Trained immunity:

immunological footprints and side-tracks

Elisabeth A. Dulfer

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Va, pensiero!

- Uit *Nabucco*

Chapter 1

General introduction and outline of this thesis

Vaccines represent one of the most remarkable achievements in modern public health. Although infectious diseases still represent a large part of mortality globally (1), the contributions of vaccines to decreasing disease burden and improving lifespan are beyond dispute. Throughout history, vaccines have played crucial roles in shaping global health, from eradicating smallpox in 1980 (2) to providing novel therapeutic options for malignancies in 2010 (3) and alleviating medical and societal turmoil during the recent SARS-CoV-2 pandemic (4). This thesis explores the wide-ranging impacts of vaccines, addressing both their specific mechanisms of protection and the broader, non-specific effects they may exert on the immune system.

Mechanisms for specific protection

The core function of vaccines is to protect recipients against infectious diseases, by eliciting a robust immune response through activation of the adaptive branch of our immune system. Upon encountering infectious intruders, such as bacteria or viruses, the innate immune system initiates a response aimed at recognizing and clearing the microbes, while also communicating the presence of a potential threat to the adaptive branch of immunity. The components of the adaptive immune system then develop into specialized cells that can recognize and neutralize the infectious agent with high specificity. Naïve B and T cells with the relevant specific antigen receptors are selected and undergo clonal expansion, resulting in large numbers of identical activated lymphocytes (5). Numerous B cells will differentiate into plasma cells to secrete antibodies to mark the invading pathogen for destruction or enhance its clearance from the host. Meanwhile, various subsets of T cells are mobilized to activate other immune cells and assist in the development of B cells (CD4-positive T helper cells) or directly target and eliminate infected cells (CD8-positive cytotoxic T cells). From both the B and T cell pool, certain cells are responsible for long-term responses by retaining a memory of past encounters with pathogens. They will become long-lived memory cells, circulating in blood and bone marrow (6, 7), essentially writing the manual on how to combat this specific attack to use for future reference. In case of a second infection, the necessary antibodies and effector cells can be mobilized quickly and counter the infection before causing significant illness.

Heterologous effects of vaccination

In contrast to what had been thought and taught for many decades, certain vaccines do not only induce protection via the adaptive immune system’s effector cells and antibodies directed at a specific pathogen. Cumulating evidence exists of vaccines exerting so-called heterologous or non-specific effects (NSEs). Live-attenuated vaccines, like many of the vaccines administered in childhood, have been associated with beneficial effects that go beyond the specific protection provided against distinct pathogens, thereby decreasing morbidity or mortality to a greater extent than initially anticipated. A striking example is the capacity of the anti-tuberculosis Bacillus Calmette-Guérin (BCG) vaccination to reduce childhood mortality up to 40%, which could not be explained by the prevention of tuberculosis alone (8, 9). Other vaccines have also been implicated in beneficial NSEs, reducing neonatal mortality rates or lowering the incidence of clinically relevant respiratory tract infections (10, 11). Even in regions of the Western world with a lower infectious burden, beneficial effects on paediatric infection-related hospital admissions have been described in the context of BCG vaccination (12). Similarly, some recent COVID-19 vaccines have been suggested to lower all-cause mortality independent of COVID-19 (13).

The dominant hypothesis explaining these NSEs uses the concept of 'trained immunity' as a framework for understanding the responses in the innate immune system following an initial trigger and subsequent heterologous exposures.

Trained innate immunity

Since the discovery of pattern-recognition receptors (PRRs) at the turn of the millennium, it is known that the innate immune system recognizes pathogens or related compounds via these receptors on innate immune cells, such as macrophages and neutrophils. Examples of these PRRs include toll like receptors (TLRs) located on cell membranes and NOD-like receptors (NLRs) located in the cytoplasm. The binding of pathogen-associated molecular patterns (PAMPs) to these receptors results in an intracellular signalling cascade that leads to the production of pro-inflammatory cytokines, essential for activating other immune cells and regulating the production of additional signalling molecules. This reaction is not specific to a certain type of pathogen, although the exact processes and involved cell types differ between microbial classes (14).

By 'training' the innate immune system with a certain stimulus, such as an infection or vaccination, the immune response to an unrelated secondary challenge can be enhanced (15). This enhanced reaction takes place after the initial stimulus has been removed or cleared. Trained immunity, or TRIM, is therefore equivalent with a *de-facto* innate immune memory [see Figure 1]. Depending on the timing and nature of the training stimulus, the ensuing reaction to subsequent triggers can also be a diminished response, referred to as 'tolerance' (15).

This innate memory is mediated by the functional reprogramming of innate immune cells upon initial exposure, with underlying epigenetic and metabolic alterations that affect their long-term responsiveness (16-18). The acquisition of certain activating or repressive epigenetic marks helps regulate the transcription of genes involved in the immune response, like the ones responsible for cytokine production, for instance by changing chromatin accessibility. In turn, metabolic rewiring provides the energy and intermediate metabolites for this reprogramming. It is imperative to emphasize that TRIM represents a concept that describes different transcriptional and functional programmes induced by various stimuli, but does not constitute one unique set of functional consequences. Accordingly, when addressing a 'trained phenotype' in organisms or cells, we do not make generalizable statements about its nature or its consequences.

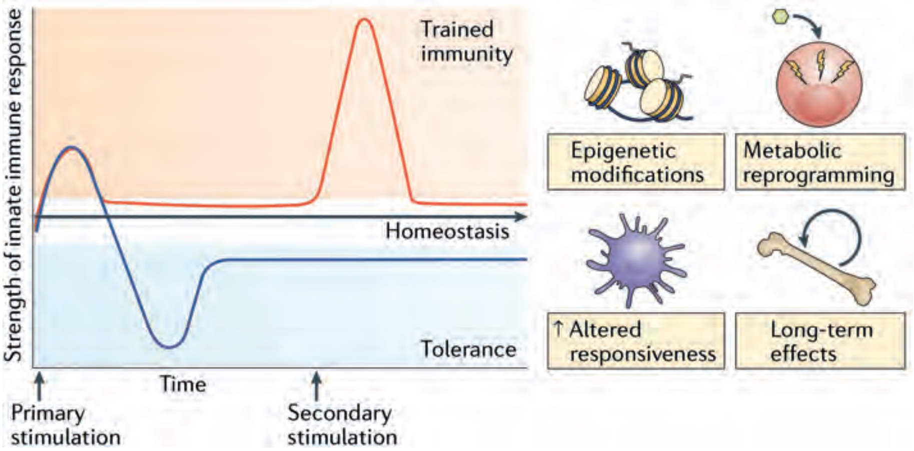

Figure 1: Trained immunity and tolerance as two opposite functional programmes of innate immunity, leading to an enhanced or repressed immune response after training (16).

The foundational demonstration of TRIM was achieved through the administration of the BCG vaccine in healthy volunteers (19). This study showed that BCG enhanced the inflammatory cytokine response to unrelated bacterial and fungal pathogens by changing histone methylation (H3K4me3) through the NOD2-receptor. These changes had been demonstrated before in immunodeficient mice and resulted in enhanced protection against disseminated candidiasis (20), a clinically relevant finding that would be corroborated and expanded in experimental and epidemiological studies in later years (21-23).

Since its initial characterization, TRIM has been shown to be inducible not only after vaccination, but upon environmental factors and natural disease as well (24-26). Inappropriate activation of trained immunity has been hypothesized to play a multitude of roles in excessive inflammation, cardiovascular disease, immune paralysis, and malignancies (15). This potential became especially clear when it was discovered that not only short-lived circulating innate immune cells could be trained, but hematologic progenitor cells in the bone marrow as well, explaining why effects of TRIM could be persistent for months or even years (27).

The ageing immune system

With advancing age, the immune system undergoes significant changes and becomes increasingly dysregulated. While certain aspects remain relatively intact, other immune functions such as chemotaxis and intracellular killing decline. Immune cell populations shrink or show a shift towards subsets that result in less functional capacity to mount an effective immune response. Conversely, the ageing immune system is characterized by a chronic, low-grade systemic inflammation termed 'inflammaging' (28). Both ineffective immune responses and inflammaging are related to the increased risk of elderly persons for age-related comorbidities (29). Despite an increased lifespan, our 'healthspan' – the period of life spent in good health – continues to lag behind.

To counter these issues, vaccines are used in older populations to prevent severe disease and complications. Although the newly developed vaccines against COVID-19 have demonstrated to elicit a robust response in individuals aged 60 and over (30), many other vaccines do not perform as effectively in this age group (31-33). The influenza vaccine, often used without adjuvants, is notoriously less effective in older adults – although it remains crucial at a population level. Some vaccines have shown potential to reduce systemic inflammation on the one hand (34, 35) and boost unspecific immune responses on the other hand, making them interesting research targets for enhancing protection in the elderly. The NSEs of vaccines in older adults are however not well-studied, as most research focuses on vaccines administered during childhood.

Knowledge gaps

Vaccines represent one of the most remarkable achievements in modern public health, paving the way for future advancements in promoting global health. By deepening our understanding non-specific effects and trained immunity, we can tailor protective efforts, particularly for vulnerable groups like senior citizens who could benefit most from enhanced protection. Several important knowledge gaps remain. First, although non-specific effects of vaccination have been increasingly reported, it is not yet fully understood how they are mediated precisely and the optimal vaccines for leveraging these effects remain unclear. Moreover, current vaccine programmes often lack solid substantiation for the choice of vaccination schedules. Moreover, the vaccines used in older individuals have often been developed for and are effective in youngsters, but less so in an older population. Finally, diseases through natural infection can induce trained immunity as well and result in maladaptive heterologous effects, potentially related to post-inflammatory complications that significantly influence clinical outcomes. The role of TRIM in these scenarios is not yet fully elucidated, precluding the development of interventions to manage such complications.

By thoroughly evaluating the benefits and side-effects of vaccines and their potential for NSEs, we could improve the protection they offer. This could involve tailoring vaccination strategies, adjusting sequences or selecting specific vaccines for vulnerable groups. Additionally, utilizing vaccines for their NSEs rather than solely for targeted protection could prove fruitful in preventing a broad range of infectious and non-infectious diseases in the future. Studying the immunomodulation provided by vaccines or natural infection could guide us towards targeted therapies that enhance or damped the immune responses as needed.

Thesis outline

In this thesis, I aimed to gain a better understanding of the broad effects of vaccination, especially mediated by the innate immune system. Accelerated by the SARS-CoV-2 pandemic, we focused our research on the potential heterologous protection offered by the BCG vaccine, influenza vaccine and the available COVID-19 vaccines. Figure 2 illustrates the interconnection among the chapters in this thesis, highlighting the interplay between different aspects of vaccine-related research.

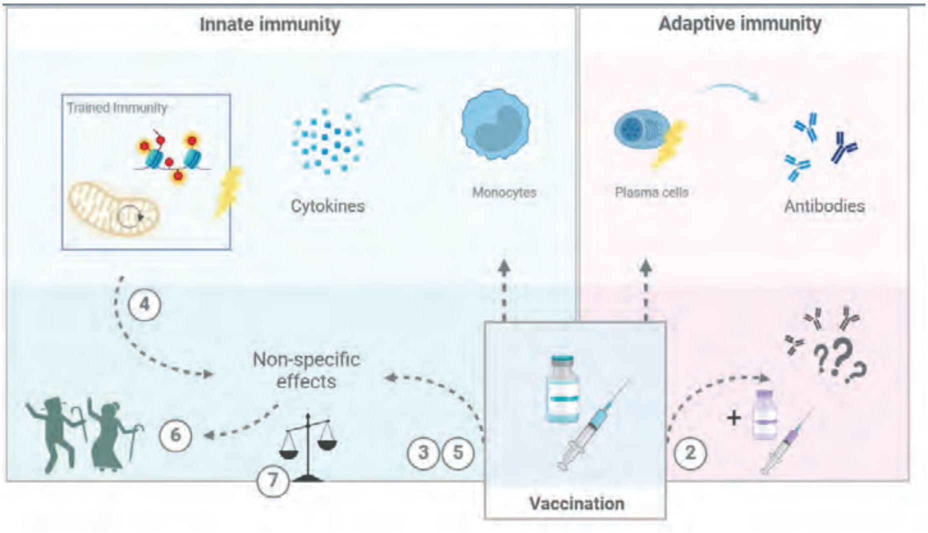

Figure 2: The role of each chapter (numbered) in understanding the interplay between vaccines and the different parts of the immune system.

Essential to informing safe and effective vaccine policy, we investigated the immunogenicity and safety of concurrent and sequential vaccination against COVID-19 and influenza. The results of this **TACTIC trial** are described in **chapter two**.

In **chapter three**, we aimed to provide an overview of the vaccines currently known to induce NSEs and which underlying mechanisms could play a role here. We assessed the evidence for both beneficial and adverse effects and how this knowledge can potentially boost vaccine efficacy.

To further contribute to our knowledge on NSEs, we compared the differential immunological effects of adenoviral and mRNA-based COVID-19 vaccines in **chapter four**. We aimed to identify immunological mechanisms that might be involved in the observed differences in all-cause mortality after COVID-19 vaccination with these distinct platforms. Employing immune cells from vaccinated individuals, we studied their transcriptional and functional responses to various stimuli.

Before specific COVID-19 vaccines were available, various trials were performed to investigate whether known TRIM-inducing vaccines could provide any protective effects. In **chapter five**, we tested the potential for heterologous protection by the inactivated influenza vaccine during the pandemic. Given the special circumstances of this trial, we also reflect on the research methodology in detail.

Although NSEs after BCG vaccination have been extensively described, this topic had remained largely unexplored in older adults. The **BCG-LongTerm study**, detailed in **chapter six**, was created to study the effects of BCG on heterologous infections and immune-mediated diseases in the elderly. We employed a questionnaire to extend the follow-up of previously randomized cohorts to two years after vaccination.

Finally, **chapter seven** analyses the NSEs following natural infection, with the objective to clarify relevant post-infectious effects and explore how this information might be utilized to manage complications in the clinics.

Collectively, these studies [summarized in Table 1] enhance our understanding of the broad effects of vaccination, particularly those mediated by the innate immune system. By investigating both the BCG and COVID-19 vaccines, we have identified potential mechanisms of heterologous protection and outlined the implications for vaccine policy and efficacy. The comprehensive reviews included in this thesis provide a detailed overview of the current state of NSE research, guiding future exploration in this field.

Table 1: Research questions per chapter.

Chapter	Main research question(s)
2. TACTIC trial	*Do COVID-19 and influenza vaccination interfere?* *Which vaccine should be given first?*
3. Innate memory for vaccine design	*Which vaccines induce NSEs and through which mechanisms?* *How can this advance vaccine design and delivery?*
4. TACTIC-II substudy	*Do mRNA and adenoviral COVID-19 vaccines exert different immunological and clinical effects?*
5. Influenza-Brazil study	*Can influenza vaccination provide protective NSEs during the COVID-19 pandemic?*
6. BCG-LongTerm study	*What are the long-term effects of BCG vaccination in older adults?*
7. Post-infectious innate immunity	*How can NSEs after natural infection be explained?*

COVID-19 = coronavirus disease 2019; NSE = non-specific effect; BCG = Bacillus Calmette-Guérin

References

1. Samadzadeh S. The unfinished agenda of communicable diseases among children and adolescents before the COVID-19 pandemic, 1990-2019: A systematic analysis of the Global Burden of Disease study 2019. Lancet. 2023;402(10398):313-35.
2. Organization WH. Declaration of global eradication of smallpox. Weekly Epidemiological Record= Relevé épidémiologique hebdomadaire. 1980;55(20):148-.
3. Cheever MA, Higano CS. PROVENGE (Sipuleucel-T) in prostate cancer: the first FDA-approved therapeutic cancer vaccine. Clin Cancer Res. 2011;17(11):3520-6.
4. Polack FP, Thomas SJ, Kitchin N, Absalon J, Gurtman A, Lockhart S, et al. Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine. New England journal of medicine. 2020;383(27):2603-15.
5. Burnet SFM. The clonal selection theory of acquired immunity: Vanderbilt University Press Nashville; 1959.
6. Chang HD, Radbruch A. Maintenance of quiescent immune memory in the bone marrow. Eur J Immunol. 2021;51(7):1592-601.
7. Okhrimenko A, Grün JR, Westendorf K, Fang Z, Reinke S, von Roth P, et al. Human memory T cells from the bone marrow are resting and maintain long-lasting systemic memory. Proc Natl Acad Sci U S A. 2014;111(25):9229-34.
8. Berendsen MLT, Øland CB, Bles P, Jensen AKG, Kofoed PE, Whittle H, et al. Maternal Priming: Bacillus Calmette-Guérin (BCG) Vaccine Scarring in Mothers Enhances the Survival of Their Child With a BCG Vaccine Scar. J Pediatric Infect Dis Soc. 2020;9(2):166-72.
9. Garly M-L, Martins CL, Balé C, Baldé MA, Hedegaard KL, Gustafson P, et al. BCG scar and positive tuberculin reaction associated with reduced child mortality in West Africa: A non-specific beneficial effect of BCG? Vaccine. 2003;21(21):2782-90.
10. Benn CS, Netea MG, Selin LK, Aaby P. A small jab - a big effect: nonspecific immunomodulation by vaccines. Trends Immunol. 2013;34(9):431-9.
11. Higgins JPT, Soares-Weiser K, López-López JA, Kakourou A, Chaplin K, Christensen H, et al. Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review. BMJ. 2016;355:i5170.
12. Stensballe LG, Ravn H, Birk NM, Kjærgaard J, Nissen TN, Pihl GT, et al. BCG Vaccination at Birth and Rate of Hospitalization for Infection Until 15 Months of Age in Danish Children: A Randomized Clinical Multicenter Trial. J Pediatric Infect Dis Soc. 2019;8(3):213-20.
13. Benn CS, Schaltz-Buchholzer F, Nielsen S, Netea MG, Aaby P. Randomized clinical trials of COVID-19 vaccines: Do adenovirus-vector vaccines have beneficial non-specific effects? iScience. 2023;26(5):106733.
14. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124(4):783-801.
15. Netea MG, Domínguez-Andrés J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, et al. Defining trained immunity and its role in health and disease. Nat Rev Immunol. 2020;20(6):375-88.
16. Arts RJW, Carvalho A, La Rocca C, Palma C, Rodrigues F, Silvestre R, et al. Immunometabolic Pathways in BCG-Induced Trained Immunity. Cell Rep. 2016;17(10):2562-71.
17. Cheng S-C, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, et al. mTOR-and HIF-1α–mediated aerobic glycolysis as metabolic basis for trained immunity. science. 2014;345(6204):1250684.

18. Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajanirefah A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. science. 2014;345(6204):1251086.
19. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LAB, Ifrim DC, Saeed S, et al. Bacille Calmette-Guérin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proceedings of the National Academy of Sciences. 2012;109(43):17537-42.
20. Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. Cell Host Microbe. 2012;12(2):223-32.
21. Arts RJ, Moorlag SJ, Novakovic B, Li Y, Wang S-Y, Oosting M, et al. BCG vaccination protects against experimental viral infection in humans through the induction of cytokines associated with trained immunity. Cell host & microbe. 2018;23(1):89-100. e5.
22. Walk J, de Bree LCJ, Graumans W, Stoter R, van Gemert G-J, van de Vegte-Bolmer M, et al. Outcomes of controlled human malaria infection after BCG vaccination. Nature communications. 2019;10(1):874.
23. Kleinnijenhuis J, Quintin J, Preijers F, Benn CS, Joosten LAB, Jacobs C, et al. Long-Lasting Effects of BCG Vaccination on Both Heterologous Th1/Th17 Responses and Innate Trained Immunity. Journal of Innate Immunity. 2013;6(2):152-8.
24. Leentjens J, Bekkering S, Joosten LA, Netea MG, Burgner DP, Riksen NP. Trained innate immunity as a novel mechanism linking infection and the development of atherosclerosis. Circulation research. 2018;122(5):664-9.
25. Christ A, Latz E. The Western lifestyle has lasting effects on metaflammation. Nature Reviews Immunology. 2019;19(5):267-8.
26. Schrum JE, Crabtree JN, Dobbs KR, Kiritsy MC, Reed GW, Gazzinelli RT, et al. Cutting edge: Plasmodium falciparum induces trained innate immunity. The Journal of Immunology. 2018;200(4):1243-8.
27. Kaufmann E, Sanz J, Dunn JL, Khan N, Mendonça LE, Pacis A, et al. BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis. Cell. 2018;172(1-2):176-90.e19.
28. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. J Gerontol A Biol Sci Med Sci. 2014;69 Suppl 1:S4-9.
29. Bowker L, Price J, Smith S. Oxford handbook of geriatric medicine: OUP Oxford; 2012.
30. Baden LR, El Sahly HM, Essink B, Kotloff K, Frey S, Novak R, et al. Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. New England journal of medicine. 2021;384(5):403-16.
31. Haq K, McElhaney JE. Immunosenescence: influenza vaccination and the elderly. Current opinion in immunology. 2014;29:38-42.
32. Vermeiren AP, Hoebe CJ, Dukers-Muijrers NH. High non-responsiveness of males and the elderly to standard hepatitis B vaccination among a large cohort of healthy employees. Journal of Clinical Virology. 2013;58(1):262-4.
33. Pereira B, Xu X-N, Akbar AN. Targeting Inflammation and Immunosenescence to Improve Vaccine Responses in the Elderly. Frontiers in Immunology. 2020;11.
34. Debisarun PA, Gössling KL, Bulut O, Kilic G, Zoodsma M, Liu Z, et al. Induction of trained immunity by influenza vaccination - impact on COVID-19. PLOS Pathogens. 2021;17(10):e1009928.
35. Koeken VA, de Bree LCJ, Mourits VP, Moorlag SJ, Walk J, Cirovic B, et al. BCG vaccination in humans inhibits systemic inflammation in a sex-dependent manner. The Journal of clinical investigation. 2020;130(10):5591-602.

Chapter 2

Timing and sequence of vaccination against COVID-19 and influenza (TACTIC): a single-blind, placebo-controlled randomized clinical trial

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Abstract

Background Novel mRNA-based vaccines have been used to protect against SARS-CoV-2, especially in vulnerable populations who also receive an annual influenza vaccination. The TACTIC study investigated potential immune interference between the mRNA COVID-19 booster vaccine and the quadrivalent influenza vaccine, and determined if concurrent administration would have effects on safety or immunogenicity.

Methods TACTIC was a single-blind, placebo-controlled randomized clinical trial conducted at the Radboud University Medical Centre, the Netherlands. Individuals ≥60 years, fully vaccinated against COVID-19 were eligible for participation and randomized into one of four study groups: 1) 0.5ml influenza vaccination Vaxigrip Tetra followed by 0.3ml BNT162b2 COVID-19 booster vaccination 21 days later, (2) COVID-19 booster vaccination followed by influenza vaccination, (3) influenza vaccination concurrent with the COVID-19 booster vaccination, and (4) COVID-19 booster vaccination only (reference group). Primary outcome was geometric mean concentration (GMC) of IgG against the spike (S)-protein of the SARS-CoV-2 virus, 21 days after booster vaccination. We performed a non-inferiority analysis of concurrent administration compared to booster vaccines alone with a predefined non-inferiority margin of -0.3 on the log10-scale.

Findings 154 individuals participated from October, 4, 2021, until November, 5, 2021. Anti-S IgG GMCs for the co-administration and reference group were 1684 BAU/ml and 2435 BAU/ml, respectively. Concurrent vaccination did not meet the criteria for non-inferiority (estimate -0.1791, 95% CI -0.3680 to -0.009831) and antibodies showed significantly lower neutralization capacity compared to the reference group. Reported side-effects were mild and did not differ between study groups.

Interpretation Concurrent administration of both vaccines is safe, but the quantitative and functional antibody responses were marginally lower compared to booster vaccination alone. Lower protection against COVID-19 with concurrent administration of COVID-19 and influenza vaccination cannot be excluded, although additional larger studies would be required to confirm this.

Funding The study was supported by the ZonMw COVID-19 Programme.

Keywords
COVID-19; SARS-CoV2 Infection; Infectious diseases; Vaccines; mRNA; Influenza

Research in context

Evidence before this study

During the COVID-19 pandemic, novel mRNA vaccines have successfully been employed to decrease morbidity and mortality worldwide. Booster vaccinations to maintain immunity over a longer time and in the context of new emerging variants were proven to be safe and effective. One of the groups most at risk for severe COVID-19 are older adults and protective efforts have been made to shield this vulnerable population. Before our study started, research had not focused on the potential co-administration of vaccination against the SARS-CoV-2 virus and the influenza virus. A collection of previous research into different vaccines suggests the possibility of interference between some vaccines, but mRNA vaccines had not been studied in this context.

Added value of this study

This study did not prove non-inferiority of concurrent administration of the BNT162b2 COVID-19 booster vaccinate and the Vaxigrip Tetra influenza vaccine, suggesting possible immune interference. To our knowledge, this is the first RCT that investigated immunogenicity of concurrent administration in a representative group of older adults with predefined non-inferiority margins and an additional focus on mucosal antibodies and systemic inflammation.

Implications of all the available evidence

The marginally lower serological responses after concurrent vaccination with a COVID-19 booster and an influenza vaccine found in this study are an import aspect to consider in public health policy and future vaccination campaigns aimed at older adults. This is of major importance for the upcoming influenza season, as well as for protection of vulnerable groups against other future pathogens. The findings of this study highlight the need for more research into the potential for immune interference prior to policy decisions concerning simultaneous administration of COVID-19 and influenza vaccines, as well as other vaccine combinations.

Introduction

The SARS-CoV-2 virus causing coronavirus disease 2019 (COVID-19) has quickly spread worldwide and caused over 6 million deaths since the first case was diagnosed in December 2019 (1). Novel viral vector vaccines (such as Ad26.COV2.S and ChAdOx1-S) and mRNA-based vaccines (such as BNT162b2 and mRNA-1237) against COVID-19 were introduced in 2021, and showed clear beneficial effects by decreasing morbidity and mortality (2, 3). Although considered successful in inducing protection against infection and severe disease, the longevity of this protection has been shown to decline over time. Antibody concentrations in the circulation of vaccinated individuals decreased in a matter of months and new virus variants emerged (4, 5). Because of these observations, many countries provided 'booster shots' to maintain immunity in the population. The Netherlands started a vaccination campaign with booster shots employing the BNT162b2 vaccine to avoid a 2021 winter surge by the then-dominant Delta variant (B.1.617.2).

The timing of this campaign coincided with the existing annual Dutch vaccination program against the influenza virus, in which more than 3 million persons at risk of severe disease are immunized every autumn. Co-administration of the vaccine against COVID-19 and influenza would provide many logistic advantages, but the combination could theoretically result in both positive and negative responses: ranging from enhanced immunity against both viruses, to inhibition of immune responses to one or both of the viruses due to immune interference. Earlier studies have investigated the co-administration of different live and inactivated vaccines, reporting variable results. In some studies, no effect on immunogenicity of vaccination with live-attenuated influenza vaccines concurrently administered with other common childhood vaccines was measured, while in other studies immune interference was found (6-8). When administering distinct types of vaccines sequentially, some sequences have been associated with reduced or increased mortality rates (9, 10). In contrast to suppression of immunogenicity or protection, it has been suggested that vaccination with an inactivated influenza vaccine could boost the immune response to SARS-CoV-2 by inducing trained immunity (11). This same study also indicated that the influenza vaccine could lower systemic inflammation, whereas concerns have been raised about increased inflammation in response to mRNA vaccines (12). The long-term inflammatory effects have not been studied in the context of co- or sequential administration of the novel COVID-19 vaccines. Long-term complications resulting from enhanced inflammation could potentially occur and would need to be ruled out.

Vaccine-induced immune interference is difficult to predict and because of their novelty, the immunological and clinical interactions between mRNA vaccines and influenza vaccines had not been studied before. Different sequences of administration may alter their potential effects. To unravel the potential immune interference between these vaccines in terms of immunogenicity and safety, and to establish an optimal vaccination strategy, the TACTIC-study was designed to assess different schemes of administration of these two vaccines. The primary aim of this study was to investigate whether influenza vaccination prior to, after, or combined with COVID-19 vaccination would influence the immune response against SARS-CoV-2 induced by the mRNA vaccine. Investigating this effect is of profound importance for the vaccination strategy in the coming years: both for the upcoming additional booster campaigns against COVID-19 (13), as well as for the use of novel vaccines in the more distant future (14).

Methods

Study design

The TACTIC study was a single-blind, placebo-controlled randomized clinical proof-of-principle trial conducted at the Radboud university medical center (Radboudumc) in Nijmegen, the Netherlands. The overall aim of the study was to evaluate immunogenicity and safety of combined influenza- and COVID-19 booster vaccinations, investigating four vaccination schemes: (1) influenza vaccination Vaxigrip Tetra followed by a BNT162b2 COVID-19 booster vaccination 21 days later (hereafter called ‘influenza first’), (2) COVID-19 booster vaccination followed by influenza vaccination 21 days later (‘booster first’), (3) influenza vaccination concurrent with a booster vaccination (‘combination’), and (4) booster vaccination only (‘booster only’). Placebo vaccines were used to prevent the participants from deducing the group they had been placed in (Figure 1).

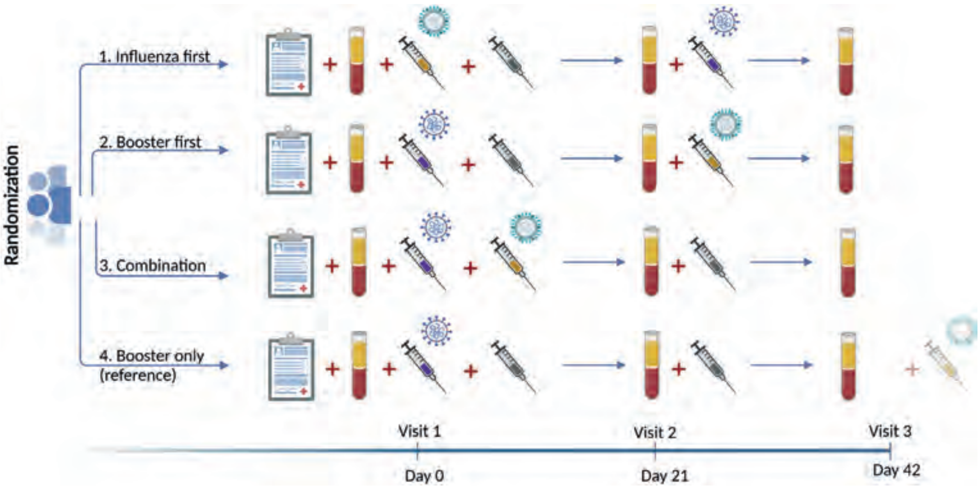

Figure 1: Study design

This study was performed in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice, as well as the local Radboudumc Research code. Approval was obtained from the competent authority (CCMO; EudraCT number 2021-002186-17) and the medical ethics committee Oost-Nederland (file number 2021-8294). Written informed consent was obtained from all study participants.

Participants

Participants were recruited by an advertisement in local newspapers, on social media and the Radboudumc research website. Volunteers aged ≥60 years who were fully vaccinated against COVID-19 at least four months prior to study start were eligible for participation (one dose of the Janssen vaccine, two doses AstraZeneca or mRNA vaccine, or one dose AstraZeneca or mRNA vaccine after previous COVID-19 infection). Details of eligibility criteria can be found in the study protocol (*attached as supplementary file*).

Randomization and masking

Castor Electronic Data Capture system (Castor EDC) randomized participants to one of the four study groups, giving equal weight to all groups and using variable block size (4, 8, 12). Participants were blinded to their group allocation by using identical syringes for all vaccines to minimize influence on reported adverse events. Trial personnel were not blinded.

Procedures

The study encompassed three study visits, each 21 days apart (*see figure 1*). During the first study visit, participants gave informed consent and baseline characteristics were recorded. Vaccines were administered intramuscularly in the upper arm during visit 1 and 2 and participants were observed 15 minutes after vaccination. We used the recommended dose of 0.5ml Vaxigrip Tetra and 0.3ml BNT162b2 COVID-19 booster vaccine, as well as 0.5ml sterile NaCl 0.9% as a placebo. In the case of two concurrently administered vaccines, two different injection sites in opposite arms were used when medically possible. Plasma, serum and mucosal lining fluid (MLF) samples were obtained during all three visits (T1 – T3). MLF was collected using Nasosorption™ FXi nasal sampling devices (Hunt Developments, UK). Participants used paper diaries to report any adverse event or possible side-effects for 14 days after each vaccination and assessed severity on a 5-point Likert-scale ('none' to 'extreme'). The side-effects listed in the diary were based on the most common side-effects associated with the used vaccines.

Outcomes

Primary endpoint

Geometric mean concentration of IgG responses against the spike (S)-protein of the SARS-CoV-2 virus in plasma, at 21 days after booster vaccination.

Secondary endpoints

- IgA responses against S-protein and IgA and IgG responses against receptor binding domain (RBD) in plasma at baseline, 21 days after each vaccination;
- IgA and IgG responses against the nucleocapsid (N)-protein to control for infection during the study;
- Seroconversion of IgG to S-protein at day 21 after the COVID-19 booster vaccine (defined as a change from seronegative at baseline (T1) to seropositive or a ≥four-fold increase);
- Neutralization of the ancestral SARS-CoV-2 Wuhan, B1.1.7 (alpha), B.1.617.2 (delta) and B.1.1.529 (omicron) variants, at 42 days after first study vaccination round;
- IgA and IgG responses against S- and N- protein in mucosal lining fluid at baseline and 21 days after each vaccination;
- Haemagglutinin inhibition titers in serum at 21 days and 42 days;
- Serious adverse events (SAEs) and other adverse events (AE);
- Local reactions at injection site or systemic reactions after vaccination.

Exploratory endpoint

Assessment of systemic inflammation by targeted proteome analysis.

Statistical analysis

Sample size calculation

The required sample size to evaluate non-inferiority of the primary endpoint had been calculated based on geometric mean IgG titers after vaccination with the BNT152b2 vaccine. The aim was to include 35 participants per intervention group, providing 90% power to evaluate non-inferiority of 'influenza first', 'booster first' and 'concomitant influenza- and COVID-19 booster vaccine', compared to a COVID-19 booster alone, considering an estimated means of -0.3 on the log10-scale as a non-inferiority margin. It must be noted that this was a conservative calculation, since the expected IgG titer variability (SD) after booster vaccination was likely to be lower than after primary vaccination, but no evidence was available at the time.

Comparative analyses

Statistical analyses were performed using GraphPad prism version 8, IBM SPSS Statistics for Windows, Version 26, R version 4.1.3 and SAS version 9.4. Analyses were performed according to the intention-to-treat principle. As no participants switched to another study group (intentionally or accidentally), these analyses equal a per-protocol population.

Baseline and safety variables were compared between participants in the different study groups, and differences were statistically tested using χ^2 tests, Fisher's exact tests (because of small numbers) or t-tests for independent samples as appropriate. For safety results, relative risks were calculated. Non-inferiority of the 'concurrent administration' was evaluated, comparing the anti-S IgG levels of that group with the reference group who received a booster vaccination only. We performed a linear mixed models analysis using Proc Mixed Model in SAS with the log transformed anti-S IgG concentrations at day 21 as outcome and group as a fixed factor. We used the Kenward-Roger method for computing the denominator degrees of freedom for the tests of fixed effects. Least squares means estimates of all groups were compared with the reference group (COVID-19 booster only) and a Dunnett correction was used to adjust for multiple testing (overall alpha=5%). For the interpretation of non-inferiority, Dunnett adjusted confidence intervals of the differences in least square means are presented in the results. If the lower limit of the adjusted confidence interval lies above the predefined non-inferiority margin of -0.3 on the log10-scale, we would conclude that the result of the corresponding group is non-inferior to the reference group.

We performed a sensitivity analysis of the primary endpoint, adjusting for differences in log transformed baseline IgG against SARS-CoV-2. As subsequent sensitivity analyses, we also adjusted for pneumococcal vaccine at baseline and baseline levels of anti-N IgG, as well as excluding participants who reported prior COVID-19. To conclude the non-inferiority analyses, we combined the 'booster first' with the 'booster only' group to create a larger reference group.

We checked the assumption of normality of residuals graphically and the residuals were normally distributed.

Antibody levels against the SARS-CoV-2 S-, RBD- and N-protein over the course of the study were measured and reported as geometric mean concentrations over time. Anti-N levels were measured to determine if any of the participants contracted a SARS-CoV-2 infection during the study. Qualitative serology titers for the reference group and different vaccination schemes were compared using Mann-Whitney U-tests. Mucosal anti-S IgG antibodies were correlated to antibodies from plasma using Pearson correlation. Protein measurements were denoted as normalized protein expression values (NPX) and analyzed by principal component analysis, including all four study groups. Participants from which one or more proteins could not be measured are not included in this PCA analysis. Wilcoxon paired signed-rank test was used to compare NPX values at 42 days after initial study vaccination to baseline, for each study group separately. Benjamini-Hochberg adjustment was used to correct the proteomics data for multiple testing. A total of 44 out of 92 measured proteins were detected in at least 70% of the plasma samples and were included in the analyses (*see supplementary methods 1*).

Effect estimates were reported with 95% confidence intervals. All statistical tests were performed in a two-sided manner and a P-value <0.05 was considered statistically significant. Given the set-up of the trial, the relative short study period and the use of established investigational products, no data monitoring committee was employed.

Laboratory analyses

Blood samples were obtained from the cubital vein and stored at -80°C prior to analysis. Mucosal lining fluid absorption strips were placed back into protective plastic tubes after sampling and stored at -20°C until further processing.

Serology and mucosal antibodies

To measure the levels of antibodies against RBD and Spike protein, a fluorescent-bead-based multiplex immunoassay (MIA) was developed as previously described by Fröberg et al. 2021, with some slight modifications.[15] The first international standard for anti-SARS-CoV-2 immunoglobulin, (20/136, NIBSC), was used to create standard curves. Next to this, four different samples from PCR-confirmed COVID-19 patients were used as quality control samples. The stabilized pre-fusion conformation of the ectodomain of the S-protein (D614G mutant) and the RBD-protein, both purchased from ExcellGene (Monthey, Switzerland), were each coupled to beads or microspheres with distinct fluorescence excitation and emission spectra. Serum samples were diluted in assay buffer (SM01/1%BSA) and incubated for 45 minutes with the antigen-coated microspheres. Following incubation, the microspheres were washed three times and incubated with phycoerythrin-conjugated goat anti-human, IgG. The data were acquired on the Luminex FlexMap3D System. Validation of the detection antibodies was obtained from a recent publication using the same antibodies and the same assay (Den Hartog et al., 2020), and specificity was checked using rabbit anti-SARS SIA-ST serum. Mean fluorescent intensities (MFI) were converted to International Units (IU/ml) by interpolation from a log-5PL-parameter logistic standard curve and log–log axis transformation, using Bioplex Manager 6.2 (Bio-Rad Laboratories) software and exported to R-studio.

Plaque reduction neutralization assays

Serum samples were tested for the presence of neutralizing antibodies against ancestral SARSCoV-2, Alpha, Delta and Omicron (BA.1) variants in a plaque reduction neutralization test (PRNT) as previously described.[16-18] Viruses were cultured from clinical specimen and were confirmed by next-generation sequencing: D614G (ancestral, GISAID: hCov19/Netherlands/ZH-EMC-2498), B.1.1.7 (alpha, GISAID: hCov-19/Netherlands/ZH-EMC-1148), B.1.617.2 (Delta, GISAID: hCoV-19/Netherlands/NB-MVDCWGS2201159/2022), and B.1.1.529 (Omicron BA.1, GISAID: hCoV-19/Netherlands/LISQD-01032/2022).

The human airway Calu-3 cell line (ATCC HTB-55) was used to grow virus stocks and for PRNT. Calu-3 cells were cultured in OptiMEM (Gibco) supplemented with Glutamax, penicillin (100 IU/mL), streptomycin (100 IU/mL), and 10% fetal bovine serum (FBS). In short, heat-inactivated sera were diluted two-fold in OptiMEM without FBS starting at a 1:10 dilution or in the case of a S1-specific antibody level >2500 BAU/mL starting at 1:80 in 60µL. 400 PFU of each SARS-CoV-2 variant in 60µL OptiMEM medium was added to diluted sera and incubated at 37°C for 1 hour.

Antibody-virus mix was transferred onto Calu-3 cells and incubated at 37°C for 8 hours. Cells were fixed in PFA and stained with polyclonal rabbit anti-SARS-CoV-2 nucleocapsid antibody (Sino Biological) and a secondary peroxidaselabeled goat-anti rabbit IgG antibody (Dako). Signal was developed with precipitate-forming 3,3′,5,5′-tetramethylbenzidine substrate (TrueBlue; Kirkegaard & Perry Laboratories) and the number of plaques per well was counted with an ImmunoSpot Image Analyzer (CTL Europe GmbH). The 50% reduction titer (PRNT50) was estimated by calculating the proportionate distance between two dilutions from which the endpoint titer was calculated. Infection controls (no sera) and positive serum control (Nanogram® 100 mg/mL, Sanquin) were included on each plate. A PRNT50 value one dilution step (PRNT50 = 10) lower than the lowest dilution was attributed to samples with no detectable neutralizing antibodies.

Hemagglutinin inhibition assays

Hemagglutination inhibition assays were performed following standard protocols [19]. Briefly, treated serum samples were serially diluted two-fold and mixed with virus stock (25 µL) containing 4 hemagglutinating units, which incubated for 30 minutes at 37 °C. Turkey erythrocyte solution (25 µL, 1%) was added and after 1 h incubation at 4 °C inhibition patterns were recorded. Titers were expressed as the value of the highest serum dilution that gave complete inhibition of agglutination.

Proteomics

Plasma proteins were measured using the Olink Inflammation panel by Olink Proteomics (Uppsala, Sweden).

Protocol amendments

The vector vaccine Ad26.COV2.s produced by Janssen was initially also included in the study, but when it became apparent this would not be used in the Dutch booster campaign, it was removed from the protocol. New emerging SARS-CoV-2 variants were added to the analysis (B.1.617.2 and B.1.1.529). Before the study started, the timepoint for primary analysis was altered from 21 days after last vaccination to 21 days after booster vaccination, as we considered this more relevant for our research question. The final study protocol can be found in a supplementary file.

Role of the funding source

The study was supported by the COVID-19 program of the Dutch Organization for Scientific Research (ZonMw). The funder had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Study population

We included 154 individuals between October 4, 2021 and November 5, 2021. 88 participants were male (56%) and the median age of volunteers was 66 years (see *Table 1*). 153 (99%) participants completed the study and received the intended vaccines in the predetermined order, according to their respective randomization (*see flow diagram in supplementary materials, figure 1*). The majority (100/154, 65.3%) had received previous SARS-CoV-2 vaccines from Pfizer/BioNTech and only 3 individuals (2%) had experienced COVID-19 before study start. The average time between the last primary SARS-CoV-2 vaccination dose and the study start was four to five months.

Table 1: Baseline characteristics

	Overall (N=154)	**1) Influenza first (N=39)**	**2) Booster first (N=39)**	**3) Combination (N=38)**	**4) Booster only (N=38)**
Demographics					
Age, years [median, (IQR)]	66.0 (64-72)	66.0 (64-73)	66.0 (62-71)	67.5 (64-74)	65.5 (63-71)
Male sex	88 (57.1%)	19 (48.7%)	21 (53.8%)	25 (65.8%)	23 (60.5%)
Actively smoking	88 (57.1%)	25 (64.1%)	19 (48.7%)	19 (50.0%)	25 (65.8%)
BMI [mean, (SD)]	25.8 (4.3)	25.4 (4.0)	25.9 (4.0)	25.7 (3.5)	26.4 (5.6)
SARS-CoV-2					
History of COVID-19	3 (1.9%)	1 (2.6%)	0 (0.0%)	0 (0.0%)	2 (5.3%)
Pfizer previously	101 (65.6%)	24 (61.5%)	28 (71.8%)	27 (71.1%)	22 (57.9%)
AstraZeneca previously	52 (33.8%)	15 (38.5%)	11 (28.2%)	11 (28.9%)	15 (39.5%)
Moderna previously	1 (0.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (2.6%)
Days since last vaccination [mean, (SD)]	146 (33)	142 (23)	154 (44)	145 (32)	143 (27)
Vaccination status					
History of BCG vaccination	51 (33.1%)	15 (38.5%)	12 (30.8%)	16 (42.1%)	8 (21.1%)
History of pneumococcal vaccination	17 (11.0%)	5 (12.8%)	0 (0.0%)	7 (18.4%)	5 (13.2%)
Influenza vaccine in season '20/'21	138 (89.6%)	35 (89.7%)	34 (7.2%)	35 (92.1%)	34 (89.5%)

IgG responses against SARS-CoV-2 S-proteinThe reference group receiving only a COVID-19 booster vaccination acquired the highest GMC of 2542.2 BAU/ml (binding antibody units) at 21 days after COVID-19 booster vaccination; the combination group with concurrent vaccination showed a lower response with a GMC of 1683.6 BAU/ml. GMCs for 'influenza first' and 'booster first' were 2347.9 and 2136.8 BAU/ml, respectively. Concurrent vaccination did not meet criteria for non-inferiority (estimate -0.1791, 95% CI -0.3680 to -0.009831). Sensitivity analyses correcting for multiple variables as explained previously, did not change this (supplementary table 1). When comparing concurrent vaccination to the merged reference group of 'booster only' and 'booster first', this did show non-inferiority (estimate -0.1165, 95% CI -0.2507 to 0.01767). The vaccination schemes incorporating 21 days in between both vaccines were both non-inferior when compared to vaccination with the booster vaccine alone.

Anti-S antibody concentrations after booster vaccination initially rose in the first 3 weeks but started declining within 42 days of vaccination, at a similar rate across all four groups (*see figure 2*). Geometric mean concentrations (GMCs) at baseline and 21 days after booster vaccination can be found in *table 2*.

Sensitivity analysis adjusting for differences in baseline IgG and previous pneumococcal vaccination showed concurrent vaccination still did not meet non-inferiority criteria when compared to vaccination with a booster only, with respect to IgG response (estimate -0.1391, 95% CI -0.3034 to 0.02510).

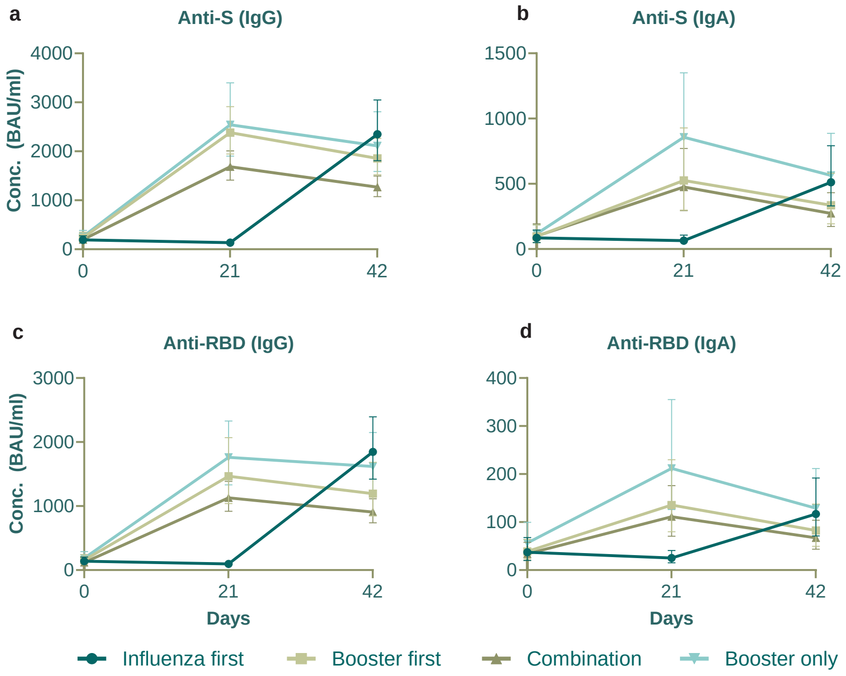

Figure 2: Geometric mean concentrations (with 95% error bars) of IgA and IgG antibodies against S-protein and RBD over the course of the study

Table 2: Geometric mean concentrations (GMCs) of anti-S IgG at baseline and at 21 days after booster vaccination

	IgG against S-protein (BAU/ml)
Influenza first (N=39)	
Baseline	190.4
Day 21	2347.9
Booster first	
Baseline (N=39)	225.1
Day 21 (N=38)	2136.8
Combination (N=37)	
Baseline	199.0
Day 21	1683.6
Booster only	
Baseline (N=38)	258.2
Day 21 (N=37)	2542.8

IgA and IgG responses against RBD-, S- and N-protein

Over the course of the study, IgA and IgG anti-RBD antibodies and IgA anti-S levels amongst all groups showed the same trend of an initial rise and subsequent decline (*see figure 2; b-f; supplementary figure 3 for individual data points*), in similar fashion to anti-S IgG. Corresponding GMCs can be found in *supplementary table 2*. No relevant differences in anti-N antibodies compared to baseline were measured, indicating that none of the participants were infected with SARS-CoV-2 during the study *(see supplementary figure 2)*.

Antibodies found in mucosal lining fluid showed patterns comparable to those found in plasma (r=0.476, p=<0.01; *see supplementary figure 4, supplementary figure 5 for individual data points)*.

Seroconversion of IgG against S-protein at 21 days after booster vaccination

All participants had been vaccinated against SARS-CoV-2 before the start of the study and baseline results showed the presence of anti-S IgG antibodies. Across all study groups, a large majority showed seroconversion at 21 days after booster vaccination: 35/39 (89 .7%) in the 'influenza first' group, 27/37 (73%) in 'booster first', 27/37 (75%) in the 'combination group', and 31/37 (83 .8%) in 'booster only'. There were no differences were between groups (Fisher's exact test, p=0.1747).

Virus neutralization

The neutralizing capacity of the induced antibodies showed comparable plaque-reducing neutralization titers for the original and delta-variant of the SARS-CoV-2 virus, but markedly lower effectivity against the omicron-variant (*see figure 3*). The 'combination group' showed significantly lower virus neutralizing capacity than the reference group, as a higher antibody concentration was needed to neutralize 50% of the viral plaque (log2 titers 1:690.5 versus 1:1530, p=0.0463 for Delta; 1:75.5 versus 1:266.5, p=0.0093 for Omicron). Neutralization results were not statistically different between the reference group and the consecutive vaccination schemes.

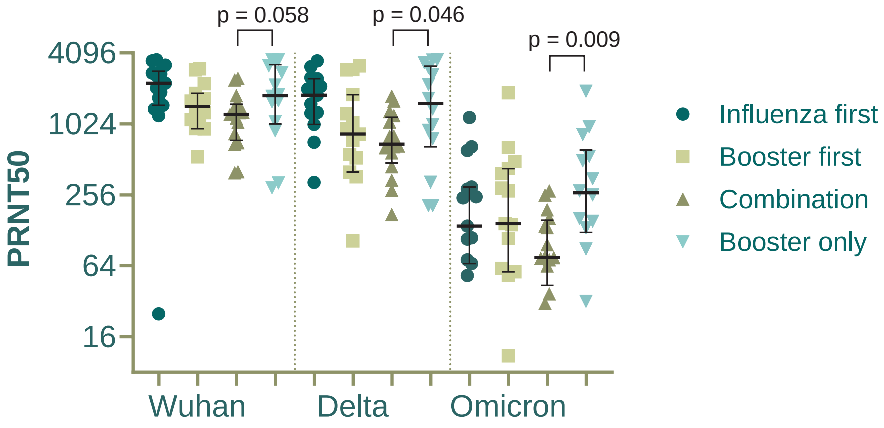

Figure 3: 50% plaque-reducing neutralization titers (PRNT-50) for the D614G, delta and omicron variant of the SARS-CoV-2 virus, compared between all groups at 42 days after first study vaccinations (Visit 3)

Hemagglutinin inhibition assays

The HAI results show the induction of antibodies against influenza for all three groups who received an influenza vaccine (*figure 4*), 21 days after vaccination. No significant difference in titers was found between these groups, notably not between the 'combination' group and the 'influenza first' group, who, at the time, only had received an influenza vaccination.

Systemic inflammation after vaccinationsPrincipal component analysis confirmed that our four study groups were generally comparable without extreme outliers and showed none of the vaccination schemes radically changed a group (*Figure 5a*). Specific comparisons of the effects of the mRNA booster vaccine showed upregulated inflammatory proteins after booster vaccination, most pronounced at 42 days after vaccination (*Figure 5; b-e*).

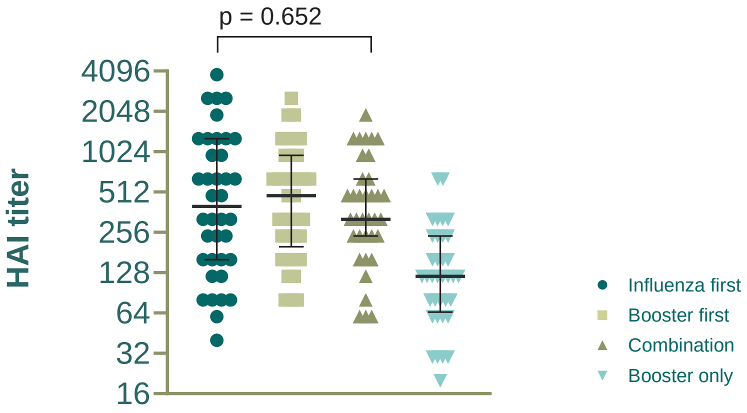

Figure 4: Hemagglutinin inhibition (HAI) titers against the H1N1pdm influenza virus at 21 days after influenza vaccination (groups 'influenza first', 'booster first', 'combination')

Safety of the vaccination schemes

One serious adverse event occurred during the study (acute cholecystectomy in the 'booster first' group) and has been assessed to be unrelated to any study procedure. After recovery, the participant took part in the final study visit.

Local and systemic side-effects

The reported side-effects were considered mild and more than 75% of symptoms resolved spontaneously after 2-3 days in all study groups. No participants sought medical advice for their symptoms and no unexpected side-effects occurred. An overview of the side-effects per group can be found in *supplementary table 3*. The most commonly reported side-effects after influenza vaccination were redness and pain at injection site, headache and fatigue. After COVID-19 booster vaccination, pain at injection site, myalgia and headache were the most prominent (*see supplementary figure 6*). Relative risks for participants in the 'combination' group compared to the reference group ('booster only') did not differ for any of the side-effects (*supplementary table 4*).

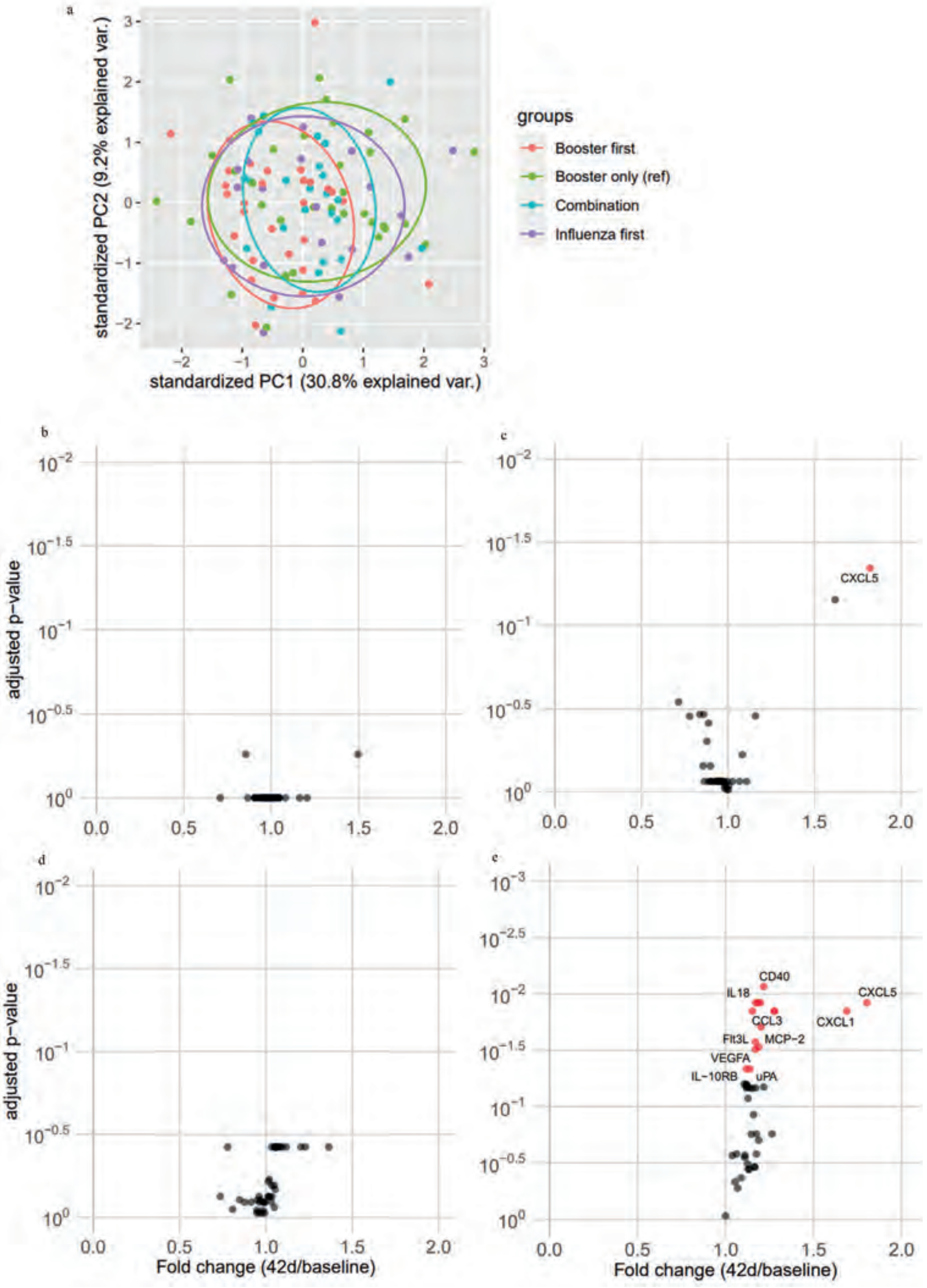

Figure 5: a): Principal component analysis of plasma proteins at 42 days after first study vaccine. (b-e): volcano plot with fold changes of proteins in all four groups, 42 after first study vaccine compared to baseline. b = Influenza first, c = Booster first, d = Combination, e = Booster only (ref)

Discussion

This study presents the results from the TACTIC trial, designed to investigate the impact of co-administration of an mRNA COVID-19 booster vaccine and an influenza vaccine on the vaccine safety and antibody responses. Based on our results, we cannot exclude non-inferiority of concurrent administration of a COVID-19 booster vaccine and an influenza vaccine, compared to COVID-19 booster vaccine only. Both quantity and functionality of the antibody response against SARS-CoV-2 was diminished when compared to receiving a booster vaccine alone or administration regimens allowing 3 weeks between the vaccines. In regards to safety of concurrent administration, we found no additional or more severe adverse events when compared to sequential administration.

The most important observation of this study is that simultaneous administration of a COVID-19 booster and an influenza vaccination results in a lower serological response against SARS-CoV-2. The predefined non-inferiority criteria for comparing antibody concentrations between the simultaneous vaccine administration and booster vaccination alone were not met in our initial or sensitivity analysis, with mean concentrations of anti-S IgG being marginally lower in the simultaneous vaccination group. Viral neutralization assays against SARS-CoV-2 also suggested potential immune interference. The clinical impact of this effect is partially uncertain, as a threshold associated with minimal protection is not yet available for COVID-19 (i.e. antibody-based correlate of protection). However, it is conceivable that a lower level of specific antibodies might result in reduced protection against COVID-19. Especially in vulnerable populations, this could be deleterious. The antibody responses measured in mucosal lining fluid were comparable to those measured in blood, which could be explained by translocation of systemically induced antibodies to the mucosal surface. Although the induction of specific mucosal antibodies after an mRNA vaccine has been demonstrated before [20], this is the first time that mucosal antibody responses are measured after a COVID-19 booster vaccination.

Furthermore, no differences in influenza titers in serum were measured between groups, indicating that possible interference does not extend to the immune response against influenza.

The safety data obtained in the present study is in line with recent research that shows no clinically relevant increase in adverse events or side-effects after concurrent administration of a COVID-19 mRNA vaccine and an influenza vaccine compared to a COVID-19 vaccine alone.[21] Although the primary outcome of that study was safety, and had been powered solely for that aim, immunogenicity was assessed as well. Concomitant vaccination of a second-dose of the primary series of COVID-19 vaccinations (not a post-primary series booster vaccination as assessed in the current study) with an influenza vaccine was presented as preserving binding antibody responses, which is not in accordance with the conclusions drawn from our study. Important discrepancies between both studies in addition to the vaccination stage (primary series versus booster) include the older age of TACTIC-participants (depending on trial arms, >10 years) and the methods used to assess immunogenicity. No mucosal antibodies or virus neutralization capacities are presented, the latter being a major influential factor in our study. Of note, the authors do not show antibody concentrations in their paper, making it difficult to assess the exact results. Another recent study that assessed the immunological interaction between another COVID-19 vaccine (mRNA-1273) and influenza vaccination did not identify any interference between a COVID-19 booster and an influenza vaccine, either.[22] However, that study by Izikson *et al.* did not perform a formal statistical comparison between various vaccination schedules, nor was the neutralizing capacity of antibodies measured. The authors excluded (among others) the use of anticoagulants or previous vaccination by a viral vector vaccine, whereas the selection of participants for the TACTIC study did not include these criteria in order to have a more representative group of older adults. The additional selection criteria might have resulted in a study population with generally better responses to vaccination overall.

In addition to our study, an investigation into the immunological effects of NVX-CoV2373 COVID-19 vaccine and seasonal influenza vaccines did show a reduction in antibody responses against SARS-CoV-2 after concurrent vaccination,[23] supporting our findings. The authors suggest that pre-existing immunological memory against the SARS-CoV-2 virus might minimize the possible interference; unfortunately, humoral immunological interference still cannot be ruled out in our booster-study. In general, the use of different vaccines in the various studies might have caused the differences in outcome.

An important aspect that remains to be studied in detail relates to the cellular and molecular mechanisms responsible for the effects observed. A possible explanation for the vaccine interference observed in our study may be the vaccination-induced type I interferons (IFNs) release, which may subsequently suppress the response to a simultaneously administered mRNA vaccine.[24] However, this may be unlikely given the time that is needed to produce IFNs and the different vaccination sites used in this study (different arms). Impaired T cell function after simultaneous presentation of closely related variant epitopes has previously been described,[25] but it remains to be demonstrated whether this mechanism might be responsible for the effects observed in the present study.

Our study also has limitations. One limitation of the TACTIC study design is the lack of epidemiological follow-up data, making it impossible to estimate vaccine effectiveness. Interpretation of the significance of serological results therefore remains an important area of research. Given that different virus variants seem to impair humoral immunity more than cellular responses,[26] the lack of neutralizing antibodies might be compensated by T cell immunity which might be less affected by concurrent vaccine administration. Another limitation is the absence of data on the T cell and memory B cells. To complement the findings from our study, future studies on T cell responses and memory B cells are warranted.

One important topic that has received little attention in vaccination studies is the long-term effect of vaccines on inflammation. Considering the known inflammatory side-effects of the novel COVID-19 vaccines, as well as rare (but sometimes severe) inflammatory complications in some vaccinated individuals,[27,28] the assessment of long-term effects of the various vaccination schedules on the systemic inflammation is important. Although low systemic inflammation is associated with poorer vaccine responses,[29] hyperinflammation can lead to more severe disease and prolonged upregulation of inflammatory markers is associated with increased cardiovascular risk.[30] We found that, in line with previously mentioned work, the mRNA COVID-19 vaccine seems to increase several proteins associated with inflammation. The differentially upregulated proteins were not found in the subpopulation who subsequently received an influenza vaccine, arguing for an anti-inflammatory role for the influenza vaccine, in line with previous research.[11] Potential long-term inflammatory effects of COVID-19 need to be considered and monitored in order to assess their relevance.

In conclusion, the TACTIC study cannot exclude the possibility of immune interference between an mRNA COVID-19 booster and an influenza vaccination when they are administered at the same time, resulting in a lower antibody concentration and reduced virus neutralizing activity against SARS-CoV-2. This is important to take into account when making public health decisions regarding vaccination schedules in populations at risk. More research is needed to understand the potential for immune interference, gain a broader understanding of the interaction between these vaccines and its clinical relevance, as well as long-term changes induced by these vaccines on the low-grade systemic inflammation.

Contributors

All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication. All authors contributed to the article and approved the submitted version.

ED	*Study conceptualization/design, collected data, performed laboratory experiments, performed the analyses, wrote the first draft*
BG	*Study design, performed laboratory experiments*
ET	*Study design, performed the analyses*
CK, HD, LE, CG, DM	*Performed laboratory experiments*
PK	*Performed non-inferiority analyses*
JD, RC, MJ	*Study conceptualization/design*
JM	*Study conceptualization/design, performed the analyses, wrote the first draft, supervised the work*
MN	*Study conceptualization/design, supervised the work*

Data sharing

Pseudonymized participant data and samples will be stored for 15 years. If consent was given previously, these data can be shared with other infectious disease researchers after permission from the principal investigator.

Declaration of interests

MGN is a scientific founder of TTxD, Lemba and BioTrip, and a member of the TTxD scientific advisory board. MGN has received research grants from TTxD and GSK. The other authors have no conflict of interest.

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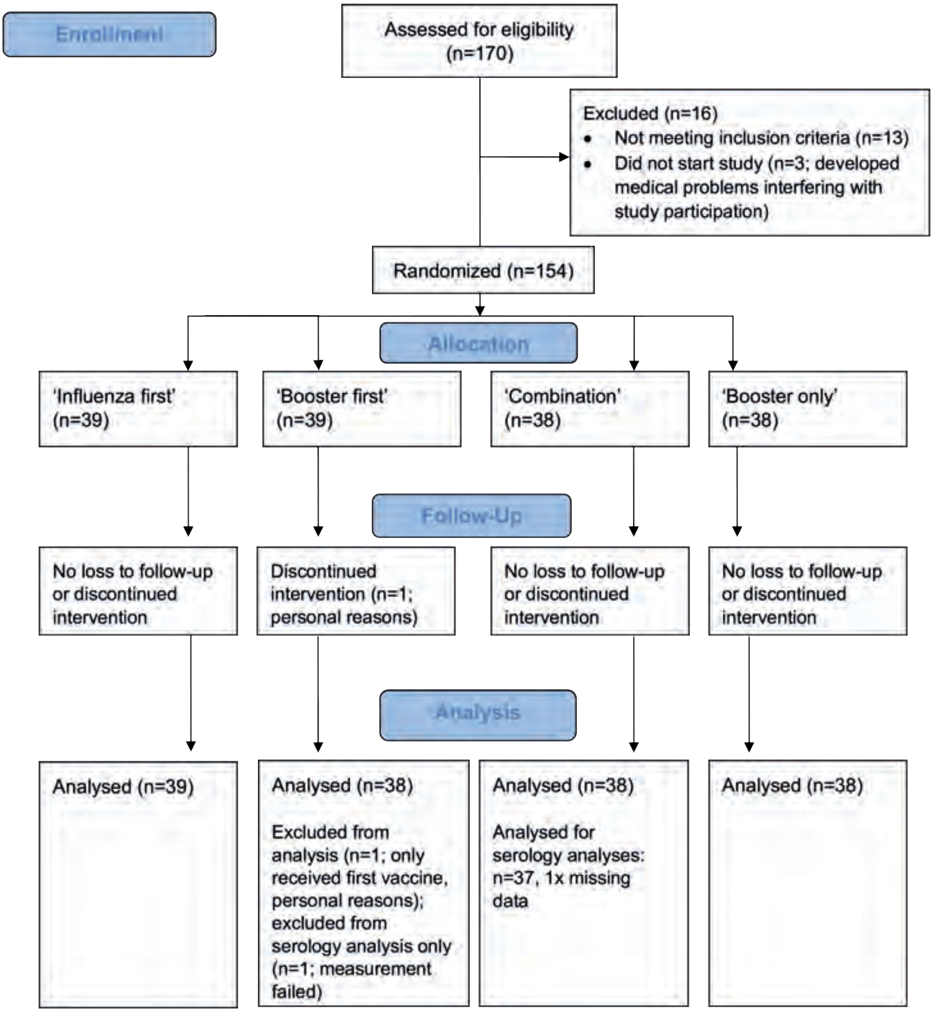
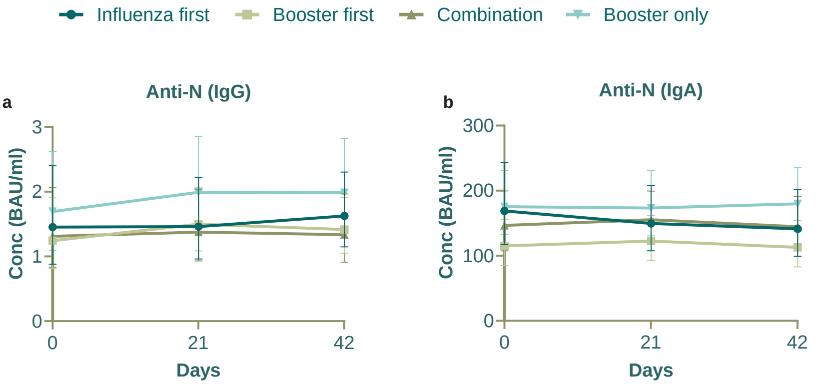
Supplementary material

1. **4E-BP1**
2. **ADA**
3. ARTN
4. AXIN1
5. Beta-NGF
6. CASP-8
7. **CCL11**
8. **CCL19**
9. **CCL20**
10. **CCL23**
11. **CCL25**
12. CCL28
13. **CCL3**
14. **CCL4**
15. **CD244**
16. **CD40**
17. **CD5**
18. CD6
19. **CD8A**
20. CDCP1
21. **CSF-1**
22. **CST5**
23. CX3CL1
24. **CXCL1**
25. **CXCL10**
26. **CXCL11**
27. **CXCL5**
28. **CXCL6**
29. **CXCL9**
30. **DNER**
31. EN-RAGE
32. **FGF-19**
33. FGF-21
34. FGF-23
35. FGF-5
36. **Flt3L**
37. GDNF
38. **HGF**
39. IFN-gamma
40. IL-1 alpha
41. IL10
42. IL-10RA
43. **IL-10RB**
44. **IL-12B**
45. IL13
46. IL-15RA
47. IL-17A
48. IL-17C
49. **IL18**
50. **IL-18R1**
51. IL2
52. IL-20
53. IL-20RA
54. IL-22 RA1
55. IL-24
56. IL-2RB
57. IL33
58. IL4
59. IL5
60. IL6
61. IL7
62. IL8
63. **TGF-beta-1**
64. LIF
65. LIF-R
66. **MCP-1**
67. **MCP-2**
68. MCP-3
69. **MCP-4**
70. **MMP-1**
71. **MMP-10**
72. NRTN
73. NT-3
74. **OPG**
75. **OSM**
76. PD-L1
77. **SCF**
78. SIRT2
79. SLAMF1
80. ST1A1
81. STAMBP
82. TGF-alpha
83. TNF
84. TNFB
85. **TNFRSF9**
86. TNFSF14
87. **TRAIL**
88. **TRANCE**
89. TSLP
90. **TWEAK**
91. **uPA**
92. **VEGFA**

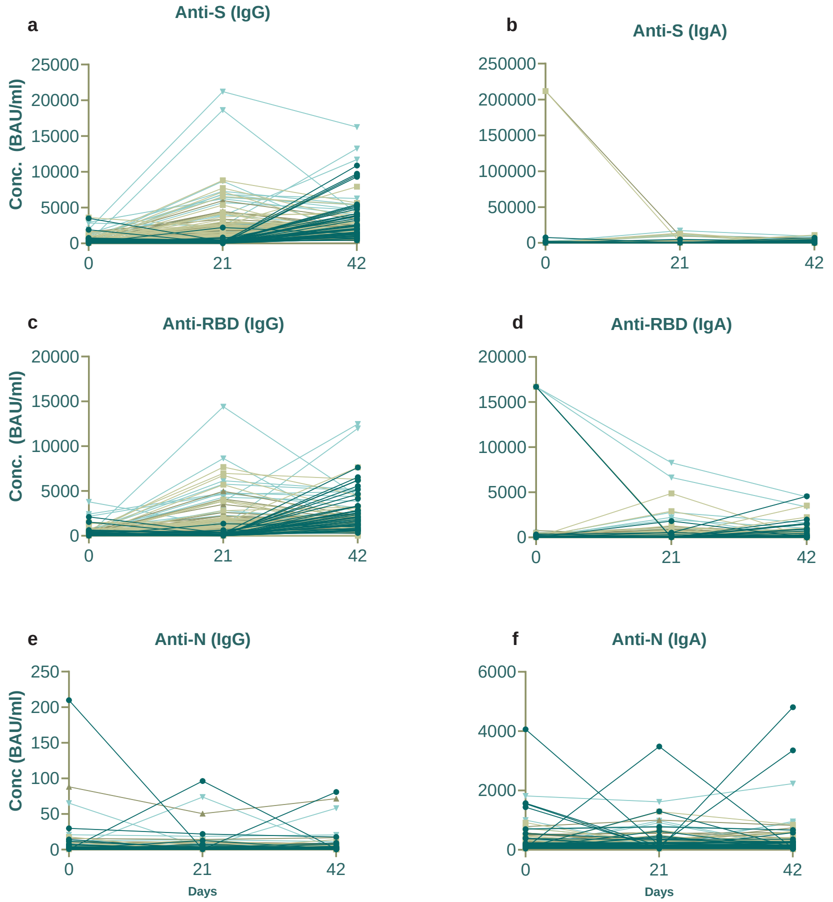
Supplementary methods 1: 92 Proteins of interested measured by the Olink targeted proteomics analysis. The proteins displayed in bold (44 in total) yielded sufficient results for analysis.



Supplementary figure 1: Study flow diagram



Supplementary figure 2: Geometric mean concentrations (with 95% error bars) of IgA and IgG antibodies against N-protein over the course of the study (a-b)



Supplementary figure 3: Concentrations of measured antibodies, induvial data points per group (a-f)

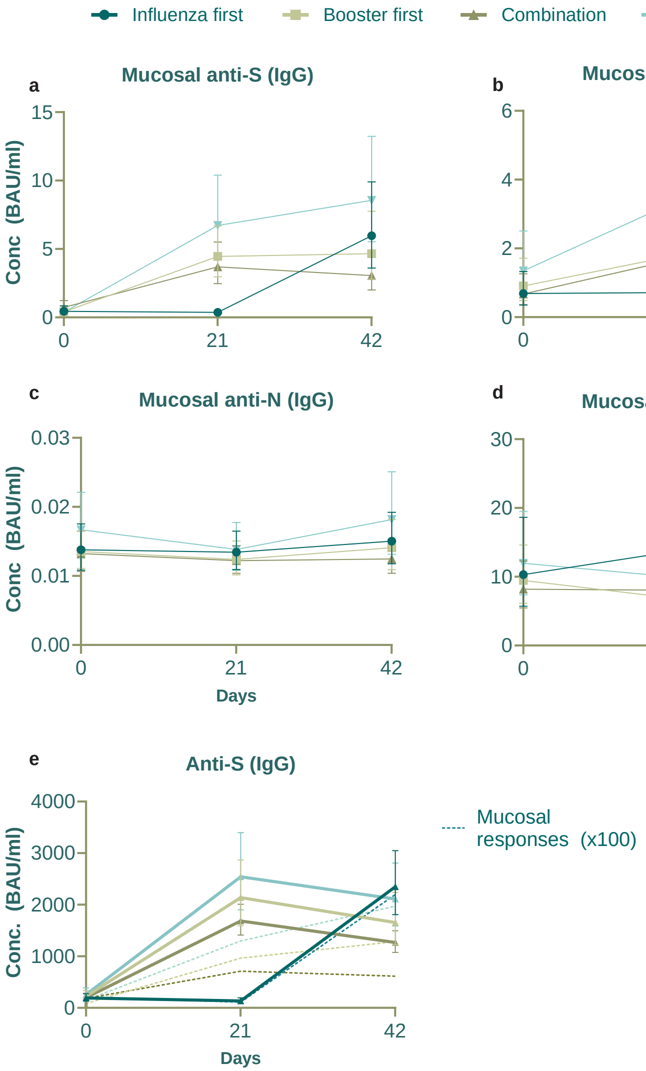
Supplementary table 1: Non-inferiority analyses comparing anti-S IgG responses, each group compared to reference group 'COVID-19 booster only'. If the lower limit of the 95% confidence interval lies above the non-inferiority margin of -0.3, the result is considered non-inferior. All results are adjusted for multiple testing using a common reference group (Dunnett's method)

Primary analyses	**Baseline GMC of anti-S IgG (BAU/ml)**	**GMC at day 21 (BAU/ml)**	**Estimate (95% CI)**
'Combination' vs Reference	199.0 (N=37)	1683.6 (N=37)	-0.17910 (-**0.3680** – 0.009831)
'Influenza first' vs Reference	190.4 (N=39)	2347.9 (N=39)	-0.03462 (-0.2211 - 0.1518)
'COVID-19 Booster first' vs Reference	225.1 (N=39)	2136.8 (N=38)	-0.07555 (-0.2632 – 0.1121)
Reference 'booster only'	258.2 (N=38)	2542.8 (N=37)	*N/A*
Sensitivity analyses comparing 'combination' to 'booster first (reference)'			
1. Adjusting for baseline anti-S IgG levels			-0.1393 (**-0.3018** – 0.02328)
2. + Adjusting for previous pneumococcal vaccination			-0.1391 (**-0.3034** – 0.02510)
3. + Adjusting for baseline anti-N IgG levels			-0.1400 (**-0.3023** – 0.02235)
4. + Excluding three participants with COVID-19 history			-0.1435 **(-0.3088** – 0.02175)
5. Combining 'booster first' with 'booster only' as one reference group			-0.1165 (-0.2507 - 0.01767)

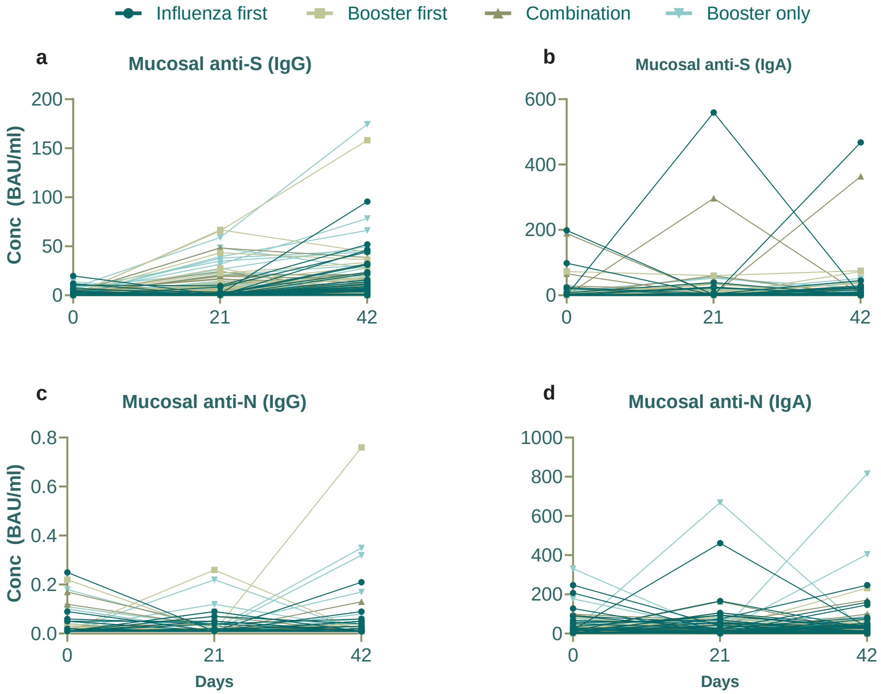
GMC = geometric mean concentration; BAU = binding antibody units

Supplementary table 2: Geometric mean concentrations (GMCs) of antibodies at baseline and at 21 days after COVID-19 booster vaccination

GMCs (BAU/ml)	**Anti-S IgA**	**Anti-N IgA**	**Anti-RBD IgA**	**Anti-N IgG**	**Anti-RBD IgG**
Influenza first					
Baseline	85.1	168.9	37.1	1.5	138.7
Day 21	511.9	141.5	116.8	1.6	1845.1
COVID-19 Booster first					
Baseline	92.0	115.0	38.7	1.2	155.2
Day 21	525.9	122.6	135.5	1.5	1466.6
Combination					
Baseline	99.5	146.6	33.8	1.3	118.5
Day 21	475.9	155.3	111.2	1.4	1129.1
COVID-19 Booster only					
Baseline	117.0	175.3	56.6	1.7	186.6
Day 21	856.6	173.5	211.8	2.0	1761.0



Supplementary figure 4: Mucosal antibody responses (a-d) and comparison of anti-S IgG to antibodies found in plasma (e)

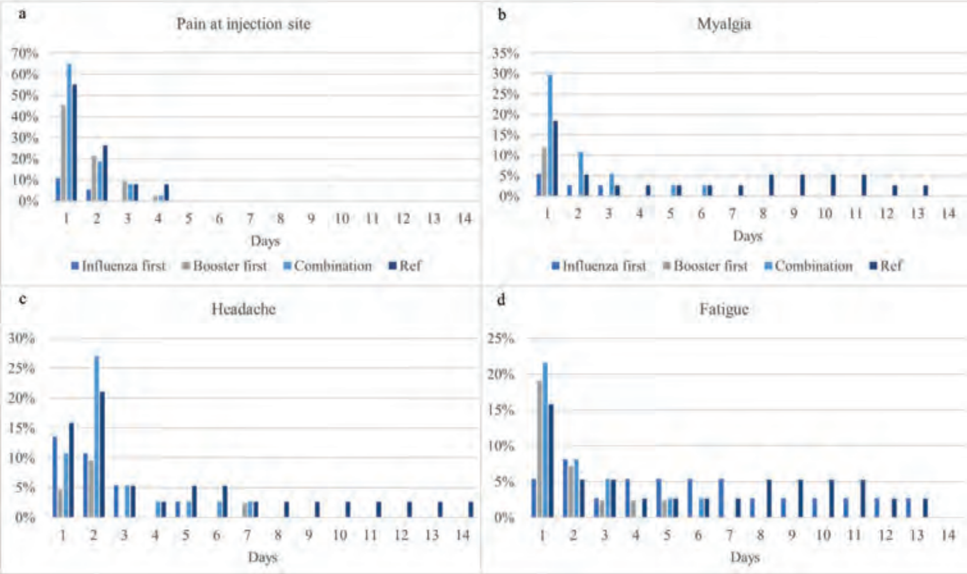


Supplementary figure 5: Individualized mucosal antibody responses (a-d)

Supplementary table 3: Occurrence of reported side-effects within 14 days after first round of vaccination. Results shown per study group

Participants reporting side-effects within 14 days after first round of vaccination (no. of participants, %)

	'Influenza first'Influenza + placebo vacc (N=38)		**'COVID-19 Booster first'Booster + placebo vacc (N=38)**		**'Combination' Booster + Influenza vacc (N=38)**		**'COVID-19 Booster only' Booster + placebo vacc (N=38)**		**p-value**
Fever	0	0%	2	5.3%	1	2.6%	3	7.9%	0.311
Redness at injection site	3	7.9%	5	13.2%	5	13.2%	3	7.9%	0.835
Pain at injection site	8	21.1%	24	63.2%	33	86.8%	27	71.1%	**0.000**
Swollen injection site	1	2.6%	9	23.7%	3	7.9%	4	10.5%	0.052
Fatigue	7	18.4%	8	21.1%	9	23.7%	6	15.8%	0.896
Myalgia	3	7.9%	7	18.4%	13	34.2%	10	26.3%	**0.034**
Joint pain	4	10.5%	3	7.9%	7	18.4%	3	7.9%	0.470
Headache	8	21.1%	6	15.8%	12	31.6%	9	23.7%	0.373
Chills	2	5.3%	4	10.5%	5	13.2%	5	13.2%	0.623
Nausea	0	0%	1	2.6%	0	0.0%	3	7.9%	0.082
Duration of side-effects (mean no. of days [range])	4.16 [0-9]		4.93 [0-7]		5.34 [0-8]		5.19 [0-11]		**0.033**
No. of participants experiencing severity score ≥ 4	3	7.9%	4	10.5%	2	5.3%	4	10.5%	0,433
Duration of severity score ≥ 4 (mean no. of days [range])	3.33 [1-8]		1 [1-1]		1 [1-1]		1.25 [1-2]		0.418



Supplementary figure 6: The four most commonly reported side-effects within 14 days after first vaccination round, divided by study group: pain at injection site (a); myalgia (b); headache (c); fatigue (d)

Supplementary table 4: Relative risks of side-effects (combination group VS reference group)

Side-effect	Relative Risk (95% CI)
Fever	0.629 (0.339-1.166)
Redness at injection site	1.333 (0.518-3.368)
Pain at injection site	1.429 (0.884-2.309)
Swollen injection site	0.836 (0.420-1.664)
Fatigue	1.271 (0.651-2.481)
Myalgia	1.173 (0.685-2.008)
Joint pain	1.719 (0.648-4.562)
Headache	1.189 (0.679-2.081)
Chills	0.969 (0.496-1.892)

References

1. WHO. COVID-19 Dashboard [Available from: https://covid19.who.int/].
2. Polack FP, Thomas SJ, Kitchin N, Absalon J, Gurtman A, Lockhart S, et al. Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. New England Journal of Medicine. 2020;383(27):2603-15.
3. Sadoff J, Gray G, Vandebosch A, Cárdenas V, Shukarev G, Grinsztejn B, et al. Safety and Efficacy of Single-Dose Ad26.COV2.S Vaccine against Covid-19. New England Journal of Medicine. 2021;384(23):2187-201.
4. Levin EG, Lustig Y, Cohen C, Fluss R, Indenbaum V, Amit S, et al. Waning Immune Humoral Response to BNT162b2 Covid-19 Vaccine over 6 Months. New England Journal of Medicine. 2021;385(24):e84.
5. Keehner J, Horton LE, Binkin NJ, Laurent LC, Pride D, Longhurst CA, et al. Resurgence of SARS-CoV-2 Infection in a Highly Vaccinated Health System Workforce. New England Journal of Medicine. 2021;385(14):1330-2.
6. Breiman RF, Brooks WA, Goswami D, Lagos R, Borja-Tabora C, Lanata CF, et al. A multinational, randomized, placebo-controlled trial to assess the immunogenicity, safety, and tolerability of live attenuated influenza vaccine coadministered with oral poliovirus vaccine in healthy young children. Vaccine. 2009;27(40):5472-9.
7. Lum LCS, Borja-Tabora CF, Breiman RF, Vesikari T, Sablan BP, Chay OM, et al. Influenza vaccine concurrently administered with a combination measles, mumps, and rubella vaccine to young children. Vaccine. 2010;28(6):1566-74.
8. Nolan T, Bernstein DI, Block SL, Hilty M, Keyserling HL, Marchant C, et al. Safety and immunogenicity of concurrent administration of live attenuated influenza vaccine with measles-mumps-rubella and varicella vaccines to infants 12 to 15 months of age. Pediatrics. 2008;121(3):508-16.
9. Do VA, Biering-Sørensen S, Fisker AB, Balé C, Rasmussen SM, Christensen LD, et al. Effect of an early dose of measles vaccine on morbidity between 18 weeks and 9 months of age: a randomized, controlled trial in Guinea-Bissau. The Journal of infectious diseases. 2017;215(8):1188-96.
10. Aaby P, Ravn H, Benn CS, Rodrigues A, Samb B, Ibrahim SA, et al. Randomized Trials Comparing Inactivated Vaccine After Medium-or High-titer Measles Vaccine With Standard Titer Measles Vaccine After Inactivated Vaccine. The Pediatric infectious disease journal. 2016;35(11):1232-41.
11. Debisarun PA, Gössling KL, Bulut O, Kilic G, Zoodsma M, Liu Z, et al. Induction of trained immunity by influenza vaccination-impact on COVID-19. PLoS pathogens. 2021;17(10):e1009928.
12. Yamamoto K. Adverse effects of COVID-19 vaccines and measures to prevent them. Virology Journal. 2022;19(1):100.
13. Gezondheidsraad. Vervolgadvies tweede boostervaccinatie tegen COVID-19. 2022 25-04-2022.
14. Kumar A, Blum J, Le TT, Havelange N, Magini D, Yoon I-K. The mRNA vaccine development landscape for infectious diseases. Nat Rev Drug Discov. 2022.
15. Fröberg, J, Gillard, J, Philipsen, R, Lanke, K, Rust, J, van Tuijl, D, et al. SARS-CoV-2 mucosal antibody development and persistence and their relation to viral load and COVID-19 symptoms. Nature Communications, 2021;12(1).
16. GeurtsvanKessel CH, Geers D, Schmitz KS, Mykytyn AZ, Lamers MM, Bogers S, et al. Divergent SARS-CoV-2 Omicron-reactive T and B cell responses in COVID-19 vaccine recipients. Sci Immunol. 2022;7(69):eabo2202.

17. Geers D, Shamier MC, Bogers S, den Hartog G, Gommers L, Nieuwkoop NN, et al. SARS-CoV-2 variants of concern partially escape humoral but not T cell responses in COVID-19 convalescent donors and vaccine recipients. Science Immunology. 2021;6(59):eabj1750.
18. Okba NMA, Müller MA, Li W, Wang C, GeurtsvanKessel CH, Corman VM, et al. Severe Acute Respiratory Syndrome Coronavirus 2-Specific Antibody Responses in Coronavirus Disease Patients. Emerg Infect Dis. 2020;26(7):1478-88.
19. World Health Organisation. Global Influenza Surveillance Network. Manual For The Laboratory Diagnosis And Virological Surveillance Of Influenza. 2011
20. Chan RWY, Liu S, Cheung JY, Tsun JGS, Chan KC, Chan KYY, et al. The Mucosal and Serological Immune Responses to the Novel Coronavirus (SARS-CoV-2) Vaccines. Front. Immunol. 2021;12(1):744887
21. Lazarus R, Baos S, Cappel-Porter H, Carson-Stevens A, Clout M, Culliford L, et al. Safety and immunogenicity of concomitant administration of COVID-19 vaccines (ChAdOx1 or BNT162b2) with seasonal influenza vaccines in adults in the UK (ComFluCOV): a multicentre, randomised, controlled, phase 4 trial. The Lancet. 2021;398(10318):2277-87.
22. Izikson R, Brune D, Bolduc J-S, Bourron P, Fournier M, Moore TM, et al. Safety and immunogenicity of a high-dose quadrivalent influenza vaccine administered concomitantly with a third dose of the mRNA-1273 SARS-CoV-2 vaccine in adults aged≥ 65 years: a phase 2, randomised, open-label study. The Lancet Respiratory Medicine. 2022;10(4):392-402.
23. Toback S, Galiza E, Cosgrove C, Galloway J, Goodman AL, Swift PA, et al. Safety, immunogenicity, and efficacy of a COVID-19 vaccine (NVX-CoV2373) co-administered with seasonal influenza vaccines: an exploratory substudy of a randomised, observer-blinded, placebo-controlled, phase 3 trial. Lancet Respir Med. 2022;10(2):167-79.
24. De Beuckelaer A, Pollard C, Van Lint S, Roose K, Van Hoecke L, Naessens T, et al. Type I Interferons Interfere with the Capacity of mRNA Lipoplex Vaccines to Elicit Cytolytic T Cell Responses. Mol Ther. 2016;24(11):2012-20.
25. Plebanski M, Lee EA, Hannan CM, Flanagan KL, Gilbert SC, Gravenor MB, et al. Altered peptide ligands narrow the repertoire of cellular immune responses by interfering with T-cell priming. Nature medicine. 1999;5(5):565-71.
26. GeurtsvanKessel CH, Geers D, Schmitz KS, Mykytyn AZ, Lamers MM, Bogers S, et al. Divergent SARS-CoV-2 Omicron–reactive T and B cell responses in COVID-19 vaccine recipients. Science immunology. 2022;7(69):eabo2202.
27. Le Vu S, Bertrand M, Jabagi M-J, Botton J, Drouin J, Baricault B, et al. Age and sex-specific risks of myocarditis and pericarditis following Covid-19 messenger RNA vaccines. Nature Communications. 2022;13(1):3633.
28. Jenny-Avital ER, Howe RA. Severe Multisystem Inflammatory Symptoms in 2 Adults after Short Interval between COVID-19 and Subsequent Vaccination. Emerg Infect Dis. 2022;28(5):1017-20.
29. Pereira B, Xu X-N, Akbar AN. Targeting inflammation and immunosenescence to improve vaccine responses in the elderly. Frontiers in immunology. 2020;11:583019.
30. Hojyo S, Uchida M, Tanaka K, Hasebe R, Tanaka Y, Murakami M, et al. How COVID-19 induces cytokine storm with high mortality. Inflammation and regeneration. 2020;40(1):1-7.

Chapter 3

Trained immunity-inducing vaccines: harnessing innate memory for vaccine design and delivery

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Abstract

While the efficacy of many current vaccines is well-established, various factors can diminish their effectiveness, particularly in vulnerable groups. Amidst emerging pandemic threats, enhancing vaccine responses is critical. Our review synthesizes insights from immunology and epidemiology, focusing on the concept of trained immunity (TRIM) and the non-specific effects (NSEs) of vaccines that confer heterologous protection. We elucidate the mechanisms driving TRIM, emphasizing its regulation through metabolic and epigenetic reprogramming in innate immune cells. Notably, we explore the extended protective scope of vaccines like BCG and COVID-19 vaccines against unrelated infections, underscoring their role in reducing neonatal mortality and combating diseases like malaria and yellow fever. We also highlight novel strategies to boost vaccine efficacy, like incorporating TRIM inducers into vaccine formulations to enhance both specific and non-specific immune responses. This approach promises significant advancements in vaccine development, aiming to improve global public health outcomes, especially for the elderly and immunocompromised populations.

Keywords
Trained immunity; Vaccines; Innate immunity; Non-specifc effects; BCG

Modern vaccinology

In the Western world, infectious diseases no longer pose the significant threats they once did[1]. The profound impact of the sanitation efforts and improved hygienic standards of the 18th and 19th centuries further evolved with the development of vaccinology in the 20th century, which helped prevent diseases or complications that used to be fatal. The morbidity rate of many diseases, now preventable, decreased by 99% in the United States between the 1900s and the early 21st century [2]. In the second part of the 20th century, the discovery and development of antibiotics unleashed a new revolution in the treatment of infections. Due to all these developments, the contribution of infectious causes to overall mortality in the Netherlands fell from 18% to 1% in the last century [3], although it still remains at a high level globally [4].

The first generation of vaccines consisted of live-attenuated or inactivated pathogens, which were then succeeded by the advent of genetic engineering that enabled the use of purified subunit or recombinant proteins. Subsequent advancements led to the creation of viral vector-based and other types of vaccines. The latest development are mRNA vaccines, with their global introduction accelerated by the SARS-CoV-2 pandemic [5].

The effectiveness of many current vaccines is beyond dispute, culminating in the eradication of smallpox in 1980 and wild poliovirus type 2 and 3 in 1999 and 2020 [6,7]. However, not all vaccines are effective, especially in certain vulnerable groups (e.g. influenza vaccination in the elderly). Certain environmental- and host-related factors can adversely affect the adequacy of vaccination responses. Other complex challenges are posed by sufficiently attenuating pathogens, or the capturing the most relevant strains of certain serotypes for specific geographical regions. From the perspective of the host, factors such as immunosenescence associated with aging and immunodeficiencies can impair immune responses and increase the risk of severe side-effects[8,9]. Many live vaccines are contra-indicated in immunocompromised individuals for this reason, further hampering adequate protection of this already vulnerable groups. Adjuvants and adjusted dosage schemes have been successful in increasing efficacy and availability of vaccines to some extent [10], but there are still large differences between specific vaccines. Ultimately, the looming threat of new pandemics poses the challenge of preparing for unknown pathogens. Given these circumstances, it becomes essential to improve vaccine responses, especially amongst the most vulnerable populations.

The induction of immunological memory with production of neutralizing antibodies and the expansion of antigen-specific T-cell clones are perceived as the core mechanisms by which vaccines elicit protection. The last decade, however, has witnessed a shift in our understanding of vaccine effects with long-term innate immune memory (also termed 'trained immunity' or TRIM) being described as an important biological effect of several vaccines. Trained immunity describes the heightened response of the innate immune system to subsequent heterologous challenges following the exposure to a previous pathogen or stimulus[11,12]. Vaccines that are known to induce TRIM open up possibilities for using this effect to enhance protection. In addition, TRIM has been proposed as a mechanism by which vaccines provide protection beyond their intended target antigens.

In this review, we will focus on the mechanisms underlying TRIM-induced heterologous protection and how to increase the efficacy of current vaccines via utilizing TRIM with novel vaccine technologies.

Non-specific effects of vaccines

An increasing body of epidemiological research has revealed that certain vaccines can extend their protection beyond their targeted pathogens[13,14]. By offering protection against unrelated infections, vaccination has led to a decrease in overall mortality. Beneficial nonspecific effects (NSEs) have been demonstrated for several live-attenuated vaccines, which will be detailed in this chapter and have been summarized in Table 1.

The bacillus Calmette-Guérin (BCG) vaccine was developed against tuberculosis and is the most used vaccine worldwide. Observational studies and clinical trials have shown reduced neonatal mortality rates after BCG vaccination by percentages that cannot be explained by preventing solely tuberculosis-related mortality alone [15–22]. Fewer cases of fatal respiratory infections and sepsis were reported after BCG vaccination, the main reason for the reduced mortality rate [15,17]. This beneficial effect seemed to decrease with age of vaccination [23], although prevention of respiratory tract infections has also been demonstrated in older adults [24–26]. In addition, BCG confers protective effects against malaria parasites and increases protection against yellow fever in controlled human models [27,28].

Sex differences in NSEs after BCG vaccination have been reported, with the protective effect being most marked in girls [29,30]. In addition to the direct protective effects of vaccinating infants themselves, Berendsen et al. found that a maternal BCG scar was associated with an additional mortality reduction of 25% in the first six weeks of life [31]. In a Danish study, BCG vaccination in children reduced neonatal infection-related hospital admissions only if the mother had also been vaccinated[19]. This suggests that maternal priming can enhance beneficial NSEs. Despite the large volume of research reporting protective effects, potential danger signals were also identified in the Danish study: a subgroup of 144 BCG-vaccinated prematurely born children had a shorter time to first infection-related hospitalization.

Table 1: Overview of vaccines that demonstrated the ability to induce trained immunity.

Vaccine	Findings
For a systematic review on heterologous effects of live-attenuated vaccines, we refer to: *Bree et al., 2018*[149] & *Higgins and Weiser et al. 2016*[23]	
BCG	First study demonstrating induction of trained immunity: *Kleinnijenhuis et al., 2012*[47] BCG vaccination-induced trained immunity protects against non-related viral infections: *Arts et al., 2018*[48] Central trained immunity is induced via the hematopoietic progenitor compartment, explaining the persistence of alterations induced by BCG: *Cirovic et al., 2020*[150] Recombinant strain vaccines are likely to induce TRIM as well: *Covián et al., 2019*[151] Mucosal recombinant BCG vaccination enhances trained immunity in mice: *Peng et al., 2023*[152]
Measles vaccine	In addition to the epidemiological indications of heterologous effects, only one paper has yet reported demonstrated induction of trained immunity by the measles-mumps-rubella vaccine: *Röring et al., 2024*[153]
Oral polio vaccine	Polio vaccines have been associated with lower non-specific mortality in children. Diphtheria-tetanus-pertussis (DTP) vaccine shows inhibitory effects on the heterologous effects associated with live-attenuated vaccines: *Aaby et al., 2004*[154] & *Øland et al., 2021*[155]
Specific COVID-19 vaccines	A single dose of viral-vector vaccine ChAdOx1 nCoV-19 induced trained immunity: *Murphy et al., 2023*[76] A study into the mRNA vaccine BNT162b2 against COVID-19 showed, in contrast, diminished cytokine production after vaccination in children: *Noé et al., 2023*[77]
Yellow fever	Higher cytokine production and NK cell activation: *da Costa Neves et al., 2009*[156] & *Gaucher et al., 2008*[157]
Influenza	Vaxigrip Tetra and Influvac Tetra can induce trained immunity: *Debisarun et al., 2021*[158]

NSEs have also been described for measles-containing vaccines (MCV), associated with lower overall mortality within the first years of life [13,29,31,32]. Maternal priming has proven relevant in this respect as well, as vaccination against measles resulted in a lower mortality rate for children who had maternal antibodies present [33]. The measles-mumps-rubella vaccine (MMR) has shown to decrease the rate of hospitalizations for off-target infections in some settings, but with different results in various geographical regions and depending on study design[34–37]. In addition, the diphtheria-tetanus-pertussis (DTP) vaccine – usually administered in combination with the inactivated or oral polio vaccine (IPV/OPV) – has shown to induce NSEs in studies in various countries, with some (albeit not all) studies suggesting potential deleterious effects [38–42]. Interestingly, the sequence in which these childhood vaccines are administered influences the mortality rates [39,43]. Administering DTP or IPV post-MCV might have increased the female-male mortality ratio in measles trials [44]. Some authors argue this this could be due to the dual effect where beneficial effects (of MCV) are more pronounced for girls, while adverse effects are higher as well [45]. Although the precise details of sex-specific differences in NSEs warrant careful investigation [46], it is beyond the scope of this review.

Vaccine-induced trained immunity

Currently, two major biological explanations for this heterologous protection are hypothesized to be cross-reactivity mediated by T cells and TRIM. TRIM describes de-facto innate immune memory, which enables the immune system to show an enhanced response upon reinfection with other pathogens in an antigen-independent manner [12]. The BCG vaccine is known to induce TRIM: peripheral blood mononuclear cells from vaccinated individuals strongly enhanced their production of pro-inflammatory cytokines upon in vitro heterologous stimulation, an effect that lasted up to three months after vaccination[47].

There is a large variety in the response to BCG, both in terms of protection against the targeted pathogen and in the induction of trained immunity [48,49]. Genetic polymorphisms, different strains used and batch effects are the predominant explanation for this variety [50], although there are other factors as well. Recently, Debisarun and colleagues showed that the induction of trained immunity was dependent on the pre-vaccination baseline cytokine production capacity of individuals [51]. The magnitude of the trained innate immune response was inversely correlated with the baseline cytokine production and associated chromatin accessibility of genes involved in trained immunity. This demonstrated that not all individuals are equally ‘trainable’. The induction of TRIM seemed independent of the doses and frequency of BCG vaccination in this study, although a higher dose boosted NK cell cytokine production in response to fungal stimulation. Given the fact that fungal infections are notoriously hard to treat in a clinical setting, this finding might have important clinical consequences. An extensive analysis of personal immune profiles in over 300 individuals further expanded the knowledge on the genetic and epigenetic predictors of immune responses after BCG[52]. A final determining factor is the route through which BCG is administered. In mice, trained immunity is only induced after BCG when injected intravascularly; not subcutaneously [53,54].

After eradicating smallpox, and being on the verge of eradicating polio and possibly other diseases, it is conceivable that current vaccine policies might change in the near future [55,56]. That might affect the protective NSEs in a negative way. Besides the direct NSEs on the vaccinees, the additional potential benefits of maternal and paternal [57] priming might be lost for future generations. Especially in low-resource countries with a poor health care system, this could result in an upsurge of infectious diseases and infant deaths. Another crucial reason to increase efforts of studying NSEs is the threat of new pandemics. As we have seen in the SARS-CoV-2 pandemic, new pathogens can emerge rapidly and overwhelm health care systems globally. A ‘bridging’ vaccine with NSEs could be useful in the future. Retrospective studies suggested a protective effect of BCG vaccine coverage on COVID-19 incidence and mortality [58–62], but recent clinical trials have shown varying results. Although some trials found a reduced risk for (severe) COVID-19 [63–66], many others did not [67–71] (despite some showing evidence that BCG did lead to a stronger induction of antibodies [72,73]). Major differences between the trials included the strains of BCG vaccines used, the duration of the follow-up and the population investigated: re-vaccination with BCG in a population that had been vaccinated at birth, seemed to have more effect than first-time vaccination. Other available live-attenuated vaccines have been proposed as a bridging vaccination as well, especially given their preexisting manufacturing capacity and stockpiles [74].

Regarding the NSEs of COVID-19 vaccines themselves, a recent study by Benn et al. sheds light on potential beneficial effects of vector-based COVID-19 vaccines compared to mRNA-based types; remarkably, on non-infectious and cardiovascular mortality [75]. Although none of the original COVID-19 vaccine studies were designed to study non-specific effects, this analysis does suggest the possibility of inducing TRIM by the ChAdOx1 vaccine. This has indeed been demonstrated in mice and humans, resulting in enhanced cytokine production up to three months after one dose [76]. Interestingly, children vaccinated with two doses of mRNA vaccine BNT162b2 showed a decreased cytokine response to heterologous stimuli after vaccination [77]. It remains to be determined whether this difference can be explained solely by the specific vaccine, or the age of the participants. Studies describing potential TRIM effects after COVID-19 vaccination have thus far been exploratory in nature and did not have the benefit of a large sample size (N < 30).

Even in the case of known pathogens, resistance offers another important reason to look at TRIM or enhanced protection. Prevention becomes increasingly important if pathogens can resist treatment by current medication and can help slow down this process. Parasites can already escape the sparse specific vaccines [78], but BCG-trained cells have been reported to enhance their killing capacities by increasing ROS production [79] and phagocytosis [78].

Despite the progress made in understanding NSE effects of vaccines, much remains to be learned. The first remarkable observation is the distinct separation between the epidemiological data pointing towards NSEs, and the lab-based research pointing towards TRIM as a mechanism. Papers integrating both epidemiological and biological data from the same individuals are lacking, leading to a lack of information whether induction of trained immunity is indeed a correlate of protection for certain vaccines. Secondly, most epidemiological research on NSEs has been performed in African countries, while the immunological studies have been done in Europe or US. In an increasingly globalized world, it is the time to expand this research further in geographic, environmental, and genetic background terms, encompassing diverse populations. Thirdly, new trials should pay extra attention to the (long-term) follow-up of their participants and include overall mortality in their design when possible [75].

Regulatory mechanisms governing trained immunity

Upon initial exposure to a TRIM-inducing pathogen or vaccine, innate immune cells undergo durable intracellular modifications which affect their long-term responsiveness, leading to an enhanced and accelerated response upon secondary heterologous stimulation. This phenomenon relies on two main pillars: epigenetic reprogramming and the metabolic rewiring of innate immune cells and their progenitors.

Epigenetic reprogramming

Epigenetic reprogramming is a key process underlying the induction of trained immunity, and it is finely regulated by various cellular programs in response to different training agents such as BCG or β-glucan. Histone modifications, such as histone 3 lysine 27 acetylation (H3K27ac) and histone 3 lysine 4 trimethylation (H3K4me3) are crucial in activating epigenetic marks that establish and maintain the memory phenotype of myeloid cells.

BCG promotes the acquisition of activating H3K27ac and H3K4me3 marks while diminishing the repressive H3K9me3 mark on the promoters of pro-inflammatory cytokine genes such as *IL6, TNFA* and *IL1B* [47,80]. BCG also alters the transcriptional profile of mouse hematopoietic stem cells (HSCs), with acetylation of histones leading to open chromatin upon exposure to BCG. After being initiated in the bone marrow, HSCs maintain these acetylation marks throughout differentiation into lymphoid or myeloid lineage, promoting myelopoiesis [54].

A meta-analysis of identified quantitative trait loci (QTLs) from BCG-vaccinated and non-vaccinated individuals revealed that KDM4 family enzymes, which demethylate H3K9, play a crucial role in the induction of TRIM [81]. Another study showed that Set7 lysine methyl transferase knock-out mice failed to exhibit a trained phenotype after β-glucan injection. Set7 was shown to regulate the innate immune memory through the activation of the metabolic genes *SDHB* and *MDH2*, illustrating the interconnected nature of epigenetic and metabolic changes regulating TRIM (Figure 1) [82].

Long non-coding RNAs (lncRNAs), specifically immune-gene priming lncRNAs (IPLs), also contribute to the regulation of immune-related gene activation or suppression [83]. IPLs facilitate the deposition of the H3K4me3 mark by bringing the histone-modifying complex near the promoters of immune-related genes through preformed chromatin loops [84].

The induction of trained immunity is also determined at the level of DNA methylation in innate immune cells. Verma et al. showed that BCG-vaccinated individuals who successfully developed TRIM (termed ‘responders’) exhibited extensive loss of DNA methylation on immune-related gene promoters compared to non-responders [85].

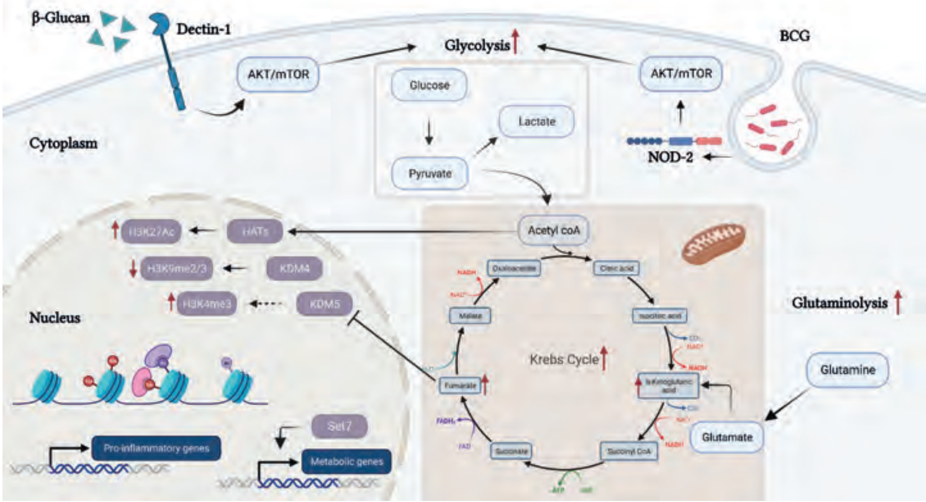

Figure 1: Intertwined metabolic and epigenetic mechanisms underlying TRIM induced by β-glucan or BCG. After the first encounter with BCG or β-glucan, innate cells undergo long-term metabolic and epigenetic changes leading to enhanced immune responses against subsequent unrelated stimuli. Changes in metabolic pathways and epigenetic modifications interactively affect each other. Alterations in metabolite levels determine the activity of histone modification enzymes such as histone demethylases and histone acetyltransferases by acting as substrates or co-factors whereas the expression of metabolic genes are regulated by the histone acetyl- or methyltransferases such as Set7.

Metabolic rewiring

The induction of TRIM in innate immune cells is also closely linked to rewiring of the cellular metabolic pathways. Several studies have highlighted the importance of glycolysis, oxidative phosphorylation (OXPHOS), TCA cycle, and lipid metabolism, as key determinants of TRIM. Enhanced glycolytic function has been observed with several training agents including BCG, β-glucan and oxLDL. These alterations were observed with monocytes, macrophages, and HSCs of mice injected with β-glucan [86]. TRIM induction depends on the AKT/mTOR/HIF1a pathway and is disrupted when this pathway is inhibited [87]. Additionally, HIF1-α knock-out mice were more susceptible to *S. aureus* infection when compared to wild-type mice, when both types of mice were trained with β-glucan [88].

Changes in glycolysis and the tricarboxylic acid (TCA) cycle not only provide the necessary energy, but also supply intermediate metabolites to support the reprogramming induced by TRIM. Elevated activation of glutaminolysis after BCG-induced training causes the accumulation of fumarate, a TCA cycle metabolite, which subsequently induces the TRIM phenotype [89]. Similarly, macrophages obtained from β-glucan-trained mice exhibited augmented levels of TCA cycle metabolites, when compared to non-trained macrophages [90]. Itaconate, another crucial metabolite exerts an anti-inflammatory effect in LPS-activated macrophages by inhibiting the succinate dehydrogenase enzyme (SDH), which converts succinate to fumarate. β-glucan reverses the tolerance induced by itaconate by inhibiting the IRG1 expression which in turn decreases itaconate levels and increases SDH activity, leading to fumarate accumulation [91,92] (Figure 1). Recently, it has also been reported that dimethyl itaconate (itaconate derivative) by itself induces TRIM and protects mice against *Staphylococcus aureus* infection[93].

Various metabolites contribute to the regulation of TRIM by serving as cofactors for histone acetyl- and methyltransferases as well as demethylases, deacetylases, and DNA methylases [80,94,95]. TCA cycle metabolite α-ketoglutarate (α-KG) acts as a cofactor for multiple enzymes involved in epigenetic modifications. High levels of α-KG derivatives (succinate, fumarate and 2-HG) similarly promote the formation of innate immune memory by reducing the KDM5 activity and retaining H3K4me3 on the promoters of pro-inflammatory genes upon training (Figure 1) [96,97]. Moreover, an elevated NAD+/NADH ratio promotes the activity of a family of mammalian histone deacetylases called sirtuins (SIRTs). Studies have shown that SIRT6 regulates TRIM induction through H3K9 deacetylation, while SIRT1 downregulation contributes to immune tolerance [98–100]. Butyrate has been shown to decrease the efficacy of training by inhibiting the HDACs [101,102].

Overall, the metabolic and epigenetic pathways involved in the initiation and persistence of TRIM are tightly interconnected and interdependent. Metabolic rewiring not only supports the energy and intermediate metabolite requirements for epigenetic and genomic alterations but also directly modulates the activity of epigenetic enzymes. Understanding the complex interplay between these pathways is crucial for advancing our knowledge of TRIM and its potential therapeutic applications.

Exploring the potential of TRIM-based approaches for the next generation vaccines

Despite the significant impact of vaccines on global health, there are still challenges in developing effective immunization strategies against global infectious diseases such as HIV, malaria, tuberculosis, and pneumococcal infections. This is particularly problematic for the elderly and immunocompromised individuals, who now comprise approximately 9% (and increasing) and 2-3% of the world population, as they often exhibit impaired immune responses to vaccines and are more vulnerable to infectious diseases. As mentioned earlier, numerous epidemiological studies have demonstrated the efficacy of live attenuated vaccines in providing nonspecific protection and reducing overall mortality rates in both children and the elderly [24–26,32,103–106]. Therefore, using the live-attenuated vaccines to learn the pathways and mechanisms necessary for broad protection will be of major importance for the development of effective vaccines and therapeutics that incorporate induction of TRIM.

BCG holds a great potential for the development of new generation vaccines because of its capacity to induce nonspecific protection and innate immune memory [47]. Recently, there have been many studies which utilize BCG in different ways such as recombinant BCG (rBCG), prime-boost studies or alternative delivery routes[107]. Delivering antigens of a pathogen via using different strains of modified BCG increases the cytokine production and T cell immunity leading to enhanced pathogen clearance. BCG carrying the HIV antigens such as HIV-gag, p24 or HTI together with chimpanzee adenovirus ChAdOx1 exerted significantly higher HIV specific CD4 and CD8 T cell immunity when compared to MVA-gag boost alone or ChAdOx1.HTI alone respectively as well as enhanced type I IFN production [108–110]. Furthermore, rBCG expressing the spike protein of SARS-CoV-2 activates the innate and virus-specific adaptive immune responses, which may lead to substantial protection against SARS-CoV-2 infection[111]. Also, intravenous BCG vaccination induced-TRIM has been shown to reduce the severity of SARS-CoV-2 infection, recruiting alveolar macrophages and reducing viral load in lungs[112,113].

Similar to viral antigens, parasite antigens delivered with rBCG also induced more effective immunization against *Trypanosoma cruzi* while BCG vaccine and β-glucan training were shown to provide non-specific protection against leishmania infections [79,114,115]. Recently, Kanno et al. showed that rBCG also induces TRIM and provides protection against unrelated diseases [116]. Another promising vaccine candidate for improved adaptive and innate immune memory is the anti-tuberculosis vaccine candidate MTBVAC. Mucosal administration of MTBVAC or BCG induces stronger trained immunity in rhesus macaques compared to intradermal vaccination [117]. Similar to BCG and MTBVAC, inactivated bacterial vaccines MV130 and MV140 provide heterologous protection against recurrent respiratory or urinary tract infections respectively [118–120]. Additionally, priming myeloid cells with *Candida albicans* V132 vaccine induces trained immunity and further increases the innate and adaptive immune responses induced by MV140 vaccine [121].

Various pattern recognition receptor (PRR) ligands can induce distinct training or tolerance programs in monocytes, depending on the concentration and duration of the initial stimulation. CpG oligonucleotides (ODNs), which activate TLR9 signaling have been used in many clinical trials and shown to exhibit adjuvant like properties and increase cell mediated and humoral immune responses upon vaccination [122,123]. Furthermore, early studies revealed that prior exposure with CpG ODNs can offer protection against various infections such as *Leishmania major*, *Francisella tularensis* and *Klebsiella pneumoniae* [124–126]. CpG ODN treatment also contributed to survival against methicillin-resistant *S. aureus* (MRSA) and viral infections as well as parasite infections [127–129]. These adjuvant-like and stand-alone immunoprotective effects of CpG ODNs that can extend at least 2 weeks, make them potential candidates as TRIM inducers for the development of TRIM-based vaccines.

Pharmacological compounds that can activate or inhibit the regulatory pathways of TRIM open new opportunities for modulating the specific and nonspecific effects of vaccines. Metformin, for instance, inhibits the induction of TRIM by BCG, while experimental studies on anticancer vaccination have demonstrated its ability to augment immune responses. Another study similarly showed that beta glucan induced-TRIM reduced tumor lung metastasis in mice, which has been shown to be mediated by the metabolite sphingosine-1 phosphate[130]. Immunomodulatory metabolites are potential targets to fine-tune the degree of TRIM. Pharmacological modulators targeting histone or metabolic modifications underlying TRIM exhibit promising potential to optimize specific and nonspecific vaccine responses, paving the way for the development of next-generation vaccines with enhanced effectiveness and broader protection.

Building a stronger defense: harnessing innovative platforms and TRIM modulation

To combat infectious diseases effectively, there is an urgent need to enhance the potency of current vaccine technologies. While early vaccine approaches utilized whole organisms or toxoids, newer technologies such as recombinant proteins, synthetic peptides, nucleic acids, recombinant viral vectors, and nanoparticles offer improved safety profiles but often exhibit lower immunogenicity due to the lack of microbe-associated molecular patterns (MAMPs) [131]. Therefore, enhancing the responses to these modern vaccine technologies is crucial to achieve robust and long-lasting protective immunity in a broad population.

By incorporating TRIM-inducers into vaccine formulations, we can potentially enhance the immune response and broaden the protective capacity of vaccines.

To achieve this, multiple strategies can be explored (Figure 2). Molecules known to induce TRIM, such as Toll-like receptor agonists or certain cytokines, can be incorporated as adjuvants into vaccine formulations. Additionally, metabolites or pharmacological compounds that enable targeted epigenetic and metabolic modulations can be considered in the formulation of novel vaccines in addition to adjuvant and antigens. A protein-free vaccine with $Al(OH)_3$, monophosphoryl lipid A and mannan formulated by Yan et al. showed improved survival up to 28 days in several bacterial pneumonia mouse models without any adaptive immune responses, although it can be discussed whether this was because of induction of TRIM or due to priming of innate immune cells [132]. β-glucan activated-TRIM involves the signaling of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-1 (IL-1) [86]. Activating or inhibiting these signals pharmacologically can interfere with the induction of TRIM. Metabolites like mevalonate or itaconate and cellular processes behind TRIM such as glycolysis, mTOR signaling, and cholesterol metabolism can be modulated by specific compounds or antibodies by being involved in novel vaccine formulations [133–135]. Metabolic modulation of immune cells also leads to changes in the epigenetic modifications, such as histone acetylation, methylation, and interactions with lncRNAs [54,84,136], which will in turn adjust the TRIM induction.

Nanomedicine approaches provide an opportunity to deliver these modulators of TRIM through the inherent interaction of nanomaterials with phagocytic myeloid cells such as monocytes, dendritic cells, or neutrophils, allowing precise control over their distribution and uptake. For example, SH2 domain-containing inositol 5′-phosphatase 1 (SHIP1) inhibits the β-glucan induced TRIM via negatively regulating the PI3K pathway activated upon dectin-1 stimulation [137]. Nanodelivery of pharmacological compounds or microRNA candidates that inhibits SHIP1 are shown to increase the training induced by β-glucan or *Candida albicans* [138–140] (Figure 3). Furthermore, Priem et al. showed that activating NOD2 signaling pathways with nanoparticles induces TRIM similar to BCG, and decreases tumor growth [47,141]. Nanoparticle platforms are also used for the delivery of CpG ODNs, indicated as potential candidates for TRIM-based vaccines, increasing the uptake of CpG ODNs and providing targeted delivery [142].

Nanodelivery can be used not only to induce TRIM but also to reduce deleterious effects of TRIM in cases of hyperactivation of innate immune responses. Studies showed effective delivery of various TLR4 antagonists via nanoparticles to lessen pro-inflammatory effects of TRIM [143,144]. Furthermore, Braza and colleagues showed that during organ transplantation in mice, enhanced macrophage activation through TLR4 and dectin-1 resulted in TRIM-induced enhancement of cytokine production, which eventually promotes allograft rejection. However, inhibiting mTOR activity using a HDL-based nano-immunotherapy modulated the glycolysis and epigenetic modifications, leading to decreased innate inflammatory responses and prolonged allograft survival in organ transplantation[143]. These studies depict the modulation of TRIM as a novel strategy of therapeutic purposes, with implications for various conditions such as cancer, autoimmune disorders, cardiovascular disease, sepsis, and transplant rejection (Figure 3).

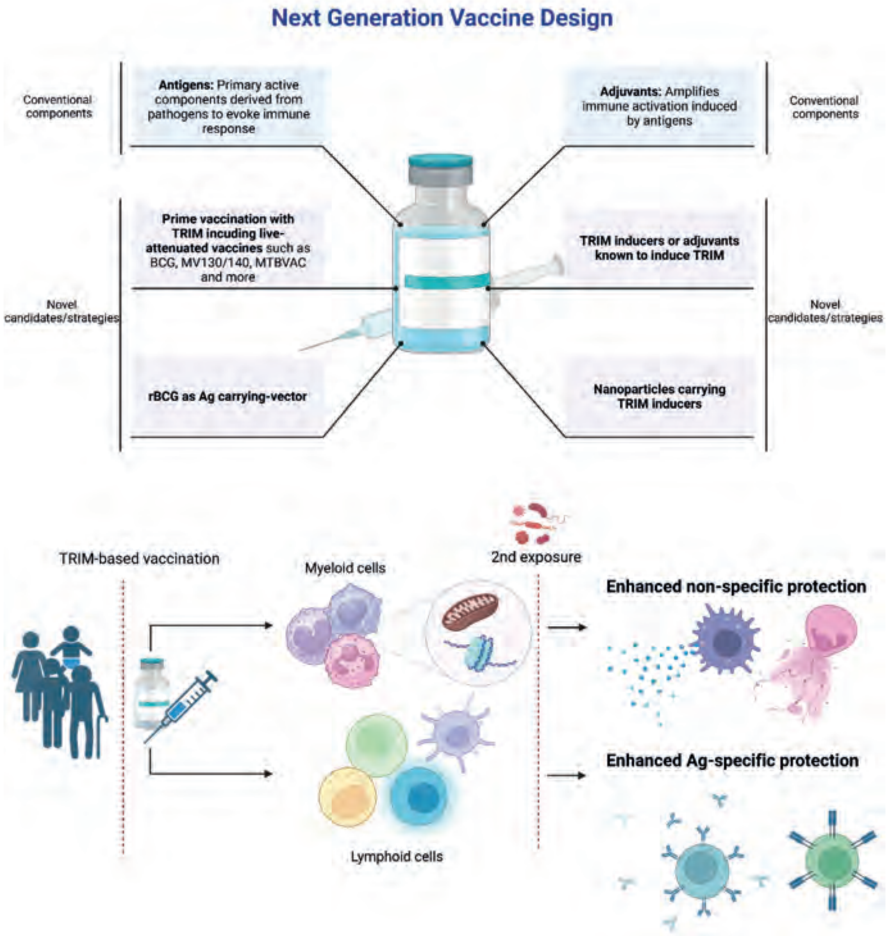

Figure 2: Developing next generation vaccines utilizing TRIM. Conventional vaccines have two main components: antigen and adjuvant. Involving a third component into novel vaccine formulations is a promising strategy in terms of building long-term innate and adaptive memory responses simultaneously. While conventional vaccines provide antigen-specific memory, next generation vaccines utilizing TRIM will additionally provide nonspecific protection which is crucial for the early onset of infections and reduced morbidity and mortality rates. Candidate components and strategies are shown in the figure.

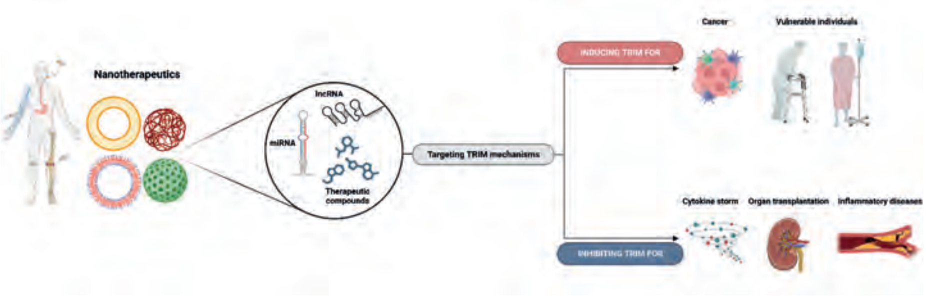

Figure 3: Targeted TRIM-based nano-delivery. For the regulation of TRIM, nanoparticles carrying the activators/inhibitors of TRIM, such as miRNAs, lncRNAs or compounds with specific therapeutic activity can be delivered to myeloid cells at specific locations such as bone marrow or certain tissues. Induction of TRIM with nanotherapeutics can provide enhanced immune responses for vulnerable populations, while inhibition of TRIM can reduce the excessive inflammation caused by exacerbated immune responses, inflammatory diseases, or organ transplantation.

A final aspect to be considered about the use of TRIM-inducing vaccines is the potential for maladaptive consequences of TRIM. Although beneficial during host defense, the effects might be detrimental during chronic inflammation which can result from western diet, chronic inflammatory diseases such as atherosclerosis and the development of inflammatory comorbidities. It has been shown that periodontitis can trigger maladaptive TRIM, which could then exacerbate inflammatory arthritis, a frequent periodontitis comorbidity [145]. Infection and atherosclerosis development have been linked in prior studies [146], but vaccines are hypothesized to reduce the infectious burden: therefore mitigating this risk. Although to date, no evidence is presented about significant inflammatory or cardiovascular problems after TRIM-inducing vaccination. On the contrary, the BCG vaccine has shown to lower systemic inflammation in elderly and reverse some effects of inflammaging [147,148]. However, this remains an important aspect to keep in mind and monitor over time.

Concluding remarks

The incorporation of TRIM modulators into modern vaccine technologies holds significant implications for vaccine development. By employing innate immune memory in the next generation vaccine formulations, we can potentially enhance vaccine efficacy against various infectious diseases, leading to improved disease control and prevention. This approach could be particularly valuable in situations in which current vaccines have shown suboptimal efficacy or when broader protection is desirable.

Although utilizing TRIM in vaccines holds great potential, certain considerations should be taken into account. Factors such as sex, age, microbiome, genetic background, dosing and vaccination schedule have influences on the induction and durability of training. Therefore, future research efforts should focus on optimizing the integration of TRIM inducers into vaccine formulations, understanding the pathways activated and their mechanisms of action, and evaluating their long-term effects on immune responses. It has been shown that TRIM is stimulated in the bone marrow and peripheral tissues. However, the differences in the training induction on different levels such as the role of trained peripheral, tissue resident or progenitor cells requires detailed investigations. Apart from beneficial effects, TRIM can lead to hyperactivation of immune cells. It is vital to investigate the effects of TRIM in the context of T cells' activation/differentiation and T cell exhaustion. Thorough preclinical and clinical evaluation is necessary to investigate the adverse effects associated with TRIM inducers to avoid potential immunopathology or interference with specific immune memory. At this point, collaborative efforts between immunologists, vaccinologists, and clinicians are essential to design and conduct well-controlled clinical trials to assess the efficacy and safety of TRIM-based vaccines. Additionally, exploring combinatorial approaches, such as prime-boost strategies with TRIM inducers and specific antigens, could further enhance vaccine effectiveness.

References

1. Armstrong, G. L., Conn, L. A. & Pinner, R. W. Trends in infectious disease mortality in the United States during the 20th century. *JAMA* 281, 61–66 (1999).
2. Impact of vaccines universally recommended for children--United States, 1990-1999. *Can. Commun. Dis. Rep.* 25, 122–127 (1999).
3. Prins, C. J. & Verhoef, R. Demografie van Nederland 1997. *Maandstat. Bevolking* 46, 8–24 (1998).
4. Communicable Disease Collaborators, A. The unfinished agenda of communicable diseases among children and adolescents before the COVID-19 pandemic, 1990–2019: a systematic analysis of the Global Burden of Disease Study 2019. *Lancet* 402, 313–335 (2023).
5. Polack, F. P. *et al.* Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *N. Engl. J. Med.* 383, 2603–2615 (2020).
6. World Health Organization. Poliomyelitis. https://www.who.int/health-topics/poliomyelitis#tab=tab_1 (2020).
7. World Health Organization. Smallpox. vol. July 2023 https://www.who.int/health-topics/smallpox#tab=tab_1 (2023).
8. Grubeck-Loebenstein, B. *et al.* Immunosenescence and vaccine failure in the elderly. *Aging Clin. Exp. Res.* 21, 201–209 (2009).
9. Liu, Z. *et al.* Immunosenescence: molecular mechanisms and diseases. *Signal Transduct. Target. Ther. 2023 81* 8, 1–16 (2023).
10. Pulendran, B., S. Arunachalam, P. & O'Hagan, D. T. Emerging concepts in the science of vaccine adjuvants. *Nat. Rev. Drug Discov. 2021 206* 20, 454–475 (2021).
11. Netea, M. G. *et al.* Trained immunity: A program of innate immune memory in health and disease. *Science* (2016) doi:10.1126/science.aaf1098.
12. Netea, M. G., Quintin, J. & Van Der Meer, J. W. M. Trained immunity: A memory for innate host defense. *Cell Host Microbe* 9, 355–361 (2011).
13. Higgins, J. P. T. *et al.* Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review. *BMJ* 355, 5170 (2016).
14. Benn, C. S., Netea, M. G., Selin, L. K. & Aaby, P. A small jab - a big effect: nonspecific immunomodulation by vaccines. *Trends Immunol.* 34, 431–439 (2013).
15. Aaby, P. *et al.* Randomized trial of BCG vaccination at birth to low-birth-weight children. *J Infect Dis* (2011).
16. Garly, M. L. *et al.* BCG scar and positive tuberculin reaction associated with reduced child mortality in West Africa: A non-specific beneficial effect of BCG? *Vaccine* 21, 2782–2790 (2003).
17. Biering-Sørensen, S. *et al.* Early BCG-Denmark and Neonatal Mortality Among Infants Weighing. *Clin. Infect. Dis.* 65, 1183–1190 (2017).
18. Jensen, K. J. *et al.* Heterologous immunological effects of early BCG vaccination in low-birth-weight infants in Guinea-Bissau: a randomized-controlled trial. *J. Infect. Dis.* 211, 956–967 (2015).
19. Stensballe, L. G. *et al.* BCG Vaccination at Birth and Rate of Hospitalization for Infection Until 15 Months of Age in Danish Children: A Randomized Clinical Multicenter Trial. *J. Pediatric Infect. Dis. Soc.* 8, 213–220 (2019).
20. Storgaard, L. *et al.* Development of BCG Scar and Subsequent Morbidity and Mortality in Rural Guinea-Bissau. *Clin. Infect. Dis.* 61, 950–959 (2015).

21. Nankabirwa, V. *et al.* Child survival and BCG vaccination: a community based prospective cohort study in Uganda. *BMC Public Health* 15, (2015).
22. Roth, A. *et al.* BCG vaccination scar associated with better childhood survival in Guinea-Bissau. *Int. J. Epidemiol.* 34, 540–547 (2005).
23. Higgins, J. P. T. *et al.* Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review. *BMJ* 355, 5170 (2016).
24. Wardhana, Datau, E. A., Sultana, A., Mandang, V. V. & Jim, E. The efficacy of Bacillus Calmette-Guerin vaccinations for the prevention of acute upper respiratory tract infection in the elderly. *Acta Med. Indones.* (2011).
25. Ohrui, T., Nakayama, K., Fukushima, T., Chiba, H. & Sasaki, H. Prevention of elderly pneumonia by pneumococcal, influenza and BCG vaccinations. *Japanese J. Geriatr.* (2005) doi:10.3143/geriatrics.42.34.
26. Nemes, E. *et al.* Prevention of Infection with Mycobacteriumtuberculosis by H4:IC31® Vaccination or BCG Revaccination inAdolescents. *N. Engl. J. Med.* 379, 138 (2018).
27. Arts, R. J. W. *et al.* BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity. *Cell Host Microbe* 23, 89-100.e5 (2018).
28. Walk, J. *et al.* Outcomes of controlled human malaria infection after BCG vaccination. *Nat. Commun.* (2019) doi:10.1038/s41467-019-08659-3.
29. Stensballe, L. G. *et al.* Acute lower respiratory tract infections and respiratory syncytial virus in infants in Guinea-Bissau: A beneficial effect of BCG vaccination for girls: Community based case-control study. *Vaccine* 23, 1251–1257 (2005).
30. Aaby, P. *et al.* The non-specific and sex-differential effects of vaccines. *Nat. Rev. Immunol. 2020 208* 20, 464–470 (2020).
31. Berendsen, M. L. T. *et al.* Journal of the Pediatric Infectious Diseases Society Maternal Priming: Bacillus Calmette-Guérin (BCG) Vaccine Scarring in Mothers Enhances the Survival of Their Child With a BCG Vaccine Scar. *J. Pediatric Infect. Dis. Soc.* 9, 166–72 (2020).
32. Aaby, P. *et al.* Non-specific effects of standard measles vaccine at 4.5 and 9 months of age on childhood mortality: randomised controlled trial. *BMJ* 341, 1262 (2010).
33. Aaby, P. *et al.* Measles vaccination in the presence or absence of maternal measles antibody: impact on child survival. *Clin. Infect. Dis.* 59, 484–492 (2014).
34. Zimakoff, A. C. *et al.* Measles, mumps, and rubella vaccine at age 6 months and hospitalisation for infection before age 12 months: randomised controlled trial. *BMJ* 381, (2023).
35. Byberg, S. *et al.* A general measles vaccination campaign in urban Guinea-Bissau: Comparing child mortality among participants and non-participants. *Vaccine* 35, 33–39 (2017).
36. Sørup, S., Jensen, A. K. G., Aaby, P. & Benn, C. S. Revaccination With Measles-Mumps-Rubella Vaccine and Infectious Disease Morbidity: A Danish Register-based Cohort Study. *Clin. Infect. Dis.* 68, 282–290 (2019).
37. Tielemans, S. M. A. J. *et al.* Non-specific effects of measles, mumps, and rubella (MMR) vaccination in high income setting: population based cohort study in the Netherlands. *BMJ* 358, (2017).
38. Vaugelade, J., Pinchinat, S., Guiella, G., Elguero, E. & Simondon, F. Non-specific effects of vaccination on child survival: prospective cohort study in Burkina Faso. *BMJ* 329, 1309–1311 (2004).

39. Aaby, P., Jensen, H., Gomes, J., Fernandes, M. & Lisse, I. M. The introduction of diphtheria-tetanus-pertussis vaccine and child mortality in rural Guinea-Bissau: an observational study. *Int. J. Epidemiol.* 33, 374–380 (2004).
40. Velema, J. P., Alihonou, E. M., Gandaho, T. & Hounye, F. H. Childhood mortality among users and non-users of primary health care in a rural west African community. *Int. J. Epidemiol.* 20, 474–479 (1991).
41. Moulton, L. H. *et al.* Evaluation of non-specific effects of infant immunizations on early infant mortality in a southern Indian population. *Trop. Med. Int. Health* 10, 947–955 (2005).
42. Lehmann, D., Vail, J., Firth, M. J., de Klerk, N. H. & Alpers, M. P. Benefits of routine immunizations on childhood survival in Tari, Southern Highlands Province, Papua New Guinea. *Int. J. Epidemiol.* 34, 138–148 (2005).
43. Aaby, P. *et al.* Early diphtheria-tetanus-pertussis vaccination associated with higher female mortality and no difference in male mortality in a cohort of low birthweight children: an observational study within a randomised trial. *Arch. Dis. Child.* 97, 685–691 (2012).
44. Aaby, P. *et al.* Sex differential effects of routine immunizations and childhood survival in rural Malawi. *Pediatr. Infect. Dis. J.* 25, 721–727 (2006).
45. Aaby, P. *et al.* Differences in female-male mortality after high-titre measles vaccine and association with subsequent vaccination with diphtheria-tetanus-pertussis and inactivated poliovirus: reanalysis of West African studies. *Lancet (London, England)* 361, 2183–2188 (2003).
46. Flanagan, K. L. *et al.* Sex differences in the vaccine-specific and non-targeted effects of vaccines. *Vaccine* 29, 2349–2354 (2011).
47. Kleinnijenhuis, J. *et al.* Bacille Calmette-Guérin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc. Natl. Acad. Sci. U. S. A.* 109, 17537–17542 (2012).
48. Arts, R. J. W. *et al.* BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity. *Cell Host Microbe* 23, 89-100.e5 (2018).
49. Koeken, V. A. C. M. *et al.* BCG vaccination in humans inhibits systemic inflammation in a sex-dependent manner. *J. Clin. Invest.* 130, 5591–5602 (2020).
50. Biering-Sørensen, S. *et al.* Variation of growth in the production of the BCG vaccine and the association with the immune response. An observational study within a randomised trial. *Vaccine* 33, 2056–2065 (2015).
51. Debisarun, P. A. *et al.* The impact of BCG dose and revaccination on trained immunity. *Clin. Immunol.* 246, 109208 (2023).
52. Moorlag, S. J. C. F. M. *et al.* Multi-omics analysis of innate and adaptive responses to BCG vaccination reveals epigenetic cell states that predict trained immunity. *Immunity* 57, 171-187.e14 (2024).
53. Hilligan, K. L. *et al.* Intravenous administration of BCG protects mice against lethal SARS-CoV-2 challenge. *J. Exp. Med.* 219, (2021).
54. Kaufmann, E. *et al.* BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis. *Cell* 172, 176-190.e19 (2018).
55. Aaby, P. & Benn, C. S. Stopping live vaccines after disease eradication may increase mortality. *Vaccine* 38, 10–14 (2020).
56. Aaby, P. *et al.* Stopping Oral Polio Vaccine (OPV) After Defeating Poliomyelitis in Low- and Middle-Income Countries: Harmful Unintended Consequences? Review of the Nonspecific Effects of OPV. *Open Forum Infect. Dis.* 9, (2022).
57. Berendsen, M. L. T. *et al.* Parental Bacillus Calmette-Guérin vaccine scars decrease infant mortality in the first six weeks of life: A retrospective cohort study. *EClinicalMedicine* 39, (2021).

58. Ebina-Shibuya, R., Horita, N., Namkoong, H. & Kaneko, T. National policies for paediatric universal BCG vaccination were associated with decreased mortality due to COVID-19. *Respirology* 25, 898–899 (2020).
59. Escobar, L. E., Molina-Cruz, A. & Barillas-Mury, C. BCG vaccine protection from severe coronavirus disease 2019 (COVID-19). *Proc. Natl. Acad. Sci. U. S. A.* 117, 17720–17726 (2020).
60. Joy, M., Malavika, B., Asirvatham, E. S., Sudarsanam, T. D. & Jeyaseelan, L. Is BCG associated with reduced incidence of COVID-19? A meta-regression of global data from 160 countries. *Clin. Epidemiol. Glob. Heal.* 9, 202–203 (2021).
61. Marín-Hernández, D., Nixon, D. F. & Hupert, N. Anticipated reduction in COVID-19 mortality due to population-wide BCG vaccination: evidence from Germany. *Hum. Vaccin. Immunother.* 17, 2451–2453 (2021).
62. Curtis, N., Sparrow, A., Ghebreyesus, T. A. & Netea, M. G. Considering BCG vaccination to reduce the impact of COVID-19. *Lancet* 395, 1545–1546 (2020).
63. Faustman, D. L. *et al.* Multiple BCG vaccinations for the prevention of COVID-19 and other infectious diseases in type 1 diabetes. *Cell reports. Med.* 3, (2022).
64. Tsilika, M. *et al.* ACTIVATE-2: A Double-Blind Randomized Trial of BCG Vaccination Against COVID-19 in Individuals at Risk. *Front. Immunol.* 13, 873067 (2022).
65. Sinha, S. *et al.* Efficacy of Bacillus Calmette-Guérin (BCG) Vaccination in Reducing the Incidence and Severity of COVID-19 in High-Risk Population (BRIC): a Phase III, Multi-centre, Quadruple-Blind Randomised Control Trial. *Infect. Dis. Ther.* 11, 2205–2217 (2022).
66. dos Anjos, L. R. B. *et al.* Efficacy and Safety of BCG Revaccination With M. bovis BCG Moscow to Prevent COVID-19 Infection in Health Care Workers: A Randomized Phase II Clinical Trial. *Front. Immunol.* 13, (2022).
67. Pittet, L. F. *et al.* Randomized Trial of BCG Vaccine to Protect against Covid-19 in Health Care Workers. *N. Engl. J. Med.* 388, 1582–1596 (2023).
68. Claus, J. *et al.* BCG Vaccination of Health Care Workers Does Not Reduce SARS-CoV-2 Infections nor Infection Severity or Duration: a Randomized Placebo-Controlled Trial. *MBio* 14, (2023).
69. Czajka, H. *et al.* A Multi-Center, Randomised, Double-Blind, Placebo-Controlled Phase III Clinical Trial Evaluating the Impact of BCG Re-Vaccination on the Incidence and Severity of SARS-CoV-2 Infections among Symptomatic Healthcare Professionals during the COVID-19 Pandemic in Poland-First Results. *Vaccines* 10, (2022).
70. ten Doesschate, T. *et al.* Bacillus Calmette-Guérin vaccine to reduce healthcare worker absenteeism in COVID-19 pandemic, a randomized controlled trial. *Clin. Microbiol. Infect.* 28, 1278–1285 (2022).
71. Koekenbier, E. L. *et al.* Bacillus Calmette-Guérin vaccine for prevention of COVID-19 and other respiratory tract infections in older adults with comorbidities: a randomized controlled trial. *Clin. Microbiol. Infect.* 29, 781–788 (2023).
72. Santos, A. P. *et al.* The effect of BCG vaccination on infection and antibody levels against SARS-CoV-2-The results of ProBCG: a multicenter randomized clinical trial in Brazil. *Int. J. Infect. Dis.* 130, 8–16 (2023).
73. Moorlag, S. J. C. F. M. *et al.* Efficacy of BCG Vaccination Against Respiratory Tract Infections in Older Adults During the Coronavirus Disease 2019 Pandemic. *Clin. Infect. Dis.* 75, E938–E946 (2022).
74. Chumakov, K. *et al.* Old vaccines for new infections: Exploiting innate immunity to control COVID-19 and prevent future pandemics. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2101718118 (2021).
75. Benn, C. S., Schaltz-Buchholzer, F., Nielsen, S., Netea, M. G. & Aaby, P. Randomized clinical trials of COVID-19 vaccines: Do adenovirus-vector vaccines have beneficial non-specific effects? *iScience* 26, (2023).

76. Murphy, D. M. *et al.* Trained immunity is induced in humans after immunization with an adenoviral vector COVID-19 vaccine. *J. Clin. Invest.* 133, (2023).
77. Noé, A. *et al.* BNT162b2 COVID-19 vaccination in children alters cytokine responses to heterologous pathogens and Toll-like receptor agonists. *Front. Immunol.* 14, 1242380 (2023).
78. Zhu, J., Liu, J., Yan, C., Wang, D. & Pan, W. Trained immunity: a cutting edge approach for designing novel vaccines against parasitic diseases? *Front. Immunol.* 14, (2023).
79. Silva, M. V. T. *et al.* The role of IL-32 in Bacillus Calmette-Guérin (BCG)-induced trained immunity in infections caused by different Leishmania spp. *Microb. Pathog.* 158, 105088 (2021).
80. Arts, R. J. W. *et al.* Immunometabolic Pathways in BCG-Induced Trained Immunity. *Cell Rep.* 17, 2562–2571 (2016).
81. Moorlag, S. J. C. F. M. *et al.* An integrative genomics approach identifies KDM4 as a modulator of trained immunity. *Eur. J. Immunol.* 52, 431–446 (2022).
82. Keating, S. T. *et al.* The Set7 Lysine Methyltransferase Regulates Plasticity in Oxidative Phosphorylation Necessary for Trained Immunity Induced by β-Glucan. *Cell Rep.* 31, (2020).
83. Chen, Y. G., Satpathy, A. T. & Chang, H. Y. Gene regulation in the immune system by long noncoding RNAs. *Nat. Immunol.* 18, 962–972 (2017).
84. Fanucchi, S. *et al.* Immune genes are primed for robust transcription by proximal long noncoding RNAs located in nuclear compartments. *Nat. Genet. 2018 511* 51, 138–150 (2018).
85. Verma, D. *et al.* Anti-mycobacterial activity correlates with altered DNA methylation pattern in immune cells from BCG-vaccinated subjects. *Sci. Rep.* 7, (2017).
86. Mitroulis, I. *et al.* Modulation of Myelopoiesis Progenitors Is an Integral Component of Trained Immunity. *Cell* 172, 147-161.e12 (2018).
87. Sohrabi, Y. *et al.* MTOR-dependent oxidative stress regulates oxLDL-induced trained innate immunity in human monocytes. *Front. Immunol.* 10, 3155 (2019).
88. Cheng, S. C. *et al.* MTOR- and HIF-1α-mediated aerobic glycolysis as metabolic basis for trained immunity. *Science (80-.).* (2014) doi:10.1126/science.1250684.
89. Arts, R. J. W., Joosten, L. A. B. & Netea, M. G. Immunometabolic circuits in trained immunity. *Seminars in Immunology* vol. 28 425–430 (2016).
90. Arts, R. J. W. *et al.* Glutaminolysis and Fumarate Accumulation Integrate Immunometabolic and Epigenetic Programs in Trained Immunity. *Cell Metab.* 24, 807–819 (2016).
91. Hooftman, A. & O'Neill, L. A. J. The Immunomodulatory Potential of the Metabolite Itaconate. *Trends Immunol.* 40, 687–698 (2019).
92. Domínguez-Andrés, J. *et al.* The Itaconate Pathway Is a Central Regulatory Node Linking Innate Immune Tolerance and Trained Immunity. *Cell Metab.* 29, 211-220.e5 (2019).
93. Ferreira, A. V. *et al.* Dimethyl itaconate induces long-term innate immune responses and confers protection against infection. *Cell Rep.* 42, (2023).
94. Saeed, S. *et al.* Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science (80-.).* 345, (2014).
95. Keating, S. T. *et al.* Rewiring of glucose metabolism defines trained immunity induced by oxidized low-density lipoprotein. *J. Mol. Med.* 98, 819–831 (2020).
96. Tahiliani, M. *et al.* Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 324, 930–935 (2009).
97. Xiao, M. *et al.* Inhibition of α-KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors. *Genes Dev.* 26, 1326–1338 (2012).

98. Yeung, F. *et al.* Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J.* 23, 2369–2380 (2004).

99. Jia, Y. *et al.* SIRT1 regulates inflammation response of macrophages in sepsis mediated by long noncoding RNA. *Biochim. Biophys. acta. Mol. basis Dis.* 1864, 784–792 (2018).

100. Zhong, L. *et al.* The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. *Cell* 140, 280–293 (2010).

101. Cleophas, M. C. P. *et al.* Effects of oral butyrate supplementation on inflammatory potential of circulating peripheral blood mononuclear cells in healthy and obese males. *Sci. Reports 2019 91* 9, 1–10 (2019).

102. Cleophas, M. C. P. *et al.* Suppression of monosodium urate crystal-induced cytokine production by butyrate is mediated by the inhibition of class I histone deacetylases. *Ann. Rheum. Dis.* 75, 593–600 (2016).

103. Aaby, P. *et al.* Randomized trial of BCG vaccination at birth to low-birth-weight children: Beneficial nonspecific effects in the neonatal period? *J. Infect. Dis.* (2011) doi:10.1093/infdis/jir240.

104. Aaby, P. & Benn, C. S. Beneficial Nonspecific Effects of Oral Polio Vaccine (OPV): Implications for the Cessation of OPV? *Clinical Infectious Diseases* vol. 65 420–421 (2017).

105. Prentice, S. *et al.* BCG-induced non-specific effects on heterologous infectious disease in Ugandan neonates: an investigator-blind randomised controlled trial. *Lancet. Infect. Dis.* 21, 993–1003 (2021).

106. Wang, Y. *et al.* Mycobacterium bovis BCG Given at Birth Followed by Inactivated Respiratory Syncytial Virus Vaccine Prevents Vaccine-Enhanced Disease by Promoting Trained Macrophages and Resident Memory T Cells. *J. Virol.* 97, (2023).

107. Kang, A. *et al.* Subcutaneous BCG vaccination protects against streptococcal pneumonia via regulating innate immune responses in the lung. *EMBO Mol. Med.* 15, (2023).

108. Chapman, R. *et al.* The Use of Directed Evolution to Create a Stable and Immunogenic Recombinant BCG Expressing a Modified HIV-1 Gag Antigen. *PLoS One* 9, e103314 (2014).

109. Kim, B. J., Kim, B. R., Kook, Y. H. & Kim, B. J. Development of a live recombinant BCG expressing human immunodeficiency virus type 1 (HIV-1) gag using a pMyong2 vector system: Potential use as a novel HIV-1 vaccine. *Front. Immunol.* 9, 309635 (2018).

110. Kilpeläinen, A. *et al.* Recombinant BCG Expressing HTI Prime and Recombinant ChAdOx1 Boost Is Safe and Elicits HIV-1-Specific T-Cell Responses in BALB/c Mice. *Vaccines 2019, Vol. 7, Page 78* 7, 78 (2019).

111. Gupta, P. K. New disease old vaccine: Is recombinant BCG vaccine an answer for COVID-19? *Cell. Immunol.* 356, 104187 (2020).

112. Singh, A. K. *et al.* Intravenous BCG vaccination reduces SARS-CoV-2 severity and promotes extensive reprogramming of lung immune cells. *iScience* 26, (2023).

113. Zhang, B. Z. *et al.* Bacillus Calmette-Guérin-induced trained immunity protects against SARS-CoV-2 challenge in K18-hACE2 mice. *JCI insight* 7, (2022).

114. Bontempi, I. *et al.* Recombinant Mycobacterium bovis BCG is a promising platform to develop vaccines against Trypansoma cruzi infection. *Clin. Exp. Immunol.* 201, 306–316 (2020).

115. dos Santos, J. C. *et al.* β-Glucan-Induced Trained Immunity Protects against Leishmania braziliensis Infection: a Crucial Role for IL-32. *Cell Rep.* 28, 2659-2672.e6 (2019).

116. Kanno, A. I., Boraschi, D., Leite, L. C. C. & Rodriguez, D. Recombinant BCG Expressing the Subunit 1 of Pertussis Toxin Induces Innate Immune Memory and Confers Protection against Non-Related Pathogens. *Vaccines* 10, (2022).

117. Vierboom, M. P. M. *et al.* Stronger induction of trained immunity by mucosal BCG or MTBVAC vaccination compared to standard intradermal vaccination. *Cell reports. Med.* 2, (2021).

118. Pérez-Sancristóbal, I. *et al.* Long-Term Benefit of Perlingual Polybacterial Vaccines in Patients with Systemic Autoimmune Diseases and Active Immunosuppression. *Biomedicines* 11, (2023).

119. Nieto, A. *et al.* Bacterial Mucosal Immunotherapy with MV130 Prevents Recurrent Wheezing in Children: A Randomized, Double-Blind, Placebo-controlled Clinical Trial. *Am. J. Respir. Crit. Care Med.* 204, 462–472 (2021).

120. Brandi, P. *et al.* Trained immunity induction by the inactivated mucosal vaccine MV130 protects against experimental viral respiratory infections. *Cell Rep.* 38, (2022).

121. Martín-Cruz, L. *et al.* Candida albicans V132 induces trained immunity and enhances the responses triggered by the polybacterial vaccine MV140 for genitourinary tract infections. *Front. Immunol.* 13, (2022).

122. Kayraklioglu, N., Horuluoglu, B. & Klinman, D. M. CpG Oligonucleotides as Vaccine Adjuvants. *Methods Mol. Biol.* 2197, 51–85 (2021).

123. Zhang, T. *et al.* Th2 and Th17-associated immunopathology following SARS-CoV-2 breakthrough infection in Spike-vaccinated ACE2-humanized mice. *J. Med. Virol.* 96, (2024).

124. Elkins, K. L., Rhinehart-Jones, T. R., Stibitz, S., Conover, J. S. & Klinman, D. M. Bacterial DNA containing CpG motifs stimulates lymphocyte-dependent protection of mice against lethal infection with intracellular bacteria. *J. Immunol.* 162, 2291–2298 (1999).

125. Deng, J. C. *et al.* CpG oligodeoxynucleotides stimulate protective innate immunity against pulmonary Klebsiella infection. *J. Immunol.* 173, 5148–5155 (2004).

126. Zimmermann, S. *et al.* CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. *J. Immunol.* 160, 3627–30 (1998).

127. Harandi, A. M., Eriksson, K. & Holmgren, J. A Protective Role of Locally Administered Immunostimulatory CpG Oligodeoxynucleotide in a Mouse Model of Genital Herpes Infection. *J. Virol.* 77, 953–962 (2003).

128. Verthelyi, D. *et al.* CpG Oligodeoxynucleotides Protect Normal and SIV-Infected Macaques from Leishmania Infection . *J. Immunol.* 170, 4717–4723 (2003).

129. Kim, T. H. *et al.* CpG-DNA exerts antibacterial effects by protecting immune cells and producing bacteria-reactive antibodies. *Sci. Rep.* 8, (2018).

130. Ding, C. *et al.* Inducing trained immunity in pro-metastatic macrophages to control tumor metastasis. *Nat. Immunol.* 24, 239–254 (2023).

131. Rueckert, C. & Guzmán, C. A. Vaccines: from empirical development to rational design. *PLoS Pathog.* 8, (2012).

132. Schäfer, A. & Martinez, D. R. “Training immunity” against nosocomial pathogens. *Sci. Immunol.* 8, eadl5685 (2023).

133. Bekkering, S. *et al.* Metabolic Induction of Trained Immunity through the Mevalonate Pathway. *Cell* 172, 135-146.e9 (2018).

134. Domínguez-Andrés, J. *et al.* The Itaconate Pathway Is a Central Regulatory Node Linking Innate Immune Tolerance and Trained Immunity. *Cell Metab.* 29, 211-220.e5 (2019).

135. Domínguez-Andrés, J., van Crevel, R., Divangahi, M. & Netea, M. G. Designing the Next Generation of Vaccines: Relevance for Future Pandemics. *MBio* 11, 1–16 (2020).

136. Natoli, G. & Ostuni, R. Adaptation and memory in immune responses. *Nat. Immunol. 2019 207* 20, 783–792 (2019).

137. Eramo, M. J. & Mitchell, C. A. Regulation of PtdIns(3,4,5)P3/Akt signalling by inositol polyphosphate 5-phosphatases. *Biochem. Soc. Trans.* 44, 240–252 (2016).

138. Saz-Leal, P. *et al*. Targeting SHIP-1 in Myeloid Cells Enhances Trained Immunity and Boosts Response to Infection. *Cell Rep.* 25, 1118–1126 (2018).

139. O'Connell, R. M., Chaudhuri, A. A., Rao, D. S. & Baltimore, D. Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc. Natl. Acad. Sci. U. S. A.* 106, 7113–7118 (2009).

140. Babar, I. A. *et al.* Nanoparticle-based therapy in an in vivo microRNA-155 (miR-155)-dependent mouse model of lymphoma. *Proc. Natl. Acad. Sci. U. S. A.* 109, E1695–E1704 (2012).

141. Priem, B. *et al*. Trained Immunity-Promoting Nanobiologic Therapy Suppresses Tumor Growth and Potentiates Checkpoint Inhibition. *Cell* 183, 786-801.e19 (2020).

142. Li, M. *et al.* Emerging nanoparticle platforms for CpG oligonucleotide delivery. *Biomater. Sci.* (2024) doi:10.1039/d3bm01970e.

143. Braza, M. S. *et al*. Inhibiting Inflammation with Myeloid Cell-Specific Nanobiologics Promotes Organ Transplant Acceptance. *Immunity* 49, 819-828.e6 (2018).

144. Bekkering, S. *et al.* Oxidized low-density lipoprotein induces long-term proinflammatory cytokine production and foam cell formation via epigenetic reprogramming of monocytes. *Arterioscler. Thromb. Vasc. Biol.* 34, 1731–1738 (2014).

145. Li, X. *et al.* Maladaptive innate immune training of myelopoiesis links inflammatory comorbidities. *Cell* 185, 1709-1727.e18 (2022).

146. Leentjens, J. *et al*. Trained Innate Immunity as a Novel Mechanism Linking Infection and the Development of Atherosclerosis. *Circ. Res.* 122, 664–669 (2018).

147. Kumar, N. P. *et al*. Effect of BCG vaccination on proinflammatory responses in elderly individuals. *Sci. Adv.* 7, (2021).

148. Giamarellos-Bourboulis, E. J. *et al.* Activate: Randomized Clinical Trial of BCG Vaccination against Infection in the Elderly. *Cell* 183, 315-323.e9 (2020).

149. de Bree, L. C. J. *et al.* Non-specific effects of vaccines: Current evidence and potential implications. *Semin. Immunol.* 39, 35–43 (2018).

150. Cirovic, B. *et al.* BCG Vaccination in Humans Elicits Trained Immunity via the Hematopoietic Progenitor Compartment. *Cell Host Microbe* (2020) doi:10.1016/j.chom.2020.05.014.

151. Covián, C. *et al.* BCG-Induced Cross-Protection and Development of Trained Immunity: Implication for Vaccine Design. *Frontiers in Immunology* (2019) doi:10.3389/fimmu.2019.02806.

152. Peng, X. *et al.* Mucosal recombinant BCG vaccine induces lung-resident memory macrophages and enhances trained immunity via mTORC2/HK1-mediated metabolic rewiring. *J. Biol. Chem.* 105518 (2023) doi:10.1016/J.JBC.2023.105518.

153. Röring, R. J. *et al.* MMR vaccination induces trained immunity via functional and metabolic reprogramming of γδ T cells. *J. Clin. Invest.* 366–374 (2024) doi:10.1172/JCI170848.

154. Aaby, P. *et al.* Oral polio vaccination and low case fatality at the paediatric ward in Bissau, Guinea-Bissau. *Vaccine* 22, 3014–3017 (2004).

155. Øland, C. B., Mogensen, S. W., Rodrigues, A., Benn, C. S. & Aaby, P. Reduced Mortality After Oral Polio Vaccination and Increased Mortality After Diphtheria-tetanus-pertussis Vaccination in Children in a Low-income Setting. *Clin. Ther.* 43, 172-184.e7 (2021).

156. da Costa Neves, P. C., de Souza Matos, D. C., Marcovistz, R. & Galler, R. TLR expression and NK cell activation after human yellow fever vaccination. *Vaccine* 27, 5543–5549 (2009).

157. Gaucher, D. *et al.* Yellow fever vaccine induces integrated multilineage and polyfunctional immune responses. *J. Exp. Med.* 205, 3119–3131 (2008).

158. Debisarun, P. A. *et al.* Induction of trained immunity by influenza vaccination - impact on COVID-19. *PLOS Pathog.* 17, e1009928 (2021).

Chapter 4

Differential heterologous immunological effects induced by ChAdOx1-S and BNT162b2 COVID-19 vaccines

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Submitted

Abstract

Background The mRNA- and adenovirus-based COVID-19 vaccines may induce different heterologous effects on non-COVID morbidity. We aimed to investigate immunological mechanisms that may account for these differences.

Methods We selected a subgroup of individuals from the TACTIC trial who had completed their COVID-19 vaccination scheme before the introduction of a BNT162b2 booster vaccine. Transcriptional activity, distribution of cell types and cytokine secretion were compared between those who were originally vaccinated with the adenovirus vaccine ChAdOx1-S, and those who had received the mRNA vaccine BNT162b2. Additionally, we investigated how these differences evolved after administration of a BNT162b2 booster vaccine.

Results 24 individuals were included in this study, with 15 volunteers (62.5%) having originally received an mRNA vaccine and 9 (37.5%) an adenovirus vector vaccine. We found that 84 gene sets were differentially expressed in PBMCs from the two vaccine groups following *ex-vivo* secondary stimulation. Although cell populations did not significantly differ, pro-inflammatory cytokine responses to most stimuli were consistently higher in the adenovirus group compared to the mRNA group. These differences decreased after an mRNA booster vaccine.

Discussion Our study findings provide additional support to the hypothesis that mRNA- and adenovirus-based vaccines differ in their broad immunological effects. Specifically, our observation that adenovirus-based vaccination tends to result in higher pro-inflammatory cytokine responses might help explain the difference in the heterologous effects of the two types of vaccines. Knowledge about NSEs is increasingly important for making public health and policy decisions, particularly for vulnerable populations.

Introduction

In May 2023, coronavirus disease 2019 (COVID-19) was declared no longer a global health emergency and its management transitioned to a long-term strategy (1). It is estimated that specific SARS-CoV-2 vaccines prevented over 14 million deaths in their first year of use alone, and effectively protected people worldwide (2). Two of the most widely used vaccine types were mRNA vaccines like BNT162b2 and those employing adenoviruses as a vector, like ChAdOx1-S. Older adults were among the first to get vaccinated, as their risk for severe illness and death is more than 20 times higher compared to adults younger than 65 years (3). Although both types of vaccine have been proven to prevent COVID-19 related morbidity and mortality to a large extent (4, 5), it has been suggested that there are potentially important differences between the two vaccines in terms of the non-specific effects (NSEs) and long-term impact on immune responses.

NSEs have been described previously for live-attenuated vaccines, which can induce a broader protection against unrelated infections and immune-mediated diseases (6). This has been described extensively for the Bacillus Calmette-Guérin (BCG) vaccine against tuberculosis, showing that neonatal mortality rates after BCG vaccination declined more than what could be explained by preventing tuberculosis alone (7). BCG has also been used to prevent non-tuberculous respiratory tract infections in the elderly (8). Benn *et al*. were the first to investigate the possibility of NSEs in COVID-19 vaccines (9). They suggest there are potential differences in cardiovascular deaths and other non-COVID-19 mortality between mRNA and vector-based vaccines, when compared to placebo. Specifically, the adenovirus-vector vaccines were associated with lower overall mortality and lower rates of non-accidental, non-COVID-19 mortality (9), suggesting that vector-based vaccines have potential beneficial effects on cardiovascular mortality.

Our objective was to investigate possible immunological mechanisms on the transcriptional and functional level that may account for this difference. In the current post-pandemic stage, in which COVID-19 is considered endemic, non-specific effects could become increasingly important for making public health and policy decisions regarding vaccination programmes – especially for the most vulnerable groups.

Methods

Study design

We used data from a clinical trial (TACTIC) that studied the effects of concurrent influenza and COVID-19 booster vaccination. In this sub-study, we used the 'COVID-19 booster-only' treatment arm to examine the differences between mRNA and vector-based primary COVID-19 vaccines. Volunteers who were fully vaccinated with any type of vaccine against COVID-19 in autumn 2021 were eligible for participation. Additional inclusion criteria were an age ≥60 years and the absence of any immunosuppressive condition (10). The study baseline (T0) started at least four months after the completion of a primary vaccination series and involved the administration of a BNT162b2 booster vaccine. Two subsequent study visits were planned after three (T1) and six weeks (T2). Blood was collected at all timepoints.

Ethical approval for the TACTIC trial was obtained from the competent authority (CCMO; EudraCT number 2021-002186-17) and the medical ethics committee Oost-Nederland (2021-8294). Written informed consent was obtained from all study participants.

Isolation of peripheral blood mononuclear cells (PBMCs)

Blood samples were collected into 10 mL EDTA-coated tubes (BD Bioscience, USA). PBMCs (peripheral blood mononuclear cells) were isolated from the blood after sampling sera and plasma from each individual. Blood was diluted 1:1 with PBS (1X) without Ca++, Mg++ (Westburg, The Netherlands, cat #LO BE17-516F). PBMCs were isolated via density gradient centrifuge using Ficoll-Paque^{TM}-plus (VWR, The Netherlands, cat #17-1440-03P). Specialized SepMate-50 tubes (Stem Cell Technologies, cat #85450) were used for the isolation according to the manufacturer's instructions. The cells were counted via Sysmex XN-450 (Japan) haematology analyser. Afterwards, $15x10^6$ /mL PBMCs were frozen in Recovery Cell Culture Freezing Medium (Thermo Fisher Scientific, USA, cat #12648010).

Cell thawing and viability assay

The PBMCs were thawed into 10 mL Dutch modified RPMI 1640 medium (Roswell Park Memorial Institute; Invitrogen, USA, cat # 22409031) containing 50 µg/mL Gentamicine (Centrafarm, The Netherlands), 1 mM Sodium-Pyruvate (Thermo Fisher Scientific, USA, cat #11360088), 2 mM Glutamax (Thermo Fisher Scientific, USA, cat #35050087) supplemented with 10% Bovine Calf Serum (Fisher Scientific, USA, cat #11551831). 12.5 µg/ml DNase (Roche, Switzerland, cat #1128493200) was added to the medium to digest extracellular DNA released from dying cells. After the cells were washed twice, they were counted via Sysmex XN-450 (Japan).

Following these procedures, a viability assay via flow cytometry was performed before stimulation. Cells ($4x10^5$) were incubated with anti-human CD45-KO (Beckman Coulter, cat#A96416) for 30 mins at room temperature, protected from light. After washing with 1% BSA in PBS, the cells were centrifuged at 500 g, 4°C, and incubated for 10 minutes with live/dead marker Helix-Nir-APC (Biolegend, cat# 425301). The stained cells were analysed using flow cytometry (Beckman Coulter, CytoFLEX) and FlowJo™.

Stimulation experiments

PBMCs ($4x10^5$ cells/well) were stimulated in sterile round bottom 96-well tissue culture treated plates (VWR, The Netherlands, cat #734-2184) in Dutch modified RPMI 1640 medium containing 50 µg/mL Gentamicine, 1 mM Sodium-Pyruvate, and 2 mM Glutamax supplemented with 10% human pooled serum. Stimulations were done with heat-inactivated SARS-CoV-2, Wuhan Hu-1 (GISAID accession number EPI_ISL_425126), ($2.8x10^3$ TCID50/mL)), influenza virus reference strain A/California/7/2009 H1N1 (11) ($3.6x10^3$ TCID50/mL), 3 µg/mL R848 (Invivogen, USA, cat #tlrl-r848), 1x 10^6/mL heat-killed *C. albicans*, 10 µg/mL Poly I:C (Invivogen, USA, cat #tlrl-pic), or 10 ng/mL *E. coli* LPS (serotype O55:B5; Sigma-Aldrich, cat #L2880, further purified as previously described (12). The PBMCs were incubated with the stimuli for 24 hours to be used for bulk RNA isolation and to detect IL-1β, TNF, IL-6, and IL-1Ra. Supernatants were collected and stored at -20 °C. Secreted cytokine levels from supernatants were quantified by ELISA (IL-1β cat # DLB50, TNF cat # STA00D, IL-6 cat# D6050, IL-1Ra cat # DRA00B, R&D Systems, USA following manufacturers' instructions.

RNA Isolation

Stimulated PBMCs were frozen in lysis buffer LBP after collection of supernatant and thawed for RNA isolation. We used the Nucleospin RNA PLUS kit (Machery-Nagel, cat # 740984.50) according to the manufacturer's instructions to isolate the RNA from a subset of 5 participants per vaccination group. Per participant, the RPMI, SARS-CoV-2 Wuhan and *C. Albicans* conditions were chosen for RNA sequencing. RNA isolation failed for one sample, resulting in 29 samples for analysis.

Bulk RNAseq analysis

Manual curation of quality control metrics and principal component analysis (PCA) was performed per timepoint and stimulation separately to identify potential outliers. 2 out of 29 samples were removed from further analysis (vector vaccination, Wuhan-stimulated).

We processed the raw bulk RNA-seq reads using the publicly available nfcore/ rnaseq v2.0 pipeline using default settings (13). Reads were aligned to the human genome (GRCh38) and genes with total read counts below 20 were filtered out.

Further downstream analyses were performed in R using DESeq2 v1.30.1 (14), with age and sex as cofactors. Gene set enrichment analysis (GSEA) was performed based on the identified differentially expressed genes (DEGs), using the fgsea package in R (15).

Flow cytometry analysis

After thawing and counting the cells as described above, 1x10^6 cells per sample were used for flow cytometry (FCM) analysis. The cells were spun down at 500g, 4°C for 5 mins and resuspended in flow cytometry buffer (filtered 0.5% bovine serum albumin, 2mM EDTA in PBS). Live/dead stain ViaKrome (Beckman Coulter, cat # C36628) diluted 1:100 was added onto resuspended cells and incubated in the dark at RT for 20 mins. After viability staining, the cells were washed and resuspended in FCM buffer. Fc receptor blocking solution TruStain (Biolegend, cat #422302) diluted 1:20 was added along with CD11c-BV605 (1:20) and incubated in the dark at 4°C for 20 mins. Afterwards, the remaining antibodies were added onto the cells. The antibodies in the panel and the final dilutions can be found in in *Supplementary table 1*. The cells were incubated for 20 more minutes in the dark at 4°C followed by washing with FCM buffer. The cells were resuspended and analysed by flow cytometry (Beckman Coulter, CytoFLEX LX) and FlowJo^{TM}. The gating strategy can be found in *Supplementary figure 1*.

Statistical analysis

Descriptive statistics are presented as medians with interquartile range (IQR) for continuous variables, and as frequency and percentages for categorical variables.

Cell populations (presented in proportions) and cytokine levels (in pg/ml) were compared between adenovirus and mRNA vaccinees at study baseline (four months after initial vaccination) using Mann-Whitney-U tests and Kruskal-Wallis followed by Dunn's multiple comparisons where more than two time points were compared. Data following mRNA booster vaccination was compared using Friedman tests and Dunn's. Given the fact that the type of initially administered COVID-19 vaccines pre-study was based on age by national policy, correcting our results for age would be a structural violation of the positivity assumption (16) (*Supplementary figure 2)*. We therefore chose to present our unadjusted data.

Statistical analyses were performed using GraphPad prism version 9 and R version 4.2.0. All statistical tests were performed in a two-sided manner and a p-value <0.05 was considered statistically significant. For gene analysis, we considered the Benjamini-Hochberg adjusted p-value.

Results

Out of the Between October and November 2021, the TACTIC trial enrolled a total of 154 immunocompetent participants (10). The present study focuses on a subset of 24 participants who received a COVID-19 booster, but no influenza vaccination, following their initial vaccinations (see *Figure 1*). Among these individuals, 15 (62.5%) had initially been vaccinated with the BNT162b2 mRNA vaccine, while 9 (37.5%) volunteers had received the vector-based ChAdOx1-S vaccine. The median ages for both groups were 70 and 64 years, respectively, and half of the study cohort was male (54%). Additional baseline characteristics are detailed in *Table 1*. The interval between the last primary SARS-CoV-2 vaccination and the collection of the first study samples was four to five months.

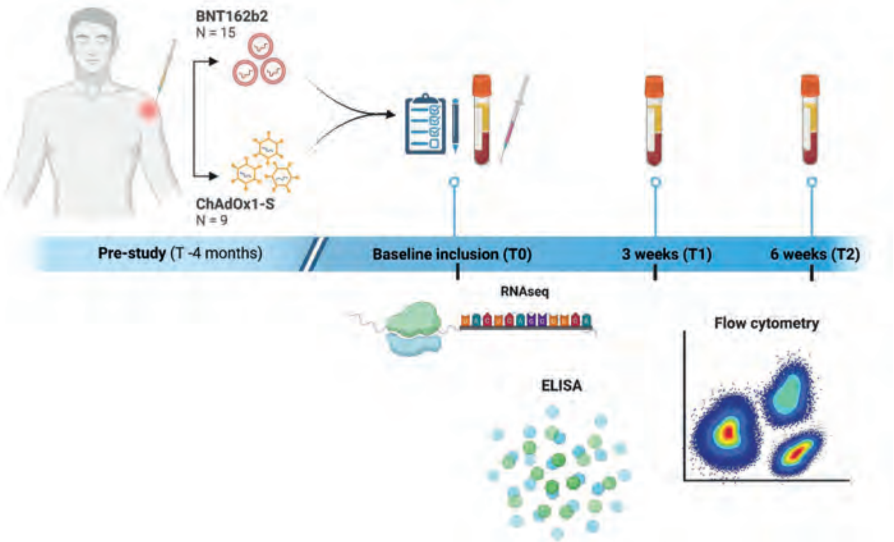

Figure 1: Study design. The volunteers who were vaccinated with either BNT162b2 or ChAdOx1-Sdonated blood four months after their primary vaccinations (study baseline). Subsequent samples were collected three and six weeks after the administered BNT162b2 booster vaccine during the first visit.

PBMCs were isolated after blood collection and cryopreserved for further functional analyses. The cryopreserved PBMC samples from the three time points were used for study baseline RNAseq analysis, flow cytometry analysis and functional ELISAs following *ex-vivo* stimulation; flow cytometry and ELISAs were performed additionally at timepoint T1 and T2 after booster vaccination.

Table 1: Study group characteristics

	mRNA vaccine (n=15)	**Adenovirus vaccine (n=9)**
Male sex (n, %)	8 (53.3%)	5 (55.6%)
Median age (IQR)	70 (68-71)	64 (62-64)
Median BMI (IQR)	23.1 (22.6-25.6)	26.8 (25.4-29.4)
Received prior influenza vaccine	13 (86.7%)	9 (100.0%)
Received prior BCG vaccine	3 (20.0%)	1 (11.1%)
Received prior pneumococcal vaccine	3 (20.0%)	0 (0.0%)
Median days between initial vaccinations and study baseline (IQR)	136 (128-152)	127 (124-147)
RNA-sequence sub-cohort	**n=5**	**n=5**
Male sex (n)	2	3
Median age (min-max)	71 (62-74)	62 (60-65)

The transcriptional landscape of stimulated PBMCs in ChAdOx1-S and BNT162b2-vaccinated individuals

We analysed the transcriptional alterations in PBMCs following stimulation with RPMI (control), heat-killed SARS-CoV-2 and heat-killed *C. albicans* at the study baseline (four months after primary COVID-19 vaccinations). Comparison of the transcriptome between the two vaccination groups showed different sets of differentially expressed genes (DEGs) depending on the stimulus employed (*Figure 2*). We found 142 DEGs when comparing RPMI-control cells between both groups and 84 after SARS-CoV-2 stimulation. Volcano plots show the sets of DEGs which were upregulated in ChAdOx1-S-vaccinated individuals compared to BNT162b2-vaccinated individuals (*Figure 2a-b*).

In RPMI-treated cells (controls), the transcription factors ZNF558 and ZSCAN26, which are involved in the regulation of immune modulation and antiviral defence (NIH gene database), were upregulated in the adenoviral vaccination group. In turn, a positive regulator of transcription, BRIX1, was downregulated in the same group. After SARS-CoV-2 stimulation, SIGLEC5 was upregulated, a known receptor that induces T-cell suppression (17).

Additionally, we performed gene set enrichment analysis (GSEA) which revealed upregulation in multiple inflammation-related pathways in RPMI-stimulated PBMCs of the ChAdOx1-S group, such as chemokines and inflammatory molecules in myeloid cells, activated (LPS) dendritic cell surface signature, TLRs and inflammatory signalling (*Figure 2c*). Notably, one of the pathways upregulated in ChAdOx1-S group was the antiviral IFN signature module, even in the absence of specific stimulations. Interestingly, after SARS-CoV-2 stimulation, the difference in upregulated pathways between the two vaccines was less pronounced. Only two pathways, enriched in monocytes and cell cycle and transcription, were upregulated In ChAdOx1-S vaccinated individuals. In contrast with significant differences to RPMI and SARS-CoV-2 stimulations, heat-killed *C. albicans* did not result in significantly distinct DEGs when comparing the two vaccine groups (*Supplementary Figure 3a*). Even though DEG profiles did not show any difference, GSEA showed up- and downregulation of several pathway modules between the vaccine groups (*Supplementary Figure 3b*).

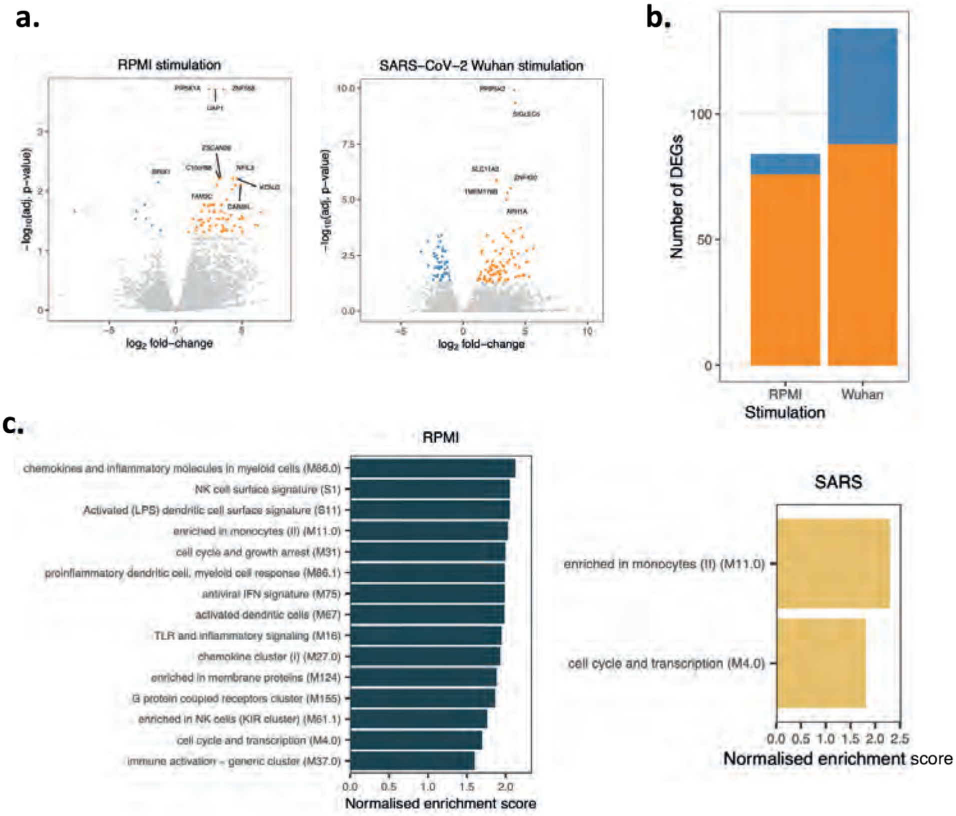

Figure 2: The transcriptomic signature of PBMCs in response to stimulation after vaccination with ChAdOx1-S or BNT162b2. Volcano plots present the DEGs in comparison with primary vaccinations BNT162b2 and ChAdOx1-S, showing the log2-fold-change (x-axis) vs. the negative log10 of the p-value (y-axis), following stimulation of the cells with SARS-CoV-2 Wuhan strain and RPMI at T0 (study baseline; before booster). (b) Barplot depicting the total number of DEGs in response to SARS-CoV-2 and RPMI at T0. (c) Gene set enrichment analysis of comparing BNT162b2 and ChAdOx1-S.

(a-c) Upregulation = upregulated in the ChAdOx1-S group *(orange)*.

Cell population distributions after ChAdOx1-S versus BNT162b2 vaccination

We performed flow cytometry to identify the cell sub-populations in PBMCs that are important for inflammatory responses. Percentages of monocytes and NK cells in viable CD45+ cells were highly comparable four months after volunteers were administered the two vaccines (*Figure 3*). However, we observed a slight but measurable 2% higher baseline percentage of CD8+ T cells in the vector group (10% vs. 8%). In addition, the baseline population of CD123$^+$ plasmacytoid dendritic cells (pDCs) was lower in the adenoviral vaccine group compared with the mRNA vaccine. These findings did not meet the statistical threshold. The percentage of B cells (CD19+, CD20-) was higher in vector vaccinated-individuals at the study baseline. This difference was not statistically significant for memory B cells or plasma cells, but clearly visible for CD19+CD20+CD38+ mature and CD19+CD20+CD38++ transitional B cells (*Supplementary Figure 4*).

Inflammatory cytokine production in response to *ex-vivo* secondary stimulation four months after primary vaccination

Next, we measured pro-inflammatory and anti-inflammatory cytokines (IL-6, IL-1β, TNF, and IL-1Ra) after *ex-vivo* stimulation of PBMCs to understand the functional differences in immune responses observed in the ChAdOx1-S and BNT162b2 groups. In general, pro-inflammatory cytokines were secreted in higher amounts from PBMCs of ChAdOx1-S-vaccinated individuals following RPMI, LPS, heat-killed SARS-CoV-2 and *C. albicans,* compared to BNT162b2-vaccinated individuals. (*Figure 4*). These differences were statistically significant after RPMI and SARS-CoV-2 stimulation for all measured pro-inflammatory cytokines, and for IL-1β after stimulation with LPS and *C. albicans*.

Stimulation of PBMCs of vaccinated individuals with other heterologous stimuli (heat-killed Influenza, poly I:C, R848) showed a largely consistent pattern of higher pro-inflammatory cytokines after ChAfdOx1-s as well. (*Supplementary Figure 5*). In general, immune cells of individuals from the ChAdOx1-Sgroup had higher cytokine production compared to their BNT162b2 counterparts. Notably, IL-1β secretion in response to LPS stimulation, TNF and IL-1β secretion in response to heat-killed Influenza stimulation, and IL-1β secretion in response to R848, were statistically significantly higher in ChAdOx1-S vaccination group (*Supplementary Figure 5*).

Longitudinal changes in PBMC cell populations

After examining the effects of ChAdOx1-S and BNT162b2 primary vaccinations, we explored if the immunological responses to a BNT162b2 booster differ depending

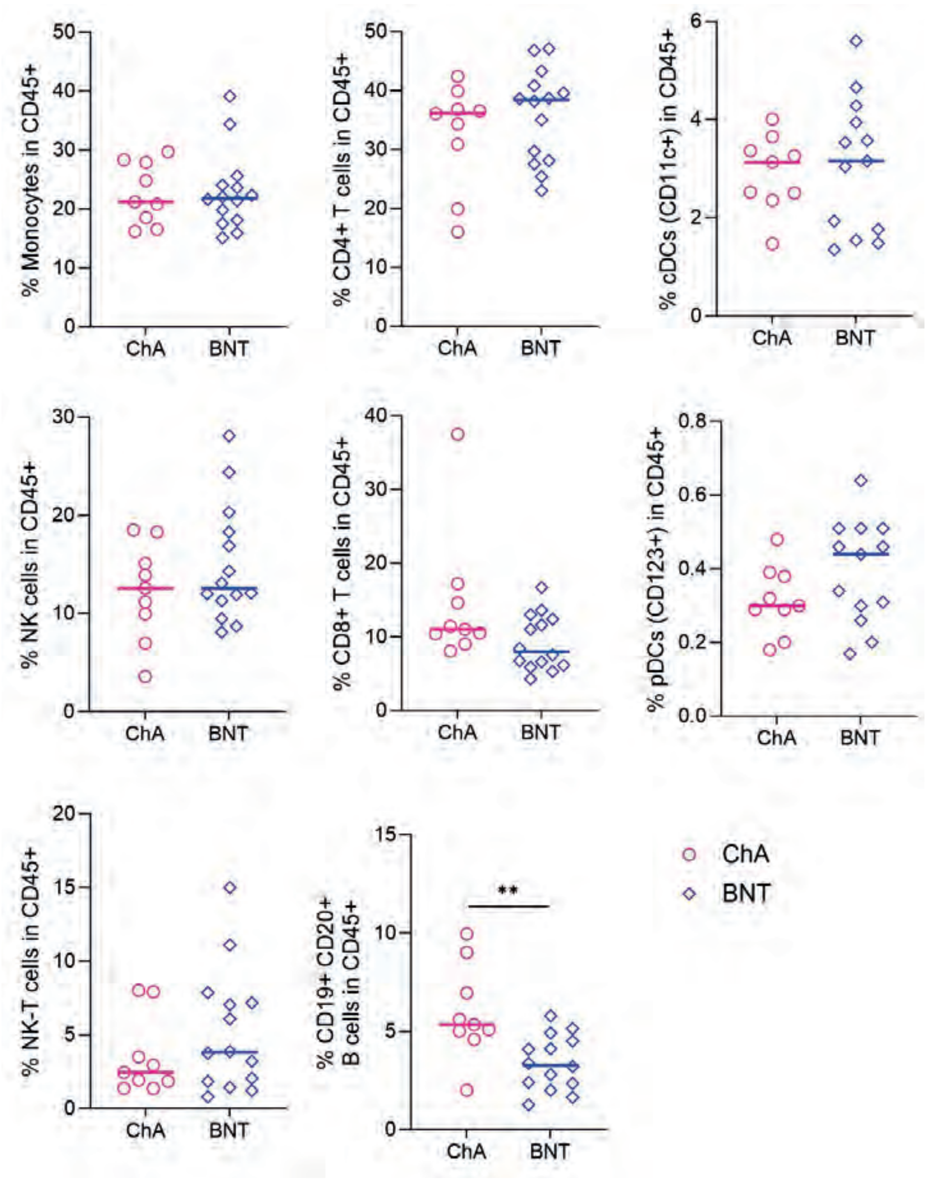

Figure 3: Immune cell populations within PBMCs four months after vaccination (T0) with ChAdOx1-S (ChA) or BNT162b2 (BNT). The flow cytometry panel shown in Supplementary Table 1 was used to detect the different cell populations. cDCs: classical dendritic cells; pDCs: plasmacytoid dendritic cells; NK: natural killer cells. Mann Whitney U tests are used to compare the cell percentages between groups. ** $p<0.01$.

on the type of primary vaccination. The cell percentages in PBMCs did not reflect any major differences in cell populations within each primary vaccination group, although the previously found difference in CD8+ T cell percentages diminished (*Figure 5*). The percentages of CD123+ pDCs in both groups were more also comparable three weeks after the booster vaccine.

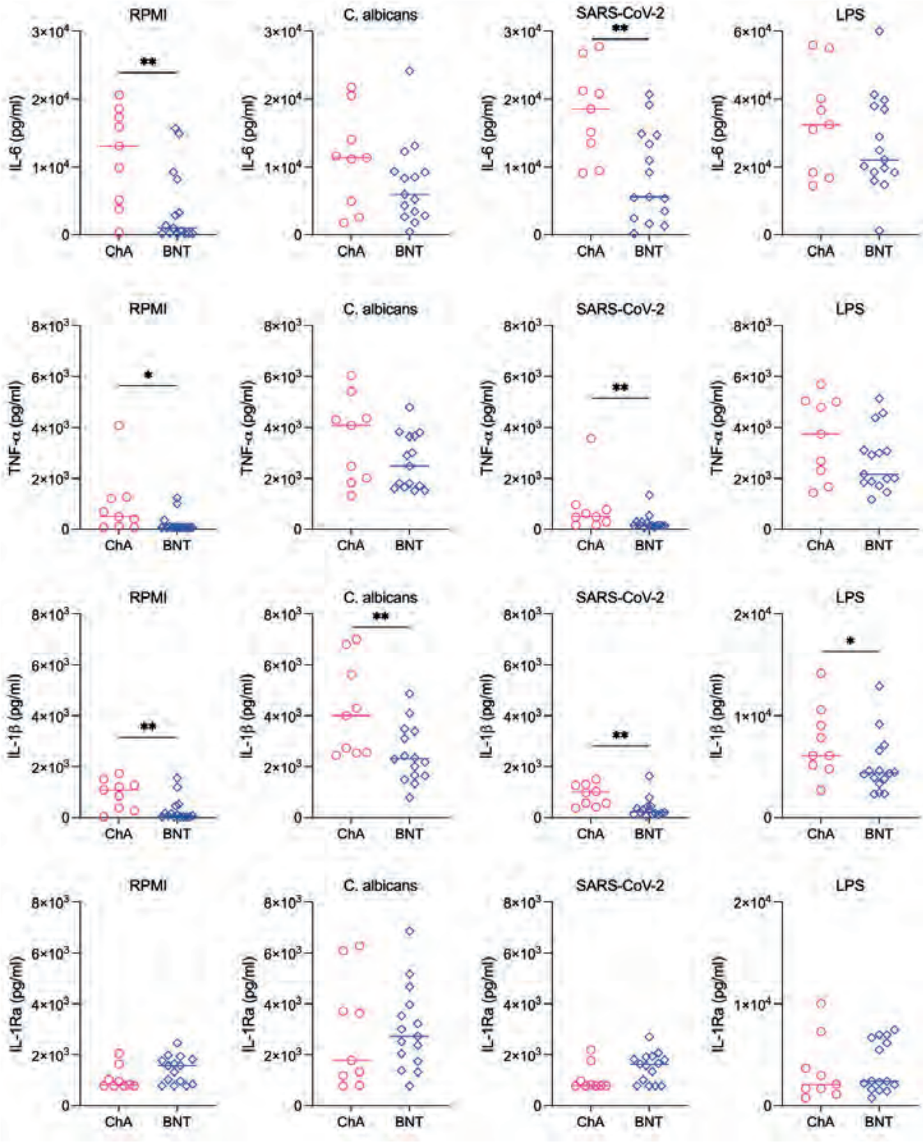

Figure 4: Pro-inflammatory and anti-inflammatory cytokine production in response to specific and non-specific stimuli, four months after vaccination (T0) with ChAdOx1-S (ChA) or BNT162b2 (BNT). IL-6, IL-1β, and IL-1Ra production by PBMCs were measured by ELISA following 24 hours *ex vivo* stimulation with RPMI, heat-killed SARS-CoV-2, *C. albicans* and LPS. Mann Whitney U tests are used to compare the values between the groups. ** $p<0.01$; * $p<0.05$.

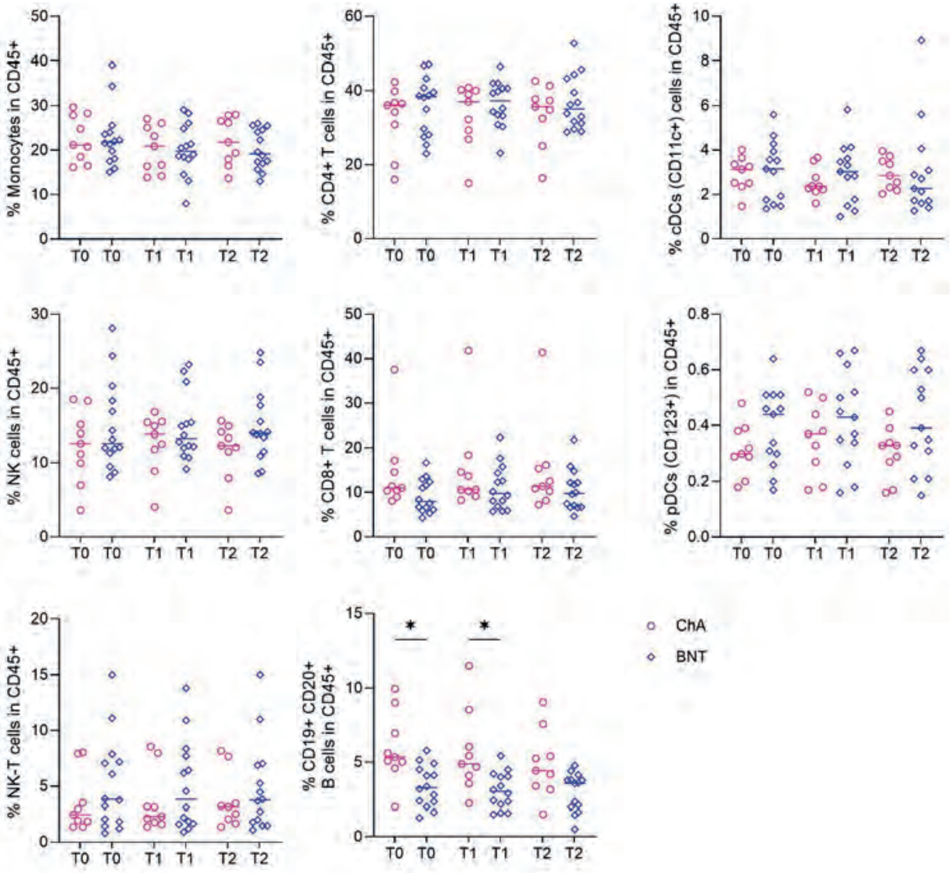

Figure 5: The effect of a BNT162b2 booster on immune cell populations after primary vaccination with ChAdOx1-S (ChA) and BNT162b2 (BNT). The flow cytometry panel shown in Supplementary Table 1 was used to detect the different cell populations through study time points (T0: study baseline; before booster, T1: 2 weeks after booster, T2: 6 weeks after booster). cDCs: classical dendritic cells; pDCs: plasmacytoid dendritic cells; NK: natural killer cells. Kruskal-Wallis test is used to compare the vaccine groups at each time point and Friedman test to compare changes over time within the primary vaccination group, both followed by Dunn's multiple comparisons. * $p<0.05$.

Inflammation-related cytokine secretion in response to stimulation after BNT162b2 booster vaccination

We measured the secreted cytokines mentioned above in response to RPMI (control), LPS, heat-killed SARS-CoV-2 and *C. albicans* following booster vaccination. Vaccination with a mRNA vaccine booster did not further change the cytokine production capacity between the two groups, but rather resulted in a converging pattern (*Figure 6*). PBMCs treated with additional heterologous stimuli followed a similar pattern (*Supplementary Figure 6*).

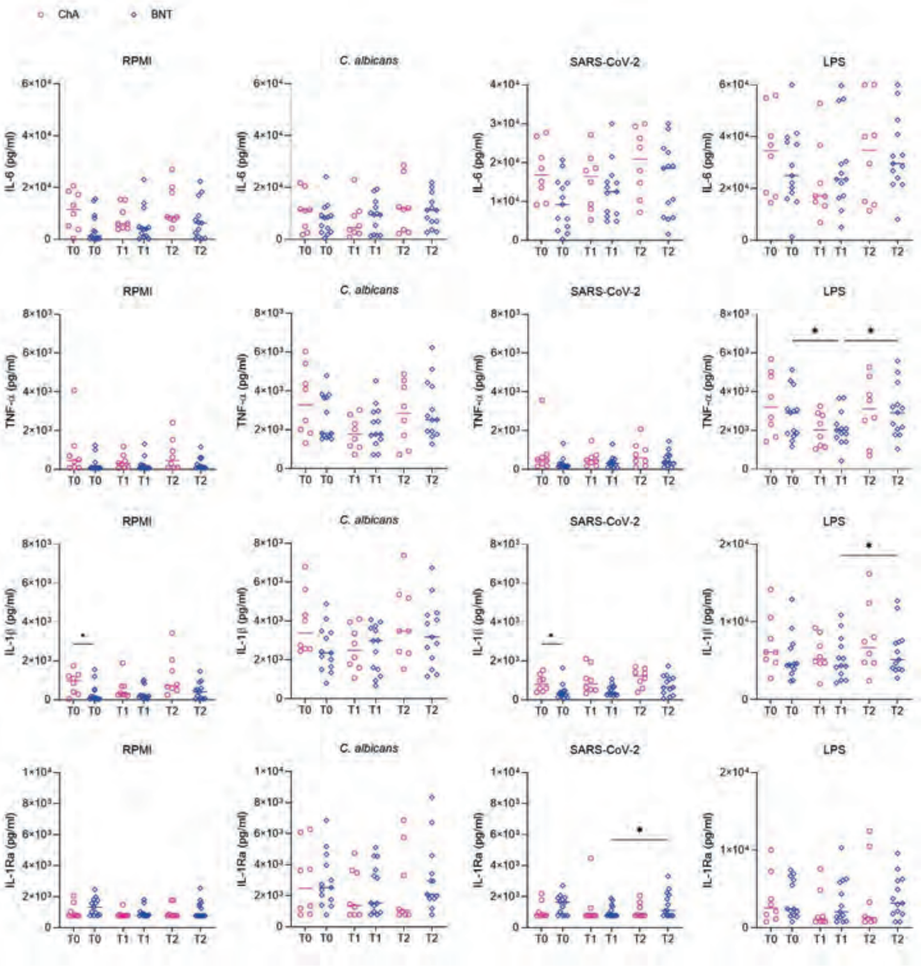

Figure 6: The effect of a BNT162b2 booster on pro-inflammatory and anti-inflammatory cytokine production in response to specific and non-specific stimuli after primary vaccination with ChAdOx1-S (ChA) and BNT162b2 (BNT). IL-6, IL-1β, and IL-1Ra production by PBMCs were measured by ELISA following 24 hours *ex vivo* stimulation with RPMI, *C. albicans,* heat-killed SARS-CoV-2 and LPS, through study time points (T0: study baseline; before booster, T1: 2 weeks after booster, T2: 6 weeks after booster). Kruskal-Wallis test is used to compare the vaccine groups at each time point and Friedman test to compare changes over time within the primary vaccination group, both followed by Dunn's multiple comparisons. * $p<0.05$.

Discussion

The present study explored the differences in the heterologous immunological effects induced by the ChAdOx1-S adenoviral and the BNT162b2 mRNA-based COVID-19 vaccine. We showed that four months after two doses of the ChAdOx1-S vaccine, the transcriptional landscape of PBMCs was distinctly different and the production of pro-inflammatory cytokines was enhanced compared to two doses of the BNT162b2 vaccine. These differences were induced despite a similar make-up of immune cell populations in the two groups. These differential profiles partially converged after the administration of an mRNA booster vaccine, leading to more comparable cytokine profiles in both groups.

Sets of differentially expressed genes were found in PBMCs from the two groups in response to stimulation, indicating a notable difference in the transcriptional landscape after primary vaccination with either an adenoviral-based vs mRNA-based vaccine. In the ChadOx1-S group, numerous inflammation-related pathways were upregulated. Without *ex-vivo* restimulation, we found regulatory changes in genes related to transcriptional activity such as ZNF558, ZSCAN26 and BRIX1 in the PBMCs of the adenoviral vaccine group. Interestingly, ZNF558 is a gene that is proposed to be involved in negative regulation of transcription by RNA polymerase II, whereas ZSCAN26 is predicted to enable DNA-binding transcription factor activity; thereby, involved in positive regulation of transcription (NIH gene database). Upregulated transcription factors for both positive and negative regulators of transcription in the unstimulated cells might suggest ChadOx1-S has more impact on the transcriptional landscape of PBMCs than BNT162b2 does. Another interesting differentially expressed gene in the RPMI-treated PBMCs of the adenoviral vaccine group was BRIX1, which is also related to transcription as it enables RNA binding activity (NIH gene database), and this gene was downregulated. Notably, gene set enrichment analysis also showed numerous upregulated pathways in RPMI-stimulated cells of the ChadOx1-S group compared to the BNT162b2 group, many of them being related to inflammatory responses. The pathways we detected in our GSEA are similar to a study by Ryan *et al.*, who show a comparison of two vaccines without stimulating PBMCs (18). They report upregulation in BTM modules of activated DCs, type I IFN response, enriched in monocytes and overall inflammatory signatures 1-2 days after BNT162b2 and/or ChadOx1-S initial vaccination. However, these results are based on comparison to baseline condition, not the comparison between two vaccines. Notably, our results indicate that these pathway associations can be still present four months after the initial ChadOx1-S vaccination.

Contrary to the unstimulated cells, SARS-CoV-2 stimulation showed fewer differences in the upregulated genes between the two vaccination groups. Among the upregulated genes of the ChAdOx1-S vaccinated volunteers, SIGLEC5 should be noted, as it encodes for the expression of a receptor that inhibits the activation of several cell types including monocytes, macrophages and neutrophils. The SARS-CoV-2 virus possesses glycans that can bind to SIGLEC5, through which the virus can induce an immunosuppressive environment (19). This finding might therefore represent a compensatory mechanism rather than a host-induced change. Taken together, these findings of differences in transcriptional programs between immune cells of the two groups of vaccinated individuals provided a rationale for investigating differences at a functional level, as transcriptional changes can be indicative of, but do not necessarily translate into, altered immune response and activity (20).

Flow cytometry analysis revealed little variation in the distribution of immune cell subsets, showing comparable percentages of monocytes and T cells after primary vaccination with both vaccines. The B cell compartment was relatively reduced after BNT162b2 vaccination, although this difference was not observed in the memory or plasma cells and did not change after the booster vaccine. Although it may be possible that the vector vaccine induced stronger B cell production or maturation, these findings are also likely to result from comparing two non-randomized and relatively small groups. The functional implications of this potential variation are uncertain, with previous studies reporting contrasting findings (18, 21, 22).

An earlier study that compared cytokine production profiles between adenoviral and mRNA vaccines employed the BNT162b2 mRNA and AZD1222 vector-based vaccine. However, that study used the vaccines as *in-vitro* stimulants rather than comparing cytokine production after *in-vivo* vaccination. The authors observed higher levels of pro-inflammatory cytokines after PBMC stimulation in the vector-group, which is in line with our results (23). Ryan *et al.* compared the cytokine profiles after two vaccine types longitudinally, but not directly between both groups, and identified changes in chemokine production (18). It is interesting to consider the mechanisms underlying the distinct cytokine profiles after both types of vaccination, as we did not see any significant shift in cell subsets. The viral vector protein itself induces a strong innate immune response by triggering PRRs, an adjuvants effect that is more intense than elicited by mRNA itself. In addition, earlier research showing elevated systemic inflammation after mRNA vaccination might explain why PBMCs exhibited a comparatively muted reaction when stimulated.

The enhanced pro-inflammatory cytokine responses align with the limited evidence supporting induction of trained immunity following vector vaccination, but not after mRNA vaccine administration, although the available studies have been inconclusive because of small size and mixed results. Increased IL-1β and IL-6 upon *ex vivo* heterologous stimulation have been reported up to three months after one dose of ChAdOx1-S vaccination (24), although this could not be replicated in another study using two doses of the vaccine (25). Antunes *et al*. discuss increased production of IL-10 as a hallmark of training after two doses of ChadOx1-s (26).

Our study provided a comprehensive overview of the innate immune system on both the transcriptional and functional level, although we could not directly study the clinical relevance of these effects in this study due to the sample size tuned for immunological assays rather than clinical outcomes. Given its exploratory nature, we employed a relatively small sample size to investigate whether the two vaccines induce different molecular and functional changes in the immune cells. As is the case for NSE-related evidence in general, future research would benefit most from randomized controlled trials rather than retrospective analyses (27). In addition, we do not know how the measured outcomes would have evolved without the administration of an mRNA booster vaccine and this would have been a useful comparison to guide recommendations about vaccine intervals and sequence. Lastly, it is important to emphasize that our study participants were older adults, and their immune response can be distinctly different from that of younger vaccinees.

The findings of this study show that the BNT162b2 and ChAdOx1-S vaccines against COVID-19 can have distinct effects on the immune system, beyond their induction of specific antibodies and T-cell responses. In contrast to the increased systemic inflammation induced by mRNA vaccination reported earlier (28), their long-term effects show decreased responsiveness of PBMCs to secondary stimulation compared to the effects induced by vaccination with a COVID-19 adenoviral vaccine. This could be reflective of a more efficient response to heterologous pathogens after vector-based vaccination, resulting in a faster resolution of inflammatory conditions. It may be tempting to speculate that these differences contribute to the reported clinical differences in all-cause mortality (9), but this remains to be demonstrated. The potential cardioprotective effects suggested for adenoviral vaccines could therefore have a biological rationale that should be considered in future policy. Certain risk groups, like those at high risk of cardiovascular disease, could benefit more from this type of vaccination, with protection going beyond the effects directly mediated by SARS-CoV-2 infection. However, the use of the adenoviral COVID-19 vaccines has definitively stopped in European countries, in favour of the mRNA vaccines, without heterologous effects having been considered. Finally, our results emphasize the relevance of investigating the broad effects of vaccines beyond their specific impact on the target disease, and provide arguments to apply that knowledge to the development of more efficacious vaccines with potential heterologous benefits.

Acknowledgements

The authors wish to thank all TACTIC study participants, without whom this research had not been possible. The assistance of Helga Dijkstra and Heidi Lemmers in the lab is highly appreciated.

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This study was conceptualized by ED, JS, RC, JD and MN. ED coordinated the TACTIC trial and collected the samples. Laboratory and statistical analyses were performed by ED and BG (RNA isolation and functional analyses), BG and LH (flow cytometry), and MZ and WL (RNA sequencing). ED and BG wrote the first draft. All authors commented on the first version and approved the final one for submission. JS, JD, YL and MN had supervisory roles.

Competing interests

MGN is a scientific founder of TTxD, Lemba and BioTrip, and a member of the TTxD scientific advisory board. All other authors declare no financial or non-financial competing interests. The TACTIC trial was conducted with the ZonMw COVID-19 programme, which had no role in the data collection, analysis or reporting.

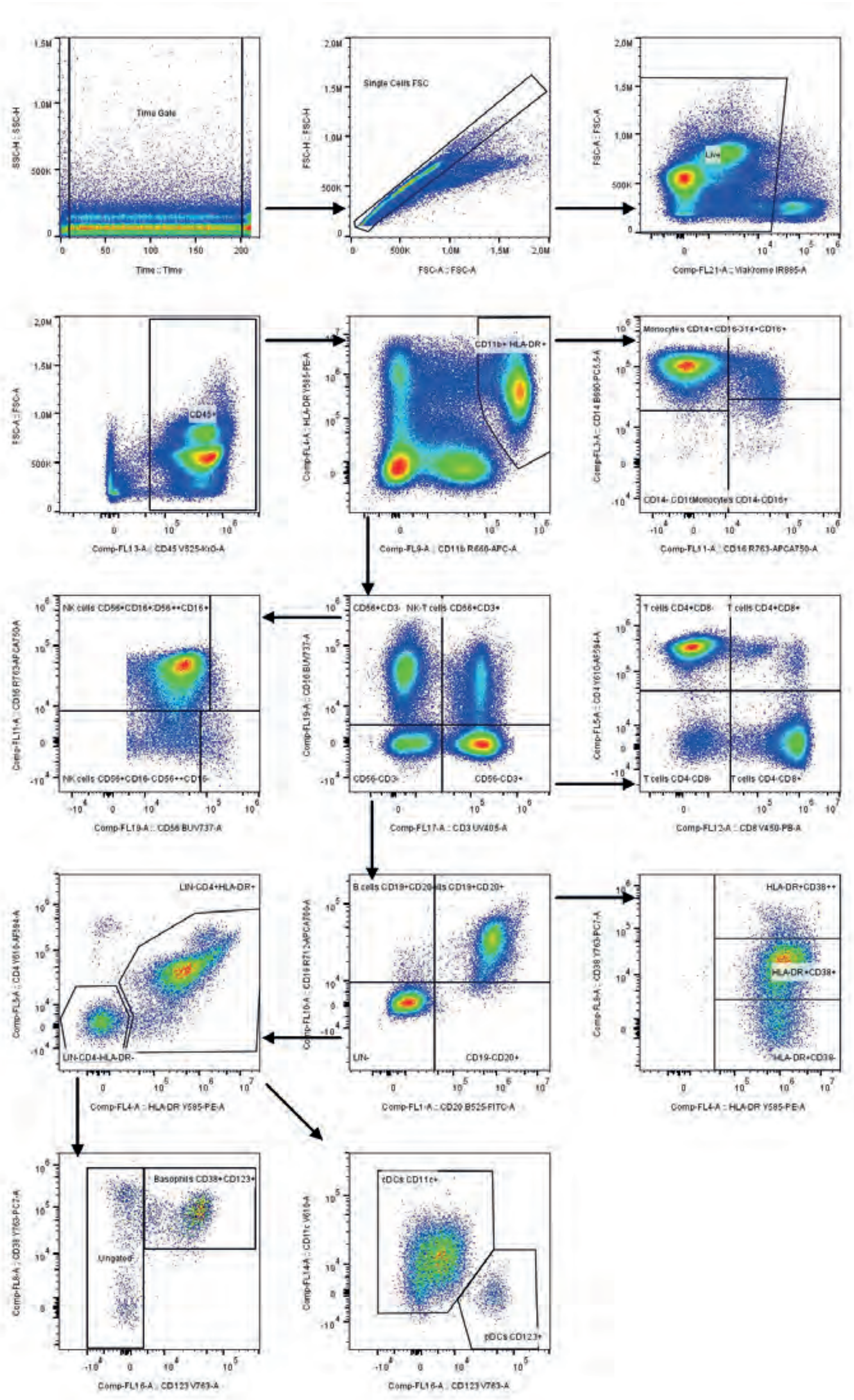
Data availability

The datasets generated and/or analysed during the current study are available, after publication of the paper, in the Radboud Data Repository, at https://doi.org/10.34973/cffd-wr15. Data can only be shared upon explicit permission of the Principal Investigator and in accordance with the signed Informed Consent forms from the original TACTIC trial.

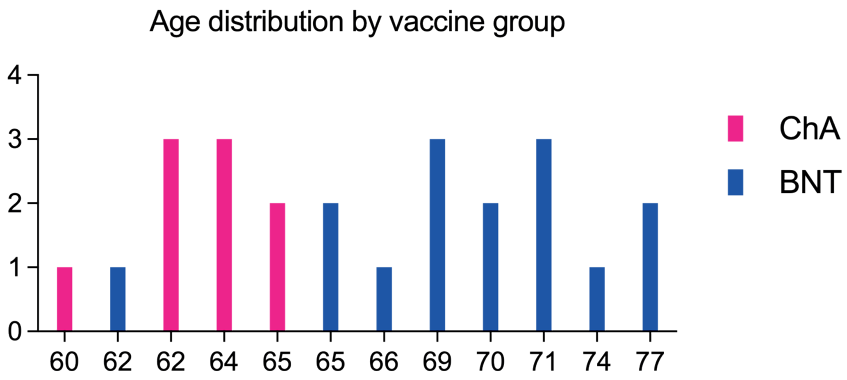
Supplementary materials

Supplementary Table 1: Flow cytometry panel

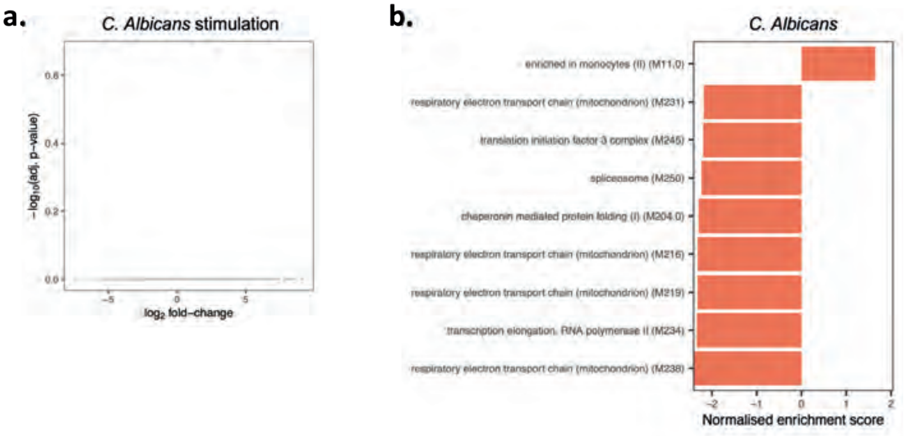
Antibody	Dilution	Company	Cat #
CD11c-BV605	1:20	Biolegend	B366366
CD3-BUV395	1:20	BD Biosciences	1214287
CD45-BV510	1:20	Biolegend	B360608
CD38-PE-Cy7	1:20	Biolegend	B361552
CD11b-APC	1:20	Biolegend	B363135
CD19-AF700	1:20	Biolegend	B363126
CD8-BV421	1:40	Biolegend	B365401
CD123-BV785	1:40	Biolegend	B361311
CD20-FITC	1:40	Biolegend	B342949
CD14-PerCPCy5.5	1:40	Biolegend	B334782
HLA-DR-PE	1:40	Biolegend	B354401
CD16 -APC-Fire750	1:40	Biolegend	B345908
CD56-BUV737	1:64	BD Biosciences	2059630
CD4-AF594	1:64	Biolegend	B329299



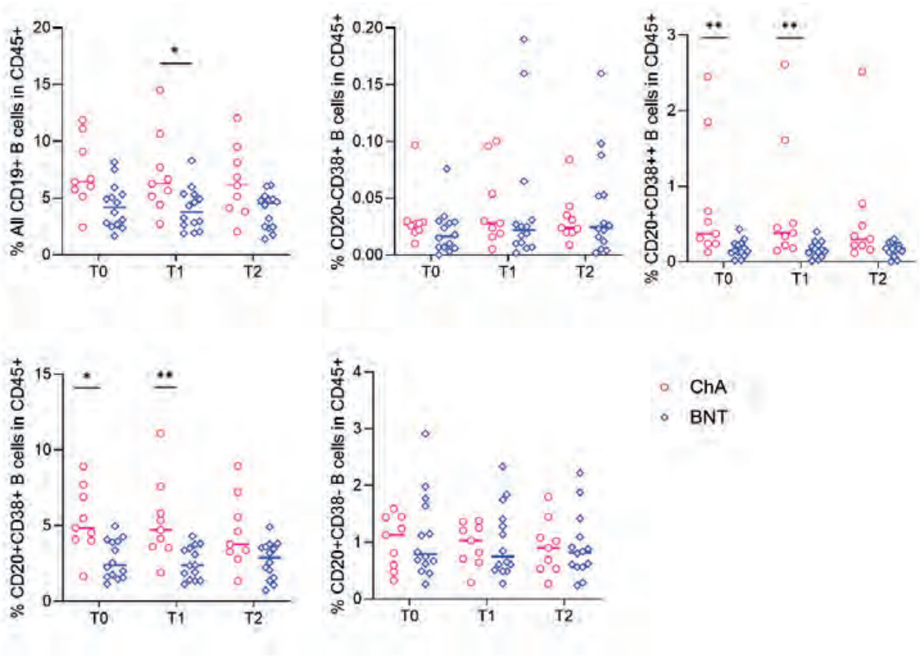
Supplementary Figure 1: Gating strategy for cell sub-populations



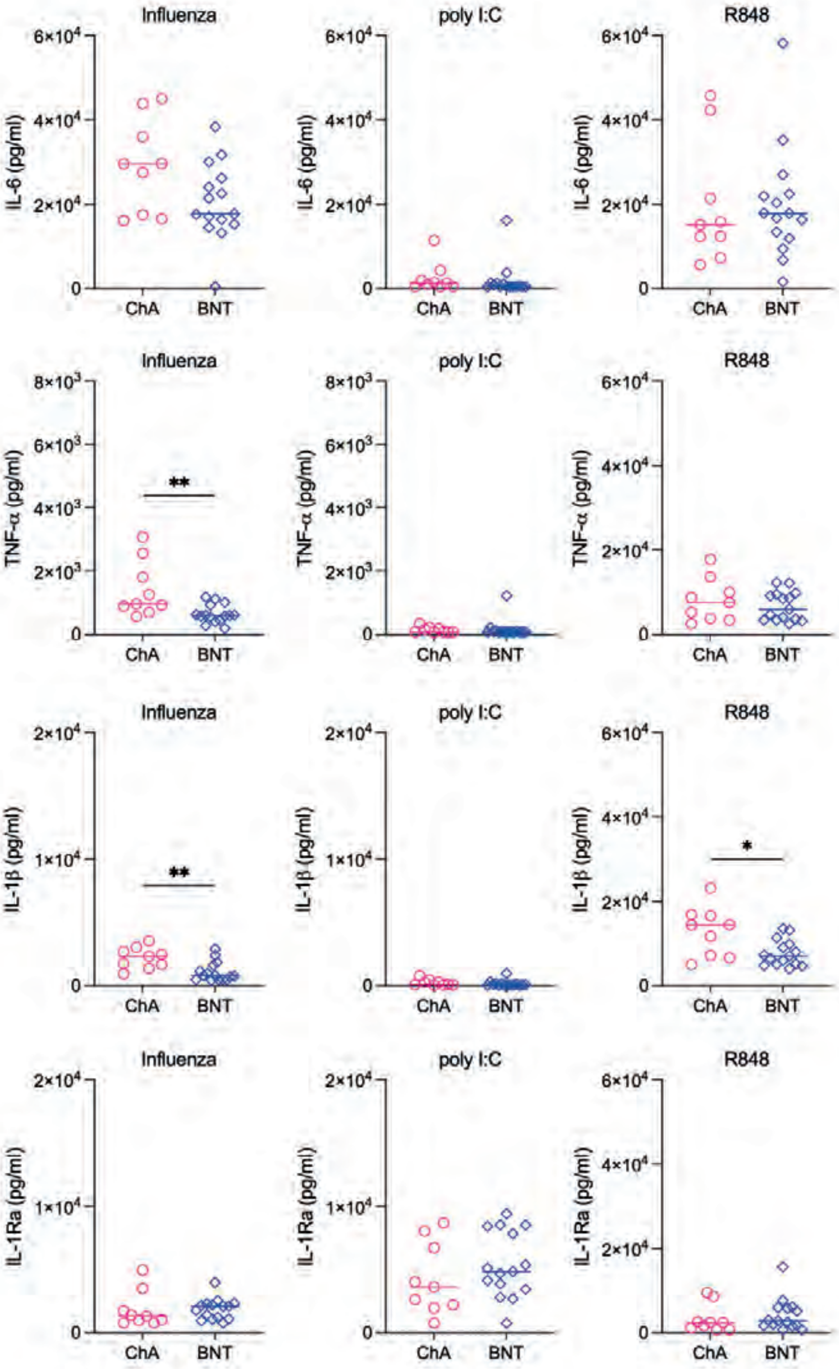
Supplementary Figure 2: Age distribution by vaccine (determined per national vaccination policy)



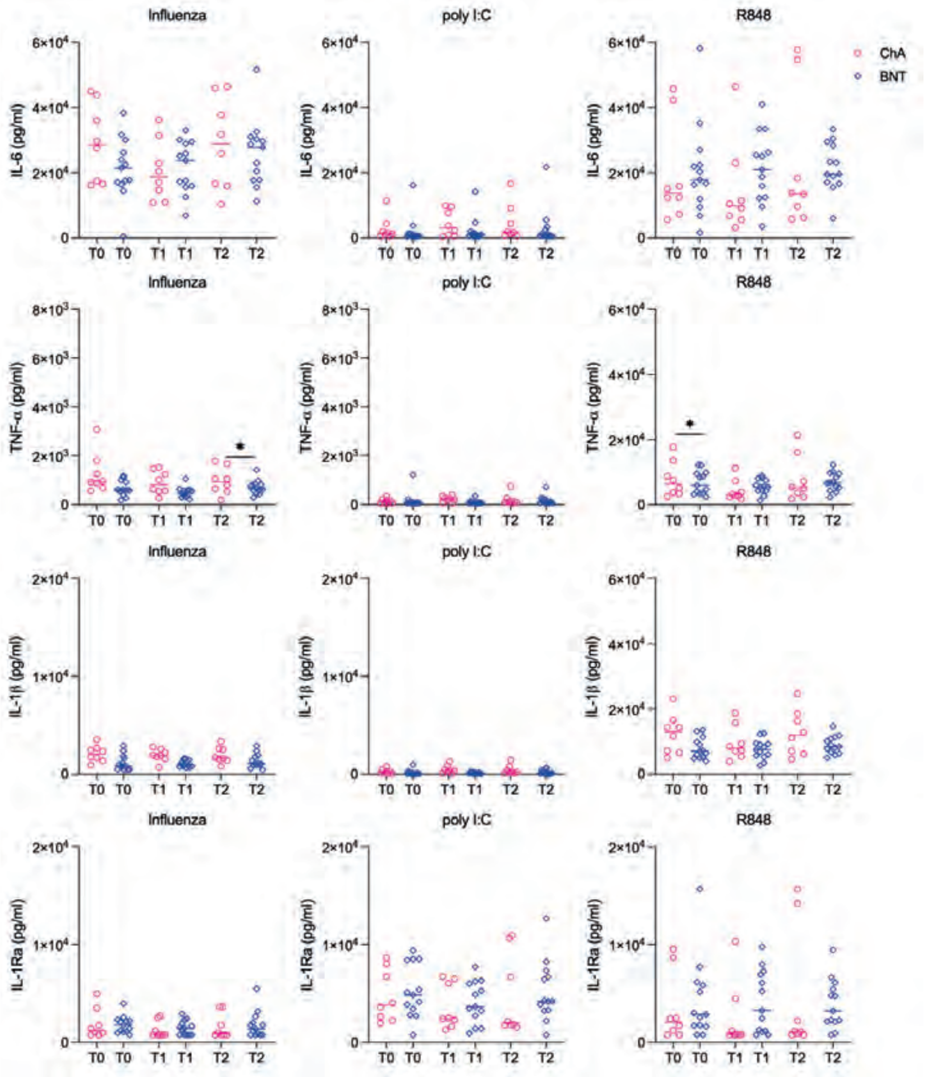
Supplementary Figure 3: The transcriptomic signature of PBMCs in response to HKCA after vaccination with ChAdOx1-S or BNT162b2



Supplementary figure 4: B cell populations within PBMCs. The flow cytometry panel shown in Supplementary Table 1 was used to detect the different cell populations through study time points (T0: study baseline; before booster, T1: 2 weeks after booster, T2: 6 weeks after booster). cDCs: classical dendritic cells; pDCs: plasmacytoid dendritic cells; NK: natural killer cells. Kruskall-Wallis test is used to compare the vaccine groups at each time point and Friedman test to compare changes over time within the primary vaccination group, both followed by Dunn's. ** $p<0.01$; $p<0.05$.



Supplementary Figure 5: Pro-inflammatory and anti-inflammatory cytokine production at baseline in response to non-specific stimuli (heat-killed Influenza, poly I:C and R848)



Supplementary Figure 6: The effect of a BNT162b2 booster on pro-inflammatory and anti-inflammatory cytokine production in response to non-specific stimuli. IL-6, IL-1β, and IL-1Ra production by PBMCs was measured by ELISA following 24 hours *ex vivo* stimulation with heat-killed Influenza, poly I:C and R848 (T0: study baseline; before booster, T1: 2 weeks after booster, T2: 6 weeks after booster). Friedman test or Kruskal-Wallis, as appropriate, is used to compare the cytokine values at different time points within the primary vaccination group, followed by Dunn's. * $p<0.05$.

References

1. Wise J. Covid-19: WHO declares end of global health emergency. BMJ. 2023;381:p1041.
2. Watson OJ, Barnsley G, Toor J, Hogan AB, Winskill P, Ghani AC. Global impact of the first year of COVID-19 vaccination: a mathematical modelling study. The Lancet Infectious Diseases. 2022;22(9):1293-302.
3. Mueller AL, McNamara MS, Sinclair DA. Why does COVID-19 disproportionately affect older people? Aging (Albany NY). 2020;12(10):9959-81.
4. Dagan N, Barda N, Kepten E, Miron O, Perchik S, Katz MA, et al. BNT162b2 mRNA Covid-19 Vaccine in a Nationwide Mass Vaccination Setting. N Engl J Med. 2021;384(15):1412-23.
5. Madhi SA, Baillie V, Cutland CL, Voysey M, Koen AL, Fairlie L, et al. Efficacy of the ChAdOx1 nCoV-19 Covid-19 vaccine against the B. 1.351 variant. New England Journal of Medicine. 2021;384(20):1885-98.
6. Higgins JP, Soares-Weiser K, López-López JA, Kakourou A, Chaplin K, Christensen H, et al. Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review. Bmj. 2016;355:i5170.
7. Aaby P, Roth A, Ravn H, Napirna BM, Rodrigues A, Lisse IM, et al. Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? J Infect Dis. 2011;204(2):245-52.
8. Wardhana DE, Sultana A, Mandang V, Jim E. The efficacy of Bacillus Calmette-Guerin vaccinations for the prevention of acute upper respiratory tract infection in the elderly. Acta Med Indones. 2011;43(3):185-90.
9. Benn CS, Schaltz-Buchholzer F, Nielsen S, Netea MG, Aaby P. Randomized clinical trials of COVID-19 vaccines: Do adenovirus-vector vaccines have beneficial non-specific effects? iScience. 2023;26(5):106733.
10. Dulfer EA, Geckin B, Taks EJ, GeurtsvanKessel CH, Dijkstra H, van Emst L, et al. Timing and sequence of vaccination against COVID-19 and influenza (TACTIC): a single-blind, placebo-controlled randomized clinical trial. The Lancet Regional Health–Europe. 2023;29.
11. Grund S, Adams O, Wählisch S, Schweiger B. Comparison of hemagglutination inhibition assay, an ELISA-based micro-neutralization assay and colorimetric microneutralization assay to detect antibody responses to vaccination against influenza A H1N1 2009 virus. Journal of virological methods. 2011;171(2):369-73.
12. Bekkering S, Blok BA, Joosten LA, Riksen NP, van Crevel R, Netea MG. In vitro experimental model of trained innate immunity in human primary monocytes. Clinical and Vaccine Immunology. 2016;23(12):926-33.
13. Ewels PA, Peltzer A, Fillinger S, Patel H, Alneberg J, Wilm A, et al. The nf-core framework for community-curated bioinformatics pipelines. Nat Biotechnol. 2020;38(3):276-8.
14. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.
15. Korotkevich G, Sukhov V, Budin N, Shpak B, Artyomov MN, Sergushichev A. Fast gene set enrichment analysis. bioRxiv. 2021:060012.
16. Westreich D, Cole SR. Invited Commentary: Positivity in Practice. American Journal of Epidemiology. 2010;171(6):674-7.

17. Vuchkovska A, Glanville DG, Scurti GM, Nishimura Michael I, White P, Ulijasz AT, Iwashima M. Siglec-5 is an inhibitory immune checkpoint molecule for human T cells. Immunology. 2022;166(2):238-48.
18. Ryan FJ, Norton TS, McCafferty C, Blake SJ, Stevens NE, James J, et al. A systems immunology study comparing innate and adaptive immune responses in adults to COVID-19 mRNA and adenovirus vectored vaccines. Cell Reports Medicine. 2023;4(3).
19. Murch SH. Common determinants of severe Covid-19 infection are explicable by SARS-CoV-2 secreted glycoprotein interaction with the CD33-related Siglecs, Siglec-3 and Siglec-5/14. Med Hypotheses. 2020;144:110168.
20. Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, et al. Global quantification of mammalian gene expression control. Nature. 2011;473(7347):337-42.
21. Liu Y, Sánchez-Ovando S, Carolan L, Dowson L, Khvorov A, Jessica Hadiprodjo A, et al. Superior immunogenicity of mRNA over adenoviral vectored COVID-19 vaccines reflects B cell dynamics independent of anti-vector immunity: Implications for future pandemic vaccines. Vaccine. 2023;41(48):7192-200.
22. Ukey R, Bruiners N, Mishra H, Mishra PK, McCloskey D, Onyuka A, et al. Dichotomy between the humoral and cellular responses elicited by mRNA and adenoviral vector vaccines against SARS-CoV-2. BMC medicine. 2022;20(1):32.
23. Jiang M, Väisänen E, Kolehmainen P, Huttunen M, Ylä-Herttuala S, Meri S, et al. COVID-19 adenovirus vector vaccine induces higher interferon and pro-inflammatory responses than mRNA vaccines in human PBMCs, macrophages and moDCs. Vaccine. 2023;41(26):3813-23.
24. Murphy DM, Cox DJ, Connolly SA, Breen EP, Brugman AA, Phelan JJ, et al. Trained immunity is induced in humans after immunization with an adenoviral vector COVID-19 vaccine. J Clin Invest. 2023;133(2).
25. Stevens NE, Ryan FJ, Messina NL, Blake SJ, Norton TS, Germano S, et al. No evidence of durable trained immunity after two doses of adenovirus-vectored or mRNA COVID-19 vaccines. The Journal of Clinical Investigation. 2023;133(17).
26. Antunes M, Sugiyama FHC, Gravina HD, Castro RC, Mercado FJR, de Lima JO, et al. COVID-19 inactivated and non-replicating viral vector vaccines induce regulatory training phenotype in human monocytes under epigenetic control. Front Cell Infect Microbiol. 2023;13:1200789.
27. WHO. Weekly Epidemiological Record (WER). 2017 02-06.
28. Giannotta G, Murrone A, Giannotta N. COVID-19 mRNA vaccines: the molecular basis of some adverse events. Vaccines. 2023;11(4):747.

Chapter 5

Non-specific effects of influenza vaccination in Brazil: clinical trial results and lessons for pandemic periods

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Submitted

Abstract

Background Before the availability of a specific COVID-19 vaccine, vaccines with non-specific effects had been proposed to potentially offer partial protection against SARS-CoV-2 through the induction of trained immunity. Quadrivalent influenza vaccines have shown to induce trained immunity, making them possible candidates as 'bridging vaccines', especially in low-resource settings with a high burden of disease but poor access to specific vaccines. However, conducting a clinical trial amidst a pandemic proves challenging and existing limitations on research infrastructure potentially further preclude common research processes.

Methods We conducted a semi-randomized, controlled trial using the Influvac Tetra vaccine and a placebo between June and December 2021 in the region of Goiás, Brazil. The primary endpoint was a microbiologically confirmed SARS-CoV-2 infection at three and six months after randomization. Secondary endpoints included incidence of other infections and infection-related hospitalisations. In addition, we evaluated challenges and lessons learnt regarding the performance of clinical trials in rapidly changing circumstances in low-resource settings.

Results 638 individuals were included, with 374 (59%) receiving an influenza vaccine. Loss to follow-up was significant (>50%) and 97% of the trial population had received a specific COVID-19 vaccine at three months after randomization. Approximately 20% of the participants developed COVID-19, with cases evenly distributed between the two groups. Main challenges were recruitment and follow-up of participants, adjusting to rapidly changing circumstances and ensuring high-quality data collection.

Conclusions This trial, performed in the midst of the COVID-19 pandemic, was unable to demonstrate that Influvac Tetra provided protection against COVID-19, severe COVID-19, or other respiratory infections. Performing a randomised clinical trial during a pandemic in low-resource settings presents major challenges, with the current study providing valuable insights for researchers working in similar circumstances.

Trial registry this trial is registered under NCT05401448 on clinicaltrials.gov

Keywords
SARS-CoV-2 pandemic; Clinical trial; Vaccine; COVID-19

Introduction

The SARS-CoV-2 virus has greatly influenced health care systems and daily life globally. Since the outbreak in 2019, it is estimated that seven millions of people have died because of COVID-19 (1). One of the countries most affected was Brazil, with one of the highest transmission rates, more than 700,000 COVID-related deaths, and overwhelmed health care facilities (2). The introduction of specific COVID-19 vaccines alleviated this situation, but the vaccines were not immediately available to the entire Brazilian population and especially not in an equal manner across different regions.

In the absence of a specific vaccine, various trials have been set up to study alternative ways to protect individuals from this severe infection. Much attention has been given to existing vaccines that are known for their heterologous, or non-specific, effects (NSEs) (3, 4). Described initially for the Bacillus Calmette-Guérin vaccine (BCG) against tuberculosis, there is growing evidence that several live-attenuated vaccines can offer protection beyond their target antigens (5, 6). Observational studies and clinical trials in neonates have shown that BCG vaccination decreased mortality rates by reducing non-tuberculous fatal respiratory infections and sepsis (7-10). In trials in an older population, BCG significantly lowered the rate of non-related respiratory tract infections (11, 12). Similar effects have been described for vaccines against measles, influenza, polio and several other diseases (13). During the pandemic, the risk of SARS-CoV-2 infection in Dutch health care workers was reported to be lower for those who had received an influenza vaccine in the previous flu season (14) and similar findings were reported in the US and Italy, which also showed improved clinical outcomes in COVID-19 patients (15-18).

The main mechanism behind these NSEs is proposed to be the induction of 'trained immunity', which describes the epigenetic and metabolic reprogramming of innate immune cells that results in an enhanced immune response upon reinfection with unrelated pathogens. An increasing list of vaccines has demonstrated the ability to induce trained immunity, including the quadrivalent influenza vaccines Vaxigrip Tetra and Influvac Tetra (13, 14). The hypothesis during the COVID-19 pandemic was that certain live-attenuated vaccines might serve a bridging function until specific vaccines would be available. Enhanced understanding of this concept is not only vital in regard to the COVID-19 pandemic, but also in light of pandemic preparedness and future emerging microbes as well.

In early 2021, a randomized, placebo-controlled trial was conducted to study the potential protective effects of an influenza vaccine on COVID-19 and other respiratory tract infections (RTIs). Given the difficult circumstances at that time in the pandemic, accessing necessary resources and executing a conventional intervention study proved to be challenging. We therefore not only present the results of this trial, but we also provide a critical reflection on the difficulties encountered, aiming to provide valuable lessons for improving future research methodologies in pandemic settings.

Methods

Study design

To study the potential protective effect of influenza vaccination on COVID-19, we designed a single-blind, placebo-controlled randomized clinical trial (registered under NCT05401448). This trial aimed to include 2000 adult participants in the Brazilian region of Goiás between 1 June 2021 and 31 December 2021, and to randomize them equally between the intervention group with an influenza vaccine, and the control group with a placebo vaccine. After vaccination at baseline, participants were contacted again for follow-up after three and six months.

The primary endpoint was a microbiologically confirmed SARS-CoV-2 infection by PCR or antibody test. Secondary endpoints included hospitalizations and ICU (intensive care unit) admissions for COVID-19, COVID-19 caused deaths, incidence of other RTIs, hospitalizations and ICU admissions for other RTIs, and hospitalizations for other infections.

This study was approved by the ethical committee of the Faculty of Military Police in Goiânia, Goiás, Brazil and registered under number 5.499.397.

Participants and randomization

This trial intended to include the adult military personnel and their relatives in the Military Police Hospital in Goiânia, Goiás, since there was a well-established routine health care system in place for a large community and invitation for study participation through e-mails and during regular consultations was considered feasible. Exclusion criteria were immunocompromised status, pregnancy, any known allergy to the influenza vaccine or its components, symptoms of an active infection, a history of documented COVID-19 diagnosis, or history of a specific COVID-19 vaccine. Importantly, as the availability of the specific vaccines improved faster than expected over the course of this study, this last exclusion criterium was changed to ‘vaccination with specific any COVID-19 vaccine in the last four weeks’ for new inclusions from the end of July.

The participants were randomized using a’ 1:1 even/odd method with printed forms where even study IDs received an influenza vaccine an uneven IDs a placebo. Since influenza vaccines are freely available in Brazil to all individuals older than 6 months of age and many people made us of this, we abandoned the randomization method at September 2021 and made use of the governmentally administered influenza vaccines: individuals who got this influenza vaccine were automatically included in the influenza-arm of this trial (thus reducing the blinding of participants), and participants without this vaccine would be placed in the placebo group. Similar to prior years, there was a large uptake of influenza vaccines in the general population to be protected against influenza infections.

Intervention

Influvac Tetra was employed as a tetravalent influenza vaccine and used in accordance with the manufacturer’s instructions (a single dose of 0.5ml reconstituted vaccine). The placebo vaccine consisted of 0.5ml 0.9% NaCl. Both were administered intramuscularly in the left upper arm.

Protocol amendments

Initially, we aimed to create three study arms to study a placebo vaccine, influenza vaccine and the measles-mumps-rubella (MMR) vaccine. For feasibility reasons, this was changed to placebo versus influenza only, since there was significant delay in the supply of MMR vaccines and their expiry date was earlier than initially anticipated.

We expanded the target population from military personnel and their families to universities in the region to increase the number of participants (Pontifícia Universidade Católica de Goiás, ethical permission registered under number 5.068.613), by announcing the study through telephone groups, TV and radio.

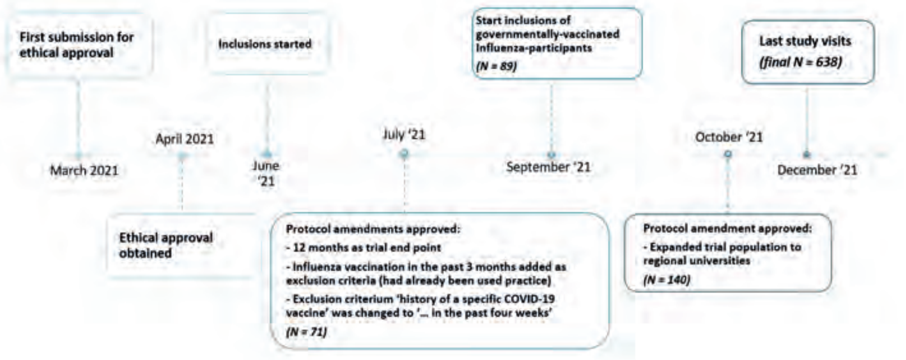

Figure 1: Modifications in study design. In the first protocol amendments, the endpoint was changed from six months to 12 months. Given the expiry dates of the vaccines, the lower number of inclusions and follow-up than anticipated, and the increasing availability of COVID-19 specific vaccines, we later chose to turn the six-months timepoint from an interim-visit into a final visit. All major changes to the study design have been summarized in Figure 1.

Data collection

During the baseline study visit, demographical and clinical data were collected (see *Supplementary Methods 1: Model eCRF*). The participants' responses were entered directly in the eCRF in CastorEDC or noted on paper before transferring the data to the eCRF. During the two follow-up time points, the research team called the participants to obtain data about infections, hospital admissions, adverse events and serious adverse events. If participants could not be reached for the follow-up after three phone calls, a reminder through a text message was sent by a team member. Reasons for loss to follow-up were recorded when possible.

Statistical analysis

Baseline and outcome variables were compared between participants in both study groups and tested using χ^2 tests or t-tests for independent samples as appropriate, in a two-sided manner. A p-value of <0.05 was considered statistically significant. When displaying percentages, missing values were excluded from the denominator. Given to only partial randomization, we chose do display p-values in our baseline demographics to enhance transparency. All analyses were performed using IBM SPSS Statistics for Windows, version 26.

Critical reflection on challenges encountered

After the study period, the research team evaluated the process and results from the trial. Colleagues from collaborating institutes that were not directly involved in this study but had experience with conducting large vaccination trials joined these discussions as well. We performed no formal qualitative analysis of these meetings, but this evaluation served as a basis for the discussion section of this paper.

Results

Trial outcomes

Between 1 June and 31 December 2021, we included 638 individuals: 374 received an influenza vaccine and 264 a placebo (*figure 2; table 1*). Approximately one-third of the participants in both groups were male and most participants were young adults (median age 24.0 years). Vaccination history at baseline including specific COVID-19 vaccines (more than 80%) was balanced between both groups. The majority of the participants was in good health and did not have underlying medical conditions or used any chronic medication.

At the three-month timepoint, the trial population consisted of 317 individuals (50.3% loss to follow-up). Primary reasons for participant withdrawal included time constraints hindering questionnaire response and difficulties in maintaining communication. The demographics and general health status of the population remained unchanged, but 97% of the participants in both groups had received a specific COVID-19 vaccine at this point (*supplementary table 1*). The interim analysis revealed no significant differences in incidence of COVID-19 (23.1% in the influenza group vs. 19.1% in the placebo group, $p = 0.37$), other RTIs or other infections between both trial arms (*table 2*). The most reported 'other infection' was dengue, diagnosed in 4 participants in each group (1.1% vs. 1.5%, $p = 0.72$). More than 90% of the COVID-19 cases had kept a symptom diary. The most common symptoms were headache, coughing, sore throat, fatigue and shivers. The frequency or duration of these symptoms did not differ between both groups. Antibiotics were prescribed in 39.5% (influenza group) and 48.7% (placebo group) of the COVID-19 cases. Only two participants were admitted to the hospital because of COVID-19 and neither of them stayed at the ICU. Other RTIs were reported in 23 cases, mostly accompanied by headache, coughing and other cold-like symptoms, and this was balanced between the vaccination groups.

Six months after baseline, 174 participants were still participating in the trial (72.2% loss to follow-up since randomization; 24/174 participants had not reported results at three months). We again observed no major shift in the population characteristics (*supplementary table 2*). Incidence and severity of the main endpoints was low and did not differ between both groups (*table 2*). No new participants were hospitalized.

Critical reflection on challenges encountered

Study personnel reported technical infrastructure as a major issue during this trial, as in many places there was no reliable internet connection and computers or tablets were not widely available. Commonly used methods for randomization and data collection could therefore not be used. Alternatives, such as the even-odd randomization method, were employed, but changed quite a few times over time, making it harder to keep a clear overview. Data collection itself was not always done uniformly. In addition, follow-up of participants was harder because of these technical limitations.

A second major challenge was the social-cultural context in which the research took place. Investigators described that it was difficult to find participants that were willing to potentially be randomized into a placebo-arm, as the concept of a controlled trial was not widely known. Researchers were met with suspicion, both towards clinical trials in general and in terms of vaccine hesitancy, and could not use monetary rewards to increase recruitment and adherence, as that would have fed suspicion even more. As one researcher put it, "we should do better as scientists" to educate and inform a broader public in more effective ways. Finally, given the pandemic situation, there were new developments each week to which the team had to adapt. At some point, this "got out of control" according to the researchers, and the research question no longer aligned with the situation in the field.

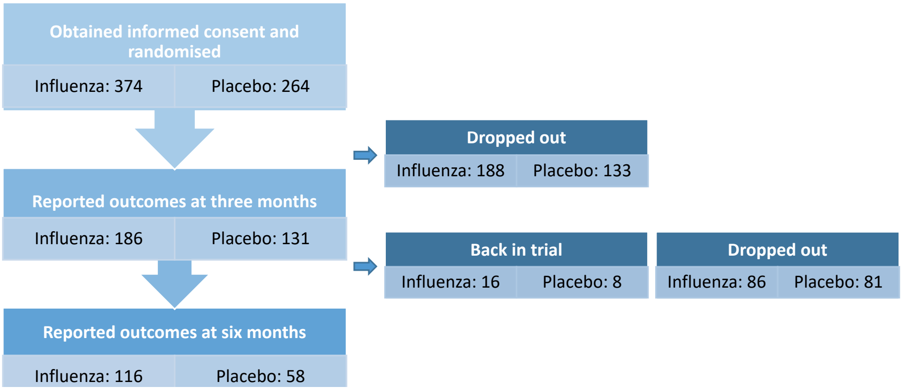

Figure 2: Inclusion flowchart

Table 1: Baseline characteristics

N=638	**Influenza vaccine (n=374)**	**Placebo (n=264)**	**p-value**
Male sex	118/374 (32%)	92/263 (35%)	0.33
Age *median (IQR)*	26.0 (18-66)	23.0 (18-50)	< 0.01
Smoking	33/370 (9%)	34/262 (13%)	0.24
Obesity	47/374 (13%)	34/264 (13%)	0.91
Ethnicity			
Pardo	138/374 (37%)	115/264 (44%)	0.09
White	174/374 (47%)	93/264 (35%)	< 0.01
Black	35/374 (9%)	38/264 (14%)	< 0.05
Latin American	10/374 (3%)	5/264 (2%)	0.52
Living condition			
With family/friends	328/374 (88%)	230/264 (87%)	0.83
Alone	42/374 (11%)	30/264 (11%)	0.96
In a community	4/374 (1%)	1/264 (0.4%)	0.33
Previous vaccinations			
Followed national programme	350/366 (96%)	253/260 (97%)	0.46
Ever BCG vaccine	362/366 (99%)	258/259 (99.6%)	0.78
BCG scar	319/360 (89%)	230/255 (91%)	0.63
Ever BCG booster	16/356 (4%)	14/282 (5%)	0.50
Hepatitis a/b	59/374 (16%)	37/264 (14%)	0.54
Yellow fever	55/374 (15%)	35/264 (13%)	0.61
Chickenpox	17/374 (5%)	16/264 (6%)	0.40
Rabies	15/374 (4%)	12/264 (5%)	0.74
Triple viral vaccine	10/374 (3%)	12/264 (5%)	0.20
Specific COVID-19 vaccine before inclusion	329/374 (88%)	219/264 (83%)	0.07
Perceived health status			
Very fit	126/374 (34%)	98/264 (37%)	0.37
Fit – managing well	232/374 (62%)	152/264 (58%)	0.26
Very mild frailty – mild frailty	14/374 (4%)	11/264 (4%)	0.79
Moderate frailty	2/374 (0.5%)	3/264 (1%)	0.40
Chronic medication use[1]			
None	221/374 (59%)	151/264 (57%)	0.63
Oral contraception	60/374 (16%)	46/264 (17%)	0.64
Other contraceptives (incl. non-specified)	9/374 (2%)	8/264 (3%)	0.63

[1] Due to small numbers, variable ‘chronic medication’ is presented per group, where ‘psychiatric medication’ consists of anxiolytics, anti-depressants, anti-psychotics and ADHD medication. ‘Cardiovascular medication’ consists of anti-hypertensives, ACE-inhibitors, anti-coagulants, statins and anti-diabetics.

Table 1: Continued

N=638	Influenza vaccine (n=374)	Placebo (n=264)	p-value
Psychiatric medication	10/374 (3%)	21/264 (8%)	< 0.01
Cardiovascular medication	22/374 (6%)	5/264 (2%)	0.01
NSAIDs	9/374 (2%)	8/264 (3%)	0.63
Corticosteroids	9/374 (2%)	5/264 (2%)	0.66
Thyroid hormones	9/374 (2%)	1/264 (0.4%)	0.04
Antibiotics (chronically)	5/374 (1%)	1/264 (0.4%)	0.22
Antibiotics >2 courses per year	61/374 (16%)	72/264 (27%)	< 0.01
Other	22/374 (6%)	28/264 (11%)	0.03
Chronic diseases			
Cardiac disorders	6/373 (2%)	2/263 (0.8%)	0.62
Hypertension	14/373 (4%)	6/262 (2%)	0.39
Asthma	10/371 (3%)	10/263 (4%)	0.70
Other pulmonary disease	5/370 (1%)	5/263 (2%)	0.84
Hematologic disease	8/373 (2%)	5/263 (2%)	0.69
Kidney disease	2/374 (0.5%)	3/262 (1%)	0.39
Liver disease	6/374 (2%)	5/264 (2%)	0.78
Asplenia	1/373 (0.3%)	2/263 (0.8%)	0.37
Neurological disease	4/371 (1%)	5/263 (2%)	0.55
Malignancy	17/373 (5%)	15/263 (6%)	0.52
Rheumatologic disease	9/370 (2%)	4/263 (2%)	0.36
Diabetes mellitus 1	5/373 (1%)	6/262 (2%)	0.37
Diabetes mellitus 2	7/373 (2%)	2/262 (0.8%)	0.24
Dyslipidaemia	24/367 (7%)	9/262 (3%)	0.11

Table 2: Outcomes at three and six months after baseline

	Influenza vaccine N=186/374 (49.7%)	Placebo N=131/264 (49.6%)	p-value
COVID-19 at 3 months	43/186 (23%)	25/131 (19%)	0.37
Symptoms			
Headache	26/39 (67%)	15/23 (65%)	0.91
Days (median)	4.5	4.9	0.25
Coughing	16/39 (41%)	13/23 (57%)	0.24
Days	4.3	4.3	0.91
Sore throat	20/39 (51%)	9/23 (39%)	0.35
Days	5.0	4.4	0.95
Other cold-like symptoms	18/39 (46%)	6/23 (26%)	0.18
Days	4.4	5.0	0.37

Table 2: Continued

	Influenza vaccine N=186/374 (49.7%)	**Placebo N=131/264 (49.6%)**	**p-value**
Cold shivers	11/39 (28%)	10/23 (45%)	0.22
Days	4.3	4.6	0.67
Fatigue	10/39 (26%)	10/23 (45%)	0.15
Days	4.2	5.0	0.10
Muscle pain	11/39 (28%)	9/23 (41%)	0.31
Days	5.4	4.8	0.46
Dyspnoea	9/39 (23%)	10/23 (44%)	0.09
Days	4.8	4.8	0.85
Fever (>38.0 *C)	10/39 (26%)	7/23 (30%)	0.91
Days	4.3	4.5	0.67
Loss of smell/taste	7/39 (18%)	6/23 (26%)	0.53
Days	5.0	5.2	0.79
Diarrhoea	4/39 (10%)	4/23 (17%)	0.45
Days	4.7	-	-
COVID-related hospital admissions	1/38 (3%)	1/18 (6%)	0.54
Medication given			
Any type of medication	17/43 (39.5%)	11/23 (48%)	0.52
Antibiotics	11/43 (25.6%)	10/23 (44%)	0.14
NSAIDs	2/43 (4.7%)	3/23 (13%)	0.33
Corticosteroids	2/43 (5%)	1/23 (4%)	1.00
Anti-viral drugs	0 (0.0%)	2/23 (9%)	0.12
Anticoagulants	0 (0.0%)	2/23 (9%)	0.12
Other	5/43 (12%)	2/23 (9%)	0.71
Other RTIs at 3 months	17/186 (9%)	6/131 (5%)	0.12
RTI-related hospital admissions	0 (0.0%)	0 (0.0%)	-
Other infections at 3 months	10/186 (5%)	7/131 (5%)	0.99
Dengue	4/186 (2%)	4/131 (3%)	0.72
	Influenza vaccine N=116/374 (31%)	**Placebo N=58/264 (22%)**	**p-value**
COVID-19 at 6 months	17/116 (15%)	12/58 (21%)	0.31
Medication given			
Any type of medication	1/17 (6.3%)	1/12 (8%)	1.00
Corticosteroids	1/17 (6.3%)	0 (0.0%)	1.00
COVID-related hospital admissions	0 (0.0%)	0 (0.0%)	-
Other RTIs at 6 months	2/116 (2%)	2/58 (3%)	0.60
RTI-related hospital admissions	0 (0.0%)	0 (0.0%)	-
Other infections at 6 months	4/116 (3%)	0 (0.0%)	0.30

Supplementary table 1: Study population characteristics at three months after baseline

N=317	**Influenza vaccine** **N=186/374 (49.7%)**	**Placebo** **N=131/264 (49.6%)**	**p-value**
Male sex	68/186 (37%)	43/130 (33%)	0.40
Age *median (IQR)*	25.0 (18-64)	23.5 (18-50)	0.52
Smoking	15/185 (8%)	18/130 (14%)	0.25
Obesity	21/186 (11%)	15/131 (11%)	0.96
Ethnicity			
Pardo	69/186 (37%)	59/131 (45%)	0.16
White	89/186 (48%)	40/131 (31%)	< 0.01
Black	16/186 (9%)	17/131 (13%)	0.21
Latin American	6/186 (3%)	5/131 (4%)	0.78
Living condition			
With family/friends	162/186 (87%)	116/131 (89%)	0.70
Alone	21/186 (11%)	13/131 (10%)	0.70
In a community	3/186 (2%)	1/131 (0.8%)	0.51
Previous vaccinations			
Followed national programme	175/184 (95%)	126/130 (97%)	0.70
Ever BCG vaccine	182/183 (99%)	128/128 (98%)	0.68
Hepatitis a/b	35/186 (19%)	21/131 (16%)	0.52
Yellow fever	31/186 (17%)	21/131 (16%)	0.88
Chickenpox	14/186 (8%)	10/131 (8%)	0.97
Rabies	11/186 (6%)	7/131 (5%)	0.83
Triple viral vaccine	8/186 (4%)	8/131 (6%)	0.47
Specific COVID-19 vacc <3m	116/156 (74%)	86/109 (79%)	0.54
Specific COVID-19 vaccine received before baseline	153/186 (82%)	100/131 (76%)	0.20
Total participants with specific COVID-19 vaccine at 3 months	181/186 (97%)	128/131 (98%)	0.82
Perceived health status			
Very fit	70/186 (38%)	44/131 (34%)	0.46
Moderate frailty	0 (0.0%)	1/131 (0.8%)	0.23
Chronic medication use			
None	110/186 (59%)	77/131 (59%)	0.95

Supplementary table 2: Study population characteristics at six months after baseline

N=174	Influenza vaccine N=116/374 (31%)	Placebo N=58/264 (22%)	p-value
Male sex	39/116 (34%)	16/58 (28%)	0.42
Age *median (IQR)*	25 (18-64)	21 (18-40)	0.03
Smoking	10/116 (9%)	8/58 (14%)	0.29
Obesity	14/116 (12%)	4/58 (7%)	0.29
Ethnicity			
Pardo	43/116 (37%)	22/58 (38%)	0.91
White	58/116 (50%)	20/58 (34%)	0.05
Black	11/116 (9%)	4/58 (7%)	0.57
Latin American	3/116 (3%)	3/58 (5%)	0.38
Living condition			
With family/friends	104/116 (90%)	47/58 (81%)	0.11
Alone	11/116 (10%)	10/58 (17%)	0.14
In a community	1/116 (0.9%)	1/58 (2%)	0.62
Previous vaccinations			
Followed national programme	110/116 (95%)	56/58 (97%)	0.61
Ever BCG vaccine	114/116 (98%)	57/58 (98%)	1.00
Hepatitis a/b	17/116 (15%)	8/58 (14%)	0.88
Yellow fever	17/116 (15%)	5/58 (9%)	0.26
Chickenpox	7/116 (6%)	1/58 (2%)	0.20
Rabies	8/116 (7%)	0 (0.0%)	0.04
Triple viral vaccine	3/116 (3%)	0 (0.0%)	0.22
Specific COVID-19 vacc <3m	57/116 (49%)	31/58 (53%)	0.55
Specific COVID-19 vaccine received before baseline	102/116 (88%)	54/58 (93%)	0.29
Total participants with specific COVID-19 vaccine at 6 months	114/116 (98%)	57/58 (98%)	1.00
Perceived health status			
Very fit	43/116 (37%)	19/58 (33%)	0.58
Moderate frailty	1/116 (0.9%)	1/58 (12%)	0.62
Chronic medication use			
None	67/116 (58%)	28/58 (48%)	0.24

Discussion

Trial results

In this paper, we presented the results of our trial investigating the protective effect of influenza vaccination on COVID-19 and other RTIs. Due to the pandemic circumstances and the intercurrent availability of the novel COVID-19 specific vaccines, this resulted in only partial randomization and large loss to follow-up, as well as a high percentage of COVID-specific vaccines during the trial, making it impossible to draw conclusions on the influence of influenza vaccine on COVID-19 incidence and severity. We did not show any additional beneficial effects of influenza vaccination in this trial.

While influenza vaccines are theoretically valuable candidates for boosting the immune system and providing heterologous protection (14), retrospective studies during the COVID-19 pandemic were subject to selection bias and confounders (15, 19, 20). Interestingly, having received an influenza vaccine a few months prior to COVID-infection seemed to decrease certain adverse clinical outcomes in COVID-19 patients, although these studies were retrospective in design as well (18, 21). Randomised trials with this intervention could not be identified in published work or trial registries. With regards to BCG, large studies from different countries did not find a protective effect of BCG vaccination on COVID-19 incidence either (22-24), although a potential beneficial effect on mortality has been suggested by summary statistics of all BCG trials performed (25).

The number of events in this study was low, which is not unexpected given the healthy and relatively young population included. It is conceivable that a possible protective effect might have been more pronounced in a more vulnerable population. A remarkable finding however was the percentage of COVID-19 cases treated with antibiotics. As reported previously in the Netherlands, empiric antibiotic use was abundant in the beginning of the pandemic, even though bacterial co-infections were rare (26). The authors suggested this could be explained by uncertainty avoidance, the fear of missing a possible developing bacterial co-infection, and calculating behaviour to avoid overloading an already crowded ICU. In this Brazilian trial, existing patterns of antibiotic decision-making could have played a role as well.

Challenges encountered

Most importantly, this trial serves as an example to show how challenging it can be to conduct a clinical trial in the difficult circumstances of a pandemic. With the aim to draw useful lessons for the future, we decided to use our findings to help improve future similar endeavours and avoid pitfalls. Most COVID-19 trials did not publish any results (27) and the reasons for this should be carefully examined.

The first main difficulty in this trial was to recruit and retain enough participants to reach a relevant sample size. This was caused by general vaccine hesitancy, a public that was not familiar with the concept of a placebo-arm and the lack of an effective incentive to participate. Secondly, the circumstances under which the trial had to be carried out where continuously changing. Despite strenuous efforts of the research team, developments unfolded rapidly and it was not always possible to adapt the study to these changing circumstances. After abandoning formal randomization and employing government-administered influenza vaccines, the study groups became too unbalanced to effectively address the research question. Thirdly, data collection and analysis in this trial was made more difficult by a suboptimal infrastructure. The eCRFs in Castor were set-up from scratch and the team lacked time to extensively test the structure. As a result, some questions and answer options were multi-interpretable and not fool-proof; subsequent questions did not always align with the given answer, making it harder to interpret the generated data.

Previous publications have focused on lessons learned during the COVID-19 pandemic, but were not directly related to trial examples and usually not specific to low- and middle-income countries. Nomali et al. emphasized the benefits of electronic technologies to aid recruitment, data collection and staff consultation (28), but initiatives like e-consent, video calls and 'online visits' need the proper facilities to successfully employ them. Effective strategies in high-income settings should often be adapted (29).

The following section of this Discussion will focus on current 'best practices' that could be translated to this situation, and are summarized in *table 4* as potential solutions for similar circumstances.

Sufficient recruitment and ensuring follow-up

Although the problem of vaccine hesitancy is beyond the scope of this paper, many authors have recently published relevant articles on how to deal with this in vaccination campaigns and in research settings (30-32). Considering the perceived problem with placebo-controlled trials, the possibility of unfavourable randomisation has indeed been identified as a factor in loss of participation, especially in combination with a lack of understanding of the reasons why a trial is being conducted (33). A change in randomisation ratio from 1:1 to smaller placebo-groups might therefore have been considered. Response-adaptive randomization, used to reduce deleterious clinical outcomes and overall sample size without meaningful loss of statistical precision, could be beneficial to optimize the necessary numbers needed to allocate to each group and avoid large numbers of those allocated to placebos (34). Being more "transparent", by making the trial open-label, has resulted in higher recruitment and retention rates in other trials but of course comes with its inherent disadvantages (35). Finally, even small monetary rewards have been found to result in significantly improved response and consent rates in Europe and the US (36); however, this was not suitable for the situation in Brazil. Providing airtime or data to lower-resource communities also did not increase participation in prior research, as the main obstacles hindering participation in these communities were a lack of hardware and privacy, which were not resolved (29).

Overall, additional recruitment strategies could have been considered to increase awareness of the trial, e.g. by using social media or matching initiatives with centralized volunteer registration. Targeted advertisements on Facebook and affiliated social media platforms have shown to improve the respondents from specific groups markedly. Importantly, associated advertisement costs were low in a study by Ali et al. (18 cents per full response), making it a conceivable option (37). Although this was a survey study, it might be a useful tool for assessing eligibility and potentially obtaining baseline information in trials as well. Ultimately, centralized volunteer registration could be useful in the future. As an example, VACCELERATE is a single-entry point for Europeans interested in participating in a COVID-19 clinical trial, which matches them to suitable trials (38). It currently operates in 12 countries and planned to be rolled-out in five more. To our knowledge, such an initiative does not exist yet in the region of Latin America.

Finally, this trial encountered the issue of representativeness (39): the ones studied were not necessarily the ones needing protection the most. Since this also meant there was very little clinical care already in place surrounding our participants, choosing a different study population might have solved two problems at the same time: easier to study since people needed medical care anyways (27), and they might have benefitted more clearly from additional interventions.

Rapidly responding to changing circumstances

Having a strong management team with clear responsibilities and daily meetings, albeit in the form of written updates, is essential to be able to timely identify and address new challenges (40). Some developments can be expected beforehand, such as the rollout of new vaccines, even if their timing is still unclear. Mapping potential developments and obstacles at the start of a trial can aid timely strategies and potentially getting ethical approval for 'in case'-scenarios beforehand (29). It can be unavoidable to change inclusion criteria halfway through the trial, in which case it is important to carefully consider if the research question can still be answered or should be adapted as well. In this regard, having a more adaptive study design could be useful. For example, more trial arms with different subpopulations could then be created during the study.

Enabling of proper data collection and analysis

A standardised manner of data collection could have facilitated faster set-up of data infrastructure and eCRFs, with endpoints comparable to other studies. For example, the forms suggested by the WHO would have needed to be adapted to the specific situation and study (41). In addition, standardised training would have been useful for the many students and researchers from non-trial backgrounds undertook great effort to perform this trial. Recorded training videos or written manuals could have helped in training many people at the same time and in a standardized manner, avoiding inconsistencies in e.g. multi-interpretable outcome questions (40). Direct means of contact between the team in the field and supervisors, e.g. via a WhatsApp group, should be implemented when possible (29). One aspect that hindered proper supervision in this trial was the collaboration between a team in Brazil that was relatively inexperienced with conducting large clinical trials, and a team in the Netherlands whose expertise was valuable but not always real-time available. A larger collaborative structure involving more local expertise could have been considered, as well as sharing resources and facilities with other parties (27, 28). Larger trials are more likely to report useful results, with the RECOVERY platform (42) study as major example. In addition, as many research projects were put on hold during the pandemic, it is advisable to carefully consider redistribution of tasks to research staff (28, 40).

To conclude, it is important to point out certain aspects of the trial that went smoothly: fast-tracked ethical approval through direct contact with the ethical board, involvement students to carry out specific research tasks, and the use of the ecological circumstances created by the pandemic that provided a context for assessing the impact of the vaccine in a way that a controlled environment might not have. The widespread prevalence of the virus, varying levels of natural immunity and ongoing public health responses have made this trial challenging, but also provided an opportunity to gain additional insights. Those factors were crucial in creating an opportunity for science during this pandemic. However, the results this trial generated could have been more robust. As the study progressed towards an increasing number of COVID-vaccinated participants and non-randomized groups, we decided prolonged study efforts would not yield more useful results and the trial was stopped after six months. Premature study termination to sacrifice statistical power for more interpretable interference, can be an acceptable option that could have been considered earlier in the process (43).

By extensively reflecting on this process, we hope to achieve two aims: help avoid future pitfalls to create better research for better care, but also to invite other scientists integrate critical reflection in their research endeavours.

Table 4: Encountered trial challenges and their associated best practices.

Sufficient recruitment and ensuring follow-up
Address vaccine hesitancy and health literacy on societal level
Consider adaptive trial designs
Use novel recruitment strategies
Be creative with incentives
Integration into regular clinical care
Rapidly responding to changing circumstances
Strong management team and daily communication
Expect the to-be-expected and plan ahead
Consider adaptive trial designs
Constantly monitor study progress and whether methods still align with the research question
Enabling of proper data collection and analysis
Standardised data collection
Training of personnel
Involve more expertise and resources in collaborative structures

Authors' contributions

LJ, MN, PH and JM conceptualized and designed the study. CG, TC, SC and PA performed the study, with assistance from JS. Data-analysis was done by ED and JM, who also wrote the first draft together. JS, MN, PA and JM had supervisory roles.

References

1. WHO. WHO Coronavirus (COVID-19) dashboard > Deaths [Available from: https://data.who.int/dashboards/covid19/deaths.
2. Mellan T, Hoeltgebaum H, Mishra S, Whittaker C, Schnekenberg R, Gandy A, et al. Estimating COVID-19 cases and reproduction number in Brazil. Imperial College London; 2020 08-05-2020.
3. Chumakov K, Avidan MS, Benn CS, Bertozzi SM, Blatt L, Chang AY, et al. Old vaccines for new infections: Exploiting innate immunity to control COVID-19 and prevent future pandemics. Proceedings of the National Academy of Sciences. 2021;118(21):e2101718118.
4. O'Neill LAJ, Netea MG. BCG-induced trained immunity: can it offer protection against COVID-19? Nature Reviews Immunology. 2020;20(6):335-7.
5. Higgins JPT, Soares-Weiser K, López-López JA, Kakourou A, Chaplin K, Christensen H, et al. Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review. BMJ. 2016;355:i5170.
6. Benn CS, Netea MG, Selin LK, Aaby P. A small jab – a big effect: nonspecific immunomodulation by vaccines. Trends in Immunology. 2013;34(9):431-9.
7. Garly M-L, Martins CL, Balé C, Baldé MA, Hedegaard KL, Gustafson P, et al. BCG scar and positive tuberculin reaction associated with reduced child mortality in West Africa: a non-specific beneficial effect of BCG? Vaccine. 2003;21(21-22):2782-90.
8. Stensballe LG, Ravn H, Birk NM, Kjærgaard J, Nissen TN, Pihl GT, et al. BCG Vaccination at Birth and Rate of Hospitalization for Infection Until 15 Months of Age in Danish Children: A Randomized Clinical Multicenter Trial. J Pediatric Infect Dis Soc. 2019;8(3):213-20.
9. Aaby P, Roth A, Ravn H, Napirna BM, Rodrigues A, Lisse IM, et al. Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? J Infect Dis. 2011;204(2):245-52.
10. Storgaard L, Rodrigues A, Martins C, Nielsen BU, Ravn H, Benn CS, et al. Development of BCG Scar and Subsequent Morbidity and Mortality in Rural Guinea-Bissau. Clin Infect Dis. 2015;61(6):950-9.
11. Wardhana DE, Sultana A, Mandang V, Jim E. The efficacy of Bacillus Calmette-Guerin vaccinations for the prevention of acute upper respiratory tract infection in the elderly. Acta Med Indones. 2011;43(3):185-90.
12. Giamarellos-Bourboulis EJ, Tsilika M, Moorlag S, Antonakos N, Kotsaki A, Domínguez-Andrés J, et al. Activate: Randomized Clinical Trial of BCG Vaccination against Infection in the Elderly. Cell. 2020;183(2):315-23.e9.
13. Baydemir I, Dulfer EA, Netea MG, Domínguez-Andrés J. Trained immunity-inducing vaccines: Harnessing innate memory for vaccine design and delivery. Clin Immunol. 2024;261:109930.
14. Debisarun PA, Gössling KL, Bulut O, Kilic G, Zoodsma M, Liu Z, et al. Induction of trained immunity by influenza vaccination - impact on COVID-19. PLOS Pathogens. 2021;17(10):e1009928.
15. Conlon A, Ashur C, Washer L, Eagle KA, Hofmann Bowman MA. Impact of the influenza vaccine on COVID-19 infection rates and severity. Am J Infect Control. 2021;49(6):694-700.
16. Marín-Hernández D, Schwartz RE, Nixon DF. Epidemiological evidence for association between higher influenza vaccine uptake in the elderly and lower COVID-19 deaths in Italy. J Med Virol. 2021;93(1):64-5.
17. Ragni P, Marino M, Formisano D, Bisaccia E, Scaltriti S, Bedeschi E, Grilli R. Association between Exposure to Influenza Vaccination and COVID-19 Diagnosis and Outcomes. Vaccines (Basel). 2020;8(4).

18. Candelli M, Pignataro G, Torelli E, Gullì A, Nista EC, Petrucci M, et al. Effect of influenza vaccine on COVID-19 mortality: a retrospective study. Intern Emerg Med. 2021;16(7):1849-55.
19. Abbasi MH, Esmaeili S, Ansarin M, Rimaz S, Faiz SHR, Riahi T, Aghakhani K. Flu Vaccination Fails to Protect Against COVID-19 Infection; A Cross-sectional Study. Journal of Iranian Medical Council. 2022;5(1):118-24.
20. Valente PK. Potential for bias in assessing risk and protective factors for COVID-19: Commentary on Conlon et al.'s "Impact of the influenza vaccination on COVID-19 infection rates and severity". American Journal of Infection Control. 2021;49(8):1089-90.
21. Taghioff SM, Slavin BR, Holton T, Singh D. Examining the potential benefits of the influenza vaccine against SARS-CoV-2: A retrospective cohort analysis of 74,754 patients. PLoS One. 2021;16(8):e0255541.
22. Schure JAt, Alexander L, Lisa B, Christine SB, Marc JMB, Jeffrey DC, et al. Bacillus Calmette-Guérin vaccine to reduce COVID-19 infections and hospitalisations in healthcare workers – a living systematic review and prospective ALL-IN meta-analysis of individual participant data from randomised controlled trials. medRxiv. 2022:2022.12.15.22283474.
23. Koekenbier EL, Fohse K, van de Maat JS, Oosterheert JJ, van Nieuwkoop C, Hoogerwerf JJ, et al. Bacillus Calmette-Guérin vaccine for prevention of COVID-19 and other respiratory tract infections in older adults with comorbidities: a randomized controlled trial. Clinical Microbiology and Infection. 2023;29(6):781-8.
24. Pittet LF, Messina NL, Orsini F, Moore CL, Abruzzo V, Barry S, et al. Randomized Trial of BCG Vaccine to Protect against Covid-19 in Health Care Workers. N Engl J Med. 2023;388(17):1582-96.
25. Aaby P, Netea MG, Benn CS. Beneficial non-specific effects of live vaccines against COVID-19 and other unrelated infections. Lancet Infect Dis. 2023;23(1):e34-e42.
26. Karami Z, Knoop BT, Dofferhoff ASM, Blaauw MJT, Janssen NA, van Apeldoorn M, et al. Few bacterial co-infections but frequent empiric antibiotic use in the early phase of hospitalized patients with COVID-19: results from a multicentre retrospective cohort study in The Netherlands. Infect Dis (Lond). 2021;53(2):102-10.
27. Janiaud P, Hemkens LG, Ioannidis JPA. Challenges and Lessons Learned From COVID-19 Trials: Should We Be Doing Clinical Trials Differently? Can J Cardiol. 2021;37(9):1353-64.
28. Nomali M, Mehrdad N, Heidari ME, Ayati A, Yadegar A, Payab M, et al. Challenges and solutions in clinical research during the COVID-19 pandemic: A narrative review. Health Sci Rep. 2023;6(8):e1482.
29. Myers B, van der Westhuizen C, Pool M, Hornsby N, Sorsdahl KR. Responding to COVID-19 threats to trial conduct: lessons learned from a feasibility trial of a psychological intervention for South African adolescents. Trials. 2021;22(1):440.
30. Gagneux-Brunon A, Ward JK, Bonneton M, Verger P, Launay O, Botelho-Nevers E. Intention to participate in COVID-19 vaccine clinical trials in May 2021: a cross-sectional survey in the general French population. Human Vaccines & Immunotherapeutics. 2022;18(5):2072630.
31. Detoc M, Gagneux-Brunon A, Lucht F, Botelho-Nevers E. Barriers and motivations to volunteers' participation in preventive vaccine trials: a systematic review. Expert Rev Vaccines. 2017;16(5):467-77.
32. Dror AA, Eisenbach N, Taiber S, Morozov NG, Mizrachi M, Zigron A, et al. Vaccine hesitancy: the next challenge in the fight against COVID-19. European Journal of Epidemiology. 2020;35(8):775-9.
33. Free C, Hoile E, Robertson S, Knight R. Three controlled trials of interventions to increase recruitment to a randomized controlled trial of mobile phone based smoking cessation support. Clinical Trials. 2010;7(3):265-73.

34. Park JJ, Thorlund K, Mills EJ. Critical concepts in adaptive clinical trials. Clin Epidemiol. 2018;10:343-51.
35. Watson JM, Torgerson DJ. Increasing recruitment to randomised trials: a review of randomised controlled trials. BMC Medical Research Methodology. 2006;6(1):34.
36. Abdelazeem B, Abbas KS, Amin MA, El-Shahat NA, Malik B, Kalantary A, Eltobgy M. The effectiveness of incentives for research participation: A systematic review and meta-analysis of randomized controlled trials. PLOS ONE. 2022;17(4):e0267534.
37. Ali SH, Foreman J, Capasso A, Jones AM, Tozan Y, DiClemente RJ. Social media as a recruitment platform for a nationwide online survey of COVID-19 knowledge, beliefs, and practices in the United States: methodology and feasibility analysis. BMC Medical Research Methodology. 2020;20(1):116.
38. Salmanton-García J, Stewart FA, Heringer S, Koniordou M, Álvarez-Barco E, Argyropoulos CD, et al. VACCELERATE Volunteer Registry: A European study participant database to facilitate clinical trial enrolment. Vaccine. 2022;40(31):4090-7.
39. Prendki V, Tau N, Avni T, Falcone M, Huttner A, Kaiser L, et al. A systematic review assessing the under-representation of elderly adults in COVID-19 trials. BMC Geriatrics. 2020;20(1).
40. Török ME, Underwood BR, Toshner M, Waddington C, Sidhom E, Sharrocks K, et al. Challenges and opportunities for conducting a vaccine trial during the COVID-19 pandemic in the United Kingdom. Clinical Trials. 2021;18(5):615-21.
41. ISARIC-WHO. [Available from: https://isaric.org/research/covid-19-clinical-research-resources/covid-19-crf/.]
42. Effect of Hydroxychloroquine in Hospitalized Patients with Covid-19. New England Journal of Medicine. 2020;383(21):2030-40.
43. Fleming TR, Labriola D, Wittes J. Conducting Clinical Research During the COVID-19 Pandemic: Protecting Scientific Integrity. JAMA. 2020;324(1):33-4.

Chapter 6

The effect of BCG vaccination in the elderly on infectious and non-infectious immune-mediated diseases

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Abstract

Introduction Previous research has suggested beneficial heterologous effects of the Bacillus Calmette-Guérin (BCG) vaccine on non-mycobacterial infections and other immune-mediated diseases. During the COVID-19 pandemic, randomized controlled trials BCG-PRIME (n=5349) and BCG-CORONA-ELDERLY (n=1907) investigated the impact of BCG on SARS-CoV-2 infections in older individuals. We extended the follow-up in these studies by one year (BCG-Long Term study), to assess the overall effects of BCG vaccination on infectious and immune-mediated diseases in individuals aged over 60.

Methods Prior participants were invited to complete a one-year follow-up survey after their completion of the original trial. Data on vaccinations, hospital admissions, infectious episodes, and new medical diagnoses were collected and compared between BCG- and placebo-vaccinated participants. Variables of interest were combined with the previous trial databases and analysed using relative risks (RR) and an adjusted Cox regression model accounting for participation probability.

Results The response in the follow-up survey was 60%, including 4238 individuals in the final analysis (2317 had received BCG and 1921 placebo). Incidence and severity of infectious diseases and other diagnoses, including cardiovascular diseases and cancer, did not differ between the groups. The proportion of individuals hospitalized for cardiac arrhythmias after BCG was two-fold higher than reported after placebo (1.6% versus 0.8%, RR 2.0 (95% confidence interval 1.1-3.6)). Cardiac arrhythmia-related hospitalizations were primarily due to exacerbation of pre-existing arrhythmias.

Conclusion The results of the present study confirm that BCG has no relevant effect on non-mycobacterial infectious diseases and other immune-mediated diseases in a population of generally mycobacteria-naïve older Dutch individuals in the two years following vaccination. However, our study suggests that BCG may aggravate pre-existing cardiac arrhythmia, which warrants further investigation.

Keywords
BCG; Non-specific effects; Trained immunity; Geriatric medicine; Infectious diseases; Immune-mediated diseases

Introduction

The innate immune system has traditionally been perceived as a non-specific defence mechanism lacking immunological memory. However, in recent years, this concept has been challenged by research demonstrating that innate immune cells have adaptive properties after encountering an insult (1). This immune process termed "trained immunity" results in an enhanced immune response to heterologous stimuli after the initial exposure of innate immune cells to a microbial ligand or infection. Long-term reprogramming of innate immune cells through epigenetic and metabolic modifications underlies mechanistically this *de-facto* innate immunological memory (2).

One of the first agents studied in this context was the Bacillus Calmette-Guérin (BCG) vaccine, originally developed to protect against tuberculosis. Kleinnijenhuis *et al*. showed an increased pro-inflammatory cytokine response in monocytes and natural killer (NK) cells of vaccinated individuals following stimulation with unrelated pathogens (3, 4), concluding that the BCG vaccine can induce trained immunity. This may explain the growing epidemiological evidence, dating back to 1920s, that suggests a non-specific protective effect of the BCG vaccine against non-mycobacterial infections and other immune-mediated diseases (5). Similar to other live attenuated vaccines, BCG vaccination in infants has been associated with decreased all-cause mortality in childhood, independent of tuberculosis infection (6). Additionally, a reduced incidence of acute respiratory tract infections after BCG has been reported in adults (7).

By reducing systemic inflammation in older individuals (8, 9), the BCG vaccine has also been linked to a decrease in a wide range of inflammatory conditions (10), including atherosclerosis (11) and autoimmune diseases such type 1 diabetes (12). The immunomodulatory effects of the BCG vaccine have been described in the context of cancer as well, which suggests a risk reduction for the development of e.g. lung cancer and childhood leukaemia (13-15). However, the long-term effects of a BCG vaccine on non-infectious diseases in an elderly population have not been studied in a randomized approach.

When the SARS-CoV-2 virus emerged in 2019, there was no specific vaccine available to decrease the associated morbidity and mortality. Because of its heterologous protective effects, we and others hypothesized that BCG as a 'bridging'-vaccine may be able to mitigate some of the detrimental effects of COVID-19 in vulnerable populations.

Although a number of studies found a protective effect of BCG (re-)vaccination (16-19) on COVID-19 incidence, this was not the case for most larger studies conducted in different countries including the BCG-PRIME and BCG-CORONA-ELDERLY trials in the Netherlands (20-24). In these two trials, more than 7000 participants were followed in detail for up to 12 months after their BCG or placebo vaccination. Both studies reported no effect on the incidence of COVID-19 and other respiratory tract infections. Regarding the severity of COVID-19, the studies were underpowered to be able to draw any conclusions for this outcome. A prolonged study period would facilitate a more comprehensive exploration of the potential long-term effects of BCG in the elderly, including its potential to influence the incidence and severity of other diseases and infections. Notably, it would also enable further investigation of findings from the BCG-PRIME study that suggested a potential adverse effect of BCG on coronary events (21).

Therefore, the BCG-Long Term (BCG-LT) study was initiated to assess the long-term, heterologous effects of BCG vaccination in individuals from the BCG-PRIME and BCG-CORONA-ELDERLY studies.

Methods

Study design and study populations

BCG-LT is a cohort study on the long-term follow up of participants from the BCG-PRIME trial (21) and the BCG-CORONA-ELDERLY trial (22). Data from both the original trials and the BCG-LT study were combined to facilitate the long-term assessment of our outcome variables.

Both original trials were randomized, controlled, multicentre trials that included immunocompetent individuals aged over 60 with (BCG-PRIME) and without (BCG-CORONA-ELDERLY) comorbidities. From the first inclusion to the end of their follow-up, these studies spanned a period between September 2020 to June 2021 and April 2020 to May 2021, respectively (see *Figure 1*). The primary endpoint of the BCG-PRIME trial was the incidence of COVID-19 and the secondary endpoints included clinically relevant respiratory tract infections (RTIs). Conversely, cumulative incidence of RTIs was the primary endpoint for BCG-CORONA-ELDERLY trial, with COVID-19 incidence serving as a secondary endpoint. For an extensive description of the methods and results of the two primary trials, we refer to the original trial publications (21, 22). The participants were unblinded for their study vaccine after they finished their participation in the original trial.

In total, 8126 elderly individuals participated in the trials, of whom 7243 gave informed consent to be approached for future research and were alive at the end of the trial period; these were invited for the BCG-LT study. Each potential participant for the BCG-LT study was invited via e-mail to complete the survey in the Castor electronic data capture environment in June 2022, followed by a reminder to reply after one week if the survey was not filled out or incomplete. The follow-up in BCG-LT ended in July 2022. The original clinical trials were approved by the Utrecht (NCT04537663) and Arnhem-Nijmegen Medical Ethics Committee (NCT04417335), respectively, and the non-applicability of the Medical Research Involving Human Subjects Act (WMO) on the BCG-LT study design was confirmed (NCT05387655). All studies were carried out in accordance with the relevant guidelines and regulations.

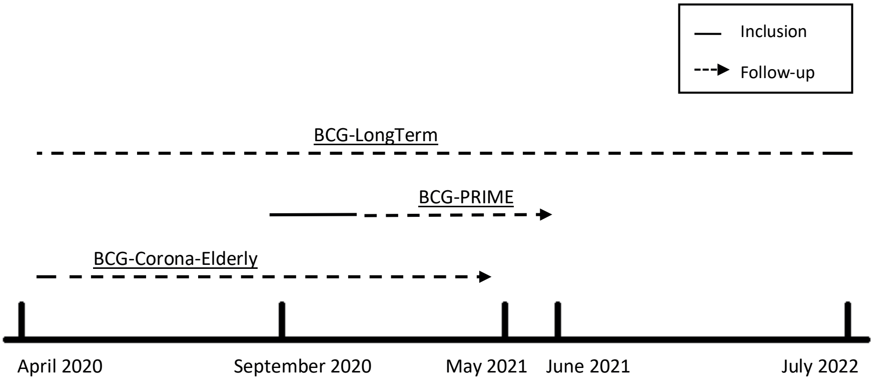

Figure 1: Scope of BCG-studies

Data collection and study variables

Baseline and early follow-up information was already available from the two original trials, including information on self-reported COVID-19 incidence and severity. The Long-Term data were collected by a questionnaire sent to consenting participants with multiple-choice questions with an open text option to specify certain answers. At the beginning of the survey participants were presented an informed consent module. When participants either never responded to the invitation or denied informed consent, they were not contacted again. The survey inquired about further vaccinations, infections (including COVID-19) and their severity, the development of other disorders, and non-elective hospital admissions (see *Supplementary Appendix 1*), that took place after participation in the original trial. Participants were repeatedly given contact details to ask for help in filling in the survey if needed.

To obtain information on participant characteristics and to consolidate events that occurred during the original study period with those that occurred during the subsequent follow-up, we combined the clinical trial databases with the newly collected data obtained by the surveys. The variable 'Prolonged COVID-19 symptoms' was defined as COVID-19 related symptoms lasting for more than 7 days. In our analyses, related conditions were grouped into overarching categories and are presented in *Supplementary Appendix 2*. Participants who reported receiving a BCG vaccine after their original trial, were excluded from the analysis (n=10).

Data cleaning

We validated the concordance of the original databases and the newly collected data during follow-up, by checking conformity of sex and height between the databases. Each completed survey was individually assessed by one of the two physician-scientists that held regular consultations. For survey replies that were ambiguous or unclear, the study team followed up with the participant by email or telephone. When participants repeatedly could not be reached for clarification, they were excluded from the study (n=104). Due to the difference in arrhythmia-related hospital admissions between the BCG and placebo groups in our BCG-LT study, we approached each of the participants with cardiac arrythmia-related hospital admission by telephone for verification. This was used to confirm whether the primary reason for admission was indeed cardiac arrhythmia, whether the admission was unplanned, and whether they had experienced arrhythmias before receiving the BCG or placebo vaccine.

Osteopontin measurements

Given our findings on cardiac arrhythmia-related hospital admissions and a recent publication showing that matricellular protein osteopontin is upregulated in patients with atrial fibrillation (25), we measured osteopontin in a subgroup of BCG-PRIME participants. Osteopontin is an antimicrobial cytokine and is induced by BCG (26). EDTA plasma was collected just before and 6 months after BCG and placebo vaccination. At the time of the BCG-PRIME study, the selection of the subgroup was based on two criteria: proximity to the laboratory and whether participants had either suffered a recent COVID-19 episode themselves or lived with someone who had. Consequently, the number of available blood samples was not equally distributed between the groups (64 BCG-vaccinated and 40 placebo-participants). However, age, sex and BMI were balanced across both groups. There were no plasma samples available from participants with an arrhythmia-related hospital admission. Concentrations were quantified by ELISA (OPN Quantikine kit, cat #DOST00, R&D Systems) following manufacturer's instructions.

Statistical analysis

The main analyses included data from the original trials combined with the survey data of the BCG-LT study, restricted to BCG-LT participants with complete responses. Descriptive statistics were presented as mean ± standard deviation (SD) or medians and interquartile range (IQR) for continuous variables, and as frequency and percentages for categorical variables. Differences in incidences of the first event between the BCG- and placebo-group were expressed as crude relative risks with 95% confidence intervals. Endpoints that showed statistically significant differences in our follow-up study or the original investigation were analysed with an in-depth Cox proportional hazards regression model. This model incorporated event data from both Long-Term participants and those who did not participate in the follow-up (see below). For all other variables, it was presumed that results would not change when analysed with the in-depth Cox model.

We have not adjusted for multiple testing to increase the power of the study to detect an effect of BCG. Consequently, the overall type-1 error rate is higher than the traditional 0.05 and significant results have to be considered as hypothesis generating.

We wished to account for the possibility that certain individuals preferentially participated in the BCG-LT study. To overcome a possible selection bias in assessing the risk of BCG for arrhythmia- or coronary events-related hospital admissions, we employed a weighted survival analysis of the time from randomization until the first event. The weight factor was derived from the inverse of the predicted probability of participants having joined the BCG-LT study from a logistic regression model incorporating the covariates 'vaccination group', 'sex', 'age', and 'experience of serious adverse events during the trial period'. The weight was applied to the BCG-LT study data, whereas for the period of the original trials a weight of '1' was assigned. We estimated hazard ratios (HR) with 95% confidence intervals (95% CI) from a Cox model with the original trial as a stratum variable, using the survey package in R, version 4.2.1.

Osteopontin concentrations between timepoints were compared with a non-parametric Wilcoxon-signed rank test. The osteopontin ELISA for the BCG group and placebo group was performed on different days, enabling us to compare circulating concentrations of osteopontin at different timepoints within one group ('before' vs 'after' vaccination), but precluding comparison between the groups.

All analyses were performed using IBM SPSS V.27, Graphpad Prism 10 and R 4.3.1 software version.

Results

Of the 7243 participants from the original trials, 4352 (60% response; 54% of the initially randomised participants) responded to the BCG-LT survey. After removing 104 unclear records and 10 individuals receiving BCG vaccination after the original trial, 4238 participants were included in the analysis: 2930/5338 (55%) from the BCG-PRIME trial and 1308/1905 (69%) from BCG-CORONA-ELDERLY (see *Figure 2*). 2317 (55%) participants of those included in the analysis had been randomised to receive a BCG vaccine. Baseline characteristics including sex, age and BMI were well-balanced between the BCG and placebo groups in the responders (*Table 1*). Compared with the invited participants who did not join the LongTerm study, the BCG-LT-population had less frequently been diagnosed with comorbidities (85.5% of the BCG-LT population, versus 91.9% of the non-respondents) while other characteristics were similar (*Supplementary table 1)*. The median time since randomisation at the time of the survey was 619 days (IQR 595-787). Most of the participants received a COVID-19 vaccine during follow-up (n=3828, 90%), with the majority having received at least one mRNA-based vaccine (n=3723, 87.8%).

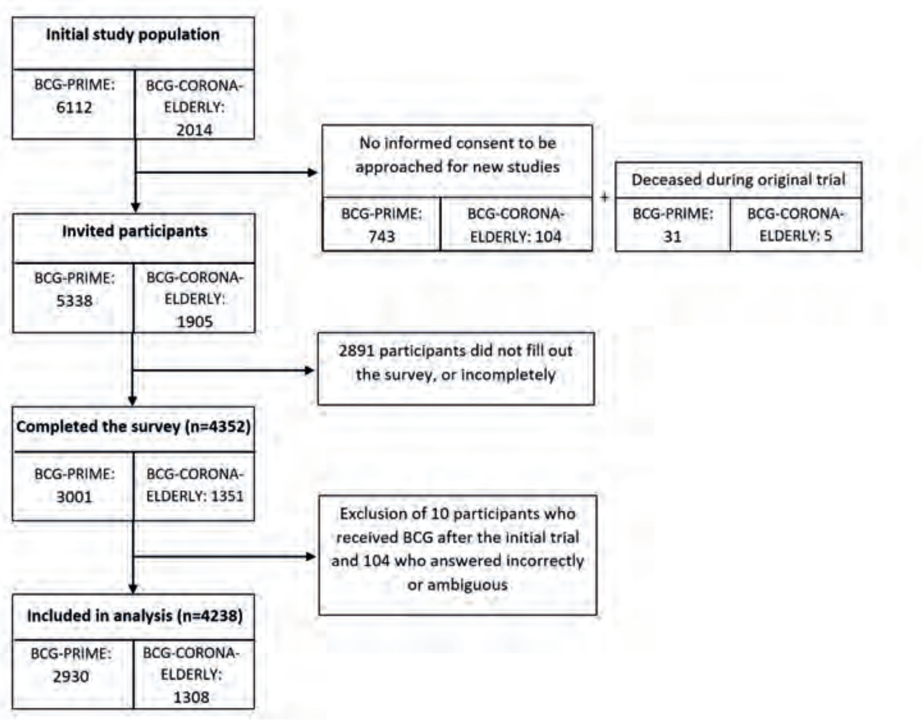

Figure 2: Inclusion flowchart

Table 1: Baseline characteristics of individuals who received BCG versus placebo vaccination

	Placebo (n=1921)	**BCG vaccine (n=2317)**
Demographics		
Male sex	1117 (58.1%)	1379 (59.5%)
Age (years) *median (IQR)*	69 (66-74)	69 (66-74)
BMI (kg/m^2) *mean (SD)*	26.4 (4.1)	26.5 (4.4)
Vaccines during follow-up		
Influenza vaccine	506 (26.3%)	687 (29.7%)
Any COVID-19 vaccine	1722 (89.6%)	2106 (90.9%)
mRNA vaccine	1676 (87.2%)	2047 (88.3%)
Viral vector vaccine	430 (22.4%)	499 (21.5%)
Protein subunit vaccine	23 (1.2%)	21 (0.9%)
Baseline comorbidities*	*N=1906*	*N=2295*
Hypertension	846 (44.4%)	1025 (44.7%)
Diabetes mellitus	291 (15.3%)	355 (15.5%)
Cardiovascular disease - other	826 (43.4%)	999 (43.5%)
Asthma	226 (11.9%)	248 (10.8%)
Pulmonary disease – other	275 (14.4%)	373 (16.3%)
Renal disease	102 (5.4%)	112 (4.9%)

Data are shown as n (%), unless otherwise indicated. BCG = bacillus Calmette-Guérin; BMI = body mass index.
** Not all baseline comorbidity values were available for all participants*

Infectious diseases and new immune-mediated diagnoses

Throughout the total follow-up period after study vaccination, there were no differences in individuals contracting any type of infection between the placebo and BCG group (*Table 2*). Specifically, we did not observe any differences in the incidence or severity of COVID-19 infections between the BCG and the placebo group. Similarly, the risk of contracting any non-COVID-19 infection or those that required antibiotics were nearly identical between both groups (RRs 1.0 and 0.9, respectively). The proportion of participants who were diagnosed with a new medical condition was low during the follow-up period (less than 10%) and similar between the two groups (*Table 3*).

Table 2: Individuals with at least one infectious episode after BCG versus placebo vaccination

	Placebo (n=1921)	**BCG vaccine (n=2317)**	**RR (95% CI)**
Any non-COVID-19 infection	266 (13.8%)	310 (13.4%)	1.0 (0.8-1.1)
Upper respiratory tract infection	67 (3.5%)	70 (3.0%)	0.9 (0.6-1.2)
Lower respiratory tract infection	84 (4.4%)	84 (3.6%)	0.8 (0.6-1.1)
Any non-COVID-19 respiratory tract infection	141 (7.3%)	151 (6.5%)	0.9 (0.7-1.1)
Urinary tract infection	82 (4.3%)	98 (4.2%)	1.0 (0.7-1.3)
Skin/wound infection	49 (2.6%)	58 (2.5%)	1.0 (0.7-1.4)
Gastro-intestinal infection	27 (1.4%)	23 (1.0%)	0.7 (0.4-1.2)
Infection with prescribed antibiotics	233 (12.1%)	256 (11.0%)	0.9 (0.8-1.1)
COVID-19 incidence	622 (32.4%)	732 (31.6%)	1.0 (0.9-1.0)
Sub analysis of COVID-19 cases below (total n=1354)			
	Placebo (n=622)	**BCG vaccine (n=732)**	
Asymptomatic	52 (8.4%)	71 (9.7%)	1.2 (0.8-1.6)
Hospitalization	12 (1.9%)	14 (1.9%)	1.0 (0.5-2.1)
Prolonged symptoms	94 (15.1%)	110 (15.0%)	1.0 (0.8-1.3)

COVID-19 = coronavirus disease 2019

Table 3: Individuals with new diagnoses after BCG versus placebo vaccination

New diagnoses	**Placebo (n=1921)**	**BCG vaccine (n=2317)**	**RR (95% CI)**
Arrhythmia	29 (1.5%)	34 (1.5%)	1.0 (0.6-1.6)
Cardiac - other	17 (0.9%)	21 (0.9%)	1.0 (0.5-1.9)
Hypertension	14 (0.7%)	10 (0.4%)	0.6 (0.3-1.3)
Diabetes mellitus	2 (0.1%)	2 (0.1%)	0.8 (0.1-5.9)
Dementia	2 (0.1%)	1 (0.0%)	0.4 (0.0-4.6)
Malignancy	44 (2.3%)	52 (2.2%)	1.0 (0.7-1.5)
Rheumatic disease	10 (0.5%)	12 (0.5%)	1.0 (0.4-2.3)
Allergy	4 (0.2%)	7 (0.3%)	1.4 (0.4-4.9)

Hospital admissions

A total of 352 participants (8.3%) reported they had experienced at least one hospital admission, which occurred more frequently in the BCG group (RR 1.2, 95% CI 1.0-1.5); *Table 4*). This difference was mainly due to the number of cardiac arrhythmia-related admissions and coronary artery events (RR 1.3, 95% CI 0.8-2.1). Other reasons for hospitalization were equally distributed between the placebo and BCG group.

Table 4: Individuals with at least one hospitalization attributed to a specific disease after BCG versus placebo vaccination

	Placebo (n=1921)	**BCG vaccine (n=2317)**	**RR (95% CI)**
Total individuals with at least one hospital admission	141 (7.4%)	211 (9.1%)	**1.2 (1.0-1.5)**
Cardiovascular-related hospital admissions			
Arrythmia	15 (0.8%)	36 (1.6%)	**2.0 (1.1-3.6)**
Coronary artery event	25 (1.3%)	39 (1.7%)	1.3 (0.8-2.1)
Cardiac – other	11 (0.6%)	22 (0.9%)	1.7 (0.8-3.4)
CVA/TIA	11 (0.6%)	13 (0.6%)	1.0 (0.4-2.2)
Total cardiovascular admissions¥	56 (2.9%)	98 (4.2%)	1.5 (1.1-2.0)
Other hospital admissions			
Infection	29 (1.5%)	42 (1.8%)	1.2 (0.8-1.9)
Pulmonary disease	13 (0.7%)	14 (0.6%)	0.9 (0.4-1.9)

¥ Also including other types of cardiovascular disease not specified in this table, e.g. peripheral artery disease

Cardiac arrhythmia-related hospital admission

The proportion of participants with at least one cardiac arrhythmia-related hospital admission was 1.6% in the BCG group (n=36) and 0.8% in the placebo group (n=15), with a relative risk of 2.0 (95% CI 1.1-3.6) and an absolute risk difference of 0.8% (95% CI 0.1-1.4). The baseline characteristics were comparable between the groups, as was the segment of participants with newly diagnosed arrhythmias (only 1.5% in both groups, *table 3*). Most of the arrhythmia-related hospital admissions were exacerbations of a pre-existing condition (80.6% and 80.0%, respectively). After adjustment of these results for the possibility of selection bias, the hazard ratio (BCG versus placebo) was 1.7 (95% CI 1.0-2.7) (see *Table 5* and *Figure 3*). Hospital admissions due to acute coronary events were numerically more frequent in the BCG group compared to the placebo group, but with wide confidence intervals (HR 1.4, 95% CI 0.8-2.4).

Table 5: Weighted analysis of cardiac arrythmia-related hospital admissions and coronary artery events

Outcome	Events in Placebo group (person-years)	Events in BCG group (person-years)	HR (95% CI)
Arrhythmia-related hospital admission *or death**			
	27 (4701)	48 (5158)	**1.7 (1.0-2.7)**
Coronary artery event hospital admission *or death**			
	24 (4706)	36 (5168)	1.1 (0.5-2.2)

* 'Death' refers to participants who died prior to the BCG-LT start only.
For survival plots, see Figure 3.

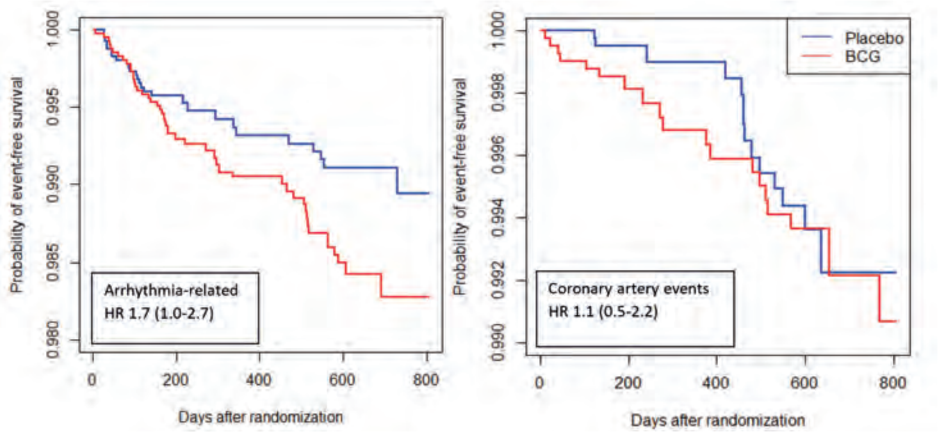

Figure 3: Survival plot of cardiac arrhythmia-related hospital admissions and coronary artery events after BCG and placebo vaccination, showing a hazard ratio of 1.7 and 1.1. See Table 5.

The median time between BCG vaccination and hospitalization for arrhythmias was 282 days, and only one individual was hospitalised within 30 days after BCG vaccination. Only two participants were admitted due to arrhythmia within one week after experiencing an infectious episode. Characteristics of the population that were admitted for arrhythmia-related reasons are presented in *Supplementary Table 2*.

To investigate a potential mechanism accounting for the differences in arrhythmia-related hospitalizations, osteopontin (OPN) concentrations were measured before and six months after BCG vaccination (n=64) and before and after placebo (n=40) (*Figure 4*). Osteopontin is a known anti-mycobacterial defence molecule, that has been recently shown to be also involved in the pathophysiology of atrial fibrillation (25). In the BCG group, 89% (57/64) of the participants showed in increase in OPN concentration 6 months after vaccination (median fold change 1.42). In comparison, higher osteopontin concentrations were measured after vaccination in 65% (26/40) of the placebo group (median fold change 1.15).

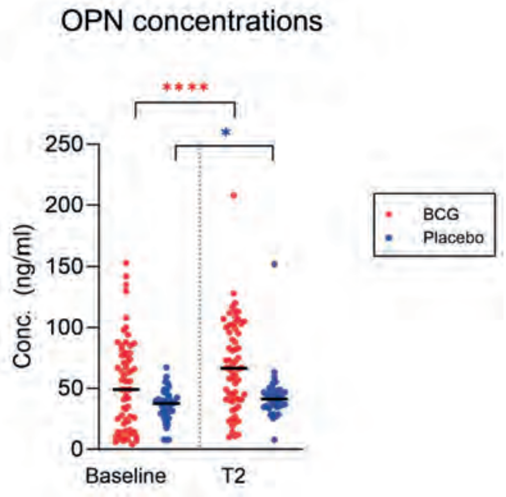

Figure 4: Osteopontin (OPN) concentrations before and after vaccination with BCG (n=64) or placebo (n=40).

Discussion

In the present study that extended the follow-up of two RCTs performed by our groups, we confirmed the absence of a measurable effect of BCG vaccination on the incidence of infectious or immune-mediated diseases in an elderly population.

Prior research has predominantly focused on evaluating the heterologous effects of BCG vaccination on respiratory infections, yielding mixed results. Our findings did not reveal any impact of BCG on COVID-19 incidence or severity. It has been suggested earlier that the immune system activation induced by BCG could also provide protection against other non-mycobacterial infections (5). However, we observed no overall differences in the incidence of infections between BCG-vaccinated and placebo-vaccinated individuals, which is different from the pre-pandemic ACTIVATE clinical trial that reported 40% less infections in BCG-vaccinated elderly compared to placebo-vaccinated individuals (27). Hospitalization rates were also largely similar between both groups, with one notable exception. We observed that BCG vaccination may potentially exacerbate pre-existing cardiac arrhythmias leading to hospital admission.

We found that the BCG group experienced nearly double the rate of admissions due to cardiac arrhythmias compared with the placebo group, although the incidence in this population was low. Upon analysing the time to the first arrhythmia-related hospitalization and incorporating weights to account for potential selection bias, the BCG-versus-placebo hazard ratio was 1.7 (95% CI 1.0-2.7). As this is a finding from a post-hoc analysis, and given the low absolute risk difference, this may well result from a chance finding. Importantly, the majority of these arrhythmias were pre-existing, which suggests that BCG may exacerbate existing arrhythmias rather than induce new ones. The fact that a significant part of the BCG-PRIME population was recruited from coagulation centres may explain the high number of pre-existing arrhythmias in our population.

The trend of a higher cardiac risk after BCG vaccination came up in the primary analysis of the BCG-PRIME study as well, albeit less pronounced, with 45 first-time cardiac arrhythmia events in the BCG group compared to 35 in the placebo group. We reviewed all these cases and found that 27/45 in the BCG group resulted in unplanned hospitalizations, while 17/35 in the placebo group required hospitalization. It is therefore important to emphasize that the BCG-PRIME findings cannot be directly compared with those of the BCG-LT-study, since both studies employed other definitions of study variables.

Besides the finding on cardiac arrhythmias, there was also an increased incidence of first-time coronary artery events in the initial follow-up of the BCG-PRIME study (21). This prompted an unplanned investigation by the Data Safety Monitoring Board (DSMB), who concluded at that time that BCG vaccination was unlikely to be the direct cause. The number of cardiac events in the BCG-CORONA-ELDERLY study was much lower due to an overall healthier population. In our current BCG-LT study, the trend of more coronary artery events was still visible, but the difference was not statistically significant between both groups. However, taking together those findings, this observation warrants further investigation and monitoring of administering BCG to elderly individuals with cardiac comorbidities, as had also been suggested by the initial DSMB of the BCG-PRIME study. It may be speculated that other BCG trials did not observe this effect due to a lower age of participants, fewer underlying comorbidities or shorter follow-up periods.

One plausible mechanistic explanation for the potential exacerbation of arrhythmias following BCG vaccination is the production of the pro-inflammatory cytokine osteopontin (26). This hypothesis is supported by findings that SSp1+ macrophages are expanded in atrial fibrillation and could be targets for treatment for those patients (25). To investigate this potential mechanism, we measured osteopontin concentrations before and after BCG/placebo vaccination in samples obtained from the PRIME study. Our findings revealed an almost 25% higher fold change following BCG vaccination than in the placebo group. The possibility of a seasonal effect, with elevated osteopontin concentrations during the winter months, cannot be entirely ruled out due to the timing of sample collection (baseline samples collected between October and December, T2 samples collected in March) and a similar trend for higher concentrations in the second sample in the placebo group. However, that does not negate the observed higher fold change in the BCG group. Unfortunately, there was no biological material available from the participants being hospitalized for arrhythmias, precluding further investigation of the role of osteopontin. This would be an important target for future research.

While the findings of our study suggest a potential detrimental rather than beneficial effect of BCG vaccination on cardiovascular disease, some experimental studies suggest the opposite. It has been demonstrated that BCG vaccination elicits reduced expression of pro-inflammatory proteins (28), which has been linked to the attenuation of systemic inflammation and subsequent cardioprotective effects (29). However, our study was unable to corroborate these findings.

To the best of our knowledge, our study represents the first investigation of the long-term heterologous effects of BCG vaccination among elderly individuals, including both beneficial and harmful outcomes. Importantly, a significant portion of the participants in the BCG-LT received specific SARS-CoV-2 vaccines in 2021, which makes it complex to distil the direct effects of BCG. In addition, a few limitations need to be acknowledged. Firstly, the response rate was 60% and there were slightly more responders from the BCG group. Although baseline characteristics were generally similar between participants and non-participants, and between the BCG and placebo groups, response may have been selective. To address this, we adjusted a time-to-event analysis to account for the varying probabilities of participation. However, medical events occurring after the original trials may have precluded participation to the survey due to death or inability, and it is not possible to detect or correct these potential sources of selection bias in case individuals receiving placebo or BCG would have a higher or lower number of complications leading to death that were not captured by the data collection.

Secondly, the participants in our study were elderly, which may limit the accuracy of some of the survey responses due to possible technical limitations filling in the online survey. Additionally, the two-year interval between the start of the main study and the follow-up survey could have led to recall problems. However, both potential issues would apply to both BCG and placebo vaccination groups equally. To mitigate these limitations, we contacted approximately 10% of the participants for clarification of their answers and found they were generally accurate. A more comprehensive approach, such as retrieving medical records from healthcare providers, was not pursued for feasibility reasons.

Lastly, despite having a large sample size of almost 4300 participants, the number of certain events is low. It is conceivable that more severely ill elderly did not fill in the survey due to physical impairment or mental overload, especially considering how the responding population was somewhat healthier compared to those who did not respond to the invitation for this study. Furthermore, we did not have access to mortality data. This may limit the generalizability of our findings to a broader population but has limited effect on the comparison of BCG versus placebo, since participants were initially well randomized and the response rate was comparable between both groups.

In conclusion, our study did not find evidence of protective effects of BCG vaccination against infectious diseases within two years of vaccination in older individuals. However, we observed that BCG vaccination may exacerbate previously existing cardiac arrhythmias, which may argue for caution when BCG vaccination would be considered in patients with arrhythmia.

Acknowledgements

ED, KF, ET, CW, MB and MGN conceptualized and designed the study. Data from previous trials was collected and analysed by KF, ET, SM, EK, CW, AH and FR. ED and KF wrote the first draft. All authors contributed to the final manuscript. JvdM, CW, JtO, JH, MB and MGN supervised the work.

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Supplementary materials

Supplementary table 1: LT-study participant baseline characteristics versus non-LT participants from original BCG-PRIME and BCG-CORONA-ELDERLY trials

	Non-LT (n=3005)	**LT (n=4238)**
Demographics		
Male sex	61.9%	59.0%
Age (years) *median (IQR)*	69 (65-74)	68 (64-72)
BMI *mean (SD)*	27.0 (4.8)	26.6 (4.4)
BCG during original trial	44.8%	54.7%
Baseline comorbidities[£]		
Hypertension	49.5%	44.6%
Diabetes mellitus	19.6%	15.3%
Cardiovascular disease - other	49.6%	43.4%
Asthma	13.3%	11.3%
Pulmonary disease - other	18.8%	14.3%
Renal disease	6.4%	5.2%

[£] A subset of the most common comorbidities are displayed here

Supplementary table 2: Arrhythmia-related hospital admissions in individuals who received placebo versus BCG vaccination

N=51	**Placebo (n=15)**	**BCG vaccine (n=36)**
Male sex	13 (86.7%)	25 (69.4%)
Age (years) *median (IQR)*	73.0 (69-76)	69.5 (66-74)
BMI (kg/m^2) *mean (SD)*	26.9 (4.3)	27.9 (5.9)
New arrythmia diagnosis	3 (20.0%)	6 (16.7%)
New hypertension diagnosis	1 (6.7%)	0 (0.0%)
New other cardiac diagnosis	1 (6.7%)	2 (5.6%)
First admission after BCG vaccine		
Days *median (IQR)*	-	282 (103-511)
Admission <180days after BCG vaccine	-	16 (44.4%)
Admission <30days after BCG vaccine	-	1 (2.8%)
First admission after COVID-19 vaccine		
N	8 (53.3%)	23 (63.9%)
Days *median (IQR)*	214 (105-324)	169 (73-319)
Admission <180 days after COVID-19 vaccine	4 (26.7%)	12 (33.3%)
Admission <30 days after COVID-19 vaccine	1 (6.7%)	4 (11.1%)
Admission <7 days after COVID-19 vaccine	0 (0.0%)	2 (5.6%)
Type of preceding COVID-19 vaccine		
mRNA vaccine	8 (53.3%)	23 (63.9%)
Viral vector vaccine	1 (6.7%)	2 (5.6%)
Protein subunit vaccine	0 (0.0%)	0 (0.0%)
COVID-19 infection before admission	1 (6.7%)	6 (16.7%)
Admission after COVID-19 infection		
Days *median (IQR)*	175 (-)	59 (38-149)
Admission <180 days after COVID-19 infection	1 (6.7%)	5 (13.9%)
Comorbidities at baseline*	*N=13*	*N=36*
Hypertension	7 (53.8%)	22 (61.6%)
Diabetes mellitus	1 (15.4%)	7 (19.4%)
Cardiovascular – other	11 (84.6%)	29 (80.6%)
Asthma	1 (15.4%)	6 (16.7%)
Pulmonary disease - other	2 (23.1%)	6 (16.7%)
Renal disease	1 (15.4%)	2 (5.6%)

* Not all baseline comorbidity values were available for all participants

Supplementary Appendix 1: Survey

(See online file)

Supplementary Appendix 2: grouping variables

Category	Includes
Coronary artery event	STEMI and non-STEMI myocardial infarct, instable angina pectoris, coronary spasms or any other event requiring acute coronary intervention
Other cardiovascular diseases	Congestive heart failure, heart valve diseases, cardiomyopathies, artery disease and pulmonary embolism
Other pulmonary diseases	Chronic obstructive pulmonary disorder, pulmonary fibrosis and hypertension, emphysema, interstitial lung disease and sarcoidosis.
Any non-COVID respiratory infection	Flu-like symptoms, bronchitis and exacerbation of pre-existing pulmonary condition
Diabetes mellitus	Both type I + II

References

1. Netea Mihai G, Quintin J, van der Meer Jos WM. Trained Immunity: A Memory for Innate Host Defense. Cell Host & Microbe. 2011;9(5):355-61.
2. Netea MG, Domínguez-Andrés J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, et al. Defining trained immunity and its role in health and disease. Nature Reviews Immunology. 2020;20(6):375-88.
3. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci U S A. 2012;109(43):17537-42.
4. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Jacobs C, Xavier RJ, et al. BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. Clin Immunol. 2014;155(2):213-9.
5. de Bree LCJ, Koeken V, Joosten LAB, Aaby P, Benn CS, van Crevel R, Netea MG. Non-specific effects of vaccines: Current evidence and potential implications. Semin Immunol. 2018;39:35-43.
6. Higgins JPT, Soares-Weiser K, López-López JA, Kakourou A, Chaplin K, Christensen H, et al. Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review. BMJ. 2016;355:i5170.
7. Wardhana DE, Sultana A, Mandang V, Jim E. The efficacy of Bacillus Calmette-Guerin vaccinations for the prevention of acute upper respiratory tract infection in the elderly. Acta Med Indones. 2011;43(3):185-90.
8. Pavan Kumar N, Padmapriyadarsini C, Rajamanickam A, Marinaik SB, Nancy A, Padmanaban S, et al. Effect of BCG vaccination on proinflammatory responses in elderly individuals. Sci Adv. 2021;7(32).
9. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. J Gerontol A Biol Sci Med Sci. 2014;69 Suppl 1:S4-9.
10. Koeken VA, de Bree LCJ, Mourits VP, Moorlag SJ, Walk J, Cirovic B, et al. BCG vaccination in humans inhibits systemic inflammation in a sex-dependent manner. J Clin Invest. 2020;130(10):5591-602.
11. van Dam AD, Bekkering S, Crasborn M, van Beek L, van den Berg SM, Vrieling F, et al. BCG lowers plasma cholesterol levels and delays atherosclerotic lesion progression in mice. Atherosclerosis. 2016;251:6-14.
12. Stienstra R, Netea MG. Firing Up Glycolysis: BCG Vaccination Effects on Type 1 Diabetes Mellitus. Trends Endocrinol Metab. 2018;29(12):813-4.
13. Usher NT, Chang S, Howard RS, Martinez A, Harrison LH, Santosham M, Aronson NE. Association of BCG vaccination in childhood with subsequent cancer diagnoses: a 60-year follow-up of a clinical trial. JAMA network open. 2019;2(9):e1912014-e.
14. Morra ME, Kien ND, Elmaraezy A, Abdelaziz OAM, Elsayed AL, Halhouli O, et al. Early vaccination protects against childhood leukemia: a systematic review and meta-analysis. Scientific reports. 2017;7(1):15986.
15. Rousseau M-C, Parent M-E, St-Pierre Y. Potential health effects from non-specific stimulation of the immune function in early age: The example of BCG vaccination. Pediatric Allergy and Immunology. 2008;19(5):438-48.
16. Tsilika M, Taks E, Dolianitis K, Kotsaki A, Leventogiannis K, Damoulari C, et al. ACTIVATE-2: A Double-Blind Randomized Trial of BCG Vaccination Against COVID-19 in Individuals at Risk. Front Immunol. 2022;13:873067.

17. Dos Anjos LRB, da Costa AC, Cardoso A, Guimarães RA, Rodrigues RL, Ribeiro KM, et al. Efficacy and Safety of BCG Revaccination With M. bovis BCG Moscow to Prevent COVID-19 Infection in Health Care Workers: A Randomized Phase II Clinical Trial. Front Immunol. 2022;13:841868.
18. Faustman DL, Lee A, Hostetter ER, Aristarkhova A, Ng NC, Shpilsky GF, et al. Multiple BCG vaccinations for the prevention of COVID-19 and other infectious diseases in type 1 diabetes. Cell Rep Med. 2022;3(9):100728.
19. Sinha S, Ajayababu A, Thukral H, Gupta S, Guha SK, Basu A, et al. Efficacy of Bacillus Calmette-Guérin (BCG) Vaccination in Reducing the Incidence and Severity of COVID-19 in High-Risk Population (BRIC): a Phase III, Multi-centre, Quadruple-Blind Randomised Control Trial. Infect Dis Ther. 2022;11(6):2205-17.
20. Schure JAt, Alexander L, Lisa B, Christine SB, Marc JMB, Jeffrey DC, et al. Bacillus Calmette-Guérin vaccine to reduce COVID-19 infections and hospitalisations in healthcare workers – a living systematic review and prospective ALL-IN meta-analysis of individual participant data from randomised controlled trials. medRxiv. 2022:2022.12.15.22283474.
21. Koekenbier EL, Fohse K, van de Maat JS, Oosterheert JJ, van Nieuwkoop C, Hoogerwerf JJ, et al. Bacillus Calmette-Guérin vaccine for prevention of COVID-19 and other respiratory tract infections in older adults with comorbidities: a randomized controlled trial. Clinical Microbiology and Infection. 2023;29(6):781-8.
22. Moorlag S, Taks E, Ten Doesschate T, van der Vaart TW, Janssen AB, Müller L, et al. Efficacy of BCG Vaccination Against Respiratory Tract Infections in Older Adults During the Coronavirus Disease 2019 Pandemic. Clin Infect Dis. 2022;75(1):e938-e46.
23. Upton CM, van Wijk RC, Mockeliunas L, Simonsson USH, McHarry K, van den Hoogen G, et al. Safety and efficacy of BCG re-vaccination in relation to COVID-19 morbidity in healthcare workers: A double-blind, randomised, controlled, phase 3 trial. EClinicalMedicine. 2022;48:101414.
24. Pittet LF, Messina NL, Orsini F, Moore CL, Abruzzo V, Barry S, et al. Randomized Trial of BCG Vaccine to Protect against Covid-19 in Health Care Workers. N Engl J Med. 2023;388(17):1582-96.
25. Hulsmans M, Schloss MJ, Lee I-H, Bapat A, Iwamoto Y, Vinegoni C, et al. Recruited macrophages elicit atrial fibrillation. Science. 2023;381(6654):231-9.
26. Khajoee V, Saito M, Takada H, Nomura A, Kusuhara K, Yoshida SI, et al. Novel roles of osteopontin and CXC chemokine ligand 7 in the defence against mycobacterial infection. Clin Exp Immunol. 2006;143(2):260-8.
27. Giamarellos-Bourboulis EJ, Tsilika M, Moorlag S, Antonakos N, Kotsaki A, Domínguez-Andrés J, et al. Activate: Randomized Clinical Trial of BCG Vaccination against Infection in the Elderly. Cell. 2020;183(2):315-23.e9.
28. Koeken V. Controlling inflammation in the elderly with BCG vaccination. Sci Adv. 2021;7(32).
29. Liu YY, Cai WF, Yang HZ, Cui B, Chen ZR, Liu HZ, et al. Bacillus Calmette-Guérin and TLR4 agonist prevent cardiovascular hypertrophy and fibrosis by regulating immune microenvironment. J Immunol. 2008;180(11):7349-57.

Chapter 7

Enduring echoes: post-infectious long-term changes in innate immunity

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Abstract

Upon encountering pathogens, the immune system typically responds by initiating an acute and self-limiting reaction, with symptoms subsiding after the pathogen has been cleared. However, long-term post-infectious clinical symptoms can manifest months or even years after the initial infection. 'Trained immunity', the functional reprogramming of innate immune cells through epigenetic and metabolic rewiring, has been proposed as a key concept for understanding these long-term effects. Although trained immunity can result in enhanced protection against reinfection with heterologous pathogens, it can also contribute to detrimental outcomes. Persisting and excessive inflammation can cause tissue damage and aggravate immune-mediated conditions and cardiovascular complications. On the other hand, suppression of immune cell effector functions by long-lasting epigenetic changes can result in post-infectious immune paralysis. Distinct stimuli can evoke different trained immunity programs, potentially resulting in different consequences for the host. In this review, we provide an overview of both the adaptive and maladaptive consequences of infectious diseases. We discuss how long-term immune dysregulation in patients can be addressed by tailoring host-directed interventions and identify areas of scientific and therapeutic potential to advance further.

Keywords
Innate immunity; Trained immunity; Infectious diseases;
Post-infectious complications

The human immune system is one of the main host defence mechanism against infections, initiating a complex response when confronted with pathogenic invaders. This response involves pathogen recognition, initiation of transcription of genes important for host defence, production of cytokines and defensins, activation of phagocytosis, and later, the initiation of adaptive immune responses. Typically, the interaction between pathogen and host results in an acute and transient inflammation, with the symptoms subsiding once the pathogen has been cleared. In the aftermath of an infection, immunological memory is very often induced, that protects the organism against reinfection. For a long time, it was believed that only adaptive immune cells such as T- and B-lymphocytes were able to build immunological memory. However, recent studies have shown that antigen-agnostic memory characteristics can also be built in cells of innate immunity, resulting in a broad, heterologous protection.

In addition, in rare instances, long-term effects can manifest themselves for weeks, months or even years after the acute phase of the infection appears to have been resolved. “Post-acute sequelae” (PAS) have been described for many infectious diseases, such as post-polio syndrome, post-sepsis syndrome, or more recently, “long COVID” (1). Although there is no clear definition of PAS, the late-occurring symptoms often include persistent fatigue, cognitive impairment, and an increased susceptibility for new infections or chronic diseases. Dysregulation of the immune system after the initial infection has been proposed as a key mechanism underpinning several of these post-infectious effects.

In this context, the concept of trained immunity (TRIM) can serve as a framework for understanding the long-term effects of an infection that are not directly related to the specific immune response to the initial pathogen. Since its first description in 2011, TRIM has been defined as a functional reprogramming of innate immune cells (2, 3). Epigenetic and metabolic modifications cause rewiring of the function of peripheral myeloid cells and their progenitors in the bone marrow, resulting in an enhanced response to subsequent infection. This has been shown to contribute to stronger protection against heterologous infections, even those unrelated to the initial pathogen. However, this augmented state can sometimes have disadvantageous consequences and cause tissue damage, long after the initial infection has been cleared. In this review, we will focus on the long-term effects of infectious diseases on the innate immune system. We provide an overview of both the protective and detrimental consequences of infection and discuss how these findings can be used in the future to improve health care.

Long-term enhancement of innate immune responses: trained immunity

Trained immunity describes the concept that certain stimuli such as microbial ligands, vaccines or infections, can lead to rewiring of the innate immune system to display an enhanced and accelerated response upon reinfection with various pathogens in an antigen-independent manner (3). The resulting 'memory response' is characterized by increased microbial pattern recognition, phagocytotic activity and cytokine production, which are all mediated by epigenetic and metabolic changes. These intracellular modifications affect the long-term responsiveness of innate immune cells.

In short, stimuli can induce a variety of epigenetic and transcriptional programmes and thereby influence immune-related gene expression – in contrast to adaptive immunity, in which gene rearrangement is the main underlying mechanism. Several epigenetic marks associated with trained immunity have been identified, such as histone modifications involving methylation and acetylation, DNA methylation and regulation of non-coding RNAs. Epigenetic marks of open chromatin at enhancer and promotor regions, like H3K4me3 and H3K27ac, are crucial to establish and maintain the memory phenotype of myeloid cells (4, 5). They can persist after removal of the initial stimulus, leading to enhanced transcriptional activity upon secondary stimulation (6). Activation or suppression of immune-related genes is also regulated by long non-coding RNAs and DNA methylation at CpG sites (7, 8).

In trained innate immune cells, changes in the cellular metabolism are intertwined with epigenetic processes. Serving as cofactors for epigenetic enzymes, various metabolites help regulate the induction of trained immunity. Examples are α-ketoglutarate and its derivates succinate and fumarate, that reduce the activity of KDM5 histone demethylase and ensure the retaining of H3K4me3 on the promotors of pro-inflammatory genes (9). Metabolic rewiring also supports the energy requirements for epigenetic alterations, e.g. by switching from oxidative phosphorylation to aerobic glycolysis to accommodate the increased metabolic needs (10, 11). Although initially described in monocytes (12), induction of a trained phenotype has been demonstrated in natural killer cells, innate lymphoid cells and dendritic cells as well (13-15). Most used inducers of trained immunity include microbial products such as LPS or β-glucan, while clinical research has centred around the bacillus Calmette-Guérin (BCG) vaccine, the most used vaccine worldwide. BCG can induce TRIM and thus provide a broader, non-specific protection against infections (16, 17).

On the one hand, induction of TRIM takes place in peripheral tissues through activation of local macrophages and dendritic cells. In the lung, but also in other organs, memory could be induced after local injury or inflammation (18, 19). On the other hand, TRIM is induced centrally at the level of the bone marrow as well (*see Figure 1*). BCG vaccination has been shown to impact haematopoietic stem and progenitor cells, which give rise to innate immune cells with a different functional profile. The alterations in cell metabolism and gene expression that result in *de facto* innate immune memory can therefore last much longer than the relatively short lifespan of circulating innate immune cells alone (20). Even though the longevity of innate immune memory remains a topic for further investigation, several studies have shown that it can last for months and even over a year (21-23). With regards to the heterologous effects of vaccines, the clinical effects have been shown to be present for several years (24, 25).

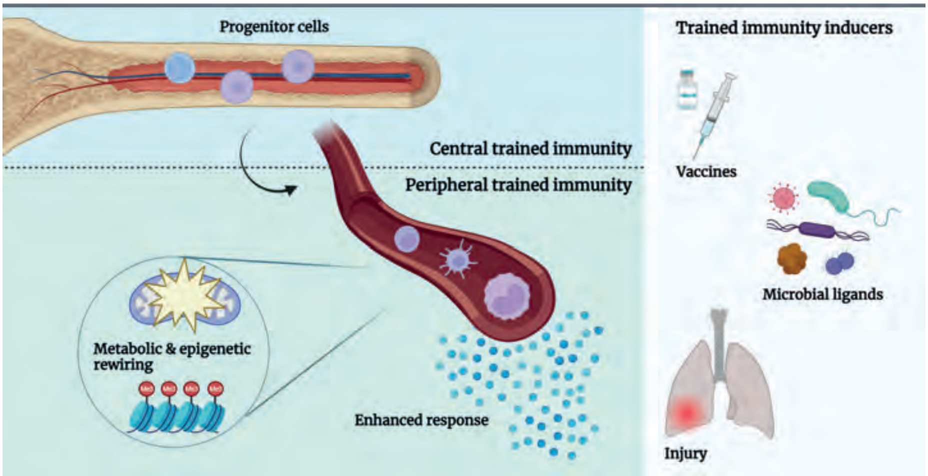

Figure 1: Induction of trained immunity in peripheral tissue and bone marrow

Infections or sterile triggers induce inflammation and the activation of immune effector mechanisms. Mediated by epigenetic and metabolic alterations, trained immunity involves reprogramming of innate immune cells, leading to enhanced responsiveness upon secondary stimulation or infection. This process can happen bot centrally at the bone marrow, involving hematopoietic stem cells and progenitor cells, and in peripheral tissue.

An interesting finding was that although lipopolysaccharide (LPS) could indeed induce a trained phenotype in innate immune cells, this seemed to be time- and dose-dependent. Higher doses and prolonged exposure resulted in the functional opposite of training: immune tolerance (26). Mediated by the silencing of genes that encode pro-inflammatory mediators (27), this protects the body against extensive tissue damage (28) but can also suppress immune cells to a point where they actively limit their production of pro-inflammatory mediators, increasing the risk for new infections. Immune paralysis is an important complication of sepsis, leading to an increased susceptibility to secondary (29). In contrast, stimulation of innate immune cells by low concentrations of LPS can lead to a trained immunity phenotype (30).

During infection, the extracellular heme released by erythrocytes or parenchymal cells can induce trained immunity in the periphery and the bone marrow (31). Mice trained with heme were protected against ensuing polymicrobial sepsis, coupled to extensive long-lasting epigenetic memory and myeloid progenitor cells. However, heme training before LPS administration increased mortality, probably by inducing a state of exaggerated inflammation while no beneficial effect of pathogen destruction could occur.

The innate immune system thus possesses the ability to retain a memory of initial microbial encounters, which modifies the functional responses to a secondary stimulus. These responses can manifest as an augmentation or suppression of immune cell effector functions. It is essential to emphasize that trained immunity is a concept, rather than a specific transcriptional or functional program: from this perspective, distinct stimuli can evoke different trained immunity programs, potentially resulting in different consequences for the host.

Adaptive and maladaptive responses

Induction of TRIM has most likely evolved to induce an improved protection against subsequent infections. For example, malaria parasites can induce a state of hyperresponsiveness in circulating innate immune cells that has been suggested to decrease the susceptibility to other pathogens (32). Similarly, Quintin et al. found that infection with *Candida albicans* or β-glucan from fungal microorganisms protected mice against reinfection by relying on epigenetic and functional reprogramming of monocytes (33). In addition, latent *Herpesviridae* increase resistance to certain bacteria in murine models, mediated by increased IFNy production and enhanced macrophage activation (34). There is an increased awareness that certain live-attenuated vaccines can have beneficial non-specific effects and have helped in decreasing neonatal and childhood mortality (35).

Although protective in this regard, it is easy to imagine this enhanced response may occur even when it is not necessary, or with undesirable magnitude. The same balance applies to tolerance, where reprogramming of innate immune cells can be beneficial to prevent collateral tissue damage at the level of mucosae or during an infection. On the other hand, inappropriate induction of tolerance could contribute to immune paralysis and result in greater susceptibility for opportunistic infections, as seen during sepsis (36).

It is also important to point out that both tolerance and trained immunity, despite having different consequences on the intensity of innate immune responses, represent outcomes of similar molecular mechanisms of long-term epigenetic reprogramming that can occur after an infection or exposure to microbial stimuli. Both are characterized by upregulation of a set of genes, and downregulation of another group of genes. The induction of LPS-induced tolerance has indeed shown to be underpinned by the downregulation of several pro-inflammatory genes, but other genes remained unaltered or were even upregulated (37). A mixture of up- and down-regulated genes was also demonstrated for trained immunity programmes induced by β-glucan or BCG (4). A strict dichotomy becomes thus less apparent at conceptual level, although they are characterized by different functional consequences.

Trained immunity can be beneficial during host defence, but could also trigger damage during chronic inflammatory conditions (*see Figure 2*). In the following paragraphs, maladaptive responses resulting from both persistent inflammation and immune suppression will be described. It is important to emphasize that both immune paralysis and inefficient hyperinflammation can be present in an individual at the same time and influence each other, resulting in a complex interplay of underlying pathways. Patients suffering from infectious diseases represent a broad group with heterogeneous immunological profiles and a multitude of factors influencing their clinical manifestation; a dysregulation of the immune response will not be necessarily found in all patients. Adequate stratification would be needed to enable meaningful descriptions and predictions for certain groups and individuals.

Long-term post-infectious hyperinflammation

After clearing the pathological agents responsible for an infectious disease, the inflammatory response is generally subsiding. However, in a minority of patients, epigenetic and metabolic rewiring due to the infection can cause innate immune cells to keep their heightened state and culminate into persistent inflammatory conditions. If inflammation is maintained after the acute phase of an infection, chronic (hyper) inflammation can contribute to various disadvantageous long-term consequences.

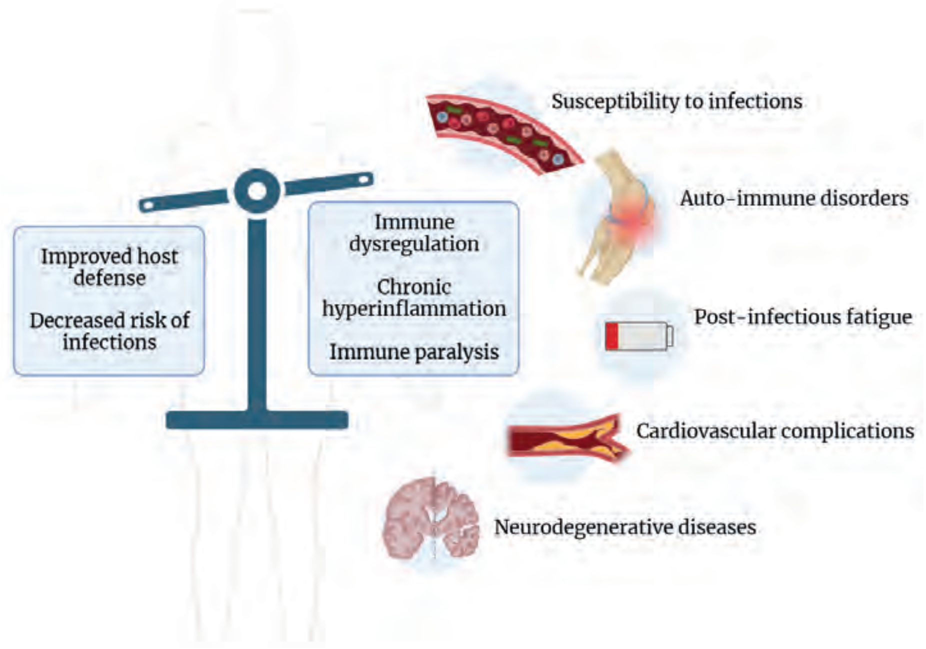

Figure 2: Adaptive and maladaptive consequences of trained immunity induction

Trained immunity programmes can provide benefits for the host, but could also prove detrimental by inducing a hyperinflammatory state or immune paralysis. Clinically, hyperinflammation mediated by trained immunity has been linked to atherosclerosis and adverse cardiovascular events, auto-immune disorders, post-infectious fatigue syndromes and neurodegenerative diseases. Paralysis of the innate immune system increases the susceptibility for recurrent or severe infections.

The most attention in this area has been given to the development of atherosclerosis and cardiovascular disease. It has been known that chronic inflammatory conditions can drive the progression of atherosclerosis. For instance, atherosclerosis rates are high in patients with rheumatoid arthritis (38). During an infectious episode such as community-acquired pneumonia, there is an increased risk of cardiovascular events and related mortality (39, 40). This can be explained by the combination of reduced oxygen supply from the lungs on the one hand, and increased oxygen requirements resulting from the inflammation on the other hand, which promotes thrombogenesis and myocardial stress (41, 42). However, persistent inflammation after pneumonia is also associated with cardiovascular disease, as well as a persistent procoagulant inflammatory state. The increased susceptibility to cardiovascular disease has been demonstrated long after the infection is cleared and can last up to 10 years (43).

Additional studies have shown that it is the cumulative infectious burden and not solely specific pathogens or inflammatory comorbidities that is mostly associated with cardiovascular disease (44). The number of infection-related hospital admissions during childhood are associated with adverse cardiovascular events and have been correlated with impaired endothelial function in young adulthood (45, 46). Trained immunity has been proposed as the link that connects infectious diseases and the prevalence of long-term cardiovascular disease.

Macrophages are the most abundant immune cell in atherosclerotic plaques, whose ruptures account for most cardiovascular events. Embryonic precursors and bone marrow-derived monocytes evolve into these arterial resident macrophages. Monocytes and macrophages are pivotal in promoting the initiation, progression and destabilization of atherosclerotic plaques via various mechanisms (47, 48). A sustained elevation in the activation of innate immune cells after infection can contribute to advancing atherosclerosis development and destabilization of plaques (47). Monocytes from patients with symptomatic atherosclerosis were found to be 'trained', as they produced higher levels of pro-inflammatory cytokines IL-6, IL-1b, IL-8, TNF and MCP-1 after stimulation than those from asymptomatic patients (49). Underlying epigenetic modifications that downregulated H3K4me3 and H3K27, as well as enhanced glycolytic activity, were identified as mechanisms for this reprogramming (50). In this regard, it might seem odd that the BCG vaccine, known for inducing trained immunity, has been associated with a reduced incidence of cardiovascular disease (51). However, this could be explained by a reduction of overall infections, thus resulting in lower infectious burden, that would have otherwise contributed to cardiovascular complications (44).

Macrophages are not only key players in atherosclerotic plaques, but are essential in all stages of the pathogenesis of fibrotic processes as well (52). Following insult or injury to the lung, exudative inflammation recruits numerous immune cells. In most cases, this response is limited and the lung returns to homeostasis. However, if the inflammation persists, the repair response can become dysregulated (53). In the case of inappropriate chronic inflammatory responses, progressive fibrosis occurs which eventually leads to organ dysfunction (54). Trained macrophages can modulate fibroblast transformation in systemic sclerosis mice models: tolerant macrophages induced a slower progression of skin thickening, while trained BCG-macrophages accelerated it (54). Of course, tissue damage due to trained immunity is not restricted to fibrosis; the continual pro-inflammatory environment also promotes apoptosis and vascular permeability via cytokine secretion and reactive oxygen species, and dysregulated immune responses can adversely target healthy tissue. Induction of central trained immunity has been shown to exacerbate emerging inflammatory arthritis in periodontitis models, demonstrating a link between maladaptive training of myelopoiesis and autoinflammatory comorbidities (55) (*see Figure 3*).

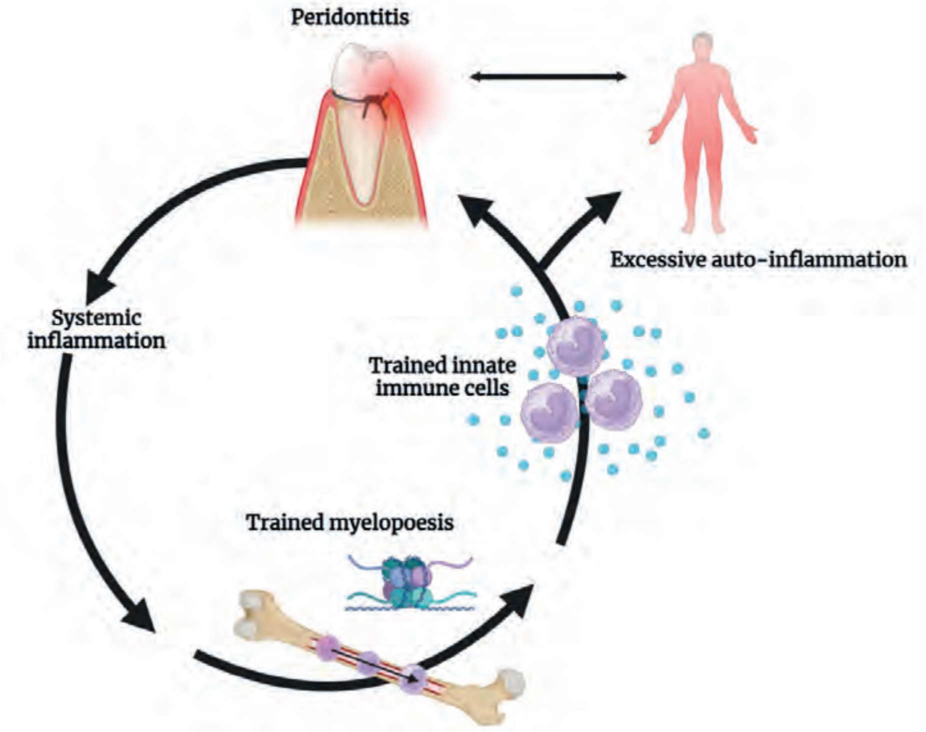

Figure 3: Maladaptive trained immunity exacerbates autoinflammation

Following chronic local inflammation, hematopoietic stem cells (HSCs) in the bone marrow are reprogrammed towards myelopoiesis. HSCs develop further into progenitor cells and eventually monocytes with enhanced effector functions, which are released into the circulation and migrate to peripheral organs. The induced hyperinflammatory phenotype exacerbates pre-existing inflammatory conditions, such as auto-immune arthritis. *Adapted from Li et al., 2022 (99)*

Certain post-acute syndromes after infectious diseases have also been linked to long-term changes in the innate immune system. Prolonged fatigue is at the core of the Q fever fatigue syndrome (QFS), in which patients retain long-term symptoms following an acute infection. Signs of chronic inflammation in QFS patients have been reported, which likely plays a role in the prolonged fatigue in this clinical condition (56, 57). Reduced expression of genes involved in the inhibition of inflammation and post-exercise recovery has also been found in monocytes (56, 57). In addition, some of these patients experience recurrent and severe upper respiratory tract infections. Raijmakers et al. reported epigenetic remodelling in genes of QFS patient that are involved in host defence (56, 57). Expression of open chromatin mark H3K4me3 was decreased at the promotor regions of several genes involving cytokine production, which resulted in lower mRNA expression and a modified cytokine response in monocytes compared to those of healthy controls. This likely leads to greater susceptibility to upper airway infections. It may be thus hypothesized that acute Q fever induces long-term changes in immune cells, and potentially in progenitor cells: this remains to be demonstrated in future studies. Importantly, QFS patients with recurring respiratory tract infections represent a distinct population, and not all QFS patients have these complaints.

Similar long-term processes seem to be induced in “long COVID”, or post-acute COVID-19 syndrome (PACS). It is estimated that up to 10% of the symptomatically SARS-CoV-2-infected population shows long-term persisting symptoms (58). The spectrum of complaints is very broad and includes persistent fatigue, dyspnoea, joint and chest pain, vertigo, attention disorders and memory problems among many others (59). A recent review by Davis et al. aimed to condensate the current literature regarding the underlying biological mechanisms of this syndrome and discusses alterations in B and T cells, autoantibodies, reactivation of underlying viruses and affected energy metabolism, as well as elevated levels of pro-inflammatory cytokines in those suffering from it (58). On an epigenetic level, PACS patients show a unique DNA methylation signature compared to controls (60). Differentially methylated CpG sites showed both hypo- and hypermethylation and could be linked to various signalling pathways including PI3 kinase, G-protein en histamine.

When recovering from a SARS-CoV-2 infection, immunophenotypic differences in NK cells, neutrophils and monocytes are still evident 16 weeks after initial infection, and RNA sequencing shows differences up until six month post infection (61). Differences in transcription factors between convalescent individuals and healthy volunteers were also described in a paper by You et al (62). They identified a trained immunity phenotype in monocytes after infection with increased chromatin accessibility and higher cytokine production after stimulation. On the one hand, this could contribute to long-term protection against reinfection; on the other hand, this could lead to hyperactivation and long-term symptoms. Similarly, a recent study by Cheong and colleagues reported long-term epigenetic memory in innate immune cells and their progenitors after COVID-19, thus demonstrating that the infection can induce a trained immunity phenotype (63). Patients developing prolonged symptoms after COVID-19 have a higher proportion of neutrophils and were significantly different on the transcriptional level at 24 weeks post infection, when most of the convalescent individuals had returned to a transcriptional baseline (60, 61). This is suggestive of ongoing immune dysregulation, highly activated immune cells and continuing inflammatory responses, which can persist for at least eight months after initial infection (64). The variation in the rate of gene expression recovery might explain the broad range of symptoms in some convalescent individuals.

Aberrant inflammatory responses potentially play a role in post-treatment Lyme disease syndrome as well (65). Patients may experience a range of neurocognitive and other symptoms, including the development of autoimmune diseases months after antibiotic-treated erythema migrans (66). Persistent transcriptional changes linked to dysregulated metabolic pathways have been proposed as a therapeutic target (67), although this concept has yet to be developed further.

Finally, a prolonged pro-inflammatory environment as a result of long-term inflammatory changes in innate immune cells after infections could potentially increase the risk for neurodegenerative syndromes, including dementia. Microglia in the central nervous system are functionally analogous to peripheral macrophages and can develop a trained immunity phenotype in mouse models in which inflammatory stimuli were applied, leading to exacerbated cerebral β-amyloidosis (68, 69). There is some evidence pointing towards exacerbated β-amyloidosis as a result of induction of trained immunity in microglia, potentially contributing to Alzheimer's disease (70). Repeated systemic LPS and *Salmonella* challenges in mice resulted in increased pro-inflammatory cytokines in the brain, contributing to local damage and the onset of neurodegenerative disease (71). Although human cohort studies with a long follow-up time are scarce, two recent study showed an association between hospitalizations for infectious diseases and an increased risk of dementia more than ten years after infection (72, 73). On the other hand, several recent epidemiological studies have shown that BCG treatment of bladder cancer is associated with a significant decrease in the incidence of Alzheimer's disease (74, 75). The mechanisms behind this effect are not known, but should be investigated in order to design new therapies for this disease.

Post-infectious immune paralysis

Whereas tolerance can be described as beneficial in the context of auto-immune reactions and preventing excessive inflammation, innate immune cells in an unresponsive state can also contribute to paralysis of the immune system. If genes involved in host defence are silenced for longer periods of time, this results in less effective immune responses with higher susceptibility to infections and decreased immunosurveillance. Epigenetic modifications associated with tolerance have been correlated to this phenomenon (76). In murine models for pneumonia, alveolar macrophages showed a tolerized phenotype up to weeks after resolution of the primary infections and had a severely diminished phagocytotic capacity (19). In addition, reprogramming was present in human alveolar macrophages and circulating monocytes for six months after systemic inflammation. The immune paralysis program was induced by the post-infectious environment rather than the infection itself.

Striking examples of post-infectious immune paralysis have been described in patients who recovered from sepsis. Even after discharge, mortality rates amongst these patients remain higher than in non-sepsis hospitalized patients for up to eight years (77, 78). The most prevalent causes of death after surviving the initial sepsis episode are cardiovascular events, new infections, and renal failure (79). Together with cognitive impairments and psychological health problems, this is termed the post-sepsis syndrome and increases the risk for hospital readmission (80). Among all sepsis survivors, readmission rates have been reported to be as high as 30%, with the majority being for recurrent sepsis (78). Persistent immune suppression plays an essential role in the infectious complications after sepsis (81). Arens et al. have shown a reduced production of pro-inflammatory cytokines in blood from sepsis survivors more than four years after discharge (82). Associated gene expression patterns were lower compared to healthy controls, indicating modifications at the epigenetic level. Scicluna et al. have proposed a stratification of septic patients based on whole-blood transcriptomics. The endotype associated with the highest mortality rates one year after discharge was identified by a decreased expression of key regulators and components of the adaptive and innate immune system, such as antigen presentation, decreased TLR expression, and nuclear factor-kB and interferon signalling (83). Treatment with IL-4-based nanotherapy inducing trained immunity has been suggested as a promising target to reverse sepsis-induced immunoparalysis, as shown in murine and *ex vivo* human sepsis models (84).

In addition to post-sepsis immune paralysis, it is important however to note that also chronic low-grade inflammation can be present in sepsis survivors (85). This contributes to the dismal outcomes of post-sepsis syndrome by inducing organ injury and injury-induced inflammation (86). Tools to adequately assess what specific immune dysregulation characterizes individual patients are urgently needed.

In addition to protection against infections, an effective activation of the immune system is essential for immunosurveillance against cancer. Protracted or excessive inflammatory responses can foster tumorigenesis by providing mediators for cell proliferation and/or survival. Pro-inflammatory cytokines such as IL-6 and IL-1b that are produced by innate immune cells are associated with increased tumorigenicity and promote metastases in several types of tumours, including oral squamous cell carcinoma and lung, kidney and breast cancer (87, 88). On the other hand, lack of immune surveillance allows malignant cells to evade detection and elimination by immune cells.

Induction of trained immunity has been suggested as a potential immunotherapy approach against cancer. BCG instillations are one of the most effective treatments of non-muscle invasive bladder cancer, and induction of trained immunity by BCG immunotherapy induces trained immunity in circulating monocytes. This leads to increased release of cytokines that help to booster T cell activation (89). Polymorphisms in autophagy genes *ATG2B* and *ATG5* that result in defective trained immunity are also associated with poorer effectiveness of BCG effects in bladder cancer (90). Notably, induction of trained immunity by BCG instillations is also associated with a lower incidence of infections (91). BCG vaccination in childhood has been associated with a lower risk of cancer, in particular leukaemia and lung cancer (92).

Interestingly, epidemiological studies have suggested different effects of acute or chronic infections for the risk of malignancies. A review by Hoption Cann et al. provided a comprehensive dataset on various types of cancers and their correlations with infectious diseases (93). They found reduced risks for melanoma and ovary cancer after acute infections. On the other hand, chronic infections were associated with elevated cancer risks. However, most evidence for a connection between infectious diseases and malignancies comes from retrospective studies – with their inherent limitations. A prospective cohort study, which started in the pre-vaccination period before 1950, found that measles and influenza were associated with decreased cancer-related mortality (94). Although pertussis was linked to a higher risk, the authors hypothesize that enhanced immunosurveillance as a result of the infections might explain their protective findings. More recently, it was proposed that γδ T cells play a role in the link between the number of febrile infections and antitumour activity (95). γδ T cells are considered a bridge between innate and adaptive immunity, as they can respond to PAMPs directly, promote B/T cell activation, and sense early changes of malignant cells. Their population expands drastically during infection, and the cells can persist in the circulation for years. It is known that BCG vaccination and viral infection can induce TRIM in γδ T cells, as evidenced by an increase in transcriptional programmes and enhanced immune responsiveness (96, 97).

Conclusions and Future perspectives

In this review, we discussed the long-term effects of infections on innate immunity and their potential consequences for adaptive and maladaptive trained immunity and tolerance programmes. We suggest that trained immunity, as an epigenetic memory of previous infections, can mediate long-term changes in the innate immune system that are increasingly linked to significant clinical effects.

Clinicians can leverage this concept to address immune paralysis or excessive activation, tailoring interventions to specific patient needs. Host-directed interventions to modulate the immune system hold great promise in this regard, and current research is focusing on translating the findings from models to the actual patients (98).

There are many remaining questions that need to be answered. A better understanding of the duration of long-term trained immunity is needed, and how its molecular mechanisms relate to long-term clinical manifestations. In addition, risk factors for long-term persistence of dysregulated immune responses should be identified to tailor therapies at an early stage. Patient stratification depending on their immune status will help us to diagnose post-infection complications, and to develop appropriate treatments.

Declaration of interest

LABJ and MGN are scientific founders of TTxD, Lemba and Salvina Therapeutics. MGN is scientific founder of Biotrip.

References

1. Hirschenberger M, Hunszinger V, Sparrer KMJ. Implications of Innate Immunity in Post-Acute Sequelae of Non-Persistent Viral Infections. Cells. 2021;10(8).
2. Netea MG, Joosten LA, Latz E, Mills KH, Natoli G, Stunnenberg HG, et al. Trained immunity: A program of innate immune memory in health and disease. Science. 2016;352(6284):aaf1098.
3. Netea Mihai G, Quintin J, van der Meer Jos WM. Trained Immunity: A Memory for Innate Host Defense. Cell Host & Microbe. 2011;9(5):355-61.
4. Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajanirefah A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. science. 2014;345(6204):1251086.
5. Novakovic B, Habibi E, Wang S-Y, Arts RJ, Davar R, Megchelenbrink W, et al. β-Glucan reverses the epigenetic state of LPS-induced immunological tolerance. Cell. 2016;167(5):1354-68. e14.
6. Netea MG, Domínguez-Andrés J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, et al. Defining trained immunity and its role in health and disease. Nature Reviews Immunology. 2020;20(6):375-88.
7. Chen YG, Satpathy AT, Chang HY. Gene regulation in the immune system by long noncoding RNAs. Nature immunology. 2017;18(9):962-72.
8. Laird PW. Principles and challenges of genome-wide DNA methylation analysis. Nature Reviews Genetics. 2010;11(3):191-203.
9. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. 2009;324(5929):930-5.
10. Cheng S-C, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, et al. mTOR-and HIF-1α–mediated aerobic glycolysis as metabolic basis for trained immunity. science. 2014;345(6204):1250684.
11. Xiao M, Yang H, Xu W, Ma S, Lin H, Zhu H, et al. Inhibition of α-KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors. Genes & development. 2012;26(12):1326-38.
12. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci U S A. 2012;109(43):17537-42.
13. O'Sullivan TE, Sun JC, Lanier LL. Natural killer cell memory. Immunity. 2015;43(4):634-45.
14. Hole CR, Wager CML, Castro-Lopez N, Campuzano A, Cai H, Wozniak KL, et al. Induction of memory-like dendritic cell responses in vivo. Nature communications. 2019;10(1):2955.
15. Placek K, Schultze JL, Netea MG. Immune memory characteristics of innate lymphoid cells. Current opinion in infectious diseases. 2019;32(3):196-203.
16. Arts RJ, Moorlag SJ, Novakovic B, Li Y, Wang S-Y, Oosting M, et al. BCG vaccination protects against experimental viral infection in humans through the induction of cytokines associated with trained immunity. Cell host & microbe. 2018;23(1):89-100. e5.
17. Walk J, de Bree LCJ, Graumans W, Stoter R, van Gemert G-J, van de Vegte-Bolmer M, et al. Outcomes of controlled human malaria infection after BCG vaccination. Nature communications. 2019;10(1):874.
18. Hoyer FF, Naxerova K, Schloss MJ, Hulsmans M, Nair AV, Dutta P, et al. Tissue-specific macrophage responses to remote injury impact the outcome of subsequent local immune challenge. Immunity. 2019;51(5):899-914. e7.

19. Roquilly A, Jacqueline C, Davieau M, Mollé A, Sadek A, Fourgeux C, et al. Alveolar macrophages are epigenetically altered after inflammation, leading to long-term lung immunoparalysis. Nature Immunology. 2020;21(6):636-48.

20. Mitroulis I, Ruppova K, Wang B, Chen L-S, Grzybek M, Grinenko T, et al. Modulation of myelopoiesis progenitors is an integral component of trained immunity. Cell. 2018;172(1):147-61. e12.

21. Yoshida K, Ishii S. Innate immune memory via ATF7-dependent epigenetic changes. Cell Cycle. 2016;15(1):3-4.

22. Khan N, Downey J, Sanz J, Kaufmann E, Blankenhaus B, Pacis A, et al. M. tuberculosis reprograms hematopoietic stem cells to limit myelopoiesis and impair trained immunity. Cell. 2020;183(3):752-70. e22.

23. Kleinnijenhuis J, Quintin J, Preijers F, Benn CS, Joosten LA, Jacobs C, et al. Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. Journal of innate immunity. 2014;6(2):152-8.

24. Benn CS, Netea MG, Selin LK, Aaby P. A small jab – a big effect: nonspecific immunomodulation by vaccines. Trends in Immunology. 2013;34(9):431-9.

25. Nankabirwa V, Tumwine JK, Mugaba PM, Tylleskär T, Sommerfelt H, for the P-EBFSG. Child survival and BCG vaccination: a community based prospective cohort study in Uganda. BMC Public Health. 2015;15(1):175.

26. Vergadi E, Vaporidi K, Tsatsanis C. Regulation of endotoxin tolerance and compensatory anti-inflammatory response syndrome by non-coding RNAs. Frontiers in immunology. 2018;9:2705.

27. Carson WF, Cavassani KA, Dou Y, Kunkel SL. Epigenetic regulation of immune cell functions during post-septic immunosuppression. Epigenetics. 2011;6(3):273-83.

28. Medzhitov R, Schneider DS, Soares MP. Disease tolerance as a defense strategy. Science. 2012;335(6071):936-41.

29. Biswas SK, Lopez-Collazo E. Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends in immunology. 2009;30(10):475-87.

30. Morris M, Li L. Molecular Mechanisms and Pathological Consequences of Endotoxin Tolerance and Priming. Archivum Immunologiae et Therapiae Experimentalis. 2012;60(1):13-8.

31. Jentho E, Ruiz-Moreno C, Novakovic B, Kourtzelis I, Megchelenbrink WL, Martins R, et al. Trained innate immunity, long-lasting epigenetic modulation, and skewed myelopoiesis by heme. Proc Natl Acad Sci U S A. 2021;118(42).

32. Schrum JE, Crabtree JN, Dobbs KR, Kiritsy MC, Reed GW, Gazzinelli RT, et al. Cutting edge: Plasmodium falciparum induces trained innate immunity. The Journal of Immunology. 2018;200(4):1243-8.

33. Quintin J, Saeed S, Martens JH, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. Cell host & microbe. 2012;12(2):223-32.

34. Barton ES, White DW, Cathelyn JS, Brett-McClellan KA, Engle M, Diamond MS, et al. Herpesvirus latency confers symbiotic protection from bacterial infection. Nature. 2007;447(7142):326-9.

35. Aaby P, Benn CS, Flanagan KL, Klein SL, Kollmann TR, Lynn DJ, et al. The non-specific and sex-differential effects of vaccines. Nature Reviews Immunology. 2020;20(8):464-70.

36. van der Poll T, Opal SM. Host–pathogen interactions in sepsis. The Lancet infectious diseases. 2008;8(1):32-43.

37. Foster SL, Hargreaves DC, Medzhitov R. Gene-specific control of inflammation by TLR-induced chromatin modifications. Nature. 2007;447(7147):972-8.

38. Mankad R. Atherosclerotic vascular disease in the autoimmune rheumatologic patient. Current atherosclerosis reports. 2015;17:1-10.

39. Corrales-Medina VF, Musher DM, Shachkina S, Chirinos JA. Acute pneumonia and the cardiovascular system. The Lancet. 2013;381(9865):496-505.

40. Musher DM, Abers MS, Corrales-Medina VF. Acute infection and myocardial infarction. New England Journal of Medicine. 2019;380(2):171-6.

41. Musher DM, Rueda AM, Kaka AS, Mapara SM. The association between pneumococcal pneumonia and acute cardiac events. Clinical infectious diseases. 2007;45(2):158-65.

42. Yende S, D'Angelo G, Mayr F, Kellum JA, Weissfeld L, Kaynar AM, et al. Elevated hemostasis markers after pneumonia increases one-year risk of all-cause and cardiovascular deaths. PloS one. 2011;6(8):e22847.

43. Corrales-Medina VF, Alvarez KN, Weissfeld LA, Angus DC, Chirinos JA, Chang C-CH, et al. Association between hospitalization for pneumonia and subsequent risk of cardiovascular disease. Jama. 2015;313(3):264-74.

44. Leentjens J, Bekkering S, Joosten LA, Netea MG, Burgner DP, Riksen NP. Trained innate immunity as a novel mechanism linking infection and the development of atherosclerosis. Circulation research. 2018;122(5):664-9.

45. Burgner DP, Cooper MN, Moore HC, Stanley FJ, Thompson PL, de Klerk NH, et al. Childhood hospitalisation with infection and cardiovascular disease in early-mid adulthood: a longitudinal population-based study. PLoS One. 2015;10(5):e0125342.

46. Burgner DP, Sabin MA, Magnussen CG, Cheung M, Sun C, Kähönen M, et al. Early childhood hospitalisation with infection and subclinical atherosclerosis in adulthood: the Cardiovascular Risk in Young Finns Study. Atherosclerosis. 2015;239(2):496-502.

47. Christ A, Bekkering S, Latz E, Riksen NP, editors. Long-term activation of the innate immune system in atherosclerosis. Seminars in Immunology; 2016: Elsevier.

48. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. Cell. 2011;145(3):341-55.

49. Bekkering S, van den Munckhof I, Nielen T, Lamfers E, Dinarello C, Rutten J, et al. Innate immune cell activation and epigenetic remodeling in symptomatic and asymptomatic atherosclerosis in humans in vivo. Atherosclerosis. 2016;254:228-36.

50. Shirai T, Nazarewicz RR, Wallis BB, Yanes RE, Watanabe R, Hilhorst M, et al. The glycolytic enzyme PKM2 bridges metabolic and inflammatory dysfunction in coronary artery disease. Journal of Experimental Medicine. 2016;213(3):337-54.

51. Rieckmann A, Villumsen M, Sørup S, Haugaard LK, Ravn H, Roth A, et al. Vaccinations against smallpox and tuberculosis are associated with better long-term survival: a Danish case-cohort study 1971–2010. International Journal of Epidemiology. 2017;46(2):695-705.

52. Novak ML, Koh TJ. Phenotypic transitions of macrophages orchestrate tissue repair. The American journal of pathology. 2013;183(5):1352-63.

53. Warheit-Niemi HI, Hult EM, Moore BB. A pathologic two-way street: how innate immunity impacts lung fibrosis and fibrosis impacts lung immunity. Clinical & translational immunology. 2019;8(6):e1065.

54. Jeljeli M, Riccio LGC, Doridot L, Chêne C, Nicco C, Chouzenoux S, et al. Trained immunity modulates inflammation-induced fibrosis. Nature communications. 2019;10(1):5670.

55. Li X, Wang H, Yu X, Saha G, Kalafati L, Ioannidis C, et al. Maladaptive innate immune training of myelopoiesis links inflammatory comorbidities. Cell. 2022;185(10):1709-27. e18.

56. Raijmakers RP, Jansen AF, Keijmel SP, Schoffelen T, Scholzen A, Van Der Meer JW, et al. Interferon-γ and CXCL10 responses related to complaints in patients with Q fever fatigue syndrome. European Journal of Clinical Microbiology & Infectious Diseases. 2018;37:1385-91.
57. Raijmakers RP, Koeken VA, Jansen AF, Keijmel SP, Roerink ME, Joosten LA, et al. Cytokine profiles in patients with Q fever fatigue syndrome. Journal of Infection. 2019;78(5):349-57.
58. Davis HE, McCorkell L, Vogel JM, Topol EJ. Long COVID: major findings, mechanisms and recommendations. Nature Reviews Microbiology. 2023;21(3):133-46.
59. Nasserie T, Hittle M, Goodman SN. Assessment of the Frequency and Variety of Persistent Symptoms Among Patients With COVID-19: A Systematic Review. JAMA Network Open. 2021;4(5):e2111417-e.
60. Nikesjö F, Sayyab S, Karlsson L, Apostolou E, Rosén A, Hedman K, et al. Defining post-acute COVID-19 syndrome (PACS) by an epigenetic biosignature in peripheral blood mononuclear cells. Clinical Epigenetics. 2022;14(1):172.
61. Ryan FJ, Hope CM, Masavuli MG, Lynn MA, Mekonnen ZA, Yeow AEL, et al. Long-term perturbation of the peripheral immune system months after SARS-CoV-2 infection. BMC medicine. 2022;20(1):1-23.
62. You M, Chen L, Zhang D, Zhao P, Chen Z, Qin E-Q, et al. Single-cell epigenomic landscape of peripheral immune cells reveals establishment of trained immunity in individuals convalescing from COVID-19. Nature cell biology. 2021;23(6):620-30.
63. Cheong J-G, Ravishankar A, Sharma S, Parkhurst CN, Grassmann SA, Wingert CK, et al. Epigenetic memory of coronavirus infection in innate immune cells and their progenitors. Cell. 2023;186(18):3882-902.e24.
64. Phetsouphanh C, Darley DR, Wilson DB, Howe A, Munier CML, Patel SK, et al. Immunological dysfunction persists for 8 months following initial mild-to-moderate SARS-CoV-2 infection. Nature immunology. 2022;23(2):210-6.
65. Aucott JN. Posttreatment Lyme disease syndrome. Infectious Disease Clinics. 2015;29(2):309-23.
66. Arvikar SL, Crowley JT, Sulka KB, Steere AC. Autoimmune Arthritides, Rheumatoid Arthritis, Psoriatic Arthritis, or Peripheral Spondyloarthritis Following Lyme Disease. Arthritis & Rheumatology. 2017;69(1):194-202.
67. Kerstholt M, Netea MG, Joosten LA. Borrelia burgdorferi hijacks cellular metabolism of immune cells: Consequences for host defense. Ticks and tick-borne diseases. 2020;11(3):101386.
68. Wendeln A-C, Degenhardt K, Kaurani L, Gertig M, Ulas T, Jain G, et al. Innate immune memory in the brain shapes neurological disease hallmarks. Nature. 2018;556(7701):332-8.
69. Haley MJ, Brough D, Quintin J, Allan SM. Microglial priming as trained immunity in the brain. Neuroscience. 2019;405:47-54.
70. He X-f, Xu J-h, Li G, Li M-y, Li L-l, Pei Z, et al. NLRP3-dependent microglial training impaired the clearance of amyloid-beta and aggravated the cognitive decline in Alzheimer's disease. Cell death & disease. 2020;11(10):849.
71. Püntener U, Booth SG, Perry VH, Teeling JL. Long-term impact of systemic bacterial infection on the cerebral vasculature and microglia. Journal of neuroinflammation. 2012;9:1-13.
72. Sipilä PN, Heikkilä N, Lindbohm JV, Hakulinen C, Vahtera J, Elovainio M, et al. Hospital-treated infectious diseases and the risk of dementia: a large, multicohort, observational study with a replication cohort. The Lancet Infectious Diseases. 2021;21(11):1557-67.
73. Bohn B, Lutsey PL, Misialek JR, Walker KA, Brown CH, Hughes TM, et al. Incidence of dementia following hospitalization with infection among adults in the atherosclerosis risk in communities (ARIC) study cohort. JAMA Network Open. 2023;6(1):e2250126-e.

74. Klinger D, Hill BL, Barda N, Halperin E, Gofrit ON, Greenblatt CL, et al. Bladder Cancer Immunotherapy by BCG Is Associated with a Significantly Reduced Risk of Alzheimer's Disease and Parkinson's Disease. Vaccines (Basel). 2021;9(5).

75. Weinberg MS, Zafar A, Magdamo C, Chung SY, Chou WH, Nayan M, et al. Association of BCG Vaccine Treatment With Death and Dementia in Patients With Non–Muscle-Invasive Bladder Cancer. JAMA Network Open. 2023;6(5):e2314336-e.

76. Rodriguez R, Suarez-Alvarez B, Lopez-Larrea C. Therapeutic epigenetic reprogramming of trained immunity in myeloid cells. Trends in Immunology. 2019;40(1):66-80.

77. Winters BD, Eberlein M, Leung J, Needham DM, Pronovost PJ, Sevransky JE. Long-term mortality and quality of life in sepsis: a systematic review. Critical care medicine. 2010;38(5):1276-83.

78. Shen H-N, Lu C-L, Yang H-H. Risk of recurrence after surviving severe sepsis: a matched cohort study. Critical Care Medicine. 2016;44(10):1833-41.

79. Wang HE, Szychowski JM, Griffin R, Safford MM, Shapiro NI, Howard G. Long-term mortality after community-acquired sepsis: a longitudinal population-based cohort study. BMJ open. 2014;4(1):e004283.

80. Mostel Z, Perl A, Marck M, Mehdi SF, Lowell B, Bathija S, et al. Post-sepsis syndrome–an evolving entity that afflicts survivors of sepsis. Molecular Medicine. 2020;26:1-14.

81. van der Slikke EC, An AY, Hancock REW, Bouma HR. Exploring the pathophysiology of post-sepsis syndrome to identify therapeutic opportunities. EBioMedicine. 2020;61:103044.

82. Arens C, Bajwa S, Koch C, Siegler BH, Schneck E, Hecker A, et al. Sepsis-induced long-term immune paralysis–results of a descriptive, explorative study. Critical care. 2016;20(1):1-11.

83. Scicluna BP, Van Vught LA, Zwinderman AH, Wiewel MA, Davenport EE, Burnham KL, et al. Classification of patients with sepsis according to blood genomic endotype: a prospective cohort study. The Lancet Respiratory Medicine. 2017;5(10):816-26.

84. Schrijver DP, Röring RJ, Deckers J, de Dreu A, Toner YC, Prevot G, et al. Resolving sepsis-induced immunoparalysis via trained immunity by targeting interleukin-4 to myeloid cells. Nat Biomed Eng. 2023;7(9):1097-112.

85. Gentile LF, Cuenca AG, Efron PA, Ang D, Bihorac A, McKinley BA, et al. Persistent inflammation and immunosuppression: a common syndrome and new horizon for surgical intensive care. Journal of Trauma and Acute Care Surgery. 2012;72(6):1491-501.

86. Hotchkiss RS, Moldawer LL. Parallels between cancer and infectious disease. New England Journal of Medicine. 2014;371(4):380-3.

87. Lee SH, Hong HS, Liu ZX, Kim RH, Kang MK, Park N-H, et al. TNFα enhances cancer stem cell-like phenotype via Notch-Hes1 activation in oral squamous cell carcinoma cells. Biochemical and biophysical research communications. 2012;424(1):58-64.

88. Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. European journal of cancer. 2005;41(16):2502-12.

89. van Puffelen JH, Keating ST, Oosterwijk E, van der Heijden AG, Netea MG, Joosten LA, et al. Trained immunity as a molecular mechanism for BCG immunotherapy in bladder cancer. Nature Reviews Urology. 2020;17(9):513-25.

90. Buffen K, Oosting M, Quintin J, Ng A, Kleinnijenhuis J, Kumar V, et al. Autophagy Controls BCG-Induced Trained Immunity and the Response to Intravesical BCG Therapy for Bladder Cancer. PLOS Pathogens. 2014;10(10):e1004485.

91. van Puffelen JH, Novakovic B, van Emst L, Kooper D, Zuiverloon TCM, Oldenhof UTH, et al. Intravesical BCG in patients with non-muscle invasive bladder cancer induces trained immunity and decreases respiratory infections. J Immunother Cancer. 2023;11(1).

92. Usher NT, Chang S, Howard RS, Martinez A, Harrison LH, Santosham M, et al. Association of BCG vaccination in childhood with subsequent cancer diagnoses: a 60-year follow-up of a clinical trial. JAMA network open. 2019;2(9):e1912014-e.
93. Hoption Cann SA, van Netten JP, van Netten C. Acute infections as a means of cancer prevention: Opposing effects to chronic infections? Cancer Detection and Prevention. 2006;30(1):83-93.
94. Tennant PWG, Parker L, Thomas JE, Craft SAW, Pearce MS. Childhood infectious disease and premature death from cancer: a prospective cohort study. European Journal of Epidemiology. 2013;28(3):257-65.
95. Kozak W, Jedrzejewski T, Pawlikowska M, Piotrowski J, Wrotek S. Toward Antitumor Immunity and Febrile Infections: Gamma/Delta (γδ) T Cells Hypothesis. The Quarterly Review of Biology. 2018;93(3):187-205.
96. Pitard V, Roumanes D, Lafarge X, Couzi L, Garrigue I, Lafon M-E, et al. Long-term expansion of effector/memory Vδ2− γδ T cells is a specific blood signature of CMV infection. Blood. 2008;112(4):1317-24.
97. Suen TK, Moorlag SJ, Li W, de Bree LCJ, Koeken VA, Mourits VP, et al. BCG vaccination induces innate immune memory in γδ T cells in humans. Journal of Leukocyte Biology. 2023:qiad103.
98. Mulder WJM, Ochando J, Joosten LAB, Fayad ZA, Netea MG. Therapeutic targeting of trained immunity. Nat Rev Drug Discov. 2019;18(7):553-66.
99. Li X, Wang H, Yu X, Saha G, Kalafati L, Ioannidis C, et al. Maladaptive innate immune training of myelopoiesis links inflammatory comorbidities. Cell. 2022;185(10):1709-27.e18.

Chapter 8

General summary and discussion

This thesis has presented new insights into the underlying mechanisms and effects of trained immunity. In particular, our research deepened the understanding of the mechanisms behind non-specific effects and explored the potential heterologous benefits of BCG and COVID-19 vaccination in older adults. We have formulated future directions for research and clinical practice concerning the dual nature of these effects following vaccination or natural infection.

The major findings from each chapter are presented in *Table 1*. Taking together the seven chapters of this thesis, we can outline three crucial remaining questions, which will be discussed after a brief review of the research in this thesis: the potential duration of TRIM effects; how to balance the dual nature of non-specific beneficial and adverse effects; and how to advance in this field by optimizing the integration of clinical endpoints and immunological correlates. In addition to deepening our biological understanding of trained immunity and its effects, the aforementioned points can help us to provide more relevant input for policy decisions regarding public health and vulnerable groups.

In the **TACTIC trial (chapter 2)**, we have studied different sequences of vaccination against COVID-19 (BNT162b2) and Influenza (Vaxigrip Tetra) to determine the optimal manner of administration from an immunological point of view (1). Our RCT involving 154 participants showed that both sequential and concurrent vaccination were safe in terms of adverse events, but decreased protection against COVID-19 after combined vaccination could not be ruled out. Both the quantitative and functional antibody responses against SARS-COV-2 were diminished in the group who received combined vaccinations in comparison to the other study arms. The pre-specified non-inferiority margin for anti-S concentrations was not met in this combination group. Biologically, a plausible mechanism for potential interference however remains dubious. The choice of a non-inferiority margin should be medically substantiated, although the current cut-off recommendations by the WHO have not been based on COVID-19 data (2) and more lenient margins could be considered if certain other benefits are provided, like logistic advantages or less side-effects. Most other RCTs on this topic did not employ a pre-specified non-inferiority value and concluded that the difference in anti-S concentrations was not likely to be clinically relevant, or used a different cut-off value (3-8). Since the TACTIC trial did not measure breakthrough infections during or after the trial, we did not have sufficient data to support or reject that conclusion with certainty.

Chapter	**Main research question(s)**	**Major findings**
2. TACTIC trial	*Do COVID-19 and influenza vaccination interfere? Which vaccine should be given first?*	1. Decreased protection against COVID-19 after concurrent vaccination with an mRNA COVID-19 booster and influenza vaccine cannot be excluded 2. The short-term safety profile of concurrent vaccination is comparable to subsequent vaccination schemes 3. Starting subsequent vaccination with a COVID-19 booster or influenza vaccine is immunologically interchangeable
3. Innate memory for vaccine design	*Which vaccines induce NSEs and through which mechanisms? How can this advance vaccine design and delivery?*	1. Many live-attenuated vaccines can induce heterologous memory in innate immune cells, mediated by various metabolites and cellular processes 2. Trained immunity and nano delivery could enhance vaccine efficacy
4. TACTIC-II substudy	*Do mRNA and adenoviral COVID-19 vaccines exert different immunological and clinical effects?*	1. Vector-based COVID-19 vaccination results in higher pro-inflammatory cytokine responses compared to mRNA-based vaccines, four months after vaccination
5. Influenza-Brazil study	*Can influenza vaccination provide protective NSEs during the COVID-19 pandemic?*	1. This study could not demonstrate any protective NSEs after influenza vaccination 2. Conducting a RCT under pandemic circumstances requires a specialized approach and careful coordination
6. BCG-LongTerm study	*What are the long-term effects of BCG vaccination in older adults?*	1. There are no differences in the incidence of (non-) infectious diseases among elderly Dutch individuals within two years after BCG or placebo vaccination 2. A higher frequency of hospitalization for pre-existing cardiac arrythmias was found after BCG, potentially mediated by osteopontin
7. Post-infectious innate immunity	*How can NSEs after natural infection be explained?*	1. Trained immunity may be involved in the pathophysiology of post-infectious clinical syndromes 2. Hyperinflammation or post-infectious immune paralysis are targets for treatment

COVID-19 = coronavirus disease 2019; NSE = non-specific effect; RCT = randomized controlled trial; BCG = bacillus Calmette-Guérin

Our conclusion regarding the safety profile of concurrent vaccination during the first month was widely supported by other RCTs and reporting systems (9, 10), some also demonstrating this over a prolonged period of time. However, the absence of serious adverse clinical events after weeks or months does not exclude the possibility of long-term effects that might be optimized by a certain sequence of vaccination. The reportedly elevated concentrations of inflammatory markers in the systemic circulation after mRNA vaccination might result in long-term complications, while an influenza vaccine has demonstrated to lower systemic inflammation (11, 12). In addition, suggested differences in all-cause mortality after an mRNA vaccine versus an adenoviral vaccine implore us to consider that for COVID-19 vaccines, differential mechanisms are at play that need to be investigated (13).

The review in **chapter 3** described the mechanisms underlying various vaccines involved in inducing trained immunity (TRIM). Certain live-attenuated vaccines can train the innate immune system to exhibit a memory-like response to subsequent infections, a process that is mediated by epigenetic and metabolic changes. Most of the vaccines studied for this goal are administered in childhood and many are included in the Dutch national immunisation programme ('Rijksvaccinatieprogramma'). The extent to which COVID-19 vaccines can induce TRIM however remains unclear (14-16), and the duration of the described TRIM effects is still much debated. Understanding this could be crucial, as TRIM might play a role in the observed differential heterologous outcomes after mRNA and adenoviral vaccines.

In the **TACTIC-II substudy (chapter 4)**, we therefore set out to study the immunological response to both types of COVID-19 vaccines on the transcriptional and functional level. All TACTIC participants (chapter 2) had been vaccinated against COVID-19 *before* the TACTIC trial started. Based on national policy at that time, ⅓ had been vaccinated with a viral vector vaccine and ⅔ with an mRNA primary series. We isolated PBMCs from a subset of TACTIC participants four months after primary vaccination ('baseline' in the trial; n=15 for BNT162b2; n=9 for ChadOx1-S) to compare their transcriptional landscape and cytokine responses.

We found that four months after the initial vaccinations, the pro-inflammatory cytokine levels upon *in vitro* stimulation were higher in adenoviral-vaccinated individuals compared to those who had received mRNA vaccines. This might indicate that vector-vaccinated individuals have a faster resolution of inflammation and could therefore be less prone to cardiovascular disease.

In the **BCG Long-Term study (chapter 6)**, we compared the incidence of infectious and immune-mediated diagnoses after randomized BCG or placebo vaccination in older adults. Over 4,000 formerly randomized participants answered a questionnaire about their health status, vaccination history and medical events. There were no differences in infectious episodes, severity of COVID-19 or new-onset chronic diseases between both groups. However, a greater proportion of BCG-vaccinated individuals experienced cardiovascular-related hospital admissions, attributed to an increase in aggravated pre-existing cardiac arrhythmias. Although the relative risk after BCG doubled, the absolute risk for these admissions remained low. This is in part due to the relatively low number of participants with pre-existing rhythm disorders, making it challenging for any kind of agent to cause ensuing high case numbers. On the other hand, as this was an exploratory study with no formal hypothesis testing, chance findings cannot be excluded.

We concluded that caution is warranted when using BCG in older adults with pre-existing cardiac problems. It is however interesting to critically regard the relevance of this warning, as BCG is not used in older adults in any clinical practice worldwide at this moment (apart from intravesical use in bladder cancer (17)), and our findings do not directly provide any rationale to change this. However, it is not inconceivable that the potential beneficial effects of BCG will be further investigated and future findings might add to existing evidence pointing towards successful prevention of respiratory tract infections in older adults (18, 19), especially since we are no longer in the midst of a pandemic that potentially interferes with patterns of circulating pathogens (20, 21). In that case, having a plausible mechanism for BCG-induced cardiac arrhythmia exacerbations would improve the reliability of this finding. We proposed that extracellular matrix protein osteopontin (OPN) might mediate this relationship and quantified OPN concentrations in a subset of available plasma samples, showing a larger over-time increase in BCG-vaccinated individuals.

OPN is a glycoprotein and pro-inflammatory cytokine, which is barely expressed under healthy conditions but increases during various pathologies and after various stimuli such as BCG (22-24). By influencing endothelial cell migration, macrophage activation and arterial calcification, OPN has been linked to cardiovascular disease. Structural cardiac remodelling and atrial fibrosis, an underlying mechanism for atrial fibrillation (AF), is mediated by OPN (24-26). A recent study found that high OPN concentrations in patients undergoing cryoablation for AF were associated with AF recurrence (27). Hulsmans et al. have shown that recruited macrophages with high expression of OPN elicit AF, and the authors successfully reduced arrhythmias in mice by inhibiting monocyte migration (28).

Given the many roles of OPN in hemostasis and disease, OPN itself does not seem to be a useful direct target for the prevention or treatment of cardiovascular disease. The mechanisms specific to OPN production in a particular condition are more promising targets. The potential role of OPN in the recurrence of cardiac arrhythmias still remains to be elucidated, as is the link between BCG and OPN in these patients.

Somewhat different from the other chapters in this thesis, **chapter 5** focused mostly on lessons learned regarding the conduct of research during a pandemic. We describe how creative recruitment strategies, preparing for different scenarios and close monitoring of changing circumstances by a dedicated team are crucial to proper vaccine research, and hope this paper will contribute to an ongoing learning process within the scientific community.

Finally, in **chapter 7**, we have outlined how trained immunity may be involved in the pathophysiology of post-infectious clinical syndromes. Although trained immunity can result in enhanced protection against reinfection with heterologous pathogens, it can also contribute to detrimental outcomes such as tissue damage and cardiovascular complications. Maladaptive consequences of infectious diseases can include long-term immune dysregulation, which often exhibits characteristics of hyperinflammation and/or immune paralysis. Adequately assessing a patient's position on this spectrum could identify promising targets for host-directed treatment.

Implications for policy and research

The results in this thesis are relevant to public health, particularly in areas such as vaccine strategy, the development of novel components, and clinical management of infectious diseases and their aftermath. While these findings offer valuable insights for policy considerations, they are not intended to dictate policy directly. Effective vaccination strategies depend not only on immunological responses: logistics, public opinion, costs, and efficiency are major factors to determine the success of vaccination campaigns and should not be overlooked. The immunological field can however take specific actions to enhance the relevance and impact of its research. To truly enable progression of this field from research to adequately informing policy, several research gaps need to be addressed.

Three of the most pressing gaps will be discussed here: the potential duration of TRIM effects; how to balance the dual nature of non-specific beneficial and adverse effects; and how to advance this field by optimizing the integration of clinical endpoints and immunological correlates. Conceptually, these three points are summarized in *Figure 1*.

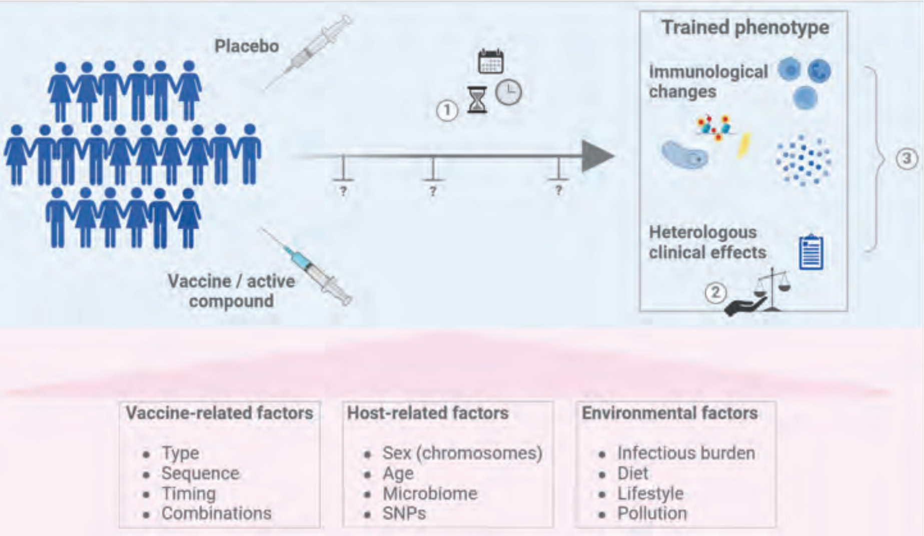

Figure 1: Conceptual study design. 1 = establishing the duration of TRIM-effects; 2 = balancing the dual nature of TRIM; 3 = integrating immunological measurements with clinical correlates. This can be adapted to a specific intervention and varying (or multiple) study populations.

1. Duration of TRIM-effects

Chapters three and seven describe possible clinical outcomes related to TRIM, although not all evidence establishes a clear causal connection. Despite the consensus that the innate immune system can be 'trained' in response to vaccination or infection, supported by a rapidly expanding body of research, questions remain regarding the duration of this phenomenon and thereby its clinical consequences. Enhanced immune responses should persist to provide long-lasting protection to subsequent challenges, so the duration of the trained immune response is a critical factor in determining its clinical utility. The relevance of the differences between two types of COVID-19 vaccines as described in chapter four would in part be determined by the period during which they remain present. Moreover, the findings regarding potential effects of BCG after two years are not easy to interpret: although studies have presented associations between childhood

BCG vaccination and cancer incidence 60 years later, it is not known whether the effects of the vaccine can truly linger for so long (29).

In *in vitro* studies, the duration of TRIM has been reported to last for several weeks (30), and animal models suggest it may extend to one year (31). The results of human trials vary, with some studies indicating effects lasting several months or longer (32), while retrospective observations suggest effects could last up to many years (33) – though these studies are based on epidemiological data that are prone to confounding and lack direct biological measurements.

Despite this abundance of research, it is complex to draw definite conclusions as study designs, types of challenge agents, and measurement techniques are not easily comparable. Moreover, most studies focus on the immediate to mid-term effects of TRIM and do not extend their observations beyond the initial exhibition of a trained phenotype. Longitudinal studies with consistent methodologies are needed to accurately determine the duration of trained immunity and the factors influencing it. Research shows that the magnitude of a TRIM response, or the 'trainability' of individuals, largely depends on the state of the chromatin state before training (34). This may also influence the duration of TRIM effects. In addition, many compounds have been found to inhibit training. Various environmental and vaccine-related factors, including sequential vaccination, could also play a role in further enhancing diminishing the effects in early phases. When investigating these factors, it is crucial to include longitudinal measurements that can capture the potentially time-dependent dynamics of trained immunity (see *Figure 1*).

Further investigation into the effects of TRIM induction might benefit from evaluating them in distinct categories: 1) epigenetic and metabolic changes, 2) immunological responses, and 3) clinically observable effects, as these processes may not occur simultaneously or progress at the same rate. For instance, immune cells might show enhanced activity shortly after vaccination, while epigenetic changes could take longer to influence clinical outcomes. Moreover, epigenetic alterations could contribute to the duration of TRIM in different ways, as DNA methylation changes are relatively stable while certain histone modifications can be rapidly reversed (35, 36). Various host-related factors that are known to impact the induction of TRIM, such as age and sex, should be considered in designing the required longitudinal studies (see *Figure 1*).

Transgenerational trained immunity

Special attention must be given to the possibility of hereditable immunological traits. Cohort studies in Guinee-Bissau have demonstrated a negative correlation between parents' BCG-vaccination status and the neonatal mortality rate of their children, independent of the children's vaccination status and after correcting for socio-economic factors (37). Given the TRIM effects of the BCG vaccine, the overall lower mortality in offspring could indicate transmission of trained immunity to the next generation. Understanding this would be crucial for developing life-course vaccination schemes and could offer additional interventions to protect children in areas with a high infectious burden. In addition, it could add incentive to intensify efforts to improve the outreach of current vaccination campaigns. Some examples include adjusting vaccination programmes to include more TRIM-inducing vaccines or to retain existing ones despite shifts in causative agents. Optimizing the timing of administering these vaccines, such as adding vaccines before reproduction, but close enough to expect ongoing TRIM effects, might also be beneficial and is in line with current calls to extend vaccine policy beyond childhood (38, 39).

Transmission of TRIM could involve the transfer of epigenetic changes via oocytes or sperm cells, although indirect inheritance via maternal immune components is investigated as well (40). Supporting the epigenetic transmission-hypothesis, a recent murine study has indeed found inheritable epigenetic changes after training with *C. Albicans* or zymosan, showing a similar 'trained' epigenetic profile in the progenitor sperm cells and two subsequent, untrained generations of offspring that showed enhanced protection against heterologous infections (41). Likely due to differences in mouse substrains and handling conditions, a similar study did not provide evidence for transmission, illustrating the prominent role of environmental factors in epigenetic inheritance (42). To advance further in this field, a randomized controlled clinical trial in healthy young adult men is underway to compare the epigenetic profile of immune cells and sperm cells after training with a BCG vaccine (NCT05766345). Assessing whether sperm cell epigenetics change in response to vaccination would be a first step in understanding the potential of epigenetic inheritance and the lasting effects of vaccination across generations.

2. Balancing the dual nature of TRIM

As discussed in detail in chapters three and seven priorly, trained immunity can provide enhanced protection but also increase the risk of adverse events, aggravate disease or paralyse key immune functions. Regulating the mechanisms driving

TRIM has been proposed as an option to treat patients where this balance has been off (43). This involves modulating or targeting specific compounds involved in TRIM pathways to restore optimal immune function, depending on the patient's state.

A notable example is the use of lipid nanoparticles encapsulating interleukin-4 (IL-4), which inhibits acute inflammation in PBMCs while paradoxically inducing trained immunity. IL-4 nanotherapy resolved immunoparalysis in murine models and experimental endotoxemia, supporting the hypothesis that regulation of trained immunity could be an effective treatment for patients at risk of complications due to immunoparalysis (44). Other compounds under investigation for clinical use include β-glucan, *Leishmania* antigens and TLR-agonists (45-48).

Strategically combining vaccines to manage potential adverse effects might also offer opportunities. While some studies have suggested that certain vaccine sequences, such as administering DTP or polio vaccines after measles vaccines, may be associated with increased mortality rates in females, this remains highly controversial as most studies contain a high risk of bias (49-52). In contrast, the TACTIC trial suggested that administering an mRNA vaccine concurrently with an inactivated influenza vaccine might reduce the risk of enhanced systemic inflammation compared to when the mRNA vaccine is given alone, though more evidence is needed to solidify these conclusions (1).

Finally, balancing the benefits and risks of TRIM modulation requires a personalized approach and involves trade-offs. This balance depends on individual patient characteristics and preferences, and can change over the course of disease. Adequate stratification into endotypes (e.g. hyperinflammatory, coagulopathic, tolerant) is therefore crucial to ensure the optimal interventions at the right moment (see *Figure 1*, characterizing immune profiles). In addition to recreating a balance within one person, the effects of TRIM could be employed for specific groups or goals. Enhancing vaccine efficacy could benefit from inducing TRIM, while it might be preferable to suppress TRIM in otherwise healthy patients with underlying cardiovascular disorders.

3. Linking immunological measurements with clinical correlates

Both for determining the duration and optimal balance of TRIM effects, it is crucial to design studies and experiments that actually measure what we need to know.

Many studies published or cited in this thesis focus on basic immunological mechanisms with limited information on clinical data, or use epidemiological findings without formulating a plausible biological mechanism due to insufficient access to useful biomaterial or techniques. Studies combining both approaches are often exploratory in nature and can therefore not offer definitive evidence to confirm or refute hypotheses. Both the TACTIC trials and BCG-LT study present relevant insights, but invite further research to provide a complete understanding.

Large vaccination trials tend to focus on assessing vaccine efficacy and do not centre on mechanisms when the effects are negative or only positive within certain groups, likely because of monetary constraints. A striking example of the added value of overcoming this has been shown in the M72/ASO1_E vaccine against active pulmonary tuberculosis in adults with a latent infection (53). Despite the moderate vaccine efficacy, immune response were detailed over a three-year follow-up period and found potential immune markers that could predict vaccine efficacy. It indicated a specific class of immune cells, polypositive CD4+ T cells, that may be pivotal in mediating protective effects, further enhancing our understanding of how to prevent active tuberculosis in the future.

A major hurdle to directly translate the findings from the TACTIC study into recommendations about the optimal sequence of vaccination is the absence of a consensus on a 'correlate of protection', or CoP, for COVID-19 vaccines (54, 55). A defined protective threshold would conveniently facilitate clearer conclusions. An immune marker – whether a serological measure or e.g. T-cell function – that reliably predicts the efficacy of preventing a certain clinical outcome, would guide public policy more effectively. In addition, a CoP could be used in experimental settings to assess new vaccines and their efficacy against new viral variants, or aid extension of approval to other population groups, without the need for a comparator vaccines or large clinical trials (55, 56).

An important reason for the lack of consensus on the CoP is that COVID-19 is caused by a mucosal infection *potentially* leading to viremia and severe systemic disease (54). Other disease, such as hepatitis or tetanus, induce more predictable immune responses with more straight-forward protection induced by neutralizing antibodies alone, or reacting to a toxin rather than the bacteria itself. An immune marker used as CoP should clearly discriminate between vaccinees with and without breakthrough disease. This has shown to be impossible for COVID-19, as despite vaccination and specific antibody production vaccinees can still develop mild disease. Ongoing discussions on the exact threshold levels of anti-spike

IgG and anti-SARS-CoV-2 neutralizing capacity that correlate with protection are primarily focused on protection against severe systemic disease. Other CoPs might be involved in protection against mucosal infection, a topic that warrants further investigation.

Although Earle at al. presented specific values of neutralizing titers and binding antibody titers, these are somewhat artificial as they had to be calculated by combining a multitude of studies that differed not only in population and vaccine type, but also in immunological analyses and measurement units (57). Similar to another study conducted around the same time (58), the authors did not provide a threshold but rather highlighted the strong correlation between vaccine efficacy and serology measures. Nonetheless, such findings provide a valuable base and can be used to predict vaccine efficacy (56). Because of differences in study population and measurement units, the results can however not be directly compared with those of the TACTIC trial, illustrating the need for more uniform outcome measures in study design and CoP evaluation (59), and underscoring the complementary effect of longitudinal clinical observations to immunological data.

The *Strategic Advisory Group of Experts on Immunization and the Development of WHO vaccine position papers* has indeed advised that additional observational studies are unlikely to further inform policy (60). High-quality RCTs should be performed to examine NSEs and all-cause mortality in vaccination programmes, with standardized immunological endpoints. This will not be equally easy in all research settings: for instance, an intensive care unit continually monitors an abundance of clinical parameters and can obtain additional biological sampling without many logistic constrains. Research originating from outpatient clinics or laboratory-associated trial units faces more significant challenges. Similarly, low- or middle-income settings with high infectious burdens but scarce resources, as previously detailed in chapter five, encounter comparable difficulties. One would therefore argue for more resources, including funding and dedicated personnel to coordinate efforts, to be dedicated to translational research involving RCTs to overcome these problems.

Beyond this thesis

In this thesis, I aimed to provide an overview of the current knowledge on the induction of trained immunity by vaccination and infectious disease. We have contributed to a better understanding of how heterologous effects are mediated and provided a starting point for researching them in a population of older adults. In addition, the relatively novel COVID-19 vaccines in this vulnerable population can now be understood on a deeper level and their immunological effects have been taken into account in recent policy advice regarding vaccination schemes (61).

I have highlighted three main routes to advance this field. Establishing the duration of various TRIM effects and balancing the beneficial and maladaptive effects will both depend on a multitude of factors, for which clinical and immunological study designs and outcomes should be integrated. This fits well within the *systems vaccinology* framework, which aims to enhance our mechanistic understanding of the variation in immune responses to vaccination and other triggers (62). Integrating our three main points into this framework is conceptually presented in *Figure 1*.

Ongoing research will be essential to clarify these mechanisms further, particularly in diverse populations and settings. Vaccines represent one of the most remarkable achievements in modern public health – and we must continue to ensure their effectiveness and utility.

References

1. Dulfer EA, Geckin B, Taks EJ, GeurtsvanKessel CH, Dijkstra H, van Emst L, et al. Timing and sequence of vaccination against COVID-19 and influenza (TACTIC): a single-blind, placebo-controlled randomized clinical trial. The Lancet Regional Health–Europe. 2023;29.
2. WHO. Guidelines on clinical evaluation of vaccines: regulatory expectations. 2020.
3. Naficy A, Kuxhausen A, Seifert H, Hastie A, Leav B, Miller J, et al. No immunological interference or concerns about safety when seasonal quadrivalent influenza vaccine is co-administered with a COVID-19 mRNA-1273 booster vaccine in adults: A randomized trial. Hum Vaccin Immunother. 2024;20(1):2327736.
4. Lazarus R, Baos S, Cappel-Porter H, Carson-Stevens A, Clout M, Culliford L, et al. Safety and immunogenicity of concomitant administration of COVID-19 vaccines (ChAdOx1 or BNT162b2) with seasonal influenza vaccines in adults in the UK (ComFluCOV): a multicentre, randomised, controlled, phase 4 trial. The Lancet. 2021;398(10318):2277-87.
5. Choi MJ, Yu YJ, Kim JW, Ju HJ, Shin SY, Yang YJ, et al. Immunogenicity and safety of concomitant bivalent COVID-19 and quadrivalent influenza vaccination: implications of immune imprinting and interference. Clin Microbiol Infect. 2024;30(5):653-9.
6. Murdoch L, Quan K, Baber JA, Ho AWY, Zhang Y, Xu X, et al. Safety and Immunogenicity of the BNT162b2 Vaccine Coadministered with Seasonal Inactivated Influenza Vaccine in Adults. Infect Dis Ther. 2023;12(9):2241-58.
7. Izikson R, Brune D, Bolduc J-S, Bourron P, Fournier M, Moore TM, et al. Safety and immunogenicity of a high-dose quadrivalent influenza vaccine administered concomitantly with a third dose of the mRNA-1273 SARS-CoV-2 vaccine in adults aged≥ 65 years: a phase 2, randomised, open-label study. The Lancet Respiratory Medicine. 2022;10(4):392-402.
8. Toback S, Galiza E, Cosgrove C, Galloway J, Goodman AL, Swift PA, et al. Safety, immunogenicity, and efficacy of a COVID-19 vaccine (NVX-CoV2373) co-administered with seasonal influenza vaccines: an exploratory substudy of a randomised, observer-blinded, placebo-controlled, phase 3 trial. The Lancet Respiratory Medicine. 2022;10(2):167-79.
9. Moro PL, Zhang B, Ennulat C, Harris M, McVey R, Woody G, et al. Safety of co-administration of mRNA COVID-19 and seasonal inactivated influenza vaccines in the vaccine adverse event reporting system (VAERS) during July 1, 2021–June 30, 2022. Vaccine. 2023;41(11):1859-63.
10. Janssen C, Mosnier A, Gavazzi G, Combadière B, Crépey P, Gaillat J, et al. Coadministration of seasonal influenza and COVID-19 vaccines: A systematic review of clinical studies. Human Vaccines & Immunotherapeutics. 2022;18(6):2131166.
11. Giannotta G, Murrone A, Giannotta N. COVID-19 mRNA vaccines: the molecular basis of some adverse events. Vaccines. 2023;11(4):747.
12. Debisarun PA, Gössling KL, Bulut O, Kilic G, Zoodsma M, Liu Z, et al. Induction of trained immunity by influenza vaccination-impact on COVID-19. PLoS pathogens. 2021;17(10):e1009928.
13. Benn CS, Schaltz-Buchholzer F, Nielsen S, Netea MG, Aaby P. Randomized clinical trials of COVID-19 vaccines: Do adenovirus-vector vaccines have beneficial non-specific effects? Iscience. 2023;26(5).
14. Yamaguchi Y, Kato Y, Edahiro R, Søndergaard JN, Murakami T, Amiya S, et al. Consecutive BNT162b2 mRNA vaccination induces short-term epigenetic memory in innate immune cells. JCI Insight. 2022;7(22).

15. Stevens NE, Ryan FJ, Messina NL, Blake SJ, Norton TS, Germano S, et al. No evidence of durable trained immunity after two doses of adenovirus-vectored or mRNA COVID-19 vaccines. The Journal of Clinical Investigation. 2023;133(17).
16. Murphy DM, Cox DJ, Connolly SA, Breen EP, Brugman AAI, Phelan JJ, et al. Trained immunity is induced in humans after immunization with an adenoviral vector COVID-19 vaccine. The Journal of Clinical Investigation. 2023;133(2).
17. van Puffelen JH, Keating ST, Oosterwijk E, van der Heijden AG, Netea MG, Joosten LA, et al. Trained immunity as a molecular mechanism for BCG immunotherapy in bladder cancer. Nature Reviews Urology. 2020;17(9):513-25.
18. Wardhana DE, Sultana A, Mandang V, Jim E. The efficacy of Bacillus Calmette-Guerin vaccinations for the prevention of acute upper respiratory tract infection in the elderly. Acta Med Indones. 2011;43(3):185-90.
19. Giamarellos-Bourboulis EJ, Tsilika M, Moorlag S, Antonakos N, Kotsaki A, Domínguez-Andrés J, et al. Activate: randomized clinical trial of BCG vaccination against infection in the elderly. Cell. 2020;183(2):315-23. e9.
20. Moorlag SJ, Taks E, Ten Doesschate T, van der Vaart TW, Janssen AB, Müller L, et al. Efficacy of BCG vaccination against respiratory tract infections in older adults during the coronavirus disease 2019 pandemic. Clinical Infectious Diseases. 2022;75(1):e938-e46.
21. Koekenbier EL, Fohse K, van de Maat JS, Oosterheert JJ, van Nieuwkoop C, Hoogerwerf JJ, et al. Bacillus Calmette-Guérin vaccine for prevention of COVID-19 and other respiratory tract infections in older adults with comorbidities: a randomized controlled trial. Clinical Microbiology and Infection. 2023;29(6):781-8.
22. Nau GJ, Liaw L, Chupp GL, Berman JS, Hogan BL, Young RA. Attenuated host resistance against Mycobacterium bovis BCG infection in mice lacking osteopontin. Infect Immun. 1999;67(8):4223-30.
23. Icer MA, Gezmen-Karadag M. The multiple functions and mechanisms of osteopontin. Clinical Biochemistry. 2018;59:17-24.
24. Shirakawa K, Sano M. Osteopontin in Cardiovascular Diseases. Biomolecules. 2021;11(7).
25. Lin R, Wu S, Zhu D, Qin M, Liu X. Osteopontin induces atrial fibrosis by activating Akt/GSK-3β/β-catenin pathway and suppressing autophagy. Life Sciences. 2020;245:117328.
26. Lau DH, Schotten U, Mahajan R, Antic NA, Hatem SN, Pathak RK, et al. Novel mechanisms in the pathogenesis of atrial fibrillation: practical applications. European heart journal. 2016;37(20):1573-81.
27. Güneş HM, Babur Güler G, Güler E, Demir GG, Kızılırmak Yılmaz F, Omaygenç MO, et al. Relationship between serum osteopontin level and atrial fibrillation recurrence in patients undergoing cryoballoon catheter ablation. Turk Kardiyol Dern Ars. 2017;45(1):26-32.
28. Hulsmans M, Schloss MJ, Lee IH, Bapat A, Iwamoto Y, Vinegoni C, et al. Recruited macrophages elicit atrial fibrillation. Science. 2023;381(6654):231-9.
29. Usher NT, Chang S, Howard RS, Martinez A, Harrison LH, Santosham M, et al. Association of BCG Vaccination in Childhood With Subsequent Cancer Diagnoses: A 60-Year Follow-up of a Clinical Trial. JAMA Network Open. 2019;2(9):e1912014-e.
30. Yoshida K, Ishii S. Innate immune memory via ATF7-dependent epigenetic changes. Cell Cycle. 2016;15(1):3-4.

31. Khan N, Downey J, Sanz J, Kaufmann E, Blankenhaus B, Pacis A, et al. M. tuberculosis Reprograms Hematopoietic Stem Cells to Limit Myelopoiesis and Impair Trained Immunity. Cell. 2020;183(3):752-70.e22.
32. Kleinnijenhuis J, Quintin J, Preijers F, Benn CS, Joosten LAB, Jacobs C, et al. Long-Lasting Effects of BCG Vaccination on Both Heterologous Th1/Th17 Responses and Innate Trained Immunity. Journal of Innate Immunity. 2013;6(2):152-8.
33. Benn CS, Netea MG, Selin LK, Aaby P. A small jab – a big effect: nonspecific immunomodulation by vaccines. Trends in Immunology. 2013;34(9):431-9.
34. Moorlag SJCFM, Folkman L, ter Horst R, Krausgruber T, Barreca D, Schuster LC, et al. Multi-omics analysis of innate and adaptive responses to BCG vaccination reveals epigenetic cell states that predict trained immunity. Immunity. 2024;57(1):171-87.e14.
35. Cedar H, Bergman Y. Linking DNA methylation and histone modification: patterns and paradigms. Nature Reviews Genetics. 2009;10(5):295-304.
36. Netea MG, Joosten LAB, Latz E, Mills KHG, Natoli G, Stunnenberg HG, et al. Trained immunity: A program of innate immune memory in health and disease. Science. 2016;352(6284):aaf1098.
37. Berendsen M, Schaltz-Buchholzer F, Bles P, Biering-Sørensen S, Jensen KJ, Monteiro I, et al. Parental Bacillus Calmette-Guérin vaccine scars decrease infant mortality in the first six weeks of life: A retrospective cohort study. EClinicalMedicine. 2021;39:101049.
38. Tate J, Aguado T, Belie JD, Holt D, Karafillakis E, Larson HJ, et al. The life-course approach to vaccination: Harnessing the benefits of vaccination throughout life. Vaccine. 2019;37(44):6581-3.
39. Scognamiglio F, Fantini MP, Reno C, Montalti M, Di Valerio Z, Soldà G, et al. Vaccinations and Healthy Ageing: How to Rise to the Challenge Following a Life-Course Vaccination Approach. Vaccines. 2022;10(3):375.
40. Dulfer EA, Domínguez-Andrés J. Mechanisms involved in the transmission of trained immunity to offspring. J Allergy Clin Immunol. 2024.
41. Katzmarski N, Domínguez-Andrés J, Cirovic B, Renieris G, Ciarlo E, Le Roy D, et al. Transmission of trained immunity and heterologous resistance to infections across generations. Nat Immunol. 2021;22(11):1382-90.
42. Kaufmann E, Landekic M, Downey J, Chronopoulos J, Teimouri Nezhad S, Tran K, et al. Lack of evidence for intergenerational inheritance of immune resistance to infections. Nat Immunol. 2022;23(2):203-7.
43. Riksen NP, Netea MG. Immunometabolic control of trained immunity. Mol Aspects Med. 2021;77:100897.
44. Schrijver DP, Röring RJ, Deckers J, de Dreu A, Toner YC, Prevot G, et al. Resolving sepsis-induced immunoparalysis via trained immunity by targeting interleukin-4 to myeloid cells. Nature Biomedical Engineering. 2023;7(9):1097-112.
45. Rodriguez JAM, Bifano M, Roca Goma E, Plasencia CM, Torralba AO, Font MS, et al. Effect and Tolerability of a Nutritional Supplement Based on a Synergistic Combination of β-Glucans and Selenium- and Zinc-Enriched Saccharomyces cerevisiae (ABB C1®) in Volunteers Receiving the Influenza or the COVID-19 Vaccine: A Randomized, Double-Blind, Placebo-Controlled Study. Nutrients. 2021;13(12):4347.
46. Dos Santos JC, Barroso de Figueiredo AM, Teodoro Silva MV, Cirovic B, de Bree LCJ, Damen M, et al. β-Glucan-Induced Trained Immunity Protects against Leishmania braziliensis Infection: a Crucial Role for IL-32. Cell Rep. 2019;28(10):2659-72.e6.

47. dos Santos JC, Moreno M, Teufel LU, Chilibroste S, Keating ST, Groh L, et al. Leishmania braziliensis enhances monocyte responses to promote anti-tumor activity. Cell Reports. 2024;43(3).
48. Owen AM, Luan L, Burelbach KR, McBride MA, Stothers CL, Boykin OA, et al. MyD88-dependent signaling drives toll-like receptor-induced trained immunity in macrophages. Front Immunol. 2022;13:1044662.
49. Aaby P, Ibrahim SA, Libman MD, Jensen H. The sequence of vaccinations and increased female mortality after high-titre measles vaccine: trials from rural Sudan and Kinshasa. Vaccine. 2006;24(15):2764-71.
50. Aaby P, Jensen H, Samb B, Cisse B, Sodemann M, Jakobsen M, et al. Differences in female-male mortality after high-titre measles vaccine and association with subsequent vaccination with diphtheria-tetanus-pertussis and inactivated poliovirus: reanalysis of West African studies. The Lancet. 2003;361(9376):2183-8.
51. Clipet-Jensen C, Andersen A, Jensen AKG, Aaby P, Zaman K. Out-of-Sequence Vaccinations With Measles Vaccine and Diphtheria-Tetanus-Pertussis Vaccine: A Reanalysis of Demographic Surveillance Data From Rural Bangladesh. Clinical Infectious Diseases. 2020;72(8):1429-36.
52. Higgins JP, Soares-Weiser K, López-López JA, Kakourou A, Chaplin K, Christensen H, et al. Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review. bmj. 2016;355.
53. Tait DR, Hatherill M, Meeren OVD, Ginsberg AM, Brakel EV, Salaun B, et al. Final Analysis of a Trial of M72/AS01_E Vaccine to Prevent Tuberculosis. New England Journal of Medicine. 2019;381(25):2429-39.
54. Goldblatt D, Alter G, Crotty S, Plotkin SA. Correlates of protection against SARS-CoV-2 infection and COVID-19 disease. Immunological Reviews. 2022;310(1):6-26.
55. Krammer F. A correlate of protection for SARS-CoV-2 vaccines is urgently needed. Nature Medicine. 2021;27(7):1147-8.
56. Gilbert Peter B, Donis Ruben O, Koup Richard A, Fong Y, Plotkin Stanley A, Follmann D. A Covid-19 Milestone Attained — A Correlate of Protection for Vaccines. New England Journal of Medicine. 2022;387(24):2203-6.
57. Earle KA, Ambrosino DM, Fiore-Gartland A, Goldblatt D, Gilbert PB, Siber GR, et al. Evidence for antibody as a protective correlate for COVID-19 vaccines. Vaccine. 2021;39(32):4423-8.
58. Khoury DS, Cromer D, Reynaldi A, Schlub TE, Wheatley AK, Juno JA, et al. Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. Nature Medicine. 2021;27(7):1205-11.
59. Gilbert PB, Fong Y, Hejazi NS, Kenny A, Huang Y, Carone M, et al. Four statistical frameworks for assessing an immune correlate of protection (surrogate endpoint) from a randomized, controlled, vaccine efficacy trial. Vaccine. 2024;42(9):2181-90.
60. WHO. Weekly Epidemiological Record (WER). 2017 02-06.
61. RIVM. COVID-19 vaccinatie uitvoeringsrichtlijn (versie 2). 2024.
62. Sugrue JA, Duffy D. Systems vaccinology studies - achievements and future potential. Microbes Infect. 2024;26(7):105318.

Appendices

Appendix I

Nederlandse samenvatting (Dutch summary)

Vaccinaties zijn een doorbraak geweest in het verminderen van sterfte door infectieziektes. Ze beschermen tegen ziekteverwekkers zoals virussen en bacteriën door een afweerreactie op te wekken zonder dat je zelf echt ziek wordt. Het afweersysteem leert zo om snel en gericht aan te vallen als het in contact komt met de ziekteverwekker.

Het afweersysteem

Het afweersysteem bestaat uit twee delen: het aangeboren deel en het verworven deel. Het verworven afweersysteem zorgt ervoor dat na vaccinatie antistoffen worden gemaakt die precies op de ziekteverwekker passen. B- en T-cellen staan op scherp en onthouden wat ze moeten doen als ze precies deze ziekteverwekker tegenkomen. Zo beschermt een vaccin heel specifiek: een vaccin tegen het mazelenvirus zorgt ervoor dat je een snelle en sterke afweerreactie krijgt als je in contact komt met het mazelenvirus. Daardoor word je niet, of minder ernstig, ziek.

Het blijkt dat sommige vaccins ook effecten hebben die *niet* specifiek op één ziekteverwekker zijn gericht. We noemen dat dan ook de niet-specifieke effecten (NSE). Veel vaccinaties die in de kindertijd worden gegeven blijken dit soort beschermende NSE te hebben. In landen met een hoge infectiedruk zijn relaties gevonden tussen deze vaccins en een afname in babysterfte, die niet verklaard kan worden door alléén hun specifieke bescherming.

Trained immunity

We proberen deze effecten te begrijpen met het concept van ‘getrainde immuniteit’, of *trained immunity*. Dat beschrijft de veranderingen in de cellen van het aangeboren afweersysteem nadat het voor de eerste keer gestimuleerd is met een bepaalde stof, zoals een ziekteverwekker of vaccinatie. Deze veranderingen zorgen ervoor dat er bij een tweede, andersoortige stimulatie, toch sneller en sterker gereageerd wordt. Een vaccin tegen het mazelenvirus zorgt dan dus niet alleen voor een specifieke afweerreactie tegen het mazelenvirus, maar zet het aspecifieke aangeboren afweersysteem ook zo ‘aan’ dat het sterk reageert op andere virussen of bacteriën. We kunnen trained immunity dus beschouwen als een soort aspecifiek ‘geheugen’ van het aangeboren afweersysteem.

Dat is een radicaal nieuwe kijk op immunologie: in de meeste leerboeken wordt een immunologisch geheugen alleen toegedicht aan het verworven afweersysteem. Als we meer over trained immunity begrijpen, zouden we die kennis kunnen gebruiken om vaccins effectiever en veiliger te maken, en betere plannen te maken om mensen te beschermen tegen ziekte.

Het eerste vaccin waarvan werd bewezen dat het trained immunity kon induceren was het BCG-vaccin tegen tuberculose. Later werd duidelijk dat niet alleen vaccins, maar ook infectieziekten zelf en omgevingsfactoren voor trained immunity konden zorgen. Een belangrijk voorbeeld is ons Westerse dieet. Onderzoekers denken nu dat trained immunity mogelijk een rol speelt in allerlei uit de hand gelopen ontstekingsreacties, cardiovasculaire problemen, ineffectieve afweer en sommige soorten kanker. We weten niet precies hoe lang de effecten na een eerste stimulans aanhouden, maar het is mogelijk maanden of zelfs jaren.

Het immuunsysteem van ouderen

Het afweersysteem verandert als we ouder worden. Aan de ene kant zijn veel soorten cellen minder goed in staat om een stevige afweerreactie neer te zetten en nemen de aantallen van bepaalde cellen af. Aan de andere kant ontstaat er een lichte ontstekingsreactie die op de achtergrond steeds aanwezig blijft, ook als er geen gevaar is. Beide factoren spelen een rol in het verhoogde risico voor oudere mensen om ziek te worden, ernstig ziek te worden of complicaties aan hun ziekte over te houden. Om dit tegen te gaan zetten we sommige vaccinaties niet in de kindertijd, maar juist bij ouderen in. Een bekend voorbeeld is de jaarlijkse 'griepprik' tegen het influenzavirus. Helaas werken niet alle vaccins even goed in ouderen als in jongere volwassenen, en weten we nog niet goed hoe dat komt. Ook weten we weinig over mogelijke niet-specifieke effecten in deze groep.

Onderzoeksvragen in dit proefschrift

Het onderzoek in dit proefschrift was bedoeld om beter te begrijpen hoe niet-specifieke effecten precies werken, wat de rol is van trained immunity die ontstaat tijdens infectieziekten en hoe we mogelijk onze manieren van vaccineren kunnen verbeteren. Daarbij denken we aan het verbeteren van vaccins zelf, maar ook aan het optimaliseren van de strategie om ze toe te dienen. Bijzondere aandacht krijgt hierbij de groep van oudere volwassenen.

In de onderzoeken is de bestaande kennis over het BCG-vaccin gebruikt om nieuwe studies op te zetten. Daarnaast lag de focus op vaccinaties tegen COVID-19, omdat deze vaccins net op de markt kwamen toen dit promotietraject begon. Hoewel de

COVID-19 vaccins veilig waren om te gebruiken en effectief beschermden tegen (ernstige) ziekte, was er nog veel onduidelijk over mogelijke andere effecten.

In **hoofdstuk twee** onderzochten we daarom of het immunologisch gezien succesvol is om een COVID-19 vaccin gelijktijdig toe te dienen met een griepvaccin, of dat deze twee vaccinaties beter een paar weken na elkaar gegeven kunnen worden. We vaccineerden ruim 150 vrijwilligers in verschillende volgordes en zagen dat gelijktijdig en sequentieel vaccineren evenveel bijwerkingen gaf, die allen na een paar dagen spontaan verdwenen. De hoeveelheid en functionaliteit van de antistoffen tegen het SARS-CoV-2 virus was verminderd bij gelijktijdige vaccinatie met het griepvaccin, hoewel deze combinatie nog steeds een sterke afweerreactie induceerde. Hoewel niet bekend is hoeveel antistoffen iemand precies nodig heeft om goed beschermd te zijn, doet dit resultaat toch vermoeden dat er mensen zullen zijn die bij gecombineerde vaccinatie net onvoldoende beschermd blijven.

Andere vaccinaties behandelen we in **hoofdstuk drie**, waarin we een overzicht geven van alle vaccins die bewezen hebben dat ze trained immunity kunnen induceren of die zijn gerelateerd aan het optreden van niet-specifieke effecten. Het bleek dat vooral levend-verzwakte vaccins deze effecten hadden op de cellen van het aangeboren afweersysteem. In dit hoofdstuk laten we zien dat dat komt doordat deze vaccins processen in de cellen herprogrammeren om te zorgen dat ze sterker kunnen reageren. Deze kennis zouden we kunnen toepassen in het verbeteren of ontwikkelen van nieuwe vaccins.

Het was nog niet bekend of vaccins tegen COVID-19 ook trained immunity konden induceren, al waren er aanwijzingen dat verschillende typen vaccins verschillende effecten konden hebben. In **hoofdstuk vier** testten we of de cellen van mensen die een mRNA-vaccin hadden gekregen, anders reageerden dan de cellen van mensen die met een vector-vaccin waren gevaccineerd. We zagen dat er verschillen waren tussen de afweer-genen die door deze vaccins 'aan' en 'uit' werden gezet. Cellen uit de vector-groep reageerden met meer ontstekingsstofjes op stimulaties in het lab, wat kan betekenen dat deze mensen een nieuwe ziekteverwekker of ontsteking sneller onder controle hebben. Dit kan een aanwijzing zijn dat vector-vaccins beschermende niet-specifieke effecten hebben die niet gelden voor de mRNA vaccinaties.

Ten tijde van **hoofdstuk vijf** waren deze specifieke COVID-19 vaccins nog niet ontwikkeld. We hebben toen onderzocht of een vaccin tegen het griepvirus niet-specifieke effecten had die beschermend zouden kunnen zijn tegen COVID-19. Dit beschermende effect hebben we in een studie in Brazilië niet kunnen aantonen.

Wel hebben we hierdoor veel kennis opgedaan over het doen van onderzoek in het midden van een pandemie, wat belangrijke leerpunten heeft opgeleverd voor toekomstige studies.

In een eerdere studie is onderzocht of een BCG-vaccin ouderen kon beschermen tegen COVID-19, wat helaas ook niet het geval bleek. Interessant was echter of er andere beschermende effecten konden zijn. **Hoofdstuk zes** gaat daarom dieper in op de niet-specifieke effecten van het BCG-vaccin bij ouderen. We vergeleken de hoeveelheid nieuwe medische diagnoses, nieuwe infecties en ziekenhuisopnames van de mensen die een BCG-vaccinatie hadden ontvangen en de mensen die een nep-vaccin (placebo) hadden gehad. Omdat deze twee groepen vóór vaccinatie precies op elkaar leken, konden we dit goed vergelijken. Twee jaar na vaccinatie was er geen verschil te vinden in de aantallen of soorten medische problemen van de deelnemers, op één specifieke categorie na. Ouderen die het BCG-vaccin hadden gekregen belandden vaker in het ziekenhuis vanwege een verergering van een hartritmestoornis. Onze hypothese is dat dit komt doordat het BCG-vaccin zorgt voor een toename van het stofje osteopontin, wat eerder in verband is gebracht met ritmeproblemen.

Ten slotte onderzochten we in **hoofdstuk zeven** wat er bekend is over niet-specifieke effecten die optreden na infectieziekten, in plaats van na vaccinatie. Bij sommige patiënten zijn de klachten namelijk niet voorbij als de ziekteverwekker al niet meer aan te tonen is, en daar speelt trained immunity mogelijk een rol in. In patiënten met bijvoorbeeld post-COVID of het post-intensive care syndroom is er soms sprake van een overdreven ontsteking of juist een lamgelegd afweersysteem: als we beter kunnen inschatten waar patiënten op dat spectrum zitten, kunnen we ze een persoonlijkere behandeling aanbieden met betere resultaten.

Ten slotte

De resultaten in dit proefschrift zijn van belang voor de ontwikkeling van nieuwe vaccins en het bepalen van optimale strategieën voor hun toepassing. Ook biedt het waardevolle inzichten voor artsen die zich bezighouden met de behandeling van infectieziekten en de gevolgen daarvan. Het is belangrijk om te benadrukken dat immunologie niet het enige vakgebied is wat zich over deze vraagstukken buigt, en dat voor het ontwikkelen van effectief beleid meer samenwerking met verschillende disciplines nodig is. Binnen de immunologie zijn specifieke ontwikkelingen nodig om trained immunity nog beter te begrijpen en te kunnen vertalen naar praktische implicaties. We doen in dit proefschrift aanbevelingen voor nieuw onderzoek om de hiaten in de huidige kennis te vullen en ervoor te zorgen dat vaccinaties een belangrijke doorbraak blijven, ook in de toekomst.

Appendix II

Dankwoord

Beste **prof. Netea**, beste Mihai, enorm bedankt voor alles in dit proefschrift en de weg daar naartoe (en omheen). Het was een eer om op dit lab te mogen werken en samen niet alleen immunologie, maar ook de staat van de wereld in het algemeen te kunnen bespreken. Je bent een van de vriendelijkste en meest toegewijde mensen die ik de afgelopen jaren heb leren kennen en ik weet zeker dat die houding veel andere artsen en onderzoekers zal blijven inspireren.

Beste **prof. Van Crevel**, beste Reinout, ons contact de afgelopen drie jaar werd vooral gekenmerkt door hoge kwaliteit. Zeer memorabel was onze onderzoeksmeeting in Cluj, die zo inspirerend was dat de hele groep er nog dagen over doorpraatte. Als enorm goede schrijver en zeker ook denker was je een fijne balans in een baan die soms toch vooral om veel en snel dreigt te gaan. Ik heb veel geleerd van je zorgvuldige feedback, niet alleen voor mezelf, maar ook om door te kunnen geven aan anderen.

Beste **dr. Van de Maat**, lieve Josephine, wat fijn om jou te hebben leren kennen en de afgelopen jaren van jou te hebben mogen leren. Je gaf me een kickstart voor dit promotietraject en een goed voorbeeld van ‘combineren kun je leren’ (toevallig ook een van mijn hobby’s). Alle dankbaarheid ten aanzien van jouw heldere schrijfstructuur, data cleaning kwaliteiten in SPSS en beleefde reply-to-reviewers-stijl ten spijt: over tien jaar denk ik waarschijnlijk vooral aan onze gesprekken over de opleiding en carrièreplanning, doorgehaalde nachten (contrasterende oorzaken), vakantieplannen, dubbele predestinatie, ratatouille, toga’s en jurkjes, koffie en de havermelkelite. Een PhD was met jou heel veel meer dan wat publicaties afvinken. Een speciaal bedankje voor de momenten met David en Flore, en ik hoop dat ze later alles gaan doen wat ze willen.

Dear **dr. Domínguez-Andrés**, querido Jorge, el español no es muy fácil pero me gusta intentarlo. Pipetear tampoco siempre fue fácil. Muchas gracias por tu ayuda estos últimos años. Gelukkig kun je ook gewoon nagenoeg-C2-Nederlands, en is onze voertaal inmiddels een leuke mix. Our first question when we hadn’t seen each other in a while was usually “what’s the tea??”, and it was great to see you being so invested not only in our research, but in our shenanigans as well. Je had de twijfelachtige eer mijn eerste aanspreekpunt te zijn als ik ergens niet meer uitkwam en besloot dat dat nu ook jouw probleem was. Gelukkig maakt een lachend rondkruipende Nico alles goed. Keep me posted for his first Vierdaagse steps.

Beste **prof. Joosten**, niet mijn supervisor, maar wat is ons L.A.B. zonder Leo? Jij was de oorzaak van veel onderdrukt gegniffel tijdens meetings die serieus pretendeerden te zijn en een onuitputtelijke bron van wat er rond ging in de literatuur en wandelgangen. Jouw wetenschappelijke expertise en enthousiasme maakten deze PhD nog leuker dan het al was.

Beste **prof. De Jonge**, de eerste studie van dit proefschrift en mijn PhD was logistiek (en socio-politiek) een uitdaging, maar dat bleek prima op te lossen als je de juiste mensen weet te treffen. Ik wil u graag bedanken voor alle hulp tijdens de TACTIC trial, het inhoudelijke meedenken over de resultaten, en vooral de zeer prettige manier waarop dat ging.

Beste **dr. Cremers**, lieve Amelieke, of het nu dokteren, onderzoeken of managen (of skiën) gaat worden, als wij af en toe nog een project samen kunnen doen, *I'm down for anything*. Heel erg bedankt voor het starten van mijn wetenschappelijke footprints en ik hoop dat we elkaar via side-tracks nog vaak weten te vinden.

Beste **dr. Koeken**, lieve Valerie, je hebt ons lab al een tijdje verlaten maar jij was de eerste (toen nog *to be*) collega die ik leerde kennen. Met jouw geduld, kennis en verhalen over Braziliaanse cocktails heb je een grotere rol gespeeld in mijn promotietraject dan je je misschien kunt voorstellen. Ik ben blij dat ik jou ook iets heb kunnen leren (over vogels, januari, en erg vroege ochtenden…) en ben heel blij dat je je plek gevonden hebt in Rotterdam en Breda.

De lijst geweldige collega's binnen en buiten het lab is (vrij letterlijk) eindeloos, maar jullie verdienen allemaal een geweldige shout-out – te beginnen met de onmisbare analisten **Helga**, **Heidi**, **Liesbeth**, **Malin** en **Hanneke**, en extra dank voor het sfeerbeheer van **Andy** en **Ilse**. Het is bijzonder hoe jullie die permanent wisselende groep onderzoekers helpen om tussen het harde werken door een sfeer te creëren waarin iedereen klaarstaat voor elkaar, alle vragen gesteld én beantwoord kunnen worden en waarin je je meteen welkom voelt. Ook veel dank aan de clinical trials unit met in het bijzonder **Sonja**, **Margot** en **Lieke**: zonder jullie was ik nu nog op zoek geweest naar noodadrenaline of een intradermale naald (of een schuilkelder, deelnemer 021…).

The lab people, whether your fav lab equipment was 'the (electronic) (multichannel) pipet' or 'Malin/the stickers', more or less kept in line by **Jaegermeister Martin**: **Adriana**, **Ajie**, **Aline**, **Alisa**, **Brenda**, **Cas**, **Corlinda**, **Daniela**, **Diletta**, **Dineke**, **Dorien**, **Eleen**, **Eveline**, **Frank**, **Harsh**, **Helin**, **Ilyas**, **Janneke**, **Jasmijn**, **Jelle**, **Jelmer**,

Jéssica, **Job**, **JuliaB**, **JuliaT**, **Kai**, **Lieke**, **Linda**, **LisaK**, **Lisanne**, **Lotte**, **Lucy**, **Maaike**, **Maartje**, **Manon**, **Margo**, **Marijn**, **Mariolina**, **Mike**, **Nadira**, **Peng**, **Prashant**, **Quirien**, **Siroon**, **Suzanne**, **Tomas**, **Twan**, **Vicky**, **Victoria**, **Wieteke**, **Wouter** and **Zara**. There is not enough space here to delve into all our adventures, but I'm sure you'll understand what I'm referring to: peacocks in Lisbon, Greek crossing techniques in Naples, aggressive wine-pouring and hushed conversations in Cluj, saving the climate in Odense and of course *that's not my name!*, Betty, het RIVM, "Teams is not letting me in", phantom-Sysmex sounds, secret cryovial stashes, the apparent attractiveness of studying sperm cells, a hundred kilos of bread, tequila is 'best ok', *C. Elegans*, anything but clothes, XXL Sup, the 4D stage at the Grote Markt, *I'm the problem it's…*, ABBA double tempo, "als ik jullie brugklasleraar was", directing pigeons / ghosts / elderly / seahorses, and many many more happy memories.

A special mention for the journal club team, captained by Jorge and **Anaísa** (also a great road trip buddy and apartment roomie), with a variable composition of **Büsra** (best training period ever), **Esther** (ups downs turn around sip mimosas repeat), **Gizem** (LEKKER MET DE MEIDEN SHINGRIX), **Özlem** (eternally impressive), **Thanasis** (never a dull moment), **Laura** (ohh but some moments…), **Ilayda** (started from a cloister now we're here), **Eli**, **Noriko**, **Titus**, **Robbin**, **Laszlo**, **Lorenzo**, **Patricia** and **Flavia** (miss you!).

Eervolle vermelding voor het **Mulderlab** en hun integratieprogramma: soms een silent partner, soms life of the party. Ik heb diep respect voor jullie werk, soms iets minder voor jullie muzieksmaak (uitzondering voor Goldband, Jeroen).

Een aantal extra bijzondere herinneringen zijn voor mijn roomies **Rutger** en **Clementine**, mijn lichtende voorbeelden in cytokines en datamanagement (grapje), waardoor dit hele avontuur vanaf het begin écht leuk was. Bijzonder trots op jullie Aussie-adventures en opleidingsplek! Het liep wat uit de hand toen jullie vervangen werden door **Pepijn** en **Leonie**. Ik weet niet of we onszelf weer veilig in de maatschappij kunnen plaatsen, maar ik weet wel dat ik me zelden met tegenzin naar onze office hoefde te slepen 's ochtends. Het liefst neem ik jullie mee naar al mijn toekomstige banen ooit. Jullie zijn bijzonder briljant (geen grapje) en blaartrekkend hilarisch. De database die ik zonder jullie consent verzameld heb is er echt eentje voor in de boeken en ik ben al helemaal ready voor volume II. **Julia H**, onze band begon met kaas-kat en heeft zich via jouw balkon, festivals, haarbanden, verschrikkelijke én leuke mannen, salsa en jazzballet ontwikkeld tot iets waarin ik jou één van mijn favoriete powervrouwen op deze wereld kan noemen. Speaking of power, **Lisa T**, that is you: great to watch you rock!

Van alle soap-taferelen op het lab hebben ook mijn parel van een huisgenoot en onze besties kunnen meegenieten: liefste **Nyn**, **Tess**, **Myr**, **Lou**, **Maaike**, **Inge**, **Linda**, **Renée**, **Lisa vL**, **Fred**, **Han**, **Maartje** en **Lau**, jullie worden ten aller-, aller diepst bedankt voor jullie geduld en adviezen (play harder!) en vriendschap die keer op keer duidelijk de landsgrenzen en mijn mooiste dromen weet te over- en ontstijgen. Jullie zijn echt heel speciaal.

Aan de **ESHPM-crew**: ja, ik mis de DAGs ook in dit boek. Daarom speciaal voor jullie, inclusief de tentamenvraag: noem ten minste zes dingen die niet kloppen in deze DAG, zet de coëfficiënten bij de juiste pijl en onderzoek etnografisch het badkuip-model. Veel liefde voor jullie en jullie inspiratie.

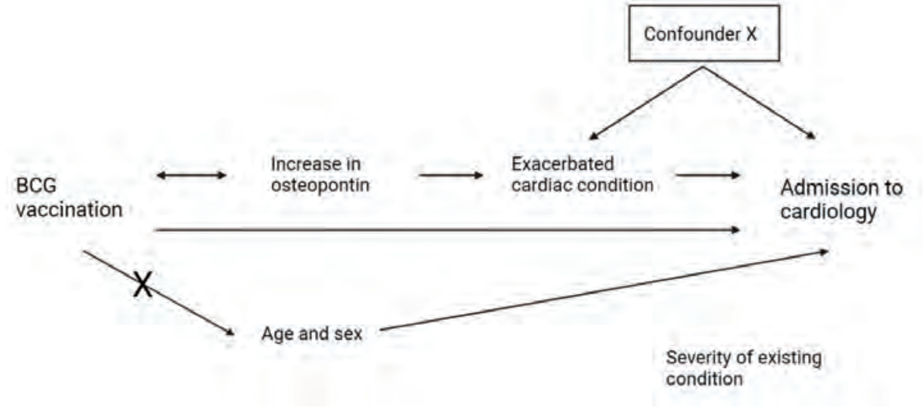

Figure 1. DAG for chapter six.

Lieve **Julia**, **Myrthe** en **Eline**, *buddy from another study*: jullie waren de gestoomde melk in mijn cappuccino en de onvolprezen interdisciplinaire PhD-oplossingscommissie. Onze PuBliBo's lopen zoals gebruikelijk weer eens hopeloos achter, dus wat enorm prettig dat we vier verdedigingen krijgen om het in te halen. Mur, lief hoe je soms door leek te hebben wat ik nodig had zonder dat ik het zei of zelf wist. Juul, speciale dank voor onze leesclub en wederzijdse kritische kanttekeningen en cheerleadergeluiden. Mijn levensgeluk en dit proefschrift gingen er op vooruit!

Lieve **Carleen**, mijn paranimf-aanzoek aan jou was hilarisch (voor Gijs), verwarrend (voor jou) en spannend (voor mij), maar gelukkig zei je 'ja'!! Als onderzoeker ben je indrukwekkend en als vriendin nog meer. Niet de hele wereld weet wat een tumormarge of monocyt is, moeten we elkaar blijven voorhouden, en sommige dingen moet je doen om ze gedaan te hebben. Het was erg fijn om samen langs vergelijkbare obstakels te navigeren, ook al lijken onze studies inhoudelijk in niks op elkaar. Heel gaaf dat je nu slechts een gebouw verderop zit te shinen. We weten allebei wat heel hard werken is en hopelijk leren we ooit nog hoe heel hard lopen voelt, maar nu eerst: heel hard ginto's drinken.

Liebe **Konstantin**, es war ein Abenteuer. Arts-zijn hier clasht soms met de onderzoekswereld, maar ook met artsen die zich daar niet fulltime in begeven. Heel erg fijn om jou daarin te gevonden te hebben en samen te kunnen spuien zonder consequenties. Soms hadden we het onszelf makkelijker kunnen maken, maar wellicht waren we dan nooit in de positie beland waar we een espresso tonic leerden kennen. Voor al het andere dat ik wil zeggen refereer ik uiteraard graag naar Sonnet XXV, XXIX en CIV. Bedankt dat jij op deze dag de AIG-kant wil vertegenwoordigen en, op alle andere dagen, ook vooral de andere aspecten van het leven!

Lieve **Moon**, ik kijk nu al uit naar het moment dat ik jouw onderzoeken kan lezen, en hoop dat ik tegen die tijd kan reproduceren wat een MOSFET is. Het lijkt me erg fijn om een PhD te behalen, maar ik weet zeker dat het nog leuker is om jouw zus te zijn. Dames Dulfer, it's a team (met flamingosokken).

Ten slotte, lieve **dokter FTD** en **dr. SFV**, oftewel, papa en mama: bedankt. Champagne. Liefs ❤️

Appendix III

List of publications

1. **Dulfer EA**, van der Meer JWM, Netea MG. Immunosenescence and infections. *Oxford Textbook of Geriatric Medicine. In press.*
2. **Dulfer EA**, Domínguez-Andrés J. Mechanisms involved in the transmission of trained immunity to offspring. *Journal of Allergy and Clinical Immunology.* 2024 Nov;154;1117-1119.
3. **Dulfer EA***, Föhse K*, Taks EJM, Moorlag SJCFM, Koekenbier EL, van de Maat JS, ten oever J, Hoogerwerf JJ, van Werkhoven CH, Bonten MJM, van HylckamaVlieg A, Rosendaal FR, Netea MG. The effect of BCG vaccination in the elderly on infectious and non-infectious immune-mediated diseases. *Journal of infection*. 2024 Oct;89;106344.
4. **Dulfer EA**, Serbée MJV, Dirkx KKT, Schaars CF, Wertheim HFL, de Jonge MI, Cremers AJH. Cardiovascular events after invasive pneumococcal disease: a retrospective cohort study. *International Journal of Infectious Diseases*. 2024 Oct;147;107185.
5. **Dulfer EA**, Joosten LAB, Netea MG. Enduring echoes: Post-infectious long-term changes in innate immunity. *European Journal of Internal Medicine*. 2024 May;123;15-22.
6. Baydemir I*, **Dulfer EA***, Netea MG, Domínguez-Andrés J. Trained immunity-inducing vaccines: Harnessing innate memory for vaccine design and delivery. *Clinical Immunology*. 2024 Apr;261;109930.
7. **Dulfer EA**, Geckin, B, Taks, EJ, GeurtsvanKessel CH, Dijkstra H, van Emst L, van der Gaast-de Jongh CE, van Mourik D, Koopmans PC, Domínguez-Andrés J, van Crevel R, van de Maat JS, de Jonge MI, Netea MG. Timing and sequence of vaccination against COVID-19 and influenza – Author's reply. *The Lancet Regional Health–Europe.* 2023 Jul;30;100669.
8. **Dulfer EA**, Geckin, B, Taks, EJ, GeurtsvanKessel CH, Dijkstra H, van Emst L, van der Gaast-de Jongh CE, van Mourik D, Koopmans PC, Domínguez-Andrés J, van Crevel R, van de Maat JS, de Jonge MI, Netea MG. Timing and sequence of vaccination against COVID-19 and influenza (TACTIC): a single-blind, placebo-controlled randomized clinical trial. *The Lancet Regional Health–Europe.* 2023 Jun;29;100628.
9. Serbée MJV, **Dulfer EA**, Dirkx KKT, Bosboom R, Robberts B, Wertheim HFL, Mulder B, de Jonge MI, Schaars CF, Swanink CMA, Cremers AJH. C-reactive protein to rule out complicated pneumococcal disease manifestations: a retrospective cohort study in adults with pneumococcal bacteraemia. *International Journal of Infectious Diseases*. 2021 Oct;111;172-178.

* These authors have contributed equally to the manuscript and share first authorship.

Acknowledged contributions

Blok B, Slagt C, van Geffen GJ, Koch R. Characteristics of trauma patients treated by Helicopter Emergency Medical Service and transported to the hospital by helicopter or ambulance. *BMC Emergency Medicine*. 2024 Sept;24;173.

Appendix IV

Data management

Part of this thesis is based on the results of research involving human participants, which were conducted in accordance with relevant national and international legislation and regulations, guidelines and the Radboudumc policy. The recognized Medical Ethics Committee 'METC Oost-Nederland' or the 'Utrecht Institutional Review Board' had given approval to conduct these studies (TACTIC: NL77590.091.21; BCG-PRIME: NL74730.041.20; BCG-CORONA-ELDERLY: NL73430.091.20). The competent authority (CCMO) approved the TACTIC trial (EudraCT number 2021-002186-17). The study presented in chapter six was not subject to the Dutch Medical Research Involving Human Subjects Act (WMO), which was confirmed by the recognized ethics committee (2022-13462).

Informed consent was obtained from all participants to collect and process their data for this research. If explicit consent was given, the study data could be re-used for new research questions and will be provided pseudonymized upon request to the Principal Investigator. The pseudonymization key was stored on a secured network drive that was only accessible to those project members whose role required this. The pseudonymization key was stored separately from the research data. Informed consent forms were stored on paper in department archive cabinets, separately from the research data.

Data for chapters two, four, five and six was obtained through direct entry of information in electronic case report forms (eCRFs) or online questionnaires in Castor EDC, which enabled monitoring and auditing. Data were converged from Castor EDC to Excel and SPSS (SPSS Inc., Chicago, Illinois, USA). Laboratory experiments in chapters two, four and six used newly obtained biomaterial from the participants and the results and analyses were stored on the departmental server with restricted access, as well as the digital research environment (DRE).

The Radboud Data Repository is used to make our study data findable and accessible, after the corresponding papers have been published and if participants have explicitly consented to this. Data on the TACTIC trials (chapters two and five) are, after publication of the corresponding manuscripts, available for re-use through persistent identifier https://doi.org/10.34973/cffd-wr15, under license RUMC-RA-DUA-01. We did not obtain consent to publicly re-share the data from BCG-LT (chapter six) or the Brazilian Influenza study (chapter five), which have not been published yet. After publication, the existence of these data will be made known via the RDR but no external access will be granted. The data will be stored in an acquisition collection after transferring from the departmental server. The TACTIC datasets were published with restricted access and requests for access will be checked by the responsible PIs against the conditions for sharing the data as described in the signed Informed Consent. The UMC is the legal owner of this data. The data comprises raw data in .csv format, read-me files in .txt and processed files in .xlsl format with a codebook attached. The read-me files provide a brief overview of the studies, variables and responsible parties. All the data can be opened with generally available software tools such as SPSS or R. All studies are published open access.

Appendix V

PhD portfolio

Department: Internal Medicine
PhD period: 01-04-2021 – 01-10-2024

PhD supervisors: prof. M. G. Netea, prof. R. van Crevel
PhD co-supervisors: dr. J. S. van de Maat, dr. J. Domínguez-Andrés

Training activities	Hours
Courses	
Radboudumc - Introduction day and course "In the lead of my PhD" (2021)	21.00
Radboudumc - eBROK course and re-registration (2021, 2024)	31.00
RU - Scientific Outreach to Children (2022)	42.00
RU – Writing scientific articles (2022)	96.00
RU – Analysing longitudinal and multilevel data using R (2022)	96.00
RU – Design and Illustration (2022)	26.00
Radboudumc - Scientific Integrity (2023)	20.00
RU – Mindfulness-Based Stress Reduction (2024)	45.00
Conferences	
32rd ECCMID, oral presentation (2022) – Lisbon	56.00
Optimmunize, poster presentation (2022) – Odense	30.00
Summer Innate Immunology Conference, poster presentation (2022) – Cluj-Napoca	24.00
5th international symposium on Trained Immunity, poster presentation (2023) – Naples	32.00
RU – PhD retreat, 2x poster and 1x oral presentation (2021, 2022, 2023)	64.00
Seminars	
Departmental Cytokine Meeting attendance and presentations (2021-2024)	130.00
CBG Collegedag: Balans in roerige tijden (2021)	7.00
Departmental Journal Club attendance and presentations (2022-2024)	30.00
Ministerie voor VWS: research presentation (2022)	8.00
Interne geneeskunde: het fundament voor de toekomst (2022)	5.00
Erasmus university ESHPM: toekomst van de Nederlandse gezondheidszorg (2022)	6.00
Workshop on COVID-19 pathways and omics data analysis (2022)	24.00
Radboud community for infectious disease Science Day, 1x laptop presentation (2022, 2024)	22.00

Lecturing	
Host-Microbe Interaction, master's course Medical Biology (2022, 2023, 2024)	32.00
Medisch-tandheelkundige interactie, bachelor's course Dentistry (2024)	16.00
Supervision	
Supervision Master review assignment (1 month) (2023)	12.00
Supervision MBO internship (4 months) (2023)	64.00
Total:	939.00

Appendix VI

Curriculum vitae

Elisabeth Astrid Dulfer (1996) werd geboren in Leiden en ruilde 18 jaar later het inmiddels zuidelijkere deel van Nederland in voor een studie Geneeskunde in Nijmegen. Haar studententijd werd gekenmerkt door grote toewijding aan zowel studie-gerelateerde zaken als extra-curriculaire projecten en avonturen. Een gecombineerd hoogtepunt was haar stage bij King's College in Londen, waar ze onderzoek deed naar regulatoire T-cellen. Tijdens haar coschappen startte zij met de deeltijdmaster Zorgmanagement aan de Erasmus Universiteit in Rotterdam.

In 2021 behaalde ze *cum laude* haar artsexamen en rondde ze haar tweede master af. Met de ambitie om "nog even iets heel gaafs te doen" begon zij aan een promotieonderzoek op het lab Algemene Interne Geneeskunde, onder leiding van prof. Netea en prof. Van Crevel. Dit proefschrift is het resultaat van haar onderzoek naar vaccinaties en trained immunity. Gedurende haar promotietraject presenteerde Elisabeth haar onderzoek op verschillende internationale congressen en gaf zij onderwijs over onderwerpen op het snijvlak van biologie en geneeskunde aan bachelor- en masterstudenten. Na een aanvullende periode als postdoctoraal onderzoeker op het gebied van immune ageing markeerde het eind van 2024 ook het eind van de tijd op het AIG lab.

Elisabeth kijkt uit naar een toekomst waarin zij optimale patiëntenzorg wil leveren in de intensieve zorg en het beschouwende veld. Daarnaast zal zij zich richten op haar interesse in de organisatie van gezondheidszorg en zich blijven inzetten voor kunst en cultuur.

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