Studies on a medicinal *Agaricus blazei* Murill based mushroom extract

Anti-inflammatory effects in vivo on healthy individuals and
patients with ulcerative colitis and Crohn's disease and cellular effects in vitro

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Preface and acknowledgments

This study was performed between 2006 and 2011 while working as a resident at the Department of Gastroenterological Surgery at Oslo University Hospital, Ullevaal. During this period, my work was equally divided between research and patient care.

The *in vitro* experiments were conducted at the Clinical Research Center, Oslo University Hospital, Ullevaal.

I am very much grateful to my main supervisor professor Egil Johnson, Department of Gastroenterological Surgery, without whom this project would never have been a reality. He stood by me during the ups and downs of this research. My thanks to assistant advisor Dr. Geir Hetland, for creative ideas.

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Finally, I wish to thank my family, my parents, and most of all, my wife Anne Marit and our daughter Helene, for their support and tolerance. Their unconditional encouragement gave me the strength and motivation to carry through this work.

Dear Anne Marit, thank you very much for your love and care!

Oslo, June 2011

Selected Abbreviations

AbM:	*Agaricus blazei* Murill
ADCC:	antibody-dependent cellular cytotoxicity
APC:	antigen-presenting cell
BRM:	biological response modifier(s)
CC:	cystein-cystein
CD:	Crohn's disease
C(3)(4)(5):	complement component (3)(4)(5)
CR3:	complement receptor 3
CXC:	cystein-x-cystein
DC:	dendritic cell
DHE:	dihydroethidium
DHR:	dihydrorhodamine 123
DNA:	deoxyribonucleic acid
EC:	endothelial cells
ELAM:	endothelial cell adhesion molecule
ELISA:	enzyme-linked immuno sorbent assay
FITC:	fluorecin isothiocyanate-conjugated
FC:	fold changes
Fc(r):	fragment crystalline (receptor)
FoxP3:	forkhead box P3
GALT:	gut associated lymphoid tissue
GCOS:	genechip operating software
G-CSF:	granulocyte colony-stimulating factor
GM-CSF	granulocyte-monocyte colony-stimulating factor
GTP:	guanosine-tri-phosphate
HLA:	human leucocyte antigen
HUVEC:	human umbilical vein endothelial cells
IBD:	inflammatory bowel disease
ICAM-1:	inter-cellular adhesion molecule 1
IFNγ:	interferon γ
Ig:	immunoglobuin
IL(r):	interleukin (receptor)
IRAK1	Interleukin-R-associated kinase 1
kD:	kilo Dalton
LAK:	lymphokine-activated killer
LAM:	leukocyte adhesion molecule
LFA:	leukocyte function antigen
LP:	lamina propria
LPS:	lipopolysaccharide
LTB4:	leukotriene B4
MAC:	membrane attack complex

MBL: mannose-binding lectin
M cell: microfold cell
MCP-1: monocyte chemotactic protein-1
MDDC: monocyte-derived dendritic cells
MFI: mean fluorecence intensity
MHC: major histocompatibility complex
MIP-(1ß) (2): macrophage inflammatory protein (1ß) (2)
MLN: mesenteric lymph node
mRNA: messenger ribonucleic acid
МΦ: macrophage
NADPH: nicotinamide adenine dinucleotide phosphate
MPO: myeloperoxidase
NF-κB: nuclear transcription factor – kappa B
NK cell: natural killer cell
NLR: nucleotide-binding oligomerization domain receptor
NOD: nucleotide-binding oligomerization domain
PAMP: pathogen associated molecular pattern
PAX: paired box
PBS: phosohate-buffered solution
PE: phycoerythrin
PLIER: probe logarithmic intensity error
PMN: polymorphonuclear granulocytes
PRM: pattern-recognition molecule
PRR: pattern-recognition receptor
RIG-1: retionoic-acid-inducible-gene1
RLR: retionoic-acid-inducible-gene1-like receptor
ROR: retinoic acid-related orphan receptor
ScR: scavenger receptor
T-bet: T-cell specific transcription factor
TGFβ: transforming growth factor β
Th cell: T helper cell
TLR: toll-like receptor
TNFα: tumor ncrosis factor α
T_{reg} cell: regulatory T cell
TSLP: thymic stromal lymphopoietin
UC: ulcerative colitis
WHO: world health organization
XBP-1: x-box binding protein-1

List of papers

I Johnson E, Førland DT, Sætre L, Bernardshaw SV, Lyberg T, Hetland G. Effect of an extract based on the medicinal mushroom Agaricus blazei Murill on release of cytokines, chemokines and leukocyte growth factors in human blood ex vivo and in vivo. Scand J Immunol 2009; 69:242-45.

II Førland DT, Johnson E, Tryggestad AMA, Lyberg T, Hetland G. An extract based on the medicinal mushroom Agaricus blazei Murill stimulates monocyte-derived dendritic cells to cytokine and chemokine production in vitro. Cytokine 2010; 49:245-50.

III Johnson E, Førland DT, Hetland G, Olstad OK, Lygerg T. Effect of an extract based on the medicinal mushroom Agaricus blazei Murill on expression of adhesion molecules and production of reactive oxygen species in human monocytes and granulocytes *in vivo.* PloS ONE Submitted April 2011.

IV Førland DT, Johnson E, Sætre L, Lyberg T, Lygren I, Hetland G. Effect of an extract based on the medicinal mushroom Agaricus blazei Murill on expression of cytokines and calprotectin in patients with ulcerative Colitis and Crohn′s disease. Scand J Immunol 2011;73:66-75.

Erratum

Paper I: In Figure 3, page 246: “Days (0,1,2)” should be “Days (0,2,12)”

General introduction

In the coastal Piedade area outside of São Paulo, Brazil, the *Agaricus blazei* Murill (AbM) mushroom grows wildly. AbM has been used as a natural food ingredient by the locals. The prevalence of serious diseases like atherosclerosis, hepatitis, hyperlipidemia, diabetes, viral infection and cancer (40;107) was lower among people in Piedade than in the general population. Accordingly, this health promoting effect may be related to intake of AbM, which belongs to the *Basidiomycetes* family. AbM has many common names, such as Royal Sun Agaricus, Sun Mushroom, Mushroom of God, Himematsutake (japanese (jp.)), Songrong (chinese (zh.)) and almond mushroom. AbM grows in soils that are rich in woody debris, mixed woods, well-composed soils, as well as along forest-field interfaces.

Spores of the mushroom were in 1966 taken to Japan for commercial cultivation and it was introduced into the health food market and later subjected especially in Asia, but also in Europe, to an increasing research effort (27). AbM exhibits biological effects foremost by interacting with cells of innate immunity including macrophages (MΦ), monocytes and natural killer cells (NK cells). Biological consequences of AbM are anti-tumor effects in rodents (27;38), but also protection against allergy (22) and lethal bacterial peritonitis in mice (38). Studies *in vitro* (5;95) demonstrated that AbM stimulates the release of pro-inflammatory cytokines from monocytes and endothelial cells. An *ex vivo* study (8) showed a pro-inflammatory effect of AbM in whole blood by increase of reactive oxygen species (ROS) in granulocytes and modulation of adhesion molecules also in monocytes.

Recently, also anti-inflammatory effects of AbM have been reported (76) after rats have ingested this mushroom extract for several weeks, measured by reduction of nystatin induced rat paw oedema, reduced neutrophil migration and arthritis. Likewise, isolated ß-glucan extract from another mushroom, *Pleurotus ostreatus* (69), reduced acetic acid induced colitis in mice when administered enterally and intraperitoneally.

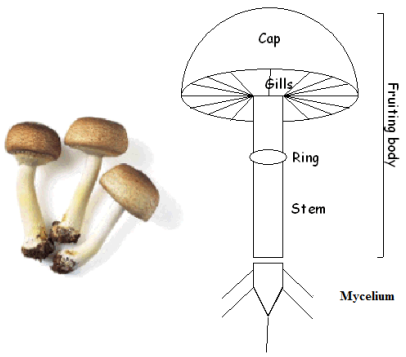
ß-glucans are considered the most potent immunomodulatory molecules in mushrooms and we were interested in studying prospective anti-inflammatory effects of the AbM based mushroom extract in both healthy individuals and in patients with inflammatory bowel disease (IBD). A potential anti-inflammatory effect in patients with ulcerative colitis (UC) and Crohn's disease (CD) could perhaps reduce the need for classical medication in these patients, which show variable effect and have side effects.

Composition of AbM and the mushroom extract AndoSan™

The mushroom extract AndoSan™ used in our experiments was obtained from the company ACE Company Ltd., Gifu-ken, Japan. The AndoSan™ extract is made of AbM mixed powder and water. The final concentration is 340 gram per litre. It contained 82.4% from the mushroom *Agaricus blazei* Murill (AbM (jp. Himematsutake)), 14,7% from the mushroom *Hericeum erinaceum (jp.* Yamabushitake) (57) and 2,9% from the mushroom *Grifola frondosa* (jp. Maitake) (1), all belonging to the *Basidiomycetes* mushroom family. The AbM mixed powder contains per 100 gram the following constituents: moisture 5,8 g, protein 2,6 g, fat 0,3 g, carbohydrates 89,4 g of which β-glucan constitutes 2,8 g, and ash 1,9 g. The amount per litre of the extract for sodium was 11 mg, phosphor 254 mg, calcium 35 mg, potassium 483 mg, magnesium 99 mg and zinc 60 mg. The content of lipopolysaccharide in AndoSan™ was measured to be a miniscule concentration of <0,5 pg/ml.

The main component AbM is rich in ß-glucans (27) composed of ß-1,6-backbone and ß-1-3-side branches (ratio of 1:2). However, AbM contains several active constituents other than β-glucans, like α-(1->4)-glucans (29), proteoglucans (51), lectins (52), ergosterol (provitamin D2) (98), agaritine (24), isoflavonoids (71) and anti-oxidant substances (46) which are of most interest. Soluble AbM contains micro particles, which was confirmed by light microscopic examination after centrifugation in our laboratory. Thus the ß-glucan fraction of AndoSan™ presumably contains a continuum of small soluble to larger insoluble fragments.

Figure 1. The medicinal mushroom, *Agaricus blazei* Murill (AbM)



The mushroom consists of a fruiting body growing above the ground. Further more the fruiting body consist of a cap and a stem. The Myceliumis the vegetative part of the mushroom growing under the ground (Fig. 1). Depending on the manufacturers, accumulation of unwanted harmful chemicals in the dried mass from the fruiting body and the mycelium vary substantially, owing to pollution of soil by heavy metals etc. which are accumulated in mushrooms and fungi. Cultivated mushrooms may generate toxic compounds from non-toxic substrates, like agaritine (36;102), which makes up approximately 1% of the dried mass of the fruiting bodies. However, it is unknown to what extent agaritine and other phenylhydrazine derivatives from the cultivated mushrooms are degraded during the manufacturing procedure. Since it is generally known that heavy metals may be accumulated in mushrooms, it is of great importance to measure concentrations of such elements and to cultivate the mushroom in unpolluted soil or substrate. However, specialized patent-protected processing treatments can remove these substances while not affecting beneficial properties of this mushroom. The elements of the AbM based mushroom extract AndoSan^{TM} that we used in our experiments, did not contain detectable Zinc (Zn), Selen (Se), Germanium (Ge) or hydrazine, according to the analysis report delivered by the Japan Food Research Laboratories, Tokyo, Japan.

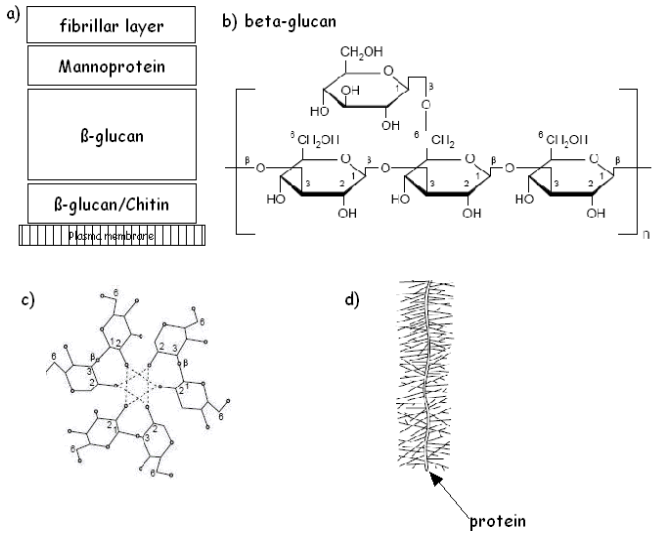
Structural and functional inter-relationship

The cell wall of the mushroom *Basidiomycetes* family and other mushrooms contains biologically active polysaccharides, in particular ß-glucans, which are recognized as biological response modifiers (BRM) (58) and act on pattern recognition receptors (PRR) on leucocytes in both innate and adaptive immunity (25). The polysaccharide BRM, which are most prominently found in the fruiting bodies of mushrooms (Fig. 1), are β-glucan and α-mannan. Such polysaccharides were also found in culture medium in which AbM was grown (72). Since the molecular structures are mainly dependant on the osmotic and chemical nature of the culture medium, the methodology in obtaining a purified form of the active substances is very important. These polysaccharides differ in chemical composition especially the β-glucans (Fig. 2). β-glucan with β-(1->3)-D-linkage is usually a main water soluble skeleton. Three β-(1->3)-D-polymers with β-(1->6)-D-branches form a triple helical structure by hydrogen bonding. The triple helical structure becomes covalently bound to chitin, which is a major soluble polymer of the cell wall (53), rendering the resulting complex insoluble in an alkaline milieu. The triple helical conformation is stable at neutral

pH, whereas decrease in pH shifts the conformation to a single helical and furthermore, to a random coil structure(58). It is generally believed that the host's immune responses to BRM are structurally specific.

Several sophisticated biotechnological methods are available for purification and extraction of AbM. However, the laboratory procedures do follow a few common and empirical methods (58;107). Briefly, the dried fruiting bodies of the mushroom is denaturated and detoxified by soluents/solvent (e.g. NaOH, EtOH, MeOH, Hexane, Chloroform) before boiling followed by additional solvents and then the freeze-drying process for development of a precipitate, from which active polysaccharides are isolated (e.g. chromatography) and tested for biological activity.

Figure 2. Structure of the outer wall of fungi including β-glucans. a) The cell wall is composed predominantly of carbohydrates that are essential for structural integrity and survival of these cells. b) β-Glucans comprise a major component of many fungal cell walls and occur in linear (β-(1->3)) or branched (β-(1->6)) forms. Innermost is the bilayered cell membrane, which also contains sterols (ergosterol), where the glucosyl units within glucans which are arranged as long coiling chains of β-(1->3)-glucan-linked residues with occasional β-(1,6)-linked side chains. c) Three β-(1->3) chains running parallel can associate to form a triple-helix, and the aggregation of helices produces a network of water-insoluble fibrils. d) Proteoglucan complex.



Effects of AbM in vitro, ex vivo and in vivo

AbM stimulates macrophages *in vitro* (95) to increase synthesis and release of interleukin-8 (IL-8), tumor necrosis faktor α (TNFα) and nitric oxide. In human monocytes and human vein endothelial cells (HUVEC), AbM induces (5) release of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNFα), but not IL-12 or the anti-inflammatory cytokine IL-10. This latter result was supported by demonstrating selective up-regulation of genes for IL-1β and IL-8, but not for IL-10 and IL-12, by using gene expression microarray analysis of promonocytic THP-1 cells (leukemic cell line) stimulated with AbM (23).

By stimulation of whole blood with the AbM extract *ex vivo* (8), expression of adhesion molecules CD62L (L-selectin) decreased and CD11b increased both on human monocytes and granulocytes. CD11b promotes complement-mediated phagocytosis in these cells. The level of reactive oxygen species (ROS), more specifically peroxynitrite ($ONOO^-$), increased moderately in granulocytes, which indicated increased potential for degradation of microorganisms. Altogether, these results demonstrated a pro-inflammatory effect *in vivo* and *ex vivo* of AbM *per se* or combined with the two other basidiomycetes mushrooms, *Hericium erinaceum* and *Grifola frondosa*, in the AndoSan™ mixture.

AbM mycelium has also been shown to inhibit the cytopathic effect of Western equine encephalitis virus on VERO cells in culture (96). Moreover, *in vitro* bactericidal and fungicidal effects of *Agaricus species* have been reported (82;105), although our group earlier found none effects when examining such properties. Although, not the topic for this thesis, anti-tumor effects of components of AbM have been reported in mouse models against fibrosarcoma, myeloma, ovarian-, lung- and prostate cancer, and in human studies against gynecological cancer (increased NK-cell activity and quality of life) and leukemia as well as in cancer cell cultures (19;98;111). In addition to ß-glucan in AbM, ergosterol and agaritine also exhibit anti-tumor activity, respectively, by oral administration in sarcoma 180 bearing mice (98) and by induction of apoptosis in leukemic cells (24). Moreover, isoflavonoids, another isolated subcomponent of AbM, had potent hypoglycaemic action as demonstrated by reduced blood glucose levels in diabetic rats (71).

In vivo, our group has previously reported that AndoSan™ given orally to mice before intraperitoneal inoculation of pneumococci (7) or feces (6), reduced subsequent degree of sepsis and increased survival of the mice. Increased levels of macrophage inflammatory protein 2 (MIP-2), the mouse analogue to human IL-8, and TNFα were detected in mice given AbM compared with placebo prior to peritonitis, which is partly thought to contribute

to the improved results in the mice using this mushroom extract. This is an intriguing finding since MIP-2 is considered to be a pro-inflammatory cytokine.

Recently, the AndoSan^{TM} mushroom extract has been shown to protect against IgE-mediated allergy in a mouse model when given orally either before or after subcutaneous sensitation of the animals (22). In supernatants of cultured spleen cells from the AbM-treated mice there was an increased T helper cell 1(Th1) response relative to the allergy-inducing T helper cell 2 (Th2) cytokine response. The observation fits with the reduced specific serum IgE levels in these animals and shows that also adaptive immunity is engaged by the mushroom. Since the original Th1/Th2 dichotomy (81) says that the anti-tumor and anti-infection Th1 response is inversely related to the Th2 response, the spleen cell finding above also helps explain the concomitant anti-allergic, anti-tumor and anti-infection effects of AbM. Moreover, this agrees with the very interesting finding that AbM extract ameliorated a skewed Th1/Th2 balance both in asthma-induced and in tumor-bearing mice (99).

In line with the anti-allergic effect induced by AndoSan™ in mice, a study in 2009 (76) where an aqueous alkaline extract of AbM was given for 1-2 weeks in mice, anti-inflammatory effects were observed in vivo. Oral intake of AbM reduced neutrophil migration to the peritoneal cavity and the degree of rat paw oedema induced by nystatin as well as reduced the extent of arthritis induced by Freund's adjuvant. It was speculated that AbM down-regulated the immune system by means of interaction with ß-glucans of the extract. The initial study demonstrating an anti-inflammatory effect of a mushroom extract, was from a ß-glucan (pleuran) isolated from the fruiting bodies of *Pleurotus Ostreatus* (69), given orally or intraperitoneally for 4 weeks in rats with experimentally acetic acid induced colitis. The colonic damage score was significantly reduced compared to placebo (cellulose). In addition, myeloperoxidase (MPO) activity was reduced in the normal mucosa of rats, without induction of colitis, treated solely with pleuran compared with cellulose. Thus, reduced MPO- activity and consequently also generation of ROS would presumably attenuate the acetic acid induced inflammatory response.

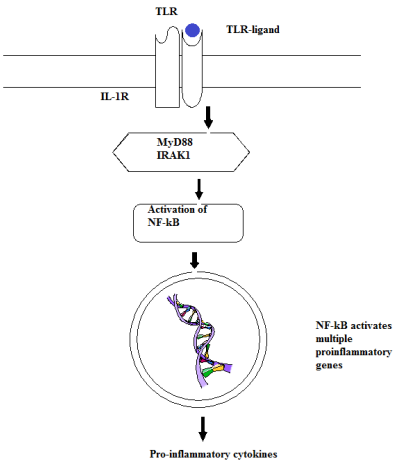
We found it intriguing, the paradoxical response of the mushroom extract being pro-inflammatory *in vitro* and *ex vivo*, and also protective against infections. It also reduced the inflammatory response *in vivo* in rodents. One contributing factor behind the anti-inflammation *in vivo*, may be the existence of low molecular weight gut absorbable antioxidant substances in AbM (46), which reduce the levels of ROS.

Mechanism for stimulation of the immune system

The reason for the forceful and swift engagement of innate immunity when encountering an edible and harmless mushroom such as AbM, is its sharing of pathogen-associated molecular patterns (PAMP) with other highly poisonous species. Such mushrooms and fungi are usually a health threat due too action of their toxins; e.g. muscimol from *Amanita muscaria* and the vasoconstrictor ergotamine from *Calviceps purpurea*, or invasion in immune deficient patients (e.g. *Aspergillus fumigatus*) or normal individuals (e.g. *Stachybotrys chartarum*). PAMP, such as β-glucans, which form the main cell wall skeleton in mushrooms and fungi and are their signature molecule, are recognized immediately by pattern recognition reseptor (PRR). More specifically, AbM acts upon cells of innate immunity like monocytes/macrophages (MΦ) (10;39), dendritic cells (DC) (33) and NK (30).

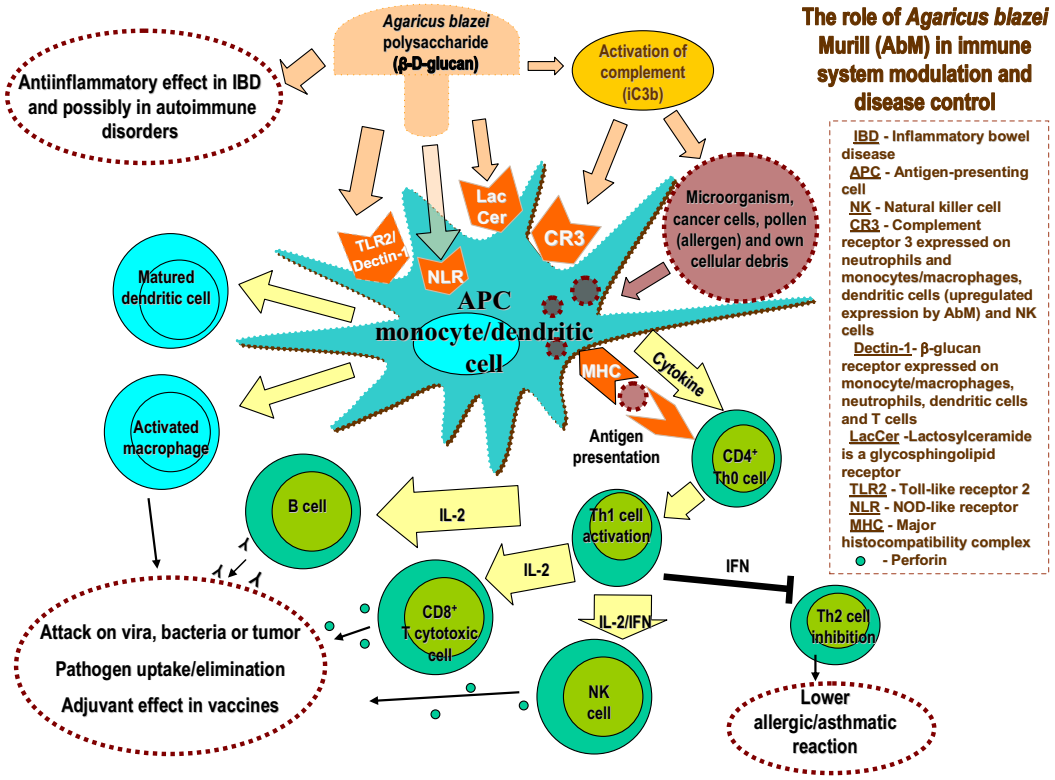
The stimulatory effect is probably mediated by binding of foremost glucans to toll-like receptor 2 (TLR2), but not TLR4 (80), the dectin-1 receptor (13), the lectin binding site for ß-glucan of complement receptor C3 (CR3) (CD11b/18) (104) and possibly complement receptor C4 (CR4) (CD11c/18) (4). Stimulation of the TLR2 induced intracellular nuclear transcript factor kappa B (NF-κB) activation (Fig. 3) leads to increase of transcription and synthesis of mainly pro-inflammatory cytokines *in vitro* (103).

Figure 3. The nuclear transcript factor kappa B (NF-κB) pathway. Triggering TLR or IL-1R recruits the adaptor molecules M_yD88 and IL-1R associated protein kinases, IRAKs, which finally translocate the NF-κB into the nucleus. This transcription factor activates transcription of pro-inflammatory genes.



Since human skin endothelial cells can express all 10 TLR genes (28), TLR-binding of AbM was probably one mechanism behind the increased synthesis of cytokines in HUVEC, which demonstrated that AbM also affects endothelial cells (EC), which are important part-takers in the innate immune response. It has also been shown that AbM affects the humoral immune system, a part of the adaptive immune system, through activation of factor C3 of the alternative complement pathway (92). The gene microarray study in AbM-stimulated promonocytic THP-1 tumor cells *in vitro* (23) also demonstrated upregulation of genes for TLR-2 and co-operative molecule MyD88, but not for TLR-4. This indicated that AbM stimulated synthesis of pro-inflammatory cytokines via interaction with TLR2. Potential immunomodulatory effects of AbM are depicted in the cartoon (Fig. 4).

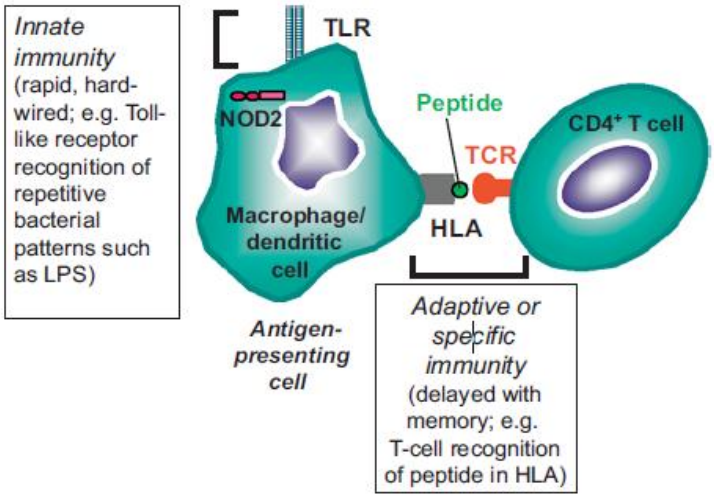
Fig. 4. An overview of AbM-mediated immunomodulatory effects, from ImmunoPharma.



The immune response

The immune response is defined by two main components innate and adaptive immunity (Fig. 5). The innate immunity represents the rapidly generated process that particularly is represented by macrophages (MΦ), dendritic cells (DC) and natural killer (NK) cells. However, minor subsets of B and T lymphocytes may also be considered as part of innate immunity. A unifying characteristic of these lymphocytes is that they express somatically rearranged receptors, like classical T and B cells, but they have limited diversity. Innate immune cells respond quickly to molecular patterns and present particular antigens. The major subtypes of cells that express pattern recognition receptors (PRR) are antigen presenting cells (APC), namely DC and MΦ. The innate immune system does not react against an individual`s own cells and molecules, partly because mammalian cells express regulatory molecules that prevent innate immune reaction. The role of the adaptive immunesystem is to recognize earlier exposure and act on its presence, mainly based on T-cells. In the adaptive immunesystem, lymphocytes are also capable of recognizing that self antibodies are produced and the cells producing self antibodies are killed or inactivated by lymphocytes. Therefore, the adaptive immune system can overreact and give rise to autoimmune disorders such as rheumatoid arthritis. Amongst inflammatory bowel diseases, Crohn's disease and ulcerative colitis have been defined as Th1- and Th2-type autoimmune diseases, respectively.

Fig. 5. Characteristics of innate and adaptive immunity, from Blumberg RS (9).

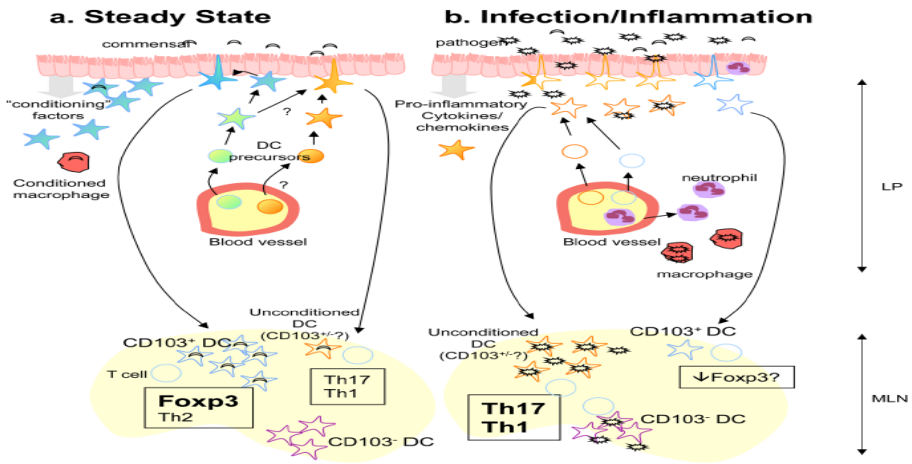


Innate immunity

The mucosal and cellular defense

The mucus layer outside the cellular layer entrap bacteria, and contains anti-bacterial peptides (e.g. α-defensins) produced by Paneth cells in the crypts (55). In addition, mucus also contains calgranulins, IgA and lysozyme that inactivate and kill bacteria. The next barrier in the gastrointestinal tract is the physical barrier of the cellular layer (32;91) that is composed of mainly epithelial cells, but also Paneth cells and mucus producing goblet cells. In addition, Microfold cells or M cells, are a specialized epithelial cell of the mucosa that has the potential to deliver antigen from gut lumen by transcellular vesicular uptake to lamina propria. Another way to sample antigen across the mucosa is via membrane protrusions of DC both between and through the epithelial cells (17). The antigens or microbes will then be further processed in the lamina propria containing lymphoid tissue (lymph nodes and Payer's patches) and different types of DC, lymphocytes, NK cells, MΦ and granulocytes. A major function of DC is their migration to mesenteric lymph nodes in order to present a wide range of antigens to T cells, leading to either a pro- or anti-inflammatory response. A situation of either steady state or inflammation of the intestinal lamina propria is shown in figure 6.

Fig. 6. Steady state and inflammatory response in the lamina propria of the gut wall, from Coombes et al (17).



Abbreviations: Lamina propria (LP), mesenteric lymph node (MLN)

In steady state with commensally bacteria in the gut lumen DC may be conditioned by epithelial-cell derived factors of anti-inflammatory nature (including IL-10, tissue growth factor ß (TGFß), retinoic acid, thymic stromal lymphopoietin (TSLP)). IL-10 and TGFß may reduce the responsiveness of DC to bacterial activation signals. Confronted with the same stimuli, TLSP can down-regulate IL-12/23p40 production and thereby Th1 responses.

The acid form of vitamin A, retinoic acid, has three important functions;

i) an enhancing effect on TGFß-mediated induction of the transcription factor called FoxP3, which turns peripheral T cells into T_{reg} cells
ii) synergy with IL-5 and IL-6 to mediate class-switching to IgA in both Peyer's patches and mesenteric lymph nodes
iii) contribution to the DC's ability to promote the expression of gut-homing receptors by lymphocytes.

This steady state and conditioned response is characterized by the presence of a certain degree of Th2 cells, downregulation of Th1- and Th17 cells/effects and upregulation of the FOXp3 T_{reg} cells. On the other hand, when the epithelial cells are exposed to pathogenic bacteria they secrete cytokines (such as IL-1, IL-6, IL-7, IL-11, and TNF), chemokine IL-8 and granulocyte-monocyte colony-stimulating factor (GM-CSF), which creates an inflammatory response that may become unbalanced and create a chronic state, as seen in IBD. This inflammatory response is characterized by an increase of Th1 and Th17 cells and their cytokines.

Receptors

Toll-like receptors

Toll-like receptor(s) (TLR), mannose receptor, scavenger receptor, retinoic-acid-inducible gene1 (RIG-1)-like receptors (RLR) and nucleotide-binding oligomerization domain (NOD) receptors are all cell membrane lined receptors. TLRs are the most important sensors of the innate immune system, recognizing microbes at the cell-surface. These receptors consist of an extracellular portion, leucine-rich repeats (LRR), which again consist of an N terminus and a C terminus, recognizing PAMP. The intracellular portion, Toll/IL-1 receptor (TIR) domains consist of Box 1-3, adapted to myeloid differentiation primary-response protein (MyD88). When MyD88 is recruited to the receptor complex it is joined by IL-1R-assosiated kinase (IRAK) and results in the activation of the NF-kB pathway and MAP-kinase pathway. NF-kB activation initiates transcription of several

genes influential on several innate immune effectors function like production of pro-inflammatory cytokines and mediating signaling pathways to the adaptive immune system, B and T cells. The LRR recognize different types of PAMP, TLR1 for lipopeptides, TLR2 for lipopeptides and peptidoglycans, TLR3 for dsRNA, TLR4 for LPS, TLR5 for flagellin, TLR6 for lipopeptides and zymosan, TLR7 and TLR8 for single-stranded ribonucleic acid (RNA), and TLR9 for CpG (cytosine-phosphate guanosine residues)-containing deoxyribonucleic acid (DNA) (61). Moreover, in response to fungal carbohydrate-PAMP, TLR2 (31) and TLR4 (64;93) have been implicated in the recognition of β-glucan and other polysaccharides (e.g. mannan and heteroglycan), respectively.

Inside the cell, in cytosol, microbial components derived from bacterial peptidoglycans are recognized by nucleotide-binding oligomerization domain (NOD) proteins, NOD1 and NOD2. NOD proteins are a part of the nucleotide-binding domain LRR-containing family (NLR), called inflammasomes. Inflammasomes are involved in the activation of pro-inflammatory cytokines, IL-1 and IL-8.

Dectin-1

Dectin-1 is the primary PRR for fungal glucans and was originally thought to be a dentritic cell (DC) specific receptor (**de**ndtritic-cell-associated **C**-type lec**tin-1**) (2). This receptor exists on many other cell types, including MΦ, monocytes, polymorphonuclear granulocytes (PMN) and a subset of T cells (13;101). Dectin-1 is found abundantly at the portals of pathogen entry (lung and intestine) (78), and its expression is influenced by various cytokines, steroids and microbial stimuli (109). The expression of dectin 1 is markedly increased by Th2 response cytokines, especially IL-4 and IL-13, whereas IL-10 and LPS down-regulate this expression (109). Dectin-1 specifically recognizes soluble and particulate β-(1-3)- and β-(1-6)-linked glucans (11;12) as well as zymosan, a stimulatory cell-wall extract of common yeast that is composed mainly of β-glucan, mannan, chitin, protein and lipids. An *in vivo* study in mice reported internalization of the receptor (75) on granulocytes and monocytes following a single injection of β-glucan which, accordingly, was internalized by circulating leucocytes and this effect prevailed for up to 7 days. When compared to β-glucans, mannan administration increased leukocyte dectin-1, thus demonstrating a differential effect on leukocyte dectin-1. However, MΦ internalization alone was not necessary to initiate an inflammatory response, but dectin-1 receptors rather than TLR2, were absolutely necessary for both efficient internalization of β-glucans and cytokine release.

Non-dectin-1 β-glucan receptors (other than TLR2/TLR6) and their protective roles

Immune cells, such as NK cells and non-immune cells like EC, alveolar epithelial cells and fibroblasts, do not express dectin-1. But they have an important role in anti-fungal immunity (26) and in mediating the protective effects of β-glucans against infectious and malignant diseases (41;79). These cells express other receptors, like CR3 (complement receptor 3, CD11b/18), lactosylceremide and scavenger receptors (ScR), which can recognize certain carbohydrates (Table 1). Binding of β-glucan to CR3 mediates, like for dectin-1, phagocytosis in mononuclear phagocytes.

The receptor binding and internalization of ligand-receptor complex prime the NF-$_K$B translocation signaling pathways, leading eventually to the production of pro-inflammatory cytokines and chemokines. These in turn activate and recruit other cells to the site of infection, resulting in the initiation of the adaptive arm of the immune response. In the innate immune system, there are mainly neutrophils, MΦ, DC and natural killer (NK) cells that are active in different stages in targeting different types of pathogens. Neutrophils constitute about 70% of blood leucocytes. These cells are short-lived (1-3 days), highly phagocytic and migrate across the blood vessel endothelium and into the tissue. They are activated by a variety of receptors like TLR receptors, lectin-, scavenger-, complement- and chemokine receptors. Phagocytosis is enhanced by antibody alone (via Fragment crystaline receptor (FcR)), complement alone (alternative pathway), antibody plus complement (classical pathway) or the lectin pathway (see; complement system).

Resting macrophages, encountering pathogens or other stimuli, will become activated and perform phagocytic killing of the pathogens and also produce inflammatory mediators to recruit other cells, e.g. IL-8 which is chemotactic factor for granulocytes. They also exhibit increased expression of MHC class II molecules necessary for presentation of antigen to T helper cells and thereby bridging innate and adaptive immunity.

Table 1. Pattern-recognition receptor PRR involved in fungal recognition, pathogen associated molecular pattern PAMP, modified from Brown GD 2006 (11)

PRR	Fungal PAMP
CD14	Glucuronoxylomannan
C3	Fungal surfaces
CR3	Mannose, β-glucan, N-acetylglucosamine, methylmannoside, Methylglucoside, complement-opsonized pathogens
DC-SIGN	Internal mannose, terminal di-mannose
Dectin-1	β-glucan
Lactosyleramide	β-glucan
Mannose receptor	Terminal mannose
MBL	Selected monosaccharides (such as mannose, fucose,glucose)
Pentraxin-3	Galactomannan, zymosan
SP-A	Selected monosaccharides (such as mannose, fucose,glucose)
SP-D	Selected monosaccharides (such as mannose, fucose,glucose)
TLR2	Phospholipomannan, zymosan, lipoproteins, lipopeptides, glycolipids
TLR4	Mannan, Glucuronoxylomannan
TLR9	CpGDNA
Mannose receptor	Mannan

Dendritic cells

DC are bone marrow-derived cells with a star-like morphology of lymphoid (including plasmacytoid) and non-lymphoid (monocytic) type (61). They are immature microbe-capturing sentinels and can shift to mature cells, activating T cells by T cell receptor (CD3) engagement via antigen presentation on self MHC (class I or class II) located on the APC with costimulation (CD28-CD80/CD86 interactions). DC are the major antigen presenting cells within the immune system and other important groups are monocytes/MΦ and B cells, which can present antigen captured on the B cell receptor (i.e. the $F(ab)_2$ part of the

immunoglobulin molecule). The DC induces then both a primary and a secondary immune response and serve as an essential link between the innate and adaptive immune system (16).

In blood and peripheral tissues immature DC are constantly sampling the antigen enviroment. These naïve DC in the environment, produces anti-inflammatory cytokine IL-10, which stimulates T-regulatory cells (T_{reg}) and inhibits Th1-, Th2- and Th17 cells and MΦ, in such a way that keeps the environment in a stable setting. With the right antigen stimuli, DC will change from naïve DC and become activated DC, and locally start secretion of a variety of pro-inflammatory mediators such as TNF-α, IL-6 (also Th2 cytokine), IL-8 and IL-12 (also Th1 cytokine). The expression of pro-inflammatory cytokines will attract and contribute to activation of eosinophils, macrophages, NK and other DC. In addition, they can kill pathogens by the production of ROS, nitric oxide and defensins (89). Mature DC will migrate to lymph nodes where they present antigens to T-cells. During maturation the DC gradually increase antigen presenting capacity at the expense of phagocytic and cytotoxic potential. The many-sided functions of the DC depends on their stage of maturation and stimulation by the microenvironment which emphasizes the cruical role of these cells in the regulation of both the innate and adaptive immune response. In conclusion, major functions of DC are i) promoting innate responses, ii) induction of peripheral immune tolerance, iii) antigen capturing, presentation and processing, iv) cytokine production and v) lymphocyte activation and differentiation.

Th0-, Th1-, Th2-, Th17- and Th_{reg} cells

There are mainly two types of T-cells involved in the inflammatory process of inflammatory bowel disease (IBD), namely Th1 and Th2 cells. However recently, Th17 cells have been described as a potential new participant in IBD (50). This new lineage gives some knowledge to aspects which are not fully explained by the Th1/Th2 paradigm (54).

The T helper cell, CD4+ Th0 cell, is a naïve cell that requires two signals in order to become activated for both cellular and humoral responses. By a combination of an APC presenting antigen in context of their MHC molecule together with accessory molecules (CD28) and a particular cytokine or combination of cytokines, the naïve T-cell will develop into specific T-cells that can help or activate naïve B-cells to become antibody-secreting plasma cells that act on the specific original antigen. For the maturation towards Th1 cells,

the naïve T-cell requires IL-12 in combination with IFNγ to activate specific signal transduction. Activation of transcription 1 and 4 (STAT1, STAT4) signal molecules in this specific signaling transcription pathway, again rapidly induces expression of T-cell specific transcription factor (T-bet). T-bet promotes Th1 lineage cytokines and suppress the development to Th2 cells.

T cell receptor (TCR) and IL-4R with its corresponding cytokine leads to the activation of STAT6, which induces the expression of the Th2 cell specific transcription factor (GATA-3) and enables the expression of the Th2 cytokines. GATA-3 promotes the Th2 lineage commitment (IL-4, IL-5, IL-13, IL-10, IL-17) and suppresses Th1 developing. It is like a "master switch" in the maturation of naïve T cells (54).

The Th17 cells do not express T-bet or GATA-3 and their differentiation is initiated by STAT3 through IL-6, TFGβ and IL-23 (56). Both retinoic acid-related orphan receptor (ROR)-γt and RORα are critical transcription factors necessary for the development of these cells. The activation of Th17 cells promote production of cytokines IL-17, IL-21, IL-22 and IL-26.

Regulatory T cells (T_{reg}) with a regulatory/suppressor function exist as various subpopulations within both CD4+ and CD8+ T cells. The most frequent naturally occurring T_{reg} is a subset of $CD4^+$ T cells and express CD25 (IL-2Rα) phenotype.

Natural killer cells

NK cells are classified as non-phagocytic large granular lymphocytes containing several azurophilic granules. They are capable of killing various target cells without the need of activation, unlike T cells that need to be activated and then need to differentiate into cells capable of killing. NK cells act together with DC and thereby regulate the adaptive immune response. NK cells act on DC via their production of IFNγ and TNFα, cytokines also important for DC maturation. Moreover, IFNγ is important for the activation of macrophages and is a coercer for the differentiation of T helper cells.

Reactive oxygen species (ROS)

In the process of phagocytosis O_2 is exitated and partially reduced intracellularly by the action of NADPH oxidase in phago-lysosomes, which gives rise to ROS like superoxide anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), hydrogen peroxide (H_2O_2), and peroxynitirite ($ONOO^-$) (100). ROS are toxic towards microorganisms, but create only a side effect

towards the host itself through peroxidation of lipids, proteins, nucleic acids and nitrosylation of proteins. ROS is generated mainly as a by-product of aerobic metabolism and PMN produce ROS more extensively than mononuclear phagocytes (20). The respiratory burst pathway which increases the production of ROS, is only induced by specific receptors like dectin-1 and the Fc receptors for IgG. Zymosan, which like AbM contain ß-glucans, induces ROS production in MΦ from the bone marrow of rats (34).

Adhesion molecules

Adhesion molecules (receptors) mediate the adhesive interactions that determine the homing of mononuclear cells to different lymphoid organs, and facilitates PMN and monocytes localization to inflammatory sites (21;42). Leukocyte migration occurs usually through paracellular endothelial cell-cell interactions, but can also occur transcellularly. In this context the functions of selectins and integrins will be briefly mention, which are the major groups of adhesion molecules.

Selectins

Selectins have three family members; E-selectin (CD62E or endothelial cell adhesion molecule (ELAM)-1), P-selectin (CD62P or granule membrane protein (GMP)-140), and L-selectin (CD62L or leukocyte adhesion molecule (LAM)-1). Selectin-mediated leukocyte adhesion is an early event responsible for the leukocyte "rolling" phenomenon in diapedesis (37). L-selectin is expressed on most peripheral blood lymphocytes, monocytes, and granulocytes. Furthermore, they are expressed in spleen, bone marrow lymphocytes, myeloid cells, and T cells (68). They also regulate lymphocyte binding to endothelium in lymph node venules and thereby regulate their trafficking through the lymphoid tissue.

Integrin receptor family

Integrins plays a critical role in the regulation of cell migration, by example in, recruitment of leukocytes into inflamed tissues. They are large membrane proteins, consisting of an α and a β subunit. At least 19 different integrins have been identified (86). They are further subclassified on the basis of structurally distinct β chains. The members of the β_2 subfamily (also known as leukocyte integrins or leukointegrins), are composed of three distinct molecules, designated as leukocyte function antigen-1 (LFA-1), Mac-1 and gp150,95. The members of the β_2 subfamily β_2 leukocyte integrins, have been classified according to their

α and β subunits as CD11/CD18 molecules by the World Health Organization (WHO). They are composed of identical β subunits (CD18) and different α subunits (CD11a for LFA-1, CD11b for Mac-1, and CD11c for gp150,95). Molecular expression of β_2 integrins on phagocytes is up-regulated when the cells are stimulated by chemotaxins (C5a, LTB4) and cytokines (TNFα). Thus, the expression of integrins on phagocyte surfaces help in locating to circulating cells to the sites of tissues injury and in the host defense against the invaders (67).

Complement system

The chemical defense mechanism of the immunesystem includes the complement system and cytokines. The complement system can be activated by three different pathways. The classical pathway is activated by certain antibodies (IgM, IgG) bound to antigens, whilst the alternative pathway and the lectin pathway are activated by the innate immune system. The alternative pathway is activated by binding to components on the microbial polysaccharides or endotoxin from the cell membrane of Gram-negative bacteria, yeast or protozoa. In Gram-positive bacteria the alternative pathway is activated by teichoic acid from the cell-wall. The lectin pathway is activated by the binding of plasma mannose-binding lectin (MBL) to mannose residues on proteins in microbes, but not on mammalian molecules.

The function of the complement system is:

i) triggering and amplification of inflammatory reactions
ii) attraction of phagocytes by chemotaxis
iii) clearance of immune complexes
iv) cellular activation
v) direct killing by lysis of invading microorganisms
vi) important role in development of antibody responses

All three pathways ends in activation of C3 and compromise a proteolytic cascade creating new complement complexes which cleave other complement proteins. The activation cascade ends with three major complexes:

i) C3a and C5a, small chemotactic and and anaphylactic fragments
ii) C3b and C4b, large opsonic fragments
iii) C5b-9_n, membranolytic membrane attack complex

Cytokines

Cytokines are small and low molecular weight proteins (8-75 kilo Dalton (kD)) synthesized by leukocytes and mediate signaling between these cells. They are both regulatory and effector molecules and are often classified into interleukines, interferons, chemokines and leukocyte growth factors. The function of the cytokines examined in this study will be presented (61).

Interleukin 1ß

Pro-inflammatory IL-1ß is produced following infection or injury by mainly mononuclear phagocytes and DC, often in synergy with TNFα (73). Immunologically activated T-cells, immune complexes, complement 5a (C5a) and interferon γ (IFNγ) can stimulate IL-1ß production. LPS from gram-negative bacteria is the major stimulant for production of IL-1ß. From the cell walls of Gram positive bacteria, exotoxins and from the cell walls of the yeast, zymosan, exotoxins and zymozan can influence the production of IL-1ß.This inflammatory cytokine can up-regulate host defenses and function as an immunoadjuvant. Crucial effects of IL-1ß are CD4+ T-cell proliferation by inducing IL-2 release, promoting B-cell growth and differentiation, inducing IL-6 synthesis and enhancing leukocyte-endothelial adhesion. IL-1ß activates T cells and in association with IL-4, B cells are activated partly by induction of IL-6, which is a B cell differentiation factor. Along with TNFα, IL-1ß -mediated induction of IL-6, induces hematopoietic growth factor production by fibroblasts, EC, and bone marrow stromal cells. The cytoplasmic structural domains of TLR are nearly identical to those of IL-1ß receptors. Furthermore, the IL-1RI and cytosolic Toll protein have similar gene organization and aminoacid homology, and trigger similar signaling cascades. In humans, IL-1ß produces fever, headache, myalgia and athralgia. IL-1ß increases the expression of adhesion molecules of EC which cause increased adherence of neutrophils, monocytes, and lymphocytes. IL-1ß induces several transcription factors, especially NF-$_K$B and also is a hepatocyte stimulating factor as shown by increased production of acute phase proteins (47).

Tumor necrosis factor α

TNFα and IL-1ß synergistically act in the inflammatory process and exhibit mainly overlapping effects. Infections, trauma, ischemia, immune-activated T cells, toxins, IL-1ß and TNFα initiate the cascade of inflammatory mediators by targeting EC, and inducing the

expression of cell membrane adhesion molecules such as intrinsic cell inter-cellular adhesion molecule (ICAM)-1. TNFα exerts *in vivo* toxic effects on cells demonstrated by the damage to tumors and blood vessels, but also induces release of chemokines and activates phagocytosis. Apoptosis is induced by TNFα through binding to its corresponding receptor which activates intracellular signals leading to programmed cell death. It is secreted mainly by mononuclear phagocytes, but also by T- and B cells and NK cells. One of its main functions together with the related cytokines is to activate the transcription of NF-κB, which has been described to be the master-switch of the immune system. Anti-TNFα is used in treatment of IBD, foremost in CD. Previously TNFα was known as cachectin and has recently been found to play a major role in cachexia together with IL-6 in driving the inflammation that is believed to be the background for this syndrome.

Interleukin 6

IL-6 is a pleiotropic type-2 pro-inflammatory cytokine which along with IL-1ß, mediates multifunctional host responses and regulates development of multiple cell types. Its role as a regulator, directing a shift from innate to adaptive immunity, is achieved through differential control of leukocyte recruitment, activation and apoptosis. It is crucial for the synthesis of acute phase proteins in the liver, mucosal production of IgA and the fever response during inflammation. IL-6 also stimulates the pathogen clearance function of neutrophils. In adaptive immunity IL-6 appears to have a major influence on end stages of B cell differentiation. However, in chronic inflammation like IBD and rheumatoid arthritis, IL-6 exhibits harmful events and inhibition of IL-6 signaling improves symptoms in arthritis patients (59). IL-6 is produced by activated monocytes, MΦ, EC, activated T-cells and liver cells in response to IL-1ß and TNFα. This activation occurs by IL-1ß and TNFα binding to a membrane bound receptor complex (glycoprotein, gp130 and IL-6R) and via naturally occurring soluble IL-6R (48).

Chemokines (IL-8, MIP-1ß, MCP-1)

Chemokines are produced as a result of acute inflammation and are known as chemotactic and activating factors of leukocytes. The primary immune function of chemokines is to mediate selective trafficking of leukocyte subsets between blood and various tissues and the recirculation of lymphocytes between the tissues and lymphatics for immune surveillance.

The cystein-x-cystein (CXC) chemokine, IL-8 acts predominantly on neutrophils and has lesser impact on monocytes and lymphocytes. Particularly good sources for IL-8 production are monocytes, EC, but also fibroblasts. Effects on *in vitro* stimulation of neutrophils include induction of shape change, respiratory burst with generation of superoxide and H_2O_2, release of lysosomal enzymes, generation of bioactive lipids and up-regulation of adhesion molecules. Transendothelial migration of neutrophils is further enhanced by down-regulation of cell-bound L-selectin by shedding (94). IL-8 also stimulates the activation and mobility of T-cells, eosinophils, basophiles and monocytes. In unstimulated EC, IL-8 increases the adhesion of neutrophils, whereas preactivated EC with IL-1, TNFα, and LPS inhibits this phenomenon (85). In B-cells, IL-8 inhibits IL-4 induced IgE production. In humans, two distinct receptors (IL-8A and B) for its analogue IL-8 have been identified and are coupled to guanosine-tri-phosphate (GTP)-binding proteins (15).

The cyctein-cystein (CC) chemokines include macrophage inflammatory protein-1ß (MIP-1ß) and monocyte chemotactic protein-1 (MCP-1), attract mostly monocytes and T cells with lesser effects on basophiles and eosinophils. In general, neutrophils are unresponsive to the group of CC chemokines. MIP-1ß also selectively attracts CD4+ cells, but not CD8+ cells. MIP-1ß acts on Th1 cells and MΦ by binding to the chemokine receptor CCR5. MCP-1 acts on the chemokine receptor CCR2 and chemoattracts and activates mainly monocytes, but also Th2 cells acting on T cells, NK cells, basophiles and immature DC. The human CC chemokines are made primarily by activated T cells and DC.

Interleukin 2

The vast majority of IL-2 is produced by Th1 cells that have been activated by a foreign antigen and stimulated with IL-1ß. Its main function is to enhance proliferation of activated Th1 cells ($CD4^+/CD8^+$) as an autocrine feedback mechanism, but also function as a chemoattractant for T-cells. Moreover, IL-2 enhances monocyte responses and stimulates B-cell proliferation and antibody production as well as proliferation of NK-cells. Synergism of IL-2 and IL-12 induces production of interferon γ (IFNγ) and TNFα in NK-cells, which then develop into lymphokine-activated killer (LAK) cells. Unlike other cytokines, IL-2 is crucial for induction of self tolerance as evidenced by development of hemolytic anemia and chronic IBD in animals lacking IL-2 and IL-2 receptor (61). In humans two low affinity receptors for IL-2 exist, IL-2Rß and IL-2Rγ, where binding of IL-2 yields low levels of signaling and further differentiation.

Interleukin 17

Interleukin-17 is mainly produced by the "new member" of T-helper cells, Th17 cells, but also by NK cells. The Th17 cells also produce IL-21, IL-22 and IL-26 (in humans) which function in synergy with IL-17. IL-17 acts on stromal cells such as keratinocytes, fibroblasts, epithelial cells and EC and stimulates them to secrete IL-6, IL-8 and G-CSF. Injection of IL-17 into mice induces IL-6-dependent increase in blood neutrophils and such mice are also resistant to virulent bacterial infections (61). High levels of IL-17 have been found in synovial tissue of patients with rheumatoid arthritis. Studies suggest that IL-17, especially at mucosal sites, plays a role in protection against both extracellular and intracellular bacterial and fungal infections (e.g. *Aspergillus* and *Candida*) (18). This protection is partly mediated through the generation of G-CSF and some CXC chemokines (54) which recruit neutrophils to the inflammatory site. IL-17 together with IL-22 can also augment the secretion of anti-microbial peptides. Examples of extracellular and intracellular infections studied are caused by S. *aureus, K. pneumonia* and M. *pneumonia, S. typhimurium,* respectively. Inadequate levels of Th17 cytokines, IL-17 included on the other hand, can result into excessive inflammatory responses with tissue destruction found in autoimmune diseased like rheumatoid arthritis and IBD (74). This "new" Th17 cell lineage offers many explanations to a porly understood area in host immunity not fully explained previously by the Th1/Th2 paradigm. IL-17 is evolutionary conserved and the gene exists in molluscs, which existed in ancient times before the development of the adaptive T-cell immunity, linking innate and adaptive immunity (54).

Interferon γ

Regardless of the types of interferon, their two major functions are antiviral activity and anti-proliferative activity. INFγ is mainly produced by activated Th1-cells and to a lesser extent by cytotoxic T-lymphocytes and NK cells. It activates MΦ to synthesize inflammatory cytokines such as TNFα, IL-1ß, IL-12 and is also responsible for intracellular generation of anti-microbial nitric oxide and ROS. Other activities of IFNγ are induction of expression of Fc receptors suitable for complement-mediated destruction and antibody-dependent cell-mediated cytotoxicity of tumor cells and microbes, the latter performed by NK cells. In the adaptive immunity INFγ up-regulates both major histocompatibility complex (MHC) class I and II expression on a wide variety of cell types. Because of its effect on synthesis of both IL-12 and IL-18 in MΦ and reduction of IL-4 synthesis in naïve

Th0 cells, Th1differentiation relative to Th2 differentiation is favored. The consequence is an immune response best-suited for fighting intracellular pathogens (e.g. virus). The IFN-γR is expressed on all cell types except erythrocytes.

Interleukin 12

IL-12 is a large disulfide-linked heterodimer composed of p35 and p40 subunits, which is unusual for cytokines. It is primarily synthesized by MΦ, but to some extent also by neutrophils, DC, monocytes and B cells. IL-12 plays a key role in the immune response by linking to MΦ and DC and thereby activate them for microbial ingestion. The innate response is activated through an NK cell activation which further differentiates naïve Th0 cells into Th1 cells. IL-12 is the major cause of IFNγ production by NK cells and Th1 cells, which is crucial for host defense against intracellular pathogens, including replicating mycobacteria. The Th1-cell response promoted by IL-12, gives rise to stimulation of antibody enhancement of isotypes IgG2a, IgG2b and IgG3, but not Th2 associated IgG1 and IgE. IL-12 may play a role in CD, which in part is considered a Th1-cell disorder. The high affinity IL-12Reseptor is expressed primarily on activated T- and NK-cells, but also by DC and B cells.

Interleukin 4

IL-4 is a powerful pleiotropic Th2 cytokine, which is mainly synthesized by $CD4^+$ T-cells, but also by basophiles, mast cells and influences all cell types through IL-4R. Key activities of IL-4 include differentiation of Th0- into Th2 cells, stimulation of B cells to synthesize IgE and IgG1, but not IgG2a, IgG2b and IgG3. IL-4 stimulates IL-12 synthesis in MΦ and DC, which also functions as a negative feedback mechanism for the Th2 cell response (increased production of IL-4, IL-5, IL-10 and IL-13). Moreover, increased IL-12 synthesis of MΦ and DC inhibits synthesis of TNFα and IL-1ß as well as adhesion molecules. Mast cells are stimulated to proliferation and degranulation which is important in allergic responses. IL-4 is also important for defense against helminth worms because the generated IgE binds to eosinophils via their fragment crystalline (Fc)ε receptors to carry out efficient antibody-dependent cellular cytotoxicity (ADCC). IL-4 effects are generally antagonistic to those of IFN-γ.

Interleukin 13

This cytokine is produced by activated T cells, in particular Th2 cells, but also basophils and mast cells. Except from differentiation from Th0 into Th2 cells, IL-13 exhibits similar activities to IL-4, but the response is smaller in magnitude. IL-13 inhibits synthesis of pro-inflammatory cytokines (IL-1ß, IL-6, TNFα and IL-8) in MΦ, but phagocytosis is not blocked, whereas antibody-dependent cellular cytotoxicity (ADCC) is reduced. IL-13 stimulates proliferation of B cells and their isotype switching to increased production of IgE. As for IL-4, IL-13 signaling at least in mice, is required for defense against parasites (nematodes).

Interleukin 10

IL-10 has either immunosuppressive or immunostimulatory effects on various cell types. The major producers of IL-10 are activated monocytes, MΦ and Th2 cells, but also DC, B cells, eosinophils, mast cells, keratinocytes, hepatocytes and other cell types as well. The main function of IL-10 is to tune down inflammatory responses by targeting mainly monocytes, MΦ, neutrophils, eosinophils and mast cells. The immunosuppressive role is by enlarge effectuated by inhibiting pro-inflammatory cytokines through inhibiting NF-κB-activated transcription of genes encoding particularly for TNFα, IL-1β, IL-6, IL-8 and IL-12. Therefore, IL-10 plays an important role for the down regulation of the massive cytokine release occurring during septic shock. The respiratory burst and ROS dependent killing of microbes by MΦ is also inhibited by IL-10. As for the immunostimulatory effect, IL-10 promotes a Th2 cell response by inhibiting secretion of IFNγ and IL-2 by Th1 cells. The two receptors for IL-10, IL-10R1 and IL-10R2 are expressed mainly on hematopoietic cells.

Interleukin 5

IL-5 is a homodimeric cytokine of particular importance for eosinophil differentiation, activation and chemotaxis. Moreover, IL-5 stimulates histamine release from mast cells and IgA synthesis in B cells as well as proliferation and differentiation of cytotoxic T lymphocytes. These effects can lead to eosinophilia and induction of asthma, but also increased cell-mediated immunity in the battle against parasitic infection. IL-5 is produced primarily by Th2 cells, and to a lesser extent by activated mast cells, eosinophils, NK cells and B cells.

Interleukin 7

IL-7 promotes lymphopoiesis and development of B-cells and T cells and is also important for generation of memory T cells. This cytokine is produced mainly by stromal cells in the bone marrow and thymus, but also by B cells, monocytes, MΦ, keratinocytes and intestinal intraepithelial lymphocytes.

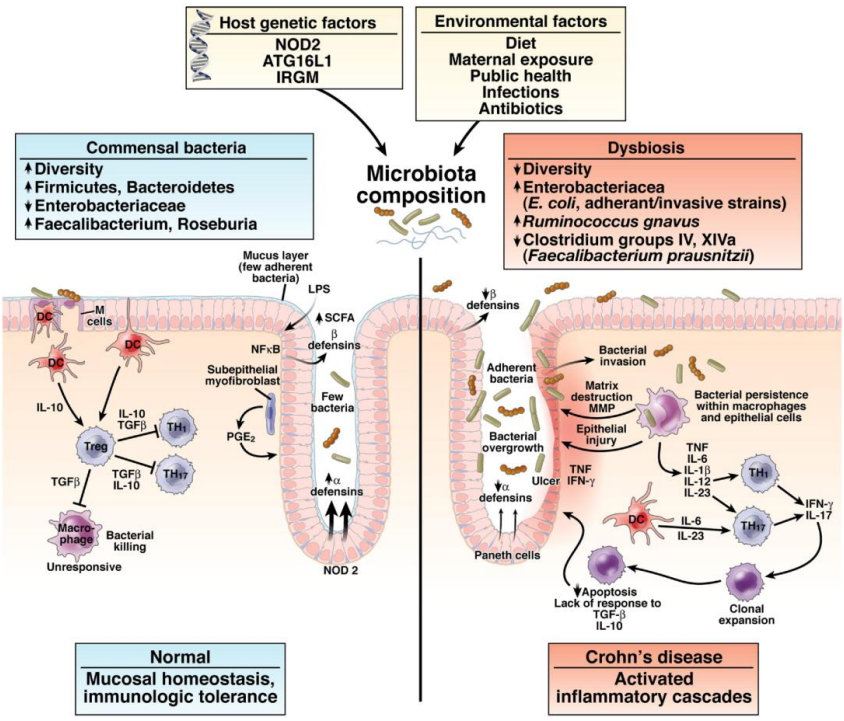
Colony stimulating factors

These factors are divided into granulocyte colony-stimulating factors (G-CSF) and granulocyte-macrophage colony-stimulating factors (GM-CSF). Both are mainly produced by stromal cells, mononuclear phagocytes, EC, activated T cells and fibroblasts. G-CSF stimulates development and recruitment of leukocytes from bone marrow to blood. It is used for recruitment of CD34+ hematopoietic stem cells from bone marrow to blood when harvesting such cells for later use in stem cell transplantation for bone marrow rescue after high-dose chemotherapy in cancer treatment. GM-CSF stimulates growth and differentiation of colonies from pluripotent hematopoietic stem cells, into neutrophils, eosinophils, basophils monocytes, megakaryocytes and erythrocytes.

Inflammatory bowel disease

In the past, inflammatory bowel disease (IBD) was almost impossible to distinguish from infectious disease of the gastrointestinal tract. The designations ulcerative colitis (UC) and Crohn's disease (CD) were presented in 1888 and 1932, respectively. Since the 1960s the term IBD has been used mainly for UC and CD that affects both sexes and mostly in the age group from 15-35 years. UC affects the mucosa whilst CD affects all layers of the intestinal wall of the large and small bowel. In UC there may be a continuous inflammation in the colon and microscopically the entire colon is often affected. In CD, healthy and diseased patches of bowel are often interspersed. In Nordic countries the incidence of UC is about 10-15 per 100.000 and 5-8 per 100.000 for CD. The diseases are characterized by chronic or relapsing inflammation that may cause anorexia, weight loss, diarrhea, pain and fever. In CD, malabsorption and subileus occurs from small bowel stenosis. The transmural inflammation can cause fistulization to other epithelial lined organs. Extraintestinal autoimmune manifestations like iridocyclitis, spondylitis and painful and inflamed joints also occur with a preponderance in CD. The development of IBD is thought to originate from a combination of genetic and environmental factors (89) (Fig. 7).

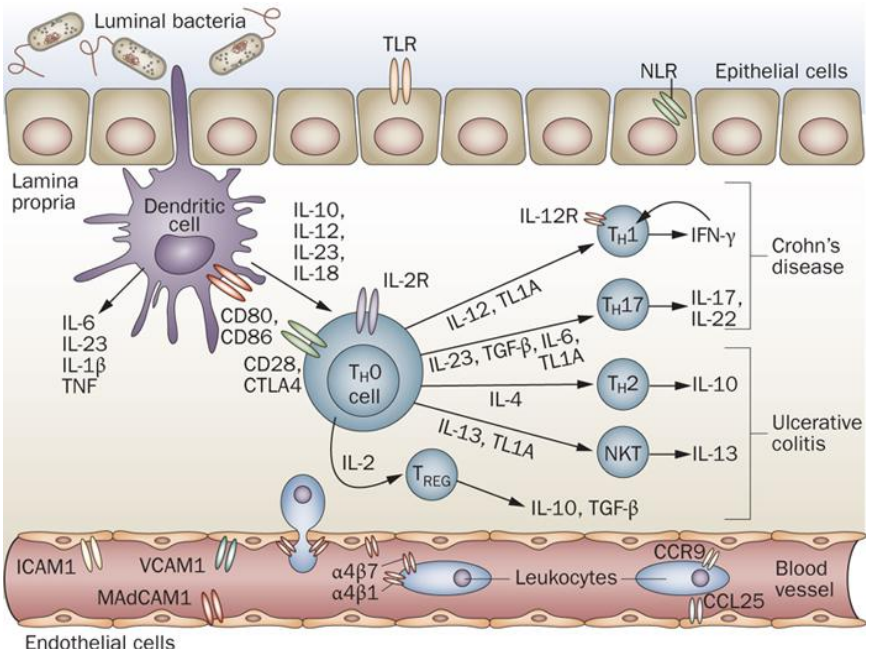
Fig. 7. Genetic and environmental factors influencing the homeostasis of the bowel mucosa (from Sartor RB (89)).



Examples of genetic aberrations that reduce host defense to bacteria are i) mutation of transcription factor x-box binding protein 1 (XBP-1) necessary for secretory function of intestinal epithelial cells and Paneth cells (e.g. anti-microbial peptides) and ii) mutation of nucleotide-binding oligomerization domain (NOD)2 resulting in reduced bacterial clearance by phagocytes. In IBD, environmental factors contribute to dysbiosis or abnormal bacterial composition of the gut, which predisposes for disease development. The diversity of bacteria has been found to be decreased in IBD. However, simultaneously there is an increase of mucosa-associated pathogenic bacteria like enterobacteria, including adherent and invasive E. coli, but also decreased clostridial bacteria, like protective Faecalibacterium prausnitzii. Reasons for disease contributing dysbiosis can be i) lack of transferral of maternal bacteria like *Lactobacilli* and *Bifidobacteria* conveyed by vaginal delivery and breast feeding, ii) aberrant composition of diet and iii) repeatedly use of antibiotics (combinations of ciprofloxacine, metronidazole and amoxicillin).

One hypothesis is that the disease process in the mucosa and lamina propria characteristically for UC is Th2 cell driven, and for CD is Th1 cell and Th17 cell driven (Fig. 8). Accordingly, modulation of these cytokines and following cellular responses towards conditioning and a steady state situation (Fig. 6 and 8) may be a therapeutic target (63).

Fig. 8. Potential cytokine profiles in UC and CD (from Melmed GY, *et al.* (63)).



Abbreviations: ICAM1/VCAM1/MadCAM1 (adhesion molecules), NLR (node-like receptor(s)), TL1A (member of family of TNF (tumor necrosis factor)), CTLA4 (cytotoxic lymphocyte-associated-protein 4).

Calprotectin, an abundant cytosolic protein in neutrophils (97), can when released to feces (83;106) be used as a sensitive and particularly suited surrogate marker for disease activity in IBD.

For patients with UC and CD an unselective increase in the colon mucosa of chemokines (MIP-1ß, MCP-1 and IL-8) (3) and cytokines IL-1ß (60), IL-6 and TNFα (110) have been demonstrated. In serum, however fewer cytokines are studied, but increased levels of IL-6 (44) and TNFα (62;66) were detected in both UC and CD, whilst increased MIP-1ß was found in a former disease of UC (90). Accordingly, more studies on levels of cytokines in

these patients are warranted. Granted that cytokine levels in blood mirror those of the gut wall, we chose to study cytokine levels in blood instead of in gut mucosa for three reasons. Firstly, blood cytokines are much easier accessible than gut located cytokines. Secondly, the representativity of a blood sample presumably is better than hitting the hot spots of disease within the mucosa of the long gut. Thirdly, endoscopy with biopsies may imply a bothersome and risky procedure (e.g. bleeding and perforation) for the patients.

Methodological considerations

Ethics

Written informed consent was obtained from all participants of the study, which was approved by the local ethical committee.

Multiplex cytokine assay

We used the multiplex bead-based sandwich immunoassay technology (Luminex, Austin, TX, USA) and a human cytokine 17-plex kit (Bio-Rad laboratories, Hercules, TX, USA), strictly following the manufacturers' instructions, to measure the concentrations in individual heparinized plasma samples of the following cytokines, chemokines and growth factors (lower detection limits (in pg/ml) in parentheses); IL-1β (2.0), IL-2 (1.2), IL-4 (0.3), IL-5 (2.3), IL-6 (2.1), IL-7 (3.0), IL-8 (1.6), IL-10 (1.8), IL-12 (3.0), IL-13 (0.9), IL-17 (2.5), G-CSF (1.9), GM-CSF (0.8), IFNγ (2.0), MCP-1 (1.7), MIP-1β (2.0) and TNF-α (5.4). Briefly an explanation of the technique, 25 µl of polystyrene micro beads (diameter 5.6 µm) coated with specific antibodies are added to each well. Then 100 µl of the sample is added and incubated for 3 h at room temperature on a horizontal orbital microplate shaker, prior to washing of the beads three times. Then 50 µl of biotinylated human antibody is added to the wells and incubated likewise for 1 h at room temperature. The biotin labeled human antibody binds to the analyte bound to the bead bound capture-antibodies. After washing of the beads to remove free antibody, 50 µl of streptavidin-PE fluorescent dye is added to the well and incubated likewise for 30 min at room temperature followed by washing of the beads. Streptavidin-PE binds to the biotinylated human antibody that again binds the corresponding antigen which is further bound via another epitope by the captured antibody that is linked to the bead. Finally, 100 µl of wash buffer is added to the wells during shaking and fluorescence signals, as measure of cytokine concentrations, are measured during 90 min using the Luminex analyzer®. The advantages of this method is mostly the

possibility to analyse precisely multiple cytokines in a small sample volume (100 µl), but also from its high sensitivity for detection of cytokines at levels of a few pg/ml.

Flow Cytometry

Flow cytometry is a rapid measurement technique used in laboratories worldwide, employing a fluid stream to carry cells through a detection counter unit. It is used to measure the relative fluorescence, size and granularity of individual cell particles in a moving suspension. Flow cytometry is also referred to as fluorescence-activated cell scanning or sorting, which can be used to harvest special cell types. We have applied this technique to measure the expression of adhesion molecules, using fluorecin isothiocyanate conjugated (FITC)- and phycoerythrin (PE)-conjugated monoclonal antibodies against CD11b, CD11c, and CD62L, and ROS in human leukocytes (8). The intracellular free radical species produced by the respiratory bursts were detected by two different probes, namely; the dihydroethidium (DHE) probe (mainly reflects superoxide anions) and the dihydrorhodamine 123 (DHR) probe (mainly reflects peroxynitrite ions). The production of ROS in leukocytes could be evaluated due to the transformation of the DHE/DHR probes from non-fluorescent to fluorescent compound by the oxidative burst intermediates within the cells. The results were always calculated as mean fluorescence intensity (MFI) of 10 000 cells. This method was also used when monocyte derived DC, after detaching from the cell wells, were labelled with FITC- or PE-labeled mouse monoclonal antibodies to markers CD1a, CD14, CD45/14, CD40, CD80, CD86 and isotype controls (IgG1,κ and IgG2b,κ), prior to analysis of fluorescence intensity of the DC by flow cytometry.

Microarray analysis

Studies on gene expression by microarray analysis (www.affymetrix.com) were performed in IBD patients and in three healthy volunteers. Blood was harvested in paired box (PAX) gene tubes specifically designed to preserve RNA from blood for microarray experiments prior to (day 0) and after (day 12) of consumption low dose (60 ml/day) of AndoSan^{TM} in IBD patients and prior (day 0) and after (day 2) of consumption high dose (360 ml/day) of AndoSan^{TM}. The PAX gene tubes were frozen and kept at -20 ºC until extraction with the PAXgene Blood RNA Kit (Qiagen) according to the manufacturer's recommendation. 2.5 µg of total RNAs were subjected to One Cycle cDNA Synthesis Kit following the manufacturer´s (Affymetrix) recommended protocol for gene expression analysis. Biotinylated and fragmented cRNA (15 µg) was hybridized to the Affymetrix HG U133

Plus 2.0 Array, representing 47000 transcripts for 38500 well-characterized human genes. The signal intensities were detected by Hewlett Packard Gene Array Scanner 3000 7G (Hewlett Packard, Palo Alto, CA). The 6 scanned images were processed using Affymetrix genechip operating software (GCOS) 1.4. The CEL files were imported into Array Assist software (v5.2.0; Iobion Informatics LLC, LaJolla, CA) and normalized using the Probe Logarithmic Intensity Error (PLIER) algorithm in Array Assist to calculate relative signal values for each probe set. In order to filtrate for low signal values, the MAS5 algorithm in Array Assist was used to create a dataset of Absolute Calls, showing the number of present and absent calls for each probe set. The filtration was performed by eliminating probe sets containing ≥4 absent calls across the data set, resulting in a reduction of probe sets from 47,000 to 28,188. For expression comparisons of different groups, profiles were compared using paired t-test. The results are expressed as fold changes (FC), e.g. ratio of the mean signals between consumption of high dose AndoSan™ for 2 days and immediately prior to AndoSan™ consumption. Gene lists were generated with the criteria of $p<0.05$ and FC of ≥|2|.

Experimental design

Ex vivo experiments**.** Heparinized blood was collected from healthy volunteers who denied smoking and use of any medication. This blood was immediately incubated with 0.1%, 0,5%, 2,0%, 5,0%, 10,0% or 15,0% of sterile AndoSan™ for 6 h at 37 °C. Plasma was then harvested and frozen until analyzed for cytokines, chemokines and growth factors (paper I).

In vivo experiments. Ten healthy individuals volunteered to participate in the study of oral intake of low dose (20 ml thrice daily) or high dose AndoSan™ (120 ml thrice daily) for 12 and 2 days, respectively. The dose of a total of 60 ml of the mushroom extract per day was chosen as this was the dose recommended by the manufacturer. Participants were asked to avoid mushroom-containing foods for 3 days prior to and during the experimental period. In the low dose experiment, the harvested blood from the participants was also incubated with LPS (1 mg/l) *ex vivo*, in order to boost the release of leukocyte derived cytokines (papers I and IV). Twelve patients with UC and patients with CD, respectively, ingested likewise the low dose AndoSan™ for 12 days, and were analysed for the same parameters in blood. In additon, the IBD patients were also analysed for amount of the inflammatory marker calprotectin in blood and feces (paper IV). Ten healthy individuals were tested for expression of ROS and adhesion molecules (CD11b, CD11c, CD62L) prior

to day 0 and during days 1, 2, 5, 8, 12 consumption of low dose (20 ml thrice daily) AndoSan™. Gene expression by microarray analysis in 15 healthy individuals were examined prior to (day 0) and after two days (day 2) intake of high dose (120 ml thrice daily) AndoSan™ (paper III).

In vitro experiments. Human monocytes were isolated from buffy coats from blood using a modified version of Sallusto's procedure (88), cultured in vitro and differentiated into DC by incubation with IL-4 and GM-CSF for 6 days, with replenishment of the culture medium at day 3. The monocytes were at day 6 differentiated into monocyte-derived DC as determined by flow cytometry assay of DC-specific surface markers (HLA DR, CD1a, CD40, CD45, CD80, and CD86, but not CD14) (77). During further experiments with the cells, IL-4 and GM-CSF was kept in the medium to prevent the cells from reverting to monocytes. The synthesis and release of cytokines were studied upon stimulation with different consentrations of AbM (paper II).

End points

The levels of the various parameters in the human studies (papers I and IV) before and after intervention with AbM were compared and tested for statistical differences. Likewise, the levels of cytokines studied were tested relative to before and after AbM stimulation.

Statistical analysis

Data are presented as mean and standard error of mean (SEM) or median (range) values comparing cytokines, calprotectin and different blood samples as a consequence of AbM stimulation. Non-parametric Wilcoxon's paired sample test was used to compare parameters from two different time-points. Analysis of variance (ANOVA) for paired data with Dunn's multiple comparisons was used for multiple comparisons of continuous variables. Pearson's product correlation was used for dose-responses. Instat for Windows™ statistics software package (Graphpad Software, San Diego, CA, USA) was used. P values below 0.05 were considered statistically significant.

Aims of the study

AbM exhibits pluripotent potential by stimulating cells of the innate immune system. Anti-tumor, pro-inflammatory, anti-allergic and even anti-inflammatory effects in rodents have been reported. However, whether AbM in humans may have an anti-

inflammatory effect has not been studied. Such an effect would particularly be of interest to further investigate in patients with IBD.

The first aim was to explore AbM`s effect in healthy individuals and in patients with IBD.

The second aim was to investigate the effect of AbM *in vitro* on the antigen presenting DC that bridge innate and adaptive immunity.

The third aim was to study the effect of AbM *in vivo* on expression of adhesion molecules and ROS in healthy individuals on and in patients with IBD.

General summary

Paper I:

In this paper we wanted to study the effect of cytokine release in healthy volunteers ingesting the mushroom extract *Agaricus blazei* Murill (AbM) for several days. AbM has been shown to stimulate mononuclear phagocytes *in vitro* to produce pro-inflammatory cytokines, and to protect against lethal peritonitis in mice. After stimulation of whole blood *ex vivo* with 0.5–5.0% of a mushroom extract, AndoSan™, there was a dose-dependent increase in all the 17 cytokines studied (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CS , IFNγ, MCP-1, MIP-1β and TNF-α), ranging from two to 399-fold (TNFα). In the eight volunteers who completed the daily intake (60 ml) of AbM extract for 12 days there was a significant reduction *in vivo* in levels of IL-1β (97%), TNF-α (84%), IL-17 (50%) and IL-2 (46%). In fifteen volunteers who completed an intake of 120 ml thrice daily for two days, there was a trend towards reduction of MIP-1β, MCP-1,IL-6 and IFNγ. We concluded that the discrepant results on cytokine release *ex vivo* and *in vivo* may partly be explained by the antioxidant activity of AbM *in vivo* and limited absorption of its large, complex and bioactive β-glucans across the intestinal mucosa to the reticuloendothelial system and blood.

Paper II:

Several studies have demonstrated stimulatory effect of *Agaricus blazei* Murill (AbM) on different immune cells. Dendritic cells are major directors of immune function and an important antigen presenting cell in the intestine. We wanted to examine the effect of AbM stimulation on cytokine release from monocyte-derived dendritic cells (MDDC). The cells were incubated with up to 10% of the AbM-based extract, AndoSan™, LPS (0.5 μg/ml) or phosohate-buffered solution (PBS) control. We found that the AbM extract promoted dose-dependent increased levels of IL-8, G-CSF, TNFα, IL-1β, IL-6 and MIP-1β and induced a 2- to 10-fold higher production than did LPS of G-CSF, TNFα and IL-1β. We conclude that stimulation of MDDC with an AbM-based extract resulted in increased production of proinflammatory, chemotactic and some Th1 type cytokines *in vitro*.

Paper III

In this foremost *in vivo* study, the aim was to examine the effect of consumption in healthy individuals of the normal dose AbM-based extract, AndoSan™, for 12 days on expression of adhesion molecules CD11b, CD11c and CD62L and ROS in blood leukocytes. There was a significant increase of CD62L expression on monocytes and granulocytes from before (day 0) compared to (day 12) after daily AbM consumption. However, only minor alterations and no clear trend in the expression of CD11b and CD11c were detected. Intracellular ROS (mainly superoxide ion) were significantly reduced in these cells from days 0 to 12. After ingestion of high dose AbM-based extract (360 ml per day) for two days, gene expression by microarray analysis based on the affymetric gene chip platform was studied. There were no alterations in gene expression, whatsoever, including TLR2, CD11b, CD11c and CD62L. These results supported that oral intake of AbM exhibited a sligth anti-inflammatory effect in humans *in vivo*.

Paper IV:

Agaricus blazei Murill (AbM) has shown to reduce blood cytokine levels in healthy volunteers, pointing to an anti-inflammatory effect. The aim was to study whether AndoSan™ had similar effects on patients with ulcerative colitis (UC) and Crohn's disease (CD). Calprotectin, a marker for inflammatory bowel disease (IBD), was also measured. Patients with CD (n=11) and with UC (n=10) consumed 60 ml/day of AndoSan™. After 12 days baseline plasma cytokine levels in UC was significantly reduced for MCP-1 in unstimulated blood, in LPS-stimulated blood for MIP-1β, IL-6, IL-1β, IL-8, G-CSF, MCP-1 and GM-CSF. There were corresponding significant reductions in CD for IL-2, IL-17 and IL-8 and in LPS-stimulated blood for IL-1β, MIP-1β, MCP-1, IL-8, IL-17, GM-CSF and G-CSF. Baseline concentrations for the 17 cytokines in the UC and CD patient groups were largely similar and elevated from ~2 to ~7-fold. Calprotectin in feces was significantly reduced in the UC patients, but not in the CD patients. Plasma calprotectin was unaltered in both diseases. Ingestion of the AbM-based medicinal mushroom extract AndoSan™ by patients with IBD resulted in interesting anti-inflammatory effects as demonstrated by declined levels of pathogenic cytokines in blood and calprotectin in feces.

Data not shown

In patients with UC (n=10) and CD (n=11) we examined, as for healthy individuals (paper I), levels of ROS and expression of adhesion molecules E-selectin (CD62L), $ß_2$ integrins (CD11b, CD11c) in granulocytes and monocytes at day 0 before and 12 days after AndoSan™ consumption. There were no striking alterations in these parameters. Five of the patients with UC and CD, respectively, were randomly selected for microarray analysis prior to and at day 12 of AndoSan™ ingestion. We were not able to demonstrate altered gene expression, whatsoever, induced by the AbM-based mushroom extract.

General discussion

The major finding in this thesis was that the *Agaricus blazei* Murill (AbM)-based mushroom extract AndoSan™ in humans, when ingested via the enteral route, exhibited an anti-inflammatory effect *in vivo* contrary to a pro-inflammatory effect *ex vivo* and *in vitro*.

This anti-inflammatory effect is based on reduction in levels of several cytokines (IL-1ß, IL-2, IL-6, IL-8, IL-17, MIP-1ß, MCP-1, G-CSF, GM-CSF) in blood of healthy individuals (paper I) and patients with UC and CD (paper IV). Further, there was a reduction of the inflammatory marker calprotectin, mainly derived from granulocytes, in feces from patients with UC but not CD. In additon, the pro-inflammatory effect of AndoSan™ *in vitro* on monocyte derived DC was demonstrated by release of cytokines (IL-8, G-CSF, TNFα, IL-1β, IL-6, MIP-1β and IFNγ) which was similar to previous studies in monocytes (5) and macrophages (95), but lesser in magnitude. As to the relevance of studying cytokines in blood in patients with IBD, the crucial finding, was that in nine (MIP-1ß, IFNγ , IL-6, TNFγ, MCP-1, IL-17, GM-CSF, IL-8 and IL-2) of the seventeen cytokines (paper IV), the levels were elevated from ~2 to ~7-fold compared with healthy individuals. Since autoimmune IBD exhibits systemic manifestations affecting the whole body, we took for granted that cytokine levels in blood mirrored those of the gut wall. There are three advantages from studying cytokine levels in blood instead of gut mucosa. Firstly, cytokines in blood are much easier accessible than gut located cytokines. Secondly, the representativity of a blood sample is presumably better than hitting the hot spots of the long gut mucosa. Thirdly, endoscopy with biopsies is bothersome and complications like bleeding and perforation may occur in a few patients.

The reduction of number of cytokines and their relative concentrations were more pronounced in healthy individuals than in IBD patients. This supported that key ingredients of the mushroom extract are absorbed into the gut wall also when confronted with an intact gut mucosa and prior to presentation to gut associated lymphoid tissue (GALT) like lymph nodes and Peyer's patches. It is commonly believed that carbohydrates larger than monosaccharides are not absorbed from the human gut. However, in murine models (14;27) uptake of ß-1,3-glucans across the gut wall, probably by microfold cells (M cells), but also by DC (17), has been demonstrated. The ß-glucan may further be transported by DC to lymphocytes in GALT, but also circulated in blood rodents (45;87). Presumably, a similar mechanism is operating in humans for intestinal absorption of small and immunomodulatory

bioactive ß-glucan fragments into the lymphoid system and blood. A key to understanding the function of down-regulating the cytokine response in the gut wall, is probably the signals mediated by DC (89). Naïve DC secrete IL-10 which stimulate T_{reg} cells which then inhibit Th1-, Th2- and Th17 cells and MΦ responses. Conversely, more mature DC, presumably like in IBD (Fig. 7 and 8) and as demonstrated *in vitro* (paper II), secrete upon stimulation pro-inflammatory mediators such as TNFα, IL-6, IL-8, IL-12 which create a chronic inflammatory cytokine driven response. In addition, AbM contains absorbable low molecular weight antioxidant substances (46), which down-regulate levels of ROS, which was seen regarding to superoxide anions in healthy individuals, but not in IBD patients. The reason for reduced superoxide anions may be related to reduction of IL-1ß because inhibitors of ROS reduce synthesis of this cytokine in macrophages (70).

The second major finding was that patients with UC had a significant reduction of fecal calprotectin after 12 days of AndoSan™ consumption, whilst calprotectin was unaltered in the CD patients (paper IV). Calprotectin is an abundant cytosolic protein mainly released from neutrophils (97), and is considered a reliable surrogate marker of disease activity in IBD (30) when released to feces. Reduction in fecal calprotectin has been detected parallel with reduced degree of inflammation in CD, but the reported initial calprotectin values were much higher (~15-fold) (106) and from more seriously affected patients than in this study (paper IV). The limited period of AndoSan™ ingestion and the difference in disease severity may contribute to explain the lack of effect on fecal calprotectin levels in our CD patients. The unaltered levels of plasma calprotectin by AbM consumption, indicated that the effect of AndoSan™ on that parameter was on granulocytes located in the colonic mucosa. During active inflammation, neutrophils infiltrate the lamina propria, the mucosal epithelium and form crypt abscesses that resolve during periods of remission (49). The IBD patients also spontaneously reported reduction of bowel movements after a few days of AndoSan™ consumption, that may in part be related to reduction of fecal calprotectin.

We were not able to demonstrate convincing anti-inflammatory effects in blood concerning altered expression of adhesion molecules E-selectin (CD62L) and $ß_2$ integrins (CD11b, CD11c) in granulocytes and monocytes in healthy individuals (paper III) and IBD patients (data not shown). However, the slight increase in E-selectin expression on granulocytes and monocytes *in vivo* as a consequence of AndoSan™ consumption in healthy individuals, may imply an anti-inflammatory effect. Neutrophils in knock-out mice (94) that lack the ability to shed E-selectin exhibited reduced migration towards a gradient of inflammatory chemokines, which suggested that L-selectin shedding is required for efficient

transendothelial migration. Concerning ROS, there was a slight, but significant reduction of DHE expression, mainly reflecting superoxide ions, in granulocytes after 12 days of AndoSan™ ingestion in healthy individuals (paper I), but not in IBD patients (data not shown). Accordingly, an anti-inflammatory effect by these parameters could generally not be demonstrated in IBD patients and only to a minor extent in healthy individuals concerning reduction of ROS and increase of E-selectin. Also, we were not able to detect any alteration of gene expression in healthy individuals after two days use of high dose (360 ml/day) AndoSan™ (paper III) and in IBD patients (data not shown) after long-term low dose (60 ml/day) consumption of the AbM mushroom extract AndoSan™, respectively.

Thus, the convincing anti-inflammatory effects demonstrated in this thesis *in vivo* are based on the impact of reductions in concentrations of cytokines and fecal calprotectin.

The effect of the AbM mushroom extract AndoSan™ on cells of innate immunity, as described in the introduction, is for ß-glucan by way of interaction of PRR on monocytes, MΦ, DC, NK cells and granulocytes. Prominent PRR are TLR 2 (103), dectin-1 receptor (13), the lectin binding site for ß-glucan in CR3 (104) and possibly CR4 (4). Effects of these receptors include induction of NF-κB mediated pro-inflammatory cytokines (TLR2, dectin-1 receptor) and binding and ingestion of microorganisms as well as degranulation and respiratory burst (CR3 and CR4) (84). The AbM mushroom extract contains soluble and larger insoluble ß-glucan fragments of several μm that may be responsible for biologic activites. Concerning anti-tumor activity in rodents (27;108), high molecular weight polysaccharide fractions (100-200 kDa) were most active *in vivo*, whereas small molecular fragments (0,5-10 kDa) showed no activity. Although AbM is the dominating mushroom (82%) of the AndoSan™ extract compared to *H. Erinacium* (15%) (57) and *G. frondosa* (3%) (1), biologic activites exerted by the latter two mushroom, isolated or in synergy, must be kept in mind.

Increased cytokine levels in serum have only been reported for IL-6 (44) and TNFα (66) in patients with UC and CD and for MIP-1ß in the former disease for UC (90). We report the novel finding of as many as six cytokines (IL-2, IL-8, IL-12, IL-17, MCP-1, GM-CSF), in addition to three known cytokines (MIP-1ß, TNFα, IL-6), being similarly elevated in UC and CD. These findings challenge the notion that UC is a Th2 cell mediated disease and that CD is a Th1 cell mediated disease, since the same type of cytokines were elevated in both diseases (paper IV). As could be expected, the anti-inflammatory effect of AndoSan™ was easier to detect in healthy individuals since there was a reduction in as many as five

cytokines (IL-1ß, TNFα, IL-2, IL-6, IL-17) in unstimulated blood versus one cytokine (MCP-1) in UC and three cytokines (IL-2, IL-8, IL-17) in CD. On the other hand, after LPS stimulation *ex vivo* of harvested blood, the corresponding number of additional cytokines reduced were four in healthy individuals, six in UC and five in CD. Using LPS stimulation, this demonstrated a considerable decrease of the intracellular stores of the crucial cytokines that substantiated the anti-inflammatory effect of the mushroom extract. Caution must be made concerning measurements of MIP-1ß after LPS stimulation of blood due to values out of range despite sample dilution 1/10.

The two cytokines which were reduced in both healthy individuals and IBD patients were IL-1ß and chemokine G-CSF, which are both crucial contributors for inflammation. Interestingly, in CD the Th1 cell cytokine IL-2 and the Th17 cell cytokine IL-17, which are considered pathogenic cytokines, were reduced with and without LPS stimulation (54;61). For UC, none of the Th2 cell cytokines (IL-4, IL-5, IL-13) were reduced, although a clear overall anti-inflammatory effect was obtained.

One healthy volunteer was excluded from the study (paper I) due to reoccurrence of herpes labialis during use of low dose AndoSan^{TM}, but other potential adverse effects were not discovered. Blood parameters testing pancreatic-, liver- and renal function were unaltered and within the normal range. It has been suggested that several months of AbM consumption was the possible cause of hepatic dysfunction, in three cancer patients, other than chemotherapy, one of whom died from hepatic failure (65). However, intake of AbM extract for 12 months normalized liver function in four patients with chronic hepatitis B (43). Moreover, one week intake of AbM in hepatitis C patients had no adverse effects on liver function (35). When considering the thousands of people consuming AbM, this extract seems harmless and definite hepatotoxicity has not been clearly demonstrated (65).

One objection to the cytokine studies in humans can be that no placebo group was included. Accordingly, the participants were their own controls and the comparison was made between before and after consumption of AndoSan^{TM}. Especially psychosomatic factors could contribute to a placebo effect independent of the AbM mushroom extract. However, since an anti-inflammatory effect was found in three groups of people with and without disease, the results must be interpreted as valid.

Conclusion

In conclusion, we have in this thesis demonstrated an anti-inflammatory effect *in vivo* as measured by reduced release of cytokines to blood and calprotectin to feces as a consequence of ingestion of the AbM-based mushroom extract AndoSan™. This effect which dampened an inflammatory response, is particularly relevant for patients with IBD and may partly be ascribed to development of tolerance mediated by the DC through stimulation of T_{reg} cells.

Future perspectives

Patients with IBD are dependent on surgical and/or medical treatment for relief of symptoms. The anti-inflammatory effect of the AbM-based mushroom extract AndoSan™ may be of potential benefit in these patients as a support therapy, thereby reducing dependence on traditional treatment. However, before AndoSan™ may be introduced as a support therapy, randomized studies comparing this mushroom extract with placebo have to be performed. Such studies should also include intake of AndoSan™ for longer time intervals.

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I

II

III

Effect of an Agaricus bM mushroom based extract on expression of adhesion molecules and production of ROS in human monocytes and granulocytes *in vivo*

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Running head: AbM, adhesion molecules and ROS *in vivo*

Abstract

Background: Oral intake (60 ml daily) over 12 days in eight healthy volunteers of an immuno-stimulatory extract based on the medicinal mushroom *Agaricus blazei* Murill (AbM), reduced monocyte and granulocyte release of mainly pro-inflammatory cytokines *in vivo,* suggesting an anti-inflammatory effect. In this foremost *in vivo* study, the aim was to examine the effect of such AbM consumption on expression of adhesion molecules CD11b, CD11c and CD62L and on production of reactive oxygen species (ROS) in leukocytes.

Methodology/Principal Findings: As shown by flow cytometry, there was a significant increase of CD62L expression on monocytes and granulocytes from before (day 0) compared to 12 days after daily AbM consumption. However, only minor alterations and no clear trend in the expression of CD11b and CD11c were detected. Intracellular ROS (mainly superoxide ion) were significantly reduced in these cells from days 0 to 12.

Conclusions/Significance: These results support that oral intake of AbM exhibits an anti-inflammatory effect in humans *in vivo*.

Key words: monocyte; granulocyte; ROS; adhesion molecules

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Introduction

The *Agaricus blazei* Murill mushroom (AbM) (jap.: Himematsutake) of the *Basidiomycetes* family grows wild in the Piedade area outside of São Paulo, Brazil. People in this area have traditionally used AbM as a health food ingredient. The frequency of serious diseases like atherosclerosis, hepatitis, hyperlipidemia, diabetes and cancer [1] were lower in Piedade than in neighbouring regions, supposedly due to the AbM intake. In 1966 the mushroom was taken to Japan and introduced to the health food market. AbM has also been subjected to an increasing research effort, especially since the early 1980s. Other interesting medicinal *Basidiomycetes* mushrooms that have been studied are *Grifola frondosa* (Gf) (Maitake) [2] and *Hericeum erinaceum* (He) (Yamabushitake) [3].

AbM, like Gf, is rich in immunostimulatory mixture of β(1-3)-, β(1-4) and β(1-6)-D-glucans with antitumor activity [4], probably secondary to modulation of NK-cells [5] and monocytes/macrophages of native immunity [6-8] .

In vitro AbM stimulates mononuclear phagocytes to secrete nitric oxide [9] and pro-inflammatory cytokines like IL-1β, IL-6, and TNFα, and chemokine IL-8 [9, 10]. The effects are probably mediated by binding of sugars in AbM to TLR-2 [11], but also to dectin-1 [12] and the lectin-binding site of CD11b/18 [13] and possibly CD11c/18 [14]. Gene microarray expression analysis of promonocytic THP-1 tumor cells [15] supported these results since stimulation with AbM upregulated genes strongly for IL-1β and IL-8, moderately for TLR-2 and MyD88 but not for TLR-4. On the other hand, daily

consumption of 60 ml of AbM for seven days in patients with chronic hepatitis C [16], had no effect *in vivo* on expression of these genes in blood cells.

Recently, we reported that AbM stimulation of whole blood *ex vivo* [17] stimulated the release of all the 17 different cytokines, chemokines and leukocyte growth factors tested. The cytokines were pro-inflammatory (IL-1β, IL-6, TNFα), anti-inflammatory (IL-10), pleiotropic (IL-7, IL-17) and of the Th1- (IL-2, IFNγ, IL-12) and Th2-types (IL-4, IL-5, IL-13). In addition, chemokines IL-8, MIP-1β, MCP-1 and leukocyte growth factors G-CSF and GM-CSF were also studied. On the other hand, when blood were harvested from volunteers prior to and 12 days after their daily intake (60 ml) of AbM, there was *in vivo* either a significant reduction in cytokine levels for IL-1β, TNFα, IL-6, IL-2 and IL-17 or unaltered levels of the remaining twelve factors. This points to a stabilising and anti-inflammatory effect of AbM *in vivo* when given via the oral route.

Ex vivo studies [18] in whole blood demonstrated that AbM decreased the expression of adhesion molecule CD62L (L-selectin) and increased the expression of the β_2 integrin CD11b on monocytes and granulocytes. These molecules are cruicial for respectively rolling and adherence to endothelial cells [19,20], and prerequisites for the ultimate transcapillary diapedesis of leukocytes to inflammatory foci in order to attack microorganisms.

Especially granulocytes, but also monocytes, generate reactive oxygen species (ROS) such as $O_2^{\cdot -}$, H_2O_2, $^{\cdot}OH$ and $ONOO^-$ upon stimulation during phagocytosis, which damages ingested microorganisms by peroxidation of lipids, proteins, and nucleic acids, and nitrosylation of proteins [21,22]. AbM stimulation of whole blood *ex vivo* [18] had no effect on ROS production detected by the dihydroethidium (DHE) probe (mainly

reflecting superoxide anion), neither in granulocytes nor monocytes. However, a slight increase in ROS detected by the dihydrorhodamine 123 (DHR) probe (mainly detecting peroxynitrite), was evident in granulocytes upon stimulation with the two highest concentrations (10%, 15%) of AbM.

Accordingly, the next step was also to study *in vivo* the effect on adhesion molecule expression (CD62L, CD11b, as well as the β_2 integrin CD11c) and intracellular ROS levels in human leukocytes after oral intake of AbM over days in healthy volunteers.

Results

Effect of AbM on expression of adhesion molecules in whole blood ex vivo

The expression of CD11c/CD18 (Fig. 1c) increased and peaked already at the lowest concentration of AbM (0.1%) and was more than two-fold higher for monocytes than for granulocytes.

Increase in CD11b expression (Fig. 1a) and a decrease in CD62L expression (Fig. 1b) on the surface of granulocytes and monocytes after stimulation of whole blood with AbM in concentrations from 0.1 to 10% were also demonstrated. The increase in CD11b was more pronounced for granulocytes than for monocytes, and reached a plateau at a lower concentration of AbM in former cell type (0.5% versus 2%). The reduction of expression of L-selectin (Fig. 1b) (CD62L) was stronger in monocytes than in granulocytes and occurred at lower concentration of AbM (0.1% versus 2%), with nadir levels at 5% and of 10%, respectively.

Effect of AbM on expression of adhesion molecules in whole blood in vivo

In eight individuals using AbM, in doses of 20 ml x 3 daily for 12 days, there was a significant reduction of CD11b expression in monocytes from day 0 to day 2 (Fig. 2a), and the levels fluctuated significantly throughout the experimental period. In granulocytes, however, there was a significantly higher amount of CD11b (Fig. 2a) compared with the monocytes, but without any significant alterations during the experimental period.

Concerning CD11c expression (Fig. 2b) and contrary to CD11b, the levels were significantly higher in monocytes than in granulocytes. However, only for granulocytes the levels increased from day 0 to day 5 and varied significantly during the experimental period only for granulocytes. There was a significant increase from day 0 to day 12 regarding expression of CD62L (Fig. 2c), and it was more pronounced for monocytes than for granulocytes.

Fifteen healthy individuals used high dose of AbM (120 ml x 3) for 2 days. When the cells were analysed for expression of CD11b, CD11c and CD62L prior to (day 0) and after intake of AbM (days 1 and 2), there were no significant alteration whatsoever at any time points tested (data not shown). However, when the cells in whole blood were stimulated for one hour with LPS (1 μg/ml) *ex vivo ,* an expected and comparable increase in CD11b and CD11c and decrease in CD62L was demonstrated (data not shown), both before and after intake of AbM in low dose for 12 days and high dose for 2 days, respectively.

Effect of AbM on ROS production in whole blood in vivo

The intracellular levels of ROS detected by the DHE-monitored assay (mainly reflecting superoxide anion) in harvested cells from eight healthy volunteers ingesting AbM, exhibited a significant reduction from prior to (day 0) and 12 days after intake (Fig. 3a). Moreover, the levels of ROS were higher in monocytes compared with granulocytes. Analysis of ROS detected by the DHR probe (mainly reflecting peroxynitrite ions), revealed some alterations throughout the experiment for monocytes

(p=0.04) but no significant difference for either cell type when comparing levels of ROS from start (day 0) till end of the experiment (day 12) (Fig. 3b).

Finally, the high concentration of AbM (120 ml x 3 daily) was used for a short period of 2 days in 15 healthy individuals. In this experimental set-up the results were unaltered for ROS detected by DHE and DHR in blood, both prior to (day 0) and after intake of AbM for 1 and 2 days (data not shown). PMA stimulation of harvested whole blood ex vivo for 60 min. from the volunteers prior to or after consumption high or low dose AbM for 2 and 12 days, respectively, revealed comparable increase of ROS levels (data not shown).

Effect of AbM on gene expression

In three healthy middle-aged males consuming high dose AndoSan^{TM} for 2 days there were no differential expression of mRNA for the following genes; TLRs- 2 and -4, CD11b (gene symbol ITGAM), CD11c (ITGAX), CD62L (SELL) and My88.

Discussion

The main aim of this study was to examine the effect of oral intake of the AbM based mushroom extract in healthy volunteers on expression of adhesion molecules and ROS production in monocytes and granulocytes in whole blood *in vivo.*

Previously [18] and more extensively in the present study also including CD11c, we demonstrate that AbM stimulation of whole blood *ex vivo* dose-dependently increased the levels of CD11b and CD11c expression and decreased the levels of C62L expression on the surface of monocytes and granulocytes (Fig. 1). From these results we propose that the AbM-stimulated leukocytes are more prone to phagocytosis via complement receptors C3 (CR3) (CD11b/CD18) [23] and C4 (CR4) (CD11c/CD18) [14], the glucan- [24] and the dectin 1 receptor [12]. Moreover, modulation of the leukocyte adhesion molecule profile [20,25] contributes to events necessary for rolling, firm adhesion and extravasation of the leukocytes through the endothelial cell layer to inflammatory foci. The inverse relationship between CD11b/CD11c and CD62L expression has also been reported [26] in a similar experimental set-up, in which whole blood was stimulated with meningococcal LPS.

On the other hand, when these adhesion molecules were analysed *in vivo* (Fig. 2) during a period of 12 days with daily intake of 60 ml of the mushroom extract, there were only minor alterations in expression of CD11b and CD11c on both on granulocytes and monocytes. However, concerning CD62L expression on the surface of the granulocytes and monocytes, there was a significant increase from baseline (day 0) to 12 days after daily consumption of AbM (Fig. 2c). This was contrary to the decrease in CD62L

expression demonstrated *ex vivo* (Fig. 1c) and explained by shedding [27] from the plasma membrane [28]. Studies in knock-out mice, which lack the ability to shed L-selectin from neutrophilic granulocytes [29], showed reduced migration towards a gradient of inflammatory chemokines. This suggests that L-selectin shedding is required for efficient transendothelial migration. Accordingly, higher levels of L-selectin on the surface of monocytes and granulocytes *in vivo* after consumption of AbM might stabilise or reduce this process, which implies an anti-inflammatory effect of AbM in the body. The lack of effect on levels of adhesion molecules after intake of high daily doses of AbM (360 ml/day) for 2 days, was probably due to the short duration of this experiment.

Regarding the generation of ROS of whole blood *ex vivo* [18], only a limited increase in mainly $ONOO^-$ (DHR probe) but not $O_2^{\cdot -}$ (DHE probe) was detected in granulocytes, upon stimulation with high concentrations of AbM (10% and 15%). However, when studying ROS *in vivo*, daily intake of low dose AbM resulted in a significant reduction in mainly $O_2^{\cdot -}$ at day 12 (Fig. 3a). This again points to an anti-inflammatory effect. Increased occurrence of cancer with age might in part be explained by damage inflicted by ROS-induced generation of oxidative stress, giving aberrant DNA methylation patterns in normal cells [30,31]. Accordingly, the AbM induced reduced production of ROS, demonstrated *in vitro* [32] and herein *in vivo,* agrees with the proposed cancer-protecting properties of AbM [8]. Recently, we reported [17] that the levels of 17 different cytokines (especially pro-inflammatory and anti-inflammatory cytokines), chemokines and growth factors increased by AbM stimulation of whole blood *ex vivo.* On the other hand, intake of low dose of AbM over 12 days *in vivo* resulted in significant reduction, from day 0 to day 12 in five of these foremost pro-inflammatory cytokines (IL-

1β, TNFα, IL-6, IL-2, IL-17). The most pronounced reduction *in vivo* was for IL-1β, which is very interesting because inhibitors of ROS (such as N-acetylcysteine) have been reported to block IL-1β production in macrophages [33]. Accordingly, since AbM also contain low molecular weight antioxidant substances [34]**,** this could contribute to the explanation of a combined reduction of ROS and IL-1β production in monocytes and granulocytes *in vivo.*

In this study, it was pertinent to examine whether genes for TLRs-2 and -4, CD11b, CD11c, CD62L as well as My88 were differentially expressed in blood cells *in vivo* as a consequence of AbM consumption in healty individuals. Similar to a recent *in vivo* study on chronic hepatitis C patients consuming low dose AbM for 7 days [19] differential effects were not demonstrated on expression of these genes as measured by mRNA in volunteers consuming high dose AbM for 2 days. The effect of AbM *in vivo* does not seem to be mediated by TLRs, especially since MyD88 expression also was unaltered, which is critical for signaling from all TLRs except TLR-3 [35]. However, when monocytic tumor like cells (THP-1) are AbM stimulated *in vitro* forementioned genes are upregulated except TLR-4 [15], as well as genes for pro-inflammatory cytokines IL-1β and IL-8.

The discrepant results obtained with AbM on expression of adhesion molecules and generation of ROS in monocytes and granulocytes *ex vivo* and *in vivo,* demands consideration. There might be a very limited absorption of large and bioactive β-glucan fragments across the intestinal mucosa to the reticuloendothelial system and to the blood, which excludes their potential prominent stimulatory effect on expression of adhesion molecules and cytokine release as demonstrated *ex vivo* [17,18]. In murine models,

however, uptake of β-1,3-glucans by gastrointestinal macrophages and shuttling to the reticuloendothelial system and bone marrow has been demonstrated [36]. As to anti-tumor activity [36,37], high molecular weigth polysaccharide fractions (100-200 kD) were most active *in vivo*, whereas smaller molecules (0.5-10 kD) exhibited no activity. Increased uptake of AbM from the gut and enhanced antitumor activity in a myeloma mouse model, has been shown after encapsulation of it AbM β-glucans in marine phospholipids [38]. On the other hand, β-glucans could have had an additional indirect effect locally in the intestines by stimulating immune cells like monocytes or dendritic cells in Peyer's patches.

We have hereby shown that AbM consumed over days by healthy volunteers increase *in vivo* the expression of L-selectin and reduce transiently that of CD11b and markedly reduce the production of ROS in monocytes and granulocytes in whole blood. Together with the reported [17] *in vivo* reduction in levels of mainly pro-inflammatory cytokines in identical experimental set-up, our results indicate a stabilising and anti-inflammatory effect of AbM *in vivo.* We plan to study whether AbM can have a clinical effect through normalization of these inflammatory parameters *in vivo* in patients with inflammatory bowel disease.

Conclusions

Oral intake over 12 days in eight healthy volunteers of an AbM based immuno-stimulatory mushroom extract resulted in reduction of ROS in monocytes and granulocytes. An increase in expression of L-selectin occurred on these cells, whilst levels of adhesion molecules CD11b and CD11c largely were unaltered. Together with reported [17] reduction *in vivo* of pro-inflammatory cytokines, the results strengthen the assumption of a stabilizing and anti-inflammatory effect of this mushroom extract. The impact of AbM on patients with inflammatory bowel disease will be tested.

Acknowledgement

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Materials and Methods

Reagents and antibodies

The mushroom extract (AndoSan^{TM}) used in our experiments was obtained from ACE Co., Ltd., Gifu, Japan. It was stored at 4°C in dark bottles and kept sterile. This *Basidiomycetes* mushroom extract is a commercial product and its exact contents a business secret, part of which has not been revealed until very recently. The AbM mixed powder contains per 100 g the following constituents: moisture 5.8 g, protein 2.6 g, fat 0.3 g, carbohydrates 89.4 g of which β-glucan constitutes 2.8 g, and ash 1.9 g. The AndoSan^{TM} extract contains 82.4% of *Basidiomycetes* mushroom derived from *Agaricus* blazei Murill, 14.7% from *Hericium erinaceum* and 2.9% from *Grifola frondosa*, and its final concentration was 340 g/l. The amount per litre of the extract for sodium was 11 mg, phosphorous 254 mg, calcium 35 mg, potassium 483 mg, magnesium 99 mg and zinc 60 mg. The LPS content of AndoSan^{TM} were found using the *Limulus* amebocyte lysate test (COAMATIC Chroma-LAL; Chromogenix, Falmouth, MA, USA) with detection limit 0.005 EU/ml (1 EU=0.1 ng/ml), to be a miniscule concentration of <0.05 pg/ml [17]. AndoSan^{TM} had been heat-sterilized (124°C for 1 h) by the producer. LPS was from *Escherichia coli* (*E. coli* 026:B6) (Sigma Co., St. Louis, MO, USA).

Fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody against β_2 integrin CD11b was obtained from Sigma (10 μl/test). The β_2 integrins are classified according to their α and β subunits as CD11/CD18 molecules. They are composed of identical β subunits (CD18) and different α subunits (CD11b/Mac-1 and CD11c/ gp150,95). Phycoerythrin (PE)-conjugated mouse monoclonal antibodies against CD62L

(L-selectin), FITC-conjugated mouse anti-CD11c (20 µl/test) and isotype IgGs for CD62L and CD11b, and CD11c were obtained from Becton Dickinson, San José, CA, USA and Sigma Co., St. Louis, MO, USA, respectively. Dihydroethidium (DHE) was obtained from Sigma, and dihydrorhodamine 123 (DHR) from Molecular Probes, Leiden, The Netherlands.

Experimental design

Ex vivo experiments

Venous EDTA-blood was collected from six healthy volunteers (four men) with median age 38.5 (range 37-61) years. In order to study the expression of leukocyte adhesion molecules (CD11b, CD11c, CD62L), aliquots of fresh whole blood (400 µl) was incubated with 0.1, 0.5, 2.0, 5.0, 10.0 or 15.0 % (final concentration) of sterile AndoSan^{TM} for 60 min at 37°C. Positive (LPS 1 µg/ml) or negative (phosphate buffered saline, PBS) controls were included.

In vivo experiments

Five healthy men and five healthy women, of median age 30.5 (range 26-51) years, volunteered to participate in the study of oral intake of low dose AndoSan^{TM}; 20 ml thrice daily for 12 days. The dose of a total of 60 ml of the mushroom extract per day was chosen because this was the dose recommended by the manufacturer and regularly used for AndoSan^{TM} as a health product. The time interval between each dose should be from 6-10 hours. Participants were asked to avoid mushroom-containing foods for 3 days prior to and during the experimental period. Three women used contraceptive drugs, one of

whom also 100 mg x 1 of iron on days 0, 7, 8 and 9, and another woman ate a pizza with mushrooms on day 1 and used medication against migraine (rizatriptan, rapitab, ibuprofen, paracetamol, phenazone-caffeine) on days 0, 5, 6 and 7. One female participant stopped the intake of AndoSan^{TM} due to reoccurrence of localized labial eruption, probably her known herpes simplex labialis. One male participant was for practical reasons unable to give the last blood sample. Accordingly, two of the 10 participants were not included in the complete analysis of the results. None of the participants reported trouble with intake of AndoSan^{TM} due to taste or volume.

Venous blood EDTA (total 10 ml) was collected on day 0 immediately prior to intake of the mushroom extract and on days 1, 2, 5, 8 and 12, and analysed for adhesion molecules (CD11b, CD11c, CD62L) and reactive oxygen species (ROS). The blood samples were each time analysed for hemoglobin, hematocrite, mean cellular volume, mean cellular hemoglobin, reticulocytes, immature reticulocytes, leukocytes including a differential count of neutrophils, basophils, eosinophils, lymphocytes and monocytes. C-reactive protein (CRP), thrombocytes, urea, creatinine, bilirubin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, γ-glutamine transferase, alkaline phosphatase, and pancreatic amylase.

Same type of *in vivo* experiment was performed in fifteen healthy persons (8 women) of median age 36 (range 23-55) years, but the dose of AndoSan^{TM} was high (120 ml thrice daily) and the experiment limited to two days. One woman used daily a 20 mg tablet of esomeprazole as anti-secretory medication. Six of these 15 persons had previously also participated in the experiment using low dose AndoSan^{TM}. By giving 720 ml of the extract to individuals of weight 60-80 kg during 2 days, the amount of

AndoSan™ taken was comparable to that given orogastrically (0.2 ml AndoSan™) to mice [23] (18-20 g) prior to experimentally infecting the animals with fecal bacteria for lethal peritonitis, which was significantly reduced by AndoSan™ pretreatment. Thus, the notion was that use of comparable amounts of the mixed mushroom extract relative to body weight (9-12 ml/kg and 10-11 ml/kg in man and mouse, respectively), would be a rational strategy for observing beneficial or possible side (toxic) effects in humans.

All the participants in the *in vivo* and *ex vivo* experiments denied regular smoking or intake of medication, unless otherwise stated.

As recently reported [17], there were no alterations whatsoever in any of the hematological parameters tested during the experimental periods of 12 days and 2 days with low and high dose AndoSan™, respectively

Preparation of blood leukocytes for flow cytometric analysis

For flow cytometric analysis of leukocyte adhesion molecules, 50 µl of whole blood (unstimulated or stimulated with AbM or LPS) in 12x75 mm polystyrene test tubes (Falcon no. 2052) was mixed with monoclonal antibodies against CD11b (10 µl), CD11c (10 µl) or CD62L (20 µl) using Vortex mixer and then incubated on ice for 30 min. Blood with 10 µl PBS served as autofluorescence control. In addition, controls containing antibody-matched isotype IgGs were used. No evidence of significant non-specific (Fc receptor-mediated) binding of IgG to AbM-LPS-stimulated granulocytes/monocytes was demonstrated (data not shown).

After finishing incubation with anti-CD11b/CD11c/CD62L or DHE/DHR (*vide infra*), respectively, erythrocytes were lysed. For lysis of erythrocytes, 1.5 ml of a lysis buffer

containing 155.5 mmol/l of NH_4Cl, 1 mmol/l of $NaHCO_3$ and 0.109 mmol/l of EDTA-Na_3 was applied for 15 min at room temperature in the dark.

The samples were then centrifuged at 300 *g* for 5 min at 4˚C, and the cells washed once in 2 ml of PBS and finally resuspended in 0.5 ml of 1% w/v paraformaldehyde (Merck, Darmstadt, Germany). The samples were kept cold and dark during the whole procedure as well as during the storage before analysis.

Flow cytometric analysis of intracellular ROS in leukocytes (whole blood), including 100 ng/ml phorbol myristate acetate (PMA) (positive control) and PBS (negative control), was performed with aliquots of 50 µl of whole blood mixed with 10 µl of DHE (mainly reflecting superoxide anion) or 10 µl of DHR (mainly reflecting peroxynitrite and hypochlorous acid), both to a final concentration of 5 µmol/l. After incubation (5% CO_2 and 95% humidified air) for 60 min at 37˚C, erythrocytes were lysed and leukocytes were washed and fixed, as described above. The production of ROS in leukocytes could be evaluated due to the transformation of the probes DHE/DHR from nonfluorescent to fluorescent compounds by the oxidative burst intermediates within the cells.

Flow cytometric analysis and data collection

The labeled samples were analyzed within 24 h in a FACSort™ flow cytometer (Becton Dickinson). The flow cytometer was equipped with a 488 nm Argon laser and CellQuest™ Software (Becton Dickinson). Ten thousand events were collected from each sample. The leukocyte subpopulations, monocytes and granulocytes, were identified by their light scatter characteristics, enclosed in electronic gates, and separately analyzed for fluorescence intensity. The results were expressed as the mean fluorescence intensity

(MFI) of 10,000 cells. It should be noted that the average MFI values after *in vitro* PMA stimulation of monocytes and granulocytes were 2.6 times and 74 times higher (DHE probe) and 6.1 times and 167 times higher (DHR probe), respectively, than the MFI values at baseline levels. The intra-assay coefficient of variation (CV) was <5% in unstimulated and <10% in PMA-stimulated samples.

Microarray analysis

Studies on gene expression by microarray analysis (www.affymetrix.com) in three of the males (ages 37, 50 and 51 years) consuming the high dose AndoSan^{TM} for 2 days, were also performed. Blood was harvested in PAX gene tubes specifically designed to preserve RNA from blood for microarray experiments prior to (day 0) and after 2 days of AndoSan^{TM} consumption. The PAX gene tubes were frozen and kept at -20 ºC until extraction with the PAXgene Blood RNA Kit (Qiagen) according to the manufacturer's recommendation. 2.5 µg of total RNAs were subjected to One Cycle cDNA Synthesis Kit following the manufacturer´s (Affymetrix) recommended protocol for gene expression analysis. Biotinylated and fragmented cRNA (15 µg) was hybridized to the Affymetrix HG U133 Plus 2.0 Array, representing 47000 transcripts for 38500 well-characterized human genes. The signal intensities were detected by Hewlett Packard Gene Array Scanner 3000 7G (Hewlett Packard, Palo Alto, CA). All data is MIAME compliant and the raw data has been deposited in a MIAME compliant database at GEO under accession number xxxxxxxxxx, as detailed on the MGED Society website http://www.mged.org/Workgroups/MIAME/miame.html.

Statistical analysis of gene expression profiling

The 6 scanned images were processed using GCOS 1.4 (Affymetrix). The CEL files were imported into Array Assist software (v5.2.0; Iobion Informatics LLC, La Jolla, CA) and normalized using the PLIER (Probe Logarithmic Intensity Error) algorithm in Array Assist to calculate relative signal values for each probe set. In order to filtrate for low signal values, the MAS5 algorithm in Array Assist was used to create a dataset of Absolute Calls, showing the number of present and absent calls for each probe set. The filtration was performed by eliminating probe sets containing ≥4 absent calls across the data set, resulting in a reduction of probe sets from 47,000 to 28,188. For expression comparisons of different groups, profiles were compared using paired t-test. The results are expressed as fold changes (FC), e.g. ratio of of the mean signals between consumption of high dose AndoSan™ for 2 days and immediately prior to AndoSan™ consumption. Gene lists were generated with the criteria of $p<0.05$ and FC of $\geq|2|$.

Statistics

All measurements were based on duplicates from six donors when blood was sampled for the *ex vivo* experiments. For the *in vivo* experiments blood was sampled from eight donors (duplicates) prior to and after intake of AndoSan™ on days 1, 2, 5, 8 and 12, or on days 1 and 2, in low and high dose experiments, respectively. Data are presented as mean ±standard error of the mean (SEM). Differences in levels of adhesion molecules (CD11b, CD11c, CD62L) and ROS were, after passage of normality test, assessed with parametric analysis of variance (ANOVA) for paired data with Dunn's test comparing the

control value with the values after stimulation *ex vivo* or *in vivo.* In order to compare whether the levels of parameters were significantly different between monocytes and granulocytes, a two-tailed paired t-test was used. The instat for Windows™ statistics software package (Graphpad Software, San Diego, USA) was used and p values of or below 0.05 were considered statistically significant.

Ethics

The study was approved by the regional ethics committee (full name: REC for Health South-Eastern HF of Norway) of the ethics committee/institutional review boardand followed the guidelines of the Helsinki declaration. The participants were informed also in written form and signed an agreement of consent for participation in the study.

Supporting Information

Figure legends

Figure 1 ***Ex vivo*** **expression of surface adhesion molecules on leukocytes.**

Expression *ex vivo* of the cell surface adhesion molecules CD11b (a), CD11c (b) and CD62L (c), measured as the mean fluorescence intensity (MFI), in leukocytes without and with AbM in increasing concentrations. Filled and open columns represent monocytes and granulocytes, respectively. Data are presented as mean values ± SEM from six healthy individuals. Significant differences from the control were set at levels of $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

Figure 2 ***In vivo*** **expression of surface adhesion molecules on leukocytes.**

Expression *in vivo* of the cell surface adhesion molecules CD11b (a), CD11c (b) and CD62L (c) in monocytes and granulocytes in healthy volunteers using 60 ml of AbM daily for 12 days. Data are presented as mean values ± SEM from eight healthy individuals. Significant differences from the control (day 0) were at $p<0.05$ (*).

Figure 3 ***In vivo*** **generation of ROS in leukocytes.** Reactive oxygen species (ROS) generation *in vivo* in monocytes and granulocytes, detected by the DHE probe (a) and the DHR probe (b), in healthy volunteers using 60 ml of AbM daily for 12 days. Data are presented as mean values ± SEM from eight healthy individuals. Significant differences from the control (day 0) were at $p<0.01$ (**).

Author Contributions

Conceived and designed the experiments: TL, EJ. Performed the experiments: DTF, LS, OKO. Analyzed the data: EJ, TL. Contributed reagents/materials/analysis tools: GH, TL. Wrote the paper: EJ, TL, GH.

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Fig. 1

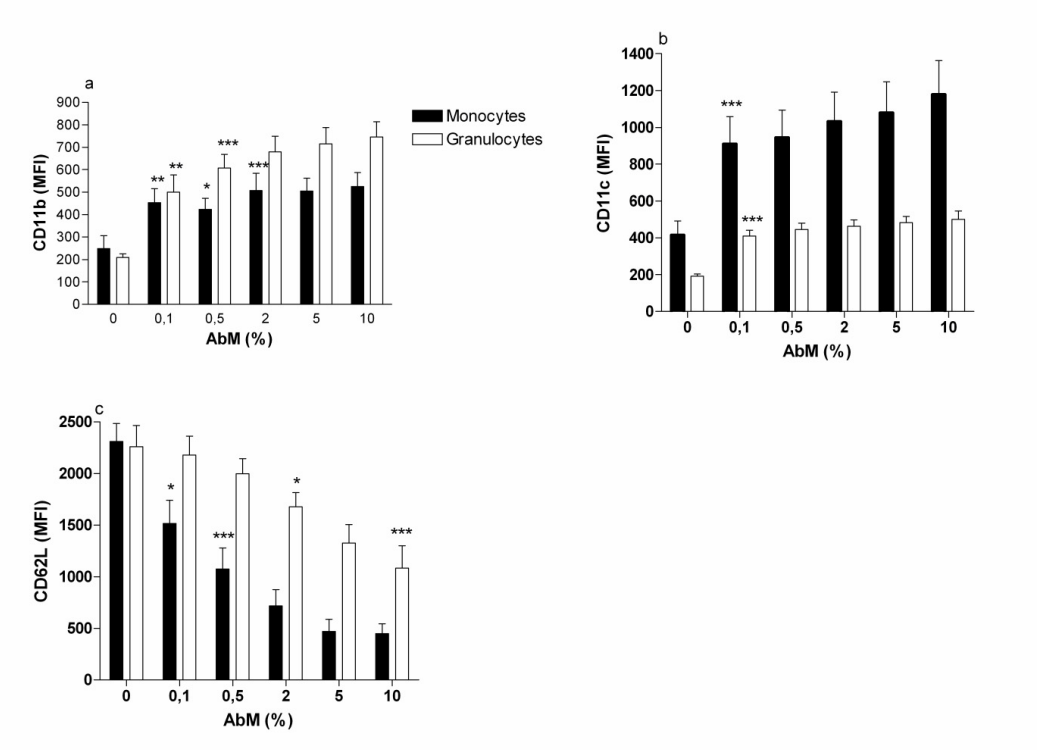

a
CD11b (MFI)
AbM (%)
Monocytes
Granulocytes
b
CD11c (MFI)
AbM (%)
c
CD62L (MFI)
AbM (%)

Fig. 2

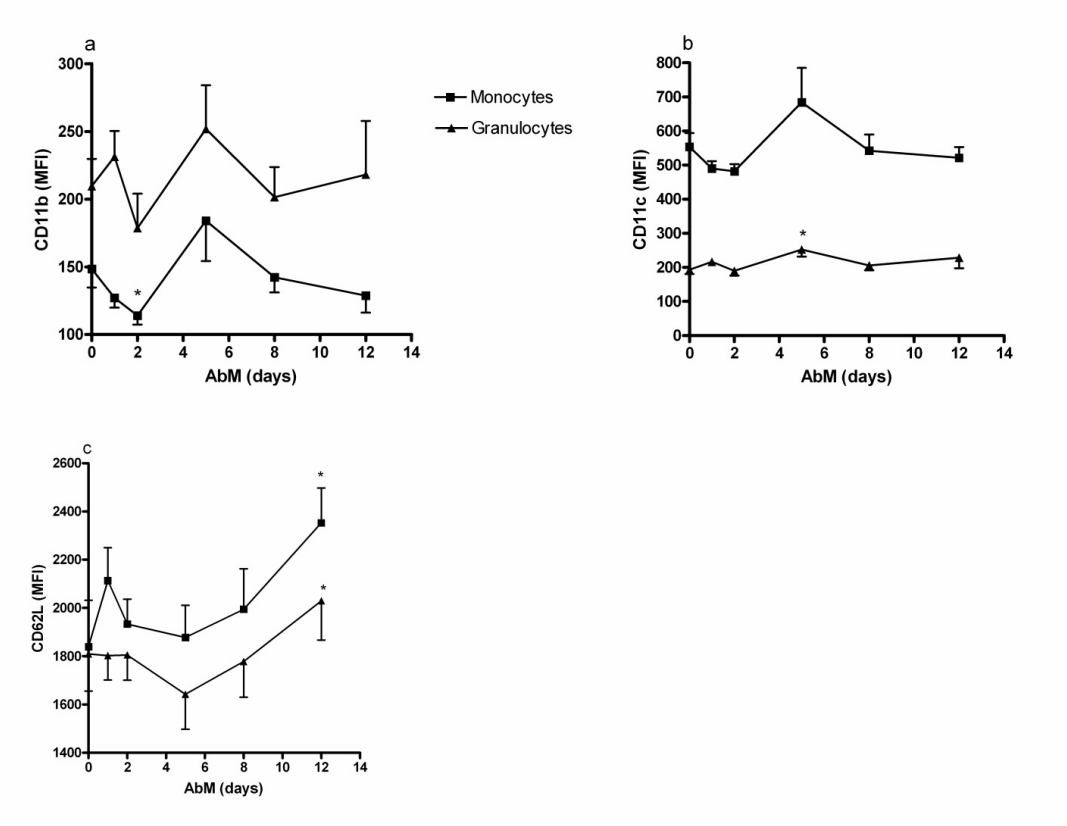
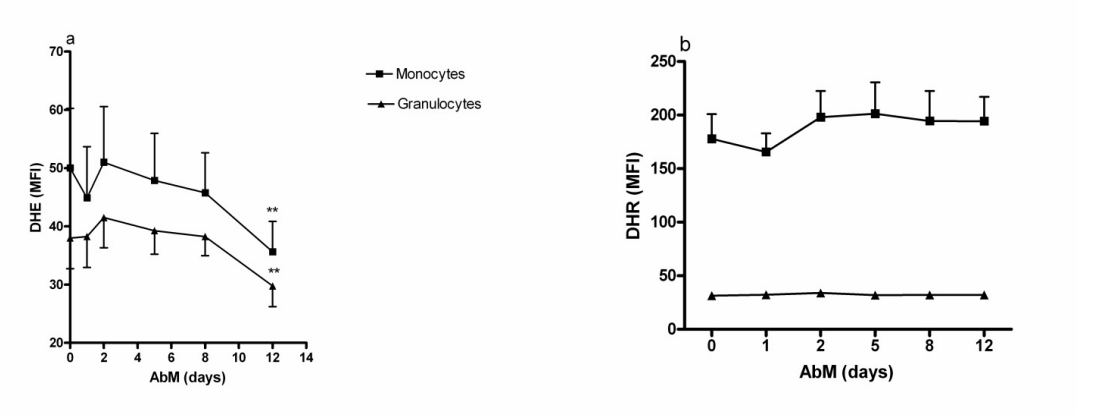

a
CD11b (MFI)
AbM (days)
Monocytes
Granulocytes
b
CD11c (MFI)
AbM (days)
c
CD62L (MFI)
AbM (days)

Fig. 3



IV