# VACCINATION OF SPECIAL POPULATIONS: PROTECTING THE VULNERABLE

EDITED BY: Paolo Palma, Viviana Moschese, Federico Martinon-Torres and Daniel O'Connor

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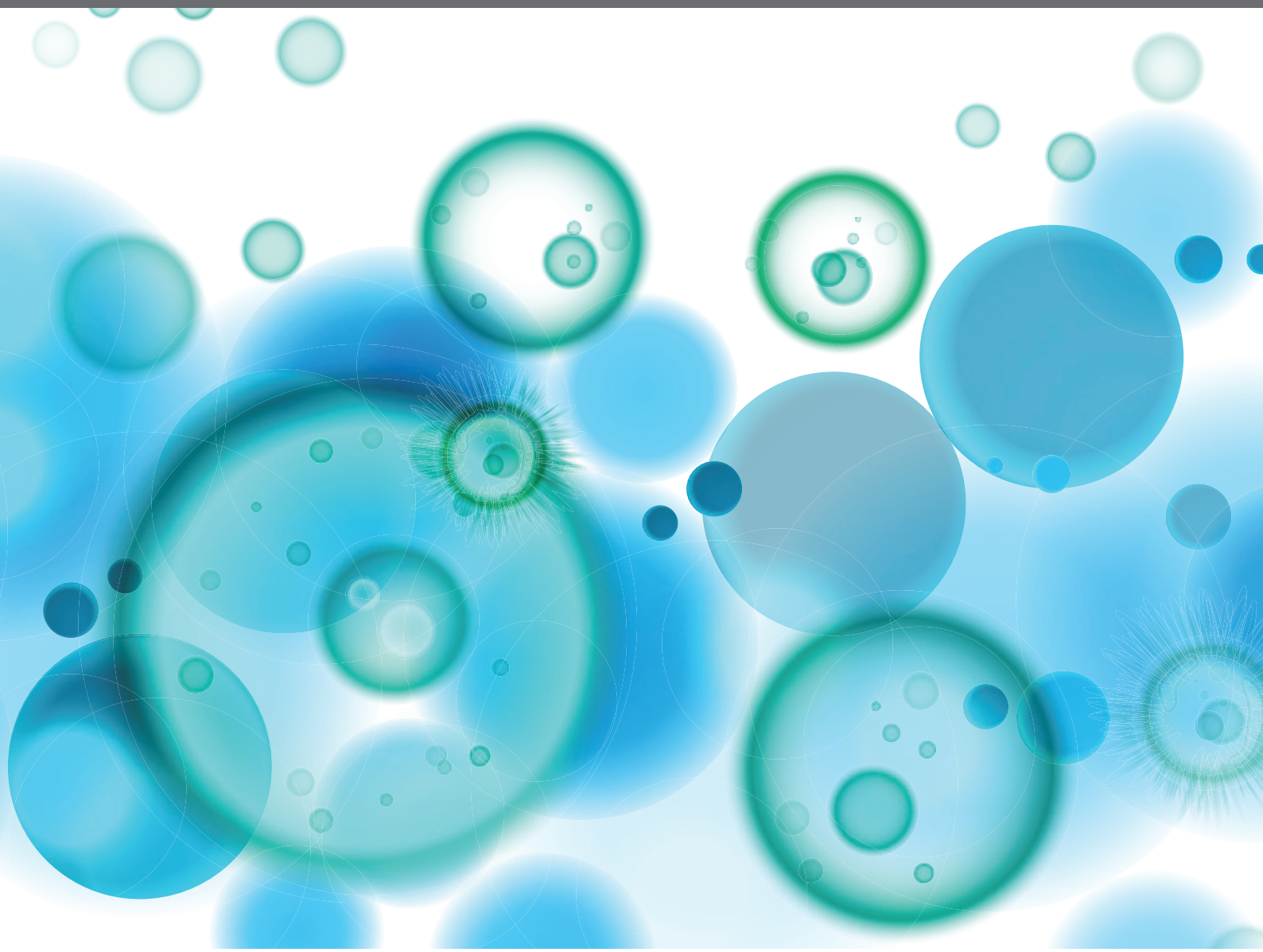

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# VACCINATION OF SPECIAL POPULATIONS: PROTECTING THE VULNERABLE

Topic Editors:
**Paolo Palma,** Bambino Gesù Children's Hospital (IRCCS), Italy
**Viviana Moschese,** University of Rome Tor Vergata, Italy
**Federico Martinon-Torres,** University Clinical Hospital of Santiago, Spain
**Daniel O'Connor,** University of Oxford, United Kingdom

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EDITORIAL
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# Editorial: Vaccination of Special Populations: Protecting the Vulnerable

*Daniel O'Connor*[1,2*], *Viviana Moschese*[3], *Federico Martinon-Torres*[4,5,6] *and Paolo Palma*[7,8]

[1] Oxford Vaccine Group, Department of Paediatrics, University of Oxford, Oxford, United Kingdom, [2] NIHR Oxford Biomedical Research Centre, Oxford University Hospitals NHS Foundation Trust, Oxford, United Kingdom, [3] Pediatric Immunopathology and Allergology Unit, Tor Vergata University Hospital, University of Rome "Tor Vergata", Rome, Italy, [4] Genetics, Vaccines and Pediatric Infectious Diseases Research Group (GENVIP), Instituto de Investigación Sanitaria de Santiago and Universidad de Santiago de Compostela (USC), Galicia, Spain, [5] Translational Pediatrics and Infectious Diseases, Hospital Clínico Universitario and Universidad de Santiago de Compostela (USC), Galicia, Spain, [6] Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBER-ES), Madrid, Spain, [7] Clinical Immunology and Vaccinology Unit, Academic Department of Pediatrics, Ospedale Pediatrico Bambino Gesù-IRCCS, Rome, Italy, [8] Chair of Pediatrics, Department of Systems Medicine, University of Rome "Tor Vergata", Rome, Italy

**Keywords: vaccines, vulnerable populations, immunity, infectious diseases, immunisation**

## Editorial on the Research Topic

## Vaccination of Special Populations: Protecting the Vulnerable

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***Edited and reviewed by:***
*Denise L. Doolan,*
*James Cook University, Australia*

****Correspondence:***
*Daniel O'Connor*
*daniel.oconnor@paediatrics.ox.ac.uk*

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Despite the remarkable success of global vaccination programmes, vulnerable populations (VPs) — who are particularly susceptible to infectious diseases — are often undervaccinated and/or exhibit reduced vaccine immunogenicity. There are relatively limited immunogenicity and safety data of vaccines in VPs, including pregnant women, newborn and preterm infants, elderly, and individuals with chronic diseases. The purpose of this special issue was to gather the latest evidence about vaccine safety and immunogenicity in VPs, including epidemiology, basic immunology, systems vaccinology and human *in vitro* modelling data — required for optimal development and utilisation of vaccines for these special populations.

Primary immunodeficiency (PID) patients represent a population with profound susceptibility to infectious diseases. PID patients are also at high-risk of severe COVID-19. Although several COVID-19 vaccines are now available, inborn errors of immunity may lead to poor vaccine responses. Amodio et al., demonstrated most their PID patients developed both specific antibody and T cell responses — albeit humoral responses were of lower magnitude — following BNT162b2 SARS-CoV-2 vaccine, with no severe adverse events reported.

People leaving with HIV (PWH) represent another group of immune-compromised individuals with impaired vaccine responses. De Armas et al., carried out a detailed analysis of transcriptomic and immunological responses to 2-doses of pandemic influenza vaccine in HIV-infected children receiving anti-retroviral therapy (ART) to suppress viral load. The authors report a baseline molecular profile — of metabolic stress and immune activation — that was associated with low responders. Conversely, increased *CXCR5* expression — a homing marker expressed on T follicular helper (Tfh) — as well as an increased peripheral blood Tfh frequency and function following vaccination was seen in high responders.

Allogeneic haematopoietic cell transplantation (HCT) recipients have an altered vaccine-induced immunity and are highly susceptible to infectious disease. Therefore, immunisation of these patients against vaccine-preventable diseases is critical. Piekarska et al., assessed humoral immunity to hepatitis B virus (HBV) in HCT recipients, following recombinant hepatitis B surface antigen (rHBsAg) vaccine. Seroconversion was achieved in all HCT vaccinees, but severe chronic graft versus host disease (cGVHD) was associated with weak responses. Conversely, prior donor rHBsAg immunisation was associated with superior vaccine responses. Authors highlighted the benefit of both donor and recipient vaccination prior to HCT and propose an intensified vaccine schedule for weak responders.

Yellow fever (YF) vaccine is one of the most effective vaccines, evoking highly durable protection in healthy individuals. However, certain populations are at increased risk of rare but severe adverse events, leading to anxiety about the use of this live-attenuated vaccine in immune-compromised individuals. Valim et al., examined primary YF vaccine safety and immunogenicity in autoimmune disease (AID) patients with low disease activity and immunosuppression. Antibody levels were lower in autoimmune disease patients than healthy controls, but most individuals did seroconvert, and only mild adverse events were observed.

The immunological mechanisms underlying rotavirus (RV) vaccine protection are unclear. Gomez-Carballa et al., explore host transcriptome responses in children immunized with a live-attenuated RV vaccine (Rotateq) in comparison with children with rotavirus infection. This oral vaccine that replicates poorly in the gut evoked measurable changes in the blood transcriptome. This study showed similar molecular responses induced by vaccine and wild-type infection, including over-expression of genes associated with gastrointestinal disease and inflammation. However, machine-learning approaches were able to use the blood transcriptome to accurately distinguish vaccination and natural infection. This type of study has the potential to reveal the mechanisms of immune protection against rotavirus as well as enable a high-resolution assessment for vaccine safety and effectiveness.

Colucci et al., evaluated the immune and vaccine competence in children with steroid-sensitive nephrotic syndrome (SSNS). SSNS can lead to leakage of proteins into the urine and reduction in serum IgG levels. Moreover, immunosuppressive therapy used to treat these patients can strongly impact vaccine responses. Authors therefore evaluated the vaccine competence of SSNS patients by measuring vaccine-specific B cell responses, prior to administration of immunosuppressive treatment. Showing that while SSNS patients have reduced anti-tetanus and anti-HBV IgG levels, they had intact vaccine-specific B cell memory compared with controls.

Several reports have suggested certain vaccines may induce non-specific immunological effects that can modify susceptibility to unrelated infections. For example, childhood BCG and measles vaccines have been associated with a reduced risk in all-cause mortality i.e., beyond that expected due to tuberculosis (Tb) and measles. "Trained immunity" has been proposed as the mechanisms underlying these associations, which has been described as the modification of innate immune responses to induce "memory" of infection that results in enhanced and non-specific immune responsiveness to unrelated pathogens. Kleen et al., review BCG as a non-specific approach to modify immune responses to COVID-19 infection in at-risk populations. Evaluating whether in the setting of an emerging pandemic — before specific vaccines are available —the immune system could be modulated to improve immune responses. Authors propose to deploy the potential properties of BCG, a vaccine that has been in use for a century and with a well-defined safety profile, in the emerging outbreak setting.

While there is evidence that BCG reshapes innate immune responses to Tb-unrelated pathogens, the underlying mechanisms in early life are obscure. Angelidou et al., show distinct age-specific effects on newborn monocytes (cord blood) compared with adult monocytes, including distinct innate cytokine responses as well as "trained immunity". Although they described greater TNF and IL-12p40 production in neonatal compared with adult monocytes following BCG stimulation, at day 7 BCG-trained adult monocytes demonstrated enhanced LPS-induced TNF production whereas newborn monocyte demonstrated tolerization. Moreover, BCG-trained newborn monocytes demonstrated an impaired BCG-induced production of lactate — a metabolite implicated in "trained immunity" in adults. These data showing age-associated differences in response to BCG may have important implications in the development of novel vaccines inducing non-specific protection.

Although the vaccine market is mostly for paediatric use, vaccine development is largely empirical and not tailored to meet the distinctions in innate and adaptive immune activation of early life. Beijnen and van Haren, evaluated vaccine-induced $CD8^+$ T cell responses in children — reviewing age-specific molecular determinants that contribute to antigen cross-presentation. Where $CD8^+$ T cells are desired, subunit-based vaccines need to be able to promote cross-presentation. There is evidence for adjuvant-induced cross-presentation, but little is known about whether and how adjuvants induce cross-presentation in early life. While young infants have higher frequency of $CD8^+$ T lymphocytes, these have less diverse TCRs, reduced development of memory cells and have reduced cytotoxicity compared with adults $CD8^+$ T cells. This review describes the sorting of soluble antigens for either classical MHC II presentation or cross-presentation on MHC class I, exploring critical steps in antigen cross-presentation and their competency in early life. Authors describe the inflammatory environment required to activate naïve CD8 T cells, and propose how adjuvant-induced cross-presentation can tailor distinct immune system early in life to induce potent $CD8^+$ T cell responses.

Similar to other conditions associated with poorly regulated glucose metabolism, type 1 diabetes (T1D) confers increased risk to infection. There are clear genetic risk factors to T1D but the trigger is unclear, with some data suggesting that viral infection may induce development of T1D. These reports have stimulated

significant debate about the use of live-attenuated viral vaccines in individuals at high-risk of T1D. Children with T1D are considered a special population and receive immunisation according to schedule recommendations for healthy subjects but with particular attention to pneumococcal and influenza vaccines. Here, Esposito et al. review the most effective and safe use of vaccines in individuals at risk of T1D or with overt T1D.

Routine childhood immunisation programmes have proven to be one of the most effective public health interventions. However, there are a number of barriers to vaccine uptake — including but not restricted to vaccine hesitancy. Olusanya et al., explored the barriers to childhood vaccine uptake and proposed recommendations for increasing vaccine compliance within the context of the COVID-19 pandemic. Authors advocate the use of more comprehensive evaluation of barriers to vaccination including social as well as individual determinants of health. Also, a multidisciplinary approach and antificial intelligence are proposed to promote optimal vaccine strategies.

Maternal immunisation offers protection to both pregnant women and their offspring, with transfer of vaccine-specific antibody to the infant reducing the incidence of neonatal tetanus and severe pertussis. Maternal immunisation has also been highlighted as strategy to prevent infant respiratory syncytial virus (RSV) and group B streptococcal (GBS) infections. Abu-Raya et al. are experts in infectious disease, vaccination, and maternal immunisation and detail a consensus paper summarizing the current literature on immunization during pregnancy and discuss current gaps related to vaccine safety and efficacy. Authors propose several priorities in future research to increase understanding of different aspects of maternal immunization to optimise protection for both the mother and child.

Qiu et al., report uptake of influenza and pertussis maternal vaccination, and factors associated with vaccine acceptance in high-income countries. Reasons for declining vaccine varied, but maternal safety concerns were a key factor associated with uptake. Knowledge gaps among pregnant women and lack of healthcare provider recommendation were important barriers for vaccine acceptance.

This Research Topic gathers some of the latest data about vaccine safety and immunogenicity in VPs, but also highlights the need for further work to improve vaccines and vaccine-uptake in these populations.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

**Conflict of Interest:** FM-T reports his institution received trial fees from the GSK group of companies, Ablynx, Abott, Jansen, Medimmune, MSD Merck, Novartis, Novavax, Pfizer, Regeneron, Roche, Sanofi Pasteur and Seqirus for activities outside the presented work. FM-T also reports his institution received research grants from AstraZeneca, Jansen, MSD Merck and Pfizer for activities outside the presented work. FM-T also reports having received personal fees / honorarium from Biofabri, Novavax, Sanofi Pasteur, Seqirus, GSK group of companies, MSD Merck and Pfizer for activities outside the presented work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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# Global Perspectives on Immunization During Pregnancy and Priorities for Future Research and Development: An International Consensus Statement

*Bahaa Abu-Raya[1], Kirsten Maertens[2], Kathryn M. Edwards[3], Saad B. Omer[4], Janet A. Englund[5], Katie L. Flanagan[6,7,8], Matthew D. Snape[9], Gayatri Amirthalingam[10], Elke Leuridan[2], Pierre Van Damme[2], Vana Papaevangelou[11], Odile Launay[12], Ron Dagan[13], Magda Campins[14], Anna Franca Cavaliere[15], Tiziana Frusca[16], Sofia Guidi[15], Miguel O'Ryan[17], Ulrich Heininger[18], Tina Tan[19], Ahmed R. Alsuwaidi[20], Marco. A. Safadi[21], Luz M. Vilca[22], Nasamon Wanlapakorn[23], Shabir A. Madhi[24,25], Michelle L. Giles[26], Roman Prymula[27], Shamez Ladhani[10], Federico Martinón-Torres[28], Litjen Tan[29], Lessandra Michelin[30], Giovanni Scambia[15], Nicola Principi[31] and Susanna Esposito[32]* on behalf of World Association of Infectious Diseases and Immunological Disorders (WAidid) and the Vaccine Study Group of the European Society of Clinical Microbiology and Infectious Diseases (EVASG)*

[1] Department of Pediatrics, University of British Columbia, Vancouver, BC, Canada, [2] Faculty of Medicine and Health Sciences, Centre for the Evaluation of Vaccination, Vaccine and Infectious Diseases Institute, University of Antwerp, Antwerp, Belgium, [3] Division of Pediatric Infectious Diseases, Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, TN, United States, [4] Department of Internal Medicine (Infectious Diseases), Department of Epidemiology of Microbial Diseases, Yale School of Medicine, Yale School of Public Health, New Haven, CT, United States, [5] Department of Pediatrics, Seattle Children's Research Institute, University of Washington, Seattle, WA, United States, [6] Faculty of Health Sciences, School of Medicine, University of Tasmania, Launceston, TAS, Australia, [7] School of Health and Biomedical Science, RMIT University, Melbourne, VIC, Australia, [8] Department of Immunology and Pathology, Monash University, Melbourne, VIC, Australia, [9] Oxford Vaccine Group, Department of Paediatrics, University of Oxford, Oxford, United Kingdom, [10] Immunisation and Countermeasures Division, National Infection Service, Public Health England, London, United Kingdom, [11] Third Department of Pediatrics, University Hospital ATTIKON, National and Kapodistrian University of Athens, Athens, Greece, [12] Université de Paris, Inserm, CIC 1417, F-CRIN I REIVAC, Assistance Publique-Hôpitaux de Paris, Paris, France, [13] The Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel, [14] Preventive Medicine and Epidemiology Department, Hospital Universitario Vall d'Hebron, Barcelona, Spain, [15] Dipartimento Scienze della Salute della Donna e del Bambino e di Sanità Pubblica, Fondazione Policlinico Universitario "A. Gemelli" IRCCS-Università Cattolica del Sacro Cuore, Rome, Italy, [16] Department of Medicine and Surgery, Obstetrics and Gynaecology Unit, University of Parma, Parma, Italy, [17] Microbiology and Mycology Program, Faculty of Medicine, Institute of Biomedical Sciences and Associate Researcher, Millennium Institute of Immunology and Immunotherapy, University of Chile, Santiago, Chile, [18] Pediatric Infectious Diseases, University of Basel Children's Hospital, Basel, Switzerland, [19] Division of Pediatric Infectious Diseases, Department of Pediatrics, Northwestern University Feinberg School of Medicine, Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL, United States, [20] Department of Pediatrics, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates, [21] Department of Pediatrics, Santa Casa de São Paulo School of Medical Sciences, São Paulo, Brazil, [22] Unit of Obstetrics and Gynecology, Buzzi Hospital - ASST Fatebenefratelli Sacco, University of Milan, Milan, Italy, [23] Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, [24] Department of Science and Technology/National Research Foundation: Vaccine Preventable Diseases, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, [25] Medical Research Council: Respiratory and Meningeal Pathogens Research Unit, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, [26] Department of Obstetrics and Gynaecology, Monash University, Melbourne, VIC, Australia, [27] School of Medicine Hradec Kralove, Institute of Social Medicine, Charles University Prague, Prague, Czechia, [28] Translational Pediatrics and Infectious Diseases, Pediatrics Department, Hospital Clínico Universitario de Santiago de Compostela, University of Santiago, Santiago de Compostela, Spain, [29] Immunization Action Coalition, St. Paul, MN, United States, [30] Infectious Diseases and Vaccinology Division, Health Sciences Post Graduation Program, University of Caxias Do Sul, Caxias Do Sul, Brazil, [31] Università degli Studi di Milano, Milan, Italy, [32] Department of Medicine and Surgery, Pediatric Clinic, Pietro Barilla Children's Hospital, University of Parma, Parma, Italy

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**Edited by:**
*Fabio Bagnoli, GlaxoSmithKline, Italy*

**Reviewed by:**
*Thorsten Demberg, Marker Therapeutics, United States*
*Selidji Todagbe Agnandji, Centre de Recherche Médicales de Lambaréné, Gabon*

***Correspondence:**
*Susanna Esposito*
*susannamariaroberta.esposito@unipr.it*

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Immunization during pregnancy has been recommended in an increasing number of countries. The aim of this strategy is to protect pregnant women and infants from severe infectious disease, morbidity and mortality and is currently limited to tetanus, inactivated influenza, and pertussis-containing vaccines. There have been recent advancements in the development of vaccines designed primarily for use in pregnant women (respiratory syncytial virus and group B *Streptococcus* vaccines). Although there is increasing evidence to support vaccination in pregnancy, important gaps in knowledge still exist and need to be addressed by future studies. This collaborative consensus paper provides a review of the current literature on immunization during pregnancy and highlights the gaps in knowledge and a consensus of priorities for future research initiatives, in order to optimize protection for both the mother and the infant.

**Keywords: group B *Streptococcus* vaccines, influenza, maternal immunization, pertussis, pregnant women, respiratory syncytial virus, tetanus**

## INTRODUCTION

Vaccination of pregnant women induces a vaccine-specific immune response in the mothers and the transfer of vaccine-specific antibodies via the placenta and breastmilk to directly protect the infant during the first months of life from the targeted pathogens (1, 2). The potential of maternal immunization in protecting young infants was made evident by tetanus vaccination during pregnancy contributing to the reduction in incidence of neonatal tetanus (3). This has also become evident by the decrease in the incidence of severe pertussis disease in young infants in countries that have implemented pertussis immunization programs in pregnancy (4–7).

During the last decade, an increasing number of countries have included vaccines for pregnant women in their national vaccination programs. Vaccination with tetanus-containing vaccines in pregnancy has been recommended for years in most low and middle -income countries (LMICs) (3), and pertussis and influenza vaccination programs for pregnant women have been more recently recommended in a number of high-income countries (HICs) and LMICs (**Figure 1**) (8, 9). Moreover, the prevention of respiratory syncytial virus (RSV) and group B *Streptococcus* (GBS) infections in infants through maternal vaccination has become a priority and a target for potential new vaccine candidates in trials and development (10–12).

To optimize the protection offered to mothers and infants by maternal immunization, several factors that can affect this strategy must be better understood (**Figure 2**). The goal of this consensus paper written by experts in infectious diseases, vaccination and maternal immunization from different world regions is to summarize current evidence in the field of immunization during pregnancy and to highlight the knowledge gaps and prioritize future research strategies in order to optimize protection for the mother, fetus and the infant.

## STUDY DESIGN

The main aim of this consensus paper is to discuss current knowledge regarding immunization during pregnancy and highlight the gaps that need to be addressed to ensure the highest protection for both the mother and their infants. References were identified through searches of PubMed for human studies published in English using the terms "immunization" or "vaccination" or "tetanus" or "tetanus disease" or "tetanus vaccine" or "pertussis" or "Tdap" or "pertussis immunization" or "pertussis vaccination" or "pertussis vaccine" or "Tdap vaccine" or "Tdap immunization" or "influenza" or "influenza vaccines" or "influenza immunization" or "maternal influenza vaccination" or "influenza vaccines in pregnancy" or "RSV" or "respiratory syncytial virus" or "GBS" or "GBS vaccine" or "Group B streptococcus" and "pregnancy." Articles resulting from these searches and relevant references cited in those articles were reviewed. References were also provided by authors. Outcomes assessed were safety, immunogenicity, efficacy, and effectiveness of immunization during pregnancy against tetanus, pertussis, influenza, RSV, and GBS diseases. After the initial review, a meeting was held in Italy to discuss the current literature and knowledge gaps. A consensus on the content was reached after multiple rounds of revision among the authors.

## ETHICS IN VACCINE TRIALS DURING PREGNANCY

Maternal immunization, and the use of medication in pregnancy in general, have been a focus of ethical deliberations for decades. Until recently, the ethical prevailing approach for immunization during pregnancy was based on the precautionary principle, which limits introduction of new intervention whose ultimate effects are uncertain. This precautionary principle-centered approach, combined with risk aversion among legal departments of vaccine manufacturers, led to exclusion of pregnant women from most vaccine trials for decades, leading to gaps in evidence of vaccine safety and efficacy among pregnant women. With an increasing focus on maternal immunization, there has been reconsideration of relevant ethical paradigms resulting in several recent developments in this area.

First, a report of the U.S. National Vaccine Advisory Committee's Working Group on Maternal Immunization

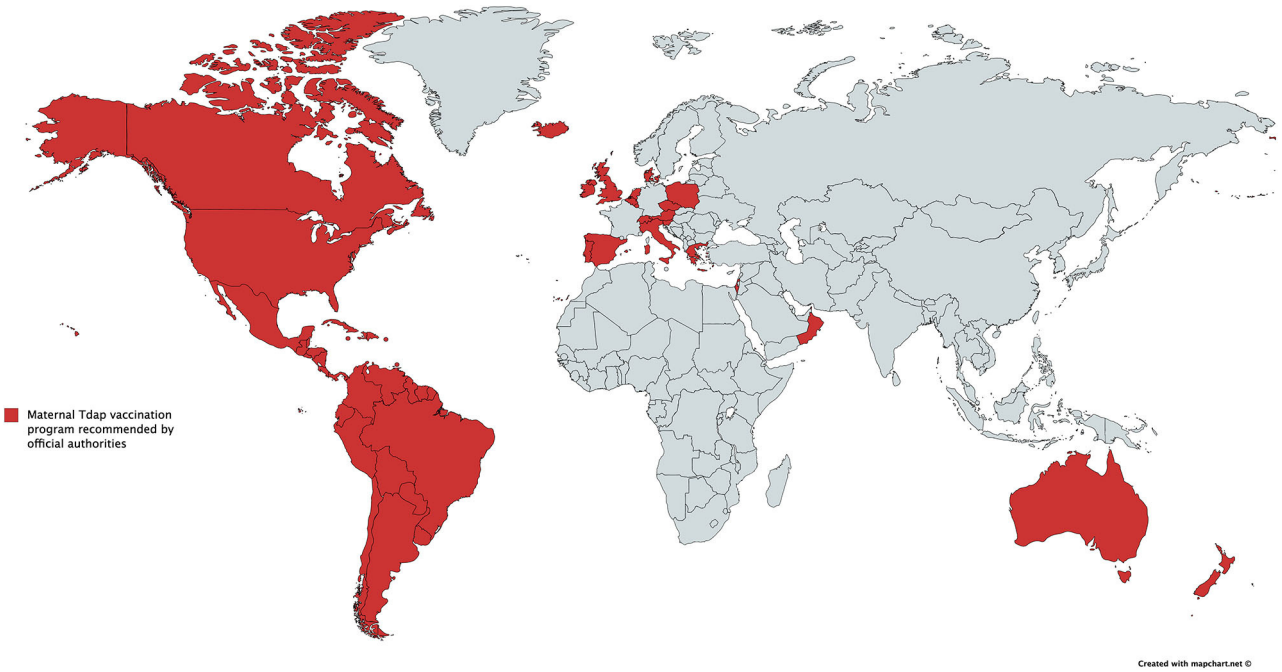


**FIGURE 1 |** Countries with recommendations for immunization against pertussis in pregnancy by official authorities (for South America, pertussis immunization during pregnancy is recommended by The Pan American Health Organization). This figure was inspired by G. Amirthalingham and K. Maertens and created by K. Maertens.

recommended that "Relevant regulations, statutes, and policies…should be modified to indicate that pregnant women are not a vulnerable population for the purposes of ethical review" (13). This recommendation and concurrent policy action led to a change in the U.S. Code of Federal Regulations which had previously classified pregnant women as being inherently vulnerable to coercion (14). Second, recognizing that conventional paradigms often treated the risks and benefits of maternal immunization to mothers and infants as independent entities, a maternal interest-based paradigm was proposed by Chamberlain et al. (15). This paradigm recognizes the legitimacy of maternal interests in protecting their infants and the legitimacy of her taking measures that benefit only the fetus/newborn even if such measures do not have direct benefits for the mother herself. The third major development was creation of the Pregnancy Research Ethics for Vaccines, Epidemics, and New Technologies (PREVENT) working group. This multidisciplinary, international team of 17 experts developed a roadmap for inclusion of the interests of pregnant women in the development and deployment of vaccines (16). The underlying goal of these recommendations was to ensure that pregnant women's inclusion in vaccine trials is the default position and that any exclusions need to be justified rather than justifications being needed for inclusion of pregnant women.

Globally, a progress has also been made in the prioritization of immunization in pregnancy and the inclusion of pregnant women in vaccine trials. The WHO Strategic Advisory Group of Experts on Immunization (SAGE) recommended in 2012 that pregnant women should be highly prioritized for influenza vaccination in countries that consider initiating or expanding of seasonal influenza vaccine programs (17). In 2015, SAGE further emphasized the importance of the platform of immunization in pregnancy, as well the need to strengthen the delivery of vaccines administered during pregnancy (18).

These and other developments in ethical considerations for maternal immunization are likely to result in a more conducive environment for maternal immunization research and deployment. However, there are a few areas that require further deliberations (**Table 1**).

## SAFETY OF IMMUNIZATION DURING PREGNANCY

Safety of vaccines administered during pregnancy needs to be evaluated for both the mother and her newborn, and is an important consideration for the mothers' willingness to receive a vaccine during pregnancy. There is a significant bulk of evidence to support the safety of immunization with tetanus toxoids (TT), the longest standing vaccine that is recommended during pregnancy. There is also an increasing body of evidence to support the safety of pertussis and influenza immunization during pregnancy (see below specific sections). However, continuous assessment and reporting of adverse events after immunization during pregnancy remains important, especially for relatively newly introduced maternal vaccines (e.g., pertussis), as it informs about rare events that might follow immunization. In addition, assessment of baseline pregnancy outcomes in unvaccinated women in different world regions

MATERNAL ACCEPTANCE
- Perception of risk / severity of infection
- Access to vaccine provider
- Cost / health insurance

HEALTHCARE WORKER ACCEPTANCE
- Knowledge of recommendations
- Vaccine access and storage
- Reimbursement

MATERNAL IMMUNE RESPONSE TO VACCINATION

TRANSPLACENTAL TRANSFER OF VACCINE-SPECIFIC ANTIBODIES AND THEIR FUNCTION

INTERFERENCE WITH SUBSEQUENT INFANT IMMUNE RESPONSE TO VACCINATION

MATERNAL CLINICAL CONDITIONS
- Malaria, HIV infection, gestational hypertension, smoking

VACCINE SAFETY / ADVERSE EVENTS

TIMING OF IMMUNIZATION
- To achieve optimal immunity in mother and /or infant

GEOGRAPHICAL LOCATION
- Different circulating pathogen strains
- Different responses to vaccination
- Different local recommendations

SEASONALITY OF PATHOGENS TARGETED BY IMMUNIZATION
- Influenza, RSV

INDUCTION OF VACCINE-SPECIFIC ANTIBODIES IN BREAST MILK

**FIGURE 2 |** A summary of the major factors affecting vaccination in pregnancy. Created by Claudio Rosa.

and settings will help in establishing baselines to assess safety outcomes against.

Furthermore, there is significant heterogeneity and lack of consensus on adverse event reporting in maternal immunization studies. This is a challenge for comparing and pooling data from different studies. In an attempt to overcome this weakness, WHO and the Brighton Collaboration worked together to provide written guidance on how to conduct safety studies in the field of maternal immunization (19). The initiative termed the Global Alignment of Immunization Safety Assessment in Pregnancy (GAIA) project worked on standardizing the assessment of safety of vaccines in pregnancy with specific focus on LMICs (20). Specifically, this initiative proposed systematic data collection, specific case definitions of key obstetric and neonatal health outcomes, ontology of key terms and a map of pertinent disease codes. More recently, case definition and guidelines for data collection, analysis and presentation has been proposed for neonatal seizures, neurodevelopmental delay, chorioamnionitis and post-partum endometritis and infection by the GAIA and Brighton collaboration working groups (21–24). Future studies assessing safety of immunization during pregnancy should use the proposed terms and definitions. In addition, currently available data on safety of vaccination in pregnancy is derived from vaccines that were initially licensed in non-pregnant populations. Future vaccine trials will likely assess vaccines intended to be licensed primarily for use in pregnant women. This further emphasizes the need to standardize reporting of safety outcomes in maternal immunization trials. Thus, we recommend following the GAIA and Brighton collaboration guidelines

**TABLE 1 |** Ethics areas related to immunization during pregnancy that require further deliberations.

| |
|---|
| Are there differential ethical considerations based on the gestational week of vaccination? |
| How is acceptable risk defined in pregnancy? |
| Can countries justify mass deployment of vaccines for use during pregnancy without an injury compensation program? |

for assessment and reporting of safety outcomes in maternal immunization trials.

# KEY FACTORS THAT INFLUENCE IMMUNOGENICITY AND EFFICACY/EFFECTIVENESS OF IMMUNIZATION DURING PREGNANCY

## Immune Responses of Pregnant Women to Vaccination

The immune system of a pregnant woman is adapted to allow for the survival of the semi-allogeneic fetus. Serum estradiol levels increase up to 500-fold during normal pregnancy (25), and the interplay between sex hormones and the maternal immune system in pregnancy is complex (**Table 2**). These changes might lead to the assumption that there are differences in immune responses to vaccines between healthy pregnant and non-pregnant women potentially leading to a lower immune response in pregnant women. However, studies comparing immunogenicity of vaccines in pregnant and non-pregnant women have generally not demonstrated decreased antibody responses in pregnant women. This has been the case for TT (40) and for the pertussis antigens in the combined tetanus, diphtheria, and acellular pertussis vaccine (Tdap) (41). However, results for influenza vaccines have been less consistent. Some studies carried out with influenza vaccines, including both the pandemic H1N1/2009 (pH1N1/2009) monovalent inactivated vaccine (MIV) and seasonal trivalent inactivated vaccine (TIV) preparations, show similar hemagglutination inhibition (HAI) seroconversion rates and antibody titers in pregnant and non-pregnant women (42–44). Other studies showed lower seroconversion rates and lower HAI geometric mean titers after vaccination of pregnant women when compared to non-pregnant women (45–47).

The effect of maternal immunization on cellular immunity has been less studied limiting conclusions. Proliferative and interferon-γ responses to the *Bordetella pertussis* (*B. pertussis*) antigens pertussis toxin (PT) 1 month after receipt of Tdap vaccine were not significantly different in pregnant and non-pregnant women and were comparable in both after 1 year (41). A small study showed that Natural killer cell and T-cell responses to inactivated influenza vaccination (IIV) were higher in a pregnant women compared to non-pregnant women (48).

**TABLE 2 |** Key changes in maternal adaptive immune system during pregnancy.

| Main changes | References |
|---|---|
| Lower B cell levels in pregnant women compared with non-pregnant women | (26) |
| B cell lymphopenia in the third-trimester of pregnancy | (25) |
| Estrogen reduces B cell lymphopoiesis during pregnancy | (27, 28) |
| Decrease in B cell function | (29) |
| Decreased total IgG levels, especially during late pregnancy | (30, 31) |
| High estradiol levels promote T helper 2 cell responses | (32–34) |
| Elevated progesterone during pregnancy inhibits T helper 1 cell immune responses | (35) |
| A progressive shift from T helper 1 cell to T helper 2 cell responses | (36) |
| Decrease in T cell function | (37–39) |

## Trans-placental Transfer of Maternal Antibodies

Immunoglobulin G (IgG) is the dominant immunoglobulin isotype that crosses the placenta and contributes to maternally derived passive immunity during early infancy. In healthy pregnant women, IgG transfer across the placenta begins toward the end of the first trimester of pregnancy and increases as pregnancy progresses. IgG concentrations in the fetus are 5–10% of the maternal levels at 17–22 weeks gestation, 50% at weeks 28–32, and usually exceed maternal levels by 20–30% at term (49–52). The transplacental transfer of IgG is mediated by the neonatal Fc receptors (FcRn), localized in the syncytiotrophoblast that covers the villous tree of the placenta (53). FcRn regulates IgG transplacental transfer through binding to its constant domain and actively transport IgG into the fetal circulation. Several factors appear to affect the transfer of IgG across the placenta. IgG subclasses have differential efficiency of transfer across the placenta, defined as the antibody levels in the newborn divided by antibody levels in the mother. Based on studies from the 1990s, IgG1 is the subclass transferred with the highest efficiency, achieving higher levels in cord compared with the maternal blood, and this subclass is induced by vaccines containing protein antigens (54). IgG2 is transferred with the least efficiency, achieving lower cord than maternal blood levels, and is the dominant antibody induced by vaccines containing polysaccharide antigens (53, 55–58). Transfer of antibodies across the placenta can also be influenced by several clinical conditions in the mother and some of these health conditions are more prevalent in certain parts of the world. For instance, cord IgG levels were lower in infants from women with human immunodeficiency virus (HIV) infection (59), malaria infection (60), and hypergammaglobulinemia (61), compared with infants from women without those conditions, and these conditions are more prevalent in LMICs. In addition, the potential effect of toxoplasma and tuberculosis infection on the transfer of maternal antibodies has not been investigated. Furthermore, other maternal conditions, that have not yet been investigated, might also affect the structure of the placenta (e.g., gestational hypertension, gestational diabetes, smoking) and the transfer of maternal antibodies.

## Timing of Immunization

A number of factors should be considered when determining the ideal timing of vaccination in pregnancy including time-dependent safety when administered at different time points in gestation, time-dependent efficiency of transplacental transfer of vaccine-induced antibodies, interference with infants' immune response to vaccination and clinical efficacy/effectiveness (62, 63). Furthermore, the optimal timing of maternal immunization varies depending on who is the target for protection and when maximal protection is desired in the mother and/or the fetus/infant (62). For example, pregnancy is a well-known risk factor for severe influenza, being most severe during the third trimester of pregnancy (64, 65). Therefore, to maximize the protection for the mother, it is best to administer the influenza vaccine early in pregnancy and ideally prior to the peak of influenza seasonal activity. If the primary goal is to protect the infant, as for pertussis, the vaccine should be administered during a time period in gestation to provide optimal trans-placental transfer of antibodies, in order to ensure maximal protection against pertussis disease in early infancy (66). The risk for premature labor should also be considered as this population is at an increased risk for severe infections, such as pertussis and might not benefit from maternal vaccination if it happens late in gestation (67–69).

Based on the literature review and consultation among authors, a consensus on priorities for future research related to factors affecting immunization during pregnancy was reached (**Table 3**).

**TABLE 3 |** Consensus on priorities for future research related to factors that influence the immunogenicity and efficacy/effectiveness of immunization during pregnancy.

| |
|---|
| **Immune responses of pregnant women to vaccination** |
| 1. Immune response (quantity and quality of cellular and antibody immune responses) of pregnant women to vaccines with potential use in pregnancy in comparison with non-pregnant women |
| 2. Immune response (quantity and quality of cellular and antibody immune responses) of pregnant women to vaccines with potential use in pregnancy at various stages of pregnancy in comparison with non-pregnant women |
| **Trans-placental transfer of maternal antibodies to fetus** |
| 1. Create a better understanding of the molecular and cellular basis of maternal antibody transfer across the placenta, based on currently available vaccines for use in pregnancy, which would help the design of future vaccines that induce antibodies with optimal characteristics for transfer to the fetus |
| 2. The induction of different vaccine-induced IgG subclasses should be evaluated early in the development of new vaccines designed for pregnant women |
| 3. The effect of maternal health conditions on the transfer of vaccine-induced IgG subclasses should be assessed early in the development of new vaccines designed for pregnant women. This is especially important for some health conditions more prevalent in low-middle income countries such as poor nutrition, human immunodeficiency infection, malaria infection and hypergammaglobulinemia |
| **Timing of immunization during pregnancy** |
| 1. The main target for protection in pregnancy (i.e., pregnant women and/or infant) and the time in gestation and/or infancy this maximal protection is desired have to be clearly defined for individual pathogens targeted for immunization |
| 2. The safety of vaccination when administered in different stages during gestation |
| 3. Time-dependent efficiency of transplacental transfer of vaccine-induced antibodies (quantity and quality) |
| 4. Time-dependent clinical efficacy/effectiveness (for both term and preterm infants) |

# VACCINES CURRENTLY RECOMMENDED FOR PREGNANT WOMEN

Vaccines currently recommended and used are aimed to protect against tetanus, pertussis and influenza diseases. Different vaccine formulations and dosages exist for use in pregnant women in selected countries in Europe, North America, South America, and Asia (**Table 4**).

## Vaccines Against Tetanus

The World Health Organization (WHO) recommends that if a pregnant woman has never received a tetanus-toxoid - containing vaccine (TT-CV) (e.g., Diphtheria-Tetanus-Pertussis [DTP], Diphtheria-Tetanus [DT], Tetanus-diphtheria [Td], TT) or her vaccination status is unknown, she should receive two TT (or Td) vaccine doses 4 weeks apart during pregnancy, with the second dose given at least 2 weeks before delivery. Based on WHO recommendations, five total doses are likely needed for protection throughout the childbearing years so a third dose is given 6 months after the second dose, and two additional doses are recommended to be given during the next 2 years or during two subsequent pregnancies (73). For women who have received 1–4 TT-CV doses prior to their pregnancy, one TT-CV dose is recommended during each subsequent pregnancy to a total of five doses. However, this vaccination schedule and policy has never been formally evaluated in clinical trials.

### Safety

Several studies have demonstrated TT-CVs to be safe in pregnancy (74–76). As the current pertussis-containing vaccines administered in pregnancy are part of multicomponent formulations that include TT, safety assessments of pertussis-containing vaccines in pregnancy also provide information on the safety of the TT component (see below discussion under pertussis vaccines) (77). Safety was demonstrated even when the most recent TT-CV was administered within 2 years prior to vaccination in pregnancy (76).

### Immunogenicity

Several studies have shown that following maternal immunization with TT-CVs, anti-TT IgG is actively transferred across the placenta, leading to protective levels in the infant (77–80). Vaccination with TT induces IgG1 (54, 81), which are efficiently transferred across the placenta. Approximately 80% of maternal antibodies remain present in infants 1 month after delivery; thus, protection is maintained until a primary vaccination course is commenced and is maximal during the most vulnerable period when umbilical infections may occur (82).

**TABLE 4 |** Formulations and dosages of common vaccines to protect against pertussis, and tetanus disease for use in pregnant women in selected countries in Europe, North America, South America, and Asia.

| Vaccine formulation | Antigen composition | References | Selected countries # |
|---|---|---|---|
| **Against tetanus** | | | |
| Td (MassBiologics) | Diphtheria toxoid: 2 Lf<br>Tetanus toxoid: 2 Lf | (70) | South America: Honduras<br>Asia: Thailand, Philippines, Malaysia<br>Africa: Egypt, Gambia, Senegal, Gabon, Cameron, Botswana |
| TT adsorbed (Serum Institute of India) | TT $\geq$ 5 Lf | (71) | |
| **Against pertussis** | | | |
| Tdap (Adacel, Sanofi Pasteur) | Diphtheria toxoid: 2 Lf<br>Tetanus toxoid: 5 Lf<br>PT: 2.5 μg<br>FHA: 5 μg<br>PRN: 3 μg<br>FIM: 5 μg | (70) | Europe: Belgium, Spain, United Kingdom, Italy<br>North America: Canada, United States of America<br>South America: Argentina, Brazil, Columbia, Chile, Mexico, Uruguay<br>Asia: Singapore<br>Africa:<br>Australia and New Zealand |
| Tdap (Boostrix, GlaxoSmithKline) | Diphtheria toxoid: 2.5 Lf<br>Tetanus toxoid: 5 Lf<br>PT: 8 μg<br>FHA: 8 μg<br>PRN: 2.5 μg | | |
| **Against influenza*** | | | |
| **Quadrivalent inactivated influenza vaccin**<br>Afluria Quadrivalent (Seqirus)<br>FluLaval Quadrivalent (GlaxoSmithKline)<br>Flucelvax Quadrivalent (Seqirus)<br>Fluzone Quadrivalent (Sanofi Pasteur) | Influenza A/Brisbane/02/2018 (H1N1)pdm09-like virus<br>Influenza A/Kansas/14/2017 (H3N2)-like virus<br>Influenza B/Colorado/06/2017-like (Victoria lineage) virus<br>Influenza B/Phuket/3073/2013–like virus (Yamagata lineage)<br>Dosage: Hemagglutinin 15 μG/dose (each virus) | (72) | Europe: Albania, Belgium, Hungary, Romania, Russian Federation, Spain, Sweden, United Kingdom, Italy<br>North America: Canada, United States of America<br>South America: Argentina, Brazil, Columbia, Ecuador, Bolivia, Mexico, Uruguay<br>Australia and New Zealand<br>Asia: Singapore, Thailand<br>Africa: South Africa, Algeria |
| **Trivalent inactivated influenza vaccine** | | | |
| Fluad (Seqirus) | Influenza A/Brisbane/02/2018 (H1N1)pdm09-like virus<br>Influenza A/Kansas/14/2017 (H3N2)-like virus<br>Influenza B/Colorado/06/2017-like (Victoria lineage) virus<br>Dosage: Hemagglutinin 15 μG/dose (each virus) | (72) | |

*TT, Tetanus toxoid; Td, Tetanus diphtheria; Tdap, tetanus-diphtheria-acellular-pertussis; Lf, limit of flocculation; PT, pertussis toxin; FHA, filamentous hemagglutinin; PRN, pertactin; FIM, fimbria 2/3.*

**Influenza vaccines compositions are reviewed each year and updated as needed. Composition presented is for 2019-20 influenza season.*

*#Source: World Health Organization website: https://apps.who.int/immunization_monitoring/globalsummary.*

If a Tdap vaccine in pregnancy is being considered to replace a single dose of TT vaccine in some settings, in order to provide dual coverage for pertussis and tetanus disease, it is important to assess the immunogenicity of Tdap in inducing anti-TT IgG compared with TT or Td formulations. In a small study from Vietnam, vaccination with Tdap in pregnancy resulted in higher cord anti-TT IgG levels compared with vaccination with TT, however, this difference did not persist at 2 months of age (83). These results are reassuring that replacing TT with Tdap is not expected to result in inferior immunogenicity against tetanus.

## Effectiveness

Both maternal and neonatal tetanus were very common in most developing countries even into the 1980's. In 1989, the WHO called for the elimination of maternal and neonatal tetanus by the end of the century. At that time, 59 countries reported maternal and neonatal tetanus. As part of the MNTE program, and along with safer birth techniques and effective immunization strategies in children and adults, more than 150 million women were vaccinated against tetanus during pregnancy. Altogether, these practices contributed to the elimination of maternal and neonatal

**TABLE 5 |** Consensus on priorities for future research related to vaccination against tetanus during pregnancy.

| **Vaccines against tetanus disease** |
|---|
| **Immunogenicity** |
| 1. The immunogenicity of different Tdap formulations in pregnancy compared with TT and Td in countries where TT/Td immunization is given in pregnancy and Tdap immunization in pregnancy is being considered |
| 2. Immunogenicity of different dosing regimens (number of doses) of tetanus vaccination during pregnancy, especially in settings where vaccination against tetanus in childhood is high |

*TT, Tetanus toxoid; Td, Tetanus diphtheria; Tdap, tetanus-diphtheria-acellular-pertussis.*

tetanus in 45/59 countries as of the end of 2018 (84, 85). However, 14 countries, mainly in Africa, still have residual maternal and neonatal tetanus, highlighting that additional efforts are required to extend maternal immunization, immunization of children and adolescents, and other hygienic measures aimed at improved cord-care. The WHOs most updated goal is to achieve maternal and neonatal tetanus elimination by 2020 which will be difficult to achieve (85).

Based on the literature review and consultation among authors, a consensus on priorities for future research related to immunization against tetanus during pregnancy was reached (**Table 5**).

## Vaccines Against Pertussis

### Safety

Data on tolerability and safety of pertussis immunization during pregnancy are reassuring (86). This has been demonstrated with different Tdap vaccine formulations regardless of the number of pertussis antigens included in the vaccines (77, 87–96). Specifically, no increased risk for the development of severe maternal adverse events (e.g., postpartum endometritis, preterm delivery, and preterm premature rupture of membranes) or fetal and neonatal outcomes (e.g., low birth weight, very low birth weight, small for gestational age, birth defects, and need for neonatal intensive care unit admission) has been reported. However, a small increased risk of chorioamnionitis among Tdap-vaccinated women (relative risk [RR] 1.19, 95% CI, 1.13 to 1.26) was documented in one study (89). In another study using the Vaccine Adverse Event Reporting System database, the majority of these women with chorioamnionitis had at least one risk factor for this complication (97). In addition, there was limited supportive evidence for a chorioamnionitis diagnosis on chart review and the risk of preterm birth (a concern after chorioamnionitis) was not higher among Tdap recipients. Therefore, the association between this complication and vaccination during pregnancy has been debated. However, a recent study reported a small increase of chorioamnionitis in pregnant women who received Tdap vaccine during pregnancy with a RR of 1.11 (95% CI: 1.07–1.15); but the absolute risk was still quite low, at 2.8% (98). Ongoing studies are currently evaluating the potential association between Tdap vaccination during pregnancy and chorioamnionitis. In view of the recommendation to vaccinate against pertussis during each pregnancy, it has been shown that repeated Tdap vaccinations in consecutive pregnancies are well-tolerated (76).

### Immunogenicity

Vaccination against pertussis in pregnancy has been achieved using Tdap formulations that include mostly three or five *B. pertussis* antigens. Antibodies against all *B. pertussis* antigens included in the Tdap vaccine have been shown to reach peak levels at the end of the second week after Tdap administration in non-pregnant women of childbearing age, and this peak is followed by a rapid decline (99). In pregnant women, studies have shown a significant increase in *B. pertussis*-specific antibody levels 1 month after Tdap vaccination, also with a significant decline, within the first year after maternal vaccination (41, 100, 101). Thus, the persistence of antibodies after a single dose of Tdap vaccine in pregnancy is short and does not probably ensure infant protection during consecutive pregnancies. Therefore, vaccination is currently recommended in every pregnancy.

Vaccination with *B. pertussis* antigens induces mainly IgG1 antibodies (102, 103) which are actively across the placenta to the newborn resulting in higher antibody levels in the term newborn than in the mother (77, 80, 104).

Pertussis toxin is a major virulence factor of *B. pertussis* and is potentially responsible for both local and systemic responses (105). Administration of humanized neutralizing anti-PT monoclonal antibodies have been shown to abolish disease manifestations in mice and non-human primates (106). Maternal immunization with a monocomponent PT vaccine protected newborn baboons against pertussis following respiratory challenge with *B. pertussis* (107). In human, low anti-PT IgG levels have been associated with high susceptibility to pertussis (108). However, antibody levels that confer protection against human pertussis disease have not been defined. In addition, the number and type of *B. pertussis* antigens are required for pregnant women in order to provide clinical protection to the infant has not been clearly established.

### Timing

A study conducted in Thailand showed that vaccination earlier in pregnancy was associated with higher *B. pertussis*-specific cord antibody levels (109). Furthermore, three other studies found that vaccination during the early third trimester of pregnancy is associated with higher cord anti-*B. pertussis*-specific IgG levels than immunization during late third trimester (110–112). In addition, one study showed that anti-PT and anti-FHA IgG levels were higher in cord blood of mothers vaccinated between 13 and 25 weeks gestation compared to those immunized after 25 weeks gestation (113). This was also observed in preterm infants (114). In addition, avidity of cord anti-PT IgG was higher when mothers were vaccinated in the early third trimester compared with late third trimester (115, 116), although this finding was not observed in a third study (117). Therefore, more data are needed to address this controversy, including also data from vaccination at earlier time points in pregnancy. Moreover, because the role of antibody levels and avidity in protection against pertussis is not conclusive to date, interpretation of the above studies requires caution.

**TABLE 6 |** Consensus on priorities for future research related to vaccination against pertussis disease during pregnancy.

| |
|---|
| **Safety** |
| 1. The association between receipt of Tdap in pregnancy and chorioamnionitis |
| **Immunogenicity** |
| 1. Assessment of immune correlates for protection against pertussis disease (e.g., *Bordetella pertussis* –specific antibody levels) |
| 2. *Bordetella pertussis* antigens to be included in pertussis vaccines for maternal immunization to provide sufficient clinical protection to the infant |
| 3. The need for immunization against pertussis disease in subsequent (3rd or more) pregnancies. |
| 4. Comparative studies comparing different pertussis vaccine formulations (e.g., Tdap vs. aP stand-alone vaccines) |
| 5. Role of previous vaccination of the mother with whole cell or aP vaccines in the immune response to maternal pertussis vaccination |
| **Timing** |
| 1. The effect of timing of vaccination on the function of anti-B*ordetella pertussis* antibodies transferred to infants |
| 2. The immunogenicity of stand-alone aP given in different times in pregnancy |
| **Effectiveness** |
| 1. Burden of pertussis disease in infancy in low and middle-income countries |
| 2. The effectiveness of maternal immunization program in low and middle-income countries if pertussis immunization in pregnancy is implemented |
| 3. Assess the eventual role of previous vaccination with whole cell or acellular pertussis to the mother on vaccine effectiveness |
| 4. Vaccine effectiveness of various Tdap formulations |

*Tdap, tetanus-diphtheria-acellular-pertussis; aP, acellular pertussis.*

### Effectiveness

Effectiveness of maternal immunization for prevention of pertussis in young infants has been well-studied. In England, vaccine effectiveness was 91% in the reduction of laboratory-confirmed cases in infants <3 months of age (6), and 93% in prevention of laboratory-confirmed cases in infants <8 weeks of age (118). In the US, effectiveness among infants <8 weeks of life ranged between 85 and 91% (7, 119, 120). In addition, disease was significantly less severe among infants from vaccinated mothers (119). In Spain, a case-control study reported VE to be 90% against laboratory-confirmed pertussis infection in infants <3 months of age (121), while in Australia it was 69% in infants <3 month of age (122). In Brazil, vaccine effectiveness was reported to be 82.6% for the prevention of clinical pertussis in infants <2 months of age, confirming the success of the maternal pertussis immunization strategy also in middle-income countries (123).

Based on the literature review and consultation among authors, a consensus on priorities for future research related to immunization against pertussis during pregnancy was reached (**Table 6**).

## Vaccines Against Influenza

### Safety

There is an extensive body of evidence in the literature from both HICs and LMICs that confirm the safety of maternal influenza vaccination (124–129) [reviewed in (130)]. During the H1N1 influenza pandemic, data from Sweden and Argentina found that both AS03-adjuvanted and MF59-adjuvanted -H1N1 influenza vaccines were not associated with increased risk for low-birth weight or preterm birth or low Apgar score (131, 132). A meta-analysis including studies using both adjuvanted and non-adjuvanted influenza vaccines found lower estimates of still birth after maternal influenza vaccination and no association with an increased risk of spontaneous abortion (133). However, a small case-control study in the US over two influenza seasons (2010–11, 2011–12) found an increased risk of early spontaneous abortion in a group of women who had received influenza vaccination in the first trimester of pregnancy, although cases had other risk factors for spontaneous abortion (older age, pervious history of spontaneous abortion, smoking); thus the causal relationship between influenza vaccination and this complication has been questioned (134). To further support the safety of influenza vaccination in pregnancy, three Bill & Melinda Gates Foundation funded studies from South Africa (135), Mali (136), and Nepal (137), and recent studies and systematic reviews found that maternal influenza vaccination was not associated with an increased risk of fetal death, spontaneous abortion, or congenital malformations (138–141).

Furthermore, concomitant or sequential vaccination with Tdap and influenza vaccines has also been shown to be safe and not associated with differences in medically attended acute events in pregnant women or adverse birth outcomes (142).

### Immunogenicity

Influenza vaccination preferentially induces IgG1 subclass antibodies (143), and studies have shown increased levels of influenza-specific hemagglutinin antibodies in neonates born to women given a monovalent (pH1N1/09) or seasonal TIV during pregnancy, suggesting efficient transplacental transfer of influenza-specific antibodies (144–146). Importantly, seroconversion rates were lower after administration of TIV in women living with HIV than in women without HIV, and hemagglutination-inhibiting antibodies (HIA) titers were lower in HIV-exposed infants (146).

The kinetics of influenza antibody decline in the infant vary according to the influenza virus and the levels of transferred antibodies, and thus the duration of protection is not precisely defined. Some data indicate that maternally-derived HIA against seasonal influenza viruses have a half-life of approximately 45 days in infants after maternal vaccination and that these antibodies decline to levels similar to those detected in infants born to unvaccinated women by 16 weeks of age (147, 148). This is consistent with higher protection from laboratory-confirmed influenza disease among infants of vaccinated mothers during the first 2–3 months of age (135, 136).

In another study, children born to mothers vaccinated with an adjuvanted pH1N1 vaccine had antibody levels that remained elevated above the correlate of protection for adults (HIA titer > 1:40) up to 5 months (149). However, the interpretation of influenza immunogenicity studies are complicated as the correlate of protection against infection in infants has not yet been established and is likely to be different and higher than the correlate of protection used for adults (150). This is an area of controversy, where more research is needed to define the correlate(s) of protection against influenza disease in infants,

which is important as currently available pediatric influenza vaccines are recommended in certain settings from 6 months of age onwards.

### Timing

The optimal timing for maternal influenza immunization has not been established, and recommendations (e.g., CDC, ECDC, WHO) allow administration at any time during pregnancy (17, 151, 152). Importantly, since influenza is a seasonal disease (except in tropical regions, where influenza disease may occur throughout the year) and the goal of vaccination also serves to protect the mother, the actual determination of timing may depend on factors other than optimizing antibody transfer to the infant. Jackson et al. reported lower antibody levels at birth in infants of mothers vaccinated earlier during pregnancy (144). On the other hand, Sperling et al. did not find a significant association between the gestational age at vaccination and the seroconversion rates following influenza vaccination in pregnant women. However, maternal seroconversion rates were slightly lower in women immunized in the first trimester than in those given the vaccine in the late third trimester (153). In another study, a higher level of transplacental transfer of antibodies was associated with a longer interval between vaccination and delivery in pregnant women vaccinated against influenza after 20 weeks gestation (146). Blanchard-Rohner et al. showed that receipt of influenza vaccine at least 2 weeks before delivery increased umbilical cord HIA titers and seroprotection rates in newborns (154). Finally, Katz et al. found no significant differences in influenza HIA titers in cord sera of women vaccinated early (17–25 weeks gestation) or later (26–34 weeks gestation) in randomized trials during pregnancy (155).

### Efficacy

Influenza can be a severe disease for pregnant women, neonates and young infants. The severity of infection increases as pregnancy advances, with the greatest maternal risk occurring during the third trimester of pregnancy (156, 157). Young infants on the other hand, have been shown to experience the highest rates of influenza-related hospitalization (158) and death (159) among children with influenza infection.

Multiple studies have shown that administration of an IIV during pregnancy reduces the risk of influenza in pregnant woman by ∼35–50% (135, 160–162). The efficacy of maternal influenza vaccination against laboratory-confirmed influenza in infants below 6 months of age also varies in different trials conducted at different geographic sites. Efficacy has been 63% (95% CI, 5–85) in Bangladesh (162), 49% (95% CI, 12–70) in South Africa (135), 33% (95% CI, 4–54) in Mali (136), and 30% (95% CI, 5–48) in Nepal (137). Efficacy against laboratory-confirmed influenza in infant was higher in the first 2–3 months of life and in the range of 70–80% in 2 RCTs from South Africa and Mali (135, 136). Observational studies carried out in the USA (163, 164) and England (165), reported reductions of laboratory-confirmed influenza in children born to vaccinated mothers ranged from 41 to 71%. A recent meta-analysis reported that maternal influenza vaccination reduced the risk of laboratory-confirmed influenza infection in infants by 48% (95% CI, 33–59) (166). In addition, maternal influenza vaccination was associated with a reduction in all-cause severe pneumonia in infants. An analysis of three Bill & Melinda Gates foundation -funded clinical trials conducted in Nepal, Mali and South Africa including 10,002 mothers and 9801 live-born eligible infants concluded that the pooled incidence rate of severe pneumonia up to 6 months of age was 20% lower in infants born to women vaccinated with IIV compared with infants born to women unvaccinated in pregnancy (incidence rate ratio [IRR]: 0.80; 95% CI: 0.66–0.99) (167). However, it should be noted that few of these cases had influenza identified despite testing suggesting that influenza vaccination during pregnancy might have benefits beyond the prevention of classical influenza disease.

The efficacy of IIV in pregnancy in the prevention of maternal and infant influenza disease varies depending on the setting as well as the match of the vaccine utilized to circulating influenza strains. The majority of efficacy data are derived from studies performed in LMICs when compared to HICs. While influenza disease is seasonal in countries with temperate climates (e.g., Europe, North America), there is no seasonal pattern in tropical countries.

Altogether, current data on safety, immunogenicity, and efficacy of maternal IIV vaccination, for the pregnant women and their infants has resulted in pregnancy as a potential indication in the vaccine label by the European Medicines Agency as of July, 2019 (168). In Australia, categorization of influenza vaccines given during pregnancy has changed to category A (no proven harmful effects) (169). Other individual countries will have their own considerations.

Based on the literature review and consultation among authors, a consensus on priorities for future research realted to immunization against influenza during pregnancy was reached (**Table 7**).

**TABLE 7 |** Consensus on priorities for future research related to vaccination against influenza disease during pregnancy.

| |
|---|
| **Immunogenicity** |
| 1. Correlate(s) of protection against influenza disease in infants |
| 2. The duration of protection conferred by vaccination in pregnancy in infants. This needs to take into account seasonality in different settings (tropical regions vs. temperate climate regions) |
| 3. Evaluation of more immunogenic influenza vaccines in pregnant women to optimize antibody transfer to their infants |
| **Efficacy/Effectiveness** |
| 1. The development of more immunogenic influenza vaccines to optimize protection of young infants |
| 2. Evaluate vaccine-efficacy against non-specific (all-cause) lower-respiratory tract infections |

## IMPACT OF MATERNAL IMMUNIZATION ON INFANTS' IMMUNE RESPONSES TO IMMUNIZATION

High levels of vaccine-induced maternally-derived antibodies have the potential to reduce the infants' humoral immune

responses by inhibiting antibody generation after the infant's own vaccination, leading to lower antibody levels/titers later on in the infant (170, 171). This phenomenon is called "interference" or "blunting" and has been described for the same vaccine antigens used by mother and infant, as well as for conjugated vaccines administered in infancy (172). Data from the 1990s showed that the administration of *Haemophilus influenzae* type b (Hib) polysaccharide or Hib conjugated vaccines in pregnant women was associated with mild inhibition of infants' immune responses to Hib conjugated vaccines (173). Differences in antibody responses in infants born to vaccinated compared with unvaccinated mothers were minimized following the booster dose. An analysis of the genetic repertoire of the light chain of antibodies to the polysaccharide vaccine demonstrated no differences between infants born to immunized women compared with non-immunized women (174). There was no evidence of inhibition of "priming" of the infants' immune system to Hib in these studies.

## Tetanus-Containing Vaccines

Most data on the impact on TT-CVs in infancy are derived from studies that used Tdap formulations in pregnancy and measured anti-TT IgG levels after infant vaccination. These studies found inconsistent results. Some showed significantly lower anti-TT levels after primary immunization in infants born to Tdap-vaccinated women compared to infants from unvaccinated women whilst other studies showed equal or even significantly higher anti-TT levels in infants born to Tdap-vaccinated women compared to infants from unvaccinated women (77, 80, 175–177). However, this inhibition found in some studies did not result in a reduction of the percentage of infants with seroprotective anti-TT antibody levels.

The effect of different TT-CV formulations used in pregnancy (Tdap vs. TT/Td) on immune responses to tetanus-containing vaccines in infancy is of importance in countries where a replacement of the existing tetanus vaccination program by a Tdap vaccination program is being considered. A small study in Vietnam reported higher anti-TT levels after primary immunization with tetanus-containing vaccines in infants born to Tdap-vaccinated pregnant women compared to infants born to TT-vaccinated pregnant women (83). A study from Canada found no difference in anti-TT levels after primary and booster immunization in infants born to Tdap-vaccinated pregnant women when compared to Td-vaccinated pregnant women (104). These data suggest that Tdap, when compared to TT or Td in pregnancy, is not associated with lower anti-TT IgG levels after primary and booster immunization with tetanus-containing vaccines in infancy. However, in order to provide a definite conclusion, formal studies should be conducted with the aim to address this question as the primary outcome.

Several vaccines are conjugated to TT as a carrier protein (e.g., Hib vaccines, meningococcal vaccines) and thus vaccine-induced immune responses to these vaccines in infant born to Tdap-vaccinated pregnant women might also be affected. Hib anti-polyribosylribitol phosphate (PRP) levels were higher after primary immunization with Hib TT-conjugated vaccine in infants born to Tdap-vaccinated pregnant women when compared to infants of unvaccinated mothers (175, 178).

One study found no differences between anti-Men C antibody levels after primary immunization with meningococcal C TT-conjugated vaccine in infant born to Tdap-vaccinated when compared to unvaccinated pregnant women (178). More studies are needed to investigate the potential effect of tetanus-containing vaccines administered in pregnancy on infants' immune response to vaccines conjugated to TT.

## Pertussis Vaccines

Studies have shown that Tdap immunization in pregnancy is associated with decreases in humoral immune responses to infants' immunization with acellular pertussis (aP) containing vaccines. Several studies describe significantly lower anti-PT IgG levels in infants born to Tdap-vaccinated pregnant women after the completion of primary immunization, while results were less consistent after booster immunization (77, 80, 83, 104, 175–177). Results from these studies showed also interference to other pertussis antigens (FHA, pertactin, fimbria 2/3) after primary immunization while results were inconsistent after booster immunization.

Most studies investigating the potential modification of infants' immune responses to aP vaccines have been performed in HICs, with the exception of one study from Vietnam (83, 177). It is important to note that the degree of reduction in immune responses to wP infant vaccines might be different than to immunization with aP infant vaccines. The use of wP vaccines but not aP vaccines was associated with a substantial reduction in the subsequent infant antibody response to PT in infants born to mothers with high levels of maternally-derived anti-PT antibodies (179). In another study, there was no correlation between low anti- *B. pertussis* antibody levels at delivery in infants born to unvaccinated women and their anti-*B. pertussis* antibody levels after wP vaccination (180).

A recent study reported that Thai infants born to unvaccinated mothers and subsequently vaccinated with wP vaccines, had higher anti-*B. pertussis*-specific antibody levels after primary and booster vaccination than infants born to women vaccinated with Tdap during pregnancy and vaccinated with wP vaccines (181). In addition, infants born to women vaccinated with Tdap in pregnancy and vaccinated with wP vaccines had lower anti- *B. pertussis*-specific antibody levels after vaccination when compared with infants born to vaccinated mothers and vaccinated with aP vaccines (181).

Altogether, these results indicate that infants born to Tdap-vaccinated mothers might be at increased risk for pertussis later in life. However, surveillance data from the US and UK did not demonstrate any increase in the number of pertussis cases later in infancy after the introduction of the maternal immunization program suggesting a possible lack of clinical significance of this interference (6). Interpretation of interference to wP immunization is more challenging in LMICS compared with HICs due to the lack of comprehensive surveillance systems in some countries (182).

Because vaccines against pertussis that are currently used in pregnancy also contain dT, interference might also be extended to

diphtheria-containing vaccines administered in infancy. Data on this respect have been inconsistent, with some studies reporting significantly lower anti-diphtheria toxin antibody levels in infants born to Tdap-vaccinated women when compared to infants born to unvaccinated women, while other studies did not report this effect (77, 80, 83, 104, 175–177). It is also important to note that Tdap immunization in pregnancy, likely due to anti-DT antibodies, is associated with lower anti-pneumococcal capsular polysaccharide levels after immunization with pneumococcal vaccines (PCVs) conjugated to a non-toxic diphtheria toxin mutant (CRM197), although, this did not result in lower seropotection levels for most serotypes (175, 183). Surveillance will be key to assess whether this interference has any impact on pneumococcal disease burden.

If long-term surveillance data would indicate that interference is clinically significant, strategies to mitigate the effect of interference will need to be evaluated. Timing of vaccination in pregnancy is an important modifiable variable and should be investigated. Delaying primary infant vaccination is another approach and has been recently implemented in The Netherlands in infants born to Tdap-vaccinated mothers. In addition, stand-alone pertussis vaccines (without TT, dT) should be investigated in clinical trials (184) as these vaccines might lessen the concern of interference to TT and DT components and vaccines conjugated to those proteins as carrier proteins.

### Influenza Vaccines

Data on the potential impact of maternal influenza immunization on the immune response of infants to their immunization against influenza are scarce as influenza vaccines are administered in infants older than 6 months, when most maternally-derived antibodies already have waned from infant's circulation. Earlier studies performed to assess immunogenicity of influenza vaccination in infants younger than 6 months old found that post vaccination seroprotection rates (titer $\geq$ 1:40) were higher in infants who received IIV at 6 months of age when compared to infants who received vaccination during 6–12 weeks of age (185). Another prospective, open-label study in which 2 doses of a TIV were administered to healthy infants aged 3–5 months found a 4-fold increase in antibody titers to be significantly more common in children who were seronegative (pre-vaccination titers <1:8) at enrollment than in those with pre-vaccination titers $\geq$1:8 (186).

### Mechanism of Interference

Mechanism of interference between maternally-derived antibodies and infant's immune responses to subsequent immunizations has not been fully explored (187). Some proposed mechanisms include inhibition of B cell response to vaccine antigens through epitope masking by maternal antibodies (172) and neutralization of vaccine antigens (187, 188). Inhibition of B cell activation through crosslinking of FcγRIIB to the B-cell receptor on B cells has also been proposed. Specifically, vaccine antigen–antibody complexes cross-link the B-cell receptor (which recognizes the variable region of the antibody) with the Fcγ receptor IIB (which recognizes the constant region of the antibody), thus inhibiting antigen specific B-cell activation (189). Furthermore, vaccine antigen-antibody complexes removal by macrophages has been suggested although no evidence has been provided to support this hypothesis. Using influenza vaccination in pregnancy as a model, it was recently shown in mice that maternal antibodies do not prevent activation of B cells or the formation of the germinal center. However, maternal antibodies reduced the number of B cells that differentiate to plasma cells and memory B cells (190). Whether these results apply to human infants and other antigens needs to be determined. Finally, while B cell responses are inhibited in the presence of maternal antibodies, scarce data support that T cell responses are detected in the presence of maternal antibodies (191).

## IMPACT OF MATERNAL IMMUNIZATION ON THE NEONATAL IMMUNE SYSTEM

The impact of maternal vaccination on the fetal/neonatal immune system, beyond the trans-placental transfer of IgG, has not been well-studied. *In utero* priming of the fetal immune system after vaccination against influenza in pregnancy has been reported. IgM antibodies against influenza vaccine antigens were detected in nearly 40% of cord blood specimens of newborns born to women vaccinated with IIV in pregnancy (192). As IgM antibodies do not cross the placenta, these antibodies are of fetal origin. In addition, using MHC tetramers, HA-specific $CD4^+$ T cells were also detected in cord blood, further supporting the "*in utero* priming hypothesis" after maternal immunization (192). Additional studies are needed to further assess the possibility of priming of fetal immune system to *B. pertussis* antigens after immunization in pregnancy.

Based on the literature review and consultation among authors, a consensus on priorities for future research related to the effect of immunization during pregnancy on infants' immune responses was reached (**Table 8**).

## FUTURE VACCINES FOR IMMUNIZATION DURING PREGNANCY

In addition to tetanus containing, pertussis containing and influenza vaccines currently used in pregnancy, multiple novel GBS and RSV candidate vaccines are under development for use in pregnant women (193). Infection with other pathogens (e.g., dengue virus, Zika virus) during pregnancy is associated with a significant risk of adverse fetal outcome (194–196), and thus vaccines developed with the goal to prevent these congenital infections might prove to be an important preventative strategy. However, these not part of this consensus paper and are reviewed elsewhere (197, 198).

### Group B *Streptococcus* Vaccines

GBS colonization in pregnant women is associated with an increased risk of premature birth, birth asphyxia, stillbirths, and invasive GBS disease in newborns during the first week of life (early-onset disease, EOD). Newborns of mothers colonized with GBS are at higher risk of developing meningitis and sepsis (199). Although intrapartum antibiotic prophylaxis is effective in

**TABLE 8 |** Consensus on priorities for future research related to the impact of maternal immunization on *in-utero* immune system and infants' immune responses to immunization.

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| **Infants' immune responses to TT-containing vaccines** |
| 1. The impact of anti-TT maternally-derived antibodies on infants' responses to tetanus-containing vaccines administered during infancy and whether this is affected by vaccine formulation given to pregnant women (TT vs. Td vs. Tdap) |
| **Infants' immune responses to DT-containing vaccines** |
| **1.** The impact of anti-DT maternally-derived antibodies on infants' responses to vaccines conjugated to DT mutants as a carrier protein (e.g., CRM197-conjugated vaccines) administered during infancy |
| **Infants' immune responses to pertussis vaccines** |
| 1. Clinical significance of interference to pertussis immunization in pregnancy |
| 2. If interference is found to be clinically significant, modifiable factors that can mitigate interference need to be explored |
| 3. The effect of timing of vaccination during pregnancy on interference |
| 4. The impact of a stand-alone pertussis vaccine (no TT, DT) on infants' immune responses to pertussis vaccine administered during pregnancy |
| **General** |
| 1. The mechanism of inhibition of maternally-derived antibodies on infants immune responses to their vaccination |
| 2. The effect of maternally derived antibodies on infant T cell responses |
| 3. The potential priming of the fetal immune system to vaccine antigens after immunization during pregnancy and its effect on training neonatal immune system |

*TT, Tetanus toxoid; Td, Tetanus diphtheria; Tdap, tetanus-diphtheria-acellular-pertussis; DT, Diphtheria toxoid.*

preventing GBS EOD, it is not effective in preventing late onset disease (LOD, >7–90 days of age) and it might be associated with dysregulation of the infants' gastro-intestinal microbiome (200). Importantly, identification and treatment of colonized mothers can be difficult and expensive, particularly in LMICs, where the incidence of neonatal invasive GBS disease is higher compared to HICs (201). Development of GBS vaccines for immunization in pregnancy and its use in LMICs has been identified as a priority by the WHO (202).

Vaccines based on the capsular polysaccharide of the most common GBS strains conjugated to a carrier protein (e.g., TT or a non-toxic mutant of diphtheria toxin) are the most studied candidate vaccines (203). A recent systematic review of clinical trials evaluating candidate GBS vaccines concluded that these candidate GBS vaccines are safe and well-tolerated in pregnant women and non-pregnant adults, may reduce vaginal colonization and induce antibody titers against the GBS strains included in the vaccine at a significantly higher level than that detected in unvaccinated controls (203). Moreover, antibodies induced by GBS vaccines showed high longevity and were able to promote GBS opsonophagocytosis *in vitro* (203).

Several challenges for the development of GBS vaccines for maternal immunization remain unsolved. There are only 10 known GBS serotypes, of which 6 are associated with 98% of all described strains that cause invasive disease and even a trivalent vaccine (Ia, Ib, and III) would provide coverage for 80% of all global invasive disease cases (204). The prevalence of different GBS serotypes may vary in different countries, however, the most common serotypes (Ia, Ib, II, III, IV, and V) are dominant globally, with only Asia reporting a slightly higher proportion of cases due to one additional serotype (VII) (205). The distribution of serotypes responsible for early and late -onset GBS disease also varies, with the most common serotypes being III and Ia (206). Correlates of protection for the different GBS serotypes against the various clinical conditions associated with the pathogen (i.e., colonization, maternal and infant disease) are not precisely defined (207), and these correlates may vary by serotype (207). Furthermore, transplacental transfer of antibodies might be affected by the characteristics of the vaccine (conjugated vs. unconjugated), the carrier protein used for conjugation, and the presence of underlying diseases in the mother which can reduce transfer, such as HIV infection (208).

Phase 1b/2 clinical trials have shown that vaccination of pregnant women with a trivalent GBS vaccine (serotype III, Ia, and Ib conjugated to CRM197) induces anti-GBS antibodies that are transferred to the newborn at delivery (208–210). Other phase 1/2 clinical trials are currently evaluating multi-serotype vaccines, including a hexavalent vaccine (serotypes Ia, Ib, II, III, IV, V) that cover 98% of strains associated with invasive GBS disease in both a non-pregnant population (NCT03170609) and in pregnant women (NCT03765073).

Finally, the clinical effectiveness of GBS vaccines in pregnant women and neonates has not been determined. Considering the relatively low incidence of invasive GBS disease, especially in HICs, the pathway of licensure of a GBS vaccine targeted at pregnant women with the main objective of protection of their infants against early and late-onset invasive GBS disease is likely to require an alternate approach than conventional efficacy trials. This would include demonstrating the safety of the vaccine in pregnant women (∼3,000–4,500 participants), and benchmarking their immune responses to a serological endpoint associated with reduced risk for invasive GBS disease. Studies are currently underway in LMICs and HICs, which are investigating the association of maternal-derived serotype-specific IgG (using a standardized assay) and threshold associated with 80–90% risk reduction for invasive GBS disease.

As current GBS vaccines that are under development are conjugated to TT or the DT mutant CRM197, it will be important to investigate whether these vaccines given to pregnant women may result in interference to infant vaccines conjugated to these carrier proteins and given in infancy (e.g., PCV, Hib, and Meningococcal vaccines). Current evidence suggests that CRM197-conjugated GBS vaccine administered in pregnancy did not affect infants' immune responses to PCVs (211).

## Respiratory Syncytial Virus

RSV is the most common cause of severe lower respiratory tract infections (LRTIs) in young children worldwide with a disproportionate high burden of disease in LMICs (e.g., higher case-fatality rate) (212). Preterm infants and infants with underlying severe chronic heart or lung disease are at higher risk of severe RSV infection, leading to hospitalization and death. A monoclonal antibody directed against the RSV fusion (F) protein has been administered to high-risk populations to prevent RSV-related morbidity in infants in high-income

countries (213, 214). However, this strategy is highly expensive and its effectiveness varies ranging between 48 and 96% in the prevention of RSV-related hospitalization in high-risk children (215, 216). In addition, overall more healthy children are infected with RSV each year than high-risk children. A novel prolonged half-life anti-RSV monoclonal antibody may prove to be more effective in preventing RSV disease in infancy (217).

Recently, several new vaccines, including live-attenuated, gene-based vector vaccines, and particle-based vaccines, have been developed and found to be safe and well-tolerated in the non-pregnant population (11, 193). Hence, as most of the cases of severe RSV infection occur in the first 3 months of life, it is unlikely that infants' immunization can provide sufficient and timely protection. Therefore, maternal immunization is considered as a suitable strategy for prevention of RSV disease in young infants (11, 218).

Studies on RSV-F protein in pregnant women have shown that these vaccines are safe and immunogenic in pregnant women (219, 220). The use of these RSV vaccines in healthy pregnant women is further supported by evidence that maternal RSV neutralizing antibodies are efficiently transferred from the mother to the newborn, with levels at delivery that are similar or higher in the cord blood compared with the maternal blood at delivery (219, 220). However, the association between higher cord RSV neutralizing antibody levels and the reduction of risk for RSV LRTI in the infant is not clear, and no definitive correlates of protection have been defined so far (221–223). Vaccines containing the RSV-F protein in pregnant women have shown that these vaccines are safe and immunogenic in pregnant women (219, 220).

A phase 3, randomized, placebo controlled trial including 4,636 pregnant women has been conducted in 11 countries with a RSV-F nanoparticle alum-adjuvanted vaccine showed that protection against RSV LRTI hospitalization was noted (44.4% vaccine efficacy, 95%CI: 19.6 to 61.5), but the primary study endpoint (per protocol analysis) for reduction of medically-significant RSV LRTI (39% vaccine efficacy; 97.5% CI: −1 to 63.7) was not met (albeit the 95% CI been 5.3 to 61.2) (224). This is the largest study so far to evaluate a vaccine primarily designed for use in pregnant women.

Multiple factors could have affected the outcomes measured in this first immunization study of a RSV vaccine in pregnancy. Pregnant women were vaccinated during 28–36 weeks gestation, and efficiency of transfer of anti-RSV antibodies were found to be higher in women vaccinated <30 weeks GA compared with women vaccinated ≥30 weeks GA. In addition, vaccine efficacy varied in different settings, being higher in middle-income countries (compared with HICs). Mathematical modeling can help predict women and infants who are expected to benefit the most from RSV vaccines. This could be achieved by defining women who are expected to deliver in RSV season and the preferred timing of vaccination to optimize protection in those infants. Ideal timing of vaccination could be predicted based on the kinetics of antibody response in mothers, the efficiency of antibody transfer and their estimated half-life, and duration of infants' exposure to seasonal RSV.

**TABLE 9 |** Consensus on priorities for future research related to vaccines against respiratory syncytial virus and Group B *Streptococcus* diseases during pregnancy.

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| **Group B *Streptococcus* vaccines** |
| 1. The epidemiology of GBS disease in early life and risk factors for GBS disease in diverse geographic settings |
| 2. Ideal composition of GBS vaccines to achieve highest protection against early and late onset GBS disease |
| 3. Correlate(s) of protection against early and late onset GBS disease |
| 4. Whether GBS vaccines given to pregnant women interfere with vaccines given in infancy and conjugated to TT and DT as carrier proteins |
| 5. Effectiveness of GBS vaccines administered during pregnancy in reduction of early and late onset GBS disease |
| **Respiratory syncytial virus** |
| 1. Definition of ideal timing of vaccination in pregnancy to achieve highest immunogenicity in infants |
| 2. Mathematical modeling to inform clinical trials design to better define infants who will benefit the most from vaccination during pregnancy |
| 3. Correlate(s) of protection against RSV disease in infancy |
| 4. Epidemiology of RSV disease in 1st and 2nd years of life in offspring of mothers vaccinated during pregnancy |

*GBS, Group B Streptococcus; TT, Tetanus toxoid; DT, Diphtheria toxoid; RSV, Respiratory Syncytial Virus.*

Based on the literature review and consultation among authors, a consensus on priorities for future research related to immunization during pregnancy against GBS and RSV was reached (**Table 9**).

## INDUCTION OF VACCINE-SPECIFIC IMMUNITY IN BREASTMILK

There is a paucity of information on the induction of antibodies in breastmilk following vaccination in pregnancy (225). Anti-*B. pertussis* secretory immunoglobulin A (sIgA) antibodies were detected in colostrum and in breast milk up to 8 weeks after delivery from women vaccinated with Tdap during pregnancy (226, 227). However, the clinical significance of these elevated *B. pertussis*-specific antibody concentrations in breastmilk has not been studied. A study from Bangladesh showed that vaccination with TIV in pregnancy induced influenza-specific sIgA levels in breastmilk for at least 6 months postpartum. In addition, breastfeeding was associated with a decrease in episodes of respiratory illness with fever in infants born to mothers vaccinated against influenza during pregnancy (228). A study from South Africa found that breastmilk sIgA against GBS was associated with lower risk for GBS LOD in young infants (229). In a study from Nepal, breastmilk RSV IgG levels, but not IgA levels, were lower in mothers of infants with RSV acute respiratory infection (230). While these studies report potential association between breastfeeding and reduction in disease risk, the casual relationship has not been confirmed.

Based on the literature review and consultation among authors, a consensus on priorities for future research related to the effect of immunization during pregnancy on the induction of vaccine-specific immunity in breast milk was reached (**Table 10**).

**TABLE 10 |** Consensus on priorities for future research related to induction of vaccine-specific immunity in breast milk.

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| 1. The additional role of breastfeeding in protection against clinical disease in infants born to mothers vaccinated against influenza, RSV and GBS during different phases of breastfeeding (colostrum, after 2–3 months of breastfeeding, etc.) |
| 2. The additional role of breastfeeding in protection against clinical disease in infants born to mothers vaccinated against pertussis in settings where vaccine effectiveness is not optimal |
| 3. Mechanisms of protection against respiratory pathogens through breastmilk |

**TABLE 11 |** Consensus on priorities for future research related to acceptance and strategies for increasing uptake of vaccines administered during pregnancy.

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| To identify strategies to increase tetanus vaccine coverage during pregnancy in low-middle income countries |
| The region and cultural specific approaches for implementation of vaccinations during pregnancy and their acceptance |
| The barriers to high maternal immunization uptake in specific populations. |
| The need of adequate education of health-care providers on maternal immunization and establishing a consensus on a minimum curriculum to be achieved during (para)medical education |
| The need of global information and awareness-raising campaigns |
| How to best inform pregnant women about new vaccines |
| The effectiveness of different strategies to increase influenza and pertussis vaccination coverage in pregnant women in different regions and cultures |
| To analyze similarities and differences in knowledge and attitudes to influenza and pertussis vaccination during pregnancy |

## ACCEPTANCE AND STRATEGIES FOR INCREASING UPTAKE OF VACCINES

The acceptance and coverage of immunization against tetanus during pregnancy in LMICs have been historically high (231). Despite recommendations by multiple health authorities worldwide, maternal immunization with influenza and pertussis vaccines has not been as widely accepted by healthcare workers or the general public, including pregnant women (232). Coverage remains suboptimal in many countries where recommendations for maternal immunization with influenza and pertussis vaccines have been in place for several years. In the US, influenza and pertussis vaccines have been recommended for all pregnant women since 2004 (233) and 2011 (234), respectively. However, during the 2017–2018 influenza season, only 49.1% of pregnant women received the influenza vaccine during the peak influenza season (235). During the same months, maternal Tdap uptake was only slightly higher (54.4%). Finally, the receipt of both vaccines was documented in only 32.8% of pregnant women (235). In the UK, where pertussis vaccine has been offered to all pregnant women since October 1, 2012, coverage during the period from April to June 2018 was 68.2% (236). In the European Union, although 90% of countries recommend vaccination against influenza for pregnant women, coverage was generally low in 2014–2015, with half of the countries reporting uptake of <10% (237). In France, during the 2015–16 season vaccine coverage was only 7.4% (238). In Switzerland influenza and pertussis immunizations in pregnancy have been recommended since 2009 and 2013, respectively. Yet, in a study performed in women who gave birth between 2013 and 2017, only five (3%) of 172 mothers had received both pertussis and influenza vaccines during pregnancy, 15 (9%) only against pertussis and 12 (7%) only against influenza (239).

Several factors can explain the poor compliance with the official recommendations. A recent literature review documented 25 individual patient-level and 24 healthcare provider-level barriers to maternal immunization uptake (240). Among the patient-identified barriers, concerns regarding safety for the mother or the newborn were very common and were cited in 6.4–71% and 2.9–77.0% of studies, respectively. Other vaccine and disease-related factors included concerns about vaccine efficacy, the belief that the disease was not sufficiently severe to require prevention, and the idea that healthy people did not need immunization. Moreover, several structural and logistical barriers were identified. Lack of insurance coverage, limited access or transportation, and the difficulty of finding a provider for vaccine administration were reported. Finally, social, psychological factors, and insufficient knowledge were listed repeatedly. Among the provider-level barriers, poor knowledge of the recommendations for immunization of pregnant women, financial concerns (inadequate reimbursement, payment, and/or complexity of billing), and inability to order, obtain and store vaccines. Globally, the lack of knowledge of vaccine recommendations seems to be the most important barrier for both health care workers (HCWs) (241–246) and pregnant women (247–252). HCWs in contact with pregnant women frequently have little experience in vaccines and therefore do not offer vaccinations to pregnant women (239, 253).

To overcome barriers to maternal immunization, both provider-focused and mother-focused interventions have been suggested, with a significant improvement in vaccine uptake has been evidenced in several cases (253). Suggested provider-focused interventions were notifying the provider of the vaccination status of pregnant women, establishing standing orders authorizing nursing staff to administer the vaccines without a medical consultation, giving provider feedback by reporting the vaccination rates of other institutions, and providing education to improve the knowledge and attitudes of HCWs toward vaccination in pregnancy. However, education of HCWs alone is probably ineffective if parental vaccine hesitancy is not addressed simultaneously.

Multiple educational efforts involving all HCWs who deliver care to pregnant women and the pregnant women themselves can yield positive results (254). This observation seems to have been confirmed by a study by Chamberlain et al., who showed that when obstetricians and women became familiar with the recommendation to promote and receive the influenza vaccine during pregnancy, the percentage of women who refused to be vaccinated declined from 88.9% in 2004 to 64.2% in 2011 (254). To overcome barriers in pregnant women, studies were planned to disseminate education and promotion of materials specifically for pregnant women by mass media campaigns

via the internet, posters and leaflets, lectures and workshops, and personalized reminders and recall system (254). Integrating maternal immunization into routine obstetric care, with vaccine availability within the obstetrical setting, appears to be the best method of improving maternal immunization as well as subsequent childhood vaccine uptake (255).

Based on the literature review and consultation among authors, a consensus on priorities for future research related to acceptance and uptake of vaccines administered during pregnancy was reached (**Table 11**).

## CONCLUSIONS

Pregnant women, their newborns and young infants are vulnerable to serious and potentially fatal infections. The new WHO goals aim to increase rates of live births and improve antenatal care for pregnant women (256), and vaccination in pregnancy is one strategy to improve health of pregnant women and their offspring. Safe and effective vaccines are already available against some diseases (tetanus, pertussis and influenza) for use during pregnancy, and these vaccines have the potential to prevent significant infectious disease morbidity and mortality in both the mothers and their offspring. In addition, new vaccines (e.g., RSV, GBS) are currently under development and are being tested in clinical trials, to be licensed and used in pregnant women. Following literature review and a consultation amongst experts in the fields of infectious diseases, vaccination and immunization during pregnancy, several gaps in knowledge and priorities for research were identified and are proposed (**Tables 3–11**). Addressing these priorities in future research has the potential to increase our understanding in different aspects of immunization during pregnancy and optimize protection for both the mother and the infant.

## AUTHOR CONTRIBUTIONS

SE proposed the project, coordinated the study group, and wrote the first draft of the manuscript. BA-R wrote the first draft of the consensus statements and revised the initial draft of the manuscript. SO wrote the first draft of the ethics section. KM produced **Figure 1**. BA-R, KM, KE, SO, JE, MDS, GA, EL, PD, VP, OL, RD, MC, AC, KF, TF, SG, LV, MO'R, UH, NP, AA, MAS, NW, SM, MG, RP, SL, LM, FM-T, and SE reviewed and edited the manuscript, provided comments, suggested references, and substantially contributed to the content of the manuscript. BA-R, KM, KE, SO, JE, MDS, GA, EL, PD, VP, OL, RD, MC, AC, KF, TF, SG, LV, MO'R, UH, NP, AA, MAS, NW, SM, MG, RP, SL, LM, FM-T, and SE approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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# Planned Yellow Fever Primary Vaccination Is Safe and Immunogenic in Patients With Autoimmune Diseases: A Prospective Non-interventional Study

*Valéria Valim[1*†], Ketty Lysie Libardi Lira Machado[1†], Samira Tatiyama Miyamoto[1], Arthur Dalmaso Pinto[1], Priscila Costa Martins Rocha[1], Erica Vieira Serrano[1], Valquiria Garcia Dinis[1,2], Sônia Alves Gouvêa[1], João Gabriel Fragoso Dias[1], Ana Carolina Campi-Azevedo[3], Andréa Teixeira-Carvalho[3], Vanessa Peruhype-Magalhães[3], Ismael Artur da Costa-Rocha[3], Sheila Maria Barbosa de Lima[4], Emily Hime Miranda[4], Gisela Freitas Trindade[4], Maria de Lourdes de Sousa Maia[4], Maria Bernadete Renoldi de Oliveira Gavi[1], Lidia Balarini da Silva[1], Ruben Horst Duque[1], Ana Paula Espíndula Gianordoli[1], Thays Zanon Casagrande[1], Karine Gadioli Oliveira[1], Bruna Costa da Mata Moura[1], Fernanda Nicole-Batista[1], Luiza Correa Rodrigues[1], Thalles Brandão Clemente[1], Enan Sales Magalhães[1], Maria de Fatima Bissoli[1], Maria da Penha Gomes Gouvea[1], Lauro Ferreira da Silva Pinto-Neto[2], Carolina Zorzanelli Costa[2], Raquel Altoé Giovelli[2], Leticia Resende Brandão[5], Elizandra Tomazela Laurenti Polito[5], Ingrid de Oliveira Koehlert[5], Brunela Passos Borjaille[5], Daniela Bergamim Pereira[5], Laiza Hombre Dias[5], Daniela Linhares Merlo[5], Luiz Fellipe Favoreto Genelhu[5], Flavia Zon Pretti[5], Maryella dos Santos Giacomin[5], Ana Paula Neves Burian[6], Francieli Fontana Sutile Tardetti Fantinato[7], Gecilmara Salviato Pileggi[8], Lícia Maria Henrique da Mota[9] and Olindo Assis Martins-Filho[3]*

[1] Divisão de Reumatologia do Hospital Universitário Cassiano Antônio de Moraes, Universidade Federal do Espírito Santo (UFES), Vitória, Brazil, [2] Escola de Ciências da Saúde da Santa Casa de Misericórdia, Vitória, Brazil, [3] Instituto René Rachou, Fundação Oswaldo Cruz (FIOCRUZ-Minas), Belo Horizonte, Brazil, [4] Instituto de Tecnologia em Imunobiológicos (Bio-Manguinhos), Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil, [5] Sociedade de Reumatologia do Espírito Santo (SORES), Vitória, Brazil, [6] Centro de Referências para Imunobiológicos Especiais (CRIE) da Secretaria de Saúde do Estado do Espírito Santo, Vitória, Brazil, [7] Departamento de Vigilância das Doenças Transmissíveis, Secretaria de Vigilância em Saúde, Ministério da Saúde, Brasília, Brazil, [8] Faculdade de Ciências da Saúde de Barretos—FACISB, Barretos, Brazil, [9] Divisão de Reumatologia do Hospital Universitário de Brasília, Faculdade de Medicina, Universidade de Brasília, Brasília, Brazil

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***Correspondence:**
Valéria Valim
val.valim@gmail.com

[†] These authors have contributed equally to this work

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Yellow Fever (YF) vaccination is suggested to induce a large number of adverse events (AE) and suboptimal responses in patients with autoimmune diseases (AID); however, there have been no studies on 17DD-YF primary vaccination performance in patients with AID. This prospective non-interventional study conducted between March and July, 2017 assessed the safety and immunogenicity of planned 17DD-YF primary vaccination in patients with AID. Adult patients with AID (both sexes) were enrolled, along with healthy controls, at a single hospital (Vitória, Brazil). Included patients were referred for planned vaccination by a rheumatologist; in remission, or with low disease activity; and had low level immunosuppression or the attending physician advised interruption of immunosuppression for safety reasons. The occurrence of AE, neutralizing antibody kinetics, seropositivity rates, and 17DD-YF viremia were evaluated at various time points (day 0 (D0), D3, D4, D5, D6, D14, and D28). Individuals evaluated ($n = 278$), including

patients with rheumatoid arthritis (RA; 79), spondyloarthritis (SpA; 59), systemic sclerosis (8), systemic lupus erythematosus (SLE; 27), primary Sjögren's syndrome (SS; 54), and healthy controls (HC; 51). Only mild AE were reported. The frequency of local and systemic AE in patients with AID and HC did not differ significantly (8 vs. 10% and 21 vs. 32%; $p = 1.00$ and 0.18, respectively). Patients with AID presented late seroconversion profiles according to kinetic timelines of the plaque reduction neutralization test (PRNT). PRNT-determined virus titers (copies/mL) [181 (95% confidence interval (CI), 144–228) vs. 440 (95% CI, 291–665), $p = 0.004$] and seropositivity rate (78 vs. 96%, $p = 0.01$) were lower in patients with AID after 28 days, particularly those with SpA (73%) and SLE (73%), relative to HC. The YF viremia peak (RNAnemia) was 5–6 days after vaccination in all groups. In conclusion, consistent seroconversion rates were observed in patients with AID and our findings support that planned 17DD-YF primary vaccination is safe and immunogenic in patients with AID.

**Keywords: yellow fever vaccine, autoimmune diseases, viremia, seroconversion, pharmacokinetics**

## INTRODUCTION

The 17DD-Yellow Fever (YF) vaccine induces safe and effective protective immunity in healthy individuals, resulting from robust humoral and cellular immune responses (1–3); however, it has been proposed that immune-compromised individuals mount suboptimal immunologic responses after vaccination (4–6). Moreover, some studies have pointed to a high prevalence of severe adverse post-vaccination events in patients with autoimmune diseases (AID), particularly systemic lupus erythematosus (SLE) and those receiving systemic corticosteroid therapy (7–10). Studies assessing the safety, effectiveness, and immunogenicity of YF vaccination in immune-compromised patients, particularly those with AID, remain scarce (4).

There is still no antiviral treatment for YF, therefore prevention actions such as mosquito control, protection from mosquito bite and vaccination are extremely necessary. A live attenuated vaccine strain 17D was developed in 1937. Two substrains are used in the vaccine today, substrains 17D-204 (Sanofi- Pasteur) and 17DD (Fiocruz), which are at passages 235–240 and 287–289, respectively, from wild-type Asibi virus (11).

The vaccine produces high level of protection that occurs in 90% of vaccines within 10 days and in nearly 100%, in 4 weeks. Immunity after a single dose is long lasting and may provide protection for life (12). The World Health Organization (WHO) recommends a single dose immunization for travelers to endemic area. However, protective cellular and humoral immunity wanes over time in some individuals (13).

YF vaccination is generally well-tolerated, adverse events are reported in only 43 per 100,000 doses and most cases are mild. "Vaccine-Associated Viscerotrophic Disease" (YEL-AVD) and "Vaccine-Associated Neurological Disease" (YEL-AND) are severe and rare adverse events, reported only in primary vaccinees, and especially in children, elderly and history of thymus disease (11, 14).

In December 2016, a YF outbreak occurred in Brazil that extended to several Eastern states, including areas not traditionally considered at risk and where, therefore, YF vaccination was not recommended to the resident populations, or travelers to those specific locations, until the outbreak. YF is a severe infectious disease and vaccination is the most important way to protect from this condition, which has high mortality rates. Soon after the first cases were reported in 2017, the Brazilian Government decided to conduct an extensive Brazilian YF vaccination campaign. Immunization was free and offered by many public services in the affected zones; consequently, numerous patients with AID were inadvertently vaccinated or remained unvaccinated and susceptible, and at risk of YF infection and its severe outcome.

Live attenuated vaccines should be used with caution in populations with AID because of the risk of adverse events (AE). The majority of guidelines generally recommend avoiding live vaccines for immunosuppressed individuals (15). The decision to be vaccinated must consider both the risks of exposure and possibility of death from YF, and the risks of complications caused by the vaccine (16). Recently, the Brazilian Society of Rheumatology, Dermatology, Bowel Inflammatory Disease have published recommendations about YF vaccination in patients with chronic immune-mediated inflammatory diseases living or traveling to YF endemic areas (17). Faced with absence of prospective studies in AID, it is necessary to establish medical evaluation criteria to allow or prohibit vaccination.

To date, there have been no studies investigating the response to, and safety of, planned 17DD-YF primary vaccination in patients with AID patients. Therefore, any effort to generate scientific evidence will contribute to development of appropriate recommendations regarding vaccination. The aims of this study were to evaluate the occurrence of AE, seroconversion rates, kinetics of neutralizing antibody production, and vaccine viremia after 17DD-YF primary vaccination of patients with AID.

## MATERIALS AND METHODS

### Study Design

This was a prospective non-interventional study, carried out between March 2017 and July 2017 in Vitória, Espírito

Santo, Brazil. All participants received the 17DD-YF primary vaccination (Bio-Manguinhos-FIOCRUZ) during the 2017 Brazilian YF vaccination campaign, coordinated by the State Government. This study is registered in the Registro Brasileiro de Ensaios Clínicos (Brazilian Registry of Clinical Trials, UTN# U1111-1217-6672).

Individuals of both sexes, aged from 18 to 88 years, with the following AID diagnoses: rheumatoid arthritis (RA), spondyloarthritis (SpA), systemic sclerosis (SSC), systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), and healthy controls (HC), were enrolled in the study. Patients with AID were attended in the Rheumatology Outpatient Unit of Hospital Universitário Cassiano Antônio Moraes/EBSERH at Universidade Federal do Espírito Santo (HUCAM-UFES/EBSERH), where the risks and safety of the YF vaccine were evaluated. The HC group consisted of individuals who attended the routine vaccination unit at HUCAM. All those did not have AID and did not meet the exclusion criteria.

The study was submitted and approved by the ethical committee of HUCAM-UFES/EBSERH (C.A.A.E 65910317.0.0000.5071, approval #2.411.738/2017). Informed consent was obtained from all participants.

## Inclusion/Exclusion Criteria

The inclusion criteria for both groups comprised: individuals > 18 years, able to understand and read the consent form, or have a legal representative to read it, and had never received YF vaccination. Moreover, in the AID group, each patient fulfilled international classification criteria for AID, according to the American College of Rheumatology and/or European League Against Rheumatism international classification criteria for RA, SpA, SSC, SLE, and SS (18–23). All patients were advised by a rheumatologist to undergo planned YF vaccination when in remission or had low disease activity; and, when using immunosuppressant or biological therapy were advised that it was safe to interrupt this by their physician. The interval between withdrawal of therapy and YF vaccination was that specified in the Brazilian Recommendations for YF vaccination in patients with AID (17), as follows: interval > 3 months for immunosuppressive oral therapy, > 5.5 half-lives for any biological therapy, and ≥ 6 months for rituximab (**Table 1**) (6, 17, 24, 25).

Exclusion criteria comprised: patients who had not been advised by a rheumatologist to receive the vaccine; did not agree to participate; immunosuppressed by other causes (HIV carriers with CD4 count < 200 cells/mm$^3$ or lymphocytes < 500 cells/mm$^3$); low IgG or IgM levels; organ transplantation history; primary immunodeficiency; neoplasia; previous history of thymus diseases (myasthenia gravis, thymoma, thymus absence, or surgical removal); high disease activity index; receiving high levels of immunosuppressive treatment with cyclophosphamide, mycophenolate mofetil, tacrolimus, cyclosporine, sirolimus, azathioprine > 2 mg/kg/day, prednisone ≥ 20 mg/day, methotrexate > 20 mg/week, or any immunobiological drug (17, 24, 25); and received another vaccine simultaneously or at an interval < 30 days. Individuals previously vaccinated against YF, according to their medical records, and those with seropositive results for anti-YF antibody by plaque reduction neutralization test (PRNT ≥ 1:50 at baseline) were also excluded.

**TABLE 1 |** Minimum period of time recommended between withdrawal of therapy and 17DD-YF vaccination for patients with AID, according to Brazilian recommendations [a].

| Drug | Interval between withdrawal and vaccination |
|---|---|
| Prednisone > 20 mg/day or pulse methylprednisolone | ≥ 1 month |
| Hydroxychloroquine, sulfasalazine, acitretin, methotrexate ≤ 20 mg/week, leflunomide 20 mg/day | Consider vaccination without interval |
| Methotrexate > 20 mg/week | ≥ 1 month |
| Azathioprine, mycophenolate, cyclosporine, tacrolimus, cyclophosphamide | ≥ 3 months |
| Tofacitinib | ≥ 2 weeks |
| Anti-cytokines and co-stimulation inhibitor | 4–5 half-lives[b] |
| B-lymphocyte depletors | 6–12 months |

[a] *The medical criteria to conduct the drug elimination protocol before vaccination are indicated (13).*
[b] *Based on pharmacological half-life, except B-lymphocyte depletors.*

## AID-Related Clinical Records

Baseline demographic data included AID classification criteria (18–23), disease duration (years), AID disease activity score (26–30), and current use of synthetic and biological disease-modifying anti-rheumatic drugs (DMARDs). Twenty-eight days after 17DD-YF primary vaccination, AID related symptoms, AID disease activity score, and AID-related symptoms were reassessed. All data collected were obtained by medical/nurse interview and current medical reports/prescriptions.

## Safety Assessment

At baseline, all patients were given a diary that contained information about all YF vaccine-related AE and were instructed to record any new symptom that presented up to 30 days after YF vaccination. They also received an appointment for a follow-up visit (D28) and examinations (as specified below). Unscheduled visits were permitted whether any new symptoms presented after vaccination. Symptoms recorded in the diary were confirmed during nurse/medical visits (unscheduled visits and/or D28 scheduled return visit). AE events were stratified by extent and severity, according to the WHO classification (31). Local AE were defined as any symptom, including pain, pruritus, hyperemia, edema, or node at the application site. Systemic AE were defined as any symptom including fever, headache, myalgia, arthralgia, weakness, tremor, urticaria, angioedema, anaphylactic reaction, jaundice, and peripheral edema. Severe AE were defined as YF vaccine-associated neurotropic disease, YF vaccine-associated viscerotopic disease, or complications that resulted in hospitalization or death. Mild AE were any other AE that did not meet the criteria for severe AE. For all

AE, participants were actively asked about the symptoms and answered "yes" or "no."

## Blood Samples

Blood samples were collected from each participant at baseline (day 0; D0) and at three subsequent scheduled time points: (i) [D3, D6, D28]; (ii) [D4, D7, D28]; or (iii) [D5, D14, D28]. Serum samples were obtained from 20 mL of whole blood collected in vacuum tubes without anticoagulant. Serum aliquots were stored at −80°C until processing for detection of neutralizing antibodies and viremia analysis.

## Analysis of YF Neutralizing Antibodies and Viremia Levels

YF vaccine immunogenicity was evaluated in serum samples by assessment of anti-YF neutralizing antibody levels using PRNT, which is the gold-standard method (32). The results are expressed as the reciprocal of serum dilution. Values above serum dilution 1:50 were considered positive. Viremia levels (YF viral RNAnemia) were quantified in serum samples by qRT-PCR assay, according to Martins et al. (33). The results are expressed as copies/mL. Samples were processed in Laboratório de Tecnologia Virológica, Bio-Manguinhos (LATEV, FIOCRUZ-RJ, Brazil).

## Statistical Analysis

Descriptive statistical analysis was conducted using Prism 5.03 software (GraphPad Software, San Diego, USA). A chi-square test was used to compare the occurrence of AE and PRNT seropositivity rates amongst groups. Comparative analysis of PRNT titers between the HC and AID groups was performed by Mann-Whitney test. Multiple comparisons of PRNT titers and viremia levels amongst HC and AID subgroups were carried out using the Kruskal-Wallis test, followed by Dunn's post-test for sequential pair-wise comparisons. In all cases, $p < 0.05$ were considered statistically significant.

# RESULTS

In total, 278 individuals were included in the study: RA ($n = 79$), SpA ($n = 59$), SSc ($n = 8$), SLE ($n = 27$), SS ($n = 54$), and HC ($n = 51$). The mean [standard deviation; SD] age of participants in the AID group was 51 (14) years and 71.8% were women. In the HC group, mean [SD] age was 56 (15) years and 56.9% were women. At baseline, all individuals were in remission, or had low disease activity, and most were under low level immunosuppression (prednisone ≤ 20 mg/day; methotrexate ≤20 mg/week, azathioprine ≤ 2 mg/kg/day; leflunomide, sulfasalazine, or hydroxychloroquine). Few were undergoing strong immunosuppression (16.75% of RA and 49% of SpA were receiving biological therapy; 11.11% were receiving cyclophosphamide in the SLE group; 14.81% were on high doses of prednisone or methylprednisolone; and 29.63% were receiving azathioprine). In these patients with very stable disease, biological therapy and immunosuppressive therapy were discontinued before vaccination, according to Brazilian recommendations (17). Detailed clinical features of participants are provided in **Table 2**. The number of participants is shown in **Figure 1**.

## Safety of the 17DD-YF Vaccine

In the present study the occurrence of adverse events in both groups, HC and AID patients, was monitored by active

**TABLE 2 |** Baseline demographic, clinical, and therapeutic characteristics.

| Features | HC ($n = 51$) | AID ($n = 227$) | RA ($n = 79$) | SpA ($n = 59$) | SSC ($n = 8$) | SLE ($n = 27$) | SS ($n = 54$) |
|---|---|---|---|---|---|---|---|
| Women, % | 57 | 72 | 82 | 52 | 75 | 100 | 98 |
| Age, mean (SD), years | 56 (15) | 51 (14) | 55 (13) | 47 (11) | 59 (7) | 45 (16) | 54 (14) |
| PRED ≤ 20 mg/d, % | – | 12.1 | 16.3 | 1.8 | 12.5 | 25.9 | 9.3 |
| MTX, % | – | 28.8 | 36.3 | 31.6 | 12.5 | 11.1 | 24.1 |
| LFN, % | – | 9.4 | 18.3 | 7.0 | 0 | 0 | 3.7 |
| HCQ, % | – | 17.1 | 13.8 | 1.8 | 0 | 44.4 | 25.9 |
| SSA, % | – | 4.9 | 2.5 | 15.8 | 0 | 0 | 0 |
| AZA, % | – | 5.9 | 0 | 0 | 12.5 | 29.6 | 7.4 |
| MMF, % | – | 1.3 | 0 | 0 | 0 | 7.4 | 1.9 |
| CSA, % | – | 0.4 | 1.3 | 0 | 0 | 0 | 0 |
| CFM, % | – | 2.3 | 1.3 | 0 | 12.5 | 11.1 | 0 |
| PRED > 20 mg/d, % | – | 2.7 | 0 | 0 | 0 | 14.8 | 3.7 |
| Biological Therapy[a], % | – | 18.4 | 16.8 | 49.1 | 0 | 0 | 0 |
| Disease Activity, mean (SD) | – | – | DAS 28 2.99 ± 0.9 | BASDAI 1.92 ± 2.1 | – | SLEDAI 1.08 ± 1.5 | ESSDAI 1.89 ± 3.2 |

*HC, healthy controls; AID, autoimmune disease patients; RA, rheumatoid arthritis; SpA, spondyloarthritis; SSC, systemic sclerosis; SLE, systemic lupus erythematosus; SS, primary Sjögren's syndrome; SD, standard deviation; PRED, prednisone; MTX, methotrexate; LFN, leflunomide; HCQ, hydroxychloroquine; SSA, sulfasalazine; AZA, azathioprine; MMF, mycophenolate; CSA, cyclosporine; CFM, cyclophosphamide; DAS 28, disease activity score; BASDAI, bath ankylosing spondylitis disease activity index; SLEDAI, systemic lupus erythematosus disease activity index; ESSDAI, EULAR Sjögren's syndrome disease activity index. [a]biological therapy included: adalimumab, etanercept, infliximab, abatacept and rituximab.*

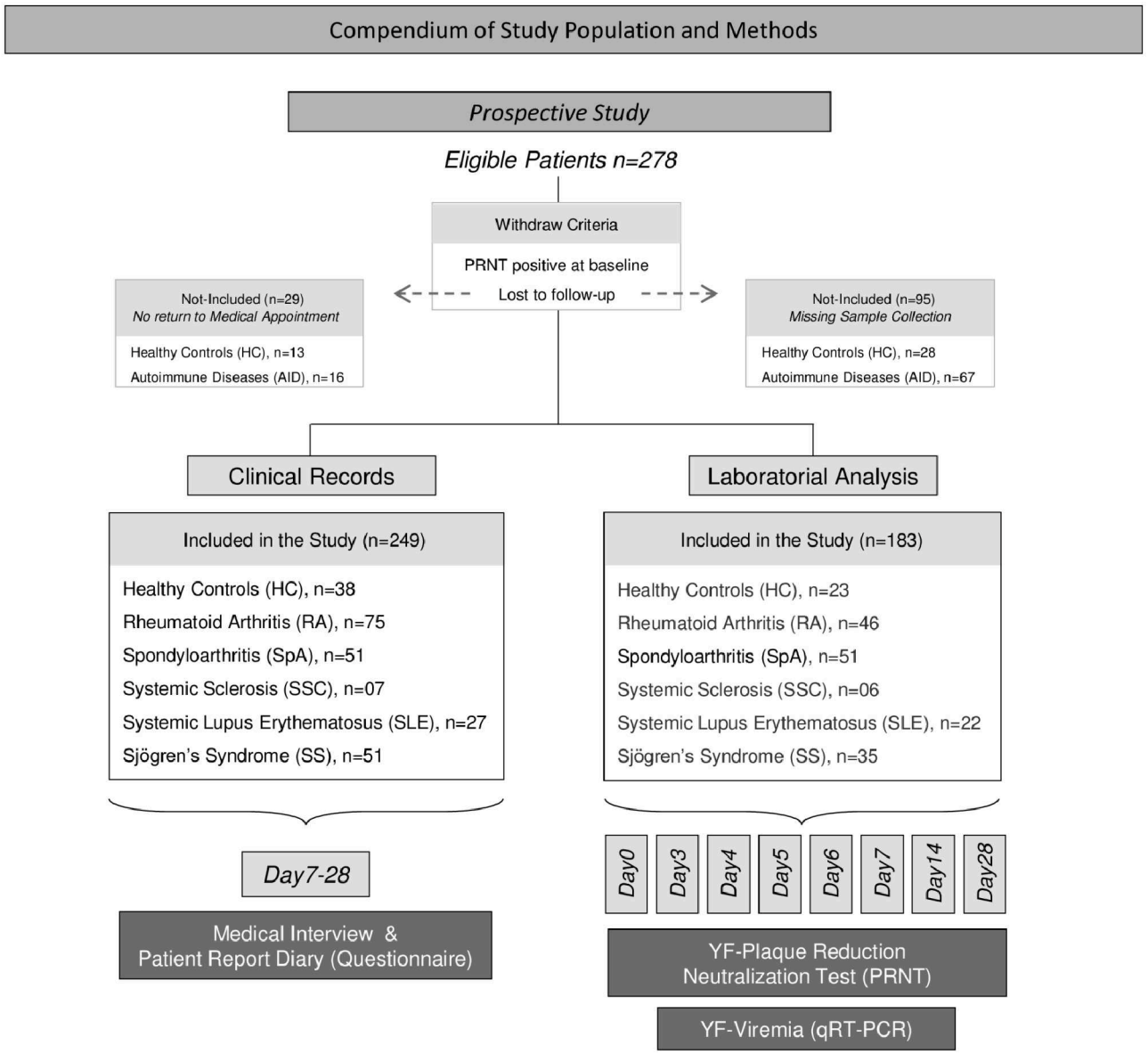


**FIGURE 1 |** Overview of the study population and methods. This was a prospective non-interventional study carried out between March and July 2017 in Vitória, Espírito Santo, Brazil. The study enrolled 278 individuals of both sexes, ranging from 18 to 88 years old. Individuals seropositive for anti-YF antibody (PRNT $\geq$ 1:50 at baseline) or those lost to follow-up were withdrawn from the study after being included. A total of 249 volunteers completed the clinical records, comprising: 75 with rheumatoid arthritis (RA), 51 with spondyloarthritis (SpA), 7 with systemic sclerosis (SSC), 27 with systemic lupus erythematosus (SLE), 51 with Sjögren's syndrome (SS), and 38 healthy controls (HC). Clinical records were obtained by medical/nurse interview, from patient report diary or previous medical reports, prescriptions, and records, from D7 to D28 after 17DD primary vaccination. For laboratory analyses, a group of 183 volunteers (RA = 46, SpA = 51, SSC = 6, SLE = 22, SS = 35, HC = 23) agreed to have blood samples collected at baseline (D0) and subsequent time-points, including: D3, D4, D5, D6, D7, D14, and D28. Laboratory analyses included YF-plaque reduction neutralization test (PRNT) and YF viremia (RNAnemia) analysis by qRT-PCR.

surveillance based on the weekly medical visit and patient diary reports up to 28 days after 17DD-YF primary vaccination. A total of 249 clinical records, including 211 from patients with AID and 38 from HC, were obtained by interview and patient diary reports. Twenty-nine individuals were lost during follow-up. The frequency of lost during follow-up was around 25% in HC and 7% in AID. The frequencies of local and systemic AE observed after 17DD-YF primary vaccination are provided in **Table 3**. Only mild AE were reported. The analysis of local and systemic AE did not reveal significant differences in AID patients relative to HC (8 vs. 10% and 21 vs. 32%; $p = 1.00$ and 0.18, respectively).

## Immunogenicity of the 17DD-YF Vaccine

Seropositivity rates and PRNT levels in patients with AID at D28 after 17DD-YF primary vaccination are presented in **Figure 2**. Seropositivity rates (PRNT $\geq$ 1:50) were lower in patients with AID than HC (78 vs. 96%, $p = 0.01$). Comparative analysis of

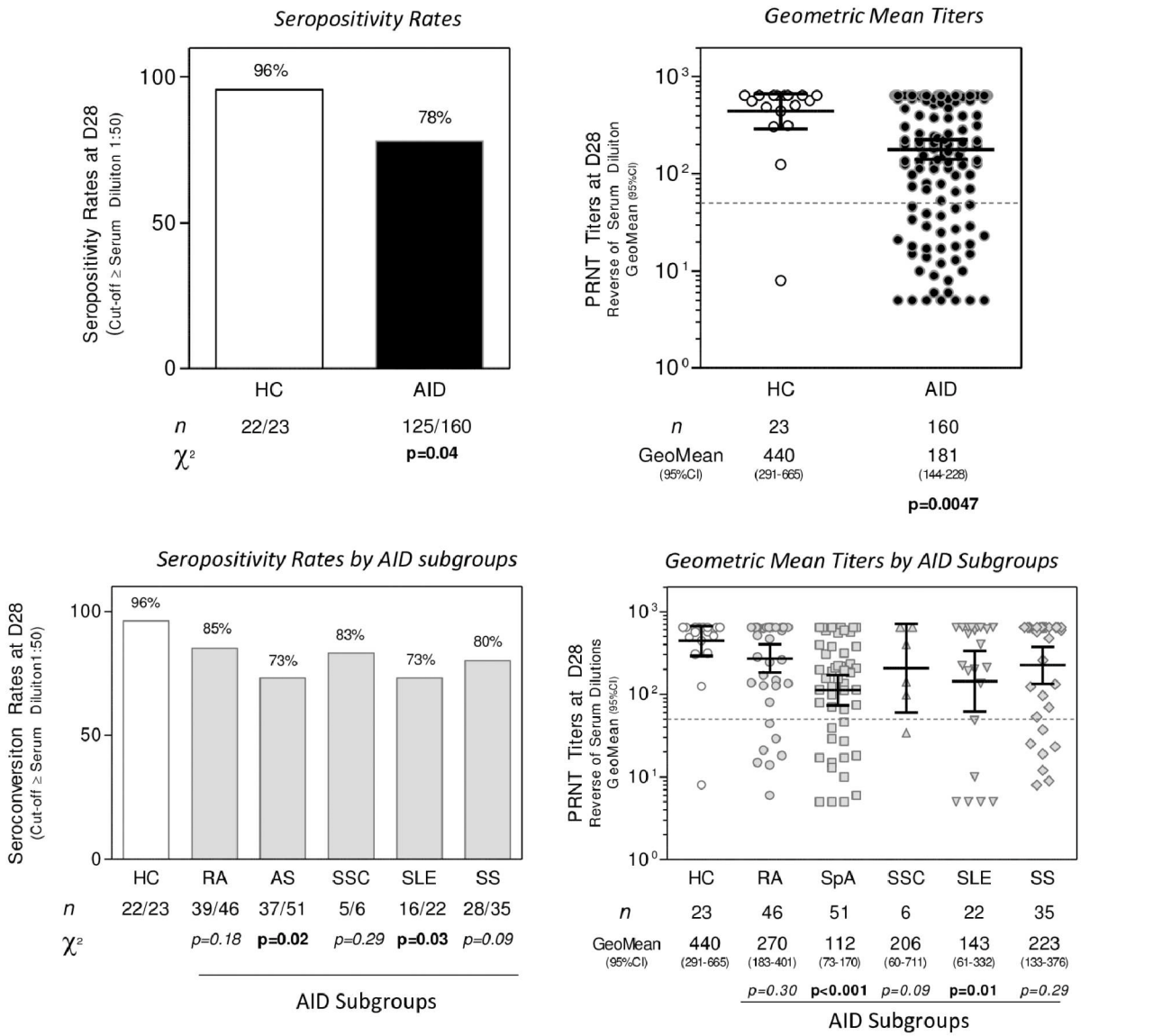


**FIGURE 2 |** Seropositivity rates and PRNT levels after 17DD-YF primary vaccination in patients with AID. Levels of 17DD-YF specific neutralizing antibodies were detected by micro-PRNT, as previously described by Simões et al. (25). Seropositivity rates were determined with serum dilution ≥ 1:50 as the cut-off criterion for PRNT positivity (dashed line). Data are presented as bar charts of proportion of seropositive results at D28 according to the cut-off of 1:50 expressed in reverse of serum dilution for HC (□), AID (■), and AID subgroups (□). The chi-square test was employed for comparative analysis of PRNT seropositivity rates amongst groups. The PRNT levels at D28 are expressed as geometric mean titer and 95% CI of reverse serum dilution, presented in scatter plots for HC (○), AID (●), RA (○), SpA (□), SSC (△), SLE (▽), and SS (◇). The cut-off of seropositivity is indicated by the dashed line (PRNT ≥ 1:50). Comparative analysis of PRNT titers between HC and AID groups was performed by Mann-Whitney test. Multiple comparisons of PRNT titers amongst HC and AID subgroups were conducted by Kruskal-Wallis test followed by Dunn's post-test for sequential pair-wise comparisons. In all cases, a threshold $p < 0.05$ was considered statistically significant. The number of samples tested for HC, AID, and AID subgroups is provided in the figure. HC, healthy contros; AID, autoimmune patients; RA, rheumatoid arthritis; SpA, spondyloarthritis; SSC, systemic sclerosis; SLE, systemic lupus erythematosus; SS, primary Sjögren's syndrome.

seropositivity rates among HC and AID subgroups demonstrated similar results for RA, SSC, and SS; however, lower seropositivity rates were observed in SpA (73%, $p = 0.02$) and SLE (73%, $p = 0.03$) relative to HC.

Analysis of PRNT levels demonstrated lower geometric mean titers in patients with AID relative to HC (181, 95% confidence interval (CI) 144–228 vs. 440, 95% CI 291–665; $p = 0.004$). Further comparative analysis among AID subgroups did not

**TABLE 3 |** Adverse events in patients with autoimmune diseases after 17DD-YF primary vaccination.

| Groups | Adverse events (AE) | | | |
|---|---|---|---|---|
| | Local[a], % (n) | *p*-value | Systemic[b], % (n) | *p*-value |
| **HC** ($n = 38$) | 8 (3) | – | 21 (8) | – |
| **AID** ($n = 211$) | 21 (44) | 1.00 | 32 (7) | 0.18 |
| **RA** ($n = 75$) | 9 (7) | 1.00 | 31 (23) | 0.37 |
| **SpA** ($n = 51$) | 4 (2) | 0.65 | 26 (13) | 0.80 |
| **SSC** ($n = 07$) | 14 (1) | 0.50 | 57 (4) | 0.07 |
| **SLE** ($n = 27$) | 4 (1) | 0.63 | 30 (8) | 0.56 |
| **SS** ($n = 51$) | 2 (1) | 0.14 | 39 (20) | 010 |

*Comparative analysis between HC and AID or AID subgroups were carried out by $\chi 2$ test. p-values are reported for comparisons to HC. [a]local AE included: pain, pruritus, hyperemia, edema, or node at the application site; [b]systemic AE included: fever, headache, myalgia, arthralgia, weakness, tremor, urticaria, angioedema, anaphylactic reaction, jaundice, peripheral edema. HC, healthy controls; AID, autoimmune disease patients; RA, rheumatoid arthritis; SpA, spondyloarthritis; SSC, systemic sclerosis; SLE, systemic lupus erythematosus; SS, primary Sjögren's syndrome.*

**TABLE 4 |** Viremia levels in patients with autoimmune diseases after 17DD-YF primary vaccination.

| Groups | Viremia peak (day after vaccine) | Viremia level at peak[a] (Mean copies/mL) | *p*-value |
|---|---|---|---|
| **HC** ($n = 07$) | Day 5 | $8.2 \pm 0.7 \times 10^3$ | |
| **AID** ($n = 42$) | Day 6 | $5.9 \pm 0.7 \times 10^3$ | 0.16 |
| **AID/PRNT(–)** ($n = 07$) | Day 5 | $1.3 \pm 0.1 \times 10^3$ | 0.18 |
| **AID/PRNT(+)** ($n = 35$) | Day 5 | $6.3 \pm 0.3 \times 10^3$ | 0.61 |
| **RA** ($n = 15$) | Day 5 | $1.6 \times 10^3$ | 0.17 |
| **SpA** ($n = 15$) | Day 5 | $11.3 \times 10^3$ | 0.56 |
| **SSC** ($n = 02$) | – | Undetectable | – |
| **SLE** ($n = 06$) | Day 5 | $4.8 \times 10^3$ | 0.25 |
| **SS** ($n = 08$) | Day 6 | $28.2 \times 10^3$ | 0.76 |

*[a]Data are reported as mean YF viral copies ± standard error (SE)/mL. Comparative analysis between HC and AID (p = 0.16) and AID/PRNT(–) and AID/PRNT(+) (p = 0.23) were carried out by Mann-Whitney test. ANOVA and multiple comparisons amongst HD and AID subgroups were performed by Kruskal-Wallis (p = 0.20), followed by Dunn's multiple comparison test. HC, healthy controls; AID, autoimmune disease patients; RA, rheumatoid arthritis; SpA, spondyloarthritis; SSC, systemic sclerosis; SLE, systemic lupus erythematosus; SS, primary Sjögren's syndrome.*

demonstrated significant differences between RA, SSC, and SS as compared to HC. Conversely, lower geometric mean titers were observed in patients with SpA (112, 95% CI 73–170; $p < 0.001$) and SLE (143, 95% CI 61–332; $p = 0.01$) relative to HC.

## Kinetic Timeline of PRNT Levels

With the aim of determining the timeline kinetics of neutralizing antibody production in patients with AID, PRNT titers were measured at sequential time points (D0, D3, D4, D5, D6, D7, D14, and D28). The results demonstrated that patients with AID had significantly lower PRNT titers at D5, D14, and D28 than HC. After correction for multiple testing the PRNT titers among AID subgroups showed that they were significantly lower at D28 relative to HC (**Figure 3**).

Seropositivity rates at D14 and D28 were further assessed, demonstrating that the seropositivity rate at D14 was significantly lower in patients with AID than those in HC (21 vs. 75%; $p = 0.04$). Comparative analysis among AID subgroups demonstrated overall impaired seropositivity rates at D14 (RA = 25%, SSC = 0%, SS = 17%) with significant differences observed for SpA (28%; $p = 0.02$) and SLE (14%; $p = 0.03$) relative to HC. Seropositivity rates at D28 showed that patients with AID presented late seroconversion profiles, regardless of subgroup, reaching 78% seroconversion relative to D14 (**Figure 4**).

## Kinetic Timeline of 17DD Viremia

Viremia profiles were analyzed at sequential time points (D0, D3, D4, D5, D6, D7, D14, and D28) and the data are presented as the percentage of maximum (**Figure 5**). Analysis of overall viremia profiles demonstrated that the YF viral RNAnemia peak and global maximum were detected around D5–D6, regardless of AID subgroup. The YF viral RNAnemia peak was slightly later and lower in patients with AID (D6 = 47%) relative to HC (D5 = 78%). Additional analysis was carried out by segregating patients with AID into two subgroups, according to their seroconversion profiles: AID/PRNT(–) and AID/PRNT(+). The day of viremia peak with global maximum values (AID/PRNT(–) = 55%; AID/PRNT(+) = 45%) was detected at D5. Comparative analyses of AID subgroups further demonstrated that global maximum values were detected at around D5 (RA = 39%; SpA = 90%; SLE = 57%) and D6 (SS = 86%). Viremia was undetectable in the SSC subgroup (**Table 4**).

# DISCUSSION

This investigation prospectively evaluated AE in response to, and efficacy of, YF primary vaccination in patients with rheumatic AID. Despite data showing that antibody levels were lower than those in controls, consistent seroconversion rates were observed in patients with AID.

A systematic review, including case reports following live vaccinations of immunosuppressed patients, showed that the rate of seroconversion of YF vaccine was high, and better than those of other live vaccines, in patients with AID (34).

Oliveira et al. (35) studied 31 individuals with AID who were inadvertently re-vaccinated. Similar to our results, they reported a seroconversion rate of 87%. Both studies suggest that, although the titers of neutralizing antibodies are lower among patients with rheumatic disease than healthy individuals, they were sufficiently high to confer a protective response (36).

A single study from the Netherlands reported 15 cases of patients with AID (rheumatoid arthritis, pyoderma gangrenosum, and psoriatic arthritis) who received primary YF vaccination, which reported 50% seroconversion (virus neutralization at serum dilution 1:50) in patients using methotrexate ($n = 8$), prednisone ($n = 1$), leflunomide ($n = 1$), and etanercept ($n = 2$) (5). We found a higher of seroconversion rate of 78% than the reported latter study; however, there are some potential reasons for the difference between these studies.

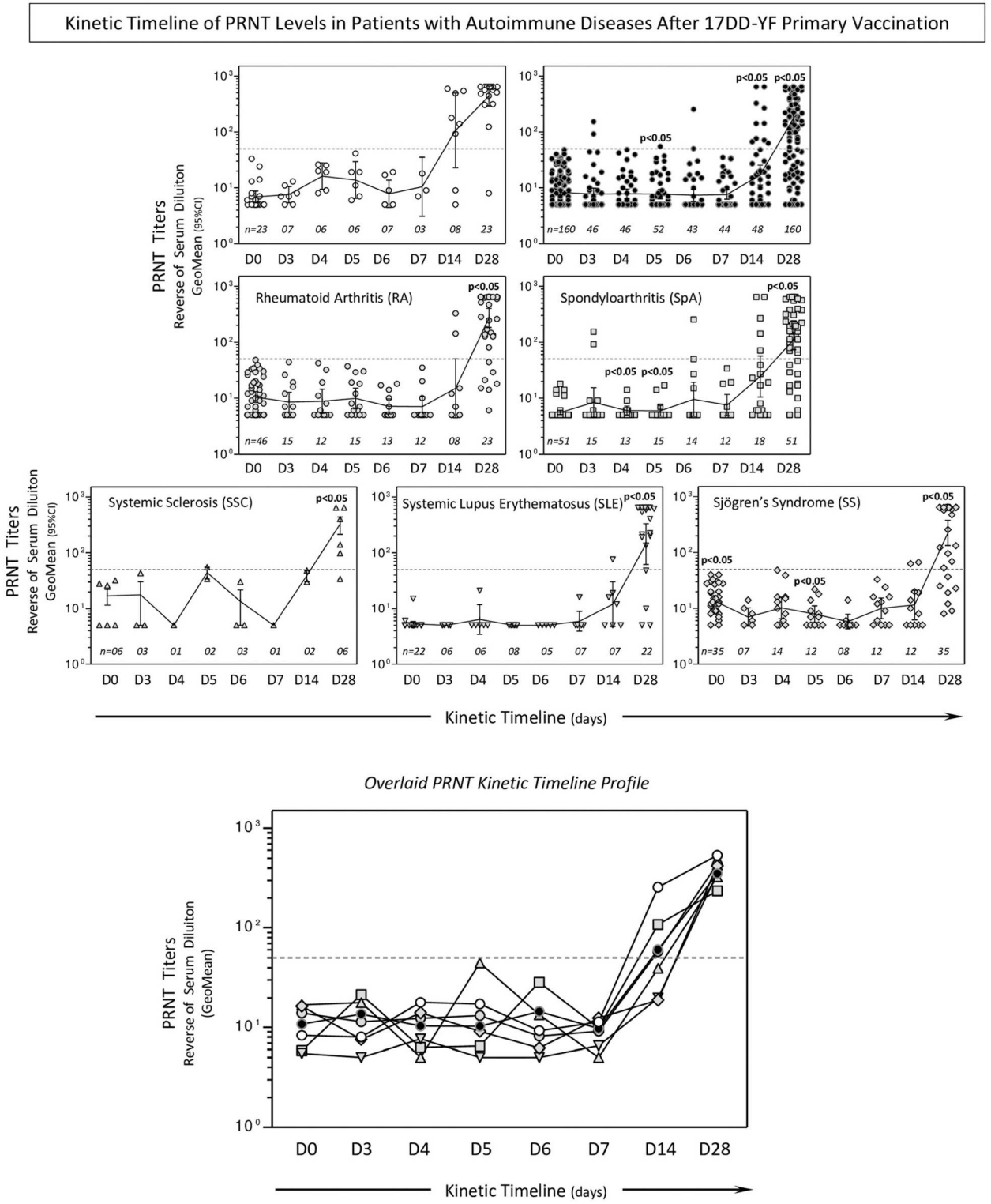


**FIGURE 3 |** Kinetic timeline of PRNT levels in patients with AID after 17DD-YF primary vaccination. Levels of 17DD-YF specific neutralizing antibodies were detected by micro-PRNT, as previously described by Simões et al. (25). Data are presented as a scatter plot over a column chart of PRNT titers, expressed as the reverse of the serum dilution and 95% CI of reverse serum dilution (HC,○; AD,●; RA,○; SpA,□; SSC,△; SLE,▽; and SS,◇) at baseline (day 0; D0) and over time after primary vaccination (D3, D4, D5, D6, D7, D14, and D28). The cut-off point (PRNT ≥ 1:50) is represented as a dashed line. Comparative analysis of PRNT titers at each time point (HC vs. AID or AID subgroups) was performed by Mann-Whitney test. A threshold $p < 0.05$ was considered statistically significant. Overlaid kinetic timeline profile of PRNT is also provided in the figure. The number of samples tested for HC, AID, and AID subgroups is provided in the figure.

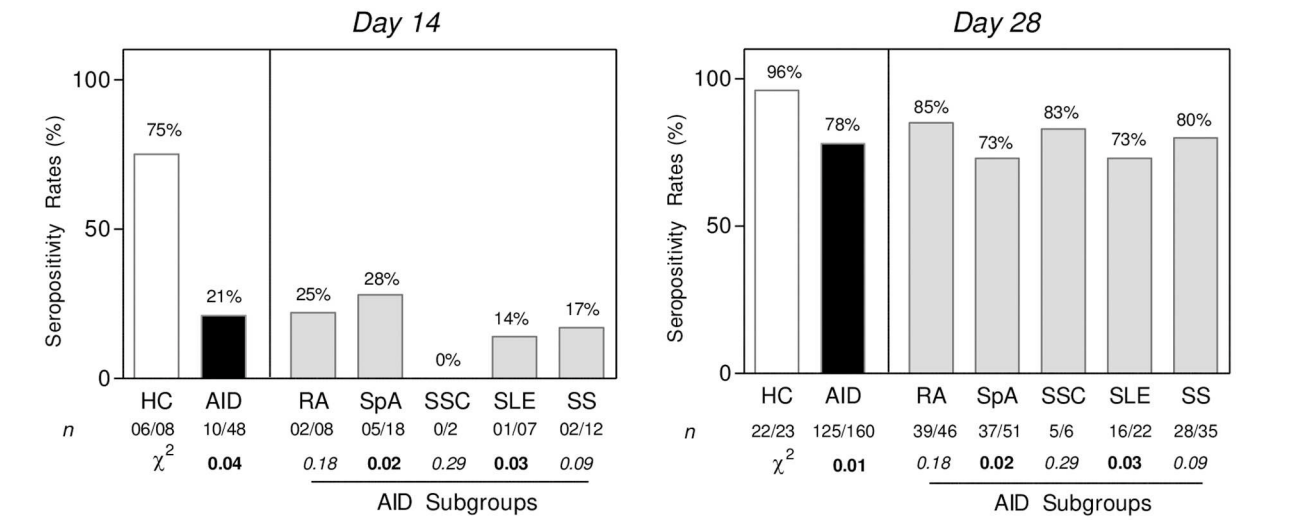


**FIGURE 4 |** PRNT seropositivity rates in patients with AID at D14 and D28 after 17DD-YF primary vaccination. Levels of 17DD-YF-specific neutralizing antibodies were detected by micro-PRNT, as previously described by Simões et al. (27). Seropositivity rates were calculated with a serum dilution ≥ 1:50 considered the cut-off criterion for PRNT positivity (PRNT ≥ 1:50). The results are presented in bar charts for HC (□), AID (■), and AID subgroups (□). A chi-square test was employed for comparative analysis of PRNT seropositivity rates among groups. A threshold $p < 0.05$ was considered statistically significant. HC, healthy contros; AID, autoimmune patients; RA, rheumatoid arthritis; SpA, spondyloarthitis; SSC, systemic sclerosis; SLE, systemic lupus erythematosus; SS, primary Sjögren's syndrome.

First, we included patients who underwent planned vaccination and were under low level immunosuppression and, second, we prospectively evaluated all participants 28 days after vaccination. In the previous study, samples were collected from 15 immune-compromised individuals, vaccinated with the 17DD-YF vaccine between 2004 and 2012, at different times after vaccination (5). The same authors reported that the percentages of early-differentiated memory cells increased over time and concluded that time since vaccination was negatively correlated with the number of specific memory cells (4).

We also evaluated the immune responses in different diseases. As expected, the response in patients with SLE was lower, probably because the disease pathology affects both innate and adaptive immune responses, particularly those of B-cells (36). A diminished response to antigenic challenge in SLE, including vaccinations, has previously been suggested (36, 37). Holvast et al. (37) evaluated 56 patients with quiescent SLE and 18 HC who received influenza vaccination. Fewer patients achieved a titer ≥ 40 to both influenza A strains (75% of patients vs. 100% of controls) (17, 36). Although the humoral response of patients with SLE is decreased, it still fulfills the criteria for influenza vaccine immunogenicity, as agreed upon by the Committee for Proprietary Medicinal Products (38). Therefore, the clinical relevance of such a decreased response remains unclear. Little is known about cell-mediated immune responses to vaccination in patients with SLE, although diminished or disturbed T helper function has been suggested (38). We considered azathioprine ≤ 2 mg/kg/d as low level immunosuppression, and one third of SLE patients were using it in our study, which may have contributed to the low humoral response observed in the SLE group.

Surprisingly, PRNT levels and the seroconversion rate were as low in the SpA group as those observed in SLE. Our hypothesis is that some patients in this group had a history of using biological therapy and that perhaps the washout time was insufficient to reconstitute an immune response (39). Ferreira et al. demonstrated earlier loss of humoral response, triggered by conventional synthetic DMARDs (csDMARDs), combined with biological DMARDs. This was confirmed by the critical decrease in PRNT seropositivity rate to 76%, observed at > 5–9 years post-vaccination in patients with RA receiving combined therapy, in contrast with the standard decline observed in controls and the csDMARD group 10 years after 17DD-YF vaccination (40).

Our study was conducted in patients under low immunosuppression. Antiproliferative drugs, mycophenolate mofetil, calcineurin inhibitors, azathioprine (> 2 mg/kg/day), prednisone (≥ 20 mg/day), methotrexate (> 20 mg/week), or any immunobiological drug were withdrawn for the minimum recommended interval, according to Brazilian guidelines (17).

In the SpA group, 49% were using biological therapies that were withdrawn after the minimum interval, and it is possible this interval (4–5 half-lives) (17) is insufficient to allow reconstitution of immune responses. Future studies of cellular immune signatures, comparing groups receiving different therapies and with various diseases, could help in understanding why patients with SLE and SpA had the lowest antibodies levels.

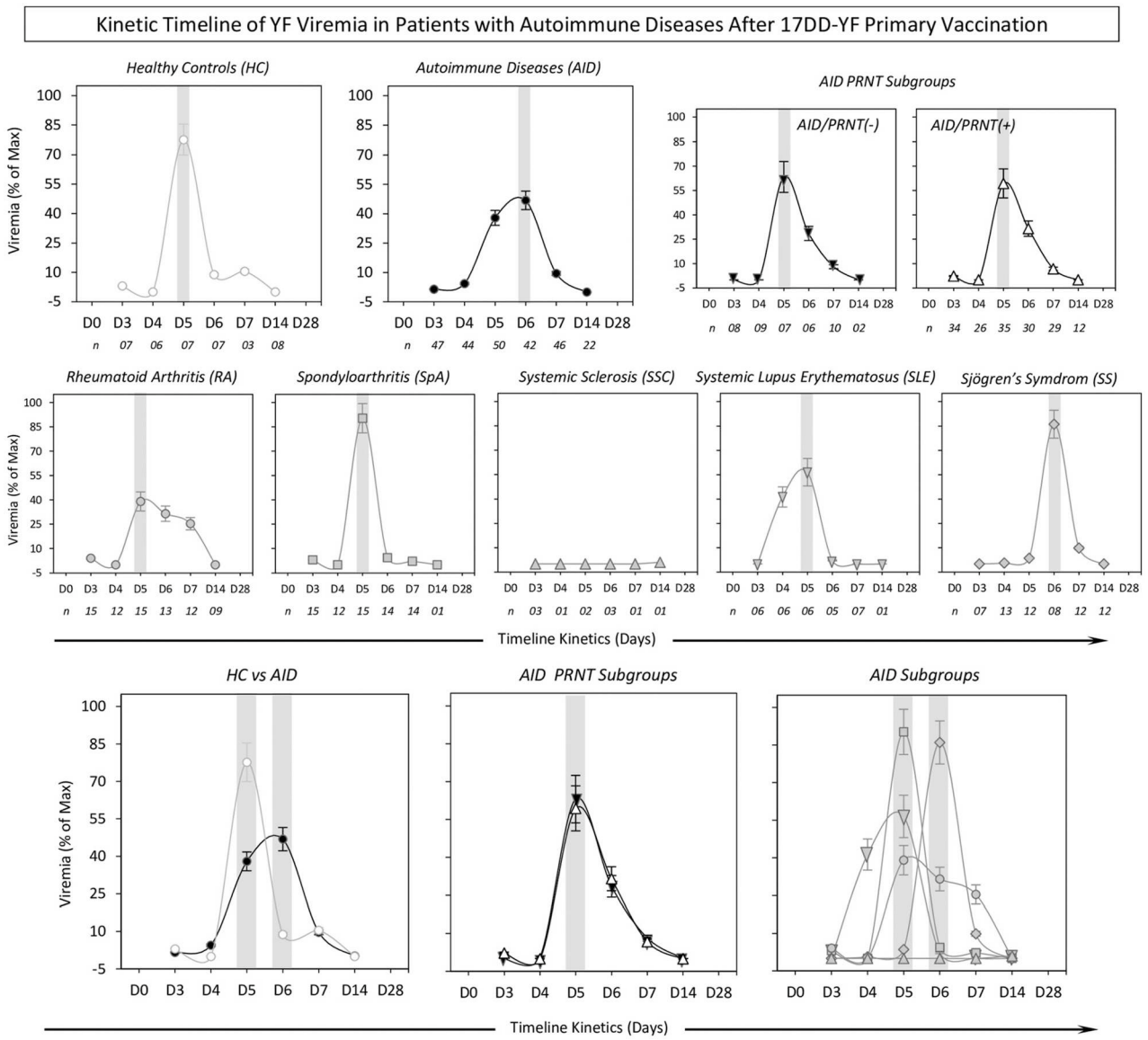


**FIGURE 5 |** Kinetic timeline of YF viremia in patients with autoimmune diseases after 17DD-YF primary vaccination. Viremia levels (YF viral RNAnemia) were quantified in serum samples by qRT-PCR assay, according to Martins et al. (28). The results are expressed as percentage of maximum viremia levels ± standard error detected for (HC,○; AID,●; AID–,▼; AID+,△; RA,○; SpA,□; SSC,△; SLE,▽; and SS,◇) over time after primary vaccination (D3, D4, D5, D6, D7, and D14). The gray background represents the day of the viremia peak. Overlaid kinetic timeline profile of YF viremia is also provided in the figure. The number of samples tested for HC, AID, and AID subgroups is provided in the figure.

Previous studies have shown that severe AE are more common in patients with AID, particularly SLE (8). Also, immunosuppressive drugs can increase the risk of AE (9, 10). We did not observe any severe AE; however, we recorded frequent mild AE (34%), which was similar in the control group and to reports from a previous study (41). We did not explore the risks associated with medication, because all patients were under low level immunosuppression.

Our study has some limitations. The number of AID/PRNT(-) is modest and further studies are required to further explore this matter. We did not analyze cellular responses, which could shed some light on the differences in immune responses observed among patients with various diseases. We were unable to analyze medication background, due to sample size restrictions. In addition, we did not include children in this study neither investigate the disease activity on follow-up. We plan to follow patients after 6 and 12 months to study disease activity, and for 5 years to determine cellular and humoral responses over time. Further studies of immunological biomarkers prior and after 17DD-YF primary vaccination would be relevant to

add new insights to explain the differences on seroconversion rates observed amongst AID patients according the subgroups of diseases.

In conclusion, our findings support the safety and efficacy of planned primary YF vaccination for patients with AID with low disease activity and receiving low level immunosuppression. These results will help to define target populations and indicators of protection, particularly in endemic countries with high historical rates of YF virus circulation in continuous expansion.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Hospital Universitário Cassiano Antônio Moraes/EBSERH at UFES. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

VV, KM, VD, LP-N, AB, and OM-F: designed the research study. VV, OM-F, AT-C, FF, and MM: acquired funding. JD, AC-A, VP-M, IC-R, SL, EM, GT, KO, MB, and SG: conducted experiments. VV, KM, SM, AP, PR, ES, VD, JD, MBG, LS, RD, AG, TZC, BM, FN-B, LR, TBC, EM, MPG, CC, RG, LB, EP, IK, BB, DP, LD, DM, LG, FP, MSG, AB, and FF: field study. VV, KM, SM, AP, PR, ES, TZC, VD, JD, IC-R, and OM-F: acquired data. VV, SM, AP, KM, JD, and OM-F: analyzed data. VV, KM, JD, and OM-F: drafted the manuscript. VV, KM, SM, AP, PR, ES, VD, SG, JD, MBG, LS, RD, AG, TZC, BM, FN, LR, EM, MPG, LP-N, CC, RG, LB, EP, IK, BB, DP, LD, DM, LG, FP, MSG, AB, FF, GP, LM, AC-A, AT-C, VP-M, IC-R, SL, EM, GT, MM, KO, MB, and OM-F: reviewed/wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Mitigating Coronavirus Induced Dysfunctional Immunity for At-Risk Populations in COVID-19: Trained Immunity, BCG and "New Old Friends"

*Thomas-Oliver Kleen[1]*, Alicia A. Galdon[2], Andrew S. MacDonald[2] and Angus G. Dalgleish[3]**

[1] Immodulon Therapeutics Limited, Uxbridge, United Kingdom, [2] Lydia Becker Institute of Immunology and Inflammation, Manchester Collaborative Centre for Inflammation Research, Faculty of Biology, Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Manchester, United Kingdom, [3] Institute for Infection and Immunity, St George's, University of London, London, United Kingdom

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United Kingdom

***Correspondence:**
Thomas-Oliver Kleen
tk@immodulon.com
Angus G. Dalgleish
dalgleis@sgul.ac.uk

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The novel, highly contagious coronavirus SARS-CoV-2 spreads rapidly throughout the world, leading to a deadly pandemic of a predominantly respiratory illness called COVID-19. Safe and effective anti-SARS-CoV-2 vaccines are urgently needed. However, emerging immunological observations show hallmarks of significant immunopathological characteristics and dysfunctional immune responses in patients with COVID-19. Combined with existing knowledge about immune responses to other closely related and highly pathogenic coronaviruses, this could forebode significant challenges for vaccine development, including the risk of vaccine failure. Animal data from earlier coronavirus vaccine efforts indicate that elderly people, most at risk from severe COVID-19 disease, could be especially at risk from immunopathologic responses to novel coronavirus vaccines. Bacterial "new old friends" such as Bacille Calmette-Guérin (BCG) or *Mycobacterium obuense* have the ability to elevate basal systemic levels of type 1 cytokines and immune cells, correlating with increased protection against diverse and unrelated infectious agents, called "trained immunity." Here we describe dysfunctional immune responses induced by coronaviruses, representing potentially difficult to overcome obstacles to safe, effective vaccine development for COVID-19, and outline how trained immunity could help protect high risk populations through immunomodulation with BCG and other "new old friends."

**Keywords: COVID-19, SARS, dysfunctional immune response, vaccine, trained immunity, BCG, IMM-101, *Mycobacterium obuense***

## INTRODUCTION

In recent months, a novel severe acute respiratory syndrome (SARS) coronavirus (CoV), SARS-CoV-2, which causes COVID-19, has spread rapidly throughout the world (1). As of July 15, 2020, more than 13 million infections and over 575,000 COVID-19 related deaths have been confirmed worldwide. Based on a chronic lack of adequate testing capabilities in many countries worldwide,

including large industrialized nations like the United States, a large amount of undiagnosed infection and mortality from COVID-19 must be assumed. The unprecedented pandemic seriously challenges the world's health care systems and available hospital capacities to treat seriously ill patients. These challenges are amplified by frequent SARS-CoV-2 infection of healthcare workers (HCW), leading to hospital-acquired infection of HCW and patients, and significant mortality within that group (2). Other high-risk groups of infection include the elderly, with age-related immunosenescence and "inflammaging" having been suggested as a mechanism responsible for lowered immunological competence and the high mortality of the elderly in the current COVID-19 pandemic (3). Age-related risks are a particular issue in assisted care facilities and individuals with serious, non-COVID underlying health conditions like cardiovascular disease, chronic kidney disease, diabetes, chronic respiratory disease, immunosuppression, and cancer (4, 5). In the case of cancer, many malignancies require active treatment, making isolation – even social distancing – impossible, based on the need to commute to the hospital regularly to receive treatments. Therefore, there is an urgent need to protect individuals aged 55 years and older with co-morbidities. Throughout the public discourse, there has been little attention given to the observations that these populations are historically the same populations that are most unlikely to develop efficient and protective immune responses to standard respiratory viruses. Consequently, this is likely to be the same case for SARS-CoV-2. Indeed, for these populations, other more potent vaccines, compared to the general population, are required, e.g., "high dose" Influenza shots for the elderly. Nevertheless, those more potent vaccines often still result in less than ideal outcomes in these vulnerable populations (6). In order to avoid the need for achieving herd immunity by infection or mass vaccinations before safely reopening societies and economies, a priority would be immunizing the most at-risk populations first. There is a justified concern that suboptimal vaccine efficacy for at-risk populations and the elderly could place the goal of not having to achieve herd immunity first in jeopardy. At the same time, a non-efficacious vaccine for at-risk populations could increase the probability of second and subsequent waves of infection in these populations (7).

Worldwide availability of safe, effective, prophylactic vaccines is likely the only approach that will ultimately control this deadly pandemic. However, such vaccines may not be available until early next year, even in the most optimistic scenarios (8). Despite numerous efforts, no vaccine, proven safe and effective in humans, has ever been developed against any coronavirus (9, 10). Questions about the potential lack of sufficient vaccine efficacy in elderly populations have not yet been widely discussed. Therefore, strategies to prevent COVID-19 morbidity and mortality in high risk groups are desperately needed in order to safeguard the most vulnerable individuals, as well as maintaining continuous patient care and functioning hospital systems.

Both humans and animals are susceptible to disease caused by CoVs. Three highly pathogenic CoVs are known, SARS-CoV, Middle East respiratory syndrome (MERS)-CoV and SARS-CoV-2. All three are now known to efficiently infect and replicate in the lower respiratory tract, frequently causing substantial immunopathology, acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and fatal pneumonia, resulting in high morbidity and mortality (11). SARS-CoV and SARS-CoV-2 are both members of the betacoronavirus genus and share more than 70% of their genetic code (12). However, it is noteworthy that SARS-CoV-2 is closest related to the bat coronavirus RaTG13, with 98% genetic similarity compared to all known genetic coronavirus sequences (13). Four additional, circulating but low pathogenic human coronaviruses (HCoV) are known and will not be reviewed here, but preexposure to them could impact the immune response to SARS-CoV-2 in patients (14). All four, HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1, display a winter seasonality, causing comparatively mild to moderate upper respiratory illnesses and only occasionally, bronchiolitis and pneumonia symptoms (15, 16). All HCoVs share a minimum of four, genome encoded, major structural proteins: the spike (S) glycoprotein, nucleocapsid (N) protein, membrane protein (M), and the envelope protein (E), all of which are required to produce a structurally complete viral particle (17).

## IMMUNE RESPONSES TO CORONAVIRUSES

The pandemic, which originally emerged from Wuhan, China, has been characterized by a rapidly increasing morbidity and mortality rate associated with older age, beginning around age 50 years (18). Multiple aspects of immunity can be influenced by ageing, prompting scrutiny of which components of the immune response might be responsible for higher mortality in older people (19). In general, an early and robust innate immune response to viral infections permits more rapid and effective viral clearance and may even prevent symptomatic infection or diminish the severity of the infection (20). No correlates of protection have yet been formally established for the recently emerged SARS-CoV-2. However, mouse model data from studies with the first SARS-CoV that emerged in 2002, suggested a delayed innate immune response during infection is linked to a more severe course, with immunopathology in the lungs and high mortality (21). Initial observational studies suggest that a failure of antiviral immunity, including depleted natural killer (NK) cells, at an early stage in COVID-19, may lead to severe clinical course and an inability to recover from infection (22). In addition, it has previously been shown that the SARS-CoV macrodomain suppresses the innate immune response during infection, whereas an early strong innate immune response can protect mice from lethal disease and prevent detrimental downstream effects on the immune system (23). On the other hand, in later stages of infection, it appears that a dysregulated immune system, including excessive inflammatory responses by innate cells in the lungs, and selective immunosuppression of the adaptive immune system, can be detrimental for the host (24, 25). Acute lung injury caused by viruses like respiratory syncytial virus, influenza A virus and SARS-CoV have been described previously (11, 26, 27). Aberrant expression of the antiviral cytokine type I interferon (IFN), interferon stimulated

genes, and other inflammatory cytokines, were observed in patients with severe SARS-CoV disease compared to healthy individuals, providing evidence that SARS-CoV is partly an innate dysregulated immune disease (28, 29).

The innate immune system recognizes pathogen-associated molecular patterns (PAMPs) of viral or bacterial intruders via pattern recognition receptors (PRRs). Toll-like receptors (TLRs), a family of type I transmembrane PRRs that consists of related, transmembrane proteins, play a central role in the initiation of inflammatory responses against pathogens, including the secretion of cytokines and chemokines. TLR4 is known to sense lipopolysaccharide (LPS) from gram-negative bacteria, but, based on its additional function as sensor for damage-associated molecular patterns (DAMPs), TLR4 has been suggested to play a central role in the induction of damaging inflammatory responses during several acute viral infections (30). In addition, oxidized phospholipids (OxPLs), DAMPs which lead to ALI in patients infected with SARS-CoV, also accumulate in lungs of patients infected with SARS-CoV-2 and activate monocyte-derived macrophages through TLR4 (31, 32). Interfering with innate cell activation by TLR4 in response to ligands such as OxPLs may therefore help prevent thrombotic complications, recently identified as a major factor in mortality of COVID-19 patients (33–35). Endothelial cell activation, infection and dysfunction has been implicated in severe COVID-19 by altering vessel barrier integrity, promoting a pro-coagulative state, inducing vascular inflammation, endotheliitis, and mediating inflammatory cell infiltration. The proposed mechanism is disruption of vascular integrity and endothelial cell death, which leads to exposure of the thrombogenic basement membrane and results in the activation of the clotting cascade (36). Altered platelet gene expression and functional responses in patients infected with SARS-CoV-2 may additionally contribute to observed hemostatic abnormalities like disseminated intravascular coagulopathy (37).

## Neutrophils

Neutrophils are an important component of the general response to infection in the respiratory system and capable of recognizing viruses via viral PAMPs (38). In the context of potentially excessive neutrophil activation in late stage COVID-19 disease, neutrophil extracellular traps (NETs) in the lungs can drive severe pathologies by accumulation of mucus in the airways of patients, contributing to ARDS (39). More importantly, NETs have been proposed to contribute to organ damage and mortality, since excess NET formation can trigger a cascade of inflammatory reactions that destroys surrounding tissues and facilitates atherosclerosis, aortic aneurysms, as well as thrombosis, including microthrombosis, in the vascular system, with devastating effects on organ function (39).

## Macrophages

Macrophages are key innate immune cells in any infection setting (40, 41). They are highly flexible innate cells that can, simplistically, be functionally and phenotypically divided into pro-inflammatory "M1" macrophages (capable producers of inflammatory cytokines and mediators, that kill infectious organisms, virus-infected cells, or tumor cells) and more regulatory "M2" macrophages (that are important for wound healing and parasite infections) (42, 43). Both activation states are needed for a "balanced" immune response, although the M1/M2 paradigm of macrophage activation is an over-simplistic definition of these complex and diverse innate cells (44, 45). During ageing and chronic inflammatory diseases, macrophages may switch to a more M2-like phenotype (46, 47). Importantly, nearly all identified high-risk factors for severe COVID-19 disease, like cardiovascular disease, diabetes, age, chronic obstructive pulmonary disease, and smoking, generally share a shift from more M1 to more M2 phenotype and function (48). Classical activation of M1 macrophages is induced by LPS/IFN-γ exposure, while alternately activated M2 macrophages are stimulated by IL-4, IL-10, IL-13 and glucocorticoids (49). The activation of innate immune cells such as macrophages can be heavily influenced by the character of the T cell response and, in particular, the cytokines produced by T cells during infection (50). SARS-CoV replication has previously been shown in human peripheral monocytes and macrophages, with varying efficacy. Importantly, the infection efficiency was shown to be donor dependent, with 100% infection in some and less than 5% in others (51).

## γδ-T Cells

In adults, Vγ9Vδ2 cells are the dominant γδ T cell population, however, in elderly individuals the variability increases (52, 53). An analysis of T cell repertoires in HCW who survived SARS-CoV infection during the 2003 outbreak revealed that an innate-like subpopulation of effector memory T cells, γδ-T cells, specifically Vγ9Vδ2 T cells, were selectively expanded approximately 3 months after the onset of disease (54). Importantly, no such expansion of non-innate αβ T cells was detected at the same time point. Furthermore, expansion of the Vγ9Vδ2 T cell population was associated with higher anti-CoV IgG titers, and *in vitro* experiments demonstrated that Vγ9Vδ2 T cells display an IFN-γ -dependent ability to directly kill CoV infected target cells. Therefore, innate-like Vγ9Vδ2 T cells may play a protective role during SARS-CoV and other CoV infections. A recent study analyzed the number and activation status of Vγ9Vδ2 T cells in hospitalized patients with COVID-19. They found significantly lower levels of Vγ9Vδ2 T cells than that of matched healthy control and concluded that this could indicate that elderly with lower frequencies of Vγ9Vδ2 T cells constitute a SARS-CoV-2 vulnerable population or that the Vγ9Vδ2 T cells in these patients have migrated to the lungs to kill SARS-CoV-2 infected cells (55).

## T Cells and NK Cells

SARS-CoV infection leads to lymphopenia and strongly reduced peripheral T cell levels, with low CD4+ and CD8+ T cell counts associated with adverse outcome, and a rapid and dramatic restoration of peripheral T cell subsets in the periphery of recovering patients (56–58). In addition, SARS-CoV can infect and replicate within PBMCs of SARS-CoV patients, with viral replication appearing to be self-limiting but leading to leukopenia or lymphopenia (59–61). Patients with clinical symptoms of severe COVID-19 also commonly present with lymphopenia,

including dramatically reduced numbers of NK cells, CD4+ T cells, CD8+ T cells and B cells, which has not been observed in mild cases (62–65). Further studies have shown exhaustion markers like NKG2A on cytotoxic lymphocytes, including NK cells and CD8+ T cells, are upregulated in patients with COVID-19, and that for recovered patients, numbers of NK cells, CD4+ T cells, CD8+ T cells, and B cells normalize, along with markers of exhaustion on cytotoxic lymphocytes (66, 67). Reduced functional diversity and increased T cell exhaustion in peripheral blood could predict severe progression in COVID-19 patients, supporting the role of functional T cells in controlling COVID-19 (67). Importantly, it was recently shown that a patient with mild to moderate COVID-19 symptoms had a broad-based robust immune response across different immune cell types, which was associated with rapid recovery (68). This observational study identified the presence of activated CD4+ T cells, CD8+ T cells, and follicular helper T cells in the blood, along with increased antibody-secreting cells and IgM and IgG antibodies. The study did not investigate the neutralization capabilities of the observed antibodies. Cell-mediated type 1 immune responses are therefore theorized to be a major component necessary to overcome COVID-19 infection (69).

This is further supported by a study that screened for the presence of SARS-specific T cells in a cohort of three SARS-CoV-recovered individuals, where CD8+ T cell responses targeting the SARS-CoV membrane and nucleocapsid proteins were found to persist up to 11 years post-infection (70). Characterization of SARS-CoV-specific memory T cells from recovered individuals 4 years after infection indicated that the majority of memory CD8+ T cells produced IFN-γ, whereas memory CD4+ T cells produced IFN-γ, IL-2, or TNF-α (71). Multiple other independent studies established that SARS-CoV specific memory CD4+ and CD8+ T cells persisted for up to 2 years after infection (72–74). S protein-derived epitopes of SARS-CoV elicited recall CD8+ T cell secretion of IFN-γ as well as intracellular production of IFN-γ, TNF-α, perforin, and granzyme A from recovered patients over 1-year post infection, indicating that SARS-CoV infection can induce strong and long-lasting cytotoxic T lymphocyte (CTL)-mediated immunity in patients (75, 76). High frequencies of CD8+ Tc1-type T cells, reactive against MERS-CoV, were observed in a large proportion of patients with severe and moderate MERS at acute stage before detection of humoral and CD4+ T cell responses. Another report emphasizing the importance of T cells demonstrated that 17 years after the 2003 SARS outbreak, SARS-CoV-recovered patients still maintained long-lasting memory T cells reactive to the N protein of SARS-CoV, which notably exhibited robust cross-reactivity to the N protein of SARS-CoV-2 (77). A recent study showed predominant Th1 responses in convalescing COVID-19 cases, with little to no Th2 responses. It demonstrated SARS-CoV-2 specific CD4+ T cells in 100% of COVID-19 convalescent patients, with the majority of responses against S protein, correlating with the magnitude of anti-SARS-CoV-2 IgG and IgA titers, but as well responses against M and N proteins in all patients, accounting for 11–27% of the total CD4 + responses. The same study found SARS-CoV-2 specific CD8+ cells against S and M proteins in about 70% of patients, and interestingly, T cell reactivity to SARS-CoV-2 epitopes was also detected in non-exposed individuals, likely cross-reactive from previous, seasonal HCoV infections (78). However, at the convalescent phase, the magnitude of the CD8+ T cell response was not increased further.

Although it seems clear that robust inflammatory and CTL responses are required to clear the invading virus, when excessive, they can also lead to lung tissue destruction and pneumonia (79). Early pathological findings of COVID-19 patients with ARDS, showed not only reduced counts of peripheral CD4+ and CD8+ T cells, but that remaining T cells were found in a hyperactivated state, with high proportions of HLA-DR and CD38 double-positive fractions (80). It is noteworthy here that, in patients hospitalized with avian H7N9, survival reflected an early, but transient, prevalence of highly activated CD8+CD38+HLA-DR+PD-1+ T cells, but prolonged CD38+HLA-DR+PD1+ co-expression predicted fatal outcomes (81). CD8+ T cells in patients that died of H7N9 were non-functional, as reflected by a lack of IFNγ production, but displayed high and continued expression of the CD38+HLA-DR+ activation markers, together with the inhibitory PD-1 immune checkpoint receptor. Similar studies in Ebola, Dengue, and pandemic H1N1 have also mentioned the presence of these "non-survival" peripheral lymphocyte populations, with high and prolonged frequency of activated CD8+CD38+HLA-DR+ cells (82–84). We hypothesize that, as suggested for H7N9 disease (81), in COVID-19 patients this could also be associated with defective T cell activation and a lack of relevant T cell receptor (TCR) specificities. It is known that infection with human immunodeficiency virus (HIV) induces broad lymphocyte activation, with an increase in T cell activation markers such as CD38 (85). Several studies have shown that such increased CD38+ expression on CD8+ T cells is a strong predictive marker for disease progression in HIV-1 infection (86). Not only does the CD8+CD38+ T cell count predict progression of HIV disease to AIDS and death, but it is also independently predictive for evaluation of high plasma virus load and low CD4+ T cell counts (87). In early HIV infection, during onset of viremia, CD8+CD38+HLA-DR+ T cells correlate inversely with viral set point. However, hyperacute HIV infection leads these cells to be short-lived effector cells that do not persist, characterized by marked apoptosis, upregulation of CD95 and failure to upregulate the IL-7 receptor CD127 (88). Strikingly, in a recent study in COVID-19 patients, considerable proportions of peripheral CD4+ and CD8+ T cells co-expressed CD38 and HLA-DR, but those cells could not be re-activated with peptide pools of the S protein *in vitro*, supporting the notion of SARS-CoV-2 specific refractory T cells and/or different specificities (14). No data about the PD-1 status of T cells was provided. The same remarkable study showed that, while the majority of S-reactive CD4+ T cells from COVID-19 patients co-expressed CD38 and HLA-DR, S-reactive CD4+ T cells from healthy donors, proposed to be cross reactive to other HCoVs, only expressed CD38 and HLA-DR at very low frequencies and co-expression was not observed. In cancer therapy models, depleting "dysfunctional" CD8+CD38hiPD-1+ cells enhanced therapeutic outcomes, and patients who did not respond to immunotherapy showed more CD8+CD38hiPD-1+ in tumor and blood compared to responders (89). The potential significance of levels and

timing of prolonged expression of CD38, HLA-DR, and PD-1 on dysregulated T cells and the utility of CD8+CD38+HLA-DR+PD-1+ T cells as a prognostic marker could be important and should be investigated in more detail. These could serve as indicators of SARS-CoV-2 immunosuppression, exhaustion and immune evasion, predicting divergent disease outcomes. The suggestion that a dysfunctional immune response is at the heart of COVID-19 pathology is further supported by the recent finding that, compared to patients with moderate disease, significantly reduced frequencies of CD8+ T cells, as well as diminished frequencies of CD4+ and CD8+ T cell subsets with activated differentiated memory/effector phenotype and migratory capacity, are found in peripheral circulation of patients with severe COVID-19 (90).

## B Cells and Antibodies

Antibody responses elicited by coronaviruses, including SARS, have been described as comparatively short lived and inconsistent (91, 92). Studies with human volunteers that were infected with a seasonal coronavirus HCoV-229E showed that individuals could get infected and display symptoms, including lymphocytopenia, regardless of preexisting antibodies (91). One study showed that six years post infection, SARS-CoV specific IgG was undetectable in 21 of 23 former patients, and no SARS-CoV specific memory B cell responses could be detected in any of the 23 patients (93). Another study revealed that SARS-CoV antibodies could be seen up to 24 months after infection (93). Interestingly, longevity of MERS-CoV antibody response correlated with disease severity. In one study, patients with severe MERS-associated pneumonia had a persistent antibody response detected for about 18 months after infection, while patients with infection limited to the upper respiratory tract or who had no clinical signs had no detectable MERS-CoV antibody response (94). In another report, the more severe the illness, the greater the antibody response, including IgM, IgG, and neutralizing Ab (NAbs). Patients in the convalescent phase, with mild or asymptomatic disease, rarely developed antibody responses (79). A strong antibody response developed in most MERS patients only after 2–3 weeks of illness, but the antibody responses were not correlated with the elimination of the virus from the body (95, 96). This was confirmed in two more studies that showed MERS infections are frequently characterized by low NAbs, despite patient recovery (93, 97–99). It is noteworthy that this was also recently shown for COVID-19 patients, where seroconversion has been observed in 9 mild to moderate cases after 6–12 days, but, despite COVID-19 antibodies arising at that time, no rapid decline of viral loads was observed, as would be expected with highly effective and neutralizing antibodies (100). Since anti-SARS-CoV antibody responses are short-lived in patients who have recovered from SARS, there are early indications that antibodies, and especially NAbs, may not be the predominant mechanism necessary for effective viral clearance and for infected individuals to overcome a COVID-19 infection (10, 101–103). This is further reinforced by the first longitudinal study in COVID-19 patients, which showed that some individuals who have recovered and displayed a strong NAb response shortly after infection, had titers fall as much as 23-fold, and in some cases back to baseline within 3 months (104). The authors speculated that the observed transient NAb response could be a feature shared by both a SARS-CoV-2 infection that causes low disease severity, and the circulating seasonal coronaviruses. Other recent data supports the notion of an unclear role of Abs, by reporting short duration of Ab and NAb titers after SARS-CoV-2 infection. Compared with responses of patients with symptoms, asymptomatic individuals (arguably with the more effective immune response), had weaker Ab responses to infection, with a reduction of IgG levels already occurring in the early convalescent phase (105); viral load and duration of infection are likely to be factors. Remarkably, in this study, 40% of asymptomatic patients had undetectable levels of protective antibodies two to three months after infection, compared to 13% of the symptomatic patients with COVID-19. An even more notable finding, further indicating a limited role for Abs in overcoming SARS-CoV-2 infection, is that intrafamilial exposure to SARS-CoV-2 induces a cellular immune response without seroconversion (106).

## SARS Vaccine Challenges

Of the different proteins that characterize coronaviruses, the S protein is an important determinant of virulence, tissue tropism and host range (107). Trimers of S form the characteristic large spikes on the coronavirus envelope and both SARS-CoV and SARS-CoV-2 use the protein angiotensin converting enzyme 2 (ACE-2) as primary receptor for docking and infecting human host cells. Priming of the virus S protein by host cell proteases is essential for entry. When SARS-CoV-2 docks to the cell via the ACE-2 receptor, the host transmembrane serine protease 2 (TMPRSS2) is responsible for cell entry (108–110). TMPRSS2 also aids the MERS-CoV to penetrate the cell (111), but its primary receptor for entry is dipeptidyl peptidase 4 (DPP4) (112). Virus S glycoproteins are postulated to elicit an immune response in humans that could protect against future infection (108, 113). Many vaccine approaches against COVID-19 that are currently in development are focusing primarily on the generation of antibody responses against the SARS-CoV-2 S protein (8). However, despite the great urgency for making an effective vaccine against COVID-19 available, this approach must be undertaken with great caution. Several SARS-CoV vaccines that initially induced antibodies and short-term protection in mouse models of SARS-CoV led to dysfunctional or type 2 helper T cell (Th2)-type immunopathology on challenge, with prominent eosinophil infiltration in the lungs, suggesting hypersensitivity to SARS-CoV components was induced (10). Several other independent studies with animal models used to develop vaccine candidates against SARS-CoV exposed signs of lethal vaccine failure based on induction of cell-mediated type 2 enhanced immunopathology, with associated eosinophilic infiltrates causing severe pneumonia, especially in aged mice. A vaccine based on SARS-CoV S protein protected against viral challenge when young mice were vaccinated, but it failed to efficiently protect older mice (114). Another study indicated poor vaccine performance as well as Th2-based eosinophilic immune pathology in the lungs that was shown to be caused by alum adjuvanted and unadjuvanted SARS-CoV vaccines in aged animals (115). All this requires that particular attention be

given to the strongly increased mortality rate already evident in older SARS-CoV-2 patients and patients with comorbidities. SARS-CoV has been shown to dysregulate the immune response in SARS patients by biased activation of a Th2 response, which can counter-regulate the type 1 response that normally attacks bacteria and viruses (97). There was a significant increase in Th2 cytokines IL-4, IL-5 and IL-10 during acute infection in fatal SARS cases, once again indicating that the character of cellular immune response induced by any COVID-19 vaccine will be critical in determining whether it will succeed (101, 116). Four earlier vaccines against MERS-CoV-2 have been tested in rhesus macaques (RM), but no reports of efficacy of a single-dose MERS-CoV vaccine in non-human primates (NHPs) had been made until a recent study reported that RM seroconverted after a single intramuscular vaccination with the experimental ChAdOx1 MERS vaccine (117). The study showed that vaccinated animals developed a neutralizing antibody response, were protected against respiratory injury and pneumonia, and showed reduced viral load in lung tissue and reduced disease severity. In addition, a Phase 1 trial in healthy individuals aged 18–50 years has been conducted, with no adverse safety signal reported (118). Neither study has provided data in either aged animals or elderly humans. Most relevant in this context are early SARS-CoV-2 vaccine trial data. A Phase 1/2 study in adults aged 18 to 55 years of a COVID-19 RNA vaccine candidate (BNT162b1), utilizing mRNA that encodes trimerized SARS-CoV-2 spike glycoprotein, showed the generation of NAb titers 28 days after the first injection and one week after the second dose (119). It is not yet known what kind of immune response the vaccine will elicit in older people or long-term. An additional mRNA nano-particle based vaccine candidate (mRNA-1273) has been reported to induce both potent Nabs and CD8+ T cell responses and to protect against SARS-CoV-2 infection in the lungs and noses of a mouse model, without evidence of immunopathology (120). Importantly, it showed spike peptide-reactive CD4+ and CD8+ T cells producing IFN-γ, IL-2, and TNF, which would be encouraging if corroborated in ongoing Phase 2 clinical trials and Phase 3 efficacy evaluation of the same vaccine candidate. Another advanced SARS-CoV-2 vaccine candidate in Phase 1 clinical studies is adenovirus-vectored vaccine ChAdOx1 nCoV-19, which has been reported to prevent SARS-CoV-2 pneumonia in RM and not to be Th2 dominated, determined by IgG subclass and cytokine expression profiling (121). Notably, no evidence of immune-enhanced disease following viral challenge twenty-eight days after vaccination was observed in the respective animals. The levels of Abs produced by the vaccine in these RM were lower than many Ab responses in humans infected with SARS-CoV-2. While the vaccine protected RM from severe infection, they became infected with evident active virus replication, which does not rule out the potential of maintained ability to transmit virus.

Despite the inherent challenges of adopting new routes of routine vaccine administration during an ongoing pandemic, recent evidence would encourage consideration of intranasal administration, inhalation or other vaccine strategies that directly target the mucosal surfaces of the airways, because of distinct functional responses by respective tissue-resident memory T cells (122). It was shown, for example, in a mouse model, that conserved epitopes shared by SARS-CoV and MERS-CoV could induce airway memory CD4+ T cells producing IFN-γ which were phenotypically and functionally different from lung-derived cells and crucial for protection against both CoVs. It is particularly noteworthy in this study that intranasal (but not subcutaneous) vaccination protected mice from pathogenic human CoVs, and that protection required IFN-γ and was depended on early induction of robust innate and virus-specific CD8+ T cells (123).

SARS-CoV-2 has shown replication, not only in human peripheral monocytes and macrophages, but also to directly infect T lymphocytes during primary infection through S protein-mediated membrane fusion, likely contributing to the severe lymphocytopenia that is a diagnostic indicator common in COVID-19 patients (51, 100, 124). SARS-CoV has also been shown to infect dendritic cells (DC), the central coordinators of the immune response, leading to impaired DC maturation and their high expression of the pro-apoptotic protein TRAIL (125). Instead of facilitating lymphocyte activation and expansion in numbers, this likely induces lymphocyte death and represents another mechanism of immune escape and intensification of the immunocompromised state of SARS-CoV patients (126). Similar mechanisms could contribute to lymphopenia and dysfunctional immune responses observed in severe COVID-19 patients. In the elderly, immune evasion by SARS-CoV-2 is probably made worse due to the reduced number and function of antigen presenting cells (APCs) (127). Multiple studies have been performed in mouse models describing the importance of type 1 CD4+ and CD8+ T cells in SARS-CoV (128, 129), with one study establishing that virus-specific memory CD8+ T cells provided substantial protection from lethal closely related SARS-CoV infection in a mouse model, emphasizing the importance of a cell-based type 1 immune response for survival of SARS infections (102). The majority of the many current vaccine strategies against SARS-CoV-2 rely on unadjuvanted or self-adjuvanted vaccines (e.g., RNA and DNA vaccines), or type 2 immune response promoting vaccines (e.g., alum adjuvanted, or unadjuvanted peptide or protein based vaccines) (113, 130, 131). Rather than promoting type 1 immunity, such approaches are likely to mostly lead to induction of type 2 responses which, as previously discussed, are unlikely to be effective against SARS-CoV-2 (100). Existing CoV antibodies have, in the case of host challenge with the same virus, enhanced viral load and disease severity in feline coronavirus or feline infectious peritonitis virus (FIPV) infections. This phenomenon is known as antibody-dependent enhancement (ADE) of viral infection (132, 133). In FIPV infection ADE can be induced by the presence of sub-neutralizing levels of anti-FIPV spike antibodies (134). Unlike in dengue virus infections, ADE in feline coronavirus infection is caused by re-infection with the identical serotype virus (124). It should be noted that mice, often used for preclinical safety evaluation of vaccines, lack FcγRIIa, the main FcγR on human cells linked to ADE induction (135, 136). Increasing viral entry into permissive cells and/or triggering excessive production of pro-inflammatory cytokines has made ADE a significant concern with several viruses, including the closely related SARS-CoV (137, 138). Concerns have also been

raised that anti-SARS-CoV-2 non-neutralizing antibodies, or even declining NAb titers over time, could lead to ADE and enhanced disease after such vaccinations, antibody-based drug therapies, or treatment with convalescent plasma from recovered patients (139, 140). However, none of the early clinical trial results of the most advanced vaccine candidates described above have reported signs of ADE (121). Demonstration of a lack of ADE induction of different experimental vaccines against SARS-CoV-2 in NHPs and humans will remain critical for other vaccines advancing through the pipeline. One recent example of the need for continued vigilance is a study using Chinese macaques indicating cause for concern by showing that vaccine-induced, S-specific immunity in the form of anti-spike IgG resulted in severe ALI by skewing macrophage responses during subsequent, acute infection with closely related SARS-CoV (139).

Given all of the above, it is likely that successful vaccines against COVID-19 will require appropriate DC activation, leading to induction of a multifaceted and long-lived type 1 immune response that includes memory CD4+ Th1 cells, CD8+ CTLs, and NAbs. Most importantly, they will need to be effectively induced and sustained in older individuals without generating type 2 responses or ADE. It may remain a challenge to achieve this formidable goal and more creative approaches to vaccination may be required, but early data from pre-clinical and clinical trials of SARS-CoV-2 vaccines seem encouraging that they will provide some protection.

## Cytokine Storm

Direct comparisons in the literature of clinical observations in COVID-19 patients with IL-6 induced "cytokine storm" or cytokine release syndrome (CRS) should be made with caution (25, 141, 142). For example, cytokine levels during hyperinflammation in COVID-19 are multiple orders of magnitude lower than has been observed during cancer treatments by adoptive cell transfer of autologous T cells modified with chimeric antigen receptors (CAR-T cell therapy), a classical example for CRS (143, 144). Although, CRS is normally treated with extensive use of steroids, the clinical evidence does not support corticosteroid treatment for COVID-19 induced lung injury and interfered with clearance (145). In SARS and MERS, corticosteroid use did not improve patient mortality and also resulted in delayed viral clearance (11). It should be noted that a recent preprint of a randomized-controlled trial observed that therapy with dexamethasone lead to a significant reduction of death in ventilated patients, as well as for patients on supplemental oxygen, while no benefit was shown in mild cases (146). A recent review of corticosteroid use in the management of COVID-19 revealed a mixed picture from five available studies. In four retrospective studies and one quasi-prospective study, three studies indicated a benefit, while the other two studies showed no benefit, and one sub-study even suggested significant harm in critical cases (147). Based on success in hematological and oncology settings, several IL-6 antagonists (tocilizumab, sarilumab as well as siltuximab) have been utilized as emergency interventions in COVID-19 patients with ARDS and hypotension, although so far with mixed results (148). IL-6 is an indispensable cytokine that initiates innate defence after pathogen invasion or tissue damage by stimulating acute phase reactions, immune responses, hematopoiesis, and activation of numerous internal organs to prepare for host defence (149). Therefore, IL-6 and other cytokines like tumor necrosis factor (TNF)-α are indispensable during functional activation of monocytes, macrophages and DCs before or early during COVID-19 disease, as they are in diseases caused by other respiratory viruses (150). However, in later disease stages increasing immune dysregulation and T cell apoptosis, macrophages and IL-6 may accelerate immune imbalance (151).

Preventing and treating coronavirus infections will likely need a multiphasic approach to prophylaxis and therapy, especially in vulnerable populations. It will be important to use the right tools at the right time to avoid unintended and potentially counterproductive consequences. The right set of immunomodulators would likely be able to prepare and boost innate immune defences to either ensure appropriate, effective responses to infection and/or guide the development of suitable, protective immunity in response to potentially suboptimal adjuvanted first generation vaccines. Antiviral treatments or combinations of them will be most useful during early infection, while a different set of immunomodulators may be needed in late stage and severe disease, where a dysregulated antiviral response can cause deadly collateral damage.

# MICROBIAL "OLD FRIENDS" AND BCG

Some microbes have existed throughout human history, with evidence of their presence in hunter-gatherer societies, shaping the evolution of the human immune system (152). Some of these microbes, branded as "old friends" or "old infections," are thought to be so intricately involved in this process that they are required for human immunity to develop and function properly (153, 154). Examples of such microbes are harmless mycobacteria that are present in the environment and used to be prevalent in water and food, where they were postulated to have a "training" impact on the human immune system (153). In addition, "paleolithic" strains of *Mycobacterium tuberculosis* (Mtb) that were less pathogenic than modern strains could have contributed to this process (152). Environmental Mycobacteria can provoke type 1 responses, as has been shown in mouse models and human cell-based *in vitro* studies for heat killed *Mycobacterium obuense,* NCTC13365 (IMM-101) and *Mycobacterium vaccae*, NCTC11659 (IMM-201) (155–158). This is also the case for the attenuated strain of *Mycobacterium bovis*, BCG (159). However, modern, urban societies are often missing frequent exposure to environmental bacteria such as *M. obuense* and *M. vaccae* – they literally have lost touch with their "old friends" and may need "new old friends," to support type 1 immune responses.

Remarkably, several observational studies have recently proposed that countries with active BCG vaccination in place had fewer confirmed COVID-19 cases and related deaths (160–162). These observational studies should be appraised with caution, since there are many confounding factors in interpreting such correlative data in the context

during the COVID-19 pandemic (163). There is no peer-reviewed data yet, or a clear scientific hypothesis about the proposed mechanism of action, to explain how decades later a single BCG vaccination could provide long lasting, heterologous protection against a viral disease. In contrast, there are evidence-based arguments, acutely relevant to the COVD-19 pandemic, regarding how BCG or type 1 immune inducing environmental Mycobacteria could provide protection against severe COVID-19 in the form of the trained immunity hypothesis.

## TRAINED IMMUNITY UTILITY FOR VACCINES

Contact with specific microbial stimuli can induce long-lasting epigenetic changes in innate immune cells, which not only results in an enhanced response to a second challenge by the same microbe, but also to unrelated microbial insults (164). Referred to as "trained" immunity or innate immune memory, this process was originally shown for the BCG vaccine (165, 166). This concept may help explain previous observations that, after infection or vaccination, prototypical innate immune cells like monocytes, macrophages and NK cells undergo long-term changes in their functional programs, promoting host resistance against a wide spectrum of pathogens, including fungi, bacteria and viruses (167). Trained immunity is thought to be responsible for the observation in clinical studies that childhood vaccination with BCG correlates with protection against 30–50% of infections with any known pathogen, including viruses (168, 169). Additionally, a reduction in childhood mortality, unrelated to the prevention of tuberculosis (TB), has been observed (169). Similar positive effects have been shown for BCG vaccinations in adults, including improving responses to Influenza vaccination (170). A study in Guinea-Bissau showed that BCG reduced the incidence of respiratory syncytial virus infection (171). Importantly for the at-risk populations for severe COVID-19, it was shown that BCG had a similar protective effect on respiratory tract infections in older individuals in Indonesia (172). In addition, a clinical trial performed in older individuals in Japan established protection against pneumonia after pneumococcal, influenza and BCG vaccinations (173). Further confirmation of this effect has been demonstrated in a randomized controlled trial in which BCG vaccination protected against experimental infection of a yellow fever virus (174). In summary, BCG vaccination has been shown to protect against a range of viral infections (175). Related to this, when vaccination against smallpox was introduced around 200 years ago, positive side-effects such as protection against measles, scarlet fever and whooping cough, among others, were noticed (176).

Monocytes from healthy human volunteers were stimulated *ex vivo* with unrelated pathogens and displayed enhanced pro-inflammatory cytokine production of IL-1β, TNF and IL-6 after BCG vaccination (165). Experimental studies in mice have delineated that some of the mechanisms by which BCG induces these protective effects. For example, in mice, reduced viral titers of influenza A virus rely on macrophages (177). Subcutaneous administration in mice of muramyl dipeptide (MDP), part of the mycobacterial cell wall, protected against vaccinia virus and herpes simplex virus type 2 (HSV2) infections (178). Newborn mice could be protected with BCG from infection by HSV2 (179). More recently, other inducers of trained immunity have also been identified, including β-glucan, which has been shown to induce protective trained immunity in human monocytes and against Mtb infection in mice (180). The combination of these observations and others led to the proposal of the development of trained immunity-based vaccines (TIbV).

TIbVs aim to induce a pre-activated or "poised" activation state in innate immune cells. In this way they are, unlike conventional vaccines, theoretically able to stimulate much broader immune responses that are not focused on just one specific pathogen (181). This capacity of TIbVs to promote responses beyond their nominal antigens may be particularly useful when conventional vaccines are not available, or when multiple co-infections and/or recurrent infections arise in susceptible individuals at the same time, as is the case in the current pandemic COVID-19 health emergency. At least six different countries, including the Netherlands and Australia, have initiated clinical trials with the intent of investigating BCG vaccination as TIbV to protect HCW from symptomatic or serious COVID-19 infections (175, 182, 183).

In general, BCG is regarded a safe vaccine in young and healthy individuals. However, as is the case with any vaccines containing live attenuated organisms, there is a possibility of adverse events, such as disseminated BCG disease, in the elderly and immunocompromised. For this reason, in cancer patients, who represent a high-risk group for severe COVID-19 infection, BCG is contraindicated in several countries highly impacted by the pandemic, including the United States and Canada (184, 185). As a result, populations likely to benefit most from the potential of TIbVs and at the highest risk of a severe COVID-19 disease (e.g., cancer patients, frail elderly, or other people with impaired immune systems), cannot be included in BCG vaccination strategies. Despite the potential promise for mitigation of the COVID-19 pandemic, a major obstacle to its quick, rational deployment is the fact that the BCG vaccine comprises of a number of genetically distinct substrains (186). These have subsequently been shown to have different immunological properties, such as variable virulence and efficacy as a tuberculosis vaccine in mice (187). This substrain diversity may also help explain some inconsistencies following BCG use, such as variable Th1 or Th2 induction and side-effects (188). In clinical use, no evidence was found that vaccination efficacy against TB was associated with a specific BCG strain; however, a Th1 or Th2 bias was not investigated in that study (189). It has also been shown that the immune response can be directed from Th1 to mixed Th1/Th2, depending on the dose of BCG used (190). Bacille Calmette-Guérin is not routinely injected more than once, but an earlier study showed that, of six patients who were given a second inoculation of the BCG vaccine, three showed persistent cutaneous granulomas (191). A recent clinical study also observed evidence of a protective effect against persistent Mtb infection after BCG revaccination

(192); although repeat treatment with BCG has been used in the past in oncology as an adjuvant to boost cell-based cancer vaccines (193).

## *Mycobacterium obuense* (IMM-101)

IMM-101 is a preparation of heat killed, whole cell, *M. obuense* National Collection of Type Cultures (NCTC) 13365, one of over 100 named species within the genus *Mycobacterium*, and an "old friend." *M. obuense* is a rapidly dividing mycobacterium that normally grows as an environmental saprophyte (194). Since IMM-101 is a heat killed preparation, treatment is not associated with the potential side-effects of delivering live or attenuated organisms (195). Moreover, one can speculate that IMM-101, by virtue of its potent type 1 inducing ability, will counter-regulate type 2 responses, helping to explain the encouraging clinical results to date in melanoma and pancreatic cancer (195, 196). An open label, Phase 2 study of IMM-101 in combination with checkpoint inhibitor therapy Nivolumab is currently underway in patients with advanced melanoma in the United Kingdom (197). The total number of patients exposed to IMM-101 across clinical trials and compassionate programs without any unexpected adverse events has been over 345. The mode of action of IMM-101 is in the process of being elucidated, but it has been shown to be a multifaceted modulator of both innate and adaptive arms of the immune system (158). Experiments with mouse and human immune cells have shown that IMM-101 is very effective in inducing cytokine expression by innate immune cells, including M1 polarization and enhanced antigen presentation by DCs, leading to a typical type 1-biased immune response (**Figures 1**, **2**) (198, 199). Systemic activation of, and IFN-γ production by, multiple immune cell types (158), including innate immune cells like NK cells, T cells expressing gamma/delta receptors (γδ-T cells) and natural killer T (NKT) cells (157, 200) (**Figure 1**), is based in part on the promotion and activation of CD4+ Th1, and CD8+ CTLs, with increased production of the cytokine IFN-γ in *in vitro* and *in vivo* (198–202). It is also possible that, in this setting, IMM-101 may act to train monocytes for enhanced M1 function (**Figure 2**). NK, γδ-T, NKT, Th1 cells, and CTLs, are well-known to play crucial roles in anti-viral and anti-tumor responses that can kill infected or tumor cells. This diverse mechanism of action of IMM-101, the safe promotion of a broad, systemic innate and adaptive type 1 immune response, may provide a rationale for considering its use against SARS-CoV-2.

Interestingly, BCG has been shown to promote activation of Vγ9Vδ2 T cells, the major subset of γδ T cell pool in human peripheral blood with a previously proposed protective role against SARS-CoV (see above) (203). Vδ2 T cells are exactly the cell-subtype that has been shown to also be activated by IMM-101 stimulation, in some experiments showing a stronger ability to do so than BCG (157). γδ T cells normally only represent a minor subset in peripheral blood, but can rapidly proliferate following infection with certain pathogens, expanding from 1% to over 50% of circulating T cells within a week (204, 205).

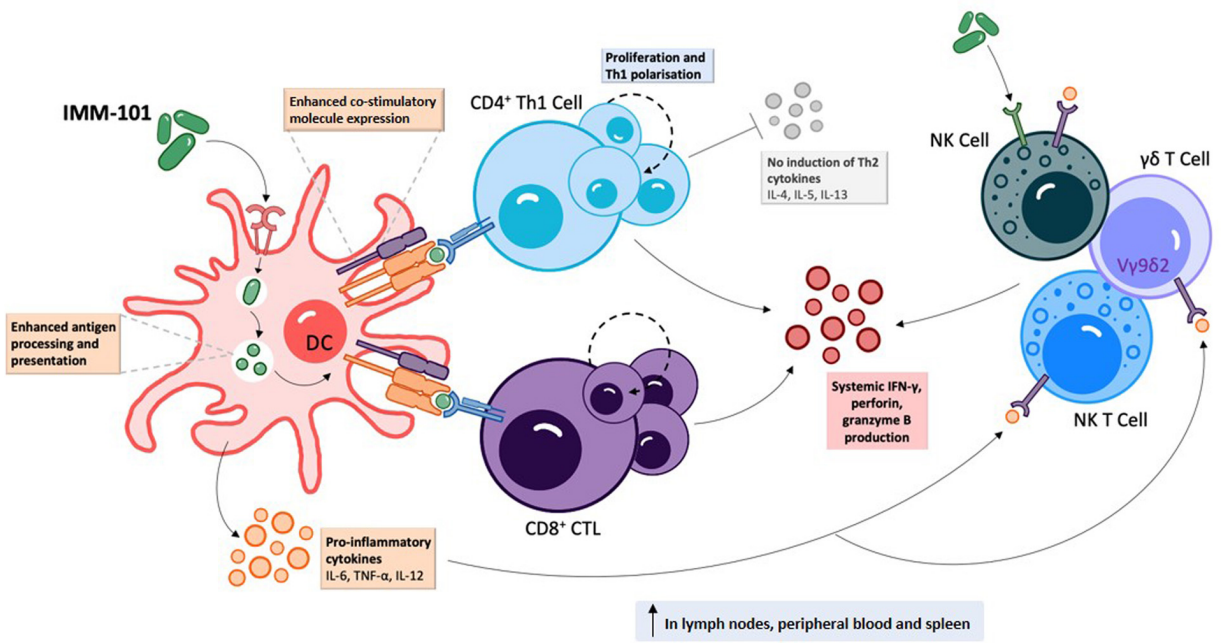


**FIGURE 1 |** IMM-101 induces a robust systemic type-1 biased immune response | Recognition of IMM-101 by DCs results in increased expression of co-stimulatory molecules, enhanced antigen processing and presentation capacity and induction of an array of pro-inflammatory cytokines 156, 158, 198–202). IMM-101 activated dendritic cells (DC) directly promote the proliferation of CD8+ cytotoxic T-lymphocytes (CTL) and type-1 polarised CD4+ T cells, whereas innate-like cells including natural killer (NK), NKT and γδ T cells can be activated either by direct interaction with IMM-101 or indirectly via recognition of DC secreted cytokines (156, 157). This local DC activation eventually leads to a systemic increase in immune cells secreting anti-viral interferon (IFN)-γ, perforin and granzyme B (158, 202). Th, helper T cell. TNF, tumour necrosis factor.

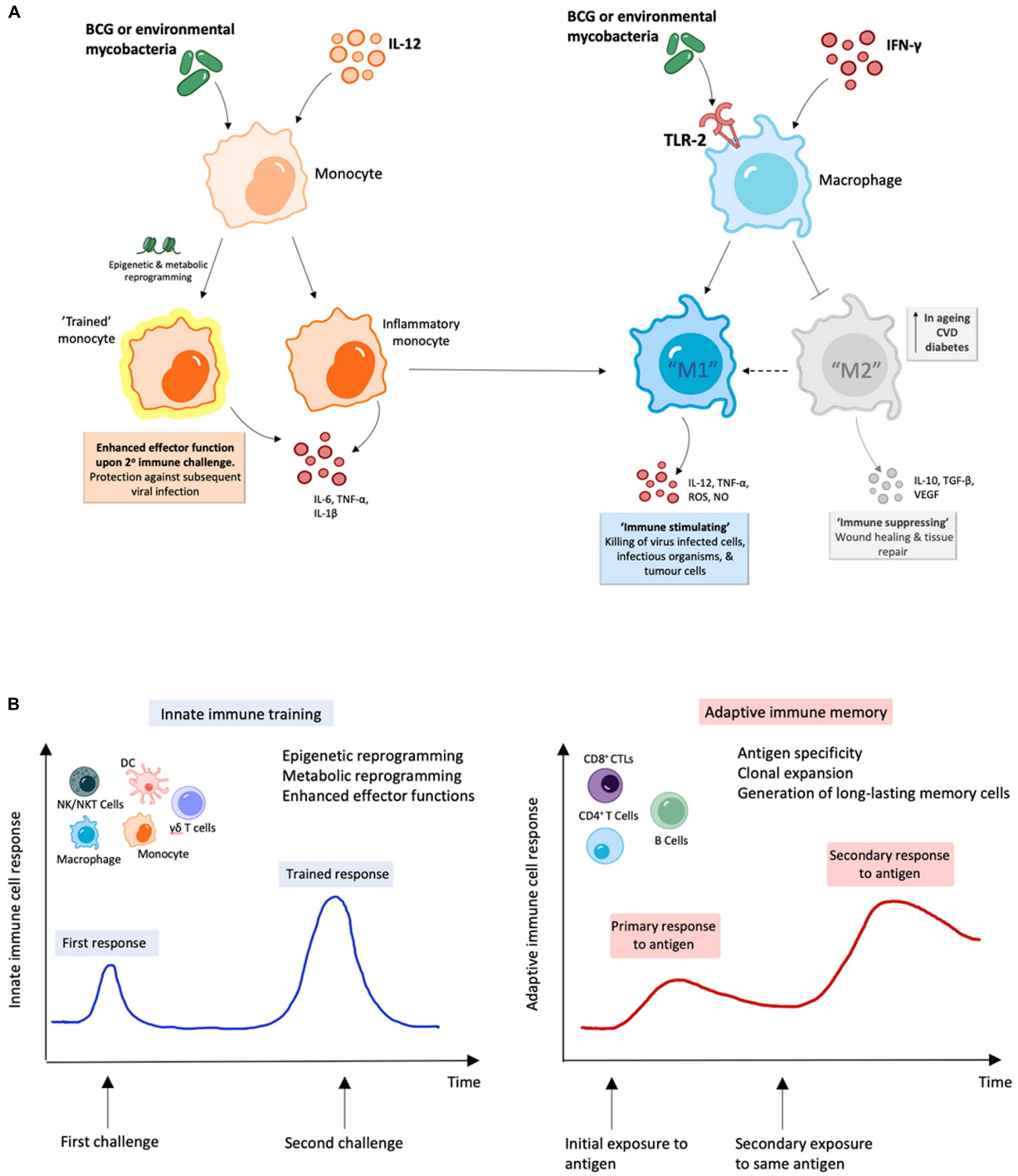


**FIGURE 2 |** BCG and environmental mycobacteria promote M1 macrophages and are likely to induce trained immunity. **(A)** Treatment with mycobacterial immunomodulators induce polarization of M1 macrophages along with "trained" inflammatory monocytes with enhanced M1 function, which can result in enhanced viral clearance (164–166, 199). **(B)** During innate immune training, innate cells undergo long-term cellular reprogramming. Unlike classical antigen-specific responses seen with adaptive immunity, this reprogramming results in increased capacity to respond to secondary challenges from a variety of pathogens and forms the basis of trained-immunity based vaccines (170–172, 181).

It is noteworthy that a large majority of Vδ2 T cells co-express Vγ9 in humans, and were shown to be important to overcome SARS-CoV infection (54, 206).

In addition to Th1 cells, CTLs and γδ T cells, NK and NKT cells also play key protective roles during viral infection (207, 208), and the potential importance of improving the NK

cell and CTL response at the early stage of SARS-CoV-2 infection has already been highlighted (22). Under the umbrella of trained immunity, broad protection could be achieved by systemically increasing the non-specific effector response of innate immune cells (e.g., macrophages, NK, NKT, and γδ T cells) while also enhancing DC activation and ability to promote adaptive T cell (e.g. Th1 and CTL) and B cell responses to both specific and non-related (bystander) antigens, all of which have been shown for IMM-101 (**Figure 1**) (198, 199).

Several studies have shown that the effects of IMM-101 are in part mediated by TLR2/1, and to a lesser extent, TLR2/6 (198, 199). TLR2 has been shown to directly trigger Th1 effector functions in mice (209). Subsequently, it was shown that IMM-101 activates human Mincle reporter cell lines (158, 227). It is noteworthy that Mincle can suppress TLR 4 activation (211) and TLR4 has been proposed to have a central role in the initiation of damaging inflammatory responses during different acute viral infections (30). In contrast to BCG, IMM-101 does not activate TLR4 (198, 199, 212). In a similar manner, Mincle suppresses Th17 immune responses, which as well have been suggested in coronavirus immunopathology and vaccine-induced immune enhancement (213, 214). It was only recently discovered that activation of the Mincle receptor is a key activation pathway for Complete Freund's Adjuvant (CFA), the "gold standard" adjuvant for eliciting cell-mediated immunity (CMI) in research models (215–217).

Effective and enhanced viral and tumor antigen cross-presentation requires TLR2 or TLR3 activation of human DCs (218). Mouse CD8α+ DCs express TLR7 and TLR9, in addition to the TLR2 family and TLR3, whereas the only relevant corresponding cross-presenting human CD141+ DCs in lymph nodes exclusively express the TLR2 family and TLR3 (218, 219). Importantly, analysis of the susceptibility of primary human DC subsets to viral infections has shown that

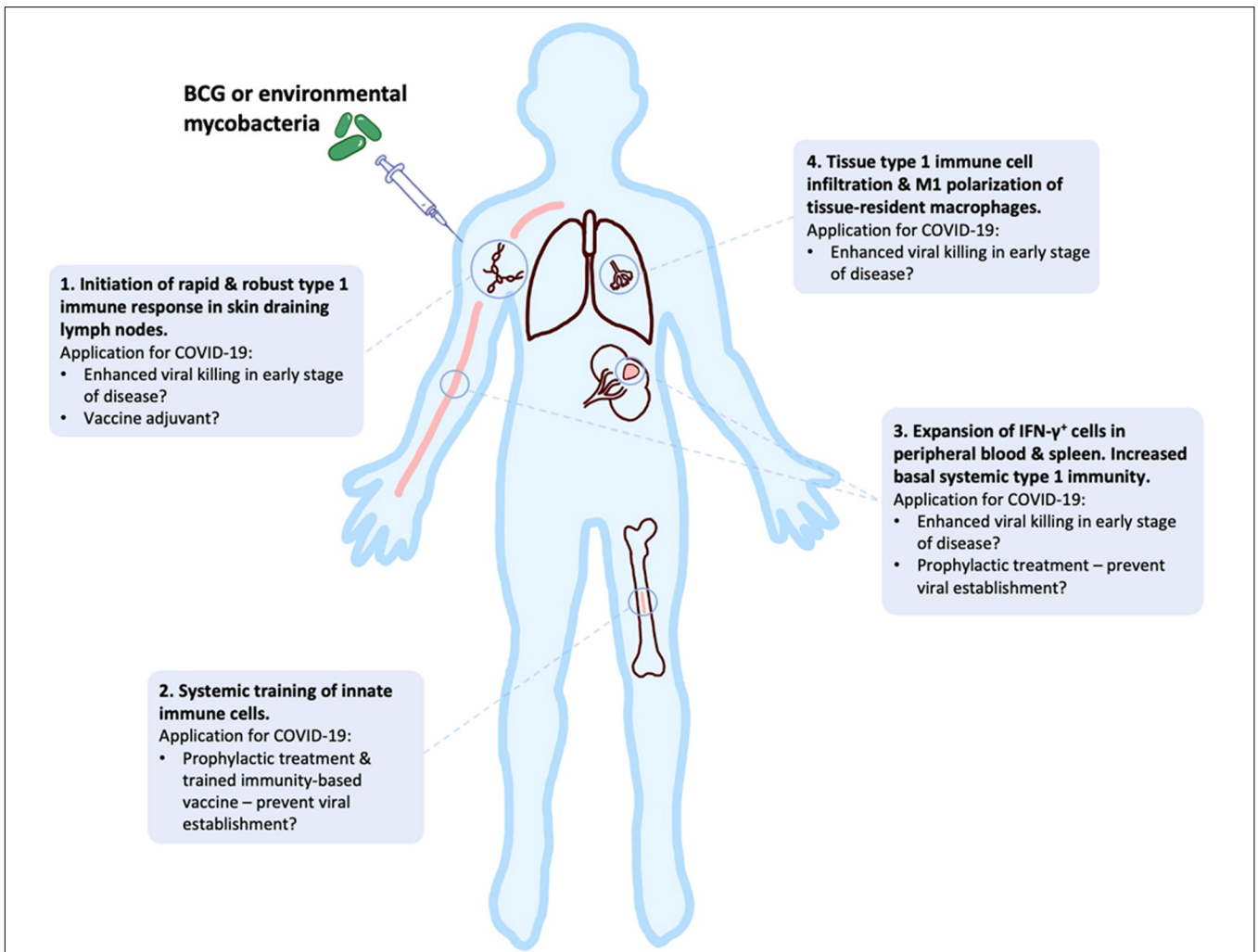


**FIGURE 3 |** BCG and "new old friends" have potential utility for prevention of severe COVID-19 in a number of ways. Bacille Calmette-Guérin (BCG) and other mycobacterial immunomodulators initiate robust type 1 immune responses and innate immune training, leading to tissue type 1 immune cell infiltration and elevated basal systemic type 1 inflammation (156–159, 164–166, 198–202). This allows for potential alteration of disease trajectory through prevention of viral establishment, enhanced viral killing or as a vaccine adjuvant to enhance immunity.

CD141+ DCs have an innate resistance to infection by a broad range of enveloped viruses, including HIV and influenza virus. In contrast, CD1c+ DCs are susceptible to infection, which enables viral antigen production, but impairs their immune function and survival. This has led to the conclusion that inclusion of TLR2 or TLR3 agonists would be the most direct mechanism to enable enhanced viral and tumor antigen cross-presentation, likely necessary for effective cancer immunotherapy (218) and viral clearance (220). Interestingly, previous work has suggested that vaccine-induced eosinophil immunopathology in the lungs after SARS-CoV infection could be avoided with the use of TLR3 agonists as adjuvants (221). However, use of TLR3 agonists may have to be viewed with caution in the context of COVID-19, based on observations of harmful contributions of TLR3 to influenza A virus-induced acute pneumonia in mice. In that scenario, TLR3-influenza A virus interaction critically contributed to the debilitating effects of a detrimental host inflammatory response (222). Further, it has been shown that TLR4 signaling induces TLR3 up-regulation in alveolar macrophages during ALI, and that TLR4 and TLR3 in macrophages are an important determinant in ALI (223), and that there is an association between respiratory syncytial virus TLR3-mediated immune responses and chronic obstructive pulmonary disease exacerbation frequency (224). TLR2 activation of macrophages leads to M1 polarization, and a shift from M2 into M1 macrophages (225). In addition, it has been shown that TLR2 activation of macrophages can impair activity of M2-like macrophages (226). IMM-101 activates TLR2 and not TLR4 and leads to M1 macrophage polarization (**Figure 2**) (198, 199). The combined characteristics of IMM-101 have led to the approval by Health Canada of a randomized, Phase 3 trial of immunization with IMM-101, versus observation, for the prevention of severe respiratory and COVID-19 related infections in cancer patients at increased risk of exposure (210).

## DISCUSSION

In this review, we have presented an overview of current knowledge of the innate, adaptive and dysfunctional immune responses to SARS-CoV-2, in relation to other closely related coronaviruses. We have outlined the responses that may be required for successful vaccine development against COVID-19, while highlighting potential risks during this development, especially for the elderly. Early clinical data look promising, but continued studies of human and NHP immune response to different SARS-CoV-2 vaccines in the pipeline are required to mitigate potential dangers of well-intended, but potentially flawed, vaccines that are being expedited to large parts of high-risk populations around the globe. In addition, the potential utility of "new old friends" as TIbVs like BCG or heat killed environmental bacteria such as IMM-101, that act as multitargeted, systemic immunomodulators of the innate and adaptive immune system have been described. Studies to show BCG's and IMM-101's potential utility for the prevention of severe COVID-19 are underway or planned, with the potential to change immune status and alter disease trajectory in multiple ways (**Figure 3**): (i) as prophylaxis, with enhanced innate memory and increased basal systemic type 1 immunity preventing viral establishment; (ii) as a treatment for patients in early stages of disease, with increased local and systemic type 1 inflammation enhancing killing of virally infected host cells; (iii) as an adjuvant for future COVID-19 vaccines. Thus, BCG and IMM-101 have the potential to be rapidly deployed to address the COVID-19 emergency and the challenge posed by the current lack of effective treatments and vaccines, leading to a high unmet medical need. With other routes of vaccine and therapy development likely to take many months or years to develop, or even reformulate, the help of "new old friends" such as BCG and IMM-101 may be precisely what we need in the current pandemic crisis.

## AUTHOR CONTRIBUTIONS

T-OK and AD conceived the idea for the review and wrote the manuscript, with constructive input from AM and AG. AG prepared display items under the supervision of AM. All authors approved the final version of the manuscript.

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# Immune Status Against Hepatitis B in Patients After Allogeneic Hematopoietic Cell Transplantation—Factors Affecting Early and Long-Lasting Maintenance of Protective Anti-HBs Titers

*Agnieszka Piekarska[1]*, Piotr Wisniewski[2], Krzysztof Lewandowski[3], Lidia Gil[4], Piotr Trzonkowski[5], Maria Bieniaszewska[1] and Jan Maciej Zaucha[1]*

*[1] Department of Hematology and Transplantology, Medical University of Gdansk, Gdansk, Poland, [2] Department of Endocrinology and Internal Diseases, Medical University of Gdansk, Gdansk, Poland, [3] Department of Laboratory Medicine, Medical University of Gdansk, Gdansk, Poland, [4] Department of Hematology and Stem Cell Transplantation, Poznan University of Medical Sciences, Poznan, Poland, [5] Department of Clinical Immunology, Medical University of Gdansk, Gdansk, Poland*

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**Edited by:**
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***Correspondence:**
*Agnieszka Piekarska*
*agnieszka.piekarska@gumed.edu.pl*
*orcid.org/0000-0002-1593-8005*

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The immunization of allogeneic hematopoietic cell transplantation (HCT) recipients against vaccine-preventable diseases is a part of posttransplantation guidelines. We conducted a prospective study to assess clinical and immunological parameters that would determine the response and long-term maintenance of protective antibody titers upon the hepatitis B virus (HBV) vaccination after HCT. The investigated variables included: vaccination of the HCT recipients and their donors prior to HCT, chronic graft versus host disease (cGVHD) and the timing of post-HCT vaccination, and B- and T-cell subtype status. Forty-two patients were immunized with three or more doses of recombinant hepatitis B surface antigen (rHBsAg) administered according to the individualized schedule of 0-1-2-6-(12) months. After vaccination, seroconversion was achieved in the whole group. The vaccines were categorized according to the antibody (Ab) titers as weak (WRs; 28.7%), good (GRs; 38%) or very good responders (VGRs; 3.3%). In multivariate logistic regression, severe cGVHD (OR= 15.5), and preceding donor immunization (OR= 0.13) were independent predictors of a weak response to vaccination. A prior belonging to the WR group impaired the durability of protection (OR= 0.17) at a median follow-up of 11.5 years. Patients with severe cGVHD showed a trend toward lower median Ab titers, although they required a higher rate of booster vaccine doses. All VGRs had CD4+ cells > 0.2 x $10^6$/L. There was a lower mean rate of CD4+IL2+ lymphocytes in WRs. Vaccination demonstrated the immunomodulatory effect on B-cell and T-cell subsets and a Th1/Th2 cytokine profile, while shifts depended on a history of severe cGVHD and the type of vaccine responder. To conclude, vaccination of HCT donors against HBV allows a better response to vaccination in the respective HCT recipients. Double doses of rHBsAg should be considered in

patients with cGVHD and in those not immunized before HCT. A dedicated intensified vaccination schedule should be administered to WRs.

**Keywords: hepatitis B vaccine, vaccination schedule, donor vaccination, hematopoietic cell transplantation (HCT), chronic graft versus host disease (GVHD)**

## INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) is a curative cellular therapy for a variety of disorders (1). In posttransplantation care, a dysfunctional immune system and infectious complications pose serious problems (2–5). Moreover, an observed gradual loss of specific postvaccination immunity after HCT necessitates the immunization of HCT recipients against vaccine-preventable diseases (6, 7). Following HCT, hepatitis B virus (HBV) can trigger serious liver complications, including fulminant hepatitis. Repetitive exposure to medical procedures poses a risk of HBV transmission. Moreover, reverse seroconversion upon immunosuppressive treatment is reported in anti-HBc-positive patients in whom HBV infection was resolved before HCT (8, 9). Therefore, in countries with a high incidence of hepatitis B, immunization with recombinant hepatitis B surface antigen (rHBsAg) and the maintenance of protective anti-HBs antibodies (Abs) are especially justified (6, 10).

Due to deficiencies in humoral and cellular immunity, as well as altered mechanisms regulating immune reactions, the overall response rate (ORR) of transplant recipients is inferior compared to that of healthy people (11–15). However, for the sake of simplicity, a universal vaccination protocol for all transplant recipients does not consider differences in immune recovery in the distinct HCT platforms (16–21). Data on the durability of postvaccine protection in HCT recipients are limited. Long-lasting immunity depends on many variables, including the immunogenicity of vaccines, immunosuppressive treatment, and chronic graft versus host disease (cGVHD) (18, 19).

The clinical presentation of cGVHD mimics autoimmune diseases, and the organ-debilitating impact does not spare the immune system (22–26). Weak granulocyte chemotaxis, low response to mitogens, defects of the primary and secondary immune response to bacterial and polysaccharide antigens as well as functional hyposplenism are well-known phenomena (27, 28). A state of chronic inflammation, maintained by IL17, may lead to immune exhaustion, while dysregulated polyclonally activated lymphocytes do not properly recognize specific antigens (23, 29). Therefore, severe cGVHD itself could deteriorate the postvaccine immune responses and the maintenance of anti-HBV immunity.

We launched a prospective study aiming at identification of clinical and immunological factors that determine the response and long-term maintenance of protective antibody titers upon individualized vaccination with rHBsAg after HCT considering: donor/recipient serological anti-HBV status, incidence and severity of cGVHD, the timing of vaccination after HCT and the patient immune reconstitution. The serological monitoring included anti-HBs Ab levels tested before HCT, and after transplantation up to rHBsAg administration and postvaccination follow-up. Subpopulations of B-cell and T-cell compartments, as well as the cytokine Th1/Th2 profile, were evaluated in the perivaccination period. An additional goal of the study was to optimize a vaccination schedule and standardize posttransplantation anti-HBV surveillance.

## MATERIAL AND METHODS

### Patients

Criteria to initiate a vaccination program included a lack of previous vaccination after allogenic HCT, the remission of any underlying disease, the discontinuation of immunosuppressive treatment for at least 2 months before vaccination, a lack of active infection and signed informed consent. Patients with a history of cGVHD were accepted, provided that they did not suffer from an active disease requiring immunosuppressive therapy. Altogether, 62 Caucasian patients qualified, but for the homogeneity of the study group, patients allotransplanted with the use of reduced intensity/toxicity conditioning regimens were excluded from the final analysis.

Standard GVHD prophylaxis consisted of cyclosporine and a short course of methotrexate. Anti-thymocyte globulin administration (7.5 mg/kg) was a component of GVHD prophylaxis in HCT from matched unrelated donors (MUDs).

A final study group consisted of 48 patients who started a vaccination program between Dec 2003 and Mar 2006, including 42 individuals requiring vaccination with rHBsAg. The study was designed to compare the quality of the immune response between patients immunized in the early (between 6 and 24 months) and late periods (> 24 months) after transplantation.

### Vaccination Protocol

Vaccination against HBV was a part of the whole immunization schedule, consisting of vaccines against poliomyelitis (3 doses), tetanus (3 doses), diphtheria (3 doses), *Haemophilus influenzae* (2 doses), and HBV, administered simultaneously in separate parts of the body. Immunization against influenza was given seasonally once a year, while immunization against *Streptococcus pneumoniae* was performed with a 23-valent polysaccharide vaccine ≥ 12 months post-HCT, as conjugated vaccines were not available at that time (11).

The recombinant surface antigen of HBV gained from *Saccharomyces cerevisiae* and absorbed on aluminum compounds was used (Engerix B; GlaxoSmithKline Biologicals). The protocol consisted of 3 or more doses of the vaccine administered intramuscularly in 4- to 6-week intervals according to the following schedule: 0-1-2-6-(12) months. The first dose of the vaccine in every case was double (40 μg) the standard dose. The titer of anti-HBs Abs was checked 4 - 6 weeks after every dose, and the administration of

subsequent doses depended on the grade of the response. A lack of seroconversion or a low titer of Abs (anti-HBs <10 mIU/ml) was followed by subsequent administration of a double vaccine dose until a titer of anti-HBs Abs >10 mIU/ml was achieved. In the case of seroconversion or an anti-HBs Ab titer >10 mIU/ml after the initial dose, the next doses were single doses (20 μg). After protocol completion, the anti-HBs Ab titer was monitored regularly during visits in the posttransplantation unit. Revaccination was prescribed in patients in whom protective immunity was lost, including patients with anti-HBc positivity.

Patients were divided into three types of responders, weak (WRs), good (GRs), and very good responders (VGRs), depending on the achieved titer of anti-HBs Abs, the administered vaccine doses, and the maintenance of protective levels of humoral anti-HBV immunity (**Table 1**).

## Chronic GVHD

Chronic GVHD diagnosis was based on data from patient medical records, and the criteria of NIH 2014 Consensus were retrospectively adopted (26).

## Flow Cytometry

Immunophenotyping was performed from heparinized peripheral blood according to standard procedures at least 3 times: before vaccination and during and after the completion of the basic vaccination protocol. Analyses were performed using triple-color flow cytometry (FC). All Abs used for immunofluorescent staining were obtained from Becton Dickinson (BD), and cells were acquired on a FACS Calibur (BD).

Cells were incubated with the following Ab-conjugates: γ1/γ2a-FITC/PE (clone X39/X40), CD3-PerCP (clone SK7), CD4-PE (clone SK3), CD4-PerCP (clone SK3), CD8-FITC (clone SK1), CD8-PerCP (clone SK1), CD45RA-FITC (clone L48), CD45RO-PE (clone UCHL-1), CD19-CyChrome (clone HIB19), CD27-PE (clone L-128), IgD-FITC (clone IA6-2), IgM-FITC (clone G20-127), IgG-FITC (clone G18-145), IFNγ-FITC (clone 25723.11), IFNγ-PE (clone 25723.11), IL2-FITC (clone 5344.111), IL2-PE (clone 5344.111), IL4-PE (clone 3010.211), IL5-PE (clone JES1.39D10), and IL10-PE (clone JES3.12G8).

Assessments of T-cell subsets were performed in whole blood, while for assessments of B-cell subsets and cytokine expression, peripheral blood mononuclear cells (PBMCs) were isolated. Cellular subpopulations were analyzed in the lymphocyte gate by positive signals above the isotype fluorescent control.

**TABLE 1** | Criteria for the WR, GR, or VGR groups depending on the achieved anti-HBs titers, the quantity of injected doses, and the maintenance of high protective immunity.

| Groups | Criteria |
|---|---|
| Weak responders (WRs) | - ≥ 4 doses to achieve anti-HBs Abs 10–100 mIU/ml or<br>- anti-HBs Abs >100 mIU/ml achieved after 2–4 doses but maintained no longer than 1 year |
| Good responders (GRs) | - anti-HBs Abs >100 mIU/ml achieved after 3–4 doses and maintained at least 1 year |
| Very good responders (VGRs) | - anti-HBs Abs >100 mIU/ml achieved after 1–2 doses and maintained at least 1 year |

### Isolation of PBMCs

PBMCs were separated by density gradient centrifugation on a Histopaq 1077 (Sigma). After washing in RPMI medium (Tominex) mixed with 10% FBS medium (Tominex), cellularity was assessed, and PBMCs were suspended in RPMI/FBS medium to achieve a lymphocyte concentration of $2x10^6$/ml. Some isolated lymphocytes were suspended in the medium for cell culture, and the rest were stained with Abs against B-cell markers.

### Stimulation of T-lymphocytes with PMA and Ionomycin

Isolated lymphocytes were incubated with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and ionomycin (Sigma-Aldrich) in the presence of an inhibitor of cytokine secretion, Brefeldin A (GolgiPlug; BD), in round-bottom 24-well plates for 5–6 h at 37°C in a 5% $CO_2$ atmosphere according to a standard protocol from Laboratoire d'Immunologie CHU Rangueil (Toulouse, France), with modifications implemented by the first author (30, 31).

### Staining of Surface Antigens and Intracellular Cytokines

The staining of membrane antigens from whole blood was preceded by double lysis. Cell suspensions were incubated with cocktails of antibodies/conjugates for 30 min in darkness and then washed in PBS (5 min; RCF 650). After centrifugation, the supernatant was removed, and specimens were ready for acquisition. Samples requiring intracellular staining were fixed with 3% formaldehyde and permeabilized with Perm2 (BD). After 30 min of incubation in darkness with cytokine-targeting Abs/conjugates, cells were washed and suspended in 200 μl of 0.5% formaldehyde solution. Acquisitions with FC were performed within 24 h.

## Ethical Approval

This study was performed in accordance with the Declaration of Helsinki and received the approval of the Independent Bioethics Committee of the Medical University of Gdansk. Informed consent forms were signed by all participating patients, and the possible consequences of the study were fully explained.

## Statistical Analysis

Data analysis involved descriptive statistics, contingency tables, Pearson's chi-square test and the Mann-Whitney-Wilcoxon U test. Logistic regression was used to examine the influence of selected predictors jointly. A p-value of less than 0.05 was considered significant. All statistical analyses, data manipulation, and graphical plots were performed using the RStudio statistical software environment (version 1.1 with R.3.6.1).

# RESULTS

## Patients' Characteristics

The median ages at transplantation and vaccination were 35 and 39 years, respectively. Forty-two patients immunized with rHBsAg were divided into the early (38%) and late vaccination groups (62%)

according to the time elapsed after HCT. **Table 2** presents detailed patient characteristics. Five patients with naturally acquired anti-HBV immunity and one patient with solely adoptive anti-HBV immunity transfer were included in serological monitoring (the so-called 'initially nonvaccinated group').

The evaluation of long-lasting anti-HBV immunity was possible in 43 patients. Five patients were excluded due to a short follow-up. The median follow-up in the analyzed vaccinated group was 11.5 (range, 5–16) years, while in the initially nonvaccinated group, it was 16.5 (range, 15–21) years.

## Immune Status Against HBV

### Serological Status of Patients and Donors

Thirty (62.5%) patients were vaccinated with rHBsAg before HCT, with an ORR of 53%, including 6 patients achieving an anti-HBs Ab range of 10–100 mIU/ml and 10 patients with anti-HBs Ab levels >100 mIU/ml. Five patients with naturally acquired immunity were anti-HBc and anti-HBs Ab positive (>100 mIU/ml). In 20 (41.7%) patients with anti-HBs Abs < 10 mIU/ml, passive immunization with anti-HBs gamma-globulin was administered.

Twenty-five (58%) of 43 matched sibling donors (MSDs) were vaccinated before donation with an ORR of 60%, and 3 donors had protective anti-HBs Ab titers following HBV infection. In 19 (44.2%) HCTs from MSDs, both donors and recipients were vaccinated.

### Maintenance of Anti-HBV Protection Post-HCT Before Vaccination

Three months, 6 months, and 1 year after HCT, anti-HBs Abs were detected in 87%, 69%, and 40% of patients, respectively (**Table 3**). Protective anti-HBs Ab levels > 10 mIU/ml were found 3 months, 6 months and 1 year after HCT in 42%, 33%, and 15% of patients, respectively.

Three months post-HCT, there were no significant differences in anti-HBV protection between those immunized actively and those immunized passively.

Six months post-HCT, in univariate analysis, anti-HBs Abs were detectable significantly more often in those vaccinated before HCT (p=0.025) or in the case of donor vaccination (p=0.006). In multivariate analysis, the odds of having anti-HBV protection (anti-HBs Abs levels > 10 mIU/ml) depended on a recipient vaccination prior to HCT (OR 8.9, 95% CI: 1.4 – 177.3; p= 0.052), or naturally acquired immunity (OR 13.1, 95% CI: 1.3–321.8; p= 0.048). Other analyzed predictors (passive immunization, donor immunization) were not significant in multivariate analysis.

In univariate analysis, the maintenance of anti-HBV immunity at one year was significantly dependent on anti-HBs Ab levels > 10 mIU/ml in donors (p=0.006) and prior effective vaccination in the patients (p=0.04). In multivariate analysis, naturally acquired anti-HBV immunity (anti-HBc positivity, anti-HBs positivity) increased the odds of maintaining protective anti-HBs titers (OR 5.09, 95% CI: 0.9–32.0; p= 0.065). The remaining predictors (passive immunization, donor's immunization) were not significant in multivariate analysis. The graphical presentation of anti-HBV protection up to one-year post-HCT with respect to recipient and donor anti-HBV immunity status is presented in **Figure 1**.

No reverse seroconversion was observed in the anti-HBc and anti-HBs Ab-positive patients. The adoptive transfer of anti-HBV immunity occurred in 10 patients with grafts from MSDs.

### Response to Vaccination With rHBsAg: Patient-, Transplant-, and Donor-Related Factors

Seroconversion was achieved in the whole group. There were 12 (28.7%) patients classified as WRs, 16 (38%) as GRs, and 14 (33.3%) as VGRs. No severe complications related to the immunization of HCT recipients were recorded.

Statistical analysis did not show the influence of various factors, including treatment with chemotherapy preceding HCT, the type of conditioning regimen, the source of hematopoietic cells, the type of donor, the patient's previous vaccinations, reactivation of cytomegalovirus, of the patient's age at transplantation and vaccination, on the results of active immunization after HCT in the study group. In univariate analysis, there was a trend toward the unfavorable impact of

**TABLE 2** | Patient characteristics.

| | |
|---|---|
| Age (at transplantation); median (range) years | 35 (16–54) |
| Age (at vaccination); median (range) years | 39 (19–57) |
| Sex: female/male | 22/26 |
| Primary disease: | |
| AML/ALL | 18 |
| CML | 26 |
| Other (MDS, PNH, CEL) | 4 |
| Chemotherapy preceding HCT: Yes/No | 18/30 |
| Conditioning regimen: TBICy/BuCy120 | 9/39 |
| Type of donor: MUD/MSD | 6/42 |
| Source of hematopoietic cells: BM/PB | 14/34 |
| CD34+ cells dose (x$10^6$/kg recipient body weight): | |
| BM median (range) | 3.3 (1.2–5.4) |
| PB median (range) | 6.5 (2.7–8.9) |
| aGvHD grade 2-3 (%) | 21 (43.8%) |
| cGVHD | 30 (62.5%) |
| cGVHD no/mild/moderate/severe | 18/9/10/11 |
| CMV reactivations/median months post-HCT (range) | 17 (35.4%)/4 (1–12) |
| Immunization with rHBsAg: total; early vs. late group | 42; 16 (38%) vs. 26 (62%) |

*AML, Acute Myeloid Leukemia; ALL, Acute Lymphoblastic Leukemia; CML, Chronic Myeloid Leukemia; MDS, Myelodysplastic Syndrome; PNH, Paroxysmal Nocturnal Hemoglobinuria; CEL, Chronic Eosinophilic Leukemia; MUD, matched unrelated donor; MSD, matched sibling donor; BM, bone marrow; PB, peripheral blood; HCT, Hematopoietic Cell Transplantation; aGVHD, acute graft versus host disease; cGVHD, chronic graft versus host disease; CMV, cytomegalovirus; rHBsAg, recombinant hepatitis B surface antigen.*

**TABLE 3** | Maintenance of anti-HBV protection in the prevaccination and pre-revaccination period post-HCT.

| Timepost-HCT | Detectable anti-HBs antibodies* | Anti-HBs 10 – 100 mIU/ml | Anti-HBs > 100mIU/ml |
|---|---|---|---|
| 3 months | 87% | 42% | 29% |
| 6 months | 69% | 33% | 12% |
| 1 year | 40% | 15% | 8% |

**Including all patients with anti-HBs titer ≥ 0.5 mIU/ml.*

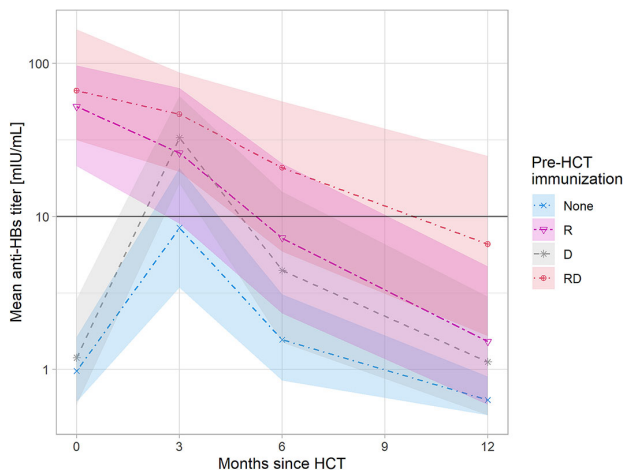


**FIGURE 1** | Anti-HBV protection up to one year post-HCT with respect to recipient and donor anti-HBV immunity. The shaded areas represent the 95% confidence intervals. Linear charts represent mean values of anti-HBs titers with respect to the anti-HBV immunity status of recipient and donor pre-HCT: - None - recipient nonimmunized actively or immunized ineffectively (anti-HBs<10 mIU/ml) and donor nonimmunized, - R - only recipient immunized, - D - only donor immunized, - RD - both recipient and donor immunized, A rise of anti-HBs titer 3 month post-HCT is caused by passive immunization with anti-HBs gamma-globulins prior to HCT administered to patients with anti-HBs<10 mIU/ml.

severe cGVHD on the results of active immunization with rHBsAg (p=0.057). Seroconversion after the first vaccine dose was significantly more frequent in patients who received transplants from donors immunized against HBV (p= 0.022). The majority (91%) of VGRs had immunized donors, in contrast to 33% of WRs (p=0.018). In 11 patients, adoptive immunity transfer was noted. Kaplan-Meier analysis indicated the positive impact of adoptive immunity transfer on postvaccination responses (p= 0.014). In the multivariate model, severe cGVHD increased the odds for WRs (OR= 15.5, 95% CI: 1.9–244.0; p= 0.02), while preceding donor immunization decreased the odds for WRs (OR= 0.13, 95% CI: 0.01–0.9; p= 0.05). A time of immunization ≥24 months after HCT (the late vaccination group) was inversely associated with weak response (OR= 0.43, 95% CI: 0.04–3.5; p= 0.4), but the effect did not reach statistical significance. Other analyzed predictors (sex, age >40, patient immunization before HCT) did not show an influence in multivariate analysis. The chart visualizing the study group and significant differences between the response groups is presented in **Figure 2**.

## Evaluation of Long-Term Immunity

In the long-term study group anti-HBs Ab concentrations >100, 50–99, and 10–49 mIU/ml were detected in 30 (69.8%), 8 (18.6%), and 5 (11.6%) patients, respectively.

In the vaccinated group, the median anti-HBs Ab titer was 230 mIU/ml (range, 11 - >1,000 mIU/ml). Thirteen (35.1%)

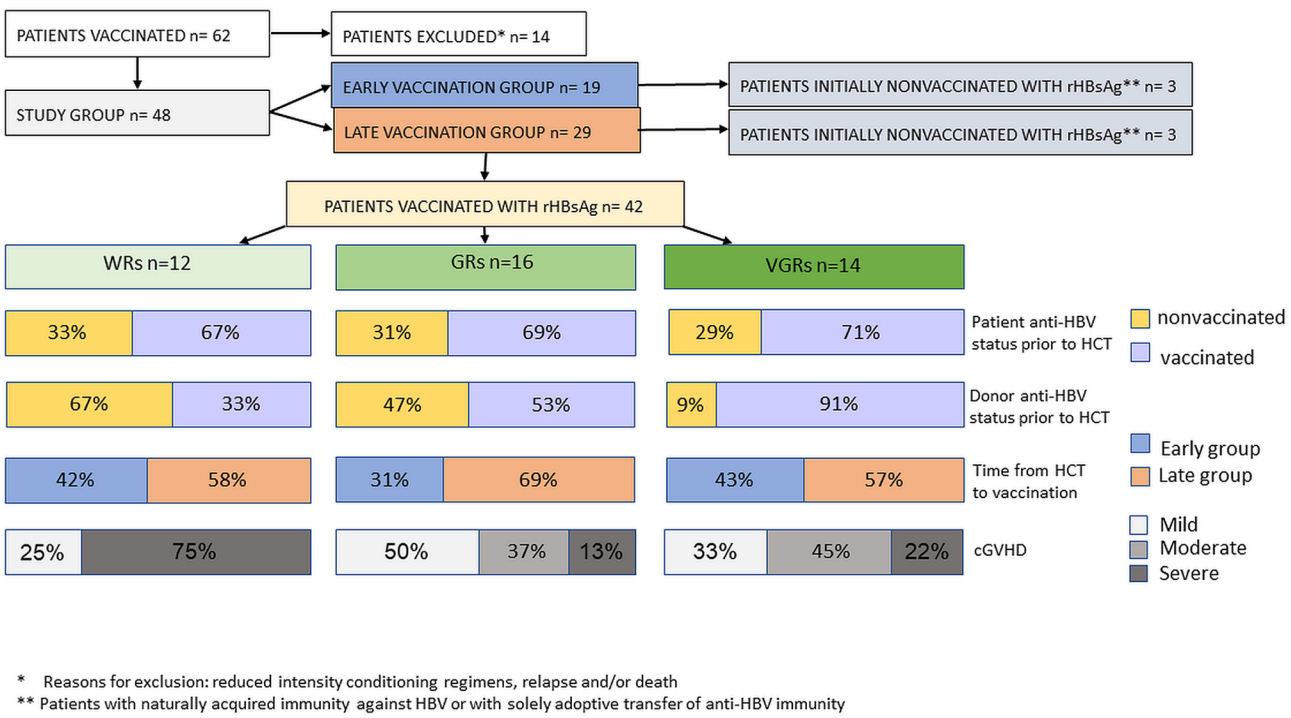


**FIGURE 2** | A structure of the study group and significant differences between the response groups with respect to patient-, transplant-, and donor-related factors.

patients required reimmunization with booster doses of rHBsAg due to a decline in Abs. In 9 patients, one booster dose was sufficient, and 2 and 3 doses were administered in 3 and 1 patients, respectively. The majority (84.6%) of patients achieved anti-HBs Ab titers >100 mIU/ml upon reimmunization. There was no case of hepatitis B in the study group in the follow-up period.

In the initially nonvaccinated group, the median anti-HBs Ab titer was 561.5 mIU/ml (range, 91 - >1,000 mIU/ml). Two patients required one booster dose of rHBsAg due to a gradual loss of immunity to approximately 30 mIU/ml, and they achieved a long-term anti-HBs Ab titer > 100 mIU/ml upon reimmunization.

In univariate analysis, a prior belonging to the WR, GR, or VGR groups had a significant impact on median anti-HBs titers that were 73, 270, and 302 mIU/ml, respectively (p= 0.05). Booster rHBsAg doses were required in 9 WR patients and in 4 GRs but in no VGR patients (p< 0.0002). In multivariate analysis, the odds for maintaining anti-HBs titers > 100 mIU/ml were lower for the WR group (OR= 0.17, 95% CI: 0.02–1.02; p= 0.059). The chart visualizing the differences in the long-term immunity between the response groups and a need for booster vaccine doses is presented in **Figure 3**.

We observed higher median anti-HBs Ab concentrations in patients with previously noted adoptive immunity transfer (363.5 vs. 240 mIU/ml), and fewer patients required a booster dose of rHBsAg (20% vs. 56.6%), but statistical significance was not achieved.

The median long-term anti-HBs Ab concentrations did not differ significantly with the use of our vaccination protocol between patients vaccinated at age of < 40 and those vaccinated at age of ≥ 40 years (224.5 vs. 263 mIU/ml, respectively) in univariate and multivariate analyses.

We observed higher median anti-HBs Ab titers in patients without a history of cGVHD or with mild cGVHD (282.5 and 270 mIU/ml, respectively) than in patients with severe cGVHD (110 mIU/ml). In multivariate analysis, the odds for maintaining anti-HBs titers > 100 mIU/ml in patients with severe cGVHD were lower than those in patients with a mild form of or without cGVHD (OR= 0.4, 95% CI: 0.04 – 3.07; p= 0.41). Although the difference was not statistically significant, in 60% of the severe cGVHD patients, the Ab titers were raised by booster doses of rHBsAg in contrast to 32% of those with a mild form of or without cGVHD (p=0.09). The remaining analyzed predictors (sex, donor immunization, vaccination timing) did not show an influence in multivariate analysis.

## Immune Reconstitution

The adoptive T-cell and B-cell immunity recover within months or years in patients after HCT, and this process is very individual. Immune reconstitution depends on the pretransplant factors like an underlying disease and its treatment, age, conditioning regimen, donor, a source of hematopoietic cells, and posttransplant factors, e.g., GVHD. The recovery of B-cells is similar to ontogeny and usually quantitatively normalizes around 1 year post-HCT. However, a lowered cumulation of hypermutations in VH genes, impaired isotype switch and IgG production, processes dependent *inter alia* on Th2 cells, are frequently observed after HCT. In contrast, T-cell reconstitution is inverted, and memory/effector T-cells dominate even many years posttransplant, while the reconstitution of naïve T-cells, which broaden the repertoire of specificities, starts not earlier than 6 months post-HCT in the case of CD4+ cells (32).

The lowered ability to produce specific antibodies in response to vaccination, observed in a substantial proportion of HCT recipients, led to the creation of intensified vaccination schedules post-HCT, including vaccination against HBV. Upon injection, rHBsAg is lysed and processed by antigen-presenting specific B-cells and presented with MHC-II molecule to Th2 cells. Activated Th2 lymphocytes induce differentiation of B-cells to plasma cells, secreting HBsAg in high quantities to stimulate immune B-cell and T-cell memory (33). All types of immune cells involved in creating anti-HBV postvaccination immunity were included into analyses, but their reconstitution depends on the time elapsed from HCT to a great extent. Therefore, the parameters of immune reconstitution were analyzed with respect

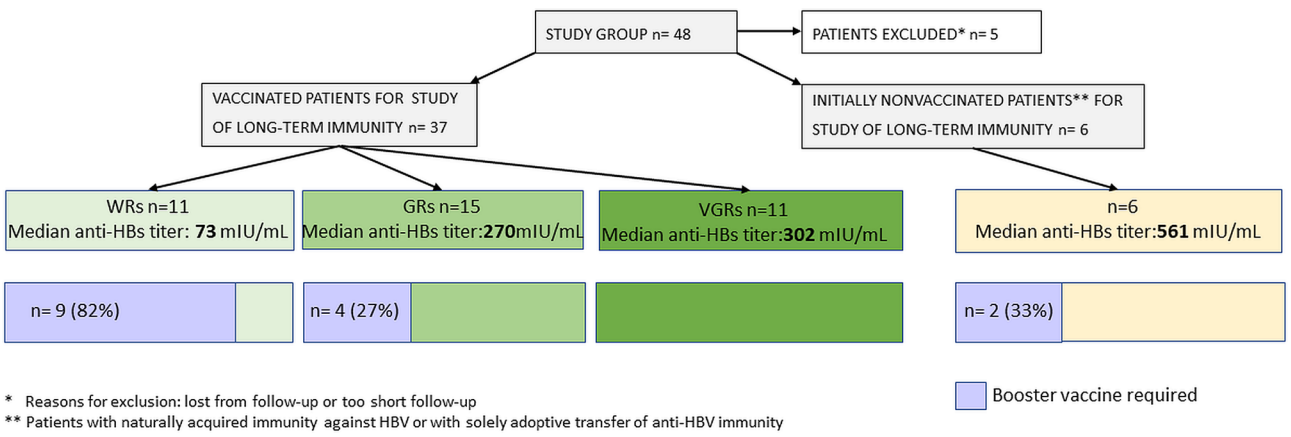


**FIGURE 3** | Differences in the long-term immunity between the response groups and a need for booster vaccine doses.

to timing from HCT and, subsequently, their impact was assessed with respect to response to vaccination with rHBsAg.

### Comparison of the Early and Late Vaccination Groups

There were no significant differences concerning absolute lymphocyte count, gamma-globulin rate or IgG and IgM levels, while the median IgA concentration was significantly higher in patients > 2 years post-HCT (2.1 vs. 1.36 g/L; p= 0.005). The summarized comparison of detailed immune parameters described below is presented in **Table 4**.

The reconstitution of B lymphocytes, including absolute counts of CD19+ cells and B-cell subpopulations with immunoglobulin IgD(+) and IgM(+) receptors, were comparable between the early and late group. The frequencies of CD19+IgG (+) cells were very low in both groups, which is why only percentages are provided. Naïve (CD27-) cells dominated over memory (CD27+) B-cells (p< 0.001), with a trend toward higher absolute values in the late group. The proportion and absolute counts of IgM(+)CD27(-) and IgD(+)CD27(-) naïve B-cells did not differ significantly between the two distinct groups, as was the case for IgM(+)CD27(+) and IgD(+)CD27(+) memory B-cells and double negative (DN) IgD(-) CD27(-) late differentiated memory B-cells.

The reconstitution of T lymphocytes did not vary concerning the proportion and absolute counts of CD3(+) and CD3(+)CD8 (+) cells, while there were higher rates and absolute counts of CD3 (+)CD4(+) cells in the late group: 18.2% (0.33 x $10^9$/L) vs. 25.6% (0.53 x $10^9$/L) (p= 0.006). The inverse CD4/CD8 ratio was more evident in the early group: 0.52 vs. 0.86 (p= 0.008). The naïve (CD45RA+CD45RO-) and memory (CD45RA-CD45RO+) subsets of CD8(+) cells were comparable, while naïve CD4(+) cells were less frequent than memory CD4(+) cells (p< 0.001). In the late group, there was a trend toward more numerous naïve CD4(+) cells (p=0.096), while memory CD4(+) cells achieved significantly higher values (p= 0.011).

The expression of cytokines from the Th1 profile (IFNγ+ and IL2+) dominated in both groups. IL2 predominated in CD3(+) CD8(-) cells while IFNγ predominated in CD3(+)CD8(+) cells with a trend to higher IFNγ expression in the early group (p=0.052). IL2(-)IFNγ(+) lymphocytes predominated in the early group (p= 0.018) and IL2(+)IFNγ(-) lymphocytes were prevalent in the late group (p=0.011). Within the Th2 cytokine profile, the expression of IL4 was higher in CD8(-) cells, and did not differ between the early and late groups. The Th1/Th2 ratio (IL2/IL4) was slightly higher in the early group: 11.94 vs. 9.62.

### Variations in Parameters of Immune Reconstitution and Cytokine Profile Upon the Receipt of Vaccination

The whole VGR group achieved an absolute count of CD3(+) CD4(+) cells > 0.2 x $10^9$/L at vaccination. Among 6 patients who had not reached this value, half turned out to be GRs, and the remaining 3 were WRs. Insight into the naïve CD4(+) T-cell subset did not show significant differences in the immune response to vaccination for the cut-off of 0.05 x $10^9$/L, while there was a trend toward a lower RR for the cut-off of 0.03 x $10^9$/L, which was not reached in 63% of WRs (p= 0.057).

Generally, the levels of either naïve or memory T-cell compartments rose gradually during the realization of the vaccination protocol (p< 0.001). However, in the WR group, naïve CD4(+) cell counts began to rise with a delay compared to those in the GR and VGR groups (p< 0.001). We did not find a negative impact of a history of cGVHD on the number of naïve CD4(+) T-cell subsets.

In turn, the numbers of CD19(+) B-lymphocyte and naïve CD27(-) and memory CD27(+) subsets had no statistical impact on the immune response to rHBsAg. Nevertheless, the rates of memory IgM(+) and IgD(+)CD27(+) B-cell subsets showed a significant rise in the second measurement in GRs when the humoral response was observed (p=0.027 and p= 0.047, respectively). Interestingly, the median percentages of DN IgD-CD27- in WRs, GRs, and VGRs were 5.77%, 4.14%, and 5.44% within CD19+ B cells, respectively, and the difference was significant between WRs and GRs (p= 0.047). Concerning a history of cGVHD, the memory B-cell subsets IgM(+) and IgD (+) increased significantly upon vaccination in the second measurement only in patients with mild cGVHD (p<0.001), in contrast to patients with a moderate or severe form. We observed slightly lower median values of DN IgD-CD27- in patients without cGVHD and with a mild form of cGVHD 4.74% and 4.51%, respectively, while in patients with a history of moderate and severe cGVHD had median values 6.27% and 5.47% without statistical significance.

The rate of CD8(-) cells with IFNγ expression had no statistical impact on vaccination efficacy and did not change during vaccination. In contrast, the mean percentage of CD8(-) IL2(+) cells differed significantly between the WR group and VGR group (18.9 vs. 26.9%; p= 0.043) and decreased upon immunization in all patients. The mean values of CD8(-)IL2 (+) cells decreased upon vaccination in patients without cGVHD and with a mild form of cGVHD (23.8 and 20.1% vs. 16.1 and 14.7%, respectively), while these values remained at a stable level of approximately 20% in patients with a history of severe cGVHD. The rates of IL4-expressing cells increased gradually in the WR group upon vaccination in subsequent analyses (2.3 vs. 2.9 vs. 3.2%; p= 0.033). In patients with severe cGVHD, a similar increase in the IL4(+) cell rate was observed during vaccination (2.6 vs. 3.3 vs. 3.5%; p= 0.012, p= 0.024). The rate of IL5(+) T lymphocytes rose already in the second measurement in GRs (p= 0.023) and VGRs (p<0.001), and a delayed rise after vaccination completion was observed in WRs (p= 0.01). In patients with mild cGVHD or without a history of cGVHD, the IL5(+) cell rate increased in the second measurement (p= 0.027 and p<0.001, respectively), whereas a delayed rise was present in patients with moderate and severe cGVHD (p= 0.017 and p= 0.038, respectively).

## DISCUSSION

Despite the high immunogenicity of the anti-HBV vaccine, approximately 5% of healthy vaccinees fail to mount an adequate humoral response. The response rate in immunocompromised patients is reported to be lower. Preceding therapy, a vaccination schedule, and a dose of antigen are postulated to have a significant impact on immune responses (34). The immunogenicity of the

**TABLE 4 |** Detailed comparison of general characteristics and parameters describing immune reconstitution between the early vaccination group and the late vaccination group.

| | Early group<24 months post-HCT | Late group>24 months post-HCT | Units | Statistics |
|---|---|---|---|---|
| General characteristics | | | | |
| Female/male | 42/58 | 47/53 | % | p = NS |
| Age at vaccination* | **35** (21–50) | **43** (20–57) | years | **p = 0.002** |
| Time after HCT* | 20 (12–24) | 56 (31–119) | months | N/A |
| Time after immunosuppression taper* | 7 (2–19) | 20 (12–31) | months | N/A |
| Immunoglobulins | | | | |
| IgG** | 11.36 (± 4.11) | 11.55 (± 2.56) | g/L | p = NS |
| IgA** | **1.36** (± 0.68) | **2.1** (± 0.97) | g/L | **p = 0.005** |
| IgM** | 0.81 (± 0.42) | 0.96 (± 0.45) | g/L | p = NS |
| Lymphocytes | | | | |
| Absolute lymphocyte count** | 2.05 (± 0.91) | 2.42 (± 0.91) | x $10^9$/L | p = NS |
| B lymphocytes CD19+* | 0.28 | 0.41 | x $10^9$/L | p = NS |
| CD19+IgD(+)* | 0.11 | 0.16 | x $10^9$/L | p = NS |
| CD19+IgM(+)* | 0.21 | 0.31 | x $10^9$/L | p = NS |
| CD19+IgG(+)* | 0.2 | 0.1 | % | p = NS |
| Naïve CD19+IgM(+) CD27-* | 50.0 | 42.6 | % | p = NS |
| | 0.14 | 0.17 | x $10^9$/L | p = NS |
| Naïve CD19+IgD(+) CD27-* | 81.0 | 83.15 | % | p = NS |
| | 0.23 | 0.34 | x $10^9$/L | p = NS |
| Memory CD19+IgG(+) CD27+* | 0.6 | 0.25 | % | p = NS |
| Memory CD19+IgM(+) CD27+* | 2.5 | 2.7 | % | p = NS |
| | 0.007 | 0.11 | x $10^9$/L | p = NS |
| Memory CD19+IgD(+) CD27+* | 2.0 | 2.7 | % | p = NS |
| | 0.006 | 0.11 | x $10^9$/L | p = NS |
| Memory CD19+IgD(-) CD27(-)* | 5.4 | 5.12 | % | p = NS |
| | 0.015 | 0.014 | x $10^9$/L | p = NS |
| T lymphocytes CD3+* | 1.07 | 1.25 | x $10^9$/L | p = NS |
| T lymphocytes CD3+CD4+* | **0.33** | **0.53** | x $10^9$/L | **p = 0.006** |
| T lymphocytes CD3+CD8+* | 0.73 | 0.7 | x $10^9$/L | p = NS |
| CD4/CD8 ratio* | **0.52** | **0.86** | | **p = 0.008** |
| Naïve CD4+CD45RA+* | 25.0 | 20.4 | % | p = NS |
| | 0.08 | 0.1 | x $10^9$/L | p =0.096 |
| Memory CD4+CD45RO+* | 61.0 | 65.9 | % | p = NS |
| | **0.21** | **0.34** | x $10^9$/L | **p = 0.011** |
| Naïve CD8+CD45RA+* | 41.1 | 44.9 | % | p = NS |
| | 0.29 | 0.3 | x $10^9$/L | p = NS |
| Memory CD8+CD45RO+* | 26.3 | 26.5 | % | p = NS |
| | 0.2 | 0.19 | x $10^9$/L | p = NS |
| Th1 cytokine expression* | | | | |
| CD3+CD8-INFγ+ | 20.34 | 20.22 | % | p = NS |
| CD3+CD8-IL2+ | 24.13 | 26.17 | % | p = NS |
| CD3+CD8+INFγ+ | **34.23** | **27.83** | % | **p=0.052** |
| CD3+CD8+IL2+ | 6.16 | 5.0 | % | p = NS |
| CD3+ IL2- INFγ+ | **49.14** | **38.13** | % | **p= 0.018** |
| CD3+ IL2+ INFγ- | **14.74** | **21.62** | % | **p=0.011** |
| Th2 cytokine expression* | | | | |
| CD3+CD8-IL4+ | 1.79 | 2.15 | % | p = NS |
| CD3+CD8-IL5+ | 0.09 | 0.15 | % | p = NS |
| CD3+CD8-IL10+ | 0.12 | 0.12 | % | p = NS |
| CD3+CD8+IL4+ | 0.41 | 0.48 | % | p = NS |
| CD3+CD8+IL5+ | 0.05 | 0.04 | % | p = NS |
| CD3+CD8+IL10+ | 0.04 | 0.03 | % | p = NS |

**Median value (range in parenthesis).*

***Arithmetic mean value.*

*Bolded values showed statistical differences.*

primary vaccination is known to last 10 to 31 years, but not in the case of immunocompromised patients (34). In the analysis performed by Kaloyannidis et al., the probability of losing HBV immunity was 100% at 5 years post-HCT for patients who received transplants from nonimmunized donors and 78% and 58% for those who received transplants from vaccinated donors and naturally immunized donors, respectively (6). In contrast to our study group, those patients were not revaccinated post-HCT, and those data cannot be directly compared to our results. The aforementioned study by Kaloyannidis et al. also reported a high

probability of reversed seroconversion, reaching 18% at 12 years (6). In the analysis by Mikulska et al., HBV reactivations were observed in 10% of patients with a median time of 19 months post-HCT (8). In our study group, from 5 patients with naturally acquired immunity, no reverse seroconversion was observed. Two patients received booster doses of rHBsAg as prevention when a gradual drop in anti-HBs Abs was observed.

A detailed analysis of our data from the early posttransplant period showed an evident impact of either patients' or their donors' vaccination status before HCT. In the case of insufficient or no protection against HBV, passive immunization remains the only solution. However, passively transferred anti-HBs Abs have a limited lifespan, as shown in our study. Therefore, repetitive infusions would be required to preserve sufficient protection. More prolonged protective anti-HBs titers were observed in patients effectively vaccinated before HCT and in those who received transplants from vaccinated donors. Moreover, donor immunization provides an additional benefit for an HCT recipient - memory B and T-cells responsible for the adoptive transfer of immunity, which can be easily recalled by a booster dose of vaccine (34, 35).

The ORR to post-HCT vaccination of 100% and the long-lasting maintenance of anti-HBV immunity might be astonishing given that these vaccinations were administered to immunocompromised patients. Similar results were reported by Machado et al. in 45 recipients immunized ≥ 1 year after allo-HCT, while in another cited study, the seroconversion rate in 168 adult patients was 59% (13, 36). We used three known strategies to improve the immune response: an increased vaccine dose to 40 μg administered until seroconversion was achieved, an intensified dosing schedule and the co-administration of rHBsAg with other vaccines from the vaccination protocol (34). A higher dose of the vaccine administered initially augments the B-cell response and increases the proportion of memory B-cells, which could also have an impact on the duration of long-lasting immunity (37). Moreover, in the study group, more than 90% of the VGRs received transplants from vaccinated donors, thereby transferring memory cells to their recipients. The positive effect of prior donor immunization was also confirmed in the multivariate analysis.

The median follow-up in our study, exceeding 10 years, enabled the monitoring of a long-lasting anti-HBV immune status in the majority of patients. The inevitable loss of anti-HBV immunity was confirmed in HCT recipients, but the rate was dependent on the degree of the immune response to the primary inoculum series (37). No patient from the VGR group required any booster dose of rHBsAg, and only 20% of patients with previously noted adoptive immunity transfer required a booster dose. In contrast, primary WRs, according to our predefined criteria, should be closely monitored as booster doses of rHBsAg are likely to be needed. However, one can expect an intense reaction of memory cells to recall antigen, as in most cases, one booster dose was sufficient to achieve anti-HBs Abs titers > 100 mIU/ml.

Upon injection, rHBsAg is lysed and processed by antigen-presenting specific B-cells and presented with MHC-II molecule to Th2 cells. Activated Th2 lymphocytes induce differentiation of B-cells to plasma cells, secreting HBsAg in high quantities to stimulate immune B-cell and T-cell memory (33). To better understand the immune response in HCT recipients, we measured several parameters of the immune system, including subpopulations of B and T-cells, with deep analysis of subsets expressing Th1 and Th2 cytokine profiles. We confirmed that it is optimal to start active immunization when the absolute CD4(+) T cell count exceeds $0.2 \times 10^9$/L. Similar to HCT recipients, in patients with HIV infection, the seroconversion rate after HBV vaccination was shown to be directly proportional to the CD4(+) cell count (34). The repertoire of naïve T lymphocytes is crucial for the optimal response to antigens and depends on thymus regeneration starting from 6 to 12 months posttransplantation (38). In our study group, there was a trend toward a weaker response in those with naïve CD4 (+) cells below $0.03 \times 10^9$/L, which was already reported by Roux et al. in HCT recipients vaccinated with tetanus toxoid (14). We did not observe a decreased proportion of Th2-like cytokine-producing CD4 + cells, while IL2-producing CD4+ cells were significantly lower in WRs, as described in "*in vitro*" studies (39). Moreover, the realization of an active immunization protocol led to a gradual increase in IL4(+) and IL5(+) cells and a decrease in IL2(+)CD4+ lymphocytes.

The early vaccination group had significantly lower IgA immunoglobulin levels than the late group, which could reflect an impaired isotype switch from naïve to memory B-cells. However, we did not confirm the influence of median IgA levels on the quality of the postvaccination humoral response (15, 16). Differences in circulating B-cells at the time of vaccination did not show significant impact on the immune response (15). However, the humoral response was parallel with the increase in memory IgM(+) and IgD(+) B-cell subsets. Interestingly, we found a significantly higher percentage of late differentiated memory DN B lymphocytes in WRs. This subpopulation is reported to reflect senescence of the immune system related to chronic inflammatory processes, e.g., HIV infection, lupus, Alzheimer's disease (40). This observation could be explained by a high rate of patients with a history of severe cGVHD in the WR group.

The comparison of the early and late groups showed some differences in immune reconstitution but these parameters had ultimately no impact on the quality of the post-vaccination response in our study group neither in the univariate nor multivariate analysis. In multivariate analysis, there was only a slight trend toward less common weak responses to immunization with rHBsAg in the late vaccination group. This is another suggestion that vaccination against hepatitis B started at 6 months post-HCT according to the international recommendations is absolutely rational.

Severe GVHD induces structural damage that has a serious and durable impact on thymus functioning and output (41–43). A direct influence of immunosuppressive agents in our study group was excluded since immunosuppression was discontinued at least 2 months before enrollment. However, a history of severe cGVHD significantly influenced the response to anti-HBV vaccination and the maintenance of protective immunity, even though immunization was performed in the nonactive phase of the disease. We also noted a delayed increase in Th2 IL4(+) and IL5(+) cells in response to rHBsAg in WRs and patients with a

history of severe cGVHD. Our observations are in line with those of the study by Jaffe et al., in which the seroconversion after 3 doses of rHBsAg was observed in 64% of patients after HCT, and the negative influence of a GVHD history was also demonstrated (13). Furthermore, Kaloyannidis et al. indicated cGVHD as an independent factor for anti-HBs Ab disappearance (6).

Guidelines for the prevention of infectious complications among HCT recipients recommend the same vaccination schedule for all HCT recipients, and active immunization of patients with cGVHD should not be postponed (18, 20, 21, 36, 38). Evidence exists that severe cGVHD and its treatment deteriorate the efficacy of active immunization (14, 18). Therefore, immunization should not begin during the exacerbation of GvHD and the escalation of immunosuppressive treatment. Instead, effective anti-infectious prophylaxis should be provided (18).

Our study has several limitations. First, a laboratory part was designed and performed when the serological assessment of antibody response was only available for vaccination against HBV in the hospital laboratory. Therefore, we did not analyze the response rates to the remaining co-administered vaccines. Second, although we did not observe any case of hepatitis B in our study group, which gives evidence for clinical protection of patients with serological response to vaccination, we did not investigate the specific cellular immunity. The modern assays such as Ag-specific cell detection, protein quantification, and transcriptomics techniques would give a deeper insight into post-vaccine immunity and potential correlation between the humoral and cellular anti-viral protection. Third, immunophenotyping was performed with the use of 3-color flow cytometry as at the time of laboratory analyses it was the only available equipment. The multicolor flow cytometry available nowadays would extend the analytic possibilities.

In summary, vaccination of recipients and their donors against HBV prior to HCT is beneficial in many aspects, including protection in the early posttransplantation period. Our results add important information that might help the clinical management of HCT recipients by implementing a double dose of rHBsAg (40 μg) in patients with a history of cGVHD and those not immunized before HCT or those who received transplants from nonimmunized donors until seroconversion is achieved. The subsequent doses might be reduced to the standard 20 μg. Second, an intensified vaccination schedule of 0-1-2-6-(12) months is advised for WRs who do not achieve a protective anti-HBs Ab titer >10 mIU/ml or in those for whom the level is between 10 and 100 mIU/ml after 3 doses of HBV vaccine. Third, a titer of anti-HBs Abs should be monitored routinely in WRs and patients with cGVHD, since a decline in specific Abs requiring reimmunization is expected. Finally, in GRs and VGRs and patients without cGVHD, the titer of anti-HBs Abs can be measured in longer (e.g., 5-year) intervals.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Independent Bioethics Committee of the Medical University of Gdansk. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

AP was involved in the conception and design of the study, the acquisition of data, statistical analysis, and the analysis and interpretation of data, and writing of the manuscript. PW took part in the statistical analysis and critical revision. LG was involved in the data analysis and critical revision. KL took part in the data analysis, drafting of the article, and critical revision. PT was involved in the data analysis and critical revision. MB took part in the data analysis and critical revision. JZ was involved in the data analysis, drafting of the article, and critical review. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Vaccine-Induced CD8$^+$ T Cell Responses in Children: A Review of Age-Specific Molecular Determinants Contributing to Antigen Cross-Presentation

*Elisabeth M. S. Beijnen*[1,2,3] *and Simon D. van Haren*[2,3]*

[1] Utrecht Institute for Pharmaceutical Sciences (UIPS), Faculty of Science, Utrecht University, Utrecht, Netherlands,
[2] Precision Vaccines Program, Division of Infectious Diseases, Boston Children's Hospital, Boston, MA, United States,
[3] Department of Pediatrics, Harvard Medical School, Boston, MA, United States

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***Correspondence:**
*Simon D. van Haren*
*simon.vanharen@childrens.harvard.edu*
*orcid.org/0000-0002-1791-0161*

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Infections are most common and most severe at the extremes of age, the young and the elderly. Vaccination can be a key approach to enhance immunogenicity and protection against pathogens in these vulnerable populations, who have a functionally distinct immune system compared to other age groups. More than 50% of the vaccine market is for pediatric use, yet to date vaccine development is often empiric and not tailored to molecular distinctions in innate and adaptive immune activation in early life. With modern vaccine development shifting from whole-cell based vaccines to subunit vaccines also comes the need for formulations that can elicit a CD8$^+$ T cell response when needed, for example, by promoting antigen cross-presentation. While our group and others have identified many cellular and molecular determinants of successful activation of antigen-presenting cells, B cells and CD4$^+$ T cells in early life, much less is known about the ontogeny of CD8$^+$ T cell induction. In this review, we summarize the literature pertaining to the frequency and phenotype of newborn and infant CD8$^+$ T cells, and any evidence of induction of CD8$^+$ T cells by currently licensed pediatric vaccine formulations. In addition, we review the molecular determinants of antigen cross-presentation on MHC I and successful CD8$^+$ T cell induction and discuss potential distinctions that can be made in children. Finally, we discuss recent advances in development of novel adjuvants and provide future directions for basic and translational research in this area.

**Keywords: vaccine, vulnerable population, CD8, children, cross-presentation**

## INTRODUCTION

British physician Edward Jenner marked the beginning of vaccinology when he developed the world's first vaccine for smallpox in 1796 (1). His invention relied foremostly on the awareness that dairymaids infected with cowpox were immune to outbreaks of smallpox. The next breakthrough occurred in 1880, when the French chemist Louis Pasteur discovered the principle of attenuation

(2). Five years later, Pasteur produced the first laboratory-developed vaccine which tremendously increased the speed of vaccine development.

Most historically developed successful vaccines use weakened or inactivated pathogens. Examples of such vaccines are whole cell pertussis vaccine, which led to large and rapid reductions in pertussis deaths in the United States after its introduction in 1914, (3, 4), or the inactivated polio vaccine which has successfully eradicated poliomyelitis (**Table 1**) (24). More recently, however, technological developments have shifted vaccine development toward the production of formulations that do not contain live material, such as nucleic acid vaccines and subunit vaccines. Subunit vaccines are comprised of purified protein or polysaccharide antigens, often combined with adjuvants, immune potentiators that are capable of stimulating the immune system (24). The first successful example is the hepatitis B subunit vaccine, derived from the hepatitis B surface antigen (HBsAg) (**Table 1**). The development of subunit vaccines has led to improved safety profiles, inclusion of immunostimulants to drive specific types of immune responses, and the opportunity for vaccine component optimization. However, the more defined composition of subunit vaccines can lead to challenges as well, as seen in the case of pertussis vaccination. Replacement of whole-cell pertussis vaccine (wP) by acellular pertussis vaccine (aP), a subunit vaccine, has led to a resurgence of pertussis due to 'waning immunity' (25, 26). The efficacy of subunit vaccines often relies on appropriate type and magnitude of immune activation by adjuvants. As the majority of the global vaccine market is for pediatric use, there is an unmet need to critically review the mechanism of action of these adjuvants in a pediatric setting. Studies on adjuvant mechanism of action in early life from our group and others thus far have focused predominantly on the induction of cytokines, antibodies and $CD4^+$ T cells (27–34), but much less is known about the activation of $CD8^+$ T cells in early life, and the ability of vaccine formulations or adjuvants to induce these.

In vaccine development, quantitative correlates of protection are often determined by quantification of serum antibody levels or neutralizing ability (35, 36). In antiviral vaccine development, however, absolute correlates of protection are not always defined, and relief of symptoms due to eradication of viral disease is a good indicator of vaccine success. Viruses are intracellular pathogens and use the host cell's machinery for internalization, translation of viral proteins and viral genome replication (37). Upon viral infection, a cell can use endogenously generated cytosolic viral proteins for antigen presentation *via* major histocompatibility complex (MHC) I molecules on its surface. MHC class I molecules can be found on the cell surface of all nucleated cells (38). $CD8^+$ T cells recognize short peptides derived from antigenic proteins presented by these molecules and, hence, play a critical role in the control and elimination of viral infections. MHC class II molecules are expressed on antigen presenting cells (APCs), such as dendritic cells (DCs) (39). $CD4^+$ T cells, which recognize peptides presented by MHC class II molecules, promote antibody production which is in many cases sufficient for protection against viruses. While other APCs such as B cells and macrophages are important during different stages of T cell activation, this review will focus on DCs and their role in the instruction of naive T cells.

Activated $CD8^+$ T cells can induce apoptotic death of virus-infected cells by the production of Tumor necrosis factor-alpha (TNF-α), Interferon-gamma (IFN-γ) and the release of cytotoxic molecules containing granzymes, perforins, and granulysin (40, 41). These effector functions directly contribute to pathogen clearance. In childhood, when the highest risk for infection

**TABLE 1** | Vaccines that are licensed in human newborns and infants in the United States.

| Licensed pediatric vaccine | Vaccine type | Antigen(s) | Type(s) of adjuvant (5–7) | Evidence of $CD8^+$ T cell mediated immunity in control of infection |
|---|---|---|---|---|
| Hepatitis A virus (HAV) | Inactivated | Inactivated hepatitis A virus (strain HM175) | Virosomes, aluminum hydroxide | Yes (limited data) (8–10) |
| Trivalent inactivated influenza vaccine (TIV) | Inactivated | Hemagglutinin | Virosomes, MF59, AS03 | No (8, 11–14) |
| Inactivated poliovirus (IPV) | Inactivated | D antigen | None | Yes (15, 16) |
| Rotavirus (RV) | Live attenuated | Spike protein | No adjuvant used | Unclear (17, 18) |
| Bacillus Calmette-Guérin (BCG) | Live attenuated | Antigen 85 | None | Yes (9, 10, 19) |
| Measles, mumps, rubella | Live-attenuated | Trivalent antigen | None | Measles: yes (18)<br>Mumps: no (20) |
| Varicella (VAR) | Live attenuated | Varicella virus live | None | Yes (11–14, 21) |
| Live attenuated Influenza vaccine (LAIV) | Live attenuated | Hemagglutinin | None | Yes (11–13, 22) |
| Hepatitis B virus (HBV) | Subunit | HBsAg | Virosomes, AS04 | Yes (16, 23) |
| Diphtheria, Tetanus & acellular Pertussis (DTaP) | Toxoid, subunit | Tetanus toxoid, diphtheria toxoid, detoxified pertussis toxin | Aluminum hydroxide | Yes (acellular Pertussis) (15, 19) |
| Pneumococcal conjugate (PCV) | Conjugate | Pneumococcal polysaccharides conjugated to a nontoxic form of diphtheria toxin CRM197 | Aluminum phosphate | No (limited data) (21, 23) |
| Haemophilus influenzae type b (Hib) | Polysaccharide conjugate | Polysaccharide conjugated to Hib bacterium | None or with aluminum hydroxide | ? |

exists, protective antibodies decline rapidly after primary vaccination (42). Newborns and infants are highly susceptible to viral infectious diseases and impaired $CD8^+$ T cell responses may lead to progressive or even fatal infection. For example, there is evidence that SARS-CoV-2 virus can infect children (43–47) and can sometimes have severe consequences, such as multisystem inflammatory syndrome in children (MIS-C) (45, 48). SARS-CoV-2-specific $CD8^+$ T cells are detectable in infected and convalescent individuals, and potentially correlate with disease outcome (49–52). Vaccine induced $CD8^+$ T cell priming may therefore improve the efficacy of immunization in infants against viral pathogens (53, 54).

Nucleic acid-based vaccines and subunit vaccines do not contain a live vector and are therefore generally more safe than inactivated and live attenuated vaccines. However, the high purity of the components can make these vaccines less immunogenic and hence potentially less effective (42), if not adjuvanted properly. Nucleic acid vaccines rely on incorporation of the genetic material into the host antigen-presenting cell genome, potentially resulting in endogenous transcription of viral proteins and therefore effective presentation on MHC class I. Subunit vaccines are composed of only antigenic viral proteins or carbohydrates and therefore the step of genome incorporation into the host is removed. As a consequence, the antigen will not gain access to the cytosol, which is known to be a critical step for MHC class I presentation and subsequent $CD8^+$ T cell activation. In general, nucleic acid vaccines are therefore more effective in eliciting $CD8^+$ T cell responses (55–57). To improve immunogenicity of subunit vaccines, adjuvants can be added to the formulation. Adjuvants promoting CD8-mediated immunity are therefore a key element for developing effective subunit vaccines against viruses. This can be accomplished by the process of cross-presentation, which enables MHC class I presentation of viral proteins, taken up from extracellular sources. Evidence of adjuvant-induced cross-presentation has been described, often including a proposed mechanism of action (58–68). However, there is to date no published data describing whether and how adjuvants induce cross-presentation in early life. In this review, we address the key concept of how adjuvants can activate $CD8^+$ T cell responses and discuss their ability to regulate key molecular pathways relating to antigen cross-presentation in early life (46). Understanding the functionality of $CD8^+$ T cells in early life and how they can be effectively induced by adjuvants directly informs the development of subunit vaccines for pediatric use.

## CHANGES IN FREQUENCY OF $CD8^+$ T CELLS WITH AGE

An important parameter for the induction of an effective antiviral response is that there is a sufficient number of $CD8^+$ T cells available to extirpate virus-infected cells. T cell precursors arise from hematopoietic stem cells (HSCs), which are composed of two main cell populations: Sca-$1^-$ lymphoid-biased stem cells, and Sca-$1^+$ myeloid-biased stem cells. Lymphocytopoiesis in infants is distinguished by the robust production of T cells, due to a relatively high number of lymphoid-biased HSCs. However, these cells decline with increasing age and as a consequence, the ratio of HSCs in adults shifts toward more myeloid biased HSCs. These cells are less efficient in creating common lymphoid progenitors with high proliferative capacity compared to their counterparts, which directly contributes to the reduction in naive T cell generation in the aged population (69). In addition to a greater influx of HSCs with lymphoid potential into the thymus in children, mouse studies have shown greater efficacy in thymopoiesis in early life (70, 71), resulting in a higher frequency of naive $CD8^+$ T cells in the periphery (72, 73). This latter observation is also seen in humans, as both the frequencies of recent thymic emigrants (RTEs) (74, 75) and of naive $CD8^+$ T cells (76) decreases with age. Other factors that affect the functioning of HSCs with increasing age are oxidative stress and reduced telomerase activity, which cause the naive $CD8^+$ T cell compartment to shrink gradually (77, 78).

In support of the foregoing, experimental data indicate that young infants exhibit higher frequencies of $CD8^+$ T cells compared to their adult counterparts. Young adults carry roughly $10^{11}$ $CD8^+$ T cells (79). Absolute values of neonatal $CD8^+$ T cells in human are absent, but limiting dilution studies have shown that the precursor frequency of $CD8^+$ cytolytic T cells in neonates is comparable to that in adults (80). In fact, Thome et al. observed that infants (0 – 2 years) express significant higher percentages of $CD8^+$ naive T cells compared to young adults (15–25 years) in circulation, lymphoid and mucosal tissues (81).

## PHENOTYPIC AND FUNCTIONAL DIFFERENCES OF $CD8^+$ T CELLS BETWEEN AGE GROUPS

### Phenotypic Differences

In addition to distinctions in frequency of total as well as naive $CD8^+$ T cells with age, the expression of certain cell surface receptors can differ between age groups as well, potentially affecting vaccine response to infection or to vaccination (**Table 2**). The main distinctions observed in receptor expression relate to the maturity or activation status of the $CD8^+$ T cells. In accordance with findings that newborns and infants have higher levels of naive $CD8^+$ T cells, a higher percentage of $CD8^+$ T cells express CD28. CD28 serves as a co-stimulator for T-cell activation and survival and is expressed on all naive T cells in newborns (87). In elderly cells, CD28 expression is diminished and sometimes even lost (**Table 2**). This likely contributes to impaired immune responses in elderly. Nevertheless, $CD28^-$ T cells express higher levels of effector molecules such as perforin and granzyme B and therefore show improved cytotoxicity (92). This supports the difference in cytotoxicity level between adults and infants, as will be discussed in the next paragraph.

Another activation marker, CD38 is also more frequently expressed on neonatal or infant $CD8^+$ T cells compared to adult $CD8^+$ T cells (**Table 2**). CD38 is expressed early in ontogeny and

**TABLE 2** | Non-exhaustive list of CD8+ T cell marker levels in different age groups.

| Phenotypic ($CD8^+$) T cell marker | Level of neonatal versus adult/elderly expression in T cells | Reference |
|---|---|---|
| αβ-TCR | Similar | (82, 83) |
| CD3 | Similar | (83–85) |
| CD5 | Similar | (83, 86) |
| CD8 | Similar | (83) |
| CD28 | Lower expression in adults; 40-50% of the elderly (age 80 and above) lack CD28 expression | (87) |
| CD38 | Higher expression in neonates | (83, 88) |
| KIR | Higher expression in adults and elderly | (89) |
| $CD45RA^{lo}CD45RO^{lo}$ | Expressed by neonates, rare or absent in adult T cells | (83, 90) |
| CD300a | Higher expression in adults | (91) |

is suggested to play an important role during T cell activation (93). In the context of human immunodeficiency virus (HIV) infection, high proportions of $CD38^+CD8^+$ T cells are associated with virologic worsening (88). However, there are studies that have observed opposite findings in children (94, 95). Thus, the significance of CD38 distinctions in the CD8 compartment with age still remains unclear and needs to be further examined in different age groups.

At baseline, children age 6–15 and age 16–17 have similar levels of central memory $CD8^+$ T cells compared to adults, but significantly less effector memory $CD8^+$ T cells (96). Upon activation with staphylococcal enterotoxin B (SEB), the increase in expression of activation marker CD69 was significantly reduced in these cells, in particular in the 6–15 age group.

Effector $CD8^+$ T cells can be distinguished by Killer cell Immunoglobulin-like Receptors (KIR) expression. $KIR^+$ cells are estimated to represent approximately 5% of the $CD8^+$ T cells in adults and can increase up to 30% in elderly individuals (89). In contrast, roughly 1.67% of $CD8^+$ T cells express KIRs in cord blood (97). $CD8^+$ T cells acquire KIRs when differentiating into effector molecules (98). This confirms that neonates have more naive T cells than their adult counterparts. The biological functions of KIRs on T cells remain poorly understood although it has been shown that these receptors enhance the efficiency of HLA class I-mediated $CD8^+$ T cell responses (99) and therefore could positively influence the outcome of viral infections.

Upon activation, $CD8^+$ T cells can introduce the expression of inhibitory molecules aiming to prevent an immoderate immune response. One of these receptors is CD300a, a transmembrane protein with immunoreceptor tyrosine-based inhibitory motifs (ITIMs) capable of conduct inhibitory signaling (100). In a comparative study exploring the CD300a expression on human neonatal versus adult immune cells, significant differences in presence of CD300 receptors on $CD8^+$ T cells derived from cord blood and adult blood were observed. The research group showed that naive and memory $CD8^+$ T cells from cord blood exhibited significant lower levels of CD300a when compared to adult T cells (91).

In summary, expression profiles of activation $CD8^+$ T cell markers correlate with age, displaying more activated T cells when older, due to repeated antigen exposure.

## Functional Differences

Neonatal and adult lymphocytes exhibit differential expression of genes involved in T cell receptor (TCR) signaling. Notably, with regards to the neonatal TCR pool, it has been proposed that neonatal T cells may be less dependent on TCR recognition than their adult counterparts (101). TCRs are integral membrane proteins, which control T cell activation through recognition of specific peptides presented by MHC molecules (102). Neonatal T cells exhibit a less diverse TCR repertoire than adult T cells due to a lag in expression of the enzyme terminal deoxynucleotidyl transferase (TdT) (101). TdT is responsible for adding nontemplated (N) nucleotides in V, D, and J gene segments of TCRs (103) and hence plays an important role in diversifying these receptors. Diversification in TCR signaling is of essence, because a larger pool of different TCRs increases the possibility of recognizing all kinds of peptide antigens. Interestingly, the diverse TCR repertoire of adult $CD8^+$ T cells diminishes with increasing age, which contributes to increased susceptibility to viral infections (104).

Upon TCR stimulation, newborn $CD4^+$ T cells favor the secretion of IL-8 but less IFN-γ secreting T-helper 1 cells are observed as compared to adult $CD4^+$ T cells (101). This is a result of impaired production of type-1-polarizing cytokines by neonatal DCs in response to stimulation through Toll-like Receptors (29, 105). This also affects the CD8 compartment, resulting in $CD8^+$ T cells with a more type-2 phenotype (Tc2), which can exacerbate allergy-type reactions in asthma or infection with respiratory syncytial virus (RSV) (106–109).Thus, the immune response generated by neonatal T cells is more of an innate nature, whereas adults produce cytokines that are typically associated with adaptive immune responses. Furthermore, neonatal T cells are less likely to secrete multiple cytokines simultaneously (110). In other words, neonatal T cells are less polyfunctional, which could subsequently lead to less potent T cell responses (96). In a recent study on HIV-1 responses by $CD8^+$ T cells, the results showed that HIV-1 specific adult $CD8^+$ T cells with high frequencies of CD300a were more polyfunctional (111). These observations are in line with the difference in CD300a expression levels between adults and neonates, as described in the previous paragraph.

Galindo-Albarrán et al. have observed that neonatal T lymphocytes are less cytotoxic than adult $CD8^+$ T cells due to lower expression of IFN-γ, a signature molecule for activating the cytolytic pathway (112). Furthermore, they showed that certain enhancers of cytotoxic genes were only expressed in adults and that neonatal $CD8^+$ T cells express only low numbers of granzyme producing cells. Interestingly, expression levels of

granzyme B by neonatal NK cells are found to be similar or even higher than adult NK cells (113). Therefore, it could be postulated that neonatal NK cells are being deployed as a compensation mechanism for having $CD8^+$ T cells bearing low cytotoxicity.

In elderly, differentiation of $CD8^+$ T cells into effector molecules has shown to be impaired in response to infection due to reduced expression of important cytokines, such as IFN-γ, TNF-α, granzyme B, and IL-2 (114). Another functional decline of the immune response in the elderly is suggested to originate from down regulation of certain genes in $CD8^+$ T cells which affect a variety of stages of gene transcription, such as transcription initiation, elongation, RNA stabilization, and protein translation and translocation (115). Certainly, more studies are required to fully understand the primary causes of the impaired gene expression that occurs in $CD8^+$ T cells in the older population and their functional consequences.

Another significant discrepancy between newborn and adult $CD8^+$ T cells is that neonatal cytotoxic T cells have higher proliferative rates than adult naive $CD8^+$ T cells and, subsequently, differentiate more rapidly into effector cells (116). As a consequence, an imbalance in effector and memory $CD8^+$ T cell formation emerges in neonatal cells, with a shift toward more $CD8^+$ T cell effector cells. Thus, newborn cells are less capable of creating immunological memory which has direct implications for creating adaptive immune responses after re-infection. It has been suggested that differences in microRNA (miRNA) expression profiles are accountable for these findings. miRNAs are non-coding mRNA molecules that modulate different aspects of immune responses, such as T cell differentiation. Wissink et al. observed that age-dependent changes in miR-29 and miR-130 in human $CD8^+$ T cells may contribute to the diminished development of neonatal memory cells (117). Further research is required to support this hypothesis.

Age-related changes in CD8+ T cell frequency and proliferation rate may also be influenced by the presence of homeostatic cytokines, such as IL-7. IL-7 plays a central role in maintaining T cell homeostasis and serves as a key factor in the proliferation and survival of naive T cells (118, 119). During thymic development, stromal and epithelial cells in the thymus produce IL-7 to promote $CD8^+$ T cell differentiation in the thymus (120). Thymic production declines with age and, as a consequence, IL-7 levels may decrease during the aging process (121). This could negatively affect CD8+ T cell expansion in response to vaccination and potentially result in failure of immunization.

Fms-like tyrosine kinase 3 ligand (FL) also functions as an important regulator of hematopoiesis and is widely distributed in both murine and human tissues (122). FL has an important role in regulating immunity, due to its capacity to stimulate the expansion of DCs (123). Its receptor, Fms-like tyrosine kinase 3 (FLT3), is mostly expressed by immature hematopoietic cells and shows similar expression patterns in newborn and adult mice (122). To our knowledge, however, no differences in FL levels among age groups have been reported.

## ONTOGENY OF VACCINE INDUCED $CD8^+$ T CELL RESPONSES

**Table 1** lists the commercially available vaccines for pediatric and adult use in the United States. The majority of the live attenuated or inactivated vaccines do induce protective $CD8^+$ T cell mediated immunity, providing empiric evidence that there is at least no impairment in MHC class I loading or $CD8^+$ T cell functionality in early life. Empiric evidence of protective $CD8^+$ T cell mediated immunity induced by protein-based vaccines is much less substantial (**Table 1**). There are different mechanisms to create $CD8^+$ T cell responses after immunization. Modern vaccines may use viral vectors or nucleic acids as a vaccine delivery system (124). These delivery systems are attractive for vaccine therapy because of their capability to provoke potent and sustained $CD8^+$ T cell responses (57, 125). However, the kinetics of nucleic acid delivery and expression of the antigen by APCs likely makes adjuvantation very challenging. Enhancement of the immune response to nucleic acid-based vaccines can be achieved by inclusion of plasmids that encode cytokines, costimulatory receptors, or Toll-like receptor (TLR) ligands (126, 127).The ability to instruct appropriate (often Th1-mediated) $CD4^+$ T cell responses in newborns and infants is impaired (128, 129) and requires adjuvantation with select molecules or combinations that have shown the ability to overcome this impairment (27, 29, 130).

An alternative method for inducing $CD8^+$ T cell responses is through the mechanism of cross-presentation in which MHC class I molecules present exogenous peptides to naive $CD8^+$ T cells. This is in contrast to classical MHC class I presentation, in which a foreign peptide will be displayed after it has arrived into the cytosol of the cell inter alia after the cell has been infected. Antigen cross-presentation has been studied for decades, since its discovery in 1976 (131, 132), but there are still many aspects of this concept which are controversial and not fully understood. However, it is clear that there are different subcellular pathways involved in cross-presentation, each consisting of crucial steps for MHC class I presentation. In order to evaluate the potential of adjuvants to induce cross-presentation in children, it is important to summarize the components and mechanisms of cross-presentation to the extent that they are currently known and understood. **Figure 1** provides an illustrated summary of the different components, cytokines, receptors, and biological processes contributing to successful vaccine-induced CD8+ T cell activation discussed in this review, and the extent to which changes with age have been observed.

## HUMAN DENDRITIC CELL SUBSETS AND CROSS-PRESENTATION

Dendritic cells are a class of bone-marrow-derived cells which can be found in blood, tissues and lymphoid organs. They are referred to as 'professional' APCs because of their unique ability to bridge the innate and adaptive immune system *via* the

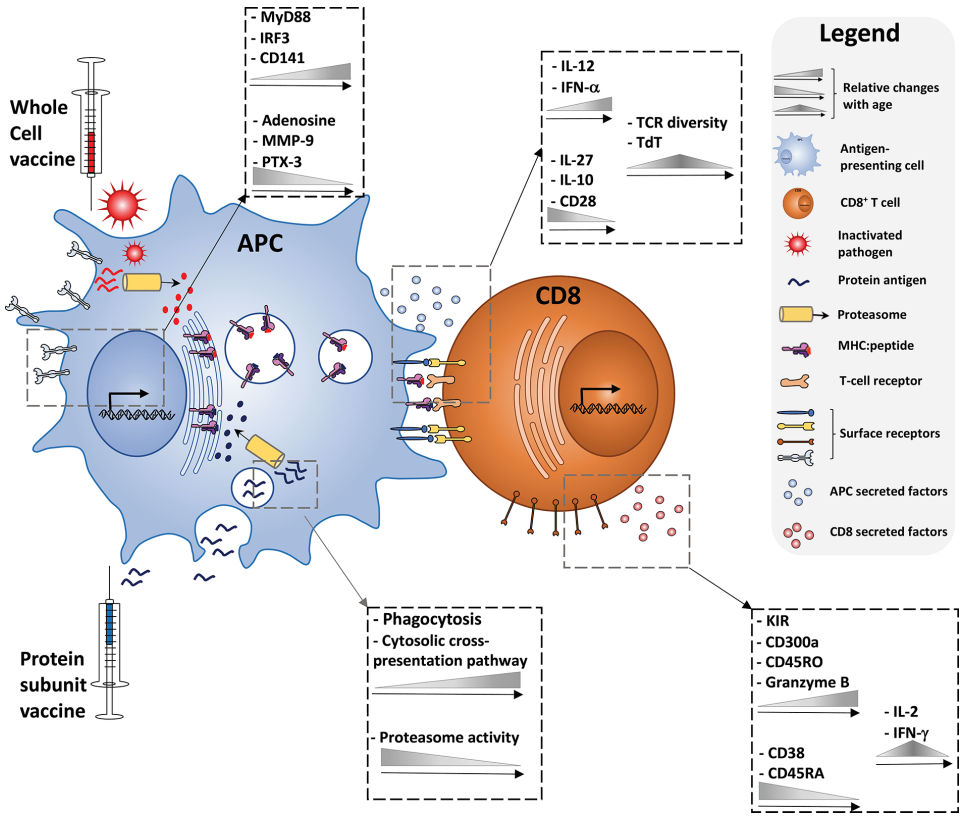


**FIGURE 1** | Immune ontogeny of a vaccine-induced CD8$^+$ T cell response. Starting with recognition of vaccine antigens by APCs (left), up to the effector phenotype of a vaccine-induced CD8$^+$ T cell (right), relative changes with age of key cytokines, receptors, and biological processes discussed in this review are depicted.

presentation of antigens to naive T cells. In human, dendritic cells are divided between two major lineages: conventional DCs (sometimes called myeloid DCs) and non-classical DCs. Based on their phenotypic and functional characteristics, these populations are further compartmentalized into several subtypes (**Table 3**). Each subset is specialized to react to particular pathogens and to interact with specific T cell subsets. In this manner, the immune system can act upon a broad spectrum of several pathogens and danger signals.

Current vaccination strategies take into account the functional specialization of different DC subsets. For example, both the CD1c$^+$ subset (also known as cDC2 DCs) and the CD141$^+$ subset (also known as cDC1 DCs) have potent capacity to induce T cell responses. Where cDC2 cells are predominantly inducers of CD4+ T cell responses, cDC1 cells are uniquely able to cross-present exogenous antigens on MHC I. Interestingly, neonatal cDC1$^+$ DCs reach adult-like levels by mid-gestation (160), and therefore, this subpopulation may be a desirable tool for vaccine development to empower antiviral immunity in early life.

In literature, the chemokine receptor XCR1 is presented as a universal surface marker on cross-presenting DCs (161) in mice as well as humans. This marker is also present on cDC1$^+$ DCs and, therefore, it is thought that XCRI$^+$ DCs are crucial in creating successful adaptive immune responses against viruses (162). In addition, pDCs, which do not express XCR1, are considered to cross-present in humans (144–146). However, the exact role of pDCs in cross-presentation remains controversial (163, 164).

Full-term newborns and adult pDCs display similar frequencies in whole blood, although subset composition between these age groups may differ (165). However, Zhang et al. observed that these differences do not affect the potency of neonatal antiviral responses (166). In contrast, pDCs from preterm newborns have shown an immature morphology and an impaired capacity to produce IFN-α (165).

It should be noted that it is difficult to determine the functional distinctions with age of DC subsets in humans. To study the characteristics of DC types *in vitro*, studies are mainly carried out with moDCs. For neonates, moDCs are generated from umbilical cord blood. One of the limitations thereof is the presence of maternal factors in the content of the blood, which may influence the characterization of neonatal DCs (167). However, due to the convenience of this method, moDCs are the main subset for studying the phenotype and function of DCs.

**TABLE 3 |** DC subsets functions and distinctive markers.

| Subset | Cross-presents? | Function(s) | Distinctive markers |
|---|---|---|---|
| cDC1 | Yes (133) | cross-presentation (134)<br>Necrotic cells uptake (135)<br>Alloactivation (136)<br>Promote Th1 polarization (137) | CD141, XCRl, CLEC9A, CADM1 |
| cDC2-A | Yes (133, 138–140) | Promote Th1/Th17 polarization (141) | CD11c, CD1c, $CD32^+$ |
| cDC2-B | Yes (133, 138, 142) | Promote Th1/Th17 polarization (141) | CD36, CD1c, CD163 |
| Dermal cDC1 | ? | ? | CD141, CD11c |
| Dermal $Langerin^-$ cDC2 | ? | ? | CD1a, CD11c |
| Dermal $Langerin^+$ cDC2 | ? | Promote Th1 polarization, inhibit Th17 cell differentiation (murine model) (143) | Langerin, CD1a, CD11c |
| pDC | Yes (144–146) | Promote antiviral immune responses (type I IFN production) (147, 148)<br>Th2 polarization (149)<br>Pathogenic functions in autoimmunity (148)<br>Tolerogenic functions: can induce suppressive responses by inducing Tregs through IDO expression (147) | CD123, BDCA2, BDCA4 |
| $CD14^+$ DC | No (134, 144, 150, 151) | Tolerogenic functions: Treg induction (152)<br>Th2 polarization (153) | CD209 (154) |
| SLAN DC | ? | Produce Th17-programming cytokines and induce Th17/Th1 cells (155)<br>Promote proliferation, cytotoxicity and IFN-$\alpha$ production by NK cells (156) | SLAN, CD16 |
| IDEC | ? | Th1 polarization, recruitment of inflammatory cells, amplification of allergic-inflammatory reactions (149) | CD1a, CD11c |
| Tip DC | ? | Might be important for immunoglobulin A production (157, 158)<br>Th1 polarization *in vitro* (159)<br>Can stimulate the differentiation and activation of Th17 cells, may participate in tumor rejection (158) | iNos, TNF |

# THE MECHANISM OF ANTIGEN CROSS-PRESENTATION

## The Role of Endocytosis: Soluble Versus Particulate Antigens

Cross-presentation of soluble and particulate antigens is regulated by distinct methods of internalization. Particulate antigens are selectively internalized by APCs through phagocytosis. Subsequently, the antigen can be presented through both MHC class I and II molecules, a time-dependent process in which the NADPH oxidase 2 (NOX2) plays a crucial part. This enzyme is found in professional phagocytes and DCs and contributes to the alkalization of phagosomes by ROS production. NOX2 is recruited to phagosomes with the help of Rab27a and Rac2 (168, 169). Thus, NOX2 prevents phagosome acidification and, consequently, abolishes lysosomal antigen degradation which then allows for cross-presentation (170). This means that when ROS production ceases and the phagosomal pH gets more acidic, the particulate antigen will be preferentially loaded onto MHC class II molecules (171).

In contrast to cell-associated antigens, soluble antigens intended for cross-presentation are internalized by endocytic receptors. Burgdorf et al. describe two different endocytic compartments for antigen processing: early endosomes and lysosomes (172). If a soluble antigen is routed into a lysosome, classical MHC II presentation will take place, whereas antigens in endosomes are targeted for presentation on MHC class I molecules. Depending on the type of endocytic receptor the antigen interacts with upon internalization, the antigen will be sorted into one of the compartments, a process taking place at the plasma membrane (172, 173). Receptors used by DCs to take up extracellular antigens and route these into endosomal compartments include the C-type lectin receptors CLEC9a, DC-SIGN, Mannose Receptor, and DEC-205 (172, 174, 175). Furthermore, molecular chaperones such as heat shock proteins (HSP) can also bind exogenous antigens for MHC class I presentation, through the scavenger receptors LOX1 and SCARF1 (176).

Interestingly, in newborns, monocytes and neutrophils exhibit a reduced ability to bind and ingest particles. This impairment is transient as neonatal phagocytic ability has shown to reach adult-like levels after a few days after birth (177). There are many factors that potentially account for this phenomenon. For example, the chemotaxis of cord blood phagocytes is decreased and Fcγ receptor expression is diminished in early life. Furthermore, newborns show reduced numbers of neutrophils with phagocytic capacity and display poor complement activity (178). Notably, it has been observed that preterm infants with low numbers of neutrophils contain higher phagocytic ability compared to term infants (179).

## The Cytosolic Pathway: Critical Steps in Antigen Cross-Presentation

The cytosolic pathway is characterized by translocation of internalized soluble or particulate antigens to the cytoplasm where they go through degradation by large protein complexes, referred to as proteasomes (59). The way antigens translocate across the endocytic membrane into the cytoplasm is still

debated. It has been suggested that proteins require an unfolding step before translocation (180). However, experimental studies observed enzymatically active proteins in cytosolic extracts, proposing that these proteins do not get unfolded (150).

A common theory is that antigens are transported into the cytosol by sec61, a member of the endoplasmic reticulum associated degradation (ERAD) machinery (181). However, there are papers that have suggested that cytosol export can be independent of sec61 (182, 183). Sec61 has additional functions relating to protein transport across ER and plasma membranes, making it challenging to explore its exact contribution to antigen cross-presentation.

As mentioned previously, low phagosomal pH prevents cross-presentation of particulate antigens. However, it should be emphasized that a slightly acidic environment in the phagosome is required for transportation into the cytosol (180). Particulate antigens can form aggregates and therefore should be processed before transportation. This means that the phagosomal pH should be strictly regulated to prevent antigens from excessive degradation but still be able to deliver them into the cytosol (184, 185).

The involvement of the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) sec22b, located in the ER-Golgi intermediate compartment (ERGIC), as a mediator of antigen export to the cytosol has been described in many papers. However, in recent literature, the role of sec22b in cross-presentation has been questioned (186, 187). Overall, whether sec22b is critical for antigen cross-presentation remains under investigation.

After antigens undergo protein degradation in the cytosol, the proteasome-generated peptides subsequently follow two possible routes: the antigens are transported back into the endosome [1] or into the ER lumen [2], of which the latter only applies to cell-associated antigens (188). The import of peptide fragments into the ER is suggested to occur *via* the transporter associated with antigen processing (TAP). This protein was also found in antigen-containing lysosomes, supporting the hypothesis that peptide loading could also occur inside the lysosomal compartment (189). Indeed, it has been observed that selective TAP deficiency in endosomes strongly impaired the ability for cross-presentation (190). However, TAP-independent pathways also have been described (191, 192). It has been observed that the majority of cytosolic peptides that are being processed TAP-independently are derived from C terminal ends of proteins or N-terminal signal sequences (193). Many proteases are thought to be involved in this process. It should be noted, however, that direct evidence for ER peptide loading is missing. This means that the exact site of peptide loading has not been clarified yet.

If peptides are routed back into the endosomal compartment, efficient cross-presentation requires the translocation of ER proteins to the endosome. ER protein trafficking takes place with the help of sec22b and syntaxin 4, a transmembrane SNARE member present on phagosomes. In this manner, ERGIC molecules such as sec61 and TAP are recruited to phagosomes and endosomes (184). Furthermore, the ER-associated aminopeptidase 1 (ERAP) and the endosomal insulin-responsive aminopeptidase (IRAP) are recruited to trim the antigens to obtain the right size for efficient MHC class I complexing (194).

With regards to newborns, Kollman et al. studied the efficacy of cross-presentation in murine neonatal dendritic cells using soluble ovalbumin (OVA) (195). Their results showed a clear reduction in neonatal MHC class I presentation of the soluble antigen, while antigen uptake in neonates and adults were similar. As OVA cross-presentation is dependent on the cytosolic pathway (180, 196), this evidence implies that the cytosolic pathway may be impaired in early life.

## The Vacuolar Pathway

Unlike the cytosolic pathway, internalized antigens that follow the vacuolar route do not reach the cytosol. Instead, the antigens are thought to be both degraded and loaded onto MHC class I molecules inside the phagosome or endosome. In literature, TAP (in)dependency is mainly used as a determining criterion to distinguish between the cytosolic and vacuolar pathway. However, as mentioned, the cytosolic pathway could also occur without the involvement of the TAP transporter. Besides, research has indicated that cross-presentation of long peptides through the vacuolar pathway can be TAP dependent (197). Therefore, it seems that this distinction does no longer holds ground.

It has been postulated that active proteases, such as the cysteine protease cathepsin S, can enter the endosome or phagosome to process internalized antigens into smaller peptides (198). However, it has been argued that the variety of hydrolases within phagosomes is too harsh for the production of 8-16 amino acid peptides, required for MHC class I loading (199). This argument might not provide sufficient grounds against the fact that there are approximately 15 degradative peptidases and over 50 acid hydrolases localized in the cytosol available for antigen processing *via* the cytosolic pathway (200).

It is not known whether the vacuolar pathway in newborns and children is fully competent. Human neonatal APCs show distinct features in terms of expression of costimulatory molecules, and therefore it has been proposed that these cells require a higher level of activation than their adult counterparts in order to create similar $CD8^+$ T cell responses (201). Considering these data, once a human neonatal APC is activated, it could still be entirely competent to induce an adaptive effector response. In support of this notion, Gold et al. found no defect in human neonatal DCs to process and present particulate antigen and concluded that cross-presentation is fully functional in human newborn DCs. However, as previously described, Kollman et al. observed otherwise (195). It could be proposed that differences between these studies might be due to dissimilarities in engagement of the vacuolar pathway. Another possibility is that these different findings are partially caused by the type of antigenic form used in the experiments. It is known that particulate and soluble antigens have distinct immunologic properties. For example, particulation ensures targeted delivery of antigens to APCs in a more concentrated form and, subsequently, results into an

adjuvant effect (202). Furthermore, the antigen within the particle is exhibited in multiple copies, leading to more robust and persisting cellular responses. In light of the foregoing, it could be possible that the intrinsic properties of particulate antigens offset the mediocre costimulatory support displayed by human neonatal APCs.

There are many other facets of cross-presentation still to be elucidated. For example, it is unknown whether the role of the TAP-transporter and sec22b are age-dependent. Furthermore, animal experiments suggest that proteasome function might be elevated in early life and decline with age (203, 204). In brief, there is an unmet need to conduct research on the MHC I pathway in early life and the age-dependent aspects of this process.

### Important Cytokines

In order to obtain a functional cytotoxic T cell response, the sole presence of antigens is not adequate. Instead, pro-inflammatory cytokines and costimulatory molecules are required to create an inflammatory environment that will activate naive $CD8^+$ T cells. Several cytokine receptors, such as IL-12R and the type I interferon receptor, are essential to activate key transcription factors that support cellular immunity. However, as mentioned earlier, the neonatal immune system demonstrates a characteristic impairment in the production of Th1 polarizing cytokines, such as IFN-α and IL-12p70, which imposes challenges on creating robust and sustained $CD8^+$ T cell responses (29, 205–213). Although cell-intrinsic components contribute to this distinct functionality of newborn DCs, elevated plasma levels of extrinsic factors such as IL-10, adenosine, MMP-9, and PTX-3 (214–216) can also play a role.

IFN-α is a type I interferon (IFN), which is predominantly produced by pDCs *in vivo*. When PRRs such as TLRs and cytosolic RIG-I-like receptors recognize viral proteins, early type I IFN production is initiated. Type I IFNs play a major role in antiviral immunity, as they are capable of upregulating MHC and costimulatory molecules on DCs (205). Besides, through direct $CD8^+$ T cell contact, type I IFNs significantly improve clonal expansion of $CD8^+$ T cells *in vivo* (206). It is well known that type I IFN levels, such as IFN-α, correlate with age. Indeed, newborns infected with RSV show a significant decline in IFN-α production compared to adults (207). It has been postulated that pDC functionality is impaired in newborns and, therefore, shows poor IFN-α induction (208).

Production levels of IL-12 are notably lower in newborns and infants compared to adults (209). Recent work showed that TCR/IL-12 stimulation can enhance expression of genes in newborns that are associated with T cell functions, including cytotoxicity genes and cell signaling genes (210). The ability of newborn dendritic cells to produce IL-12p70 in response to TLR agonists proposedly can be overcome by combined stimulation through TLR4 and Dectin-1 (213). In this study, however, dendritic cells were generated from cord blood monocytes (moDCs) in the presence bovine serum before activation. We have previously demonstrated that the ability of newborn moDCs to produce IL-12p70 is highly reduced by soluble factors present in cord plasma, and impaired Th1 induction was instead overcome independently of IL-12p70 production (29).

Another member of the IL-12 family, IL-27, is a cytokine which consists of both inflammatory and immunosuppressive capabilities. One of its functions is to promote the survival and differentiation of $CD8^+$ T cells, thereby contributing to their effector functions (211). IL-27 secretion by dendritic cells is highest in childhood, while adults' levels are low (212). Interestingly, Il-27 helps drive T helper 1 (Th1) cell differentiation, while newborns are impaired in inducing this type of immune response. The pleiotropic nature of IL-27 could make it difficult to determine its contribution to the impaired Th1 response observed in newborns.

## ADJUVANT-INDUCED CROSS-PRESENTATION

Several studies have described potential mechanisms of cross-presentation induced by clinically relevant adjuvants, such as aluminum, saponin and toll like receptor agonists. The next paragraphs elaborate on the molecular pathways of these adjuvants. However, very little is known about these mechanisms in newborns, and therefore, more research is required in order to comment on potential age-dependent differences between these adjuvants.

### Aluminum-Based Adjuvants

Insoluble aluminum (alum) salts are the most broadly used classical adjuvants in human vaccines (217). Alum is known for its ability to provoke strong T helper 2 (Th2) responses but does not typically enhance $CD8^+$ T cell-mediated immunity.

Alum salts are particulate adjuvants comprised of crystalline structures, which are thought to be central to their adjuvanticity. It has been shown that alum induces the production of uric acid (218). Uric acid can precipitate into crystals of monosodium urate (MSU), which can be phagocytosed by APCs. Phagocytosis of particulate matter, such as alum or MSU, can trigger disruption of the phagosomal membrane, resulting into the activation of the NOD-like receptor protein 3 (NLP3) inflammasome. In addition, alum has also been shown to induce cell death, leading into the release of danger signals like DNA and uric acid. These components are also able to activate the NLP3 inflammasome (218). However, the role of NLP3 in cross-presentation is likely to be limited since NLPR3 is a transcriptional regulator of Th2 differentiation (219). In support of this notion, alum has shown to be capable of inducing a $CD8^+$ T cell response without the involvement of the inflammasome (217).

Interestingly, alum-based nanoparticles in combination with the TLR ligand cpG showed enhanced cross-presentation by DCs (220). With the use of endocytic pathway inhibitors, it was observed that the scavenger receptor A was responsible for internalization of the alum-polymer particles. The nanoparticles were both found in the lysosome and cytosol, indicating lysosomal escape. In addition, both brefeldin A, which

inhibits ER transport to the Golgi apparatus, and MG-132, a proteasome inhibitor, reduced alum-induced cross-presentation in DCs. A potential reason for this enhancement in response could be the involvement of both the cytosolic and vacuolar pathway. This is, because it has been speculated that TLR ligands potentially use the vacuolar pathway (58), while alum-based adjuvants seem to follow the cytosolic pathway. Activating both routes of cross-presentation may enhance MHC class I restricted presentation and, thus, promote $CD8^+$ T cell mediated immunity. There are many other factors that could play a role, such as particle size and manufacturing conditions.

## Saponin-Based Adjuvants

Saponins are triterpene plant glycosides that exhibit different biological and pharmacological properties. There are several saponins that can stimulate the immune system which has led to significant interest in their potential as vaccine adjuvants (221). The most extensively investigated saponin adjuvant is QS-21, a purified fraction from the soap bark tree (*Quillaja Saponaria*) (222).

The molecular composition of QS-21 revealed that its aldehyde group is key in inducing cellular immunity. This is, because it was observed that after reduction of the aldehyde moiety into a secondary amine, adjuvanticity was lost (223). The immune stimulating role of aldehyde-containing adjuvants has been previously described, such as in case of lipidated tucaresol (224). QS-21 is thought to provide a costimulatory signal to the T cell through imine formation from its aldehyde and the primary amine on the T cell, most likely CD2 (221). However, the aldehyde group is not likely to play a role in cross-presentation because tucaresol is not able to induce $CD8^+$ T cell immunity by itself. Furthermore, there are also existing triterpene saponins that lack imine-forming structural groups but still induce cytotoxic T cells against exogenous antigens (223).

Saponin-antigen complexes enter the APC by endocytosis in a cholesterol-dependent way (221). Den Brok et al. proposed that, once the antigen-saponin complex is engulfed by the membrane, MHC class I presentation is induced through lipid body formation (225). As previously described, LBs potentially facilitate antigen export to the cytosol and would therefore play an important role in inducing $CD8^+$ T cell responses. LB formation destabilizes the membrane and, therefore, allows the antigen to escape the endosome early (221). Thus, antigen translocation into the cytosol occurs in a proteasome-independent matter. Indeed, saponin-induced cross-presentation was not compromised by different NAPDH oxidases and several ROS scavengers.

Surprisingly, pharmacological inhibition of LB induction did not reduce antigen export to the cytosol. However, pharmacological and genetic interreference with lipid body formation did abrogate saponin-induced cross-presentation. Thus, LBs might contribute to saponin-mediated $CD8^+$ T cell immunity in a different yet undefined matter.

## TLR-Based Adjuvants

DCs express different subtypes of TLRs on their surface. TLRs recognize various PAMPs and therefore play an important role in immunosurveillance. Increasing evidence shows that TLR signaling is involved in multiple steps in cross-presentation. It was found that TLR activation controls several aspects of phagocytosis like internalization and phagosome maturation. For example, TLR signals accelerate both phagocytosis and phagolysosomal fusion (226). DC activation status plays a critical role in this process. Indeed, it was shown that activation of DCs with TLR3 and TLR4 ligands significantly reduced the uptake and subsequent cross-presentation of particulate antigen compared with immature DCs (227). This phenomenon was not observed with TLR2 and TLR7 ligands. Another potential explanation for this difference is that TLR3 and TLR4 signaling require Trif as essential adapter, whereas the other TLRs operate Trif independent (228).

TLRs may also contribute to cross-presentation *via* MHC I enrichment, a process which is suggested to occur in a phagosome-autonomous way (59). Gupta et al. observed that TLR4 stimulation in murine BMDCs enhanced the recruitment of MHC class I molecules to phagosomes (229). In their work, they showed that these molecules were not derived from the ERGIC machinery, since recruitment of ERGIC components to phagosomes happened in a TLR-independent matter. This also suggests that TLRs are not involved in TAP recruitment, as proposed in literature (190). Instead, they suggested that MHC I molecules are recruited from the endosomal recycling compartment (ERC), regulated by the activity of rab11a. TLRs would manage this process through TLR-MyD88-IKK2-dependent phosphorylation of phagosomal SNAP-23.

Cross-presentation may be further enhanced through TLR mediated antigen export. Antigen transport from the phagosome to the cytosol was increased after TLR4 stimulation with LPS (227). This would suggest that TLR adjuvanticity favors the cytosolic pathway. However, this would not explain the previous described enhancement in MHC class I molecules in the phagosome, which suggests phagosomal loading instead of ER loading. Furthermore, TLRs accelerate phagosome maturation in the first hours after antigen uptake (230). Phagosome maturation in DCs allows antigens to be processed for antigen presentation. In this way, antigen degradation would not include the proteasome and, therefore, it could be argued that TLR ligands follow the vacuolar pathway. However, evidence points to the contrary, as many papers observed that the cytosolic pathway is ruling in TLR-mediated cross-presentation (68, 231, 232). Very little is known about the underlying molecular mechanism of adjuvant-induced cross-presentation in newborns. However, the type and magnitude of CD4 T cell activation by licensed adjuvants often differs, due to distinct signaling requirements in newborn antigen-presenting cells (233–237). To induce cross-presentation in neonates, TLR-adjuvants are interesting candidates for adjuvant application. TLR expression and downstream signaling have been well studied in newborns and although age distinctions have been observed, specific TLR agonists or combinations have been identified that can induce adult-like levels of pro-inflammatory cytokines such as type I IFNs and Il-12, which are important for cross-presentation and are generally not highly produced in

newborn cells. Furthermore, TLR ligands appear to induce a similar degree of polyfunctionality compared to adults (110). However, IRF3 activation by TLR3 and TLR4 is reduced in newborns (238). This process is Trif-dependent and, as described in the previous chapter, TLR3 and TLR4 ligands showed reduced antigen uptake and cross-presentation, indicating that adjuvants stimulating these receptors will not induce cross-presentation in newborns as effectively.

Most TLR signaling is dependent on the adaptor protein myeloid differentiation primary response 88 (MyD88). It has been suggested that MyD88 functioning in neonatal DCs is impaired (239). As described above, MHC class I upregulation may take place in a MyD88 dependent way and, therefore, it could be postulated that TLR-mediated MHC I enrichment in newborns is reduced, possibly resulting in impaired cross-presentation. However, it has been shown that newborn cells can increase MyD88 mRNA expression after bacterial infection (240), and potent nuclear translocation of NF-kB can be achieved using TLR7/8 agonists rather than TLR3 or TLR4 agonists. Whether this would also happen upon viral infection is unknown. Even though alum-adjuvants are probably less suitable candidates in early life, because of their propensity to be Th2 skewing, combinations of alum with TLR adjuvants have shown promise, as described above.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

This paper highlights key differences between the neonatal, infant, and adult immune system and aims to underline that our understanding of vaccine mediated $CD8^+$ induction in early life requires further investigation.

Most commercially available vaccines for pediatric use consist of attenuated or inactivated pathogens. While these vaccines are mostly competent in stimulating $CD8^+$ T cell immunity, modern vaccine development is shifting toward subunit and nucleic acid vaccines and, consequently, has imposed major challenges on inducing adequate cellular immunity. Therefore, subunit vaccines often depend on immune activation by adjuvants. Little is known about $CD8^+$ T cell induction by adjuvants, for example, *via* cross-presentation, in newborns and infants. Adding to the complexity, in early life, many aspects of the immune system correlate with age. Even though neonates and infants have enough naive $CD8^+$ T cells to create a robust antiviral response, they exhibit several functional differences compared to adults that may have direct implications for their ability to cross-present antigens. As a result, their $CD8^+$ T cells have reduced cytotoxicity and are biased toward type 2 immunity. And neonatal APCs receive weak costimulatory stimulation. Altogether, this means that a vaccinated child will produce less pro-inflammatory cytokines important for cross-presentation, does not receive the same stimulation as an adult and shows poor $CD8^+$ T cell effector properties. To overcome these hurdles in the pediatric population, adjuvants should be tailored to their distinct immune system.

Future research should examine whether cross-presentation mechanisms in neonates and infants are fully operational, and aim to identify adjuvants that can induce potent $CD8^+$ T cell responses For example, using adjuvant combinations that employ both the vacuolar and cytosolic pathway or use different mechanisms for antigen export to the cytosol may enhance MHC class I presentation. Furthermore, antigen particulation can boost the adjuvant effect and outbalance poor neonatal APC costimulation. Besides, extra stimulation of cytokines such as Il-12 may enhance neonatal cytotoxicity and, thus, improve the antiviral response.

To date, however, it is unknown how adjuvants contribute to cross-presentation in neonates. For example, do TLR adjuvants also enhance antigen uptake and phagolysosomal fusion in newborns or is this an age-dependent process? Do adjuvants use similar cross-presentation pathways in newborns as they do in adults? Refining our understanding of adjuvant-induced $CD8^+$ T cell immunity will further improve vaccine formulations in the pediatric setting and, hopefully, create more robust and sustained responses to protect this vulnerable population.

## AUTHOR CONTRIBUTIONS

EB performed literature study. EB and SH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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# Host Transcriptomic Response Following Administration of Rotavirus Vaccine in Infants' Mimics Wild Type Infection

*Alberto Gómez-Carballa[1,2,3†], Ruth Barral-Arca[1,2,3†], Miriam Cebey-López[1,2,3], Maria José Currás-Tuala[1,2,3], Sara Pischedda[1,2,3], José Gómez-Rial[1,3], Dominic Habgood-Coote[4], Jethro A. Herberg[4], Myrsini Kaforou[4], Federico Martinón-Torres[1,2†] and Antonio Salas[1,3*†]*

[1] Genetics, Vaccines and Pediatric Infectious Diseases Research Group (GENVIP), Instituto de Investigación Sanitaria de Santiago (IDIS) and Universidad de Santiago de Compostela (USC), Santiago de Compostela, Spain, [2] Translational Pediatrics and Infectious Diseases, Department of Pediatrics, Hospital Clínico Universitario de Santiago de Compostela, Santiago de Compostela, Spain, [3] Unidade de Xenética, Instituto de Ciencias Forenses (INCIFOR), Facultade de Medicina, Universidade de Santiago de Compostela, and GenPoB Research Group, Instituto de Investigacinó Sanitaria (IDIS), Hospital Clínico Universitario de Santiago (SERGAS), Santiago de Compostela, Spain, [4] Section of Pediatric Infectious Diseases, Imperial College London, London, United Kingdom

**Background:** Rotavirus (RV) is an enteric pathogen that has devastating impact on childhood morbidity and mortality worldwide. The immunologic mechanism underlying the protection achieved after RV vaccination is not yet fully understood.

**Methods:** We compared the transcriptome of children affected by community-acquired RV infection and children immunized with a live attenuated RV vaccine (RotaTeq®).

**Results:** RV vaccination mimics the wild type infection causing similar changes in children's transcriptome, including transcripts associated with cell cycle, diarrhea, nausea, vomiting, intussusception, and abnormal morphology of midgut. A machine learning approach allowed to detect a combination of nine-transcripts that differentiates vaccinated from convalescent-naturally infected children (AUC: 90%; 95%CI: 70–100) and distinguishes between acute-infected and healthy control children (in both cases, AUC: 100%; 95%CI: 100–100). We identified a miRNA hsa-mir-149 that seems to play a role in the host defense against viral pathogens and may have an antiviral role.

**Discussion:** Our findings might shed further light in the understanding of RV infection, its functional link to intussusception causes, as well as guide development of antiviral treatments and safer and more effective vaccines. The nine-transcript signature may constitute a marker of vaccine protection and helps to differentiate vaccinated from naturally infected or susceptible children.

**Keywords: biomarkers, RNA-seq, transcriptomics, vaccination, miRNA, rotavirus, machine learning, intussusception**

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*Linda Saif, The Ohio State University, United States*

***Correspondence:**
*Antonio Salas*
*antonio.salas@usc.es*

*†These authors have contributed equally to this work*

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## BACKGROUND

Infectious acute gastroenteritis is one of the major causes of hospitalization in children, with rotavirus (RV) being the most frequent etiologic agent in severe disease (1). RV is also one of the leading causes of infant death in developing countries; it was estimated that RV was responsible for the death of more than 600,000 children per year worldwide before the introduction of vaccines, and 128,000 after the introduction of vaccines in children younger than five years (2–4). As there are no antiviral therapies available, the treatment of RV infection is based on avoiding dehydration and replacing the electrolyte losses of affected children. The development and introduction of RV vaccines have resulted in significant fewer cases of severe gastroenteritis in those countries where RV vaccination is included in the routine schedule (5, 6).

Two different vaccines are licensed in Europe for the immunization against RV: (a) the live attenuated pentavalent human-bovine reassorted vaccine RotaTeq® (RV5, Merck and Co, Inc, Pennsylvania, USA), and (b) the live attenuated human vaccine Rotarix™ (RV1, GSK Biologicals, Rixensart, Belgium) (7). RV5 is composed of a combination of five human/bovine reassorted RV that replicate poorly in the gut (3). RV1 is made from a single human live attenuated strain that replicates easily in the intestine (3, 7). Both vaccines confer protection and have shown real-life effectiveness and impact; however, the exact immunologic mechanism conferring protection against RV gastroenteritis is not fully understood (8). The development of future RV vaccines or the improvement of current formulations is limited by our incomplete knowledge of the mechanisms responsible for RV pathogenesis and the host susceptibility (9). Possible heterologous effects of RV vaccination are also the focus of attention (see (10–12) and references therein). It has been recently reported that RV infection is able to provoke global changes in the transcriptome of infected cells to evade the innate host response; likewise, the host develops mechanisms to avoid viral invasion, including a strong inhibition of glycophorin genes (13).

Despite the importance of these interactions and the burden that RV means to human health, only a few human blood gene expression studies have been published to date (13, 14); none of them have investigated how vaccines influence the blood transcriptome. There is therefore a lack of knowledge on how RV interacts with the host (13) and the mechanism that underlies the acquired immunity after RV vaccination.

To the best of our knowledge, this is the first transcriptomic investigation of RV vaccine response in whole blood, and we present a comparison of vaccinated infants *vs.* wild type RV infected children and age-matched healthy controls.

## METHODS

### Samples and Ethical Approval

The Spanish cohort of 32 western-European children, prospectively collected between 2013 and 2014 at the Hospital Clínico Universitario of Santiago de Compostela (Galicia; Western Spain) (**Figure 1A**) comprised: (*i*) six healthy age-matched controls (with all the vaccines of the Spanish immunization schedule up to date but no rotavirus vaccine), (*ii*) 14 RV5 vaccinated infants, *i.e.*, all the regular vaccines up to date plus three RV5 doses (RV5V group), and (*iii*) six RV infected children required medical attention due to moderate or severe symptomatology (RVinf group) at two different time-points, namely, acute (during medical attendance) and convalescent phases (40 ± 10 days after clinical recovery) (**Table S1**). A blood sample was obtained from these children using a PAXgene RNA tube (PreAnalytiX GmbH). Ages ranged from nearly 2 to 34 months (male/female ratio = 0.77). The mean time elapsed from hospital admission to blood collection in infected children was three days; whereas, in RV vaccinated children the blood sample was taken prior to vaccination and one month after the last RV5 dose. There were no remarkable clinical features in the individuals recruited. A subset of these controls and infected children were previously analyzed in (13). We used RV5 in our study instead of RV1 because it was the only RV vaccine available in Spain at the time of sample collection (2013-2014) (15).

All researchers were specifically trained in the study protocol for patient recruitment, sampling processing, and storage. The study was conducted following the principles of Good Clinical Practice and of the Declaration of Helsinki. Written informed consent was obtained from a parent or legal guardian for each subject before study inclusion. The project was approved by the Ethical Committee of Clinical Investigation of Galicia (CEIC ref. 2012/301).

### Quality Control of Total RNA, Library Preparation, and RNA-Seq

We followed the same quality standards described in Salas et al. (13). Bioanalyzer 2100 and Qubit 2.0 were employed to evaluate the quality and the quantity of the collected RNA. Globin mRNA (which can make up to about 70% of the mRNA in blood) can compromise the detection of other specific mRNAs from leukocytes. We reduced the amount of globin RNA using GLOBINclearTM-Human Blood Globin Reduction Kit (Life Technologies; CA, USA) to obtain a clearer signal from mRNAs from leukocytes. Then, Poly(A) + RNA was isolated on poly-T oligo-attached magnetic beads and chemically fragmented prior to reverse transcription and cDNA generation. The cDNA fragments subsequently went through an end repair process, the addition of a single 'A' base to the 3′ end, and then ligation of the adapters. Finally, the products were purified and enriched with PCR to create the indexed final double stranded cDNA library. High sensitivity assay and quantification of libraries were determined by real-time PCR in LightCycler 480 (Roche). Equimolar pooling of the libraries was performed before clusters' generation. Clonal clusters from single molecule DNA templates were created using *cBot* (Illumina). The *cBot* system isothermally amplifies cDNA fragments covalently bound to the flow cells to create hundreds of millions of clusters, with around ~1,000 identical

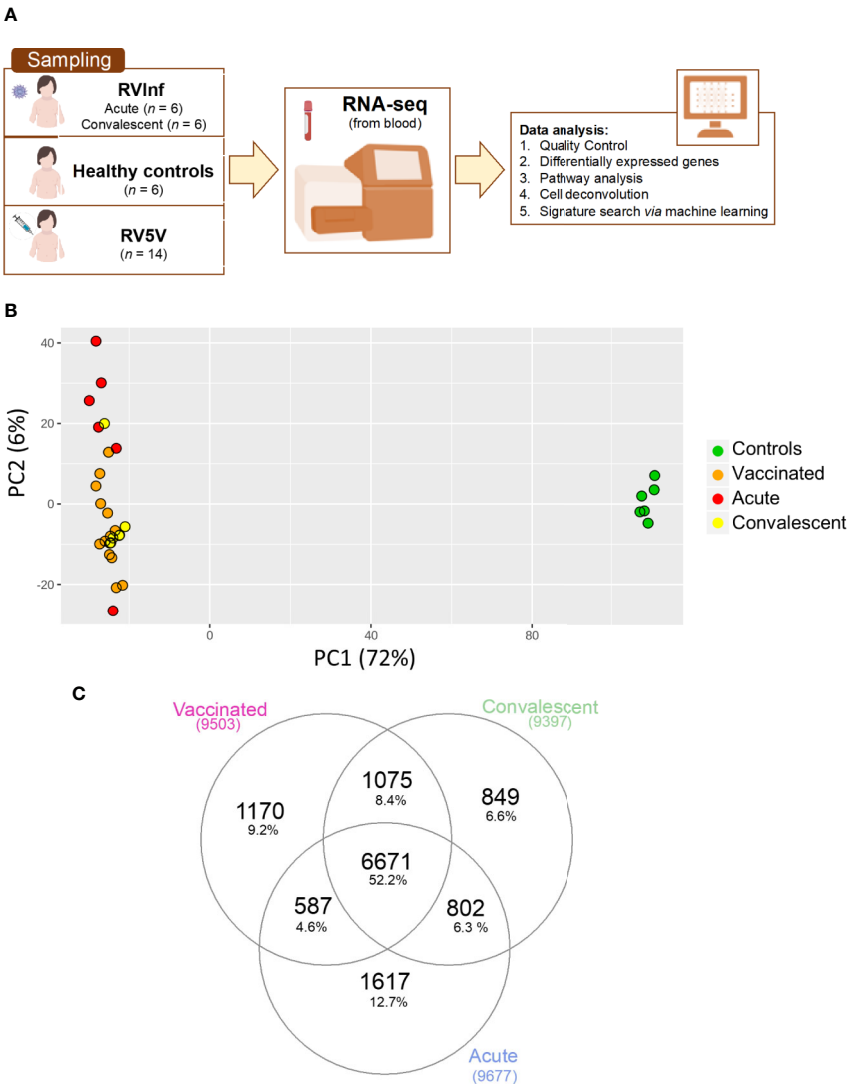


**FIGURE 1** | **(A)** Scheme of sampling and project design; **(B)** Principal component analysis (PCA) built with the top 500 most highly expressed genes; **(C)** Venn plot of the DEGs when comparing healthy controls *vs*. vaccinated children and healthy controls *vs.* acute and convalescent infected (community-acquired) children, corrected by age and gender.

copies of a single template. An Illumina HiSeq 2000 sequencer was used to sequence the pool of cDNA libraries using paired-end sequencing (100 × 2).

## RNA-Seq Bioinformatic Analysis

RNA-seq quality data analysis was carried out following the recommendations described in Conesa et al. (16). We first performed the quality control of the raw data from single samples using *FastQC* (17) to ensure the optimal quality of the reads and avoid potential technical biases due to low quality samples in the dataset which may affect the downstream analysis. Next, FastQC output from single samples were analyzed together using *MultiQC* (18) to create a single report across the samples. Afterward, the whole transcriptome paired-end reads were mapped against the human genome provided by Ensembl v. GRCh37_r87/release 87 using the aligner *STAR* (https://github.com/alexdobin/STAR). We used *STAR* to generate the raw count expression matrix with the number of reads that map to each gene. Normalization of raw data is an essential step to obtain comparable samples, and it is of key importance to accurately interpret the results in transcriptomics. For this reason, we tested different normalization methods with the raw count data using R v3.4.3 (http:/www.r-project.org), including the following: Reads Per Million Mapped reads (RPKM) (19) and Trimmed Means of M values (TMM) (20) both implemented in the *edgeR* package (21); and Conditional Quantile Normalization (CQN) (22) and *Deseq2* (23) using the library *tweeDEseq* package (24).

As all tested normalization methods yielded virtually the same result, we choose the one implemented in *Deseq2* since it is a well-known and popular tool for differentially expression

gene analysis of RNA-seq data. *Deseq2* package was also used to perform the differential expression analysis.

The samples were previously analyzed for their ancestral background in Barral-Arca et al. (25) indicating their main European ancestry, then matching the self-reported ethnicity.

## Statistical Analysis

In order to obtain differentially expressed genes (DEGs) between cohorts we used the Negative Binomial distribution (20) implemented in the *DESeq* package. Gender and age were included in the model as known covariates in order to account for differences in gene expression from age and sex related genes. In addition, we also used the Surrogate Variable Analysis (SVA) method implemented in the *sva* R package to estimate potential hidden and unwanted variation that might be affecting many or all of the genes in the dataset. The surrogate variables obtained from the analysis were also used as covariates in the model to adjust for unknown or unmodeled sources of noise. A generalized linear model was fitted in each cohort, and a t-statistic was calculated for each gene. *P*-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) approach.

We used Principal Component Analysis (PCA) to visualize the global transcriptome patterns of RNA-seq data and to identify outliers. PCA was undertaken using the *DESeq2* R package. In addition, we carried out a Permanova analysis as implemented in the *vegan* R package to assess statistical differences between clusters.

We also carried out an over-representation and pathways analysis using the DEGs obtained from different comparisons through a hypergeometric test that calculates the probability that the proportion of genes within a given function/pathway might be found by chance within our selection of genes. We used two different public databases: (*i*) the Gene Ontology Project [GO (26)], and (*ii*) the Kyoto Encyclopedia of Genes and Genomes or KEGG (27). Ingenuity Pathway Analysis (IPA; https://www.qiagenbioinformatics.com/) tool was used to estimate the most significantly altered pathways and generate networks of biomarkers. Among the DEGs between RV5V and controls, we focused on those reported to be associated with intussusception according to the Disgenet database (28), namely: *STK11*, *PTEN* and *ARID1B*. We also investigated the *APC* gene as it was also reported to be associated to intussusception in the literature (29).

The R package *CORNA* (30) was used to investigate if microRNAs (miRNAs) can be regulating mRNA expression levels between the genes differentially expressed in vaccinated children.

The two-way hierarchical clustering analysis heatmaps of the genes associated with nausea, vomiting, and diarrhea according to Ingenuity® and the genes associated with hsa-mir-149 according to CORNA were generated using hierarchical clustering and the R package *gplots*.

We used a linear discriminant analysis to identify a transcript signature that distinguishes unvaccinated children from vaccinated children using Parallel Regularized Regression Model Search or PReMS (31). The ability of the predicted model to discriminate vaccinated children was assessed by computing the Area Under the Curve (AUC) and the sensitivity, and the specificity at the optimal cutpoint according to the Youden index was calculated with the R package *Optimal Cutpoints* (32). PReMS was initially built splitting the whole dataset into a training set (80% of the samples) and a test set (20% of the samples taken at random).

The performance of the proposed signatures was evaluated using Receiver Operating Characteristic (ROC) curves that represent the true positive rate (TPR) against the false positive rate (FPR) at different threshold cutpoints. ROC curves were built in R using the package *pROC* (33).

Boxplots were built using the R package *beeswarm* (https://cran.r-project.org/web/packages/beeswarm) to represent the total score for the transcript signature in the different groups analyzed. The total score was obtained using the same approach as the described in (34–36) for Disease Risk Score (DRS) calculation.

The proportions of different cell types in peripheral blood may differ naturally, and in consequence, mRNA measurements can vary as well (37). We used the Cell-type COmputational Differential Estimation (CellCODE) (38) method implemented in the R package of the same name, which assigns expression alterations to their cell type of origin with high accuracy, to analyze if there were any difference between the cell-type proportions in the blood of our three groups under study.

The data generated in this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB41347 (https://www.ebi.ac.uk/ena/browser/view/PRJEB41347).

# RESULTS

## RNA-Seq Results

To study the changes experienced in the transcriptome of RV5V and RVinf we performed large-scale expression screening using RNA-seq. A PCA of the whole transcriptome identified one outlier among the acutely infected children, which was eliminated from the followed-up analysis. After eliminating this outlier, the first principal component of the PCA (PC1; accounting for most of the variation, 72%; **Figure 1B**), shows two main significant clusters (Permanova *P*-value = 0.001) separating healthy controls from vaccinated children plus infected children, suggesting that both RV wild type and the vaccine attenuated virus modify the global transcriptome in a similar manner.

We obtained 9,503 DEGs in the vaccinated *vs.* controls comparison, and 8,958 in the infected children (RVinf acute phase and RVinf convalescent phase) *vs.* controls (**Table S2**, **Figure 1C**). It is interesting to note that more than half (~52%; **Figure 1C**) of the DEGs of vaccinated children against healthy controls overlap with those differentially expressed in infected children against healthy controls (**Figure 1C**).

Three out of four genes related to intussusception (*ARID1B*, *APC*, *PTEN, and STK11*) according to Disgenet database were significantly differentially expressed between RV5V and controls (**Figure 2**). The three genes were up-regulated in the RV5V group: *ARID1B* lLog Fold Change [logFC]: 0.76; *P*-value $2.1 \times 10^{-11}$), *PTEN* (logFC: 0.64; *P*-value = $3.7 \times 10^{-5}$), and *APC* (logFC: 1.32; *P*-value = $7.7 \times 10^{-14}$).

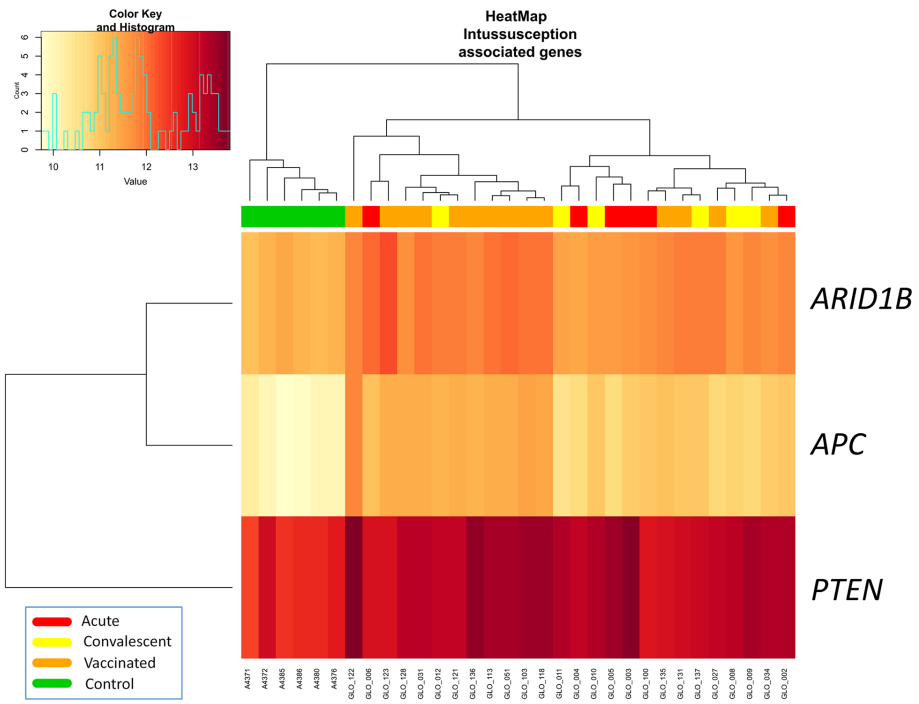


**FIGURE 2** | Two-way hierarchical clustering analysis heat map of DEGs associated with intussusception according to IPA. Each row represents one transcript; each column represents one patient, with a red bar above indicating the sample status red (acute), yellow (convalescent), orange (vaccinated), green (control). Expression intensity is indicated by color (high expression in red; low expression in yellow).

RVinf convalescence samples have a large number of DEGs when compared against controls, showing a persistence of transcriptomic signals even after clinical recovery. It has been recently reported that viruses can stimulate the immune system and affect gene expression during long periods (39).

We detected 480 DEGs when comparing RVinf acute phase and vaccinated group, whereas only 25 were found when comparing RVinf convalescent phase with vaccinated, pointing to a similar systemic transcriptomic pattern generated by the vaccine and the virus in the convalescence phase of the disease. Only 80 out of the 480 DEGs between RVinf acute phase and vaccinated children (**Table S2**) were over-expressed (12 genes with logFC > 2.5), while 400 were under-expressed (logFC < 2.5), suggesting a higher systemic response of patients to the vaccine than to the infection. In addition, among those DEGs with the lowest significant values ($<10^{-3}$) and logFC in the range >|2.5|, all genes but three were found to be under-expressed (logFC values ranging from −2.6 to 4.4), and from these three over-expressed genes, the glutathione S-transferase mu 1 (*GSTM1*) gene has by far the highest logFC value (*P*-adjusted value of $4.07 \times 10^{-5}$ and logFC = 9.8).

When comparing RVinf in acute phase against RVinf in convalescence phase we detected 675 DEGs, all of them over-expressed in acute against convalescence samples. DEGs with the higher logFC (>2.5) correspond to genes that are involved in leukocyte mediated immunity process (GO:0002443; *P*-adjusted value = $7.06 \times 10^{-3}$).

## Pathway Analysis

Analysis of differential regulation using Ingenuity Pathway Analysis® (IPA) showed that many of the DEGs in vaccinated *vs.* healthy children were associated with gastrointestinal disease, inflammatory disease, organ injury and abnormalities (*P*-value = $3.3 \times 10^{-4}$; **Table S3; Figure 3**), including fecal incontinence (*P*-value = $3.1 \times 10^{-3}$; **Figure 3B**), diarrhea (*P*-value = $1.7 \times 10^{-2}$; **Figure 3C**), and nausea and vomiting (*P*-value = $2.7 \times 10^{-2}$; **Figure 3D**). Two-way hierarchical clustering analysis heat maps highlights the differential expression patterns of the genes involves in these pathways between cohorts. Furthermore, IPA also identified a statistically significant over-expression of pathways and genes associated to the humoral immunity component of the adaptive immune system which is responsible for secreting antibodies with respect to controls (**Figure 4**) (*PTPRJ*, *IKZF3*, *TNFRSF1* genes). This result is consistent with the Fisher analysis showing that there is an enrichment in genes associated with the immune system in both comparisons RV5V *vs.* controls (*P*-value [Fisher exact test] = $5.5 \times 10^{-14}$; OR = 2.12) and RVinf *vs.* controls (*P*-value [Fisher exact test] = $8.1 \times 10^{-15}$; OR = 2.20).

In addition, IPA also identified (**Figure S1**) the pathway "abnormal morphology of midgut" (nine genes involved) as significantly enriched in RV5V *vs.* controls (*P*-value = $2.0 \times 10^{-3}$); the heatmap of **Figure S1** shows the differential expression of these genes.

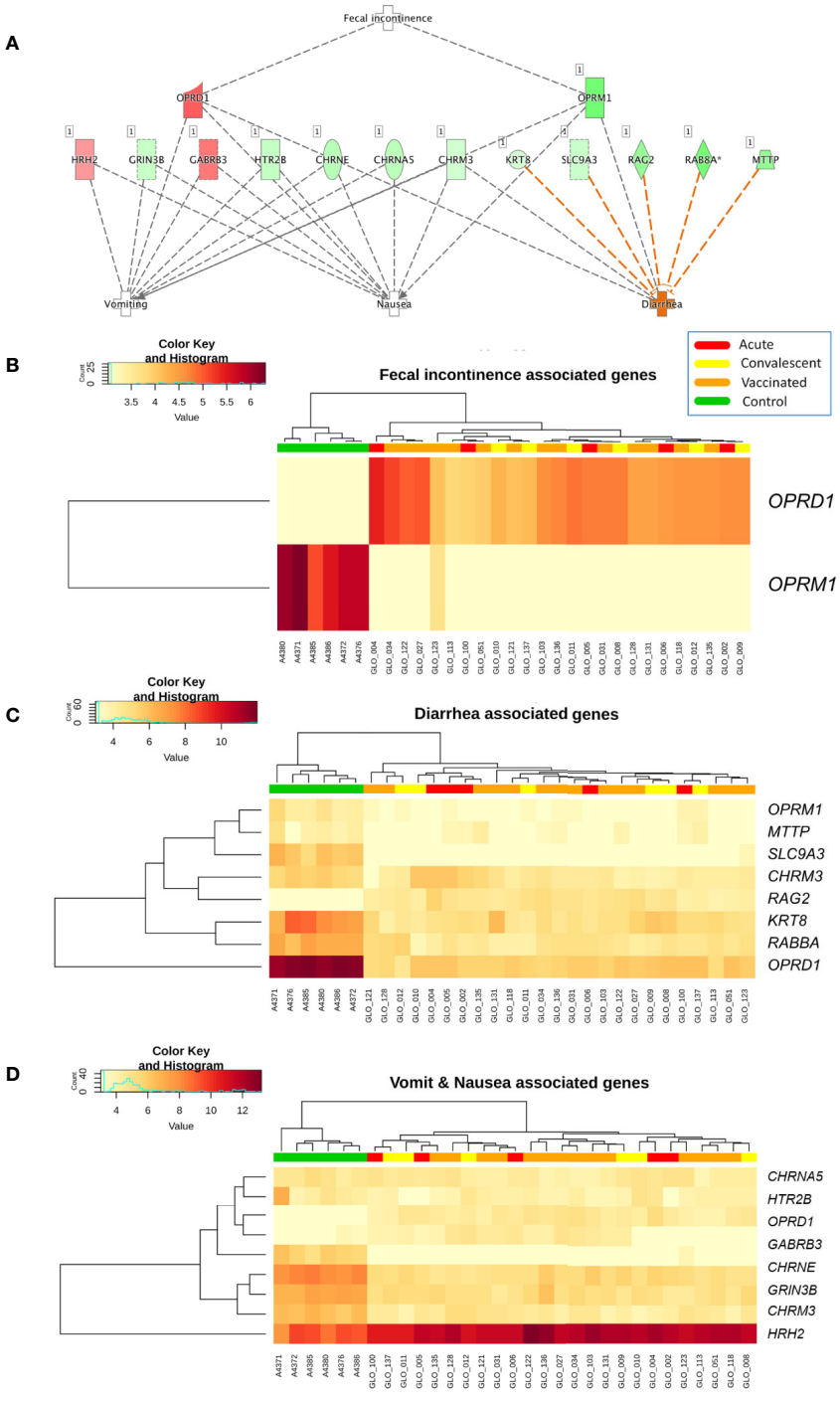


**FIGURE 3** | **(A)** Network of biomarkers associated to fecal incontinence, diarrhea, nausea, and vomiting within the genes differentially expressed between vaccinated and healthy controls according to IPA. The genes in red background are upregulated and the green ones are downregulated; **(B)** Two-way hierarchical clustering analysis heat map of the genes associated to fecal incontinence within the DEGs between vaccinated and healthy controls according to IPA; **(C)** Two-way hierarchical clustering analysis heat map of the genes associated to diarrhea within the DEGs between vaccinated and healthy controls according to IPA; and **(D)** Two-way hierarchical clustering analysis heat map of the genes associated to nausea and vomit within the genes differentially expressed between vaccinated and healthy controls according to IPA. See **Figure 2** for more information on color legend.

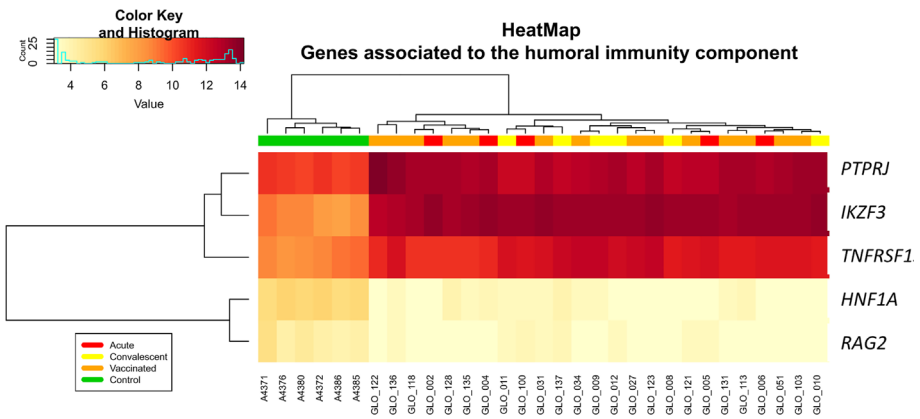


**FIGURE 4** | Two-way hierarchical clustering analysis heat map of genes associated with humoral immunity according to IPA. See **Figure 2** for more information on color legend.

Enrichment of humoral immunity component of the adaptive immune system is also present when comparing RVinf against controls (**Figure 4**).

Overall, the results suggest that the activation of the immune system produced by the vaccine is comparable to the one caused by the wild type infection.

GO analysis indicates the over-expression of biomarkers associated with gastrointestinal injury and abnormalities (**Table S4**), including bacterial invasion of the epithelium (hsa05100, hsa05120) and a noticeable down-expression of genes associated to cell-to-cell adhesion: GO:0007155, GO:0022610, GO:0016337, hsa04540, hsa04530, hsa04520.

Furthermore, the pathway analysis results yielded by KEGG (**Table S5**) and GO ontology (**Table S4**) showed an enrichment of genes related to the regulation of cell cycle (hsa04110, GO:0051726, GO:0007049).

## Cell Deconvolution

Cell deconvolution analysis indicates a statistically significant increase of B and T lymphocytes in vaccinated children compared to controls (CD4T: $P$-value = $8.4 \times 10^{-5}$; CD8T: $P$-value = $2.0 \times 10^{-3}$; B cells: $P$-value = $5.0 \times 10^{-3}$; **Figure S2**), in agreement with the IPA results. (**Table S3**).

The results also indicate that the relative proportion of innate and adaptive immune cells of the infected against the vaccinated children is statistically significant in several cell types (**Figure S2**); in particular when comparing convalescent-infected children against vaccinated.

## MiRNA Enrichment Analysis

The association test for over-representation of microRNA-target between vaccinated children and controls yielded one remarkable result: from the 9,503 DEGs obtained, there were a total of 216 (**Figure 5**) that are targets of the microRNA hsa-mir-149 ($P$-value = $3.7 \times 10^{-2}$; Expectation = 173; Observations = 216). It is worth mentioning that these target genes also showed differences between infected patients and controls in the two-way hierarchical clustering analysis heat maps.

## A Nine-Transcript RNA Signature to Differentiate Vaccinated *vs.* Unvaccinated

We used the PReMS algorithm (31) to create the minimum gene signature able to distinguish between vaccinated and unvaccinated children (including healthy controls, RV acute and convalescent children). The algorithm found a nine-transcript signature (**Table 1**) that allows to accurately separate these three classes (**Figure 6A**). ROC curves indicate that our model differentiates correctly between vaccinated and unvaccinated group as the AUC was 100% for the training set and >90% for the test set (**Figure 6A**). When we look at the individual comparisons (**Figure 6B**) we found AUC values ranging from 100% (95%CI: 100–100) for vaccinated *vs.* RVinf in acute phase patients and vaccinated *vs.* controls, to 90% (95% CI: 70–100) for convalescent children *vs.* vaccinated groups. These latter two classes are more difficult to differentiate using this nine-transcript signature.

At the optimal cutpoint and according to the Youden statistic (−1.9967), the sensitivity was 100%; whereas the specificity was 97% with an AUC of 98% (95%CI: 94–100) when comparing vaccinated against unvaccinated children in the whole dataset.

Boxplot of the total score calculated from the nine-transcript signature in the different groups clearly shows its potential to differentiate between vaccinated cohort and those samples from infected and control subjects (**Figure 6C**).

# DISCUSSION

RV vaccination causes global long-lasting changes in the transcriptome of peripheral blood cells, affecting the expression of more than 9,000 genes. Although the vast majority of children do not experience any adverse effects after vaccination (40), we found altered expression of biomarkers associated with vomit, diarrhea, fecal incontinence, and nausea. This suggests that the vaccine actually mimics a mild version of the disease.

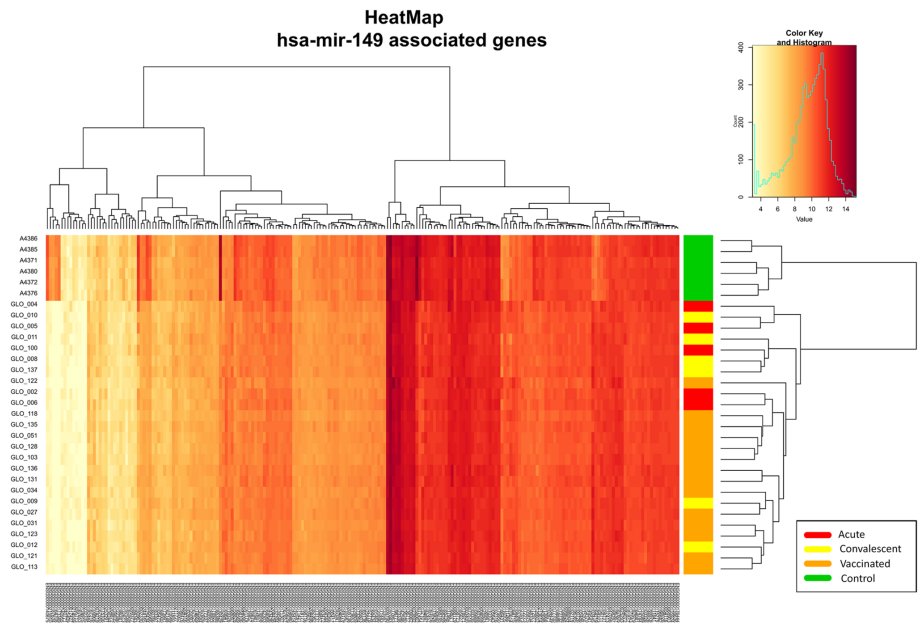


**FIGURE 5** | Two-way hierarchical clustering analysis heat map of the genes regulated by the miRNA hsa-mir-149 within the DEGs between vaccinated and healthy controls. See **Figure 2** for more information on color legend.

Due to the reported association of intussusception and earlier RV vaccines in the past [risk of 1.5 [95%CI: 0.2–3.2] with the first dose according to Yih et al. (41)], large safety studies were conducted on the current vaccines RV5 and RV1 before they were approved. Nevertheless, the link between RV and intussusception remains unclear, up to the point that several studies have not found an increase in intussusception cases after administration of RotaTeq® (42). There is now a general agreement in the medical community indicating that the benefits of RV vaccination substantially surpass the low risk of intussusception that might be associated with vaccination (43). We found that several DEGs between RV5V, and control children have been reported to be associated with intussusception (**Figure 2**) and abnormal morphology of midgut (**Figure S1**); *e.g.* gene *APC*, that is up-regulated in RV5V and RVinf, has been described to play a role in the development of a jejunal adenoma causing intussusception (29). This gene expression pattern may contribute to explain the reported increase of intussusception risk in vaccinated children. These genes could be targeted for the development of future safer vaccines and specifically analyzed in those children experiencing intussusception after vaccination. In addition, we have observed that *GSTM1* gene is among the DEGs with the most significant signal and was found to be strongly under-expressed in comparisons between RVinf acute children and vaccinated group. This gene encodes for a protein involved in detoxification of electrophilic compounds; the glutathione detoxifying system is important in maintaining intestinal barrier protection by attenuating enterocyte death (44). Glutathione S-transferase has been also previously proposed as a potential marker of intestinal epithelial cell damage (45).

**TABLE 1** | Genes included in the nine-transcript signature.

| Ensembl ID | Gene name | Gene | LR coefficient |
|---|---|---|---|
| ENSG00000118113 | *MMP8* | Matrix metalloproteinase-8 | $-7.31\times10^{-03}$ |
| ENSG00000128512 | *DOCK4* | Dedicator of cytokinesis 4 | $5.80\times10^{-03}$ |
| ENSG00000131142 | *CCL25* | C-C motif chemokine ligand 25 | $-5.54\times10^{-02}$ |
| ENSG00000172738 | *TMEM217* | Transmembrane protein 217 | $-9.37\times10^{-02}$ |
| ENSG00000175894 | TSPEAR | Thrombospondin type laminin G domain and EAR repeat | $2.41\times10^{-02}$ |
| ENSG00000196565 | *HBG2* | Hemoglobin subunit gamma 2 | $-6.18\times10^{-06}$ |
| ENSG00000197768 | *STPG3* | Sperm-tail PG-rich repeat containing 3 | $-2.41\times10^{-01}$ |
| ENSG00000198435 | *NRARP* | Notch-regulated ankryrin repeat protein | $-1.63\times10^{-01}$ |
| ENSG00000255423 | *EBLN2* | Endogenous Bornavirus like nucleoprotein 2 | $2.24\times10^{-02}$ |

*Genes with positive logistic regression coefficient values are upregulated in vaccinated children relative to unvaccinated, whereas genes with negative values are downregulated. LR, logistic regression.*

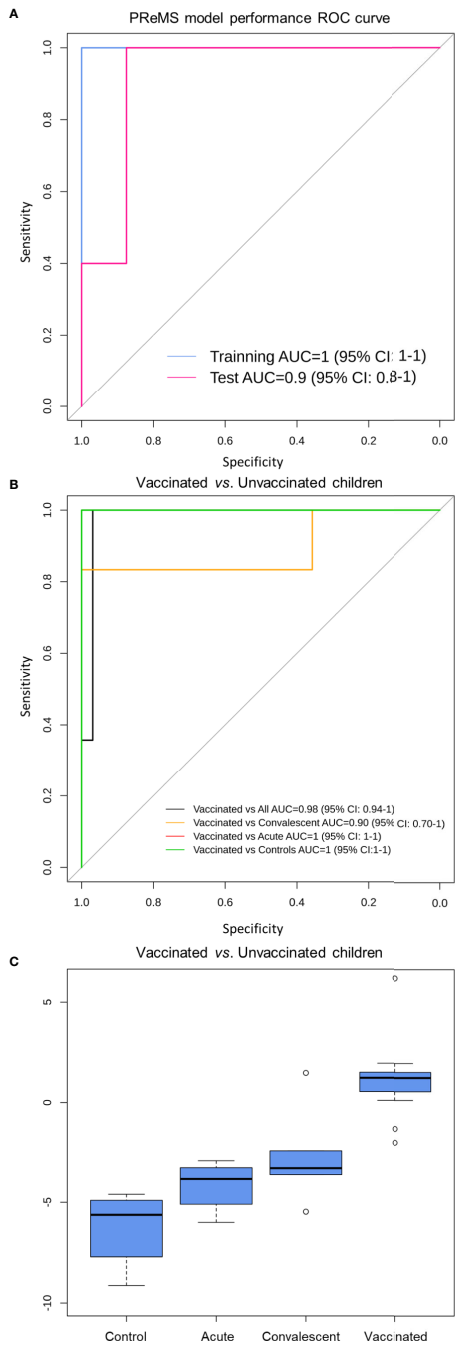


**FIGURE 6** | Classification performance to distinguish RV5 vaccinated group from control children based on a nine-transcript model. **(A)** ROC curve of the model to distinguish between vaccinated and unvaccinated children (including healthy controls, RV acute and convalescent children). The blue curve corresponds to the training set whereas the pink curve to the test set. **(B)** ROC curve of the model to distinguish between vaccinated, controls and convalescent children (note that some curves overlap). **(C)** Box and whisker plot of the model: the horizontal lines in the boxes indicate the median of each group; the lower and upper edges of boxes reflect interquartile ranges, and the whiskers are <1 times the interquartile range. Total score value from nine-transcript signature calculated as in (34–36) is represented in the y-axis.

Children vaccinated against RV over-expressed cell cycle related genes, this mechanism is used by many other viruses to facilitate their replication (46). Transcription of these genes may be a consequence of the increase of B2 lymphocytes observed in RV5V children (**Figure S2**; **Table S3**). As RV5 is a live-attenuated vaccine whose viral particles replicate in the gut, these results are in good agreement with our previous findings indicating that the host cell cycle is affected by RV infection (13, 47).

Previous studies suggested that antibody-based responses are necessary for acute control of RV infection, and for immunological memory (48). We found that vaccinated children have a significant increase in B cell proportion in peripheral blood (IPA analysis [**Table S3**]: $P$-value = $2.7 \times 10^{-2}$; cell deconvolution analysis [**Figure S2**]: $P$-value = $5.0 \times 10^{-3}$). This signal persists for a month after the last dose of the vaccine (the time the sample was taken in vaccinated children), in concordance with the role of B cells in long term protection against RV reinfections. Several studies claim that both B cells and CD8+-T cells play an important role in long term protection against RV reinfection (48–50). Consistently, our results also indicate that vaccinated children have higher levels of T cells (**Figures S2B, C**) compared to the healthy controls. Furthermore, vaccinated children express biomarkers associated with the differentiation of pre-T lymphocytes *CEBPA*, *MYH11*, *RAG2* and T cell receptor signaling (hsa04660) (**Tables S3** and **S5**). Also interesting is the fact that in general the innate and adaptive response of convalescent infected children seem to be more remarkable that the response provoked by the vaccine (see B-cells and natural killer in **Figure S2**); this can be due to (*i*) the stronger impact on the immune system of the wild infection compared to the vaccine, and/or (*ii*) the fact that the sampling time point for convalescent is about 3.7 months while for vaccinated children is roughly 5.2 months. In this time period, we cannot discard the possibility of new infections among convalescents. It is expected however, that such reinfections would modify the transcriptome in the same direction as the transcriptome of acute infected children; actually, this might be the case of one of the convalescent children in the PCA plot (see yellow dot within the cluster of infected children; **Figure 1B**).

Response to RV vaccination is also characterized by an over-expression of genes associated with gastrointestinal disease and inflammation (**Table S3**). RV5, like the RV, has a lytic cycle that burst epithelial cells to liberate the viral particles. Therefore, the presence of those biomarkers in vaccinated children possibly reflects that the intestinal barrier is being compromised due to the attenuated RV virus replication. This hypothesis is also supported by the fact that several pathways associated to cell–cell adhesion (*e.g.* GO:0007155, GO:0016337, hsa04530, hsa04520, hsa04540) are significantly down-regulated in the vaccinated cohort (**Tables S4** and **S5**).

Bioinformatic miRNA target enrichment analysis showed that the expression levels of >200 DEGs between RV5V and healthy controls (**Figure 5**) can be explained by the regulatory effects of the miRNA hsa-mir-149. Hsa-mir-149 is known to target the HIV gene *Vpr* (51) and also RV genes (52). Most recently, it has been

described that hsa-mir-149 is able to significantly reduce polio replication within host cells (53). Further investigation of the relationship between RV and host mirna hsa-mir-149 may elucidate mechanisms of RV pathogenesis.

While RV5 is an oral vaccine containing reassorted RV strains that replicate poorly in the gut, we were able to see its effects in the blood transcriptome. This fact strengthens the hypothesis that RV causes a systematic infection, rather than one limited to the intestine (54, 55).

The PReMS method yielded a nine-transcript signature that distinguished vaccinated and unvaccinated children with an accuracy ~90%. Although the signature shows a good performance in the training and test sets, it would be necessary to validate this signature in an external cohort of vaccinated children. A signature that identifies children who have mounted a successful vaccine response might be of particular interest to detect vaccine failures, to prevent severe RV reinfections, to perform epidemiological control, and to evaluate immune response in the development of new RV vaccines. While the number of transcripts might be too large for a ready to use qPCR assay (36), other technologies would allow to easily test a 9-transcript panel that could be used for epidemiological surveillance or vaccine research purposes.

The present study has a few limitations: *i*) the results were derived from a limited number of subjects, even though the sample size lies within the standard range of transcriptome functional studies (56); *ii*) there is a lack of serological information of patients that might be useful for a more complete comprehension of the transcriptomic findings; *iii*) the results represent a cohort of South-European origin; therefore, additional analysis should ideally be carried out in other cohorts under the assumption that vaccines effectiveness could vary significantly *e.g.* in patients from low- and middle-income countries (57) or when considering other ancestral backgrounds (25); and *iv*) our results were obtained with children vaccinated with the RV5 vaccine (the only one available at the time of sample recruitment); therefore, it would be convenient to explore the impact of other RV vaccines on transcriptome. Finally, we analyzed the transcriptome of peripheral blood samples, away from the principal target of infection on the intestinal epithelium; therefore, it would be of particular interest to compare the impact of RV vaccination on these different tissues.

To conclude, the response to RV vaccination is characterized by the over-expression of genes associated with gastrointestinal disease, inflammation, activation of the immune system and gene over-expression of the cell cycle. Although the alterations of the transcriptome caused by RV vaccination strongly resemble the ones caused by community-acquired disease, there are DEGs that allow accurate discrimination of vaccinated and acute/convalescent infected children. Further research on these differences may help to unravel the molecular mechanisms of immune protection against RV, heterologous effects of the vaccine (58), and key features that allow the development of safer and more effective vaccines and novel antiviral drugs. Finally, we describe a nine-transcript signature/panel able to distinguish vaccinated children from unvaccinated, which may aid in the detection of vaccination failures.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: European Nucleotide Archive, study accession number is: PRJEB41347.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of Clinical Investigation of Galicia (CEIC ref. 2012/301). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

AS and FM-T conceived the study and gave financial support to the project. AG-C, MC-L, MC-T, SP, and J-GR were involved in sampling recruitment and laboratory analyses. AS, RB-A, DH-C, JH, and MK were involved in the data analysis. AS, RB-A, and AG-C wrote the first draft of the manuscript, which was revised by FM, MK, and JH. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.580219/full#supplementary-material

**SUPPLEMENTARY FIGURE 1 |** Two-way hierarchical clustering analysis heat map of genes associated to abnormal morphology of the midgut according to IPA. Each row represents one transcript; each column represents one patient, with a red bar above indicating the sample status red (acute), yellow (convalescent), orange (vaccinated), green (control). Expression intensity is indicated by color (high expression in red; low expression in yellow).

**SUPPLEMENTARY FIGURE 2 |** Box and whiskers plots of the proportion of blood cells according to cell deconvolution analysis. **(A)** Dendritic cells, **(B)** CD4T lymphocytes, **(C)** CD8T lymphocytes, **(D)** plasma cells, **(E)** monocytes, **(F)** natural killer cells, **(G)** B lymphocytes, and **(H)** neutrophils. For clarity, statistically significant values are only given for comparisons between all conditions (acute and convalescent infected and healthy controls) against vaccinated children.

**SUPPLEMENTARY TABLE 1 |** Detailed sample information.

**SUPPLEMENTARY TABLE 2 |** Differentially expressed genes between controls and vaccinated/RV infected according to *Deseq2* corrected by age and gender.

**SUPPLEMENTARY TABLE 3 |** Ingenuity canonical pathway analysis of the differentially expressed genes between controls and vaccinated children. The top diseases and functions are indicated as well as a detailed list of pathways specifically related to gastrointestinal and immunological diseases.

**SUPPLEMENTARY TABLE 4 |** KEGG pathway enrichment analysis of the differentially expressed genes between controls and vaccinated children.

**SUPPLEMENTARY TABLE 5 |** GO pathway enrichment analysis of the differentially expressed genes between controls and vaccinated children.

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# Evaluation of Immune and Vaccine Competence in Steroid-Sensitive Nephrotic Syndrome Pediatric Patients

*Manuela Colucci[1]*, Eva Piano Mortari[2], Federica Zotta[3], Francesco Corrente[4], Carlo Concato[5], Rita Carsetti[2,4], Francesco Emma[1,3] and Marina Vivarelli[1,3]*

*[1] Renal Diseases Research Unit, Genetics and Rare Diseases Research Area, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy, [2] Diagnostic Immunology Research Unit, Multimodal Medicine Research Area, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy, [3] Division of Nephrology, Department of Pediatric Subspecialties, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy, [4] Diagnostic Immunology Unit, Department of Laboratories, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy, [5] Division of Virology, Department of Laboratories, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy*

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**Edited by:**
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*James McCaffrey, Cambridge University Hospitals, United Kingdom*

***Correspondence:**
*Manuela Colucci*
*manuela.colucci@opbg.net*

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Idiopathic nephrotic syndrome is a childhood renal disease characterized by a damage of the glomerular filtration barrier leading to an intense leakage of proteins into the urine. This severe proteinuria causes a transient but strong reduction of serum IgG. Therefore, evaluation of vaccine competence by measuring serum levels of protective antibodies can be misleading in nephrotic syndrome, especially during the active phase of disease. To overcome this issue, in parallel to measuring serum antigen-specific IgG, we quantified by ELISPOT the number of antigen-specific memory B cells induced by previous immunization with tetanus and hepatitis B virus (HBV) in 11 steroid-sensitive nephrotic syndrome (SSNS) pediatric patients at onset before any immunosuppressive treatment (mean age 5.1±0.9 years). Five age-matched children with non-immunomediated nephro-urologic disorders were also enrolled as controls (mean age 6.9±2.3 years). Low total serum IgG levels (<520 mg/dl) were found in all the analyzed SSNS patients. In parallel, median levels of anti-tetanus and anti-HBV IgG were significantly reduced compared to controls [0.05 (0.03–0.16) vs. 0.45 (0.29–3.10) IU/ml and 0.0 (0.0–0.5) vs. 30.3 (5.5–400.8) mIU/ml, respectively; $p = 0.02$ for both], with serum IgG titers below protective threshold in 7/11 SSNS patients for tetanus and in 9/11 SSNS patients for HBV. In contrast, all SSNS patients had a competent B-cell response, showing an amount of total IgG-secreting B cells >1,000 counts/$10^6$ stimulated cells. The amount of anti-tetanus and anti-HBV IgG-secreting B cells was also comparable to that of controls ($p = 0.24$, $p = 0.32$, respectively), with a frequency of memory anti-tetanus and anti-HBV IgG secreting B cells >0.1% of total IgG secreting B cells. In conclusion, SSNS children at disease onset pre-immunosuppressive therapy showed a competent immune and vaccine response against tetanus and HBV, which can be

correctly evaluated by quantification of antigen-specific memory B cells rather than by measuring serum IgG levels. This approach allows early identification of the impairment of immune and vaccine competence, which may derive from protracted use of different immunosuppressive drugs during disease course.

**Keywords: vaccine competence, IgG, pediatric nephrology, steroid-sensitive nephrotic syndrome, immune competence, ELISPOT**

## INTRODUCTION

Idiopathic nephrotic syndrome (INS) is the most frequent glomerular disease in childhood. However, it has a rare incidence (1–17 cases per 100.000 children per year) (1). INS is characterized by a damage of the glomerular permeability barrier, which causes a severe leakage of proteins into the urine, associated with hypoalbuminemia and edema (1). A strong reduction of serum IgG associated with increased serum IgM levels is also frequent during the active phase of disease and sometimes persists also during remission (2, 3). Whether it depends on an impairment of the immune homeostasis or just on the intense proteinuria is debated (2–6). Several T-cell dysregulations have indeed been described both in relapse and in remission (7, 8) and altered levels of memory B cells have been observed already at disease onset, before any immunosuppressive therapy (9). The reduction of protective antibodies observed in INS patients can also be dependent on the prolonged and intense immunosuppression administered in severe forms of the disease, increasing the risk for these patients to develop severe infections (10, 11). At disease onset, patients are treated with a standardized protocol of oral prednisone therapy, to which most patients respond within 4–6 weeks (defined as "steroid-sensitive nephrotic syndrome" patients, SSNS). Within the majority of pediatric patients affected by SSNS, clinical evolution can be extremely heterogeneous, ranging from non-relapsing to severely steroid-dependent forms, which require repeated cycles of steroid therapy and further immunosuppression with one or more steroid-sparing drugs, including anti-proliferative agents, calcineurin inhibitors and B-cell depleting drugs (1). This intense and prolonged immunosuppression can strongly impact immune and vaccine competence in severe forms of SSNS (10, 11).Whether this competence of SSNS pediatric patients is impaired only by the intense and prolonged immunosuppression required to maintain the disease remission or whether the intrinsic immune dysregulation can contribute to this impairment is not clear. Whatever the mechanism behind the lowering of serum IgG titers, this reduction hampers the correct evaluation of the immune and vaccine competence which is usually based on the dosage of total and antigen-specific serum IgG titers.

The aim of this pilot observational study is to evaluate the immune and vaccine competence of SSNS pediatric patients at disease onset, prior to any immunosuppressive treatment. To this purpose, we quantified antigen-specific memory B cells in parallel to the dosage of serum protective IgG.

## MATERIALS AND METHODS

### Study Patients

This monocentric observational study was conducted among INS pediatric patients followed from July 2018 to June 2020 at the Bambino Gesù Children's Hospital, IRCCS in Rome, Italy. The study was approved by our Ethics Committee and was conducted in compliance with the declaration of Helsinki. Written informed consent on behalf of the minors/children enrolled was obtained from parents. All patients at disease onset accessing our clinic in the study period and consenting to participate in the study were enrolled before starting oral prednisone therapy at a standard protocol of 60 mg/m$^2$/daily for 6 weeks followed by 40 mg/m$^2$/every other day for 6 weeks. Patients were then monitored for the response to prednisone therapy and defined "steroid-sensitive" (SSNS) if they responded within 4 weeks by showing negative proteinuria on urine dipstick for $\geq$ 3 days (12). Patients who did not respond to the standardized prednisone therapy within 4 weeks [defined as "steroid-resistant" (12)] were subsequently excluded. Excluding criteria were also chronic infections, previous treatment with immunosuppressive drugs (excluding low dose steroids for periods <3 months), age >18 years. Renal biopsy was considered only for patients $\leq$1 or $\geq$12 years old, sustained elevation of serum creatinine or findings indicative of another immune-mediated disorder (1). Age-matched non immune-mediated nephro-urologic disorders were also evaluated as controls (CTRL). Clinical and demographical characteristics were registered.

### Cell Collection

An additional blood sample to perform the evaluation of immune and vaccine competence was obtained at the first hospital admission for SSNS patients and during routine visits for CTRL. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus (Amersham Biosciences) density-gradient centrifugation and cryopreserved in liquid nitrogen up to analysis.

### CpG Stimulation and ELISPOT

This assay was performed on previously anonymized samples in a blinded fashion in order to minimize a potential bias of data analysis. Immune and vaccine competence were determined by evaluating the ability of stimulated B cells to produce total and antigen-specific immunoglobulins, respectively.

PBMCs were cultured in complete medium at a concentration of $1\times10^6$ cells/ml. Complete medium was prepared as follows: RPMI-1640 (Euroclone), 10% heat inactivated fetal bovine

serum (FBS, Hyclone Laboratories), 1% L-Glutammine (GIBCO BRL); 1% Penicillin/Streptomicin 100× (Euroclone), 1% sodium pyruvate (GIBCO BRL).

Cells were stimulated for 5 days with 0.35 μM TLR9 agonist CpG-B ODN2006 (Hycult Biotech) plus 20 ng/ml rhIL-21 (Milteny) and 20 ng/ml rhIL-4 (Milteny).

For the simultaneous detection of IgM and IgG we used the Human IgG/IgM Dual-Color B Cell ELISpot Kit (R&D System). This kit is designed for the simultaneous detection of total and antigen specific IgM and IgG.

For the detection of total IgG and IgM polyclonal antibodies specific for human IgG and IgM, respectively, were coated onto a polyvinylidene difluoride (PVDF)-backed microplate following manufacturer's instructions. For the detection of antigen specific memory B cells microplate were coated overnight with recombinant hepatitis B surface Ag (HbsAg adw), (Prospec) and with synthetic tetanus toxin peptide (C-term), (OriGene).

PBMCs stimulated for 5 days, as described before, were collected, counted and seeded in the coated plates. Plates were left at 37°C, 2% $CO_2$ for overnight to allow antibody secretion. A total of three 1:2 serial dilutions were done starting in the first well with $2\times10^4$ cells for detection of total IgG and IgM. A total of $2\times10^5$ cells were seeded in the first dilution well (three 1:2 serial dilutions) for the detection of B cells secreting specific antibodies.

After washing, a horseradish peroxidase-conjugated polyclonal antibody specific for IgG and a biotinylated polyclonal antibody specific for human IgM were added to the wells. Following a wash, alkaline-phosphatase conjugated to streptavidin was added and a substrate solution (BCIP/NBT) was added. After washing the BCIP/NBT from the wells with deionized water, an AEC chromogen solution was added to the wells. A red precipitate and a blue-black colored precipitate formed and appeared as spots, with each red spot representing an individual IgG secreting cell and each blue spot representing an individual IgM secreting cell. Plates were left to dry before counting with an ELISCAN (A-EL-VIS).

## Laboratory Analytes

Hematology (serum protein, serum albumin, serum creatinine, C reactive protein) and urinary (protein-to-creatinine ratio) parameters and serum IgG, IgA, IgM, anti-tetanus IgG and anti- hepatitis B virus (HBV) IgG were measured as routine analysis. Normal ranges for serum IgG (520–1,500 mg/dl), IgA (36–320 mg/dl) and IgM (35–155 mg/dl) as well as antibody titers determining sufficient (protective) immunization against HBV (>10 mIU/ml) and tetanus (>0.6 IU/ml) were indicated in the diagnostic laboratory of Bambino Gesù Children's Hospital – IRCCS. Range for antibody titers representing an existing (but not sufficient) immunization against tetanus (0.1–0.6 IU/ml) was also reported (13).

## Statistical Analyses

This is a single center, pilot study. As there is no null hypothesis to test, no formal sample size calculation was performed. Continuous data were expressed as mean ± standard error of the mean (SEM) if they passed normality test (Shapiro-Wilk test), or medians and interquartile range otherwise; categorical data were represented as numbers and percentages. Differences between groups were compared by unpaired *t*-test for normally distributed data or by Mann-Whitney U test for non-parametric data; Fisher exact test was used to compare proportions of patients in different categorical variables. *P*-values<0.05 were considered significant. Analyses were performed through the software GraphPad Prism 6.

# RESULTS

## Patient Characteristics

Twelve INS patients (five males and seven females) at disease onset were enrolled for the current study. One patient who did not respond to steroid treatment within 4 weeks was subsequently excluded from the analysis, which was performed on the remaining 11 SSNS patients. Five age-matched controls (three males and two females) with non-immune-mediated nephro-urologic disorders (one chronic kidney disease, one kidney stone, one kidney hypodysplasia, two nephrocalcinosis with hypercalcemia) were also enrolled. Mean time to remission of SSNS patients was 8.0±0.6 days from starting prednisone treatment. **Table 1** summarizes demographical and clinical characteristics. Mean age was 5.1±0.9 years for SSNS patients and 6.9±2.3 years for CTRL. Only one SSNS patient underwent renal biopsy since he was ≥12 years old at onset and presented a histological pattern suggestive of minimal change disease. As expected, serum protein and serum albumin were significantly lower and proteinuria was significantly higher in SSNS patients compared to CTRL (**Table 1**). Serum

**TABLE 1 |** Characteristics of study patients.

| Parameter | Unit | SSNS at onset (*n* = 11) | CTRL (*n* = 5) | *P*-value |
|---|---|---|---|---|
| **Demographics** | | | | |
| Age | Years | 5.1 ± 0.9 | 6.9 ± 2.3 | 0.38 |
| Male sex | *N* (%) | 5 (45) | 3 (60) | 1.0 |
| **Clinical characteristics** | | | | |
| Serum Protein | g/dl | 4.1 ± 0.1* | 6.9 ± 0.2 | **<0.001** |
| Serum albumin | g/dl | 2.2 ± 0.1* | 4.7 ± 0.1 | **<0.001** |
| Serum Creatinine | mg/dl | 0.3 [0.2–0–4] | 0.4 [0.3–1.8] | 0.11 |
| C reactive protein >0.5 mg/dl | *N* (%) | 0 (0) | 0 (0) | - |
| Urinary protein-to-creatinine ratio | mg/mg | 18.3 ± 4.1 | 0.2 ± 0.1 | **0.01** |
| Time to remission | Days | 8.0 ± 0.6 | - | - |

*Continuous data are expressed as mean ± standard error of the mean or median [interquartile range] and compared by unpaired t test or Mann-Whitney U test, respectively. Categorical values are indicated as absolute count and percentage, compared by a Fisher's exact test. SSNS, steroid-sensitive nephrotic syndrome; CTRL, control group. *All SSNS patients had already received albumin infusions at time of sampling. The bold numbers highlight the significant differences.*

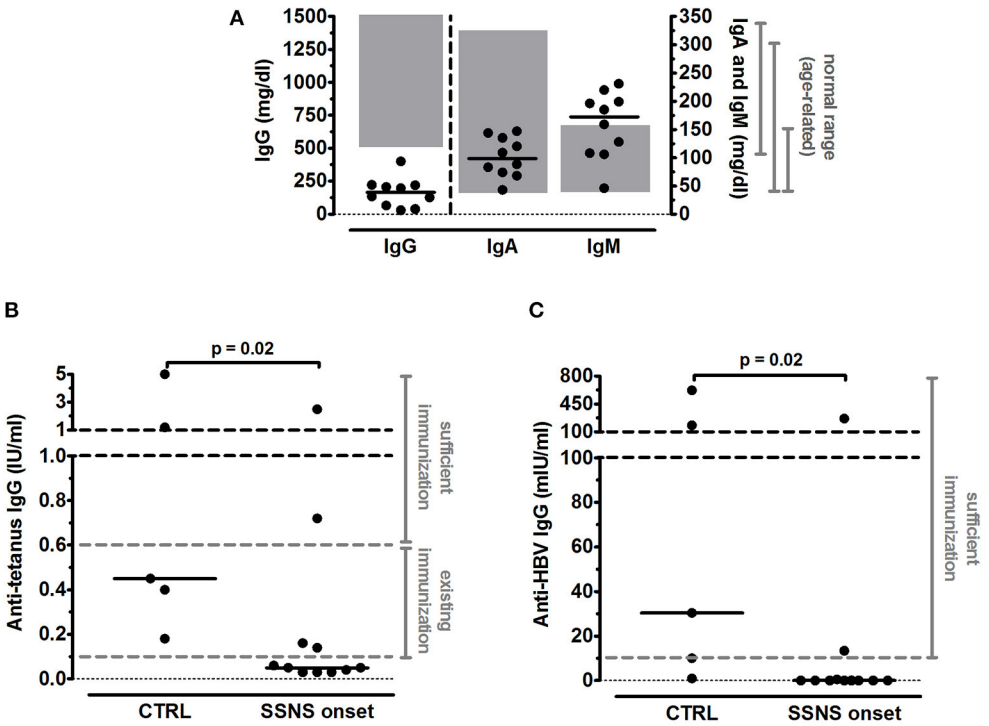


**FIGURE 1 |** Serum immunoglobulin levels in steroid-sensitive nephrotic syndrome pediatric patients at onset. **(A)** Levels of total serum IgG, IgA and IgM were measured in steroid-sensitive nephrotic syndrome pediatric patients at disease onset (SSNS, *n*=10/11) and expressed as mg/dl. In one patient serum immunoglobulin levels were not determined. Each plot represents a different patient. Gray areas represent the age-related normal range as indicated by the diagnostic laboratory of Bambino Gesù Children's Hospital, IRCCS. **(B,C)** Antigen-specific IgG titers against **(B)** tetanus and **(C)** hepatitis B virus (HBV) were measured in SSNS pediatric patients at onset (*n*=11) and in age-matched controls (CTRL, *n*=5) and expressed as IU/ml and mIU/ml, respectively. Protective levels identified by dashed gray lines were indicated in the diagnostic laboratory of Bambino Gesù Children's Hospital, IRCCS. Horizontal lines indicate the medians and differences between groups were compared using the Mann–Whitney *U* test.

levels of C-reactive protein were normal (<0.5 mg/dl) in all patients.

## Serum Immunoglobulin Levels and Immune and Vaccine Competence

All patients were previously vaccinated against tetanus and HBV as per national requirements (14): in the first year of age, all children received three doses of both tetanus and HBV vaccines; a fourth booster dose of tetanus vaccine was administered in 4 SSNS patients and in 2 CTRL who were older than 6 years. Mean time elapsed since last immunization was not significantly different between the two groups both for tetanus (2.5±0.5 years for SSNS patients vs. 4.2±1.2 years for CTRL, $p = 0.13$) and HBV (4.1±0.9 years for SSNS patients vs. 6.0±2.3 for CTRL, $p = 0.38$). Serum IgG were below the age-related normal range (520–1,500 mg/dl) in 10/10 analyzed SSNS patients (mean levels = 164.3±35.0 mg/dl) and serum IgM were higher than age-related normal range (35–155 mg/dl) in 6/10 SSNS patients (mean levels = 157.8±18.8 mg/dl) (**Figure 1A**). In contrast, no alteration was observed in serum IgA levels (mean levels = 101.1±11.2 mg/dl) (**Figure 1A**).

Serum anti-tetanus IgG titers were below the level of sufficient protection (0.6 IU/ml) in 9/11 SSNS patients and below the existing protection (0.1 IU/ml) in 7/11 SSNS patients, respectively (**Figure 1B**) and median levels were significantly reduced in SSNS patients compared to CTRL (0.05 [0.03–0.16] vs. 0.45 [0.29–3.10] IU/ml, $p = 0.02$; **Figure 1B**). In parallel, serum anti-HBV IgG titers were undetectable in 9/11 SSNS patients (**Figure 1C**) and were significantly lower in SSNS patients compared to CTRL [0.0 (0.0–0.5) vs. 30.3 (5.5–400.8) mIU/ml, $p = 0.02$, **Figure 1C**].

In contrast to the reduced levels of serum IgG, SSNS patients showed an intact B-cell memory pool as demonstrated by the competent immune response to polyclonal stimulation (**Figure 2A**). The amount of total IgG-secreting B cells was > 1,000 counts/$10^6$ stimulated cells in all SSNS patients and, despite interpersonal variability, no significant difference was observed as compared to CTRL ($p = 0.78$, **Figure 2A**). In parallel, also a competent memory B-cell response against tetanus and HBV was observed in SSNS patients compared to CTRL ($p = 0.24$, **Figure 2B** and $p = 0.32$, **Figure 2D**, respectively), with a frequency of memory anti-tetanus and anti-HBV IgG secreting B cells >0.1% of total IgG secreting B cells ($p = 0.14$ and p=0.17, respectively, compared to CTRL, **Figures 2C,E**). A competent response was observed also for IgM-secreting B cells (> 10,000 counts/$10^6$ stimulated cells of total IgM-secreting B cells in both groups; $p$=0.99,

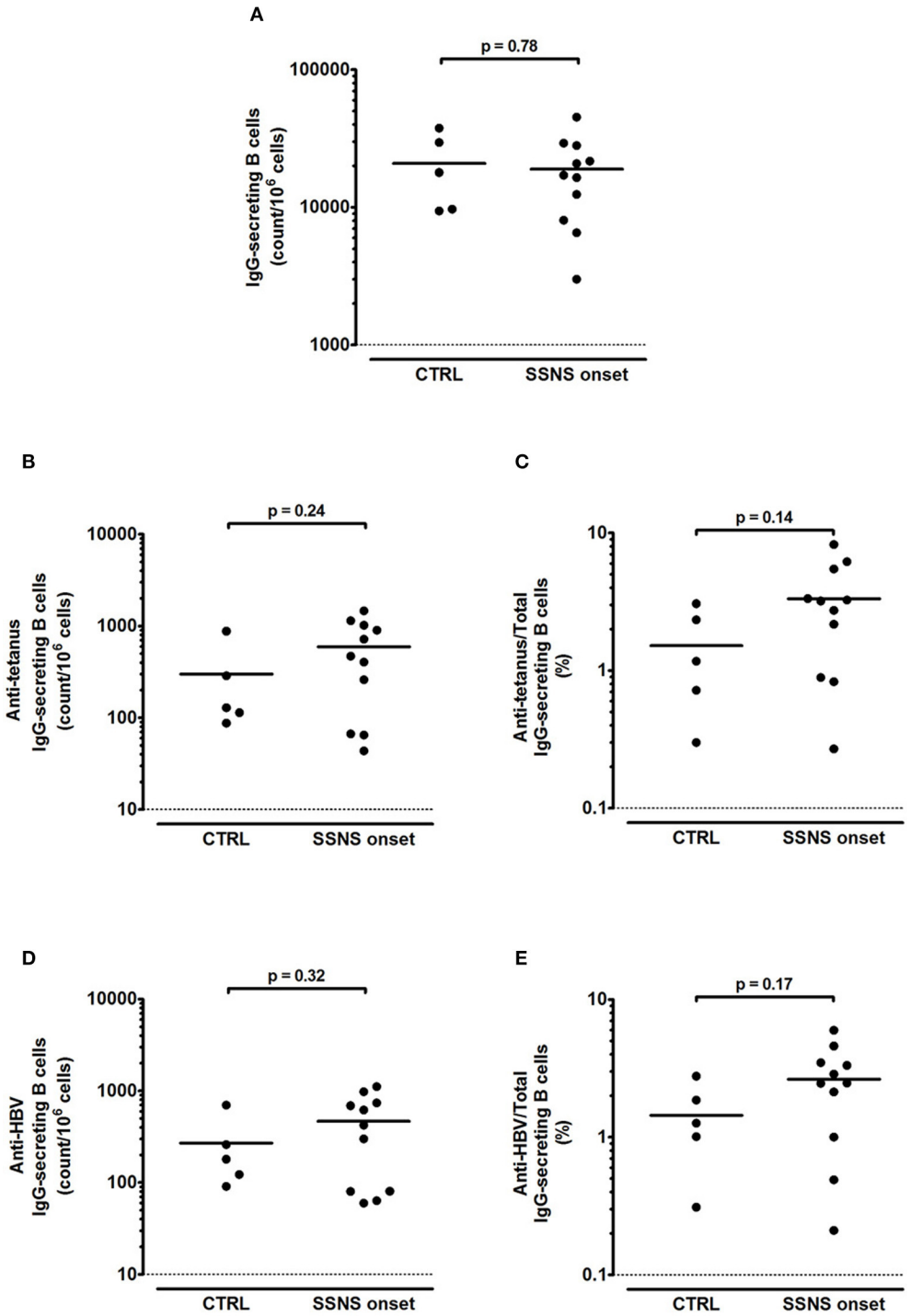


**FIGURE 2 |** Total and antigen-specific IgG-secreting B cells in steroid-sensitive nephrotic syndrome pediatric patients at onset. **(A–E)** Isolated PBMCs were stimulated for 5 days with CpG plus rhIL-21 and rhIL-4. Following stimulation, **(A)** total, **(B,C)** anti-tetanus and **(D,E)** anti-HBV IgG-secreting B cells were enumerated by ELISPOT in steroid-sensitive nephrotic syndrome pediatric patients at disease onset (SSNS, $n$=11) and in age-matched controls (CTRL, $n$=5). Antigen-specific memory B cells were represented as **(B,D)** absolute count/$10^6$ cells and as **(C,E)** percentage of total IgG-secreting B cells. Each plot represents a different patient. Horizontal lines indicate the means and differences between groups were compared using the unpaired $t$ test.

SSNS vs. CTRL), with a frequency of memory anti-tetanus and anti-HBV IgM secreting B cells >4% of total IgM secreting B cells ($p$=0.83, SSNS vs. CTRL for both vaccine-specific responses).

## DISCUSSION

The current study focuses on SSNS pediatric patients at disease onset, prior to any immunosuppressive treatment, in order to investigate the immune and vaccine competence of SSNS patients without confounding effects exerted by an intense immunosuppression steroids, anti-proliferative agents, calcineurin inhibitors and/or B-cell depleting drugs, usually administered in severe forms of SSNS to avoid recurrence of the disease (1). Many reports already investigated the response to previous and subsequent vaccination in INS children and found a reduction of seroprotection induced by previous immunization and an impaired immunogenicity of vaccines administered following the onset of the disease (15–19). However, most of these studies evaluated the levels of vaccine-specific antibodies of INS patients who were under an intense immunosuppression, which can strongly impact the immune response (10). As reported, high-dose prednisone or steroid-sparing agents administered at time of HBV vaccination impair the antibody response (16, 17). In contrast, patients who were vaccinated before starting immunosuppression partially preserve protective titers of anti-HBV IgG (16). However, anti-HBV and anti-tetanus antibodies induced by previous immunization are strongly reduced by a prolonged and intense immunosuppression and by B-cell depletion in INS children (11, 20). B-cell depleting agents are indeed able to efficiently eliminate the circulating memory B-cell subsets, especially in INS patients who received this treatment at an early age (11). Of note, re-immunization following B-cell depletion (after B-cell reappearance) can be effective in restoring vaccine competence in treated patients (11). Another factor that confounds the evaluation of protective antibodies in INS is the reduction of serum IgG that can be dependent on the leakage of immunoglobulins into the urine during the active phase of disease or on an intrinsic immune dysregulation specific of INS patients (2–7, 9). Accordingly, we observed reduced total and anti-tetanus and anti-HBV IgG titers. To overcome this relevant bias, in parallel to the determination of serum vaccine-specific IgG titers, we quantified the number of vaccine-specific memory B cells by an ELISPOT assay. With this approach, we found that circulating B cells in our cohort were highly effective in responding to polyclonal stimulation by producing a large amount of total IgG and IgM. We also observed a competent vaccine-specific memory B-cell response against previous tetanus and HBV immunization. Our study demonstrates that SSNS patients have a competent immune response and a preserved immune memory to previous vaccination against tetanus and HBV at disease onset, before any immunosuppressive therapy.

The main limitation of this study is the limited number of the enrolled patients at disease onset, due to the rarity of the disorder and to the monocentric nature of this pilot study. However, the selection of SSNS patients at onset, prior to any immunosuppression, was necessary to avoid confounding effects of immunosuppressive therapy. More importantly, the experimental approach to quantify the amount of IgG-secreting memory B-cells permitted to overcome the bias of leaked serum IgG into the urine and to correctly evaluate the immune and vaccine competence of the study cohort.

In conclusion, our study demonstrates that SSNS pediatric patients show a preserved immune and vaccine competence at disease onset, which can be efficiently evaluated by quantifying antigen-specific memory B cell response rather than by measuring serum IgG titers. This approach allows early identification of the impairment of the immune and vaccine competence that a protracted use of different immunosuppressive drugs may determine during disease course. Moreover, it overcomes the bias deriving from urinary leakage of serum protein, given that the amount of memory B cells is not affected by proteinuria. Further investigations are necessary to validate our results in a larger cohort of SSNS patients at disease onset and to identify which immunosuppressive drugs affect the vaccine-specific memory B-cell response.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Bambino Gesù Children's Hospital, IRCCS, Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

MC designed the study, performed data analysis, and manuscript preparation. EP performed data experiments, data analysis, and helped with manuscript preparation. FZ helped with collection of study samples, clinical information, and manuscript preparation. FC and CC helped with experiments and data interpretation. RC, FE, and MV helped with study design, data interpretation, and manuscript preparation. All authors approved the final version of the manuscript.

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# Addressing Parental Vaccine Hesitancy and Other Barriers to Childhood/Adolescent Vaccination Uptake During the Coronavirus (COVID-19) Pandemic

*Olufunto A. Olusanya*[1]*, *Robert A. Bednarczyk*[2], *Robert L. Davis*[1] *and Arash Shaban-Nejad*[1]*

[1] Oak Ridge National Laboratory, Center for Biomedical Informatics, Department of Pediatrics, College of Medicine, University of Tennessee Health Science Center, Memphis, TN, United States, [2] Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, GA, United States

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***Correspondence:**
*Olufunto A. Olusanya*
*oolusan1@uthsc.edu*
*Arash Shaban-Nejad*
*ashabann@uthsc.edu*

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Routine childhood immunizations are proven to be one of the most effective public health interventions at controlling numerous deadly diseases. Therefore, the CDC recommends routine immunizations for children and adolescent populations against vaccine-preventable diseases e.g., tetanus, pertussis, diphtheria, etc. This current review sought to examine barriers to pediatric vaccine uptake behaviors during the COVID-19 pandemic. We also explored the implications for parental vaccine hesitancy/delay during an ongoing health crisis and proposed recommendations for increasing vaccine confidence and compliance. Our review determined that the receipt for vaccinations steadily improved in the last decade for both the United States and Tennessee. However, this incremental progress has been forestalled by the COVID-19 pandemic and other barriers i.e. parental vaccine hesitancy, social determinants of health (SDoH) inequalities, etc. which further exacerbate vaccination disparities. Moreover, non-compliance to routine vaccinations could cause an outbreak of diseases, thereby, worsening the ongoing health crisis and already strained health care system. Healthcare providers are uniquely positioned to offer effective recommendations with presumptive languaging to increase vaccination rates, as well as, address parental vaccine hesitancy. Best practices that incorporate healthcare providers' quality improvement coaching, vaccination reminder recall systems, adherence to standardized safety protocols (physical distancing, hand hygiene practices, etc.), as well as, offer telehealth and outdoor/drive-through/curbside vaccination services, etc. are warranted. Additionally, a concerted effort should be made to utilize public health surveillance systems to collect, analyze, and interpret data, thereby, ensuring the dissemination of timely, accurate health information for effective health policy decision-making e.g., vaccine distribution, etc.

**Keywords: vaccine hesitancy, vaccine confidence, vaccine compliance, vaccine disparity, COVID-19, SARS-COV-2, Social Determinants of Health (SDoH)**

## INTRODUCTION

In the United States (U.S.) and globally, routine prophylactic childhood immunizations are established as public health interventions that are most effective and cost-beneficial at significantly preventing numerous infectious diseases and premature mortalities (1). In the pre-vaccine era and before the 1963 measles vaccination programs, there were roughly 6,000 deaths attributed to the measles virus each year (2). Moreover, between 1964 and 1965, an epidemic of the rubella virus resulted in an estimated 2,000 neonatal deaths and 11,000 fetal miscarriages (3). Accordingly, it has been projected that over 100 million cases of vaccine-preventable illnesses i.e. measles, mumps, rubella, pertussis, etc., have been prevented in the U.S. (4). Between 1994 and 2013, an enormous financial burden to the tune of approximately $402 billion and $1.5 trillion was prevented in direct and societal costs (5). Concurrently, within Tennessee, a racially and economically diverse state ranking 16$^{th}$ most populous in the U.S, vaccine-preventable diseases have significantly decreased. This is due to widespread institutional policies to increase vaccine uptake (to meet Tennessee Immunization Program (TIP)'s 90% goal), as well as, wider acceptance of healthcare providers' recommendations (6). Overall, vaccinations continue to serve an essential role in protecting vulnerable individuals from potentially deadly vaccine-preventable illnesses.

Moreover, the scientific community supports the consensus that the highly contagious coronavirus disease 2019 (COVID-19), which was declared a pandemic by the World Health Organization (WHO) in March 2020, can be controlled with an effective COVID-19 vaccine (7). The COVID-19 presents with a continuum of respiratory tract symptoms such as fever, shortness of breath, pneumonia, influenza-like illness, etc., and is caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-COV-2) (8, 9). In the U.S., the COVID-19 has resulted in 28,405,925 incident cases and 511,839 mortalities. Whereas, in Tennessee, 2.7% of total U.S. cases (775,693) and 2.2% of total U.S. deaths (11,421) have been recorded (10). Following mutations in their virus genome, new variants of the SARS-COV-2 have begun to emerge with alterations to their features. Concerningly, these genetic variants may increase disease severity and infectivity as well as change treatment and vaccine efficacy (11). Consequently, this ongoing public health crisis from COVID-19 has had devastating impacts on *every* aspect of human life causing significant morbidity and mortality, adverse psychological outcomes, and growing socioeconomic losses. Additionally, the pandemic has disrupted the hard-earned progress made in the last decade to improve vaccination rates. This current review sought to explore the barriers to pediatric vaccine uptake behaviors (e.g., vaccine hesitancy), as well as, propose recommendations for increasing vaccine confidence and compliance to immunization schedules within the context of the COVID-19 pandemic.

## VACCINATION COVERAGE AMONG CHILDREN (19–35 MONTHS) AND ADOLESCENTS (13–17 YEARS) IN TENNESSEE AND THE U.S.

The CDC's Advisory Committee on Immunization Practice (ACIP) recommends routine immunizations against diseases e.g., measles, whooping cough for children ages through 2 years. For the 78.6 million children born between 1994 and 2013 in the U.S., routine childhood vaccinations have prevented an estimated 322 million illnesses, 21 million hospitalizations and 732,000 untimely deaths from measles (70,748), varicella (68,445), pertussis (54,406), mumps (42,704), and rubella (36,540) (10). Consequently, within the last decade i.e. 2009-2017, coverage for the combined 7-vaccine series[1] among children ages 19-35 months has risen comparably in the U.S. and Tennessee from 44.3% to 72.2% and 44.8% to 79.3%, respectively (12) (**Figure 1**).

Concomitantly, among adolescents, vaccinations are recommended to prevent illnesses such as human papillomavirus (HPV), whooping cough, and meningococcal disease. As portrayed in **Figure 2**, HPV coverage; diphtheria, tetanus, and acellular pertussis (Tdap); and meningococcal conjugate vaccines have steadily increased in the last decade within the U.S. and Tennessee (12). Although, while the prevalence for all specified vaccines varied slightly within a 10%-point range in 2008, HPV vaccination rates have continued to significantly lag behind that of Tdap and meningococcal conjugate vaccines in recent years. While national vaccination rates for Tdap and meningococcal conjugate vaccines have reached or exceeded the HealthyPeople 2030 set-goal of 80% for vaccine coverage among adolescents (13 to 17 years), immunization with the HPV vaccine remains considerably low. See **Figure 2**. Despite current ACIP protocols and improving trends for other vaccines, the 2019 coverage for ≥1 HPV vaccine among male and female adolescents was estimated at 71.5% in the US and 9.6 percentage points lower (61.9%) in Tennessee (13).

## BARRIERS TO CHILDHOOD/ ADOLESCENT VACCINATION UPTAKE DURING THE COVID-19 PANDEMIC

### Impacts of the COVID-19 Pandemic on Vaccination Rates

As part of the efforts to "flatten the curve" and control the rapid spread of SARS-COV-2 during the COVID-19 pandemic, numerous policies and preventive public health measures including shelter-in-place, stay-at-home orders, social distancing, lockdowns, and other quarantine measures, were imposed (13). These precautionary measures, which have disrupted healthcare systems and health personnel services, have ultimately led to sub-optimal vaccine delivery services and vaccination rates (14). In the U.S., after the national emergency declaration, the aggregate count for pediatric vaccine doses procured by the Vaccine-for-Children (VFC) providers substantially declined (15). Similarly, the WHO recorded a 28-year reduction in global coverage for the Tdap

[1] ≥ 4 doses of diphtheria, tetanus toxoid and pertussis, ≥ 3 doses of polio, ≥ 1 measles-containing vaccine, Haemophilus influenza type b full series, ≥ 3 hepatitis b, ≥ 1 varicella and ≥ 4 pneumococcal vaccine.

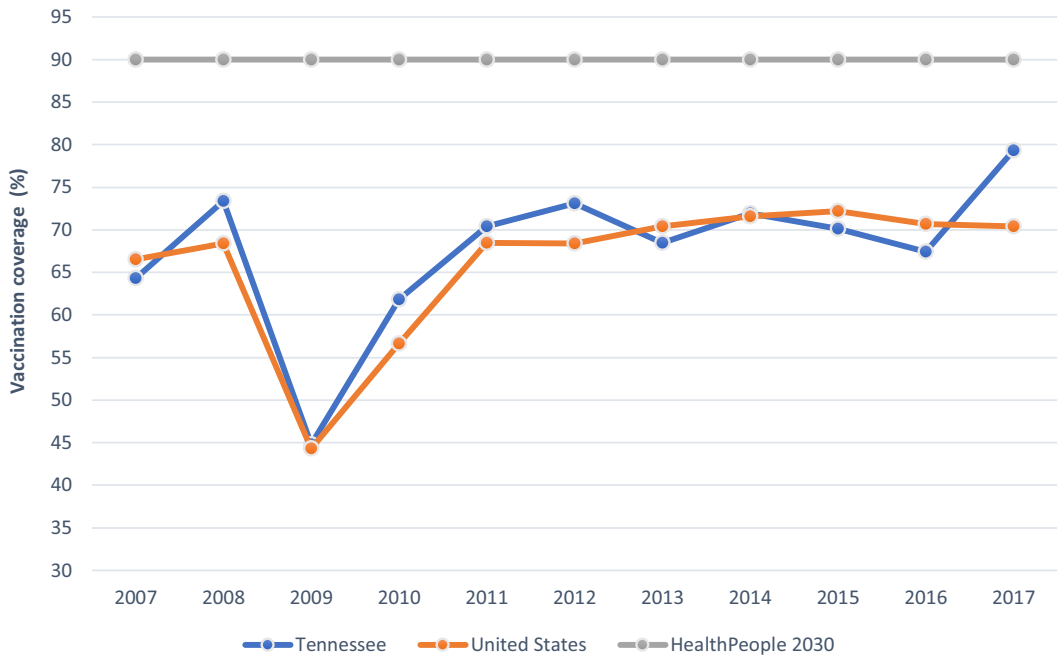


**FIGURE 1** | Combined 7-Vaccine Series Coverage (%) by Year among Children ages 19-35 months in Tennessee and the United States in Relation to the HealthyPeople2030 Goal.Combined 7-vaccine series: ≥4 doses of diphtheria, tetanus toxoid, and pertussis, ≥3 doses of polio, ≥1 measles-containing vaccine, influenza 1b full series, ≥3 hepatitis b, ≥1 varicella and ≥4 pneumococcal vaccine. Data source: National Center for Immunization and Respiratory Diseases. Retrieved October 13, 2020

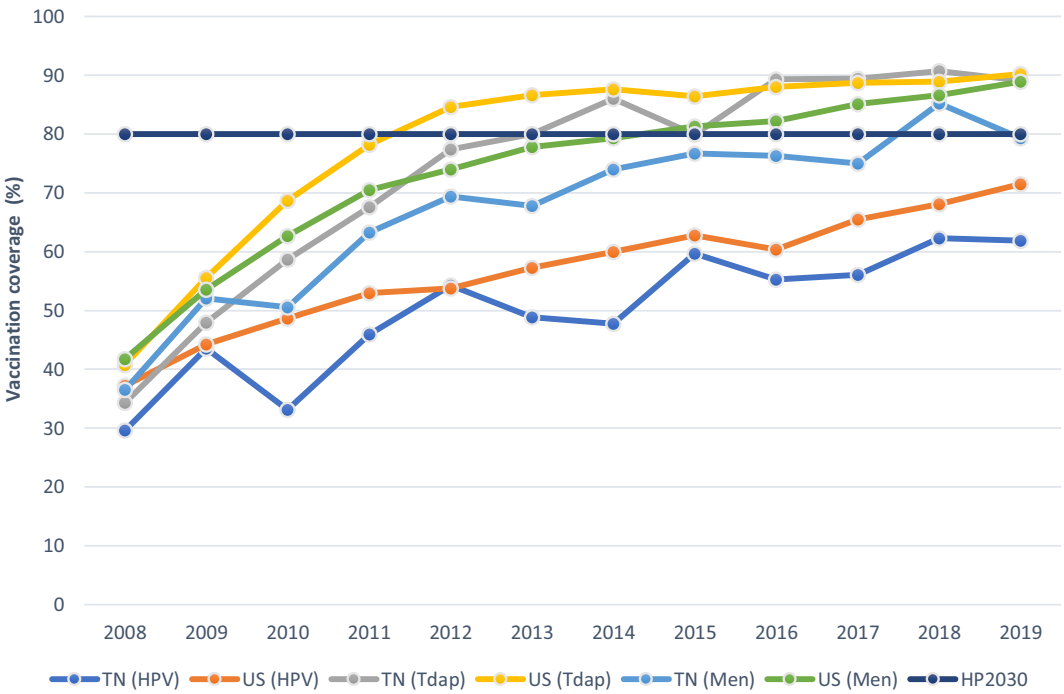


**FIGURE 2** | Estimated Vaccine Coverage (%) by Year among Adolescents ages 13-17 years in Tennessee and the United States in Relation to the HealthyPeople2030 Goal.HPV= human papillomavirus vaccine; Tdap= tetanus toxoid, reduced diphtheria toxoid, and acellular pertussis vaccine; Men= meningococcal conjugate vaccine, TN=Tennessee, US=United States. HPV, Tdap, and meningococcal conjugate vaccinations are depicted as the receipt of ≥1 dose of vaccine Data source: National Center for Immunization and Respiratory Diseases. Retrieved October 13, 2020

vaccine (16). Also, notable was the collective shift in focus on routine vaccinations to respond urgently to the ongoing health crisis (17). These events have been exacerbated by the adverse rippling effects of other COVID-19 pandemic sequelae which include overwhelmed healthcare systems; inequalities in healthcare delivery; financial recession and job losses; worries about vaccine costs; inadequate personal protective equipment for healthcare workers; severe shortages in testing modalities and treatment therapies; long-term school closures; contradictory messages from health agencies/authorities; as well as; disruptions to transportation and travel restrictions. Additionally, parental concerns regarding exposure to the COVID-19 have discouraged individuals who would otherwise have utilized vaccination services, thus, resulting in postponed/canceled medical appointment visits. Moreover, restrictions on routine in-person office visits due to physical distancing protocols have limited health care providers' communications promoting vaccine uptake to patients (18).

Overall, the existing COVID-19 pandemic has forestalled the painstaking but incremental progress made in the last decade to improve uptake for HPV and other vaccines. Disruptions to vaccine delivery services have negatively impacted timely immunizations leaving children/adolescents susceptible to vaccine-preventable diseases. As the United Nations Children's Fund (UNICEF) Executive Director Henrietta Fore aptly describes it, "COVID-19 has made previously routine vaccination a daunting challenge…" (16). An outbreak of vaccine-preventable diseases during the COVID-19 pandemic would only worsen the already strained health care system due to rising hospitalization and death rates.

## Impacts of Social Determinants of Health on Vaccination Rates

In research, individual and interpersonal level approaches have long been utilized to examine and facilitate behavior change. However, this approach is limited as it fails to integrate societal components that influence health outcomes. More recently, factors that facilitate and/or hinder the implementation of health behaviors are addressed using a novel approach - Social Determinants of Health (SDoH) (19). The SDoH encompasses elements in an individual's neighborhood, community, and environment as determined by where that individual is born, resides, learns, works, worships, etc. Consequently, the HealthyPeople 2030 SDoH Framework classifies SDoH indicators into five categories: social and community context, education, economic stability, neighborhood, and built environment, and health and health care (19). SDoH include access to education, affordable housing and health services, public safety, food security, etc. (20).

SDoH are impacted by the distribution of resources that improve the quality of life and public health outcomes. For instance, in the U.S., individuals who reside in certain metropolitan statistical areas (MSA), non-MSA (mostly rural), and without health insurance are disproportionately less likely to be vaccinated (21). Additionally, parental education; household living conditions and income; healthcare access; philosophical and cultural beliefs; religious affiliations; and urban Vs. rural residence, are some of the SDoH that influence childhood vaccination rates (22). In recent times, the COVID-19 pandemic has underscored the importance of incorporating SDoH into health systems and health service delivery. While only a few studies have examined the impacts of SDoH on vaccinations, it is likely the COVID-19 pandemic has exacerbated the adverse effects of some SDoH on vaccination uptake behaviors e.g., employment, poverty, healthcare access, food insecurity, education, etc.

## Impacts of the Vaccine Hesitancy/Refusal on Vaccination Rates

The WHO describes vaccine hesitancy as the, "delay in acceptance or refusal of vaccines despite availability of vaccine services" and categorizes it within the top ten threats to global health (23). This phenomenon also incorporates the antivaccine movement as well as parents' adoption of alternate, non-standardized vaccination schedules. Parent's hesitancy, refusals, and delays in adhering to routine childhood immunizations are largely responsible for a significant number of unvaccinated/under-vaccinated children, disease outbreaks, co-morbidities (e.g., meningitis, pneumonia, HPV-related cancers), as well as, untimely deaths. Vaccine hesitancy and refusal have mostly occurred due to state/local policies that have allowed parents to decline routine childhood vaccinations based on non-medical exemptions (24). These non-medical exemptions occur in the form of religious exemptions e.g., due to an individual's religious beliefs which oppose the use of fetal tissue for vaccines and personal belief exemptions e.g., due to an individual's logical reasoning which disapproves the use of non-natural products for vaccines (24). Prevalence estimates for vaccination exemptions are currently 2.5% and 1.9% nationally and in Tennessee, respectively (25). Currently, in the U.S., 45 states and Washington D.C. permit religious exemptions while 15 states allow philosophical exemptions from childhood vaccinations.

Due to parental concerns on vaccine safety/side effects, some studies which implied a link between the measles-mumps-rubella (MMR) vaccine and autism, played a significant role in vaccine hesitancy and refusal (26, 27). However, Wakefield et al. was retracted due to methodological deficits and data misrepresentation (28). Following larger multiple studies and a wealth of scientific evidence, this hypothesized link was disproved and the safety of the MMR vaccine reinforced (29, 30). Likewise, fears regarding administering multiple vaccines concurrently in a child, unverified sources, and misinformation campaigns from the internet/media have served to dissuade parents from seeking child vaccination services (22). Nevertheless, evidence-focused literature has debunked numerous myths and misinformation citing that recommended vaccines are too many; contain unfavorable ingredients e.g., mercury, aluminum, DNA fragments; damage immune and neurologic systems; and display life-threatening side-effects, etc. (31).

## IMPLICATIONS OF NON-ADHERENCE TO VACCINATION PROTOCOLS

Overall, a major accomplishment of universal vaccine coverage has been to markedly reduce and/or eradicate transmittable diseases that would ultimately have led to premature mortalities in the pre-vaccine era. Despite these advances, however, sporadic outbreaks within communities have continued to occur and coincide with pockets of low community vaccination rates and limited ability for vaccines to elicit immune responses (32). The majority of recent outbreaks have occurred among unvaccinated individuals particularly those exposed to illnesses imported from other countries, as well as those who claimed religious or personal exemptions or had missed immunization opportunities (33, 34).

As a result, outbreaks, incidence, prevalence, and transmission of illnesses e.g., measles virus are seeing an increasing trend in the U.S. (35). Between January and December 2019, there were 1,282 confirmed cases of measles reported in 31 states. This is significantly higher than the 375 cases seen in 2018 and represents the highest prevalence reported since 1992 (36). Moreover, in 2019, almost half of the 14 counties that granted non-medical vaccination exemptions to parents of kindergarten school-aged children (37) experienced the measles outbreak (38). Accounts of other vaccine-preventable outbreaks have occurred for the Haemophilus influenza type B (39) and pneumococcal infections (40).

In addition, adolescents engaging in risky sexual behaviors e.g., multiple sexual partners, and unprotected sexual intercourse, are susceptible and considered high risk for acquiring HPV infections (41). Between 2013 and 2017, there were an estimated 45,300 HPV-associated cancers recorded consisting of cervical (12,143), oropharyngeal (19,775), and anal (7,083) cancers (42). More than 90% of all HPV-associated cancers (e.g., cervical, vulvar, vaginal, and anal cancers) are preventable through receipt of the HPV vaccine (43).

## RECOMMENDATIONS TO RESTORE PARENTAL VACCINE CONFIDENCE DURING THE COVID-19 PANDEMIC

Despite the disruption to health amenities during the COVID-19 pandemic, the continuity of immunization services for children and adolescents is pertinent to enable progress in vaccination trends as well as deter vaccine-preventable diseases and outbreaks. Against the backdrop of the COVID-19 pandemic and vaccine non-compliance/refusal, the pediatrician and other healthcare providers are uniquely qualified to promote vaccinations achieved through the use of strong, presumptive languaging (44) to offer effective, consistent recommendations that emphasize disease/cancer prevention. The healthcare professionals' reluctance to share recommendations that facilitate vaccine uptake could result in parental hesitancy, refusal, and delay. In 2019, national HPV coverage among adolescents with a provider's recommendation (74.7% CI:73.3-76.0) was almost twice as those without one (46.7% CI:43.8-49.6) (21). Concurrently, in Tennessee, coverage was 72.4% (CI:64.3-79.2) for those who received advice from their providers as opposed to those without (28.2% CI:16.6-43.6) (21). This supports the notion that recommendations offered by health providers could significantly predict vaccine uptake, thereby reinforcing the need for personalized patient-provider interactions.

Consequently, interventions and training should empower healthcare providers to disseminate evidence-based advice on vaccines. Specifically, quality improvement coaching such as the CDC's Assessment, Feedback, Incentives, and eXchange (AFIX) program which facilitates provider's education and feedback through face-to-face coaching has been shown to improve immunization rates (45). Also, campaigns should aim to increase providers' self-efficacy and confidence to address parental concerns on vaccine's efficacy, side effect(s), lack of health insurance as well as adopt the use of electronic medical records (EMRs), immunization information systems, and medical practice alerts to remind parents about scheduled regular in-patient visits (46). Parents without health insurance should receive information on reduced out-of-pocket costs and publicly-funded vaccines available through the VFC program (13). For parents with religious or philosophical beliefs, healthcare provider's information on the fewer components of proteins and polysaccharides in vaccines could serve to allay fears (38). For others, communication on societal norms that promote routine vaccination as a social responsibility could increase vaccine uptake (47). Moreover, addressing parental concerns for needle pain, skin reactions, and sensitivity as well as the adoption of motivational interview techniques (i.e. acceptance, compassion, collaboration, etc.) could be impactful (38).

Furthermore, best-practices that facilitate adherence to standardized safety protocols, beneficence, and non-transmission of the COVID-19 should be employed e.g., physical distancing, mask usage, hand hygiene practices, etc. Training and instructions on disease/infection prevention and control should be incorporated into the continuing medical education (CME) curriculum for health professionals (48). Wellness-child visits through telemedicine video conferencing; administering of vaccines through outdoor/curbside/drive-through services; vaccine delivery in alternative settings e.g., pharmacies, schools; minimizing on-site patient visit at any single point in time; delineating specific, well-ventilated rooms for wellness visits, vaccine-only visits, etc., should be implemented to tackle the current COVID-19 health crisis (14). Overall, clinicians should work in synergy with other healthcare team members to maximize scheduled wellness/immunization visits, and other routine medical checkups particularly in places with a low prevalence of health provider's recommendations e.g., rural areas. While mandatory vaccination policies have been shown to be associated with higher vaccine acceptance rates (49), these should be reinforced with patient-provider interactions that address parental concerns. Additionally, nonmedical exemption laws should be reviewed to ensure that in places where they have not been prohibited, there should be in place effective administrative controls so that exemptions do not become easier defaults when compared to vaccinations (50, 51). Government health officials, as

well as, the school districts should continue to enforce and maintain up-to-date immunization records. The catch-up vaccination protocols issued by the CDC to facilitate coverage for children with missed appointments during the pandemic should be implemented (52). Education campaigns should also be tailored to engage local and religious leaders, be culturally appropriate and address specific concerns from vaccine-hesitant populations.

Ultimately, a multifaceted, multidisciplinary approach involving science, engineering, and social sciences should be incorporated to explore facilitators and barriers to childhood vaccine uptake as well as comprehend the drivers for vaccine hesitancy, refusal, and delay. Accordingly, the application of Machine Learning and Artificial Intelligence (53) would be beneficial to (a) identify trends, patterns, and prevalence of childhood vaccine uptake and vaccine-preventable illnesses; (b) investigate psychosocial factors and disparities influencing the receipt of vaccines; as well as (c) examine the interface between vaccine-preventable disease outbreaks and vaccine hesitancy/refusal. Specifically, more concerted efforts should be made to implement *Personal Health Libraries* (54) along with *Public Health Observatories* (55) for vaccine acceptance surveillance (56) on a national scale and within the state of Tennessee. These intelligent tools can facilitate precision health promotion to increase vaccination rates (57) as well as examine causal associations between predictors (e.g., SDoH, COVID-19 pandemic policies, etc.) and outcomes (e.g., vaccine uptake, vaccine hesitancy). In addition to facilitating linkages between healthcare systems, these applications could ensure timely access to accurate health information crucial for effective decision-making regarding vaccine access, allocation services, etc. Health policy-driven changes that address vaccine hesitancy, SDoH inequalities, and disparities in vaccination access would be advantageous. Finally, more research that qualitatively examines barriers to vaccine uptake behaviors, as well as drivers to vaccine hesitancy among specific populations, would be beneficial.

## AUTHOR CONTRIBUTIONS

OO: writing original draft, review and editing, visualization, and conceptualization. RB and RD: review and editing, and conceptualization. AS-N: review and editing, conceptualization, obtained funding, and supervision. All authors contributed to the article and approved the submitted version.

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# Transcriptional and Immunologic Correlates of Response to Pandemic Influenza Vaccine in Aviremic, HIV-Infected Children

*Lesley R. de Armas[1], Varghese George[1], Abdelali Filali-Mouhim[2], Courtney Steel[3], Anita Parmigiani[1], Coleen K. Cunningham[4], Adriana Weinberg[5], Lydie Trautmann[6], Rafick-Pierre Sekaly[7], Mark J. Cameron[8] and Savita Pahwa[1]**

[1] Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL, United States, [2] Department of Neurosciences, CRCHUM University of Montreal, Montreal, QC, Canada, [3] Collaborative Genomics Center, Vaccine and Gene Therapy Institute, Port St. Lucie, FL, United States, [4] Department of Pediatrics, Duke University School of Medicine, Durham, NC, United States, [5] Departments of Medicine, Pathology, and Pediatric Infectious Diseases, University of Colorado School of Medicine, Aurora, CO, United States, [6] Vaccine and Gene Therapy Institute, Oregon Health and Science University, Portland, OR, United States, [7] Department of Pathology, Emory University School of Medicine, Atlanta, GA, United States, [8] Department of Population and Quantitative Health Sciences, Case Western Reserve University, Cleveland, OH, United States

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***Correspondence:**
*Savita Pahwa*
*spahwa@med.miami.edu*

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People living with HIV (PWH) often exhibit poor responses to influenza vaccination despite effective combination anti-retroviral (ART) mediated viral suppression. There exists a paucity of data in identifying immune correlates of influenza vaccine response in context of HIV infection that would be useful in improving its efficacy in PWH, especially in younger individuals. Transcriptomic data were obtained by microarray from whole blood isolated from aviremic pediatric and adolescent HIV-infected individuals (4-25 yrs) given two doses of Novartis/H1N1 09 vaccine during the pandemic H1N1 influenza outbreak. Supervised clustering and gene set enrichment identified contrasts between individuals exhibiting high and low antibody responses to vaccination. High responders exhibited hemagglutination inhibition antibody titers >1:40 post-first dose and 4-fold increase over baseline. Baseline molecular profiles indicated increased gene expression in metabolic stress pathways in low responders compared to high responders. Inflammation-related and interferon-inducible gene expression pathways were higher in low responders 3 wks post-vaccination. The broad age range and developmental stage of participants in this study prompted additional analysis by age group (e.g. <13yrs and ≥13yrs). This analysis revealed differential enrichment of gene pathways before and after vaccination in the two age groups. Notably, *CXCR5*, a homing marker expressed on T follicular helper (Tfh) cells, was enriched in high responders (>13yrs) following vaccination which was accompanied by peripheral Tfh expansion. Our results comprise a valuable resource of immune correlates of vaccine response to pandemic influenza in HIV infected children that may be used to identify favorable targets for improved vaccine design in different age groups.

**Keywords: pandemic, influenza, vaccine, pediatric, HIV, microarray, systems vaccinology**

## INTRODUCTION

It is well established that very young, elderly and immune compromised individuals including people living with HIV (PWH) are at higher risk of influenza infection and related complications, underscoring the need for effective vaccination in these populations (1, 2). The Centers for Disease Control recommends seasonal influenza vaccination for all persons above six months of age (3), but seasonal vaccines have shown modest efficacy (4) and low antibody titers are generated in the elderly (over age 60 years) (5, 6) and in PWH (7). In particular, children and adolescents living with perinatally acquired HIV infection have impaired responses to vaccinations, including influenza vaccination, despite successful viral suppression by combination anti-retroviral therapy (ART) (8–10).

Influenza vaccination confers protection primarily *via* humoral immunity (11, 12). In response to natural infection, neutralizing antibodies are critical for blocking infection while cell-mediated immunity clears the virus (13, 14). Molecular and immunological factors contributing to protection induced by vaccines have been studied amply in recent years. Systems biology approaches have been used to evaluate immune responses to vaccines, e.g. yellow fever (15–17), meningococcus (18, 19), pneumococcal (18, 20) and influenza (21–23) and have been powerful tools for elucidating immunological correlates of vaccine responses. In the context of seasonal influenza vaccination, gene sets related to immunoglobulins, complement proteins, and cellular proliferation are strongly enriched in vaccine responders compared to non-responders 7 days post-vaccination (22). Ex vivo studies show that antibody-secreting B cells exhibit peak proliferation around day 7 post-vaccination (24–26), thereby validating transcriptomic analyses in vaccine biology. Based on gene signatures alone, transcriptomic analysis from pre-vaccination samples across multiple cohorts was used to predict response to influenza vaccination with accuracy above 83% (27). However, the majority of these studies focus on healthy, young adults leaving many questions still unanswered regarding PWH and other immune-compromised populations.

In 2009, the WHO declared the pandemic influenza A H1N1 swine-origin influenza virus a novel strain. Children were found to have no pre-existing immunity to the new strain but older adults (over age 60 years) had some degree of immunity attributed to cross reactivity to past influenza strains (28). A clinical trial (P1088) launched by the International Maternal Pediatric and Adolescent Clinical Trials (IMPAACT) Network evaluated safety and efficacy of a monovalent pandemic H1N1 (pH1N1) vaccine in perinatally HIV-1-infected children and adolescents (29). We utilized a systems biology approach to evaluate gene signatures from peripheral blood before and after pH1N1 vaccination in participants of the IMPAACT P1088 study with integration of serum antibody titer data from the same individuals. Multiple gene set enrichment databases were used to correlate gene expression patterns with antibody titers induced by vaccination and create this resource for this unique patient cohort. In light of the SARS-CoV-2 pandemic beginning in 2019, this study may have further relevance to the study of vaccine responses to novel antigens in children and adolescents living with HIV infection.

## MATERIALS AND METHODS

### IMPAACT P1088 Clinical Study Participants and Immunogenicity Assessments

Specimens from the P1088 clinical trial "Safety of and Immune Response to an H1N1 Influenza Virus Vaccine in HIV Infected Children and Youth", aged 4-24 years (n=40, mean age 13.7 yrs, 17 females and 23 males), were obtained from IMPAACT sites in the United States and Puerto Rico. All participants in the current study were HIV positive and receiving stable ART for at least 90 days before entry and had HIV RNA copies/ml ≤50. Other exclusion and inclusion criteria were described in the original study (29). In the trial, 155 participants received two doses (30ug) of 2009 Novartis influenza A (H1N1) monovalent vaccine separated by 21-28 days, each delivered as two 0.5 ml (15ug) injections into the thigh muscle. This study used blood samples collected pre-vaccination (baseline, BL) and 21-28 days post-first vaccination (visit 1, V1). Blood was processed for PBMC and plasma and an aliquot (2.5ml) was collected in PAXgene tubes and shipped overnight to the Miami IMPAACT laboratory at room temperature. Immunogenicity was determined by specific hemagglutination inhibition (HAI) titers in serum. The HAI assay was adapted from previously described methods (30).

### Microarray Experiments on Whole Blood

Total RNA was isolated using PreAnalytix PAXgene Blood RNA Isolation Kits (Qiagen), globin removed using GLOBINclear Kit (Ambion), and the quantity and quality of the RNA was confirmed using a NanoDrop 2000c (Thermo Fisher Scientific) and an Experion Electrophoresis System (BioRad). Samples (50 ng) were amplified using Illumina TotalPrep RNA amplification kits (Ambion). The microarray analysis was conducted using 750 ng of biotinylated complementary RNA hybridized to HumanHT-12_V4 BeadChips (Illumina) at 58°C for 20 h. The arrays were scanned using Illumina's iSCAN. All microarray data is available under GEO reference number GSE167893.

### PBMC Culture and Flow Cytometry

Cryopreserved PBMC from BL and V1 were thawed and allowed to rest overnight at 37°C in culture medium (RPMI containing 10% FBS and pen/strep). For surface staining: PBMC were labeled with fluorescently-conjugated antibodies to human CD3, CD4, CD8, CD38, CD45RO, CXCR5 and HLA-DR. For 12 hr stimulation and intracellular cytokine staining: PBMC were cultured with or without 5ug/ml H1N1 (A/California/09) for 12hr at 37°C prior to the staining procedure. Data was acquired on BD Fortessa Instrument and analyzed using FlowJo software version 9.7.6 (TreeStar).

## Statistics

Quantile normalization, followed by a log2 transformation using the Bioconductor package LIMMA was applied to process microarrays. The LIMMA package was used to fit a linear model to each probe and perform (moderated) *t* tests or *F* tests on the groups being compared. To control the expected proportions of false positives, the FDR for each unadjusted *P* value was calculated using the Benjamini and Hochberg method implemented in LIMMA. Multidimensional scaling was used as a dimensionality reduction method in R to generate plots for evaluation of similarities or dissimilarities between datasets. For data mining and functional analyses, genes that satisfied a *p*-value (<0.05) with ≥ 1.3 fold change (up or down) were selected. The differentially expressed genes selected based on above criteria were mapped to ingenuity pathway knowledge base with different colors (red: up-regulated; blue: down-regulated). Significance of the association between the dataset and canonical pathway was measured in two ways (1): A ratio of the number of genes from the dataset that map to the pathway divided by the total number of genes that map to the canonical pathway; (2) over-representation analysis where Fisher's test was used to calculate a *p*-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

# RESULTS

## Baseline Molecular Profiles Are Associated With pH1N1 Responsiveness

The primary goal of our study was to identify gene expression signatures that correlated with immunogenicity of pH1N1 monovalent vaccine in PWH of younger age groups. Vaccine response was determined by measuring hemaglutination inhibition (HAI) titers in serum before and after immunization (**Figure 1**). High responders were distinguished by exhibiting a ≥ 4-fold increase at week 3 (visit 1, V1) compared to week 0 (baseline, BL), while low/non-responders failed to increase titer at least 4-fold between these timepoints (31).

To investigate gene expression profiles predictive of antibody response to vaccination, we performed regression analysis using microarray data from BL samples against fold change pH1N1 titers (V1/BL). Two-way hierarchical cluster analysis of the top genes correlating with responder status (**Supplementary Table S1**) divided participants into 2 distinct clusters (**Figure 2A**). Downstream gene set enrichment analysis (GSEA) analyses of differentially expressed genes was performed on participants in the clusters using Ingenuity Pathway Analysis (IPA) and the immunologic signature module from Molecular Signatures Database (MSigDB), the latter of which contains published, manually curated gene sets from the Gene Expression Omnibus (GEO) that represent cell types, states, and perturbations of the human and mouse immune system. We found that the Low/non-responder (LNR) group exhibited pathway enrichment in mitochondrial dysfunction, oxidative phosphorylation, cytokine signaling modulation (LTB, IL-4), macrophage signaling (Fc-gamma receptor-mediated phagocytosis), and EIF2 signaling (stress-related signaling) at BL compared to high responder (HR) group (**Figure 2B**). In MSigDB analysis several gene sets identified were derived from studies with Flu-vaccinated HIV-negative adults (GSE29617) (21) and day 21 yellow fever vaccine responses in human PBMCs (GSE13485) (**Figure 2C**). The gene *BCL21L* encoding the cell death inhibitor protein Bcl-2 like protein was highly enriched in HR. HR displayed gene signatures resembling pre-vaccination signatures from HIV-negative individuals (GSE29617_CTRL_VS_TIV_FLU_VACCINE_PBMC_ 2008). Some genes from this pathway (*ATP5J, UQCRQ, PSMA4, and NDUFB10*) overlapped with IPA analysis as members of the mitochondrial dysfunction and oxidative phosphorylation pathways. Overall, the BL gene expression data suggests that enrichment of mitochondrial or oxidative stress transcriptional pathways at the time of vaccine administration may confer poor responses to vaccination.

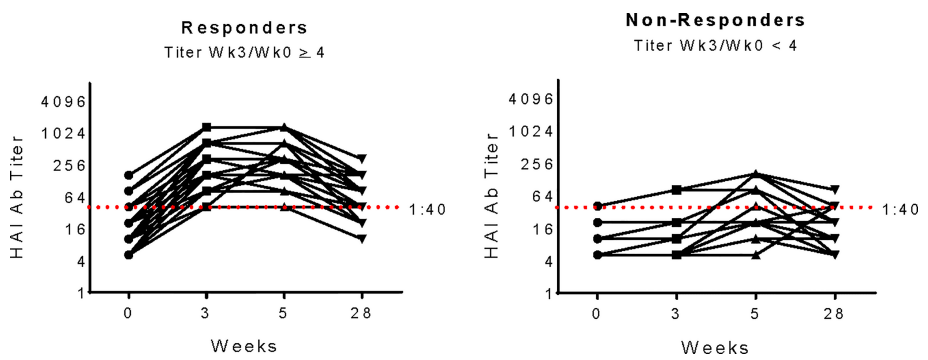


**FIGURE 1** | pH1N1 Serology for Study participants. pH1N1 Ab titers as determined by Hemagluttination Inhibition Assay (HAI) in study participants from Responder and Non-responder groups at each timepoint in the IMPAACT P1088 study. Responders (n=29, left panel) were defined as exhibiting a 4-fold increase at week 3 compared to week 0 and Non-responders (n=11right panel) failed to increase titers at least 4-fold between week 0 and 3. The red dashed line at titer 1:40 shows the accepted threshold for sero-protection.

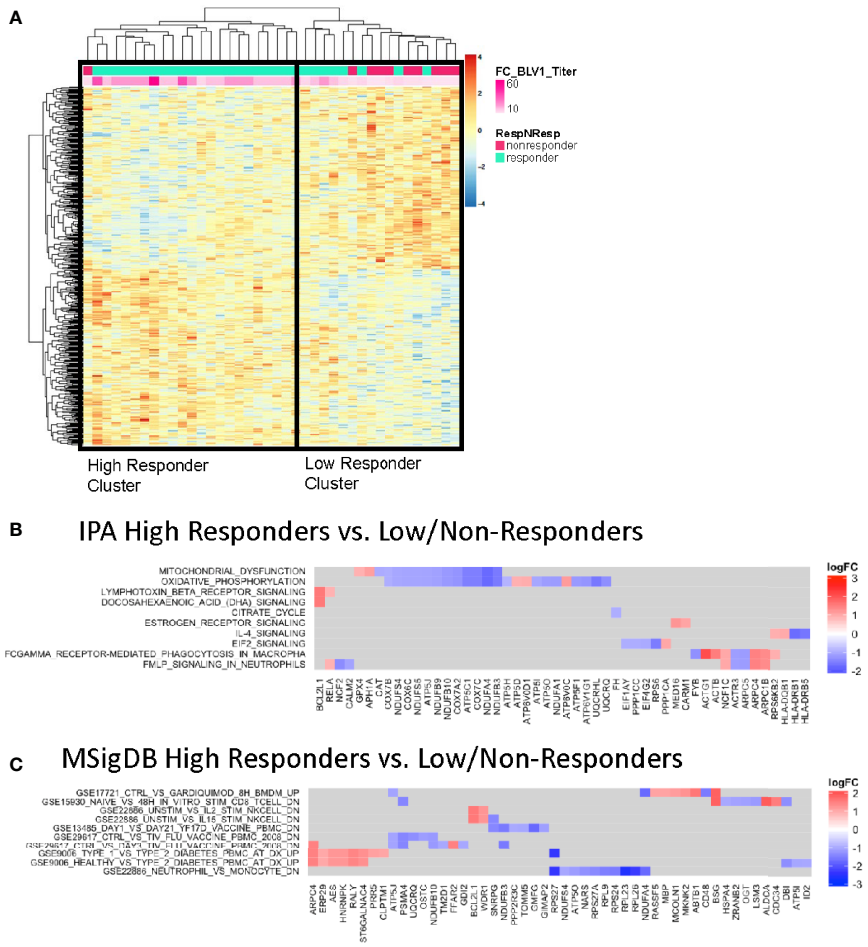


**FIGURE 2** | Baseline Enriched Molecular Pathways Associated with Antibody Titer Response. **(A)** Heatmap representation of the top 1,000 significantly correlated transcripts by regressing baseline gene expression from all participants against V1 pH1N1 titer. The expression intensities are represented using a blue-white-red color scale. Rows correspond to probes and columns correspond to profiled samples ($p \leq 0.01$). High responder and Low/non responder clusters were compared and subjected to gene set enrichment analysis using the IPA database **(B)** and MSigDB immunologic signature model **(C)**.

## Post-Vaccination Molecular Profiles Associated With pH1N1 Responsiveness

Given that pH1N1 was a novel antigen in the P1088 study cohort, participants received a boost at 21-28 days after the first vaccination. We investigated molecular signatures at this timepoint using differentially expressed gene profiles from PBMC at V1 prior to the boost vaccination. Regression analysis was performed and supervised based on the fold change in pH1N1 titer (V1/BL), as in **Figure 2** (top genes listed in **Supplementary Table S2**). This analysis also generated two clusters with one containing all non-responders and some low responders and the other with high responders (**Figure 3A**). IPA analysis of the top correlating genes revealed lower expression of the activation marker *CD69* (as a member of the 'Crosstalk between Dendritic cells and Natural Killer cells' pathway) and *LY96*, whose protein associates with TLR4 to respond to LPS in HR compared with LNR (**Figure 3B**). MSigDB analysis (**Figure 3C**) showed that expression of multiple IFN-inducible genes (*IFI16, IFI27, IFI44, IFI44L, IFIT1, ISG15, OAS1, OAS2, MX1*) were higher in LNR. These genes are upregulated in PBMC during acute viral and bacterial infections (GSE6269) (32).

## GSEA Using Cell-Type Specific Gene Database

A caveat of systems biology approaches using whole blood samples is the inability to evaluate the contribution of specific cell populations to the observed transcriptomic profiles. We employed a cell-type specific database for gene set enrichment (21) to clusters identified in previous analysis that associated with Responder groups at each timepoint. Gene signatures related to B cells, NK, and monocytes were enriched in HR at BL, while DC subsets and T cells were enriched in LNR (**Figure 4A**). At V1, DC subsets were enriched in HR along with B cells, NK, and monocytes, while T cell signatures remained enriched in LNR (**Figure 4B**). Monocyte-associated gene expression

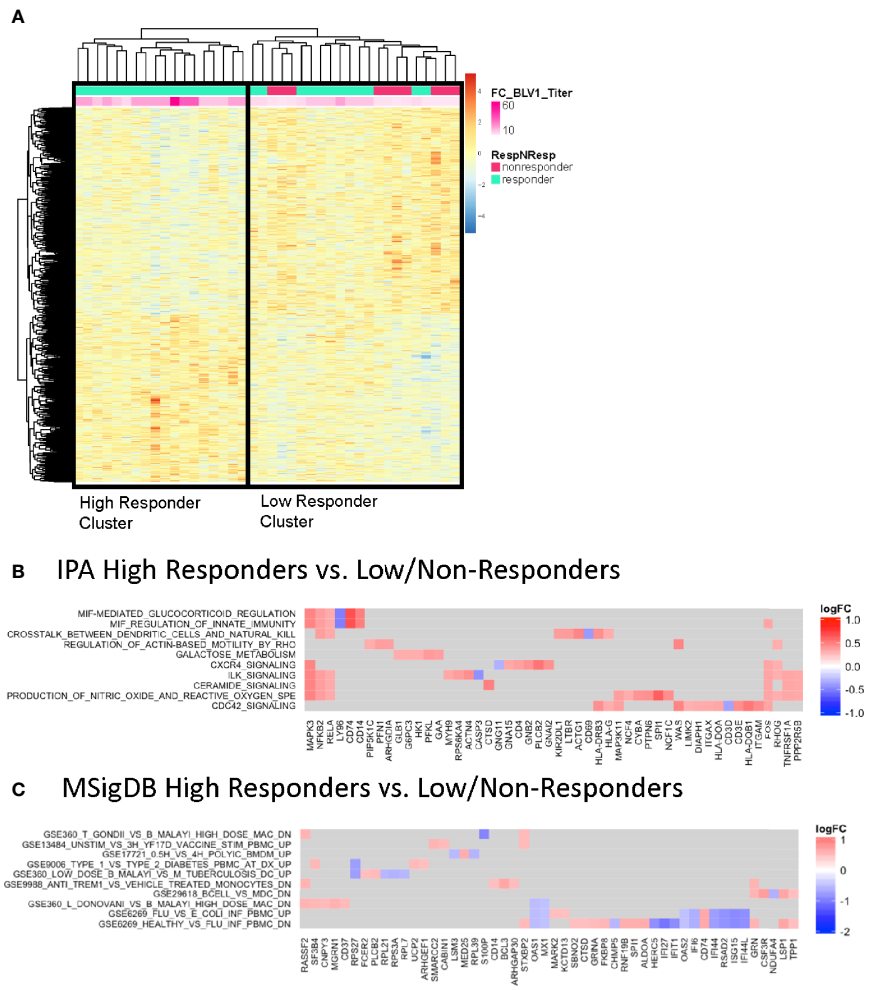


**FIGURE 3** | Correlates of Vaccine-induced Antibody Responses Post-Vaccination. **(A)** Heatmap representation of the top significantly correlated transcripts from regressing baseline expression from all participants and fold change difference in pH1N1 antibody titers at V1 compared to BL. The expression intensities are represented using a blue-white-red color scale. Rows correspond to probes and columns correspond to profiled samples ($p \leq 0.05$). "High" responder and "low/non" responder clusters were compared and subjected to gene set enrichment analysis using the IPA database **(B)** and MSigDB Immunologic signature model **(C)**.

accounted for most of the observed genes and showed enrichment at BL and V1 in HR (**Figures 4C, D**, respectively). This signature shared multiple genes (*ACTG1, ALDOA, ATP6V06, CD151, CSF3R, CTSD, FKBP8, GRN, MARK2, SLC6A10P, TSPO, TYMP, and UBXN6*) between the two timepoints (**Figure 4B**).

## Pathway Analysis by Age of Study Participants

The P1088 study enrolled participants representing a broad age range from 4 to 24 years old, however age was not associated with Responder status (29). In the subset of participants analyzed by microarray, there was similarly no correlation between age and fold change of antibody titers (r=0.015). However, we reasoned that puberty may affect gene expression profiles in HIV-infected children and adolescents and therefore divided the donors into two age groups for further pathway analyses: 4-12 years ("children"; n=16) and 13-24 years ("adolescents", n=24). For this analysis individuals were compared in each age group based on responder status: fold change of ≥ 4 were considered high responders (HR) and < 4 were considered low/non-responders (LNR). IPA analysis of gene expression at BL revealed a group of molecular pathways that were induced in both age groups (e.g. age-independent) as well as age-dependent pathways for each age group (**Figure 5**). Age-independent pathways enriched in HR were related to metabolic pathways (Pentose Phosphate-Oxidative branch, Aryl hydrocarbon receptor, Vitamin D/Retinoic acid receptor), and cell survival and protein synthesis pathways (PI3K/AKT, eIF4 and p70S6K, mTOR) confirming data from regression and cluster analysis in **Figure 2**. HR in the adolescent group demonstrated enrichment in more pathways than children including numerous pathways involved in cell

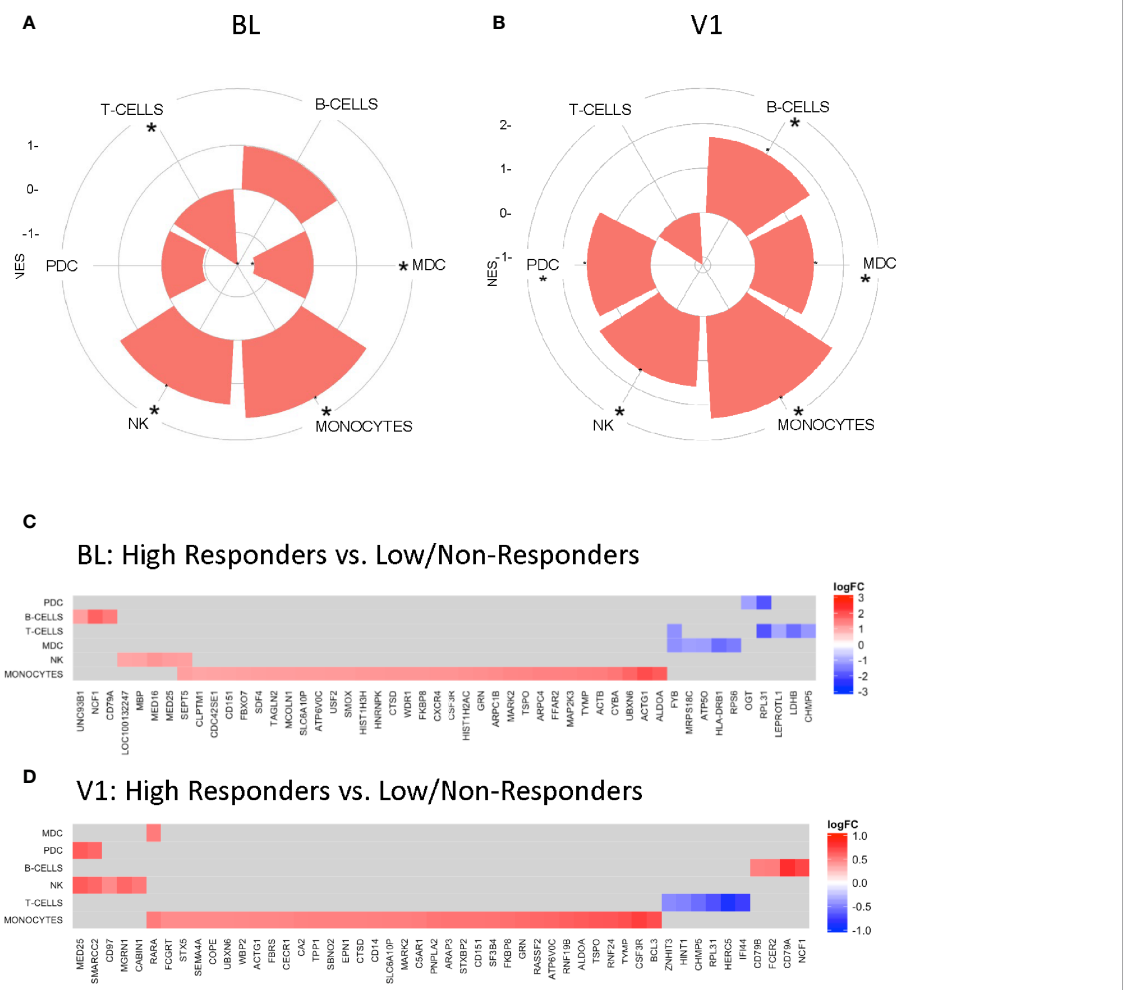


**FIGURE 4** | Gene Set Enrichment using Nakaya.NatImmunology Cell Specific Signatures. "High" responder and "low" responder clusters from regression analysis with baseline gene expression **(A**, **C)** and V1 gene expression **(B**, **D)** and fold change difference in pH1N1 titers (V1/BL) were subjected to gene set enrichment analysis using the Nakaya modules (21). **(A**, **B)** Radial plots illustrating selective enrichment in major PBMC cell types high responders compared to low/non-responders. **(C**, **D)** Genesets induced in a specific subset are significantly enriched (adjusted p-value <0.05 denoted by *) among genes upregulated or downregulated with respect to the enrichment score – (NES) between groups.

growth (RAR activation, Cdc42, Rho, G beta gamma signaling), cell adhesion and mobility (remodeling of Epithelial Adherens Junctions, Integrin and Tight Junction signaling) and hormonal and growth factor signaling (prolactin, IGF-1, NGF, BMP and GNRH signaling).

Consistent with BL data, HR in the younger age group exhibited distinct molecular signatures from the adolescent subset at V1, sharing only one gene; Forkhead box O3 (*FOXO3*) amongst the top 10 enriched pathways (**Figure 6**). Molecular pathways related to cell cycle and protein translation were enriched in children HR including EIF2 signaling, an indicator of ER stress and unfolded protein response (UPR) (**Figure 6A**). In the adolescent group, classic inflammatory markers such as *TNF*, *FASLG*, and *CXCL10* had higher expression in LNR compared to HR (**Figure 6B**). In the younger group, 'classical' inflammatory markers were not identified, however other inflammation-related genes such as *ADAM17, GSTP1*, and *PPP1R15A* were upregulated in LNR, suggesting that different, age-dependent mechanisms of inflammation may be responsible for poor influenza vaccine responses.

## Markers of T Follicular Helper Cells Are Enriched in High Responders to pH1N1 Vaccine

Higher expression of the CXC chemokine receptor type 5 (*CXCR5*) was noted at V1 in HR (**Figure 6B**). CXCR5 is a homing marker of T follicular helper cells (Tfh), a CD4+ T cell subset essential for supporting B cell function and differentiation *via* abundant production of IL-21 in germinal centers (GC). A proportion of

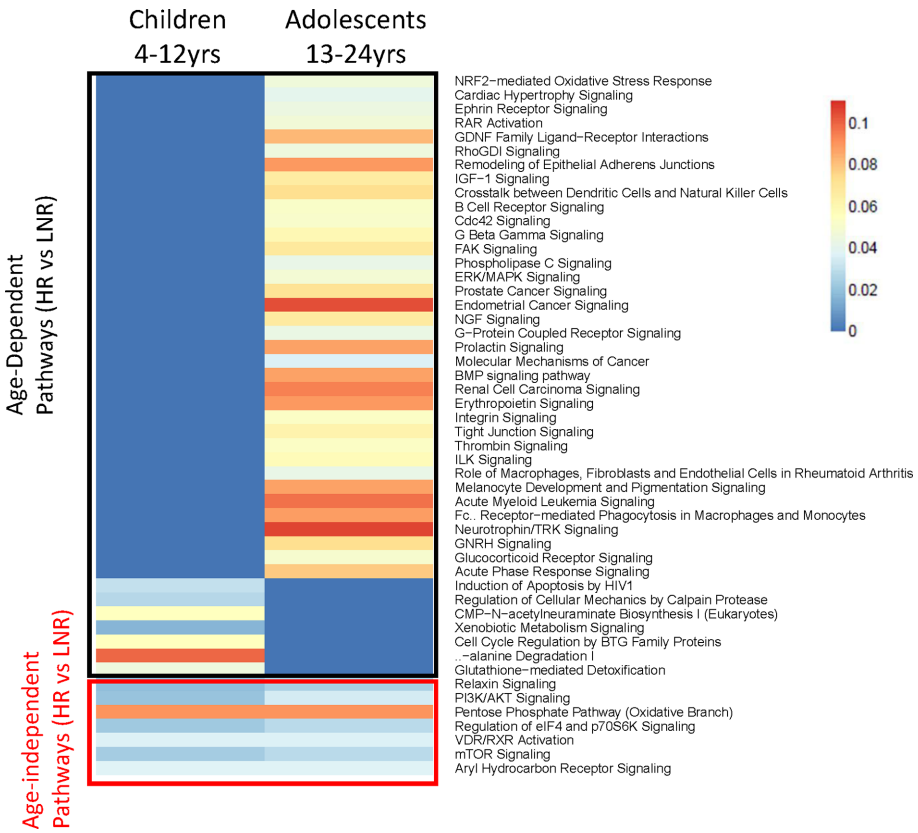


**FIGURE 5** | Pathway Analysis in two Age Groups at Baseline. Heatmap showing statistically significant canonical pathways (IPA) (both uniquely and commonly) regulated in high responders versus low/non-responders in the two age groups (4-12 years and 13-24 years) at baseline. Genes with an adjusted p-value <0.05, |FC|>1.3 and associated with canonical IPA pathway were used for analysis. Heat scaling refers to results from over-representation test performed using Fisher Exact Test (red indicating greater gene enrichment in the pathway). All pathways shown are statistically significant (p value < 0.05) in one or both groups.

circulating CD4+ T cells express CXCR5 and exhibit functional properties of GC Tfh (33–35). To explore the significance of *CXCR5* expression in the microarray data in HR at V1, we investigated peripheral (pTfh) frequencies and function by flow cytometry. We did not observe differences in the frequency of pTfh prior to vaccination, however frequencies of pTfh (CD4+CD45RO +CXCR5+) were significantly higher at V1 compared to BL in HR only (**Figure 7A**). Upon *in vitro* stimulation of PBMC with pH1N1 antigen, pTfh from HR produced significantly more IL-21 at V1 compared to LNR and frequency of IL-2-producing pTfh positively correlated with HAI titer at V1 (**Figures 7B, C**, respectively).

To investigate the relationship immune activation and vaccine response, we measured co-expression of CD38 and HLA-DR on CD4+ T cells. At BL, LNR exhibited increased frequencies of CD38+HLADR+ CD4+ T cells compared to HR (2.5 +/-0.29 vs. 1.6 +/-0.26, respectively, p=0.04). CD38 +HLADR+ CD4+ T cells at BL showed negative correlations with IL-21-producing pTfh and pH1N1 titer at V1 (**Figures 7D, E**, respectively). These data provide a link between known immunological correlates of influenza vaccine response to immunological and transcriptional signatures at pre-vaccination.

## DISCUSSION

In this study, transcriptomic analyses of whole blood were applied to identify gene signatures related to novel pH1N1 vaccine responses in HIV-infected children and adolescents under suppressive ART. We used multiple GSEA platforms to generate a comprehensive resource of the transcriptomic changes related to antibody responses in young PWH. Our hypothesis was that chronic immune activation would influence vaccine responses (36). Previously, our group has shown that prior to vaccination markers of immune activation, including CD38+ HLA-DR+ T cells and serum levels of TNF and other inflammatory markers, negatively correlate with antibody responses to seasonal influenza vaccination (including pH1N1) in multiple cohorts of HIV-infected ART-treated adults (37–40).

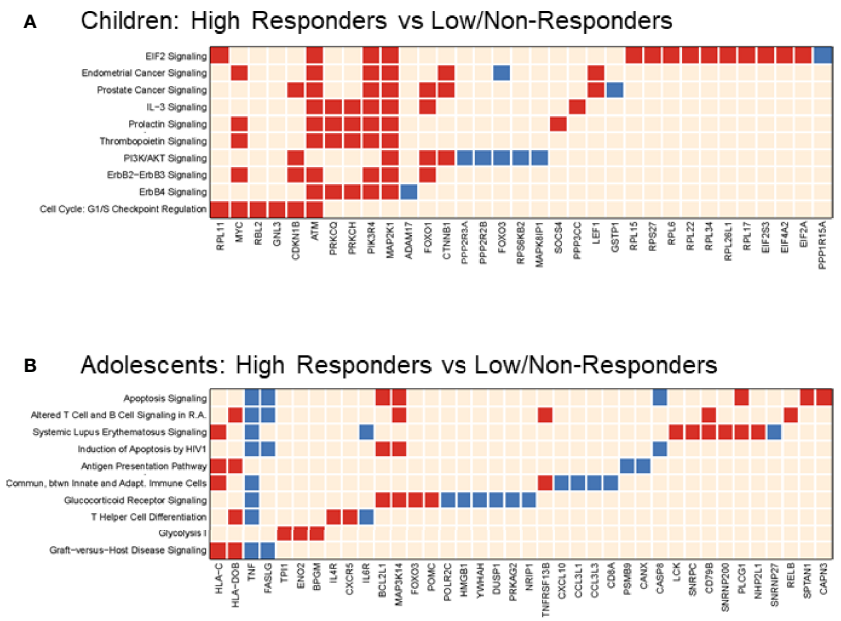


**FIGURE 6** | Pathways Analysis in two Age Groups at Post-Vaccination. Top 10 (or top selected) significant pathways and their gene members enriched when comparing high responder versus low/non-responder at Visit 1 in 4-12yr group **(A)** and 13-24yr group **(B)**. Each row is a regulated canonical pathway (ingenuity software); each column represents an up-(red) or down-(blue) regulated genes (p-value <= 0.05 and |FC|>1.3) induced in 1 or more pathways(s). Over representation test was performed using Fisher Exact Test; significance, displayed on the right, is achieved for p<0.05 (-log(p)1.3).

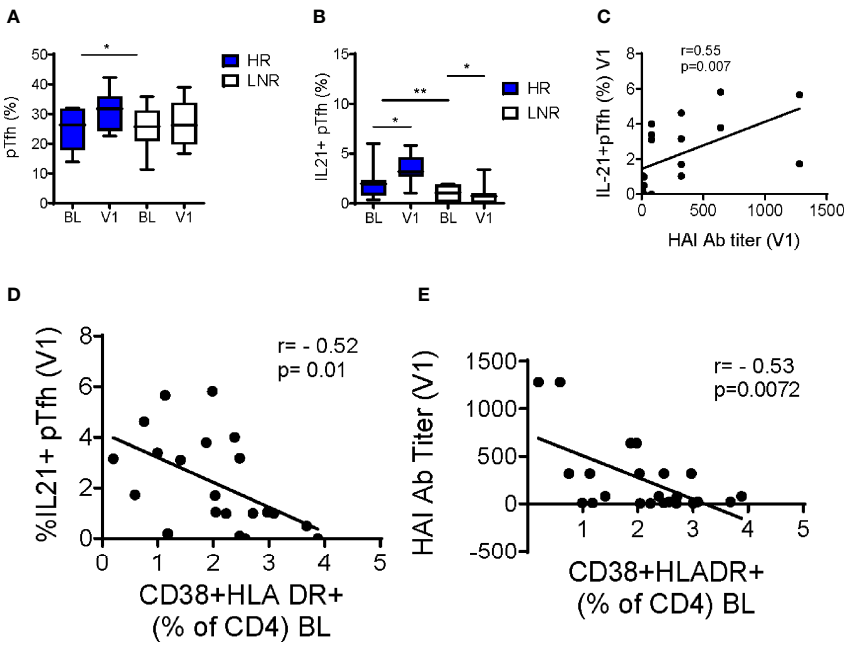


**FIGURE 7** | Increase in pTfh Frequency and Function Post-Vaccination in pH1N1 Responders. **(A)** Frequencies of CD4+CD45RO+CXCR5+ peripheral T follicular helper cells (pTfh) without stimulation and **(B)** IL-21+ pTfh after 12-hour stimulation with H1N1 from responders (n=14) and non-responders (n=14) at baseline (BL) and post vaccination (visit 1, V1). Pearson correlations between **(C)** IL-21+pTfh at V1 following H1N1 stimulation with HAI titers at V1 **(D)** IL-21+pTfh at V1 following H1N1 stimulation with baseline CD4 Immune activation **(E)** HAI titers at V1 with baseline CD4 Immune activation. P-values were calculated with Student's t-test or Mann-Whitney test as appropriate. Box plots include median with 25th and 75th percentile borders, and error bars represent 10th and 90th percentiles. Stars indicate the level of significance: *p < 0.05, **p < 0.01.

The current study in a pediatric cohort confirmed these observations as we demonstrated increased immune activation shown by negative correlations of the CD38+HLA-DR+ CD4 T cell frequencies with serum antibody titers, and this is further supported by GSEA showing DC and T cell related genes significantly enriched in LNR at pre-vaccination. Additionally, we observed higher CD69 following vaccination in LNR which is an early activation marker expressed on leukocytes (especially T and NK cells) and its dysregulation is associated with multiple inflammatory diseases (41).

Metabolic stress pathways (mitochondrial dysfunction and oxidative phosphorylation) were differentially expressed in transcriptional analysis at baseline in high and low/non-responders and this result was consistent regardless of whether participants were grouped by age. The link between metabolic programs and immune function have been described (42–44). Mitochondria have well-characterized roles in cellular energy and apoptosis and have been shown to play an important role in priming the innate immune system in the context of viral and bacterial infection (45). Our findings were supportive of this link with the observation that the potent anti-apoptotic protein, *BCL2L1*, was enriched in HNR at baseline and this gene has been shown to be regulated by mitochondrial transcription factor A (46). Moreover, ART is associated with numerous side effects, of which mitochondrial toxicity is one. The mechanisms of toxicity *in vivo* are unclear and controversial due to the use of multiple drugs and classes of drug by each patient and the reliance on *in vitro* data for determining drug effects on mitochondria (47). The question remains how alterations in mitochondrial function and quality relate to chronic immune activation and vaccine responses.

In the present study, GSEA using cell specific gene signatures confirmed that B cells were enriched post-vaccination in high responders, but our analysis did not identify them as a predictive cell-type (**Figure 3**). UPR is upregulated in plasmablasts in order to support high levels of antibody production (48, 49), therefore this signature may represent an ongoing or residual antibody response in responders to pH1N1 vaccination. Typically, the plasmablast response to influenza vaccination peaks at 7 days (50), however given that pH1N1 was a novel antigen in the participant group it is possible the response was delayed to remain detectable 3 weeks post-infection. The B cell compartment is highly heterogeneous and the methodology used here was not sensitive enough to detect a rare predictive subset however, peripheral T follicular helper (pTfh) cells have been shown to correlate with vaccine-induced antibody responses in HIV-infected and HIV negative populations. Specifically, IL-21 producing pTfh are a strong immunological correlate of T-dependent B cell responses against influenza antigens (51, 52), as well as HIV (53) and malaria vaccine antigens (54).

Monocyte signatures were significantly enriched before and after vaccination and may provide a target for predicting vaccine effectiveness. Indeed, pre-vaccination expression of costimulatory molecules, CD80 and CD86 on TLR-activated monocytes from elderly and young HIV-uninfected adults was shown to associate with vaccine responses to influenza (55). Our results did not address monocyte function since samples were analyzed without stimulus; however, we found increased IFN-inducible gene expression in LNR at V1 by multiple analysis platforms (IPA, MSigDB, and Age-specific) which could be attributed to the monocyte population. Proteins encoded by IFN-inducible genes are essential antiviral effectors with capabilities to block at various steps of the viral life cycle (56), however unregulated IFN responses can lead to immune dysfunction. The role of type I IFN in HIV infection is complex; it is important in controlling viral replication very early following infection while contributing to pathogenesis in chronic infection (57). Because the difference in IFN-inducible gene expression was not present prior to vaccination, our findings beg the question of what effect routine vaccination is having on viral replication and the existing HIV infection. Influenza vaccination has been shown to increase plasma viremia transiently following vaccination (peak 2 weeks post-vaccination) (58). Future studies monitoring vaccine responses in PWH on ART should consider a possible effect on viral recrudescence. it will be important to evaluate the role of monocytes in influencing antibody-driven vaccine responses. *CSF3R* encodes the receptor for granulocyte-colony stimulating factor (G-CSF) and is expressed on circulating, classical monocytes (CD14+CD16-) (59) suggesting that enrichment of this predominant monocyte subset could be an immunological correlate for antibody responses as well as a biomarker or predictor of response.

Despite the intriguing findings in this cohort there were several limitations inherent in the present study. The study design catered to blood sampling coincident with important timepoints for measuring serum antibody responses. The inclusion of blood sampling at an early timepoint post-vaccination (e.g. day 2-7) would have allowed for evaluation of the innate immune response to the vaccine, however the innate response to seasonal influenza vaccine (trivalent-inactivated) has been studied extensively in HIV-uninfected populations (18, 21, 22, 60, 61). Thus, our study is focused on creating a resource of later adaptive immune biomarkers that correlate with serum antibody titers to a novel influenza antigen and, therefore, response. As one of the few transcriptomic studies in perinatal HIV infected children and adolescents, the data presented herein will undoubtedly serve as a novel resource for further immune monitoring studies in this population and may especially be important in light of the current pandemic SARS-CoV-2.

Unexpectedly, this cohort had relatively high baseline antibody responses to pH1N1 despite no documented exposure to the antigen (29), while other cohorts with participants at similar ages displayed low baseline titers and a lack of cross-reactivity (62). Despite the complexity in using systems biology to predict immunogenicity to influenza vaccines due to individual infection and vaccination histories, we believe that our transcriptomic study in young PWH fills an age gap and has yielded a valuable resource likely to provide insight into favorable and negative targets for improving vaccine design and assessment of vaccine responses in young PWH subjects.

## AUTHOR'S NOTE

Parts of the work described in this manuscript have been presented at Conference of Retrovirus and Opportunistic Infections 2013 and American Association of Immunologists 2014 as poster presentations by A.P. and L.D., respectively.

## DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Gene Expression Omnibus data repository (https://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE167893.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by International Maternal Pediatric Adolescent AIDS Clinical Trials Network. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

SP, AW, CC, R-PS, and MC were involved in conception of the work. CS, AP, VG, and AF-M were involved in data collection. LD, CS, AP, VG, AF-M, R-PS, MC, and SP, data analysis and interpretation. LD, MC, and SP drafted the article. LD, CS, VG, AW, CC, R-PS, MC, and SP provided critical revision of the article. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.639358/full#supplementary-material

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MINI REVIEW
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# Barriers and Facilitators Associated With Vaccine Acceptance and Uptake Among Pregnant Women in High Income Countries: A Mini-Review

*Xiao Qiu*[1], *Heather Bailey*[1] *and Claire Thorne*[2]*

[1] UCL Institute for Global Health, University College London, London, United Kingdom, [2] UCL Great Ormond Street Institute of Child Health, University College London, London, United Kingdom

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***Correspondence:**
*Claire Thorne*
*claire.thorne@ucl.ac.uk*

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Vaccination during pregnancy is a safe and effective intervention to protect women from potentially severe consequences of influenza and reduce risk of influenza and pertussis in their infants. However, coverage remains variable. In this mini-review we update findings from a 2015 systematic review to describe results from recent studies in high income countries on the uptake of influenza and pertussis vaccination in pregnancy, reasons for vaccine hesitancy and barriers to increasing uptake, from maternal and healthcare provider (HCP) perspectives. Studies reported highly variable uptake (from 0% to 78%). A main facilitator for uptake among pregnant women was receiving a recommendation from their HCP. However, studies showed that HCP awareness of guidelines did not consistently translate into them recommending vaccines to pregnant women. Safety concerns are a well-established barrier to uptake/coverage of maternal immunization; 7%-52% of unvaccinated women gave safety concerns as a reason but these were also present in vaccinated women. Knowledge/awareness gaps among pregnant women and lack of confidence among HCPs to discuss vaccination were both important barriers. Several studies indicated that midwives were more likely to express safety concerns than other HCPs, and less likely to recommend vaccination to pregnant women. Women who perceived the risk of infection to be low were less likely to accept vaccination in several studies, along with women with prior vaccine refusal. Findings highlight the importance of further research to explore context-specific barriers to vaccination in pregnancy, which may include lack of vaccine confidence among pregnant woman and HCPs, and policy and structural factors.

**Keywords: vaccination, pregnancy, acceptance, hesitancy, influenza, pertussis**

## INTRODUCTION

Vaccination in pregnancy was first implemented in the 1960s with tetanus toxoid immunization, with strategies of maternal immunization to protect pregnant women and their infants against influenza and pertussis more recently introduced. The potentially severe consequences of influenza in pregnancy (1) and the efficacy of maternal influenza immunization in preventing infection in young infants (2, 3), alongside reassuring safety data (4–7) have driven recommendations for its widespread use (8, 9). In the last decade, maternal pertussis vaccination programs have also been implemented in high income countries (HICs) to protect neonates, who have high risk of severe complications, through passively transferred maternal antibodies, with high effectiveness (10).

Concerns around vaccination remain an issue despite robust evidence on the safety and benefits of vaccination. The term 'vaccine hesitancy' is used to refer to "*delay in acceptance or refusal of vaccines despite availability of vaccination services. Vaccine hesitancy is complex and context specific varying across time, place and vaccines. It includes factors such as complacency, convenience and confidence*" (11, 12). A systematic review on vaccine acceptance in pregnancy in 2015 found that concern about vaccine safety was the main factor contributing to vaccine hesitancy, with other common barriers being lack of recommendation from health care providers (HCPs) and poor vaccine knowledge (13).

Our aim is to update these findings with recent studies conducted in HICs in order to describe the uptake of influenza and pertussis vaccination in pregnancy, explore reasons for vaccine hesitancy and discuss barriers to increasing uptake, from maternal and HCP perspectives. We have therefore focused our narrative review on papers published from April 2015 to July 2020.

## UPTAKE OF INFLUENZA AND PERTUSSIS VACCINATION IN PREGNANCY

Ten studies reported on uptake, mainly based on maternal self-report. For maternal influenza vaccination the highest uptakes of 78% and 76%, were reported among 984 women in a US study and 101 women in a New Zealand study (14, 15), with a Spanish study reporting 62% uptake among 683 women (16). Two large studies from France with 2045 and 1194 women reported uptakes of 36% and 22% respectively (17, 18), with uptake of 45% among 823 women in Belgium (19) and 16.2% among 197 women in Greece (20). The lowest coverage was found in a study of 743 women in Italy at 6.5% (21). The latter study also had a low pertussis vaccine uptake, at 4.8%. Pertussis vaccination uptake in other studies ranged from 74% in a large survey of 1809 pregnant women in Taiwan (22) and 61% in an Australian study of 537 women (23) to 64% in the study in Belgium (19) to 0% in the study from Greece (20).

Studies on vaccine acceptance (combining intention to vaccinate with actual uptake at the time of survey) included a multi-site UK survey of around 300 pregnant women, where 38% and 56% had been vaccinated for influenza and pertussis respectively, with a further 40% and 36% intending to be vaccinated (24). In a similar sized survey in the USA, acceptance rates were 71% and 76% respectively for influenza and pertussis vaccination (25). In another US study, with a convenience sample of 316 pregnant women in the public health system, 82% said they had received the vaccine or intended to get the vaccine that day (26). A study of 113 pregnant women in Ireland found uptake rates of 31% for pertussis and 42.5% for influenza, with 29% of unvaccinated women reporting that they would take up if discussed and offered (27). Studies based on acceptance rates need careful interpretation because they may over-estimate final uptake, as demonstrated by Bettinger and colleagues' finding that 36% of women who stated intention to have the influenza vaccine had not done so by delivery (28). Self-reported actual uptake may also be over-reported (15). Discrepancies are likely to reflect various factors, including social desirability bias and unforeseen barriers to uptake.

## INFLUENCE OF HCP OFFER AND RECOMMENDATION

Knowledge of availability of influenza and pertussis vaccination in pregnancy is a pre-requisite for women to decide to vaccinate or not, and for a high proportion of women such knowledge is only gained when they are offered vaccination by an HCP. Consistent with previous findings (13), receiving a HCP recommendation was a main facilitator of vaccine uptake among pregnant women in recent studies, and its absence was the pre-eminent barrier reported among unvaccinated women (18, 20, 21, 25, 28–31). To illustrate, in an Italian survey 62% of vaccinated women said that HCP recommendation was the main facilitator of vaccination, whilst 81% of unvaccinated women reported no HCP recommendation as the main barrier experienced (21), while influenza vaccination uptake was 47% in women who reported being recommended to vaccinate by a HCP versus 3% in those who did not in a French study (17). Two studies (from Australia and the US) found that women receiving an HCP recommendation for pertussis vaccination had 10-fold greater odds of being vaccinated compared with those who did not (23, 25).

The importance of HCPs' recommendation has led several recent studies to specifically investigate recommendation behaviors among HCPs, and vaccine knowledge and attitudes that may underpin these. **Table 1** shows the proportion of HCPs reporting that they recommend influenza and/or pertussis vaccinations to pregnant women or informed their patients about these vaccines in each study. Comparisons between studies are complicated by differences in HCP roles and responsibilities regarding recommendation/administration of vaccinations during pregnancy, differing national guidelines and study methodologies (e.g. capturing whether vaccines were mentioned/discussed versus recommended) and some very low response rates, suggesting that HCP samples may be non-representative with respect to vaccine recommendation behaviors.

**TABLE 1 |** Self-reported behavior of HCPs in recommending influenza and pertussis vaccinations to pregnant women, or discussing with or informing pregnant women about these vaccinations.

| Setting | HCP *n* and group | Vaccine | % HCPs who recommended vaccination to pregnant women, or who discussed with or informed pregnant women about vaccinations | Reference |
|---|---|---|---|---|
| **Studies that reported on HCP recommendations to pregnant women** | | | | |
| Israel<br>Multi-site (6 hospitals in Northern and Central Israel) | **150 HCPs:**<br>54% gynecologists<br>25% family practitioners<br>21% Master of Public Health students who work in medical system | Pertussis<br>Influenza | 68% implemented recommendation<br>70% implemented recommendation | (32) |
| **US**<br>Multi-site (Texas, New York, Illinois, Pennsylvania) | **76 HCPs:**<br>Included ob-gyn, nurse practitioners, physician assistants, nurse midwives | Influenza | All recommended vaccine (90.7% in any trimester; 9.3% after first trimester) | (15) |
| **US**<br>Pennsylvania | **24 HCPs:**<br>All obstetric care providers | Pertussis | All said that they recommended | (25) |
| **France**<br>Paris | **208 HCPs:**<br>All midwives | Influenza | 81% ever informed their patients that a vaccine was available;<br>17% systematically recommended that their patients were vaccinated. | (33) |
| **Georgia**<br>Multi-site (8 cities: Tbilisi, Rustavi, Batumi, Caspi, Kutaisi, Tskaltubo, Gori and Kobuleti) | **278 HCPs:**<br>All ob-gyn | Influenza | 43% recommended influenza vaccination during pregnancy;<br>18% reported vaccinating any pregnant patients during last influenza season | (34) |
| **Spain**<br>Catalonia region | **194 HCPs:**<br>70% midwives<br>30% ob-gyn | Influenza | 40.8% of ob-gyn and 44.1% of midwives recommended during first trimester<br>85.7% of ob-gyn and 84.8% of midwives recommended during second/third trimester | (35) |
| | | Pertussis | 95.9% of ob-gyn and 97.9% of midwives recommended | |
| **Belgium**<br>Flanders region | **261 HCPs:**<br>61% GPs<br>29% midwives<br>10% gynecologists | Influenza<br>Pertussis | 72% recommend always, 11.1% sometimes<br>75.1% recommend always, 6.1% sometimes | (19) |
| **France**<br>Loire-Atlantique | **694 HCPs:**<br>57% physicians, family or general medicine;<br>10% physicians, ob-gyn;<br>22% midwives;<br>8% midwifery students<br>4% unknown | Pertussis | 93% indicated would follow recommendation for anti-pertussis vaccination of pregnant women if this was introduced in France | (18) |
| **Studies that reported on HCPs discussing with or informing pregnant women about vaccinations** | | | | |
| **Ireland**<br>County Cavan | **50 HCPs:**<br>70% midwives<br>30% hospital doctors | Pertussis and influenza | 52% ever discussed both vaccinations with antenatal patients during consultations | (27) |
| **Germany**<br>National | **867 HCPs:**<br>Gynecologists in private practice | Pertussis | 82% informed pregnant patients about vaccine (of these, 18.6% on patient request only) | (36) |
| | | Influenza | 98.5% informed pregnant patients about vaccine (8.6% of these on patient request only) | |

Overall, studies showed that HCPs' awareness of guidelines did not consistently translate into recommendations to pregnant women. In a study in Israel, over a quarter of 150 HCP respondents indicated that they did not recommend influenza and pertussis vaccines to pregnant women despite awareness of their recommendation in guidelines (32) while among 208 midwives in France, 91% were aware that vaccination against influenza was recommended during pregnancy but only 17% recommended this systematically (33). Among 50 HCPs in Ireland (70% midwives), 48% never discussed these vaccines with pregnant patients despite almost all being aware that guidelines existed (27). Conversely, a study in Germany found that although lack of an official recommendation about pertussis vaccination in pregnant women was the main barrier to providing vaccination (cited by 40% of HCPs), 59% reported administering vaccines anyway (36).

Several studies found that midwives were less likely to discuss vaccinations with pregnant women and recommend these than other HCPs, as were less experienced HCPs; e.g. in a French study, 42% of midwives recommended maternal influenza vaccination versus 63% of other HCPs (18), a study in Belgium found that while 78% of gynecologists and GPs recommended

both influenza and pertussis vaccines, this was true for only 24% of midwives (19), while another study of midwives in France found that 50% of those with at least ten years of experience often or always suggested influenza vaccine compared with only 29% of those less experienced (33).

## PREGNANT WOMEN: KNOWLEDGE AND INFORMATION PROVISION

Recently published studies have identified some important knowledge gaps among pregnant women regarding vaccines in pregnancy (including availability). In a multi-center Italian study, 44% and 49% of women unvaccinated for influenza and pertussis respectively were unaware that vaccination in pregnancy would provide protection for their baby from the infection in early life, and receipt of vaccination was associated with such knowledge (21), whilst single center surveys in Rome, Italy and Riyadh, Saudi Arabia found that 35% and 46% of pregnant women respectively were unaware of the elevated risk of complications associated with influenza in pregnancy (30, 37). However, this lack of understanding should be considered as a marker of the absence of advice and recommendation from HCPs (as for 82% of women in the multi-center Italian study and 99% and 97% respectively in the Rome and Riyadh surveys) rather than as an important barrier to vaccine acceptance per se.

Several recent studies have examined information provision on vaccination in pregnancy, including how this should be disseminated. In the Rome survey above, only 6% of pregnant women correctly identified the current national recommendation for influenza vaccination, despite this survey being conducted during a vaccination campaign (30). A small Canadian mixed-methods study found that around 40% of women who did not receive the influenza vaccine, including some who had intended to be vaccinated, reported not having enough information to make a decision (28). Similarly, in a large survey in Taiwan, 55% of recently delivered women who declined Tdap vaccination said that they had received insufficient information to make an informed decision and 77% said that they did not trust the information they had been given (22), whilst an Irish study found that 59% of unvaccinated women stated that inadequate information was a reason for their lack of pertussis vaccine uptake (27). In contrast, only 16% and 7% of pregnant women who intended not to receive pertussis or influenza vaccination respectively in the UK cited insufficient information as a reason (24).

Regarding information provision, a generally negative response to leaflets was found in a qualitative study in Northern Ireland, with preference for face-to-face discussion with a HCP, although most felt that insufficient time was given by HCPs for such discussions and some reported that their HCP was unable to address all their questions (38). Studies reported that pregnant women obtained information on vaccines from the media, family and friends, plus HCPs. The latter were the most common source in both a large French study of pertussis vaccination (18) and a study in New Zealand addressing influenza vaccine uptake (14). The importance of family, friends and the media as information sources among unvaccinated women varied by setting: in the New Zealand study, 20-25% cited these as having influenced their decision (14), whilst fewer than 5% of pregnant women intending not to be vaccinated in a UK study cited concerns about information in the media or the influence of family and friends as a reason (24).

Recent studies have shown sometimes significant knowledge gaps among HCPs regarding maternal immunization (18, 27, 33). Confidence to advise pregnant women also differed by profession and experience; e.g. in a UK study, only 59% of HCPs overall were extremely/moderately confident to advise pregnant women on influenza vaccine and 57% for pertussis, with midwives less confident than obstetricians (55% vs 68%) (24). In a French study, 37% of midwives self-reported limited knowledge of influenza vaccination, of whom only 13% proposed the vaccine to patients, as compared with 90% of the 9% who self-reported high knowledge (33). Only 43% of almost 300 obstetricians/gynecologists in a study in Georgia recommended influenza vaccination to patients, with 75% stating that there was insufficient evidence to support vaccination, but 93% were receptive to receiving additional education (34). Over 90% of 194 maternal care providers in Spain (mostly midwives) agreed that vaccination training for HCPs could be a strategy to improve uptake of vaccines (along with official recommendations) (35). The need for effective communication is underscored by one US study in which all HCPs reported recommending vaccination but only 85% of women reported receiving this (15); time needed to effectively counsel women about vaccination was perceived as a barrier to recommendation by HCPs in some studies (35, 36).

## SAFETY CONCERNS AS A BARRIER TO UPTAKE

A consistent barrier to vaccine uptake across studies was the fear of potential harm to woman or baby. Among recent studies where reasons for declining vaccination in pregnancy were examined, the proportion of women citing concerns about safety as influencing this decision varied substantially (**Table 2**). Maternal perception of the frequency of vaccine complications was associated with uptake in a French study (17): uptake was 55% among women who thought frequency of fetal/infant complications was very low compared with 35% in those who thought these were very common, but lowest uptake (21%) was in women who thought there was a medium rate of complications. Such findings are an important reminder that some pregnant women accept vaccination despite safety concerns. This was also highlighted by a mixed methods study in Canada, with the authors noting that for most women "the unknown risks from the vaccine did not outweigh the benefits of vaccination" (28); focus groups also identified concerns regarding potential delayed discovery of vaccine-related adverse effects, consistent with another qualitative study in Northern Ireland where some unvaccinated women were worried about long-term adverse effects (38). The latter study also found that maternal vaccination was thought by some to be inconsistent with warnings around using medications whilst

**TABLE 2 |** Prevalence of safety concerns as a reason given by women for not taking up influenza and/or pertussis vaccination in pregnancy.

| Setting | Number of unvaccinated women responding to survey | Vaccine | % unvaccinated women reporting safety concerns as a reason for declining vaccination | | | Reference |
|---|---|---|---|---|---|---|
| | | | For woman | For fetus/infant | Non-specified | |
| **Australia** | 95 | Influenza | | 10% | | (23) |
| Melbourne | 46 | Pertussis | | 7% | | |
| **France** | 1320 | Influenza | 13% | 24% | | (17) |
| Lille | | | | | | |
| **Greece** | 164 | Influenza or Pertussis | 2% | 8% | | (20) |
| Athens | | | | | | |
| **Italy** | 682 | Influenza or Pertussis | | 17% | | (21) |
| Multi-center (Milan, Rome, Jesi) | | | | | | |
| **New Zealand** | 16 | Influenza | 38% | 31% | | (14) |
| Wellington | | | | | | |
| **Spain** | 262 | Influenza | | | 21% | (16) |
| Valencian Community | | | | | | |
| **Taiwan** | 473 | Pertussis | | 44% | | (22) |
| Multi-site (8 hospitals) | | | | | | |
| **UK** | 68 | Influenza* | 29% | 31% | | (24) |
| Multi-site (Southampton, Bristol, Oxford, London) | 24 | Pertussis* | 28% | 52% | | |
| **USA** | 91 | Influenza* | | | 17% | (25) |
| Pennsylvania | 43 | Pertussis* | | | 12% | |

**Women indicating intent not to vaccinate.*

pregnant. An Australian survey found that fewer migrant women (comprising 69% of the sample) believed that Tdap is safe during pregnancy than Australian-born women (53% versus 65%, $p$=0.01) and that, overall, maternal belief that the vaccine was safe for the baby was the key factor associated with uptake (23).

Safety concerns among HCPs are also barriers to vaccine recommendation. In a study of HCPs in Israel, around a third reported that Tdap and influenza vaccines were unsafe in pregnancy or controversial (32), while in a US study a similar proportion (32%) reported being concerned or very concerned about the safety of influenza vaccine in the first trimester (15). Among around 200 midwives in France, only 73% agreed that influenza vaccine was safe in pregnancy; 39% had been vaccinated themselves and this group were more likely to recommend vaccination to patients (33). Among HCPs giving reasons for not recommending vaccines in pregnancy in a study in Spain, concerns relating to adverse events were more common among midwives than obstetricians/gynecologists (30.8% vs 10% respectively) (35). Of note, in one US study 71% of obstetric care providers were concerned about the safety of influenza vaccination in the first trimester and 46% about the safety of Tdap, but all still recommended vaccination, indicating that as for women, concerns about safety do not necessarily preclude vaccination recommendations.

## PERCEPTION OF RISK OF INFECTION, AND SEVERITY, IN PREGNANCY AND INFANCY

Perception of susceptibility and severity are constructs that influence health behaviors according to the Health Belief Model. Lefebvre and colleagues in a French study reported that women who perceived risk of acquisition of pertussis to be non-existent or low were significantly less likely to accept vaccination than those who perceived risk to be high (adjusted OR 0.44 [0.31,0.62]) (18). Similarly, in the Taiwanese study discussed above, 18% of women declining pertussis vaccination reported that the main factor in their decision was their belief that pertussis is not a severe disease in newborn infants; conversely, multivariable analysis showed that rating pertussis among young infants as highly severe was significantly associated with acceptance of the vaccine (22). A Canadian study also found that women's opinions on vulnerability to influenza and its severity were central factors regarding uptake, with most women in this qualitative study not perceiving themselves or their infants to be at high risk of infection. Of note, there was sub-group of women who noted their increased vulnerability (e.g. due to occupational exposure or because of conditions such as asthma) and this group had high vaccine uptake (28).

## OTHER FACTORS INFLUENCING ACCEPTANCE

Prior vaccination experience was an important factor influencing uptake in several studies, both with vaccine-experienced women being more likely to take up influenza vaccination (17, 21) and with history of no previous vaccination and/or past refusal being associated with non-acceptance of vaccines in pregnancy (16, 18). With respect to maternal socio-demographics, higher maternal education level was associated with pertussis but not with influenza vaccine uptake in two studies (21, 23), while a Belgian study found education level to be associated with coverage of both vaccines (19). Another study found that

parity was associated with uptake, with women with two or more previous deliveries less likely to receive influenza vaccine than women with fewer (17), possibly reflecting access challenge relating to childcare responsibilities.

## COVID-19 CONSIDERATIONS

Vaccine development is essential to the COVID-19 response, with rapid progress of Phase III clinical trials (all excluding pregnant women), licensing and roll-out (39, 40). The pandemic may modify perceptions and/or health seeking behaviours regarding vaccination for respiratory infections, as shown by a study examining online interest in COVID-19 and vaccinations worldwide through Google Trends, which found an upsurge in interest in influenza and pneumococcal vaccines concurrent with the first pandemic wave (41). An important impact of COVID-19 on vaccination to date has been the world-wide disruption to routine immunisations for reasons including reduced access to services during lockdowns, HCP capacity issues, reluctance to attend health services for vaccinations (e.g., due to fears about exposure to SARS-CoV-2, or due to confusing messaging around "protecting" health services) (42–44). Results from an international survey of clinicians in April 2020 showed that 50% of respondents had problems regarding maternal immunisation delivery (43). More research is needed to understand the collateral damage inflicted by the pandemic on maternal immunisation, as the impact on vaccination rates remains unknown. There is also the question of COVID-19 vaccination in pregnancy, currently a focus of the Pregnancy Research Ethics for Vaccines, Epidemics and New Technologies (PREVENT) group (45). In the absence of trial data among pregnant women, guidance from governments and professional bodies is highly variable with respect to pregnancy and vaccination, and subject to change; many currently recommend an individual risk-benefit approach which is challenging given the evidence gap (46). The recent announcement of a Phase 2/3 study to assess the safety, tolerability, and immunogenicity of the Pfizer-BioNTech COVID-19 vaccine (BNT162b2) in preventing COVID-19 in healthy pregnant women is therefore very welcome (ClinicalTrials.gov Identifier: NCT04754594).

## DISCUSSION

Understanding reasons for vaccine hesitancy and/or low coverage in pregnancy (which may be related to the individual woman, to the vaccinator, to policies or to structural factors) is a pre-requisite for addressing them. The recent studies examined here have provided useful information for policymaking in vaccine delivery. However, study limitations should be considered, including a high proportion of single site studies, use of convenience samples and general reliance on self-reported vaccination status. The findings also underscore the importance of context, with highly variable uptake rates reported ranging from 0% to 78%. This limits comparisons between studies and precludes summary estimates of vaccine uptake or of HCP provider behavior such as vaccine recommendation.

Despite maternal vaccines for influenza and pertussis being safe and effective, safety concerns among women and some HCPs are well-established barriers to uptake/coverage (13). Recent studies have continued to examine perceptions and beliefs of pregnant women regarding vaccine safety for themselves and/or their baby. Between 7% and 52% of unvaccinated women gave safety concerns as a reason for decline in reviewed studies, but some did not investigate the association between presence of safety concerns and actual uptake. Qualitative studies tended to provide richer data on the precise nature of women's concerns whilst overall findings underscored that maternal worries about safety are not necessarily incompatible with acceptance of a vaccine in pregnancy. A greater understanding of what facilitates HCP recommendation of vaccination in pregnancy and what prevents them from doing so in different settings/contexts is needed. More research on specific factors shaping maternal confidence in vaccines, to incorporate the potential influence of COVID-19, is also required.

## AUTHOR CONTRIBUTIONS

XQ, HB and CT developed the search strategy, XQ conducted the literature search and did the initial screen of articles. XQ, HB and CT wrote the manuscript together. All authors contributed to the article and approved the submitted version.

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# Human Newborn Monocytes Demonstrate Distinct BCG-Induced Primary and Trained Innate Cytokine Production and Metabolic Activation *In Vitro*

*Asimenia Angelidou[1,2,3]*, Joann Diray-Arce[2,3], Maria-Giulia Conti[2,4], Mihai G. Netea[5,6], Bastiaan A. Blok[5], Mark Liu[2], Guzman Sanchez-Schmitz[2,3], Al Ozonoff[2,3], Simon D. van Haren[2,3†] and Ofer Levy[2,3,7†]*

[1] Department of Neonatology, Beth Israel Deaconess Medical Center, Boston, MA, United States, [2] Precision Vaccines Program, Division of Infectious Diseases, Boston Children's Hospital, Boston, MA, United States, [3] Department of Pediatrics, Harvard Medical School, Boston, MA, United States, [4] Department of Maternal and Child Health, Sapienza University of Rome, Rome, Italy, [5] Department of Internal Medicine and Radboud Center for Infectious Diseases, Radboud University Medical Center, Nijmegen, Netherlands, [6] Department for Genomics & Immunoregulation, Life and Medical Sciences Institute (LIMES), University of Bonn, Bonn, Germany, [7] Broad Institute of MIT & Harvard, Cambridge, MA, United States

**Background:** Newborns exhibit distinct immune responses and are at high risk of infection. Neonatal immunization with BCG, the live attenuated vaccine against tuberculosis (TB), is associated with broad protection against a range of unrelated pathogens, possibly reflecting vaccine-induced training of innate immune cells ("innate memory"). However, little is known regarding the impact of age on BCG-induced innate responses.

**Objective:** Establish an age-specific human monocyte *in vitro* training platform to characterize and compare BCG-induced primary and memory cytokine responses and immunometabolic shifts.

**Design/Methods:** Human neonatal and adult CD33-selected monocytes were stimulated for 24h with RPMI (control) or BCG (Danish strain) in 10% autologous serum, washed and cultured for 5 additional days, prior to re-stimulation with the TLR4 agonist LPS for another 24h. Supernatants were collected at Day 1 (D1) to measure *primary* innate responses and at Day 7 (D7) to assess *memory* innate responses by ELISA and multiplex cytokine and chemokine assays. Lactate, a signature metabolite increased during trained immunity, was measured by colorimetric assay.

**Results:** Cytokine production by human monocytes differed significantly by age at D1 (primary, BCG 1:750 and 1:100 vol/vol, $p<0.0001$) and D7 (innate memory response, BCG 1:100 vol/vol, $p<0.05$). Compared to RPMI control, newborn monocytes demonstrated greater TNF (1:100, 1:10 vol/vol, $p<0.01$) and IL-12p40 (1:100 vol/vol, $p<0.05$) production than adult monocytes (1:100, $p<0.05$). At D7, while BCG-trained adult monocytes, as previously reported, demonstrated enhanced LPS-induced TNF production, BCG-trained newborn monocytes demonstrated tolerization, as evidenced by significantly diminished

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***Correspondence:**
*Asimenia Angelidou*
*Asimenia.Angelidou@childrens.harvard.edu*

*†These authors have contributed equally to this work and share senior authorship*

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subsequent LPS-induced TNF (RPMI vs. BCG 1:10, p <0.01), IL-10 and CCL5 production (p<0.05). With the exception of IL-1RA production by newborn monocytes, BCG-induced monocyte production of D1 cytokines/chemokines was inversely correlated with D7 LPS-induced TNF in both age groups (p<0.0001). Compared to BCG-trained adult monocytes, newborn monocytes demonstrated markedly impaired BCG-induced production of lactate, a metabolite implicated in immune training in adults.

**Conclusions:** BCG-induced human monocyte primary- and memory-innate cytokine responses were age-dependent and accompanied by distinct immunometabolic shifts that impact both glycolysis and training. Our results suggest that immune ontogeny may shape innate responses to live attenuated vaccines, suggesting age-specific approaches to leverage innate training for broad protection against infection.

**Keywords: Bacille Calmette-Guérin (BCG) vaccine, cord blood, cytokines, lactate, newborn monocytes, immunometabolism, trained immunity**

## INTRODUCTION

As compared to other age groups, human newborns are highly susceptible to infections due in part to functionally distinct innate (1) and adaptive immunity (2). Epidemiologic studies have linked early life BCG immunization to an unanticipated reduction (~50%) in all-cause mortality, greatly exceeding that attributable to tuberculosis (TB) (3, 4). These observations suggest BCG induces heterologous protection against antigenically diverse, unrelated pathogens. One of the suggested mechanisms for heterologous protection against infection in the context of BCG vaccination is the novel concept of innate immune memory, also termed as "trained immunity" (5). Trained immunity is the ability of innate immune cells to mount an altered response against infection following a previous unrelated infection or vaccination.

Several lines of evidence suggest that trained immunity occurs in newborns (6). In mice, pre-treatment with Toll-like receptor (TLR) agonists enhances subsequent responses to polymicrobial sepsis (7) and treatment with BCG results in enhanced emergency granulopoiesis (8). Evidence that such trained immunity occurs in human newborns includes: (a) critically ill preterm newborns demonstrate enhanced pathogen-specific mononuclear cell pattern recognition receptor (PRR) expression in the setting of Gram-positive or Gram-negative bacteremia (9); and (b) histologic chorioamnionitis affecting preterm infants is associated with a significantly reduced risk of late onset bacterial sepsis (10). These observations suggest the existence of neonatal innate memory that alters responses to subsequent unrelated microbial challenges (11).

Early life immunization in Guinea-Bissau with BCG had beneficial effects on overall mortality, especially when provided at birth, with the largest effect seen in low birth weight newborns and during the first 2 months of life (12–14). In addition to reduced mortality, heterologous beneficial BCG effects in early life include reductions in respiratory infections and sepsis, in both high- and low-income settings (15, 16). *In vitro*, stimulation of BCG-trained adult peripheral blood mononuclear cells (PBMCs) with heterologous TLR agonists and bacteria led to increased production of TNF (17). BCG, given 1 month prior to an infectious challenge, enhanced clearance of yellow fever vaccine strain viremia, an effect that correlated with higher pro-inflammatory cytokine production (TNF, IL-1β, IL-6) from BCG-vaccinated adult volunteers, with a crucial role for IL-1β production (18). Such trained immunity effects have been ascribed to genome-wide epigenetic reprogramming of monocytes (Mos), which in adults is accompanied by metabolic rewiring, crucial for the induction of the histone modifications and functional changes underlying BCG-induced trained immunity in adults (19). Ongoing clinical trials are underway to assess BCG pathogen-agnostic protection against COVID-19 (20).

The extent, mechanism and ontogeny of trained immunity in early life remain incompletely defined. Understanding how BCG-induced innate immune engagement, including the enhancement of Th-polarizing cytokine production by antigen-presenting cells, varies by age, is of basic and translational importance (21, 22). In this study, we compared the impact of BCG stimulation on innate cytokine and chemokine responses by CD33+ monocytes characterizing both primary and memory innate responses of human newborn and adult monocytes to a subsequent stimulation with lipopolysaccharide (LPS). We found that in marked contrast to increased cytokine induction by BCG-trained adult monocytes, BCG-trained newborn monocytes mounted a tolerogenic response to endotoxin. Remarkably BCG-induced cytokines and chemokines at Day 1 inversely correlated with subsequent LPS-induced TNF production on Day 7. Moreover, human newborn monocytes failed to produce lactate in response to BCG, suggesting distinct immunometabolism in early life that could contribute to age-dependent effects of BCG.

## METHODS

### Human Blood Collection

In accordance with approved protocols from the Institutional Review Board (IRB) of the Beth Israel Deaconess Medical Center,

Boston, MA (protocol number 2011P-000118) and The Brigham & Women's Hospital, Boston, MA (protocol number 2000-P-000117), human cord blood samples were collected from healthy full-term cesarean deliveries (>37 weeks gestational age). All de-identified blood samples from adult (age 18–40 years old) participants were collected with approval from the IRB of Boston Children's Hospital, Boston, MA (protocol number 307-05-0223), after written informed consent. Small blood samples (10-15ml) were collected in vacutainer serum collection tubes (BD Biosciences; San Jose, CA) to secure autologous serum from each participant, and the remaining blood volume was anti-coagulated with 15 U/ml of pyrogen-free heparin sodium (Sagent Pharmaceuticals; Schaumburg, IL, USA) and assayed within 4h. Prior to study blood collection, none of the study participants had ever received BCG.

## Autologous Serum Preparation

Blood collected in vacutainer serum collection tubes was left undisturbed at room temperature for 30 min and allowed to clot, then centrifuged at 1500 × *g* for 10 minutes. Serum was maintained at 2-8°C during handling or apportioned in 0.5-1 ml aliquots and stored in -20°C until use.

## Isolation of Human Mononuclear Cells and Monocytes

Heparinized cord blood from newborns and peripheral blood from adults was centrifuged for 10 min at 500 × *g*, then the upper layer of clear yellow plasma was removed. The remaining blood was reconstituted to its original volume by resuspending in Dulbecco's Phosphate Buffered Saline (DPBS, Life Technologies, Carlsbad, CA). Then, 25 ml of reconstituted blood was layered on to 15 ml of Ficoll-Hypaque gradients (Ficoll-Paque PREMIUM; GE Healthcare, Waukesha, WI) and centrifuged for 30 min at 500 × *g*. After Ficoll separation, the mononuclear cell fraction was collected. Monocytes were then isolated from mononuclear cell fractions by positive selection with magnetic CD33 MicroBeads, performed according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). CD33 *vs.* CD14 was chosen in order to avoid induction of non-physiological activation and provide more natural monocyte heterogeneity (23, 24). Purity was checked by flow cytometry and was always > 90%.

## Trained Immunity Assay

Following isolation, monocytes were counted and re-suspended in a Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with L-glutamine, penicillin, streptomycin and 10% autologous serum at a concentration of 1 million monocytes/ml. After a 1h resting phase, monocytes were stimulated with BCG or RPMI (negative control) in duplicate: one singlicate was used to study primary innate responses and supernatants were filtered and harvested after 24h for cytokine measurements and lactate measurements. The other singlicate was used to study trained immunity: After 24h, BCG was washed out with a calcium- and magnesium-free PBS [PBS(−)], medium was replenished, and BCG-trained or control monocytes were incubated at 37°C for 5 days with an interim medium replenishing step on Day 3 of culture. On Day 6, supernatants were collected and a second stimulus, lipopolysaccharide from *Salmonella minnesota* (LPS-SM Ultrapure, Invivogen; San Diego, CA) was added. After 24 h incubation, supernatants were harvested on Day 7 of culture for cytokine and lactate measurements (**Figure 1**).

## Cytokine/Chemokine Assays

Supernatants from the trained immunity assay were analyzed for TNF with a human ELISA kit (BD Opteia ELISA set, BD Biosciences; San Jose, CA) as per the manufacturer's directions. ELISA plates were read on a Versamax microplate reader with SoftMax Pro Version 5 (both from Molecular Devices; Sunnyville, CA). A fluorescent bead-based multianalyte xMAP technology cytokine kit (Milliplex Human Cytokine/Chemokine Immunoassay, Millipore Corp; Billerica, MA) was employed to measure the concentration of 41 analytes including Th1-, Th2-, and Th17- cytokines, chemokines and hematopoietic factors. Assays employed a Luminex 200 Bioanalyzer (Luminex Corp; TX, USA) set to acquire at least 50 events per cytokine. Multiplex cytokine/chemokine data were analyzed using *BeadView* multiplex Data Analysis Software (v.1), according to the manufacturer's instructions (Millipore).

## Lactate Measurements

Lactate concentrations in culture supernatants were measured post-primary stimulation (Day 1) and post-secondary stimulation (Day 7) using a colorimetric assay (Lactate Kit II, Biovision; Milpitas, CA).

## Statistical Analysis

Cytokine/chemokine concentrations were normalized to RPMI control, $\log_2$-transformed and represent log-fold-change over RPMI. To assess statistical significance, differences between individual treatment conditions (for example BCG 1:10 vs RPMI) were evaluated by Student's t-test, while differences between age groups and across BCG concentrations were evaluated by ANOVA.

Lactate production was normalized to the vehicle condition on Day 1 and $\log_2$-transformed. The paired Student's t-test was used for comparison of trained *vs.* untrained conditions and within each age group over time. Unpaired Student's t-test was used for comparison of similar conditions between age groups.

We used Pearson correlation to evaluate associations between primary cytokine/chemokine concentrations (Day 1) and subsequent LPS-induced TNF trained immune responses (Day 7). A $p$-value <0.05 was considered statistically significant. Statistical and graphical analysis was performed using *Prism* 7 software (Graph Pad Software Inc; La Jolla, CA), R version 4.0 and geepack v 1.3-2 package.

# RESULTS

A human neonatal *in vitro* trained immunity platform was designed based on studies of adult monocyte BCG-induced trained immunity *in vitro* assays, which were used as a benchmark (25). In accordance with these studies, we employed

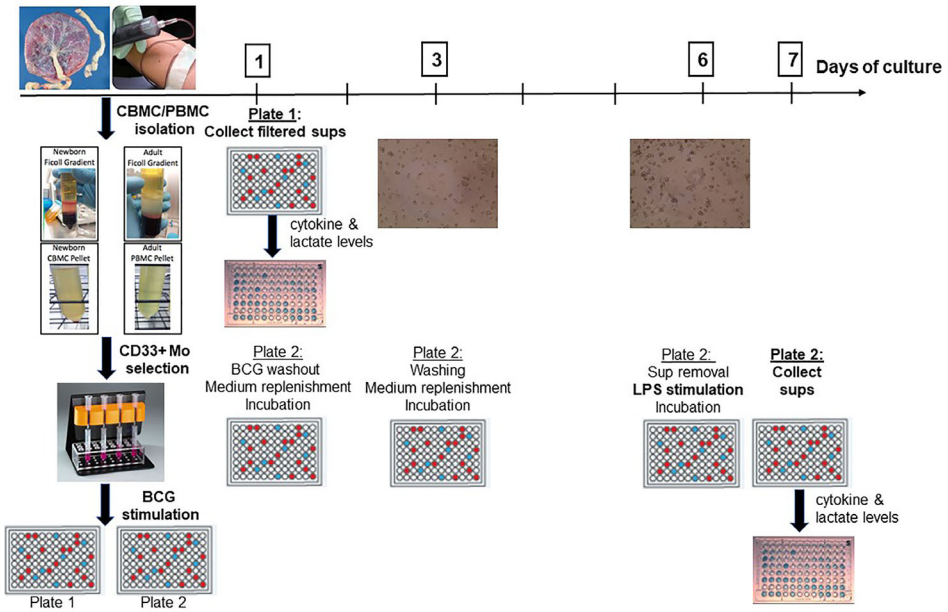


**FIGURE 1** | A human *in vitro* platform to assess age-dependent BCG training. Peripheral venous blood was drawn from healthy adult volunteers and cord blood was collected from healthy term (≥ 37 weeks gestation) elective cesarean deliveries. After PBMC and CBMC isolation, pure CD33+ Mos were isolated by immunomagnetic separation and plated in two separate 96-well plates. After 1h of resting, Mos were stimulated with control RPMI medium or with BCG. Supernatants were harvested from the first plate at 24h post-stimulation for Day 1 cytokine and lactate measurements. The second plate was further cultured after BCG was filtered out at 24h to allow time for immune training. After intermediate washing and culture medium replenishment steps, trained and untrained (control) monocytes were stimulated at Day 6 with LPS for 24h at which point supernatants were harvested for cytokine and lactate measurements. PBMC, peripheral blood mononuclear cells; CBMC, cord blood mononuclear cells; Mo, monocyte; sups, supernatants.

LPS, a TLR4 agonist which is not present on the mycobacterial surface, as our heterologous stimulus of choice. We defined Day 0 as the day of blood collection and primary monocyte stimulation. We confirmed that assessment of a trained immunity response was optimal after 7 days of culture and used this as our timepoint of choice.

Primary BCG-induced TNF production by human CD33+ Mos isolated from adult peripheral and cord blood mononuclear cells was assessed after *in vitro* stimulation. Newborn Mos stimulated with increasing concentrations (vol/vol 1:750, 1:100, 1:10) of BCG-Denmark generated concentration-dependent increases in TNF production compared to control RPMI medium (RM 1-way ANOVA, ***p=0.0002; **Figure 2**). Although adult Mos also exhibited concentration-dependent increases in TNF production compared to control RPMI medium (RM 1-way ANOVA, *p=0.01), they produced significantly lower BCG-induced TNF compared to newborn Mos for each BCG concentration and overall (2-way ANOVA, *p=0.01) (**Figure 2**). Absolute TNF concentrations are shown in **Supplementary Figure 1**. Additional analyses fitting a linear trend showed that TNF values increase 80% on average for each step up in concentration among newborns (p<0.0001) vs. 56% increase for each step up in concentration among adults (interaction p=0.02). Overall, adult Mos produce 56% lower TNF on average compared to newborn Mos (p=0.002).

The effect of BCG on production of a range of cytokines and chemokines was further examined in human Mos stimulated with a low or high BCG concentration- 1:750 (vol/vol) and 1:100 (vol/vol), respectively (**Supplementary Table 1**). Primary (24h after BCG stimulation) BCG-induced Mo cytokine/chemokine production significantly differed by age (RM-ANOVA, BCG 1:750, p<0.01; BCG 1:100, p <0.001). Specifically, after stimulation with the low BCG concentration, newborn Mos demonstrated higher log-fold increases for the vast majority of cytokine/chemokine concentrations as compared to RPMI than adult Mos (**Figure 3A**). Cytokines that were significantly elevated in newborn Mos compared to RPMI included IL-1β, TNF, IL-12p70, IL-6 and IL-10 (**Supplementary Figure 2**).

In contrast to neonatal Mos, adult Mos demonstrated higher log-fold increases for the vast majority of cytokine/chemokines as compared to RPMI than newborn Mos after stimulation with the high BCG concentration (**Figure 3B**). Cytokines that were significantly elevated compared to RPMI in adult Mos included TNF, IFNγ, IFNα2 and IL-4. Several chemoattractants were also significantly elevated in the adult Mos only, such as CXCL1, CCL11 (formerly eotaxin-1), CCL3 and CCL4. At the high BCG dose, the age-differential effect of BCG was particularly evident for IL-12p40 and CXCL10 with the same or opposite directionality of production in newborns and adults, respectively.

To assess the effects of BCG on Mo innate immune memory, control (RPMI)- or BCG- stimulated human Mos underwent a procedure to filter out BCG at 24h post-stimulation (as described in the *Methods* section) and were then cultured in parallel for

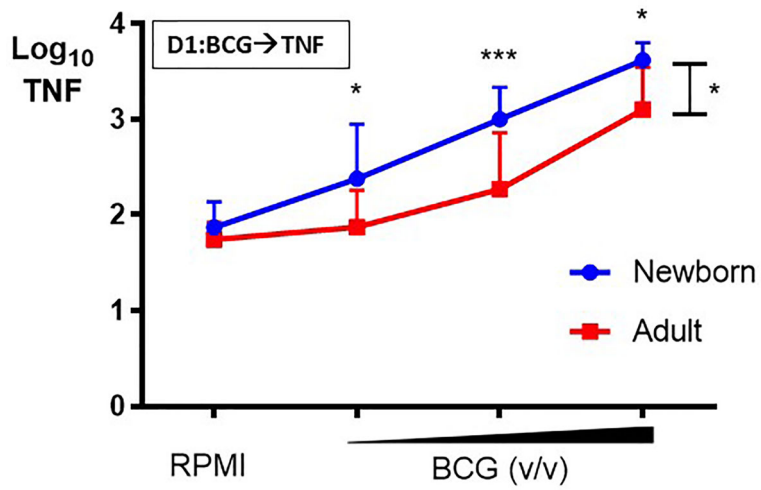


**FIGURE 2** | Human newborn monocytes demonstrate distinct BCG-induced primary TNF responses. Human newborn and adult CD33+ monocytes were cultured *in vitro* as described in **Figure 1**. In contrast to adult monocytes, neonatal monocytes demonstrated relatively greater primary TNF responses to BCG. Results are shown as $log_{10}$ cytokine concentrations due to skewed distribution of values. N = 7 newborns, 9 adults. D, Day; v/v, volumetric concentrations. Bars indicate mean + SD. Repeated-measures 1-way ANOVA was used for comparisons across BCG concentrations and 2-way ANOVA was used for comparisons between age groups. *$p < 0.05$; ***$p < 0.001$.

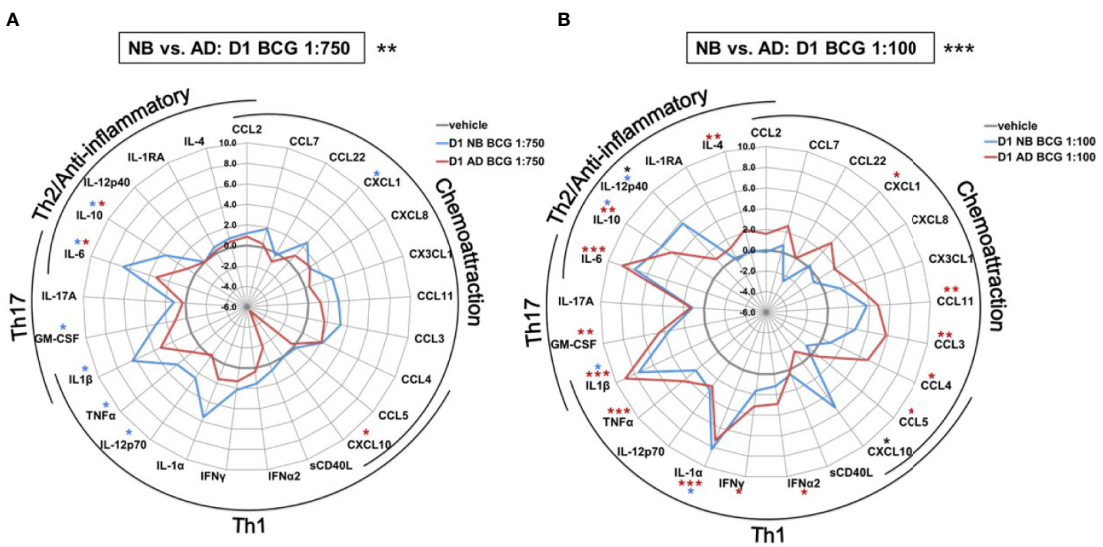


**FIGURE 3** | Human newborn and adult monocytes demonstrate distinct primary BCG-induced cytokine and chemokine production at Day 1 of culture. Human newborn and adult CD33+ monocytes were cultured *in vitro* as described in **Figure 1** then stimulated for 24 hours with **(A)** low (1:750 vol/vol) or **(B)** high (1:100 vol/vol) concentrations of BCG prior to measurement of cytokine and chemokine production in supernatants using a multiplex assay as described in *Methods*. Data was normalized to RPMI control, $log_2$ transformed, and represents $log_2$ fold-change. N = 5 newborns and 7 adults; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (blue stars: NB *vs.* vehicle; red stars: AD *vs.* vehicle; black stars: NB *vs.* AD). Vehicle-(re)stimulated conditions shown in gray.

5 more days to allow for *in vitro* BCG training. At Day 6 of culture, control and BCG-trained Mos were stimulated with RPMI (to allow for background correction of TNF concentrations at Day 6 of culture) or a heterologous stimulus, LPS, and resulting TNF production was measured 24h later at Day 7 of culture. Compared to the control RPMI-trained adult Mos, BCG-trained adult Mos demonstrated enhancement of subsequent LPS-induced TNF production for 2 of the 3 BCG concentrations tested (**Figure 4**). Remarkably, in contrast, BCG-trained newborn Mos demonstrated a dose dependent *decrease* in LPS-induced TNF production compared to the RPMI-trained (control) newborn Mos for all BCG concentrations tested.

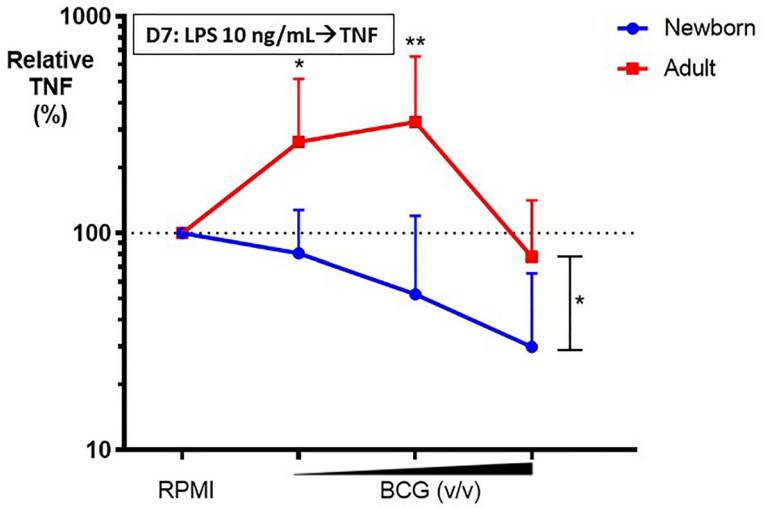


**FIGURE 4** | BCG-training induces enhanced LPS-induced TNF responses in adult monocytes but diminished TNF responses in newborn monocytes. Human newborn and adult CD33+ monocytes were trained with BCG as described in **Figure 1**. At Day 7 of culture, monocytes were stimulated by LPS prior to collection of supernatants for TNF ELISA. Relative TNF is the calculated ratio of trained vs. untrained TNF concentrations. N = 7 newborns, 9 adults. D, Day; v/v, volumetric concentrations. Bars indicate mean + SD. Repeated-measures 1-way ANOVA was used for comparisons across BCG concentrations and 2-way ANOVA was used for comparisons between age groups. *$p < 0.05$; **$p < 0.01$.

The effect of BCG-training on TNF responses was significantly different for 2 of the 3 BCG concentrations tested (BCG 1:750 and BCG 1:100 v/v) in newborn *vs.* adult Mos and had the opposite direction between age groups (**Figure 4**). Decrease in TNF concentration at the highest BCG concentration (BCG 1:10 v/v) was accompanied by a relative loss of Mo viability, which was of similar magnitude in both age groups (**Supplementary Figure 3).** Limited immunophenotyping assessment of Mo cell surface markers CD14, CD11b and TLR4 by flow cytometry raised the possibility of distinct BCG-induced phenotypic changes between newborn and adult Mos (data not shown), but further study of this phenomenon will be required given the limited nature of this data set. Notably, vaccination of adult study participants with BCG *in vivo* was associated with a decrease in Mo TLR4 expression 2 weeks post-immunization (17), suggesting early immunophenotypic changes in trained immunity.

In addition to the aforementioned differences in TNF production between newborns and adults the impact of BCG training on a variety of cytokine responses also differed markedly by age (**Figure 5** and **Supplementary Table 2**). Effects were significantly different between newborns and adults after priming with the high BCG dose and restimulation with LPS [BCG 1:100; RM-ANOVA, p=0.01]; **Figure 5B**). IL-10 concentrations were not only significantly different, but also divergent between newborns and adults with a recorded 5 $\log_2$-fold decrease in newborn IL-10 concentrations relative to RPMI control. The directionality of change in newborns was opposite from that in adults and statistically significant for CCL5. Production of IL-12p40, which was significantly upregulated by primary BCG stimulation, was significantly attenuated by LPS restimulation of BCG pre-exposed newborn Mos.

We next assessed whether BCG-induced Mo primary cytokine/chemokine responses on Day 1 correlated with subsequent trained LPS-induced TNF responses at Day 7. BCG-induced adult Mo production of IL-1RA on Day 1 inversely correlated with subsequent LPS-induced TNF on Day 7 (R=-0.56, *p=0.04), while newborn Mos demonstrated a positive correlation for this cytokine (R +0.25) (**Figure 6).** Using the signed rank test, correlation coefficients of Day 1 cytokines/chemokines and subsequent Day 7 LPS-induced TNF production were generally negative in both age groups (p<0.001 for both age groups; **Supplementary Figure 4**).

As newborns have distinct immunobiology, immunity, and metabolism (26, 27), as well as BCG-induced primary and trained cytokine production, we hypothesized that BCG priming may have distinct immunometabolic effects towards newborn vs adult Mos. To test this hypothesis, we measured lactate production in supernatants from our Mo training assay. BCG-trained adult Mos subsequently treated with LPS produced 2 to 3 $\log_2$-fold more lactate compared to RPMI control (**Figure 7**), as expected given that glycolysis is associated with innate immune activation in adults (28). In marked contrast to their adult counterparts, newborn Mos did not exhibit any significant increase in lactate from baseline but rather trended towards diminished lactate concentrations, suggesting absence of a metabolic switch toward increased glycolysis (**Figure 7**).

## DISCUSSION

While there is growing evidence that BCG re-shapes innate immune responses to tuberculosis-unrelated pathogens

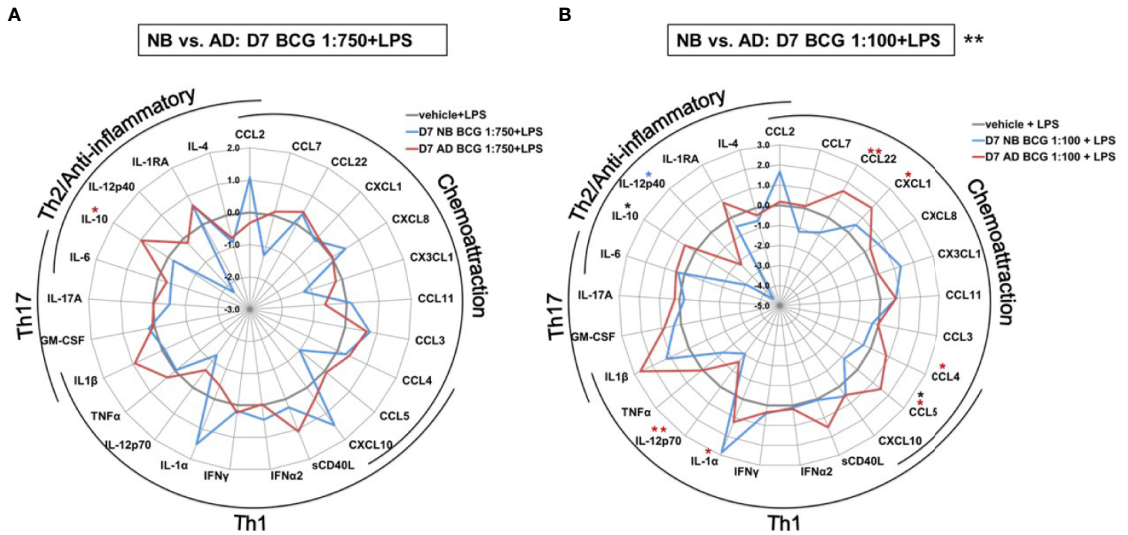


**FIGURE 5** | Distinct LPS-induced cytokine production by BCG-trained human newborn vs. adult monocytes at Day 7 of culture. Human newborn and adult CD33+ monocytes were cultured *in vitro* as described in **Figure 1**. Cytokines and chemokines were measured by Multiplex assay. Data shown for BCG concentrations **(A)** 1:750 vol/vol and **(B)** 1:100 vol/vol. Data was normalized to RPMI control, $\log_2$ transformed, and represents $\log_2$ fold-change. N = 5 newborns and 7 adults; $^*p < 0.05$; $^{**}p < 0.01$ (blue stars: NB *vs.* vehicle; red stars: AD *vs.* vehicle; black stars: NB *vs.* AD). Vehicle-(re)stimulated conditions shown in gray.

potentially accounting for pathogen- agnostic protection and clinical benefit, the underlying mechanisms for these heterologous effects in early life are incompletely characterized (29). Herein, we demonstrate for the first time that BCG has distinct age-specific effects on human newborn Mos, including distinct primary innate cytokine responses as well as trained immunity.

To characterize age-specific effects of BCG, we utilized a human *in vitro* trained immunity platform using primary human Mos cultured in autologous serum to compare newborn and adult Mo primary and trained responses to BCG. We view the use of autologous plasma or serum (intact, i.e. not heat-treated and from the same individual), repleted with age-specific immune factors such as maternal antibodies, adenosine and prostaglandins (30), as an important element of our design which strives to remain faithful to physiologic conditions that are relevant *in vivo*.

Compared to their adult counterparts, human newborn Mos responded to BCG in a fundamentally distinct manner. With respect to the primary response to BCG, newborns responded more robustly to the low concentration (1:750 vol/vol) relative to RPMI, as demonstrated by significantly enhanced production of Th1 (IL-12p70 and TNF), Th2 (IL-6 and IL-10) and Th17-(IL-1ß and GM-CSF) polarizing cytokines, as well as of the chemoattractant CXCL1. A possible explanation for the enhanced response of newborn Mos to low-dose BCG compared to adult Mos could be age-dependent differences in the magnitude of TLR responses (31–33), bearing in mind that BCG activates TLR8 (34) that is a power activating pathway in the newborn (35). The complex nature of BCG as a live mycobacterial stimulus that activates multiple PRRs (36) likely explains primary production of broadly acting cytokines. IL-12p70 induces cytotoxic T cell responses as well as high and broad humoral immune responses (37). TNF concentrations *in vitro* have been used as a benchmark cytokine for BCG-trained immunity in adults (17), while IL-1β production is implicated in BCG-trained innate immunity in adults and low birth weight infants (4, 38). GM-CSF may contribute to the host response against mycobacterial infection by favoring macrophage M1 polarization after *Mycobacterium bovis* BCG infection (39), as well as regulating the neutrophil-mediated inflammatory response, which mediates BCG-induced protection in a mouse model of neonatal polymicrobial sepsis (8).

Adults overall responded more robustly to the higher concentration of BCG (1:100 vol/vol), and specifically exceeded neonatal responses in production of IL-12p40 and CXCL10 (formerly IP10). In addition to its chemotactic properties, CXCL10 is also involved in the stimulation of natural killer and T-cell migration in response to *Mycobacterium tuberculosis* infection (40). Selective induction of the IL-12p40 component of the IL-12 cytokine and subsequent development of T-follicular helper cells in the lymph node *via* upregulated IL-12-receptor signaling is a unique feature of live vaccines. Such BCG-induced IL-12 pathway activation is mediated *via* sensing of viability by TLR8 whose functional alleles correlate with protection vs. pulmonary TB in BCG-immunized adults, and is not observed with killed vaccines (34).

With respect to BCG-trained Mo responses to subsequent stimulation with LPS, neonatal Mos demonstrated a distinct profile. The directionality and magnitude of cytokine production of BCG-primed/LPS-restimulated newborn Mos was BCG-concentration dependent. Specifically, unlike BCG-trained

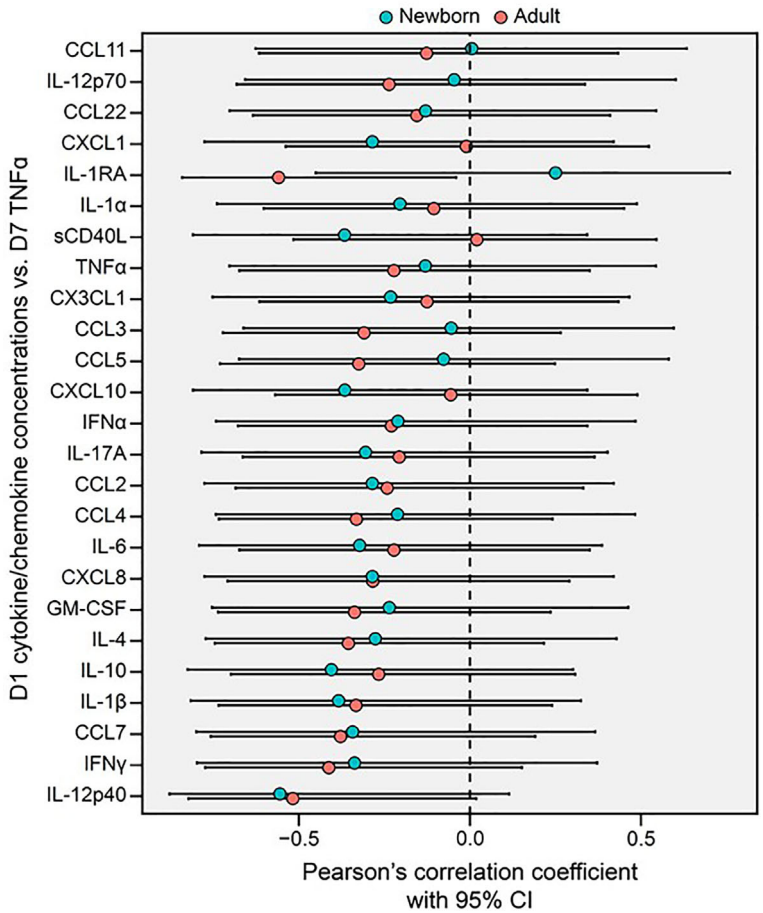


**FIGURE 6** | Primary BCG-induced cytokine/chemokine concentrations (Day 1, D1) of human monocytes inversely correlate with their trained TNF cytokine concentrations (Day 7, D7) in newborns and adults. A Forest plot depicts pairwise comparisons between newborn and adult D1 cytokine/chemokine *vs.* D7 TNF correlations. Correlations between the cytokine and chemokine data depicted in **Figures 3**, **5** were quantified using Pearson's coefficient. Error bars represent the associated 95% CI.

adult Mos that demonstrated enhanced LPS-induced cytokine and chemokine production, BCG-trained newborn Mos displayed decreased LPS-induced TNF production. Multiplex analysis revealed that at the low concentration (1:750 vol/vol), BCG-trained adult Mos demonstrated greater LPS-induced IL-10 production. In contrast, at the high BCG concentration (1:100 vol/vol), BCG-trained newborn Mos demonstrated diminished LPS-induced IL-10 and CCL5 production compared to adult Mos, and significantly decreased IL-12p40 production compared to RPMI control-treated Mos. A similar tolerogenic response has been previously reported in whole blood of BCG-vaccinated infants, who demonstrated increased production of IFN-γ in response to mycobacterial stimulation, but decreased production of IFN-γ in response to subsequent heterologous stimulation and TLR agonists, as compared to BCG-naïve infants (41).

Correlations between BCG-induced human Mo production of individual cytokines/chemokines on Day 1 and subsequent LPS-induced TNF production on Day 7, could serve as novel cytokine/chemokine biomarker signatures of BCG-induced training in adults and tolerance in newborns. We found a significant moderate negative correlation in adults between primary IL-1RA and trained TNF production, while this correlation was positive in newborns. As IL-1β has an established role in trained immunity in adults (38), and IL-1RA may prevent IL-1β binding to its receptor, the interplay between these two cytokines in trained immunity and in particular their role in neonatal trained immunity is worthy of further exploration. In neonates, BCG vaccine induces production of IL-12, the primary cytokine that drives CD4+ T cell Th1 differentiation, in a TLR2-dependent manner (42). Our results indicate that early interaction of BCG with Mos shapes their subsequent responses to LPS, a heterologous innate stimulus and raises the possibility that the cytokines induced early may engage counter-regulatory pathways.

Overall, to the extent that our *in vitro* results are relevant *in vivo*, our observations suggest that BCG-induced trained immunity in the neonate may attenuate an overwhelming inflammatory response to potentially noxious subsequent stimuli. With respect to TNF production, after the initial phase of primary innate immune

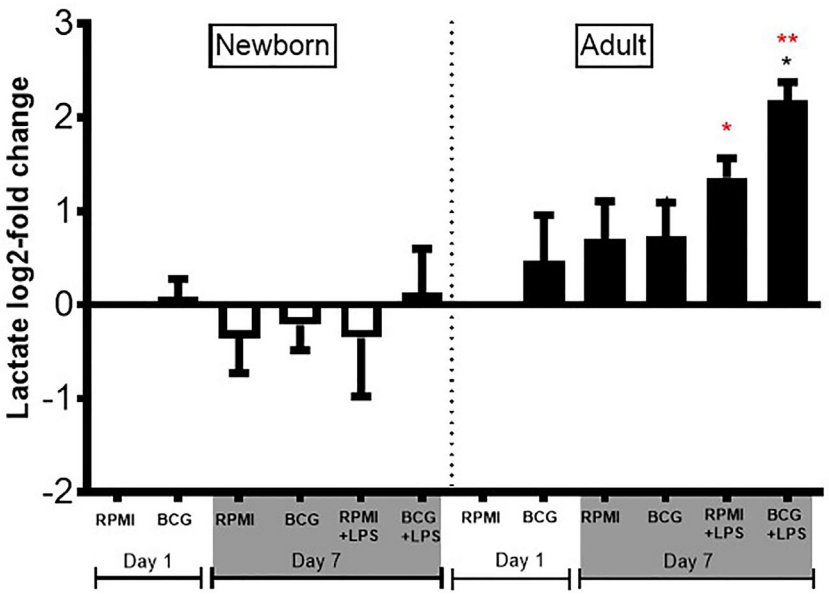


**FIGURE 7** | Diminished lactate production in human newborn vs. adult monocytes. Human newborn and adult CD33+ monocytes were cultured *in vitro* as described in **Figure 1**. Lactate was measured in culture supernatants post-primary BCG stimulation (Day 1) and post-secondary LPS stimulation (Day 7) using a colorimetric assay as described in *Methods*. Lactate production was normalized to the vehicle condition on Day 1 and $log_2$-transformed. N = 4 newborns and 4 adults. Bars indicate mean + SD. $^*p \leq 0.05$, $^{**}p \leq 0.01$ (black star: $log_2$-fold change compared to untrained control RPMI+LPS at Day 7; red stars: NB *vs.* AD for the respective conditions).

activation resulting in increased production and enhanced chemotaxis, BCG appears to re-wire neonatal Mos for a tolerogenic response to subsequent stimulation, as occurs in bacterial sepsis. However, the immune system likely seeks homeostasis and one way to prevent immunoparalysis, the extreme version of immune tolerance, is through concomitant decreased production of anti-inflammatory cytokines such as IL-10 and IL-12p40. Concurrently, decreased chemotaxis and angiogenesis may prevent untoward inflammatory sequelae. The overall pattern of BCG-trained Mo cytokine and chemokine responses suggests that neonatal Mo responses may serve a different purpose compared to adult Mo responses, where training seems to cause Th1 polarization of the innate immune response and enhancement of the inflammatory response.

A prior *in vitro* study comparing human cord blood Mos and adult peripheral blood Mos suggested similar cytokine production after BCG priming and LPS restimulation (43). Multiple differences in study design between our study and the prior one could account for our distinct findings, including a different study population (US-based cohort in our study *vs.* Norwegian in the prior study), use of different BCG formulations (BCG-Denmark in our study *vs.* BCG-Bulgaria in the prior), blood collection from BCG-naïve adult study participants in our study *vs.* previously BCG-immunized in the prior, the use of heparin *vs.* citrate (a calcium chelator) for blood collection, the use of untreated autologous newborn *vs.* adult serum in our study vs. 10% pooled sterile serum from humans of undescribed age in the prior study, which could obscure soluble plasma-based ontogenic differences that shape immune responses (30), and the method for Mo isolation (isolation of Mos by gradient-centrifugation in our study vs. adherence, which is an activating step, in the prior study). Different licensed BCG formulations vary substantially in their immune-stimulating capacity, including in induction of IL-1β, a cytokine key to trained immunity (38), correlating with differences in viability (44). This is especially notable in light of growing literature regarding differences between BCG vaccine formulations/strains in effectiveness in preventing tuberculosis and unrelated infections (45), with BCG-Denmark being the most frequently studied formulation for its trained immunity inducing properties *in vivo* and *in vitro* (29). Overall these multiple differences in study design, could have contributed to the prior study not demonstrating differences in cytokine production between newborns and adults.

Upon training with BCG and subsequent stimulation with LPS, whereas adult Mos demonstrated robust ~2 to 3 $log_2$-fold higher lactate production compared to RPMI, newborn Mos demonstrated little lactate production, almost comparable to vehicle control (RPMI). Similar age-dependent immunometabolic differences were recently observed in another study of activated human cord blood and adult macrophages (46). Of note, mean lactate production of neonatal Mos at baseline is slightly lower than that of adult Mos and directly correlated with pyruvate kinase activity, which is diminished in newborn vs. adult Mos but reaches adult levels halfway through infancy (47). Overall, these observations collectively suggest that glycolytic metabolism of newborn Mos differs from that of adult Mos, possibly contributing to the distinct age-specific BCG-induced Mo training. Global metabolomic

profiling of human newborn Mos may provide immunometabolic signals unique to BCG-trained immunity (48).

To the extent our *in vitro* results reflect the effects of BCG *in vivo*, the protective effects of BCG may in part rely on attenuating inflammatory responses to microbial products that signal *via* PRRs. The induction of training or tolerance appears to be dependent on the type and quantity of the microbial stimulus and host factors. BCG is a live and complex microbial stimulus that activates multiple PRRs, including TLRs (49–51), C-type lectin receptors (CLRs) (52), and NOD-like receptors (NLRs) (17). BCG training of adult Mos was associated with NOD-2-dependent epigenetic reprogramming (17). Among the PRRs, TLRs appear to play a prominent role in neonatal responses to immunization/infection as suggested by: a) an association between TLR polymorphisms and altered responses to neonatal BCG immunization (53), and b) selective predisposition to bacterial infection in young but not older children with genetic defects affecting TLR downstream signaling (IRAK-4, MyD88) (54). Of note, a microbial stimulus can elicit different responses when engaging different receptors. For example, LPS induces immunosuppressive effects when engaging TLRs vs. immunopotentiating effects when engaging NLRs (55). Innate immune memory responses are complex and depend on the age of the exposed, timing of exposure and properties of the stimulus. Of note, while neonatal Mos express similar quantities of TLRs as their adult counterparts (55, 56) the downstream consequences of TLR activation are distinct with age (8). Whether the BCG-induced tolerance to LPS in neonatal Mos observed in our study is related to TLR-mediated epigenetic reprogramming will be an important area of future investigation.

Our study features multiple strengths, including (a) use of species (human)- and age (newborn)-specific Mos cultured in autologous serum, (b) Mo-selection through CD33 instead of CD14 to avoid activation, (c) study of a licensed WHO-prequalified BCG formulation/strain, (d) assessment of BCG concentration-dependent effects, (e) study of both primary (24-hour stimulation) and trained (LPS-induced cytokine production at Day 7) immune effects, and (f) measurement of metabolic activation in the form of lactate production.

As with any research, our study also has some limitations including (a) an *in vitro* approach that likely does not capture all of the immunologic effects of BCG *in vivo* (17), (b) an exclusive focus on myeloid CD33+ mononuclear cells which, although important to BCG responses *in vitro* and *in vivo* (51), will not capture the full range of relevant human leukocyte responses to this live vaccine, and (c) a focus on LPS as a secondary stimulus which may not reflect responses to other PRR agonists. Spontaneous *in vitro* differentiation of Mos over time towards macrophage phenotypes is possible and has been previously described in culture medium supplemented with autologous serum (57). Detailed immunophenotyping assessment of BCG-treated human newborn and adult Mos should be pursued in future studies to provide a fuller picture of BCG's age-dependent effects. Given the marked variability between BCG formulations/strains (44), future studies should also directly compare and characterize the impact of a range of BCG formulations/strains on the subsequent responses of a range of human leukocytes and innate stimuli.

In summary, BCG-induced training of human Mos is age-dependent, suggesting that immune ontogeny may shape primary and trained innate cytokine responses to BCG. Much remains to be learned about alterations in neonatal immune function following infection/vaccination during this critical period of immune system adaptation and development. Using BCG as a model to characterize distinct trained immunity in newborns may inform discovery and development of novel adjuvants, vaccines and immunotherapies for this vulnerable population (56).

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Raw data files for cytokines, chemokines and lactate concentrations were deposited in ImmPort under accession number: SDY1790.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board (IRB) of the Beth Israel Deaconess Medical Center, The Brigham & Women's Hospital, Boston, MA and Boston Children's Hospital, Boston, MA. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

AA was the project lead and wrote the manuscript draft. AA, MGC, ML, and SvH collected and processed the samples and generated the data. MGN and BAB assisted with establishment of the trained immunity assay. AA, JD-A and AO analyzed the data. AA, SvH and OL interpreted the results. GS-S provided key intellectual input and edited the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.674334/full#supplementary-material

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**Conflict of Interest:** OL is a named inventor on vaccine adjuvant patent applications as well as an issued patent on an *in vitro* microphysiologic tissue construct platform for vaccine evaluation.

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REVIEW
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# Childhood Vaccinations and Type 1 Diabetes

*Susanna Esposito*[1]*, *Elena Mariotti Zani*[1], *Lisa Torelli*[1], *Sara Scavone*[1], *Maddalena Petraroli*[1], *Viviana Patianna*[1], *Barbara Predieri*[2], *Lorenzo Iughetti*[2] *and Nicola Principi*[1]

[1] Paediatric Clinic, Department of Medicine and Surgery, University Hospital, University of Parma, Parma, Italy, [2] Pediatric Unit, Department of Medical and Surgical Sciences of the Mothers, Children and Adults, University of Modena and Reggio Emilia, Modena, Italy

Type 1 diabetes (T1D) is the most common paediatric endocrine disease, and its frequency has been found to increase worldwide. Similar to all conditions associated with poorly regulated glucose metabolism, T1D carries an increased risk of infection. Consequently, careful compliance by T1D children with schedules officially approved for child immunization is strongly recommended. However, because patients with T1D show persistent and profound limitations in immune function, vaccines may evoke a less efficient immune response, with corresponding lower protection. Moreover, T1D is an autoimmune condition that develops in genetically susceptible individuals and some data regarding T1D triggering factors appear to indicate that infections, mainly those due to viruses, play a major role. Accordingly, the use of viral live attenuated vaccines is being debated. In this narrative review, we discussed the most effective and safe use of vaccines in patients at risk of or with overt T1D. Literature analysis showed that several problems related to the use of vaccines in children with T1D have not been completely resolved. There are few studies regarding the immunogenicity and efficacy of vaccines in T1D children, and the need for different immunization schedules has not been precisely established. Fortunately, the previous presumed relationship between vaccine administration and T1D appears to have been debunked, though some doubts regarding rotavirus vaccines remain. Further studies are needed to completely resolve the problems related to vaccine administration in T1D patients. In the meantime, the use of vaccines remains extensively recommended in children with this disease.

**Keywords: autoimmunity, infection, type 1 diabetes, vaccination, vaccine**

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*Novella Rapini,*
*Bambino Gesù Children Hospital (IRCCS), Italy*

***Correspondence:**
*Susanna Esposito*
*susannamariaroberta.esposito@unipr.it*

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## BACKGROUND

The anti-infective vaccines included in the immunization schedule adopted by most countries for protecting children can cause several problems when these prophylactic measures have to be given to subjects at risk of or with overt type 1 diabetes (T1D). T1D is the most common paediatric endocrine disease, and its frequency has been found to increase worldwide with relevant medical, social and economic issues (1). According to the International Diabetes Federation, it was estimated that more than 1.1 million children and adolescents around the world were living with T1D in 2019 compared to 860,000 in 2013 (2). Similar to all conditions associated with poorly regulated glucose

metabolism and persistent hyperglycaemia, including type 2 diabetes (T2D), T1D carries an increased risk of infection. Consequently, careful compliance by T1D children with schedules officially approved for child immunization by national governments is strongly recommended by scientific societies (3).

A list of vaccines for the prevention of the most common infectious diseases diagnosed in adults with T1D and T2D is recommended for children with T1D (4). However, because patients with T1D show persistent and profound limitations in immune function (5, 6), vaccines may evoke a less efficient immune response, with corresponding lower protection. A larger use of boosters to maintain elevated protection has been suggested for some vaccines (3). Moreover, T1D is an autoimmune condition (7–11) that develops in genetically susceptible individuals (12), when epigenetic or environmental factors act as triggers and modulate the penetrance of susceptibility genes (13). For example, associations of T1D development with some nutrients such as cow's milk and gluten as well as increased maternal age and rate of postnatal growth, vitamin D deficiency, chemical exposure, and gut dysbiosis have been suggested (14–18). Nevertheless, most data regarding T1D triggering factors appear to indicate that infections, mainly those due to viruses, play a major role. Enteroviruses and herpesviruses have frequently been associated with T1D development, but other viruses, including some of those used to prepare vaccines such as rotavirus, influenza viruses, rubella and mumps viruses, have also been found to cause pancreatic infection and autoimmunity (19). Accordingly, the use of vaccines is being debated, as this hypothesis is reinforced by some epidemiological evidence (20–22). In this narrative review, these issues are discussed to define the most effective and safe use of vaccines in patients at risk of or with overt T1D.

# INFECTIONS IN TYPE 1 DIABETES PATIENTS

## Risk of Infection

Together with multisystem microangiopathy and macrovascular disease (23), immune compromise is the most common complication of poor glycaemic control. The immune response is disrupted in any type of diabetes, and both the innate and adaptive immune systems are impaired. Defects in pathogen recognition, suppression of cytokine production, poor neutrophil and macrophage recruitment and function, alteration in natural killer cell activity, and inhibition of antibodies and complement effectors have been repeatedly reported in both experimental animals and humans with T1D and T2D (24). Indeed, despite a few exceptions (25, 26), studies involving adults have clearly shown that patients with T1D are at increased risk of infection (27–29). In a 12-month prospective cohort study carried out in the Netherlands from May 2000 through April 2002, it was demonstrated that the incidence of lower respiratory tract infections was significantly higher among 705 T1D patients than among 18,911 controls (adjusted odds ratio [AOR], 1.42; 95% confidence interval [CI] 0.96–2.08), with urinary tract infection (AOR, 1.96 [95% CI 1.49–2.58]), bacterial skin and mucous membrane infections (AOR, 1.59; 95% CI 1.12–2.24) and mycotic skin and mucous membrane infection (AOR, 1.34; 95% CI 0.97–1.84) being common (30).

Furthermore, risk increased with recurrence. These findings were confirmed by a more recent study performed using English primary care data collected during 2010-2015 in which the incidence and outcome of infections were related to the degree of T1D severity measured through HbA1c evaluation (31). In this study, 5,863 T1D patients were matched with 8,231 controls, and patients requiring a prescription or hospitalization or who died were compared. The results showed that long-term infection risk rose with increasing HbA1c for most outcomes. Hospitalization for infection was significantly more frequent in patients with T1D than in controls (incidence rate ratio [IRR] 3.34; 95% CI 2.82–3.96), and poor glycaemic control was associated with an increased risk: subjects with HbA1c ≥11% had an IRR of 8.47 (95% CI 5.86-12.24), whereas those with optimal control had an IRR of 1.41 (95% CI 1.36–1.47). The largest relative associations between the poorest level of glycaemic control and optimal control were seen for bone and joint infections, endocarditis, and sepsis. In addition, a strict relationship between poor glycaemic control and infection severity has been recently shown in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-infected patients. T2D was found to be among the most common medical condition in adult patients developing COVID-19 (32), and it was associated with an almost fourfold greater risk for severe disease and death (OR 3.68, 95% CI 2.68–5.03; $P < 0.001$) (33).

Unfortunately, the incidence of infections in T1D paediatric patients has been poorly studied. Most of the available evidence comes from studies conducted in adults and the evidence suggesting a higher risk of infections in children with T1D is extremely weak. On the other hand, it is common experience of clinicians looking after these patients that they do not show any increased risk of infections, especially severe infections. Nonetheless, as children with poor glycaemic control have immune and metabolic disorders similar to those found in adults, it seems likely that children may have a risk of infection substantially similar to that in adults. Support for this hypothesis can be found in a retrospective study in the USA using data collected from 2008 to 2014 at 44 freestanding children's hospitals across the country (34). The authors analysed the clinical characteristics of children and adolescents with T1D who presented to the emergency department (ED) or were hospitalized for infection management. A total of 104,739 cases were studied: 34,332 visited the ED, and 60,407 visited the hospital. The data showed that medical attention for infections is routinely given to paediatric patients with T1D and that the need for assistance for these patients increases over time in parallel with the increase in T1D cases, with a relevant impact on assistance costs that increased from $189 to $218 million dollars per year. Considering COVID-19, overall, the accumulating evidence suggests that children with T1D infected with severe

acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have similar disease outcomes as peers without diabetes (35, 36).

### Type of Infections in Patients With Type 1 Diabetes (T1D)

As previously highlighted, some infections are common in T1D patients. Respiratory infections caused by *Mycobacterium tuberculosis, Staphylococcus aureus*, gram-negative bacteria and fungi may occur with an increased frequency (37, 38). Infections due to *Streptococcus pneumoniae* (39) and influenza viruses deserve particular attention because they are extremely more common than in healthy subjects and are associated with a significantly increased risk of hospitalization and death (40, 41). Skin and soft tissue infections ranging from folliculitis, furunculosis, and subcutaneous abscesses to necrotizing fasciitis are frequently caused by methicillin-resistant *S. aureus* and *Staphylococcus epidermidis* (42). Urinary tract infections are described in diabetic subjects up to 10 times more frequently than in healthy subjects. Moreover, these infections are 4 times more commonly associated with bacteraemia than in healthy subjects and are frequently due to multi-drug-resistant microbes (43). Among gastrointestinal diseases, hepatitis B (HB) (44), hepatitis C (45) and oral and oesophageal candidiasis (46) are common in those with T1D.

## IMMUNE RESPONSE AND CLINICAL EFFICACY OF RECOMMENDED VACCINES IN PATIENTS WITH TYPE 1 DIABETES (T1D)

Children and adolescents with T1D are considered a special population requiring vaccination according to the immunization schedule recommended for healthy subjects, with particular attention to pneumococcal and influenza vaccines. Boosters of pneumococcal vaccines may be necessary, and influenza vaccines must be rigorously administered each year (3). In adults with T1D, there are specific recommendations for the administration of the hepatitis B (HB) vaccine, tetanus/diphtheria/acellular pertussis vaccine, pneumococcal vaccine, influenza vaccine and herpes zoster vaccine according to age and previous immunizations (4).

However, despite the well-known impairment of immune system function in T1D patients, the immune response to commonly recommended vaccines in these subjects has been poorly studied. Moreover, the results of the few available studies are conflicting and do not allow us to draw definitive conclusions regarding the real protection offered by the different vaccines in a single T1D patient. Evaluation of the immune response of 20 T1D patients to hepatitis A (HA), diphtheria and pneumococcal polysaccharide vaccines showed that T1D patients had a significantly impaired primary antibody response to HA vaccine ($P = 0.017$) and diphtheria toxoid ($P = 0.004$) compared to healthy controls but that the response to pneumococcal polysaccharide was normal (47). In another study enrolling 36 children with T1D and a similar number of age-matched healthy controls in which the immune response to conjugate pneumococcal vaccine, Hib vaccine and tetanus/diphtheria vaccine was compared, no difference in antibody levels against the antigens tested was found between the groups. However, after a booster dose, the median level against pneumococcal serotypes was significantly lower in the T1D patients than in the controls (2.3 g/mL [range, 0.05 to 664.7 g/mL] and 6.1 g/mL [0.12 to 203.36 g/mL]), respectively, suggesting reduced immune memory in the former (48).

Regarding the immune response to the HB vaccine, studies carried out approximately 20 years ago showed that both the immediate and long-term immune responses to the HB vaccine in T1D patients did not differ from those of healthy subjects (49–54). Paradigmatic in this regard are the studies of Marseglia et al., who monitored HBsAb titres immediately after the usual schedule of immunization (0, 1 and 6 months) as well as 4 years later in T1D children/young adults and healthy subjects (4.5 to 27.5 years of age). In both cases, the immune response was similar in the T1D patients and controls. A few weeks after the booster dose (49), 3 (4.6%) of 65 T1D patients and 3 (1.7%) of 174 age- and sex-matched healthy subjects were considered to have a low (HBsAb titre = 10 IU/L) or no (HBsAb titre, < 2 IU/L) response. Moreover, the median HBsAb titre was similar in the responding patients (120 IU/L) and controls (125 IU/L). There were no significant correlations between antibody titre and age, diabetes duration, or HbA1c or insulin requirement. After 4 years, mean anti-HBs log-titres were 1.95 ± 0.88 in T1D patients and 2.18 ± 0.64 in controls (P=0.11). Additionally, the number of subjects with protective antibody concentrations (anti-HBs >10 IU/l) was 50/54 (92%) among T1D patients and 67/70 (96%) among controls (P=0.70) (50). More recent studies have shown the opposite. Leonardi et al. reported significantly more common detection of protective serum anti-HBs antibody levels in previously immunized children among healthy subjects (84%) than patients with diabetes (58.2%) ($P < 0.0001$), regardless of age or duration and metabolic control of T1D. Moreover, among children with antibodies, the T1D children had significantly lower antibody values (58 ± 112.9 mIU/mL *vs* 266.49 ± 335.85 mIU/mL, respectively; $P < 0.0001$) (55). Similar results were reported by Elrashidy et al., who found protective anti-HBs levels in only 30.2% of children with the disease compared to 60% of healthy controls ($P < 0.001$), which was independent of the age of patients and the duration of T1D (56). Finally, by analysing the serological response to HBV vaccine in 69 T1D patients and 79 healthy controls who had received the third dose 6.8 and 4.7 years prior, Zanoni et al. (57) showed that although the total number of subjects with protective antibody levels was quite similar in both groups (72% *vs* 77%, respectively), mean serum anti HBs antibody concentrations were lower in the patients than in the controls (75 ± 149 mIU/mL *vs* 169 ± 268 mUI/mL, respectively; $P = 0.0068$).

Dissimilar results were also reported when the influenza vaccine was evaluated. A study enrolling 105 T1D subjects aged 9-30 years who were randomized to receive either a virosomal or a standard subunit influenza vaccine showed that

serum haemagglutinin inhibition antibody titres against the three viruses included in the vaccines at one month post vaccination met the requirements for immunogenicity, with high seroprotection rates (>95%) for strains A/H1N1 and A/H3N2 and seroprotection of 73% and 70% for the virosomal and subunit vaccine for strain B, respectively (58). Similar results were obtained by the same authors in a further study in which T1D paediatric patients who received an influenza MF59-adjuvanted vaccine were evaluated (59). However, in a previous study, it was found that the incidence of non-response to the H3N2 and influenza B components of a trivalent vaccine was substantially lower in T1D patients than in healthy controls (100% *vs* 78% and 80 *vs* 44%, respectively, $p<0.05$). Moreover, the delayed-type hypersensitivity reaction to influenza antigen was significantly decreased in patients with worse glycaemic control ($P < 0.01$) (60).

Overall, the real efficacy of immunization in T1D patients has not been established. As most T1D paediatric patients receive the recommended vaccines at the proper time, the effect of no vaccination cannot be easily evaluated. In fact, in a systematic review and meta-analysis of the effectiveness of influenza vaccines in patients with diabetes published in 2015 (61), no data for children with T1D could be analysed because relevant studies were unavailable. Furthermore, studies carried out in adults generally consider patients with T1D and T2D together, and the importance of T1D in conditioning the efficacy of vaccines has not been evaluated. Regardless, the previously cited systematic review and meta-analysis indicates that most studies to date have a very low quality that makes it impossible to determine to what extent vaccines are effective, even though they suggest some beneficial effects of influenza immunization for patients with diabetes.

## RISK OF TYPE 1 DEVELOPMENT AFTER ADMINISTRATION OF VACCINES

A number of experimental and clinical observations have suggested a potential relationship between infection and T1D development. In experimental animals, viral infections, particularly those due to coxsackieviruses, may cause pancreatic infections and lead to T1D development. In humans, the association between recurrent respiratory tract infections in the first semester of life and the development of pancreatic islet autoimmunity with overt T1D at approximately 8 years of age have been reported (62, 63). Enterovirus (EV) epidemics have also been associated with an increased incidence of T1D. For instance, evidence of infection and detection of EV in the blood and stool were several times more common in children with T1D than in controls (64–67). Similar, although less stringent, results have been obtained for Epstein-Barr virus (68, 69). Among viruses included in vaccines, influenza (70), rubella (71), mumps (72) and rotavirus (73) were initially considered potential triggers of T1D, though the results of recent studies seem to exclude this risk for influenza (74) as well as mumps and rubella (75). Doubt remains with regard to rotavirus, though a higher incidence of T1D among children with clear evidence of a previous rotavirus infection has been reported (76).

Four mechanisms have been proposed to explain how viruses lead to autoimmunity: molecular mimicry, in which virus proteins bearing similar sequences to pancreatic beta cell components activate autoreactive T cells (77); bystander activation, in which beta-cell proteins released during viral infection are captured by antigen-presenting cells that present host epitopes and activate immune response (78); epitope spreading, in which immune responses to endogenous epitopes secondary to the release of self-antigens during viral induced chronic inflammatory pancreatic disease are the basis of autoimmunity (79); and cryptic antigens, in which cryptic self-determinants are presented to T cells in amounts sufficient to induce autoimmunity (80). For molecular mimicry, this hypothesis has been substantiated by several clear lines of evidence. As an example, potential cross-reactivity between structural components of coxsackievirus and human cytomegalovirus and a pancreatic beta-cell component has been reported (81, 82) as well as between the VP1 protein of enterovirus and the beta-cell antigen tyrosine phosphatase IA-2 (83). There are also similarities between islet antigen-2 (IA-2), an autoantigen associated with T1D, and the VP7 protein of a human G3P rotavirus strain. Moreover, cross-reactivity of T cells generated against rotavirus VP7 peptide with IA-2, and vice versa, has been reported (84).

The hypothesis that vaccines might have the same potential role already reported for some viruses and trigger T1D development was initially strongly substantiated by a number of studies. In most cases, the temporal association between vaccine introduction in the immunization schedule of infants and children and the sudden increase in T1D incidence in the same paediatric population was considered key for demonstrating that vaccines might cause T1D. For example, clustering of T1D cases at approximately 2-4 years after *Haemophilus influenzae* type B (Hib) vaccine, pertussis vaccine, combined measles, mumps, rubella (MMR) vaccine, and BCG vaccine administration has been reported (85). As the time distance between the onset of autoantibodies against pancreatic beta cells and the development of overt T1D is generally the same, this was considered strong evidence of the risk related to vaccine use. A relationship between vaccine and T1D development and the time of the first vaccine administration was proposed. Certain vaccines, such as the HB vaccine and BCG vaccine, might decrease the risk of developing T1D if given at birth; first vaccination at 2 months of life or later might also increase the risk (86). However, most experts did not attribute significant importance to these findings, and recommendations for infant and child immunization were not modified. The results of these studies were debated mainly because most of them had significant methodological limitations, enrolling a small number of unvaccinated subjects or being statistically underpowered. Moreover, these reports were counterbalanced by a large number of studies showing that vaccines were safe and not associated with an increased risk

of T1D development, even when infectious agents included in the vaccines had been found to be associated with this disease. A protective effect was even evidenced in some studies (75). The findings of these studies can be illustrated by some examples. In a case-control study carried out in Sweden that included 339 cases and 528 controls (87), BCG, smallpox, pertussis, tetanus, rubella, and mumps vaccines had no influence on T1D epidemiology, whereas measles vaccine was associated with protection from T1D development (OR = 0.69; 95% CI 0.48-0.98). In a retrospective study carried out in Canada in which BCG vaccination was evaluated, a trend in favour of a protective effect of the vaccine was found, even though the small number of children receiving the BCG vaccine did not allow for drawing firm conclusions (88). Among children vaccinated at birth, only one (3.3%) was diagnosed with T1D by the age of 5 years, compared with 52 (24.5%) who had not been vaccinated ($P < 0.01$) (88). A 10-year follow-up study carried out in Finland, where a relationship between Hib vaccine and T1D development had been speculated a few years after introduction of the vaccine (83), did not implicate this vaccine regard and showed no significant difference in risk between children vaccinated against Hib at the age of 3 months and at the age of 24 months (89). A large, population-based, case-control study carried out in the USA reported that none of the evaluated vaccines was associated with an increased risk of T1D. The OR for the association with T1D was 0.28 (95% CI 0.07–1.06) for the whole cell pertussis vaccine, 1.36 (95% CI 0.70–2.63) for MMR, 1.14 (95% CI 0.51–2.57) for Hib, 0.81 (95% CI 0.52–1.27) for the HB vaccine, 1.16 (95% CI 0.72–1.89) for the varicella vaccine, and 0.92 (95% CI 0.53–1.57) for acellular pertussis-containing vaccines. Regarding the HB vaccine, it was shown that the vaccine was safe and that the risk of T1D did not differ between children at birth and those vaccinated later (90). A study in children with an increased genetic risk for T1D who received the influenza vaccine during the A/H1N1 2009 pandemic showed that this vaccine was not associated with an increased risk of islet autoimmunity, multiple islet autoantibodies or type 1. The hazard ratio [HR] (95% CI) for the appearance of at least one islet autoantibody was 0.75 (0.55-1.03), for at least two autoantibodies was 0.85 (0.57-1.26) and for T1D was 0.67 (0.42-1.07) (90). Regarding the HPV vaccine, no risk of T1D was found after HPV vaccine administration in two French studies (OR 1.2; 95% CI 0.4-3.6 in the first and HR 1.07; 95% CI 0.87-1.31 in the second) (91, 92). Similar results were reported in a retrospective cohort study carried out in the USA in which no increased risk of T1D associated with the HPV vaccine was found over the 10 years of the study period when comparing vaccinated with unvaccinated subjects (HR 1.21; 95% CI 0.94-1.57) (93). Moreover, autoimmune-specific safety analyses performed separately as part of this larger safety study noted a decreased association between HPV and new-onset T1D (HR 0.57; 95% CI 0.47-0.73) (94).

All these findings seem to indicate that the vaccines usually recommended for child protection are safe and not associated with the risk of T1D development, though it was not definitively established whether a certain degree of protection might be associated with very early administration of one or more vaccines. Some doubts may still exist for rotavirus vaccines. In general, the results of recently performed studies are conflicting, and a global evaluation of available data does not allow for firm conclusions, though the populations included in each database are quite different, as are the assumptions, inclusion criteria, and methods used for analysis. Moreover, it cannot be excluded that differences among studies are related to population variations in genetic background or other factors found to be associated with an increased risk of T1D development. Two studies carried out in Finland comparing the incidence of T1D in children with or without rotavirus vaccination showed no difference between the groups at short or long-time frames since immunization. The first study examined children at 4-6 years of age, and the absolute risk reduction of T1D development was 0.91 (95% CI 0.69–1.20) (95). In the second study enrolled children who had received the vaccine 11-14 years before, and the prevalence of T1D was similar in both groups, at 0.97% (25 of 2,580 children) in the control group and 1.04% (33 of 3,184 children) in the vaccine group ($P = 0.810$) (96). Conversely, completely different results have been reported by other studies. In studies carried out in Australia (97) and in the USA (98), vaccines were found to exert a protective effect, as the incidence of T1D measured before and after vaccine introduction decreased by 15% (relative risk [RR] 0.86; 95% CI 0.74-0.99) and 33% (HR 0.67; 95% CI 0.54-0.83), respectively, in vaccinated children. However, in the USA, differences between vaccines were attributed to a stronger effect of the pentavalent vaccine compared to the monovalent vaccine. Moreover, two very recent studies in which several sensitivity analyses to reduce the risk of bias were carried out did not find any influence of rotavirus vaccines on the risk of T1D (99, 100).

## CONCLUSIONS

T1D is not a rare disease. Nevertheless, several problems related to the use of vaccines in children with this disease have not been completely resolved, making administration of vaccines a challenge. T1D is considered a risk factor for infection development, and based on the incidence of infections in adults, mainly those with T2D, it is presumed that children are also at an increased risk of infection. However, data in this regard are scant, and the infections that must be monitored in children have not been established. To reduce the risk of infection, vaccines are strongly recommended in children with T1D. However, there are few studies regarding the immunogenicity and efficacy of vaccines in T1D children, and the need for different immunization schedules has not been precisely established. Fortunately, the previous presumed relationship between vaccine administration and T1D development appears to have been debunked, though some doubts regarding rotavirus vaccines remain. Further studies are needed to completely resolve the problems related to vaccine administration in T1D patients. In the meantime, the use of vaccines remains extensively recommended in children with this disease.

## AUTHOR CONTRIBUTIONS

SE designed the project, co-wrote the first draft of the manuscript and supervised the activities. EMZ, LT, and SS participated in the preparation of the manuscript and literature review. MP and VP performed the literature review. BP and LI gave a substantial scientific contribution. NP co-wrote the first draft, revised the manuscript and made substantial scientific contributions. All authors contributed to the article and approved the submitted version.

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# Humoral and Cellular Response Following Vaccination With the BNT162b2 mRNA COVID-19 Vaccine in Patients Affected by Primary Immunodeficiencies

*Donato Amodio[1†], Alessandra Ruggiero[1,2†], Mayla Sgrulletti[3,4,5], Chiara Pighi[1], Nicola Cotugno[1,3], Chiara Medri[1], Elena Morrocchi[1], Luna Colagrossi[6], Cristina Russo[6], Salvatore Zaffina[7], Gigliola Di Matteo[3,8], Cristina Cifaldi[8], Silvia Di Cesare[1,3], Beatrice Rivalta[3,5,8], Lucia Pacillo[3,5,8], Veronica Santilli[1], Carmela Giancotta[1], Emma Concetta Manno[1], Marta Ciofi Degli Atti[9], Massimiliano Raponi[10], Paolo Rossi[3,8], Andrea Finocchi[3,8], Caterina Cancrini[3,8], Carlo Federico Perno[6,11], Viviana Moschese[3,4,12‡] and Paolo Palma[1,3*‡]*

[1] Academic Department of Pediatrics (DPUO), Research Unit of Clinical Immunology and Vaccinology, Bambino Gesù Children's Hospital, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Rome, Italy, [2] Department of Neuroscience, Biomedicine and Movement Sciences, University of Verona, Verona, Italy, [3] Chair of Pediatrics, Department of Systems Medicine, University of Rome "Tor Vergata", Rome, Italy, [4] Pediatric Immunopathology and Allergology Unit, Policlinico Tor Vergata, Rome, Italy, [5] PhD Program in Immunology, Molecular Medicine and Applied Biotechnology, University of Rome Tor Vergata, Rome, Italy, [6] Microbiology and Diagnostic Immunology Unit, Bambino Gesù Children's Hospital, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Rome, Italy, [7] Occupational Medicine Unit, Bambino Gesù Children's Hospital, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Rome, Italy, [8] Academic Department of Pediatrics (DPUO), Immune and Infectious Diseases Division, Research Unit of Primary Immunodeficiencies, Bambino Gesù Children's Hospital, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Rome, Italy, [9] Clinical Pathways and Epidemiology Unit—Medical Direction, Bambino Gesù Children's Hospital, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Rome, Italy, [10] Medical Direction, Bambino Gesù Children's Hospital, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Rome, Italy, [11] Multimodal Medicine Research Area, Bambino Gesù Children's Hospital, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Rome, Italy, [12] UniCamillus—Saint Camillus International University of Health Sciences, Rome, Italy

Mass SARS-Cov-2 vaccination campaign represents the only strategy to defeat the global pandemic we are facing. Immunocompromised patients represent a vulnerable population at high risk of developing severe COVID-19 and thus should be prioritized in the vaccination programs and in the study of the vaccine efficacy. Nevertheless, most data on efficacy and safety of the available vaccines derive from trials conducted on healthy individuals; hence, studies on immunogenicity of SARS-CoV2 vaccines in such populations are deeply needed. Here, we perform an observational longitudinal study analyzing the humoral and cellular response following the BNT162b2 mRNA COVID-19 vaccine in a cohort of patients affected by inborn errors of immunity (IEI) compared to healthy controls (HC). We show that both IEI and HC groups experienced a significant increase in anti-SARS-CoV-2 Abs 1 week after the second scheduled dose as well as an overall statistically significant expansion of the Ag-specific CD4+CD40L+ T cells in both HC and IEI. Five IEI patients did not develop any specific CD4+CD40L+ T cellular response, with one of these patients unable to also mount any humoral response. These data raise immunologic concerns about using Ab response as a sole metric of

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*Sunil Kumar Saini, Technical University of Denmark, Denmark*

***Correspondence:**
*Paolo Palma*
*Paolo.palma@opbg.net*

*†These authors have contributed equally to this work*

*‡These authors share last authorship*

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protective immunity following vaccination for SARS-CoV-2. Taken together, these findings suggest that evaluation of vaccine-induced immunity in this subpopulation should also include quantification of Ag-specific T cells.

**Keywords: BNT162b2 mRNA COVID-19 vaccine, Comirnaty, SARS-CoV-2, COVID-19, inborn errors of immunity, vaccine efficacy, antigen-specific T cell, SARS-CoV-2 antibody**

## INTRODUCTION

Since the rapid spread of COVID-19 across the globe and the identification of SARS-CoV-2 genomic sequence, enormous international scientific and economic efforts have been made to develop safe and effective vaccines. In fact, in the absence of a specific treatment against the SARS-CoV-2, except for monoclonal antibodies that are licensed for few selected clinical conditions, the only strategy to combat the virus and control the pandemic is to vaccinate the population (1, 2). To date, in Europe, four vaccines against SARS-CoV-2 have been approved by the EMA agency; two mRNA vaccines and two attenuated adenovirus vector vaccines. Despite this, the vaccination campaign is proceeding in a non-homogeneous manner with significant differences among countries. The identification of at-risk categories led to prior vaccine administration to vulnerable populations, especially elderly and patients with comorbidities.

Individuals with inborn errors of immunity (IEI) have an increased susceptibility to infections that often affects the clinical outcome; thus, routine immunization represents a critical issue in this population and a precise vaccine schedule is recommended (3). Indeed, vaccine response may vary depending on the type of immune disorder; however, with caution for live attenuated vaccines where data are limited, an overall protective effect has been demonstrated with significant reduction of morbidity and mortality and of healthcare cost containment.

Theoretically, primary immunodeficient patients are assumed to be at high risk of developing severe COVID-19. Most studies have described the course of SARS-CoV-2 infection in antibody deficiency (4–8). More recently, Meyts et al. described a large international cohort of children and adults with IEI mostly experiencing a mild course of disease, although a higher frequency of young individuals admitted to ICU compared to the general population was observed (9).

Since IEI consist of more than 450 monogenic defects, and these reports only partially cover the broad spectrum of IEI disorders (10, 11).

In accordance with interim indications for primary immunodeficient patients (3), COVID-19 vaccines should be advised according to national vaccine schedule, unless contraindicated. IEI patients are characterized by a generally reduced or completely absent vaccine response, depending on the type of immune disorders (12). Given the heterogeneity of IEI disorders with various degrees of immune impairment, it is not possible to define general recommendations regarding immunization. As for routine immunization, it is reasonable to speculate that in these patients, the anti-SARS-CoV-2 vaccine response might be suboptimal, due to the impaired immune system. Thus, vaccination schedule in such vulnerable population needs an accurate assessment of risk-benefits to grant both the best possible protection and avoid unnecessary adverse events. Additional knowledge on the safety and effectiveness of SARS-CoV-2 vaccines in such vulnerable population is paramount since most data come from healthy subjects (13, 14). Indeed, in-depth immunological evaluations following SARS-CoV-2 vaccination might establish correlates of protection other than SARS-CoV-2-specific serology and translate these data for the benefits of other cohorts, i.e., transplanted patients and/or patients receiving chemotherapy, immunosuppressive therapies, or biologic response modifiers.

Given the lack of information on the safety and effectiveness of vaccines in general and anti-SARS-CoV-2 in particular in IEI patients, in this work, we seek to describe the effect of the BNT162b2 mRNA COVID-19 vaccine in this cohort. We first analyzed the humoral response by the mean of two different assays. Despite the development of the humoral response following vaccination, we deeply explored the cellular response by focusing on the SARS-CoV-2-specific CD4+ T cells, which are known to be fundamental for the production of effective neutralizing antibodies (Abs) in both convalescent adults and children and vaccinated healthy controls (HC) (15–17).

## MATERIALS AND METHODS

### Study Participants

Twenty-one patients with IEI were enrolled from February to March 2021 at Bambino Gesù Children's Hospital and Tor Vergata University Hospital, in Rome (**Table 1**). According to national regulations, only Latium region residents were eligible for vaccination in our centers with few exceptions. This prospective observational study included patients aged 16–59 years affected by IEI, according to ESID criteria (19). All patients were naïve to SARS-CoV-2 infections as demonstrated by the absence of SARS-CoV-2 Abs both anti-spike and anti-nucleocapsid protein and received the BNT162b2 mRNA COVID-19 vaccine, with a schedule of two doses of 30 μg 21 days apart (20). Longitudinal blood samples were collected on day of vaccination (D0), 21 days after the first dose (D21), and 7 days after the second dose (D28). All patients had negative serology and/or molecular tests for SARS-CoV-2 by nasopharyngeal swabs prior to vaccination. HC with no comorbidities aged <60 who received the BNT162b2 mRNA COVID-19 vaccine were also investigated. Healthy vaccinated gender-matched donors were used as controls. HC were older than IEI ($p$ = 0.003 and $p$ = 0.0001 for cellular and humoral

**TABLE 1 |** Demographics, diagnosis, and clinical and genetic data of IEI cohort.

| Pt code | Gender (M/F) | Age (years) | Diagnosis | Clinical phenotype | Comorbidities | Genetics | IVIG (Y/N) | Other treatments | Past treatments | Vaccine side effects |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 45 | XLA | LRTI; skin infections; | COPD, chronic pancreatitis, sclerosing cholangitis | Hemizygous *BTK* missense VoUS: c.1078 A>G, p.T316A | Y | Antibiotic prophylaxis; ICS-LABA | / | Myalgia |
| 2 | F | 48 | CVID | URTI; LRTI; UTI; vaginal candidiasis; Rheumatoid Arthritis; | Headache; Fibromyalgia; | Heterozygous *IKBKB* missense VoUS: c.1465A>G: p.S489G | Y | Sarilumab, SSZ | Anti-TNF agents, RTX - DMARDS | Fever |
| 3 | M | 32 | unPAD | URTI; LRTI; Rheumatoid Arthritis | / | Negative * | Y | SSZ, Hydroxychloroquine | Anti-TNF agents, RTX - DMARDS | Fever and myalgia |
| 4 | M | 51 | unPAD | URTI; LRTI; past gastric non-Hodgkin lymphoma | Asthma | Negative * | Y | / | R-CHOP chemotherapy (2006) | Fever and malaise |
| 5 | M | 18 | CVID | Past thrombocytopenia and neutropenia; URTI; LRTI | / | Negative ** | Y | / | Parenteral corticosteroids and high dose IVIG | Fever and malaise |
| 6 | F | 32 | WHIM-like | HPV infections | Pelvic inflammatory disease | WES in progress | N | / | / | Local pain |
| 7 | F | 51 | CVID | LRTI; chronic sinusitis; *S. epidermidis* superinfection on surgical wound; ulcerative colitis and spondyloarthritis | Asthma | Heterozygous *NFKB1* missense VoUS: c.1501C>G: p.L501V heterozygous *TCF3* missense VoUS: c.931G>C: p.V311L and c.920A>G: p.H307R | Y | SSZ | Betamethasone, methylprednisolone | Myalgia |
| 8 | M | 34 | CVID | GI; UTI; arthritis | / | Negative *** | Y | / | / | Not referred |
| 9 | M | 20 | unPAD | URTI | Allergic rhinitis, headache, anxiety disorder | Negative *** | N | Antihistamine; valproic acid | / | Malaise and fever |
| 10 | F | 21 | CVID | ITP; Hashimoto thyroiditis | Allergic rhinitis | Negative * | Y | Antihistamine | Parenteral corticosteroids, high-dose IVIG, MMF | Not reported |
| 11 | F | 31 | CVID | Past ITP | / | Heterozygous *PTPN22* missense VoUS: c.1858C>T p.R620W | Y | / | Parenteral CCS, high dose IVIG, RTX | Fever |
| 12 | F | 38 | CVID | Vitiligo; Hashimoto thyroiditis | / | Heterozygous *TNFRSF13B* (TACI) missense VoUS: c.512T>G; p.L171R | Y | / | / | Not referred |
| 13 | F | 25 | CVID | GI; URTI; HP infection; recurrent abdominal pain | Chronic sinusitis | Heterozygous *TNFRSF13B* (TACI) missense VoUS: c.58C>T; p.R20C | Y | / | / | Fever, myalgia and headache |
| 14 | F | 59 | unPAD | Legionella pneumonia; | Chronic renal failure, hypertension, obesity | CVID NGS panel in progress | Y | Antibiotic prophylaxis | / | Not referred |
| 15 | F | 33 | unPAD | URTI; LRTI; | Allergic rhinitis and asthma | NGS analysis progress | N | ICS-LABA; antihistamine | NO | Fever |
| 16 | M | 16 | CVID | Past Burkitt lymphoma; URTI, LRTI | / | NGS analysis progress | Y | / | R-CODOX M chemotherapy | Local pain |
| 17 | M | 21 | CVID | LRTI; chronic sinusitis; mild | Severe hypermetropia; | WGS in progress | Y | / | / | Not referred |

*(Continued)*

**TABLE 1** | Continued

| Pt code | Gender (M/F) | Age (years) | Diagnosis | Clinical phenotype | Comorbidities | Genetics | IVIG (Y/N) | Other treatments | Past treatments | Vaccine side effects |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | asymptomatic thrombocytopenia | psychomotor delay, dysgenesis of corpus callosum, cysts of arachnoid mater | | | | | |
| 18 | M | 21 | CVID | URTI; recurrent laryngospasm; splenomegaly; atypical mycobacterial infection; ITP | Preclinical diabetes; symmetric axonal sensitive polyneuropathy | Heterozygous *TNFRSF13C* (BAFFR) missense VoUS: c.C475T: p.H159Y | Y | / | / | Not referred |
| 19 | M | 20 | CVID | URTI; chronic rhinosinusitis; Hashimoto thyroiditis | Obsessive–compulsive disorder | Novel unpublished *NFKB1* variant: functional test ongoing | N | / | / | Not referred |
| 20 | M | 21 | CVID | ITP; LRTI; URTI; bronchiectasis; bone marrow hypoplasia; mycobacterial infection; vitiligo | / | Novel unpublished *NFKB1* variant: functional test ongoing | Y | / | RTX | Not referred |
| 21 | F | 36 | CVID | Thrombocytopenia | Past melanoma | Heterozygous *CTLA4* missense VoUS: c.224G>A; p.R75Q and heterozygous *PTEN* missense VoUS: c.596T>C p.M199T | N | / | / | Rhinitis and sore throat |

*XLA, X-linked agammaglobulinemia; unPAD, unclassified antibody deficiency; LRTI, lower respiratory tract infection; COPD, chronic obstructive pulmonary disease; ICS-LABA, inhaled corticosteroid and long-acting β2-agonist; CVID, common variable immunodeficiency; URTI, upper respiratory tract infection; UTI urinary tract infection; SSZ, sulfasalazine; Anti-TNF agents, anti-tumor necrosis factor agents; DMARDs, disease-modifying anti-rheumatic drugs; RTX, rituximab; R-CHOP chemotherapy, (Rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy; IVIG, intravenous immunoglobulin; HPV, human papilloma virus; ITP, immune thrombocytopenia; MMF, mycophenolate mofetil; GI, gastrointestinal infection; HP, Helicobacter pylori; R-CODOX M, R-Rituximab C—cyclophosphamide and cytarabine O—vincristine, also known as oncovin DOX—doxorubicin M—methotrexate chemotherapy.*

**CVID NGS panel available on Cifaldi et al., 2019 https://doi.org/10.3389/fimmu.2019.00316.*

***CID NGS panel available on Cifaldi et al., 2019 https://doi.org/10.3389/fimmu.2019.00316.*

****Haloplex NGS panel available on Cifaldi et al., 2019 https://doi.org/10.3389/fimmu.2019.00316 (18).*

analysis, respectively, **Table 2**). All participants received a survey reporting any adverse events and side effects following each dose of vaccine.

All procedures performed in the study were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. A local ethical committee approved the study and written informed consent was obtained from all participants or legal guardians. Age, gender, clinical, and routine laboratory characteristics of the cohort are described in **Table 1**.

## Sample Collection and Storage

Venous blood was collected in EDTA tubes and processed within 2 h. Plasma was isolated from blood and stored at −80°C. Peripheral blood mononuclear cells (PBMCs) were isolated from blood of all patients with Ficoll density gradient and cryopreserved in FBS 10% DMSO until analysis, in liquid nitrogen.

## Humoral Response

For serology test, we used different chemiluminescence test performed on an automated analyzer following the manufacturer's instructions.

**TABLE 2** | Cohort characteristics.

| | HC—cellular response (*N* = 65) | HC—humoral response (*N* = 18) | IEI | *p*-value |
|---|---|---|---|---|
| Age, years (mean) | 43.7 | 45.0 | 32.0 | $p = 0.003$ HC cell *vs.* IEI, $p < 0.0001$ HC humoral *vs.* IEI |
| Sex (M/F) | 7/11 | 19/46 | 11/10 | ns |
| Ethnicity | All Caucasian | All Caucasian | All Caucasian | ns |

*ns, not significant.*

Anti-SARS CoV-2 IgG Ab titers were measured at D0, D21, and D28. In particular, we measured Abs against the S1-receptor-binding-domain (RBD) (Roche, cutoff: 0.8 U/ml) and anti-trimeric SARS-CoV-2 Ab (LIAISON® SARS-C0V-2 DiaSorin, cutoff: 13 AU/ml).

The LIAISON® SARS-CoV-2 TrimericS IgG (DiaSorin—Saluggia TO) is an indirect chemiluminescent immunoassay (CLIA) intended for the qualitative and semi-quantitative detection of anti-trimeric spike protein specific IgG antibodies to SARS-CoV-2 in human serum, used on the LIAISON® XL platform Analyzer. The test detects IgG antibodies against the Trimeric complex, which includes the RBD and NTD sites from the three subunit S1 (the Trimeric complex). Test results are reported as positive or negative along with a numeric value for semi-quantitative measurement for values between 13 AU/ml and 800 AU/ml. TrimericS IgG assay has a quantification range between 4.81 BAU/ml and 2,080 BAU/ml (dilution factor 1:20).

Elecsys anti- SARS-CoV-2 and Elecsys anti-SARS-CoV-2 S (Roche Diagnostics) test on a Cobas e801 analyzer have been used.

The Elecsys® Anti-SARS-CoV-2 is an immunoassay for the *in vitro* qualitative detection of a mix of antibodies (including IgA, IgM, and IgG) to SARS-CoV-2 in human serum and plasma. In order to investigate a broad-spectrum immune response, we use two types of Roche antibody assays using a recombinant protein, respectively, for the S antigen and for the nucleocapsid (N) antigen in a double-antigen sandwich assay format. Results for anti-N antibodies are expressed as "present" or "absent" on the basis of a cutoff index (COI) ≥ 1.0 and COI < 1.0, respectively. Titer for Anti-S Ab was interpreted as absent when <0.8 U/ml (<0.8 BAU/ml) and as present when ≥0.80 U/ml (≥0.8 BAU/ml). When antibody titer was higher than 250 U/ml (250 BAU/ml), the instrument automatically executed a 20-fold dilution, ranging the upper limit of quantification to 5,000 U/ml (5,000 BAU/ml).

## CD4 Ag-Specific T-Cell and B-Cell Phenotype

SARS-CoV-2-specific CD4+CD40L+ T cells were identified, as previously described (17). Briefly, thawed PBMCs were plated ($1.5 \times 10^6$/aliquot/200 µl) in 96-well plates containing CD154-PE (CD40L, BD PharMingen, Franklin Lakes, NJ, USA) and anti-CD28 (1 mg/ml) in the presence or absence of 0.4 mg/ml PepTivator SARS-CoV-2 Prot_S (Miltenyi Biotec, Bergisch Gladbach, Germany). Following 16 h incubation at 37°C/5% $CO_2$, PBMCs were centrifuged and stained with LIVE/DEAD fixable NEAR-IR dead cell stain kit (for 633 or 635 nm excitation, ThermoFisher, Waltham, MA, USA) 1 µl per $10^6$cells/ml for 15 min at room temperature (RT), protected by light. Surface staining was performed using the following antibodies: CD3 PE-CF594 [clone UCHT1, BD (562280)], CD4 APC-Cy7 [clone RPA-T4, BD (557871)], CD27 FITC [clone M-T271, BD (555440)], CD45RO PE-Cy5 (clone UCHL1, BD), CD185 BV605 (CXCR5, clone RF8B2, BS), CD10 BV510 (clone HI10a, BD 563032), CD19 APC-R700 (clone SJ25C1, BD 659121), CD21 APC (clone B-Ly4, BD 559867), and IgD BV421 (clone IA6-2, BD 565940). T- and B-cell population and SARS-CoV-2-specific CD40L+CD4+ T cells were gated as previously reported (17).

Due to limited sample available for testing and in accordance with the evidence of a Th1 response following both the disease and vaccination and our previous work showing that interaction between CD4 T cell and B cell is critical in order to mount specific neutralizing antibodies, we decided to focus our efforts on CD4 T-cell response (17, 20–22).

## Quantification and Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA). Statistical significance was set at $p < 0.05$, and the test was two-tailed. All data were analysed by D'Agostino-Pearson to assess normality. As indicated in figure legends, paired and non-paired non-parametric tests were used to assess differences between Ab load at the different time points, and between HC and IEI, respectively. Spearman's correlation was used to compute the association between variables. GraphPad Prism 8 software was used for statistical analysis of cell-type distribution and serological parameters for demographic and routine laboratory blood tests.

# RESULTS

## Study Population

The study included 21 IEI patients aged 16–59 years (mean age, 32 years). Patient samples were collected right before the first vaccine dose (D0), for baseline immunological investigation, at the second dose (D21), and 1 week after the second dose (D28). In line with previous evidence showing an increase in specific ab titer and cellular responses 7 days following the second dose (20, 23), we decided to consider the same time points. Demographics, diagnosis, and clinical and genetic data are reported in **Table 1**. The study cohort included patients affected by common variable immunodeficiency (CVID, $n = 14$) and unclassified antibody deficiency (unPAD, $n = 5$) and two patients affected by X-linked agammaglobulinemia (XLA) and WHIM-like disorder, respectively (**Table 1**). Recurrent infections represented the most common clinical manifestations of these patients (17/21, 81%), followed by autoimmunity, mainly cytopenias, arthritis, and autoimmune thyroiditis, (13/21, 62%) and neoplasia (3/21, 14%). Allergic diseases, such as allergic rhinitis and asthma, represented common comorbidities. Sixteen out of 21 patients (76%) are currently receiving Ig replacement therapy and 6/21 (28%) have been treated or are still being treated with immunosuppressant drugs or biologics. Two patients previously received chemotherapy due to neoplasia. Routine immunological evaluation at baseline is reported in **Table 3**. Healthy vaccinated age- and gender-matched donors were used as controls.

## Vaccine Side Effects

As reported in **Table 1**, no severe adverse events following vaccination have been observed. The most common side effect

**TABLE 3 |** Routine immunological evaluation at baseline.

| | Pt1 | Pt2 | Pt3 | Pt4 | Pt5 | Pt6 | Pt7 | Pt8 | Pt9 | Pt10 | Pt11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Hb (mg/dl)** | 15.1 | 16.9 | 15.5 | 14.3 | 15.2 | 12.9 | 14.1 | 15.4 | 15.2 | 12.2 | 14.4 |
| **PLT (10x3/ml)** | 123 | 174 | 214 | 245 | 129 | 228 | 259 | 165 | 218 | 253 | 192 |
| **WBC (10x3/ml)** | 9.07 | 6.22 | 7.6 | 5.39 | 4.06 | 9.01 | 4.76 | 4.42 | 5.79 | 3.73 | 4.52 |
| **Eosinophils (% and 10x3/ml)** | 1.3/120 | 2.9/180 | 2.5/190 | 0.4/20 | 4.4/180 | 7.3/660 | 1.3/60 | 2.3/100 | 5.7/330 | 1.1/40 | 3.5/220 |
| **Total lymphocytes (10x3/ml)** | 1.05 | 2.76 | 2.6 | 2.7 | 1.6 | 4.8 | 2.2 | 1.2 | 2.3 | 1.2 | 1.1 |
| **CD3+ (% and cell/µl)** | 87/913 | 81/2235 | 83/2158 | 64.7/1746 | 77/1232 | 86/4128 | 58/1276 | 70/840 | 70/1610 | 73/876 | 82.7/909 |
| **CD4+ (% and cell/ µl)** | 46/483 | 65/1794 | 36/936 | 37/999 | 27/432 | 38/1824 | 36.7/807 | 48/576 | 37/851 | 40.6/487 | 39.7/436 |
| **CD8+ (% and cell/ µl)** | 41/430 | 15/414 | 44/1144 | 24/648 | 45/720 | 31.7/1521 | 17/374 | 20/240 | 25/575 | 17/204 | 42.1/463 |
| **CD19+ (% and cell/µl)** | 5.5/52 | 9/248 | 6.7/174 | 17/459 | 12/192 | 6.3/302 | 26/572 | 18/216 | 17/391 | 19.8/237 | 8/88 |
| **CD16+56+ (% and cell/µl)** | 6.5/68 | 8.8/243 | 9.9/257 | 15.6/421 | 7/112 | 7.8/374 | 8.7/191 | 11/132 | 9/207 | 12.2/146 | 6.1/66 |
| **CD3+CD4+CD27+CD45RO- Naïve T cell (%)** | 26 | 68.6 | 52.9 | 64.2 | 41.9 | 79 | 70.5 | 66.5 | 70 | 27.2 | 48 |
| **CD3+CD4+CD27+CD45RO+ Tcm (%)** | 49.9 | 25.4 | 33.2 | 25.9 | 49.1 | 12.2 | 22.2 | 26.8 | 21.7 | 68.6 | 41.1 |
| **CD3+CD4+CD27-CD45RO+ Tem (%)** | 4.52 | 1.43 | 1.14 | 1.98 | 2.54 | 3.79 | 1.13 | 1.94 | 2.73 | 0.31 | 1.88 |
| **CD3+CD4+CD27-CD45RO- Temra (%)** | 17.2 | 2.37 | 8.74 | 6.08 | 5.5 | 3.93 | 3.11 | 2.99 | 3.54 | 2.73 | 6.59 |
| **CD3+CD4+CD27+CD45RO +CXCR5+ pTfh (%)** | 28.9 | 24.9 | 18.7 | 24.3 | 45.5 | 8.22 | 23.3 | 12.8 | 17 | 53.9 | 30.3 |
| **CD27-IgD- Double negative B cells (%)** | 16.5 | 0.86 | 2.11 | 6.61 | 1.13 | 3.77 | 2.58 | 8.29 | 8.62 | 7.48 | 1.56 |
| **CD27+IgD+ Unswitched memory B cells (%)** | 3.46 | 0.39 | 0.56 | 11.9 | 3.46 | 4.99 | 0.85 | 9.73 | 11.8 | 14 | 2.23 |
| **CD27+IgD- Switched memory B cells (%)** | 13.8 | 0.054 | 0.25 | 8.11 | 0.13 | 5.5 | 1.26 | 5.19 | 5.49 | 4.16 | 0.83 |
| **CD27-CD21+ Naïve B cells (%)** | 50.4 | 96.7 | 85.1 | 66.4 | 83.6 | 34.6 | 91.5 | 63 | 62.6 | 37.6 | 90.5 |
| **CD27-CD21- TLM B cells (%)** | 27.1 | 1.75 | 13.5 | 5.59 | 10.2 | 49 | 5.22 | 13 | 10.8 | 33.9 | 4.27 |
| **CD21+ (RM) Switched memory B cells (%)** | 76.2 | 67.9 | 70.3 | 84.6 | 72.5 | 94.2 | 84.7 | 88.1 | 90.1 | 62.9 | 70.3 |
| **CD21- (AM) Switched memory B cells (%)** | 19.5 | 32.1 | 26.6 | 10.4 | 26.4 | 4.79 | 13.2 | 8.62 | 7.86 | 33 | 27.7 |
| **IgG (mg/dl)** | 1090 | 993 | 723 | 1420 | 1132 | 1132 | 1165 | 1159 | 698 | 1180 | 708 |
| **IgA (mg/dl)** | 33 | 50 | 22 | 5 | 5 | 356 | 113 | 45 | 91 | 290 | 45 |
| **IgM (mg/dl)** | 10 | 7 | 55 | 131 | 5 | 284 | 45 | 23 | 24 | 25 | 23 |
| **IgE (kU/L)** | 1 | 1.67 | 9.94 | 142 | 1 | 2.5 | 2 | 3 | 207 | 2.47 | 1 |
| **IgG anti-tet** | NA | NA | NA | R | NR | R | R | TR | R | NA | TR |
| **IgG anti-pneumo** | NA | NA | NA | R | NR | R | R | TR | R | NA | TR |
| **TCR α/β** | NA | 96.5% | 98.3% | 96.5% | 89.6% | 78.2% | 91.6% | 96.4% | 80.8% | 89.6% | 96% |
| **TCR γ/δ** | NA | 3.3% | 1.5% | 3% | 8.6% | 21.5% | 6.4% | 1.2% | 17.7% | 10.2% | 3.6% |

| **continued** | **Pt12** | **Pt13** | **Pt14** | **Pt15** | **Pt16** | **Pt17** | **Pt18** | **Pt19** | **Pt20** | **Pt21** |
|---|---|---|---|---|---|---|---|---|---|---|
| **Hb (mg/dl)** | 13.4 | 13.3 | 13.8 | 12.1 | 15.6 | 13.8 | 14.2 | 16.3 | 13 | 12.9 |
| **PLT (10x3/ml)** | 241 | 169 | 339 | 218 | 232 | 109 | 129 | 173 | 84 | 128 |
| **WBC (10x3/ml)** | 3.81 | 4.82 | 7.95 | 7.6 | 5.15 | 7.00 | 5.73 | 4.99 | 2 | 4.61 |
| **Eosinophils (% and 10x3/ml)** | 0.5/20 | 1.7/80 | 1.5/120 | 2.2/170 | 2/100 | 1.2/80 | 1.9/108 | 2/100 | 0.7/14 | 4/0.18 |
| **Total lymphocytes (10x3/ml)** | 0.8 | 1.6 | 2.1 | 2.0 | 1.8 | 2290 | 1.19 | 1.65 | 0.51 | 0.83 |
| **CD3+ (% and cell/µl)** | 75/600 | 61.3/981 | 90.6/1902 | 88.5/1770 | 85/1530 | 90.6/2074 | 87/1035 | 83.7/1981 | 96.6/493 | 70.9/586 |
| **CD4+ (% and cell/ µl)** | 51/408 | 35.2/563 | 53.7/1127 | 57/1140 | 30/540 | 54/1236 | 50.3/599 | 41.8/690 | 56.2/287 | 43.5/361 |
| **CD8+ (% and cell/ µl)** | 17/136 | 20/320 | 29.7/623 | 25.5/510 | 43/774 | 32.5/744 | 32/381 | 24.4/403 | 35.2/179 | 25/207 |
| **CD19+ (% and cell/µl)** | 10.5/84 | 11.3/176 | 3.9/82 | 9.8/196 | 6.3/113 | 1.4/32 | 3.1/178 | 9.4/155 | 0.2/1 | 13.4/111 |
| **CD16+56+ (% and cell/µl)** | 11.6/93 | 26.2/419 | 5.1/107 | 3.7/74 | 7.5%/135 | 7.4/169 | 9.8/561 | 6.2/102 | 2.7/14 | 14.4/119 |
| **CD3+CD4+CD27+CD45RO- Naïve T cell (%)** | 55.9 | 46.2 | 24.5 | 36.9 | 51.1 | 68.8 | 26.5 | 81 | 49.4 | 32.2 |
| **CD3+CD4+CD27+CD45RO+ Tcm (%)** | 38.6 | 47.5 | 46 | 44.3 | 23.8 | 24.4 | 56.7 | 16.2 | 37.6 | 57.3 |
| **CD3+CD4+CD27-CD45RO+ Tem (%)** | 0.088 | 0.12 | 0.5 | 2.64 | 0.61 | 0.47 | 0.97 | 1.46 | 1.21 | 0.37 |
| **CD3+CD4+CD27-CD45RO- Temra (%)** | 3.22 | 2.72 | 26.6 | 12.9 | 23.4 | 4.74 | 13.4 | 0.55 | 10.2 | 8.46 |
| **CD3+CD4+CD27+CD45RO +CXCR5+ pTfh (%)** | 30.2 | 38.1 | 19.2 | 23.4 | 18.5 | 15 | 30.2 | 15.1 | 14.7 | 28 |

*(Continued)*

**TABLE 3** | Continued

| | Pt12 | Pt13 | Pt14 | Pt15 | Pt16 | Pt17 | Pt18 | Pt19 | Pt20 | Pt21 |
|---|---|---|---|---|---|---|---|---|---|---|
| **CD27-IgD- Double negative B cells (%)** | 5.42 | 5.79 | 9.72 | 15.5 | 3.87 | 3.85 | 1.2 | 3.27 | 33.3 | 4.77 |
| **CD27+IgD+ Unswitched memory B cells (%)** | 4.18 | 4.9 | 6.63 | 3.31 | 3.57 | 0.41 | 3.16 | 2 | 0 | 6.57 |
| **CD27+IgD- Switched memory B cells (%)** | 2.26 | 1.52 | 12.7 | 10 | 0.84 | 0.36 | 0.094 | 0.8 | 0 | 0.72 |
| **CD27-CD21+ Naïve B cells (%)** | 82.1 | 74.7 | 57.2 | 62.9 | 85.2 | 91.6 | 69.4 | 87.9 | 33.3 | 31.9 |
| **CD27-CD21- TLM B cells (%)** | 6.32 | 13.4 | 15.9 | 14.4 | 7.39 | 5.22 | 23.1 | 5.89 | 33.3 | 53.7 |
| **CD21+ (RM) Switched memory B cells (%)** | 72 | 77.9 | 88.8 | 74 | 81.3 | 73.3 | 50 | 66.9 | 0 | 79.1 |
| **CD21- (AM) Switched memory B cells (%)** | 21 | 16.3 | 8.71 | 19.4 | 15.7 | 13.3 | 50 | 21 | 0 | 19.4 |
| **IgG (mg/dl)** | 1021 | 1197 | 655 | 618 | 1052 | 876 | 325 | 726 | 746 | 289 |
| **IgA (mg/dl)** | 5 | 16 | 550 | 96 | 5 | <4 | <4 | 91 | <4 | 17 |
| **IgM (mg/dl)** | 8 | 34 | 87 | 189 | 25 | <5 | 10 | 87 | <5 | 37 |
| **IgE (kU/L)** | 1 | 1 | 3.78 | 211 | 92 | NA | NA | NA | 2 | NA |
| **IgG anti-tet** | R | R | NA | NA | NR | NA | NA | R | NA | TR |
| **IgG anti-pneumo** | NR | NR | NA | R | NR | NA | NA | R | NA | R |
| **TCR α/β** | 97.5% | 93.4% | 92.5% | 97% | 87.6% | NA | 96.9% | 83.6% | 94.6% | 92.3% |
| **TCR γ/δ** | 3.3% | 5.3% | 5.9% | 2.8% | 12.1% | NA | 2.3% | 9.8% | 3.4% | 2.3% |

*WBC, White blood cells; PLT, Platelets; HB, Hemoglobin; Tcm, central memory T cells; Tem, effector memory T cells; Temra, terminally differentiated effector memory T cells; pTfh, peripheral follicular helper memory T cells; TLM, tissue like memory B-cells; RM, resting memory B-cells; AM, activated memory B-cells.*

*NA, Not available; NR, Not Responder; R, Responder; TR, Transient Responder.*

*Serum immunoglobulin concentrations from Whelan MA et al., J. Clin Immunol 2006 (24); T-cell subsets from Schatorie E.J.H. et al., Clin Immunol 2011 (25); B-cell subsets from Piatosa B. et al., Cytometry part B, Clinical Cytometry 2010 and Duchamp M et al., Immunity, Inflammation and Disease 2014 (26, 27); Regulatory T-cell subsets from van Gent R. et al., Clinical Immunology 2009 (28).*

was fever (8/21, 38%), followed by myalgia and malaise (4/21, 19% and 3/21, 14% respectively). Local pain at the site of injection was reported by two patients (9%) and headache by one patient (5%).

## Humoral Response

We evaluated the humoral response before (D0) and after the first (D21) and second dose (D28, 1 week after the second dose administration) of vaccine in both IEI and HC. Results are summarized in **Figures 1A, B**. Overall, both HC and IEI groups experienced an increase in anti-SARS-CoV-2 Abs between D21 and D28, with only one patient for each category lacking anti-RBD Abs at D28. At D21, Ab levels were similar in the two groups, while at D28, patients with IEI showed lower median specific antibody levels measured as both anti-RBD (**Figure 1A**) and anti-trimeric Abs (**Figure 1B**). In particular, HC showed a higher increase in both anti-RBD titer and anti-trimeric S titer compared to IEI, $p = 0.0060$ and $p < 0.0001$ respectively. Of note, at the end of vaccine schedule at D28, 3/21 (14%) IEI patients had undetectable levels of anti-trimeric Abs, whereas all HC had measurable levels (**Figure 1B**).

## Cellular Response

### T-Cell Response

Gating strategy for SARS-COV-2-specific T cells (CD4+CD40L+) is summarized in **Supplementary Figure S1**. When we evaluated the Ag-specific cellular response at D0 and D28, we found an overall statistically significant expansion of the CD4+CD40L+ T cells in both HC ($p < 0.001$) and IEI ($p = 0.002$) patients (**Figure 1C**), with different levels at baseline (D0) and D28 between the two groups. However, in 5/21 (24%, Pt14, Pt17, Pt19, Pt20, and Pt21) IEI patients, no increase in the proportion of the Ag-specific T cells could be observed. In one of these five non-responsive patients, Pt20, humoral correlates were also lacking (**Figure 1D**). The remaining four out of five seroconverted at similar levels to HC (**Figure 1E**).

We further explored CD4 T cells and CD4 memory subsets for these patients at D0 (**Figures 2A–E**). When analyzing these patients in comparison to the rest of the cohort, we did not observe any statistically significant difference in terms of frequency of T cell maturation subset (**Figure 2F**). We then explored the T- and B-cell phenotype for the entire IEI cohort (**Figures 3A–E**) and we observed changes in the frequency of T-cell memory subsets at D28 compared to baseline values. In particular, we observed a reduction of Naïve T cells ($p = 0.002$) in favor of an expansion of central memory (Tcm) ($p = 0.009$) and effector memory (Tem) ($p = 0.002$) (**Figure 3C**), following vaccination. On the other hand, frequency of peripheral T follicular helper cells (pTFH) (CD3+CD4+CD27+CD45RO+CXCR5+) did not vary at D28 (**Figure 3D**).

### B-cell Response

We further explored the phenotypic maturation profile of B-cell subsets in IEI patients, and no variation of this compartment upon vaccination was found (**Figures 3E–H**). Overall, accordingly due to their immune impairment, these patients appeared to have very few switched B cells (mean = 3.4%, SD = 4.176%, **Figure 3G** and **Table 3**).

# DISCUSSION

This work represents the first longitudinal immunological study on the efficacy of the BNT162b2 mRNA COVID-19 vaccine in a

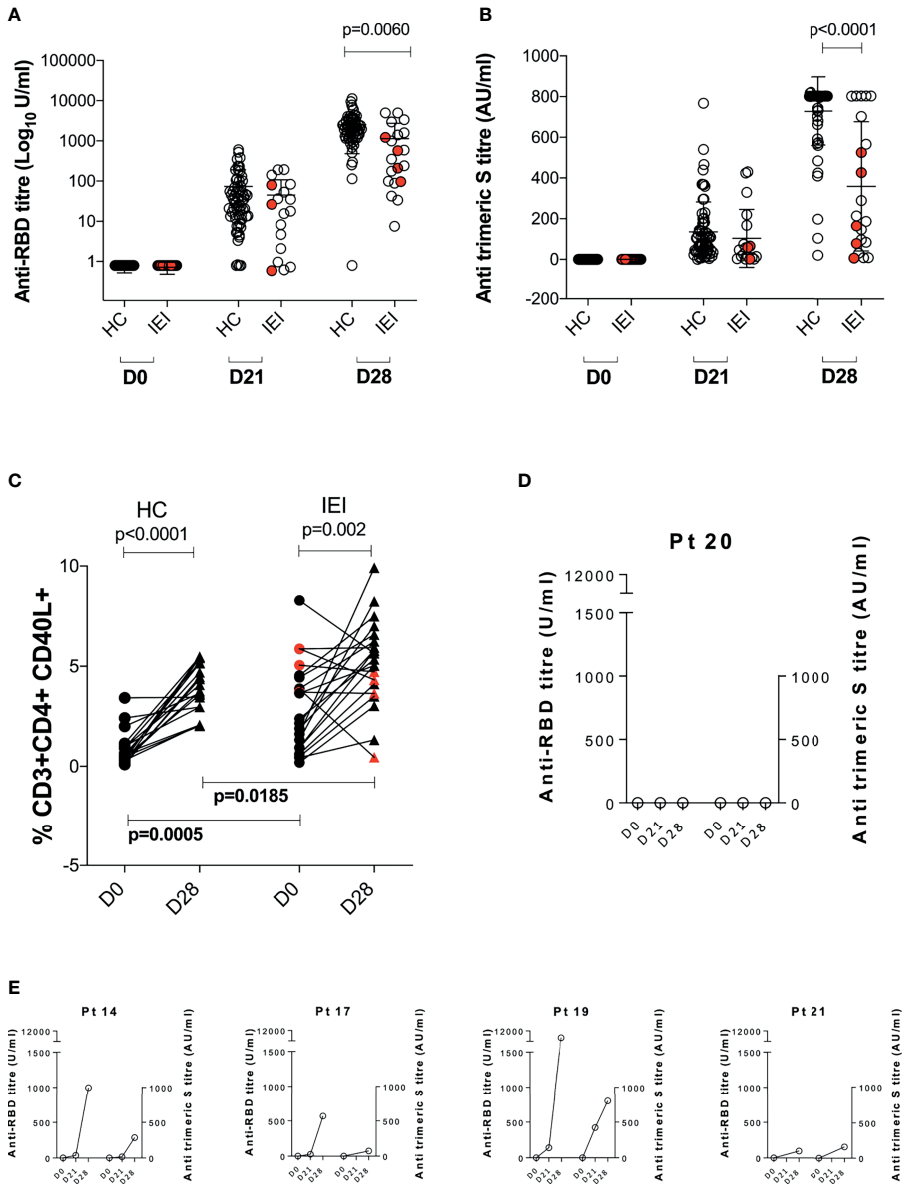


**FIGURE 1** | Anti-SARS-CoV-2 immune response before (D0) and at 21 days (D21) after the 1° dose and at 7 days (D28) after the 2° dose of the BNT162b2 mRNA COVID-19 vaccine. Humoral response is represented in **(A)** anti-RBD Ab ($Log_{10}$ U/ml) and **(B)** anti-trimeric S titer (AU/ml). Cellular response is depicted in **(C)**. Humoral response of five patients lacking any cellular response is reported in **(D, E)**. Non-paired non-parametric Mann–Whitney test was used in **(A, B)**; paired non-parametric Wilcoxon tests were used to assess differences between D0 and D28 in HC and IEI in **(C)**. HC, healthy controls; IEI, inborn errors of immunity patients.

European cohort of 21 patients affected by IEI compared to HC. Our data show that patients with IEI are able to develop specific anti-spike antibody response in terms of both anti-trimeric S IgG and anti-S1-RBD IgG following vaccination, although at a significant lower magnitude ($p < 0.0001$) compared to HC (**Figures 1A, B**). The age was not associated with humoral response measured with anti-trimeric S antibodies or with the cellular response in both HC and IEI. On the other hand, we observed a weak significance between age and anti-RBD titers, indicating a lower humoral response in the older population compared to the younger one in HC, as widely documented by other groups (29–31). This result warrants a dedicated discussion because it does reinforce our findings. Indeed, despite the presence of an older group in HC that probably dragged down the median levels of Ab titer in HC, we were able to detect a weaker Abs response in IEI at D28. Long-term studies are needed to evaluate a potential early waning of vaccine-induced antibodies in IEI patients. Furthermore, we demonstrated that IEI are capable of sustaining generation of Ag-specific T cells after 1 week from the completion of the vaccination schedule.

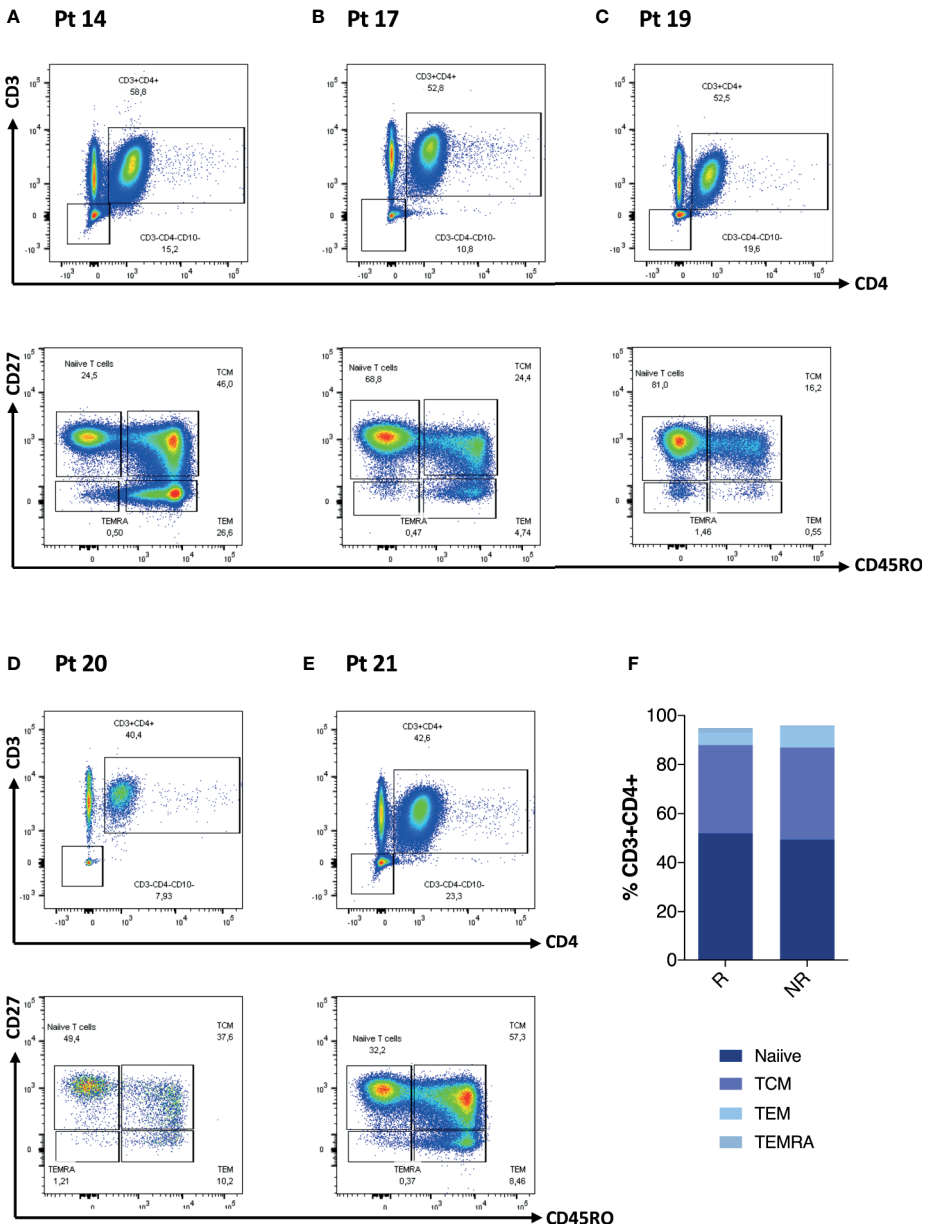


**FIGURE 2** | CD4 T-cell phenotype characteristics of five patients lacking any cellular response is reported in **(A–E)**. Non-paired non-parametric Mann–Whitney test was used to assess differences in the levels of each sub-population in **(F)**.

Given that the robust elicitation of Ag-specific T cells represents the major correlate of mRNA-based vaccine efficacy (20), we further evaluated the SARS-CoV-2-specific T-cell response showing the ability of patients with IEI to increase the frequency of Ag-specific T-cell response upon vaccination. Of note, the CD4+CD40L+ T-cell subset was higher at D0 and D28 in IEI compared to HC as seen by the compensatory T-cell function in patients with primary B-cell defect (32). Despite the fact that the majority of IEI showed increased levels of Ag-specific T cells following vaccination, we observe that five patients failed to mount any cellular response (Pt14, Pt17, Pt19, Pt20, and Pt21), as usually observed in healthy individuals (20, 30), with Pt20 also lacking a specific humoral response. This rate of "non-responders" is in line with the only available study by Hagin et al. conducted on a similar cohort of Israelian IEI patients (33). We then explored to what extent this lack of response could be due to patients' clinical condition. Pt19 and Pt20 are two siblings affected by a novel NFKB1 mutation (functional tests are in progress). Nuclear factor kB subunit 1 mutation represents one of the most common cause of CVID (34) with a wide range of clinical phenotypes (35–37). NF-kB is a key regulatory transcription factor involved in several aspects of the immune response including the development of specific immune responses (38).

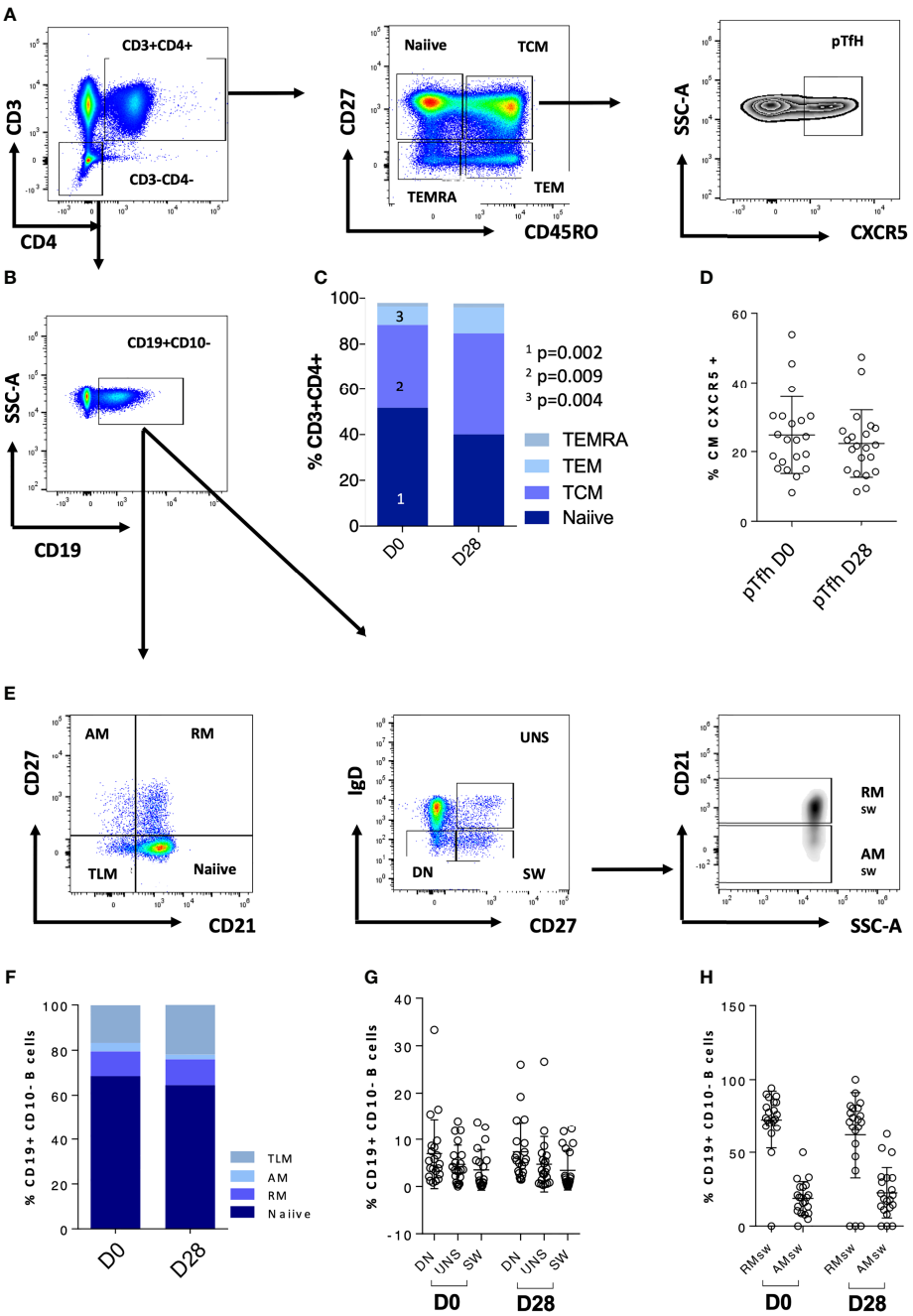


**FIGURE 3** | CD4 T-cell and B-cell phenotype characteristics of the whole cohort are reported. **(A)** Gating strategy for CD4 T-cell populations; **(B)** gating strategy for the CD19+ population used to analyze the B-cell subsets. **(C)** CD4 T-cell populations at D0 and D28; **(D)** pTfh at D0 and D28. **(E)** Gating strategy for the B-cell populations. **(F–H)** B-cell populations at D0 and D28. Non-paired non-parametric Mann–Whitney test was used to assess differences in the levels of each sub-population in **(C, D, F–H)**. TCM, central memory T cells; TEMRA, terminally differentiated T cells; TEM, effector memory; pTfh, peripheral follicular T cells; TLM, tissue-like memory; AM, activated memory; RM, resting memory; UNS, unswitched; DN, double negative; SW, switched; $RM_{SW}$, resting memory switched B cells; $AM_{SW}$, activated memory switched B cells.

Pt21 is affected by a heterozygous mutation of CTLA4 (c.G224A) and PTEN. PTEN is one of the major regulators of phosphoinositide 3-kinase (PI3K) signaling pathway playing a critical role in modulating T-cell activity (39, 40). In addition, CTLA-4 may also play a role in PI3K signals as well as in regulatory T-cell function (41), autoimmunity, and cancer (42, 43).

Altogether, these mutations could explain the impairment of specific T-cell response upon vaccination. For Pt14 and Pt17, a genetic diagnosis is not available yet.

Most of our patients have an immunodeficiency that mainly impairs the B-cell compartment. Our data show their relative ability to mount a specific humoral response upon two doses, although at a lower magnitude in comparison to HC. In this contest, the evaluation of Ag-specific T-cell response seems to be critically important to analyze vaccine-induced protection in this cohort. A discordant immune response as defined by the presence of humoral response in the absence of specific T-cell response was observed in roughly one-quarter of the IEI patients. These data raise immunologic concerns on the sole use of Ab response as a metric of protective immunity following anti-SARS-CoV-2 vaccine. Indeed, after natural infection, T-cell responses have been reported as a finer marker than Ab response (21, 44–46).

Patients with IEI are prone to develop persisting viral shedding, probably due to their impaired B- and/or T-cell function with subsequent higher risk of persistent viral replication and mutation within the host (47). Indeed, most variants were first described in immunocompromised patients (48). Moreover, specific immunomodulatory treatment could affect the immune response following vaccination (49). Taken together, these findings suggest that the evaluation of vaccine-induced immunity should also include quantification of Ag-specific T cells.

The following study limitations need to be mentioned: (a) the paucity of the sample size due to the Italian national regulation, which did not allow vaccine administration to patients living outside the Latium region where the two hospitals are located; (b) the short time of observation; (c) the lack of real-life data of protection against the different SARS-CoV-2 strains despite vaccination; and (d) variability of immune defects among subjects with IEI and within the same IEI condition.

In conclusion, our findings confirm the good safety and immunogenicity profile of the BNT162b2 mRNA COVID-19 vaccine in IEI patients and reinforce current national and international vaccine recommendation against COVID-19. The observation of an appropriate vaccine response in most patients should support trust on vaccination and immunization programs for distinct immune disorders. Studies of specific correlates to monitor persistence of vaccine-induced immunity will further support the design of tailored vaccine schedules for the benefit of these patients and the community.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local ethical committee from Bambino Gesù Children's Hospital and Policlinico Tor Vergata. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

DA, AR, VM, and PP designed the project. AR and CP performed the experimental analysis. LC and CR performed Anti-SARS Cov-2 IgG Ab assays. DA, AR, and MS wrote the original draft. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.727850/full#supplementary-material

**Supplementary Figure 1 |** Experimental approach to define SARS-CoV-2 specific CD4+CD40L+ T-cells. **(A)** Gating strategy for CD3+CD4+ T cells; **(B)** design and gating to analyze the CD40L+ T cells.

**Supplementary Figure 2 |** Correlation between Age and humoral response or cellular response in HC and IEI.

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