Mucosal Barrier Injury, Innate Immunity, and Stem Cell Transplantation

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Mucosal Barrier Injury, Innate Immunity, and Stem Cell Transplantation

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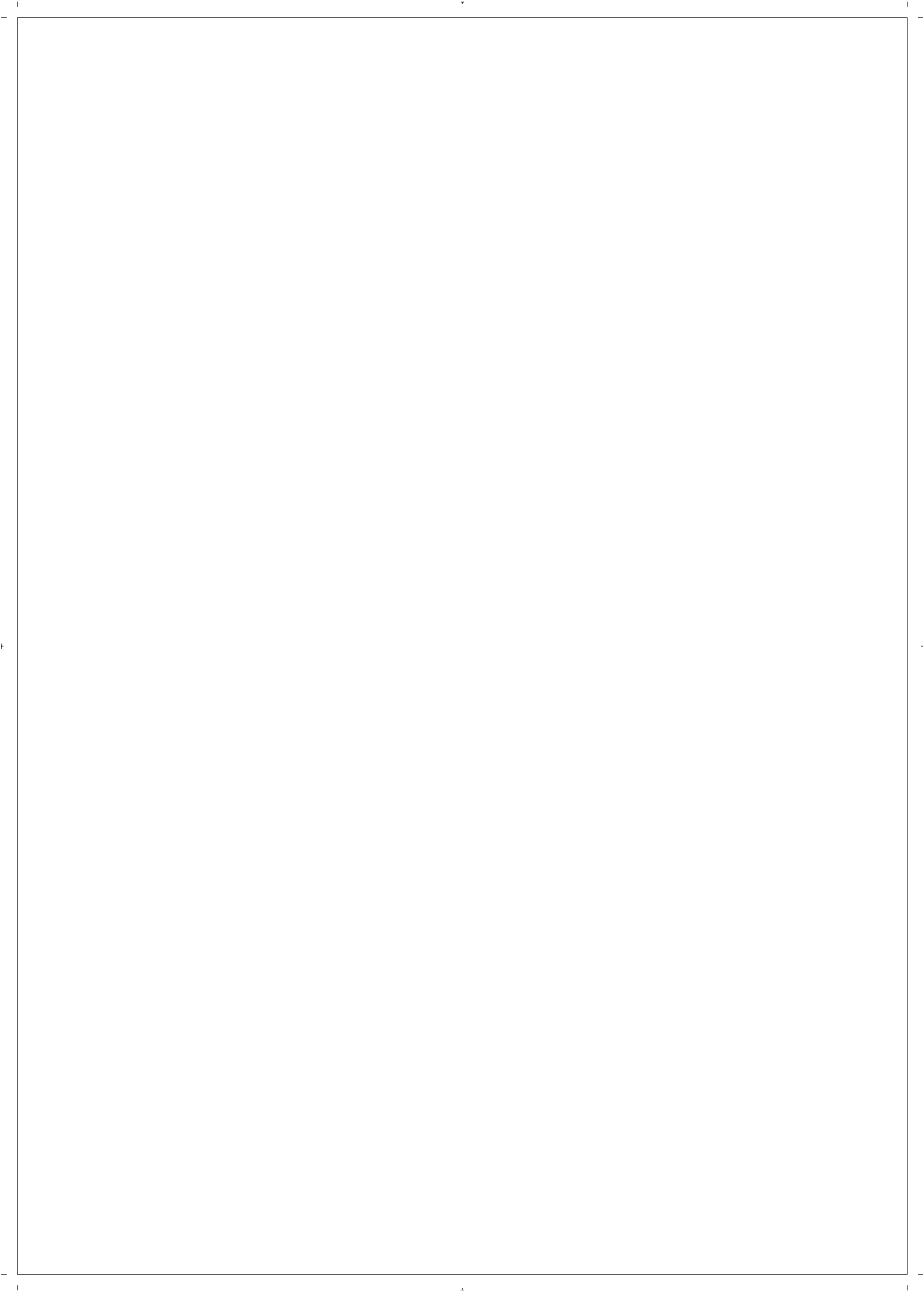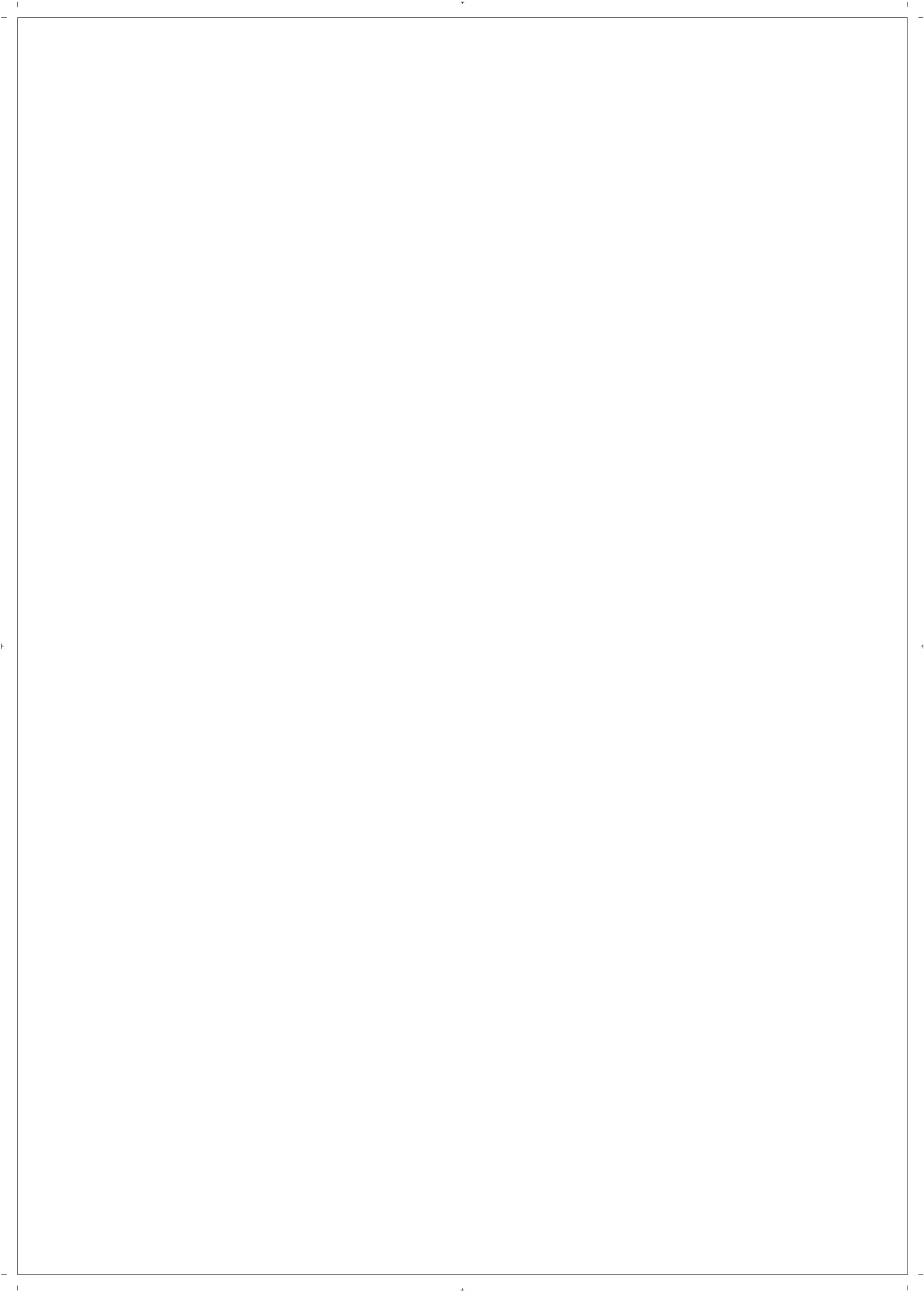**Copromotoren**

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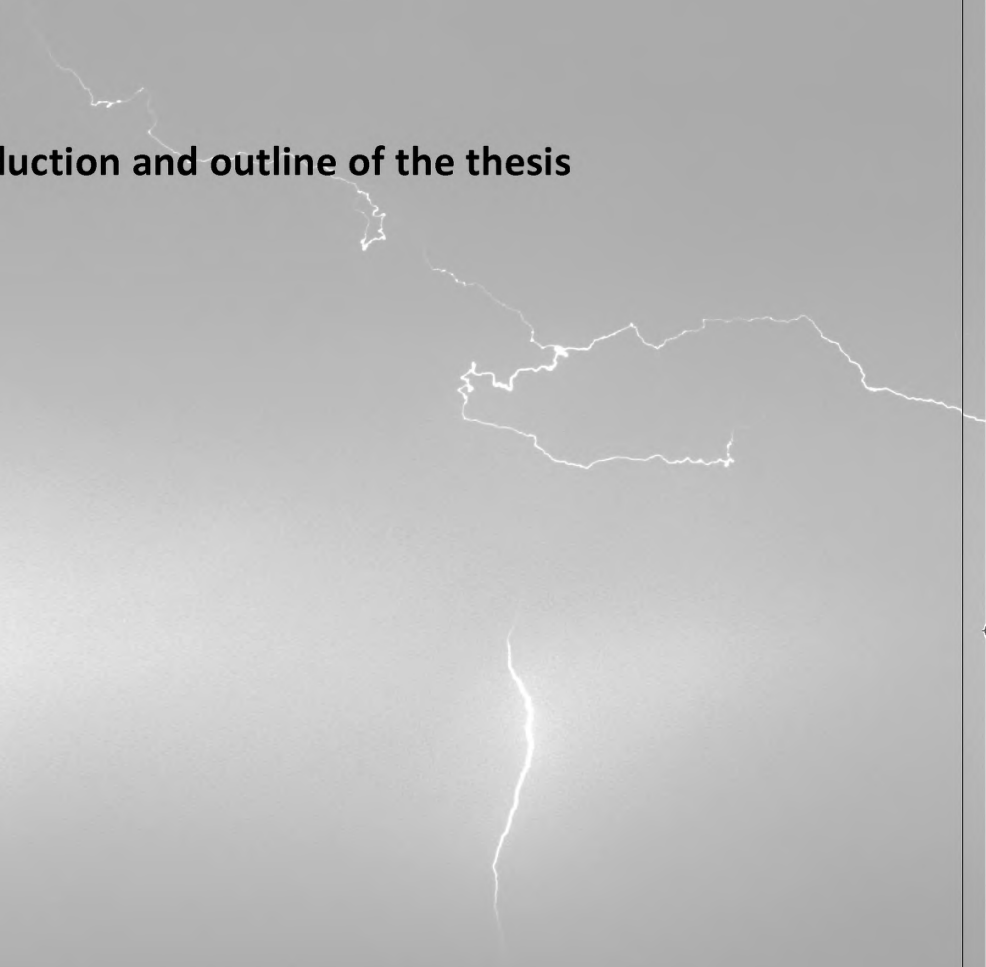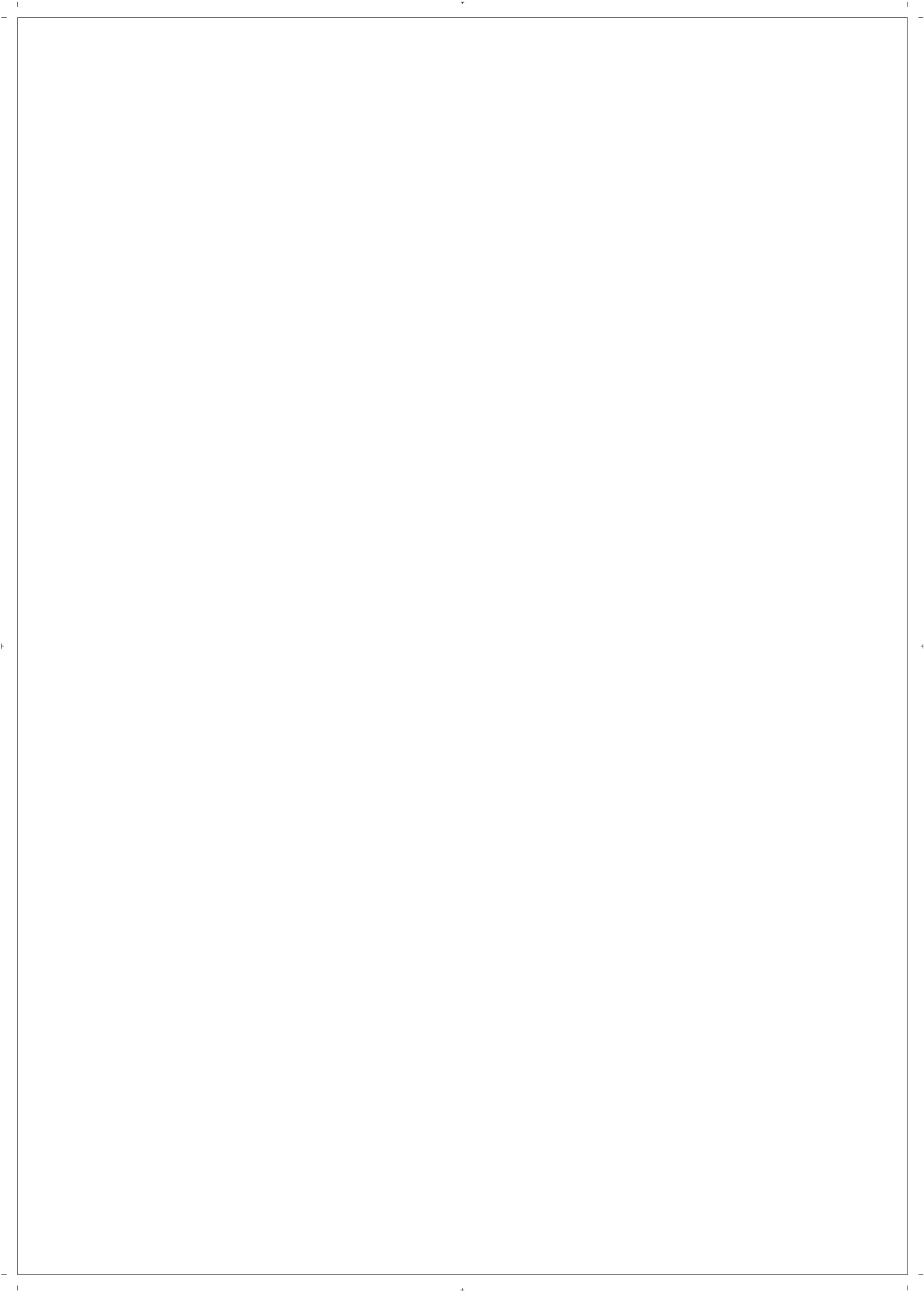Prof. dr. O. Levy (Harvard Medical School, USA)

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Introduction and outline of the thesis

Introduction

Hematopoietic stem cell transplantation (SCT) is highly effective for treating hematological malignancies, and other disorders. However, preparation for SCT is still accompanied by significant morbidity and mortality resulting from uncontrolled inflammatory conditions that occur at several stages of the treatment [1]. These inflammatory conditions consist of mucosal barrier injury (MBI), manifesting clinically as mucositis, acute and chronic graft-versus-host disease (GvHD), idiopathic pneumonia syndrome and infections. The pathogenesis of MBI [2;3] and gastrointestinal acute GvHD [4;5] have striking similarities with inflammatory bowel diseases, including Crohn's disease, in which the host-microbial homeostasis in the gastrointestinal tract becomes disturbed resulting in uncontrolled mucosal inflammation [6-9].

The skin and gastrointestinal tract are fascinating organs, because their epithelial barriers are normally in a state of immunological tolerance, and homeostasis is present, despite the constant exposure to billions of microorganisms and foreign antigens (e.g. food, drugs, pollutants), for which strict and careful regulation of the immune system is essential [9;10]. There has to be a balance between tolerance to the 'normal' commensal flora and intolerance to pathogenic microorganisms, which requires the generation of an effective and swift immune response. Exciting new discoveries have been made in recent years, providing greater insight in the complex regulatory mechanisms facilitating "barrier homeostasis". It appears that constant interaction and "cross-talk" between intestinal epithelial cells (IECs), immune cells (monocytes, macrophages and dendritic cells) on the one hand and microbes (normal flora and pathogens) on the other hand are necessary to determine exactly what is going on at the mucosal surface and to direct immune activity.

The innate immune system plays a central role in keeping the balance, since it is the first to come in contact with and to react to microorganisms, and also orchestrates the adaptive immune system. In this regard pattern recognition receptors (PRRs), the "sensors" of innate immune cells, are key players in the regulation of mucosal immunity [11;12]. In addition, antimicrobial peptides (AMPs) play an important role, as they are widely expressed by the skin and gastrointestinal tract and regulate the composition and quantity of the microbial flora present at the barriers.

Studies on inflammatory bowel diseases and psoriasis have identified several factors involved in the disruption of epithelial homeostasis, including altered microbial composition, over-activation of certain cytokine pathways (interleukin (IL-) 12/Th1, IL-23/Th17), and defective PRRs, autophagy and AMPs [8;13-15]. These insights have already allowed the development of new therapeutic and preventive interventions for these auto-immune diseases, such as cytokine modulation,

pre-biotics and pro-biotics [16], PRR modulation [17-20] and the use of AMPs. The similarities in pathogenesis between inflammatory bowel diseases and MBI and acute GvHD, suggest that these beneficial developments might be translated to the setting of SCT and to ameliorate morbidity and reduce mortality.

Innate immunity

The immune system has historically been divided into the innate ('natural') and adaptive ('acquired') immune system to highlight the difference in primitive and more sophisticated responses. Current knowledge suggests that the division between the innate and adaptive immune systems is blurred as there is considerable overlap in function, and both are highly interlinked. Importantly it is now clear that the innate immune system actually orchestrates the adaptive immune system, which, in turn, controls and modulates the innate immune responses [21;22]. Nevertheless, the dichotomy is still useful if only for the sake of clarity.

Innate immunity is a primary highly conserved immune system which can been found in most living organisms, from plants, insects, fish, birds, and mammals [23]. Indeed innate immunity is the only immune system many organisms have. It is only the more developed organisms, such as mammals, that also harbor an adaptive immune response. The innate immune system brings about the first contact with microorganisms and foreign molecules and initiates a primary response to react quickly to external threats. Besides speed, the response is characterized by being coarse, non-specific, and lacks "memory" so that on re-challenge the same response ensues. In contrast, the adaptive immune system is refined, recognizes specific foreign molecules, and shows a better response with every re-challenge.

The innate immune system consists of several humoral and cellular components [23]. It comprises the epithelial surfaces which result in a direct physical barrier that has also been referred to as the integument. Humoral factors consist of the complement systems, AMPs, acute phase proteins (e.g. C-reactive protein) and mucosal secretions (mucins, saliva). Cellular components consist of natural killer cells (NK) and phagocytic cells such as monoyctes, macrophages, polymorphonuclear neutrophils (PMN) and dendritic cells (DC). These classical immune cells, are, in fact, complemented by epithelial cells, endothelial cells and fibroblasts which are now recognized as being essential cellular components of the innate immune system. For instance, IECs recognize microbes, produce cytokines and AMPs, phagocytize and present antigens [24-26].

Pattern recognition receptors: key players of the innate immune system

The important discovery in the drosophila fly of the pattern recognition receptor named *Toll* receptor led the way to the discovery of the Toll-like receptors (TLRs) which explained how innate immune cells recognize foreign molecules [27;28]. This gave a major boost to the research in innate immunity which resulted in the discovery of many different PRRs in humans. Several families of PRR have been identified, the most important ones being the TLRs, the Nod-like receptors (NLRs), RIG-like receptors (RLRs), and the family of C-type lectin receptors (CLRs) (Table 1) [11;29;30].

PRRs are expressed on nearly every human cell ranging from blood cells to epithelial and endothelial cells and recognize the so called microbe-associated molecular patterns (MAMPs; lipids, proteins and nucleic acids) that are evolutionary conserved molecular patterns of the cell wall of microbes (Figure 1). Some PRRs are present on the cell surface, e.g. TLR1, TLR2, TLR4, TLR6, dectin-1 and the mannose receptor, while others reside in the endosome/lysosome compartment or the cytosol, e.g. TLR3, TLR7, TLR9 and NOD2 [11]. The site and degree of expression is highly regulated and, far from being constant, increases in the face of infection and under inflammatory conditions. The spatial distribution is also important, for instance, stimulation of TLR9 at the apical or basolateral site of epithelial cells respectively inhibits or activates NF-κB [31]. Microbial components possess different motifs that are recognized by different PRRs, with a certain degree of specificity, which is actually advantageous as the system is still capable of preventing infection even when a given PRR fails to function. PRRs were originally thought to discriminate "self" from "non-self" [32], but they also recognize endogenous ligands, released after tissue damage including heparan-sulphate, high-mobility group box 1 (HMGB-1), fibrinogen, uric acid and heat shock proteins (HSPs), so called danger-associated molecular patterns (DAMPs) [33]. This enables the innate immune system to respond to "danger" in general, whether or not it stems from infection [34].

PRRs exercise broad regulatory functions in homeostasis and disease [12;35]. In homeostasis sensing of commensal flora contributes to the maintenance of barrier integrity (mediated by TLR2) [36] and maturation of the immune system e.g. by promoting the development of IgA+ plasma cells (TLR5) [37], lymphoid follicles (NOD1) [38] and Peyer's patches (NOD2) [39]. When faced with pathogenic microorganisms, or barrier disruption, PRRs sense MAMPs and DAMPs to elicit powerful protective immune responses in an attempt to restore homeostasis. When infection develops or tissue damage occurs, the ensemble of activated PRRs and subsequently activated intracellular signaling pathways results in the release of a cocktail of cytokines and the activation of different signaling pathways. The resulting "cytokine-profile" defines the inflammatory response and orchestrates the development of the adaptive immune response towards T-helper 1 (Th1), Th2, Th17 or regulatory T cell activity [22;40;41]. The simultaneous activation of multiple

Table 1 Overview of pattern recognition receptors, ligands, and characteristics.

Family of PRR	Name	Adaptors/ signaling proteins	Cellular compartment	Microbial ligands	Micro-organisms	Endogenous ligands
Toll-like receptors (TLR)	TLR-2 (TLR-1 and TLR-6)	MyD88/Tirap	Membrane	Glycolipids, lipoproteins, phospholipomannan, LTA, PGN Pam$_3$Cys, zymosan	Most bacteria Mycobacteria Fungi	HMGB-1 HSP
	TLR-3	TRIF	Endosome/lysosome	Poly I:C dsRNA	Viruses	--
	TLR-4	MyD88/Tirap TRIF TRAM	Membrane	LPS, lipid-A Mannan (O-linked) F-protein for RSV	Gram-negative bacteria Fungi, mycobacteria, RSV	HMGB-1, defensins, heparin, hyaluronate, HSP, fibrinogen
	TLR-5	MyD88	Membrane	Flagellin	Flagellated bacteria	--
	TLR-7	MyD88	Endosome/lysosome	ssRNA	Viruses	--
	TLR-8	MyD88	Endosome/lysosome	ssRNA	Viruses	--
	TLR-9	MyD88	Endosome/lysosome	CpG DNA	Bacteria and mycobacteria Viruses, fungi	--
Nod-like receptors (NLR)	NOD-1	RIP-2 (RICK)	Cytoplasmic	DAP (PGN)	Gram-negative bacteria	--
	NOD-2	RIP-2 (RICK)	Cytoplasmic	MDP (PGN)	Gram-negative and positive bacteria, mycobacteria	--
	NLRP-1-14	Asc, caspase-1 (inflammasome)	Cytoplasmic	MDP (NLRP1, NLRP3) β-glucan (NLRP3)?	Many bacteria, viruses and fungi	Uric acid, alum, silica, hyaluronate, amyloid, cholesterol crystals

	IPAF		Cytoplasmic	Flagellin	Pseudomonas, Legionella Flagellated bacteria	
	NAIP		Cytoplasmic	Flagellin	Legionella, flagellated bacteria	
C-type lectin receptors (CLR)	Dectin-1	Syk/CARD9 RAF-1	Membrane	β-glucan	Fungi, mycobacteria	--
	Dectin-2	Syk/CARD9	Membrane	Mannan (N-linked)	Fungi	--
	DC-sign	RAF-1	Membrane	Mannan (N-linked)	Fungi, mycobacteria	Glycoproteins
	Mannose receptor (MR)	?	Membrane	Mannan (N-linked)	Fungi, mycobacteria	Glycoproteins
RIG-like receptors (RLR)	RIG-I	Cardif	Cytoplasmic	dsRNA	Viruses	--
	MDA-5	Cardif	Cytoplasmic	dsRNA	Viruses	--
Secreted PRR	MBL	--	Secreted	Mannan	Fungi	--
	PGRP	--	Secreted	PGN	Bacteria	--

HMGB-1 = high-mobility group box 1, HSP = heat shock protein, PGN = peptidoglycan, RSV = respiratory syncytial virus.

Figure 1 Microbe-associated molecular patterns (MAMPs) of gram-negative and gram-positive bacteria.

Gram-negative bacteria | Gram-positive bacteria

Lipoproteins (TLR2)
Bacterial DNA (TLR9)
Lipoteichoic acid (TLR2)
Lipopolysaccharide (TLR4)
Peptidoglycan (TLR2)
Flagellin (TLR5)

TLR = Toll-like receptor. Figure adapted from van der Poll and Opal Lancet Infect Dis 2008[128].
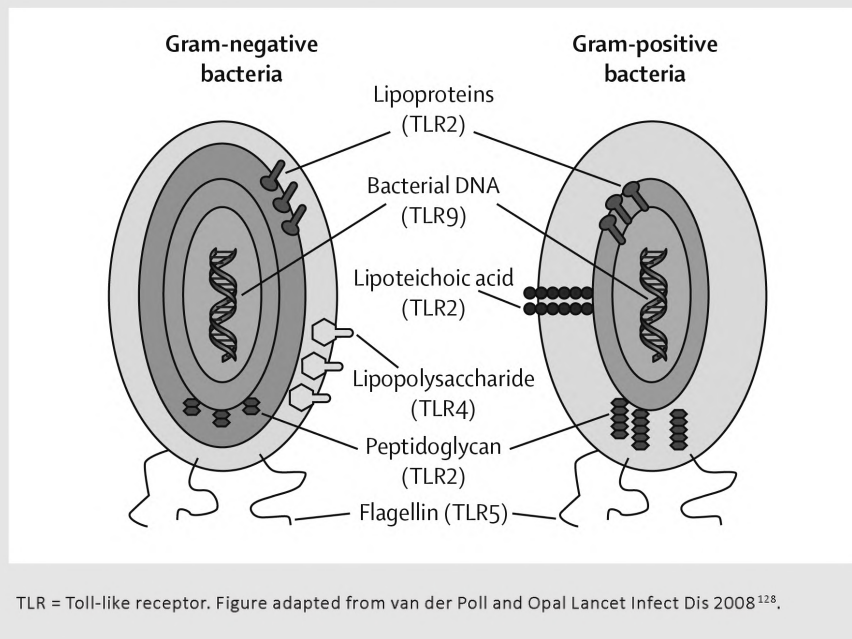
PRRs provides endless possibilities for tailoring this response against a broad range of microbes. However, overwhelming infection, deregulated expression or activation of PRRs, and failing negative feedback mechanisms can result in the collapse of this finely tuned system resulting in infectious disease as well as uncontrolled inflammatory responses that become manifest as auto-immune diseases, the acute respiratory distress syndrome (ARDS) and the systemic inflammatory response syndrome (SIRS) [42-45].

Antimicrobial peptides: nature's antibiotics
Antimicrobial peptides are evolutionarily conserved elements of innate immunity and probably evolved along with the host and its commensal microorganisms and pathogens, by exercising strict control over the pathogenic microbes while preserving beneficial commensal bacteria. More than 700 AMPs have been identified and they are widely distributed in nature, being found in plants, insects, and mammals [46]. In general AMPs are small (peptides containing 12-50 amino acids), amphipatic (having hydrophobic and hydrophilic charged patches), and contain at least 2 positively moieties (arginine or lysine residues). AMPs are produced mainly

by epithelial cells, in particular the Paneth cells of the small intestine, as well as by PMNs, and can be constitutively expressed or induced by the activation of PRRs and pro-inflammatory cytokines. The defensins and cathelicidins are the best characterized in humans and are of major importance in the mucosal host defenses (Table 2).

Pleiotropic functions of AMPs have been demonstrated in the context of host immunity, although not all AMPs share the same set of activities [46;47]. Direct antimicrobial activity is related to the charge and amphipatic structure of these peptides facilitating interaction with microbial cell membranes ultimately resulting in pore formation and subsequent cell death [48]. Other mechanisms of action have also been described, and some AMPs increase microbial clearance by opsonization, facilitating chemotaxis, and by activating phagocytes. Other immunomodulatory activities such as increased production of cytokines by stimulating PRRs, and increased differentiation, maturation and antigen presentation of DCs have been described [46;48]. Notably, deficiencies in, and dysfunction of, AMPs have been associated with an increased risk of infections [49;50] and the occurrence of auto-immune diseases of the skin and gastrointestinal tract [51;52]

The functional profile of AMPs makes them attractive for further investigations as potential agents for treating infectious complications that arise from treating patients for solid tumors and hematological malignancies [46]. Several AMPs are now under investigation as antimicrobial and immunomodulatory agents in an attempt to stave off the increasing resistance of microorganisms to important antimicrobial drugs including quinolones, azoles and cephalosporins, which hampers effective control of bacterial and fungal infections in cancer patients (Table 2).

Innate immunity and SCT

It is important to understand the role of innate immunity in the setting of SCT, because most complications result from treatment effects on host immunity and consist of infectious complications and inflammatory syndromes. These contribute to both the morbidity and mortality of SCT recipients and treating these complications generates considerable costs [53-56]. Therefore exploring ways to modulate the innate immune components involved in these pathological processes could prove to be beneficial by improving SCT outcome and reducing costs.

Infections
After conditioning with chemotherapy and radiotherapy the human immune system is severely perturbed. Conditioning aims to achieve myeloablation to reduce disease burden, and, together with immunosuppression, attempts to create 'room' for the

Table 2 Overview of antimicrobial peptides and their properties.

	Group	Name	Source	Antimicrobial activity	Pleiotropic effects
Natural antimicrobial cationic peptides	α-defensins	HNP-1,2,3,4	Neutrophil granules	Broad spectrum antibacterial Antiviral activity	Chemotactic activity, induction of IL-8 LPS neutralization Promotion of phagocytosis
	α-defensins	HD-5,6	Epithelial cells (intestinal (Paneth cells), urogenital)	Broad spectrum antibacterial	Induction IL-8 Decreased IL-1β release LPS neutralization? Classical complement pathway inhibition
	β-defensins	HBD-1,2,3,4	Epithelial cells (intestinal, respiratory, urogenital, skin)	Broad spectrum antibacterial Anti-Candida activity	Chemotactic activity, induction of IL-8 Activation TLR4 LPS neutralization
	Cathelicidins	LL-37	Neutrophil granules, monocytes Epithelial cells (skin and lung)	Broad spectrum antibacterial Anti-Candida activity	Chemotactic activity, induction of IL-8 Production of mucins LPS and LTA neutralization Promotion of phagocytosis Differentiation iDCs from monocytes
	Histatins	Histatin-5	Salivary glands	Antibacterial Anti-Candida activity	--
Synthetic commercial antimicrobial peptides	Group	Derivative	Intended use	Antimicrobial activity	Pleiotropic effects
	Human lactoferrin	hLF1-11, LFcinH	Treatment of bacteremia and fungal infections in SCT (hLF1-11)	Broad spectrum antibacterial, antifungal, and antiviral activity	Promotion of phagocytosis Increased IL-10 production LPS neutralization (LFcinH)

Bovine lactoferrin	LFcinB, LFampin	Antimicrobial agent
IDR	IDR-1, IDR-1002	Antimicrobial agent
Indolicidin	Omiganan	Prevention catheter infections
Protegrin	Iseganan	Oral mucositis
BPI	$rBPI_{21}$	Endotoxin neutralization in SCT

IDR = innate defense regulator, BPI = bactericidal/permeability-increasing protein.

Broad spectrum antibacterial, antifungal , and antiviral activity	LPS and CpG DNA neutralization Classical complement pathway inhibition
Broad spectrum antibacterial, however indirect	Induction IL-8 and MCP-1 Increased IL-10 production
Broad spectrum antibacterial and antifungal activity	--
Broad spectrum antibacterial and antifungal activity	--
Gram-negative bacteria	LPS neutralization

stem cell graft and prevent its rejection. Consequently, there is a prolonged period of severe immune failure. Use of immunosuppressants for acute GvHD prophylaxis contributes further to this immunocompromised state, particularly with respect to the acquired immune system. After allogeneic SCT reconstitution of acquired immunity occurs slowly, and can last up to a year or even longer [57;58]. During this period patients are at risk of developing infections due to bacteria, viruses, fungi, and other opportunistic pathogens [59-61].

Acquired immunity is effectively absent for a prolonged period leaving only the remnants of the innate immune system to defend patients against infection [62]. However, the innate immune system also suffers damage from conditioning that results in neutropenia, epithelial barrier damage, and lower production of AMPs and salivary secretions, although its duration is much shorter, lasting mostly 3-4 weeks [58;63;64]. The risk of developing infectious complications is at its highest during this time.

Current knowledge is limited as to which components of the immune system remain sufficiently intact to contribute to the antimicrobial defenses after SCT, although it is generally believed that the macrophages and antigen presenting cells (APCs) that reside in the tissues remain after conditioning. Furthermore, Paneth cells in the small intestine are mostly spared from chemotherapy-induced damage, how well they still function is not known [65]. What exactly happens at the epithelial barriers of gastrointestinal tract and the lung remains largely speculative, as investigations of the mucosal surfaces during SCT in humans have not been performed primarily because of technical difficulty.

An essential question remains why there is so much difference in inter-individual risk for infectious complications even to patients treated within the same protocols. This probably relates to differences in the damage inflicted upon the innate immune system, the magnitude of MBI, patient factors such as age, gender and co-morbidities, prior treatments, but also differences in interventions such as the use of prophylactic antimicrobials. However, differences in innate immune genes might also explain, in part, the apparent susceptibility of individual patients [62;66].

Inflammatory complications
Inflammatory complications occurring during SCT constitute a group of overlapping and successive disorders designated MBI [2], sepsis, acute GvHD [67], idiopathic pneumonia syndrome [68], and engraftment syndrome [69]. The induction by the conditioning treatment of tissue damage, especially of epithelial barriers of the gut and lung, appears central to the pathogenesis that leads to the unrestrained inflammation resulting from excessive release of pro-inflammatory cytokines elicited by activation of PRRs by various microbial motifs and endogenous ligands (Figure 2 and 3). The role of innate immunity and PRR-induced inflammation has also been implicated in the process of graft rejection [70].

Mucosal barrier injury

Chemotherapy and radiotherapy damage the oral and gastrointestinal mucosa initiating an inflammatory cascade that culminates in MBI, which manifests itself clinically as mucositis. The pathogenesis of MBI is thought to consist of five phases [2;3;71]: 1) the activation of nuclear factor-κB directly by chemo/radiotherapy and indirectly from formation of reactive oxygen species (ROS), DNA, and non-DNA damage [72], 2) production and release of pro-inflammatory cytokines and chemokines (IL-1, IL-6, IL-8, tumor necrosis factor (TNF) α, IL-23, interferon (IFN) γ) by macrophages, IECs and endothelial cells [72-77], 3) positive feedback loop of TNFα, epithelial cell apoptosis and increased mucosal permeability [78;79], 4) translocation of microbes or microbial wall components aggravating inflammation ("cytokine storm") [80-82], and 5) repair and healing. Although the impact of microbes and their cell wall components on the inflammatory response is of secondary importance, stimulation of PRRs by MAMPS translocating the disrupted mucosal barrier, or the invasion of microorganisms leading to bacteremia and endotoxemia, appear to aggravate inflammation (Figure 2). This could also so be the case for DAMPs, which are released as a result of tissue damage, and in this regard the endogenous ligands HMGB-1 and HSP might be of particular interest (Figure 2) [83].

Graft-versus-host disease

Acute GvHD results from an allo-immune response directed at host antigens after engraftment and is accompanied by the activation of alloreactive T lymphocytes that attack the host tissues. However, MBI following conditioning is also thought to play an important role in the initiation phase of acute GvHD [5;74]. Chemotherapeutic agents, radiation, pro-inflammatory cytokines (IL-1β, TNFα, IFNγ) and stimulation of PRRs by DAMPs and MAMPs activate both APCs and epithelial cells which increase their expression of co-stimulatory cytokines, minor histocompatibility complex antigens (miHags) and major histocompatibility complex (MHC) molecules [70;82;84-89]. Furthermore, chemokines (CXCL10, CXCL8) are released to attract alloreactive T- and NK-cells to the secondary lymphoid tissues of target organs [90;91]. In the second phase activated host APCs, and to a lesser extent, donor APCs, present host antigens to alloreactive T-lymphocytes [84;92-94]. Activation and proliferation of T lymphocytes, predominantly Th1 lymphocytes and probably Th17, then ensues and is crucial to the pathogenesis of GvHD. In addition, APCs imprint alloreactive T-cells with more or less tissue specific homing molecules [95]. During the last phase trafficking of alloreactive T- and NK-cells to inflamed tissues occurs [91;96] leading to their damage as result of the cytotoxic effects and the release of Th1 and Th17 type cytokines [67;93]. Subsequently, at least in intestinal GvHD, mucosal damage again leads to amplification of inflammation, through translocation of bacterial products, increasing cytokine release and stimulating Th1 and Th17 responses [70;82;97;98] (Figure 2 and 3).

Figure 2 Mucosal barrier and innate immunity.
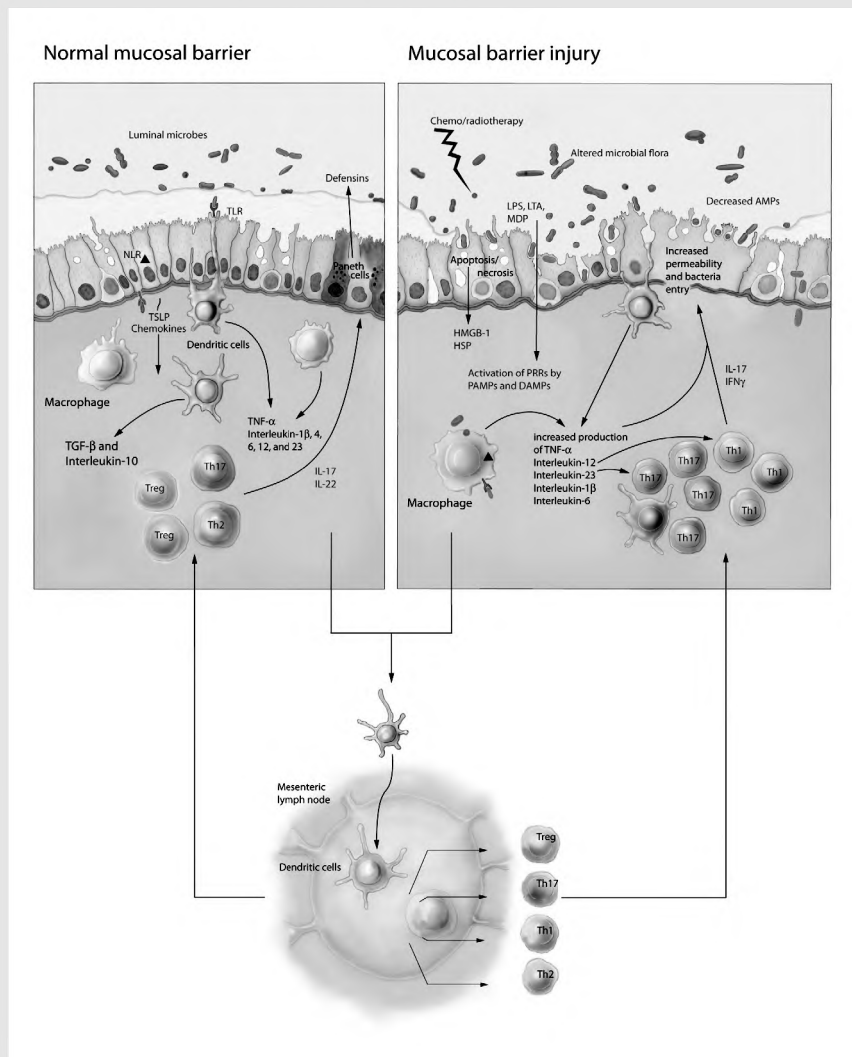
In health there is a tightly controlled immune balance at the mucosal barriers with tolerance to commensal flora, and absence of tissue damage. PRRs sensing MAMPs play an import role in maintaining this homeostasis. The microbial flora is kept in check, for instance through the release of AMPs, and immune responses are dampened by preservation of the physical barrier, selective expression of PRRs, and beneficial properties of commensal microorganisms. There is a low state of immune activation and inflammation ('physiological inflammation'), that contributes to the development of a healthy local and systemic immune system and keeps the immune system on stand-by (left panel). During health, innate immune responses modulate the acquired immune responses towards a tolerogenic profile contributing to the homeostasis.

Due to MBI resulting from cytotoxic therapy and mucosal damage resulting from GvHD the mucosal barriers and immune homeostasis are perturbed. Microorganisms and MAMPs are able to translocate and induce inflammation by activating PRRs. This is aggravated by the release of DAMPs released on the occurrence of tissue damage. The microbial flora is not kept in check with an increase in pathogenic microorganisms (dysbiosis) (right panel). Deregulated innate immune responses contribute to the development of inflammatory complications by eliciting pro-inflammatory acquired immune responses which can further damage the mucosal barriers. TLR = Toll-like receptor, NLR = Nod-like receptor, TSLP = thymic stromal lymphopoietin, TGF-β = transforming growth factor beta, TNFα = tumor necrosis factor alfa, IFNγ = interferon gamma, IL = interleukin, HMGB-1 = high mobility group box 1, HSP = heat shock protein, LPS = lipopolysaccharide, LTA = lipoteichoic acid, MDP = muramyl dipeptide, Th = T helper lymphocyte, Treg = regulatory T cell, PAMP = pathogen-associated molecular pattern, DAMP = danger-associated molecular pattern, and AMP = antimicrobial peptide. Illustration by Tine Thörig wetenschappelijke illustraties.

The inflammatory conditions mainly involve the gastrointestinal tract, highlighting the importance of host-microbial interactions in creating the risk and defining the magnitude of these complications. Some patients suffer from severe inflammation and acute GvHD while others do not. These inter-individual differences result from variation in the magnitude of damage inflicted on the innate immune system, the composition of the microbial flora [99;100], mismatches in MHC and miHags, the severity of MBI [101], and also differences in the genes for innate immunity and other non-HLA genes involved in the expression and function of PRRs, cytokines, and NK cell activation.

Non-HLA immunogenetics in SCT

Individual differences in transplant-related complications, has stimulated the growing interest in the impact of single nucleotide polymorphisms (SNPs) in non-HLA immune genes on the occurrence of infections, MBI and acute GvHD in SCT [102-105]. The genes of particular interest are those involved in the production and activity of cytokines and chemokines, innate immune genes such as the PRRs and AMPs, and killer cell immunoglobulin-like receptor (KIR) genes in NK cells. The concept of environmentally determined genetic expression (EDGE) was introduced by *Kallianpur et al.* to account for the observations [106]. EDGE is based on the following observations: 1) genetically encoded variations in expressed proteins have different effects in different environmental contexts; 2) a disease phenotype is determined by both the functional magnitude of the genetic change and the severity of the environmental change; 3) rare genetic disorders (e.g. inborn errors of metabolism) represent one extreme with little contribution from the environment, while massive environmental insults result in phenotypes independent of genetic variation; and 4) most diseases/phenotypes fall between these extremes (Figure 4).

Figure 3 Hypothesis on the relationship between MBI and inflammatory complications post SCT.
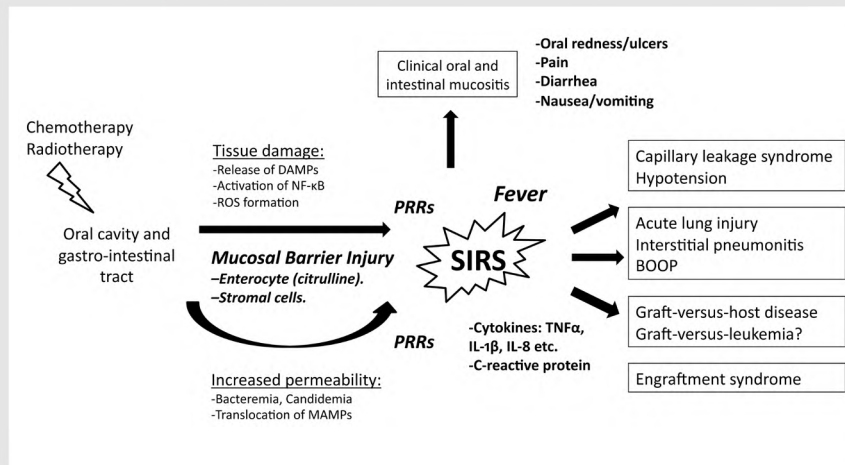
Chemotherapy and radiotherapy induce tissue damage in particular to the mucosal barriers of the oral and intestinal tracts. Activation of NF-κB, ROS formation, release of DAMPs and increased mucosal permeability resulting in translocation of MAMPs which activate PRR-pathways ultimately result in a systemic inflammatory response which is associated with the occurrence of complications including the systemic inflammatory response syndrome, acute lung injury, and graft-versus-host disease. SIRS = systemic inflammatory response syndrome, BOOP = bronchiolitis obliterans organizing pneumonia, PRR = pattern recognition receptor, MAMP = microbe-associated molecular pattern, DAMP = danger-associated molecular pattern

Exposure to high-dose chemotherapy presents a considerable insult that may unmask the effects of normally silent genetic polymorphisms.

Many studies have been performed on the impact of SNPs in non-HLA genes in SCT, especially regarding cytokines, PRRs, and the complement system (mannose-binding lectin (MBL)) [102-104]. However, contradictory results and a lack of consistency make drawing firm conclusions difficult, and impede the development of strategies to ameliorate SCT-related complications in clinical practice. This arises from problems associated with research into genetic linkage, including sample size, selection bias, genetic diversity/heterogeneity between populations, and clinical context [107;108]. Nevertheless, at least for the time being, studying these polymorphisms provides insight into the pathogenesis of the complex immunological processes that take place after SCT. In the future detection of genetic polymorphisms that are consistently associated with disease could result in therapeutic and preventive approaches, such as altered donor selection, risk adapted antimicrobial prophylaxis, and use of agents modulating PRR-pathways. Then, screening for SNPs

Figure 4 The concept of environmentally determined genetic expression (EDGE).
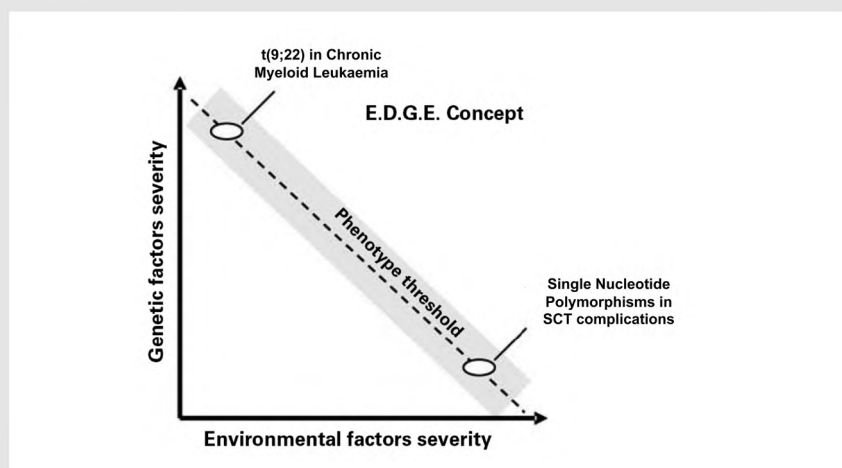
The role of genetic defects on the development of diseases depends at the one hand on the degree of functional consequences of a particular defect and on the other hand on environmental factors and confounders. Figure adapted from Kallianpur *et al*. Bone Marrow Transplant 2005 [106].

in non-HLA genes should be implemented in clinical practice. At present, the most promising candidates are gene polymorphisms in NOD2/CARD15 [109], TLR4, the IL-23 receptor [110], MBL [111;112], IL-10 and TNFα [113-115]. However, many others genes remain to be evaluated and investigated in SCT as new PRRs, cytokines, and complement molecules are discovered every year.

At the same time, developments in genetic research in other areas of medicine should be followed closely. For instance, new interesting SNPs and mutations have been found in various diseases with the help of genome-wide association studies [108;116]. Since many genes are related to host immunity any new findings might be of interest in the setting of SCT.

Antimicrobial peptides in SCT

In current practice, supportive care measures in SCT are aimed at preventing and treating infections and inflammatory complications using antimicrobial agents, granulocyte colony-stimulating factors (G-CSF), corticosteroids, and other immuno-modulators. Using reduced intensity conditioning regimens, mucosal protectants

or growth factors such as keratinocyte growth factors (KGF) [117] are also ways of attenuating the tissue damage resulting from conditioning, although with variable success. Strategies are often rather artificial, or at best remedies, so approaches in which the physiological state of innate immunity is preserved, improved or restored are preferred. The application of growth factors such as G-CSF and KGF is a good example of this.

Introducing AMPs in the setting of SCT would be of particular interest for several reasons [46;118;119]. Firstly, decreased expression of AMPs has been shown to be linked to the occurrence of human diseases [120;121]. For instance, SNPs in genes of the Paneth cells, the most important source of AMPs in the intestine, result in decreased production of both α-defensins (HD-5 and 6) and β-defensins (hBD2) and have been linked to the pathogenesis of inflammatory bowel diseases [122;123]. This has been confirmed for SNPs in NOD2, the PRR for muramyl dipeptide, and SNPs in the Wnt signaling pathway, a pathway important in Paneth cell development and homeostasis [124;125]. Therefore, administering AMPs to correct the deficit, especially in those individuals who have a genetic disadvantage, might be therapeutically valuable. Secondly, these naturally occurring peptides possess several important attributes that could be exploited in the setting of SCT. The broad spectrum antimicrobial activity of AMPs with antibacterial, antiviral, and antifungal activity, even against multi-resistant microorganisms, would be of great importance in the treatment of infections after SCT. At the same time AMPs can dampen potentially harmful pro-inflammatory responses by scavenging MAMPs, that translocate across the damaged gastrointestinal tract during MBI and acute GvHD. In addition, the immune enhancing activity of AMPs might contribute to the prevention of infections and help accelerate immune reconstitution.

Many cationic AMPs have been developed and studied extensively *in vitro*; however few have been studied *in vivo* and even fewer in humans (Table 2). One of the exceptions, iseganan, an analog of protegrin, has been tested in phase III clinical trials for the prevention and treatment of radiotherapy- and chemotherapy-induced oral mucositis, but the results were disappointing [126;127]. In fact, despite their promise *in vitro*, none of the commercially developed AMPs has yet proved safe and efficacious in the treatment of any human diseases. So, their introduction into clinical practice is likely a long way away as studies are just starting to be designed to generate the answers we need before we can employ AMPs as new clinical therapeutics.

Aims and outlines of this thesis

The thesis consists of three complementary parts addressing the theme of innate immunity and host-microbial interactions in the setting of hematopoietic SCT with a special focus on infectious and inflammatory complications.

In the first part 'the stage was set' by investigating the factors that contribute to the inflammatory response and the complications seen after conditioning with chemo- and radiotherapy to prepare for a SCT. Historically, the focus has been on neutropenia and the resulting infectious complications, but in recent years the role of MBI and alterations in host-microbial interactions at mucosal barriers have gained prominence. Our hypothesis was that the degree of MBI, rather than neutropenia, determines the onset and extent of inflammation and influences the incidence of complications post-SCT. We also wanted to better define the role of bacteremia in this process. We describe the results of two retrospective studies of patients who had received an autologous or allogeneic SCT in our hospital (**chapter 2 and 3**).

In the second part of the thesis we explored the impact of SNPs in two different innate immune genes, namely, NOD2 and dectin-1, on the outcome of SCT recipients. Both NOD2 and dectin-1 are PRRs involved in the sensing of microorganisms and MAMPs at epithelial and mucosal surfaces of the human body. In previous studies it has been shown that the presence of NOD2 polymorphisms, which were originally described in Crohn's disease, exerted a significant impact on the incidence of severe acute GvHD and treatment-related mortality (TRM). Besides supporting the role for host-microbial interactions in SCT, these results suggest that looking for these polymorphisms might have clinical consequences with respect to antimicrobial therapy and prophylaxis. One particular problem with SNP studies has been that documented associations are very much context dependent and therewith often difficult to confirm and reproduce. Therefore, we set out to confirm the impact of NOD2 polymorphisms on severe acute GvHD and TRM, especially as we perform partial T cell-depletion, and looked for the impact of NOD2 polymorphisms on bacteremia. We analyzed the data from a homogenous cohort of patients who all had received myeloablative conditioning containing idarubicin for SCT (**chapter 4**).

The dectin-1 polymorphism Y238X was shown to result in a "loss-of-function" with defective *Candida* binding and cytokine production and had been recently discovered in a Dutch family with several subjects suffering from recurrent mucocutaneous candidosis. We investigated the impact of this polymorphism on *Candida* colonization, systemic *Candida* infections, and invasive mould infections occurring after SCT (**chapter 5**) to see whether it was possible to better tailor prophylaxis to the individual patient's needs. SNPs associated with the occurrence of fungal infections, if consistent and confirmed, could be used to perform risk stratification.

Dectin-1 is activated by the fungal cell wall component β-glucan which results in both innate and acquired immune responses. Therefore, we studied the impact of the dectin-1 status as well as *Candida* colonization on the occurrence of acute and chronic GvHD, relapse, relapse-related mortality, and overall survival (***chapter 6***).

In the third part we investigated the role of the antimicrobial protein human lactoferrin and a peptide derivative - human lactoferrin 1-11 (hLF1-11) - in the treatment of SCT recipients. Human lactoferrin is a natural defense protein present in body fluids and secretions as well as neutrophils, and has pleiotropic functions including anti-microbial activity, anti-tumor activity, regulation of cell growth and differentiation, and modulation of inflammatory, humoral and cellular immune responses. Levels of lactoferrin are decreased following SCT, contributing to the overall immune deficiency, so correcting this deficit might enhance immunity in SCT recipients. Preclinical studies showed promising antimicrobial activity of hLF1-11 even in immunodeficient mice, and being a derivative of a 'natural' protein it might have fewer disadvantages like side effects and occurrence of antimicrobial resistance. We first described the theoretical background, summarizing the potential roles of lactoferrin, derivatives and other AMPs in the treatment of SCT-related complications, including infections, MBI and GvHD (***chapter 7***). Next, after confirming that the drug was safe in healthy volunteers, we investigated the tolerability and safety of a single dose of hLF1-11 in a clinical trial involving patients receiving an autologous SCT (***chapter 8***). At the same time *in vitro* studies were performed to determine the immunomodulatory activity of hLF1-11, and establish the presence of any of the pleiotropic activities possessed by lactoferrin and other AMPs (***chapter 9***).

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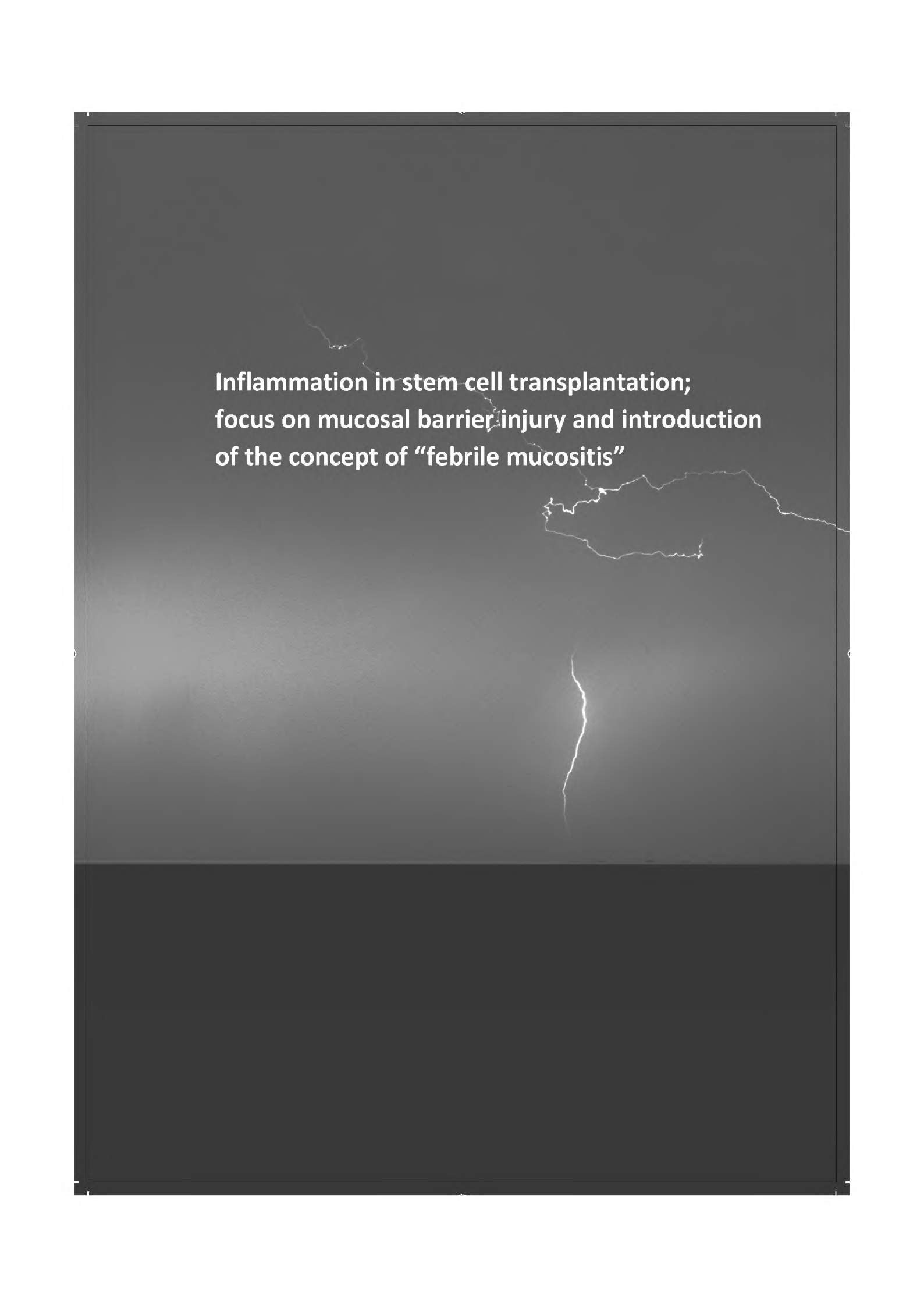
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Inflammation in stem cell transplantation;
focus on mucosal barrier injury and introduction
of the concept of "febrile mucositis"

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Febrile mucositis in hematopoietic stem cell transplantation recipients

W.J.F.M. van der Velden, N.M.A. Blijlevens, T. Feuth, and J.P. Donnelly

Abstract

We undertook a retrospective analysis of a cohort of sixty-seven patients with multiple myeloma who had received an autologous HSCT following high dose melphalan to explore the impact of mucositis on the systemic inflammatory response. A homogenous group of 16 patients without a documented infection and a group of 30 patients with bacteremia were identified for whom complete data on neutropenia, an inflammatory response, infectious complications and mucositis were available. All patients showed a similar course of events with an inflammatory response coinciding with the occurrence of significant mucositis, regardless the presence or absence of infection. The only differences between the two groups were significantly higher maximum C-reactive protein (CRP) levels and lower citrulline levels for patients with bacteremia, suggesting a causative role for mucositis in the occurrence of bacteremia. Statistical analysis showed a significant association over time between citrulline levels, to a lesser extent bacteremia, but not neutropenia, and the inflammatory response measured by CRP. These data suggest that the inflammatory response after conditioning for a HSCT is the result of the chemotherapy-induced mucositis and independent of neutropenia. Though primary inflammation appeared due to mucositis, infections resulting from mucosal barrier injury and neutropenia aggravated the inflammatory response.

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Introduction

Four decades have passed since Bodey *et al.* [1] reported that neutropenia accompanied by subsequent infectious complications was the main determinant of fever during neutropenia ("febrile neutropenia"). However, they also showed that this relationship did not hold for febrile patients who were not infected, suggesting that neutropenia was not the sole determinant of fever [1]. Nonetheless, febrile neutropenia has been regarded one of the most important complications of cancer therapy and therefore prevention and treatment with anti-microbial drugs and granulocyte colony stimulating factors have been studied extensively [2-5]. However, many of these studies also reported that a substantial number of patients remained febrile without a infection ever being documented [2]. Hence, such episodes of fever were designated "unexplained fever" [6].

Fever is a consequence of the release of cytokines. Many different inducers of these cytokines exist in hematopoietic stem cell transplantation (HSCT) recipients, and by no means are all of these related to infections. Chemotherapy and radiotherapy used in conditioning therapy damage the oral and gastrointestinal mucosa initiating an inflammatory cascade, culminating in mucosal barrier injury (MBI), which manifests itself clinically as mucositis. The pathogenesis of MBI is thought to consist of five phases: 1) the activation of nuclear factor-κB, 2) release of pro-inflammatory cytokines, 3) epithelial cell apoptosis and increased mucosal permeability, 4) translocation of microbes or microbial wall components and 5) repair and healing [7-9]. Disruption of the mucosal barrier is also an important risk factor for the occurrence of bacteremia [10-12] and candidemia [13]. However, the impact of microbes on the inflammatory response may not be necessary since MBI alone appears able to elicit a systemic inflammatory response evidenced by elevated C-reactive protein (CRP) levels and fever. This is supported by clinical data showing that patients receiving chemotherapy experience more fever with or without infections in the presence of severe mucositis [14]. This suggests mucositis to be a significant independent risk factor for the occurrence of fever and infections of HSCT recipients.

In order to determine the impact of mucositis on the systemic inflammatory response, and the occurrence of "febrile neutropenia", a retrospective analysis was conducted in a cohort of recipients of an autologous HSCT following high dose melphalan therapy (HDM) who had no identifiable infectious complications. We compared the course of inflammatory events in this group with those having a bacteremia during their HSCT.

Methods

Study population

We performed a retrospective analysis of 67 consecutive patients with multiple myeloma who had been given an autologous HSCT following HDM in our hospital from May 2004 to December 2006. Data on mucositis, inflammation and infections had been prospectively gathered from the day of starting chemotherapy (day 1) until discharge.

All patients were treated according to the same protocol and managed with a central venous catheter (CVC). The preparative regimen consisted of HDM (200 mg/m^2) administered over two days on days 1 and 2. On day 4, all patients were given an autologous HSCT containing at least 2.0 x 10^6 CD34+ cells per kg. Patients did not receive hematopoietic growth factors and anti-microbial prophylaxis consisted of 500 mg ciprofloxacin given twice daily and 500 mg valaciclovir given three times daily. Fluconazole was given at 200 mg a day only to those who were colonized with *Candida albicans*. Blood (10 ml) was drawn for aerobic culture each Monday and Thursday from each lumen of the CVC. A blood culture was considered positive if one or more bottles yielded a microorganism, except for coagulase-negative staphylococci, for which two separate positive blood cultures yielding the same strain were required to be considered to represent bacteremia [15].

Plasma was obtained on the same days to detect *Aspergillus* galactomannan-antigen (Platelia Aspergillus EIA test; Biorad Laboratories, Veenendaal, The Netherlands). Axillary temperature was recorded four times a day and fever was defined by a single axillary temperature ≥ 38.5°C. At the onset of fever 40 ml of peripheral blood was obtained for culture together with 10 ml from each lumen of the catheter, the patients were examined for any signs of local infection, empirical therapy was started with 2000 mg ceftazidime given three times daily intravenously, and a chest X-ray was ordered. Blood cultures were obtained again on the 4th day of empirical therapy and a thorough clinical examination was done. A high-resolution computerized tomography scan of the lungs was undertaken for patients who were still febrile without there being an obvious cause to detect any signs of invasive fungal disease. Where possible, bronchoscopy was carried out if indicated to obtain a bronchoalveolar lavage, which was then subjected to a comprehensive microbiological examination including detection of *Aspergillus* antigen.

Neutrophil count

The absolute neutrophil count (ANC) was determined daily and neutropenia was defined as an ANC ≤ 0.5 x10^9/l, with profound neutropenia being defined as an ANC ≤ 0.1 x10^9/l. The duration and depth of neutropenia was recorded for each patient.

Inflammatory response

CRP levels (mg/l) were determined every day and the maximum CRP (CRP max) and day of CRP max were recorded.

Mucosal barrier injury assessment

Oral mucositis was graded daily according to the validated Nijmegen Nursing Mucositis Scoring System (NNMSS) [16]. Also the maximum value and day of maximum value were recorded. Plasma citrulline was determined to estimate gut mucositis before the start of HDM and three times weekly thereafter until discharge [17]. The day of nadir and the lowest citrulline levels were recorded for each patient.

Study group selection

Two patient groups were selected. The first group consisted of patients for whom no microbiologically defined or a clinically defined infection had been identified according to the Consensus definitions of Immunocompromised Host Society [6]. The second group consisted of patients with bacteremia according to the same consensus definitions.

Data analysis

We employed descriptive statistics for fever, neutrophil count, CRP level, NNMSS and citrulline levels which were expressed as mean values together with the 95% confidence interval, with the use of SPSS v 14.0. Comparison of the variables was done using the independent t-test, Mann-Whitney U-test, χ^2-test or Fisher's exact test when appropriate.

Citrulline was measured three times weekly; therefore the real citrulline nadir might be attained between two measurements and hence remained unobserved. To study the mean of the possibly unobserved citrulline nadirs and the day of occurrence we studied the course of citrulline as a function of time during the first 18 days by developing a cubic linear mixed model with first, second and third power of time as predictors.

To describe the relationship of neutrophil count, mucositis (NNMSS and citrulline) and bacteremia on CRP we used several linear mixed models for the first 18 days and implemented the logarithmic transformed outcome variable *log10* CRP. The covariance structure could adequately be modeled with the heterogeneous compound symmetry.

Because the citrulline levels were measured three times weekly, this variable had missing values. The mechanism of these missing data was considered to be completely at random. Therefore, we used the SAS procedure PROC MIXED, which handles missing data without introducing bias.

A P-value of <0.05 was considered to indicate statistical significance.

Results

Study population:
In the total cohort of 67 subjects 19 patients had no documented infection (Table 1). However, data on citrulline levels were lacking for 3 patients, leaving 16 patients eligible for analysis. A total of 40 patients had a microbiologically defined infection, 37 with bacteremia due to Gram positive bacteria (Table 1). Data on citrulline levels were missing in 7 cases resulting in a final cohort of 30 patients with bacteremia. There were no statistically significant differences in age, body mass index, gender distribution or dose per kg of melphalan (Table 2).

Table 1 Study population.

Total study population	N = 67	
No infection	N = 19	
Microbiologically defined infection*:	N = 40	
- OVS bacteremia.	-	N = 17
- OVS and CoNS bacteremia.	-	N = 12
- CoNS bacteremia.	-	N =5
- Bacteremia (*Aerococcus*, *Gemella*, *Bacillus*).	-	N =3
- Fusariosis.	-	N =1
- Candidemia.	-	N =1
- Generalized herpes zoster infection.	-	N =1
Clinically defined infection*:	N = 7	
- Pneumonia.	-	N = 6
- Thrombophlebitis.	-	N = 1
Non-infectious inflammatory condition.	N = 1	

*Definitions according to the Immunocompromised Host Society definitions[6].
OVS = oral viridans streptococcus and CoNS = coagulase negative staphylococcus.

Neutrophil count
All patients experienced neutropenia for a mean duration of 9 days, which was profound in all cases for a mean duration of 6 days (Figure 1a). No significant differences existed between patients without infections and those with bacteremia (Table 2). Neutropenia with ANC $\leq 0.5 \times 10^9$/l occurred a mean of 9 days after starting chemotherapy (5 days post-HSCT).

Inflammatory response, fever and bacteremia
The course of mean daily CRP levels during HSCT is illustrated in Figure 1b. CRP levels were elevated for all patients in both study groups starting on average on

Table 2 Patient characteristics and results.

Characteristics	No infection N=16 Mean (95% CI) Unless stated otherwise	Bacteremia N=30 Mean (95% CI) Unless stated otherwise	P-value
Age (years)	57 (53-61)	55 (52-58)	0.343[a]
Gender: male	56.3%	63.7%	0.639[c]
HDM mg/Kg	4.9 (4.7-5.1)	5.0 (4.8-5.2)	0.373[a]
BMI	26.3 (24.7-27.9)	26.6 (25.0-28.2)	0.922[a]
Fever (%)	69%	100%	0.003[d]
Day fever	11.9 (10.9-12.9)	12.0 (11.5-12.5)	0.799[a]
Neutrophils ≤ 0.5 x10^9/l (days)	9.2 (8.4-10.0)	8.6 (8.1 -9.2)	0.240[a]
Neutrophils ≤ 0.1 x10^9/l (days)	6.0 (5.5-6.5)	5.6 (5.2-6.0)	0.228[a]
Day neutrophils ≤ 0.5 x10^9/l	8.9 (8.5-9.3)	8.5 (8.2-8.8)	0.144[a]
Day CRP max	14.2 (13.3-15.1)	14.1 (13.5-14.6)	0.768[a]
Mean CRP max (mg/l), median (range)	94 (30-153)	193 (54-384)	0.003[b]
Day peak mucositis	11.9 (10.9-12.8)	11.8 (11.4-12.2)	0.898[a]
Mean max mucositis	8.3 (6.9-9.7)	8.4 (7.8-8.9)	0.950[a]
Day nadir citrulline (observed)	13.6 (12.4-14.7)	13.5 (12.9-14.2)	0.827[a]
Day nadir citrulline (predicted)	14.1 (13.7-14.5)*	14.1 (13.8-14.4)*	1.0[e]
Citrulline nadir (μmol/l) (observed)	7.3 (5.8-8.7)	5.2 (4.6-5.9)	0.004[a]
Citrulline nadir (μmol/l) (predicted)	6.9 (6.0-7.8)	5.4 (4.7-6.1)	<0.0001[e]

a: independent *t*-test, b: Mann-Whitney U-test, c: χ^2-test, d: Fisher's exact test, e: *P*-value resulting from linear mixed model. *Confidence intervals based on bootstrap percentiles (150 replications). A *P*-value of <0.05 was considered statistically significant. HDM = high dose melphalan, CRP = C-reactive protein, BMI = body mass index.

day 10 (6 days post-HSCT), and reaching a maximum on day 14 (10 days post-HSCT). The course was similar but there was a significant difference in the mean CRP max (193 vs. 94 mg/l (*P* =0.003)) (Table 2).

All patients with bacteremia became febrile as did 69% of patients without infections (*P* =0.003), though the mean onset of fever was 12 days (8 days post-HSCT) in both cases. Although not febrile according to our definition five patients without fever had an elevated body temperature from baseline up to 38.0°C.

Bacteremia occurred a mean of 12 days after starting chemotherapy in the cohort of infected patients, mostly 2 days after the start of the inflammatory response.

Figure 1 Course of neutrophil count, mucositis and inflammatory response following HSCT after HDM conditioning in patients without infectious complications (closed lines) and in patients with bacteremia (dashed lines).
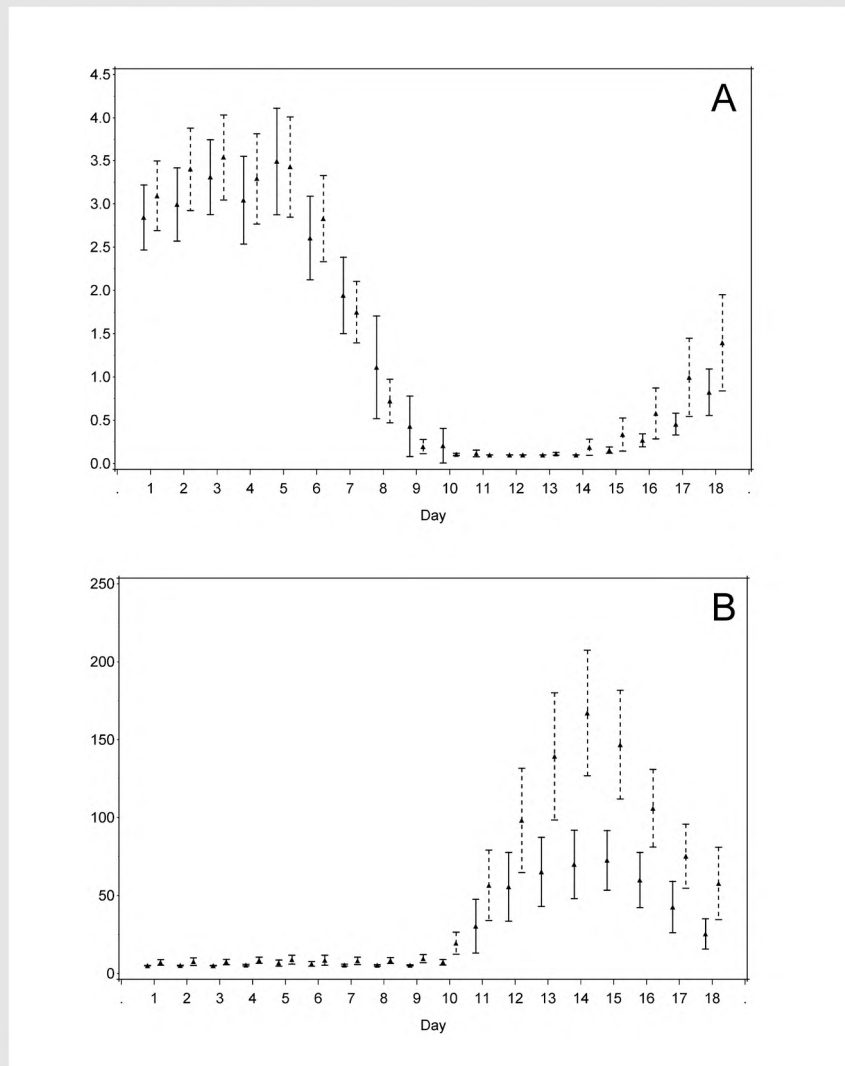
Course of neutrophil count (x10⁹/l) (a), C-reactive protein (CRP) levels (mg/l) (b) mucositis score according to the Nijmegen Nursing Mucositis Scoring System (NNMSS) (c) and citrulline levels (µmol/l) (d) following HSCT after conditioning with HDM. Day 1 is the day of start of chemotherapy. Day 4 is the day of transplantation. Values are expressed as mean values with 95% confidence interval (CI).

2

Figure 1 Continued.
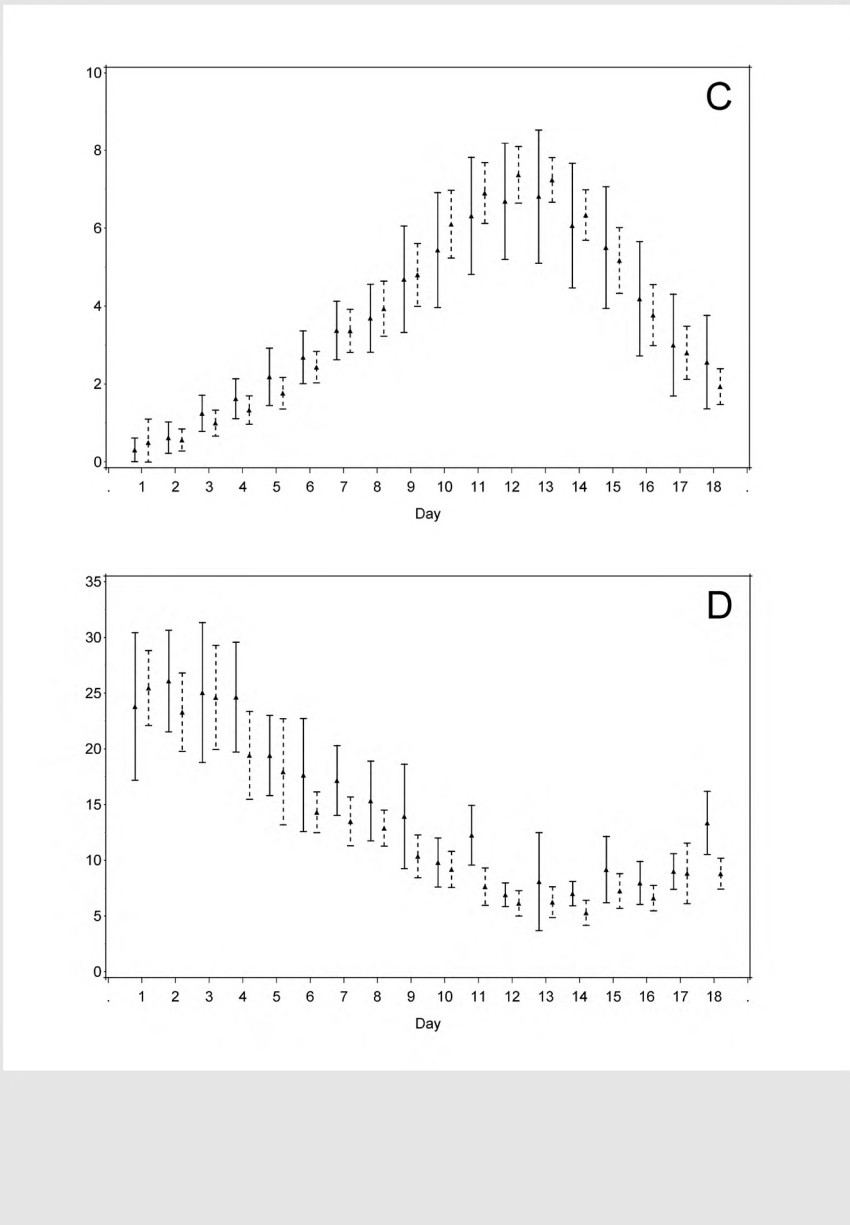
Mucosal barrier injury

Patients in both groups suffered from oral mucositis with a mean maximum score of 8.3 and 8.4, both on day 12 (8 days post-HSCT, Figure 1c).

In both study groups mean baseline citrulline levels were 25.5 and 23.8 µmol/l for those without infections and those with bacteremia respectively, and there was a marked decrease with nadir occurring on day 14 (10 days post-HSCT, Figure 1d, Table 2). Importantly, in both study groups on day 10 most patients had already passed the citrulline threshold of 10 µmol/l indicating severe mucosal atrophy [18]. Furthermore, there was a statistically significant difference in the nadir of citrulline between the two groups (6.9 vs. 5.4 µmol/l), with a *P*-value of <0.0001.

Relationship variables and CRP

In longitudinal univariate analysis, citrulline and NNMSS score were significantly associated with *log10* CRP (both *P* <0.0001), as opposed to neutrophil count (*P* =0.11). However, in the model with both citrulline and NNMSS as predictors for *log10* CRP, only citrulline contributed significantly (*P* <0.0001 vs. *P* =0.69). Models with neutrophil count combined with citrulline, NNMSS, or both, came up with non-significant estimates of the regression coefficients for neutrophil count. We concluded that the best model to predict the CRP values in a patient from day 1 until 18 only uses citrulline levels.

Although bacteremia is significantly associated with increased CRP levels (*P* =0.003), in a model with citrulline and bacteremia as independent variables and outcome *log CRP* the contribution of bacteremia becomes insignificant (*P* =0.939). This implies that given the value of citrulline, the presence or absence of bacteremia does not influence the value of CRP, but given the status of bacteremia, citrulline values are still significantly negatively correlated with CRP.

Discussion

In our study, patients receiving an autologous HSCT, who were free of clinically or microbiologically defined infections [6], still elaborated a significant inflammatory response as evidenced by marked elevations of CRP and an incidence of fever of 69%. All patients suffered from significant mucositis of the oral cavity and gut that coincided with the occurrence of the inflammatory response (Figure 1). Five patients without fever never required empirical anti-microbial therapy and their clinical course was uneventful despite the presence of profound neutropenia. These data therefore suggest that the inflammatory response of these patients developed after conditioning for a HSCT in response to chemotherapy-induced mucositis. This was supported by statistical analysis showing a significant correlation

between mucositis measured through citrulline, but not neutropenia, and inflammation determined by CRP.

In the patients with bacteremia due to gram-positive bacteria, the course of events were very similar to that of the patients without infections suggesting they also primarily suffered from inflammation related to mucositis, but that a superimposed infection aggravated the response with higher maximal CRP levels and an increased incidence of fever (Figure 1). An important observation was that the inflammatory response already preceded the bacteremia by 2 days. This has been previously observed in the setting of allogeneic HSCT [19]. It is consistent with a study on rats exposed to chemotherapy in which the release of pro-inflammatory cytokines was associated with evolving mucositis and preceded microbial translocation [20]. In addition, statistical analysis revealed a stronger association over time between citrulline levels and CRP than between bacteremia and CRP, underlining a predominant role for mucositis in the occurrence of inflammation.

Interestingly, for patients developing bacteremia the citrulline level was significantly lower than was that of those without infection, suggesting that the degree of mucositis was a risk factor for bacteremia. This is in line with earlier studies which have shown bacterial translocation from the mucosal surfaces to be related to the degree of mucositis [12]. No difference was seen in oral mucositis score but that is probably explained by the more subjective nature of these measurements. Future studies are required to determine more precisely the relationship between mucositis measured by citrulline and the occurrence of bacteremia. However, other factors such as differences in gastrointestinal bacterial colonization, innate immunity and neutropenia may also play a role in the occurrence of bacteremia [21], although in our study no difference in neutropenia was found between the two patient groups.

Currently the neutrophil count is used to predict the risk period for patients receiving a HSCT. Our data suggest that monitoring citrulline levels provides additional information to predict more precisely the onset of the inflammatory response and when bacteremia is likely to occur. Although most patients had reached a citrulline level of 10 µmol/l before developing fever and/or bacteremia, a definite cut off value could not be established, an issue that should be addressed in future studies.

In most patients baseline citrulline levels were below the normal value [18]. Probably the prior use of chemotherapy and other factors such as malnutrition already resulted in some mucosal atrophy. If patients with a low base line citrulline level are more at risk for severe mucositis is an important question, which remains to be answered.

Data from other studies indicate that mucositis is an important cause of fever and infections in HSCT, supporting our findings. Fact remains that despite use of

empirical antibiotics there remains a large group of patients with fever without documented infection [2]. Efforts to reduce the occurrence of fever with pre-emptive antibiotics and G-CSF have yielded largely unsatisfactory results [22;23]. One study even showed that the highest predictor for infection was grade II-IV mucositis, concluding that only reducing of neutropenia with G-CSF is insufficient to prevent fever and infections [23]. In addition, other studies have shown that ameliorating mucositis, with the use of recombinant keratinocyte growth factor (Palifermin®) or using reduced intensity conditioning regimen, also resulted in a decreased incidence of fever, despite unchanged neutropenia [24;25].

Our study is limited by being retrospective, although the data had been collected prospectively. Nevertheless, the data show a clear pattern of an inflammatory response, irrespective of the presence or absence of infection, and that this coincides with the occurrence of mucositis. Hence, at least for some HSCT recipients, the inflammatory response and fever are the direct consequence of mucositis alone, and neutropenia plays a minor role or none at all. The magnitude of the inflammatory response can however be aggravated by intercurrent infections arising from the loss of the mucosal barrier and simultaneous neutropenia. Consequently, the term "febrile neutropenia" might be better replaced by the term "febrile mucositis". Future research should therefore focus on strategies directed at the prevention and treatment of mucositis.

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3|

Intestinal damage determines the inflammatory response and risk for early complications in patients receiving conditioning for a hematopoietic stem cell transplantation

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Abstract

Background
Stem cell transplantation (SCT) is still complicated by the occurrence of fever and inflammatory complications attributed to neutropenia and subsequent infectious complications. The role of mucosal barrier injury (MBI) of the intestinal tract therein has received little attention.

Methods
We performed a retrospective analysis in 163 SCT recipients of which data had been collected prospectively on intestinal damage (citrulline), inflammation (C-reactive protein), and neutrophil count. Six different conditioning regimens were studied; 5 myeloablative (MA) and 1 non-myeloablative (NMA). Linear mixed model multivariate and AUC analyses were used to define the role of intestinal damage in post-SCT inflammation. We also studied the relationship between the degree of intestinal damage and the occurrence of early post-SCT complications.

Results
In the 5 MA regimen there was a striking pattern of inflammatory response that coincided with the occurrence of severe intestinal damage. This contrasted with a modest inflammatory response seen in the NMA regimen in which intestinal damage was limited. With linear mixed model analysis the degree of intestinal damage was shown the most important determinant of the inflammatory response, and both neutropenia and bacteremia had only a minor impact. AUC analysis revealed a strong correlation between citrulline and CRP (Pearson correlation r = 0.96). Intestinal damage was associated with the occurrence of bacteremia and acute lung injury, and influenced the kinetics of acute graft-versus-host disease.

Conclusion
The degree of intestinal damage after myeloablative conditioning appeared to be the most important determined of the inflammatory response following SCT, and was associated with inflammatory complications. Studies should explore ways to ameliorate cytotoxic therapy-induced intestinal damage in order to reduce complications associated with myeloablative conditioning therapy.

Introduction

Treating patients with hematological malignances by use of a hematopoietic stem cell transplantation (SCT) is still complicated by the occurrence of infections and inflammatory complications including sepsis, acute lung injury, and graft-versus-host disease (GvHD). Historically the focus was on neutropenia and fever ("febrile neutropenia") and its relation to infections [1]. However, a substantial number of SCT recipients develop fever unrelated to infection ("unexplained fever") [2], resulting from other causes including cytotoxic therapy-induced mucosal barrier injury (MBI) [3;4]. Many studies have shown associations between the magnitude of the C-reactive protein (CRP) response and cytokine release and post-SCT complications [5-9], and these complications might therefore best be regarded as manifestations of a systemic inflammatory response syndrome (SIRS) [8]. Other studies have shown that infections may contribute to non-infectious complications including acute GvHD [10;11]. However, few if any of these studies addressed the role of MBI *per se* as an isolated cause of inflammation and risk factor for infections, nor its role in the pathogenesis of inflammatory complications. Animal models have enhanced our understanding of the inflammatory processes that take place in the intestine following chemotherapy [12-14], and in the clinical setting of SCT the relationship between intestinal damage and the inflammatory response has become better appreciated [4;15]. Mucosal damage and deregulated host-microbial interactions have also been shown to contribute to SIRS and post-SCT complications such as acute GvHD [16;17]. Therefore, we hypothesized that intestinal damage could be the most important determinant of early SIRS following conditioning with chemotherapy and radiotherapy and that the degree of damage correlates with the occurrence of post-SCT complications.

Studying damage to the gastro-intestinal tract during SCT remains difficult, since it remains hidden and hitherto only indirect and non-specific tests were available [18;19]. Measuring serum or plasma citrulline levels provides a more direct and specific way of determining intestinal damage of certain conditions that are accompanied by gut failure [20]. Recently, citrulline-based assessment of intestinal damage has also shown to be objective, reproducible, specific, and reliable in the setting of SCT [21;22].

To test our hypothesis we studied the relationship between the magnitude of the inflammatory response and the degree of intestinal damage as measured by citrulline, the duration of neutropenia, and the occurrence of bacteremia. To achieve this we selected recipients of a SCT for which 5 cohorts of patients had been given different myeloablative (MA) conditioning regimens, and a single cohort had received a non-myeloablative (NMA) conditioning regimen. We also investigated whether we could determine a relationship between the degree of intestinal damage and the occurrence of early post-SCT complications.

Materials and methods

Study population

This was a retrospective analysis of 163 patients who had received an autologous or allogeneic SCT in our hospital from May 2004 to December 2007. Plasma had been collected prospectively and stored for later analysis of citrulline, but other data including CRP, temperature, and clinical and microbiological infections had been prospectively gathered from the day of starting chemotherapy. Patients had given their informed consent to the prospective collection of data and plasma samples for investigational use. The local ethics committee (CMO Regio Arnhem-Nijmegen) judged that no formal approval for this study was necessary regarding the fact that data were used anonymously and the analysis would not reveal results harming contributing patients.

Conditioning regimens and stem cell transplantation

The MA and NMA regimens are depicted in Table 1. All patients received a stem cell graft on the day scheduled. Autologous SCT grafts contained at least 2.0×10^6 CD34+ cells per kg, and allogeneic SCT partially T cell-depleted grafts contained on average 3.0×10^6 CD34+ cells per kg and 0.5×10^6 CD3+ cells per kg.

Treatment protocol

All patients were treated according to the same protocol, which has been described earlier [23]. GvHD prophylaxis consisted of cyclosporine only. Anti-microbial prophylaxis consisted of ciprofloxacin and valacyclovir. Fever was defined by a single axillary temperature $\geq 38.5^\circ$C. At the onset of fever 40 mL of peripheral blood was obtained for culture together with 10 mL from each lumen of the catheter, patients were examined for any sign of local infection, and empirical therapy with ceftazidime was started [24].

Neutropenia was defined as an ANC $\leq 0.5 \times 10^9$/L , and the duration and depth was recorded. CRP levels (mg/L) were determined every day and the maximum CRP (CRP_{max}) recorded. Plasma citrulline was determined to estimate intestinal damage before the start of conditioning and 3 times weekly thereafter until discharge. Citrulline concentrations (μmol/L) were measured by a standard procedure for determining amino acids using high-performance liquid chromatography [21]. Citrulline levels below 10 μmol/L were deemed to indicate hypocitrullinemia, and were regarded as reflecting severe intestinal damage [20].

Definition of stem cell transplantation complications

Clinical and microbiologically defined infections were defined according to the Consensus definitions of Immunocompromised Host Society [2]. A blood culture was

Table 1 Conditioning regimens.

Regimen	Doses	Frequency	Days	Type of conditioning	Type of SCT, day
HDM				MA	Autologous, day 4
- Melphalan	100 mg/m²	Od	1, 2		
BEAM				MA	Autologous, day 7
- Carmustine (BCNU)	300 mg/m²	Od	1		
- Etoposide.	100 mg/m²	Bd	2-5		
- Cytarabine	100 mg/m²	Bd	2-5		
- Melphalan	140 mg/m²	Od	6		
Ida-Cyclo-TBI:				MA	Allogeneic Matched related donor, day 13
- Idarubicine	42 mg/m²	Infusion over 48 h	1		
- Cyclophosphamide	60 mg/kg	Od	7, 8		
- TBI	4.5 Gy	Od	11, 12		
Cyclo-ATG-TBI				MA	Allogeneic Matched unrelated donor, day 9
- Cyclophosphamide	60 mg/kg	Od	1, 2		
- Thymoglobuline	2 mg/kg	Od	3-6		
- TBI	4.5 Gy	Od	7, 8		
Cyclo-TBI:				MA	Allogeneic Matched related donor, day 7
- Cyclophosphamide	60 mg/kg	Od	1, 2		
- TBI	4.5 Gy	Od	5, 6		
Cyclo-Flu:				NMA	Allogeneic Matched related donor, day 7
- Cyclophosphamide	1200 mg/m²	Od	1-4		
- Fludarabine	30 mg/m²	Od	1-4		

Abbreviations: od; once daily, bd; two times daily, TBI = total body irradiation, MA = myeloablative, NMA = non-myeloablative.

considered to represent bacteremia if one or more bottles yielded a microorganism, except in the case of coagulase-negative staphylococci (CoNS), which required recovery of the same strain from two separate positive blood cultures [24]. The incidence of bacteremia that occurred on the day of fever was documented and compared among the regimens. Invasive fungal diseases were scored according to the EORTC/MSG consensus guidelines [25]. Acute lung injury (ALI) was defined according to current guidelines [26]. Acute GvHD, GvHD occurring the first 100 days after SCT, was graded according to the criteria of *Przepiorka et al.* [27]. Early mortality related to SCT complications was defined as any death occurring within 30 days following SCT (day +30), but unrelated to the underlying disease.

Data analysis

We employed descriptive statistics for fever, neutrophil count, CRP levels, and citrulline levels which were expressed as mean values together with the 95% confidence interval (Table 2). As citrulline was measured three times weekly, the real nadir might have been attained between two measurements and hence was likely missed. To compensate for this and study the true length of time in which citrulline levels were below 10 μmol/L we modeled the course of citrulline as a function of time during the first 30 days by developing a linear mixed model using first, second, third and fourth power of time as fixed factors to predict the citrulline levels after taking into account the within-person correlations by incorporating a random patient intercept. To describe the relationship of CRP to the neutrophil count, intestinal damage (citrulline concentration) and bacteremia we used several linear mixed models for the first 30 days with random patient effect and the logarithmic transformed CRP (*log* CRP) as the outcome variable.

To assess the impact of conditioning on intestinal damage and CRP we performed an area under the curve (AUC) analyses. Per patient, the CRP_{AUC} was defined as the sum of the 30 estimated CRP values, resulting from a piecewise linear mixed model which uses a linear time component for day 1-10 and a cubic time component for day 11-30. The conditioning regimen and the interactions between the particular regimen and time were also part of this model that also accounted for within person correlations by virtue of a random intercept. Likewise, the $citrulline_{AUC}$ per patient was defined as the sum of the 30 estimated 10/citrulline values. We used the citrulline levels estimated by the linear mixed model described above and transformed these values into 10 times the inverse of the estimated value.

Comparisons between the impact of the different conditioning regimens on neutropenia, CRP_{AUC} and $citrulline_{AUC}$ were studied using the Kruskal-Wallis test. The correlation between the degree of neutropenia and CRP_{AUC} versus $citrulline_{AUC}$ and CRP_{AUC} was studied by Pearson correlation over the different regimens

Comparison of the mean onset of acute GvHD between the different regimens was done using one-way ANOVA. Comparison of the incidence of ALI in relation to OVS between the different regimens was done using the χ^2-test.

Analyses were performed using SAS 8.2 and a *P*-value of <0.05 was considered to indicate statistical significance.

Results

Study population and patient characteristics
Seventy-seven (77) patients received an autologous and 86 an allogeneic SCT (Table 2). All but 14 patients received MA conditioning. Autologous SCT was performed for patients with multiple myeloma and non-Hodgkin lymphoma, but allogeneic SCT was employed for a greater variety of diagnoses including acute and chronic lymphatic and myeloid leukemia and myelodysplastic syndrome. NMA conditioning was employed to prepare patients who had received an autologous SCT 4-6 months earlier for MM.

Intestinal damage
MA conditioning was associated with severe and prolonged intestinal damage shown by a rapid decline in citrulline to < 10 μmol/L, a mean of 10 days after starting chemotherapy. The mean nadir of citrulline was 4.5-7.0 μmol/L, and hypo-citrullinemia lasted for more than one week in most patients (Figure 1A-E, 2A, Table 2). Hypocitrullinemia was most pronounced in patients receiving idarubicin in their conditioning, lasting approximately 18 days. In contrast, an early and short drop of citrulline level was noticed for NMA conditioning, but hypocitrullinemia was not evident for most patients (Figure 1F, 2A, Table 2).

Inflammatory response measured by C-reactive protein and fever
The course of CRP during SCT of the different conditioning regimens is illustrated in Figure 1 and 2B. Within each type of MA conditioning, patients showed similar patterns of inflammatory response, although there was some variation in the precise onset, peak and resolution of CRP levels. Those without bacteremia did not have a different course when compared to those with; although in general CRP levels were lower (data not shown). As for intestinal damage, the CRP response was highest in patients receiving idarubicin. Resolution of inflammation occurred with engraftment and restoration of the intestinal damage defined by rising citrulline levels. In Cyclo-ATG-TBI conditioning the first peak of CRP was related to ATG induced lymphocyte depletion and cytokine release, but the second peak resembled that seen for the other MA regimens. Also some patients treated with Cyclo-TBI

Table 2 General characteristics.

Conditioning	HDM (N=56)	BEAM (N=21)
Age, mean (range), years	56 (33-65)	47 (18-65)
Gender: M/F	35/21	17/4
Diagnoses: -MM -NHL/CLL -AML -ALL -MDS -CML/MPD	56 (100%) - - - - -	- 21 (100%) - - - -
Type of conditioning	MA	MA
Type of SCT	Autologous	Autologous
Fever (axillary temperature ≥ 38.5^{0}C)	88.0%	90.5%
Fever, day from start conditioning, mean (95%CI)	11.8 (11.4-12.2)	13.0 (12.2-13.9)
Neutrophils < 0.5 x10^9/L, days (95%CI)	8.4 (8.0-8.7)	9.5 (8.5-10.5)
Citrulline < 10 μmol/L, number of patients, (%)	51 (91%)	21 (100%)
Measurements with citrulline <10 μmol/L, mean (95%CI)*#	3.5 (3.2-3.9)	4.7 (4.0-5.3)
Observed citrulline nadir μmol/L, mean (95%CI)*	6.0 (5.4-6.6)	4.3(3.5-5.1)
Citrulline <10 μmol/L, days, mean (95%CI)&#	7.9 (7.1-8.7)	11.2 (9.6-12.9)
Citrulline nadir μmol/L, mean (95%CI)&	6.5 (5.7-7.2)	4.9 (3.7-6.0)
CRP$_{max}$ (mg/L), mean (95%CI)	163 (136-189)	202 (160-246)

Characteristics of patients, stem cell transplantation and general outcome measures of intestinal damage (citrulline), inflammation (CRP and fever), and neutropenia (neutrophil count ≤ 0.5 x10^9/L) for each conditioning regimen. *Citrulline was measured 3 times weekly. # Only those patients included with citrulline levels below 10 μmol/L. & Based on estimated values. MA = myeloablative, NMA = non-myeloablative, CRP = C-reactive protein, MM = multiple myeloma, NHL = non-Hodgkin lymphoma, CLL = chronic lymphatic leukemia, AML/ALL = acute myeloid and lymphatic leukemia, MDS = myelodysplastic syndrome, CML/MPD = chronic myeloid leukemia/myeloproliferative disease.

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	Ida-Cyclo-TBI (N=28)	Cyclo-ATG-TBI (N=34)	Cyclo-TBI (N=10)	Cyclo-Flu (N=14)
	46 (18-64)	43 (20-58)	50 (38-59)	54 (39-65)
	13/15	21/13	8/2	10/4
	-	-		14 (100%)
	7 (25%)	13 (38%)	8 (80%)	-
	12 (42.5%)	8 (23.5%)	2 (20%)	-
	3 (10.5%)	4 (11.75%)	-	-
	6 (22%)	4 (11.75%)	-	-
	-	5 (15%)	-	-
	MA	MA	MA	NMA
	Matched sibling allogeneic	Matched unrelated allogeneic	Matched sibling allogeneic	Matched sibling allogeneic
	100%	Early: 73.5% Late: 100%	100%	Early: 28.6% Late: 78.6%
	12.9 (12.2-13.6)	4.1 (3.7-4.5) 14.1 (13.0-15.1)	13.4 (12.0-14.8)	3.0 (1.7-4.3) 16.0 (14.4-17.5)
	15.8 (14.6-17.0)	15.5 (14.6-16.4)	11.1 (9.8-12.4)	12.4 (10.8-14.1)
	26 (93%)	30 (88%)	10 (100%)	4 (29%)
	6.2 (4.6-7.8)	4.8 (4.0-5.7)	3.5 (2.4-4.6)	3.0 (1.1-4.9)
	4.6 (3.9-5.3)	5.6 (4.9-6.3)	5.6 (4.2-7.0)	10.8 (8.9-12.6)
	17.7 (15.6-19.8)	14.6 (13.3-15.9)	11.0 (7.9-14.1)	7.5 (5.4-9.6)
	4.5 (3.5-5.7)	7.0 (6.1-7.9)	6.6 (5.1-8.1)	12.4 (10.2-14.6)
	257 (222-291)	188 (162-213)	211 (154-269)	66 (38-95)

Figure 1 Course of citrulline and CRP in time after start of conditioning.
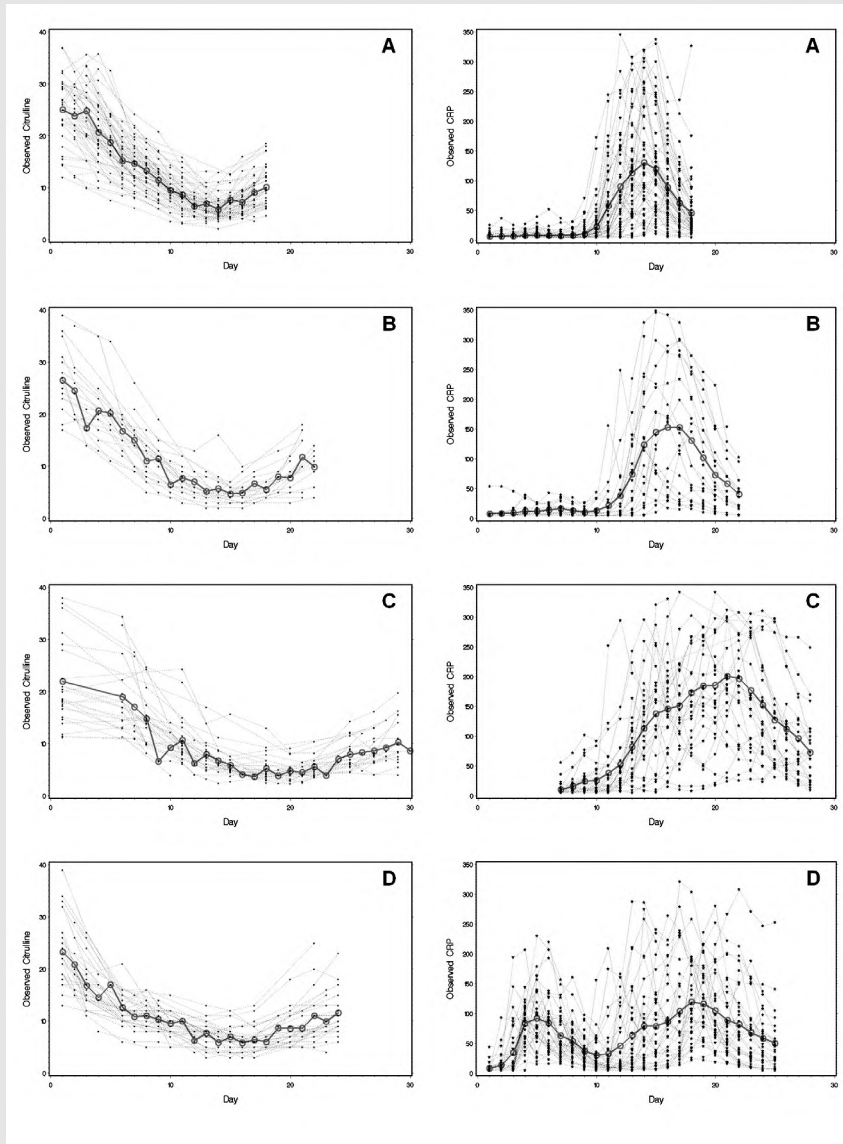
Five MA and one NMA conditioning regimens are shown; A = HDM, B = BEAM, C = Ida-Cyclo-TBI, D = Cyclo-ATG-TBI, E = Cyclo-TBI, F = Cyclo-Flu. Observed values (•), mean values (◦).

Figure 1 Continued.
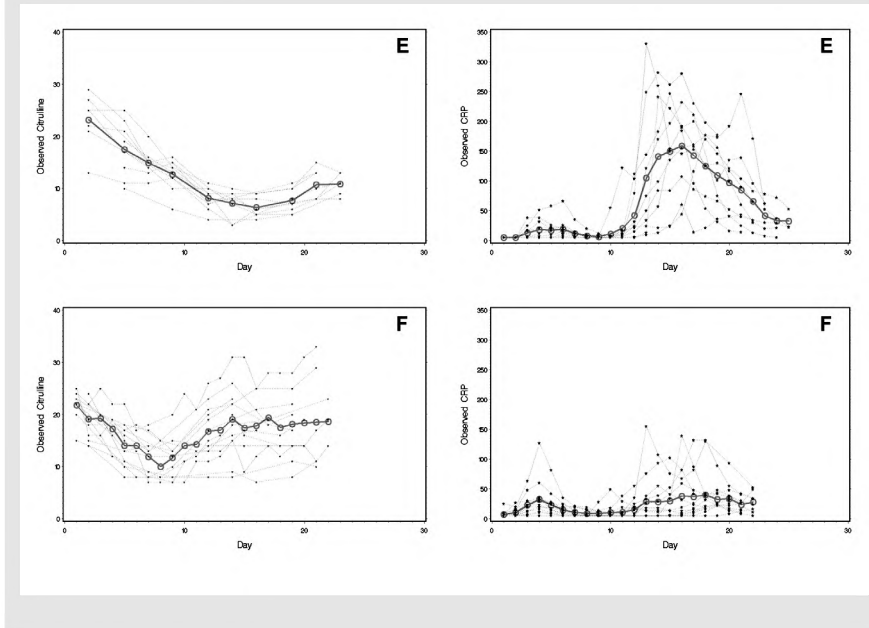
and BEAM had an early peak in CRP during conditioning, which was probably related to chemotherapy-induced cytokine release.

Only a moderate inflammatory response occurred after NMA (Figure 1F and 2B). Also, the timing was different when compared to MA regimens with a peak occurring early during conditioning and a second peak much later. The latter occurred during engraftment and thereby resembled to some extent the inflammatory complication designated engraftment syndrome [28].

Virtually every patient who had received MA developed fever as did 80% of those given NMA conditioning. Some patients receiving Cyclo-ATG-TBI and Cyclo-Flu also experienced an early episode with fever during conditioning (25 and 4 patients, respectively). In MA regimens fever occurred on days 12-14, 2-3 days after CRP had become elevated (Figure 1, Table 2). By contrast, fever occurred late during engraftment at a mean of day 16 after starting NMA conditioning.

Figure 2 Summary of the time course of citrulline (A) and CRP (B) for all 6 regimens.
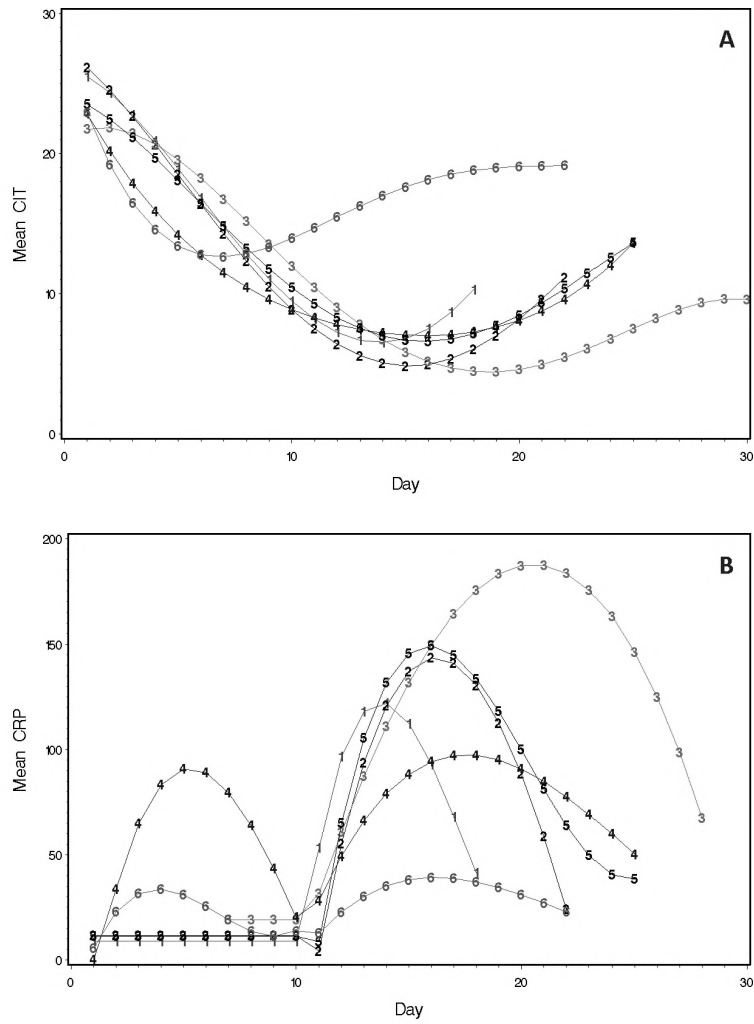
Day 1 is the day of start of conditioning. To correct for unobserved citrulline and CRP values we modeled the course of citrulline and CRP as described in methods. 1 = HDM, 2 = BEAM, 3 = Ida-Cyclo-TBI, 4 = Cyclo-ATG-TBI, 5 = Cyclo-TBI, 6 = Cyclo-Flu. Mean CRP in mg/L, mean citrullline in μmol/L.

Relation intestinal damage to inflammation

In MA conditioning CRP levels started to increase from 10-11 following the start of conditioning which coincided with the development of hypocitrullinemia (Figure 1A-E and 2). The peak of the inflammatory response coincided with the nadir of citrulline levels. Although interindividual differences existed the occurrence of inflammation was related to the development of intestinal damage in almost every patient.

Additional AUC analysis was used to grade the impact of conditioning on intestinal damage and CRP. There were significant differences in both CRP_{AUC} and $citrulline_{AUC}$ between the various conditioning regimens (Kruskal-Wallis P <0.001), except between BEAM, Cyclo-TBI, and Cyclo-ATG-TBI. Interestingly, a very strong correlation between the degree of intestinal damage and the inflammatory response was seen for the different regimens (Pearson correlation 0.96, Figure 3). By contrast, there was only a weak correlation between the duration of neutropenia and inflammation (Pearson correlation 0.48, Figure 3).

Figure 3 Pearson correlation between the mean degrees of neutropenia (NP in days) and inflammation (CRP_{AUC}) versus intestinal damage ($Citrulline_{AUC}$) and inflammation (CRP_{AUC}) over the different regimens.
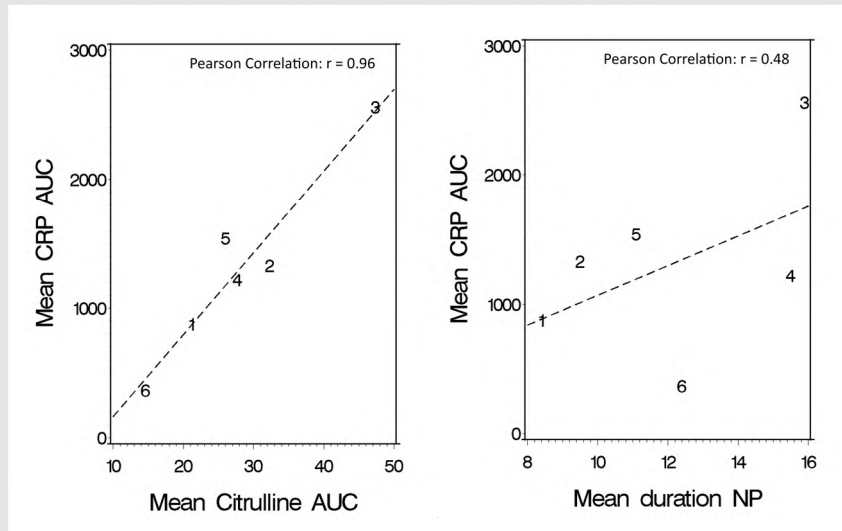
1 = HDM, 2 = BEAM, 3 = Ida-Cyclo-TBI, 4 = Cyclo-ATG-TBI, 5 = Cyclo-TBI, 6 = Cyclo-Flu.

Table 3 Stem cell transplantation complications.

SCT complications	HDM (N=56)	BEAM (N=21)
Bacteremia on day of fever -OVS -CoNS -Other	29 22 (39.3%) 12 (21.4%) 2	10 6 (28.5%) 7 (33.3%) -
Concomitant OVS/CoNS	7	3
Candidemia	1	0
Clinically defined infection: -Phlebitis superficial vein. -Tunnel infection/infected thrombosis -Pneumonia. -Probable/Proven IA.	5 - 1 4 -	5 - 3 2 -
ALI ALI following OVS bacteremia	4/56 (7.1%) 3/22 (13.6%)	2/21 (9.5%) 1/6 (16.7%)
Early mortality (day +30 post SCT) -ALI. -Acute GvHD	0 - -	1 1 -

aGvHD I-IV, all (N, %)
    -    Grade II-IV
    -    Grade III-IV
	NA	NA

Onset from day SCT, mean (95%CI)	NA	NA

OVS = oral viridians streptococci, CoNS = coagulase-negative staphylococci, IA = invasive *Aspergillosis*, ALI = acute lung injury, acute GvHD = acute graft-versus-host disease. Grading of acute GvHD was done according to the criteria of *Przepiorka* et al.[27] and probable/proven IA was defined according to EORTC/MSG consensus definitions[25].

In univariate linear mixed model analysis, 10/citrulline, the type of conditioning regimen, neutropenia and bacteremia were significantly associated with *log* CRP. In multivariable analyses only citrulline and type of conditioning regimen remained important.

Stem cell transplantation complications

Bacteremia

There was a significant difference between MA and NMA regarding bacteremia on the day of fever (Table 3) with up to 85% of patients experiencing bacteremia predominantly due to oral viridans streptococci (OVS) and CoNS after MA conditioning, compared with none of those receiving NMA (*P* <0.001). OVS was

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	Ida-Cyclo-TBI (N=28)	Cyclo-ATG-TBI (N=34)	Cyclo-TBI (N=10)	Cyclo-Flu (N=14)
	18	29	6	0
	7 (25%)	13 (38%)	5 (50%)	-
	12 (46.5%)	21 (65%)	3 (20%)	-
	1	1	-	-
	2	6	2	NA
	3	1	0	0
	9	7	3	3
	-	-	-	1
	5	6	1	-
	2	-	1	2
	1	1	1	-
	6/28 (21.5%)	4/34 (11.8%)	2/10 (20%)	0
	4/7 (57.1%)	4/13 (30.8%)	2/5 (40%)	
	2	1	2	0
	1	-	1	-
	1	1	1	-
	13/28 (46%)	12/34 (28%)	6/10 (60%)	6/14 (43%)
	8 (28.5%)	7 (20.5%)	3 (30%)	4 (28.5%)
	2 (7%)	2 (6%)	1 (10%)	-
	26 (16-36)	46 (29-63)	23 (19-27)	40 (19-61)

recovered with CoNS in 20/55 (36%) of cases. A minority of patients experiencing a bacteremia with CoNS on the day of fever had any clinical or radiological signs of a CVC related tunnel infection or thrombophlebitis at the same time (5/55 (9%)).

Acute lung injury
The overall incidence of ALI was 18/163 (11%), with 14/18 (78%), being associated with OVS bacteremia. Conversely, ALI affected 14/53 (26.4%) patients with OVS bacteremia. However, this incidence varied significantly between conditioning regimens and was related to the severity of intestinal damage as ALI occurred in 3/22 (13.6%), 4/13 (30.8%) and 4/7 (57.1%) in patients receiving HDM, Cyclo-ATG-TBI, and Ida-Cyclo-TBI conditioning, respectively (*P* =0.03) (Table 3).

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Acute graft-versus-host disease
No differences were seen in the total incidence of acute GvHD, although there were no cases of acute GvHD III-IV in the group with NMA; with only skin acute GvHD being encountered. However, there was a significant difference in the onset of acute GvHD. In Ida-Cyclo-TBI and Cyclo-TBI, despite receiving a partially T-cell-depleted graft, acute GvHD occurred early with a mean onset on day +25 post SCT. In both Cyclo-ATG-TBI and Cyclo-Flu the onset was delayed, with a mean onset on day +46 and +40 post SCT, respectively (*P* =0.02).

Early mortality
Overall, early mortality was low 6/163 (3.7%), and related to ALI and acute GvHD, and all but one death occurred following MA conditioning for an allogeneic SCT.

Discussion

In this study we show the course and extent of intestinal damage and inflammatory responses following various conditioning regimens used to prepare for a hematopoietic SCT. There was a striking pattern of inflammatory response coinciding with the occurrence of severe intestinal damage for patients receiving MA conditioning, defined by hypocitrullinemia [20]. Moreover, the degree of intestinal damage seemed the most important determinant of inflammation and was highly correlated with the magnitude of the inflammatory response measured by CRP. Neither neutropenia nor bacteremia had a major impact on this. The close relationship between intestinal damage and inflammation was further underscored by the fact that NMA resulted in only a modest inflammatory response, with a completely different time course, and the virtual absence of severe intestinal damage. Consequently, intestinal damage appears the primary determinant of inflammation following myeloablative conditioning with chemo- and radiotherapy in the setting of autologous and allogeneic SCT.

While there are limitations associated with retrospective analysis and the potential for bias resulting from selection, the relationship of intestinal damage to SCT complications was remarkable. As expected, there was a significant difference in occurrence of bacteremia between those receiving MA and NMA conditioning [29]. The similar duration of neutropenia and the marked difference in intestinal damage, suggest that the gut may have been the origin of bacteremia [30]. Moreover, most pathogens recovered from blood cultures are known residents of the gut [31;32]. Notably, a considerable proportion of patients had bacteremia with both CoNS and OVS, which was probably due to simultaneous intestinal translocation [31].
A strong relationship has been found between the occurrence of OVS bacteremia

and septic shock and ALI in neutropenic patients, which was explained by virulence factors and pulmonary cytotoxicity of chemotherapy [33-35]. Interestingly, we saw that in patients with OVS bacteremia the incidence of ALI was related to the degree of intestinal damage. It is known that barrier dysfunction facilitates bacterial translocation, but intestinal damage also seems to determine the resulting inflammatory response. This was confirmed in our linear mixed model, which showed citrulline but not bacteremia related to the CRP response. Although ALI seems directly associated with OVS bacteremia this might be only coincidental, as both complications are consequences of severe intestinal damage. So intestinal damage 'primes' the immune system with subsequent aggravated cytokine release following activation of pattern recognition receptors from translocating microbial motifs [12].

This 'priming' of the immune system also applies to the occurrence acute GvHD in which the role of intestinal damage has been acknowledged [16]. Although we found no apparent differences in the citrulline and CRP levels between patients with and without acute GvHD within any given regimen, between regimens there was a clear difference in the kinetics of acute GvHD. In addition severe acute GvHD did not develop after NMA, as opposed to 6-10% after MA, and GvHD of liver or gut did not occur. The early onset of acute GvHD after Ida-Cyclo-TBI and Cyclo-TBI suggests that the tissue inflammation, resulting from the profound intestinal damage, contributes to the accelerated allo-reactive T-cell response [36], even in the setting of partial T cell-depletion. The delay in onset of acute GvHD in patients conditioned with ATG results from additional *in vivo* T cell-depletion creating a 'time-window' between the intestinal damage-induced inflammation and T cell recovery. After NMA we also saw a delay in the onset of acute GvHD, which is in accordance with previous data from studies in mice [37] and humans [38]. This altered kinetics of acute GvHD was, at least in part, related to the absence of significant intestinal damage and tissue inflammation. Differences in the kinetics of acute GvHD in NMA have been largely attributed to alterations in antigen presenting cell chimerism, T cell chimerism and regulatory T cell activation [37], but our data underscore the role con-ditioning-induced intestinal damage plays in the complex pathogenesis of acute GvHD.

Several studies have shown correlations between CRP and the occurrence of SCT complications but they all used different cut-off values [5;7]. CRP is not a specific marker since chemotherapy and ATG, and the process of engraftment itself, elicit inflammatory responses. Hence it is not possible to identify who is at risk or when that risk might occur. Citrulline could provide an alternative, because it is a specific marker of enterocyte mass, which decreases only when there is intestinal damage. Furthermore, citrulline levels correspond with the inflammatory responses following MA conditioning, and more importantly with SCT complications. Clearly

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it is necessary to confirm the predictive value of citrulline for individual patients and to define cut-off values more precisely. Classifying other conditioning regimens, by means of measuring citrulline can already help determine the need for antimicrobial prophylaxis, hospital care, and the use of anti-inflammatory treatment.
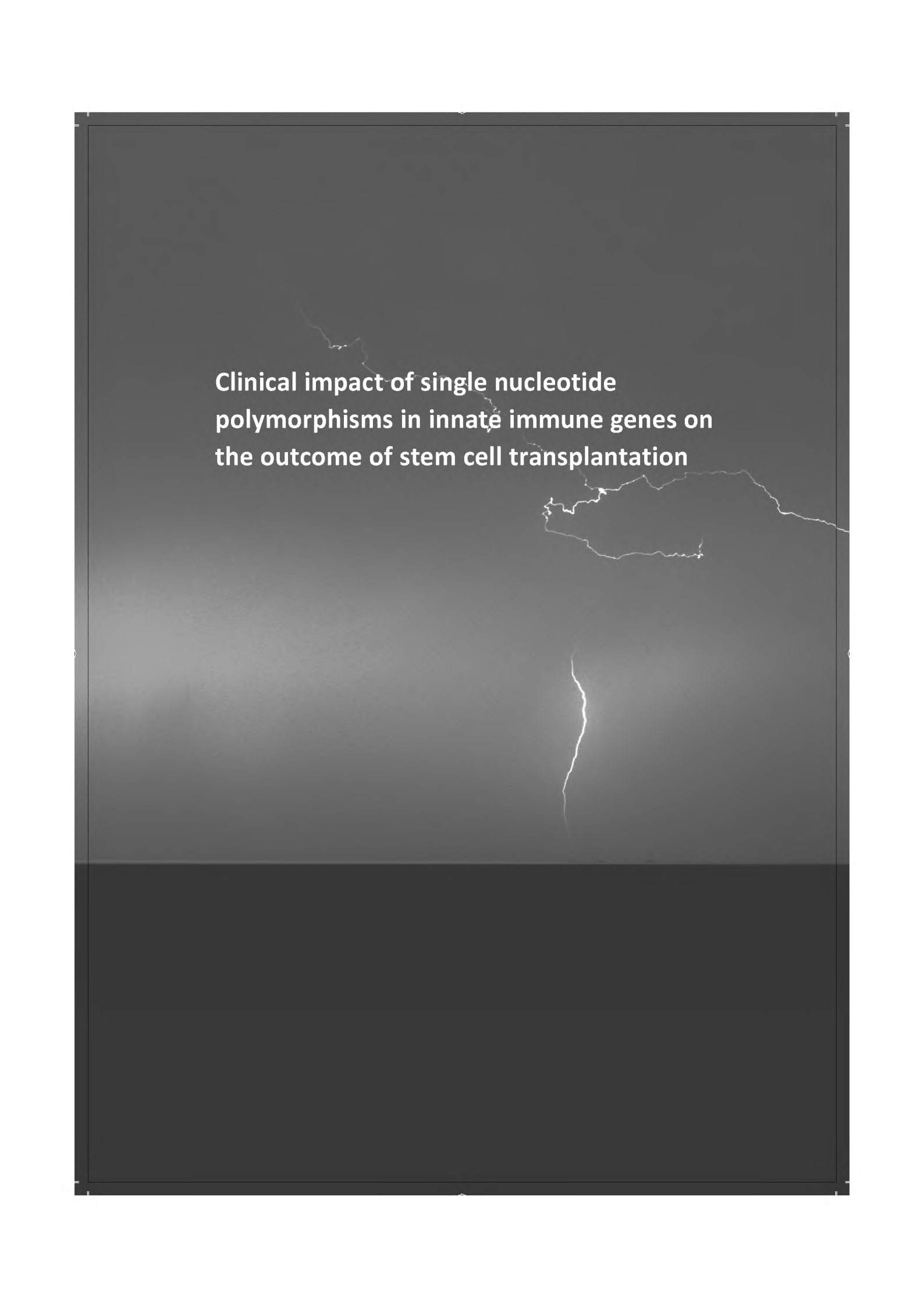
Given the role of intestinal MBI in complications after SCT studies should explore ways to ameliorate cytotoxic therapy-induced intestinal damage in order to reduce inflammatory complications associated with myeloablative conditioning therapy.

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3

Clinical impact of single nucleotide polymorphisms in innate immune genes on the outcome of stem cell transplantation

4 |

NOD2 polymorphisms predict severe acute graft-versus-host and treatment-related mortality in T-cell-depleted hematopoietic stem cell transplantation

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Abstract

Single nucleotide polymorphisms (SNPs) in the *NOD2* gene have significant impact on both treatment-related mortality (TRM) and acute graft-versus-host disease (aGvHD) in hematopoietic stem cell transplantation (HSCT). The effect of these polymorphisms when using T-cell-depleted grafts has been poorly studied. We retrospectively analyzed NOD2 polymorphisms in a cohort of 85 patients and donors that received a HLA identical sibling partially T-cell-depleted HSCT (0.5 x 10^6 CD3+ T-cells/kg) following idarubicin-containing conditioning regimens. NOD2 polymorphisms were present in 14 of 85 (16.5%) of patients and 18 of 85 (21%) of donors. The risk of severe aGvHD (grade III-IV) and the 1-year TRM was significantly higher in the presence of NOD2 polymorphisms (hazard ratio (HR) 6.0, *P* =0.02 for severe aGvHD and HR 3.3, *P* =0.02 for TRM, respectively) and was most prominent in cases where patient and donor both had a polymorphism (HR 10.5, *P*=0.002 and HR 3.9, *P* =0.002). There was also a trend towards increased risk of bacteremia due to coagulase-negative staphylococci in patients with a NOD2 polymorphism. We conclude that NOD2 polymorphism screening should be used to optimize donor selection and antimicrobial prophylaxis in order to reduce the occurrence of aGvHD and TRM following allogeneic HSCT.

Introduction

Acute graft-versus-host disease (aGvHD), infections and other inflammatory complications following conditioning therapy still have considerable impact on morbidity and mortality in hematopoietic stem cell transplantation (HSCT). An uncontrolled inflammatory response as a result of the mucosal barrier injury (MBI) induced by cytostatic chemotherapy and subsequent microbial translocation, are thought to play an important role in the initiation of aGvHD [1-3]. Polymorphisms of innate immune genes (non-HLA genes) can affect the inflammatory responses and therefore the incidence of aGvHD [4;5]. Any polymorphism that predicts complications should therefore be screened for to select the most suitable stem cell donor.

A robust association between single nucleotide polymorphisms (SNPs) in NOD2/ CARD15 and the outcome of allogeneic HSCT has been reported by Holler *et al.* [6]. NOD2 is as an intracytosolic pattern recognition receptor that senses muramyl dipeptide (MDP) of bacterial cell walls, and is expressed in Paneth cells, dendritic cells, neutrophils, and monocytes [7]. Altered function due to NOD2 polymorphisms can result in uncontrolled inflammation of the gut mucosa and can result in Crohn's disease [8]. The pathogenesis is not fully understood but there are several hypotheses involving the loss of anti-microbial activity and a deregulated immune response. Reduced antimicrobial activity results from decreased production of antimicrobial peptides [9;10], decreased autocrine cytokine release [11] and increased gut permeability [12], leading to bacterial translocation that elicits an immune response through toll-like receptor (TLR) signaling. This response is deregulated with increased production of Th1, and probably Th17 cytokines, due to reduced inhibition of TLR-mediated cytokine release ("loss of tolerance") [13-16] or decreased production of IL-10 [17].

Several reports, but not all, have shown that NOD2 polymorphisms influence the evolution of aGvHD, the occurrence of treatment-related mortality (TRM) [6], the incidence of disease relapse [18], and the development of bronchiolitis obliterans [19]. This underlines the importance of the specific conditions surrounding HSCT. For instance, T-cell-depleted HSCT with delayed T-cell recovery could reduce the impact of NOD2 polymorphisms on aGvHD. Therefore, we undertook a retrospective analysis on the impact of NOD2 polymorphisms in a homogenous cohort of Dutch recipients of a partially T-cell-depleted HSCT at our hospital.

Patients, materials and methods

Patients and donors
One hundred and sixteen (*n*=116) Dutch patients and their donors were initially included in the study. All had participated in supportive care studies and been

admitted to our transplant unit between May 1996 and November 2005 for an HLA-identical sibling, T-cell-depleted allogeneic HSCT. To obtain the most homogenous cohort possible, we selected 85 patients who had been given idarubicin in their conditioning regimen. The characteristics of patients, donors, and HSCT procedures are depicted in Table 1.

Table 1 Characteristics of patients, donors, and HSCT procedures.

	No polymorphism (n=63)	Any polymorphism (n=22)	*P*-value
Patient characteristics			
Age at transplantation, year (range)	46.9 (20-62)	43.7 (16-60)	0.274
Sex no female/no male	26/37	6/16	0.243
Disease:			
- Acute leukemia, no (%)	33 (52)	13 (59.5)	0.894
- CML/MPS, no (%)	13 (21)	3 (13.5)	
- Lymphoma, no (%)	7 (11)	3 (13.5)	
- MDS, no (%)	10 (16)	3 (13.5)	
Donor characteristics			
Age at donation, year (range)	47.1 (21-69)	43 (20-60)	0.188
Sex no. female/no. male	29/34	11/11	0.748
Stem cell transplantation			
Gender combination: male/female, no (%)	16 (25%)	8 (36%)	0.325
Disease status at HSCT: advanced, no (%)	12 (19%)	3 (14%)	0.567
Stem cell source:			
- Peripheral blood, no (%)	23 (37)	13 (59)	0.065
- Bone marrow, no (%)	40 (63)	9 (41)	
Conditioning regimen:			
- Ida-Cyclo-TBI, no (%)	53 (84)	20 (91)	0.432
- Ida-Bus-Cyclo, no (%)	10 (16)	2 (9)	
T-cell-depletion:			
- CD34 selection, no (%)	32 (51)	13 (59)	0.146
- Counterflow elutriation, no (%)	22 (35)	8 (36)	
- CD3/CD19 selection, no (%)	9 (14)	1 (5)	

The study group consisted of 85 HSCT recipients and their donors. All received a HLA matched sibling T-cell-depleted HSCT following conditioning with an idarubicin-containing regimen. GvHD prophylaxis consisted of cyclosporine only. Characteristics are expressed in absolute numbers and percentage. Age is expressed as mean value with the total range. Differences between the study groups were compared using the Pearson's χ^2-test or Fisher's exact test and with the use of the independent *t*-test.

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Treatment protocol

All patients had been treated according to the same protocol and received a central venous catheter (CVC). The conditioning regimen consisted of idarubicin (42 mg/m^2 in 48 hours), cyclophosphamide (60 mg/kg for 2 days) and either total body irradiation (TBI 4.5 Gy for 2 days) or busulfan (4 mg/kg for 4 days). Idarubicin was used in this conditioning regimen to reduce the risk of relapse in the setting of T-cell-depleted HSCT [20]. Partial T-cell-depletion was achieved by counter-flow elutriation, CD34 selection or CD3/CD19 depletion. On day 0, all patients were given a graft containing 3.0 x 10^6 CD34+ cells/kg (range 0.8-11.4; BM or PBSC) and 0.5 x 10^6 CD3+ cells/kg (range 0.3-0.7) achieved by dosed T-cell ad back. GvHD prophylaxis consisted of cyclosporine A (CsA) only.

GvHD was diagnosed by clinical signs, or pathological examination of biopsies of skin, gut or liver. GvHD was graded according to the criteria of Glucksberg *et al.* [21]. The score was decreased by one stage when there were other causes, such as drug reactions and infections, that might contribute to the clinical signs of skin, gut or liver [22].

Antimicrobial prophylaxis consisted of 500 mg ciprofloxacin given twice daily and 500 mg valaciclovir given three times daily. Fluconazole was only given at a dose 200 mg per day orally to those who were considered colonized by *Candida albicans.* Patients received trimethoprim-sulphamethoxazole 480 mg per day orally at discharge, as prophylaxis against infections due to *Pneumocystis jirovecii.*

Twice weekly blood was drawn for aerobic culture from each lumen of the CVC. A blood culture was considered positive if a microorganism was recovered from one or more bottles, with the exception of coagulase-negative staphylococci (CoNS), for which two separate positive blood cultures with the same strain were required [23]. At the onset of fever, defined as an axillary temperature $\geq$ 38.5°C, empirical therapy was started with 2000 mg ceftazidime given three times daily intravenously after blood cultures were obtained from each catheter lumen as well as from peripheral blood. Twenty-four patients (*n*=24) had received meropenem pre-emptively starting the day of HSCT.

Genotyping for NOD2 polymorphisms

DNA from patients and donors was extracted by standard procedures (QIAamp DNA blood mini kit, Qiagen) from peripheral blood cells collected before HSCT. Patients and their donors had given their informed consent to the prospective collection of DNA samples for investigational use. We assayed SNP8 (Arg702Trp), 12 (Gly908Arg) and 13 (Leu1007fsinsC) using a real-time TaqMan PCR. Control samples that had been confirmed by sequencing were kindly supplied by Dr. E. Holler and J. Brenmoehl (University Hospital of Regensburg). Probes and primers were synthesized by Applied Biosystems (Lingley House, Warrington, UK), with

reporter dye 6-FAM or VIC linked at the 5' end and the quencher dye TAMRA at the 3' end. The final reaction mixture of 50 µl consisted of Master Mix (MgCl$_2$ 5 mM, 10 x TaqMan buffer 5 µl, dNTP 250 µM, AmpliTaq Gold 1.25 mU), 300 nM of each forward and reverse primer, 200 nM of each fluorescent probe and 50 ng of DNA. PCR amplification and fluorescence detection were achieved using an ABI Prism 7700 (Applied Biosystems).

Statistical analysis

We used descriptive statistics for the study group characteristics regarding the HSCT with values expressed in absolute numbers and percentage. Differences between patient/donor pairs with and without NOD2 polymorphisms were compared using the Pearson's χ^2-test or Fisher's exact test for proportions and with the independent t-test for numerical data (SPSS version 14.0).

The outcome variables aGvHD, TRM at 1 year, and the occurrence of bacteremia were analyzed in relation to NOD2 polymorphisms.

The time to clinical event (1-year TRM, aGvHD) was determined from the date of HSCT. The time span for aGvHD extended to day 100, and that for 1-year TRM to day 365 after HSCT. The incidence of bacteremia was determined from start of chemotherapy (day -12) until 14 days after transplantation.

The cumulative incidence was estimated to reflect the competing risks of death within 100 days from other toxicities or relapse for aGvHD, death from relapse for TRM, and treatment related death within 14 days without bacteremia for bacteremia. Groups were formed according to occurrence of any polymorphism vs. wild-type and polymorphisms in both patient and donor vs. wild-type to allow analysis of polymorphisms in relation to aGvHD and TRM. In the analysis of bacteremia only recipient status was considered of importance and patients with a polymorphism were compared with wild-types.

The Gray test was used to evaluate the differences between cumulative incidence curves in these groups. These differences were also quantified by calculating the hazard ratios (HR) that resulted from the Fine and Gray models that take into account competing risks. These models were also used to adjust these HRs for age of the patient and donor, gender combination, the underlying hematological disease and status at HSCT, the T-cell-depletion technique, and the stem cell source. Because the study group was small we had to limit the number of variables included in the multivariable analysis to the risk factors most associated with aGvHD and TRM.

The competing risks analyses were performed using the *cmprsk* package of open source language R version 2.6.2. (www.r-project.org). *P*-value <0.05 was considered to indicate statistical significance.

Results

NOD2 polymorphism frequency

The frequency of any polymorphism among patients was 16.5, and 21% among donors (Table 2). Allele frequencies were 6.2, 0.6 and 3.2% for SNP8, 12 and 13 respectively, similar to the general Dutch population (5.9, 0.7 and 1.9) [24]. There were four pairs (4.7%) in which only the patient, 8 pairs (9.4%) in which only the donor and 10 pairs (11.8%) in which both had a SNP. There were no significant differences in the characteristics of patient/donor pairs with and without a polymorphism (Table 1).

Table 2 Frequency of SNPs in patients and donors.

NOD2 status	Study group (n=85)	
	Patient no (%)	Donor no (%)
Wt/wt	71 (83.5)	67 (79)
SNP8/wt	9 (10.5)	12 (14)
SNP12/wt	0 (0)	1 (1)
SNP13/wt	3 (3.5)	5 (6)
SNP13/SNP13	1 (1)	0
SNP12/SNP13	1 (1)	0

NOD2 polymorphisms and outcome

Acute Graft-versus-Host disease

In the analysis of aGvHD, four patients, one with a polymorphism, were excluded because of death before engraftment. The overall incidence of aGVHD grade II-IV and grade III-IV was 32.1% and 7.4%, respectively.

We first compared the incidence of aGvHD in pairs with any polymorphism present with the outcome of wild-type patient/donor pairs and found no significant difference in grade II-IV aGvHD (cumulative incidence 43 vs. 30%, HR 1.5 95%CI: 0.7-4.6, *P* =0.30). However, an effect of NOD2 polymorphisms was seen for severe aGvHD with a cumulative incidence of 19% in pairs with any polymorphism compared to 3% for pairs with wild type status (HR 6.0, 95%CI: 1.1-100, Gray test *P* =0.02). The magnitude of this difference was accounted for by the higher incidence of severe aGvHD in the subgroup of pairs in which both had a polymorphism, with an incidence of 33% (HR 10.5, 95%CI: 1.8-100, Gray test *P* =0.002, Figure 1). Furthermore, multivariable analysis, including patient and donor age, gender

Figure 1 Cumulative incidence of severe acute graft-versus-host disease (aGvHD grade III-IV) in relation to NOD2 polymorphisms.
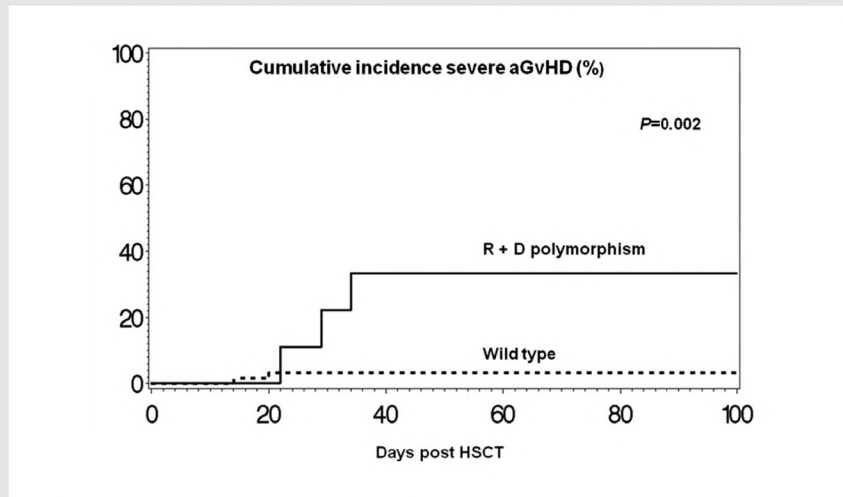
R + D polymorphism indicates recipient and donor both having a NOD2 polymorphism, wild type; both recipient and donor without polymorphism. Comparison of R + D vs. wild type showed a statistically significant increased risk of severe aGvHD associated with NOD2 polymorphisms. *P*-value Gray test.

combination, stem cell source, and T-cell-depletion technique, showed that polymorphisms in NOD2 were the only significant predictor of the occurrence of severe aGvHD (*P* <0.001).

Treatment related mortality and relapse
The overall 1-year TRM was 16.5%. The presence of a polymorphism was a significant factor with a cumulative incidence of 32% compared to 11% for wild-type status (HR 3.3, 95%CI: 1.2-9.1, Gray test *P* =0.02). Again this was mostly accounted for by patient/donor pairs both bearing a polymorphism with an incidence of 50% (HR 5.9, 95%CI: 1.9-18.0, Gray test *P* =0.002, Figure 2). Mortality was predominantly attributed to aGvHD and infections, especially fungal infections. In pairs with any polymorphism mortality was in 42.9% related directly to aGvHD compared to 0% for the wild-types.
In multivariable analysis, including patient age and gender, underlying disease and disease status at HSCT, this impact remained with a HR of 3.8 for all polymorphisms and a HR of 7.8 for pairs both having a polymorphism.

Figure 2 One year treatment-related mortality (1-year TRM).
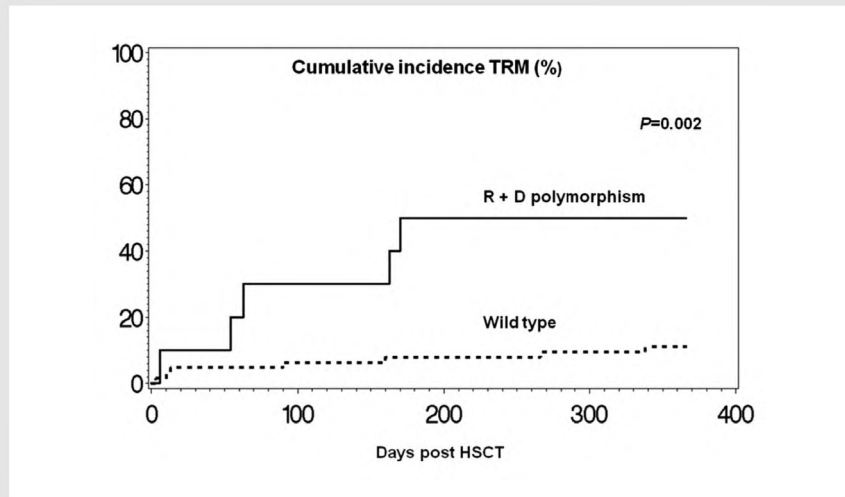
R + D polymorphism indicates recipient and donor both having a NOD2 polymorphism; wild type, both recipient and donor without polymorphism. Comparison of R + D vs. wild type showed a statistically significant increased 1-year TRM associated with NOD2 polymorphisms. *P*-value Gray test.

No differences in the occurrence of disease relapse at 1- and 3-year follow-up were found in either univariable or multivariable analysis (data not shown).

Bacteremia
Bacteremia occurred in 68% of patients, with an incidence of bacteremia due to coagulase-negative staphylococci (CoNS) of 45.9% and that due to oral viridans streptococci (OVS) of 25.9%. Analysis of the OVS bacteremia was not possible as 24 patients had received meropenem. There was a cumulative incidence of CoNS bacteremia until day 14 of 64.3 vs. 36.6% (HR 1.85; 95%CI 0.95-3.60, *P* =0.07) among patients with a polymorphism compared to wild-types.

Discussion

We show that there was a significant impact of NOD2 polymorphisms on treatment outcome, in particular severe aGvHD and TRM, among recipients of T-cell-depleted HSCT. The impact was most pronounced when both the patient and donor had a

NOD2 polymorphism, suggesting that a "continuous" NOD2 dysfunction in both epithelia and immune cells with subsequent defective antimicrobial activity and immune deregulation results in a worse clinical outcome. This finding was similar to the results reported by Holler *et al.* [6]. However, the subgroups were too small to determine the impact when only a patient or donor had a polymorphism. Our data suggest that screening for NOD2 polymorphisms in this patient population could aid in improving the outcome after HSCT. This especially applies to the setting of HLA-identical sibling HSCT, because of a considerable chance of both patient and donor bearing a polymorphism, with significant consequences. Selecting a wild-type NOD2 sibling is preferred, but this might prove difficult because of the lack of matched siblings. Therefore, other approaches might be to select a voluntary unrelated donor without NOD2 polymorphisms or to adapt antimicrobial prophylaxis.

Our results contrast with those of Granell *et al.* who found no impact of NOD2 polymorphisms on aGvHD and associated TRM in T-cell-depleted HSCT [25]. There are several explanations for these different results. The most important is the higher overall incidence of aGvHD (grade II-IV 32.1 vs 10%) which probably relates to the use of idarubicin-containing regimen, as this induces more MBI and aGvHD [20]. Furthermore, the use of partially T-cell-depleted grafts, with a mean of 0.5×10^6 CD3+ T-cells/kg, contributes to the higher incidence of aGvHD in our patients. There was also a higher allele frequency of NOD2 polymorphisms in our cohort (9.7 vs. 5.1%). This results from true genetic heterogeneity between the Dutch and Spanish populations [26], but could also be influenced by chance. Consistent with the increased incidence of severe aGvHD, in patients with NOD2 polymorphisms, TRM was related to aGvHD and infectious complications resulting from immunosuppressive therapy, especially fungal infections. No specific increase in mortality related to pneumonia was found contrary to the study of Granell *et al.*.

In the setting of HSCT using unrelated donors discrepancies in the impact of NOD2 polymorphisms on TRM and relapse have been attributed to the use of T-cell-depletion with alemtuzumab (MabCampath®) or not [27]. This underlines the necessity to take "the context" of studies into account, especially with respect to the level of T-cell-depletion and the overall incidence of aGvHD. As always, local data are required before adopting general conclusions and screening proposals.

We saw more bacteremia due to CoNS in patients with a NOD2 polymorphism than was found among wild-types, although the difference did not reach statistical significance. We were unable to determine the impact on bacteremia due to OVS, because 24 patients had received meropenem and after correction the study group was too small to draw firm conclusions. NOD2 senses the cell wall constituent MDP which is present in the gram-negative and gram-positive bacteria that colonize body surfaces or cause infection. NOD2 is important in the antibacterial defenses

especially by regulating the production of defensins at the epithelial barriers of gut and lung [9;28;29]. Defensins possess anti-staphylococcal and anti-streptococcal activity [30] and, in the gut, are elaborated predominantly by Paneth cells. Their production has been shown decreased when there are NOD2 polymorphisms [9;10]. The increased incidence of CoNS bacteremia found in patients with a NOD2 polymorphism suggests that lower number or activity of the peptides influence infectious complications in HSCT. This might explain how the gut may be an important origin of staphylococcal and streptococcal bacteremias [31;32]. Consequently, polymorphisms in NOD2 predispose to infections with gram-positive bacteria when patients are given prophylaxis with fluoroquinolones while suffering from MBI and lung epithelial damage.

Importantly, sensing bacteria or derived MDP by NOD2 has an impact on the incidence of aGvHD, since the impact of NOD2 polymorphisms on aGvHD disappears with the use of comprehensive prophylaxis [33]. MBI is induced by myeloablative conditioning therapy [1], and is an inflammatory condition of the gut associated with the occurrence of aGvHD [2;3]. Inflammation is exacerbated by the translocation of bacteria and microbial products which elaborate cytokine release through the stimulation of TLRs. In case of NOD2 polymorphism reduced defensin production could contribute to increased bacterial translocation. Additionally "loss of tolerance" increases TLR activity and cytokine release [13-16]. Consequently, reducing the microbial burden of the gut using antimicrobial prophylaxis that provides better gram-positive and anaerobic coverage could prove of benefit for patients bearing a NOD2 polymorphism to reduce the risk of severe aGvHD and therewith TRM. This would especially apply to those patients in whom no wild-type donor can be selected.

The role of donor polymorphisms is less easy to explain, especially in the setting of T-cell-depletion. However, donor immune cells can indirectly lower the production of defensins [34] and also suffer from the "loss of tolerance", so they could influence incidence and severity of aGvHD.

Our study is limited by being of a retrospective nature and the fact that the study group was small. Nevertheless, in this very homogenous cohort of Dutch patients we were able to detect a significant impact of NOD2 polymorphisms in T-cell-depleted HSCT with regard to severe aGvHD and TRM and showed a higher incidence of gram-positive bacteremia. We conclude that in our population NOD2 polymorphism screening should be used to optimize donor selection and antimicrobial prophylaxis to reduce the occurrence of aGvHD and TRM following allogeneic HSCT.

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Early stop polymorphism in human Dectin-1 is associated with increased Candida colonization in hematopoietic stem cell transplant recipients

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Abstract

Background: Intensive treatment of hematological malignancies with hematopoietic stem cell transplantation (HSCT) is accompanied by a high incidence of opportunistic invasive fungal infections, but individual risk varies significantly. Dectin-1, a C-type lectin which recognizes 1,3-β-glucans from fungal pathogens, including *Candida* species, is involved in the initiation of the immune response against fungi.

Methods: Screening for the *DECTIN-1* Y238X polymorphism within a group of 142 patients receiving HSCT was correlated with *Candida* colonization and candidemia. Furthermore, functional studies were performed on the consequences of the polymorphism.

Results: Patients bearing the Y238X polymorphism in the *DECTIN-1* gene were more likely to be colonized with *Candida* species compared with patients bearing wild-type *DECTIN-1*, necessitating more frequent use of fluconazole in the prevention of systemic *Candida* infection. Functional assays demonstrated a loss-of-function phenotype of the polymorphism, as shown by the decreased cytokine production by immune cells bearing this polymorphism.

Conclusions: The Y238X polymorphism is associated with increased oral and gastrointestinal colonization with *Candida* species. This suggests a crucial role played by dectin-1 in the mucosal antifungal mechanisms in immunocompromised hosts. The finding that *DECTIN-1* polymorphisms rendered HSCT recipients at increased risk for fungal complications may contribute to the selection of high-risk patients who should be considered for antifungal prophylaxis to prevent systemic candidiasis.

Introduction

The treatment of patients with hematological malignancies with a hematopoietic stem cell transplantation (HSCT) following myeloablative conditioning is accompanied by complications that include mucosal barrier injury, prolonged neutropenia and graft-versus-host disease (GvHD), all of which contribute to fungal and other opportunistic infections [1;2]. Traditional risk factors are predictive for the incidence of invasive fungal disease in general, but the individual risk is more difficult to determine, although this is needed for a more guided use of antifungal prophylaxis and therapy.

Dectin-1 is a member of the C-type lectin receptor family that recognizes the β-1,3-glucan motif of the cell wall of pathogenic fungi [3]. Dectin-1 is mainly expressed by immune cells of the myeloid lineage (neutrophils, macrophages and dendritic cells). Several studies have shown that dectin-1 belongs to the armamentarium of immune cells against fungal pathogens, including *Candida* species and *Aspergillus* species [4;5]. Furthermore, dectin-1 synergizes with TLR2 and TLR4 signals and promotes Th1 and Th17 responses to activate antifungal host defense [6-8].

Recently, we have demonstrated that a polymorphism in *DECTIN-1* (Y238X, rs16910526) is responsible for recurrent mucocutaneous fungal infections in a Dutch family [9]. This polymorphism resulted in an early stop codon which leads to the loss of the last 10 amino acids of the extracellular domain, and in a diminished capacity to bind β-glucans.

To test the hypothesis that *DECTIN-1* variants influence the susceptibility to fungal infections in general and *Candida* infections in particular of patients receiving HSCT, we first assessed the frequency of *DECTIN-1* polymorphisms in a healthy Dutch population. We identified the Y238X mutation as an important polymorphism, and the only one with significant functional consequences for the recognition of *Candida* species. Subsequently, we have investigated the impact of this *DECTIN-1* polymorphism on the incidence of mucosal *Candida* colonization and the occurrence of candidemia and other invasive fungal infections in 142 patients receiving a sibling T-cell-depleted allogeneic HSCT.

Patients, materials and methods

Genetic screening for DECTIN-1 polymorphisms

DNA template of the *DECTIN-1* gene (also named *CLEC7A*) was taken from GenBank, chromosome position 12p13, NC_000012.10. Sequencing of the exonic and nearby intronic regions of the *DECTIN-1* gene in the 138 healthy volunteers was performed by applying the primers and conditions depicted in table 1. Genotyping

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Table 1 Primers and PCR conditions applied to amplify every exon and proximal intronic regions of the *DECTIN-1* gene, to ultimately perform sequence analysis.

Exon	Primer	Sequence (5'- 3')	[MgCl$_2$] (mM)	Annealing temperature
1	Forward	TTTCACCACGTTAGCCAAGCT	2.5	52°C
	Reverse	CTGAAATAGTTTGCATCGGTT		
2	Forward	CCCTTTATAAGTGAAATGGGC	1.75	60°C
	Reverse	ACCGTGCAAGGCCAGATTTT		
3	Forward 1	GCCAGTGATAAATCAGTTACT	3.5	56°C
	Reverse 1	TTCTTCTTCTCCACCTTCTT		
	Forward 2	TGGCAACATTTTCCCTTCTT	3.5	56°C
	Reverse 2	GGCAAGGGCATAGTTAAAGG		
4	Forward	TCATTACCTGGAATCTCCCTCT	2.5	56°C
	Reverse	TGGCAACTAATTGGTTATTTCA		
5	Forward	GCTGCTCGACAGAGGTTTTC	1.75	62°C
	Reverse	GGATGGTCTCGATCTCCTGA		
6	Forward	AATCACAGCCTCTCCCTTCA	2.5	60°C
	Reverse	GATTTAAGCCTCCTTTTCCAA		

For all amplicons sequence analyses was carried out with the forward and the reverse primer. For the sequences of exon 3 technical difficulties were encountered. Therefore we amplified exon 3 with two different primer pairs and aligned both forward assays after sequence analysis.

for the presence of the Y238X polymorphism in the patient and donor groups was performed by applying the TaqMan single-nucleotide polymorphism (SNP) assay C_33748481_10 on the 7300 ABI Real-Time PCR system (Applied Biosystems). Patients, donors, and healthy volunteers had given informed consent to prospective collection of DNA samples for investigational use.

Flow cytometry

For staining of membrane-bound dectin-1, monocytes were incubated with 5 μg/mL murine anti-dectin-1 directed towards the stalk region (BD6) conjugated with biotin, or mouse IgG2b isotype control, followed by streptavidin–allophycocyanin conjugated goat anti-mouse antibody (Pharmingen). Dectin-1 expression was determined by flow cytometry (FACScalibur; BD Biosciences).

Confocal microscopy

Confocal laser scanning microscopy was performed as described by Meyer-Wentrup *et al.* [10]. Cells were stained with 10 µg/mL mouse anti-dectin-1 (clone 259931; R&D systems) or mouse IgG2b isotype control, followed by goat anti-mouse-Alexa647-conjugated secondary antibody (Molecular Probes). Samples were mounted in mowiol and analyzed by confocal laser scanning microscopy (Olympus FV1000).

Cytokine stimulation assays

Isolation of mononuclear cells was performed as described previously [8]. Cells were incubated at 37°C for the indicated duration (4 h or 48 h) with either culture medium or the various stimuli: 10^5 heat-killed *Candida albicans* (heat-killed by incubation at 56°C for 30 min), the TLR2 agonist Pam3Cys (10 µg/mL) and β-glucan (10 µg/mL) or a combination of Pam3Cys and β-glucan. In a part of the experiments, after peripheral blood mononuclear cells (PBMC) isolation, monocytes were purified by CD14+ MACS MicroBeads (Miltenyi Biotec) and stimulated as described above. Cytokine production was measured by ELISA (R&D Systems).

Patients and donors

We performed a retrospective analysis in 142 Dutch patients undergoing HSCT due to hematological malignancies. The patients and their donors were consecutively admitted at our transplant unit between May 1996 and September 2007 for a human leukocyte antigen-identical sibling, partially T-cell-depleted allogeneic HSCT. The characteristics of patients, donors and HSCT procedures are depicted in table 2.

Treatment protocol

The treatment protocol has been previously described in detail [11]. Conditioning regimen consisted of cyclophosphamide (60 mg/kg for 2 days) in combination with either total body irradiation (4.5 Gy for 2 days) or busulfan (4 mg/kg for 4 days). Idarubicin (42 mg/m^2 in 48 h) was often added in these conditioning regimen to reduce the risk of relapse in the setting of T-cell-depleted HSCT [12]. On day 0, all patients were given an allogeneic HSCT containing 3.2×10^6 CD34+ cells/kg (range 0.6-11.6) and 0.5×10^6 CD3+ cells/kg (range 0.1-0.8).

Antimicrobial prophylaxis consisted of 500 mg ciprofloxacin given twice daily and 500 mg valaciclovir given 3 times daily. Surveillance cultures for *Candida* were collected twice weekly from hospital admission until hospital discharge, with the first cultures taken on the day of admission before the start of conditioning. Fluconazole (200 mg daily) was only prescribed to those who were colonized with *Candida albicans, C. tropicalis* or *C. parapsilosis (not C. krusei* or *C. glabrata)* when the yeast was present in both fecal cultures and mouth washes obtained on the

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Table 2 Clinical characteristics of the study group.

Recipient, donor and HSCT characteristics	Homozygous wild-type for *DECTIN-1*	Heterozygous for *DECTIN-1* Y238X	*P*-value
Number of subjects			
Recipients	126	15	
Donors	116	22	
Gender (% male)			
Recipients	65%	60%	**0.78**
Donors	56%	64%	**0.64**
Age, mean (range)			
Recipient	47.5 (18.5-64.4)	42.8 (19.2-59.8)	**0.11**
Donor	47.4 (14-75.6)	43.3 (23.8-67.6)	**0.24**
Diagnosis:			
-AML/ALL no (%)	60 (47.6)	8 (53.3)	**0.79**
-CML/MPS, no (%)	26 (20.6)	3 (20)	
-MDS, no (%)	23 (18.3)	-	
-Lymphoma/CLL, no (%)	17 (13.5)	4 (26.7)	
Conditioning regimen:			
-Ida-Cyclo-TBI, no (%)	79 (62.7)	10 (66.7)	
-Ida-Cyclo-Bus, no (%)	15 (11.9)	1 (6.7)	
-Cyclo-TBI, no (%)	27 (21.4)	1 (6.7)	
-Cyclo-Bus, no (%)	5 (4.0)	3 (20)	
TBI, no (%)	106 (84.1)	11 (73.3)	**0.25**
Stem cell source:			
-Peripheral blood, no (%)	72 (57.1)	9 (60.0)	**1.0**
-Bone marrow, no (%)	54 (42.9)	6 (40.0)	
T-cell-depletion:			
-CD34 selection, no (%)	61 (48.5)	9 (60.0)	
-Counterflow elutriation, no (%)	41 (32.5)	4 (26.7)	
-CD3/CD19 selection, no (%)	24 (19.0)	2 (13.3)	**0.43**
Duration of neutropenia $\leq 0.1 \times 10^9/l$ in days(range)	11.9 (6-20)	11.0 (6-15)	**0.23**
Acute GvHD:			
-Grade 0-I, no (%)	81 (64.3)	11 (73.3)	**0.77**
-Grade II-IV, no (%)	39 (31.0)	4 (26.7)	
-Grade III-IV, no (%)	10 (7.9)	1 (6.7)	
-NA	6 (4.7)	-	

All patients received an HLA matched sibling partially T-cell-depleted HSCT. GvHD prophylaxis consisted of only cyclosporine in all patients. Characteristics are expressed in absolute numbers and percentages. Age and neutropenia are expressed as mean value with the total range. Differences between the study groups were compared using the Pearson's χ^2 test or Fisher's exact test and with the use of the independent *t*-test where appropriate. No significant differences were obtained. Abbreviations: AML/ALL = acute myeloid and lymphatic leukemia, CML/MPS = chronic myeloid leukemia/myeloproliferative syndrome, MDS = myelodysplastic syndrome, CLL = chronic lymphatic leukemia, Ida = idarubicin, Cyclo = cyclophosphamide, Bus = busulphan, TBI = total body irradiation, GvHD = graft-versus-host-disease, NA = not applicable.

same day, or when obtained from the same site on two consecutive occasions [13]. Invasive fungal infections were defined according to the European Organization for the Treatment of Cancer/Mycoses Study Group consensus guidelines, designating invasive fungal disease as possible, probable or proven [14]. Oral mucositis was graded daily according to the validated Nijmegen Nursing Mucositis Scoring System (NNMSS) [15]. Acute GvHD was diagnosed by clinical signs or pathological examination of biopsies of skin, gut or liver and graded according to the criteria of Glucksberg *et al.* [16].

Statistical analysis

In multivariable logistic regression analyses, we investigated the association of *DECTIN-1* status of the patient with *Candida* colonization, controlling for underlying hematological disease, age and gender of the patient. In the analysis on the impact of early candidemia the *DECTIN-1* status of only patients, but not of the donors, was considered of importance. Early candidemia was defined as occurring on day 21 or earlier. In myeloablative stem cell transplantation, monocyte recovery usually occurs only 3-4 weeks after HSCT [17], and therefore the genetic make-up of the donor is considered as not relevant for the susceptibility to early candidemia. The association between *DECTIN-1* status of the patient and the occurrence of early candidemia was studied using logistic regression models accounting for confounding by including age of patient, the underlying disease, the duration of neutropenia, presence of colonization on admission and the presence of GvHD. In contrast, in the analysis on the impact of proven and probable invasive mould infections up to day 100, *DECTIN-1* status of both patients and donors was included.

To compare percentages between two independent groups we used the χ^2 test, or the Fisher's exact test when appropriate. Differences in the cytokine production capacity were tested by the Student's *t* test. $P < 0.05$ was considered to represent a statistically significant difference.

Results

DECTIN-1 polymorphism screening in healthy individuals

Genetic variation in the *DECTIN-1* gene was investigated in 138 Dutch healthy volunteers, which revealed the polymorphisms depicted in figure 1. A small number of polymorphisms were identified with the Y238X (rs16910526) in exon 6 being the only exonic polymorphism. This polymorphism was present in 19 (13.8%) out of 138 individuals, all of whom were heterozygous, resulting in an allele frequency of 6.9%. This polymorphism was identified earlier in a family previously analyzed for mucocutaneous *Candida* infections [9]; 3 members of this family were homozygous for the Y238X polymorphism.

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Figure 1 Schematic drawing of the *DECTIN-1* gene (also known as *CLEC7A*), consisting of an N-terminal cytoplasmatic tail (Cyto), encoded by exon 1, a transmembrane region (TM, exon 2), a stalk region (exon 3) and the carbohydrate recognition domain (CRD, exon 4-6). Intronic polymorphisms were detected in 5'UTR, intron 2, intron 3, exon 6 and 3'UTR. All variations are depicted together with their corresponding chromosome location and rs number, if available.
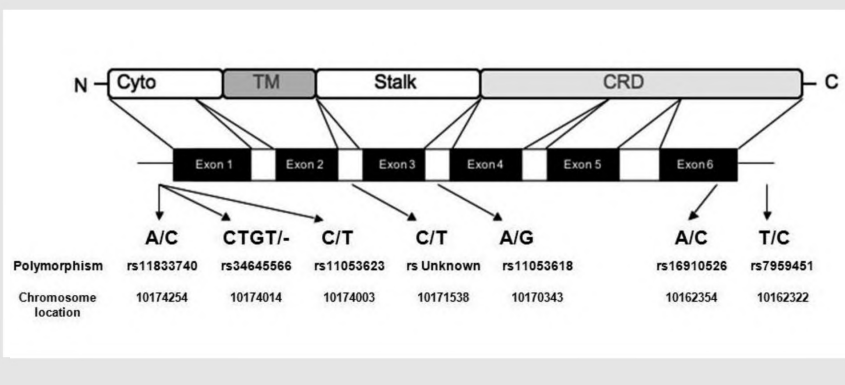
Dectin-1 protein expression

We investigated the consequence of the polymorphism on dectin-1 expression and localization at the protein level. Monocytes isolated from individuals homozygous for the wild-type *DECTIN-1* allele and from individuals heterozygous or homozygous for the Y238X polymorphism were analyzed for dectin-1 expression by flow cytometry and confocal microscopy. Monocytes from the individuals homozygous for the Y238X polymorphism exhibited no dectin-1 expression on the cell surface, whereas cells from individuals heterozygous for the Y238X polymorphism had intermediate cell surface expression compared to cells from individuals with the wild-type allele (figure 2*A*). In line with this, no cell surface expression of dectin-1 could be detected on monocytes isolated from individuals homozygous for the Y238X polymorphism in contrast to wild-type cells, when monocytes were analyzed by confocal microscopy (figure 2*B*). However, dectin-1 mRNA expression was demonstrated to be equal between the genotypes (data not shown). Thus, these findings demonstrate that dectin-1 protein expression is absent from the cell membrane of monocytes from the individuals homozygous and intermediate on monocytes from individuals heterozygous for the Y238X polymorphism.

Figure 2 (**A**) Flow cytometry graphs of extracellular dectin-1 staining, and (**B**) fluorescent confocal staining of dectin-1 (right panel) and light microscopic image (left panel) on unstimulated monocytes derived from individuals homozygous for the wild-type *DECTIN-1* allele (WT) and heterozygous (HET) or homozygous for the *DECTIN-1* Y238X polymorphism (HOM).

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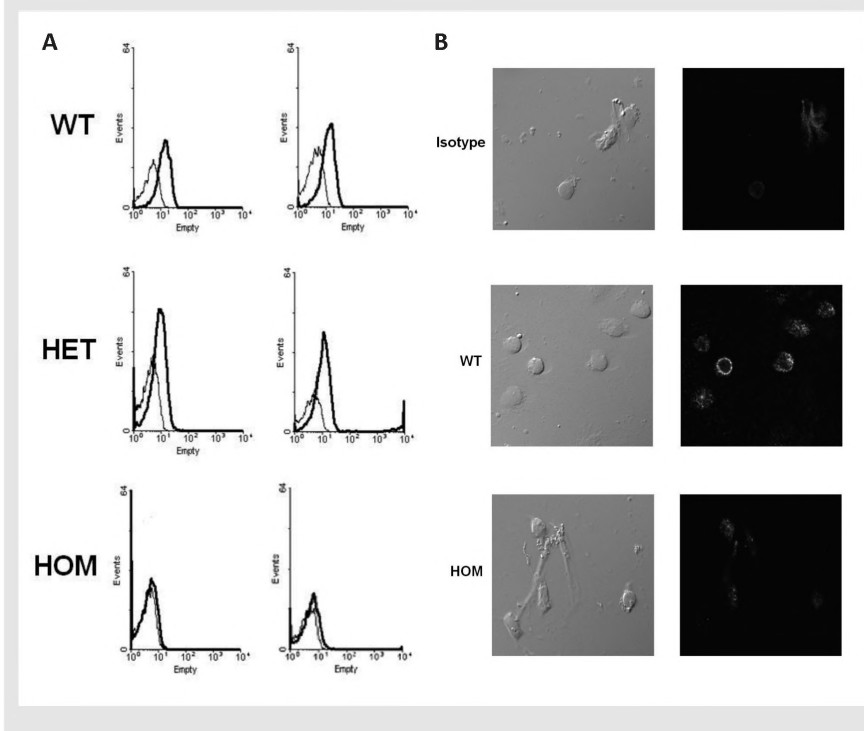
Cytokine production

Functional consequences of the Y238X polymorphism were investigated in monocytes and PBMCs isolated from individuals bearing only the wild-type *DECTIN-1* allele and individuals being heterozygous or homozygous for the Y238X polymorphism. Interleukin (IL)-1β induction by *C. albicans* was lower in cells from individuals bearing the Y238X polymorphism (figure 3*A*, *P* <0.05). Dectin-1 has been previously demonstrated to amplify TLR2 signaling [8]; this effect was absent in cells isolated from individuals homozygous for the Y238X allele (figure 3*B*). In contrast, IL-18 and interferon (IFN)-γ production was not defective in cells isolated from these individuals (figure 3*C*, *P* = not significant). Although a tendency towards a lower IL-18 production capacity has been observed in cells from individuals heterozygous

for Y238X compared with cells bearing only the wild-type *DECTIN-1* allele, this did not reach statistical significance. IL-18 production in cells of individuals homozygous for the Y238X mutation was similar to production in cells from individuals with the wild-type allele. Furthermore, IFN-γ production capacity is practically equal between the genotypes.

DECTIN-1 Y238X polymorphism in patients and donors
The *DECTIN-1* genetic status could be determined in 141 patients and 138 donors. Insufficient amounts of DNA precluded the determination in 1 patient and 4 donors. Fifteen (10.6%) of 141 patients and 22 (15.9%) of 138 donors had the *DECTIN-1* Y238X polymorphism; all individuals were heterozygous. In 9 patient-donor pairs, both individuals were heterozygous for the polymorphism. There were no statistically significant differences between the clinical characteristics of patients with and without the *DECTIN-1* Y238X polymorphism (table 2). No difference was detected in the severity of mucositis between patients with or without the *DECTIN-1* Y238X polymorphism, with a mean NNMSS score of 3.8 vs. 3.9 on day 0 and 7.2 vs. 7.6 on day 7 (*P* = not significant).

Candida species colonization
Seven patients who received secondary antifungal prophylaxis at hospital admission were excluded from the study, as were 11 patients with missing colonization data, leaving 124 patients eligible for analysis of *Candida* colonization. At hospital admission, 46 (37.1%) of 124 patients were colonized with *Candida* species. Patients who were heterozygous for the Y238X polymorphism were statistically significantly more often colonized than were patients with *DECTIN-1* wild-type alleles (11 (84.6%) of 13 vs. 35 (31.5%) of 111; *P* <0.001) (odds ratio (OR), 11.9; 95% confidence interval (CI), 2.5–56.8). After adjusting for diagnosis, the OR was 12.2 (95% CI, 2.5–59.7); after adjusting for patient age and sex, the OR was 12.0 (95% CI, 2.5–57.1). On the day of HSCT (day 0), this difference persisted: 12 (92.3%) of 13 patients vs. 50 (45.1%) of 111 patients (*P* =0.001). The unadjusted OR was 14.6 (95% CI, 1.8–116.5) and the OR adjusted for diagnosis, patient age, and sex was 15.5 (95% CI, 1.9–125.6).

Patients with a *DECTIN-1* polymorphism were more likely than other patients to receive fluconazole (9 (69.2%) of 13 vs. 42 (37.8%) of 111; *P* =0.03) (table 3). Among those patients who received fluconazole, eradication was achieved in 1 (11.1%) of 9 patients who were heterozygous for the Y238X polymorphism, compared with 14 (33.3%) of 42 patients who bore only the wild-type *DECTIN-1* allele (*P* =0.25). Colonizing species were *C. albicans* (87% of patients), *C. glabrata* (8.1%), and sporadically, *C. krusei, Candida kefyr, Candida parapsilosis,* and *Candida dubliniesis* (1.6% each). No difference in the frequency of colonization with particular *Candida*

Figure 3 Cytokine production capacity of IL-1β, IL-18 and IFN-γ after stimulation of monocytes during 4 hours (panel A) or PBMCs during 48 hours with heat-killed *C. albicans* (panel C) or with β-glucan, Pam3Cys or β-glucan /Pam3Cys (panel B). Cells were obtained from individuals being wild-type (WT, n=5), heterozygous (HET, n=4) and homozygous (HOM, n=3) for the *DECTIN-1* Y238X polymorphism. Data are percentages compared to wild-type (WT) ± SD (*P < 0.05).
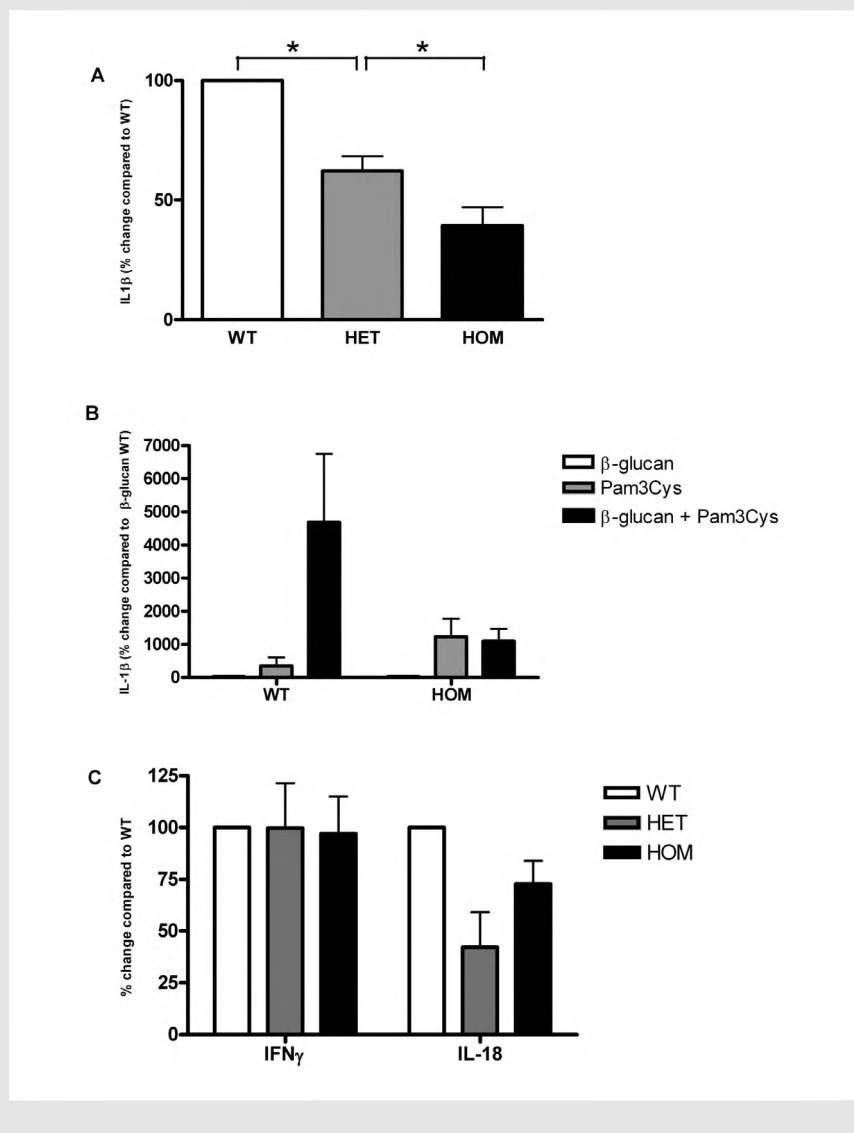

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Table 3 Observed *Candida* colonization and invasive fungal disease in HSCT patients. For details see material and methods.

Clinical outcome	Recipients (+ donors*) homozygous wild-type for *DECTIN-1*	Recipients (+ donors*) heterozygous for *DECTIN-1* Y238X	*P* values	OR
Candida colonization on admission	31.5% (35/111)	84.6% (11/13)	<0.001	11.9
Candida colonization day of HSCT (day 0)	45.1% (50/111)	92.3% (12/13)	0.001	14.6
Surveillance-culture guided fluconazole therapy	37.8% (42/111)	69.2% (9/13)	0.03	3.7
Early candidemia, ≤ day 21	8.0% (9/112)	18.2% (2/11)	0.26	2.5
Invasive mould disease ≤ day 100	3.0% (3/100)*	5.0% (1/20)*	0.52	1.7

*The genetic DECTIN-1 status of the patient-donor couples (either patient, donor or both heterozygous for the Y238X polymorphism) was included in this analysis of the impact of the polymorphism on the occurrence of invasive mould disease ≤ day 100.

species was observed between patients who were heterozygous for Y238X and patients who bore only the wild-type *DECTIN-1* allele.

Invasive fungal disease

Patients who received early antifungal prophylaxis with either itraconazole, voriconazole, or posaconazole, starting from day 0 or earlier and until day 21, were excluded (18 patients, 7 of whom had received secondary prophylaxis and 11 of whom had participated in antimicrobial studies). The overall incidence of candidemia until day 21 was 8.9% (11 of 123 patients). There was no statistically significant difference in the incidence of early candidemia between patients with and patients without the *DECTIN-1* Y238X polymorphism (2 (18.2%) of 11 vs. 9 (8.0%) of 112; *P* =0.26) (table 3); the OR was 2.5 (95% CI, 0.5–13.6). However, this study was not designed to detect differences in the risk of developing invasive candidiasis, because patients who were colonized were prescribed fluconazole to prevent systemic *Candida* infection, and the study was underpowered to find a difference. Candidemia was caused by *C. albicans* in 7 of 11 cases, with the remainder of the cases being due to *C. glabrata* (1 case), *C. parapsilosis* (2), and *C. dubliniesis* (1). Eight (15.7%) of 51 patients who were colonized and received fluconazole experienced candidemia. Two (3.6%) of 55 patients who were not colonized, and who therefore received no fluconazole developed candidemia.

The incidence of proven and probable invasive mold infection up to day 100 was 3.3% (4 of 120 patients), with 4 probable and no proven mold infections. Three probable cases were due to *Aspergillus* species, and 1 probable case was due to *Rhizomucor* species. There was no statistically significant difference between pairs with and without a *DECTIN-1* Y238X polymorphism; 1 (5.0%) of 20 patients with the Y238X polymorphism had a probable case, compared with 3 (3.0%) of 100 patients with the *DECTIN-1* wild-type allele (P =0.52) (table 3). However, the study was underpowered to detect a difference, especially because of the very low incidence of mold infection.

Discussion

In this study, we demonstrate that a newly characterized polymorphism in *DECTIN-1* is associated with increased susceptibility to fungal colonization among HSCT recipients. As a consequence, among patients who were immunocompromised as a result of HSCT, the need to prescribe fluconazole to prevent systemic *Candida* infection was, in part, defined by the presence of this polymorphism.

Dectin-1 is one of the most important pattern recognition receptors for fungal pathogens in general, and *Candida* species in particular. Polymorphisms in pattern recognition receptors are known to be associated with an increased susceptibility to fungal infections [18;19]. We hypothesized that genetic variants of *DECTIN-1* could influence susceptibility to *Candida* colonization and infection in HSCT recipients.

Screening of all 6 exons of the *DECTIN-1* gene in a healthy Dutch population revealed 1 exonic polymorphism and several intronic SNPs. Since the intronic SNPs are not likely to affect dectin-1 function, we considered the Y238X polymorphism as the only polymorphism that could alter dectin-1 function and could influence susceptibility to fungal infections.

To characterize the functional consequences of the Y238X polymorphism in more detail, flow cytometry and confocal microscopy were performed. Expression of dectin-1 was absent on the cell membrane of cells isolated from individuals homozygous for the Y238X allele, wich suggested a defective transport of the mutated form of dectin-1 to the cell membrane. Accordingly, dectin-1 expression was intermediate on cells from individuals heterozygous for the polymorphism.

After stimulation with heat-killed *C. albicans* or β-glucan, IL-1β secretion was intermediate in cells isolated from individuals heterozygous for the Y238X polymorphism and low in cells from individuals homozygous for this polymorphism, compared with individuals who where homozygous for the wild-type allele. Moreover, the previously described synergism between TLR2 and dectin-1 signals [8] was completely absent in individuals homozygous for the Y238X polymorphism.

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These data demonstrate the loss-of-function effect of the Y238X polymorphism for dectin-1 activity. However, no difference in production of IL-18 and IFN-γ could be observed between the different *DECTIN-1* genotypes. This could well be due to a certain redundancy of dectin-1 signaling in production of these cytokines, and this is accompanied by a normal *Candida* killing activity by neutrophils from individuals bearing the Y238X polymorphism (data not shown), resulting in an adequate host defense in systemic infections.

In the follow-up of these genetic and immunological studies, we demonstrate that this polymorphism has a significant impact on oral and gastrointestinal mucosal colonization with *Candida* species in HSCT recipients, which defined the need for the use of fluconazole in our approach. This is in line with the finding of recurrent mucocutaneous candidiasis in a family with individuals who where homozygous for the *DECTIN-1* polymorphism [9]. The overall rate of fungal colonization at admission was comparable to that in earlier studies, with colonization rates of 28-57% [20;21]. These data strongly support a role of dectin-1 mediated mechanisms for mucosal anti-*Candida* defense.

The mechanisms of the increased susceptibility to mucosal colonization with *Candida* in individuals bearing the *DECTIN-1* polymorphism cannot be definitively pinpointed, although the defective cytokine responses are probably involved. Although monocytes are important for mucosal defenses, epithelial cells may also contribute to these effects. Interestingly, the expression of dectin-1 on epithelial cells has been recently demonstrated and the interaction of these cells with fungal pathogens leads to chemokine release [22]. One has to consider however, that in the setting of HSCT, both monocytes and epithelial cells of the mucosa are damaged for a prolonged period, although largely intact on admission and during the first days of conditioning. On the other hand, the residential macrophages of the mucosa are less affected, and it is most likely that the impact of the *DECTIN-1* polymorphism is exerted at their level.

The presence of the *DECTIN-1* polymorphism did not result in a significantly higher incidence of candidemia, although that might have been expected since mucosal colonization is associated with systemic candidiasis in HSCT recipients who experience mucosal barrier injury [23;24]. Importantly, in this cohort patients were prescribed oral fluconazole when colonized, which is known to reduce the incidence of candidemia [21;25]. Therefore, fluconazole was most likely a confounding factor, precluding a definitive conclusion regarding the role of the *DECTIN-1* Y238X polymorphism for susceptibility to early candidemia, although our data may suggest an increased risk (OR 2.5, 95%CI 0.5-13.6). In addition, our study was underpowered to study the impact of the polymorphism on the occurrence of invasive mould infections because of the low incidence of these infections.

In our culture-guided approach fluconazole could safely be withheld for those

individuals who were not colonized with *Candida* species, preventing overtreatment and accompanied side effects and costs. However, those individuals who were colonized remained at significant risk for candidemia, necessitating better ways to predict, determine and treat colonization at an early stage. Determining the *DECTIN-1* status before HSCT might be a factor contributing to a more risk adapted prophylactic approach. Because *Candida* colonization is associated with invasive disease in HSCT, we propose that patients bearing the polymorphism should be considered for antifungal prophylaxis to prevent systemic candidiasis. Nevertheless, future studies in prospective trials are necessary to further confirm the impact of this newly characterized *DECTIN-1* polymorphism and to define its role in selecting adequate prophylaxis or early treatment for HSCT recipients.

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The incidence of acute graft-versus-host disease increases with Candida colonization depending the dectin-1 gene status

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Abstract

Dectin-1 plays an important role in antifungal immunity. The dectin-1 Y238X polymorphism, which results in decreased Th17 responses, is associated with increased *Candida* colonization of stem cell transplantation (SCT) recipients. In this study we found no impact of the polymorphism on the incidence of graft-versus-host disease (GvHD), or on disease-free and overall survival in these SCT recipients. However, patients from patient-donor pairs bearing the wild-type allele who where colonized with *Candida* had a significant increased incidence of acute GvHD compared to non-colonized patients (OR=2.6, *P*=0.04). The fact that was not the case in patients from pairs with the Y238X polymorphism (OR=1.2, ns) suggests that despite increased colonization defective dectin-1 signaling might have prevented an impact of *Candida* colonization on the incidence of acute GvHD to occur. These are the first human data showing a role for *Candida* in the pathogenesis of acute GvHD. The mechanism could involve C-type lectin receptor mediated Th17 responses.

Introduction

We have recently established a role of the pattern recognition receptor (PRR) dectin-1 for the mucosal immunity against *Candida spp* in SCT recipients by showing increased *Candida* colonization among patients bearing the dectin-1 Y238X polymorphism [1]. This early stop-codon polymorphism results in the loss of the last ten amino-acids and an altered three-dimensional structure of the extracellular domain of dectin-1, resulting in the reduced ability to bind its ligand β-glucan and impaired induction of pro-inflammatory cytokines [1].

Besides its role in antifungal immunity, dectin-1 exhibits a broader function in immunity. Stimulation of dectin-1 with either curdlan or β-glucan affects antigen presentation [2], modulates T-lymphocytic (CD4+, both Th1 and Th17, and CD8+) and B-lymphocytic responses [3;4], and induces cytokine production including interleukin (IL) 10, IL-12 and IL-23. These specific T-cell responses and cytokines are of particular interest in SCT because they are involved in graft-versus-leukemia (GvL) effects, as well as in the pathogenesis of graft-versus-host disease (GvHD), although the role of the IL-23/Th17 response has only recently been explored in animal models [5-7] and in human studies [8]. Moreover, activation of PRRs by microbial components can modify allo-reactive immune responses with implications for the outcome of SCT [9]. In addition, with respect to T-cell responses, β-glucan shows promising in vitro and in vivo activity as an immune adjuvant in the treatment of solid and hematological malignancies. The anti-tumor activity is directly related to signaling through dectin-1 [4;10], suggesting that the cellular immunity elicited by β-glucan could be of therapeutic value, possibly by eliciting GvL reactions in SCT.

Therefore, our hypothesis was that changes in dectin-1 activation either by a "loss-of-function" resulting from the Y238X polymorphism or by *Candida* colonization could have an impact on the immunological responses following SCT and the outcome. We performed a retrospective study on the impact of the Y238X polymorphism and *Candida* colonization in the setting of matched related, partially T cell-depleted SCT, with the focus on GvHD and relapse (GvL).

Patients and methods

Patients
We performed a retrospective analysis among 140 patients who had been consecutively admitted for a matched related, partially T cell-depleted, allogeneic SCT. The characteristics of patients, donors and SCT procedures are depicted in Table 1.

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Table 1 Clinical characteristics of the study groups.

Recipient, donor and SCT characteristics	Dectin-1 wt pairs (n=112)	Dectin-1 Y238X pairs (n=28)	P-value
Subjects heterozygote Y238X			
Patients	0	15	
Donors	0	22	
Gender (no, % male)			
Patients	70 (62.5%)	18 (64.3%)	ns
Donors	63 (56.3%)	17 (60.7%)	ns
Age, mean (range)			
Patients	47.6 (18.5-64.4)	43.8 (19.1-59.8)	P=0.09
Donors	47.7 (14.0-75.6)	43.7 (21.7-68.3)	ns
Diagnosis			
-AML, no (%)	40 (35.7%)	13 (46.4%)	ns
-ALL, no (%)	12 (10.7%)	4 (14.3%)	
-CML/MPS, no (%)	23 (20.5%)	4 (14.3%)	
-MDS, no (%)	17 (15.1%)	2 (7.1%)	
-NHL/CLL, no (%)	20 (17.9%)	5 (17.9%)	
Conditioning regimen			
-Ida-Cyclo-TBI, no (%)	75 (67.0%)	15 (53.6%)	
-Ida-Cyclo-Bus, no (%)	11 (9.8%)	5 (17.9%)	
-Cyclo-TBI, no (%)	21 (18.8%)	5 (17.9%)	
-Cyclo-Bus, no (%)	5 (4.5%)	3 (10.7%)	
TBI	96 (85.7%)	20 (71.4%)	P=0.09
Stem cell source			
-PB, no (%)	58 (51.8%)	17 (60.7%)	ns
T cell-depletion			
-CD34 selection, no (%)	50 (44.6%)	17 (60.7%)	ns
-Counterflow elutriation, no (%)	38 (33.9%)	7 (25.0%)	
-CD3/CD19 depletion, no (%)	24 (21.4%)	4 (14.3%)	
Male patient/female donor, no (%)	31 (27.6%)	6 (21.4%)	ns
Prophylactic DLI, no (%)	31 (27.7%)	12 (42.9%)	ns

All patients received an HLA matched sibling partially T cell-depleted SCT. Characteristics are expressed in absolute numbers and percentages. Age is expressed as mean value with the total range. Differences between the study groups were compared using chi-squared test or Fisher's exact test and with the use of the independent t-test where appropriate. No significant differences were found; P-values < 0.10 are shown. Abbreviations: AML/ALL = acute myeloid and lymphatic leukemia, CML/MPS = chronic myeloid leukemia/ myeloproliferative syndrome, MDS = myelodysplastic syndrome, CLL = chronic lymphatic leukemia, Ida = idarubicin, Cyclo = cyclophosphamide, Bus = busulphan, TBI = total body irradiation, DLI = donor lymphocyte infusion.

Transplantation procedure

All patients had been treated according to the same protocol as previously has been described [1;11]. The myeloablative conditioning regimen consisted mainly of idarubicin and cyclophosphamide in combination with either total body irradiation (TBI) or busulphan. GvHD prophylaxis consisted of three months of cyclosporine only. Patients who had remained free of GvHD three months after stopping cyclosporine were eligible for prophylactic donor lymphocyte infusion (DLI).

Surveillance cultures for *Candida* were collected twice weekly from admission until discharge, with the first cultures taken on the day of admission before the start of conditioning. Fluconazole (200 mg daily) was only prescribed to those who were colonized with *Candida albicans, C. tropicalis* or *C. parapsilosis* (not *C. krusei* or *C. glabrata)* when the yeast was present in both fecal cultures and mouth washes obtained on the same day, or when obtained from the same site on two consecutive occasions.

Genetic screening for Dectin-1 polymorphism

Genotyping for the presence of the Y238X polymorphism was performed by applying TaqMan SNP assay C_33748481_10, as previously described [1]. Patients and donors had given informed consent to collecting DNA samples for investigational use.

Definitions and grading of outcome measures

Acute and chronic GvHD were classified respectively according to the criteria of *Glucksberg et al.* [12] and to *Shulman et al.* [13]. Disease-free survival (DFS), relapse-related mortality (RRM), overall survival (OS), and non-relapse mortality (NRM) were defined according to standard criteria.

Statistical analysis

The end-points GvHD, DFS, RRM, NRM and OS were analyzed in relation to dectin-1 genetic status. Study groups were formed according to presence of polymorphism Y238X, comparing patient-donor pairs with at least one bearing the polymorphism with pairs comprising the wild-type. The time-to-clinical event was determined from the date of SCT. Because the study groups were small, we limited the number of variables included in the multivariable logistic and Cox regression analysis to known risk factors and those variables having P-values ≤0.20 in univariable analysis. Hence, the conditioning regimen, NOD2 status and gender combination were included in the analysis of acute and chronic GvHD. Disease-status, diagnosis, conditioning regimen, and use of prophylactic DLI were included in the analysis of DFS and RRM, disease status, diagnosis, conditioning regimen and acute GvHD in the analysis of NRM and OS.

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In addition, acute GvHD was analyzed in relation to *Candida* colonization on the day of SCT. Patient-donor pairs bearing only the wild-type dectin-1 allele and pairs with at least one dectin-1 polymorphism were analyzed separately. Conditioning regimen, NOD2 status and gender-combination were included in multivariable logistic regression analysis.

The chi-squared test or the Fisher's exact test was employed to compare proportions between two independent groups, depending upon which was more appropriate. *P* <0.05 was considered to represent a statistically significant difference.

Results and discussion

Among the 140 patient-donor pairs, the allele frequency of the Y238X polymorphism was 6.6%, with 10.7% of patients and 15.7% of donors bearing the Y238X polymorphism. All were heterozygous and at least one polymorphism was present in 28 of 140 (20%) patient-donor pairs.

The mean duration of follow-up after SCT was 37.5 months (range 0.1-132.0). DFS, RRM, NRM and OS at 5-years were 37.8%, 25.6%, 20.7% and 53.6%, respectively. Acute GvHD grades II-IV affected 30.7% of patients and 7.9% had severe acute GvHD (grades III-IV). Chronic GvHD occurred in 28.6% of patients, being limited in 10.7% and extensive in 17.9%. No significant differences were seen in DFS, RRM, NRM and OS between pairs with or without the Y238X polymorphism both in univariable and multivariable analysis (Table 2). Also the occurrence of acute GvHD and chronic GvHD did not differ between couples with or without the polymorphism (Table 2).

Interestingly, *Candida* colonization during aplasia was associated with an increased risk of acute GvHD. This however seemed to depend on the dectin-1 status. Data on *Candida* colonization had been collected in 124 patients (97 wild type pairs and 27 pairs with at least one Y238X polymorphism). On the day of SCT, 63% of the patients from patient-donor pairs with a dectin-1 polymorphism were colonized vs. 44.5% among wild type pairs. This difference was predominantly due to the significantly increased colonization in patients bearing the Y238X polymorphism [1]. Colonized patients received fluconazole, although rather late in the second and third week of SCT, and full eradication of *Candida* was rarely achieved. Fluconazole use was similar (≈ 80%) in colonized patients with and without the polymorphism. The incidence of acute GvHD was increased among patients from wild-type dectin-1 pairs who where colonized with *Candida spp* compared to non-colonized patients (41.9% vs. 20.4%, OR=2.6, 95%CI: 1.02-6.58, *P*=0.04, multivariable analysis, Fig. 1). This effect on acute GvHD was however not seen in patients from pairs bearing at least one Y238X polymorphism (23.5% colonized vs. 20% not colonized, OR=1.2, ns, Fig. 1).

Table 2 Outcome of SCT in dectin-1 wild type (wt) vs. dectin-1 Y238X patient-donor pairs.

Outcome variable	Dectin-1 wt pairs (N=112)	Dectin-1 Y238X pairs (N=28)	Hazard ratio or Odds ratio (95%CI)	P-value
Overall survival, 5 year	56.2%	42.8%	1.5 (0.8-2.8)*	P=0.18
Disease free survival, 5 year	36.7%	40.6%	0.9 (0.5-1.6)*	P=0.75
Non-relapse mortality, 5 year	20.5%	21.4%	1.1 (0.5-2.7)*	P=0.82
Relapse-related mortality, 5 year	23.3%	35.8%	2.1 (0.9-4.7)*	P=0.09
Acute GvHD	33.3%	25.9%	0.9 (0.3-3.0)**	P=0.89
Chronic GvHD	28.5%	29.2%	1.0 (0.3-3.0)**	P=0.98

These are the results of the multivariable Cox regression analysis (HR)* and multivariable logistic regression analysis (OR)**.

Figure 1 The impact of Candida colonization and dectin-1 status on the incidence of acute GvHD grade II, III and IV.
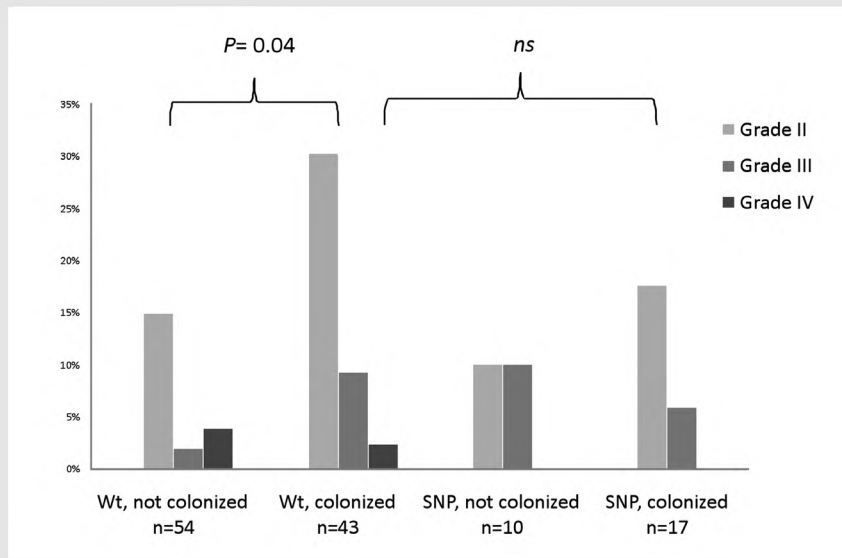

Wt = wild type dectin-1, SNP = Y238X polymorphism, ns = not significant.

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Looking only at the patients colonized with *Candida*, the presence of the Y238X polymorphism reduced the impact of colonization on the incidence of acute GvHD (41.9% wild-type vs. 23.5% Y238X, *P*=0.18).

A role for micro-organisms residing at mucosal surfaces has long been implicated in the pathogenesis of acute GvHD [9]. Use of antimicrobial agents in order to achieve gut decontamination has already proved to decrease the incidence and severity of acute GvHD [14]. Although the focus so far has been on gut bacteria, our data underscore the importance of fungi as well. Moreover, in a study by *Marr et al.* a decreased incidence of gut acute GvHD was observed when using early fluconazole prophylaxis [15], supporting the association between *Candida* colonization and acute GvHD.

Th17 responses have been shown to play an important role both mucosal and systemic anti-*Candida* host defenses in mice [16;17] and humans [18;19]. These responses are induced through the activation of different PRRs, most importantly dectin-1, dectin-2, mannose receptor, and TLR-2. At the same time, recent studies have directed at a previous unrecognized role for Th17/IL-23 in the pathogenesis of GvHD, especially acute GvHD [6-8]. Therefore the link between *Candida* colonization and acute GvHD might prove to be the induction of Th17/IL-23 responses by the fungus. Previously, we have shown that the Y238X polymorphism results in a decreased Th17 response [20], explaining the mechanism of increased *Candida* colonization in patients bearing the polymorphism. The fact that colonization in patient-donor pairs with the polymorphism did not result in increased acute GvHD but did in wild-type pairs, suggests that despite increased colonization defective dectin-1 signaling prevented an increase in Th17-mediated acute GvHD (Fig. 2). However, the limited sample size of this study precludes firm statements regarding the role of dectin-1, especially since other PRRs are also important for anti-*Candida* immunity. Therefore, additional studies are necessary to confirm our results and these studies should incorporate measurements of cytokines and Th-subsets.

Nevertheless, our study presents the first human in vivo data showing a clear link between *Candida* colonization and acute GvHD. In addition, our data support that the mechanism could involve C-type lectin mediated Th17 responses, which have recently been implicated in the pathogenesis of acute GvHD.

Figure 2 Simplified hypothesis on the role of *Candida* colonization in the pathogenesis of acute GvHD.
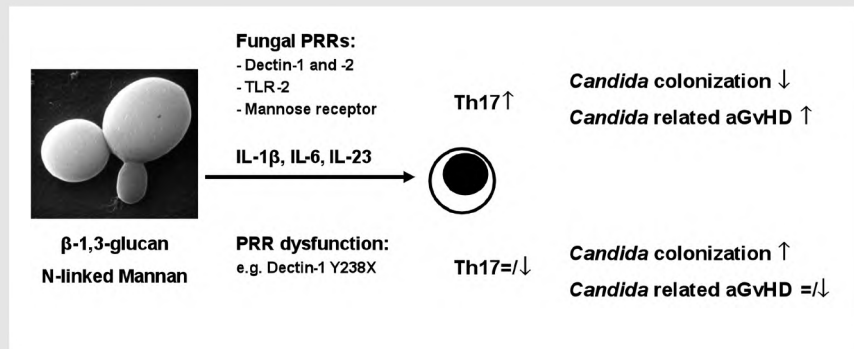
GvHD = graft-versus-host disease, TLR = Toll-like receptor, MAMPs = microbe-associated molecular patterns.

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The role of antimicrobial peptides
with innate defense regulatory functions
in stem cell transplantation

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The potential role of lactoferrin and derivatives in the management of infectious and inflammatory complications of hematology patients receiving a hematopoietic stem cell transplantation

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Abstract

Human lactoferrin is a natural defense protein belonging to the innate immune system present in several body fluids and secretions as well as in the secondary granules of polymorphonuclear neutrophils. Lactoferrin and its derivatives have pleiotropic functions including broad-spectrum anti-microbial activity, anti-tumor activity, regulation of cell growth and differentiation, and modulation of inflammatory as well as humoral and cellular immune responses. This is the reason why much research has addressed the potential therapeutic activity of these molecules in different clinical settings, especially regarding infectious diseases and uncontrolled inflammatory conditions. In patients with hematological malignancies treated with a hematopoietic stem cell transplantation (HSCT) morbidity and mortality due to infections and uncontrolled inflammation remains high, despite many advances in supportive care. These life threatening complications are a result of the damage caused by the conditioning regimens to the mucosal barrier, and the innate and adaptive, humoral and cellular immune defenses. These complications necessitate the continued exploration of new treatment modalities. Systemic and probably local levels of lactoferrin are decreased following HSCT. Therefore, the use of lactoferrin, or short peptide derivatives that retain the cationic N-terminal moiety that is essential for the anti-microbial and anti-inflammatory activity, may prove to be a promising versatile class of agents for managing the complications that arise from HSCT.

Introduction

Because mammalian neonates of primates depend on milk for their nutritional needs as well as for their immune protection much research has addressed the beneficial components of milk. One of these components, lactoferrin, was first identified in 1939, isolated in human milk in 1969 [1] and later detected in the granules of human polymorphonuclear neutrophils (PMNs) [2]. Since then this protein has been extensively studied. In 1978 Broxmeyer identified lactoferrin as a regulator of myelopoiesis inhibiting the production of granulocyte-macrophage colony stimulating factor by monocytes and macrophages. Because PMNs in chronic myeloid leukemia were shown deficient of lactoferrin, a role in the pathogenesis of leukemia through defective growth inhibition was suggested, implying a potential therapeutic effect of lactoferrin [3]. This provided an impulse for further investigations, reviewed in 1984, on the biological significance of lactoferrin in hematology [4]. However, many conflicting data existed which resulted in an extensive discussion regarding the potential role of lactoferrin in myelopoiesis, designated "the lactoferrin controversy" [5]. This discussion was not resolved and interest in lactoferrin from a hematological point of view appeared to subside. In that same period, a promising report suggested that lactoferrin given orally as anti-bacterial prophylaxis in neutropenic patients deserved further study [6]. But measurement of the plasma level of lactoferrin as an early means of monitoring hematopoietic regeneration following bone marrow transplantation seemed to be the only application [7].

However, interest in the hematological value of lactoferrin re-emerged in a report of children with refractory graft-versus-host disease (GVHD) following hematopoietic stem cell transplantation (HSCT) where there was dramatic improvement seen in four of the seven cases following oral treatment with lactoferrin [8].

Research has mainly focused on the anti-microbial activity of lactoferrin, recognizing it as a powerful anti-infective protein in synergy with other anti-microbial peptides and proteins released from PMNs and Paneth cells such as defensins, lysozyme and bactericidal/permeability-increasing protein [9]. Recently, an extensive review was published on lactoferrin emphasizing its important role in host defenses and immune responses suggesting a potential therapeutic benefit, especially in treating infectious diseases [10-15]. Therefore, it seems appropriate to reassess the biological significance of lactoferrin for hematology patients in general and HSCT recipients in particular.

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Lactoferrin

Molecular structure and production

Lactoferrin belongs to the family of transferrins, glycoprotein's that binds ferric ions. It is a protein weighing 80 kDa consisting of a single polypeptide chain comprising 692 amino acid residues. This chain forms two homologous domains, the so-called N- and C-lobe each binding a ferric ion, which are connected by a α-helix (Fig. 1) [10].

Figure 1 Molecular structure of lactoferrin.

Lactoferrin contains two high affinity binding sites for iron and therefore seems important in iron homeostasis. The N-terminal moiety of lactoferrin, especially the first five amino acids (^{1}Gly-Arg-Arg-Arg-Arg^{5}), denoted with N, has a high cationic charge allowing binding to negatively charged molecules including lysozyme, DNA and bacterial products. This moiety is of particular interest for the anti-microbial and immune-modulating actions of lactoferrin.

Besides being present in human milk, lactoferrin has been found in tears, saliva and secretions of the nasal, urogenital, respiratory and biliary tract [11]. The production and secretion seems to be located in the glands of mucosal surfaces where it contributes to the barrier defenses. Limited evidence exists that lactoferrin is produced by the intestinal epithelium, especially Paneth cells, but lactoferrin mRNA expression has been shown to occur in small intestinal cells [16].

The PMNs are another major source of lactoferrin [2], which is stored in secondary granules and released on neutrophil activation. Serum lactoferrin mirrors the release from neutrophils, normally being low with concentrations ranging from 0,1-1,0 μg/mL, but increasing enormously during active inflammation, to up to 20 μg/mL [17].

Functional properties of lactoferrin

The most striking physiochemical feature of lactoferrin is its high affinity for iron. Its role in iron homeostasis is emphasized by a study on iron deficiency anemia in pregnant women in which lactoferrin increased hemoglobin and total serum iron [18]. However, lactoferrin possesses pleiotropic functions independent of its iron binding capacity that have been attributed to several factors. Specific 'classical' receptors (LfRs) have been determined on many cell types including intestinal epithelial cells (IECs), respiratory epithelial cells, lymphocytes, monocytes and macrophages, thrombocytes and hepatocytes [13]. Current knowledge about the intracellular effects following receptor binding is modest, although lactoferrin is shown to activate different intracellular signaling pathways [19].

More important, most functional properties of lactoferrin result from interaction of its N-terminal moiety with both soluble and membrane bound molecules (Fig. 1). This N-terminal moiety, in particular the first five amino acids 1-5 (^{1}Gly-Arg-Arg-Arg-Arg^5), has a high cationic charge allowing binding to negatively charged molecules including heparin, DNA and bacterial products such as lipopolysaccha-rides (LPS, also known as endotoxin) and CpG motifs of bacterial DNA (CpG-DNA) [20;21]. Furthermore the N-terminal moiety of lactoferrin facilitates the interaction with sulphated proteoglycans. These proteoglycans function as co-receptors of LfRs or as aspecific receptors themselves, mediating the internalization and transport of lactoferrin into the nucleus, leading to the N-terminal moiety being christened the 'nuclear localization signal' of lactoferrin [22]. In the nucleus lactoferrin modulates transcription of genes, e.g. related to cytokine production [23;24]. Therefore, short peptides such as human lactoferrin 1-11 (hLF1-11) and lactoferricin (Lfcin) containing this N-terminal moiety have been developed and are currently under investigation [25;26].

The list of lactoferrin's properties (Table 1) now includes anti-microbial activity [12;14], regulation of cell growth and differentiation [27] and modulation of inflammatory, humoral and cellular immune responses [11;28]. Furthermore, induction of apoptosis has been shown in solid tumor and leukemia cell lines [12].

The timing and its abundant release from PMNs during inflammation suggest lactoferrins function might be predominantly one of negative feedback regulation to prevent uncontrolled inflammation. Indeed, several studies have supported this by showing lactoferrin to possess anti-inflammatory activity [23;24;29-33] and exhibit

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Table 1 Functional properties of lactoferrin and lactoferrin derivatives.

Functional properties
Anti-microbial activity
Modulation of inflammatory reactions
Modulation of humoral immune responses
Modulation of cellular immune responses
Immune reconstitution following chemotherapy
Anti-cancer activity: solid tumors, leukemia
Intestinal epithelial regeneration
Iron homeostasis

inhibition of the effector-phase of adaptive cellular immune reactions [8;28;33-35]. Therefore lactoferrin seems to be part of the innate defenses attenuating inflammation and adaptive cellular immune responses, thereby promoting resolution and repair. Paradoxically, lactoferrin can also act as an immune stimulant though this predominantly occurs in the context of inactive immune systems [36-38] underlining the importance of the immune status upon the eventual outcome.

Lactoferrin production in hematopoietic stem cell transplantation
The systemic production and secretion of lactoferrin decreases in parallel to the declining PMN count following myeloablative therapy from normal concentrations of 0,5 μg/mL to 0,05 μg/mL [7] as a result of the absence of functional neutrophils during neutropenia. Much less is known about local levels of lactoferrin. Following cytotoxic chemotherapy and radiotherapy changes in saliva composition occur, at first with elevated salivary lysozyme and lactoferrin secretion, probably mirroring acinary cell damage, followed by reduced secretion [39]. Furthermore GVHD of the gut is associated with salivary dysfunction [40] probably altering lactoferrin production and secretion, although no proof exists whether the local levels are indeed diminished.

Inflammatory complications of hematopoietic stem cell transplantation

HSCT is still accompanied by many complications resulting in an overall survival rate of 60% after 5 years [41]. Most of these complications are caused by the conditioning regimens necessary to prepare for HSCT impairing barrier defenses and innate and

acquired immune systems both local and systemic favoring infections and causing uncontrolled inflammation.

Infections
Bacterial pathogens account for most of the infections occurring shortly after transplantation during neutropenia, when mucosal barrier injury (MBI) is most pronounced. While patients are likely receiving anti-bacterial prophylaxis with fluoroquinolones, the majority of infections are caused by gram-positive bacteria (65-75%), mainly viridans group streptococci and coagulase-negative staphylococci, and a minority by gram-negative pathogens, mostly Enterobacteriaecae. The incidence of fungal infections also remains relatively high affecting up to 15% in allogeneic HSCT, with *Candida* species and *Aspergillus fumigatus* predominating. Finally infections with herpesvirus, when patients are not receiving prophylaxis with acyclovir, or other members of this class, and respiratory viruses also contribute to the infectious complications in HSCT recipients.

Mucosal Barrier Injury
Uncontrolled activation of inflammation of the mucosal membranes of the alimentary tract is thought to be the direct results of the MBI induced by cytotoxic chemotherapy. The pathogenesis of MBI is regarded a multiphase process with successive events eventually resulting in an uncontrolled inflammatory reaction [42]. Activation of nuclear factor kappa B (NF-κB), directly by cytotoxic chemotherapy and radiotherapy and indirectly through reactive oxygen species (ROS), up-regulates the production of pro-inflammatory cytokines (interleukin-1 (IL-1), IL-6, tumor necrosis factor alpha (TNFα)) and chemokines (IL-8) by IECs, endothelial cells and macrophages [43;44]. Simultaneously apoptosis in crypt epithelial stem cells is induced resulting in hypoplasia and villous atrophy with increased mucosal permeability [45]. Translocation of bacteria and their wall components, such as LPS, occurs [46], stimulating the cytokine production of previously primed macrophages, augmenting the inflammatory response ('cytokine storm') with possibly detrimental systemic effects including high fever, acute respiratory distress syndrome, thrombotic microangiopathy, veno-occlusive disease, multi-organ failure and central nervous system disorders [47;48].

Acute Graft-versus-Host Disease
Another type of uncontrolled inflammation is GVHD, which still causes significant morbidity and mortality in allogeneic HSCT recipients. Approximately 30% will develop grade 2 or worse acute GVHD involving the skin and gastrointestinal tract [49]. The pathogenesis of GVHD is generally described in 3 phases, the first 2 designated the afferent and the third the effector phase [49;50]. The first phase is dominated by

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the release of pro-inflammatory cytokines following cytotoxic chemotherapy and radiotherapy inducing NF-κB, aggravated by the translocation of bacteria and their products such as LPS [43;44;50;51]. These cytokines increase expression of co-stimulatory molecules, adhesion molecules and major histocompatibility complex type II (MHC-II) antigens critical for the induction of the adaptive cellular immune response. The extent of mucosal damage and inflammation is related to the intensity of the conditioning regimens and has been correlated with the occurrence and severity of GVHD [43;44]. In the second phase activation of antigen presenting cells occurs which present host antigens to T-lymphocytes. Subsequent activation and proliferation of T-lymphocytes, predominantly Th1-lymphocytes, ensues, crucial to the pathogenesis of GVHD. Finally, the third phase concerns the occurrence of damage to host cells as a result of the cytotoxic effects of activated T-lymphocytes and natural killer cells as well as increased release of Th1 type cytokines. Subsequently, gut mucosal damage leads to amplification of inflammation, through translocation of bacterial products such as LPS, again increasing cytokine release and stimulating Th1 responses [52].

Lactoferrin and management of infectious complications

Anti-microbial activity
In 1982 the importance of lactoferrin in host defense was acknowledged in a report of a patient with lactoferrin deficiency who suffered from recurrent bacterial infections [53]. Neutropenic patients, following chemotherapy, showed a lower incidence of predominantly gram-negative bacteremia if treated orally with lactoferrin [6].

Lactoferrin has both bacteriostatic and bactericidal activities as well as antifungal properties (Fig. 2) [12]. Bacteriostasis is attributed to the chelating property of lactoferrin which deprives bacteria from iron thereby inhibiting their metabolic activities. The bactericidal effects are considered also very important, because the cationic charge of the N-terminal moiety of lactoferrin facilitates the binding to anionic microbial wall components such as LPS and lipoteichoic acid (LTA) [54;55] resulting in membrane disruption and subsequently osmotic shock. Lactoferrin binds to the outer membrane of gram-negative bacteria resulting in rapid release of LPS, exposing the underlying peptidoglycan (PGN) to the digestive action of lysozyme [55]. Similarly, through binding to LTA lactoferrin disrupts the membrane of gram-positive bacteria [54].

Other antibacterial activities described comprise induction of autolytic cell wall enzymes, proteolysis of virulence factors (serine protease activity), inhibition of biofilm formation, altering bacterial metabolic activity and mitochondrial ROS generation and gene transcription. By contrast bacteria of the gut commensal flora *Bifidobacterium*

Figure 2 Anti-microbial activities of lactoferrin.
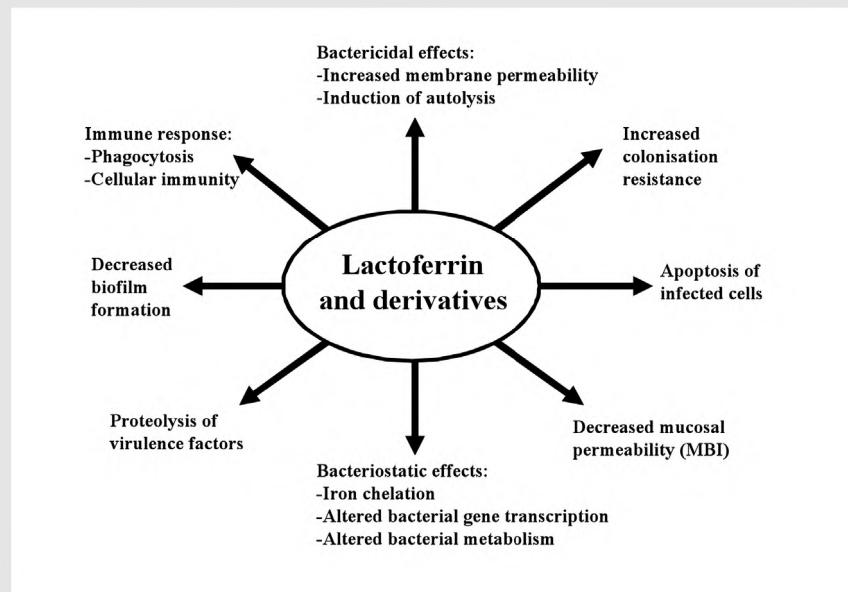
Several bacteriostatic and bactericidal properties have been described for lactoferrin as well as the potential to decrease the production of several virulence factors and bacterial defense mechanisms. Furthermore, lactoferrin enhances immune responses directed against microbes for instance through increased phagocytosis and stimulated cellular immune responses. Lactoferrin could also prevent infections by maintaining an intact mucosal barrier and accelerating immune reconstitution following treatment with chemo- and radiotherapy.

bifidum and *Lactobacillus acidophilus* are resistant against lactoferrin favoring their survival resulting in enhanced colonization resistance [56]. Direct anti-fungal activity of lactoferrin and derivatives result from comparable mechanisms [57-59].

Immunomodulatory effects

Lactoferrin accelerates the immune reconstitution following intensive treatments with cytotoxic chemotherapy and radiotherapy and shortens the neutropenic phase in animal models. In mice given cyclophosphamide and methothrexate immune recovery was accelerated resulting in enhanced clearance of *Escherichia coli* and *Staphylococcus aureus* [60].

Furthermore, lactoferrin promotes phagocytosis through direct opsonin-like activity [61] and increased complement deposition, and enhances intracellular killing activity of phagocytes e.g. through increased superoxide generation [37].

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Increased adaptive cellular immune responses by increased IL-12, IL-18 and IFNγ production, activating Th1 responses directed against yeasts, viruses and bacteria, with orally administered lactoferrin have been reported [14;36-38]. These effects were probably mediated through altered cytokine production by IECs [38].

Lactoferrin and Mucosal Barrier Injury

As described earlier, MBI is an inflammatory condition initiated by chemo- and radiotherapy and aggravated by the translocation by bacterial components. Therefore it is important, regarding a potential role for lactoferrin in attenuating MBI, that most studies on the anti-inflammatory effect of lactoferrin have examined the inhibition of inflammatory responses mediated by bacterial components especially LPS, but also CpG-DNA and LTA.

In vitro investigations in mice and humans show reduced LPS-induced release of pro-inflammatory cytokines IL-1, IL-6, IL-8, and TNFα by monocytes and macrophages [23;24;29], IL-6 and IL-8 by IECs [29;30] and IL-8 by the endothelium [62]. Other studies showed inhibition of LPS-mediated expression of the endothelial adhesion molecules E-selectin and ICAM-1 [63] and reduced LPS-mediated reactive oxygen production by leukocytes [64]. These effects involved inhibition of NF-κB activation and as a consequence altered mRNA expression and the production of several pro- and anti-inflammatory cytokines.

There is uncertainty about the precise mechanism through which lactoferrin facilitates this inhibition. Elass-Rochard *et al.* have suggested that direct binding of lactoferrin to LPS, the so-called LPS-scavenging (or endotoxin antagonism), prevents the interaction of LPS with membrane bound CD14, the co-receptor of toll-like receptor 4 (TLR-4), thereby inhibiting the associated intracellular signaling pathways [65] (Fig. 3). The same investigator also showed reduced IL-8 expression on endothelial cells as a result of lactoferrin preventing interaction between LPS and soluble CD14 [62]. Similarly, CpG-DNA induced immune responses via TLR-9 are inhibited by the scavenging properties of lactoferrin [66].

However, others suggested that lactoferrin inhibits cytokine production regardless of its LPS scavenging properties [23;24]. They demonstrated that lactoferrin was taken up by human monocytes and transported into the nucleus with subsequent decreased binding of NF-κB to the TNFα promoter resulting in decreased expression and production of pro-inflammatory cytokines (Fig. 3). Since internalization and nuclear localization of lactoferrin is documented in IECs this mechanism of NF-κB inhibition might influence their cytokine release [13]. Therefore, lactoferrin could reduce inflammation in 'sterile' inflammation, as is the case in the early phases of MBI before the mucosal barrier is injured and microbes translocate.

Figure 3 Anti-inflammatory properties of lactoferrin.
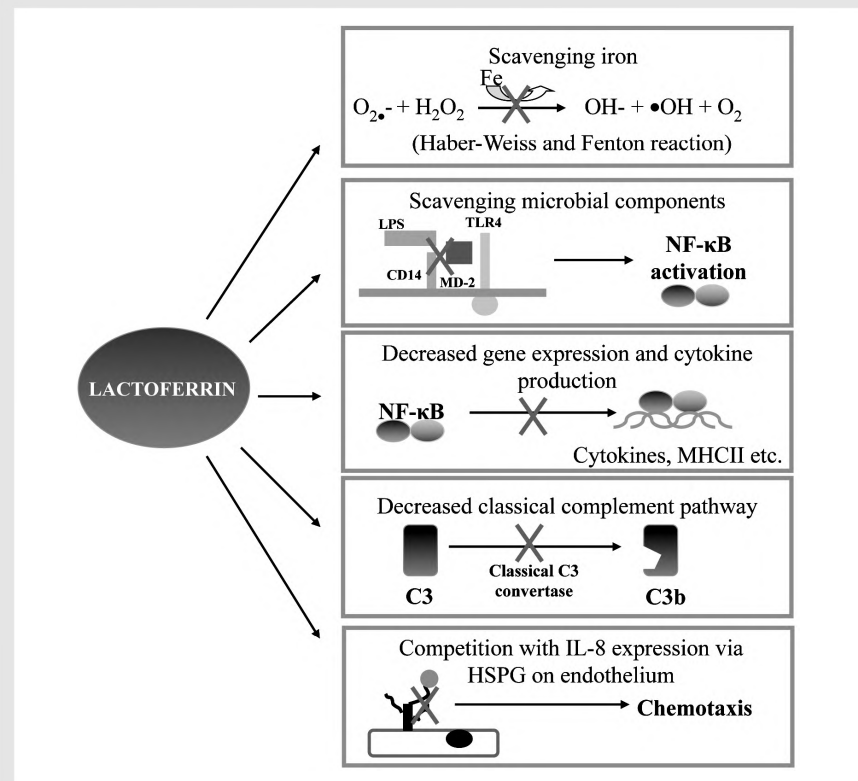
Several anti-inflammatory mechanisms of lactoferrin are described, although not all are completely understood. Through iron scavenging lactoferrin decreases the production of reactive oxygen species, thus protecting tissues from oxidative damage resulting from for instance chemo- and radiotherapy. The scavenging of microbial components, such as LPS and CpG-DNA, prevents generation of pro-inflammatory cytokines normally resulting from the activation of pattern recognition receptors including TLR-4. Furthermore, intracellular inhibition of NF-κB decreases pro-inflammatory responses. Other anti-inflammatory actions involving the complement system and chemotaxis have been described.

Other anti-inflammatory mechanisms of lactoferrin involve direct anti-oxidative effects, inhibitory effect on the complement system and competition with chemokines (Fig. 3). Ferric (Fe^{3+}) ions are at the centre of ROS generation through their ability to catalyze the Haber-Weiss reaction. Lactoferrin binds Fe^{3+} with high affinity thereby functioning as an iron scavenger decreasing inflammation through reduced ROS production. High levels of non-transferrin-bound iron resulting in

increased ROS production are often present in recipients of HSCT and are associated with transplantation-related complications. Lactoferrin might therefore attenuate oxidative stress and subsequent inflammation in these patients.

Conflicting data exist on the modulation of the complement system by lactoferrin, although most data support a down-regulatory effect on the classical complement pathway, with reduced C3 and C5 deposition, through decreased C3-convertase formation [67].

Lactoferrin inhibits IL-8-mediated chemotaxis through competition for binding sites on heparan sulphate proteoglycans involved in IL-8 expression on endothelial cells [62].

The broad array of anti-inflammatory actions associated with lactoferrin might have a protective effect for patients who develop MBI following cytotoxic chemotherapy. In fact, the protective activity of orally administered lactoferrin on the gut mucosa was shown in animals with various inflammatory conditions such as NSAID-related enteropathy [68], LPS induced mucositis [31], dextran sulfate-induced colitis [33] and acute gut GVHD [8]. Furthermore, recently human lactoferrin has been shown to protect IECs from oxidative damage induced by hydrogen peroxide [32]. In addition, lactoferrin might also attenuate MBI through the stimulation of mucosal regeneration, similar to keratinocyte growth factor [27].

Lactoferrin and acute Graft-versus-Host Disease

Attenuating the uncontrolled inflammation following myeloablative conditioning for HSCT makes lactoferrin a potentially attractive agent for the prevention of acute GVHD, when administered during the initial phase (see the role of lactoferrin in MBI).

In the effector phase lactoferrin could also attenuate the inflammatory response through several direct and indirect mechanisms. The role of endotoxin translocation promoting local and systemic cytokine release amplifying GVHD is accepted, suggesting that LPS antagonists such as lactoferrin might be useful for treatment [52]. Lactoferrin also directly down-regulates LPS- and CpG-DNA-mediated Th1 responses. Decreased expression of MHCII and co-signaling molecules (CD40, CD86), necessary for Th1 responses, was shown in dendritic cell cultures stimulated with LPS following treatment with lactoferrin. Further studies have shown that lactoferrin can shift adaptive cellular immune responses from Th1 towards Th2, e.g. by increasing IL-10 and decreasing IL-12 and IL-18 secretion, which would be beneficial during the effector phase of GVHD [28;33;34].

However, in the second phase, lactoferrin might pose a risk by promoting acute GVHD. For instance, lactoferrin was shown to stimulate Th1-lymphocytes and induce delayed type hypersensitivity responses in mice [69]. Lactoferrin increased

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the release of IL-12 and IL-18 in animal models of infections and cancer [37;38], both cytokines known to promote Th1 immune responses in GVHD. Furthermore, lactoferrin promotes lymphopoiesis, lymphocyte differentiation and restoration of adaptive cellular immune responses [19;60].

Inoue *et al.* showed attenuation of gut GVHD in 4 of 7 children after HSCT suffering from gut GVHD refractory to conventional immunosuppressive therapy, suggesting that lactoferrin might contribute to the prevention and treatment of GVHD in humans [8]. Indirect evidence from studies in patients contact dermatitis suggest that lactoferrin could also attenuate skin GVHD since the pathogenesis of skin GVHD and contact dermatitis are regarded as being similar [35]. Taken together these data suggest lactoferrin might prove a logical and potent adjunctive therapeutic agent for managing GVHD, although timing seems of crucial importance.

Future directions

Lactoferrin holds promise for the future as a therapeutic agent for treating complications arising during the different phases of HSCT (Fig. 4). Its pleiotropic properties, especially the anti-microbial and anti-inflammatory activities, are of interest because infections and uncontrolled inflammation pose the greatest threats to HSCT recipients. Correcting lactoferrin deficiency seems a logical and simple treatment option. Currently only 2 small studies have shown promising results [6;8]; therefore further studies are necessary.

Several lactoferrin derivatives have been developed as powerful anti-microbial peptides such as Lfcin and hLF1-11 [12;25;26], all containing the essential cationic N-terminal moiety of lactoferrin. These derivatives have physical and functional properties comparable to that of the short cationic peptides present in virtually every life form, such as defensins, protegrins and cathelicidins, which function as 'nature's antibiotics' but also as immune modulators [70]. The use of these derivatives is preferred because of superior anti-microbial activity as has been shown for hLF1-11 [57]. Furthermore, these derivatives exhibit as broad an anti-microbial spectrum of activity as natural lactoferrin [25;57;71;72], a very desirable quality in the light of growing antibiotic and anti-fungal resistance. Finally, the anti-inflammatory and anti-tumor activities are also retained [12;24;73]. This is not to say that derivatives posses all the activities present in the intact lactoferrin molecule.

Although most data on lactoferrin derive from studies with lactoferrin given orally the intravenous route seems the most logical and practical for HSCT recipients because oral administration might not prove feasible owing to reduced oral intake, dysphagia and mucosal absorption associated with extensive mucositis. Systemic availability, therefore, can be better achieved through intravenous use. Pharma-

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Figure 4 The potential role for lactoferrin in the treatment of HSCT recipients.
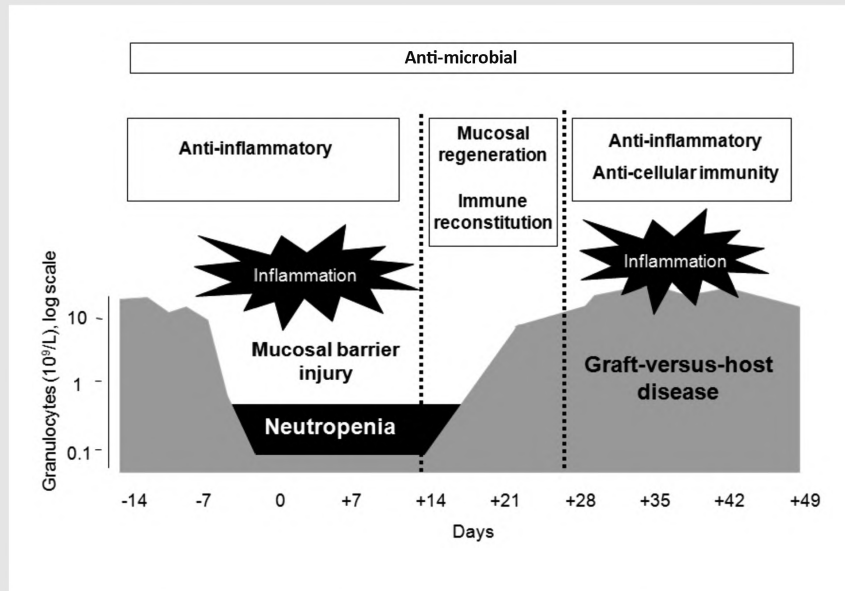
HSCT comprises three phases. The first phase is the period of neutropenia following conditioning with chemo- and radiotherapy with increased risk of infectious complications. Simultaneously, MBI occurs resulting in an inflammatory response and disruption of the normal anatomical barriers further increasing the risk of infection. The second phase is the phase of mucosal regeneration and immune reconstitution accompanied by clinical recovery. In the third phase patients receiving a HSCT are at risk of developing complications from graft-versus-host disease (GVHD). GVHD is an inappropriate cellular immune response directed at the patients skin, liver and gastro-intestinal tract resulting in an inflammatory response and disruption of the gut barrier. In combination with the necessity for immune-suppressive therapy and with still recovering immunity risk of infections is increased. In phase one and three lactoferrin's anti-microbial and anti-inflammatory properties might prove beneficial attenuating inflammation and preventing and decreasing mucosal barrier injury as well as infectious complications. In the second phase accelerated immune reconstitution might prove of benefit, although theoretically initiation and aggravation of GVHD might pose a threat in this phase. Day 0 is the day of HSCT.

cokinetic data show clearance of lactoferrin and derivates through the liver with biliary secretion and enterohepatic circulation [74-76]. Consequently lactoferrin reaches the sites where it is needed, namely, the gut where microbial translocation takes place and inflammation originates.

Because lactoferrin is a natural component of the human innate immunity direct adverse events would not be expected from intravenous administration, however the safety and tolerability of these derivatives has to be established in humans.

These and their clinical efficacy can only be evaluated by formal trials in the clinical setting of HSCT.

In summary, despite many advances in supportive care, transplantation-related morbidity and mortality due to infections and uncontrolled inflammation remains high, necessitating the continued exploration of new treatment modalities. Against this background and with their anti-microbial and anti-inflammatory effects, lactoferrin derivatives that retain the N-terminal moiety of the parent molecule may prove promising for managing the complications that arise following HSCT.

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Safety and tolerability of antimicrobial peptide human lactoferrin 1-11 (hLF1-11)

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Abstract

Background: The treatment of patients with hematological malignancies by means of hematopoietic stem cell transplantation (HSCT) is often accompanied by life threatening infections. With emerging antimicrobial resistance there is an increased need for new agents, with a beneficial safety profile. Therefore we evaluated the safety of the promising new antimicrobial peptide human lactoferrrin 1-11 (hLF1-11) in healthy volunteers and patients.

Methods: We undertook a sequential, randomized, double-blinded, placebo-controlled study using ascending single (0.005, 0.05, 0.5, 5 mg) and multiple intravenous doses (0.5, 5 mg) in healthy volunteers, and open-label, single intravenous 5 mg dose in autologous hematopoietic stem cell transplant (HSCT) recipients.

Results: Single and multiple doses of hLF1-11 were tolerable up to 5 mg intravenously in healthy volunteers, while 5 mg single dose was tolerable in patients. Elevations in transaminases possibly related to treatment were reversible and not serious.

Conclusions: The new antimicrobial hLF1-11 is well-tolerated in healthy volunteers with repeated daily doses up to 5 mg. The side-effect profile is very favorable for an antimicrobial, the only undesirable effect being a possible elevation of transaminases, which may be related to hLF1-11 although the current data do not allow conclusive interpretation of treatment relationship. A lower dose is recommended for the forthcoming multiple dosing studies in HSCT patients.

Background

The treatment of patients with hematological malignancies with hematopoietic stem cell transplantation (HSCT) is often accompanied by life threatening complications as a result of the damage caused by the conditioning regimens to the mucosal barrier, and the innate and adaptive, humoral and cellular immune defenses [1-3]. Despite many advances in supportive care, transplantation-related morbidity and mortality due to bacterial and fungal infections and uncontrolled inflammation remains high [4,5]. A troublesome fact is the increasing resistance against several important antimicrobial drugs including quinolones, azoles and cephalosporins, making control of bacterial and fungal infections in HSCT a difficult task [6-8]. Therefore, the discovery of a broad array of naturally occurring antimicrobial peptides (AMPs) is interesting, although few AMPs have been studied so far and even less have been studied in clinical settings [9-11].

Human lactoferrin (hLF) is a natural defense protein present in body fluids and secretions as well as neutrophils [12,13], and has pleiotropic functions including broad spectrum antimicrobial activity, antitumor activity, regulation of cell growth and differentiation, and modulation of inflammatory, humoral and cellular immune responses [14-17]. Levels of lactoferrin are decreased following HSCT [18], contributing to the overall immune deficiency. Correcting this deficit might ameliorate immunity in HSCT recipients [19].

Human lactoferrin 1-11 (hLF1-11) is a lactoferrin derivative being developed for the treatment of bacterial and fungal infections in HSCT recipients. It contains the N-terminal moiety, consisting of eleven amino acids, of hLF that is essential for the antimicrobial and anti-inflammatory activity [15,17]. Preclinical studies have shown promising antimicrobial activity even in the setting of immunodeficiency justifying further investigation for clinical application [20-24]. Being a derivative of a 'natural' human protein hLF1-11 might have the advantage of fewer side effects and less formation of antibodies and antimicrobial resistance, especially since antimicrobial peptides are unlikely to induce resistance because of the evolutionary difficulty in changing bacterial membrane structure [11].

We report on the first three studies conducted in humans with ascending doses of hLF1-11 in healthy volunteers and in patients receiving autologous HSCT following conditioning with high-dose melphalan (HDM) for multiple myeloma or lymphoplasmocytic lymphoma.

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Methods

Study design
The 3 studies were conducted sequentially and included a total of 56 subjects (placebo: 12; hLF1-11: 44) as follows. Study 1: single intravenous (IV) administration of ascending hLF1-11 doses (0.005, 0.05, 0.5, 5 mg) in healthy volunteers; study 2: multiple IV administration of 2 ascending hLF1-11 doses (0.5, 5 mg daily for 5 days) in healthy volunteers; study 3: single intravenous (IV) administration of a fixed hLF1-11 dose (5 mg) in patients undergoing an autologous HSCT (Table 1).

Blinding and subject selection
Studies 1 and 2 were conducted in healthy volunteers, both were randomized, placebo-controlled, enrolled 48 volunteers in total (placebo: 12; antimicrobial peptide (AP): 36) (Table 1) and were conducted at the Phase-I Clinical Pharmacology Unit, Xendo Drug Development BV, Groningen, The Netherlands, with prior approval by the appropriate Institutional Review Board (IRB). Entry criteria were similar for studies 1 and 2, namely subjects considered healthy during medical screening by a qualified physician, medical history, physical examination, vital signs, blood and urine evaluations, and 12-lead electrocardiogram (ECG). Age and body mass index (BMI) entry criteria were 18-45 years (study 1) and 1-65 years (study 2), while body mass index (BMI) was 18-30 kg/m^2 (study 1) and 18-28 kg/m^2 (study 2). All volunteers provided written informed consent and the studies were conducted in compliance with current Guidelines on Good Clinical Practice [25]. Enrolment took place between March-April 2005 (study 1) and August-September 2005 (study 2). The aims of both studies were to evaluate the safety, tolerability and pharmacodynamics of intravenous administration of hLF1-11. Safety parameters were adverse events, vital signs, changes in ECG, hematology, clinical chemistry, urinalysis and immunogenicity. Pharmacodynamics evaluations were conducted during multiple dosing (study 2) at baseline, days 1 and 5: cytokine release (Interleukin(IL)6, IL10, and tumor necrosis factor (TNF) α) after *ex vivo* stimulation with lipopolysaccharide (LPS) in whole blood.
Study 3 was conducted in autologous HSCT recipients. The study was open, enrolled 8 patients (Table 1) between March-October 2006 at the Department of Hematology, Radboud University Nijmegen Medical Centre, The Netherlands, with prior approval by the hospital's IRB. Study conduct was in compliance with current Guidelines on Good Clinical Practice [25]. Entry criteria were males/females aged 18 years or older who were admitted to hospital for autologous HSCT after myeloablative therapy with high dose melphalan HDM for multiple myeloma or lymphoplasmocytic lymphoma, BMI <30 kg/m^2, without serious other pathologies, or history of hepatitis B or C, or HIV infection. The aims of study 3 were to evaluate the safety and

Table 1 Entry demographics and dosing schedule.

	Subjects	Age (SD) years	Height (SD) cm	Weight (SD) Kg	BMI (SD) Kg/m²
Study 1	Healthy volunteers	24 (5)	185 (6)	79 (9)	23 (3)
Study 2	Healthy volunteers	32 (12)	183 (7)	78 (12)	23 (3)
Study 3	HSCT patients	53 (8)	178 (7)	78 (14)	24 (3)

BMI: body mass index
HSCT: hematopoietic stem cell transplantation

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Male / Female N	Dosing	Dose (mg)	Placebo, n	hLF1-11, n	ALL, n
32 / 0	Single	0.005	2	6	8
		0.05	2	6	8
		0.5	2	6	8
		5.0	2	6	8
16 / 0	Multiple	0.5	2	6	8
		5.0	2	6	8
7 / 1	Single	5.0	-	8	8
			12	44	56

tolerability hLF1-11 given as single IV administration (5 mg) on the day of transplantation. Safety parameters were adverse events, vital signs, changes in ECG, hematology, clinical chemistry, urinalysis, and immunogenicity. Safety and tolerability were evaluated by adverse event reporting, vital signs, changes in ECG, hematology, clinical chemistry, and urinalysis. Adverse events were graded according the to the Common Terminology Criteria for Adverse Events (CTCv3.0, National Cancer Institute, Bethesda, MD, USA) [26].

Blinding in studies 1 and 2 was assured by central allocation of randomization codes in sealed envelopes, audited on completion of each study and by supply of study medication in indistinguishable form.

For the pharmacodynamics evaluations, cytokine measurements were analyzed in study 2 using validated assays for each cytokine once prior to dosing and at six time-points post-dosing (5 min, 30 min, 2 h, 4 h, 8 h, and 24 h) on days 1 and 5, while antibodies against hLF1-11 were measured in all three studies as specific IgG anti-HLF1-11 and IgE anti-hLF1-11 once prior to dosing and at three time-points post-dosing (days 2 and 3, 5 to 8 and 14 and 15) by validated enzyme-linked immunosorbent assay (ELISA), centrally by Xendo Biotech Centre, Groningen, The Netherlands.

Study medication (active and placebo) was supplied by AM-Pharma BV (Bunnik, the Netherlands) as lyophilized powder for solution in normal saline (0.9% NaCl solution) according to dose.

Statistics, assignment and analysis

Treatment allocation was sequential in all studies, with randomization lists issued as single blocks of eight (two placebo and six active) for studies 1 and 2, while there was no randomization in study 3. The intention-to-treat (ITT) population for safety evaluations was defined in all studies as all randomized subjects who received any medication. Data for studies 1 and 2 were analyzed by Xendo Drug Development, Groningen, using SAS version 8.2 (SAS, Cary, NC, USA) and for study 3 by CRM Biometrics GmBH (Bonn, Germany) who also checked the databases for the other two studies. Means, standard deviations, medians, ranges, upper and lower quartiles were calculated, and parameters were listed by subject, summarized and evaluated using descriptive statistics.

Results

Subject characteristics and progress through studies

Demographic data for all three studies at entry are displayed in Table 1, with pathological characteristics of patients in study 3 presented in Table 2. One volunteer in study 2, originally allocated to the placebo group (multiple dosing) received one dose of

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Table 2 Hematopoietic stem cell transplant patients' disease characteristics.

Patient no.	Age (y)	Gender	Diagnosis, stage*	Paraprotein type	Bence Jones	Prior treatment**	Status***	Melphalan dose (mg/kg)
SC001	61	M	Multiple myeloma, III-A	IgG kappa	Not present	VAD, CAD	Partial response	3.92
SC002	62	F	Multiple myeloma, III-A	IgG kappa	Present	VAD, CAD	Progressive disease	5.61
SC003	50	M	Multiple myeloma, III-A	Light chain kappa	Present	VAD, CAD	Minimal response	5.19
SC004	55	M	Multiple myeloma, III-A	IgG kappa, light chain kappa	Not present	VAD, CAD	Partial response	5.13
SC005	45	M	Multiple myeloma, III-A	Light chain kappa	Present	PAD, CAD	Partial response	6.25
SC006	39	M	Multiple myeloma, III-B	IgG kappa	Not present	VAD, CAD	Minimal response	4.37
SC007	54	M	Multiple myeloma, II-A	IgG kappa	Not present	Thalidomide, dexamethasone, CAD	Stable disease	5.07
SC008	61	M	Lymphoplasmocytic lymphoma, IV-A	IgG kappa, light chain kappa	Present	VAD, CAD	Minimal response	5.13

*: Staging by Durie & Salmon criteria [41]
**: VAD: vincristine, adriamycin, dexamethasone, CAD: cyclophosphamide, adriamycin, dexamethasone, PAD: bortezomib, adriamycin, dexamethasone.
***: Status after previous treatment by International Bone Marrow Transplant Registry and Autologous Blood, and Marrow Transplant Registry criteria [42]

hLF1-11 (5 mg) on day 4 due to an administrative error, and his safety data were computed in the active group. All other subjects in the studies received study medication as planned and yielded complete datasets for safety and other analyses.

Safety and tolerability results

The main adverse events from the safety evaluations are presented in Table 3 for studies 1 and 2 and, Table 4 for study 3. Overall, intravenous administration of hLF1-11 did not raise safety concerns in either volunteers or patients. During single dosing in volunteers, all events blindly rated as possibly-related to treatment were reported once and occurred on placebo and the lowest hLF1-11 dose (0.005 mg), none being reported on the two higher doses. During multiple dosing, the commonest reported events on hLF1-11 were elevations in liver enzymes (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)), which were below twice the upper level of normal range (ULN: 40 U/l for ALT; 45 U/l for AST) in one volunteer on 0.5 mg and in two volunteers on 5 mg. The third event was below three times ULN. Detailed analysis of daily measurements of liver enzymes, regardless of levels being reported as adverse events, recorded enzymes levels above ULN in 1/2 placebo volunteers and in 3/6 volunteers on 0.5 mg dose, while levels above ULN were recorded in 6/7 volunteers on 5 mg. All but one of the daily measurements were below twice ULN (one ALT measurement was 127 U/l on day 6; 5 mg dose). All levels were in the normal range within 7 days thereafter.

In HSCT recipients after conditioning with HDM, as expected, several events were recorded, ranging from 5 to 35 events per patient (Table 4). Four serious events (SAEs) were reported, none of which was considered to be related to hLF1-11, while four non-serious events were reported in one patient, considered possibly related to hLF1-11, none being reported for the remaining seven patients.

Other clinical laboratory (hematology, biochemistry, and urinalysis) did not suggest any treatment-related abnormalities in any of the three studies. No abnormalities in coagulation tests were seen. Hemodynamic (blood pressure, heart rate) and 12-lead ECGs (including QT interval) did not yield any treatment-related effects. None of the HSCT recipients developed signs of hemolysis or unexpected cytopenias and no abnormalities regarding engraftment were seen.

Pharmacodynamics evaluations

There were no changes in any of the pharmacodynamics evaluations in volunteers during single or multiple dosing or in patients after single dosing. Cytokine measurements in study 2 (healthy volunteers) showed high variability intra- and

Table 3 Adverse events in healthy volunteers (n=48).

Study 1 (single dosing)	Placebo, n (%)	hLF1-11 0.005 mg, n (%)	hLF1-11 0.05 mg, n (%)	hLF1-11 0.5 mg, n (%)	hLF1-11 5 mg, n (%)	Study 2 (multiple dosing)	Placebo, n (%)	hLF1-11 0.5 mg, n (%)	hLF1-11 5 mg, n (%)
Subjects per Group	8 (100)	6 (100)	6 (100)	6 (100)	6 (100)	Subjects per Group	3* (100)	6 (100)	7* (100)
Diarrhea	-	1 (16.7)	-	-	-	ALT increase	-	2 (33.3)	3 (42.9)
Dizziness	-	-	-	-	1 (16.7)	AST increase	-	-	1 (14.3)
Epistaxis	1 (12.5)	-	-	-	-	Dry skin	1 (33.3)	1 (16.7)	-
Feeling cold	-	1 (16.7)	-	-	-	Hyperhydrosis	-	1 (16.7)	-
Flatulence	-	-	-	-	1 (16.7)	Injection site erythema	1 (33.3)	-	-
Headache	1 (12.5)	1 (16.7)	-	-	1 (16.7)	Injection site pain	1 (33.3)	-	-
Increased appetite	1 (12.5)	-	-	-	-	Injection site reaction	-	2 (33.3)	1 (14.3)
Phlebitis	-	-	-	-	1 (16.7)	Malaise	-	1 (16.7)	-
Purpura	-	-	-	-	1 (16.7)	Nausea	-	1 (16.7)	-
Somnolence	1 (12.5)	-	-	-	1 (16.7)				

Obs: all listed events were rated blindly as possibly-related to treatment.

*: One subject in the placebo group (#016) received one dose of hLF1-11 5 mg (Day 4) due to administrative error, therefore events were computed in the 5 mg group.

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Table 4 Adverse events in HSCT patients (n=8).

All recorded events (n)	**187**
All events possibly treatment-related (n)	**4**
Events per patient (min-max) (n)	**5-35**
Severity (% of events)	
Mild (%)	58
Moderate (%)	25
Severe (%)	17
Non-serious events possibly treatment-related (n)*	**4**
Supraventricular extrasystoles	1
ALT increased	1
AST increased	1
Gamma-GT increased	1
Serious Adverse Events (SAEs) (n)**	**4**
Heart failure/pulmonary edema	1
Pulmonary infiltrates	1
Hypoxemia/Respiratory insufficiency	2
Treatment-related SAEs (n)	**0**

*: All 4 events occurred in one patient (no. 001)
**: The 4 SAEs occurred in 2 patients (no. 002 and no. 005)
HSCT: hematopoietic stem cell transplant

interindividually. Levels of IL10 were undetectable in all samples. Release of IL6 and TNFα on LPS stimulation seemed attenuated with 0.5 and 5 mg; however this was neither clinically nor statistically significant. No antibodies against hLF1-11 were detected (IgG anti-hLF1-11 or IgE anti-hLF1-11) in volunteers or patients.

Discussion

Bacterial pathogens account for most infections occurring shortly after transplantation during neutropenia, when mucosal barrier injury is most pronounced. Whilst most patients receive standard antibacterial prophylaxis with fluoroquinolones, the majority of infections are caused by gram-positive bacteria (65-75%), mainly *viridans* group streptococci and coagulase-negative staphylococci as observed in all our patients, and a minority by gram-negative pathogens, mostly *Enterobacteriaecae* [27]. The incidence of fungal infections also remains relatively high affecting up to 15% in allogeneic HSCT, with *Candida* species and *Aspergillus*

fumigatus predominating [27]. Lactoferrin or derivatives may prove to be a promising versatile class of agents for managing infectious complications that arise from HSCT, because of their broad antimicrobial activity especially in the context of emerging antimicrobial resistance to currently used antimicrobial agents. Additionally, immune modulating and anti-inflammatory properties might attenuate mucosal barrier injury and graft-versus-host disease in HSCT, although for now this remains speculative [19].

The N-terminal moiety of hLF, in particular the first five amino acids (Gly-Arg-Arg-Arg-Arg), has a high cationic charge allowing binding to negatively charged molecules including microbial products such as lipopolysaccharide (LPS) and CpG motifs of bacterial DNA (CpG-DNA) [28;29]. These interactions result in microbial cell wall disruption and direct microbial killing [30]. Additionally, indirect antimicrobial activity is seen through the intermediary of cells mainly phagocytic cells (polymorphnuclear cells, macrophages) probably as result of opsonization and other not yet fully determined mechanisms [31-33]. Anti-inflammatory and immune-modulatory properties have also been largely related to the N-terminal moiety of hLF [15;17;34-36].

hLF1-11, derived from the active N-terminal moiety of hLF, has been tested *in vitro* and *in vivo* showing broad spectrum activity against the pathogens commonly involved in infections after HSCT, both bacterial and fungal, similar to human lactoferrin. The activity in preclinical studies was even superior to that of hLF [37]. *In vivo* animal experiments indicated that hLF1-11 is highly effective against *Staphylococcus aureus*, *Listeria monocytogenes* and antibiotic resistant *Acinetobacter baumannii*, reducing bacterial loads in infected organs by 2 to 3 log [20;23;24;37]. Furthermore, hLF1-11 was effective against invasive infections fluconazole resistant and sensitive *Candida* species even in neutropenic and lymphopenic mice [21;22;38]. Regarding immune-modulatory properties, as for the antimicrobial activity, the activity of hLF1-11 is expected to be similar albeit more potent than those reported for hLF, although this has to be studied in more detail.

In this study, for the first time, the antimicrobial peptide hLF1-11 has been tested in healthy volunteers and autologous HSCT recipients. In both populations the drug was well tolerated with few possibly related side effects. The reported events consisted predominantly of discomfort at the injection site, commonly reported with intravenous drugs. No changes on electrocardiography and in particular no increase in QT interval was seen. All adverse events were mild in intensity, reversible without clinical sequel, not necessitating any intervention. No signs of cytotoxicity were seen, consistent with earlier *in vitro* data [39], and in HSCT recipients no changes in engraftment occurred. Pharmacodynamic evaluation revealed no apparent changes in the cytokine profiles, suggesting that the immune-modulatory effects of hLF1-11 do not result in an unexpected increase in pro-inflammatory responses.

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During multiple dosing, elevations in transaminase levels were detected and considered related to the study drug in five subjects, because use of other drugs or alcohol was not allowed during the study, although elevations were observed on placebo as well. Similar abnormalities were observed in one patient receiving the 5 mg dose, followed by spontaneous and complete recovery. According to the CTCv3.0 [26], the maximum elevation was observed once in one patient and was moderate (grade 2/3). Since this patient was receiving a number of other drugs known to affect transaminases (Figure 1), this occurred after single dose and no effects on transaminases had been reported during single dosing in healthy volunteers but mild elevations had been recorded after multiple dosing, we considered this event as possibly-related to treatment. A safety advisory board (independent of investigators) also evaluated the liver enzyme results of all studies and could not establish a definite relationship between hLF1-11 and elevated transaminases, although it did not rule out such an effect and advised that a lower dose (0.5 mg) should be used in the next, multiple dosing study in patients with close monitoring of liver parameters. This lower dose is expected not to interfere with the antimicrobial activity of hLF1-11, shown in preclinical data to be active in the µg/kg range [22]. Moreover, a recent study emphasized that even at very low concentrations AMPs with strong membrane-binding activity could disrupt bacteria by reaching higher membrane-bound concentrations than intuitively expected [40].

One important evaluation that is missing in our studies is the pharmacokinetics of hLF1-11, as to date the detection of the peptide is impossible to quantify in humans, which makes the safety evaluations all the more important. Inability to measure hLF1-11 in plasma is partly due to the doses being so small (0.005-5 mg) that the concentrations in plasma are undetectable by usual methods. In animals, [99mTc]-labelled hLF1-11 was rapidly removed from the circulation mainly via the liver and to a lesser extent via the kidneys. The quantity of [99mTc]-labelled peptide in the liver remained stable during the first hour after injection, while the plasma half-life (in mice) was estimated at approximately 30 min, yet this may be a underestimation since some of the peptide may be receptor bound and an unknown amount may be quickly metabolized. Studies are ongoing with internally labeled (C_{14} and tritium) peptide to determine distribution and metabolism in animals, while the quantification in humans remains undetermined. Nevertheless, the results of this new antimicrobial in preclinical studies merit further investigations on the applicability of hLF1-11 in humans, particularly in patients with hematological malignancies, and were the motivation for the current studies.

Figure 1 Serum alanine aminotransferase (ALT) levels in hematopoietic stem cell transplantation patients.
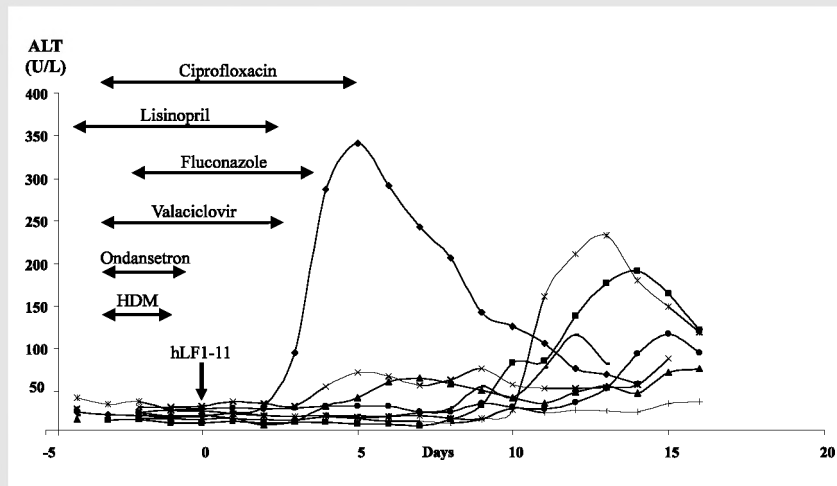
The drugs depicted in figure are the drugs used by the patient experiencing elevated transaminases. Day 0 = day of hematopoietic stem cell transplantation. HDM: high-dose melphalan.

Conclusions

The new antimicrobial drug hLF1-11 was well-tolerated in healthy volunteers with repeated daily doses up to 5 mg. Owing to elevations in transaminase levels being possibly related to treatment caution is warranted in further studies, although this potential effect was regarded not clinically serious and reversible without intervention in all cases to date. Nevertheless, as a precaution, further testing will be conducted with a lower dose of 0.5 mg in the forthcoming multiple dosing study in HSCT patients.

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In vitro immunomodulatory effects
of antimicrobial peptide human lactoferrin
1-11 (hLF1-11)

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Abstract

Background

A short peptide derivative of human lactoferrin, hLF1-11, has been developed as an anti-microbial peptide for prophylactic and therapeutic use in the setting of hematopoietic stem cell transplantation (SCT). It's efficacy in animal models has suggested additional immune enhancing properties on top of the anti-microbial activity. Because other immunological processes during SCT, like graft-versus-host disease (GvHD), could also be influenced by the peptide, we wanted to test the *in vitro* effects of hLF1-11 on immune cells involved in transplant immunology.

Methods

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of healthy human volunteers. T lymphocytes, natural killer (NK) cells, and dendritic cells (DC) were isolated using standard procedures. The effects of different concentrations of hLF1-11 (range 0-100 µg/ml) were studied in standardized experimental models on proliferation, differentiation and activation of these different immune cells.

Results

No phenotypic alterations were found in DCs cultured in the presence of hLF1-11, but adding hLF1-11 during the final step of DC maturation showed absence of podosome loss, a sign of inhibited LPS-induced maturation. Proliferation of stimulated NK cells and T lymphocytes was not affected by hLF1-11, and hLF1-11 exposure did not result in toxic lysis of these cells. NK cell cytotoxicity and regulatory T cell activity was unaltered, but allogeneic PBMC stimulated CD4+ memory T cells showed a decreased release of pro-inflammatory cytokines IFNγ and IL-17 (Th1 and Th17 pathway, respectively).

Conclusion

The *in vitro* studies did not reveal unexpected immune responses that evoke concerns regarding the safety of hLF1-11 in the clinical setting of allogeneic SCT. The observed immunosuppressive effects of the peptide might prove beneficial with regard to acute GvHD, but this needs confirmation. Because data on *in vivo* immune effects of hLF1-11 are still lacking caution is warranted during future studies in SCT patients.

Introduction

Human lactoferrin (hLF) is a natural defense protein belonging to the innate immune system, and is present in several body fluids and secretions as well as in the secondary granules of neutrophils [1]. Lactoferrin and its derivatives have pleiotropic functions including anti-microbial activity, anti-tumor activity, regulation of cell growth and differentiation, and modulation of inflammatory as well as humoral and cellular immune responses [1-4]. Most of these properties have been attributed to the N-terminal moiety of the molecule, which contains a high cationic charge responsible for the interactions with microbe-associated molecular patterns (MAMPs), heparin, and proteoglycans [5].

A short peptide derivative of human lactoferrin, hLF1-11, has been developed as an anti-microbial peptide (AMP), which consists of the cationic N-terminal 11 amino acids of hLF. Results from animal models representing systemic fungal and bacterial infection have shown impressive *in vivo* efficacy even in the setting of neutropenia [6;7]. Because of its anti-microbial potential, hLF1-11 could be a promising peptide in the treatment of patients receiving high-dose chemotherapy and a stem cell transplantation (SCT), especially considering the increasing resistance against several important anti-microbial drugs including quinolones, cephalosporins and azoles, which makes treating bacterial and fungal infections in SCT a difficult task. Being a derivative of a 'natural' human protein hLF1-11 might have the advantage of less side effects and antimicrobial resistance, because AMPs are unlikely to induce resistance because of the evolutionary difficulty in changing bacterial membrane structure [8].

In animal models, efficacy of hLF1-11 and other AMPs was seen at very low concentrations suggesting that additional immune enhancing properties might have contributed to the anti-microbial effects of hLF1-11 [6;9;10]. Indeed, *in vitro* data have already confirmed immunomodulatory effects of hLF1-11 on monocytes and macrophages. These cells exerted enhanced effector functions including enhanced recognition and clearance of pathogens and increased MAMP-induced cytokine production [11]. These effects could play an important role by stimulating immune responses in immunocompromised SCT recipients suffering from mucosal barrier injury and neutropenia.

Currently, little is known about the immunomodulatory effects of hLF1-11 on other immune cells like T lymphocytes, natural killer (NK) cells, and dendritic cells (DC), which play a key role in both innate and adaptive immunity. This is important because these cells are involved in allo-reactive immune responses and immune reconstitution[12;13]. Before applying hLF1-11 in SCT, more data are necessary on the possible effects on graft-versus-host disease (GvHD) and graft-versus-tumor (GvT) immunity, since activation or inhibition of the immune system by hLF1-11 could have both beneficial and detrimental consequences.

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In order to further study the issues regarding safety, but also potential beneficial immune properties of hLF1-11, we conducted *in vitro* studies analyzing the effect of hLF1-11 on the different immune cells involved in innate and acquired immune responses during conditioning and SCT.

Material and methods

Lactoferrin and derived peptide hLF1-11

Human lactoferrin was purchased from Sigma Aldrich, Zwijndrecht, the Netherlands. The synthetic peptide corresponding to residues 1-11 of hLF, representing the cationic domain of hLF, was provided by AM-Pharma BV, Bunnik, the Netherlands. This peptide was designated hLF1-11 (amino acids, GRRRRSVQWCA; molecular mass, 1375 DA). The purity of these peptides exceeded 97%, as determined by reverse-phase high-performance liquid chromatography. The peptides were stored and used as previously described [6].

Cell source

In all experiments peripheral blood mononuclear cells (PBMCs) were used and isolated from buffy coats from healthy human volunteers upon written informed consent according to the Declaration of Helsinki with regard to scientific use.

Dendritic cell culture

The generation and culture of monocyte-derived DCs has previously been described by Hobo *et al.* [14]. Immature DCs were generated by culturing monocytes in X-VIVO-15 medium supplemented with 2% human serum (HS), 500 U/ml interleukin (IL)-4, and 800 U/ml GM-CSF. After 2-3 days, half of the medium was replaced with fresh X-VIVO-15/2% HS medium, containing 1000 U/ml IL-4 and 1600 U/ml GM-CSF. During the culture process hLF1-11 was added at concentrations ranging from 0 to 100 µg/ml. Maturation of DCs was induced at day 6-7 by culturing 0.5×10^6 immature DCs/ml in 6-well plates in X-VIVO-15/2% HS containing 500 U/ml IL-4, 800 U/ml GM-CSF, 5 ng/ml IL-1β, 15 ng/ml IL-6, 20 ng/ml TNF-α, and 1.0 µg/ml prostaglandin E_2 (PGE$_2$) or 2.5 µg/ml lipopolysaccharide (LPS). At day 8, mature DCs were harvested and analyzed by FACS analysis for surface marker expression with the emphasis on expression of pattern recognition receptors (PRRs) including CD282 (Toll-like receptor (TLR) 2), CD284 (TLR4), dectin-1, CD206 (mannose receptor (MR)), and CD209 (DC-SIGN).

Podosome formation by DCs

The technique used has previously been described by van Helden *et al.* [15]. Plastic-adherent monocytes were cultured in X-VIVO 15 medium (BioWhittaker) supplemented

with 2% HS (PAA Laboratories), IL-4 (500 U/ml), and GM-CSF (800 U/ml), or in RPMI 1640 medium supplemented with 10% (v/v) FCS, IL-4 (500 U/ml), and GM-CSF (800 U/ml). Immature DCs (iDCs) were harvested on day 6. Day 6 iDCs were seeded on fibronectin-coated 12 mm coverslips. The cells were maintained in 0.5 ml of RPMI 1640 supplemented with 10% FCS, 1% ultraglutamine, IL-4 and GM-CSF. After the cells had adhered (approximately 3 hrs), stimuli were added consisting of LPS (2 µg/ml), PGE2 (10 µg/ml), hLF (10, 50 and 100 µg/ml), hLF1-11 (1, 2, 5, 10, 50 en 100 µg/ml), or the combination of LPS (2 µg/ml) and hLF or hLF1-11 (100 µg/ml). The iDCs were cultured overnight and fixed and stained the next day. The coverslips were then analyzed with a standard fluorescent microscope and percentage of cells containing podosomes was calculated.

T cell stimulation assay

T cell isolation and culture were performed as described by Koenen et al. [16]. Briefly, memory T cells were generated by depletion of CD25+ and CD45RA+ cells. To assess the impact on proliferation, measured by 3H-Thymidine incorporation, CD4+ T cells were stimulated with allogeneic stimulator PBMCs (allo-PBMCs) or CD3/CD28 beads in the presence of different concentrations of hLF1-11 (range 0-100 µg/ml). Cytokine production (IFNγ and IL-17) was measured after stimulation of CD4+ memory T cells with allo-PBMCs in the presence of polarizing cytokines and 10 or 100 µg/ml of hLF1-11. Cells were analyzed by flowcytometry on day 8 of culture. Regulatory T cells (Treg), CD4+CD25^{high} T cells, were isolated by flowcytometric cell sorting. These cells were stimulated with allo-PBMC in the presence of polarizing cytokines and 10 or 100 µg/ml of hLF1-11. Cells were analyzed by flowcytometry on day 8 of culture on the expression of Foxp3.

Natural killer cell assay

NK cells were isolated through negative selection, using an antibody cocktail of CD3, CD4, CD19 and CD33. NK^{dim} cells were isolated from negatively isolated NK cells, through positive selection with CD16 micro-beads. NK cells were cultured in culture medium (RPMI 1640 with glutamax supplemented with pyruvate (0,02 mM)), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Paisley, UK) and 10% HS at 37°C, 95% humidity and 5% CO_2, in 96 wells round bottom plates (Greiner, Frickenhausen, Germany). The leukemic K562 cell line, was cultured in culture medium (RPMI 1640 with glutamax supplemented with pyruvate (0,02 mM)), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Paisley, UK) and 10% Fetal Calf Serum (FCS) at 37°C, 95% humidity and 5% CO_2, in T75 tissue culture flasks (Greiner, Frickenhausen, Germany). After overnight stimulation with 100 U/ml rIL-2 and 10 ng/ml rIL-15, hLF1-11 was added to the cell culture in titrated concentrations. NK cell proliferation was measured after day 1, 3, 5, and 7 of treatment with hLF1-11

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by 3H-Thymidine incorporation. The cytotoxic capacity was examined by ^{51}Cr-release of ^{51}Cr-labeled K562 cells (100 µCi* ^{51}Cr (Amersham, UK)), which were used as target cells, 1000 cells/well. Different effector/target (E:T) ratios were tested. Cytotoxic capacity was shown as percentage specific lyses of the target cells. Activation of NK cells was determined by measuring the CD69 expression on day 1, 3, 5, and 7 of treatment with hLF1-11 using flowcytometry.

Flow cytometry

NK, T cells and DCs were phenotypically analyzed by 4- or 5-color flow cytometry. The following conjugated mAbs were used: CD3-(UCHT1), CD4-(MT310), CD8-(DK25), CD27-(M-T271), CD45RA-(4KB5), CD45RO-(UCHL1) FITC- or PE-labeled (DAKO), CD25-(M-A251) PE, CD127-(M21) PE, CCR4-(1G1) PeCy7, CCR6-(11A9) PE- or biotin-labeled, CD206-(19.2) PE, CXCR3-(1C6/CXCR3) PeCy5 (BD Biosciences), CXCR4-(12G5) PeCy5 (eBioscience, Uithoorn, The Netherlands), CCR7-(150503) FITC or PE, Dectin-1-(259931) PE (R&D Systems), CD4-(T4) ECD, CD4-(T4) PeCy5, and CD62L-(DREG54) ECD, CD80-(MAB104) PE, CD83-(HB15a) FITC, CD86-(HA5.2B7) PE (Beckman Coulter), CD209-(9E9A8) PE, CD282-(TL2.1) PE, CD284-(HTA125) PE (Biolegend), and isotype controls IgG1 FITC/PE dual-color control (DAKO) and IgG2b PE (Beckman Coulter). Intracellular analysis of Foxp3-(PCH101) FITC or PE and IL-17-(6CAP17) PE (eBioscience) and IFNγ was performed after fixation and permeabilization, using Fix and Perm reagent (eBioscience). Before intracellular cytokine measurements, the cells were stimulated for 4 hours with PMA (12.5 ng/ml) plus ionomycin (500 ng/ml) in the presence of Brefeldin A (5 µg/ml; Sigma-Aldrich).

Results and discussion

hLF1-11 does not alter surface marker expression in monocyte-derived DCs

DCs cultured and matured in the absence of hLF1-11 showed the expected pattern with high expression of CD83, CD86, and CCR7, low and decreasing expression of MR, DC-SIGN, dectin-1, TLR2, and TLR4 (data not shown). Addition of hLF1-11 to the culture at doses up to 100 µg/l did not result in altered expression patterns of any of these surface markers, although a slight but non-significant decrease in the expression of CCR7 was noted with 100 µg/l hLF1-11. No differences were seen between maturation induced by PGE2 or LPS.

These results are different from those in monocytes exposed to hLF1-11 which did not show an altered expression of surface markers and PRRs in the un-stimulated, but increased expression of TLR4 in the LPS-stimulated setting [17]. This altered expression profile also resulted in a more pro-inflammatory phenotype of these monocytes on MAMP stimulation [17]. The same study group reported similar results

in macrophages cultured from monocytes during hLF1-11 exposition, showing an increased expression of PRRs, mainly TLR4 and dectin-1, with enhanced phagocytic activity [11]. These contradicting results might be caused by differential effects of hLF1-11 on the different cell types studied, although these cells all originate from the same myeloid lineage.

Because DCs play an important role in post SCT immunological processes like GvHD, immune reconstitution and GvT reactivity the current data do not seem to raise concerns on adverse effects of hLF1-11 regarding these processes. The DC phenotype remained unaltered, and preliminary data suggest inhibitory effects of hLF1-11 on the cytokine release of DCs (data not shown). Since the peptide is designed as an anti-microbial peptide for use in the early phase of SCT, where mainly inflammatory complications occur, this anti-inflammatory effect might even proof to be beneficial.

Clearly, more studies are necessary to delineate the effects of hLF1-11 on DC subsets and the impact of timing, dosage, and co-stimulation of hLF1-11 exposition. Also, the effects of the peptide on other DC functions including cytokine release, T cell skewing and T cell activation remain to be determined in future studies.

hLF1-11 impairs LPS-mediated podosome down-regulation in immature DCs

In the assay on podosome down-regulation through a TLR4-mediated pathway, we found an impaired loss of podosomes on exposition to hLF and hLF1-11 (Figure 1). It is important to notice that the DCs were not cultured under the influence of these proteins, like in the previous DC study, as these had been added only in the final stimulation step. The effects suggest that hLF and hLF1-11 interfere more or less directly with LPS signaling, but the mechanisms by which they do so are not easily deductable. For hLF many studies have shown a LPS-scavenging effect, purely based on charge-dependent interactions [18]. This could be the mechanism in the impaired podosome loss with hLF in this assay. However, how hLF1-11 brought about a similar inhibitory effect remains speculative. hLF1-11 is thought to be too small a peptide for direct LPS-scavenging and it has been proposed as for other small cationic AMPs that cell penetration and intracellular translocation are of utmost importance for their biological activity although the intracellular target is currently not known [19]. Unfortunately, we have not been able to perform additional mechanistic studies on the effects of hLF1-11 in DCs.

Podosome loss during DC maturation results in increased mobility of DCs and migration to lymph nodes where mature DCs present antigen to lymphocytes and induce additional immune responses [20]. Persisting of the podosomes under the influence of hLF and hLF1-11 possibly limits immune activation as has been shown in other models in which hLF reduced DC-mediated cellular immune responses and diseases [21;22]. Theoretically inhibition of DC function during the early phase of SCT

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**Figure 1** Effect of hLF1-11 on podosome formation and expression.
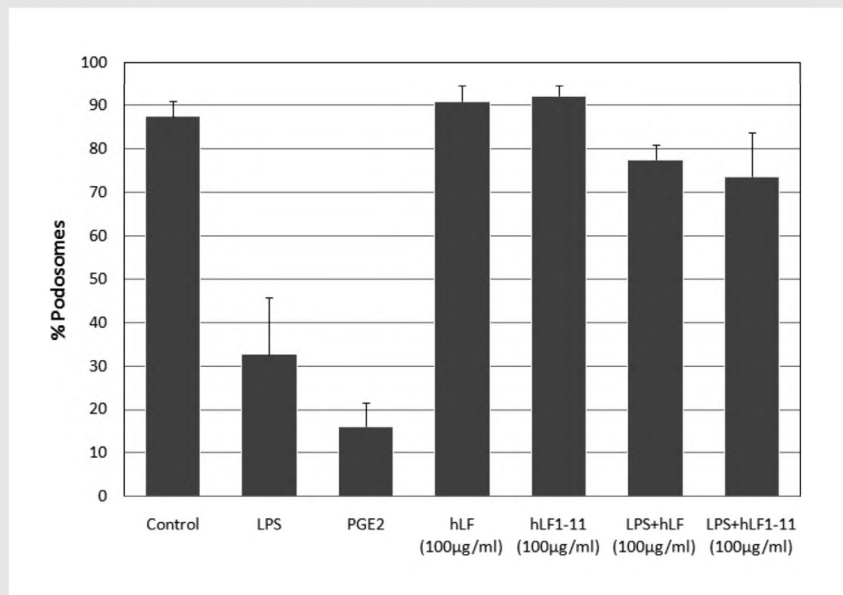
Day 6 immature DCs were seeded on fibronectin-coated 12 mm coverslips. After the cells had adhered, stimuli were added consisting of LPS (2 µg/ml), PGE2 (10 µg/ml), hLF (100 µg/ml), hLF1-11 (100 µg/ml), or the combination of LPS (2 µg/ml) and hLF or hLF1-11 (100 µg/ml). The iDCs were cultured overnight and fixed and stained the next day. The coverslips were then analyzed with a standard fluorescent microscope and percentage of cells containing podosomes was calculated. The mean values of 3 independent experiments are shown. The LPS-induced loss of podosomes was significantly reduced in the presence of hLF and hLF1-11 (P = 0.03 and P = 0.01 respectively), according to the Tamhane correction for multiple testing, not assuming equal variances [34].

might prove beneficial as host APCs are powerful inducers of GvHD, although they also play a role in the generation of GvT responses [23].

hLF1-11 does not alter NK cell proliferation and effector functions

NK cell proliferation was measured after day 1, 3, 5, and 7 of treatment with hLF1-11 by 3H-Thymidine incorporation. A wide range of hLF1-11 concentrations (0.01-5.0 µg/ml) did not impact NK cell proliferation (data not shown). Additional assays also revealed no changes in NK cell activation and cytotoxicity with hLF1-11 concentrations up to 1 µg/ml (Figure 2). In addition, no direct *in vitro* toxic effect of hLF1-11 on NK cells was observed. These results suggest the absence of direct effects of hLF1-11 on NK cells at concentrations within the therapeutic range used in animal and human studies [24].

Figure 2 Effect of hLF1-11 on NK cell function.
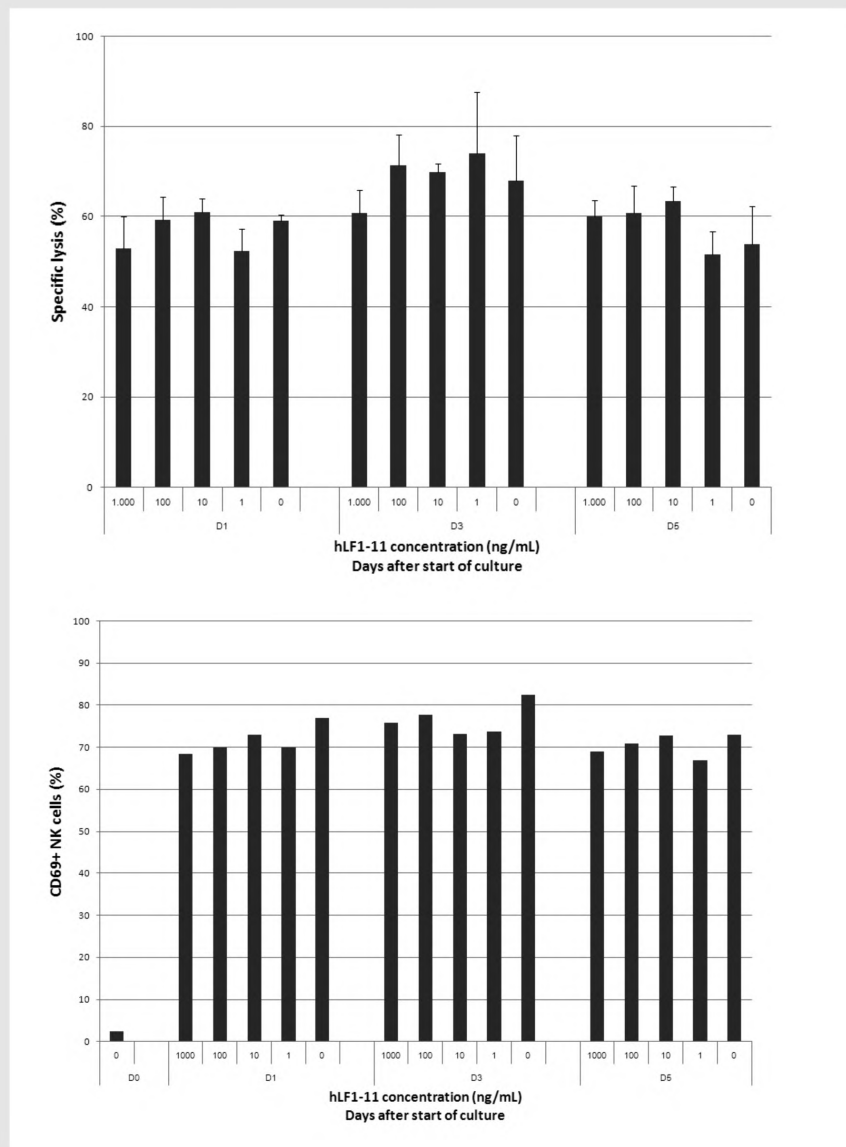
The effect of hLF1-11 on the cytolytic capacity of NK cells against K562 was studied with the use of 51Cr-release assay (**A**), and the effect on NK cell activation was measured by the CD69 expression (**B**). No significant changes were noticed using a wide range of hLF1-11 (0-1,0 μg/ml). One representative experiment out of 3 is shown.

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No other data on hLF derivatives like hLF1-11 are currently available and few exist on the effects of hLF on NK cells. Damiens *et al.* found increased NK cell mediated anti-tumor effects against epithelial and hematological cancer cells with hLF concentrations of 10 μg/ml [25], but higher doses of hLF resulted in NK cell death. In that study the anti-tumor effects resulted from both direct modulation of NK cell cytotoxicity and increased target cell sensitivity. The NK modulation was achieved by direct binding of hLF to the NK cells. However, the binding site or receptor for hLF on NK cells has not been identified. Most other studies regarding NK cell modulation involved *in vivo* animal models in which oral administration of hLF was investigated. These studies showed enhanced NK cell activation and anti-tumor activity resulting from hLF increasing the release of IL-18 from intestinal epithelial cells [26].

The role of NK cells in the setting of SCT is increasingly appreciated as they contribute to early immune reconstitution and GvT activity [27]. In this regard the absence of inhibitory or cytotoxic effects of hLF1-11 on these cells is reassuring, although the effects of higher doses remain to be established.

hLF1-11 affects memory but not regulatory T cell function
In two different assays the effect of hLF1-11 on T cell proliferation was studied, and stimulated CD4+ T cells did not display altered proliferation on exposure to hLF1-11. No direct toxic effect of hLF1-11 on T cells was observed at concentrations lying within the therapeutic range [24]. Memory CD4+ T cells stimulated with allo-PBMC exhibited significantly decreased production of both IFNγ and IL-17 in the presence of hLF1-11 at concentrations of 10 and 100 μg/ml (Figure 3). No effect was seen on regulatory CD4+CD25^{high} T cells differentiation measured by the expression of Foxp3 (Figure 4).

Few studies have addressed the effects of hLF on T lymphocytes and the different subsets. Lymphocytes do express receptors for hLF, and one specific receptor is even designated the lymphocytic (LC)-LfR [28]. Although increased hLF-induced proliferation, CD4 expression, and IFNγ production has been suggested in some studies, these results have been contradicted by others, the substantial differences in methodology, use of bovine versus human LF, and settings explaining these discrepancies [21;29;30]. More consistent data come from *in vivo* animal studies which propose an immune enhancing and T cell activating (both CD4 and CD8) effect of orally administered hLF [31]. Similar to NK cell activation, as mentioned earlier, these effects seem to result from altered IL-18 release from intestinal epithelial cells.

Interestingly, one study showed an inhibitory effect of hLF on T cell responses with reduced proliferation, chemokine receptor expression, and Th1-cytokine release, comparable to, but less efficient than, Cyclosporin A [32]. More standardized studies are necessary to clarify the direct and indirect effects of hLF and derivatives on

Figure 3 Effect of hLF1-11 on IFNγ and IL-17 production by activated T cells.
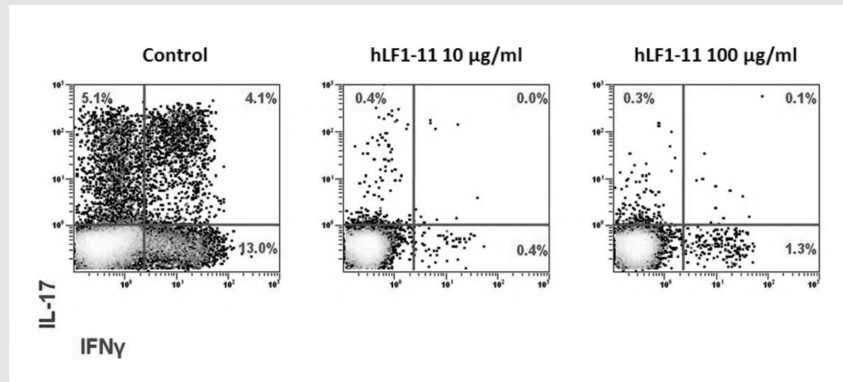
Cytokine production was measured after stimulation of memory CD4+ T cells with allo-PBMC in the presence of polarizing cytokines and 10 or 100 µg/ml of hLF1-11. Cells were analyzed by flowcytometry on day 8 of culture. A significant reduction in the production of both IL-17 and IFNγ was noticed in the presence of hLF1-11 ($P = 0.01$)[34]. One representative experiment is shown.

Figure 4 Effect of hLF1-11 on Foxp3 expression of regulatory T cells.
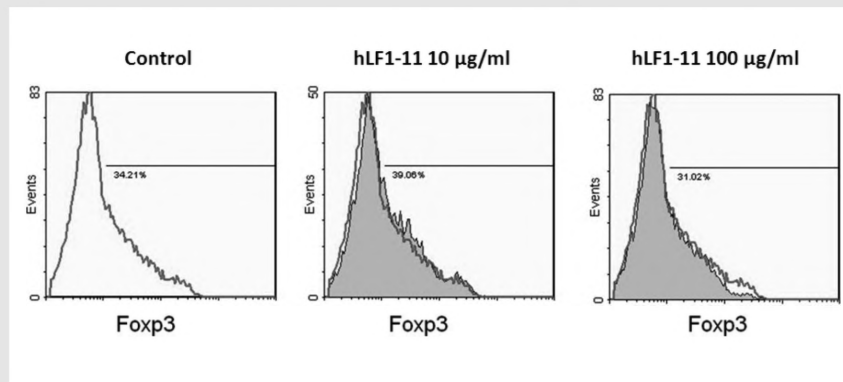
Regulatory T cells (Treg) CD4+CD25^{high} T cells were isolated by flowcytometric cell sorting and stimulated with allogeneic stimulator PBMC in the presence of polarizing cytokines and 10 or 100 µg/ml of hLF1-11. Cells were analyzed by flowcytometry on day 8 of culture on the expression of Foxp3. No changes were noticed on exposure to hLF1-11. One representative experiment is shown.

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T cells and T cell-mediated immune responses. The observed inhibitory effect of hLF1-11 on IFNγ and IL-17 release by T lymphocytes is however reassuring, since both Th1 and Th17 responses have been implicated in the origin of acute GvHD. Hence no increase in the incidence of acute GvHD would be expected with the use of this AMP. Nevertheless, the effects on GvT induction and adaptive anti-microbial immunity remain to be determined, although similar to Cyclosporin A, the inhibition of T cell responses by hLF1-11 are probably only temporary and reversible. Since cytotoxic T lymphocytes (CD8+) and naïve T lymphocytes also play a crucial role in GvHD and GvT additional studies should address the effects of hLF1-11 on APC-mediated activation of naïve T lymphoyctes.

Comprehensive summary

The current *in vitro* studies did not reveal unexpected immune responses of hLF1-11 that evoke concerns regarding safety and therewith do not preclude further testing of this new anti-microbial drug in the clinical setting of allogeneic SCT, especially considering the fact that the peptide hLF1-11 was tested at the expected *in vivo* therapeutic concentration of hLF1-11. Nevertheless, because many immunological processes change during an allogeneic SCT, and data on *in vivo* effects of hLF1-11 on immune cells in humans are lacking, there still needs to be caution during future studies with this anti-microbial peptide in patients receiving an allogeneic SCT. In addition, immunomodulating effects of AMPs, including hLF1-11, seem dependent on the cell type studied and the timing, duration and dosage of the AMP tested [33]. Nevertheless, the observed inhibitory effect of hLF1-11 on LPS-induced DC maturation and decreased Th1 and Th17 cytokine release by CD4+ memory T cells also suggests possible additional beneficial activities of hLF1-11 during SCT with regards to acute GvHD. These observations need to be confirmed but at least warrant further testing.

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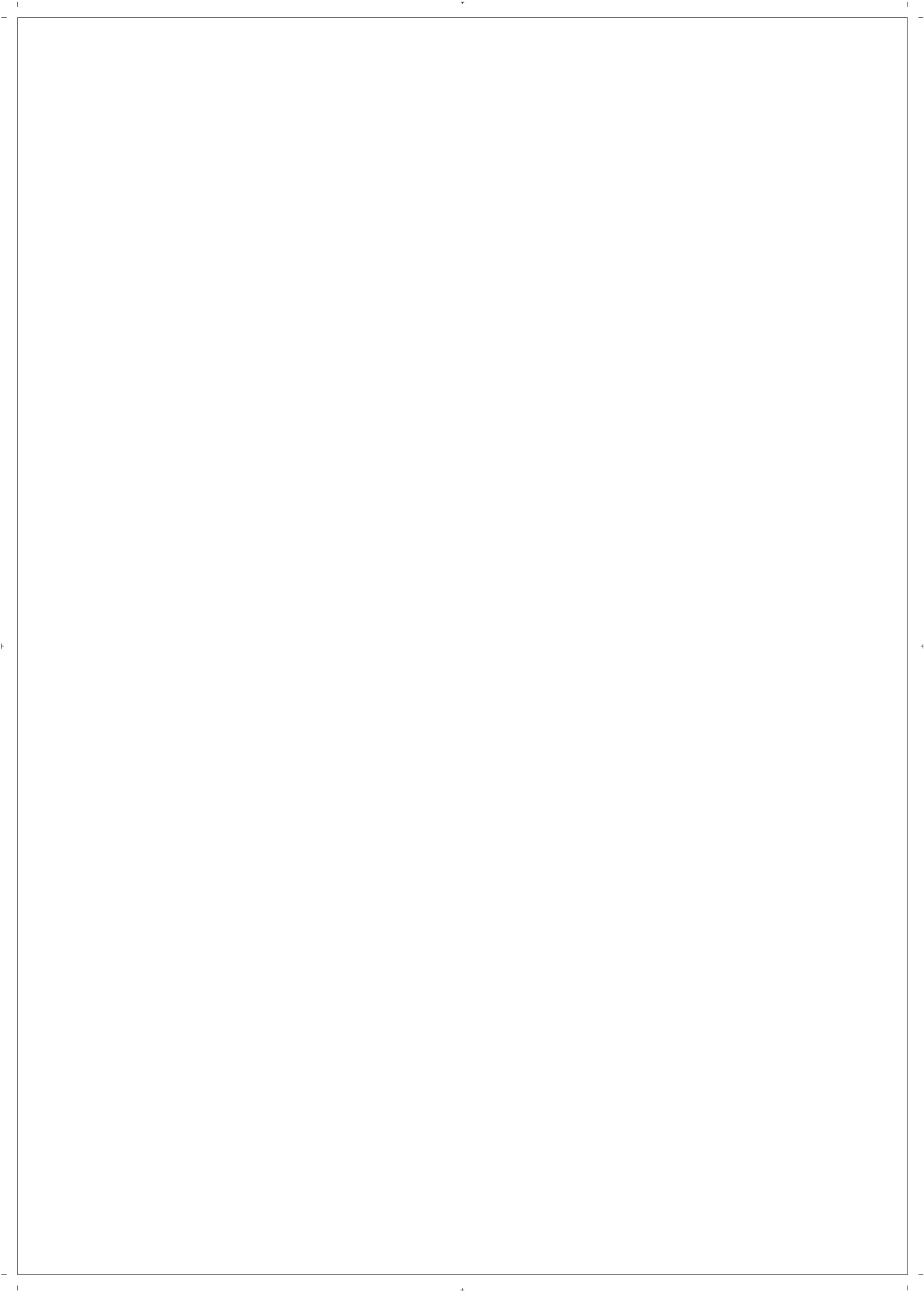26. Kuhara T, Yamauchi K, Tamura Y, Okamura H. Oral administration of lactoferrin increases NK

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Summary and future perspectives

Summary

For many patients with hematological malignancies intensive therapy with high-dose chemotherapy alone or combined with radiotherapy followed by hematopoietic stem cell transplantation (SCT) is the only way to cure their disease. However, therapy is almost invariably complicated by the occurrence of infectious and non-infectious inflammatory complications, though considerable inter-individual variability is seen. Traditionally the focus has been on blood cells, or rather their absence, regarding the pathogenesis of these inflammatory complications. Fever is the most frequent complication and it generally occurs as the granulocytes or neutrophils reach their nadir. This complication has been christened 'febrile neutropenia' designating neutropenia as the most important factor in the occurrence of these complications. More recently greater emphasis has been placed on the (sub)mucosal damage of the mouth and gastro-intestinal tract following cytotoxic therapy, and is more generally referred to as mucosal barrier injury (MBI). The emphasis on MBI has helped to highlight the role of the intestinal innate immune system in post SCT inflammatory complications.

The mucosal barrier is extraordinarily complex plying host to more than 10^{14} microbes that reside in close proximity to a single layer of intestinal epithelial cells. This symbiosis is normally mutually beneficial, but it requires the tight control of the immune system to prevent infection and microbial invasion as well as the occurrence of uncontrolled inflammation. Innate immunity plays a crucial role herein, and the perturbation of the delicate balance after cytotoxic therapy leads to alterations in host-microbial interactions which in turn influence early SCT complications that extend beyond the occurrence of infection. Especially in the development of graft-versus-host disease (GVHD) the occurrence of MBI, disrupted interactions between host and micro-organisms, and deregulated mucosal immunological processes play important roles. However, these new insights have not yet resulted in a greater acceptance of the role that MBI plays in post SCT complications, and research still focuses predominantly on immune cells rather than epithelial surfaces.

In the first part of this thesis we hypothesize that MBI is an important determinant of the systemic inflammatory response, as measured by C-reactive protein (CRP), and contributes to the occurrence of inflammatory complications following conditioning with chemotherapy with or without irradiation in the preparation for a SCT. In *chapter 2* we performed a retrospective study in a homogenous group of autologous SCT recipients who had been given high-dose melphalan as conditioning regimen. We found a striking pattern of inflammation, and the occurrence of fever, coinciding with the occurrence of MBI, as measured by citrulline. This affected all patients without exception regardless of the presence or absence of bacteremia.

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With additional statistical analysis we were able to underscore this by showing that citrulline levels best predicted the course of CRP. Bacteremia possibly aggravated the inflammatory response but did not trigger it, and neutropenia did not significantly contribute to the inflammation at all. So we were able to strengthen our hypothesis and suggested that the paradigm 'febrile neutropenia' could in fact be replaced by a new paradigm of 'febrile mucositis'.

In *chapter 3* we elaborated on this topic by studying the natural history of MBI and inflammation associated with another five conditioning regimens used for autologous and allogeneic SCT. The inclusion of a non-myeloablative regimen allowed us to estimate the sole impact of MBI on inflammation since treatment was accompanied by significantly less MBI, modest inflammation and no bacteremia, despite profound neutropenia. We also found a strong correlation between the degree of MBI elicited by the different regimen and the grade of inflammation. Moreover, we were able to link these findings with the occurrence of SCT complications, including the occurrence of bacteremia and acute lung injury. In addition we established a relationship between MBI and the kinetics of acute GvHD. We consider that our data underline that MBI plays a central role in the triad of MBI, inflammation and bacteremia. The extent of MBI determines the loss of physical barrier which facilitates bacteremia due to micro-organisms arising from the gut and the translocation of microbe-associated molecular patterns (MAMP). This was confirmed by the observation of a significant lower citrulline level in those with bacteremia as opposed to those without. In addition, MBI determines the magnitude of the inflammatory response. Chemotherapy and radiotherapy induce tissue damage and appear to 'prime' the innate immune system and it is the degree of activation status that ultimately determines the magnitude of the inflammatory responses elicited by host-microbial interactions once the mucosal barrier is breached.

In the second part of the thesis we performed retrospective analyses on the impact of single nucleotide polymorphisms (SNPs) in innate immune genes on the outcome of SCT, to explain partially the differences in the occurrence of post SCT complications and to further establish the importance of host-microbial interactions. In *chapter 4* we confirmed the impact of polymorphisms in the intracellular pattern recognition receptor (PRR) nucleotide-binding oligomerization containing protein (NOD)2 on the incidence of severe acute GvHD and 1-year treatment-related mortality in the setting of partially T cell-depleted myeloablative allogeneic SCT. This result contradicts the findings of *Granell et al.* who did not find an impact of these SNPs in T cell-depleted SCT in a Spanish cohort. The differences between the two studied cohorts emphasize the importance of context in judging on associations between gene polymorphisms and clinical outcomes. We conclude that in our particular clinical setting NOD2 polymorphisms could be used to optimize therapy in SCT recipients. For instance donor selection, antimicrobial prophylaxis, and acute GvHD management could be

tailored on the basis on the NOD2 status of both patient and potential donors. In addition, the large impact of NOD2 polymorphisms in SCT supports the concept that host-microbial interactions at the epithelial and mucosal barriers are important in the pathogenesis of SCT complications.

In *chapter 5*, we showed an association between *Candida* colonization in SCT recipients and the newly discovered 'loss-of-function' polymorphism Y238X in the C-type lectin PRR dectin-1, which is involved in the recognition of β-1,3-glucan. Colonization would probably have resulted in an increased incidence of candidemia whilst patients were neutropenic and suffered from MBI had we not employed prophylaxis with fluconazole.

In both association studies the cohorts were rather small with consequently some difficulty employing multivariable analyses. Nonetheless, there is a fair chance that the genetic associations we report are genuine and clinically relevant since the pre-study probability of an association between these SNPs and SCT outcome was high because of the existence of other confirmatory studies and biological plausibility. The biological plausibility is high since the functions of both NOD2 and dectin-1 have been extensively characterized and a 'loss-of-function' phenotype established in the presence of the polymorphisms we studied.

Interestingly, in the analysis of SNP Y238X, we noted that *Candida* colonization seemed to increase the incidence of acute GvHD, described in *chapter 6*. In retrospect this should not come as a total surprise since gram-negative bacilli are known to play a role in the development of acute GvHD and antimicrobial prophylaxis during neutropenia reduces the incidence of acute GvHD. We concluded that the presence of *Candida* species on the mucosa during MBI and neutropenia can contribute to the pathophysiology of acute GvHD, probably through activation of fungal PRRs by motifs possessed by the *Candida* species. Based on our findings we speculated that the common link between *Candida* colonization and acute GvHD might be the interleukin (IL)-23/T helper lymphocyte (Th)17 axis. Th17 is involved in the mucosal immunity to many pathogens and is implicated in the pathogenesis of several auto-immune diseases, including Crohn's disease. Mucosal immunity against *Candida* has recently been shown to be mediated by Th17, whereas systemic responses rely more on Th1 responses. Furthermore, a role for Th17 has recently been suggested in animal studies of acute GvHD pathogenesis, although thus far the data are inconclusive on the exact impact in relation to Th1 responses. However, a link between *Candida* colonization and Th1/Th17 has also been suggested in the pathogenesis of Crohn's disease. Nevertheless, the triad of *Candida*, acute GvHD and Th1/Th17 should be further investigated which, if confirmed, could have clinical consequences. For instance, fluconazole prophylaxis would then be reconsidered for all SCT recipients, not solely to prevent systemic *Candida* infections but also to reduce acute GvHD. In addition, that proving Th17 contributes to the

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pathogenesis of acute GvHD in humans opens new avenues for the treatment of this complication.

In the third part of the thesis we focus on new therapeutic options for post-SCT complications, in essence trying to learn from the natural innate immune system, so to say, 'nature knows best'. Pleiotropic molecules like antimicrobial peptides (AMPs) are employed by the host defenses, but also have a secondary role in damage control and negative feedback. Hence, these molecules seem exceptionally suitable candidates for treating infections without increasing inflammation. In *chapter 7* we described the theoretical background for the use of human lactoferrin (hLF) and its short-peptide cationic derivatives to treat SCT-related complications. The potential beneficial properties of hLF in SCT are diverse and include stimulation of growth and repair of epithelial cells, broad-spectrum antimicrobial activity, anti-inflammatory activity and immune modulation. These properties are less well defined and characterized for the hLF-derived AMPs, but most AMPs contain the essential hLF domains necessary for interactions with microbial motifs and hLF receptors. One of these AMPs is human lactoferrin 1-11 (hLF1-11), developed by the pharmaceutical company AM-Pharma. Pre-clinical research has shown broad activity against pathogens involved in infections encountered in SCT recipients, with MICs ranging from 3.1 to 25 mg/L for different strains of bacteria and fungi (although in sub-physiological salt concentrations). Moreover, with animal studies antimicrobial activity was confirmed *in vivo*, even in neutropenic and immunocom-promised subjects, at remarkably low concentrations (0.4 μg/kg), compared with the *in vitro* activity. hLF1-11 was well tolerated by animals, with no adverse events being seen after a daily dose of 10 mg/kg. These results encouraged and stimulated the further development of hLF1-11 for use in human subjects.

In *chapter 8* we reported that the drug was well tolerated and safe after being given in single and sequential doses up to 5 mg to healthy volunteers. These data were complemented with those from our safety study of a single dose of 5 mg hLF1-11 given intravenously to 8 SCT recipients. The drug was well tolerated and safe and no severe adverse events related to the study drug occurred. One concern arising from these studies was the increase in transaminase levels seen in both healthy volunteers and a single subject from our clinical trial. These abnormalities were transient, resolved completely, and were generally moderate, without any apparent clinical consequences. The use of other potentially hepatotoxic drugs probably explained the occurrence of the abnormalities experienced by the subject in our trial. Nevertheless, a safety board ordered a reduction of the dose to 0.5 mg in the planned multiple dose safety study in SCT recipients. However the study was cancelled when the company decided not to pursue the drug any further for clinical use.

The high efficacy of hLF1-11 in animal models suggested immune enhancing properties in addition to direct antimicrobial activity. This might give rise to safety

concerns because in SCT immunological processes such as GvHD, immune reconstitution and graft-versus-leukemia could also be influenced by the peptide. Therefore in *chapter 9* we performed additional *in vitro* experiments on T lymphocytes, natural killer (NK) cells, and dendritic cells (DC), all key players in transplantation immunology. There were no unexpected immune responses to hLF1-11. Data on the *in vivo* effects of hLF1-11 on NK-cells, lymphocytes, and DCs in humans are lacking so caution should be exercised in future studies of SCT recipients. The observed inhibitory effect of hLF1-11 on lipopolysaccharide-induced DC maturation and decreased Th1 and Th17 cytokine release by CD4+ memory T cells suggests possible additional beneficial activities during SCT with regards to acute GvHD that needs confirmation.

Future perspectives

Mucosal innate immunity plays an important role in cytotoxic therapy-induced MBI and GvHD, and hence has an impact on SCT outcome. Future studies are needed to gain further insights into the complex processes involved in mucosal immunology so as to develop new preventive and treatment strategies for improving the outcome of SCT. With the inflammatory response during MBI being unrelated to infections as such there needs to be a shift in focus from infections towards mucosal barrier integrity and innate immune defenses. The interactions between PRRs and MAMPs and danger-associated molecular patterns (DAMPs), and the antimicrobial and immunomodulatory activity of natural AMPs, may provide better insight into the way we approach uncontrolled inflammatory post SCT and indicates the direction in the development of treatment strategies for acute GvHD and other complications.

Therefore, future studies should address questions regarding pathogenesis, prevention and treatment of MBI in SCT recipients. There is a need to know more about what is happening at the mucosal surfaces during SCT. Which cells are involved in MBI and inflammation, - macrophages, epithelial cells, fibroblasts, or endothelial cells? The role of MAMPs needs to be explored in more detail, and the threshold for translocation needs to be determined. This also applies to DAMPs, which role is just started to be addressed in the setting of SCT. Ultimately, the most important question is if we can ameliorate MBI and the following inflammatory response and prevent and treat GvHD for instance by using AMPs or PRR-agonists and antagonists.

The role of citrulline as a biomarker of conditioning-induced tissue damage should be more precisely defined. Prospective studies are necessary to confirm the value of citrulline for the individual patient in predicting the occurrence and extent of

MBI, but more important subsequent complications including bacteremia and GvHD. In the meantime, classifying the degree of MBI of conditioning regimens, including the so-called reduced intensity and non-myeloablative regimens, by means of measuring citrulline and/or CRP, could be used in clinical practice. For instance, it could help determine the need for antimicrobial prophylaxis, hospital admission, and use of preventive and therapeutic anti-inflammatory therapies for a certain regimen.

The value of SNP screening in the clinical setting of SCT remains to be determined, but looks promising. To study and detect genes of interest in the future, two general approaches exist, - genome-wide association studies and a more step-wise approach focusing on pathogenic mechanisms. Screening individual genes or complete signaling pathways involved in antimicrobial peptide and cytokine synthesis, autophagy, PRR activation and signaling would be an attractive approach in SCT. The first goal is to enhance the insight into the contributing genes in the pathophysiology of inflammatory complications such as GvHD, but ultimately the goal is to find genetic associations which open ways for preventive or therapeutic interventions. The next step would then be designing prospective studies to determine clinical applicability of SNPs which are consistently and repeatedly associated with SCT outcome. However, studying the role of SNPs in the setting of SCT can only be done using large cohorts of patients, and this can only be achieved when individual institutions collaborate in national or international consortia.
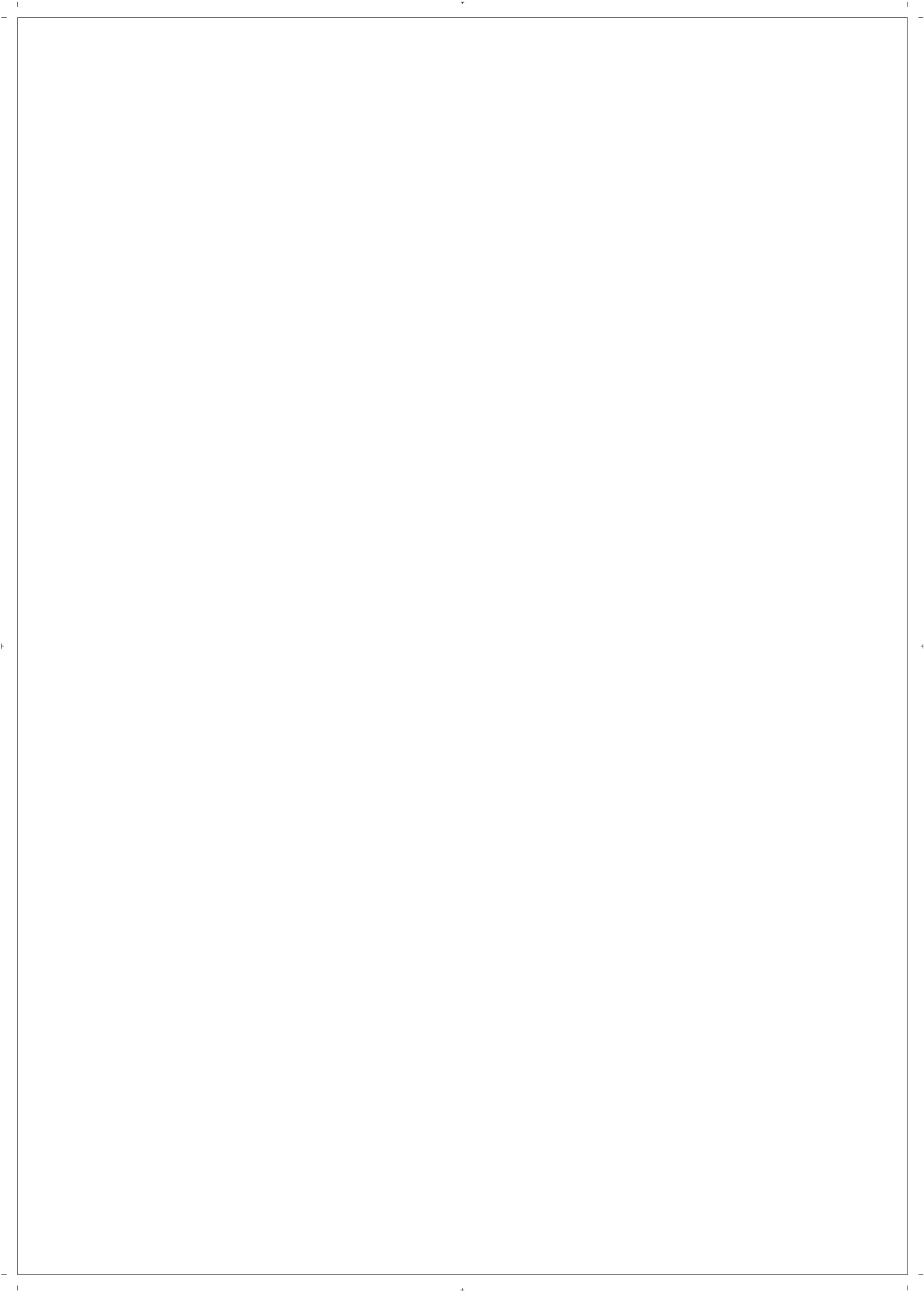
Nederlandse samenvatting

Dankwoord

List of abbreviations

List of publications

Curriculum vitae

Samenvatting

Voor veel patiënten met een hemato-oncologische ziekte blijft intensieve therapie met chemotherapie alleen, of in combinatie met radiotherapie, gevolgd door een stamceltransplantatie (SCT) de enige manier om van hun ziekte te kunnen genezen. Echter, de behandeling wordt bijna altijd gecompliceerd door het optreden van infectieuze en niet-infectieuze ontstekingprocessen, hoewel er een aanzienlijke inter-individuele variabiliteit bestaat. Van oudsher is de focus gericht op bloedcellen, of liever gezegd het ontbreken daarvan, met betrekking tot de pathogenese van deze complicaties. Koorts is de meest frequent voorkomende reactie, die optreedt als de granulocyten of neutrofielen hun dieptepunt hebben bereikt. Deze complicatie wordt 'febriele neutropenie' genoemd. Recent is er echter meer interesse gekomen en onderzoek gedaan naar de rol van weefselbeschadiging na cytotoxische therapie bij het ontstaan van ontstekingscomplicaties na SCT, in het bijzonder van de slijmvliezen van de mond en het maag-darmkanaal. Deze chemotherapie en/of radiotherapie geïnduceerde slijmvliesschade wordt aangeduid als mucositis of 'mucosal barrier injury' (MBI).

De barrièrefunctie van de darmwand is buitengewoon complex, zeker als men beseft dat de menselijke gastheer samenleeft met meer dan 10^{14} micro-organismen, het overgrote deel bestaand uit bacteriën, die slechts gescheiden worden door een enkele laag darmepitheelcellen. Deze samenleving is normaal gesproken voor zowel gastheer en micro-organismen voordelig, maar het vereist een strikte controle door het immuunsysteem. Enerzijds moet microbiële invasie en infectie voorkomen worden en anderzijds moeten commensale micro-organismen getolereerd worden zonder dat het leidt tot het optreden van ongecontroleerde ontstekingsreacties die vervolgens weer tot weefselschade kunnen leiden. De aangeboren immuniteit ('innate immunity') speelt een cruciale rol bij het behoud van deze homeostase. Bij verstoring van deze delicate balans na cytotoxische therapie ontstaan veranderingen in de interacties tussen gastheer en micro-organismen die invloed hebben op het optreden van ontstekingscomplicaties na SCT die verder gaan dan het optreden van infecties alleen. Helaas hebben deze nieuwe inzichten nog niet geresulteerd in een grotere acceptatie van de rol van MBI tijdens SCT, en de focus van huidig onderzoek richt zich toch nog voornamelijk op bloedcellen in plaats van epitheelcellen en de complexe mucosale immunologie.

In het eerste deel van dit proefschrift onderzochten wij de hypothese dat MBI, ontstaan na de behandeling met chemotherapie met of zonder bestraling, een belangrijke onafhankelijke factor is bij het optreden van systemische ontstekings-reacties, gemeten door middel van het C-reactief proteïne (CRP), en bijdraagt aan het ontstaan van ontstekingscomplicaties na SCT. In *hoofdstuk 2* hebben we een retrospectief onderzoek verricht in een homogene groep van patiënten die een

autologe SCT ondergingen na conditionering met een hoge dosis melfalan. We vonden een opvallend patroon van systemische ontsteking en optreden van koorts die samenhing met het optreden van significante MBI van het maag-darmkanaal. Dit was vastgesteld door sequentiële metingen van citrulline, een aminozuur dat sterk correleert met de hoeveelheid aanwezige darmepitheelcellen. Deze samenhang werd zonder uitzonderingen gezien bij alle patiënten, ongeacht of er wel of geen bacteriëmie optrad. Met aanvullende statistische analyses konden we de relatie tussen darmschade en ontsteking bevestigen door aan te tonen dat citrulline de beste voorspeller was van het CRP beloop en de hoogte van het CRP. Het optreden van bacteriëmie droeg mogelijk wel bij aan de mate van ontstekings-reactie, maar leek een secundaire en minder belangrijke factor te zijn. Belangrijker nog, neutropenie bleek geen significante bijdragende rol te hebben bij het optreden van ontsteking na SCT. Dus we waren in staat om onze hypothese te bevestigen en stelden daarom voor om het paradigma 'febriele neutropenie' te verlaten en te vervangen door het nieuw paradigma 'febriele mucositis'.

In *hoofdstuk 3* hebben we het onderwerp, 'febriele mucositis', verder uitgewerkt door het natuurlijke beloop van MBI en ontsteking te bestuderen in nog eens vijf andere conditioneringregimes die gebruikt worden bij de voorbereiding voor een autologe of allogene SCT. Het meenemen van een zogenaamd niet-myeloablatief regime maakte de impact van MBI op ontsteking extra zichtbaar. Deze behandeling ging namelijk gepaard met slechts geringe MBI en een beperkte ontstekingsreactie, zonder het optreden van bacteriemie, ondanks een langdurige en diepe neutropenie. We vonden een sterke correlatie tussen de ernst van MBI en de hoogte van de systemische ontstekingsreactie. Bovendien konden we een duidelijke relatie leggen tussen het optreden van MBI en post SCT complicaties, zoals bacteriëmie, acute longschade en acute graft-versus-host disease (GvHD).

Wij zijn van mening dat onze gegevens het concept versterken dat MBI een centrale rol speelt in de trias van MBI, ontsteking en bacteriëmie. De mate van MBI bepaalt de ernst van de verstoring van de fysieke barrière, die vervolgens translocatie van zogenaamde microben-geassocieerde moleculaire patronen (MAMP) en darmbacteriën mogelijk maakt. Dit werd bevestigd door de waarneming dat patiënten met een bacteriemie aanzienlijk lagere citrulline waarden hadden dan degenen zonder bacteriëmie. Bovendien blijkt MBI de mate van de systemische ontstekingsreactie te beïnvloeden. Chemotherapie en radiotherapie induceren weefselschade en activeren het aangeboren immuunsysteem ('primen'). Het is deze 'priming' die uiteindelijk de ernst van de ontstekingsreactie bepaalt.

In het tweede deel van het proefschrift hebben we diverse retrospectieve analyses uitgevoerd naar de impact van enkel-nucleotide polymorfismen (single nucleotide polymorphisms (SNP)) van genen van het aangeboren immuunsysteem op de uitkomsten van een SCT. Dit is enerzijds gedaan om een deel van het inter-individu-

ele verschil in het optreden van complicaties te kunnen verklaren en anderzijds om het belang van eerder benoemde interacties tussen gastheer en micro-organismen te karakteriseren in de context van SCT. In *hoofdstuk 4* hebben we de invloed van polymorfismen in de intracellulaire patroon herkenningreceptor (PRR) NOD2 op de incidentie van ernstige acute GvHD en de 1-jaars therapiegerelateerde mortaliteit bevestigd, maar nu binnen de context van partieel T-cel gedepleteerde allogene SCT. Dit resultaat was in tegenspraak met de bevindingen van een andere studie, in een Spaans cohort, waarbij geen effect van deze SNPs gevonden werd in een T-cel gedepleteerde setting. De verschillen tussen de twee bestudeerde cohorten benadrukken het belang van de context in de beoordeling van de gevonden associaties tussen gen-polymorfismen en klinische uitkomstmaten. We concluderen dat in onze Nijmeegse klinische setting NOD2 polymorfismen wel kunnen worden gebruikt om de therapie voor patiënten die SCT ondergaan te optimaliseren. Zo kan bijvoorbeeld de selectie van donoren, de antimicrobiële profylaxe en het beleid bij acute GvHD worden toegespitst op basis van de NOD2 status van zowel de patiënt als de donor(en). Bovendien ondersteunt de invloed van NOD2 polymorfismen op de uitkomstmaten van een SCT het concept dat interacties tussen gastheer en micro-organismen ter plaatse van de epitheliale en mucosale barrières belangrijk zijn in de pathogenese van post-SCT complicaties.

In *hoofdstuk 5* hebben we een verband kunnen aantonen tussen toegenomen *Candida* kolonisatie bij SCT patiënten en een recent ontdekt Y238X polymorfisme in de C-type lectine PRR, dectin-1, die betrokken is bij de herkenning van β-1,3-glucan. De toegenomen kolonisatie zou waarschijnlijk hebben geresulteerd in een verhoogde incidentie van candidemie tijdens de fase van neutropenie en MBI, ware het niet dat bij vaststelling van *Candida* kolonisatie gestart werd met behandeling met oraal fluconazol.

De beide studies werden verricht in relatief kleine cohorten met daardoor enige beperkingen in de multivariate analyse. Niettemin achten wij de kans reëel dat de door ons gevonden genetische associaties daadwerkelijk bestaan en klinisch relevant zijn. De associaties tussen NOD2 en SCT uitkomsten zijn al aangetoond in vergelijkbare cohorten met patiënten die getransplanteerd werden met een verwante donor. Verder is de zogenaamde biologische plausibiliteit van de associaties hoog. Zo zijn de biologische functies bij processen, zoals ontsteking en infectie van zowel NOD2 als dectin-1, uitgebreid gekarakteriseerd en zijn de functionele gevolgen van de bestudeerde polymorfismen, beiden hebben een verlies van functie ('loss-of-function') tot gevolg, geassocieerd met ontregeling van deze processen.

Interessant is dat wij bij de analyse van SNP Y238X vaststelden dat *Candida* kolonisatie de incidentie van acute GvHD leek te verhogen, zoals beschreven in *hoofdstuk 6*. Achteraf is dit ook niet een onverwachte bevinding aangezien al veel

langer de rol van Gram-negatieve bacteriën bij het ontstaan van acute GvHD bekend is en aangetoond is dat antimicrobiële profylaxe tijdens neutropenie de incidentie van acute GvHD vermindert. We concludeerden dat de aanwezigheid van *Candida* soorten op het slijmvlies tijdens MBI en neutropenie ook kan bijdragen aan de pathogenese van acute GvHD, waarschijnlijk door het activeren van receptoren die betrokken zijn bij de herkenning van gisten en schimmels. Op basis van onze bevindingen speculeren wij dat mogelijk de nieuw ontdekte interleukine (IL)-23/T helper lymfocyt (Th)17-as het gemeenschappelijke verband kan zijn tussen *Candida* kolonisatie en acute GvHD. Th17 reacties zijn betrokken bij mucosale immuniteit gericht tegen verschillende micro-organismen, inclusief *Candida*, en zij spelen ook een rol bij het ontstaan van auto-immuun ziekten, waaronder de ziekte van Crohn. Bovendien is er een rol voor Th17 gesuggereerd bij het ontstaan van acute GvHD in dier studies, alhoewel tot nu toe de gegevens niet eenduidig zijn over de exacte impact, onder andere in verhouding tot Th1 reacties. Bovendien is het verband tussen *Candida* kolonisatie en Th1/Th17 ook al eens gesuggereerd bij de ziekte van Crohn, een ziekte waarbij, net als bij GvHD, verstoorde interacties tussen gastheer en micro-organismen een grote rol spelen in de pathogenese. Daarom moet de trias van Candida, Th1/Th17 en GvHD verder onderzocht worden. Indien dit bevestigd wordt, heeft dit klinisch consequenties. Fluconazol profylaxe voor alle allogene SCT patiënten zou dan opnieuw overwogen moeten worden, tijdens de SCT en het eerste jaar erna, niet alleen om systemische *Candida* infecties te voorkomen, maar ook om de incidentie en ernst van acute GvHD te verminderen. Bovendien, als Th17 daadwerkelijk blijkt bij te dragen aan de pathogenese van acute GvHD, dan opent dit nieuwe wegen voor de behandeling van deze complicatie.

In het derde deel van dit proefschrift concentreerden we ons op nieuwe therapeutische opties voor vroege SCT complicaties. Daarbij imiteerden we het natuurlijke aangeboren immuunsysteem. In de natuur komen namelijk veel antimicrobiële peptiden (AMP) voor met een breed scala aan functies waartegen, door de eeuwen heen, klaarblijkelijk weinig resistentie is ontwikkeld door de micro-organismen. Vandaar dat deze peptiden geschikte kandidaten lijken te zijn bij de bestrijding van infecties, zeker ook bij patiënten met een immuunstoornis. In **hoofdstuk 7** beschrijven we de theoretische achtergrond voor het gebruik van humaan lactoferrine (hLF) en de daarvan afgeleide kationische peptiden bij de behandeling van SCT gerelateerde complicaties. De potentieel gunstige eigenschappen van hLF in SCT zijn divers en bestaan uit stimulering van de groei en herstel van epitheelcellen, breedspectrum antimicrobiële activiteit, ontstekings- remmende activiteit en immuunmodulatie. Deze eigenschappen zijn minder goed gedefinieerd voor de AMPs afgeleid van hLF, maar de meeste AMPs bevatten de essentiële domeinen van het moedermolecuul, die nodig zijn voor microbiële interacties en binding aan hLF receptoren. Een van deze AMPs, is het humaan

lactoferrine 1-11 (hLF1-11), ontwikkeld door het farmaceutische bedrijf AM-Pharma BV (Bunnik, Nederland). Uit preklinisch onderzoek is antimicrobiële activiteit gebleken tegen ziekteverwekkers, die betrokken zijn bij infecties die veel voorkomen bij SCT patiënten. In diverse *in vivo* dierstudies is de antimicrobiële effectiviteit van hLF1-11 getest en bevestigd, zelfs bij neutropene en immuungecompromitteerde dieren en bij opvallend lage concentraties (0,4 μg/kg). hLF1-11, oplopend tot dagelijkse doseringen van 10 mg/kg, werd goed verdragen door de proefdieren, zonder noemenswaardige bijwerkingen. Deze resultaten hebben de weg vrijgemaakt voor de verdere ontwikkeling van hLF1-11 voor humaan gebruik.

In *hoofdstuk 8* rapporteren wij de eerste data van hLF1-11 toegepast bij mensen. Dit betrof in eerste instantie veiligheidsstudies in gezonde vrijwilligers. hLF1-11, intraveneus toegediend met enkelvoudige en meervoudige doseringen tot 5 mg, werd goed verdragen en veilig bevonden in gezonde vrijwilligers. Deze gegevens werden aangevuld met gegevens uit onze eerste klinische studie naar de veiligheid van een eenmalige dosis van 5 mg intraveneus toegediend in 8 SCT patiënten. Het peptide werd goed verdragen en er traden geen ernstige bijwerkingen op die verband hielden met het studie-peptide. Een zorg die voortvloeide uit deze studies was het optreden van parenchymateuze levertestafwijkinen (verhoogd ASAT en ALAT) na hLF1-11, bij zowel gezonde vrijwilligers als ook bij één patiënt uit onze klinische trial. Deze levertestafwijkingen waren voorbijgaand, volledig omkeerbaar, mild van aard en hadden voor zover te beoordelen geen duidelijke klinische consequentie. Het gelijktijdig gebruik van andere potentieel hepatotoxische geneesmiddelen verklaart hoogstwaarschijnlijk het ontstaan van de levertestafwij-kingen bij de patiënt uit de klinische studie. Toch heeft een onafhankelijke veilig-heidscommissie de opdracht gegeven de dosis hLF1-11 te verlagen naar 0,5 mg bij de vervolgstudie met meervoudige doses bij SCT patiënten. Deze geplande studie werd helaas geannuleerd op het moment dat het bedrijf besloot af te zien van verdere ontwikkeling van hLF1-11 voor humaan gebruik.

De hoge effectiviteit van hLF1-11 in diermodellen heeft geleidt tot de suggestie dat het peptide additionele immuunstimulerende eigenschappen bezit naast de directe antimicrobiële activiteit. Dit roept vragen op betreffende de veiligheid van dit peptide bij SCT patiënten, omdat tijdens een SCT vele immunologische processen zoals alloreactieve T-cel reacties (GvHD, graft-versus-leukemia) en immuunrecon-stitutie een rol spelen en beïnvloed kunnen worden door dit peptide. Daarom beschrijven we in *hoofdstuk 9* de aanvullende *in vitro* experimenten die uitgevoerd zijn met T-lymfocyten, natural killer (NK) cellen en dendritische cellen (DC), belangrijke spelers in de transplantatie-immunologie. Er traden geen onverwachte immuunreacties op na blootstelling van deze cellen aan hLF1-11. Gezien het feit dat gegevens over de *in vivo* effecten van hLF1-11 nog onbekend zijn moet voorzichtig-heid worden betracht in toekomstige studies bij patiënten. De waargenomen

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remmende effecten van hLF1-11 op DC maturatie en cytokine productie (IL-17, IFNγ) door CD4+ T-cellen suggereren mogelijk additionele activiteiten van hLF1-11 die van belang kunnen zijn bij de behandeling van specifieke SCT gerelateerde complicaties, zoals acute GvHD.
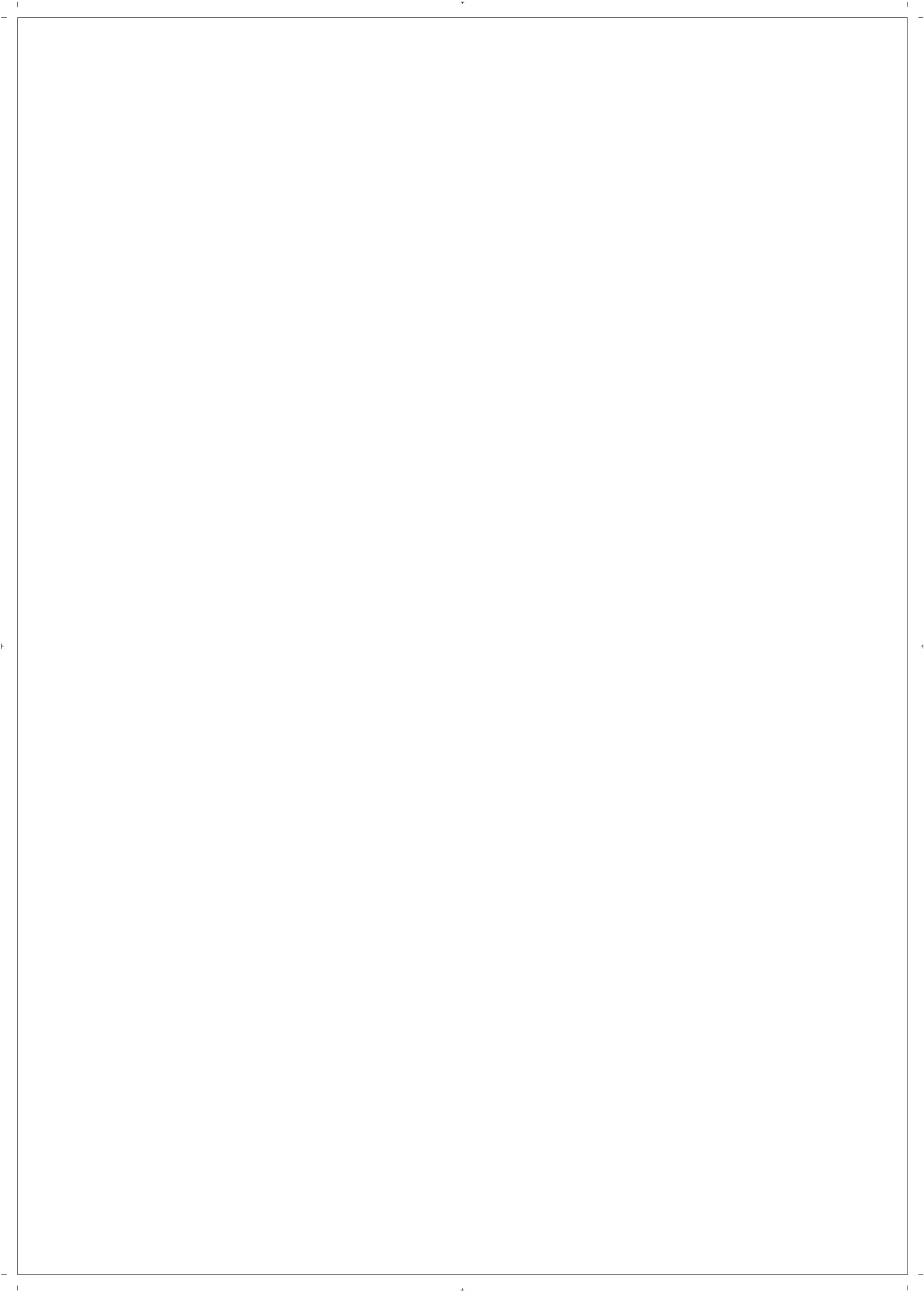
Toekomstperspectief

Het aangeboren immuunsysteem van de slijmvliezen van mond en maag-darmkanaal speelt een belangrijke rol bij de door cytotoxische therapie veroorzaakte MBI en de daaruit volgende ontstekingscomplicaties, zoals acute longschade en GvHD. Deze complicaties hebben een grote invloed op de uiteindelijke uitkomst van een SCT. Verdere studies zijn dan ook nodig om meer inzicht te krijgen in de complexe processen die betrokken zijn bij de mucosale immunologie, zodat nieuwe preventieve en therapeutische strategieën ontwikkeld kunnen worden, waardoor de morbiditeit en mortaliteit na een SCT verminderd kan worden. Omdat de systemische ontstekingsreactie, die gezien wordt na de conditionering voor een SCT, weinig relatie heeft met de neutropenie en geassocieerde infecties en meer het gevolg blijkt te zijn van MBI moet er een verschuiving plaatsvinden in de focus naar de mucosale barrière en het aangeboren immuunsysteem. Inzichten, enerzijds in de interacties tussen PRRs en MAMPs en gevaar-geassocieerde moleculaire patronen (danger-associated molecular patterns; DAMPs) en anderzijds in de antimicrobiële en immunomodulerende eigenschappen van natuurlijke kationische AMPs, kunnen zorgen voor nieuwe invalshoeken bij de preventie en behandeling van de ontregelde ontstekingsreacties en complicaties die optreden na een SCT.

Toekomstig onderzoek moet zich richten op vraagstukken betreffende de pathogenese, preventie en behandeling van MBI bij SCT patiënten. Er is een grote behoefte om te weten wat er allemaal gebeurt ter plaatse van de mucosale barrières van de mond en darm in de vroege fase na cytotoxische therapie. Welke cellen zijn nog aanwezig na de conditionering en betrokken bij MBI en de ontstekingsreactie tijdens SCT; macrofagen, epitheelcellen, fibroblasten of endotheelcellen? Om dit te bepalen zal weefselonderzoek van de mucosa tijdens mucositis onontbeerlijk zijn. Verder moet de precieze rol van de diverse MAMPs en DAMPs worden onderzocht, alsook de drempel voor translocatie bepaald worden. Uiteindelijk blijft de belangrijkste vraag hoe wij MBI en de daar op volgende ontstekingsreacties en complicaties, zoals acute GvHD, kunnen voorkomen en behandelen, bijvoorbeeld door het toepassen van AMPs of door beïnvloeding van de activatie status van PRRs met agonisten of antagonisten.

De rol van citrulline als een biomarker van chemo- en radiotherapie geïnduceerde darmschade moet nauwkeuriger worden vastgesteld. Prospectieve studies zijn

uiteindelijk nodig om de waarde van citrulline te bepalen voor de individuele patiënt bij het voorspellen van MBI en de daaraan gerelateerde complicaties zoals bacteriëmie en GvHD. In de tussentijd kan men met het meten van citrulline en CRP in principe alle conditioneringregimes in kaart brengen en graderen naar de impact op MBI en ontsteking. Die informatie kan gebruikt worden in de dagelijkse klinische SCT praktijk bij de keuzes die gemaakt worden betreffende het gebruik van antimicrobiële profylaxe, noodzaak voor opname in het ziekenhuis en totale parenterale voeding en het gebruik van preventieve en therapeutische ontstekings-remmende therapieën.

De waarde van het screenen op SNPs moet voor de klinische setting van SCT nog worden vastgesteld, maar lijkt veelbelovend. Bij het bestuderen en ontdekken van genen van belang in de toekomst zijn er twee algemene benaderingen mogelijk; enerzijds de genoomwijde associatie studies en anderzijds de meer stapsgewijze benadering gericht op bekende pathogenetische mechanismen. Het onderzoeken van afzonderlijke genen of volledige signalering pathways betrokken bij de antimicrobiële peptiden, cytokine productie, autofagie, PRR activering en signalering zou een aantrekkelijke aanpak in SCT kunnen zijn. Het eerste doel daarbij is om het inzicht te vergroten in de genen die bijdragen aan het ontstaan van ontstekingscomplicaties zoals GvHD, maar het uiteindelijke doel is het vinden van genetische associaties die de weg vrijmaken voor preventieve of therapeutische interventies. De volgende stap zou dan het ontwerpen van prospectieve studies moeten zijn naar de klinische toepasbaarheid van de SNPs die geassocieerd zijn met SCT uitkomsten. Echter, bij het bestuderen van de rol van deze SNPs in de context van SCT zijn grotere en meer homogene cohorten van patiënten nodig. Daarom kunnen deze studies alleen verricht worden indien nog beter samengewerkt wordt binnen de nationale en internationale consortia.

Dankwoord

Promoveren is een groepsgebeuren. Een groot aantal mensen heeft dan ook bijgedragen aan het tot stand komen van dit proefschrift. Ik prijs mijzelf gelukkig met alle hulp en steun en wil iedereen daarvoor hartelijk bedanken.

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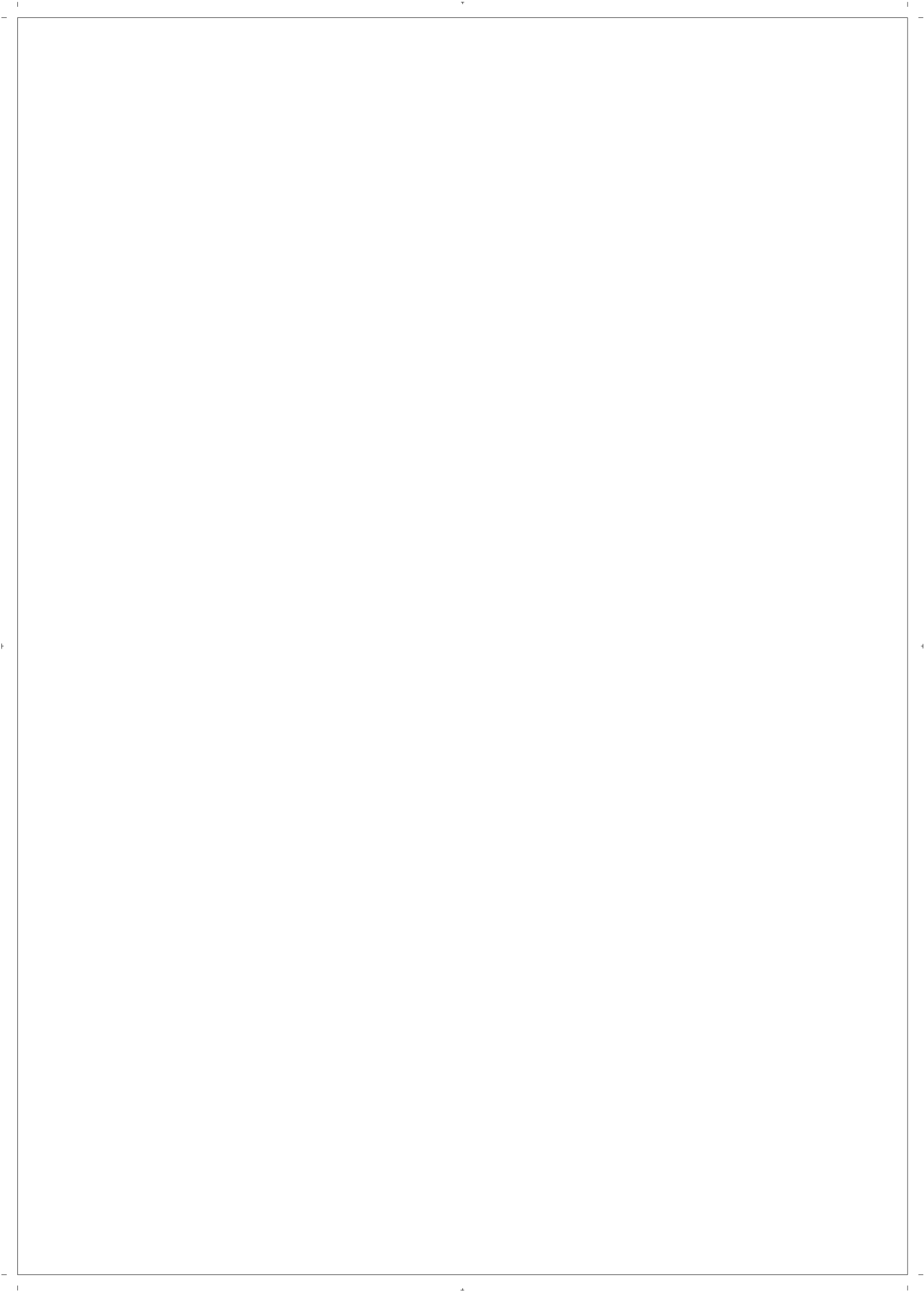
Abbreviations

ALI	Acute lung injury
ALL	Acute lymphoblastic leukemia
ALT	Alanine aminotransferase
AML	Acute myeloid leukemia
AMP	Antimicrobial peptide
ANC	Absolute neutrophil count
APC	Antigen presenting cell
ARDS	Acute respiratory distress syndrome
AST	Aspartate aminotransferase
ATG	Antithymocyte globulin
AUC	Area under the curve
BEAM	BCNU, etoposide, cytarabine, melphalan
BMI	Body mass index
BOOP	Bronchiolitis obliterans organizing pneumonia
BPI	Bactericidal/permeability-increasing protein
Bus	Busulphan
CD	Cluster of differentiation
CLL	Chronic lymphocytic leukemia
CLR	C-type lectin receptor
CML	Chronic myeloid leukemia
CoNS	Coagulase-negative staphylococcus
CpG	C-phosphate-G
CRP	C-reactive protein
CsA	Cyclosporin A
CTC	Common toxicity criteria
Cyclo	Cyclophosphamide
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DFS	Disease-free survival
DLI	Donor lymphocyte infusion
EDGE	Environmentally determined genetic expression
ELISA	Enzyme-linked immunosorbent assay
Flu	Fludarabine
G-CSF	Granulocyte-colony stimulating factor
GvHD	Graft-versus-host disease
GvL	Graft-versus-leukemia
GWAS	Genome-wide association study
hBD2	Human beta defensin 2

HD-5	Alfa defensin 5
HD-6	Alfa defensin 6
HDM	High dose melphalan
HET	Heterozygote
hLF	Human lactoferrin
hLF1-11	Human lactoferrin 1-11
HLA	Human leukocyte antigen
HOM	Homozygote
IBD	Inflammatory bowel disease
Ida	Idarubicin
IDR	Innate defense regulator
IEC	Intestinal epithelial cell
IFNγ	Interferon gamma
IL	Interleukin
IPS	Idiopathic pneumonia syndrome
KIR	Killer cell immunoglobulin-like receptor
LfR	Lactoferrin receptor
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MA	Myeloablative
MAMP	Microbe-associated molecular pattern
MBI	Mucosal barrier injury
MBL	Mannose-binding lectin
MDP	Muramyl dipeptide
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MiHag	Minor histocompatibility antigen
MM	Multiple myeloma
NF-κB	Nuclear factor kappa B
NHL	Non-Hodgkin lymphoma
NK	Natural killer cell
NLR	Nod-like receptor
NMA	Non-myeloablative
NNMSS	Nijmegen Nursing Mucositis Scoring System
NOD2	Nucleotide-binding oligomerization domain containing protein 2
NRM	Non-relapse mortality
OS	Overall survival
OVS	Oral viridans streptococcus
PAMP	Pathogen-associated molecular pattern
PMN	Polymorphonuclear neutrophil

PMBC	Peripheral blood mononuclear cell
PRR	Pattern recognition receptor
RLR	RIG-like receptor
ROS	Reactive oxygen species
RRM	Relapse-related mortality
SAE	Serious adverse event
(H)SCT	(Hematopoietic) stem cell transplantation
SIRS	Systemic inflammatory response syndrome
SNP	Single nucleotide polymorphism
TBI	Total body irradiation
Th	T helper lymphocyte
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alfa
Treg	Regulatory T cell
TRM	Treatment-related mortality
ULN	Upper limited of normal
WT	Wild-type
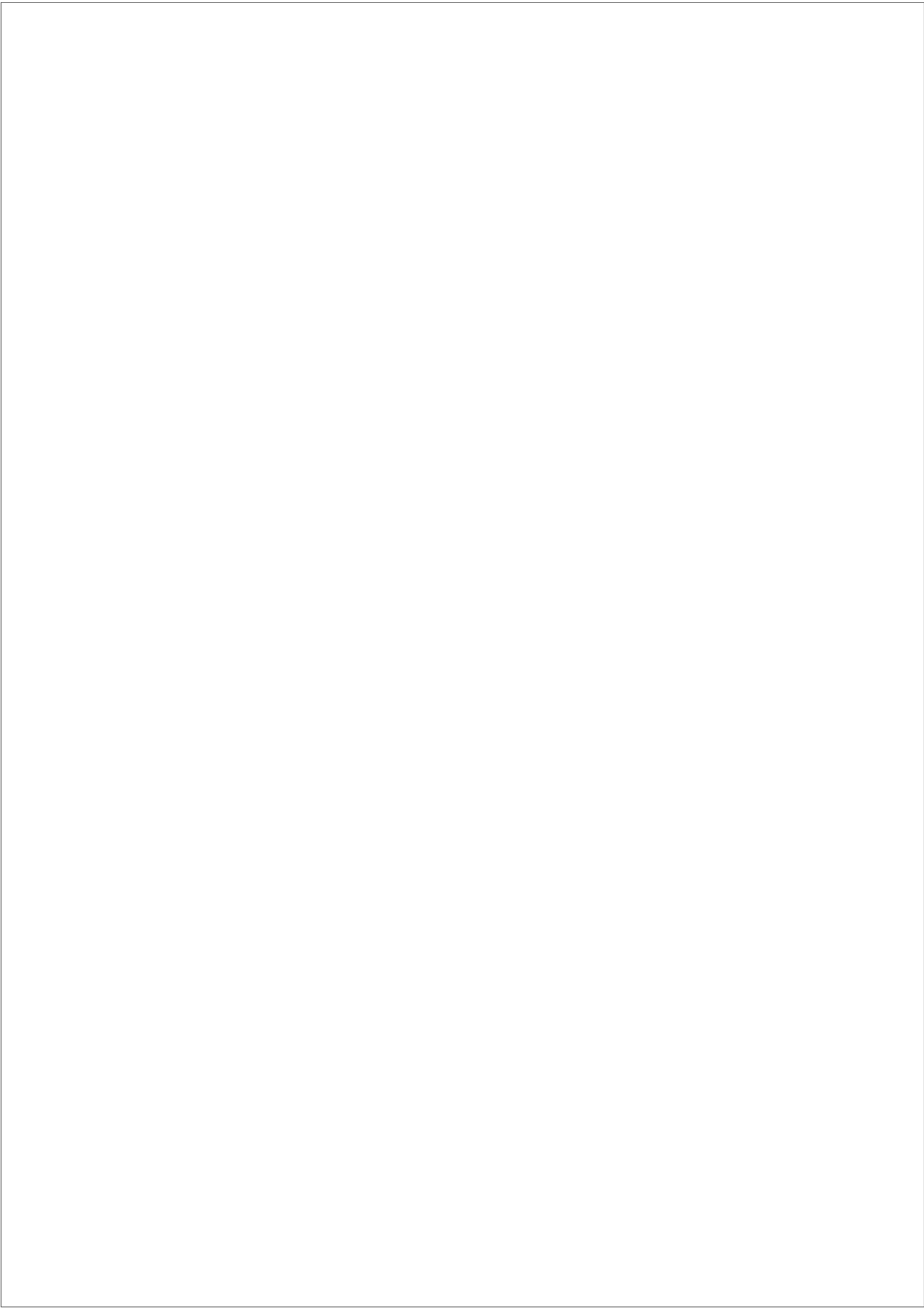
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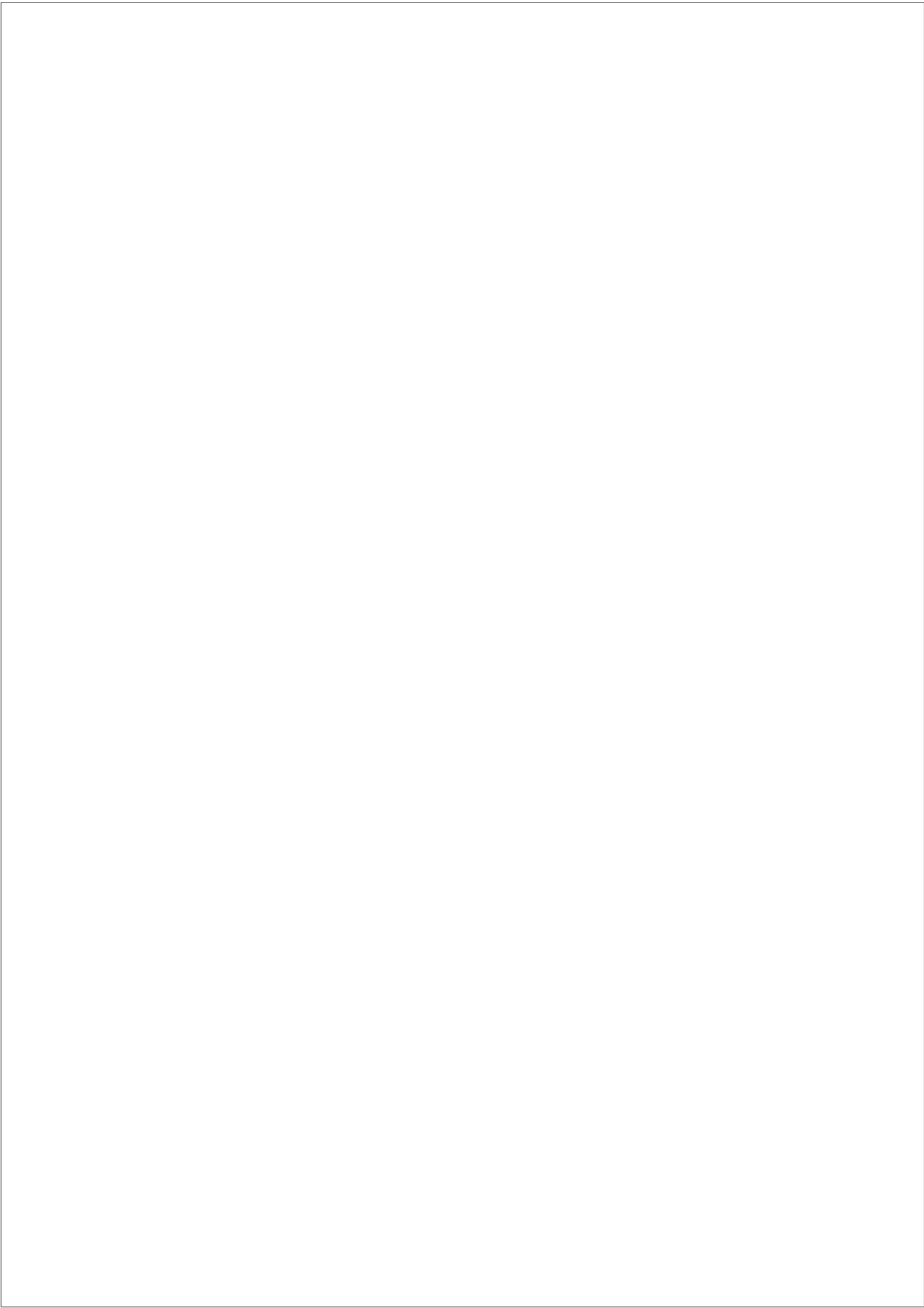
Curriculum Vitae

Walter van der Velden werd op 17 mei 1975 geboren in Oss. Na het behalen van zijn VWO diploma (cum laude) aan het Rivendell, later Comenius, College te Uden (1993) ging hij in Nijmegen aan de Radboud Universiteit geneeskunde studeren. In 1997 haalde hij zijn doctoraal examen en in 1999 zijn arts examen. Begin 2000 begon hij als arts niet in opleiding op de afdeling interne geneeskunde van het Canisius Wilhelmina Ziekenhuis in Nijmegen. Aldaar begon in 2001 zijn opleiding interne geneeskunde (opleiders Dr. R. de Koning en Dr. A.S.M. Dofferhoff). Vanaf april 2004 werd de opleiding voorgezet in het Universitair Medisch Centrum St Radboud (opleiders Prof. dr. P.M.J. Stuyt, dr. J. de Graaf en Prof. dr. J.W.M. van der Meer). In juli 2006 rondde hij zijn opleiding af en is hij geregistreerd als algemeen internist.

Na zijn opleiding interne geneeskunde is hij in juli 2006 gaan werken op de afdeling hematologie waar ook de opleiding tot hematoloog plaatsvond (opleider Prof. T.J.M. de Witte). Tegelijkertijd startte hij met zijn promotieonderzoek bij Prof. T.J.M. de Witte, dr. N.M.A. Blijlevens en dr. J.P. Donnelly resulterende in meerdere publicaties die vermeld zijn in dit proefschrift. In november 2009 is hij geregistreerd als hematoloog. Sindsdien werkt hij als staflid op de afdeling hematologie van het Universitair Medisch Centrum St Radboud. Zijn aandachtsveld is de supportieve zorg in de intensieve hemato-oncologie en de (mucosale) immunologie in het kader van stamceltransplantatie.