In vitro vs. *in vivo*: Is it time to reduce dependence on the latter?

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Abstract

Biological 'wet lab' research can broadly be classified into *in vivo*, *ex vivo* and *in vitro* categories. So far, *in vivo* experimentation involving animals has been essential before a vaccine or drug candidate could be moved to a clinical trial phase but because of the cost, time, resource and ethical concerns involved with those *in vivo* models, not to mention the chance that clinical trials do not always have the same success rate has made *in vivo* experiments cumbersome. Hence, there is an urgent need to look towards *in vitro* models and measure their reliability.

The research questions explored in this thesis are : (1*) to what extent can in vitro studies substitute in vivo studies* and, (2) *can data from in vitro studies be credible enough to reduce the dependence on in vivo studies?*

The two main *in vitro* models that are in focus are the peripheral blood mononuclear cell (PBMC) model and the modular immune *in vitro* construct (MIMIC) system. The PBMC model involves separating PBMCs from whole blood via density gradient centrifugation or leukapheresis, subsequently stimulating those cells with a vaccine, adjuvant or drug candidate of interest and performing functional assays on them. The MIMIC system is divided into four parts that can broadly be explained as the collection of white blood cells, the Peripheral Tissue Equivalent (PTE) module that mimics innate immune response, the Lymphoid Tissue Equivalent (LTE) module that mimics adaptive immune response and functional assays to gauge the effectiveness of that response.

Past and current literature points out that *in vitro* data obtained from research on diseases like influenza and tetanus is already in line with what *in vivo* data claims about the same, thus establishing its credibility. Hence, researchers can use the aforementioned models and the data they are generating to reduce the dependency on *in vivo* animal models. Unfortunately, for research on malaria, that is not the case yet and more investigation needs to be done before the same can be said. It holds promise, nonetheless.

The most optimal way to move forward in this direction of research is to optimise both *in vivo* and *in vitro* models and make the two work in synergy because at the present juncture of research, neither can do without the other. Further refining of *in vivo* animal models would ensure they consume less time, money, and resources and deliver a better representation of human clinical trials and the same for *in vitro* models would ensure the generation of credible data that will help ease the current heavy dependence on *in vivo* animal models.

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Introduction

The past two years have been a glaring proof to academia and the world that vaccines and their study are the cornerstones of public health and crucial at this juncture of human history. To gain a deeper understanding of the proceedings in the domain of vaccinology, extensive research is required before an idea and/or product can be applied commercially. In this vast field, experiments can broadly be classified into three subtypes: *in vivo*, *ex vivo* and *in vitro*. *In vivo* (*within the living* in Latin) refers to experiments on host organisms or cells in the same biological context as found in nature. *Ex vivo* (*out of living* in Latin) refers to the experimentation carried out with tissues outside the biological host body. *Ex vivo* experiments grant finer control and optimisation over *in vivo* at the cost of changing the natural biological context to better suit the needs of the research. *In vitro* (*in glass* in Latin) refers to experimentation done with microorganisms or cells outside their biological habitat like in a test tube or a petri dish. (7)

In vivo studies are an essential part of the long scientific process that takes a biological solution like a vaccine or a drug from the laboratory bench to a pharmacy. Presently, clinical trials are not pursued unless reliable, good quality, affirmative *in vivo* data from animal models are at hand. The scope of exploration in this mode is endless wherein the effects of certain biological molecules or disease progression can be studied extensively inside a living organism. (1) However, such an expansive mode is not free of shortcomings. *In vivo* studies are expensive, time-consuming and raise various ethical concerns regarding the usage of live animals for the experiments. Another pressing issue has been the translation of the accuracy of *in vivo* data to clinical trials. Mouse, pig or chimpanzee models might closely mimic human physiology but there are still ample chances that the data that is obtained from animal models could not be replicated in human models with the same success rate.

In vitro experiments allow scientists to evaluate individual biological phenomena in cells of interest without the interference of co-existing variables often present in living organisms. (1) They are also quicker to replicate and less expensive.

The PBMC model and its more sophisticated version, the MIMIC system designed by scientists at VaxDesign, are two such examples of *in vitro* systems that act as a surrogate to the human immune system and allow immunologists to test out the immune response to a drug, vaccine, biological molecule or other substances directly.

However, a petri dish or a test tube cannot replicate the natural biological context in its entirety, hence *in vitro* data must be assessed and used with forethought. (7)

This thesis aims to study the reliability of two *in vitro* experimental systems mainly- the PBMC model and the MIMIC system, by comparing the data generated by them with corresponding *in vivo* data and answer the following research questions: (1*) to what extent can in vitro studies substitute in vivo studies* and, (2) *can data from in vitro studies be credible enough to reduce the dependence on in vivo studies?*

Description of the *in vitro* models

Before citing examples, finding out which *in vitro* models are available, which mechanisms they operate on and what information they can deliver is of the essence.

In silico models

The term *in silico* (*in silicon* in pseudo-Latin) in biology generally refers to the experiments carried out via computer simulations emulating a pharmacologic or physiologic process. Advancements in the field of information technology and biotechnology have enabled the representation and investigation of the pathophysiology of biological systems via computer and mathematical models. The digital nature of *in silico* models bypasses the ethical concerns, time and cost factors involved in *in vivo* experiments. (4)
In silico modelling has been extensively used in the field of pharmacokinetics and drug discovery to understand drug metabolism, zero in on a lead compound for a potential drug from a wide array of compound databases using high throughput molecular docking (30), screen for epitopes, do protein docking, assess ligand affinity and sometimes go beyond the scope of *in vitro* studies by introducing an array of multiple parameters in the experiment that can be hard to observe in an *in vitro* set up. *In silico* setups are also useful in the domain of systems biology, genetics and cellular models. Digital genetic sequences are stored in databases, analysed, altered and used as templates for modelling new DNA or RNA strands and primers.

The human immune system is an inordinately complicated web of various cellular participants cross-interacting in multiple layers. Computational modelling is one practical way to study the contributions of those participants in an integrated way. Other *in vitro* models work with a reductionist approach where certain cell subpopulations are separated from the rest to be studied exclusively. But, often it is the interaction of the multiple cell subpopulations of the immune system within themselves and with other systems of the host body that affects the final result. The results become hard to interpret when the immune cell subpopulations are studied independently without taking those interactions into account. *In silico* models cater to that interdisciplinary interest wherein those interactions are mapped, their parameters manipulated and probed digitally to generate extensive data about the same. The models help immunologists frame informed hypotheses before beginning ‘wet lab’ testing, have a neat, comprehensive chart on the desegregated performance of the human immune system and with identification of prospective targets for clinical manipulation of an immune response. (11)

The PBMC system

PBMCs are a heterogeneous group of mononuclear leukocytes that make up an integral part of innate and adaptive immunity. These cells offer protection against bacterial, viral and parasitic attacks as well as the destruction of foreign substances and tumour cells. PBMCs differentiate into the myeloid (monocytes, macrophages, dendritic cells) and lymphoid (T cells, B cells, natural killer cells) lineage from hematopoietic stem cells (HSCs) via hematopoiesis. In adult humans, the PBMC population consists of 70-90% lymphocytes, 10-20% monocytes and 1-2% DCs. Within the lymphocytic population, 70-85% are CD3+ T cells (composed of CD4+ and CD8+ T cells in a 2:1 ratio), 5-10% B cells and 5-20% natural killer (NK) cells. (14)

Monocytes circulate in the blood waiting to be stimulated and differentiated into macrophages that have phagocytic functions or into dendritic cells (DCs).
Antigen-presenting cells (APCs) like DCs work as an interface between the innate and adaptive immune systems. DCs play an integral role in the activation of the adaptive immune system (B and T cells). B cells are activated in two ways- T cell-dependent and T cell-independent. T cell-dependent reactions that are responsible for generating the majority of long-lived plasma cells (a subset of mature B cells that produce antibodies) via involvement of the germinal center (specialised compartments in secondary tissues of lymph nodes) are slow. A faster-acting protection, in the meantime, is offered by the T cell-independent response that produces short-lived plasma cells. Not only do DCs present antigenic matter to T cells to initiate the T cell-dependent pathway of B cell differentiation, but recent studies have shown that certain subsets of APCs also release signals to control B cell proliferation, survival, differentiation and class switching in a T cell-independent manner. (29)

Hematopoietic stem cells (about 0.5% of the PBMC population), are present in blood and bone marrow and responsible for giving birth to all kinds of blood cells, not just lymphocytes. (28)

Lymphocytes are important for cell-mediated and humoral immune responses and consist of B cells, T cells and NK cells. The majority of T cells exist in a naïve condition initially in lymph nodes. Naïve T cells are activated once they undergo antigen recognition and initiate a cell-mediated immune response that targets infected cells. (15) First exposure to an antigen gives birth to effector cells (CD4+ helper T cells, CD8+ cytotoxic T cells and plasma B cells) and inactive memory cells. When the host body encounters the same antigen the second time, the long-lasting memory cells initiate a chain of immune responses that generate a new set of effector and memory cells. This is also known as immunological memory and often gives us lifelong protection against many diseases. (27)
CD4+ T cells do not attack the pathogens directly. They are activated and start proliferating when APCs have presented the antigenic material to their receptor with the help of class II major histocompatibility complex molecules, and a second signal involving cytokines or a costimulatory reaction between the CD28 receptor protein on the surface of the helper T cell and B7 signalling protein on the surface of the APC has been achieved. CD4+ helper T cells help activate the CD8+ cytotoxic T cells or stimulate the B cells to differentiate into antibody-secreting plasma cells via the production of cytokines. One of those cytokines, interleukin-2, makes cytotoxic or regulatory T cells active and proliferate. CD8+ killer T cells subsequently attack the target antigenic cell and kill them. Regulatory T cells reduce the severity of the immune response later once the foreign antigen has been eliminated by inducing apoptosis of effector cells. (27)

Similarly, CD19+ B cells wait for their receptor to recognise and bind to an antigen. But, B cell activation is also dependent on stimulation from CD4+ T cells, as mentioned above. An activated CD4+ T cell can stimulate a B cell that has encountered the same antigen via CD40 ligand - CD40 protein interaction, with the former on the surface of helper T cells and the latter on the surface of B cells. Antigens that initiate an immune response this way are called T cell-dependent antigens. Some antigens, however, trigger B cell stimulation without the help of T cells. These T cell-independent antigens are large polymers with repeating, identical antigenic motifs. A high concentration of those motifs is enough to stimulate B cells in a T cell-independent manner. (27)

Stimulated B cells differentiate and proliferate into immunoglobulin (Ig) producing plasma cells. Each plasma cell secretes thousands of Igs per minute into the bloodstream and continues doing so for many days. Inactive B cells express IgM and IgD but activated B cells can express IgA, IgE, and IgG or maintain IgM expression. The Ig production is gradually slowed down as the host body recovers from the infection.
The aforementioned process described the activity of circulating B cells. For memory B cells, exposure to antigen occurs in the germinal centre. Memory B cells do not secrete antibodies. They remain in the tissues and bloodstream for a long time scouting for the same antigen, with a high-affinity receptor. These memory B cells launch a quick recall response when the antigen has attacked the host body the second time. That is the core concept of vaccination. (27) A mature naïve B cell expressing IgM and IgD accounts for 50% of the total B cell population in adults. (21)

NK cells are a kind of cytotoxic lymphocyte and a part of the innate immune system. Unlike B and T cells, NK cells are nonspecific. They do not need the presence of an antigen to work and mainly offer protection against tumour cells. (20)

Two techniques are most commonly used to separate PBMCs from whole peripheral blood- gradient centrifugation and leukapheresis. The gradient centrifugation process works on the principle of difference in the densities of the different cell factions present in whole peripheral blood. Whole blood is layered over or under a suitable medium and centrifuged to separate PBMCs from red blood cells and granulocytes, as shown in Figure 1.

Figure 1: Separation of PBMCs from whole peripheral blood using gradient centrifugation. (22) *The tube on the left consists of the density gradient liquid (Ficoll) only. The tube in the middle has whole peripheral blood layered over the gradient liquid, before centrifugation. The tube on the right is the result observed after centrifugation. The red blood cells are observed to be settled at the bottom as a dark red faction because they are the*

heaviest. PBMCs are found 'sandwiched' in the middle in the form of a cloudy ring. They can be extracted by careful pipetting.

Leukapheresis is carried out in real-time inside a machine that separates the white blood cells from blood simultaneously with the inflow of whole blood employing high-speed centrifugation and sends the rest of the cell factions back to the donor. 1 blood volume of Leukopak, the product of leukapheresis, isolates up to 20 times more B cells than is present in one unit of blood and is ideal for large scale clinical trials. (8)

PBMCs are indispensable to the immune system since they are capable of eliciting specific responses against foreign pathogens and rogue cancerous cells. Therefore, these cells are highly studied in the domains of immunology, infectious disease study, haematological malignancies and vaccine development to name a few. *In vitro* PBMC models are used to study cell functions, biomarkers, disease modelling and response to drugs and/or vaccines. PBMCs are also used in the *in vivo* analysis of humanised mice. Immunocompromised mice are reconstituted with human PBMCs to mimic the human immune system and study its response to pathogens, toxins, drugs, vaccines and cancer in an *in vivo* model.
PBMCs are also being used in the field of advanced and more precise human medicine. Genome editing techniques like CRISPR/Cas9 have been used to transform PBMCs (T cells) into CAR-T cells which made it possible to treat cancers even without a donor match.
In the field of immunology, PBMCs have been used for vaccine development, especially via studies on B and T cell responses wherein they are exposed to vaccine candidates of interest and their activity studied to gain deeper insights into the adaptive immune response. *These in vitro* studies are particularly helpful in bridging the gap between success rates of *in vivo* animal studies and human clinical trials by helping narrow in on potential vaccine candidates. In-depth insight into ruminant innate immunity has been possible after studying cytokine production and activity using sheep PBMCs. (18) The research on persistent aftereffects of combination antiretroviral therapy in HIV (human immunodeficiency virus) which include immune hyperactivation is being studied with the help of PBMCs. (17) PBMCs are also used to study the up- and/or downregulation of biomarkers and measure the reaction of lymphocytes and monocytes to biological compounds.
Through methodical analysis of PBMCs, scientists can study immune responses and gain deeper insights into the human immune system and apply that knowledge to find efficient cures for diseases, with the least number of side effects.

<u>The MIMIC system</u>

A more sophisticated and modular version of the PBMC system that relies on the isolation and stimulation of PBMCs too was developed by scientists at VaxDesign called the MIMIC system. The MIMIC system tries to emulate clinical trials in a test tube that simulates the human immune response via a high throughput method. These predictions can dramatically reduce the cost and time required to use such products commercially.

The MIMIC system can broadly be divided into four steps – blood collection and preparation, peripheral tissue equivalent (PTE) module, lymphoid tissue equivalent (LTE) module and functional assays. (12) In the first step of blood collection and preparation, white blood cells are collected from pre-screened donors (shown in Figure 2. a). The white blood cells are then processed into either purified PBMCs or isolated subpopulations of the PBMCs such as

monocytes, T cells or B cells. The second step in the MIMIC system is the construction of the PTE module (shown in Figure 2. b). This module mimics peripheral tissue for the representation of innate immune responses. Well plates are fitted with a collagen layer covered with human umbilical vein endothelial cells (HUVEC) to allow the transmigration of DCs. Each of the wells effectively acts as one human test subject. The purified PBMCs or the monocytes generated in the previous step are placed on top of this endothelial layer. The monocytes selectively migrate through the endothelium and subsequently undergo differentiation into DCs and macrophages. The excess cells that are left behind are washed off after an hour. The DCs reverse migrate to the other side of the endothelium mimicking traffic across the lymphatics. After this reverse transmigration, the DCs are exposed to immunogens, drugs or vaccine candidates of interest and extracted with a pipette. The third step is the LTE module which involves a co-culture of T cells, B cells and follicular DCs that emulates the environment of a lymph node (adaptive immune system), shown in figure 2. c. When the potent antigen-presenting DCs from the PTE module step present their antigen to the right receptor matched T cells, a chain reaction of triggering an adaptive immune response involving B and T cells, as described in the PBMC system section previously, is initiated. Both cellular and humoral immune responses can be examined in this module. The immunocytes and biomolecules are removed for analysis. The final step is the characterisation of these immunocytes and biomolecules using a wide array of functional assays (shown in figure 2.d) such as enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunosorbent spot (ELISpot), flow cytometry, intracellular cytokine staining, cytotoxic T cell assays, micro neutralisation assays, hemagglutination inhibition assay, neutralising antibody assay and disease modelling.

The MIMIC system can do everything that the PBMC system can. Because of the multidimensional nature of the setup, it can potentially emulate a clinical trial with insights into the effect of immunotherapy on human subpopulation donor groups which can embody genetic diversity, HLA typing data, age (6) and gender biases and autoimmunity status. These datasets can hopefully be used to construct quick and perceptive clinical trials as well overcome the need to depend on *in vivo* animal models in prognosticating the immunogenic potential of a drug or vaccine candidate. (12)

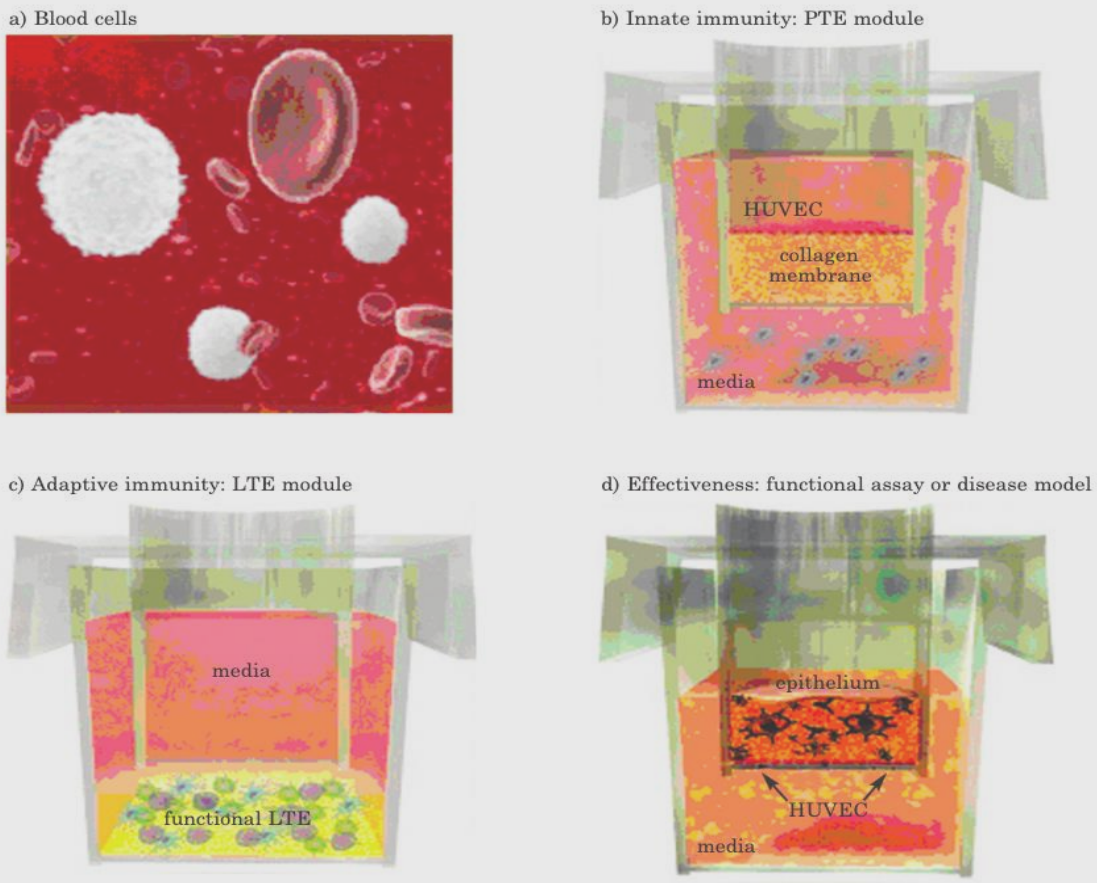

Figure 2: The four modules of the MIMIC system. (12) *(a) Collection of white blood cells from pre-screened donors and their preservation. (b) The PTE module that emulates the innate immune response. It comprises a single layer of HUVEC cultured on top of a cellulose matrix on which the PBMCs are released. (c) The LTE module emulates the adaptive immune response that takes place in the lymph nodes. DCs, follicular DCs, T cells and B cells are applied in the right order to mimic the in vivo immune response. (d) Functional assays are used to determine if the produced immune response (for example antibodies and cytokines) in the previous steps is potent against the stimulant or pathogen of interest.*

Comparative examples of usage of *in vitro* models found in the scientific literature

Malaria:

To understand elemental aspects of parasitic biology, disease progression and immune response in the case of malaria, establishing quality experimental models is of the essence.

The only commercially available malaria vaccine, Mosquirix™, while generating a strong humoral immune response, is deficient in offering long-lasting protection and is restricted by strain variation. Noe et. al (19) used *in silico* modelling to predict and analyse human

leukocyte antigen (HLA) restricted class II epitopes from *Plasmodium falciparum* circumsporozoite protein (PfCSP) intending to improve PfCSP centred malaria vaccines. The authors aimed to develop a workflow for the identification of peptide sequences that can bind the HLA-DR complex while achieving wide human population coverage. A subset of these class II epitopes was synthesised as peptides and their affinity for HLA-DR binding was studied *in vitro*. Furthermore, the functional capacity of these peptides to initiate an immune response in PBMCs was studied by looking at the cytokine response profile in the MIMIC system. (19)

The authors used *in vitro* HLA-DR allele binding data to validate HLA binding predictions obtained from *in silico* analysis to identify clusters of HLA-DR restricted epitopes in CD4+ T cells which play an essential role in activating a humoral immune response. An accuracy rate of 79% was found between *in silico* HLA-DR binding predictions and HLA-DR binding for the peptides tested *in vitro*. Eleven class II epitope clusters were tested with the MIMIC system on their initiation of a recall immune response. The LTE module allowed DC antigen presentation and T cell priming using cells of naïve PfCSP donors to the selected peptides. The recall multifunctional cytokine response can then be assessed using the primed T cell/DC co-culture. The frequency and magnitude of cytokine-producing helper T cells were recorded for five cytokines- interferon-gamma, tumour necrosis factor-alpha, interleukin-2 (IL-2), IL-4 and IL-10. By observing the percentage of recall responses in individual donors and the magnitude of those responses, four clusters were shortlisted. (19)

The process was further optimised by predicting class I HLA epitopes and testing their binding affinity *in vitro*. 58% of the predicted epitopes that were assessed bound to the class I HLA allele *in vitro*. Three of them known to be CD8+ T cell determinants were tested via the MIMIC system for their killer T cell response. One showed a high multifunctional cytokine recall response across a panel of donors. The authors of this paper have highly recommended including B cell epitopes in the next step of this research and studying T cell responses using human PBMCs as a part of the vaccine construct before moving on to advanced stages of PfCSP-based vaccine development. (19)

Radiation-attenuated sporozoites also offer protection against malaria but it is limited due to the presence of multiple parasite strains. Killer T cells make for good targets while developing a malaria vaccine strategy but that must be accompanied by studies on conserved class I HLA epitopes to provide cross-strain immunity. Tucker et. al. (26) aimed to increase the number of validated T cell epitopes that can be included in subunit vaccine development strategies targeted toward pre-erythrocytic malaria. They chose conserved *P. falciparum* proteins using *in silico* models, that were predicted to have a high frequency of T cell epitopes across six class I HLA allele supertypes. Five proteins were shortlisted based on the density and frequency of epitopes and the epitopes synthesized into peptides to check if they bind to class I HLA epitopes *in vitro*, as a way to verify the *in-silico* predictions. The same peptides were used to stimulate T cells in the MIMIC system to study immunogenicity. The authors used human dendritic cells (DCs) and autologous CD8 T cells in the MIMIC system to model initial T cell priming and to study the recollection of the immune response against epitopes from *Plasmodium falciparum* protozoa and were able to shortlist peptides that initiated an immune response in PBMC samples of three or more donors. Multiple donors were used for each HLA allele in the MIMIC system panel. Out of sixty-seven peptide-HLA combinations as observed from the binding analysis and data from MIMIC, forty-five had at

least one donor PBMC sample with the expected HLA allele reacting to the corresponding peptide. Fifteen peptides were identified that stimulated T cells across all six HLA alleles that represent six different supertypes of a wider human population. (26) However, this study only tested less than 10% of all the predicted epitopes so it is highly likely that each protein has a greater number of effective epitopes that can be discovered upon further investigation.

The data obtained in the domain of malaria research from these two papers are promising however, not close to replacing the need for *in vivo* or *ex vivo* experimentation. Not only do these experiments need to be repeated over a broader variety of donors but also scaled up in quantity to obtain a larger, more inclusive data set. Right now, at this stage, they are adequate as the first stepping stone in the process of vaccine development and as the transitory phase between *in silico* and *in vivo* experimentation.

Influenza:

Vaccine development strategies are heavily reliant on *in vivo* animal models and as discussed in the introduction section, come with their set of drawbacks. *In vitro* assay systems can contribute to the vaccine candidate selection screening process before an expensive clinical trial is pursued. Tapia-Calle et. al (25) studied memory T cell responses stimulated by H5N1 derived whole inactivated virus (WIV) vaccine and split vaccine using the PBMC system to demonstrate that these vaccine candidates induced antigen-specific T cell recall responses. The model was further validated for a peptide-based formulation.
This model uses long-standing cultures of unfractionated PBMCs as opposed to harvesting them from peripheral blood. Not only is this simpler and involves less number of steps but also lets the authors study the interaction between the different subpopulations of immune cells. (25)
The following key observations were drawn in this paper: (1) the WIV stimulated cultures initiated T cell proliferation and generated interferon-gamma, (2) T cells in the cultures responded to all three types of formulations, the WIV, split and peptide vaccine with WIV showing the most pronounced reaction, (3) effector memory T cells (TEM) elicited the most responses for both CD4+ and CD8+ T cells and (4) the vaccine candidates increased the number of follicular helper T cells (TFH) secreting IL-21. (25)
It was observed that antigen-specific T cells underwent a strong expansion after seven days, which is in line with previous literature. (16) What was new, was that these expansions took place even without the presence of exogenous cytokines like IL-2 and IL-21. It is in line with past studies that emphasized on the role of T cell receptor signalling as the reason behind T cell proliferation. (1) In this setup, cytokines might not be essential, but for de novo antigens, it would be interesting to include them in the framework.
The greater immunogenicity of WIV over the split vaccines is in agreement with previous *in vivo* studies on the same. (5,10) as is the prevalence of TEM cells over central memory T cells in both CD4+ and CD8+ T cell populations.
Vaccine candidate exposure did not change the overall number of TFH cells but increased the number of active ones. TFH cells play a role in generating and maintaining germinal center reactions and in the production of high-affinity antibodies. Past studies have revealed that the frequency of active TFH cells in blood was highest on day seven after vaccination and correlated with a high level of antibody titers and high avidity antibodies. Hence, the level of activated TFH can be used as a parameter to judge vaccination success. (25)

A drawback of this study was the suitability is limited to recall response, that is, antigens encountered before. Naïve T cell response was weak indicating the need for further optimization for de novo antigens. This model will work efficiently to screen booster vaccines that will improve pre-existing immunity against a particular antigen. This system helps compare vaccine candidates and zero in on the ones that hold the most potential and can be used for clinical trials.

Stoel et. al. (23) has compared the effects of influenza WIV and subunit (SU) vaccines on cultured murine DCs. Their first aim was to explain how capable the formulations were in initiating an immune response in terms of cytokine secretion and gene expression of the DCs studied *in vitro*. The second aim was to compare the DC response observed *in vitro* with the differential potency observed *in vivo*. The authors found out both vaccine candidates induced distinct responses in the murine DCs. The authors have also been able to successfully demonstrate that WIV had a significantly stronger effect on the cultured murine DCs than SU, which is in line with *in vivo* studies done earlier. (9, 11) A similar observation has been made in the case of monocyte-derived DCs as well wherein WIV showed a stronger reaction than SU. (24) More genes were differentially regulated when stimulated with WIV than SU. Moreover, WIV activated a higher number of signalling pathways for a longer duration, as compared to SU. These differences can be explained by the presence of single-stranded viral RNA in WIV which activated TLR7 and is present in low amounts in SU. Qualitative and quantitative differences were observed in gene expression of DCs stimulated with WIV or SU *in vitro* (23)

Influenza virus-like particles (VLPs) are a potential alternative to conventional influenza vaccine formulations. They mimic the biological virus without the genetic material, are safe and recognized by the human immune system. Buffin et. al. (2) used a mammalian cell platform to produce influenza VLPs containing hemagglutinin (HA), neuraminidase (NA) and matrix M1 proteins. Immunogenicity studied *in vivo* demonstrated VLPs administered intramuscularly were quite immunogenic at low doses, without adjuvant. Functional antibodies against HA and NA were successfully induced. Similar data were obtained *in vitro* in the MIMIC system. In fact, the authors have mentioned that surface assisted HA inhibition assay (SA-HAI) employed by the MIMIC system is more sensitive than the classical HA inhibition assay. (2) The classical HAI assay involves serial dilution of viral samples filled in wells of a microtiter plate mixed with antiviral antibodies to act as inhibitors to the agglutination process. Aliquots of red blood cells are added to it and if the concentration and binding affinity of the antibodies were strong enough, it would prevent clumping. The titer value is the inverse of the dilution factor of the last sample that fully inhibited hemagglutination. The SA-HAI is a more sensitive version of the HAI that involves transferring the hemagglutination reaction from the solution to the activated surface of an opsonized plate and using superior standardization of the classic HAI assay that reduces the variance in the HAI data. (13) The authors have successfully developed a flexible VLP platform that is based on the purification and characterization of 293T cells using techniques like mass spectrometry and cryogenic electron microscopy. The resulting VLP were observed to be very immunogenic in *in vivo* mice models without any adjuvant and in the MIMIC system *in vitro*. (2)

In keeping with the literature reviewed for influenza, it can be said that *in vitro* data on dendritic cells and T cell recall response to vaccine candidates has already been derived

reliably (the *in vitro* data is in line with *in vivo* data derived in the past), and should be used to support *in vivo* data to further reduce the dependence on the same. If not completely, *in vitro* data can partially fill the need for future *in vivo* experimentation for influenza.

Tetanus:

The MIMIC system has been used by Byers et. al. (3) to perform a preliminary screening for testing the efficacy of potential tetanus vaccine candidates. Purified CD4+ helper T cells and B cells were combined with autologous tetanus vaccine pulsed DCs to produce specific antibodies. Tetanus specific IgG antibody-secreting plasma cells were counted with ELISpot and a significant rise in the magnitude was observed post-vaccination (*in vitro*). The tetanus specific antibody response observed *in vitro* was in line with tetanus specific IgG serum titer profiles measured in the same donor *in vivo*. The data obtained from tetanus specific antibody response measured by ELISA also recapitulated *in vivo* data.
Most vaccines provide long-lasting immunity by producing antibodies specific to the targeted antigen. The authors have proved (using ELISpot) that the MIMIC system, in this case, correctly represented memory B cell response. Memory B cells might not correlate directly with serum antibody titer profiles but higher levels of it are indeed related to the immunogenicity of the vaccine candidate. Hence, the MIMIC system can be considered a reliable system for monitoring vaccine quality. The authors claimed to have unpublished data that showed the use of purified lymphocytes and autologous DCs in MIMIC generating more sensitive data than PBMC assays for both B and T cells. (3)

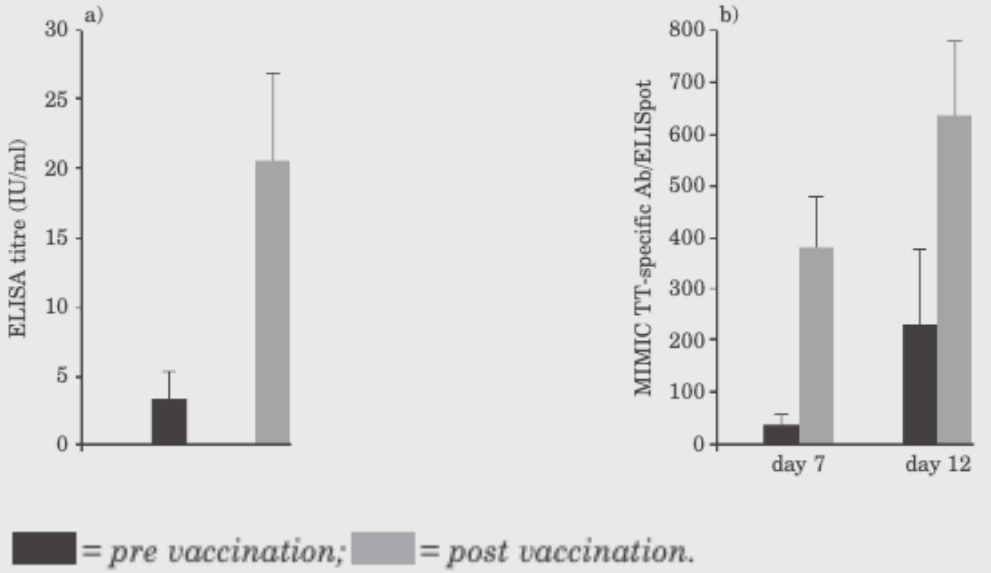

Figure 3: Specific immune response to tetanus vaccine in vivo and in vitro in the MIMIC model. (12) *Blood samples were collected from thirteen individuals before and after vaccination with a commercial tetanus vaccine. (a) The graph shows levels of tetanus specific antibodies in the individuals' sera which were determined by ELISA, pre- (black) and post-vaccination (grey). (b) The graph shows the number of tetanus specific antibody-producing plasma cells counted by ELISpot assay. PBMCs purified from both pre- (black) and post-vaccination (grey) blood samples of individual donors, determined using the MIMIC model, were used.*

Discussion

The research goal of this thesis was to look at the reliability of *in vitro* data and gauge how much it can measure up to *in vivo* data. For all the diseases that were used as an example to frame the argument, *in vitro* data has fared well. For research in malaria, the *in vitro* data might not have been as reliable and is still in a transitory phase (19,26) but for research in influenza and tetanus, the *in vitro* data is already in line with *in vivo* data obtained in the past when studying certain immune cell subpopulations, hence to a degree, reliable. (2,3,9,23,24,25) Plenty of *in vivo* data is available from past research work for these diseases and moving forward, further *in vivo* experimentation can be minimised by testing drug compounds and vaccines *in vitro* first. *In vitro* data with time and further optimisation is becoming quite a credible source of supporting evidence. However, it is not at the stage where it can fully satisfy the need to conduct *in vivo* experiments. *In vivo* experimentation is still very much needed before a vaccine or drug candidate can move to a clinical trial stage but with time, the reliance on those resource-heavy procedures may be lessened.

PBMC and MIMIC, both *in vitro* systems have a similar end goal – generating reliable data so as to reduce the cost and time factor associated with *in vivo* setups. Both are used to study immune responses and gain a deeper insight into the workings of the same. Both systems are highly modular wherein the components involved can be regulated quite easily to suit the needs of the research goal. Both are quite fast when it comes to evaluating different components of the immune system like a variety of cytokines and cell biomarkers as compared to the time it takes to generate *in vivo* data.
The PBMC system gives more control over the stimulation process since it is an independent setup by itself unlike a module in a cascade of procedures in MIMIC. It has less number of steps and is relatively simple. It is suitable to measure immune responses of cell subpopulations with minimal manipulation. Once the PBMCs have been isolated and stimulated, the functional assays can be started right away.
A number of questions can already be answered by the PBMC system but further refining of that data can be done via MIMIC. MIMIC employs the basic principles of the PBMC system but in a more sophisticated way. The MIMIC system emulates the *in vivo* environment of a lymph node more closely because of the LTE module. (12) The dataset generated is also much larger since every single well of the 96 well plates can be treated as a separate test subject. While the data obtained from MIMIC cannot replace *in vivo* experimentation yet, it is reliable enough to reduce the dependence on *in vivo* data as we have observed with influenza and tetanus studies. It can be applied in the analysis of adjuvant or antigenic compounds or even vaccines. It can serve as the first line of testing for vaccine efficacy to identify the most optimal formulation for human consumption. (3)

There is no one “clear winner” in this debate of *in vitro* vs *in vivo*. *In vitro* models might not be as mature enough to replace *in vivo* models completely but *in vivo* models themselves come with a plethora of time, resources and ethical issues. The best way to move forward is to optimise both to work best in tandem with each other. Past *in vivo* data can be used to refine *in vitro* experiments and reduce the need to rely on further *in vivo* experimentation to generate reliable data. As is observed in the case of influenza and tetanus, if there is *quality in vitro* data available, it can already help in screening vaccine candidates and bring down the time and cost needed to move it to a clinical trial phase. Moving forward, researchers can

work on the lines of refining *in vivo* experiments to be more resource-efficient, *in vitro* experiments to be more precise and an accurate representation of the immune system and for both to work harmoniously.

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