014, **20**:4540-4548.

69. Tschumper RC, Asmann YW, Hossain A, Huddleston PM, Wu X, Dispenzieri A, Eckloff BW, Jelinek DF: **Comprehensive assessment of potential multiple myeloma immunoglobulin heavy chain V-D-J intraclonal variation using massively parallel pyrosequencing**. *Oncotarget* 2012, **3**:502-513.

70. Ladetto M, Bruggemann M, Monitillo L, Ferrero S, Pepin F, Drandi D, Barbero D, Palumbo A, Passera R, Boccadoro M *et al.*: **Next-generation sequencing and real-time quantitative PCR for minimal residual disease detection in B-cell disorders**. *Leukemia* 2014, **28**:1299-1307.

71. Galson JD, Pollard AJ, Truck J, Kelly DF: **Studying the antibody repertoire after vaccination: practical applications**. *Trends Immunol* 2014, **35**:319-331.

72. Calis JJ, Rosenberg BR: **Characterizing immune repertoires by high throughput sequencing: strategies and applications**. *Trends Immunol* 2014. (Epub ahead of print).

73. Mattoo H, Mahajan VS, Della-Torre E, Sekigami Y, Carruthers M, Wallace ZS, Deshpande V, Stone JH, Pillai S: **De novo oligoclonal expansions of circulating plasmablasts in active and relapsing IgG4-related disease**. *J Allergy Clin Immunol* 2014, **134**:679-687.

74. Srivastava R, Aslam M, Kalluri SR, Schirmer L, Buck D, Tackenberg B, Rothhammer V, Chan A, Gold R, Berthele A *et al.*: **Potassium channel KIR4.1 as an immune target in multiple sclerosis**. *N Engl J Med* 2012, **367**:115-123.

75. Reardon DA, Freeman G, Wu C, Chiocca EA, Wucherpfennig KW, Wen PY, Fritsch EF, Curry WT Jr, Sampson JH, Dranoff G: **Immunotherapy advances for glioblastoma**. *Neuro Oncol* 2014, **16**:1441-1458.

76. World Health Organization (WHO): *Use of Convalescent Whole Blood or Plasma Collected From Patients Recovered from Ebola Virus Disease for Transfusion, as an Empirical Treatment during Outbreaks*. 2014:. WHO/HIS/SDS/2014.8.