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REVIEW ARTICLE

New Technologies for Vaccine Development: Harnessing the Power of Human Immunology

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Abstract | Our understanding of human immune responses is increasing at an unprecedented scale. Cutting edge new technologies are allowing us to disregard a piecemeal approach and analyze human immune responses in a comprehensive and cohesive manner. Innovative approaches to analyze immune cell phenotype, genotype, function, and associated soluble factors have provided us with a new hope for making vaccines against diseases that have been historically impossible. This review recaps a few state-of-the-art tools and technologies that have helped us harness the power of human immunology for vaccine design, evaluation, and testing.

1 Introduction

Vaccines are the most successful, cost-effective tools to prevent or significantly reduce the incidence of disease and mortality.[1] However, many infections that have complex host–pathogen interactions are still not vaccine preventable and continue to cause high morbidity and mortality in both India and worldwide.

Since the advent of mankind, life-long immunity against communicable diseases was achieved empirically. One of the first historical written documentation of such empirically acquired immunity to infectious disease is recorded in the description of the plague that devastated the city–state of Athens, Greece in 430 bce, killing one-third of its population. However, it was observed at that time that individuals who survived the plague never suffered from it again, which indicated to the concept of life-long immunity after exposure.[2] This practice of prophylactically exposing healthy individuals to natural infections was continued until about the 1950s what was known as 'measles parties', where in healthy children are invited to the house, where a child has measles to increase probability of exposure.[3]

Even before Edward Jenner's landmark discovery of 'vaccination' with cowpox in 1796, the first reports of immunization for small pox date are as far back as the tenth century China.[4] Pustules from individuals with mild small pox were dried and nasally administered to uninfected individuals or inoculated into the skin through variolation. This was also likely customary in Africa, India and Central Asia long before it was introduced in Europe from Istanbul in 1714 by Emanuel Timoni and Giacomo Pilarino through separate but simultaneous letters to the Royal Society of London.[5] The earliest reports of variolation of an individual of European decent comes from Lady Montague who decided to inoculate her 5-year-old son in 1718 in Istanbul, followed by her 4-year-old daughter upon her return to London in 1721.[5]

However, this process of variolation not only caused very severe disease manifestations, but also resulted in Death of some individuals. Thus, Jenner's revolutionary observation that milkmaids that handled cows with similar small pox like lesions did not suffer from the perils of the deadly disease, laid the foundation for the use of an attenuated pathogens to provide long-term protective immunity. Therefore, this hallmark experiment is considered to be the official birth of vaccines and vaccinations, which lead to the ultimate global eradication of the deadly small pox in 1980.[6]

Through the years, Louis Pasteur who developed the first rabies vaccine in 1885[7] then refined Edward Jenner's work that resulted in the first generation of vaccines, which were based on, attenuated or killed pathogens (e.g., BCG; plague; pertussis, etc.). These innovations were then

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closely followed by the use of live attenuated strains to make vaccines such as the Sabin oral polio, Hepatitis A and MMR and are categorized as the second generation of vaccines. The discovery and use of these traditional vaccines that are listed in Table 1 have saved millions of lives and decreased morbidity over the last century (http://www.who.int/immunization/diseases/en/).

Unfortunately, these traditional methods have proven ineffective for pathogens that have complex host–parasite interactions (dengue/chikungunya/influenza), establish chronic infection (TB) or pathogens that cannot be cultured in vitro such as *Mycobacterium leprae*. Moreover, increasing number of host-related issues such as having an immature immune system such as during infancy,[8] aging,[9] non-communicable diseases that affect immune responses such as diabetes,[10], etc. further add to challenges in vaccine design and development. A few of them are listed in Table 2 that allow us to appreciate the hurdles that need to be overcome for any new vaccine.

Moreover, most successful vaccines to date heavily rely on the generating a high titer antibody response. With progress in vaccine research, it is becoming more evident, that an antibody-based vaccine alone cannot overcome diseases that fall into the spectrum of having complex host–parasite interphases. In fact, going back to some of the most successful vaccines and comprehensively analyzing both arms of the adaptive immune response (humoral and cell mediated), we have come to realize that though empirically, only antibody titers were analyzed, there is a strong T-cell response to the vaccine that heavily contribute the life-long protective immunity.[11], [12] Therefore, it is evident that we cannot make protective vaccines against these challenging diseases if we do not engage the entire immune response as a whole.

For the most part, most of our understanding of the formation of immunological memory comes from model antigens and animal models.[13] This is an reasonable approach, and has worked for filling gaps in our knowledge of the cellular and molecular basis of many infections; but in diseases that do not have reliable animal models (such as dengue) or have complex host–pathogen interphases and the immune response is highly multifaceted, the use of animal models with model antigens is rarely predictive of human vaccine responses. Thus, the biggest challenge that we face today is that we do not holistically understand what the immune response in human's looks like during a natural infection or when a successful vaccine is administered. Therefore, there is a tremendous need to develop cutting edge approaches that help us better understand how the immune system functions during an infection to make better vaccines for improving prevention and control.

Understanding several characteristics of human immunology in infected individuals and or vaccines that will ultimately improve the health and well-being of human society in the future. Some of these aspects are molecular signatures of innate immune responses; B-cell responses, antibody secreting cell response; CD4 and CD8 T-cell responses and define correlates of protective immunity in cases of natural infections and successful vaccines. Reproducing these positive characteristics that ultimately result in a successful and protective immune response will allow us to better test and evaluate novel vaccines tested in pre-clinical and clinical studies (Fig. 1).

Table 1: Available vaccines.

Small pox	Mumps
Rabies	Pertussis
Diphtheria	Pneumococcal disease
Hepatitis A	Poliomyelitis
Hepatitis B	Rotavirus
Hepatitis E	Rubella
Haemophilus influenza	Tetanus
Human papillomavirus	Tick-borne encephalitis
Japanese encephalitis	Typhoid
Measles	Yellow Fever
Meningococcal meningitis	Tuberculosis
Malaria*	Influenza
Dengue*	*Not used globally

Table 2: Hurdles of the complex host–pathogen interphase.

Pathogen-related	Host-related
Immune evasion	Immature immune system
Intracellular life cycle	Elderly
Complex life cycle	Immuno-compromised
Multiple serotypes	Non-communicable/life-style diseases
Mutability	Other diseases that affect immune responses
Latency	Immune pathology
Cannot be cultured in vitro	Chronic infections

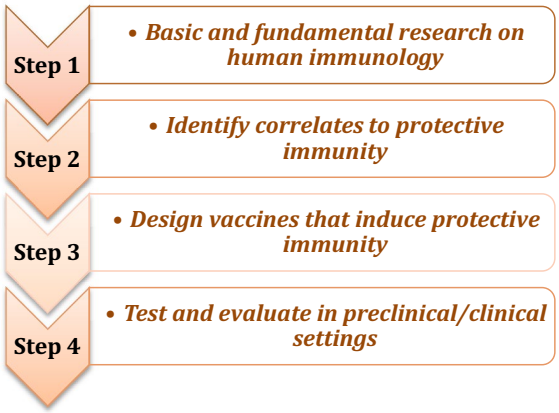

Figure 1: Pathway to the development of a successful vaccine.

Until recently, we could only take a piecemeal approach to understand human immune responses to infections and vaccines. However, recent advances in innovative technologies in human immunology have allowed us to fill some of the gaps in our understanding of the innate and adaptive immune system allowing us to have a more rational approach towards vaccine design, testing, and evaluation. Now, multi-level high-throughput analysis can be performed at both a bulk and a single-cell level to comprehensively profile human immune responses. The advances in multi-parametric flow cytometry have allowed us better quantitate immune phenotype and function of a cell at a single-cell level; advances in systems biology have allowed us to holistically review the entire immune system in its totality in many cell types at a time or even at a single-cell level; advances in next-gen sequencing have permitted us to analyze B- and T-cell receptor repertoires to a single-cell level. Therefore, in the past decade, considerable strides have been made to identify correlates and molecular signatures that predict long-lived memory responses. This information has been very crucial to fill our gaps in understanding of what it takes to make a successful vaccine and helps us in future rational vaccine design, testing, and evaluation.

This review will discuss key technological advances in the light of human immunology that will ultimately allow for rational vaccine designs.

2 Understanding the Complex Human Immune Response: New Avenues for Vaccine Development

The challenge for modern vaccines, especially the ones with complicated host–pathogen interactions, is to elicit the multiple steps necessary for immune activation that will make long-lived protective immune memory. A successful immune response that results in long-lived immunological memory that is protective towards re-challenge from the infecting pathogen begins from the first recognition of the presence of a non-self substance by the innate immune response by a process called pattern recognition. These pattern recognition innate systems recognize and respond to invasion by a various infectious agents by being strategically located at distinct sites within the tissues, cellular spaces, and sub-cellular compartments. Pattern recognition receptors (PPRs) present on innate immune cells such as monocytes, macrophages, neutrophils, and dendritic cells then recognize 'pathogen associated molecular patterns' (PAMPs) that could be varied from cell wall components, lipoproteins, lipopolysaccharides, DNA and RNA of bacteria, viruses, protozoan, and fungi.[14–16] The receptor–ligand interactions activate different signaling cascades and elicit downstream immune responses. These downstream signals include chemokines, cytokines, and activation of complement and recruitment of other immune cells, all of which result in local inflammation.[17] The activated innate immune cells carrying the antigen then migrate to local secondary lymphoid tissue to initiate the adaptive immune response. The manner in which the innate cells induce the adaptive immune response has profound implications on the induction of immunological memory.[18]

The formation of immunological memory occurs largely by T- and B-cell responses. Once B cell recognizes a specific antigen, they differentiate into short-lived plasma cells or plasmablasts and secrete antigen-specific antibodies.[19] Some of these B cells that enter the follicles and get CD4

T cell help from the T follicular helpers, become antibody secreting cells that produce high affinity class switched antibodies and also form a pool of memory B cells.[20] On the other hand, both CD4 and CD8 T cells recognize antigens that are processed and presented to them by antigen presenting cells.[21] Upon antigen encounter, CD4 T cells can differentiate into different populations and are categorized based on the their functions into Th1, Th2, Th17, TFH, etc.[22] and CD8 T cells differentiate into cytotoxic cells.[23], [24] CD4 T cell help also vital for survival and functional responsiveness of long-lived CD8 T cells.[25], [26] Depending on the orchestration of multiple positive events results in the formation of long-lived immunological memory, whose hallmark is the ability to be rapidly activated upon re-challenge.[27], [28]

For these complex, multi-level pathways to be optimally triggered that ultimately result in long-lived immunological memory responses requires the initiation of an optimal innate immune response that directs the adaptive immune response towards efficient activation in an antigen-specific manner.[29]–[31] This critical knowledge has allowed us to make huge strides in tailoring vaccines. For example, purified polysaccharide vaccines are poorly immunogenic and do not induce a robust T-cell response.[32], [33] The addition of a protein carrier to such polysaccharide molecules resulted in the activation of innate cells that could then present the antigen to T cells allowing for the development of long-term B- and T-cell immunity. This was only possible because of the knowledge of the workings of an optimal immune response. Such protein-conjugate vaccines have revolutionized our fight against childhood bacterial infections such as *Haemophilus influenza*, *Neisseria meningitides,* and *Streptococcus pneumoniae*.[34]

3 Generating an Optimal Innate Immune Response: In Search of Adjuvants in Vaccine Development

The knowledge of how vital innate immune responses are for a robust adaptive immune response and formation of immunological memory, the role of adjuvant in vaccinology is extremely sought-after. The word 'adjuvant' means 'to help/aid', because it was originally thought that adjuvants improve vaccine efficiency by prolonging antigen exposure and delivery to the immune system. However, it is now recognized that most efficient adjuvants activate the innate immune pathways either directly or indirectly by inducing other cellular components and pattern recognition that further activate innate cells.[35] Therefore, there is a huge push for innovative 'immune potentiators' or adjuvants that can influence the type, magnitude, and quality of the downstream adaptive T- and B-cell responses. The most important functional criteria are required for a successful adjuvant is overcoming poor immunogenicity, increasing breadth and durability to enhance the outcome of certain vaccines such as subunit vaccines that are weakly immunogenic by themselves as they are designed to include only antigens required for protective immunity and do not have any self-adjuvanting properties that would otherwise be present in whole molecular attenuated or killed vaccines.[36], [37] Due to these features, it also allows for reducing the total antigen quantity that goes into a preparation thus increasing the number of vaccine doses that can be achieved from a finite amount of antigen. Moreover, because they also enhance the innate immune responses by providing the much-need 'signal 3', Signal 3 are cytokines such as IL-12 or type I IFNs that support the expansion and differentiation of T cells. Therefore, in addition to Signal 1 (TCR engagement) and Signal 2 (co-stimulation), an inflammatory stimulus which is the third signal greatly improves the functional quality of an immune response.[38–40] Therefore, incorporation of adjuvants improves immune responses in individuals such as the elderly and infants who have reduced responses to some vaccines.[41] Another favorable aspect of adjuvants is that their effects are short-lived and are not systemic and typically limited to the site of injection and regional lymph nodes.[42]

Aluminum (Alum) was the first adjuvant that was licensed for human use in 1932 and was the only adjuvant in use for almost 70 years. It was extremely successful for vaccines that were protective due to a large antibody response, but was ineffective in situations, where T-cell responses were needed.[43] This was specially true in case of the RTS,S malaria vaccine, where in alum failed as an adjuvant in GSK's pre-clinical and human challenge studies in the 1980s.[44] It was then that 'immune stimulating complexes' (ISCOM) were combined with the RTS,S antigen. ISCOMs are essentially liposome-based adjuvants. wherein the antigen is mixed with saponin, cholesterol, and phospholipid.[45], [46] AS01 is one such liposome adjuvant that has demonstrated increased RTS,S vaccine efficacy in Phase III trials in vaccinated children.[47], [48]

The first vaccine to be licensed for human use that contained an adjuvant that was not alum but an ISCOM was the hepatitis A vaccine in the

mid 1990s.[49] Since then many adjuvants have been included in newer vaccine formulations and enhance both an antibody and T-cell response.[50] The hepatitis B vaccine that now uses AS04 as an adjuvant is a classic example of how adjuvants enhance immune responses in individuals that are immunologically compromised, such as those with end-stage renal disease.[51]

There are several challenges that need to be overcome to find improved adjuvants and formulations. Apart from the understanding of mode of action, we need to ensure that there are no unacceptable side effects, toxicity, and are safe for use in pediatric vaccines.[52] MF59 is one such oil-in-water emulsion that is now licensed in more than 20 countries.[53] After a safety and immunogenicity study in MF59 adjuvanted influenza vaccine in children, it has also been cleared for pediatric vaccine use. Monophosphoryl lipid A (MPL) is a non-toxic derivative of LPS of Salmonella and is a potent inducer of Th1 responses and is now included in the AS04-adjuvanted HPV vaccine and will be the first TLR agonist licensed for human use.[54] Other adjuvants that stimulate the toll-like receptor pathway using TLR 3, 5, and 9 are currently at different phases of clinical trials. Unmethylated CpG that is a TLR9 agonist enhances antigen-specific CD4 and cytotoxic CD8 T-cell responses and the production of pro-inflammatory cytokines.[55] HEPLISAV-B by Dynavax is Hepatitis B vaccine candidate that is currently in Phase 3 clinical trials.[56] Poly I:C binds to TLR 3 and is a potent inducer of Type I interferons which are imperative for a successful adaptive immune response. Unfortunately, Poly I:C by itself suffered from issues of instability and toxicity. Thus, discovery of its analogue Ampligen was timely and has now been considered as an adjuvant for H5N1 intranasal vaccinations.[57] Another TLR agonist that is in clinical trials for Influenza vaccine is a flagellin-based TLR5 agonist.[58]

We have come a long way, since the approval of Alum as an adjuvant in human vaccines. With better tools and technologies available to dissect the human immune response, following candidate adjuvant administration has allowed us to identify novel combination of adjuvants and formulations that are capable for inducing strong long lasting humoral and cellular immune responses in humans.

Table 3 provides some of the adjuvants currently being tested or are licensed.

There is a constant quest for discovering new adjuvants and this research is happening in parallel to antigen discovery in the field of vaccine development. However, even here, the biggest hurdle has been the gaps in our knowledge on how the immune system responds to adjuvants. The classic example is of alum that has been in use for decades and we still have very little understanding of its mode of action until recently, where in it was demonstrated that Alum introduces host DNA into the cytoplasm of antigen-bearing dendritic cells that results in increased

Table 3: Adujvants, mode of action and status.

Adjuvant	Description	Phase	Refs.
Aluminum	Aluminum salts that increase local inflammation	In use for DPT, IPV, hepatitis A & B, HPV, meningococcal and pneumococcal vaccines	59
Virosomes	Lipid vesicles that increase uptake by APCs	In use for hepatitis and influenza	60
MF59	Squalene. Increases APC recruitment, activation and increases antigen uptake	Seasonal and pandemic influenza	61
CpG	TLR 9 agonist, enhances antibody titer, TH1 and CD8 responses	Phase 3, hepatitis B	56
Poly I:C	TLR 3 agonist, enhances antibody titer, TH1 and CD8 responses	Phase I, cancer immuno-therapies	62
Flagellin	TLR5 agonist, enhances antibody titer, TH1 and CD8 responses	Phase I, cancer immuno-therapies	58
AS01	Monophosphoryl lipid A	Phase 3, RTS,S	63
AS02	Squalene	Phase 3, pneumococcal vaccine	64
AS03	Alpha-tocopherol, Squalene and surfactant polysorbate 80 promotes local production of cytokines	Pandemic influenza	65
AS04	3-deacyl-monophosphoryl lipid A derived from LPS of Salmonella Minnesota. Stimulates TLR4	In use for hepatitis B and HPV	66

MHC Class II expression and thereby antigen presentation.[67]

Since an optimal immune response not only requires cellular factors but requires multiple soluble signals in the form of cytokines and chemokines. Based on the type of cytokine or chemokine, secreted results in target cell activation, proliferation, differentiation, migration, and mounting antigen-specific responses. Therefore, using new technologies such as xMAP luminex that allow us to analyze almost 500 unique markers has given us predictive value of the type of immune response that will be generated in presence of a particular adjuvant.[68]

As advances are made in human immunology, we hope that we will have a deeper understanding of the mode of action of adjuvants on the innate and adaptive immune response and fast-track towards a rationale vaccine design for diseases such as dengue, HIV, etc.

Cutting edge human immunology research on successful vaccines such as the yellow fever vaccine have allowed us to get a glimpse into what the appropriate quality of a successful immune response looks like.[11, 12, 69] Moreover, progress in genomics has allowed us to process large amounts of data in a high-throughput manner from human blood samples and use sophisticated systems biology approaches to identify molecular signatures and immunological correlates of protection.[70] Refinement of technologies such as flow cytometry and addition of new dimensions to classic flow-cytometry analysis has opened our avenues to perform improved analysis on human immune responses and are discussed in the sections below.

4 The Power of Multi-parametric Flow Cytometry for Human Immunophenotyping: Implications for Vaccine Design

One of the most successful technologies that revolutionized the analysis of human immune responses is multi-color and multi-dimensional flow cytometry. This innovative technique uses antigen-specific antibodies typically coupled to a fluorochrome to detect expression of surface markers and intracellular proteins. One of the major advantages of this technique is that it is high-throughput and allows for the analysis of approximately 10,000 cells/s to sorting individual cells to analyze at a single-cell level. The number of parameters that this technology can accommodate depends on the number of lasers and filters that the machine used can incorporate, for e.g., a machine with 4 lasers and 5 filters per laser can determine up to 20 parameters simultaneously. Flow cytometry is the mainstay of vaccine research and development because it has for the first time allowed us to monitor multiple phenotypic and functional changes during complex immune responses.

Because we now know that heavy reliance on generation of an antibody-based protective immunity may not always be successful, there is a renewed interest in understanding T-cell responses that can be channeled in a vaccine design. Flow cytometry allows us to do this. Studies with yellow fever vaccine is classic example due to which we now a lot more about CD8 T-cell responses in successful vaccines and gives us immune correlates of protection.[11, 12]

Intracellular cytokine assays by flow cytometry have permitted us to study the ability of immune cells to make multiple cytokines ex vivo in response to a vaccine challenge. Due to this powerful technique, researchers could find prospective evidence of correlates to protection in CD4 T-cell responses for an intracellular parasite *Leishmania major*.[71]

Another important aspect of effector CD8 T cells is that they are cytotoxic and have the ability to kill infected cells. To do this, CD8 T cells degranulate which can be studied by CD107a (LAMP1) expression by flow cytometry. This protein is normally expressed in the internal granular membranes and is transiently expressed on the cell surface when CD8 T cells undergo degranulation and the granule membrane merges with the cell membrane. Addition of the anti-CD107a fluorescently labeled antibody to the culture at the time of stimulation allows us to gauge cytotoxic CD8 T-cell responses by flow cytometry.[72] Simultaneous measurement of other cytotoxic molecules ex vivo such as Perforin and Granzymes allows researchers to gauge the level of cytotoxic T-cell responses after natural infections and vaccinations more accurately.[73]

In addition, when naïve cells encounter cognate antigen during vaccination or natural infections, they undergo massive proliferation to form effector cells. Flow cytometry has transformed our ability to study this proliferation trait. Dyes such as carboxyfluorescein succinimidyl ester (CFSE), PKH-26 or DNA labeling by bromodeoxyuridine (BrdU) that can be analyzed on a flow cytometer have completed eliminated our need to use radioactive thymidine.[74] Combining lymphoproliferation with phenotype and functional assays has given us the power to analyze the evolution of an immune response in context of time.

Cytotoxicity can also be measured by in vitro killing assays that use effector CD8 T cells co-cultured with peptide-labeled target cells. Originally developed as an radioactivity based assay, where in the release of radioactive chromium from dying target cells was measured.[75] However, these assays were not very sensitive and more importantly the quality of the T cells could not be assessed. Due to flow cytometry, it is now possible and safer to use fixable live dead stains or CFSE labeled target cells whose gain or loss respectively can be measured along with qualitative measurement of the effector cells.[76] Correspondingly, other functional assays such as activation of caspases,[77] TCR signaling[78] and phosphorylation of proteins[79] can be analyzed reproducibly with a flow cytometer. Similarly, other important correlates of protection such as 'antibody dependent cell mediated cytotoxicity' (ADCC) or activation of complement pathway can also be tailored for flow-cytometry analysis.[80] This has been used extensively to analyze the therapeutic potential of anti-cancer antibodies through their ability to initiate ADCC or cell mediated lympholysis (CML).[81] Researchers have also analyzed the role of granulocyte phagocytosis in pneumococcus vaccinations as a correlate of protection using flow cytometry.[82, 83] Finally, for analysis of T-cell functionality, intracellular cytokine assays by flow cytometry are a goldmine that have given us the ability to analyze immune cells making multiple cytokines ex vivo in response to a vaccine challenge or infection.[84, 85]

Thus, multi-parametric flow cytometry has revolutionized vaccine research because it has provided us with the unique ability to analyze different parameters such as proliferation, phenotype, cytokine production, intracellular protein expression, phosphorylation, etc. in a high-throughput manner but continuing to retain the ability of single-cell analysis. This technology has single-handedly revolutionized design, testing and evaluation of candidate vaccines.

Almost all successful vaccines induce a potent and long-lived serological response.[86] Recently, a population of antibody secreting B cells were identified that transiently appears the blood of subjects that are infected or vaccinated. These plasmablasts, are mostly antigen-specific and make antibodies only to the current infection or vaccination.[69, 87, 88] Human immune phenotyping allowed for identification of these cells by their expression of unique surface markers. Studies performed with yellow fever virus vaccine[69]; influenza vaccine[87, 89]; tetanus vaccine[90] and dengue infections[91] all identified these cells appearing during the acute phase of the antigen-specific immune response. Therefore, this transient nature of the appearance of plasmablasts in the periphery is now a key parameter that can be used to gauge the responsiveness of subjects to any vaccine. In addition, apart from analysis of serum antibody titers and neutralizing ability of the antibodies, responsiveness to new vaccines can be judged based on burst of the plasmablast response and their functionality and

Figure 2: Flow-cytometry-based technologies are at the center stage of vaccine research and discovery.

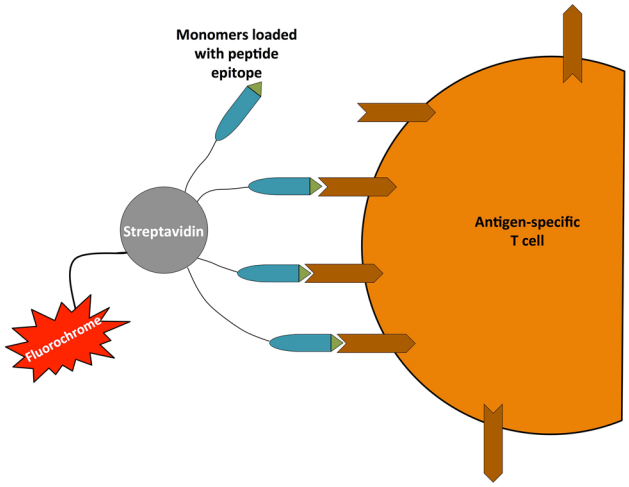

Figure 3: MHC tetramer technology allows us to accurately detect and track antigen-specific T cells.

holds predictive value during vaccine testing and evaluation.

Because flow cytometry is restricted to the number of fluorochromes, lasers and filter combination another advanced technique that has made multiple measurements of complex immune responses feasible is cytometry by time-of-flight (CyTOF). This innovative technique uses advantages of flow cytometry to perform single-cell analysis and combines it with mass cytometry.[68, 92] This is achieved by not using fluorochrome labeled antibodies but heavy metal tags allowing 45–50 measurements at a time with very little spectral overlap. Therefore, using CyTOF multi-parametric vaccine responses can be measured to characterize phenotype, function, antigen-specificity, signaling, phosphorylation, proliferation, cell cycle phases, and numbers in a single stain which is invaluable to vaccine research where in multi-dimensional analysis is needed to identify novel cellular functions that mark correlates of protective immunity[93] (Fig. 2).

5 Finding Needles in a Haystack: Tetramer Technology

After initial encounter with antigen and formation of effector cells, only a very small number of immune cells survive and form memory cells that typically homes to the bone marrow[28, 94]. To find those small frequencies of memory cells that may be circulating in the peripheral blood after vaccination or a natural infection requires very sensitive and high-throughput methods. One of the cutting edge technologies that has facilitated this analysis is the ability to stain and track antigen-specific T cells that are responding to a particular peptide/antigen of interest. This innovation is the MHC tetramer technology that was first pioneered in 1996 when soluble MHC–peptide complexes were made that were fluorescently tagged and thus could be detected by a flow cytometer[95]. Since the development of this technology, researchers have had the power to identify, visualize, enumerate, phenotype and isolate antigen-specific T cells[96–100]. The technology exploits the presence of alpha–beta T-cell receptors on the surface of T cells that are specific to the molecular complex of a particular MHC bound peptide. Biotinylation followed by conjugation to a fluorescent streptavidin allows the MHC–peptide monomers to get tetramerized that increases specific binding and decreases internalization of the TCR[101] (Fig. 3). Tetramer labeled cells can be analyzed for further phenotypic and functional characteristics because the staining can be combined with both surface and intracellular staining protocols. Therefore, the use of both MHC Class I and II tetramers has allowed researchers to monitor antigen-specific T cells generated during vaccine responses and in natural infections. Most importantly, because tetramer staining relies on the MHC–peptide–TCR complex, it is very specific and thus few numbers of memory cells can also be enumerated months after vaccinations or natural infections.[11, 12] The key ingredients required for tetramers is the knowledge of the antigenic regions or peptides that the T cells may be responding to in context of its HLA. This is typically done through a process called epitope mapping. Recently, using biotin conjugated–tetramerized proteins have allowed for the

detection of small numbers of circulating memory B cells after tetanus vaccination.[102]

The key to making good memory cells after vaccination is to identify the antigenic regions that result in a strong effector and memory response. In case the immuno-dominant epitope is hidden in the natural conformation of the protein, using whole protein in vaccine formulations result in sub-optimal immunity. Therefore, for an intelligent vaccine design, it is important to identify and incorporate the immuno-dominant regions in a candidate antigen. Identification of regions or epitopes that generate maximal immune responses has been possible by the process of epitope mapping.

6 Immuno-dominant Epitope Discovery by High-Throughput Mapping

Identification immuno-dominant epitopes is required not only to identify antigen-specific cells through tetramer technology, but more importantly to ensure that these epitopes are accessible in the candidate vaccine for an optimal immune response. Thus, mapping of immuno-dominant epitopes is crucial for T-cell-based vaccine development. This process was historically challenging because one needed to identify regions of T-cell reactivity across entire pathogen proteomes. However, with improved tool and technologies to sequence entire genomes it has become a lot more feasible to generate synthetic overlapping peptides that span the entire proteome or candidate protein(s) of a pathogen. Utilizing these in the form of peptide matrixes such that two pools contain only a single peptide in common made this method more high-throughput.[103, 104] Such overlapping peptide screens have been used for both T cell and antibody mapping and thus allow dissection of both B- and T-cell responses in infected subjects or vaccinees and thus allow for predicting what sequences must be present in the candidate vaccine to ensure a robust T-cell response.[105, 106] Similar approaches can also be used to identify linear epitopes that induce humoral or antibody responses. In fact, screening of human sera samples, or purified antibodies has been made extremely rapid and high-throughput by modern methods that allow us to globally profile antibody responses. These systems use highly purified whole protein or peptide blotted on microarray chips and represents either the entire pathogen proteome or certain sections of it.[107–110] The diversity and breakdown of the antibody responses can then rapidly measured to select for antigens or proteins that have the maximum immune responses and can be further explored as vaccine candidates.

7 Antibody and T-Cell Repertoire Analysis for Vaccine Testing and Evaluation

The ultimate goal of vaccines is to generate an adaptive immune response capable of detecting and rapidly eliminating the pathogen upon encounter. To achieve this, both T- and B-cell responses must be generated in diverse configurations that allow for the detection of vast number of antigens. This is called the repertoire of the immune response. Another cutting edge new technology that has deeply impacted vaccine design and research is nucleic acid sequencing. Recent advances made that allow for high-throughput next generation sequencing of T- and B-cell repertoires have allowed us to analyze the diversity, clonal expansion, immune variability of both B and T cells in context of a vaccine responses.[111–113] A recent development that has extended NGSs usefulness in vaccine responses further is the ability to sequence single responding T- and B-cell ex vivo.[114] Individual cells are sorted from human blood such that every single cell is immediately lysed and the TCR or Ig chains are amplified. After amplification all products from the single cell are ligated to a 'barcode' that allows all products from the same cell to be re-grouped informatically at a later stage and thus complete information of heavy and light chains from a single antibody secreting cell or plasmablast or alpha/beta TCRs of effector T cells can be reconstructed.[115] Thus, NGS is becoming a vital technology in vaccine research and analysis with the ultimate goal to identify signals, factors or pathways that result in a protective immune response with the idea to potentially specifically enhance those protective responses during a vaccination regime.[105, 116]

8 Antibody Therapies: An Alternative to Traditional Vaccination Approaches?

There are instances especially in cases of sudden outbreaks in naïve unexposed populations that administration of vaccines may be too little and too late to help the population. In such cases, and in absence of drugs that can help infected individuals, therapeutic antibody treatments are a promising route. Moreover, though antigen discovery that induces broadly neutralizing antibodies has been the goal of almost all vaccine discovery efforts, it is not always possible to reconfigure the epitopes to which the most neutralizing antibody

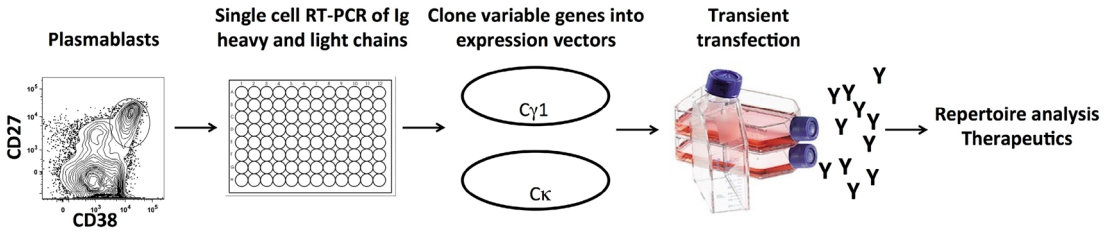

Figure 4: High-throughput production of human monoclonal antibodies.

responses are directly. This is specifically true in cases if the neutralizing antibody response is directed towards a conformational epitope that requires the native antigen to be in a 3D confirmation that cannot be achieved in a recombinant vaccine. It also becomes challenging in case the target pathogen has multiple serotypes/genotypes that have to be incorporated into the vaccine to generate the optimal response. In such cases, it is judicious to reverse the approach and generate a class of broadly neutralizing by isolating the plasmablasts that are making the antibody of interest. Such passive immunization techniques can be traced back to the early twentieth century where in sera from infected and recovered humans was used to provide protective immunity to naïve-uninfected subjects.[117] This serum therapy was gradually replaced by the use of antibodies purified from pooled sera for intravenous immune globulin (IVIG) and has had a major impact in the treatment of many immunological, neurological, dermatological illnesses, etc.[118] This method soon took a back seat in 1975 when a method to generate large quantities of murine monoclonal antibodies was discovered.[119] However, due to the lack of myeloma fusion partners that could be used for human B cells, the use of this technique for immune prophylaxis was minimal. Several methods were then discovered and one of the most successful ones were to use phage display libraries, where VH and VL variable regions from immune, vaccinated, or naturally infected and recovered individuals were cloned.[120]–[122] However, it was because the heavy and light chain pairing was random and did not reflect the true antibody repertoire that was generated in vivo the use of these antibodies therapeutically was minimal.

The game changer technology that was first reported in 2009 was making human monoclonal antibodies directly from circulating antibody secreting cells or memory B-cell ex vivo. This was rapid and high-throughput where in heavy and light chains were cloned from the same single plasmablast cell sorted ex vivo and produced in a standard 293T culture system. The antibody secreting cells were identified by surface marker expression or antigen bait, and then sorted into single cells using a flow cytometer. The heavy and light chains of the Fab fragment were then cloned from the same cell to generate clones of the antibodies that were being originally produced in vivo by that plasmablast.[123], [124] This method is highly throughput, since the cells are sorted in 96 well plates and can result in hundreds of potential antibodies in a short duration that can be then tested for their functional ability (Fig. 4).

Moreover, since these antibodies are completely human they are potentially safer to be used as immuno-therapies of prophylaxis against infections. Lastly, since the technology is also high-throughput, monoclonal can be made in a very short duration of time and thus potentially be available to treat or prevent infections with the period of an epidemic with a novel pathogen.

Many other novel technologies are currently under investigation in the field of vaccine research and development. Advances in the state-of-the-art techniques such as X-ray crystallography, Nuclear Magnetic Resonance (NMR) and cyro electron microscopy that won the nobel prize for chemistry this year, have paved the way for structural vaccinology.[3], [125], [126] These cutting edge techniques allow a deeper characterization of antigenic regions and neutralizing epitopes of pathogens by crystal structures is a revolutionary technique that allows rationale and intelligent design of vaccines for pathogens that are inherently difficult to manipulate by classical biochemical methods.

Therefore, the combination of human immunology, structural biology and bioinformatics is allowing us to make major strides in filling the gaps in our understanding of immune responses to natural infections and vaccines. These powerful techniques are now providing us with strategies for rationale vaccine design that generate broad and effective immunity to pathogens that have historically proven to be challenging. This multi-pronged approach to understand the complexities

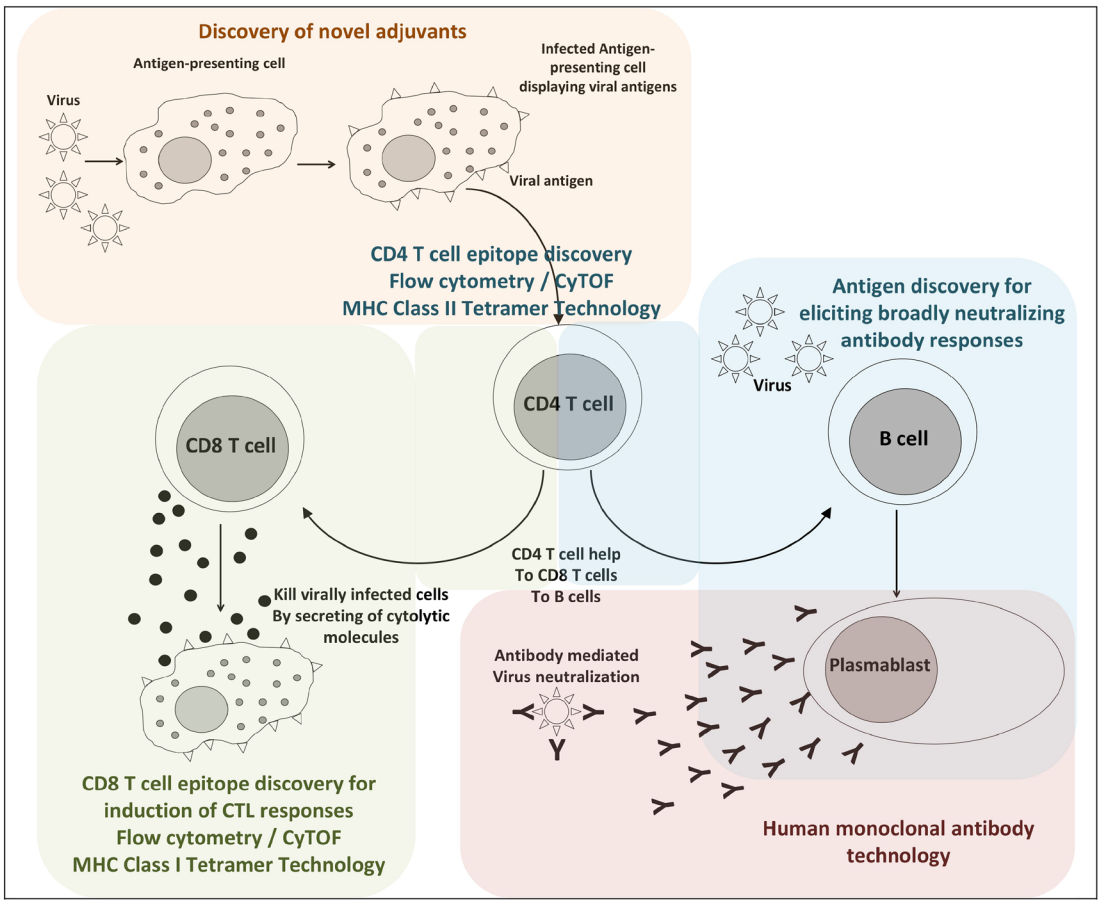

Figure 5: Different arms of the immune system targeted in vaccine discovery.

of the immune system will eventually allow us to design vaccines that will contribute to a significantly long and disease free life.

9 Conclusions

Our understanding of human immune responses is increasing at an unprecedented scale. Cutting edge new technologies are allowing us to disregard a piecemeal approach and analyze human immune responses in a comprehensive and cohesive manner. Innovative approaches to analyze immune cell phenotype, genotype, function, and associated soluble factors have provided us with a new hope for making vaccines against diseases that have been historically impossible.

Analysis of the complexity of the immune response using multi-parametric innovative approaches is the most likely be key to in discovery of novel vaccines and therapeutics. The advent of these new technologies has allowed for advances in human immunology and development of vaccines that would have not been possible otherwise. Bringing together all the knowledge that we gain from different angles of the human immune response, we can hope to make novel and improved vaccines and therapies for diseases such as HIV, Dengue, Chikungunya that are urgently needed for improvement of health and well-being of society (Fig. 5).

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