IMMUNOMODULATION IN SEVERE INFECTIONS

Jenneke Leentjens
 IMMUNOMODULATION IN SEVERE INFECTIONS

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Colofon

Immunomodulation in severe infections

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IMMUNOMODULATION IN SEVERE INFECTIONS

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by

Jenneke Leentjens

Born on February 23, 1983
in Kerkrade (the Netherlands)
And above all, watch with glittering eyes the whole world around you because the greatest secrets are always hidden in the most unlikely places.

- Roald Dahl
Part Three
New immunomodulatory approaches

Chapter 7  
Gamma-irradiated bacille Calmette-Guerin vaccination does not modulate the innate immune response during experimental human endotoxemia in adult males.  
*J Immunol Res.* 2015; 261864

Chapter 8  
BCG vaccination enhances the immunogenicity of subsequent influenza vaccination in healthy volunteers: a randomized placebo-controlled pilot study.  
*J Infect Dis.* 2015; Epub 2015/06/12

Chapter 9  
The effects of orally administered Beta-glucan on innate immune responses in humans, a randomized open-label intervention pilot-study.  
*PloS one.* 2014;9(9):e108794

Part One
Immunomodulation as adjunctive treatment for sepsis

Chapter 2  
Immunotherapy for the adjunctive treatment of sepsis: from immunosuppression to immunostimulation. Time for a paradigm change?  

Chapter 3  
*Am J Respir Crit Care Med.* 2012;186(9):838-45

Part Two
Immunomodulation as adjunctive treatment for opportunistic infections

Chapter 4  
Interferon-gamma as adjunctive immunotherapy for invasive fungal infections: a case series.  
*BMC Infect Dis.* 2014;14:166

Chapter 5  
Interferon-gamma immunotherapy in a patient with refractory disseminated candidiasis.  
*J Ped Infect Dis.* 2015; Epub 2015/09/15

Chapter 6  
Interferon-gamma immunotherapy in a patient with progressive cerebral *Nocardia* abscesses.  
Submitted

Chapter 10  
Summary

Chapter 11  
General discussion and future perspectives

Chapter 12  
Nederlandse samenvatting

Dankwoord

List of Publications

Curriculum Vitae
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
<th>Description</th>
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<tbody>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>AU</td>
<td>Arbitrary units</td>
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</tr>
<tr>
<td>BAL</td>
<td>Broncho alveolar lavage</td>
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<tr>
<td>BCG</td>
<td>Bacille Calmette-Guérin</td>
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<tr>
<td>BG</td>
<td>Beta-glucan #300</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>bpm</td>
<td>Beats per minute</td>
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<tr>
<td>BTLA</td>
<td>B and T lymphocyte attenuator</td>
<td></td>
</tr>
<tr>
<td>cALL</td>
<td>Common acute lymphoblastic leukaemia</td>
<td></td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
<td></td>
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<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
<td></td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptors</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
<td></td>
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<tr>
<td>CTLA</td>
<td>Cytotoxic t-lymphocyte antigen</td>
<td></td>
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<tr>
<td>CTMM</td>
<td>Center for translational molecular medicine</td>
<td></td>
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<tr>
<td>DAMP</td>
<td>Damage-associated molecular patterns</td>
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<tr>
<td>EBV</td>
<td>Epstein Barr virus</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
<td></td>
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<tr>
<td>FasFP</td>
<td>Fas-receptor fusion protein</td>
<td></td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
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<tr>
<td>FLT-3L</td>
<td>Fms-like tyrosine kinase-3 ligand</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
<td></td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally recognized as safe</td>
<td></td>
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<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
<td></td>
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<tr>
<td>H1N1pdm09</td>
<td>Pandemic H1N1</td>
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<tr>
<td>H3-K4me3</td>
<td>H3K4 trimethylation</td>
<td></td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>HMGB-1</td>
<td>High-mobility group protein B1</td>
<td></td>
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<tr>
<td>HR</td>
<td>Heart rate</td>
<td></td>
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<tr>
<td>ICD</td>
<td>Implantable cardioverter-defibrillator</td>
<td></td>
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<tr>
<td>ICU</td>
<td>Intensive care unit</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IL-1ra</td>
<td>IL-1 receptor antagonist</td>
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<tr>
<td>IQR</td>
<td>Interquartile range</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>mAB</td>
<td>Monoclonal antibodies</td>
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<tr>
<td>MAP</td>
<td>Mean arterial blood pressure</td>
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<tr>
<td>MEB</td>
<td>Dutch medicines evaluation board</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
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<tr>
<td>mHLA-DR</td>
<td>HLA-DR expression on monocytes</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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</tr>
<tr>
<td>MTB</td>
<td>Mycobacterium Tuberculosis</td>
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</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
<td></td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide oligomerization domain-like receptors</td>
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<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
<td></td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
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<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PET-CT</td>
<td>Positron emission tomography-computed tomography</td>
<td></td>
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<tr>
<td>PD-1</td>
<td>Programmed death-1</td>
<td></td>
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<tr>
<td>PD-L</td>
<td>Programmed death-ligand</td>
<td></td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemaglutinin</td>
<td></td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
<td></td>
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<tr>
<td>PUFAs</td>
<td>Polyunsaturated fatty acids</td>
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<tr>
<td>RCT</td>
<td>Randomized controlled trial</td>
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<tr>
<td>rHL-7</td>
<td>Recombinant human IL-7</td>
<td></td>
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<tr>
<td>rIFN-gamma</td>
<td>Recombinant Interferon-gamma</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>sCD163</td>
<td>Soluble haemoglobin scavenger receptor</td>
<td></td>
</tr>
<tr>
<td>sFas</td>
<td>Human soluble Fas</td>
<td></td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
<td></td>
</tr>
<tr>
<td>spp</td>
<td>Species</td>
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</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>Th-17</td>
<td>T-helper 17</td>
<td></td>
</tr>
<tr>
<td>TMP-SMX</td>
<td>Cotrimoxazole</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
<td></td>
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<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
<td></td>
</tr>
<tr>
<td>TREM-1</td>
<td>Triggering receptor expressed on myeloid cells 1</td>
<td></td>
</tr>
<tr>
<td>Tregs</td>
<td>T regulatory cells</td>
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CHAPTER 1

General introduction, aim and outline of this thesis
GENERAL INTRODUCTION

When invading microorganisms have passed the first hurdle represented by the physical barriers of the host, the innate immune system is responsible for the initial recognition of, and defense against pathogenic microorganisms. In past decades it has become clear that innate immune activation occurs through recognition of recurring structural patterns of microbial components, the so-called pathogen-associated molecular patterns (PAMPs), or through recognition of host molecules released from the extracellular matrix or from damaged or stressed cells, the so-called damage-associated molecular patterns (DAMPs) (1). Examples of PAMPs include endotoxin (lipopolysaccharide [LPS]), lipoproteins, flagellin, fimbriae, peptidoglycans, peptidoglycan-associated lipoproteins, lipoteichoic acid, and bacterial/viral DNA or RNA fragments. Examples of DAMPs include heat-shock proteins, hyaluronic acid, uric acid, HMGB1, and mitochondrial DNA fragments(1). PAMPs and DAMPs are recognized by specific pattern recognition receptors (PRRs) present on innate immune cells such as macrophages, monocytes, and dendritic cells. Binding of a PAMP/DAMP to a PRR results in activation of intracellular signaling cascades that subsequently leads to activation of the innate immune system. In case of infection, a pro-inflammatory response is mounted, which activates and potentiates phagocytosis and/or killing of the pathogen. In case that innate immune defenses do not eliminate the pathogen, a second line of defense is activated. This second line of defense is the adaptive immune response, which becomes prominent after several days and usually leads to strong immunological memory.

Simultaneously with this pro-inflammatory response, an anti-inflammatory response is mounted as well. This response is generally regarded to represent a compensatory mechanism with two aims: firstly, to prevent the proinflammatory response from derailing uncontrollably and causing extensive tissue damage, and secondly, to restore homeostasis (2). Depending on a number of factors, including pathogen load, pathogen virulence, host comorbidities, and host genetic factors, the magnitude of both responses varies (Figure 1). An adequate pro- and anti-inflammatory immune response will eradicate all pathogens without causing serious harm to the host. However, when an inadequate immune response is
mounted (e.g. due to a suppressed immune system) serious infections may occur. For example, sepsis patients were thought to succumb to an overwhelming pro-inflammatory response. However, numerous trials have failed to improve outcome by dampening the pro-inflammatory immune response in these patients, and recently subsequent suppression of the immune system was identified to be the overriding immune dysfunction. In these cases, increasing the effectiveness of the immune response is a promising approach to prevent or combat life-threatening secondary infections.

This thesis describes the effects of several immune stimulatory compounds on the immune response of humans following severe bacterial, fungal or viral infections in pre-clinical and clinical studies. In the remainder of this introduction the pathophysiology of infectious disease-specific immune dysfunction will be discussed. Furthermore, the rationale of the administered immune stimulatory compounds is detailed.

IMMUNE DYSFUNCTION IN INFECTIOUS DISEASES

Opportunistic infections in patients with a suppressed immune system
Some patients have genetic defects in their immune system (so-called “primary or inherited immunodeficiency”), but most in most cases, immunodeficiency is acquired and caused, among others, by administration of immunosuppressive agents, chemotherapy, and bone marrow diseases (so-called “secondary immunodeficiency’s”). Many patients with immunodeficiency’s (either primary or secondary) are particularly vulnerable to pathogens that usually do not cause infections in patients with a healthy immune system (so-called “opportunistic infections”). The incidence of opportunistic infections is steadily increasing due to more frequent use of invasive medical procedures and immunosuppressive treatment modalities. Furthermore, despite development of new classes of antimicrobial agents, opportunistic infections remain associated with unacceptable high mortality rates (5). As such, new treatment modalities to treat or prevent opportunistic infections are highly warranted. Because all patients suffering from opportunistic infections display a more or less impaired immune system, adjunctive immunotherapy to improve host defence is an attractive strategy to improve patient’s outcome.

Secondary infections in sepsis patients
A recently discovered particular cause of secondary immunodeficiency is sepsis. Sepsis is defined as systemic inflammation caused by an infection. In combination with hypoperfusion or dysfunction of at least one organ system, a diagnosis of severe sepsis is made (6). If hypotension or signs of hypoperfusion occur and persists despite adequate fluid therapy, the patient is in septic shock (6). Conservative estimates of the incidence of sepsis are 20 million cases per year worldwide, accounting for more casualties than breast cancer, lung cancer, and prostate cancer combined (7). Especially septic shock is associated with high mortality rates, not only in immune compromised patients, but also in previously healthy patients with an adequate immune system. It was thought for decades that septic patients succumb to an uncontrolled pro-inflammatory immune response to invading microorganisms, resulting in multi-organ failure and death induced by hyperinflammation. However, in recent years it has become clear that the vast majority of septic patients do not die from the initial pro-inflammatory hit, but at a later time point in an immunocompromised state which reduces the patient’s ability to eradicate the initial infection, and/or renders them more vulnerable to secondary infections (8, 9). This is probably the main reason behind the fact that all clinical trials that investigated anti-inflammatory strategies in patients with sepsis have failed to achieve a significant reduction in important endpoints such as mortality, hospital length of stay, or occurrence of secondary infections. Therefore, adjunctive immunotherapy to improve host defense appears to represent a valid and promising strategy to improve outcome of septic patients.

Primary infections in otherwise healthy subjects
In addition to the described opportunistic infections in immune compromised patients, no antimicrobial therapy is currently available for some pathogens (especially viruses: e.g. influenza virus), and in these patients life-threatening infections can develop even with an adequate immune response. For instance,
influenza virus infection is a global problem which annually affects an estimated 5%–10% of adults and 20%–30% of children. The influenza epidemics are estimated to result in about 3 to 5 million cases of severe illness worldwide, and account for approximately 500,000 deaths annually (10). The magnitude and quality of the vaccination-induced antigen-specific antibody titers is considered to be the primary correlate of protection against influenza infection (11, 12), although protective antibody titers are often not reached, especially not in the elderly (13). Moreover, various studies suggest that cellular immunity is likely to play a significant role in pathogen clearance as well, directly and through indirect effects on antibody responses (14, 15). Also in these cases, potentiating the immune response is an attractive strategy to improve outcome, which could have enormous socio-economic impact because very large patient groups could benefit.

**IMMUNOMODULATORY COMPOUNDS**

In spite of the clear rationale, immunostimulatory treatment in infectious diseases is still in its infancy. To date, only a few small studies have investigated the administration of immunostimulatory compounds in patients suffering from life-threatening infectious diseases, but large randomized placebo-controlled clinical trials are lacking. The immunostimulatory compound that has been studied most extensively in the context of opportunistic infections is Interferon-gamma (IFN-γ), which protects against these infections in patients with chronic granulomatous disease (CGD) (16), Human Immunodeficiency Virus (HIV) (17), leukemia (18), and organ transplants (18, 19). In sepsis patients, IFN-γ and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) are the most extensively studied immunostimulatory compounds (20-22). Both of these treatments have been demonstrated to enhance the function of immune cells in vitro, in vivo in animals, and ex vivo in humans (20-22). However, effects of these compounds on the immune response in vivo in humans have never been studied. Nevertheless, IFN-γ is a registered treatment for prevention of infections in patients with CGD and osteopetrosis, while GM-CSF is registered for prevention of infections in patients with neutropenia following chemotherapy. However, the severity of

![Figure 1. Global representation of possible immunological responses in patients; Depending on a number of factors, including bacterial load, pathogen virulence, host comorbidities, and host genetic factors, the magnitude of this response may vary. Panel A. An adequate pro-inflammatory response (grey line) to eradicate all bacteria (dashed line) combined with an adequate non-sustained anti-inflammatory response (black line) to prevent tissue damage due to excessive pro-inflammation (for example a healthy young adult who fully recovers from erysipelas with, except for antibiotics, no need for any supporting therapy); Panel B. Preexistent impaired immune functions (grey and black lines) resulting in opportunistic infections (for example a patient on high dose steroid therapy who develops cerebral Nocardiosis); Panel C. A normal pro-inflammatory immune response in a healthy individual which nevertheless does not result in eradication of overwhelming amounts of pathogens and/or highly virulent pathogens (for example a previously healthy patient with dengue infections, or an infection with a highly virulent influenza strain); Panel D. An overwhelming pro-inflammatory response (grey line) in combination with an adequate anti-inflammatory response (black line) likely eradicates all bacteria (dashed line), but will also cause pronounced tissue damage and ultimately multi-organ failure (for example a previously healthy young adult with meningococcemia); Panel E. An appropriate pro-inflammatory response (grey line) combined with a pronounced and/or sustained anti-inflammatory state (black line), resulting in immunoparalysis with ongoing bacterial infection (dashed line) or secondary (opportunistic) infections (dotted line) (for example a patient who develops candidemia following postoperative abdominal sepsis). Adapted from (8).](image-url)
potential side-effects and the substantial costs of these compounds (e.g. one dose of GM-CSF costs €1500) highlights the need for development of novel immunostimulatory treatment modalities. Thereto, new immunomodulatory approaches emerge because in recent years there has been a tremendous progress in the understanding of immune mechanisms.

One of these completely new immunomodulatory approaches is based on the recently discovered concept of “trained innate immunity”. For example, recent studies have shown that production of pro-inflammatory cytokines, such as IFN-γ, TNF-α, and IL-1β by monocytes upon ex vivo stimulation with non-related pathogens was enhanced after vaccination with the live attenuated BCG vaccine, even months after vaccination. These effects were based on “training” of the innate immune system, as they were independent of B- and T-cells (23, 24). Therefore, compounds that can induce trained immunity could enhance human host defences against unrelated pathogens, possible with profound therapeutical or prophylactic implications. Next to BCG, Beta-glucan has also been identified as a compound that induces trained innate immunity (25). Beta-glucans are ubiquitous naturally occurring carbohydrates that are already used for centuries in alternative medicine all over the world for their presumed immune enhancing effects, and it is widely offered on the internet as a dietary supplement for humans. Due to the fact that it is inexpensive and well tolerated, oral Beta-glucan might represent a promising candidate to enhance the immune response, although its efficacy has not yet been established in humans.

In this thesis, we investigated whether well-known immunostimulatory compounds can reverse sepsis-induced immunoparalysis, and whether they are also able to enhance immune responses in patients with invasive opportunistic infections. In addition, we investigated whether the innate immune system can be trained in vivo in humans by live attenuated BCG, inactivated BCG and orally administered commercially available Beta-glucan.

**AIM AND OUTLINE OF THIS THESIS**

The aim of this thesis was to perform translational, clinically relevant studies regarding immunomodulatory therapies for the adjunctive treatment of infectious diseases.

In the first part of this thesis, immunotherapy for the adjunctive treatment of sepsis is assessed. The rationale for immunostimulatory therapy as adjunctive treatment for sepsis is presented in a review in chapter 2. In chapter 3, the effects of the immunostimulatory compounds IFN-γ and GM-CSF on immunoparalysis are studied in a randomized, placebo-controlled manner using the human endotoxemia model, a model for sepsis-induced immunoparalysis in humans in vivo.

The second part of this thesis focuses on the effects of immunotherapy for the adjunctive treatment of opportunistic infections. In chapter 4, the immunostimulatory effects of adjunctive IFN-γ therapy are investigated in patients suffering from invasive fungal infections. Chapters 5 and 6 are case studies describing the effects of adjunctive IFN-γ therapy in a pediatric patient with progressive invasive Candida infection and in an adult patient with progressive cerebral nocardiosis.

In the third part of this thesis new immunomodulatory modalities are investigated. Chapter 7 describes the non-specific effects of live attenuated BCG vaccination on cellular and antibody responses induced by a subsequent influenza vaccination in vivo in humans. In chapter 8, the potential immunostimulatory effects of gamma-irradiated BCG on the innate immune response in vivo in humans are studied using the human endotoxemia model. Chapter 9 describes the effects of orally administered Beta-Glucan on ex vivo innate immune responses in humans.

Finally this thesis is concluded with a summary in chapter 10 and a general discussion of the findings and future perspectives in chapter 11.
REFERENCES


Immunomodulation as adjunctive treatment for sepsis

Part

ONE
CHAPTER 2

Immunotherapy for the adjunctive treatment of sepsis: from immunosuppression to immunostimulation. Time for a paradigm change?

Jenneke Leentjens
Matthijs Kox
Johannes G. van der Hoeven
Mihai G. Netea
Peter Pickkers
ABSTRACT

Sepsis is the leading cause of death in the ICU, and ranks in the top 10 causes of death in general worldwide. Proinflammatory mediators are related to symptoms observed early in sepsis patients such as fever and hemodynamic instability. However, in recent years it has become clear that most septic patients do not die from an overwhelming proinflammatory immune response, but in an immunosuppressive state, which can last for days or even weeks, that results in increased susceptibility to secondary (opportunistic) infections. Although infection control and supportive therapies will remain the cornerstone of treatment, especially in the early phase of sepsis, the identification of this so-called “immunoparalysis” is currently causing a paradigm shift in the adjunctive treatment of sepsis from therapies that suppress the immune system towards immunostimulation. In this Critical Care Perspective we will give an overview of the pathophysiology of sepsis with a focus on immunosuppressive mechanisms that play an important role in outcome. In addition, we will present an appraisal of the recent advances in immunotherapy as an adjunctive treatment for sepsis.

INTRODUCTION

Sepsis is one of the leading causes of death among hospitalized patients, and a frequent cause of admission to intensive care units (ICUs) (2). Despite advances in medical care, mortality rates of sepsis remain high. In addition, the incidence of sepsis in the United States has increased annually from 1975 to 2000 by approximately 9%, to 240 cases per 100,000 persons (2). Because the incidence is likely to increase even further due to several reasons (increasing age, immunosuppressive therapies, invasive procedures), and due to the high mortality rate, reaching 30-40% in severe sepsis, new therapeutic perspectives are highly warranted.

The classical type of therapeutical immunomodulation assessed repeatedly during the last decades was based on inhibition of inflammatory mediators such as cytokines. This approach has been investigated because of the beneficial effects of cytokine blockade in animal models of sepsis (3). However, the traditional approach of suppressing the immune system as an adjunctive treatment of sepsis has proven to be unsuccessful in numerous clinical trials (see supplementary Table 1 in the online supplement). An important reason for this might be that the vast majority of septic patients do not die due to complications resulting from an overwhelming proinflammatory immune response, but due to secondary/opportunistic infections resulting from a severely suppressed immune response (4). In this Critical Care Perspective we will translate new insights in the pathophysiology of sepsis into a better understanding of the current treatment of septic shock, and possible future therapeutical immunomodulating approaches.

PATHOPHYSIOLOGY OF SEPSIS AND DETERMINANTS OF OUTCOME

GENERAL

A simplified overview of the innate immune response in sepsis is depicted in Figure 1. When invading microorganisms have overtaken the first hurdle represented by the physical barriers of the host, the nonspecific or “innate” immune
system is responsible for the initial recognition of, and defense against pathogenic microorganisms. In past decades it has become clear that innate immune activation occurs through recognition of recurring structural patterns of microbial components, the so-called pathogen-associated molecular patterns (PAMPs). These PAMPs may be surface molecules of microorganisms (such as endotoxin [lipopolysaccharide (LPS)], lipoprotein, flagellin, fimbriae, peptidoglycans, peptidoglycan-associated lipoprotein and lipoteichoic acid), but also intracellular patterns that are released by lysis of bacteria (such as heat-shock proteins, and DNA or RNA fragments) (9). PAMPs are recognized by specific pattern recognition receptors (PRRs) present on, among others, macrophages, monocytes and dendritic cells. Four families of PRRs have been described to date: Toll-Like receptors (TLRs), Nucleotide Oligomerization Domain-like receptors (NLRs), C-type lectin receptors (CLRs) and RigI-helicases (g). Binding of a PAMP to a PRR results in activation of intracellular signaling cascades that subsequently leads to activation of the innate immune system. Depending on a number of factors, including bacterial load, pathogen virulence, host comorbidities, and host genetic factors, the magnitude of this response may vary, and can ultimately lead to systemic inflammation and the subsequent signs and symptoms that define the sepsis syndrome as an entity (6).

PRO-INFLAMMATION
While highly variable between patients, the proinflammatory response often predominates in the early phase of an infection. This is commonly regarded as the hyperinflammatory phase of sepsis. The ultimate goal of this proinflammatory reaction is the eradication of pathogens. This phase is characterized by production of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IFN-γ, and chemokines, including neutrophil and macrophage chemotactic factors such as CCL2 (MCP-1), CCL3 (MIP-1α), CCL6 (C10), and CXCL8 (IL-8) (7). The proinflammatory cytokines stimulate the effector functions of neutrophils, macrophages, and Th1-cells, exacerbating cellular immunity. These responses are induced in a context in which the complement system and the coagulation cascade are also strongly activated, and this can ultimately result in the typical septic shock symptoms, such as hemodynamic instability, coagulation abnormalities and end-organ dysfunction (7). If patients succumb to sepsis in the first few days, it is therefore most likely attributable to an overwhelming proinflammatory cytokine response. For instance, in certain forms of sepsis such as meningococcemia (Figure 2, panel A), circulating proinflammatory cytokine levels are high and correlate with (early) mortality (8).

However, in sepsis patients in general, circulating levels of proinflammatory cytokines are much lower (9), and signs of immune suppression are often observed from very early on. For example, lymphocyte apoptosis is already observed upon patient admission in the hospital (10), and similar early effects have been observed regarding monocyte deactivation (11). In this respect it is interesting to.
observe that therapies aimed to suppress proinflammatory mediators in sepsis failed to achieve a significant reduction in important endpoints such as mortality and hospital length of stay. Some interventions even led to an increased mortality (supplementary Table 1 in the online supplement), that could be attributed to increased susceptibility towards secondary infections (22). The necessity of adequate levels of proinflammatory mediators like TNF-α in combating infection has been underscored further by the finding that sepsis and other infectious complications are more likely to develop in patients with rheumatoid arthritis who are treated with TNF antagonists (33).

ANTI-INFLAMMATION AND ASSOCIATED IMMUNOPARALYSIS
As mentioned earlier, simultaneous with the proinflammatory response upon infection, an anti-inflammatory reaction is mounted, presumably to curtail inflammation and thereby prevent collateral tissue damage (Figure 2, panel B). However, a too pronounced or sustained anti-inflammatory response can lead to generalized and long-term impairment of the host’s immune function, known as "immunoparalysis" (6). This profound state of immunosuppression, that can last days to weeks after the initial infectious hit, results in increased vulnerability towards secondary (opportunistic) infections (14). There is increasing evidence that immunoparalysis worsens outcome in the later phase of sepsis, with multiple studies suggesting that not the presence of immunosuppressive processes “per se”, but the lack of sufficient recovery of the immune response is the main contributor to late mortality (10, 15). To date, there is no evidence that early anti-inflammatory immune responses in sepsis are responsible for an impaired early outcome. Recently, it was found that, compared with patients who died of non-sepsis etiologies, isolated splenic and pulmonary immune cells from deceased sepsis patients exhibit clear signs of severe immunosuppression (4); In another autopsy study of patients who died from sepsis or septic shock, a continuous septic focus was observed in 63 of the 71 patients (88.7%) who were treated for more than 7 days, suggesting that the "paralyzed" immune system is unable to eradicate the invading microorganisms (16). The clinical relevance of immunoparalysis is also apparent from the frequent occurrence of infections and reactivation of relatively less virulent bacteria, viruses or fungi, including Acinetobacter, Enterococcus, cytomegalovirus, herpes simplex virus, and Candida spp. (14, 17-19). Furthermore, it has been reported that reduced expression of HLA-DR on monocytes (mHLA-DR), a marker currently used to identify patients with immunoparalysis, is associated with reduced survival in patients with sepsis in most (20-22), though not all (23), studies. Likewise, attenuated TNF-α production by ex vivo LPS-stimulated monocytes, another mean of identifying immunoparalysis, has been associated with impaired survival (24). Finally, the lack of positive effects of anti-inflammatory therapies on sepsis outcome has played an important role in the current paradigm shift towards more attention for the immunosuppressive phase of sepsis (6). Hence, we can conclude that immunoparalysis is an important cause of the persistent adverse outcome of sepsis patient in the post-acute phase, even if the primary infection has been completely eradicated. As a result, secondary hospital acquired infections can develop despite aggressive source control, broad spectrum antibiotics, and the best available supportive care (Figure 2, panel C). Importantly, with some pro-inflammatory mediators such as HMGB-1, sTREM-1, or C5a increasing in the subacute phase of sepsis, it has become clear that the pro- and antiinflammatory phase of sepsis are not distinct, but may occur simultaneously with a more pronounced role for anti-inflammation in the later phases (25).

Several mechanisms appear to be involved in the development of sepsis-induced immunoparalysis (see supplementary Table 2 in the online supplement for a schematic overview and relevant references). A number of functional defects in leukocytes isolated from sepsis patients have been characterized. These defects include impaired neutrophil (chemotactic) responses (26), diminished expression of important cell surface antigens like HLA-DR on monocytes causing alterations in antigen-presenting ability, and enhanced apoptosis, which has also been implicated to contribute to leukocyte deactivation by rendering specific leukocyte subpopulations unable to react and perform their normal cellular functions. Moreover, recent studies suggest that expression of inhibitory receptors like programmed death-1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4), and B and T lymphocyte attenuator (BTLA) play a role in the exhaustion of lymphocytes. Furthermore, several lymphocyte subpopulations such as CD4+CD25+
T regulatory (Treg) cells, NK-T cells, and CD8+ T cells that are known to actively suppress the adaptive immune response may also play an important role in septic immune dysfunction. Others have found that a subset of mature human neutrophils, capable of suppressing human T cell proliferation, could be involved. In a murine model of sepsis, it was demonstrated that dysfunctional dendritic cells also play an important role in the development of sepsis-induced immunoparalysis (at least intrapulmonary) (27, 28). Immunoparalysis is also characterized by a dysregulated cytokine production, favoring the expression of antiinflammatory cytokines (e.g. IL-10, IL-4, IL-13, transforming growth factor-β (TGF-β), and interleukin 1 receptor antagonist) relative to the production of proinflammatory cytokines.

At the intracellular level, recent studies have indicated that long-term cellular inhibition is due to epigenetic changes, leading to the suppression of proinflammatory gene transcription (extensively reviewed in (29)). In short, epigenetic regulation of gene transcription occurs through numerous mechanisms, but can be generally regarded as the regulated organization of gene loci into transcriptionally active or silent states. Transcriptionally active chromatin (euchromatin) is accessible to transcription factors and polymerases, while transcriptionally silent chromatin (heterochromatin) is sequestered from these factors. It has now become clear that TLR-induced chromatin modifications are responsible for transient silencing of tolerizable (T) genes (including those encoding proinflammatory mediators), and for priming of non-tolerizable (NT) genes (including those encoding antimicrobial peptides). The T genes are transiently inactivated to prevent pathology associated with excessive inflammation, while the NT genes remain inducible to provide continuous protection from infection and tissue repair (Figure 3). For example, it was demonstrated that selective histone deacetylation contributes to the silencing of class T genes (e.g. IL-6) in endotoxin-tolerance in macrophages (a LPS-induced refractory state towards a subsequent LPS challenge, bearing resemblance to sepsis-induced immunoparalysis).

Figure 2. Global representation of possible immunological responses in patients with sepsis. Depending on a number of factors, including bacterial load, pathogen virulence, host comorbidities, and host genetic factors, the magnitude of this response may vary. (A) An antiinflammatory response (orange line) in combination with an overwhelming proinflammatory response (red line), which is likely to eradicate all bacteria (purple line) but will also lead to pronounced tissue damage and ultimately multiorgan failure (for example, a previously healthy young adult with meningococcemia). (B) An adequate proinflammatory response (red line) to eradicate all bacteria (purple line) combined with an adequate nonsustained antiinflammatory response (orange line) to prevent tissue damage due to excessive proinflammation (for example, a healthy young adult who fully recovers from erysipelas without the need of any supporting therapy but antibiotics). (C) A proinflammatory response (red line) combined with a pronounced and/or sustained antiinflammatory state (orange line) resulting in immunoparalysis with ongoing bacterial infection (purple line) or secondary (opportunistic) infections (purple dotted line) (for example, a patient who develops candidemia after postoperative abdominal sepsis). Adapted by permission from Reference 5.
observed after recovery of mice from caecal ligation and puncture, when mice are highly susceptible to secondary infections. Recent studies have demonstrated that epigenetic regulation of inflammatory genes, with reciprocal changes in histone 3 lysine 4 (H3K4) trimethylation and H3K27 dimethylation underlies post-sepsis immunosuppression (30, 31). In addition, it was recently demonstrated that negative TLR regulators such as IRAK-M and SHIP-1, might also participate in the development of immunoparalysis (32). For example, IRAK-M, a protein that negatively regulates LPS-induced inflammatory responses, is upregulated in monocytes isolated from septic patients (33). Moreover, chromatin remodeling and epigenetic gene silencing by IRAK-M has been shown to be mediated by reduced acetylation (AcH4) and methylation (H3K4) of specific histones in lung macrophages (34).

**SUITABILITY OF MARKERS OF IMMUNOPARALYSIS TO GUIDE IMMUNOSTIMULATORY TREATMENT**

The apparent detrimental effects associated with the immunoparalytic phase of sepsis suggests that immunostimulation may be an attractive new treatment strategy (discussed in the next paragraph). However, caution should be taken that this approach is not applied when proinflammation predominates. Although there is no clinical evidence to date to support this notion, immunostimulation during proinflammatory predominance could theoretically result in an augmented proinflammatory response leading to more collateral tissue damage. As mentioned earlier, up till now, mHLA-DR expression and the production of cytokines by leukocytes that are ex vivo stimulated with Toll-like receptor agonists, two parameters that are highly correlated (29, 35), are mostly used to identify immunoparalysis. It was demonstrated that sepsis patients with reduced expression of HLA-DR on monocytes or reduced production of TNF-α by ex vivo stimulated leukocytes are significantly more likely to develop secondary infections (22). Interestingly, recent studies indicate that, instead of a single value at a given time point, the slope of recovery of mHLA-DR may better reflect susceptibility towards secondary infections and therefore may more accurately predict

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Figure 3. Epigenetic modifications. (A) In the DNA helix, inactive DNA is wrapped around histones, so that genes are inaccessible for transcription. After uncoupling of the histone (A, middle), genes become accessible. Transcriptionally active chromatin (euchromatin) is accessible to transcription factors and polymerases, whereas transcriptionally silent chromatin (heterochromatin) is sequestered from these factors. Histones are proteins responsible for packaging and organizing the lengthy DNA strand(s) into organized chromatin. Modifications to the protein tails of histone core components can direct the winding or unwinding of the associated DNA. The activating or repressing nature of these histone tail modifications is dependent on both the chemical nature of the modification and the location of the modification on the histone tail. For example, as depicted in B, acetylation of histone tails at specific amino acid residues are believed to act as an activation histone mark. In contrast, methylation of histone tails can be an activation or repressing transcriptional mark, depending on both the type and the location of the modification. It was demonstrated that in endotoxin tolerance, toll-like receptor–induced chromatin modifications are responsible for transient silencing of tolerizable (T) genes (including those encoding proinflammatory mediators) and for priming of non tolerizable (NT) genes (including those encoding antimicrobial peptides). The T genes are transiently inactivated to prevent pathology associated with excessive inflammation, whereas the NT genes remain inducible to provide continuous protection from infection (58). Panel A reprinted with permission from G. Almouzni, Institut Curie-CNRS; watercolor by Nicolas Bouvier.
outcome (35). Nevertheless, the potential value of mHLA-DR expression and/or ex vivo cytokine production as indicators of a patient’s “immune status” remains to be demonstrated because sepsis patients often show evidence of immune suppression on admission, indicating that initiation of immunostimulatory therapy based on these markers may be questionable. Furthermore, these markers only reflect the status of the blood/leukocyte compartment and not the in vivo immune status of the patient, which is predominantly mediated by tissue-resident macrophages. This was illustrated by a murine model of immunoparalysis showing that impaired bacterial clearance was largely attributable to impaired function of tissue-resident macrophages (36), and findings of elevated cytokine levels in LPS-challenged mice that lack circulating immune cells (37). Moreover, the difference in duration of ex vivo and in vivo endotoxin tolerance and a lack of correlation between ex vivo (including HLA-DR expression) and in vivo responses to endotoxin in humans (38-40) suggest that tissue macrophages rather than circulating cytokines are the main effector cells in sepsis. An important argument to support an immunoparalytic status of tissue macrophages has been provided by a recent autopsy study who demonstrated defective cytokine production capacity of tissue macrophages in patients deceased due to sepsis compared with matched control patients that died of non-septic etiologies (4).

Other potentially suitable biomarkers that have been linked to patients’ immune status include plasma IL-10 levels and TNF-α/IL-10 ratio, markers of anti-inflammatory macrophages (Soluble haemoglobin scavenger receptor [sCD163]), markers of lymphocyte apoptosis (human soluble Fas [sFas], Fas ligand [FasL], and sFas/FasL ratio), and markers of T-cell exhaustion (programmed cell death 1 [PD-1], and PD ligand 1) (see supplementary Table 4 in the online supplement for a schematic overview and relevant references). However, it remains to be determined whether these markers are truly representative of a patient’s in vivo immune status and thus if they are suitable to guide immunostimulatory treatment. With regard to the heterogeneity of the immune response in sepsis, combination of several biomarkers (in panels) may prove to be of additional value towards a more individualized, goal-directed therapy. Possibly, the type of underlying infection (infected tissue), or causative microorganism(s) should be taken into account as immunosuppressive mechanisms can vary between these (41). In the meantime, the use of surrogate markers to determine whether a patient has passed the proinflammatory phase, such as recovery of hemodynamic stability indicated by decreased inotropic requirements, might be considered.

**THERAPY**

**CURRENT APPROACH**

In patients who meet the criteria for septic shock, the first approach is focused on careful monitoring, hemodynamic and organ function support, in addition to source control. The swift (if necessary surgical) removal of infected tissues in combination with antibiotics is the key to success in the treatment of sepsis (42). Prospective cohort studies showed a 4- to 8-fold higher mortality when inadequate initial antibiotics were administered (43, 44), and a more recent observational study showed that in septic shock patients, the risk of death increases by about 8% with each hour passing without antibiotics (42). It is beyond the scope of this review to describe other potential supportive measures like vasopressor therapy, glycemic regulation, renal replacement therapy, or ventilation modes; these are extensively reviewed elsewhere (45).

**NEW INSIGHTS**

Although infection control and supportive therapies, especially in the early phase of sepsis, will remain the cornerstone of sepsis treatment, new adjuvant treatments are warranted to prevent or treat the infaust effects of the immunoparalytic phase of sepsis. Various immune stimulants have shown favorable results in vitro, ex vivo (such as effects on monocyte HLA-DR expression and/or TNF-alpha production), and in vivo in animals. The therapies studied and their mechanisms of action are depicted in Figure 1 (for a schematic overview and relevant references see supplementary Table 4 in the online supplement), and include the anti-apoptotic and immune stimulatory cytokine IL-15, the anti-apoptotic Fas-receptor fusion protein (FasFP) (46), caspase inhibitors, the anti-apoptotic and negative regulatory molecule blocking anti-PD-L1 antibody, the dendritic cell growth...
factor Fms-like tyrosine kinase-3 ligand (Flt-3L), the glucocorticoid receptor antagonist mifepristone (RU486), the IL-10 synthesis inhibitor ammonium trichloro(dioxoethylene-o,o’)-tellurate (AS101), the anti-regulatory T-cell and anti-IL-10 monoclonal antibodies, the anti-transforming growth factor-β monoclonal antibodies, T-cell anti-CD25 antibodies, the negative regulatory molecule blocking anti-CTLA4, myeloid cell function augmenting Granulocyte-Colony stimulating factor (G-CSF), and immuno-modulating diets (such as diets containing high concentrations of omega-3 polyunsaturated fatty acids (PUFAs) derived from fish oil, or health food supplements such as the proinflammatory cytokine enhancing supplement Beta-glucan). However, clinical application of these therapies still requires extensive clinical studies to test their clinical safety and efficacy.

A promising candidate to reverse sepsis-induced immunoparalysis that is already available for human use and has an excellent safety profile is IL-7. IL-7 is a potent anti-apoptotic cytokine that enhances immune effector cell function and is essential for lymphocyte survival (47), and IL-7 has been shown to improve survival in murine models of sepsis (48). Furthermore, it was recently demonstrated that lymphocyte function of septic patients was restored by ex vivo stimulation with IL-7 (49). Interestingly, subcutaneous IL-7 administration in HIV patients reversed signs of immune dysfunction similar to those observed in immunoparalyzed septic patients (50). In these patients, IL-7 increased circulating lymphocyte numbers and restored their function, reflected by increased cytokine production upon ex vivo stimulation (50). Of note, IL-7 robustly enhances IFN-γ production, which might play an important role in its immunostimulatory effects (further discussed below) (49, 50).

The two most studied therapies to reverse immunoparalysis to date are interferon-gamma (IFN-γ) (51-56), and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) (52, 53, 55, 56). These agents also represent the only two immune stimulants that have already been tested in clinical trials in septic patients. A one-armed intervention study showed that subcutaneous administration of IFN-γ for an average of 6 days to patients with septic shock increased mHLA-DR expression and the production of TNF-α by leukocytes stimulated ex-vivo. Furthermore, 8 out of the 9 patients recovered from severe sepsis (35). In case of GM-CSF, a meta-analysis of 4 randomized, placebo-controlled clinical trials in septic patients (total n=195; GM-CSF treated n=75) showed an increase in clearance of infection (57). However, no beneficial effect of GM-CSF on 28-day mortality could be demonstrated. This was probably because of limited power, but one has to be also aware that other factors besides immunoparalysis may account for patient mortality. One of these randomized clinical trials used mHLA-DR expression to guide treatment of septic patients with GM-CSF (58). In this study, all 19 patients treated with GM-CSF showed normalization of mHLA-DR expression, while this was only the case in 3 of the 19 placebo-treated patients. Moreover, in GM-CSF-treated patients, time-on-ventilator was reduced, as was ICU and hospital length of stay (58). In addition, one study has shown that immunostimulatory therapies can actually reverse immunoparalysis determined not only in vitro, but also in vivo in humans. Repeated administration of endotoxin to healthy volunteers (experimental human endotoxemia) was used as a model to mimic sepsis-induced immunoparalysis. A second endotoxin administration within a relatively short time-frame results in a greatly suppressed innate immune response compared to the response after the first endotoxin administration. Using this model it was demonstrated that, compared with placebo, treatment with IFN-γ in between endotoxin administrations (separated by one week) not only increased mHLA-DR expression, but also restored in vivo production of the proinflammatory cytokine TNF-α, while further attenuating the production of the anti-inflammatory cytokine IL-10 upon the second endotoxin administration. Similar, but less pronounced effects were found in subjects treated with GM-CSF (40).

Recent in vitro studies have shed light on the mode of action by which IFN-γ reverses immunoparalysis. It was shown that IFN-γ is able to bypass the effects of negative regulators of the LPS signaling pathway contributing to immunoparalysis at the mRNA level (54). Others have found that IFN-γ does not alter proximal LPS signaling defects in tolerized monocytes, but reverses tolerance-associated epigenetic modifications that suppress TNF-α and IL-6 transcription (52). However, although promising, treatment with immune-stimulatory agents in sepsis is still in the experimental phase. New clinical trials are currently underway to confirm the promising results of IFN-γ (NCT01649921) and GM-CSF (NCT01653665).
CONCLUSION

In recent years, it has become increasingly clear that immunoparalysis plays an important detrimental role in the morbidity and mortality of sepsis. Although infection control and supportive therapies will remain the cornerstone of sepsis treatment, especially in the initial phase of sepsis, the future of adjunctive treatment of sepsis may prove to be stimulation, rather than suppression, of the immune system in selected patients. Promising immune stimulants such as IFN-γ and GM-CSF are currently under investigation in clinical trials. However, with a wide range of possible immune responses between patients, and the interaction of proinflammatory processes and immune defects, both at the onset of infection and in a later phase, adequate stratification of sepsis patients is of primordial importance. When clear signs of immunoparalysis are present, and if positive effects of immune stimulatory therapy are demonstrated on clinically relevant endpoints, adjunctive tailor-made immunotherapy may finally come to age for sepsis patients. Nevertheless, immunointerventions need to be cautiously analyzed in order to prevent the same errors that were made in the past using anti-inflammatory treatments.

REFERENCES


Supplementary Table 2. Potential mechanisms involved in the development of sepsis-induced immunoparalysis.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diminished expression of important cell surface antigens like HLA-DR on monocytes [7]</td>
<td>Alterations in antigen-presenting ability</td>
</tr>
<tr>
<td>Enhanced apoptosis of leukocytes [8]</td>
<td>Rendering specific leukocyte subpopulations unable to react and perform their normal cellular functions</td>
</tr>
<tr>
<td>Expression of inhibitory receptors [9]</td>
<td>Rendering specific leukocyte subpopulations unable to react and perform their normal cellular functions</td>
</tr>
<tr>
<td>Appearance of immune suppressive lymphocyte/neutrophil subpopulations [10, 11]</td>
<td>Active suppression of the immune response</td>
</tr>
<tr>
<td>Dysregulated cytokine production [12]</td>
<td>Favoring the expression of antiinflammatory cytokines relative to the production of proinflammatory cytokines</td>
</tr>
</tbody>
</table>

HLA, Human Leukocyte Antigen.

Supplementary Table 3. Potentially suitable biomarkers that have been linked to patients’ immune status.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10 levels [14-17]</td>
<td>Marker of anti-inflammatory cytokine response</td>
</tr>
<tr>
<td>TNF-α/IL-10 ratio [18, 19]</td>
<td>Marker of anti-inflammatory cytokine balance</td>
</tr>
<tr>
<td>sCD163</td>
<td>Markers of anti-inflammatory macrophages</td>
</tr>
<tr>
<td>sFas, FasL, and sFas/FasL ratio [21]</td>
<td>Markers of lymphocyte apoptosis</td>
</tr>
<tr>
<td>PD-1, and PD ligand 1 [22, 23]</td>
<td>Markers of T-cell exhaustion</td>
</tr>
<tr>
<td>Reduced expression of HLA-DR on monocytes [24, 48]</td>
<td>Marker of reduced antigen presenting capacity of monocytes</td>
</tr>
<tr>
<td>Attenuated TNF-α production by ex vivo LPS-stimulated monocytes [29]</td>
<td>Marker of reduced capacity of proinflammatory cytokine production</td>
</tr>
</tbody>
</table>

IL, interleukin; TNF, tumor necrosis factor; sCD163, Soluble haemoglobin scavenger receptor; sFas, Human soluble Fas; FasL, Fas ligand; PD-1, Programmed cell death 1, HLA, Human Leukocyte Antigen; LPS, lipopolysaccharide.

ONLINE SUPPLEMENT

Supplementary table 1. Overview of anti-inflammatory strategies.

<table>
<thead>
<tr>
<th>Drug category</th>
<th>Mechanisms of action</th>
<th>Number of RCT's</th>
<th>Odds ratio survival (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosteroids [1]</td>
<td>Anti-inflammatory effects and reversal of relative adrenal cortical insufficiency</td>
<td>5</td>
<td>0.91 (0.97–1.25)</td>
</tr>
<tr>
<td>Mediator-specific anti-inflammatory [2]</td>
<td>Antibody-specific inactivation TNF-α</td>
<td>9</td>
<td>1.10 (0.97–1.25)</td>
</tr>
<tr>
<td>Soluble TNF receptors</td>
<td>Receptor-specific inactivation/enzyme degradation of PAF</td>
<td>7</td>
<td>1.09 (0.93–1.30)</td>
</tr>
<tr>
<td>Mediator-specific anti-inflammatory [2]</td>
<td>Receptor-specific inactivation circulating TNF</td>
<td>3</td>
<td>0.95 (0.78–1.16)</td>
</tr>
<tr>
<td>Mediator-specific anti-inflammatory [2]</td>
<td>Receptor-specific inactivation IL-1</td>
<td>3</td>
<td>1.48 (1.37–1.61)</td>
</tr>
<tr>
<td>Prostaglandin antagonists</td>
<td>Cyclooxygenase inhibition of prostaglandin production</td>
<td>3</td>
<td>1.22 (0.78–1.38)</td>
</tr>
<tr>
<td>Bradykinin antagonists</td>
<td>Inhibition of the kallikrein-kinin cascade</td>
<td>2</td>
<td>0.93 (0.65–1.32)</td>
</tr>
<tr>
<td>Anticoagulants</td>
<td>Anticoagulatory + anti-inflammatory</td>
<td>13</td>
<td>0.96 (0.89–1.03)</td>
</tr>
<tr>
<td>Tissue factor pathway inhibitor [2]</td>
<td>Anticoagulatory + anti-inflammatory</td>
<td>2</td>
<td>0.91 (0.84–1.25)</td>
</tr>
<tr>
<td>Activated protein C [4]</td>
<td>Anticoagulatory + anti-inflammatory</td>
<td>5</td>
<td>0.97 (0.78–1.22)</td>
</tr>
<tr>
<td>Other</td>
<td>Inhibition of the TLR4-MD2 interaction, anti-inflammatory</td>
<td>1</td>
<td>1.38 (0.75–2.53)</td>
</tr>
</tbody>
</table>

RCT, randomized controlled trial; TNF, tumor necrosis factor; mAb, monoclonal antibodies; PAF, platelet activating factor; IL, interleukin; LPS, lipopolysaccharide.
Supplementary Table 4. Potential proinflammatory therapies in septic patients.

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-15 [39]</td>
<td>Anti-apoptotic and stimulation of proinflammatory cytokines</td>
</tr>
<tr>
<td>IL-7 [30-31]</td>
<td>Anti-apoptotic, stimulation of proinflammatory cytokines, and promotion of lymphocyte survival</td>
</tr>
<tr>
<td>FasFP [33]</td>
<td>Anti-apoptotic</td>
</tr>
<tr>
<td>Caspase inhibitors [34]</td>
<td>Prevention of lymphocyte apoptosis</td>
</tr>
<tr>
<td>Anti-PD-L1 antibody [35]</td>
<td>Anti-apoptotic and blocking of negative regulatory molecules</td>
</tr>
<tr>
<td>FLT-3L [36]</td>
<td>Dendritic cell growth factor</td>
</tr>
<tr>
<td>Miliepristone (RU486) [37]</td>
<td>Glucocorticoid receptor antagonist</td>
</tr>
<tr>
<td>Ammonium trichloro(dioxoethylene-o,o')tellurate (AtSau) [38]</td>
<td>Inhibition of IL-10 synthesis</td>
</tr>
<tr>
<td>Anti-regulatory T-cell monoclonal antibodies [39]</td>
<td>Blocking of regulatory T-cells</td>
</tr>
<tr>
<td>Anti-IL-10 monoclonal antibodies [39]</td>
<td>Blocking of IL-10</td>
</tr>
<tr>
<td>Anti-transforming-growth factor-β monoclonal antibodies [40]</td>
<td>Blocking of transforming-growth factor-β</td>
</tr>
<tr>
<td>Anti-CTLA4 [41]</td>
<td>Inhibition of T-cells</td>
</tr>
<tr>
<td>Anti-CD25 antibodies [40]</td>
<td>Blocking of negative regulatory molecules</td>
</tr>
<tr>
<td>G-CSF [42]</td>
<td>Augmentation of myeloid cell functions</td>
</tr>
<tr>
<td>Immuno-modulating diets (especially PUFAs) [43]</td>
<td>Decreased production of proinflammatory arachidonic acid derived mediators</td>
</tr>
<tr>
<td>Beta-glucan [44]</td>
<td>Stimulation of proinflammatory cytokines</td>
</tr>
<tr>
<td>GM-CSF [45-48]</td>
<td>Stimulation of proinflammatory cytokines and enhancement of antigen presenting capacity of monocytes</td>
</tr>
<tr>
<td>Interferon (IFN)-gamma [45-49]</td>
<td>Stimulation of proinflammatory cytokines and enhancement of antigen presenting capacity of monocytes</td>
</tr>
</tbody>
</table>

FasFP, Fas-receptor fusion protein; PD, programmed death; FLT-3L, Fms-like tyrosine kinase-3 ligand; G-CSF, Granulocyte-Colony stimulating factor; PUFAs, poly unsaturated fatty acids; GM-CSF, Granulocyte Macrophage-colony stimulating factor; IFN, Interferon.

REFERENCES


CHAPTER 3

Reversal of immunoparalysis in humans in vivo: A double-blind, placebo-controlled, randomized pilot study

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Mihai G. Netea
Peter Pickkers
**ABSTRACT**

**Rationale:** Reversal of sepsis-induced immunoparalysis may reduce the incidence of secondary infections and improve outcome. While Interferon-gamma (IFN-γ) and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) restore immune competence of ex vivo stimulated leukocytes of septic patients, effects on immunoparalysis in vivo are not known.

**Objectives:** To investigate the effects of IFN-γ and GM-CSF on immunoparalysis in vivo in humans.

**Methods:** We performed a double-blind placebo-controlled randomized study in 18 healthy male volunteers that received E. coli endotoxin (LPS, 2 ng/kg, intravenously) on days 1 and 7 (visits 1 and 2). On days 2, 4 and 6 subjects received subcutaneous injections of IFN-γ (100 μg/day, n=6), GM-CSF (4 μg/kg/day, n=6), or placebo (NaCl 0.9%, n=6).

**Measurements and main results:** In the placebo group, immunoparalysis was illustrated by a 60% [48-71] reduction of LPS-induced TNF-α plasma concentrations during visit 2 (p=0.03), whereas the anti-inflammatory IL-10 response was not significantly attenuated (39% [2-65], p=0.15). In contrast, in the IFN-γ group, TNF-α concentrations during visit 2 were not significantly attenuated (28% [1-47], p=0.09), while the IL-10 response was significantly lower (reduction of 54% [47-66], p=0.03). Compared with the placebo group, the reduction in the LPS-induced TNF-α response during visit 2 was significantly less pronounced in the IFN-γ group (p=0.02). Moreover, compared with placebo, treatment with IFN-γ increased monocyte HLA-DR expression (p=0.02). The effects of GM-CSF tended in the same direction as IFN-γ, but were not statistically significant compared with placebo.

**Conclusions:** IFN-γ partially reverses immunoparalysis in vivo in humans. These results suggest that IFN-γ is a promising treatment option to reverse sepsis-induced immunoparalysis.

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**AT A GLANCE COMMENTARY**

**Scientific Knowledge on the Subject**

Suppression of the immune system, so-called ‘immunoparalysis’, is increasingly recognized as the overriding immune dysfunction in septic patients, rendering them vulnerable for secondary infections associated with impaired outcome. Trials in septic patients have demonstrated that treatment with IFN-γ or GM-CSF restores the cytokine response of ex vivo stimulated leukocytes, however the effects of IFN-γ or GM-CSF on immunoparalysis in vivo in humans have not yet been established.

**What this Study Adds to the Field**

We report that endotoxin administration in humans results in immunoparalysis reflected by attenuated plasma cytokine concentrations following the second endotoxin challenge. Treatment with IFN-γ augments endotoxin-induced TNF-α and further attenuates IL-10 concentrations, indicating partial reversal of immunoparalysis in vivo. The effects of GM-CSF tended in the same direction as IFN-γ, but were not statistically significant compared with the placebo group. These results implicate that immunoparalysis is relevant in vivo in humans and that immunostimulation represents a promising treatment option to reverse sepsis-induced immunoparalysis in critically ill patients.
INTRODUCTION

Sepsis is the leading cause of death in the ICU with an estimated 6 million victims per year worldwide (1). Although septic shock is traditionally viewed as an excessive systemic inflammatory reaction to invasive microbial pathogens, pharmacological interventions aimed at suppression of the immune response in sepsis have proved to be unsuccessful (2). An important reason for this might be that the vast majority of septic patients survive the initial pro-inflammatory hit, but die at a later time point from secondary/opportunistic infections in an immunosuppressed state (3-5). This so-called sepsis-induced 'immunoparalysis' is increasingly recognized as the overriding immune dysfunction in septic patients (6), and is characterized by impaired innate and adaptive immune responses, including enhanced apoptosis and dysfunction of lymphocytes, impaired phagocyte functions, and decreased ex vivo cytokine production (7). A widely used marker of immunoparalysis is monocyte HLA-DR (mHLA-DR) surface expression, reflecting monocyte (de)activation (8). Prolonged downregulation of mHLA-DR expression is associated with a higher risk of secondary infections (9) and reduced survival in septic patients (9, 10).

In light of the deleterious effects of sepsis-induced immunoparalysis on outcome, the sepsis research field is shifting towards novel therapies aimed at restoring immune competence in the later phase of sepsis (11). Recent case series and pilot trials indicate that long-lasting monocyte deactivation in sepsis can be reversed by treatment with Interferon gamma (IFN-γ) or Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) (12-16), reflected by restoration of TNF-α production by ex vivo stimulated leukocytes and increased mHLA-DR expression (17, 18). These compounds therefore appear promising candidates to reverse sepsis-induced immunoparalysis. However, as indicated above, all studies to date have analyzed the 'immune status' of the patients using ex vivo stimulation of leukocytes or flow cytometric analysis of HLA-DR expression on circulating monocytes. We have recently demonstrated that during systemic inflammation as observed during experimental human endotoxemia (lipopolysaccharide [LPS]) administration in healthy volunteers), the response of ex vivo LPS-stimulated leukocytes does not correlate with the in vivo LPS-induced inflammatory response in the same subject (19, 20). These data indicate that the in vivo response to endotoxin is mainly mediated by tissue-resident cells and that ex vivo measurements do not accurately reflect a subject or patient's in vivo immune status. Hence, it is unknown whether pharmacological interventions that restore the ex vivo immune competence of leukocytes, such as IFN-γ and GM-CSF, are effective in restoring immune competence in vivo.

As experimental human endotoxemia leads to pronounced immunosuppression, so-called endotoxin tolerance, both in vivo and ex vivo (20-23), it can serve as a model for sepsis-induced immunoparalysis. In the present proof-of-principle study we investigated whether treatment with IFN-γ or GM-CSF restores immune competence following experimental endotoxemia in humans in vivo.

METHODS

Subjects
This study was registered at ClinicalTrials.gov as NCT01374711. After approval from the local ethics committee of the Radboud University Nijmegen Medical Centre, 18 healthy, non-smoking, male volunteers gave written informed consent to participate in the experiments. All experiments were in accordance with the declaration of Helsinki. Subjects were screened before the start of the experiment and had a normal physical examination, electrocardiography, and routine laboratory values (including serology on HIV and hepatitis B). Subjects with febrile illness during the two weeks before the experiment were excluded. Subjects were not allowed to take any prescription drugs and asked to refrain from caffeine and alcohol intake 24 hours before the start of the experiment. Furthermore, subjects refrained from food 12 hours before the start of each endotoxemia experiment.

Study design
We performed a parallel double-blind placebo-controlled randomized study. The study design is depicted in Figure 1. Briefly, all subjects received an intravenous injection of lipopolysaccharide (LPS) on two occasions (visits 1 & 2) separated...
by 6 days. In between, on days 2 (t=24), 4 (t=76), and 6 (t=120), subjects were randomized to receive subcutaneous injections of either 100 μg IFN-γ (Immukine; Boehringer Ingelheim Alkmaar, the Netherlands, n=6), 4 μg/kg GM-CSF (Leukine/Sargramostim; Bayer Healthcare Pharmaceuticals, Seattle, United States, n=6), or placebo (NaCl 0.9%, n=6), in a double-blind fashion using the sealed envelope method. Endotoxemia experiments were conducted as described previously (20), a detailed description is provided in the online supplement. The dosages were based on previous studies in septic patients (15, 27). Since GM-CSF and IFN-γ had different administration volumes, a double dummy was used to ensure adequate blinding.

**Cytokine measurements**

For plasma cytokine analysis, EDTA anticoagulated blood was centrifuged immediately after withdrawal at 2000g at 4°C for 10 minutes, after which plasma was stored at -80°C until analysis. Concentrations of tumor necrosis factor-alpha (TNF-α), Interleukin (IL)-6, IL-10, IL-1 receptor antagonist (IL-1ra), IFN-γ, and GM-CSF were analyzed batch-wise by a Luminex assay according to the manufacturer’s instructions (Milliplex, Millipore, Billerica, MA, USA).

**Flow cytometric analysis of mHLA-DR expression and lymphocyte subset counts**

EDTA anticoagulated blood was stored at 4°C immediately after withdrawal and analyzed by flow cytometry (see online supplement for details). mHLA-DR expression was determined by calculating % HLA-DR-positive cells within CD14-positive cells within the CD45-positive leukocytes and HLA-DR mean fluorescence intensity (MFI) within these CD14-positive cells. Lymphocyte subsets were defined as: T-cells (CD45+CD3+), T-helper cells (Th, CD45+CD3+CD4+), cytotoxic T-cells (Tc, CD45+CD3+CD8+), B-cells (CD45+CD19+), and NK-cells (CD45+CD3-CD56+). Subset counts were calculated by multiplying the percentage gated cells by the total lymphocyte count.

**Calculations and statistical analysis**

In view of the small sample size, normality of distribution was not assumed. Area under curve (AUC) during both visits 1 and 2, representing an integrated measure of the LPS-induced responses, was calculated using time points 0-24 hours (cytokines) and 0-8 hours (symptom score, heart rate, mean arterial pressure [MAP], and temperature). Comparisons were made using Wilcoxon’s matched pairs (within-group comparisons, 2 groups), and Mann-Whitney U (between-group comparisons, 2 groups), or Kruskal-Wallis (between-group comparisons, 3 groups) tests as appropriate. A p-value of <0.05 was considered statistically significant. Data are expressed as median [interquartile range]. Calculations and statistical analyses were performed using Graphpad Prism version 5.0 (Graphpad Software, San Diego, CA, USA).

**RESULTS**

**Demographic characteristics**

Demographic characteristics of the study population are listed in Table 1. There were no significant differences in baseline characteristics between the three study groups. No serious adverse events occurred during the trial. During the treatment period (starting just before the first IFN-γ/GM-CSF/placebo administration [visit 1 t=24] until visit 2 t=0), one subject in the placebo group reported mild headaches and muscle pains, whereas 5 subjects in the IFN-γ group and 3 subjects in the GM-CSF group reported mild complaints ranging from headaches and muscle pains to fatigue.
Table 1. Demographic characteristics obtained during the screening visit

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=6)</th>
<th>GM-CSF (n=6)</th>
<th>IFN-γ (n=6)</th>
<th>Total (n=18)</th>
<th>P value between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, kg</td>
<td>81 [89-75]</td>
<td>72 [67-85]</td>
<td>72 [68-80]</td>
<td>74 [70-86]</td>
<td>0.24</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>96 [82-104]</td>
<td>89 [88-98]</td>
<td>98 [91-99]</td>
<td>95 [88-99]</td>
<td>0.75</td>
</tr>
</tbody>
</table>

BMI: body mass index; HR: heart rate; MAP: mean arterial blood pressure; bpm: beats per minute. Data are presented as median and interquartile range.

Plasma cytokines

As expected, during visit 1 there was a typical endotoxemia-induced transient increase in all measured cytokines, which was similar in the three experimental groups (Figure 2). In the LPS-placebo group, the area under the curve (AUC) of the LPS-induced plasma concentration of the archetypal pro-inflammatory cytokine TNF-α was 60% [48-71] (p=0.03) lower on visit 2 compared with visit 1, indicating immunoparalysis. In the IFN-γ and GM-CSF groups, this decrease in LPS-induced TNF-α release during the second LPS infusion was less pronounced and no longer significant (28% [1-47] (p=0.09) and 38% [-2-63] (p=0.16) lower on visit 2, respectively). Furthermore, compared with placebo, the reduction in AUC for TNF-α on visit 2 compared with visit 1 was significantly less pronounced in the IFN-γ group (p=0.01). Similarly, in the placebo-treated group, plasma IL-6 levels were suppressed (74% [65-83], p=0.03) following the second LPS challenge. Treatment with IFN-γ (62% [42-77], p=0.03) or GM-CSF (70% [-27-85], p=0.09) did not prevent the attenuated response of IL-6 during repeated endotoxemia. In the placebo and GM-CSF groups, no significant attenuation of the plasma concentrations of the anti-inflammatory cytokine IL-10 between visits 1 and 2 were observed (39% [2-65] (p=0.25) and 48% [-6-67] (p=0.21), respectively). In contrast, in IFN-γ-treated subjects, the IL-10 response was significantly attenuated upon the second LPS administration (54% [47-66], p=0.03). Finally, in the placebo group, the LPS-induced IL-1ra response was attenuated by 63% [52-86] (p=0.03), while in the IFN-γ and GM-CSF groups, IL-1ra levels were 44% [35-67] (p=0.03) and 41% [30-76] (p=0.06) lower on visit 2 compared with visit 1, respectively. This reduction was not significantly different compared with placebo-treated subjects.
mHLA-DR expression
In the placebo-group, the percentage HLA-DR positive monocytes (%mHLA-DR+) decreased from 73% [68-84] at t=0 to 61% [54-71] (p=0.03) during the first LPS challenge (Figure 3). A similar reduction was observed in the IFN-γ and GM-CSF groups during visit 1 (prior to the administration of study medication). Throughout the treatment period %mHLA-DR+ remained stable in the placebo and GM-CSF groups (from 80% [74-84] to 76% [72-80], p=0.30 and from 85% [80-92] to 94% [82-95] (p=0.40), respectively). Conversely, in the IFN-γ group, mHLA-DR expression tended to increase during the treatment period, from 83% [79-92] to 98% [94-99] (p=0.06). Histograms of a representative subject from the IFN-γ group are shown in Supplementary Figure 1 in the online supplement. The effects of IFN-γ on %mHLA-DR+ during the treatment period were significantly different compared with placebo (p=0.02). Moreover, %mHLA-DR+ in the IFN-γ group remained above 85% in the acute phase (8 hours) following the second LPS administration, while it dropped to 65-70% in the other two experimental groups (Supplementary Table 1 in the online supplement). MFI data showed a similar pattern as %mHLA-DR+ (Supplementary Table 1 in the online supplement).

Hematological parameters
Following the first LPS administration, total leukocyte counts showed the characteristic biphasic pattern in all three experimental groups, a leukopenic phase at t=1 (p<0.01 compared with t=0 for all three groups, Figure 4). In the placebo group, the total leukocyte count tended to decrease during the treatment period (a reduction of 24% [6-31], p=0.06, whereas in the IFN-γ group, the effects were highly variable between subjects (median reduction of 32% [-22-54], p=0.25). In the GM-CSF treated group, there was a clear increase in leukocyte counts during the treatment period (increase of 21% [3-36], p=0.22; p=0.009 compared with placebo). This effect was almost completely attributable to an increase of neutrophils (p=0.02 compared with placebo). In the placebo and GM-CSF groups, the LPS-induced leucopenia 1 hour after the first LPS-administration was less pronounced and no longer significant after the second LPS-administration (p=0.19 and p=0.44, respectively), while in the IFN-γ group, no attenuation of the LPS-induced effects occurred and again a distinct trend towards leucopenia was observed following the second LPS-administration (p=0.06). The subsequent leukocytosis was similar between the three experimental groups during visit 2, with a comparable distribution of leukocyte subpopulations, except for eosinophils which showed a pronounced rise in the GM-CSF treated group during visit 2 (p=0.03). Lymphocyte subset analysis of T-cell (including T-helper and cytotoxic T-cells), B-cell, and NK cell counts by flow cytometry revealed no differences between groups (Supplementary Table 2 and Supplementary Figure 2 in the online supplement).
Clinical parameters

The first LPS administration expectedly elicited transient flu-like symptoms, which were comparable between all three experimental groups. (Figure 5, upper panel). Symptoms were significantly less pronounced in the placebo and GM-CSF groups following the second LPS administration (reduction in AUC symptom score of 72% [48-87] (p=0.03) and 50% [29-75] (p=0.03), respectively). In the IFN-γ group however, the symptom score did not significantly differ between visit 1 and visit 2 (reduction AUC of 9% [-21-49], p=0.48), an effect that was significantly different compared with the placebo group (p=0.02). In both the placebo and GM-CSF groups, the LPS-induced increase in heart rate was significantly attenuated after the second LPS administration (reduction in AUC heart rate of 10% [7-14] (p=0.03) and 4% [1-13] (p=0.03), respectively, Figure 5, lower panel). In the IFN-γ group, heart rate response was not significantly attenuated during the second LPS challenge (7% [-11, p=0.15]). In all three groups, there was a significant LPS-induced rise in temperature and a decrease in MAP during both visits (p<0.05 in all three groups for visits 1 and 2, data not shown). No differences in temperature and MAP (based on AUC) between visits 1 and 2 were observed in any of the experimental groups.

DISCUSSION

Immunoparalysis is increasingly recognized as the overriding immune response observed in sepsis patients and the interest in pharmacological interventions aimed at the prevention and treatment of immunoparalysis is growing. This is the first study showing that IFN-γ partially restores in vivo production of the pro-inflammatory cytokine TNF-α, while this is accompanied by further dampening of the anti-inflammatory IL-10 response following a second LPS administration. The more pro-inflammatory spectrum during the second LPS challenge indicates that IFN-γ partially reverses LPS-induced immunoparalysis in vivo in humans. In accordance, IFN-γ prevented the reduction in symptom score and heart rate response to a second LPS administration and significantly increased mHLA-DR cell-surface expression, reflecting monocyte function improvement. The effects of GM-CSF tended in the same direction as IFN-γ, but were not statistically significant compared with the placebo group.

In sepsis patients, the term immunoparalysis is commonly used to indicate reduced responsiveness of leukocytes upon ex vivo stimulation with LPS. Data on the ‘immune status’ of tissue-resident cells, which play a more important role in host defense compared with leukocytes, is limited due to the lack of access to these cells/tissues in patients. However, there is a strong relation between ex vivo responsiveness and susceptibility towards secondary infections and outcome in sepsis patients (24, 25). Furthermore, a very recent study demonstrated that immunosuppression was also found in splenocytes and lung tissue of sepsis patients (6). We (20-22), and others (26, 27) have demonstrated that endotoxin administration in healthy volunteers leads to a profound suppression of cytokine production, both in ex vivo LPS-stimulated leukocytes and in vivo. This indicates that the human endotoxemia model and the associated development of endotoxin tolerance (20, 21) represents a suitable model for sepsis-induced immunoparalysis. To the best of our knowledge, we are the first to investigate reversal of immunoparalysis in vivo, and the result of this study suggest that repeated LPS administration with an interval of one week is a suitable model to investigate possible interventions to reverse sepsis-induced immunoparalysis.
IFN-γ is a well-known activator of monocytes. It increases monocyte antigen-presenting capacity by upregulating costimulatory and HLA molecules (28). Furthermore, previous studies have demonstrated that IFN-γ reverses LPS-tolerance; it restores inflammatory cytokine production in vitro, and in vivo in mice (29-31). In contrast, IFN-γ failed to restore TNF-α production by ex vivo LPS-stimulated leukocytes obtained four hours after LPS administration in vivo, possibly related to the short IFN-γ incubation time (32). Interestingly, a case series report revealed that, when administered to septic patients with low mHLA-DR expression, IFN-γ treatment resulted in restoration of mHLA-DR expression and ex vivo LPS-induced TNF-α production by monocytes. Moreover, the recovery of monocyte function resulted in resolution of sepsis in eight of nine patients (33). More recently, two case reports showed similar results (9). It is clear that more studies are needed to confirm these observations. Our data expand on these studies by showing for the first time that IFN-γ can indeed restore immune competence in vivo.

We found differential effects of IFN-γ on the various cytokines studied. In agreement with our results, earlier work has demonstrated that IFN-γ enhances TNF-α production (34, 35, 36) and suppresses IL-10 release by primary human monocytes in vitro (37). In contrast to our data, it was recently shown that IFN-γ prevents LPS-induced tolerization for IL-6 in primary human monocytes in vitro (38). We do not have a clear explanation for the lack of an effect of IFN-γ on IL-6 in our study, although in vitro/in vivo differences may play a role. It should be noted, however, that the absence of restoration of the IL-6 response does not necessarily reflect a less pro-inflammatory spectrum in IFN-γ-treated subjects, because IL-6 has been shown to possess both pro- and anti-inflammatory properties (39, 40). No effects of IFN-γ on IL-1ra levels were observed in our study. Reports regarding the direct effects of IFN-γ on IL-1RA production are conflicting (37-39). Moreover, while generally regarded as an anti-inflammatory cytokine, IL-1ra production is induced by both TNF-α and IL-6 (40, 41). Therefore, the lack of an effect of IFN-γ on IL-6 levels in our study might have diluted its effects on IL-1ra production through enhanced TNF-α release. In this respect, it is of note that we did observe a trend towards restoration of IL-1ra.

Similar to IFN-γ, GM-CSF is known for its potent immunostimulatory effects. In a recent meta-analysis of four placebo-controlled randomized controlled trials in septic patients (total n=395), treatment with GM-CSF was associated with a significantly increased rate of reversal from infection (16). Not surprisingly, no beneficial effect of GM-CSF on 28-day mortality could be demonstrated because of limited power. Recently, a double-blind, randomized, placebo-controlled trial used mHLA-DR expression to guide treatment of septic patients with GM-CSF (35). In a selected patient population with very low mHLA-DR expression, GM-CSF restored mHLA-DR expression and TNF-α production of ex vivo LPS-stimulated monocytes. Moreover, the results suggested that GM-CSF may shorten the time on mechanical ventilation and hospital/intensive care unit length of stay. In our study, GM-CSF tended to increase mHLA-DR expression, although to a much lesser extent than IFN-γ. Furthermore, GM-CSF prevented the attenuation in TNF-α, IL-6, and IL-1ra plasma levels following the second LPS-administration observed in the placebo group, but, unlike IFN-γ, this difference was not statistically significant compared with the placebo group for any of the studied cytokines. The small sample size in our study is a possible explanation for this lack of significance. An alternative explanation is that the immunoparalysis evoked by repeated LPS administrations is relatively mild, thereby limiting possible beneficial effects of GM-CSF. This is illustrated by the fact that in the aforementioned studies in septic patients, treatment was started when mHLA-DR expression was below 50% (15) or even 30% (17), while mHLA-DR expression in our subjects never dropped below 61%.

The promising results of IFN-γ in this study as well as previous case series warrant a larger trial in septic patients. However, care should be taken with regard to the timing of this intervention in the clinical setting. The more pronounced pro-inflammatory response in the early phase of sepsis might be amplified by IFN-γ, thereby possibly enhancing shock/tissue damage (42). Although no evidence of detrimental effects of IFN-γ are available, there is a clear need for suitable biomarkers to identify a patient’s ‘immune status’. Currently, mHLA-DR expression and the production of cytokines by ex vivo stimulated leukocytes, two parameters that are highly correlated (35, 17), are used to this end. Decreased expression of mHLA-DR has been reported to be associated with reduced survival in septic patients (50, 43–46) by some, but not all (47, 48). Recent studies indicate that, instead of a single
value at a given time point, the slope of recovery of mHLA-DR may better reflect susceptibility towards secondary infections and therefore may more accurately predict outcome (9, 49, 50). Likewise, attenuated TNF-α production by ex vivo LPS-stimulated monocytes is associated with a higher incidence of clinical infection (24). Moreover, in a recent study in trauma patients suffering from sepsis, attenuated TNF-α production by ex vivo LPS-stimulated monocytes was shown to be an earlier predictor of clinical outcome than mHLA-DR expression (23). However, the potential value of mHLA-DR expression and/or ex vivo cytokine production as predictors of a patient's immune status remains questionable because they only reflect the status of the blood leukocyte compartment. Recent studies of our group indicate that this compartment barely contributes to the in vivo immune response to LPS (19, 20). In accordance, in the present study HLA-DR expression of circulating monocytes returned to normal levels within 24 hours, while the in vivo immune response was still severely blunted 6 days later. These results indicate that tissue-resident immune cells, such as macrophages, are the predominant mediators of the in vivo immune response. In this respect, the slow turnover rate of tissue-resident macrophages, as opposed to that of circulating cells, is a plausible explanation for the lengthy in vivo refractory state towards a second LPS challenge. The fact that the function of tissue-resident immune cells, like their counterparts in the blood compartment, is severely compromised in septic patients was recently confirmed (5). It was shown that postmortem obtained tissue-resident cells of patients who die of sepsis exhibit a distinct immunosuppressed phenotype compared with cells obtained from patients who die of etiologies not related to sepsis. Taken together, novel markers to assess a patient’s in vivo immune status are highly warranted, but their identification and applicability is hampered by the lack of access to tissue-resident immune cells. In the meantime and with regard to the timing of immunostimulatory therapy, the use of surrogate markers to determine whether a patient is out of the initial overwhelming pro-inflammatory phase, such as recovery of hemodynamic stability indicated by decreased inotropic requirements, might be considered.

Our study has several limitations. First, because of the small sample size, some of the trends described did not reach statistical significance, likely due to a type 2 error. Despite the small sample size, the significant primary end-points of our study illustrate the extent of the effects of IFN-γ. Second, experimental human endotoxemia remains a model of sepsis-induced immunoparalysis, rather than completely mimicking the complex pathologic clinical condition of sepsis, and our study population is distinctively different from ICU patients suffering from sepsis. Furthermore, inherent to the administration of purified LPS, this model is limited to activation of the immune system by the Toll-like receptor 4 pathway, whereas in the pathophysiology of sepsis, a combination of recognition pathways can be engaged by a large variety of microorganisms.

In conclusion, we demonstrate that IFN-γ partially reverses immunoparalysis in vivo in humans. These results implicate that IFN-γ-mediated immunostimulation could represent a promising treatment option to reverse sepsis-induced immunoparalysis in critically ill patients.

ACKNOWLEDGEMENTS

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REFERENCES


ONLINE SUPPLEMENT

METHODS

Endotoxicemia experiments

After admission to the research intensive care unit of the Radboud University Nijmegen Medical Centre, purified LPS (US Standard Reference Endotoxin Escherichia Coli O:113) obtained from the Pharmaceutical Development Section of the National Institutes of Health (Bethesda, MD) was administered at a dose of 2 ng/kg body weight. In all subjects, heart rate (5-lead electrocardiogram) and blood pressure (20-gauge radial artery catheter) were monitored starting 2 hours before administration of LPS until discharge 8 hours after LPS administration. A cannula was placed in an antecubital vein to permit infusion of prehydration fluid (1.5 L 2.5% glucose/0.45% saline 1 hour before LPS administration), endotoxin, and continuous infusion of 2.5% glucose/0.45% saline (150 mL/hour during 8 hours after LPS administration) to ensure optimal hydration status. Body temperature was measured using an infrared tympanic thermometer (FirstTemp Genius, Sherwood Medical, Crawley/Sussex, UK). The course of endotoxin-induced flu-like symptoms (headache, nausea, shivering, and muscle and back pain) was scored every 30 minutes on a 6-point Likert scale (0=no symptoms, 5=very severe symptoms), resulting in a total score of 0 to 25.

Flow cytometric analysis of mHLA-DR expression and lymphocyte subset counts

To ascertain that expression levels did not change due to a delay between withdrawal and analysis, we performed separate experiments on 5 different blood samples. Expression was determined immediately after withdrawal and after 24 hours storage at 4°C. When samples were immediately stored at 4°C after withdrawal and analyzed within 24 hours, we did not observe significant differences in % or MFI compared with samples that were immediately analyzed after withdrawal. Therefore, analysis was performed within 24 hours after immediate storage at 4°C. After withdrawal, 100 μl blood was incubated with the following fluorochrome-conjugated monoclonal antibodies, for 15 minutes protected from light at 4°C. After erythrocyte lysis (NH₄CL: 180 mL + 20 mL lysis stock dilution [BD Pharm-Lyse, BectonDickinson]), cells were washed three times in PBS and monocytes and lymphocytes were identified in a 8-color immunophenotyping (NAVIOS flow cytometer, BeckmanCoulter, Miami). Monocytes and lymphocytes were identified by forward and side scatter and by cell-specific binding. The following monoclonal antibodies were used for monocyte HLA-DR analysis: HLA-DR-PE (Immu-357), CD14-ECD (RMO52), CD45-KO (J33). Lymphocyte subpopulations were identified by gating on the lymphocyte population in the CD45/SS plot followed by a gating on CD3-APC (UCHT1), CD4-PECy5.5 (13B8.2), CD8-APCAlexa700 (B9.11), CD19-APCAlexa750 (HD37) and CD56-PECy7 (N901) to determine the helper T cells, cytotoxic T cells, B cells and NK cells within the lymphocyte gate (all MoAbs were obtained from Beckman Coulter, Marseille, France).
### Supplementary Table 1. Monocyte HLA-DR expression.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Placebo</th>
<th>MFI</th>
<th>IFN-γ</th>
<th>GM-CSF</th>
</tr>
</thead>
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<tr>
<td>Visit 1 t=4</td>
<td>0.23 (0.12-0.34)</td>
<td>7.4 (5.0-10.2)</td>
<td>7.0 (5.0-10.2)</td>
<td>9.6 (5.0-14.2)</td>
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<tr>
<td>Visit 1 t=8</td>
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<td>5.2 (4.0-6.4)</td>
<td>5.2 (4.0-6.4)</td>
<td>5.2 (4.0-6.4)</td>
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<tr>
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<td>0.13 (0.07-0.26)</td>
<td>4.1 (2.0-6.4)</td>
<td>4.1 (2.0-6.4)</td>
<td>4.1 (2.0-6.4)</td>
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<tr>
<td>Placebo</td>
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<td>5.2 (4.0-6.4)</td>
<td>5.2 (4.0-6.4)</td>
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<tr>
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<td>5.2 (4.0-6.4)</td>
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</tbody>
</table>

% indicates % HLA-DR-positive monocytes, MFI indicates monocyte HLA-DR mean fluorescent intensity. Values are presented as median (interquartile range) of 6 subjects per group.

### Supplementary Table 2. Lymphocyte subset counts.

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<tr>
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<th>GM-CSF</th>
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<td>7.0 (5.0-10.2)</td>
<td>9.6 (5.0-14.2)</td>
</tr>
<tr>
<td>Visit 1 t=8</td>
<td>0.21 (0.13-0.24)</td>
<td>5.2 (4.0-6.4)</td>
<td>5.2 (4.0-6.4)</td>
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Lymphocyte counts determined by flow cytometry. T-cells (CD45+CD3+); T-helper cells (Th, CD45+CD3+CD4+); cytotoxic T-cells (Tc, CD45+CD3+CD8+); B-cells (CD45+CD19+); NK-cells (CD45+CD56+). Values are presented as median (interquartile range) of 6 subjects per group.

### Supplementary Figure 1 (1/2). mHLA-DR expression histograms of a representative subject from the IFN-γ group.
Supplementary Figures 1 and 2. Lymphocyte subset counts determined by flow cytometry. T-cells (CD45+CD3+); T-helper cells (Th, CD45+CD3+CD4+); cytotoxic T-cells (Tc, CD45+CD3+CD8+); B-cells (CD45+CD19+); NK-cells (CD45+CD3-CD56+). Values are presented as median of 6 subjects per group.

Supplementary Figure 1 (2/2). mHLA-DR expression histograms of a representative subject from the IFN-γ group.
Immunomodulation as adjunctive treatment for opportunistic infections

Part TWO
Interferon-gamma as adjunctive immunotherapy for invasive fungal infections: a case series

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ABSTRACT

Background: Invasive fungal infections are very severe infections associated with high mortality rates, despite the availability of new classes of antifungal agents. Based on pathophysiological mechanisms and limited pre-clinical and clinical data, adjunctive immune-stimulatory therapy with interferon-gamma (IFN-γ) may represent a promising candidate to improve outcome of invasive fungal infections by enhancing host defence mechanisms.

Methods: In this open-label, prospective case series, we describe eight patients with invasive Candida and/or Aspergillus infections who were treated with recombinant IFN-γ (rIFN-γ, 100 μg s.c., thrice a week) for 2 weeks in addition to standard antifungal therapy.

Results: Recombinant IFN-γ treatment in patients with invasive Candida and/or Aspergillus infections partially restored immune function, as characterized by an increased HLA-DR expression in those patients with a baseline expression below 50%, and an enhanced capacity of leukocytes from treated patients to produce proinflammatory cytokines involved in antifungal defence.

Conclusions: The present study provides evidence that adjunctive immunotherapy with IFN-γ can restore immune function in fungal sepsis patients, warranting future clinical studies to assess its potential clinical benefit.

Trial registration: ClinicalTrials.gov - NCT01270490

INTRODUCTION

The incidence of fungal infections is steadily increasing in the last years due to invasive medical diagnosis and immunosuppressive treatment modalities. Despite development of new classes of antifungal agents (1), the invasive fungal infections remain associated with unacceptable high mortality rates and represent a major cause of death worldwide (2-7). The emergence of significant resistance to the currently available antifungal therapies emphasizes the need for novel approaches to treat invasive fungal infections (8, 9). Invasive fungal infection are most commonly observed in individuals with immune defects or a compromised immune system, and the number of these patients is steadily increasing (10). Therefore, adjunctive immunotherapy to improve host defence is an attractive strategy to improve the outcome of patients with disseminated fungal infections.

In the past decade, major progress in the understanding of anti-fungal host responses has enabled the development of a number of novel molecular and cell-based immunotherapeutic approaches for invasive fungal infections (11). Although invasive candidiasis and aspergilliosis are rather different in their pathogenesis, the major protective host response against both fungi is the effective induction of Th1 and IFN-γ responses (12-16). The Th1 cytokine response activates effector phagocytic cells that kill the fungus (17). Interestingly, Th1 immunity against A. fumigatus was demonstrated to be cross-protective against C. albicans (18).

Interferon-gamma (IFN-γ), the prototype Th1 cytokine, promotes Th1 differentiation and skews the immune response towards a protective Th1 phenotype (19). As such, it has been implicated as a treatment option in (invasive) fungal infections (20, 21). Moreover, limited evidence suggests that recombinant IFN-γ (rIFN-γ) has a beneficial effect on the outcome of fungal infections in patients with chronic granulomatous disease (CGD) (22), HIV (23-25), leukemia (26, 27), and in patients receiving organ transplants (28). However, it has not been investigated whether rIFN-γ actually enhances the immune response in these patients to explain these beneficial clinical effects.
In this report we describe a series of patients with invasive Candida and/or Aspergillus infections in whom we investigated the effects of treatment with rIFN-γ on the host innate and adaptive immune responses.

MATERIALS AND METHODS

Patients and treatment
To assess the feasibility and preliminary efficacy of IFN-γ in combination with anidulafungin for the treatment of candidemia, a single-centre, prospective, randomized open-label pilot (Phase IIb) study was conducted. This study was registered at ClinicalTrials.gov (NCT01270490) and approved by the local ethics committee of the Radboud University Medical Center. Due to slower than anticipated enrollment rates (from August 2010 until March 2013, only 12 patients could be screened, of which 6 were eligible and provided informed consent [Figure 1]), the study was terminated early. However, during this period, several other patients presented with invasive fungal infections which had an insufficient response to standard antifungal therapy. Although these patients did not meet the inclusion criteria (i.e. presenting with one or more positive cultures of blood or normally sterile tissue growing Candida spp.), they were deemed to benefit from adjunctive immunotherapy as “therapy of last resort” as decided by the attending physician. Within the parameters of standard clinical care these patients were treated according to the same protocol as the patients enrolled in the study, and were therefore included in the present case series. All patients with a history of documented epileptic seizures, pre-existent severe renal impairment (creatinine clearance <30 mL/min) or severe liver failure (defined as a spontaneously increased prothrombin time) were excluded. After obtaining informed consent, eight patients (3 study patients, 5 last resort patients) were treated with rIFN-γ (Immukine, Boehringer Ingelheim, 50 µg/m² body surface, subcutaneously, three times a week) in addition to standard antifungal therapy as recommended by national and international treatment guidelines (29, 30). Three patients who were included in the Phase IIb Candida pilot-study were assigned to the control group and did not receive rIFN-γ.

Blood sampling
Plasma, serum and whole blood specimens were collected at baseline (BL) and serially after the start of antifungal therapy (days 1, 2, 3, 7, 14 and 28). Blood cultures were performed as part of routine care.

Leukocyte populations and surface HLA-DR expression
Heparin anticoagulated blood was stored at 4°C immediately after withdrawal and analyzed by flow cytometry. To determine the extent of immune suppression, HLA-DR expression was determined by calculating % HLA-DR-positive cells and HLA-DR mean fluorescence intensity (MFI) within CD14+ cells and various lymphocyte subsets within CD45+ leukocytes (see online supplement and supplemental figure 1 for details and a representative flow diagram). Lymphocyte subsets were defined as: T-cells (CD45+CD3+),...
T-helper cells (Th, CD45CD3CD4), cytotoxic T-cells (Tc, CD45CD3CD8), B-cells (CD45CD20), and NK-cells (CD45CD3CD56). Subset counts were calculated by multiplying the percentage of gated cells by the total lymphocyte count. Patients with <50% HLA-DR positive monocytes at baseline were considered to exhibit immune paralysis. This threshold of 50% is well below the lower bound of the 99% confidence interval obtained in healthy volunteers in an earlier study of our group using the same methodology in the same laboratory (32). Therefore mHLA-DR expression levels below 50% are likely to represent immunoparalysis.

Cytokine assays
Venous blood was drawn into 10 mL EDTA tubes, after which peripheral blood mononuclear cells (PBMCs) were isolated as described previously (32). In short, blood was diluted in phosphate buffered saline (PBS) (1:1) and fractions were separated by Ficoll (Ficoll-Paque Plus, GE healthcare, Zeist, The Netherlands) density gradient centrifugation. Cells were washed twice with PBS and resuspended in RPMI-1640+ (RPMI-1640 Dutch modification supplemented with 10µg/mL gentamicin, 10mM L-glutamine, and 10mM pyruvate) (Gibco, Invitrogen, Breda, The Netherlands). The PBMCs were counted using a particle counter (Beckmann Coulter, Woerden, The Netherlands) and were plated in 96 well round-bottom plates (Corning, NY, USA) at a final concentration of 2,5x10⁶/mL, in a total volume of 200 µL. The PBMCs were stimulated for 24 hours, 48 hours, and 7 days with medium alone, or medium containing E. coli lipopolysaccharide (LPS; 10 ng/mL), phytohaemaglutinin (PHA; 10µg/mL), heat-inactivated Candida albicans blastoconidia (1x10⁶/ml) or heat-inactivated Candida albicans hyphae (derived from 1x10⁶/m conidia). After stimulation, cell culture supernatant was collected and stored at -20°C. When all samples were collected, cytokines were measured using commercially available ELISAs (R&D Systems, MN, USA and Sanquin, Amsterdam, The Netherlands) according to the protocols supplied by the manufacturer. Ex-vivo production of cytokines was assessed at timepoints at which their production has been shown to peak (33). Monocyte derived cytokines such as Interleukin (IL)-1β and tumour necrosis factor (TNF)-α were measured in culture supernatants of 24 hour cultures, IL-10 was measured in culture supernatants of 48 hour cultures. T-cell derived cytokines IL-17 and IL-22 were measured in culture supernatants of 7 day cultures.

Statistical analysis
In view of the small sample size, normality of distribution was not assumed. Comparisons of baseline with follow up time points were made using Wilcoxon signed rank test (within-group comparisons, 2 groups). A p-value of <0.05 was considered statistically significant. Data are expressed as means and standard error of the mean. Calculations and statistical analyses were performed using GraphPad Prism v 5.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Patient characteristics
The patients treated with rIFN-γ (5 men, 3 women) had a median age of 49.5 [IQR 28.5-68.8] years. The three female patients in the control group were 36, 51 and 73 years old. Clinical characteristics of the participants are listed in Table 1. Of the 6 patients included in the pilot study, three patients had a positive blood culture for C. albicans, two patients for C. glabrata, and one patient for C. tropicalis. During randomization, the three patients with C. albicans cultures were assigned to rIFN-γ treatment, whereas the two patients with C. glabrata and one with C. tropicalis cultures were assigned to the control group. However, no pathophysiological evidence currently exists to suggest that rIFN-γ therapy would have a different effect on the immune system in case of albicans vs. non-albicans Candida infections. Of the other 5 patients treated with rIFN-γ as therapy of last resort, one patient had proven acute aspergillosis, one had probable acute aspergillosis, and one probable chronic aspergillosis according to the EORTC/MSG criteria (34). One patient had a positive blood culture for C. tropicalis associated with osteomyelitis. This patient developed new suspected lesions on positron emission tomography-computed tomography (PET-CT) while receiving antifungal treatment. In another patient, CT-scan revealed progression of suspected hepatic Candida lesions during antifungal treatment. All patients included suffered some degree of immunosuppression: the 6 patients with positive blood cultures for Candida spp. had impaired physical barriers due to the presence of indwelling venous catheters (for the need of recurrent blood sampling or total parental nutrition),
We monitored the fold change in cytokine production compared with baseline (before start of treatment). IL-1β and TNF-α are pro-inflammatory cytokines of the innate immune system crucial in the induction and maintenance of the anti-fungal immune response (35-40). Before IFN-γ treatment, inter-patient variability in cytokine production was high (e.g. TNF-α median [IQR] concentration after stimulation with LPS was 792 pg/mL [314-2005]). Nevertheless, in all patients an increase in the capacity to induce different cytokines was observed in the first two days after initiation of IFN-γ treatment, independent of their baseline values (group data shown in figure 2), at subsequent time points only a trend towards increased could be observed. In contrast, the placebo-treated patients IL-1β and TNF-α responses over time remained similar to baseline. The response against hyphae of *C. albicans* was highly variable between patients. Some rIFN-γ-treated patients demonstrated a profound increase of TNF-α production after treatment (up to 70 fold), whereas other patients showed no relevant change in TNF-α production. Cytokine production remained similar in patients in the control group.

**Clinical outcome**

The three patients in the control group and five out of eight patients treated with rIFN-γ recovered uneventfully from the fungal infection. Two patients with invasive aspergillosis that were already admitted to the ICU at the time of treatment died due to infectious complications of severe pulmonary aspergillosis, despite rIFN-γ treatment. The patient with a *Candida* endocarditis, who despite rIFN-γ treatment developed intracerebral mycotic aneurysm, could be discharged from the hospital 93 days after onset of invasive candidiasis.

In all patients treated, rIFN-γ was well tolerated. Five patients reported moderate fever upon administration of rIFN-γ, which responded well to acetaminophen. Two patients developed liver enzyme abnormalities for which tuberculostatic antibiotics and voriconazole were temporarily discontinued, resulting in recovery of the liver enzyme abnormalities while rIFN-γ treatment was continued. No other significant adverse events were observed.

**Effect of rIFN-γ on ex-vivo IL-1β and TNF-α production**

To assess the effect of rIFN-γ on the capacity of PBMCs to produce pro-inflammatory cytokines, cells were isolated and stimulated before, during, and after treatment.
Figure 2. Effect of rIFN-γ on ex-vivo IL-1β and TNF-α production
PBMCs of patients were isolated at baseline and day 1, 2, 7, 14 and 28 after rIFN-γ administration. Isolated PBMCs were stimulated for 24 hours with LPS, PHA, C. albicans blastoconidia, or C. albicans hyphae. IL-1β(a) and TNF-α(b) concentrations were measured in culture supernatants. Baseline concentrations were used as control and set at 1; subsequent measurements are plotted as the mean relative fold change ±SEM. Significant change from baseline was determined by subjecting the data to Wilcoxon signed rank test. (*=p<0.05; **=p<0.01).

Figure 3. Effect of rIFN-γ on ex-vivo IL-17 and IL-22 production
PBMCs of patients were isolated at baseline and day 1, 2, 7, 14 and 28 after rIFN-γ administration. Isolated PBMCs were stimulated for 7 days with PHA, C. albicans blastoconidia, or C. albicans hyphae. IL-17(a) and IL-22(b) concentrations were measured in culture supernatants. Baseline concentrations were used as control and set at 1; subsequent measurements are plotted as the mean relative fold change ±SEM. Significant change from baseline was determined by subjecting the data to Wilcoxon signed rank test. (*=p<0.05).
Effect of rIFN-γ on ex-vivo IL-10 production

In addition to pro-inflammatory cytokines, the capacity to produce anti-inflammatory cytokines can also influence disease outcome. In particular the anti-inflammatory cytokine IL-10 has been associated with protection against immunopathology during severe infections. IL-10 production in response to stimulation with LPS, PHA and Candida was highly variable between patients and did not show a distinct pattern following rIFN-γ treatment (figure 4). No relevant differences compared to the placebo-treated patients were observed.

HLA-DR expression

The numbers of HLA-DR-positive monocytes, a marker of immunosuppression, varied substantially between patients at baseline (39.05% [27.5-61.6] vs. 90.6 [88.7-92.5] in IFN-γ-treated patients and placebo-treated patients, respectively). Five out of eight IFN-γ treated patients exhibited HLA-DR positive monocyte levels below the “immunoparalysis threshold” of 50% and in these patients, an increase of HLA-DR-positive monocytes after IFN-γ treatment between 10% and 44% was observed which persisted throughout the study period (Figure 5). Patients with a baseline HLA-DR expression higher than 50% did not show a change in expression. The patient with a HLA-DR-expression <50% who did not show increased levels of HLA-DR positive monocyte numbers at any time point, was one of the two patients who died due to infectious complications. No correlation was found between the level of mHLA-DR expression and TNF-α production of LPS-stimulated PBMCs. An inverse correlation of baseline mHLA-DR levels with severity of underlying illness and tissue involvement was found (with higher mHLA-DR levels in patients with only impaired physical barriers, e.g. due to indwelling catheters, compared to patients with impaired immune responses, e.g. due to chemotherapy, immune suppressive agents, bone marrow disease,; data not shown because this compromises patients anonymity).

Cell populations

There were no significant changes in the total leukocyte and granulocyte numbers in rIFN-γ treated patients (supplemental Figure 2A). Monocyte counts significantly
circulating granulocyte numbers. It is not known whether this reduction is due to activation and migration into the infected tissue, or whether a true decrease in granulocyte generation was induced by the treatment. Although the decrease in granulocyte numbers was slight, the fact that granulocytes, and especially neutrophils, are crucial in the antifungal host defence warrant careful monitoring of granulocyte numbers during IFN-γ treatment.

Several clinical studies and case reports have previously demonstrated beneficial effects of rIFN-γ in combination with antifungal therapy on outcome of fungal infections (for example in patients with CGD ($n=130$) (22, 46, 47), HIV ($n=173$) (23-25), leukaemia ($n=5$) (26, 27), and transplant patients ($n=7$) (28), in a patient with $S. aureus$ liver abscess and invasive $C. albicans$ infection (48), in a patient with intracerebral aspergillosis (49), in two patients with progressive chronic pulmonary aspergillosis (50), and in two patients with idiopathic CD4 lymphopenia and cryptococcal meningitis (51)). However, in contrast to our study, ex-vivo immune responses in these patients were not investigated. Due to the limited number of patients and the very heterogeneous population, we could not assess clinical endpoints, although a mean mortality of 25% in the IFN-γ treated patients lies below the mean 40% estimated in patients with invasive fungal infections (10, 52).

To the best of our knowledge, we are the first to describe mHLA-DR expression, a widely used marker of immunosuppression in (bacterial) sepsis patients (53)), in patients with invasive fungal infections. In all IFN-γ treated patients who showed baseline mHLA-DR levels below the immunoparalysis threshold of 50% and survived, IFN-γ mediated upregulation of mHLA-DR expression was observed. In agreement with the data presented in this case series, rIFN-γ has been shown to significantly increase numbers of HLA-DR-positive monocytes both in a human preclinical bacterial sepsis model and in septic patients (31, 54). Reduced production of TNF-α by leukocytes ex-vivo stimulated with LPS has also been shown to be marker of immunoparalysis in sepsis patients. In contrast to our study, mHLA-DR expression and ex-vivo TNF-α production were found to be highly correlated in bacterial sepsis patients (54, 55). A possible explanation for this discrepancy is that, in contrast with the emerging consensus that immunoparalysis renders

**DISCUSSION**

While several small clinical trials illustrated the beneficial clinical effects of adjuvant treatment with IFN-γ, the proposed immunostimulating effect of IFN-γ as the mechanism of action has not been investigated. In this case series we demonstrate for the first time that adjunctive immunotherapy with rIFN-γ improves the leukocyte immune responses in patients with severe invasive fungal infections. This was primarily reflected by increased ex-vivo pro-inflammatory cytokine responses of the innate immune system such as IL-1β or TNF-α, as well as an increased production of the T-cell cytokines IL-17 and IL-22, which are known to play an important role in the anti-fungal host defence (35, 41-45), and by an increase in HLA-DR expression in mHLA-DR expression in those patients with a low cellular expression as a measure of their immune suppression.

In addition to enhanced ex-vivo responses, subtle changes in the leukocyte differentiation were observed following IFN-γ treatment. Although there were no significant differences in total leukocyte numbers after treatment with rIFN-γ, shifts in leukocyte subpopulations such as increased monocyte and lymphocyte counts were apparent. While lymphocyte numbers increased after rIFN-γ therapy, it could not directly be attributed to a specific subset as all of them showed increased values. The most significant increase was that of CD8 cells one week after initiation of rIFN-γ therapy. Monocytes and lymphocytes are known to be crucial cells in the host defence against fungal infections. However, the increase of monocytes and lymphocytes during rIFN-γ therapy was accompanied by slightly decreased circulating granulocyte numbers. It is not known whether this reduction is due to activation and migration into the infected tissue, or whether a true decrease in granulocyte generation was induced by the treatment. Although the decrease in granulocyte numbers was slight, the fact that granulocytes, and especially neutrophils, are crucial in the antifungal host defence warrant careful monitoring of granulocyte numbers during IFN-γ treatment.
patients more vulnerable to opportunistic infections in general (53), different defects in immune defences may be responsible for enhanced susceptibility towards different pathogens.

Based on the apparent inverse correlation of baseline mHLA-DR levels with severity of underlying illness and tissue involvement, mHLA-DR levels seem to reflect disease severity and general immune status, and not specific immune defects per se. Hence, patients with invasive fungal infections and associated impaired anti-fungal immune responses will probably benefit more from immunostimulatory treatment compared to patients with only impaired physical barriers, e.g. due to indwelling catheters and apparent intact anti-fungal immune responses. Biomarkers reflecting the capacity of specific anti-fungal immune defences are required to identify patients who suffer from invasive fungal infections due to impaired cell-mediated immunity. It is important to identify such patients and attempt a tailored immunotherapeutic approach guided by the actual level and type of immunoparalysis of that specific patient. A blood based assay has been described that demonstrates a failure to induce IFN-γ expression in renal transplant patients and differences in IL-10 and TNF-α expression (56), which could be promising biomarkers to identify patients who could benefit from adjunctive immunotherapy.

The intracellular mechanism(s) through which the beneficial effects of IFN-γ are mediated remain to be elucidated. Recently it was proposed that IFN-γ exerts its effects at the transcription level (57), while others have demonstrated that IFN-γ reverses tolerance-associated epigenetic modifications (58). Another possible mechanism involved in the IFN-γ mediated reversal of immunoparalysis is the downregulation of negative TLR regulators such as IRAK-M, a protein that negatively regulates LPS-induced inflammatory responses and contributes to the development of immunoparalysis (59).

Administration of rIFN-γ was tolerated well. Several patients developed a mild fever upon administration, which responded well to acetaminophen treatment. No other side effects were observed. The most important limitation of the present study is the limited number of patients studied. Because the control group consisted of only three patients, no statistical analysis between the treatment and control groups could be performed. However, despite the small sample size, the increase in HLA-DR expression in patients with mHLA-DR expression levels below 50% and the increased ex-vivo response of several cytokines that are crucial in antifungal host defence is a promising observation that underlines the potential of immunotherapy. The slow enrolment of patients presenting with candidemia was the main factor contributing to the decision to terminate the phase IIib Candida pilot-study early. With a reported incidence of 2.5-11 per 100,000 persons in Europe (60), and based on previous epidemiological data in our hospital this low enrollment was not expected at the time of the initiation of the study. The much lower incidence of candidemia in the last two years in our hospital is most likely due to a new antibiotic stewardship introduced recently in our hospital, which has reduced the incidence of opportunistic infections. The cut-off value of mHLA-DR expression levels of 50% to distinguish between immunoparalyzed and immunocompetent patients is another limitation of this study, as this is an arbitrary value chosen. We chose this value because it is well below the 99% CI of mHLA-DR values in healthy volunteers (31). Therefore, patients with mHLA-DR below 50% do have an impaired antigen presenting capacity of their monocytes which we show to be enhanced by IFN-γ therapy. Whether this cut-off value truly represents immunoparalysis, reflected by enhanced susceptibility to secondary infections or reduced capacity to clear opportunistic infections, remains to be investigated. Furthermore, the use of a standardized analysis technique to quantify mHLA-DR, such as the Quantibrite method, is preferable, because it facilitates an objective comparison of mHLA-DR expression levels between studies and aids in the definitive establishment of a cut-off value to identify immunoparalyzed patients. Larger studies are required to confirm the data obtained here. To do so, multicentre studies should be facilitated in order to fully explore the potential of IFN-γ immunotherapy.

Our data indicate that adjunctive immunotherapy with rIFN-γ in patients with invasive fungal infections partially restores cell-mediated immunity. This suggests that IFN-γ treatment enhances anti-fungal immunity and larger studies are...
warranted to validate the findings reported here and to assess the impact of IFN-γ treatment on clinical outcome. Biomarkers of impaired anti-fungal immunity should be further investigated in order to identify patients who will benefit most from immunostimulatory therapy.

ACKNOWLEDGEMENTS

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REFERENCES


**ONLINE SUPPLEMENT**

**METHODS**

Flow cytometric analysis of mHLA-DR expression and lymphocyte subset counts

To ascertain that expression levels did not change due to a delay between withdrawal and analysis, we performed separate experiments on 5 different blood samples. Expression was determined immediately after withdrawal and after 24 hours storage at 4°C. When samples were immediately stored at 4°C after withdrawal and analyzed within 24 hours, we did not observe significant differences in % or MFI compared with samples that were immediately analyzed after withdrawal. Therefore, analysis was performed within 24 hours after immediate storage at 4°C. After withdrawal, 100 μl blood was incubated with the following fluorochrome-conjugated monoclonal antibodies, for 15 minutes protected from light at 4°C. After erythrocyte lysis (NH4Cl: 180 mL + 20 mL lysing solution stock dilution [BD Pharm-Lyse, BectonDickinson]), cells were washed three times in PBS and monocytes and lymphocytes were identified in a 8-color immunophenotyping (NAVIOS flow cytometer, Beckman Coulter, Miami). Monocytes and lymphocytes were identified by forward and side scatter and by cell-specific binding. The following monoclonal antibodies were used for monocyte HLA-DR analysis: HLA-DR-PE (Immu-357), CD14-EC (RMO52), CD45-KO (J33). Lymphocyte subpopulations were identified by gating on the lymphocyte population in the CD45/SS plot followed by a gating on CD3-APC (UCHT1), CD4-PECy5.5 (13B8.2), CD8-APCAM5 (BD Pharm-Lyse, Becton Dickenson). Cells were washed three times in PBS and monocytes and lymphocytes were identified in a 8-color immunophenotyping (NAVIOS flow cytometer, Beckman Coulter, Miami). Monocytes and lymphocytes were identified by forward and side scatter and by cell-specific binding. The following monoclonal antibodies were used for monocyte HLA-DR analysis: HLA-DR-PE (Immu-357), CD14-EC (RMO52), CD45-KO (J33). Lymphocyte subpopulations were identified by gating on the lymphocyte population in the CD45/SS plot followed by a gating on CD3-APC (UCHT1), CD4-PECy5.5 (13B8.2), CD8-APCAM5 (BD Pharm-Lyse, Becton Dickenson, Miami). Cells were washed three times in PBS and monocytes and lymphocytes were identified in a 8-color immunophenotyping (NAVIOS flow cytometer, Beckman Coulter, Miami).
Supplementary Figure 1. Representative flow diagram of monocyte HLA-DR measurements. Heparin blood was first analysed on forward- and side scatter to exclude cell debris and erythrocytes (A). Subsequently, CD45+ cells were selected (B) and within the CD45+ fraction was gated for CD14+ cells (C). The CD45+ CD14+ cells (D) were analysed for the percentage of HLA-DR positivity (E).

Supplementary Figure 2. Changes in immune cell populations. Total leukocyte numbers (a) and numbers of granulocytes (b), monocytes (c) and lymphocytes (d) measured in peripheral blood. Numbers of CD4 lymphocytes (e), B-lymphocytes (f), CD8 lymphocytes (g) and NK cells (h) within the lymphocyte population were quantified using flowcytometry.
Interferon-gamma immunotherapy in a patient with refractory disseminated candidiasis

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**INTRODUCTION**

Patients suffering from leukemia are prone to invasive fungal infections because of the presence of indwelling catheters in combination with an immunocompromised immune status due to prolonged periods of neutropenia and mucosal breakdown associated with intensive chemotherapeutic regimens (1-3). *Candida* spp. are the most frequent fungal opportunistic pathogen in such patients (4), with 30 day mortality rates reaching 40% in adult patients (5, 6). In the pediatric population prognosis of invasive candida infections is slightly better, but particularly in cases with poor response to antifungal therapy, mortality remains unacceptably high (7-9).

Adjuvant interferon-gamma (IFN-γ), an immunostimulatory cytokine, may improve outcome of fungal infections in adult patients with immune paralysis (10-12). This was recently substantiated in a small case-series which showed that IFN-γ partially restored anti-fungal immune function in patients with invasive fungal infections (13). Therefore, in adult patients who deteriorate despite optimal antifungal treatment, adjuvant IFN-γ immunotherapy could be considered as adjuvant salvage therapy (12). Here we report the clinical case of a three-year old patient with severe systemic *C. dubliniensis* infection and poor clinical response to standard antifungal therapy. Adjuvant therapy with IFN-γ restored immune function and likely contributed to clinical improvement.

**CASE REPORT**

A previously healthy three-year-old boy was diagnosed in June 2014 with common acute lymphoblastic leukaemia (cALL), with CNS status CNS-2 and the favourable t(12;21) translocation. He started induction chemotherapy the day after his diagnosis according to protocol ALL-11 (Dutch Childhood Oncology Group study for children with ALL; [https://www.skion.nl/workspace/uploads/Onderzoeksprotocol-ALL11-version-4-2-september-2014.pdf](https://www.skion.nl/workspace/uploads/Onderzoeksprotocol-ALL11-version-4-2-september-2014.pdf)). On day 15 after start of chemotherapy he developed neutropenic fever, for which empirical antibiotics were started (ceftazidim and vancomycin). Despite antibiotic treatment, fever persisted, and a blood culture drawn on day 28...
was positive for *Candida dubliniensis*, with good *in vitro* sensitivity to micafungin, amphotericin B and voriconazole. Micafungin and G-CSF were started on day 30 and the central venous catheter (CVC) was removed on day 36. Ultrasound investigations of the abdomen, retinal inspection by the ophthalmologist and magnetic resonance imaging (MRI) of the brain did not show any evidence for disseminated candida infection. Neutrophil counts recovered and G-CSF was stopped on day 39.

Because of progressive fever and a further increase in serum C-reactive protein, antifungal therapy was switched to liposomal amphotericin B and voriconazole on day 39. The following days the patient continued to deteriorate and was transferred to the intensive care unit for respiratory support. Imaging of the abdomen and brain now showed multiple nodular lesions in the brain, kidneys, liver and spleen, with a radiological appearance suggestive of disseminated *Candida* infection (Figure 1A). Ultrasound guided biopsy of the liver (histology and PCR) confirmed the presence of *Candida* spp. in the nodular hepatic lesions (Figure 1B). Flucytosine was added to the antymycotic regimen for better central nervous system penetration. The patient continued to deteriorate and on day 66, micafungin was re-added to the treatment. HLA-DR expression on monocytes was extremely low at that time, demonstrating immunoparalysis. Granulocyte oxidative burst, phagocytosis and chemotaxis were normal.

Because of the immunoparalysis, IFN-γ (Immukine, Boehringer Ingelheim, 50 μg/m² body surface, subcutaneously, three times/week) was started as salvage therapy on day 74. Initially, the clinical condition and appearance of nodular lesions on imaging analysis did not improve, after which it was decided to increase the dose to daily injections. After five days of daily IFN-γ administration, pericardial effusion developed. IFN-γ administration was reverted to three times per week, with spontaneous disappearance of pericardial effusion. Finally, monocyte HLA-DR expression increased and the clinical condition of the patient improved. Imaging of the brain revealed complete resolution of lesions and abdominal ultrasound showed markedly diminished kidney, liver and spleen lesions. Despite IFN-γ treatment, the patient suffered from several episodes of CVC-associated bacterial sepsis. IFN-γ treatment was stopped on day 115, liposomal amphotericin B on day 134 and micafungin on day 143. Nine months after the leukaemia diagnosis, reinduction chemotherapy was started. Bone marrow biopsies were regularly performed, which showed that the leukemia remained in remission. As a precautionary measure, voriconazole was continued and micafungin restarted.
**DISCUSSION**

Here we describe a child with proven progressive disseminated invasive candidiasis. Despite receiving multiple antifungal agents with low minimal inhibitory concentrations to the cultured *C. dubliniensis*, the patient deteriorated. Last resort adjunctive immunotherapy with IFN-γ resulted in normalization of mHLA-DR expression, and cytokine responses were also partly restored. This restoration of innate immune functions paralleled and likely contributed to clinical recovery.

To adequately eradicate *Candida* spp., innate immunity has to be intact (15, 16). In our patient chemotherapy-induced neutropenia resolved, but innate immune function was severely impaired as demonstrated by very low levels of mHLA-DR expression (reflecting diminished antigen presenting capacity). In adult patients, mHLA-DR expression is currently the most widely used marker of impaired innate immunity, but its accuracy still lacks solid evidence (12). Although reference values are not available for the pediatric population, we observed an extremely low level of mHLA-DR (both MFI and % positivity), which increased dramatically during IFN-γ treatment. This implies that mHLA-DR expression is likely to be a useful biomarker for innate immune function in children as well, but further studies in pediatric patients are highly warranted.

Our assessment of *ex vivo* cytokine responses to *Candida* revealed multiple effects of IFN-γ therapy on the capacity of PBMCs to mount adequate cytokine responses. After initiation of IFN-γ therapy, we observed enhanced production of TNF-α of PBMCs *ex vivo* stimulated with both *Candida* yeast and hyphae, which is similar to what we have previously observed in a case series of patients with invasive fungal infections treated with adjunctive IFN-γ immunotherapy (13). In addition, IFN-γ treatment resulted in a slight restoration of the IL-17 response to *Candida* hyphae (of which we assume that it reflects the response to *Candida* spp. in the infected tissues). Because IL-17 is of primordial importance in the anti-fungal immune defense (17), it is tempting to speculate that long-term IFN-γ treatment could lead to a full recovery of IL-17 responses, although...
this has to be determined in future studies. However, one must be cautious in interpreting the effects of IFN-γ adjunctive therapy on cytokine production capacity, as these have been done in only one patient with only one (not age-matched) control, which may explain that some of the results are not fully consistent (e.g. increased of IL-17 production capacity upon stimulation with Candida hyphae, but not for yeasts). More studies are therefore needed to fully decipher the immunologic effects of IFN-γ adjunctive therapy.

Experience with IFN-γ treatment in children is limited (18), but it is well tolerated in children with chronic granulomatous disease, a rare inherited disorder of leukocyte function (19). Life-threatening side-effects are rare, although in early clinical trials deterioration of pre-existing heart disease was observed in patients who were treated with doses of 250µg/m² or higher, which spontaneously reverted when treatment was discontinued. In our patient, pericardial effusion occurred during daily injections, which resolved after reverting to the usual three times per week. and therefore very likely could be attributed to the IFN-γ therapy. Hence, this case underscores that close monitoring is warranted when IFN-γ dose is increased, or when heart disease is already present, because side effects can be potentially life-threatening.

CONCLUSION

Treatment of a leukemic child with refractory disseminated candidiasis with adjuvant IFN-γ increased innate immune activation and altered cytokine responses. These improvements paralleled clinical improvement, indicating this immunotherapeutic approach may have contributed to the favorable clinical outcome. This observation warrants further study in pediatric patients with invasive fungal infections who deteriorate despite optimal antifungal treatment, in which immunotherapy with IFN-γ may be considered as adjuvant salvage therapy.

ACKNOWLEDGMENTS

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ONLINE SUPPLEMENT

Flow cytometric analysis of mHLA-DR expression

We used to methods to determine mHLA-DR expression. First, we determined Mean Fluorescence Intensities as described earlier (2). Briefly, EDTA anticoagulated blood was stored at 4°C immediately after withdrawal and analyzed by flow cytometry. After withdrawal, 100 μl blood was incubated with the following fluorochrome-conjugated monoclonal antibodies, for 15 minutes protected from light at 4°C. After erythrocyte lysis (NH4CL: 180 mL + 20 mL lysis stock dilution [BD Pharm-Lyse, BectonDickinson]), cells were washed three times in PBS and monocytes and lymphocytes were identified in an 8-color immunophenotyping (NAVIOS flow cytometer, Beckman Coulter, Miami). Monocytes and lymphocytes were identified by forward and side scatter and by cell-specific binding. The following monoclonal antibodies were used for monococyte HLA-DR analysis: HLA-DR-PE (Immu-357), CD14-ECD (RMO52), CD45-KO (J33). Lymphocyte subpopulations were identified by gating on the lymphocyte population in the CD45/SS plot followed by a gating on CD3-APC (UCHT1), CD4-PECy5.5 (13B8.2), CD8-APCAlexa700 (B9.11), CD19-APCAlexa750 (HD37) and CD56-PECy7 (N901) to determine the helper T cells, cytotoxic T cells, B cells and NK cells within the lymphocyte gate (all MoAbs were obtained from Beckman Coulter, Marseille, France). mHLA-DR expression was determined by calculating HLA-DR mean fluorescence intensity (MFI) within CD14-positive cells. Second, we determined which percentage of CD14-positive monocytes was HLA-DR positive, using HLA-DR positive B-cells as an internal positive control.

Cytokine assays

Ex-vivo cytokine production was determined as described earlier (2). In short, blood was diluted in phosphate buffered saline (PBS) (1:1) and fractions were separated by Ficoll (Ficoll-Paque Plus, GE healthcare, Zeist, The Netherlands) density gradient centrifugation. Cells were washed twice with PBS and resuspended in RPMI-1640 (RPMI-1640 Dutch modification supplemented with 10μg/mL gentamicin, 10mM L-glutamine, and 10mM pyruvate) ( Gibco, Invitrogen, Breda, The Netherlands). The PBMCs were counted using a particle.
counter (Beckmann Coulter, Woerden, the Netherlands) and were plated in 96 well round-bottom plates (Corning, NY, USA) at a final concentration of 2.5x10^6/mL, in a total volume of 200 L. The PBMCs were stimulated for 24 hours, 48 hours, and 7 days with medium alone, or medium containing *E. coli* lipopolysaccharide (LPS; 10 ng/mL), phytohaemaglutinin (PHA; 10μg/mL), heat-inactivated *Candida albicans* yeast (1x10^6/mL) or heat-inactivated *Candida albicans* hyphae (derived from 1x10^6/mL yeast). After stimulation, cell culture supernatant was collected and stored at -20°C. When all samples were collected, cytokines were measured using commercially available ELISAs (R&D Systems, MN, USA and Sanquin, Amsterdam, The Netherlands) according to the protocols supplied by the manufacturer. Ex-vivo production of cytokines was assessed at time points at which their production has been shown to peak (3). Monocyte derived cytokines such as tumour necrosis factor (TNF-α) were measured in culture supernatants of 24 hour cultures, IFN-γ was measured in culture supernatants of 48 hour cultures. T-cell derived cytokine IL-17 was measured in culture supernatants of 7 day cultures.

REFERENCES


**Supplementary Figure 1. Effect of IFN-γ on ex-vivo cytokine responses**

PBMCs of the patient were isolated before initiation of IFN-γ therapy and 8 days later. Panel A. TNF-α production of PBMCs ex-vivo stimulated with LPS, *C. albicans* yeast, or *C. albicans* hyphae for 24 hours. Panel B. IL-17 production of PBMCs ex-vivo stimulated with *C. albicans* yeast, or *C. albicans* hyphae for 7 days. Panel C. IFN-γ production of PBMCs ex-vivo stimulated with *C. albicans* yeast, or *C. albicans* hyphae for 48 hours.
Interferon-gamma immunotherapy in a patient with progressive cerebral *Nocardia* abscesses

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ABSTRACT

Despite advances in medical care, mortality due to cerebral Nocardia abscesses remains unacceptably high. In this report, we present a typical immunocompromised patient, who deteriorated clinically despite optimal antimicrobial treatment. Adjuvant immunotherapy with IFN-γ resulted in partial restoration of the immune response, and paralleled clinical and radiographic recovery.

What was known on this topic?
Nocardiosis is a rare opportunistic Gram-positive bacterial infection. Despite optimal antimicrobial treatment, the mortality rate remains very high, especially in patients with multiple brain abscesses.

What does this add?
In patients with intracerebral Nocardia abscesses who deteriorate despite optimal antimicrobial treatment, immunotherapy with IFN-γ could be considered as adjuvant salvage therapy.

INTRODUCTION

Nocardiosis is a rare Gram-positive bacterial infection caused by aerobic actinomycetes of the genus Nocardia. Nocardia species (spp.) can cause both localized and disseminated infection (1), and they are responsible for approximately 2% of all brain abscesses (2). Mortality rates up to 66% have been reported in patients with multiple lesions (3). Because nocardiosis is most commonly observed in individuals with immune defects (4), adjunctive immunotherapy is a potentially attractive strategy to improve host defense and the outcome of patients with disseminated nocardiosis. Interferon-gamma (IFN-γ), a well-known immunostimulatory cytokine (4), has shown promising results in the adjunctive treatment of other opportunistic infections (5-7). Moreover, IFN-γ is used as prophylactic treatment for prevention of Nocardia infection in patients with chronic granulomatous disease (CGD) (8). Therefore, IFN-γ represents a promising candidate to improve the outcome of invasive Nocardia infections. However, to date, no reports of adjunctive treatment with IFN-γ for patients with Nocardia infections have been reported.

CASE REPORT

A 50-yr-old male patient with multiple Nocardia cerebral abscesses was referred to our university hospital because of clinical deterioration despite optimal antimicrobial treatment. Six months earlier, the patient was referred to a pulmonologist because of recurrent pneumonia, and unidentified interstitial pneumonitis was diagnosed per exclusionem. Corticosteroid treatment was initiated and administered in a dose varying between 5 to 30 mg per day. Two months prior to admission at our centre, he had been admitted to a non-university hospital with a one-month history of fever, behavioral changes, progressive unilateral muscle weakness, nausea and headache. At this admission, cerebral magnetic resonance imaging (MRI) showed two annular contrast enhancements in the left gyrus frontalis intermedius and in the right centrum semiovale, with both lesions showing pronounced vasogenic edema (MRI of the largest lesion is depicted in Figure 1, Panel A). At day 7 of admission, stereotactic aspiration of the largest lesion yielded 3 ml purulent discharge with
The patient was deemed to benefit from adjunctive immunotherapy as “last resort therapy”. Therefore, after referral to our centre and obtaining informed consent, IFN-γ (Boehringer-Ingelheim, Arnhem, The Netherlands) treatment was initiated at day 55 of admission (50 μg/m² subcutaneously, thrice weekly). In addition, two days after initiation of IFN-γ therapy, ceftriaxone was started next to TMP-SMX and meropenem because of persistent fever. Four days later, meropenem was stopped, as recent cultures were negative for multi-resistant bacteria, and there were no clear effects on the clinical course despite treatment for 14 days. Compared with the MRI performed one week before initiation of IFN-γ treatment, the MRI performed 14 days later showed no new lesions and a decrease of the right paraventricular lesion (Figure 1, Panel E). Fever disappeared 12 days after initiation of IFN-γ treatment. However, despite combination antimicrobial therapy (TMP-SMX and ceftriaxone), disappearance of fever, and ameliorating radiography, cerebrospinal fluid (CSF) remained purulent and amikacin was added, administered intrathecally through an Ommaya catheter. Within two weeks, the headache subsided and CSF became normal, and amikacin could be discontinued. Nausea persisted slightly longer, but one week later only trunk balance impairment and vertigo with a tendency to fall remained. Combination antimicrobial therapy and adjunctive IFN-γ treatment were well tolerated and were continued. The condition of the patient further improved and 3 and a half months after initiation of IFN-γ treatment he was discharged, being also able to walk. MRI showed further improvement (Figure 1, Panel F). He was discharged on TMP-SMX and with IFN-γ treatment.

However, 4 days after discharge patient was readmitted because of a Non-ST-Elevation myocardial infarction. Coronary angiography showed no stenosis eligible for intervention and medication was optimized. The same day chest X-ray followed by a CT-scan showed free subdiaphragmal air with pneumatosis intestinalis, a rare complication of high dose corticosteroid administration. Abdominal surgery showed no additional cause for free air. The patient was admitted at the Intensive Care Unit in need of ventilator support and inotropic medication. He died seventeen days later due to cardiovascular causes that were not directly related to the *Nocardia* infection, which was confirmed on autopsy.

![Figure 1. Studies of the largest right paraventricular brain lesion by magnetic resonance imaging (MRI). Panel A shows a T1-weighted contrast enhanced hyperintense MRI image of the right brain lesion at admission on January 12th 2012 (sized 25x22x20 mm, with extensive surrounding edema). Culture guided initial treatment with cotrimoxazole resulted in an initial decrease in size and reduction of edema (Panel B; size of lesion: 17x15x17 mm). However, subsequent imaging showed little to no further reduction of the abscess (Panel C; size of lesion: 15x13x19 mm, and D; size of lesion: 16x13x19 mm). Therefore, adjunctive immunotherapy with IFN-γ treatment was initiated at March 6th. MRI performed 12 days later showed a decrease of the right paraventricular lesion (Panel E: 12x13x17 mm). Further ameliorating of MRI images was observed three weeks later (Panel F; size of lesion: 12x9x12 mm). All images are published with permission of legal representative.](image-url)
Immunological analysis
Besides corticosteroid use, personal and family history was negative for immune deficiencies. However, additional testing revealed a CD4 lymphopenia (140 cells/mm³) in the absence of HIV infection. This may be related to the steroid treatment, but a primary cause cannot be excluded. Together with routine clinical blood withdrawal, additional blood was collected to analyze immune responses before and during IFN-γ treatment (see online supplement for methods of blood sampling and cytokine assays). Before IFN-γ treatment, the capacity of peripheral blood mononuclear cells (PBMCs) to produce cytokines upon ex vivo stimulation with Candida albicans, lipopolysaccharide (LPS), and phytohaemagglutinin (PHA) was severely blunted (Figure 2, Panel A to F). IFN-γ treatment was associated with an increased production of Interleukin (IL)-1β, Tumor Necrosis Factor (TNF)-α, IL-6, and IL-10 by PBMCs stimulated with all three stimuli (Figure 2, Panel A to D). C. albicans–induced ex-vivo production of the T-helper 17 (Th17) cytokines IL-17 and IL-22, was also increased, although to a lesser extent (Figure 2, Panel E and F).

DISCUSSION
In this case report we describe that adjunctive immunotherapy with IFN-γ can result in augmentation of the innate immune response in a patient with progressive Nocardia abscesses, and very likely contributed to clinical recovery.

Patients with impaired cell-mediated immunity (including hematopoietic stem cell transplant patients and patients receiving long-term treatment with steroids) have a particularly high risk to develop opportunistic infections, including nocardiosis (1). Immunotherapy to improve cell-mediated immunity is therefore a promising therapy to improve outcome in these patients. It has previously been demonstrated that IFN-γ has favorable effects on outcome of sepsis patients who exhibit clear signs of suppression of cellular immune responses, which renders them more vulnerable to opportunistic infections (4). In addition, IFN-γ restored the suppressed cytokine production in vivo in humans in an experimental sepsis model (4), and in patients with severe invasive fungal infections (7). Furthermore, IFN-γ has a beneficial effect on the outcome of opportunistic infections in immunocompromised patients with HIV (5), or leukemia (6). However, whether IFN-γ restored the impaired cellular immune responses in these patients was not investigated. In the present study, we demonstrate that IFN-γ improved cellular immune responses in a patient with cerebral nocardiosis, and this correlated with improvement of the clinical outcome.
CONCLUSION

Optimal antimicrobial treatment in patients with nocardiosis should be individualized according to species identification, resistance profile, and CNS penetration capacity of the antimicrobial agent used. In patients with intracerebral Nocardia abscesses who deteriorate despite optimal antimicrobial treatment, immunotherapy with IFN-γ could be considered as adjuvant salvage therapy.

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METHODS

Blood sampling

Together with routine blood sampling, additional blood was drawn to analyse immune responses. Specimens were collected at baseline (day 0) and at several timepoints after start IFN-γ immunotherapy (days 1, 2, 7 and 14).

Cytokine assays

Venous blood was drawn into 10 mL EDTA tubes, after which peripheral blood mononuclear cells (PBMCs) were isolated. In short, blood was diluted in phosphate buffered saline (PBS) (1:1) and fractions were separated by Ficoll (Ficoll-Paque Plus, GE healthcare, Zeist, The Netherlands) density gradient centrifugation. Cells were washed twice with PBS and resuspended in RPMI-1640+ (RPMI-1640 dutch modification supplemented with 10µg/mL gentamicin, 10mM L-glutamine, and 10mM pyruvate) (Gibco, Invitrogen, Breda, The Netherlands). The PBMCs were counted using a particle counter (Beckmann Coulter, Woerden, The Netherlands) and were plated in 96 well round-bottom plates (Corning, NY, USA) at a final concentration of $2.5 \times 10^6$/mL, in a total volume of 200 µL. The PBMCs were stimulated for 24 hours, 48 hours, and 7 days with medium alone, or medium containing *E. coli* lipopolysaccharide (LPS; 10 ng/mL), phytohaemaglutinin (PHA; 10µg/mL), or heat-inactivated *Candida albicans* blastoconidia ($5 \times 10^6$/mL). After stimulation, cell culture supernatant was collected and stored at -20°C. 7 day cultures were supplemented with 10% human pooled serum.

After all samples were collected, cytokines were measured using commercially available ELISAs (R&D Systems, MN, USA and Sanquin, Amsterdam, The Netherlands) according to the protocols supplied by the manufacturer. Interleukin (IL)-1β, Tumor Necrosis Factor (TNF)-α, and IL-6 were measured in culture supernatants of 24 hour cultures, IFN-γ and IL-10 were measured in culture supernatants of 48 hour cultures, and IL-17 and IL-22 were measured in culture supernatants of 7 day cultures.
New immunomodulatory approaches

Part THREE
Gamma-irradiated Bacille Calmette-Guérin vaccination does not modulate the innate immune response during experimental human endotoxemia in adult males

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INTRODUCTION

Sepsis is a clinical condition that represents a major medical challenge due to its high mortality rate. Related to this, it is a major clinical challenge also because it may be difficult to diagnose in due time, and difficult to treat. Previous adjunctive therapeutic strategies, aiming to treat sepsis by inhibition of pro-inflammatory mediators have failed, likely related to the recent insight that the majority of septic patients do not succumb to the initial pro-inflammatory “hit”, but die at a later time-point in a pronounced immunosuppressive state [1-3]. This so-called ‘sepsis-induced immunoparalysis’ results from counter-regulatory anti-inflammatory pathways that are activated simultaneously with pro-inflammatory mechanisms [2-4]. This renders patients unable to clear the initial infection and increased vulnerable to secondary infections [2, 3, 5]. As a consequence, reconstitution of immunocompetence is emerging as a new and promising therapeutic target to improve outcome in sepsis patients [2, 3, 6, 7].

ABSTRACT

Bacille Calmette-Guérin (BCG) vaccine exerts non-specific immunostimulatory effects, and may therefore represent a novel therapeutic option to treat sepsis-induced immunoparalysis. We investigated whether BCG vaccination modulates the systemic innate immune response in humans in vivo during experimental endotoxemia. We used inactivated gamma-irradiated BCG vaccine because of the potential risk of disseminated disease with the live vaccine in immunoparalyzed patients. In a randomized double blind placebo-controlled study, healthy male volunteers were vaccinated with gamma-irradiated BCG (n=10) or placebo (n=10) and received 1 ng/kg lipopolysaccharide (LPS) intravenously on day 5 after vaccination to assess the in-vivo immune response. Peripheral blood mononuclear cells were stimulated with various related and unrelated pathogens 5, 8 to 10, and 25 to 35 days after vaccination to assess ex-vivo immune responses. BCG vaccination resulted in a scar in 90% of vaccinated subjects. LPS administration elicited a profound systemic immune response, characterized by increased levels of pro-and anti-inflammatory cytokines, hemodynamic changes, and flu-like symptoms. However, BCG neither modulated this in-vivo immune response, nor ex-vivo leukocyte responses at any time-point. In conclusion, gamma-irradiated BCG is unlikely to represent an effective treatment option to restore immunocompetence in patients with sepsis-induced immunoparalysis.
innate immune cells, as BCG vaccination enhances resistance against *Candida* infection and increased lipopolysaccharide (LPS)-induced cytokine production in splenocytes of mice lacking T- and B-cells [17].

Considering its potentiating effects on host defense, BCG could represent a therapeutic option to prevent or treat sepsis-induced immunoparalysis. Nevertheless, as patients with sepsis have an increased susceptibility to secondary infections, vaccination with live BCG may be associated with unwarranted risks for dissemination [22]. As recent data showed that gamma-irradiated BCG has similar potentiating effects on trained immunity *in vitro* (Arts et al, submitted), but does not present any risk for infection, this inactivated form of BCG represents a clinically relevant alternative in these patients. However, the effects of BCG vaccination on the immune response in humans have hitherto only been shown *ex vivo* [17, 21]. It has yet to be established whether these findings can be extrapolated to the human *in-vivo* situation, because *ex-vivo* data might not always reflect *in-vivo* responses [23, 24]. The human endotoxemia model, in which healthy volunteers are administered a low dose of LPS, represents a unique model to study modulation of the systemic inflammation in humans *in vivo* in a safe, highly standardized, and reproducible manner [25].

The aim of the present study was to investigate the effects of vaccination with gamma-irradiated BCG on the systemic innate immune response in adult males *in vivo* during experimental endotoxemia.

**METHODS**

**Subjects**

After approval from the Arnhem-Nijmegen Ethics Committee, 20 healthy non-smoking male volunteers gave written informed consent to participate in this study that was registered at ClinicalTrials.gov as NCT02085590. Subjects were screened before the start of the experiment and had a normal physical examination, electrocardiography, and routine laboratory values. Exclusion criteria were febrile illness during the 2 weeks before start of the study, prior BCG-vaccination, any vaccination other than BCG within 3 months before start of the study, and a tuberculin skin test within 1 year prior to the start of the study. Throughout the study period, subjects were not allowed to take any drugs, including acetaminophen, and were asked to refrain from alcohol and caffeine 24 hours, and from food 12 hours before the start of the endotoxemia experiment. All study procedures were conducted in accordance with the declaration of Helsinki including current revisions and Good Clinical Practice guidelines.

**Study design and procedures**

We performed a randomized double-blind placebo-controlled study. The study design is schematically depicted in Figure 1. For reasons detailed in the introduction, gamma-irradiated (and therefore inactivated) BCG vaccine was used in this study. Irradiated BCG was cultured for 6 weeks using Mycobacteria Growth Indicator Tubes according to Dutch national guidelines to confirm inactivation, and no growth was observed. Subjects were randomly assigned to receive either 0.075 mg (0.1 ml) gamma-irradiated BCG-vaccine intracutaneously (BCG-vaccine SSI; Statens Serum Institut, gamma-irradiation [25-30 kGy] performed by Synergy Health Ede, The Netherlands; *n* = 10) or 0.1 ml placebo (BCG-reconstitution fluid: diluted Sauton 1+3; Statens Serum Institut; *n* = 10) in a double-blind fashion. Five days after vaccination, all subjects received an intravenous injection of LPS (lipopolysaccharide derived from Escherichia coli O:113, Clinical Center Reference Endotoxin, National Institutes of Health [NIH], Bethesda, MD; 1 ng/kg. Endotoxemia experiments were conducted as described previously [24]. Heart rate (three-lead electrocardiogram), blood pressure, respiratory rate, and oxygen saturation (pulse oximetry) data were recorded from a Philips MP50 patient monitor every 30 seconds by a custom in-house–developed data recording system. LPS-induced flu-like symptoms (headache, nausea, shivering, muscle and back pain) were scored every 30 min on a six-point Likert scale (0 = no symptoms, 5 = worst ever experienced), resulting in a total score of 0–25 points. After the endotoxemia experiment, additional blood samples were drawn on days 8-10 and days 25-30 after vaccination. During the last visit, BCG scar formation was measured by an independent research nurse (to maintain blinding) using a centimeter ruler.
Mycobacterium tuberculosis (MTB) (1 μg/ml end protein concentration, strain H37Rv), Escherichia coli lipopolysaccharide (LPS; 1 ng/ml; Sigma-Aldrich, St. Louis, Mo., USA), heat-killed Staphylococcus aureus (1 x 10^6 microorganisms/ml, clinical isolate), or heat-killed Candida albicans (1 x 10^6 microorganisms/ml, strain UC820). After 24h (for determination of TNF-α, IL-1β, and IL-6) or 48h (for determination of IFN-γ and IL-10) of incubation, plates were centrifuged and supernatants were stored at -20°C until analysis. Cytokines were measured batch-wise using commercially available ELISAs (R&D Systems, MN, USA and Sanquin, Amsterdam, The Netherlands) according to the protocols supplied by the manufacturers.

Calculations and Statistical Analysis
Data are represented as median and interquartile range or mean and SEM, based on their distribution (calculated by the Shapiro–Wilk test). The area under the curve (AUC) of cytokine levels during experimental endotoxemia, representing an integrated measure of the cytokine response, was calculated using time points 0-8 hours post-LPS. Comparisons were made using Mann-Whitney U tests (non-normally distributed data, between-group comparisons) or repeated measures two-way ANOVA (normally distributed data, where the time factor represents differences across both groups over time and the interaction factor represents between-group differences over time). Ex-vivo cytokine data were log-transformed to obtain a normal distribution. A P-value < 0.05 was considered statistically significant. Calculations and statistical analyses were performed using Graphpad Prism version 5.0 (Graphpad Software, San Diego, CA, USA).

RESULTS
Baseline characteristics
No differences in baseline characteristics between both groups were present (Table 1). Gamma-irradiated BCG vaccination resulted in a scar at the vaccination site in 9 out 10 subjects (median [range] size of 6 [1-9] mm). In the placebo group, 1 subject developed a small scar (1 mm). BCG vaccination did not result in fever or other clinical symptoms and no serious adverse events occurred during the trial.
very low levels (approximately 10 pg/mL) were found, but no clear patterns over time or differences between groups were observed.

As described earlier, scar size differed substantially between BCG-vaccinated subjects, which might represent vaccination efficacy, and is associated with non-specific beneficial effects of BCG [27, 28]. Therefore, we stratified the BCG-vaccinated group according to scar size (≤5 mm, n=5; >5 mm, n=5). These stratified analyses did not reveal notable differences in cytokine responses either (supplementary Figure 1).

After LPS administration, transient leukocytosis developed, reaching maximum levels at T=8 hours, with no differences between groups (mean ± SEM of BCG and placebo groups, respectively: 10.2 ± 0.7 vs. 9.7 ± 0.7 X 10⁹/L, p=0.62). At the first visit after the endotoxemia day (day 8-10), leukocyte numbers were normalized in both groups (5.2 ± 0.4 vs. 5.7 ± 0.6 X 10⁹/L in the BCG and placebo groups, respectively, p=0.50).

Cytokine production by peripheral blood mononuclear cells
Five days after vaccination with BCG or placebo, but before LPS administration in vivo, there were no differences between groups in ex-vivo cytokine responses induced by specific (Mycobacterium tuberculosis) or unrelated (LPS, Staphylococcus aureus, and Candida albicans) pathogens or stimuli (fold change data [compared with baseline] of IFN-γ, TNF-α, and IL-1β are depicted in Figure 4, and fold change data of IL-6 and IL-10 in supplementary Figure 2. Absolute values of all cytokines are depicted in supplementary Figure 3). Similar to previous endotoxemia experiments [24, 29], four hours after LPS administration, an overall profound decrease in ex-vivo cytokine production was observed, indicative of immunoparalysis. BCG vaccination did not influence the development or magnitude of immunoparalysis. Likewise, no differences between groups in ex-vivo cytokine responses to any of the pathogens or stimuli were found on days 8-10 and 25-35 after vaccination. Of note, LPS-induced production of IFN-γ, as well as LPS- and Mycobacterium tuberculosis-induced production of IL-10, was absent in many subjects and very low in others. Therefore, the endotoxemia-induced decrease in ex-vivo cytokine production was less noticeable and did not always reach statistical significance for these combinations. Staphylococcus aureus- and Candida albicans-induced IL-10 production was absent in virtually all subjects and was therefore not analyzed.
Figure 4. Production of IFN-γ, TNF-α, and IL-1β by peripheral blood mononuclear cells stimulated ex vivo with Mycobacterium tuberculosis (MTB), LPS, Staphylococcus aureus (SA), and Candida albicans (CA) of subjects vaccinated with gamma-irradiated BCG or placebo. Data expressed as median and interquartile range of the fold change compared with day 1 (before vaccination) (n=10 per group). p-values calculated using repeated measures two-way analysis of variance (ANOVA, time and interaction terms) on log-transformed data. Day 6 was the endotoxemia experiment day.

Figure 3. Plasma cytokine concentrations in subjects vaccinated with gamma-irradiated BCG or placebo. In the panels A-D, median values of pro-inflammatory cytokines TNF-α, IL-6, IL-8, and MCP-1 are depicted while in panels E and F median values of anti-inflammatory cytokines IL-10 and IL-1RA are shown (n=10 per group). Panels G-L depict median ± interquartile range of area under curve (AUC) the respective cytokines (n=10 per group). P values calculated using Mann-Whitney U-tests.
DISCUSSION

In the present study, we demonstrate that gamma-irradiated BCG vaccination does not influence the LPS-induced innate immune response in adult males in vivo five days later. Furthermore, no effects of BCG vaccination on cytokine production of leukocytes stimulated ex vivo with specific and unrelated pathogens were observed.

As all measured parameters were similar between groups, we can conclude that five days after vaccination, gamma-irradiated BCG has no effect on the innate immune system and therefore does not induce trained immunity. This is evident from both the lack of an effect on LPS-induced plasma cytokine levels in vivo, as well as from the similar ex-vivo innate cytokine responses (TNF-α, IL-1β, IL-6, and IL-10) against unrelated pathogens five days after vaccination in both groups. Previous epidemiological studies have shown that scar formation after vaccinia or BCG vaccination is associated with improved survival, possibly related to improved resistance against infections [27, 28]. Therefore, we stratified subjects based on scar size, but no effects were found in these analyses either. Furthermore, no effects indicative of trained immunity induction were found at later time-points, ranging from 8-10 days to 25-35 days after vaccination.

Our results are different from previous studies that used the live attenuated BCG vaccine [17, 21] instead of the gamma-irradiated BCG. There are several reasons and/or limitations of the present study that might explain these differences. First and foremost, we used gamma-irradiated BCG in the present study because our target treatment population consists of immunoparalyzed septic patients who may be at risk for disseminated mycobacterial infection [22]. We hypothesized that gamma-irradiated BCG would be effective in inducing trained immunity in vivo because recent unpublished data of our group showed that gamma-irradiated BCG exerts monocyte training in vitro. Furthermore, previous in-vitro studies showed that monocytes could be trained with live BCG, as well as with the inert NOD2 ligand MDP [17], highlighting that live BCG persistence is not mandatory for inducing trained immunity in vitro. Nevertheless, inactivating the vaccine could have reduced or abrogated the “training capacity” of BCG. While live vaccines can replicate and/or disseminate in the host’s body and thereby trigger the immune response to a greater extent, inactivated vaccines only activate immune responses locally [30]. Although the scar formation in gamma-irradiated BCG-vaccinated subjects indicates a local immune response, it could be envisioned that possible training effects of gamma-irradiated BCG are much less sustained and widespread, and thus less pronounced. Along these lines, it was demonstrated that two and four weeks after vaccination with live BCG, 83 and 50% of individuals still displayed viable BCG at the vaccination site [31], respectively, indicative of a relatively long-lasting “active infectious pool” of bacteria (or their products) and/or cytokines that trigger a variety of responses. This is likely not relevant for the in-vitro situation, where cells are continuously exposed to bacteria and/or their products irrespective of whether they are alive or inactivated.

Also, others have shown that viable bacteria elicit more potent immune responses compared to killed bacteria, due to recognition of so called ‘vita-PAMPs’ such as prokaryotic mRNA by innate immune cells [32]. The absent effects of gamma-irradiated BCG on ex-vivo cytokine responses to stimulation with M. tuberculosis further substantiate the hypothesis that gamma-irradiation results in functional inactivation of BCG, resulting not only in abrogation of trained immunity but also of “classic” specific protection against M. tuberculosis.

Secondly, the timing of the interventions in our study might have precluded effects of gamma-irradiated BCG. We chose to assess in-vivo and ex-vivo responses already five days after vaccination, in order to assess potential short-term effects that may be most relevant during sepsis. We hypothesized that this period would be sufficient to induce trained immunity based on the fact that in-vitro training by BCG only takes one day and that non-specific beneficial effects of BCG-vaccination in neonates were already apparent within 3 days [15]. Nevertheless, in previous studies enhancing effects of BCG on leukocytes were found 2 weeks, 3 months, and one year after vaccination [17, 21]. No earlier time-points were assessed in these earlier studies.

Thirdly, we only included young male volunteers in this study. There are considerable differences in the cytokine response to LPS between males and females [33]. This is likely influenced by menstrual cycle-related hormonal variations that can affect the immune response. Because we wanted our study...
population to be as homogenous as possible, we therefore included only males, analogous to virtually all of our previous endotoxemia studies. This might have biased our results, because the majority of the studies on non-specific effect of the BCG vaccine point to important sex-differential non-specific effects, and often the most pronounced effects were observed among females [12, 34, 35]. Non-specific effects may also vary with age, nevertheless, live BCG exerted profound effects in a similarly aged study population [37, 21].

Fourthly, possible training effects of gamma-irradiated BCG on monocytes in the long-term might have been obscured by the LPS administration five days after vaccination. BCG-induced trained immunity has been shown to be mediated through epigenetic reprogramming of monocytes [17]. Interestingly, exposure to LPS results in opposite epigenetic changes in monocytes and/or macrophages [20]. Therefore, possible training effects induced by gamma-irradiated BCG might have been nullified by the LPS administration.

Fifthly, the human endotoxemia model employed in this study is relatively mild and does not replicate the severe sepsis-induced immunoparalysis observed in actual patients. Therefore, although unlikely based on the complete absence of effects in the present study, we cannot exclude the possibility that gamma-irradiated BCG exerts immunomodulatory effects in a true model of immunoparalysis or in immunoparalyzed septic patients.

Finally, our study is limited by the fact that, apart from medical history, we did not screen for previous exposure to *Mycobacterium tuberculosis*. We chose not to perform a tuberculin skin test since this could trigger trained immunity effects on its own which would confound our study results. However, the infectious pressure of tuberculosis in the Netherlands is very low [36], and this possibility is unlikely to explain the absent effects of gamma-irradiated BCG.

In view of the points raised above, a study using live BCG and possibly other timing of the interventions could be considered. While such as study would be warranted to elucidate the mechanisms behind the important nonspecific beneficial effects of BCG vaccination in neonates [9-15], it may be less relevant with regard to sepsis patients, in which the use of live BCG vaccine would be associated with too high risks.

**CONCLUSIONS**

Gamma-irradiated BCG does not modulate the in-vivo innate immune response in adult male volunteers five days after vaccination. Furthermore, vaccination did not induce trained immunity ex vivo. Therefore, gamma-irradiated BCG is unlikely to represent a viable treatment option to restore immunocompetence in patients with sepsis-induced immunoparalysis.

**ACKNOWLEDGEMENTS**

The authors thank the research nurses (Marieke van der A, Chantal Luijten-Arts, Hellen van Wezel) of the ICU department. M.G.N. was supported by a Vici grant of the Netherlands Organization for Scientific Research and an ERC Consolidator Grant (#310372).
ONLINE SUPPLEMENT

Supplementary Figure 1. Area under curve (AUC) of plasma concentrations of pro-inflammatory cytokines TNF-α, IL-6, IL-8, and MCP-1, and anti-inflammatory cytokines IL-10 and IL-1RA in subjects vaccinated with gamma-irradiated BCG stratified according to vaccination scar size (≤5 mm or >5 mm, n=5 per group). Data are presented as median ± interquartile range of the respective cytokines. P values calculated using Mann-Whitney U-tests.

Supplementary Figure 2. Production of IL-6 and IL-10 by peripheral blood mononuclear cells stimulated ex vivo with Mycobacterium tuberculosis (MTB), LPS, Staphylococcus aureus (SA), and Candida albicans (CA) of subjects vaccinated with gamma-irradiated BCG or placebo. Data expressed as median and interquartile range (n=10 per group). Day 6 was the endotoxemia experiment day. For statistical analyses, see Figures 4 and supplementary Figure 2, which depict the same data expressed as fold change compared with day 1.

Supplementary Figure 3. Production of IFN-gamma, TNF-α, IL-1β, IL-6, and IL-10 by peripheral blood mononuclear cells stimulated ex vivo with Mycobacterium tuberculosis (MTB), LPS, Staphylococcus aureus (SA), and Candida albicans (CA) of subjects vaccinated with gamma-irradiated BCG or placebo. Data expressed as median and interquartile range (n=10 per group). Day 6 was the endotoxemia experiment day. For statistical analyses, see Figures 4 and supplementary Figure 2, which depict the same data expressed as fold change compared with day 1.
CHAPTER 8

BCG-vaccination enhances immunogenicity of subsequent influenza vaccination in healthy volunteers: a randomized placebo-controlled pilot study

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Jelle Gerretsen
Reinout van Crevel
Dimitri A. Diavatopoulos
Guus F. Rimmelzwaan
Peter Pickkers
Mihai G. Netea
ABSTRACT

Background: Influenza-related morbidity and mortality remain high. Seasonal vaccination is the backbone of influenza management but does not always result in protective antibody titres. Non-specific effects of BCG-vaccination related to enhanced function of myeloid antigen-presenting cells have been reported. We hypothesized that BCG vaccination could also enhance immune responses to influenza vaccination.

Methods: Healthy volunteers received either live attenuated BCG vaccine (n=20) or placebo (n=20) in a randomized fashion, followed by intramuscular injection of trivalent influenza vaccine 14 days later. Hemaglutination inhibiting (HI) antibodies and cellular immunity measured by ex-vivo leukocyte responses were assessed.

Results: In BCG-vaccinated subjects, HI antibody responses against the 2009 pandemic H1N1 vaccine strain were significantly enhanced compared with the placebo group, and there is a trend towards more rapid seroconversion. Additionally, apart from enhanced pro-inflammatory leukocyte responses following BCG vaccination, nonspecific effects of influenza vaccination were also observed, with modulation of cytokine responses against unrelated pathogens.

Conclusion: BCG vaccination prior to vaccination with influenza vaccine results in a more pronounced increase and accelerated induction of functional antibody responses against the 2009 pandemic H1N1 influenza vaccine strain. These results may have implications for the design of vaccination strategies and could lead to improvement of vaccination efficacy.

INTRODUCTION

Annually, influenza virus infection leads to millions of cases of severe illness worldwide and up to an estimated 500,000 deaths (1). The potential for the sudden emergence of pandemic influenza strains represents an incessant threat on even a larger scale: it is estimated that if a strain with similar virulence to the 1918 ‘Spanish flu’ emerged today, it could kill between 50 and 80 million people (2). With very few therapeutic options available, seasonal vaccination is the backbone of influenza management. High-affinity antibodies play a key-role in the protective immune response against influenza virus infection (3). However, antibodies generated by vaccination most often do not effectively neutralize emergent strains due to the high mutation rate of the influenza viral genome (4). In addition, vaccination is not always effective, as 85% of healthy adults and only 40-60% of elderly people mount a protective antibody response, due to original antigenic sin (5), and an age-related decline in immune function (a process called immunoscenescence) (6). As a result, particularly in high-risk groups, the protective effects of influenza vaccination are limited, and strategies to improve host immune defences against influenza virus infection and the response to influenza vaccination are highly warranted (7).

In addition to protection against tuberculosis (TB), vaccination with Bacille Calmette-Guérin (BCG) provides protection against other infectious diseases (8). Murine studies have shown that vaccination with BCG results in protection not only against secondary infections with Candida albicans (9), Schistosoma mansoni (10), but also against influenza virus (11). Moreover, non-specific beneficial effects of BCG-vaccination on mortality of young children were demonstrated in observational studies (12), and several randomized studies demonstrated reduced overall mortality in BCG-vaccinated neonates, which could not be explained by TB prevention (13, 14). The underlying immunologic mechanisms responsible for the non-specific effects of BCG are currently being unravelled, and they may be mediated by both induction of trained innate immunity and heterologous adaptive immune responses. Assessment of trained immunity in BCG-vaccinated individuals has recently shown that monocytes undergo epigenetic reprogramming towards an enhanced pro-inflammatory phenotype (15, 16). This results in increased production of pro-
inflammatory cytokines, such as IFN-γ, TNF-α, and IL-1β upon ex vivo stimulation with unrelated pathogens, even up to one year after BCG vaccination (15, 16).

As the nonspecific immunomodulatory effects of BCG vaccination increase the function of myeloid immune cells with antigen-presenting properties, we hypothesized that BCG vaccination could enhance immune responses to other vaccines in general, and to influenza vaccination in particular. In the present randomized trial, we investigated the effects of BCG-vaccination on the immunogenicity of a trivalent influenza vaccine in healthy volunteers.

MATERIALS AND METHODS

Subjects

This study was registered at ClinicalTrials.gov as NCT02114255. After approval from the Arnhem-Nijmegen Ethics Committee, 40 healthy, non-smoking, male volunteers gave written informed consent to participate in the study, which took place from May to July 2014. All experiments were conducted in accordance with the declaration of Helsinki. Subjects were screened before the start of the experiment and had a normal physical examination. Subjects who were vaccinated with BCG before, received influenza vaccination in the previous year, or had febrile illness during the two weeks before the experiment were excluded. Subjects were not allowed to use any prescription drugs.

![Figure 1. A schematic representation of the study design. BCG, Bacille Calmette-Guérin; HI: Hemagglutination inhibition.](image)

**Study design**

The design of this placebo-controlled randomized trial is depicted in Figure 1. Briefly, subjects were randomized using the sealed envelope method to receive intradermal injections of either 0.1 ml of live attenuated BCG vaccine (BCG-vaccin SSI/Danish strain 1331; Bilthoven Biologicals, Bilthoven, the Netherlands; n=20) or placebo (NaCl 0.9%; n=20), in a double-blinded fashion. Fourteen days later all subjects received an intra-muscular injection of 0.5 ml trivalent influenza vaccine season 2013-2014 (containing A/California/7/2009 (pandemic H1N1, H1N1pdm09) derived strain, Victoria/365/2011 related strain derived from A/Texas/50/2012 (A/H3N2/2012), and B/Massachusets/2/2012 (B/2012) derived strain surface antigens, and no adjuvants; Batrevac; Abbot biologicals B.V., Weesp, the Netherlands). Adverse effects were recorded after day 0, and antibody titres and cytokine production capacity were assessed before BCG vaccination (day -14), before influenza vaccination (day 0) and 7, 14, and 28 days after influenza vaccination. The primary study endpoint was the difference in HI antibody titres over time after influenza vaccination. Secondary endpoints were the proportion of participants in each group who achieve seroconversion (defined by a ≥4-fold rise in antibody titre) over time after influenza vaccination, and cytokine responses of leukocytes ex-vivo stimulated with various influenza-related and unrelated stimuli over time after BCG vaccination.

**Hemagglutination inhibition (HI) assay**

HI assays were performed according to standard procedures which are detailed in the Online Supplement. Every sample was run in duplicate and Geometric Mean Titres were determined by calculating the mean of the log-transformed duplicate titres followed by back-transformation \(10^x\), where \(x\) equals mean log-transformed titre). Seroconversion was defined as ≥ 4-fold titre increase compared with baseline. Antibody titres were similar between day -14 (before BCG/placebo-vaccination) and day 0 (before influenza vaccination) within groups for all three vaccine strains in the placebo group, and for two vaccine strains in the BCG-group (a significant difference in the A/H3N2/2012 strain was found, see Supplementary Table S1). This variability could due to assay variation.
or from a non-specific boosting effect of BCG on plasma cells. We calculated relative increases in antibody titres compared to titres at day 0, just before influenza vaccination.

Peripheral blood mononuclear cell stimulation and cytokine measurements

Venous blood was drawn into EDTA tubes, after which peripheral blood mononuclear cells (PBMCs) were isolated and stimulated with various stimuli, after which cytokines were determined in supernatants. A detailed description is provided in the Online Supplement.

Statistical analyses

All data were not normally distributed (determined using Kolmogorov-Smirnov tests). Demographic data were analyzed using Mann-Whitney U tests. Differences between the two groups over time were calculated using Mann-Whitney U tests of Area Under the Curve (AUC) calculated from the fold-change data (to correct for baseline differences). Within-group differences in cytokine production over time were calculated using Friedman tests. Within-group differences in cytokine production between day 0 and day 14, and antibody titres between day -14 and day 0 were calculated using Wilcoxon matched-pairs tests. Stratified HI assay analysis according to baseline titers was based on routine dilutions used to assess HI titers. Finally, differences in seroconversion rate between groups over time were calculated using log-rank tests. A p-value of <0.05 was considered statistically significant. We calculated that, in order to be able to detect a two-fold difference in HaNa titre increase between the BCG and placebo groups with a power of 80%, an SD of 113% (17), and a two-sided alpha of 0.05, 20 subjects per group were needed. Calculations and statistical analyses were performed using Graphpad Prism version 5.0 (Graphpad Software, San Diego, CA, USA).

Table 1. Demographic characteristics

<table>
<thead>
<tr>
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<th>Placebo (n=20)</th>
<th>BCG (n=20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>20.5 [20.3-25]</td>
<td>21 [20-24]</td>
<td>0.35</td>
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<tr>
<td>Height (cm)</td>
<td>178 [175-188]</td>
<td>183 [180-190]</td>
<td>0.13</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>74.4 [66.2-82.8]</td>
<td>80.2 [72.3-93.4]</td>
<td>0.06</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>22.3 [21.1-24.7]</td>
<td>24.5 [22.2-27.0]</td>
<td>0.08</td>
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</tbody>
</table>

Data are presented as median and interquartile range. P-values were calculated using Mann-Whitney U-tests.

Table 2. Baseline (day 0) antibody titres

<table>
<thead>
<tr>
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<th>Placebo (n=20)</th>
<th>BCG (n=20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/H1N1/2009</td>
<td>132.7 [68.71-257.04]</td>
<td>42.31 [22.5-103.3]</td>
<td>0.13</td>
</tr>
<tr>
<td>A/H3N2/2012</td>
<td>66.4 [32.1-137.4]</td>
<td>115.9 [57.9-231.2]</td>
<td>0.33</td>
</tr>
<tr>
<td>B/2012</td>
<td>39.0 [20.5-74.3]</td>
<td>40.9 [20.9-78.0]</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Data are presented as Geometric Mean Titres [95%CI]. P-values were calculated using Mann-Whitney U-tests.

RESULTS

Demographic characteristics and side effects

Baseline characteristics were similar in both groups (Table 1). No serious adverse events occurred during the study. None of the subjects in the placebo group and 10 subjects in the BCG group reported a local inflammatory reaction at the injection site, which resolved in all cases within four weeks after injection. After influenza vaccination, six placebo-vaccinated subjects and six BCG-vaccinated subjects reported mild complaints (including fatigue, headache, malaise and muscle pain at the injection site), which resolved within two days after vaccination in all cases.
Influenza antibody titres

There were no baseline differences in antibody titres between groups for the three influenza strains (Table 2). In BCG-vaccinated subjects, HI antibody responses against the pandemic H1N1pdm09 vaccine strain was markedly enhanced compared with the placebo-treated group, and there is a trend towards more rapid seroconversion (Figure 2A). No significant differences between groups were observed regarding HI antibody responses against the A/H3N2 and B vaccine strains (Figure 2, panels B and C). Stratified analyses according to baseline antibody titres revealed similar patterns compared with the overall analysis presented in Figure 2 (Supplementary Figures S2-S5). As expected, HI antibody responses induced by influenza vaccination were much more pronounced in subjects with low baseline antibody titres. Accordingly, subjects with high baseline antibody titres in both groups barely attained seroconversion defined by 4-fold increase from baseline. These data indicate that differences between groups were mainly based on responses of subjects with low baseline antibody titres. When subjects with high baseline antibody titres (set as the titre for which ≤ 25% of subjects in both groups attained seroconversion) were excluded, the potentiating effect of BCG vaccination on antibody responses against the H1N1pdm09 strain became more apparent (Supplementary Figure S4, panel A). In addition, attainment towards enhanced antibody responses against the influenza B strain in the BCG group also emerged when groups were stratified (Supplementary Figure S4, panel C), while antibody responses against the A/H3N2 strain remained similar between groups (Supplementary Figure S4, panel B).

Cytokine responses upon ex vivo stimulation of PBMCs with influenza-related stimuli

There were no differences in baseline ex vivo cytokine responses between groups (data not shown). In both groups, the production of IFN-γ upon stimulation with influenza vaccine was enhanced after influenza vaccination (Figure 3, Panel A). Likewise, IFN-γ production upon stimulation with live influenza virus increased in both groups after vaccination, although this did not reach statistical significance in the placebo group (Figure 3, Panel B). No effect was observed on IFN-α production upon stimulation with influenza vaccine in both groups (Figure 3, Panel C), whereas production of this cytokine upon stimulation with live influenza virus was enhanced in both groups, although this did not reach statistical significance in the BCG group (Figure 3, Panel D). No significant differences between treatment groups were observed, apart from a trend towards enhanced and more sustained IFN-γ production upon stimulation with influenza vaccine in the BCG-group.
There were no differences in baseline ex vivo cytokine responses between groups (data not shown). As expected, enhanced production of IFN-γ and IL-6 was found upon stimulation with *Mycobacterium tuberculosis* (MTB) in BCG-vaccinated subjects compared with the placebo group (Figure 4). This enhanced cytokine production was already present at day 0 (just before influenza vaccination), thereby indicating that these effects are mediated by BCG. For MTB-induced TNF-α and IL-1β production, a trend towards enhanced responses was observed in BCG-vaccinated subjects (Figure 4), while no effects were observed for the anti-inflammatory cytokine IL-10 (data not shown).

To investigate the effects of influenza vaccination on ex-vivo cytokine responses to unrelated pathogens, we assessed changes in cytokine responses within the placebo group between day 0 (before influenza vaccination) and day 14 (Figure 5, Panel A). Influenza vaccination on its own resulted in enhanced TNF-α and IL-6 production upon stimulation with LPS. Furthermore, upon stimulation with *C. albicans*, enhanced production of TNF-α and reduced production of IL-10 was observed. However, IFN-γ and IL-1β production were also decreased upon stimulation with *C. albicans*. Stimulation with *S. aureus* also resulted in reduced expression of IFN-γ, IL-1β, and IL-10, which was also the case for IL-1β and IL-10 production upon stimulation with MTB.
DISCUSSION

In the present study, we investigated the capacity of BCG vaccination to modulate the immune response to subsequent vaccination with a trivalent influenza vaccine. We demonstrate that BCG vaccination not only modulates innate immune responses upon ex-vivo stimulation with unrelated pathogens, as previously reported (15, 16), but also enhances functional antibody responses against the pandemic H1N1 2009 strain induced by subsequent influenza vaccination, reflected by a more pronounced increase in antibody titres and a trend towards a more rapid seroconversion.

In addition to its effects on the severe clinical forms of tuberculosis, BCG vaccination also beneficially influences morbidity and mortality due to other infections (18). This is accompanied by non-specific stimulatory effects on the function of both myeloid and lymphoid cells (15, 19). These epidemiological and immunological data formed the basis of the hypothesis that BCG vaccination may also potentiate the function of antigen-presenting cells, and thus improve the response to other vaccines. This hypothesis is supported by the increase in the titres of neutralizing antibodies against the pandemic H1N1 2009 influenza strain, while a similar tendency was observed for the responses to an influenza B strain, especially in individuals with initial low antibody titres. A potentiating effect of BCG on the response to other vaccines is also supported by observational studies in infants, in which BCG increased heterologous responses to poliovirus vaccination (20), responses to anti-pneumococcus, anti-Haemophilus type B and anti-tetanus toxoid vaccines (21), and responses to hepatitis B vaccine (22).

BCG vaccination influenced both humoral and cellular responses to influenza vaccination. The magnitude and quality of antigen-specific antibody titres is considered to be the primary correlate of protection against most pathogens/viruses that infect the host through mucosal surfaces, such as influenza virus (23, 24). The effects of BCG-vaccination on antibody responses to subsequent influenza vaccination observed in this study demonstrate that the immunological history
effects the humoral immune response to subsequent infections/vaccinations in a clinically relevant manner. Moreover, a trend towards enhanced and more sustained IFN-γ production upon ex-vivo stimulation with influenza vaccine was also observed in the BCG-vaccinated group.

The percentage subjects who achieve a four-fold increase of baseline antibodies in this study is in line with the influenza vaccination-induced increase previously observed in healthy male volunteers (29). Not surprisingly, subjects with low baseline anti-influenza virus antibody titres displayed the strongest increase in antibody titres following influenza vaccination. Nevertheless, an increase in antibody titre was also observed in subjects with baseline antibody titres higher than 1:40 (26). Moreover, in these subjects, BCG-vaccination still exerted a potentiating effect on antibody responses. Earlier studies demonstrated that a progressive increase in protection is reached with increased titres, rather than that protection is attained above a discrete threshold that is applied to each individual (26). The increase in protection is particularly important for titres up to 1:160 (26), which applies to our study.

The reason for the significant effects of BCG vaccination on the response to the pandemic H1N1 2009 influenza strain, but not to the H3N2 strain, remains unknown. Previous studies have reported differential effects of BCG on different antigens from the same vaccination as well (21), but no clear mechanism has been proposed. The absence of BCG-induced effects on H3N2 antibody titres (see also below), could be explained from an immunological point-of-view. Previous work has shown differences in the immunopathology caused by pandemic and seasonal strains, related to the strain virulence and the localization of the inflammatory response (27, 28). In this respect, infections with the pandemic H1N1 strain result in expression of viral antigens in mucosal epithelial cells of the airways (from the nasopharynx to the bronchioles), but also in alveolar macrophages, and pneumocytes (27, 28). In contrast, infections with the H3N2 strain show viral antigens primarily in mucosal epithelial cells of the larger airways (27, 28). It is tempting to hypothesize that the potentiating effects of BCG on antibody responses against the H1N1pdm09 strain, but not against the A/H3N2 strain, may be due to differences in cells responsible for the recall response caused by the different localization of the primary infection, but this remains to be demonstrated by future studies.

In addition to the effects of antibody titers, BCG-vaccination also modulated the effects on cytokine production capacity induced by the influenza vaccination. Interestingly, our data indicate that trivalent influenza vaccine exerts non-specific effects as well, although different from those observed following BCG vaccination (15). BCG-vaccination exerted an overall immunostimulatory effect on the cytokine production modulated by influenza vaccine. In contrast, influenza-vaccination results in enhanced responses against certain pathogens, but impaired responses against others. These findings are in support of the hypothesis that infectious/vaccine history affects immune status in a clinically relevant manner.

This study has several limitations. Firstly, the relatively small sample size could be a reason for a type 2 error, resulting in the fact that some of the effects observed did not reach statistical significance, most importantly the enhanced antibody production against the B/2012 strain in the BCG-vaccinated subjects when stratified according to baseline antibody titres. Nevertheless, the significant effects on antibody titres to the pandemic strain and the ex-vivo cytokine responses illustrate the extent to which BCG-vaccination impacts on unrelated adaptive and innate immune responses. Secondly, although BCG vaccination potentiates antibody responses, humoral immunity is not the only mechanism involved in the protection against influenza infection. Upon vaccination, not only an adequate antibody response, but also generation of specific memory cytotoxic T lymphocytes (CTLs) contributes to protection against influenza virus (29). It has even been suggested that in the elderly CTL immune responses are better predictors of immunity than antibody titres (30, 31). We only studied IFN-γ responses as a surrogate of T-cell function, and direct CTL assays could be also considered in future studies to understand how BCG affects the response to influenza vaccination. Finally, we based our 14-day interval between BCG and influenza vaccination on previous studies in mice, in which viral challenges were performed 14 to 49 days after BCG vaccination (11), and in humans, in whom non-
specific effects of BCG were demonstrated ex-vivo 14 days after vaccination (15). However, there is little knowledge on the optimal timing of BCG vaccinations in the context of influenza virus vaccination and it is possible that a different time-interval between BCG and influenza vaccination could prove to be even more effective.

CONCLUSIONS

In the present study, we demonstrate that BCG vaccination followed by trivalent influenza vaccination significantly improves the magnitude and possibly also the swiftness of the antibody responses against the pandemic H1N1 2009 influenza virus in humans in vivo. In addition, this study validates the previously observations that vaccination exerts non-specific effects on cytokine responses against unrelated pathogens. In line with this, our data indicate that modular effects on innate immunity are not restricted to BCG, but that trivalent influenza vaccination also exerts non-specific effects on cytokine responses elicited by various pathogens. Overall, our data further support the concept that trained immunity effects on myeloid APCs can influence the specific response to other vaccines, and the hypothesis that vaccine history affects immune status in a clinically relevant manner. This is the first randomized trial showing that BCG can potentiate the responses to other vaccines. These results open the door to improve vaccination strategies in at-risk groups such as neonates or the elderly.

ACKNOWLEDGMENTS

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REFERENCES

Peripheral blood mononuclear cell (PBMC) isolation
Venous blood was drawn into EDTA tubes, after which peripheral blood mononuclear cells (PBMCs) were isolated as described previously (1). In short, blood was diluted in phosphate buffered saline (PBS) (1:1) and fractions were separated by Ficoll (Ficoll-Paque Plus, GE healthcare, Zeist, The Netherlands) density gradient centrifugation. Cells were washed twice with PBS and resuspended in RPMI-1640+ (RPMI-1640 Dutch modification supplemented with 10µg/mL gentamicin, 10mM L-glutamine, and 10mM pyruvate) (Gibco, Invitrogen, Breda, The Netherlands). PBMCs were counted using a particle counter (Beckmann Coulter, Woerden, The Netherlands) and were seeded in 96-well round-bottom plates (Corning, NY, USA) at a final concentration of 2.5x10^6/mL, in a total volume of 200 µL. PBMCs were stimulated for 24 hours (for measurement of TNF-α, IL-1β, and IL-6) and 48 hours (IL-10 and IFN-γ) with *E. coli* lipopolysaccharide (LPS; 10 ng/mL), heat-inactivated *Candida albicans* blastoconidia UC820 (1 x 10^6 microorganisms/mL), sonicated *Mycobacterium tuberculosis* (MTB) H37Rv (1 μg/mL), heat-killed *Staphylococcus aureus* (1 x 10^6 microorganisms/mL) or RPMI culture medium (medium controls). Furthermore, we stimulated PBMCs for 48 hours with medium alone, or medium containing live swine-origin influenza virus strain A/Netherlands/602/2009(H1N1) (2), at a multiplicity of infection (MOI) of 1 plaque forming unit (PFU) per cell. In separate wells, PBMCs were also stimulated with influenza vaccine (gathered from the same batch of trivalent influenza vaccine that was administered to the healthy volunteers) (1µg/ml, Batrevac; Abbot biologicals B.V., Weesp, the Netherlands). After incubation at 37°C and 5% CO₂, cell culture supernatants were collected and stored at -20°C. After all samples were collected, cytokines were measured batch-wise using commercially available ELISAs (R&D Systems, MN, USA and Sanquin, Amsterdam, The Netherlands) according to the protocols supplied by the manufacturer.

**Hemagglutination inhibition (HI) assay**
HI assays were performed according to standard procedures (3). Briefly, two-fold serial dilutions of the serum samples, starting at a 1:10 dilution, were mixed with...
25 µl of a virus stock containing four hemagglutinating units of the virus strains present in the vaccine, and were incubated at 37°C for 30 min. Subsequently, 25 µl of 1% turkey erythrocyte solution was added, and the mixture was incubated at 4°C for 1 h. The HI titre is expressed as the reciprocal value of the highest serum dilution that completely inhibited hemagglutination. Titres of less than 10 were recorded.

**REFERENCES**


**Supplementary Table 1.**

<table>
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<th>BCG</th>
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Difference in antibody titre within groups between visit 1 (day -14, before BCG vaccination) and visit 2 (day 0, before influenza vaccination) per serotype. Data are presented as median [IQR]. P-values were calculated using Wilcoxon matched-pairs tests.

**Supplementary Figure 1.** Antibody titres and percentage seroconversion over time for the pandemic H1N1 2009 strain, stratified by baseline antibody titre. Left panels: Geometric Mean Antibody Titres. To correct for baseline differences, baseline titres are plotted as 1, and subsequent time-points are plotted as fold changes of baseline values. Data are presented as median ± range. Right Panels: Percentage of subjects that attain seroconversion (defined as ≥4-fold titre increase compared with baseline) over time.
Supplementary Figure 2. Antibody titres and percentage seroconversion over time for the A/H3N2/2012 strain, stratified by baseline antibody titre. Left panels: Geometric Mean Antibody Titres. To correct for baseline differences, baseline titres are plotted as 1, and subsequent time-points are plotted as fold changes of baseline values. Data are presented as median ± range. Right Panels: Percentage of subjects that attain seroconversion (defined as ≥4-fold titre increase compared with baseline) over time.

Supplementary Figure 3. Antibody titres and percentage seroconversion over time for the B/2012 strain, stratified by baseline antibody titre. Left panels: Geometric Mean Antibody Titres. To correct for baseline differences, baseline titres are plotted as 1, and subsequent time-points are plotted as fold changes of baseline values. Data are presented as median ± range. Right Panels: Percentage of subjects that attain seroconversion (defined as ≥4-fold titre increase compared with baseline) over time.
Supplementary Figure 4. Panels A-C. Antibody titres and percentage seroconversion over time for pandemic H1N1 2009, A/H3N2/2012, and B/2012 strains in subjects with relatively low baseline antibody titres (arbitrarily set as the titre for which >25% of subjects in both groups attained seroconversion) that were vaccinated with BCG or placebo followed by influenza vaccination. Left panels: Geometric Mean Antibody Titres. To correct for baseline differences, baseline titres are plotted as 1, and subsequent time-points are plotted as fold changes of baseline values. p-values were calculated using Mann-Whitney U tests on Area under the curve (AUC) of subjects in both groups. Right Panels: Percentage of subjects that attain seroconversion (defined as ≥4-fold titre increase compared with baseline) over time. p-values were calculated using log-rank tests.
CHAPTER 9

The effects of orally administered Beta-glucan on innate immune responses in humans, a randomized open-label intervention pilot-study

Jenneke Leentjens
Jessica Quintin
Jelle Gerretsen
Matthijs Kox
Peter Pickkers
Mihai G. Netea
ABSTRACT

Rationale: To prevent or combat infection, increasing the effectiveness of the immune response is highly desirable, especially in case of compromised immune system function. However, immunostimulatory therapies are scarce, expensive, and often have unwanted side-effects. β-glucans have been shown to exert immunostimulatory effects in vitro and in vivo in experimental animal models. Oral β-glucan is inexpensive and well-tolerated, and therefore may represent a promising immunostimulatory compound for human use.

Methods: We performed a randomized open-label intervention pilot-study in 15 healthy male volunteers. Subjects were randomized to either the β-glucan (n=10) or the control group (n=5). Subjects in the β-glucan group ingested β-glucan 1000 mg once daily for 7 days. Blood was sampled at various time-points to determine β-glucan serum levels, perform ex vivo stimulation of leukocytes, and analyze microbicidal activity.

Results: β-glucan was barely detectable in serum of volunteers at all time-points. Furthermore, neither cytokine production nor microbicidal activity of leukocytes were affected by orally administered β-glucan.

Conclusion: The present study does not support the use of oral β-glucan to enhance innate immune responses in humans. This study was registered at ClinicalTrials.gov as NCT01727895.

INTRODUCTION

Defense mechanisms against invading pathogens are of vital importance to our survival. Therefore, to prevent or combat infection, increasing the effectiveness of the immune response is highly desirable. However, immunostimulatory therapies are scarce, expensive, and often have unwanted side-effects [1].

“Medicinal” mushrooms are used in alternative medicine throughout the world for their presumed enhancing effect on the immune system [2,3]. Although a number of fungal components have been implicated in these properties, β-glucans (naturally occurring carbohydrates) have attracted the most attention [4]. Since the early 1900s, numerous in vitro and animal studies have demonstrated immunostimulatory effects of β-glucans [5]. In addition, the advent of molecular immunology has provided rigorous mechanistic explanations for how humans recognize glucans and how this may influence the immune system [6]. β-glucan is already applied as a food additive in animal feed to enhance the immune response [7] and it is also widely offered on the internet as a dietary supplement for humans, advertised to have beneficial immunostimulatory effects. Due to the fact that it is inexpensive and well tolerated, oral β-glucan appears as a promising candidate to enhance the immune response. However, there are no studies to substantiate the putative immunostimulatory effects of orally administered β-glucan in humans. The only evidence of immunological effects of oral β-glucan in humans to date is derived from a study in patients with advanced breast cancer, in which oral β-glucans enhanced expression of surface molecules associated with macrophage proliferation and activation in peripheral blood mononuclear cells (PBMCs) [8].

In the present study we investigated the effects of a commercially available orally administered water-insoluble β-glucan on immune responses of ex vivo-stimulated leukocytes in healthy volunteers.
METHODS

Subjects
This study was registered at ClinicalTrials.gov as NCT01727895. After approval from the local Ethics Committee of the Radboud University Nijmegen Medical Centre, 15 healthy male volunteers gave written informed consent to participate in the experiments which took place from May 2013 until July 2013 (Figure 1). All experiments were in accordance with the declaration of Helsinki. Subjects were screened before the start of the experiment. Subjects with febrile illness during the two weeks before the experiment were excluded, and subjects were not allowed to take any prescription drugs. Throughout the study period, subjects documented their consumption of β-glucan-containing foods in a “food-diary”, and the amount of consumed β-glucan-containing foods was limited in order to minimize the inter-individual variability.

Study design
We performed an open-label, intervention pilot-study in 15 healthy human volunteers. The study design is depicted in Figure 2. Briefly, subjects were randomized to either the β-glucan group (n=10) or the control group (n=5) using the sealed envelope method. Subjects in the β-glucan group ingested β-glucan (water-insoluble β-glucan derived from bakers yeast [S. cerevisiae] sold as a dietary supplement [Glucan #300, BG; Biothera, Eagan, Minnesota, USA, for Transferpoint, Columbia, USA, hereafter designated as BG]) 1000 mg once daily as recommended by the manufacturer, for 7 days. This preparation, has a purity of at least 83% guaranteed by the manufacturer. Before the first BG ingestion (on day 0) as well as 3, 6, and 24 hours afterwards, subjects came to the hospital for blood sampling and reporting of side-effects. This sampling schedule was repeated on day 6 (before and after the seventh BG ingestion). A final single sampling time-point took place on day 20. The subjects in the control group (n=5) did not take BG, but the sampling schedule was otherwise identical.

Cytokine measurements
Venous blood was drawn into EDTA tubes, after which peripheral blood mononuclear cells (PBMCs) were isolated as described previously [9]. In short, blood was diluted in phosphate buffered saline (PBS) (1:1) and fractions were separated by Ficoll (Ficoll-Paque Plus, GE healthcare, Zeist, The Netherlands) density gradient centrifugation. Cells were washed twice with PBS and
resuspended in RPMI-1640+ (RPMI-1640 Dutch modification supplemented with 10µg/mL gentamicin, 10mM L-glutamine, and 10mM pyruvate) (Gibco, Invitrogen, Breda, The Netherlands). PBMCs were counted using a particle counter (Beckmann Coulter, Woerden, The Netherlands) and were plated in 96 well round-bottom plates (Corning, NY, USA) at a final concentration of 2.5x10⁶/mL, in a total volume of 200 µL. The PBMCs were stimulated for 24 hours, 48 hours, and 7 days with medium alone, or medium containing E. coli lipopolysaccharide (LPS; 10 ng/mL), heat-inactivated Candida albicans blastoconidia UC820 (1 x 10⁶ microorganisms/mL), Pam3Cys 1µg/mL (EMC Microcollections), sonicated mycobacterium tuberculosis (MTB) H37Rv (1 µg/mL), poly(I:C) 50 µg/mL (Invivogen), S. aureus (1 x 10⁷ microorganisms/mL), antiCD3/antiCD28 2.5x10⁵ beads/well (Miltenyi Biotec). After stimulation, cell culture supernatant was collected and stored at -20°C. When all samples were collected, cytokines were measured using commercially available ELISAs (R&D Systems, MN, USA and Sanquin, Amsterdam, The Netherlands) according to the protocols supplied by the manufacturer.

**Microbicidal activity assay**

Microbicidal activity assay was performed as previously described, using the fungal microorganism Candida albicans as a model pathogen [10]. Briefly, C. albicans UC820 yeast suspension was incubated with PBMCs isolated from the volunteers at day 0 (t=0) and day 6 (t=0) at a MOI of 1:5 or 1:50 in RPMI in a 96 wells plate. C. albicans UC820 suspension was obtained from an overnight culture in 25ml liquid Sabouraud at 29°C. The C. albicans solution was washed three times with PBS, and the number of yeast cells counted in a hemacytometer. C. albicans were then incubated with PBMCs or no cells (control well) for 5 hours at 37°C. The amount of C. albicans added to the wells was determined by plating serial dilutions on Sabouraud plates in duplo. After a 5–hour incubation, the content of each well was recovered in sterile water and serially diluted. Serial dilutions were plated on Sabouraud plates in duplo. After 24 hours at 29°C, the CFU were counted. The candidacidal activity was calculated as a ratio of Candida growth with PBMCs vs. control wells (Candida alone, no cells).

**RESULTS**

**Demographic characteristics**

Demographic characteristics of the study population are listed in Table 1. There were no significant differences in baseline characteristics between the two study groups. No serious adverse events occurred during the trial. BG intake was well tolerated, with no side effects reported besides one subject who reported a flare of acne one week after discontinuation of BG for which he contacted his general practitioner.

<table>
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<th>Control (n=5)</th>
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</table>

BMI: body mass index. Data are presented as median and interquartile range. P-values calculated using Mann-Whitney U-tests.

β-glucan levels were determined for all subjects on several time points using the Fungitell kit (Associates of Cape Cod, Inc., Cape Cod, MA), according to the manufacturer’s instructions [11]. Lower and upper detection limits were 31.25 and 500 pg/mL, respectively.

**Calculations and statistical analysis**

Distribution of data was determined using Kolmogorov-Smirnov tests. Demographic data were analyzed using Mann-Whitney U tests. Comparisons between the two groups over time were made using repeated measures two-way analysis of variance (ANOVA, interaction term). Ex vivo stimulation cytokine data were log-transformed to obtain a normal distribution. A p-value of <0.05 was considered statistically significant. Calculations and statistical analyses were performed using Graphpad Prism version 5.0 (Graphpad Software, San Diego, CA, USA).
(1→3)-β-D-glucan serum levels
Following oral administration, β-glucan was barely detectable in serum of volunteers at all time-points, and this was not significantly different from the β-glucan levels observed in the placebo group (data not shown).

Cytokine production by ex vivo-stimulated PBMCs
The production of the archetypal proinflammatory cytokine tumor necrosis factor (TNF-α) after incubation with various stimuli at different time points showed considerable interindividual variation, but it was not affected by β-glucan intake (Figure 3). Furthermore, no relevant differences in TNF-α production compared with the control group were observed. Likewise, no effects of BG intake within the β-glucan group or between the β-glucan group and the control group were observed with regard to the production of interleukin (IL)-6, IL-1β, IL-10, Interferon (IFN)-γ, IL-17 and IL-22 (Figure S1, Panel A-D, in the online supplement).

Microbicidal activity
We assessed the candidacidal properties of PBMCs isolated from volunteers before and 6 days after the administration of β-glucan, and at the same time-points in the control group. No significant differences could be observed in candidacidal activity in both groups, using multiplicity of infection (MOI) of either 1:50 or 1:5 (Figure 4).

Figure 3. Effect of oral β-glucan on ex vivo TNF-α production by PBMCs stimulated for 24 hours with lipopolysaccharide (LPS), Pam3Cys, Poly(I:C), S. aureus, C. albicans, or M. tuberculosis (MTB). To correct for possible baseline differences between groups, concentrations at day 0 are set at 1, and concentrations at subsequent time-points are plotted as ratios (median and interquartile range). Baseline (day 0) TNF-α concentrations in pg/ml [interquartile range] of subjects in the β-glucan and control groups were: LPS: 691 [276-2649] and 749 [287-1160], Pam3Cys: 855 [590-1727] and 1242 [622-3567], Poly(I:C): 107 [78-399] and 125 [78-250], S. aureus: 21948 [14347-41751] and 19424 [12140-31722], C. albicans: 10875 [5860-14159] and 6208 [3924-14943], and MTB: 424 [285-1980] and 1153 [218-2461]. P values between groups were calculated using repeated measures two-way analysis of variance (ANOVA, interaction term) on log transformed data.
the class of most active β-glucans. In addition, in an evaluation of four different orally administered commercially available β-glucans in mice, BG was shown to be the most potent in increasing phagocytosis capacity of peripheral blood cells and IL-2 production of splenic cells [10]. Based on these data, and because BG is rated ‘GRAS’ (Generally Recognized As Safe) by the Food and Drug Administration (FDA) as a dietary food additive supplement [14], and safe and non-toxic by the Dutch Medicines Evaluation Board (MEB) [15], we deemed this preparation of BG representative of commercially available oral β-glucans. Because of the complete absence of any effect on cytokine production or microbicidal activity, although investigated in a small number of subjects for only a limited period, we conclude that BG or similar glucan products do not appear to be viable approaches for immunostimulation in humans.

The lack of immunostimulatory effects of β-glucan in our study could have several reasons. First, it could be due to the absence of adequate absorption of β-glucans from the intestinal tract in healthy volunteers, which is in agreement with the undetectable serum levels found in various other studies showing that enteral administration of insoluble β-glucans from different sources result in low systemic blood levels (less than 0.5%) in mice [16], and weaned pigs [17]. Nevertheless, despite these low systemic blood levels, significant systemic immunomodulating effects in terms of humoral and cellular immune responses were demonstrated. It was speculated that these effects are exerted by enterocytes that facilitate the transportation of β-glucans and similar compounds across the intestinal cell wall into the lymph fluid, where they interact with macrophages and thereby activate the immune system despite low serum levels [18]. Given the lack of effects of BG in our study, it is unlikely that these local effects also take place to a significant degree in humans.

A second explanation might be related to the water solubility of β-glucans, because previous studies have demonstrated that water soluble β-glucans interact with the immune system differently compared with their insoluble counterparts. Soluble β-glucans activate the immune system in a complement mediated manner, depending on specific antibodies [19], while insoluble β-glucans, like BG used in this study, activate both the innate and the adaptive immune responses...
CONCLUSION

Despite promising results obtained in *in vitro* and animal studies, our study demonstrates that the use of oral β-glucan does not enhance the responsiveness of the immune system in humans. Future efforts should concentrate on assessing the immunological and clinical effects of intravenous β-glucan preparations.

ACKNOWLEDGEMENTS

M.G.N. was supported by a Vici grant of the Netherlands Organization for Scientific Research.

via the dectin-1 receptor pathway [19,20]. As such, it has been suggested that soluble β-glucans possess less biological activity than their insoluble counterparts. Therefore, although we did not investigate a soluble formula, it is unlikely that orally administered water soluble β-glucans will have a more pronounced immunostimulatory effect in humans than the BG used in this study. However, future studies are warranted to assess this aspect.

Third, the lack of effects might be related to the fact we used a commercially available β-glucan preparation sold as a dietary supplement, with a relatively modest purity of at least 83%. It is possible that a highly purified pharmaceutical preparation optimized for oral delivery could have immunostimulatory effects in humans.

Finally, we only investigated one dose of BG, based on the recommendation of the manufacturer, which is not supported by empirical data. Nevertheless, this dose is similar to that used in two previous studies, where it was demonstrated that oral ingestion of 900 mg water-insoluble β-glucan daily for 16 or 26 weeks reduced the incidence in common cold episodes during the cold season [21,22]. Of note, endpoints in both studies were based on questionnaires, no immunological endpoints were assessed. Also, the dose used in the present study is higher than the dose rated ‘GRAS’ by the FDA and MEB (375 mg per day) [14] [15], which was based on the mean background intake of β-glucans from other dietary sources.

While our study does not support use of oral β-glucan to enhance the immune response, it does not exclude that parental administration of β-glucan, as currently performed in clinical trials (e.g. NCT01269385, NCT00002099), could represent a valuable immunostimulatory therapy. It has been reported that β-glucan from *S. cerevisiae* administered intravenously to high-risk surgical patients resulted in decreased infection incidence, reduced need for antibiotics, shortened intensive care unit length stay, and ultimately improved survival compared with placebo [23]. In addition, several clinical trials in East Asia have proposed immunomodulatory and antioncogenic effects of β-glucan in cancer patients [24-26]. Larger studies are warranted to confirm these data, investigate the biological mechanisms though which effects are induced, and to fully explore the therapeutic potential of β-glucans.
REFERENCES


7. www.myfreeequine.com/betaglucan.htm


14. FDA. http://www.fda.gov/Food/FOODenovels/General/GRAS/gottabeta-d-glucan.htm


Supplementary Figure 1A. Effect of oral β-glucan on *ex vivo* IL-6 production by PBMCs stimulated for 24 hours with lipopolysaccharide (LPS), Pam3Cys, Poly(I:C), *S. aureus*, *C. albicans*, or *M. tuberculosis* (MTB).

To correct for possible baseline differences between groups, concentrations at day 0 are set at 1, and concentrations at subsequent time-points are plotted as ratios (median and interquartile range). Baseline (day 0) IL-6 concentrations in pg/ml [interquartile range] of subjects in the β-glucan and control groups were: LPS: 22862 [25882-12146] and 24536 [39860-17357], Pam3Cys: 32933 [59532-40943] and 12298 [59532-22042], Poly(I:C): 5908 [1668-8228] and 937 [468-2964], *S. aureus*: 19277 [12123-2401] and 12585 [8093-2036], *C. albicans*: 12120 [8899-17594] and 8244 [5268-10417], and MTB: 21977 [10416-3546] and 12470 [12232-1556]. P values between groups were calculated using repeated measures two-way analysis of variance (ANOVA, interaction term) on log transformed data.

Supplementary Figure 1B. Effect of oral β-glucan on *ex vivo* IL-β production by PBMCs stimulated for 24 hours with lipopolysaccharide (LPS), Pam3Cys, Poly(I:C), *S. aureus*, *C. albicans*, or *M. tuberculosis* (MTB).

To correct for possible baseline differences between groups, concentrations at day 0 are set at 1, and concentrations at subsequent time-points are plotted as ratios (median and interquartile range). Baseline (day 0) IL-β concentrations in pg/ml [interquartile range] of subjects in the β-glucan and control groups were: LPS: 5600 [2604-11284] and 4061 [2322-10436], Pam3Cys: 9295 [3688-11162] and 5189 [3303-6686], Poly(I:C): 233 [39-412] and 142 [39-266], *S. aureus*: 24141 [20536-29528] and 15456 [14697-24582], *C. albicans*: 6975 [5327-8954] and 6187 [3832-8224], and MTB: 2574 [1680-7120] and 3313 [1211-6465]. P values between groups were calculated using repeated measures two-way analysis of variance (ANOVA, interaction term) on log transformed data.
Supplementary Figure 1C. Effect of oral β-glucan on ex vivo Interleukin-10 and Interferon-gamma (IFN) production by PBMCs stimulated for 7 days with S. aureus, C. albicans, or anti-CD3CD28. To correct for possible baseline differences between groups, concentrations at day 0 are set at 1, and concentrations at subsequent time-points are plotted as ratios (median and interquartile range). Baseline (day 0) IL-10 concentrations in pg/ml [interquartile range] of subjects in the β-glucan and control groups were: S. Aureus: 245 [116-470] and 200 [70-563], C. Albicans: 885 [606-1621] and 940 [720-1365], and anti-CD3CD28: 488 [219-580] and 360 [235-1268]. Baseline (day 0) IL-22 concentrations in pg/ml [interquartile range] of subjects in the β-glucan and control groups were: S. Aureus: 2620 [1089-12670] and 6145 [2140-9165], C. Albicans: 2730 [1694-9335] and 6875 [5588-10440], and anti-CD3CD28: 250 [147-1623] and 1175 [383-1225]. P values between groups were calculated using repeated measures two-way analysis of variance (ANOVA, interaction term) on log transformed data.

Supplementary Figure 1D. Effect of oral β-glucan on ex vivo Interleukin-17 and -22 production by PBMCs stimulated for 7 days with S. aureus, C. albicans, or anti-CD3CD28. To correct for possible baseline differences between groups, concentrations at day 0 are set at 1, and concentrations at subsequent time-points are plotted as ratios (median and interquartile range). Baseline (day 0) IL-17 concentrations in pg/ml [interquartile range] of subjects in the β-glucan and control groups were: S. Aureus: 245 [116-470] and 200 [70-563], C. Albicans: 885 [606-1621] and 940 [720-1365], and anti-CD3CD28: 488 [219-580] and 360 [235-1268]. Baseline (day 0) IL-22 concentrations in pg/ml [interquartile range] of subjects in the β-glucan and control groups were: S. Aureus: 2620 [1089-12670] and 6145 [2140-9165], C. Albicans: 2730 [1694-9335] and 6875 [5588-10440], and anti-CD3CD28: 250 [147-1623] and 1175 [383-1225]. P values between groups were calculated using repeated measures two-way analysis of variance (ANOVA, interaction term) on log transformed data.
SUMMARY

In this thesis, preclinical, translational and clinical studies investigating immunomodulatory therapies for the adjunctive treatment of life-threatening infectious diseases are described. We focused on the effects of the well-known immunostimulatory compounds Interferon-γ (IFN-γ) and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) as adjunctive treatment of sepsis, and IFN-γ as adjunctive treatment of invasive opportunistic infections. In addition, the effects of new immunomodulatory approaches based on the recently discovered concept of “trained innate immunity” (memory of the innate immune system), were explored in humans.

In chapter 1 a short introduction on the innate immune system with focus on the pathophysiology of infectious disease-specific immune dysfunction is given. First, opportunistic infections in patients with a pre-existing suppressed immune system are discussed, followed by an introduction in secondary infections in patients with sepsis-induced “immunoparalysis”. Finally, the rationale behind the immune stimulatory compounds used in this thesis is provided.

PART ONE: IMMUNOMODULATION AS ADJUNCTIVE TREATMENT FOR SEPSIS

Chapter 2 provides an overview of the pathophysiology of sepsis, with a focus on recent insights in immunosuppressive mechanisms that play an important role. Despite advances in medical care, sepsis remains a major cause of death worldwide. In recent years it has become clear that most septic patients do not die from an overwhelming initial pro-inflammatory immune response, but in the subsequent immunosuppressive phase, called “immunoparalysis”, which is characterized by a compromised ability to clear the initial infection, and increased susceptibility to secondary and opportunistic infections. Although infection control and supportive therapies, especially in the early phase of sepsis, will remain the cornerstone of sepsis treatment, the discovery
of immunoparalysis and its detrimental consequences is currently causing a profound shift in the sepsis research field. Hitherto, research related to treatment for sepsis predominantly focused on suppression of the immune system (for example with anti-cytokine and anti-Toll-like receptor therapies), but more recently, sepsis research increasingly focuses on immunostimulatory treatment options. In this chapter an overview of mechanisms behind sepsis-induced immunoparalysis and possible markers to identify immunoparalyzed patients is provided (including the most studied markers HLA-DR expression on monocytes (mHLA-DR), and ex vivo leukocyte responses). In addition, the few studies that have evaluated immunostimulatory therapies in sepsis are discussed, and new compounds that seem promising candidates to reverse sepsis-induced immunoparalysis are proposed.

In chapter 3 the effects of IFN-γ and GM-CSF on immunoparalysis in vivo in humans are described. Repeated administration of endotoxin to healthy volunteers was used to mimic sepsis-induced immunoparalysis and it was demonstrated that a second endotoxin administration a week after the first results in a profoundly suppressed innate immune response. This indicates that this approach represents a suitable model for sepsis-induced immunoparalysis and, importantly, provides a timeframe to administer immunostimulatory therapy in between endotoxin administrations. In a few small studies it was already demonstrated that both IFN-γ and GM-CSF are able to enhance ex vivo immune responses of septic patients. However, using the repeated human endotoxin model it was already demonstrated that ex vivo measurements do not necessarily reflect the in vivo immune status. We demonstrated that, compared with placebo, treatment with IFN-γ in between endotoxin administrations not only increased expression of the well-known immunoparalysis marker mHLA-DR, but indeed also restored in vivo production of the pro-inflammatory cytokine TNF-α, while further attenuating the production of the anti-inflammatory cytokine IL-10 upon the second endotoxin administration. Similar, but less pronounced, effects were found in subjects treated with GM-CSF, thereby demonstrating that immunoparalysis in vivo in humans can be partially reversed by IFN-γ and, to a lesser extent, by GM-CSF.

PART TWO: IMMUNOMODULATION AS ADJUNCTIVE TREATMENT FOR OPPORTUNISTIC INFECTIONS

Because most patients suffering from opportunistic infections have an impaired immune system, albeit to varying degrees, adjunctive immunotherapy to improve host defence is theoretically an attractive strategy to improve patient’s outcome. In chapter 4 we describe a case series of patients with invasive fungal infections in which we demonstrate that adjunctive immunotherapy with IFN-γ improves leukocyte immune responses ex vivo in these patients. This was reflected by increased production of innate pro-inflammatory cytokines, such as IL-1β or TNF-α, as well as increased production of T-cell cytokines IL-17 and IL-22, which are known to play an important role in the anti-fungal host defence. Furthermore, IFN-γ treatment increased mHLA-DR expression in a subset of severely immunosuppressed patients with very low baseline mHLA-DR expression. These data indicate that adjunctive immunotherapy with IFN-γ in patients with invasive fungal infections partially restores cell-mediated immunity, and suggest that IFN-γ treatment can enhance anti-fungal immunity and thereby may improve outcome of these patients.

Chapters 5 and 6 describe the effects of adjunctive last-resort immunotherapy with IFN-γ in a pediatric patient with progressive invasive Candida infection after remission-induction chemotherapy for Acute Lymphoblastic Leukemia, and in a patient with cerebral Nocardiosis. Both patients deteriorated despite optimal antimicrobial treatment. IFN-γ therapy resulted in augmentation of the innate immune response in both patients, and was associated with clinical and radiographic recovery. In the absence of randomized clinical trials, these case reports suggest that in patients with invasive opportunistic infections, who deteriorate despite optimal antimicrobial treatment, immunotherapy with IFN-γ could be considered as adjuvant salvage therapy.
PART THREE: NEW IMMUNOMODULATORY APPROACHES

Non-specific effects of BCG-vaccination were long ignored, and only in the last decade interest in these non-specific effects revitalized. Several randomized studies demonstrated reduced mortality in early BCG-vaccinated neonates (which could not be explained by TBC prevention, as TBC is a rare cause of death in neonates) (1-5). Additionally, it was demonstrated in vitro and ex vivo that BCG provides protection against unrelated infections later on. Moreover, experimental studies suggested that these unrelated beneficial effects were independent of B- and T-cells, and consequently based on “training” of the innate immune responses. However, the immune modulating effects of BCG vaccination in humans have hitherto only been shown ex vivo using stimuli of bacterial and fungal origin. In chapter 7 we describe that prior vaccination with live attenuated BCG leads results in a more swift and pronounced induction of antibodies following administration of trivalent influenza vaccine in healthy volunteers. In addition, we confirmed the non-specific effects of BCG on cytokine production upon ex vivo stimulation of PBMC’s with non-related pathogens, and demonstrated that trivalent influenza vaccination also exerts non-specific effects on cytokine responses elicited by various pathogens. Overall, our data further support the hypothesis that vaccination history of the host affects the immune status in a clinically relevant manner, and as such, it opens up new avenues towards improved vaccination strategies. Because live attenuated BCG is not a treatment option in patients with impaired immune functions due to the associated risk of dissemination, we investigated the trained-immunity effects of gamma-irradiated (and thus inactivated) BCG. However, in chapter 8 we demonstrate that gamma-irradiated BCG does not induce trained immunity ex vivo. Furthermore, it does not potentiate the innate immune response in vivo during immunoparalysis induced by repeated human entotoxemia. This is likely due to the inactivation of the vaccine. Therefore, Gamma-irradiated BCG is unlikely to represent a possible treatment option for the reversal of sepsis-induced immunoparalysis.

In addition to BCG, Beta-glucan has also been demonstrated to induce trained immunity in vitro and in vivo in animals. Furthermore, oral Beta-glucan is widely offered on the internet as a dietary supplement for humans claiming to improve the immune system. However, its immunostimulatory effects in humans have never been investigated. In chapter 9 we describe that daily intake of 1000 mg commercially available oral Beta-glucan for 7 days does not result in detectable serum Beta-glucan levels in healthy human volunteers, and that it does not modulate the innate immune response, as reflected by leukocyte-derived cytokine production upon ex vivo stimulation with various pathogenic stimuli and microbicidal activity. We therefore cannot substantiate the putative immunostimulatory effects of commercially available orally administered Beta-glucan.
CHAPTER 11

General discussion and future perspectives
GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The backbone of infectious disease management consists of source control, through administration of antimicrobial/antifungal/antiviral therapy and surgical removal of infected tissue if necessary. However, despite advances in medical care, infectious diseases still account for unacceptably high mortality worldwide (6), and the ever increasing resistance to the currently available antimicrobial therapies emphasizes the need for novel approaches to treat infectious diseases. Because a well functioning immune system is of primordial importance for the adequate eradication of invading pathogens (7), increasing the effectiveness of the immune response is a very attractive strategy to improve outcome. This is especially true for patients with a compromised immune system, such as those suffering from opportunistic infections and sepsis-induced immunoparalysis. However, immune stimulation as adjunctive treatment for infectious diseases is only in its infancy. As evidenced in this thesis, the currently available immunostimulatory therapies IFN-γ and GM-CSF appear promising strategies to potentiate the immune system of these patients. However, these therapies are expensive, have potentially serious side effects, and might not always be effective. Furthermore, new molecular and transcriptional pathways in the context of disease pathogenesis are constantly being discovered. Therefore, novel immunomodulatory therapies are highly warranted.

In this final chapter, we discuss the findings described in this thesis, also in light of recent studies in the field that were performed during the conduct of our own research, present future perspectives, and draw conclusions.

Immunoparalysis in sepsis

In the first part of this thesis, we presented an overview of recent insights in the pathophysiology of sepsis with a focus on sepsis-induced immunoparalysis, which plays an important role in the outcome of these patients. Furthermore, we demonstrated that IFN-γ is able to partially restore immunocompetence in a model for sepsis-induced immunoparalysis in humans in vivo.
The clinical relevance of sepsis-induced immunoparalysis is illustrated by an observational study in surgical patients who died from sepsis or septic shock (10). In 63 of 71 patients (89%) who were treated with antibiotics for more than 7 days a continuous septic focus was found, illustrating that these patients were unable to eradicate their infection (10). Furthermore, other reports have shown that during the late phase of sepsis, infections due to opportunistic bacteria increase from 9 to 18%, and *Candida*-infections from 13 to 30% (11). Also, many pathogens responsible for nosocomial sepsis in ICUs are weakly virulent opportunistic pathogens usually isolated only from severely immunodepressed patients (12). Further evidence for a severely immunosuppressed state is derived from a recent study that reported reactivation of latent viruses in 43% of 560 sepsis patients (13). Of note, Epstein Barr virus (EBV) and cytomegalovirus (CMV) detection in sepsis patients was similar to those reported in stem cell and organ transplant patients (13). However, there is still much debate about the clinical relevance of immunoparalysis in septic patients (14-16), and many clinicians argue that they do not recognize it to be a relevant problem in their septic patient population. These doubts are further substantiated by the fact that a causative link or mechanism between immunoparalysis and the development of organ damage and lethality has not yet been established.

Of interest, very recent studies provide the first evidence that could link immunoparalysis and cell dysfunction leading to multi-organ failure in sepsis. It was demonstrated that impaired bioenergetics and mitochondrial dysfunction, leading to ATP depletion, may be an important feature of sepsis-induced immunoparalysis (17-21), that could also contribute to organ dysfunction (19). A decrease in metabolic activity reduces energy requirements and result in a new hibernating steady-state in which the cell does not longer perform its specialized function but at the same time does not allow ATP to decrease to levels that trigger cell death. Such a decrease in cell functionality, if sufficiently severe, will be manifest as decreased functionality of organs, which may represent a mechanism to cope with a severe and/or prolonged inflammatory insult, but will eventually lead to the death of the patient (22). Interestingly, every hallmark of failed cellular homeostasis (e.g. inadequate contraction of myocardial cells [myocardial depression]; impaired constriction of vascular smooth muscle cells [hypotension]; failure of the cell-cell...
Independent of the impaired bioenergetics hypothesis proposed above, the initial excessive degree of inflammation in response to the infectious insult remains the obvious trigger for these and other downstream pathways, and, in selected cases, is responsible for distinct collateral damage (e.g. in meningococcal sepsis or infectious diseases causing macrophage activation syndromes such as dengue (25), severe influenza infections (26), malaria (27)). In addition, the impaired immune signaling and effector functions described in chapter 2 of this thesis are a clear hallmark of sepsis. Therefore, finding ways to orchestrate the inflammatory response in a tailored fashion could be of great therapeutic value: potentiation when necessary to eliminate microorganisms, dampening in case of potential collateral tissue damage. Hence, accurate biomarkers that can identify a patient's immune status are of primordial importance for the future of immunotherapy in sepsis. Biomarkers may serve both as a means to select patients eligible for immunomodulatory therapies, as well as for monitoring the response to treatment. Furthermore, biomarkers may identify high risk patients that are potentially eligible for therapies associated with higher risks, or to exclude patients who are anticipated to have a good clinical outcome with standard therapy. The goal of stratification would thus be to target a distinct biological process, while at the same time maximizing the benefit-risk ratio. In order to achieve routine clinical applicability, an accurate biomarker should provide reliable and reproducible results within a short time-frame and at low costs. HLA-DR expression on monocytes measured by flow cytometry, and *ex vivo* leukocyte responses to LPS, both discussed in chapter 2 of this thesis, currently represent the most widely used markers for immunoparalysis (28-30). However, they reflect only the immune status of the blood compartment, and pre-analytical and analytical issues inherent to these markers limit their use in large multicentered clinical studies and/or on a routine basis. There is hope that improved molecular diagnostics and prognostics based on a systems biology approach (combining genomic, transcriptomic, metabolomic, proteomic, and/or epigenetic data), will eventually result in new diagnostic tools, although up till now, these are largely unstudied in the context of sepsis research (31-34).

Until then, clinical trials investigating immune modulatory therapies should stratify sepsis patients according to their immune status using the tools currently available.
Immunomodulation in opportunistic infections

In the second part of this thesis we demonstrated that IFN-γ immunotherapy is able to enhance mHLA-DR expression and/or ex vivo leukocyte responses, and very likely contributed to clinical and radiographic recovery, in patients with invasive opportunistic infections.

In contrast with the earlier described sepsis patients who can either suffer from a too pronounced immune response or a severely suppressed one, non-septic patients suffering from non-resolving opportunistic infections represent a more uniform group in terms of their immune response as these infections are virtually always related to impaired host defense mechanisms. Nevertheless, although most patients are likely to benefit of adjunctive therapy, similar to sepsis, there is no established marker that can be used to identify patients who would benefit or not of adjunctive therapy, or that can be used to monitor treatment responses. In chapter 4 and 5 of this thesis we described for the first time that mHLA-DR may serve this purpose as mHLA-DR expression levels inversely correlated with disease severity. In these patients, immunoparalysis was illustrated by very low expression of mHLA-DR at baseline, while this significantly increased after initiation of IFN-γ therapy. Although these results from case series/studies are promising, larger clinical trials are warranted to confirm our results. Also, the pitfalls of these markers described before should also be taken into account in this patient group. Nevertheless, in both patients described in chapter 5 and 6 of this thesis, monitoring of the immune responses by mHLA-DR expression and/or ex-vivo cytokine responses proved to be of great value in the guidance of immunomodulatory treatment. Similar to sepsis, this biomarker-guided approach should be applied in future larger (multicentre) clinical trials powered on both hard clinical endpoints and immunological effects to substantiate the future of adjunctive immunotherapy in invasive opportunistic infections. National and international collaboration between infectious-disease specialists, intensivists, oncologists and translational immunologists will be required, as studies in these patients are clearly challenging.
The results described in chapter 7 are the first non-observational data that show that BCG vaccination has immune modulating effects against unrelated pathogens in vivo in humans. Moreover, the fact that trivalent influenza vaccination also exerts non-specific effects on cytokine responses against several other pathogens indicates that modulation of innate immune parameters is not restricted to BCG. This makes sense from an evolutionary perspective, but also underscores that trained immunity completely changes the dogma of innate immunity lacking any form of adaptation. These data implicate that all vaccinations (and probably also all infectious diseases encountered during lifetime) will affect the immune status in a non-specific manner. However, the concept of trained immunity has only recently been proposed, and as such, the underlying mechanisms remain to be fully elucidated. Recently, it has been demonstrated that epigenetic reprogramming plays a central role not only in the induction of trained immunity resulting in an enhanced immunological phenotype, but also in the induction of LPS-tolerance, which can be regarded as the opposite (33). As described in chapter 2 of this thesis, epigenetic regulation of gene transcription occurs through several mechanisms but can be regarded as the regulated organization of gene loci into transcriptionally active or silent states in which H3K4 methylation and H3K27 acetylation play an important role (Figure 2).

New immunomodulatory approaches

In the final part of this thesis we demonstrated that live attenuated BCG vaccination resulted in enhanced antibody responses induced by administration of trivalent influenza vaccine, and that trivalent influenza vaccine itself also exerts non-specific effects on cytokine responses against several other pathogens. Furthermore, we demonstrated that vaccination with gamma-irradiated (and thus inactivated) BCG, and oral administration of commercially available Beta-glucan, did not induce training of the innate immune system.

Of interest, for patients with invasive fungal infections, improvements in the field of molecular diagnostics and prognostics based on a systems biology approach already resulted in important discoveries. For instance, the type I IFN pathway was identified as the most prominent Candida-specific transcriptional response, and Candida-induced type I IFN induction modulates Th1/Th17 responses in vitro, resulting in increased IFN-γ production while IL-17 production is suppressed (42). Moreover, in the first genome-wide association study (GWAS) assessing genetic susceptibility to candidaemia (42), three novel risk factors for susceptibility to candidaemia were identified. Carrying two or more risk alleles from these loci increases the risk for candidaemia by 19-fold. In addition, the unexpected identification of CD8 as an important risk factor for candidaemia resulted in the discovery of its role in inhibiting Candida germination at the level of the phagosome and involvement of Candida-induced TNF-α production (42). These findings illustrate the importance of systems biology approaches, even in smaller patient groups. Using this approach, screening strategies based on SNPs to identify patients at risk who could benefit from prophylactic treatment can be developed. Furthermore, it will provide more insight into immunological pathways and identify new pathways that can contribute to a better understanding of host defenses (including the mechanism of action of IFN-γ). Taken together, a systems biology approach represents a powerful tool for the discovery of more accurate immune status biomarkers as well as for the design of future immunotherapeutic strategies.

Figure 2. Schematic overview of epigenetic reprogramming underlying trained immunity. BCG, Bacille Calmette-Guérin; H3-K4me3, H3K4 trimethylation. Copyright by Siroon Bekkering.
In addition, it has recently been demonstrated that autophagy plays a central role in BCG-induced trained immunity (43). However, signalling and molecular mechanisms responsible for the induction of trained immunity remain subject of future studies, and molecular mechanisms through which autophagy is related to epigenetic changes remain subject of future studies. Moreover, the extent to which trained immunity affects immune responses (e.g. the nature of effects of a given vaccine against specific pathogens) and the optimal timing for therapeutic use (the timeframe, but also the sequence of vaccine administration could be important in order to achieve the intended effect), are unknown. In addition, in addition to the age-specific aspects regarding the immunological response to vaccines discussed in chapter 7, there are studies indicating that non-specific effects of vaccines are also gender-specific (44, 45). All these aspects have to be unravelled before we can fully understand to which extent trained immunity will affect daily clinical practice. The proof-of-principle data discussed in chapter 7 of this thesis suggest that (the cost-effectiveness of) vaccination strategies could be greatly improved, and that vaccination therapy could also be used for the prevention of non-related infectious diseases.

As such, this concept could have a major impact on public health. For instance, the combination of BCG and influenza vaccination could reduce the dose influenza vaccine needed to mount adequate antibody responses, which could save many lives in case of a new influenza pandemic, as the amount of influenza vaccine available is limited. Larger randomized clinical trials are warranted to investigate these BCG-influenza specific effects in which the aforementioned age- and gender-specific differences should also be taken into account. Thereto, (non-specific) trained immunity effects could also be employed for the adjunctive treatment of diseases associated with primary or secondary immunodeficiencies. However, in these patients only compounds that are not associated with risk of dissemination (as is the case for live attenuated BCG) can be used. The lack of effects of gamma irradiated (thus inactivated) BCG vaccination concerning the induction of trained immunity in vivo or ex vivo indicates that both live attenuated and inactivated BCG vaccination do not represent viable treatment options for the reversal of sepsis-induced immunoparalysis. For this patient group, future studies should focus on other immunostimulatory compounds.

The lack of any effects of orally administered commercially available Beta-glucan on ex vivo leukocyte innate immune responses in chapter 9 emphasize the need for thorough clinical research of claimed health food effects, especially because these oral Beta-glucan formulations are sold all over the world as a health food supplement claiming to enhance the immune system. Despite the absence of effects in our study, immunological training effects of Beta-glucan are clearly demonstrated in vitro (17, 33, 46). A very recent study, using a systems biology approach, demonstrated that Beta-glucan training of monocytes results in a long-lasting exclusive epigenetic signature, revealing a complex network of promoters and enhancers linked to genomic coordinates (33). Furthermore, again by a systems biology approach, it was demonstrated that cAMP mediated molecular signal transduction is important for Beta-glucan-induced training. This study further highlights the importance of these combined approaches and provides a resource to further understand the biological mechanisms through which Beta-glucan-induced training effects are mediated. Together with this growing basic knowledge, clinical studies are warranted to confirm the beneficial immunostimulatory effects of Beta-glucan in vivo. However, future studies should not use commercially available orally administered compounds, but rather focus on formulas of which it is demonstrated that they are absorbed and/or penetrate target tissues, e.g. compounds approved for parental administration.

CONCLUSION

The past few years of research have dramatically changed our perspective of infection-related immune responses. The identification of sepsis-induced immunoparalysis, probably an important determinant of mortality, has resulted in a real paradigm shift in the general thinking and experimental immunomodulatory treatment of sepsis. Trained immunity challenged our perspective of the innate immune system lacking immunological memory, because it provides innate protection against re-infection by related as well as unrelated pathogens. The results of the studies described in this thesis endorse these theories. We demonstrated that immunoparalysis can be reversed by IFN-γ in a model for...
sepsis-induced immunoparalysis in humans, that BCG vaccination enhances antibody responses induced by a subsequent influenza vaccination, and that, next to BCG, influenza vaccination has non-specific effects in terms of modulated cytokine responses against unrelated pathogens as well. Apart from this, we also demonstrated that IFN-γ is able to enhance immune responses in patients with invasive opportunistic infections.

However, beneficial effects of these approaches have to be confirmed on clinical relevant endpoints in larger randomized clinical trials. To facilitate these large trials, accurate biomarkers that are able to identify patients who may benefit from immunomodulatory therapies, and to monitor treatment responses, are highly warranted. Although suboptimal, mHLA-DR expression and ex-vivo cytokine responses are currently used to this end. Hopefully, recent technical advances in molecular diagnostics and prognostics, and the use of a systems biology approach, will result in identification of novel pathways and thereby contribute to a better understanding of host defenses. In turn, this may serve as a starting point for the discovery of more accurate immune status biomarkers and aid the design of future immunotherapeutic strategies. Although it has the potential to radically change treatment of infectious diseases, the finding that inactivated BCG vaccination and orally administered commercially available Beta-glucan do not induce trained immunity in vivo and/or ex vivo underscore that there is still a lot of work ahead before trained immunity can be used for the benefit of patients in daily clinical practice.

REFERENCES

CHAPTER 12

Nederlandse samenvatting
Het aspecifieke, aangeboren, "innate" afweersysteem is verantwoordelijk voor de initiële herkenning van, en verdediging tegen, pathogene micro-organismen die door de fysieke barrières van de gastheer heen zijn gedrongen. De activatie van het aangeboren afweersysteem wordt gekarakteriseerd door secretie van onder andere pro-inflammatoire cytokines, chemokines, en vaso-actieve stoffen. Dit leidt onder meer tot koorts, T-cel activatie (met als gevolg activatie van het specifieke immuunsysteem), activering van pijnreceptoren, en vasodilatatie (1, 2). In een vroeg stadium zijn deze pro-inflammatoire mediatoren verantwoordelijk voor de typische symptomen waarmee een patiënt met bloedvergiftiging (ook sepsis genoemd) zich zal presenteren (koorts, hemodynamische instabiliteit, stollingsstoornissen, eind-orgaan dysfunctie, etc.). Gelijktijdig met deze pro-inflammatoire respons wordt er ook een anti-inflammatoire reactie opgestart, waarin anti-inflammatoire cytokines een belangrijke rol spelen die ervoor moet zorgen dat de pro-inflammatoire afweerrespons niet uit de hand loopt (3). Een adequate pro- en anti-inflammatoire afweerrespons zal pathogene micro-organismen elimineren zonder dat het lichaam van de gastheer schade wordt berokkend. Levensbedreigende situaties kunnen ontstaan wanneer een inadequade afweerrespons ervoor zorgt dat organen van de gastheer worden aangetast (zoals het geval is bij een uit de hand gelopen pro-inflammatoire afweerrespons), maar ook dat de pathogene niet uit het lichaam verwijderd wordt (zoals het geval is bij zeer pathogene microorganismen zoals het grieppivirus, maar ook bij patiënten met een onderdrukte pro-inflammatoire afweerrespons of een te uitgesproken anti-inflammatoire afweerrespons). In de laatste jaren is duidelijk geworden dat onderdrukking van de afweerrespons bij septische patiënten in de dagen tot weken na de eerste sepsis symptomen zeer vaak voor problemen zorgt. Deze zogenaamde “immuunparalyse” zorgt er namelijk voor dat deze patiënten minder goed hun primaire infectie kunnen klaren en bovendien zijn ze veel vatbaarder voor secundaire infecties.

Dit proefschrift bevat preklinische, translationele en klinische studies gericht op modulatie van de afweerrespons voor de aanvullende behandeling van ernstige
Infecties. We hebben ons gefocust op de effecten van Interferon-gamma (IFN-γ) en Granulocyt Macrofaag-kolonie Stimulerende Factor (GM-CSF) (middelen bekend om hun stimulerende effect op de afweerrespons) als aanvullende behandeling van sepsis, en op de effecten van IFN-γ als aanvullende behandeling van infecties die alleen voorkomen in patiënten met een onderdrukt afweersysteem (de zogenaamde "opportunistische infecties"). Daarnaast hebben we nieuwe immuunmodulerende methoden onderzocht die gebaseerd zijn op de recente ontdekking dat het aangeboren immuunsysteem, net als het specifieke immuunsysteem, ook getraind kan worden.

Hooftstuk 1 is een korte inleiding over het aangeboren immuunsysteem maar richt zich vooral op de afwijkingen in de afweerrespons die verantwoordelijk zijn voor een ernstiger beloop van bepaalde infecties. Als eerste worden opportunistische infecties besproken die alleen voorkomen bij patiënten met een onderdrukte afweerrespons, waarna een korte introductie volgt over infecties die voorkomen bij patiënten met sepsis-geïnduceerde immuunparalyse. Al laatste wordt uitgelegd waarom we voor de immunstimulerende middelen hebben gekozen die we hebben gebruikt in de studies die in dit proefschrift beschreven zijn.

DEEL 1: MODULATIE VAN DE AFWEERRESPONS ALS AANVULLENDE BEHANDELING VOOR SEPSIS

Hooftstuk 2 geeft een overzicht van de pathofysiologie van sepsis en focust met name op de recente ontdekking dat niet de pro-inflammatoire afweerrespons, maar juist de anti-inflammatoire afweerrespons belangrijk is voor het ziektebeloop van septische patiënten. Deze zogenaamde "immuunparalyse" zorgt er namelijk voor dat patiënten minder goed hun primaire infectie kunnen klaren en dat zij bovendien veel vatbaarder zijn voor secundaire infecties. Hoewel broncontrole en organa-ondersteunende behandelingen, zeker in de vroege fase van sepsis, de hoeksteen van de behandeling van septische patiënten zal blijven, is de ontdekking van deze immuunparalyse de reden dat er momenteel een verschuiving optreedt in onderzoek naar de advjuvante behandeling van sepsis. Waar de nadruk voorheen lag op onderdrukken van het immuunsysteem met bijvoorbeeld anti-cytokine therapieën of corticosteroïden, ligt de nadruk nu meer en meer op het stimuleren van het immuunsysteem. In dit hoofdstuk geven we dan ook een overzicht van de mechanismen die ten grondslag liggen aan immuunparalyse en van mogelijke markers die patiënten met immuunparalyse kunnen identificeren. Verder bespreken we het kleine aantal klinische studies die stimulatie van de afweerrespons hebben onderzocht bij septische patiënten, en bespreken we nieuwe immunstimulerende middelen die veelbelovend lijken voor het behandelen van immuunparalyse.

In hoofdstuk 3 beschrijven we de effecten van IFN-γ en GM-CSF op immuunparalyse in vivo bij de mens. In deze studie werd gebruik gemaakt van het experimentele humane endotoxemie-model, een humaan preklinische model dat gebruikt kan worden om de reactie van het immuunsysteem te onderzoeken. In dit model wordt endotoxine (een stukje van de celwand van een Gram-negatieve bacterie) toegediend aan gezonde vrijwilligers waardoor een systemische immuunreactie ontstaat die veel overeenkomsten vertoont met de immuunreactie tijdens sepsis. Wanneer endotoxine na een week voor een 2e keer wordt toegediend aan dezelfde proefpersoon, is de reactie van het aspecifieke immuunsysteem veel minder uitgesproken dan na de eerste endotoxine-toediening. Deze zogenaamde "endotoxine-tolerantie" vertoont veel overeenkomsten met sepsis-geïnduceerde immuunparalyse. Gebruikmakend van dit model werd aangetoond dat behandeling met IFN-γ niet alleen de cytokineproductie van witte bloedcellen in het laboratorium, maar ook de in vivo secretie van het pro-inflammatoire cytokine TNF-α stimuleerde, terwijl het de productie van het anti-inflammatoire cytokine IL-10 onderdrukte na herhaalde toediening van endotoxine. IFN-γ veroorzaakt dus een duidelijke verschuiving van de immunrespons richting een meer uitgesproken pro-inflammatoire reactie. Ook verhoogde IFN-γ de expressie van HLA-DR op monocytten (mHLA-DR; een belangrijke marker van immuunparalyse). In een derde groep proefpersonen die behandeld werd met GM-CSF werden vergelijkbare, maar minder uitgesproken effecten gevonden.
DEEL 3: NIEUWE IMMUUNMODULERENDE STRATEGIEËN

Bacille Calmette Guérin (BCG) is een vaccin tegen tuberculose dat gemaakt wordt van de bij runderen voorkomende tuberculosebacterie, Mycobacterium bovis. In grote epidemiologische studies werd jaren geleden al aangetoond dat BCG niet alleen beschermd tegen tuberculose, maar ook tegen andere niet verwante infecties. Lange tijd zijn deze aspecifieke effecten van BCG vaccinatie genegeerd, pas in de laatste 10 jaar is hier belangstelling voor gekomen. Recent werd in vitro en ex vivo aangetoond dat blootstelling aan BCG niet alleen bescherming biedt tegen tuberculose, maar ook tegen ander (niet-verwante) pathogenen. Bovendien werd aangetoond dat deze niet–tuberculose-gerelateerde gunstige effecten onafhankelijk waren van B- en T-cellen, en daarom veroorzaakt lijken te worden door “training” van het aangeboren afweersysteem. Desalniettemin zijn tot op heden deze aspecifieke effecten van BCG bij de mens enkel ex vivo aangetoond. In hoofdstuk 7 beschrijven we dat bij gezonde vrijwilligers, vaccinatie met levend afgezwakte BCG leidt tot snellere en meer uitgesproken antistofvorming uitgelokt door een daaropvolgende toediening van trivalent griep vaccin. Verder werden de aspecifieke effecten van BCG op cytokine productie van ‘witte bloedcellen die ex vivo gestimuleerd werden door niet verwante pathogenen bevestigd, en hebben we bovendien aangetoond dat het griepvaccin zelf ook niet-specifieke effecten heeft op cytokine productie uitgelokt door verschillende pathogenen. In het geheel ondersteunen deze resultaten de hypothese dat de afweerstatus op een klinisch relevante manier wordt beïnvloed door de vaccinatievoorgeschiedenis, en door de mogelijkheid de immuunrespons op vaccinaties te verbeteren kan deze kennis bijvoorbeeld gebruikt worden om vaccinatiestrategieën te verbeteren.

In hoofdstuk 5 en 6 beschrijven we dat aanvullende behandeling met IFN-γ, ingezet als laatste redmiddel bij een patiëntje met progressieve invasieve Candida infectie na remissie-inductie chemotherapie voor acute lymfoblastische leukemie, leidde tot verbetering van hun aangeboren afweerrespons, en meer waarschijnlijk ook bijdroeg aan radiografische en klinische verbetering. Dit wijst erop dat aanvullende behandeling met IFN-γ en de vaccinatie BCG leidt tot een beter functioneren van hun afweersysteem. Deze resultaten wijzen erop dat aanvullende behandeling met IFN-γ ook de afweer tegen schimmels zou kunnen verbeteren.
BCG is niet de enige stimulus waarvan werd aangetoond dat het aangeboren afweersysteem hierdoor getraind kan worden, ook van Beta-glucan werd dit in vitro en in vivo bij dieren aangetoond. Beta-glucanen zijn natuurlijke koolhydraten die wijd verspreid voorkomen, waarvan geclaimd wordt dat ze een versterkende werking op het afweersysteem hebben, en die daarom wereldwijd op het internet aangeboden worden als voedingsadditief. Omdat oraal Beta-glucan bovendien goedkoop is en zeer goed verdragen wordt, is het een veelbelovende kandidaat om het afweersysteem te versterken, alhoewel dit nog niet bij mensen werd onderzocht. In hoofdstuk 9 beschrijven we dat dagelijkse inname van 1000mg commercieel verkrijgbaar oraal Beta-glucan gedurende 7 dagen bij gezonde vrijwilligers niet leidt tot aantoonbare serum spiegels van Beta-glucan, en dat het aangeboren afweersysteem er ook niet door beïnvloed wordt; we vonden namelijk onveranderde microbicidle activiteit en ook de cytokineproductie van witte bloedcellen die ex vivo gestimuleerd werden met verschillende pathogenen was niet anders na behandeling met Beta-glucan. De geclaimde afweersysteem-stimulerende effecten van commercieel verkrijgbare en oraal toegediende Beta-glucan kunnen wij dan ook niet bevestigen.

**ALGEMENE CONCLUSIE EN TOEKOMSTPERSPECTIEVEN**

In het eerste deel van dit proefschrift hebben we een overzicht gegeven van recente inzichten in de pathofysiologie van sepsis. Hierbij hebben we de nadruk gelegd op sepsis-gedeïnduceerde immuunparalyse omdat dit een belangrijke rol speelt in de prognose van septische patiënten. Verder hebben we aangetoond dat IFN-γ in staat is om immuunparalyse gedeeltelijk op te heffen in een onderzoeksmodel voor sepsis-gedeïnduceerde immuunparalyse bij de mens.

Een groot probleem binnen het sepsisonderzoek is het feit dat sepsispatiënten een zeer heterogene groep vormen. Dit wordt op de eerste plaats verklaard door de definitie van sepsis (Tabel 1), die zeer ruime klinische en laboratorium parameters (zoals verandering in hartslag, temperatuur, ademhaling, of aantal witte bloedcellen in aanwezigheid van een infectie) gebruikt, waardoor vrijwel alle patiënten met een ontstekingsreactie hieraan voldoen, zonder onderscheid te maken tussen veroorzaakende pathogenen, plaats van infectie of ernst van de ziekte (4). Deze heterogeniteit maakt het dan ook waarschijnlijk dat per patiënt verschillende afweermechanismen betrokken kunnen zijn, waardoor evaluatie van een enkel mechanisme ernstig bemoeilijkd wordt. Zoals beschreven in hoofdstuk 2 van dit proefschrift vinden inderdaad zowel pro- als anti-inflammatoire processen tegelijkertijd plaats, en afhankelijk van verschillende individuele factoren zal een pro- of anti-inflammatoir overwicht de overleving van de individuele patiënt in gevaar brengen. Door de vooruitgang in orgaanondersteunende therapieën en doordat patiënten steeds ouder worden zal immuunparalyse waarschijnlijk in het merendeel van de patiënten de overleving bedreigen, en niet de primaire pro-inflammatie. Dit laatste is dan ook zeer waarschijnlijk een van de belangrijkste redenen van het mislukken van alle voorgaande studies die geprobeerd hebben om de overleving van septische patiënten te verbeteren door het onderdrukken van de afweerrespons.

**Tabel 1.** Definities voor SIRS, sepsis, ernstige sepsis en septische shock

| SIRS | Temperatuur <36°C of >38.3°C Hartfrequentie > 90 min⁻¹ Ademhalingsfrequentie >20 min⁻¹ of een PaCO₂ <32 mmHg (4.3 kPa) WBC < 4000 μL⁻¹ of >12000 μL⁻¹ of normaal WBC met > 10% immature vormen |
| Sepsis | SIRS + bewezen of waarschijnlijke infectie |
| Severe sepsis | Sepsis + orgaandysfunctie |
| Septic shock | Sepsis + hypotensie of verminderde weefselperfusie, ondanks adequate volume resuscitatie |

SIRS, systemisch inflammatie-respons syndroom; WBC, witte bloed cel; adequate volume resuscitatie: ≥20 ml/kg per ideaal lichaamsgewicht isotoon crystalloïde vocht, of een centraal veneuze druk ≥8 mmHg of een pulmonale capillaire wedge druk van ≥12 mmHg.

Hoewel het in verschillende observationele studies al werd aangetoond dat immuunparalyse klinische relevant is, zeggen veel clinici dat zij immuunparalyse niet herkennen als een relevant probleem bij hun septische patiënten. Deze twijfels worden verder ondersteund door het feit dat er nog geen directe relatie tussen immuunparalyse en orgaanfalen/mortaliteit is aangetoond. Recente studies hebben hier echter verandering in gebracht: inzichten in de vermindering van bio-energetica en mitochondriële dysfunctie die leiden tot ATP-depletie zorgt
voor de eerste link tussen immuunparalyse en cel dysfunctie die resulteert in multi-
orgaanfalen (5-9). Een afname in metabole activiteit zal ervoor zorgen dat een cel
minder energie nodig heeft. Hierdoor zal de cel minder goed functioneren, maar zal
de cel ook kunnen overleven door een kritische daling in ATP. Elk van de kenmerken
van deze maladaptieve cellulaire homeostase zorgt voor een kenmerkende
eigenschap van sepsis (bijvoorbeeld het niet goed contraheren van myocardcellen
zorgt voor myocarddepressie, het niet goed contraheren van spiercellen in de
vaatwand leidt tot hypotensie, enz.) (10). Dit is bovendien volledig in lijn met autopsie
studies waarbij gevonden werd dat bij overleden septische patiënten nagenoeg
geen apoptose (geprogrammeerde cel dood) gevonden kan worden, behalve in de
lymfocyten compartimenten (11, 12). Hiermee wordt dan ook meteen de link met
immuunparalyse verklaard: cellen in de lymfocyten compartimenten die in apoptose
zijn gegaan zullen leiden tot immuunparalyse. Dit zal tot gevolg hebben dat septische
patiënten hun initiële infectie minder makkelijk kunnen klaren, en dat zij vatbaar
zijn voor secundaire infecties wat dan weer gepaard kan gaan met voortgaande
inflammatie die leidt tot mitochondriële dysfunctie en een vicieuze cirkel zal ervoor
zorgen dat cellen niet in staat zijn om te herstellen (Figuur 1).

Onafhankelijk van deze bio-energetische hypothese zal de buitensporige pro-
inflammatoire reactie op een binnendringende pathogeen de uitlokkende factor
zijn van een cascade van gebeurtenissen. De overmatige pro-inflammatie kan
in bepaalde gevallen zelf ook voor veel bijkomende schade en zelfs overlijden
zorgen (zoals het geval is bij bijvoorbeeld meningokokkensepsis of infecties
die voor een macrofaag activatie syndroom zorgen (bijvoorbeeld ernstige
influenza infecties, dengue of malaria)). Daarnaast zijn de in hoofdstuk 2 van dit
proefschrift beschreven onderdrukte afweerfuncties ook duidelijk aangetoond bij
septische patiënten in het algemeen. Vandaar dat het van grote therapeutische
waarde zal zijn als we manieren vinden om de afweer respons “op maat” aan te
pakken: de afweer respons verhogen als deze niet in staat is om binnendringende
micro-organismen te elimineren en deze onderdrukken als deze zo overmatig
is dat het voor veel bijkomende schade zal zorgen. Accurate biomarkers die
de afweerstatus van een patiënt kunnen identificeren zijn daarom bijzonder
belangrijk voor de toekomst van immuuntherapie bij sepsis. Biomarkers kunnen
zowel patiënten identificeren die baat zouden kunnen hebben van stimulatie van
het afweersysteem, maar ze zouden ook gebruikt kunnen worden om de respons
op therapie te monitoren. Op dit moment worden mHLA-DR en de respons van
witte bloedcellen op ex vivo stimulatie met lipopolysaccharide (LPS), hiervoor
het meest gebruikt. Zij geven echter alleen de immuun status van het bloed, en
niet van de weefsels, weer en bovendien zorgen pre-analytische en analytische
kwesties die inherent zijn aan hun analyse ervoor dat zij slechts beperkt bruikbaar
zijn in grote internationale studies of routinematig in de kliniek zoals besproken in
hoofdstuk 2 van dit proefschrift. Er is hoop dat verbeterde moleculaire methodes
gebaseerd op een “systeembiologie” aanpak (een combinatie van genomische,
transcriptomische, metabolomische, proteomische en/of epigenetische
gegevens) uiteindelijk zal leiden tot nieuwe diagnostische instrumenten, hoewel
dit wat betreft sepsis nog in de kinderschoenen staat (13-16). Omdat de huidig
beschikbare biomarkers suboptimaal zijn, moeten toekomstige klinische studies
een panel van markers gebruiken om hun patiënten te stratificeren waarbij gebruik
gemaakt kan worden van mHLA-DR en witte bloedcel responsen in combinatie
met klinische data (zoals een duidelijke afname van vasopressiebehoeft als
surrogaat marker van pro-inflammatie). Hopelijk voorkomt deze biomarker-

Figuur 1. Vereenvoudigde weergave van de verminderde bio-energetische hypothese.
gestuurde stratificatie nog meer mislukte klinische studies bij septische patiënten, en zal hierdoor het uitvoeren van grote klinische studies vergemakkelijken worden.


In het tweede deel van dit proefschrift hebben we aangetoond dat immuunstimulatie met IFN-γ bij een reeks patiënten met invasieve schimmelinfecties leidde tot verhoogde productie van cytokines door witte bloedcellen die ex vivo gestimuleerd werden met verwante stimuli, en dat het mHLA-DR expressie duidelijk verhoogde bij patiënten met lage baseline mHLA-DR waarden. Daarnaast beschreven we dat aanvullende behandeling met IFN-γ bij een patiëntje met refractaire invasieve Candida infectie en een patiënt met refractaire cerebrale Nocardiose leidde tot verbetering van de aangeboren immuun respons en zeer waarschijnlijk heeft bijgedragen aan radiografische en klinische verbetering.

Ook bij patiënten met opportunistische infecties ontbreekt een adequate biomarker die patiënten kan identificeren die baat zouden kunnen hebben bij immuunstimulerende therapie of die de respons op behandeling kan monitoren. In hoofdstuk 4 en 5 van dit proefschrift hebben we voor de eerste keer beschreven dat naast ex vivo cytokineresponsen ook mHLA-DR bij deze patiënten als marker voor hun immunestatus zou kunnen dienen: mHLA-DR expressie was omgekeerd gecorreleerd met ziekte ernst en bij patiënten met lage baseline waarden kon de mHLA-DRexpressie duidelijk verhoogd worden door toediening van IFN-γ. Ondanks dat deze resultaten veelbelovend zijn zullen zij eerst bevestigd moeten worden in grote klinische studies waarbij bovendien rekening gehouden moet worden met de beperkingen van mHLA-DRmetingen die al beschreven werden bij septische patiënten. Desalniettemin was monitoring van de immuunrespons met ex vivo cytokine responses en/of mHLA-DR expressie bij de in hoofdstuk 5 en 6 beschreven patiënten van grote meerwaarde in het sturen van de immuunmodulerende behandeling. Deze biomarker-gestuurde benadering zal ook toegepast moeten worden bij toekomstige grotere (multi-center) klinische studies bij patiënten met invasieve opportunistic infecties om immunotherapie bij deze patiënten te onderbouwen door het bevestigen van gunstige immunologische maar ook klinische effecten. Dit zal nationale en internationale samenwerking vereisen tussen infectiologen, intensivisten, oncologen en immunologen, aangezien onderzoek bij deze patiënten zeer uitdagend is vanwege de lage incidentie.

In het laatste deel van dit proefschrift hebben we onderzocht of het aangeboren immuunsysteem ook in vivo bij mensen getraind kan worden en of deze nieuwe benadering kan leiden tot nieuwe immuunmodulerende strategieën. In hoofdstuk 7 van dit proefschrift tonen we aan dat vaccinatie met levend afgezwakte BCG leidt tot snellere en meer uitgesproken antistofvorming uitgelokt door daaropvolgende toediening van een trivalent griep vaccin aan gezonde vrijwilligers, en dat niet alleen BCG maar ook het griepvaccin zelf niet-specific effecten heeft op cytokineresponsen van witte bloedcellen die ex vivo gestimuleerd werden met niet verwante stimuli. Dit is niet alleen logisch vanuit een evolutionair perspectief, maar het onderstreep ook hoe totaal de dogma van het niet-specific zijn van het aangeboren immuunsysteem verandert door de ontdekking dat het dit systeem toch getraind kan worden: alle vaccinaties (en waarschijnlijk ook alle doorgemaakt
infecties) zullen het immuunsysteem beïnvloeden op een niet-specifieke en klinisch relevante wijze. Nochtans staat dit onderzoek nog in de kinderschoenen en het ontrafelen van de onderliggende mechanismen is pas net begonnen. Onlangs werd aangetoond dat epigenetische veranderingen niet alleen ten grondslag liggen aan de training van het aangeboren immuunsysteem, maar ook aan de tolerantie die ontstaat na blootstelling aan LPS (15). Zoals beschreven in hoofdstuk 2 van dit proefschrift, is epigenetische regulatie van gentranscriptie door talrijke mechanismen gemedieerd, maar in het algemeen kan het beschouwd worden als de gereguleerde organisatie van genloci in transcriptioneel actief of inactieve toestand waarin H3K4 methylering en H3K27 acetylering een rol spelen (Figuur 2). Daarenboven werd recent aangetoond dat autofagie ook een belangrijke rol speelt bij het trainen van het aangeboren immuunsysteem door BCG (22). Desalniettemin zijn de moleculaire机制en die ten grondslag liggen aan het trainen van het aangeboren immuunsysteem nog verre van ontrafeld. Ook de mechanismen waardoor autofagie epigenetische veranderingen zou kunnen medieren is nog niet bekend. De mate waarin het trainen van het aangeboren immuunsysteem het immuunsysteem beïnvloed (bijvoorbeeld welk effect welk vaccin heeft tegen welke pathogenen), en wat de optimale timing is voor behandeling (niet alleen het tijdsbestek maar ook de volgorde van toediening kan van belang zijn) is bovendien ook nog steeds niet bekend. Al deze aspecten moeten eerst ontrafeld worden voordat we volledig kunnen begrijpen in welke mate de klinische praktijk beïnvloed zal worden door de ontdekking dat het aangeboren immuunsysteem getraind kan worden. De data die in hoofdstuk 7 van dit proefschrift beschreven worden geven een eerste hint dat vaccinatie strategieën veel efficiënter gemaakt zouden kunnen worden en dat vaccinaties ook gebruik zouden kunnen worden in het voorkomen van niet-gerelateerde infecties. Zo zou BCG vaccinatie er bijvoorbeeld voor kunnen zorgen dat maar de helft van de dosis van het griepvaccin nodig zou zijn om mensen tegen de griep te beschermen.

In het geval van een grieppandemie zou dit veel levens kunnen redden aangezien de beschikbare hoeveelheid griepvaccin beperkt is. Voor het zover is zullen er echter veel grotere studies uitgevoerd moeten worden om deze BCG-griep effecten te bevestigen.

Training van het aangeboren afweersysteem zou bovendien niet alleen gebruikt kunnen worden in het voorkomen van infecties maar ook voor de behandeling van infecties bij mensen met een onderdrukt afweersysteem. Bij deze patiënten zullen echter alleen middelen gebruikt kunnen worden die niet het risico hebben om zelf ernstige en verspreide infecties te veroorzaken (zoals wel het geval is bij levend afgezwakt BCG). In hoofdstuk 8 van dit proefschrift beschrijven we echter dat gamma-bestaalde (en dus geïnactiveerde) BCG niet in staat is om in of ex vivo training van het aangeboren afweersysteem te veroorzaken bij het eerder beschreven humaan endotoxinemie model. Daarom zijn zowel levend afgezwakt als gamma-bestaalde BCG geen goede opties om sepsis-geïnactiveerde immuunparalyse te behandelen. Onderzoek bij deze patiënten zal zich moeten focussen op andere immuunstimulerende middelen.

Tenslotte hebben we in hoofdstuk 8 van dit proefschrift aangetoond dat oraal toegediend commercieel verkrijgbaar Beta-glucan geen effect heeft op de functie van witte bloedcellen bij mensen. Deze gegevens benadrukken de behoefte aan gedegen klinisch onderzoek en bevestigen dat de gezondheidsclaims die bij dit soort middelen vaak gedaan worden niet altijd voor waarheid moeten worden aangenomen.
REFERENCES

Dankwoord
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List of Publications
LIST OF PUBLICATIONS


CURRICULUM VITAE


Jenneke is getrouwd met Bas Dirken. Zij wonen in Heesch en hebben twee kinderen (Finne en Nout).