Engaging innate immunity in perioperative strategies:

mechanisms, biomarkers, and clinical observations

Leonie Suzanne Helder

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Title: Engaging innate immunity in perioperative strategies: mechanisms, biomarkers, and clinical observations

Radboud Dissertations Series
ISSN: 2950-2772 (Online); 2950-2780 (Print)

Published by RADBOUD UNIVERSITY PRESS
Postbus 9100, 6500 HA Nijmegen, The Netherlands
www.radbouduniversitypress.nl

Design: Proefschrift AIO | Guus Gijben
Cover: artificially generated by Leonie Helder
Printing: DPN Rikken/Pumbo

ISBN: 9789465150123
DOI: 10.54195/9789465150123
Free download at: www.boekenbestellen.nl/radboud-university-press/dissertations

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Engaging innate immunity in perioperative strategies: mechanisms, biomarkers, and clinical observations

Proefschrift ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.M. Sanders,
volgens besluit van het college voor promoties
in het openbaar te verdedigen op

woensdag 18 december 2024
om 10.30 uur precies

door

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Dissertation to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.M. Sanders,
according to the decision of the Doctorate Board
to be defended in public on

Wednesday, December 18, 2024
at 10.30 am

by

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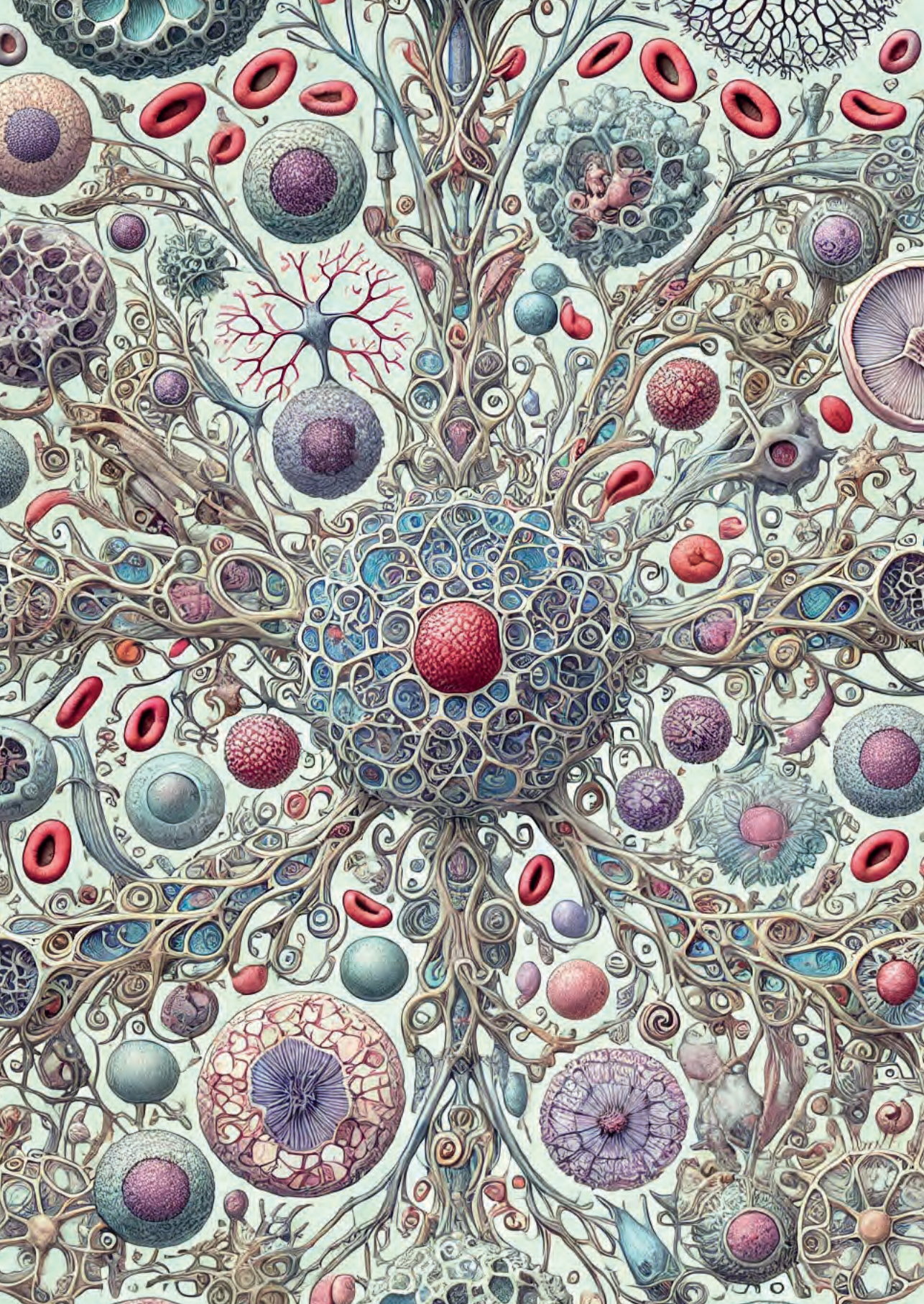

Chapter 1

General introduction, aim and outline of the thesis

General introduction

The Immune Response to Surgery, Trauma, and Sterile Inflammation

The human immune system consists of a complex network of cells and molecules that play a critical role in protecting the body from pathogens and facilitating tissue repair after damage. Surgery and trauma induce a robust immune response primarily mediated by the release of danger-associated molecular patterns (DAMPs). These endogenous molecules are released from damaged or stressed cells and serve as danger signals, triggering a sterile inflammatory response (1). Unlike pathogen-associated molecular patterns (PAMPs), which originate from infectious agents such as bacteria and viruses, DAMPs are derived from the host's own cells. Common DAMPs include interleukin-1α (IL-1α), heat shock proteins (HSPs), high mobility group box 1 (HMGB1), and extracellular ATP, which are released upon cellular injury or stress (2-4).

Cells of the innate immune system, such as monocytes, macrophages, and dendritic cells (DCs), recognize DAMPs through pattern recognition receptors (PRRs) expressed on the cell surface and in the cytoplasm. Activation of PRRs by DAMPs initiates sterile inflammation, involving the secretion of pro-inflammatory signaling molecules or cytokines, such as IL-1β, IL-6, and tumor necrosis factor (TNF). These cytokines promote the recruitment and activation of other immune cells to the site of injury, with the aim of clearing damaged cells and initiating tissue repair. In addition, the influx of immune cells offers protection against potential infectious pathogens that may invade the site of injury. However, in surgical settings, excessive or prolonged inflammation can lead to tissue damage and contribute to postoperative complications (5). Major surgical procedures are intrinsically linked to tissue damage and cellular stress (6). The extent of surgical trauma, which is influenced by the magnitude, invasiveness, and duration of surgery, causes a significant release of DAMPs. This release is associated with dysregulation of the immune response to surgery, via an initial pro-inflammatory response and a subsequent compensatory state of immune suppression (7). The resulting immune suppression has been shown to increase the risk of postoperative infections (8).

The inflammatory response to surgery and trauma is a double-edged sword: while necessary for tissue repair and initial host-defense from infection, it can also predispose patients to secondary infections and prolonged immune dysfunction (figure 1). The balance between effective immune defense and excessive inflammation is crucial in determining patient outcomes following surgery.

Modulation of the Immune Responses by Anesthesia

Anesthesia, while essential for performing surgical procedures, can attenuate and modulate the immune response (9). Anesthetic agents have been shown to influence both the innate and adaptive immune systems, with potential implications for patient outcomes. The immunomodulatory effects of anesthesia are complex and multifaceted, involving both pro-inflammatory and anti-inflammatory mechanisms (10). Different anesthetic agents have been shown to exert varying impacts on immune function. For example, volatile anesthetics like sevoflurane and isoflurane can suppress the activity of immune cells such as neutrophils, macrophages, and natural killer (NK) cells, potentially reducing the body's ability to control infections (11). Analgesic compounds like opioids have also been shown to inhibit multiple functions of the innate and adaptive immune systems (12).

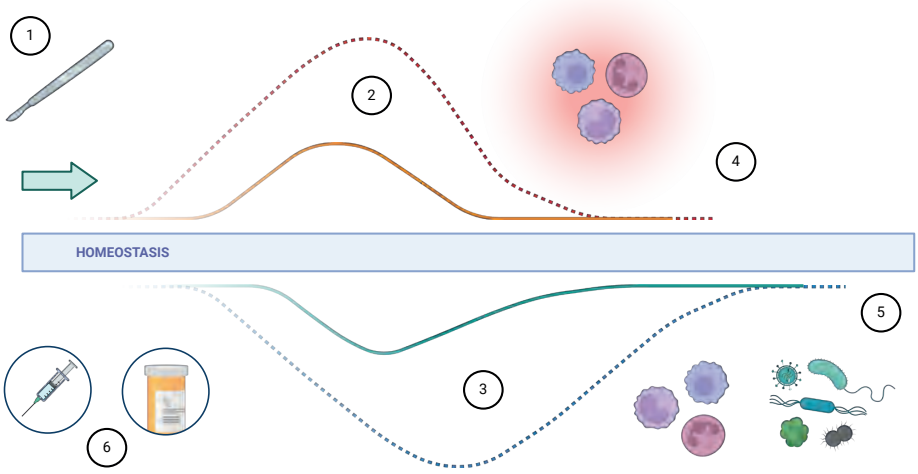

Figure 1. The immune response to surgery. Schematic representation of the dysregulation of inflammatory responses induced by surgical tissue injury. Disruption of the tissues (1) results in the release of DAMPs by damaged cells and tissue, initiating an early pro-inflammatory response (2). A congruent phase of immune suppression, characterized by anti-inflammatory responses (3), follows. In normal situations, these subsequent pro- and anti-inflammatory responses lead to a resolution of inflammation, the initiation of wound healing, and a return to homeostasis. However, excessive release of pro-inflammatory mediators can exacerbate inflammatory responses, resulting in systemic inflammation (4). Conversely, prolonged suppression of the immune response can increase susceptibility to opportunistic secondary infections (5). Intraoperative factors, such as anesthesia, can modulate the immune response, exerting a variety of beneficial as well as detrimental effects depending on the context (6). Figure created with BioRender.com.

Conversely, some anesthetics may exert protective effects by reducing excessive inflammation and promoting the resolution of inflammation, which can be beneficial in preventing tissue damage and improving outcomes in critically ill patients (13). In addition to the immunomodulatory effects of anesthetic medications, other perioperative interventions, including nutritional care (14), intraoperative fluid management (15), mechanical ventilation during surgery (16) and the administration of blood products (17) may all substantially alter immune cell function. Thus, the perioperative period is particularly critical for modulating the immune response. The combination of surgical trauma and anesthesia can dysregulate the systemic inflammatory response, affecting patient outcomes. Characterizing and understanding the mechanisms by which surgery and anesthesia affect the immune system is therefore crucial for developing strategies to mitigate these effects and improve patient outcomes.

Trained Immunity or Innate Immune Memory in Response to PAMPs and DAMPs

Recent advances in immunology have expanded our understanding of the immune response beyond the classical concepts of innate and adaptive immunity, revealing complex interactions between the different components of the immune system. Classically, immune responses have been divided into innate and adaptive immune responses. Innate immunity represents a first line of host defense, and involves cells such as monocytes, macrophages, and granulocytes (18). Activation of the innate immune response through PRR signaling results in the production of cytokines, reactive oxygen species (ROS), and the induction of phagocytosis and pathogen killing. Innate immune responses are rapid and non-specific, meaning that the mechanisms of defense are effective against a broad range of pathogens. When the innate immune response does not fully clear an infection, the second line of host defense, called the adaptive immune system, is activated. The adaptive immune system, consisting of T- and B-cells, takes much longer to mount an effective response, but is highly specific (19,20). Furthermore, the adaptive immune response establishes immunological memory, conferring lifelong protective immunity against specific pathogens. This mechanism forms the basis for classical vaccination (21).

Immunological memory was long considered a trait exclusive to the adaptive immune response. However, recent evidence suggests that cells of the innate immune system can also exhibit immunological memory-like properties (22). This innate immune memory, also referred to as 'trained immunity', refers to enhanced responsiveness of innate immune cells, such as monocytes, macrophages, and NK cells. Unlike adaptive immune memory, this heightened state of readiness is not

specific to a particular pathogen but represents a general enhanced innate immune response (figure 2). Trained immunity may be induced by various stimuli, including PAMPs from pathogens or vaccines (23), as well as DAMPs such as IL-1α released during tissue damage (24,25).

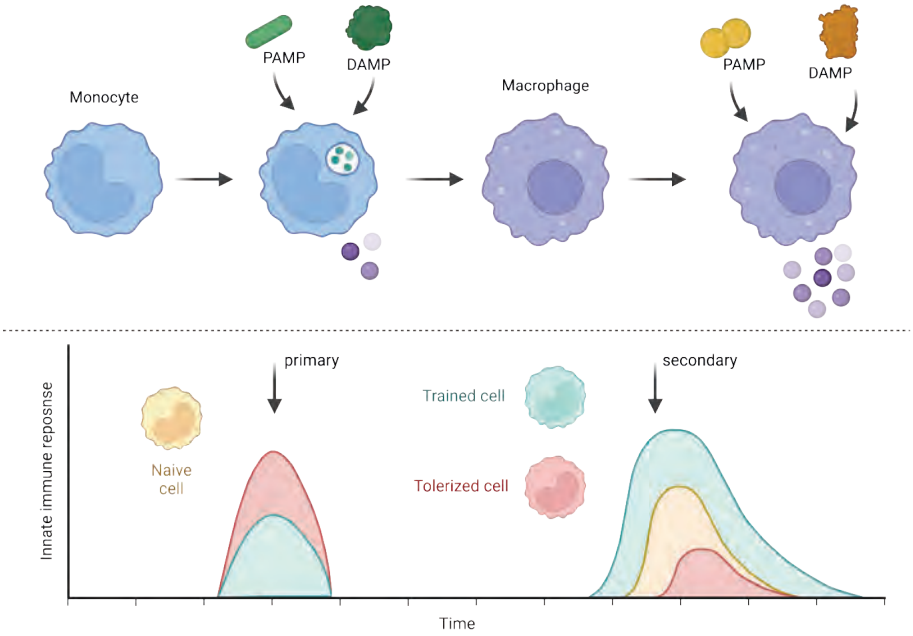

Figure 2. Trained innate immunity responses. A simplified graphical overview of trained immunity and tolerance in innate immune cells. Upon exposure of naïve cells (yellow) to PAMPs or DAMPs, a primary inflammatory response is induced. Concomitant to this proinflammatory response, anti-inflammatory mechanisms are triggered to prevent exacerbated inflammation and tissue damage. Induction of trained immunity in innate immune cells allows for enhanced responses to subsequent heterologous stimulation (green). Maladaptive innate immune memory responses, resulting in a persistent state of immunological tolerance (red), lead to dampened inflammatory responses upon secondary stimulation, increasing the risk of secondary infections. Figure created with BioRender.com.

This concept has significant implications for understanding the immune response in surgical settings. The release of DAMPs during surgery and trauma could potentially induce trained immunity, enhancing the body's ability to respond to subsequent opportunistic infections. However, this heightened state of immune activation might also contribute to maladaptive innate immune responses if not properly regulated (26,27). In another scenario of immune memory adaptation, it is feasible that excessive DAMP release could result in innate immune 'tolerance'. This phenotype represents the opposite of trained innate immunity and is characterized by a loss of immune cell plasticity, inability to activate gene transcription programs,

and loss of antimicrobial function. Often seen in sepsis following repeated or persistent exposure to lipopolysaccharide (LPS), this state of immune paralysis is associated with T cell exhaustion, suppression of monocyte-derived cytokines, and increased susceptibility to secondary infections (28).

Therapeutic Implications of Trained Immunity in the Surgical Setting

Trained immunity represents a paradigm shift in our understanding of the innate immune system's capabilities. Unlike adaptive immunity, which is specific to antigens, trained immunity provides a non-specific enhancement of the immune response. This heterologous protection can be particularly beneficial in defending against various pathogens, including those not previously encountered by the host. The induction of trained immunity involves epigenetic and metabolic reprogramming of innate immune cells. Modifications in the chromatin structure of innate immune cells, such as histone methylation and acetylation, facilitate sustained expression of genes involved in the immune response. These epigenetic changes allow for the rapid production of cytokines and other immune mediators upon secondary exposure to stimuli (27). Trained immune cells also undergo metabolic reprogramming, shifting metabolic pathways towards glycolysis, providing the necessary energy to support potentiated production of cytokines and other effector molecules (29). Previous research has shown that peripheral trained immunity can be induced in tissue resident (macrophages) and circulating (monocytes) immune cells. In addition, changes in the hematopoietic stem and progenitor cells (HSPCs) in the bone marrow result in long-term maintenance of central trained immunity, offering protection for at least 3 months and up to 1 year, although the precise duration of heterologous protection against infections remains the subject of current investigation (30).

The concept of trained immunity opens new avenues for therapeutic interventions in the surgical setting (31). One potential application is the implementation of trained immunity to prevent immune suppression and improve patient outcomes following surgery. An inherent aspect of surgical procedures is the disruption of physical barriers that prevent infection, such as the skin, leaving patients at increased risk of infections. In situations involving significant tissue damage, the release of DAMPs can lead to a state of immune suppression, predisposing the patient to postoperative opportunistic infections. Trained immunity provides a mechanism for enhancing the body's defense against these secondary infections. By inducing a heightened state of readiness in innate immune cells, trained immunity can facilitate a more rapid and effective response to pathogens. This non-

specific protection can be particularly valuable in the early postoperative period when patients are most vulnerable to infections (32).

On the other hand, while trained immunity can enhance the immune response to infections, it can also contribute to chronic inflammation and tissue damage if not properly regulated (26). Suppression of trained immunity may therefore be a viable treatment modality for surgical cases associated with a hyperinflammatory status. The therapeutic potential of the promotion or inhibition of trained immunity is an area of active research. A thorough understanding of the immune response to surgery and anesthesia is crucial for the development of therapeutic strategies to harness trained immunity. Developing treatments that target DAMPs or other stimuli known to induce trained immunity, as well as the use of immunomodulatory agents to support or suppress the trained immune response are promising avenues for improving patient outcomes in the surgical setting and beyond.

In summary, the immune response to surgery is a complex interplay of pro-inflammatory and anti-inflammatory processes, influenced by factors such as DAMPs, anesthesia, and potentially by the induction of trained immunity. Understanding these processes is crucial for developing strategies to optimize immune function, prevent complications, and improve patient outcomes.

Aim and outline of the thesis

A significant challenge in the investigation of the effects of anesthesia on the immune system is that these medications are usually not given without subsequent surgical intervention. Conversely, surgery is not performed without adequate anesthesia and analgesia. Therefore, isolating the effects of anesthesia on the immune system from the effects of the surgical stress response is difficult in clinical settings. The studies presented in this thesis sought to make use of translational studies, derived from laboratory investigations as well as from clinical trials. The aim of this thesis is to investigate the modulatory effects of perioperative measures on the host response towards microbial challenges *in vivo* and *in vitro* (figure 3).

Part I of this thesis explores the mechanisms of innate immune dysregulation in patients undergoing surgery and investigates perioperative strategies to modulate the surgical stress response.

Chapter 2 presents an investigation of monocyte epigenomic changes, as well as a proteomic analysis of a cohort of patients undergoing colorectal surgery. The role of epigenetic adaptations of monocytes in response to surgical stress in the dysregulation of postoperative immune responses were investigated.

Chapter 3 describes the impact of the extent of surgical trauma on innate immune responses in a cohort of breast cancer surgery patients. Breast-conserving surgery was compared to mastectomy in terms of DAMP release, postoperative immune suppression, and intraoperative sympathetic activation.

In **Chapter 4**, we performed a thorough exploration of the mechanisms underlying innate immune cell function, such as immunometabolism and mitochondrial adaptations. In addition, we assessed the effects of an integrated prehabilitation program on immune cell responses to perioperative stress. To this end, patients undergoing elective bladder cancer, rectal cancer, or esophageal cancer surgery were included in the study.

Part II of the thesis is focused on trained immunity, the impact of medications on innate immune function, and the potential for therapeutic interventions that leverage these insights.

Chapter 5 details our investigations into the immunomodulatory effects of intravenous anesthetic agent propofol. Utilizing an *in vitro* model, we demonstrate that propofol induces trained immunity in monocytes, and that these cells exhibit enhanced antimicrobial functions.

We explore another previously unknown inducer of trained immunity in **Chapter 6**. Antifungal drug amphotericin B was shown to induce trained immunity through epigenetic and metabolic reprogramming. The resulting functional adaptations of macrophages contributed to enhanced host defense against heterologous pathogens, and better infection clearance *in vitro*.

Lastly, in **Chapter 7** of this thesis, we explore the interactions between the innate and adaptive immune responses in the context of trained immunity. We describe that trained immunity enhances antigen presentation capacities of macrophages. When trained macrophages were co-cultured with T cells, this resulted in more T-cell derived cytokine production, as well as augmented T cell proliferation and polarization *in vitro*.

The concepts detailed in this thesis, summarized in **Chapter 8**, will be critical for advancing clinical practice and improving the care of patients undergoing surgery and trauma. This thesis explores several key concepts relevant to the immune response in surgical and trauma settings, the role of anesthesia in modulating immune responses, and the phenomenon of trained immunity, which could hold significant implications for both infection prevention and immune suppression in clinical practice. A general discussion of the findings and future perspectives is found in **Chapter 9**.

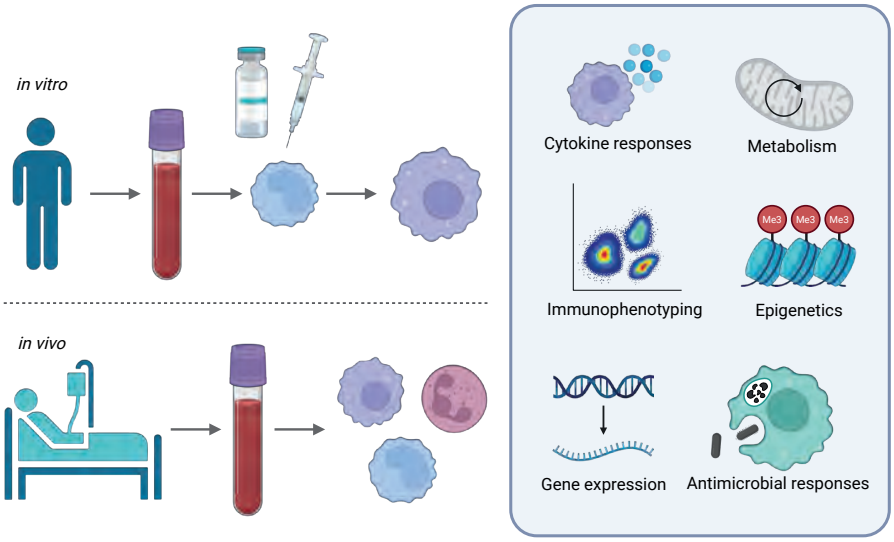

Figure 3. Schematic overview of experimental approach in this thesis. Chapters 2, 3, and 4 were performed using clinical samples from patients undergoing surgery. Chapters 5 through 7 were performed using immune cells from healthy donors to study individual stimuli, including anesthetics, medications, and compounds inducing trained immunity *in vitro*. Regulation of the immune responses was profiled at multiple levels. Figure created with BioRender.com.

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Part I

Mechanisms of innate immune dysregulation in patients undergoing surgery

Chapter 2

Postoperative innate immune dysregulation, proteomic and monocyte epigenomic changes after colorectal surgery: a substudy of a randomized controlled trial

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Abstract

Background. Colorectal surgery is associated with moderate to severe postoperative complications in over 25% of patients, predominantly infections. Monocyte epigenetic alterations leading to immune tolerance could explain postoperative increased susceptibility to infections. This research explores whether changes in monocyte DNA accessibility contribute to postoperative innate immune dysregulation.

Methods. Damage associated molecular patterns (DAMPs) and *ex vivo* cytokine production capacity were measured in a randomized controlled trial (n=100) in colorectal surgery patients, with additional exploratory subgroup proteomic (proximity extension assay; Olink) and epigenomic analyses (Assay for Transposase-Accessible Chromatin; ATAC sequencing). Monocytes of healthy volunteers were used to study the effect of High Mobility Group Box 1 (HMGB1) and Heat Shock Protein 70 (HSP70) on cytokine production capacity *in vitro*.

Results. Plasma DAMPs were increased after surgery. HMGB1 showed a mean 235% increase from before (preop) to the end of surgery (95% CI [166-305], p<.0001) and 90% increase (95% CI [63-118], p=.0004) preop to postoperative day 1 (POD1). HSP70 increased by a mean 12% from preop to the end of surgery (95% CI [3-21], NS) and 30% to POD1 (95% CI [18-41], p<.0001). nDNA increases by 66% (95% CI [40-92], p<.0001) at the end of surgery and 94% on POD1 (95% CI [60-127], p<.0001). mtDNA increases by 370% at the end of surgery (95% CI [225-515], p<.0001) and by 503% on POD1 (95% CI [332-673], p<.0001). *In vitro* incubation of monocytes with HSP70 decreased cytokine production capacity of TNF by 46% (95% CI [29-64], p<.0001), IL-6 by 22% (95% CI [12-32], p=.0004) and IL-10 by 19% (95% CI [12-26], p=.0015). *In vitro* incubation with HMGB1 decreased cytokine production capacity of TNF by 34% (95% CI [3-65], p=.0003), IL-1β by 24% (95% CI [16-32], p< .0001), and IL-10 by 40% (95% CI [21-58], p= .0009). Analysis of the inflammatory proteome alongside epigenetic shifts in monocytes indicated significant changes in gene accessibility, particularly in inflammatory markers such as CXCL8 (IL-8), IL-6, and IFN-γ. A significant enrichment of interferon regulatory factors (IRFs) was found in loci exhibiting decreased accessibility, whereas enrichment of Activating Protein 1 (AP-1) family motifs was found in loci with increased accessibility.

Conclusions. These findings illuminate the complex epigenetic modulation influencing monocytes' response to surgical stress, shedding light on potential biomarkers for immune dysregulation. Our results advocate for further research

into the role of anesthesia in these molecular pathways and the development of personalized interventions to mitigate immune dysfunction following surgery.

Key points

- Question: Does colorectal surgery induce epigenetic changes in monocytes that lead to postoperative immune tolerance?
- Findings: Post-surgery, patients exhibited elevated plasma DAMPs and monocytes showed altered cytokine production and significant epigenetic changes in genes related to inflammation.
- Meaning: The study highlights the role of epigenetic regulation of monocytes in response to surgical stress, suggesting new potential biomarkers for immune dysregulation and paving the way for personalized treatment to reduce postoperative complications.

Introduction

Colorectal surgery is associated with a high postoperative complication rate, 27% of patients develop moderate to severe 30-day complications (1). The majority of these complications are infectious, not only surgical-site and wound infections, but also distant infections such as pneumonia and urinary tract infections (1,2). The capacity to elicit an inflammatory response is an important predictor of postoperative complications and survival after colorectal surgery (3). It is well known that the early postoperative immune response is predominantly suppressive, leaving patients more vulnerable to secondary infections (4). Moreover, immune dysregulation plays an important role in growth and spread of many cancers, including colorectal carcinoma. Early postoperative innate immune dysregulation and the immune phenotype associated with a higher risk of infections are currently not well understood.

Our research group recently published that decreasing surgical tissue injury by lowering intra-abdominal pressure (IAP) during laparoscopic colorectal surgery can preserve innate immune homeostasis and decrease the incidence of postoperative infectious complications (5). Undoubtedly IAP is only one of several determinants, as surgical tissue injury and anaesthesia both constitute a hit to immune homeostasis. Innate immune cells possess a memory capacity referred to as trained immunity when it results in higher responsiveness and innate tolerance when cells become less responsive (6). The underlying metabolic and epigenetic reprogramming of immune cells occurs in reaction to stimulation. This concept has mainly been investigated and characterized in vaccinations, trauma, infectious diseases, sepsis, and atherosclerotic cardiovascular disease, but may also be relevant in the perioperative period. Further exploration of the innate immune phenotype, trained immunity and innate tolerance in the perioperative period may identify patients more likely to develop susceptibility to infection. Perioperative epigenetic alterations could explain the prolonged postoperative immune effects and, if identified, can provide opportunity for early intervention to decrease postoperative infections. These epigenetic alterations consist of changes in chromatin structure and conformation, that regulate how easily genes are transcribed reviewed in (7). Potential immune influencing factors are challenging to separate or control for in perioperative clinical trials. Moreover, quantifying surgical tissue injury is challenging. There are numerous different damage-associated molecular patterns (DAMPs; endogenous proteins or fragments of cells that are released in circulation when a cell is damaged, or can be expressed upon the threat of damage), and a few have been linked to immune dysregulation in surgical patients (8). Nonetheless, which DAMPs are most influential and the

extent of their impact is still uncertain. Therefore, analysis of individual possible determinants of immune dysregulation in *in vitro* experiments can prove valuable.

A better understanding of perioperative innate immunity is crucial in order to diminish the number of infectious complications after colorectal surgery. As monocytes are known to have an important role in the immune response associated with (surgical) trauma (9,10) we aim to explore if epigenetic alterations in monocytes in response to surgery could explain increased postoperative susceptibility to infections. To this end, we will investigate: (1) pre- to postoperative DAMP kinetics as well as the impact of DAMPs on innate immune dysregulation, and (2) the pre- to postoperative proteomic and epigenomic changes in the innate immune response. Given the smaller sample size for the subgroup analyses, these results should be interpreted as hypothesis-generating.

Methods

The RECOVER PLUS study (clinicaltrials.gov NCT03572413, principal investigator Michiel Warlé) is an immunological substudy of the multicenter double-blinded randomized RECOVER trial (clinicaltrials.gov NCT03608436), assessing the effects of low intra-abdominal pressure facilitated by deep neuromuscular blockade on quality of recovery and innate immune homeostasis. Trials were registered prior to patient enrolment on 18-06-2018. The RECOVER trial included 178 patients in three different teaching hospitals, the first 100 patients enrolled at the Canisius Wilhelmina Hospital were included in the RECOVER PLUS substudy. Patients in the substudy were not treated differently, but blood was drawn before surgery and on postoperative day (POD) 1 and 3. The complete study protocol (11) and primary analysis of the RECOVER trial (5) were published previously. This manuscript adheres to the applicable Equator guidelines. An overview of the RECOVER PLUS analyses is shown in figure 1, relevant methods are summarized below. The study was approved by the Medical Research Ethics Committee 'CMO region Arnhem-Nijmegen' and the competent authority (Central Committee on Research Involving Human Subjects). All participants provided written informed consent for study participation. Additional *in vitro* experiments were performed in monocytes isolated from buffy coats from healthy volunteers obtained after written informed consent (Sanquin blood bank, Nijmegen, the Netherlands).

Intervention and patient samples

Patients undergoing elective colorectal surgery were randomized in a 1:1 fashion to low intra-abdominal pressure (8mmHg) and deep neuromuscular blockade (PTC 1-2) or standard intra-abdominal pressure (12mmHg) and moderate neuromuscular blockade (TOF 1-2). Blood was drawn for all 100 substudy patients by venepuncture before surgery, at the end of surgery after neuromuscular block reversal, and on POD 1 and 3 if patients were still admitted at that time. Lithium heparin (LH) and ethylenediaminetetraacetic acid (EDTA) anticoagulated blood were centrifuged and plasma was stored at -80°C until analysis.

DAMPs, RNA expression, and *ex vivo* cytokine production capacity

Plasma DAMPs were measured from doubly centrifuged EDTA anti-coagulated blood as previously described (8,12). In short, concentrations of HSP70 (R&D systems, Minneapolis, MN, USA) and HMGB1 (IBL International GmbH, Hamburg, Germany) were measured batchwise by ELISA according to manufacturer's instructions. DNA was isolated with the QIAamp DNA Blood Midi Kit (Qiagen, Valencia, CA, USA) and levels of nDNA and mtDNA were determined by qPCR on a CFX96 Real-Time PCR Detection System using SYBR green reagents (Bio-Rad Laboratories, Hercules, CA, USA) and expressed as fold change relative to preoperative values of the same patient using the formula $2^{\Delta Ct}$. Levels of nDNA were quantified using primers for GAPDH: forward 5'-AGCACCCCTGGCCAAGGTCA-3' and reverse 5-CGGCAGGGAGGAGCCAGTCT-3'. For the quantification of mtDNA levels, primers for MT-ND1 were used: forward 5'-GCCCCAACGTTGTAGGCCCC-3' and reverse 5'AGCTAAGGTCGGGGCGGTGA-3'. RNA was isolated from blood collected in Paxgene RNA tubes using the Paxgene Blood RNA kit (Qiagen, Valencia, CA, USA) and transcribed into cDNA using the iScript cDNA Synthesis kit (Bio-rad, Hercules, CA, USA). Levels of RNA were quantified using 5'-AGGGCAGAATCATCACGAAGT-3' forward primers and 5'-AGGGTCTCGATTGGATGGCA-3' reverse primers for VEGFA and '5-AGTCCCTGTGCTAGGATTTTTCA-3' forward and '5-ACATAAACTCGCCTGATTGGTC-3' reverse primers for HLA-DRA. 18S was used as a reference gene with forward 5'-AAACGGCTACCACATCCAAG-3'and reverse'5-CGCTCCCAAGATCCAACTAC-3'primers.

Whole blood *ex vivo* cytokine production capacity upon endotoxin stimulation was quantified as previously described (10,11). Briefly: 0.5mL of LH anticoagulated whole blood was added to preprepared tubes with 2mL culture medium (negative control) and 2mL culture medium supplemented with 12.5ng/mL *Escherichia coli* lipopolysaccharide (serotype O55:B5 Sigma Aldrich, St Louis, MO) resulting in a final concentration of 10ng/mL. After mixing, tubes were cultured for 24 hours at 37°C, then centrifuged for 5 minutes at 1500 rpm and supernatants were stored

at –80°C until analysis. Cytokines were measured in the supernatant by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

In vitro experiments

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation and washed 3 times with phosphate-buffered saline (PBS) at 4°C. Monocytes were further purified from the PBMC fraction using the Pan-Monocyte Isolation Kit (MACS Miltenyi) according to manufacturer's instructions. All experiments were performed in Roswell Park Memorial Institute (RPMI) 1640 Dutch Modified (Gibco, Thermo Scientific) cell culture medium, supplemented with gentamycin 50µg/ml, pyruvate 1mM, and GlutaMAX 2mM. Cells were cultured at 5x10^5/well in a 96-well plate and incubated with culture medium only (negative control), HMGB1 (Merck) and HSP70 (Enzo Life Sciences) at various concentrations. Directly after, cells were stimulated with 10ng/mL *E. coli* lipopolysaccharide (LPS; serotype O55:B5; Sigma-Aldrich, St. Louis, MO, USA) for 24 hours. Supernatants were stored at -20°C until analysis of IL-1β, IL-6, IL-10 and TNF by ELISA (R&D systems, Minneapolis, MN).

Proteomics

Preoperative and POD1 plasma samples were used for commercial targeted plasma proteomics analysis (Olink, Uppsala, Sweden). Olink Target 96 Inflammation panel was used to measure 92 inflammation protein biomarkers by multiplex proximity extension assays, as quantified by real-time PCR (qPCR; 13). The thirty patients consisted of the 8 patients from epigenomic analysis and 22 patients randomly selected from all patients enrolled in the initial RCT.

Epigenomics

Genome-wide profiling of chromatin accessibility landscapes was performed on isolated monocytes using the Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) as previously described (14). This technique can identify where the conformational changes in chromatin occur and whether it leaves the surrounding genes more ('open') or less ('closed') accessible for transcription. Comparing the chromatin structure from before and after surgery allows to determine which genes are influenced by surgery and/or anesthesia. Peripheral blood mononuclear cells were isolated, monocytes were purified and ~50.000 were tagmented for each sample. Libraries were sequenced with Nextseq 500, FASTQ files were processed with the ENCODE pipeline on TERRA. Differentially accessible regions were identified by 'edgeR', peaks were annotated using 'ChIPseeker' and

gene ontology enrichments with 'clusterProfiler'. Processed promoter capture Hi-C data was downloaded from https://osf.io/u8tzp, coordinates were converted from hg19 to hg38 with liftOver tools. HOMER was utilized for motif enrichment. Differentially accessible regions were used as foreground and all detected peaks were used as background. The 8 patients were 4 consecutive patients from the low-pressure arm and 4 consecutive patients from the standard pressure arm.

Statistical analyses

All statistical analyses were performed using Statistical Package for the Social Sciences (IBM SPSS Statistics version 27; Armonk, NY). Pre- to postoperative levels of DAMPs (HMGB1 and HSP70) and *in vitro* cytokine production capacity after incubation with HMGB1 and HSP70 were compared with a Friedman test with post-hoc Dunn's multiple comparisons test. For analysis of *ex vivo* cytokine production capacity, a mixed-effects model analysis was used due to the missing values on POD3. qPCR fold-change values (nDNA, mtDNA, HLA-DR and VEGFA) were compared by a paired Wilcoxon signed rank test. Pre- to postoperative differences of the Olink analysis were compared by a paired Wilcoxon signed-rank test and the false discovery rate (FDR) was adjusted using the Benjamini-Hochberg procedure. A p-value of < 0.05 was considered significant and indicated in the graphs as *, $p \leq .01$ as **, $p \leq .001$ as *** and $p \leq .0001$ as ****.

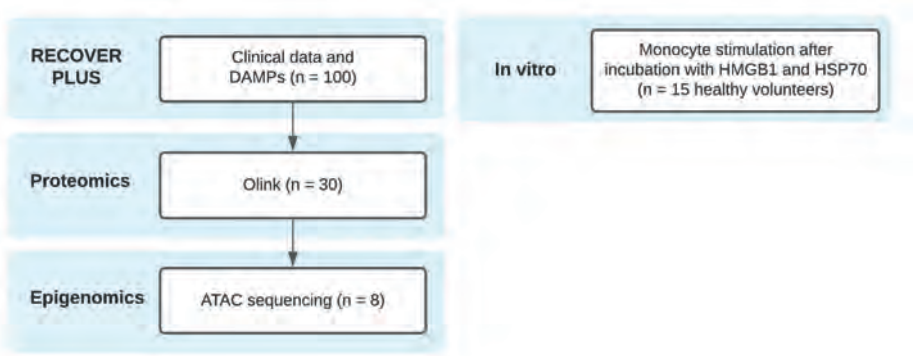

Figure 1. Overview of the RECOVER PLUS analyses and *in vitro* experiments. ATAC = assay for transposase-accessible chromatin, DAMPs = damage associated molecular patterns, HMGB1 = high-mobility group box 1, HSP70 = Heat shock protein 70, Olink = proximity extension immunoassay.

Results

The screening and treatment allocation flowchart of the RECOVER study was included in the primary publication (5). For the substudy, 100 patients were analyzed irrespective of treatment allocation in order to assess pre- to postoperative differences. The patient characteristics of all patients, patients included in the proteomic and epigenomic analysis are displayed in table 1A, infectious complications are presented in table 1B.

Table 1A. Characteristics of all substudy patients and patients included in the proteomic and epigenomic analyses.

	All patients N = 100	**Proteomic analysis N = 30**	**Epigenomic analysis N = 8**
Gender (Male / Female)	64 / 36	19 / 11	5 / 3
Age (years)	69 ± 9	69 ± 10	67 ± 9
BMI [a] (kg/m²)	26.6 ± 4.4	25.5 ± 3.6	25.7 ± 2.1
ASA [b] (I / II / III)	22 / 60 / 18	7 / 20 / 3	3 / 4 / 1
Intra-abdominal pressure (8 / 12 mmHg)	50 / 50	16 / 14	4 / 4
Type of surgery			
Sigmoid resection	39	10	2
Right hemicolectomy	30	8	5
LAR [c]/ PME [d]	18	7	1
Left hemicolectomy	8	3	0
Ileocecal resection	3	2	0
Right hemicolectomy and sigmoid resection	2	0	0
Malignancy (yes / no)	87 / 13	25 / 5	8 / 0

Presented values are absolute numbers or mean ± SD. [a] Body Mass Index, [b] American Society of Anesthesiologists classification, [c] low anterior resection, [d] partial mesorectal excision.

Table 1B. Infectious complications.

Infectious complications	**Total**	**In proteomic analysis**	**In epigenomic analysis**
Infected hematoma	3	1	1
Pneumonia	3	1	0
Abdominal infection/abscess	3	2	0
Anastomotic leak	2	1	0
Wound infection	2	2	0
Urinary tract infection	2	1	0
Total	15	8	1

DAMPs and *ex vivo* cytokine production capacity

Figure 2A shows a significant increase in circulating concentrations of HMGB1, HSP70, nDNA and mtDNA at the end of surgery (OR) compared to before surgery (preop). HMGB1 showed a mean 235% increase from preop to the end of surgery (95% CI [166-305], p< .0001) and 90% increase (95% CI [63-118], p= .0004) preop to postoperative day 1 (POD1). HSP70 increased by a mean 12% from preop to the end of surgery (95% CI [3-21], NS) and 30% to POD1 (95% CI [18-41], p< .0001). nDNA increases by 66% (95% CI [40-92], p< .0001) at the end of surgery and 94% on POD1 (95% CI [60-127], p< .0001). mtDNA increases by 370% at the end of surgery (95% CI [225-515], p< .0001) and by 503% on POD1 (95% CI [332-673], p< .0001). Figure 2B illustrates a significant postoperative decrease in cytokine production capacity compared to before surgery for TNF, IL-1β, IL-6 and IL-10 on POD1 and POD3.

In vitro effects of HMGB1 & HSP70 on monocyte function

To investigate the effects of circulating DAMPs on cytokine production, we pre-incubated monocytes of healthy volunteers (n=15) with different concentrations of HSP70 and HMGB1 (figure 3). *In vitro* incubation of monocytes with 30ng/ml HSP70 decreased cytokine production capacity of TNF by 46% (95% CI [29-64], p< .0001), IL-6 by 22% (95% CI [12-32], p= .0004) and IL-10 by 19% (95% CI [12-26], p= .0015). *In vitro* incubation with 100 ng/ml HMGB1 decreased cytokine production capacity of TNF by 34% (95% CI [3-65], p= .0003), IL-1β by 24% (95% CI [16-32], p< .0001), and IL-10 by 40% (95% CI [21-58], p= .0009).

Pre- to postoperative proteomic changes

Using the Olink proteomics inflammation panel, 92 protein biomarkers were measured in the preoperative and POD1 plasma samples of 30 patients. The comparison of relative expression levels of inflammatory proteins before and following colorectal surgery are represented in the volcano plot of proteins as displayed in figure 4A. The X-axis denotes relative fold change in expression compared to preoperative levels, whereas the Y-axis represents the degree of statistical significance. Proteins with a significantly changed expression from pre- to postoperative are marked by name and in red. Figure 4A illustrates the elaborate surgical signature of simultaneous up and downregulation of inflammatory proteins. Next, RNA expression of antigen presenting MHC class II receptor Human Leukocyte Antigen – DR isotype (HLA-DR) and vascular endothelial growth factor A (VEGFA) were determined in blood from 100 patients obtained preoperatively, as well as on POD1 and POD3. RNA expression of HLA-DR was significantly decreased on POD1 and POD3 (Figure 4B). RNA expression of VEGFA was only elevated on POD3 (Figure 4C).

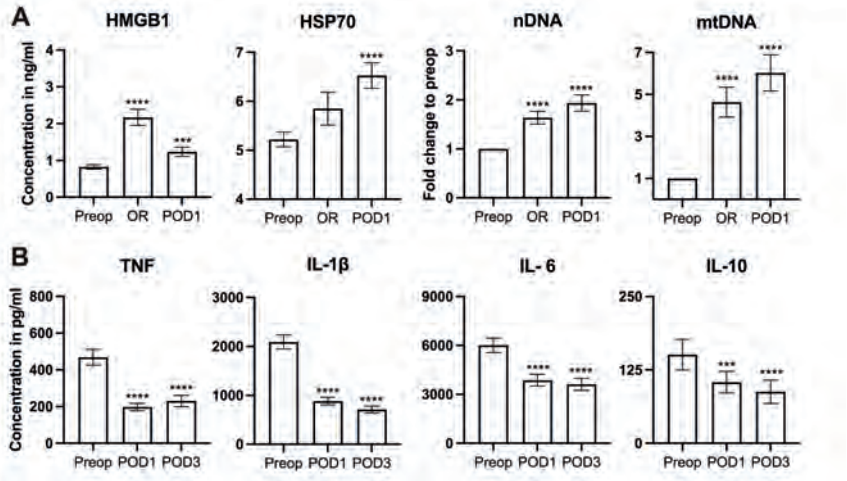

Figure 2. Plasma DAMP concentrations and cytokine production capacity. [A] Changes in plasma HMGB1 and HSP70 from preoperative (preop) to the end of surgery (OR) and postoperative day 1 (POD1) (comparisons were made with preoperative by Friedman test with post-hoc Dunn's multiple comparisons test), and fold change in relation to preoperative values (Wilcoxon signed rank test) for serum nDNA and mtDNA (n=100). [B] *Ex vivo* cytokine production capacity upon stimulation of whole blood with lipopolysaccharide (LPS) preop (n=100), POD1 (n=99) and POD3 (n=67) (comparisons were made with preoperative by mixed-effects model analysis with post-hoc Dunnett's multiple comparisons test, *** = $p \leq .001$, **** = $p \leq 0.0001$). Error bars display the SEM.

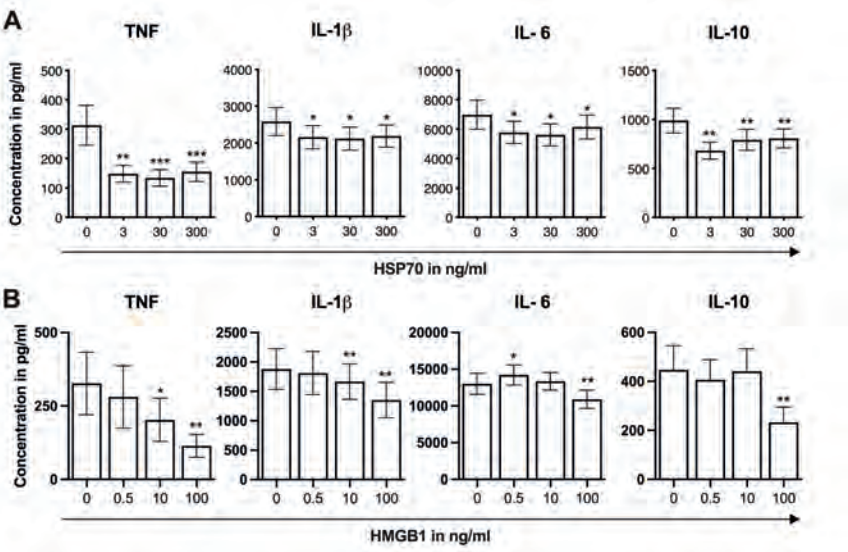

Figure 3. Effect of DAMPs on cytokine production. [A] *in vitro* cytokine production capacity of healthy donor monocytes upon LPS stimulation after pre-incubation with HSP70 (n=15) and [B] HMGB1 (n=15) (comparisons were made with baseline by Friedman test with post-hoc Dunn's multiple comparisons test, * = $p < 0.05$, ** = $p \leq .01$, *** = $p \leq .001$, **** = $p \leq .0001$). Error bars display the SEM.

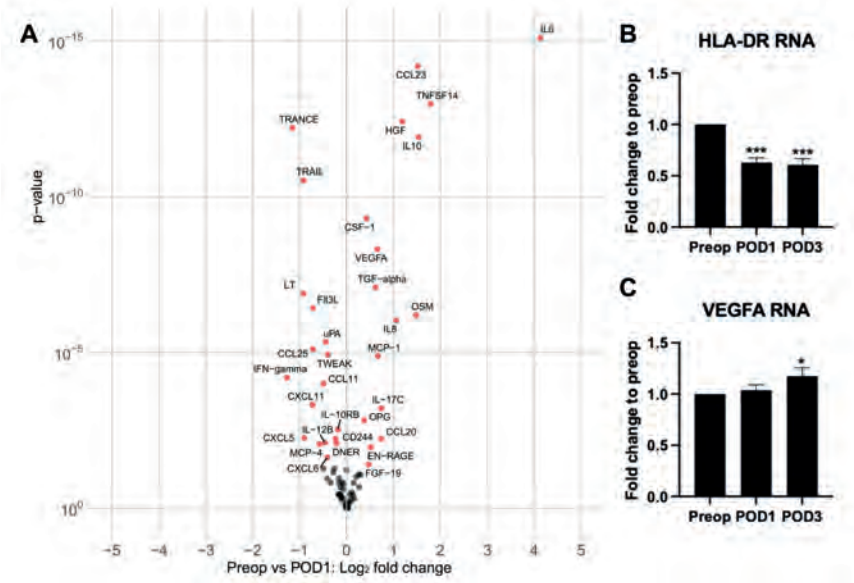

Figure 4. Analysis of pre- to postoperative plasma proteomics. [A] volcano plot of the pre- to postoperative differences in proteins measured with the Olink inflammation panel, the X-axis denotes relative fold change in expression compared to preoperative levels, the Y-axis represents the degree of statistical significance as tested with a paired Wilcoxon signed rank test, corrected for multiple testing by the Benjamini-Hochberg procedure. The proteins marked in red are statistically significant at a FDR-adjusted p-value of < 0.05, the proteins marked in black are not. [B] RNA levels of HLA-DR decrease from preoperative (preop) to postoperative day 1 (POD1) and 3 (POD3). [C] RNA levels of VEGFA are increased on POD3 compared to preoperative (Preop) (Wilcoxon signed-rank test; n =100, * = $p < 0.05$, *** = $p \leq .001$). Error bars display the SEM.

Pre- to postoperative epigenomic changes

In order to elucidate the mechanisms governing the observed alterations in gene expression, we performed the Assay for Transposase-Accessible Chromatin (ATAC) sequencing on isolated monocytes from eight patients, both preoperatively and on POD1. By assessing the differences in chromatin structure, we can assess genomic accessibility, as the conformational changes leave certain DNA regions more or less accessible for transcription. By investigating whether this occurs for inflammatory genes, we can deduct whether these changes underlie postoperative immune suppression. This analysis revealed 2,793 loci exhibiting increased accessibility following surgery, while 610 regions demonstrated decreased accessibility. Differentially accessible loci were predominantly located in intergenic regions as compared to other genomic regions (figure 5A), potentially suggesting that changes in enhancer accessibility are instrumental in driving postoperative gene expression alterations. In alignment with the Olink data, our findings indicate significant changes in accessibility of inflammatory genes, including CXCL8 (IL-8)

(Figures 5B; shows which genes are up- and downregulated, the same direction in Olink and ATAC analysis, 5C & D). Conversely, we observed reduced accessibility of antigen presentation genes in postoperative samples (Figures 5B & D).

Including patients from both the low and standard pressure arm may have introduced heterogeneity to these results. Although the number of investigated patients was small, we explored whether the intervention was of influence by principal component analysis (supplemental Figure 1). Only 56 loci (out of 157,471 consensus peaks in autosome; False Discovery Rate < 0.01) were differentially accessible when comparing low versus standard pressure. Whereas 3,602 loci (out of the consensus peaks) were differentially accessible from pre- to post-surgery. Low and standard pressure conditions did not lead to easily distinguishable changes in the epigenomic landscape as evidenced by the close proximity and overlap of the data points representing the two conditions.

To identify whether enhancers play a role in modulating the identified genes to have an altered accessibility following surgery, we utilized publicly available promoter capture Hi-C (PHi-C) data to determine whether differentially accessible enhancers are predictive of contacting the promoter of one gene with enhanced accessibility (CXCL8) and one with reduced accessibility (HLA-DPA1) (Figure 5D). The 4 out of the 5 enhancers predicted to interact with the CXCL8 promoter were also shown to have an increase in differential accessibility (blue bars). Meanwhile for HLA-DPA1, 1 out of the 4 predicted enhancers showed a change in accessibility. Transcription factors (TFs) are proteins that modulate gene expression and chromatin accessibility through direct DNA binding. Given that TFs recognize specific DNA sequences, we assessed whether recognition sequences for any particular TF were enriched within differentially accessible loci (Figure 5E). Our analysis revealed a significant enrichment of AP-1 family motifs in loci exhibiting increased accessibility in postoperative samples. Conversely, we identified a high enrichment of interferon regulatory factor (IRF) family protein motifs in loci exhibiting decreased accessibility.

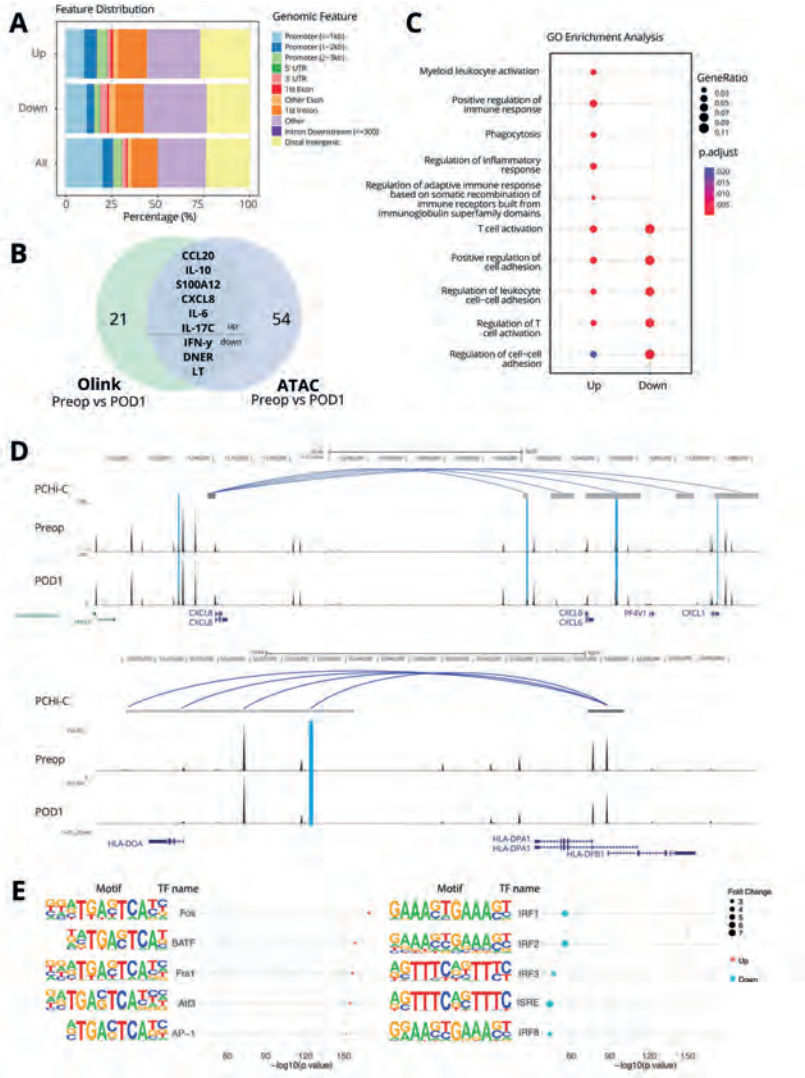

Figure 5. ATAC-seq analysis reveals differential accessibilities and key transcription factors. [A] Genomic distribution of differential accessible regions in loci exhibiting increased (up) or decreased (down) accessibility. [B] Of the 21 significantly changed proteins identified with Olink and 54 significantly altered genes identified with ATAC seq, 9 overlapped. [C] GO enrichment analysis of differentially accessible genes following surgery. The size of the dots indicates gene ratio and the color indicates adjusted p-value. [D] genomic view around CXCL8 (IL-8) and HLA-DPA1, the blue shaded regions indicate differential accessibility (more accessible for CXCL8 and less accessible for HLA-DPA1 The PCHi-C track indicates interactions between the promoter of CXCL8 and HLA-DPA1 and other elements. Dark grey bars are bait regions and light grey bars are open end regions. The interactions with CHICAGO score > 5 in monocytes are only shown. Accessibilities of pre- and post-operation in monocytes from one of the patients are shown below PCHi-C track. [E] Top 5 enriched motifs in differentially accessible regions. X axis indicates -log10 transformed p-value. The size of dots means fold enrichment.

Discussion

In this substudy, we show the major pre- to postoperative changes in DAMPs and inflammatory mediators after laparoscopic colorectal surgery. For several of these circulating inflammatory markers we have identified concomitant epigenetic alterations in monocytes. This supports our hypothesis that surgical tissue injury and/or anesthesia induce epigenetic and functional changes (immune tolerance) in monocytes, which play a substantial role in postoperative immune dysregulation. If these alterations last for days to several months, as described for other stimuli (15), these monocyte pathways could provide leads for treatment targets to prevent infectious complications after colorectal surgery.

It is well known that surgery with general anesthesia leads to immune dysregulation. Nevertheless, it remains difficult to determine the cause: is it mainly tissue injury, the anesthetics and analgesics, perioperative stress and pain, or a combination of all? The theory that DAMPs as ligands of Toll-like receptors (TLRs) on immune cells acts as danger signals and modify the innate immune response was already presented almost three decades ago by Polly Matzinger (16). This association has been confirmed in patient studies (8,17) and *in vitro* experiments (18,19). It is well established that DAMPs can induce trained immunity through epigenetic regulation of transcriptional programs (20).

Here, we show a reduction in cytokine production capacity of human monocytes for TNF, IL-6 and IL-10 upon incubation with HSP70, and of TNF, IL-1β and IL-10 after incubation with HMGB1. In postoperative patients, we found mean circulating HSP70 levels above 6ng/ml, whereas *in vitro* the significant suppressive effects on TNF, IL-1β, IL-6 and IL-10 production capacity are already seen at 3ng/ml. Several of the measured changes are not large. Nonetheless, they are comparable with changes in DAMPs and cytokines reported after HIPEC surgery, where such changes were associated with immune suppression and infectious complications (8). The physiological relevance of these changes cannot be definitively established. However, it is important to consider the cumulative and synergistic effects of multiple DAMPs and cytokines *in vivo*. Each measured DAMP and cytokine is one of many, and although only a small selection is presented here, there are hundreds of different DAMPs that bind to pattern recognition receptors like Toll-like receptors. A collective increase in these DAMPs can lead to significant downstream effects.

The significantly altered proteins overlapping from proteomic and epigenomic analyses are IL-6, IL-10, CXCL8 (IL-8) and IL-17C, CCL20, S100A12 (EN-RAGE), IFN- γ

and lymphotoxin (LT). Multiple studies highlight the association between higher postoperative increases of interleukins IL-6, IL-8 and IL-10 concentrations and increased risk of sepsis (21,22). A systematic review of IL-6 in over 5000 surgical patients with cancer of the gastrointestinal tract reveals high IL-6 is associated with impaired overall survival (23). IL-17C is involved in host defense and mainly produced by epithelial cells upon bacterial challenge or inflammatory stimuli, IL-17C deficient mice are highly resistant to endotoxin (LPS) induced septic shock (24). Chemokine C-C motif ligand 20 (CCL20) is a ligand for C-C chemokine Receptor 6 (CCL6) expressed in colon, liver and skin. Binding and activation of the CCL20-CCL6 axis is not only associated with inflammation and infections but also with cancer proliferation by modulation of the tumor microenvironment through immune cell control (25). Alarmin S100A12 positively correlates with length of ICU stay, 28-day mortality and in-hospital mortality after major abdominal surgery (26). IFN-γ and LT concentrations are downregulated while they seem to exert protective properties against postoperative immune suppression (27). Patients with LT polymorphisms undergoing major gastrointestinal surgery have a higher risk of postoperative complications and mortality for patients with postoperative sepsis (28). For all of these factors, more profound dysregulation is associated with a worse patient outcome. Accordingly, potential interventions should be aimed at reducing postoperative immune dysfunction.

In critical illness, innate immune dysfunction is often quantified by two parameters: LPS induced whole-blood *ex vivo* cytokine production capacity and monocyte MHC class II (like HLA-DR) expression (29). The identified enrichment of IRF family protein motifs in loci exhibiting decreased accessibility and activating protein-1 (AP-1) family motifs in loci exhibiting increased accessibility can contribute to postoperative impairment in both parameters. IRF's regulate Toll-like receptor (TLR) and interferon (IFN) signaling, and thereby have a crucial role in the response to pathogen infection, inflammation, antigen-presentation, anti-microbial defense and tumor suppression (reviewed in 30). IRF's recognize the interferon-stimulated response element (ISRE, which also exhibits decreased accessibility) in promoters of target genes (31). Binding of Pathogen- or Damage- Associated Molecular Patterns (PAMPs or DAMPs) to TLRs or IFN to the IFN receptors induces IRF activation and translocation to the nucleus where they interact with co-acting transcription factors like STAT, NF-kB and PU (28). Overexpression of IRF's is implicated in auto-immune disease (32), the postoperative decreased accessibility of IRF's observed in this study likely contributes to immunosuppression. Interferon signaling activates IRF family proteins, expression and activation of IRF1, 2 and 8 is dependent on IFN-γ (33). Proteomic and epigenomic analysis revealed a postoperative decrease in IFN-γ expression. The top 5 enriched

motifs in loci exhibiting increased accessibility (Fos, BATF, FRA1 Atf3 and AP-1) are all subunits or dimers of the AP-1 protein family (34). AP-1 activity is regulated by physiological and pathological stimuli like cytokines, stress signals, infections and oncogenic stimuli. AP-1 transcription factors are regulators of the immune response, they cooperate with transcription factor Nuclear Factor of activated T-cells (NFAT) to regulate cytokine production and T-cell activation and play a vital role in inflammatory disorders (35). Hyperinflammation and immune suppression often coincide during immune dysregulation, and likely exacerbate each other (36). Both IRF's and AP-1 are referred to as a double-edged sword with respect to the immune response (37,38), as both a shortage and surplus can negatively impact outcomes. This emphasizes the importance of only reducing excessive dysregulation without tipping the balance to the other side.

A limitation of this study is that the exploratory proteomic and epigenomic analyses were only performed in a small subgroup, as they are time consuming and costly. Therefore, the results need to be interpreted as hypothesis-generating. Nevertheless, the trial findings are theoretically founded and confirmed by independent *in vitro* experiments which substantiate the conclusions. The subset of patients analyzed with Olink technology contained patients with and without postoperative infections, however, the number of patients was too small for reliable comparisons. RNA levels of HLA-DR showed a significant reduction, indicative of immune suppression and aligning with our clinical expectations. However, it is important to remember RNA levels do not always correlate with protein levels. Flow cytometry of HLA-DR could have provided superior insights by directly quantifying the protein expression levels, offering a more precise assessment of immune competence.

In conclusion, our study endorses the relevance of epigenomic alterations in monocytes to immune dysregulation after laparoscopic colorectal surgery. Next, it is paramount to investigate how long postoperative innate immune tolerance lasts and to explore whether we can meaningfully modulate dysregulation through the identified pathways. Conceivably, not all patients will benefit from the same therapy as individual patient's immune response and training or tolerance develops based on previous encounters with stimuli and pathogens. Moreover, immune training and tolerance seem to occur simultaneously in different pathways. First, it could prove valuable to investigate the effects of anesthetics on IRF and AP-1 accessibility. This is still relatively unexplored; sevoflurane seems to affect AP-1 expression *in vitro* in human kidney cells (39), while propofol upregulates IFN-γ production in cultured NK cells (40). The knowledge on blocking or administering immune proteins has

greatly advanced in recent years and is already being applied in several infectious diseases, like IL-6 blockade and IFN-γ administration in COVID-19 (41,42). Moreover, AP-1 inhibitors are increasingly studied as a potential cancer treatment (34,43). It is key to investigate how we can identify which patients could benefit from what treatment. Ideally in the future, we will be able to identify patients at higher risk for postoperative infectious complications and create a personalized perioperative plan to minimize postoperative immune dysregulation.

Declarations

Funding

RECOVER and RECOVER PLUS were supported by two research grants from the Investigator Initiated studies Program from Merck Sharpe & Dohme, reference numbers #55890 and #57675. Sugammadex was provided for all study patients by Merck Sharpe & Dohme.

Conflicts of Interest

Michiel Warlé received grants from Merck Sharp & Dohme for investigator-initiated studies. The remaining authors declare that they have no financial conflict of interest with regard to the content of this report.

Clinical trial number and registry URL

The original RCT and immunological substudy (RECOVER and RECOVER PLUS) were registered at clinicaltrials.gov on 18-6-2018 under NCT03608436 and NCT03572413, respectively.

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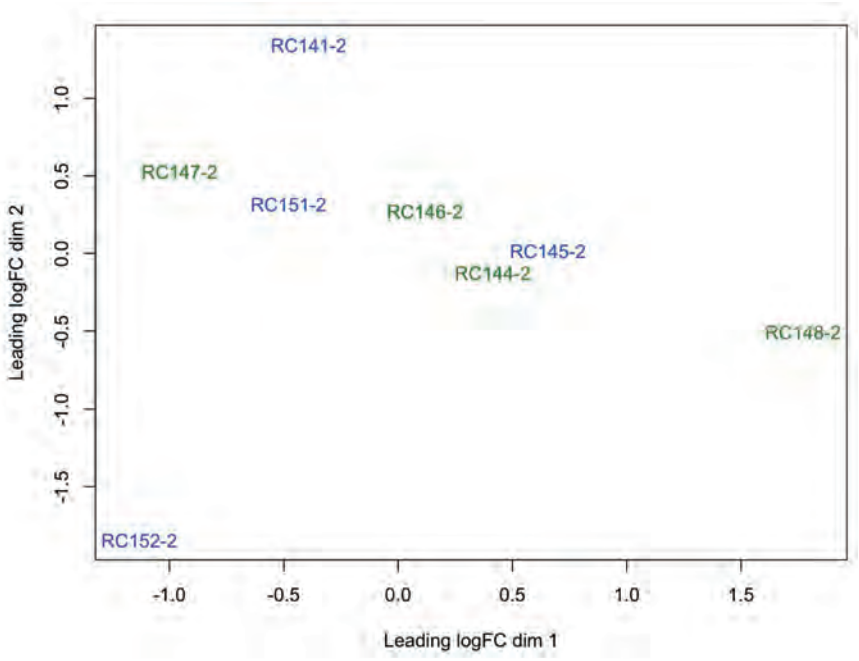
Supplementary material



Supplemental Figure 1. Principal component analysis of low and standard pressure. Low (blue text) and standard pressure (green text) conditions did not lead to easily distinguishable changes in the epigenomic landscape as evidenced by the close proximity and overlap of the data points representing the two conditions. Only 56 loci (out of 157,471 consensus peaks in autosome; False Discovery Rate < 0.01) were differentially accessible when comparing low versus standard pressure. Whereas 3,602 loci (out of the consensus peaks) were differentially accessible from pre- to post-surgery.

Chapter 3

The role of surgical tissue injury and intraoperative sympathetic activation in postoperative immunosuppression after breast-conserving surgery versus mastectomy: a prospective observational study

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Abstract

Background. Breast cancer is the second most common cause of death from cancer in women worldwide. Counterintuitively, large population-based retrospective trials report better survival after breast-conserving surgery (BCS) compared to mastectomy, corrected for tumour- and patient variables. More extensive surgical tissue injury and activation of the sympathetic nervous system by nociceptive stimuli are associated with immune suppression. We hypothesized that mastectomy causes a higher expression of plasma damage associated molecular patterns (DAMPs) and more intraoperative sympathetic activation which induce postoperative immune dysregulation. Immune suppression can lead to postoperative complications and affect tumour-free survival.

Methods. In this prospective observational study, plasma DAMPs (HMGB1, HSP70, S100A8/A9 and S100A12), intraoperative sympathetic activation (Nociception Level (NOL) index from 0 to 100), and postoperative immune function (plasma cytokine concentrations and *ex vivo* cytokine production capacity) were compared in patients undergoing elective BCS (n = 20) versus mastectomy (n = 20).

Results. *Ex vivo* cytokine production capacity of TNF, IL-6 and IL-1β was nearly absent in both groups one hour after surgery. Levels appeared recovered on postoperative day 3 (POD3), with significantly higher *ex vivo* production capacity of IL-1β after BCS (p = .041) compared to mastectomy. Plasma concentration of IL-6 was higher one hour after mastectomy (p = .045). Concentrations of plasma alarmins S100A8/A9 and S100A12 were significantly higher on POD3 after mastectomy (p = .003 and p = .041, respectively). Regression analysis showed a significantly lower percentage of NOL measurements ≤ 8 (absence of nociception) during mastectomy when corrected for norepinephrine equivalents (36% versus 45% respectively, p = .038). Percentage of NOL measurements ≤ 8 of all patients correlated with *ex vivo* cytokine production capacity of IL-1β and TNF on POD3 (r = .408; p = .011 and r = .500; p = .001, respectively).

Conclusions. This pilot study revealed substantial early postoperative immune suppression after BCS and mastectomy that appears to recover in the following days. Differences between BCS and mastectomy in release of DAMPs and intraoperative sympathetic activation could affect postoperative immune homeostasis and thereby contribute to the better survival reported after BCS in previous large population-based retrospective trials. These results endorse further exploration of (1) S100 alarmins as potential therapeutic targets in breast cancer surgery and (2) suppression of intraoperative sympathetic activation to substantiate the observed association with postoperative immune dysregulation.

Introduction

Breast cancer is the most common cancer among women and is the second most common cause of death from cancer among women in the world (1). Several large population-based retrospective trials have investigated survival rates between breast-conserving surgery (BCS) and mastectomy. Most of these trials report superior survival in the breast-conserving surgery group (2-6). However, it is critical to acknowledge the potential for selection bias inherent to these retrospective analyses, as they may not fully account for patient-specific factors such as mobility and frailty which can influence treatment decisions. This discovery, while reported consistently, is not geographically bound, nor age dependent, and has been adjusted for all tumour- and patient variables available in the cancer registration database (7). Nonetheless, the retrospective nature of these trials necessitates a cautious interpretation of this association.

There is no straightforward explanation why limited surgery for early-stage breast cancer could lead to a better survival in comparison to mastectomy, and thus these findings should be considered hypothesis-generating rather than conclusive. A possible contributing factor to this observation is radiotherapy, since the majority of patients undergoing BCS receive radiotherapy, as opposed to fewer patients after mastectomy (8). Furthermore, there is a larger degree of surgical trauma in mastectomy, which is associated with increased odds of developing a postoperative complication (9). Complication rates are already low after breast cancer surgery. Still, a recent study also showed that mastectomy has higher medical and surgical postoperative complication rates than BCS (10). A plausible hypothesis emerging in recent literature is that more extensive surgical trauma could lead to immune dysregulation. Cell damage leads to the release of damage-associated molecular patterns (DAMPs), substances either actively released by cells under threat, or components of the cell exposed when a cell is injured (11,12). DAMPs function as ligands for Toll-like receptors that, upon binding, induce inflammation followed by a compensatory state of immune suppression (11,13). In addition, activation of the sympathetic nervous system by nociceptive stimuli is known to induce immune suppression (14,15). More surgical trauma could lead to more pain and a higher degree of intraoperative sympathetic activation, which can easily be quantified with a non-invasive monitor (16). Fragidiakis *et al.* describe a strong correlation between immune status and recovery from surgery (17). In trauma patients, the suppressed immune state has been linked to infectious complications and mortality (18-20).

Several DAMPs have been linked to postoperative immune suppression and infectious complications (High mobility group box 1 (HMGB1) and Heat shock protein 70 (HSP70) (21), and breast cancer (alarmins S100A8/A9 and S100A12) (22-24). While functions and interactions of individual DAMPs have not been comprehensively characterized, in general HMGB1 and HSP70 reflect the degree of surgical injury or tissue damage, while increased S100A8/9 and S100A12 are associated with morbidity and mortality (25) and cancer progression (26-30). The aim of this pilot study was to further explore the role of these DAMPs and intraoperative sympathetic activation in immune suppression after breast cancer surgery, and to investigate whether these factors are likely to contribute to the previously reported difference in survival between BCS and mastectomy. We hypothesized that more extensive surgical trauma and sympathetic activation during mastectomy are associated with more immune suppression, which in turn may lead to postoperative complications and affect survival.

Materials and methods

Study population

Women undergoing elective BCS or mastectomy at the Canisius Wilhelmina Hospital (CWZ) in Nijmegen in the Netherlands were included in this single-centre prospective pilot study in 2021-2022. Patients were excluded if they were under 18 years of age or unwilling to give informed consent. The study protocol was approved by the Medical Research Ethics Committee "CMO region Arnhem-Nijmegen" (NL65918.091.18). Randomization was not applicable as only patients who were already scheduled to undergo either BCS or mastectomy were included. Participation in the study did not alter or delay the already scheduled treatment in any way. Written informed consent was obtained from all study participants before the start of any study-related procedures. Determinations and data handling were performed in agreement with the guidelines of The National Institutes of Health and in accordance with the declaration of Helsinki and its later amendments.

Sample and data collection

Baseline-, tumour-, and treatment characteristics and perioperative parameters were obtained from digital patient files in the programme Healthcare Information eXchange (HiX). Pain scores (Numerical Rating Scale (NRS), 0–10, 10 worst pain score) at one hour after surgery were obtained by attending nurses at the recovery. Blood samples were taken before surgery, 1 hour after surgery, and 3 days after surgery. Lithium heparin (LH) anti-coagulated blood was drawn for

ex vivo endotoxin stimulation of leukocytes immediately after sampling and ethylenediaminetetraacetic acid (EDTA) anti-coagulated blood was obtained to measure plasma DAMP levels and cytokine concentrations. After blood withdrawal, both LH and EDTA anti-coagulated blood samples were centrifuged at 1,600 RCF at 4°C for 10 min. EDTA anti-coagulated plasma samples were centrifuged again at 16,000 RCF at 4°C for 10 min to remove potential remaining cells and cell debris. Plasma was stored at -80°C until further analysis.

Plasma DAMP and cytokine concentrations

Plasma concentrations of HMGB1 were measured batchwise using the HMGB1 Express ELISA according to the manufacturers protocol (TECAN, Männedorf, Switzerland, catalogue number 30.164.033). HSP70, S100A8/A9, and S100A12 plasma concentrations were measured using Human HSP70/HSPA1A, Human S100A8/S100A9 Heterodimer, and Human EN-RAGE DuoSet ELISAs according to the manufacturer's protocol (R&D systems, Minneapolis, MN, USA, catalogue numbers DY1663-05, DY8226-05, and DY1052-05 respectively). Concentrations of TNF, IL-6 and IL-10 were measured batchwise in plasma from EDTA anti-coagulated blood using a simultaneous Luminex assay (Milliplex; Millipore, Billerica, MA) according to the manufacturer's instructions.

Ex vivo cytokine production upon whole blood stimulation

Ex vivo cytokine production capacity upon lipopolysaccharide (LPS) stimulation was measured as previously described (19,21,31). In short, 0.5mL whole LH anti-coagulated blood was added to tubes with 2mL Roswell Park Memorial Institute (RPMI) medium as negative control or 2mL RPMI culture medium supplemented with 12.5ng/mL *Escherichia coli* LPS (serotype O55:B5 Sigma Aldrich, St Louis, MO, USA), end concentration 10ng/mL. These tubes were incubated at 37°C with 5% CO_2 for 24 hours. Next, the samples were centrifuged at 3000 RPM and 24°C for 5 min and supernatants were stored at -80°C until further analysis. Concentrations of inflammatory cytokines IL-1β, TNF, IL-6 and anti-inflammatory cytokine IL-10 in the supernatants were measured using Human Bio-Techne R&D DuoSet ELISA according to the manufacturer's protocol (R&D systems, Minneapolis, MN, USA). The plates were read at 450nm using a ELx808 BioTek plate reader.

Anaesthesia and nociception Level

Anaesthesia was given according to the local standardized hospital protocol for breast surgery and consisted of total intravenous anaesthesia (TIVA) with remifentanil and propofol. Analgesia consisted of acetaminophen, diclofenac and intravenous morphine. During surgery, all patients were connected to a Nociception Level

(NOL) monitor (Medasense Biometrics Ltd, Ramat Gan, Israel) by a finger probe that can detect and quantify mild to intense noxious stimulation. Using a validated algorithm, this monitor combines the physiological parameters heart rate, heart rate variability, plethysmograph wave amplitude, skin conductance level, number of skin conductance fluctuations, and their time into a single index: the NOL index (32). Index values ranging from 0 (no nociception) to 100 (extreme nociception) were measured every 5 seconds and collected on the hard disc of the monitor until they were extracted for analysis. Generally, NOL values below 10 are considered absence of nociception (33,34). As median NOL values of 11–13 are reported during surgery (34), a NOL ≤ 8 was chosen as the cut-off value for this trial. The anaesthesiologist was blinded to the NOL index, measurements were not used to guide treatment in any way. Use of vasopressors (ephedrine, phenylephrine and norepinephrine were converted to norepinephrine equivalents (35)) was recorded to correct for increases in the NOL index secondary to the resulting increase in blood pressure and/or heart rate in ANCOVA.

Statistical analysis

This pilot study will be used to determine estimated effects of BCS and mastectomy on measures of interest and plan subsequent studies. Therefore, no sample size calculation was performed. Data presented in tables and text are presented as mean with standard deviation (SD). Data presented in figures are expressed as mean with standard error of the mean (SEM). Independent samples T-tests and chi-squared tests were used to determine differences between the groups (BCS versus mastectomy) for each of the time points. Linear regression analysis was used to determine differences in nociception level index corrected for use of vasopressors. Furthermore, repeated measures ANOVAs with Bonferroni correction were performed to determine differences between the different timepoints within each group. Cytokine levels below the detection limit as determined by ELISA were considered equal to the lowest detectable concentration because these values were expected to be very low. Haemolytic blood samples were excluded from the analysis of DAMP levels because haemolysis has been described to influence the accuracy of the results and the reliability of laboratory testing (36,37). Correlations were determined using Pearson's correlation.

All figures were made using GraphPad Prism version 5.03 and statistical analyses were performed using GraphPad Prism version 9.4.1 and SPSS version 27. P-values ≤ 0.05 were considered statistically significant.

Results

Patient characteristics

From February 2021 until June 2022, 20 women undergoing BCS and 20 women undergoing mastectomy at the Canisius Wilhelmina Hospital in the Netherlands were included in this study. Baseline characteristics age, BMI, and ASA score were similar between groups (Table 1). Incidence of invasive lobular carcinoma was more prevalent in the mastectomy group while invasive carcinoma of no special type (NST) was more prevalent in the breast-conserving surgery group. Neo-adjuvant chemotherapy occurred more frequently in the mastectomy group while frequency of administration of hormone therapy was similar between groups.

Table 1. Baseline characteristics.

	Breast conserving surgery (n=20)	Mastectomy (n=20)	P
Patient characteristics			
Age (years)	67.7 ± 9.0	63.1 ± 12.7	.193
BMI (kg/m^2)	27.8 ± 4.9	26.1 ± 4.5	.257
ASA (I / II / III)	6 / 13 / 1	5 / 14 / 1	.770
Tumour characteristics			
Indication for surgery			.019
Invasive carcinoma NST	19	12	
Invasive lobular carcinoma	0	6	
Carcinoma in situ	1	2	
Unilateral / bilateral	19 / 1	19 / 1	1.000
Sentinel node excision (Y / N)	15 / 5	16 / 4	.705
Oestrogen receptor (+/-/unknown)	18 / 2 / 0	16 / 3 / 1	.589
Progesterone receptor (+/-/unknown)	15 / 5 / 0	14 / 5 / 1	.925
Her2Neu receptor (+/-/unknown)	2 / 18 / 0	1 / 16 / 4	.686
Additional treatment			
Neo-adjuvant chemotherapy (Y / N)	0 / 20	6 / 14	.008
Letrozole (Y / N)	4 / 16	4 / 16	1.000

*ASA = American Society of Anaesthesiologists classification, BMI = body mass index, NST = no special type*Anaesthesia and pain

Total doses of anaesthesia and analgesia in units per hour administered during surgery did not differ between the groups (Table 2). However, duration of surgery was significantly longer for mastectomy compared to breast-conserving surgery (89 versus 58 min, P < .001). Pain and morphine consumption at the recovery room were significantly higher after mastectomy (Table 2).

Table 2. Anaesthesia and pain.

	Breast conserving surgery (n=20)	Mastectomy (n=20)	P
Duration of surgery (min)	58 ± 20	89 ± 27	<.001
Propofol (mg/kg/h)	9.7 ± 2.1	9.4 ± 1.9	.692
Propofol (mg/kg IBW/h)	12.6 ± 2.4	11.6 ± 1.9	.157
Remifentanil (ug/kg/h)	10.2 ± 3.7	10.8 ± 3.0	.570
Remifentanil (ug/kg IBW/h)	13.2 ± 4.4	13.3 ± 3.5	.866
Lidocaine (mg)	46	58	.226
Ketamine (mg)	1	4	.137
Ropivacaine (mg)	38	44	.371
Morphine OR (mg)	3.5	4.3	.237
Norepinephrine equivalents (ug/kg IBW/h)	1.2 ± 0.9	1.0 ± 1.1	.608
iv. morphine equivalents OR (fentanyl + remifentanil + morphine in mg/kg IBW/h) (38)	1.4 ± 0.4	1.4 ± 0.3	.990
Pain at the recovery room (NRS)	2.0 ± 1.1	3.5 ± 1.8	.004
Morphine at the recovery room (IV in mg)	0.9 ± 2.0	3.4 ± 4.5	.037

IBW = ideal body weight, IV = intravenous, NRS = numeric rating scale, OR = operating room

Innate immune function

Concentrations of DAMPs in plasma did not differ between the groups at baseline and one hour after surgery. However, on postoperative day 3 (POD3), concentrations of S100A8/A9 and S100A12 were significantly higher in patients after mastectomy (Fig. 1). Plasma concentrations of IL-6 were significantly higher one hour after mastectomy compared to BCS (Fig. 2). No other differences in plasma cytokine concentrations were detected between groups. In all patients collectively, strong immunosuppression was observed at one hour after surgery indicated by a significant decrease to nearly absent *ex vivo* production capacity of TNF, IL-6 and IL-1β. *Ex vivo* cytokine production capacity appeared restored three days after surgery, *ex vivo* production of IL-1β upon endotoxin stimulation was significantly

higher in the mastectomy group on POD3 (Fig. 3). No baseline differences were found for the assessed parameters between patients with and without neo-adjuvant chemotherapy, exclusion of patients with neo-adjuvant chemotherapy did not alter the results.

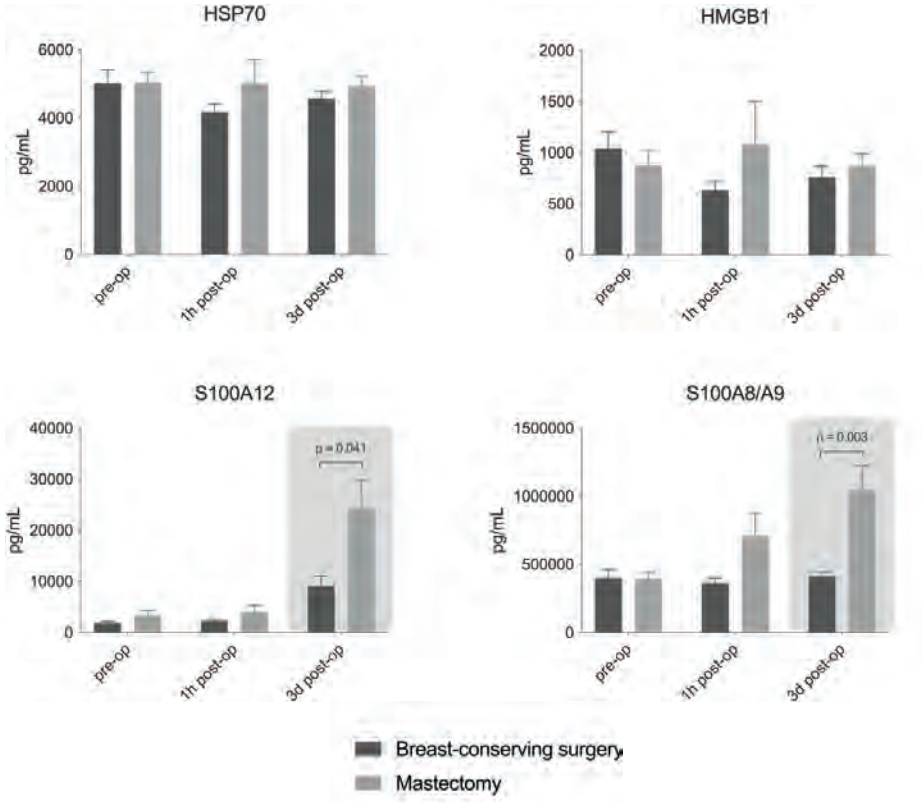

Figure 1. Concentrations of damage associated molecular patterns (DAMPs) in plasma. Before surgery, one hour (1h) and three days (3d) after surgery. Data are presented as mean ± standard error. HSP70: heat shock protein 70, HMGB1: high mobility group box 1, S100A12: S100 calcium-binding protein A12, S100A8/A9: calprotectin.

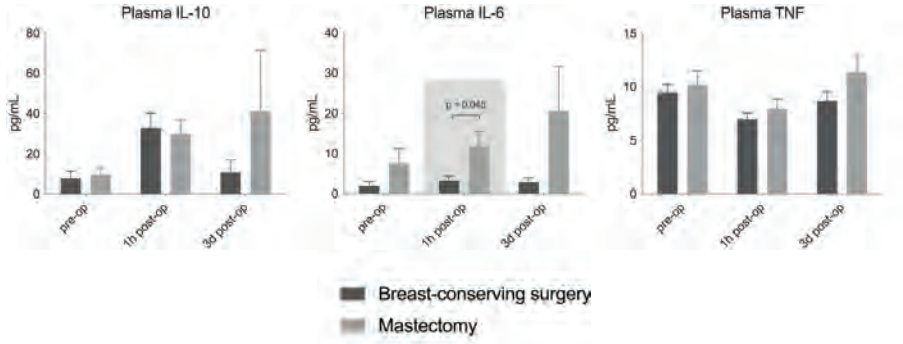

Figure 2. Plasma cytokine concentrations before surgery, one hour (1h) and three days (3d) after surgery. Data are presented as mean ± standard error. IL: interleukin; TNF: tumour necrosis factor.

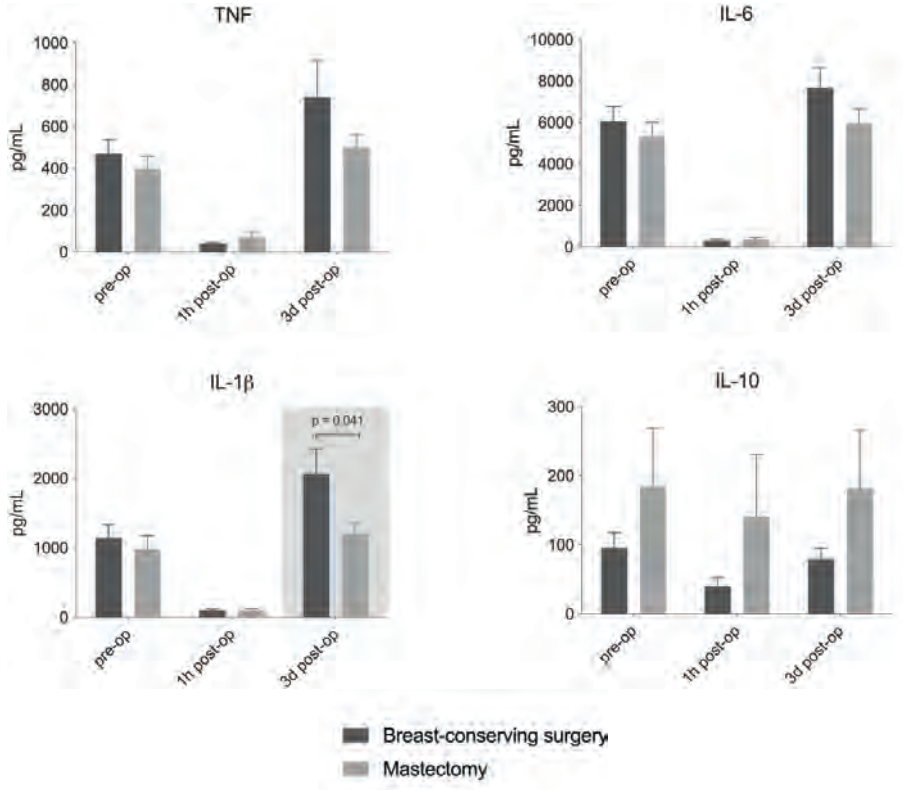

Figure 3. *Ex vivo* cytokine production capacity of leukocytes. Whole blood stimulation with *E. coli* lipopolysaccharide (LPS) before, one hour (1h) and three days (3d) after surgery. Data are presented as mean ± standard error. IL: interleukin; TNF: tumour necrosis factor.

NOL index

The mean observed NOL index for all 40 patients combined was 13 ± 5. The percentage of NOL measurements ≤ 8 correlated with *ex vivo* cytokine production capacity of IL-1β and TNF on POD3 ($r = .408$, $p = .011$ and $r = .500$, $p = .001$, respectively). Linear regression analysis shows patients undergoing mastectomy had a significantly lower percentage of NOL measurements ≤ 8 compared to patients undergoing BCS (36% versus 45%, respectively, $p = .038$) when corrected for norepinephrine equivalents by ANCOVA. Patients who developed a wound infection within 30 days after surgery ($n = 3$, one in the BCS group and two in the mastectomy group) had a significantly lower percentage of NOL measurements ≤ 8 (19% versus 42%, $p = .023$). The mean NOL and percentage of NOL measurements ≤ 8 did not correlate with pain scores at the postanaesthetic care unit (PACU).

Discussion

In this pilot study, the role of DAMPs and intraoperative sympathetic activation in postoperative immune suppression after breast cancer surgery were explored to investigate whether these factors are likely to contribute to greater survival after BCS compared to mastectomy, as reported in previous large population based retrospective trials.

Plasma alarmins S100A12 and S100A8/A9 were significantly increased on postoperative day 3 after mastectomy as compared to BCS. During inflammation, S100A12 and S100A8/A9 are known to modulate the immune inflammatory response by stimulation of leukocyte recruitment and induction of cytokine secretion (39,40). The increased levels after mastectomy could be related to wound healing as there is a greater wound surface after mastectomy. Considering the association of high S100A12 and S100A8/A9 with worse prognosis in breast cancer and other types of cancer (25-29), this could be a contributing factor to the observed difference in survival in previous observational studies. Regulation of S100 alarmins as therapeutic targets in inflammatory disease is an upcoming area of research (41). For both groups, HMGB1 and HSP70 were not significantly increased one hour and 3 days after surgery compared to before surgery. This was an unexpected finding, as several previous studies report an increase in these DAMPs after different types of surgery (21,42-44). Either the expression of these DAMPs is simply not increased after breast cancer surgery, or possibly the window of increase was outside the measured timepoints. There were also no differences between BCS and mastectomy for HMGB1 and HSP70.

Plasma IL-6 was significantly higher 1 hour after surgery for the mastectomy group. IL-6 is a major regulator of myeloid-derived suppressor cells (MDSCs) that suppress the anti-tumour functions of T and NK-cells. High plasma IL-6 is therefore associated with a worse prognosis as it leads to increased tumour cell proliferation and metastasis (45-47). Additionally, more extensive surgical trauma and sympathetic activation during mastectomy may cause greater adrenergic and prostaglandin responses, which also affect immune function. Acute stress can promote the release of pro-inflammatory cytokines, such as IL-6 and IL-1β, while chronic stress can also contribute to immunosuppression (48). This is in line with the current study as higher NOL values and higher plasma concentrations of IL-6 1 hour after mastectomy were found compared to breast-conserving surgery. Plasma TNF and IL-10 were not significantly different between BCS and mastectomy 1 hour and 3 days after surgery.

It is well recognized that the early postoperative immune response is predominantly immunosuppressive (49). Leijte *et al.* (21) and Albers *et al.* (44) have previously described the association between DAMPs and *ex vivo* cytokine production capacity. Nonetheless, the fact that the *ex vivo* cytokine production capacity (regarded as the capacity to elicit an inflammatory response when encountering a pathogen (50) is nearly absent one hour after surgery has not been described before. The observed levels were comparable to levels in patients displaying a state of immunoparalysis after sepsis (50,51). Whether this is the direct result of anaesthetics or intraoperative circulating DAMPs is still unknown. Anaesthetics commonly used in surgery have a direct effect on the functions of immunocompetent cells (52). Propofol impairs several monocyte and neutrophil functions and remifentanil also presents strong immunomodulatory effects (53). In general, the effects of anaesthetics appeared to be moderate to even negligible to the effects of surgical trauma in healthy patients anesthetized for short procedures (53,54). However, the newly identified tremendous depression of cytokine production capacity directly after surgery in patients with cancer and often other comorbidities perhaps calls for a re-evaluation of the role of anaesthetics in postoperative complications. Curiously, while *ex vivo* production capacity of IL-1β is approximately back to baseline on POD3 for mastectomy, it is significantly higher on POD3 after BCS. IL-1β, being a potent pro-inflammatory cytokine crucial to the host-defence against invading pathogens, is usually not detectable in plasma of healthy individuals (55,56). Higher production capacity upon endotoxin stimulation therefore indicates improved protection against postoperative infectious complications. IL-1β appears to have opposing functions in breast cancer, as high levels in the tumour micro-environment inhibit tumour growth while on the other hand it seems to enhance metastasis in bone (57).

Altogether, this study investigates only a small subset of DAMPs and a fraction of the innate immune response. Considering the small sample size and the pilot character of the study there is no foundation for hard conclusions. Also, our *ex vivo* findings should be interpreted with caution as this approach does not take in to account all factors present in the *in vivo* situation, such as stress hormones (58). Nonetheless, the significant immune-related differences identified between BCS and mastectomy do suggest the type of surgery is substantive for the postoperative course and prognosis.

Intra-operative activation of the sympathetic nervous system (e.g. stress or nociception during general anaesthesia) could very well also contribute to the observed differences between BCS and mastectomy. More extensive surgical tissue injury understandably leads to more nociceptive activation. Morisson *et al*. previously described that the percentage of NOL measurements < 10 during surgery was predictive for pain at the PACU (34). We did not find the same association for pain scores in the recovery room. However, the percentage of NOL measurements ≤ 8 during surgery did correlate with the *ex vivo* cytokine production capacity of IL-1β and TNF on POD3. Moreover, patients undergoing mastectomy had a significantly higher percentage of NOL measurements above this nociception threshold. While numbers are very small, patients who suffered a postoperative wound infection (n = 3) had a significantly lower percentage of NOL measurements ≤ 8. The current manufacturer guidelines direct that a NOL index of 0–25 represents an appropriately suppressed physiological response to noxious stimuli and adequate analgesia (59). While the manufacturer's instructions suggest a prolonged NOL < 10 may indicate excessive analgesia, these results and results of Morrison *et al*. support that striving for a lower threshold for absence of nociception could improve clinical outcomes. The connection between early postoperative pain and infectious complications is well established in breast cancer- and other types of surgery (60–62). The optimal depth of anaesthesia as quantified by the bispectral index (BIS) had been long debated before the Balanced Anaesthesia. A previous study revealed no differences in one year mortality or severe adverse events between light and deep anaesthesia (BIS 50 versus 35, respectively) (63). Relevant differences may however be present for the analgesia pillar of the triad of anaesthesia. These data support that a lower intraoperative NOL index is associated with less postoperative immune suppression and could lead to a lower risk of postoperative infections. A randomized trial investigating whether enhanced suppression of nociception with multimodal analgesia can reduce postoperative immune suppression is warranted.

There are certain limitations of this study that readers need to consider when interpreting the results. First, this study does not discriminate between the effect of surgical tissue injury and the duration of surgery. An extensive meta-analysis revealed that the likelihood of complications increased significantly with prolonged operative duration (64). However, as no differences in the use of anaesthetics were found between the groups, we consider the larger extent of surgical tissue injury the most important consequence influence immune function due to longer duration of surgery. Second, this study did not take into account the effect of radiation, which can also influence postoperative survival.

In conclusion, our study has identified potential differences between BCS and mastectomy in the release of DAMPs and intraoperative sympathetic activation. These differences may influence postoperative immune homeostasis. While these findings could provide a partial explanation for the improved survival outcomes associated with BCS as observed in previous large population-based retrospective trials, it is imperative to recognize the limitations inherent in such retrospective analyses, including possible selection bias and the confounding role of adjuvant therapies. Therefore, our results should be interpreted as preliminary evidence that contributes to a growing body of research. These results endorse further exploration of (1) S100 alarmins as potential therapeutic targets in breast cancer surgery and (2) suppression of intraoperative sympathetic activation to substantiate the observed association with postoperative immune dysregulation.

Declarations

Author contributions

KA, LS, and MW contributed to the study design. KA, LJ, JK, and LS contributed to inclusions and data collection. LJ and LH analysed the blood samples. LJ, KA and MW contributed to the data analysis. All authors contributed to the interpretation of the data. LJ, KA, and MW wrote the first draft of the manuscript. All authors reviewed the manuscript.

Ethical Approval

The study protocol was approved by the Medical Research Ethics Committee "CMO region Arnhem-Nijmegen" (NL65918.091.18) and all study procedures were performed in accordance with the Declaration of Helsinki.

Consent to participate

Written informed consent was obtained from all research participants.

Funding

This research received no funding.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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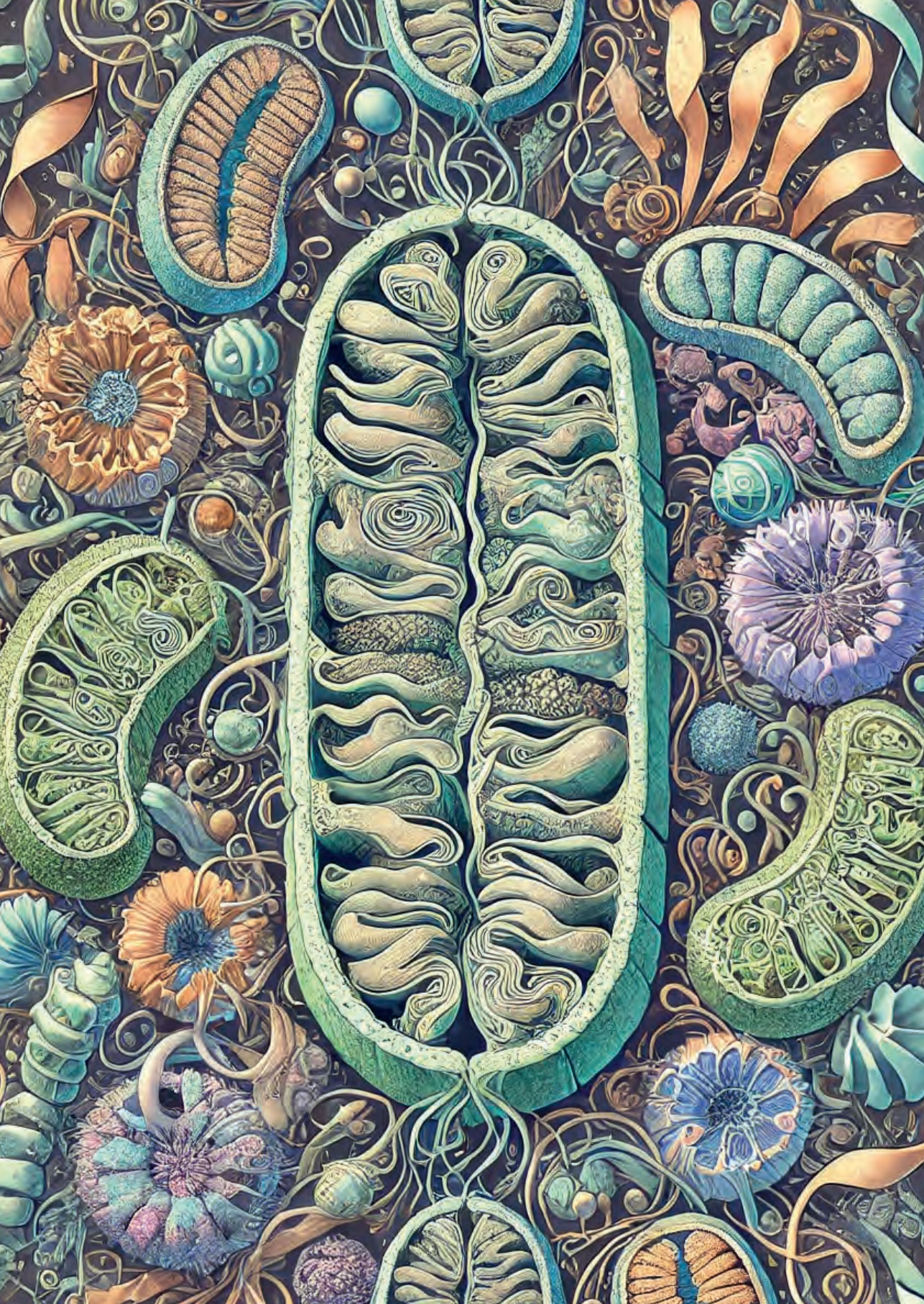

Chapter 4

Effects of multimodal prehabilitation on mitochondrial fitness and immunometabolism in surgical patients: a substudy of the F4S PREHAB trial

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Abstract

Background. Surgery represents a significant physiological stressor, disrupting homeostasis and potentially dysregulating processes essential for immunoprotection and recovery. Recent studies have proposed prehabilitation as a possible intervention strategy to enhance surgical stress endurance capacity, but the effects on the immune system are unexplored. Mitochondria play a central role in regulating immune cell energy metabolism, providing the energy necessary for host defense mechanisms. Mitochondrial capabilities can be substantially enhanced by physical activity, bolstering their integrity, operational efficiency, and dynamic response to stress. Given the critical function of mitochondrial dynamics in immune responses, enhancing mitochondrial fitness through prehabilitation could be a decisive factor in postoperative inflammation.

Methods. The F4S PREHAB trial integrates nutrition, exercise, smoking and alcohol cessation, and stress-reduction prehabilitation strategies to boost patients' functional capacity pre-surgery. In this sub-study, we explore the impact of prehabilitation on mitochondrial fitness and immunometabolism in innate immune cells.

Results. Prehabilitation was found to reduce circulating monocyte counts, lactate production and glucose consumption, as well as interleukin (IL)-18 and interferon (IFN)-γ production. Monocyte mitochondrial membrane potential (MMP) was increased after prehabilitation, whereas mitochondrial reactive oxygen species (mtROS) production and mitochondrial mass were decreased postoperatively in the prehabilitation group. Expression of genes involved in the regulation of metabolic pathways, mitochondrial dynamics, and immune activation was increased after prehabilitation, with a central role for HIF-1α in prehabilitation.

Conclusions. We describe broad anti-inflammatory effects of multimodal prehabilitation, such as decreased circulating monocyte cell count, cytokine and metabolite production, as well as mitochondrial adaptations and reduced oxidative stress post-surgery. Optimizing cellular responses to perioperative stress through prehabilitation could positively affect immune function, which may in turn improve patient recovery after surgery.

Introduction

Surgical intervention, while essential for the treatment of various conditions, can significantly stress the body, provoking a complex immune and inflammatory response. This response is influenced by patient-specific characteristics such as age and sex, and pre-existing conditions like frailty, which can dramatically affect outcomes (1,2). For instance, older adults and those identified as frail exhibit heightened inflammatory responses, potentially complicating recovery post-surgery (3-5). Prehabilitation programs that include physical exercise and nutritional optimization before surgery are increasingly recognized for their potential to enhance patient outcomes. In colorectal cancer surgery (6,7), orthopedic surgery (8), urologic cancer surgery (9), and other types of cancer surgery (10), severe complications after surgery may be reduced through the implementation of prehabilitation, though the underlying mechanisms remain poorly understood. Exercise and nutrition are crucial components of prehabilitation programs. Exercise, in particular resistance training, has been shown to modulate immune cell function (11,12), stimulate mitochondrial biogenesis, improve antioxidant capacity (13-15), and affect cellular respiration of peripheral blood mononuclear cells (PBMCs) (16). Nutritional interventions, particularly those supplementing essential amino acids and antioxidants, have also been shown to support immune cell function and reduce oxidative stress (17-19).

Immunometabolism refers to the interplay between metabolic processes and immune cell function. Surgical tissue damage triggers a systemic immune response characterized by a shift in cellular metabolism towards catabolism, to meet the increased energy demands for immune cell activation, proliferation, and function (20). This metabolic reprogramming, which includes heightened glycolysis, oxidative phosphorylation, and fatty acid oxidation, is intimately tied to innate immune responses (21,22), and can significantly influence the extent and resolution of inflammation (23,24).

A key factor in the regulation of immune responses through metabolic pathways is the functionality of mitochondria. Besides their role in cellular energy provision, mitochondria are crucial for maintenance of cellular homeostasis and function during infection and inflammation through a variety of processes like mitochondrial fission, fusion, and biogenesis (25). The importance of mitochondria is further highlighted by the direct implication of mitochondrial dysfunction in situations where the immune response is dysregulated, such as in sepsis (26,27) and severe SARS-CoV-2 infection (28,29). Mitochondrial dysfunction, characterized by diminished mitochondrial biogenesis, accumulated mitochondrial DNA

mutations, and altered mitochondrial dynamics is also a well-described hallmark of aging (30,31) and contributes to reduced cellular energy production and increased oxidative stress (32). These factors could compromise immune defense mechanisms in elderly patients, particularly in response to surgical stress.

As mitochondrial dysfunction can compromise immune function, targeting mitochondrial pathways through physical exercise may be a therapeutic strategy to modulate the inflammatory response to surgery, supporting better overall recovery from surgical intervention. Therefore, this study seeks to provide an initial exploration of the effects of multimodal prehabilitation on the metabolism and immune responses to surgical stress.

Methods

Study Design

The current study is a sub-study of the F4S PREHAB trial, a single-center stepped wedge trial performed in the Radboud university medical center (Radboudumc) in Nijmegen, the Netherlands (33). The study protocol was approved by the Central Committee on Research Involving Human Subjects (CCMO) in the Netherlands (NL73777.091.20).

Study population

Patients participating in the F4S PREHAB trial, aged sixteen years and over, undergoing elective bladder cancer, rectal cancer, or esophageal cancer surgery at the Radboudumc were included. Eligibility criteria for the F4S PREHAB trial excluded patients with impaired mobility or premorbid conditions (cardiorespiratory disease) that contraindicate high-intensity exercise, cognitive disabilities, inability to read and understand the Dutch language, chronic kidney disease stage ≥3, and American Society of Anesthesiologists (ASA) score ≥4.

Randomization was not performed as all patients undergoing rectal or esophageal surgery were invited to the Fit4Surgery prehabilitation program, which was a prerequisite for participation in the study. The control group was therefore comprised of cystectomy patients only. Baseline characteristics of patients included in the study are detailed in supplementary table 1. Written informed consent was obtained from all study participants before the start of any study-related procedures. Study procedures and data handling were performed in agreement

with the guidelines of The National Institutes of Health and in accordance with the declaration of Helsinki and its later amendments.

Multimodal prehabilitation intervention

Patients who participated in the control group received standard preoperative care. Patients in the intervention cohort underwent a multimodal prehabilitation program in addition to standard preoperative care. Multimodal prehabilitation comprised of four distinct components: an exercise program (endurance and resistance training), a nutritional intervention including Whey protein supplementation, psychological support, and smoking and alcohol cessation (34). Data regarding these outcomes, such as VO_2 peak and muscle strength (indirect 1RM), was gathered prior to the prehabilitation program and shortly before surgery (after prehabilitation). Parameters of intervention outcomes are detailed in supplementary figure 1 (S1). In the prehabilitation group, 3 patients were excluded from the analysis due to not having participated in the exercise intervention.

Sample and data collection

Blood samples were taken at baseline, before start of any prehabilitation activity (T1), before surgery (T2), and at postoperative day 1 (POD1, T3). For *ex vivo* endotoxin stimulation, lithium heparin (LH) anti-coagulated blood was drawn. Ethylenediaminetetraacetic acid (EDTA) anti-coagulated blood was obtained to evaluate whole blood counts, collect plasma, perform flowcytometric analysis, and isolate PBMCs. After blood withdrawal, LH blood was aliquoted for whole blood stimulation with 10ng/mL *Escherichia coli* lipopolysaccharide (LPS; serotype O55:B5; Sigma) or medium control. For all cell culture experiments, RPMI 1640 cell culture medium (Dutch modified) containing 11mM glucose (ThermoFischer) supplemented with 50mg/mL gentamicin (Centrafarm), 2mM GlutaMAX (Gibco) and 1mM pyruvate (Gibco) was used. Whole blood stimulations were incubated at 37°C, 5% CO_2 in a humidified incubator for 24 hours. Supernatants were collected after centrifugation at 1,600 RCF for 10 minutes and stored at -80°C until further analysis.

EDTA blood was aliquoted for whole blood count and flowcytometric analysis of mitochondrial function. Whole blood cells counts were determined via Sysmex XN-450 (Japan) hematology analyzer. Remaining LH and EDTA blood was centrifuged at 1,600 RCF at 4°C for 10 minutes. Plasma was collected and stored at -80°C until further analysis. After plasma sampling, the remaining EDTA blood was used to isolate PBMCs. PBMCs were isolated by means of Ficoll-Paque (GE Healthcare) density gradient centrifugation, and washed 3 times with PBS at

4ºC. A total of 5x10^6 PBMCs were lysed in LBP buffer (Macherey-Nagel) and stored at -80ºC until RNA extraction.

Ex vivo cytokine and metabolite production upon whole blood stimulation

Concentrations of cytokines IL-18 and IFN-γ in supernatants were quantified using human DuoSet ELISA's according to the manufacturer's protocol (R&D systems). Glucose and lactate concentrations in supernatants were determined using a glucose oxidase (Sigma), and lactate oxidase (Sigma) based enzymatic reaction. H_2O_2 from the reaction was coupled to the conversion of Amplex Red reagent (Life Technologies) by horseradish peroxidase (Sigma). The resulting fluorescence of resorufin (excitation/emission of 570/585nm) was then measured on a 96-well plate reader (BioTek). Glucose consumption was calculated by subtracting the glucose concentration measured in supernatants from the concentration measured in culture medium. For glucose and lactate measurements, supernatants were pretreated with perchloric acid to remove proteins from supernatants that could interfere with the assay.

Flowcytometric analysis of monocyte mitochondrial function

All measurements of mitochondrial function were performed within 1 hour of blood withdrawal, blood was kept at RT for the duration. EDTA blood was treated with red blood cell lysis buffer, washed once at RT with PBS, and stained with anti-human CD14-PerCP-Cy5.5 antibody, clone 63D3 (Biolegend). Cells were then incubated with either 5μM MitoSOX Red (Life Technologies) in HBSS with 1mM Calcium, 100nM tetramethylrhodamine ethyl ester perchlorate (TMRE, Sigma) in RPMI culture medium supplemented with or without 10μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, Sigma), or 500nM MitoTracker Deep Red (Life Technologies) in RPMI. These dyes allow for the evaluation of mtROS production, MMP, and mitochondrial mass, respectively. Cells were incubated for 20 minutes at room temperature, then measured on a CytoFLEX flowcytometer (Beckman Coulter). Data were analyzed using FlowJo (v10.8.1). Representative gating strategy is detailed in supplementary figure S2. To calculate the MMP, the median fluorescence intensity of TMRE was divided by the MFI of TMRE+FCCP.

Plasma proteomics

EDTA plasma from cystectomy patients in control and prehabilitation groups was collected at baseline, after prehabilitation (preoperative), and at POD1 and used for commercial targeted plasma proteomics analysis by Olink multiplex proximity

extension assays (Uppsala, Sweden). 92 Inflammation protein biomarkers were measured ('Inflammation' panel).

RNA isolation and quantification

RNA was extracted from PBMCs stored in lysis buffer with the NucleoSpin® RNA Plus kit (Macherey-Nagel) according to the manufacturer's instructions. Briefly, DNA was removed from the lysate by centrifugation over a gDNA removal column, after which RNA was purified and transcribed into cDNA using the iScript cDNA Synthesis kit (Bio-Rad). Levels of RNA were quantified with qRT-PCR using PowerUp Sybr Master Mix (Life Technologies), on a Quantstudio 3 PCR machine (Applied Biosystems). Analysis was performed using the $2^{-\Delta\Delta Ct}$ method, normalized against expression of endogenous genes *ACTB* and *B2M*. Primers used in this study were designed to be intron spanning and are listed in supplemental table 2.

In vitro experiments

PBMCs from healthy volunteers were isolated from buffy coats (Sanquin, Nijmegen, the Netherlands) by density centrifugation over Ficoll-Paque (GE Healthcare). Isolated cells were resuspended in Dutch modified RPMI 1640 culture medium (Gibco, Thermo Scientific) supplemented with 2mM glutaMAX, 1mM pyruvate, and 50µg/mL gentamycin. PBMCs were seeded at 5x10^5 cells in round-bottom 96-well culture plates and incubated at 37°C, 5% CO_2. To assess the HIF-1α/mTOR pathway, specific inhibitors were added. GM-CSF (1ng/mL, Miltenyi Biotec), cobalt (II) chloride ($CoCl_2$; 100µM, Sigma), or vehicle control were added to PBMCs. For cytokine and lactate measurements, cells were pre-incubated for 2 hours with compounds, after which LPS (10ng/mL) was added for another 24 hour incubation period. For flowcytometry analysis, cells were pre-incubated for 24 hours with compounds, then stained and measured as described above.

Statistical analysis

Data presented in figures are expressed as mean with standard error of the mean (SEM), unless stated otherwise. To determine differences within patient groups over time, repeated measures ANOVA with Šídák's multiple comparisons test was used. When comparing between control and intervention groups per timepoint, a Mann Whitney test was used. GraphPad Prism (v10.1.2) was used for all statistical analyses. Two-tailed p-value of < 0.05 was considered statistically significant. Differences between control and prehabilitation groups in the Olink analysis were compared by an unpaired Wilcoxon signed-rank test. Differences between timepoints within groups were compared by a paired Wilcoxon signed-rank test. The false discovery rate (FDR) was adjusted using the Benjamini-Hochberg procedure where indicated.

Results

We conducted a prospective, exploratory clinical trial investigating the effects of prehabilitation on innate immune cell metabolic adaptation. Patients scheduled to undergo elective cancer surgery participated in a multimodal prehabilitation program for a period of 2-6 weeks (fig. 1A-B). All participants were between 39 and 84 years of age, and there were no differences in baseline age, weight, or BMI between groups, though there were notable differences in the male/female distribution between patient groups (table S1).

Effects of prehabilitation and surgery on circulating leukocyte counts

Whole blood counts (WBC) increased significantly post-surgery (fig. 1C), corresponding to systemic inflammation after surgical insult. At T2, after prehabilitation, WBC was significantly lower in the prehabilitation group compared to control. Changes in WBC counts at T3 were mainly driven by an increase in neutrophils and monocytes. No significant changes in neutrophil counts or neutrophil-to-lymphocyte ratio (NLR) were observed between control and prehabilitation groups (data not shown). In prehabilitated patients, we observed an increase in lymphocyte-to-monocyte ratio (LMR) at T2, which was not seen in control group patients (1D). This shift in LMR was caused by a decrease in absolute monocyte counts post-prehabilitation (1E), while lymphocyte counts remain unchanged (1F).

Circulating biomarkers of inflammation during prehabilitation and surgery

Proteomic analysis of cystectomy patient plasma revealed an increase at T2 in IL-18 receptor 1 (IL-18R1) in the prehabilitation group, compared to control group (fig. 2A). Comparing plasma proteome composition between baseline (T1) samples and T2 samples indicated lower inflammation at T2 in both groups, the highest number of changes were seen in the control group, trending towards downregulation of inflammation. Specifically, factors related to T cell activation (IFN-γ, CD40, PD-L1) as well as IL-18R1 and IL-18 were among the decreased proteins in control group (2B), but not in the prehabilitated group (2C). A decrease in circulating IL-18R1, IL-18, and IFN-γ was observed prior to surgery at T2 in control patients, contrasting to stable levels seen in prehabilitated patients (2D-F). Significant reductions in POD1 circulating IFN-γ levels were seen in both control and intervention groups. Additional proteomic analysis figures are included in supplementary figure S3.

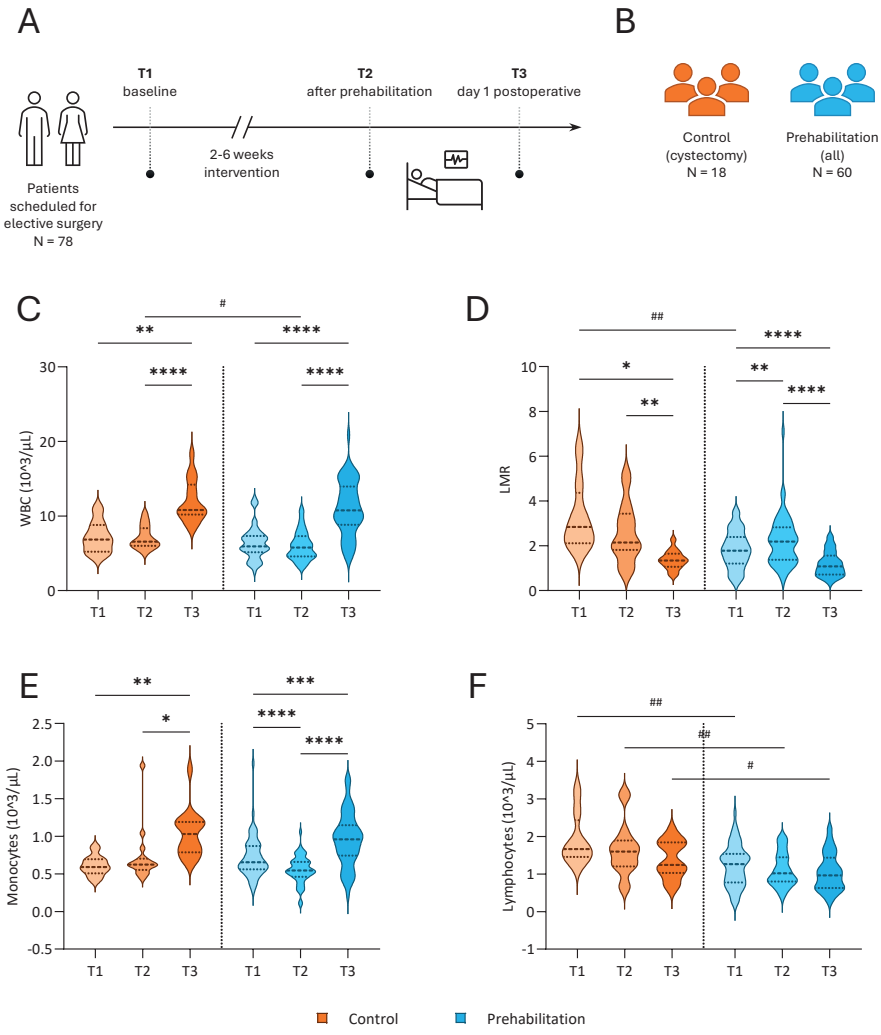

Figure 1. Changes in white blood cell populations during prehabilitation and surgery. [A] Schematic overview of study design. Patients scheduled for elective cancer surgery were included in the study. Blood was drawn at baseline (T1), after 2-6 weeks of prehabilitation or control (T2), and at POD1 (T3). [B] Cystectomy patients not participating in the prehabilitation program (n=18) were included in the study as a control group. Patients scheduled to undergo cystectomy (n=17), colorectal (n=18), and esophageal (n=25) surgery participated in the prehabilitation program (total n=60). Whole blood counts (WBC) in control group versus prehabilitation group [C]. Lymphocyte-to-monocyte ratio (LMR) in control versus prehabilitation group [D]. Absolute monocyte count [E] and absolute lymphocyte count [F] in control versus prehabilitation group. Repeated measures ANOVA with Šídák's multiple comparisons test, * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Mann Whitney test, # indicates $p < 0.05$, ## $p < 0.01$. Data are represented as median with quartiles.

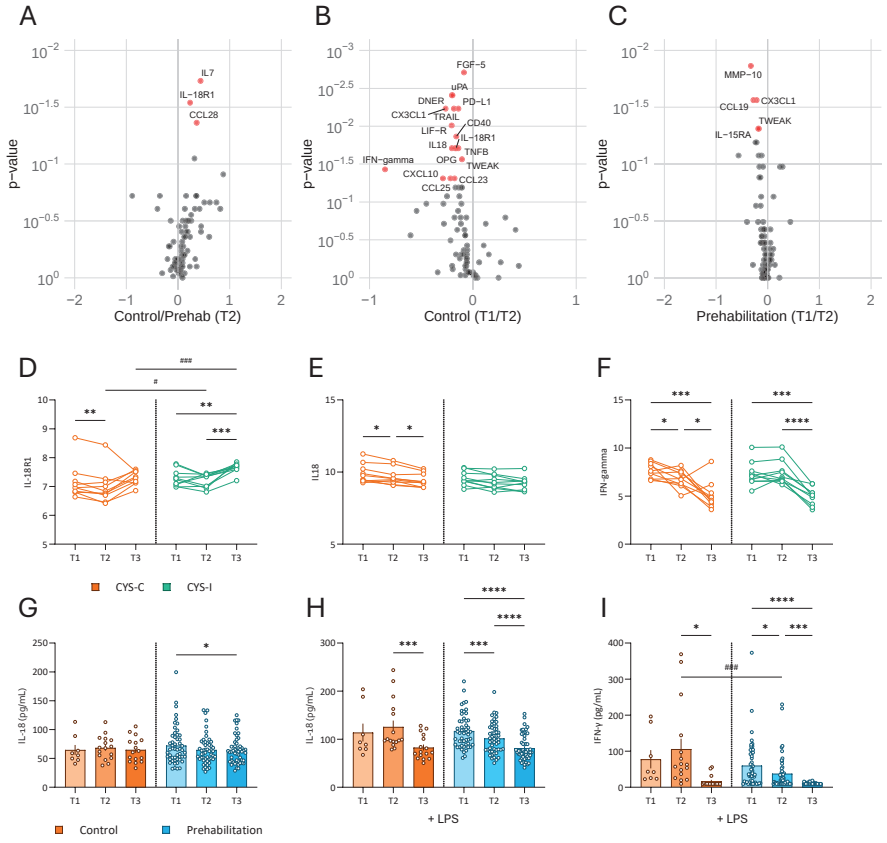

Figure 2. Plasma proteomic analysis of baseline, post-prehabilitation, and POD1 plasma samples. Volcano plot of differences at T2 between control and prehabilitation groups [A]. The effect of prehabilitation within groups was explored by volcano plots detailing the differences between T1 and T2 in control group [B], and prehabilitation group [C]. Y-axis represents the degree of statistical significance as tested with an unpaired [A] or paired [B, C] Wilcoxon signed rank test, not corrected for multiple testing. Proteins marked in red are statistically significant at a p-value of < 0.05. NPX values of proteins of interest were plotted, showing differences in IL-18R1 [D], IL-18 [E], and IFN-γ [F]. Repeated measures ANOVA with no correction for multiple comparisons, * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Mann Whitney test, # indicates $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. Data are represented as before-after (individual values), n=10 for control group and n=10 for cystectomy prehabilitation group. *Ex vivo* production of IL-18 and IFN-γ were compared over time. Levels of IL-18 were measured in unstimulated whole blood [G], and in whole blood stimulated with LPS [H]. IFN-γ was measured in stimulated whole blood [I]. Repeated measures ANOVA with Šídák's multiple comparisons test, * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Mann Whitney test, # indicates $p < 0.05$, ### $p < 0.001$. Data are represented as mean ± SEM with individual values, n=16 in control group, n=51 in prehabilitation group.

To further investigate the role of these inflammatory cytokines in prehabilitation, levels of IL-18 and IFN-γ were assessed in unstimulated and LPS-stimulated whole blood by ELISA. In unstimulated whole blood, IL-18 levels were significantly lower after surgery in prehabilitated patients, but not in the control group (fig. 2G). Upon LPS stimulation, IL-18 levels were significantly lower after surgery in both prehabilitation and control groups (2H). In prehabilitated patients, this decrease was already seen after prehabilitation at T2. No IFN-γ was detected in unstimulated whole blood supernatants (data not shown). In LPS stimulated whole blood supernatants, a significant reduction in postoperative IFN-γ levels was observed. At T2, IFN-γ production was significantly lowered in prehabilitated patients, compared to control (2I). Additionally, we observed a reduction in IFN-γ production between T1 and T2 in prehabilitated patients, but not in control patients.

Levels of glucose and lactate in response to surgical stress and prehabilitation

To assess metabolic activity of white blood cells during prehabilitation and surgery, we measured levels of metabolites glucose and lactate in whole blood supernatants. In unstimulated whole blood supernatants, prehabilitation resulted in lower lactate levels at T2 compared to baseline (fig. 3A). Postoperative lactate levels were higher in the prehabilitation group compared to control. When stimulated with LPS, a similar decrease in lactate production after prehabilitation was observed in the prehabilitation group (3B).

We further investigated the metabolic changes after prehabilitation by assessing the glucose consumption dynamics in the perioperative period. Glucose consumption in unstimulated whole blood was not affected by prehabilitation, and remained stable throughout the perioperative period (3C). However, upon stimulation with LPS, we observed a decrease in glucose consumption in the prehabilitation group at T2, which increased again after surgery at T3 (3D).

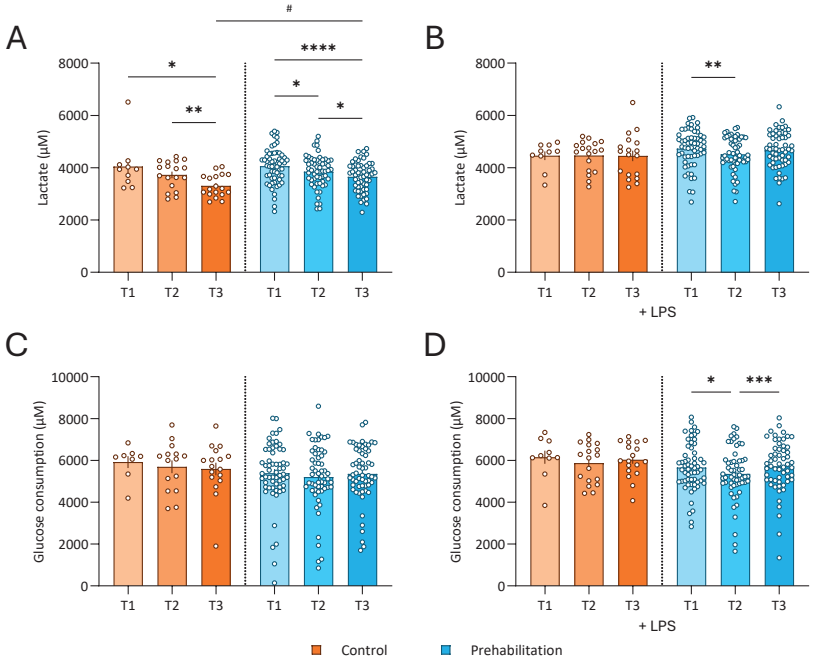

Figure 3. Lactate production and glucose consumption dynamics in whole blood. Lactate production in unstimulated whole blood [A] and in LPS-stimulated whole blood [B] in control versus prehabilitation groups. Glucose consumption in unstimulated whole blood [C] and after LPS stimulation [D]. Repeated measures ANOVA with Šídák's multiple comparisons test, * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Data are represented as mean ± SEM with individual values, n=18 in control group and n=58 in prehabilitation group.

Monocyte mitochondrial dynamics and gene expression

Given that our circulating leukocyte data indicate a specific effect of prehabilitation on the monocyte immune cell compartment, we sought to further investigate the potential changes in monocytes regarding metabolic adaptations. To this end, monocyte mitochondrial dynamics were evaluated by means of flowcytometry. In addition, transcription of key regulatory genes in metabolism, inflammation, and mitochondrial adaptations was investigated by qRT-PCR.

Mitochondrial ROS (mtROS) production, as measured by MitoSOX dye, was significantly decreased in monocytes after surgery in prehabilitated patients, but not in the control group (fig. 4A). Monocyte mitochondrial mass, as measured by MitoTracker dye, was significantly lower at POD1 in the prehabilitated patient group (fig. 4B). No effect of prehabilitation on mitochondrial mass was observed. Most notably, we observed that prehabilitation increased the monocyte MMP, as measured by TMRE dye, at T2 (4C).

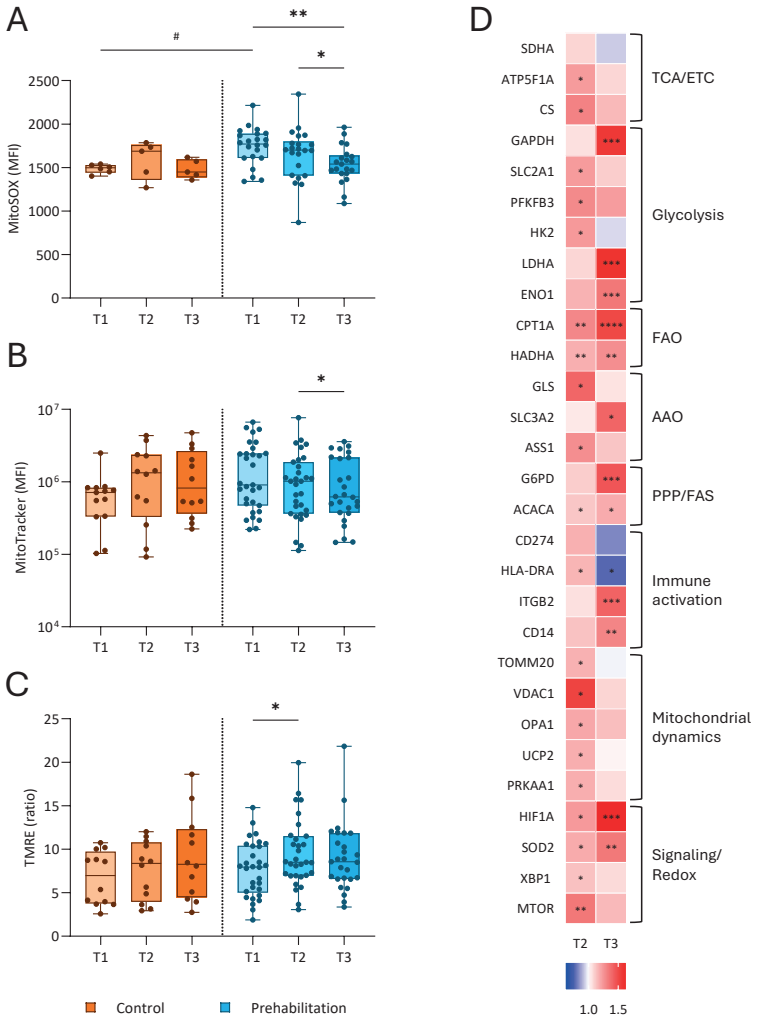

Figure 4. Mitochondrial dynamics in monocytes during prehabilitation and surgery. In CD14+ monocytes, mitochondrial ROS production was measured by MitoSOX fluorescent dye [A]. MitoTracker dye was used to assess monocyte mitochondrial mass [B]. Mitochondrial membrane potential was assessed in monocytes by incubation with TMRE dye and TMRE+FCCP [C]. Repeated measures ANOVA with no correction for multiple comparisons, * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Mann Whitney test, # indicates $p < 0.05$, ## $p < 0.01$. Data are represented as median fluorescence intensity with min to max error bars, showing individual values, n=12 in control group and n=31 in prehabilitation group. Expression of genes involved in metabolic adaptations and signaling pathways was quantified in mRNA isolated from PBMCs from prehabilitated patients at all 3 timepoints. mRNA levels were normalized to ACTB and B2M expression and are shown as fold change (FC) to T1. Heatmap shows the relative gene expression in total prehabilitation group (n=24) [D]. Repeated measures ANOVA with no correction for multiple comparisons, * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Data are represented as geometric mean, with red color indicating a FC > 1, and blue color indicating a FC < 1.

Transcription of an array of metabolic genes related to the TCA cycle, glycolysis, amino acid and fatty acid metabolism were all found ob e affected by prehabilitation (4D), indicating a shift towards increased metabolic capacity and efficiency. Transcription of genes involved in regulating glycolysis, such as *SLC2A1*, *HK2* and *PFKFB3*, was increased at T2. In addition, *CPT1A* and *HADHA*, essential genes for the regulation of mitochondrial oxidation of long-chain fatty acids, and genes important for amino acid metabolism, such as *GLS* and *ASS1*, were found ob e significantly upregulated after prehabilitation. Most notably, several genes involved in the regulation of mitochondrial dynamics were upregulated after prehabilitation, specifically *VDAC1*, *OPA1*, *TOMM20*, *UCP2*, and *PRKAA1*. In addition, we found increases in transcription of *HIF1A* and *MTOR*, important regulatory ob e hat link metabolism to inflammation. *XBP1* and *SOD2*, crucial genes for sensing and remediating oxidative stress, were found ob e upregulated after prehabilitation.

Correlation between prehabilitation, inflammation, and metabolism

To explore if prehabilitation parameters are associated with changes in composition of immune cells in blood, mitochondrial dynamics, or production of lactate, glucose, or cytokines, changes in these parameters between T1 and T2 (Δ) were correlated with changes in BMI, weight, VO_2 max, 1RM, number of training sessions, and duration of the intervention (fig. 5A).

Correlation analyses of the cohort revealed that a higher number of training sessions during the prehabilitation period was associated with an increase in *UCP2* gene expression, an increase in LPS-induced lactate production, and a decrease in IL-18 levels in whole blood. We observed an association between loss of body weight and increased *OPA1* gene transcription. An increase in peak VO_2 after prehabilitation showed a positive correlation with *VDAC1* gene expression. Increased in muscle strength (RM1) was associated with decreased levels of IL-18 in whole blood, and with increased *UCP2* gene expression. We observed an association between higher IL-18 production upon LPS stimulation with increased circulating monocytes, and with increased glucose consumption. Furthermore, LPS-induced IL-18 levels negatively correlated with *HIF1A* and *SOD2* gene expression, and mitochondrial mass, as measured by MitoTracker dye.

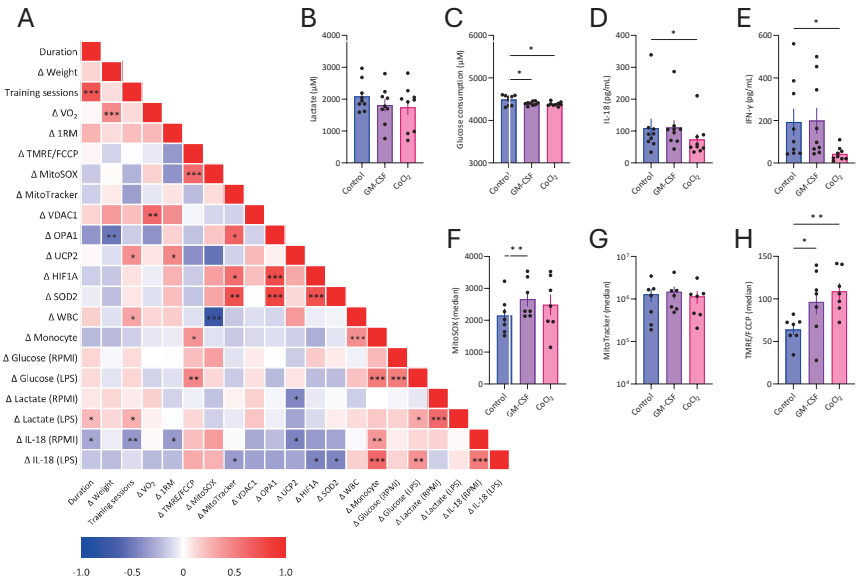

Figure 5. Associations between study parameters and *in vitro* validation. [A] Association heatmap of changes between T1 and T2 (Δ) in prehabilitation parameters and metabolic and mitochondrial readouts, showing Spearman correlation coefficients and their significances. Red color indicates a positive correlation, and blue a negative correlation. Color intensity indicates the Spearman correlation coefficient ®, * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Metabolic and signaling pathways of interest were validated *in vitro* with PBMCs isolated from healthy donors. PBMCs were incubated with vehicle control, GM-CSF (1ng/mL), or $CoCl_2$ (100μM) for 24 hours. For lactate [B], glucose [C], IL-18 [D] and IFN-γ [E] measurements, cells were pre-incubated with compounds for 2 hours, then stimulated with LPS (10ng/mL) or medium control for 24 hours. Monocyte mitochondrial ROS production [F], mitochondrial mass [G], and TMRE/TMRE+FCCP ratio [H] were measured after 24 hour stimulation with compounds. Repeated measures ANOVA with no correction for multiple comparisons, * indicates $p < 0.05$, ** $p < 0.01$. Data are represented as mean ± SEM showing individual values, n=9 for lactate, glucose, IL-18 and IFN-γ measurements, n=7 for mitochondrial flowcytometry experiments.

In vitro validation of regulatory pathways

To further explore the interactions between the regulatory pathways highlighted in our gene expression data and metabolic adaptations in immune cells, we performed a series of *in vitro* validation experiments in PBMCs from healthy donors using pharmacological inhibition or activation of HIF-1α and mTOR (fig. 5B-H). Chemical induction of HIF1-α using cobalt chloride ($CoCl_2$) was found to decrease glucose consumption, IL-18, and IFN-γ production (5C-E), while concurrently increasing the MMP (5H). The activation of the mTOR pathway through stimulation with GM-CSF resulted in lowered glucose consumption (5C), but significantly increased mtROS production (5F), and MMP (5H). No effect of GM-CSF was observed on lactate, IL-18, or IFN-γ production.

Discussion

The integration of prehabilitation strategies aimed at improving clinical outcomes through physical conditioning and nutritional optimization prior to surgery has been the subject of extensive study regarding feasibility, implementation, and clinical efficacy (10,35). However, the underlying mechanisms, particularly in regards to the metabolic and immunological shifts that may occur during prehabilitation and surgery, remain poorly understood. This pilot study sought to explore the multifaceted roles of immunometabolism, mitochondrial dynamics, and metabolic pathway regulation in patients following a multimodal prehabilitation program.

Prehabilitation was found to significantly affect both the number and the composition of circulating leukocytes. After prehabilitation, absolute counts of white blood cells were significantly lower in the prehabilitation group, compared to control group. Although we did not observe significant changes in circulating neutrophils or lymphocytes, absolute monocyte counts were decreased after prehabilitation. This resulted in a proportional increase in the lymphocyte to monocyte ratio (LMR) in the prehabilitation cohort. The LMR is derived from differential white blood cell (WBC) counts, and reflects systemic immunity. The prognostic role of the LMR has been examined in a variety of patient cohorts, including urothelial bladder cancer, esophageal cancer, colorectal cancer, and other malignancies. Low LMR is frequently associated with poor prognosis of several types of tumors (36-38), as well as COVID-19 pneumonia progression (39). A previous study in gastrointestinal cancer patients showed that preoperative nutritional support, a component of multimodal prehabilitation programs, increased total lymphocyte count (40). We found that prehabilitation may specifically affect circulating monocyte counts, possibly affecting immunological readiness for surgery by modulating the composition of the innate and adaptive immune cells. Whether these shifts in cellular composition translate to altered inflammatory responses seen after surgery needs to be investigated more thoroughly.

Significantly altered proteins between the control and prehabilitation group, as determined by proteomic analysis, were IL-18R1, IL-18, IFN-γ. In the control group, these 3 proteins were all decreased between T1 and T2, in the absence of any intervention, whereas in the prehabilitation group they remained stable between T1 and T2. These results indicate that circulating inflammation-related proteins tended to be better preserved during prehabilitation. The relationship between exercise and inflammatory cytokine signaling (reviewed in 41) is well established and evidence has shown that both pro- and anti-inflammatory cytokines may be produced in

response to exercise. The immunosuppressive effects of surgery have been described in various studies (42,43). The precise cause of immunological dysfunction following surgery remains a subject of active investigation, though it is likely the result of a complex interplay between medications including anesthesia and analgesia, the release of danger-associated molecular patterns (DAMPs) through surgical injury, the activation of the sympathetic nervous system through stress and nociception, and many other factors (44-46).

Postoperative immune suppression is evidenced by a strong decrease in production of IL-18 and IFN-γ upon LPS stimulation in both control and prehabilitation groups. Interestingly, the decrease in IFN-γ and IL-18 production was already observed after prehabilitation. Given the strong correlation between circulating monocyte counts and IL-18 production, it is possible that this decrease in cytokine production is partly due to the decrease in circulating monocytes after prehabilitation, though no causal relation can be inferred from our data. IL-18 has been associated with type II muscle atrophy (47), and is known to activate NF-κB, a major proinflammatory transcription factor that mediates the effects of proinflammatory cytokines but also exacerbates inflammation. Li *et al*. show that intensive lifestyle intervention consisting of nutritional supplementation and exercise can decrease IL-18 levels, alleviating inflammation in sarcopenia patients whilst simultaneously leading to the recovery of muscle mass (48). A meta-analysis of the effects of exercise training on biomarkers of cardiometabolic health identified significantly lower levels of IL-18 in exercise groups, along with several biomarkers of insulin resistance and hemostatic factors (49). The utilization of IL-18 as a biomarker of effective exercise training intervention should be further investigated.

IL-18 is known to directly induce IFN-γ production (50). Accordingly, we found that both cytokines were reduced after prehabilitation. The role of IFN-γ in exercise-induced inflammation in muscles is described in numerous studies (summarized in 51). An evaluation of acute and chronic exercise models in mice revealed that elevated IFN-γ production directly impairs muscle mitochondrial function, which limits the performance-enhancing benefits of exercise (52). Other research has shown that lifelong aerobic exercise training ameliorates many age-associated pro-inflammatory cytokines, including IFN-γ, and protects against multiple cancer types in mice (53). However, more research is needed to establish whether shorter periods of exercise could reduce IFN-γ production capacity in a similar manner. IFN-γ is a crucial cytokine in the regulation of both innate and adaptive immune responses, playing an important role in host-defense against opportunistic infections. The clinical implications of reduced IFN-γ production capacity after prehabilitation must be further evaluated.

Upon our investigation of the immunometabolic effects of prehabilitation, we observed that prehabilitation reduces lactate production in both stimulated and unstimulated whole blood. This change was accompanied by a concomitant decrease in glucose consumption in stimulated whole blood samples in prehabilitated patients. Changes in glucose consumption and lactate production serve as a first indication of altered glycolytic flux, which is generally associated with increased inflammatory signaling. Taken together, these fluctuations reflect changes in cellular metabolism in response to prehabilitation and surgical stress, underscoring the impact of prehabilitation on metabolic adaptability. Dynamic metabolic capacity contributes to effective mobilization of energy reserves upon surgical stress, promoting a rapid return to homeostasis (54,55). Along with systemic fluctuations in metabolites, we found changes in transcription of genes related to various metabolic pathways, including TCA cycle, glycolysis, amino acid metabolism, and fatty acid metabolism. These changes further elucidate the shift in metabolic capacity of leukocytes during prehabilitation and surgery. Furthermore, we found that prehabilitation resulted in altered transcription of genes that regulate immunometabolic pathways, mitochondrial adaptations, and cellular stress responses.

The link between physical exercise and mitochondrial adaptations is well established in literature, both in skeletal muscle cells (56), and in peripheral immune cells (13,57,58). When investigating monocyte-specific mitochondrial changes, we observed that prehabilitation increased mitochondrial membrane potential. A reduction in mitochondrial ROS production was observed after surgery in the prehabilitation group, but not in the control group. This decrease in mitochondrial ROS production could be related to the increased transcription of *UCP2* gene, as well as the *SOD2* gene. Uncoupling protein 2 (UCP-2) regulates mitochondrial ATP production via the TCA cycle, lowers the ATP/ADP ratio, MMP, and mtROS production (59,60), facilitates glycolysis (61), and ameliorates mitochondrial dysfunction in acute inflammation in certain mouse models (62), offering cytoprotective effects under conditions of oxidative stress. The *SOD2* gene encodes the mitochondrial variant of superoxide dismutase, clearing mitochondrial ROS and thereby protecting the cell from oxidative stress (63). These mechanisms may underlie the reduced mtROS production in prehabilitated patients after surgery, though more research is needed to establish the exact pathways involved.

Our results indicate that prehabilitation increased transcription of several mitochondrial genes, including *VDAC1* and *OPA1*, among others. Voltage-dependent anion channel 1 (VDAC1), a mitochondrial outer membrane protein,

plays an important role in the regulation of energy production, mitochondrial oxidase stress, Ca^{2+} signaling, TCA cycle, glycolysis, lipid metabolism and mitophagy (64,65). Optic atrophy 1 (OPA1), a mitochondria-shaping protein, is a key metabolic driver in myeloid cells, orchestrating mitochondrial fusion, biogenesis, and TCA cycle respiration in myeloid cells (66). Transcription of these genes was found to be associated with clinical outcomes of prehabilitation VO_2 peak and change in body weight, respectively, potentially identifying these proteins as targets for future mechanistic studies.

The upregulation of genes involved in mitochondrial dynamics and immune activation post-prehabilitation further supports the role of metabolic conditioning in enhancing immune responses and cellular resilience to surgical stress. We observed an increased transcription of gene *XBP1*, which modulates the cellular response during endoplasmic reticulum (ER) stress (67). Gene expression of mTOR, another key modulator of cellular responses to stress, was also elevated at T2. mTOR acts as a sensor of the metabolic environment, and may enhance transcription of HIF-1α mRNA (68), driving a metabolic shift towards glycolysis. In our dataset, we saw that *HIF1A* gene expression was indeed elevated at T2 and remained elevated at day 1 post-surgery. mTOR may be suppressed through AMPK signaling, under conditions of limited energy or glucose (69). The gene expression patterns observed after prehabilitation, combined with the measurements of monocyte mitochondrial function by flowcytometry suggest enhanced mitochondrial dynamics, translating to reduced oxidative stress, and maintenance of cellular energy production through various metabolic pathways. This suggests a shift towards more efficient cellular energy utilization, which could enhance cell survival and function under stress and in conditions of limited energy supply.

To gain further insight into the immunometabolic pathways highlighted in our gene expression data, we performed a series of *in vitro* experiments, utilizing PBMCs from healthy donors. We selected HIF-1α and mTOR, since there is a vast body of research supporting the central regulatory function of these genes in both inflammatory and metabolic reprogramming (70,71). Using $CoCl_2$ and GM-CSF as chemical inducers of the HIF1-α and mTOR pathways, we confirmed that $CoCl_2$ increases the MMP, while simultaneously decreasing glucose consumption, and IL-18 and IFN-γ production. It must be noted however that GM-CSF in particular may have additional off-target effects on immune cell function besides mTOR pathway activation (72). These findings closely match our prehabilitated patient data, suggesting a potential role for HIF1-α in prehabilitation. These findings highlight the complex interplay between metabolic pathways regulation and immune cell function.

Our study is limited to the measurement of systemic immunological adaptations and circulating factors, not exploring the immunological changes that may occur in the tissues affected by exercise interventions, such as the muscle and adipose tissues. It is described that exercise induces transient inflammation in skeletal muscles, followed by metabolic adaptation, tissue repair, and performance enhancement (52). More research on the potential cross-talk between these localized immunometabolic changes and circulating factors is needed to fully understand the effects of prehabilitation, and the implications on surgery-induced physiological changes.

We did not incorporate postoperative clinical outcomes in our dataset. Therefore, we cannot draw conclusions about the causal relation between mitochondrial fitness and the improved postoperative functional capacity associated with multimodal prehabilitation (6). Lastly, due to the exploratory design of this study, some of the analyses performed consist of a small number of patients. Therefore, care should be taken to interpret the results as hypothesis-generating. Still, the findings of this research are consistent with literature and with basic principles of immunology, and were validated through a series of *in vitro* experiments to support the major outcomes.

Conclusions

This study represents an opportunity to establish a framework of understanding for the biological mechanisms that underlie the beneficial effects of prehabilitation programs. We find that prehabilitation profoundly affects immune cell metabolism and function, resulting in a shift in metabolic pathways, altered cytokine production, and changes in mitochondrial function. The mechanisms explored here may contribute to the development of targeted therapies, and generate hypotheses for future research. The data generated in this study provides an initial exploration of the effects of prehabilitation on immunological outcomes and contributes to the rationale for implementation into the standard of care.

Acknowledgments

We wish to thank the participants of this study for their efforts. We thank Liesbeth van Emst for performing the Olink proteomics assays.

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Supplemental material

Supplementary Table 1. Baseline characteristics of study participants.

Characteristic*	Cystectomy control (n=21)	Cystectomy prehabilitation (n=22)	Rectum (n=20)	Esophagectomy (n=26)
Age (years)	72 (57 - 78)	74 (65 - 77)	65 (62 - 73)	69 (63 - 72)
Weight (kg)	83.7 (77.3 - 92.2)	83.9 (66.4 - 94.1)	77.9 (64.2 - 90.0)	78.5 (64.3 - 87.8)
BMI (kg/m^2)	28.9 (25.9 - 31.5)	25.9 (22.2 - 28.2)	26.2 (23.0 - 29.0)	24.6 (21.3 - 27.8)
Sex, female	4 (23.5 %)	1 (5.6 %)	9 (45.0 %)	6 (25.0 %)
Training sessions (*n*)	N/A	7 (5 - 10)	8 (6 - 10)	7 (5 - 10)
Time T1 to T2 (days)	17 (13 - 24)	29 (22 - 35)	31 (21 - 36)	27 (17 - 36)

* Median with IQR for continuous variables and n (%) for categorical variables

Supplementary Table 2. Primer sequences used in the study.

Gene	FW primer (5' to 3')	RV primer (5' to 3')	Function
ACTB	TGGCACCACACCTTCTACAA	CCAGAGGCGTACAGGGATAG	Housekeeping
B2M	CTTTCTGGCCTGGAGGCTATC	ACCAGTCCTTGCTGAAAGACA	Housekeeping
HLA-DRA	AGTCCCTGTGCTAGGATTTTTCA	ACATAAACTCGCCTGATTGGTC	Immune activation
ITGB2	AAGTGACGCTTTACCTGCGAC	AAGCATGGAGTAGGAGAGGTC	Immune activation
CD274	GGGCATTCCAGAAAGATGAGG	GACAATTAGTGCAGCCAGGTC	Immune activation
CD86	GGATGAGTGGGGTCATTTCCA	GGCAGGTCTGCAGTCTCATTG	Immune activation
CD14	CCGCTGTGTAGGAAAGAAGC	GCAGCGGAAATCTTCATCGT	Immune activation
SDHA	CAGCATGTGTTACCAAGCT	GGTGTCGTAGAAATGCCAC	TCA cycle / ETC
IDH2	TGCCGACAAAAGGATCAAGG	ATCAGTCTGGTCACGGTTTGG	TCA cycle / ETC
CYCS	GTTCGTTGTGCCAGCGACTA	ATTGGCGGCTGTGTAAGAGT	TCA cycle / ETC
ATP5F1A	GTGAAGAGGACAGGAGCCAT	CATTGGTTCCCGCACTGAAA	TCA cycle / ETC
CS	GGGGCCATTGACTCTAACCT	ACAGGTAAGGGTCGGAAAGG	TCA cycle / ETC
GAPDH	CACATCGCTCAGACACCATG	TGACGGTGCCATGGAATTTG	Glycolysis
SLC2A1	GTGGGCCTTTTCGTTAACCG	CCCAGTTTCGAGAAGCCCAT	Glycolysis
PFKFB3	ATTGCGGTTTTCGATGCCAC	GCCACAACTGTAGGGTCGT	Glycolysis
HK2	TTGACCAGGAGATTGACATGGG	CAACCGCATCAGGACCTCA	Glycolysis
LDHA	CCGGATCTCATTGCCACGC	GCACCAACCCCAACAACTGTA	Glycolysis
SLC16A1	TGTCAGGCTGTGGCTTGATT	GCCAATGGTCGCCTCTTGTA	Glycolysis
ENO1	CGGGAATCCCACTGTTGAGG	TTCTTGCTAACCAGGGCAGG	Glycolysis
CPT1A	CGGTTGCTGATGACGGCTAT	CCAGCAGCTCCAGTGGAATTA	Fatty acid oxidation
ACADM	GGGAGAATGACTGAGGAGCC	TCTGGATCAGAACGTGCCAA	Fatty acid oxidation
HADHA	CAAGGGAGTGATGCCGGTTA	CCCTGCACCAAGAATAGCCA	Fatty acid oxidation
GLS	AGGGTCTGTTACCTAGCTT	ACGTTCGCAATCCTGTAGA	Amino acid metabolism
SLC3A2	CTGAAGGTGAAGGGCCTTGT	ACCCCGGTAGTTGGGAGTAA	Amino acid metabolism
SLC1A5	CCTCTTCACCCGCAAAAACC	TGAAACGGCTGATGTGCTTG	Amino acid metabolism
ASS1	GTGTGAATTTGTCCGCCACT	AGTGACCTTGCTCTGGAGAC	Amino acid metabolism
PPARGC1A	CTGCTCGGAGCTTCTCAAAT	GTCATTTGGTGACTCTGGGGT	Mitochondrial dynamics
TOMM20	AGAGAAGATGGTGGGTCGGA	TTGGAAAGCCCAGCTCTCTC	Mitochondrial dynamics

Supplementary Table 2. Continued.

Gene	FW primer (5' to 3')	RV primer (5' to 3')	Function
VDAC1	CAGGCTCCTGTGTCTGCTG	GGCTGAGCCTGAGCTTGTAA	Mitochondrial dynamics
OPA1	AACCACAGTCCGGAAGAACC	TGCGCTGTATACGCCAAAAC	Mitochondrial dynamics
UCP2	GTCCGATTCCAAGCTCAGGC	CACCAGCTCAGCACAGTTGA	Mitochondrial dynamics
PPARGC1B	CCCACTTGCTCTGACCACTG	ATGCTTGGCGTTCTGTCTGA	Mitochondrial dynamics
PRKAA1	TTGAAACCTGAAAATGTCCTGCT	GGTGAGCCACAACTTGTTCTT	Mitochondrial dynamics
HIF1A	CATAAAGTCTGCAACATGGAAGGT	ATTTGATGGGTGAGGAATGGGTT	Signaling/Redox
GSS	GAACCGTTCGCGGAGGAAA	GAATGGGGCATAGCTCACCA	Signaling/Redox
XBP1	GAGTTAAGACAGCGCTTGGG	CTGGGGAAGGGCATTTGAAG	Signaling/Redox
PRDX2	CGAGATCATCGCGTTCAGCA	TCTGTTTTCAGCACGCCGTA	Signaling/Redox
MTOR	CCTGCCTTTGTCATGCCTTT	CTGGGTTTGGATCAGGGTCT	Signaling/Redox
G6PD	CGAGGCCGTCACCAAGAAC	GTAGTGGTCGATGCGGTAG	Pentose phosphate pathway
ACACA	TCGCTTTGGGGGAAATAAAGTG	GTGTGACCATGACAACGAATCTA	Fatty acid synthesis

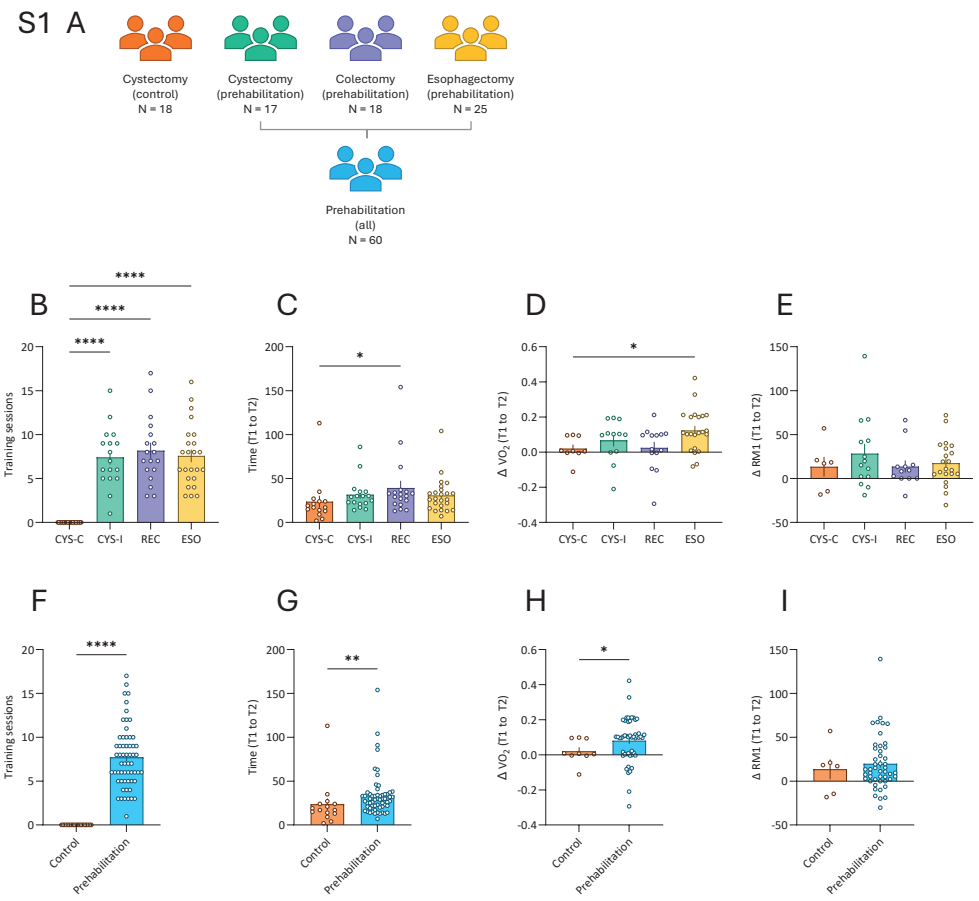


Figure S1. Prehabilitation variables of study cohort. [A] Composition of the prehabilitation group, consisting of cystectomy (n=17), colectomy (n=18), and esophagectomy patients (n=25). The number of training sessions [B and F], time (in days) between T1 and T2 [C and G], ΔVO_2 max between T1 and T2 [D and H], and ΔRM1 between T1 and T2 [E and I]. Figures in top row show patient groups, and bottom row compares all prehabilitated patients with control group. Mann Whitney test, * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are represented as mean ± SEM with individual values, n=16 in control group, n=13, n=16, and n=22 in cystectomy, colorectal, and esophageal prehabilitation groups, respectively.

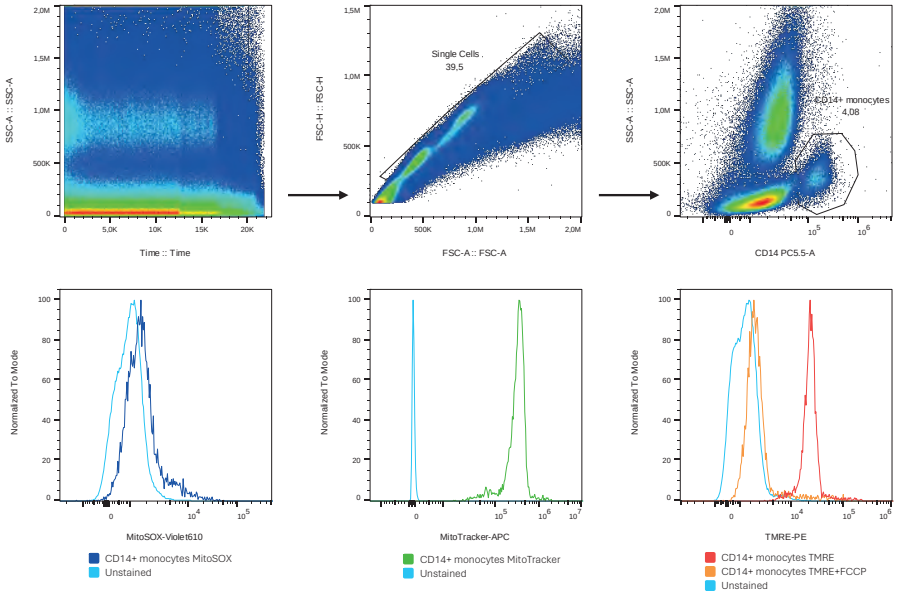
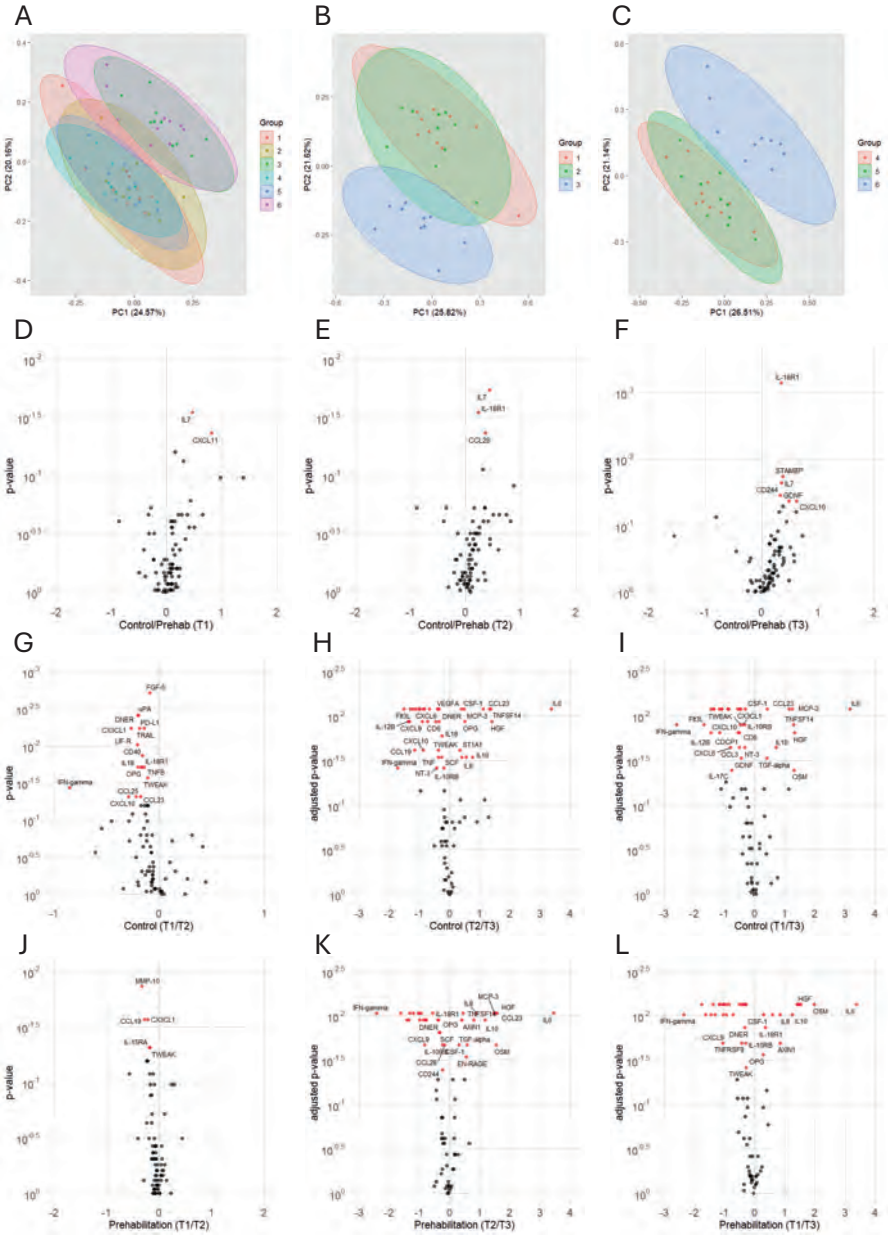


Figure S2. Representative gating strategy for flow cytometry based assays of mitochondrial dynamics. After selection for single cells, CD14+ monocytes were gated. In CD14+ monocyte population, median fluorescence intensity (MFI) of MitoSOX, MitoTracker, or TMRE dye was measured.


A
B
C
Group
PC1 (24.57%)
PC2 (20.16%)
PC1 (25.82%)
PC2 (21.52%)
PC1 (26.51%)
PC2 (21.14%)
D
E
F
p-value
Control/Prehab (T1)
Control/Prehab (T2)
Control/Prehab (T3)
G
H
I
adjusted p-value
Control (T1/T2)
Control (T2/T3)
Control (T1/T3)
J
K
L
Prehabilitation (T1/T2)
Prehabilitation (T2/T3)
Prehabilitation (T1/T3)

< Figure S3. Additional proteomic analyses. [A] Principal component analysis (PCA) of 77 inflammatory proteins comparing control and prehabilitation samples at all three timepoints. Groups 1, 2 and 3 represent T1, T2 and T3 of control group, respectively [A, B]. Groups 4, 5 and 6 represent T1, T2 and T3 of prehabilitation group [A, C]. Volcano plots comparing log-fold changes of differentially expressed proteins between control and prehabilitation groups at T1 [D], T2 [E], and T3 [F]. Within the control group, differentially expressed proteins between T1 and T2 [G], T2 and T3 [H], and T1 and T3 [I] are shown in volcano plots. Within the prehabilitation group, differentially expressed proteins between T1 and T2 [J], T2 and T3 [K], and T1 and T3 [L] are shown in volcano plots. Y-axis represents the degree of statistical significance as tested with an unpaired or paired Wilcoxon signed rank test as appropriate, not corrected for multiple testing, or corrected for multiple testing by the Benjamini-Hochberg procedure [H, I, K, L]. Proteins marked in red are statistically significant at a p-value of < 0.05 or an FDR-adjusted p-value of < 0.05, as indicated in the graphs.

Part II

Protective effects of trained immunity against unrelated infections

Chapter 5

Propofol induces trained immunity resulting in increased inflammation and pathogen killing via augmented fatty acid metabolism

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Abstract

Background. Propofol is an intravenous anesthetic agent that has been described to have immunomodulatory effects. Here, we determined the long-term effects of propofol on monocyte and macrophage effector functions *in vitro*, and investigated the role of fatty acid metabolism in these effects.

Methods. Isolated primary human monocytes were exposed to propofol for 2-24 hours, and re-stimulated with LPS either immediately or following propofol wash-out and culture for 5 days. Levels of inflammation-related gene expression were measured by qPCR, and protein concentrations of IL-1β, TNF, and IL-6 were assessed via ELISA. Real-time metabolic rates were assessed by Seahorse analysis. Production of reactive oxygen species (ROS) was assessed by luminol-based ROS assay and by mitoSOX-based mtROS assay. A microbicidal activity assay was performed with *S. aureus*, *P. aeruginosa*, and *E. coli*, to assess *in vitro* antimicrobial function.

Results. Acute exposure to propofol induced pro-inflammatory cytokine production, while suppressing oxidative phosphorylation and glycolytic metabolism in primary human monocytes. Propofol induced a trained immunity phenotype *in vitro* in human primary monocytes, accompanied by increased production of IL-1β, TNF, and IL-6. Propofol-trained macrophages had elevated oxygen consumption, relying in part on fatty acid metabolism. Co-incubation with pharmacological inhibitors of fatty acid metabolism prevented propofol-induced trained immunity. Macrophages trained with propofol had heightened microbicidal activities despite impaired generation of reactive oxygen species.

Conclusions. This study demonstrates that propofol-trained macrophages exhibit enhanced antimicrobial functions that conform to the trained immunity phenotype, and highlights fatty acid oxidation as a novel metabolic pathway for trained immunity. Further unraveling the possible role of trained immunity in the pathophysiology underlying propofol infusion syndrome could help to identify new targets for therapeutic strategies in clinical settings. Taken together, our data contribute to the understanding of the effects of propofol on the innate immune system.

Introduction

Intravenous propofol (2,6-diisopropylphenol) is a common anesthetic used for the induction and maintenance of general anesthesia and sedation in surgery, critical care patients, and routine outpatient procedures (1). Though widely used, evidence is mounting that brief exposure to propofol may have repercussions for a patient's susceptibility to microbial infection, as the drug has been linked to acute immunomodulatory effects in both *in vitro* and *in vivo* models (2-5).

Several studies have reported impaired neutrophil effector functions, including respiratory burst (6), chemotaxis (7), and phagocytosis (8). When investigating lymphocytes, proliferation and cytokine production were not significantly affected by propofol (9). The mechanisms contributing to propofol-induced anti-inflammatory immunomodulation remain unclear, though there may be a role for NF-κB (5). In addition, propofol infusion syndrome (PRIS) is a rare but severe complication that has been known to occur when patients receive high doses of propofol for prolonged periods of time (10). Even though the underlying mechanisms of PRIS remain uncertain, some evidence suggests that this form of propofol toxicity is linked to impaired mitochondrial function (11-14).

Recent studies have shown that following brief exposure to endogenous atherogenic particles, such as lipoprotein (a), oxidized low-density lipoprotein (oxLDL), and aldosterone, monocytes can adopt a long-term pro-inflammatory phenotype, a process termed trained immunity (15). Of note, mitochondrial metabolic reprogramming was recently highlighted as a crucial component of the induction of trained immunity (16). In monocytes and macrophages, inflammatory phenotypes are intimately tied to distinct metabolic profiles. In the context of trained immunity, several metabolic pathways including glycolysis, the TCA-cycle, fatty acid synthesis and oxidative phosphorylation have all been implicated as critical pathways for the induction of trained immunity (17-21).

The oxidation of fatty acids is an important source of energy for macrophages when initiating an inflammatory response (22). Fatty acids are shuttled over the mitochondria by conjugation to carnitine, where they are catabolically broken down by a process termed β-oxidation into acetyl-CoA, which then enters the TCA cycle for further metabolism. Fatty acid oxidation (FAO) has been described to regulate immune cell function to some degree, being the primary source of energy production in 'M2-like' macrophages (23-24). Conversely 'M1-like' macrophages downregulate FAO in favor of glycolytic metabolism. However, recent research

has suggested a more complex understanding of macrophage metabolism during activation and differentiation (25-27). The role of fatty acid metabolism in trained immunity has been the focus of recent studies, demonstrating that fatty acid synthesis (FAS), and lipoxygenase pathways appear to play essential roles in the induction of trained immunity (15,28).

Due to the lipophilic nature of propofol, the clinical formulation contains long-chain polyunsaturated fatty acid triglycerides, phospholipids, and glycerol, with the primary lipid components being palmitic acid, stearic acid, oleic acid, and linoleic acid (29). Some studies have shown that the immunomodulatory properties of propofol on neutrophils are related to this lipid carrier vehicle (30). Given the potential broad clinical implications of long-term immunomodulation by propofol, we sought to investigate whether transient exposure of monocytes to propofol can affect key features of innate immunity, and whether this treatment results in a trained immunity phenotype with long-term modulation of immune responsiveness. Elucidating the cellular mechanisms underlying propofol-induced immunomodulation could offer novel targets for pharmacotherapy.

Methods

Reagents and inhibitors

Clinical formulation of propofol (Fresenius Kabi GmbH, Austria) was used in all experiments. Fatty acid oxidation inhibitor etomoxir (ETO), glycolysis inhibitor 2-deoxyglucose (2-DG) and ATP synthase inhibitor oligomycin (OLI) were purchased from Sigma. 2,6-diisopropylphenol was purchased from Sigma (cat. number D126608) and dissolved in DMSO. Palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1; all from Sigma) were dissolved in 100% ethanol and conjugated with human albumin (Albuman 200g/L, Sanquin) as described previously (31). Ultra-pure lipopolysaccharide from *E. coli* (LPS, serotype O55:B5; Sigma) was used for stimulation experiments.

Isolation of primary human immune cells

Buffy coats from healthy volunteers were obtained after written informed consent (Sanquin Blood Bank, Nijmegen, the Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated by means of Ficoll-Paque (GE Healthcare) density gradient centrifugation, after which the monocyte fraction was further enriched with hyper-osmotic Percoll gradient (Sigma). Percoll monocytes (1x10^5 cells/well) were seeded in a flat-bottom 96-wells plate for 1 hour at 37°C, 5% CO_2. Remaining

lymphocytes and non-adherent monocytes were removed by washing with warm PBS. Cells were cultured in Dutch Modified RPMI 1640 (Life Technologies) supplemented with 50µg/mL gentamicin (except for microbicidal experiments; Centrafarm), 2mM GlutaMAX (Life Technologies), 1mM pyruvate (Life Technologies), and 10% pooled human serum, referred to as medium.

Stimulation experiments and trained immunity experiments in adherent monocytes

Adherent Percoll monocytes were pre-incubated with propofol (25-200µM) for up to 6 hours, then stimulated for 24 hours with LPS (10ng/mL). Cell viability was assessed using CytoTox 96 non-radioactive cytotoxicity assay (Promega), measuring cell-death mediated release of lactate dehydrogenase (LDH). Supernatants were collected and stored at -20°C until analysis.

For trained immunity experiments, monocytes were incubated for 24 hours in medium (negative control), 1µg/mL β-glucan (positive control), and various concentrations of propofol (25-100µM). In inhibition experiments, cells were pre-treated for 30 minutes with oligomycin (1µM), etomoxir (100µM), 2-DG (1µM), or with medium or medium + DMSO as vehicle control, after which they were stimulated as described above. After initial stimulation, cells were washed with PBS, and incubated in medium for 5 days. These monocyte-derived macrophages were subsequently restimulated with medium alone or LPS (10ng/mL), according to the established *in vitro* model of trained immunity (32,33). When oligomycin (1µM), etomoxir (10µM), 2-DG (1µM), or palmitic acid (25µM) were used in combination with LPS restimulation on day 6, these compounds were added 30 minutes before LPS. Supernatants were collected and stored at -20°C until analysis.

Cytokine and lactate measurements

Enzyme-linked immunosorbent assays were performed following the manufacturer's protocols for measuring cytokines in supernatants after 24 hour stimulation with LPS. The concentrations of tumor necrosis factor (TNF), interleukin-6 (IL-6), and IL-1β were measured in supernatants. Additionally, IL-1β (R&D Systems) was measured after lysis of cells with 0.5% triton-x, to determine intracellular levels. Lactate was measured in supernatants from 24 hour LPS stimulation as described previously (34). Briefly, a lactate oxidase (Sigma) based enzymatic reaction with horseradish peroxidase (Sigma), and Amplex Red reagent (Life Technologies) was incubated for 20 minutes. The fluorescence of resorufin (excitation/emission of 570/585nm) was then measured on a 96-well plate reader (Biotek).

Reactive oxygen species measurements

For measurement of ROS production, a luminol-based luminescence assay was used. Primary monocytes or trained monocyte-derived-macrophages were added to a white 96-well assay plate (Corning) and stimulated with PMA (phorbol-12-myristate-13-acetate, 100nM; Sigma). After addition of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; 100μM; Sigma), luminescence was measured for 1 hour. Primary monocytes (1x10^5/well) were pre-incubated with propofol (50-100μM) for up to 24 hours, then used for ROS production measurement. Trained monocyte-derived-macrophages (day 6, 3x10^5/well) were directly restimulated with PMA and used for ROS production assay.

Flowcytometric analysis of monocyte mtROS

Primary monocytes or trained monocyte-derived-macrophages were stained with anti-human CD45-APC (clone HI30; Biolegend), anti-human CD14-Pacific Blue (clone RMO52; Beckman Coulter), and anti-human CD16-FITC (clone 3G8; Beckman Coulter), and washed once. Cells were incubated with 5μM MitoSOX Red Mitochondrial Superoxide Indicator (Life Technologies) in HBSS with 1mM Calcium for 20 minutes at room temperature, then measured on a CytoFLEX flowcytometer (Beckman Coulter). Data were analyzed using FlowJo (v10.8.1). Representative gating strategy is detailed in supplementary figure 3A. Briefly, after exclusion of debris and doublet events, CD45+ cells were classified as monocytes based on expression of CD14 and CD16. Geometric mean fluorescence intensity (gMFI) of MitoSOX dye was determined for total monocyte population, as well as for CD14+/CD16-, CD14+/CD16+, and CD14-/CD16+ monocyte subsets.

Extracellular flux measurements

Real-time oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of monocytes and trained monocyte-derived-macrophages were evaluated using an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience). 1x10^5 Primary monocytes or 3x10^5 trained monocyte-derived-macrophages were added per well to overnight-calibrated cartridges and incubated in assay medium (DMEM supplemented with 2mM L-glutamine, 11mM D-glucose and 1mmol/L pyruvate; pH adjusted to 7.4) for 1 hour in a non-CO_2 regulated incubator at 37°C. OCR and ECAR were measured using a Mitochondrial Stress test, with final concentrations of 1μM oligomycin, 1μM carbonyl cyanide-4-(tri-fluoromethoxy) phenylhydrazone (FCCP), and 0.5μM rotenone/antimycin A. For the modified Mitochondrial Stress test, 4μM etomoxir was added first, after which the standard sequence of inhibitors was injected.

RNA isolation and qRT-PCR

Trained cells were cultured as described above. On day 6, after 4 hours of LPS stimulation, total mRNA was extracted and purified using the RNeasy mini kit (Qiagen). Reverse transcription into cDNA was performed using iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was done using a SYBR Green PCR master mix (Life Technologies). Primer sequences used here are presented in Supplementary table 1. Primer pairs were intron-spanning. Analysis was performed using the $2^{-\Delta\Delta Ct}$ method, normalized against endogenous control gene *HPRT*.

Microbicidal activity assay

Propofol-trained day 6 macrophages were cultured for 4 hours in the presence of live *Escherichia coli* (*E. coli*; ATCC35218), live *Staphylococcus aureus* (*S. aureus*; ATCC25923), or live *Pseudomonas aeruginosa* (*P. aeruginosa*; ATCC27853) with a MOI of 2:1 in RPMI supplemented with 10% pooled human serum. After 4 hours, samples were diluted 100x in sterile H_2O and counted using a CASY cell counter with a range of 0.69 - 4.00µM to determine concentrations of bacteria. The percentage of killing was calculated from samples of bacteria cultured for 4 hours without macrophages co-culture.

Statistical analysis

All experiments were performed in a minimum of n=6, number of donors is stated in figure legends. To compare between groups, we used repeated measures one-way or two-way ANOVA, where appropriate. P-values of < 0.05 were considered statistically significant. Statistical analysis was performed using Graphpad Prism 5.03. Data are presented as mean ± SEM unless stated otherwise.

Results

Propofol acutely induces inflammatory cytokine production and augments cell metabolism in primary human monocytes

Upon LPS stimulation, the production of pro-inflammatory cytokines TNF, IL-6, and IL-1β increased significantly in adherent monocytes pre-incubated with propofol in a dose-dependent manner (fig. 1A-C). A key feature of activated immune cells is an augmentation of cellular metabolism following stimulation (35). Therefore, we sought to determine whether propofol treatment alters the metabolism of adherent monocytes. At higher concentrations, propofol seemed to suppress maximal oxygen consumption rate (OCR; fig. 1E) and spare respiratory capacity (SRC; fig. 1F) after 6 hour pre-incubation, but did not affect basal OCR (fig. 1D). Similarly, basal and

maximal extra-cellular acidification rate were diminished at higher concentrations of propofol after 6 hour pre-incubation (fig. 1G-H). ATP production was significantly decreased at 4 hour per-incubation, but was not consistently suppressed (fig. 1I). Incubation with concentrations up to 100μM propofol was not cytotoxic, as indicated by LDH cytotoxicity assay, therefore these concentrations were used in further experiments (supplementary fig. 1A).

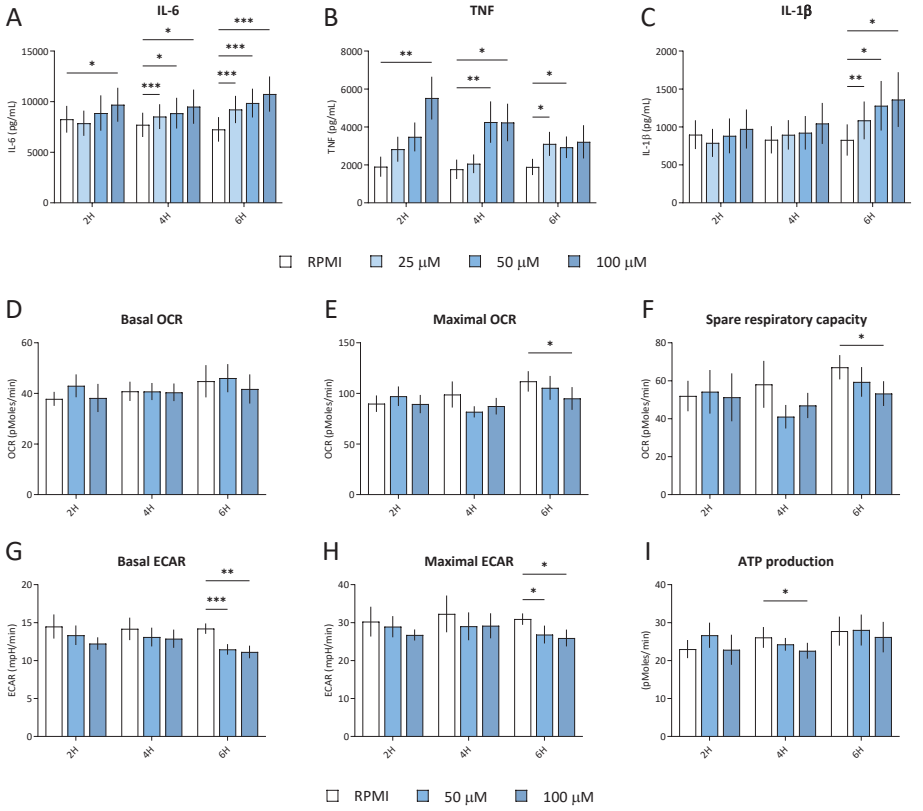

Figure 1. Propofol acutely enhanced cytokine production and attenuated cell metabolism in monocytes. Monocytes derived from healthy donors were exposed to propofol (25-100μM) for up to 6 hours, after which cells were stimulated with LPS for TNF (A), IL-6 (B), and IL-1β (C) measurement (n=9), or used in mitochondrial stress test (D-I). OCR stands for oxygen consumption rate, ECAR for extra-cellular acidification rate (n=6, mean ± SEM, * p<0.05, ** p<0.01, two-way ANOVA, compared to RPMI control).

Transient exposure to propofol induces trained immunity in human monocytes

Utilizing the established model for *in vitro* trained innate immunity, we explored the effects of propofol on monocyte training (Figure 2A). 24 hour incubation with propofol followed by wash-out and resting for 5 days in culture medium, induced a dose-dependent increase in the production of pro-inflammatory cytokines TNF, IL-6, and IL-1β upon day 6 re-stimulation with the TLR4 ligand LPS (fig. 2C-E), as well as a significant increase in expression of the corresponding genes *TNFA*, *IL6*, and *IL1B* (fig. 2B). Propofol also altered day 6 gene expression of *HIF1A* and *MTOR*, but not *NLRP3* (fig. 2B). A significantly increased cytokine production was observed in TNF, IL-6, and IL-1β production for propofol concentrations of 50 and 100μM. Based on these results, subsequent trained immunity experiments were performed using these concentrations. Day 6 propofol-trained cells also exhibited higher basal and maximal OCR, SRC and ATP production (fig. 2F-I). No consistent effects of propofol-induced trained immunity on glycolytic metabolism was seen at day 6 (supplementary figure 2).

Fatty acid metabolism drives propofol–induced trained immunity in monocytes

The role of various metabolic pathways in the enhanced cytokine responsiveness seen in propofol-trained macrophages was investigated by inhibition of oxidative phosphorylation (OXPHOS) with oligomycin (OLI), fatty acid oxidation by etomoxir (ETO), and glycolysis by 2-deoxyglucose (2-DG), both during initial propofol stimulation (fig. 3A) and during LPS re-stimulation (fig. 3B). These inhibitors were not found to be cytotoxic (supplementary fig. 2F). In terms of TNF production upon LPS re-stimulation at day 6, etomoxir seemed to partially abolish induction of propofol training when present during the initial 24 hour propofol exposure. When present during secondary LPS stimulation, most inhibitors showed a trend to lowered propofol training, but only etomoxir significantly reduced TNF production.

The role of fatty acid metabolism in the long-term pro-inflammatory effects of propofol was further explored using a modified mitochondrial stress test on the Seahorse XF analyzer, with subsequent injections of etomoxir, oligomycin, FCCP and rotenone/antimycin A. As seen previously, day 6 propofol-trained cells exhibited higher basal OCR. Upon injection of etomoxir, the basal OCR did not change for RPMI cells, but was significantly lowered in the 50 and 100μM propofol trained cells (fig. 3C).

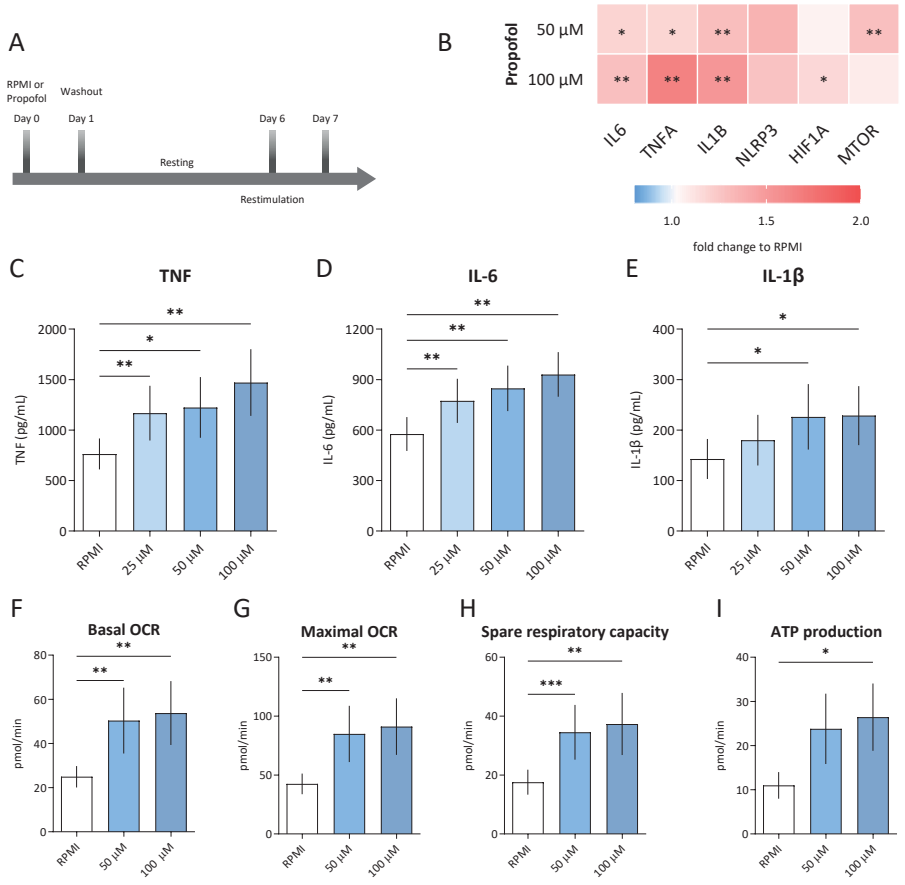

Figure 2. Transient exposure to propofol induced trained immunity in primary human monocytes. Schematic of *in vitro* trained immunity setup (A). Monocytes derived from healthy individuals were incubated with propofol (25-100µM) for 24 hours, after which cells were washed and rested for 5 days. Cells were re-stimulated on day 6 with LPS, and relative mRNA expression (B) was determined in 4 hour stimulated cells (n=10). TNF (C), IL-6 (D), and intracellular IL-1β (E) were measured in 24 hour LPS stimulated cell supernatants (n=12). Basal oxygen consumption rate (F), maximal oxygen consumption rate (G), spare respiratory capacity (H), and ATP production (I) were determined in a mitochondrial stress test (n=16, mean ± SEM, * p<0.05, ** p<0.01, *** p<0.001, one-way ANOVA, compared to RPMI control).

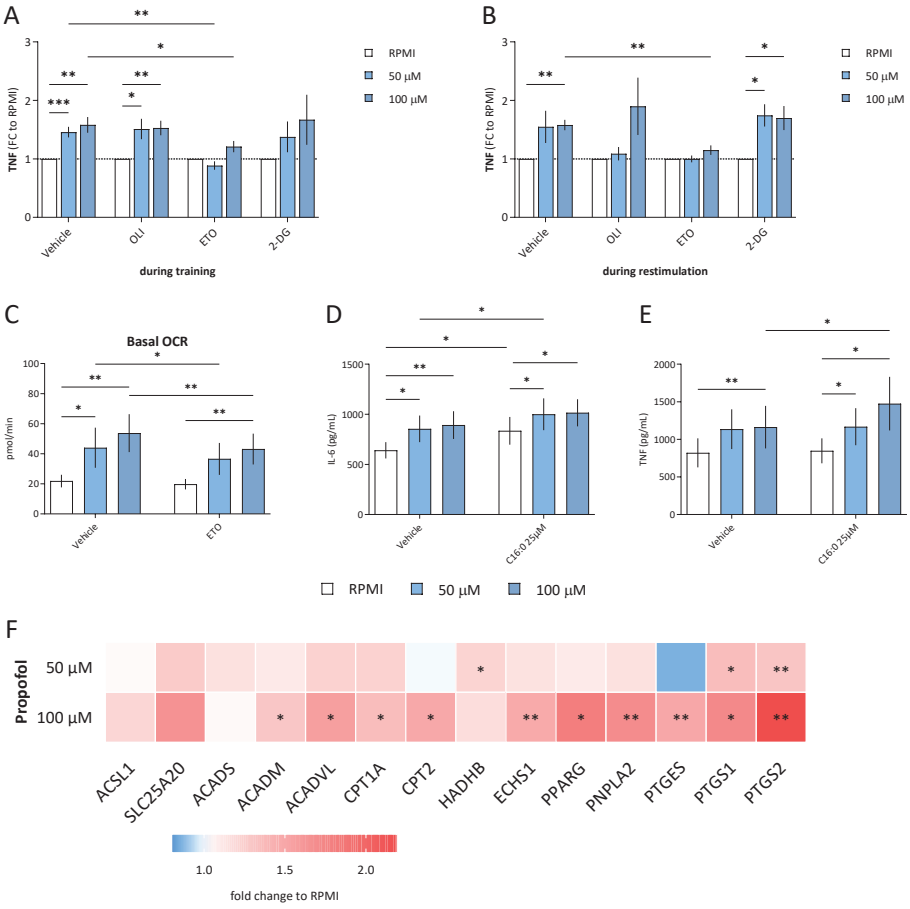

Figure 3. Role of fatty acid metabolism in propofol-induced trained immunity. Monocytes derived from healthy individuals were pre-incubated with oligomycin (OLI), etomoxir (ETO), 2-deoxy glucose (2-DG), or vehicle control (DMSO), prior to incubation with propofol (A) or prior to secondary LPS stimulation on day 6 (B, n=9, 2-way ANOVA). The effects of inhibition of fatty acid oxidation on trained macrophages was further investigated by injection of etomoxir (ETO) (C) (n=10, 2-way ANOVA) during measurement of oxygen consumption rate. Additionally, propofol-trained cells were supplemented with palmitate (C16:0) or vehicle control (D, E) prior to secondary stimulation with LPS. TNF and IL-6 were measured in 24 hour supernatants (n=9, 2-way ANOVA). Relative mRNA expression (F) of fatty acid metabolism related genes was determined in 4 hour LPS stimulated cells (n=10, one-way ANOVA; mean ± SEM, * p<0.05, ** p<0.01, *** p<0.001, compared to RPMI control).

We further assessed the role of fatty acid oxidation by supplementation of palmitate (C16:0) prior to restimulation with LPS (fig. 3D-E). Our results indicate that palmitate boosted IL-6 production upon LPS stimulation in all conditions, and significantly increases TNF production capacity in 100μM propofol-trained cells. Furthermore, propofol-treated cells significantly upregulated mRNA expression of fatty acid metabolism genes, including enzymes conjugating long-chain fatty acids to carnitine (*CPT1A*, *CPT2*), and genes encoding β-oxidation catalysts acyl-CoA dehydrogenases (*ACADM*, *ACADVL*), enoyl-CoA hydratase (*ECHS1*), and β-ketothiolase (*HADHB*). In addition, genes regulating fatty acid storage (*PPARG*) and adipose triglyceride lipase (ATGL; *PNPLA2*), a lipid-droplet associated protein responsible for the degradation of triglycerides in macrophages, were significantly upregulated in propofol trained macrophages (fig. 3F). We also observed increased transcription of *PTGES*, *PTGS1* (COX-1), and *PTGS2* (COX-2), genes involved in the biosynthesis of prostaglandins.

Previous research has suggested a role for the lipid components in clinical formulations of propofol in the immunomodulatory effects (30). We observed that propofol, when dissolved in DMSO instead of lipid vehicle, was capable of inducing trained immunity to a similar degree (supplementary fig. 1B-C). We also determined that the primary fatty acids in propofol, namely palmitic acid and stearic acid, but not oleic acid, can independently induce trained immunity in high concentrations (supplementary fig. 1D-E).

Propofol-induced trained immunity heightens microbicidal responses

To assess the potential effects of propofol-induced trained immunity on host-defense, the *in vitro* killing of *S. aureus*, *P. aeruginosa*, and *E. coli* bacteria was determined by incubating day 6 trained macrophages with live bacteria for 4 hours, after which remaining colony forming units (CFU) were counted. The growth of all 3 bacteria was significantly reduced when cultured with propofol-trained macrophages, as compared to RPMI macrophages (fig. 4A-C).

The underlying functional phenotype of propofol-trained macrophages was further investigated by measurement of oxidative burst upon PMA stimulation at various timepoints. A significant reduction in ROS production capacity was seen after 2 and 24 hours pre-incubation, as well as at day 6 (fig. 4D). Monocyte subsets were not significantly affected by propofol treatment (supplementary fig. 3B). In the total monocyte population, mtROS levels were significantly affected by propofol treatment, showing increased production after 2 hours of pre-incubation with propofol, and at

day 6 (4E). No significant changes in mRNA levels of ROS contributing genes such as *NOX2*, *NOX4*, and *NOS2* were observed, though the expression of antioxidant glutathione synthetase (*GSS*) mRNA was significantly upregulated. Propofol trained cells expressed higher levels of antimicrobial peptides cathelicidin (*CAMP*) and lysozyme (*LYZ*) mRNA, but not β-defensins (*DEFB1-4*) (fig. 4F).

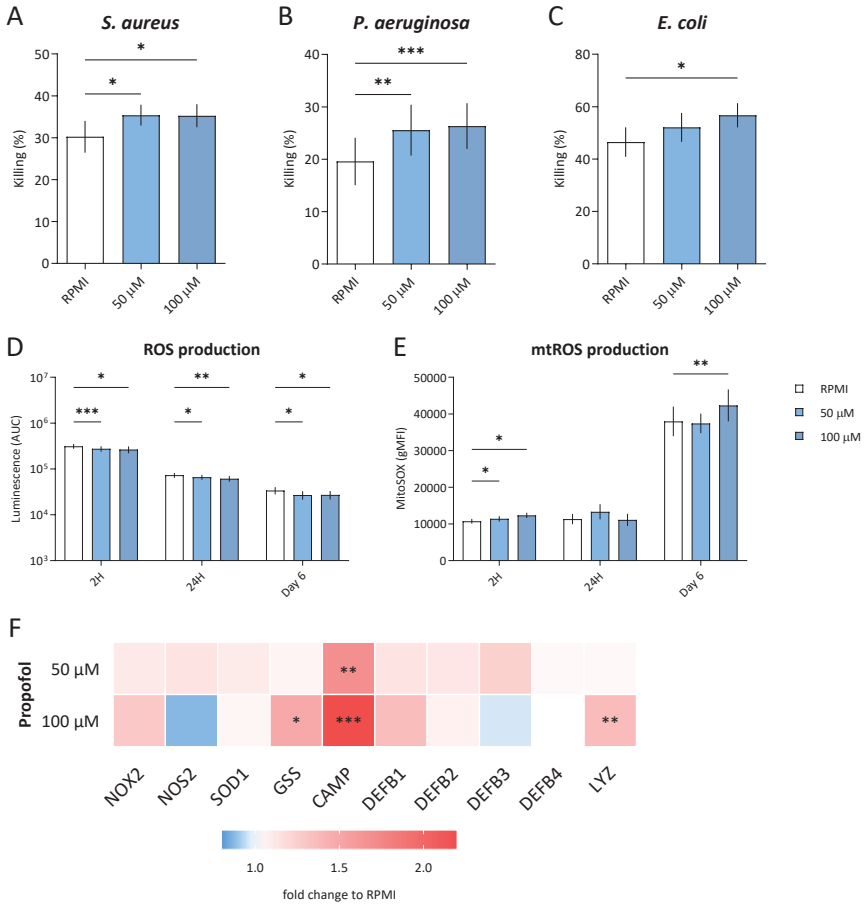

Figure 4. Propofol induced trained immunity protects against infection *in vitro*. Monocytes were trained with propofol (50-100µM) as described above. *S. aureus* (A), *P. aeruginosa* (B) and *E. coli* (C) growth in the presence of day 6 RPMI or propofol trained macrophages was determined after 4 hour co-culture with an MOI of 2:1 (n=9, mean ± SEM, * p<0.05, ** p<0.01 , one-way ANOVA, compared to RPMI control). ROS production capacity (D) was assessed in monocytes pre-incubated with propofol for 2 hours (n=6), 24 hours (n=8), and in day 6 trained macrophages (n=6) by stimulation with PMA (mean ± SEM, * p<0.05, ** p<0.01, two-way ANOVA, compared to RPMI control). Mitochondrial ROS (mtROS) production was determined at 2 hours, 24 hours, and day 6 (all n=9) by staining with mitoSOX dye (E). Relative mRNA expression (F) of oxidative stress and microbicidal molecule genes was determined in 4 hour LPS stimulated cells (n=10, mean ± SEM, * p<0.05, ** p<0.01, one-way ANOVA, compared to RPMI control).

Discussion

General anesthesia may modulate the immune response in the perioperative period, which could be a crucial factor in maintaining homeostasis upon surgical stress and inflammation. Dysregulation of the inflammatory processes surrounding surgery could affect wound healing, susceptibility to infections, and overall patient recovery. This study explored the immunomodulatory effects of propofol, one of the most commonly used anesthetics, both in acute and long-term settings. Previous publications have shown conflicting data on the pro- and anti-inflammatory effects of propofol (2-5). The bulk of research on the immunomodulatory effects of propofol has been conducted in polymorphonuclear leukocytes (PMNs), with limited studies done in monocytes and macrophages.

In this study, we describe that pre-incubation with propofol acutely increases the release of pro-inflammatory cytokines by monocytes in both a time and dose-dependent manner upon stimulation with LPS. This pro-inflammatory effect of propofol on monocyte-derived cytokines is of interest, since previous literature establishes the anti-inflammatory effects on PMNs. When investigating monocyte oxidative burst, we did observe a reduction in ROS production capacity after propofol treatment, similar to previously published data in neutrophils (6).

We explored the acute effects of propofol on monocyte metabolism, which demonstrated that high concentrations of propofol did not affect the basal OCR, but suppressed the maximal OCR and spare respiratory capacity (SRC) after 6 hours of pre-incubation. In addition, the ECAR, an indicator of glycolytic activity, was also reduced after 6 hour incubation with propofol. ATP production was not significantly affected by propofol pre-incubation, though similar trends towards decreasing production rates were observed. Defective production of ATP is one of the proposed mechanisms underlying propofol infusion syndrome (PRIS) (36). Whilst the exact mechanisms remain unclear, our data support the hypothesis that propofol acutely suppresses monocyte metabolism.

Our investigation into the long-term effects of propofol treatment on monocytes revealed that short-term incubation with propofol increased the production of pro-inflammatory cytokines upon day 6 restimulation with LPS. A closer examination of the metabolic adaptations of propofol-treated cells showed that day 6 monocyte-derived macrophages were profoundly affected by transient exposure to propofol. Oxygen consumption rates, ATP production, and SRC were all increased on day 6. In addition, we observed increased ECAR and higher lactate production in propofol-

treated macrophages. This increase in cell metabolism at day 6 is a complete reversal from the acute suppressing effects of propofol on monocyte metabolism we initially observed, indicating there is a possible compensatory mechanism following acute propofol exposure. The link between macrophage metabolic profiles and immunological activation and functioning is well established in literature. Pro-inflammatory macrophages are generally considered to be more reliant on glycolytic pathways, whereas pro-resolving (anti-inflammatory) macrophages are associated with oxidative phosphorylation and fatty acid oxidation pathways (37). We find that the metabolic phenotype of propofol-treated macrophages does not conform to the traditional M1/M2 categorization, instead more closely resembling the rewiring of metabolic pathways that define trained immunity (21).

Metabolic inhibitors were selected to establish which metabolic pathways contribute to this trained phenotype induced by propofol treatment. Etomoxir, when applied both during the initial propofol challenge, and during the secondary LPS challenge, inhibited trained immunity in our model, and reduced the increase in OCR induced by propofol. Etomoxir is an inhibitor of fatty acid metabolism, binding to CPT1, thereby preventing fatty acid β-oxidation in the mitochondria (38). Supplementation with palmitic acid significantly increased TNF production by propofol-trained macrophages, further indicating that fatty acid metabolism is essential in the induction of trained immunity by propofol, and for the resulting increase in pro-inflammatory cytokine production. Supportive evidence for the role of fatty acid metabolism in propofol comes from qPCR analysis of fatty acid oxidation-associated genes *ACADM*, *ACADVL*, *CPT1A*, *CPT2*, *HADHB*, *PPARG*, and *PNPLA2 (*ATGL), showing increased expression after propofol treatment on day 6. In addition, we saw upregulation of *PTGES*, *PTGS1* (COX-1), and *PTGS2* (COX-2) in propofol-trained macrophages, possibly indicating increased prostaglandin biosynthesis. Prostaglandins are associated with proinflammatory activity, enhancing both innate and adaptive immune functions, though under certain conditions they also contribute to tissue repair (39).

Under inflammatory conditions, macrophages convert fatty acids to triglycerides, which are subsequently stored in lipid droplets, or to acylcarnitine through CPT1 in the outer mitochondrial membrane (40). A study by Wolf *et al.* (41) demonstrated that propofol increases production of malonylcarnitine, an inhibitor of CPT1, leading to specific disruption of fatty-acid oxidation in the mitochondria. Inhibition of CPT1 might lead to increased lipid droplet formation in propofol treated cells, promoting the inflammatory capacity of macrophages (42), which corresponds to the increased cytokine responsiveness we observed. This CPT1 inhibitory

mechanism leads to accumulation of fatty acids in mitochondria, which is exacerbated by the presence of additional fatty acids in the clinical formulation of propofol. This process ultimately leads to disruption of the ETC and a defect in the production of ATP, a process that is thought to contribute to the pathophysiology of propofol infusion syndrome (36).

We assessed mitochondrial function during acute propofol exposure, and 6 days after. We found that mitochondrial reactive oxygen species (mtROS) were increased in monocytes after 2 hours of propofol exposure, as well as in day 6 monocyte-derived macrophages. Previous studies have reported conflicting data on the effects of propofol on mitochondrial ROS. Propofol inhibited ROS production and mitochondrial depolarization, conferring neuroprotective benefits during ischemia-reperfusion injury in a mouse model (43). Conversely, a study by Sumi *et al.* (14) showed that propofol induced ROS generation, while suppressing oxygen metabolism and boosting glycolysis in a neuroblastoma cell line. These studies were conducted after short-term exposure to propofol in cell lines. To our knowledge, this is the first study reporting on the long-term effects of propofol on cellular metabolism and function. Monocytes and macrophages are characterized by their high plasticity and heterogeneity, covering a wide array of functions and phenotypes in tissues depending on the environmental stimuli they encounter (44,45). It is therefore feasible that mtROS induced by transient exposure to propofol could initiate compensatory mechanisms that lead to mitochondrial adaptations resulting in long-term increases in mtROS production. Controlled production of mtROS is required for induction of immune responses, while excessive production of mtROS may lead to mitochondrial damage and cell death (46). Although we did not observe any significant propofol-induced cytotoxicity, even with the supraclinical concentrations used in this study, more research is needed to fully characterize the contribution of this long-term elevation of mtROS after propofol treatment to clinical manifestations of propofol toxicity.

Opportunistic infections are a life threatening condition following surgery. With the clear immune stimulatory effects of propofol on monocytes, we hypothesized that propofol could skew monocyte-derived-macrophages to a pathogen controlling phenotype. We observed profound changes in the antimicrobial response of propofol-trained macrophages, which was assessed by microbial killing assays with *S. aureus*, *P. aeruginosa*, and *E. coli* bacteria, three pathogens frequently associated with hospital acquired infections (47). The growth of all 3 bacteria was significantly reduced when co-cultured with propofol-trained macrophages. This enhanced killing capacity was likely not ROS-dependent, as we observed that propofol-trained cells

produced fewer ROS than control cells after 6 days of culture. Previous research has shown that trained immunity induced by β-glucan (48), oxLDL (49), or BCG (32), leads to an increase of ROS release. We observed higher levels of gene expression in propofol trained cells for cathelicidin antimicrobial peptide (*CAMP*). Production of cathelicidin, which exhibits both direct antimicrobial activities, but also influences and modulates the TLR-mediated responses of monocytes and macrophages, could contribute to enhance killing capacity of propofol-treated cells (50).

The conclusions in this study regarding the immune effects of propofol have been derived from *in vitro* experiments, which limits one-to-one translational potential of the results. Human clinical studies are complex due to their integration of many confounding variables, including type of surgery, patient characteristics, influence of other medications, and possible surgical complications (51). It is therefore challenging to distill the effects of propofol alone on the patient's immune system in an *in vivo* setting. The effects of medications, such as anesthetics, opiates, NSAID's, and corticosteroids, as well as the influence of perioperative factors like surgical tissue injury, mobilization, stress, and pain should be further investigated for their individual and cumulative contributions to postoperative immune function.

Although the immunological effects of propofol could be considered to be of minor consequence in immunocompetent patients, the modulation of immune cell metabolism and effector functions described in this study may be of particular relevance in immunocompromised patients. Aging, comorbidities such as diabetes mellitus or cancer, acute immune dysregulation following trauma or sepsis, malnutrition, the use of immunosuppressive medicines, or genetic predispositions, may all contribute to immune impairment which could be worsened or improved by immunomodulatory anesthetics (51). As such, assessment of the immune status of individual patients could be valuable for appropriate selection of anesthetic agents.

Conclusion

This study characterizes the long-term immunomodulatory effects of propofol on the innate immune response. We demonstrate that monocyte-derived-macrophages that have been exposed to propofol gain a long-term pro-inflammatory phenotype, which is characterized by increased cytokine production and altered cellular metabolism. We explore the role of fatty acid oxidation as an important mechanism for trained immunity induced by propofol. Propofol-trained macrophages exhibit enhanced antimicrobial functions that conform to the trained

immunity phenotype. It remains to be investigated to what extent the results reported here reflect propofol activity *in vivo*, and whether the effects on clinical outcomes can be attributed to the same mechanisms described here.

Acknowledgements

LH, JvH, and LG conceived and designed the experiments. LH and JvH performed the experiments. LH analyzed the results and wrote the manuscript. All authors critically read the manuscript. GJS and LJ share senior authorship.

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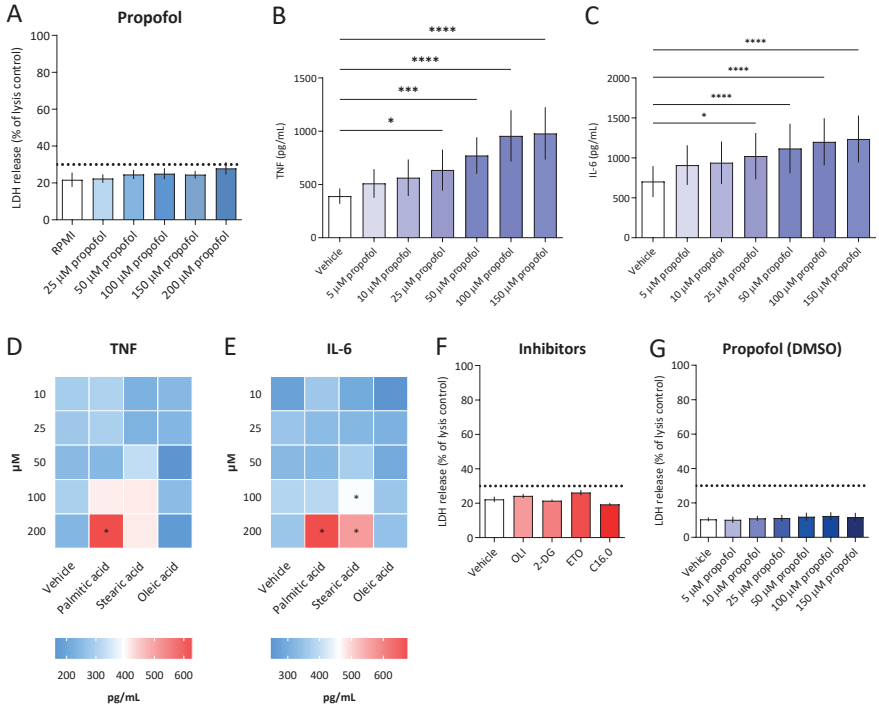
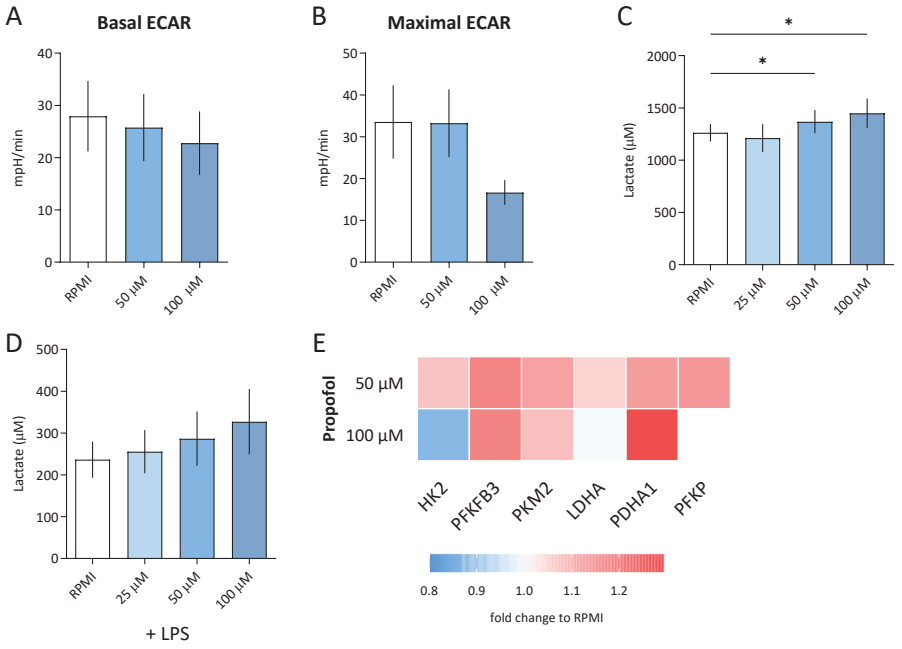
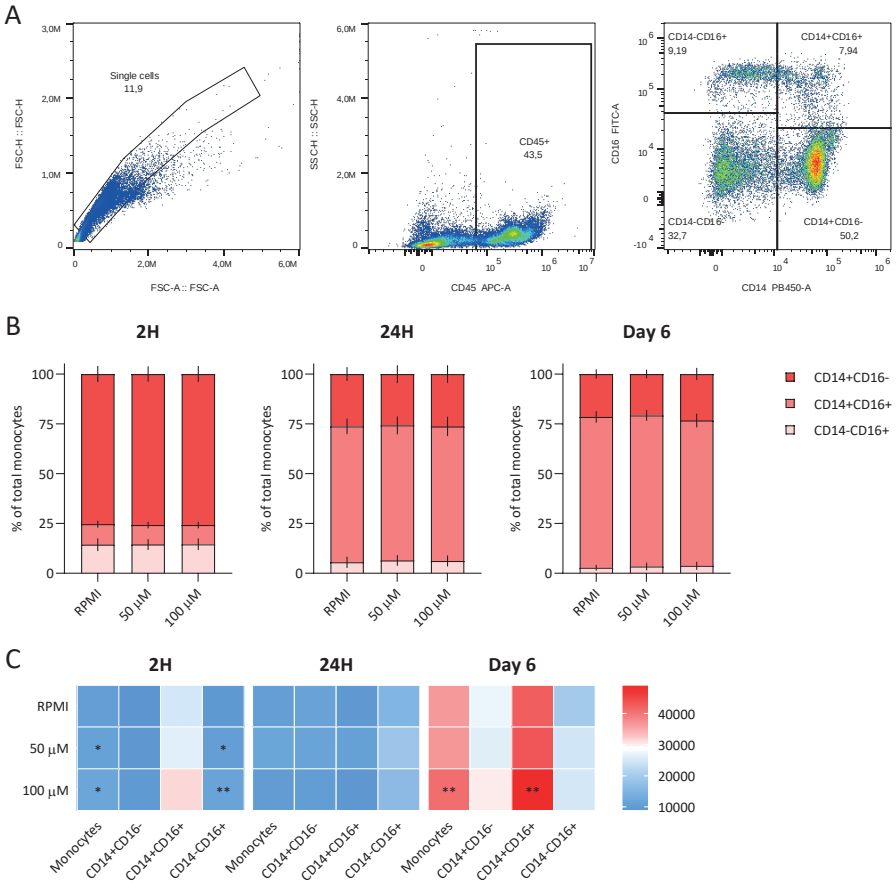
Supplemental material



Supplementary figure S1. Cytotoxicity of propofol and inhibitors. Lactate dehydrogenase (LDH) levels were measured in supernatant of monocytes treated with propofol (25-200µM), for 24 hours (A). Relative LDH release to positive control (lysed monocytes) was calculated (n=6). Induction of trained immunity with 2,6-diisopropylphenol dissolved in DMSO in terms of TNF (B) and IL-6 (C) production (n=9). Induction of trained immunity with fatty acids palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1) in doses ranging from 10-200µM (D-E; n=9). Cytotoxicity of inhibitors used (F) lactate dehydrogenase levels in supernatant from monocytes treated with oligomycin (1µM), 2-DG (1mM), etomoxir (100µM) C16:0 (25µM), or vehicle control for 24 hours (n=3). Cytotoxicity of propofol 2,6-diisopropylphenol dissolved in DMSO (G; n=6). Mean ± SEM, * p<0.05, ** p<0.01, *** p<0.001, one-way ANOVA, compared to vehicle control.



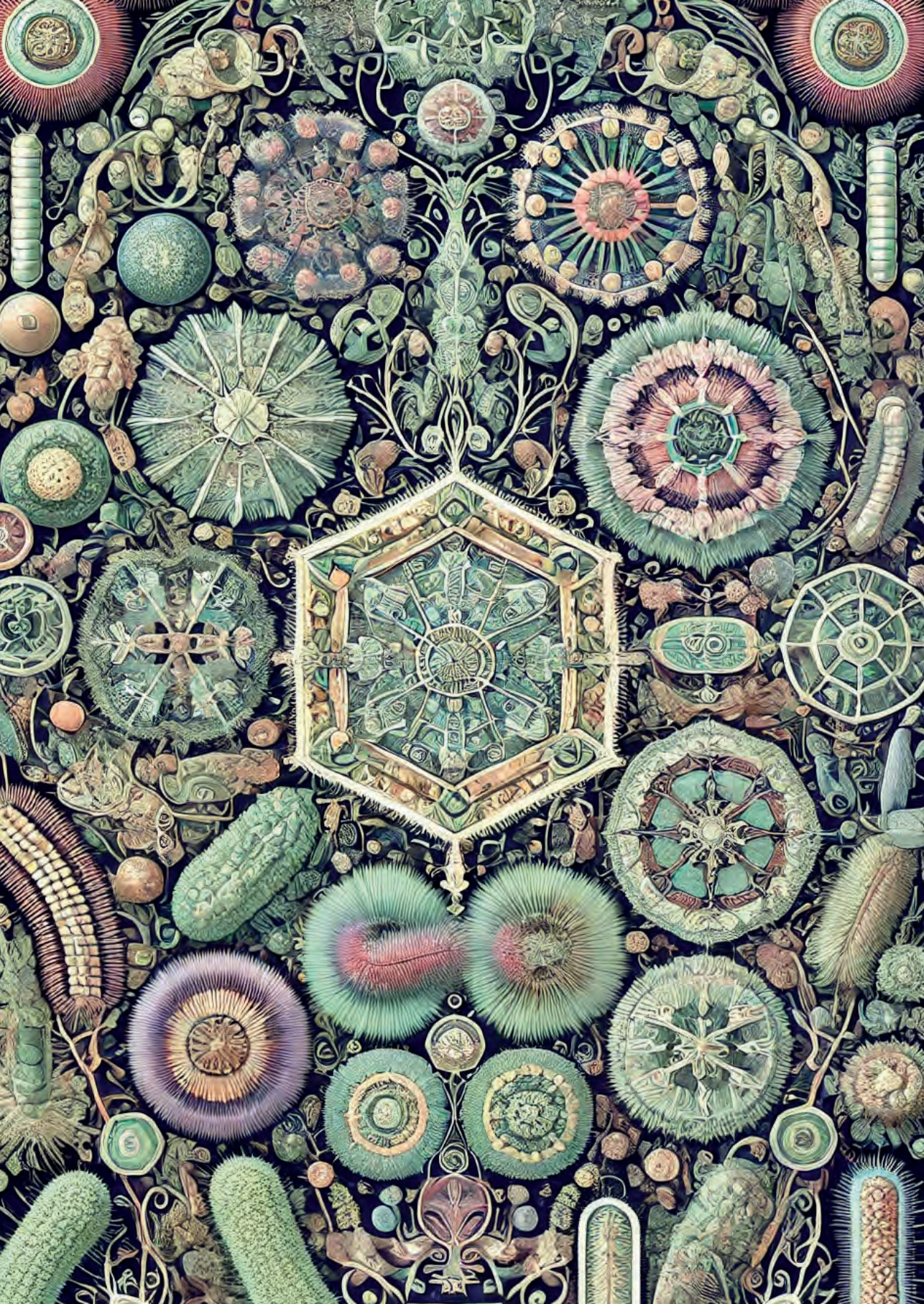
Supplementary figure S2. Role of glycolysis in propofol-induced trained immunity. Monocytes were trained with propofol (25-100µM) as described above. On day 6, basal extracellular acidification rate (A) and maximal extracellular acidification rate (B) were determined in a mitochondrial stress test (n=16). Concentrations of lactate were measured in day 6 supernatants prior to restimulation with LPS (C) (n=10) and in 24 hour LPS stimulated samples (D) (n=12, mean ± SEM, * $p<0.05$, ** $p<0.01$, one-way ANOVA, compared to RPMI control). Relative mRNA expression (E) of glycolytic genes was determined in 4 hour LPS stimulated cells (n=10, mean ± SEM, one-way ANOVA, compared to RPMI control).



Supplementary figure S3. Flowcytometric analysis of propofol-treated monocytes and macrophages. Monocytes were trained with propofol (50-100µM) or RPMI control, and stained with mitoSOX dye to evaluate mitochondrial ROS (mtROS) production after 2 hours, 24 hours, and at day 6. Representative gating strategy for flowcytometry analysis of mitoSOX in monocyte subsets (A). Propofol did not affect monocyte subsets (B) at any timepoint measured. Propofol increased mtROS production in total monocyte population, and in CD14-CD16+ subset after 2 hours pre-incubation (C). At day 6, mtROS production was increased in total monocyte population, and in CD14+CD14+ subset, following treatment with 100µM propofol (n=9, mean ± SEM, one-way ANOVA, compared to RPMI control).

Supplementary table 1. Primer sequences.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
HPRT	CCTGGCGTCGTGATTAGTGAT	AGACGTTCAGTCCTGTCCATAA
IL6	GGCACTGGCAGAAAACAACC	GCAAGTCTCCTCATTGAATCC
TNFA	TGTTGTAGCAAACCCTCAAGC	GAGGTACAGGCCCTCTGATG
IL1B	ACAGATGAAGTGCTCCTTCCAG	CATGGCCACAACAACTGACG
NLRP3	GATCTTCGCTGCGATCAACA	GGGATTCGAAACACGTGCATTA
HIF1A	CATAAAGTCTGCAACATGGAAGGT	ATTTGATGGGTGAGGAATGGGTT
MTOR	TCCGAGAGATGAGTCAAGAGG	CACCTTCCACTCCTATGAGGC
ACSL1	CTTCTGGTACGCCACGAGAC	GTCGCTGTCAAGTAGTGCG
SLC25A20	CGACCAGCCAAAACCCATCA	ATTCCCCGATATAGCCCCGT
ACADS	GGAACATCTCTTCCCAGCGG	TTCAAGATGGGCCCCAGGTA
ACADM	GGGAGAATGACTGAGGAGCC	TCTGGATCAGAACGTGCCAA
ACADVL	CTGGCACGGATGGTTATGCT	ATCTCGGAGCACACGCTCTA
CPT1A	CGGTTGCTGATGACGGCTAT	CCAGCAGCTCCAGTGGAATTA
CPT2	GAGCTTCAGCAGATGATGGTTG	CTCGTGGACAGGACATTGTG
HADHB	TGTTGCATCGGACCAGTGTC	CTGGCCAGAAGCAATCAAGC
ECHS1	ATCGCTGCTGTCAATGGCTA	GGTCACCAGTGAGGACCATC
PPARG	TTGCAGTGGGGATGTCTCAT	TTTCCTGTCAAGATCGCCCT
PNPLA2	GGTGGCATTTCAGACAACCTG	GTATCCCTGCTTGCACATCTC
PTGES	GATGCCCTGAGACACGGAG	AGGCGACAAAAGGGTTAGGA
PTGS1	CGCCAGTGAATCCCTGTTGTT	AAGGTGGCATTGACAAACTCC
PTGS2	CTGGCGCTCAGCCATACAG	CGCACTTATACTGGTCAAATCCC
NOS2	AATGTGGAGAAAGCCCCCTG	GGAGACTTCTTTCCCGTCTCC
SOD1	GGTGGGCCAAAGGATGAAGAG	CCACAAGCCAAACGACTTCC
NOX2	ACCGGGTTTATGATATTCCACCT	GATTTCGACAGACTGGCAAGA
NOX4	AAGCAGGAGAACCAGGAGATTG	AATAGCACCACCACCATGCAG
GSS	GAACCGTTCGCGGAGGAAA	GAATGGGGCATAGCTCACCA
CAMP	CGGATGCTAACCTCTACCGC	AGGGTCACTGTCCCCATACA
DEFB1	ATGAGAACTTCCTACCTTCTGCT	TCTGTAACAGGTGCCTTGAATTT
DEFB2	GGTGTTTTTGGTGGTATAGGCG	AGGGCAAAAGACTGGATGACA
DEFB3	TAGCAGCTATGAGGATCCA	CTTCGGCAGCATTTTCGG
DEFB4	CATCAGCCATGAGGGTCT	AGGCAGGTAACAGGATCG
LYZ	GGCCAAATGGGAGAGTGGTT	TTGTGGATCACGGACAACCC
HK2	TGCCACCAGACTAAACTAGACG	CCCGTGCCCACAATGAGAC
PFKFB3	ATTGCGGTTTTCGATGCCAC	GCCACAACTGTAGGGTCGT
PKM2	ATAACGCCTACATGGAAAAGTGT	TAAGCCCATCATCCACGTAGA
LDHA	CGTGTTATTGGAAGCGGTTG	TTCATTCCACTCCATACAGGC
PDHA1	TGGTAGCATCCCGTAATTTTGC	ATTCGGCGTACAGTCTGCATC
PFKP	CTCTCCCATCGCAGGGTAAC	GCCAGCTCCACGTACTGAAA



Chapter 6

Antifungal drug amphotericin B activates trained immunity, promoting heightened cytokine production capacity, metabolism, oxidative burst, and antimicrobial activity

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Abstract

Background. Amphotericin B is an antifungal drug originally derived from *Streptomyces nodosus*, active against clinically relevant yeasts and molds. Amphotericin B has been described to modulate innate immune cell function, though the long-term effects are not well understood. Monocytes may adopt a long-term pro-inflammatory phenotype after transient exposure to microbial pathogens, resulting in trained immunity. Trained immunity confers non-specific protection against secondary infections via a process dependent on metabolic and transcriptional reprogramming.

Methods. Using the established model of *in vitro* trained immunity, this study aimed to investigate whether amphotericin B can induce trained immunity in primary human monocytes, and to explore which host defense factors and inflammatory responses are affected by this process.

Results. Using human primary monocytes, we find that therapeutic concentrations of amphotericin B deoxycholate and liposomal amphotericin B induce trained immunity, resulting in potentiated cytokine and chemokine responses, and increased oxidative burst upon secondary stimulation. We also demonstrate augmented transcriptional accessibility and metabolic plasticity in amphotericin B trained cells. In addition, we show that amphotericin B trained immunity enhanced macrophage effector functions, including phagocytosis, antigen presentation, and pathogen killing, which are essential for host defense to infections. Using pharmacological inhibition experiments, it was revealed that amphotericin B induction of trained immunity was dependent on epigenetic regulation of gene transcription.

Conclusions. Amphotericin B induces trained immunity *in vitro* through epigenetic and metabolic reprogramming of monocytes, leading to functional adaptation of macrophages. These findings provide insight into a parallel mechanism by which amphotericin B contributes to host defense and infection clearance, in addition to its primary antifungal effects.

Introduction

Amphotericin B is an antifungal medication used for a wide range of fungal infections and leishmaniasis (1-4). First isolated from *Streptomyces nodosus*, amphotericin B interferes with fungal cell membranes by binding to ergosterol, causing pore formation and oxidative stress, leading to fungal cell death. Mammalian cell wall membranes contain cholesterol, which is structurally similar to ergosterol. As such, binding of amphotericin B to cholesterol in human cells can result in cytotoxicity under certain conditions (5,6). Due to its insolubility in saline, several formulations exist, including amphotericin B deoxycholate (AMB) and lipid-based formulations such as liposomal amphotericin B (AMB-L). Although there is no difference in efficacy between these formulations, liposomal variants appear to be better tolerated by patients (7,8).

Infusion of AMB is associated with innate immune production of pro-inflammatory cytokines, which is thought to contribute to side effects of the treatment (9). AMB is a microbial product and has been shown to stimulate immune cells via Toll-like receptor 2 (TLR2), TLR4, and cluster of differentiation 14 (CD14) (10,11). The resulting production of pro-inflammatory cytokines, chemokines, and other molecules such as prostaglandins and nitric oxide has been demonstrated both *in vitro* (12,13) and *in vivo* (14). Conversely, AMB has been demonstrated to inhibit the production of the anti-inflammatory cytokine interleukin-1 receptor antagonist (IL-1Ra) *in vitro* (15). These studies have demonstrated the acute pro-inflammatory effects of AMB; however, no data regarding the possible long-term effects of AMB on innate immune cells exist at present.

Recent literature describes that common antimicrobial medications, including AMB, are often contaminated with β-glucan particles (16,17). These trace amounts of β-glucan can interfere with commercially available diagnostic tests for serum levels of β-glucan (18,19). Amphotericin B deoxycholate reportedly contained 1315 ± 88.6pg/mL of β-glucan, with liposomal amphotericin B containing 712 ± 50.4pg/mL. β-glucan is a potent immunomodulatory compound, which binds to the dectin-1 receptor (20), enhances the functional activity of myeloid cells (21-24), and stimulates the production of pro-inflammatory molecules such as complement components (25,26), cytokines and chemokines (27,28), and eicosanoids (29). Consistent with these findings, β-glucans have also been shown to protect against or mitigate the deleterious effects of bacterial and other infections in animal models (30-32). In addition, emerging evidence has shown that β-glucan can induce long-term functional changes in innate immune cells, resulting in increased

responsiveness upon heterologous secondary stimulation and enhanced capacity to eliminate infection (33-35). This enhanced state of activation of innate immune cells is known as “trained immunity” and is a consequence of epigenetic and metabolic reprogramming (36-38).

We therefore hypothesized that exposure of monocytes to AMB could induce trained immunity, either through direct activation of inflammatory mechanisms or due to contamination with β-glucan. Trained immunity confers non-specific protection against infections (39-41) which could offer immunotherapeutic benefits to patients receiving AMB. This study explores if AMB can induce trained immunity and whether this provides increased responsiveness against subsequent infections, possibly leading to better protection.

Methods

Stimuli

Pharmaceutical grade, endotoxin-free amphotericin B deoxycholate (AMB; Fungizone, Bristol-Myers Squibb Co) and liposomal AMB (AMB-L; AmBisome, Fujisawa Healthcare) were commercially obtained and reconstituted in sterile water per the manufacturer’s instructions. To determine whether our preparations contained trace amounts of β-glucan, preparations of AMB were tested for their 1,3-β-D-glucan (BDG) content using the Fungitell assay (Associates of Cape Cod, MA, USA) according to the manufacturer’s instructions. In brief, 5µL of the preparation was pretreated with 20µL of alkaline reagent, then incubated with 100µL of Fungitell reagent at 37°C and monitored at 405nm kinetically for 40 minutes. BDG contamination was not detected in our AMB preparations using this assay (supplementary table 1).

Cells were cultured in Dutch Modified RPMI 1640 (Life Technologies) supplemented with 50µg/mL gentamicin (except for microbicidal experiments; Centrafarm), 2mM GlutaMAX (Life Technologies), 1mM pyruvate (Life Technologies), and 10% pooled human serum, referred to as medium, at 37°C, 5% CO_2. The fungal cell wall component β-glucan was used as a positive control, kindly provided by Professor David Williams (East Tennessee State University, USA). For pharmacological inhibition experiments, cells were pre-incubated with R406 (50nM; Sigma), GW5074 (1µM; Sigma), PF06650833 (100nM; Sigma), diclofenac (6.5µg/mL; commercially obtained), 5'-deoxy-5'(methylthio) adenosine (MTA, 1mM; Sigma), cyproheptadine (CPH, 200µM; Selleckchem), zaragozic acid A (ZA, 5µM; Santacruz Biotechnology) or

fluvastatin sodium hydrate (FS, 20μM; Sigma) for 1 hour prior to stimulation with β-glucan or AMB. To test AMB preparations for LPS contamination, stimuli were pre-incubated with polymyxin B (2μg/mL; Merck) for 2 hours before adding to cells. For cytokine, lactate, and gene expression transcription assays, cells were stimulated with *E. coli* LPS (10ng/ml, serotype O55:B5; Sigma).

In vitro trained immunity model

We utilized the established model of *in vitro* trained immunity, as described previously (42). In brief, buffy coats of healthy blood donors (Sanquin, Nijmegen, the Netherlands) were obtained after written informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (GE healthcare) density gradient centrifugation. For assays with adherent cells (cytokines, lactate, RNA, chromatin immunoprecipitation; ChIP), monocytes were obtained by layering hyper-osmotic Percoll solution (Sigma) on PBMCs. Percoll monocyte purity was further enhanced by letting the cells adhere to flat-bottom culture plates ($1x10^5$ cells/well) for 1 hour, and washing once with warm phosphate-buffered saline (PBS) to remove non-adherent cells.

For assays with non-adherent cells (extracellular flux measurement, flowcytometry, reactive oxygen species production; ROS, antimicrobial activity assay), monocytes were isolated from the PBMC fraction using pan-monocyte magnetic beads (MACS Miltenyi). Cells were counted using the Sysmex XN-450 automated differential hematology analyzer (Sysmex Corporation, Japan), whereafter monocytes were cultured in suspension in tubes (1x10^6 monocytes/tube). Monocytes were cultured at 37°C, 5% CO_2 for 24 hours with medium control, β-glucan (1μg/mL), and AMB or AMB-L. After 24 hour incubation, monocytes were washed once with warm PBS to remove the stimuli, and were rested in medium for 5 days, during which they differentiated to macrophages. Culture medium was changed once on day 3 (fig. 1A). Cytotoxicity of AMB and AMB-L was determined via CytoTox 96 non-radioactive cytotoxicity assay (Promega). Supernatants after 24 hour incubations were collected to measure LDH release, according to the manufacturer's instructions.

Cytokine and lactate measurements

For cytokine and lactate measurements, supernatants were collected after 24 hours of stimulation with medium control or LPS and stored at −20°C until further analysis. Cells were lysed in 0.5% Triton X-100 (Sigma) and stored at -20°C for intracellular IL-1β measurements. Levels of tumor necrosis factor (TNF), IL-6, IL-1β, monocyte chemoattractant protein 1 (MCP-1), and IL-8 (R&D Systems) were determined in supernatants by enzyme-linked immunosorbent assay (ELISA) according to the

manufacturer's instructions. Lactate concentrations were measured in supernatants of cells stimulated with LPS or medium control. Samples were incubated with 10mM Amplex Red reagent (Life Technologies), 100U/mL lactate oxidase (Sigma), 10U/mL horseradish peroxidase (Sigma), and PBS for 20 minutes at room temperature (RT), after which fluorescence (at 570/585nm) was measured.

RNA Extraction, Reverse Transcription, and qRT-PCR

For RNA isolation, cells were collected after 4 hour stimulation with LPS, lysed with buffer RLT, and stored at -20°C until RNA extraction. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed into cDNA using the iScript kit (Bio-Rad). Gene expression was determined by qRT-PCR (StepOnePlus qPCR system, Applied Biosystems) using SYBR green reagents (Applied Biosystems), and relative expression was calculated using the $2^{-\Delta\Delta CT}$ method against the endogenous control gene 18S. Primers used in this study are listed in supplementary table 2.

Chromatin Immunoprecipitation (ChIP)

Day 6 macrophages were cross-linked in 1% methanol-free formaldehyde. Fixed cells were sonicated using the Diagenode Bioruptor Pico sonicator and ChIP was performed with antibody against H3K4me3 (Cell Signaling technology). Immunoprecipitated chromatin was subsequently processed for qRT-PCR analysis using the MinElute DNA purification kit (QIAGEN). Samples were analyzed with a comparative Ct method on the StepOne PLUS qPCR machine (Applied Biosystems) using SYBR green (Invitrogen) in accordance with the manufacturer's instructions. Myoglobin (MYO) was used as a negative control, and H2B was used as a positive control for H3K4me3 enrichment. Primers used in the reaction are listed in supplementary table 3.

Extracellular flux measurements

Real-time oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of trained monocyte-derived macrophages were evaluated using an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience). Day 6 macrophages were suspended in assay medium (RPMI with 0.6mM glutamine, 5mM glucose and 1mM pyruvate; pH adjusted to 7.4) and 1x10^5 cells were plated in quadruplicate in calibrated cartridges, and incubated in a non-CO_2 37°C incubator for 1 hour. OCR and ECAR were measured using a Cell Mito Stress Kit, using oligomycin (1µM), FCCP (1µM), and rotenone/antimycin A (0.5µM).

Reactive oxygen species (ROS) production

For measurement of ROS production on day 6, a luminol-enhanced luminescence assay was used. Macrophages were plated in triplicate at 3x10^5 per well in white 96-well assay plates (Corning). Cells were stimulated with 1mg/mL plasma-opsonized zymosan. Luminol (5-amino-2,3,dihydro-1,4-phtalazinedione) was added at 0.1mM in HBSS, and chemiluminescence was measured for 1 hour at 142 second intervals at 37°C. Relative light units (RLU) per second were used to calculate the area under the curve (AUC). Opsonized zymosan particles were prepared by incubation of zymosan derived from *Saccharomyces cerevisiae* (Sigma) in human plasma for 30 min at 37°C, after which the particles were washed twice in PBS and suspended in PBS.

Microbicidal activity assays

For phagocytosis assays, day 6 trained macrophages were incubated with fluorescein isothiocyanate (FITC)-labelled *Candida albicans* (*C. albicans*) conidia (heat-killed; strain UC820) with an multiplicity of infection (MOI) of 5:1 for 1 and 2 hours at 37°C. Cells were then washed in PBS + 1% bovine serum albumin (BSA) and stained with mouse anti-human CD45-allophycocyanin (APC; Beckman Coulter) for 30 min at 4°C. Fluorescent signal of non-phagocytosed *C. albicans* was quenched using 0.2% trypan blue. Cells were measured on a CytoFLEX flowcytometer (Beckman Coulter). Surface expression of HLA-DR was assessed by staining with mouse anti-human CD45-APC (Beckman Coulter) and mouse anti-human HLA-DR-BV421 (Becton Dickinson) and measured as described above. Data were analyzed using FlowJo software (v10.10.0). Representative gating strategies are detailed in supplementary figure S1A for HLA-DR measurements, and figure S1B for phagocytosis assays.

Antimicrobial activity of trained cells was investigated with a pathogen killing assay. Day 6 trained macrophages were collected and washed in PBS to remove residual culture medium. Cells were subsequently co-incubated with live *C. albicans* conidia (MOI of 5:1; strain UC820), live *Staphylococcus aureus* (*S. aureus*, MOI 2:1; strain ATCC25923), or live *Aspergillus fumigatus* (*A. fumigatus*) conidia (MOI 5:1; strain V05-27). After 4 hours, macrophages were lysed in H_2O and supernatants were serially diluted and plated on Sabouraud glucose agar (Becton Dickinson) for *A. fumigatus* and *C. albicans*, or Columbia III W/5% SB agar plates (Becton Dickinson) for *S. aureus*. As a negative control, samples containing pathogens but no macrophages were incubated and processed the same way. Plates were incubated for 24 hours at 37°C and the number of colony forming units (CFU) was enumerated.

Microbicidal activity was assessed by calculating CFUs in macrophage co-culture samples as a percentage of CFU in negative control samples.

Microbicidal activity against *Leishmania braziliensis* (*L. braziliensis*, strain MHOM/BR/2003/IMG) was investigated by adhering day 6 trained macrophages on 12mm coverslips, followed by infection with stationary-phase *L. braziliensis* promastigotes (MOI 5:1) for 2 hours. Afterwards, cells were washed and incubated for 4 and 48 hours, and subsequently fixed with methanol. Cells were stained with Giemsa (Merck Millipore) and analyzed under a light microscope to determine the infection index as described previously (33). One hundred cells were analyzed to determine the percentage of infected cells as well as the number of intracellular parasites per infected cell. The infection index was calculated as the percentage of infected cells multiplied by the mean number of parasites per infected cell.

Statistical analysis

In vitro trained immunity experiments were performed in at least 6 donors from at least 2 individual experiments. Data are shown as means ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism software (version 10). The Wilcoxon matched-pairs signed-rank test was used for comparisons between trained macrophages to control. Two-sided p-values < 0.05 were considered statistically significant. Indications are * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

AMB and AMB-L treatment of primary monocytes results in trained immunity

We adopted a previously established *in vitro* experimental model of trained immunity in monocytes (fig. 1A). In short, primary human monocytes were incubated with culture medium, β-glucan (1µg/mL), AMB (0.3 - 300µg/mL), or AMB-L (3 - 60µg/mL) for 24 hours (initial stimulus). After a 5-day resting period, day 6 macrophages were exposed to the secondary heterologous stimulus LPS for 24 hours to assess pro-inflammatory cytokine production. Cytokines detected in 24 hour supernatants after initial stimulation with AMB showed a small increase in IL-6 production (supplementary fig. 2D).

In accordance with previous studies, β-glucan induced a trained immunity phenotype characterized by a significant increase in production of pro-inflammatory cytokines upon LPS restimulation (fig. 1C). In the same model, monocyte-derived

macrophages exposed to clinically relevant (43,44) concentrations of AMB (3µg/mL) also demonstrated a potentiated cytokine and chemokine production upon restimulation, demonstrated both in gene expression (fig. 1B) and on protein level (fig. 1C). This AMB-induced trained immunity phenotype was dose-dependent (fig. 1D) and was not due to contamination of AMB preparations with LPS, as pre-incubation with polymyxin B did not prevent induction of trained immunity (supplementary figure S2E). However, concentrations of AMB above the clinically relevant range induced cytotoxicity (fig. S2A-B).

AMB trained macrophages exhibited upregulated gene expression of *TLR2* and *CLEC7A*, but not *TLR4* (fig. 1B). AMB-L was also capable of inducing trained immunity (fig. 1E), although the concentrations needed to significantly increase cytokine production (≥15µg/mL) were much higher than with conventional AMB. Pharmacokinetic studies have shown that AMB-L has higher maximum concentrations in plasma than conventional AMB (22.9±10µg/mL) (44). However, no significant increases in TNF were seen in AMB-L trained macrophages upon LPS stimulation. We therefore proceeded with subsequent experiments using conventional AMB only. No significant cytotoxicity was observed in AMB-L treated monocytes (fig. S2C).

AMB induces epigenetic modifications to inflammatory genes

We explored changes in trained macrophages at the level of gene regulation, where epigenetic changes to chromatin modifications are a key component in the development of trained immunity (34,45,46). We investigated whether chromatin modifications characteristic of trained immunity, specifically activating mark histone 3 lysine 4 trimethylation (H3K4me3), could also be found in AMB trained cells. We observed that AMB enhances accumulation of H3K4me3 at the promotor regions of *IL-6* (fig. 2A), *TNF* (fig. 2B), and *IL-1B* (fig. 2C). Though not significant, this trend towards enrichment of H3K4me3, combined with gene expression and cytokine data, suggests that AMB treatment contributes to epigenetic reprogramming of monocytes.

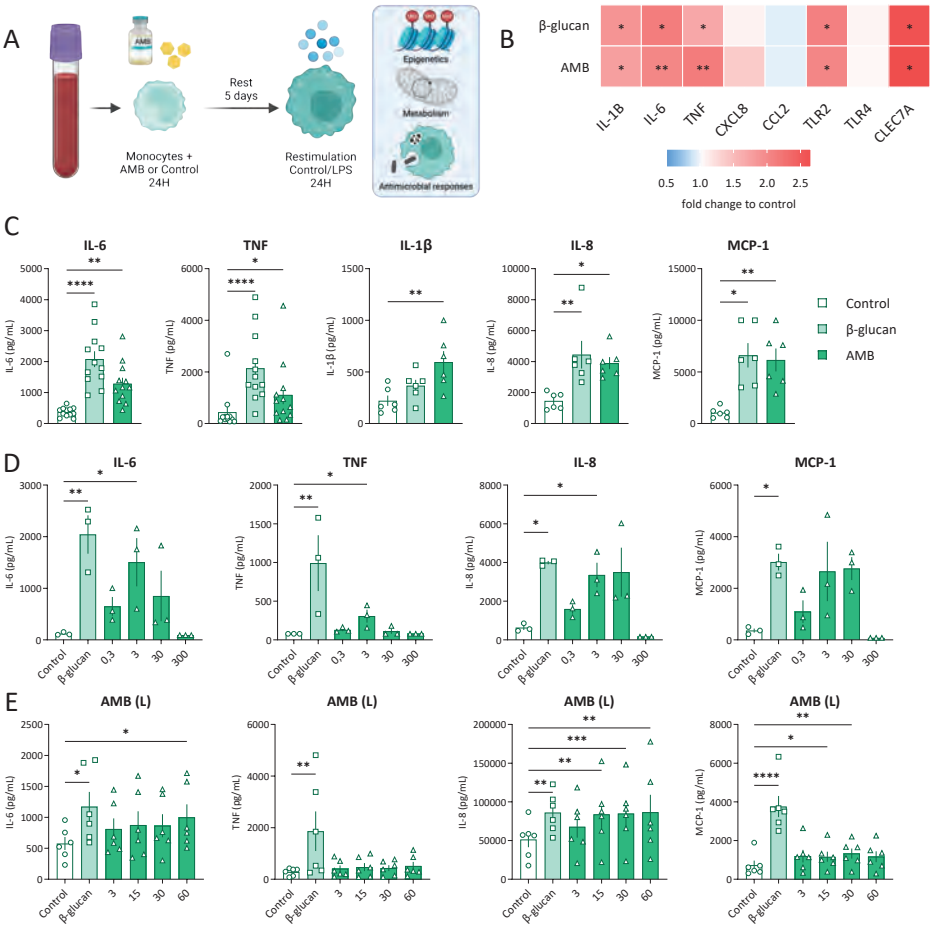

Figure 1. Short-term exposure of monocytes to clinically relevant concentrations of AMB and AMB-L induces trained immunity. Schematic of *in vitro* cell culture methods (A). Primary monocytes were incubated for 24 hour with AMB, AMB-L, or β-glucan as a positive control. After a 5 day rest period, macrophages were stimulated with LPS for 4 or 24 hours. Relative mRNA expression (B) of genes was determined in 4 hour LPS stimulated cells (n=8). Cytokine production upon 24 hour restimulation with LPS (C); levels of IL-6, TNF (n=6), IL-8, and MCP-1 (n=6) were determined in supernatants; intracellular IL-1β was measured in cell lysates (n=6). Dose response experiments using AMB (n=3; D) and AMB-L (n=6; E) determined the optimal concentration for induction of trained immunity (in μg/mL). Figure A was created with BioRender.com.

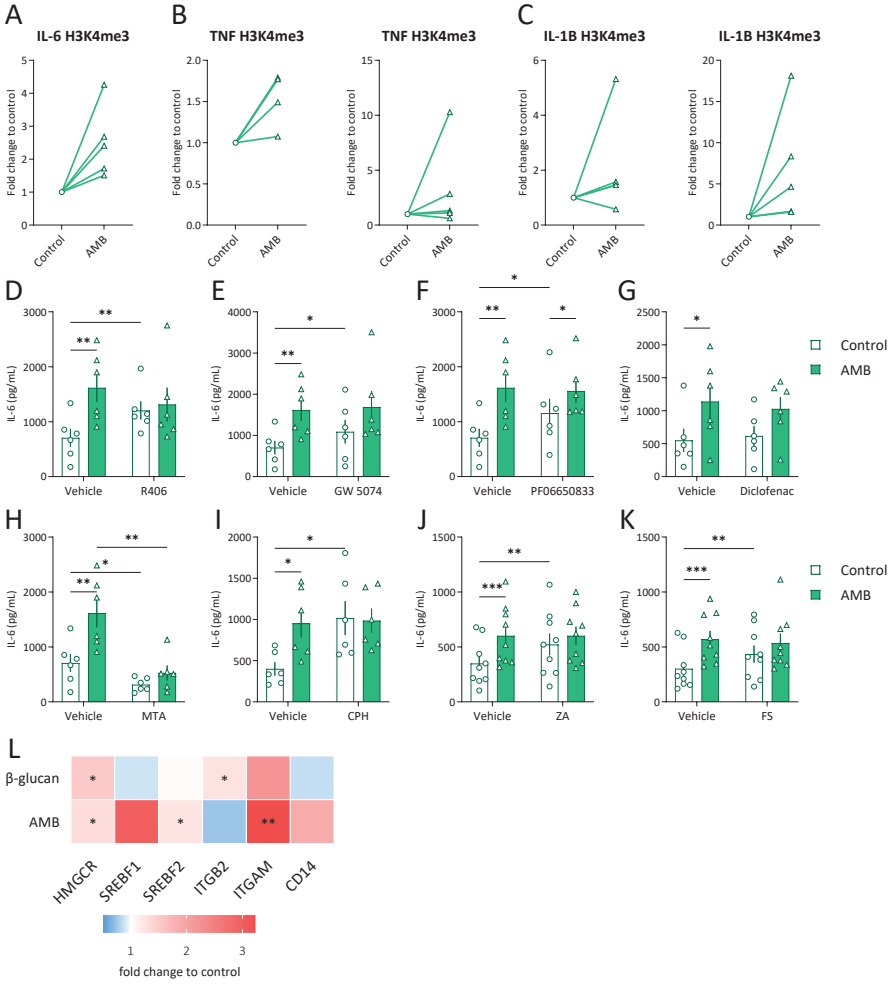

Figure 2. Epigenetic changes of pro-inflammatory cytokine genes characterize AMB-induced trained immunity. H3K4me3 enrichment was determined at the promoter regions of *IL-6* (A), 2 regions of *TNF* (B), and 2 regions of *IL-1B* (C) using immunoprecipitated chromatin from AMB-trained cells on day 6 (n=5). Pharmacologic inhibition of AMB training, as illustrated by IL-6 production capacity, was attained by pre-incubation with R406 (D), GW5074 (E), PF06650833 (F), diclofenac (G), MTA (H), CPH (I), zaragozic acid A (ZA; J), or fluvastatin (FS; K) for 1 hour prior to AMB stimulation (n=6). Relative mRNA expression (L) of genes in 4 hour LPS stimulated cells (n=8).

To identify other pathways with a potential role in AMB-trained immunity, additional pharmacological inhibition experiments were performed using Syk inhibitor R406 (fig. 2D), RAF-1 inhibitor GW5074 (fig. 2E), IRAK-4 inhibitor PF06650833 (fig. 2F), and COX1/2 inhibitor diclofenac (fig. 2G). Of these inhibitors, R406 was shown to inhibit AMB trained immunity. Syk-dependent pathways can be triggered through the interaction with C-type lectin CLEC7A (dectin-1), as well as TLR4 signaling pathways (47). Inhibition of these pathways may result in the suppression of inflammatory responses in monocytes necessary for AMB training. Inhibition of RAF-1, IRAK-4, or COX1/2 reduced the AMB trained immunity response, but a trend towards increased IL-6 production was still observed.

To further investigate the role of epigenetic reprogramming in AMB-trained macrophages, we inhibited chromatin modifications during the initial 24 hour exposure to AMB by pre-incubation with either MTA or CPH. MTA is a pan-histone methyltransferase inhibitor, whereas CPH is a Set7 methyltransferase activity inhibitor. We observed that pharmacological inhibition of chromatin modification by MTA or CPH prevented induction of AMB trained immunity (fig. 2H-I). These data support a role for methyltransferase activity in AMB-induced trained immunity. Given the potential interaction between AMB and the cell membrane lipid structures, we investigated the role of the cholesterol synthesis pathway in AMB training. Pre-incubation with zaragozic acid A, a squalene synthase inhibitor, inhibited the induction of pro-inflammatory cytokine production (fig. 2J). In addition, inhibition of high-mobility group (HMG)-coenzyme A (CoA)-reductase by fluvastatin prevented the induction of trained immunity by AMB (fig. 2K). Furthermore, AMB trained macrophages showed increased gene expression of *HMGCR*, *SREBF2*, and *SREBF1*, which contribute to cholesterol homeostasis (fig. 2L). Combined, these data indicate that the cholesterol synthesis pathway plays a significant role in AMB-induced trained immunity.

Glycolysis and oxidative phosphorylation metabolism in macrophages trained with AMB

Previous studies have identified that metabolic changes support the trained phenotype induced by β-glucan and other compounds (36,48). This is characterized by intracellular metabolic reprogramming, including increased oxidative and glycolytic metabolism, and accumulation of certain intracellular metabolites (45,49). To establish the significance of metabolic changes in AMB-trained macrophages, cells were assessed using Seahorse extracellular flux analysis.

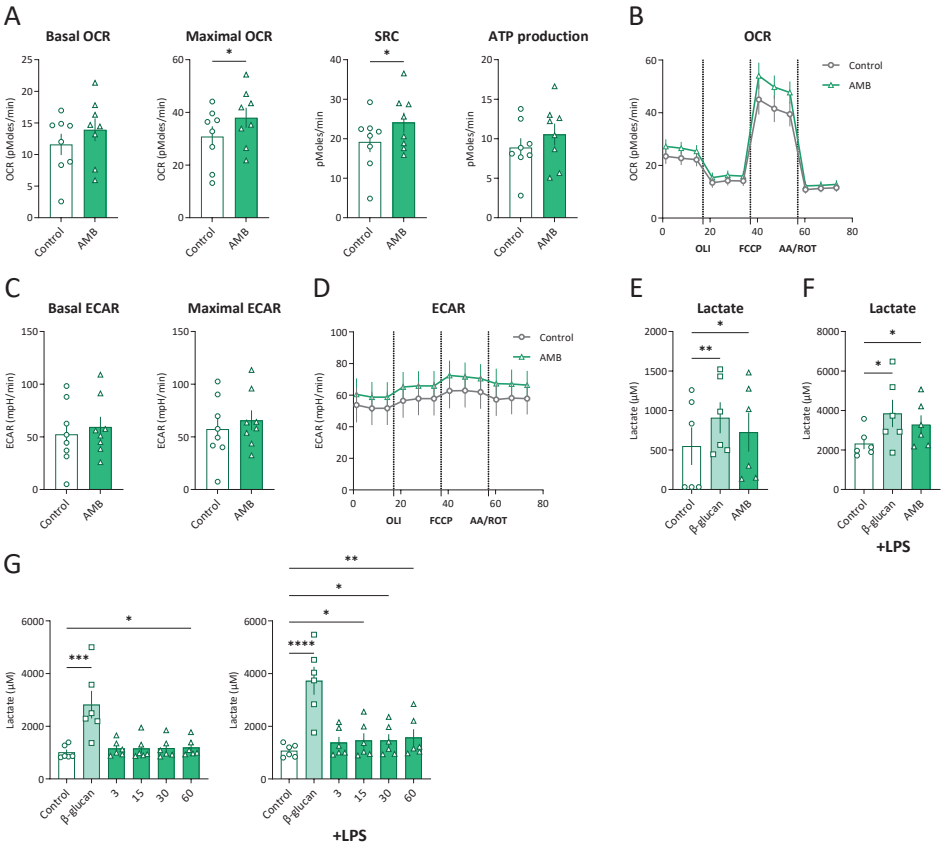

Figure 3. Amphotericin B alters both oxidative phosphorylation and glycolysis. Day 6 AMB trained cells were harvested and ECAR and OCR were determined by Seahorse XF (n=8). Maximal respiration as well as spare respiratory capacity (SRC) were significantly increased (A-B). Basal and maximal glycolytic metabolism did not significantly differ between control and AMB (C-D). Lactate was measured in supernatants of AMB trained cells (n=6) after 24 hour stimulation with medium (E), or LPS (F), and in AMB-L-trained cell supernatants (G; n=6).

We observed that transient exposure to AMB induced an increased maximal oxygen consumption rate (OCR; fig. 3A) and spare respiratory capacity (SRC), indicating that mitochondrial respiration is enhanced by AMB trained immunity (fig. 3B). The basal and maximal extracellular acidification rate (ECAR), serving as a measure of glycolysis, did not differ significantly between AMB and control macrophages when assessed with Seahorse technology (fig. 3C-D). Interestingly, when measured after 24 hour stimulation medium control (fig. 3E) or LPS (fig. 3F), we observed a significant increase in lactate in the supernatants of AMB-trained cells. These

findings are compatible with the previously formulated hypotheses that trained immunity increases glycolytic metabolism (50). AMB-L induced a similar increase in lactate production upon LPS stimulation (fig. 3G).

AMB training boosts key antimicrobial effector functions of macrophages

To explore whether our *in vitro* model of trained immunity resulted in enhanced antimicrobial defense, key effector functions of macrophages, such as antigen presentation, phagocytosis, oxidative burst, and microbicidal activity were investigated. Antigen-presenting capacity of AMB-trained macrophages was expanded, as demonstrated by an increased expression of MHC-II molecule HLA-DR on the cell surface, both in percentage of positive cells (fig. 4A), as well as fluorescence intensity (fig. S3C). This finding was corroborated by increased *HLA-DRA* gene transcription (fig. 4F). In addition, the induction of a trained immunity phenotype by β-glucan and AMB was accompanied by significantly more phagocytic activity, assessed as the percentage of macrophages that engulfed FITC-labelled heat-killed *C. albicans* conidia after 2 hours (fig. 4B) and 1 hour (supplementary fig. S3D) of co-culture. Gene expression analysis indicated that phagosome composition and maturation was altered upon induction of trained immunity by β-glucan or AMB. We investigated three genes representing the various stages of fusion and fission with endocytic components, including *RAB5A* (an early endosome marker), *RAB7A* (a late endosome marker), and *LAMP1* (a lysosomal membrane protein). Taken together, the upregulation of these genes in AMB trained macrophages suggests enhanced degradative capacity and antimicrobial activity of the phagosome, possibly leading to more efficient pathogen clearance (fig. 4F).

Cells trained with β-glucan and AMB were also found to produce significantly higher levels of ROS upon stimulation with opsonized zymosan (fig. 4C). Having established increased production of ROS, we sought to determine transcription of genes encoding the NADPH oxidase (NOX) family of enzymes, which catalyze the production of various ROS. Training with β-glucan and AMB increased transcription of *NOX1* and *NOX2* (fig. 4G). Expression of anti-oxidative genes, including glutathione synthetase (*GSS*), paraoxonases (*PON1*), peroxiredoxins (*PRDX1*, *PRDX5*), and superoxide dismutases (*SOD1*, *SOD2*) was also found to be affected by trained immunity induced by β-glucan and AMB, trending towards elevated levels of mRNA, though this effect did not reach statistical significance for each gene (fig. 4G).

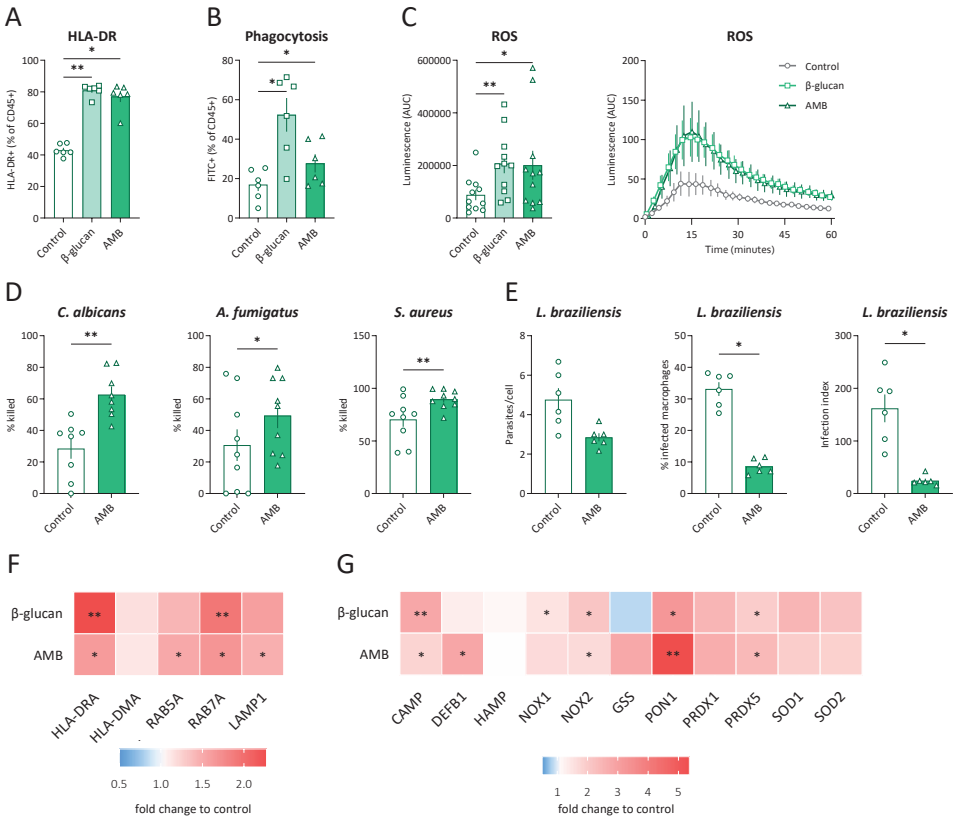

Figure 4. AMB-trained immunity increases macrophage effector functions and antimicrobial activity against fungi, bacteria, and parasites. Macrophages trained with β-glucan or AMB expressed significantly more HLA-DR molecules on the cell surface (A; n=6). Phagocytosis of FITC-labelled *C. albicans* was enhanced after 2 hour co-incubation (B; n=6). ROS production upon macrophage stimulation with opsonized zymosan was measured over 1 hour (n=11), and area under the curve (AUC) was calculated (C). Microbicidal activity against *C. albicans* (n=8), *A. fumigatus* (n=9), and *S. aureus* (n=9) was determined after 4 hour of co-culture with AMB-trained macrophages or control (D). Trained macrophages were infected with stationary-phase *L. braziliensis* promastigotes for 2 hours, washed, and incubated for another 4 hours (E). Number of parasites per infected cell, percentage of infected macrophages, and infection index were determined after 4 hours of incubation (n=6). mRNA levels in AMB trained cells show a trend towards increased transcription of genes related to phagocytosis, the production of antimicrobial peptides and ROS, as well as increased expression of the genes protecting against oxidative stress (F-G; n=8).

Enhanced microbicidal activity against fungi, bacteria, and protozoa in AMB trained macrophages

To establish whether the potentiating effects of AMB training on macrophage effector functions would translate to enhanced antimicrobial activity, a pathogen killing assay was performed using fungi, bacteria, and protozoa. Microbicidal capacity of day 6 AMB-trained and medium control macrophages was assessed by co-incubation with *C. albicans*, *A. fumigatus*, and *S. aureus*. AMB-induced trained immunity significantly increased killing of all three pathogens, illustrated by a higher percentage of CFUs killed (fig. 4D). Similar trends were seen in absolute CFU/mL counts (fig. S3A). Upon co-incubation with *L. braziliensis*, we observed that a lower percentage of AMB-trained macrophages had been infected after 4 hours (fig. 4E), as well as after 48 hours (supplementary fig. 3B). In addition, the number of parasites in each infected macrophage tended to be lower after AMB training, though this effect did not reach statistical significance. The infection index, calculated as the percentage of infected cells multiplied by the mean number of parasites per infected cell, was significantly lower in AMB trained macrophages after 4 hours, but not after 48 hours.

To investigate possible mechanisms of this enhanced antimicrobial activity, we explored two classes of cationic antimicrobial peptides, cathelicidins and β-defensins, which can exert antimicrobial functions. Expression of the *CAMP* gene, which encodes the precursor for cathelicidin antimicrobial peptides LL-37 and FALL-39, was found to be elevated in β-glucan and AMB-trained macrophages, compared to control cells (fig. 4G). In addition, we observed increased expression of β-defensin 1 (*DEFB1*) in AMB-trained macrophages. A similar though not significant effect was seen in β-glucan trained cells. Furthermore, we assessed gene expression levels of hepcidin antimicrobial peptide (*HAMP*), and iron transporter molecule *SLC40A1*, which play an essential role in regulating cellular iron homeostasis, but no significant changes were seen in AMB or β-glucan trained macrophages (fig. 4G).

Discussion

In this study, we assessed the long-lasting effects of AMB on primary human monocytes. Using the previously established model of *in vitro* trained immunity, we find that both AMB and AMB-L formulations were capable of inducing a trained immunity phenotype. Trained immunity is mediated through epigenetic reprogramming at the histone methylation level (51) and is characterized by profound changes in intracellular metabolic pathways, including glycolysis,

oxidative phosphorylation, and lipid synthesis (52). We observed that short-term exposure of monocytes to clinically relevant concentrations of AMB induced epigenetic modifications, translating to enhanced gene expression and subsequently leading to more pro-inflammatory cytokine production. AMB-trained immunity is characterized by enrichment of the activating mark histone 3 lysine 4 trimethylation (H3K4me3). These modifications are known to modulate transcription of innate immune genes, leading to the adoption of a pro-inflammatory phenotype during monocyte-to-macrophage differentiation (51). Pharmacological inhibition of methyltransferases resulted in failure to induce AMB-trained immunity, illustrating that the potentiated cytokine responses are dependent on epigenetic modifications. Additionally, we observed that AMB treatment increased oxygen consumption rates and lactate production, illustrating shifts in metabolic pathways that are crucial characteristics of trained immunity.

Contamination of AMB with β-glucan has been described in recent literature (16,17). β-glucans have been described as potential contaminants of multiple pharmaceutical products, including materials used in dialysis and surgery, monoclonal antibodies, antibiotic and antifungal medications, and certain blood products (53,54). Efforts to minimize this contamination have been proposed, given the potential for clinically significant immunological side-effects. Conversely, β-glucan's intrinsic immunostimulatory properties have made it an attractive candidate for vaccine adjuvants (55-57). Thus, the question of beneficial versus adverse effects of β-glucan contamination seems dependent on the intended application of the product, and the immune status of the patient receiving it (58). Upon our investigation of AMB and AMB-L preparations, we found no detectable levels of 1,3-β-D-glucan (BDG) contamination.

Trained immunity induced by β-glucan is dependent on the Dectin-1/Raf-1/mTOR signaling axis (34,59). Inhibition of the Raf-1 kinase signaling pathway was previously shown to significantly decrease the priming effect of β-glucan, but was not able to fully prevent AMB training. However, pharmacological inhibition of the Syk kinase pathway resulted in clear inhibition of the AMB-induced trained immunity phenotype. Syk-dependent pathways can be triggered through the interaction with C-type lectin CLEC7A (dectin-1), as well as TLR4 signaling pathways (47). Sau *et al*. found a minor role for TLR4 signaling pathway in AMB-related infusion toxicity (11). We demonstrate increased expression of *CLEC7A* and *TLR2*, but not *TLR4* in AMB trained macrophages, indicating that TLR2 and CLEC7A signaling pathways contribute to the long-term immunomodulatory effects of AMB. Addition of polymyxin B prior to AMB did not affect AMB-induced responses, demonstrating that the AMB-dependent changes in

cytokine responses were not due to endotoxin contamination. We found that high concentrations of AMB (>30µg/mL) induced significant cytotoxicity. When used in clinically relevant concentrations, no cytotoxicity was observed in our model, verifying that the immunomodulatory effects of AMB were not dependent on cell death. Taken together, our results show that there is no indication that the trained innate immune phenotype conferred by AMB is a consequence of contamination by β-glucan or endotoxin, or due to acute cytotoxicity.

In our search for a possible mechanism for AMB-induced trained immunity, we noted that the conventional AMB induced a stronger phenotype than liposomal AMB. AMB and AMB-L show no significant difference in clinical efficacy against pathogens (8). Conventional AMB has been linked to acute inflammatory toxicity via stimulation of pro-inflammatory cytokines, whereas AMB-L does not elicit cytokine production (11). AMB-L is formulated in small, unilamellar vesicles, which bypass interaction with host-cell receptors, but do still interact with lipid bilayer structures (44,60). We found that AMB-L is still capable of inducing trained immunity, albeit to a lesser extent compared to conventional AMB. We therefore postulate that AMB interaction with plasma membrane cholesterol contributes to the induction of trained immunity, but is not the only mechanism involved. Cholesterol is an essential lipid, serving both as a plasma membrane component, and as a precursor of steroid hormones and bile acids (61). The cholesterol synthesis pathway is therefore tightly regulated to maintain homeostasis (62). The role of the cholesterol synthesis pathway in trained immunity was established in previous research (63), demonstrating that the mevalonate pathway, but not cholesterol synthesis itself, plays a key role in the induction of trained immunity by β-glucan. We show here that AMB trained immunity is inhibited by pharmacological inhibition of rate-limiting enzyme high-mobility group (HMG)-coenzyme A (CoA)-reductase with fluvastatin. In addition, inhibition of downstream synthesis of squalene with zaragozic acid A also prevented the induction of AMB trained immunity. Rate-limiting enzymes in cholesterol synthesis pathway are encoded by *SREBP2* and *HMGCR* (64). We found that these genes were upregulated in AMB-trained macrophages at day 6. Our findings support a crucial role for the cholesterol synthesis pathway in the induction of trained innate immunity by AMB.

To investigate the extent to which AMB trained immunity affected macrophage effector functions, we investigated antigen presentation capacity, phagocytosis, and oxidative burst. In their role as antigen-presenting cells (APCs), macrophages must preserve peptides for presentation to T cells (65). Day 6 AMB-trained macrophages expressed more MHC-II molecule HLA-DR on their cell surface. HLA-DR functions both as an APC molecule but has also been described as a

general marker of activation for macrophage. Reduced expression of HLA-DR on macrophages is a hallmark of sepsis and immune suppression (66) and is associated with an increased risk of secondary bacterial infections (67). We also observed increased phagocytosis efficiency in AMB-trained cells, resulting in the uptake of more *C. albicans* particles compared to control cells. In addition, we describe that AMB-induced gene expression patterns suggesting enhanced coordination of phagosome composition and maturation. Gene expression of three markers of phagosome maturation were evaluated, including *RAB5A*, *RAB7A*, and *LAMP1*, which were all increased after AMB training. Levels of the gene encoding GTPase molecule Rab5, which is acquired after fusion with early endosomes, was used as a marker for newly formed phagosomes. Subsequent conversion from Rab5 to Rab7 marks the transition from early to late phagosome. Further gradual maturation of the phagosome leads to acquisition of LAMP-1, which is required for the development of the phagolysosome (68). Taken together, the induction of these key genes indicates an acceleration of phagosome maturation, which could enhance the degradative capacity and antimicrobial activity of the phagosome, possibly leading to more efficient pathogen clearance.

In contrast to previous articles investigating the effect of trained immunity on ROS production, we found an increased capacity in both β-glucan- and AMB-trained cells. Both Bekkering *et al*. (69) and Dos Santos *et al*. (33) described lower levels of ROS production in β-glucan trained cells, compared to control cells. We attribute this inconsistency with previous data to different methods of cell culture. Previous studies used macrophages cultured from adherent monocytes that were detached on day 6 by mechanical scraping of the cells, a technique known to induce ROS release in macrophages (70,71). We hypothesize that this method of harvesting the cells diminishes ROS production induced by subsequent stimulation with opsonized zymosan or other stimuli. We demonstrate that trained macrophages cultured in suspension and not mechanically detached do in fact show increased ROS production, compared to control cells, a finding confirmed in other trained immunity research (72). ROS can exert fundamental immunological functions both as signaling molecules and as an antimicrobial defense mechanism. Properly balanced oxidative and anti-oxidative responses are essential for immune cell function and antimicrobial defense mechanisms, while preventing free-radical toxicity (73). We found that AMB trained cells show enhanced expression of genes contributing to ROS production (*NOX2*) as well as regulating and reducing oxidative stress (*PRDX1/5*).

As both phagocytosis and ROS production are well-established factors influencing pathogen clearance (74,75), we performed a killing assay in AMB-trained cells. We used established (33,76) *in vitro* infection models to investigate whether AMB possessed long term immunostimulatory properties in defense against bacteria, fungi, and parasites. We demonstrate that AMB-trained macrophages exhibit significantly higher microbicidal activity against *C. albicans*, *A. fumigatus*, *S. aureus*, and *L. braziliensis*. These data further support that the development of trained immunity by AMB could improve general protection against infection, which is not specific to individual pathogens. Another intriguing mechanism of non-specific protection may be the production of antimicrobial peptides such as β-defensins and cationic antimicrobial peptides (CAMPs). These peptides, primarily stored in the lysosomes of macrophages and neutrophils, provide a critical first line of host defense (77). CAMPs and β-defensins are induced during infection and exert their antimicrobial effector function by permeating the microbial cell membrane (78). We describe that gene expression of CAMPs (*CAMP*) and β-defensins (*DEFB1*) was elevated in AMB-trained macrophages, indicating another mechanism by which trained macrophages may enhance their pathogen clearance efficiency. Though we did not observe significant changes in hepcidin antimicrobial peptide (*HAMP*) expression in AMB-trained cells, we see a trend towards enhanced expression in β-glucan macrophages, although this effect did not reach significance due to high donor variability. Similarly, we observed upregulation of iron transporter molecule *SLC40A1* in AMB trained cells, though again there was large variation between donors. Upregulation of *HAMP* and *SLC40A1* leads to iron retention in macrophages, which is a mechanism by which macrophages limit iron access to extracellular pathogens, impeding their growth (79). The role for trained immunity in the iron metabolism mechanism of macrophages warrants further investigation. To summarize, induction of AMB trained immunity resulted in enhanced non-specific antimicrobial activity *in vitro*.

A hallmark of trained immunity is the establishment of non-specific protection (80). Our data support increased host defense mechanisms against a broad range to pathogens, including fungi, bacteria, and protozoa. Thus, AMB treatment may modulate the innate immune system in patients and provide additional non-specific protection against infections with heterologous pathogens. In certain scenarios, such as in immunocompromised patients, enhancing the functional status of monocytes and macrophages by the induction of trained immunity may be helpful. We acknowledge that *in vitro* models of monocyte trained immunity may not be equivalent to *in vivo* treatment with AMB or other stimuli that confer a trained phenotype. The immunomodulatory effects of AMB have been described

in numerous studies, but we did not identify any previous works examining the long-term effects of AMB treatment. More studies are required to elucidate the complexity of the immune mechanisms behind the protective effect of AMB, and whether inducing trained immunity by AMB could provide immunocompromised patients with an extra protective measure against infections.

Conclusion

In summary, our findings show that AMB is capable of inducing increased cytokine production, evocative of trained immunity, in primary human monocytes. These data explore the long-term implications of AMB treatment for several crucial host defense mechanisms. We demonstrate activation of PRR signaling pathways, the accumulation of epigenetic modifications, enhanced inflammatory gene transcription, and augmented macrophage effector functions, ultimately resulting in increased microbicidal activity. We demonstrate that the cholesterol synthesis pathway is required to successfully induce potentiated cytokine responses. Collectively, the evidence presented here underlines that AMB is able to exert metabolic and epigenetic immunomodulatory effects through mechanisms that partially overlap with β-glucan trained immunity, but with distinctive features involving the cholesterol synthesis pathway. Thus, in addition to its direct antifungal activity, AMB induces trained immunity in human monocytes, conferring non-specific protection against infections *in vitro*.

Acknowledgements

We would like to thank M.C. Tehupeiory-Kooreman (Radboudumc) for performing the Fungitell BDG measurements. We thank M. Jaeger (Radboudumc) for providing FITC-labelled *C. albicans* (clinical isolate 10061110). *L. braziliensis* promastigotes (strain MHOM/BR/2003/IMG) were kindly provided by J.C. dos Santos (Radboudumc). L.H., N.J., and L.G. conceived and designed the experiments. L.H. performed the experiments, analyzed the results and wrote the manuscript. All authors critically read the manuscript. G.J.S. and L.J. share senior authorship.

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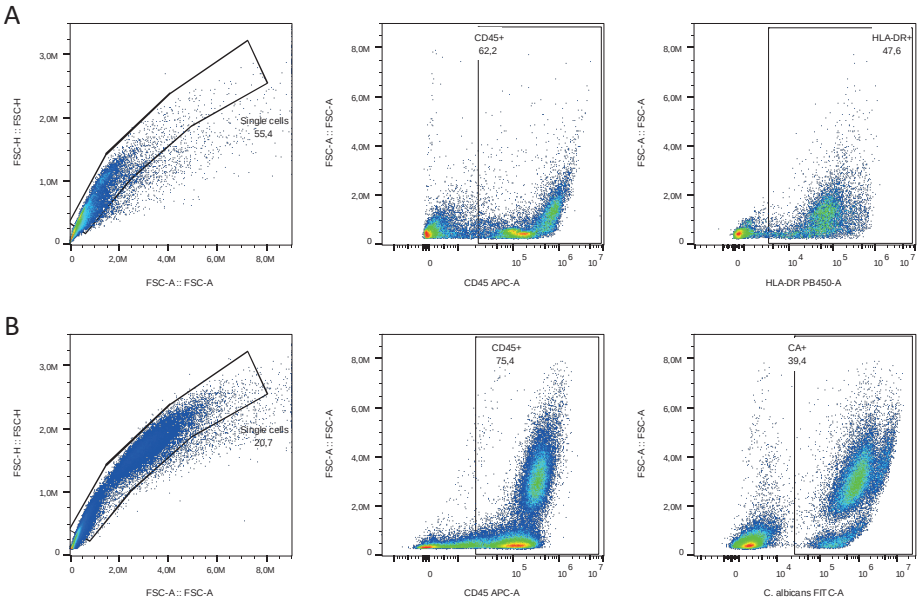
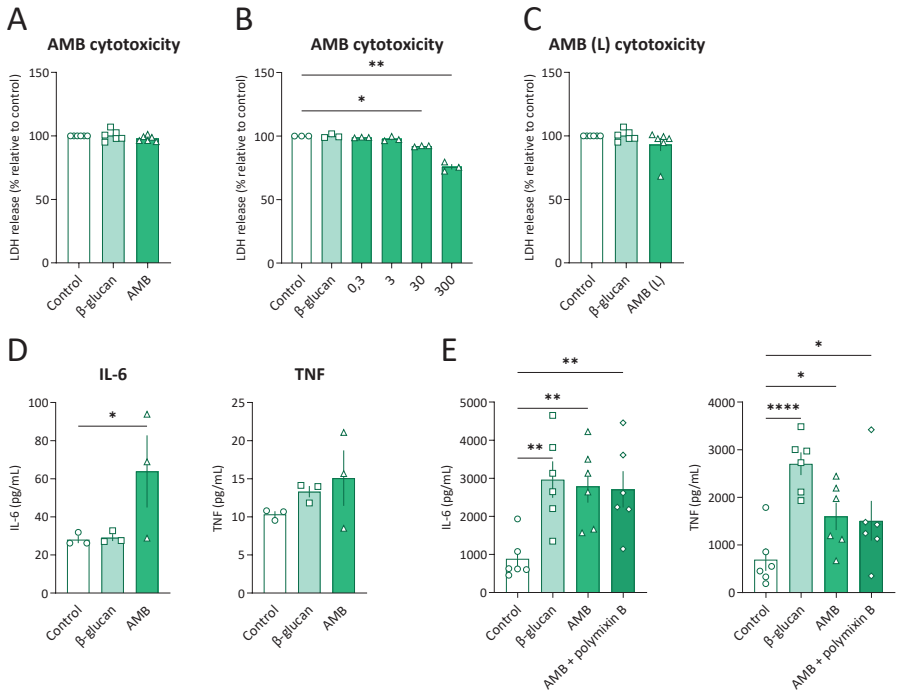
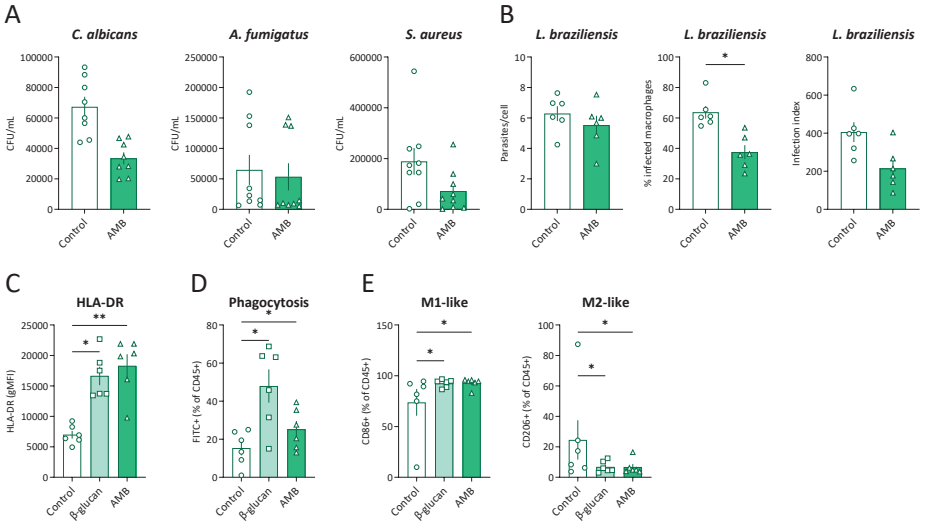
Supplementary material



Supplementary figure S1. Flowcytometric analysis of AMB-trained macrophages. Monocytes were trained with AMB or RPMI control, and evaluated for cell surface HLA-DR expression and phagocytic activity. Representative gating strategies for flowcytometry analysis of HLA-DR in macrophages (A), and for phagocytosis of *C. albicans* (CA; B).



Supplementary figure S2. Cytotoxicity of AMB, AMB-L and inhibitors. Lactate dehydrogenase (LDH) levels were measured in supernatant of monocytes treated with AMB (0.3 - 300µg/mL), for 24 hours (A-B), or with AMB-L (30µg/mL; C), LDH release relative to negative control (culture medium) was calculated (n=6). 24 hour stimulation with AMB resulted in minor release of cytokines (D; n=3). Induction of trained immunity with AMB (3µg/mL) was not affected by pre-incubation with polymyxin B (E; n=6).



Supplementary figure S3. Additional microbicidal assays. CFU/mL of *C. albicans* (n=8), *A. fumigatus* (n=9), and *S. aureus* (n=9) after 4 hours of co-culture with AMB trained macrophages or medium control macrophages (A). Trained macrophages were co-incubated with stationary-phase *L. braziliensis* promastigotes for 2 hours, washed, and incubated for another 48 hours (B). Number of parasites per infected cell, percentage of infected macrophages, and infection index were determined after 48 hours of culture (n=6). AMB or β-glucan trained macrophages expressed higher HLA-DR (gMFI) on the cell surface (C; n=6). Phagocytosis of FITC-labelled *C. albicans* was enhanced after 1 hour of co-incubation (D; n=6).

Supplementary table 1. BDG content results from Limulus Amebocyte Lysate (LAL) assay (Fungitell).

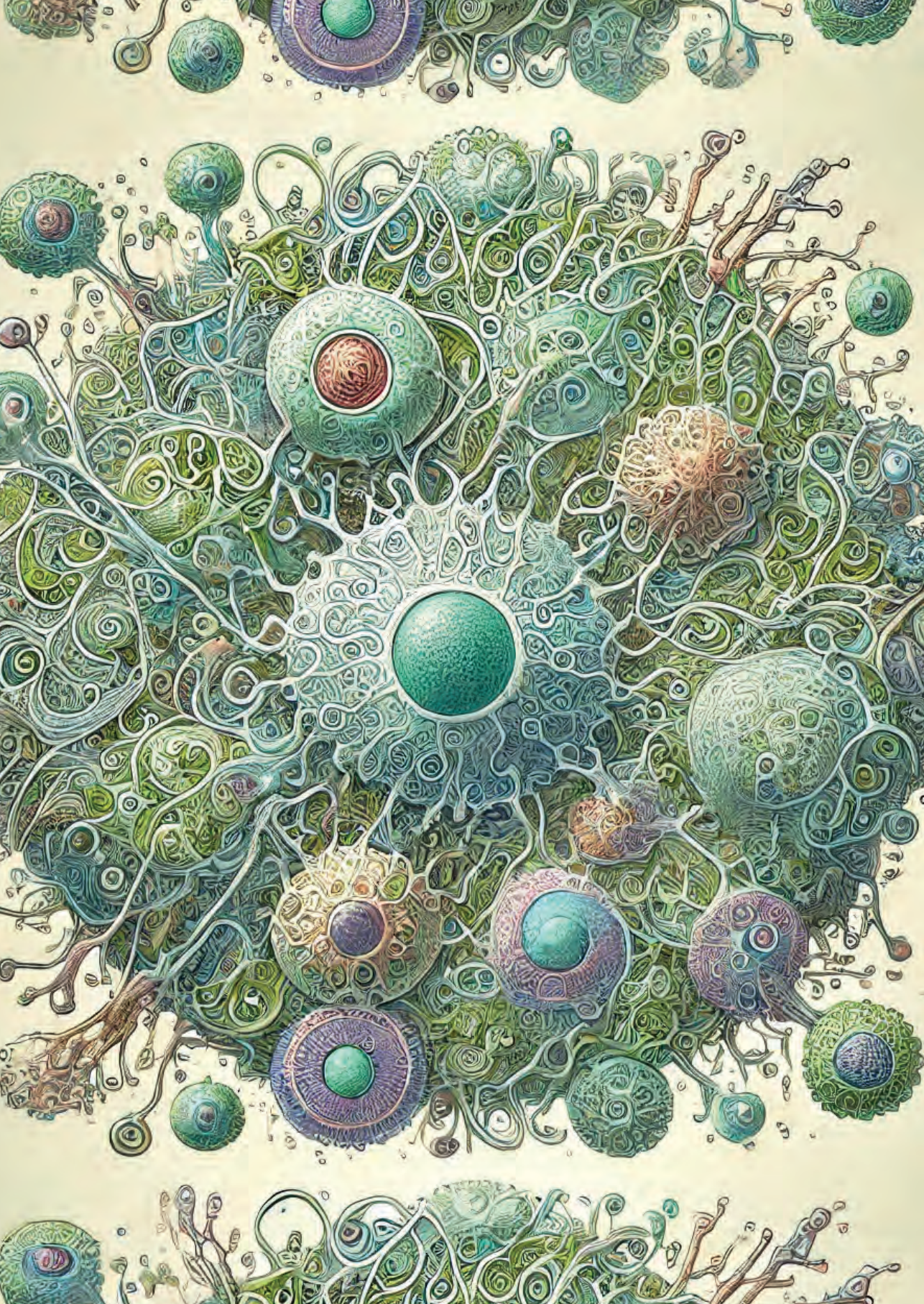
Substance	BDG (pg/mL)
RPMI medium control	<30
Amphotericin B 5 mg/mL	<30
Amphotericin B liposomal 4 mg/mL	<30

Supplementary table 2. mRNA primer sequences.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
18S	AAACGGCTACCACATCCAAG	CGCTCCCAAGATCCAACTAC
IL1B	GCCCTAAACAGATGAAGTGCTC	GAACCAGCATCTTCCTCAG
IL6	AATTCGGTACATCCTCGACGG	GGTTGTTTTCTGCCAGTGCCT
TNF	AACGGAGCTGAACAATAGGC	TCTCGCCACTGAATAGTAGGG
CXCL8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTC
CCL2	CCAGTCACCTGCTGTTATAAC	TGGAATCCTGAACCCACTTCT
TLR2	CCCAGGAGCTCTTAGTGACC	CTGAGCTGCCCTTGCAGATA
TLR4	GGCATGCCTGTGCTGAGTT	CTGCTACAACAGATACTACAAGCACACT
CLEC7A	ACAATGCTGGCAACTGGGCT	GCCGAGAAAGGCCTATCCAAAA
HMGCR	TGATTGACCTTTCCAGAGCAAG	CTAAAATTGCCATTCCACGAGC
SREBF1	ACAGTGACTTCCCTGGCCTAT	GCATGGACGGGTACATCTTCAA
SREBF2	AACGGTCATTCACCCAGGTC	GGCTGAAGAATAGGAGTTGCC
ITGB2	AAGTGACGCTTTACCTGCGAC	AAGCATGGAGTAGGAGAGGTC
ITGAM	TGGTGGCTTCCTTGTGGTTC	TGCGTTCTCTTGGAAGGTCA
CD14	CCGCTGTGTAGGAAAGAAGC	GCAGCGGAAATCTTCATCGT
HLA-DRA	AGTCCCTGTGCTAGGATTTTTCA	ACATAAACTCGCCTGATTGGTC
HLA-DMA	CCTGCACACAGTGTACTGC	CACCCGAGTGTTCTGGGAA
RAB5	AGACCCAACGGGCCAAATAC	GCCCCAATGGTACTCTCTTGAA
RAB7	GTGTTGCTGAAGGTTATCATCCT	GCTCCTATTGTGGCTTTGTACTG
LAMP1	CAGATGTGTTAGTGGCACCCA	TTGGAAAGGTACGCCTGGATG
NOX1	GCACACCTGTTTAACTTTGACTG	GGACTGGATGGGATTTAGCCA
NOX2	ACCGGGTTTATGATATTCCACCT	GATTTCGACAGACTGGCAAGA
SOD1	GGTGGGCCAAAGGATGAAGAG	CCACAAGCCAAACGACTTCC
SOD2	AAATTGCTGCTTGTCCAAATCAGGA	AGTAAGCGTGCTCCCACACATCAA
GSS	GAACCGTTCGCGGAGGAAA	GAATGGGGCATAGCTCACCA
PON1	CCATCCAGATGCCAAGTCCA	TACGACCACGCTAAACCCAA
CAMP	CGGATGCTAACCTCTACCGC	AGGGTCACTGTCCCCATACA
DEFB1	ATGAGAACTTCCTACCTTCTGCT	TCTGTAACAGGTGCCTTGAATTT
HAMP	CCACAACAGACGGGACAACT	GGGCAGGTAGGTTCTACGTC
SLC40A1	TCCTTGGCCGACTACCTGAC	TCCCTTTGGATTGTGATTGC
PRDX1	CCACGTAGGTGCGGGAAC	AAAGCAATGATCTCCGTGGGG
PRDX5	GGGTATGGGACTAGCTGGCG	TCCCCTTCAAACACCTCCAC

Supplementary table 3. DNA primer sequences.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
IL6	AGGGAGAGCCAGAACACAGA	GAGTTTCCTCTGACTCCATCG
TNFA_1	CAGGCAGGTTCTCTTCCTCT	GCTTTCAGTGCTCATGGTGT
TNFA_2	TGATGGTAGGCAGAACTTGG	ACTAAGGCCTGTGCTGTTCC
IL1B_1	AATCCCAGAGCAGCCTGTTG	AACAGCGAGGGAGAAACTGG
IL1B_2	CTGGCGAGCTCAGGTACTTC	ACACATGAACGTAGCCGTCA
H2B	TGTACTTGGTGACGGCCTTA	CATTACAACAAGCGCTCGAC
MYO	AGCATGGTGCCACTGTGCT	GGCTTAATCTCTGCCTCATGAT



Chapter 7

BCG-induced innate immune memory potentiates T_H17 responses *in vitro*

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Abstract

This study tested the ability of trained macrophages to phenotypically and functionally alter T cells *in vitro*. Trained immunity, also known as innate immune memory, results in augmented innate immune responses. Macrophages play an important role in presenting antigens to T cells, which results in their activation and antigen-specific clonal expansion, however, few studies have investigated if trained immunity contributes to an altered T cell activation. Here, through surface marker analysis of naive, β-glucan, and BCG-trained macrophages, we identified 8 distinct macrophage clusters following trained immunity induction, with population-wide increases in surface activation markers CD40 and CD86, as well as MHC molecules. Co-culture of T cells with autologous BCG-trained macrophages *in vitro* resulted in a skewing towards T_H17 cells, which was also observed after *in vivo* BCG vaccination. This was determined to be antigen non-specific and not to be the result of increased proliferation of T_H17. In this line, T cells and BCG macrophages co-cultures responded with greater levels of interferon-γ (IFN-γ) and interleukin-17 (IL-17) when stimulated. However, no clear shifts towards effector or memory cells were observed. The T cell exhaustion marker PD-1 was also reduced in T cells co-cultured with BCG-trained macrophages. In conclusion, this study provides the first evidence that BCG-trained macrophages can directly alter T cell function, suggesting that BCG trained immunity has the potential to enhance not only innate immune responses but also to modify adaptive T cell immunity.

Introduction

Immune memory is classically associated with the adaptive immune system whereby specific defenses develop after exposure to pathogens. In addition to B cells and antibodies, T cells are a key feature of adaptive immune memory. Antigen-presenting cells (APCs), such as macrophages and dendritic cells, present processed antigens on their surface via MHC molecules, which are recognized by T cell receptors (TCR) on naïve T cells. This interaction, along with co-stimulatory signals, activates the T cells, leading to their clonal expansion and differentiation into effector T cells. Through this expansion, memory T cells are also generated, which persist long-term to provide rapid and robust responses upon re-exposure to the same antigen (1). Vaccines exploit the adaptive immune system to confer long-lasting memory and protection against infections. Namely, tuberculosis Bacillus Calmette-Guérin (BCG) vaccine induces the activation of CD4+ helper and CD8+ cytotoxic T cells and the production of IFN-γ (2,3). Upon infection with *Mycobacterium tuberculosis*, these specific memory T cells become highly proliferative and reduce and control bacterial dissemination (2,3).

Interestingly, the innate immune system likewise has features of memory, termed trained immunity. Epidemiological studies have shown that vaccines, notably in live attenuated forms, such as measles, polio oral vaccine, or BCG, elicit non-specific effects that confer long-term protection against diseases other than the target pathogen (4,5). Trained immunity involves epigenetic and metabolic reprogramming of innate immune cells, leading to enhanced responses upon subsequent, heterologous stimuli (6). This reprogramming can occur peripherally, within tissues and circulating immune cells, and in the bone marrow, affecting newly generated myeloid cells. Trained immunity is initiated by primary stimuli such as β-glucan (7), lipopolysaccharide (LPS) (8), or the BCG vaccine (9), which result in potentiated immune responses to unrelated future challenges. Indeed, monocytes, macrophages, neutrophils and natural killer cells can develop features of trained immunity, and can thereby confer non-specific protection (10,11,12).

Trained immunity augments different effector functions in innate immune cells, such as cytokine and ROS production. However, the influence of trained immunity on adaptive immune responses has not been extensively explored. Therefore, in the present study we sought to investigate whether trained macrophages are capable of altering autologous T cell function *in vitro*, by studying key effector functions of macrophages such as antigen presentation, chemokine secretion, and induction of T cell responses.

Methods

Isolation of peripheral blood mononuclear cells and monocytes

Buffy coats from healthy donors were obtained after written informed consent (Sanquin Blood Bank, Nijmegen, the Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by dilution in phosphate buffered saline (PBS) and density centrifugation over Ficoll-Paque PLUS (GE Healthcare Biosciences, Chicago, IL, USA). A portion of this PBMC fraction was frozen in Recovery™ cell culture freezing medium (Gibco, ThermoFisher Scientific, Waltham, MA, USA) at -80°C for later isolation of T lymphocytes. Monocytes were subsequently obtained from the remaining PBMC fraction using negative pan-monocyte magnetic beads (MACS Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Cells were cultured in RPMI 1640 Dutch-modified culture medium (Gibco) supplemented with 50µg/mL gentamicin (Centrafarm, Etten-Leur, the Netherlands), 2mM glutamax (Gibco), 1mM pyruvate (Gibco), and 10% human pooled serum.

Stimulation and training experiments

β-glucan (β-1,3-(D)-glucan) was kindly provided by Professor David Williams (College of Medicine, Johnson City, USA). Cells were cultured at 37°C, 5% CO_2. Monocytes were stimulated either with culture medium only, as a negative control, or with 1µg/mL of β-glucan, 5µg/mL BCG SSI (AJ vaccines, Copenhagen, Denmark), for 24 hours (in 10% pooled human serum). Cells were washed once with warm PBS and incubated for 5 days in culture medium with 10% pooled human serum. Culture medium was changed once on day 3. Cells were collected on day 6 and counted with a Sysmex automated hematology analyzer (XN-450; Sysmex Corporation, Kobe, Japan). Macrophages were seeded in flat-bottom 96-well plates and re-stimulated with 10ng/mL LPS from *E. coli* (serotype 055:B5, Sigma-Aldrich, Saint Louis, MO, USA), according to the established *in vitro* model of human monocyte training (13). After 24 hours, supernatants were collected and stored at -20°C until cytokine and chemokine measurements.

T cell activation experiments

Trained monocytes cultured for 6 days were seeded at 1x10^5 cells per well and pulsed with keyhole limpet hemocyanin (KLH; 10µg/mL, biosyn, Arzneimittel GmbH, Fellbach, Germany), *S. aureus* (1x10^6/mL, heat-killed, strain ATCC25923) and R848 (Resiquimod, 10µg/mL, InvivoGen, San Diego, CA, USA) for 1 hour, or left unstimulated. Autologous PBMCs were then thawed out and CD3+ T cells were isolated with magnetic CD3 beads according to the manufacturer's instructions

(MACS Miltenyi, Bergisch Gladbach, Germany). CD3+ cells were labeled with the CellTrace Violet cell proliferation kit (ThermoFisher Scientific, USA) according to manufacturer's instructions, counted and seeded with autologous day 6 monocytes at 5x10^5 per well (ratio monocytes to CD3+ cells 1:5) and cultured for 14 days in culture medium with 10% fetal calf serum. Every 2 days, supernatants were collected and culture medium was refreshed. Cells were protected from light during culture. When indicated, T cells and macrophages at day 14 of co-culture were stimulated with phytohaemagglutinin (PHA; 10µg/mL, Sigma-Aldrich).

Cytokine and chemokine measurement

Cytokine and chemokine concentrations were determined in supernatants using commercial enzyme-linked immunosorbent assay (ELISA) kits for TNF, IL-6, IL-1β, CCL2 (MCP-1), IL-4, IL-17, IL-22, and IFN-γ (R&D Systems, MN, USA), following the instructions of the manufacturers.

Flow cytometric analysis

For immunophenotyping of day 6 trained macrophages, cells were stained with ViaKrome-808 viability dye (Beckman Coulter, Brea, CA, USA), Fc receptors were blocked with TruStain FcX (Biolegend, San Diego, CA, USA), and cells were stained with mouse anti-human monoclonal antibodies (Table S1A). For additional intra- and extracellular HLA-molecule expression analysis, macrophages were fixed and permeabilized, and stained with mouse anti-human monoclonal antibodies (Table S1B). For evaluation of KLH antigen presentation, macrophages were stained with mouse anti-human CD45-APC (clone HI30; Biolegend) and polyclonal FITC-conjugated rabbit anti-KLH antibody (Abcam, Cambridge, UK). For macrophage flow cytometry experiments, the applied gating strategy was as follows; after exclusion of debris, doublets, and dead cells, macrophages were selected in the FSC/CD45+ plot, identifying macrophages as CD45+ cells with macrophage scatter properties (Figure S4).

For T cell proliferation and immunophenotyping analyses, T cells were stained at baseline and after 14 day co-culture with macrophages. For T cell proliferation assays, cells were incubated with Zombie Aqua viability dye (Biolegend) and stained using monoclonal anti-human antibodies (Table S2A). For additional T cell immunophenotyping, cells were incubated with ViaKrome-808 viability dye (Beckman Coulter) and stained with mouse anti-human monoclonal antibodies (Table S2B). Cells were then added to pre-formulated DURA Innovations tubes (Beckman Coulter) containing dried formulations of additional antibodies (Table S2C). For T cell flow cytometry experiments, the applied gating strategy

was as follows; after exclusion of debris, doublets, and dead cells, T cells were selected in the FSC/CD3+ plot. In the CD3+CD4+ and CD3+CD8+ cell populations, the percentages of gated T cell subsets, effector (T_{EFF}, CD45RA+CD197-), effector memory (T_{EM}, CD45RA-CD197-), central memory (T_{CM}, CD45RA-CD197+) and naïve (T_N, CD45RA+CD197+) were used for analyses (Figure S5). Additionally, in the CD3+CD4+ cell population, T_H1 (CD183+CD196-), T_H2 (CD183-CD196-), and T_H17 (CD183-CD196+) subsets were used for analyses. Identification of T cell subsets follows current recommendations (14). Samples were assessed using either a CytoFLEX or CytoFLEX-LX flow cytometer (Beckman Coulter). Data were analyzed using FlowJo (v10.8.1).

In vivo model of trained immunity

The 300BCG cohort consists of 325 adults from the Netherlands (44% males and 56% females, age range 18–71 years), as previously described (15) Briefly, healthy adults were vaccinated with a standard dose of 0.1mL BCG (BCG-Bulgaria, InterVax) intradermally, and blood was collected before, 14 and 90 days after vaccination. Individuals were genotyped using the Infinium Global Screening Array MD v1.0 genotyping microarray (Illumina). Immune cell frequencies with no missing values were obtained for 323 (day 0), 314 (day 14), and 301 (day 90) individuals. Cell percentages were determined according to surface marker expression for identification of T_H1 (CD4+CD196-CD183+CD194-), T_H2 (CD4+CD196-CD183-CD194+), T_H17 (CD4+CD196+CD183-CD194+) and $T_H1/17$ (CD4+CD196+CD183+CD194-) as measured with the Navios flow cytometer (Beckman Coulter).

Also, at each visit, 5×10^5 PBMCs were stimulated for 24 hours or 7 days with heat-killed *S. aureus* (1x10^6 CFU/mL, clinical isolate) or *C. albicans* (1x10^6 CFU/mL, strain UC820). Cytokine production was determined using commercial kits for IL-1β, IL-6, and TNF and IFN-γ (ELISA, R&D Systems) and IL-17 (Luminex ProcartaPlex, Thermo Fisher Scientific). The measurements of cytokine production after stimulation were filtered and batch-corrected, resulting in high-quality data for 317 (day 0), 307 (day 14), and 292 (day 90) individuals. The batch-corrected log10 data were transformed to a log2 scale. Cytokine fold changes were calculated as the log2 ratio of the values of each individual at day 90 or 14 compared to day 0. The fold change of cytokine production was mapped to genotype data using a linear regression model with age, sex, time of blood drawing and log2 fold change of PBMC cell type fractions as covariates. R-package Matrix-eQTL 2.370 was used for cytokine QTL mapping and visualization was performed in R (3.6.3). The 300BCG study was approved by the Arnhem-Nijmegen medical ethical committee

(NL58553.091.16) and conducted in accordance with the Declaration of Helsinki. All study participants gave written informed consent during the first visit.

Statistical analysis

Experiments were performed with at least 6 biological replicates, pooled from at least 2 independent experiments. Data were analyzed using a Friedman test followed by Dunn's multiple comparisons test or a two-way ANOVA followed by Dunnett's multiple comparisons test, where appropriate, as indicated in the figure legends. A P-value below 0.05 was considered statistically significant (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Data are shown as mean ± SEM unless stated otherwise. All data were analyzed using GraphPad Prism 5.03.

Results

BCG or β-glucan trained macrophages are prepared for antigen presentation

To gain insights into the surface marker expression profile of macrophages trained with β-glucan and BCG, we performed a trained immunity *in vitro* protocol as described previously (13) (Figure 1A). Trained macrophages were harvested for flow-cytometry analysis of activation and antigen presenting markers; HLA-DR, CD86, CD273, CD40, CD163, CD80, CD4, CD274, CD206, CD38, CD16, CD11b, CD11c, and CD14. t-distributed stochastic neighbor embedding (tSNE) clustering analysis revealed a large degree of variability in macrophage populations (Figure 1B). In total we identified 8 distinct populations with diverse surface marker expression (Figure 1C), most of which were modulated upon trained immunity. β-glucan trained macrophages showed elevations in population 0, 1 and 6, and reductions in 3 and 5. Of particular interest is the increase in population 6, representing 4% of β-glucan trained macrophages, which is characterized by high expression of CD11b, CD273 (PD-L2) and CD274 (PD-L1).

Differences between training stimuli were most prominent for macrophages trained with BCG which showed a proportionally smaller population 7, and a larger population 1 and 2 (Figure 1D, E). Population 1 seemed to be affected similarly by both β-glucan and BCG induction of trained immunity, possibly representing common features of trained macrophages. This population, representing ~20% in β-glucan and ~34% in BCG, was characterized by high levels of HLA-DR, CD11b and CD14, as well as moderate to high expression of CD86, CD40 and CD80 (Figure 1C). Overall analysis of surface expression of activation and antigen

presentation markers in macrophages revealed that the activation markers CD40 and CD86 were elevated in β-glucan trained macrophages, and that antigen presentation molecules HLA-A/B/C, HLA-DR, HLA-DM, and CD74 were increased in both β-glucan and BCG trained macrophages (Figure 1F, G).

Co-culture with trained macrophages results in less T cell proliferation

Having determined that a higher proportion of β-glucan and BCG trained macrophages express antigen presenting and activation molecules, we assessed whether these trained macrophages could activate autologous T cells *in vitro*. We co-culture β-glucan and BCG trained macrophages with autologous CD3+ T cells for 14 days (Figure 2A). No differences were seen in the percentages of CD4+ and CD8+ T cell populations after co-culture with β-glucan and BCG trained macrophages when compared to co-culture with stimulus naïve macrophages (Figure 2B). The analysis of 16 different markers, allowed us to perform a cluster analysis of the T cell population revealing 13 distinct populations of T cells (Figure S1A), with shifts in the proportion of different populations (Figure S1B, C, D), with β-glucan trained macrophages inducing the most significant shifts in T cell populations (Figure S1D). The distribution of CD4+ and CD8+ naive, central memory, effector memory, and effector T cells after co-culture with trained macrophages, was not changed, with the exception of reduced levels of CD8+ T_{EM} cells (Figure S1E).

Although CD4+ and CD8+ T cell percentages were not altered, we observed changes in T helper subsets. We observed a reduction in the percentage of CD4+ T_H1, and a concomitant increase in CD4+ T_H17 cells in T cells co-cultured with BCG trained macrophages (Figure 2C). In accordance, the degree of proliferation in T_H1 cells was reduced in BCG trained macrophages (Figure 2D, E). However, in contradiction to T_H17 cells making up a relatively larger proportion of T cells in the final T cell population after culture with BCG trained macrophages, the degree of proliferation of T_H17 cells was reduced (Figure 2D, E). Additionally, it is relevant to note that the presence of naive macrophages influenced T cell polarization and proliferation when compared to T cells cultured alone, revealing the expected influence of macrophages on T cell function (Figure S2A-D).

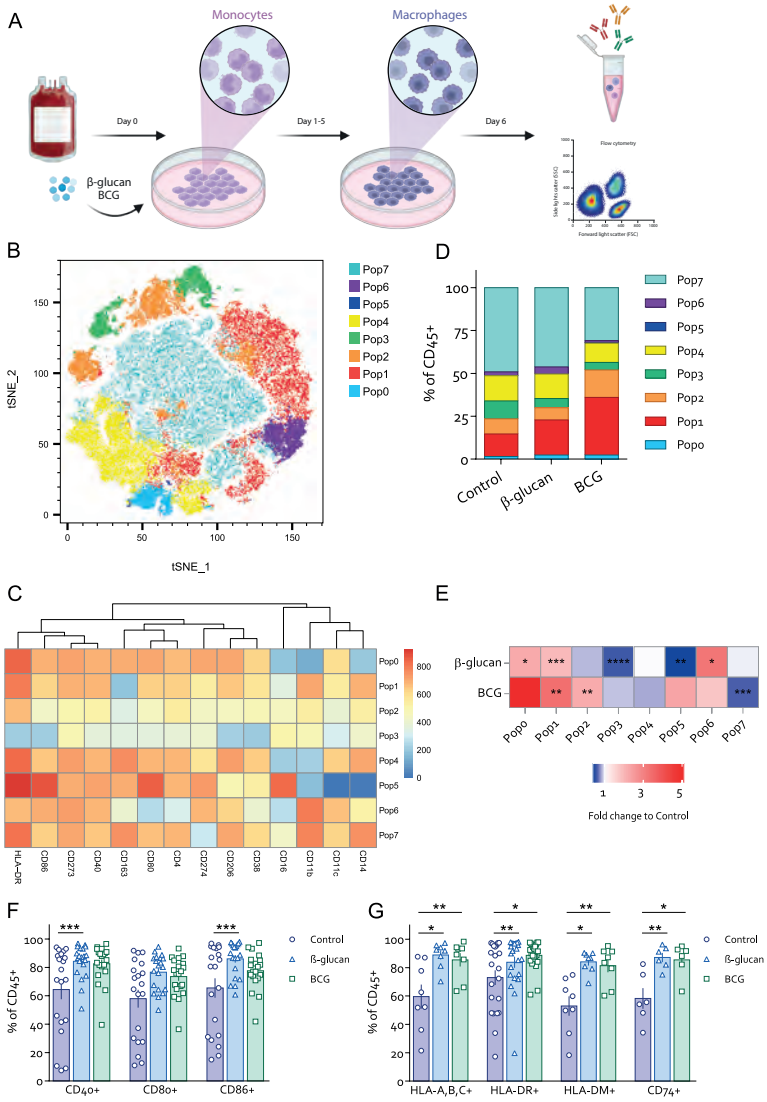

Figure 1. *In vitro* macrophages trained with β-glucan and BCG present distinct populations, with increased levels of activation markers and antigen presenting molecules. (A) Schematic overview of *in vitro* macrophage trained immunity experiments. (B) tSNE analysis based on HLA-DR, CD86, CD273, CD274, CD40, CD80, CD163, CD206, CD38, CD16, CD11b, CD11c, CD14 reveals different clustering features for control, β-glucan and BCG trained macrophages, overlay represents the FlowSOM (self-organizing maps of flow cytometry data) clusters. (C) Heatmap of FlowSOM clustering analysis. (D) FlowSOM analysis reveals 8 macrophage subpopulations. (E) Heatmap of FlowSOM analysis by training stimulus. (F) Percentage of macrophages trained with β-glucan or BCG that express the activation markers CD40, CD80 and CD86, (G) and the antigen presenting molecules HLA-A, B, C, HLA-DR, HLA-DM and CD74. Data shown as mean + SEM, n=14 biological replicates, pooled from 4 independent experiments. Friedman test, followed by Dunn's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Figure A was created with BioRender.com.

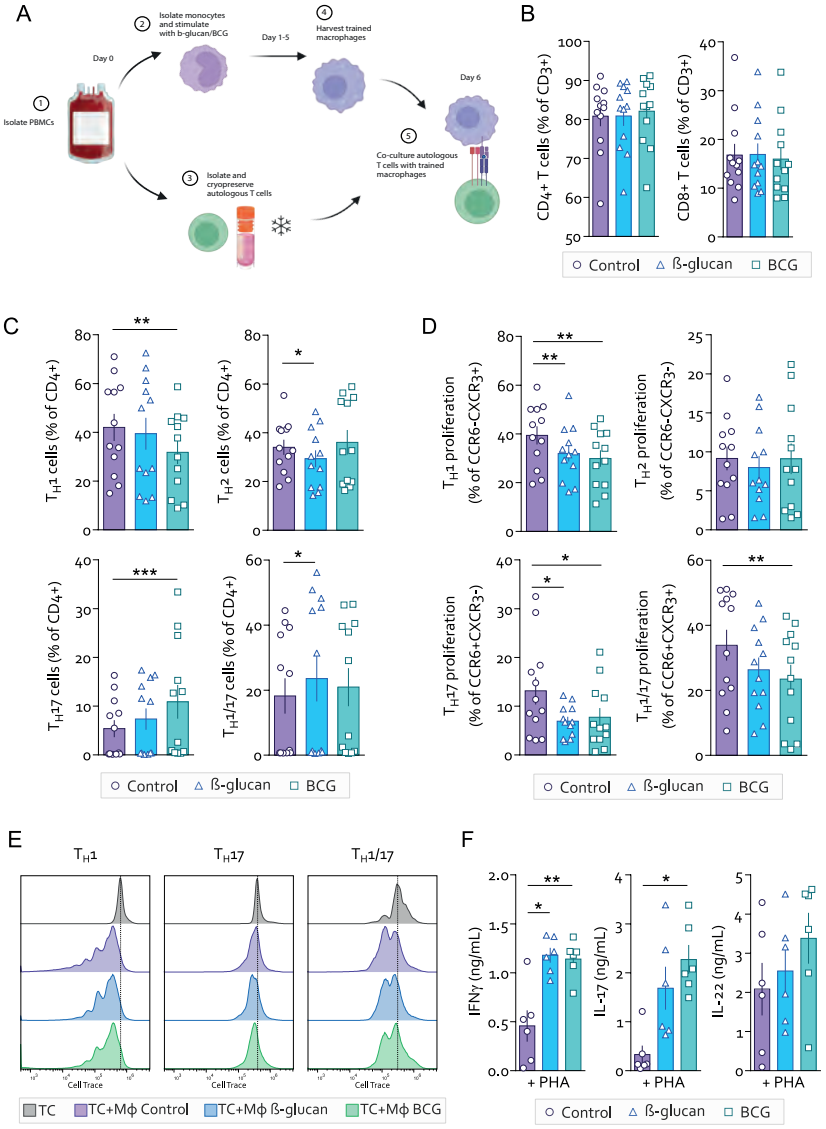

Figure 2. T cells exposed to trained macrophages present a skewed phenotype towards T_H17. (A) Schematic overview of experiment of T cell and macrophage co-culture. (B) Percentage of CD4+, CD8+ T cells after two weeks of co-culture with control, β-glucan or BCG trained macrophages. (C) T_H17, T_H2, T_H17 and $T_H1/17$ subtypes of CD4+ T cells after two weeks of co-culture with β-glucan or BCG macrophages. Percentage (D) of T_H1, T_H2, T_H17 and $T_H1/17$ subtypes of CD4+ T cells in proliferation after two weeks of co-culture with control, β-glucan or BCG macrophages, and representative histogram (E). (F) IFN-γ, IL-17 and IL-22 production after two weeks of co-culture of T cells with control, β-glucan or BCG trained macrophage and upon PHA restimulation for 24 hours. Data shown as mean + SEM, n=6-12 biological replicates, pooled from 2-4 independent experiments. Friedman test, followed by Dunn's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001). Figure A was created with BioRender.com.

T cells co-cultured with trained macrophages produce more cytokines upon stimulation in an antigen-agnostic manner

To determine whether T cells co-cultured with trained macrophages are also functionally altered, T cells and trained macrophages co-cultures were further stimulated with PHA for 24 hours. T cells that were co-cultured with β-glucan or BCG trained macrophages showed robust increase in IFN-γ production (Figure 2F), while only BCG trained macrophages influenced T cells to a higher IL-17 secretion; in line with the elevated proportion of T_H17 cells resulting from these co-cultures (Figure 2D). It is important to note that this increase in IFN-γ and IL-17 production were only observed upon PHA stimulation. The basal production of IFN-γ and IL-17 were instead decreased when T cells were cultured with BCG or β-glucan trained macrophages, respectively (Figure S3A, B). Most interestingly, allied to the decrease in basal T cytokine production and the increase in CD274 (PD-L1) expression by trained macrophages (Figure 1 C, Figure S3C), the expression of the T cell exhaustion marker PD-1 was reduced in CD4+ T cells after T cell co-culture with BCG trained macrophages (Figure S3D).

To further functionally profile the T cells following co-culture with trained macrophages, macrophages were exposed to *S. aureus* or R848 prior to addition of the autologous T cells (Figure 3A). Stimulating macrophages with *S. aureus* and R848 prior to addition of T cells lead to global reductions in the percentages of CD4+ T cells irrespective of macrophage training condition (Figure 3B). However, the degree of activated CD4+ (CD38+HLA-DR+) T cells and T_H17 cells was enhanced upon co-culture with *S. aureus* stimulated macrophages (Figure 3C, D). The viral mimicking particle R848, often used for inducing antigen presentation in DCs, failed to increase the percentage of T_H17, yet enhanced the percentage of activated CD4+ T cells in control and β-glucan trained macrophages.

Next, we aimed to test the ability of trained macrophages to mount an antigen specific response *in vitro*. To accomplish this, the antigen keyhole limpet hemocyanin (KLH) was used to pulse the macrophages prior to addition of T cells (Figure 3A). We determined that a higher percentage of β-glucan trained macrophages are positive for KLH surface expression, and a similar trend was observed for BCG trained macrophage (Figure 3E), which supports our previous finding that trained immunity promotes the expression of MHC molecules. The addition of KLH to stimulus naïve macrophages led to the increase of T_H17 percentages, however, the skewing of T cells towards the T_H17 subset by β-glucan or BCG trained macrophages was not altered (Figure 3F). Thus, suggesting that the effects of trained macrophages on T cell skewing are not dependent on specific antigen presentation. No effects were observed for other T_H subsets (data not shown).

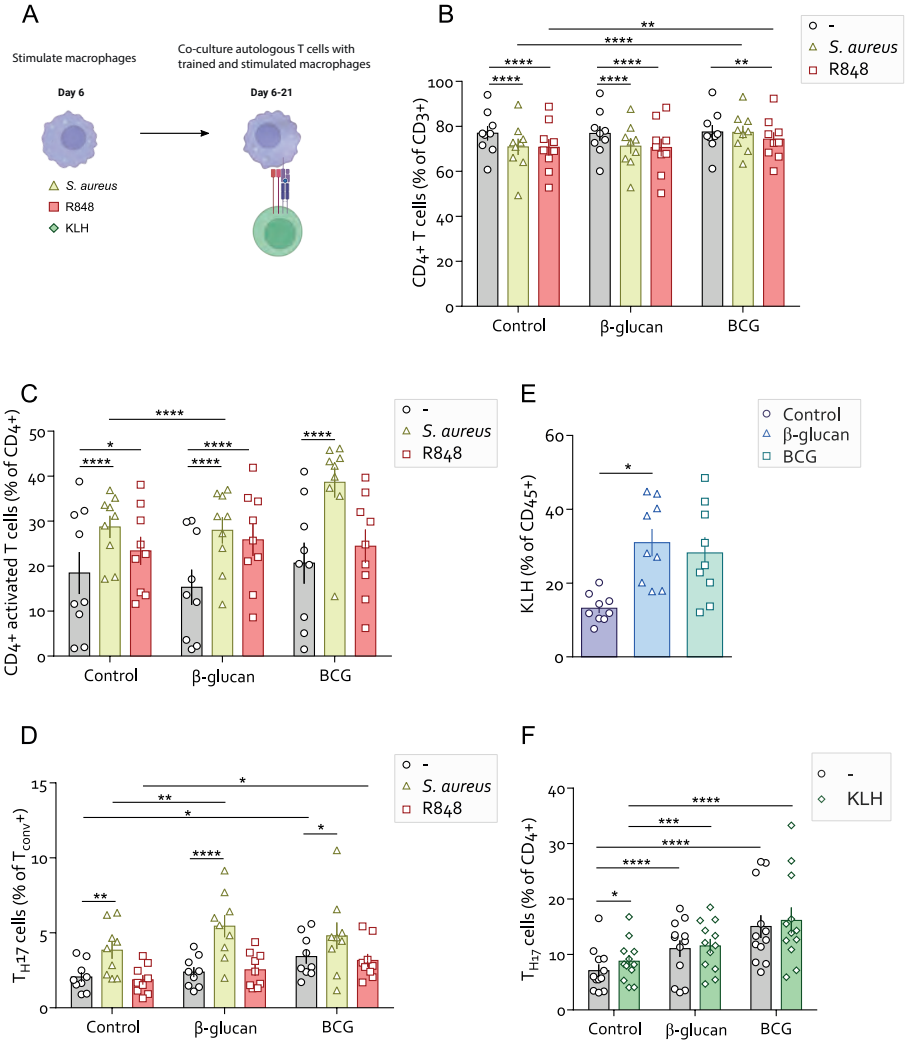

Figure 3. *S. aureus* and KLH stimulation of macrophages increases T_H17 skewing. (A) Schematic overview of experiment of T cell and macrophage co-culture after exposure with *S. aureus*, R848 or KLH. Percentage of CD4+ T cells (B), activated CD4+ (CD38+HLA-DR+) T cells (C) and T_H17 cells (D) after two weeks of co-culture with control, β-glucan or BCG trained macrophages and stimulated with *S. aureus* or R848. (E) Percentage of KLH+ macrophages after control, β-glucan or BCG trained macrophages were pulsed with KLH. (F) Percentage of T_H17 cells after two weeks of co-culture with control, β-glucan or BCG trained macrophages and pulsed with KLH. Data shown as mean + SEM, n=9-12 biological replicates, pooled from 3-4 independent experiments. (C-D, F) Two-way ANOVA, followed by Dunnett's multiple comparisons test. (E) Friedman test, followed by Dunn's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Figure A was created with BioRender.com.

Human BCG vaccination recapitulates in vitro co-culture features of T_H17 cell potentiation

To validate our *in vitro* findings, we took advantage of data obtained from the 300BCG cohort, where 323 healthy individuals were vaccinated with BCG (15). These individuals were genotyped and their PBMCs were evaluated for immune cell frequency and cytokine production capacity (Figure 4A).

Firstly, we assessed whether common single nucleotide polymorphisms (SNPs) (MAF > 0.05) in a window of 150 kb around the activation markers genes *CD40*, *CD80* and *CD86* were associated with changes in cytokine production. We identified various SNPs within 150 kb of the genes of interest that were suggestively associated ($p < 0.05$) with changes in the production of the T cell cytokines IFN-γ and IL-17 and the innate cytokines IL-1β, IL-6 and TNF upon *C. albicans* or *S. aureus ex vivo* stimulation, both 14 (Figure 4B) and 90 days (Figure 4C) after vaccination. The most strongly associated SNPs are highlighted in the scatter plots. Specifically, the variation in *CD40* (rs8123864, $p = 0.0007$) was associated with the fold increase of IL-17, 90 days after vaccination and upon *ex vivo* stimulation with *C. albicans* (Figure 4D, right panel). The *CD86* (rs2681416, $p = 0.00003$) was associated with the potentiation of IFN-γ, 90 days after vaccination and upon *ex vivo* stimulation with *S. aureus* (Figure 4D, left panel).

Additionally, immune cell frequency analysis was performed on the PBMCs collected from the 300BCG cohort. In accordance with our *in vitro* data, BCG vaccination recapitulated the observed reduction in T_H1 cells and increase in T_H17 cell percentages 90 days post vaccination (Figure 4E). Interestingly, an unexpected increase was observed for T_H2 cells 90 days post vaccination with BCG.

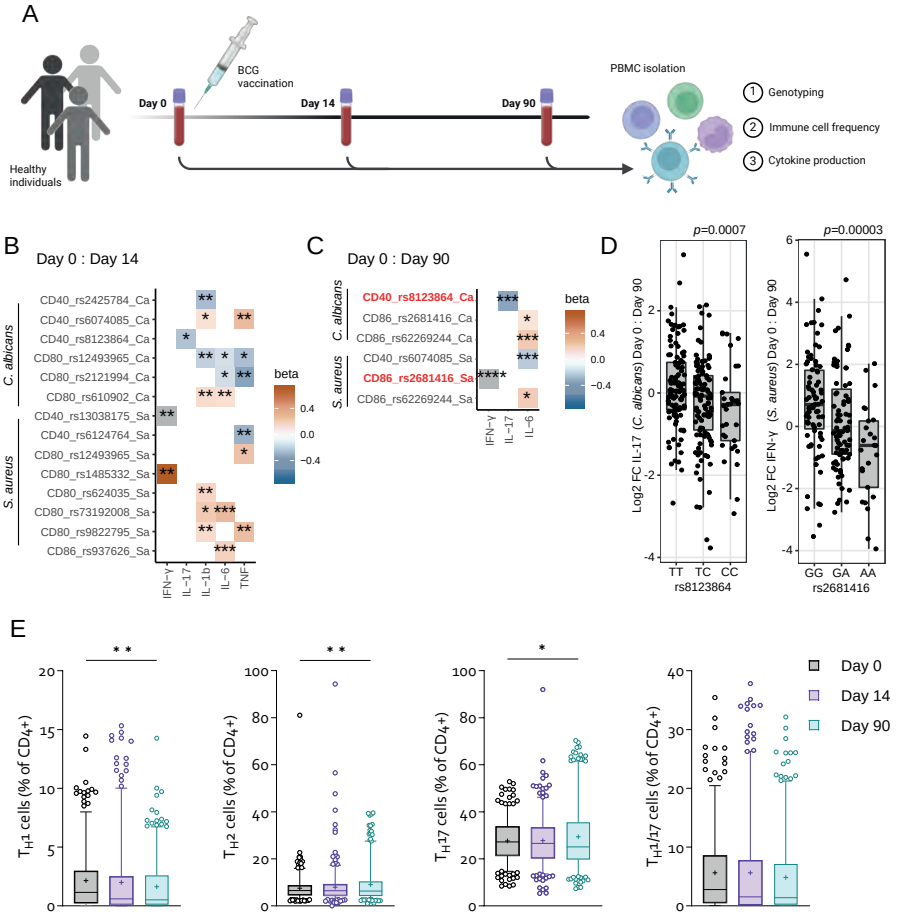

Figure 4. BCG vaccination of healthy individuals leads to T_H17 skewing. (A) Schematic overview of the 300BCG cohort. (B-C) Heatmaps of the beta coefficients of the associations between SNPs around *CD86*, *CD80* and *CD40* genes (+/- 150kb) and the fold change of the production of IL-17, IFN-γ, IL-1β, IL-6 and TNF after *C. albicans* or *S. aureus* stimulation (B) 14 days or (C) 90 days after BCG vaccination (n=317). (D) Beta coefficients are derived from a linear model using age, sex, time of blood drawing and log2 fold change of PBMC cell type fractions as covariates (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Example boxplot of SNP with the lowest p-value stratified according to genotype. (E) Boxplot shows median, upper and lower quartiles, whiskers extending to the most extreme points less than 1,5x the interquartile range from the box. T_H1, T_H2, T_H17 and $T_H1/17$ subtypes of CD4+ T cells prior, 14 days and 90 days after BCG vaccination (n=319). Boxplots show median with quartiles, with the whiskers extending from the 5th – 95th percentiles, showing individual points for extremes and + for the mean. Friedman test, followed by Dunn's multiple comparisons test (*p<0.05, **p<0.01). Figure A was created with BioRender.com.

Discussion

The current study demonstrates that BCG and β-glucan increase the expression of activation and antigen presentation molecules of macrophages, which in turn promote T cell functional and phenotypical changes.

We show that trained macrophages skew T cells to a T_H17 subset, which secrete more IFN-γ and IL-17 upon stimulation. In line with this finding, our group has previously demonstrated that BCG vaccination led to a greater production of IFN-γ and IL-17 by stimulated PBMCs up to 1 year post-vaccination (9,16). This increase in cytokine secretion was seen in *Mycobacterium tuberculosis* (MTB) stimulated cells, but also upon exposure to unrelated pathogens *C. albicans* and *S. aureus*, suggesting an antigen non-specific effect of BCG trained immunity on T_H1 and T_H17 cell responses. Similarly, our *in vitro* approach, where T cells were co-cultured with BCG trained macrophages, also revealed that non-specific stimulation with PHA increases both IFN-γ and IL-17 secretion. Given that T_H1 percentages were decreased while T_H17 cells' percentages were elevated upon co-culture with BCG-trained macrophages, and that T_H17 cells have the capacity to secrete both IL-17 and IFN-γ (17,18), we hypothesize that this cell population is responsible for secreting both cytokines. Interestingly, we also observed this shift, from T_H1 to T_H17 polarization, in the peripheral blood of a cohort of healthy individuals vaccinated with BCG.

A particularly surprising finding is that co-culture with BCG-trained macrophages led to an elevated proportion of T_H17 cells while concomitantly decreasing T_H17 proliferation. This phenomenon needs further exploration, however this may be the result of a tightly regulated maturation of T_H17 cells, as opposed to antigen-specific activation that would lead to their clonal expansion. Additionally, previous *in vitro* and *in vivo* models have shown that trained macrophages are enriched for IL-17 signaling pathway (19), highlighting the potential of this macrophage phenotype to drive T cells towards T_H17 polarization. Differentiation to T cell effector or T cell memory subsets was not modulated by trained macrophages in our model. This finding highlights the ability of trained macrophages to enhance T cell cytokine production without driving specific effector or memory T cell differentiation. However, it is important to note that this study was performed with *ex vivo* circulating cells, while tissue trained innate immune cells may contribute towards adaptive immunity differently. Notably, in a mouse model of influenza A infection, BCG increased CD4+ effector T cells in the lung which limited viral infection in an antigen-independent manner (20).

We explored the trained macrophage phenotype that could underlie this T_H17-promoting capacity. We show that *in vitro* trained macrophages could be separated into 8 distinct populations by surface marker expression. This is in line with previous research that identified 11 distinct populations through single-cell RNA sequencing of *in vitro* trained macrophages (19). Specifically, we show that trained macrophages were enriched for macrophage activation markers CD40 and CD86 as well as MHC-complexes HLA-A/B/C, HLA-DR, HLA-DM, and CD74. This unique fingerprint of activation markers and MHC molecules, as well as yet-to-be-identified soluble factors, may be responsible for how BCG-trained macrophages exhibit a T_H17 cell-skewing capacity. Additionally, in a cohort of BCG vaccinated individuals, we have identified genetic variations in the proximity of macrophage activation marker genes which were suggestively associated with changes in the *ex vivo* production of IFN-γ and IL-17. Taken together, the activation profile of trained macrophages appears to contribute to their capacity to potentiate T_H17 responses.

In the context of vaccine development, our findings support the potential of trained immunity to enhance overall immune responsiveness. BCG vaccination has been shown to improve the effectiveness of other vaccines, as was demonstrated for the H1N1 influenza vaccine, where prior BCG vaccination led to enhanced cytokine responsiveness against non-specific *ex vivo* stimulation with a faster and more resilient induction of functional antibody responses to H1N1 vaccination (21). This enhanced non-specific immune state was shown to be beneficial against experimental infectious challenge with yellow fever viremia (22). However, the potential for off-target effects, as observed in some COVID-19 vaccines (23), necessitates careful consideration during vaccine design.

BCG vaccination has also been implicated in cancer therapy (24,25). Intravesical BCG administration has long been used for the treatment of bladder cancer, which has recently been demonstrated to have carry-over benefits against respiratory viral infections (26). We now show that BCG-trained macrophages downregulated PD-1 expression on T cells. PD-1 is a critical immune checkpoint molecule that contributes to T cell exhaustion, a hallmark of tumor immune-evasion (27). By reducing PD-1 expression on T cells, BCG-trained macrophages may have the potential to reverse T cell exhaustion and improve the efficacy of cancer immunotherapy strategies that target the PD-1/PD-L1 pathway. To our knowledge, no studies have directly aimed to study the reversal of T cell exhaustion in cancer by the induction of *in vivo* trained immunity with BCG. However, reversing the pro-tumor phenotype of tumor associated macrophages via targeted induction of trained immunity with HDL-based nano biologics loaded with a modified muramyl-dipeptide

has been shown to effectively improve tumor killing. This therapeutic strategy made the tumors more sensitive to checkpoint blockade immunotherapy against PD-1 and CTLA-4 (28). Similarly, β-glucan has been shown to synergize with PD-L1 blockade therapy prolonging the survival of a murine pancreatic ductal adenocarcinoma model (29).

Defining the effects of trained immunity on T cell responses in various disease contexts is crucial for understanding its clinical relevance. Exploring the interactions between trained immunity and adaptive responses could aid in the development of better therapeutic interventions and vaccine strategies, particularly for vulnerable populations like infants, the elderly, and the immunocompromised (30).

Conclusion

In conclusion, this study provides the first evidence that BCG-trained macrophages can directly enhance T cell function. Our findings suggest that BCG-trained macrophages promote a T_H17 cell response and downregulate the T cell exhaustion marker PD-1. This research highlights the potential of trained immunity to enhance overall immune responsiveness, influencing both innate and adaptive immunity.

Acknowledgements

We thank Hans Jacobs for consulting on the design of our *in vitro* T cell activation model. M.N. and L.J. received funding from the European Union's Horizon 2020 research and innovation program (grant agreement no. 667837). M.N. was supported by an ERC Advanced Grant (European Union's Horizon 2020 research and innovation program, grant agreement no. 833247) and a Spinoza grant from the Netherlands Organization for Scientific Research (NWO). L.H., L.G. and A.V.F., conceived, designed, and performed the experiments. L.H. analyzed the results. L.H., L.G. and A.V.F., wrote the manuscript. All authors critically read the manuscript.

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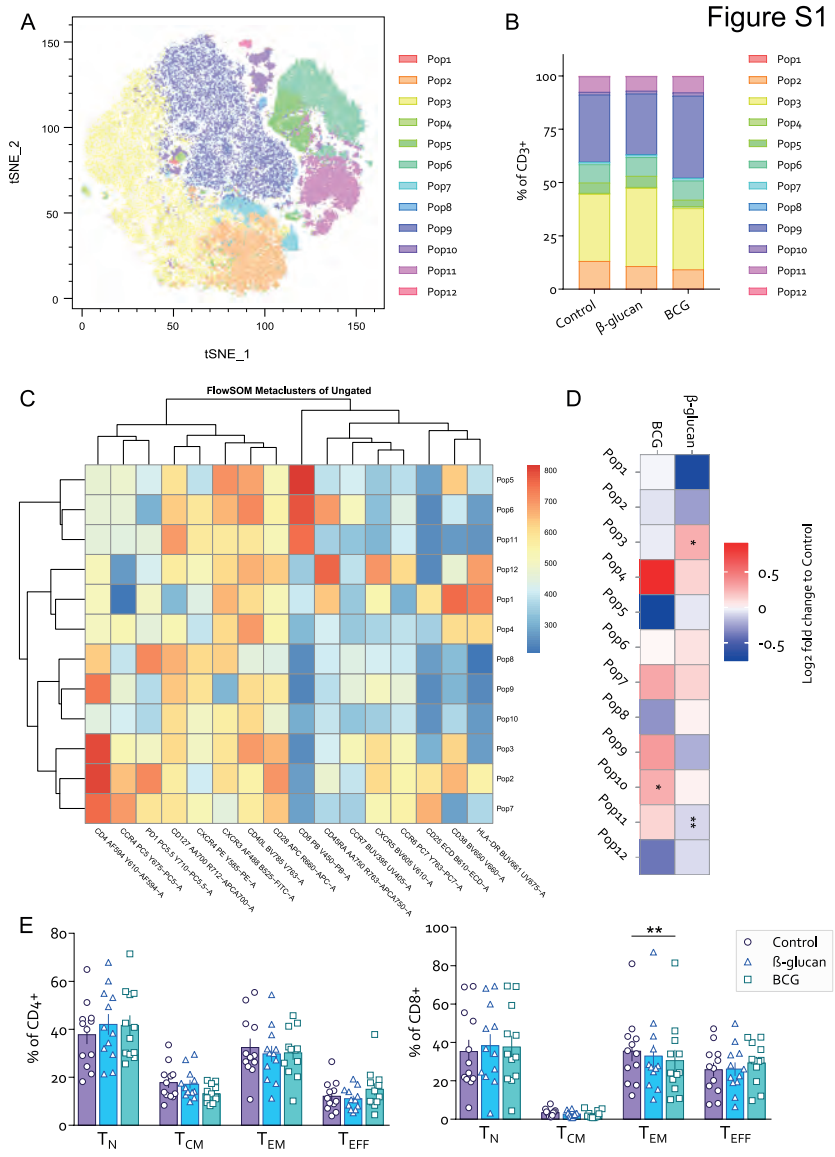
Supplemental material

Table S1. List of antibodies used for macrophage immunophenotyping

S1A. General macrophage immunophenotyping			
Antibody	*Fluorophore*	*Clone*	*Manufacturer*
HLA-DR	BUV661	G46-6	Becton Dickinson
CD86	BUV737	FUN-1	Becton Dickinson
CD40	BV421	5C3	Biolegend
CD45	BV510	HI30	Biolegend
CD11c	BV605	3.9	Biolegend
CD80	BV650	L307.4	Becton Dickinson
CD274	BV785	29E.2A3	Biolegend
CD273	KB520	24F.10C12	Biolegend
CD14	PerCP-Cy5.5	63D3	Biolegend
CD206	PE	15-2	Biolegend
CD4	AF594	RPA-T4	Biolegend
CD163	PE-Cy5	GHI/61	Biolegend
CD38	PE-Cy7	HB-7	Biolegend
CD11b	APC	ICRF44	Biolegend
CD16	APC-Fire750	3G8	Biolegend
S1B. Intra- and extracellular HLA molecule measurements			
Antibody	*Fluorophore*	*Clone*	*Manufacturer*
CD45	APC	J33	Beckman coulter
HLA-A,B,C	FITC	W6/32	Biolegend
HLA-DR,DP,DQ	BV421	Tu39	Becton Dickinson
HLA-DM	PE	MaP.DM1	Biolegend
CD74	FITC	MB741	Becton Dickinson

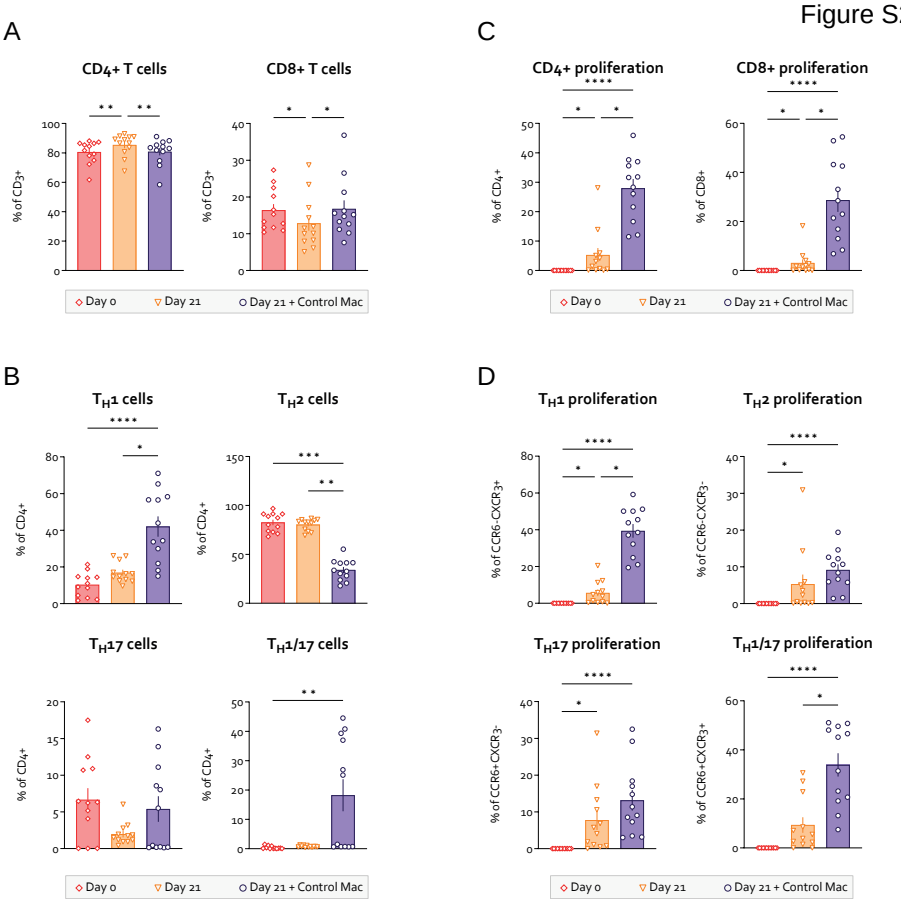
Table S2. List of antibodies used for T cell proliferation assay and immunophenotyping

S2A. T cell proliferation assay			
Antibody	*Fluorophore*	*Clone*	*Manufacturer*
CD197 (CCR7)	FITC	3D12 (rat anti-human)	Becton Dickinson
CD3	APC-AF750	UCHT1	Beckman Coulter
CD8	PE-Cy7	RPA-T8	Becton Dickinson
CD4	APC	RPA-T4	Biolegend
CD45RA	PE	HI100	Biolegend
CD196 (CCR6)	BV650	G034E3	Biolegend
CD183 (CXCR3)	BV785	G025H7	Biolegend
S2B. General T cell immunophenotyping - part I			
Antibody	*Fluorophore*	*Clone*	*Manufacturer*
CD28	APC	CD28.2	Biolegend
CD4	AF594	RPA-T4	Biolegend
CD3	PerCP	UCHT1	Biolegend
CD38	BV786	HB-7	Biolegend
CD154 (CD40L)	BV650	24-31	Biolegend
CD185 (CXCR5)	BV605	J252D4	Biolegend
CD56	BUV737	NCAM16.2	BD Biosciences
HLA-DR	BUV661	G46-6	BD Biosciences
CD197 (CCR7)	BUV395	2-L1-A	BD Biosciences
S2C. General T cell immunophenotyping - part II (DURA Innovations)			
Antibody	*Fluorophore*	*Clone*	*Manufacturer*
CD8	PB	B9.11	Beckman Coulter
CD45	KrO	J33	Beckman Coulter
CD183 (CXCR3)	AF488	G025H7	Beckman Coulter
CD25	ECD	B1.49.9	Beckman Coulter
CD184 (CXCR4)	PE	12G5	Beckman Coulter
CD194 (CCR4)	PC5	L291H4	Beckman Coulter
CD279 (PD-1)	PC5.5	PD1.3	Beckman Coulter
CD196 (CCR6)	PC7	B-R35	Beckman Coulter
CD127	APC-A700	R34.34	Beckman Coulter
CD45RA	APC-A750	2H4LDH11LDB9	Beckman Coulter



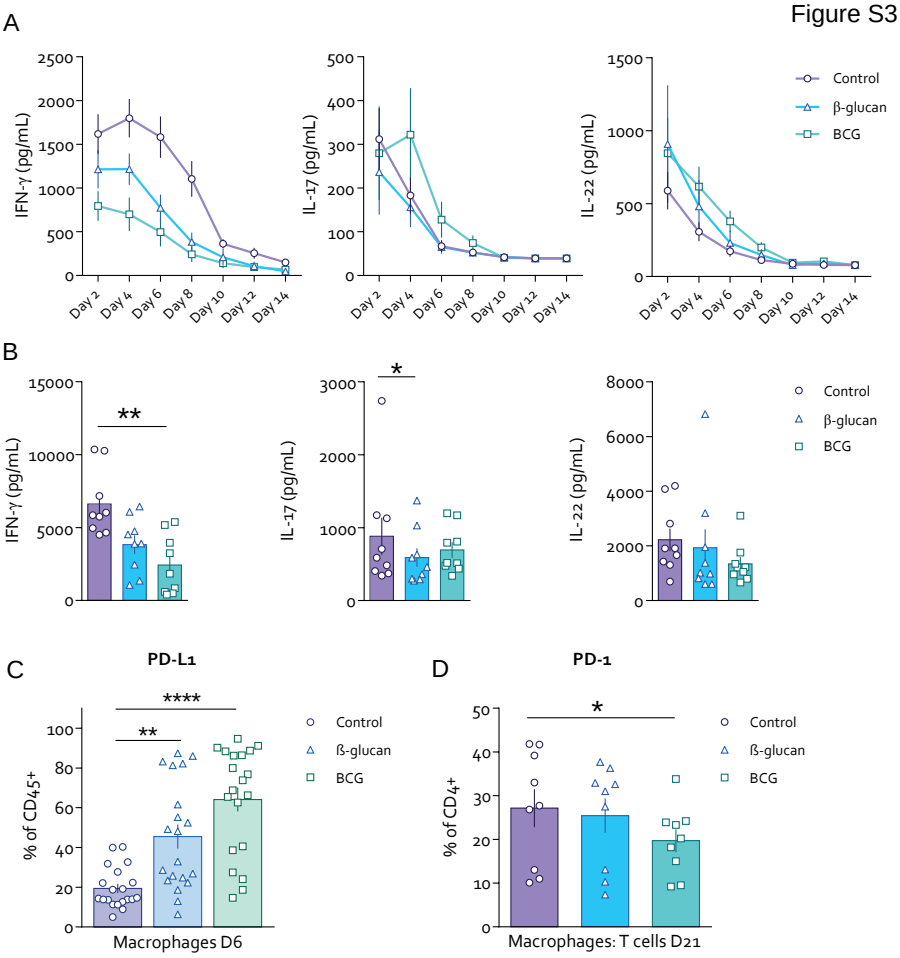
Supplemental Figure S1. T cells co-cultured *in vitro* with macrophages trained with β-glucan and BCG present distinct populations. (A) tSNE analysis based on HLA-DR, CD38, CD25, CCR6, CXCR5, CCR7, CD45RA, CD8, CD28, CD40L, CXCR3, CXCR4, CD127, PD1, CCR4 and CD4 reveals different clustering features for T cells co-cultured with control, β-glucan or BCG trained macrophages. (B) FlowSOM (self-organizing maps of flow cytometry data) analysis reveals 12 CD3+ T cell subpopulations. (C) Heatmap of FlowSOM clustering analysis. (D) FlowSOM analysis by training stimulus. (E) Naive, effector, central memory and effector memory subsets of (left panel) CD4+ T cells and (right panel) CD8+ T cells after two weeks of co-culture with control, β-glucan or BCG trained macrophages. Data shown as mean + SEM, n = 12 biological replicates, pooled from 4 independent experiments. Friedman test, followed by Dunn's multiple comparisons test (*p<0.05, **p<0.01).

Figure S2

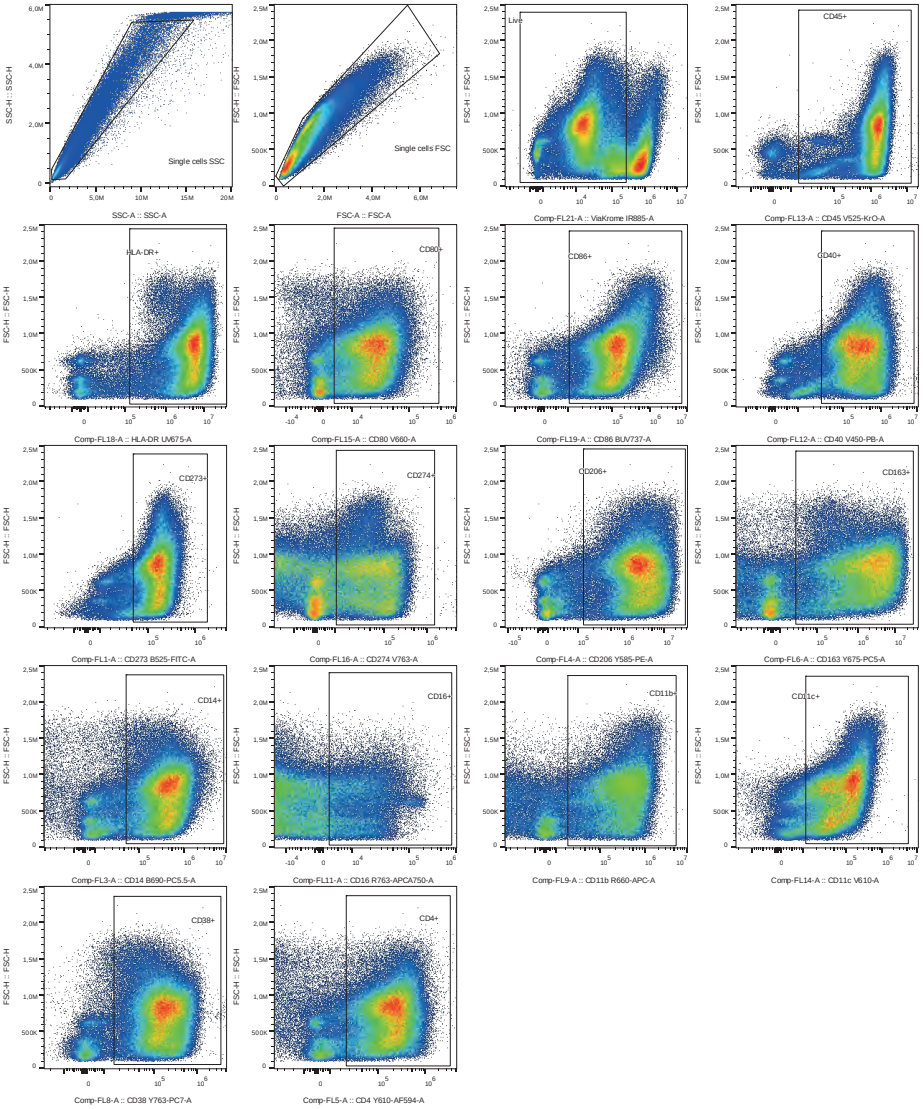


Supplemental Figure S2. T cells co-cultured *in vitro* with autologous macrophages exhibit changes in T cell subpopulations and proliferation. (A,B) Incubation with macrophages did not change ratio of CD4+ or CD8+ T cell populations (A), but affected T_H subset populations (B). (C) Without adding stimuli, macrophage co-incubation resulted in T cell proliferation, which was seen in CD4+ and CD8+ T cells, (D) as well as in T_H subsets. Data shown as mean + SEM, n = 12 biological replicates, pooled from 4 independent experiments. Friedman test, followed by Dunn's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

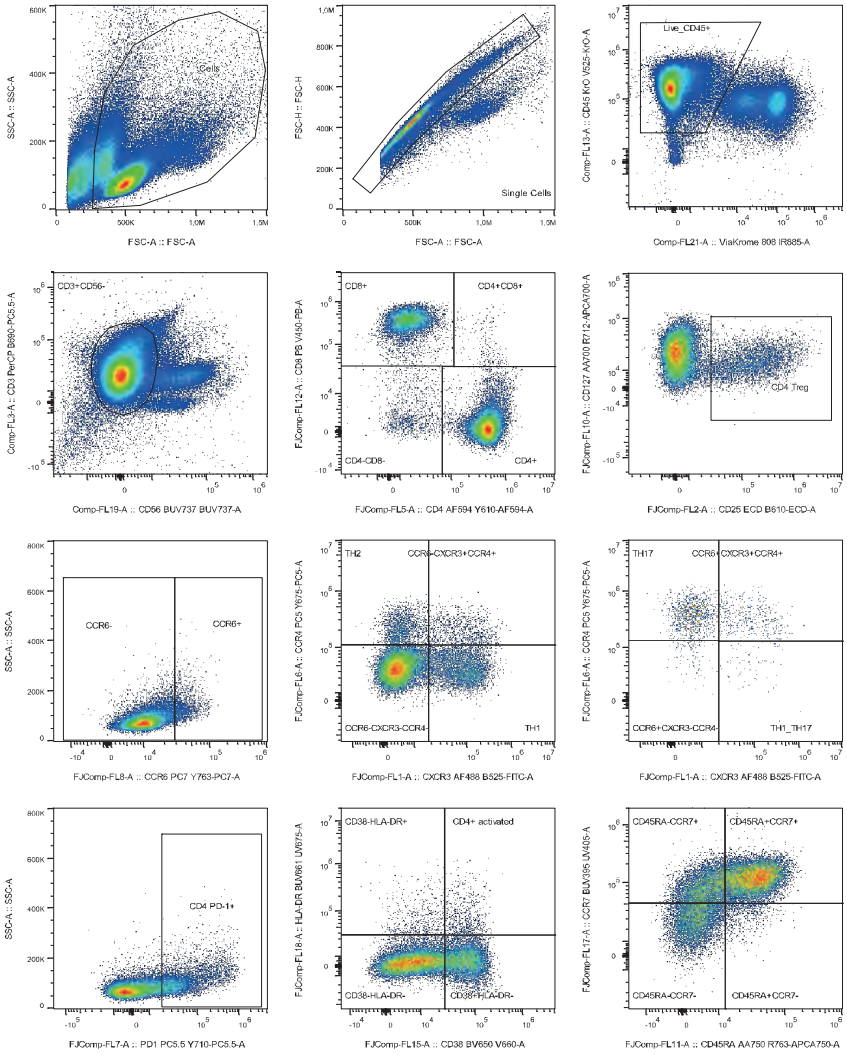
Figure S3



Supplemental Figure S3. Cytokine production and PD-(L)1 expression upon T cell and trained macrophages co-culture. (A,B) Basal production of IFN-γ, IL-17, and IL-22 during 14 day co-culture (n=9). (C) Expression of CD274 (PD-L1) in control, β-glucan or BCG trained macrophages (n=14). (D) Expression of PD-1 in CD4+ T cells after 14 day co-culture with control, β-glucan or BCG trained macrophages (n=9). Data shown as mean + SEM. Friedman test, followed by Dunn's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).



Supplemental Figure S4. Macrophage phenotyping gating strategy.



Supplemental Figure S5. T cell immunophenotyping gating strategy.

Chapter 8

Summary of the thesis

The chapters in this thesis provide a combination of exploratory, translational, and clinical research, investigating the dysregulation of inflammation in response to surgery and anesthesia, and how it relates to the incidence of postoperative complications. We focus on inflammatory mechanisms, epigenetic alterations, and metabolic adaptations of innate immune cells, aiming to enhance our understanding of immunomodulation in the perioperative period.

Chapter 1 discusses the general outline of the thesis. We present a brief overview of the different factors influencing the immune response to surgery, including surgical trauma, release of inflammatory mediators, and the effects of anesthesia. We outline how surgical injury can trigger subsequent pro- and anti-inflammatory immune responses via the release of danger-associated molecular patterns (DAMPs) from damaged tissues. We introduce several pre-, intra-, and post-operative factors that can modulate the immune response. Finally, we examine the potential role of innate immune memory, or trained immunity, in the responsiveness of innate immune cells after surgery. This chapter sets the stage for exploring the effects of perioperative measures on immune responses, particularly focusing on monocytes and the therapeutic potential of trained immunity.

Colorectal surgery has a high rate of postoperative infectious complications. The early postoperative immune response is predominantly suppressive, increasing vulnerability to infections. In **Chapter 2** of this thesis, we examined how surgical trauma and anesthesia induce epigenetic and functional changes in monocytes, contributing to postoperative immune dysregulation. Understanding the innate immune phenotype and the impact of surgical tissue injury on immune homeostasis is crucial. Decreasing surgical tissue injury, such as by lowering intra-abdominal pressure during laparoscopic surgery, can preserve innate immune homeostasis and reduce complication rates.

The study found significant increases in circulating DAMPs and inflammatory mediators post-surgery, while the *ex vivo* cytokine production capacity was suppressed. We demonstrate that exposing healthy monocytes to DAMPs *in vitro* results in a decrease in cytokine production capacity, supporting the hypothesis that surgical injury directly suppresses the innate immune system. Analysis of changes in the epigenetic landscape in monocytes alongside the inflammatory proteome revealed that inflammatory markers IL-6 and S100A12 (among others) were increased after surgical intervention, while IFN-γ was decreased. It remains to be determined whether these changes in gene accessibility are transient, and to what extent they represent a causal factor in post-operative immune suppression.

In **Chapter 3**, we investigate the role of surgical tissue injury in postoperative immune suppression in breast cancer patients. Breast-conserving surgery (BCS) and mastectomy show different survival rates, potentially due to varying degrees of surgical trauma and subsequent immune dysregulation. Mastectomy involves more extensive tissue damage, translating to higher levels of DAMPs and sympathetic activation, which could suppress the immune response.

We found that levels of alarmins S100A12 and S100A8/A9 were increased post-mastectomy compared to BCS, correlating with higher immune suppression and complications. The study supports the idea that more extensive surgical trauma leads to greater immune suppression, impacting survival rates. In this cohort, HMGB1 and HSP70 did not show significant changes, suggesting different factors may influence immune responses in different types of surgery. The study also examined the role of intraoperative activation of the sympathetic nervous system in postoperative immune suppression, highlighting the role of postoperative pain and immune suppression in the development of infectious complications.

In **Chapter 4** of the thesis, we move on from observational studies to evaluate the effects of a multimodal prehabilitation program, including exercise and nutritional optimization, on immune function and mitochondrial fitness. This chapter explores the underlying mechanisms of how these interventions affect immune responses to surgical stress. Exercise and nutritional interventions are known to modulate immune cell function in healthy individuals, but the effects on immune responses to surgical stress have not been explored.

We demonstrate that prehabilitation significantly affected the number and composition of circulating leukocytes, potentially affecting immunological readiness for surgery. Proteomic analysis revealed that prehabilitation preserved inflammation-related proteins better than the control group. We also found that prehabilitation increased mitochondrial membrane potential and reduced ROS production post-surgery, indicating enhanced cellular resilience through mitochondrial and metabolic adaptations. The potential of prehabilitation to modulate immune responses and improve surgical outcomes through immunometabolic conditioning warrants further investigation.

In **Chapter 5** and **Chapter 6**, we examine immunomodulatory effects of medications and their potential to induce trained immunity. Propofol, a commonly used anesthetic, has been linked to acute immunomodulatory effects. **Chapter 5** investigates whether brief exposure to propofol can induce long-term immune

changes, known as trained immunity, which could affect inflammatory responses after surgery and susceptibility to infections. Understanding how propofol influences immune responses can help optimize anesthesia protocols, aiming to minimize postoperative complications. Trained immunity could play a crucial role in providing enhanced defense against infections but may also lead to excessive inflammation if not regulated.

We established that propofol acutely modulates the immune response, but also induced a trained immunity phenotype in monocytes, enhancing cytokine production, cellular metabolism, and pathogen killing. We highlight the role of augmented fatty acid metabolism in the induction of trained immunity by propofol. The potentially broad clinical implications of these findings, particularly in immunocompromised patients, underline the need for a more complete understanding of the mechanisms and long-term effects of propofol-induced trained immunity.

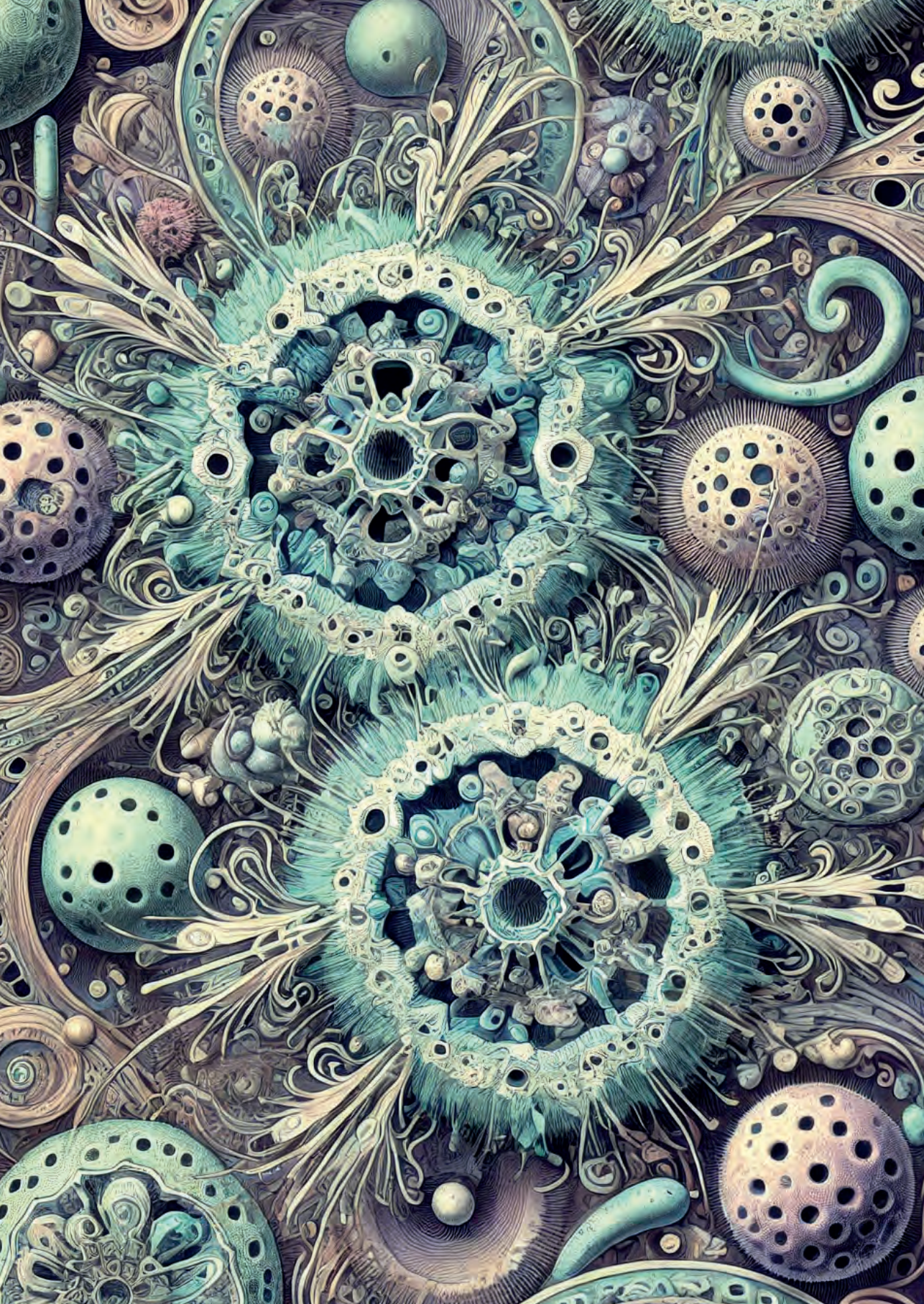
Chapter 6 provides a thorough exploration of the immunostimulatory properties of antifungal drug Amphotericin B (AMB). This chapter explores whether AMB can induce trained immunity, and the implications for long-term immune responsiveness and pathogen clearance. Inducing trained immunity with AMB resulted in enhanced cytokine production, metabolism, oxidative burst, and antimicrobial activity. The study also found that the cholesterol synthesis pathway played a crucial role in AMB-induced trained immunity. We emphasize that AMB-induced trained immunity could offer immunotherapeutic benefits, particularly in enhancing host defense mechanisms against a broad range of pathogens. This could be valuable in immunocompromised patients.

In the final chapter of this thesis, we sought to investigate how trained immunity influences T cell function and polarization. Understanding the interplay between trained innate immunity and adaptive responses can improve therapeutic interventions and vaccine strategies. In **Chapter 7**, we demonstrated that BCG-trained macrophages skew T cells towards a T_H17 phenotype, enhancing cytokine production by these cells. This effect was linked to changes in the antigen-presentation machinery of trained macrophages.

Our study highlights the potential of trained immunity to augment non-specific adaptive immune responsiveness, reducing expression of T cell exhaustion markers. This could have implications for designing vaccines and therapies that seek to

harness trained immunity to boost their effectiveness. However, the potential for off-target effects necessitates careful consideration in therapeutic applications.

Collectively, the research in this thesis highlights the significant impact of surgical trauma, anesthesia, and perioperative interventions on immune responses. Understanding the mechanisms behind immune modulation can lead to better strategies for enhancing patient outcomes. Key findings include the insights in immune suppression resulting from surgery and anesthesia in multiple patient cohorts, the potential of trained immunity to provide enhanced defense against infections, and the importance of balancing immune responses to prevent excessive inflammation or immune suppression.



Chapter 9

General discussion and future perspectives

General discussion

The surgical stress response is a complex interplay of tissue damage, immunomodulation by various medications and interventions, and dysregulation of the immune response. In this thesis, several research questions regarding host immune responses in the surgical setting were investigated. While each chapter examines a different aspect of the intricacy of the immune system's response to surgery and anesthesia, we find persistent pathways of immune activation and suppression that indicate ubiquitous mechanisms in the surgical stress response (figure 1). The most important findings of this thesis will be discussed in the context of two crucial aspects of immunomodulation in the perioperative period; in **part I**, the mechanisms of innate immune dysregulation in patients undergoing surgery, and in **part II**, the potentially protective effects of trained immunity against unrelated infections.

Part I: Mechanisms of innate immune dysregulation in patients undergoing surgery

Advances in our knowledge of immunomodulatory effects of the surgical stress response

We have demonstrated that surgical stress responses result in the suppression of the innate immune system. In several clinical trials targeting various groups of patients with diverse indications for undergoing surgery, co-morbidities, and perioperative factors, we consistently show that the surgical stress response suppresses the function of innate immune cells.

In **Chapter 2**, we establish that laparoscopic colorectal surgery results in postoperative innate immune dysregulation. The release of DAMPs and inflammatory mediators was shown in a large cohort of patients, and we described a direct effect of DAMPs HSP70 and HMGB1 on the suppression of immune cell responsiveness *in vitro*. A key finding in this chapter is the concurrent changes in monocyte epigenetic modifications and circulating inflammatory markers, suggesting a key role for monocytes in immune suppression following surgical tissue injury and anesthesia. Gene accessibility after surgery remains a largely underexplored area of research. However, in many other studies involving critically ill patients, e.g. in COVID-19, sepsis, and other severe infections, it has been made evident that epigenetic changes contribute to dysregulated host immune responses, and can sustain prolonged effects on immune cells, resulting in a long-term immunosuppressed state (1-3). At this time, we do not know if surgical

intervention could lead to similar long-term epigenomic changes, and how this might affect clinical outcomes.

To further explore the role of DAMPs in post-operative immune suppression, **Chapter 3** explores the differential immune responses in patients undergoing breast-conserving surgery (BCS) versus mastectomy. We demonstrate once more that surgical tissue injury and anesthesia result in increased plasma DAMPs and inflammatory cytokines, and a profound suppression of cytokine production capacity. In addition, differences in the immune response between BCS and mastectomy patients indicate that more extensive tissue injury results in higher concentrations of DAMPs, higher severity of inflammation, and lower cytokine production capacity. In addition, sympathetic nervous system activation, an indication of intraoperative stress and nociception, was higher in mastectomy patients, and was found to coincide with a higher risk of postoperative infections. These findings suggest that the magnitude of surgical tissue injury directly influences the degree of immune suppression, possibly contributing to the differences in clinical outcomes (4). Indeed, previous research has revealed that impaired postoperative *ex vivo* cytokine production capacity is associated with the development of infectious complications (5). Similar associations have been established between early postoperative pain and development of infectious complications (6).

Novel insights into the management of surgical stress

Chapter 4 of this thesis investigates the impact of multimodal prehabilitation on inflammation, immunometabolism, and mitochondrial dynamics in surgical patients. Composition of circulating immune cell populations, intracellular metabolic pathways, and mitochondrial function were all affected by prehabilitation, suggesting that lifestyle interventions could affect immune readiness for surgery. We observed features of immune suppression, such as lymphopenia and reduced *ex vivo* cytokine production capacity, in both prehabilitation and control groups. However, we report that prehabilitation preserved circulating inflammation-related proteins, induced mitochondrial adaptations, and increased metabolic pathway activation. Furthermore, we found that production of IL-18 and IFN-γ was decreased by prehabilitation, identifying these cytokines as potentially valuable biomarkers. Given that previous research on the effects of exercise training on cardiometabolic health also identifies IL-18 as a biomarker of interest (7), we encourage further investigation into the utilization of IL-18 in the assessment of prehabilitation interventions.

Prehabilitation is one of the few cost-effective (8) strategies aiming to improve surgical outcomes and reduce complication rates, complementing the 'enhanced recovery after surgery' (ERAS) guidelines (9). Together, these programs strengthen patient's physiological reserve, enhance surgical resilience, and optimize perioperative care (10). Here, we describe additional immunometabolic effects of prehabilitation strategies, which could benefit patients' metabolic and immune profiles before surgery. In the future, it would be of interest to fully evaluate the effects of prehabilitation on the immune system in a larger cohort of patients.

The central concepts contributing to postoperative immune dysregulation explored in this thesis are illustrated in figure 1, panel A. In **Chapter 2** and **Chapter 3**, the influence of pain and DAMP release on the immune response is investigated. We describe that the surgical stress response has broad implications for leukocyte populations and functionality, and we investigate the mechanisms involved in changes in immune responsiveness, including mitochondrial dysfunction (**Chapter 4**), antigen presentation capacity (**Chapters 2 and 4**), and the epigenetic landscape in immune cells (**Chapter 2**). Dynamic inflammatory mechanisms orchestrate the postoperative immune response, affecting pathogen clearance efficiency, wound healing, and even cancer recurrence. These mechanisms regulate immune homeostasis, a tightly controlled process balancing protection from pathogens, clearance of dead cells and tissues, and resolution of inflammation after surgery.

Part II: Protective effects of trained immunity against unrelated infections

Considering the immunomodulatory effects of medication

Tissue damage, stress responses, and pain are intrinsic aspects of surgical intervention. Although we can minimize the impact of surgery, we cannot fully prevent the effects of the surgical stress response. However, there are intraoperative factors that are more easily controlled, such as the choice of anesthesia and analgesia.

In **Chapter 5** of this thesis, we explore the immunomodulatory effects of propofol, demonstrating that this anesthetic induces a trained immunity phenotype in human primary monocytes. We observed that acute exposure to propofol increased pro-inflammatory cytokine production, but suppressed metabolic activity. Long-term exposure, on the other hand, enhanced both metabolic activity and cytokine responsiveness. The induction of trained immunity by propofol involves significant metabolic adaptations, including increased oxidative phosphorylation and fatty acid oxidation. We show how propofol affects the mitochondria in monocytes,

resulting in increased mitochondrial ROS production. These metabolic features are shared by other canonical inducers of trained immunity (11). Our data reveal that propofol-treated macrophages exhibit enhanced antimicrobial activity against various clinically relevant pathogens. We propose that propofol's ability to induce trained immunity could be leveraged to enhance postoperative immune responses and reduce infection risks. However, a more complete understanding of the mechanisms and pathways involved in propofol-induced trained immunity *in vivo* is needed to evaluate the clinical potential of propofol for potentiating innate immune functions.

Though not necessarily used in perioperative settings, amphotericin B (AMB) is another drug that has been described to modulate immune responses (12). **Chapter 6** discusses the induction of trained immunity by AMB, highlighting its effects on metabolism, epigenetic modifications, and macrophage effector functions. AMB induces long-lasting changes in monocytes that result in enhanced cytokine production and antimicrobial activity. The study identifies the cholesterol synthesis pathway as a key mechanism of AMB-induced trained immunity. AMB's immunomodulatory effects could be harnessed to improve immune responses in patients at risk of infections. Further research is needed to explore the potential clinical applications of AMB-induced trained immunity, particularly in immunocompromised patients.

Epigenetic regulation, metabolic adaptation, and potentiated immune responses

Over the past decade, emerging research has found that exogenous signals, such as bacterial, fungal, and parasitic cell wall components (LPS, β-glucan, *Leishmania* lysates), as well as certain vaccines (BCG), can lead to a change in chromatin state in myeloid cells and result in a deviation from steady-state immune function (13-16). This phenomenon is known as innate immune memory, with the best-characterized outcomes being sepsis-associated endotoxin tolerance or trained innate immunity (17,18).

In addition to pathogen-derived exogenous stimuli, innate immune memory can also be triggered by self-derived DAMPs released during tissue damage and sterile inflammation (19). In surgical settings, sterile inflammation can result from several factors, including tissue dissection, ischemia-reperfusion injury, and mechanical ventilation (20). Although it is feasible that the release of DAMPs during surgery could induce a trained or tolerized immune phenotype, the epigenetic landscape in surgery remains largely unexplored. In this thesis, we show that surgery affects the monocyte epigenome, resulting in reduced accessibility of antigen presentation genes and T cell activation genes, indicating the acquisition of an

immunosuppressed phenotype (**Chapter 2**). This was also reflected in the reduced RNA expression of the antigen-presenting MHC class II molecule HLA-DR (**Chapters 2 and 4**). Specifically, these data support that surgical intervention diminishes monocyte-T cell interactions. Additional mapping of the epigenome of patients undergoing surgery may allow for the identification of regulatory regions that mediate immune activation and suppression after surgery, providing novel targets for therapy in the future.

Antigen presentation and T cell activation are fundamental functions of macrophages and dendritic cells. In **Chapter 7** of this thesis, we assess mechanisms by which macrophages interact with T cells, and we establish the effects of trained immunity on antigen presentation, T cell activation, and T cell polarization. We demonstrate that the induction of trained immunity in macrophages results in distinct changes in surface protein expression profiles, including activation markers, MHC molecules, and co-stimulatory molecules. These findings suggest that trained macrophages exhibit features that can enhance overall immune responsiveness in T cells. Given that our research indicates that surgery impairs monocyte-T cell interaction (**Chapter 2**), trained immunity offers an interesting mechanism by which these mechanisms could be restored, boosting immune responses in postoperative immune suppression but also in various other clinical settings, including vaccination and cancer immunotherapy. Understanding the molecular mechanisms underlying trained immunity in monocytes/macrophages and T cell activation will enable the development of targeted therapies to enhance immune function.

Another feature of trained immunity that might confer beneficial effects in surgical settings is the metabolic rewiring described in innate immune memory adaptations. Cellular metabolism has been correlated to immune cell function, but intracellular metabolites also play an important role as signaling molecules, cofactors, and substrates for chromatin modifying enzymes, highlighting the interplay between metabolic pathways adaptations and epigenetic reprogramming (21). In **Chapter 4** of this thesis, we explore metabolic pathways involved in immunological adaptation after prehabilitation and surgery. We provide evidence that multimodal prehabilitation induces immune cell mitochondrial adaptations, resulting in differential expression of metabolic pathway genes. In particular, we found upregulation of genes involved in glycolysis, oxidative phosphorylation, amino acid, and fatty acid metabolism. Functionally, this translated to lower levels of circulating lactate after prehabilitation, both in steady-state and in stimulated cells. Potentiation of metabolic pathways allows immune cells to enter a highly energetic

state, which may be beneficial in situations that require a substantial amount of available energy, such as surgical trauma and infection.

In figure 1, panel B, strategies to modulate the immune response to surgery are explored. As discussed in **Chapter 2** and **Chapter 3**, the surgical stress response can be minimized through low-impact surgery and adherence to enhanced recovery after surgery (ERAS) guidelines. Selection of medication should involve a consideration of the immunomodulatory effects (**Chapters 5 and 6**). Enhancing patient physiologic reserve and capacity pre-surgery by means of prehabilitation (**Chapter 4**) or trained immunity (**Chapter 7**) may improve immunological resilience to surgical stress.

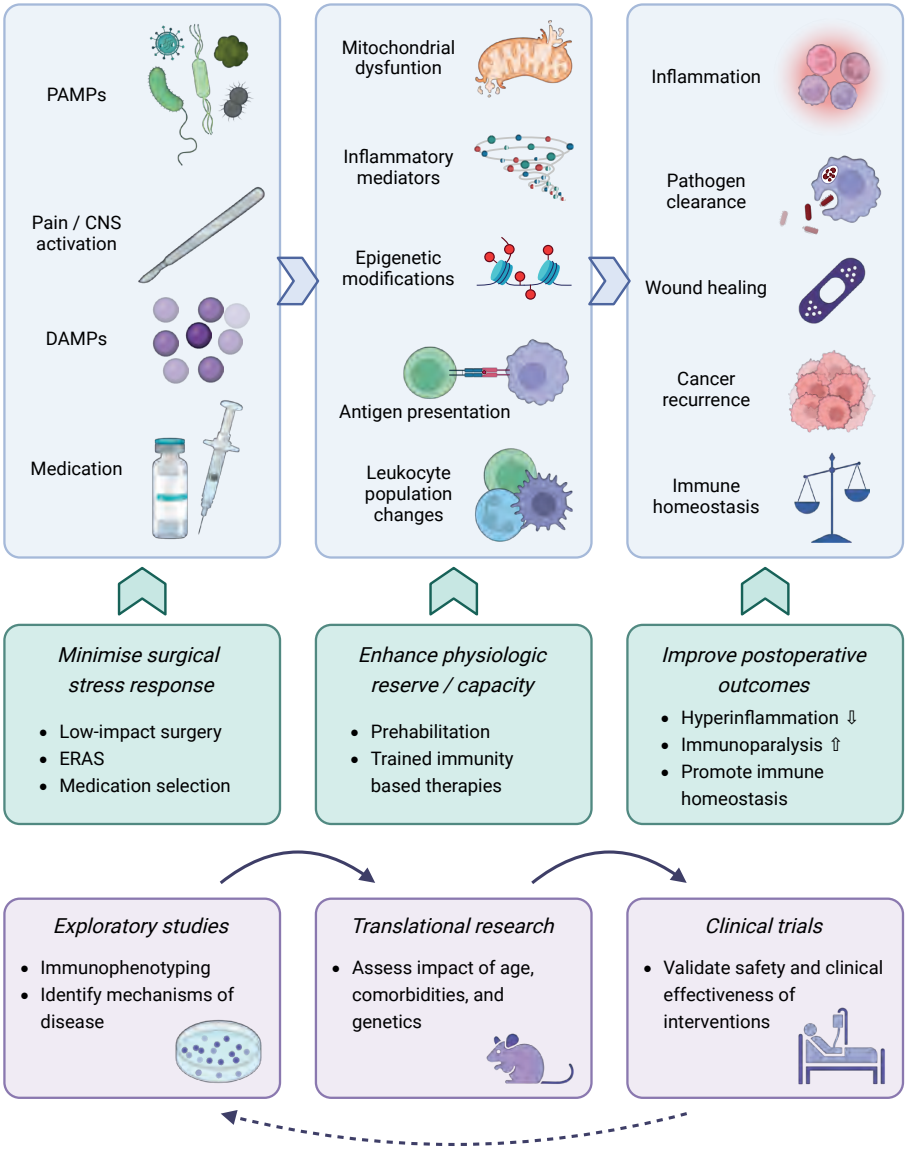


Figure 1. Schematic outline of the thesis. Mechanisms involved in post-operative immune responses addressed in this thesis (A). Hypothesized therapeutic approaches explored in this thesis (B), such as minimizing the surgical stress response, enhancing the physiological capacity of the patient, and addressing postoperative hyperinflammation or immune suppression. A proposed future research agenda for the investigation of postoperative inflammation (C) combines exploratory studies, translational research, and clinical trials. Figure created with BioRender.com

Future perspectives

The research outlined in this thesis examines the inflammatory mechanisms in the perioperative period, and explores the therapeutic potential of trained immunity. We have provided several novel scientific insights in the field of surgical immune responses, yet several important questions remain to be addressed.

Additional research is necessary to translate the mechanisms described in this thesis into clinical settings, especially in regard to modulation of the immune response. Modulating the immune response is not without risk. Attempts to boost postoperative immune responses could result in inadvertent induction of hyperinflammation, which may lead to worsening of tissue pathology, impaired wound healing, and prolonged pain (22). Conversely, heavy-handed suppression of the surgical stress response could impair the body's ability to mount an adequate immune response, which exposes patients to increased risk of developing secondary infections and even tumor recurrence (23).

In this section, I will outline future areas of research that will contribute to our understanding of postoperative immune dysregulation. These areas can be generally divided into three strategies for improving postoperative outcomes, namely [**I**] identifying predictive factors for postoperative outcomes, [**II**] improving the preoperative immune status of the patient, and [**III**] directly modulating the immune system after surgery.

Part I: Prediction of postoperative outcomes

Previous research has claimed that preoperative immunological status is a valuable predicting factor for postoperative complications (24). Thus, by building an accurate profile of the preoperative immune status of the patient, it may be possible to predict the direction of the surgical stress response (hyperinflammation versus immune suppression). To refine the stratification of a patient's immune status, biomarkers that identify overactive or suppressed immune responses are highly warranted, in addition to general patient characteristics such as age, medication use, and co-morbidities. Identifying those patients that are at increased risk of developing postoperative complications will allow for the development and implementation of personalized therapeutic interventions, also called precision medicine.

The concept of precision medicine has been a prominent feature in sepsis, cancer and COVID-19 immunotherapy (25,26). However, personalized care pathways

could be beneficial to surgical patients as well, given the commonalities regarding dysregulated immune responses in surgery and in critical illness. In sepsis and septic shock, several circulating immune biomarkers are currently utilized to gauge the immune status of the patient, including monocyte HLA-DR expression (27), lymphopenia (28), ferritin (29), C-reactive protein, procalcitonin, and IL-6 (30), among many others (31).

Larger cohorts of immunological functional data are needed to establish if these biomarkers could have predictive or monitoring value in surgical patients with regards to postoperative complications. Additionally, the influence of patient characteristics, such as age, sex, and co-morbidities, need to be further explored in the context of surgery-induced immune suppression. In the future, this may allow for the implementation of host-directed immunotherapies, which could provide more targeted, safe, and cost-effective therapies.

Part II: Optimizing preoperative patient health and immune status

Elective surgical intervention remains a primary treatment for cancer. The procedural nature of the perioperative period provides ample opportunity for implementation of preoperative interventions, aiming to better prepare patients for surgery. One such intervention is prehabilitation, combining nutritional support, physical exercise, and smoking and alcohol cessation. Multiple systematic reviews have demonstrated that various prehabilitation strategies reduce postoperative complications, length of hospital stay, and all-cause perioperative mortality (32,33). More prospective clinical trials, carefully designed to compare and combine various treatment modalities, are warranted to investigate the effects of prehabilitation on immunological outcomes. In addition, more mechanistic studies at a cellular level are needed in order to optimize the effects of prehabilitation on the immune status of surgical patients. Specifically, more research should be done to investigate the effects of prehabilitation on immune cell metabolism.

Another prospective intervention that may moderate postoperative immune responses is the induction of trained immunity. It remains undecided if building a trained immunity phenotype would be beneficial or detrimental to clinical outcomes after surgery. Although trained immunity can result in enhanced protection against infection, under certain circumstances it can also contribute to detrimental outcomes, such as persisting and excessive inflammation, and aggravated immune-mediated complications (34).

We have provided several results indicating that the postoperative immune response can generally be described as suppressed, such as the acquisition of epigenetic modifications evocative of innate immune tolerance, and impeded cytokine production capacity. However, additional features of immune cells should be investigated to establish with certainty that surgical intervention results in a suppressed immune state, including intracellular metabolism and transcriptional profiles. Preventing the acquisition of this immunosuppressed phenotype could be an interesting approach to maintain immune homeostasis after surgery. Previous studies describing the induction of innate immune tolerance in sepsis (35) and in cancer (36,37) provide insights into promising therapeutic targets for modulation.

Dynamic changes in the epigenetic landscape of immune cells represent a crucial stage in the activation of gene transcription upon induction of inflammatory responses. It has been hypothesized that blocking epigenetic modulation during surgery could maintain balance in the immune system, possibly resulting in favorable immunological outcomes. Several therapeutic strategies could be employed to achieve this. Bromodomain and extraterminal domain (BET) inhibitors are of particular interest, as they have been shown to effectively modulate inflammatory immune responses (38). For example, I-BET151, a clinically relevant small molecular histone mimic BET inhibitor, was able to prevent the induction of trained immunity, and was also capable of preventing innate immune tolerance (39,40). In addition, several other small-molecule inhibitors of epigenetic mechanisms like histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) are continuously being developed (41). These compounds could be interesting prospects for modulating the immune response to surgery, though more research needs to be conducted to develop targeted epigenetic therapies.

Similarly, targeting intracellular metabolic pathways provides another potential avenue to modulate or prevent the induction of innate immune memory. In this regard, a key consideration in the design of therapies that target metabolic pathways is that they should be specific to immune cells. Given that activated immune cells demand high amounts of energy, they are relatively easy to target with metabolic strategies. In addition, recent advances in the targeting of myeloid cells by nanoparticles pave the way for cell-specific delivery of therapeutics, which could include metabolic pathway inhibitors (42). Several pathways are essential for the induction of innate immune memory, including the mTOR–HIF-1α pathway, which can be suppressed by compounds like rapamycin or metformin (43). Additional metabolic strategies that may inhibit the induction of trained immunity are targeting of the mevalonate pathway (44), glycolysis (45), and lipid metabolism

(46). Important advancements in the prevention of immune suppression after surgery could be achieved by enhancing our knowledge of the delicate balance between molecular, metabolic, and epigenetic changes after surgery.

Alternative approaches to prevent or reverse postoperative immune suppression could be conceived by harnessing trained immunity's therapeutic potential. Inducing trained immunity before surgery could moderate the immune response to surgical stress and anesthesia, resulting in maintenance of immune responses, better protection from opportunistic pathogens, and enhanced recovery after surgery. To our knowledge, there are currently no human trials that support beneficial effects of inducing trained immunity before surgery. However, evidence from exploratory animal studies shows that trained immunity confers broad protection against secondary infection by several clinically relevant pathogens, including *S. aureus* skin infection (47), *E. coli* surgical wound infection (48), pneumococcal pneumonia (49) and other bacterial infections (50).

We propose that the negative effects of postoperative immunosuppression might be negated by the increased responsiveness of trained innate immune cells, providing additional protection from infection and complementing traditional forms of surgical prophylaxis (51). In theory, there is a chance that induction of trained immunity before surgery could result in the presence of hyperresponsive immune cells in peripheral tissues, which could exacerbate systemic inflammatory responses upon surgical trauma. However, insights from sepsis research have suggested that efficient cytokine release during acute inflammation could result in rapid clearance of invading pathogens, expediting the return to steady-state inflammation (52). Similar effects of enhanced immune responses could be expected to aid in the clearance of damaged tissue after surgery. Currently available data does not indicate either beneficial or detrimental effects of trained immunity induction for the prevention of postoperative immune suppression. Thus, this concept will need to be explored in future studies.

In addition to preventative applications of trained immunity, it has also shown considerable potential in the reversal of immunosuppressed states, such as in cancer and in sepsis (53). Trained immunity induces myelopoiesis in the bone marrow, conferring anti-tumor capabilities to monocytes and macrophages, which can overcome the immunosuppressive tumor microenvironment (54). For example, it has been described that trained immunity can exert potent anti-tumor capabilities in *in vivo* models of lymphoma (15). These findings complement clinical studies that demonstrate that intravesical BCG immunotherapy reduces the risk

of bladder cancer recurrence, a mechanism surmised to involve the induction of trained immunity (55). These studies are highly relevant in the context of cancer surgery, given that resection of the tumor may lead to shedding of cancer cells into the circulation (56). Suppression of the immune response at this crucial time may contribute to tumor cell evasion of the immune system, leading to accelerated growth of residual cancer cells (57).

The induction of trained innate immunity was also shown to be a viable therapeutic strategy in *in vitro* and *in vivo* models of LPS-induced endotoxemia to reverse tolerant immunophenotypes towards a more responsive immune status (40,58). The studies highlighted here indicate that induction of trained innate immunity may have potential therapeutic value both in the prevention of immune suppression, and in the reversal of immunosuppressed phenotypes. However, several important questions regarding trained immunity must be addressed that could limit potential utility in clinical settings.

First, we must consider optimal methods to induce trained immunity. BCG vaccine has been studied for the induction of trained immunity in humans in multiple clinical trials (reviewed in 59). However, live attenuated vaccines such BCG may not be safe in immunocompromised patients (60). Furthermore, while BCG vaccination effectively induces trained immunity in infants (61) and young people (62), the effectiveness in older people is known to be limited (63). Thus, BCG may not be the optimal candidate for induction of trained immunity in surgical patients.

Topical, oral, or intravenous administration of β-glucan may represent another potential route of inducing trained immunity. Topical β-glucan application on wounds has been extensively studied in multiple human clinical studies, and is generally considered a safe and promising treatment of chronic wounds (64). However, applying β-glucan on wounds would limit the use to postoperative treatment only. Oral β-glucan supplementation did not effectively induce trained immunity in humans (65), but several clinical and preclinical studies have explored the use of BTH-1677, an intravenous formulation of yeast-derived β-glucan, as an adjuvant therapy to cancer immunotherapy (66).

As we continue to identify other inducers of trained immunity, we propose that medications that are already approved for use in humans may require specific consideration. In this thesis, we newly identified two medications that can induce trained immunity. We do not consider amphotericin B (AMB) an attractive option for inducing trained immunity in patients preparing for surgery, due to the possible

negative side effects of AMB infusion. Additionally, we do not advocate for the use of antimicrobial medications without indication of medical necessity, given the potential development of drug-resistant microorganisms (67,68).

Propofol is also not likely to be utilized solely for its capability to induce trained immunity, given the dose-dependent effects on consciousness (69). However, one could argue that both AMB and propofol are given in situations where the immune system faces substantial challenges, and could benefit from the boosting properties these drugs. Induction of trained immunity provides a parallel mechanism by which AMB may aid in the clearance of systemic fungal infections. In a similar vein, having established that surgery suppresses the immune response, we speculate that the immunostimulatory effects of propofol may act as a balancing factor in this process. However, additional studies are needed to explore the complex interactions between these medications and the immune response. In contrast to BCG vaccine, this thesis puts forward the concept that trained immunity occurs as a side effect of medications not originally developed to target immune responses.

Second, the benefits of choosing induction of trained immunity-based interventions over lifestyle-based interventions have not been investigated. Further research is needed to investigate the potentially additive effects of inducing trained immunity, and to establish possible interactions between prehabilitation programs, anesthetics, and other factors on the immune response. This knowledge will be crucial for developing optimized therapeutic interventions and improving surgical outcomes.

Part III: Direct modulation of the immune response in the perioperative period

Next to the development of interventions that aim to indirectly improve host immune responses, such as prehabilitation and trained immunity-based interventions, treatments that directly modulate the immune response could be beneficial in resolving postoperative inflammation. Timing and type of treatment are crucial factors in determining which course of action will lead to favorable outcomes for the patient. For example, early hyperinflammation caused by the surgical stress response and DAMP release could be counteracted by anti-inflammatory treatments. Such interventions could target cytokine and DAMP signaling pathways, immunometabolic changes, mitochondrial dynamics, and epigenetic modulation to reduce postoperative immune dysfunction.

Targeting immunometabolism in critically ill patients has been described in the context of COVID-19 (70), infection (71), sepsis (72,73), and cancer (74). Modulating mitochondrial dynamics has been the subject of investigation in adaptive and maladaptive stress responses (75), specifically cancer (76), sepsis (77,78), COVID-19 (79,80), and cardiovascular disease (81,82). Examples of treatments that could dampen the immune response to surgery include administration of systemic corticosteroids (83), recombinant anti-IL-6 receptor antibody tocilizumab (84), or recombinant IL-1 receptor antagonist Anakinra (85). Immune checkpoint inhibitors and cytokine signaling pathway modulators have been evaluated for their effectiveness in fine-tuning the immune response in cancer and inflammatory diseases (86-89). In later stages of the immune response to surgery, postoperative immune suppression may be countered or reversed by administration of immunostimulatory agents. Multiple compounds have been investigated in the context of reversing sepsis-induced immunoparalysis. Noteworthy immunostimulatory therapies currently under investigation include GM-CSF, anti-PD-(L)1, and recombinant IFN-γ (90-92).

Therapeutic strategies involving blockade or administration of immune proteins have seen continuous advancement over the past few decades (reviewed in 93). However, clinical trials have often produced conflicting results on the efficacy of these treatments. To this day, no efficacious immunotherapy against sepsis is available, and mortality rates remains high (94,95). It has become clear that many yet unknown factors may influence the timeline and severity of pro- and anti-inflammatory conditions, making direct modulation of inflammation a less desirable approach for the immediate future. To this end, establishing an *in vitro* model of surgical immune suppression would aid in the investigation of modulating therapies. As has been described in the field of sepsis research, the heterogeneity of immune responses presents significant challenges to the design of universal treatments (90). Optimal treatment modalities should aim to rebalance postoperative immune responses, which will depend on individual patient characteristics, accurate preoperative assessment of risk factors, and monitoring of biomarkers to detect dynamic changes in the perioperative period. With this in mind, more direct modulation of the inflammatory pathways affected by surgery and anesthesia may become possible in the future.

In figure 1, panel C, we propose a combined approach of exploratory studies, translational research, and clinical trials, which will aid in the discovery of molecular mechanisms and host-factors that influence surgical stress responses. Future studies should extensively evaluate the effects of immunomodulatory treatments

on immunological characteristics *in vitro* and *in vivo*, followed by robust clinical studies evaluating the safety, feasibility, and effectiveness of such interventions. In time, we hope this will lead to the discovery of preventive and therapeutic immunological interventions, aiming to temper inflammation while preserving host-defense mechanisms, promoting rapid resolution of inflammation, and supporting wound healing.

Concluding remarks

The findings in this thesis contribute to unraveling the complexity of the immune response to surgery and anesthesia. By combining relevant observations from the clinic with exploratory studies in the laboratory setting, we have advanced our understanding of the multifaceted roles of the innate immune system in surgical stress responses.

I surmise that the concepts addressed in this thesis will offer new avenues for the investigation and discovery of optimized preventive and treatment strategies for managing inflammation in patients undergoing surgery. I hope that clinical translation of these results will contribute to a decrease in infectious burden in surgical patients in the future. This thesis explores conceptual and mechanistic insights into the role of trained immunity in the surgical setting, and the protective effects imparted by trained immunity. The quest for novel treatment strategies in surgical patients, including modulating inflammation, promoting wound healing, and protecting against secondary infections, is ongoing.

Collectively, I hope to underscore the intricate balance in immune responses required to optimize postoperative outcomes. The biomarkers and pathways identified in this thesis may provide a foundation for developing targeted interventions, aimed at monitoring and reducing immune dysregulation. Future research should focus on personalizing therapeutic strategies based on individual patient profiles, to minimize postoperative complications and enhance recovery. The novel insights from proteomic, epigenomic, and immunometabolic analyses will be the basis for innovative treatments that improve surgical outcomes and patient care in the near future.

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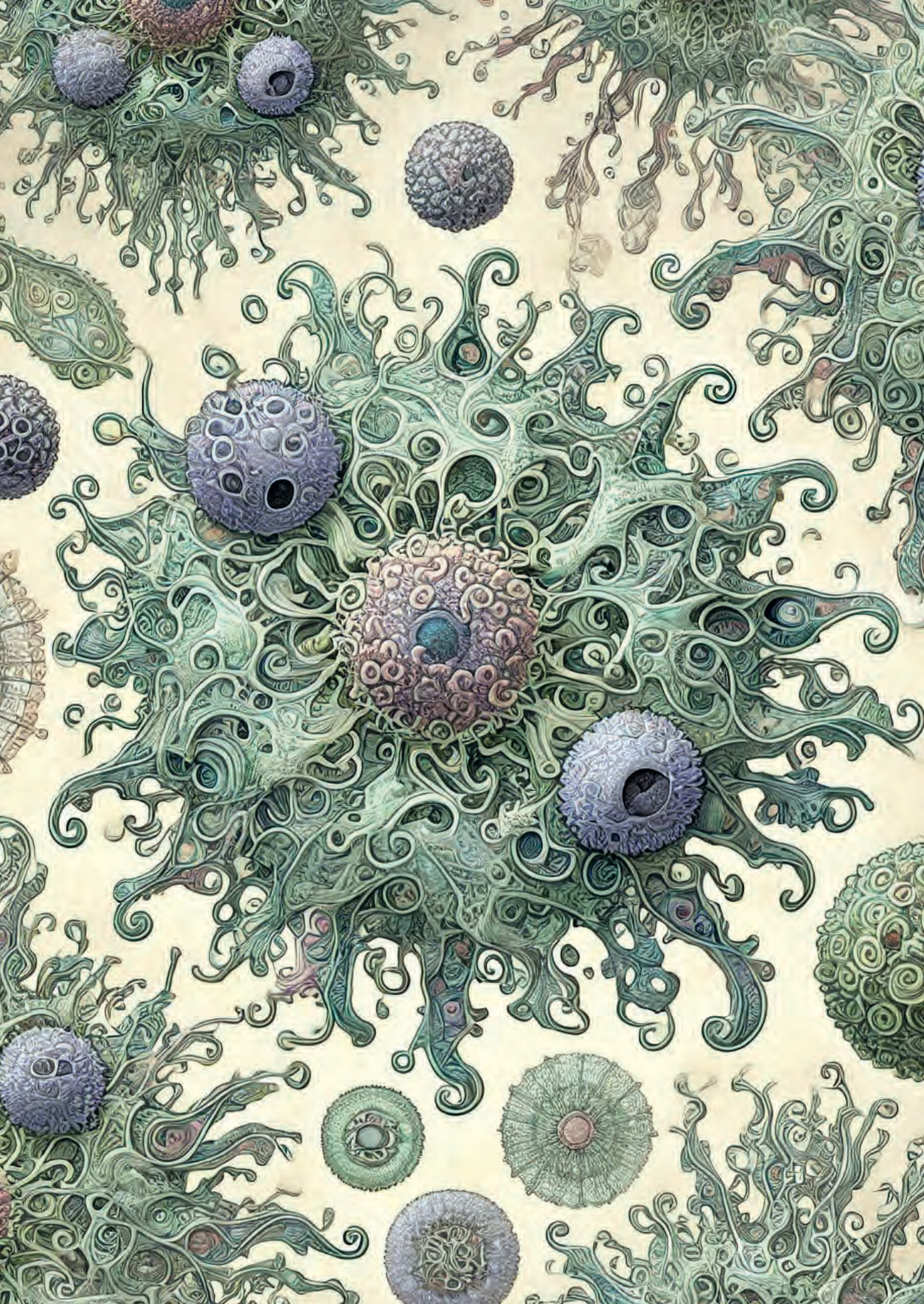


Chapter 10

Appendix

I Dutch summary | Nederlandse samenvatting

II Research data management

III List of publications

IV PhD portfolio

V Acknowledgements | Dankwoord

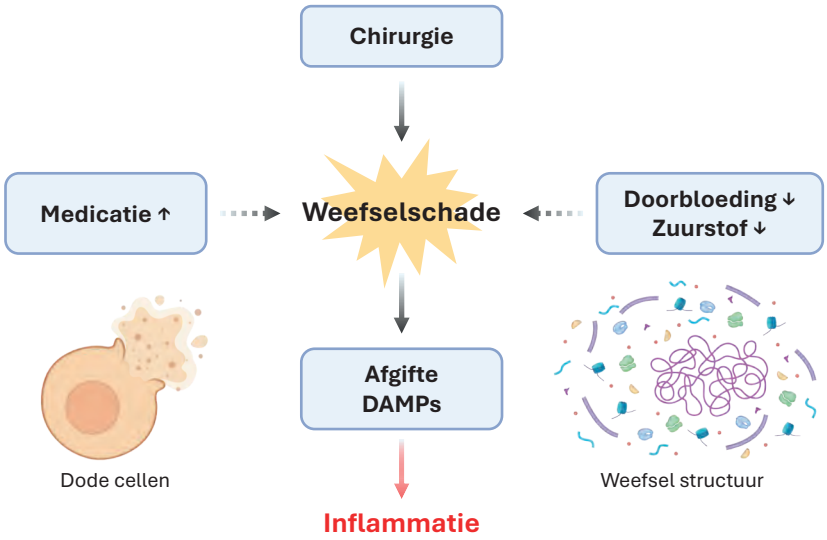
VI Curriculum vitae

Appendix I Nederlandse samenvatting

Chirurgie veroorzaakt schade aan het lichaam, dat is helaas onvermijdelijk. Tijdens een operatie worden weefsels zoals de huid, spieren en soms ook organen beschadigd. Door deze schade raken de weefsels ontregeld, beschadigt de lokale structuur en raken cellen gestrest, waardoor ze beginnen af te sterven. De inhoud van deze cellen, zoals eiwitten, DNA en RNA, komt daardoor vrij in het lichaam (Figuur 1). Deze vrijgekomen moleculen worden samen 'DAMPs' genoemd, wat staat voor 'Damage Associated Molecular Patterns'. Normaal gesproken komen DAMPs niet voor in de bloedsomloop. Wanneer ze wel vrijkomen, worden ze door cellen van het immuunsysteem gezien als een teken van gevaar. Dit zorgt ervoor dat immuuncellen zich uit het bloed bewegen naar de plaats van de schade. Deze immuuncellen produceren vervolgens grote hoeveelheden ontstekingssignalen, waaronder cytokinen. Onder normale omstandigheden is deze immuunrespons in balans en gaat gepaard met een tegenwerkende, ontstekingsremmende respons.

Soms kan het echter gebeuren dat de immuunrespons te sterk is, wat leidt tot een situatie van overmatige ontsteking, ook wel hyperinflammatie genoemd. Aan de andere kant kan het ook zijn dat de ontstekingsremmende respons te sterk is, wat resulteert in immuunsuppressie, oftewel een onderdrukt immuunsysteem. Beide situaties zijn risicovol: een te sterke ontstekingsreactie kan pijn, zwelling, en zelfs orgaanschade veroorzaken, terwijl een te onderdrukt immuunsysteem het risico op infecties vergroot. Het immuunsysteem is namelijk cruciaal voor het bestrijden van ziekteverwekkers die tijdens of na een operatie het lichaam kunnen binnendringen. Bovendien speelt het immuunsysteem een belangrijke rol bij het opruimen van dode cellen en het genezen van wonden. Daarom is het essentieel om een goede balans te vinden tussen de ontstekingsbevorderende en ontstekingsremmende immuunrespons.

Het doel van dit proefschrift was om beter te begrijpen hoe het immuunsysteem ontregeld raakt door chirurgische ingrepen. We hebben factoren onderzocht die de immuunrespons kunnen verzwakken of versterken, zoals weefselschade, pijn, medicatie, en interventies voor en na de operatie. Het doel was om onze kennis over hoe we het immuunsysteem in deze periode kunnen beïnvloeden te vergroten.



Figuur 1. De ontstekingsreactie op een operatie. Tijdens een operatie raken de lokale weefsels beschadigd. Dit kan leiden tot zuurstofgebrek, omdat de doorbloeding tijdelijk verminderd is. Medicijnen die tijdens de operatie worden toegediend, kunnen dit proces ook beïnvloeden. Uit dode cellen en beschadigd weefsel komen stoffen vrij die DAMPs worden genoemd. Deze stoffen zorgen ervoor dat er een ontstekingsreactie (inflammatie) in het lichaam op gang komt.

In **Hoofdstuk 1** geef ik een overzicht van de opzet van het proefschrift. Ik bespreek kort de verschillende factoren die de immuunrespons na een operatie kunnen beïnvloeden, zoals de schade die door de operatie wordt veroorzaakt, het vrijkomen van ontstekingsstoffen, en de effecten van anesthesie en andere medicijnen. Ik leg uit hoe chirurgische weefselschade kan leiden tot het vrijkomen van DAMPs, en hoe het immuunsysteem daarop reageert met zowel ontstekingsbevorderende als ontstekingsremmende reacties. Verder bespreek ik de verschillende factoren die de immuunrespons kunnen beïnvloeden, zowel voor, tijdens als na de operatie.

In het eerste deel van mijn proefschrift onderzoeken we het immuunsysteem van verschillende groepen patiënten die een operatie ondergaan. We proberen te begrijpen welke mechanismen bijdragen aan veranderingen in de immuunrespons na de operatie. In mijn onderzoek hebben we ons vooral gericht op immunologische uitkomsten, zoals de aanwezigheid van ontstekingseiwitten in het bloed, veranderingen in het aantal immuuncellen, en hoe effectief het immuunsysteem ziekteverwekkers bestrijdt. We verzamelden bloed van de patiënten zowel vóór als na de operatie om deze met elkaar te vergelijken. Uiteraard kunnen we een patiënt niet opzettelijk infecteren om de immuunreactie te bestuderen, daarom gebruikten

we een infectiemodel in het laboratorium. Hierbij infecteerden we het afgenomen bloed met bacteriën om de immuunrespons te bestuderen. We keken naar verschillende factoren, zoals de productie van zuurstofradicalen, signaalstoffen zoals cytokinen, en het energieverbruik van immuuncellen.

In **Hoofdstuk 2** van dit proefschrift onderzochten we de effecten van DAMPs op de immuunrespons. Bij 100 patiënten die een darmoperatie ondergingen, maten we de DAMPs in hun bloed. We zagen dat direct na de operatie veel DAMPs aanwezig waren in het bloed, en dat sommige DAMPs zelfs nog hoger waren op de dag na de operatie. In dezelfde studie onderzochten we ook hoe goed de immuuncellen infecties konden bestrijden na de operatie. We ontdekten dat de immuunrespons na de operatie sterk was onderdrukt, wat zou kunnen betekenen dat patiënten na een operatie vatbaarder zijn voor infecties. Om te zien of er een verband was tussen de hoeveelheid DAMPs in het bloed en de immuunrespons, voerden we experimenten uit in het lab. Hieruit bleek dat een hogere concentratie DAMPs direct leidde tot een lagere ontstekingsreactie van het immuunsysteem.

In **Hoofdstuk 3** bestuderen we de rol van chirurgische weefselschade bij de immuunrespons na de operatie bij borstkankerpatiënten. We vergeleken twee soorten operaties: borstsparende chirurgie en mastectomie. Bij borstsparende chirurgie wordt minder weefsel verwijderd, wat resulteert in minder vrijkomende DAMPs na de operatie vergeleken met een mastectomie. Deze studie onderzocht ook de rol van pijn na de operatie en de onderdrukking van het immuunsysteem. We vonden een verband tussen weefselschade, pijn na de operatie, en onderdrukking van de immuunrespons.

In **Hoofdstuk 4** onderzoeken we of prehabilitatie invloed heeft op het immuunsysteem. Prehabilitatie is een programma dat bestaat uit verschillende interventies, zoals fysieke training, aangepaste voeding, mentale begeleiding, en stoppen met roken en alcohol, om de conditie van patiënten te verbeteren vóór een operatie. We onderzochten welke immuunmechanismen een rol spelen bij prehabilitatie en chirurgische schade. We ontdekten dat prehabilitatie het aantal immuuncellen in het bloed kan beïnvloeden en dat er verschillen zijn in de ontstekingseiwitten in het bloed van patiënten voor en na het prehabilitatie programma. We vonden ook dat prehabilitatie invloed heeft op een specifiek onderdeel van immuuncellen, de mitochondriën, die verantwoordelijk zijn voor het maken van energie voor de cel. Verder onderzoek moet uitwijzen of deze veranderingen invloed hebben op de uitkomsten van de operatie.

In het tweede deel van mijn proefschrift onderzoeken we de effecten van specifieke medicijnen op het immuunsysteem. Het is lastig om de invloed van medicatie op de immuunrespons te onderzoeken met bloed van patiënten, omdat zij vaak meerdere medicijnen tegelijk krijgen. Hierdoor is het moeilijk om te bepalen welk medicijn welke effecten veroorzaakt. Daarom gebruiken we in dit onderzoek bloed van gezonde mensen en stellen we de immuuncellen in het laboratorium bloot aan verschillende soorten medicijnen. Dit geeft ons meer mogelijkheden om te experimenteren met hogere doseringen of combinaties van medicijnen die niet bij patiënten gebruikt kunnen worden.

In **Hoofdstuk 5** onderzoeken we de effecten van propofol, een veelgebruikt anestheticum, op de immuunrespons. We ontdekten dat propofol een sterk effect heeft op de productie van pro-inflammatoire cytokinen, stoffen die een ontstekingsreactie in het lichaam veroorzaken. Ook toonden we aan dat propofol langetermijneffecten heeft op de functie van immuuncellen. Cellen die aan propofol zijn blootgesteld, produceren zelfs een week later meer pro-inflammatoire cytokinen. Daarnaast lijkt de functie van deze cellen in de verdediging tegen ziekteverwekkers te zijn verbeterd. Het is belangrijk om in toekomstig onderzoek te kijken of deze effecten ook bij patiënten optreden.

In **Hoofdstuk 6** richten we ons op een ander medicijn, amfotericine B, dat wordt gebruikt voor de behandeling van schimmelinfecties. Dit middel heeft ook effecten op het immuunsysteem. We laten zien dat amfotericine B invloed heeft op het metabolisme, de eiwitsynthese en de cytokineproductie van immuuncellen. Daarnaast zagen we dat immuuncellen na blootstelling aan amfotericine B betere verdedigingsmechanismen hebben tegen verschillende soorten ziekteverwekkers. Deze mechanismen kunnen belangrijk zijn voor de immunologische verdediging tegen infecties.

In het laatste onderzoek van dit proefschrift hebben we gekeken naar hoe verschillende soorten immuuncellen met elkaar communiceren. In **Hoofdstuk 7** bestuderen we de interactie tussen het aangeboren immuunsysteem (monocyten en macrofagen) en het verworven immuunsysteem (T-cellen). We ontdekten dat monocyten en macrofagen kunnen worden 'getraind', waardoor ze beter in staat zijn T-cellen aan te sturen. Deze inzichten in de samenwerking tussen het aangeboren en het adaptieve immuunsysteem kunnen in de toekomst helpen bij het verbeteren van medicijnen en vaccinaties.

Conclusie

De onderzoeken in dit proefschrift laten zien dat chirurgische schade, anesthesie en perioperatieve interventies een grote impact kunnen hebben op de immuunrespons. Ontregeling van de immuunrespons kan leiden tot negatieve klinische uitkomsten voor de patiënt. Daarom is het belangrijk om te begrijpen welke factoren hierbij een rol spelen en welke nieuwe behandelmethoden mogelijk zijn.

In **Hoofdstuk 8** geef ik een samenvatting van de verschillende hoofdstukken van dit proefschrift. Tot slot worden de bevindingen van dit proefschrift besproken in **Hoofdstuk 9**. Hier plaats ik ons onderzoek in de context van andere wetenschappelijke studies en bespreek ik de toekomst van onderzoek naar de immuunrespons na een operatie. Het vertalen van experimentele resultaten naar de klinische praktijk blijft een uitdaging. Hopelijk leiden de bevindingen in dit proefschrift tot meer innovatieve onderzoeken die bijdragen aan betere zorg voor patiënten.

Appendix II Research Data Management

Ethics and privacy

Use of human material was conducted in accordance with the principles of the Declaration of Helsinki (1). Clinical studies were subject to the Medical Research Involving Human Subjects Act (WMO) and were performed in accordance with the ICH-GCP guidelines (Good Clinical Practice). All participants gave written informed consent before participating in the research. Some participants gave additional written informed consent to be contacted again. The recognized Medical Ethics Review Committee 'METC Oost-Nederland' has given approval to conduct these studies.

RECOVER+ study	NL65290.091.18
BREAST study	NL65918.091.18
F4S PREHAB study	NL73777.091.20
300BCG study	NL58553.091.16

The privacy of participants was ensured through the use of encrypted and pseudonymized individual subject codes. The pseudonymization key was stored on a secured network drive that was only accessible to members of the project who needed access to it because of their role within the project. The pseudonymization key was stored separately from the research data.

Data collection and storage

Raw and processed data produced at the Radboud University Medical Center is recorded in lab journals stored at the department of Internal Medicine, and on the Radboudumc server of the department of Internal Medicine. This server is backed up daily and is only accessible by project members working at the Radboudumc. These secure storage options safeguard the availability, integrity and confidentiality of the data.

- Data for chapters 2 and 3 were extracted from (electronic) health records (HIX).
- Data for chapter 4 were extracted from (electronic) health records (EPIC).
- Data for chapters 2, 3, 4, 5, 6 and 7 was obtained through laboratory experiments involving anonymous or non-human materials.

- Data for chapters 2, 3, and 4 was collected through electronic Case Report Forms (eCRF) of a prospective data collection in Castor EDC. Data were converged from (electronic) health records or Castor EDC to SPSS (SPSS Inc., Chicago, Illinois, USA).
- Data from chapters 2, 3, 4, 5, 6, and 7 were stored and analyzed on the department of Internal Medicine server and are only accessible by project members working at the Radboudumc.

Data sharing

Chapter 3 is published open access. Data generated or analyzed in this thesis that are part of published articles (chapters 2 and 3) are published in the Radboud data Repository. Participants in these studies did not give permission to reuse the data, therefore the data are published in a closed access Data Acquisition Collection (DAC).

RECOVER+ study	ru.rumc.recover_t0000107a_dac_831
BREAST study	ru.rumc.breast_t0000108a_dac_286

The data underlying chapters 2, 4, 5, 6, and 7 will be published without restrictions, only after an embargo period of 12 months to enable publication of new results based on the data. After publication, the datasets from these chapters will be made available in Data Sharing Collections (DSC's) in the Radboud Data Repository. The proteomics data generated in chapters 2 and 4 will be published in the PRIDE repository after publication of the articles. The ATAC sequencing data generated in chapter 2 will be shared in a repository such as GEO or EGA after publication of the article.

Chapter 7 is partially based on existing data from the 300BCG study, which was obtained from the original authors and is available for reuse via https://doi.org/10.1016/j.immuni.2023.12.005 upon request. All data will remain archived for at least 15 years after termination of the studies.

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1. World Medical Association. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA. 2013 Nov 27;310(20):2191-4. doi: 10.1001/jama.2013.281053.

Appendix III List of publications

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Keating ST, Groh L, van der Heijden CDCC, Rodriguez H, Dos Santos JC, Fanucchi S, Okabe J, Kaipananickal H, van Puffelen JH, **Helder L**, Noz MP, Matzaraki V, Li Y, de Bree LCJ, Koeken VACM, Moorlag SJCFM, Mourits VP, Domínguez-Andrés J, Oosting M, Bulthuis EP, Koopman WJH, Mhlanga M, El-Osta A, Joosten LAB, Netea MG, Riksen NP. The Set7 Lysine Methyltransferase Regulates Plasticity in Oxidative Phosphorylation Necessary for Trained Immunity Induced by β-Glucan. Cell Rep. 2020 Apr 21;31(3):107548.

Domínguez-Andrés J, Arts RJW, Bekkering S, Bahrar H, Blok BA, de Bree LCJ, Bruno M, Bulut Ö, Debisarun PA, Dijkstra H, Cristina Dos Santos J, 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 Protoc. 2021 Feb 24;2(1):100365.

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Appendix IV Ph.D. portfolio of Leonie Helder

Department: Anesthesiology

Ph.D. period: 15-11-2017 to 15-11-2023

Ph.D. supervisors: Prof. Dr. Gert Jan Scheffer, Prof. Dr. Leo A.B. Joosten

Training activities	Hours
Courses	
Radboudumc - General Radboudumc introduction for research personnel (2018)	9.00
RIMLS - Introduction course "In the lead of my PhD" (2018)	15.00
Radboudumc - eBROK course (2018)	42.00
Erasmus MC - Basic course on R software (2018)	40.00
RTC Clinical Studies - Basic monitoring course (2019)	28.00
RU - Scientific writing for PhD candidates (2019)	84.00
Radboudumc - Scientific Integrity (2020)	20.00
Radboudumc - Re-registration BROK (2022)	5.00
RU - Scientific Outreach to Children (2022)	42.00
RU - Grant writing and Presenting for Funding Committees (2022)	18.00
RU - Education in a Nutshell (2022)	28.00
Seminars	
Cytokine meeting presentations (2018 ^, 2019 ^, 2019 ^, 2019 ^, 2021 ^, 2021 ^, 2022 ^, 2023 ^)	64.00
Cytokine meeting attendance (2017-2023)	80.00
Science Café Anesthesiology (2019 ^, 2021 ^, 2023 ^)	24.00
Symposium 'Hoe anesthesiologie en intensive care persoonlijk werd' (2023) ^	8.00
Conferences	
Retreat Radboudumc - AMC (2017)	28.00
New Frontiers in Innate Immunity and Inflammation (2018) ^ Cluj-Napoca, Romania	21.00
29th ECCMID (2019) ^ Amsterdam, the Netherlands	28.00
4th International Conference on Innate Immune Memory (2019) ^ Nijmegen, the Netherlands	28.00
RIMLS PhD retreat (2018, 2019, 2021 ^)	60.00
Euroanaesthesia (2022) ^ Milan, Italy	24.00
Summer Innate Immunology Conference (2022) ^ Cluj-Napoca, Romania	20.00

Teaching activities	
Host-Microbe Interaction course (2023)	16.00
Supervision of internships / other	
Supervision Master Thesis (3 month) (2020)	30.00
Supervision Master Internship (3 month) (2020)	30.00
Supervision Master Internship (6 month) (2021)	60.00
Supervision Master Internship (3 month) (2021)	30.00
Supervision Master Internship (6 month) (2023)	60.00
Total:	**942.00**

^ Indicates oral or poster presentation

Appendix V Acknowledgements | Dankwoord

Dear reader,

Thank you for your interest in my PhD thesis. Completing this project has been a transformative journey, filled with challenges and rewarding moments alike. It has been a period of tremendous growth, both as a researcher and as a person, and I am deeply grateful to those who helped make it possible. I owe a great deal to my supervisors, colleagues, friends, and family, whose support, encouragement, and guidance have been invaluable. Without them, this thesis would not have been realized, and for that, I extend my heartfelt thanks.

Graag wil ik mijn oprechte dank uitspreken aan mijn promotoren, prof. dr. **Gert Jan Scheffer** en prof. dr. **Leo Joosten**, voor hun waardevolle begeleiding tijdens dit project.

Beste **prof. Scheffer**, beste Gert Jan, als eerste wil ik jou ontzettend bedanken. Jouw enthousiaste betrokkenheid, ruime ervaring en het vertrouwen dat je in mij hebt gesteld, hebben mij enorm gemotiveerd om het beste uit mezelf te halen. Vooral tijdens uitdagende periodes, zoals de COVID-crisis, was jouw voortdurende steun van onschatbare waarde. Ik bewonder je kalme en positieve begeleiding, en je leiderschap heeft me door het hele PhD-traject heen gedragen. Je scherpe wetenschappelijke inzichten en pragmatische aanpak bij complexe vraagstukken hebben me geholpen om realistische doelen te stellen en oplossingen te vinden die haalbaar zijn. Je directe stijl en aanmoedigende gesprekken hebben me niet alleen wetenschappelijk, maar ook persoonlijk enorm laten groeien. Dank je wel voor de kansen die je me hebt geboden en voor de fijne samenwerking in deze bijzondere jaren. Ik ben dankbaar voor alles wat ik van je heb geleerd.

Beste **prof. Joosten**, beste Leo, ook jou wil ik graag uitgebreid bedanken voor de geweldige jaren. Na mijn masterstage kwamen we in contact en bespraken we de mogelijkheid voor een gedeeld promotietraject bij de afdelingen Interne Geneeskunde en Anesthesiologie. Het voelde meteen goed, en ik hoefde dan ook niet lang na te denken om ja te zeggen. Bedankt voor deze fantastische kans die je me hebt geboden. Ik heb ontzettend genoten van onze levendige en inhoudelijke discussies over allerlei zaken. Jouw openheid en het vertrouwen dat je me gaf om zelfstandig mijn weg te vinden, hebben me enorm geholpen in mijn ontwikkeling als onderzoeker. Mede dankzij jou heb ik zoveel plezier en interesse gekregen in immunologisch onderzoek, en je hebt een grote rol gespeeld in het creëren

van de unieke sfeer op lab EIG. Bedankt voor de kansen, het vertrouwen, en de gezelligheid. Het was een voorrecht om met jou te werken!

Daarnaast wil ik prof. dr. **Mihai Netea** van harte bedanken voor zijn waardevolle bijdrage aan dit project. Beste Mihai, hoewel je formeel niet in mijn promotieteam zat, heb ik ontzettend veel geleerd van jouw deskundigheid, zowel op klinisch als experimenteel gebied. Je enthousiasme voor de wetenschap is aanstekelijk en heeft me vaak geïnspireerd. Na onze overleggen zat mijn hoofd altijd vol nieuwe ideeën en motivatie om verder te gaan. Je diplomatieke leiderschap en talent om mensen samen te brengen in diverse projecten zijn onmisbaar, en ik bewonder je efficiëntie, analytisch vermogen en creativiteit. Ondanks je drukke agenda was je altijd bereid om tijd te maken voor advies en gedetailleerde vragen. Dat heb ik enorm gewaardeerd. Daarnaast wil ik je bedanken voor het vertrouwen dat je in mij stelt voor het aankomende project in Nederland en Roemenië. Dit geeft me de energie en motivatie om met hernieuwde focus verder te gaan. Bedankt voor je steun en begeleiding, en voor de mooie jaren op het lab EIG.

Geachte leden van de manuscriptcommissie, prof. dr. **Peter Pickkers**, prof. dr. **Gosse Adema**, en dr. **Gertrude Nieuwenhuijs-Moeke**, hartelijk dank voor het kritisch lezen en beoordelen van mijn manuscript.

Lieve paranimfen, Elisabeth, Julia en Pepijn. Lieve **Julia**, bedankt dat je altijd zo lekker vrolijk bent! Ik bewonder jouw bevlogenheid en ruimdenkendheid als wetenschapper en als mens. Bedankt voor de gezelligheid, de outfit *inspo*, de mentale support, en je positieve instelling. Nog maar even voordat wij het hele lab kunnen overnemen! Lieve **Elisabeth**, you can manage my health anytime. Tussen de masterstudies en de klinische studies door is het altijd gezellig als jij er bij bent! Bedankt voor de leuke gesprekken, de constructieve klaagsessies over de organisatie van het gezondheidssysteem, de excursies in de diverse bars van Nijmegen om deelnemers te werven voor de NEXT studie, de uitjes naar de 7de verdieping of het fertiliteitslab, en de vele gezellige borrels. Jij bent een echte wervelwind! Lieve **Pepijn**, jij haalt echt het beste en soms ook het slechtste in mij naar boven! Samenwerken met jou is altijd een feestje, zelfs tijdens serieuze momenten in het lab of de sportschool. Toch geniet ik misschien nog wel meer van onze lunches, borrels en gezellige gesprekken. Jouw humor, zelfvertrouwen, vastberadenheid en gedrevenheid maken je tot een geweldige collega en vriend. Bedankt voor alle gezelligheid, je steun, een luisterend oor, en al het ongevraagde kledingadvies (ik leg het naast me neer, oké?).

Ook wil ik graag mijn dank uitspreken aan de medewerkers van de afdeling Anesthesiologie voor hun hulp en begeleiding tijdens dit project. In het bijzonder wil ik **Lucas van Eijk**, **Rebecca Koch** en **Michiel Vaneker** bedanken voor hun enthousiasme voor het onderzoek binnen de afdeling, en voor hun scherpe inzichten in de klinische gang van zaken. Hun bereidheid om samen te werken in verschillende ambitieuze projecten heeft dit onderzoek verrijkt en naar een hoger niveau getild. Daarnaast wil ik graag mijn waardering uitspreken voor de medewerkers van de researchunit Anesthesiologie, **Jackie van Gemert** en **Ilona van der Brink**, voor hun onmisbare ondersteuning. Jullie hulp bij het regelen van de administratieve aspecten van het onderzoek heeft ervoor gezorgd dat ik me volledig kon richten op de inhoudelijke kant. Bovendien waardeer ik jullie oprechte interesse in mijn werk en jullie gezelligheid tijdens de monitorvisites op de afdeling. Dankzij jullie gezamenlijke inzet, betrokkenheid en samenwerking is dit project succesvol verlopen. Ik ben jullie allemaal enorm dankbaar voor jullie tijd, inzet en het vertrouwen. Jullie begeleiding en ondersteuning hebben een groot verschil gemaakt. Hartelijk dank!

Aan de medewerkers van afdeling Heelkunde: het was altijd een genoegen om met jullie samen te werken! Kim en Michiel, ik wil jullie in het bijzonder bedanken. Tijdens ons eerste overleg was ik nog nerveus dat onze projecten elkaar in de weg zouden zitten, maar al snel bleek dat we elkaar juist perfect aanvulden. **Kim**, ik bewonder je enorm voor je doorzettingsvermogen en toewijding, ongeacht de omstandigheden. Zelfs tijdens de COVID lockdown hebben wij nog samples kunnen verwerken van de RECOVER studie, en ook de vele ELISA dagen, DNA en RNA isolaties tijdens de kerstvakantie of in de weekenden staan mij nog goed bij. Het was allemaal een stuk gezelliger met jou er bij! **Michiel**, jouw drive om de IMPACT onderzoekslijn op te zetten, en jouw focus op het ondersteunen van je team zodat iedereen optimaal kan presteren, maken jouw een geweldige team leader! Naast het harde werken, was er gelukkig ook altijd ruimte voor ontspanning. De diverse wijn- en pizza-avonden met het team zijn onvergetelijk en hebben nog maar eens bewezen dat het niet altijd over onderzoek hoeft te gaan. Ook wil ik graag **Gaby** en **Veerle** bedanken voor hun waardevolle bijdrage aan dit gezellige team.

Matthijs en **Jelle**, bedankt voor de fijne samenwerking de afgelopen jaren, het delen van jullie expertise in het lab, en al jullie waardevolle suggesties voor onze immuunsuppressie experimenten.

Yutaka, **Mumin**, and **Musa**, thank you for the wonderful collaboration and your contributions to the RECOVER+ study and the INFLUENZA-SHINGRIX trial, as well as the many inspiring scientific discussions.

INFLUENZA-SHINGRIX team, in particular Gizem, Esther, Elisabeth and Malin. **Gizem**, together we tackled the INFLUENZA-SHINGRIX trial, though to be honest you did most of the work! I'm happy we were able to work together on this project, as you are a dedicated researcher and a wonderful person in general (except when you are absolutely crushing me in a boardgame). **Esther**, thank you for your contributions to the design of the study and for helping out with the flow cytometry experiments. **Elisabeth**, your support during the study has been instrumental in the completion of this project. Only you would interrupt a dinner to cycle back to the lab with me to shut down the CytoFLEX! **Malin**, you are simply delightful! Thanks for being so pretty. I'm happy that we continue to work together on all things flow cytometry. I would also like to thank **Lieke** and **Margot**, for all their help with the participant inclusions in the INFLUENZA-SHINGRIX trial.

Ook wil ik graag de onderzoekers van de F4S PREHAB trial bedanken, met name **Lotte**, **Luuk** en **Dieuwke,** maar natuurlijk ook dr. **Baukje van den Heuvel** en prof. dr. **Kees van Laarhoven**. Dank voor jullie interesse en medewerking aan de substudie!

To my fellow **BCG-PRIME project** members: Andy, Thanasis, and Anaisa. **Thanasis**, through this project and the collaboration with you, I've been able to learn a lot, and I really appreciate the effort you put into this research project. DJ **Andy**, you are a menace inside the lab and outside! Thank you for always bringing positive energy and helpful insights to any project we work on. **Anaisa**, I always enjoyed working with you, on this project but of course also on the APC paper. I'm happy we were able to finally (almost) finish it. I hope your journey to the USA brings you everything you wanted and more!

During my PhD project, I have occupied 3 different offices in 3 separate buildings. I'd like to thank my roommates for all the fun times and interesting discussions we have had over the last few years. **Jorge**, you were a constant presence in my first office in the Buitenhoek, and I take it as a personal achievement that you had to move to the postdoc office after a while to 'be more productive'. Thank you for your mentorship over the years. My thanks as well to **Dennis** and **Inge** for completing the Buitenhoek office. From there on, we were relocated to the offices above the West entrance, where I was joined by **Linda**, **Julia**, **Konstantin** and **Elke** in our cozy

corner office. Thank you for the fun discussions! Last but not least, **Elisabeth** and **Pepijn**, our office at the Internal Medicine lab is always busy, although I can never seem to get any work done there. I want to thank you all for the great times!

To my fellow **AIG lab** members. I cannot express how much these fun, challenging, and exciting years have been shaped by your presence. Working in the AIG lab has been an incredible experience, and I am deeply grateful to everyone who has been part of this journey. Spending so much time in one lab means there are too many of you to name individually, but to every single person who passed through the lab: thank you for the support, the laughter, the insightful discussions, and the camaraderie. From the moment I joined, the lab felt like home, and I have been continually inspired by so many of you. Special thanks to **Jelmer, Lisa T., Viola, Siroon, Charlotte, Julia T., Helin, Valerie, Margo, Intan, Maartje, Anna, Marijn, Wieteke, Martin, Zara, Michelle, Collins, Frank, Julia B., Vicky, Jessica, Özlem**, and of course my dear friend **Büşra**. I'll always cherish the crazy brainstorm sessions, the endless PBMC isolations, trained immunity discussions, and unforgettable trips like Milan, the Ardennes, and Cluj. I wish you all the best in your future research endeavors, and I'm excited to see what you will accomplish!

Adriana, I remember the first time I spoke to you about applying the 2000HIV flow cytometry panels to the INFLUENZA-SHINGRIX study. You told me 'Leonie, you are going to suffer', and you were right. Thank you for all your support, I'm glad at least I don't have to suffer alone.

Beste analisten van het lab EIG, **Heidi**, **Helga**, **Cor**, **Anneke**, **Andy**, **Liesbeth**, **Ilse**, **Malin** en **Hanneke**, ik wil jullie graag enorm bedanken voor de fijne samenwerking en de gezellige tijd op het lab. Jullie maken het leven in het lab een stuk makkelijker en staan altijd klaar om te helpen met experimenten. Vanaf het begin van mijn PhD hebben jullie me veel geleerd over de verschillende lab technieken, en gedurende de jaren daarna hebben jullie me altijd met geduld en precisie ondersteund. Dankzij jullie deskundigheid en gestructureerde aanpak heb ik enorm veel geleerd. De gezellige gesprekken op het lab en in de koffiekamer maakten de lange dagen een stuk aangenamer. Het was een plezier om met jullie samen te werken. Bedankt voor jullie kennis, geduld en vooral voor de gezellige sfeer die jullie creëerden!

Mariolina, Diletta, Cas, Harsh and **Dogukan**, thank you for being such wonderful friends and travel companions throughout our journey across Romania, from Bucharest to Cluj (the scenic route). Your humor, adventurous spirit, and fun personalities made every moment memorable! I'd also like to thank the amazing

people that I travelled with from Budapest to Cluj, another unforgettable excursion. **Jelmer, Michelle, Vera, Inge** and **Viola**, thanks for being such wonderful colleagues and travelling companions!

To the members of the Josefowicz lab in New York: Thank you for the amazing collaboration! Conducting the neonatal BCG study with all its challenges was quite a feat. Without your expertise and pragmatic solutions, it would certainly have been impossible to design and conduct the study. I want to thank you for welcoming me into your lab, I gained a lot of knowledge and experience by working with you all. **Chenyang, Jin, Andrew, Tori, Alexia** and **Lucy**, thank you for all your valuable help! **Rachel** and **Steve**, thank you so much for welcoming me so warmly into your home and for all the tips on interesting outings, great coffee/lunch spots, and must-sees in New York. I hope to see you soon in Nijmegen or somewhere else!

I extend my sincere thanks to my Romanian colleagues in Craiova and Cluj for the inspiring collaboration, insightful discussions, and invaluable feedback. **Anca**, **Andra**, and **Ioana**, it's been a pleasure working with you, and I look forward to continuing our work together on future exciting projects!

I would also like to thank the dedicated colleagues at the Tumor Immunology lab, the Department of Laboratory Medicine, and the RTC Flow Cytometry. **Sophie**, I am grateful for your insights into the subtle differences between monocyte-derived macrophages and monocyte-derived dendritic cells; your perspective was invaluable. **Dimitri**, **Evi**, and **Lucille**, thank you for your guidance on the T cell activation assay design—it made a crucial difference. **Hans** and **Bram**, your expertise in flow cytometry was instrumental in developing the BCG-PRIME panel. **Rob**, **Thessa**, and **Tereza**, your support with the INFLUENZA-SHINGRIX measurements was greatly appreciated. Thank you all!

Aan de leden van de enige echte AIG feestcommissie, **Job, Jeroen, Andy, Marijn, Elisabeth** en **Julia**: met jullie erbij is er altijd leven in de brouwerij! Dank voor de fijne tijd, de positieve energie en het vele lachen. Het is altijd gezellig om met jullie samen activiteiten te plannen, ook al doen we het hoogstens 1 keer per jaar. Laten we binnenkort weer iets organiseren!

Dagmar, **Sam**, en **Lissy**, hartelijk dank voor jullie inzet, enthousiasme en harde werk tijdens jullie stages! Bedankt voor jullie geduld met mijn soms wat chaotische planning en beperkte uitleg. Ik hoop dat jullie iets van mij hebben geleerd, want ik heb zeker veel van jullie geleerd. Ik wens jullie het allerbeste voor de toekomst!

Lotte, ooit begon je als student op het lab, maar inmiddels heb je me al bijna ingehaald als PhD! Het verbaast me niet, want jouw doorzettingsvermogen is bewonderenswaardig. Ik herinner me nog goed hoe je (lopend) door weer en wind samples van de RECOVER-studie van het CWZ naar het Radboudumc kwam brengen. Onze samenwerking verloopt altijd soepel dankzij jouw nauwkeurigheid, integriteit en optimisme. Ik weet zeker dat er een prachtige toekomst voor je ligt!

My dear fellow horror movie lovers, **Nico, Ruiqi, Fadel, Rutger**, and **Anouk**—thank you for the unforgettable Halloween movie nights! I'll always cherish the memories of your inventive costumes (Rutger's 'reviewer #2'), your terrified screams (Fadel), and your scare tactics (Nico haunting our house). Academia may lead us in different directions, but I'm grateful for your friendship and these fun memories. Here's hoping we'll reunite in person one day!

Lieve **Wessel, Zino, Stijn** en **Ties**, dank jullie wel voor de geweldige jaren en de bijzondere vriendschap. Jullie hebben de gave om zelfs de meest wilde plannen werkelijkheid te maken, ook al verloopt dat niet altijd vlekkeloos. Ik waardeer onze avonturen enorm en kijk uit naar nog veel meer memorabele momenten samen. Op naar de volgende avonturen!

Lieve **Phelan, Guus, Tom, Fee** en **Yannick**, wat ben ik blij met zo'n heerlijk chaotisch stel vrienden! Met jullie erbij is er nooit een saaie dag, en ik wil jullie enorm bedanken voor de geweldige filmavonden, feestjes en de vele avonturen die we samen hebben beleefd. Jullie enthousiasme, energie en gezelligheid hebben me door de uitdagende momenten van dit proefschrift geholpen en gaven me telkens weer motivatie. Bedankt voor al het lachen en de mooie jaren samen. Op naar nog meer geweldige avonturen in de toekomst!

Lieve **Evy**, wat ben ik dankbaar dat jij al ruim 10 jaar een van mijn beste vriendinnen bent! Jouw samenvattingen waren tijdens onze bachelor BMW mijn redding, en ik ben zo blij dat onze vriendschap al die tijd is gebleven. Samen hebben we zoveel mooie en hilarische momenten beleefd—van feestjes en etentjes tot onze slechte grappen en de eindeloze verhalen die je altijd met me deelt. Je staat altijd voor me klaar, en jouw steun en warmte betekenen de wereld voor me. Ik ken niemand die zo lief en zorgzaam is als jij. Bedankt voor alles, voor de fijne tijd, de gezelligheid, en voor al het lachen samen. Ik kijk uit naar nog veel meer mooie momenten met jou!

Lieve **Jeroen**, ook wij kennen elkaar nu al een hele lange tijd, sinds de start van onze studie BMW in Nijmegen, en wat ben ik blij met al die jaren vriendschap. Vanaf het begin hadden we dezelfde humor en hebben we samen ontzettend veel gelachen. Jouw kennis, onmisbare grappen en verrassend wijze levenslessen maken je uniek. Je bent er altijd geweest als luisterend oor, vooral op momenten dat ik gefrustreerd was over werk. Ik ben enorm trots op wat je al bereikt hebt, eerst als PhD en nu als postdoc—je bent echt een voorbeeld voor me. Bedankt voor alle mooie jaren samen, en op nog veel mooie momenten in de toekomst!

Lieve **Merel**, ik ben heel blij dat we via Evy bevriend zijn geraakt en inmiddels zo'n goede vriendschap hebben opgebouwd! Jij brengt altijd positieve energie mee, en ik kan heerlijk met je lachen. Je bent een fantastische luisteraar en weet altijd goede gesprekken op gang te brengen. Jouw adviezen hebben me enorm geholpen, en ik waardeer het ontzettend dat je me steeds aanmoedigt om nieuwe dingen te proberen. Dankzij jou leer ik dingen vanuit een ander perspectief te zien, en je geduld daarin betekent veel voor me. Ik ben ook ontzettend trots op hoe jij steeds meer jezelf durft te zijn; het is prachtig om te zien. Bedankt voor de fijne momenten samen en voor je vriendschap. Ik wens je al het moois voor de toekomst!

Lieve meiden van het Staring College, **Eline, Chloe, Jessica, Dorien, Jorien, Sophia, Laura** en **Paulien**. We kennen elkaar nu al zo'n 18 jaar, en wat ben ik dankbaar voor onze vriendschap! Het maakt niet uit hoe lang we elkaar niet zien—het voelt altijd als gisteren. Samen hebben we zoveel mooie momenten beleefd, van lachen op school en knutseldagen tot vakanties en nu zelfs bruiloften en babyshowers. Eline, Jessica en Sophia, jullie weten natuurlijk als geen ander hoe een PhD zijn ups en downs heeft, en in tijden van stress kon ik altijd vertrouwen op jullie steun. Jullie vrolijkheid, luisterende oren, en de vele momenten samen hebben me enorm geholpen om deze PhD af te ronden. Ik ben ontzettend dankbaar voor jullie en weet zeker dat we nog veel mooie momenten tegemoet gaan. Laten we er altijd voor elkaar blijven, zoals jullie er altijd voor mij waren. Bedankt voor de gezelligheid en voor jullie vriendschap door al die jaren!

Dear **Petra**, thank you for your constant cheerfulness, support, and warmth. **Kippy, Jesse,** and **Rob**, I'm incredibly grateful for the wonderful memories we created together in St. Martin. Your amazing hospitality and kindness made that time unforgettable. Thank you all for your encouragement and support over the years!

Lieve familie, tante **Linda** en oom **John**, **Maud** en **Benato**, tante **Brenda** en oom **Fanis**, natuurlijk mijn geweldig lieve **Oma**, en ook **Ron** en **Carla**: Heel veel dank

voor jullie onvoorwaardelijke steun en liefde die mij door mijn PhD-traject hebben geholpen. In stressvolle tijden kon ik altijd bij jullie terecht voor een gezellig etentje of een goed gesprek. Die momenten waren van onschatbare waarde en gaven me steeds weer de moed om door te gaan. Ik koester de mooie herinneringen aan de tijd die we samen hebben doorgebracht en ben dankbaar voor alle liefde en ondersteuning via telefoontjes, kaartjes en mailtjes. Bedankt voor jullie steun, liefde en waardevolle adviezen. Ik had het niet zonder jullie kunnen doen!

Lieve **Eric**, wat bof ik toch met een grote broer zoals jij! Hoewel we elkaar vroeger soms in de haren zaten, hebben we ook veel mooie tijden samen gehad, zowel in Zwitserland als nu in Nederland. Nu we ouder zijn, waardeer ik onze band alleen maar meer. Ik ben ontzettend trots op de weg die je hebt gevonden in het leven en wens jou en **Scarlet** nog heel veel mooie jaren samen. Weet dat ik altijd voor je klaarsta, ook al wonen we niet om de hoek en hebben we allebei drukke agenda's. Dankjewel voor alles, grote broer!

Dear **Laszlo**, it's hard to capture in words what you mean to me. I met you on my first day as a PhD, and from that moment on I have admired your wisdom and experience. Our friendship quickly grew into a relationship that's become a truly meaningful partnership. There are countless reasons why I admire you, but I want to especially thank you for your unwavering patience, understanding, and support over these last few years. Your words, 'don't let great be the enemy of good,' have kept me grounded, and you've introduced me to so many new experiences—from travel and food (not too spicy) to spooky movie nights and philosophical debates. You are my lab partner, rabbit co-parent, sparring partner, co-author, traveling companion, trusted confidant, and so much more. Thank you for being all that you are. I couldn't imagine a better partner to share these memories and adventures with.

Mijn geweldige ouders, lieve **pap en mam**. Als jullie enige (en dus favoriete) dochter heb ik altijd geprobeerd om jullie trots te maken. Al op jonge leeftijd was ik in de badkamer bezig met 'wetenschappelijke experimenten', namelijk het bij elkaar mixen van verschillende zeepjes en shampoos. Jullie stonden altijd klaar om mijn nieuwsgierigheid en creativiteit aan te moedigen, wat uiteindelijk heeft bijgedragen aan mijn keuze om onderzoeker te worden. Jullie steun en interesse in mijn studie en werk hebben me altijd gemotiveerd om door te zetten en mijn best te doen. Van jullie heb ik waardevolle lessen geleerd: hard werken, vertrouwen op mezelf, en de balans vinden om niet teveel hooi op mijn vork te nemen. Jullie hebben me altijd vrijgelaten om mijn eigen weg te vinden, en het was een enorme

geruststelling te weten dat ik altijd bij jullie terecht kon, dat ik altijd een veilige thuishaven had. Ik kan jullie niet genoeg bedanken voor de liefdevolle basis die jullie me gegeven hebben en voor jullie onvoorwaardelijke steun, zowel tijdens de hoogte- als dieptepunten. Bedankt dat jullie er altijd voor mij zijn. Ik hou ontzettend veel van jullie!

Appendix VI Curriculum vitae

Leonie Suzanne Helder was born in Baden, Switzerland on March 16th, 1994. After moving to the Netherlands in 2000, she graduated from the Staring College in Lochem in 2012. That same year, she started her Bachelor studies in Biomedical Sciences at the Radboud University in Nijmegen. During her Bachelor, she performed an internship at the department of Cell Biology under supervision of Dr. Ineke van der Zee, where she investigated the role of Ehmt1 protein in the developing brain.

During her Master program, she majored in Human Pathobiology and Toxicology. Her first Master internship was completed at the Department of Biomaterials in the Radboudumc, under supervision of Dr. Fang Yang. Here she investigated the role of polymer end groups on drug-delivery properties of electrospray-generated microspheres. During this internship, she visited the lab of Dr. Mingshi Yang at the Department of Pharmacy, University of Copenhagen. For her senior Master internship, she investigated the role of dynamic cell culture conditions in tissue engineered scaffolds for urinary diversion, under supervision of Dr. Egbert Oosterwijk at the Department of Experimental Urology in the Radboud Institute for Molecular Life Sciences.

Her PhD project was conceptualized as a collaboration between the Department of Anesthesiology and the Department of Internal Medicine, under supervision of Prof. Dr. Gert Jan Scheffer and Prof. Dr. Leo Joosten, respectively. The main focus of her PhD research was to investigate the dysregulated immune response after surgery, and to elucidate the possible role of trained immunity. To this end, she collaborated with the Department of Surgery in multiple research projects. The results of these studies were presented at multiple international conferences. In addition to the research documented in this thesis, she collaborated with other researchers on several research projects including the BCG-PRIME cohort, the INFLUENZA-SHINGRIX study, and the BCG-CORONA cohort.

During the final months of her PhD, she visited the lab of Dr. Steven Josefowicz at Weill Cornell Medicine in New York to investigate the effects of BCG vaccination on hematopoietic stem cell development in neonates. Leonie continues to pursue her interests in immunology, immunotherapy, and immune aging as a postdoctoral researcher in the Department of Internal Medicine, under the supervision of Prof. Dr. Mihai Netea.

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