A study on immunomodulating beta-glucan: Effects of oral application on inflammation, tissue injury, and the mucosal immune system in experimental animals

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Abbreviations

Ab(s)	Antibody(ies)
AB/PAS	Alcian blue / periodic acid-Schiff
Ag(s)	Antigen(s)
ALAT	Alanine aminotransferase
ANOVA	Analysis of variance
APC(s)	Antigen presenting cell(s)
ASAT	Aspartate aminotransferase
BRM(s)	Biological response modifier(s)
CARD	Caspase recruitment domain
CD	Crohn's disease
CLP	Cecal ligation and puncture
CR	Complement receptor
CV	Animals with conventional microbial flora
DAMP(s)	Damage-associated molecular pattern(s)
DC(s)	Dendritic cell(s)
DSS	Dextran sulphate sodium
FAE	Follicle-associated epithelium
FcRn	Neonatal Fc receptor
GALT	Gut-associated lymphoid tissue
GF	Germ-free
H&E	Hematoxylin and eosin
IBD	Inflammatory bowel disease
IEC(s)	Intestinal epithelial cell(s)
IEL(s)	Intra-epithelial lymphocyte(s)
IFN	Interferon
Ig(s)	Immunoglobulin(s)
IL	Interleukin
ILF(s)	Isolated lymphoid follicle(s)
I/R	Ischemia-reperfusion
ITAM	Immunoreceptor tyrosine-based activation motif
KO	Knockout
LN(s)	Lymph node(s)
LP	Lamina propria
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MAMP(s)	Microbe-associated molecular pattern(s)
MAP	Mean arterial blood pressure
MHC	Major histocompatibility complex
MLN(s)	Mesenteric lymph node(s)
MODS	Multiple organ dysfunction syndrome
NF	Nuclear factor
NK	Natural killer
PAMP(s)	Pathogen-associated molecular pattern(s)
PBMC(s)	Peripheral blood mononuclear cell(s)
PC(s)	Plasma cell(s)
pIgR	Polymeric immunoglobulin receptor
PP(s)	Peyer's patch(es)
PRR(s)	Pattern recognition receptor(s)
RA	Retinoic acid
SAb(s)	Secretory Ab(s)
SBG	Soluble beta-glucan
SC	Secretory component
SED	Subepithelial dome
SIgA	Secretory IgA
SIgM	Secretory IgM
Syk	Spleen tyrosine kinase
TCR(s)	T cell receptor(s)
TGF	Transforming growth factor
Th	T helper cell
TLR(s)	Toll-like receptors
TNF	Tumor necrosis factor
$T_{reg(s)}$	Regulatory T cell(s)
UC	Ulcerative colitis

Papers included

The presented thesis is based on the following papers:

I. **ORAL AND SYSTEMIC ADMINISTRATION OF β-GLUCAN PROTECTS AGAINST LIPOPOLYSACCHARIDE-INDUCED SHOCK AND ORGAN INJURY IN RATS**

Sandvik A., Wang Y.Y., Morton H.C., Aasen A.O., Wang J.E. and Johansen F-E.

Clin Exp Immunol. 2007, 148: 168-177. Erratum in: *Clin Exp Immunol*. 2007, 149: 399.

II. **SOLUBLE β-GLUCAN PROTECTS AGAINST EXPERIMENTAL ULCERATIVE COLITIS**

Sandvik A., Grzyb K., Reikvam D.H., Erofeev A., Jahnsen F.L. and Johansen F-E.

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III. **EFFECTS OF ORAL ADMINISTRATION OF SOLUBLE β-GLUCAN ON THE GUT AND GUT-ASSOCIATED LYMPHOID TISSUE IN MICE**

Sandvik A., Bækkevold E.S., Jahnsen F.L. and Johansen F-E.

Manuscript, 2008.

1 Introduction

1.1 The immune system – an overview

The immune system has evolved primarily to protect the host against invading pathogens including viruses, bacteria, yeasts, protozoa and multi-cellular parasites. Ever-present microorganisms and parasites are relentlessly trying to make a meal or breading ground out of a suitable host organism. To enhance the odds of survival and consequently the prospect to reproduce, complex defense systems and counter measures have co-evolved in an evolutionary arms-race between invaders and host organisms.

The immune system is divided into the innate and the adaptive immune systems. The innate system is phylogenetically conserved and components of this system can be found in practically all multi-cellular organisms, whereas an adaptive immune system is found in vertebrates only (1). Characteristics of the mammalian, and particularly the human, immune system will be discussed briefly in this section.

Epithelial cells, lining the skin and the mucosal surfaces provide a physical barrier segregating the tightly regulated internal compartment of the host and the external milieu. Infections occur when invading pathogens succeed in colonizing the epithelium and breach the barrier. Some intestinal pathogens can cause an infection without breaching the barrier, but these typically secrete toxins or interact directly with the epithelial cells. Located immediately behind the first line of defense, awaiting intruders, are key players of the cellular innate immune system; namely the phagocytic macrophages. Activated macrophages release cytokines and other mediators ultimately leading to inflammation which involves recruitment of neutrophils and plasma proteins to the site of infection to facilitate elimination of the pathogen.

A fundamental feature of the immune system is its ability to discriminate between foreign structures originating from invading organisms and innocuous environmental factors or self antigens (Ags). Cells of the innate immune system rely on a limited repertoire of promiscuous germ-line encoded pattern recognition receptors (PRRs) that recognize conserved pathogen-associated molecular patterns (PAMPS), or more generally microbe-associated molecular patterns (MAMPS) to make this discrimination (2;3). Importantly, the immune system also sense endogenous alarm or danger signals from infected or damaged host tissues, many of which signal though the same receptors as do MAMPS. The danger model (4;5) put forward the idea that the governing signals of immune reactions are solely endogenous signals emanating from stressed or injured tissues. Poly Matzinger, the mother of the danger model, recently suggested that the innate immune system evolved to detect damage-associated molecular patterns (DAMPS); "*Any molecule that is not normally exposed can be a DAMP if it is revealed during, after, or because of injury or damage*" (6).

The innate defense system is fully functional immediately following recognition of infection or tissue damage. In addition to providing a crucial first line of defense, usually sufficient to fight off most pathogens on its own, the innate immune system alarms and recruits the adaptive immune system if required. The hallmark of adaptive immunity is the ability to tailor an Ag-specific response. Specific defense is based primarily on the presence of immunoglobulins (Igs) and T cell receptors (TCRs) on B- and T-lymphocytes, respectively. Each lymphocyte, or clone thereof, expresses a unique Ig or TCR generating an enormous pool of specificities with the potential to recognize practically any Ag. In order to activate the adaptive immune system, Ags must be internalized and processed by antigen-presenting cells (APCs), i.e. dendritic cells (DCs), macrophages and B cells that migrate to regional lymph nodes (LN). Here, the Ag is presented to T cells in the context of a peptide - major histocompatibility complex (MHC) class I or II molecule complex. The adaptive

immune response is characterized by clonal expansion of activated lymphocytes, generation of B cells (plasma cells) producing Ag-specific antibodies (Abs), generation of $CD4^+$ T helper (Th) cells (of critical importance for plasma cell development and Ab production) and generation of cytotoxic $CD8^+$ T cells. In contrast to the innate defense, the Ag-specific adaptive defense mechanisms needs time to get operational. Another key feature of the adaptive immune system is immunological memory. A pool of Ag-specific lymphocytes is maintained after an infection is cleared, allowing faster and more robust recall responses upon re-challenge with the same pathogen at a later time.

This oversimplified overview of the intimately interacting innate- and adaptive immune systems only serves as a brief introduction to selected terms and mechanisms discussed in this thesis. The complex and fascinating biology of the immune system has been thoroughly reviewed elsewhere (7-11) and detailed expertly in several textbooks (12;13).

1.2 Mucosal defense – selective border control

The mucosal surfaces of the gut, airways, urogenital tracts, and ducts of exocrine glands are lined by a monolayer of epithelial cells that form crucial barriers. The vast and vulnerable surface of the human mucosa, estimated to be approximately 200-times larger than that of the skin, is the major port of entry for microorganisms and exogenous materials (14). The mucosae of the airways and gut are faced with a paradoxical challenge, namely the task of allowing gas exchange and nutrient uptake, respectively, and at the same time selectively exclude entry of harmful agents such as pathogens and toxins. The task appears particularly challenging for the gut mucosa. The complex microflora of the human intestine, estimated to contain more than 1000 bacterial species, comprise a total microbial load of 10^{13}-10^{14} microorganisms (15-17). Thus, the indigenous microbiota is a plentiful source of potentially

harmful organisms and biologically active products with potential to challenge the epithelial integrity and consequently the host's wellbeing.

An array of constitutive non-specific mucosal defence features, including production of mucus and anti-microbial peptides (e.g. defensins), intestinal peristalsis and respiratory ciliary movement, and constant epithelial shedding and renewal, contributes to Ag elimination and host resistance (18-20). Moreover, a specialized mucosal defence system has evolved to protect these vulnerable surfaces, handle the immense antigenic load and maintain homeostasis.

1.2.1 Inductive sites – sampling, priming and homing

The mucosal immune system is frequently divided into inductive sites and effector sites (Figure 1). The principle inductive site is organized mucosa-associated lymphoid tissue (MALT). Gut-associated lymphoid tissue (GALT) comprise Peyer's patches (PPs) of the small intestine, numerous scattered isolated lymphoid follicles (ILFs), predominantly in the distal gut, local and regional draining LN and the appendix. PPs and ILFs do not have afferent lymphatics. Instead, microfold (M) cells of follicle-associated epithelium (FAE) serve as entry portals for luminal Ags. The underlying subepithelial dome (SED) is populated by APCs that process and present Ags to naïve lymphocytes (reviewed in 21;22;23). In addition intestinal APCs, particularly DCs, may sample luminal contents directly by extending transepithelial protrusions (24). Stimulated lymphocytes drain to mesenteric LNs (MLNs), and subsequently enter the blood stream via the thoracic duct before they eventually migrate ("home") to the appropriate effector sites where terminal differentiation takes place (21-23). In general, primed immune cells preferentially home to effector sites corresponding to the inductive sites where they initially were activated by Ags. Homing to the gut is governed by tissue-specific imprinting and depends on specific

interactions between endothelial cells and leukocytes as well as soluble tissue-produced chemotactic cytokines (chemokines) (25;26).

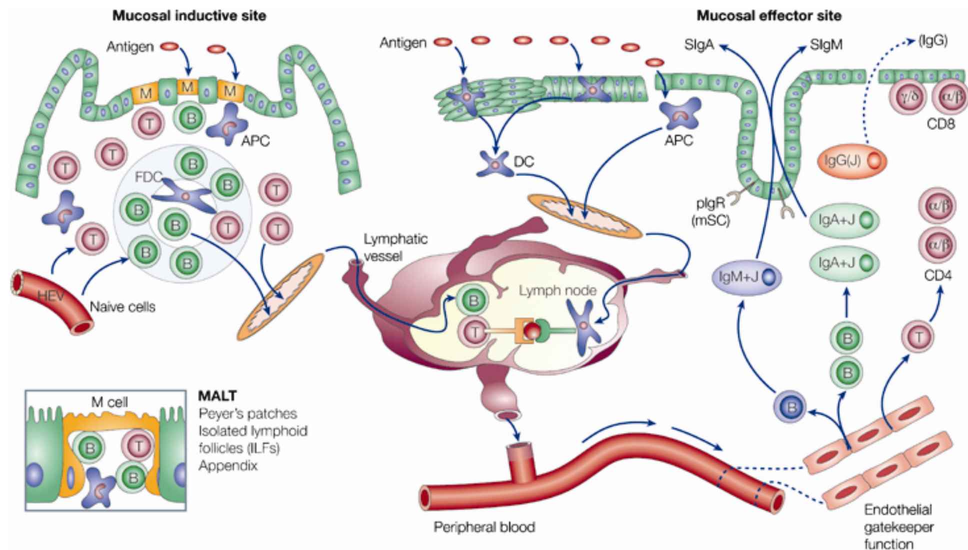

Figure 1. The mucosal immune system.
MALT are the inductive sites for mucosal immunity and are composed of B-cell follicles, T cell zones and FAE with M cells (detailed in insert) through which exogenous Ags are actively transported to reach APCs, including DCs, macrophages, B cells, and folicular DCs (FDCs) in the SED region. In addition, quiescent intra- or subepithelial DCs may capture Ags at effector sites and migrate via draining lymphatics to local/regional LNs where they stimulate T cells for productive or suppressive immune responses. Naïve B and T cells enter MALT (and LNs) via high endotelial venules (HEVs). Primed memory/effector B and T cells migrate from MALT and lymph nodes to peripheral blood for subsequent extravasation at mucosal effector sites. The gut LP contains abundant T cells (mainly $CD4^+$) and mostly IgA PCs with lower abundance of IgM and IgG PCs. J-chain expression in PCs mediates polymerization of IgA and IgM and is required for pIgR-mediated epithelial transport and generation of SIgA and SIgM. IgG is also transferred into the lumen paracellularly (broken arrow) or via neonatal Fc receptor (FcRn)-mediated transport (not depicted). The distribution of IELs (mainly T-cell receptor α/β^+CD8^+ and some γ/δ^+ T cells) is also depicted.

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1.2.2 Effector sites – secretory immunity and immune exclusion

A network of scattered immune cells throughout the lamina propria (LP) and the epithelium constitute the effector sites of the intestinal immune system (Figure 1). The epithelium is populated by intra epithelial lymphocytes (IELs), predominantly cytotoxic $CD8^+$ T cells,

whereas the LP harbors CD4$^+$ T cells, numerous APCs and vast numbers of Ab-secreting plasma cells (PCs) (reviewed in 28).

IgA$^+$ B cells, primed in PPs, home to the intestinal LP where they undergo terminal differentiation to IgA-producing PCs. The primary role of LP PCs is to produce mainly dimeric IgA complexed with J chain. The polymeric immunoglobulin receptor (pIgR) on secretory epithelial cells mediates transepithelial transport of this complex to the lumen of the intestine. The ectodomain of pIgR, known as secretory component (SC), is cleaved of coupled to its cargo at the apical surface to form secretory IgA (SIgA). pIgR also exports J chain-containing pentameric IgM to produce secretory IgM (SIgM), and cleaved, unoccupied pIgR is released to secretions as free SC (reviewed in 22). Secretory Abs (SAbs) perform multi-layer immune exclusion of exogenous Ags: (I) Coating of luminal microorganisms and their products reduces access to the epithelial surface and hence protects against invasion and overgrowth, (II) SAbs that encounter epithelial invaders en route to the lumen may neutralize these intracellularly, and (III) penetrating Ags in the LP may be shuttled back out to the lumen bound to polymeric Igs by pIgR-mediated export (22). The importance of the non-inflammatory, secretory immune system is illustrated by the striking fact that approximately 80% of all PCs reside in the LP of the gut, 90% of which are dimeric IgA producers (29). Mucosal secretions also contain IgG. Transepithelial transport of IgG is mediated by the neonatal Fc receptor (FcRn). FcRn also contribute to luminal Ag sampling by recycling IgG immune complexes across the intestinal epithelial barrier for processing by LP APCs (30;31).

1.3 Immune regulation – battle strategy and peace keeping

The immune system utilizes a broad arsenal of powerful and lethal “arms” to combat invaders, many of which may harm the host itself. It is of decisive importance for the

function of the immune defense, and thus the wellbeing of the host, that the "warfare" is tightly regulated. A well functioning immune system handles the following key challenges appropriately: What, when, where and how to attack? And just as important: When to "cease fire"?

1.3.1 Self tolerance and regulatory T cells

To prevent an attack on healthy self tissues, self-reactive T- and B lymphocytes are eliminated during development in the thymus and bone marrow, respectively. Despite the strict selection process, known as central tolerance, self-reactive T cells are found in the T-cell repertoire of healthy individuals. However, autoimmune disease is relatively rare, indicating that peripheral regulatory mechanisms suppress the potentially pathogenic effect of these cells (32;33). This regulatory effect is now largely ascribed to a subset of $CD4^+$ T cells, known as regulatory T cells (T_{regs}). T_{regs} are frequently divided into naturally occurring T_{regs} and adaptive/inducible T_{regs} which develop in the thymus or are induced in the periphery, respectively. Recently, multiple subpopulations of T_{regs} have been characterized. Although the expression pattern is not exclusive for T_{regs} or include all T_{regs}, T_{regs} are frequently characterized by surface expression of CD25 and high expression of the transcription factor FoxP3 (reviewed in 34).

T_{regs} are emerging as instrumental regulators of immune responses with a range of functions including suppression of immune cell proliferation and differentiation, suppression of effector T-cell cytokine production/release, inhibition of cytotoxic $CD8^+$ T cell degranulation and of B-cell maturation and Ab production. Accordingly, T_{regs} have the capacity to turn off immune responses following clearance of pathogens and hence, avoid chronic, pathogenic, immune activation. Regulation of target cells may be mediated directly by the T_{regs} or indirectly via T_{reg} modulation of Th cells and APCs (reviewed in 35).

Although the mechanisms of T_{reg} action remain debated, direct cell-cell contact, competition for growth factors and release of soluble factors have been suggested (reviewed in 36).

The most prominent mediators produced by T_{regs} include transforming growth factor (TGF)-β and interleukin (IL)-10. Belonging to a diverse class of soluble signaling components known as cytokines, TGFβ and IL-10 have pleiotropic effects on the immune system and are central to immune regulation and homeostasis (37). These regulatory cytokines contributes to tolerance to self Ags and harmless environmental Ags and control inflammatory responses by targeting both effector T cells (paracine effect) and T_{regs} (autocrine effect) (37).

Recently it has become evident that T_{regs} also interact with APCs. In general, APCs respond to T_{regs} by down-regulation of Ag-presentation function and concurrent expression of immunosuppressive surface molecules and cytokines. Conversely, APCs have an important effect on the peripheral pool of T_{regs} as they have been demonstrated to induce *de novo* generation of peripheral T_{regs} by conversion of naïve T cells (38). Thus the mutual interaction between APCs and T_{regs} is crucial for the maintenance of peripheral tolerance. T_{reg} conversion by mucosal DCs is discussed in some detail below (section 1.3.2).

1.3.2 Oral tolerance and ignorance to commensal bacteria

Priming of lymphocytes in the intestinal mucosa may lead to three major immunological events: (I) local SIgA production, (II) priming of systemic immunity, both of which produce a protective responses to pathogens, and importantly (III) induction of immunological tolerance. Tolerance induction to harmless dietary Ags and the normal harmless microflora (commensal bacteria) is a fundamental event in gut immune regulation. Oral tolerance, defined as suppression of an Ag-specific immune reaction by prior oral administration of the same Ag, preserve systemic hyporesponsiveness to the ever present food proteins and

commensal flora. While the precise mechanisms remain elusive, it is generally accepted that the orally induced hyporesponsiveness involves T cell deletion, anergy and/or the induction of T_{reg} (reviewed in 39;40).

Although systemic tolerization may take place in peripheral LNs, the key site for induction of oral tolerance is the MLNs (41;42). Tolerization in the MLNs relies on Ag-loaded DCs migrating from the LP and PPs (42). How then do the tolerogenic DCs communicate that they carry innocuous Ags rather than Ags of pathogenic origin to the MLN T cells? Accumulating evidence supports the idea that resident mucosal DCs are quiescent, possibly due to conditioning in a suppressive environment shaped by stromal cells. Furthermore, activation and Ag-processing in the absence of inflammation or danger signals results in Ag presentation in the context of low levels of costimulatory molecules and concomitant production of immunomodulating cytokines, eventually causing tolerance induction rather than productive immunity (reviewed in 43;44).

Recent data demonstrates that GALT DCs express retinaldehyde dehydrogenases (RALDH) enabling them to metabolize vitamin A in the diet to retinoic acid (RA). RA has emerged as a pivotal mediator of TGFβ-dependendt conversion of T cells into $FoxP3^+$ T_{regs} (45-48). RA-signaling is also essential for gut homing imprinting, preferentially to the small intestine, and is implicated in IgA class switching. Thus, mucosal DCs orchestrate the mucosal immune response and homeostasis in part via RA (reviewed in 25). The mechanisms by which the mucosal DCs themselves are “coached” are only partly understood, but involve factors present in the local environment, including intestinal epithelial cells (IEC), IEC-derived thymic stromal lymphopoietin (TSLP) and RA, bacterial products, IL-10, TGFβ and peroxisome-proliferative-activated receptor (PPAR)γ-ligands (reviewed in 49).

In healthy individuals the systemic compartment of the immune system is unresponsive to the large and complex commensal microflora present in the intestine. Interestingly, the same bacteria may easily prime systemic immunity when administered intravenously. This compartmentalized organization of the immune system facilitate production of a strong, non-inflammatory, mucosal response without the requirement of establishing systemic tolerance which would in turn suppress the ability to respond to invading commensals (e.g. opportunistic pathogens) or closely related pathogens causing sepsis (41). MLNs are essential in preserving systemic ignorance to commensal microorganisms. DCs that have sampled commensals from the intestine primarily induce mucosal IgA responses in the PP. Importantly, DCs also carry commensals to the MLNs. Commensal-laden DCs do not leave the MLN via efferent lymphatics and thus live commensals do not gain access to systemic circulation (50). Hence, the MLNs function as a firewall that eliminates constant penetration and systemic priming by intestinal microbes "astray" (41).

1.3.3 Immunopathology

Inappropriate immune reactions may cause severe immunopathology in genetically predisposed individuals. A dysregulated immune system may potentially cause hypersensitivity, autoimmunity, immunodeficiency or exaggerated host responses, all of which are associated with morbidity and mortality. In the case of hypersensitivity, loss of tolerance induction or break of tolerance cause adverse immune reactions to innocuous environmental Ags such as dietary- or inhaled Ags evident in food allergy and allergic asthma, respectively. Inappropriate immunologic ignorance on the other hand, may lead to functional immunodeficiency with increased risk of infections. In autoimmune diseases, including multiple sclerosis, rheumatoid arthritis and type I diabetes, the immune system

direct an assault to self Ags, causing persistent inflammation and associated tissue- and organ injury.

In sepsis, a systemic host response to infection, the release of potent mediators of inflammation may result in an exaggerated pathogenic response in the circulation and vital organs (51). An experimental model of endotoxic shock, which mimics the dysregulated systemic inflammation seen in severe sepsis and associated organ failure, has been employed in this thesis (Paper I).

In inflammatory bowel disease (IBD), epithelial barrier dysfunction and abrogated systemic ignorance to commensal bacteria contributes to chronic inflammation in the gut (17). An animal model of IBD has been employed in this thesis (Paper II). Sepsis, IBD and the applied animal models are discussed in some detail below (section 1.5) and in Paper I and II, respectively.

1.4 Beta-glucans – immunomodulating polysaccharides

Medicinal bioprospecting, the search for natural products useful in preventing or treating disease, has proven to be a valuable strategy in the discovery and development of novel drugs. Celebrated examples include antibiotics and immunosuppressive drugs isolated from fungi (e.g. penicillin from *Penicillium notatum* and cyclosporine A from *Tolypocladium inflatum*, respectively) (52;53).

Attempts to manipulate the immune system to improve health, has led to the discovery of a range of promising natural compounds including immunomodulating polysaccharides. In particular, β-glucans, a diverse group of glucose polymers, have attracted considerable interest (54).

1.4.1 History of β-glucans – Initial interest and early exploration

Interest in β-glucans as so called biological response modifiers (BRMs), with beneficial effects on human and animal health, has two major historical origins (reviewed in 54). In the 1940s Pillemer and Ecker (55) prepared and studied zymosan, a crude mixture of polysaccharides, proteins and lipids, isolated from the cell wall of the yeast *Saccharomyces cerevisiae*. Zymosan proved to be a potent stimulator of macrophages and mediated the release of various cytokines, contributing to enhancement of non-specific immune reactions (56). Subsequent research identified β-glucan as the primary zymosan component responsible for the observed biological effects. While early β-glucan research in the United States and Europe was founded on the zymosan story, efforts in Asia, primarily Japan, originated in traditional medicine (54). In Asian folk medicine, consumption of medicinal mushrooms (e.g. shiitake, *Lentinula edodes*; maitake, *Grifola frondosa*; and reishi, *Ganoderma lucidum*) and mushroom-derived extracts has a long standing tradition (57-59). When the composition and biological effects of these mushrooms were investigated, β-glucan was once again found to be the main immunomodulating constituent.

Since the pioneering work by DiLuzio (e.g. 60-63) and Chihara (e.g. 64-66) in the 1960s and 1970s on yeast- and mushroom-derived β-glucans, respectively, numerous beneficial effects of various β-glucan preparations have been reported (reviewed in section 1.4.3). Currently the research focus is on elucidating the cellular and molecular mechanisms by which purified β-glucans exert their immunomodulating properties.

1.4.2 Structure and source of β-glucans

β-Glucans are carbohydrate polymers consisting entirely of glucose (Figure 2). These glucose homopolymers make up a highly heterogeneous group differing in glycosidic bond arrangement (67). This thesis deals with a major class of β-glucans known as β-1,3-glucan

(also known as β-1→3-glucan) and other β-glucan classes are not discussed herein. In the following the term "β-glucan" refers to β-1,3-glucan.

The D-glucose monomers are connected by β-1,3 linkages to form a helical polymer backbone (Figure 2A). Adding to the structural complexity, β-glucans may also contain branches connected to the backbone (e.g. by β-1,6 linkages) (Figure 2B) or display mixed backbone glycosidic bond composition (e.g. β-1,3/1,4)(67).

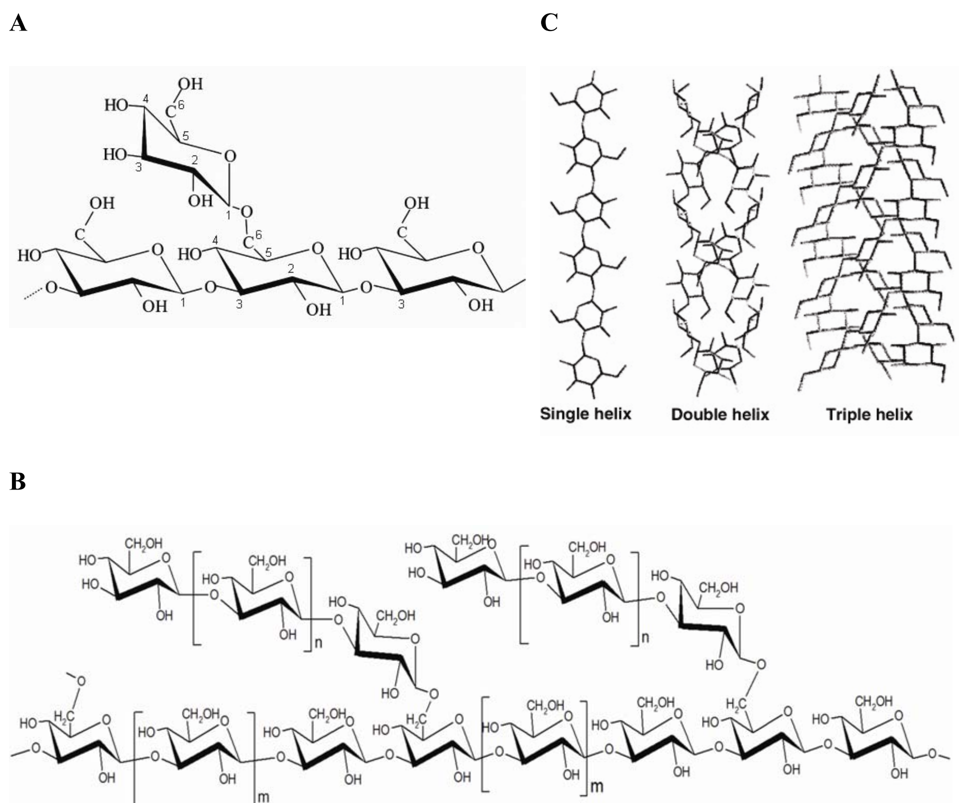

Figure 2. General structure of β-1,3/1,6-glucan.
A) β-1,3/1,6-glucans are polymers with a backbone consisting of glucose residues linked by β-glycosidic bonds between carbon atoms in position 1 and 3. Side chain residues are bound to the backbone by β-glycosidic bonds between carbon atoms in position 1 and 6. Adapted from *Biopolymers* (M. Sletmoen *et al.*) 2008 (68). B) Example of a branched β-1,3/1,6-glucan. Backbone length and distance between branches (m≥1) as well as side chain length (n≥1) may vary considerably depending on the source of origin and the extraction method. Figure reprinted with permission from Biotec Pharmacon ASA. C) β-Glucans form higher order structures. Schematic representation of single-, double- and triple helical β-glucan structures. The glucose ring is rigid and β-glucan polymer flexibility arises from rotation around the glycosidic bond, creating a pseudo-helical structure. Inter strand hydrogen bonds in the helical core contribute to formation and stability of dynamic higher order polymers with side branches exposed to the exterior (68). Reprinted by permission from Elsevier, *International Immunopharmacology* (M.F. Moradali *et al.*) © 2007 (69). The figure has been modified.

These diverse polymers can exist as single strands or as higher complexes such as triple helices (68;69) (Figure 2C). The nature of the backbone, the nature and frequency of branches, tertiary structure, molecular weight, solubility and polymer charge are all physicochemical characteristics that influence the potency of a given β-glucan as a BRM (54;68;70;71). Despite extensive research, structure – function prediction is not straight forward. Differences in macromolecular structure, and thus biological activity, largely depend on the isolation procedure and source of origin.

β-Glucans are found in a variety of organisms as structural components of the cell wall, polysaccharide storage or secretory products (67). The major source of β-glucan is fungi, including yeast and mushrooms. This thesis is based on work employing a *Saccharomyces cerevisiae*-derived, soluble, branched β-1,3/1,6-glucan (SBG, Biotec Pharmacon ASA, Tromsø, Norway). Other examples of yeast derived β-1,3/1,6-glucan products include poly-1,6-β-D-glucopyranosyl-1,3-β-D-glucopyranose (PGG) (72) and whole glucan particle (WGP) (73). β-Glucans isolated from medicinal mushrooms include Lentinan (shiitake, *Lentinula edodes*) and Grifolan (maitake, *Grifola frondosa*), both of which are examples of branched β-1,3/1,6-glucans (74;75). β-Glucans are also prepared from plants. Cereal grains, including oat (*Avena sativa*) and barly (*Hordeum vulgare*) have been demonstrated to contain linear β-glucans with mixed β-1,3/1,4 backbone composition (76-78). Cereal-derived β-glucans have attracted considerable interest due to their importance as widely used components in human- and livestock nutrition. Furthermore, β-1,3-glucans have been isolated form algae and bacteria including Laminarin (e.g. Phycarine, *Laminaria digitata*) and Curdlan (*Agrobacterium* sp.), respectively (79;80). Thus, β-glucans are found in a broad range of organisms belonging to different taxonomic groups including both prokaryotes and eukaryotes.

Interestingly, β-glucan is generally not found in organisms belonging to the animal kingdom. One sole exception, *Hyalinoecia tubicola* – a polychaete worm, has been reported to secrete β-1,3-glucan (81). β-Glucan is a potential source of glucose, provided the presence of an appropriate digestive machinery. The digestive tract of invertebrates contains β-1,3-glucan specific hydrolases, but whether these are products of the animal itself or of its microflora is debated. β-Glucan hydrolases are generally not found in vertebrates, humans included, thus β-1,3-glucan digestion in higher animals is likely to be caused by gut microorganisms (81).

1.4.3 Immunomodulating effects of β-1,3-glucans

β-Glucans have been reported to modulate cytokine profiles and phagocyte activity, enhance protection against sepsis, infections and tumor development, and promote wound healing. Furthermore, effects on non-immune parameters including cholesterol reduction and blood glucose control have been reported. Although the accumulating literature on the properties of β-glucans is extensive, the cellular and molecular mechanisms behind the reported effects remain unclear. Contradictory findings and discrepancies in the literature, contributes to the lack of mechanistic understanding.

Selected examples of biological effects of β-1,3-glucans *in vitro*, in animal models and in humans are discussed in some detail below. The literature on β-glucans as BRMs has been thoroughly reviewed elsewhere (54;70;71;78;82-84).

1.4.3.1 Effects of β-1,3-glucan: highlights from *in vitro* studies

Although major discrepancies exist, β-glucan treatment *in vitro* in general enhances the response of leukocytes. Increased production of pro-inflammatory cytokines, exemplified by

tumor necrosis factor (TNF)α from rat alveolar macrophages (85), and TNFα, interleukin (IL)-6, IL-8, IL-10 and tissue factor production by human peripheral blood mononuclear cells (PBMCs) (86), has been reported. However not all β-glucan preparations mediate release of pro-inflammatory cytokines (72). In fact, β-glucans have also been demonstrated to suppress pro-inflammatory cytokine production in response to a secondary challenge (87). In addition, β-glucan treatment *in vitro* has proved to enhance phagocyte activity, antimicrobial capacity, and cytotoxicity including increased oxidative burst activity (83). Furthermore, β-glucan treatment has been reported to influence neutrophil migration (88), DC maturation (89) and epithelial chemokine production (90) *in vitro*.

1.4.3.2 Effects of β-1,3-glucans following parenteral administration

β-1,3-Glucans have been studied extensively in animal models and to a much lesser extent in clinical trials. In most of these studies the study drug has been administered via parenteral routes.

Systemic administration of β-glucans enhances host protection against infections. Intravenous (i.v.) and intra nasal administration of lentinan significantly reduced viral titers in the lung and enhanced survival in an influenza infection model (91). Furthermore, intraperitoneal (i.p.) administration of lentinan has been demonstrated to confer protection against *Mycobacterium tuberculosis* in mice (92). Soluble β-1,3-glucan from *Sclerotinia sclerotiorum* delivered i.p. had both curative and prophylactic effects on experimental *Streptococcus pneumoniae* infection (93). Intramuscular (i.m.) and i.v. administration of *S. cerevisiae*-derived β-glucan enhanced clearance of *Staphylococcus aureus* and *Escherichia coli* in animal models (94;95). Moreover, parenteral β-glucan administration increased host resistance to fungal pathogens (96) and protozoal infections (97).

Parenteral administration of β-glucan reportedly mediate protection against sepsis and associated multiple organ dysfunction syndrome (MODS), and prolong survival in experimental models (98-100). In line with the animal studies, i.v. β-1,3-glucan treatment decreased post-surgery infection incidence, reduced the duration of intensive care, decreased septic morbidity and increased survival in critical care patients (101-104). Glucan phosphate delivered i.p. mediated organ protection following myocardial ischemia-reperfusion (I/R) injury, further supporting the idea that β-glucan therapy may prove useful in prevention of surgical complications (105).

β-Glucans protects against neoplastic transformation and cancer progression and have been demonstrated to reduce established tumors in animal models (106-108). Accordingly, β-glucans have attracted considerable interest as anti-cancer remedies and several β-glucan preparations are currently approved for clinical cancer therapy (57;58). Additionally, systemic β-glucan administration has been demonstrated to alleviate adverse reactions associated with radiation and chemotherapy (107;109). The anti-cancer activity has in part been credited to the modulating effect on the Th1/Th2 balance. β-Glucan treatment reportedly result in Th1 skewing and thus, dominance of a cell-mediated immune response (110-113).

Th1 profile predisposition suggests a possible role for β-glucan in allergic disease management. Also of note, β-glucans used as vaccine adjuvant increased specific Ab titers, reduced pathogen load and decreased mortality in animal models (114-116). Moreover, topical and i.v. application of β-glucans have proved to enhance wound healing in animal models and humans (117-119) and SBG is currently in clinical trials for topical treatment of chronic ulcers in diabetic patients (120).

1.4.3.3 Effects of β-1,3-glucans following oral administration

Oral drug administration is attractive and remains the preferred delivery route despite challenges associated with low bioavailability due to poor intestinal barrier penetration and gastrointestinal degradation. Although parenteral delivery has dominated in the field of β-glucan research, orally administered β-1,3-glucans clearly mediates beneficial effects on human and animal health. Essential literature on the effects of p.o. and intragastric administration of β-1,3-glucans is summarized below.

Like parenteral delivery, oral and intragastric β-glucan treatment reportedly enhanced host resistance to viral, bacterial, fungal and protozoal infections (121-123). Also, oral pretreatment with β-glucans mediated protection against sepsis and MODS in animal models (124 and Paper I). Oral β-glucan prophylaxis attenuated I/R-injury in animal models of kidney failure and pressure ulcer formation (125;126). Supporting the clinical relevance of pre-surgery β-glucan treatment, orally administered β-glucan attenuated I/R-injury and exhibited cardioprotective properties in patients subjected to coronary artery bypass grafting (127). Dietary β-1,3-glucans, especially those derived form cereal grains, may reduce blood glucose and cholesterol levels, indicating a possible role for β-glucans in management of diabetes and hypercholesterolemia (84;128-136).

It has been speculated whether β-1,3-glucans may enhance the mucosal immune system. In support of this idea, Lehne et al. (137) reported that orally administered SBG increased saliva IgA- but not IgG-levels, without affecting the level of antibodies in circulation. Moreover, evidence of increased numbers of IELs in the small intestine following p.o. β-glucan administration was presented by Tsukada and co-workers (138). In line with these reports, data presented in Paper III indicate that oral β-1,3-glucan administration had an effect on MLNs and PPs, key inductive sites for mucosal immunity. Furthermore, oral and intracolonic β-1,3-glucan pretreatment protected against chemically-

induced mucosal injury in animal models of acute colitis (139 and Paper II), possibly by improving epithelial restitution and thus, barrier integrity (Paper III).

Oral β-glucan treatment enhances wound healing. Impaired wound healing is a major complication of diabetes mellitus (140). Interestingly, *Sparassis crispa*-derived β-glucan delivered orally accelerated skin wound healing in diabetic rats (141). Also, bowel anastomosis wound healing, impaired by anti-inflammatory drugs, was significantly improved in rats pretreated with β-glucan orally (142).

Allergic rhinitis, conjunctivitis, asthma and food allergy are IgE mediated diseases, largely driven by a Th2-biased immune response to an allergen (143). Accordingly, a shift towards a Th1 profile emerges as a useful strategy to treat these prevalent conditions. Interestingly, oral β-glucan treatment improved allergic symptoms, reduced total- and specific IgE levels, decreased eosinophil counts and mediated a rise and fall in Th1 and Th2 cytokine levels, respectively, in pollen allergy clinical trials (144;145). In a mouse model of food allergy, similar effects of oral β-glucan administration were reported (146).

Early evidence that orally administered β-1,3-glucan has immunomodulating properties and the potential to inhibit tumor growth was provided by Suzuki and colleagues (147). Subsequently, β-glucans delivered p.o. have attracted some interest as anti-cancer remedies on their own (148-150). However, major attention is now given to orally administered β-glucans as an adjuvant in cancer therapy. β-1,3-Glucans reportedly synergized with, and enhanced the therapeutic effect of anti-tumor monoclonal antibodies (73;151-154).

Chemo- and radiation therapy frequently results in leukopenia, rendering patients immunocompromised and at risk of acquiring treatment-associated complications. Oral and parenteral β-1,3-glucan therapy may increase patient tolerance to irradiation and chemotherapy by stimulating hematopoietic activity and increase leukocyte numbers (155).

The cellular- and molecular mechanisms behind the health promoting effects of β-1,3-glucans are discussed in section 1.4.4 below.

1.4.3.4 Adverse reactions

Purified β-1,3-glucans generally show low or no toxicity and yeast β-glucans recently received the "generally recognized as safe" (GRAS) rating by the U.S. Food and Drug Administration (FDA) (156). However, there are reports of adverse events, primarily in relation to parenteral delivery of insoluble, particulate or impure β-glucan preparations. Unexpectedly, non-steroidal anti-inflammatory drugs (NSAIDs) in combination with β-glucans increased mortality in several mouse strains (157;158). Furthermore, Di Luzio and colleagues reported that parenteral administration of particulate, but not soluble, β-glucans caused granuloma formation and hepatosplenomegaly in a mouse model (159). Interestingly, although the data is inconsistent, inhalation of environmental β-1,3-glucan aerosol (e.g. molds and house dust) has been suggested to contribute to airway inflammation and allergy (reviewed in 160).

1.4.4 Mechanisms of β-1,3-glucan action

Numerous reports advocate the use of β-1,3-glucans to treat or prevent various medical conditions. However, the current knowledge of the mechanisms of action and pharmacokinetics remains insufficient. The fact that the β-glucan literature is inconsistent, and frequently contradictory, complicates delineation of underlying mechanisms. Heterogeneity in the physicochemical characteristics of the β-glucans studied, source of origin, purity, presence of contaminants and the experimental model systems employed all

contribute to the current confusion. Clearly, increased mechanistic understanding is desirable. Proposed mechanisms of action are discussed here.

1.4.4.1 Bioavailability - Uptake, clearance and pharmacokinetics

Information about the persistence and clearance of β-glucans from circulation, and the significance of molecular weight, degree of branching and solution conformation on pharmacokinetics is limited. Rice and colleagues were the first to demonstrate that the physicochemical characteristics of the β-glucan critically influenced pharmacokinetics (161). In line with previous reports, Rice et al. concluded that various β-glucans administered i.v. have similar plasma half lives (161-164). Estimation of bioavailability is complicated by the fact that β-glucans are internalized by cells in circulation and solid organs (e.g. liver and spleen) and remains cell-associated for an extended period of time, thus plasma levels only tell part of the story (123;165-167).

The uptake of orally administered β-glucans, and biological effect thereof, has been highly controversial. Although it is now widely accepted that orally administered β-glucans may enhance host immunity, it is still not established whether β-glucans may act directly on the gastrointestinal mucosa or if entry to the blood stream is feasible and required to mediate biological effects. Gastrointestinal absorption of orally administered β-glucans has been addressed in a very limited number of publications, including Paper I. Hong et al. reported uptake of particulate β-glucan from the gut mediated by intestinal macrophages that internalized the β-glucan particle, circulated throughout the body, and subsequently released bioactive soluble β-glucan into circulation (153). A recent paper by Rice et al. convincingly demonstrated that three structurally distinct β-1,3-glucans were internalized by a subset of epithelial cells (possibly M cells), GALT cells (i.e. macrophages and DCs) and rapidly entered circulation following a single oral dose (123). We too detected β-glucan in

circulation following oral administration and speculated whether gastrointestinal uptake is (in part) mediated by macrophages and DCs that sample β-glucan from the lumen by extending trans-epithelial protrusions (Paper I). Although the uptake mechanism remains elusive, Rice and coworkers points out that several cells in the gastrointestinal tract are capable of interacting with β-glucans and suggest that β-glucan loaded cells may serve as a reservoir (123). Of note, we reported that the immunomodulating capacity of SBG does not appear to correlate strictly with plasma levels, indicating that different mechanisms of action may be at play depending on the route of delivery (Paper I).

β-Glucan glucanase deficiency, and hence lack of rapid degradation, in vertebrates contributes to the longevity of β-glucans. Clearance of low and high molecular weight β-glucans are believed to occur primarily via glomerular filtration and liver oxidation, respectively (167-169).

1.4.4.2 β-Glucan receptors, signaling pathways and downstream consequences

β-Glucans are believed to exert their immunomodulating properties through specific receptors. Multiple cell-surface receptors, including Dectin-1, complement receptor 3 (CR3 a.k.a. CD11b/CD18), scavenger receptors and lactosylceramide, have been implicated in β-glucan recognition (83;170). These widely expressed receptors are found at varying expression levels on macrophages, DCs, neutrophils, natural killer (NK) cells, mast cells, microglia, B cells and a subset of T cells (83;171-173) and on non-immune cells including epithelium (90;174;175), endothelium (176), fibroblasts (177) and pituitary cells (178).

Accumulating evidence suggests that Dectin-1 is the primary β-glucan receptor responsible for the immunomodulating effects of β-glucans (179-181). Furthermore, Dectin-1-deficient mice are susceptible to fungal infections, indicating an important role in anti-fugal defence (182;183). Dectin-1 is highly specific for glucans with a β-1,3-backbone and

differentially recognizes a variety of β-1,3-glucans demonstrated by a great range of binding affinities (184). The minimal structural component recognized by Dectin-1 is a glucose-heptamer backbone with at least one glucose side branch (184). Low molecular weight β-glucans are generally considered to be biologically inactive (170), suggesting that receptor cross-linking (i.e. formation of homo- or hetero multi-receptor complexes) is required for bioactive β-glucans to exert their effects (185). Systemic administration of β-1,3-glucan phosphate resulted in prolonged reduction of membrane-associated Dectin-1 in peripheral leukocytes, whereas oral administration caused increased surface expression on GALT macrophages (123;165). Thus, Dectin-1-positive cells respond differentially to β-1,3-glucan exposure depending on tissue localization and drug delivery route.

Dectin-1 engagement leads to phosporylation of the immunoreceptor tyrosine-based activation motif (ITAM) present in the cytoplasmic tail of the receptor resulting in a number of cellular responses including; ligand-receptor complex endocytosis, enhanced phagocytosis, nuclear factor (NF)κB activation ultimately leading to production of cytokines and chemokines, and respiratory burst (production of reactive oxygen species; ROS) (reviewed in 71;181;186;187). Although the mechanistic details are still lacking and multiple (possibly cell-type specific) downstream signalling pathways exist, spleen tyrosine kinase (Syk) and caspase recruitment domain (CARD) 9 appears to be central (71;181;186-188) (Figure 3).

Interestingly, Dectin-1 has been demonstrated to collaborate with Toll-like receptors (TLRs) in a MyD88 and Syk-dependent manner (180;188-192) (Figure 3). Blocking of Dectin-1, using specific mAbs, abrogated the anti-tumor effect of Schizophyllan β-1,3-glucan, indicating a critical role for Dectin-1 in the observed cancer-protective capacity of β-glucans (193). Research employing Dectin-1 knock out (KO) animal models (or tissue/cell-specific KO models) will shed additional light on the precise mechanisms and the

role of Dectin-1. It is conceivable that Dectin-1 contributes to many different immune system-mediated responses to β-1,3-glucans including increased resistance to infections and tumor development, and modulation of inflammation.

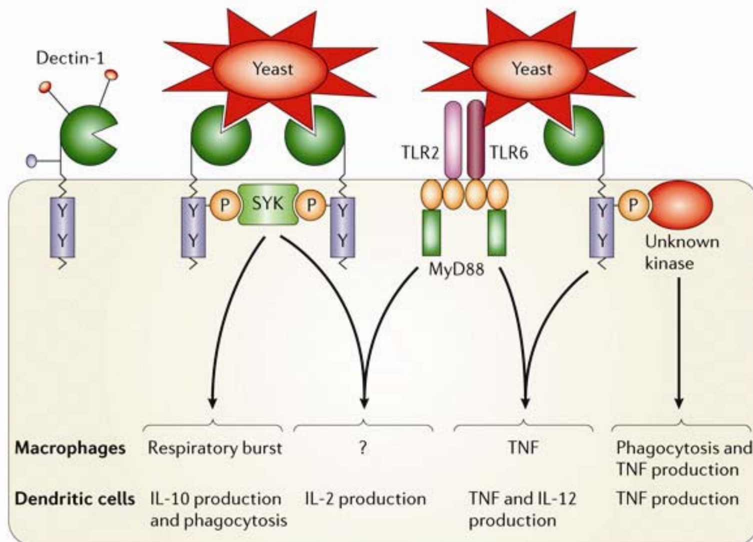

Figure 3. Dectin-1 is a major β-glucan receptor; signal transduction and TLR collaboration.
Ligand binding, e.g. by β-glucan containing yeast, to the extra-cellular carbohydrate recognition domain of Dectin-1 triggers tyrosine phosphorylation of the cytosolic ITAM motif, presumably by Src kinases, and induces an intracellular signaling cascade that results in various cell-type specific responses. Despite requiring only the membrane-proximal tyrosine for signaling, the cytoplasmic tail of Dectin-1 can interact with spleen tyrosine kinase (Syk). The nature of this interaction is unknown but it is proposed to occur by bridging of two Dectin-1 molecules. Whereas interactions with Syk can directly induce cellular responses, such as the respiratory burst and IL-10 production, signals from the TLR-pathway are also required for the production of IL-2. Syk contributes to phagocytosis in DCs, but it is not required for Dectin-1 induced phagocytosis in macrophages, which occurs through an uncharacterized mechanism possibly involving an unidentified kinase. Through this novel pathway, Dectin-1 also collaborates with the TLRs to induce pro-inflammatory responses (e.g. TNFα production), although Dectin-1 might be able to directly induce TNFα in certain cells.

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However, β-glucans may also work through other receptors than Dectin-1. Such mechanisms include potentiation of anti-tumor mAbs via β-glucan priming of CR3 (153;194), causing complement enhanced Ab-dependent cell-mediated cytotoxicity and/or

CR3-dependent cell-mediated cytotoxicity (195). Moreover, activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway by β-glucans (possibly involving Dectin-1 or class A scavenger receptor) has been demonstrated to ameliorate septic, inflammatory and I/R injury (reviewed in 196).

The molecular mechanism underlying the beneficial effects of orally administered β-glucans on hypercholesterolemia and blood glucose levels remains blurred. Suggested mechanisms include delayed stomach emptying, binding to bile, increased small intestine viscosity, suppression of glucose absorption and enhanced production of short-chain fatty acids (71;84;197). Finally, β-glucans have been proposed to have prebiotic properties, promoting health indirectly by affecting the composition of the intestinal commensal microbiota. Indeed, work by Snart et al demonstrated that a β-glucan supplemented diet resulted in *Lactobacillus*-enriched cecal microbiota in a rat model (198). Interestingly, beneficial effects of *Lactobacillus*-based probiotics have been demonstrated in animal models as well as in clinical trials (199).

1.5 Model diseases and experimental animal models

A rat model of endotoxin-induced shock and multiple organ dysfunction syndrome (MODS) and a mouse models of IBD were adopted to confirm SBG bioactivity and to investigate effects of β-1,3-glucan on mucosal inflammation, respectively. The animal models employed in this thesis, and the human diseases they intend to mimic, are outlined below.

1.5.1 Sepsis, shock and MODS

Sepsis is defined as a systemic inflammatory response to infection. The diagnosis represents a continuum of severity, ranging from minor symptoms to life-threatening conditions.

Severe sepsis is defined as sepsis accompanied by hypoperfusion or dysfunction in at least one organ system (multiple organ dysfunction syndrome, MODS) and septic shock is defined as sever sepsis accompanied by hypotension requiring circulatory support therapy (200;201). The reported incidence rate of sepsis varies considerably (202-210), nevertheless frequently sited papers by Martin et al. (202) and Dombrovskiy et al. (203) estimated the incidence of sepsis and severe sepsis to be approximately $240/10^5$ and $132/10^5$, respectively. The mortality rate in patients hospitalized with sever sepsis is decreasing. Despite this decrease a considerable increase in the incidence rate causes the over all number of sepsis-related deaths to increase, thus sepsis remains a leading cause of death (202;203).

The pathophysiology of sepsis involves pathologic processes triggered by microbial pathogens that are exacerbated by the host attempt to produce a protective immune response (Figure 4). Sepsis is a complex and multifactorial syndrome. Innate recognition of bacterial components, including lipopolysaccharide (LPS), lipoteichoic acid or peptidoglycan, classical PAMPs, by TLRs and other PRRs triggers the release of potent mediators of inflammation. The pro-inflammatory cytokines TNFα and IL-1β in particular, but also IL-6, IL-8 and high-mobility group box-1 protein (HMGB-1) are thought to be important mediators during the early stage of sepsis and MODS development. Synergistic effects between cytokines, chemokines, acute phase proteins, complement, coagulation factors, eicosanoids, proteases and reactive oxygen intermediates, have been shown to be critically involved in the pathogenesis of sepsis and MODS. Induction of inducible nitric oxide synthase (iNOS) expression (i.e. by LPS, TNFα or IL-1β), predominantly in macrophages, leads to excessive production of nitric oxide (NO) which cause vasodilatation, reduced cardiac contraction and consequently, reduced blood pressure and organ perfusion, ultimately causing MODS and shock to develop. Also of note, dysregulated coagulation and

increased capillary leakage further ads to hemodynamic disturbances and eventually to shock (reviewed in 51;211-214) (Figure 4).

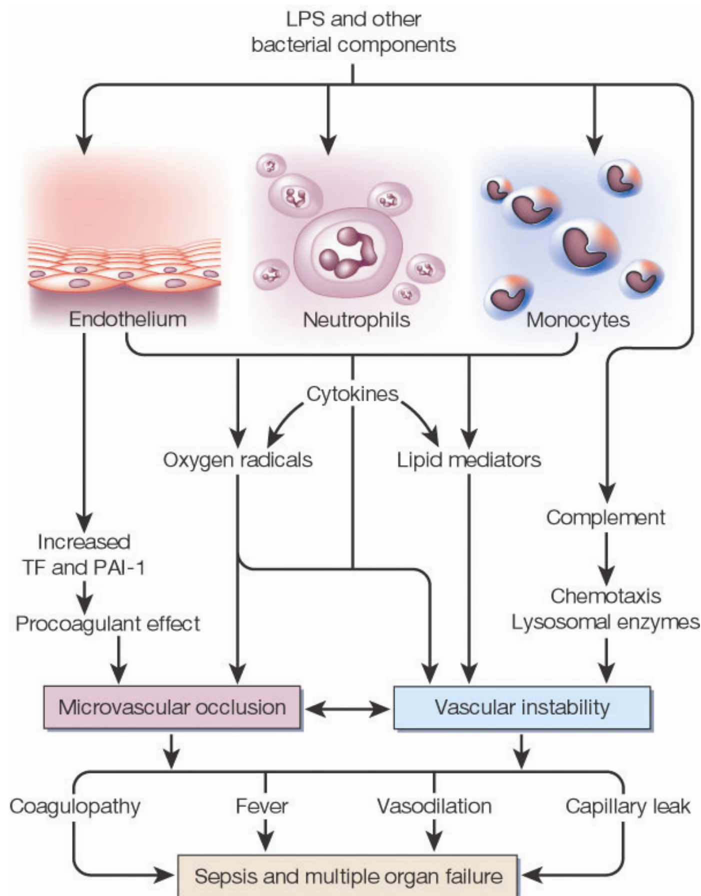

Figure 4. Pathogenetic networks in sepsis, shock and organ failure.
LPS and other PAMPs simultaneously activate multiple parallel cascades that contribute to the pathophysiology of systemic inflammation, shock and MODS. Explosive release of cytokines and other pro-inflammatory mediators and activation of complement and coagulation cause an exaggerated state of inflammation ultimately leading to hemodynamic disturbances, hypoperfusion, multiple organ dysfunction, septic shock and eventually death.

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Clearly, there is a need for novel and alternative treatment and prevention strategies. The use of BRMs, such as β-glucans, to modulate the host immune response may prove to be a promising therapeutic approach.

1.5.1.1 Endotoxin-induced shock and shock-associated organ failure

Systemic injection of LPS (a.k.a. endotoxin), a key mediator in Gram-negative sepsis, has been demonstrated to produce pathophysiological alterations, including systemic inflammation, hemodynamic disturbance and organ dysfunction, similar to those reported for septic patients (216). LPS activates an immense range of genes, causing an explosive release of pro-inflammatory cytokines and additional mediators with the capacity to cause injury to vital organs (217). Importantly, LPS-induced shock does not fully recapitulate what happens in the septic patients. Acute endotoxemia is a widely used model and it represents a valid tool to study systemic inflammation and the resulting sepsis-like symptoms, although confirmation in a more clinically relevant model is desirable. The LPS-model and alternative sepsis models are discussed briefly in the methodological consideration section (4.1.1) and in Paper I.

1.5.2 Inflammatory bowel disease (IBD)

IBD refers to two related chronic inflammatory disorders characterized by acute flares followed by remission: ulcerative colitis (UC) and Crohn's disease (CD). UC affects the inner lining of the colon which becomes inflamed and develops ulcers. UC generally involves the distal part of the colon but may progress proximally to pan-colitis. CD tends to involve the entire bowel wall and commonly affects the terminal ileum and parts of the colon, but may affect any part of the gastrointestinal tract (reviewed in 218). A major complication of CD, affecting 35% of CD patients, is the formation of fistulas (219). Furthermore, UC and CD may be considered systemic disorders as more than 35% of IBD patients experience extraintestinal complications, with musculoskeletal, dermatologic and ocular symptoms predominating (220). The incidence of IBD varies considerably world wide with incidence rates between 0.5-24.5/10^5 and 0.1-16/10^5 inhabitants for UC and CD,

respectively. The highest rates are reported in Northern and Western Europe as well as North America (221). The etiology and pathophysiology of both UC and CD is complex. Accumulating evidence suggests that an inappropriate immune response to non-pathogenic microbes of the intestine and other luminal antigens plays a critical role in the initiation and pathogenesis of IBD (17;222;223).

Mucosal homeostasis is a balancing act between effector cells and regulatory cells and a shift in the balance may result in mucosal inflammation. IBD is associated with over expression of pro-inflammatory cytokines including TNFα, interferon (IFN)γ, IL-1β and IL-6. CD and UC have been considered Th1 and Th2 driven diseases, respectively, although the picture now appears more complex. Th17 cells, a distinct subset of CD4+ Th cells characterized by abundant IL-17 production, are associated with intestinal inflammation and tissue pathology and have attracted considerable attention recently. IL-23, a cytokine that shares the p40 subunit with IL-12 (which drives Th1 differentiation), is central in promoting Th17 function and earlier blocking studies of p40 may have affected both Th1 and Th17 effector T cells (reviewed in 224;225). The above mentioned cytokines and their producers may prove to be attractive therapeutic targets.

Familial aggregation suggests that IBD has a heritable component. The discovery of susceptibility genes has demonstrated the importance of innate and adaptive immune responses and epithelial barrier integrity in IBD pathogenesis (Figure 5). Since NOD2 (a.k.a. CARD15 and IBD1), encoding a intracellular gene product involved in innate sensing of microbes and release of anti-microbial peptides, was described as the first susceptibility gene associated with CD, additional IBD-associated genes have been described (reviewed in 226;227;228). Positive family history of IBD is generally more frequent in CD than in UC.

Genome-wide association studies have significantly increased the number of known genetic risk factors in CD. Currently more than 30 genes are described as risk factors associated with CD development. Selected examples of recently identified genetic risk factors include ATG16L1, ATG5 and IRGM which are involved in autophagy, handling of gut microbes and macrophage function, MST1 and its receptor which limit inflammatory responses, PTGER4 which is involved in epithelial restitution, and IL23R which play a central role in maintenance and expansion of Th17 cells (reviewed in 229) (Figure 5).

Importantly, IBD-prone genotypes results from multiple genetic variants that each exerts a minor effect on the overall risk of disease development. The increased IBD incidence over the past decades, particularly in developing countries, suggests that environmental factors, are implicated in IBD development (221).

IBD is routinely treated with antibiotics, immunosuppressive- and anti-inflammatory drugs. Promising antibody-based therapeutics blocking key cytokines, interfering with T-cell activation and migration of inflammatory cells emerge as potent alternative therapies for IBD (230). Although these strategies may prove effective, available therapeutics are associated with considerable adverse reactions (230) including opportunistic infections (231).

Patients with severe IBD, refractory to medical treatment or with neoplastic transformation, require surgery. Surgery continues to have an important role in IBD treatment as 30-40% of UC patients and 70% of CD patients require surgical intervention at some point (232). Total colectomy, the only cure for UC, is indicated in approximately 25% of UC patients (233). Notably, as many as 40-60% of IBD patients respond poorly to current standard therapy, indicating a considerable need for new, more effective and safe therapies (234).

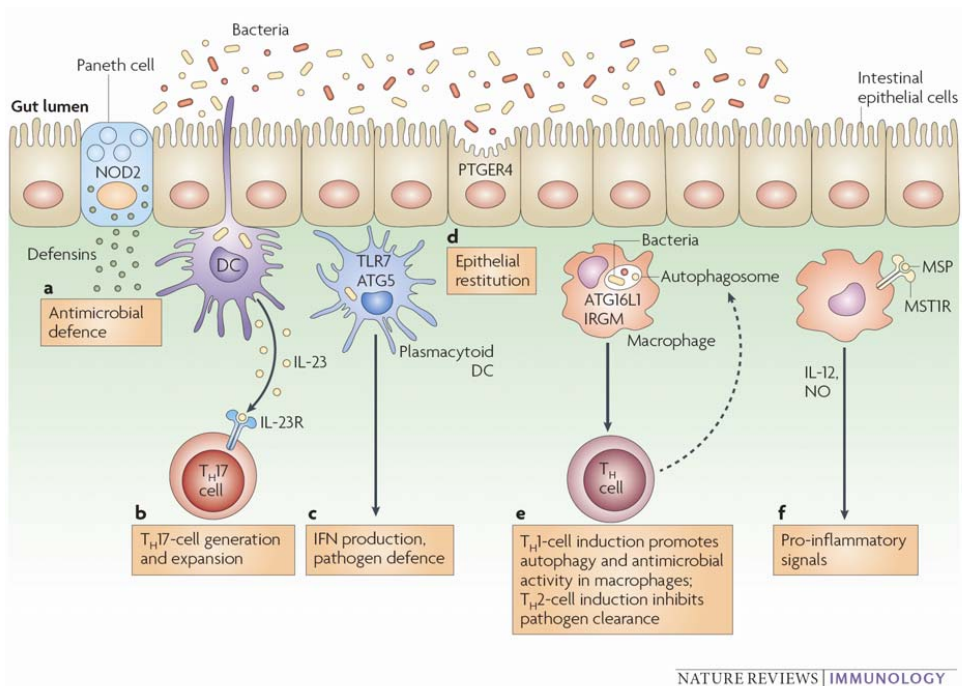

Figure 5. Selected CD-associated susceptibility genes and cell-specific signaling pathways. The mucus layer and tight junctions associated with intestinal epithelial cells maintain barrier integrity under homeostatic conditions. Disruption of this dynamic balance between host-defence immune responses and luminal enteric bacteria at the mucosal frontier is central to the pathogenesis of IBD. Recent genetics studies implicate alterations in autophagy, innate immunity and the balance between pathogenic and regulatory T-cell populations as risk factors for Crohn's disease. Signalling pathways involved in inflammation and the potential roles of proteins encoded by disease-associated genes are depicted. a) Nucleotide-binding oligomerization domain protein 2 (NOD2) has a role in the sensing of cytosolic microbial ligands and in the release of antimicrobial peptides. b) Dendritic cells (DCs) extend dendrites between epithelial cells and can sense changes in luminal contents. Interleukin-23 receptor (IL23R) has a role in maintaining Th17 cells. c) Autophagy (involving ATG5) is required for the delivery of nucleic acids to endosomal Toll-like receptor 7 (TLR7) and the downstream activation of type 1 interferon (IFN) signalling in plasmacytoid DCs. d) Prostaglandin E receptor 4 (PTGER4) is involved in promoting epithelial restitution. e) Autophagy 16-like 1 (ATG16L1) and immunity-related GTPase family member M (IRGM) are involved in autophagy, anti-microbial defence, macrophage stimulation and stimulation of adaptive immune responses. f) Macrophage-stimulating protein (MSP) and its receptor, MST1 receptor (MST1R), have a role in inhibiting inflammatory responses. NO, nitric oxide.

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1.5.2.1 Dextran sulfate sodium-induced colitis

We have employed the widely used dextran sulfate sodium (DSS) model to study the effect of SBG (soluble β-1,3-glucan) on intestinal inflammation (Paper II). Oral exposure to water-dissolved DSS induces a reproducible acute colitis in rodents. Although the precise molecular mechanisms remains elusive, DSS appears to have a toxic effect on the basal crypt epithelium, causing reduced mucosal barrier function, subsequently resulting in colonic inflammation and ulceration (235). Resembling UC, DSS-induced colitis is characterized by diarrhoea, bloody stool and body weight loss. Histological hallmarks include superficial mucosal erosions, ulcer formation and inflammatory cell infiltration to the colonic lamina propria. Furthermore, colon shortening and thymic involution are known features of DSS-induced colitis. As in UC, pathology is predominant in the distal colon with no involvement of the small intestine. Importantly, DSS-induced murine colitis is treatable with drugs frequently used to control human UC (235; and reviewed in 236;237).

Available animal models do not fully recapitulate all the traits of human IBD. Still, they are important tools developed to evaluate new treatments and to elucidate the etiology and pathophysiology of IBD. The DSS-model, and alternative IBD models, are discussed briefly in the methodological considerations section (section 4.1.2) and in Paper II and have been thoroughly reviewed elsewhere (236-240).

2 Aims of the study

β-1,3-Glucans have proven to be potent immunomodulators with obvious clinical potential. Yet, the current understanding of the underlying mechanisms of action remains insufficient. The main aim of this study was to establish *in vivo* models amenable to experimental manipulations in order to study the health-promoting effects of β-glucans with a focus on oral administration and mucosal biology.

A major objective was to compare systemic- and oral SBG prophylaxis in a rat model of shock and shock-associated organ failure.

Specific objectives were to:

- Quantify the extent of absorption of orally given SBG to systemic circulation.
- Determine the effect of SBG on hemodynamics during shock development.
- Investigate organ protection by SBG prophylaxis.
- Analyze the effect of SBG prophylaxis on endotoxin-induced cytokine storm.

A second major objective was to evaluate the effect of oral SBG administration on mucosal inflammation in a mouse model of inflammatory bowel disease.

Specific objectives were to:

- Determine if oral SBG reduced colitis-associated morbidity and mortality.
- Examine and quantify the effect of oral SBG on intestinal histopathology.
- Analyze the effect of oral SBG on colitis-associated systemic inflammation.

A third major objective was to analyze effects of oral SBG on mucosal inductive and effector sites in the intestine of healthy mice.

Specific objectives were to:

- Identify qualitative and quantitative changes in GALT (inductive sites).
- Analyze colonic epithelial proliferation, goblet cell and IEL numbers.

3 Summary of results

3.1 Paper I

Oral and systemic administration of β-glucan protects against lipopolysaccharide-induced shock and organ injury in rats.

Sandvik A., Wang Y.Y., Morton H.C., Aasen A.O., Wang J.E. and Johansen F-E.

Systemic administration of β-glucan reportedly mediates protection against experimental sepsis, associated shock and organ failure. Furthermore, oral β-glucan administration enhances host resistance, although the extent and significance of gastrointestinal absorption remains unclear. We set out to confirm the immunomodulating capacity of SBG in a rat model of LPS-induced shock and shock-associated organ injury, comparing systemic- to oral β-glucan administration. Rats were pretreated with SBG or placebo orally or subcutaneously, anaesthetized and subjected to endotoxemia by intravenous infusion of LPS or saline (sham).

Oral SBG prophylaxis produced plasma β-glucan levels significantly higher than what we observed in the placebo group, indicating gastrointestinal absorption. Still, the reported β-glucan content in plasma was only a minute fraction of a single oral dose and approximately 40-fold less than what we observed in rats administered SBG subcutaneously, despite the fact that the daily oral dose was 10-fold higher. Oral and systemic SBG prophylaxis enhanced mean arterial blood pressure recovery following LPS-induced blood pressure collapse. Furthermore, SBG treatment attenuated LPS-induced organ injury. Oral treatment with SBG conferred relative protection of the liver and kidneys whereas only hepatic protection was observed in rats treated subcutaneously. We observed a moderate increase in baseline plasma IL-1β levels following subcutaneous SBG

administration and a moderate reduction in plasma levels of proinflammatory cytokines in both SBG-treated groups compared to placebo.

In conclusion, we demonstrated a striking positive effect of SBG on LPS-induced hemodynamic disturbances and on shock-associated organ injury. Oral administration of SBG had a more pronounced effect than subcutaneous injection. Additionally, SBG prophylaxis caused subtle changes in the cytokine profile, including attenuated levels of mediators of inflammation subsequent to endotoxin challenge.

3.2 Paper II

Soluble β-glucan protects against experimental ulcerative colitis.

Sandvik A., Grzyb K., Reikvam D.H., Erofeev A., Jahnsen F.L. and Johansen F-E.

Treatment of IBD is associated with significant adverse reactions and unsatisfactory efficacy, thus new therapies are wanted. β-Glucans reportedly modulate inflammation and mediate wound healing, suggesting a potential role for β-glucans in IBD therapy. We investigated the effect of oral SBG administration on experimental UC. Mice were pretreated with SBG-supplemented drinking water prior to colitis induction and SBG was continued throughout the experiment. Mice supplied with regular drinking water or SBG only served as controls. Colitis was induced by exposing mice to DSS orally.

Oral SBG prophylaxis reduced colitis-associated body weight loss and mortality. Furthermore, SBG treatment attenuated DSS-induced colonic inflammation and tissue damage. Also, colitis-associated colon shortening and thymic involution was attenuated by SBG treatment. Finally, colitis-associated systemic inflammation was attenuated in SBG treated mice.

In conclusion, we demonstrated a beneficial effect of oral SBG administration on all investigated parameters in experimental UC and propose that SBG has potential as a therapeutic agent in IBD management.

3.3 Paper III

Effects of oral administration of soluble β-glucan on the gut and gut-associated lymphoid tissue in mice.

Sandvik A., Bækkevold E.S., Jahnsen F.L. and Johansen F-E.

β-Glucans may stimulate the mucosal immune system when administered orally. We sought to identify effects of oral β-glucan administration on gut-associated lymphoid tissue and the intestinal epithelium of healthy mice. Mice were provided SBG-supplemented drinking water for 20 days. Control animals were provided regular drinking water.

SBG was well tolerated and no clinical signs of morbidity were noted. Oral SBG administration increased the number of macroscopically visible Peyer's patches (PPs) and enlarged the mesenteric lymph nodes (MLNs) without altering the composition of major lymphocyte subsets. Also, increased intestinal epithelial proliferation, possibly related to improved epithelial barrier function, was observed in SBG-treated mice.

In conclusion we demonstrated that mucosal application of SBG stimulated inductive sites of immune responses (PPs and MLNs) as well as effector sites of immune defense (mucosal epithelium). Our data supports the hypothesis that β-glucans may enhance host protection, in part, by effects on the mucosal immune system.

4 Methodological considerations

Detailed descriptions of methods are provided in the respective papers included in this thesis. General aspects of the chosen methods, their advantages and limitations are discussed in this section.

4.1 Animal models

Laboratory mice and rats reflect human biology remarkably and animal models have contributed immensely to increased understanding of human biology as well as to the development of new therapies and diagnostic tools (241;242). Mice have become the animal of choice in immunological research due to the availability of well characterized strains and the relative ease by which mice can be managed and genetically manipulated. However, separated by 65 million years of evolution, essential differences in physiology as well as in both innate and adaptive immunity exists between the human and murine immune system (243). Thus, extrapolating data form animal studies to humans require caution and awareness of the limitations of the applied animal model.

For ethical reasons, and as required by animal rights legislation, we have aimed at minimizing the number of experimental animals employed. Experiments have been designed according to the reduction, refinement and replacement principles (reviewed in 244). All use of laboratory animals was approved by the National Animal Research Authority (Forsøksdyrutvalget) and conducted in accordance with the Norwegian Animal Welfare Act and the Norwegian Regulation on Animal Experimentation.

4.1.1 LPS-induced shock and shock-associated organ failure in rats

Systemic and oral administration of β-glucan has previously been demonstrated to mediate protection against sepsis and associated organ injury (100;124). Several sepsis models exist, but no one fully resembles the timing of disease onset, progression and the use of supportive intervention in clinical human sepsis. We employed a rat model of endotoxin-induced shock to investigate whether the β-glucan studied (SBG) may in fact mediate biological responses. LPS infusion is a reductionistic model, designed to mimic the systemic host response to Gram-negative sepsis. Recognition of LPS, by the innate immune system triggers the release of potent mediators of inflammation that may result in an exaggerated pathogenic response. Pathophysiological alterations, similar to those reported for septic patients, include systemic inflammation, hemodynamic disturbances, organ dysfunction and shock (216).

The trachea, right carotid artery, jugular vein and urine bladder of anesthetized rats were cannulated to facilitate respiration, blood pressure monitoring and blood sampling, administration of LPS or vehicle, and to facilitate urine flow, respectively. In addition to LPS infusion, the surgical trauma, open wounds in a non-sterile environment, repeated blood sampling and extended anesthesia, may contribute to pathology. The experimental procedures are detailed in Paper I.

Although the clinical relevance of a bolus injection of LPS is questionable, the explosive release of pro-inflammatory cytokines and additional mediators of inflammation observed shortly after LPS-infusion are potential drug targets. Indeed, numerous preclinical studies have successfully targeted cytokines (e.g. TNFα and IL-1β). However, the cytokine storm triggered by LPS does not mirror the human sepsis situation well and attempts to block these mediators therapeutically has regrettably largely failed in the clinic (216;245).

The endotoxin model is of limited clinical relevance compared to models based on live polymicrobial sepsis, such as the cecal ligation and puncture (CLP) model, which is considered the current gold standard (245-247). Pure LPS engages a limited number of PRRs (i.e. TLR4) in contrast to the complex picture in septic patients (and in CLP) challenged with propagating Gram-positive, Gram-negative and fungal pathogens, all of which display multiple PAMPs that eventually affect the host response (248;249).

We chose to investigate the outcome of oral prophylactic β-glucan treatment in the applied model because the time of onset, the amount of circulating endotoxin and the severity of the sepsis-like reaction is tightly controlled. On the downside, the model is labour intensive and does not have survival as an outcome. From an ethical perspective, on the other hand, acute experiments are favourable as post-surgery distress is eliminated.

It should be noted that rodents are relatively resistant to LPS compared to humans (250). Nevertheless, unexpected, premature mortality, likely to be associated with varying LPS-potency, was observed. It is well known that experimental animals exposed to sublethal doses of LPS exhibit markedly reduced mortality when rechallenged with a normally lethal injection of endotoxin, a phenomenon known as endotoxin tolerance (251;252). Concerns that the observed protective effect of SBG against LPS-induced shock and MODS was due to induction of endotoxin tolerance by contaminating LPS rather than mediated by the β-glucan itself may duly be expressed. However, we find this scenario unlikely as SBG is certified to be essentially endotoxin free. Certainly, the concern is irrelevant for the animals administered SBG orally, as the gastrointestinal tract is constantly exposed to LPS.

The clinical relevance of prophylactic drug administration, with respect to sepsis management, can certainly be debated. We and others provide evidence and propose that pretreatment with β-glucans may in fact be a constructive strategy to reduce the risk of

complications associated with scheduled surgery (in high risk patients), including post-operative hospital-acquired infections, sepsis, organ failure and death (102;104;127;196).

4.1.2 DSS-induced colitis in mice

DSS-induced colitis is one of the most frequently used models of intestinal inflammation. DSS appears to have a direct toxic effect on the basal crypt epithelium, leading to reduced mucosal barrier function, subsequently resulting in inflammation and ulceration (235). Colitis induction depend on IFNγ expression and additional pathogenic mechanisms include impaired macrophage phagocytosis (253;254). Recently, DCs were demonstrated to be critical in the development of acute colitis (255). Furthermore, colitis induction is independent of T cells, B cells and NK cells as severe combined immunodeficient (SCID) mice develop colitis in response to DSS (256-258).

Although, oral DSS-exposure causes a reproducible colitis resembling UC, outcome prediction is complicated by the fact that DSS concentration, quality and exposure time; animal strain, age/size and gender; as well as environmental conditions all affect the result. Consequently, considerable inter-lab variation and variation with time can be expected. To further complicate the picture, the microbial environments in which the experiments are conducted, and the microbiota of the experimental animals, are also of decisive importance. Accordingly, the model requires laborious optimization of experimental conditions, including titration of DSS. Results from pilot studies illustrate how surprisingly narrow the DSS-concentration window may be. In our hands 1.5 and 2.0 % (w/v) DSS for 7 days followed by 7 days of regular drinking water produced moderate, sub lethal, colitis in male C57BL/6 mice, whereas 1.5 and 3% DSS proved lethal in a different microbial environment ("unclean" vs. "clean" side of the animal facility, respectively). Repeated dose titration demonstrated that 1.5% DSS caused a sever colitis with acceptable mortality in male

BALB/c mice, in the latter environment, thus these conditions were chosen for the SBG intervention study in Paper II. Bearing in mind that DSS concentrations of 3-10% is regularly used by others (259) 1.5% DSS must be considered a rather mild regiment.

Inter-animal fluid consumption variation has been reported, thus the observed differences in colitis severity may simply be a dose dependency phenomenon reflecting variations in the daily fluid consumption. Egger *et al*. addressed this key question and concluded that acute DSS-induced colonic mucosal injury is directly dependent on the concentration of DSS rather than the total DSS dose consumed (238). This supports the robustness of the model as minor differences in fluid consumption is unlikely to significantly affect the severity of the colitis induced.

Although the model has contributed to increased insight into the pathophysiology of IBD, particularly on the role of the epithelial barrier, it can be argued that DSS-induced acute colitis is an invalid model of human UC as UC is a chronic relapsing disease. Thus, it should be mentioned that chronic colitis may be achieved in selected mouse strains (260) or by the use of repeated cycles of DSS exposure (235). In support of the clinical relevance of the acute DSS model, gene expression profiling revealed that of 32 genes known to change transcriptional activity in IBD, 15 are differentially expressed in DSS-colitis (240). DSS-colitis resembled human IBD more closely than the widely used 2,4,6-trinitrobenzene sulfonic acid (TNBS) model (2/32 genes), but proved inferior to a model relying on transfer of a T-cell population depleted of T_{regs} to immunocompromised recipients (30/32 genes), in terms of gene expression matching (240).

Oral DSS administration causes intestinal inflammation and ulceration affecting the colon exclusively, despite exposure of the entire gastrointestinal tract. This phenomenon may in part be explained by the high degree of water re-absorption naturally occurring in this anatomical segment of the intestine (261), causing particularly high DSS exposure to

the colonic mucosa. Also, the high density and complexity of the large bowel microflora, compared to the relatively sparsely colonized stomach and small bowel (17), may add to the explanation of the observed segmentation of the intestinal injury. In favor of the DSS-model, with respect to clinical relevance, altered microflora composition is observed both in UC and DSS-induced colitis (17;235).

The role played by the commensal microbiota in DSS-induced colitis is unclear, largely due to conflicting results from different research groups. Microbes are not of critical importance for colitis induction as germ-free mice (GF) develop intestinal inflammation in response to DSS, but disease characteristics in GF mice differ from traits seen in conventional (CV) animals with intact microbiota (262;263). Kitajima *et al*. found sever rectal bleeding and an enlarged cecum filled with blood on day 1, followed by sever anemia and death as early as day 3 in GF mice given water with 5% DSS, whereas no mortality and only moderate changes was recoded in CV mice (262). Mortality in GF mice was not associated with colonic lesions indicative of colitis, while colitis with crypt loss, inflammatory cell infiltration and ulceration was observed in identically treated CV mice (262). Furthermore, a low dose DSS (1%) gave no observed colitis in CV mice, while rectal bleeding, body weight loss, anemia and mortality was delayed in GF mice compared with 5 % DSS (262). Similarly, Bylund-Fellenius and co-workers reported 75% mortality in GF mice vs. no mortality in CV mice following exposure to 5% DSS for 6 days, but their histopathological findings were similar in surviving GF and CV animals (263). Contrary to these two reports, a paper by Hudcovic and colleagues reported that 2.5% DSS caused severe UC in CV mice, but not in GF mice (in both acute and chronic models), indicating an important role for the microbiota in DSS-induced UC after all (258). These inconsistencies may possibly be explained by heterogeneity in the DSS products, doses and animal strains employed.

Supporting the clinical relevance of the model, both human UC and DSS-induced chronic colitis is associated with development of colorectal cancer (264). Moreover, DSS-induced colitis is successfully treated with drugs frequently used to treat human IBD including antibiotics, immunosuppressive- and anti-inflammatory drugs as well as so called biologic therapeutics such as cytokine-blocking Abs (265-269).

4.2 Plasma β-glucan quantitation

The planning and execution of work in this thesis has been hampered by the lack of readily available reagents to detect, trace and quantitate β-glucans. To the best of my knowledge, the only reagent commercially available for β-glucan measurement is an assay known as Fungitell (or Glucatell) which is currently marketed as a diagnostic tool for fungal infections. The assay is based on Factor G, a soluble PRR isolated from horseshoe crab (*Limulus polyphemus*) amebocytes, which specifically recognize β-1,3-glucans. The assay is sensitive (pg/ml range) and thus at risk of contamination from environmental β-1,3-glucan during sampling or analysis. We used Fungitell to determine the plasma β-glucan levels in blood samples collected approximately 1 hour after the final SBG dose was administered. Although repeated blood sampling was performed, kinetic analysis was not performed. The reported cut off value for a positive test in fungal diagnostics varies between 60-120 pg/ml (270-272) and we considered plasma levels below 80 pg/ml to be background signal.

4.3 Clinical chemistry analysis – organ injury assessment

Biochemical analysis of plasma for indicators of organ dysfunction and injury was carried out on an automated clinical chemistry analyzer by an accredited hospital laboratory (Paper I). Elevated plasma levels of alanine aminotransferase (ALAT), aspartate aminotransferase

(ASAT), bilirubin and γ-glutamyl-transferase (γ-GT) were considered markers of hepatic dysfunction and liver injury. Whereas ALAT is considered a specific marker for parenchymal injury, contribution to ASAT levels may originate from muscle cells, kidneys, erythrocytes and the brain in addition to injured hepatocytes (273). Although ALAT and ASAT levels were modulated by SBG, bilirubin and γ-GT, markers of hepatic secretory dysfunction and liver injury, respectively, were not altered by neither oral nor subcutaneous prophylaxis with SBG. Although the experimental animals were outbred we did not expect the broad range of aminotransferase values obtained.

Elevated plasma levels of urea and creatinine, were used as indicators of renal dysfunction. However, these commonly used markers are not ideal as they need time to accumulate in blood, fail to reflect dynamic changes in glomerular filtration rate and do not reflect genuine kidney injury (274).

Also of note, blood chemistry may not always correlate well with clinical disease as abnormal plasma levels of disease markers may be reported without signs of disease and *vice versa*. Histological analysis to confirm the proposed organprotective effect of SBG was not performed.

4.4 Multiplex bead array cytokine/chemokine measurement

Cytokine- and chemokine levels in rat plasma and mouse serum were measured using species-specific multiplex bead-based array assays from Bio-Rad Laboratories on the Luminex 100 technology platform. The assays were carried out according to manufacturer's instructions. The assay is based on dyed beads as the solid phase for sandwich immuno-detection with cytokine/chemokine specific pairs of mAbs. Beads are classified based on their dye intensity and one bead population is coupled with one target specificity. Multiple bead populations, each conjugated with different capture mAbs, may be mixed to allow

simultaneously detection of up to 100 different analytes. The corresponding reporter mAbs are biotinylated for subsequent labeling with a fluorescent reporter and the recorded reporter intensity correlates with the amount of a given analyte in the sample.

Collection of data from numerous beads per analyte provides statistical rigor to the reported signal intensity. The method requires only small samples (<10μl), is semi-automated, requires limited sample handling and generates a lot of data from each sample. The assays include standards for direct calculations of concentrations, are sensitive (<10pg/ml) and have a large dynamic range (5 log) which reduce the need for sample dilution and optimization. Potential cross reactivity is clearly an issue when performing multiplex analysis. Importantly, the assays employed have been validated by the manufacturer and cross reactivity is certified to be negligible. Repeated freezing and thawing of the samples as well as high lipid content and hemolysis will affect the analysis negatively.

Of note, comparison of results obtained using bead-based multiplex arrays with enzyme-linked immunosorbent assays (ELISAs) or bead arrays form other manufacturers or alternative technology platforms is not straight forward. Although a good correlation is reported, quantitative values may differ significantly. However, variability is reduced if identical pairs of mAb and similar diluents and standards are employed (275-277).

4.5 Histological assessment

Histology, the study of the microscopic anatomy of cells and tissues, is a powerful diagnostic tool that permits studies of changes in tissues *in situ*. Tissue samples collected for histological analyses were fixed in neutral buffered formalin, which preserve the tissue morphology well, processed using an automated tissue processor and embedded in paraffin.

Hematoxylin and eosin (H&E) stained sections of colon segments were evaluated, for the degree of inflammation, extent of tissue damage and presence of epithelial regeneration based on a semi-quantitative scoring system adapted from Siegmund *et al*. (278) (Paper II, Table 1). Although multiple (proximal, medial and distal) colon segments from each experimental animal were scored separately, we can not rule out that patchy distribution of diseased tissue may have affected the results. Ideally, multiple serial sections within each colon segment, alternatively a longitudinally oriented section, should have been analyzed to address this potential pitfall.

MLN cross section area, a measure of MLN size, was calculated by analyzing microphotographs of H&E sections using a build in feature in the microscope imaging software (Paper III). Considering that the MLNs are asymmetrical structures of variable shapes, the orientation of the LN is of importance when cutting sections for morphological assessment and size estimation. Also of note, repeated sectioning produce sections with gradually increasing or decreasing area depending on the starting point of the first section and this is likely to vary from sample to sample. Although, systematic serial sectioning of entire MLNs was not carried out, repeated sectioning produced results with the same tendency.

Alcian blue (AB) and periodic acid-Schiff (PAS) staining, marking acidic- and neutral mucopolysaccharides, respectively, enhanced recognition of mucus secreting goblet cells in the epithelium of the distal colon (Paper III). The number of AB/PAS positive cells (goblet cells) in a representative area of a section was counted and expressed as the average number per 20 crypts. The intra crypt distribution of goblet cells was expressed as the number of AB/PAS positive cells present in the lower-, central- and upper 1/3 of the crypts.

To eliminate bias in the morphometric examinations, all microscopy, inflammation grading and image analysis was performed by an examiner blinded to the sample identity.

4.6 Immunofluorescence assays

Immunofluorescence assays exploit the antigen specificity and target binding properties of Abs, which are directly or indirectly labeled with fluorophors that can be readily detected. Immunofluorescence assays are commonly divided into histochemistry- and cytochemistry assays; dealing with tissue samples or cell smears and suspensions, respectively. In Paper III we took an immunofluorescence histochemistry approach to study intestinal epithelial proliferation and IELs, whereas immunofluorescence cytochemistry was employed to address the leukocyte composition of secondary lymphoid organs and whole blood after oral SBG treatment.

4.6.1 Immunohistochemistry

Ki67 antigen is a cell cycle related nuclear protein expressed in all phases of the active cell cycle and hence, a commonly used proliferation marker (279). CD3, a pan-T cell marker, was used to identify T cells in the epithelium. Both primary Abs employed were raised against antigens of human origin, but was confirmed to cross react with several species, including mice. Ki67-antigen and CD3 expression was visualized using a fluorophor conjugated secondary detection Ab. Naturally, the specificity of the Abs employed is of critical importance. Also of decisive importance is the antigen epitope accessibility. Tissue fixation in formalin leads to protein-protein and protein-nucleic acid cross-linking by formation of methylen bridges (280). Although this contributes to excellent morphology conservation, antigenic epitopes may become “masked”, rendering the material largely unavailable for Ab binding and thus, immunohistochemistry analysis. Fortunately, antigen retrieval procedures may reverse epitope distortion (280). We successfully used the Ki67 and anti-CD3 Abs on deparafinized formalin fixed sections following antigen retrieval in

Paper III. Immunohistochemistry is a relatively insensitive method and the evaluation of specific staining relies on the signal-to-noise ratio. Thus, careful titration of Abs and the use of irrelevant negative control Abs is essential. We titrated the primary Abs using serial sections of the study biopsies and employed a primary irrelevant antibody at the same concentration. Unspecific staining was negligible.

Sections stained with anti-CD3 Ab were studied directly in a fluorescence microscope and digital photomicrographs of Ki67 stained sections were evaluated by an examiner blinded to the sample identity. CD3 positive cells clearly located within the epithelium were considered to be IELs. IELs were scarce in the distal colon and the entire circumference of the colon section was screened for positive cells.

Analysis of topographic cell distribution depends on proper tissue orientation. Photomicrographs of intact and well oriented crypts were chosen for analysis of epithelial proliferation. The number of Ki67 positive cells was counted and expressed as the average number per crypt, counting 8 or more crypts. The distribution of Ki67 positive cells, a measure of the proliferating zone, was expressed as the height of the Ki67 positive zone in relation to the total crypt height.

4.6.2 Flow cytometry

In Paper III, flow cytometry analysis was used to study the distribution of major leukocyte populations in dispersed cells isolated from secondary lymphoid organs (PP, MLN, inguinal LN, spleen) and whole blood. Flow cytometry is more sensitive than is immunohistological techniques and by far more suited for objective cell enumeration. The instrument can handle large cell numbers in a short period of time which adds statistical rigor to the analysis. Detection of multiple fluorophores allows for simultaneous analysis of multiple cell subsets. A major drawback of flow cytometry analysis on dispersed cells from solid organs is the

lack of information on the spatial distribution of the various cell types within the organ from which they originated. In this respect flow cytometry analysis is complementary to immunohistochemistry. One should also be aware of the potential pitfall that the cell isolation procedure may affect the cells, consequently changing the expression profiles of molecular markers selected for analysis. To minimize sample handling bias, all samples were treated identically. All antibodies were titrated, using splenocytes, to identify the optimal working dilutions. Unstained cells from all tissues analyzed served as controls.

4.7 Statistics

The statistical analysis is detailed in each paper. In general measurements involving three or more experimental groups and parameters evaluated at consecutive time points, including body weight data, fluid consumption, mean arterial blood pressure and cytokine measurements were analyzed by two-way analysis of variance (ANOVA) with Bonferroni post test. Measurements involving three or more experimental groups and parameters evaluated at one time point only, including indicators of organ dysfunction and injury, were analyzed by ANOVA with Bonferroni post test. Plasma β-glucan level data failed the D'Angostino & Pearson Omnibus Normality Test and were analyzed by non-parametric ANOVA (Kruskall-Wallis Test) with Dunn's Multiple Comparison Test. Categorical data on mortality was expressed as percent survival and analyzed with the log-rank test. Measurements involving comparison of two experimental groups, including histopathology score, colon length, thymus weight, PP number, MLN cross section area, goblet cell number, epithelial proliferation and IEL number and lymphocyte composition were analyzed using the non-parametric Mann-Whitney test.

We have studied a limited number of experimental animals, and infer that what we observe in our samples is also true for the populations from which the samples were taken.

The presence of outliers, i.e. data points far from neighbouring data points, may seriously bias or distort data analysis, making generalizations about the population invalid. In particular, outliers may bias data analysis when studying small sample sizes. Obviously, suspect outliers can represent genuine and exiting findings or originate from abnormal experimental animals, but can also simply be an error of unknown origin. There may be good reasons both to keep and eliminate outliers, thus handling of outliers is a delicate business. Highly suspect outlier values, unlikely to represent random sampling from a Gaussian population, were identified by Grubbs' outlier detection test and excluded from further analysis (281).

All statistical analysis was carried out using GraphPad Prism, version 4 (GraphPad Software, San Diego, CA, USA). Differences at $P<0.05$ were considered statistically significant.

5 General discussion

A well functioning immune system is a powerful tool to preserve homeostasis and host wellbeing. A dysregulated immune system, on the other hand, may cause sever pathology. Accordingly, modulation of host immunity emerges as a valuable approach to prevent and treat disease. β-Glucans are potent immunomodulators with evident clinical potential. However, our comprehension of the underlying mechanisms explaining how β-glucans modulate host immunity remains insufficient. The objective of this investigation was to study how β-glucans work, with emphasis on oral drug administration and mucosal biology. To this end we examined the effect of the study drug on healthy experimental animals and in two animal models of disease.

The rational for focusing on oral administration and mucosal biology was dual. First, from a drug delivery point of view the oral route is desirable. It is non-invasive, easily permit repeated drug administration and self medication with minimal user supervision. Hence, confirmation of oral efficacy is of great interest. Secondly, oral administration deposits the drug in proximity to inductive sites and effector sites of the mucosal immune system. We proposed that the effect of the study drug on the gut and mucosal immune system could shed additional light on the mechanisms by which β-glucans work.

It should be stressed that we have only studied one specific β-glucan product, namely SBG, a *Saccharomyces cerevisiae*-derived water-soluble β-1,3/1,6-glucan provided by Biotec Pharmacon ASA. Importantly, β-glucans are heterogeneous by nature, standard assays to validate β-glucan bioactivity are lacking and so are systematic head-to-head comparisons of various β-glucan preparations. Thus, we can not infer from our data, with confidence, that what we observed with SBG is also true for β-glucans in general.

In Paper I, we set out to investigate the effect of SBG in a rat model of endotoxemia and shock-associated organ injury, as a benchmark test for biological response modifier potential. We compared the efficacy of systemic vs. oral drug delivery and sought to establish whether orally administered SBG was absorbed from the gastrointestinal tract to the circulation. Parenteral delivery has dominated in the field of β-glucan research. Efforts following the pioneering work by Suzuki and co-workers (147) in the late 1980's, has established that β-glucans are potent biological response modifiers also when delivered via the oral route. However, the extent of absorption and translocation from the gastrointestinal tract to blood, and the functional importance of this, remains poorly understood.

We report in Paper I that plasma from animals given SBG orally were clearly positive for β-glucan content. However, plasma levels indicated that only a very small fraction of the orally administered SBG translocated to systemic circulation. On the other hand, we can not rule out that absorbed SBG is rapidly eliminated from plasma. In fact, Ozment-Skelton *et al.*, recently demonstrated that β-glucan was cleared from systemic circulation by circulating peripheral leukocytes, splenocytes, and peritoneal cells (165). Hence, plasma levels might not be a meaningful measure of uptake.

Our finding is in line with absorption data published by Hong *et al*. and Rice *et al*., but deviates from what Lehne *et al*. reported for SBG in humans (123;137;153). It should be stressed that, although we detected β-glucan in plasma following oral administration and found this to be instructive, a kinetic study is warranted to examine gastrointestinal absorption of SBG in detail. Also, our study was designed to compare subcutaneously treated rats and rats given SBG by oral gavage in a rat model of endotoxemia and was inadequate for pharmacokinetic comparison between the two routs of delivery.

β-Glucans reportedly mediate protection against sepsis and sepsis-associated organ injury following both systemic and oral administration (100;124;282). In line with these

reports, SBG attenuated critical liver and kidney injury mediated by the septic shock-like reaction induced by LPS infusion. One mechanism of organ injury in sepsis is oxidative damage due to the generation of free radicals (reviewed in 283). Sener *et al.*, proposed that β-glucans are organoprotective by counteracting oxidative injury in sepsis (124) and, although controversial, β-glucans have been suggested to have anti-oxidant properties (284).

Hypoperfusion is a major contributor to shock-associated MODS (285). Interestingly, SBG produced a beneficial effect on mean arterial blood pressure (MAP) in endotoxaemic rats. This is a novel asset of β-glucans, first described in Paper I. We propose that SBG protects against LPS-induced organ injury, in part, by improving organ perfusion. The mechanism underlying the effect of SBG on hemodynamics remains unknown. We speculate that SBG modulate the release of inflammatory mediators causing indirect protective downstream effects on vascular stability and thus, blood pressure.

We identified a discrepancy between the effects of oral and subcutaneous administration of β-glucan. Renal protection was only seen following oral SBG treatment. Furthermore, the 40-fold higher β-glucan plasma level obtained using systemic drug delivery did not correlate with superior protection of the liver, recovery of MAP or modulation of cytokines seen with oral administration. Although we have not performed a proper dose-response study, our data suggests that the plasma level does not correlate with the beneficial effect of SBG for any of the parameters tested. This suggests to us that discrete cellular and molecular mechanism may be involved in the mode of action depending on the route of delivery.

In Paper II we investigated the effect of SBG on experimental colitis. Nosalova *et al.* reported that Pleuran, a water insoluble β-glucan from *Pleurotus ostreatus*, protected against colon injury induced by instillation of acetic acid (139). In our study, SBG delivered orally mediated a protective effect on all investigated parameters of disease severity in DSS-

induced colitis. Colitis-associated body weight loss and mortality was significantly reduced in SBG treated mice. Furthermore, thymic involution, colon shortening and histological inflammation and intestinal ulceration were attenuated. DSS induces colitis via toxic effects on the epithelium, resulting in reduced mucosal barrier function and subsequent inflammation and ulceration (235). We hypothesize that SBG protects against DSS-induced experimental UC, in part, via beneficial effects on the epithelial barrier. In Paper III we demonstrate that oral SBG administration increased colonic epithelial proliferation in healthy mice. Our data suggests that SBG may enhance epithelial restitution and we find it plausible that increased epithelial proliferation may reinforce intestinal barrier function and thus resistance to DSS and influx of luminal content.

SBG emerge as a promising drug candidate for IBD therapy in humans. IBD is a chronic-relapsing disease associated with impaired intestinal barrier function and it remains to be established whether SBG therapy is beneficial in established or chronic colitis. Although clinical trials are warranted, we hypothesize that SBG therapy may prolong the remission phase in IBD patients, consequently reducing the need for harsh treatments, including immunosuppressive drugs, associated with adverse reactions. It is well established that chronic colitis is a major risk factor in development of colorectal cancer (reviewed in 286). Furthermore, β-glucans are known to posses anti-tumor properties (reviewed in 108). Accordingly, although speculative, we propose that SBG might have dual protective effects in IBD patients by (I) limiting intestinal injury and inflammation associated with cancer development, and (II) by direct anti-cancer effects.

SBG administration attenuated systemic inflammation both in LPS-induced shock (Paper I) and DSS-induced colitis (Paper II). Release of mediators of inflammation, including cytokines, is a central event in the pathogenesis of sepsis and colitis. In Paper I we reported that LPS-induced rise in plasma IFNγ and IL-6 was attenuated at later time points

following both oral and systemic SBG administration, whereas the rise in IL-1β and IL-2 levels was attenuated in the oral group only. Our data suggests that these mediators implicated in the early phase of sepsis may return more rapidly to baseline levels in SBG treated rats. It has been suggested that the organ-protective capacity of β-glucans in sepsis and trauma models is associated with reduction in TNFα levels (124;287). We did not identify a significant effect of SBG on plasma TNFα levels in Paper I, regardless of delivery route. The subtle changes in the cytokine profile are unlikely to explain the beneficial effect of SBG on organ function and hemodynamics in the endotoxaemic rat. However, our data convincingly demonstrate that SBG prophylaxis modulated systemic inflammation induced by LPS infusion.

DSS-induced colitis is associated with systemic inflammation. In Paper II we demonstrate that oral SBG therapy limited the release of key cytokines implicated in IBD pathogenesis (288), including TNFα, IFNγ, IL-1β, IL-6, IL-17. Interestingly, several of these pro-inflammatory mediators are targets for novel IBD therapies (289). In general SBG treated colitic mice had a cytokine profile very similar to that of healthy control animals. We believe that the systemic inflammation is secondary to the DSS-induced intestinal inflammation. The reduced level of inflammatory mediators in circulation in SBG treated colitic mice is likely to reflect the beneficial effect of SBG on the integrity of the intestine.

Importantly, data from the LPS-shock model and DSS-colitis model, on the effect of SBG on markers of systemic inflammation, correspond very well. Interestingly SBG did not have a major impact on cytokine profiles in the absence of inflammation. With one exception, a small increase in IL-1β following subcutaneous SBG injection, none of the cytokines or chemokines studied was significantly altered by SBG administration to healthy animals. The literature on the effects of β-glucans on cytokines is contradictory and the

mechanisms by which β-glucans modulate cytokine expression are only partly understood (see section 1.4.4).

β-Glucans reportedly enhance the mucosal immune system (137;138). Moreover, Rice *et al.*, demonstrated that orally administered β-glucans are captured and internalized by intestinal epithelial cells and GALT cells (123). We sought to identify effects of oral SBG administration on GALT and the colonic epithelium, mucosal inductive- and effector sites, respectively. In Paper III we demonstrate that oral SBG stimulated expansion of PPs and MLNs. Although speculative, we find it plausible that β-glucan-laden cells migrating from the intestinal epithelium to GALT may contribute to the observed expansion of MLNs and PPs. The nature and maturation/activation status of these cells remains unknown. Interestingly, Rice *et al*. reported that PP macrophages and DCs up-regulate Dectin-1 and TLR2 expression, respectively, in response to β-glucan internalization (123). Conceivably, these cells may modulate the inductive events in GALT, and consequently impact the resulting mucosal immune response. Follow-up studies are warranted to further characterize this novel finding and to establish whether GALT expansion results in superior immune defense.

Tsukada *et al*., reported that oral β-glucan administration increase the number of small intestine IELs, mucsal effector cells (138). IELs were scarce in the distal colon and, in contrast to Tsukada, we did not observe a difference between SBG-treated mice and controls. Furthermore, oral SBG administration did not change the number or distribution of goblet cells in the colon.

Oral SBG delivery significantly increased colonic epithelial proliferation. To the best of our knowledge, we are the first to report that β-glucans stimulate intestinal epithelial proliferation. Further investigations are warranted to elucidate how SBG stimulate epithelial

renewal. Data in Paper III supports the hypothesis that β-glucans may enhance host protection, in part, by effects on the mucosal immune system.

6 Concluding remarks and future perspectives

β-Glucans continue to attract considerable interest due to their evident potential for use in human and veterinary medicine. Efforts to characterize the underlying mechanisms of action have advanced basic research on these BRMs. I hope that this thesis add new knowledge to this exiting field of study and contribute to move it forward.

A dysregulated immune system cause severe pathology in septic shock with MODS and in IBD. We have employed animal disease models, mimicking key features of these inflammatory disorders, as well as healthy experimental animals in an attempt to elucidate how β-glucans, SBG explicitly, modulate host immunity and thereby promote health.

The present study demonstrates that:

- SBG was well tolerated in the animal models employed.
- Only a minute fraction of orally administered SBG translocated to systemic circulation.
- Oral and systemic SBG administration attenuated LPS-induced shock and shock-associated organ injury.
- SBG had a beneficial effect on hemodynamics in endotoxaemic rats suggesting that the mechanism for organ protection involves superior organ perfusion.
- The level of protection was not strictly dependent on β-glucan plasma levels.
- SBG prophylaxis attenuated systemic levels of mediators of inflammation both in endotoxic rats and colitic mice.
- Oral SBG administration protected against mortality and morbidity in experimental UC.
- Oral SBG administration had an effect on mucosal inductive- (PPs and MLNs) and effector (intestinal epithelium) sites.

We propose that SBG have a potential for clinical application in pre-/post surgery care. For instance as prophylactic treatment in high risk patients scheduled for surgery, to reduce the risk and severity of post surgery complications like severe sepsis. Although clinical trials are warranted, we suggest that SBG has a potential for use in IBD treatment. Furthermore, SBG affected essential inductive sites of the mucosal immune system, suggesting that, although speculative, SBG may have adjuvant properties that can boost the effect of mucosal vaccines. Our data support the idea that β-glucans enhance host protection, in part, by effects on the mucosal immune system. Yet, the precise mechanisms by which β-glucans work remains unclear.

Future plans and work in progress aims at elucidating the cellular and molecular mechanisms behind our findings following SBG administration. We aim at identifying β-glucan target cells by employing experimental animals that lack essential immune cells and/or by tracing the fate of orally administered SBG.

7 References

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Paper I

ORAL AND SYSTEMIC ADMINISTRATION OF β-GLUCAN PROTECTS AGAINST LIPOPOLYSACCHARIDE-INDUCED SHOCK AND ORGAN INJURY IN RATS

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Oral and systemic administration of β-glucan protects against lipopolysaccharide-induced shock and organ injury in rats

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Summary

β-Glucans are glucose polymers with a variety of stimulatory effects on the immune system. The objective of this study was to determine the effect of prophylactic oral administration of soluble *Saccharomyces cerevisiae*-derived β-1,3/1,6-glucan (SBG) on the outcome of experimental endotoxaemia and shock-associated organ injury. Male Wistar rats were pretreated with SBG orally (SBGpo, 20 mg/kg/day) for 14 days, subcutaneously (SBGsc, 2 mg/kg/day) for 3 days, or vehicle (placebo). Rats were anaesthetized and subjected to endotoxaemia by intravenous infusion of *Escherichia coli* lipopolysaccharide (LPS) (6 mg/kg) or saline infusion (sham). We observed significant levels of plasma β-glucan in the SBGpo group ($P < 0{\cdot}5$), although the SBGsc group had levels approximately 40-fold higher despite a 10-fold lower dose. SBG prophylaxis caused enhanced blood pressure recovery following LPS-induced blood pressure collapse. Oral treatment with SBG attenuated the LPS-induced rise in plasma creatinine levels ($P < 0{\cdot}05$), indicating protection against renal injury. SBG also attenuated the plasma levels of aspartate aminotransferase and alanine aminotransferase (SBGpo, $P < 0{\cdot}01$; SBGsc, $P < 0{\cdot}01$), indicating protection against LPS-induced hepatic injury. A moderate increase in baseline interleukin (IL)-1β levels was observed in the SBGsc group ($P < 0{\cdot}05$). In the LPS-challenged rats, plasma levels of proinflammatory cytokines was moderately reduced in both SBG-treated groups compared to placebo. SBG treatment, particularly oral administration, had a striking effect on the haemodynamics of LPS-treated rats, although only a minute fraction of the orally administered β-glucan translocated to the circulation. Enhanced organ perfusion may thus be responsible for the attenuated levels of indicators of kidney and liver injury seen in SBG-treated rats.

Keywords: β-glucan, endotoxaemia, shock, multiple organ dysfunction, syndrome (MODS), immunomodulation

Introduction

Sepsis, a systemic host response to infection, with multiple organ dysfunction syndrome (MODS), continues to be the main cause of morbidity and mortality in intensive care units [1]. Systemic administration of β-glucans has been shown to mediate protection against sepsis and MODS [2,3], modulate cytokine profiles [4,5] and prolong survival [5–8] in experimental animal models. β-Glucans have also been shown to possess an array of beneficial properties, including enhancing protection against infections [6,8,9], tumour development [10,11] and radiation injury [12,13], lowering plasma lipids [14,15], increasing salivary IgA secretion [16], promoting wound healing [17], mediating protection against myocardial ischaemia and reperfusion injury [18] as well as restoring haematopoiesis following bone marrow injury [19]. These heterogeneous glucose polymers consist of a backbone of β-(1→3)-linked β-D-glucopyranosyl units with β-(1→6)-linked side chains of varying length and distribution. They are major cell wall structural components in fungi and are also found in some bacteria and plants (reviewed in [20]).

Recognition of bacterial components such as Gram-negative lipopolysaccharide (LPS) by the innate immune system trigger the release of potent mediators of inflammation that result in an exaggerated pathogenic inflammatory

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response in the circulation and vital organs. The proinflammatory cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-1α and IL-6 are thought to be important mediators during the early stage of sepsis and MODS. Synergistic effects between these cytokines, secondary inflammatory mediators and reactive oxygen intermediates have been shown to be involved in the pathogenesis of sepsis and associated organ injury (reviewed in [21,22]).

The immunomodulating potential of β-glucans has been attributed to their ability to prime and activate leucocytes. Several receptors, expressed both by immune and non-immune cells, have been implicated in recognition of β-glucans, including the type 3 complement receptor, scavenger receptors, lactocylceramide and dectin-1 (reviewed in [23]). Several reports indicate that orally administered β-glucans may exert biological effects [2,10,11,24–26]. However, no reports on the absorption and pharmacokinetics of orally administered soluble glucans have been available until recently [8].

We designed this study to investigate the protective capacity of orally administered soluble β-glucan in an experimental rat model of LPS-induced shock and shock-associated organ injury. The specific objectives of the investigation were: (1) to establish whether an orally administered *Saccharomyces cerevisiae*-derived water-soluble β-glucan was absorbed from the gastrointestinal tract and translocated to systemic circulation, (2) to examine the effect of this β-glucan on haemodynamic parameters during the progression of endotoxin-induced shock, (3) to investigate whether β-glucan administration mediate protection against development of organ injury/dysfunction and (4) to study the effect of β-glucan on systemic inflammation.

Materials and methods

Materials

Endotoxin free (< 0·5 EU/ml) SBG, a *S. cerevisiae*-derived water-soluble β-1,3/1,6-glucan was provided by Biotec Pharmacon ASA (Tromsø, Norway). LPS from *Escerichia coli* (B6:026, chromatography purified) was from Sigma (St Louis, MO, USA).

Animals

Male Wistar rats (Taconic Europe, Denmark) were maintained in the minimal disease unit at the Centre for Comparative Medicine at Rikshospitalet-Radiumhospitalet University Hospital, Oslo, Norway for at least 1 week before they were entered into experiments. Animals were supplied with water (reversed osmosis and ionic-exchange-treated) and fed conventionally (Rat and Mouse no. 3 Breeding, Special Diets Services, Witham, Essex, UK) *ad libitum*. Cages were kept at 21 ± 1°C and 55 ± 10% relative humidity. Light conditions consisted of alternating 12-h light/dark cycles with 1 h dusk and dawn with gradual decrease or increase of light intensity. The present investigation was approved by the national ethics committee for animal experiments.

Surgical procedure and sample collection

Rats [275 (244–306) g; mean and (range)] were anaesthetized with thiopental sodium [intraval sodium, 120 mg/kg, intraperitoneally (i.p.)]. Anaesthesia was maintained by supplementary injections of thiopental sodium as required and the rectal temperature was maintained at 37°C using a homeothermic blanket (Harvard Apparatus, Holliston, MA, USA). Surgery was carried out essentially as described previously [27]. Briefly, the trachea was cannulated to facilitate respiration. The right carotid artery was cannulated to facilitate repeated blood sampling and connected to a pressure transducer (Harvard Apparatus) for monitoring of mean arterial blood pressure. The jugular vein was cannulated for the administration of LPS or vehicle (saline). The urine bladder was also cannulated to facilitate urine flow. Cardiovascular parameters were allowed to stabilize for approximately 30 min before animals were subjected to endotoxaemia. Rats were given saline 1 ml/kg/h intravenously (i.v.) throughout the experiment for fluid resuscitation. Blood samples were collected from the cannulated artery immediately prior to LPS or saline (sham) infusion and at 1, 3 and 6 h thereafter. Blood samples were collected in heparinized microcentrifuge tubes and plasma was obtained by immediate centrifugation at 2200 ***g*** for 3 min at room temperature. Plasma samples were stored at –70°C for subsequent analysis.

Experimental design

Rats were divided into the following experimental groups:

1. SBGpo group: SBG was administered orally to rats by tube feeding [20 mg/kg body weight (bw), 400–600 μl of a 10-mg/ml solution] daily for 14 days prior to surgery. Following surgery, LPS (6 mg/kg) ($n = 8$) or vehicle (sham, $n = 5$) was administered via the jugular vein over a 10-min period.
2. SBGsc group: SBG was administered in three subcutaneous injections (2 mg/kg bw, 100–120 μl of a 5-mg/ml solution) 48 h, 24 h and immediately prior to surgery. Following surgery, LPS (6 mg/kg) ($n = 8$) or vehicle (sham, $n = 5$) was administered via the jugular vein over a 10-min period.
3. Placebo group: rats received subcutaneous injections or oral instillation of equal volumes of vehicle [phosphate buffered saline (PBS)] as described above for SBG. Following surgery, LPS (6 mg/kg) ($n = 8$) or vehicle (sham, $n = 10$) was administered via the jugular vein over a 10-min period.

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Measurement of biochemical indicators of organ dysfunction and injury

Liver injury was assessed by measuring rise in plasma levels of alanine aminotransferase (ALAT, a specific marker for parenchymal injury), aspartate aminotransferase (ASAT, a non-specific marker for parenchymal injury), bilirubin (a marker of hepatic secretory dysfunction) and γ-glutamyltransferase (γ-GT, and indicator of liver dysfunction and liver injury). Renal dysfunction was assessed by measuring the rises in serum levels of urea and creatinine (an indicator of reduced glomerular filtration rate), whereas pancreatic injury was assessed by measuring amylase. All the above-mentioned organ function markers were measured by enzymatic photometric assays (Roche Automated Clinical Chemistry Analyser; Roche Diagnostics, Indianapolis, USA).

Measurement of plasma cytokines

Plasma cytokine levels were measured with a rat-specific Bio-Plex multiplex suspension array assay (Bio-Rad Laboratories, Hercules, CA, USA). Measurements and data analysis were performed on a Bio-Plex system, powered by xMAP technology by Luminex, operated with Bio-Plex Manager 4·0 software (Bio-Rad Laboratories). The instrument was calibrated with the CAL2 settings (LOW RP1 target value) using Bio-Plex calibration beads (Bio-Rad Laboratories). All samples were diluted 1 : 4 in Bio-Plex rat serum sample diluent buffer (Bio-Rad Laboratories) and the assays carried out according to manufacturer's instructions. Plasma samples were analysed as single samples, whereas standards were analysed in duplicate.

Measurement of plasma β-glucan

Plasma β-glucan levels were determined with Fungitell™, a 1,3-β-D-glucan specific protease zymogen-based colorimetric assay according to the manufacturer's instructions (Associates of Cape Cod Inc., East Falmouth, MA, USA). Samples and standards were analysed in duplicate.

Statistical analysis

Plasma β-glucan levels are expressed as mean ± standard error of the mean (s.e.m.) values. These data failed the D'Angostino and Pearson omnibus normality test and were analysed by non-parametric analysis of variance (ANOVA) (Kruskall–Wallis test) with Dunn's multiple comparison test. Mean arterial blood pressure and cytokine measurements are expressed as mean ± s.e.m. values and analysed by two-way ANOVA with Bonferroni's multiple comparison test. Data representing plasma levels of indicators of organ dysfunction and injury are analysed by ANOVA with Bonferroni's multiple comparison test. Highly suspect outlier values were identified by Grubb's outlier detection test and excluded from further analysis. All statistical analysis was carried out using GraphPad Prism, version 4 (GraphPad Software, San Diego, CA, USA). Differences at $P = 0{\cdot}05$ were considered statistically significant.

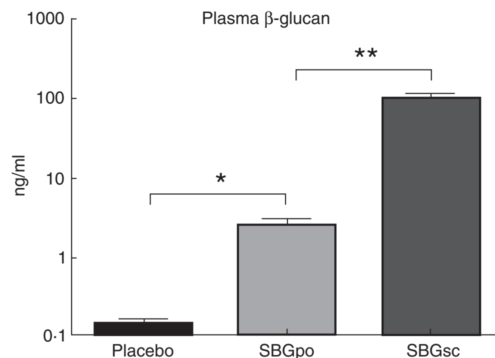

Fig. 1. Plasma β-glucan concentration following oral and subcutaneous soluble *Saccharomyces cerevisiae*-derived β-1,3/1,6-glucan (SBG) administration. SBG, a *S. cerevisiae*-derived water-soluble β-glucan, was administered by oral gavage [20 mg/kg body weight (bw)] daily for 14 days (SBGpo, $n = 13$) or as subcutaneous injections (2 mg/kg bw) on 3 consecutive days (SBGsc, $n = 13$) to male Wistar rats. Placebo control animals received corresponding volumes of phosphate-buffered saline ($n = 17$). Blood samples were collected from the cannulated carotid artery. Plasma β-glucan levels were measured with a 1,3-β-D-glucan specific, protease zymogen-based, colorimetric assay. Data are presented as mean ± s.e.m. on a log scale. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ as determined by non-parametric analysis of variance with Dunn's multiple Comparison Test.

Results

Absorption and translocation of orally administered SBG

Male Wistar rats were divided into arbitrarily three groups: group 1 received 20 mg SBG/kg bw daily via feeding tube for 14 days prior to surgery (SBGpo); group 2 received 2 mg SBG/kg bw subcutaneously 48 h, 24 h and immediately prior to surgery (SBGsc); and group 3 received PBS orally or subcutaneously prior to surgery (placebo). To determine the extent of β-glucan absorption into circulation in the SBGpo group, plasma concentration of β-glucan was examined by means of the Fungitell™ method. We found significant levels of plasma β-glucan in the SBGpo group compared with placebo ($P < 0{\cdot}05$), although the SBGsc group had levels approximately 40-fold higher despite a 10-fold lower dose (Fig. 1).

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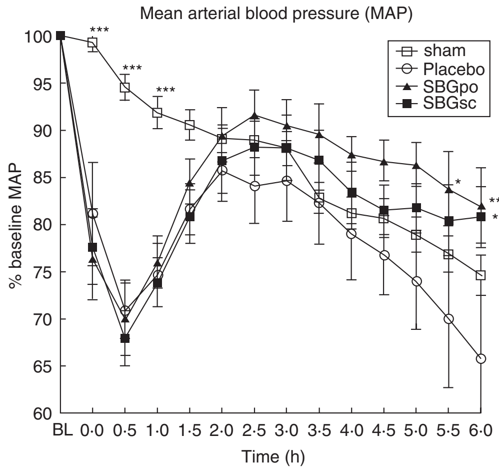

Fig. 2. Effect of soluble *Saccharomyces cerevisiae*-derived β-1,3/1,6-glucan (SBG) administration on mean arterial blood pressure (MAP) in endotoxaemia. Rats were pretreated with SBG or placebo as described, followed by anaesthesia and general surgery to catheterize the jugular vein and carotid artery. MAP was allowed to stabilize for approximately 30 min before intravenous infusion of lipopolysaccharide (LPS) (SBGpo $n = 8$, SBGsc $n = 8$ and placebo $n = 8$) or vehicle (sham $n = 20$) over a 10-min period. The end of the infusion defined the 0 h time-point. MAP was recorded at baseline (BL), 0 h and every 30 min until the 6 h time-point at which the rats were sacrificed. Overall mean MAP at baseline was 142 ± 2 mmHg, with variation between all groups of animals ranging from 136 ± 5 to 147 ± 7 mmHg. No statistically significant differences were observed between the sham animals, regardless of SBG or placebo pretreatment (SBGpo $n = 5$, SBGsc $n = 5$ and placebo $n = 10$) (data not shown), thus the data were combined to one sham group. MAP dynamics are presented as percentage of BL, mean ± s.e.m. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$ *versus* placebo as determined by two-way analysis of variance with Bonferroni's multiple comparison test.

Effect of SBG on mean arterial blood pressure in endotoxaemia

To investigate the effect of prophylactic SBG-treatment on haemodynamics during LPS-induced shock, mean arterial blood pressure (MAP) was monitored. Baseline levels of MAP for all groups of animals ranged from 136 ± 5 to 147 ± 7 mmHg, and did not differ significantly between groups. MAP in sham-control animals decreased gradually throughout the experiment (Fig. 2). SBG administration, mucosal or systemic, did not affect MAP of the sham-control animals significantly (data not shown). Systemic administration of LPS caused an immediate and dramatic decrease in MAP. Approximately 40 min after initiation of the LPS infusion, MAP levels started to increase and continued to do so until the 2·5 h time-point at which the blood pressure again started to decrease slowly (Fig. 2). Oral administration of SBG enhanced the recovery of MAP compared with placebo treatment and MAP remained higher in this group throughout the experiment ($P < 0{\cdot}05$ at 5·5 h and $P < 0{\cdot}01$ at 6 h *versus* placebo). Subcutaneous injection of SBG also resulted in enhanced MAP recovery ($P < 0{\cdot}05$ at 6 h *versus* placebo), although to a lesser extent than did oral SBG prophylaxis.

Effect of SBG on indicators of organ injury

Organ injury was assessed by quantification of plasma level of various biochemical markers 6 h following intravenous LPS administration. LPS infusion caused a twofold increase in levels of plasma creatinine compared to sham ($P < 0{\cdot}001$). The creatinine level in the SBGpo group was reduced by approximately 25% compared to the placebo group $P < 0{\cdot}05$). This effect was not observed in the SBGsc group (Fig. 3a). LPS infusion also caused a 3·5-fold increase in the plasma urea level compared to sham ($P < 0{\cdot}001$). Although not statistically significant, the SBGpo group had a minor reduction in plasma urea compared to placebo control animals after LPS administration ($P = 0{\cdot}07$). The plasma urea level in the SBGsc group remained at the placebo level and was significantly higher than in the SBGpo group ($P < 0{\cdot}05$) (Fig. 3b).

LPS-infusion to placebo-treated rats caused an approximately 10-fold elevation in ASAT level compared to sham ($P < 0{\cdot}001$). Both the SBGpo group ($P = 0{\cdot}01$) and the SBGsc group ($P = 0{\cdot}01$) had significantly attenuated plasma ASAT levels compared with placebo following LPS infusion (Fig. 4a). Intravenous LPS administration caused an approximately 25-fold increase in mean plasma ALAT level compared to sham animals ($P < 0{\cdot}001$). Mean ALAT values in both SBG-treated groups were significantly reduced compared to the placebo-treated animals (SBGpo; $P < 0{\cdot}01$, SBGsc; $P < 0{\cdot}01$) (Fig. 4b).

Administration of LPS also caused increased plasma levels of γ-GT, bilirubin and pancreatic amylase compared to sham animals. However, these variables were not altered by either oral or subcutaneous prophylaxis with SBG (data not shown).

Effect of SBG on baseline plasma cytokine levels

Prophylactic treatment with SBG resulted in a statistically significant increase in plasma level of IL-1α in the SBGsc group ($P < 0{\cdot}05$) compared to placebo-treated animals prior to LPS infusion (Fig. 5a). A slight elevation in IL-2, IL-6, IL-10 and IFN-γ levels was also observed, whereas TNF-α levels were modestly reduced following SBG treatment. These changes were, however, not statistically significant (Fig. 5).

Effect of SBG on plasma cytokine levels following LPS infusion

Blood samples were collected 1, 3 and 6 h after intravenous infusion of LPS, and plasma levels of cytokines were analysed. Administration of LPS caused an immediate and

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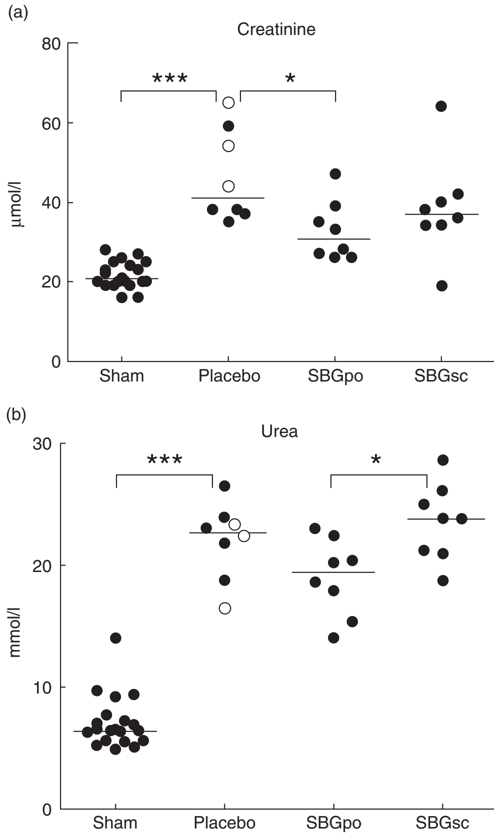

Fig. 3. Effect of soluble *Saccharomyces cerevisiae*-derived β-1,3/1,6-glucan (SBG) prophylaxis on indicators of kidney injury and renal dysfunction in the endotoxic rat. Rats were pretreated with SBG or placebo and underwent surgery as described. At the experimental end-point, 6 h after intravenous infusion of lipopolysaccharide (LPS) (SBGpo $n = 8$, SBGsc $n = 8$ and placebo $n = 8$) or vehicle (sham $n = 20$), plasma samples were collected and examined for levels of creatinine (a) and urea (b) by automated enzymatic photometric assays. No statistically significant differences were observed between the sham animals, regardless of SBG or placebo pretreatment (SBGpo $n = 5$, SBGsc $n = 5$ and placebo $n = 10$) (data not shown), thus the data were combined to one sham group. In the placebo group oral and subcutaneous pretreatment is indicated by open (○) and closed circles (●), respectively. Each data point represents one animal, bars represent median values. *$P < 0{\cdot}05$, ***$P < 0{\cdot}001$ as determined by analysis of variance with Bonferroni's multiple comparison test.

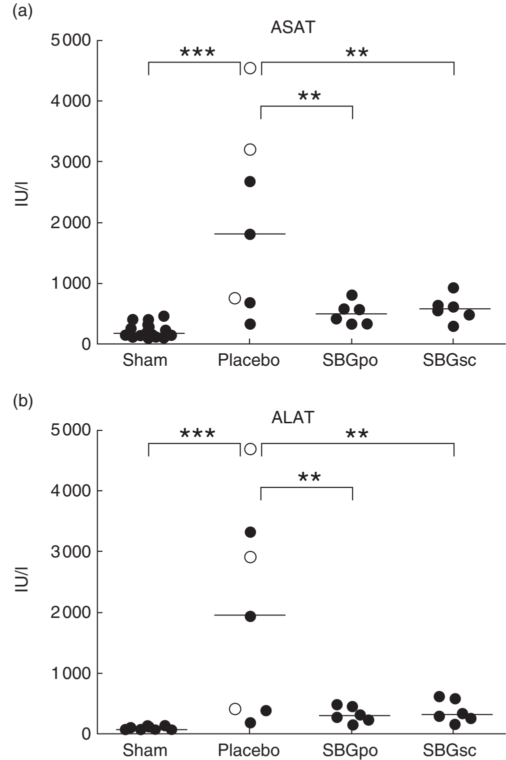

Fig. 4. Effect of soluble *Saccharomyces cerevisiae*-derived β-1,3/1,6-glucan (SBG) prophylaxis on indicators of liver injury and hepatic dysfunction in the endotoxic rat. Rats were pretreated with SBG or placebo and underwent surgery as described. At the experimental end-point, 6 h after intravenous infusion of lipopolysaccharide (LPS) (SBGpo $n = 8$, SBGsc $n = 8$ and placebo $n = 8$) or vehicle (sham 20), plasma samples were collected and examined for levels of aspartate aminotransferase (ASAT) (a) and alanine aminotransferase (ALAT) (b) by automated enzymatic photometric assays. No statistically significant differences were observed between the sham animals, regardless of SBG or placebo pretreatment (SBGpo $n = 5$, SBGsc $n = 5$ and placebo $n = 10$) (data not shown), thus the data were combined to one sham group. In the placebo group oral and subcutaneous pretreatment is indicated by open (○) and closed circles (●), respectively. Each data point represents one animal, bars represent median values. **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$ as determined by analysis of variance with Bonferroni's multiple comparison test.

substantial rise in plasma level of TNF-α (Fig. 5b). Subsequently, levels decreased gradually but remained considerably higher than baseline values even after 6 h. Conversely, the plasma level of IFN-γ increased gradually and remained high at the 6 h time-point (Fig. 5c). Following LPS infusion, plasma levels of IL-1α, IL-2, IL-6 and IL-10 increased rapidly, levelled off and remained at an elevated level throughout the experiment (Fig. 5).

In the SBGsc group, the LPS-induced rise in IFN-γ and IL-6 levels were significantly attenuated (IFN-γ; $P < 0{\cdot}001$,

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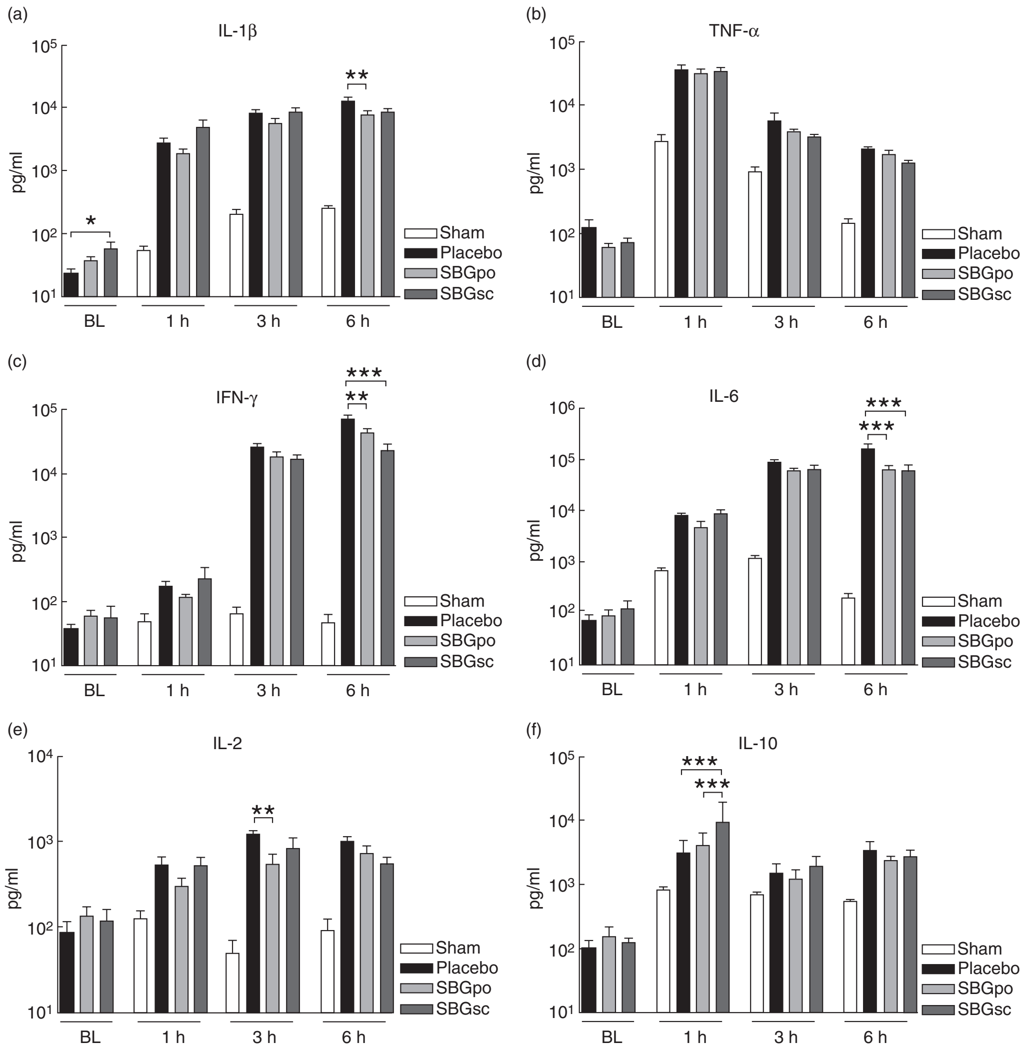

Fig. 5. Effect of soluble *Saccharomyces cerevisiae*-derived β-1,3/1,6-glucan (SBG) administration on plasma cytokine levels. Rats were pretreated with SBG or placebo and underwent surgery as described. Blood samples were collected from the cannulated carotid artery prior to administration of lipopolysaccharide (LPS) or vehicle, BL, and 1, 3 and 6 h after infusion. Plasma levels of tumour necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-6, IL-1-α, IL-2, and IL-10 were analysed using a multiplex bead-array assay. Samples collected prior to LPS injection included SBGpo ($n = 13$), SBGsc ($n = 10$) and placebo ($n = 14$) experimental groups. Samples collected 1, 3 and 6 h after LPS challenge included SBGpo ($n = 8$), SBGsc ($n = 8$) and placebo ($n = 8$) experimental groups. No statistically significant differences were observed between the animals receiving vehicle (sham $n = 20$), regardless of SBG or placebo pretreatment (SBGpo $n = 5$, SBGsc $n = 5$ and placebo $n = 10$) (data not shown), thus the data were combined to one sham group. Data are presented as mean ± s. e.m. *$P < 0{\cdot}05$, **$P< 0{\cdot}01$, ***$P < 0{\cdot}001$ as determined by two-way analysis of variance with Bonferroni's multiple comparison test.

IL-6; $P < 0{\cdot}001$) compared to placebo-treated animals at 6 h (Fig. 5c,d). SBGsc-treated animals also had significantly higher plasma levels of IL-10 compared to both placebo ($P < 0{\cdot}001$) and SBGpo (0·001) at 1 h (Fig. 5f).

In the SBGpo group, levels of IFN-γ ($P < 0{\cdot}01$) and IL-6 ($P < 0{\cdot}001$) were also significantly attenuated compared to placebo control rats at 6 h (Fig. 5c,d). Furthermore, levels of IL-1α ($P < 0{\cdot}01$) and IL-2 ($P < 0{\cdot}01$) were significantly

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attenuated compared to placebo control rats at 6 and 3 h, respectively (Fig. 5a,e).

Neither SBGsc nor SBGpo treatment reduced the elevated levels of TNF-α significantly, although a tendency towards reduction in TNF-α level was observed at 3 and 6 h (Fig. 5b). In the sham animals, no significant differences in plasma levels between placebo and SBG treatment were observed for any of the studied cytokines at 1, 3 and 6 h (data not shown).

Discussion

In this report we demonstrate for the first time that prophylactic treatment with orally administered water-soluble β-1,3/1,6-glucan produces a beneficial effect on haemodynamics and attenuates critical organ injury in the LPS-challenged rats.

Several animal sepsis models exist, but none fully resemble the timing of disease onset, progression and the use of supportive therapeutic intervention in clinical human sepsis. Systemic injection of LPS, a key mediator in Gram-negative sepsis, has been demonstrated to produce pathophysiological alterations, including systemic inflammation, haemodynamic disturbance and organ dysfunction similar to those reported for septic patients. We chose to investigate the outcome of oral prophylactic β-glucan treatment in the applied endotoxin-based model because the time of onset, the amount of circulating endotoxin and the severity of the sepsis-like reaction is tightly controlled [28].

Whether β-glucans are absorbed from the gastrointestinal tract has been a matter of dispute. Recently, Rice *et al.* [8] administered fluorescently labelled β-glucans orally to rats and detected fluorescence in plasma shortly after administration, suggesting rapid uptake. However, they did not address whether the detected fluorescence originated from fluorochromes associated with β-glucan in plasma or from detached fluorochromes. Conversely, in a recent phase I clinical trial Lehne *et al.* [16] reported lack of systemic absorption of orally administered soluble β-glucan. We found that oral administration of SBG to rats produced plasma levels of β-glucan 17-fold higher than that observed by us in the placebo control animals. The total amount of β-glucan in plasma was estimated to be approximately 30 ng following 14 consecutive days of oral administration of 5–6 mg per day. Thus only a minute fraction of a single oral dose of SBG was translocated to plasma and the biological relevance of intestinal absorption remains uncertain. Pharmacokinetic analysis and further comparisons between the two chosen routes of delivery were beyond the scope of this work. Tissue levels of SBG were not quantified due to methodological limitations.

Dendritic cells (DCs) sample constitutively the intestinal mucosa for food- and environmental antigens, commensal microbes and their products. These DCs migrate from the intestinal epithelium and Peyer's patches to the mesenteric lymph nodes (MLNs), where they are involved in the development of oral tolerance and systemic immunity [29]. We speculate that mucosal DCs sample or interact with soluble β-glucan locally via projections across the epithelium and then migrate via afferent lymphatics to the MLNs, where immune modulation is initiated. In support of this hypothesis, Rice *et al.* [8] reported recently that orally administered β-glucan was bound and internalized by intestinal epithelial cells and gut-associated lymphoid tissue leading to increased dectin-1 and Toll-like receptor 2 expression associated with increased survival in experimental sepsis. Furthermore, Hong *et al.* [11] demonstrated that fluorescent β-glucan particles were taken up by gastrointestinal macrophages and shuttled to the spleen, lymph nodes and the bone marrow.

In severe sepsis and septic shock the release of proinflammatory mediators leads to haemodynamic disturbances. The present investigation demonstrated clearly that oral, as well as subcutaneous, administration of SBG had beneficial effects on the mean arterial blood pressure in rats after experimental induction of endotoxaemia. The protective effect became pronounced as the shock response progressed and at the 5·5- and 6-h time-points, MAP in the orally treated group was significantly higher than in the placebo control group. MAP in the subcutaneously treated group was significantly elevated at 6 h compared to placebo.

We observed a gradual decrease in blood pressure in the sham animals, similar to what has been described in this model previously [30]. We find it plausible that the gradual fall in MAP is caused by the stress of the surgery in a non-sterile environment: cannulation, repeated blood sampling and the fact that the animals are under anaesthesia for more than 6 h. Reduced blood pressure in sham animals over time resulted in no statistically significant difference in MAP between placebo and sham-treated animals at the late time-points, although a clear trend was observed. Furthermore, a single animal with vastly improved MAP dynamics was observed in the placebo group, explaining the large s.e.m. value. Nevertheless, indicators of organ injury and cytokine levels for this animal indicated development of a significant shock reaction with organ injury.

The cellular and molecular mechanisms behind the observed haemodynamic protection effect of orally administered SBG on MAP remain unknown. Although controversial, β-glucans have been demonstrated to hold anti-oxidant properties [31]. Accordingly, SBG treatment may affect the oxidative status, and hence mediate indirect effects on myocardial- and smooth muscle contraction and consequently enhance blood pressure.

Infusion of LPS to rats resulted in increased plasma levels of creatinine and urea, indicating impaired glomerular filtration due to renal injury. Here we demonstrated that oral administration of SBG significantly attenuated plasma creatinine levels and also resulted in a minor reduction of plasma urea in the endotoxic rats. However, subcutaneous delivery

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of the soluble β-glucan did not mediate the same level of protection against renal injury.

To monitor liver injury we measured plasma levels of ALAT and ASAT, caused by hepatocyte leakage, although ASAT may also be released from skeletal and heart muscle cells (reviewed in [32]). Accordingly, our observation that SBG significantly attenuated the plasma levels of ASAT and ALAT indicated strongly that SBG had a protective effect against LPS-induced hepatic injury. Both subcutaneous and oral administration of soluble β-glucan mediated protection against liver injury and dysfunction in contrast to the better efficacy of oral β-glucan in reducing kidney injury. The mechanism behind the organ-protective capacity of β-glucan, however, remains elusive. We hypothesize that the superior blood pressure in the SBG-treated rats may contribute to the reduced kidney and liver injury, reflecting the benefit of enhanced organ perfusion.

Our observations on the organ-protective effect of SBG are in agreement with recent papers. Sener *et al.* [2] and Toklu *et al.* [26] demonstrated reduced TNF-α levels following administration of β-glucan in animal models of sepsis, suggesting that the organ-protective capacity of SBG may be due to modulation of the cytokine profile in the endotoxic rat. We found that prophylactic subcutaneous treatment with SBG produced a moderate increase in baseline plasma levels of IL-1α, whereas the expression level for the other cytokines studied was not significantly changed, regardless of delivery route. The literature on the effects of β-glucan on cytokine expression is inconsistent, probably reflecting a complex biological interplay as well as the use of different experimental systems and a variety of β-glucan preparations. In agreement with our findings, Rasmussen and coworkers [7] demonstrated that treatment with aminated soluble β-glucan and β-glucan-derivatized microbeads resulted in increased levels of IL-1 but no change in TNF-α in a murine model. Although Engstad *et al.* [33] found that soluble β-glucan induced production of IL-8 and monocyte tissue factor as well as minor amounts of TNF-α, IL-6 and IL-10 in human whole blood cultures, Wakshull and coworkers [34] found no production of inflammatory cytokines following glucan exposure in a similar assay. β-Glucan treatment of isolated leucocytes and monocytic cell lines has, to a variable degree, induced or had no effect on production of inflammatory cytokines [4,35]. Furthermore, conflicting data exist regarding the activation or inhibition of the transcription factors nuclear factor-kappa B (NF-κB) and NF-interleukin-6 (IL-6) transcription factor by β-glucan [5,35]. These inconsistencies contribute to the enigma associated with the mechanisms by which β-glucans work.

LPS activates an immense range of genes, including an array of inflammatory mediators with the capacity to cause injury to vital organs (reviewed in [36]). In contrast to Sener *et al.* [2] and Toklu *et al.* [26], we did not observe a statistically significant reduction in TNF-α levels following SBG treatment. Thus, the observed organo-protective effect of SBG appears not to be coupled solely to the modulation of this key mediator of inflammation. We found that LPS-induced increase in plasma IL-1α, IFN-γ, IL-6 and IL-2 levels were attenuated at later time-points following prophylactic treatment with SBG, suggesting that these mediators in the early phase of sepsis may return more rapidly to baseline levels in SBG-treated rats. In accordance with our data, Nakagawa *et al.* [37] reported that soluble β-glucan extracted from *Candida albicans* significantly suppressed endotoxin-induced IL-6, IL-2 and IFN-γ production in cultures of human monocytes or peripheral blood mononuclear cells. In contrast to our observations, Soltys and Quinn [4] reported a reduction in TNF-α production and described a substantial increase in IL-6 production from lymphocytes and monocytes isolated from β-glucan-treated mice following a subsequent challenge with LPS *in vitro*.

The subtle changes in the cytokine profile reported here are unlikely to explain solely the observed beneficial effect of SBG on organ function and haemodynamics in the endotoxaemic rat. Nevertheless, the cytokine data demonstrate that SBG prophylaxis does affect the systemic inflammation induced by LPS infusion. Notably, this is the case even after oral applications that produce low plasma levels of SBG. The contribution of this cytokine modulation on the observed organoprotective and haemodynamic effect remains unknown.

The present work identified a discrepancy between the effects of oral and subcutaneous administration of β-glucan. Plasma IL-10 and urea levels were significantly higher in the subcutaneously treated group at 1 and 6 h, respectively, compared to the orally treated animals. Interestingly, renal protection was seen only in the SBGpo group, despite the fact that the plasma level of SBG in the SBGsc group was much higher. Moreover, the higher plasma level of SBG in the SBGsc group did not lead to superior protection of the liver, recovery of MAP or modulation of cytokines compared to oral administration. Thus, it seems that the plasma level does not correlate with the observed effect of SBG for any of the parameters tested. This suggests that discrete cellular and molecular mechanisms may be involved in the mode of action, depending on the route of delivery. Further research is required to elucidate this discrepancy.

In conclusion, we demonstrated a striking positive effect on the haemodynamics during the progression of LPS-induced shock in rats that had received prophylactic SBG treatment. In spite of the fact that only a small fraction of orally administered β-glucan was absorbed from the gastrointestinal tract to the circulation, oral administration of SBG had a more pronounced effect than subcutaneous injection. We observed attenuated levels of indicators of kidney and liver injury following SBG treatment, which may be due to enhanced organ perfusion. Additionally, SBG prophylaxis caused subtle changes in the cytokine profile, including attenuated levels of mediators of inflammation subsequent to endotoxin challenge. Characterization of how

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β-glucans exert their biological effects may add to the understanding of the workings of the innate immune system and may be helpful in identifying new targets and applications for β-glucans in human therapy.

Acknowledgements

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Corrigenda

In [1], **IL-1β** is erroneously named **IL-1α** on several occasions in the text.

IL-1β is used correctly in the abstract (p. 168) and in the relevant figure heading (Fig 5a, p. 173).

IL-1α is incorrectly used on seven (7) occasions throughout the paper:
- p 169, Top left column (Introduction), line 3.
- p 171, Bottom right column (Results), line 40.
- p 172, Bottom right column (Results), line 1.
- p 173, Bottom right column (Results), line 4.
- p 173, Figure legend, figure 5.
- p 175, Center left column (Discussion), line 26.
- p 175, Top right column (Discussion), line 3.

IL-1α should be substituted with IL-1β in all these occurrences.

In [1], an extra comma was introduced in the keywords list: 'multiple organ dysfunction, syndrome (MODS)'. This is incorrect and should read 'multiple organ dysfunction syndrome (MODS)'.

We apologize for these errors.

Reference

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Paper II

SOLUBLE β-GLUCAN PROTECTS AGAINST EXPERIMENTAL ULCERATIVE COLITIS

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SOLUBLE β-GLUCAN PROTECTS AGAINST EXPERIMENTAL ULCERATIVE COLITIS

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Short running head: β-Glucan protects against experimental colitis

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Key words:

beta-glucan, colitis, inflammatory bowel disease, dextran sulphate sodium

Abstract

Background and objective: Treatment of inflammatory bowel disease (IBD) is associated with significant adverse reactions and unsatisfactory efficacy, thus new therapies are welcomed. β-Glucans are glucose polymers with an array of stimulatory effects on the immune system. We set out to investigate the effect of soluble *Saccharomyces cerevisiae*-derived β-1,3/1,6 glucan (SBG) on experimental ulcerative colitis (UC). **Methods:** Experimental colitis was induced by exposing mice to dextran sulphate sodium (DSS, 1.5% w/v) for 7 days followed by regular drinking water for 4 days. Some mice were pretreated for 7 days with SBG-supplemented drinking water (100mg/L), and SBG was supplied throughout the experiment for these mice. Mice supplied with regular drinking water or SBG only served as controls. **Results:** Oral SBG administration reduced colitis-associated mortality and body weight loss. Furthermore, SBG treatment attenuated DSS-induced colonic inflammation and tissue damage, characterized by lowered histopathology score. Also, colitis-associated colon shortening and thymic involution was attenuated by SBG treatment. Finally, colitis-associated systemic inflammation was attenuated in SBG treated mice. **Conclusion:** We demonstrate a beneficial effect of oral SBG administration on key parameters in experimental UC and propose that SBG have a potential as a therapeutic agent in future IBD management. It remains unanswered, however, whether SBG enhance mucosal barrier function or modulate the inflammation secondary to epithelial injury.

Introduction

Inflammatory bowel disease (IBD) refers to two related chronic relapsing inflammatory disorders: ulcerative colitis (UC) and Crohn's disease (CD). UC affects the inner lining of the colon which becomes inflamed and develops ulcers while CD tends to involve the entire bowel wall and commonly affects the terminal ileum and parts of the colon, but may affect any part of the gastrointestinal tract (1). The incidence of IBD varies considerably world wide with incidence rates between 0.5-24.5/10^5 and 0.1-16/10^5 inhabitants for UC and CD, respectively. The highest rates are reported in Northern and Western Europe as well as North America (2).

The etiology of both UC and CD is complex. The discovery of susceptibility genes has demonstrated the importance of innate and adaptive immune responses and epithelial barrier integrity in IBD pathogenesis. Environmental factors, including commensal bacteria, are implicated in IBD development. Accumulating evidence suggests that an inappropriate immune response to non-pathogenic microbes of the intestine and other luminal antigens plays a critical role in the initiation and pathogenesis of IBD (3, 4).

IBD is routinely treated with antibiotics, immunosuppressive- and anti-inflammatory drugs, and more recently by promising antibody-based therapeutics. Although these strategies may prove effective, available therapeutics are associated with considerable adverse reactions (5) including opportunistic infections (6). Surgery continues to have an important role in IBD treatment as 30-40% of UC patients and 70% of CD patients require surgical intervention (7). Notably, as many as 40-60% of IBD patients respond poorly to current standard therapy, indicating a considerable need for new, more effective and safe therapies (8).

Several animal models have been developed to investigate the etiology and

pathophysiology of IBD and to evaluate new treatments. Experimental models of IBD can be roughly divided into three main categories: models that are based on genetic defects in immune regulation (spontaneous or engineered), models based on transfer of colitogenic cells to immunocompromised hosts, and models that rely on a chemical insult to the mucosa (reviewed in 9, 10). We have employed the widely used dextran sulfate sodium (DSS) model. Oral exposure to water-dissolved DSS induces a reproducible acute colitis in rodents. Although the precise molecular mechanism behind DSS-induced colitis remains elusive, DSS appears to have a direct toxic effect on the basal crypt epithelium, leading to reduced mucosal barrier function, subsequently resulting in inflammation and ulceration (11).

β-Glucans are glucose polymers with a variety of stimulatory effects on the immune system. β-Glucan administration has been reported to modulate cytokine profiles, enhance protection against sepsis, infections and tumor development and promote wound healing (12). Although most studies on β-glucan *in vivo* have employed systemic delivery, we recently demonstrated that oral feeding of soluble *Saccharomyces cerevisiae*-derived β-1,3/1,6 glucan (SBG) provided better protection against experimental endotoxemia and associated multiple organ dysfunction syndrome in rats than subcutaneous injection of SBG (13).

Because β-glucan may modify inflammation, enhance wound healing and possibly function as a pre-biotic (affect the composition of indigenous gut microbiota) (14), we postulated that SBG would have a protective effect on experimental IBD. The aim of this study was to investigate the protective capacity of orally administered SBG in a murine model of UC (11). We provide evidence here that oral SBG administration ameliorated key parameters of DSS-induced colitis, indicating a potential for novel use of β-glucan in IBD management.

Materials and Methods

Materials

Dextran Sulfate Sodium (DSS, MW 36,000-50,000) was purchased from MP Biomedicals, Inc. (Solon, Ohio, USA). Endotoxin free (<0.5EU/mL) SBG, a *Saccharomyces cerevisiae*-derived water-soluble β-1,3/1,6-glucan was provided by Biotec Pharmacon ASA (Tromsø, Norway). Anesthesia: Hypnorm® (fentanyl citrate 0.315 mg/mL and fluanison 10 mg/mL, VetaPharma Ltd, Leeds, UK) and midazolam (5 mg/mL, B. Braun Meslungen AG, Germany) were diluted 1:1 in sterile water and subsequently combined in a 1:1 ratio.

Animals

Male BALB/c mice were purchased from Taconic Europe (Ejby, Denmark) and maintained in the minimal disease unit at the Centre for Comparative Medicine at Rikshospitalet University Hospital, Oslo, Norway for at least one week before they were entered into experiments. Animals were housed 2 mice per cage, supplied with water and conventionally fed (Rat and Mouse No.3 Breeding, Special Diets Services, Witham, Essex, UK) *ad libitum*. Cages were kept at 21±1°C and 55±10% relative humidity. Light conditions consisted of alternating 12h light/dark cycles with one hour dusk and dawn. The present investigation was approved by the National Animal Research Authority and conducted in accordance with the Norwegian Animal Welfare Act and the Norwegian Regulation on Animal Experimentation.

Induction and treatment of colitis

Experimental colitis was induced by exposure to DSS dissolved in the drinking water for 7 days. Animals were pretreated as indicated below for 7 days before induction of colitis. Mice were sacrificed following an acute-/recovery phase of 4 days.

Mice [22.3 (19.4-24.9) g; mean and (range)] were randomly distributed to four experimental groups:

1. **Control** (**Ctr**) animals (n=12): Regular drinking water was provided throughout the experiment.
2. **SBG** treated animals (n=12): SBG (100mg/L) was provided in the drinking water throughout the experiment.
3. **DSS** treated animals (n=16): Regular drinking water was provided in the pretreatment phase (7 d). DSS (1.5% w/v) was administered during the induction period (7 d) and regular drinking water was continued in the acute-/recovery phase (4 d).
4. **SBG+DSS** treated animals (n=15): SBG (100mg/L) was provided in the drinking water in the pretreatment phase (7 d). DSS (1.5% w/v) dissolved in water containing SBG (100mg/L) was administered during the induction period (7 d) and SBG (100mg/L) in the drinking water was continued in the acute-/recovery phase (4 d).

Termination and necropsy

Main criterion for humane endpoint was body weight reduction of >20% of baseline weight. Mice assessed as clearly moribund, without meeting the weight criterion, were also euthanized for animal welfare reasons.

Animals were anesthetized by subcutaneous injection of Hypnorm® and midazolam (50-75µL/10g body weight) prior to cardiac puncture. Postmortem mice were soaked in 70% ethanol and fixed to a dissection board. The abdomen and thorax was opened and the colon and thymus were excised. The colon was flushed with cold PBS and partitioned into proximal-, medial- and distal colon segments prior to fixation. All tissue samples were kept on ice and fixed in 10 % formalin for 24 h at 4 ^{o}C. Fixed tissue samples were transferred to PBS with 0.1% formalin and stored at 4 ^{o}C for subsequent preparation and analysis. Blood collected by cardiac puncture at termination was allowed to clot on ice. Serum was harvested following centrifugation and stored at -70 ^{o}C for subsequent analysis.

Clinical evaluation

Body weight, fluid consumption and general observation

Body weight and fluid consumption was monitored and recorded daily. Mice were monitored for signs of rectal bleeding, diarrhea, and general signs of morbidity, including hunched posture and failure to groom.

Colon length and appearance

Colon was excised and left to rest on a non-absorbing surface to reduce tension before the total colon length was measured and photographed. Colon appearance, including absent or unformed stool and macroscopic bowel thickening, was recorded.

Thymus assessment

Formalin fixed thymus were blotted onto absorbing paper to remove excess liquid. Both thymus lobes were weight separately (Ohaus Explorer®, d= 0.1mg, Ohaus, Switzerland) and the average lobe weight was calculated and recorded.

Histological scoring

Formalin fixed biopsies were processed using an automated tissue processor (Leica TP1050, Leica Instruments GmbH, Nussloch, Germany) and subsequently embedded in paraffin. Sections were cut at 4µm (Leica RM 2135, Leica Instruments GmbH), stained with hematoxylin and eosin (H&E), and evaluated by a trained pathologist (K.G.) blinded to the sample identity and study groups.

Histopathology grading of colitis was determined largely as described in (15), with some adaptations (Table 1). The tissue damage score was adjusted by multiplying the score with a factor corresponding to the extent of ulceration (Table 1). Proximal-, medial- and distal colon segments were assessed separately.

Cytokine analysis

Serum cytokine levels were measured using a mouse-specific Bio-Plex 23-plex suspension array assay (BioRad Laboratories, Hercules, CA, US). All samples were diluted 1:4 in Bio-Plex species specific serum sample diluent buffer (BioRad Laboratories) and the assay carried out according to manufacturer's instructions. Measurements and data analysis were performed on a Bio-Plex system, powered by xMAP technology by Luminex, operated with Bio-Plex Manager 4.1 software (BioRad Laboratories). The instrument was calibrated with the CAL2 settings (LOW RP1 target value) using Bio-Plex calibration beads (BioRad Laboratories). Samples were analyzed as single samples, whereas standards were analyzed in duplicates. Analytes below detection level or unchanged in all experimental groups were not included in the figure (Fig. 6).

Statistical analysis

Body weight data are expressed as mean ± standard error of the mean (SEM) values and analyzed using two-way analysis of variance (ANOVA) with Bonferroni posttest. Categorical data on mortality was expressed as percent survival and analyzed with the logrank test. Histopathology score, colon length and thymus weight data were expressed as median values and analyzed using the Mann-Whitney test. Cytokine data are expressed as mean ± standard deviation (SD) values and analyzed using one-way ANOVA with Bonferroni's multiple comparison test. Data presented are pooled from two independent experiments. All statistical analysis was carried out using GraphPad Prism, version 4 (GraphPad Software, San Diego, CA, USA). Differences at $P<0.05$ were considered statistically significant.

Results

Oral SBG administration attenuates colitis-associated body weight loss.

To evaluate a possible protective role of SBG in murine DSS colitis we designed a treatment regimen consisting of a prophylactic phase (day -7 to day 0), an induction phase (day 0 to day 7), and an acute phase/recovery period (day 7 to day 11). We randomly divided wild type BALB/c mice into four experimental groups as detailed in the methods section: **Ctr**, **SBG**, **DSS** and **DSS+SBG.**

Body weight and fluid consumption was recorded daily during pretreatment, colitis induction, and for 4 days after DSS termination. Water consumption was 5-7 mL/mouse/day, corresponding to a daily SBG dose of 20-30mg/kg (data not shown). There was no significant difference in fluid consumption between the experimental groups (data not shown). We observed a dramatic weight loss of approximately 15% between day 6 and 10 in DSS-treated animals. Also the DSS+SBG group experienced colitis-associated weight loss but was significantly protected compared to the DSS group (Fig. 1). Furthermore, the onset of weight reduction appeared delayed in the SBG+DSS group compared to the DSS group. Control animals increased steadily in weight throughout the experiment with no difference in body weight dynamics between the SBG-treated animals and mice receiving regular drinking water (Fig. 1).

Oral SBG administration improves survival in experimental colitis.

DSS exposure induced clinical symptoms including bloody stool, diarrhea, rectal bleeding, inactivity, failure to groom, and in some severe cases hunched posture and trembling. Moribund animals and mice experiencing weight loss exceeding 20% of base line weight were euthanized for humane reasons. In the DSS+SBG group only 1 out of 15 animals was euthanized compared to 6 out of 16 mice in the DSS group. Thus, oral SBG treatment protected against colitis-associated mortality (Fig. 2).

Oral SBG administration attenuates DSS-induced colonic inflammation and tissue damage.

To further investigate whether SBG administration could protect against DSS-induced inflammation and ulceration, histology sections of the proximal, medial and distal colon were examined. No apparent pathology was observed in the Ctr- or SBG groups. In the DSS group, on the other hand, considerable inflammatory cell infiltration extending into the submucosa, and in several cases with transmural involvement, was observed. Severe distortion of the mucosal architecture, including lack of distinct crypts and goblet cells, moderate to extensive ulceration, in some cases with total lack of epithelium, was disclosed. Mucosal edema and signs of bowel wall thickening was also identified (Fig. 3A). In accordance with previous publications (11), inflammation and tissue damage was more prominent and consistent in distal than in proximal sections of the colon (data not shown). Therefore, we compared distal sections from the DSS+SBG and DSS groups and found a significantly lower histopathology score in the SBG-treated group (Fig. 3B).

Oral SBG administration reduces colitis-associated colon shortening.

Colon shortening is a well established disease-associated characteristic of DSS-induced colitis (11). To further evaluate the protective capacity of SBG on experimental colitis, colons were excised and the length was measured. Oral SBG administration alone did not have an effect on colon length. In the DSS group colons were approximately 30% shorter than colons from control animals. The colon length in the DSS+SBG group was clearly affected by DSS-exposure, but was significantly longer than colons in the DSS group (Fig. 4 A and B). The 6 shortest colons in the DSS group all originated from euthanized animals, suggesting that shortening of the colon correlated well with colitis-associated morbidity. We also observed that colons from the DSS group contained largely unformed stool as opposed to fecal pellets in the DSS+SBG group and control groups (Fig. 4A). Macroscopic wall thickening distally and loss of bowel transparency was apparent in both DSS- and DSS+SBG groups although it appeared more striking in the DSS group.

Oral SBG administration protects against thymus involution in acute colitis.

Thymic involution has been associated with experimental colitis including DSS-induced colitis (16). To investigate whether oral SBG administration had an impact on thymic involution in acute colitis, the thymus was collected post mortem. Mice euthanized due to the severity of colitis had reduced thymic mass compared to control animals, suggesting that reduced thymic weight correlated with colitis-associated morbidity (Fig. 5, open symbols). Interestingly, oral SBG administration significantly limited colitis-associated thymus involution (Fig. 5).

Oral SBG administration limits systemic inflammation in acute colitis.

To investigate whether the reduced inflammation observed in the colon is mirrored in the systemic compartment, serum samples were analyzed for levels of cytokines and chemokines. DSS-induced intestinal inflammation was associated with increased systemic levels of key mediators and regulators of inflammation (Fig. 6). In the DSS+SBG group serum levels of interleukin (IL)-1β, IL-3, IL-6, IL-10, IL-17, monocyte chemotactic protein (MCP)-1 and interferon (IFN)γ were significantly lower than what we observed in the DSS group (Fig. 6). In most instances these cytokines/chemokines were not above baseline levels in SBG+DSS animals while they were significantly increased in the DSS group. Furthermore, a tendency towards reduction in tumor necrosis factor (TNF)α, IL-1α, IL-5, IL-13, MIP-1β and GM-CSF was observed in the DSS+SBG group compared to the DSS-group. Serum IL-2 was significantly elevated in both the DSS- and the DSS+SBG groups. No difference between the Ctr group and SBG group was observed for any of the assessed analytes (Fig. 6 and data not shown).

Discussion

DSS-induced colitis is a widely used mouse model of human IBD. Here, we demonstrate that oral administration of soluble *S. cerevisiae*-derived β-1,3/1,6-glucan (SBG) mediated a protective effect on all investigated parameters of disease severity in this experimental colitis model. Oral SBG treatment reduced colitis-associated weight loss, mortality, colon shortening and thymic involution and attenuated colonic inflammation and tissue damage in the experimental animals. To our knowledge, this is the first study demonstrating a protective effect of soluble β-glucan in experimental colitis.

Although animal models of UC and CD do not fully recapitulate all the traits of human IBD, they are valuable tools to delineate molecular mechanisms of IBD pathogenesis and to evaluate new therapeutic strategies. In a recent study te Velde and co-workers (17) compared the colonic gene expression profiles in 3 mouse models of IBD: (I) Transfer of colitogenic CD45RB T-cells to immunodeficient hosts, (II) DSS-induced colitis and (III) 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. They demonstrated that of 32 genes known to be differentially expressed in IBD, 30 were up- or down regulated in the T-cell transfer model, compared to 15 and 2 in the DSS and TNBS models, respectively, inferring that the chronic colitis induced in the T-cell transfer model most closely mimics human IBD (17). Oral exposure to water-dissolved DSS induces a reproducible acute colitis in rodents without affection of the small intestine. Resembling UC, the hallmark of DSS-induced colitis is diarrhea, bloody stool, body weight loss, mucosal ulcer formation and inflammatory cell infiltration to the colonic lamina propria. Furthermore, colon shortening and thymic involution are characteristics of DSS-induced colitis. Importantly, DSS-induced murine colitis is treatable with drugs frequently used to control human UC (9-11).

SBG administration alone did not affect body weight or any other parameter that we measured compared to control animals receiving regular drinking water. Oral pretreatment and continued SBG exposure significantly reduced the severity of all investigated parameters of experimental colitis. Thus, SBG had a protective effect against DSS-induced colitis. This is in agreement with Nosál'ová *et al.* (18), which found that pleuran, an insoluble β-glucan derived from oyster mushrooms, reduced colonic injury in rats injected with acetic acid to induce acute colitis.

Although the majority of studies concerning the properties of β-glucans *in vivo* have employed systemic delivery, several studies have demonstrated that orally administered β-glucan mediate biological responses. We recently demonstrated that oral administration of SBG mediated protection against experimental endotoxemia and shock-associated organ failure in rats (13). Uptake of orally administered β-glucans from the gastrointestinal tract has been a matter of dispute. Lehne *et al.* (19) reported lack of systemic uptake following oral SBG administration in humans. Employing a structurally related, fluorescently labeled β-glucan, Rice *et al.* (20) reported rapid systemic uptake following oral administration. In our previous study, we found detectable plasma levels of the ~20kDa β-glucan following three weeks of feeding, but only a minute fraction of the total SBG dose was detected in circulation. Furthermore, the biological activity of fed SBG in modulating endotoxemia could not be accounted for by the plasma levels of β-glucan suggesting that mechanisms of β-glucan action may depend on the route of delivery (13). Thus, the relevance as well as the mode of gastrointestinal β-glucan uptake remains to be determined. Considering that β-glucan action does not necessarily correlate with plasma levels, we did not quantify the level of SBG in circulation or in tissue samples in the present work.

A broad range of DSS concentrations, exposure times and exposure cycles and assorted mouse strains with diverse susceptibility have been employed to study pathogenesis

of colitis and to evaluate new therapeutics in this model (21, 22). In the present study a relatively mild regimen, 1.5% DSS for 7 days in male BALB/c mice, caused severe disease, demonstrating the importance of appropriate dose titration. The sensitivity to DSS appeared to depend on microbial or other environmental factors as we have observed different susceptibility in genetically identical mice with known differences in their commensal intestinal flora (unpublished observations).

Reduced mucosal barrier function, as a consequence of DSS feeding, causes colonic inflammation with inflammatory cell infiltration and ulceration. Our histopathology analysis demonstrated that SBG administration reduced colitis-associated inflammation and tissue damage, supporting the notion that β-glucan may protect against colitis development and progression. In accordance with Okayasu (11) and others we observed that the colonic inflammation and tissue damage was more pronounced in the distal part of the colon, with moderate inflammation in the medial segment and no apparent pathology proximally.

We demonstrate that β-glucan treatment protected against colitis-induced thymic involution. Involution may be due to increased thymocyte migration associated with cell recruitment to the site of inflammation and SBG therapy may attenuate thymus involution by limiting the initial DSS-induced inflammation. Alternatively, one may speculate that SBG-stimulated dendritic cells (DCs) or macrophages interact with thymocyte proliferation and maturation directly or indirectly. Reid *et al.*. demonstrated strong dectin-1 expression, a major β-glucan receptor, on subpopulations of DCs and macrophages in the medullary and corticomedullary regions of the thymus, suggesting that dectin-1 play a role in leukocyte interactions and T-cell development (23).

As expected, serum levels of pro-inflammatory cytokines and chemokines were elevated in the DSS group. Also, systemic IL-10, a key regulatory cytokine, was found to be elevated in this group probably reflecting an attempt to resolve the inflammation. TNFα has

been demonstrated to be a major player in intestinal inflammation and a promising target in IBD therapy (reviewed in 24). In the present study a significant difference in serum TNFα levels between the study groups was not revealed by the statistical analysis with correction for multiple comparisons. In a separate analysis, however, comparing the DSS- and DSS+SBG groups only, p-values of <0.05 were obtained also for TNFα, IL-1α and IL-13. In line with the clinical histopathology data presented, oral SBG administration appeared to attenuate systemic inflammation. We believe that the systemic inflammation is secondary to the DSS-induced intestinal inflammation. Thus, the reduced serum levels of inflammatory mediators in the DSS+SBG group is likely to reflect the beneficial effect of SBG on the integrity of the intestine in DSS-exposed mice.

IBD pathogenesis is associated with elevated levels of potent pro-inflammatory cytokines including IL-1β, IL-6, IL-12, IL-17, IL-23, IFNγ and TNFα (reviewed in 25). Targeting of these key mediators of inflammation has proven to be a promising strategy in IBD management (26). Also, the important roles of IL-10 and transforming growth factor (TGF)β, key regulatory cytokines in mucosal homeostasis, have been evidenced by the development of spontaneous colitis in IL-10 deficient mice and mice with defective TGFβ signaling, respectively (27, 28). β-Glucan administration reportedly modulate cytokine profiles in various animal models of inflammation. Bedirli *et al.* reported that β-glucan treatment reduced plasma levels of IL-1β, IL-6 and TNFα associated with attenuated lung injury in a rat model of sepsis (29). Breivik *et al.* demonstrated enhanced TGFβ induction by peritoneal lipopolysaccharide (LPS) injection in rats orally pre-treated with SBG (30). We recently demonstrated that SBG administration attenuated a LPS-induced rise in plasma levels of IFNγ, IL-1β, IL-2 and IL-6 in a rat model of endotoxin shock. We also reported a significant increase in plasma IL-10 levels (13).

In UC and DSS-induced colitis mucosal erosions and ulcers develop. Ulceration represented a major part of the reported histopatology score and the lower score in the DSS+SBG vs. DSS group was largely due to lower extent of ulceration. Although we hypothesize that SBG treatment limited the initial formation of ulcers in the inductive phase rather than enhanced mucosal recovery only after ulcer development, it is possible that β-glucan may have beneficial effects on epithelial regeneration and healing of established wounds. In support of this hypothesis, SBG has been demonstrated to enhance healing of chronic ulcers in diabetic patients (31). Moreover, Portera *et al.* demonstrated that intravenous injection of β-glucan phosphate increased wound tensile strength, correlated with collagen biosynthesis, in experimental skin- and colon wound models (32).

Patients with severe UC, refractory to medical treatment or with neoplastic transformation, require surgery and colectomy is ultimately indicated in approximately 25% of UC patients (33). Anastomotic leakage is a common adverse event following colectomy. Furthermore, corticosteroid exposure, which frequently is the case in UC, leads to impaired wound healing in bowel anastomosis. Interestingly, oral β-glucan treatment has been demonstrated to improve colonic anastomotic wound healing impaired by long-term medication in a rat model (34).

The precise cellular and molecular mechanisms behind the immunomodulating properties of β-glucans remain elusive. Several β-glucan receptors with a wide distribution profile on both immune and non-immune cells have been described (12). Accumulating evidence suggests that dectin-1 is the primary receptor that mediates host responses to β-glucans (35, 36). Dectin-1 has been proposed to collaborate with Toll-like receptors (TLRs) in a MyD88 and Syk dependent manner (37-41 and reviewed in 42). Dectin-1 activation without concomitant TLR activation results in expression of IL-10 and IL-2 mediated by activation of Syk (43). It is possible that SBG, which is a pure soluble β-glucan preparation

free of detectable protein, is particularly useful in this regard as it would presumably fail to engage TLRs.

The dynamic interplay between the intestinal microflora and the host mucosal immune system plays a pivotal role in the initiation and pathogenesis of IBD. Thus, animal models with defective immune regulation completely rely on intestinal colonization for colitis development (44, 45). Also, altered flora composition in IBD patients and the therapeutic benefit of antibiotics, probiotics and prebiotics supports the notion that intestinal flora composition is of critical importance (46, 47). Interestingly, β-glucans have been proposed to have prebiotic properties, affecting the composition of the commensal flora. Work by Snart *et al.* demonstrated that a β-glucan supplemented diet resulted in *Lactobacillus*-enriched cecal flora in a rat model (14). Beneficial effects of *Lactobacillus*-based probiotics have been demonstrated in animal models of IBD as well as in clinical trials (47).

In conclusion, we demonstrate that oral administration of soluble β-glucan mediated protection against DSS-induced colitis evidenced by beneficial effects on all major parameters of experimental colitis. However, it remains to establish whether SBG therapy is beneficial in established or chronic colitis. Also it remains unanswered whether SBG enhance mucosal barrier function or modulate the inflammation secondary to epithelial injury. Despite increased understanding of the biology behind IBD and efforts to exploit this knowledge in the development of new therapies, there is still a considerable need for new effective and safe drugs to treat IBD patients. In light of our findings, and previously reported beneficial effects of β-glucan, SBG appear to be a promising drug candidate for IBD therapy in humans. Work in progress aims at elucidating the cellular and molecular mechanisms behind these promising findings.

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Study Highlights

What is current knowledge

- IBD treatment is associated with considerable adverse reactions and unsatisfactory efficacy, thus new therapies are welcomed.
- Accumulating evidence suggests that an inappropriate/dysregulated immune response plays a critical role in the instigation and pathogenesis of IBD.
- β-Glucans mediates an array of stimulatory effects on the immune system.

What is new here

- Oral administration of soluble β-glucan (SBG) produced beneficial effects on all investigated parameters of experimental colitis.
- We propose that SBG has a potential as a novel therapeutic agent in future IBD management.

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Conflict of Interest

Guarantor of the article: Anders Sandvik

Specific author contributions: Anders Sandvik conceived and designed the study, carried out the experiments, analyzed the data and wrote the manuscript. Finn-Eirik Johansen conceived and designed the study, supervised data analysis, and contributed to writing of the manuscript. Krzysztof Grzyb conducted the histopathology scoring, reviewed the scoring criteria and contributed to writing of the manuscript. Dag Henrik Reikvam and Alexander Erofeev established the animal model, contributed to pilot experiment design and data acquisition and writing of the manuscript. Frode Jahnsen contributed to study design, data analysis and writing of the manuscript. All authors have approved the final draft submitted.

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Potential competing interest: None.

Figure legends

Figure 1. Effect of oral SBG administration on weight loss in acute colitis.

Mice were pretreated with SBG or regular drinking water for 7 days (day -7 to 0), prior to induction of acute colitis by oral exposure to DSS for 7 days (day 0 to 7). Body weight was recorded daily during pretreatment, colitis induction, and for 4 subsequent days during the acute- and initial recovery phase (Acu/Rec; day 7 to 11), after which the animals were sacrificed. Body weight is expressed as percentage of base line (BL) values, mean ± SEM. E = Euthanized mice: body weight reduction >20% or moribund; weight at time of euthanasia was extrapolated to subsequent days. *P<0.05, **P<0.01, ***P<0.001, DSS+SBG vs. DSS as determined by two-way analysis of variance with Bonferroni posttest. Data presented are pooled from two independent experiments.

Figure 2. Effect of oral SBG administration on survival in acute colitis.

Mice were pretreated with SBG or regular drinking water prior to induction of acute colitis by oral exposure to DSS as described. Mortality was recorded in the acute-/recovery phase following DSS-removal (from day 7 to planned termination at day 11) and expressed as percent survival. Humane endpoint criterion was body weight loss >20% of baseline weight. Unmistakably moribund animals not meeting the weight loss criterion were also euthanized. *P<0.05, DSS+SBG vs. DSS as determined by log-rank test. Data presented are pooled from two independent experiments.

Figure 3. Effect of oral SBG administration on colonic inflammation and tissue damage in acute colitis.

Mice were pretreated with SBG or regular drinking water prior to induction of acute colitis by oral exposure to DSS as described. Postmortem, colons were excised, flushed with PBS and prepared for histological analysis. Formalin fixed, paraffin embedded, H&E stained sections were examined for inflammatory cell infiltration, tissue damage and the absence or presence of epithelial regeneration (Table 1). A) Representative distal colon H&E sections from control animals (Ctr), SBG-treated animals (SBG), DSS-treated animals (DSS) and DSS and SBG combination-treated animals (DSS+SBG). Original magnification 100x. B) Distal colon histopatology score. Each data point represents one animal. Open symbols indicate animals euthanized for animal welfare reasons. Bars represent median values. *$P<0.05$, DSS+SBG vs. DSS as determined by Mann-Whitney test. Data presented are pooled from two independent experiments.

Figure 4. Effect of oral SBG administration on colitis-associated colon shortening.

Mice were pretreated with SBG or regular drinking water prior to induction of acute colitis by oral exposure to DSS as described. Postmortem, colons were excised and the colon length was measured. A) Representative pictures of colons excised from control animals (Ctr), SBG-treated animals (SBG), DSS-treated animals (DSS) and DSS and SBG combination-treated animals (DSS+SBG). Images have been digitally enhanced (Adobe Photoshop CS 8.0, Adobe Systems Inc., San Jose, CA, USA). B) Colon length in mm. Each data point represents one animal. Open symbols indicate animals euthanized for animal

welfare reasons. Bars represent median values. *P<0.05, DSS+SBG vs. DSS as determined by Mann-Whitney test. Data presented are pooled from two independent experiments.

Figure 5. Effect of oral SBG administration on thymus weight in acute colitis.

Mice were pretreated with SBG or regular drinking water prior to induction of acute colitis by oral exposure to DSS as described. Postmortem, thymus was excised. Following formalin fixation, weight (mg) was recorded. The average weight of one thymus lobe is presented. Each data point represents one animal. Open symbols indicate animals euthanized for animal welfare reasons. Bars represent median values. *P<0.05, DSS+SBG vs. DSS as determined by Mann-Whitney test. Data presented are pooled from two independent experiments.

Figure 6. Effect of oral SBG administration on indicators of systemic inflammation in acute colitis.

Mice were pretreated with SBG or regular drinking water prior to induction of acute colitis by oral exposure to DSS as described. Blood, collected by cardiac puncture at sacrifice, was allowed to clot before centrifugation and serum collection. Serum cytokines and chemokine levels (pg/ml) were analyzed using a multiplex bead-array assay as detailed in the methods section. Data presented are mean ± SD values, 4-6 mice per group. * P<0.05, ** P<0.01, *** P<0.001 as determined by one-way ANOVA with Bonferroni's multiple comparison test. # P<0.05 DSS+SBG vs. DSS as determined by Mann-Whitney test. The dotted line indicates the estimated detection limit of the assay.

Table 1

Histopathology scoring criteria

Inflammatory cell infiltration	**Score 0-3**
Presence of occasional inflammatory cells in the lamina propria	0
Increased numbers of inflammatory cells in the lamina propria	1
Confluence of inflammatory cells extending into the submucosa	2
Transmural extension of inflammatory infiltrate	3
Tissue damage	**Score 0-3**
No mucosal damage	0
Lymphoepithelial lesions	1
Surface mucosal erosion or focal ulceration	2
Extensive mucosal damage and extension into deeper structures of the bowel wall	3
Extent of ulceration; epithelial surface area	**Factor 1-4**
0 - 25%	1
25 - 50%	2
50 - 75%	3
75 - 100%	4
Epithelial regeneration	**Score 0-1**
Absent	0
Present	1

Figure 1

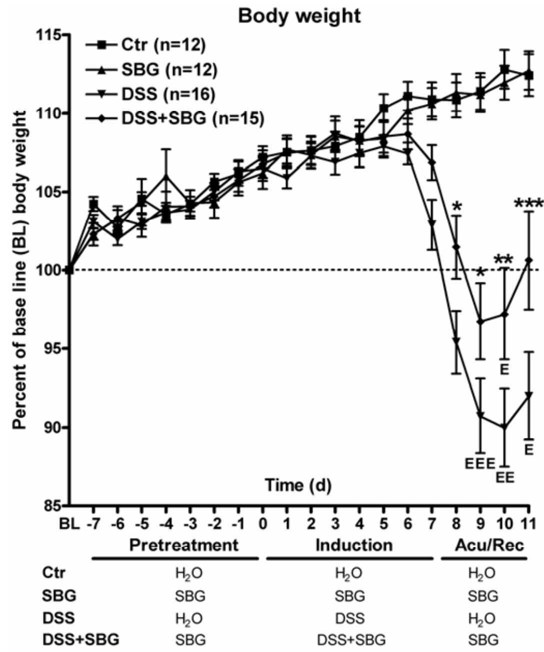

Figure 2

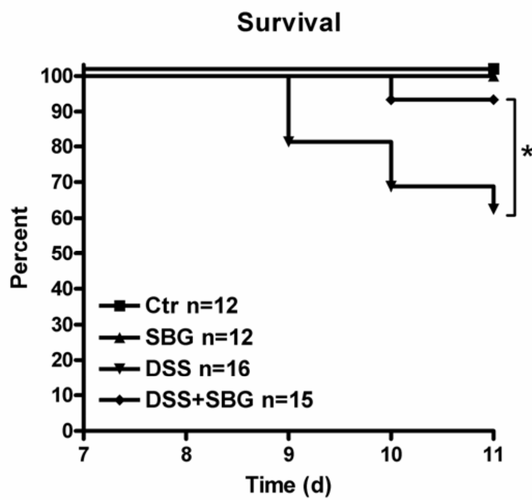

Figure 3

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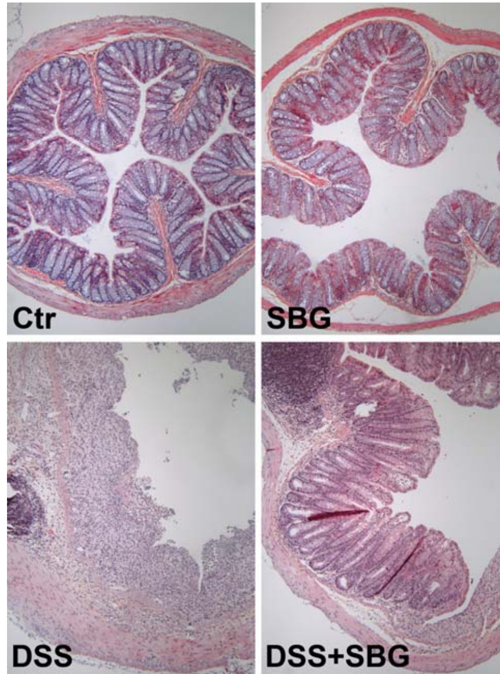

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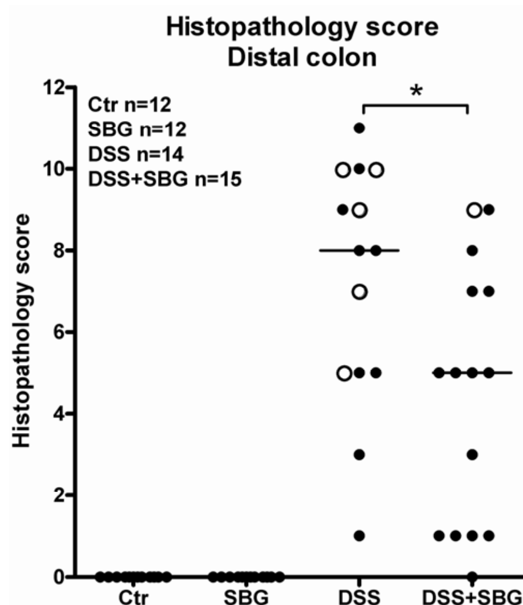

Figure 4

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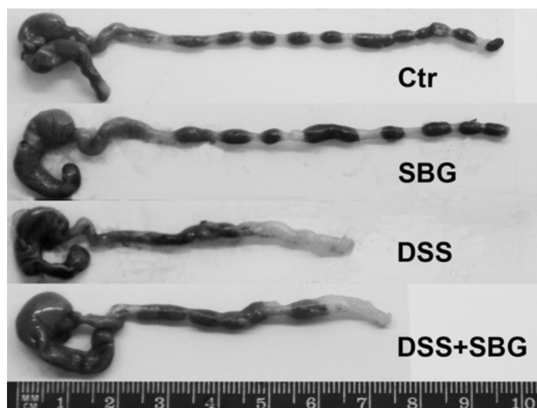

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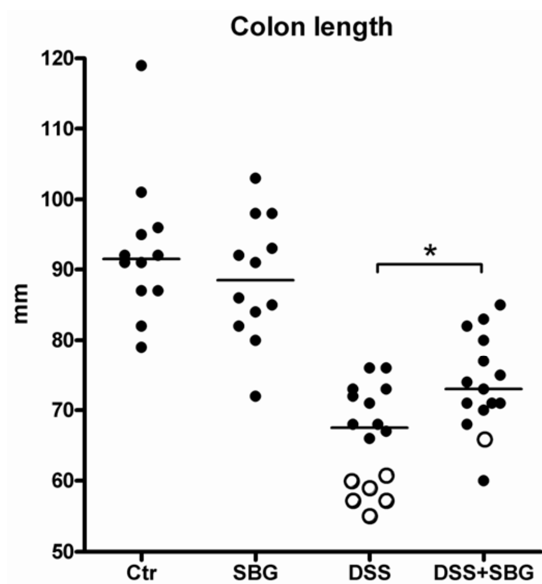


Figure 5

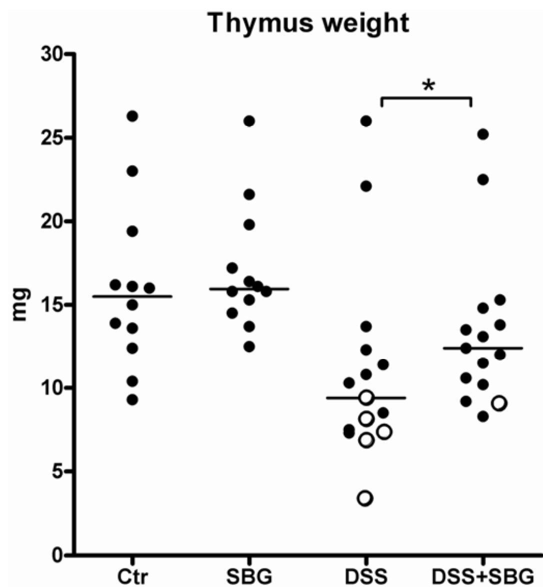
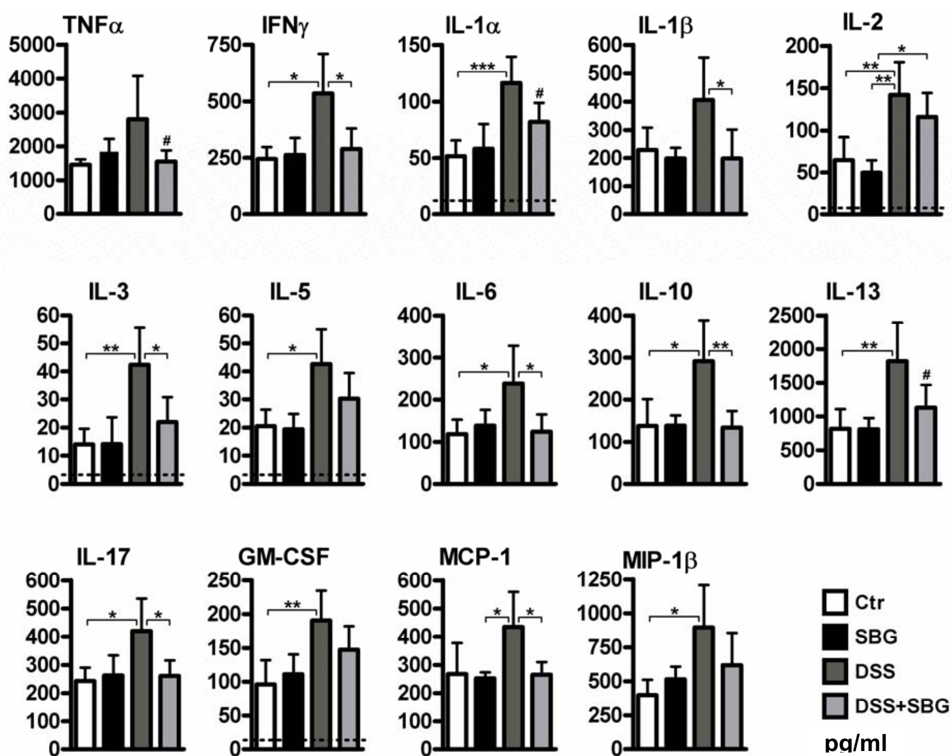


Figure 6



Paper III

EFFECTS OF ORAL ADMINISTRATION OF SOLUBLE β-GLUCAN ON THE GUT AND GUT-ASSOCIATED LYMPHOID TISSUE IN MICE

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EFFECTS OF ORAL ADMINISTRATION OF SOLUBLE β -GLUCAN ON THE GUT AND GUT-ASSOCIATED LYMPHOID TISSUE IN MICE

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Abstract

β-Glucans are glucose polymers with a variety of stimulatory effects on the immune system. Although little is known about the cellular and molecular mechanisms behind the beneficial effects, it has been suggested that β-glucans may stimulate the mucosal immune system when administered orally. **Aim:** To identify effects of oral β-glucan administration on gut-associated lymphoid tissue (GALT) and the intestinal epithelium of healthy mice. **Methods:** Male BALB/c mice were provided drinking water supplemented with SBG, a water-soluble *Saccharomyces cerevisiae*-derived β-glucan, *ad libitum* for 20 days. Control animals (Ctr) were provided regular drinking water. **Results:** SBG was well tolerated and no clinical signs of morbidity were noted. In the SBG group, the number of macroscopically visible Peyer's patches (PPs) was approximately 40 % higher than what we observed in the Ctr group ($P<0.01$). Furthermore, the cross section area of isolated mesenteric lymph nodes (MLNs) was approximately 35 % larger than in the Ctr group ($P<0.05$). Despite evident changes in GALT, lymphocyte composition was not altered. Moreover, the number of proliferating epithelial cells was 37 % higher in the SBG group ($P<0.01$) and the size of the proliferative zone was 25 % larger ($P<0.001$) compared to Ctr. **Conclusions:** Mucosal application of SBG stimulate formation and/or expansion of PPs and MLNs. Increased epithelial proliferation suggests that SBG may also affect intestinal barrier function and/or restitution. Our data supports the hypothesis that β-glucans may enhance host protection, in part, by effects on the mucosal immune system. Such effects may be mediated both on the inductive sites of immune responses (PPs and MLNs) as well as the effector sites of immune defense (mucosal epithelium).

Introduction

Attempts to manipulate the immune system with natural compounds to prevent or treat disease, has led to the exploration of immunomodulating polysaccharides. β-Glucans are heterogeneous glucose polymers with a variety stimulatory effects on the immune system (reviewed in 1). β-Glucans reportedly improve host responses to infection (2-4), enhance protection against tumor development (5;6), sepsis (7-9), ischemia/reperfusion injury (10-12) and colitis (13 and Sandvik *et al*., manuscript), promote wound healing (14-16) have adjuvant properties (17-19) and stimulate hematopoietic activity (20). Furthermore, effects on non-immune parameters including cholesterol reduction and blood glucose control (reviewed in 21) as well as pre-biotic properties (22) have been reported. β-Glucans are thought to exert their immunomodulating properties through specific receptors. Multiple cell-surface receptors, including Dectin-1, complement receptor 3, scavenger receptors and lactosylceramide, expressed on both immune- and non-immune cells, have been implicated in β-glucan recognition (reviewed in 23;24). Accumulating evidence points to Dectin-1 as the primary β-glucan receptor (25-27), but clearly alternative pathways exists (28;29).

The uptake of orally administered β-glucans from the intestine to systemic circulation, and the biological effect thereof, has been controversial. Yet, it is now recognized that β-glucans enhance host defence when delivered orally (e.g. 4;5;9;28). Although we and others recently demonstrated limited translocation to blood following oral β-glucan administration to experimental animals (4;9;28), it remains unclear whether β-glucans may act directly on the gastrointestinal mucosa or if entry to the blood stream is required to mediate biological effects.

The intestinal epithelial barrier, consisting of a monolayer of epithelial cells, is crucial in confining the large intestinal microbiota and potentially harmful environmental

antigens to the luminal compartment while allowing nutrient absorption. The intestinal mucosa is the major port of entry for microorganisms and ingested exogenous materials. Consequently, a specialized defence system has evolved to fortify the vulnerable surface, handle the immense antigenic load and maintain homeostasis (30). The mucosal immune system is frequently divided into inductive sites and effector sites. The principle inductive site is organized mucosa-associated lymphoid tissue (MALT). Gut-associated lymphoid tissues (GALT) comprise Peyer's patches (PPs), isolated lymphoid follicles (ILFs), local and regional draining lymph nodes (LN), including the mesenteric LNs (MLNs), and the appendix (31;32). Class switch recombination to IgA, the major antibody isotype and principal effector molecule in mucosal secretions (i.e. secretory IgA; SIgA), primarily takes place in the PPs, MLNs and ILFs (reviewed in 33). Furthermore, MLNs constitute a functional firewall between gut and systemic immune systems and are necessary for induction of oral tolerance and preservation of systemic ignorance to commensal microorganisms (34;35).

A network of immune cells scattered throughout the lamina propria (LP) and the epithelium constitute the effector sites of the intestinal immune system. The epithelium is populated by intra epithelial lymphocytes (IELs), predominantly cytotoxic CD8^{pos} T cells, whereas the LP harbors CD4^{pos} T cells, numerous antigen presenting cells (APCs) and vast numbers of Ab-secreting, primarily IgA^{pos}, plasma cells (reviewed in 36). IELs, frequently divided into two main groups based on the nature of their T cell receptor (αβ vs. γδ), are strategically located in proximity to the antigen-rich intestinal lumen where they serve on the first line of defense by eliminating infected or transformed epithelial cells in a highly regulated fashion (37). Additional non-specific and constitutive defense mechanisms, including secretion of mucus by goblet cells in the crypts of the intestine, production of anti-

bacterial peptides (e.g. defensins) by paneth cells in the crypt base and constant epithelial shedding and renewal, contributes to host resistance (38-40).

It has been investigated whether β-glucans enhance the mucosal immune system. Lehne *et al*. reported that orally administered water-soluble β-glucan significantly increased saliva IgA- but not IgG-levels, without affecting the level of antibodies in circulation in healthy humans (41). Furthermore, Tsukada *et al*. found that oral β-glucan treatment increased the number of small intestine IELs (42).

The overall aim of this work was to identify and examine physiological changes potentially contributing to the health-promoting effects of β-glucans. Specifically we aimed at identifying effects of oral administration of SBG, a *Saccharomyces cerevisiae*-derived water-soluble β-glucan, on mucosal inductive- and effector sites in the intestine of healthy mice. We show a stimulatory effect of oral SBG on mucosal inductive sites which may modulate host immune responses. Furthermore, orally administered β-glucans enhanced epithelial proliferation, and thus conceivably may reinforce intestinal repair.

Materials and Methods

Materials

Endotoxin free (<0.5EU/mL) SBG, a *Saccharomyces cerevisiae*-derived water-soluble β-1,3/1,6-glucan was provided by Biotec Pharmacon ASA (Tromsø, Norway). Anesthesia: Hypnorm® (fentanyl citrate 0.315 mg/mL and fluanison 10 mg/mL, VetaPharma Ltd, Leeds, UK) and midazolam (5 mg/mL, B. Braun Meslungen AG, Germany) were diluted 1:1 in sterile water and subsequently combined in a 1:1 ratio. Antibodies for immunohistochemistry (IHC): Polyclonal rabbit IgG anti-human Ki67 (Ab15580; Abcam, Cambridge, UK), reported to cross react with murine Ki67 antigen, polyclonal rabbit anti-human CD3 (A0452; Dako Cytomation, Glostrup, Denmark), reported to cross react with murine CD3, rabbit IgG anti-hemocyanin control antibody (H0892; Sigma-Aldrich, St. Louis, MO, US) and Alexa Fluor 488 conjugated goat anti-rabbit IgG secondary reporter antibody (A11034; Invitrogen, Carlsbad, CA, US). Primary and secondary antibodies were diluted to working concentrations in phosphate buffered saline (PBS) supplemented with 1.25% and 12.5% bovine serum albumin (BSA) (A7511; Sigma-Aldrich, St. Louis, MO, US), respectively. Citraconic anhydride (CA) buffer (0.05%, pH7.4) (27430; Sigma-Aldrich, St. Louis, MO, US) was used for IHC antigen retrieval. Flow cytometry analysis medium (FM) was made up of PBS supplemented with 5% heat inactivated fetal calf serum and 0.01% sodium azide. OptiLyse B lysing solution (Beckman Coulter, Fullerton, CA, US) was used to lyse whole blood erythrocytes prior to flow cytometry analysis. PE-Cy7 conjugated rat anti-mouse CD4 (RM4-5), Pacific Blue conjugated rat anti-mouse CD8α (53-6.7), PerCP-Cy5.5 conjugated rat anti-mouse CD11b (M1/70), APC conjugated hamster anti-mouse CD11c (HL3), PE conjugated rat anti-mouse CD19 (ID3), biotinylated rat anti-mouse I-A/I-E (MHC class II) (2G9) antibodies and APC-Cy7 conjugated streptavidin were

all from BD Pharmingen (BD Biosciences, San Jose, CA, US) and FITC conjugated rat anti-mouse Dectin-1 (2A11) antibody was provided by AbD Serotec (Kidlington, UK).

Animals

Male BALB/c mice were purchased from Taconic Europe (Ejby, Denmark) and maintained in the minimal disease unit at the Centre for Comparative Medicine at Rikshospitalet University Hospital, Oslo, Norway for at least one week before they were entered into experiments. Animals were housed 2 mice per cage, supplied with water and conventionally fed (Rat and Mouse No.3 Breeding, Special Diets Services, Witham, Essex, UK) *ad libitum*. Cages were kept at 21±1°C and 55±10% relative humidity. Light conditions consisted of alternating 12h light/dark cycles with one hour dusk and dawn. The present investigation was approved by the National Animal Research Authority and conducted in accordance with the Norwegian Animal Welfare Act and the Norwegian Regulation on Animal Experimentation.

Experimental groups and treatment

Mice [22.4 (20.3-24.9) g; mean and (range)] were randomly distributed to two experimental groups **SBG** treated mice and **control (Ctr)** mice receiving SBG-supplemented water (100mg/L) or regular drinking water, respectively, *ad libitum* for 20 days (0-19). Body weight and fluid consumption was recorded and mice were monitored for clinical signs of morbidity throughout the experiment.

Three identical, but separate, experiments were performed. Tissue samples conserved for subsequent histological investigation were collected in the two first experiments, whereas fresh material for flow cytometric analysis was collected in the third experiment.

Termination and necropsy

Animals were anesthetized by subcutaneous injection of Hypnorm® and midazolam (50-75µL/10g body weight) prior to cardiac puncture. Postmortem mice were soaked in 70% ethanol and fixed to a dissection board. The abdomen was opened and the, MLNs, inguinal lymph nodes (ILNs), spleen and intestine were excised. The small intestine was examined for macroscopically visible PPs and identified PPs were excised. The colon was flushed with cold PBS to remove fecal contents prior to fixation. Tissue samples collected for subsequent histological analysis were kept on ice and fixed in 10 % formalin for 24 h at 4 ^{o}C. Fixed tissue samples were transferred to PBS with 0.1% formalin and stored at 4 ^{o}C for subsequent preparation and analysis. PPs, MLNs, ILNs and spleens isolated for subsequent flow cytometric analysis were transferred to ice cold FM. Blood collected by cardiac puncture, in lithium heparin vacuum tubes (Venoject, Terumo, Tokyo, Japan), was kept on ice until subsequent analysis.

Histochemistry and immunohistochemisty

Formalin fixed biopsies were processed using an automated tissue processor (Leica TP1050, Leica Instruments GmbH, Nussloch, Germany) and subsequently embedded in paraffin. Sections were cut at 4µm (Leica RM 2135, Leica Instruments GmbH) and placed on polysine coated slides (Menzel GmbH & Co KG, Braunschweig, Germany). Microscopy and image analysis was performed by an examiner blinded to the sample identity (A.S.).

MLN cross section area

MLN sections were manually stained with hematoxylin and eosin (H&E) and examined in a light microscope fitted with a digital camera and imaging software (Olympus BX51, ColorView IIIu, AnalySISpro 5.0, Olympus Soft Imaging Solution GmbH, Münster, Germany). The MLN cross section area was calculated by analyzing microphotographs

using a build in feature in the microscope imaging software. Briefly, the perimeter of the MLN section was marked using an interpolating drawing tool, and the area was calculated based on the number of pixels included.

Goblet cell count

Sections from the distal colon were deparafinized in xylene and ethanol and rehydrated in distilled water before staining with hematoxylin, alcian blue and periodic acid Schiff reagent (AB/PAS) in an automated tissue stainer (NexES special stains, Ventana Medical Systems Inc., Tucson, AZ, US). Colonic sections were examined in a light microscope (Leitz Dialux 20, Leica Microsystems GmbH, Weltzar, Germany). The number of goblet cells was determined by counting AB/PAS positive cells in 20 well oriented crypts, displaying the intact crypt height, and expressed as the mean number of positive cells per crypt. Intra crypt distribution of goblet cells was indicated as the number of positive cells in the basal-, central- and top 1/3 of the crypt. Illustration microphotographs were acquired using a light microscope fitted with a camera (Olympus BX51, ColorView IIIu, AnalySISpro 5.0).

Immunohistochemistry

Formalin fixed sections from distal colon biopsies were deparafinized in xylene and ethanol, rehydrated in PBS, and boiled in CA antigen retrieval buffer for 20 minutes. Sections were incubated with primary antibodies or concentration- and isotype-matched control antibodies over night at 4 °C. Following washing in PBS, sections were incubated with fluorochrome-conjugated secondary antibody for 3 h at room temperature. Nuclei were stained with Hoechst stain. Sections were examined in a fluorescence microscope (Nikon Eclipse E800, Nikon, Tokyo, Japan) fitted with a digital camera and imaging software (AnalySISpro 3.2, Olympus Soft Imaging System GmbH, Münster, Germany).

The number of proliferating Ki67 positive epithelial cells and the size of the proliferative zone were determined by analysis of digital images (Microsoft Office Picture Manager, Microsoft Corporation, Redmond, WA, US). Areas of the section displaying intact crypt height were chosen for analysis. Cell count was expressed as the mean number of positive cells per crypt, counting ≥ 8 crypts, and the proliferative zone was expressed as a percentage of the total crypt height.

IEL numbers were determined by counting CD3 positive cells clearly located within the epithelium. The entire circumference of a colon section was screened directly in the fluorescence microscope (Nikon Eclipse E800).

Flow cytometry

Spleens, MLNs, ILNs and PPs were disrupted and grinded between two sheaths of nylon mesh in FM buffer using flat spatula-tip tweezers. The homogenate was filtrated over a fresh nylon mesh, centrifuged (1400 rpm/410 g, at 4 ^{o}C for 4 min, Kubota 8800, Kubota Manufacturing Corp., Tokyo, Japan) and washed in FM to produce single cell suspensions. 1 million MLN-, ILN- and PP cells and 300µL whole blood were incubated with 100 µL of a staining cocktail, consisting of antibodies (listed in the material section) and 0.1 mg rat IgG per 100 µL in FM buffer, for 30 min on ice in the dark. Cells were washed in FM, centrifuged as described above and incubated with APC-Cy7 conjugated streptavidin for 20 min on ice in the dark to label the biotinylated antibody employed. Following washing in FM, tissue-derived single cells were resuspended in paraformaldehyde (1% in PBS) and incubated for 5 min on ice in the dark for fixation. The fixative was removed and cells were resuspended in FM and stored in the dark at 4 ^{o}C for subsequent flow cytometry analysis. Leukocytes were prepared for analysis from the whole blood staining reaction by lysis of erythrocytes in OptiLyse B according to manufacturer's instructions. The lysis solution was removed by centrifugation, cells were fixed, resuspended in FM, and stored for analysis as

described above. Unstained spleen, MLN, ILN, PP and OptiLyse B treated whole blood cells served as controls. Cell suspensions were analysed on a flow cytometer (BD LSR II, BD FACSDiva analysis software 5.0.3, BD Biosciences, San Jose, CA, US).

Statistical analysis

Body weight and fluid consumption data were expressed as mean values with standard deviation of the mean (SD) and analyzed using two-way analysis of variance (ANOVA) with Bonferroni post test. PP number, MLN cross section area, goblet cell numbers, epithelial proliferation and IEL numbers were expressed as median values and analyzed using the Mann-Whitney test. Flow cytometry data on lymphocyte composition was expressed as mean values with standard deviation (SD) and analyzed using the Mann-Whitney test. Highly suspect outlier values, unlikely to represent random sampling from a Gaussian population, were identified by Grubb's outlier detection test and excluded from further analysis. All statistical analysis was carried out using GraphPad Prism, version 4 (GraphPad Software, San Diego, CA, USA). Differences at $P<0.05$ were considered statistically significant.

Results

Effect of SBG supplementation on body weight and fluid consumption

Male BALB/c mice were randomly distributed into two experimental groups: A group receiving SBG-supplemented drinking water (SBG) and a control group receiving pure drinking water (Ctr). To monitor the overall health condition of the experimental animals in response to oral SBG administration, body weight was recorded. The mice steadily gained weight and no difference in body weight dynamics between Ctr and SBG treated animals was observed (Figure 1 A). SBG appeared to be well tolerated and no clinical signs of morbidity were noted.

To further investigate the effect of SBG supplementation on appetite and overall activity, and importantly to estimate the daily and total SBG dose acquired, the average fluid consumption per mouse was calculated. Fluid consumption was approximately 4-7 ml/mouse/day, corresponding to a daily β-glucan dose of 15-30 mg/kg body weight in the SBG group. No difference in fluid consumption between the experimental groups was recorded (Figure 1 B). Fluid consumption showed a minor fall immediately after transfer to clean cages, replacement of bottles with fresh ones or addition of fresh drinking water with or without SBG (days 0, 4, 6 and 13) (Figure 1 B).

Oral SBG administration affect mucosal inductive sites

To investigate the effect of oral SBG administration on GALT, PPs and MLNs, essential mucosal inductive- and regulatory sites, were examined. In the SBG group, the median number of macroscopically observable PP in the small intestine was approximately 40 % higher than what we observed in the Ctr group ($P<0.01$) (Figure 2 A). Furthermore, we identified a significant increase in the MLN size in SBG-treated mice. In the SBG group, the

median cross section area of isolated MLNs was approximately 35 % larger than what we observed in the Ctr group (P<0.05) (Figure 2 B). Assuming sphereoid LNs, this corresponds to an estimated volume increase of 50-60 %.

Despite the evident changes in GALT following oral SBG administration, characterization of the major lymphocyte populations (CD4^{pos}, CD8^{pos}, CD19^{pos} cells) by flow cytometry analysis revealed no differences between the experimental groups neither for the PPs nor the MLNs (Figure 3). Similarly, lymphocyte composition in blood leukocytes, spleen- and ILN single cell suspensions, representing the systemic compartment, was not altered by oral SBG administration (data not shown). Flow cytometry analysis revealed the presence of Dectin-1^{pos} cells, primarily MHC class II^{pos}, CD11b^{pos} or CD11c^{pos} cells, i.e. macrophages and dendritic cells (DCs), in all cell preparations examined. However, oral SBG administration did not appear to change the expression profile of this β-glucan receptor. Of MLN single cells isolated from SBG treated mice, 3.5 ±1.8 % were Dectin-1 positive vs. 3.3 ±1.2 % in Ctr animals (mean ± SD). The corresponding numbers for PP were 0.22 ±0.04 % vs. 0.23 ±0.05 %, for ILN 3.2 ±1.2 % vs. 3.4 ±0.7 %, for spleen 6.5 ±1.6 % vs. 7.1 ±1.2 %, and for blood leukocytes 7.9 ±1.6 % vs. 8.0 ±1.9 %.

Oral SBG administration increase epithelial proliferation

To examine natural defense functions mediated by the different intestinal epithelial cell types, we first analyzed the number and distribution of mucus producing goblet cells. Oral SBG administration did not affect the number or intra crypt distribution of goblet cells in the distal colon. The number of AB/PAS positive goblet cell per crypt in SBG treated mice was 6.6 [3.1-8.8] compared to 7.6 [5.4-10.3] in the Ctr group (mean and [range]). We also stained sections for IELs, but very few IELs were identified in the distal colon sections and no difference in IEL numbers between SBG treated mice and controls was revealed. The

number of $CD3^{pos}$ IELs per section in SBG treated mice was 12.4 [5.0-21.0] compared to 12.3 [6.5-21.5] in the Ctr group (mean and [range]).

Next, we investigated the effect of oral SBG administration on the intestinal epithelium, a mucosal effector site. In mice treated with SBG the number of proliferating epithelial cells in the distal colon was significantly higher than what we observed in control animals (Figure 4). The median number of $Ki67^{pos}$ cells per crypt in the SBG group was 37 % higher than in the Ctr group ($P<0.01$). Also, the median size of the proliferative zone was 25 % larger in the SBG group ($P<0.001$) (Figure 4).

Discussion

Numerous reports advocate the use of β-glucans to treat or prevent various medical conditions and several β-glucan preparations are currently in clinical trials (43). Yet, the current understanding of the mechanisms of action remains inadequate. Here we demonstrate for the first time that orally administered SBG stimulate GALT and epithelium, vital inductive- and effector sites of the mucosal immune system, respectively.

Although β-glucans have attracted interest as growth performance enhancers in livestock (44-46), we did not observe any difference in body weight gain between SBG-treated mice and controls over the 20 day experimental period. Yeast β-glucans are listed as "generally recognized as safe" (GRAS) by the US Food and Drug Administration (FDA) (47). We observed no clinical signs of morbidity and no change in appetite, fluid consumption or general behavior, thus the study drug appeared to be well tolerated. Whether the minor decrease in fluid consumption in both experimental groups following transfer to clean cages, replacement of bottles with fresh ones or addition of fresh drinking water with or without SBG represents a methodological problem or reflects a biological phenomenon remains unclear. The calculated daily SBG dose range (15-30mg/kg) was previously demonstrated to mediate protection against experimental colitis (Sandvik *et al*., manuscript). Furthermore, in a recent clinical trial, Lehne *et al*. reported increased saliva IgA levels in healthy volunteers administered 400 mg SBG orally for 4 consecutive days (5.7 mg/kg/day, assuming 70kg body weight) (41). Thus, it is conceivable that the dose range used in this study is sufficient to mediate biological responses in experimental animals as well as in humans.

Although it is now acknowledged that oral β-glucan therapy enhance host immunity, it remains unclear whether β-glucans act on the gastrointestinal mucosa or require

absorption to the blood stream to mediate protective effects. Lehne and co-workers suggests that systemic uptake is not a prerequisite for host responses as serum samples were β-glucan negative (41). In line with this finding, we recently published evidence suggesting that the degree of protection does not depend on the β-glucan serum levels and that the mechanism of action may depend on the route of delivery (9).

In this study we demonstrate that oral administration of soluble β-glucan has an effect on GALT (PP and MLN) size. Hong and co-workers reported that particulate β-glucan delivered by intragastric gavage was internalized by gastrointestinal macrophages that traffic to the spleen, peripheral lymph nodes and bone marrow (28). Furthermore, Rice and co-workers showed that intestinal epithelial cells, possibly M cells of follicle associated epithelium, and PP cells bound and internalized water-soluble β-glucans delivered orally (4). Epithelial uptake was demonstrated to be independent of Dectin-1 expression (4). In a previous study we speculated whether mucosal macrophages and DCs could sample β-glucan from the intestinal lumen via transepithelial protrusions (9). Presumably, β-glucan-laden cells migrate from the gut via GALT and lymphatics to reach the systemic compartment. Although speculative, we find it plausible that β-glucan-laden cells migrating from the intestinal epithelium to GALT may contribute to the observed expansion of MLNs and PPs.

Increased numbers of macroscopically visible PPs in SBG treated mice may theoretically be the result of (I) expansion of invisible PPs or PP anlagen or (II) genuine secondary lymphoid organ neogenesis. While we have not addressed this question experimentally, the current view is that PPs show considerable plasticity but form at predetermined primitive anlagen that develop during embryogenesis or early post natal life (48). It remains to be explored whether PP and MLN expansion is due to increased cell influx, reduced efflux, or proliferation. If proliferation is taking place, central questions

arising include; is the response mono- or polyclonal, what is the antigen, and is the propagation driven by SBG-primed APCs? In general purified β-glucans are considered poor immunogens. Hence, we find it unlikely that SBG administration results in a SBG specific immune response, especially when delivered via the oral route. Also, the maturity/differentiation status of the GALT cells and the presence/number of germinal centres remain unexplored. Follow-up studies are required to establish whether GALT expansion results in superior immune defence. Nevertheless, we hypothesize that SBG modulation of mucosal inductive sites, may shape the immune response and, consequently, contribute to improved host wellbeing.

We characterized the relative content of $CD4^{pos}$ and $CD8^{pos}$ T cells and $CD19^{pos}$ B cells, major lymphocyte populations, of MLNs and PPs by flow cytometry and found no significant difference between SBG treated mice and controls. Rice *et al*., found that oral β-glucan administration resulted in increased Dectin-1 expression on PP macrophages as well as up regulated Toll-like receptor (TLR) 2 expression on PP DCs. Thus, oral β-glucan delivery modulated expression of central pattern recognition receptors in GALT (4). Of note, Dectin-1 and TLR2 have been demonstrated to collaborate (49). In contrast to Rice and co-workers we observed no change in Dectin-1 expression in response to oral SBG administration.

Goblet cells are mucus producing cells of epithelial origin with an important role in natural defence at the mucosal effector site. Goblet cells are abundant in the densely colonized colon where secreted mucins add strength to the epithelial barrier and prevent adhesion of intestinal microbes to the epithelium (50). Oral SBG administration did not change the number and distribution of goblet cells in the colon. The effect of oral SBG treatment on production of antimicrobial peptides, including defensins from Paneth cells – yet another cell type of epithelial origin, and the mucus composition, was not examined. Of

note, we only addressed the distal colon and do not know whether our observations are representative for other segments of the intestine.

Tsukada *et al*. reported that oral β-glucan administration increased the number of IELs in the small intestine in mice, indicating that oral β-glucan delivery may reinforce effector mechanisms of the mucosal immune system (42). We did not observe that SBG caused altered IEL numbers. IELs were scarce in the distal colon and we can not rule out that a difference between the groups may be apparent in the small intestine which is more densely populated with IELs. What is more, the distribution of IEL subsets in the large and small intestine differs (51). In contrast to Tsukada *et al*., Van Nevel and co-workers, studying a different β-glucan, reported that a β-glucan supplemented diet resulted in reduced numbers of IELs and possibly reduced mucosal turnover rate in newly weaned piglets (52).

We recently demonstrated that oral SBG prophylaxis protected against dextran sulfate sodium (DSS)-induced colitis (Sandvik *et al*., manuscript). DSS is believed to induce intestinal inflammation primarily by disrupting the epithelial barrier (53). Here we report that SBG increased the number of proliferating epithelial cells as well as the size of the proliferative zone in the colon when administered orally. Data presented here strongly suggests that the protective effect of SBG in experimental colitis is, in part, due to stimulatory effects on epithelial proliferation and thus conceivably on epithelial barrier restitution and function. In support of this idea, Laukoetter *et al*. demonstrated that increased intestinal epithelial proliferation in Junctional Adhesion Molecule A (JAM-A) deficient mice was associated with improved colon histology following DSS-induced colitis (54). To the best of our knowledge, we are the first to report that oral β-glucan administration stimulate intestinal epithelial proliferation. Of note, in the mouse, the small intestine epithelium renews approximately every 5 days (55). Thus, the baseline rate of proliferation is high and continuous epithelial shedding and renewal is part of the innate

defense system. It remains to establish whether the SBG-mediated increase in proliferation improve the intestinal barrier integrity.

We have not addressed whether oral SBG administration have stimulatory effects on the lamina propria (LP). Lehne *et al*. demonstrated that oral SBG caused increased levels of IgA in saliva, indicating stimulatory effects on mucosal inductive sites and/or IgA^{pos} plasma cells in the LP (41). In a pilot experiment, we too examined IgA levels in saliva and feces, following intra gastric SBG administration. In contrast to Lehne and colleagues, we did not observe any difference in total IgA levels between SBG treated mice and controls (unpublished observation). Furthermore, we have not addressed whether oral SBG may affect intestinal DCs and regulatory T cells, key regulators of intestinal defense and homeostasis.

In conclusion, we demonstrate that oral administration of SBG, a *Saccharomyces cerevisiae*-derived water-soluble β-glucan, stimulated formation and/or expansion of PPs and MLNs. Furthermore, SBG stimulated proliferation of mucosal epithelial cells, suggesting that SBG may also affect intestinal renewal and/or barrier function. Conceivably, β-glucans may enhance host protection, in part, by effects on the mucosal immune system. The stimulatory effects may be mediated both on the mucosal inductive sites of immune responses as well as the effector sites of immune defense. Further work is required to explain how these changes in health mice may contribute to the reported health promoting effects of β-glucans.

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Figure legends

Figure 1. Effect of SBG supplementation on body weight and fluid consumption.

Male BALB/c mice were randomly assigned to two experimental groups. One group received SBG supplemented drinking water (SBG; closed symbols) and the control group received pure drinking water (Ctr; open symbols). Mice and drinking bottles were weight regularly throughout the experiment. A) Body weight was recorded and B) daily fluid consumption per mouse was calculated. Body weight is expressed as percentage of base line (BL) values, and fluid consumption is expressed as ml/mouse/day, mean values and SD.

Figure 2. Oral SBG administration increases the number of Peyer's patches and cross section area of mesenteric lymph nodes.

Mice were treated with SBG (SBG; closed symbols) or regular drinking water (Ctr; open symbols) as described. At termination, anesthetized mice were sacrificed and the small intestine and the mesenteric lymph nodes (MLNs) were excised. Sections of formalin fixed, paraffin embedded MLNs were stained with hematoxylin and eosin and examined in a light microscope. A) The number of macroscopically visible Peyer's patches (PP) in the small intestine was recorded. B) The MLN cross section area was calculated by software assisted image analysis. Bars represent median values. *$P<0.05$, **$P<0.01$, as determined by Mann-Whitney test. Highly suspect outlier values, unlikely to represent random sampling from a Gaussian population, were identified by Grubb's outlier detection test and excluded from further analysis.

Figure 3. Oral SBG administration does not affect PP and MLN lymphocyte composition.

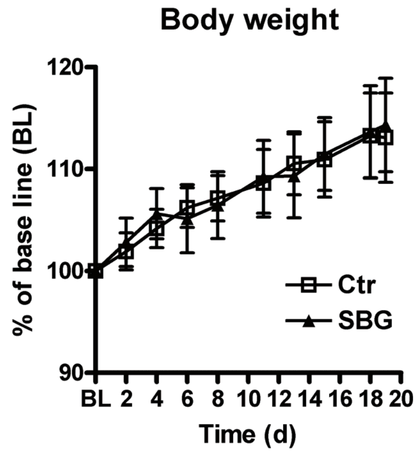
Mice were treated with SBG (SBG; closed bars) or regular drinking water (Ctr; open bars) as described. Freshly isolated Peyer's patches (PPs) and mesenteric lymph nodes (MLNs) were homogenized and single cell suspensions were stained for flow cytometry analysis as described in the materials and methods section. Lymphocytes were identified and gated on based by their forward-, side scatter properties. Back gating confirmed that the selected cell population was in fact lymphocytes. The proportion of major lymphocyte populations ($CD4^{pos}$ and $CD8^{pos}$ T cells and $CD19^{pos}$ B cells) in A) PPs and B) MLNs were determined. Mean values and SD are presented (n=6/group).

Figure 4. Oral SBG administration increase epithelial proliferation

Mice were treated with SBG (SBG; closed symbols) or regular drinking water (Ctr; open symbols) as described. Formalin fixed distal colon specimens were prepared for immunohistochemistry as described in the materials and methods section. Briefly, Ki67 was used as a proliferation marker and positive cells were visualized using an Alexa Fluor 488 conjugated secondary antibody. A) The number of Ki67 positive epithelial cells expressed as the mean number of positive cells per crypt, counting ≥8 intact crypts. B) The size of the proliferative zone expressed as a percentage of the total crypt height. Proliferation was quantified by analysis of digital images. Bars represent median values. **P<0.01, ***P<0.001, as determined by Mann-Whitney test.

Figure 1.

A



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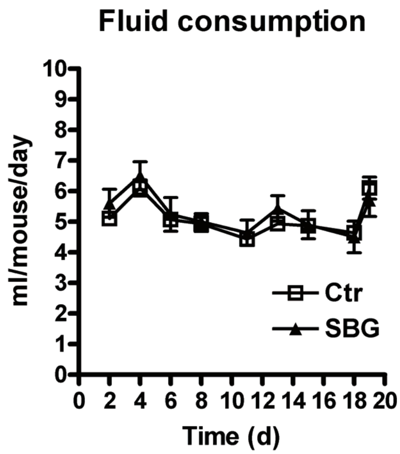

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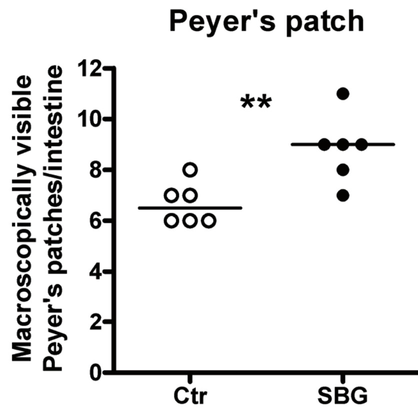

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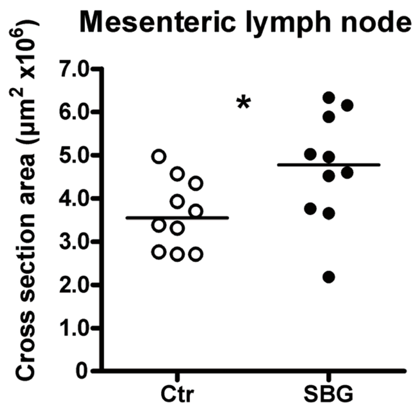

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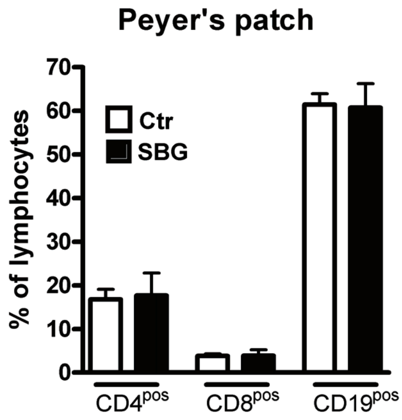

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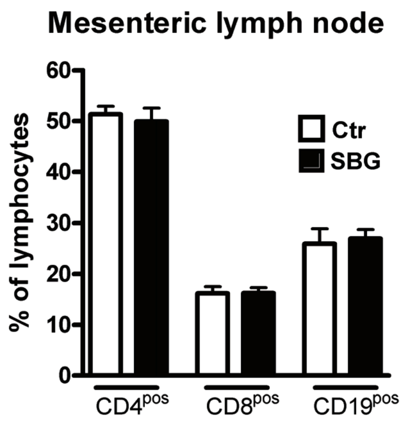

Figure 4.

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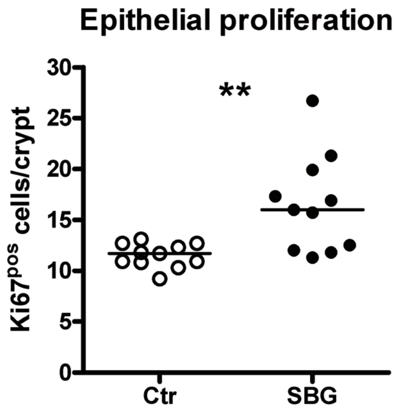

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