Immunosequencing: Accelerating discovery in immunology and medicine

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ABSTRACT
Recently developed sequencing technologies have increased both the sensitivity and depth at which large number of T cell and B cell receptors can be identified and quantitated from different biological tissues. High-throughput sequencing of B cell and T cell receptors (immunosequencing) makes it possible to not only identify target-specific receptors, but to accurately track the frequencies of individual T and B cell clonotypes over time. Immunosequencing is being used to better understand antigen specificity of adaptive immune responses to infectious agents and autoimmunity, to identify new tumor antigens, and to monitor responses to immunotherapies. In this review we will discuss the current uses and potential future applications of immunosequencing.

KEYWORDS: TCR sequencing, cancer, immune profiling, autoimmunity

INTRODUCTION
With the advent of DNA sequencing, the scientific community has forever changed the way we monitor the immune system. Through sequencing across the genetic rearrangements of the T cell receptor (TCR) or the B cell receptor (BCR, Ig), we have significantly increased our capabilities for identifying receptors important to the immunobiology of multiple threats including pathogens, autoimmunity, and cancer [1-3]. However, traditional methods of cloning and sequencing are notoriously time consuming and laborious. In addition, traditional sequencing misses many clones that are less abundant, typically recognizing only the most robust responses [4]. In the last few years, emerging technologies that allow for the in-depth sequencing of the immune system (Immunosequencing) have changed the way researchers are using the genome in research and medicine. Immunosequencing is capable of sequencing the T cell receptor or B cell receptor from virtually every T or B cell present in a sample, increasing the sensitivity at which researchers are able to probe the immune system. In this review, we will discuss the advancements made in high-throughput immunosequencing of the T cell receptor repertoire and the implementation of this technology in both primary research and the development of immunotherapies and vaccines.

Immunosequencing, trends and technologies
Technological advances have been made to increase our understanding of the TCR repertoire. Multiple mouse models have been produced that use truncated TCR loci to decrease the diversity of TCR rearrangements on T cells and more easily assess the potential of the TCR repertoire [5-8]. As an example, the mini-TCR mouse, which expresses a transgenic TCRβ chain and truncated TCRα loci (limited number of TCRVα and TCRJα segments), has been used by multiple groups to assess the diversity and similarity in the TCR repertoires of CD4 FoxP3+ conventional and CD4 FoxP3+ regulatory T cells [7, 9]. More recently, a large-scale study used immunosequencing of conventional and regulatory T cells to show that TCR sequence

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overlap between conventional and regulatory T cells is limited and revealed that T cells sharing T cell receptors are derived from common progenitors [5]. This technology has allowed for the comparison of TCR repertoires expressed by different subsets of T cells. However, these types of analyses are only available for studies in mice.

In addition to mouse models, antigen-specific T cell recognition technology is advancing. Use of human leukocyte antigen (HLA) or major histocompatibility complex (MHC) multimers or tetramers (HLA/MHC molecules loaded with peptide) has been a popular method for identifying antigen-specific T cells. Multimers are being used to identify, and in some cases purify, pathogen and tumor-specific T cells in order to sequence and identify the specific TCRs. This is a versatile technology that is capable of quickly identifying antigen-specific T cells and immunogenic peptides [10, 11]. However, the technology is limited by the fact that the identity of the peptides in context of a specific MHC/HLA molecule must be known, and some recombinant molecules, especially MHC class II molecules, have been notoriously difficult to express as multimers [12].

High-throughput sequencing is a powerful new tool allowing for broad in-depth sequencing of the TCR repertoire [13]. By using primers specific for every individual TCR/BCR V gene segment and TCR/BCR J gene segment and sequencing across the complementarity-determining region 3 (CDR3) region, the ultra-deep sequencing dubbed “immunosequencing”, is able to identify virtually every TCR/BCR-chain present in a sample [14]. The inclusion of synthetic DNA templates into samples during amplification and sequencing corrects for any potential amplification bias by normalizing sequence output against the amplification of the synthetic DNA templates [15]. Immunosequencing can provide sequences from millions of cells and given the correct sets of controls, can be used to computationally identify TCR sequences from antigen-specific T cells. Additionally, immunosequencing can be combined with more traditional immune assays such as multimer staining and T cell activation assays to discover antigen-specificity [16]. Currently, high-throughput sequencing is only able to analyze a single chain (TCRβ and IgH) of the T cell and B cell receptors, meaning that TCRα and TCRβ pairs or IgH and IgL pairs are not known, but technologies are becoming available that are able to perform concurrent high-throughput sequencing and TCRαβ pairing [17].

**Immune profiling in infectious disease and vaccine studies**

Using immunosequencing, significant advances in understanding the dynamics of the T cell response to pathogens have been made. Recently, deep sequencing of the TCR repertoire of individuals with chronic HIV infection displayed a significant decrease in TCR sequence diversity that was maintained even after anti-retroviral therapy. The loss of TCR diversity was accompanied by decreased TCR sequence sharing between repertoires of HIV+ individuals. The loss of diversity was attributed to the loss of CD4+ T cells and non-traditional T cells such as mucosal-associated invariant T-cells (MAIT cells) [18]. Immunosequencing from peripheral blood mononuclear cells (PBMCs) showed a significant loss of HIV-specific clonotypes during the course of therapy. In addition, depleted MAIT cell populations, which are thought to be specific for intracellular pathogens, never reconstituted during anti-retroviral therapy [18, 19]. This is of interest as MAIT cell TCR repertoires in healthy individuals are less diverse compared to conventional αβ T cells but are remarkably stable over time.

In models of chronic infection, such as with *M. tuberculosis*, identification of immunodominant T cell responses is correlated with both protection and disease progression [20]. Unlike in response to pathogens with high mutation rates, T cell responses to pathogens like *M. tuberculosis*, while still broad, are concentrated on a smaller number of immunodominant epitopes [21]. Paradoxically, despite being biased towards a small number of epitopes, immunosequencing data shows very few public TCRs have been identified even between multiple tuberculosis-induced granulomas in the same individual. However, it was observed that while the TCR sequences in tuberculosis-specific T cells are unique, common motifs within the CDR3 amino acid sequences confer specificity for the same immunodominant antigen(s) [22-24]. Thomas et al. attempted to identify tuberculosis-specific TCR repertoires in mice immunized with heat-inactivated tuberculosis to develop a diagnostic
Immune profiling in immunology and medicine

assay to distinguish infected vs. uninfected mice. However, direct sequence comparisons failed to distinguish immunized vs. unimmunized mice; though when using the conserved CDR3 amino acid motifs as a classifier, authors were able to correctly segregate immunized from unimmunized mice [24].

When considering the TCR repertoire as a tool for identifying T cell specific responses, whether it is for designing a diagnostic assay or vaccine, it is becoming clear that it is important to consider how the primary and memory responses differ between antigen-naïve and antigen-experienced individuals. In cytomegalovirus-experienced HLA-A2+ individuals, virus-specific T cells are reactive to the immunodominant epitope “NVL” from the pp65 protein [25]. Yet, immunosequencing of cytomegalovirus-naïve and -experienced individuals showed that T cells from naïve individuals responded to less common “atypical” pp65 epitopes and failed to raise a measurable response to the immunodominant NLV epitope [26]. TCR repertoire analysis of CMV-positive individuals does show the presence of TCRs specific for the atypical epitopes but were found at low frequencies and were less responsive to peptide compared to T cells from CMV-negative individuals. Data from these experiments, in addition to murine CMV infection models, show us that there can be significant disparity in antigen-specificity between the long-term persistent response and the T cells responsible for the initial primary response [27]. These considerations are important in future vaccine/drug development as we come to understand what is required to elicit long-term immunity.

**Immunosequencing to understand autoimmunity**

In the field of autoimmunity, there is an ongoing search for antigens responsible for the activation of the self-reactive T cells [28]. The effort is confounded by the fact that such an antigen might be present on a transient foreign pathogen that is cleared or in a tissue compartment separate from the diseased tissue. As an example, infection with the Epstein-Barr virus has been associated with the onset of multiple sclerosis, an autoimmune-driven inflammation of the central nervous system [29]. Immunosequencing of the T cell population(s) present in the cerebrospinal fluid of patients with multiple sclerosis displayed a significant enrichment of T cells expressing EBV-reactive TCRs [30]. In this study, Lossius et al. (2016), identified multiple TCRβ sequences from CD8+ T cells in the cerebrospinal fluid of multiple sclerosis patients matching known Epstein-Barr virus-specific TCR sequences. Additionally, sequencing of the Ig heavy chain displayed increased anti-Epstein-Barr virus-specific antibodies [31]. The authors concluded that further understanding of the relationship between Epstein-Barr virus infection and multiple sclerosis pathology could lead to the development of a vaccine that could prevent the onset of autoimmunity.

Currently one of the more effective treatments for more severe multiple sclerosis involves the autologous transplantation of hematopoietic stem cells after immune depletion. Immunosequencing of the CD4+ and CD8+ T cell compartments shows that following transplantation, while previously dominant CD4+ clones were undetectable, the bulk of the reconstituted CD8+ T cell repertoire was created through clonal expansion of cells present prior to immune depletion [32]. In individuals who displayed diminished CD4+ and CD8+ TCR diversity after treatment, it was shown that there is increased risk of developing secondary autoimmunity [33].

In rheumatoid arthritis, immunosequencing of the TCR repertoire in inflamed synovium and blood showed that only a small number of expanded clonotypes in the synovium could be identified in the blood (4%), in contrast to 34% of clonotypes being shared in multiple joints of the same patient [34]. Interestingly, there were neither indications of public TCRs among the expanded clones nor any significant variance in the VJ combination usage between synovial-resident and blood TCR sequences. Using immunosequencing, Bending et al. showed that the expanded clones in synovial fluid of juvenile idiopathic arthritis patients are predominately effector T cells and the clones are not commonly found in the synovial-resident FOXP3+ regulatory T cell population [35]. In addition, it was recently published that the synovial-resident Tregs in arthritis patients have an activated phenotype (CD45RA+FOXP3+) and TCR sequences from activated Tregs in the blood are shown to be shared with the synovial-resident Tregs in arthritis [36]. The authors conclude that the data depicted a
scenario in which synovial Tregs are activated but fail to control the T cell-driven inflammation. The authors hypothesize that normalizing the inflamed synovial microenvironment may recover Treg-driven suppression of the effector T cell response.

In patients with ulcerative colitis, a recent study revealed that the TCR repertoires in autologous sections of inflamed colon bore less TCR clonotypic overlap than did sections of non-inflamed colonic tissues [37]. And while it is understood that a higher proportion of CD4⁺ T cells residing in inflamed sections of colon express FOXP3 [38, 39], immunosequencing data shows that the TCR repertoires of the regulatory (CD4⁺FOXP3⁺) and effector (CD4⁺FOXP3⁻) populations are distinct, suggesting unique clonal lineages [37]. These are important discoveries, as the apparent increase in TCR diversity in inflamed colonic tissue is atypical of what we know in more traditional autoimmune disease(s), such as type-1 diabetes, wherein TCR repertoires from islet-infiltrating lymphocytes display limited TCR diversity [40]. Similarly, immunosequencing data from human patients with lupus and mouse models of autoimmune diabetes display significantly lower recombinatorial diversity compared to healthy controls [41, 42]. In the non-obese diabetic mouse (NOD) model of type-1 diabetes, auto-reactive T cells predominately express TCRs that are considerably restricted to very few TRBV chains (TRBV1, 13-3, and 19) [42, 43]. Targeted depletion of the TRBV13-3 (Vβ8.2) in NOD mice was able to protect mice from the onset of type-1 diabetes [42, 44]. These observations have significant value as not only a method to halt the progression of disease, but also as a possible diagnostic tool for the early detection of autoimmunity.

**Immune profiling in cancer and cancer therapy**

One of the largest issues in using the TCR repertoire as a tool for following disease progression in cancer is the lack of TCR sequence overlap between tumor-infiltrating lymphocytes and circulating PBMCs. Multiple groups have shown that the TCR repertoires present in tumors are distinct from the repertoires found in the blood [2, 45]. As an example, Hsu et al. (2016) reported that immunosequencing data from glioma-infiltrating T cells and matched PBMCs displayed less TCR repertoire overlap in the glioma tissue and circulating PBMCs when compared between low- (greater overlap) and high-grade glioma (less overlap) patients. The extent of TCR overlap between the tumor-infiltrating lymphocytes and PBMCs is indicative of disease progression and survival, with increased overlap equating to slowed disease progression and longer survival [46]. While immunosequencing the tumor-infiltrating lymphocytes via tumor biopsies is the most direct path for monitoring the T cell response to the tumor, it is highly invasive and not optimal for long-term patient monitoring. Ideally, identifying biomarkers in the circulating PBMCs would be the most non-invasive option for long-term monitoring. A recent publication by Sims et al. (2016) found that a “signature set” of commonly identified public TCRs present in the periphery of low-grade glioma patients is depleted in high-grade glioma patients [45]. By measuring for the presence of non-tumor specific TCRs rather than tumor-specific sequences in the blood of glioma patients, these early results suggest that it may be possible to monitor disease progression by immunosequencing of the PBMCs’ TCR repertoire.

While the issue of distinct TCR repertoires from tumor-infiltrating lymphocytes and blood is a major complication in most cancers, for disseminated or non-tumor forming cancers such as leukemia, immunosequencing is already being demonstrated as a powerful tool for identifying cancerous cells as well as for the detection and quantitation of leukemia-derived TCR sequences [4]. Clinical management of patients with leukemia relies on an accurate assessment of the possibility of relapse [47]. Proper monitoring is vital in determining the intensity of therapy. Currently, the most important predictor for risk-classification assessments in measuring residual leukemia levels is the “minimum residual disease” (MRD) [48, 49]. Classic techniques to monitor MRD in leukemia focused on detection of abnormal phenotypes by flow cytometry or measurement of a leukemia-associated immunoglobulin or TCR allele by PCR called “allele-specific oligonucleotide PCR”. These techniques are able to detect leukemic cells as rare as 1:10,000 and 1:100,000 respectively, and while they have proven reliable in the past, these methodologies have inherent restrictions, limiting their effectiveness. Flow cytometry is the least
sensitive and allele-specific PCR is laborious and time-consuming as reagents and conditions have to be developed for each individual patient [49-51]. Immunosequencing has been used to identify leukemic cells as rare or rarer than 1:1,000,000 cells, has been shown to be both more sensitive and specific than previous methods, and is able to more accurately predict potential relapse [4, 52, 53]. Immunosequencing of the Ig or TCR repertoires as a method of determining the MRD in cancer is currently being implemented in multiple disease models in addition to leukemia such as mantle cell lymphoma and multiple myeloma [54-59]. Immunotherapies targeting the adaptive immune response have been showing great progress in recent years. As an example, adoptive transfer of tumor antigen-specific T cells has been shown to be effective in delaying disease progression. Unfortunately complete regression is rare due to inhibited T cell activation and a short in vivo half-life [60, 61]. However, treatment with antibody blocking cytotoxic T lymphocyte (CTL)-associated 4 (CTLA4) has been shown to prevent the inhibitory signals that prevent T cell activation after TCR binding and has been shown to be effective in 22% of metastatic melanoma patients [62]. In a single case study, a combination therapy of the adoptive transfer of melanoma-specific T cells and anti-CTLA4 treatment achieved complete remission. Immunosequencing was used to monitor and track the melanoma-specific T cells which persisted at least 4 years after treatment [63]. Alternatively, other groups have had success by eliminating the need for TCR-MHC interaction all together. Use of immunosequencing to identify tumor-specific antibodies has led to the engineering of cancer targeting chimeric antigen receptors. Chimeric antigen receptors, or CARs, are engineered molecules composed of the T cell receptors’ transmembrane and intracellular signaling domains with an immunoglobulin binding region. T cells from cancer patients are being engineered to express cancer-specific CARs. CAR-expressing T cells are able to target cancer cells without MHC-interaction, preventing activation inhibition [64]. Currently, there has been success using CARs targeting the CD19 antigen in leukemia [65]. Additionally, immunosequencing allows for the determination of residual disease through monitoring the expansion or contraction of the engineered T cells [4, 52, 53]. Here we have described the roles

Immunosequencing is currently playing in both cancer research and immunotherapy. As the use of immunosequencing in cancer research continues to expand, we predict seeing its use in personalized-patient immunotherapies and as a method of monitoring patient health.

Future directions

Despite the progress that has been made thus far using immunosequencing, there are still many questions that remain unanswered. Currently, new immunosequencing technologies and applications are being developed. As mentioned previously, a new method for the simultaneous sequencing, and pairing, of both the TCRα and TCRβ chains is being developed [17]. The technology works by dividing the T cells from a population into multiple wells, with cells in each well receiving a unique bar code. After PCR amplification and sequencing, TCRα and TCRβ sequences that carry with them identical barcodes and are found paired together in multiple wells are determined to be paired. Howie et al. sequenced the TCRα and TCRβ sequences from PBMCs and tumor infiltrating lymphocytes and was able to correctly identify TCRαβ pairs with >99% accuracy. The accuracy of the method was confirmed by spiking samples with a subset of clonal TCRα and TCRβ sequences and measuring the method’s ability to identify biologically paired sequences [17]. Future applications of this technology could lead to rapid identification of antigen-specific TCR sequences, which would accelerate the development of antigen-specific T cell therapies like those currently being studied as potential cancer immunotherapies [63].

In vaccine design and antigen-specific targeted therapy, one of the greatest challenges is identifying the specific peptides or epitopes that are recognized by the immune system. Traditional methods such as multimer staining, Enzyme-Linked ImmunoSpot (ELISPOT), intracellular cytokine staining, and proliferation or activation assays have made great contributions to the understanding of the immune response. However, these methods can lack sensitivity, detecting only the most robust response, and require knowledge of the antigens recognized by the immune response [66]. Using a unique multiplex approach, advancements are being made in identifying pathogen-specific T cells and their cognate antigen
immunosequencing, the high-throughput sequencing of the Ig or TCR repertoires, is an emerging powerful tool capable of both accurate sequencing of millions of Ig or TCR CDR3 regions as well as measuring the frequency or abundance at which each clonotypes is found in a sample [13]. This technology is allowing researchers the ability to computationally identify both high-frequency and rare antigen-specific clonotypes [1]. Additionally, immunosequencing is currently being combined with traditional immune assays to provide a new level of sensitivity for identifying antigen-specific T cells in models of infection, autoimmunity, and cancer [16, 23, 69, 70]. With the sensitivity to identify TCRs rarer than 1:1,000,000 cells, immunosequencing is becoming the gold standard for minimal residual disease detection in patients with leukemia [4]. Finally, immunosequencing of the TCR repertoire can evaluate vaccine efficacy by measuring the induction of antigen-specific effector and memory T cells [1]. This technology has the potential to significantly accelerate vaccine development and further the development of future immunotherapies in both autoimmunity and cancer.

CONCLUSION

Immunosequencing, by using a combination of immune assays and receptor sequencing. By incubating pools of T cells with unique peptide libraries and purifying activated T cells, immunosequencing is being used to accurately determine antigen specificity in the reactive T cells [16, 66]. The potential of this technology is far-reaching. Using this technology, we believe it will become much easier to correctly identify both antigen-specific TCRs and immunogenic epitopes in tumors, emerging pathogens, and vaccines. Understanding which epitopes provide the strongest immunogenic response is of great value in vaccine development. Conversely, pooled immunogenic peptides from an array of pathogens could be used to develop diagnostic assays for determining individuals’ immunological history.

As immunosequencing technologies continue to grow, so does the need for high order analytics. The breadths of the TCR and Ig repertoires are vast and raw sequencing data becomes cumbersome to analyze using traditional tools [67]. The depth of the data sets gives rise to a higher degree of type-II error, requiring the incorporation of more advanced statistical analyses [1]. New methods and software for analyzing these large data sets continue to be developed as the technology becomes more widely used [68].

CONFLICT OF INTEREST STATEMENT

All individuals named as authors on this manuscript certify that this material has not been and will not be submitted for publication in any other publications. Additionally, no author has any affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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Immune profiling in immunology and medicine


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Immunology and medicine


