Host and Environmental Factors Modulating Innate Immune Responses

Gizem Kilic

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"Nature does not hurry, yet everything is accomplished."

Lao Tzu

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Eurasian blue tit
Mavi baştankara
Pimpelmees

CHAPTER 1

Introduction, aim, and outline of the thesis

The Immune System

The immune system is a vital part of our body that keeps us alive against the usually invisible dangers of the world. It has a crucial role in host defense against dangerous pathogens, performs immune surveillance against cancer, and contributes to tissue homeostasis and regeneration. During host defense, the immune system employs a complex array of physical barriers, diverse proteins, and various cell populations to combat and eliminate toxins or pathogens. The immune system has been traditionally divided into two components based on their specificity to antigens: the innate immune system, which recognizes a limited number of evolutionarily conserved molecules and mounts a rapid response during minutes and hours, and the adaptive immune system, whose response is specifically tailored to the threat by recognizing specific antigens of the pathogens and develops more slowly during days and weeks.

The innate immune system relies on physical and chemical barriers and cellular mechanisms mediated by both myeloid cells such as monocytes/macrophages, neutrophils, and dendritic cells (DCs), as well as lymphoid cell populations such as natural killer (NK) cells, and immunoregulatory molecules produced by these cells. [1]. The cells of the innate immune system recognize evolutionarily conserved structures of the microorganisms called pathogen-associated molecular patterns (PAMPs) through their receptors like Toll-like receptors (TLRs) and NOD-like receptors (NLRs). In certain conditions, recognition of self-structures of the host itself, called danger-associated molecular patterns (DAMPs), can also stimulate innate immunity.

The adaptive immune system, on the other hand, is characterized by its ability to generate specific responses. It consists of two main types of cells: T cells and B cells. Depending on their subset, T cells are responsible for killing infected cells directly (CD8 lymphocytes) or for helping other cells to get activated and regulating the response (CD4 lymphocytes) [2]. The adaptive immune system is extremely specific due to the vast array of antigen receptors on B and T cells, each being capable of recognizing a unique antigen. Upon such recognition, the corresponding cell undergoes rapid proliferation and expansion, ensuring an adequate response.

The innate and adaptive immune systems were considered separate entities for many years. Dr. Charles Janeway was the first to propose in 1989 that they are interconnected and that the pattern recognition receptors (PRRs) on innate immune cells detect conserved structures of the microorganisms, subsequently activating the adaptive immune system [3]. In fact, this led the way to the discovery of the Toll-like receptors (TLRs) and other components of the innate immune system that play

essential roles in initiating and shaping adaptive responses. Since then, the concept of the interconnection between innate and adaptive immune responses has been developed: the innate immune system provides the initial defense and activates the adaptive system, while the adaptive immune system promotes and regulates innate responses.

Trained Immunity

The idea of innate and adaptive immune systems being independent was not the only big misconception in the immunology field. Originally, immunological memory was attributed only to the adaptive immune system. However, recent studies have challenged this concept, showing the capacity of the innate immune system cells, e.g., NK cells, monocytes and neutrophils, to exhibit immunological memory. Recent studies have shown that certain infections or vaccinations reprogram the innate immune cells and their progenitors to respond stronger and better upon future heterologous encounters. This phenomenon, termed trained immunity, confirmed and explained the epidemiological observations reporting a lower all-cause mortality in individuals vaccinated with measles, oral polio, or BCG vaccines in countries such as Guinea-Bissau and Nigeria [4]. Several DAMPs (oxLDL, uric acid, etc.), PAMPs (low dose LPS, β-glucan), and particularly live-attenuated vaccines have been reported to induce trained immunity. Among these, BCG is a well-studied vaccine that induces trained immunity.

Different epigenetic, metabolic, and transcriptomic changes are involved in the development of trained immunity. Trained cells show epigenetic rewiring at both histone and DNA levels. As an example, after exposure to the BCG vaccine, monocytes exhibited increased H3 lysine 4 trimethylation (H3K4me3) and H3 lysine 27 acetylation (H2K27ac) at the promoters of pro-inflammatory genes [5]. Furthermore, BCG-vaccinated infants showed a persistent BCG-associated DNA methylation signature in their monocytes, which was associated with viral response pathways [6].

Changes in cellular metabolism are another molecular mechanism supporting increased responsiveness of the trained innate immune cells. BCG vaccination leads to the upregulation of multiple metabolic pathways, including aerobic glycolysis, oxidative phosphorylation, cholesterol synthesis, and glutaminolysis [7]. Importantly, studies performed at the single-cell level have demonstrated the existence of specific myeloid cell sub-populations that respond with different trained immunity programs geared towards improved responses to bacterial or viral pathogens [8].

Moreover, as monocytes in the circulation have only a short lifetime, studies have shown that vaccination with BCG resulted in a persistent transcriptional program in the hematopoietic stem and progenitor cells (HSPCs) linked with the myeloid compartment [9] that ensures long-term effects.

On the one hand, the induction of trained immunity has significant benefits for vulnerable populations, specifically for immunodeficient individuals, fragile older adults, and children in areas with high infectious disease burden, as it reduces morbidity and mortality to a broad range of infections. On the other hand, trained immunity might have harmful effects if not adequately regulated. For instance, inappropriately increased immune responsiveness induced by DAMPs could lead to tissue damage, chronic inflammatory diseases and autoimmune disorders [10, 11].

Factors Affecting Innate and Trained Immunity

The innate immune system, along with trained immunity, is significantly influenced by a combination of internal and external factors. Among the host factors, age, sex, and genetic variation impact innate immune responses. Environmental influences extend to, but are not limited to, seasonal variations, diet, lifestyle, infections and vaccinations (termed immunobiography), and the use of medications.

Host-Related Factors

Aging of the immune system is characterized by higher systemic inflammation, decreased adaptive immune responses, and impaired PRR activation and signaling. Hence, older individuals exhibit dysregulated immune responses upon infections and poor vaccination efficacy [12]. Despite the defective innate immune system and responses, older individuals can develop trained immunity [13]. Moreover, the induction of trained immunity by BCG was shown to reduce acute upper respiratory infections in older individuals [14]. How aging impacts the immune system, vaccine responses, and trained immunity is explained in more detail in **Chapter 2**.

Sex has a considerable impact on immune responses. Genetics, sex hormones and environmental influences are the main drivers of the immune differences between males and females [15]. *TLR7* gene expression, type I IFN activity, antibody production, as well as $CD4^+$ T and B cell numbers are typically higher in females than males. In line with this, females have more robust vaccination responses and are more prone to developing autoimmune diseases. On the other hand, males produce more pro-inflammatory cytokines from their macrophages, have higher NK and

$CD8^+$ T cell counts, and have an increased risk of developing atherosclerosis and non-reproductive cancers [16]. Furthermore, epidemiological studies suggested that vaccines' heterologous effects might differ in females and males. As an example, a non-live malaria vaccine was associated with two times more all-cause mortality in girls compared to boys [17]. A similar trend was also observed after DTP and hepatitis B vaccination in girls. Sex differences should be further investigated and considered when designing vaccination schedules or immunotherapy treatment.

Humans have tremendous genetic diversity, which contributes to the modulation of the immune system and responses. A study performed on 497 adult female twins reported that 76% of the immune traits were determined by genetics, while 24% were influenced by the environment [18]. Furthermore, this study showed that genetics has a more considerable influence on adaptive immune traits, whereas environment is more important for innate immune traits. Genetic variations also contribute to the strength of BCG-induced trained immunity responses. A recent study showed that polymorphisms associated with trained immunity after BCG vaccination are enriched in pathways related to cellular metabolism [19]. Another research demonstrated the importance of genetic variants in the SIGLEC14 and KDM4 genes for trained immunity responses [20].

Environmental Factors

Seasonality is an important parameter shaping the immune system's function and responses. Seasonal factors include UV, humidity, and temperature. A study performed in the UK reported the seasonal expression profile of more than 4000 protein-coding genes with inverted patterns in two hemispheres [21]. During the European winter, a higher C-reactive protein (CRP) and soluble IL-6 receptor abundance were found in the circulation. Seasonal variation was also seen in circulating IL-18, IL-18BP, resistin, and α-1 antitrypsin concentrations, the cytokine production capacity of PBMCs upon different pathogens, and immune cell population counts [22]. Some studies suggest the seasonality of vaccine-induced antibody production, although the effects are mild or moderate. For instance, summer vaccination led to a slower decrease in antibody production in boys after DTP vaccination compared to winter vaccination, whereas no significant difference was observed in girls [23]. As many innate immune parameters are influenced by season, it is rational to propose that trained immunity induction follows a seasonal pattern. Indeed, a recent study showed the seasonality of BCG-induced innate immune memory with a higher response during the winter months [19].

The history of past infections and vaccinations is a significant determinant shaping the immune system's response to future encounters. The term “immunobiography” was proposed in 2017 by Dr. Claudio Franceschi and defines the combination, dose and sequence of antigenic stimuli to which we are exposed throughout life [24]. This concept emphasizes how these immunological experiences regulate the adaptation of the immune system continuously, creating heterogeneity in its responses. Each antigenic encounter might shift the baseline parameters of the immune system for a short or long period, influencing the subsequent responses. As an example, sepsis leaves epigenetic marks on alveolar macrophages even after recovery, rendering the host more susceptible to subsequent infections [25]. Prior mild COVID-19 infection shifted the immune system in a sex-dependent manner and influenced the response after influenza vaccination [26]. Moreover, incubation with IFNβ and IFNγ resulted in epigenetic modifications and transcriptional memory in mouse embryonic fibroblasts and bone marrow-derived macrophages, enhancing the defense after viral stimulation [27]. These changes can explain how various infections change the immune response to following infections.

The impact of medication use on the immune system can be profound, often leading to unexpected or poorly understood effects. Various drugs have immunomodulatory properties; therefore, it is plausible to hypothesize that certain pharmaceuticals may positively or negatively influence the induction of trained immunity. For example, metformin, mainly used for type II diabetes, has anti-inflammatory properties [28]. In a proof-of-principle clinical trial, metformin use was shown to decrease the cytokine production capacity of PBMCs trained with BCG [29]. Anakinra (interleukin-1 receptor antagonist, IL-1Ra) was also reported to attenuate central trained immunity in bone marrow progenitors in mice [30]. These studies suggest that the effects of certain drugs could be partially mediated by controlling trained immunity responses.

Aim and Outline of the thesis

Over the last 100 years, the fields of immunology and medicine have significantly progressed, thanks to new methods and discoveries about the human physiology and immune system. Despite these advancements, a substantial knowledge gap persists regarding the influence of host-related and environmental elements on innate and trained immunity responses. Understanding the interplay between these factors and immune responses is critical for the development of novel pharmaceuticals and vaccines, as well as for the improvement of the effects of existing ones. This thesis explores how different internal and external factors contribute to the functioning of the innate immune system and modulate trained immunity responses following BCG vaccination.

Chapter 2 summarizes the immunological changes observed in people with advanced age at the innate and adaptive levels and how these changes contribute to specific and non-specific vaccine responses. Furthermore, it discusses how trained immunity can be employed as a novel approach to improve immune responses and protect against various infections.

The COVID-19 pandemic showed that the disease severity is variable among different populations. **Chapter 3** investigates why the elderly and males are more susceptible to developing severe COVID-19. In this study, circulating inflammatory proteins and immune cell populations associated with severe infection were compared between young and older individuals as well as males and females. Moreover, PBMCs of female, male, young, and old subjects isolated during different seasons were stimulated with heat-inactivated SARS-CoV-2 to investigate the impact of seasons on SARS-CoV-2-induced immune responses.

Chapter 4 explores the genetic factors influencing trained immunity responses after BCG vaccination. First, the relationship between polymorphisms (SNPs) in the *RORA* gene and the strength of BCG-induced trained immunity was investigated in a cohort of healthy individuals. Then, the role of RORα was further analyzed in an *in vitro* model of trained immunity: The hallmarks of trained immunity were studied using a RORα inverse agonist in the presence or absence of BCG.

In **Chapter 5**, the trained immunity response in individuals vaccinated with BCG during winter and spring was compared. Three months after vaccination, isolated PBMCs and NK cells were incubated with several inflammatory stimuli, and cytokine responses were analyzed. Additionally, the effects of winter and spring vaccination on monocyte and NK cell epigenome and monocyte transcriptome were investigated.

A multi-omics study was designed to understand whether mild SARS-CoV-2 infection leaves marks on innate and adaptive immunity after recovery, and its results were presented in **Chapter 6**. Healthy controls and recovered individuals around 4 weeks after having a mild/moderate SARS-CoV-2 infection were included in the study. Single-cell RNA sequencing, single-cell ATAC sequencing, genome-wide DNA methylation profiling, flow cytometry and functional validation experiments were performed to examine how mild/moderate COVID-19 impacts the immune system after convalescence.

Chronic use of medication is highly prevalent, especially in older adults. Having insights into how certain drugs interact with trained immunity inducers can give valuable information about designing and improving the efficacy of these inducers. In **Chapter 7**, how a tablet of alendronate impacts BCG-induced trained immunity was investigated. Cytokine responses of PBMCs upon incubation with heterologous stimuli were analyzed. Furthermore, RNA sequencing and flow cytometry were performed to determine the changes in gene expression and immune cell subsets after alendronate use.

Lastly, the findings of this thesis were summarized and discussed in **Chapter 8**.

References

1. Chaplin DD. Overview of the immune response. J Allergy Clin Immunol 2010; 125(2 Suppl 2): S3-23.
2. Bonilla FA, Oettgen HC. Adaptive immunity. J Allergy Clin Immunol 2010; 125(2 Suppl 2): S33-40.
3. Janeway CA, Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 1989; 54 Pt 1: 1-13.
4. Goodridge HS, Ahmed SS, Curtis N, et al. Harnessing the beneficial heterologous effects of vaccination. Nat Rev Immunol 2016; 16(6): 392-400.
5. Arts RJW, Moorlag S, Novakovic B, 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.
6. Bannister S, Kim B, Dominguez-Andres J, et al. Neonatal BCG vaccination is associated with a long-term DNA methylation signature in circulating monocytes. Sci Adv 2022; 8(31): eabn4002.
7. Ferreira AV, Domiguez-Andres J, Netea MG. The Role of Cell Metabolism in Innate Immune Memory. J Innate Immun 2022; 14(1): 42-50.
8. Li W, Moorlag S, Koeken V, et al. A single-cell view on host immune transcriptional response to in vivo BCG-induced trained immunity. Cell Rep 2023; 42(5): 112487.
9. Cirovic B, de Bree LCJ, Groh L, et al. BCG Vaccination in Humans Elicits Trained Immunity via the Hematopoietic Progenitor Compartment. Cell Host Microbe 2020; 28(2): 322-34 e5.
10. Netea MG, Dominguez-Andres J, Barreiro LB, et al. Defining trained immunity and its role in health and disease. Nat Rev Immunol 2020; 20(6): 375-88.
11. Ochando J, Mulder WJM, Madsen JC, Netea MG, Duivenvoorden R. Trained immunity - basic concepts and contributions to immunopathology. Nat Rev Nephrol 2023; 19(1): 23-37.
12. Weyand CM, Goronzy JJ. Aging of the Immune System. Mechanisms and Therapeutic Targets. Ann Am Thorac Soc 2016; 13 Suppl 5(Suppl 5): S422-S8.
13. Gill PS, Ozment TR, Lewis NH, Sherwood ER, Williams DL. Trained Immunity Enhances Human Monocyte Function in Aging and Sepsis. Front Immunol 2022; 13: 872652.
14. Wardhana, Datau EA, Sultana A, Mandang VV, 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
15. Jacobsen H, Klein SL. Sex Differences in Immunity to Viral Infections. Front Immunol 2021; 12: 720952.
16. Klein SL, Flanagan KL. Sex differences in immune responses. Nat Rev Immunol 2016; 16(10): 626-38.
17. Benn CS, Fisker AB, Rieckmann A, Sorup S, Aaby P. Vaccinology: time to change the paradigm? Lancet Infect Dis 2020; 20(10): e274-e83.
18. Mangino M, Roederer M, Beddall MH, Nestle FO, Spector TD. Innate and adaptive immune traits are differentially affected by genetic and environmental factors. Nat Commun 2017; 8: 13850.
19. Moorlag S, Folkman L, Ter Horst R, 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.
20. Moorlag S, Matzaraki V, van Puffelen JH, et al. An integrative genomics approach identifies KDM4 as a modulator of trained immunity. Eur J Immunol 2022; 52(3): 431-46.

21. Wyse C, O'Malley G, Coogan AN, McConkey S, Smith DJ. Seasonal and daytime variation in multiple immune parameters in humans: Evidence from 329,261 participants of the UK Biobank cohort. iScience 2021; 24(4): 102255.
22. Ter Horst R, Jaeger M, van de Wijer L, et al. Seasonal and Nonseasonal Longitudinal Variation of Immune Function. J Immunol 2021; 207(2): 696-708.
23. Abreu TC, Boshuizen H, Mollema L, Berbers GAM, Korthals Altes H. Association between season of vaccination and antibody levels against infectious diseases. Epidemiol Infect 2020; 148: e276.
24. Franceschi C, Salvioli S, Garagnani P, de Eguileor M, Monti D, Capri M. Immunobiography and the Heterogeneity of Immune Responses in the Elderly: A Focus on Inflammaging and Trained Immunity. Front Immunol 2017; 8: 982.
25. Roquilly A, Jacqueline C, Davieau M, et al. Alveolar macrophages are epigenetically altered after inflammation, leading to long-term lung immunoparalysis. Nat Immunol 2020; 21(6): 636-48.
26. Sparks R, Lau WW, Liu C, et al. Influenza vaccination reveals sex dimorphic imprints of prior mild COVID-19. Nature 2023; 614(7949): 752-61.
27. Kamada R, Yang W, Zhang Y, et al. Interferon stimulation creates chromatin marks and establishes transcriptional memory. Proc Natl Acad Sci U S A 2018; 115(39): E9162-E71.
28. Bai B, Chen H. Metformin: A Novel Weapon Against Inflammation. Front Pharmacol 2021; 12: 622262.
29. Arts RJW, Carvalho A, La Rocca C, et al. Immunometabolic Pathways in BCG-Induced Trained Immunity. Cell Rep 2016; 17(10): 2562-71.
30. Mitroulis I, Ruppova K, Wang B, et al. Modulation of Myelopoiesis Progenitors Is an Integral Component of Trained Immunity. Cell 2018; 172(1-2): 147-61 e12.

1

PART I
HOST-RELATED FACTORS

European robin
Kızılgerdan
Roodborst

CHAPTER 2

Overcoming immune dysfunction in the elderly: trained immunity as a novel approach

Ozlem Bulut*, Gizem Kilic*, Jorge Domínguez-Andrés, and Mihai G. Netea

* These authors share first authorship.

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Abstract

People with advanced age have a higher susceptibility to infections and exhibit increased mortality and morbidity as the ability of the immune system to combat infections decreases with age. While innate immune cells display functional defects such as decreased phagocytosis, chemotaxis and cytokine production, adaptive immune cells exhibit reduced receptor diversity, defective antibody production and a sharp decline in naive cell populations. Successful responses to vaccination in the elderly are critical to prevent common infections such as influenza and pneumonia, but vaccine efficacy decreases in older individuals compared with young adults. Trained immunity is a newly emerging concept that showed that innate immune cells possess non-specific immunological memory established through epigenetic and metabolic reprogramming upon encountering certain pathogenic stimuli. Clinical studies suggest that trained immunity can be utilized to enhance immune responses against infections and improve the efficiency of vaccinations in adults; however, how trained immunity responses are shaped with advanced age is still an open question. In this review, we provide an overview of the age-related changes in the immune system with a focus on innate immunity, discuss current vaccination strategies for the elderly, present the concept of trained immunity and propose it as a novel approach to enhance responses against infections and vaccinations in the elderly population.

Introduction

Rapid aging of the world population is one of the most crucial social shifts taking place in the twenty-first century with an extensive impact on different fields, including economics and health care. According to the United Nations Population Division, the number of people over 60 years of age in urban areas increased by 68% between 2000 and 2015 [1]. This number is predicted to grow by another 56% until 2030, reaching 1.4 billion. By 2050, the population over 60 years will more than double its current size, exceeding 2 billion people.

As humans age, their immune system undergoes age-related changes that are collectively termed immunosenescence [2]. Besides other age-related conditions such as Alzheimer's disease and cardiovascular diseases, aging of the immune system leads to increased susceptibility to infections and autoimmune diseases, and poor response to vaccination, followed by high hospitalization and increased mortality rates [3]. Morbidity associated with infectious diseases in the elderly population is a significant burden on the healthcare systems and economies of countries all around the globe. Because of these reasons, counteracting immunosenescence and developing new immunization strategies for elderly people are considered priority research areas by the World Health Organization [4]. Understanding the mechanisms of immunosenescence and developing counteractive measures are of great importance.

Here we describe the mechanisms of immunosenescence, with a particular emphasis on the innate immune system. We then review the impact of vaccine responses in the elderly and the current approaches to improve vaccine efficacy. Lastly, we describe the concept of trained immunity, the adaptation of innate host defense that leads to non-specific immunological memory in innate immune cells through epigenetic and metabolic reprogramming [5]. We finally detail recent studies utilizing trained immunity to boost vaccine responses and propose trained immunity as a promising approach to increase vaccine efficiency in the elderly population.

Aging of the immune system: a brief overview

The most established features of immunosenescence—the dysregulated state of an aged immune system—include short-lived memory responses, defective response to new antigens, higher disposition to autoimmunity and the chronic low-grade systemic inflammation that is termed inflammaging [6]. The main cellular culprits behind these dysregulated responses are a sharp decrease of naive T- and B-cell pools with increasing age, reduced natural killer (NK) cell cytotoxicity, impaired signaling and decreased function of some innate immune cell subsets [2] (**Fig. 1**).

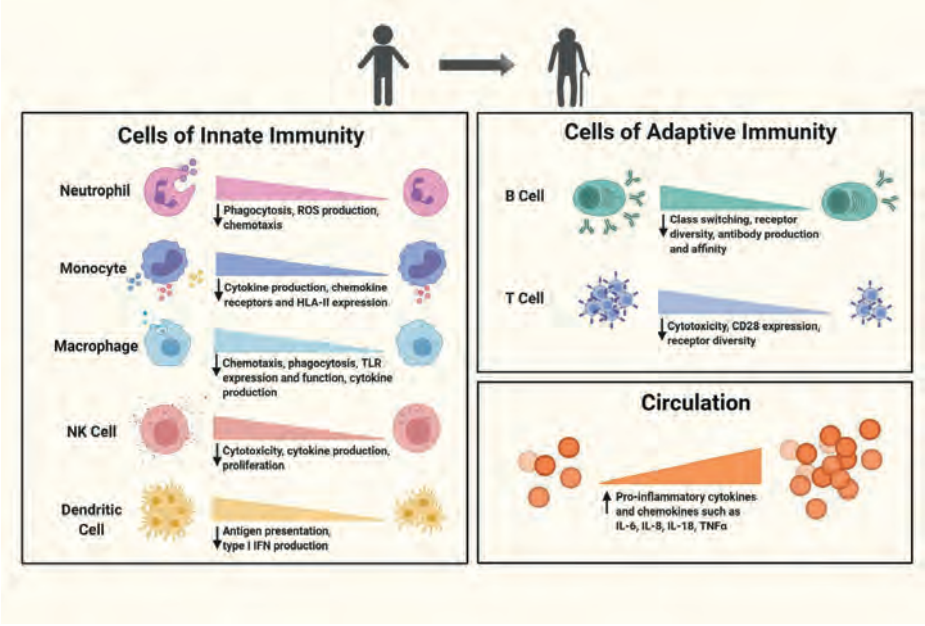

Figure 1. Age-associated functional changes in the immune system

Both innate and adaptive immune systems undergo age-related alterations in terms of cell numbers and functions toward the later decades of human life. Multiple human and murine studies revealed that the cells of innate immunity such as neutrophils, monocytes, macrophages, dendritic cells and NK cells display impaired receptor expression, chemotaxis, phagocytosis, antigen presentation, cytotoxicity, ROS and cytokine production. Adaptive immune cells (B cells and T cells) experience shifts in sub-populations such as the depletion of naive cell pools and accumulation of late-differentiated effector and memory cells. Apart from those, both display reduced receptor diversity. Functionally, expression of the co-stimulatory molecule CD28 is critically diminished in T cells while B cells become weaker in class-switching and affinity maturation. Numbers of plasma cells and production of antibodies also decrease. Despite these functional down-regulations at the cellular level, levels of pro-inflammatory cytokines and chemokines are elevated in circulation with advancing age.

Lingering inflammation causes tissue damage and contributes to the development and progression of age-related diseases. Elevated circulating levels of pro-inflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor α (TNFα) along with C-reactive protein (CRP) are some of the most reliable markers of inflammaging, their circulating concentrations predicting frailty and mortality in the elderly [7, 8]. Inflammaging is the result of the accumulated long-term stimulation of the innate immune system with increasing age. As the current life expectancy of humans exceeds the life span that characterized human evolution for hundreds of thousands of years, beneficial physiological responses may become damaging as humans age [9].

One of the mechanisms proposed to drive inflammaging is the accumulation of damage-associated molecular patterns (DAMPs), which are essential for effective tissue repair and inflammatory response against pathogens, but can also cause maladaptive responses and chronic disease, as disposal of the accumulating material by autophagy or mitophagy declines with age [10]. Another likely source is the senescence-associated secretory phenotype (SASP) of senescent epithelial and endothelial cells which secrete pro-inflammatory cytokines and modify the response of neighboring cells [11, 12]. Products of microbiota might also contribute to inflammaging. As the body ages, the gut is less efficient in sequestering microbes and their products [13]. Contents of the gut microbiota change with age as well, becoming more inflammatory [14]. Age-related expansion of Proteobacteria and a decline in butyrate-producing bacteria, for example, have been correlated to increased IL-6 and IL-8 levels [15].

Age-related changes in innate immunity

Hematopoietic stem cells (HSCs) in the bone marrow increase in number with age and become more likely to commit to the myeloid lineage, which gives rise to the majority of the innate immune cells including dendritic cells (DCs), monocytes, macrophages, mast cells and granulocytes (e.g. neutrophils) [16, 17]. However, despite the skewing to the myeloid lineage in the bone marrow, numerous age-related declines in terms of cell number and function have been described for the cells of innate immunity. On the one hand, elderly people tend to develop low-grade systemic inflammation although their immune cells present defective capacities of migration, phagocytosis and cytokine production [18]. Impaired functions of innate immunity can further exacerbate the flaws in adaptive immunity, for instance by not providing efficient antigen presentation to T cells. Here, we detail the age-related changes in different innate immune cell subsets and their consequences.

Neutrophils

Neutrophils are the most abundant type of immune cell in circulation. They internalize pathogens through phagocytosis and destroy them using reactive oxygen species (ROS) and degradative proteases, while also recruiting and activating DCs, monocytes and lymphocytes [19]. Neutrophils also efficiently trap and kill extracellular pathogens by forming neutrophil extracellular traps composed of web-like structures of chromatin and proteases [20].

Many functions of neutrophils including chemotaxis, phagocytosis, ROS production, signal transduction and apoptosis have been reported to be dysfunctional in the elderly [21–25]. However, neutrophil numbers are mostly preserved during aging [21]. Healthy centenarians—the people aged 100 years or older—have well-conserved neutrophil functions [26]. Increased activation of constitutive phosphoinositide 3-kinase (PI3K) was associated with impaired chemotaxis in the elderly [22]. Expression of CD16, an Fc receptor, is low in neutrophils of people aged over 65, which potentially restricts Fc-mediated phagocytic activity [24]. Intracellular killing of the phagocytosed pathogens is also defective in the elderly [27, 28]. Defects in ROS production have been linked to the changing composition of cell membranes with age [29]. Moreover, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-2 have anti-apoptotic effects on neutrophils of young adults but not in adults over 65 years of age [30, 31]. Increased neutrophil susceptibility to apoptosis might also contribute to the weakened response of the elderly against pathogens [3].

Monocytes

Monocytes present a large range of functions, including phagocytosis, cytokine production and antigen presentation. They circulate in the blood and migrate into tissues in response to infection or tissue damage where they can differentiate into macrophages or DCs [32]. In humans, there are three major monocyte subsets with different functions, identified based on their CD14 and CD16 expression: $CD14^{+}$ $CD16^{-}$ classical monocytes, $CD14^{+}$ $CD16^{+}$ intermediate monocytes and $CD14^{dim}$ $CD16^{+}$ non-classical monocytes [33].

Circulating monocyte numbers are stable with advancing age [21]. However, the ratios of different monocyte subsets are altered. Classical monocytes are reportedly reduced, while intermediate and non-classical monocytes are increased, with age [34]. Of note, expanding non-classical monocytes present lower expression of the CX3CR1 chemokine receptor and the human leukocyte antigen class II molecule HLA-DR, whereas macrophages derived from monocytes of elderly subjects display intact cytokine production [35, 36]. Monocytes from subjects over 60 years old present higher Toll-like

receptor 5 (TLR5) expression, produce more IL-8 and show increased phosphorylation of mitogen-activated protein kinases (MAPKs) p38 and extracellular signal-regulated kinase (ERK) upon activation with TLR ligands, but they are defective in activation of nuclear factor κB (NF-κB) [37].

Another study reported lower TLR1 expression, less ERK1/ERK2 phosphorylation upon TLR1/TLR2 activation, and reduced IL-6 and TNFα production in monocytes of people over 66 years of age [38]. A recent study investigating innate immune responses in a healthy population of individuals of various ages has shown intact cytokine production capacity and normal numbers of innate immune cells in the circulation [39]. Moreover, the production of some of the inflammatory cytokines was even higher in the elderly, underscoring the development of inflammaging.

Macrophages

Macrophages are phagocytic cells present in nearly all tissues where they contribute to tissue homeostasis, tissue repair and host defense [40]. They exhibit high plasticity and heterogeneity, and secrete a wide variety of cytokines and chemokines upon recognition of pathogen-derived or damage-associated signals [41].

Although circulating monocyte numbers are stable through life, numbers of macrophage precursors are reportedly reduced with advancing age [42, 43]. Similar to neutrophils, macrophages display age-related defects in chemotaxis, TLR expression and function, signal transduction, phagocytosis and superoxide production [21]. Upon lipopolysaccharide (LPS) stimulation, peritoneal macrophages of aged mice had 70% decreased p38 MAPK and c-Jun N-terminal kinase (c-JNK) activation, which are critical for TLR-mediated responses [44, 45]. Decreased expression of inducible nitric oxide synthase (iNOS) and impaired production of nitric oxide were also observed in macrophages from aged mice [46].

Aged-mouse macrophages also had less major histocompatibility complex (MHC) class II expression, lower levels of TLRs and reduced IL-6 and TNFα production upon stimulation with TLR ligands [47, 48]. MHC II expression on the surface was 50% less in macrophages of old mice following interferon γ (IFNγ) stimulation [49]. LPS-induced IL-1β and IL-12 production was also reduced in splenic macrophages from aged mice [50]. Macrophages in aged mice were also less capable of clearing apoptotic debris [51].

Most published studies investigating age-related changes in macrophages are murine studies, because of difficulties in obtaining tissue macrophages from humans. Nevertheless, there are a few studies suggesting decreased macrophage function in

the elderly. The numbers of bone marrow macrophages in the later decades of life were found to be comparable to younger adults [52]. Monocyte-derived macrophages from the elderly produced less TNFα, IL-6, IL-8 and IL-1β when incubated with *Streptococcus pneumoniae*, even though their phagocytic ability seemed to be intact [53]. This functional defect was linked to impaired PI3K–AKT (Ak-strain thymoma oncogene; also called protein kinase B) signaling. Another study with monocyte/macrophage cultures infected with dengue virus revealed lower TNFα, IL-6 and IL-1β production by cells of elderly subjects over 65 years old compared with younger adults [54].

Dendritic cells

DCs are very potent antigen-presenting cells (APCs) that are usually considered as the bridge between innate and adaptive immunity [55, 56]. The two main subsets of DCs are myeloid DCs (mDCs) or conventional DCs (cDCs) of myeloid origin and plasmacytoid DCs (pDCs) of lymphoid origin, which are crucial for anti-viral defense [57].

Total peripheral DC and mDC numbers are lower in people aged over 60 years although pDC numbers remain stable [58]. Thymic DCs are also reduced in the elderly and are less efficient in stimulating T cells [59]. Even though pDC numbers do not change with age, they have lower type I IFN-releasing capacity due to impaired interferon regulatory factor 7 (IRF7) phosphorylation, which is associated with a reduced response to influenza virus [60]. Their antigen-presentation capacity is also decreased. mDCs of elderly people have restricted migratory and phagocytic capacities [61].

People over 60 years of age present higher production of TNFα and IL-6 by mDCs upon TLR4 stimulation, despite defective AKT phosphorylation and PI3K signaling [62, 63]. Also, when derived from elderly individuals, mDCs that appear to have a more mature phenotype produced less IL-12 upon LPS stimulation [58]. In addition, Langerhans cells, which are specialized DCs in epidermis and are critical for skin immunity, are lower in number in elderly people and migrate less in response to TNFα [64].

NK cells

NK cells are cytotoxic cells that are heavily studied in the context of anti-tumor responses, but they also exert cytotoxic activity upon recognition of infected cells, particularly in viral infections, or cytokines such as IL-2, IL-12, IL-15 and IL-18 [65, 66].

Studies reported increased or maintained NK numbers in the elderly although proliferation rates appear to be decreased [67, 68]. This is suggestive of the existence of long-lived NK cells. Recently, memory-like NK cells, defined as $NKG2C^{+}$ $CD57^{+}$, were indeed described in people with cytomegalovirus (CMV) infection and were also detected in CMV^{-} individuals later [69, 70].

Despite some studies reporting preserved NK cytotoxicity, it is considered to be impaired on a per cell basis [71, 72]. Low NK cytotoxicity is associated with higher infection rates and infection-related deaths in the elderly [73]. Higher NK cytotoxic activity is also linked to higher antibody titers after influenza vaccination in people over 65 years of age [74]. Production of IFNγ and proliferation upon IL-2 stimulation were also reduced in this group [71]. The NK cell receptor repertoire was also found to be altered with age [75]. Additionally, the $CD56^{bright}$ NK cell subset, which constitutes around 10% of peripheral NK cells, was critically diminished in the elderly [76].

Age-related changes in adaptive immunity

Antigen-specific adaptive immunity with memory-generating capabilities is crucial for responding against tumors, allergens and pathogens. The most profound changes in the immune system related with aging are observed in adaptive immunity. In the following paragraphs, we summarize the age-related defects in T cells and B cells.

T cells

T cells, through their diverse range of antigen receptors [T-cell receptors (TCRs)], recognize pathogenic or tumor-derived antigens and develop antigen-specific memory or tolerance [77]. Upon recognition of antigen and receiving co-stimulatory signals, naive T cells differentiate into effector cells. Most of the effector cells are short-lived; however, a portion persists as memory cells and establish long-term immunity. The two main lineages of T cells are $CD4^+$ helper and $CD8^+$ cytotoxic T cells [78].

Maturation and selection of T cells take place in the thymus. Thymic involution—the gradual atrophy of the thymus with age—starts from the first year of life and progresses until the end of life [79]. The thymopoietic space, where T-cell maturation occurs, is shrunk to <10% in volume by the age of 70 [80]. Processes underlying this include loss of thymic epithelium, reduced IL-7 production by thymic epithelium, which is essential for the maturation of thymocytes, and defective rearrangement of the TCR β-chain [81, 82]. People who had undergone thymectomy in early childhood show a premature immunosenescent phenotype [83].

The typical immunosenescent profile includes reduced output of naive T cells and a T-cell pool consisting mostly of differentiated effector cells and memory cells [84]. It is important to note that most age-related changes in T-cell profiles are either only seen in, or are more pronounced in, individuals seropositive for CMV, which is a chronic infection present in almost 70% of people over 60 years of age [85, 86]. Among $CD8^+$

T cells, the $CD28^-$ effector population is markedly increased in the elderly [87, 88]. In contrast, the naive $CD8^+$ T-cell pool is depleted with age [89]. Loss of CD28, which plays a critical role in T-cell activation in effector cells, is among the hallmarks of immunosenescence in T cells [90].

Furthermore, the limited number of existing $CD28^+$ cells have a more restricted TCR repertoire and shorter telomeres in people over 65 years of age [91]. Clonal expansion of $CD28^-$ $CD8^+$ T cells was inversely correlated with antibody production against influenza vaccination [92]. Because of the extreme expansion of these cells and the reduced naive T-cell output, the T-cell repertoire diversity is restricted and susceptibility to novel infections is increased [93].

The naive $CD4^+$ T-cell pool does not undergo such a critical change as $CD8^+$ T cells, although there is a decline in numbers [94, 95]. Upon probing with novel antigens, IL-2 production by naive $CD4^+$ T cells of elderly people was also comparable to young individuals [96], even though there is defective TCR-induced ERK signaling [97]. In contrast to naive cells, central memory $CD4^+$ cells accumulate in people over 65 years of age [94, 98]. Effector memory cells, on the other hand, are found at a lower frequency in the elderly and their numbers were correlated with anti-influenza response upon vaccination [98]. The accumulation of effector cells and loss of CD28 seen in $CD8^+$ T cells are not pronounced in $CD4^+$ cells [95].

B cells

B cells mediate humoral immunity against pathogens and allergens by producing antibodies with high specificity and affinity [99]. The B-cell antibody response is one of the crucial outcomes that vaccination strategies strive to achieve. B cells develop and mature in the bone marrow. In contrast to T cells, whose output is severely affected by thymic involution, B-cell lymphopoiesis continues throughout life, but B-cell precursor numbers in the bone marrow and the antibody-producing plasma cells decrease with age [100, 101].

Similar to T cells, accumulation of memory B cells with restricted receptor diversity was reported in the elderly [102]. Impaired class-switching and somatic recombination along with lower diversity of antibodies are also observed in this group, leading to weak antibody responses with low affinity [103]. Age-related alterations in number and size of germinal centers, where B cells proliferate and undergo somatic hypermutation, partly contributed by sub-optimal T-cell help underlie these defects [104, 105]. The percentage of switched memory B cells, which have been positively correlated with influenza vaccine responses, also declines significantly with age [106–108].

This population has very short telomeres in the elderly compared with younger individuals [109]. In contrast, late exhausted memory B cells are expanded in the elderly, filling up the immunological space [109]. Another age-related change is the increase of auto-antibodies in the elderly, likely contributing to prevalence of autoimmune diseases [110].

Vaccine responses in the elderly and current improvement strategies

In order to prevent and reduce the number of infections in elderly people, vaccines are the most cost-effective and safe approach. However, the overall vaccination efficiency of currently available vaccines remains low in the elderly population, because of the impaired ability of their immune system to respond to immune stimulation [111].

Influenza is one of the major infections worldwide, and it represents a considerable threat for vulnerable populations such as elderly and young children. There are up to 500 000 deaths reported every year in people aged >65 years because of influenza [112]. Along with increased risk of hospitalization and deaths linked with influenza-associated respiratory diseases, vaccine efficiency is also lower at 17–53% in the elderly, compared with the 70–90% efficacy in young adults [113]. Suggested reasons for the impaired influenza vaccine response included decreased somatic mutations in B cells [114], an increased regulatory T cell (Treg) population [115], impaired expression of the co-stimulatory molecule CD28 in T cells [116], the reduced antigen-presenting capacity of pDCs [60] and low NK cell cytotoxicity [21].

Currently, there are two commonly available influenza vaccines: inactivated vaccines and live-attenuated vaccines. A high-dose inactivated vaccine with 60 μg hemagglutinin (HA) antigen from each strain demonstrated improved antibody responses with 24.2% more efficiency in people over 65 years of age compared with the 15 μg standard dose [117–119]. In 2019, a high-dose influenza vaccine was approved by the Food and Drug Administration (FDA) for use in people older than 65 years, reported as well-tolerated and more effective [120]. Nonetheless, vaccination of elderly with the high-dose vaccine still induced lower antibody responses and Th1 T-cell responses in comparison with young adults vaccinated with the standard dose [121].

Another study demonstrated that intra-dermal injection instead of intramuscular injection significantly improved antibody titers in people over 65 years of age; however, intra-dermal injection of the high-dose (60 μg) influenza vaccine was not significantly different than that of the normal dose (15 μg) in terms of protection [119].

Adjuvanting the vaccines is another promising strategy to boost immune responses in the elderly. Adjuvants are a crucial part of vaccines, contributing to better vaccine responses by increasing antigen presentation and activating the innate immune system [122]. Considering that antigen presentation, responsiveness and chemotaxis of immune cells are mostly impaired in old individuals, improvements in adjuvant systems would increase the efficacy of vaccinations.

MF59 ® (Fluad), an emulsion-based adjuvant, was significantly immunogenic, and it reduced influenza-related hospitalizations by 25% in the elderly in comparison with non-adjuvanted influenza vaccine [123–125]. MF59 has been reported to increase viral antigen uptake and antigen presentation, hence enhancing immunization efficacy. Additionally, the MF59-adjuvanted subunit influenza vaccine induced antibody responses against non-specific seasonal viral strains [126]. TLR ligands are also utilized as adjuvants. A phase 2b/3 trial demonstrated that topical application of the synthetic TLR7/TLR8 agonist imiquimod prior to intra-dermal trivalent influenza vaccination significantly elevated the immunogenicity of vaccine in the elderly [127].

Streptococcus pneumoniae is another prevalent cause of severe infections in the elderly that might result in several complications such as upper respiratory disease, bacteremia and meningitis [128]. There are two commonly used vaccines: a 23-valent pneumococcal polysaccharide vaccine (PPSV23), which is mostly used for adults and the elderly; and a 13-valent pneumococcal conjugate vaccine (PCV13) for children older than 2 years of age [129].

Although PPV23 has been recommended for a long time to vaccinate the elderly, a meta-analysis assessing vaccine efficiency showed that PPV23 had a moderate effect on invasive pneumococcal disease while it was not potent against pneumococcal pneumonia [130]. On the other hand, PCV13 has been reported as partly effective against pneumococcal diseases in old individuals; however, age still influences the potency of PCV13 with efficacy of 65% and 40% in 65-year-old and 75-year-old participants, respectively [131]. A study argued that the combination of PCV13 with PPV23 possibly enhances protection in the elderly; however, clinical data demonstrating elevated antibody production and reduced disease incidence are still missing [132].

Varicella zoster virus (VZV) is another important pathogen affecting the elderly. This virus remains latent in the nerve cells of infected individuals after an episode of chickenpox in early life [133]. Herpes zoster or shingles is caused by reactivation of latent VZV, and the risk of developing shingles increases with age because of the reduced activity of cell-based immunity [134]; therefore, most of the cases that require hospitalization are people older than 50 years [135].

Two vaccines are licensed for usage against shingles: a live-attenuated vaccine (Zostavax™) developed by Merck; and a subunit zoster vaccine (Shingrix™) formulated by GSK. A double-blind, placebo-controlled study with people older than 60 years showed that the live-attenuated vaccine lowered the burden of illness by 61.1% and prevalence of herpes zoster by 51.3% [136].

The novel adjuvant AS01b, consisting of MPL (3-O-desacyl-monophosphoryl lipid A), a TLR4 agonist as a derivative of LPS from *Salmonella minnesota*, and saponin QS-21, has been shown to effectively promote antigen presentation and $CD4^+$ T-cell-mediated immune responses, and demonstrated high efficacy in combination with different vaccines in clinical trials [137]. An inactivated vaccine utilizing AS01b as a liposome-based adjuvant exhibited promising results in elderly people, with 97.2% efficacy in people over 50 years of age [138]. Of note, the vaccine potency did not decrease with age; the efficiency in people older than 70 years of age is similar to that in people between 50 and 70 years old. Additionally, vaccine-induced antibody production was still higher than the pre-vaccination level even after 9 years [139]. A phase II trial comparing the AS01b-adjuvanted vaccine with non-adjuvanted vaccine reported that immunogenicity of the viral subunit vaccine increased with the adjuvant in a dose-dependent manner [140]. The very special behavior of this AS01-containing vaccine with high efficacy in the elderly provides a potential tool to investigate the mechanisms needed to induce proper vaccination responses in the elderly and gives hope that similar levels of efficacy may be achieved with other vaccines as well.

Trained immunity and vaccination in the elderly

For a long time, the development of immunological memory was solely attributed to adaptive immunity, which is maintained by antigen-specific long-lasting memory lymphocytes upon recognition of a pathogen. On the other hand, innate immune responses are mediated by non-specific effector molecules and have been considered as being devoid of memory properties. However, recent studies consistently reported the capacity of the innate immune system to develop memory-like features [141–144].

Our group and others showed that, following an insult with certain infections or vaccinations, members of the innate immune system, for example monocytes, DCs and NK cells, exhibit enhanced responsiveness to a second infection that might be the same or a different pathogen. This phenomenon was later termed as 'trained immunity' or 'innate immune memory' [144]. Although the concept of trained immunity was first demonstrated and mostly studied in monocytes, there is

evidence that memory-like properties are also present in other innate immune cells. For instance, *ex vivo* stimulation of human NK cells with heterologous pathogens 3 months after Bacillus Calmette–Guérin (BCG)—a live-attenuated vaccine against tuberculosis [145]—results in enhanced pro-inflammatory cytokine production but not IFNγ production compared with before vaccination [146].

Notably, BCG neither induced NK cell expansion nor altered the expression of NK cell markers. A recent study suggested that DCs from immunized mice showed a long-term memory response upon fungal challenge that was mediated by specific epigenetic modifications [147].

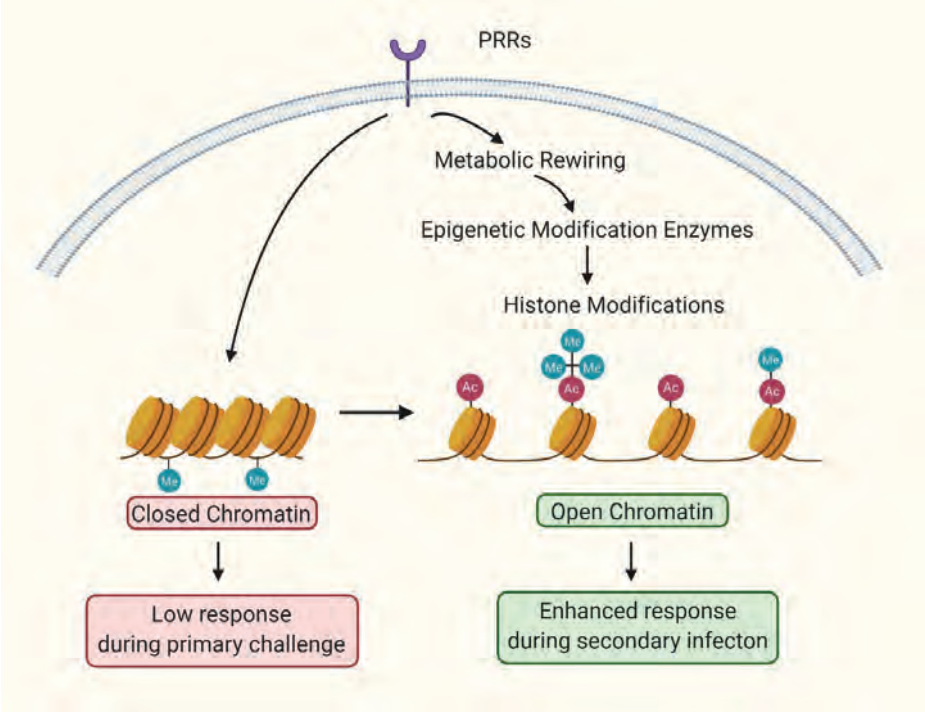

Figure 2. Overview of fundamental mechanisms in trained immunity

Certain infections and vaccinations alter metabolic pathways, leading to histone modifications that enable chromatin regions to be more open for transcription. Increased gene expression results in improved responses against pathogens during secondary infection.

The underlying mechanisms of trained immunity are explained by epigenetic and metabolic reprogramming (**Fig. 2**). Immunological signal pathways, for example pattern-recognition receptors (PRRs) engaged with DAMPs or bacterial products, induce

epigenetic changes (i.e. increase in H3K4me3, H3K4me and H3K27Ac and removal of H3K9me3) at the promoter and enhancer sites of genes coding for pro-inflammatory cytokines and metabolic rewiring such as up-regulation of glycolysis, cholesterol synthesis and glutaminolysis [148–151]. Certain metabolites of these pathways, such as α-ketoglutarate and fumarate, subsequently modulate the activities of epigenetic remodeling enzymes, such as histone demethylases or histone acetyltransferases [5]. As a result, increased chromatin accessibility of pro-inflammatory genes eventually leads to elevated pro-inflammatory cytokine production when a secondary challenge occurs. Another remarkable finding is that memory-like properties can persist for a long time beyond the limited life spans of immune cells, owing to the reprogramming of HSCs and myeloid progenitors in the bone marrow [152, 153].

Trained immunity can be used as an effective way to boost vaccine responses by conferring wide protection against a diverse range of pathogens [154]. For instance, trained immunity induced by β-glucan protected mice against bacterial infections causing peritonitis, enteritis and pneumonia by increasing inflammatory monocyte and granulocyte numbers and IL-1β production [155].

Clinical trials and epidemiological studies revealed that certain vaccines such as vaccinia, BCG and measles have non-specific protective effects [156, 157]. Among them, BCG is the most extensively studied vaccine for its heterologous protective effects. It has been used for treatment and decreasing the progression of non-muscle invasive bladder cancer for >40 years, although its mode of action has not been fully understood yet [158–160]. The wide range of protection conferred by BCG is mainly attributed to increased cytokine production as a result of metabolic and epigenetic reprogramming of innate immune cells. It is also important to point out that in addition to induction of trained immunity, BCG and other live attenuated vaccines can induce heterologous adaptive immune responses, such as Th1-dependent IFNγ production [161, 162]. It is conceivable that the complete beneficial effects of BCG vaccination are due to a combination of trained immunity and heterologous T-cell immunity. In addition, it is important to note that BCG vaccination prior to influenza and childhood vaccines can also act as an adjuvant and enhance antibody responses; however, the mechanisms in play are yet to be established [163, 164].

Evidence from several animal and human studies suggests that BCG vaccination is also effective to protect against *Leishmania spp*. and *Plasmodium falciparum* infections [165]. In a double-blinded, placebo-controlled study, individuals vaccinated with BCG 1 month before experimental viral infection induced by yellow fever vaccine displayed less viremia in their blood compared with people vaccinated with placebo. Protection against yellow

fever virus was reported to be associated with epigenetic modifications of monocytes, and high IL-1β production was inversely related to viremia [166]. In a clinical trial investigating protective effects of BCG on malaria infection, BCG-vaccinated subjects 5 weeks prior to controlled malaria infection presented early activation of NK cells and monocytes which were correlated with lower parasitemia [167].

Remarkably, heterologous protection by BCG was not limited to an enhanced innate immune/trained immunity response. It has been shown that BCG vaccination induced heterologous Th1 and Th17 responses even 1 year after immunization [168]. Another study from our group demonstrated that BCG vaccine could be used to improve the beneficial effects of diphtheria tetanus pertussis (DTP) and influenza vaccines. BCG vaccination prevented the immunosuppressive effects of acellular diphtheria tetanus pertussis combined vaccine (DTaP) and induced trained immunity in adults when it was given concurrently with or 3 months after DTaP [169]. BCG vaccination 2 weeks before trivalent influenza vaccination significantly boosted HA-inhibiting antibody production in healthy adults. Moreover, BCG-priming induced higher production of pro-inflammatory cytokines after *ex vivo* stimulation of peripheral blood mononuclear cells (PBMCs) with unrelated pathogens such as *Candida albicans* and *Staphylococcus aureus* [164].

Although literature for trained immunity in the elderly is very scarce, a few studies in the elderly suggest that not only children and adults, but also the elderly, might benefit from protection against heterologous infections. It has been recently shown that BCG-vaccinated individuals in Guinea-Bissau who are older than 50 years of age displayed increased pro-inflammatory cytokine production following *ex vivo* stimulation with heterologous stimuli 2 months after vaccination [170]. Considering the impaired ability of innate immune cells to respond against infections in the elderly, this study suggests that trained immunity could indeed be induced in elderly people and might be utilized as a powerful tool to increase vaccine responses and protect this vulnerable population from various infections by counteracting the effects of immunosenescence.

Another clinical study, in which participants between 60 and 75 years old received BCG once a month for 3 months, demonstrated that BCG vaccination significantly prevented acute upper respiratory tract infections while increasing IFNγ and IL-10 production [171]. Furthermore, the scar diameter at the vaccination site was correlated to the circulating IFNγ levels. Another study performed in Japan with elderly people indicated a lower risk of pneumonia following immunization with BCG [172].

Utilizing the trained-immunity response to increase resistance and defense against infections is advantageous in many settings. First of all, since trained immunity confers a broad range of protection, it might be useful in illnesses in which secondary infections or co-infections play a role. As an example, bacterial infections following influenza can worsen the outcome by increasing morbidity and mortality [173, 174]. As viruses frequently undergo mutations, conventional vaccines remain ineffective in some cases. Therefore, trained immunity can be employed to protect people from newly emerged bacterial or viral strains. Lastly, clinical conditions such as immunoparalysis could be rescued by inducing trained immunity [175].

Improving innate immune responses to provide protection is crucial for vulnerable populations such as the elderly and people with immune deficiencies. In a recent review by Sánchez-Ramón et al., approaches to employ trained immunity in vaccine formulations were explicitly discussed [154]. According to that, it was suggested that trained immunity inducers can be used as immunostimulants and adjuvants, the former promoting innate and adaptive immune responses leading to enhanced protection against bystander pathogens, while the latter delivered with a specific antigen further enhance adaptive immune response against that specific pathogen.

It is important to note that trained immunity might be damaging in situations where people have excessive inflammation as a result of endogenous and exogenous stimuli, and thus vaccines based on trained immunity should be mainly aimed for groups at high risk of infections. Indeed, people with atherosclerosis and hyper-IgD syndrome have been shown to have chronic inflammation due to continuously active trained immunity [176, 177].

The prolonged presence of certain DAMPs induces reprogramming of innate immune cells by providing a basis for sustained low-grade and chronic inflammation. For instance, pre-incubation of splenocytes with high-mobility group box protein 1 (HMGB1) was shown to increase TNFα production after secondary infection, indicating that HMGB1 might prime the cells to protect against infections [178]. Another molecule, oxidized low-density lipoprotein (oxLDL), leads to epigenetic reprogramming of monocytes, eventually causing long-term elevated pro-inflammatory cytokine production [179]. Similarly, pre-treatment of healthy PBMCs with soluble uric acid induced cytokine secretion that was mediated by histone methylation [180]. Along with advanced age, accumulation of DAMPs—for example, HMGB1, sodium monourate and uric acid crystals—results in sterile inflammation, which is one of the underlying causes of several diseases including but not limited to atherosclerosis, cardiovascular diseases, gout and ischemia–reperfusion injury [10, 181, 182].

Nevertheless, our group demonstrated that BCG vaccination lowers systemic inflammation by decreasing circulating inflammatory markers in healthy individuals while enhancing cellular responses (L. C. J. de Bree et al., unpublished data); therefore, it would serve to reduce chronic inflammation while overcoming functional impairments at a cellular level.

Conclusions

Age-related alterations in the immune system result in high susceptibility to infections, increased risk of hospitalization and mortality. Defects in adaptive immunity underlie the markedly low vaccine efficiency in the elderly. Additionally, many functional defects in chemotaxis, phagocytosis, antigen presentation, ROS production, TLR signaling and cytokine production are present in aged innate immune cells such as neutrophils, monocytes, macrophages, DCs and NK cells. Despite reduced cellular functions, a systemic increase in inflammatory markers, so-called inflammaging, is observed in aged individuals.

In addition to numerous efforts underway to develop new vaccines with higher efficacy in the elderly, novel approaches targeting innate immunity to improve host responses are crucial to evade the consequences of the aged immune system. It is an emerging concept that innate immune cells can manifest memory-like properties that are not antigen-specific and exhibit enhanced responsiveness upon later challenges with heterologous stimuli. This concept of 'trained immunity' has been reported to enhance immunization efficiency. However, whether trained immune responses change as people age is yet to be explored. Further investigation is crucial to understand if and how trained immunity can be employed to protect the elderly from a broad range of infections. Besides the possibility that impaired innate immune cell functions could be reversed by inducing trained immunity, recent data suggest that BCG down-regulates circulating inflammatory markers, which would help alleviate the detrimental effects of inflammaging in the elderly. Therefore, it would be worthwhile to explore the potential of trained immunity for overcoming age-related immune dysregulation and protecting the vulnerable elderly population against infections.

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Conflicts of interest statement

M.G.N. is a scientific founder of TTxD. The other authors declare that they have no conflicts of interest.

References

1. United Nations, 2015. World population ageing. Department of Economic and Social Affairs.
2. Grubeck-Loebenstein, B., Della Bella, S., Iorio, A. M., Michel, J. P., Pawelec, G., and Solana, R. 2009. Immunosenescence and vaccine failure in the elderly. Aging clinical and experimental research 21:201.
3. Crooke, S. N., Ovsyannikova, I. G., Poland, G. A., and Kennedy, R. B. 2019. Immunosenescence: A systems-level overview of immune cell biology and strategies for improving vaccine responses. Experimental gerontology 124:110632.
4. Thomas-Crusells, J., McElhaney, J. E., and Aguado, M. T. 2012. Report of the ad-hoc consultation on aging and immunization for a future WHO research agenda on life-course immunization. Vaccine 30:6007.
5. Netea, M. G., Dominguez-Andres, J., Barreiro, L. B., Chavakis, T., Divangahi, M., Fuchs, E., Joosten, L. A. B., van der Meer, J. W. M., Mhlanga, M. M., Mulder, W. J. M., Riksen, N. P., Schlitzer, A., Schultze, J. L., Stabell Benn, C., Sun, J. C., Xavier, R. J., and Latz, E. 2020. Defining trained immunity and its role in health and disease. Nature reviews. Immunology.
6. Goronzy, J. J. and Weyand, C. M. 2013. Understanding immunosenescence to improve responses to vaccines. Nature immunology 14:428.
7. Puzianowska-Kuznicka, M., Owczarz, M., Wieczorowska-Tobis, K., Nadrowski, P., Chudek, J., Slusarczyk, P., Skalska, A., Jonas, M., Franek, E., and Mossakowska, M. 2016. Interleukin-6 and C-reactive protein, successful aging, and mortality: the PolSenior study. Immunity & ageing : I & A 13:21.
8. Xia, S., Zhang, X., Zheng, S., Khanabdali, R., Kalionis, B., Wu, J., Wan, W., and Tai, X. 2016. An Update on Inflamm-Aging: Mechanisms, Prevention, and Treatment. Journal of immunology research 2016:8426874.
9. Franceschi, C., Garagnani, P., Parini, P., Giuliani, C., and Santoro, A. 2018. Inflammaging: a new immune-metabolic viewpoint for age-related diseases. Nature reviews. Endocrinology 14:576.
10. Franceschi, C., Garagnani, P., Vitale, G., Capri, M., and Salvioli, S. 2017. Inflammaging and 'Garb-aging'. Trends in endocrinology and metabolism: TEM 28:199.
11. Coppe, J. P., Desprez, P. Y., Krtolica, A., and Campisi, J. 2010. The senescence-associated secretory phenotype: the dark side of tumor suppression. Annual review of pathology 5:99.
12. Campisi, J. and d'Adda di Fagagna, F. 2007. Cellular senescence: when bad things happen to good cells. Nature reviews. Molecular cell biology 8:729.
13. Biagi, E., Candela, M., Franceschi, C., and Brigidi, P. 2011. The aging gut microbiota: new perspectives. Ageing research reviews 10:428.
14. Biagi, E., Candela, M., Fairweather-Tait, S., Franceschi, C., and Brigidi, P. 2012. Aging of the human metaorganism: the microbial counterpart. Age 34:247.
15. Biagi, E., Nylund, L., Candela, M., Ostan, R., Bucci, L., Pini, E., Nikkila, J., Monti, D., Satokari, R., Franceschi, C., Brigidi, P., and De Vos, W. 2010. Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. PloS one 5:e10667.
16. Pang, W. W., Price, E. A., Sahoo, D., Beerman, I., Maloney, W. J., Rossi, D. J., Schrier, S. L., and Weissman, I. L. 2011. Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. Proceedings of the National Academy of Sciences of the United States of America 108:20012.

17. Beerman, I., Bhattacharya, D., Zandi, S., Sigvardsson, M., Weissman, I. L., Bryder, D., and Rossi, D. J. 2010. Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. Proceedings of the National Academy of Sciences of the United States of America 107:5465.

18. Fulop, T., Larbi, A., Dupuis, G., Le Page, A., Frost, E. H., Cohen, A. A., Witkowski, J. M., and Franceschi, C. 2017. Immunosenescence and Inflamm-Aging As Two Sides of the Same Coin: Friends or Foes? Frontiers in immunology 8:1960.

19. Nathan, C. 2006. Neutrophils and immunity: challenges and opportunities. Nature reviews. Immunology 6:173.

20. Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., and Zychlinsky, A. 2004. Neutrophil extracellular traps kill bacteria. Science 303:1532.

21. Solana, R., Pawelec, G., and Tarazona, R. 2006. Aging and innate immunity. Immunity 24:491.

22. Sapey, E., Greenwood, H., Walton, G., Mann, E., Love, A., Aaronson, N., Insall, R. H., Stockley, R. A., and Lord, J. M. 2014. Phosphoinositide 3-kinase inhibition restores neutrophil accuracy in the elderly: toward targeted treatments for immunosenescence. Blood 123:239.

23. Wenisch, C., Patruta, S., Daxbock, F., Krause, R., and Horl, W. 2000. Effect of age on human neutrophil function. Journal of leukocyte biology 67:40.

24. Butcher, S. K., Chahal, H., Nayak, L., Sinclair, A., Henriquez, N. V., Sapey, E., O'Mahony, D., and Lord, J. M. 2001. Senescence in innate immune responses: reduced neutrophil phagocytic capacity and CD16 expression in elderly humans. Journal of leukocyte biology 70:881.

25. Sauce, D., Dong, Y., Campillo-Gimenez, L., Casulli, S., Bayard, C., Autran, B., Boddaert, J., Appay, V., and Elbim, C. 2017. Reduced Oxidative Burst by Primed Neutrophils in the Elderly Individuals Is Associated With Increased Levels of the CD16bright/CD62Ldim Immunosuppressive Subset. The journals of gerontology. Series A, Biological sciences and medical sciences 72:163.

26. Alonso-Fernandez, P., Puerto, M., Mate, I., Ribera, J. M., and de la Fuente, M. 2008. Neutrophils of centenarians show function levels similar to those of young adults. Journal of the American Geriatrics Society 56:2244.

27. Schroder, A. K. and Rink, L. 2003. Neutrophil immunity of the elderly. Mechanisms of ageing and development 124:419.

28. Gocer, P., Gurer, U. S., Erten, N., Palanduz, S., Rayaman, E., Akarsu, B., Karan, A., and Cevikbas, A. 2005. Comparison of polymorphonuclear leukocyte functions in elderly patients and healthy young volunteers. Medical principles and practice : international journal of the Kuwait University, Health Science Centre 14:382.

29. Fulop, T., Larbi, A., Douziech, N., Fortin, C., Guerard, K. P., Lesur, O., Khalil, A., and Dupuis, G. 2004. Signal transduction and functional changes in neutrophils with aging. Aging cell 3:217.

30. Fortin, C. F., Larbi, A., Dupuis, G., Lesur, O., and Fulop, T., Jr. 2007. GM-CSF activates the Jak/STAT pathway to rescue polymorphonuclear neutrophils from spontaneous apoptosis in young but not elderly individuals. Biogerontology 8:173.

31. Fulop, T., Jr., Fouquet, C., Allaire, P., Perrin, N., Lacombe, G., Stankova, J., Rola-Pleszczynski, M., Gagne, D., Wagner, J. R., Khalil, A., and Dupuis, G. 1997. Changes in apoptosis of human polymorphonuclear granulocytes with aging. Mechanisms of ageing and development 96:15.

32. Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M., and Ley, K. 2010. Development of monocytes, macrophages, and dendritic cells. Science 327:656.

33. Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D. N., Leenen, P. J., Liu, Y. J., MacPherson, G., Randolph, G. J., Scherberich, J., Schmitz, J., Shortman, K., Sozzani, S., Strobl, H., Zembala, M., Austyn, J. M., and Lutz, M. B. 2010. Nomenclature of monocytes and dendritic cells in blood. Blood 116:e74.

34. Hearps, A. C., Martin, G. E., Angelovich, T. A., Cheng, W. J., Maisa, A., Landay, A. L., Jaworowski, A., and Crowe, S. M. 2012. Aging is associated with chronic innate immune activation and dysregulation of monocyte phenotype and function. Aging cell 11:867.

35. Seidler, S., Zimmermann, H. W., Bartneck, M., Trautwein, C., and Tacke, F. 2010. Age-dependent alterations of monocyte subsets and monocyte-related chemokine pathways in healthy adults. BMC immunology 11:30.

36. Villanueva, J. L., Solana, R., Alonso, M. C., and Pena, J. 1990. Changes in the expression of HLA-class II antigens on peripheral blood monocytes from aged humans. Disease markers 8:85.

37. Qian, F., Wang, X., Zhang, L., Chen, S., Piecychna, M., Allore, H., Bockenstedt, L., Malawista, S., Bucala, R., Shaw, A. C., Fikrig, E., and Montgomery, R. R. 2012. Age-associated elevation in TLR5 leads to increased inflammatory responses in the elderly. Aging cell 11:104.

38. Nyugen, J., Agrawal, S., Gollapudi, S., and Gupta, S. 2010. Impaired functions of peripheral blood monocyte subpopulations in aged humans. Journal of clinical immunology 30:806.

39. Ter Horst, R., Jaeger, M., Smeekens, S. P., Oosting, M., Swertz, M. A., Li, Y., Kumar, V., Diavatopoulos, D. A., Jansen, A. F. M., Lemmers, H., Toenhake-Dijkstra, H., van Herwaarden, A. E., Janssen, M., van der Molen, R. G., Joosten, I., Sweep, F., Smit, J. W., Netea-Maier, R. T., Koenders, M., Xavier, R. J., van der Meer, J. W. M., Dinarello, C. A., Pavelka, N., Wijmenga, C., Notebaart, R. A., Joosten, L. A. B., and Netea, M. G. 2016. Host and Environmental Factors Influencing Individual Human Cytokine Responses. Cell 167:1111.

40. Wynn, T. A., Chawla, A., and Pollard, J. W. 2013. Macrophage biology in development, homeostasis and disease. Nature 496:445.

41. Gordon, S. 2007. The macrophage: past, present and future. European journal of immunology 37 Suppl 1:S9.

42. Plowden, J., Renshaw-Hoelscher, M., Engleman, C., Katz, J., and Sambhara, S. 2004. Innate immunity in aging: impact on macrophage function. Aging cell 3:161.

43. Takahashi, I., Ohmoto, E., Aoyama, S., Takizawa, M., Oda, Y., Nonaka, K., Nakada, H., Yorimitsu, S., and Kimura, I. 1985. Monocyte chemiluminescence and macrophage precursors in the aged. Acta medica Okayama 39:447.

44. Boehmer, E. D., Goral, J., Faunce, D. E., and Kovacs, E. J. 2004. Age-dependent decrease in Toll-like receptor 4-mediated proinflammatory cytokine production and mitogen-activated protein kinase expression. Journal of leukocyte biology 75:342.

45. Dong, C., Davis, R. J., and Flavell, R. A. 2002. MAP kinases in the immune response. Annual review of immunology 20:55.

46. Kissin, E., Tomasi, M., McCartney-Francis, N., Gibbs, C. L., and Smith, P. D. 1997. Age-related decline in murine macrophage production of nitric oxide. The Journal of infectious diseases 175:1004.

47. Herrero, C., Sebastian, C., Marques, L., Comalada, M., Xaus, J., Valledor, A. F., Lloberas, J., and Celada, A. 2002. Immunosenescence of macrophages: reduced MHC class II gene expression. Experimental gerontology 37:389.

48. Renshaw, M., Rockwell, J., Engleman, C., Gewirtz, A., Katz, J., and Sambhara, S. 2002. Cutting edge: impaired Toll-like receptor expression and function in aging. Journal of immunology 169:4697.

49. Herrero, C., Marques, L., Lloberas, J., and Celada, A. 2001. IFN-gamma-dependent transcription of MHC class II IA is impaired in macrophages from aged mice. The Journal of clinical investigation 107:485.

50. Chelvarajan, R. L., Collins, S. M., Van Willigen, J. M., and Bondada, S. 2005. The unresponsiveness of aged mice to polysaccharide antigens is a result of a defect in macrophage function. Journal of leukocyte biology 77:503.

51. Aprahamian, T., Takemura, Y., Goukassian, D., and Walsh, K. 2008. Ageing is associated with diminished apoptotic cell clearance in vivo. Clinical and experimental immunology 152:448.

52. Ogawa, T., Kitagawa, M., and Hirokawa, K. 2000. Age-related changes of human bone marrow: a histometric estimation of proliferative cells, apoptotic cells, T cells, B cells and macrophages. Mechanisms of ageing and development 117:57.

53. Verschoor, C. P., Johnstone, J., Loeb, M., Bramson, J. L., and Bowdish, D. M. 2014. Anti-pneumococcal deficits of monocyte-derived macrophages from the advanced-age, frail elderly and related impairments in PI3K-AKT signaling. Human immunology 75:1192.

54. Valero, N., Mosquera, J., Levy, A., Anez, G., Marcucci, R., and Alvarez-Mon, M. 2014. Differential induction of cytokines by human neonatal, adult, and elderly monocyte/macrophages infected with dengue virus. Viral immunology 27:151.

55. Rossi, M. and Young, J. W. 2005. Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. Journal of immunology 175:1373.

56. Steinman, R. M. and Nussenzweig, M. C. 2002. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. Proceedings of the National Academy of Sciences of the United States of America 99:351.

57. Mildner, A. and Jung, S. 2014. Development and function of dendritic cell subsets. Immunity 40:642.

58. Della Bella, S., Bierti, L., Presicce, P., Arienti, R., Valenti, M., Saresella, M., Vergani, C., and Villa, M. L. 2007. Peripheral blood dendritic cells and monocytes are differently regulated in the elderly. Clinical immunology 122:220.

59. Varas, A., Sacedon, R., Hernandez-Lopez, C., Jimenez, E., Garcia-Ceca, J., Arias-Diaz, J., Zapata, A. G., and Vicente, A. 2003. Age-dependent changes in thymic macrophages and dendritic cells. Microscopy research and technique 62:501.

60. Sridharan, A., Esposo, M., Kaushal, K., Tay, J., Osann, K., Agrawal, S., Gupta, S., and Agrawal, A. 2011. Age-associated impaired plasmacytoid dendritic cell functions lead to decreased CD4 and CD8 T cell immunity. Age 33:363.

61. Agrawal, A. and Gupta, S. 2011. Impact of aging on dendritic cell functions in humans. Ageing research reviews 10:336.

62. Agrawal, A., Agrawal, S., Cao, J. N., Su, H., Osann, K., and Gupta, S. 2007. Altered innate immune functioning of dendritic cells in elderly humans: a role of phosphoinositide 3-kinase-signaling pathway. Journal of immunology 178:6912.

63. Janssen, N., Derhovanessian, E., Demuth, I., Arnaout, F., Steinhagen-Thiessen, E., and Pawelec, G. 2016. Responses of Dendritic Cells to TLR-4 Stimulation Are Maintained in the Elderly and Resist the Effects of CMV Infection Seen in the Young. The journals of gerontology. Series A, Biological sciences and medical sciences 71:1117.

64. Bhushan, M., Cumberbatch, M., Dearman, R. J., Andrew, S. M., Kimber, I., and Griffiths, C. E. 2002. Tumour necrosis factor-alpha-induced migration of human Langerhans cells: the influence of ageing. The British journal of dermatology 146:32.

65. Hamerman, J. A., Ogasawara, K., and Lanier, L. L. 2005. NK cells in innate immunity. Current opinion in immunology 17:29.

66. Abel, A. M., Yang, C., Thakar, M. S., and Malarkannan, S. 2018. Natural Killer Cells: Development, Maturation, and Clinical Utilization. Frontiers in immunology 9:1869.

67. Panda, A., Arjona, A., Sapey, E., Bai, F., Fikrig, E., Montgomery, R. R., Lord, J. M., and Shaw, A. C. 2009. Human innate immunosenescence: causes and consequences for immunity in old age. Trends in immunology 30:325.

68. Zhang, Y., Wallace, D. L., de Lara, C. M., Ghattas, H., Asquith, B., Worth, A., Griffin, G. E., Taylor, G. P., Tough, D. F., Beverley, P. C., and Macallan, D. C. 2007. In vivo kinetics of human natural killer cells: the effects of ageing and acute and chronic viral infection. Immunology 121:258.

69. Bayard, C., Lepetitcorps, H., Roux, A., Larsen, M., Fastenackels, S., Salle, V., Vieillard, V., Marchant, A., Stern, M., Boddaert, J., Bajolle, F., Appay, V., and Sauce, D. 2016. Coordinated expansion of both memory T cells and NK cells in response to CMV infection in humans. European journal of immunology 46:1168.

70. Lopez-Verges, S., Milush, J. M., Schwartz, B. S., Pando, M. J., Jarjoura, J., York, V. A., Houchins, J. P., Miller, S., Kang, S. M., Norris, P. J., Nixon, D. F., and Lanier, L. L. 2011. Expansion of a unique CD57(+)NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. Proceedings of the National Academy of Sciences of the United States of America 108:14725.

71. Solana, R. and Mariani, E. 2000. NK and NK/T cells in human senescence. Vaccine 18:1613.

72. Ogata, K., Yokose, N., Tamura, H., An, E., Nakamura, K., Dan, K., and Nomura, T. 1997. Natural killer cells in the late decades of human life. Clinical immunology and immunopathology 84:269.

73. Ogata, K., An, E., Shioi, Y., Nakamura, K., Luo, S., Yokose, N., Minami, S., and Dan, K. 2001. Association between natural killer cell activity and infection in immunologically normal elderly people. Clinical and experimental immunology 124:392.

74. Mysliwska, J., Trzonkowski, P., Szmit, E., Brydak, L. B., Machala, M., and Mysliwski, A. 2004. Immunomodulating effect of influenza vaccination in the elderly differing in health status. Experimental gerontology 39:1447.

75. Almeida-Oliveira, A., Smith-Carvalho, M., Porto, L. C., Cardoso-Oliveira, J., Ribeiro Ados, S., Falcao, R. R., Abdelhay, E., Bouzas, L. F., Thuler, L. C., Ornellas, M. H., and Diamond, H. R. 2011. Age-related changes in natural killer cell receptors from childhood through old age. Human immunology 72:319.

76. Le Garff-Tavernier, M., Beziat, V., Decocq, J., Siguret, V., Gandjbakhch, F., Pautas, E., Debre, P., Merle-Beral, H., and Vieillard, V. 2010. Human NK cells display major phenotypic and functional changes over the life span. Aging cell 9:527.

77. Kumar, B. V., Connors, T. J., and Farber, D. L. 2018. Human T Cell Development, Localization, and Function throughout Life. Immunity 48:202.

78. Germain, R. N. 2002. T-cell development and the CD4-CD8 lineage decision. Nature reviews. Immunology 2:309.

79. Steinmann, G. G., Klaus, B., and Muller-Hermelink, H. K. 1985. The involution of the ageing human thymic epithelium is independent of puberty. A morphometric study. Scandinavian journal of immunology 22:563.

80. Flores, K. G., Li, J., Sempowski, G. D., Haynes, B. F., and Hale, L. P. 1999. Analysis of the human thymic perivascular space during aging. The Journal of clinical investigation 104:1031.

81. Andrew, D. and Aspinall, R. 2002. Age-associated thymic atrophy is linked to a decline in IL-7 production. Experimental gerontology 37:455.

82. Agarwal, S. and Busse, P. J. 2010. Innate and adaptive immunosenescence. Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology 104:183.

83. Sauce, D., Larsen, M., Fastenackels, S., Duperrier, A., Keller, M., Grubeck-Loebenstein, B., Ferrand, C., Debre, P., Sidi, D., and Appay, V. 2009. Evidence of premature immune aging in patients thymectomized during early childhood. The Journal of clinical investigation 119:3070.

84. Weinberger, B., Herndler-Brandstetter, D., Schwanninger, A., Weiskopf, D., and Grubeck-Loebenstein, B. 2008. Biology of immune responses to vaccines in elderly persons. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 46:1078.

85. Chidrawar, S., Khan, N., Wei, W., McLarnon, A., Smith, N., Nayak, L., and Moss, P. 2009. Cytomegalovirus-seropositivity has a profound influence on the magnitude of major lymphoid subsets within healthy individuals. Clinical and experimental immunology 155:423.

86. Derhovanessian, E., Maier, A. B., Beck, R., Jahn, G., Hahnel, K., Slagboom, P. E., de Craen, A. J., Westendorp, R. G., and Pawelec, G. 2010. Hallmark features of immunosenescence are absent in familial longevity. Journal of immunology 185:4618.

87. Olsson, J., Wikby, A., Johansson, B., Lofgren, S., Nilsson, B. O., and Ferguson, F. G. 2000. Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study. Mechanisms of ageing and development 121:187.

88. Wikby, A., Johansson, B., Olsson, J., Lofgren, S., Nilsson, B. O., and Ferguson, F. 2002. Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study. Experimental gerontology 37:445.

89. Almanzar, G., Schwaiger, S., Jenewein, B., Keller, M., Herndler-Brandstetter, D., Wurzner, R., Schonitzer, D., and Grubeck-Loebenstein, B. 2005. Long-term cytomegalovirus infection leads to significant changes in the composition of the CD8+ T-cell repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons. Journal of virology 79:3675.

90. Moro-Garcia, M. A., Alonso-Arias, R., and Lopez-Larrea, C. 2013. When Aging Reaches CD4+ T-Cells: Phenotypic and Functional Changes. Frontiers in immunology 4:107.

91. Pfister, G., Weiskopf, D., Lazuardi, L., Kovaiou, R. D., Cioca, D. P., Keller, M., Lorbeg, B., Parson, W., and Grubeck-Loebenstein, B. 2006. Naive T cells in the elderly: are they still there? Annals of the New York Academy of Sciences 1067:152.

92. Saurwein-Teissl, M., Lung, T. L., Marx, F., Gschosser, C., Asch, E., Blasko, I., Parson, W., Bock, G., Schonitzer, D., Trannoy, E., and Grubeck-Loebenstein, B. 2002. Lack of antibody production following immunization in old age: association with CD8(+)CD28(-) T cell clonal expansions and an imbalance in the production of Th1 and Th2 cytokines. Journal of immunology 168:5893.

93. Weng, N. P. 2006. Aging of the immune system: how much can the adaptive immune system adapt? Immunity 24:495.

94. Koch, S., Larbi, A., Derhovanessian, E., Ozcelik, D., Naumova, E., and Pawelec, G. 2008. Multiparameter flow cytometric analysis of CD4 and CD8 T cell subsets in young and old people. Immunity & ageing : I & A 5:6.

95. Czesnikiewicz-Guzik, M., Lee, W. W., Cui, D., Hiruma, Y., Lamar, D. L., Yang, Z. Z., Ouslander, J. G., Weyand, C. M., and Goronzy, J. J. 2008. T cell subset-specific susceptibility to aging. Clinical immunology 127:107.

96. Gomez, I., Marx, F., Gould, E. A., and Grubeck-Loebenstein, B. 2004. T cells from elderly persons respond to neoantigenic stimulation with an unimpaired IL-2 production and an enhanced differentiation into effector cells. Experimental gerontology 39:597.

97. Li, G., Yu, M., Lee, W. W., Tsang, M., Krishnan, E., Weyand, C. M., and Goronzy, J. J. 2012. Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity. Nature medicine 18:1518.

98. Kang, I., Hong, M. S., Nolasco, H., Park, S. H., Dan, J. M., Choi, J. Y., and Craft, J. 2004. Age-associated change in the frequency of memory CD4+ T cells impairs long term CD4+ T cell responses to influenza vaccine. Journal of immunology 173:673.

99. Ademokun, A., Wu, Y. C., and Dunn-Walters, D. 2010. The ageing B cell population: composition and function. Biogerontology 11:125.

100. Frasca, D., Landin, A. M., Riley, R. L., and Blomberg, B. B. 2008. Mechanisms for decreased function of B cells in aged mice and humans. Journal of immunology 180:2741.

101. Caraux, A., Klein, B., Paiva, B., Bret, C., Schmitz, A., Fuhler, G. M., Bos, N. A., Johnsen, H. E., Orfao, A., Perez-Andres, M., and Myeloma Stem Cell, N. 2010. Circulating human B and plasma cells. Age-associated changes in counts and detailed characterization of circulating normal CD138- and CD138+ plasma cells. Haematologica 95:1016.

102. Siegrist, C. A. and Aspinall, R. 2009. B-cell responses to vaccination at the extremes of age. Nature reviews. Immunology 9:185.

103. Frasca, D., Riley, R. L., and Blomberg, B. B. 2005. Humoral immune response and B-cell functions including immunoglobulin class switch are downregulated in aged mice and humans. Seminars in immunology 17:378.

104. Johnson, S. A. and Cambier, J. C. 2004. Ageing, autoimmunity and arthritis: senescence of the B cell compartment - implications for humoral immunity. Arthritis research & therapy 6:131.

105. Eaton, S. M., Burns, E. M., Kusser, K., Randall, T. D., and Haynes, L. 2004. Age-related defects in CD4 T cell cognate helper function lead to reductions in humoral responses. The Journal of experimental medicine 200:1613.

106. Frasca, D., Diaz, A., Romero, M., Phillips, M., Mendez, N. V., Landin, A. M., and Blomberg, B. B. 2012. Unique biomarkers for B-cell function predict the serum response to pandemic H1N1 influenza vaccine. International immunology 24:175.

107. Frasca, D., Landin, A. M., Lechner, S. C., Ryan, J. G., Schwartz, R., Riley, R. L., and Blomberg, B. B. 2008. Aging down-regulates the transcription factor E2A, activation-induced cytidine deaminase, and Ig class switch in human B cells. Journal of immunology 180:5283.

108. Shi, Y., Yamazaki, T., Okubo, Y., Uehara, Y., Sugane, K., and Agematsu, K. 2005. Regulation of aged humoral immune defense against pneumococcal bacteria by IgM memory B cell. Journal of immunology 175:3262.

109. Colonna-Romano, G., Bulati, M., Aquino, A., Pellicano, M., Vitello, S., Lio, D., Candore, G., and Caruso, C. 2009. A double-negative (IgD-CD27-) B cell population is increased in the peripheral blood of elderly people. Mechanisms of ageing and development 130:681.

110. Ma, S., Wang, C., Mao, X., and Hao, Y. 2019. B Cell Dysfunction Associated With Aging and Autoimmune Diseases. Frontiers in immunology 10:318.

111. Weinberger, B. 2018. Vaccines for the elderly: current use and future challenges. Immunity & ageing : I & A 15:3.

112. Haq, K. and McElhaney, J. E. 2014. Immunosenescence: Influenza vaccination and the elderly. Current opinion in immunology 29:38.

113. Goodwin, K., Viboud, C., and Simonsen, L. 2006. Antibody response to influenza vaccination in the elderly: a quantitative review. Vaccine 24:1159.

114. Henry, C., Zheng, N. Y., Huang, M., Cabanov, A., Rojas, K. T., Kaur, K., Andrews, S. F., Palm, A. E., Chen, Y. Q., Li, Y., Hoskova, K., Utset, H. A., Vieira, M. C., Wrammert, J., Ahmed, R., Holden-Wiltse, J., Topham, D. J., Treanor, J. J., Ertl, H. C., Schmader, K. E., Cobey, S., Krammer, F., Hensley, S. E., Greenberg, H., He, X. S., and Wilson, P. C. 2019. Influenza Virus Vaccination Elicits Poorly Adapted B Cell Responses in Elderly Individuals. Cell Host Microbe 25:357.

115. Herrero-Fernandez, I., Rosado-Sanchez, I., Alvarez-Rios, A. I., Galva, M. I., De Luna-Romero, M., Sanbonmatsu-Gamez, S., Perez-Ruiz, M., Navarro-Mari, J. M., Carrillo-Vico, A., Sanchez, B., Ramos, R., Canizares, J., Leal, M., and Pacheco, Y. M. 2019. Effect of homeostatic T-cell proliferation in the vaccine responsiveness against influenza in elderly people. Immunity & ageing : I & A 16:14.

116. Goronzy, J. J., Fulbright, J. W., Crowson, C. S., Poland, G. A., O'Fallon, W. M., and Weyand, C. M. 2001. Value of immunological markers in predicting responsiveness to influenza vaccination in elderly individuals. Journal of virology 75:12182.

117. DiazGranados, C. A., Dunning, A. J., Kimmel, M., Kirby, D., Treanor, J., Collins, A., Pollak, R., Christoff, J., Earl, J., Landolfi, V., Martin, E., Gurunathan, S., Nathan, R., Greenberg, D. P., Tornieporth, N. G., Decker, M. D., and Talbot, H. K. 2014. Efficacy of high-dose versus standard-dose influenza vaccine in older adults. N Engl J Med 371:635.

118. DiazGranados, C. A., Dunning, A. J., Jordanov, E., Landolfi, V., Denis, M., and Talbot, H. K. 2013. High-dose trivalent influenza vaccine compared to standard dose vaccine in elderly adults: safety, immunogenicity and relative efficacy during the 2009-2010 season. Vaccine 31:861.

119. Tsang, P., Gorse, G. J., Strout, C. B., Sperling, M., Greenberg, D. P., Ozol-Godfrey, A., DiazGranados, C., and Landolfi, V. 2014. Immunogenicity and safety of Fluzone((R)) intradermal and high-dose influenza vaccines in older adults >/=65 years of age: a randomized, controlled, phase II trial. Vaccine 32:2507.

120. U.S. Food and Drug Administration (FDA). 2019. Fluzone, Fluzone High-Dose and Fluzone Intradermal. Available at: https://www.fda.gov/vaccines-blood-biologics/vaccines/fluzone-fluzone-high-dose-and-fluzone-intradermal

121. Chen, W. H., Cross, A. S., Edelman, R., Sztein, M. B., Blackwelder, W. C., and Pasetti, M. F. 2011. Antibody and Th1-type cell-mediated immune responses in elderly and young adults immunized with the standard or a high dose influenza vaccine. Vaccine 29:2865.

122. Leroux-Roels, G. 2010. Unmet needs in modern vaccinology: adjuvants to improve the immune response. Vaccine 28 Suppl 3:C25.

123. Tsai, T. F. 2013. Fluad(R)-MF59(R)-Adjuvanted Influenza Vaccine in Older Adults. Infect Chemother 45:159.

124. Puig Barbera, J. and Gonzalez Vidal, D. 2007. MF59-adjuvanted subunit influenza vaccine: an improved interpandemic influenza vaccine for vulnerable populations. Expert Rev Vaccines 6:659.

125. Camilloni, B., Basileo, M., Valente, S., Nunzi, E., and Iorio, A. M. 2015. Immunogenicity of intramuscular MF59-adjuvanted and intradermal administered influenza enhanced vaccines in subjects aged over 60: A literature review. Hum Vaccin Immunother 11:553.

126. Ansaldi, F., Zancolli, M., Durando, P., Montomoli, E., Sticchi, L., Del Giudice, G., and Icardi, G. 2010. Antibody response against heterogeneous circulating influenza virus strains elicited by MF59- and non-adjuvanted vaccines during seasons with good or partial matching between vaccine strain and clinical isolates. Vaccine 28:4123.

127. Hung, I. F., Zhang, A. J., To, K. K., Chan, J. F., Li, P., Wong, T. L., Zhang, R., Chan, T. C., Chan, B. C., Wai, H. H., Chan, L. W., Fong, H. P., Hui, R. K., Kong, K. L., Leung, A. C., Ngan, A. H., Tsang, L. W., Yeung, A. P., Yiu, G. C., Yung, W., Lau, J. Y., Chen, H., Chan, K. H., and Yuen, K. Y. 2016. Topical imiquimod before intradermal trivalent influenza vaccine for protection against heterologous non-vaccine and antigenically drifted viruses: a single-centre, double-blind, randomised, controlled phase 2b/3 trial. Lancet Infect Dis 16:209.

128. Fedson, D. S. and Scott, J. A. 1999. The burden of pneumococcal disease among adults in developed and developing countries: what is and is not known. Vaccine 17 Suppl 1:S11.

129. Centers for Disease Control and Prevention (CDC). 2019. Available at: www.cdc.gov/pneumococcal/vaccination.html

130. Melegaro, A. and Edmunds, W. J. 2004. The 23-valent pneumococcal polysaccharide vaccine. Part I. Efficacy of PPV in the elderly: a comparison of meta-analyses. Eur J Epidemiol 19:353.

131. van Werkhoven, C. H., Huijts, S. M., Bolkenbaas, M., Grobbee, D. E., and Bonten, M. J. 2015. The Impact of Age on the Efficacy of 13-valent Pneumococcal Conjugate Vaccine in Elderly. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 61:1835.

132. Hayward, S., Thompson, L. A., and McEachern, A. 2016. Is 13-Valent Pneumococcal Conjugate Vaccine (PCV13) Combined With 23-Valent Pneumococcal Polysaccharide Vaccine (PPSV23) Superior to PPSV23 Alone for Reducing Incidence or Severity of Pneumonia in Older Adults? A Clin-IQ. J Patient Cent Res Rev 3:111.

133. Arvin, A. M. 1996. Varicella-zoster virus. Clin Microbiol Rev 9:361.

134. Oxman, M. N. 2009. Herpes zoster pathogenesis and cell-mediated immunity and immunosenescence. J Am Osteopath Assoc 109:S13.

135. Kawai, K., Gebremeskel, B. G., and Acosta, C. J. 2014. Systematic review of incidence and complications of herpes zoster: towards a global perspective. BMJ Open 4:e004833.

136. Oxman, M. N., Levin, M. J., Johnson, G. R., Schmader, K. E., Straus, S. E., Gelb, L. D., Arbeit, R. D., Simberkoff, M. S., Gershon, A. A., Davis, L. E., Weinberg, A., Boardman, K. D., Williams, H. M., Zhang, J. H., Peduzzi, P. N., Beisel, C. E., Morrison, V. A., Guatelli, J. C., Brooks, P. A., Kauffman, C. A., Pachucki, C. T., Neuzil, K. M., Betts, R. F., Wright, P. F., Griffin, M. R., Brunell, P., Soto, N. E., Marques, A. R., Keay, S. K., Goodman, R. P., Cotton, D. J., Gnann, J. W., Jr., Loutit, J., Holodniy, M., Keitel, W. A., Crawford, G. E., Yeh, S. S., Lobo, Z., Toney, J. F., Greenberg, R. N., Keller, P. M., Harbecke, R., Hayward, A. R., Irwin, M. R., Kyriakides, T. C., Chan, C. Y., Chan, I. S., Wang, W. W., Annunziato, P. W., Silber, J. L., and Shingles Prevention Study, G. 2005. A vaccine to prevent herpes zoster and postherpetic neuralgia in older adults. N Engl J Med 352:2271.

137. Didierlaurent, A. M., Laupeze, B., Di Pasquale, A., Hergli, N., Collignon, C., and Garcon, N. 2017. Adjuvant system AS01: helping to overcome the challenges of modern vaccines. Expert Rev Vaccines 16:55.

138. Lal, H., Cunningham, A. L., Godeaux, O., Chlibek, R., Diez-Domingo, J., Hwang, S. J., Levin, M. J., McElhaney, J. E., Poder, A., Puig-Barbera, J., Vesikari, T., Watanabe, D., Weckx, L., Zahaf, T., Heineman, T. C., and Group, Z. O. E. S. 2015. Efficacy of an adjuvanted herpes zoster subunit vaccine in older adults. N Engl J Med 372:2087.

139. Schwarz, T. F., Volpe, S., Catteau, G., Chlibek, R., David, M. P., Richardus, J. H., Lal, H., Oostvogels, L., Pauksens, K., Ravault, S., Rombo, L., Sonder, G., Smetana, J., Heineman, T., and Bastidas, A. 2018. Persistence of immune response to an adjuvanted varicella-zoster virus subunit vaccine for up to year nine in older adults. Hum Vaccin Immunother 14:1370.

140. Chlibek, R., Bayas, J. M., Collins, H., de la Pinta, M. L., Ledent, E., Mols, J. F., and Heineman, T. C. 2013. Safety and immunogenicity of an AS01-adjuvanted varicella-zoster virus subunit candidate vaccine against herpes zoster in adults >=50 years of age. The Journal of infectious diseases 208:1953.

141. Milutinovic, B. and Kurtz, J. 2016. Immune memory in invertebrates. Seminars in immunology 28:328.

142. Quintin, J., Saeed, S., Martens, J. H. A., Giamarellos-Bourboulis, E. J., Ifrim, D. C., Logie, C., Jacobs, L., Jansen, T., Kullberg, B. J., Wijmenga, C., Joosten, L. A. B., Xavier, R. J., van der Meer, J. W. M., Stunnenberg, H. G., and Netea, M. G. 2012. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. Cell Host Microbe 12:223.

143. Netea, M. G., Quintin, J., and van der Meer, J. W. 2011. Trained immunity: a memory for innate host defense. Cell Host Microbe 9:355.

144. Netea, M. G., Joosten, L. A., Latz, E., Mills, K. H., Natoli, G., Stunnenberg, H. G., O'Neill, L. A., and Xavier, R. J. 2016. Trained immunity: A program of innate immune memory in health and disease. Science 352:aaf1098.

145. Dockrell, H. M. and Smith, S. G. 2017. What Have We Learnt about BCG Vaccination in the Last 20 Years? Frontiers in immunology 8:1134.

146. Kleinnijenhuis, J., Quintin, J., Preijers, F., Joosten, L. A., Jacobs, C., Xavier, R. J., van der Meer, J. W., van Crevel, R., and Netea, M. G. 2014. BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. Clinical immunology 155:213.

147. Hole, C. R., Wager, C. M. L., Castro-Lopez, N., Campuzano, A., Cai, H., Wozniak, K. L., Wang, Y., and Wormley, F. L., Jr. 2019. Induction of memory-like dendritic cell responses in vivo. Nat Commun 10:2955.

148. Arts, R. J., Joosten, L. A., and Netea, M. G. 2016. Immunometabolic circuits in trained immunity. Seminars in immunology 28:425.

149. van der Heijden, C., Noz, M. P., Joosten, L. A. B., Netea, M. G., Riksen, N. P., and Keating, S. T. 2018. Epigenetics and Trained Immunity. Antioxid Redox Signal 29:1023.

150. Arts, R. J., Novakovic, B., Ter Horst, R., Carvalho, A., Bekkering, S., Lachmandas, E., Rodrigues, F., Silvestre, R., Cheng, S. C., Wang, S. Y., Habibi, E., Goncalves, L. G., Mesquita, I., Cunha, C., van Laarhoven, A., van de Veerdonk, F. L., Williams, D. L., van der Meer, J. W., Logie, C., O'Neill, L. A., Dinarello, C. A., Riksen, N. P., van Crevel, R., Clish, C., Notebaart, R. A., Joosten, L. A., Stunnenberg, H. G., Xavier, R. J., and Netea, M. G. 2016. Glutaminolysis and Fumarate Accumulation Integrate Immunometabolic and Epigenetic Programs in Trained Immunity. Cell Metab 24:807.

151. Arts, R. J. W., Carvalho, A., La Rocca, C., Palma, C., Rodrigues, F., Silvestre, R., Kleinnijenhuis, J., Lachmandas, E., Goncalves, L. G., Belinha, A., Cunha, C., Oosting, M., Joosten, L. A. B., Matarese, G., van Crevel, R., and Netea, M. G. 2016. Immunometabolic Pathways in BCG-Induced Trained Immunity. Cell Rep 17:2562.

152. Mitroulis, I., Ruppova, K., Wang, B., Chen, L. S., Grzybek, M., Grinenko, T., Eugster, A., Troullinaki, M., Palladini, A., Kourtzelis, I., Chatzigeorgiou, A., Schlitzer, A., Beyer, M., Joosten, L. A. B., Isermann, B., Lesche, M., Petzold, A., Simons, K., Henry, I., Dahl, A., Schultze, J. L., Wielockx, B., Zamboni, N., Mirtschink, P., Coskun, U., Hajishengallis, G., Netea, M. G., and Chavakis, T. 2018. Modulation of Myelopoiesis Progenitors Is an Integral Component of Trained Immunity. Cell 172:147.

153. Kaufmann, E., Sanz, J., Dunn, J. L., Khan, N., Mendonca, L. E., Pacis, A., Tzelepis, F., Pernet, E., Dumaine, A., Grenier, J. C., Mailhot-Leonard, F., Ahmed, E., Belle, J., Besla, R., Mazer, B., King, I. L., Nijnik, A., Robbins, C. S., Barreiro, L. B., and Divangahi, M. 2018. BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis. Cell 172:176.

154. Sanchez-Ramon, S., Conejero, L., Netea, M. G., Sancho, D., Palomares, O., and Subiza, J. L. 2018. Trained Immunity-Based Vaccines: A New Paradigm for the Development of Broad-Spectrum Anti-infectious Formulations. Frontiers in immunology 9:2936.

155. Ciarlo, E., Heinonen, T., Theroude, C., Asgari, F., Le Roy, D., Netea, M. G., and Roger, T. 2019. Trained immunity confers broad-spectrum protection against bacterial infections. The Journal of infectious diseases.

156. Benn, C. S., Netea, M. G., Selin, L. K., and Aaby, P. 2013. A small jab - a big effect: nonspecific immunomodulation by vaccines. Trends in immunology 34:431.

157. de Bree, L. C. J., Koeken, V., Joosten, L. A. B., Aaby, P., Benn, C. S., van Crevel, R., and Netea, M. G. 2018. Non-specific effects of vaccines: Current evidence and potential implications. Seminars in immunology 39:35.

158. Alhunaidi, O. and Zlotta, A. R. 2019. The use of intravesical BCG in urothelial carcinoma of the bladder. Ecancermedicalscience 13:905.

159. Fuge, O., Vasdev, N., Allchorne, P., and Green, J. S. 2015. Immunotherapy for bladder cancer. Res Rep Urol 7:65.

160. Kamat, A. M., Flaig, T. W., Grossman, H. B., Konety, B., Lamm, D., O'Donnell, M. A., Uchio, E., Efstathiou, J. A., and Taylor, J. A., 3rd. 2015. Expert consensus document: Consensus statement on best practice management regarding the use of intravesical immunotherapy with BCG for bladder cancer. Nat Rev Urol 12:225.

161. Kleinnijenhuis, J., Quintin, J., Preijers, F., Joosten, L. A., Ifrim, D. C., Saeed, S., Jacobs, C., van Loenhout, J., de Jong, D., Stunnenberg, H. G., Xavier, R. J., van der Meer, J. W., van Crevel, R., and Netea, M. G. 2012. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proceedings of the National Academy of Sciences of the United States of America 109:17537.

162. Gil, A., Kenney, L. L., Mishra, R., Watkin, L. B., Aslan, N., and Selin, L. K. 2015. Vaccination and heterologous immunity: educating the immune system. Trans R Soc Trop Med Hyg 109:62.

163. Ritz, N., Mui, M., Balloch, A., and Curtis, N. 2013. Non-specific effect of Bacille Calmette-Guerin vaccine on the immune response to routine immunisations. Vaccine 31:3098.

164. Leentjens, J., Kox, M., Stokman, R., Gerretsen, J., Diavatopoulos, D. A., van Crevel, R., Rimmelzwaan, G. F., Pickkers, P., and Netea, M. G. 2015. BCG Vaccination Enhances the Immunogenicity of Subsequent Influenza Vaccination in Healthy Volunteers: A Randomized, Placebo-Controlled Pilot Study. The Journal of infectious diseases 212:1930.

165. Dos Santos, J. C., Vilela Teodoro Silva, M., Ribeiro-Dias, F., and Joosten, L. A. B. 2019. Non-specific effects of BCG in protozoal infections: tegumentary leishmaniasis and malaria. Clin Microbiol Infect 25:1479.

166. Arts, R. J. W., Moorlag, S., Novakovic, B., Li, Y., Wang, S. Y., Oosting, M., Kumar, V., Xavier, R. J., Wijmenga, C., Joosten, L. A. B., Reusken, C., Benn, C. S., Aaby, P., Koopmans, M. P., Stunnenberg, H. G., van Crevel, R., and Netea, M. G. 2018. BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity. Cell Host Microbe 23:89.

167. Walk, J., de Bree, L. C. J., Graumans, W., Stoter, R., van Gemert, G. J., van de Vegte-Bolmer, M., Teelen, K., Hermsen, C. C., Arts, R. J. W., Behet, M. C., Keramati, F., Moorlag, S., Yang, A. S. P., van Crevel, R., Aaby, P., de Mast, Q., van der Ven, A., Stabell Benn, C., Netea, M. G., and Sauerwein, R. W. 2019. Outcomes of controlled human malaria infection after BCG vaccination. Nat Commun 10:874.

168. Kleinnijenhuis, J., Quintin, J., Preijers, F., Benn, C. S., Joosten, L. A., Jacobs, C., van Loenhout, J., Xavier, R. J., Aaby, P., van der Meer, J. W., van Crevel, R., and Netea, M. G. 2014. Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. J Innate Immun 6:152.

169. Blok, B. A., de Bree, L. C. J., Diavatopoulos, D. A., Langereis, J. D., Joosten, L. A. B., Aaby, P., van Crevel, R., Benn, C. S., and Netea, M. G. 2020. Interacting, Nonspecific, Immunological Effects of Bacille Calmette-Guerin and Tetanus-diphtheria-pertussis Inactivated Polio Vaccinations: An Explorative, Randomized Trial. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 70:455.

170. Berendsen, M., Bles, P., de Bree, C. et al. . 2020. BCG vaccination induces innate immune training in adults over 50 years of age. Optimmunize: improving the beneficial effects of vaccines Cambridge, UK. Abstract no:P3. Available at: https://coursesandconferences.wellcomegenomecampus.org/wp-content/uploads/2020/02/Optimmunize-Abstract-Book-v2.pdf

171. Wardhana, Datau, E. A., Sultana, A., Mandang, V. V., and Jim, E. 2011. The efficacy of Bacillus Calmette-Guerin vaccinations for the prevention of acute upper respiratory tract infection in the elderly. Acta Med Indones 43:185.

172. Ohrui, T., Nakayama, K., Fukushima, T., Chiba, H., and Sasaki, H. 2005. [Prevention of elderly pneumonia by pneumococcal, influenza and BCG vaccinations]. Nihon Ronen Igakkai Zasshi 42:34.

173. Morris, D. E., Cleary, D. W., and Clarke, S. C. 2017. Secondary Bacterial Infections Associated with Influenza Pandemics. Front Microbiol 8:1041.

174. MacIntyre, C. R., Chughtai, A. A., Barnes, M., Ridda, I., Seale, H., Toms, R., and Heywood, A. 2018. The role of pneumonia and secondary bacterial infection in fatal and serious outcomes of pandemic influenza a(H1N1)pdm09. BMC Infect Dis 18:637.

175. Novakovic, B., Habibi, E., Wang, S. Y., Arts, R. J. W., Davar, R., Megchelenbrink, W., Kim, B., Kuznetsova, T., Kox, M., Zwaag, J., Matarese, F., van Heeringen, S. J., Janssen-Megens, E. M., Sharifi, N., Wang, C., Keramati, F., Schoonenberg, V., Flicek, P., Clarke, L., Pickkers, P., Heath, S., Gut, I., Netea, M. G., Martens, J. H. A., Logie, C., and Stunnenberg, H. G. 2016. beta-Glucan Reverses the Epigenetic State of LPS-Induced Immunological Tolerance. Cell 167:1354.

176. Leentjens, J., Bekkering, S., Joosten, L. A. B., Netea, M. G., Burgner, D. P., and Riksen, N. P. 2018. Trained Innate Immunity as a Novel Mechanism Linking Infection and the Development of Atherosclerosis. Circ Res 122:664.

177. Bekkering, S., Arts, R. J. W., Novakovic, B., Kourtzelis, I., van der Heijden, C., Li, Y., Popa, C. D., Ter Horst, R., van Tuijl, J., Netea-Maier, R. T., van de Veerdonk, F. L., Chavakis, T., Joosten, L. A. B., van der Meer, J. W. M., Stunnenberg, H., Riksen, N. P., and Netea, M. G. 2018. Metabolic Induction of Trained Immunity through the Mevalonate Pathway. Cell 172:135.

178. Valdes-Ferrer, S. I., Rosas-Ballina, M., Olofsson, P. S., Lu, B., Dancho, M. E., Li, J., Yang, H., Pavlov, V. A., Chavan, S. S., and Tracey, K. J. 2013. High-mobility group box 1 mediates persistent splenocyte priming in sepsis survivors: evidence from a murine model. Shock 40:492.

179. Bekkering, S., Quintin, J., Joosten, L. A., van der Meer, J. W., Netea, M. G., and Riksen, N. P. 2014. 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.

180. Crisan, T. O., Cleophas, M. C., Oosting, M., Lemmers, H., Toenhake-Dijkstra, H., Netea, M. G., Jansen, T. L., and Joosten, L. A. 2016. Soluble uric acid primes TLR-induced proinflammatory cytokine production by human primary cells via inhibition of IL-1Ra. Ann Rheum Dis 75:755.

181. Gong, T., Liu, L., Jiang, W., and Zhou, R. 2020. DAMP-sensing receptors in sterile inflammation and inflammatory diseases. Nature reviews. Immunology 20:95.

182. Rock, K. L., Latz, E., Ontiveros, F., and Kono, H. 2010. The sterile inflammatory response. Annual review of immunology 28:321.

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CHAPTER 3

The immunological factors predisposing to severe COVID-19 are already present in healthy elderly and men

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Abstract

Male sex and old age are risk factors for COVID-19 severity, but the underlying causes are unknown. A possible explanation for this might be the differences in immunological profiles in males and the elderly before the infection. With this in mind, we analyzed the abundance of circulating proteins and immune populations associated with severe COVID-19 in 2 healthy cohorts. Besides, given the seasonal profile of COVID-19, the seasonal response against SARS-CoV-2 could also be different in the elderly and males. Therefore, PBMCs of female, male, young, and old subjects in different seasons of the year were stimulated with heat-inactivated SARS-CoV-2 to investigate the season-dependent anti-SARS-CoV-2 immune response. We found that several T cell subsets, which are known to be depleted in severe COVID-19 patients, were intrinsically less abundant in men and older individuals. Plasma proteins increasing with disease severity, including HGF, IL-8, and MCP-1, were more abundant in the elderly and males. Upon *in vitro* SARS-CoV-2 stimulation, the elderly produced significantly more IL-1RA and had a dysregulated IFNγ response with lower production in the fall compared with young individuals. Our results suggest that the immune characteristics of severe COVID-19, described by a differential abundance of immune cells and circulating inflammatory proteins, are intrinsically present in healthy men and the elderly. This might explain the susceptibility of men and the elderly to SARS-CoV-2 infection.

Introduction

Having emerged in China in December 2019, the coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has become a major health crisis. As of early June, 2021, SARS-CoV-2 has led to over 171 million infections and more than 3.5 million deaths worldwide [1].

The most vulnerable groups are people older than 70 years old and adults with underlying health conditions such as chronic respiratory problems and diabetes [2]. While age is the strongest predictor of death from COVID-19, the sharp increase in fatality after 50 years of age is more critical in men [3, 4]. Association of male sex with higher mortality has been consistently reported in different populations [5–7].

The factors underlying the impact of age and sex on the susceptibility to severe COVID-19 are, however, incompletely understood. Most studies to date have focused on the differences between either young and old or men and women during the disease process. A recent study has shown that male COVID-19 patients had higher circulating concentrations of cytokines such as IL-8 and IL-18, as well as a greater abundance of non-classical monocytes [8]. In contrast, a higher degree of T cell activation was observed in females than males during SARS-CoV-2 infection.

We previously analyzed 269 circulating proteins to identify COVID-19 severity markers by comparing patients in the intensive care unit (ICU) to patients who do not require ICU admission [9]. Several cytokines and chemokines such as IL-8 and monocyte chemoattractant protein-3 (MCP-3), and growth factors, e.g., hepatocyte growth factor (HGF), were increased in ICU patients, whereas stem cell factor (SCF) and several TNF-family proteins, e.g., TNF-related activation-induced cytokine (TRANCE) were decreased in ICU patients. Besides, frequently reported severe COVID-19 characteristics include elevated TNFα, IL-6, IL-7, MCP-1, IP-10, G-CSF, and IL-10 concentrations, lower numbers and activity of $CD4^+$, $CD8^+$ cells, Tregs, B cells, and NK cells, increased number of plasmablasts and neutrophils, lower antigen presentation, and downregulated type I interferon signaling [10–15]. Changes in cell populations and plasma proteins related to COVID-19 severity are also summarized in **Supplementary Table 1**. However, it is unknown whether such differences are induced by the disease severity itself, or the potential to respond differently was already present in the healthy steady-state condition.

In this study, we analyzed some of the immune cell populations and circulating proteins linked to COVID-19 severity (**Supplementary Table 2**) in two Dutch cohorts

of healthy individuals. Our reasoning for choosing these parameters is based on the type of data available from the two cohorts. We investigated if healthy men and individuals over 50 years old already have an immunological profile that predisposes them to severe COVID-19 progression upon SARS-CoV-2 infection. Although the pandemic's seasonal character is not completely clear, several studies reported links to temperature and humidity [16, 17]. Therefore, we also hypothesized that immunological differences induced by the seasons could influence the disease outcome. Hence, we investigated SARS-CoV-2-induced immune responses in healthy individuals *in vitro* at different time points of a year using cryo-preserved PBMCs from one of the cohorts. This study provides new insights into how age, sex, and seasons influence COVID-19 response (**Figure 1**).

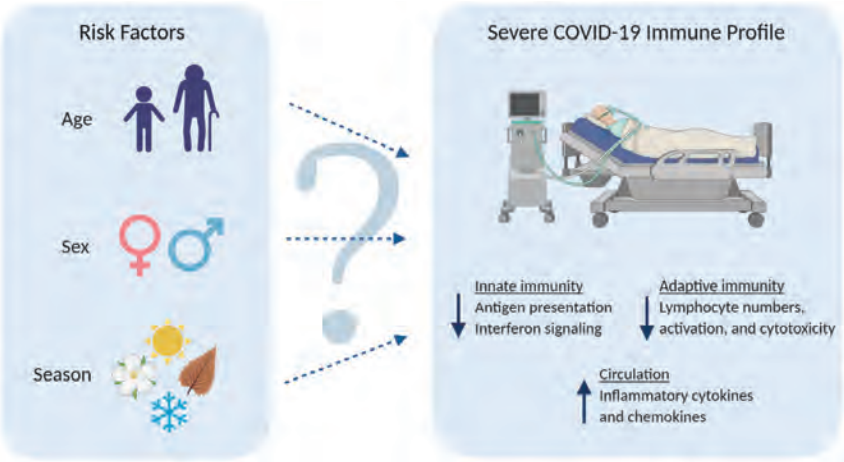

Figure 1. (Left) Potential COVID-19 risk factors investigated in this study. (Right) Frequently reported immunological characteristics of severe COVID-19 patients.

Downward arrows depict a decline, while upward arrow represents an increase. (Created with BioRender.com).

Materials and methods

Study Cohorts

534 healthy individuals of Western European origin were included in Cohort 1 (500 Functional Genomics Project, see www.humanfunctionalgenomics.org) at the Radboud University Medical Center between August 2013 and December 2014. 45 volunteers were initially excluded due to medication use and chronic diseases, while 37 participants were later excluded from the analysis because one or more

measurements were unavailable. Data of 452 participants, 229 females and 223 males with age ranges of 18-70 and 18-75, respectively, were used for analysis.

324 healthy individuals of Western European origin, Cohort 2, were included from April 2017 until June 2018 at the Radboud University Medical Center. 183 participants were female, and 141 were male with age ranges of 18-62 and 18-71, respectively. This cohort served as a validation cohort for proteomics.

Both studies were approved by the Arnhem-Nijmegen Medical Ethical Committee (NL42561.091.12 and NL58553.091.16). Inclusion and experimentation procedures were conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all volunteers before sample collection.

Proteomics

Plasma proteins were measured using the Proximity Extension Assay (PEA) by Olink Proteomics (Uppsala, Sweden). The Olink Inflammation Panel consisting of 92 inflammation-related biomarkers was measured. This assay provides relative protein quantification expressed as normalized protein expression (NPX) values on a log2 scale. The proteins for which the missing data frequency was over 20% were excluded from the analysis. The remaining data under the detection limit was replaced with the lower limit of detection for each protein. Measurements were normalized according to inter-plate controls.

Flow Cytometry

Immune cell types in Cohort 1 were measured from whole blood by 10-color flow cytometry with Navios flow cytometer (Beckman Coulter, CA, USA). Staining and gating strategies were previously described in detail by Aguirre-Gamboa et al. [18].

In Vitro Stimulations and Cytokine Measurements

From the 452 individuals in Cohort 1, a sub-cohort of 50 people were asked to donate blood at 4 different time points in a year. Peripheral blood mononuclear cells (PBMCs) were collected and cryo-preserved between February 2016 and February 2017 to assess the seasonality of immune responses. From those 50, we selected 20 individuals considering the optimal age and sex matching of young-old and male-female comparisons. Therefore, cells isolated from 5 young males, 5 old males, 5 young females, and 5 old females were included for an *in vitro* study assessing seasonality's impact on the cytokine responses to SARS-CoV-2. Cohort demographics are shown in **Supplementary Table 3**. Upon thawing, PBMCs were stimulated with heat-inactivated SARS-CoV-2 at a concentration of 3.3×10^3 TCID50/mL and heat-inactivated Influenza

A H1N1 (California strain) at a concentration of 3.3×10^5/mL. To measure the innate immune response, the cytokines TNFα, IL-6, IL-1β, and IL-1RA were measured after 24 hours incubation with SARS-CoV-2 and influenza A, whereas IFNγ response was measured after a 5-day incubation with the viruses. A control condition without a stimulus was included in the experiment. Cytokine concentrations in the supernatants were measured with DuoSet® ELISA kits (R&D Systems, MN, USA) according to the manufacturer's protocols.

Statistical Analyses

Statistical analyses were performed using R 3.6.1 (www.R-project.org) and GraphPad Prism 8 (GraphPad Software Inc., CA, USA). After adjusting the data for the covariate sex using linear regression, correlation of cell numbers or protein levels with age was done using Spearman's rank-order correlation (**Figures 2, 4**). Correction for sex was not applied in the heatmaps where two sexes were analyzed separately. After adjusting the data for the covariate age, differential protein expression or cell numbers between males and females was tested using the Mann-Whitney test (**Figures 3, 5**). For box plots comparing different age groups, the two sexes, or four seasons, the Mann-Whitney test was used between any two groups. The Benjamini-Hochberg procedure was employed to correct multiple testing errors for the heatmaps and volcano plots. False discovery rate (FDR)-adjusted p-values smaller than 0.05 were considered statistically significant.

Results

Age-Dependent Changes in Immune Cell Populations Linked to COVID-19 Severity

Various immune cell sub-types were correlated with age in Cohort 1. Non-classical monocytes increased with advancing age in both sexes, but intermediate and classical monocyte numbers were not significantly correlated with age. $CD56^{bright}$ NK population decreased with age in females while other NK cell types remained unchanged (**Figure 2A**). Previously, these results were partly reported by our group [18].

Total T cell count was negatively correlated with age, indicating the lymphopenia already experienced by the elderly, even in health, is likely to predispose to COVID-19 severity. Naïve T cells, especially $CD8^+$, exhibited the most striking age-dependent decline in both sexes (**Figures 2B, C**). Naïve regulatory T cells (Tregs) also decreased considerably with age in males, whereas memory Treg numbers were elevated. Total and naïve B cell numbers were negatively correlated with age. Plasmablasts similarly

declined significantly in females. No significant age-related changes were observed among the other B cell sub-types.

Overall, these results demonstrate that naïve $CD4^+$, $CD8^+$, Treg, and B cell pools, as well as $CD56^{bright}$ NK cells, which are all depleted in severe COVID-19, also decrease with age. The differences are clear even from the age of 50.

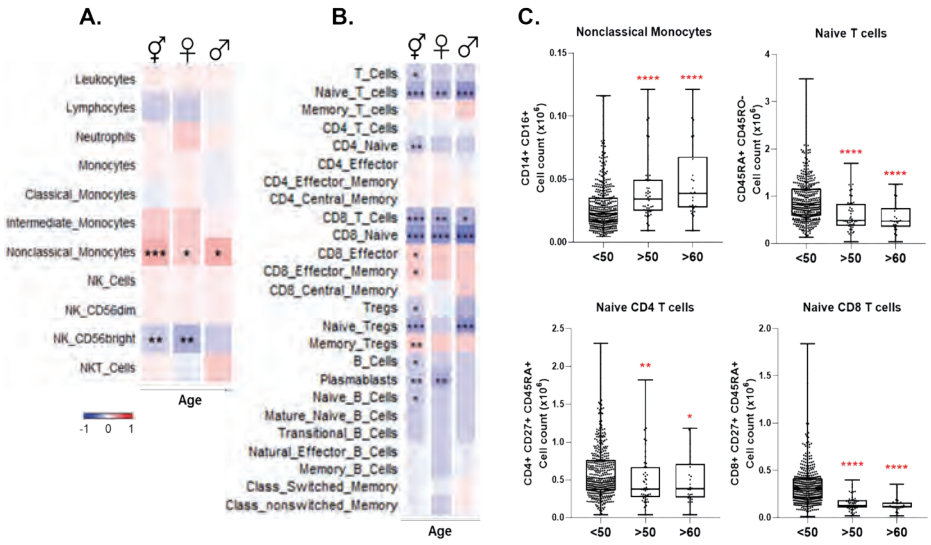

Figure 2. Heatmaps depicting Spearman correlation of age with cell numbers in (A) Cohort 1 and (B) Cohort 2

Red depicts positive correlation while blue depicts negative correlation. Data were controlled for the covariate sex when analyzing the whole cohort. **(C)** Exemplary bar plots of selected cell populations in individuals aged less than 50, more than 50, and more than 60. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. ⚥ whole cohort, ♀ females (n = 229), ♂ males (n = 223).

Sex-Dependent Patterns of Immune Cell Populations Linked to COVID-19 Severity

Next, we investigated the differential abundance of the same immune cell populations influencing COVID-19 severity in females vs. males. Only $CD8^+$ effector memory T cells were significantly more abundant in males (**Figure 3A**). Almost all cell types, including neutrophils, naïve $CD4^+$ and $CD8^+$ T cells, memory T cells, class-switched memory B cell, and $CD56^{bright}$ NK cell counts, were significantly higher in females (**Figures 3A, B**). The T cell types and $CD56^{bright}$ NK cells, which are depleted in severe COVID-19 and elderly healthy people, are also apparently less abundant in males.

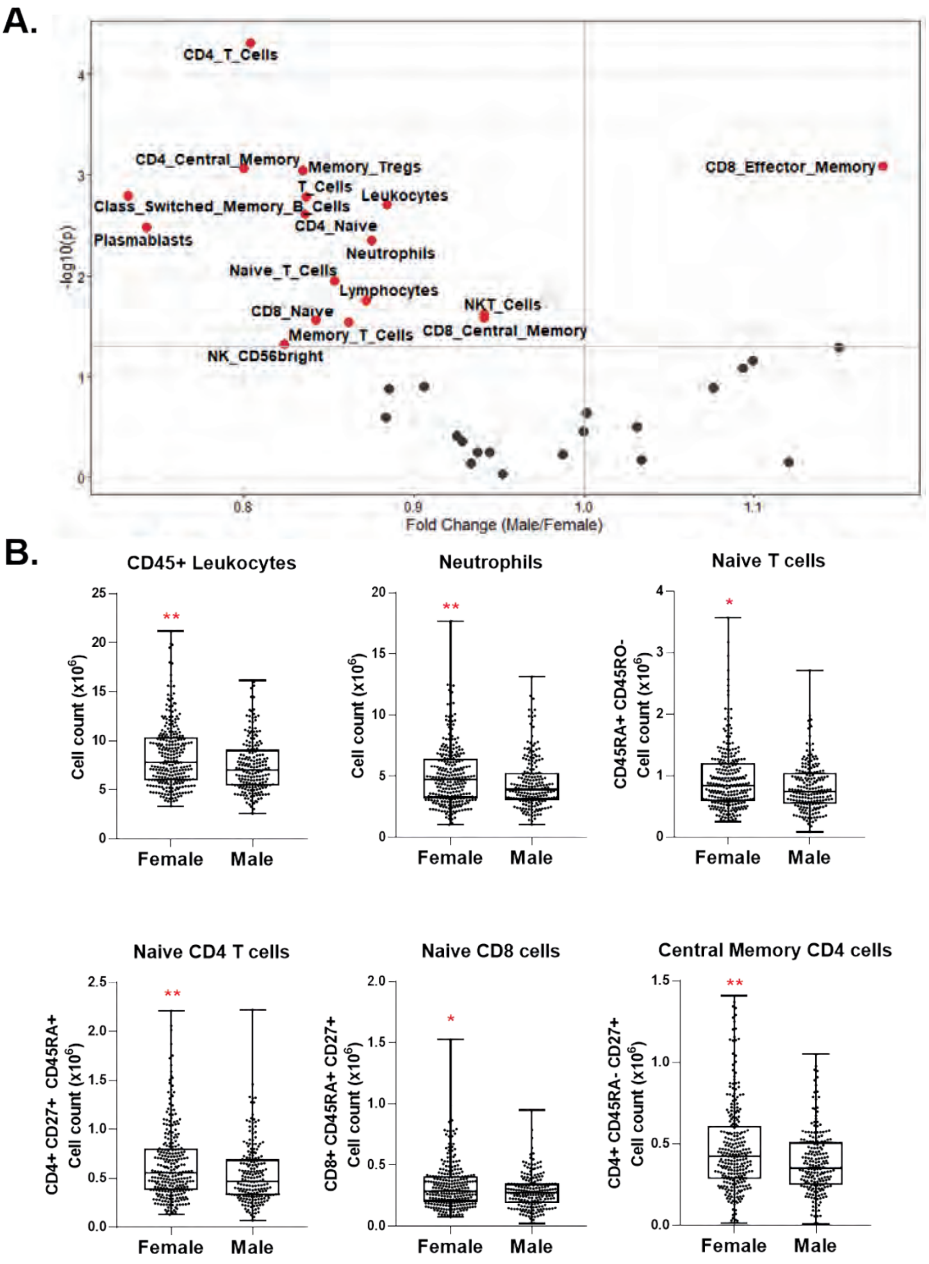

Figure 3. (A) Volcano plot depicting differential numbers of cell populations depending on sex in Cohort 1. Significant results are depicted in red. Data were controlled for the covariate age. **(B)** Exemplary bar plots of selected cell populations in females and males. *$p \leq 0.05$, **$p \leq 0.01$.

Age-Dependent Changes in Immune Mediator Proteins Linked to COVID-19 Severity

We selected 28 proteins whose plasma concentrations have been associated with severe COVID-19 and correlated them with age in healthy cohorts. Circulating IL-6 concentrations increased with age in healthy women, while IL-18 concentrations were higher in healthy men with advancing age in Cohort 1 (**Figure 4A**). IL-8 concentrations positively correlated with old age in both females and males; however, it was only significant when sexes were combined. Among investigated chemokines, only MCP-1 was positively correlated with age in females in Cohort 2 (**Figure 4B**).

TNF-family proteins in plasma also changed with increasing age: TNF and TNFB concentrations were significantly lower in the circulation of older men in Cohort 1 and 2, respectively (**Figures 4A, B**). TRANCE sharply declined in older individuals, more strikingly in males, while TWEAK was positively correlated only in females with advancing age. Moreover, aging in males was associated with elevated osteoprotegerin (OPG) concentrations, and HGF concentrations exhibited a considerable age-dependent increase in females in both cohorts.

In summary, several proteins in plasma that are increased in severe COVID-19 patients, such as IL-6, IL-8, IL-18, MCP-1, OPG, and HGF, are more abundant in healthy elderly compared to young individuals (**Figures 4A–C**). Furthermore, proteins that are lower in severe COVID-19, e.g., TRANCE, decline with age.

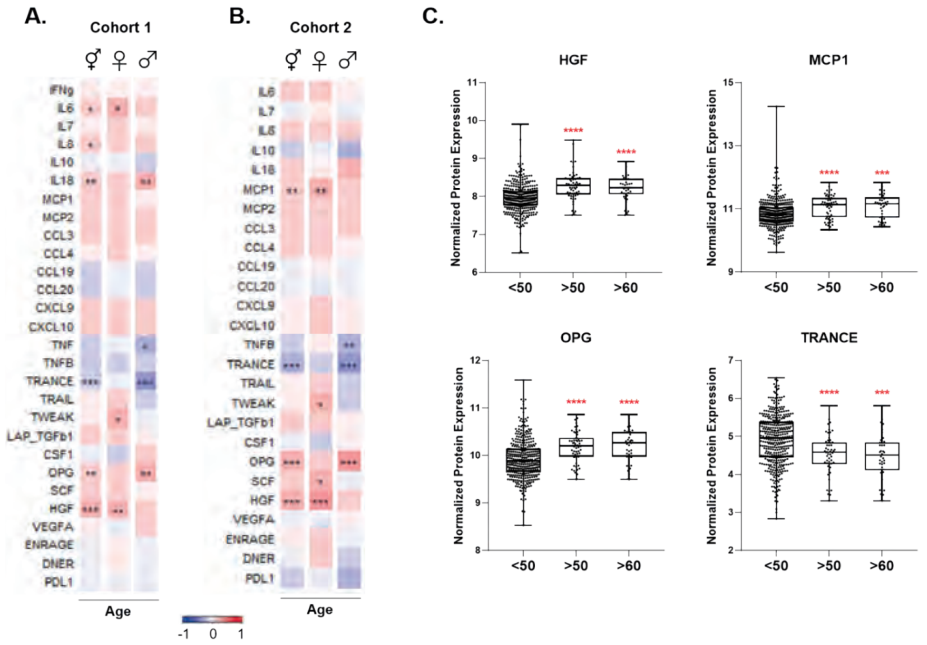

Figure 4. Heatmaps depicting Spearman correlation of age with plasma levels of proteins linked to COVID-19 severity in (A) Cohort 1 and (B) Cohort 2

Red depicts positive correlation while blue depicts negative correlation. Data were controlled for the covariate sex when analyzing the whole cohort. **(C)** Exemplary bar plots of plasma levels of selected proteins in individuals aged less than 50, more than 50, and more than 60. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. ⚥ whole cohort, ♀ females (nCohort1 = 229, nCohort2 = 183), ♂ males (nCohort1 = 223, nCohort2 = 141).

Sex-Dependent Patterns of Immune Mediator Proteins Linked to COVID-19 Severity

We also compared plasma protein concentrations between sexes. It must be noted that Koeken et al. previously reported that males and females of Cohort 2 exhibit differences in baseline levels of many inflammatory markers [19]. Here we provide a more detailed analysis of COVID-19-related proteins among those in Cohorts 1 and 2. We observed a similar sex-dependent trend in both cohorts (**Figures 5A, B**). Only OPG and colony-stimulating factor-1 (CSF-1), related to severe COVID-19, were significantly higher in women (**Figure 5C**). On the other hand, plasma concentrations of other severity markers such as IL-8, IL-18, MCP-1, MCP-2, CCL3, and CCL4 were all higher in men. Furthermore, TRAIL, TWEAK, and TRANCE, which are all lower in COVID-19 patients in ICU, were more abundant in males [9]. Males exhibited more anti-inflammatory proteins, e.g., PD-L1 and IL-10. Growth factors HGF and SCF were also more abundant in male plasma. These analyses show that most of the inflammatory mediators playing a role in infection severity are already higher in the circulation of healthy men.

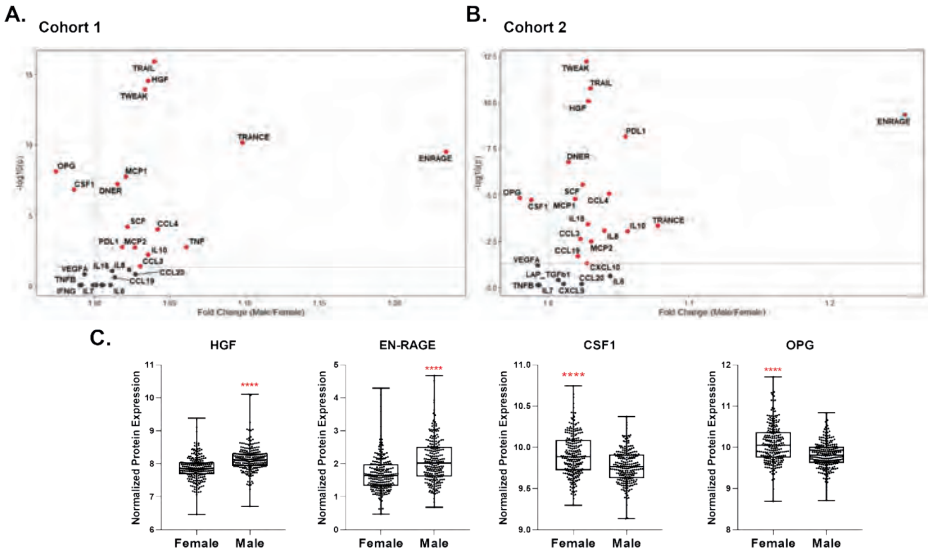

Figure 5. Volcano plots depicting differential plasma levels of proteins linked to COVID-19 severity depending on sex in (A) Cohort 1 and (B) Cohort 2

Significant results are depicted in red. Data were controlled for the covariate age. **(C)** Exemplary bar plots of selected proteins in females and males.****$p \leq 0.0001$.

We hypothesized that circulating inflammatory proteins could be related to impaired cytokine response against the virus. Therefore, we correlated SARS-CoV-2-induced *in vitro* cytokine productions with baseline circulating protein concentrations in a sub-cohort of Cohort 1. Indeed, PBMCs of the individuals with higher baseline plasma levels of MCP-2 and IL-8 produced more IL-1RA against SARS-CoV-2 *in vitro* (**Supplementary Figure 1**). Furthermore, MCP-1 was negatively correlated with IFNγ production. The data indicate that higher baseline plasma concentrations of MCP-2, IL-8, and MCP-1 are associated with the inability to produce an optimal defense against SARS-CoV-2 infection.

Sex, Age, and Season as Influencing Factors of Immune Response Against SARS-CoV-2

Next, we investigated the impact of seasonality on the SARS-CoV-2-induced immune response and assessed the contribution of age and sex. To this end, we selected 20 individuals from Cohort 1, for which cryo-preserved cells collected at 4 roughly equidistant time points in one year were available. We stimulated their PBMCs with heat-inactivated SARS-CoV-2 and influenza A H1N1.

We found that cytokine production upon SARS-CoV-2 stimulation did not substantially vary during the year, considering all 20 individuals (**Supplementary Figure 2**). However, SARS-CoV-2-induced cytokine production did differ for different age groups and sexes throughout the year. Cytokines of the IL-1 biological pathway were higher in the elderly: IL-1β production tended to be greater in the elderly than in young individuals (**Figure 6A**), while SARS-CoV-2 induced more IL-1RA all-year-round in the old individuals (**Figure 6B**). Of note, basal IL-1RA production in the absence of any stimulus was also significantly higher in the elderly, but this was not the case for IL-1β **(Supplementary Figures 3A, B**). Interestingly, IFNγ production upon stimulation with SARS-CoV-2 had a different seasonal profile in the young and elderly: young individuals produced more IFNγ in the summer and fall (**Figure 6C**). Remarkably, the elderly did not display this seasonal effect, with low IFNγ production throughout the year. In contrast to IL-1RA, the basal IFNγ production of the young and the elderly was very low and not different between groups (**Supplementary Figure 3C**). TNFα and IL-6 productions upon stimulation were similar in the young and the elderly (**Supplementary Figure 4A, B**). In addition, IL-1β production in response to SARS-CoV-2 in spring and summer was higher in females; however, the average yearly response failed to reach statistical significance (**Figure 6D**). IL-1RA and IFNγ production in males and females were comparable (**Figures 6E, F**).

Stimulating PBMCs using another RNA virus, influenza H1N1, resulted in a similar pattern to SARS-CoV-2 stimulation regarding the age and sex effects. The elderly tended to produce more IL-1β and IL-1RA, while young individuals could produce higher IFNγ amounts upon influenza stimulation (**Supplementary Figure 5**). Similar amounts of IL-1β, IL-1RA, and IFNγ were induced in males and females on average, with few exceptions (**Supplementary Figures 5D–F**).

These data show that individuals of distinct ages and sexes respond differently to SARS-CoV-2 infection depending on the seasons of the year.

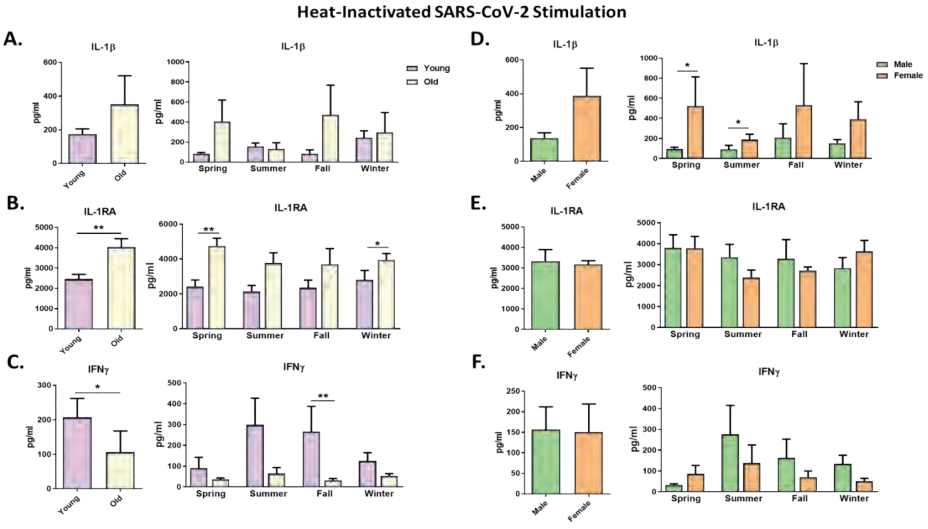

Figure 6. Cytokine responses against heat-inactivated SARS-CoV-2 in healthy individuals

Each panel's left graphs show the yearly average production, while the right graphs demonstrate cytokine production in every season. Responses were compared between young and old individuals (**A–C**) and between males and males (**D–F**). IL-1β and IL-1RA cytokine levels were measured after 24 hours, while IFNγ was measured after 5 days. *p ≤ 0.05, **p ≤ 0.01. n = 8-10. Error bars depict the standard error of the mean (SEM).

Discussion

Old age and male sex are important risk factors for COVID-19 severity. Several studies have investigated whether immune responses during SARS-CoV-2 infection are influenced by demographic factors such as age and sex [20, 21]. Although they identified immune profiles associated with severe COVID-19, they could not assess whether these were secondarily induced by the disease, or due to *a-priori* immune differences between different groups. In the present study, we show that the immune characteristics associated with severe COVID-19, such as specific changes in cell populations and circulating inflammatory proteins, are already present in healthy elderly and men. Interestingly, while the season did not impact the immune response to SARS-CoV-2 stimulation in the entire group, there was a clear difference in the responses between the young and old. Young individuals, but not the elderly, improve their IFNγ responses to SARS-CoV-2 during the summer.

COVID-19 progression to a severe clinical picture is related to the depletion of several immune cell types, including naïve $CD4^+$ and $CD8^+$, and $CD56^{high}$ NK cells. Our study

demonstrates the age-related decline of these cells in healthy individuals even before the infection, which likely contributes to their incapacity to eliminate the virus. These data are supported by studies suggesting that some of these cell types are scarcer in uninfected elderly and males [22, 23]. Naïve B lymphocyte numbers were also reduced with advanced age, which would undermine the development of adaptive immunity and antibody production upon infection [24]. The aging process does not only alter cell numbers, but also the functions (**Figure 6C**) [25]. All these might cumulatively disrupt the response against SARS-CoV-2 infection.

Sex differences in immune cell types have been documented before [26]. In line with the literature, we showed that one of the striking differences in immune cell types between males and females was the number of $CD4^+$ T cells. Although we cannot rule out that significant differences in cell populations might not determine the disease severity, SARS-CoV-2-specific $CD4^+$ T cells were strongly linked with milder COVID-19, unlike antibodies and $CD8^+$ T cell numbers [27]. Fast induction of $CD4^+$ T cells was related to a milder disease, while defects in inducing SARS-CoV-2-specific $CD4^+$ T cells were associated with severe or fatal COVID-19.

An exaggerated systemic inflammation has been associated with severe COVID-19, mirrored by high circulating concentrations of pro-inflammatory mediators [28]. Among those, IL-8, IL-18, and MCP-1 have been frequently reported, and the first two characterize the immune response of men with a severe outcome [8]. Notably, their concentrations are already higher in both men and the elderly in our healthy cohorts. Another protein displaying the same pattern is HGF, acting on epithelial and T cells promoting migration [29], which has higher concentrations in the circulation of severe patients [30]. The circulating HGF concentrations are higher in men, but also increases with age in women.

Chemokines are critical inflammatory mediators, and MCP-2, CCL3, CCL4, CCL19, and CXCL10 are all more abundant in males. ENRAGE (S100A12), produced by neutrophils and monocytes, is also higher in healthy males than females. Monocytes expressing high S100A12 and IL-8 are linked to COVID-19 severity [31]. Additionally, anti-inflammatory proteins IL-10 and PD-L1 are elevated in healthy males and severe COVID-19. Initially considered a negative feedback mechanism for infection-induced inflammation, there are arguments suggesting these proteins as biomarkers of immune exhaustion, which is likely to play a substantial role in the pathophysiology of COVID-19 [32, 33]. Notably, the association of early IL-10 production with COVID-19 severity supports this idea [34].

Severity markers increasing with old age, but not affected by sex, include IL-6 and OPG. IL-6 secreted by hyperactive monocytes contributes to low HLA-DR expression and lymphopenia in severe COVID-19 [35]. TNF-family cytokine receptor OPG, abundant in ICU patients, increases with old age in our healthy cohorts [9]. High OPG concentrations in females are likely due to estrogen's effects promoting OPG expression to inhibit bone resorption [36]. Another TNF-family member, TRANCE (RANKL), which is lower in severe COVID-19 cases, also declines with advancing age in healthy individuals. T cells are one of the primary TRANCE sources, which might explain its scarcity in the elderly and severe COVID-19 patients with lymphopenia [37].

Of note, concentrations of circulating IL-7 and IFNγ, which are increased in severe COVID-19, are similar in men and women. Therefore, T cell numbers being higher in women is unlikely due to IL-7-induced lymphopoiesis, whereas higher T cell numbers do not necessarily lead to more circulating IFNγ. An overview of the age- and sex-dependent immune profiles in healthy individuals potentially predisposing to severe COVID-19 upon infection is provided in **Figure 7**.

CELL TYPE	HIGH IN SEVERE COVID-19	HEALTHY COHORTS: INCREASES WITH AGE	HEALTHY COHORTS: HIGHER IN MALES
Memory Tregs	✓	✓	
PROTEIN			
IL6	✓	✓	
IL8	✓	✓	✓
IL10	✓		✓
IL18	✓	✓	✓
MCP1	✓	✓	✓
MCP2	✓		✓
CCL3	✓		✓
CCL4	✓		✓
CCL19	✓		✓
CXCL10	✓		✓
PDL1	✓		✓
HGF	✓	✓	✓
OPG	✓	✓	
ENRAGE	✓		✓

CELL TYPE	LOW IN SEVERE COVID-19	HEALTHY COHORTS: DECREASES WITH AGE	HEALTHY COHORTS: HIGHER IN FEMALES
CD56^{high} NK cells	✓	✓	✓
T cells	✓	✓	✓
Naive CD4 T Cells	✓	✓	✓
Naive CD8 T Cells	✓	✓	✓
Tregs	✓	✓	
Naive Tregs	✓	✓	
B Cells	✓	✓	
Naive B Cells	✓	✓	
PROTEIN			
TRANCE	✓	✓	

Figure 7. Age- and sex-dependent factors in healthy individuals that are in-line with the severe COVID-19 phenotype

Aging is already known to alter the immune system in numerous ways. The collective impairments in the aging immune system, termed immunosenescence, and the chronic systemic inflammatory state called inflammaging renders the elderly more susceptible to infections [38, 39]. Some of the hallmarks of immune aging observed in both sexes, such as the decline in $CD8^+$ naïve T cells and elevated circulating levels of IL-6 and IL-8, are shared with the pathophysiology of severe COVID-19. Thus, the high COVID-19-related morbidity and mortality observed in the elderly is pathophysiologically plausible. On top of that, in this study, we identified new factors in the elderly, such as elevated OPG and decreased TRANCE, that are associated with COVID-19 severity but not classically linked to immunosenescence.

Immune responses are more robust and homogenous in young adults, while heterogenous and more variable in the elderly. Along with the age-dependent changes in the immune system, immunobiography of individuals, which is defined by the dose, magnitude, and type of the antigen that each person is exposed to during their lifetime, can explain the large heterogeneity in immune responses, especially in older people [40]. Although history of infections affects the immune system of both males and females, our group and others identified a clear distinction between the aged immune systems of men and women [41]. As an example, older males have higher pro-inflammatory cytokine levels along with more innate immune activity, but a lower adaptive immune function compared to older females. These variations surely underlie the differences in susceptibility of elderly males and females to infections, including COVID-19.

Environmental factors are also known to affect immune responses. Certain infections such as influenza follow a seasonal pattern [42], and the evolution of the pandemic last year also suggested that COVID-19 incidence might follow a seasonal variation [16]. Therefore, we questioned whether this might be due to seasonal changes in the immune response to the virus. In the entire group, we found no clear seasonal response against SARS-CoV-2, although this could be due to the limited sample size. Interestingly, young individuals improve their IFNγ response to SARS-CoV-2 during the summer, while the elderly do not. Further research with larger cohorts is required to validate seasonality's full impact on anti-SARS-CoV-2 host defense.

Our results indicate that the immune response upon *in vitro* SARS-CoV-2 stimulation varies depending on age and sex. The response of the elderly is characterized by low IFNγ and elevated IL-1RA production. IFNγ is crucial for an effective response of T- and NK cells to viral infections, and its deficiency is associated with severe COVID-19 [43]. Depleted NK and T cell pools might explain why the elderly are less capable of

producing IFNγ upon SARS-CoV-2 stimulation. Poor IFNγ response, especially in the fall, might put the elderly at higher risk for severe COVID-19. Men and women produce comparable amounts of IFNγ, although the T cell numbers are higher in women. Other roles of T cells besides IFNγ production may contribute to the better prognosis of women with COVID-19. One important point is that these defects are present in the whole population, not at the individual level: while the elderly as a group have lower immune responses, there are certainly aged individuals who have effective immune reaction. This inter-individual variability due to immunobiography of each person could be the reason why some elderly or some men have good responses against SARS-CoV-2 and develop only mild disease.

In COVID-19, an overproduction of pro-inflammatory cytokines contributes to the pathophysiology late in the disease [44]. On the other hand, cytokines such as IL-1β might also be crucial for an early anti-viral response. The deficiency of IL-1β or its receptor causes higher viral load and mortality in murine models [45]. Furthermore, genetic variants in *IL1B* contribute to influenza susceptibility in humans [46]. We observed higher IL-1β production in women in response to SARS-CoV-2 *in vitro*, arguing that an initial potent anti-viral defense is essential to prevent severe disease. Moreover, IL-1RA is an antagonist of IL-1 bioactivity, helping prevent excessive inflammation [47]. However, early IL-1RA production in patients is associated with COVID-19 severity [34], while our finding of higher IL-1RA production in the elderly suggests that IL-1RA might be hindering their ability to mount an optimal immune response against SARS-CoV-2. Alternatively, high IL-1RA production with increasing age might mirror the general inflammatory profile of elderly individuals, as we have shown that baseline IL-1RA produced by PBMCs is higher in the elderly compared to young individuals.

Due to limited statistical power, it was not possible to assess the impact of season depending on both age and sex of the volunteers, e.g. comparing old men, old women, young men, and young women. Assessing how age, sex, and season collectively affect the anti-SARS-CoV-2 response of each subgroup should be assessed in larger future studies. An additional aspect to be assessed in future studies is the potential contribution of innate immune cell reprogramming (also called 'trained immunity') to the hyperinflammation in COVID-19. Trained immunity can be beneficial for the host in terms of protection against heterologous infection, but dysregulated trained immunity response could result in pathological conditions as it was described in autoinflammatory diseases [48, 49]. A recent study has described induction of trained immunity by SARS-CoV-2 infection [50], and it has been proposed that this contributes to the acute dysregulation during the disease both an endothelial and immune cell level [51].

In conclusion, our findings shed light on the immunological factors that might explain why men and the elderly have a higher risk of developing severe COVID-19. These results also emphasize the importance of the IL1β/IL-1RA axis and IFNγ in the anti-SARS-CoV-2 response. We propose that intrinsically different immune characteristics, including plasma inflammatory mediators and immune cell populations, in healthy people would influence their immune response upon SARS-CoV-2 infection and the severity of the disease. Results of this study inform prophylactic and therapeutic efforts.

Data Availability Statement

The data analyzed in this study is subject to the following licenses/restrictions: The data is available on request through http://www.humanfunctionalgenomics.org. Requests to access these datasets should be directed to http://www.humanfunctionalgenomics.org.

Ethics Statement

The studies involving human participants were reviewed and approved by The Arnhem-Nijmegen Medical Ethical Committee: NL42561.091.12 and NL58553.091.16. The patients/participants provided their written informed consent to participate in this study.

Author Contributions

GK, OB, and MGN conceptualized and designed the study. MJ, RH, SJCFMM, VACMK, and CB conducted the cohort studies and provided the data. GK and OB performed the analyses and *ex vivo* experiments. LABJ and MGN provided funding. GK and OB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest

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

References

1. European Center of Disease Prevention and Control. COVID-19 Situation Update Worldwide. Available at: www.ecdc.europa.eu/en/geographical-distribution-2019-ncov-cases (Accessed June 2, 2021).
2. Wolff D, Nee S, Hickey NS, Marschollek M. Risk Factors for Covid-19 Severity and Fatality: A Structured Literature Review. Infection (2021) 49(1):15–28. doi: 10.1007/s15010-020-01509-1
3. Pastor-Barriuso R, Perez-Gomez B, Hernan MA, Perez-Olmeda M, Yotti R, Oteo-Iglesias J, et al. Infection Fatality Risk for SARS-CoV-2 in Community Dwelling Population of Spain: Nationwide Seroepidemiological Study. BMJ (2020) 371:m4509. doi: 10.1136/bmj.m4509
4. Meng Y, Wu P, Lu W, Liu K, Ma K, Huang L, et al. Sex-Specific Clinical Characteristics and Prognosis of Coronavirus Disease-19 Infection in Wuhan, China: A Retrospective Study of 168 Severe Patients. PLoS Pathog (2020) 16(4):e1008520. doi: 10.1371/journal.ppat.1008520
5. Suleyman G, Fadel RA, Malette KM, Hammond C, Abdulla H, Entz A, et al. Clinical Characteristics and Morbidity Associated With Coronavirus Disease 2019 in a Series of Patients in Metropolitan Detroit. JAMA Netw Open (2020) 3(6):e2012270. doi: 10.1001/jamanetworkopen.2020.12270
6. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, et al. Clinical Characteristics of 113 Deceased Patients With Coronavirus Disease 2019: Retrospective Study. BMJ (2020) 368:m1091. doi: 10.1136/bmj.m1091
7. Gupta S, Hayek SS, Wang W, Chan L, Mathews KS, Melamed ML, et al. Factors Associated With Death in Critically Ill Patients With Coronavirus Disease 2019 in the US. JAMA Intern Med (2020) 180(11):1436–46. doi: 10.1001/jamainternmed.2020.3596
8. Takahashi T, Ellingson MK, Wong P, Israelow B, Lucas C, Klein J, et al. Sex Differences in Immune Responses That Underlie COVID-19 Disease Outcomes. Nature (2020) 588(7837):315–20. doi: 10.1038/s41586-020-2700-3
9. Janssen NAF, Grondman I, de Nooijer AH, Boahen CK, Koeken V, Matzaraki V, et al. Dysregulated Innate and Adaptive Immune Responses Discriminate Disease Severity in COVID-19. J Infect Dis (2021) 223(8):1322–33. doi: 10.1093/infdis/jiab065
10. Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, et al. Dysregulation of Immune Response in Patients With Coronavirus 2019 (COVID-19) in Wuhan, China. Clin Infect Dis (2020) 71(15):762–8. doi: 10.1093/cid/ciaa248
11. Wilk AJ, Rustagi A, Zhao NQ, Roque J, Martinez-Colon GJ, McKechnie JL, et al. A Single-Cell Atlas of the Peripheral Immune Response in Patients With Severe COVID-19. Nat Med (2020) 26(7):1070–6. doi: 10.1038/s41591-020-0944-y
12. Yao C, Bora SA, Parimon T, Zaman T, Friedman OA, Palatinus JA, et al. Cell Type-Specific Immune Dysregulation in Severely Ill COVID-19 Patients. Cell Rep (2020) 34(1):108590. doi: 10.1101/2020.07.23.20161182
13. Mann ER, Menon M, Knight SB, Konkel JE, Jagger C, Shaw TN, et al. Longitudinal Immune Profiling Reveals Key Myeloid Signatures Associated With COVID-19. Sci Immunol (2020) 5(51):eabd6197. doi: 10.1126/sciimmunol.abd6197
14. Bergamaschi L, Mescia F, Turner L, Hanson A, Kotagiri P, Dunmore BJ, et al. Longitudinal Analysis Reveals That Delayed Bystander CD8+ T Cell Activation and Early Immune Pathology Distinguish Severe COVID-19 From Mild Disease. Immunity (2021) 54(6):1257–75. doi: 10.1016/j.immuni.2021.05.010
15. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical Features of Patients Infected With 2019 Novel Coronavirus in Wuhan, China. Lancet (2020) 395(10223):497–506. doi: 10.1016/S0140-6736(20)30183-5

16. McClymont H, Hu W. Weather Variability and COVID-19 Transmission: A Review of Recent Research. Int J Environ Res Public Health (2021) 18(2):396. doi: 10.3390/ijerph18020396

17. Rayan RA. Seasonal Variation and COVID-19 Infection Pattern: A Gap From Evidence to Reality. Curr Opin Environ Sci Health (2021) 20:100238. doi: 10.1016/j.coesh.2021.100238

18. Aguirre-Gamboa R, Joosten I, Urbano PCM, van der Molen RG, van Rijssen E, van Cranenbroek B, et al. Differential Effects of Environmental and Genetic Factors on T and B Cell Immune Traits. Cell Rep (2016) 17(9):2474–87. doi: 10.1016/j.celrep.2016.10.053

19. 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. doi: 10.1172/JCI133935

20. Scully EP, Haverfield J, Ursin RL, Tannenbaum C, Klein SL. Considering How Biological Sex Impacts Immune Responses and COVID-19 Outcomes. Nat Rev Immunol (2020) 20(7):442–7. doi: 10.1038/s41577-020-0348-8

21. Chen Y, Klein SL, Garibaldi BT, Li H, Wu C, Osevala NM, et al. Aging in COVID-19: Vulnerability, Immunity and Intervention. Ageing Res Rev (2021) 65:101205. doi: 10.1016/j.arr.2020.101205

22. Huang W, Berube J, McNamara M, Saksena S, Hartman M, Arshad T, et al. Lymphocyte Subset Counts in COVID-19 Patients: A Meta-Analysis. Cytometry A (2020) 97(8):772–6. doi: 10.1002/cyto.a.24172

23. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, et al. Clinical and Immunological Features of Severe and Moderate Coronavirus Disease 2019. J Clin Invest (2020) 130(5):2620–9. doi: 10.1172/JCI137244

24. Akkaya M, Kwak K, Pierce SK. B Cell Memory: Building Two Walls of Protection Against Pathogens. Nat Rev Immunol (2020) 20(4):229–38. doi: 10.1038/s41577-019-0244-2

25. Bulut O, Kilic G, Dominguez-Andres J, Netea MG. Overcoming Immune Dysfunction in the Elderly: Trained Immunity as a Novel Approach. Int Immunol (2020) 32(12):741–53. doi: 10.1093/intimm/dxaa052

26. Gubbels Bupp MR, Potluri T, Fink AL, Klein SL. The Confluence of Sex Hormones and Aging on Immunity. Front Immunol (2018) 9:1269. doi: 10.3389/fimmu.2018.01269

27. Sette A, Crotty S. Adaptive Immunity to SARS-CoV-2 and COVID-19. Cell (2021) 184(4):861–80. doi: 10.1016/j.cell.2021.01.007

28. Del Valle DM, Kim-Schulze S, Huang HH, Beckmann ND, Nirenberg S, Wang B, et al. An Inflammatory Cytokine Signature Predicts COVID-19 Severity and Survival. Nat Med (2020) 26(10):1636–43. doi: 10.1038/s41591-020-1051-9

29. Komarowska I, Coe D, Wang G, Haas R, Mauro C, Kishore M, et al. Hepatocyte Growth Factor Receptor C-Met Instructs T Cell Cardiotropism and Promotes T Cell Migration to the Heart via Autocrine Chemokine Release. Immunity (2015) 42(6):1087–99. doi: 10.1016/j.immuni.2015.05.014

30. Yang Y, Shen C, Li J, Yuan J, Wei J, Huang F, et al. Plasma IP-10 and MCP-3 Levels Are Highly Associated With Disease Severity and Predict the Progression of COVID-19. J Allergy Clin Immunol (2020) 146(1):119–27 e4. doi: 10.1016/j.jaci.2020.04.027

31. Schulte-Schrepping J, Reusch N, Paclik D, Bassler K, Schlickeiser S, Zhang B, et al. Severe COVID-19 Is Marked by a Dysregulated Myeloid Cell Compartment. Cell (2020) 182(6):1419–40 e23. doi: 10.1016/j.cell.2020.08.001

32. Blackburn SD, Wherry EJ. IL-10, T Cell Exhaustion and Viral Persistence. Trends Microbiol (2007) 15(4):143–6. doi: 10.1016/j.tim.2007.02.006

33. Lu L, Zhang H, Dauphars DJ, He YW. A Potential Role of Interleukin 10 in COVID-19 Pathogenesis. Trends Immunol (2021) 42(1):3–5. doi: 10.1016/j.it.2020.10.012

34. Zhao Y, Qin L, Zhang P, Li K, Liang L, Sun J, et al. Longitudinal COVID-19 Profiling Associates IL-1RA and IL-10 With Disease Severity and RANTES With Mild Disease. JCI Insight (2020) 5(13):e139834. doi: 10.1172/jci.insight.139834

35. Giamarellos-Bourboulis EJ, Netea MG, Rovina N, Akinosoglou K, Antoniadou A, Antonakos N, et al. Complex Immune Dysregulation in COVID-19 Patients With Severe Respiratory Failure. Cell Host Microbe (2020) 27(6):992–1000 e3. doi: 10.1016/j.chom.2020.04.009

36. Bord S, Ireland DC, Beavan SR, Compston JE. The Effects of Estrogen on Osteoprotegerin, RANKL, and Estrogen Receptor Expression in Human Osteoblasts. Bone (2003) 32(2):136–41. doi: 10.1016/s8756-3282(02)00953-5

37. Ono T, Hayashi M, Sasaki F, Nakashima T. RANKL Biology: Bone Metabolism, the Immune System, and Beyond. Inflammation Regen (2020) 40:2. doi: 10.1186/s41232-019-0111-3

38. Pawelec G. Age and Immunity: What is "Immunosenescence"? Exp Gerontol (2018) 105:4–9. doi: 10.1016/j.exger.2017.10.024

39. Franceschi C, Garagnani P, Parini P, Giuliani C, Santoro A. Inflammaging: A New Immune-Metabolic Viewpoint for Age-Related Diseases. Nat Rev Endocrinol (2018) 14(10):576–90. doi: 10.1038/s41574-018-0059-4

40. Franceschi C, Salvioli S, Garagnani P, de Eguileor M, Monti D, Capri M. Immunobiography and the Heterogeneity of Immune Responses in the Elderly: A Focus on Inflammaging and Trained Immunity. Front Immunol (2017) 8:982. doi: 10.3389/fimmu.2017.00982

41. Marquez EJ, Chung CH, Marches R, Rossi RJ, Nehar-Belaid D, Eroglu A, et al. Sexual-Dimorphism in Human Immune System Aging. Nat Commun (2020) 11(1):751. doi: 10.1038/s41467-020-14396-9

42. Lofgren E, Fefferman NH, Naumov YN, Gorski J, Naumova EN. Influenza Seasonality: Underlying Causes and Modeling Theories. J Virol (2007) 81(11):5429–36. doi: 10.1128/JVI.01680-06

43. van der Made CI, Simons A, Schuurs-Hoeijmakers J, van den Heuvel G, Mantere T, Kersten S, et al. Presence of Genetic Variants Among Young Men With Severe COVID-19. JAMA (2020) 324(7):663–73. doi: 10.1001/jama.2020.13719

44. Tang Y, Liu J, Zhang D, Xu Z, Ji J, Wen C. Cytokine Storm in COVID-19: The Current Evidence and Treatment Strategies. Front Immunol (2020) 11:1708. doi: 10.3389/fimmu.2020.01708

45. Gram AM, Frenkel J, Ressing ME. Inflammasomes and Viruses: Cellular Defence Versus Viral Offence. J Gen Virol (2012) 93(Pt 10):2063–75. doi: 10.1099/vir.0.042978-0

46. Liu Y, Li S, Zhang G, Nie G, Meng Z, Mao D, et al. Genetic Variants in IL1A and IL1B Contribute to the Susceptibility to 2009 Pandemic H1N1 Influenza A Virus. BMC Immunol (2013) 14:37. doi: 10.1186/1471-2172-14-37

47. Dinarello CA. Overview of the IL-1 Family in Innate Inflammation and Acquired Immunity. Immunol Rev (2018) 281(1):8–27. doi: 10.1111/imr.12621

48. Flores-Gomez D, Bekkering S, Netea MG, Riksen NP. Trained Immunity in Atherosclerotic Cardiovascular Disease. Arterioscler Thromb Vasc Biol (2021) 41(1):62–9. doi: 10.1161/ATVBAHA.120.314216

49. Bekkering S, Arts RJW, Novakovic B, Kourtzelis I, van der Heijden C, Li Y, et al. Metabolic Induction of Trained Immunity Through the Mevalonate Pathway. Cell (2018) 172(1-2):135–46 e9. doi: 10.1016/j.cell.2017.11.025

50. You M, Chen L, Zhang D, Zhao P, Chen Z, Qin EQ, et al. Single-Cell Epigenomic Landscape of Peripheral Immune Cells Reveals Establishment of Trained Immunity in Individuals Convalescing From COVID-19. Nat Cell Biol (2021) 23(6):620–30. doi: 10.1038/s41556-021-00690-1

51. Shao Y, Saredy J, Xu K, Sun Y, Saaoud F, Drummer C, et al. Endothelial Immunity Trained by Coronavirus Infections, DAMP Stimulations and Regulated by Anti-Oxidant NRF2 May Contribute to Inflammations, Myelopoiesis, COVID-19 Cytokine Storms and Thromboembolism. Front Immunol (2021) 12:653110. doi: 10.3389/fimmu.2021.653110

Supplementary Information

Supplementary Tables

Supplementary Table 1. Plasma proteins and immune cell populations linked with severe COVID-19

Reference	Severe COVID-19 Immune Profile
Janssen et al. (2021) *J Infect Dis*	Higher CRP, D-dimer, TNFα, IL-6, IL-8, IL-18, HGF, CCL3, CCL19, CCL20, ENRAGE, MCP3, VEGFA, and CD40. Lower SCF, DNER, TRAIL, TRANCE, TNFB, VEGFD, and HLA-DR expression.
Qin et al. (2020) *Clin Infect Dis*	Higher TNFα, IL-6, IL-8, IL-10, CRP, ferritin, memory $CD4^+$ T cells, and neutrophils. Lower lymphocytes, T cells, $CD4^+$ T cells, and Tregs.
Huang et al. (2020) *Cytometry A*	Lower lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells, $CD56^+$ NK cells and B cells.
Yang et al. (2020) *J Allergy Clin Immunol*	Higher IP-10, MCP-3, HGF, MIP-1α, IL-1RA, and CSF1.
Bergamaschi et al. (2021) *MedRxiv*	Higher plasmablasts, classical monocytes, and neutrophils. Lower $CD4^+$ naïve, $CD4^+$ central memory, $CD4^+$ effector memory, Tregs, $CD4^+$ follicular helper, $CD8^+$ naïve, $CD8^+$ effector memory, B cells, transitional B cells, memory B cells, pDCs, and non-classical monocytes.
Chen et al. (2020) *BMJ*	Higher lactate dehydrogenase, D-dimers, CRP, IL-6, IL-10, TNFα, and neutrophils. Lower lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells, and naïve T regs.
Huang et al. (2020) *Lancet*	Higher IL-2, IL-7, IL-10, IP-10, MCP-1, MIP1α, TNFα, and G-CSF.

Supplementary Table 2. List of immune cell types and circulating proteins investigated in the study. These have been previously linked to COVID-19 severity

Immune cell types	Circulating proteins
Leukocytes	Interferon gamma (IFNγ)
Lymphocytes	Interleukin 6 (IL-6)
Neutrophils	Interleukin 7 (IL-7)
Monocytes	Interleukin 8 (IL-8)
Classical Monocytes	Interleukin 10 (IL-10)
Intermediate Monocytes	Interleukin 18 (IL-18)
Non-classical Monocytes	Monocyte chemoattractant protein 1 (MCP-1)
Natural Killer (NK) Cells	Monocyte chemoattractant protein 2 (MCP-2)
$CD56^{dim}$ NK Cells	Chemokine (C-C motif) ligand 3 (CCL3)
$CD56^{bright}$ NK Cells	Chemokine (C-C motif) ligand 4 (CCL4)
Natural Killer T Cells	Chemokine (C-C motif) ligand 19 (CCL19)
T Cells	Chemokine (C-C motif) ligand 20 (CCL20)
Naïve T Cells	Chemokine (C-X-C motif) ligand 9 (CXCL9)
Memory T Cells	Chemokine (C-X-C motif) ligand 10 (CXCL10)
$CD4^+$ T Cells	Tumor necrosis factor (TNF)
Naïve $CD4^+$ T Cells	Tumor necrosis factor beta (TNFB)
Effector $CD4^+$ T Cells	TNF-related activation-induced cytokine (TRANCE)
Effector Memory $CD4^+$ T Cells	TNF-related apoptosis-inducing ligand (TRAIL)
Central Memory $CD4^+$ T Cells	TNF-related weak inducer of apoptosis (TWEAK)
$CD8^+$ T Cells	Latency associated peptide - transforming growth factor beta (LAP-TGFB1)
Naïve $CD8^+$ T Cells	Osteoprotegerin (OPG)
Effector $CD8^+$ T Cells	Colony stimulating factor 1 (CSF1)
Effector Memory $CD8^+$ T Cells	Stem cell factor (SCF)
Central Memory $CD8^+$ T Cells	Hepatocyte growth factor (HGF)
Regulatory T cells (Tregs)	Vascular endothelial growth factor alpha (VEGFA)
Naïve Tregs	Extracellular newly identified RAGE-binding protein (EN-RAGE)
Memory Tregs	Delta and Notch-like epidermal growth factor-related receptor (DNER)
B Cells	Programmed death-ligand 1 (PD-L1)
Plasmablasts	
Naïve B Cells	

Supplementary Table 2. Continued

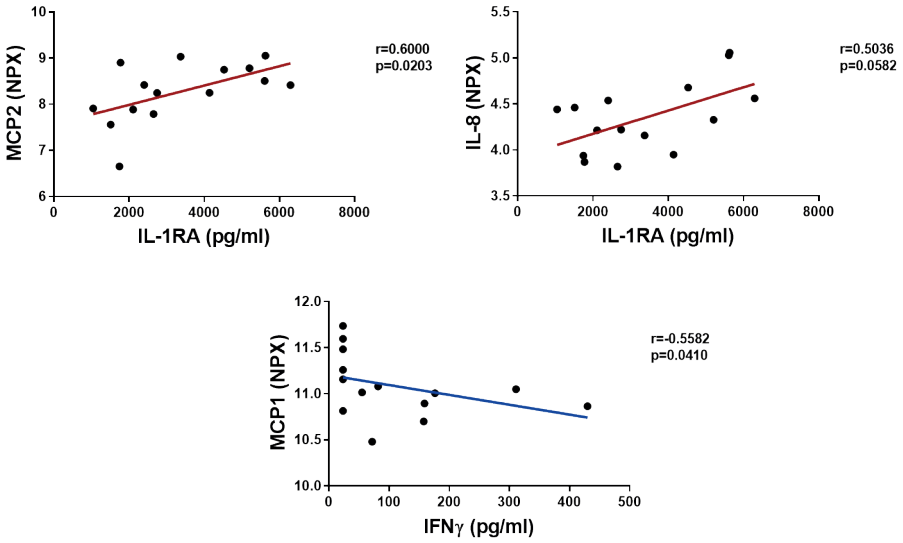
Immune cell types	Circulating proteins
Mature Naïve B Cells	
Transitional B Cells	
Natural Effector B Cells	
Memory B Cells	
Non-class-switched Memory B Cells	
Class-switched Memory B Cells	

Supplementary Table 3. Demographics of the 20 individuals selected from Cohort 1 for seasonality analysis

Group (n=5)	Age	Body mass index (BMI)
Young male	23.2 ± 3.11	24.46 ± 1.47
Old male	64.6 ± 3.65	24.39 ± 2.46
Young female	23.8 ± 3.11	22.40 ± 1.63
Old female	60.8 ± 6.22	24.07 ± 3.70

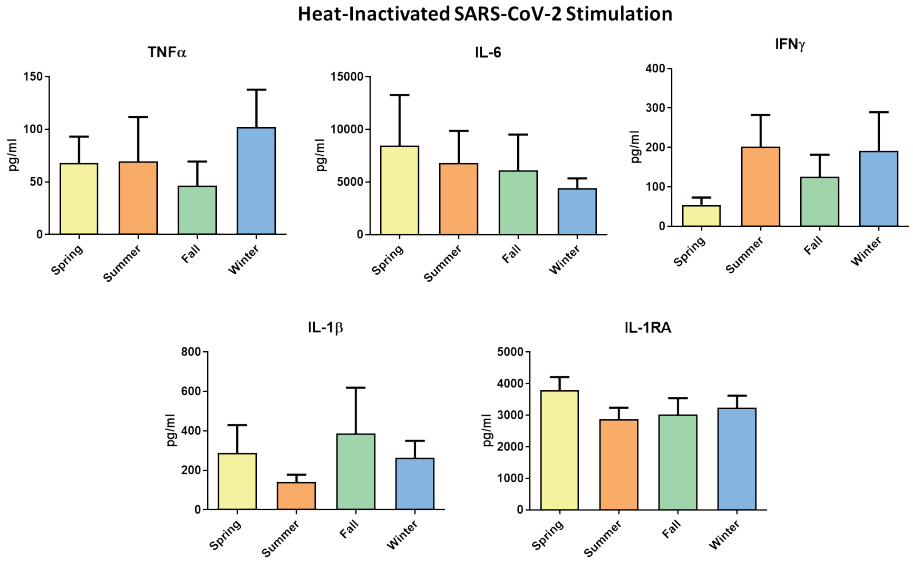
Age and BMI are depicted as mean ± standard deviation.

Supplementary Figures



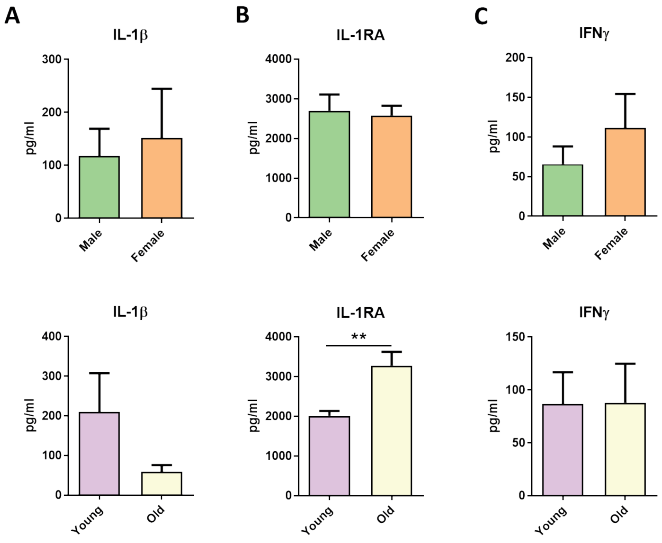
Supplementary Figure 1. Correlation between baseline plasma protein levels and cytokine productions against in vitro SARS-CoV-2 stimulation in healthy individuals

The x-axis shows the cytokine productions of PBMCs after stimulation with heat-inactivated SARS-CoV-2 while the y-axis demonstrates the baseline plasma protein levels of healthy individuals. Red indicates a positive correlation whereas blue indicates a negative correlation. NPX: normalized protein expression, r = Spearman correlation coefficient, n=14-15.



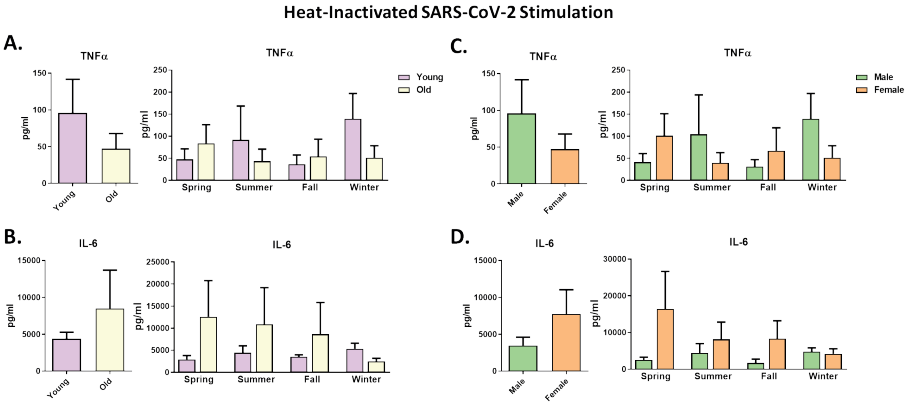
Supplementary Figure 2. The cytokine productions of PBMCs that were stimulated with heat-inactivated SARS-CoV-2

The PBMCs from healthy individuals were collected and frozen at different times of the year. n=7-20. Error bars depict the standard error of the mean (SEM).



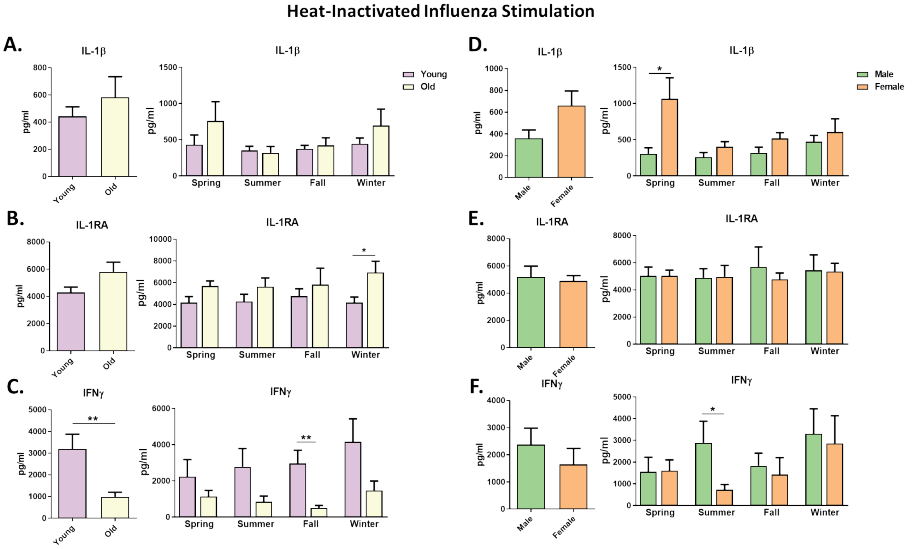
Supplementary Figure 3. Basal cytokine responses of healthy individuals in the absence of any stimulus

Responses were compared between males and females (upper row) and between young and old individuals (lower row). The graphs show the yearly average production. **(A)** IL-1β and **(B)** IL-1RA cytokine levels were measured after 24 hours, while **(C)** IFNγ was measured after 5 days. **$p \leq 0.01$. n=8-10. Error bars depict the SEM.



Supplementary Figure 4. Cytokine responses against heat-inactivated SARS-CoV-2 in healthy individuals

Each panel's left graphs show the yearly average production, while the right graphs demonstrate cytokine production in every season. Responses were compared between young and old individuals (**A-B**) and between males and females (**C-D**). Cytokine levels were measured after 24 hours. n=3-8. Error bars depict the SEM.



Supplementary Figure 5. Immune responses against heat-inactivated influenza A (H1N1) in healthy individuals

Each panel's left graphs show the yearly average production, while the right graphs demonstrate cytokine production in every season. Responses were compared between young and old individuals (**A-C**) and between males and females (**D-F**). IL-1β and IL-1RA cytokine levels were measured after 24 hours, while IFNγ was measured after 5 days. *p ≤ 0.05, **p ≤ 0.01. n=5-10. Error bars depict the SEM.

Atlantic puffin
Bayağı deniz papağanı
Papegaaiduiker

CHAPTER 4

RORα negatively regulates BCG-induced trained immunity

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Submitted

Abstract

Trained immunity is a long-lasting change in the responsiveness of innate immune cells, leading to a stronger response upon an unrelated secondary challenge. Epigenetic, transcriptional, and metabolic reprogramming contribute to the development of trained immunity. By investigating the impact of gene variants on trained immunity responses after Bacillus Calmette–Guérin (BCG) vaccination, we identified a strong association between polymorphisms in the *RORA* gene and BCG-induced trained immunity. RORα, encoded by the *RORA* gene in humans, is a nuclear receptor and a transcription factor, regulating genes involved in circadian rhythm, inflammation, cholesterol, and lipid metabolism. We found that natural RORα agonists in the circulation negatively correlate with the strength of trained immunity responses after BCG vaccination. Moreover, pharmacological inhibition of RORα in human PBMCs led to higher cytokine production capacity and boosted trained immunity induction by BCG. Blocking RORα activity also resulted in morphological changes and increased ROS and lactate production of BCG-trained cells. Blocking lactate dehydrogenase A (LDHA) and glycolysis with sodium oxamate reduced the cytokine production capacity of cells trained with a combination of BCG and the RORα agonist. In conclusion, this study highlights the potential role of RORα in trained immunity, and its impact on human vaccination and diseases should be further investigated.

Introduction

The innate immune cells have the capacity to identify pathogenic structures, initiating a broad initial defense by generating effector molecules. Furthermore, multiple studies over the past few years have consistently revealed the innate immune system's capability to retain an epigenetic memory after infections and vaccinations. This enables the innate immune system to mount a more robust and effective response upon encountering unrelated pathogens. The memory-like characteristics of the innate immune system, termed trained immunity, were primarily demonstrated by increased cytokine production, epigenetic and metabolic programming, and transcriptional changes [1]. Microbial stimuli, including live attenuated vaccines, e.g., Bacille Calmette-Guerin (BCG) and the oral polio vaccine (OPV), as well as non-microbial stimuli, such as oxidized low-density lipoprotein (oxLDL) and uric acid, are among the known inducers of trained immunity [2]. BCG was shown to induce pro-inflammatory cytokines, e.g., TNFα, IL-6 and IL-1β from monocyte-derived macrophages upon a secondary stimulation in both *in vivo* and *in vitro* settings. This increased responsiveness is accompanied by epigenetic rewiring at the chromatin level and increased glycolysis and oxidative phosphorylation, allowing cells to respond strongly [3].

Many factors can shape immune responses, e.g., age, sex, genetics, and seasons. The same elements were recently demonstrated to influence and predict trained immunity responses [4]. Regarding genetic factors, specific variations known as single nucleotide polymorphisms (SNPs) have been shown to regulate trained immunity response. For instance, genetic polymorphisms in the genes of the IL-1β pathway, which plays a crucial role in the generation of trained immunity response, affect the response induced by BCG vaccination [5]. Several SNPs linked to glutathione metabolism have been associated with increased cytokine production after training with BCG [6]. Additionally, SNPs in the autophagy genes *ATG2B* and *ATG5* impacted both the *in vitro* and *in vivo* training effect of BCG [7].

Retinoid-related orphan receptor α (RORα or NR1F1), encoded by the *RORA* gene in humans, belongs to the nuclear hormone receptor superfamily. It regulates gene transcription by recognizing and attaching to particular sequences of DNA called ROR response elements (ROREs) [8]. The binding of RORα within the promoter region of a target gene leads to the recruitment of cofactors and altered gene transcription. For example, RORα controls the expression of two circadian rhythm genes, *BMAL1* and *CLOCK*, and genes such as *SHH*, *SLC1A6* and *ITPR1* regulating Purkinje cell maturation by binding to their ROREs [9]. Although RORα is still named as an orphan receptor with no precisely identified ligand, cholesterol and its derivatives were suggested to bind RORα, regulating the protein's activity and transcription [10].

RORA is expressed in a wide range of cell types in humans, from glial cells to epithelial cells to immune cells [11]. With its broad expression, RORα has multiple functions, such as regulating the circadian rhythm, cholesterol metabolism, and immune system [8]. Many studies have shown that RORα controls inflammation in different tissues. Multiple layers of evidence support this finding both *in vitro* and *in vivo*: mice with a spontaneous mutation on *Rora* ($Rora^{sg/sg}$) showed increased airway inflammation after LPS challenge, evident from higher neutrophil recruitment and cytokine production [12], whereas overexpression of *Rora* protected mice from LPS-mediated organ injury by lowering inflammatory cell recruitment and increasing survival [13]. Similarly, TNFα-induced cytokine release was reduced in *RORA* overexpressing human aortic primary smooth muscle cells [14]. On the other hand, the deletion of RORα led to a dramatic increase in the production of pro-inflammatory cytokines in THP-1 monocytes [15].

Although the evidence regarding the immunomodulatory effects of RORα is growing, whether and how it regulates trained immunity is unknown. In this study, we first investigated whether SNPs around the *RORA* gene modulate trained immunity induced by BCG vaccination in a healthy cohort (300BCG). Subsequently, we confirmed the regulatory role of RORα in an *in vitro* trained immunity model: inhibition of RORα with an inverse agonist, SR3335, increased cytokine production upon heterologous stimulation with LPS, promoted lactate release and ROS production after BCG training.

Materials and methods

Cohort Information

To study the genetic factors modulating the BCG-induced trained immunity response, the SNPs data, cytokine measurements, and baseline metabolite concentrations from the 300BCG cohort were used. 325 individuals were included in the cohort, of which 44% are males and 56% are females (age range 18-71 years) from the Netherlands with Western European genetic background. Exclusion criteria were previous BCG vaccination, history of tuberculosis, illness within 4 weeks before participation to the study, administration of any vaccine within 3 months before the study, immunodeficiency, use of antibiotics and use of a systemic medication except for oral contraceptives and acetaminophen. After receiving the written informed consent, the blood was drawn and the individuals were vaccinated with a standard dose of 0.1 mL of BCG (BCG vaccine strain Bulgaria; Intervax, Canada) intradermally in the left upper arm. The study participants were invited for another blood collection 3 months after BCG vaccination. The study was approved by the METC Oost-Nederland (NL58553.091.16).

Ex vivo stimulation of the 300BCG cohort

As reported in [16], PBMC isolation was performed before and 3 months after BCG vaccination from EDTA whole blood using Ficoll-Paque (GE Healthcare) density gradient separation. After washing the cells twice with cold PBS, PBMCs were counted with a hematology analyzer (Sysmex, Japan). $5x10^5$/well PBMCs were cultured in a final 200 µl/well volume in round-bottom 96-well plates (Greiner, Austria) and stimulated with heat-killed *S. aureus* ($1×10^6$ CFU/ml). Cytokine production (IL-1β, IL-6, and TNFα) was measured after 24-hour stimulation from cell-free supernatant. The fold change in cytokine production (after vaccination compared to baseline) was used as a measurement of the magnitude of the trained immunity response.

Genotyping of the 300BCG cohort

As described in [17], the DNA of 325 individuals was isolated from whole blood and genotyped using the commercially available SNP chip, Infinium Global Screening Array MD v1.0 from Illumina (CA, USA). After standard quality control per SNP and sample as described previously [17], approximately 4 million SNPs with minor allele frequency >5% were tested for association with trained immunity.

Quantitative Trait Loci (QTL) Mapping

Both genotype and cytokine data on trained immunity responses were available for a total of 296 individuals. Genetic outliers and outliers due to medication use and the onset of type 1 diabetes during the study were excluded as previously described [17]. In addition, to exclude the possible influence of the circadian rhythm, 18 evening-vaccinated individuals were excluded, resulting in 278 samples before QTL mapping. The fold change of cytokine production was then log-transformed and mapped to genotype data using a linear regression model with age and sex as covariates. R-package Matrix-eQTL was used for cytokine QTL mapping [18].

Pathway enrichment analysis

To investigate whether the QTLs associated with trained immunity ($p<0.01$) were enriched for specific pathways, we performed pathway enrichment analysis using Pascal (Pathway scoring algorithm). This tool allows gene and pathway-level analysis of GWAS association results by accounting for genes in linkage disequilibrium (LD) as single entities, so it does not "over-count" gene association signals due to the presence of genes in LD [19]. LD information from 1000 Genomes was employed by PASCAL in order to consider the linkage between SNP markers for each gene. Results were reported using REACTOME as a database. The Bonferroni correction sets the significance cut-off at $1.37 x10^{-5}$ (0.05 / 3,651 genes). This number of genes includes all genes from QTL loci at $p<0.01$.

Metabolomics measurements from the 300BCG cohort

Untargeted metabolomics was performed from plasma by the General Metabolics (Switzerland) using flow injection time-of-flight mass spectrometry, as described in [20]. Annotation was done using the Human Metabolome Database.

In vitro experiments

Reagents

For the *in vitro* trained immunity experiments, BCG-Denmark (5 μg/ml, AJ Vaccines, Denmark), SR3335 (0.5 or 5 μM, MedChemExpress, NJ, USA), and E. coli-derived LPS (10 ng/ml, Sigma-Aldrich, MI, USA) were used. Glycolysis inhibition experiments were performed with sodium oxamate (5 mM, Sigma-Aldrich) and 2-DG (1 mM, Sigma-Aldrich).

PBMC isolation

PBMC isolation was performed from the buffy coats of healthy donors after written informed consent (Sanquin Blood Bank, the Netherlands). Blood was diluted with PBS, and density-gradient centrifugation was performed using Ficoll-Paque (Cytiva, MA, USA). After centrifugation, the middle layer containing PBMCs was collected, and the cells were washed 3 times with cold PBS. PBMCs were resuspended in RPMI 1640 Dutch modified (Invitrogen, MA, USA) supplemented with 2 mM Glutamax (Gibco, MA, USA), 1 mM sodium pyruvate (Gibco, MA, USA) and 5 μg/ml gentamicin (Centrafarm, the Netherlands).

In vitro training of adherent PBMCs

In vitro training was performed as described previously [21] with minor modifications. Briefly, PBMCs were seeded as $5x10^5$/well on 96-well flat bottom plates (Sarstedt, Germany). After incubation for at least 1 hour at 37°C in 5% CO2, non-adherent cells were removed by washing with warm PBS, and then monocyte-enriched adherent cells were incubated with DMSO, SR3335 and/or BCG for 24 hours in the presence of 10% human pooled serum. Following the 5-day resting period, cells were restimulated with 10 ng/ml LPS for 24 hours, and the cell-free supernatants were collected.

Cytotoxicity and viability assays

The amount of LDH released to the cell supernatant after 24h incubation with stimuli, as a measure of cytotoxicity, was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, WI, USA), following the manufacturer's protocol.

Cell viability was assessed on day 6 of the *in vitro* trained immunity experiment, before LPS restimulation, using the CellTiter-Glo One Solution Assay Kit (Promega), per the manufacturer's instructions.

Cytokine and lactate measurements

TNFα and IL-6 concentrations in the cell-free supernatants were measured using the TNFα and IL-6 DuoSet ELISA kits (R&D Systems, MN, USA), according to the manufacturer's instructions.

Lactate production was measured from the cell-free supernatants on day 6 of the training experiment before LPS restimulation. First, perchloric acid precipitation was performed to minimize the interference of serum in the cell culture media to the lactate concentrations. Then, the supernatants were neutralized with NaOH and incubated with lactate oxidase (Sigma-Aldrich), horseradish peroxidase (HRP, Sigma-Aldrich) and Amplex Red (Thermo Fisher Scientific, MA, USA) for 20 minutes. The lactate levels were determined by measuring the fluorescence with filters 530/±25 and 590/±35.

ROS measurements

PBMCs were added in a white 96-well assay plate (Corning, NY, USA) ($5x10^5$/well) for the assay. ROS measurements were performed on day 1 (24 hours after DMSO, SR3335 and/or BCG stimulation) and day 6 (after a 5-day resting period, before LPS restimulation). Serum opsonized-zymosan (from *S. cerevisiae*, Sigma-Aldrich, 30 μg/ml) and luminol solution (Sigma-Aldrich, 145 μg/ml) in Hank's Balanced Salt Solution (HBSS) and 0.5% Bovine Serum Albumin (BSA) were added to the cells, and chemiluminescence was measured at 37 °C every 142 seconds for 1 hour. The assay was performed in quadruplicates.

Metabolic analyses

On day 6 of the training protocol, adherent cells were scraped with cold PBS, counted, and seeded 10^5/well in quintuplicates to overnight-calibrated cartridges, which were incubated in a non-CO2 incubator at 37°C. The assay medium is DMEM supplemented with 2 mM L-glutamine, 11 mM D-glucose, and 1 mM pyruvate, with pH adjusted to 7.4 for the oxygen consumption rate (OCR) measurements, and DMEM supplemented with 1 mM L-glutamine for the extracellular acidification rate (ECAR) measurements.

ECAR and OCR were measured using the Glycostress and Cell Mito Stress Kit, respectively, in an XFe96 Extracellular Flux Analyzer (Seahorse, Agilent Technologies, CA, USA). The manufacturer's protocol was followed to perform the assays.

Quantitative RT-PCR

On day 1 and day 6 of the *in vitro* training protocol, adherent PBMCs trained with BCG (5 μg/ml) were collected and lysed using the lysis buffer LBP (Macherey-Nagel, Germany). RNA was isolated with the NucleoSpin RNA kit per the manufacturer's protocol. Then, cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad, CA, USA). RT-qPCR was performed with the SYBR Green method (Applied Biosystems, MA, USA) on an Applied Biosystems StepOne qPCR machine. Relative expressions, calculated by the Livak-Schmittgen equation [22] $r=2^{-\Delta\Delta Cq}$, were determined over a housekeeping gene. *HPRT* was selected as the housekeeping gene, as it was shown to be stable in monocytes and PBMCs [23, 24]. The primer sequences used in the study are listed in **Supplementary Table 1**.

Microscopy

Cell morphology during *in vitro* training was observed using light microscopy at 20X magnification (Leica, Germany), and pictures were taken using Leica LAS EZ software (Leica).

Statistical analyses

Data analyses were performed with GraphPad Prism 8 (MA, USA) and R Studio version 4.2.3 (MA, USA). Baseline metabolites and fold change of cytokines were correlated using Spearman correlation. Statistical comparisons were performed using a Wilcoxon signed-rank test for paired samples and a Kruskal Wallis test for unpaired samples. Data are represented as mean ± SEM, unless otherwise specified. P values lower than 0.05 were considered statistically significant.

Results

Polymorphisms in the *RORA* gene modulate the trained immunity response induced by BCG

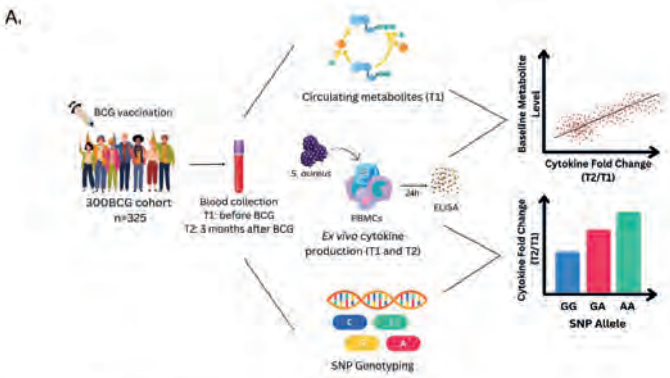
To investigate the genetic factors influencing trained immunity responses in humans, we used the genetic, cytokine, and metabolomics data from 325 healthy adults vaccinated with BCG (**Figure 1A**). In this study, blood was collected from these volunteers before BCG and 3 months after BCG vaccination. PBMCs isolated from the blood before and after BCG vaccination were incubated with heat-killed *S. aureus* to determine the trained immunity response, which was assessed by the increased cytokine production capacity (the ratio between cytokine production after vs. before BCG administration). Genotype information was obtained on approximately

4 million SNPs. Furthermore, metabolite levels were measured at baseline (before BCG vaccination) from participants' plasma.

First, we mapped QTLs using the genetic and *ex vivo* cytokine data from the 300BCG cohort (n=278) and performed a pathway enrichment analysis with genes from QTL loci that showed an association with trained immunity response after BCG vaccination ($p<0.01$, **Figure 1B**). As a result, we identified several pathways that are substantially enriched as being associated with BCG-induced trained immunity, including homeostasis ($p=1.95x10^{-7}$), metabolism of lipids and lipoproteins ($p=1.95x10^{-7}$), and adaptive immune system ($p=2.91x10^{-7}$). As certain lipids and lipid metabolism are already known to play a crucial role in immune responses and trained immunity induction [25-27], we decided to focus on the top gene of these pathways, *RORA*.

We then investigated whether polymorphisms around the *RORA* gene affect the trained immunity responses. As shown in **Supplementary Figure 1**, we identified several SNPs in a window of 250 kb around *RORA* that associate with BCG-mediated trained immunity responses ($p<0.05$). Two of these SNPs, rs17204973 and rs4774392 were significantly associated with cytokine production capacity to heterologous stimulation after BCG vaccination (**Figure 1C**). These results suggest that the *RORA* gene could modulate trained immunity induced by BCG vaccination.

A.



B.

Pathways	Chromosome	Gene ID	Gene Symbol	#SNPs	p-value
REACTOME_SIGNALLING_BY_NGF	chr9	5125	*PCSK5*	45	2.35E-08
REACTOME_HEMOSTASIS	chr6	2162	*F13A1*	35	1.95E-07
REACTOME_METABOLISM_OF_LIPIDS_AND_LIPOPROTEINS	**chr15**	**6095**	***RORA***	**73**	**1.95E-07**
REACTOME_FATTY_ACID_TRIACYLGLYCEROL_AND_KETONE_BODY_METABOLISM	**chr15**	**6095**	***RORA***	**73**	**1.95E-07**
REACTOME_PPARA_ACTIVATES_GENE_EXPRESSION	**chr15**	**6095**	***RORA***	**73**	**1.95E-07**
REACTOME_PLATELET_ACTIVATION_SIGNALING_AND_AGGREGATION	chr6	2162	*F13A1*	35	1.95E-07
REACTOME_HEMOSTASIS	chr10	5592	*PRKG1*	26	2.91E-07
REACTOME_ADAPTIVE_IMMUNE_SYSTEM	chr10	5592	*PRKG1*	26	2.91E-07
REACTOME_RAP1_SIGNALLING	chr10	5592	*PRKG1*	26	2.91E-07
REACTOME_IMMUNE_SYSTEM	chr10	5592	*PRKG1*	26	2.91E-07

C.

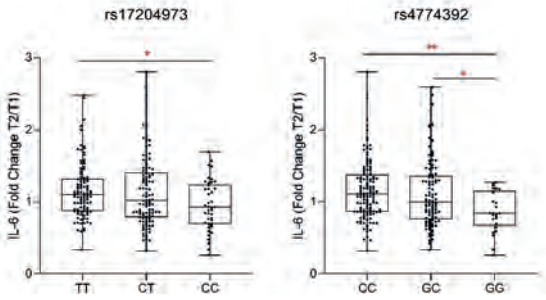


Figure 1. Summary of the experiments using the 300BCG cohort and identification of the *RORA* gene as a modulator of BCG-induced trained immunity

A) 325 healthy individuals were vaccinated with BCG, and blood was collected before and 3 months after vaccination. Metabolite levels in the plasma were measured from baseline samples, and DNA was isolated from blood to perform SNP genotyping. Furthermore, Isolated PBMCs at baseline and 3 months post-BCG were stimulated with heat-killed *S. aureus* (10^6 CFU/ml), and cytokine production was measured. Cytokine fold changes (T2/T1) were correlated with baseline metabolites and analyzed based on SNPs of interest. **B)** Pathway analysis of genes from QTL loci associated with trained immunity responses ($p<0.01$). The top genes from the pathways and the number of SNPs associated with that gene were indicated in the table. **C)** Two examples of SNPs associated with *RORA* and their allele-dependent BCG-induced trained immunity response, labeled in the regional association plot in Supplementary Figure 1. Comparisons were made using the Kruskal-Wallis test. *$p<0.05$, **$p<0.01$.

RORα ligands cholesterol and cholesterol sulfate in the circulation are negatively correlated with trained immunity response

Cholesterol and cholesterol sulfate are natural RORα ligands and agonists, increasing the protein's activity [10]. Therefore, we determined whether the abundance of cholesterol and cholesterol sulfate in the circulation is correlated with the increase of cytokine production capacity induced by BCG vaccination in humans.

We observed a weak but significant negative association between baseline cholesterol concentrations in plasma with fold change in the capacity of TNFα and IL-1β production (**Figure 2**). Similarly, cholesterol sulfate was negatively correlated with trained immunity responses. After analyzing males and females separately, we found that the association between baseline cholesterol levels and BCG-induced trained immunity was stronger and significant only in males (IL-1β and IL-6, **Supplementary Figure 2**). These results suggest that baseline levels of the natural RORα ligands present in the circulation might modulate the strength of the trained immunity response achieved by BCG vaccination, particularly in males.

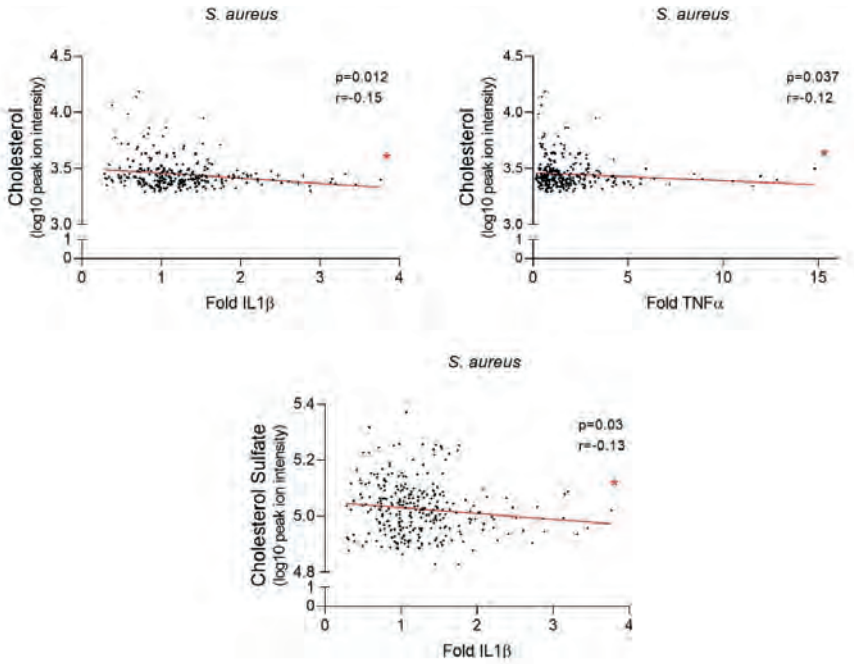


Figure 2. Correlation of BCG-mediated trained immunity response with baseline plasma cholesterol and cholesterol sulfate levels

Spearman correlation of baseline cholesterol and cholesterol sulfate levels and PBMCs fold cytokine responses after 24-hour incubation with heat-killed *S. aureus* (10^6 CFU/ml) (T2/T1). *p<0.05, **p<0.01. r: Spearman's correlation coefficient.

RORα inhibition induces trained immunity and promotes trained immunity by BCG ex vivo

Next, we sought to determine the role of RORα in an *in vitro* model of trained immunity [21]. First, we incubated adherent PBMCs with BCG for 24 hours. Then, we washed the cells and let them rest in a fresh cell culture medium for 5 days. We collected the cells after 24 hours and 6 days and analyzed the *RORA* expression. The *RORA* expression was similar between RPMI and BCG on day 1; however, on day 6, *RORA* expression was significantly lower in the BCG group compared to the RPMI group (**Figure 3A**).

Subsequently, we performed the *in vitro* trained immunity protocol to determine the effect of a RORα inhibitor SR3335 on trained immunity induced by BCG challenge. The experimental protocol and time point of each assay are summarized in **Figure 3B**.

We checked whether acute inhibition of RORα with a RORα inverse agonist, SR3335, leads to pro-inflammatory cytokine production in adherent PBMCs with or without BCG. Incubation of PBMCs with SR3335 for 24 hours did not stimulate TNFα and IL-6 production (**Figure 3C**). Furthermore, SR3335 did not influence the acute induction of cytokine production mediated by BCG. Of note, incubation with SR3335 was not toxic to PBMCs after 24 hours (**Supplementary Figure 3**).

Interestingly, exposure of immune cells to SR3335 alone increased their cytokine production capacity upon heterologous stimulation by LPS (**Figure 3D**). RORα inhibition by SR3335 also enhanced BCG-induced trained immunity, evident by the higher fold change over the control group (vehicle) in TNFα and IL-6. 0.5 μM of the inhibitor alone led to a greater fold change in TNFα and IL-6 release; however, only 5 μM SR3335 concentration promoted the training effect of BCG. Therefore, we used 5 μM SR3335 in the follow-up experiments.

Trained monocytes change their morphology and increase in size [28]. We observed the morphology of the cells on day 1 (after the first stimulation), day 6 (at the end of the resting period), and day 7 (24h after LPS restimulation) using light microscopy. On day 1, the cell morphology of different groups appeared similar (**Supplementary Figure 4**). In contrast, on day 6, cells in the SR3335, BCG+vehicle, and BCG+SR3335 groups had a bigger size than those in the vehicle group. This change in morphology has been previously associated with a trained immunity phenotype and is in accordance with the increase in cytokine production capacity induced by these stimulations observed in **Figure 3D**.

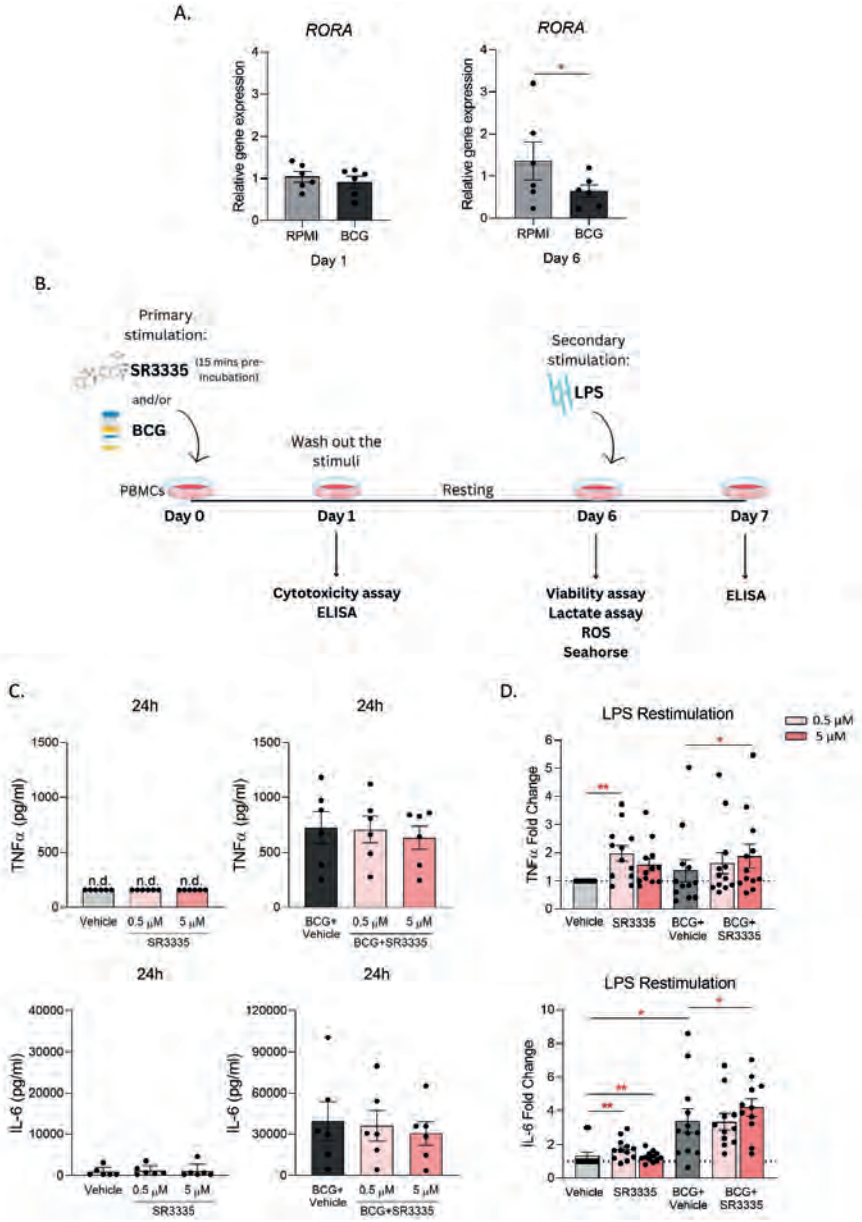


Figure 3. *In vitro* induction of trained immunity using BCG and/or the RORα inhibitor SR3335

A) Expression of *RORA* in adherent PBMCs after 24-hour or 6-day incubation with BCG (5 μg/ml) relative to the housekeeping gene *HPRT*. **B)** Summary of the *in vitro* trained immunity experiment and assays performed at different time points. **C)** TNFα and IL-6 production of adherent PBMCs following 24-hour stimulation with BCG (5 μg/ml) and/or vehicle/SR3335 (0.5 μM and 5 μM). **D)** Fold changes of TNFα and IL-6 production over the vehicle of adherent PBMCs following 24-hour stimulation with LPS (10 ng/ml) on day 6 of training with BCG (5 μg/ml) and/or SR3335 (5 μM). The Wilcoxon matched-pairs signed rank test was used to compare the experimental groups. *p<0.05, **p<0.01. Vehicle: DMSO

SR3335 leads to functional changes in BCG-trained immune cells

Subsequently, we investigated whether trained immunity triggered by RORα inhibition and BCG leads to alterations in reactive oxygen species (ROS) and lactate production, as well as shifts in the rate of glycolysis and oxidative phosphorylation. SR3335, in combination with BCG, significantly increased the production of ROS on day 1 and day 6, compared to BCG alone **(Figure 4A**). On the other hand, SR3335 alone did not impact ROS production. On day 6, SR3335 resulted in significantly higher lactate production by immune cells, both in the absence and presence of BCG (**Figure 4B**).

We also performed a Seahorse assay to measure the glycolysis and oxidative phosphorylation (OXPHOS) in SR3335- and BCG-trained cells. Surprisingly, the level of glycolysis was similar in the cells stimulated with SR3335 compared to the control group despite the higher lactate release on day 6 (**Figure 5A, 4B**). Similarly, there was no considerable difference in OXPHOS levels upon RORα inhibition. Of note, BCG training led to more glycolysis and OXPHOS, confirming the earlier reports [29], while SR3335 failed to boost BCG-induced glycolysis and oxidative phosphorylation. RORα inhibition did not considerably affect the basal or maximal glycolysis, the glycolytic and non-glycolytic capacity of cells (**Supplementary Figure 5A**). On the other hand, inhibition of RORα alone significantly reduced the spare respiratory capacity of the cells (**Supplementary Figure 5B**).

To rule out the possibility that the observed increase in lactate and ROS production on day 6 and cytokine production on day 7 are simply due to higher cell numbers in the SR3335 groups, we performed an ATP-based luminescent viability assay on day 6 of the trained immunity protocol. Neither BCG nor SR3335 improved or declined cell survival compared to the vehicle group (**Supplementary Figure 6**). Therefore, the functional differences in SR3335-trained cells could not stem from different cell numbers.

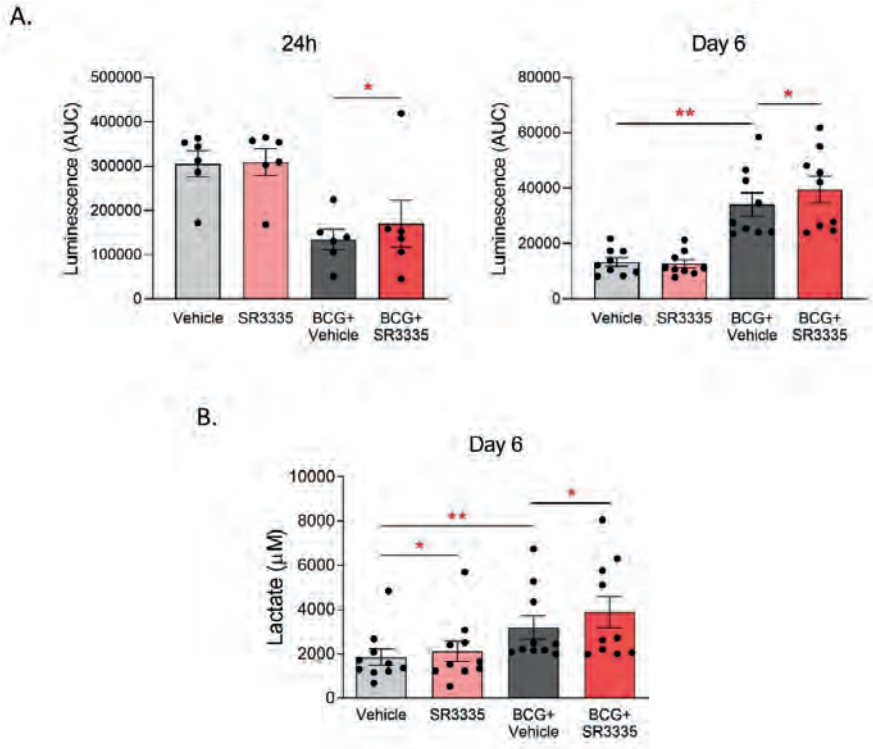


Figure 4. ROS and lactate production of adherent PBMCs upon training with BCG and/or SR3335

A) Adherent PBMCs were trained with BCG (5 µg/ml) in the presence or absence of SR3335 (5 µM)/vehicle. On day 1 and day 6, luminescence was measured for 1 hour. Each dot shows the average of quadruplicates. **B)** Lactate production was measured on day 6 of training with BCG (5 µg/ml) and/or SR335 (5 µM)/vehicle. The Wilcoxon matched-pairs signed rank test was used to compare the experimental groups. *p<0.05, **p<0.01. Vehicle: DMSO

Trained immunity induced by BCG+SR3335 is partially dependent on lactate dehydrogenase A activity

As the increase in lactate secretion suggested an impact on RORα modulation of trained immunity, we inhibited the glycolytic pathways in adherent PBMCs using two pharmacological inhibitors: hexokinase inhibition by 2-DG and lactate dehydrogenase A (LDHA) inhibition by sodium oxamate during the first 6 days of the *in vitro* trained immunity protocol with BCG and/or SR3335 (**Figure 5B**).

Inhibition of hexokinase activity resulted in higher cytokine production in the vehicle and SR3335 groups (**Figure 5C**). On the other hand, the presence of BCG together with SR3335 or vehicle prevented an increase in cytokine production when 2-DG is present, suggesting that BCG training is influenced by the first step of glycolysis [29].

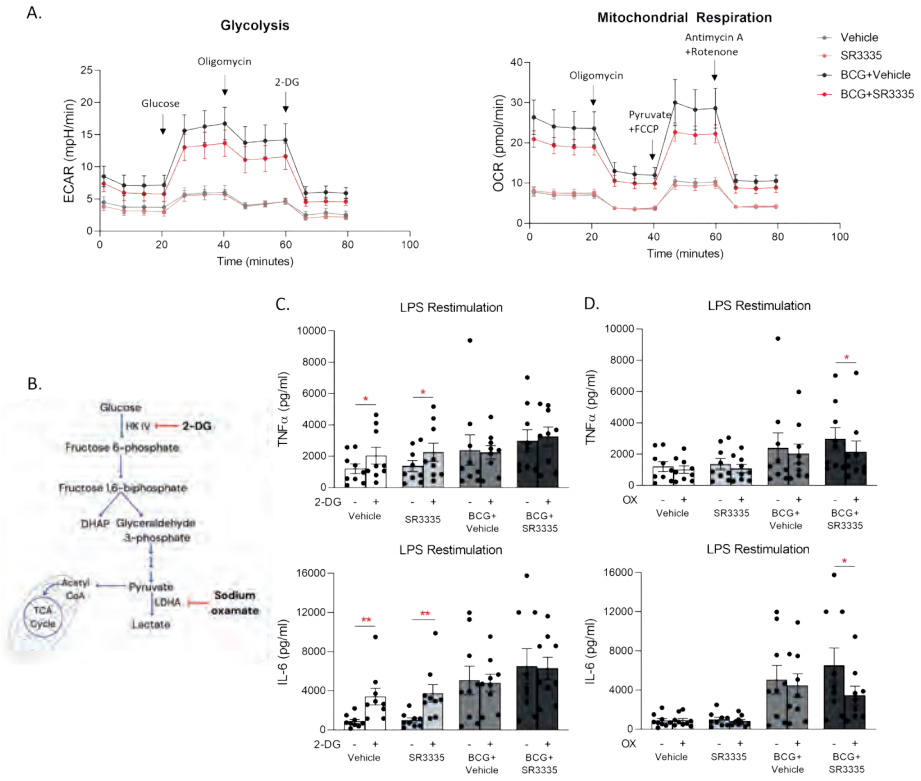


Figure 5. Glycolysis and mitochondrial respiration in trained PBMCs and the effect of glycolysis inhibition on trained immunity by SR3335 and/or BCG

A) Adherent PBMCs were trained with BCG (5 μg/ml) in the absence or presence of SR3335 (5 μM)/vehicle. On day 6, glycolysis and mitochondrial respiration were measured by Seahorse, using the Glyco Stress and Mito Stress Test Kits, respectively. **B)** The first and last steps of glycolysis were blocked with 2-DG (1 mM) and sodium oxamate (5 mM) for 6 days, and the cells were restimulated with LPS. **C-D)** TNFα and IL-6 production was measured by ELISA 24-hour after incubation with LPS on 7 of the trained immunity protocol. The Wilcoxon matched-pairs signed rank test was used to compare the experimental groups. *p<0.05, **p<0.01. Vehicle: DMSO

Blocking LDHA by sodium oxamate significantly decreased the TNFα and IL-6 secretion in cells trained with BCG+SR3335, whereas the cytokine production of other groups remained unaffected (**Figure 5D**). This suggests that the trained immunity induction by BCG and RORα inhibition partially depends on pyruvate-to-lactate conversion.

Discussion

Genetic factors are essential regulators of vaccine and immune system responses, as well as trained immunity. In this study, we reported that genetic polymorphisms of the *RORA* gene affect the strength of the trained immunity response induced by BCG vaccination. Moreover, pharmacological inhibition of RORα increased cytokine production capacity in adherent PBMCs and boosted BCG-induced trained immunity. The RORα inverse agonist SR3335 promoted functional changes triggered by BCG in the trained immune cells, such as ROS and lactate production. Lastly, we identified LDHA as a modulator of trained immunity induced by BCG and RORα inhibition.

BCG is one of the most well-studied trained immunity inducers, both *in vivo* and *in vitro*. Previous findings reported that BCG vaccination leads to persistent epigenetic and metabolic reprogramming, resulting in a robust response from the innate immune cells against future pathogenic encounters [29, 30]. Our study confirmed the functional and metabolic changes reported in the literature after BCG training, such as higher cytokine, lactate, and ROS production and increased glycolysis and oxidative phosphorylation. Incubation of PBMCs with BCG for 24 hours did not impact *RORA* expression; however, BCG significantly reduced *RORA* on day 6, when the trained immunity phenotype was established. The lower *RORA* expression on day 6 after BCG exposure likely contributes to the increased responsiveness of the innate immune cells upon secondary stimulation with LPS, thus promoting trained immunity development. This hypothesis is strengthened by the fact that inhibition of RORα alone, even in the absence of BCG, leads to significantly higher TNFα and IL-6 production after secondary stimulation with LPS compared to the vehicle group. The RORα inverse agonist SR3335 and BCG had a synergistic effect on trained immunity induction: ROS, lactate and cytokine productions were higher in the combination group compared to BCG alone.

RORα regulates inflammatory responses via the NFκB pathway. Studies showed that it downregulates NFκB expression by reducing the nuclear translocation of its p65 subunit while increasing the inhibitor of NFκB (IκB) expression [13, 14, 31]. This leads to lower expression and secretion of pro-inflammatory cytokines, such as TNFα, IL-6, and IL-1β. Interestingly, we did not observe any difference in cytokine production after blocking RORα for 24 hours in human immune cells: all effects were seen at later time points, after epigenetic reprogramming leads to induction of trained immunity [29]. In line with this, a secondary challenge with LPS after the training and resting period induced a more robust cytokine production.

It was previously reported that RORα induces epigenetic reprogramming by recruiting histone deacetylase 3 (HDAC3) on the p65 subunit of NFκB, decreasing H3K9ac deposition, which, in turn, attenuates NFκB target gene expression, e.g., *Il6*, *Tnf*, and *Il1b* [32]. In our *in vitro* trained immunity model, blocking RORα might have increased H3K9ac deposition on p65, upregulating pro-inflammatory gene transcription upon secondary stimulation with LPS. Future studies should investigate how RORα influences epigenetic modifications in the context of trained immunity and the persistence of these changes.

Changes in cellular metabolism, especially glycolysis and oxidative phosphorylation, are an essential component of trained immunity induction. Intriguingly, human adherent cells trained with BCG+SR3335 released more lactate; however, their extracellular acidification rate (ECAR), measured by Seahorse, did not change. To investigate further whether immune cell training by SR3335 relies on glycolysis, we blocked the glycolysis pathway using 2-DG and sodium oxamate. 2-DG inhibits the conversion of glucose to glucose-6-P [33], while oxamate competes with pyruvate and inhibits LDHA [34]. We found that vehicle (DMSO)- and SR3335-trained cells increased cytokine production when 2-DG was present, while BCG blocked this increase.

Inhibiting LDHA activity by sodium oxamate significantly reduced the increase in cytokine production capacity in BCG+SR335-trained immune cells. This could reveal a possible role for pyruvate-to-lactate conversion in trained immunity triggered by BCG+SR3335 synergy. LDHA catalyzes the conversion of pyruvate to lactate, sustaining the glycolysis pathway [35]. Transcription of LDHA is controlled by several transcription factors, including c-Myc, HIF-1α, POU1F1, and KLF4 [36]. Among these, RORα targets c-Myc and HIF-1α to decrease their activation in cancer [37, 38]. Therefore, inhibition of RORα might have led to increased LDHA activity through c-Myc and HIF-1α, favoring lactate production. How RORα modulates the glycolysis pathway should be addressed in more detail.

We reported that while blocking RORα improves trained immunity response, the abundance of natural RORα ligands in the circulation is negatively associated with the strength of BCG-induced trained immunity. Interestingly, this negative correlation was sex-dependent and stronger in males rather than in females. Lower cholesterol concentrations in the circulation might be associated with lower activity of RORα and higher trained immunity response conferred by BCG. Cholesterol metabolism, but not cholesterol itself, is important in BCG-induced trained immunity [39]. Further studies are needed to investigate whether cholesterol levels change after BCG training and whether cholesterol influences the strength of trained immunity through RORα activity.

Although trained immunity could be beneficial in protecting vulnerable populations from infections and mortality, it might result in pathological outcomes when it is not adequately regulated [40]. It was recently proposed that endogenous stimuli, e.g., lipoproteins and adrenal hormones, that contribute to the progression of atherosclerosis, could also lead to the development of trained immunity [41]. RORα might be a link connecting atherosclerosis with trained immunity. Mice with a dysfunctional *Rora* gene (Rora$^{sg/sg}$ mice) are more susceptible to atherosclerosis on a high-fat diet [42]. Moreover, it was shown that *RORA* expression is decreased in human atherosclerotic plaques [43]. It could be hypothesized that downregulated *RORA* expression increases inflammation and amplifies the induction of trained immunity by endogenous ligands, contributing to immune cell recruitment and pathogenesis of atherosclerosis. Further investigation of the role of RORα on atherosclerosis development and trained immunity would eventually open up more treatment possibilities.

This study explored how RORα modulates trained immunity in adherent PBMCs, which are mainly monocytes. It is known that RORα is present in a wide range of immune cells, with a higher expression in NK cells and T cells than in monocytes/macrophages [44]. As NK cells have a prominent role in BCG-mediated trained immunity [45], the importance of RORα in NK-cell-mediated trained immunity should be studied.

Overall, we identified RORα as an important protein regulating the trained immunity response by BCG vaccination in humans. The concentration of the natural RORα agonists in the circulation was negatively correlated with fold cytokine production after BCG. In contrast, blocking RORα promoted BCG-induced trained immunity *in vitro*. The suppression of LDHA activity decreased cytokine production during BCG+SR3335 training, making LDHA a possible contributor to trained immunity development mediated by BCG and RORα.

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Author contributions

Gizem Kilic: Conceptualization, Investigation, Data curation, Visualization, Formal analysis, Writing-original draft, Writing-review&editing. **Vasiliki Matzaraki:** Formal analysis, Visualization, Writing-review&editing. **Ozlem Bulut, Ilayda Baydemir, Anaisa V. Ferreira, Katrin Rabold:** Investigation, Writing-review & editing. **Simone J.C.F.M. Moorlag, Valerie A.C.M. Koeken, L. Charlotte J. de Bree, Vera P. Mourits:** Investigation, Resources, Data curation, Writing-review&editing. **Leo A.B. Joosten:** Supervision, Writing-review&editing. **Jorge Dominguez-Andres:** Investigation, Supervision, Funding acquisition, Writing-review&editing. Conceptualization, Supervision, Funding acquisition, Writing-review&editing.

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Conflict of interest

MGN is a scientific founder and is on the scientific advisory board of Trained Therapeutix Discovery (TTxD), and he is a scientific founder of Lemba Therapeutics, Salvina Therapeutics and Biotrip. LABJ is a scientific founder of TTxD, Lemba Therapeutics and Salvina Therapeutics. The other authors declare that they have no conflicts of interest.

Data availability

Data will be made available upon request.

References

1. Netea MG, Joosten LA, Latz E, et al. Trained immunity: A program of innate immune memory in health and disease. Science 2016; 352(6284): aaf1098, https://doi.org/10.1126/science.aaf1098.
2. Bekkering S, Dominguez-Andres J, Joosten LAB, Riksen NP, Netea MG. Trained Immunity: Reprogramming Innate Immunity in Health and Disease. Annu Rev Immunol 2021; 39: 667-93, https://doi.org/10.1146/annurev-immunol-102119-073855.
3. Dominguez-Andres J, Dos Santos JC, Bekkering S, et al. Trained immunity: adaptation within innate immune mechanisms. Physiol Rev 2023; 103(1): 313-46, https://doi.org/10.1152/physrev.00031.2021.
4. Moorlag S, Folkman L, Ter Horst R, 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, https://doi.org/10.1016/j.immuni.2023.12.005.
5. Arts RJW, Moorlag S, Novakovic B, 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, https://doi.org/10.1016/j.chom.2017.12.010.
6. Ferreira AV, Koeken V, Matzaraki V, et al. Glutathione Metabolism Contributes to the Induction of Trained Immunity. Cells 2021; 10(5), https://doi.org/10.3390/cells10050971.
7. Buffen K, Oosting M, Quintin J, et al. Autophagy controls BCG-induced trained immunity and the response to intravesical BCG therapy for bladder cancer. PLoS Pathog 2014; 10(10): e1004485, https://doi.org/10.1371/journal.ppat.1004485.
8. Solt LA, Burris TP. Action of RORs and their ligands in (patho)physiology. Trends Endocrinol Metab 2012; 23(12): 619-27, https://doi.org/10.1016/j.tem.2012.05.012.
9. Lee JM, Kim H, Baek SH. Unraveling the physiological roles of retinoic acid receptor-related orphan receptor alpha. Exp Mol Med 2021; 53(9): 1278-86, https://doi.org/10.1038/s12276-021-00679-8.
10. Ladurner A, Schwarz PF, Dirsch VM. Natural products as modulators of retinoic acid receptor-related orphan receptors (RORs). Nat Prod Rep 2021; 38(4): 757-81, https://doi.org/10.1039/donp00047g.
11. Karlsson M, Zhang C, Mear L, et al. A single-cell type transcriptomics map of human tissues. Sci Adv 2021; 7(31), https://doi.org/10.1126/sciadv.abh2169.
12. Stapleton CM, Jaradat M, Dixon D, et al. Enhanced susceptibility of staggerer (RORalphasg/sg) mice to lipopolysaccharide-induced lung inflammation. Am J Physiol Lung Cell Mol Physiol 2005; 289(1): L144-52, https://doi.org/10.1152/ajplung.00348.2004.
13. Han S, Li Z, Han F, et al. ROR alpha protects against LPS-induced inflammation by down-regulating SIRT1/NF-kappa B pathway. Arch Biochem Biophys 2019; 668: 1-8, https://doi.org/10.1016/j.abb.2019.05.003.
14. Delerive P, Monte D, Dubois G, et al. The orphan nuclear receptor ROR alpha is a negative regulator of the inflammatory response. EMBO Rep 2001; 2(1): 42-8, https://doi.org/10.1093/embo-reports/kve007.
15. Nejati Moharrami N, Bjorkoy Tande E, Ryan L, Espevik T, Boyartchuk V. RORalpha controls inflammatory state of human macrophages. PLoS One 2018; 13(11): e0207374, https://doi.org/10.1371/journal.pone.0207374.
16. Koeken VA, de Bree LCJ, Mourits VP, et al. BCG vaccination in humans inhibits systemic inflammation in a sex-dependent manner. J Clin Invest 2020; 130(10): 5591-602, https://doi.org/10.1172/JCI133935.
17. Moorlag S, Matzaraki V, van Puffelen JH, et al. An integrative genomics approach identifies KDM4 as a modulator of trained immunity. Eur J Immunol 2022; 52(3): 431-46, https://doi.org/10.1002/eji.202149577.
18. Shabalin AA. Matrix eQTL: ultra fast eQTL analysis via large matrix operations. Bioinformatics 2012; 28(10): 1353-8, https://doi.org/10.1093/bioinformatics/bts163.

19. Lamparter D, Marbach D, Rueedi R, Kutalik Z, Bergmann S. Fast and Rigorous Computation of Gene and Pathway Scores from SNP-Based Summary Statistics. PLoS Comput Biol 2016; 12(1): e1004714, https://doi.org/10.1371/journal.pcbi.1004714.
20. Koeken V, Qi C, Mourits VP, et al. Plasma metabolome predicts trained immunity responses after antituberculosis BCG vaccination. PLoS Biol 2022; 20(9): e3001765, https://doi.org/10.1371/journal.pbio.3001765.
21. Dominguez-Andres J, Arts RJW, Bekkering S, et al. In vitro induction of trained immunity in adherent human monocytes. STAR Protoc 2021; 2(1): 100365, https://doi.org/10.1016/j.xpro.2021.100365.
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25(4): 402-8, https://doi.org/10.1006/meth.2001.1262.
23. Geiss C, Alanis-Lobato G, Andrade-Navarro M, Regnier-Vigouroux A. Assessing the reliability of gene expression measurements in very-low-numbers of human monocyte-derived macrophages. Sci Rep 2019; 9(1): 17908, https://doi.org/10.1038/s41598-019-54500-8.
24. Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques 2004; 37(1): 112-4, 6, 8-9, https://doi.org/10.2144/04371RR03.
25. van der Heijden C, Keating ST, Groh L, Joosten LAB, Netea MG, Riksen NP. Aldosterone induces trained immunity: the role of fatty acid synthesis. Cardiovasc Res 2020; 116(2): 317-28, https://doi.org/10.1093/cvr/cvz137.
26. Sohrabi Y, Sonntag GVH, Braun LC, et al. LXR Activation Induces a Proinflammatory Trained Innate Immunity-Phenotype in Human Monocytes. Front Immunol 2020; 11: 353, https://doi.org/10.3389/fimmu.2020.00353.
27. Ferreira AV, Alarcon-Barrera JC, Dominguez-Andres J, et al. Fatty acid desaturation and lipoxygenase pathways support trained immunity. Nat Commun 2023; 14(1): 7385, https://doi.org/10.1038/s41467-023-43315-x.
28. 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. Clin Vaccine Immunol 2016; 23(12): 926-33, https://doi.org/10.1128/CVI.00349-16.
29. Arts RJW, Carvalho A, La Rocca C, et al. Immunometabolic Pathways in BCG-Induced Trained Immunity. Cell Rep 2016; 17(10): 2562-71, https://doi.org/10.1016/j.celrep.2016.11.011.
30. Kleinnijenhuis J, Quintin J, Preijers F, 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, https://doi.org/10.1073/pnas.1202870109.
31. Friesenhagen J, Viemann D, Borgeling Y, et al. Highly pathogenic influenza viruses inhibit inflammatory response in monocytes via activation of rar-related orphan receptor RORalpha. J Innate Immun 2013; 5(5): 505-18, https://doi.org/10.1159/000346706.
32. Oh SK, Kim D, Kim K, et al. RORalpha is crucial for attenuated inflammatory response to maintain intestinal homeostasis. Proc Natl Acad Sci U S A 2019; 116(42): 21140-9, https://doi.org/10.1073/pnas.1907595116.
33. Pajak B, Siwiak E, Soltyka M, et al. 2-Deoxy-d-Glucose and Its Analogs: From Diagnostic to Therapeutic Agents. Int J Mol Sci 2019; 21(1), https://doi.org/10.3390/ijms21010234.
34. Zhao Z, Han F, Yang S, Wu J, Zhan W. Oxamate-mediated inhibition of lactate dehydrogenase induces protective autophagy in gastric cancer cells: involvement of the Akt-mTOR signaling pathway. Cancer Lett 2015; 358(1): 17-26, https://doi.org/10.1016/j.canlet.2014.11.046.

35. Sharma D, Singh M, Rani R. Role of LDH in tumor glycolysis: Regulation of LDHA by small molecules for cancer therapeutics. Semin Cancer Biol 2022; 87: 184-95, https://doi.org/10.1016/j.semcancer.2022.11.007.

36. Dai M, Wang L, Yang J, et al. LDHA as a regulator of T cell fate and its mechanisms in disease. Biomed Pharmacother 2023; 158: 114164, https://doi.org/10.1016/j.biopha.2022.114164.

37. Xiao W, Geng W, Zhou M, et al. POU6F1 cooperates with RORA to suppress the proliferation of lung adenocarcinoma by downregulation HIF1A signaling pathway. Cell Death Dis 2022; 13(5): 427, https://doi.org/10.1038/s41419-022-04857-y.

38. Park SC, Park IG, Kim H, Lee JM. N-Terminal Domain Mediated Regulation of RORalpha1 Inhibits Invasive Growth in Prostate Cancer. Int J Mol Sci 2019; 20(7), https://doi.org/10.3390/ijms20071684.

39. Bekkering S, Arts RJW, Novakovic B, et al. Metabolic Induction of Trained Immunity through the Mevalonate Pathway. Cell 2018; 172(1-2): 135-46 e9, https://doi.org/10.1016/j.cell.2017.11.025.

40. Netea MG, Dominguez-Andres J, Barreiro LB, et al. Defining trained immunity and its role in health and disease. Nat Rev Immunol 2020; 20(6): 375-88, https://doi.org/10.1038/s41577-020-0285-6.

41. Flores-Gomez D, Bekkering S, Netea MG, Riksen NP. Trained Immunity in Atherosclerotic Cardiovascular Disease. Arterioscler Thromb Vasc Biol 2021; 41(1): 62-9, https://doi.org/10.1161/ATVBAHA.120.314216.

42. Mamontova A, Seguret-Mace S, Esposito B, et al. Severe atherosclerosis and hypoalphalipoproteinemia in the staggerer mouse, a mutant of the nuclear receptor RORalpha. Circulation 1998; 98(24): 2738-43, https://doi.org/10.1161/01.cir.98.24.2738.

43. Besnard S, Heymes C, Merval R, et al. Expression and regulation of the nuclear receptor RORalpha in human vascular cells. FEBS Lett 2002; 511(1-3): 36-40, https://doi.org/10.1016/s0014-5793(01)03275-6.

44. Uhlen M, Karlsson MJ, Zhong W, et al. A genome-wide transcriptomic analysis of protein-coding genes in human blood cells. Science 2019; 366(6472), https://doi.org/10.1126/science.aax9198.

45. Kleinnijenhuis J, Quintin J, Preijers F, et al. BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. Clin Immunol 2014; 155(2): 213-9, https://doi.org/10.1016/j.clim.2014.10.005.

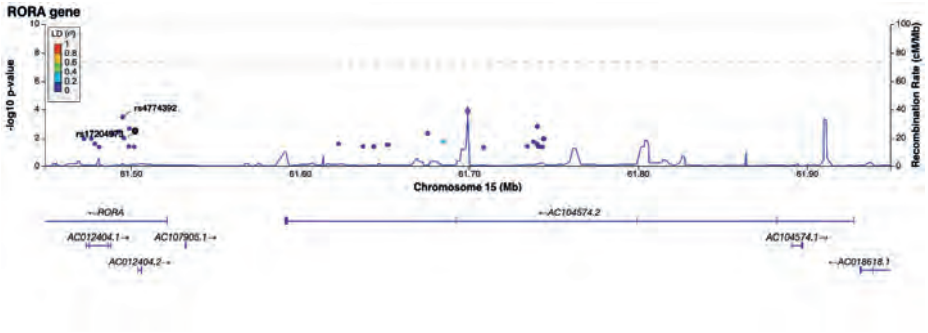
Supplementary Information

Supplementary Tables

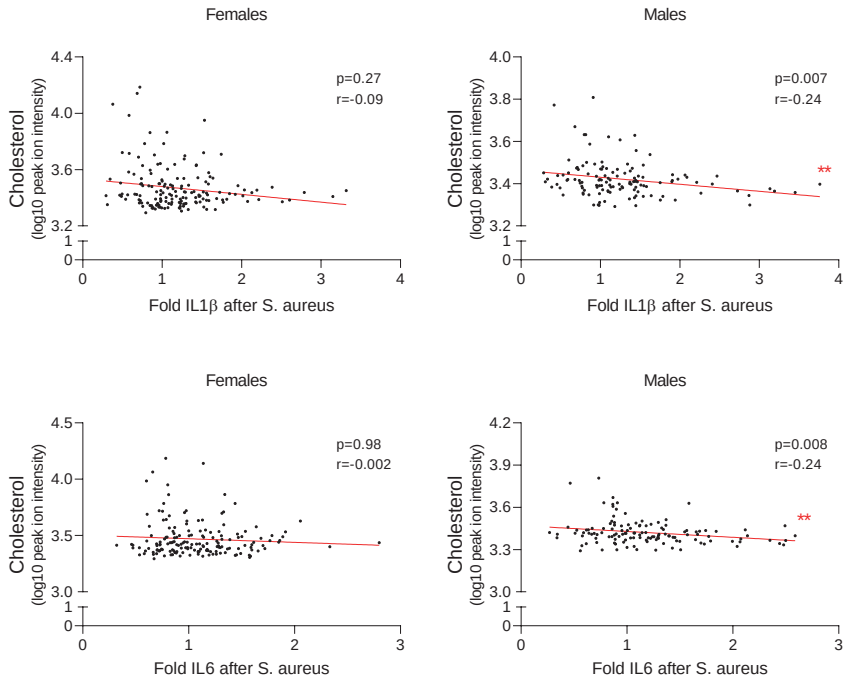
Supplementary table 1. Primer sequences used in the study

Primer Name	Forward primer (5'-3')	Reverse Primer (5'-3')
h***RORA***	GTTCACCAACGGCGAGACTTCC	TCTTCTCTCAAGTATTGGCAGGTTTC
h***HPRT***	CCTGGCGTCGTGATTAGTGAT	AGACGTTCAGTCCTGTCCATAA

Supplementary Figures

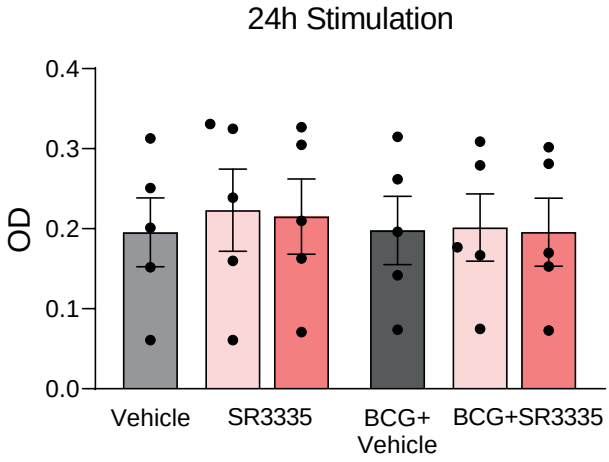


Supplementary Figure 1.Regional association plot of trans QTLs (p<0.05) around the *RORA* gene in a window of 250 kb that influence IL-6 production fold change upon BCG vaccination



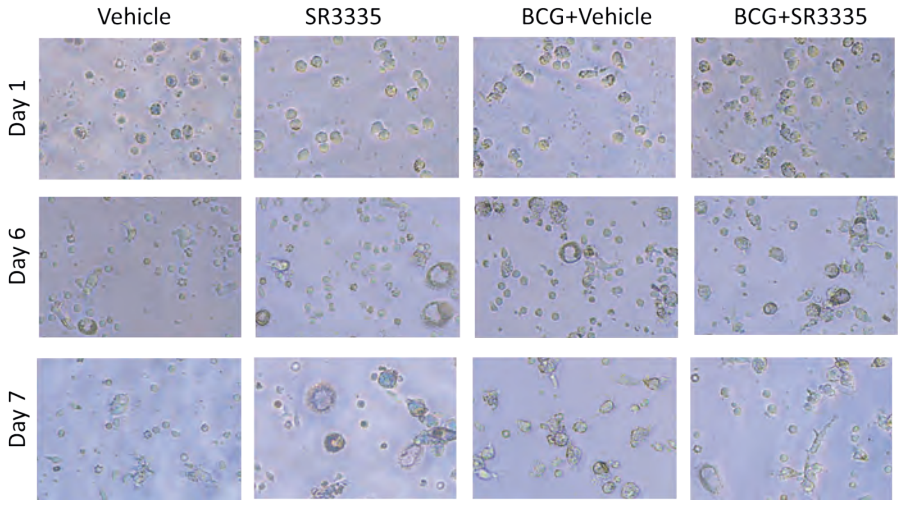
Supplementary Figure 2. Sex-dependent correlation of baseline cholesterol levels with trained immunity response after BCG vaccination

Baseline cholesterol levels of females and males were correlated with their fold TNFα, IL-6, and IL-1β responses upon incubation with heat-killed *S. aureus* (10^6 CFU/ml) (T2/T1). r: Spearman's correlation coefficient. *p<0.05, **p<0.01.



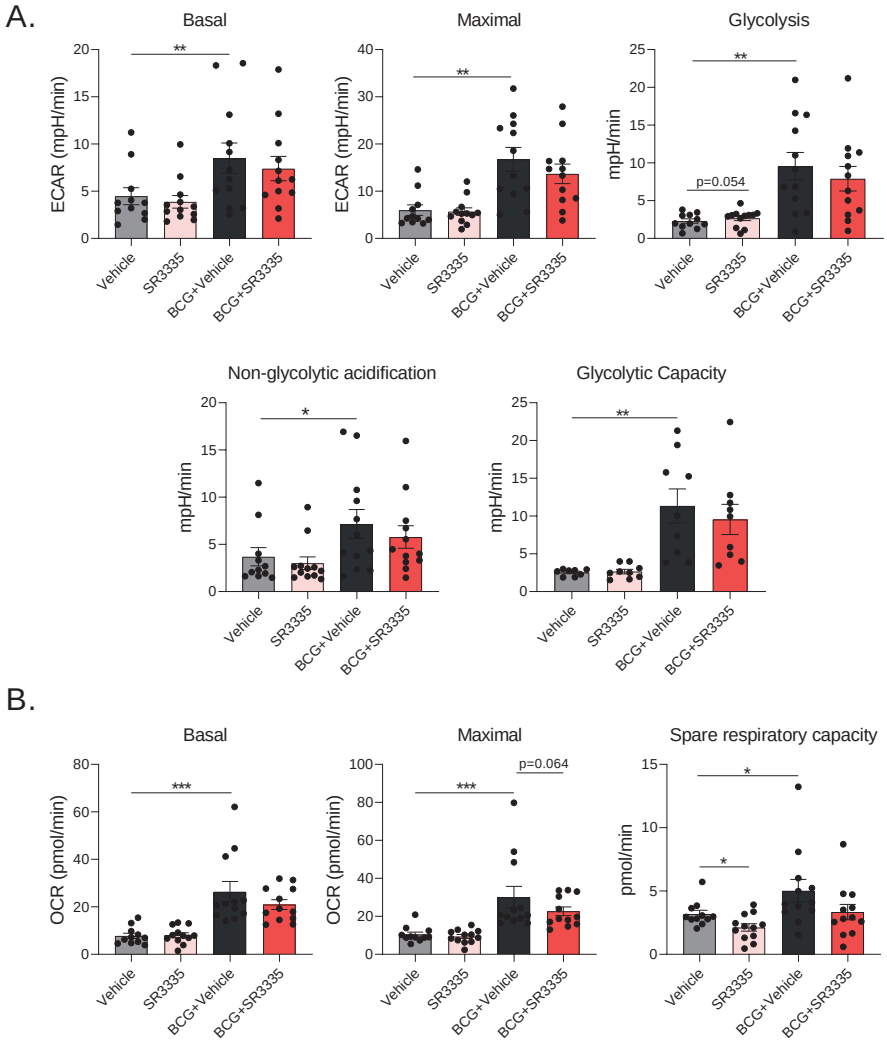
Supplementary Figure 3. Cytotoxicity of BCG and SR3335 on adherent PBMCs after incubation for 24 hours

Adherent PBMCs were incubated with BCG (5 μg/ml) and/or SR3335 (5 μM)/vehicle for 24 hours. LDH levels were measured from cell supernatant as a measure of cytotoxicity. Vehicle: DMSO



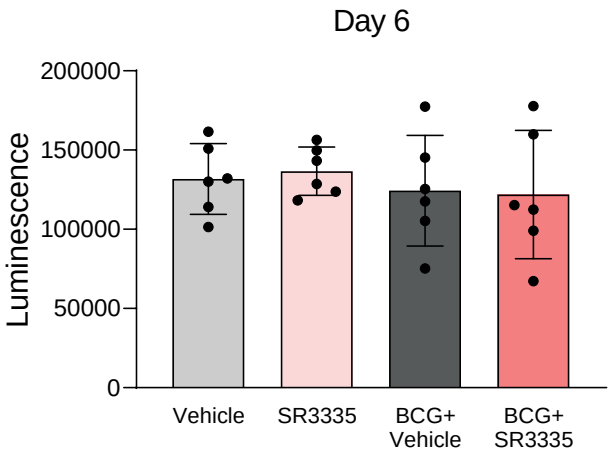
Supplementary Figure 4. Cell morphology of adherent PBMCs on day 1, day 6, and day 7 of the *in vitro* trained immunity protocol

Adherent PBMCs were stimulated with BCG (5 μg/ml) and/or SR3335 (5 μM)/vehicle for 24 hours, and the cells were rested for 5 days. Then, PBMCs were restimulated with LPS (10 ng/ml) for 24 hours. Pictures were taken with light microscopy at 20x magnification on day 1, day 6 (end of the resting period), and day 7 (24 hours after LPS).



Supplementary Figure 5. Measurement of glycolysis and oxidative phosphorylation in PBMCs trained with BCG and/or the RORα inhibitor SR3335

Adherent PBMCs were trained with BCG (5 μg/ml) in the absence or presence of SR3335 (5 μM)/vehicle. On day 6, glycolysis and mitochondrial respiration were measured by Seahorse. **A)** Basal and maximal extracellular acidification rate, glycolysis, non-glycolytic acidification, and glycolytic capacity of the trained cells, measured using the Glyco Stress Test. **B)** Basal and maximal oxygen consumption rate, spare respiratory capacity, non-mitochondrial oxygen consumption, and maximal respiration of the trained cells, measured using the Mito Stress Test. Wilcoxon matched-pairs signed rank test was used to compare the experimental groups. *p<0.05, **p<0.01, ***p<0.001. Vehicle: DMSO.



Supplementary Figure 6. Viability of adherent PBMCs on day 6 of the *in vitro* trained immunity protocol

Adherent PBMCs were stimulated with BCG (5 μg/ml) and/or SR3335 (5 μM)/vehicle for 24 hours. Then, cells were washed with PBS, cell culture medium was refreshed, and cells were rested for 5 days. ATP content of the cells was quantified as a measure of cell viability at the end of the resting period. Vehicle: DMSO.

PART II
ENVIRONMENTAL FACTORS

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CHAPTER 5

Seasonal variation in BCG-induced trained immunity

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Submitted

Abstract

The Bacille Calmette-Guerin (BCG) vaccine is a well-established inducer of innate immune memory (also termed *trained immunity*), causing increased cytokine production upon heterologous secondary stimulation. Innate immune responses are known to be influenced by season, but whether seasons impact induction of trained immunity is not known. To explore the influence of season on innate immune memory induced by the BCG vaccine, we vaccinated healthy volunteers with BCG either during winter or spring. Three months later, we measured the *ex vivo* cytokine responses against heterologous stimuli, analyzed gene expressions and epigenetic signatures of the immune cells, and compared these with the baseline before vaccination. BCG vaccination during winter induced a stronger increase in the production of pro-inflammatory cytokines by peripheral blood mononuclear cells (PBMCs) upon stimulation with different bacterial and fungal stimuli, compared to BCG vaccination in spring. In contrast, winter BCG vaccination resulted in lower IFNγ release. Furthermore, NK cells of the winter-vaccinated people had a greater pro-inflammatory cytokine and IFNγ production capacity upon heterologous stimulation. BCG vaccination had only minor effects on the transcriptome of monocytes 3 months later. In contrast, we identified season-dependent epigenetic changes in monocytes and NK cells induced by BCG vaccination, partly explaining the higher immune cell reactivity in the winter BCG vaccination group. These results suggest that BCG vaccination during winter is more prone to induce a robust trained immunity response by activating and reprogramming the immune cells, especially NK cells. (Dutch clinical trial registry no. NL58219.091.16)

Introduction

Variability in immune responses among individuals is influenced by host and environmental factors. Host factors include age, sex, and genetics, while environmental factors encompass infections, lifestyle, and seasonality [1, 2]. Seasonal variations significantly affect immune system responsiveness, altering circulating inflammatory protein levels and immune cell function throughout the year. In addition, certain microbes have a better chance to survive, proliferate, and infect during certain periods of the year. A Danish study measured the annual indoor and outdoor abundance of fungi, bacteria and endotoxin [3], and they found that seasons influence the concentrations of both indoor and outdoor microbial exposures. Due to several seasonal factors, the incidence and severity of some infectious and autoinflammatory diseases are different throughout the year [4, 5]. For instance, patients with rheumatoid arthritis (RA) report seasonal fluctuations in joint symptoms, with increased disease activity during spring and a decrease during fall [6]. Autoimmune type 1 diabetes in children shows a seasonal pattern, with lower summer incidence and autumn-winter peaks in the northern hemisphere [7]. Moreover, communicable diseases like influenza, respiratory syncytial virus (RSV), tuberculosis, and vector-borne diseases also follow seasonal trends, varying by geographical location [8, 9].

Vaccine responses are also impacted by the changing seasons. Several studies documented increased seroconversion rates after winter inoculation compared to summer or spring inoculation with measles-mumps-rubella (MMR) or influenza vaccines [10-12]. In sub-Saharan Africa, BCG vaccination induces stronger responses in infants born in the wet season, similar to vaccines against pertussis and pneumococcus [13]. For others, such as Hepatitis-B, tetanus and meningitis-C containing vaccines, the effect of the season remains unclear or limited [8].

The notion that the innate immune system, vaccine responses and antibody production can be affected by season, leads to the hypothesis that the season of vaccine administration may also influence innate immune memory function, or trained immunity. Trained immunity refers to the ability of certain vaccines or infections to stimulate memory characteristics in the innate immune system that lead to increased resistance to subsequent heterologous infections [14]. Trained immunity is induced through metabolic and epigenetic modifications of myeloid and NK cells [15], leading to increased cytokine production capacity and protection against a broad array of unrelated microorganisms [16]. Trained immunity can boost the function of the immune system, but it has particular relevance in infection-prone populations

such as children, the elderly, and immunocompromised individuals. BCG vaccine, known for inducing trained immunity, reduces all-cause mortality in children from high-infection areas, particularly against respiratory infections and sepsis [17]. In studies from West Africa, the effect of BCG on all-cause neonatal mortality was particularly beneficial when administered from November to January, coincident with peaking malaria infections [18]. During these months, BCG was also associated with stronger pro-inflammatory responses to heterologous challenge [18, 19]. Furthermore, a recent study suggested a seasonal pattern in BCG-induced trained immunity response in adults [20], however, how seasons can modulate trained immunity is yet to be known.

In the present study, we investigated the influence of BCG administration during winter and spring on the induction of trained immunity in young, healthy individuals. We assessed functional changes by measuring cytokine production in PBMCs and NK cells after stimulation with various stimuli, as well as transcriptional and epigenetic differences of immune cells, to identify a possible seasonal pattern for BCG-induced reactivity (**Figure 1A**).

Materials and methods

Study Design and Subjects

This study was performed in a cohort of healthy adults in the Netherlands (BCG-booster cohort, no. NL58219.091.16) at the Radboud University Medical Center. The local ethics committee METC Oost-Nederland gave ethical approval for the study. Inclusion and experimentation procedures were conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all volunteers. The participants in this cohort had no prior BCG vaccination, and they did not receive other vaccinations during the study period. Volunteers had not experienced acute illness (e.g., infection) for 2 weeks before or during the study. Subjects did not use any drugs except oral contraceptives. Pregnant women were excluded from participation.

The study cohort consisted of 51 participants. Among them, 17 had received a BCG vaccine (intradermal injection of 0.1 ml, 0.75 mg/ml, *M. bovis*, BCG Denmark, AJ Vaccines) in the winter (December-February), while 29 participants had received a BCG vaccine in the spring (April-May). All vaccinations were performed with the same BCG batch. The 5 participants in the placebo group had received a placebo vaccination (intradermal injection of 0.1 ml of vaccine diluent) twice, first in the

winter and then in the spring. As this study was not originally designed to assess the seasonality of trained immunity, the winter and spring vaccination groups were not equal in sample size. Blood collection was done before and 3 months after vaccination. For the placebo group, blood was drawn before and 3 months after each placebo vaccination.

PBMC Isolation

Venous blood was collected into EDTA-coated tubes (BD Bioscience, USA), used for cell counting and PBMC isolation. To count the number of immune cells in the whole blood, a hematology analyzer (Sysmex, Japan) was used.

Blood was diluted with calcium/magnesium-free phosphate-buffered saline (PBS) in a 1:1 volume ratio and layered on Ficoll-Paque (GE Healthcare, USA). After density gradient centrifugation, the middle layer containing PBMCs was taken. The collected cells were washed three times with cold PBS. PBMCs were resuspended in RPMI 1640 (Dutch-modified) culture medium (Invitrogen, Thermo Fisher Scientific, USA) supplemented with 50µg/ml gentamycin (Centrafarm), 2mM GlutaMAX (Thermo Fisher Scientific, USA) and 1mM sodium pyruvate (Thermo Fisher Scientific, USA).

Monocyte and NK cell isolation

Monocytes were purified from freshly isolated PBMCs using magnetic-activated cell sorting with MACS pan monocytes isolation kit (Miltenyi Biotec, Germany), according to the manufacturer's instructions. 1-2x10^6 monocytes with 90-95% purity (assessed by a hematology analyzer) were resuspended in the RLT buffer (Qiagen, Germany) and stored at -800C until RNA isolation and RNA sequencing.

NK cells were isolated from PBMCs using magnetic-activated cell sorting with MACS NK cell isolation kit (Miltenyi Biotec, Germany) with approximately 90% purity (assessed by flow cytometry, data not shown), according to the manufacturer's instructions. A part of the NK cells was used for *ex vivo* stimulation and cytokine measurements. 5x10^4 NK cells were processed further to perform ATAC-seq (the assay for transposase-accessible chromatin using sequencing) later.

Ex vivo stimulation

Freshly isolated PBMCs were seeded on 96-well round bottom plates (Corning, USA) 5x10^5cells/well and incubated with RPMI containing 10% human pooled serum and supplemented with gentamicin, glutamax, and pyruvate, as previously described. Cells were stimulated with 10 ng/ml LPS (Sigma Aldrich, USA), 10^6/ml heat-killed *Escherichia coli*, 10^6/ml heat-killed *Staphylococcus aureus*, 5 µg/ml heat-killed

Mycobacterium tuberculosis, 10^6/ml heat-killed *Candida albicans*, $5x10^6$/ml heat-killed *Streptococcus pneumoniae*, and 10 µg/ml PHA for 24 hours and 7 days, respectively. Cell-free supernatants were collected and stored at -20 °C.

All NK cell stimulations were performed in the presence of 10 ng/ml recombinant human IL-15 (R&D Systems, USA) in the cell culture media. NK cells were seeded on 96-well round bottom plates (Corning) 10^5 cells/well and incubated with RPMI (with 10% human pooled serum and supplemented as previously described), 10 ng/ml LPS (Sigma Aldrich), 10^6/ml *E. coli*, 10^6/ml *S. aureus*, 5 µg/ml *M. tuberculosis*, 10^6/ml *C. albicans*, 10 µg/ml Pam3Cys4, 50 ng/ml PMA+ 1 µg/ml ionomycin for 48 hours. Cell-free supernatants were collected and stored at -20°C.

Cytokine measurements

After stimulation of PBMCs and NK cells, TNFα, IL-6, IL-1β and IFNγ cytokine levels were determined using DuoSet® ELISA kits (R&D Systems), following the manufacturer's instructions.

RNA isolation

RNA was isolated from the monocytes stored in the RLT buffer by RNeasy Kit (Qiagen), according to the manufacturer's instructions.

RNA sequencing

Total RNA was used to prepare the RNA sequencing libraries using the KAPA RNA HyperPrep Kit with RiboErase (KAPA Biosystems, USA). Oligo hybridization and rRNA depletion, rRNA depletion cleanup, DNase digestion, DNase digestion cleanup, and RNA elution were performed according to protocol. Fragmentation and priming were performed at 94°C for 6 min. First-strand synthesis, second-strand synthesis and A-tailing were performed according to protocol. For the adaptor ligation, ligation buffer with a 1.5 µM concentration was used (NextFlex DNA barcodes, Bio Scientific). The first and second post-ligation cleanup was performed according to protocol. A total of 11 PCR cycles were performed for library amplification. The library amplification cleanup was done using a 0.8x followed by a 1.0x bead-based cleanup. Library size was determined using the High Sensitivity DNA bioanalyzer kit, and the library concentration was measured using the dsDNA High Sensitivity Assay (DeNovix, USA). Paired-end sequencing of reads of 50 bp was generated using an Illumina NextSeq 500.

Tagmentation and Elution of DNA

Tagmentation of the purified monocyte and NK cell-DNAs was performed using Tagment DNA TDE1 Enzyme and Buffer Kit (Illumina, USA). Briefly, $5x10^4$ monocytes or NK cells were incubated at 37°C for 30 minutes in the following mix: 12.5 µl Tagment DNA buffer, 2 µl TDE1 Tagment Enzyme, 0.25 µl Digitonin and 10.25 µl water per reaction. After this step, DNA was purified and eluted in 12 µl elution buffer using the MinElute Kit (Qiagen), following the manufacturer's instructions.

ATAC sequencing of monocytes and NK cells

Tagmented DNA was amplified by PCR using Nextera primers (Illumina) and KAPA HiFi HotStart ReadyMix. Purification was performed using SPRI beads before a second PCR amplification was performed to amplify the tagmented DNA further. DNA concentrations were measured with a DeNovix dsDNA HS Assay Kits (Life Technologies, USA) on a DeNovix Spectrophotometer/Fluorometer (DS-11) (DeNovix). Library size distribution was measured using High Sensitivity DNA analysis (Agilent) on an Agilent 2100 Bioanalyzer. The libraries were sequenced using an Illumina NextSeq 500 system.

Data analysis

The demographics of the study participants were compared using Fisher's exact test. Differences in cell numbers before BCG (T1) and 3 months after BCG (T2) within the same group were compared using the Wilcoxon signed-rank test. Fold cytokine changes (T2/T1) between the two groups were analyzed using the Mann-Whitney U test. Differences in fold changes between the placebo groups were analyzed using the Wilcoxon signed-rank test. P-values below 0.05 were considered statistically significant and were indicated with asterisks (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

RNA and ATAC sequencing data were first processed using NEXTFLOW pipelines (version 21.04.3) [21]. Out of this, feature count tables were normalized and loaded to the Deseq2 tool (version 1.40.1) for differential expression analysis [22]. Genes and peaks were selected for functional enrichment when the statistical significance of the differential expression was less than 0.05. Age and sex of the subjects were considered as covariates during differential analysis. Functional enrichment analysis of the selected genes derived from the expression data or corresponding to peaks was conducted by an over-representation analysis tool from the ConsensusPathDB-human platform (Release 35) [23]. The q-value in the enrichment results indicates a hypergeometric test false discovery rate-adjusted-p-value. The statistical analyses for RNA-seq and ATAC-seq data were performed using the R programming language

(version 4.1.2, R Core Team). Volcano plots were made using the *ggplot2* and *ggrepel* packages. GraphPad Prism 8 (GraphPad Software Inc., USA) was used to perform statistical analyses to compare cell counts and cytokine production.

Results

Baseline characteristics

To investigate whether the season of vaccination influences trained immunity responses induced by BCG, we assessed the *ex vivo* pro-inflammatory cytokine production and the transcriptomic profile in the winter and spring of BCG-vaccinated and placebo-vaccinated individuals (**Figure 1A**). All 51 subjects were of Western-European descent and between 18 and 50. There were no significant differences in age, sex and BMI between intervention groups (**Table 1**).

Table 1. Demographics of Study Participants

	Placebo (n=5)	Winter BCG (n=17)	Spring BCG (n=29)	p-value
Age	22.8±2.8	24.5±7.4	24.0±5.9	0.87
Sex (F/M)	4/1	13/4	14/15	0.11
BMI	21.9±3.96	22.9±2.5	23.8±2.9	0.30

Spring BCG vaccination increases the total number of leukocytes and lymphocytes

First, we determined how BCG or placebo vaccination during winter and spring affects the immune cell composition. For this, we measured the white blood cell, neutrophil, lymphocyte, and monocyte counts using a hematology analyzer before (T1) and 3 months after vaccination (T2). We found that participants vaccinated with BCG during spring had significantly more white blood cells 3 months later, unlike individuals vaccinated with BCG during winter, primarily due to increased lymphocyte numbers after vaccination compared to baseline (**Figure 1B**). The number of circulating monocytes in the spring-vaccinated BCG group was slightly higher, but not significant than those in the winter-vaccinated BCG group 3 months after vaccination. Of note, the baseline cell counts of winter and spring vaccinated individuals were similar (**Supp. Figure 1**).

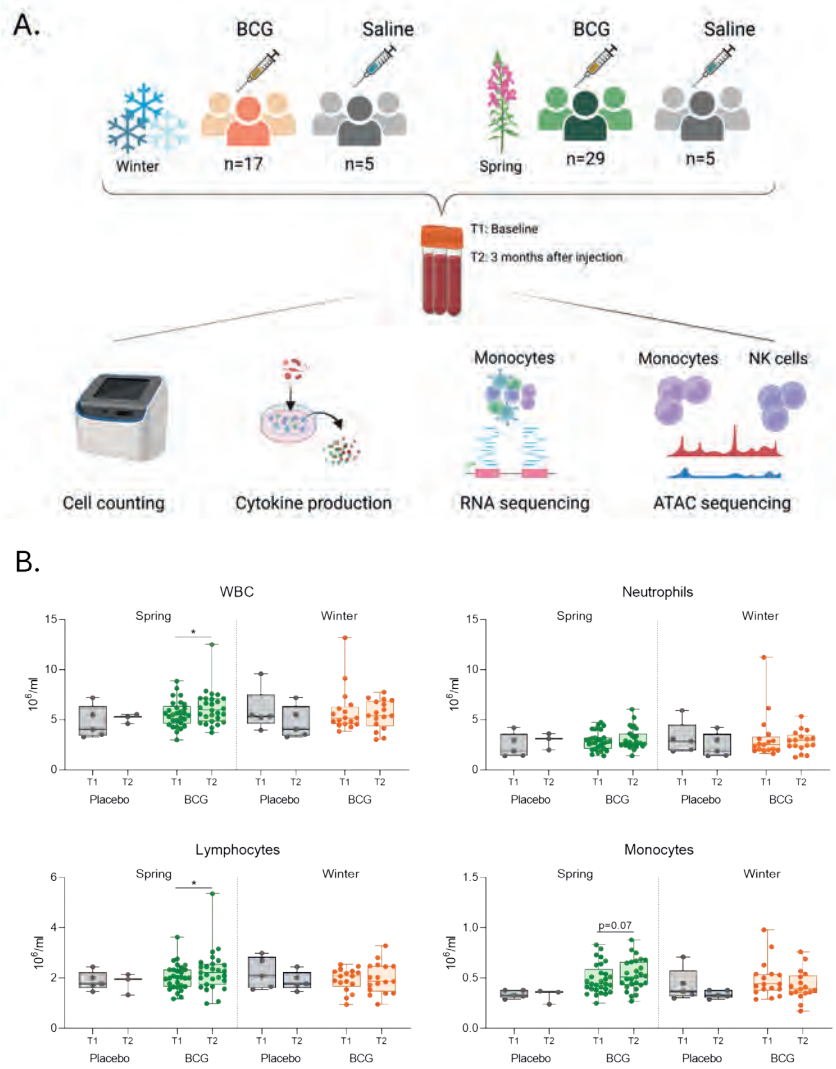

Figure 1. Experimental design and immune cell counts in the blood

A) Design of the clinical study and summary of the assays. **B)** White blood cell, neutrophil, lymphocyte, and monocyte counts are measured from whole blood using a hematology analyzer before (T1) and 3 months after placebo or BCG vaccination (T2). Differences in cell counts between T1 and T2 were analyzed using the Wilcoxon signed-rank test. *p<0.05.

Baseline cytokine production during winter and spring

Next, we measured cytokine production by PBMCs and NK cells of individuals vaccinated either in the winter or spring after challenge with specific and heterologous stimuli. PBMCs isolated in the winter produced more TNFα after E. coli and less TNFα after *M. tuberculosis* incubation compared to spring PBMCs, although these differences did not reach statistical significance (**Supp. Figure 2A**). Moreover, winter PBMCs released significantly more IL-6 and IL-1β following *S. aureus* stimulation compared to spring PBMCs (**Supp. Figure 2B and C**). On the other hand, winter PBMCs were less responsive to *C. albicans*, evident from lower TNFα and IL-1β production compared to spring PBMCs. IFNγ levels produced after pathogen stimulations were comparable between the winter and spring groups (**Supp. Figure 2D**). In contrast to a few seasonal differences in cytokine production on PBMCs, NK cells isolated during winter released similar amounts of IFNγ, TNFα and IL-6 compared to NK cells from the spring. (**Supp. Figure 3A-C**).

Winter BCG leads to higher pro-inflammatory cytokine production capacity from myeloid-derived cells

Subsequently, we assessed whether the strength and degree of BCG-induced trained immunity differ depending on the vaccination season. Therefore, we calculated the fold change in the cytokine production capacity (T2/T1) before and 3 months after BCG in the spring and winter groups. The PBMCs of the participants vaccinated with BCG in the winter were more reactive in terms of IL-6 production induced by *E. coli*, *S. aureus*, *M. tuberculosis*, and IL-1β after *E. coli* stimulation (**Figure 2A-C**). The fold changes of TNFα, IL-6 and IL-1β after PHA stimulation were significantly higher in the winter-vaccinated BCG group compared to the spring group. Notably, the fold induction of IL-6 following *E. coli*, *M. tuberculosis* and PHA incubation in the spring group after BCG vaccination was significantly lower compared to its corresponding placebo group (**Figure 2B**). Furthermore, BCG vaccination in the winter led to a higher IL-6 induction upon C. albicans stimulation, but not TNFα and IL-1β. (**Supp. Fig 4A-C**).

Intriguingly, incubation of PBMCs with *S. pneumoniae* resulted in more TNFα and less IL-6 release in individuals vaccinated with BCG during spring.

Spring BCG induces more IFNγ production from T cells compared to winter vaccination

We also measured the T-cell-derived IFNγ production from PBMCs following 7-day incubation with various stimuli. Interestingly, spring BCG-vaccinated individuals produced significantly more IFNγ following incubation with *S. aureus* (Mann-Whitney

p<0.0001) and *C. albicans* (Mann-Whitney p=0.035), compared to the winter group (**Figure 3**). The same pattern was observed after PHA stimulation (**Supp. Fig. 4D**). Conversely, IFNγ release upon the homologous stimulus *M. tuberculosis* was similar in the spring and winter BCG groups and was not significantly higher than their corresponding placebo.

Overall, these data show that BCG vaccination in the winter generally induces more inflammatory cytokines and less type II interferon production against heterologous stimuli compared to BCG vaccination in the spring.

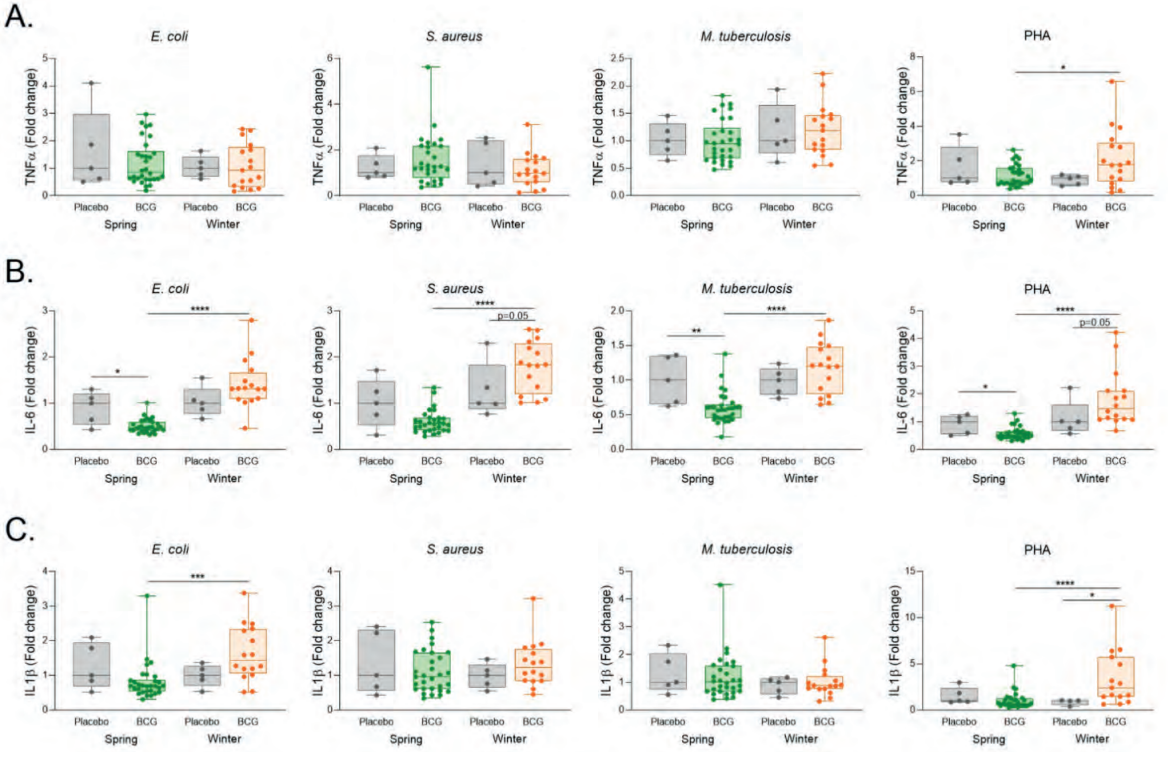

Figure 2. Monocyte-derived cytokines produced by PBMCs

PBMCs isolated before (T1) and 3 months after placebo or BCG vaccination (T2) were stimulated with *E. coli*, *S. aureus*, *M. tuberculosis*, and PHA. **A)** TNFα, **B)** IL-6 and **C)** IL-1β productions from PBMCs were measured from cell-free supernatants following 24-hour incubation, and fold changes (T2/T1) were shown in the graphs. Differences in fold changes between the two groups were analyzed using the Mann-Whitney U test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

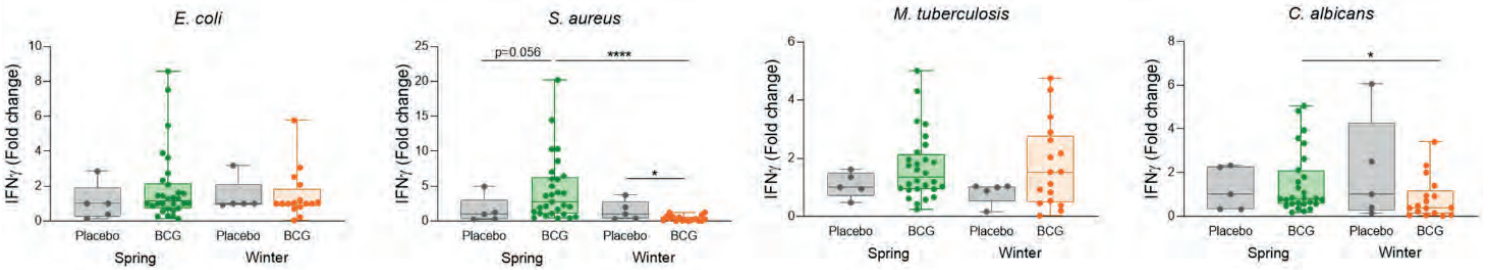

Figure 3. T cell-derived cytokines produced by PBMCs

PBMCs isolated before (T1) and 3 months after placebo or BCG vaccination (T2) were stimulated with *E. coli*, *S. aureus*, *M. tuberculosis*, and *C. albicans*. IFNγ production from PBMCs was measured from cell-free supernatants following 7-day incubation, and fold changes (T2/T1) were shown in the graphs. Differences in fold changes between the two groups were analyzed using the Mann-Whitney U test. *p<0.05, ****p<0.0001.

Winter BCG leads to a higher cytokine production capacity in NK cells

As BCG vaccination also induces trained immunity responses in NK cells [24], we assessed whether the season of vaccination influences the strength of cytokine production in NK cells. We determined the fold changes as a measure of the cytokine production capacity (T2/T1) of NK cells upon BCG or placebo vaccination in the winter and spring groups. In contrast to our observations in the PBMCs, NK cells from individuals receiving BCG in the winter produced more IFNγ after stimulation with *E. coli* and *C. albicans* (**Figure 4A**). Furthermore, stimulation with *E. coli, M. tuberculosis* and *C. albicans* led to significantly higher TNFα fold inductions from NK cells of individuals vaccinated with BCG in the winter (**Figure 4B**). Lastly, a stronger IL-6 induction was observed after winter-BCG vaccination in NK cells incubated with *E. coli* and *S. aureus* (**Figure 4C**). No seasonal difference was observed in NK cell-derived cytokine production capacity following stimulation with Pam3csk4 and PMA-Ionomycin (**Supp. Figure 5**). Our results indicate that BCG vaccination during winter leads to more robust NK cell activation against heterologous stimuli and is superior to spring vaccination.

Spring-vaccinated individuals exhibit more transcriptional changes 3 months after vaccination

Next, we investigated whether the seasonal differences in cytokine production induced by BCG vaccination are modulated via transcriptomic and epigenetic changes. Therefore, we performed RNAseq from monocytes, and ATACseq from monocytes and NK cells from randomly selected 5 winter-vaccinated and 5 spring-vaccinated individuals from our cohort (**Supplementary Table 1**). Initially, we compared the baseline differentially expressed genes (DEGs) of winter- and spring-vaccinated individuals. No significant DEGs were identified at the threshold of FDR=0.05. However, some differences in gene expression were noted at the threshold of nominal p=0.05, including a few immune-related genes. (**Supp. Figure 6A**).

After BCG vaccination in the winter, 66 genes were suggestively upregulated and 35 genes were suggestively downregulated compared to the baseline. (**Figure 5A**). Among the upregulated genes during winter were *AIM2* (cytoplasmic sensor), *IL7* (growth factor for T and B cells) and *CXCL10* (chemokine ligand). On the other hand, 233 upregulated and 83 downregulated genes were identified after spring vaccination (**Figure 5B**). *LY6G5B* (gene in the MHC class III region), and *CLEC4C* (C-type lectin receptor) were two examples of upregulated immune-related genes, while *CXCL9* (chemokine ligand), *GZMH* (granzyme), and *TIGIT* (T cell immunoreceptor) were less expressed in the spring group 3 months after BCG vaccination.

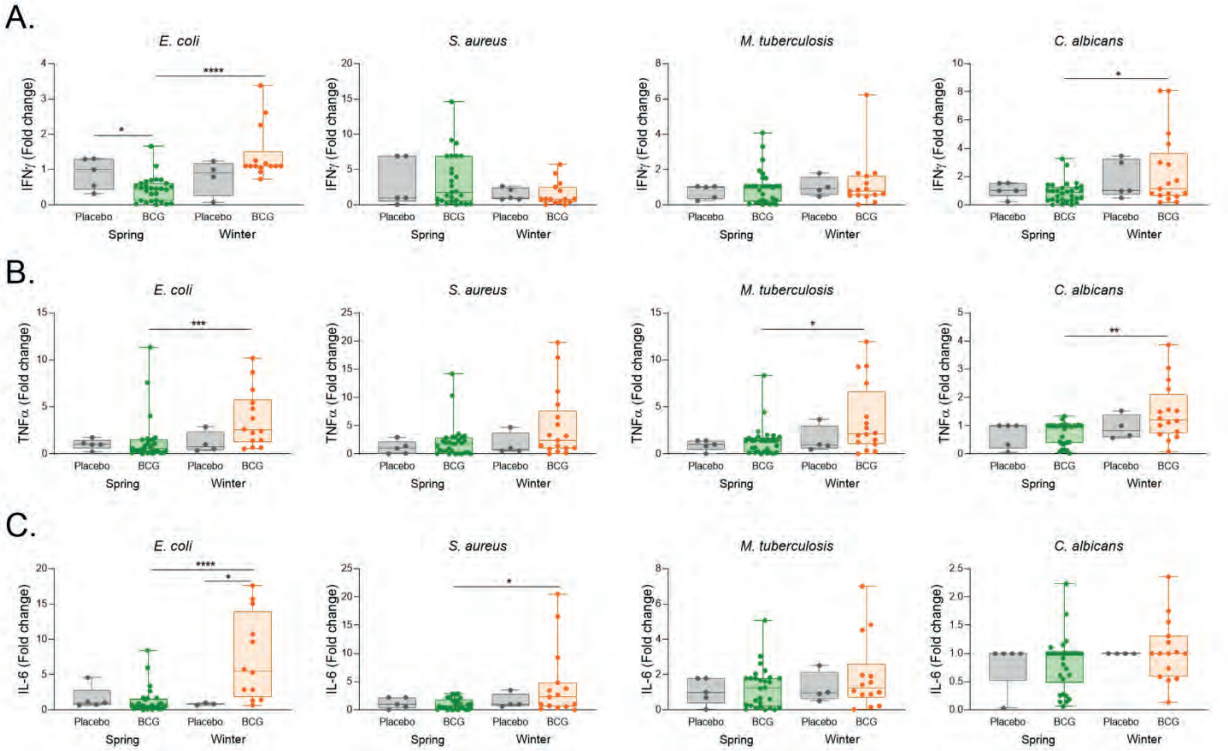

Figure 4. Cytokines produced by NK cells

NK cells purified from PBMCs before (T1) and 3 months after placebo or BCG vaccination (T2) were stimulated with *E. coli*, *S. aureus*, *M. tuberculosis*, and *C. albicans*. **A)** IFNγ, **B)** TNFα, and **C)** IL-6 productions were measured from cell-free supernatants after 48-hour incubation, and fold changes (T2/T1) were shown in the graphs. Differences in fold changes between the two groups were analyzed using the Mann-Whitney U test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Comparison between post-vaccination seasons revealed gender-based differences, with *XIST* significantly upregulated in winter due to more women in the group (**Supp. Figure 6B**). Pathway analysis of 578 suggestively significant DEGs showed that regulation of neutrophil degranulation, activation and neutrophil-mediated immunity pathways and pentose biosynthetic processes were enriched in the spring BCG group, while type I and type II interferon and cytokine production-related pathways were upregulated in the winter group.

Overall, transcriptomic analyses suggested mild baseline differences in the gene expression profiles of the winter and spring groups. Only modest changes in immune-related gene expression were observed following BCG vaccination during winter, however, spring vaccination led to more pronounced transcriptomic alterations even after 3 months. Furthermore, spring-vaccinated subjects exhibited more neutrophil-prominent transcriptomic changes, while winter-vaccinated subjects showed more interferon and cytokine production-related modifications in gene expression based on the top genes.

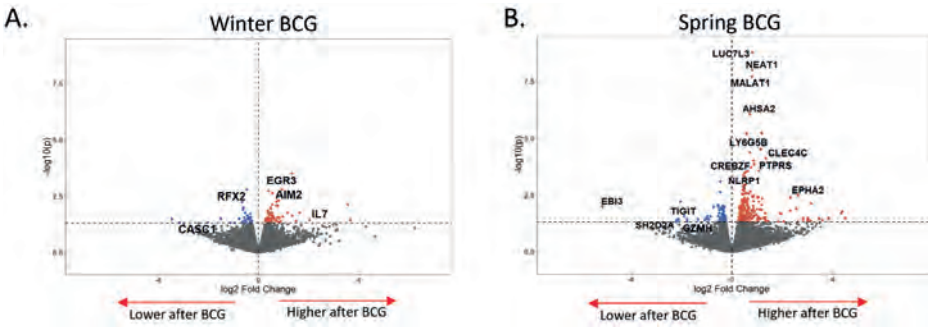

Figure 5. Volcano plots depicting differentially expressed genes 3 months after BCG vaccination in the winter and spring

Gene expressions of monocytes isolated from BCG-vaccinated individuals during **A)** winter and **B)** spring before and 3 months after vaccination. Blue dots show significantly downregulated genes after BCG vaccination, while red dots indicate significantly upregulated genes.

BCG vaccination has a greater effect on NK cell epigenome compared to monocytes

ATACseq was performed on monocytes and NK cells from the same 5 winter and 5 spring vaccinated individuals as in the RNA-seq study to investigate seasonal variations in the epigenetic landscape following BCG vaccination.

Initially, no significant DARs were found between monocytes from winter and spring groups at baseline at an FDR of 0.05. However, nominal differences ($p<0.05$) revealed 273 more accessible and 338 less accessible regions in the winter group, associated with more accessible genes in RNA splicing, and mRNA processing, while spring showed changes in endothelial and chondrocyte differentiation pathways (**Supp. Figure 7A**). BCG vaccination during winter revealed 169 suggestive DARs at the nominal p-value threshold of 0.05, of which 79 were more accessible and 90 were less accessible in monocytes 3 months later (**Figure 6A**). Epithelial cell proliferation and fat cell differentiation pathways were enriched after vaccination, while the response to vitamins and chemokine-mediated signaling pathways were less accessible. In monocytes of the spring group, 194 suggestive DARs were more accessible and 204 suggestive DARs were less accessible for transcription 3 months after BCG (nominal p-value<0.05, **Figure 6B**). Pathways with the suggestively more accessible genes include response to sterols and cholesterol and protein kinase B signaling, while those with the suggestively less accessible genes were negative regulation of phosphate and phosphorus metabolic processes. On the other hand, when BCG vaccination in the winter versus spring was compared in monocytes, we found that 390 regions were suggestively differentially accessible in the spring group, while this number was 657 in the winter (nominal p-value<0.05, **Figure 6C**). Several lipid-related pathways and smooth muscle cell-matrix adhesion were upregulated upon winter BCG, whereas cell cycle-related pathways were enriched after spring BCG.

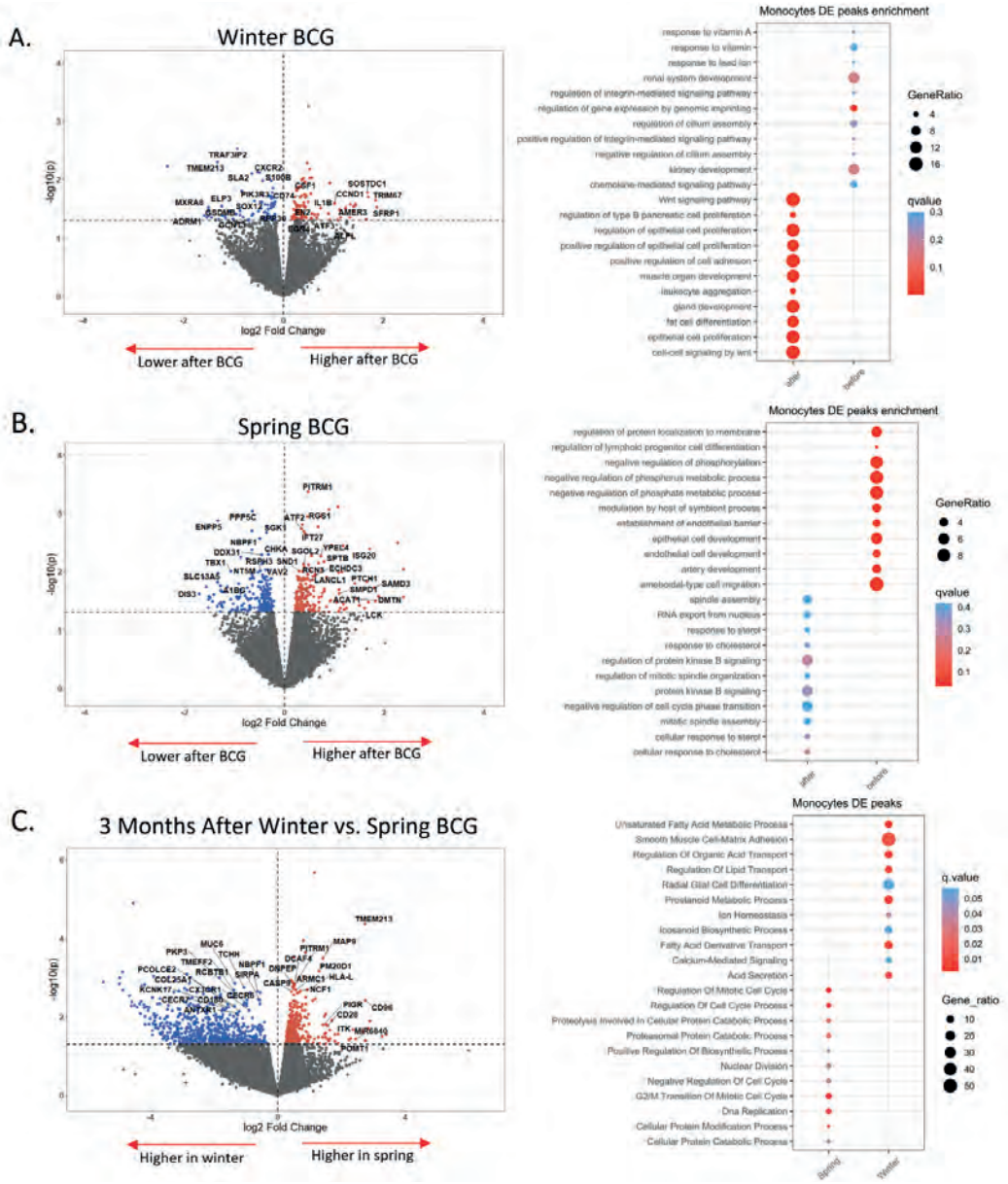

Figure 6. Volcano plots showing differentially accessible genes (on the left panels) and respective pathway enrichment analyses (on the right panels) of purified monocytes upon BCG vaccination in the winter and spring

The left panels depict volcano plots of gene accessibility in monocytes comparing **A)** before and 3 months after winter BCG, **B)** before and 3 months after spring BCG, **C)** 3 months after winter BCG and 3 months after spring BCG. Blue dots show less accessible genes after BCG vaccination (for panels **A** and **B**) and more accessible genes after winter BCG (for panel **C**), while red dots indicate more accessible genes after BCG vaccination (for panels **B** and **C**) and more accessible genes after spring BCG (for panel **C**). GO pathway enrichment analysis (biological pathways) of each volcano are displayed on the right panels.

The season had an even greater effect on NK cell epigenome: at baseline, 1058 suggestive DARs in the spring and 1430 suggestive DARs in the winter were identified (nominal p-value<0.05, **Supp. Figure 7B**). Like the monocytes, genes mediating the RNA splicing and processing pathways were more accessible for transcription during winter. Additionally, NK cell-mediated immunity and activation, and leukocyte-mediated immunity and cytotoxicity pathways were enriched during winter. In spring, genes playing a role in the Wnt signaling pathways were more accessible compared to winter.

BCG vaccination in the winter led to 1002 suggestively more accessible and 1497 suggestively less accessible regions in NK cells (nominal p-value<0.05, **Figure 7A**). Pathway analysis showed that winter vaccination led to the enrichment of genes related to the regulation of chromosome segregation and organization. Among the downregulated pathways were T-cell differentiation, leukocyte-mediated immunity and cytotoxicity, and NK cell activation.

In contrast to winter, BCG vaccination in the spring caused fewer changes in accessibility: 74 suggestive regions were more and 185 suggestive regions were less accessible in NK cells (nominal p-value<0.05, **Figure 7B**). Genes involved in PI3K/MAPK signaling pathways were more open for transcription, whereas genes playing a role in the pathways related to the activation of immune responses, bacterial responses and phagocytosis were less open.

Comparing winter and spring vaccination after 3 months revealed 820 suggestive DARs in total (nominal p-value<0.05, **Figure 7C**). During spring, genes of the pathways related to neuronal development were more accessible, whereas in the winter, several immune regulating pathways linked to IL-1 production, cytokine signaling, cytokine production and neutrophil immunity were more accessible.

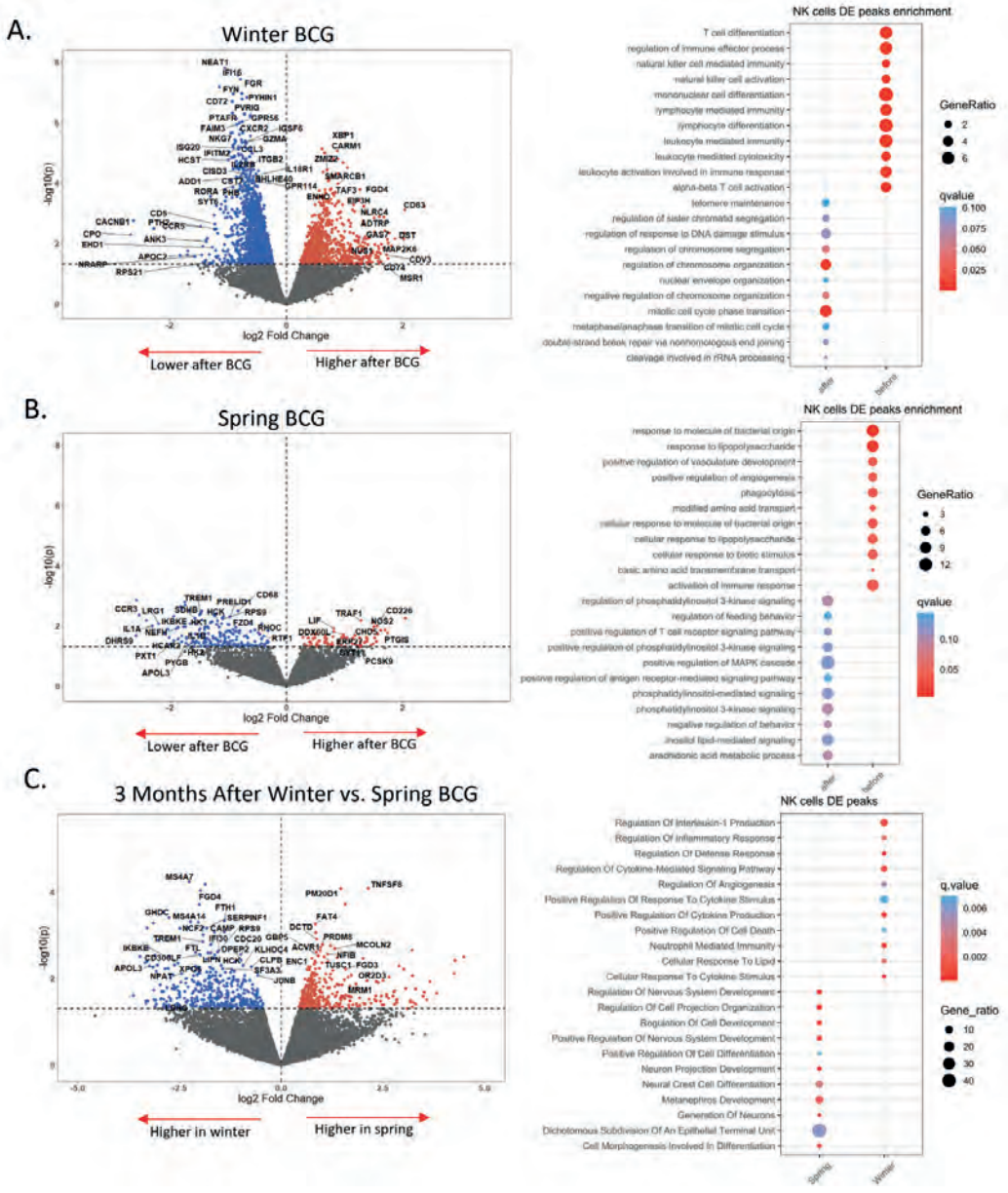

Figure 7. Volcano plots showing differentially accessible genes (on the left panels) and pathway enrichment analyses (on the right panels) of purified NK cells upon BCG vaccination in the winter and spring

The left panels depict volcano plots of gene accessibility in NK cells comparing **A)** before and 3 months after winter BCG, **B)** before and 3 months after spring BCG, **C**) 3 months after winter BCG and 3 months after spring BCG. Blue dots show less accessible genes after BCG vaccination (for panels **A** and **B**) and more accessible genes after winter BCG (for panel **C**), while red dots indicate more accessible genes after BCG vaccination (for panels **B** and **C**) and more accessible genes after spring BCG (for panel **C**). GO pathway enrichment analysis (biological pathways) of each volcano are displayed on the right panels.

Discussion

This study delved into the seasonal variation in trained immunity responses following BCG vaccination through functional, transcriptomic, and epigenetic analyses. We found that peripheral blood immune cell composition, as well as the immune cell activity, was affected by the season. We partly corroborated these findings on a transcriptional and epigenetic level.

Despite similar baseline leukocyte counts between seasons, BCG vaccination showed moderate seasonal effects, particularly on lymphocyte counts. As intra-individual variation in immune cell subsets is limited, changes observed are likely due to external factors such as seasonality and circadian rhythms [25]. For instance, more extended daylight in spring may promote increased lymphocyte egression from lymph nodes [26, 27], contributing to a seasonal pattern in lymphocyte numbers. Additionally, enhanced expansion capacity of T cells, particularly $CD4^+$ T cell subsets, due to higher vitamin D levels during spring and summer, could also explain the rise in lymphocyte counts [28]. It is plausible that BCG vaccination might further influence lymphocyte trafficking, potentially contributing to seasonal regulation of susceptibility to infection. Alternatively, lymphocyte counts may inherently be higher during spring, independent of BCG vaccination. It is worth noting that immune cell frequencies are also affected by sex, age and are moderately heritable [29]. However, our study did not reveal significant differences in baseline cell counts among participants.

Seasonal effects were also observed in immune cell bioactivity. Compared to spring vaccination, PBMCs of winter-vaccinated individuals were more prone to produce monocyte-derived cytokines for various stimuli, especially IL-6, whereas IL-1β and TNFα were affected to a lesser extent. A similar trend was observed in the NK cells. Of note, in most cases, there was no significant difference between the placebo and winter groups, likely due to the low sample size in the placebo. Furthermore, spring vaccination was associated with increased PBMC-derived IFNγ production, possibly linked to the higher lymphocyte numbers post-vaccination in spring. Our results indicate that seasonal immune modulation of trained immunity is important, favoring winter BCG vaccination over spring. Though seasons cannot be directly compared, these results align with observations in Guinean infants, where BCG vaccination during November-January led to lower all-cause neonatal mortality and stronger immune responses to non-specific challenges [18]. Of note, although most BCG-vaccinated individuals produced IFNγ after *M. tuberculosis* stimulation, the difference was not statistically significant compared to placebo. As protection against tuberculosis involves a combination of innate and adaptive immune responses

and the formation of granulomas, the measurement of a single cytokine might not capture the full complexity of the immune response [30].

The molecular basis of trained immunity involves epigenetic and metabolic reprogramming, enhancing chromatin accessibility for gene transcription and protein synthesis upon activation, while gene expression remains relatively unchanged in unstimulated cells [31]. In line with this, we only observed minor effects of vaccination at the transcriptome level, as RNAseq analyses were performed on unstimulated cells. Nevertheless, after heterologous stimulation, winter-vaccinated monocytes showed upregulated pathways related to type I and type II interferon signaling compared to spring-vaccinated monocytes, which may explain the higher cytokine production capacity observed in the winter BCG group. Interferon signaling activates downstream pathways, such as STAT and PI3K, inducing more interferon and cytokine production [32]. It is important to note that the potential effects of season on gene expression, independent of BCG administration, cannot be ruled out, as many genes exhibit seasonal expression patterns [33].

Significant epigenetic modifications were found in monocytes, where winter vaccination led to increased chromatin accessibility in lipid transport and metabolism pathways, crucial for trained immunity. Lipid metabolism supports trained immunity induction, as mevalonate accumulation [34] and activation of LXR, a regulator of cholesterol and fatty acid homeostasis [35], are important for trained immunity responses in monocytes. Therefore, BCG-induced modulation of lipid-related pathways could explain the higher monocyte-derived cytokine production observed in winter.

An intriguing finding of the study is the more extensive epigenomic differences in NK cells compared to monocytes after vaccination, especially in winter. Spring BCG vaccination led to lower accessibility of genes in pathways related to responses against bacterial components and phagocytosis compared to before BCG, supporting the findings of lower reactivity of NK cells after spring vaccination. Additionally, NK cells isolated three months after winter BCG had more accessible genes related to cytokine production, lipid metabolism, and defense responses than NK cells three months after spring BCG. Although these results should be carefully interpreted as these differences combine season and vaccination, they suggest that NK cells are more active following winter vaccination. Overall, we found more prominent changes in the epigenome of NK cells compared to monocytes upon BCG vaccination, especially in winter. Given the crucial role of NK cells in the induction of trained immunity [24], these data suggest a possible role of season in influencing NK cell reactivity, leading to stronger cytokine induction and trained immunity.

Seasonality significantly influences immune responses, potentially due to factors such as ultraviolet radiation (UVR), vitamin D levels, humidity, circulating microorganisms, and annual vaccinations. Low-dose UV radiation seems to increase innate and adaptive cell activation, whereas higher doses have immunosuppressive effects [36]. An Israeli study found that children vaccinated against rubella in winter had a stronger antibody response than those vaccinated in summer, suggesting UVR levels during vaccination could affect immunogenicity. In the Netherlands, where this study was conducted, low UVR during winter [37] could have promoted the beneficial effects of BCG on the immune system (i.e., increased cytokine production capacity). UVR, however, would likely not explain the observations in Guinean babies, since UVR is still relatively high from November-January, and lower during the rainy season from June-October.

Vitamin D is another factor related to both seasonality and UVR. It is well known that vitamin D influences immune cell function *in vivo* and *in vitro*, however, there are conflicting reports on the effects of vitamin D deficiency and supplementation in disease susceptibility and protection [38]. Although we did not measure circulating vitamin D concentrations in our study participants, they likely differ between the winter and spring months, impacting trained immunity responses.

Humans are exposed to microbes in all seasons, however, some infections peak in specific seasons. Infections could also affect the baseline immune parameters of the host, influencing the vaccine responses [39]. Our findings of winter BCG vaccination upregulating pathways related to interferon and cytokine production might benefit the immune system's defense against circulating microbes during winter. Furthermore, annual and seasonal vaccines such as influenza, and COVID-19 could have long-term immunomodulatory effects. For example, we have shown that a quadrivalent inactivated influenza vaccine induces trained immunity [40]. Furthermore, BCG vaccination 14 days before influenza vaccine administration enhanced immune responses against certain pathogens, but impaired responses to others [41]. Although we do not have the influenza vaccination status of our study participants, it is plausible that the annual influenza vaccination in the fall primes the innate immune system and boosts the trained immunity response induced by BCG during winter.

While our study suggests that the seasonal timing of BCG vaccination affects trained immunity, it has some limitations. The placebo group consists of only five individuals who received a placebo both in winter and spring. This limits the power of comparison with the corresponding BCG groups. As this study was not originally designed to assess the seasonality of trained immunity, the winter and spring vaccination groups were not equal in sample size. A future study with more individuals in placebo and BCG groups

would be needed to confirm the results of this research. Furthermore, winter vaccinated subjects had their blood collected for RNAseq after 3 months, when it was already spring. Lastly, the impact of seasonal vaccination on infection protection remains to be explored, despite evidence from Guinean infants indicating stronger mortality reduction and cytokine responses after vaccination between November and January [18]. A larger and longitudinal study assessing the rate and severity of infections in people vaccinated with BCG in winter and spring would be necessary to investigate this.

Overall, our findings highlight the complex interplay between seasonality and trained immunity, suggesting that the timing of BCG vaccination can modulate immune responses. Winter BCG vaccination is associated with increased innate immune cell responsiveness with the potential role of epigenetic modifications, with monocytes and NK cells exhibiting season-specific epigenetic signatures. The findings of this study add to the growing body of knowledge on the regulation of trained immunity. Trained immunity is not solely a static phenomenon but appears to be modulated by seasonal cues, adding another layer of complexity to the understanding of immune memory and responses. This research opens new avenues for further investigation into the mechanisms underlying trained immunity and how they can be leveraged to improve vaccination outcomes.

Author contributions

All authors attest that they meet the ICMJE criteria for authorship. GK, PAD, LCJdB, JDA, and MGN conceptualized the study. GK, PAD, MPB, LAL, HD, HL, and JHAM performed the experiments and curated the data. Data analysis was performed by GK, PAD, AA, YL, and CX. Project administration and oversight were done by PAD and MGN. GK and PAD prepared the original draft. All authors read, edited and accepted the manuscript.

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Data sharing statement

Upon reasonable request, the data supporting the findings of this study are available from the corresponding author (MGN).

Declaration of interests

MGN is a scientific founder of TTxD, Lemba and Biotrip. All other authors declare that they have no competing interests.

References

1. Liston A, Carr EJ, and Linterman MA. Shaping Variation in the Human Immune System. Trends Immunol. 2016;37(10):637-46.
2. Ter Horst R, Jaeger M, Smeekens SP, Oosting M, Swertz MA, Li Y, et al. Host and Environmental Factors Influencing Individual Human Cytokine Responses. Cell. 2016;167(4):1111-24 e13.
3. Frankel M, Beko G, Timm M, Gustavsen S, Hansen EW, and Madsen AM. Seasonal variations of indoor microbial exposures and their relation to temperature, relative humidity, and air exchange rate. Appl Environ Microbiol. 2012;78(23):8289-97.
4. Fisman DN. Seasonality of infectious diseases. Annu Rev Public Health. 2007;28:127-43.
5. Watad A, Azrielant S, Bragazzi NL, Sharif K, David P, Katz I, et al. Seasonality and autoimmune diseases: The contribution of the four seasons to the mosaic of autoimmunity. J Autoimmun. 2017;82:13-30.
6. Iikuni N, Nakajima A, Inoue E, Tanaka E, Okamoto H, Hara M, et al. What's in season for rheumatoid arthritis patients? Seasonal fluctuations in disease activity. Rheumatology (Oxford). 2007;46(5):846-8.
7. Moltchanova EV, Schreier N, Lammi N, and Karvonen M. Seasonal variation of diagnosis of Type 1 diabetes mellitus in children worldwide. Diabet Med. 2009;26(7):673-8.
8. Paynter S, Ware RS, Sly PD, Williams G, and Weinstein P. Seasonal immune modulation in humans: observed patterns and potential environmental drivers. J Infect. 2015;70(1):1-10.
9. Tedijanto C, Hermans S, Cobelens F, Wood R, and Andrews JR. Drivers of Seasonal Variation in Tuberculosis Incidence: Insights from a Systematic Review and Mathematical Model. Epidemiology. 2018;29(6):857-66.
10. Linder N, Abudi Y, Abdalla W, Badir M, Amitai Y, Samuels J, et al. Effect of season of inoculation on immune response to rubella vaccine in children. J Trop Pediatr. 2011;57(4):299-302.
11. Shadrin AS, Marinich IG, and Taros LY. Experimental and epidemiological estimation of seasonal and climato-geographical features of non-specific resistance of the organism to influenza. J Hyg Epidemiol Microbiol Immunol. 1977;21(2):155-61.
12. Zykov MP, and Sosunov AV. Vaccination activity of live influenza vaccine in different seasons of the year. J Hyg Epidemiol Microbiol Immunol. 1987;31(4):453-9.
13. Zimmermann P, and Curtis N. Factors That Influence the Immune Response to Vaccination. Clin Microbiol Rev. 2019;32(2).
14. 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.
15. Li W, Moorlag S, Koeken V, Roring RJ, de Bree LCJ, Mourits VP, et al. A single-cell view on host immune transcriptional response to in vivo BCG-induced trained immunity. Cell Rep. 2023;42(5):112487.
16. Bekkering S, Dominguez-Andres J, Joosten LAB, Riksen NP, and Netea MG. Trained Immunity: Reprogramming Innate Immunity in Health and Disease. Annu Rev Immunol. 2021;39:667-93.
17. Benn CS, Fisker AB, Rieckmann A, Sorup S, and Aaby P. Vaccinology: time to change the paradigm? Lancet Infect Dis. 2020;20(10):e274-e83.
18. Jensen KJ, Biering-Sorensen S, Ursing J, Kofoed PL, Aaby P, and Benn CS. Seasonal variation in the non-specific effects of BCG vaccination on neonatal mortality: three randomised controlled trials in Guinea-Bissau. BMJ Glob Health. 2020;5(3):e001873.

19. Berendsen ML, van Gijzel SW, Smits J, de Mast Q, Aaby P, Benn CS, et al. BCG vaccination is associated with reduced malaria prevalence in children under the age of 5 years in sub-Saharan Africa. BMJ Glob Health. 2019;4(6):e001862.

20. Moorlag S, 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.

21. 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.

22. Love MI, Huber W, and Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.

23. Kamburov A, Pentchev K, Galicka H, Wierling C, Lehrach H, and Herwig R. ConsensusPathDB: toward a more complete picture of cell biology. Nucleic Acids Res. 2011;39(Database issue):D712-7.

24. 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.

25. Carr EJ, Dooley J, Garcia-Perez JE, Lagou V, Lee JC, Wouters C, et al. The cellular composition of the human immune system is shaped by age and cohabitation. Nat Immunol. 2016;17(4):461-8.

26. Druzd D, Matveeva O, Ince L, Harrison U, He W, Schmal C, et al. Lymphocyte Circadian Clocks Control Lymph Node Trafficking and Adaptive Immune Responses. Immunity. 2017;46(1):120-32.

27. Wyse C, O'Malley G, Coogan AN, McConkey S, and Smith DJ. Seasonal and daytime variation in multiple immune parameters in humans: Evidence from 329,261 participants of the UK Biobank cohort. iScience. 2021;24(4):102255.

28. Khoo AL, Koenen HJ, Chai LY, Sweep FC, Netea MG, van der Ven AJ, et al. Seasonal variation in vitamin D(3) levels is paralleled by changes in the peripheral blood human T cell compartment. PLoS One. 2012;7(1):e29250.

29. Orru V, Steri M, Sole G, Sidore C, Virdis F, Dei M, et al. Genetic variants regulating immune cell levels in health and disease. Cell. 2013;155(1):242-56.

30. de Martino M, Lodi L, Galli L, and Chiappini E. Immune Response to Mycobacterium tuberculosis: A Narrative Review. Front Pediatr. 2019;7:350.

31. Zhang B, Moorlag SJ, Dominguez-Andres J, Bulut O, Kilic G, Liu Z, et al. Single-cell RNA sequencing reveals induction of distinct trained-immunity programs in human monocytes. J Clin Invest. 2022;132(7).

32. McNab F, Mayer-Barber K, Sher A, Wack A, and O'Garra A. Type I interferons in infectious disease. Nat Rev Immunol. 2015;15(2):87-103.

33. Dopico XC, Evangelou M, Ferreira RC, Guo H, Pekalski ML, Smyth DJ, et al. Widespread seasonal gene expression reveals annual differences in human immunity and physiology. Nat Commun. 2015;6:7000.

34. Bekkering S, Arts RJW, Novakovic B, Kourtzelis I, van der Heijden C, Li Y, et al. Metabolic Induction of Trained Immunity through the Mevalonate Pathway. Cell. 2018;172(1-2):135-46 e9.

35. Sohrabi Y, Sonntag GVH, Braun LC, Lagache SMM, Liebmann M, Klotz L, et al. LXR Activation Induces a Pro-inflammatory Trained Innate Immunity-Phenotype in Human Monocytes. Front Immunol. 2020;11:353.

36. Cui J, Yang G, Pan Z, Zhao Y, Liang X, Li W, et al. Hormetic Response to Low-Dose Radiation: Focus on the Immune System and Its Clinical Implications. Int J Mol Sci. 2017;18(2).

37. den Outer PN, Slaper, H., Tax, R. B. UV radiation in the Netherlands: Assessing long-term variability and trends in relation to ozone and clouds. JGR Atmospheres. 2005;110(D2).

38. Martens PJ, Gysemans C, Verstuyf A, and Mathieu AC. Vitamin D's Effect on Immune Function. Nutrients. 2020;12(5).

39. Sparks R, Lau WW, Liu C, Han KL, Vrindten KL, Sun G, et al. Influenza vaccination reveals sex dimorphic imprints of prior mild COVID-19. Nature. 2023;614(7949):752-61.

40. Debisarun PA, Gossling KL, Bulut O, Kilic G, Zoodsma M, Liu Z, et al. Induction of trained immunity by influenza vaccination - impact on COVID-19. PLoS Pathog. 2021;17(10):e1009928.

41. Leentjens J, Kox M, Stokman R, Gerretsen J, Diavatopoulos DA, van Crevel R, et al. BCG Vaccination Enhances the Immunogenicity of Subsequent Influenza Vaccination in Healthy Volunteers: A Randomized, Placebo-Controlled Pilot Study. J Infect Dis. 2015;212(12):1930-8.

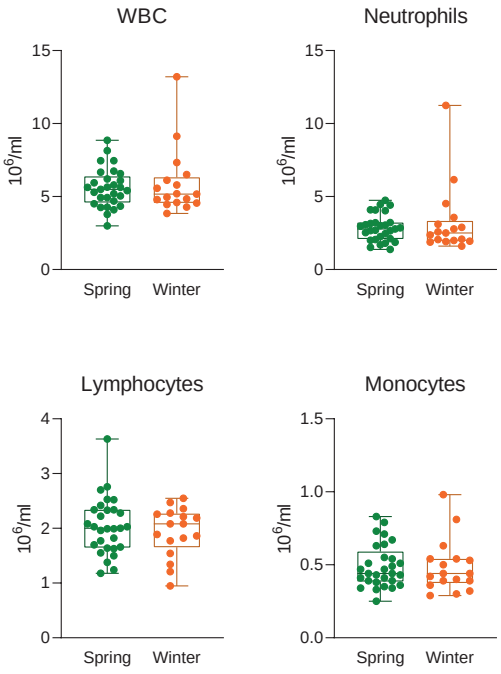
Supplementary Information

Supplementary Table

Supplementary Table 1. Demographics of the study participants who were selected for RNA and ATAC sequencing. The Fisher's exact test was used to compare the demographics between the groups

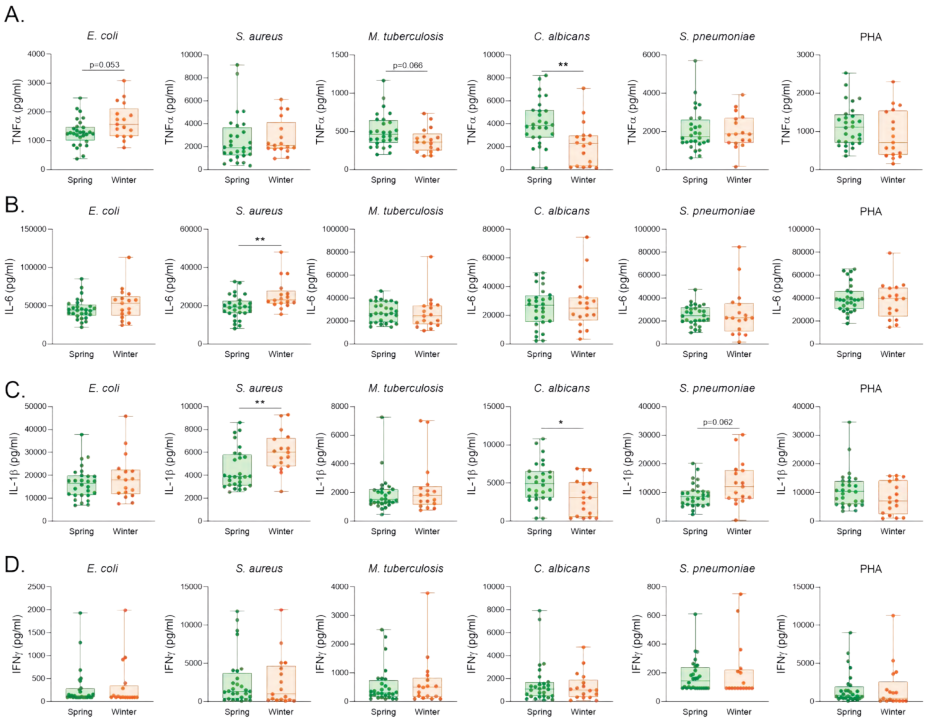
	Winter BCG (n=5)	Spring BCG (n=5)	p-value
Age	22.2±2.3	21.6±2.1	0.71
Sex (F/M)	4/1	2/3	0.52
BMI	23.5±3.9	24.5±3.3	0.41

Supplementary Figures



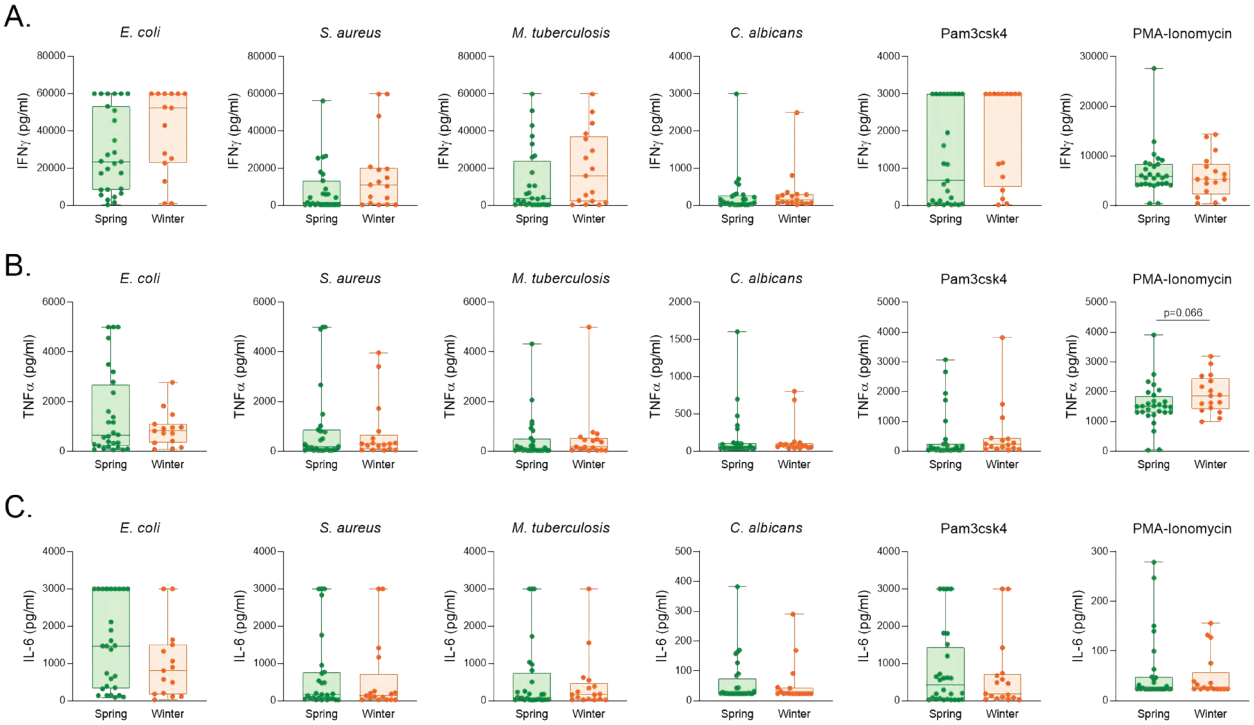
Supplementary Figure 1. Baseline immune cell counts

Before vaccination, white blood cells, neutrophils, lymphocytes and monocytes were counted from whole blood using a hematology analyzer.



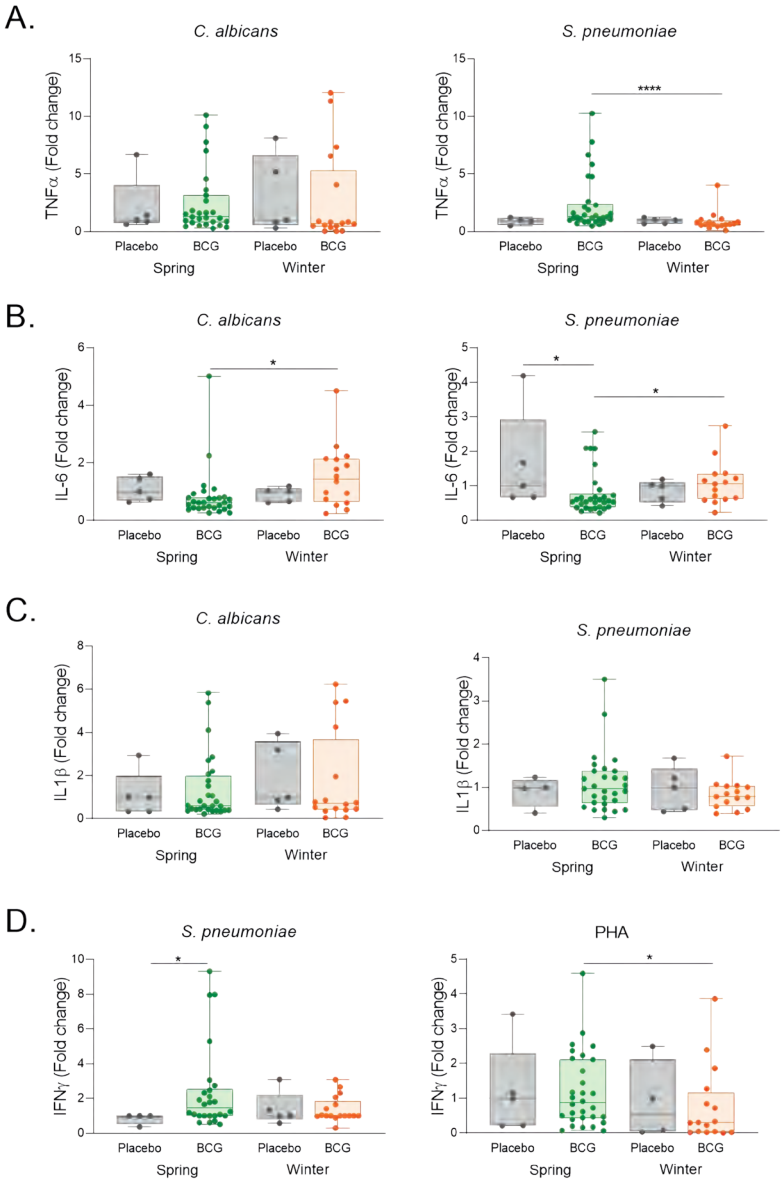
Supplementary Figure 2. Cytokine productions of PBMCs before BCG vaccination

PBMCs isolated before BCG vaccination were stimulated with *E. coli*, *S. aureus*, *M. tuberculosis*, *C. albicans*, *S. pneumoniae*, and PHA. **A)** TNFα, **B)** IL-6, **C)** IL-1β levels were measured from the cell-free supernatants following 24-hour incubation while **D)** IFNγ production was measured after 7-day incubation with the stimuli. Differences between two seasons were analyzed using the Mann-Whitney U test. *p<0.05, **p<0.01.



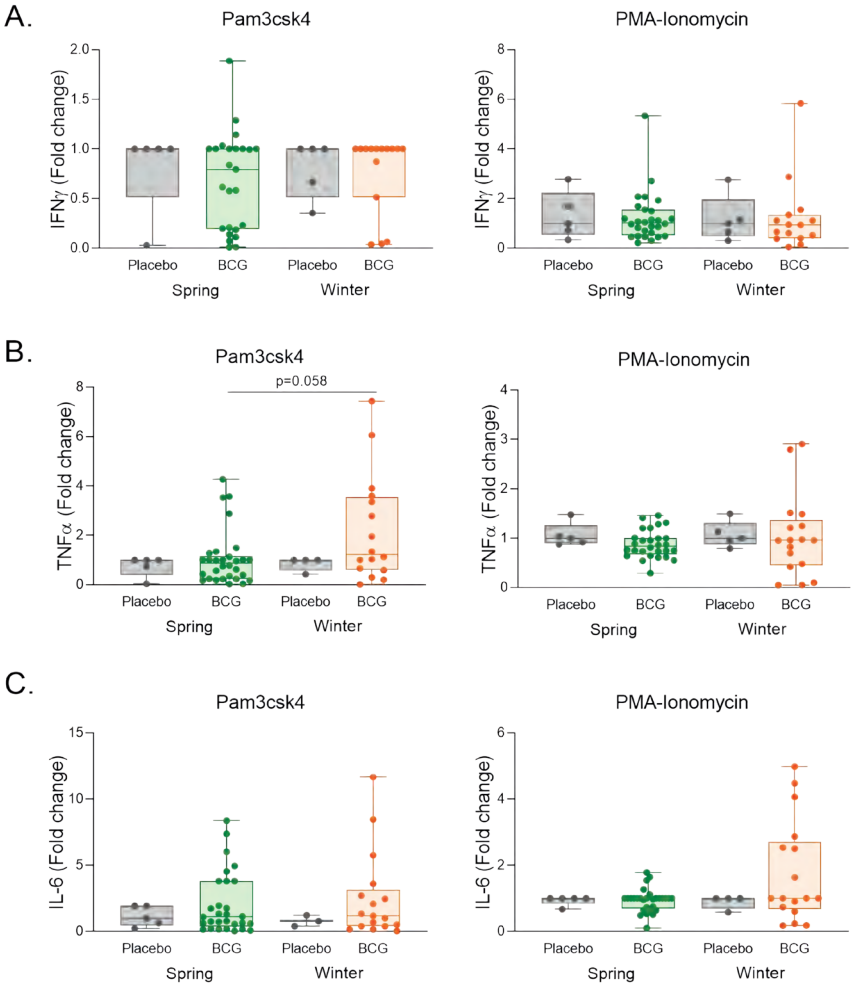
Supplementary Figure 3. Cytokine productions of NK cells before BCG vaccination

Purified NK cells before BCG vaccination were stimulated with *E. coli*, *S. aureus*, *M. tuberculosis*, *C. albicans*, Pam3csk4 and PMA-Ionomycin. **A)** IFNγ, **B)** TNFα, **C)** IL-6 productions were measured from the cell-free supernatants following 48-hour incubation. Differences between two seasons were analyzed using the Mann-Whitney U test.



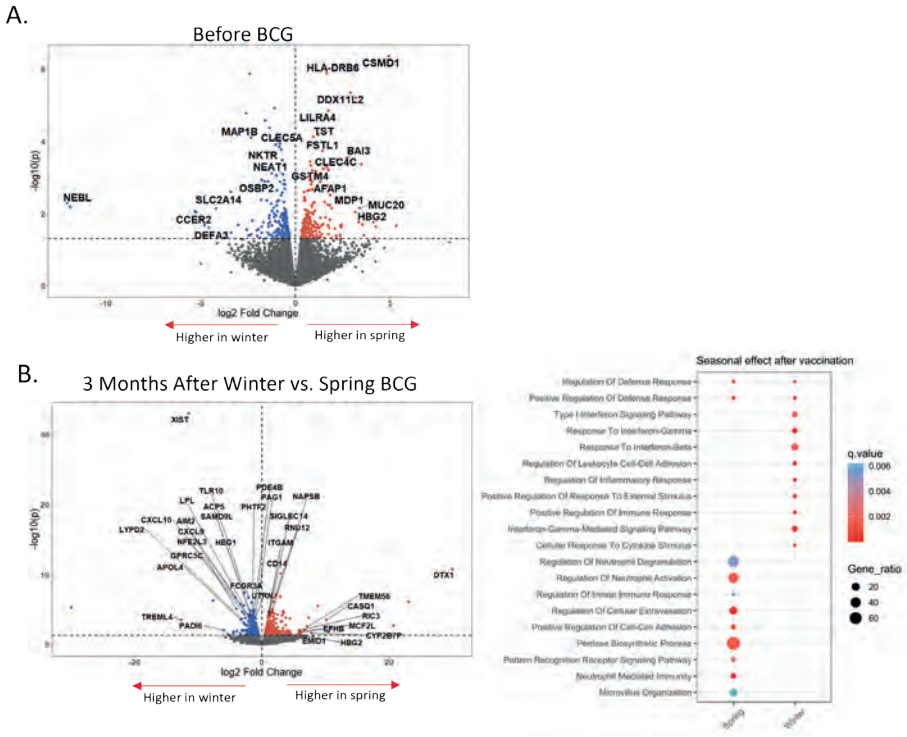
Supplementary Figure 4. Cytokines produced by PBMCs after stimulation with *C. albicans* and *S. pneumoniae*

PBMCs isolated before (T1) and 3 months after placebo or BCG vaccination (T2) were stimulated with *C. albicans* and *S. pneumoniae*. **A)** TNFα, **B)** IL-6, **C)** IL-1β levels were measured following 24-hour incubation while **D)** IFNγ production was measured after 7-day incubation with the stimuli. Fold changes (T2/T1) were shown in the graphs. Differences in fold changes between two groups were analyzed using the Mann-Whitney U test. *p<0.05, ***p<0.001.



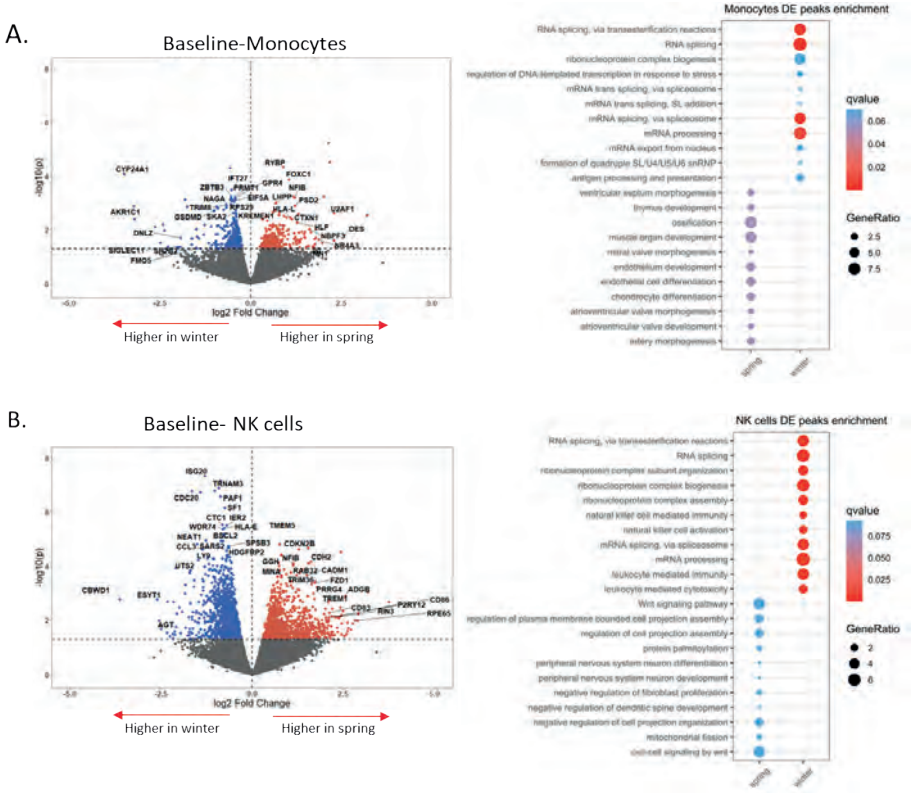
Supplementary Figure 5. Cytokines produced by NK cells after stimulation with Pam3csk4 and PMA-Ionomycin

NK cells purified from PBMCs before (T1) and 3 months after placebo or BCG vaccination (T2) were stimulated with Pam3csk4 and PMA-Ionomycin for 48 hours. **A)** IFNγ, **B)** TNFα, **C)** IL-6 productions were measured from the cell-free supernatants. Fold changes (T2/T1) were shown in the graphs. Differences in fold changes between two groups were analyzed using the Mann-Whitney U test.



Supplementary Figure 6. Baseline gene expression differences in monocytes of the spring and winter groups and comparison of gene expression profiles of the winter and spring BCG vaccination 3 months later

Left panels show volcano plots comparing **A)** baseline gene expressions in the winter and spring and **B)** gene expressions 3 months after winter with 3 months after spring BCG vaccination. Blue dots show upregulated genes in the winter group, while red dots show upregulated genes in the spring group. GO enrichment analysis (biological pathways) of the volcano on the panel B is given on the right panel.



Supplementary Figure 7. Baseline gene accessibility differences of the spring and winter groups in A) monocytes and B) NK cells on the left panel, and their respective pathway analyses on the right

Blue dots show significantly more accessible genes in the winter group, while red dots show significantly more accessible genes in the spring group. GO enrichment analysis (biological pathways) of the volcanos are given on the right panel.

Eurasian jay
Bayağı alakarga
Vlaamse gaai

CHAPTER 6

Multi-omics integration reveals only minor long-term molecular and functional sequelae in immune cells of individuals recovered from COVID-19

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Abstract

The majority of COVID-19 patients experience mild to moderate disease course and recover within a few weeks. An increasing number of studies characterized the long-term changes in the specific anti-SARS-CoV-2 immune responses, but how COVID-19 shapes the innate and heterologous adaptive immune system after recovery is less well known. To comprehensively investigate the post-SARS-CoV-2 infection sequelae on the immune system, we performed a multi-omics study by integrating single-cell RNA-sequencing, single-cell ATAC-sequencing, genome-wide DNA methylation profiling, and functional validation experiments in 14 convalescent COVID-19 and 15 healthy individuals. We showed that immune responses generally recover without major sequelae after COVID-19. However, subtle differences persist at the transcriptomic level in monocytes, with downregulation of the interferon pathway, while DNA methylation also displays minor changes in convalescent COVID-19 individuals. However, these differences did not affect the cytokine production capacity of PBMCs upon different bacterial, viral, and fungal stimuli, although baseline release of IL-1Ra and IFN-γ was higher in convalescent individuals. In conclusion, we propose that despite minor differences in epigenetic and transcriptional programs, the immune system of convalescent COVID-19 patients largely recovers to the homeostatic level of healthy individuals.

Introduction

Coronavirus disease 2019 (COVID-19), caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in a considerable global morbidity and mortality [1]. The majority of infected individuals experience mild to moderate symptoms and recover within several weeks after infection [2]. Earlier studies have reported that lymphopenia and a higher level of T cell exhaustion serve as the hallmarks of severe infection [3, 4]. The release of inflammatory cytokines, e.g., interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, and IL-8, tend to rise during this viral infection, determining severe inflammatory complications [5, 6]. Elevated neutrophil count with higher neutrophil-to-lymphocyte ratio (NLR) as well as reduced monocyte, basophil, and eosinophil counts were also reported in peripheral blood from COVID-19 patients, with NLR being used as an early warning marker for COVID-19 severity [7].

Changes at the transcriptomic level were also observed in immune cells isolated from COVID-19 patients, with the response to interferon (IFN)-α and inflammatory pathways being significantly upregulated [8]. On the other hand, during severe COVID-19 infection, patients experienced reduced IFN-α and IFN-β production and activity, which leads to a high viral load and imbalanced inflammatory response [9]. Moreover, lower expression of HLA-DR molecules was observed on monocytes and dendritic cells from COVID-19 infected patients, a sign of immune paralysis [10–12].

SARS-CoV-2 also hijacks the host epigenetic processes and may subsequently alter the transcriptome to evade the host immune defense. Recent studies reported hypermethylation in the gene regions related to IFN response and significant hypomethylation in the gene regions related to inflammation and cytokine production in severe COVID-19 cases, resulting in the shutoff of antiviral IFN response, uncontrolled inflammation, and cytokine storm [13, 14]. One recent study found that the DNA methylation signatures from 44 5'-C-phosphate-G-3'(CpG) sites could predict COVID-19 disease severity [15].

While much has been done to understand the activation of immune responses during SARS-CoV-2 infection, much less is known about the long-term immunological sequelae of these processes. On the one hand, COVID-19 induces a robust adaptive immune memory response, which is characterized by an increased number of effector and memory T cells and antibody-producing plasma cells [16]. Whether the immunological effects of COVID-19 extend beyond adaptive immune memory and also incorporate changes in innate and heterologous adaptive immunity is not known. Multi-omics integration can provide a deeper understanding of the immune system [17].

We, therefore, investigated whether COVID-19 continues to affect the immune system's functioning after recovery. To this end, we integrated multi-omics studies and functional assays to explore if SARS-CoV-2 infection induces any persistent changes at the transcriptome, epigenome, or chromosome accessibility level in convalescent COVID-19 individuals.

Materials and methods

Study Subjects

The ethical approval for the study was obtained from CMO Arnhem-Nijmegen (NL32 357.091.10). All participants gave written consent for their participation in the study. In total, 14 convalescent COVID-19 patients and 15 sex-matched healthy donors (controls) from Radboudumc, the Netherlands, participated in this study. Convalescent patients were included in the study based on self-reported symptoms and subsequent confirmation of the presence of IgA, IgG, and IgM antibodies against the SARS-CoV-2 Spike protein in the circulation. All controls were at healthy status during sampling and did not experience COVID-19 based on antibody level (**Figure S1**). At the time of inclusion, patients had no reported COVID-19 symptoms or any other infections. Inclusions took place between April and June of 2020, during the first wave of the pandemic in the Netherlands. The demographic and clinical characteristics of the participants are provided in **Table 1.**

Table 1. General characteristics of the study population

	Convalescence (n=14)		**Control (n=15)**	**P value**
Age/years (mean±sd)	60 ± 11		50 ± 14	0.054
Gender (# M / # F)	7 / 7		7 / 8	1
BMI (kg/m2)	22 ± 2.53		22 ± 1.85	0.97
Symptom (# of having symptom / # all individuals)	Fever and headache	12 / 14		
	sleepy and tired	7 / 14		
	no taste	6 / 14		
	no smell	5 / 14		
	cough	4 / 14		
	pain	4 / 14		
	weight loss	2 / 14		
	nausea	1 / 14		
	no appetite	1 / 14		

Isolation and Cryopreservation of Peripheral Blood Mononuclear Cells (PBMCs)

Whole blood was diluted with phosphate-buffered saline (PBS), and PBMC isolation was performed by density gradient centrifugation using Ficoll-Paque (GE Healthcare, IL, USA). The PBMC fraction was collected and washed three times with PBS. The cells were resuspended in RPMI 1640 Medium (Dutch modification) (Thermo Fisher Scientific, MA, USA) supplemented with 1 mM sodium pyruvate (Thermo Fisher Scientific), 2 mM GlutaMAX supplement (Thermo Fisher Scientific), and 5 µg/mL gentamicin (Centraform, Netherlands). For DNA methylation, single cell RNA sequencing (scRNA-seq), and single cell ATAC sequencing (scATAC-seq) analysis, and future stimulation assays, cells were cryopreserved in RPMI containing 40% bovine calf serum (Cytiva, MA, USA) and 15% dimethyl sulfoxide (VWR, PA, USA).

Flow Cytometry From Whole Blood

After erythrocyte lysis in isotonic NH4CL buffer and washing twice with PBS, total leukocytes were obtained. White blood cell counts were determined by a cell counter (Coulter Ac-T Diff® cell counter; Beckman Coulter, CA, USA) and used to calculate the absolute numbers of $CD45^+$ leukocytes identified by flow cytometry as previously described in detail [18]. Briefly, half a million of total leukocytes per staining panel were used to analyze surface markers with the Navios™ flow cytometer (Beckman Coulter). Cells were transferred to a V-bottom 96-well plate and washed twice with PBS containing 0.2% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, USA), stained for 20 minutes at room temperature in the dark with the staining panels of interest and washed twice with PBS containing 0.2% BSA. The conjugated antibodies specific for human cells are given in **Table S1**. The gating strategy used in flow cytometry analyses was provided in **Figure S2**. Data analysis was performed using the Kaluza 2.1® software (Beckman Coulter).

scRNA Sequencing

Frozen cells were rapidly thawed at 37 °C and transferred into 50 mL centrifuge tubes. Subsequently, cells were counted using an automated cell counter (Thermo Fisher Scientific). An equal number of cells (3,300 per individual) from 4 different individuals were pooled together and loaded into a 10X Chromium Controller to generate Gel Beads-in-emulsion (GEMs). scRNA-seq libraries were generated according to the manufacturer's instructions (Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) User Guide, Rev A, CG000315 Rev A). In brief, all the loading cells were separated into nanoliter-scale droplets. Within each droplet, cDNA was generated via reverse transcription, adding a cell barcoding sequence and unique molecular identifier (UMI) to each cDNA molecule. Library quality per pool

was examined using the Agilent Bioanalyzer High Sensitivity DNA kit. Sequencing was carried out on NovaSeq 6000, having a 50,000 reads depth per cell. DNA was isolated from PBMCs and then subjected to genotyping by Illumina Global Screening Array Beadchip to demultiplex the pooled samples.

scRNA Sequencing Data Analysis

The Cellranger (v.4.0.0) utility of 10X genomics was used to process the scRNA-seq data. The standard protocol of the Cellranger pipeline consists of mapping sequenced reads and measuring gene expression [19]. The human reference genome (GRCh38) was used for mapping sequence reads. Later, demultiplexing of individuals mixed in the same pool was done by using the genotype-free clustering method souporcell [20]. Further downstream analyses were performed using the Seurat package V4 [21] in R following its default pipelines, i.e., quality filtering, data normalization, and scaling. During quality filtering, mitochondrial and ribosomal genes along with cells having mitochondrial reads > 25% and expressed genes > 5000 or < 250 were removed. Data normalization was done using a global-scaling normalization method, namely "LogNormalize". Subsequently, data was scaled prior to dimensionality reduction through principal component analysis (PCA) using the top 2,000 variable features detected using the vst method. Uniform Manifold Approximation and Projection (UMAP) technique was utilized for cell clustering. The "FindAllMarkers" function was used for identifying marker genes for each cluster. Using publicly available information, each cluster was annotated based on top marker genes (having the lowest adjusted p-value and positive logFC). Differentially expressed genes (DEGs) between convalescent COVID-19 patients and controls were detected through the "FindMarkers" function using the MAST test [22] adjusted by age and sex. Genes that were expressed in at least 10% cells and with adjusted P-value (after Bonferroni post-hoc correction) < 0.05 were considered significant. Gene Ontology (GO) enrichment analysis of significant DEGs was done using the "clusterProfiler" package in R [23]. Later, for cross-validation, a matrix containing the total gene count per sample was generated from Seurat by summing up all the read counts per gene, and the pseudo-bulk RNA analysis was performed through DESeq2 [24].

scATAC Sequencing

Cryopreserved PBMCs were thawed at 37°C and transferred into 50 mL centrifuge tubes. PBMCs were washed in PBS through centrifugation at 400G for 5 minutes at 4 °C and lysed for 3 minutes on ice. After discarding the supernatant, lysed cells were diluted within 1× diluted nuclei buffer (10X Genomics) prior to counting with trypan blue using a Countess II FL Automated Cell Counter to validate lysis. An equal number of nuclei (approximately 3000 nuclei per sample) from four individuals were pooled

together and then loaded into the Chromium Next GEM Chip H based on the user guides from 10X genomics (Chromium Next GEM Single Cell ATAC Reagent Kits v1.1 User Guide, CG000209 Rev D). After breaking the emulsion, the barcoded tagmented DNA was purified and amplified for sample indexing and generation of scATAC-seq libraries. The final libraries were quantified using the Agilent Bioanalyzer High Sensitivity DNA kit. Sequencing was performed on NovaSeq 6000 with a depth of 25,000 reads per nuclei.

scATAC Sequencing Data Analysis

Using the cellranger-atac mkfastq (10X Genomics, v.1.2.0) utility, raw sequencing data were converted to FASTQ format. Reads were aligned based on the human reference genome (GRCh38). Demultiplexing and doublets removal was done using souporcell (20). Each scATAC-seq library fragment file was utilized further for downstream analysis. Employing the ArchR package (v.1.0.1) within R, quality control (cells having a transcription start site (TSS) enrichment score < 4 and unique nuclear fragments < 1000 were filtered), dimensionality reduction (iterativeLSI function, default parameters), harmony batch correction [25] (addHarmony function, default parameters), clustering (addClustersfunction, method = "Seurat", resolution = 1, UMAP) and cluster visualization (addUMAP function) were done [26]. The default parameter of ArchR was used to calculate gene expression by computing a "gene score" based on chromatin accessibility within a gene body and distally & proximally from the transcription start site (TSS). A gene score matrix was created using the gene score profiles for all cells. Later, the wilcoxon rank-sum test was used to identify cell type specific marker genes. Gene having adjusted P-value (Benjamini-Hochberg) < 0.05 and logFC >= 0.05 were considered as marker genes. Each cluster was annotated based on cell type specific marker genes and cluster specific marker genes retrieved from scRNA-seq data. Further, using the method ("addGeneIntegrationMatrix" function) developed by Stuart [27], the gene score matrix was integrated with scRNA expression data. MACS2 [28] strategy (getMarkerFeatures function, useMatrix = "PeakMatrix") was adapted on an integrated dataset to call peaks in each cluster. Peaks having logFC greater than 0.05 and P-value < 0.05 were considered as marker peaks. For cross-validation, a peak matrix per sample was generated, and the pseudo-bulk ATACseq analysis was performed through ArchR.

In Vitro Measurement of Interferon Response

Six healthy controls and six convalescent COVID-19 patients were used to assess interferon response. Cryopreserved PBMCs were stimulated with 10 ng/ml recombinant human IFN-α2 (R&D Systems), 1000 U/ml IFN-β (R&D Systems), and 50 ng/ml IFN-γ1b (Miltenyi Biotec, Germany) for 24 hours. Stimulations were performed in U-bottom

96-well tissue culture plates with $5x10^5$ PBMCs per well. RNA isolation was carried out using the RNeasy Mini kit (Qiagen, Germany), and cDNAs were generated with the iScript cDNA synthesis kit (Bio-Rad Laboratories, CA, USA) according to the instructions of the manufacturers. RT-qPCR for the interferon-response genes *IFI44L*, *IFI6*, *IRF3*, *IRF7*, *IRF9*, *ISG15*, and *OAS2* was performed with StepOnePlus PCR System (Applied Biosystems, MA, USA). *HPRT1* was used as the housekeeping control gene. Primers and reaction conditions are provided in **Tables S2, S3**.

DNA Methylation Analysis

DNA was isolated from whole blood using QIAamp DNA Micro Kit (Cat: 56304, Qiagen, Hilden, Germany), and the concentration was determined using a NanoDrop spectrophotometer at 260 nm. The high-quality DNA from 20 samples (11 convalescent COVID-19 and 9 controls) were obtained successfully for the genome-wide DNA methylation profiling through the Illumina Infinium© MethylationEPIC array (~850,000 CpG sites). The DNA methylation values were gained from the raw IDAT files using the minfi package in R (v.4.0.3) [29]. Initially pre-processing was performed to filter bad quality probes with a detection P-value > 0.01, cross-reactive probes, polymorphic probes [30], and probes in the sex chromosome. Subsequently, after stratified quantile normalization [31], the comparison was made between convalescent COVID-19 individuals and controls to detect the relative proportion of cell types and differentially methylated CpG sites. Based on methylation value, cell proportion was estimated using modified Housman's method implemented in the estimateCellCounts2 function of the FlowSorted.Blood.EPIC R package [32]. Later, differential analysis of cell-type proportions was done using the regression model of the DirichletReg package of R. Differentially methylated CpG sites were detected by a linear regression model using the limma R package [33], with age and sex as covariates. The Spearman correlation was calculated between the cell proportion and the top 150 CpG differentially methylated sites. DMRs analysis was done by using combp R package [34].

PBMC Stimulations and Cytokine Measurements

$5x10^5$ PBMCs per well were seeded in U-bottom 96-well tissue culture plates (Greiner Bio-One, Austria) and stimulated with 10 ng/ml lipopolysaccharides (LPS) derived from *Escherichia coli* O55:B5 (Sigma-Aldrich, MO, USA), 10^6/ml heat-killed *Staphylococcus aureus*, 10^6/ml heat-killed Candida albicans, R848 (InvivoGen), or heat-inactivated (30 minutes, 60°C) SARS-CoV-2 Wuhan-Hu-1 strain (NRW-42 isolate, GISAID accession number: EPI_ISL_425126) [35] with 1 µg/ml soluble purified mouse anti-human CD28 (BD Biosciences, New Jersey, USA), for 24 hours and 7 days in the presence of 10% pooled human serum. A control condition without any stimulant was included. Cells were incubated at 37°C with 5% CO_2. Stimulations with R848

and SARS-CoV-2 were performed later with cryopreserved cells, while the rest of the stimulations were done with freshly isolated PBMCs. Concentrations of TNF-α, IL-6, IL-1β, IL-1Ra, and IFN-γ in culture supernatants were determined using DuoSet ELISA kits (R&D Systems, MN, USA). IFNα concentrations were determined using VeriKine Human Interferon Alpha ELISA Kit (PBL Assay Science, NJ, USA) according to the manufacturer's instructions. Supernatants from 7 days stimulations were used for IFN-γ, and the remaining cytokines were measured in 24-hour supernatants.

Results

Study Design

To characterize the immunological features of convalescent COVID-19 patients, we collected blood from 14 convalescent COVID-19 patients and 15 healthy donors. At the time of sampling, none of the control individuals experienced any symptoms or signs of infection, including COVID-19. All convalescent COVID-19 patients had recovered from SARS-CoV-2 infection. Out of the 14 patients, only one convalescent COVID-19 patient had experienced a severe COVID-19 and was admitted to an intensive care unit (ICU), while the remaining 13 experienced mild infections. The recovery time for most of the convalescent COVID-19 patients (9 out of 14) was ca. one month, only one patient with one-week recovery time and two patients with around two-month recovery time. Sex was distributed almost equally in both convalescent and healthy individuals. The mean age of convalescent COVID-19 patients and control individuals were 60 ± 11 years and 50 ± 14 years, respectively (**Table 1**).

To explore the effect of the SARS-CoV-2 infection on the immune system after recovery, we have performed a comprehensive set of assays and analyses, which were summarized in **Figure 1A**.

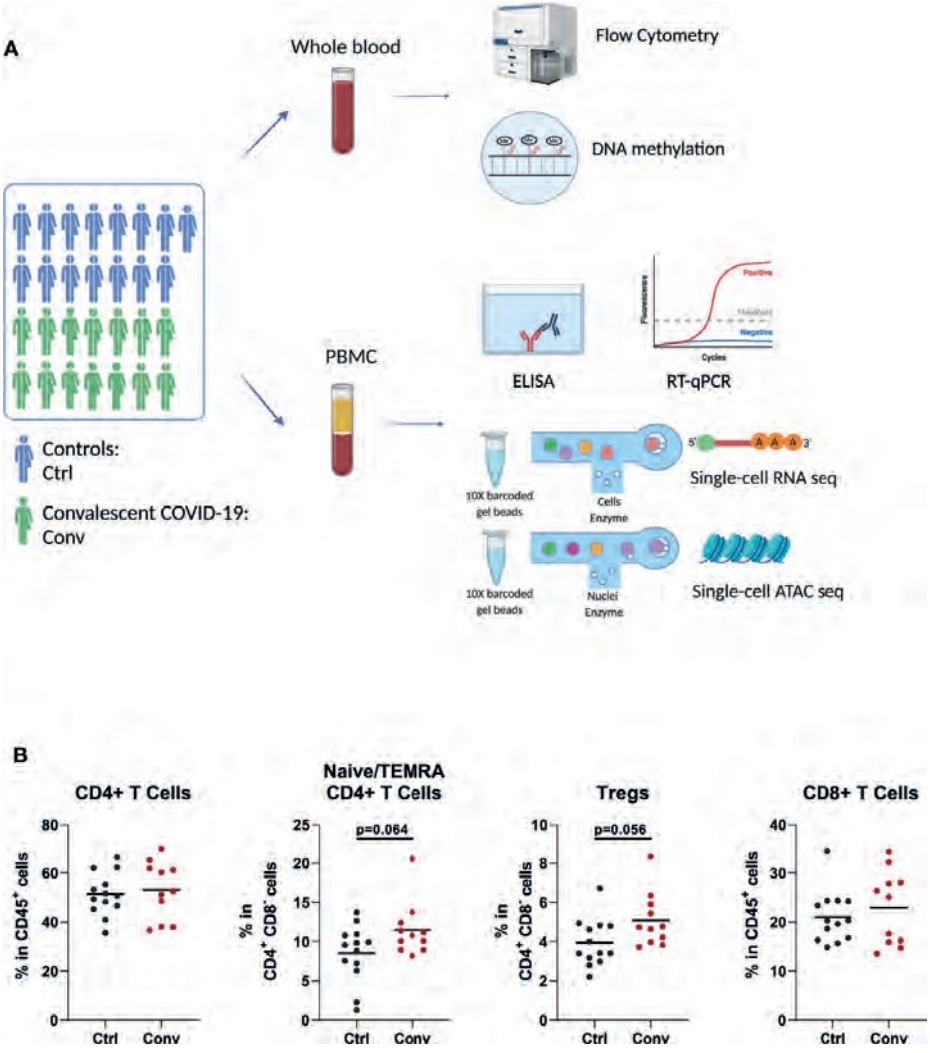


Figure 1. Study design and immune cell populations of healthy controls and convalescent COVID-19 patients

(A) The summary of the experiments performed in the study. Blood was drawn from 15 healthy controls and 14 convalescent COVID-19 patients. Flow cytometry, RT-qPCR, and ELISA were performed to determine the cell counts, assess interferon stimulated gene expressions, and measure the cytokine levels in culture supernatants, respectively. Single-cell RNA sequencing was used to determine the transcriptome. Single-cell ATAC sequencing and whole-genome methylation assay were performed to analyze the epigenetic differences between convalescent COVID-19 individuals and controls (This figure was created with BioRender.com). **(B)** Dot plots showing proportions of T cell subsets between the convalescent COVID-19 individuals and controls from flow cytometry. Mann-Whitney test was used to analyze the differences between controls (black dots) and convalescent patients (red dots).

Subtle Differences of Immune Cell Composition in COVID-19 Convalescence

Since acute SARS-CoV-2 infection leads to striking changes in immune cell types in the blood, flow cytometry analysis was performed to assess whether the abundance of various immune cell subsets in convalescent COVID-19 individuals is still different from those of healthy controls. The absolute numbers and percentages of most immune cell subsets, such as neutrophils, natural killer (NK) cells, B cells, and T cells, were similar between convalescent patients and controls (**Figures S3A–C**). Although COVID-19 leads to a dramatic decline in $CD4^+$ T and $CD8^+$ T cells in the acute phase, these cell populations in convalescent patients were not significantly different from those of uninfected people, indicating the full recovery from lymphopenia (**Figure 1B**). We observed that regulatory T cells (Treg) and $CD4^+$ naive/terminally differentiated effector memory cell (naïve/TEMRA) populations were somewhat more abundant in the convalescent COVID-19 individuals compared to controls, but these differences were not statistically significant (**Figure 1B**).

Single-Cell RNA-Seq and ATAC-Seq Analysis of PBMCs in Convalescent COVID-19 Individuals

Next, we performed an integrative analysis of scRNA-seq and scATAC-seq in convalescent and healthy individuals. For scRNA-seq analysis, we examined 66, 753 single cells from 21 individuals (12 convalescent and 9 controls) after QC (**Figure S4A**). Uniform manifold approximation and projection (UMAP) was used for cell clustering. In total, we detected 11 immune cell types, namely, $CD4^+$ T cells ($IL7R^+$), $CD8^+$ T cells ($CD8A^+CD8B^+GZMK^+$), B cells ($CD79A^+$), NK cells ($NKG7^+GZMB^+$), classical monocytes ($CD14^+LYZ^+$), non-classical monocytes ($FCGR3A^+$), platelets ($PPBP^+$), and monocyte-derived dendritic cells (mDCs, $HLA\text{-}DP^{high}$, $HLA\text{-}DR^{high}$) **(Figures 2A, B)**. Subsequently, we analysed 38,186 nuclei from 16 individuals (9 convalescent and 7 controls) in scATAC-seq analysis (**Figures 2C, D** and **Figure S4B–D**). It is pertinent to note that a strong correlation of the marker genes between scRNA-seq and scATAC-seq analysis was detected (**Figure S4E**). Subsequently, we compared the cell compositions of both scRNA-seq and scATAC-seq. No statistically significant difference was detected between the convalescent COVID-19 patients and controls (**Figures 2E, F**). This supports the findings of the flow cytometry measurements showing full recovery of the immune cell subsets.

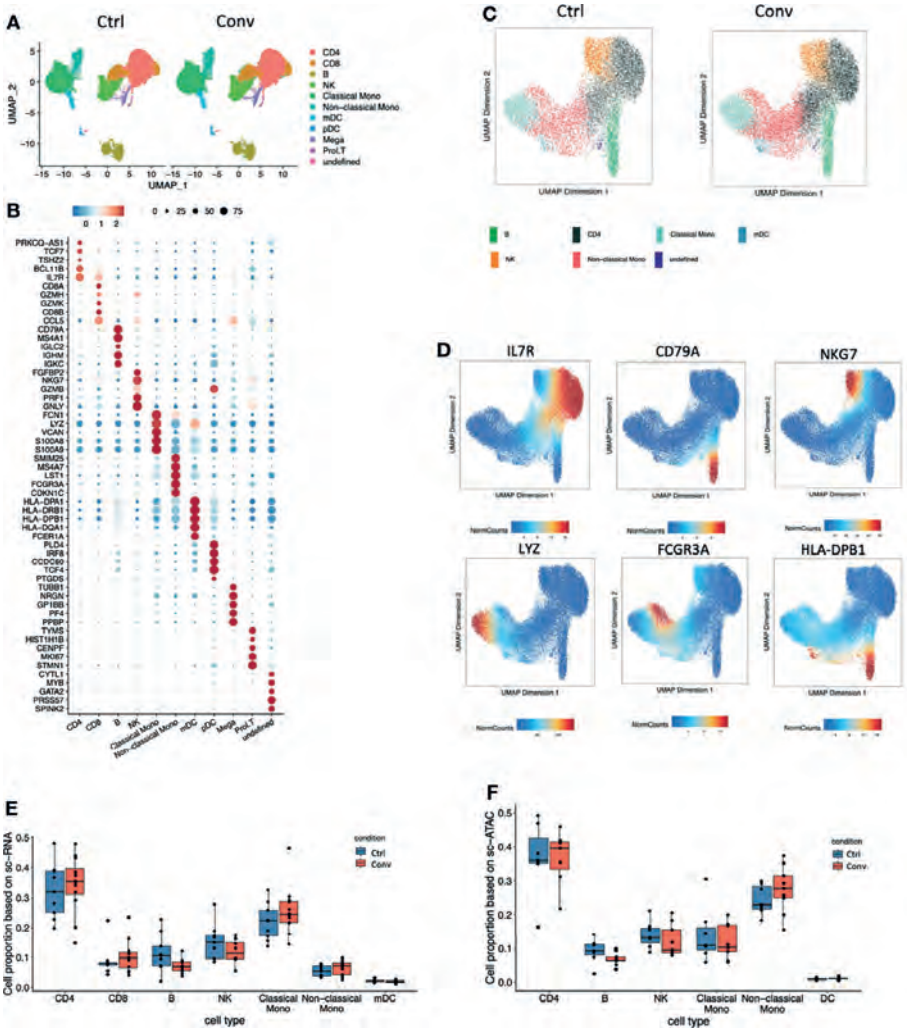


Figure 2. Single-cell transcriptome and epigenome analysis defined major immune cell subsets in convalescent COVID-19 individuals

(A) A total of 41486 and 25266 single-cell transcriptomes from COVID-19 convalescent and control samples were obtained respectively. Using the uniform manifold approximation and projection (UMAP) method, we captured 11 major cell types based on the canonical markers in the dot plots **(B)**. **(B)** Dot plot showing the expression level of the marker genes in each cell type. Dot size reflects the proportion of cells expressing the indicated gene; the color encodes the average expression level (low=blue, high=red). **(C)** A total of 21102 and 17084 nuclei from 9 COVID-19 convalescent and 7 control samples were obtained for scATACseq analysis, respectively. Seven major cell types were captured based on the canonical markers shown in the feature plot **(D)**. **(D)** The UMAP showing the labelled gene score for each cell. Blue represents the minimum gene score while red represents the maximum gene score for the given gene. The minimum and maximum scores are shown in the bottom of each panel. The gene of interest are shown in the upper of each panel. Cell proportions from **(E)** single-cell RNA sequencing and **(F)** single-cell ATAC dataset between convalescent COVID-19 and controls in the seven main cell types, $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells, classical monocytes, non-classical monocytes, and mDCs.

Pathways of Antigen Processing and Presentation via MHC II Remain Downregulated in Convalescent COVID-19 Individuals at Transcriptional Level

To identify if any differences exist at the transcriptomic level between immune cells of convalescent COVID-19 patients and controls, we performed a differential expression analysis. After correcting for age and sex, 907 differentially expressed genes (DEGs) were identified with false discovery rate (FDR) < 0.05 and absolute log-fold change (logFC) > 0.05 (**Figure S5A**).

DEGs were found predominantly in monocytes (both classical (DEGs=579) and non-classical (DEGs=62)) and $CD4^+$ T (DEGs=172) cells. Moreover, we assessed the number of DEGs at different logFC thresholds: we identified 293 DEGs with FDR < 0.05 and absolute logFC > 0.1, and 30 DEGs with FDR < 0.05 and absolute logFC > 0.25. Although the number of DEGs decreased with higher logFC thresholds, most of them predominantly existed in monocytes and $CD4^+$ T cells (**Figure S5A**). This indicates that the observed differences in the transcriptional profile of convalescent COVID-19 patients were mainly in monocytes and $CD4^+$ T cells. Additionally, we compared the peak accessibility in each cell type based on scATAC-seq data. After multiple testing correction, none of the peaks were significant, which indicated that the open chromatin accessibility between convalescence patients and healthy controls did not show significant difference. The differential expressed genes were mainly found in classical monocytes. In classical monocytes, for peaks in the proximity (500kb) of up-regulated DEGs in convalescence, 7205 peaks were up-regulated (mean log2FC = 1.077) in convalescence while 7371 peaks were down-regulated (mean log2FC = -1.073). For peaks in the proximity (500kb) of down-regulated DEGs in convalescence, 10252 peaks were down-regulated (mean log2FC = -1.078) in convalescence while 9777 peaks were up-regulated (mean log2FC = 1.086). None of these peaks were significantly different between convalescence and controls (P-value FDR adjusted > 0.5).

Differential expression analysis in the scRNA-seq revealed that MHC class II genes, such as *HLA-DRB5, HLA-DPB1, HLA-DPA1, HLA-DRB1,* and *HLA-DRA*, were downregulated in convalescent COVID-19 individuals (**Figure 3A**). Moreover, antigen processing and presentation via the MHC II pathways were downregulated in $CD4^+$ T cells (**Figure 3B**). On the other hand, no significant difference in the surface expression of *HLA-DR* in $CD4^+$ T cells and monocytes between healthy and convalescent individuals was observed (**Figure S5B**).

Interferon Pathway in Convalescent COVID-19 Patients and Healthy Volunteers

Since we identified DEGs mainly in classical monocytes (**Figure S5A**), we performed a sub-clustering analysis of these cells. Initially, using UMAP, we captured 6 sub-clusters in classical monocytes. Further inspection revealed that one healthy control, HC04, was markedly different from others (**Figures S5C, D**). Classical monocytes-specific pseudo-bulk RNA analysis also showed that HC04 was separated from the main group in a PCA plot (**Figure S5E**). Therefore, we temporarily removed HC04 while performing differential expression analysis within the classical monocytes. After discarding HC04, we re-performed sub-clustering and detected five sub-clusters (**Figure 3C**). The top markers for each sub-cluster are shown in **Figure 3D**. DEGs between convalescent individuals and controls mainly occurred in cluster 0 (S100A^{high}) and cluster 1 (IL7R^{high}) with FDR < 0.05 and logFC > 0.05 (**Figure S5F**). Subsequently, GO enrichment analysis was performed using the DEGs present in cluster 0 (812 DEGs) and cluster 1 (33 DEGs), respectively. The upregulated DEGs in convalescent individuals were enriched in antigen processing and presentation via MHC I, while the down-regulated DEGs were enriched in response to the interferon pathway in the S100A^{high} classical monocytes. No enriched pathway was found in DEGs from cluster 1 (**Figure 3E**). The genes engaged in the down-regulated interferon response pathway included interferon regulatory factor 3 (*IRF3*), *IRF7*, *IRF9*, 2'-5'-Oligoadenylate Synthetase 2 (*OAS2*), interferon-alpha inducible 6 (*IFI6*), interferon-alpha inducible ligand 44 (*IFI44L*), interferon-stimulated gene 20 (*ISG20*), and *ISG15* (**Figure 3F**). These genes were thus selected for the *in vitro* validation of the sequencing results.

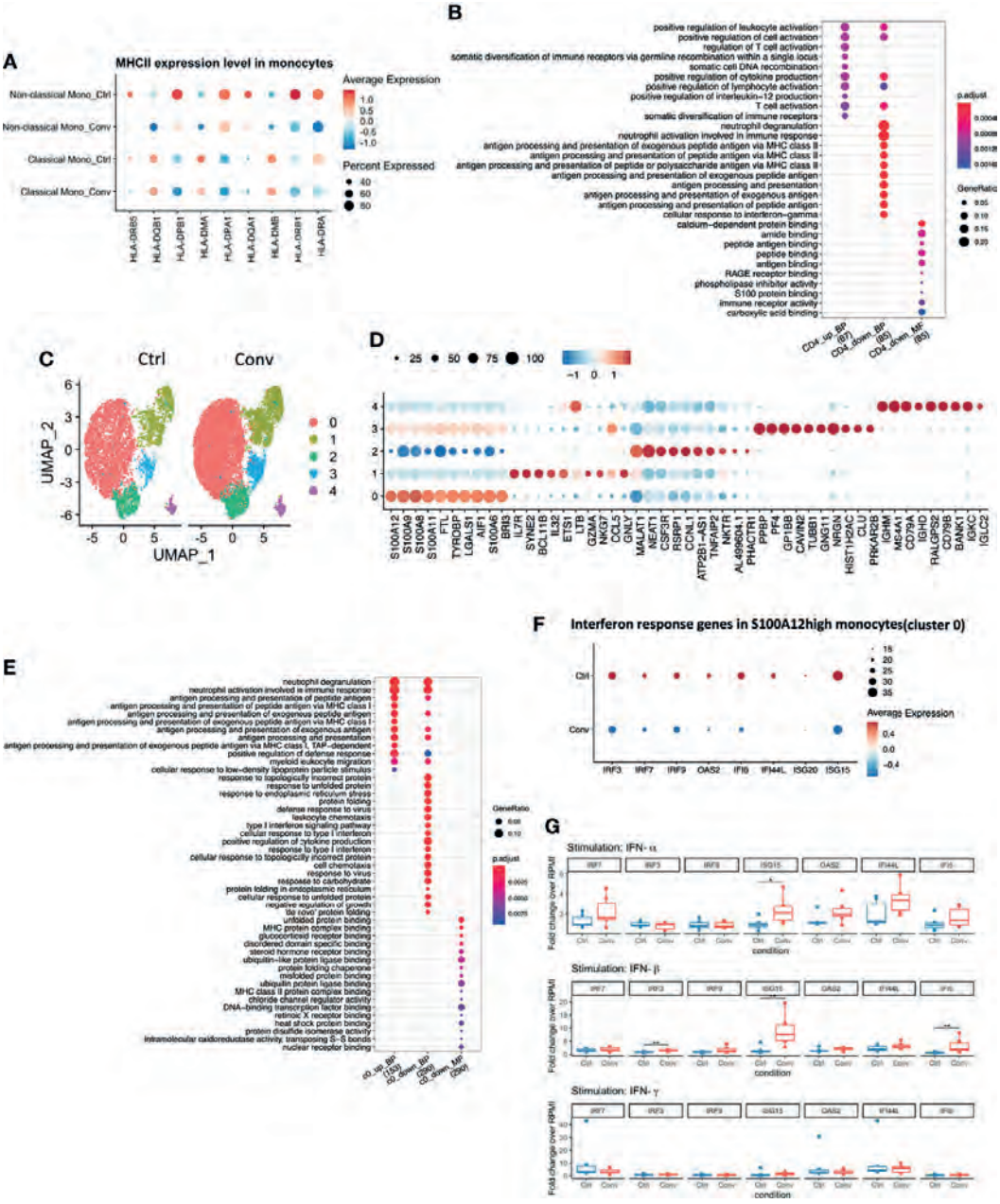
IFN production constitutes the major first line of defense against viruses. Type I and type II IFNs induce hundreds of antiviral effectors, or ISGs, to achieve a cell-intrinsic state of viral resistance [36]. Since IFN response pathway genes were downregulated in monocytes of convalescent patients, we hypothesized that expressions of those genes would be different between healthy and convalescent COVID-19 individuals after stimulation with IFN-α, IFN-β, and IFN-γ. Therefore, PBMCs of 6 healthy and 6 convalescent individuals were stimulated with type I and II interferons, and genes involved in IFN response were analyzed via RT-qPCR. The baseline gene expressions were not different between controls and convalescent individuals (**Figure S5G**). On the other hand, we found that expression of three ISGs, namely, *IRF3*, *ISG15*, and *IFI6*, were significantly higher in convalescent COVID-19 individuals after stimulation with IFN-β, IFN-α/β, and IFN-β, respectively (**Figure 3G**), indicating that the degree of induction by type I IFNs is higher for some of the IFN regulated genes in convalescent individuals compared to healthy volunteers. We performed

pseudo-bulk RNA analysis between convalescent COVID-19 individuals and controls and found no significant difference, in line with the RT-qPCR results from non-stimulated PBMCs (**Table 2**). Consistently, pseudo-bulk ATAC analysis also revealed no significant difference in the chromatin accessibility of ISGs associated with the interferon response pathway in PBMC between conditions (**Table 2**). These results suggest that the down-regulation of the interferon pathway is specific to monocytes, while at the PBMC level, this pathway is not changed.

Table 2. Expressions of interferon-stimulated genes analyzed by pseudo-bulk RNAseq and pseudo-bulk ATACseq

Gene	**pseudo bulk RNA**		**pseudo bulk ATAC**	
	log2FC	**raw P**	**log2FC**	**raw P**
ISG15	0.66	0.055	0.14	0.513
IFI6	1.04	0.021	0.04	0.693
IFI44L	1.18	0.285	0.31	0.048
IRF7	0.41	0.262	-0.29	0.249
OAS2	0.46	0.314	-0.46	0.088
IRF9	0.16	0.617	0.05	0.903
IRF3	-0.006	0.984	0.17	0.209

*Wald test was used to analyze the differences between controls and convalescent patients


A
MHCII expression level in monocytes
Non-classical Mono_Ctrl
Non-classical Mono_Conv
Classical Mono_Ctrl
Classical Mono_Conv
HLA-DRB5
HLA-DQB1
HLA-DPB1
HLA-DMA
HLA-DPA1
HLA-DQA1
HLA-DMB
HLA-DRB1
HLA-DRA
Average Expression
Percent Expressed
B
positive regulation of leukocyte activation
positive regulation of cell activation
somatic diversification of immune receptors via germline recombination within a single locus
somatic cell DNA recombination
positive regulation of cytokine production
positive regulation of lymphocyte activation
positive regulation of interleukin-12 production
T cell activation
somatic diversification of immune receptors
neutrophil degranulation
neutrophil activation involved in immune response
antigen processing and presentation of exogenous peptide antigen via MHC class II
antigen processing and presentation of peptide antigen via MHC class II
antigen processing and presentation of peptide or polysaccharide antigen via MHC class II
antigen processing and presentation of exogenous peptide antigen
antigen processing and presentation
antigen processing and presentation of exogenous antigen
antigen processing and presentation of peptide antigen
cellular response to interferon-gamma
calcium-dependent protein binding
amide binding
peptide antigen binding
peptide binding
antigen binding
RAGE receptor binding
phospholipase inhibitor activity
S100 protein binding
immune receptor activity
carboxylic acid binding
CD4_up_BP (87)
CD4_down_BP (85)
CD4_down_MF (85)
p.adjust
GeneRatio
C
Ctrl
Conv
UMAP_2
UMAP_1
D
S100A12
S100A9
S100A8
S100A11
FTL
TYROBP
LGALS1
AIF1
S100A6
BHB
ILTR
SYNE2
BCL11B
IL32
ETS1
LTB
GZMA
NKG7
CCL5
GNLY
MALAT1
NEAT1
CSF3R
RSRP1
CCNL1
ATP2B1-AS1
TNFAIP2
NKTR
AL499604.1
PHACTR1
PPBP
PF4
GP1BB
CAVIN2
TUBB1
GNG11
NRGN
HIST1H2AC
CLU
PPEF1
PRKAR2B
IGHM
MS4A1
CD79A
IGHD
RALGPS2
CD79B
BANK1
IGKC
IGLC2
E
neutrophil degranulation
neutrophil activation involved in immune response
antigen processing and presentation of peptide antigen
antigen processing and presentation of peptide antigen via MHC class I
antigen processing and presentation of exogenous peptide antigen via MHC class I
antigen processing and presentation of exogenous antigen
antigen processing and presentation
antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent
positive regulation of defense response
myeloid leukocyte migration
cellular response to low-density lipoprotein particle stimulus
response to topologically incorrect protein
response to unfolded protein
response to endoplasmic reticulum stress
protein folding
defense response to virus
leukocyte chemotaxis
type I interferon signaling pathway
cellular response to type I interferon
positive regulation of cytokine production
response to type I interferon
cellular response to topologically incorrect protein
cell chemotaxis
response to virus
response to carbohydrate
protein folding in endoplasmic reticulum
cellular response to unfolded protein
negative regulation of growth
tat rovs' protein folding
unfolded protein binding
MHC protein complex binding
glucocorticoid receptor binding
disordered domain specific binding
steroid hormone receptor binding
ubiquitin-like protein ligase binding
protein folding chaperone
misfolded protein binding
ubiquitin protein ligase binding
MHC class II protein complex binding
chloride channel regulator activity
DNA-binding transcription factor binding
retinoic X receptor binding
heat shock protein binding
protein disulfide isomerase activity
intramolecular oxidoreductase activity, transposing S–S bonds
nuclear receptor binding
GeneRatio
p.adjust
F
Interferon response genes in S100A12high monocytes(cluster 0)
Ctrl
Conv
IRF3
IRF7
IRF9
OAS2
IFI6
IFI44L
ISG20
ISG15
Average Expression
G
Stimulation: IFN-α
Stimulation: IFN-β
Stimulation: IFN-γ
Fold change over RPMI
condition

< Figure 3. Antigen processing and presentation via MHC II were downregulated in convalescent COVID-19 individuals, while baseline expressions of Interferon-Stimulated Genes (ISGs) in PBMCs are comparable to uninfected individuals

(A) Dot plot showing the MHC II expression levels across different conditions. The size of the dot depicts the percentage of cells within classical monocytes or non-classical monocytes; the color encodes the average expression level (low=blue, high=red). **(B)** Dot plot showing the GO enrichment analysis of DEGs in $CD4^+$ T cells. The color and size of the dot indicate the significance (adjusted P-value) and the percentage of DEGs in the given GO term, respectively (BP, biological process, MF, molecular function). Top 10 enriched categories were shown. **(C)** UMAP of single-cell RNA sequencing data in classical monocytes in convalescent COVID-19 (n = 11) and control (n=9) samples. 5 cell clusters were identified. **(D)** Dot plot showing the top 10 markers for each cell cluster using the Wilcoxon Rank Sum test. The 0-4 in the y-axis means the cell clusters from**(C)**. The color and size of the dot indicate the gene expression level (low=blue, high=red) and percentage of cells which expressing these markers. **(E)** Dot plot showing the GO enrichment categories of the DEGs found in each cluster. **(F)** Dot plot showing the interferon response genes expression levels across different conditions. The size of the dot depicts the percentage of cells expressing the indicated genes within cluster0; the color encodes the average expression level (low=blue, high=red). **(G)** Box plots showing the gene expressions of the seven interferon-stimulated genes (IRF7, IRF9, IRF3, ISG15, OAS2, IFI44L, and IFI6) 24 hours after stimulation with recombinant human IFN-α, IFN-β, and IFN-γ. Fold changes over RPMI (unstimulated condition) were reported. Mann-Whitney test was used to analyze the differences between controls and convalescent patients (n = 6, *P value < 0.05, **P value < 0.001).

The Differences in DNA Methylation Sites in Convalescent COVID-19 Patients Mainly Originated From Monocytes and $CD4^+$ T Cells

Earlier studies have reported that the host cell epigenetic landscape of DNA methylation changes during SARS-COV2 infection [14]. Therefore, we performed DNA methylome profiling from high-quality DNA of 20 whole blood samples (11 convalescent COVID-19 and 9 controls). Initially, pre-processing of the dataset scanned 794,745 good-quality probes. Cellular deconvolution analysis using modified Housman's method [32, 37, 38] detected six cell-types, namely NK, $CD8^+$ T, $CD4^+$ T, B cells, neutrophils, and monocytes, which were present in both convalescent COVID-19 individuals and controls. The relative proportion of these six cell types did not significantly differ (**Figure 4A**). This result, consistent with our flow cytometry, scRNA-seq and scATAC-seq analyses, indicated that the cell proportions returned to normal levels after recovery from SARS-CoV-2 infection.

Furthermore, epigenome-wide association analysis (EWAS) was performed to assess the difference in genome-wide DNA methylation profiles between convalescent COVID-19 individuals and controls. No significant differentially methylated CpG sites (DMSs) (FDR < 0.05) were identified (**Figures S6A-C**). Subsequently, to detect any minor changes at the molecular level, we scanned the top 50, 75, 100, and 150 DMSs. A prevalence of hypermethylated and demethylated CpG sites was consistently observed at these top DMSs in convalescent COVID-19 patients compared with those in healthy controls (**Figure 4B**). Thus, genome-wide DNA methylation profiles of convalescent COVID-19 individuals show only minor differences compared to controls.

Next, we aimed to detect individual cell types where the methylation signal was the strongest. We performed a correlation analysis between the top 150 DMSs (94 downregulated and 56 upregulated) having the lowest P-value and the estimated cell proportions from the above-mentioned deconvolution analysis. We observed that the upregulated CpG sites were positively correlated with $CD4^+$ T cells, monocytes, and neutrophils (**Figure 4C**). Conversely, the downregulated CpG sites showed a negative correlation with $CD4^+$ T cells, monocytes, and neutrophils (**Figure 4D**), suggesting that majority of the top differentially DNA methylation signatures in convalescent COVID-19 individuals mainly originated from monocytes and $CD4^+$ T cells, and somewhat less neutrophils. These results could be replicated using the top 100 or top 75 DMSs (**Figures S6D, E**). However, we could not identify enriched epigenetic changes around the DEGs (+/- 250kb window) in monocytes observed from the scRNA-seq, compared to randomly selected CpG sites (**Figure S6F**).

Differential methylation regions (DMRs) have identified 30 significant DMRs between convalescent COVID-19 individuals and controls (**Table S4**). The genes annotated to these DMRs were enriched in the following biological pathways: glycosphingolipid biosynthesis, telomere maintenance, recognition of DNA damage, sialic acid metabolism, chromosome maintenance and TGF-Ncore.

Cytokine Production Capacity Is Not Impaired in People Recovered From COVID-19

Lastly, we measured the cytokine production capacity of PBMCs upon incubation with different viral, bacterial, and fungal stimuli to assess whether the differences in transcriptome influence immune responses upon recovery from COVID-19. PBMCs were either left unstimulated or stimulated with LPS, *S. aureus*, *C. albicans*, viral RNA mimic R848, and heat-inactivated SARS-CoV-2 (original Wuhan-Hu1 strain) for 24 hours to measure TNF-α, IL-6, IL-1β, IFNα and IL-1Ra and seven days to measure IFN-γ. Anti-CD28 agonistic antibodies were combined with inactivated SARS-CoV-2 to better activate T cell responses, particularly in convalescent COVID-19 patients. TNF-α and IL-6 production upon different stimuli were similar between healthy individuals and convalescent COVID-19 patients (**Figures 5A, B**). Another proinflammatory cytokine, IL-1β, was also produced similarly in healthy and convalescent individuals upon R848 and *S. aureus* stimulation (**Figure 5C**). Furthermore, R848 and SARS-CoV-2 induced IFNα production from PBMCs did not differ between the groups (**Figure 5D**). Intriguingly, baseline IL-1Ra secretion of convalescent COVID-19 patients was significantly higher than healthy controls, although IL-1Ra production following R848 or heat-inactivated SARS-CoV-2 stimulation was similar (**Figure 5E**). Furthermore, PBMCs from convalescent individuals produced significantly more IFN-γ without any stimulation

(**Figure 5F**). As expected, incubation of convalescent COVID-19 patient PBMCs with heat-inactivated SARS-CoV-2 resulted in higher IFN-γ production, indicating the presence of T cell memory after recovery. Lastly, IFN-γ secretion did not differ between healthy and convalescent upon incubation with viral RNA mimic R848 (**Figure 5F**).

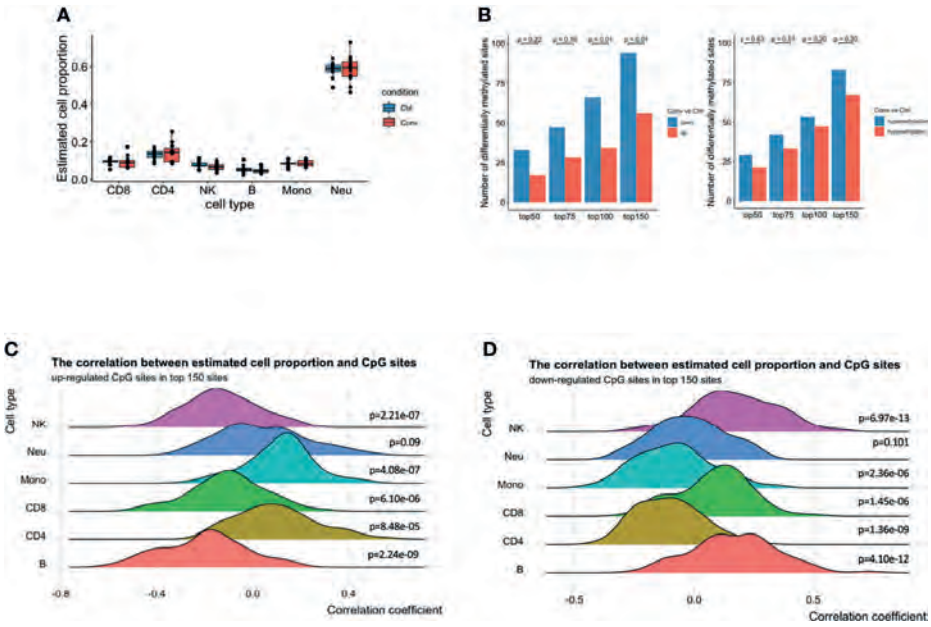


Figure 4. A predominance of down-regulated methylation sites was observed in convalescent COVID-19 patients, mainly originating from monocytes and CD4+ T cells

(A) Box plot showing the estimated cell proportions in convalescent COVID-19 (n = 11) and control (n = 9) samples based on the methylation. No significant difference was observed in estimated cell proportions between the two conditions. **(B)** The number of up-/down-regulated and hyper/hypomethylated CpG sites in top 150 differentials methylated CpG sites ordered by raw P value from the linear regression model. A preponderance of hypermethylated sites and down-regulated methylation value was consistently observed at various thresholds compared with those in healthy controls. Fisher's exact test was used for the statistical analysis. **(C)** The density ridgeline plots showing the correction between the estimated cell proportions and the up-regulated CpG sites in the top 150 differentially methylated CpG sites. **(D)** The density ridgeline plots showing the correction between the estimated cell proportions and the down-regulated CpG sites in the top 150 differentially methylated CpG sites. One side Wilcoxon test was used to calculate the significance of the correlation.

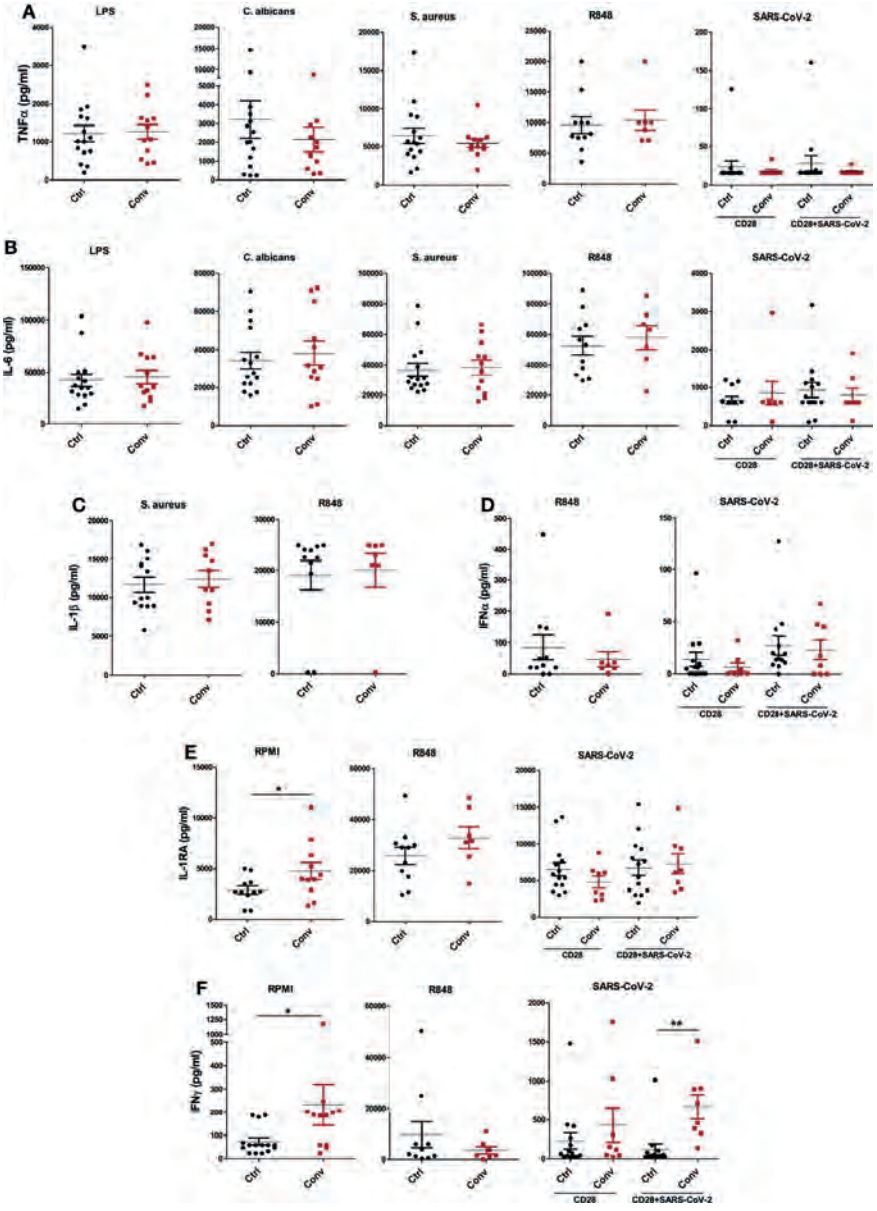


Figure 5. Cytokine production capacity is intact in people recovered from COVID-19

The concentrations of **(A)** TNF-α and **(B)** IL-6 after stimulation of PBMCs with LPS, C. albicans, *S. aureus*, R848, and heat-inactivated SARS-CoV-2. **(C)** IL-1β production following *S. aureus* and R848 stimulation. **(D)** IFNα levels after incubation with R848 and heat-inactivated SARS-CoV-2. **(E)** IL-1Ra and **(F)** IFN-γ at the baseline (RPMI condition) and stimulation with R848 and SARS-CoV-2. TNF-α, IL-6, IL-1β, IFNα and IL-1Ra productions were measured after 24 hours, while IFN-γ was measured after 7 days of incubation. Stimulations with R848 and SARS-CoV-2 were performed later with cryopreserved cells, while the rest of the stimulations were done with freshly isolated PBMCs. Mann-Whitney test was used to analyze the differences between controls and convalescent patients (*P value < 0.05, **P value < 0.001).

Discussion

In the present study we provided a detailed insight into the immunological and molecular features of immune responses in the convalescent COVID-19 individuals by integrating multi-omics data. Furthermore, we assessed whether SARS-CoV-2 infection influences immune functions in terms of *in vitro* cytokine production capacity of recovered individuals. We found only minor differences in the transcriptome and DNA methylation profiles of immune cells in convalescent COVID-19 individuals, and these differences were consistently more pronounced in monocytes and CD4+ T cells compared to other immune cell populations. However, these changes did not seem to affect the cytokine production capacity of immune cells, although they were accompanied by a higher homeostatic release of IL-1Ra and IFN-γ.

Results obtained from flow cytometry, scRNA-seq, scATAC-seq, and DNA methylation analyses consistently revealed that immune cell proportions were at the normal levels in convalescent COVID-19 individuals within one-week to 6-month timeframe. One earlier study reported a higher percentage of $CD4^+$ T cells and DCs and lower numbers of NKT-like cells in patients two months after COVID-19 recovery compared to uninfected controls, while other cell types remained similar between groups [39]. We observed no major difference in cell populations, only slightly higher levels of regulatory T cells (Tregs) in convalescent individuals. Tregs play a critical role in the maintenance of self-tolerance and immunological homeostasis by negatively regulating the activation, proliferation, and effector functions of a wide variety of immune cells [6]. They can also prevent cytokine storm [40] and repress the formation of lung inflammatory disorders [41, 42]. Although there are conflicting reports, most studies report decreased circulating Treg levels, especially in severe COVID-19 cases [42]. Thus, the comparable numbers of Tregs as well as other immune cell subsets between healthy and recovered COVID-19 individuals in our study suggest that the immune cells go back to the level of healthy individuals after recovery. We also found that naive/TEMRA cell subset was somewhat more abundant in convalescent patients, although not statistically significant. TEMRA cells are known to expand following pathogens, such as the Dengue virus, and play a protective role during infections [43]. Increased ratio of $CD4^+$ TEMRA cells in moderate and severe COVID-19 was previously reported [44]. The same study also showed that $CD4^+$ TEMRA population stayed higher even after patients' recovery, however, sampling time after recovery was not indicated.

Transcriptomic analysis revealed that most of the DEGs were detected in classical monocytes. Although the differences between convalescent individuals and controls were modest, and we did not find a distinct expression in the surface *HLA-DR*

expression of monocytes and $CD4^+$ T cells, the downregulation of the HLA class II pathway in convalescent COVID-19 patients is important to be noted. Broad MHC II downregulation during COVID-19 infection was also reported in previous studies [45]. Our findings suggest that the attenuated immune function via MHC II downregulation during viral infection is still present at the transcriptomic level after recovery, and that may influence the capacity of myeloid cells to respond during infections a long time after SARS-CoV-2 was eliminated. Moreover, sub-clustering revealed upregulated MHC class I-dependent antigen processing and presentation pathways and downregulated interferon pathway in classical monocytes. However, the baseline expression of interferon pathway genes was not significantly different in the entire PBMC population, as evident from RT-qPCR, pseudo-bulk RNA, and pseudo-bulk ATAC sequencing analyses. Thus, these two pathways seem to be differentially regulated only in monocytes of convalescent COVID-19 individuals and neither represent the changes in the PBMC population nor translate into impaired *in vitro* response to IFNs. Notably, type I IFNs led to a significant increase in *IRF3*, *ISG15*, and *IFI6* expressions in convalescent COVID-19 patients compared to healthy controls, suggesting a higher degree of activation. On the other hand, stimulation with different antigens did not result in significantly different cytokine production, showing that changes in interferon pathway related-gene activation do not influence cytokine production capacity, including IFNα, in recovered COVID-19 individuals.

A recent study reported important epigenetic and functional changes in monocytes isolated from convalescent COVID-19 patients, especially in the IL-1 pathway and chemokines, suggesting a trained immunity phenotype [16]. We did not identify similar changes in our individuals, suggesting potential heterogeneity between different populations, disease severity, or sampling timepoints. Similarly, we did not find significant changes in $CD8^+$ T cell transcriptome in convalescent COVID-19 individuals compared to healthy controls, although such changes were reported during disease course, including upregulated CD8 expression, and an hyperactivated and exhausted phenotype [46, 47]. However, since $CD8^+$ T cells are a major source of IFN-γ production [48], higher IFN-γ production of non-stimulated PBMCs in convalescent COVID-19 patients might indicate that this hyperactive state of $CD8^+$ T cells could persist during the convalescent phase of the disease. In our study, the main changes in transcriptome are the decreased expression of MHC class II and type I interferon pathways in monocytes, but these were not associated with functional defects. In contrast, immune cells from convalescent individuals released more IFN-γ and IL-1Ra compared to healthy controls. The release of IFN-γ in individuals recovering from COVID-19 may reduce the susceptibility of host cells to secondary infections [49], while IL-1Ra might have an important role in the rebalancing of

inflammatory responses. The discrepancy between transcriptomic and functional data may denote that we did not capture the entire functional spectrum of immune cells in our study, but may also underline the fact that cell function is likely regulated at multiple additional levels after gene transcription such as translation, processing, and post-transcriptional modifications (glycosylation, etc).

EWAS analysis reveals that, though not genome-wide significant, there is a preponderance of hypermethylated sites and down-regulated methylation in convalescent COVID-19 patients, explicitly originating from monocytes and $CD4^+$ T cells. DNA methylation changes around DEGs in monocytes from scRNA-seq were not significantly different from the random set. This is likely due to the fact that the methylation was measured in the whole blood, which limits the power to detect the difference present in e.g. monocytes. In the future, further investigation focusing on monocytes may provide monocytes specific epigenetic change in convalescent individuals. Earlier studies have shown the incidence of hyper and demethylated CpG sites within COVID-19 patients: significant hypermethylation in regulatory regions of the genes related to the type I interferon response and first-line antiviral defense genes, like *ISG20* and *IFITM1*, are associated with disease severity in COVID-19 patients [14]. Another study observed that systemic lupus erythematosus (SLE) patients are more likely to develop SARS-CoV-2 symptoms, not because of a weakened immune system, but because of overexpression of *ACE2* in the lung, and hypomethylation of the *ACE2* gene, as well as a high level of demethylation of interferon genes [50]. Retrotransposon upregulation, which is commonly experienced after coronavirus infection, is also reported to be possibly due to increased global DNA demethylation activity [51]. These suggested that SARS-CoV-2 infection may have long-term effects, including irreversible genome modification among patients with prolonged recovery. Thus, genome-wide DNA methylation profiles of convalescent COVID-19 individuals are different from controls, which may be associated with clinical complication experienced by convalescent COVID-19 patients.

However, there are several limitations of this study to consider. Firstly, it is essential to note that the participants included in the study had no symptoms and they did not develop symptoms associated with the long term COVID-19 syndrome. Therefore, we could not associate the epigenetic and transcriptional signatures retained in convalescent patients with lingering symptoms, and these changes may not mirror functional effects. A recent study reported that 31 people with long COVID-19 symptoms exhibited less naïve T and B cells and higher plasma type I and type III interferons, even 8 months after the infection [52]. Nevertheless, larger studies should explore whether these changes can be identified in larger populations and assess their clinical relevance.

Our cohort mainly consisted of recovered COVID-19 patients from mild/moderate disease. The immunological differences we reported could be more apparent in the patients recovered from severe COVID-19. We have only one individual recovered from severe infection, however, we did not find differences compared with other participants in both transcriptome and methylome level (**Figures S7A–B**). Furthermore, this study was performed during the first wave of the pandemic, during which the original virus strain dominated the infections. Therefore, how the new variants affect the immune system after recovery remains elusive and must be investigated.

In summary, a comprehensive epigenetic, transcriptional and functional assessment shows that the immune responses of patients recovering from mild-to-moderate COVID-19 largely return to normal a few weeks to months after recovery. However, minor differences persist at the transcriptomic and epigenetic levels, especially in classical monocytes. Although MHC class II gene expressions and IFN response pathway were downregulated in monocytes of convalescent patients, those changes were not reflected in basal expressions of interferon-stimulated genes and cytokine production capacity of PBMCs. Therefore, our study shows that the immune system of patients who recover from mild/moderate COVID-19 largely return to normal, but future studies should investigate potential disturbances in individuals infected with different SARS-CoV-2 variants and patients suffering from long term COVID-19.

Data Availability Statement

The datasets presented in this study have been deposited at the European Genome-phenome Archive (EGA), under accession number EGAS00001005529.

Ethics Statement

The studies involving human participants were reviewed and approved by CMO Arnhem-Nijmegen (NL32 357.091.10). The patients/participants provided their written informed consent to participate in this study.

Author Contributions

YL, MGN, and C-JX conceptualized and designed the study. ZL, GK, WL, BZ, OB, HP, YM, and CQ performed the data analysis supervised by YL, C-JX, and MGN. GK, OB, AVF, CM, and JD-A recruited the participants and collected the biological material. GK, OB, HC-T, ZL, AVF, CM, CS, JD-A, BC, HK, ES, DD, MJ, and MC performed or supported the experiments. AvdV, FV, RC, BE-V, LJ, and MGN helped with participant recruitment and interpretation of the data. PO, LM, and HS provided the heat-inactivated SARS-CoV-2. YL, MGN, C-JX, ZL, GK, OB, and MG wrote the manuscript with input from all the authors. All authors contributed to the article and approved the submitted version.

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Conflict of Interest

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

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References

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A Novel Coronavirus From Patients With Pneumonia in China, 2019. N Engl J Med (2020) 382(8):727–33. doi: 10.1056/NEJMoa2001017
2. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical Features of Patients Infected With 2019 Novel Coronavirus in Wuhan, China. Lancet (2020) 395(10223):497–506. doi: 10.1016/S0140-6736(20)30183-5
3. Bonifacius A, Tischer-Zimmermann S, Vogel A, Dragon AC, Krettek U, Gödecke N, et al. Covid-19 Immune Signatures Reveal Stable Antiviral T-Cell Function Despite Declining Humoral Responses. Immunity (2021) 54(2):340-54.e6. doi: 10.1016/j.immuni.2021.01.008
4. Diao B, Wang C, Tan Y, Chen X, Liu Y, Ning L, et al. Reduction and Functional Exhaustion of T Cells in Patients With Coronavirus Disease 2019 (COVID-19). Front Immunol (2020) 11:827. doi: 10.3389/fimmu.2020.00827
5. Lagunas-Rangel FA. Neutrophil-to-Lymphocyte Ratio and Lymphocyte-to-C-Reactive Protein Ratio in Patients With Severe Coronavirus Disease 2019 (COVID-19): A Meta-Analysis. J Med Virol (2020) 92(10):1733–4. doi: 10.1002/jmv.25819
6. Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, et al. Dysregulation of Immune Response in Patients With Coronavirus 2019 (COVID-19) in Wuhan, China. Clin Infect Dis (2020) 71(15):762–8. doi: 10.1093/cid/ciaa248
7. De Zuani M, Lazničková P, Tomašková V, Dvončová M, Forte G, Stokin GB, et al. High CD4-to-CD8 Ratio Identifies an at-Risk Population Susceptible to Lethal COVID-19. Scand J Immunol (2021) 95(3):e13125. doi: 10.1111/sji.13125
8. Zhang J-Y, Wang X-M, Xing X, Xu Z, Zhang C, Song J-W, et al. Single-Cell Landscape of Immunological Responses in Patients With COVID-19. Nat Immunol (2020) 21(9):1107–18. doi: 10.1038/s41590-020-0762-x
9. Hadjadj J, Yatim N, Barnabei L, Corneau A, Boussier J, Smith N, et al. Impaired Type I Interferon Activity and Inflammatory Responses in Severe COVID-19 Patients. Science (2020) 369(6504):718–24. doi: 10.1126/science.abc6027
10. Arunachalam PS, Wimmers F, Mok CKP, Perera RAPM, Scott M, Hagan T, et al. Systems Biological Assessment of Immunity to Mild Versus Severe COVID-19 Infection in Humans. Science (2020) 369(6508):1210–20. doi: 10.1126/science.abc6261
11. Giamarellos-Bourboulis EJ, Netea MG, Rovina N, Akinosoglou K, Antoniadou A, Antonakos N, et al. Complex Immune Dysregulation in COVID-19 Patients With Severe Respiratory Failure. Cell Host Microbe (2020) 27(6):992–1000.e3. doi: 10.1016/j.chom.2020.04.009
12. Moratto D, Chiarini M, Giustini V, Serana F, Magro P, Roccaro AM, et al. Flow Cytometry Identifies Risk Factors and Dynamic Changes in Patients With COVID-19. J Clin Immunol (2020) 40(7):970–3. doi: 10.1007/s10875-020-00806-6
13. Bernardes JP, Mishra N, Tran F, Bahmer T, Best L, Blase JI, et al. Longitudinal Multi-Omics Analyses Identify Responses of Megakaryocytes, Erythroid Cells, and Plasmablasts as Hallmarks of Severe COVID-19. Immunity (2020) 53(6):1296–314.e9. doi: 10.1016/j.immuni.2020.11.017
14. Corley MJ, Pang APS, Dody K, Mudd PA, Patterson BK, Seethamraju H, et al. Genome-Wide DNA Methylation Profiling of Peripheral Blood Reveals an Epigenetic Signature Associated With Severe COVID-19. J Leukoc Biol (2021) 110(1):21–6. doi: 10.1002/JLB.5HI0720-466R

15. Castro de Moura M, Davalos V, Planas-Serra L, Alvarez-Errico D, Arribas C, Ruiz M, et al. Epigenome-Wide Association Study of COVID-19 Severity With Respiratory Failure. EBioMedicine (2021) 66:103339. doi: 10.1016/j.ebiom.2021.103339

16. 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. Nat Cell Biol (2021) 23(6):620–30. doi: 10.1038/s41556-021-00690-1

17. Kuijpers Y, Chu X, Jaeger M, Moorlag SJCFM, Koeken VACM, Zhang B, et al. The Genetic Risk for COVID-19 Severity Is Associated With Defective Innate Immune Responses. medRxiv (2020) 2020.11.10.20229203. doi: 10.1101/2020.11.10.20229203

18. Aguirre-Gamboa R, Joosten I, Urbano PCM, van der Molen RG, van Rijssen E, van Cranenbroek B, et al. Differential Effects of Environmental and Genetic Factors on T and B Cell Immune Traits. Cell Rep (2016) 17(9):2474–87. doi: 10.1016/j.celrep.2016.10.053

19. Lee J, Geng S, Li S, Li L. Single Cell RNA-Seq and Machine Learning Reveal Novel Subpopulations in Low-Grade Inflammatory Monocytes With Unique Regulatory Circuits. Front Immunol (2021) 12:627036. doi: 10.3389/fimmu.2021.627036

20. Heaton H, Talman AM, Knights A, Imaz M, Gaffney DJ, Durbin R, et al. Souporcell: Robust Clustering of Single-Cell RNA-Seq Data by Genotype Without Reference Genotypes. Nat Methods (2020) 17(6):615–20. doi: 10.1038/s41592-020-0820-1

21. Hao Y, Hao S, Andersen-Nissen E, Mauck WM, Zheng S, Butler A, et al. Integrated Analysis of Multimodal Single-Cell Data. Cell (2021) 184(13):3573–87.e29. doi: 10.1016/j.cell.2021.04.048

22. Finak G, McDavid A, Yajima M, Deng J, Gersuk V, Shalek AK, et al. MAST: A Flexible Statistical Framework for Assessing Transcriptional Changes and Characterizing Heterogeneity in Single-Cell RNA Sequencing Data. Genome Biol (2015) 16(1):278. doi: 10.1186/s13059-015-0844-5

23. Yu G, Wang L-G, Han Y, He Q-Y. Clusterprofiler: An R Package for Comparing Biological Themes Among Gene Clusters. Omics J Integr Biol (2012) 16(5):284–7. doi: 10.1089/omi.2011.0118

24. 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. doi: 10.1186/s13059-014-0550-8

25. Korsunsky I, Millard N, Fan J, Slowikowski K, Zhang F, Wei K, et al. Fast, Sensitive and Accurate Integration of Single-Cell Data With Harmony. Nat Methods (2019) 16(12):1289–96. doi: 10.1038/s41592-019-0619-0

26. Turner AW, Hu S, Mosquera JV, Ma WF, Hodonsky CJ, Wong D, et al. Cell-Specific Chromatin Landscape of Human Coronary Artery Resolves Regulatory Mechanisms of Disease Risk. bioRxiv (2021) 2021.06.07.447388. doi: 10.1101/2021.06.07.447388

27. Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM, et al. Comprehensive Integration of Single-Cell Data. Cell (2019) 177(7):1888–902.e21. doi: 10.1016/j.cell.2019.05.031

28. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, et al. Model-Based Analysis of ChIP-Seq (MACS). Genome Biol (2008) 9(9):R137. doi: 10.1186/gb-2008-9-9-r137

29. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, et al. Minfi: A Flexible and Comprehensive Bioconductor Package for the Analysis of Infinium DNA Methylation Microarrays. Bioinformatics (2014) 30(10):1363–9. doi: 10.1093/bioinformatics/btu049

30. Pidsley R, Zotenko E, Peters TJ, Lawrence MG, Risbridger GP, Molloy P, et al. Critical Evaluation of the Illumina MethylationEPIC BeadChip Microarray for Whole-Genome DNA Methylation Profiling. Genome Biol (2016) 17(1):208. doi: 10.1186/s13059-016-1066-1

31. Touleimat N, Tost J. Complete Pipeline for Infinium ® Human Methylation 450k BeadChip Data Processing Using Subset Quantile Normalization for Accurate DNA Methylation Estimation. Epigenomics (2012) 4(3):325–41. doi: 10.2217/epi.12.21

32. Salas LA, Koestler DC, Butler RA, Hansen HM, Wiencke JK, Kelsey KT, et al. An Optimized Library for Reference-Based Deconvolution of Whole-Blood Biospecimens Assayed Using the Illumina HumanMethylationEPIC BeadArray. Genome Biol (2018) 19(1):64. doi: 10.1186/s13059-018-1448-7

33. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. Limma Powers Differential Expression Analyses for RNA-Sequencing and Microarray Studies. Nucleic Acids Res (2015) 43(7):e47–7. doi: 10.1093/nar/gkv007

34. Pedersen BS, Schwartz DA, Yang IV, Kechris KJ. Comb-P: Software for Combining, Analyzing, Grouping and Correcting Spatially Correlated P-Values. Bioinformatics (2012) 28(22):2986–8. doi: 10.1093/bioinformatics/bts545

35. Ramani A, Müller L, Ostermann PN, Gabriel E, Abida-Islam P, Müller-Schiffmann A, et al. SARS -CoV-2 Targets Neurons of 3D Human Brain Organoids. EMBO J (2020) 39(20):e106230. doi: 10.15252/embj.2020106230

36. Park A, Iwasaki A. Type I and Type III Interferons – Induction, Signaling, Evasion, and Application to Combat COVID-19. Cell Host Microbe (2020) 27(6):870–8. doi: 10.1016/j.chom.2020.05.008

37. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA Methylation Arrays as Surrogate Measures of Cell Mixture Distribution. BMC Bioinf (2012) 13(1):86. doi: 10.1186/1471-2105-13-86

38. Koestler DC, Jones MJ, Usset J, Christensen BC, Butler RA, Kobor MS, et al. Improving Cell Mixture Deconvolution by Identifying Optimal DNA Methylation Libraries (IDOL). BMC Bioinf (2016) 17(1):120. doi: 10.1186/s12859-016-0943-7

39. Liu J, Yang X, Wang H, Li Z, Deng H, Liu J, et al. Analysis of the Long-Term Impact on Cellular Immunity in COVID-19-Recovered Individuals Reveals a Profound NKT Cell Impairment. mBio (2021) 12(2):e00085-21. doi: 10.1128/mBio.00085-21

40. Gogishvili T, Langenhorst D, Lühder F, Elias F, Elflein K, Dennehy KM, et al. Rapid Regulatory T-Cell Response Prevents Cytokine Storm in CD28 Superagonist Treated Mice. PloS One (2009) 4(2):e4643. doi: 10.1371/journal.pone.0004643

41. McKinley L, Logar AJ, McAllister F, Zheng M, Steele C, Kolls JK. Regulatory T Cells Dampen Pulmonary Inflammation and Lung Injury in an Animal Model of Pneumocystis Pneumonia. J Immunol (2006) 177(9):6215–26. doi: 10.4049/jimmunol.177.9.6215

42. Rahimzadeh M, Naderi N. Toward an Understanding of Regulatory T Cells in COVID-19: A Systematic Review. J Med Virol (2021) 93(7):4167–81. doi: 10.1002/jmv.26891

43. Tian Y, Babor M, Lane J, Schulten V, Patil VS, Seumois G, et al. Unique Phenotypes and Clonal Expansions of Human CD4 Effector Memory T Cells Re-Expressing CD45RA. Nat Commun (2017) 8(1):1473. doi: 10.1038/s41467-017-01728-5

44. Odak I, Barros-Martins J, Bošnjak B, Stahl K, David S, Wiesner O, et al. Reappearance of Effector T Cells Is Associated With Recovery From COVID-19. EBioMedicine (2020) 57:102885. doi: 10.1016/j.ebiom.2020.102885

45. Wilk AJ, Rustagi A, Zhao NQ, Roque J, Martínez-Colón GJ, McKechnie JL, et al. A Single-Cell Atlas of the Peripheral Immune Response in Patients With Severe COVID-19. Nat Med (2020) 26(7):1070–6. doi: 10.1038/s41591-020-0944-y

46. Chen Z, John Wherry E. T Cell Responses in Patients With COVID-19. Nat Rev Immunol (2020) 20(9):529–36. doi: 10.1038/s41577-020-0402-6

47. Ganji A, Farahani I, Khansarinejad B, Ghazavi A, Mosayebi G. Increased Expression of CD8 Marker on T-Cells in COVID-19 Patients. Blood Cells Mol Dis (2020) 83:102437. doi: 10.1016/j.bcmd.2020.102437

48. Bhat P, Leggatt G, Waterhouse N, Frazer IH. Interferon-γ Derived From Cytotoxic Lymphocytes Directly Enhances Their Motility and Cytotoxicity. Cell Death Dis (2017) 8(6):e2836–6. doi: 10.1038/cddis.2017.67

49. Kang S, Brown HM, Hwang S. Direct Antiviral Mechanisms of Interferon-Gamma. Immune Netw (2018) 18(5):e33. doi: 10.4110/in.2018.18.e33

50. Sawalha AH, Zhao M, Coit P, Lu Q. Epigenetic Dysregulation of ACE2 and Interferon-Regulated Genes Might Suggest Increased COVID-19 Susceptibility and Severity in Lupus Patients. Clin Immunol (2020) 215:108410. doi: 10.1016/j.clim.2020.108410

51. Yin Y, Liu X, He X, Zhou L. Exogenous Coronavirus Interacts With Endogenous Retrotransposon in Human Cells. Front Cell Infect Microbiol (2021) 11:609160. doi: 10.3389/fcimb.2021.609160

52. 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. Nat Immunol (2022) 23(2):210–6. doi: 10.1038/s41590-021-01113-x

Supplementary Information

Supplementary Tables

Supplementary table 1. Flow cytometry antibodies used for the analysis of immune cells

Reagent Or Resource	Source	Identifier
Granulocyte panel		
anti-CD16 FITC (clone 3G8)	Beckman Coulter	Cat#B49215; RRID: AB_2848116
anti-CD10 PE (HI10A)	BioLegend	Cat#312203; RRID: AB_314914
anti-CD11b PE-Dazzle (ICRF44)	BioLegend	Cat#301347; RRID: AB_2564080
anti-CD14 PE-Cy5.5 (clone M5E2)	BioLegend	Cat#301847; RRID: AB_2564058
anti-CD62L PE-Cy7 (clone DREG-56)	BioLegend	Cat#304821; RRID: AB_830800
anti-PD-L1 APC (clone MIH1)	ThermoFisher	Cat#17598342; RRID: AB_10597586
anti-CD66b AF700 (G10F5)	BioLegend	Cat#305113; RRID: AB_2566037
anti-CD15 Brilliant Violet 421 (clone W6D3)	BioLegend	Cat#323039; RRID: AB_2566519
anti-CD45 Krome Orange (clone J33)	Beckman Coulter	Cat#A96416; RRID: AB_2833027
General panel		
anti-CD16 FITC (clone 3G8)	Beckman Coulter	Cat#B49215; RRID: AB_2848116
anti-HLA-DR PE (clone immu-357)	Beckman Coulter	Cat#IM1639; RRID: AB_131284
anti-CD14 ECD (clone RM052)	Beckman Coulter	Cat#B92391; RRID: AB_130853
anti-CD4 PE-Cy5.5 (clone 13B8.2)	Beckman Coulter	Cat#B16491; RRID: Unknown
anti-CD25 PE-Cy7 (clone M-A251)	BD Biosciences	Cat#557741; RRID: AB_396847
anti-CD56 APC (clone N901)	Beckman Coulter	Cat#IM2474; RRID: AB_130791
anti-CD8 APC-AF700 (clone B9.11)	Beckman Coulter	Cat#B49181; RRID: AB_2750854
anti-CD19 APC-AF750 (clone J3-119)	Beckman Coulter	Cat#A94681; RRID: AB_2833030
anti-CD3 Pacific Blue (clone UCHT1)	Beckman Coulter	Cat#A93687; RRID: AB_2728095
anti-CD45 Krome Orange (clone J33)	Beckman Coulter	Cat#A96416; RRID: AB_2833027
B cell panel		
anti-IgD FITC (clone IADB6)	Southern Biotech	Cat#2032-02; RRID: AB_2687521
anti-IgM PE (clone SA-DA4)	Beckman Coulter	Cat#B30657; RRID: unknown
anti-CD3 ECD (clone UCHT1)	Beckman Coulter	Cat#A07748; RRID: unknown
anti-CD27 PE-Cy5.5 (clone 1A4CD27)	Beckman Coulter	Cat#B21444; RRID: unknown
anti-CD38 PE-Cy7 (clone LS198-4-3)	Beckman Coulter	Cat#B49198; RRID: unknown
anti-CD24 APC (clone ALB9)	Beckman Coulter	Cat#A87785; RRID: unknown

Supplementary table 1. Continued

Reagent Or Resource	Source	Identifier
anti-CD5 APC-AF700 (clone BL1a)	Beckman Coulter	Cat#A78835; RRID: unknown
anti-CD19 APC-AF750 (clone J3-119)	Beckman Coulter	Cat#A94681; RRID: AB_2833030
anti-CD20 Pacific Blue (clone B9E9)	Beckman Coulter	Cat#B49208; RRID: unknown
anti-CD45 Krome Orange (clone J33)	Beckman Coulter	Cat#A96416; RRID: AB_2833027
Senescence panel		
anti-CD57 FITC (clone NC1)	Beckman Coulter	Cat#IM0466U; RRID: unknown
Anti-CD28 PE (clone CD28.1)	DAKO	Cat#R7164; RRID: AB_579570
Anti-CD45RA ECD (clone 2H4LDH11LDB9)	Beckman Coulter	Cat#B49193; RRID: unknown
Anti-KLRG1 PerCp-Cy5.5 (clone 2F1)	Biolegend	Cat#138418; RRID: AB_2563014
Anti-CD279 PE-Cy7 (clone EH12.2H7)	Biolegend	Cat#329918; RRID: AB_2159324
Anti-CD25 APC (clone 2A3)	BD Biosciences	Cat#340907; RRID: AB_2819021
Anti-CD4 AF700 (clone RPA-T4)	eBioscience	Cat#56-0049-42; RRID: AB_11219085
anti-CD8 APC-AF750 (clone B9.11)	Beckman Coulter	Cat#A94683; RRID: unknown
Anti-CD197 BV421 (clone G043H7)	Biolegend	Cat#353208; RRID: AB_11203894
anti-CD45 Krome Orange (clone J33)	Beckman Coulter	Cat#A96416; RRID: AB_2833027

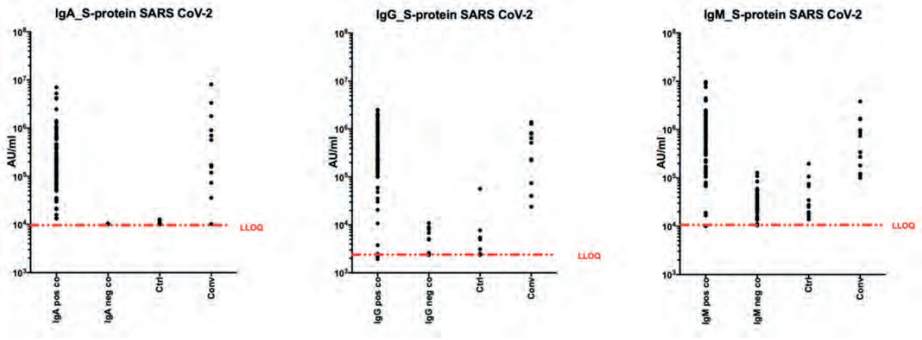
Supplementary table 2. Human primers used for RT-qPCR

Gene	Primer	Sequence
HPRT1	Forward	CCTGGCGTCGTGATTAGTGAT
	Reverse	AGACGTTCAGTCCTGTCCATAA
IFI44L	Forward	AGGGAATCATTTGGCTCTGTAGA
	Reverse	AGCCGTCAGGGATGTACTATAAC
IFI6	Forward	GGTCTGCGATCCTGAATGGG
	Reverse	TCACTATCGAGATACTTGTGGGT
IRF3	Forward	AGAGGCTCGTGATGGTCAAG
	Reverse	AGGTCCACAGTATTCTCCAGG
IRF7	Forward	GCTGGACGTGACCATCATGTA
	Reverse	GGGCCGTATAGGAACGTGC
IRF9	Forward	GATACAGCTAAGACCATGTTCCG
	Reverse	TGATACACCTTGTAGGGCTCA
ISG15	Forward	CGCAGATCACCCAGAAGATCG
	Reverse	TTCGTCGCATTTGTCCACCA
OAS2	Forward	CTCAGAAGCTGGGTTGGTTTAT
	Reverse	ACCATCTCGTCGATCAGTGTC

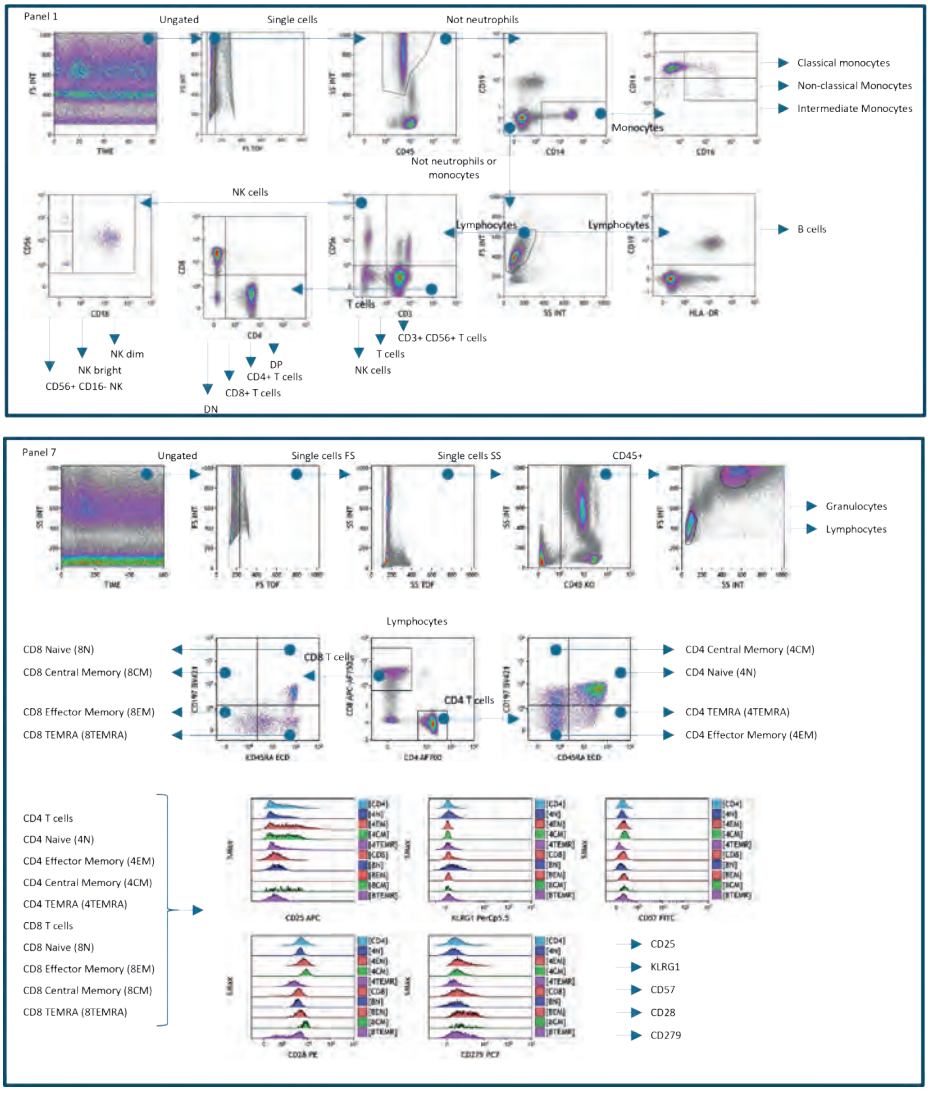
Supplementary table 3. Conditions of the RT-qPCR reaction

Step	Temperature	Duration	Cycle #
Initial Denaturation	95°C	10 minutes	1
Denaturation	95°C	15 seconds	40
Annealing/Extension	60°C	60 seconds	
Hold	4°C	∞	

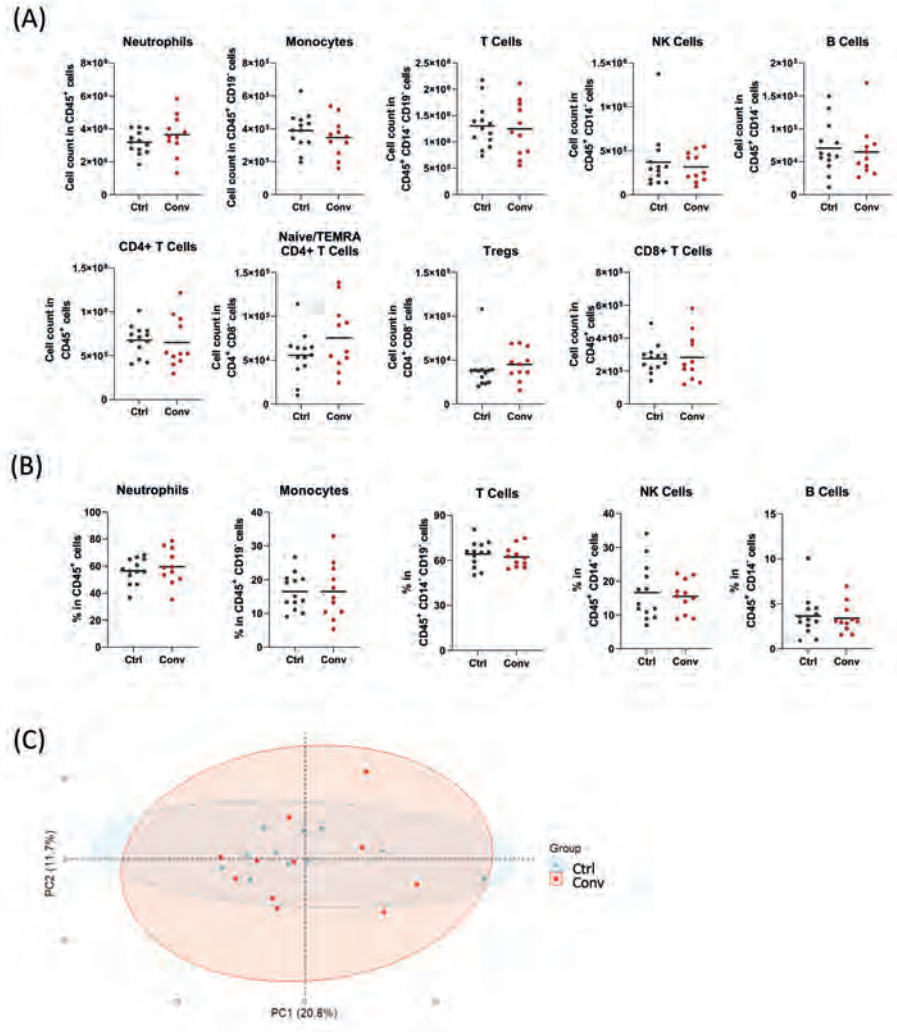
Supplementary Figures



Supplementary figure 1. IgA, IgG, and IgM antibodies against spike protein in the study participants

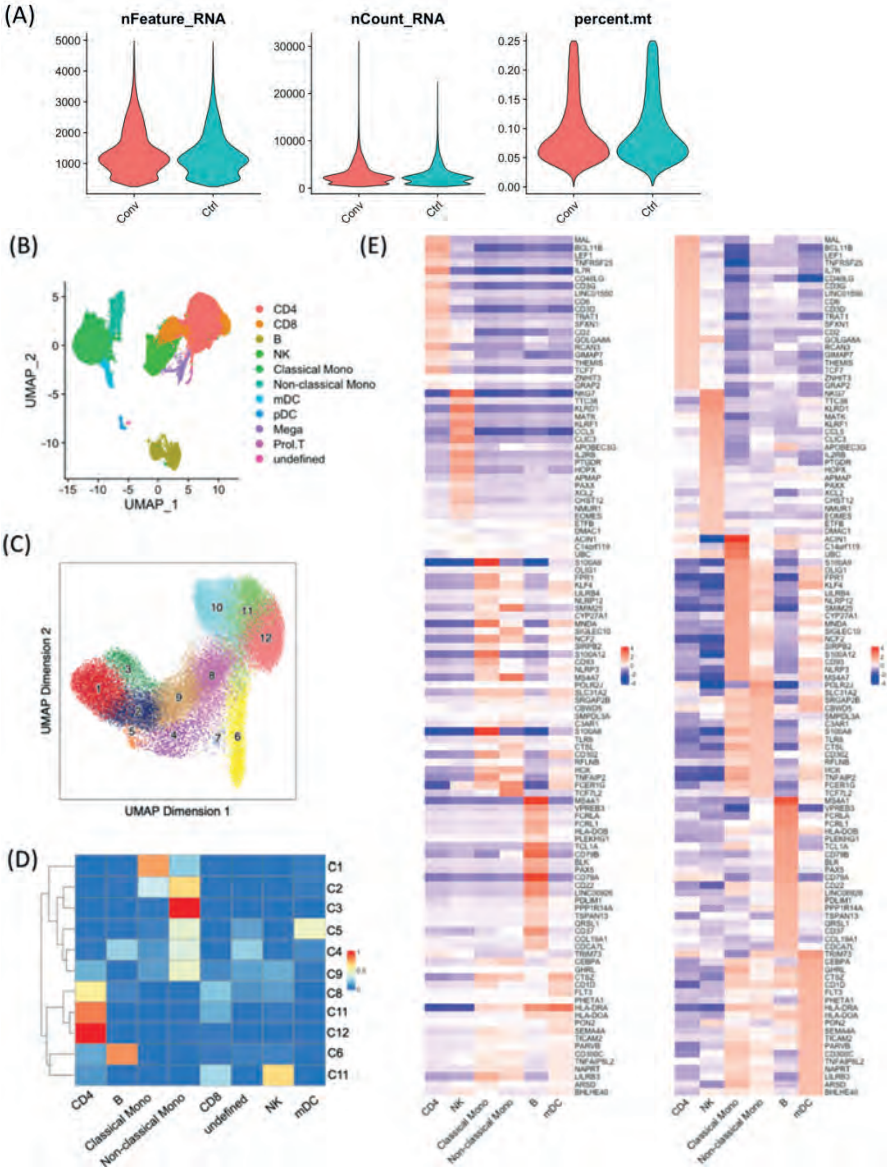


Supplementary figure 2. Gating strategy used in flow cytometry analyses



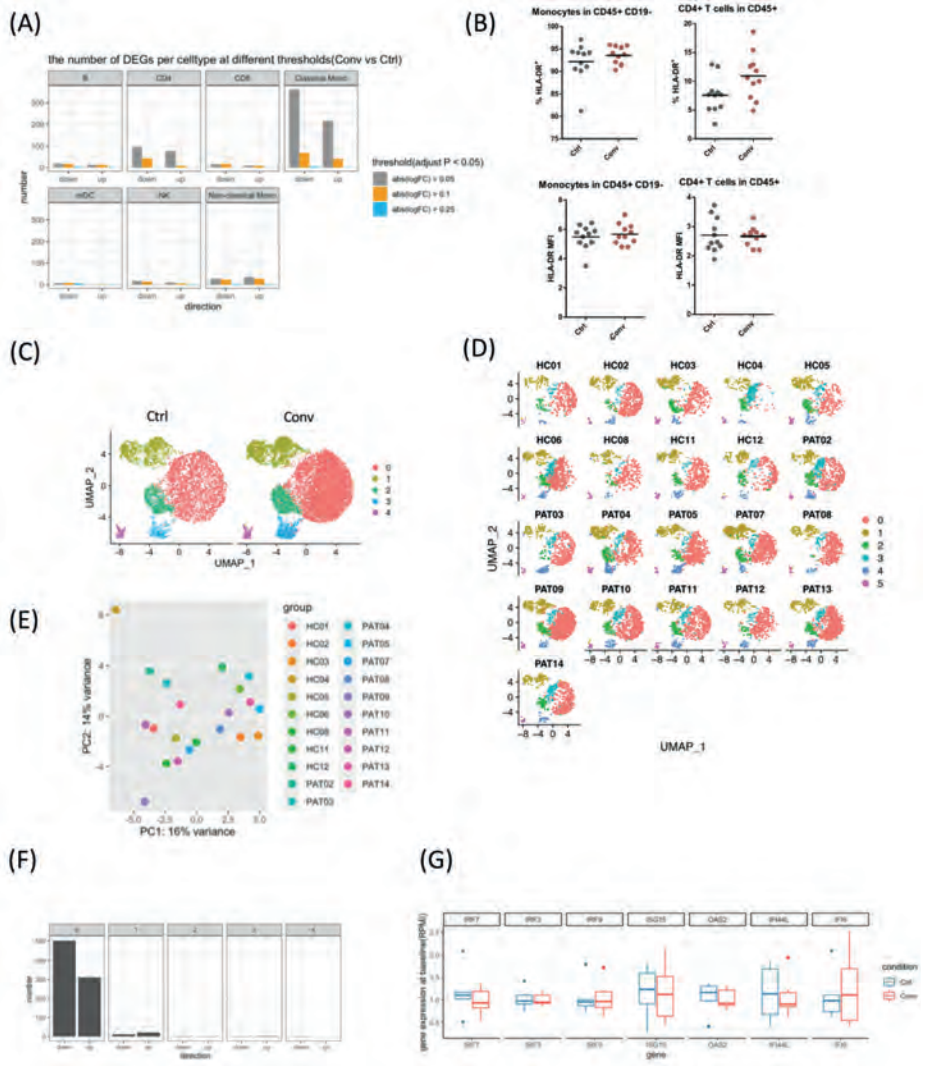
Supplementary figure 3. Flow cytometry analysis of cell populations in the blood

(A) Dot plots showing the absolute cell counts of immune cell populations in the blood. **(B)** Dot plots showing the percentages of the main cell populations in the blood of healthy controls and convalescent COVID-19 patients. **(C)** Principal component analysis of the cell populations based on the absolute cell counts.



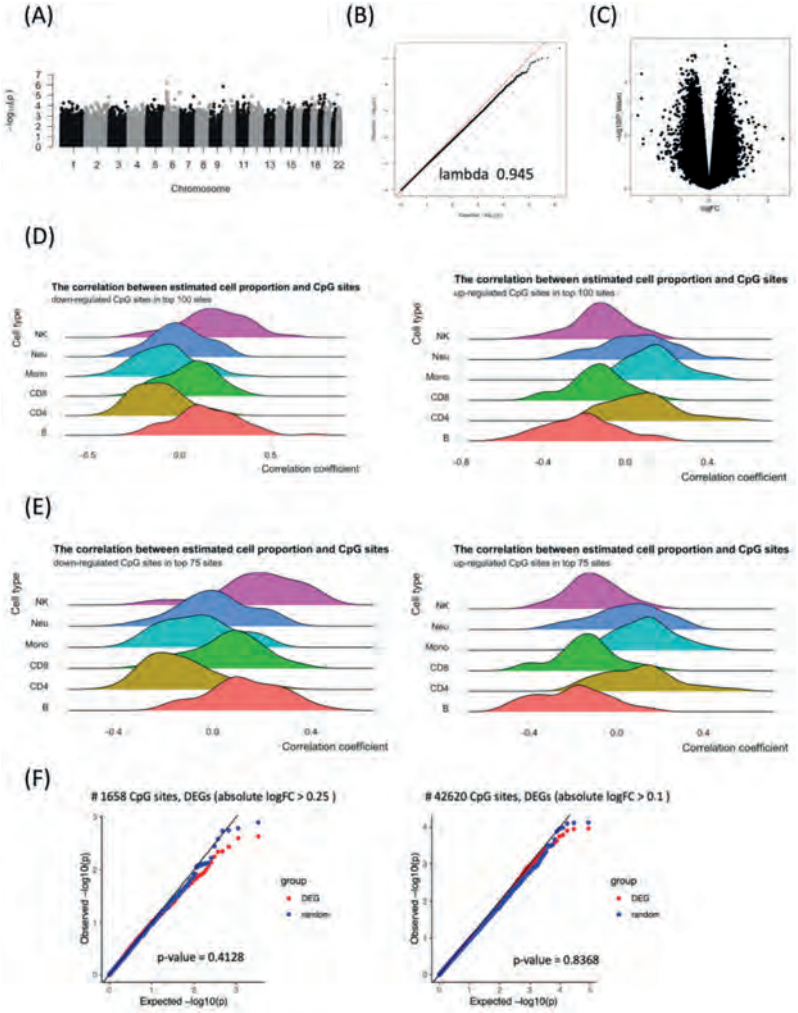
Supplementary figure 4. Single-cell RNA-seq and ATAC-seq analysis of PBMCs in convalescent COVID-19 individuals

(A) Number of RNA features, counts, and mitochondria percentage after QC for the scRNA-seq dataset. **(B)** UMAP visualization of scRNA-seq profiling in both convalescent COVID-19 patients and controls. 11 cell clusters were annotated, each color indicated one cell type. **(C)** UMAP visualization of scATAC-seq profiling in both convalescence COVID-19 and controls. 12 cell clusters were identified. **(D)** Heatmap showing the integration results of scATAC-seq and scRNA-seq data. Color indicates the level of overlapping between two clusters from scATAC-seq and scRNA-seq. C1- C12 were corresponding to the cell clusters labelled in **(C)**. **(E)** Heatmap showing the expression level of the top overlapping markers in scATAC-seq (left) and scRNA-seq (right).



Supplementary figure 5. Transcriptional changes from single-cell RNA and ISG expression at baseline level

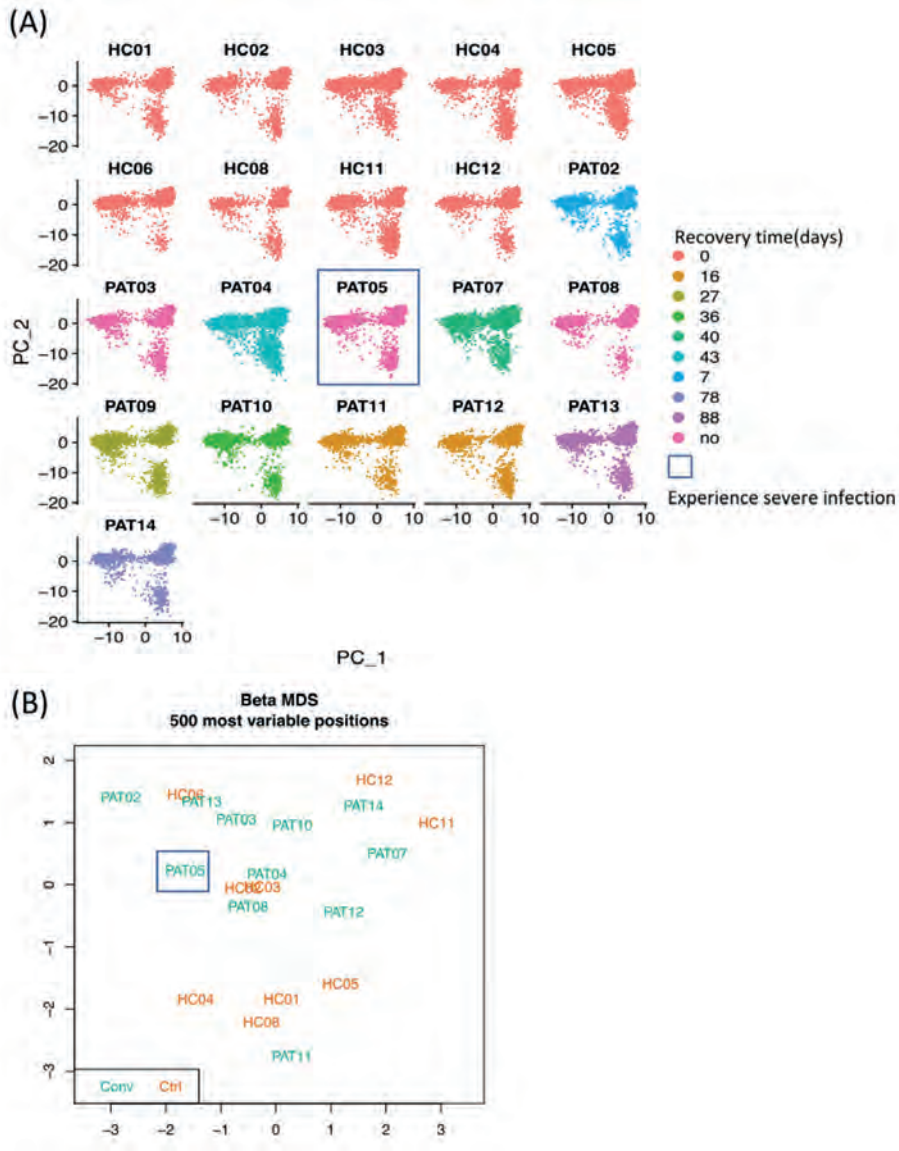
(A) The number of down- or up-regulated DEGs found in each cell type. The color for each bar depicts different thresholds of the log fold change. **(B)** Surface expression of HLA-DR in monocytes and CD4+ T cells, analyzed by flow cytometry. Both HLA-DR+ cells and the mean fluorescent intensity (MFI) of HLA-DR were shown. **(C)** UMAPs showing the sub- clusters in classical monocytes. **(D)** UMAPs showing the sub-clusters in classical monocytes split by samples. **(E)** PCA on classical monocytes specific-pseudo-bulk RNA result. **(F)** The number of DEGs between convalescence COVID-19 and controls were identified in each cluster. **(G)** Baseline ISG expressions level between controls and convalescent individuals.



Supplementary figure 6. Epigenome-wide association analysis of convalescent COVID-19 and healthy controls

(A) The Manhattan plot showing the epigenome-wide association analysis (EWAS) results comparing DNA methylation profiles between convalescent COVID-19 individuals and controls. The x-axis is the chromosomal position, and the y axis is the significance on a – log 10 scales. No significant CpG sites were identified with FDR < 0.05. **(B)** The quantile-quantile (Q-Q) plot showing the distribution of the -log10 (P value) from the EWAS result and the expected - log10(P value). The lambda value is 0.945. **(C)** The volcano plot showing the log fold change and the -log10(P value) for each CpG site. **(D)** The density ridgeline plots showing the correction between the estimated cell proportions and the down-regulated(left) or up-regulated(right) CpG sites in the top 100 differentially methylated CpG sites. **(E)** The density ridgeline plots showing the correction between the estimated cell proportions and the down-regulated(left) or up-regulated (right) CpG sites in the top 75 differentially methylated CpG sites. **(F)** The quantile- quantile (Q-Q) plot showing the distribution of the -log10(P value) and the expected -log10 (P value) for the CpG sites in the proximity (+/- 250kb) of the DEGs from monocytes and those random choose from our EWAS result. Red dot represented the CpG sites around DEGs, Blue dot represented the random chosen CpG sites. Two-

sample Kolmogorov-Smirnov test was used to compare the distributions of the p values from two groups.



Supplementary figure 7. Dimensional reduction plot in single-cell RNA and methylation data

(A) PCA plot in each individual. Recovery time was defined as the disappearance of symptoms to sample collection. "o" represents the healthy control group. "no" in the legend indicates the missing value in our clinical information. **(B)** Multi-dimensional scaling (MDS) plots showing a 2-d projection of distances between participants from methylation data.

King penguin
Kral penguen
Koningspinguïn

CHAPTER 7

Alendronate modulates cytokine responses in healthy young individuals after BCG vaccination

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Abstract

Bacillus Calmette–Guérin (BCG) vaccination induces memory characteristics in innate immune cells and their progenitors, a process called trained immunity mediated by epigenetic and metabolic reprogramming. Cholesterol synthesis plays an amplifying role in trained immunity through mevalonate release. Nitrogen-containing bisphosphonates (N-BPs), such as alendronate, can inhibit cholesterol synthesis. We explored their effects on trained immunity induced by BCG in a placebo-controlled clinical study (NL74082.091.20) in young, healthy individuals. Participants receiving single-dose oral alendronate on the day of BCG vaccination had more neutrophils and plasma cells one month after treatment. Alendronate led to reduced proinflammatory cytokine production by PBMCs stimulated with heterologous bacterial and viral stimuli one month later. Furthermore, the addition of alendronate transcriptionally suppressed multiple immune response pathways in PBMCs upon stimulation. Our findings indicate that N-BPs modulate the long-lasting effects of BCG vaccination on the cytokine production capacity of innate immune cells.

Introduction

The innate immune system responds early and rapidly after an infection. Although able to distinguish self from non-self, responses generated by innate immunity are antigen-agnostic [1]. Traditionally, only the adaptive immune system was thought capable of developing immunological memory. However, more recent research revealed that the innate immune system also mounts a memory-like response through epigenetic and metabolic programming of innate immune cells, which subsequently exhibit a more robust response to secondary infections. This trait, also termed trained immunity, can be induced by several live-attenuated vaccines, including Bacillus Calmette–Guérin (BCG), measles, and oral polio, that were shown to induce protection against heterologous infections [2].

Initially developed against tuberculosis, BCG also reduces all-cause childhood mortality through protection against a wide range of infections [3]. This non-specific protection is partly mediated by the changes in the epigenetic regulation of the function of innate immune cells and metabolic reprogramming marked by an increase in glycolysis and oxidative phosphorylation [4]. Moreover, BCG vaccination leads to persistent transcriptomic changes in human hematopoietic stem and progenitor cells and a myeloid differentiation bias in the bone marrow [5]. However, the efficacy of inducing trained immunity differs between individuals [6], and strategies to improve trained immune responses are needed.

Bisphosphonates (BPs) are a class of drugs mainly used to treat and prevent bone resorption [7]. They have an affinity for the bone and work as a calcium-chelating agent. So far, there are around ten BPs for human use in different conditions, varying from osteoporosis to Paget's disease to cancer. Some BPs, including alendronate, contain nitrogen in their side chains and have a different mechanism of action compared to simple BPs. Aside from targeting bone-resorbing osteoclasts, nitrogen-containing BPs (N-BPs) are internalized by monocytes and macrophages [8-10], and they inhibit cholesterol synthesis by blocking the activity of farnesyl pyrophosphate (FPP) synthase [11]. This inhibition leads to the accumulation of isopentenyl diphosphate and dimethylallyl diphosphate in peripheral blood mononuclear cells (PBMCs), with immunomodulatory activities such as activation of γδ T cells [8, 12].

Blockade of FPP synthase by alendronate can also lead to the accumulation of metabolites in the mevalonate pathway. Mevalonate accumulation in monocytes was previously linked to a stronger trained immunity phenotype [13]. Furthermore, another N-BP zoledronate drove both peritoneal and tumor-associated macrophages

toward the pro-inflammatory and tumoricidal M1 phenotype in a mouse model of breast cancer [14]. A few observational studies found a lower risk of infections and cancer in people with chronic use of N-BPs [15-17]. Due to its low adverse event profile and potential immune-stimulating properties, we hypothesized that N-BPs could be a good candidate to improve BCG vaccine efficacy.

To test the potential of N-BPs as an adjuvant to improve trained immunity, we designed a clinical study in which the participants received either a placebo vaccination, the BCG vaccine, or the BCG vaccine together with oral alendronate tablets. We collected blood samples from participants before and one month after the intervention and measured cytokine responses following *ex vivo* stimulation of PBMCs with various stimuli. Furthermore, we performed flow cytometry from the whole blood to identify immune cell subsets and RNA sequencing (RNAseq) from PBMCs to assess the transcriptional responses.

Materials and methods

Study design and subjects

Healthy adults were recruited between June and August 2020 at the Radboud University Medical Center. Study subjects did not use any chronic medication except for oral contraceptives, did not have comorbidities, and were BCG-naïve by the time of inclusion. No power calculation was possible due to the absence of information on alendronate's potential effect size; therefore, this clinical trial was designed as an exploratory study. Participants were randomized as 1:1:1 to each group using the Castor electronic data capture platform to receive 9 mg/ml intradermal sodium chloride (Centrafarm, Netherlands) in 0.1 ml as placebo for BCG vaccination, 0.75 mg/ml intradermal BCG vaccine (AJ Vaccines, Denmark) in 0.1 ml, or intradermal BCG vaccine right after orally ingesting a 70 mg alendronate tablet (Aurobindo Pharma, India). 18, 21 and 18 participants completed the study in each arm, respectively. Age, sex, and BMI distributions in each group are provided in **Table 1**. Blood was collected from participants before and 1 month after vaccination. Ethical approval for the study (NL74082.091.20) was granted by the local ethics committee CMO region Arnhem-Nijmegen.

Table 1. Demographics of the study participants

	Placebo (n=18)	BCG (n=21)	BCG+Alendronate (n=18)	p-value
Age (years, mean±sd)	26.9±7.4	26.9±8.1	30.5±9.5	0.296
Sex (F/M)	7/11	16/5	8/10	0.039
BMI (kg/m2, mean±sd)	23.6±1.8	22.9±3.1	23.8±3.7	0.604

Flow cytometry from whole blood

Before flow cytometry staining, the number of immune cells in the whole blood was determined using a hematology analyzer (Sysmex, Japan). The hematology analyzer relies on flow cytometry principles to identify different cell populations in the blood: the forward scatter light determines cell volume, the side scatter light gives information about cell nuclei and granules, while the side fluorescence indicates nucleic acids and organelles. As a result, cells having similar properties appear in a cluster.

For flow cytometry staining, erythrocytes were first lysed in isotonic NH4CL buffer and washed twice with PBS. White blood cell counts were determined by Coulter Ac-T Diff® cell counter (Beckman Coulter, CA, USA) and used to calculate the absolute numbers of $CD45^+$ leukocytes identified by flow cytometry. 500.000 total leukocytes were used per staining panel. Cells were transferred to a V-bottom 96-well plate, washed twice with PBS + 0.2% bovine serum albumin (BSA) (Sigma-Aldrich, MO, USA), and stained in the dark for 20 minutes at room temperature. Afterward, cells were washed twice more with PBS + 0.2% BSA and measured with the Navios™ flow cytometer (Beckman Coulter). Details of the panels and antibodies are provided in **Supp. Table 1**. Kaluza 2.1® software (Beckman Coulter) was used for data analysis.

PBMC isolation and *ex vivo* stimulation

Whole blood was diluted with PBS, and PBMCs were isolated using density gradient centrifugation with Ficoll-Paque (GE Healthcare, IL, USA). The middle PBMC fraction was collected and washed three times with cold PBS. The cells were resuspended and stimulated in RPMI 1640 Medium (Dutch modification) (Thermo Fisher Scientific, MA, USA) supplemented with 1mM sodium pyruvate (Thermo Fisher Scientific), 2mM GlutaMAX supplement (Thermo Fisher Scientific), and 50 µg/mL gentamicin (Centrafarm, Netherlands). The PBMCs were isolated and used for experiments within 4 hours after blood collection.

500.000 PBMCs per well were stimulated with 10 ng/ml E. coli-derived LPS, 10^6/ml *S. aureus*, 10 µg/ml poly(I:C) (Invivogen, CA, USA), 3 µg/ml R848 (Invivogen), $3.3x10^5$ K/mL TCID50 heat-inactivated influenza A H1N1 and $1.4x10^3$ K/mL TCID50 heat-

inactivated SARS-CoV-2 Wuhan strain for 24 hours or 7 days in the presence of 10% pooled human serum at 37°C with 5% CO_2. Virus inactivation was performed at 60°C for 30 minutes. After stimulation, cytokine levels in supernatants were measured using DuoSet® ELISA kits (R&D Systems, MN, USA) following the manufacturer's protocols. Only for IFNα, Human IFN-Alpha ELISA Kit from PBL Assay Science (NJ, USA) was used.

RNA isolation and sequencing

10 subjects from each intervention group were selected for RNA sequencing. The demographics of the subjects were given in **Supp. Table 2**. Cryopreserved PBMCs were thawed and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Corning, NY, USA) and 2 mM L-glutamine (Thermo Fisher) for 2 hours. After incubation, samples were washed with PBS, filtered, and counted. For samples which were also stimulated with Poly(I:C) (limited to 5 individuals per group with sufficient remaining cells, details in **Supp. Table 3**), an additional 1 million cells were plated into one well of a 12-well plate and stimulated with 20 ug/mL poly(I:C) (Invivogen) for 4 hours. For each sample, 1 million cells were lysed in RLT buffer (Qiagen, MD, USA) and stored at -80°C for RNA extraction and sequencing. RNA extractions were performed using the miRNeasy mini or miRNeasy micro kits (Qiagen). RNA quality was evaluated with the 2100 Bioanalyzer (Agilent Technologies).

RNA library preparations were carried out on 100-500 ng of RNA using the Illumina TruSeq Stranded Total RNA Sample preparation kit, according to the manufacturer's instructions. The libraries were size-selected using Ampure XP Beads (Beckman Coulter) and quantified using the KAPA Library Quantification kit – Universal (KAPA Biosystems). The RNA-Seq libraries were sequenced on the Illumina NovaSeq 6000 system using 100-bp single-end sequencing.

The quality of raw sequencing reads was assessed using FastQC v0.11.5 [18]. Reads were mapped to GENCODE human genome model (GRCh38 V34) using STAR 2.7.9a [19]. Gene transcripts were assembled and quantified on their corresponding human genome using the count-based method featureCounts [20] available in R from package Subread 2.0.3.

Differential gene expression and gene set enrichment analyses

Gene expression levels across all samples were first normalized using the calcNormFactors function implemented in the edgeR R package (version 3.34.0), which utilizes the TMM algorithm (weighted trimmed mean of M-values) to compute normalization factors. Then, the voom function implemented in the limma package (version 3.38.3) was used to

log-transform the data and to calculate precision weights. A weighted fit using the voom-calculated weights was performed with the lmFit function from limma.

To investigate the impact of vaccination on baseline (before intervention) PBMC gene expression, normalized, log-transformed gene expression levels of unstimulated samples were fit to the linear model Expression ~ 1 + individual + timepoint:vaccination, which corrects for natural differences in baseline gene expression between individuals and therefore captures the independent effect of each vaccination condition on gene expression after 1 month.

To investigate the impact of vaccination on the PBMC response to poly(I:C) stimulation, we fit normalized, log-transformed gene expression levels to the linear model Expression ~ 1 + individual + timepoint + stimulus:timepoint and used the makeContrasts and contrasts.fit functions implemented in limma to compare the gene expression response to poly(I:C) before vaccination, with the response to poly(I:C) one month post-vaccination.

Gene set enrichment analyses (GSEA) were performed using the fgsea R package (version 1.18.0) with parameters: minSize = 15. To investigate biological pathway enrichments among genes responsive to vaccination (placebo, BCG, or BCG+Alendronate) or with altered responses to poly(I:C) stimulation before compared to after vaccination (placebo, BCG, or BCG+Alendronate), genes were ordered by the rank statistic: -log10(pvalue)*logFC and compared with the Hallmark gene sets from the MSigDB collections.

IL-6 concentration measurements in plasma

Whole blood in EDTA tubes was centrifuged at 3800 RPM for 10 minutes to obtain plasma. The plasma samples were then stored at -80 until testing. IL-6 levels in the plasma samples were measured using the Human IL-6 Quantikine HS ELISA (R&D Systems) according to the manufacturer's instructions.

Statistical analyses

Statistical analyses apart from RNA sequencing were performed using GraphPad Prism 8 (GraphPad Software Inc., CA, USA) or R 3.6.1 (www.R-project.org). Comparisons between the two time points were performed with the Wilcoxon matched-pairs signed rank test. Mann–Whitney U test was used for comparisons between treatment groups. p values below 0.05 were considered statistically significant.

Results

Study design and demographics

Eighteen, twenty-one, and eighteen participants who completed the study were randomized to receive a placebo vaccine, BCG vaccine, and BCG vaccine with an oral alendronate tablet, respectively. Venous blood was collected before the intervention and during the follow-up visit one month later. Flow cytometry was performed to analyze the changes in the immune cell populations in the blood. PBMCs isolated from blood were incubated with different bacterial and viral stimuli for 24 h and 7 days, and cytokine levels were measured. The study design is visually presented in **Fig. 1**.

The participant demographics are given in **Table 1**. There were no significant age and BMI differences between the intervention groups; however, there were more females in the BCG group compared to the other study groups.

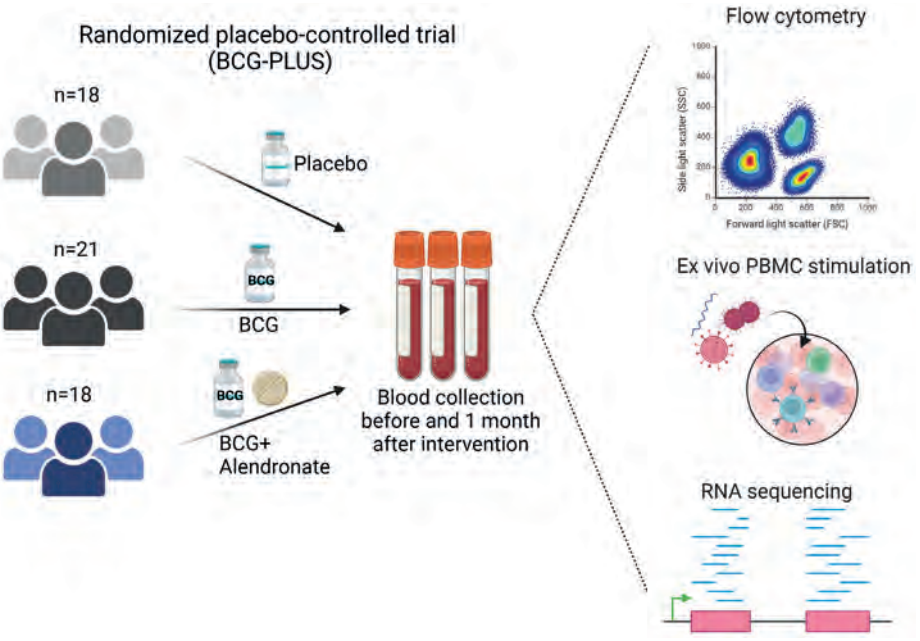

Figure 1. Study Design

Blood was collected from the study participants before and 1 month after the intervention. Flow cytometry was performed using whole blood. Following PBMC isolation from the blood, *ex vivo* stimulations were performed to measure cytokine production, and cells were stored for RNA sequencing.

Alendronate used together with BCG vaccination led to increased plasma cell and neutrophil numbers in the circulation

Using different cell surface markers, we identified circulating immune cell populations in the blood by flow cytometry. Principal component analysis (PCA) showed that the baseline immune cell counts were similar between the groups (**Fig. 2A, left**). One month after treatment, there was no significant difference in immune cell populations between different treatment groups (**Fig. 2A, right**). Although the abundance of immune cells at the baseline and after treatment remained similar between the groups, the size of plasma and naïve B cell populations exhibited differences after treatment compared to baseline (**Fig. 2B**). The number of naïve B cells increased after the intervention in all groups, including the placebo, although the increase was higher in the BCG and BCG+alendronate groups. Furthermore, the number of plasma cells in the blood became significantly higher in the BCG+alendronate group after the treatment compared to the other groups. We found no additional differences in immune cell numbers after the interventions, except for more intermediate monocytes and regulatory T cells (Tregs) one month after treatment in all groups (**Supp. Fig. 1A**). The reason for this is unclear, but the fact that it was observed in all groups argues for either a seasonality effect or batch-effect between time points.

Since neutrophils were not included in the flow cytometry panel, we also used the whole blood counts obtained with a hematology analyzer. Interestingly, we found that the total numbers of white blood cells and neutrophils were significantly increased only in the BCG+alendronate group one month after treatment compared to baseline **(Fig. 2C).** Monocyte and lymphocyte numbers did not change after the treatments **(Supp. Fig. 1B).** These data show increased peripheral neutrophil and plasma cell counts one month after receiving the BCG vaccine and oral alendronate treatment.

Combining alendronate with BCG vaccination reduced TNFα production against bacterial and viral stimuli

BCG vaccine can improve cytokine response to unrelated pathogens starting from 2 weeks up to a year after vaccination [21,22]. However, the effects of BCG vaccination in the present study were lower, and IL-6, TNFα, and IL-1RA production did not significantly increase one month after BCG vaccination (**Fig. 3**). Without any secondary stimulation, BCG vaccination (with or without alendronate) led to a trend of higher basal IL-1RA production, but no statistical significance was reached (**Supp. Fig. 2**). Of note, when sexes were analyzed separately, BCG vaccination's impact was more apparent in female participants, although statistical significance was reached only for the TNFα response against poly(I:C) (**Supp. Fig. 3**). However, this might simply be due to the low number of BCG-vaccinated male participants in the study.

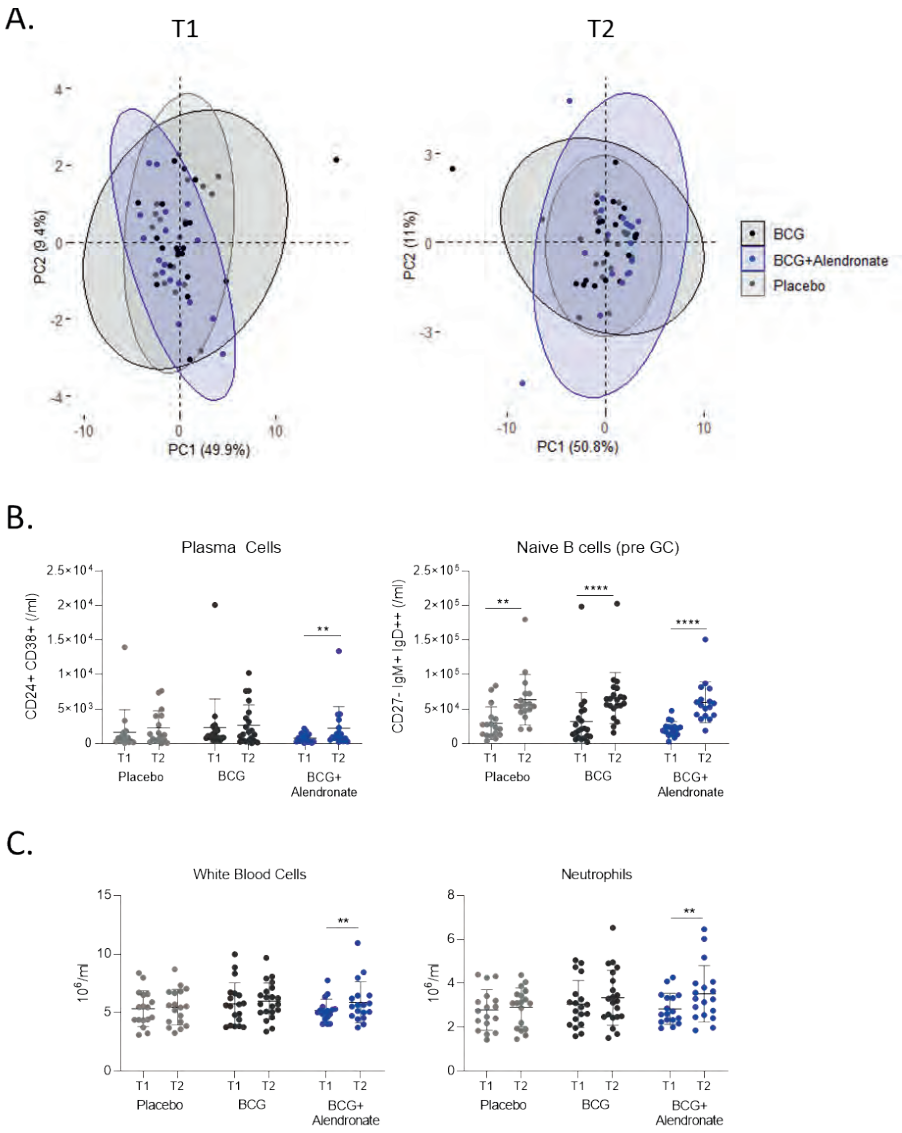

Figure 2. Immune cell counts measured by flow cytometry and a hematology analyzer before (T1) and after (T2) treatment

A) Principal component analyses of the immune cell populations before (left) and one month after (right) treatment. **B)** Plasma and naïve B cell counts measured using flow cytometry. **C)** Total white blood cell and neutrophil counts measured by a hematology analyzer. Cell numbers before and after treatment were compared using the Wilcoxon matched-pairs signed-rank test. $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$.

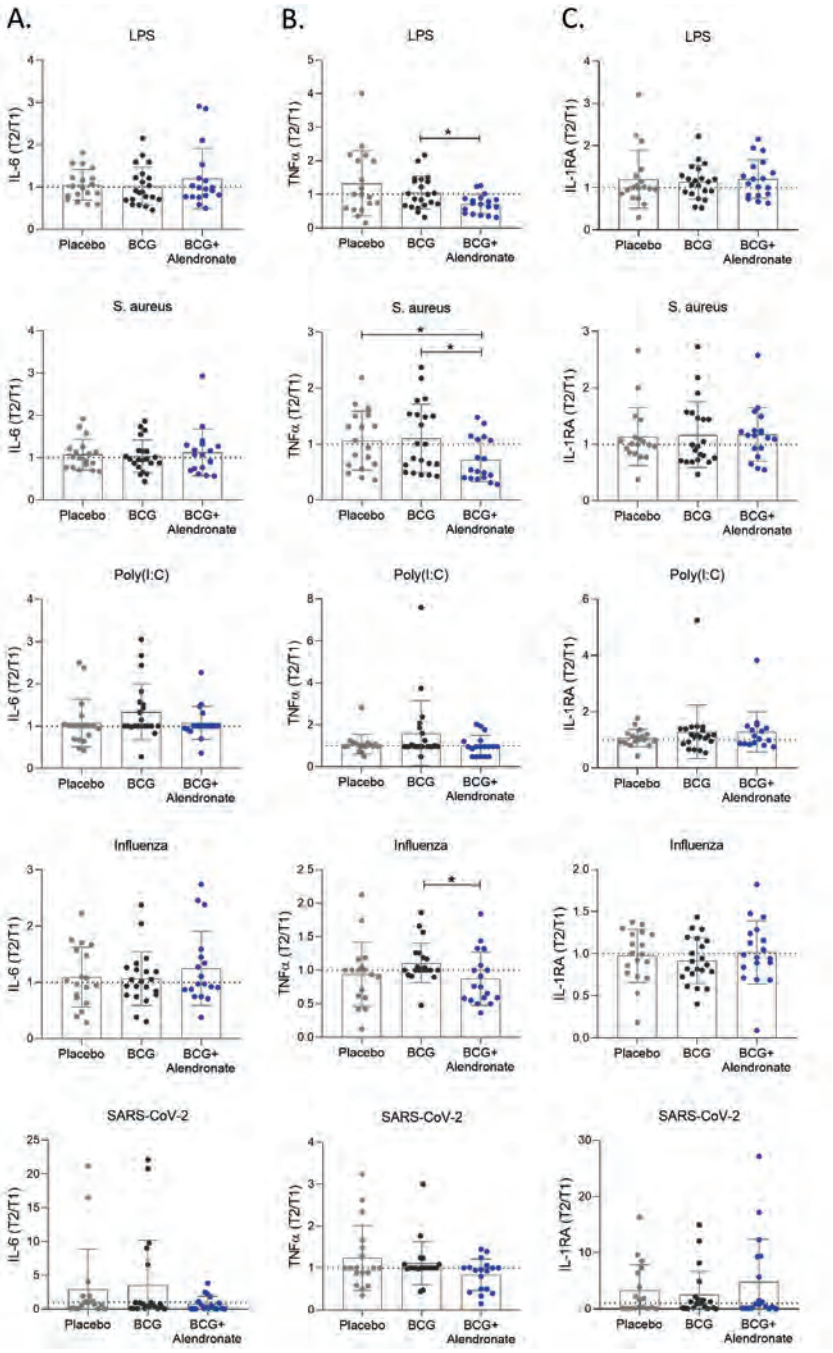

Figure 3. Fold changes (T2/T1) of A) IL-6, B) TNFα, and C) IL-1RA production by PBMCs upon bacterial and viral stimulation

Dotted lines depict the fold change of 1. Groups were compared with the Mann-Whitney U test. T1: before the intervention, T2: one month after the intervention. *$p \leq 0.05$.

The simultaneous administration of alendronate and BCG did not significantly impact IL-6 production (**Fig. 3A**). Upon LPS or *S. aureus* stimulation, TNFα production was lower in the group that received alendronate combined with BCG compared to the group receiving BCG alone (**Fig. 3B**). Alendronate also decreased the TNFα response against the Influenza A virus in the combination group compared to the BCG-alone group. IL-1RA production was similar between groups in all stimulation conditions (**Fig. 3C**).

Next, we assessed the impact of BCG vaccination and alendronate on the modulation of interferon responses. Generally, BCG vaccination led to a trend of enhanced IFNα and IFNγ production at baseline and after viral stimulation, although statistical significance was not reached (**Supp. Fig. 2 and Fig. 4**). IFNα and IFNγ production was overall not significantly modulated by alendronate (**Fig. 4**). However, sex-specific analyses revealed a significant reduction of IFNα production against poly(I:C) in females of the combination group compared to the placebo (**Supp. Fig. 4**).

Lastly, we compared the cytokine productions at baseline and one month later for each group with a paired analysis to complement the fold change analysis (**Supp. Fig. 5 and 6**). Although most data were consistent, we have observed a few differences between these two representations: IL-6 production was significantly lower one month later after SARS-CoV-2 stimulation only in the combination group (**Supp. Fig. 5A**), while the fold changes of the treatment groups were not statistically different (**Fig. 3A**). **Fig. 3B** showed a lower TNFα production capacity in the combination group than in the BCG-only group after LPS stimulation. **Supp. Fig. 5B** showed no statistical significance on TNFα production against LPS. Finally, the placebo group produced more IFNα after poly I:C, and less IFNα after influenza and SARS-CoV-2 stimulation 1 month after treatment. However, there was a stronger decline in IFNα after influenza in the BCG+alendronate group (**Supp. Fig. 6**).

Single alendronate treatment suppressed transcriptional priming by BCG vaccination

RNA sequencing was performed with PBMCs from 10 individuals per group before and one month after the interventions to investigate if any transcriptional differences were present. When baseline gene expression patterns were compared, BCG and BCG+alendronate treatments led to similar upregulation of pathways, including glycolysis, inflammatory response, and IFNγ response (**Fig. 5A**). However, the addition of alendronate together with BCG vaccination led to higher TNFα signaling and IFNα response in the unstimulated condition.

We also wanted to compare the transcriptomic changes in a stimulated condition. Only 5 participants per group with adequate number of cells for the stimulation were included in this analysis. Upon poly(I:C) stimulation, alendronate treatment led to the striking downregulation of the pathways upregulated by BCG (**Fig. 5B**). These pathways included cholesterol homeostasis, glycolysis, IL-2/STAT5 signaling, IL-6/JAK/STAT3, inflammatory response, IFNα and IFNγ responses, and reactive oxygen species pathway. When the TNFA and IL1B gene expressions were individually analyzed, priming by BCG compared to placebo and its reversal by alendronate was evident, although not statistically significant due to the low sample size (**Fig. 5C-D**). Overall, the RNA sequencing data support the functional observations suggesting that alendronate suppresses the induction of trained immunity by BCG.

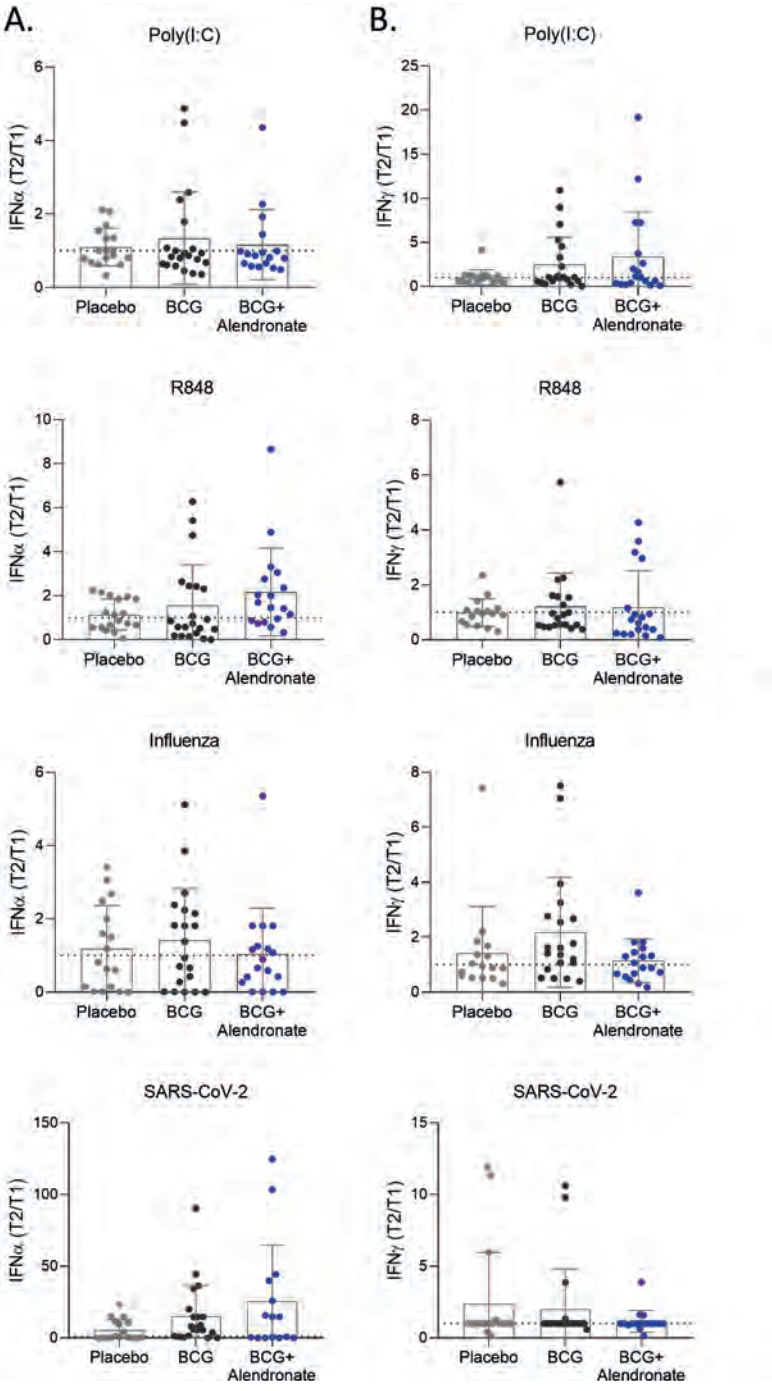

Figure 4. Fold changes (T2/T1) of A) IFNα and B) IFNγ production by PBMCs upon viral stimulation

Dotted lines depict the fold change of 1. Groups were compared with the Mann-Whitney U test. T1: before the intervention, T2: one month after the intervention.

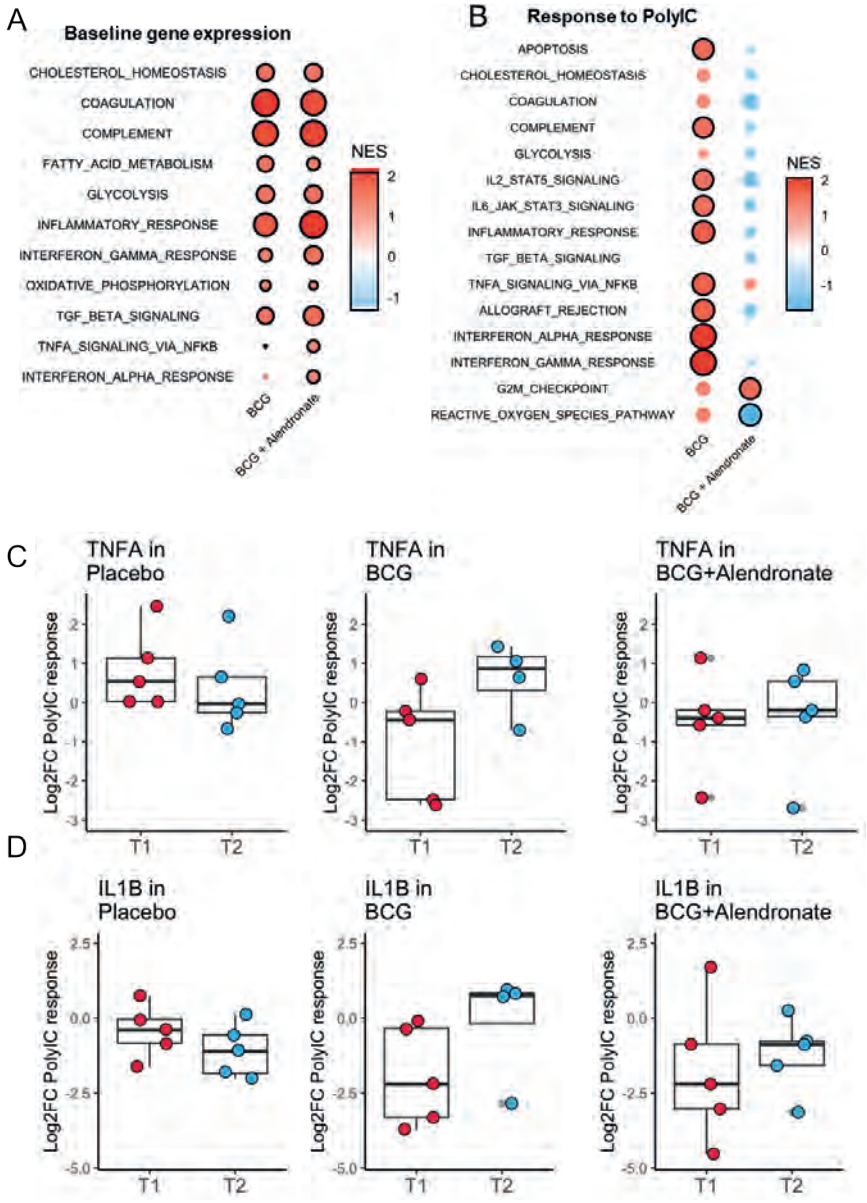

Figure 5. Gene set enrichment analysis (GSEA) from A) unstimulated and B) poly(I:C)-stimulated PBMCs of treatment groups compared to placebo, and example fold change plots of C) TNFA and D) IL1B expression upon poly(I:C) stimulation

Genes were ordered by the rank statistic –log10(pval)*logFC for the effect of priming on baseline gene expression and for response to poly(I:C) in the indicated primed condition and compared against Hallmark gene sets. Circle size and shading are scaled to a normalized enrichment score (NES). All circles with black border have padj ≤ 0.05. On the boxplots showing log2FC change in gene expression to poly(I:C), padj > 0.05 (non-significant) for all panels. T1: before the intervention, T2: one month after the intervention.

Discussion

In this study, we explored the effect of alendronate on the induction of trained immunity responses by BCG vaccination in healthy individuals. We observed that oral administration of alendronate simultaneous with BCG vaccination reduced the cytokine production capacity of PBMCs upon immunological challenge with various stimuli. At the transcriptional level, PBMCs of the individuals who received BCG with alendronate showed downregulated immune-related pathways, such as IFNα and IFNγ response and TNFα, IL-2, and IL-6 signaling upon activation with poly(I:C), compared to the individuals who received only BCG.

Furthermore, BCG vaccination combined with oral alendronate treatment increased white blood cell, neutrophil, and plasma cell numbers in circulation. In a study of patients with chronic idiopathic neutropenia-associated osteopenia or osteoporosis taking 10 mg of alendronate daily for one year, neutrophil counts were elevated at one month and continued to rise throughout the year [23]. Together with our observations, this suggests a stimulatory effect of alendronate on granulopoiesis in the bone marrow. A recent murine study showed that bisphosphonates promote B cell proliferation and antibody production after antigen encounter [24]. Although the study was primarily on clodronate, alendronate was also shown to improve antibody responses. This corroborates the findings that subjects receiving alendronate had significantly higher numbers of plasma cells one month later compared to baseline.

Bisphosphonates are heavily negatively charged molecules that cannot easily permeate the cell membrane. Fluid-phase endocytosis is required for their intracellular uptake [25]. Immune cells such as neutrophils, monocytes, and macrophages are capable of fluid-phase endocytosis and, therefore, could be susceptible to alendronate's immunomodulatory actions. Alendronate's effects on innate immune cells have not been thoroughly investigated, and the existing literature is inconsistent. A few studies have explored alendronate uptake by macrophages and its functional consequences. However, these were mostly performed with macrophage-like J774 or RAW 264 cell lines and not primary human cells [11,26,27]. Simple BPs such as clodronate, etidronate, and nitrogen-containing BP pamidronate suppressed LPS-induced IL-1β, IL-6, and TNFα production from RAW 264 cells [26]. Of note, pamidronate was cytotoxic in high concentrations. In another study with RAW 264 cells, alendronate also proved to be cytotoxic, but it enhanced LPS-induced IL-1β, IL-6, and TNFα production [28]. In J774.1 cells, alendronate increased lipid A-induced IL-1β production and caused cell death dependent on the activation of the Smad3/NLRP3/

ASC axis [29]. A study using PBMCs and monocytes from healthy humans showed that a wide dose range of alendronate inhibited PBMC proliferation in response to lectins or tetanus toxoid (TT) and suppressed IL-1β production from monocytes after LPS or TT stimulation [30].

Our study is the first to explore the effect of alendronate on trained immunity induced *in vivo* by BCG vaccination. Surprisingly, we have not observed a significant trained immunity response one month after BCG vaccination in this study. A reason for this could be the time of vaccination: the participants were vaccinated with BCG during the summer. It was recently shown that the training capacity of BCG is the highest in the winter, while it is lower in the summer [31]. Despite weak BCG-induced training, the results suggest an overall inhibitory effect of alendronate on proinflammatory cytokine production by innate immune cells, particularly TNFα. The observed effects could be considered relatively small, as they generally amounted to 20–25 % differences in the group that received alendronate and BCG compared to the group receiving BCG alone. However, while this may be a relatively small difference at the individual level, inhibition of BCG effects in this range in patients with bladder cancer treated with BCG immunotherapy [32] could have significant deleterious effects at an epidemiological level in large cohorts.

The transcriptome of PBMCs after poly(I:C) stimulation also corroborates our findings on decreased cytokine production capacity. In the unstimulated condition, we did not observe significant differences between the gene expression in PBMCs of the BCG vs. BCG+alendronate group. However, several pathways related to innate and adaptive immune response and signaling were transcriptionally downregulated in the BCG+alendronate group after incubation of PBMCs with poly(I:C). This suggests that specific genes might be epigenetically modulated by alendronate treatment, but future studies are needed to characterize the context and duration of these effects.

The observed decrease in the cytokine production capacity of immune cells when volunteers were vaccinated with BCG in the presence of alendronate administration is somewhat surprising: inhibition of FPP synthase by alendronate leads to the accumulation of mevalonate, and this was reported to enhance trained immunity responses [13]. Further studies are needed to study the possible molecular mechanisms of these effects of alendronate on BCG-induced immune modulation.

Alendronate is used in both men and women with osteoporosis, although it is more common in women. The tolerability profile and side effects were similar between the two sexes [33], as well as the drug's effectiveness [34]. However, it is not known

if bisphosphonates have sex-specific effects on the molecular level. Considering that bisphosphonates, including alendronate, have immunomodulatory effects and considerable differences exist between men's and women's immune responses [35], it would be plausible that alendronate would differentially influence the male and female immune responses. Although this study was not designed to answer how alendronate influences the immune system of young or older men and women, future studies must address this question. Within the limited sample size of our study, we observed a greater suppressive effect of alendronate on LPS-induced TNFα and poly(I:C)-induced TNFα and IFNα in women. Since a significantly improved cytokine response upon BCG vaccination was also only observed in women, the impact of alendronate might be sex-specific. However, these findings need to be confirmed in larger studies.

An increasing body of research suggests a lower risk of infections and cancer associated with bisphosphonate use. Injection of N-BPs 3 days after infection with influenza resulted in protection against influenza in mice through the expansion of γδ T cells [16]. N-BP use was also linked to a reduced rate of epithelial ovarian cancer compared to no use in women over 50 [17]. An observational study in people with hip fractures reported a lower risk of pneumonia and pneumonia mortality in people using N-BPs than those using non-NB-P anti-osteoporosis medications [15]. On the other hand, a study assessing the incidence of COVID-19 hospitalization and mortality in people using N-BPs found no effect of bisphosphonate use on the risk of getting severe COVID-19 [36]. As these studies are only observational and cannot prove causality, randomized controlled trials are needed to determine whether bisphosphonate use has beneficial or detrimental effects on immunity. Our results indicate a lower *ex vivo* cytokine production against viral and bacterial stimuli in individuals receiving alendronate with BCG, but whether this translates to clinical outcomes such as infection incidence and severity remains to be investigated.

Though this study investigated alendronate's immunological effects on healthy young individuals, alendronate is primarily used to prevent and treat osteoporosis, a disease of old age [37]. Immune cell numbers and functions are compromised in advanced age, accompanied by sustained low-grade systemic inflammation, which leads to increased susceptibility to infections, higher morbidity, and mortality [38]. Induction of trained immunity is a promising approach to overcoming immune dysregulation in the elderly and relieving the healthcare burden due to infections. BCG vaccination improves cytokine responses of innate immune cells while decreasing systemic inflammation in healthy elderly [39]. Our observations show that even a single use of oral alendronate tablets together with BCG vaccination can have

long-term modulatory effects on cytokine and interferon responses against bacterial, viral, and fungal pathogens. Whether this effect would be beneficial or deleterious remains to be investigated in larger studies. As a systemic inflammation marker, we also measured IL-6 concentrations in the plasma before and after placebo, BCG, or BCG+alendronate treatment. However, no differences existed between time points in any group (**Supp. Fig. 7**). Whether chronic use of alendronate in older individuals would impact systemic inflammation, prevent the induction of trained immunity, or further suppress the host response to pathogens are important open questions to be considered.

One limitation of this study was the sample size. Although decreasing cytokine production patterns were observed after the simultaneous use of BCG and alendronate compared to the BCG-only group, these changes sometimes failed to reach statistical significance. A study with a larger sample size is necessary to further validate the effects of alendronate on trained immunity induction.

In conclusion, we show that simultaneous administration of BCG with oral alendronate reduces the cytokine production capacity of PBMCs against heterologous stimuli one month later in young, healthy individuals. These findings align with the transcriptome of poly(I:C)-stimulated PBMCs where BCG+Alendronate treatment results in downregulated inflammatory pathways. These observations could have implications for BCG vaccination in the elderly with chronic use of N-BPs, and larger studies investigating the effect of long-term alendronate use on BCG-induced trained immunity are required.

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CRediT authorship contribution statement

Ozlem Bulut: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Gizem Kilic:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Priya A. Debisarun:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Software. **Rutger Jan Röring**: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Software.

Sarah Sun: Data curation, Formal analysis, Investigation, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. **Manon Kolkman:** Data curation, Formal analysis, Investigation, Resources. **Esther van Rijssen:** Data curation, Formal analysis, Investigation, Resources. **Jaap ten Oever:** Conceptualization, Project administration, Supervision. **Hans Koenen:** Resources, Supervision. **Luis Barreiro:** Resources, Supervision, Writing – review & editing. **Jorge Domínguez-Andrés:** Resources, Supervision. **Mihai G. Netea:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mihai G. Netea reports financial support was provided by European Research Council. Mihai G. Netea reports financial support was provided by Dutch Research Council. Jorge Dominguez-Andres reports financial support was provided by Dutch Research Council. Mihai G. Netea reports a relationship with Trained Therapeutix Discovery (TTxD) that includes: board membership and equity or stocks. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

1. Akira, S., S. Uematsu, and O. Takeuchi, Pathogen recognition and innate immunity. Cell, 2006. 124(4): p. 783-801.
2. Geckin, B., et al., Trained immunity: implications for vaccination. Curr Opin Immunol, 2022. 77: p. 102190.
3. de Bree, L.C.J., et al., Non-specific effects of vaccines: Current evidence and potential implications. Semin Immunol, 2018. 39: p. 35-43.
4. Dominguez-Andres, J., et al., Trained immunity: adaptation within innate immune mechanisms. Physiol Rev, 2023. 103(1): p. 313-346.
5. Cirovic, B., et al., BCG Vaccination in Humans Elicits Trained Immunity via the Hematopoietic Progenitor Compartment. Cell Host Microbe, 2020. 28(2): p. 322-334 e5.
6. Moorlag, S., et al., Safety and COVID-19 Symptoms in Individuals Recently Vaccinated with BCG: a Retrospective Cohort Study. Cell Rep Med, 2020. 1(5): p. 100073.
7. Russell, R.G., et al., Bisphosphonates: an update on mechanisms of action and how these relate to clinical efficacy. Ann N Y Acad Sci, 2007. 1117: p. 209-57.
8. Miyagawa, F., et al., Essential requirement of antigen presentation by monocyte lineage cells for the activation of primary human gamma delta T cells by aminobisphosphonate antigen. J Immunol, 2001. 166(9): p. 5508-14.
9. Munoz, M.A., et al., Bisphosphonate drugs have actions in the lung and inhibit the mevalonate pathway in alveolar macrophages. Elife, 2021. 10.
10. Roelofs, A.J., et al., Fluorescent risedronate analogues reveal bisphosphonate uptake by bone marrow monocytes and localization around osteocytes in vivo. J Bone Miner Res, 2010. 25(3): p. 606-16.
11. Luckman, S.P., et al., Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. J Bone Miner Res, 1998. 13(4): p. 581-9.
12. Thompson, K. and M.J. Rogers, Statins prevent bisphosphonate-induced gamma,delta-T-cell proliferation and activation in vitro. J Bone Miner Res, 2004. 19(2): p. 278-88.
13. Bekkering, S., et al., Metabolic Induction of Trained Immunity through the Mevalonate Pathway. Cell, 2018. 172(1-2): p. 135-146 e9.
14. Coscia, M., et al., Zoledronic acid repolarizes tumour-associated macrophages and inhibits mammary carcinogenesis by targeting the mevalonate pathway. J Cell Mol Med, 2010. 14(12): p. 2803-15.
15. Sing, C.W., et al., Nitrogen-Containing Bisphosphonates Are Associated With Reduced Risk of Pneumonia in Patients With Hip Fracture. J Bone Miner Res, 2020. 35(9): p. 1676-1684.
16. Tu, W., et al., The aminobisphosphonate pamidronate controls influenza pathogenesis by expanding a gammadelta T cell population in humanized mice. J Exp Med, 2011. 208(7): p. 1511-22.
17. Tuesley, K.M., et al., Nitrogen-based Bisphosphonate Use and Ovarian Cancer Risk in Women Aged 50 Years and Older. J Natl Cancer Inst, 2022. 114(6): p. 878-884.
18. Andrews, S., FastQC A Quality Control tool for High Throughput Sequence Data. 2012.
19. Dobin, A., et al., STAR: ultrafast universal RNA-seq aligner. Bioinformatics, 2013. 29(1): p. 15-21.
20. Liao, Y., G.K. Smyth, and W. Shi, featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics, 2014. 30(7): p. 923-30.

21. de Bree, L.C.J., et al., Circadian rhythm influences induction of trained immunity by BCG vaccination. J Clin Invest, 2020. 130(10): p. 5603-5617.
22. Kleinnijenhuis, J., et al., Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. J Innate Immun, 2014. 6(2): p. 152-8.
23. Papadaki, H.A., et al., Alendronate reduces serum TNFalpha and IL-1beta, increases neutrophil counts, and improves bone mineral density and bone metabolism indices in patients with chronic idiopathic neutropenia (CIN)-associated osteopenia/osteoporosis. J Bone Miner Metab, 2004. 22(6): p. 577-87.
24. Tonti, E., et al., Bisphosphonates target B cells to enhance humoral immune responses. Cell Rep, 2013. 5(2): p. 323-30.
25. Thompson, K., et al., Cytosolic entry of bisphosphonate drugs requires acidification of vesicles after fluid-phase endocytosis. Mol Pharmacol, 2006. 69(5): p. 1624-32.
26. Pennanen, N., et al., Effect of liposomal and free bisphosphonates on the IL-1 beta, IL-6 and TNF alpha secretion from RAW 264 cells in vitro. Pharm Res, 1995. 12(6): p. 916-22.
27. Coxon, F.P., et al., Visualizing mineral binding and uptake of bisphosphonate by osteoclasts and non-resorbing cells. Bone, 2008. 42(5): p. 848-60.
28. Toyras, A., et al., Inhibition of mevalonate pathway is involved in alendronate-induced cell growth inhibition, but not in cytokine secretion from macrophages in vitro. Eur J Pharm Sci, 2003. 19(4): p. 223-30.
29. Tamai, R. and Y. Kiyoura, Alendronate augments lipid A-induced IL-1beta release and Smad3/NLRP3/ASC-dependent cell death. Life Sci, 2018. 198: p. 8-17.
30. Sansoni, P., et al., Inhibition of antigen-presenting cell function by alendronate in vitro. J Bone Miner Res, 1995. 10(11): p. 1719-25.
31. Moorlag, S., 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): p. 171-187 e14.
32. van Puffelen, J.H., 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).
33. Sharpe, M., S. Noble, and C.M. Spencer, Alendronate: an update of its use in osteoporosis. Drugs, 2001. 61(7): p. 999-1039.
34. Sawka, A.M., et al., A systematic review of the effect of alendronate on bone mineral density in men. J Clin Densitom, 2005. 8(1): p. 7-13.
35. Klein, S.L. and K.L. Flanagan, Sex differences in immune responses. Nat Rev Immunol, 2016. 16(10): p. 626-38.
36. Degli Esposti, L., et al., The Use of Oral Amino-Bisphosphonates and Coronavirus Disease 2019 (COVID-19) Outcomes. J Bone Miner Res, 2021. 36(11): p. 2177-2183.
37. Pietschmann, P., et al., Osteoporosis: an age-related and gender-specific disease--a mini-review. Gerontology, 2009. 55(1): p. 3-12.
38. Bulut, O., et al., Overcoming immune dysfunction in the elderly: trained immunity as a novel approach. Int Immunol, 2020. 32(12): p. 741-753.
39. Pavan Kumar, N., et al., Effect of BCG vaccination on proinflammatory responses in elderly individuals. Sci Adv, 2021. 7(32).

Supplementary Information

Supplementary Tables

Supplementary Table 1. Flow cytometry antibodies used in the study

Antibody	Source	Identifier
General panel		
anti-CD16 FITC (clone 3G8)	Beckman Coulter	Cat#B49215; RRID: AB_2848116
anti-HLA-DR PE (clone immu-357)	Beckman Coulter	Cat#IM1639; RRID: AB_131284
anti-CD14 ECD (clone RM052)	Beckman Coulter	Cat#B92391; RRID: AB_130853
anti-CD4 PE-Cy5.5 (clone 13B8.2)	Beckman Coulter	Cat#B16491; RRID: Unknown
anti-CD25 PE-Cy7 (clone M-A251)	BD Biosciences	Cat#557741; RRID: AB_396847
anti-CD56 APC (clone N901)	Beckman Coulter	Cat#IM2474; RRID: AB_130791
anti-CD8 APC-AF700 (clone B9.11)	Beckman Coulter	Cat#B49181; RRID: AB_2750854
anti-CD19 APC-AF750 (clone J3-119)	Beckman Coulter	Cat#A94681; RRID: AB_2833030
anti-CD3 Pacific Blue (clone UCHT1)	Beckman Coulter	Cat#A93687; RRID: AB_2728095
anti-CD45 Krome Orange (clone J33)	Beckman Coulter	Cat#A96416; RRID: AB_2833027
B cell panel		
anti-IgD FITC (clone IADB6)	Southern Biotech	Cat#2032-02; RRID: AB_2687521
anti-IgM PE (clone SA-DA4)	Beckman Coulter	Cat#B30657; RRID: unknown
anti-CD3 ECD (clone UCHT1)	Beckman Coulter	Cat#A07748; RRID: unknown
anti-CD27 PE-Cy5.5 (clone 1A4CD27)	Beckman Coulter	Cat#B21444; RRID: unknown
anti-CD38 PE-Cy7 (clone LS198-4-3)	Beckman Coulter	Cat#B49198; RRID: unknown
anti-CD24 APC (clone ALB9)	Beckman Coulter	Cat#A87785; RRID: unknown
anti-CD5 APC-AF700 (clone BL1a)	Beckman Coulter	Cat#A78835; RRID: unknown
anti-CD19 APC-AF750 (clone J3-119)	Beckman Coulter	Cat#A94681; RRID: AB_2833030
anti-CD20 Pacific Blue (clone B9E9)	Beckman Coulter	Cat#B49208; RRID: unknown
anti-CD45 Krome Orange (clone J33)	Beckman Coulter	Cat#A96416; RRID: AB_2833027

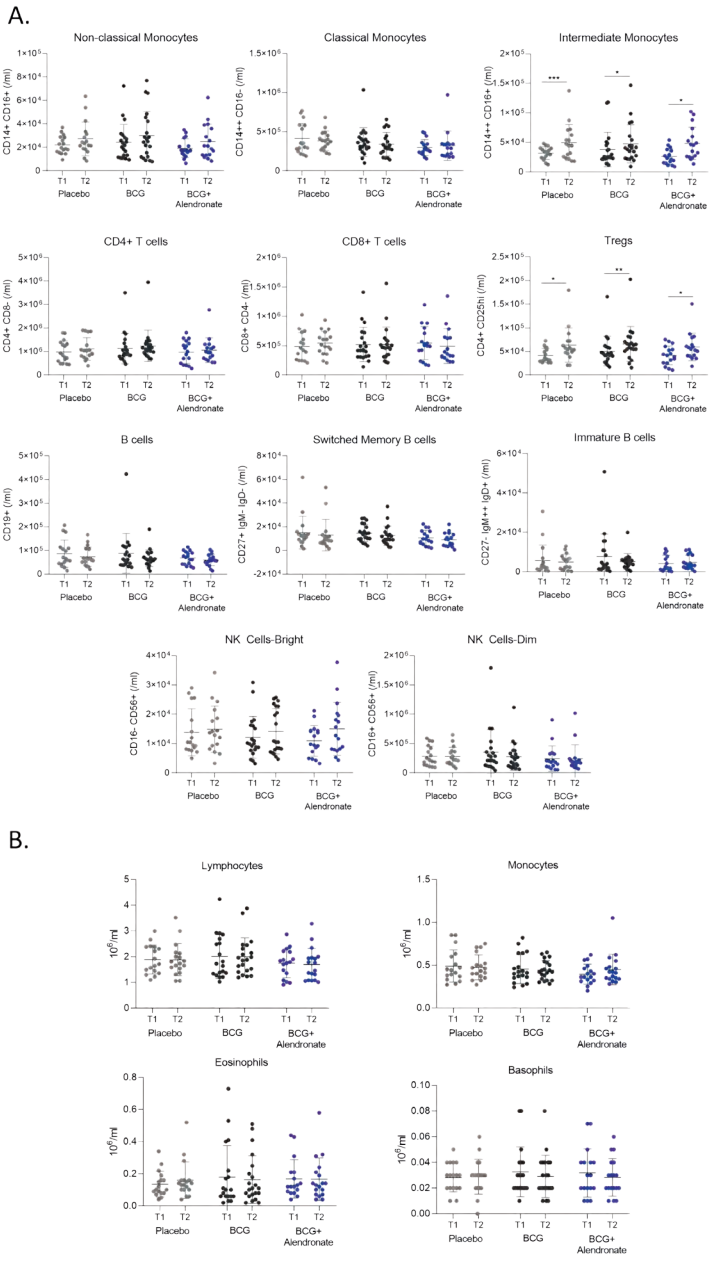
Supplementary Table 2. Demographics of the study participants who were selected for RNA sequencing

	Placebo (n=10)	BCG (n=10)	BCG+Alendronate (n=10)	p-value
Age (years, mean±sd)	27.7±7.0	25.6±7.7	27.6±8.6	0.79
Sex (F/M)	5/5	7/3	8/2	0.35
BMI (kg/m2, mean±sd)	23.7±1.7	21.3±1.8	23.0±3.5	0.11

Supplementary Table 3. Demographics of the study participants for whom RNA sequencing was possible upon poly(I:C) stimulation

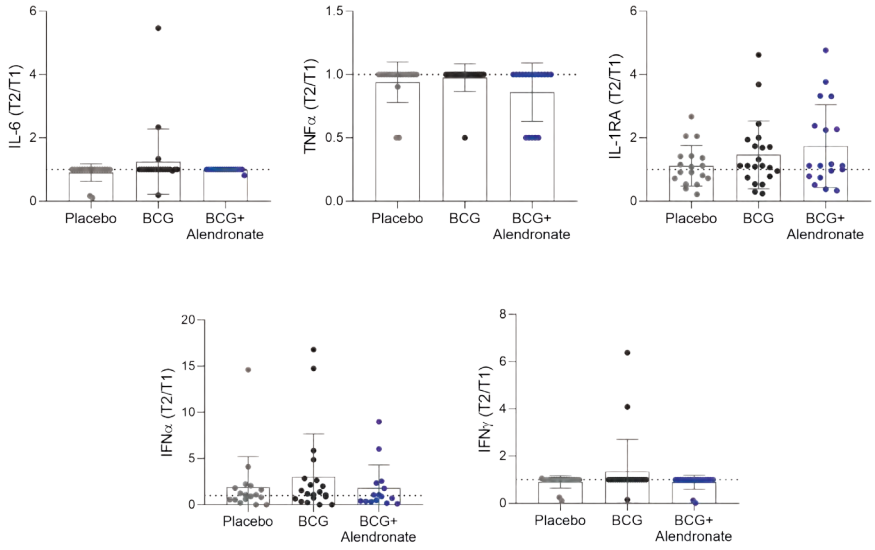
	Placebo (n=5)	BCG (n=5)	BCG+Alendronate (n=5)	p-value
Age (years, mean±sd)	27.2±6.1	28.6±9.8	28.4±10.1	0.96
Sex (F/M)	1/4	5/0	4/1	0.02
BMI (kg/m2, mean±sd)	24.6±1.8	21.4±2.2	23.1±3.9	0.23

Supplemetary Figures



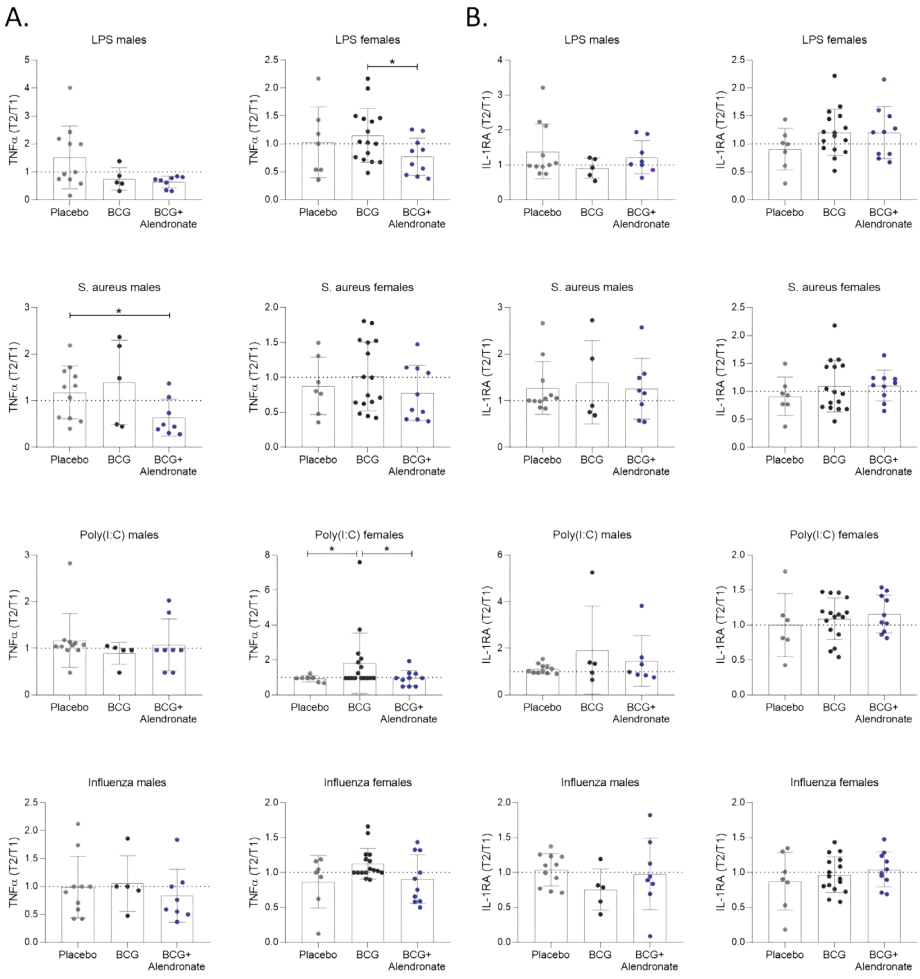
Supplementary Figure 1. Numbers of the immune cell populations in the blood measured by A) flow cytometry and B) a hematology analyzer before and after treatment

Cell numbers before (T1) and one month after (T2) treatment were compared with the Wilcoxon matched-pairs signed rank test. *p<0.05, **p<0.01, ***p<0.001.



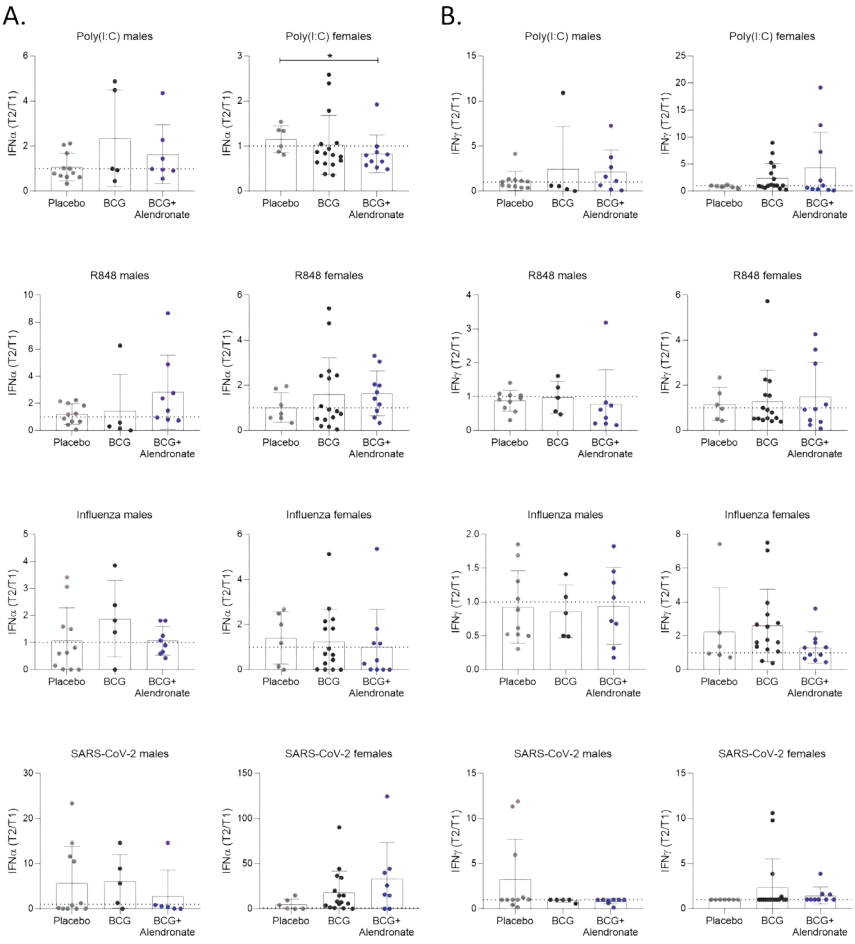
Supplementary Figure 2. Fold changes of cytokine levels in the absence of stimuli

Dotted lines depict the fold change of 1. Groups were compared with the Mann-Whitney U test. T1: before the intervention, T2: one month after the intervention. *p<0.05.



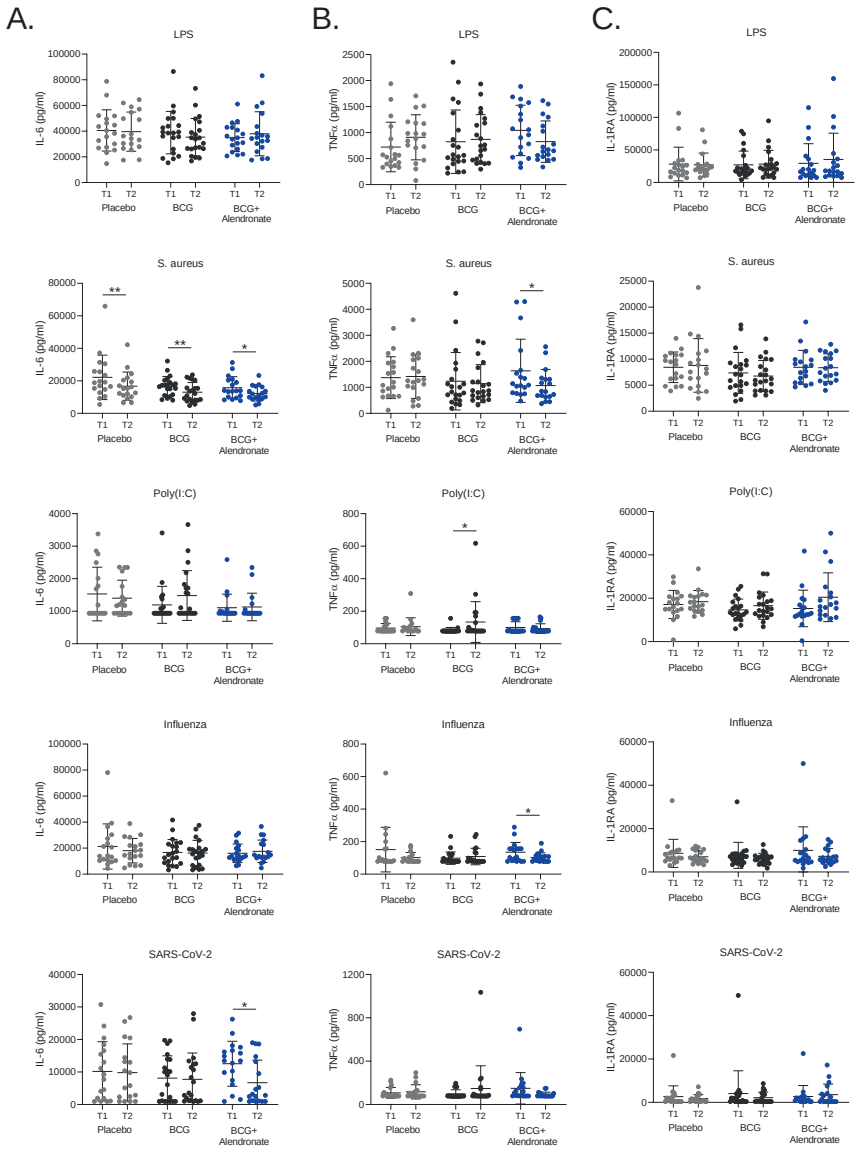
Supplementary Figure 3. Fold changes (T2/T1) of A) TNFα and B) IL-1RA production by PBMCs upon bacterial and viral stimulation in males and females

Dotted lines depict the fold change of 1. Groups were compared with the Mann-Whitney U test. T1: before the intervention, T2: one month after the intervention. *p≤0.05.



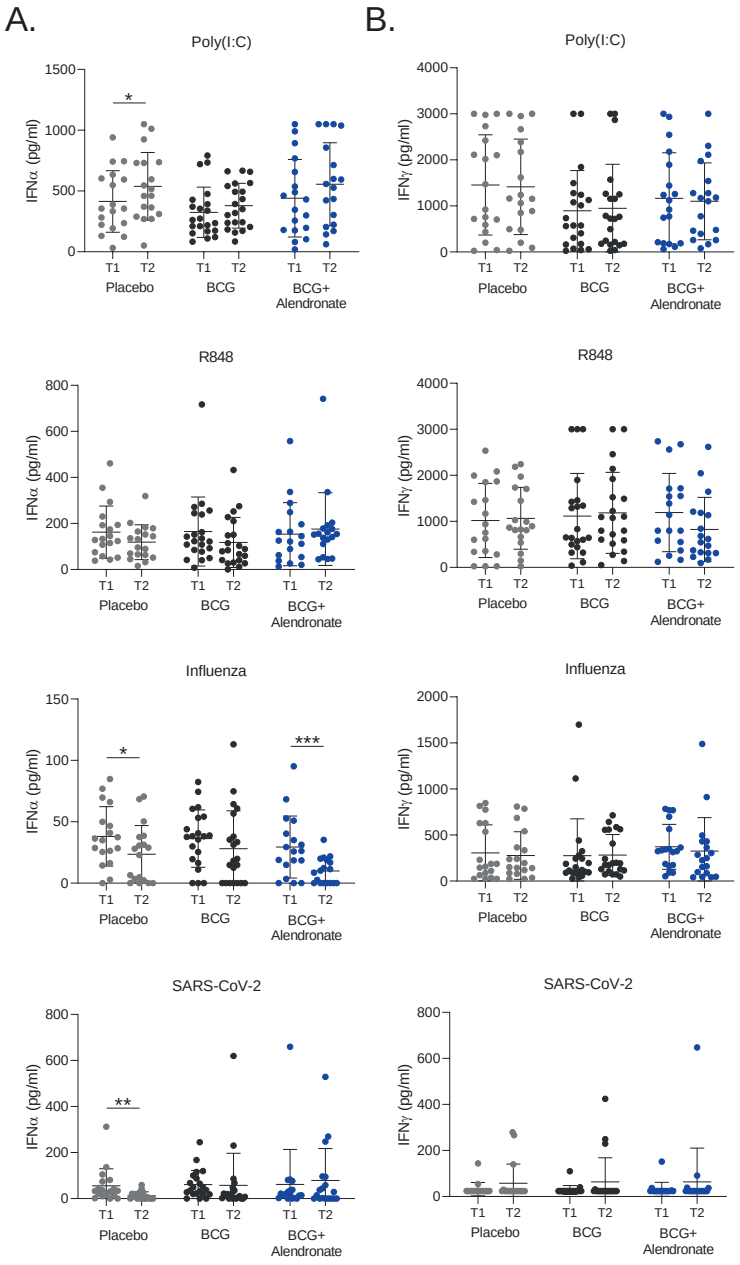
Supplementary Figure 4. Fold changes (T2/T1) of A) IFNα and B) IFNγ production by PBMCs upon viral stimulation in males and females

Dotted lines depict the fold change of 1. Groups were compared with the Mann-Whitney U test. T1: before the intervention, T2: one month after the intervention. *p≤0.05.



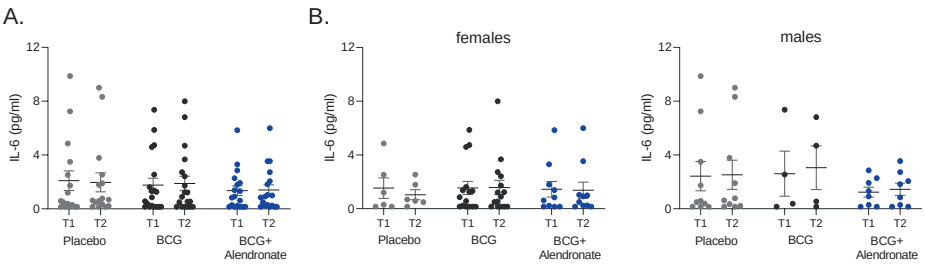
Supplementary Figure 5. A) IL-6, B) TNFα, and C) IL-1RA production by PBMCs before and 1 month after intervention upon stimulation

Time points within the same group were compared with the Wilcoxon matched-pairs signed rank test. T1: before the intervention, T2: one month after the intervention. *p≤0.05.



Supplementary Figure 6. A) IFNα and B) IFNγ production by PBMCs before and 1 month after intervention upon stimulation

Time points within the same group were compared with the Wilcoxon matched-pairs signed rank test. T1: before the intervention, T2: one month after the intervention. *p≤0.05, **p<0.01, ***p<0.001.



Supplementary Figure 7. Plasma IL-6 levels of A) all participants and B) females and males separately before (T1) and one month after (T2) treatment

T1 and T2 levels were compared with the Wilcoxon matched-pairs signed rank.

Common blackbird
Karatavuk
Merel

CHAPTER 8

Summary and general discussion

Summary

The immune system is a complex and dynamic network of various cell populations and humoral mediators, characterized by significant interindividual variability, which is subject to the influence of both intrinsic and extrinsic factors. This variability in immune responses plays a significant role in vaccine efficacy, disease incidence and severity, and response to therapeutic interventions at an individual level. Therefore, a deeper understanding of the factors impacting the immune system could help develop more effective therapies and vaccines and optimize existing ones. This thesis examines six of these variables, which are age, sex, genetic predispositions, prior exposure to pathogens, pharmacological interventions, and seasonal variations, to elucidate their roles in modulating immune responses, with a focus on innate immunity. The findings of this research add to the evidence of the significant impact of these factors on the functionality of the immune system.

The thesis is structured into two sections: The first section explores the intrinsic or host-related factors shaping the immune responses, namely age, sex and genetics. The second section addresses extrinsic or environmental factors, such as previous infections, medication use and seasonality.

Chapter 1 provides a general introduction to immune system dynamics and the concept of trained immunity. It also presents an overview of the variables that influence immune system responses and which were further discussed in this thesis. **Chapter 2** focuses on the aging of the immune system and the impact of aging on immune functionality and vaccine responsiveness. Age alters both the quantitative and qualitative aspects of innate and adaptive immune cells. Cells of innate immunity, e.g., neutrophils, monocytes, DCs and NK cells, exhibit impaired functional responses in cytokine production, phagocytosis, antigen presentation and more, while aged adaptive immune cells are characterized by reduced receptor diversity and naïve cell numbers. Furthermore, people with advancing age have elevated concentrations of pro-inflammatory cytokines and chemokines in the circulation, contributing to decreased vaccine efficacy and more severe infection outcomes. Given the critical role of a well-regulated innate immune response in activating the adaptive immune system and optimizing immune response, I proposed the use of trained immunity-based interventions as a strategy to protect the elderly against infections. An example is an adjuvanted shingles vaccine (Shingrix), demonstrating around 97% efficacy in individuals over 50. Although how the Shingrix vaccine counteracts immunosenescence in the elderly is yet to be known, it was associated with a 16% lower risk of COVID-19 diagnosis and a 32% lower risk of hospitalization,

suggesting a role of trained immunity [1]. As live-attenuated vaccines pose potential risks for people with immunodeficiency and the frail elderly, adjuvanting vaccine formulations to induce an effective innate immune activation emerges as a safe and effective method for enhancing vaccine efficacy among these vulnerable groups.

Recent evidence, including the outcomes of the COVID-19 pandemic, highlights that not only older adults, but also males are more prone to getting severe infections [2, 3]. In **Chapter 3**, I hypothesized that disparities in SARS-CoV-2 infection outcomes and severity could be attributable to intrinsic differences in the immune system of the elderly and males. Indeed, I found that plasma proteins linked to severe COVID-19 outcomes, such as HGF, IL-8, IL-18, and MCP-1, were present in higher concentrations in the circulation of healthy males and older individuals. Moreover, fewer Tregs, naïve $CD4^+$ and $CD8^+$ T and plasmablasts were observed in these populations compared to females and younger adults, which were linked to severe SARS-CoV-2 infection. Additionally, this study investigated the impact of seasons on SARS-CoV-2 immune responses across different age and sex groups. While the overall cohort did not display a significant difference in immune responses throughout the year, the elderly showed a lower IFNγ and higher IL-1Ra response compared to younger adults. Moreover, females produced more IL-1β during the spring and summer months than males. Collectively, these findings underscore the significance of intrinsic factors and seasonality in response to SARS-CoV-2 infection and disease severity.

Human genetics is highly diverse and contributes not only to the individual variability in immune responses but also to the differential susceptibility to infectious diseases and the varying efficacy of vaccines. By analyzing the genetic polymorphisms influencing trained immunity responses after BCG vaccination in **Chapter 4**, I identified significant associations with pathways, including homeostasis, metabolism of lipids and lipoproteins and adaptive immune system. Given the repeatedly demonstrated significance of lipids and lipid metabolism in trained immunity [4-6], our research focused on the top gene of the lipid-related pathways, *RORA*, and discovered a negative association of cholesterol and cholesterol sulfate abundance in circulation, acting as RORα agonists, with BCG-induced trained immunity. In line with this, a RORα inverse agonist SR3335 induced trained immunity in human PBMCs by itself and amplified the training effect of BCG. I observed notable changes in cell morphology alongside increased lactate and ROS production in cells trained with BCG+SR3335. Although no increase in glycolysis and oxidative phosphorylation was observed upon RORα inhibition, blocking LDHA using sodium oxamate significantly decreased the cytokine production capacity after BCG+SR3335 training. This research suggests that RORα may serve as a modulator in BCG-induced trained immunity

responses and could be a potential therapeutic target for cardiovascular diseases characterized by inappropriately activated trained immunity.

In the second part of this thesis, I first explored the seasonal variation in trained immunity responses following BCG vaccination in **Chapter 5**. For this study, participants were vaccinated with a placebo or BCG vaccine during winter or spring. PBMCs from young adults vaccinated during winter produced more TNFα, IL-6, and IL-1β and less IFNγ after incubation with various viral, bacterial, and fungal stimuli. Notably, winter vaccination led to elevated pro-inflammatory cytokine and IFNγ release from NK cells. While BCG had a minimal effect on the monocyte transcriptome 3 months post-vaccination, it induced a season-dependent epigenetic signature in monocytes and NK cells. In summary, BCG vaccination during winter induces a more robust immune response from human immune cells, including NK cells, which is partly attributable to epigenetic modifications.

Immunogenic encounters shift the baseline status of the immune system and determine the immune responses against subsequent infections and vaccinations. In the first months of the COVID-19 pandemic, before the availability of vaccines, we had a chance to investigate whether a mild/moderate SARS-CoV-2 infection impacts the immune system of healthy individuals post-recovery, with a focus on innate immunity. The results in **Chapter 6** revealed minor differences at the transcriptomic and epigenetic levels of the convalescent individuals compared to uninfected adults. Although a downregulation in interferon response pathways was observed in recovered individuals, their functional immune response was comparable to that of healthy controls. Additionally, I did not detect any considerable difference in circulating immune cell subsets and cytokine production capacity of PBMCs upon stimulation with various bacterial, viral and fungal agents. Notably, without any stimulation, IFNγ and IL-1Ra production of recovered adults was higher than that of healthy adults. Collectively, these findings suggest that the immune parameters and responses of recovered COVID-19 patients largely return to the level of healthy individuals, although slight differences in DNA methylation and transcription persist after the resolution of infection.

The safety and efficacy of medications must be well documented in order to be used in humans. However, whether or not medications have non-specific long-term effects is largely an unknown area. In **Chapter 7**, I explored the influence of alendronate, a commonly prescribed osteoporosis medication, on the trained immunity responses elicited by BCG vaccination. The results demonstrated that concurrent BCG and oral alendronate administration diminished pro-inflammatory cytokine production

capacity. The combination treatment was associated with an increase in neutrophils and plasma cells one month post-treatment, suggesting alendronate's potential long-term immunomodulatory effects. Additionally, poly I:C stimulation led to an upregulation of genes involved in pathways related to inflammatory responses, as well as interferon-alpha and -gamma responses in the group receiving BCG vaccination alone. However, this pronounced enrichment in these pathways was not observed in the group treated with both BCG and alendronate. These findings propose that alendronate, and possibly other bisphosphonates, could modulate the immune responses and trained immunity after BCG vaccination.

General discussion

The immune system plays an essential role in survival by recognizing the threat, mobilizing a response, and eliminating the pathogen. In addition to host defense, immune cells provide immunosurveillance against malignant processes and contribute to tissue repair. The immune system has two main branches: innate and adaptive immunity. Innate immunity refers to the body's initial defense against pathogens using physical barriers besides immune cells that can recognize the invaders and respond quickly [7]. On the other hand, adaptive immunity has a specialized set of immune cells involving lymphocytes, recognizing specific pathogens and conferring a long-lasting response [8]. A more recent concept of adaptation within innate immunity has been described, also termed *trained immunity*, that explains the antigen-agnostic protection against heterologous infections provided by some vaccines [9]. This gives the innate immune system the ability to remember and react strongly upon subsequent exposures, highlighting a more dynamic immune system than previously understood. Considering that the immune system constantly interacts with other cells, tissues, and external factors, these influences naturally shape the immune characteristics and response. This section will discuss the complexity and interactions of these factors with the immune system, focusing on innate immunity.

Aging is recognized as a prominent risk factor for various infections, including SARS-CoV-2. Epidemiological research indicates that individuals older than 65 years of age accounted for 80% of COVID-19-related hospitalizations and had a 23-fold higher risk of death compared to those younger than 65 [10]. Furthermore, the risk of severe infections and death increases gradually with age [11]. In general, age-dependent impairments cause an imbalance in the innate immune response and elevate the inflammatory molecules in the circulation [12]. Such systemic inflammation lessens the functioning of both innate and adaptive immunity, creating a cycle of inflammation that renders the elderly more susceptible to infections and results in lower vaccine efficacy. **Chapter 3** shows that people have shown signs of inflammaging from 50 years of age, which aligns with the signature linked to severe COVID-19. PBMCs of older adults displayed higher IL-1Ra and lower IFNγ production after SARS-CoV-2 stimulation, indicating impaired innate and adaptive immune functionality. A recent study found a positive association between IL-1Ra concentrations with disease severity and organ failure in COVID-19 [13]. Additionally, given the critical role of IFNγ in controlling viral load and the COVID-19 severity, insufficient production of IFNγ in the elderly could contribute to their increased vulnerability to the infection [14].

Although this study did not specifically investigate sex differences in the elderly population due to the small sample size, studies suggest that males are at a higher risk of getting severe SARS-CoV-2 infection than females, regardless of age [15]. Another study argued that higher low-grade systemic inflammation, impaired type I IFN response, downregulation of ACE2, and faster biological aging lead to severe COVID-19 in males [16]. Hormonal, biological, and genetic factors might explain the sex differences causing differences in COVID-19 severity. Notably, Kuijpers et al. found no significant association between sex and genetic risk score of COVID-19 severity, although sex chromosomes were not investigated in their study [17]. It was previously shown that the *TLR7* gene on the X chromosome is vital to develop an immune response to the SARS-CoV-2 infection [18]. As it is known that the *TLR7* gene can escape X inactivation [19], two copies of the gene likely give females an advantage in generating an early and robust immune response [20, 21].

Aside from age and sex differences, we found some seasonal differences in response against SARS-CoV-2 in different age and sex groups. Female PBMCs isolated during spring and summer produced higher IL-1β than male PBMCs isolated in the same period. Furthermore, younger people had a lower IL-1Ra and higher IFNγ response throughout the year, with IFNγ being higher during summer and fall. These findings are partially corroborated by Agarwal et al., who reported a significant increase in IFNγ production from PHA-stimulated blood during summer in young adults [22]. These results demonstrate the influence of seasonality on immune responses, which manifests itself distinctly across different age and sex groups.

The spring and summer months are associated with an enhanced adaptive immune response, potentially due to higher lymphocyte numbers and homing and egression of lymphocytes through the lymphatic system and other tissues [23, 24]. Consistent with this notion, we observed a higher T cell-, but not NK cell-, derived IFNγ response after heterologous stimulation of young adults vaccinated with BCG during spring months compared to winter (**Chapter 5**). Additionally, spring vaccination increased lymphocyte numbers three months post-BCG, which might be driven solely by seasonal factors rather than the vaccination itself. Contrary to the enhanced heterologous IFNγ response in the spring-vaccinated group, the winter-vaccinated group exhibited a stronger pro-inflammatory cytokine response by innate immunity against non-specific pathogens. These findings suggest that BCG vaccination in the winter months is associated with a more effective induction of trained immunity.

A recent study identified seasonal variations in cytokine responses to various pathogens, with pro-inflammatory cytokine production being generally lower in

summer and higher in winter [25]. Additionally, research in the UK revealed that various immune-related genes exhibit seasonal fluctuations. The authors found a more inflammatory profile during winter in European individuals, characterized by increased gene transcription in innate immunity genes and monocyte and neutrophil counts in the blood [26]. Given the lower reactivity of adaptive immune responses during winter, it is plausible to propose that the innate immune system becomes more readily inducible in this season. This heightened inducibility may provide sufficient signals to activate adaptive immunity or compensate for its reduced activity during the colder months. Overall, this could be the reason for more robust trained immunity in winter months.

Humans continuously engage with their environment and the microbes it harbors. While some of these microbes lead to disease or severe infections, others have negligible impact on health. Nevertheless, whether significant or minor, each interaction has the potential to imprint a short or long-lasting effect on the immune system, thereby shaping its response to subsequent encounters. For instance, a gastrointestinal infection of mice with *Y. pseudotuberculosis* disrupts the immune homeostasis even after recovery, resulting in sustained inflammation and lymphatic leakage of dendritic cells [27]. Additionally, measles virus infection is known to have deleterious effects on the adaptive immune system: infection eliminates existing antibody repertoire against other infections, leading to increased mortality and morbidity rates after infection [28]. In **Chapter 6**, I aimed to investigate the impact of mild/moderate COVID-19 infection on the immune system, focusing on innate immunity, and found only minor differences at epigenetic and transcriptional levels. On the other hand, some studies found distinct differences even after mild SARS-CoV-2 infection. One notable study reported an inflammatory imprint of mild COVID-19 on monocytes and macrophages, which lasts up to 12 months [29]. Another research showed the sex-dimorphic impact of mild infection after recovery [30]. Here, the authors highlighted a male-specific increase in monocyte counts, besides a more pronounced T cell activation signature in the $CD8^+$ effector memory cells.

Severe infection likely leaves a more substantial mark as the prolonged and heightened inflammatory response damages the body and the immune system more. A study demonstrated the chromatic remodeling in the innate and adaptive immune cells of convalescent individuals from moderate/severe COVID-19, indicating the development of immunological memory. [31]. Furthermore, persistent immune-related changes were identified up to one year post-recovery, including epigenetic reprogramming of the hematopoietic stem progenitor cells, myelopoiesis and altered immune responses [32]. Although various studies have found an impact of SARS-CoV-2 infection on the immune system, how it influences future disease risk and severity is unclear.

This thesis investigated how various host-related and environmental factors influence the innate immune system, providing valuable insights into its modulation. The research in the thesis also has translational and clinical implications, which are discussed in the following sections.

Development and Repurposing of Vaccines and Drugs

Drug and vaccine repurposing is an innovative process of identifying new uses for existing products beyond their original indications. This strategy has recently gained significant attention, particularly during the COVID-19 pandemic, as a cost-effective and time-efficient approach to speed up the availability of therapies by bypassing the early stages of drug development. At the same time, developing new drugs and vaccines continues to be crucial as a solution to existing health challenges. The research in this thesis has implications for the development of new drugs as well, as repurposing the existing ones for more diseases and conditions.

Coronary heart diseases are the primary cause of death worldwide. Atherosclerosis, as a cause of coronary artery disease, is a chronic inflammatory disease leading to the narrowing of the arterial lumen [33]. In **Chapter 4**, I found RORα as a genetic factor influencing the strength of BCG-induced trained immunity. Modulating RORα could have implications not only in the context of trained immunity but also in atherosclerosis. Recent studies demonstrated the link between atherosclerosis development and trained immunity, and how dysbalanced trained immunity responses drive the pathology of atherosclerosis [34]. In this aspect, RORα could be an important regulator of both processes. Indeed, a study done on mice reported that RORα expression in macrophages regulates inflammation during atherosclerosis development [35]. Moreover, *RORA* expression was found to be lower in patients with atherosclerosis and coronary artery disease [36, 37]. There are RORα inhibitors and activators used in mice for various diseases: however, no trials have tested them in human trials thus far.

Another option would be to investigate the potential use of melatonin for the treatment of atherosclerosis and overactive trained immunity. Melatonin, secreted from the pineal gland, was reported to regulate RORα and its functions, acting as a positive regulator [38, 39]. Mice studies demonstrated that melatonin protects against ischemic heart disease and atherosclerosis development through RORα by decreasing inflammation and oxidative stress [40-43]. Melatonin is mainly regarded as an anti-inflammatory molecule [44, 45]. Therefore, it can be speculated that melatonin attenuates trained immunity induction, possibly through RORα activation, and it could be a useful treatment strategy against cardiovascular diseases.

As it has a good safety profile and is being used in many clinical trials, from sleep disorders to acute myocardial infarction and periodontitis, its potential in chronic inflammatory diseases with overactive trained immunity, including atherosclerosis, is worth investigating.

Chronic medication use is widespread among the elderly, however, the non-specific long-term implications of such treatments remain unknown for most of the drugs. With the emergence of the concept of trained immunity, understanding the effects of medications on innate immune memory has become increasingly important. For example, metformin decreased BCG-induced trained immunity, possibly by blocking the mTOR pathway [46]. **Chapter 7** showed that even a single dose of the chronic osteoporosis medication alendronate can diminish the training effect induced by BCG vaccination in young adults. While the mechanism of this inhibition remains unclear, the findings underscore the need for further research into the long-term impacts of medications, especially those used chronically, on the immune system.

Alendronate, along with other bisphosphonates, is not metabolized in the body and directly targets the bone [47]. It has a very low gastrointestinal absorption profile, and the unabsorbed portion is excreted through urine. Interestingly, bisphosphonates are used as an option for treating bone marrow edema syndrome, a condition marked by increased fluid in the bone marrow and accumulation of immune cells, such as macrophages and T and B cells [48]. This raises the possibility that bisphosphonates could create an anti-inflammatory milieu and induce long-term changes in the bone marrow. With their great safety profile and tolerability, alendronate and other bisphosphonates are already used outside their indication, and they emerge as possible candidates for drug repurposing, although their bone affinity may reduce their efficacy. Bisphosphonate derivatives without such affinity for the bone may be needed to be developed. A better understanding of the non-specific effects of bisphosphonates could facilitate the use of their immunomodulatory properties in inflammatory diseases, where trained immunity also contributes to disease progress.

Timing of Vaccination

The timing of vaccination is a critical factor that can significantly influence the efficacy of the vaccine and the overall health outcomes for individuals. For example, morning vaccination was shown to induce higher B and follicular helper T cell activation after an inactivated COVID-19 vaccination [49]. On the other hand, another study suggests that adults vaccinated with a pneumococcal vaccine in the morning have similar levels of antibody production and T cell activity compared to the evening vaccinated adults [50]. Regarding heterologous benefits of vaccination,

BCG vaccination in the morning was associated with more robust trained immunity induction [51]. The season of the year also impacts the heterologous and homologous protection of vaccines [52-54].

In this context, timing can be broadly defined and is not only limited to the time of the day or the year but also includes periods following drug administration, another vaccination, or infection recovery. As the baseline status of the immune system is essential for vaccine responses [55], identifying the optimal timing for vaccine administration is crucial.

The research in this thesis is informative about the timing of vaccination to induce an optimal trained immunity response. In **Chapter 5**, I demonstrated that the season of BCG vaccination influences the trained immunity response. Moreover, **Chapter 6** shows that the immune system of convalescent patients turns back to normal functioning after a mild SARS-CoV-2 infection. Although I did not find considerable functional changes in response against pathogens tested between convalescent and healthy subjects, these minor differences could influence future vaccination responses. In line with this hypothesis, a study assessing the influenza vaccine responses in recovered patients after COVID-19 found that even a mild infection shifts the immune baseline of individuals and shapes the vaccination response [30]. Of note, how this altered vaccine response correlates with the influenza vaccine's efficacy in the real world is unclear. Lastly, **Chapter 7** reports the attenuating impact of alendronate use on BCG-induced trained immunity. These findings have implications, especially for the elderly using alendronate for a prolonged period.

It is essential to emphasize that the influence of factors such as seasonality, duration post-vaccination, medication usage, and infection recovery on vaccine efficacy must be only considered in scenarios where vaccinations are not imperative and urgent. In the future, where trained-immunity-based vaccines are developed and used to increase the immune defense, especially in immunodeficient and frail individuals, optimizing the timing for administration of these vaccines will be advantageous for maximizing the induction of trained immunity.

Limitations and Future Directions

In this thesis, I provide insights into how various host and environmental factors influence innate immunity, as well as trained immunity induction. While the research presented here contributes significantly to understanding the relationship between the immune system and these factors, studies performed here have limitations. These constraints open avenues for further investigation.

Numerous parameters influence the immune and vaccine responses, some of which are not in the context of this thesis, including co-morbidities, microbiota, and lifestyle. Importantly, many of these parameters are interrelated. For instance, the aging process leads to the emergence of co-morbidities, while co-morbidities may also accelerate the aging of the immune system. Moreover, genetic diversity is one of the contributing factors to the sex differences in the immune system. Lastly, it is important to acknowledge that seasonality impacts human behavior and lifestyle, including alterations in diet and exercise patterns, among other factors. It should be emphasized that unraveling the sole impact of any one factor is challenging due to the intertwined nature of these parameters.

A bigger sample size is needed for future validation studies to confirm the findings and reach a more robust statistical significance. In **Chapter 3**, the findings do not provide a direct causal link between high systemic inflammation and aberrant immune cell frequencies with severe COVID-19, but show that the homeostatic state of healthy elderly and males is linked with severe infection. This suggests a need for further research to explore the underlying mechanisms and potential causal links.

Regarding the genetic determinants influencing BCG-induced trained immunity discussed in **Chapter 4**, it is essential to conduct in-depth mechanistic studies investigating which metabolic and epigenetic pathways contribute to enhanced trained immunity during RORα inhibition. Additionally, exploring the involvement of RORα in the atherosclerosis-trained immunity axis is crucial for a deeper understanding of its therapeutic potential.

The research presented in **Chapters 5, 6**, and **7** does not answer how the findings are reflected in real life. For instance, a longitudinal study involving a large cohort should compare the outcomes of winter and spring BCG vaccination on the incidence and severity of non-specific infections, but this is difficult to perform. In **Chapter 6**, even though minor differences were observed after COVID-19 recovery compared to healthy controls, the incidence and severity of subsequent infections following COVID-19 recovery should be investigated. Lastly, given the limited existing research on the long-term immunomodulatory effects of bisphosphonates, future studies should explore the impact of chronic alendronate use in a larger population of older individuals, especially regarding infectious disease incidence and severity.

Conclusion

This thesis investigates key host-related and environmental factors to explore their influence on modulating immune responses, particularly on innate immunity. The

findings provide substantial evidence of the crucial role these parameters play in impacting the immune system's functionality and trained immunity responses. Furthermore, it opens up new avenues for the development of new treatments, the potential repurposing of existing drugs, and offer strategies to improve public health outcomes. As the field of immunology moves forward, a deeper understanding of this dynamic relationship between different parameters and the immune system will be essential to developing more effective disease prevention and treatment approaches.

References

1. Bruxvoort, K.J., et al., Recombinant Adjuvanted Zoster Vaccine and Reduced Risk of Coronavirus Disease 2019 Diagnosis and Hospitalization in Older Adults. J Infect Dis, 2022. 225(11): p. 1915-1922.
2. Dias, S.P., M.C. Brouwer, and D. van de Beek, Sex and Gender Differences in Bacterial Infections. Infect Immun, 2022. 90(10): p. e0028322.
3. Jacobsen, H. and S.L. Klein, Sex Differences in Immunity to Viral Infections. Front Immunol, 2021. 12: p. 720952.
4. Ferreira, A.V., et al., Fatty acid desaturation and lipoxygenase pathways support trained immunity. Nat Commun, 2023. 14(1): p. 7385.
5. Bekkering, S., et al., Metabolic Induction of Trained Immunity through the Mevalonate Pathway. Cell, 2018. 172(1-2): p. 135-146 e9.
6. Sohrabi, Y., et al., LXR Activation Induces a Proinflammatory Trained Innate Immunity-Phenotype in Human Monocytes. Front Immunol, 2020. 11: p. 353.
7. Chaplin, D.D., Overview of the immune response. J Allergy Clin Immunol, 2010. 125(2 Suppl 2): p. S3-23.
8. Bonilla, F.A. and H.C. Oettgen, Adaptive immunity. J Allergy Clin Immunol, 2010. 125(2 Suppl 2): p. S33-40.
9. Netea, M.G., et al., Trained immunity: A program of innate immune memory in health and disease. Science, 2016. 352(6284): p. aaf1098.
10. Mueller, A.L., M.S. McNamara, and D.A. Sinclair, Why does COVID-19 disproportionately affect older people? Aging (Albany NY), 2020. 12(10): p. 9959-9981.
11. Herrera-Esposito, D. and G. de Los Campos, Age-specific rate of severe and critical SARS-CoV-2 infections estimated with multi-country seroprevalence studies. BMC Infect Dis, 2022. 22(1): p. 311.
12. Shaw, A.C., D.R. Goldstein, and R.R. Montgomery, Age-dependent dysregulation of innate immunity. Nat Rev Immunol, 2013. 13(12): p. 875-87.
13. Jakobs, K., et al., Disease Severity in Moderate-to-Severe COVID-19 Is Associated With Platelet Hyperreactivity and Innate Immune Activation. Front Immunol, 2022. 13: p. 844701.
14. Hilligan, K.L., et al., Bacterial-induced or passively administered interferon gamma conditions the lung for early control of SARS-CoV-2. Nat Commun, 2023. 14(1): p. 8229.
15. Jin, J.M., et al., Gender Differences in Patients With COVID-19: Focus on Severity and Mortality. Front Public Health, 2020. 8: p. 152.
16. Bonafe, M., et al., Inflamm-aging: Why older men are the most susceptible to SARS-CoV-2 complicated outcomes. Cytokine Growth Factor Rev, 2020. 53: p. 33-37.
17. Kuijpers, Y., et al., The Genetic Risk for COVID-19 Severity Is Associated With Defective Immune Responses. Front Immunol, 2022. 13: p. 859387.
18. van der Made, C.I., et al., Presence of Genetic Variants Among Young Men With Severe COVID-19. JAMA, 2020. 324(7): p. 663-673.
19. Souyris, M., et al., TLR7 escapes X chromosome inactivation in immune cells. Sci Immunol, 2018. 3(19).
20. Gomez-Carballa, A., et al., Sex-biased expression of the TLR7 gene in severe COVID-19 patients: Insights from transcriptomics and epigenomics. Environ Res, 2022. 215(Pt 2): p. 114288.
21. Spiering, A.E. and T.J. de Vries, Why Females Do Better: The X Chromosomal TLR7 Gene-Dose Effect in COVID-19. Front Immunol, 2021. 12: p. 756262.

22. Agarwal, S., D.T. Nguyen, and E.A. Graviss, Season Is Associated with Interferon Gamma Measured in QuantiFERON Gold In-Tube Test. Open Forum Infect Dis, 2017. 4(Suppl 1): p. S621.
23. Druzd, D., et al., Lymphocyte Circadian Clocks Control Lymph Node Trafficking and Adaptive Immune Responses. Immunity, 2017. 46(1): p. 120-132.
24. He, W., et al., Circadian Expression of Migratory Factors Establishes Lineage-Specific Signatures that Guide the Homing of Leukocyte Subsets to Tissues. Immunity, 2018. 49(6): p. 1175-1190 e7.
25. Ter Horst, R., et al., Seasonal and Nonseasonal Longitudinal Variation of Immune Function. J Immunol, 2021. 207(2): p. 696-708.
26. Dopico, X.C., et al., Widespread seasonal gene expression reveals annual differences in human immunity and physiology. Nat Commun, 2015. 6: p. 7000.
27. Fonseca, D.M., et al., Microbiota-Dependent Sequelae of Acute Infection Compromise Tissue-Specific Immunity. Cell, 2015. 163(2): p. 354-66.
28. Mina, M.J., et al., Measles virus infection diminishes preexisting antibodies that offer protection from other pathogens. Science, 2019. 366(6465): p. 599-606.
29. Bohnacker, S., et al., Mild COVID-19 imprints a long-term inflammatory eicosanoid- and chemokine memory in monocyte-derived macrophages. Mucosal Immunol, 2022. 15(3): p. 515-524.
30. Sparks, R., et al., Influenza vaccination reveals sex dimorphic imprints of prior mild COVID-19. Nature, 2023. 614(7949): p. 752-761.
31. You, M., et al., Single-cell epigenomic landscape of peripheral immune cells reveals establishment of trained immunity in individuals convalescing from COVID-19. Nat Cell Biol, 2021. 23(6): p. 620-630.
32. Cheong, J.G., et al., Epigenetic memory of coronavirus infection in innate immune cells and their progenitors. Cell, 2023. 186(18): p. 3882-3902 e24.
33. Jebari-Benslaiman, S., et al., Pathophysiology of Atherosclerosis. Int J Mol Sci, 2022. 23(6).
34. Riksen, N.P., et al., Trained immunity in atherosclerotic cardiovascular disease. Nat Rev Cardiol, 2023. 20(12): p. 799-811.
35. Molendi-Coste, O., Fleury, S., Bouchaert, E., Pineau, L., Staels, B., Dombrowicz, D., Macrophage-specific inactivation of the nuclear receptor ROR-alpha leads to increased atherosclerosis in mouse. Atherosclerosis, 2014. 235(2): p. E20.
36. Bai, Z., Y. Luo, and L. Tian, ERCC5, HES6 and RORA are potential diagnostic markers of coronary artery disease. FEBS Open Bio, 2022. 12(10): p. 1814-1827.
37. Besnard, S., et al., Expression and regulation of the nuclear receptor RORalpha in human vascular cells. FEBS Lett, 2002. 511(1-3): p. 36-40.
38. Lardone, P.J., et al., Melatonin synthesized by T lymphocytes as a ligand of the retinoic acid-related orphan receptor. J Pineal Res, 2011. 51(4): p. 454-62.
39. Wiesenberg, I., et al., Transcriptional activation of the nuclear receptor RZR alpha by the pineal gland hormone melatonin and identification of CGP 52608 as a synthetic ligand. Nucleic Acids Res, 1995. 23(3): p. 327-33.
40. Li, J., et al., Melatonin ameliorates Parkinson's disease via regulating microglia polarization in a RORalpha-dependent pathway. NPJ Parkinsons Dis, 2022. 8(1): p. 90.
41. Ma, S., et al., Melatonin Ameliorates the Progression of Atherosclerosis via Mitophagy Activation and NLRP3 Inflammasome Inhibition. Oxid Med Cell Longev, 2018. 2018: p. 9286458.
42. Zhao, Y., et al., Novel protective role of the circadian nuclear receptor retinoic acid-related orphan receptor-alpha in diabetic cardiomyopathy. J Pineal Res, 2017. 62(3).

43. He, B., et al., The nuclear melatonin receptor RORalpha is a novel endogenous defender against myocardial ischemia/reperfusion injury. J Pineal Res, 2016. 60(3): p. 313-26.
44. Cho, J.H., et al., Anti-inflammatory effects of melatonin: A systematic review and meta-analysis of clinical trials. Brain Behav Immun, 2021. 93: p. 245-253.
45. Mauriz, J.L., et al., A review of the molecular aspects of melatonin's anti-inflammatory actions: recent insights and new perspectives. J Pineal Res, 2013. 54(1): p. 1-14.
46. Arts, R.J.W., et al., Immunometabolic Pathways in BCG-Induced Trained Immunity. Cell Rep, 2016. 17(10): p. 2562-2571.
47. Porras, A.G., S.D. Holland, and B.J. Gertz, Pharmacokinetics of alendronate. Clin Pharmacokinet, 1999. 36(5): p. 315-28.
48. Baumbach, S.F., et al., How We Manage Bone Marrow Edema-An Interdisciplinary Approach. J Clin Med, 2020. 9(2).
49. Zhang, H., et al., Time of day influences immune response to an inactivated vaccine against SARS-CoV-2. Cell Res, 2021. 31(11): p. 1215-1217.
50. Whittaker, A.C., S. Gallagher, and M. Drayson, Time of day of vaccination does not relate to antibody response to thymus-independent vaccinations. Vaccine X, 2022. 11: p. 100178.
51. de Bree, L.C.J., et al., Circadian rhythm influences induction of trained immunity by BCG vaccination. J Clin Invest, 2020. 130(10): p. 5603-5617.
52. Moorlag, S., 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): p. 171-187 e14.
53. Jensen, K.J., et al., Seasonal variation in the non-specific effects of BCG vaccination on neonatal mortality: three randomised controlled trials in Guinea-Bissau. BMJ Glob Health, 2020. 5(3): p. e001873.
54. Linder, N., et al., Effect of season of inoculation on immune response to rubella vaccine in children. J Trop Pediatr, 2011. 57(4): p. 299-302.
55. Tsang, J.S., et al., Improving Vaccine-Induced Immunity: Can Baseline Predict Outcome? Trends Immunol, 2020. 41(6): p. 457-465.

8

Eurasian magpie
Saksağan
Ekster

CHAPTER 9

Nederlandse samenvatting
Türkçe özet

Nederlandse samenvatting

Het afweersysteem of immuunsysteem is het beveiligingsteam van ons lichaam. Het beschermt ons tegen schadelijke ziekteverwekkers door onder andere te patrouillereren op kankercellen, weefsel te genereren en de homeostase te behouden. Het heeft twee componenten: aangeboren en adaptieve afweer. De eerste verdedigingslinie is het aangeboren immuunsysteem, met barrières als de huid en specifieke cellen die snel bedreigingen kunnen herkennen en aanvallen. Vervolgens betrekt het adaptieve immuunsysteem een meer gespecialiseerde groep cellen, genaamd lymfocyten. Deze cellen hebben het opmerkelijke vermogen om specifieke ziekteverwekkers – die ze eerder zijn tegengekomen – te onthouden. Hierdoor genereren ze een sterkere reactie bij de tweede ontmoeting, hoewel trager dan het aangeboren immuunsysteem.

Getrainde afweer, een nieuwer concept dat nog geen 15 jaar geleden is gedefinieerd, verwijst naar het vermogen van de aangeboren immuuncellen om een soort geheugen te vormen. Dit stelt aangeboren immuuncellen in staat om patronen te herkennen en krachtiger te reageren als ze later een ander microbiologisch patroon tegenkomen. Getrainde afweer biedt bescherming tegen een breed scala aan ziekten en infecties door het verhoogde vermogen van de aangeboren immuniteit om sterk te reageren. Bijvoorbeeld, het Bacillus Calmette-Guérin (BCG) vaccin biedt bescherming tegen tuberculose door het induceren van adaptief immuungeheugen. Echter, het induceert ook getrainde afweer en biedt bescherming tegen verschillende ontstekings- en ademhalingsziekten.

Het immuunsysteem werkt niet op zichzelf; het is constant in interactie met en wordt beïnvloed door alles om ons heen en in ons lichaam. De interne factoren die de activiteit van het immuunsysteem beïnvloeden, zijn onder andere leeftijd, geslacht en genetische achtergrond. Aan de andere kant zijn voorbeelden van externe factoren dieet, geschiedenis van vaccinaties en infecties, medicijngebruik en de tijd van het jaar.

In de afgelopen eeuw hebben de immunologie en geneeskunde grote vooruitgang geboekt. We hebben meer geleerd over hoe ons lichaam en immuunsysteem werken. Desalniettemin is er nog veel onbekend. Met name over hoe interne- en omgevingsfactoren beïnvloeden hoe het lichaam infecties afweert en reageert op vaccinaties. Deze gaten in onze kennis zijn belangrijk, omdat ze de sleutel vormen tot de ontwikkeling van nieuwe behandelingen en vaccins. Dit kan ons ook helpen onze therapie- en preventieopties te verbeteren.

In deze scriptie heb ik onderzocht hoe verschillende interne en externe factoren het aangeboren immuunsysteem en de getrainde immuniteit beïnvloeden. In het eerste deel van de scriptie, dat de **hoofdstukken 2, 3** en **4** omvat, heb ik het effect van leeftijd, geslacht en genetische achtergrond op de functie van het immuunsysteem als interne factoren onderzocht. In het tweede deel, dat bestaat uit de **hoofdstukken 5, 6** en **7**, heb ik bewijs geleverd over hoe de tijd van het jaar, een eerdere infectie of medicijngebruik de immuunrespons en de inductie van getrainde afweer beïnvloeden.

Hoofdstuk 2 bespreekt de veranderingen in het immuunsysteem naarmate we ouder worden, wat invloed heeft op het vermogen om te reageren op ziekteverwekkers en vaccinaties. Zowel het aantal als de functie van de immuuncellen verandert tijdens het verouderingsproces, wat leidt tot een zwakkere immuunrespons en lagere vaccinatie-efficiëntie. Om hiermee te helpen, heb ik voorgesteld om getrainde afweer-gebaseerde vaccins bij ouderen te gebruiken.

Oudere mensen lopen ook een hoger risico op ernstige infecties en ziekenhuisopnames. De recente COVID-19-pandemie toonde nogmaals aan dat mensen op latere leeftijd vatbaarder zijn voor het ontwikkelen van ernstige ziekten. Bovendien lopen niet alleen oudere volwassenen, maar ook mannen, ongeacht hun leeftijd, meer risico op ernstige COVID-19. In **Hoofdstuk 3** heb ik gevonden dat mannen en oudere volwassenen een immuunprofiel hebben dat al gerelateerd is aan ernstige infecties. Verder hebben we inzichten geboden in hoe de tijd van het jaar de immuunrespons van mensen op het SARS-CoV-2-virus kan beïnvloeden. Ouderen hadden het hele jaar door een zwakkere respons, en het patroon tussen mannen en vrouwen verschilde. Deze studie suggereert dat oudere individuen en mannen intrinsiek vatbaarder zijn voor het ontwikkelen van ernstige COVID-19, en hun respons op dit virus verandert afhankelijk van het seizoen.

Hoofdstuk 4 verkent hoe onze genetica een rol speelt in de getrainde immuniteitsrespons na BCG-vaccinatie. In het bijzonder hebben we gevonden dat een gen genaamd *RORA* een factor is die bijdraagt aan de door BCG geïnduceerde getrainde immuniteitsrespons. Wanneer de activiteit van dit gen wordt geblokkeerd, induceert het getrainde immuniteit op zichzelf, en deze blokkade verbetert ook het trainingseffect van BCG. Het blokkeren van RORα, het eiwit gecodeerd door *RORA*, veranderde de vorm van de cellen en verhoogde de productie van reactieve zuurstofsoorten en lactaat, wat wijst op de inductie van getrainde immuniteit. We hebben ook aangetoond dat een eiwit genaamd LDHA de getrainde immuniteit die door BCG en de RORα-remmer wordt verleend, kan moduleren. Dit hoofdstuk benadrukt dat het *RORA*-gen kan worden gebruikt om de vaccinreacties van BCG te verbeteren. En mogelijk om cardiovasculaire ziekten te behandelen die gekoppeld zijn aan overactieve immuunresponsen en getrainde immuniteit.

De tijd van het jaar bleek de functionaliteit van het immuunsysteem te beïnvloeden. In **Hoofdstuk 5** was ik benieuwd of de verschillende seizoenen de getrainde immuniteitsreacties op BCG-vaccinatie beïnvloedt. Om dit te onderzoeken, ontvingen jonge en gezonde deelnemers in de winter- of lentemaanden een placebo of BCG. Ik vond dat de immuuncellen geïsoleerd uit het bloed van de in de winter gevaccineerde individuen meer pro-inflammatoire cytokinen produceerden dan die van de in de lente gevaccineerde proefpersonen. Interessant is dat de IFNγ-productie van de lentegroep hoger was dan die van de wintergroep, wat wijst op een sterkere adaptieve immuunrespons. Ik heb verschillende epigenetische veranderingen in de monocyten en NK-cellen van de twee groepen geïdentificeerd, wat suggereert dat de seizoensfactoren de epigenetica van de immuuncellen kunnen beïnvloeden.

Een andere omgevingsfactor die het immuunsysteem beïnvloedt, zijn eerdere infecties. Elke ontmoeting, of het nu een vaccinatie of een ziekteverwekker is, zal waarschijnlijk een kort- of langetermijneffect op het immuunsysteem achterlaten. In **Hoofdstuk 6** heb ik het mogelijke effect van milde/matige COVID-19 op de functionaliteit van het immuunsysteem na herstel onderzocht, met de focus op aangeboren immuniteit. We vonden dat een belangrijk pad in de virale verdediging, de respons op interferon, is zwakker bij herstellende individuen. Echter, toen ik de immuuncellen van deze deelnemers stimuleerde met verschillende ziekteverwekkers en interferonen, zag ik geen verschil in hun immuunrespons vergeleken met de gezonde controlegroep. De hoeveelheid immuuncelsubsets in het bloed was vergelijkbaar tussen gezonde en herstellende proefpersonen. Aan de andere kant was, zonder enige stimulatie, de IL-1Ra- en IFNγ-productie van de herstelde patiënten significant hoger dan die van gezonde proefpersonen. Deze resultaten tonen aan dat het immuunsysteem van mensen die herstellen van milde/matige COVID-19 terugkeert naar een gezond niveau, hoewel lichte verschillen op epigenetisch en transcriptomisch niveau blijven bestaan in vergelijking met niet-geïnfecteerde personen.

Medicijngebruik komt veel voor bij ouderen en kan invloed hebben op hoe het aangeboren en adaptieve immuunsysteem reageert op bepaalde ziekteverwekkers of vaccinaties. In **Hoofdstuk 7** heb ik onderzocht of alendroninezuur, vaak voorgeschreven voor osteoporose, de BCG-geïnduceerde getrainde immuunrespons bij jongvolwassenen kan beïnvloeden. In deze studie ontvingen deelnemers een placebo, BCG-vaccin of oraal alendroninezuur samen met BCG-vaccinatie. We vonden een verlaagde TNFα na heterologe stimulatie bij proefpersonen die BCG plus alendroninezuur ontvingen in vergelijking met de groep die enkel BCG ontving. De combinatiebehandeling resulteerde ook in een hoger aantal neutrofielen en

plasmacellen in de circulatie. Hoewel BCG-vaccinatie leidde tot de opregulatie van paden gerelateerd aan ontsteking en interferon alpha- en gammareacties na poly I:C stimulatie van immuuncellen, werd deze patroon niet gevonden in de combinatiegroep. Samengevat wijzen deze bevindingen op de mogelijke impact van alendroninezuurgebruik op getrainde immuniteitsreacties op de lange termijn.

Samenvattend benadrukt het onderzoek in deze scriptie hoe verschillende intrinsieke en extrinsieke factoren de manier waarop het immuunsysteem werkt kunnen beïnvloeden. Deze kennis is cruciaal, omdat het wetenschappers informeert over nieuwe manieren om ziekten te behandelen en bestaande geneeswijzen op andere gebieden toe te passen. Naarmate we meer leren over hoe al deze factoren het immuunsysteem beïnvloeden, kunnen we nieuwe en betere manieren vinden om ziekten te voorkomen en te behandelen.

Türkçe özet

Bağışıklık sistemi, vücudumuzun güvenlik timidir ve zararlı mikrop ve patojenlere karşı bizi korur. Bağışıklık sisteminin kanser hücrelerini fark etmeye, doku yenilenmesine ve homeostazına yardımcı olmak gibi diğer işlevleri de vardır. Bu sistem iki bileşenden oluşur, bunlar doğuştan gelen ve edinilmiş bağışıklık olarak adlandırılır. Doğuştan gelen bağışıklık sistemi, cilt gibi bariyerleri ve tehditleri hızla tanıyıp saldırabilecek özel hücreleri kullanan ilk savunma hattıdır. Öte yandan, edinilmiş bağışıklık sistemi, lenfositler olarak adlandırılan daha uzmanlaşmış bir hücre grubunu içerir. Bu hücreler, daha önce karşılaştıkları mikropları hatırlama yeteneğine sahiptirler. Bu sayede edinilmiş bağışıklık sistemi aynı mikropla ikinci karşılaşmada daha güçlü bir tepki geliştirir, ancak bu tepki yavaş oluşur.

Bunun yanında, eğitilmiş doğal bağışıklık 15 yıldan az bir süre önce tanımlanmış bir kavramdır ve doğal bağışıklık hücrelerinin belli patojenik molekülleri tanıyarak bir tür hafıza oluşturma kapasitesine denir. Kısacası, enfeksiyon ya da aşılanma sonrasında doğal bağışıklık hücreleri yeniden programlanır ve sonraki enfeksiyonlara karşı daha güçlü bir tepki oluşturmak için hazır hale gelir. Eğitilmiş bağışıklık, doğuştan gelen bağışıklığın güçlü tepki verme kapasitesini arttırdığı için birçok hastalık ve enfeksiyona karşı koruma sağlayabilir. Örneğin, Bacillus Calmette-Guerin (BCG) aşısı, edinilmiş bağışıklık yoluyla tüberküloza karşı koruma sağlar. Ancak, aynı zamanda eğitilmiş doğal bağışıklığı da harekete geçirir ve farklı iltihaplara ve solunum hastalıklarına karşı koruma sağlar.

Bağışıklık sistemi izole bir şekilde çalışmaz; sürekli olarak etrafımızdaki ve vücudumuzdaki her şeyle etkileşim halindedir ve bunlardan etkilenir. Bu etkileşimler, bağışıklık sistemimizin çalışma kapasitesini değiştirip geliştirebilir ya da azaltabilir. Bağışıklık sistemi aktivitesini etkileyen iç faktörler arasında yaş, cinsiyet ve genetik bulunur. Öte yandan, diyet, mevsimsel değişimler, aşılanma ve enfeksiyon geçmişi ve ilaç kullanımı dış faktörlere örnek olarak verilebilir.

Geçtiğimiz yüzyılda, immünoloji ve tıp alanında büyük ilerlemeler kaydedildi. Bunun yanında vücudumuzun ve bağışıklık sisteminin nasıl çalıştığı hakkında birçok şey keşfedildi. Ancak bağışıklık sistemimizin iç ve dış faktörlerden nasıl etkilendiği ve bu faktörlerin vücudun enfeksiyonlarla savaşma yeteneği ve aşıların etkinliğini nasıl etkilediği konularında hala çok fazla bilinmeyen var. Bu konularda daha fazla bilgi sahibi olmak önemlidir çünkü bunlar yeni tedavilerin ve aşıların geliştirilmesinde anahtardır. Bunlar, aynı zamanda var olan tedavi aşıları (örneğin BCG aşısı gibi eğitilmiş doğal bağışıklığı güçlendiren) iyileştirmemize de yardımcı olabilir.

Bu tezde, çeşitli iç ve dış parametrelerin doğuştan gelen bağışıklık sistemi ve onun hafıza kapasitesine (eğitilmiş doğal bağışıklık) olan etkilerini inceledim. Tezin ilk kısmı olan, **2., 3. ve 4. bölümlerini** kapsayan kısımda, iç faktörlere örnekler olarak ilerleyen yaş, cinsiyet ve genetiğin bağışıklık sistemi işlevselliğine olan etkilerini araştırdım. İkinci kısımda, yani **5., 6. ve 7. bölümde**, mevsimler, geçmiş bir enfeksiyon veya ilaç kullanımının bağışıklık yanıtı ve eğitilmiş bağışıklığın uyarılması üzerindeki etkilerine dair kanıtlar sundum.

Tezin **2. bölümü**, yaşlandıkça bağışıklık sistemimizde meydana gelen değişiklikleri ve bu farklılaşmanın patojenlere ve aşılara yanıt verme yeteneğini nasıl etkilediğini tartışmaktadır. Yaşlanma sürecinde hem bağışıklık hücrelerinin sayısı hem de işlevi değişir, bu da daha zayıf bir bağışıklık yanıtına ve aşıların ileri yaştaki insanlarda daha az etkili olmasına yol açar. Bununla başa çıkmak ve daha güçlü bir bağışıklık yanıtı oluşturmak için yaşlılarda eğitilmiş doğal bağışıklık temelli aşıların kullanılmasını önerdik.

Yaşlı insanlar ayrıca ciddi enfeksiyonlara yakalanma ve hastaneye kaldırılma konusunda daha büyük risk altındadır. COVID-19 pandemisi, ileri yaşlardaki insanların ağır enfeksiyon geçirme olasılığının daha yüksek olduğunu bir kez daha gösterdi. Ayrıca, sadece yaşlı yetişkinler değil, yaşları ne olursa olsun erkekler de ağır COVID-19 hastalığı geçirme riski taşır. Ağır derecede COVID-19 geçiren insanlarda kanda belirli proteinlerin ve bağışıklık hücrelerinin düşük veya yüksek miktarda olduğu tanımlanmış bir durumdur. **3. bölümde**, ağır enfeksiyonla ilişkili bu daha önce tanımlanmış bağışıklık sistemi profilinin sağlıklı erkeklerde ve yaşlı yetişkinlerde enfeksiyon geçirmeden bile zaten mevcut olduğunu buldum. Ayrıca, mevsimlerin insanların SARS-CoV-2 virüsüne karşı olan bağışıklık sistemi yanıtını nasıl etkileyebileceği hakkında içgörüler sağladım. Yaşlı insanlar, gençlere göre yıl boyunca bu virüse karşı daha zayıf bir bağışıklık sistemi yanıtı gösterdi ve erkekler ile kadınlar arasındaki bağışıklık sistemi cevabı mevsimlere bağlı olarak birbirinden farklıydı. Bu çalışma, yaşlı bireylerin ve erkeklerin ağır bir COVID-19 enfeksiyonuna yakalanma konusunda doğal olarak daha savunmasız olduğunu ve bu virüse karşı tepkilerinin mevsime bağlı olarak değiştiğini öne sürmektedir.

4. bölüm, genetiğimizin BCG aşısı sonrası eğitilmiş doğal bağışıklık yanıtında nasıl bir rol oynadığını araştırmaktadır. Öncelikle, *RORA* adlı bir genin BCG tarafından uyarılan eğitilmiş bağışıklık yanıtına katkıda bulunan bir faktör olduğunu buldum. Bu genin çalışmasının engellenmesi, eğitilmiş bağışıklığı kendiliğinden uyardı ve bu aynı zamanda BCG'nin eğitilmiş bağışıklığı uyarma etkisini de artırdı. *RORA* geni tarafından kodlanan RORα proteininin çalışmasını engellemek, hücrelerin dış

görünüşünü etkiledi ve reaktif oksijen türleri ile laktat üretimini artırarak eğitilmiş bağışıklığı uyardı. Ayrıca, LDHA adlı bir proteinin BCG ve RORα inhibitörünün aktifleştirdiği eğitilmiş bağışıklıkta etkisinin olabileceğini gösterdik. Bu bölüm, *RORA* geninin BCG'nin sağladığı eğitilmiş bağışıklık yanıtını düzenlemek ve aşırı aktif eğitilmiş bağışıklıkla bağlantılı kardiyovasküler hastalıkları tedavi etmek amacıyla kullanılabileceğini vurgulamaktadır.

Mevsim değişikliklerinin bağışıklık sisteminin işlevselliğini etkilediği gösterilmiştir. **5. bölümde**, farklı mevsimlerde yapılan BCG aşılamasının eğitilmiş bağışıklık yanıtlarını nasıl etkilediğini araştırdım. Bunun için, genç ve sağlıklı katılımcılar kış veya ilkbahar aylarında plasebo ya da BCG ile aşılandılar. Kış aylarında aşılanmış bireylerin kanından elde edilen bağışıklık hücrelerinin, ilkbaharda aşılanmış olanların hücrelerine göre daha fazla pro-enflamatuar sitokin ürettiğini gözlemledim. İlginç bir şekilde, ilkbahar grubunun IFNγ üretimi kış grubundan daha yüksekti, bu da ilkbahar aylarında daha güçlü bir edinilmiş bağışıklık yanıtını gösteriyor olabilir. İki grubun monositleri ve doğal öldürücü hücrelerinde farklı epigenetik değişiklikler tespit ettim, bu durum mevsimsel faktörlerin bağışıklık hücrelerinin epigenetiğini etkileyebileceğini öne sürmektedir.

Bağışıklık sistemini etkileyen başka bir çevresel faktör geçirilmiş enfeksiyonlardır. Aşılanma veya mikroplarla her karşılaşma, muhtemelen bağışıklık sistemi üzerinde kısa veya uzun vadeli bir iz bırakacaktır. **6. bölümde**, hafif/orta dereceli COVID-19 hastalığından iyileşen insanların bağışıklık sistemini sağlıklı ve enfeksiyonu daha önce hiç geçirmemiş (sağlıklı) insanlarla karşılaştırdım ve bu çalışmada özellikle doğuştan gelen bağışıklık üzerine odaklandım. Viral savunmada çok önemli olan interferona yanıt yolağının, hastalık sonrası iyileşmiş bireylerde düşük olduğunu gözlemledim. Ancak, bu katılımcıların bağışıklık hücreleri çeşitli patojenler ve interferonlarla uyarıldığında, sağlıklı kontrollerle karşılaştırılınca immün yanıtlarında bir fark yoktu. Ayrıca, kanda bulunan bağışıklık sistemi hücre tipleri ve bu hücrelerin sayısı enfeksiyonu daha önce geçirmemiş ve geçirip iyileşen bireyler arasında benzerlik gösterdi. Öte yandan, herhangi bir uyarım olmadan, iyileşen hastaların IL-1Ra ve IFNγ üretimi sağlıklı insanlardan önemli ölçüde daha yüksekti. Bu sonuçlar, hafif/orta dereceli COVID-19 hastalığından iyileşen kişilerin bağışıklık sisteminin bu hastalığı hiç geçirmemiş olanların seviyesine neredeyse geri döndüğünü, ancak epigenetik ve transkriptomik seviyelerde küçük farklılıkların hala olabildiğini göstermektedir.

Yaşlılarda ilaç kullanımı yaygındır ve ilaç kullanımı belirli patojenlere karşı bağışıklık yanıtını ya da aşıların etkinliğini etkileyebilir. **7. bölümde**, osteoporoz için yaygın

olarak reçete edilen alendronatın, genç yetişkinlerde BCG tarafından uyarılan eğitilmiş bağışıklık yanıtını nasıl etkileyebileceğini araştırdım. Bu çalışmada, katılımcılar plasebo, BCG veya BCG aşısıyla birlikte alendronat tableti aldı. BCG+alendronat alan bireylerde, BCG grubuna kıyasla değişik patojenlerle uyarılma sonrası TNFα üretiminin az olduğunu bulundu. BCG grubuyla karşılaştırıldığında, kombinasyon tedavisi ayrıca dolaşımdaki nötrofil ve plazma hücresi sayısını artırdı. BCG aşısı, periferik kan mononükleer hücrelerinin bir viral çift sarmallı RNA olan poli I:C ile uyarımı sonrası immün cevabı ve interferon alfa ve gamma yanıtlarıyla ilgili yolakların artmasına yol açarken, bu sonuçlar kombinasyon grubunda tespit edilmedi. Sonuç olarak, bu bulgular alendronat kullanımının uzun vadede eğitilmiş bağışıklık yanıtları üzerindeki olası etkisine işaret etmektedir.

Özetle, bu tezdeki araştırmalar, çeşitli iç ve dış faktörlerin bağışıklık sisteminin işleyişi üzerindeki etkisini vurgulamaktadır. Bu bilgi, bilim insanlarını yeni tedavi yöntemleri bulma ve mevcut olanları yeniden kullanma konusunda bilgilendirmektedir. Tüm bu faktörlerin bağışıklık sistemini nasıl etkilediğini daha iyi öğrendikçe, hastalıkları önleme ve tedavi etme konusunda yeni ve daha iyi yollar keşfedebiliriz.

Hummingbird
Sinek kuşu
Kolibrie

Appendices

Research data management
PhD portfolio
List of publications
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Curriculum vitae

Research data management

The data obtained in this thesis are archived according to the Findable, Accessible, Interoperable, and Reusable (FAIR) principles.

This thesis is based on the results of human studies and existing data from published studies, which were conducted following the principles of the Declaration of Helsinki. The clinical studies were approved by the CMO Region Arnhem-Nijmegen, and the registration numbers can be found in the methods section of each chapter. Informed consent was obtained from all research participants. The privacy of participants was ensured by the use of encrypted and unique subject codes.

Raw and processed data are stored in lab journals or digitally on a local server of the Department of Internal Medicine, which is backed up daily on the Radboud University Medical Center server. Case report files of the clinical studies are stored in Castor EDC. The data will be saved for 15 years after clinical studies are terminated. The datasets presented in Chapter 6 have been deposited at the European Genome-phenome Archive (EGA), under accession number EGAS00001005529. All other data are furthermore available upon request to the corresponding author(s) of each chapter.

A

PhD portfolio of Gizem Kilic

Department: **Internal medicine**
PhD period: **01/07/2019 – 31/12/2023**
PhD supervisor: **prof. M. G. Netea**
PhD co-supervisor: **Assist. Prof. J. Domínguez-Andrés**

Training activities	Hours
Courses	
• Basic course for clinical investigators - BROK (2019)	42.00
• Radboudumc - Scientific integrity (2020)	20.00
• Science Journalism and Communication (2020)	84.00
• RIMLS - Introduction course "In the lead of my PhD" (2021)	15.00
• RIMLS PhD course (2021)	21.00
• RU - Design and Illustration (2021)	28.00
• RU - Analytic Storytelling (2021)	28.00
• RU - Entrepreneurship and Innovation for PhD-students (2023)	84.00
• RU - The Art of Finishing Up (2023)	10.00
• Radboudumc - Re-registration BROK (2023)	5.00
• The next step in my career (2024)	24.00
Seminars	
• Investigating cellular pathways driving severe COVID-19: Fresh insights from longitudinal plasma proteomics (2020)	2.80
• How to get published: Workflows and writing tips (2020)	2.80
• How to sell your science (2020)	8.40
• Immune Responses to SARS-CoV-2 (2021)	2.80
• Olink Proteomics & Gene Expression Profiling (2021)	2.80
• Immunology in the time of COVID-19: achievements, challenges and opportunities (2021)	4.00
• Meet the Expert; How to Write a Rebuttal (2021)	1.00
• Research Integrity Round – The dark side of science (2021)	1.00
• Research Integrity Round – The challenges of collaboration with profit and non-profit organisations" (2021)	1.00
• Immune responses to SARS-CoV-2 (2022)	1.00
• Research Integrity Round – Publication ethics: Promises, problems and perspectives (2022)	1.00
• You and your PhD - Essential career advice for doctoral students (2022)	1.00
• Research Integrity Round – Artificial Intelligence and Research Integrity: a good marriage? (2024)	1.00

Conferences	
• 4th International Conference on Innate Immune Memory (2019) – *Poster presentation*	56.00
• Optimmunize 2020 (2020) - *Oral presentation*	84.00
• PhD Retreat (2020)	14.00
• ImmunoMetNet (2020)	7.00
• Biomarkers of Paris 2020 (2020)	7.00
• International Symposium on Innate Immunity and COVID-19 (2020)	2.80
• Sex Differences in the Immune System (2021)	21.00
• 31st European Congress of Clinical Microbiology & Infectious Diseases (ECCMID) (2021) *-Short oral presentation*	28.00
• Secondary Effects of Antigen Specific Vaccines (2021)	12.00
• PhD Retreat (2021)	7.00
• New Frontiers in Translational Glycoscience Symposium (2021)	10.00
• PhD Retreat (2022) – *Oral presentation*	42.00
• Summer Innate Immunology Conference, Cluj (2022) – *Poster presentation*	28.00
• Optimmunize 2022 (2022) – *Oral presentation*	35.00
• 5th International Symposium on Trained Immunity (2023) – *Poster presentation*	28.00
Other	
• Cytokine Meeting (2019-2023) – *6x Oral presentation*	138.00
Teaching activities	
Supervision of internships / other	
• Student internship coaching (2021)	56.00
Total	**967.40**

List of publications

1. **Kilic G**, Debisarun PA, Alaswad A, Baltissen MP, Lamers LA, de Bree CJ, Benn CS, Aaby P, Dijkstra H, Lemmers H, Martens JHA, Domínguez-Andrés J, van Crevel R, Li Y, Xu CJ, Netea MG. Seasonal Variation in BCG-Induced Trained Immunity. (submitted)
2. **Kilic G**, Matzaraki V, Bulut O, Baydemir I, Ferreira AV, Rabold K, Moorlag SJCFM, Koeken VACM, de Bree CJ, Mourits VP, Joosten LAB, Domínguez-Andrés J, Netea MG. RORα negatively regulates BCG-induced trained immunity. (submitted)
3. Bulut O, **Kilic G**, Debisarun PA, Röring RJ, Sun S, Kolkman M, van Rijssen E, Ten Oever J, Koenen H, Barreiro L, Domínguez-Andrés J, Netea MG. Alendronate Modulates Cytokine Responses in Healthy Young Individuals After BCG Vaccination. *Immunology Letters*. 2024 Mar 11:106851. DOI: 10.1016/j.imlet.2024.106851
4. Röring RJ, Debisarun PA, Botey-Bataller J, Suen TK, Bulut O, **Kilic G**, Koeken VA, Sarlea A, Bahrar H, Dijkstra H, Lemmers H, Gössling K, Rüchel N, Ostermann PN, Müller L, Schaal H, Adams O, Borkhardt A, Ariyurek Y, de Meijer EJ, Kloet SL, ten Oever J, Placek K, Li Y, Netea MG. MMR vaccination induces trained immunity via functional and metabolic reprogramming of γδ T cells. *The Journal of Clinical Investigation*. 2024 Jan 30. DOI: 10.1172/JCI170848
5. Glymenaki M, Hahn AM, Hausmann A, Kempkes RW, **Kilic G**, Kulsvehagen L, Mooslechner AA, Mossadegh-Keller N, Nabhan M, Bechara R. Young EFIS: Three years of progress-Reflecting on achievements and embracing new horizons. *European journal of immunology*. 2023 Dec;53(12):e2350749. DOI: 10.1002/eji.202350749
6. Ferreira AV, Alarcon-Barrera JC, Domínguez-Andrés J, Bulut Ö, **Kilic G**, Debisarun PA, Röring RJ, Özhan HN, Terschlüsen E, Ziogas A, Kostidis S, Mohammed Y, Matzaraki V, Reniers G, Giamarellos-Bourboulis EJ, Netea MG, Giera M. Fatty acid desaturation and lipoxygenase pathways support trained immunity. *Nature Communications*. 2023 Nov 15;14(1):7385. DOI: 10.1038/s41467-023-43315-x
7. Geckin B, **Kilic G**, Debisarun PA, Föhse K, Rodríguez-Luna A, Fernández-González P, Sánchez AL, Domínguez-Andrés J. The fungal-derived compound AM3 modulates pro-inflammatory cytokine production and skews the differentiation of human monocytes. *Frontiers in Immunology*. 2023 Oct 9;14:1165683. DOI: 10.3389/fimmu.2023.1165683
8. Föhse K, Debisarun PA, **Kilic G**, van Dodewaard-de Jong JM, Netea MG. Evaluation of the safety and immunological effects of Bacillus Calmette–Guérin in combination with checkpoint inhibitor therapy in a patient with neuroendocrine carcinoma: a case report. *Journal of Medical Case Reports*. 2023 Sep 4;17(1):377. DOI: 10.1186/s13256-023-04117-3
9. Ferreira AV, Kostidis S, Groh LA, Koeken VA, Bruno M, Baydemir I, **Kilic G**, Bulut Ö, Andriopoulou T, Spanou V, Synodinou KD, Gkavogianni T, Moorlag SJCFM, de Bree LC, Mourits VP, Matzaraki V, Koopman WJH, van de Veerdonk FL, Renieris G, Giera M, Domínguez-Andrés J. Dimethyl itaconate induces long-term innate immune responses and confers protection against infection. *Cell Reports*. 2023 Jun 27;42(6). DOI: 10.1016/j.celrep.2023.112658
10. Geckin B, Zoodsma M, **Kilic G**, Debisarun PA, Rakshit S, Adiga V, Ahmed A, Parthiban C, Kumar NC, D'Souza G, Baltissen MP, Martens JHA, Domínguez-Andrés J, Li Y, Vyakarnam A, Netea MG. Differences in immune responses in individuals of Indian and European origin: relevance for the COVID-19 pandemic. Microbiology Spectrum. 2023 Apr 13;11(2):e00231-23. DOI: 10.1128/spectrum.00231-23
11. Debisarun PA, **Kilic G**, de Bree LC, Pennings LJ, van Ingen J, Benn CS, Aaby P, Dijkstra H, Lemmers H, Domínguez-Andrés J, van Crevel R, Netea MG. The impact of BCG dose and revaccination on trained immunity. *Clinical Immunology*. 2023 Jan 1;246:109208. DOI: 10.1016/j.clim.2022.109208

12. Bulut O, **Kilic G**, Domínguez-Andrés J. Immune memory in aging: a wide perspective covering microbiota, brain, metabolism, and epigenetics. *Clinical Reviews in Allergy & Immunology*. 2022 Dec;63(3):499-529. DOI: 10.1007/s12016-021-08905-x

13. Zoodsma M, de Nooijer AH, Grondman I, Gupta MK, Bonifacius A, Koeken VA, Kooistra E, **Kilic G**, Bulut O, Kox M, Domínguez-Andrés J, van Gammeren AJ, van der Ven JAM, Pickers P, Blasczyk R, Behrens G, van de Veerdonk FL, Joosten LAB, Xu CJ, Eiz-Vesper B, Netea MG, Li Y. Targeted proteomics identifies circulating biomarkers associated with active COVID-19 and post-COVID-19. *Frontiers in Immunology*. 2022 Nov 3;13:1027122. DOI: 10.3389/fimmu.2022.1027122

14. Bannister S, Kim B, Domínguez-Andrés J, **Kilic G**, Ansell BR, Neeland MR, Moorlag SJ, Matzaraki V, Vlahos A, Shepherd R, Germano S, Bahlo M, Messina NL, Saffery R, Netea MG, Curtis N, Novakovic B. Neonatal BCG vaccination is associated with a long-term DNA methylation signature in circulating monocytes. *Science Advances*. 2022 Aug 5;8(31):eabn4002. DOI: 10.1126/sciadv.abn4002

15. Liu Z, **Kilic G**, Li W, Bulut O, Gupta MK, Zhang B, Qi C, Peng H, Tsay HC, Soon CF, Mekonnen YA, Ferreira AV, van der Made CI, van Cranenbroek B, Koenen HJPM, Simonetti E, Diavatopoulos D, de Jonge, MI, Müller L, Schaal H, Ostermann PN, Cornberg M, Eiz-Vesper B, van Crevel R, Joosten LAB, Domínguez-Andrés J, Xu CJ, Netea MG, Li Y. Multi-omics integration reveals only minor long-term molecular and functional sequelae in immune cells of individuals recovered from COVID-19. *Frontiers in Immunology*. 2022 Apr 6;13:838132. DOI: 10.3389/fimmu.2022.838132

16. Zhang B, Moorlag SJ, Dominguez-Andres J, Bulut Ö, **Kilic G**, Liu Z, van Crevel R, Xu CJ, Joosten LA, Netea MG, Li Y. Single-cell RNA sequencing reveals induction of distinct trained-immunity programs in human monocytes. *The Journal of clinical investigation*. 2022 Apr 1;132(7). DOI: 10.1172/JCI147719

17. Debisarun PA, Gössling KL, Bulut O, **Kilic G**, Zoodsma M, Liu Z, Oldenburg M, Rüchel N, Zhang B, Xu CJ, Struycken P, Koeken VACM, Dominguez-Andres J, Moorlag SJCFM, Taks E, Ostermann PN, Müller L, Schaal H, Adams O, Borkhardt A, ten Oever J, van Crevel R, Li Y, Netea MG. Induction of trained immunity by influenza vaccination-impact on COVID-19. *PLoS pathogens*. 2021 Oct 25;17(10):e1009928. DOI: 10.1371/journal.ppat.1009928

18. **Kilic G**, Bulut O, Jaeger M, Koeken VA, de Bree C, Domínguez-Andrés J, Joosten LA, Netea MG. The immunological factors predisposing to severe covid-19 are already present in healthy elderly and men. *Frontiers in immunology*. 2021 Aug 9;12:720090. DOI: 10.3389/fimmu.2021.720090

19. Bechara R, Cela C, Hahn A, **Kilic G**, Laudisi F, McAllister E, Mossadegh-Keller N. One year of Young EFIS: achievements and future directions. *European journal of immunology*. 2021 Aug;1875-1878. DOI: 10.1002/eji.202170085

20. Domínguez-Andrés J, Arts RJ, Bekkering S, Bahrar H, Blok BA, de Bree LC, Bruno M, Bulut Ö, Debisarun PA, Dijkstra H, Dos Santos JC, Ferreira AV, Flores-Gomez D, Groh LA, Grondman I, Helder L, Jacobs C, Jacobs L, Jansen T, **Kilic G**, Klück V, Koeken VACM, Lemmers H, Moorlag SJCFM, Mourits VP, van Puffelen JH, Rabold, K, Röring RJ, Rosati D, Tercan H, van Tuijl J, Quintin J, van Crevel R, Riksen NP, Joosten LAB, Netea MG. In vitro induction of trained immunity in adherent human monocytes. *STAR protocols*. 2021 Mar 19;2(1):100365. DOI: 10.1016/j.xpro.2021.100365

21. Bulut O, **Kilic G**, Domínguez-Andrés J, Netea MG. Overcoming immune dysfunction in the elderly: trained immunity as a novel approach. *International immunology*. 2020 Dec;32(12):741-53. DOI: 10.1093/intimm/dxaa052

22. Yazar V, **Kilic G**, Bulut O, Canavar Yildirim T, Yagci FC, Aykut G, Klinman DM, Gursel M, Gursel I. A suppressive oligodeoxynucleotide expressing TTAGGG motifs modulates cellular energetics through the mTOR signaling pathway. *International immunology*. 2020 Jan;32(1):39-48. DOI: 10.1093/intimm/dxz059

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Curriculum Vitae

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