Cellular Biomarkers: Predicting Clinical Trial Outcomes Through the Understanding of T-cell Responses in the Human Challenge Model

Every year, there are three to five million severe cases of influenza with a global mortality of as many as 640,000 people. While influenza vaccines are effective, there have been problems with mismatch to circulating influenza strains and there is a need for next-generation influenza vaccines that provide broader and more durable protection against multiple strains of the virus, alongside carefully designed clinical trials to test their efficacy.

Human challenge studies allow researchers and drug developers to assess vaccine efficacy in terms of route of inoculation, and environmental- or vaccine-associated immune priming providing proof of principle protection data. In this complex field, the ability to directly measure cellular and humoral correlates of efficacy in challenge studies—and equate them to observed outcomes related to effectiveness—is invaluable and can assist in rapid candidate selection and go/no-go decisions for novel vaccines, such as those that protect against emergent, potentially pandemic influenza strains.

Screening for Susceptibility
Prior to enrollment into a human challenge trial, it is essential to screen individuals for susceptibility to the virus or viruses to be used in the study. Identifying a gender-balanced cohort of young, healthy adults (ages 18–65) with no serological evidence of exposure to the challenge strain to be employed is usually the preferred approach. Such serological eligibility (serosusceptibility) has traditionally been assessed by measuring antibodies to the influenza virus haemagglutinin surface antigen. Screening for antibodies that can inhibit haemagglutination by the haemagglutination inhibition (HAI or HI) assay is a traditional way to test for susceptibility to challenge viruses. However an improved assay, the microneutralization test (MNT), which directly assesses the titre of directly neutralizing antibodies in patient serum, is seen as a more relevant way to determine susceptibility to currently circulating flu strains; especially those that do not possess the ability to haemagglutinate (e.g., H3N2 virus strains). In addition to the above, the single radial haemolysis (SRH) antibody assay is still considered a good measure of immunity against influenza (see Figure 1).

The presence of high titres of anti-HA stalk antibodies and neuraminidase antibodies may also be associated with protection from influenza strains used in challenge studies, although they may not serve as a reliable predictor of reduced severity of illness (1). More complicated still is defining the role of nasal IgA in protection against influenza. Some human challenge studies have shown that individuals with high secretory IgA levels may be less likely to shed virus in the nasal cavity (2). Thus, high sIgA may be associated with protection from influenza. This appears to have been corroborated by community studies of Live Attenuated Influenza Vaccine programs in children performed in the UK (3).
The Importance of CD4 and CD8 Cells

CD4 T lymphocytes (CD4 cells) help manage the body’s immune response by stimulating other immune cells, such as macrophages, B lymphocytes (B cells), and CD8 T lymphocytes (CD8 cells) to kill virus-infected cells. Researchers have found an inverse correlation between viral shedding and CD8 T-cells in a large cohort of seronegative adults (4). Subsequent studies showed that CD8 cells secreting interferon gamma (and not IL-2) were associated with protection and milder symptoms if the virus were contracted (5).

In the human challenge model, pre-existing influenza-specific CD4 T-cells correlated with disease protection (6). CD4 cells that recognize NP and M1 (i.e., internal viral antigens that do not undergo rapid or progressive antigenic change like the surface haemagglutinin and neuraminidase glycoproteins) were associated with lower symptom scores and a shorter duration of illness (Figure 2). With H3N2 and H1N1 viruses, T-cell responses are mainly seen to be toward the nucleoprotein and the M1 or matrix protein; such responses have also been seen to be dominant following experimental challenge of adults.

Overall, these human studies suggest CD4 and/or CD8 T-cells can provide protection irrespective of antibodies.

Adjuvanted Vaccine Studies

A Phase 1 clinical trial of bird flu H5N1 vaccine with a Matrix-M
adjuvant was conducted at the University of Bergen. Following the first vaccination (prime), researchers found a significant increase in CD4 Th1-cells. After the second vaccination, the adjuvant boosted those responses (Figure 3).

In a longer-term study, 250 healthcare workers were recruited as part of an AS03 adjuvanted vaccine study (7). A group was vaccinated annually for five years. Over the five-year period, there was an increase in the magnitude of T-cells, which plateaued after the third and fourth vaccination. Of note, repeated annual vaccination improved the multifunctional, triple-producing CD4 T-cells (Figure 4). The multifunctional memory CD4 T-cells (i.e., of the IFN-γ, TNF-α, IL-2) increased over time and there was significant increase in the double producers as well. This indicates the importance of looking at the kinetics and specificity of the T-cell response to ensure that human challenge is performed at a suitable time after vaccination.

**Human versus Animal Models**

**Human condition.** In looking at the human model of influenza infection, humans exhibit a primarily upper respiratory tract infection with replication of the virus in the nasopharynx (and limited dissemination to the lungs). Influenza antigens are located within the nasopharyngeal-associated lymphoid tissue (NALT) within 48 hours of infection. The NALT serves as an induction site for T-effector memory (Tem) cells but does not support activation of CD8 T-cells, which are primarily from the lymph nodes and derived from memory cells. Antigen-presenting cells, including macrophages, B cells, and dendritic cells, stimulate T-helper cells to promote the CD8 T cells and B-cell proliferation and differentiation in the cervical lymph nodes.

Mucosal T-cells may offer a heterosubtypic immunity, providing protection against drifted and shifted reassortant strains. This may be important for later immunity if there is a pandemic or radically drifted influenza strains predominate, such as the current H3N2 that has resulted in recent vaccine failures. However, cell-mediated immunity declines within six months following primary infection despite circulating

**Figure 3:** Higher numbers of multifunctional T cell after adjuvanted vaccine.

![Figure 3](image1.png)


**Figure 4:** Repeated vaccination increases multifunctionality of T cell responses.

![Figure 4](image2.png)

Source: Barr et al., NPJ Vaccines, 2018; 3:45
T-central memory (Tcm) and Tem cells; secretory IgA and circulating IgA decrease after three to five months.

Finally, with regard to the human model, pulmonary, but not systemic challenge, generates resident memory (T_{RM}) cells that are embedded in the large airways. These cells may be effective if a pulmonary infection follows an influenza infection, and may dampen lung inflammation to reduce the incidence of secondary bacterial pneumonias.

Boosting Tcm may lead to accelerated recruitment of T-cells in mucosal tissues where they are needed to protect against the virus. Tcm has a longevity of about 12 months and can differentiate and migrate to the nasal cavity and lungs, unlike resident memory cells that remain in the proximal tissues and do not recirculate; these cells also have a much shorter lifespan. Tcm works in concert with the Tem cells as soon as an individual becomes infected. Scientific studies have shown repeatedly that intramuscular (IM) vaccination does not boost T_{RM} cells in the mucosa. T_{RM} cells are the prime effector mechanism for T-cell protection against mucosal virus infections. However, T_{cm} cells are short-lived, surviving up to 90 days, which is possibly related to the high turnover rate of mucosal tissues.

T_{cm} cells have been under studied until very recently. They are generated through a distinct, tissue-induced differentiation program. Antigens are transferred from the skin to the draining lymph nodes, where responding T cells are generated (Figure 5). Pathogenic T-cells can be stimulated or differentiate in the presence of disease to become effectors of viral control. However, some of these T-cells undergo a distinct differentiation, and become resident memory cells when they locate within the peripheral tissues. They then provide rapid local immune protection against re-infection.

**Mouse model.** Figure 6 provides a synopsis of the differences in mouse versus human immunity. For instance, mouse models are very effective for looking at certain T-cell responses and influenza disease.

Compared to humans, the commonly used pre-clinical model for studying infection has substantially greater bronchus-associated lymphoid tissues, which differ in terms of both their design, distribution and germinal centers. Additionally, the lymphocyte/neutrophil balance is seen to be different with mice having considerably more lymphocytes’ whereas humans tend to be neutrophil rich.

Also, while human T-cells may express both MHC Class I and MHC Class II (specialized antigen-presenting cells), mice T-cells express MHC I alone—leading to a class restriction and subsequent effects on humoral versus T-cell activation and function. Possibly as the result of such class restriction, influenza infection in mice leads to a rapid dissemination and replication of the virus in the lower respiratory tract (lung) and thus does not provide a comparable model for normal community-acquired infections in humans where up to one-third of infections are asymptomatic and the majority of symptomatic disease occurs in the upper-respiratory tract.

Mice may also show a profound difference in susceptibility to influenza challenge, dependent on both the mouse variety and the influenza strain. For example, if a BALB-C mouse is challenged with PR8 or WSN (both are H1N1 flu strains), the mouse will almost certainly progress to a profound lung infection resulting in death of the animal. However, if a BALB-C MX1 mouse is challenged with Avian H5N1, survival rates substantially improve. Thus, different strains of virus may induce profoundly different disease states.

Global differences in ethnicity or gender do not seem to provide data supporting such “strain variation” in human communities; however, age differential rather than a strain differential is known to exist with both the very young and the very old disproportionately affected by influenza; also, pregnancy raises the risk of hospitalisation during flu-illness by a factor of 7.

Physiological factors may also contribute to limitations in mouse-modelling; for example, there is a predominance of α2,3 sialic acid moieties in the lower respiratory tract in mice. Humans (and pigs) mostly have α2,6 sialic moieties, located in the upper respiratory tract. All human flu viruses have their origins in avian viruses, usually circulating in wildfowl
populations. Such wild type influenza viruses attach to α2,3 moieties in the avian hosts upper respiratory tract but, in mice, will preferentially descend into the lungs of mice. It usually requires multiple passages of novel avian flu strains through pigs or other closely related mammals to allow them to consistently cross the species barrier into humans—and even then the virus remains antigenically restricted to certain tissue types by reason of the attachment proteins.

### Mice vs. Man: Vaccine Responses

IM vaccination against flu in mice usually does not protect against lethal influenza challenge, whereas appropriate intranasal (IN) vaccination may. This points to an inducible, localized immune response protecting the mucosa from pathogen associated inflammation and/or disease. This may possibly be attributed to the MHC class restriction as described previously.

When considering studies regarding routes of challenge agent or vaccine administration in animals, the following caveats should be adjusted for:

- Infection in humans is primarily localized to the upper respiratory tract. IM vaccinations in humans may result in delays (5–9 days) in T-effector cells recruitment from central germinal centres to the site of disease.
- IM vaccination in animals results primarily in a B-cell response. It is possible that the upper respiratory tract is already overloaded with virus-infected cells by time the T cells reach the respiratory system and begin to differentiate into effector cells.
- IN vaccinations may offer a more profound effect on influenza-associated disease as local T_{rm} are recruited within hours after infection and damp-down replication/disease.
- IM vaccination in humans provides reduced protection from disease but offers better long-term protection from homologous virus.
- IN vaccines promote localized neutralizing antibody and T-cell mediated reductions in symptomatic disease (even from heterologous virus), but such local immunity may be short-lived.

A comparative analysis of murine and human models with regard to vaccine responses illustrates how the differences in the dissemination times of challenge virus plus the primary site of infection may distort measures of efficacy and thus detract from prognoses of effectiveness in the field. The presence of α2,3 sialic acid moieties in the lungs of mice can lead to a delay of five to seven days before

![Figure 6: Differences in mouse vs human immunity.](image)
consistent shedding is observed; this, in turn, may allow any potential vaccine a larger window to promote antibody and cellular responses. Vaccines in mice may have two or three extra days, compared to humans, to both recruit and differentiate effector T-cells. In infected humans in the general population, symptomatic disease develops more rapidly than in mice, starting two to three days after the initial inoculation. To avoid this “immune-lag,” vaccine efficacy may be more accurately modeled via IN inoculations in ferrets, or in human volunteers in a controlled human infection model, than in IM mouse studies.

To ensure more accurate prediction of potential efficacy of vaccines in the real-world and to reduce the failure rate of new vaccines as well as improve the performance of current, seasonal vaccines, we must ask: “Can any animal model predict efficacy in humans?”

To a certain extent, the answer must be yes. However, the inoculation site for the candidate vaccine and the titre and route of any challenge agent must be considered as much in animal studies as in human trials. For example, intratraacheal (IT) inoculation of influenza is often used in ferrets as it emulates a descending infection with lung involvement similar to severe influenza. However, the kinetics of an IT injection of pathogenic virus are likely to be artificially rapid relative to normal intranasal/nasopharyngeal infection in humans, giving the vaccines a smaller window of opportunity to promote protective responses. Note that ferrets, like mice, are immunonaive by nature (i.e., they have not seen influenza virus before and therefore do not have immunological memory of protective priming when challenged). Pre-immune ferrets and also immunocompromised animals are available, but are not the norm for most pre-clinical vaccine studies. Also, artificially high titres of challenge virus are frequently employed (107–109), which may overwhelm innate responses and lead to exacerbations of disease. Finally, IN vaccination has a good correlation to protection from disease following IN viral challenge, therefore we need to use such studies to better understand and align vaccination correlates if we are to develop products that promote long-lasting immunity to infection and disease.

To optimize the prognostic potential of the controlled human infection model, it may be necessary to more fully characterize pre-existing immunity in subjects entering such studies and to better define the individuals’ susceptibility to infection prior to challenge. To better “base-line” cohorts, simple antibody assays, such as HI, microneutralization, nasal sIgA and neuraminidase titres may be augmented with cellular response assays such as interferon gamma ELISPOT assays, T-cell flow cytometry and the omics to better understand the host’s antigenic history and the imprint such antigenic events have left on the adaptive immune system.

It remains important to bear in mind that the physical age and social setting of the subject in concert with the relative timing of flu epidemics, pandemics and vaccination events for outwardly healthy individuals are critical in determining the susceptibility of cohorts and severity of subsequent disease upon exposure to challenge virus.

While mouse and ferret studies may be suitable for certain influenza vaccine and drug tests, they still remain limited for a variety of reasons. As vaccine studies move from the pre-clinical to clinical setting, the controlled human infection model may offer substantial benefits with regard to predictive efficacy in addition to being host-appropriate:

- Vaccine efficacy has been effectively modeled via IN inoculation in human volunteers and such efficacy may be translatable into field effectiveness.
- A controlled human infection model has demonstrated a high negative predictive value for vaccines, which translates to a rapid candidate selection.
- A controlled human infection model provides similar symptom and seroconversion rates compared to community-acquired infections.
- A controlled human infection model provides shedding and symptom data consistent with normal influenza disease in a healthy population.

Summary

There is an urgent need to fully map and translate the immunological correlates of protection against influenza using both existing and novel technologies. Genomic, proteomic, lipidomic, metabolomic and inflammasomic data from human challenge studies will help shed light on the complex immunology of influenza infection in the context of shifting viral antigenicity and on-going seasonal vaccine failures. Allied to exponential leaps in processing power, large datasets may be analyzed to look for relatedness of individual markers to outcomes or to model (e.g., gene) signals against immunological pathways, cascades or binding events with physical motifs (structural biology).

References

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Contact Information

Tel: +32 15 27 32 45
clinicalresearch@sgs.com
www.sgs.com/cro
Follow us on LinkedIn:
www.sgs.com/linked-in-life