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MEETING REPORT

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The SeroNet Clinical and Translational Serology Task Force (CTTF) SARS-CoV-2 mucosal immunity methodological considerations and best practices workshop

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ABSTRACT

SARS-CoV-2 persists in certain populations, even with vaccination and boosters. Emerging evidence suggests that reductions in virus transmission and infection will likely require involvement of the mucosal immune system, especially secretory antibodies in the upper respiratory tract. The Clinical and Translational Serology Task Force (CTTF) within The National Cancer Institute (NCI)'s Serological Sciences Network for COVID-19 (SeroNet) hosted a workshop to review the status of development and standardization of mucosal sample collection methods and assays, identify challenges, and develop action plans to bridge gaps. Speakers presented data underscoring a role for secretory IgA in protection, mucosal markers as correlates of protection, methods for tracking and assessing mucosal antibodies, and lessons learned from other infectious agents. Perspectives from regulators and industry were put forward to guide mucosal vaccine development. Methodological considerations for optimizing collection protocols and assays and harmonizing data were highlighted. Rigorous studies, standardized protocols, controls, standards, and assay validation were identified as necessary to gain momentum in expanding SARS-CoV-2 vaccines to the mucosa.

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Introduction

While SARS-CoV-2 is no longer considered a public health emergency, the virus and its variants persist in certain populations even in the face of vaccination and boosters. It is becoming apparent that serum antibody levels do not tell the full story on factors contributing to immunity to SARS-CoV-2. Rather, the mucosal immune system and secretory antibodies specifically are likely playing a central role in preventing viral transmission and blocking early stages of infection. Measuring secretory antibodies in mucosal compartments is fraught with intrinsic and extrinsic challenges. Recognizing these challenges, NCI's Serological Sciences Network for COVID-19 (SeroNet) and the NCI Serology Program Clinical and Translational Serology Task Force (CTTF) organized a workshop to discuss key findings, current challenges, and limitations, as well as encourage best practices. Such efforts are essential as second generation, improved antiviral vaccine strategies are soon to be under investigation.[1,2]

From within SeroNet, NCI and the Frederick National Laboratory for Cancer Research (FNLCR) established CTTF to implement standardized serology testing and catalyze "translation of research findings into public health changes."[3]

On January 17th, 2023, CTTF cochair Dr. Ligia Pinto, in collaboration with SeroNet members Dr. Nicholas Mantis and Dr. Christopher D. Heaney, hosted a workshop titled "Mucosal Immunity to SARS-CoV-2: Methodological Considerations and Best Practices" to review standardization of oral fluid and nasal swab collection methods and assays, identify remaining challenges, and develop action plans to bridge gaps. Session 1 underscored the role of mucosal adaptive and innate immune responses in the oral cavity and nasopharyngeal space in blocking SARS-CoV-2 infection and limiting transmission. Highlighted in this session was emerging evidence that locally produced secretory IgA plays a significant role in preventing SARS-CoV-2 reinfection. Session 2 detailed the gaps in assay and data standardization, sampling, and populations. Here, scientists with experience in mucosal sampling and analysis of viral immunity shared how they adjusted their protocols to study SARS-CoV-2 immune responses, as well as their "wish-lists" for expanding and standardizing the field. This session also included a summary of the role and status of the World Health Organization (WHO) SARS-CoV-2 serology International Standard (IS) and secondary standards. Session 3 focused on the identification of gaps and next steps in expanding our current knowledge in mucosal immunity, especially as nasal vaccines come to the forefront of public health.

The goal of the workshop was to identify potential mucosal correlates of protection and knowledge gaps in assay standardization and data harmonization to optimize and standardize immunological assays and reagents. The hope is to eventually develop a core for large-scale standards production and testing support for clinical trials to drive innovative, integrative

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studies and proposals to investigate mucosal immune responses. Ultimately, the organizers compiled the results of the workshop into this publication with the goal of pushing the field of mucosal viral immunology forward.

The virtual workshop was attended by more than 240 individuals from around the world.

Session 1: setting the stage on mucosal immunity

The speakers in Session 1 made a case for the importance of both adaptive and innate immunity in modulating SARS-CoV-2 infection and transmission. The session highlighted evidence implicating mucosal markers as correlates of protection against infection, methods for tracking and assessing mucosal antibodies, and lessons learned from other infectious agents (Human Immunodeficiency Virus 1 [HIV-1] and Human Respiratory Syncytial Virus [RSV]) as they pertain to COVID-19. A final speaker provided perspective on the industry requirements for using mucosal immunology and serology data to inform regulatory and public health decisions.

Dr. Charlotte Thålin (Karolinska Institutet) provided some of the strongest evidence to date for the importance of mucosal IgA in preventing SARS-CoV-2 infection, detailing a longitudinal study (starting April 2020) of over 2000 individuals in a Healthcare worker (HCW) cohort at the Karolinska Institutet.[4] Mucosal IgA levels in the upper quartile were associated with protection against omicron breakthrough infection and higher levels of IgA appeared to limit viral load.[5] Protection lasted through 8 months, and preliminary data suggested mucosal IgA protection against the new variants in a compartment-specific response to SARS-CoV-2 infection.[5] Omicron infection elicited high mucosal IgA levels in both previously wild-type infected and previously uninfected individuals that endured at low (but higher than baseline) levels.[5] Apparent determinants of higher mucosal IgA levels included prior infection, higher serum IgG, and less time since infection (mucosal IgA was elevated up to 22 months post infection), while vaccine dose or number of infections had no significant effect on IgA levels. Secretory antigen-specific IgA correlated strongly with total antigen-specific IgA, suggesting that assays detecting IgA can be used as a proxy for secretory IgA in the nasal compartment. Parental boosters appeared to help prevent severe disease but did not impact mucosal IgA and block infection.

Dr. Peter Openshaw of Imperial College London demonstrated that both adaptive and innate immune responses may influence disease severity, discussing lessons learned from using mucosal sampling in RSV and influenza. Based on his experience of mucosal immunology studies in RSV and influenza, Dr. Openshaw believed that mucosal IgA (in addition to the status and exposure history of the mucosa) affects whether a virus causes symptomatic disease.[6] He highlighted the need for mucosal vaccines to enhance mucosal immunity against SARS-CoV-2 to prevent infection, lessen the risk of emerging variants, and prevent post-acute sequelae. As human infection models had previously revealed an RSV lag phase in disease pathogenesis that can only be studied using deliberate challenge studies with infection of volunteers, Openshaw co-led infection challenge studies of SARS-CoV-2 in young volunteers with no risk factors.[7,8] Rapid antigen tests were predictive of viable virus release if performed frequently. There was no correlation between viral load and symptoms, but there was a correlation between inflammation and symptom severity. In pre-vaccinated, uninfected volunteers, cross-reactive antibodies appear to modulate infection onset, while protection appears to be more of an active, local/mucosal T cell boost-mediated process. In addition, innate mucosal immunity may be key to abortive infection. In follow-up studies, SARS-CoV-2-specific nasal IgA waned 9 months after hospitalization with COVID-19 and was not induced by subsequent vaccination.[9]

Dr. Maria Lemos from Fred Hutchinson Cancer Center discussed correlates of recovery from SARS-CoV-2 infection, biodistribution of neutralizing monoclonal antibodies (VRC01) in mucosal surfaces based on lessons from HIV, and Singulex/SMCx Pro technology for the quantitation of low concentration antibodies in mucosal samples.[10,11] Disease severity was significantly lower in vaccinated patients, while breakthrough infections generated early IgG and IgA responses and strong inhibition of Angiotensin Converting Enzyme 2 (ACE-2). Vaccine breakthroughs also developed a memory response that mobilized quickly in blood. The best negative correlate of symptom duration was specific systemic IgA, which correlated with saliva IgA (collected with synthetic swab; $r = 0.850$; $p = .001$). Studies on monoclonal neutralizing antibody distribution in mucosal surfaces during HIV-infection showed that rectal compartments had lower monoclonal antibody levels (localized mainly to the lamina propria) compared to blood, and anti-HIV antibodies were differentially distributed in multiple mucosal layers.[10,11] SARS-CoV-2 localization in the mucosa revealed infection primarily in the supra-basal epithelium of the mucosa, salivary ducts, and acini, suggesting that the best correlates of protection could be antibodies specifically at the sites and layers of infection.[12]

Dr. Jeffrey Roberts (formerly of the Food and Drug Administration [FDA] and currently employed by MERCK) commented on critical considerations from the regulatory and industry perspective. Normalization and standardization are of high importance when presenting findings to the FDA. The effects of the sample matrix, dilution factors, perturbation of the mucosal surfaces by sampling method, and medical history (e.g. allergic rhinitis, polyposis) must be considered, as well as validation and reproducibility of assays. Compared with academia, where investigators are motivated to explore complex, high-variability assays, industry is more likely to adopt assays that can be thoroughly validated and operationalized at scale. Regulators are sensitive to the differences between cases, diseases, assays, and protocols, and they will be reluctant to accept data from one pathogen to model the immune response to another. Regulatory bodies will also tend to view claims based on a specific assay as applicable only to the specific candidate product developed using that assay (i.e., until multiple sets of data establish the performance of a specific assay across different vaccines, assay results will be applied in a product-specific, not class-wide, manner). All sponsors can expect scrutiny with respect to the device and collection technique, user factors, and sample collection standardization (especially in self-collection

protocols). In general, regulators will defer to sponsors in choosing the assay(s) they think will demonstrate the best association with protection from disease, although they will insist on more rigorous validation as development proceeds to late phase trials, especially for those trials that will be pivotal to support licensure.

Discussion centered around the regulatory and technical challenges to developing a new IgA assay without a gold standard or controls. The speakers agreed that there is a need for more evidence: clarity is key when performing assays in a clinical setting, and there are still many gaps in the field. Whether mRNA vaccines elicit effective mucosal antibody responses, how those responses compare with those after natural infection, and how long such responses last are still unknown. Since then, several studies have been published addressing these questions, with varying results.[13–15]

Participants also discussed the best strategy for mucosal vaccination, and how effective could it be without frequent boosts for new viral variants? If an effective mucosal vaccine could be developed, it would be easier to boost than with systemic vaccination, but one mucosal vaccination is unlikely to be sufficient. However, there is evidence that patients with prior wildtype virus infection exhibited effective mucosal IgA cross-binding with more recent variants, suggesting that nasal vaccination with ancestor strains may protect against future variants.

Session 2: assay and data standardization, sampling, and populations

The second session focused on the needs and obstacles for standardization of assays and harmonization of mucosal antibody measures, summarizing sample types, collection procedures, and key variables, discussing key markers, and defining data normalization procedures. Speakers detailed their experiences with troubleshooting sample collection procedures and assays to produce reliable and accurate data while building creative techniques for new questions involving the role of mucosal immunity in SARS-CoV-2 infection and disease. Important considerations in standardizing protocols were emphasized, and this session highlighted the many practical challenges behind standardization and normalization of mucosal immunological data.

Dr. Georgia Tomaras and her laboratory team (Caroline Brackett, Dr. Kelly Seaton, and Dr. Nicole Yates) from Duke University presented techniques, assays, and data developed to define antibody correlates of protection and disease outcome for influenza and SARS-CoV-2, with a focus on secretory IgA (SIgA). The team rigorously standardized their assays for the detection of IgA and SIgA and developed acceptance criteria for sample qualification. Their assays are customized to sensitively detect different antibody isotypes or subclasses against many pathogens for clinical trial endpoints. For a COVID-19 cohort, SARS-CoV-2 specific secretory antibody was consistently detectable throughout acute infection in nasal wash samples collected from patients over a series of visits occurring immediately post diagnosis, allowing for comprehensive analysis alongside serum and plasma data to determine correlates of virus shedding duration. The team noted a continued need for polyclonal mucosal samples negative and positive for influenza and SARS-CoV-2 as standards for qualification and validation assays in preparation for clinical trial evaluation.

Dr. Giada Mattiuzzo from the Medicines and Healthcare products Regulatory Agency, UK, introduced the WHO International Standards, which are the highest order of reference material (measured in International Units [IUs]) for the calibration of assays worldwide to ensure harmonization and comparability. The WHO IS is a primary reference material, while national standards or certain industrial standards are secondary reference materials.[16] The material used for the production of a WHO IS is often similar to a clinical sample (e.g. convalescent plasma or serum for antibody standards). The added value comes from the process of their development; it starts with an endorsement from the WHO Expert Committee on Biological Standardization (ECBS), which highlights the public health need for the standard. The candidate material is formulated in a stable form (i.e. lyophilized) and sent to 15–20 laboratories internationally to prevent site bias, assess interlaboratory and inter-assay suitability, and assure standard quality and useability.[17] Secondary standards are calibrated against the WHO IS, creating greater quantities of calibrated material than would be available from the limited WHO IS. High uptake of the IS coupled with improper standard use resulted in depletion of the WHO IS for SARS-CoV-2 by August 2021, and a second SARS-CoV-2 IS was produced.[18,19] The recommendations for the second standard are the same as they were for the first, and can be used to calibrate secondary standards for IgA in Binding Antibody Units (BAU)/mL.[18]

Dr. Michal Tal from the Massachusetts Institute of Technology reviewed the optimization of a multiplex assay using beads bought from a commercial vendor and precoated with spike, Receptor-Binding Domain (RBD), and other epitopes and a neutralization assay for saliva via fluorescent (Green Fluorescent Protein [GFP]) recombinant Vesicular Stomatitis Virus (rVSV) containing the original wild-type spike. She also compared saliva collection devices, highlighting that multiplexing allows for a broader examination of different aspects of saliva. Analysis of the impact of heat inactivation on different antibody isotypes showed that while IgG is thermostable, other isotypes such as IgE are not. Finally, with the goal of using viral neutralization to elucidate the optimal nasal versus systemic vaccination order-of-administration, Dr. Tal's group developed a real-time-imaging-based neutralization assay to visualize cellular infection with and without the presence of neutralizing antibodies.[20] This real-time technique was used to demonstrate the waning of mRNA primary plus mRNA booster vaccination-induced protection in the saliva after 3–6 months. ChAdOx1-S+mRNA booster vaccination was roughly equivalent to mRNA vaccination plus mRNA booster, and Ad26.COV2.S induced no salivary antibody response, even when boosted with mRNA vaccination. Their data highlighted the importance of neutralizing antibodies in preventing infection, as well as demonstrated the necessity of studying vaccine-administration protocols from an immunological efficacy stand-point.

Dr. Jennifer Gommerman from the University of Toronto presented her team's techniques for measuring the kinetics, magnitude, and durability of rotavirus infection in the mouse gut in relation to mucosal immunity.[21] The team pivoted at the beginning of the pandemic to use the same techniques to study SARS-CoV-2, focusing on antibodies in saliva in humans after infection with SARS-CoV-2.[22,23] Dr. Gommerman turned the discussion to questions relevant to IgA mucosal immunity, describing her laboratory's pilot work in Salivette® tubes and noting considerations for saliva ELISAs such as alternatives to heat inactivation, evaluating and choosing the most sensitive plates, reducing sample background, and choosing appropriate sample concentrations. Using these optimized protocols, experiments studying local antibody responses to systemic vaccination or infection revealed that, while nearly all adult participants were positive for IgA after the first dose of vaccine, IgA levels were undetectable in the majority of participants after dose 2. Meanwhile, anti-spike and anti-RBD IgA increased in individuals after Omicron breakthrough infection compared to patients who received the booster, suggesting a mechanism of immunity not replicated in systemic vaccination. Dr. Gommerman also discussed how breakthrough vs. non-breakthrough samples had significantly different levels of IgA, complicating normalization against the WHO IS. Albumin and other blood proteins were suggested, so long as the assay has a good signal-to-noise ratio.

Finally, the discussion turned to different methodological considerations such as best sampling locations, the use of protease inhibitors, isotype competition, and possibilities for practical and renewable IgA standards.

As the session closed, the importance of studying mucosal immunology against SARS-CoV-2, as well as the great need for direct steps toward sampling and assay standardization, were extremely evident.

Session 3 and concluding remarks: next steps and goals

The objectives of this final session were to determine the next steps in standardizing mucosal immunity testing and accelerating the development of SARS-CoV-2 mucosal vaccines. In addition, attendees hoped to identify critical gaps and define priorities to address them.

There was a consensus among discussants that producing and implementing assay standards was critical to assessing the feasibility of harmonization and the ability to compare results from different laboratories currently using different protocols.

The secondary priority identified was more rigorous studies to define the clinical relevance of mucosal antibodies in the context of vaccine trials or large, well-designed epidemiological studies. These studies would be necessary to foster further investments in mucosal vaccine development.

Members also highlighted that a critical gap is the lack of clinically annotated saliva and nasal biospecimens: there is a need for saliva and nasal swab biobanks that can be used by developers for assay development and validation. These materials were hard to find pre-COVID, and having these samples both available and profiled would be extremely useful. There was discussion on how this could be achieved in practical terms, considering all the differently used collection and processing methods. Regardless of methodology, all agreed that the existing collection methods must be optimized, compared and standardized.

Finally, attendees stated on a need to demonstrate the validity of mucosal biomarkers. This could be done by building on the success of the recent RSV vaccine development program, which demonstrated immunity by comparing neutralization antibody levels to other known-to-be-effective vaccines.[24] Building on this momentum will speak to regulators and developers on the validity of mucosal biomarkers as correlates of protection.

Addressing the identified priorities: how can we envision making a standard available?

The first consideration lies in what collection method and device to use. Secretions are hugely variable (whereas serum is more constant), and the concentration of IgA in saliva varies inversely with the flow rate. Anything inserted into the mouth to measure antibody levels will promote gingival crevicular fluid collection, which will vary depending on gingival health. In addition, nasal washing introduces an unknown dilution. Consequently, mucosal immune measurements must be normalized against the total recoverable immunoglobulin. A comparison study with existing optimized protocols and selected assays could help understanding the impact of these variables on results and building best practices.

Finally, participants noted the need for additional mucosal research, standardization and validation work to build the appropriate strong infrastructure to generate reliable data at different mucosal sites. Such data can support novel vaccine recommendations and aid in understanding their correlation with serum biomarkers.

Concluding remarks

The upper respiratory tract represents the site of the first encounter between viruses like SARS-CoV-2 and components of the human immune system, as well as the departure point for virus transmission in the form of respiratory droplets. Emerging evidence suggests that local concentrations of virus-specific IgG and SIgA antibodies, developed in response to vaccination or previous infection, influence an individual's likelihood of reinfection and possibly even limit person-to-person transmission. Critical to examining this question at a population level is the need to optimize and standardize collection protocols, make antibody standards and critical reagents available to the community, and harmonize data analysis. As SARS-CoV-2 mucosal vaccines undergo development, the ability to assess vaccine effectiveness and infection immunity is key.

The motivation of this workshop, in addition to a review of the status of knowledge regarding mucosal immunity to infection and vaccination and identifying key needs in the field to facilitate data collection and harmonization, was to specify next steps and goals in filling those existing gaps. Publication of this manuscript was identified as a key step in addressing these goals, in hopes of facilitating larger standardization efforts that will eventually impact vaccine approvals and implementation.

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