Antitumor and immunomodulating effects of the mushroom product Andosan^{TM}, based on the *Basidiomycota* mushroom *Agaricus blazei* Murill, with special focus on multiple myeloma.

Ph. D. thesis

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This study was performed in the period 2010 – 2019 within the framework of the Research Group Transfusion, Immunomodulation, and Allergology, led by Professor Geir Hetland, Department of Immunology and Transfusion Medicine, Institute of Clinical Medicine, Faculty of Medicine, University of Oslo. In the period 2010-2015 my time was equally divided between working as a senior consultant at The Department of Hematology and The National CBRNE Medical Advisory Centre, Oslo University Hospital (OUH). In addition, I was doing research. After retirement from my position at OUH in 2015, I have worked as a consultant on contract basis at The National CBRNE Medical Advisory Centre, while continuing working with the research project presented in this thesis.

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Conflict of interest

Professor Geir Hetland is a shareholder in the company Immunopharma AS, Oslo, Norway, which imports Andosan™.

Contents

List of papers

1. Tangen JM, Tryggestad AMA , Hetland G. Stimulation of human monocytic cells by the medicinal mushroom *Agaricus blazei* Murill induces expression of cell surface markers associated with activation and antigen presentation. Applied Scientific reports 214, doi:10.7243/2054-9903-1-1.

2. Tangen JM, Tierens A, Caers J, Binsfeld M, Olstad OK, Siebke Trøseid A-M, Wang J, Tjønnfjord GE, Hetland G. Immunomodulatory effect of the *Agaricus Blazei* Murill-based mushroom extract AndoSan in patients with multiple myeloma undergoing high dose chemotherapy and autologous stem cell transplantation: A randomized, double-blinded study. Biomed Res Int 2015.dx.doi.org/10.1155/2015/728539.

3. Tangen JM, Holien T, Mirlashari MH, Misund K, Hetland G. Cytotoxic effect on human myeloma cells and leukemic cells by the *Agaricus blazei* Murill based mushroom extract, Andosan^{TM}. Biomed Res Int 2017. doi:10.1155/2017/2059925

4. Hetland G, Eide DM, Tangen JM, Haugen MH, Mirlashari MR, Paulsen JE. The *Agaricus blazei*-based mushroom extract Andosan^{TM} protects against intestinal tumorigenesis in the A/J Min + Mouse. Plos One.doi:10.1371/journal.pone.0167754. December 21, 2016.

5. Tangen JM, Tjønnfjord GE, Gulbrandsen N, Gedde-Dahl T, Stormorken E, Anderson K, Dao Vo C, Schjesvold. Improved outcome in patients following autologous stem cell transplantation for multiple myeloma in South Eastern Norway 2001-1010. A retrospective, population-based analysis. BMC Cancer 2018 Aug 8;18(1):801. doi:10.1186/s1228-018-4722-x.

Selected abbreviations

AbM-	*Agaricus blazei* Murill
APC-	Antigen presenting cell
ASCT-	Autologous stem cell transplantation
DC-	Dendritic cell
G-CSF-	Granulocyte- colony stimulating factor
IBD-	Inflammatory bowel disease
IFN-	Interferon
IL-1β-	Interleukin 1β
IL-1βr-	Interleukin 1β receptor
IL-2-	Interleukin 2
IL-4-	Interleukin 4
IL-5-	Interleukin 5
IL-6-	Interleukin 6
IL-7-	Interleukin 7
IL-10-	Interleukin-10
IL-8-	Interleukin -8
IL-12-	Interleukin 12
IL-17-	Interleukin 17
LPS-	Lipopolysaccharide
MCP-	Macrophage chemoattractant protein

MGUS-	Monoclonal component with unknown significance
MM-	Multiple myeloma
MIP-β-	Macrophage inflammatory protein-β
MDDC-	Monocyte derived dendritic cell
NK cell-	Natural killer cell
OUH-	Oslo University Hospital
SMM-	Smoldering multiple myeloma
Th1-	T helper cell1
Th2-	T helper cell 2
Th17-	T helper cell 17
T_{reg} -	Regulatory T- cell
TLR-	Toll like receptor
TNF-	Tumor necrosis factor

Introduction

From the early days of civilization, man has used edible mushrooms for medicinal purposes. Historically, the use of higher mushrooms for medicinal purposes has been documented in

many countries (1). For instance, there are Chinese reports from around 500 BC, which mention the nutritional, medicinal and anti-cancer properties of the *Basidiomycota* mushroom *Ganoderma lucidum* (known in China as Reishi) (2). There are also descriptions from the Byzantine period, from the 4th century AD to the 15thcentury AD, relating the use of *Agaricus* mushrooms as a remedy against cancer of the larynx (3). In modern times, the Nobel prize laureate Aleksander Solzhenitsyn describes in his novel "The Cancer Ward" how extracts from the *Basidiomycota* mushroom *Chaga (Inonotus obliquus)*, which grows on the birch tree, is used to treat cancer in rural Russia (4).

With the advent of molecular biology, a large number of edible mushrooms have been the subject of scientific research to clarify their medicinal value, among them the *Basidiomycota* mushrooms *Agaricus blazei* Murrill*, Ganoderma lucidum, Grifola frondosa, Lentula etodes*, and others. A large number of biological actions, including immunomodulation, anticancer, antibacterial, antioxidant effects, and others, have been reported from these investigations. A significant part of these effects has been linked to the immunomodulating actions of different types of polysaccharides, in particular, β-glucans, which are constitutional part of the mushroom fruiting bodies (5). Besides, β-glucans have been found to have a cytotoxic effect on malignant cells through the induction of apoptosis and formation of reactive oxygen species (ROS)(6-11). In addition to polysaccharides, a large number of bioactive substances, showing notably tumoricidal, antimicrobial and anti-inflammatory effects have been isolated from edible mushrooms. Among such compounds are proteins, i.e., lectins, sterols- in particular, ergosterol, and phenols (12,13). Most of the research on biological effects of edible mushrooms has focused on *in vitro* investigations and animal models. A limited number of studies concerning clinical effects have been published, and only a few of them have shown benefits for mushroom extracts (13,14). Among these are studies showing increased survival or improved quality of life with the use of mushroom extract in addition to chemotherapy in cancer patients (15-17), and studies showing improvement in clinical symptoms and quality of life in patients with inflammatory bowel disease (IBD) (18,19).

In Norway, research on the biological effect of edible mushrooms was introduced in 2004 with an investigation of possible anti-infective effects of five commercial Japanese mushroom products, containing different *Agaricus blazei* Murill (AbM) extracts, in a mouse

model for pneumococcal sepsis. Only one of these products, which contains 82,4% of AbM and also the other *Basidiomycota* mushrooms, *Hericium erinaceus* (*14.7%*) and *Grifola Frondosa* (*2,9%*), was shown to have a significant anti-infective effect in this model (20). This study became the starting point for further investigations on this mushroom product, which is imported to Norway as a food supplement. In recent years this product has been marketed under the name of Andosan™. These studies were primarily initiated by scientific interest, but may also be regarded as pilot studies in an attempt to explore if Andosan™ might have potential as a medicinal product.

Subsequent studies on Andosan™ have confirmed the antibacterial effect of this product in mouse models (21,22). Also, strong pro-inflammatory effects have been found on cells of the innate immune system *in vitro* (23,24). In contrast, a predominantly anti-inflammatory effect was noted *in vivo* when Andosan™ was ingested by healthy volunteers (25) and by patients with inflammatory bowel disease (IBD) (26,27).

Norwegian legislation for food supplements.

The registration and marketing of food supplements are regulated by The National Regulation on Food Supplements (Forskrift om kosttilskudd), issued by The Ministry of Health and Care Services on 25.05.2004. Food supplements are distinguished from medicinal products by criteria stated in The Norwegian Medicinal Products Regulation (Legemiddelforskriften). In short, food supplements are defined as nutritional products, which are primarily aimed at supporting the body's physiological processes, while medicinal products are defined as "substances produced with the aim of preventing or treat diseases, easing symptoms or pain, or influencing physiological functions". In general, food supplement may not claim an ability to prevent or treat diseases. However, it is recognized that food supplements may claim biological effects in general. Food supplements may be the subject of scientific research, including clinical studies, to clarify if they have medicinal effects and thus have the potential to be classified as medicinal products. The decision whether to classify a given product as a food supplement or a medicinal product is taken by The Norwegian Medicines Agency based on of the contents of the product and

documentation of the effects claimed by the manufacturer (www.legegmiddelverket.no/Documents/Godkjenning/klassifisering/198029-syseutredningen.pdf).

Aims

The primary aims of the thesis are

a: to further investigate the immunomodulating effects of Andosan™ *in vitro* by studying the ability of Andosan™ to stimulate the expression of cell surface markers on monocyte-derived dendritic cells (MDDC) (Paper 1), and *in vivo*, by giving this product in addition to high dose chemotherapy with autologous stem cell support (ASCT) to patients with multiple myeloma (MM) in a clinical, randomized study (Paper 2). These studies were both performed at Oslo University Hospital (OUH).

b: to study the cytotoxic effect of Andosan™ on myeloma cell lines and leukemia cell lines *in vitro* (Paper 3). These studies were performed at the Department of Immunology and Transfusion Medicine, Oslo University Hospital, The Laboratory of Hematology, University of Liege, Belgium and at the Institute of Cancer Research and Molecular Medicine, NTNU, Trondheim (studies on patient myeloma cells from The Norwegian Myeloma Biobank).

c: to investigate the preventive effect of Andosan™ against the development of intestinal tumors in a *preclinical model*, i.e., in A/J Min/+ mouse (Paper 4). This animal study was performed at Norwegian Institute of Public Health, Oslo and the Norwegian University of Life Sciences, Oslo.

Secondary project- aim: to investigate the survival of patients receiving ASCT for MM at Oslo University Hospital in a nine year period, starting in 2001.

Background: The clinical study on MM patients (Paper 2), showed a markedly increased survival in patients receiving ASCT compared to survival in MM patients reported from two earlier Nordic studies conducted in the 1990-ies.

It was therefore decided to investigate the survival of MM patients receiving ASCT in the period 2001-2010 in OUH, to clarify if a similar improvement of survival could be detected in this patient group in general. (Paper 5).

Methodology

Paper 1: This is an *in vitro* study, where the expression of cell surface markers and translocation of NF-κB from cytosol to nucleus after stimulating MDDC or THP-1 cells, respectively, with Andosan™ , was measured by flow cytometry and Western blot, respectively.

Paper 2: This clinical, randomized, double-blind study, including 40 consecutive patients, was aimed at showing differences in laboratory parameters, e.g., serum levels of cytokines, gene expression in bone marrow cells, and quantity of different cell subgroups in the leukapheresis products. The sample size of the study was not calculated to show statistical differences in survival. Survival data are therefore reported only as a point of interest.

In a preliminary experiment, cytotoxicity analysis of Andosan™ against a mouse myeloma cell line was performed, using a ^{3}H- thymidine incorporation assay.

Paper 3: Cytotoxicity of Andosan™ in malignant human blood cell lines was investigated using the CellTiter-Glo Assay (myeloma cell lines), the Nucleo Counter assay (leukemia cell lines) and the ScanR automated image acquisition and analysis (primary myeloma cells).

Paper 4. This study was performed in A/J Min +/ mice, which have a genetic defect predisposing to the development of intestinal tumors. One group of animals received regular drinking water, while the other group received drinking water with the addition of Andosan™ 10%. After 28 days, the animals were then sacrificed, and the number of intestinal tumors was counted. The number of tumors in animals who received Andosan™ was compared to the number of tumors in animals, which had received only regular drinking water.

Paper 5. This retrospective investigation of patient survival was based on a search in the patient registry at OUH to identify patients discharged with a diagnosis of multiple myeloma (C90.0 in the ICD 10 diagnostic system) in the period from 01.01.2001 to 31.12.2009. Subsequently, the patient records of all MM patients who were assessed for ASCT were reviewed, and data concerning survival and relapse treatment were noted. On the basis of these data, survival rate calculations were performed.

Fungi/ Mushrooms in general

Fungi were defined initially by the Linnea system as lower plants, but are currently classified in a separate fungal kingdom, the kingdom *Mycetea*. Mushrooms are a subgroup of fungi, which has been defined as ".. macrofungi with a distinct fruiting body which can either be epigeous (located above ground) or hypogeous (located underground), and which are large enough to be seen by the naked eye and to be picked by hand"(28). Fungi lack the ability to manufacture their food from inorganic material and must, therefore derive nutrition from dead or living organic material from plants or animals. The number of mushroom species in the world is estimated to be about 140.000. However, only about 10% are currently identified. Approximately 3000 mushroom species are regularly consumed for their nutritional and culinary value (29). Higher mushrooms are divided into two Divisions: *Basidiomycota* (example: A*garicus* species), and *Ascomycota* (examples: *Morchella* (morels) species and *Tuber* (truffles) species). Most *Basidiomycota* mushrooms have an umbrella-shaped fruiting body, which is situated over the ground and is composed of a stem and a cap (pileus). Spores are produced in lamellae, located on the underside of the cap (29). The vegetative part of mushrooms is called mycelia, which grows underground by creating a network of filaments (hyphae). The fruiting body, which is the reproductive phase, subsequently develops from the mycelia.

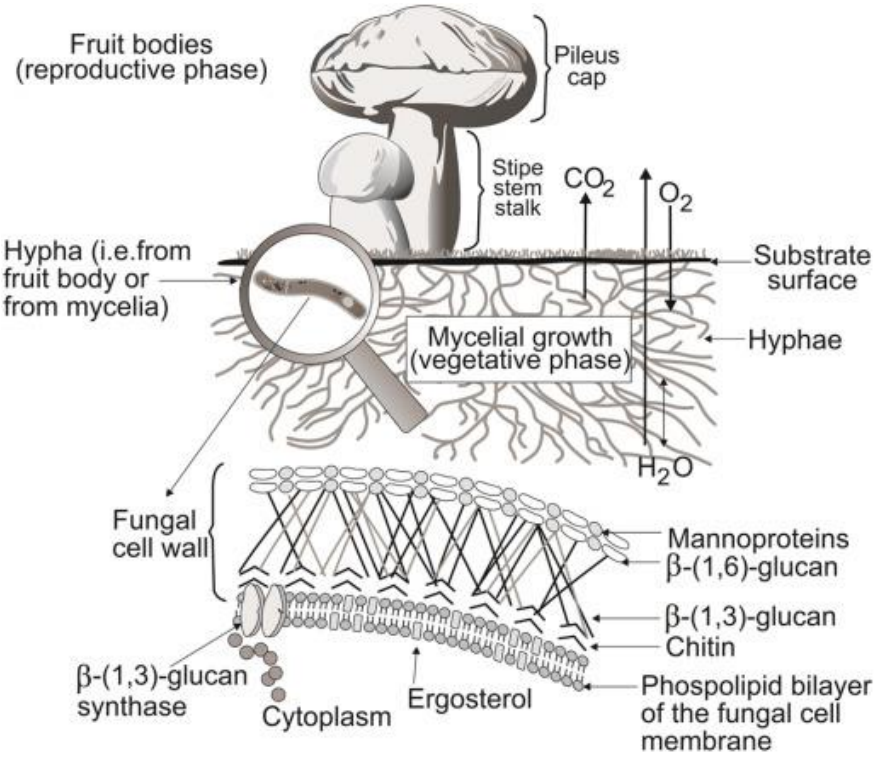


Figure 1. **Upper part:** Schematic drawing of a mushroom with the fruiting body (reproductive phase) overground and the mycelia (vegetative phase) underground.

Lower part: The main components of the fungal cellular wall.

From: Sanchez C. Reactive oxygen species and antioxidant properties from mushrooms. Synthetic and Systems Biotechnology 2017;2:13-22

The structure of the fruiting body cell wall may be regarded as analogous to reinforced concrete, where 35% consists of an “armature” of fibrils of β-glucans embedded in an amorphous mass of "concrete," consisting of β-glucans, mannoproteins, and chitin (a polysaccharide) (Figure 1). Mushrooms are therefore rich in carbohydrates (from 46 to 81% of dry weight). Furthermore, they contain large quantities of proteins, essential amino acids, vitamins (especially vitamin B_1, vitamin B_2, and vitamin C), fibers and minerals. Edible mushrooms have therefore from ancient times been consumed for their nutritional value (29). In modern times, lower fungi have been the source of drugs, which have had a major impact in medicine, i.e., penicillin, produced by *Penicillium chrysogenum* (30), ciclosporin (31), produced by *Tolypoladium inflatum*, and statins, which were originally developed from *Penicillium citrinum* (32). Edible mushrooms have also been the subject of extensive research in an attempt to identify compounds with medicinal effects. Such mushrooms

products would then combine nutritional and medicinal properties, so- called "*nutraceuticals"*. However, to date, no product from higher, edible mushrooms has been approved for medicinal use in Western medicine (33). Artificial cultivation of mushrooms for nutritional and medicinal purposes started around A.D. 600. An important advance in this field took place in the 17th century with the start of cultivation on composts (i.e., decaying organic substrates) (34). At present, about 60 mushroom species are regularly commercially cultivated, about ten on an industrial scale. The most cultivated and consumed mushroom in the world is *Agaricus bisporus* (often referred to as simply "champignon"), because of its nutritional and culinary values (35)(Figure 2).

Figure 2. *Agaricus bisporus*

Artificial cultivation of mushrooms is time and labor consuming, requiring close control of the composition of the compost and of climate conditions, which all must be continuously adapted to the need of the different phases of the development (34). Cultivation in submerged cultures, where the mycelia are grown in a liquid medium, is often preferred for the production of mushroom juices, mixtures with "mushroom flavor" for culinary use and cosmetic products. This method is easier to control and standardize, more economical and more readily adaptable to production on an industrial scale (35). China is the world's largest producer of cultivated mushrooms, with an estimated production in 2000 of 6.6 million tons. In the same year, the value of the sale of mushroom products in the US was estimated at 14 billion dollars (35).

Biological effects of selected mushrooms used in traditional eastern medicine

Lentinus edodes (in Chinese: *Shittake*) is growing in the wild in Eastern Asia, where it is also has been cultivated artificially from ancient times. This mushroom is widely consumed as a food product and has also traditionally been used in traditional herbal medicine for a variety of conditions, including heart and lung diseases and cancer. In modern times research on *Lentinus edodes* has focused on a β- glucan with a β- (1-3) glucose backbone and β- (1-6) side branches (similar to AbM), called lentinan, which has been shown to have strong immunostimulatory and tumoricidal effects *in vitro* and in preclinical models (36,37). Several clinical trials, conducted in China and Japan, have reported effect of lentinan as adjuvant treatment to chemotherapy in advanced cancer, in particular in gastrointestinal cancer and lung cancer (16,38,39).

Ganoderma lucidum (in Chinese: Lishi, in Japanese: Reshi) has since ancient times been used in herbal medicine, where it is believed to have effect against a number of diseases, in particular, against diseases of the chest, and to give «life-energy» in general. In recent years, two biologically active compounds have been isolated from *Ganoderma lucidum*, β-glucans, and triterpenoids. The latter is a precursor to steroids and is composed of three terpene units. In particular, research has focused on group of derivates from triterpenoids, called ganoderic acids, which have been shown to have immunomodulating and cytotoxic effect *in vitro* and preclinical models (40). However, no certain clinical effects have been documented (41).

Grifola frondosa (in Japanese: Maitake) grows on trees in America and the Far East. It is widely used in Japan, China and Korea as a food additive because of its culinary qualities. The fruiting body *of Grifola frondosa* is rich in β- glucans. Research has particularly focused on a proteoglycan from this mushroom, called D-fraction, which, is composed by protein-bound β-1,6 glucans and β-1,3 glucans. D- fraction has been shown to have immunomodulatory effects, cytotoxic, and antioxidant effects *in vitro* and preclinical studies, but clinical effects have not been documented (42, 43).

Hericium erinaceus (Lion's mane) grows on hardwood trees in America, Europe, and Asia. Extracts from this mushroom have been found to have antibacterial and anticancer effect in *in vitro,* which has been attributed to β- glucans. *Hericium erinaceus* also contains the cyanthane derivates hericenons and erinacines (44). These compounds have been found to have a function as nerve growth factors. They also have a protective effect against oxidative stress on nerve cells *in vitro*. Hericenons and erinacines may therefore have a potential in the treatment of neuro-degenerative diseases, such as Alzheimer's disease (45).

Agaricus blazei Murill

Agaricus blazei Murill *(*AbM) is an edible mushroom of the *Basidiomycota* family, which grows naturally in the Piedade Area, near São Paulo, Brazil. It has traditionally been used as a food ingredient, and also as a remedy against a wide range of diseases, in particular against infection and cancer. The mushroom was first described in 1967 by the Belgian botanist Heineman, who named it after the American mycologist William Murill. In Brazil, AbM is known under the names of *Cogumelo Piedade* (The mushroom from Piedade) and *Cogumelo do Sol* (The mushroom from the Sun), and others (46). *Agaricus* species are saprophytic mushrooms, which grow both in America, Europe and Asia (46). Genetic studies have shown that AbM is closely related- and possibly identical- to the North American mushroom *Agaricus subrufescence* (47). Formally, AbM is classified as belonging to the kingdom Fungi, phylum *Basidiomycota*, subdivision *Agaricomycotina*, subclass *Agaricomycetidae*, order *Agaricales* and family *Agaricaceae* (Index Fungorum). AbM is characterized by a short stem and a brownish-gold, fleshy, convex cap with a diameter between 7 and 12 cm (Figure 3).

Figure 3. The mushroom *Agaricus blazei* Murill.

In 1965 spores from AbM were brought to Japan, where artificial cultivation was undertaken (48). AbM is cultivated in cases with composts, containing organic material, such as rice, wheat, and straw, in a temperature around 30° C (48). Since the 1990-ies a large number of reports have been published regarding different biologic actions of AbM, in particular immunomodulating, cytotoxic, anti-diabetic and anti-cholesterol effects, in *in vitro* studies and preclinical models (49-52). However, a major difficulty in evaluating these reports is the fact that the part of the mushroom involved (mycelia or fruiting body), as well as the type of extraction procedures from the raw mushroom used, vary greatly between the different investigations. In general, the biological effects of AbM have mainly attributed to the immunomodulating action of polysaccharides, in particular, β-glucans (53,54).

β-*glucans.*

β-glucans are naturally occurring polysaccharides, which are present in plants, bacterias, and fungi. They consist of chains of D-glucose linked by β-type glycosidic bonds, forming a backbone, often with branching side chains. β- glucans differ significantly with regard to length and branching structure. Their molecular weight varies from a few kilodaltons (kDa) to several thousand kDa. In aqueous solutions, β- glucans change from a linear conformation to triple helix or coil conformations. Their biologic effects vary with their solubility, structure, molecular weight and tertiary conformation (55,56). It has been suggested that the immunomodulatory and cytotoxic effects of β- glucans is related to the degree of complexity of its structure (46). The β- glucans predominating in fungi have been found to have a 1-3-β backbone with 1-6-β-side-branches (46)(Figure 4).

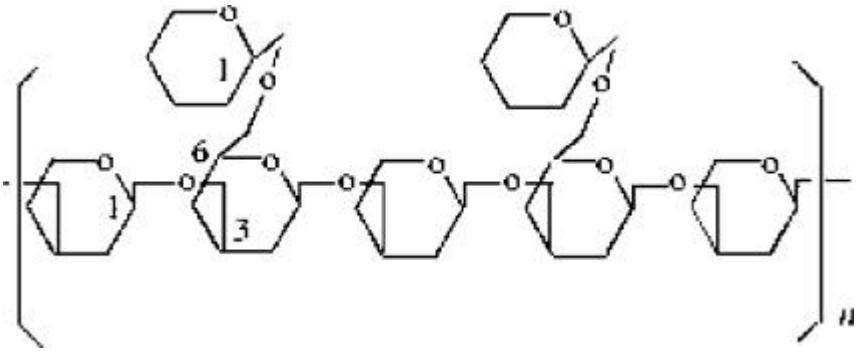


Figure 4. β-glucan from AbM. (1-3)-β backbone structure of carbohydrate molecules with (1-6)-β side branches. From Firenzuoli F, Gori Lombardo G. The medicinal mushroom *Agaricus blazei* Murill: Review of literature and pharmaco-toxicological problems. eCAM 2008;5(1):3-15. (ref. 46).

β- glucans have a strong immunomodulating effect, primarily through activation of the innate immune system (57). A number of publications have also reported a direct cytotoxic effect of β-glucans (7,36,43), but the concept of a direct cytotoxic effect of β-glucans on malignant cell lines has been challenged by other authors (56).

β glucans are indigestible carbohydrates, and it has traditionally been considered that they must be fermented by the intestinal flora before uptake from the intestine can take place (58). However, animal studies have shown that β-glucans are also captured directly by macrophages in the intestine, where they are fragmented into smaller sized β- glucans and subsequently released into the circulation and later taken up by circulating monocytes, granulocytes and dendritic cells (DC), thus eliciting an immune response (59).

Just a few investigations have been published regarding the structure of the mycelium of AbM. Extracts from the mycelium have been found to contain different types of polysaccharides, and one of them was estimated to be a small β- glucan with a molecular weight of only 2.1kDa (60-61).

Antitumor effects of AbM extracts.

In 1994, the first report regarding biologic actions of AbM was published, showing that a water-soluble fraction of the fruiting body of the mushroom, which contained a protein- β-glucan complex, had tumoricidal effect when injected subcutaneously adjacent to a human sarcoma cell tumor, xenografted on mice (62). It was shown that this effect was associated

both with stimulation of the immune system, especially stimulation of macrophages and NK cells, and with apoptosis of tumor cells (63). The cytotoxic effect of two different polysaccharides, extracted from the mycelium of AbM, i.e., a glucomannan and a polysaccharide-protein complex named Antitumor Organic Substance Mie (ATOM), was also demonstrated in this model (64). In a publication by Kobayashi et al. (65), it was shown that a β-(1-6)-glucan extracted from the fruiting body of AbM had a cytotoxic effect on human ovarian cancer cells in a mouse model. Li et al. reported the apoptotic effect of a polysaccharide from the fruiting body of AbM via the caspase 3- dependent pathway in human leukemia cells (7). Furthermore, Murakawa et al. demonstrated the effect of β-(1-6) glucans from AbM in combination with marine phospholipids on myeloma cells in a mouse model (66).

In addition to β-glucan, which is an integral part of the mushroom, AbM contains a number of metabolic substances, which exhibit cytotoxic effects. Among them are the steroid blazein, the ergosterol derivate agarol and the fenolhydrazine-containing compound agaritine (67- 71).

In a clinical trial, one hundred patients receiving chemotherapy for gynecological cancer were randomized to receive either adjuvant treatment with the commercial mushroom extract Agaricus blazei Murill Kyowa (ABMK) from the fruiting body of AbM, or no adjuvant treatment. The NK cell activity was found to be significantly higher and quality of life significantly better in the ABMK group (17).

Genotoxicity. Different fractions of AbM extracts have been investigated for genotoxicity. In a report by Bellini et al. (72), methanolic- (ME), hexanic- (HE), n-butanolic- (BE) and aqueous extracts (AE) of AbM were all shown to have an antimutagenic effect on the mammalian cell line CHO-K1 exposed to the DNA damage-inducing agent ethyl methone sulphonate (EMS). In the mammalian drug- metabolizing cell line HTC, the BE extract was found to be genotoxic, while the other AbM extracts exerted antimutagenic effects (73). Thus, AbM was found to have a predominantly antigenotoxic effect, although a mutagenic effect was reported in one case.

S*afety:* Three cases of severe hepatic dysfunction have been reported in cancer patients using an oral AbM extract as adjuvant treatment (74). This is the only known report of a putative toxic effect of AbM. However, other causes of hepatic dysfunction could not be ruled out in these cancer patients. AbM has been found to inhibit cytochrome P-450 and the trans-membrane-efflux pump P-glycoprotein (P-gp) in a mild degree (similar to green tea). These two factors play an important role in drug metabolism and may thus interact with a number of drugs. Therefore, although clinically relevant interactions with cytochrome p-450 are considered unlikely (75), AbM should as a precaution not been used together with drugs that are P-gp substrates, such as vinblastine, vincristine, digitoxin, cyclosporine, loperamide, verapamil and quinidine (76).

Andosan™

The commercial mushroom extract Andosan™, which has been used in investigations included in this thesis, was produced and heat sterilized by ACE Co Ltd, Gifu-ken, Japan, and GMP certified by Meiji Co. This product contains fermented extracts from the mycelia of *Agaricus blazei* Murill (82,4%), *Hericeum erinaceus* (He) (44,45) (14.7%) and *Grifola frondosa* (Gf) (2.9%)(42,43), which are all grown in dry cultures (commercial information). Andosan™ is imported to Norway as a food supplement (mushroom juice) by the company Immunopharma AS (organization no. 994924273), Oslo, Norway. The lipopolysachharide (LPS) content of Andosan™, using the Limulous amebocyte lysate test (COAMAIC Chromo LAL; chromogenix, Falmouth MA, USA), was found to be lower than the detection level (0,5 pg/ml) of this test. Any possible contamination of LPS in this product is therefore in any case minuscule and should not influence the biological effect of Andosan™ in *in vitro*, preclinical or clinical investigations. Repeated analyzes, last performed in February 2016, by The Japan Food Research Laboratories, Tokyo, have not detected heavy metals, such as zinc, selenium or germanium in Andosan™. In addition, tests for coliform bacteria, yeast, and aerobic spores were in normal range (77). Also, Andosan™ has been tested by an approved antidoping laboratory in Oxford, UK, and found to not contain any of the 130 substances listed by the World Anti Doping Agency (WADA) (78). The exact content of Andosan™ has not been communicated by the producer. However, an independent investigation showed that this product contains only 2.0 % of carbohydrates in dry weight, corresponding to 0.09

% β- glucan (79). It is therefore unlikely that the biologic effects documented from Andosan™ may be attributed to polysaccharides or, specifically, β- glucans, alone.

Safety: No subjective side effects or effects on hematologic parameters, electrolyte balance, liver, pancreatic and renal function were noted when Andosan™ was consumed by healthy volunteers or patients with IBD (25,18-19).

The immune system

The immune system is a collection of specialized cells and humoral factors, aiming at protecting an individual from both outside invaders and its own altered cells (i.e., cancer cells). The cells of the immune system are, together with platelets and red blood cells, derived from pluripotent hematopoietic stem cells in the bone marrow. The immune system is divided into two functional parts, the innate and the adaptive immune system (80). **The innate immune system** is the first line of host defense and is rapidly activated. It is based on epithelial barriers, secretion of antimicrobial proteins, various humoral systems and the action of phagocytic cells, which ingest and kill invading pathogens such as bacteria, virus, and fungi. Macrophages are the most important phagocytic cells at homeostasis. They are found in large numbers in the mucosal layer of the gastrointestinal tract, in the alveoli of the lungs, around blood vessels in the liver and the spleen. During infection, macrophages can also develop from circulating monocytes. The second most important type of phagocytic cells is neutrophils. They are found only in the circulation at homeostasis but migrate to infected tissues as a part of the inflammatory response in order to engulf and destroy pathogens. A third class of phagocytic cells is dendritic cells (DC), which reside in lymphoid organs and lymphatic tissues. The main task of DC is to act as antigen presenting cells (APC), i.e., they process phagocytic material into peptides, which may subsequently, in combination with molecules of the Major Histocompatibility Complex, be presented as antigens and activate cells of the adaptive immune system. Also, various humoral systems, in particular, the complement system and the interferons, are part of the innate immune system. Activated complement binds to invading organisms and facilitate their phagocytosis by immune cells, including APC, while the main function of interferons is stimulation of the early immune response against virus infections (81). Activation of lymphocytes (B and T

cells) of the adaptive immune system leads to a more specific immune response, which also confers to the immune system the function of memory for a particular pathogen and the distinction between self and non-self antigens (82). The cross-talk of different cellular elements of the immune system is mediated by signal molecules, called cytokines, which are divided into interleukins, chemokines and growth factors.

Activation of the innate immune system results in an inflammatory process, mediated by pro-inflammatory cytokines and characterized by increased production of phagocyting cells and attraction of immune cells to areas of inflammation by chemokines (83). Furthermore, activated phagocytic cells excrete proteolytic substances, like nitric oxide and hydrogen peroxide, which directly kill invading microbes (84). Clinical signs of inflammation are increased temperature, edema (swelling), redness and pain. The activation of the innate immune system is based on the presence of ancient germline-encoded receptors, i.e., Pathogen Recognizing Receptors (PRR), located on the surface of immune cells, in particular on DC and macrophages. PRRs are divided into two types: Membrane receptors (phagocytic receptors and signaling receptors) and cytoplasmatic receptors. PRR recognize specific molecular structures, called Pathogen Associated Molecular Patterns (PAMP), which are parts of the surface of invading pathogens, such as bacteria and fungi. Also, structures on non- pathogenic agents, such as polysaccharides from edible mushrooms, exhibit PAMPs and may initiate an immune response (85). Besides, some PRR react with self-molecules, which are produced as a result of physical or microbial cell damage, so-called Damage Associated Molecular Patterns (DAMP). One of the most important groups of PRR is the Toll-like receptors (TLRs) (86). In humans, 13 Toll-like receptors, have been discovered, which all play a role in the defense against microbial infections.

The TLRs, which interact with β-glucans, are mainly TLR-2/ TLR-6 and TLR-4 (87,88). In addition, the receptor dectin-1 (**de**ndritic-cell-associated **C**-type lec**tin-1**) (89), the lectin binding site for β-glucan of complement receptor C3 (CR3) (CD11b/CD18) (90), the LacCer (lactosylceramide-glycosphingolipid) receptor (91) and the scavengers receptors (92), have all been shown to be activated by β-glucans. Furthermore, the cytosolic receptor NOD (nucleotide- binding and oligomerization domain) also play a role as PRR for β-glucans (93). The binding of an invading pathogen to a PRR results in triggering of a cascade of intracellular signaling proteins. Several pathways of signal transduction may be triggered by

β-glucans, but most important are pathways leading to activation and translocation of the transcription factor Nuclear Factor Kappa B (NF-κB) from the cytosol to the nucleus, resulting in production of cytokines.

The binding of β-glucan to the TLR4 and or to the TLR-2/6 receptors triggers the NF-κB pathway through recruitment of the adaptor molecule MYD88 (Myeloid Differentiation factor 88) and the signal molecule TRAF-6 (TNF Receptor Associated Factor-6). The binding of β-glucan to the Dectin-1 receptor, the SIGNR1 (Specific Intracellular adhesion molecules 3 Grabbing N Related-1) receptor, and the C3 receptor, activates the syk (spleen tyrosine kinase) pathway, the signal molecules card 9 (caspase recruitment domain-containing protein 9) and NFAT (Nuclear Factor of Activated T-cells). Also, the binding of β-glucan to the CR3 receptor may activate the complement system. The binding of β-glucan to the LacCer receptor activates the MIP2 (Myocardial Ischemic Preconditioning upregulated protein 2) pathway, which in turn activates the signaling molecule PKC (Protein Kinase C). Binding of β-glucan to Scavenger receptor leads to activation of the MAPK (Mitogen-Activated Protein Kinase) pathway by recruitment of the signal molecules PI3K (Phosphatidyl Inositol 3 Kinase) and akt (ak strain transforming factor) (56) (Figure 5).

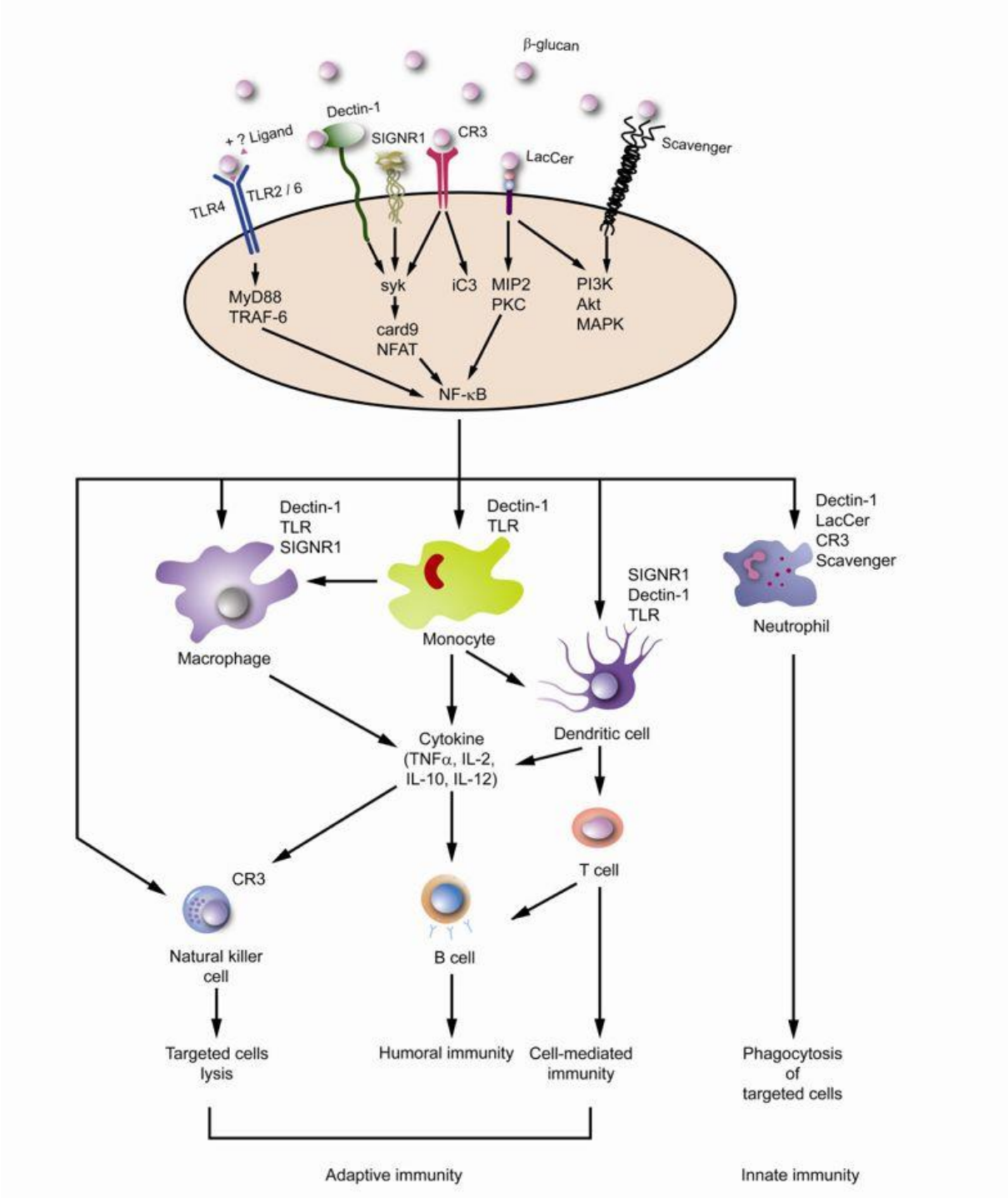


Figure 5. Immune activation induced by β-glucans can act on a variety of membrane receptors found on different phagocyting cells. β-glucans may act singly or in combination with other ligands. Various signaling pathways lead to activation of Nuclear factor kappa B (NF-κB). Their respective downstream signaling molecules are shown. Abbreviations: TLR – Toll-like receptors. Dectin- 1 - **de**ndritic-cell associated **C**-type lec**tin-1.** SIGNR1- Specific

Intercellular adhesion molecules 3 Grabbing Related-1. CR3- complement receptor 3. LacCer- Lactosylceramide. MyD88 – Myeloid Differentiation factor 88. TRAF-6- TNF Receptor Associated Factor 6. Syk- spleen tyrosine kinase. Card 9- caspase recruitment domain-containing protein 9. NFAT-Nuclear Factor of activated T- cells. MIP2- Myocardial Ischemic Preconditioning upregulated protein 2. PKC- Protein Kinase C. PI3K- Phosphatidyl Inositol 3 Kinase. Akt- Ak strain transforming. MAPK (Mitogen-Activated Protein Kinase).

From: Chan GC-F, Chan WK, Sze D M-Y. The effects of β-glucan on human immune and cancer cells. Journal of Hematology & Oncology 2009,2:25 doi: 10.1186/1756-8722-2-25 (ref. 56).

NK (Natural Killer) cells is a particular subgroup of lymphocytes, which belongs to the innate immune system. These cells have distinctive cytoplasmic granules, containing cytotoxic proteins. Morphologically, they are therefore designated as " large granular lymphocytes." NK cells are triggered by germline-encoded receptors that recognize molecules on the surface of microbes or malignantly transformed cells. NK cells do not have a phagocyting or antigen presenting function but are capable of killing various target cells without prior activation. They play an important role in the first line of defense against malignant cells, virus-infected cells, bacteria, and protozoa. NK cells express Fc receptors. Binding of antibodies to these receptor triggers the release of cytotoxic granules. This process is known as antibody-dependent cellular cytotoxicity (ADCC). NK cells may also act together with DC and thereby play a role in the regulation of the adaptive immune response (94).

Cytokines primarily secreted by cells of the innate immune system

Cytokines are a group of small protein molecules (about 25 kDa), mediating signaling between cells of the immune system. Cytokines act by binding to a receptor on a responsive target cell. The action may occur in an *autocrine* way (reaction with the same cell), *paracrine* way (reaction with a nearby cell) or in an *endocrine* way (reaction with a distant cell). Cytokines may interact in a *pleiotropic* manner (different effect on different target cells), a *redundant* manner (various cytokines have the same effect) or an *antagonistic* manner

(inhibition of the effect of another cytokine(s)). Most cytokines are secreted transiently, for instance in connection with exposure to a pathogen (95).

IL-1β(Interleukin-1β) is a pro-inflammatory cytokine, which is produced primarily by macrophages and neutrophils, often in synergy with TNF-α (96). The main stimulants of IL-1β production are microbial products, such as LPS. Furthermore, activated T helper cells also produce IL-1β (94). The most important effects of IL-1β are CD4+ T cell proliferation, inducing the release of IL-2, and induction of the synthesis of the IL-6, IL-8 and TNF-α by monocytes, macrophages, and neutrophils. In low concentrations, IL-1β promotes the expression of adhesion molecules, such as intrinsic cell intercellular adhesion molecule (ICAM-1), on endothelial cells, which facilitates the adherence and subsequent transmigration of neutrophils, monocytes, and lymphocytes. In high concentrations IL-1β also becomes a systemic inflammatory activator, affecting the function of the hypothalamus, leading to a rise in body temperature. Furthermore, IL-1β also causes a series of clinical symptoms characteristic of inflammation, such as hyperalgesia, vasodilatation, bradycardia and hypotension (97).

IL-1ra (IL-1 receptor antagonist) is a naturally occurring circulating competitor for IL-1β, primarily produced by mononuclear phagocytes in response to bacterial or viral products. The main stimulants of production of IL-1ra are LPS, IL-4, and GM-CSF. One of the main roles of IL-1ra is to modulate IL-1 activity. Thus, it exerts mainly an anti-inflammatory activity (97).

TNF-α (Tumor necrosis factor-α), like IL-1β, is mainly produced by macrophages and neutrophils, but also to some extent by T-cells, B- cells and NK cells. TNF-α acts in synergy with IL-1β. TNF-α induces the production of the pro-inflammatory cytokines IL-1 and IL-6 and stimulates antimicrobial activities of monocytes/ macrophages. Furthermore, TNF-α promotes the expression of adhesion molecules on endothelial cells, stimulates the production of acute phase proteins by the liver and causes clinical symptoms of inflammation. Furthermore, TNF-α activates apoptosis (cell death). TNF-α has also a pro-coagulant effect, and a hematopoietic effect by mediating the production of granulocyte-colony stimulating factor (G-CSF), leading to increased production of neutrophil granulocytes. Together with IL-1 TNF-α is involved in bone remodeling by depressing osteoblast activity and stimulating osteoclast activity (96,97).

IL-6 is a pleiotropic cytokine with both pro-inflammatory and anti-inflammatory actions. IL-6 is produced mainly by activated monocytes, macrophages and endothelial cells in response to IL-1 and TNF-α. The functions of IL-6 include synthesis of acute phase proteins in the liver and mucosal production of IgA. IL-6 also stimulates end-stage differentiation of B-cells. Furthermore, IL-6 exerts an anti-inflammatory effect by inhibition of TNF-α and IL-1 activity, and also by stimulation of the anti-inflammatory cytokines IL-10 and IL-1ra (96,97).

IL-7 is produced mostly by the stromal cells in the bone marrow and thymus, as well as B-cells, monocytes, and macrophages. IL-7 is critical for γδT cell development and important for B-cell development (97).

IL-1β, TNF-α, and IL-6 all act on hepatocytes, changing the profile of proteins secreted by the liver, so-called acute phase proteins. This reaction is called an **acute phase response** (96,97).

IL-10 is a pleiotropic cytokine, which is primarily secreted by activated monocytes and macrophages. The major role of IL-10 is to reduce inflammatory responses by inhibiting the NF-κB activated transcription of genes coding for the pro-inflammatory cytokines TNF-α, IL-1, IL-6, IL-8, and IL-12. In this way, IL-10 has a protective function against the massive cytokine release connected with endotoxic shock. IL-10 also inhibits the secretion of IFN-γ and IL-2 by Th1 cells. On the other hand, IL-12 exerts an immunostimulatory effect by supporting the proliferation and cytotoxic activity of CD8+ T cells and by stimulating NK cells (96,97).

IL- 12 is mostly secreted by activated macrophages and monocytes, dendritic cells (DC), neutrophils, and B-cells. IL-12 plays an important role by stimulating macrophages and DC to microbial phagocytosis. IL-12 is the major stimulator for INF-γ production by NK cells. Furthermore, IL-12 promotes the differentiation of Th0 cells into Th1 cells and stimulates Th1 cells to produce IFN-γ. Th1- cell response promoted by IL-12 stimulates the production of the immunoglobulin isotypes Ig2a, Ig2b, and Ig3, but not Th2 associated Ig1 and IgE (96,97).

G-CSF (Granulocyte- colony-stimulating factor) is a growth factor produced mainly by mononuclear phagocytes, activated T- cells, fibroblasts, and endothelial cells. Its function is to stimulate hematopoietic precursor cells into production of neutrophils (97).

CXCL8 (IL-8), MIP (Macrophage inflammatory Protein) -1β and MCP (Macrophage Chemoattractant Protein) -1 are chemokines, which are produced by monocytes, macrophages, and epithelial cells. Their main function is to attract neutrophils and T cells to a site of infection (96,97).

Interferons (IFN) are antiviral proteins, which are mainly secreted by plasmocytic dendritic cells (pDC), but may also be produced by activated T- cells, cytotoxic T- lymphocytes and NK cells. IFN-α and IFN-β induce resistance to virus replication, increase MHC class I expression and antigen presentation, activate DC and macrophages (96,97).

Besides triggering production of cytokines, the inflammatory response is also accompanied by the expression of cell-adhesion molecules, facilitating extravasation and recruitment of phagocytes to a site of infection. **Integrins** are immunoglobulin-like receptors, situated on leucocytes. The leucocyte integrins responsible for extravasation are **LFA (leucocyte functional antigen)-1** also designated as **CD 11a/CD18, C3 (complement receptor 3),** also designated as **CD 11b/CD18** and **CD 11c.** These receptors react with **intercellular adhesion molecules (ICAMs)** situated on endothelial cells (96,97).

Unlike innate immune responses, the responses of **the adaptive immune system** are highly specific to the particular pathogen that induced them. Whereas the receptors on the cells in the innate immune system are encoded in a predetermined form by a germline genome, receptors on cells in the adaptive immune system are encoded by gene elements that continuously rearrange to obtain an antigen binding structure adapted to each specific foreign element (98). Activation of the adaptive immune system, which takes place primarily in the secondary lymphatic organs, spleen and lymph nodes, is triggered by the encounter of a cutaneous or mucosal APC, migrating from peripheral sites of the body, with lymphocytes of the adaptive immune system. After activation, lymphocytes migrate to other parts of the body to exert specific effector functions (99).

The functions of the adaptive immune system are carried out by different subgroups of lymphocytes, i.e., T- lymphocytes, which mature in the thymus, and B- lymphocytes, which rise in the bone marrow. Both groups of lymphocytes ultimately migrate to the lymphatic organs, where they are activated. The diversity of B- cell receptors is assured by

recombinations of receptor genes, i.e., genes coding for the immunoglobulin light chain and heavy chain. Further diversification of the receptor takes place by *somatic hypermutation* under the influence of an antigen encountered in peripheral lymphatic organs. The maturation of T- cell receptors (TCR) takes place by receptor gene rearrangement in a way similar to the B- cell receptors, but somatic hypermutation does not take place. T-cells are classified according to the structure of the proteins chains constituting the TCR. In the great majority of T- cells TRC is constituted by α and β chains, but in a small minority, TCR is constituted by γ and δ chains (γδT- cells). T-cells are also classified according to surface markers, so-called Cluster of Differentiation (CD) and are divided into CD 4+ cells (T-helper cells) and CD 8+ cells (cytotoxic T-cells)(100).

The driving force in the engagement of the adaptive immune system is the presentation of an antigen by an APC together with an MHC-molecule, in combination with specific costimulatory molecules, to a T- cell receptor expressed on naïve T- helper cells (CD4+ cells)(Th0 cells), thus forming an immunologic synapsis (98) (Figure 6).

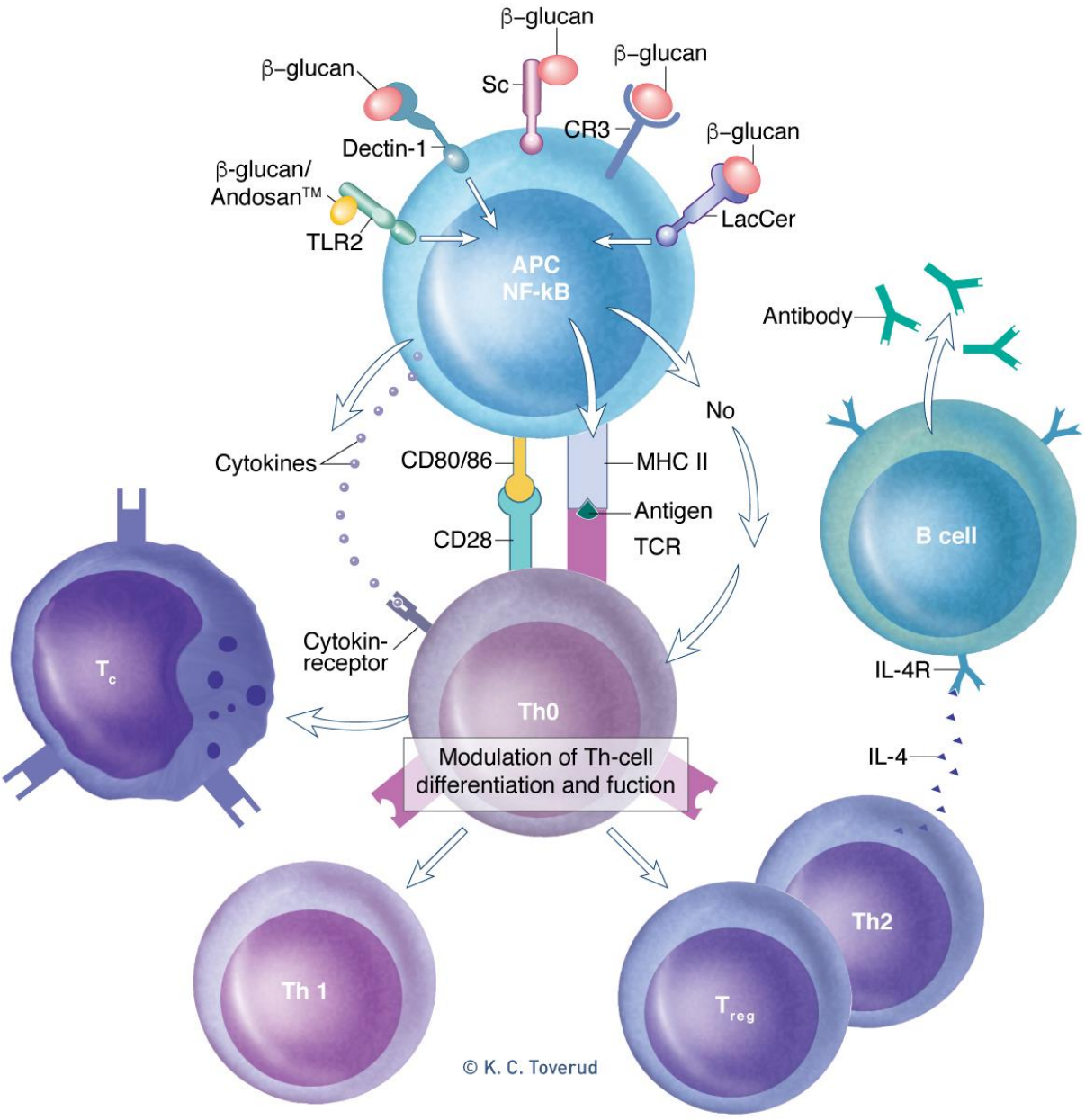


Figure 6. Activation of the innate and adaptive immune system by β-glucan/Andosan™ via antigen presentation by an APC (dendritic cells, monocytes, and macrophages), followed by activation of effector cells in the adaptive immune system (B-cells and cytotoxic T cells).

MCH II- Major Histocompatibility System type II, TCR- T-cell receptor, CD80/86 and CD28- surface receptors, IL-4- interleukin 4, IL-4R- interleukin 4 receptor, No- Nitric oxide. Tc- cytotoxic T cell, T_{reg} - regulatory T cells. Drawing: ©Kari C. Toverud CMI.

After the initial activation Th cells develop into specialized Th cells, i.e., Th1 cells, Th2 cells, Th17 cells and regulatory T cells (T_{reg}). Th1 cells, which mainly secrete IFN-γ and IL-12, develop from Th0 cells by the influence of IL-12 and by activation of the T- box transcription factor T-bet (T-**b**ox transcription factor **e**xpressed in **t-** cells). Th1 cells are primarily involved in activating cell-mediated responses against intracellular pathogens. The Th2 cells, which are producers of IL-4, IL-5, and IL-13, are driven by IL-4 and the transcription factor GATA-3 (named after the nucleotide sequence G(uanine), A(denosine), T(hymidine), A(denosine) in the target gene). Th2 cells are involved in hypersensitivity and parasite induced immune response. In accordance with the Th1/ Th2 dichotomy, there is an inverse relationship between Th1 and Th2 responses ("The Ying and Yang of Th cell differentiation")(101). Th17 cells are driven by TGF-β, IL-6, IL-21 and IL-22, which activate the transcription factor RORγt (retinoic acid-related orphan receptor γt). Th17 cells produce IL-17A, IL-17F, IL-22, and IL-23. Their main functions are control of extracellular pathogens and immunity, by activating macrophages, NK cells and cytotoxic T cells (CD8+ cells). T_{reg} cells develop by the influence of IL-21 and TGF-β (Transforming Growth Factor-β), activated by the transcription factor FoxP3 (Forkhead box P3). These cells play a role in immunosuppression and tolerance (Figure 7). There is a balance between the Th responses in such a way that Th1 inhibits Th2, which again inhibits the Th17and Treg response. "Over-shoot" of Th1 and Th17 responses in susceptible individuals may result in autoimmune disorders. Activation of Treg cells results in immunosuppression and tolerance (102).

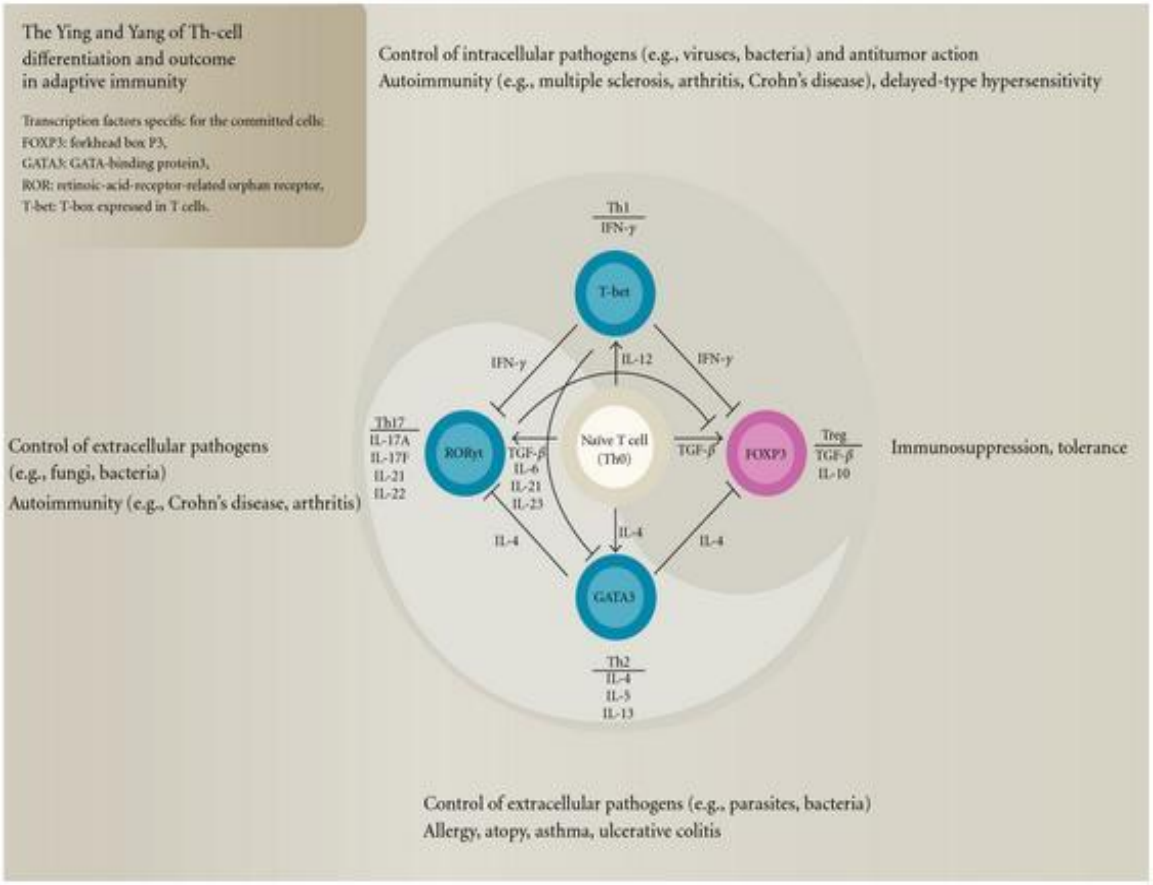


Figure 7. The development of subgroups of Th lymphocytes.

From: Hetland G, Johnson E, Lyberg T, Kvalhem G. The mushroom *Agaricus blazei* Murill elicits a medicinal effect on tumor, infection, allergy, and inflammation through its modulation of the innate immunity and amelioration of Th1/Th2 imbalance and inflammation. Advances in Pharmacological Sciences Volume 2011, Article ID 157015 (102).

CD8+ T cells (cytotoxic T lymphocytes), like CD4+ T helper cells, are generated in the thymus and express T- cell receptor. CD8+ cells can be activated through antigen recognition and by cytokines secreted by Th1 and Th17 cells (Figure 6). Their main function is to kill infected and malignant cells through secretion of cytokines, primarily TNF-α and IFN-γ, by the release of cytotoxic granules, and by activation of the caspase cascade, resulting in apoptosis of the target cells (103).

B-lymphocytes and their derivatives, plasma cells, are responsible for mediating the humoral immunity by the production of antigen-specific immunoglobulin (Ig), after stimulation by T helper cells, or by direct contact with an antigen (104).

Selected cytokines involved in the adaptive immune system

IL-2 is produced by activated Th1 cells and mainly stimulates the proliferation of CD4+ and CD8+ cells**.** Moreover, IL-2 functions as a chemoattractant for T cells, enhances monocyte responses and stimulates the proliferation of B-lymphocytes and NK cells. In synergy with IL-12, IL-2 stimulates the production of IFN-γ and TNF-α in NK cells. Furthermore, IL-2 induces transformation of NK cells to tumor killing LAK cells (96,97).

IL-4 is secreted mainly by Th2 CD4+ T cells. As IL-4R is widely expressed, IL-4 is a powerful pleiotropic cytokine. The main function of IL-4 is to stimulate the growth of Th2 cells. IL-4 inhibits the secretion of pro-inflammatory cytokines, such as TNF-α and IL-1β and blocks the IFN-γ induced expression of cellular adhesion molecules (96,97).

IL-5 is primarily produced by Th2 cells and plays a role in the activation of eosinophils and chemotaxis. Also, IL-5 stimulates histamine release from mast cells, IgA synthesis, and proliferation of cytotoxic T- lymphocytes. In particular, IL-5 is engaged in Th2 –mediated allergic and asthmatic responses, and also in cell-mediated immunity against parasitic infections (96,97).

IL-13 is secreted by activated T- cells, in particular by Th2 cells, basophils and mast cells. Its functions are similar to the functions of IL-4, i.e., it inhibits synthesis and secretion of pro-inflammatory cytokines. Moreover, IL-13 stimulates the proliferation of B-cells and promotes the isotype switching of immunoglobulins to IgE (96,97).

IL-17 is produced mainly by Th17 cells during memory CD4+ responses. It acts on stromal cells, such as keratinocytes, fibroblasts, and epithelial cells and stimulates them to produce IL-6, IL-8, and G-CSF. Besides, IL-17 stimulates CD34+ hematopoietic precursor cells to production of neutrophil granulocytes, thereby increasing the resistance to bacterial infections (96,97).

Apoptosis

Apoptosis is a form of programmed, energy-dependent cell death, which under physiological conditions occurs as a part of the cell turnover related to the normal development and functioning of the human body (105). It is also a defense mechanism which eliminates cells that have either undergone malignant transformation or are infected or damaged by toxic agents (106). Apoptosis is distinguished from necrosis, which is an uncontrolled, passive process due to cellular injury caused by external factors, like toxic substances or deprivation (107). Inappropriate function of apoptosis is related to a series of diseases. In particular, inhibition of apoptosis may lead to autoimmune diseases and cancer, while excessive apoptosis leads to neurodegenerative diseases like Parkinson's disease and Alzheimer's disease. Apoptosis is initiated by two main signaling pathways, the *extrinsic* and the *intrinsic pathways.* These two pathways are linked and may influence each other (108). In addition, a third pathway, the *perforin/ granzyme* pathway has been described. Each of these pathways results in the activation of *caspases,* i.e., proteins which in turn activate the destructive enzymes *proteases* and *nucleases*, which are situated in the cytosol and the nucleus, respectively (108).

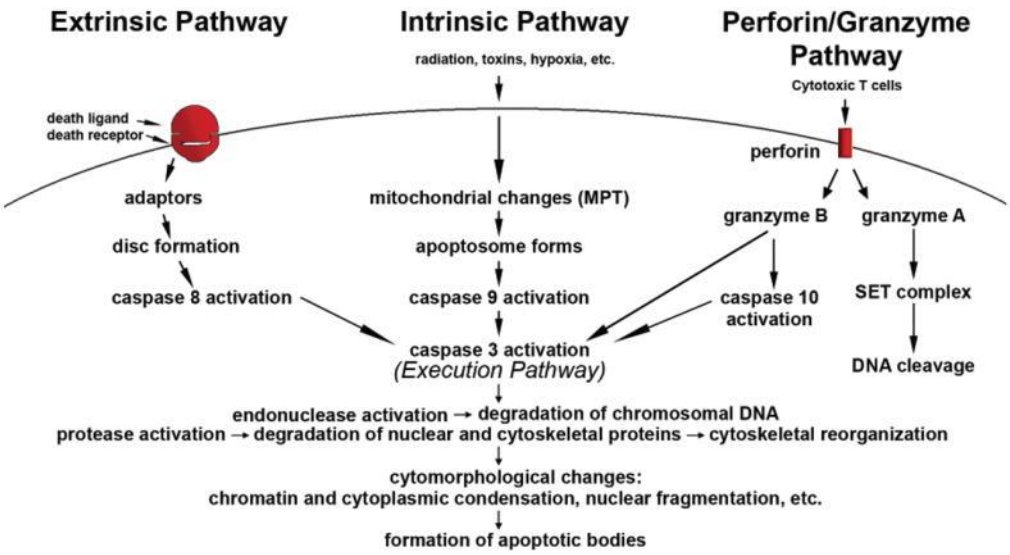


Figure 8. Schematic representation of apoptotic events. From Elmore S. Apoptosis: A review of programmed cell death. Toxicol Pathol 2007;35(4):495-516. (ref. 108). For description - see text.

The extrinsic pathway is activated when the ligands TNF-α (mainly produced by macrophages) and soluble FasL (Fas ligand), which is formed by cleavage of transmembrane FasL, found on cytotoxic T- lymphocytes, react with their corresponding receptors on the cell surface –so-called "death receptors". After recruitment of the adaptor molecule *Fas-associated death domain (FADD),* the *death-inducing signaling complex, DISC*, is formed. DISC then activates caspase 8. The intrinsic pathway is activated when stimuli, like radiation, toxins, hyperthermia, and viral infections create widening of the mitochondrial permeability transition (MPT) pores inside the cell. This causes the release of pro-apoptotic proteins, in particular *Cytochrome- C,* from the mitochondria into the cytosol. The reaction of cytochrome- C with Apaf-1 (Apoptotic protease activity factor-1) and procaspase-9 results in the formation of an *apoptosome*, which in turn activates caspase-9. The perforin/ granzyme pathway is activated by the secretion of the transmembrane pore-forming molecule *perforin* by cytotoxic T- lymphocytes, followed by the release of the serine proteases *granzyme B* and *granzyme A* through these pores and into the target cell. Granzyme B then activates caspase 10. In this way, the extrinsic, intrinsic and the perforin/ granzyme pathways activate caspase 8,9 and 10, respectively, which all, in turn, activate the executioner protein caspase 3. Besides, granzyme B may activate caspase 3 directly. Also, granzyme A may activate a caspase-independent apoptotic pathway. Activated caspase 3 triggers the execution pathway, leading to endonuclease activation, which causes degradation of chromosomal DNA, and to protease activation, which leads to degradation of nuclear and cytoskeletal proteins (108).

Morphologically, early signs of ongoing apoptosis are rounding of the cell, increased density of the cytoplasm and chromatin condensation. In the next stage, nuclear fragmentation and irregular buds on the cell membrane, called *buds*, will occur. In a more advanced stage, there will be a formation of apoptotic membrane protrusions containing nuclear fragments. According to their morphology, they are classified as *microtubule spikes, apoptopodia* (feet of death) and *beaded apoptopodia*, respectively (109).

The end stage of apoptosis is fragmentation of the cell into vesicles called apoptotic bodies, which will be ultimately removed by phagocytosis (Figure 9) (109).

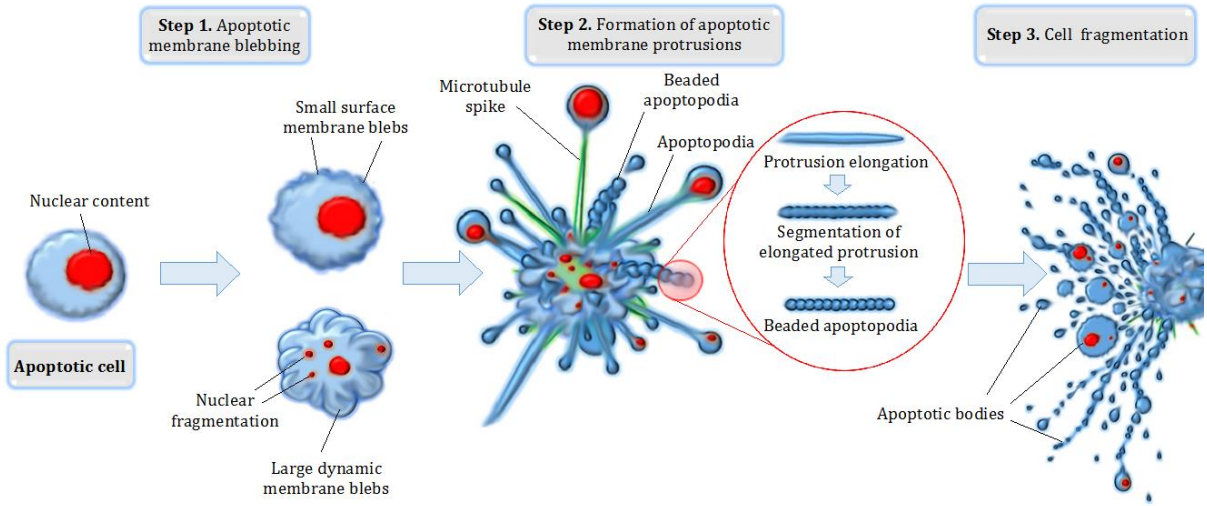


Figure 9. Different steps in apoptotic cell disassembly. From Tixeria R, Caruso S, Paone S, Baxter AA, Atkin-Smith GK, Hulett MD, Poon IK. Defining the morphologic features and products of cell disassembly during apoptosis. Apoptosis 2017 March;22(3):475-477 (ref. 109).

Inhibition of apoptosis by interference with one of the signaling apoptotic pathways is one of the main mechanisms behind the development of cancer (110). In particular, mutations of the *p53* tumor suppressor gene have been found to play an important role in carcinogenesis. In physiological conditions, the *p53* tumor suppressor gene regulates the cell cycle and activates DNA repair. Mutated *p53* increases the expression of the anti-apoptotic protein Bcl-2 and decreases the expression of the pro-apoptotic protein Bax (111).

Also, interference with the Fas ligand/Fas receptor reaction, which initiate the extrinsic pathways, is one other mechanism of dysregulation of apoptosis in cancer (112).

Previous studies on Andosan™

Antimicrobial effects

The antimicrobial effect of β-glucans has been demonstrated in both extracellular infection (e.g., *Streptococcus pneumoniae* (113) and *E. coli*) (114)) and in intracellular infections (e.g., *Mycobacterium tuberculosis* (115) and *Mycobacterium bovis BCG* (116). On this background, it was decided to investigate the possible antibacterial effect of Andosan™, given orally, in a mouse model. In a first experiment NIH/OlaHsd mice, inoculated with *S. pneumoniae* 6B intraperitoneally, were given 200µl PBS or PBS containing 10% Andosan™ via a gastric catheter. A lower degree of bacteremia and an increased survival rate were found in animals that received Andosan™. Furthermore, an increase in the serum levels of the pro-inflammatory cytokines MIP-2 and TNF-α were found in the animals in the treatment group. There was no effect of Andosan™ on the growth of these bacteria *in vitro.* It was concluded that the protective effect of Andosan™ was most probably due to activation of the immune response against this microbe, i.e., a pro-inflammatory effect (22). In a second experiment, BALB/c mice received 200µl PBS or PBS containing 10% Andosan™ by orogastric intubation 24 h prior to being inoculated intraperitoneally with a fecal solution containing coliform bacteria, enterococci, α-hemolytic streptococci, and non-hemolytic streptococci. It was shown that the level of bacteremia as lower and the survival was better in animals receiving prophylactic treatment with Andosan™ (21).

Immunomodulating effects

In vitro studies

In a study by Bernardshaw et al. (23) human monocytes and human umbilical vein endothelial cells (EC) were suspended in culture media with Andosan™, in concentrations from 1% to 15%, or with PBS (control). After incubation for 18-24 hrs, a dose-dependent increase of the levels the pro-inflammatory cytokines TNF-α (>4500-fold increase), IL-1β, IL-6, and IL-8, was observed for both cell types, reaching a plateau at 10% concentration of Andosan™. There was no increase in the levels of the anti-inflammatory cytokine IL-10 and Th1 cytokine IL-12.

In another experiment on human monocytes and granulocytes (117) it was found that stimulation with Andosan™ *in vitro* led to an increased expression of the surface receptor β-integrin (CD11b). Furthermore, there was a decreased expression of CD62L (L-selectin), which was interpreted as being due to shedding of the receptor from the cell surface. A similar reverse relationship between the expression of CD11b and CD62 L has earlier been described in leucocytes stimulated with meningococcal LPS. An increased production of reactive oxygen species (ROS) was found in granulocytes but not in monocytes.

In a third experiment, it was found that Andosan™ stimulation of monocyte-derived dendritic cells (MDDC) promoted a dose-dependent increase of the levels of IL-8, G-CSF, TNF-α, IL-1β, IL-6 and MIP1-β in the culture. In particular, it was noted that Andosan™ induced higher levels of G-CSF, TNF-α, and IL-1β than did stimulation with LPS (24).

Ex vivo experiment

Stimulation of whole blood with Andosan™ *ex vivo* led to a dose-dependent increase in the Th1 cytokines (IL-2, IFN-γ, IL-12), the Th2 cytokines (IL-4, IL-5, IL-13), the pleiotropic cytokines IL-7 and IL-17, the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, the anti-inflammatory cytokines/ chemokines IL-8, MIP-1β and MCP-1, and the leukocyte growth factors C-CSF and GM- CSF. Thus, there was a simultaneous stimulation of both pro-inflammatory and anti-inflammatory cytokines, but with a predominantly *pro-inflammatory* effect. (NB. The use of pure AbM in the same concentration as in Andosan™ gave identical results, indicating that AbM is the main component in this product)(25).

In vivo experiments

In twelve healthy volunteers who ingested Andosan™ 60 ml daily for 12 days a significant *increase* of CD62 L expression was found on monocytes and granulocytes, while no change in the expression of CD11b or increase in ROS production was observed. Thus, the findings *in vivo* were different from those, which were found *in vitro* and were most compatible with an anti-inflammatory effect (118).

Furthermore, in eight healthy volunteers who ingested Andosan™ 60 ml daily for 12 days a significant reduction was observed in the serum levels of IL-1β, TNF- α, IL-17 and IL-8, indicating an anti-inflammatory effect (25).

The reasons for the discrepancy between the effect of Andosan™ on the cytokine levels in the *ex vivo* and *in vivo* studies are unclear. One possible mechanism may be reduced absorption of large, bioactive β-glucans from the GI tract in favor of easily absorbable low molecular weight antioxidant substances, which may inhibit the production of cytokines by macrophages. This is supported by a previous observation that Andosan™ dialyzed against a membrane with a cut-off of 12.5 kD to remove small molecular weight substances lost its previous anti-allergic effect in a mouse model for allergy (119).

Effects on gene expression

The effect of Andosan™ on gene expression was investigated on the human monocyte line THP-1. Stimulation with LPS was included for comparison. Stimulation with both these agents led to a marked upregulation of the pro-inflammatory genes IL-1β and IL-8, and for the genes for TLRs and MyD88 (120). However, in another study on gene expression in peripheral blood cells in patients with hepatitis C virus infection who ingested Andosan™ 60 ml daily for 7 days, upregulation of genes involved in cell cycling and transcriptional regulation was found, but not an increased expression of pro-inflammatory genes (121).

Effect in inflammatory bowel disease

On the background of the predominately anti-inflammatory effects of Andosan™ found *in vivo* in healthy volunteers, it was decided to investigate the clinical effect of Andosan™ in patients with inflammatory bowel disease (IBD). In all, 21 patients with ulcerative colitis (UC)(n=10) and Crohn's disease (CD)(n=11) received Andosan™ 60 ml daily per os alone, or in addition to standard medical treatment for 12 days. At the end of the study plasma baseline levels for MCP-1 was reduced in UC patients, and also the levels of MIP-1β, IL-6, IL-1β, IL-8, G-CSF, MCP-1 and GM-CSF in LPS stimulated blood. In CD patients reduction of plasma levels of IL-2, IL-17 and IL-8 were noted, as well as decrease of IL-1β, MIP-1, IL-8, IL-17 and G-CSF in LPS stimulated blood. Furthermore, fecal calprotectin was reduced in the UC group (26). In another clinical trial, 50 patients with UC and 50 patients with CD were randomized to receive either Andosan™ or placebo for 21 days. Plasma levels of IL-5 were reduced in the UC group, and plasma levels of IL-2, IL-5, and MIP-β were decreased in the

CD group (27). In both UC and CD patients improvement of clinical symptoms and quality of life was found with Andosan™ treatment (18,19). It is concluded that Andosan™ has a predominantly anti-inflammatory effect in IBD.

Effects against allergy

In a murine allergy model, the animals were given Andosan™ 200 µl or PBS by a gastric tube one day before (group 1) or three weeks after immunization (group 2) with the model allergen ovalbumin. Lower levels of serum IgE antibodies against ovalbumin were found in both treatment groups compared to the placebo group. Also, in spleen cell cultures an overall reduction of the levels of the cytokines IL-4 and IL-5 and an increase in the levels of the Th1 cytokines INF-γ and IL-2 were noted (119). Thus, an anti-allergic effect was seen both when Andosan™ was given prophylactically and when this product was given after exposure to the allergen. Similar results have been reported in asthma-induced and in tumor-bearing mice which received AbM (122). The findings in these studies are in line with the Th1/Th2 paradigm, which states that an increased Th1 response is accompanied by a reduced Th2 response (101). In a very recent randomized, placebo-controlled clinical trial in pollen allergic and asthmatic blood donors, Andosan™ supplementation during the pollen season reduced symptoms, specific IgE levels and basophil allergen sensitivity (F. Mahmood et al. accepted abstract at EAACI meeting, June 2019).

The A/J Min/+ mouse

The multiple intestinal neoplasia (Min/+) mouse model was developed as a result of a random mutation in the tumor suppressor gene for adenomatous polyposis coli (APC), caused by exposure to the mitogen ethyl nitrose urea. A Min/ + mouse model with A/J genetic background has been found to be particularly suitable as a model for colon cancer because the animals develop neoplastic lesions both in the intestine and in the colon. These neoplastic lesions have a high susceptibility for developing further to adenocarcinomas.

Three stages of tumorigenesis are noted in these animals: 1) Formation of so-called flat aberrant crypt foci (ACF), characterized by thickening of the epithelial lining, irregular

lumens and increased pericryptal space, 2) Tumors, which resemble flat ACF, but contain ≥ 30 aberrant crypts and 3) Carcinoma. The tendency to develop ACF/ tumors is age-related and especially increasing from the age of mature adult (13-24 weeks). With age, the number of flat ACF decrease and the number of tumors increase. The tumors are characterized by an increased cytoplasmocytic β- catenin accumulation, indicating Wnt (**W**ingless+I**nt** genes) signaling activation. Also, an increased frequency of the proliferation marker Ki67 is noted in the nuclei in the tumors (Figure 8). Transformation of the tumors to carcinomas starts after the age of approximately 30 weeks (124,125).

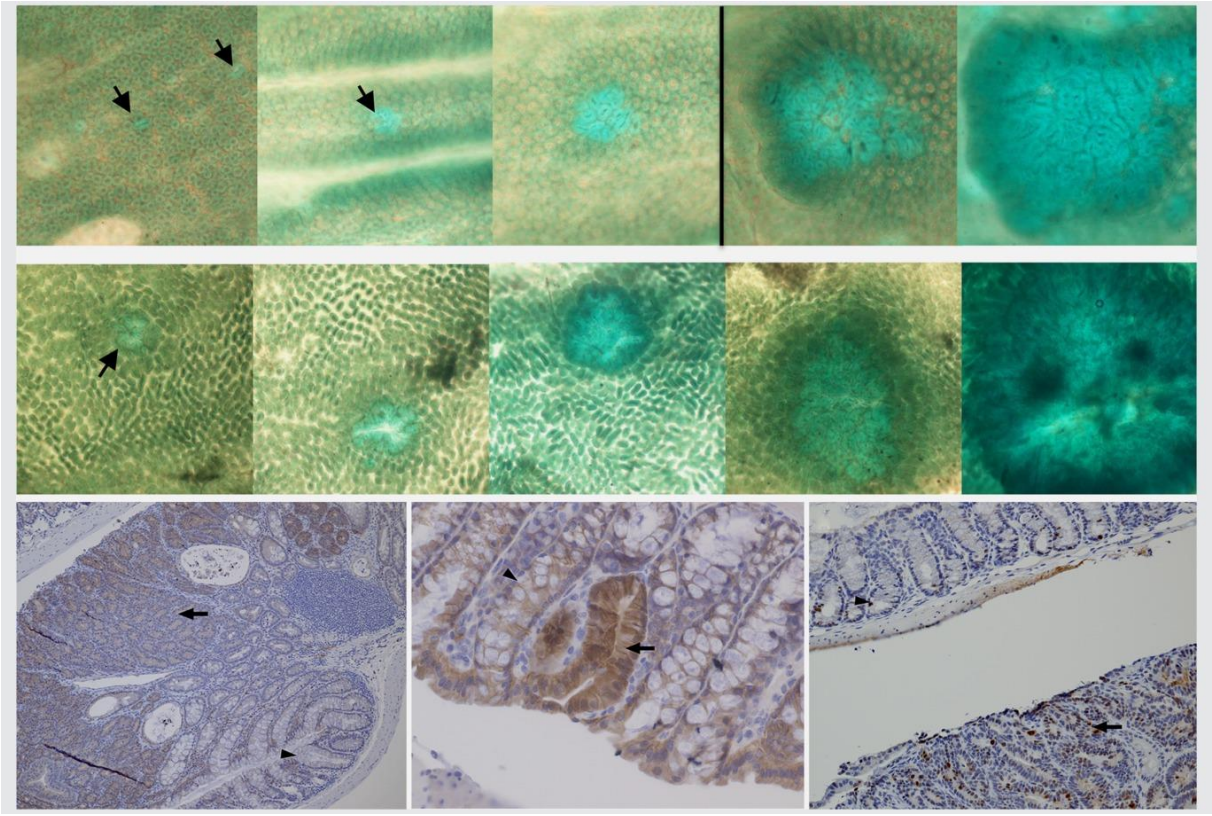


Figure 8

Morphological features of intestinal lesions occurring spontaneously in the A/J Min/+ mouse. Upper and middle panel show methylene blue-stained sections of biopsies.

Upper panel- colon: Left of the vertical bar: Flat ACF. Right of the vertical bar: Tumors. (Magnification x 100).

Middle panel: Small intestine: Tumors. (Magnification x 40)

Lower panel: Left and middle picture: β-catenin stained sections. Left picture- β-catenin accumulation in the tumor (Magnification x 100). Middle picture: β- catenin accumulation in a single crypt of a flat ACF (Magnification x 400). Right picture: Staining of tumors with the proliferation marker Ki67 shows numerous Ki67 positive nuclei throughout the tissue of tumors (bottom right- arrow) but only in scattered cells at the bottom of the crypts in normal tissue (top left, arrowhead)(magnification x 200).

From: Sødring M, Gunnes G, Paulsen JE. Spontaneous initiation, promotion and progression of colorectal cancer in the novel A/J Min + mouse. Int J Cancer 2016;138:1936-1946. (124).

Multiple myeloma

Multiple myeloma (MM) is a neoplastic disorder caused by malignant transformation of B-lymphocytes in the post-germinal centers of the lymph nodes. These cells then migrate to and proliferate in the bone marrow. MM is therefore mainly confined to the bone marrow in the early stages of the disease and extra-medullary manifestations are seen only in advanced stages. In the majority of cases, the disease is accompanied by monoclonal immunoglobulins in serum and urine, secreted by clonally transformed B- lymphocytes. The major clinical features of the disease are defined by the CRAB symptoms: hyperCalcemia, Renal insufficiency, Anemia or Bone lesions (126). The median age at diagnosis is approximately 70 years (127). With the advent of autologous stem cell transplantation (ASCT) and the introduction of new drugs, like immunomodulatory drugs (IMiDs) and proteasome inhibitors, mean overall survival has increased from 3 years to 6 years for younger patients in recent years. However, MM is at present still regarded as an incurable disease (126,128).

Epidemiology

In the period 2001-2005 the incidence of MM in Norway was 9.7/100 000 in males and 6.2/100 000 in females. Mean age at diagnosis was 71.2 years in males and 69.3 years in females (Cancer Registry of Norway).

Oncogenesis

Chromosomal abnormalities play an important role in the oncogenesis of MM. Most important are translocations involving the immunoglobulin switch region (IgH) on chromosome 14 (q32). As a result, oncogenes on the other chromosomes involved in the translocation are juxtaposed to the IgH switch region. The main IgH translocations representing primary oncogenic events in multiple myeloma are t(14q32;11q13), involving the gene CYCLIN D, t(14q32;16q21), involving the gene MAF, and t(14q23;4p16), involving the gene MMSET/FGFR3. Secondary gene mutations involved in disease progression of MM include abnormalities in the MYC gene, activation of NRAS and KRAS genes, mutations in the FGFR3 and TP53 genes, and inactivation of the cyclin-dependent kinase inhibitor genes CDKN2A and CDKN2C (129,130).

Pathogenesis

The interaction between the myeloma cells and the bone marrow microenvironment is the basis for the pathogenesis of MM. This cross-talk is mediated by adhesion molecules, cytokines, and growth factors, regulated by autocrine and paracrine loops. In particular, adhesion of myeloma cells to hematopoietic and stromal cells leads to the secretion of cytokines and growth factors like IL-6, vascular endothelial growth factor (VEGF), insulin growth factor, and others (131). Bone lesions are one of the main features of MM. They are caused by the suppression of osteoblasts, in particular, by the increased production of dickkopf homolog1 (DKK1), and by increased osteoclast activity, mainly caused by enhanced production of RANK ligand (RANKL) and reduced production of osteoprotegerin (OPG). In addition, increased production of the molecular inflammatory protein 1α (MIP1α) also activates osteoclasts (132). The interaction between plasma cells and the bone marrow is illustrated in Figure 8.

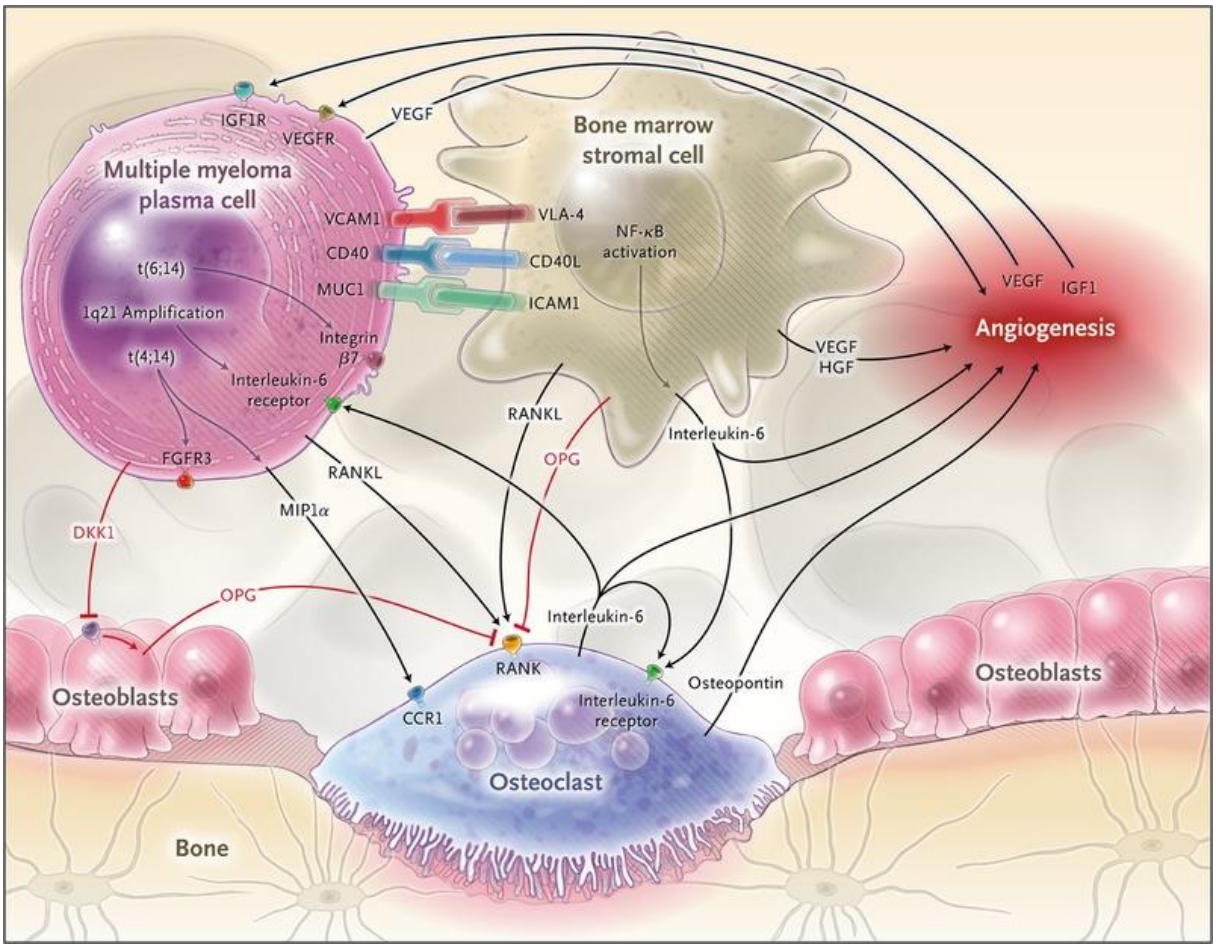


Figure 8. **Interaction between plasma cell and bone marrow in multiple myeloma.**

Adhesion between plasma cells and stromal cells is mediated by cell-adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM1) and integrin alpha 4 (VL4-4). This interaction increases the production of growth factors, such as IL-6 and vascular endothelial growth factor (VGEF), which stimulate both plasma cell proliferation and angiogenesis. Increased osteoclast activity is in particular due to an imbalance in the ratio between receptor activator of nuclear factor κB (RANK) and osteoprotegerin (OPG), which occurs as a result of enhanced production of RANK ligand (RANKL) and reduced production of OPG. Also, osteoblast activity is suppressed by the increased production of dickkopf homolog1 (DKK1) by plasma cells. Moreover, plasma cells inhibit a key transcription factor for osteoblasts, runt-related transcription factor 2, causing a reduction in differentiation from precursors to mature osteoblasts. The adhesion of plasma cells to stromal cells upregulates many cytokines with angiogenic activity, in particular, IL- 6 and VGEF. Osteoclasts that are activated by stromal cells can also sustain angiogenesis by secreting osteopontin.

Chromosomal abnormalities can cause overexpression of receptors on myeloma cells. The 1q21 amplification causes an increase in IL-6 receptor and, consequently, an increase in the growth of myeloma cells mediated by IL-6. Abbreviations: CCR1 denotes chemokine receptor 1, CD40L= CD 40 ligand, FGFR3= fibroblast growth factor receptor 3, HGF= hepatocyte growth factor, ICAM1= intracellular adhesion molecule 1, IGF1= insulin-like growth factor 1, MIPα= macrophage inflammatory protein 1α, MUC1= cell-surface-associated mucin 1, and NF-κB= nuclear factor κB.

Reproduced with permission from Palumbo A, Anderson K. Multiple myeloma. N Engl J Med 2011;364:1046-60 (128). Copyright Massachusetts Medical Society.

Immune responses to MM

MM is accompanied by a state of inflammation, which is primarily mediated by activated macrophages. Myeloma-associated macrophages stimulate the production of IL-6 by stromal cells. Furthermore, they stimulate vasculogenesis by secreting pro-angiogenic factors, such as VEGF and TNF-α. Also, myeloma-associated macrophages protect myeloma cells from apoptosis by interaction between P-selectin glycoprotein -1 (PSG-1) on macrophages with its ligand, PSGL-1, on MM cells, and by the interaction between CD 18 on macrophages and ICAM1/CD18 on MM cells (133). Myeloma associated macrophages, together with myeloid-derived suppressor cells, also exert immunosuppressive effects, resulting, in particular, in defective functions of B-, T-, and NK cells (134,135).

NK cells play a key role in immune surveillance in cancer. They express a range of receptors which allow them to recognize tumor cells. NK cells directly kill target cells by secreting lytic granules (94). Paradoxically, there is an elevated number of circulating NK cells in MM. This may be seen as compensation for a decreasing number of NK cell receptor ligands on myeloma cells (136). Also, CD4/CD8 T cell ratios are decreased in the blood in MM patients and the Th1/Th2 ratio is skewed towards Th1 cells (137).

Diagnosis and treatment

MM develops from a precursor phase, called monoclonal gammopathy with unknown significance (MGUS). Genomic studies indicate that several subsets of clonally different

plasma cells are present already in the MGUS phase. Thus, MM is characterized from the early stage by clonal diversity. This may explain the occurrence of biclonal disease and the occurrence of clonal shifts, which are sometimes seen in the course of the disease (126).

The diagnosis of MM is based on the presence of at least 10% clonal plasma cells in the bone marrow and monoclonal protein in the serum or urine. In non-secretory MM (10% of all patients), the diagnosis is based on at least 30% monoclonal plasma cells in the bone marrow or the presence of plasmacytomas. Indications for treatment of MM are signs of end-organ damage, i.e., the CRAB criteria (138). Patients are classified into three risk categories according to the International Staging System, which is based on serum levels of albumin and β-globulin. Besides, high-risk chromosome abnormalities, such as t(4;14), del 17p13 and del 1q21, as diagnosed by FISH, are taken into account (139). Smoldering MM (SMM) is an intermediate stage between MGUS and MM, where the formal criteria for MM are fulfilled, but CRAB criteria are absent. Recently, The International Myeloma Working Group has published additional myeloma defining events, i.e., ≥ 60% plasma cells in the bone marrow, κ/λ or λ/κ ratio ≥100, and ≥2 bone lesions on MR or PET- CT scan, which define high risk SMM. It is recommended that these patients should receive up-front treatment (140).

Until the 1990-ies the cornerstone of the treatment for MM was the combination melphalan and prednisolone. With this treatment, a median overall survival (OS) around 36 months was reached (141). From the 1990-ies high dose melphalan with autologous stem cell transplantation (ASCT) was introduced in the treatment of MM for patients ≤ 65 years (142). The superiority of ASCT over conventional chemotherapy was first demonstrated in a French study, showing a median OS of 57 months in patients receiving ASCT *versus* 44 months in patients receiving conventional chemotherapy (143). A similar result was subsequently reached in a British study (144). Since the late 1990-ies, ASCT has been standard first-line treatment for myeloma patients ≤65 years. This recommendation is based both on clinical efficacy (142) and on a positive effect on quality of life (145). The Nordic Myeloma Study Group conducted in the period 1994-1997 a population-based clinical study in patients ≥ 60 years with MM in Denmark, Norway, and Sweden, which showed a median OS of 63 months in patients receiving ASCT and a median OS of 39 months in historical controls (146).

From the late 1990-ies, several new drugs have been introduced in the treatment of MM, in particular, the immunomodulatory drugs (IMiDs) thalidomide, lenalidomide, and pomalidomide, and the proteasome inhibitors bortezomib and carfilzomib (147). Thalidomide was the first IMiD to be introduced in the treatment of MM (148). Subsequently, thalidomide, in combination with dexamethasone, became an established treatment both in refractory and newly diagnosed MM patients (149,150). However, treatment with thalidomide is accompanied by several adverse effects, including numbness/ tingling, tremor, and somnolence. In the new generation IMiDs, such as lenalidomide and pomalidomide, subjective adverse effects are much less pronounced. The main effect of IMiDs is inhibition of the production of pro-inflammatory cytokines from activated macrophages (151). Furthermore, IMiDs have been shown to inhibit multiple myeloma-induced osteoclast formation by influencing the RANKL/OPG ratio in the myeloma environment, and by inhibiting the secretion of osteoclast activating factors, such as MIP1a, B cell activating factor (BAFF) and RANKL by myeloma cells (152). Besides, IMiDs have a direct toxic effect on myeloma cell, which has been attributed to inhibition of NF-κB and increased expression of pro-apoptotic factors (153). Moreover, IMiDs have been found to stimulate the production of human natural killer T cells (154), and to inhibit the proliferation and function of T regulatory cells (155).

Proteasome (PI) inhibitors is a class of drugs that interfere with the ubiquitin-proteasome pathway, which plays a central role in the protein catabolism. Three catalytic subunits (β1,β2, and β5) have been identified in the proteasome. Inhibition of the ubiquitin-proteasome pathway leads to accumulation of proteins. This is especially important in myeloma cells, which produce large quantities of immunoglobulins. Such a build-up of proteins leads to inhibition of NF-κB, which results in downregulation of angiogenesis, cytokine signaling and cell adhesion in the microenvironment (156). Bortezomib, which was the first PI to be approved in routine treatment of MM, targets primarily the β5 subunit, but may also target non proteasomal proteases. The next generations of PIs, carfilzomib, and ixazomib, have a higher selectivity for the β5 subunit and less off-target activity against non-proteasomal proteases (157).

In recent years, the introduction of novel drugs in induction therapy before ASCT has been shown to increase response rate, depth of response and progression free survival. A three

drug (triplet) combination, including dexamethasone, bortezomib, and a third drug (an IMiD, cyclophosphamide, or others) is at present recommended induction therapy (158). Maintenance therapy after ASCT is aimed at increasing treatment response and survival. Several studies have investigated the use of thalidomide in this setting. Although a prolonged progression-free survival (PFS) has been demonstrated, the results are conflicting regarding benefit in OS (159). Moreover, a negative impact on quality of life was noted with this treatment (160). In contrast, a recent meta-analysis of clinical trials concluded shows that maintenance therapy with lenalidomide after ASCT is associated with an OS benefit of up to two years (161). This treatment is therefore recommended in the recent ESMO guidelines for MM (162). The use of bortezomib as maintenance treatment has also been extensively investigated. Although a prolonged OS has been found in some investigations (163,164), the role of bortezomib as maintenance treatment is at present not clarified.

First line treatment of patients who are not candidates for ASCT is decided individually on the basis of clinical condition, prognostic group, and comorbidities (164). In the Norwegian treatment guidelines the following treatment regimens are proposed for these patients: Repeated courses with VCD (bortezomib, cyclophosphamide, dexamethasone), VTD (bortezomib, thalidomide, dexamethasone) and VRD (bortezomib, lenalidomide, dexamethasone)(165).

Relapse treatment after ASCT is also given on an individual basis, based on the degree and duration of the response to first-line treatment, type of induction before ASCT, whether maintenance treatment was given, age at progression and the presence of co-morbidities (165). A second ASCT is one of the main options in this setting. According to the Norwegian treatment guidelines a second ASCT may be considered if the response to the first ASCT has exceeded two years (165).

New drugs: In recent years the histone deacetylase (HDAC) inhibitor, panobinostat, and the monoclonal antibodies elotuzumab and daratumumab, have been approved in Norway for selected MM patients after relapse (165).

In recent years, coinciding with the introduction of novel agents, a continuous improvement of response rate and survival in MM patients has been documented (166,167). In particular, the improvement of prognosis in patients receiving ASCT has been

demonstrated both in single-center studies (167,168) and in population-based studies (169,170).

Summary of the papers

Paper 1

Stimulation of human monocytic cells by the medicinal mushroom *Agaricus blazei* Murill induces expression of cell surface markers associated with activation and antigen presentation. (Tangen J-M, Tryggestad AMA, Hetland G).

It has previously been shown that Andosan™ stimulates the production of pro-inflammatory cytokines and the chemokine MIP-β-1 in MDDC *in vitro* (24). It was therefore decided to study also the modulatory effect of Andosan™ on cell surface markers of MDDC.

MDDC were stimulated with Andosan™ 10% for 24h and 48h, and the expression of a range of cell surface markers associated with maturation, activation or antigen presentation were examined by flow cytometry. Stimulation with Andosan™ resulted in neo-expression of CD69, increased expression of CD1a, CD14, CD40, CD80, CD83 and CD86, and down-regulation of the expression of CD11c. Andosan™ stimulation for 48h led to a higher degree of upregulation of the cell surface markers than stimulation for 24h in most of the cases. Interestingly, stimulation with this mushroom product induced a similar neo-expression of CD69 (associated with activation) and up-regulation of CD86 (associated with antigen presentation) as did stimulation with *E.coli* LPS. These findings are in line with the results from the previous experiment, showing that stimulation of MDDC with Andosan™ increases the production of pro-inflammatory cytokines in a similar manner as stimulation with LPS (24). Furthermore, upregulation of CD14 was also noted. The CD14 molecule is associated with TRL4 binding of LPS (169). The increased CD14 and CD86 expression could therefore together indicate an enhanced ability of these cells to fight infection with Gram-negative bacteria. This may be a possible explanation for the protective effect reported earlier on Andosan™ against Gram-negative sepsis in a mouse model (22). CD 69, which is found on activated T, B, and NK cells, was found to be expressed *de novo* on Andosan™ stimulated MDCC. Together with the upregulation of CD86, this suggests an enhanced antigen presenting function of these cells. Somewhat surprisingly, downregulation of CD

11c was found in this investigation. A technical explication may be suggested for this phenomenon, i.e., binding of Andosan™ to an antigen epitope, which may block later binding of a fluoresence labelled antibody for flow cytometry examination.

In conclusion, the experiment shows that Andosan™ has a predominately activating effect on cell surface activation markers of MDDC *in vitro*. As the content of LPS in Andosan™ is in any case miniscule, i.e. not detected with the Limoulous amebocyte lysate test (dection level 0,5 pg/ml), it is presumed that contamination with LPS is not a factor in the stimulating effect of Andosan™ in this experiment.

In a second experiment, promonocytic THP-1 cells were incubated with 10% Andosan™, nuclear and cytoplasmatic fractions were isolated, and a Western blot was performed in the absence or presence of either anti-TLR2, anti-TLR4, or IgG2a (isotype control) antibodies. This experiment showed translocation of NF-κB from cytosol to the nucleus, which was inhibited by preincubation with anti-TLR2 antibody but not anti-TLR4 antibody, showing that the NF-κB translocation was mainly induced via TLR2.

NB: After scrutiny of this paper, a technical flaw was recognized in this experiment, as α-tubulin, which is only present in the cytosol, was erroneously used as loading control for the nuclear fraction. A different compound, specific for the nucleus, such as lamin, should have been used in this case. However, this technical flaw does not appear to have a decisive impact on the results of the experiment. Also, flow cytometry data plots with gating of the analyzed cells, should have been included in the paper.

NB! Errata: The 5th line in the **Discussion** should read "… CD83 and CD 86 (**Figures 2A-2F**)"

The x- axis in figure **2F** should have the designation "CD86".

Paper 2

Immunomodulatory effects of the *Agaricus blazei* Murill-based mushroom extract AndoSan in patients with multiple myeloma undergoing high dose chemotherapy and autologous stem cell transplantation: A randomized, double-blinded study. (Tangen J-M,

Tierens A, Caers J, Binsfeld M, Olstad OK, Siebke Trøseid A-M, Wang J, Tjønnfjord G, Hetland G).

Background

The main part of Paper 2 concerns an investigation of the immunomodulating effects in MM patients receiving Andosan™ as adjuvant treatment to ASCT. The reasons for initiating this study were: 1 An AbM extract has earlier been shown to have effect on MM in a mouse model (66). 2. Andosan™ was shown to have a dose-related cytotoxic effect on mouse myeloma cells (MOPC315.BM cells) in a preliminary experiment. These cells have a tropism for the bone marrow, causing a clinical picture in mice similar to MM in humans (172). 3. MM is characterized by a pro-inflammatory state (134), and Andosan™ has earlier been shown to have an anti-inflammatory effect in healthy volunteers (25).

Formal permissions

The study was approved by the Regional Committee for Medical and Health Research Ethics (REK South East). The Norwegian Medicines Agency was notified of the intent to conduct the study. The study was evaluated after its publication by this Agency and found to be compliant with the guidelines for clinical investigations of food supplements.

Conduction of the study

Forty consecutive MM patients with a median age of 57,3 years, scheduled to undergo ASCT at Oslo University Hospital (OUH) in the period from 01.05.2009 until 31.11.2010, were randomized in a double-blinded fashion to receive adjuvant treatment with Andosan™ 60 ml daily, or placebo. The guidelines for treatment of MM, issued by the Norwegian Health Directorate, states that ASCT is the recommended first-line treatment for patients $\leq$ 65 years, unless there are contraindications. In general, it is emphazised, that the use of any other treatment than those recommended by this Directorate should be justified and the reason documented in the patients record (165). On this background, it is presumed that the great majority of the patients $\leq$ 65 years with newly diagnosed MM are referred to the regional University Hospital for assessment for ASCT. OUH is the reference center for South East Norway. Intake of the study product started on the day of stem cell

mobilizing chemotherapy and continued until the end of aplasia after high dose chemotherapy, a period of about seven weeks. All 40 patients were evaluable for survival endpoints, while 33 patients were evaluable for both laboratory and clinical endpoints. Calculation of the sample size of the study was related to laboratory endpoints and based on the results from an earlier study of the effect of Andosan™ on serum cytokine levels in healthy volunteers. The sample size was not calculated for clinical endpoints because of a lack of any background data, which could be used as the base for such a calculation.

A biobank was created for the study, with the permission of REK, for the keeping of samples for genetic investigations of bone marrow aspirates, serum cytokine level analyses, and flow cytometry of cells in the harvested leukapheresis product. These analyses were performed at the end of the study. A quality of life study was performed prospectively. Clinical data (as, for instance, date of diagnose, date of start of second line therapy, date of follow-up, date of death, and others) were noted retrospectively from the patient records.

Results

In the leukapheresis product harvested after stem cell mobilization, increased percentages of T_{reg}s and plasmacytoid dendritic cells (pDC) were found in patients receiving Andosan™. T_{reg}s have been found to play a role in reducing immune surveillance in cancer. In a clinical study on MM serum levels of T_{reg}s were found to increase with increased disease activity. However, these findings were not confirmed in MM patients receiving ASCT (173). pDC stimulate both the innate and the adaptive immune system. In clinical studies, pDC levels have been found to be lower in MM patients compared to controls. The finding of higher pDC levels in the leukapheresis product in patients receiving Andosan™ may be therefore be interpreted as a positive effect (174). Furthermore, in patients receiving Andosan™ a significant increase of serum levels of IL-1**ra** (**r**eceptor **a**ntagonist), IL-5 and IL-7 were found at the end of the treatment period. IL-1ra is a natural inhibitor of the pro-inflammatory cytokine IL-1β. On this background, the elevated serum levels of IL-1ra found in MM patients treated with Andosan™ may be interpreted as an anti-inflammatory effect (175). In a clinical trial, treatment with recombinant IL-1ra in patients with SMM was associated with a decreased myeloma proliferative rate. The main role of IL-5 is to stimulate the production of eosinophils. It has recently been shown that eosinophils can stimulate the growth of malignant plasma cells (176). The elevated serum IL-5 levels found in the

Andosan™ group may therefore be interpreted as a negative factor. IL-7 is a strong stimulator of both B-lymphocytes and T- lymphocytes (177). In this perspective, the elevated IL-7 levels found in the Andosan™ group may be interpreted as a positive factor. Whole genome microarray of the bone marrow aspirate showed increased expression of immunoglobulin genes, Killer Immunoglobulin Receptor (KIR) genes and HLA genes in the Andosan™ group, which may be interpreted as signs of immunoactivation.

Trends for a longer median time to second line treatment and a longer mean (OS), (37.5 months vs. 31.4 months (p=ns) and 50.7 months vs. 47.4 months (p=ns), respectively), were noted in patients receiving Andosan™ at follow-up 58 months after start of the study. Median OS was not reached in any of the groups. However, as the study was not designed to show survival differences, the survival data are presented only as a point of information. An assessment, performed at Oslo Centre for Biostatistics and Epidemiology after the end of the study (in August 2014), showed that in order to detect a statistically significant difference in median OS, based on the data from the study, it would have been necessary to include in the order of 160 patients.

Also, a shorter time with i.v. antibiotics during aplasia (8.6 days v.s. 10.0 days (p=ns)) were found in the Andosan™ group compared to the placebo group. Quality of life studies showed no differences between the groups.

Conclusions

In a preliminary study, a ^{3}H-thymidine incorporation assay showed that Andosan™ has a dose-related cytotoxic effect on mouse myeloma cells (MOPC315.BM cells).

Furthermore, in the clinical study, changes in a number of immunological parameters in patients receiving Andosan™ were found. In particular, an increase in the serum levels of Il-1ra may indicate an anti-inflammatory effect. However, the clinical significance of these findings can not be assessed.

No conclusion can be drawn regarding effect on survival because of the restricted number of patients included in the study.

Addendum: At a follow-up at 01.05.2017, 95 months after start of the study, mean OS was 71.4 months in the Andosan™ group and 60.4 months in the placebo group (p=0.32).

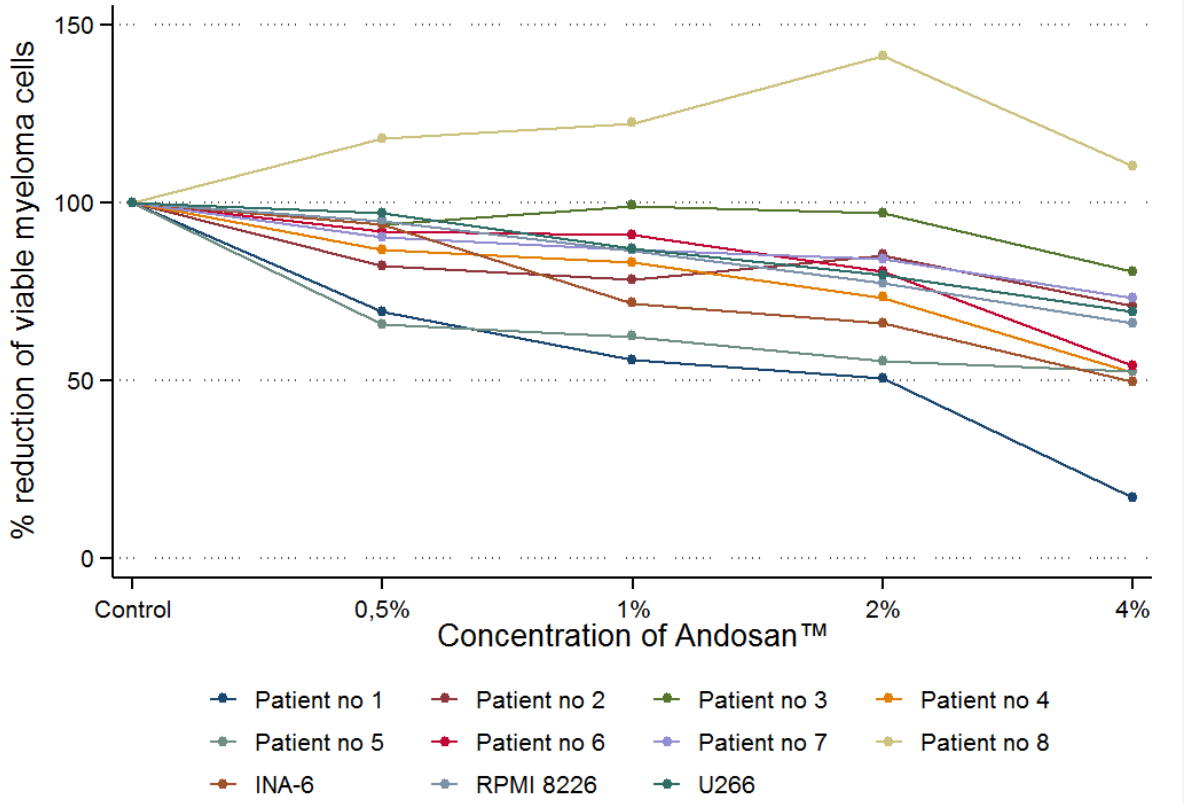
Median OS was not reached in the Andosan™ group, (12/19 patients were alive), while median OS was 53.5 months in the placebo group (8/21 patients were alive). For all patients together, median OS was 87.9 months (unpublished data), which is a markedly higher than the median OS found in a Nordic population-based study in MM patients ≤ 60 years receiving ASCT, conducted in the period 1994-1997 (63 months)(146). This may indicate that a general improvement of survival in this group of MM patients had taken place during this period.

Paper 3

Cytotoxic effect on human myeloma cells and leukemic cells by the *Agaricus blazei* Murill based mushroom extract Andosan™. (Tangen JM, Holien T, Mirlashari MR, Misund K, Hetland G).

In in a preliminary experiment, presented in paper 2, it was shown that Andosan™ had a dose-dependent inhibitory effect on the proliferation of mouse myeloma cells. On this background it was decided to investigate the cytotoxic effect of Andosan™ in human malignant cell lines, i.e. human myeloma cell lines, primary myeloma cells and human leukemic cell lines, as well as on peripheral blood mononuclear cells. In a first experiment, the human myeloma cell lines RPMI-8226, U-226 and INA-6 were cultured for 72 hrs with Andosan™ in concentrations from 0.5% to 4%, or with PBS (control). Using the CellTiter-Glo assay, which measures the cells' ATP content, it was found that Andosan™ had a dose-related cytotoxic effect on these myeloma cell lines. Furthermore, in a cell cycle analysis, a decrease in the DNA content in G1 phase and an increase of the DNA content in S phase and in G2-M phase, indicating cell cycle arrest. In a third experiment primary CD138 + myeloma cells from ten patients from The Norwegian Myeloma Biobank were cultured for 72 hrs together with Andosan™ in concentrations from 0.5% to 4%, or control (PBS). The cells were then stained with fluorescent markers for viability and counted directly using ScanR automated image acquisition and analysis. In eight of the ten patients, the results were evaluable. In one of these eight patients, the cell viability *increased* after incubation with Andosan™, while for the other seven patients a dose-dependent cytotoxic effect of

Andosan™ was seen. See Figure below:



Cytotoxic effect on Andosan™ on the myeloma cell lines INA-6, RPMI 8226, U266, and myeloma cells from patients no 1-8.

In a fourth experiment the human leukemic cell lines KG1a (Acute myelocytic leukemia, immature phenotype), HL 60 (Acute promyelocytic leukemia) and Meg 01 (Acute megakaryocytic leukemia) were cultured with Andosan™ in the concentrations 5.0% and 10.0%, or control (PBS) for 96 hrs and analysed for viability, using the NucloCounter method. Finally, peripheral blood mononuclear cells from three healthy blood donors were cultivated with Andosan™ in similar concentrations, or PBS (control), for 96 hrs and also analyzed for viability using the NucleoCounter method. No cytotoxic effect was seen by Andosan™ on these cells.

In conclusion, these experiments showed a dose- dependent inhibitory effect of Andosan™ on human myeloma cell lines, primary myeloma cell lines and human leukemic cell lines.

For myeloma cell lines, cell cycle arrest was shown. No cytotoxic effect was found on peripheral mononuclear blood cells.

Paper 4

The *Agaricus blazei*-based mushroom extract, Andosan™, protects against intestinal tumorigenesis in the A/J Min/+ mouse. (Hetland G, Eide DM, Tangen JM, Haugen MH, Mirlashari MR, Paulsen JE).

In a preliminary study, dose-related cytotoxic effect of Andosan™ was found on human epithelial colorectal adenocarcinoma cells (Caco-2) in culture. The results are presented in Figure 1 in this article.

NB! Erratum: The term "viability" should replace the term "proliferation" in the annotation in Figure 1.

Secondly, a flow- cytometric analysis of annexin-V binding of the Caco-2 cells to assess early apoptosis, and incorporation of a second dye, (7-ADD), as a marker for late apoptosis (necrosis), was performed. The results are presented in Figure 2. The degree of total apoptosis (early apoptosis+ late apoptosis (necrosis)) was 13 % of the cells in controls, 34 % of the cells in cultures with 1% Andosan™, and 76 % of the cells in cultures with 5% Andosan™.

Thus, taken together, these investigations indicate a dose-related cytotoxic effect of Andosan™ on Caco-2 cells, connected to apoptosis.

In a second experiment, 46 A/J Min/+ mice and 30 A/J wild type (wt) mice, received drinking water with or without the addition of Andosan™ 10%. The multiple intestinal neoplasia (Min+) mice of A/J genetic background have a mutation in the tumor suppressor gene *APC*, which leads to the development of adenomas in the small intestine and colon (123) These tumors may later progress to carcinoma, starting approximately after the age of 30 weeks (124). The animals were sacrificed after 22 weeks, the colon and intestine were examined by light microscopy and the number of tumors counted. Furthermore, in 28/46 A/J Min/+ mice (14 receiving Andosan™ and 14 receiving regular drinking water) and in 30 wt A/J mice (14 receiving Andosan™ and 16 receiving regular drinking water), serum levels of cytokines were measured.

Ethical approval*:* The animal investigation was considered by the local representative of the Norwegian Food Safety Authority at The Norwegian Institute of Public Health before its start in 2012. As the only intervention between the animal groups, which were normally bred, was differences in alimentation (feeding), it was considered that the experiment was exempt from approval from The Norwegian Food Safety Authority. Reference: The Norwegian Law-Regulations for animal experiments- Introductory chapter- point d).

Results*:* Among the A/J Min/+ mice receiving AndosanTH (n=24) the mean total number of tumors/mouse was 23, while the corresponding number in A/J Min/+ mice receiving regular drinking water was 34. The difference was found to be statistically significant (Lower part of table 1). However, the differences failed to reach statistical significance after adjusting for the effect of sex and litter. The main reason for this was found to be the fact that there was a higher number of tumors in female mice (mean total number of tumors/ mouse= 34)(n= 19) compared to male mice (mean total number of tumors/mouse= 25) (n=27), both treatment groups analyzed together. The A/J Min/+ mice are considered to be practically isogenetic (JE Paulsen, personal information). Consequently, the possibility for a litter effect is considered to be negligible. A sex difference in the tendency to develop intestinal tumors has previously not been observed in A/J Min/+ mice (124,125). The finding of a sex difference in this study may therefore be coincidental and attributable to the restricted number of animals included.

The cytokine analyses showed a statistically significant increase in Th1 type cytokine IL-12p70, and in the pro-inflammatory cytokines IL-1β, MCP-1 and TNFα in the 14 A/J Min/+ mice + 14 A/J wt mice receiving Andosan^{TM}, compared to the 14 A/J Min/+ mice + 16 A/J wt mice receiving only water (upper part of table 1). However, when adjusting for sex and litter, the differences were no longer statistically significant (see discussion above). In all, the cytokine analyses show a predominantly pro-inflammatory effect of Andosan^{TM}. This agrees with the results observed earlier in a sepsis mouse model (23), but are contrary to the effect of Andosan^{TM} in humans, where a predominantly anti-inflammatory effect of Andosan^{TM} is reported (27). The reason for this difference is unknown. One may be speculate that it may be due to differential uptake from the intestine of immunomodulating polysaccharides, or antioxidant substances, in mice and humans. In

addition, an increase in the Th1 type cytokine IL-12p70 was noted, which may be associated with an anti-tumor effect. A decreased expression of the tumor-associated protease legumain was observed by immunofluorescence tissue staining of intestines from animals that had received Andosan™, compared to controls. This is a semi-quantitative method, and the results are presented without claim to statistical significance. However, this observation is in line with the results of an earlier study, showing that an ethanol insoluble fraction of Andosan™, which contained 10% glucose, significantly inhibited the activity of the proteolytic enzyme legumain in macrophages (79).

In conclusion, a protective effect of orally administered Andosan™ against the development of gastrointestinal tumors was found in the A/J Min/+mouse model in this pilot study. Possible mechanisms are stimulation of the immunological defence against cancer by a pro-inflammatory effect and by a relative shift in the Th1/ Th2 balance towards Th1 lymphocytes, inhibition of tumor cell growth by induction of apoptosis, and inhibition of legumain production by intestinal macrophages.

Paper 5

Improved outcome in patients following autologous stem cell transplantation for multiple myeloma in South Eastern Norway 2001-2010.A retrospective, population-based analysis. (Tangen JM, Tjønnfjord GE, Gulbrandsen N, Gedde-Dahl T, Stormorken E, Anderson K, Dao Vo C, Schjesvold F).

In a follow-up 95 months after start of the study investigating the effect of Andosan™ as adjuvant treatment to ASCT in 40 MM patients (Paper 2), a median OS of 87.9 months was found (unpublished data), which is markedly higher than the median OS found in a previous Nordic population-based study (146). On this background, it was decided to investigate the general survival in patients receiving ASCT for MM in OUH in recent years. The investigation concerns MM patients ≤ 65 years, referred to OUH for assessment for ASCT in the period 01.01.2001 - 31.12.2009. The guidelines for treatment of MM, issued by the Norwegian Health Directorate, state that ASCT is the recommended first-line treatment for patients ≤ 65 years, unless there are contraindications (165). A choice of any different treatment should be justified and the reason documented in the patients record (165). For this reason, it is assumed that the great majority of MM patients where ASCT is indicated according to the national guidelines, are referred to a regional reference center to be

assessed for this treatment. The authors have therefore chosen to name this study "population-based", although, formally, it is not based on data from a population- based patient registry. The study was a retrospective analysis, based on a search in the patient registry in OUH of patients (in-patients and out-patients) entered with a diagnose code of C90.0 (MM) in the ICD 10 diagnostic system. The study was approved by the Data Protection Officer at OUH as a quality control of ASCT for MM in this hospital.

The patients were divided in two groups according to the date of their first entry in the patient registry; patients registered from 01.01.2001 until 31.06.2005 (Period 1), and patients registered from 01.07.2005 until 31.12.2009 (Period 2). A comparison of treatment results between these two periods is considered to be valid for two reasons: 1) The distribution of patients in different clinical stages were comparable between the periods (data not shown), 2) There were no substantial changes in the procedure for ASCT (induction treatment, mobilization and harvest of hematopoietic stem cells, and conditioning), or supportive care, during these two periods. A total of 623 patients $\leq$ 65 years were registered with the diagnosis C90.0 in the period 01.01.2001-31.12.2009. Of these, 355 patients were found to have a confirmed diagnose of MM with indications for treatment. In all, 296 patients received ASCT, while 62 patients (17%) received other treatments (medical contraindications: 58 patients, no consent for ASCT: 4 patients). See figure 1:

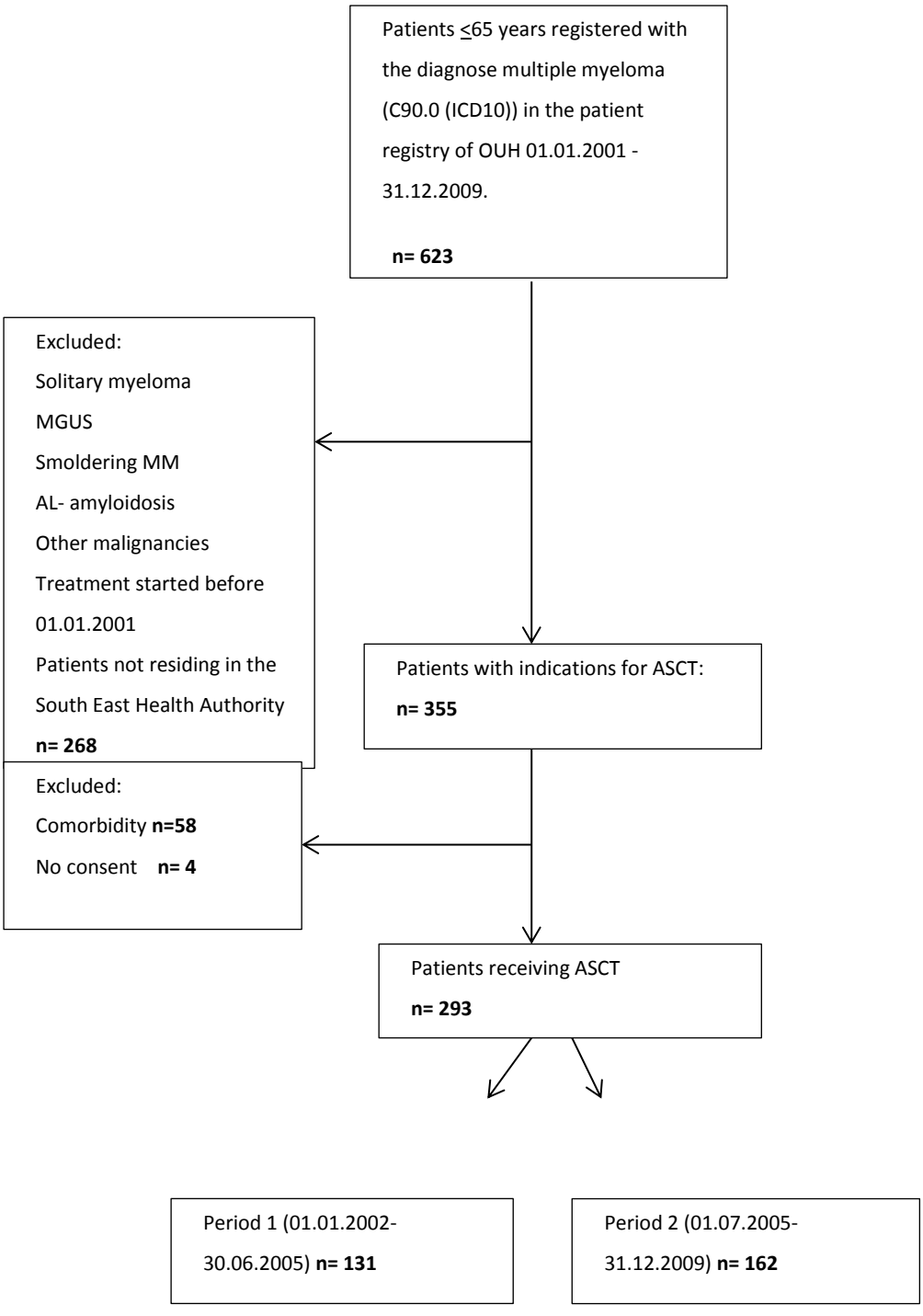

Patients ≤65 years registered with the diagnose multiple myeloma (C90.0 (ICD10)) in the patient registry of OUH 01.01.2001 - 31.12.2009.
n= 623
Excluded:
Solitary myeloma
MGUS
Smoldering MM
AL- amyloidosis
Other malignancies
Treatment started before 01.01.2001
Patients not residing in the South East Health Authority
n= 268
Patients with indications for ASCT:
n= 355
Excluded:
Comorbidity n=58
No consent n= 4
Patients receiving ASCT
n= 293
Period 1 (01.01.2002-30.06.2005) n= 131
Period 2 (01.07.2005-31.12.2009) n= 162

Figure 1. Flowchart for inclusion of patients in the study: Tangen JM et al. Improved outcome in patients following autologous stem cell transplantation for multiple myeloma in south eastern Norway 2001-2010: a retrospective, population-based analysis.

Results: For all 355 patients together, OS was 75.4 months, 82.9 months for patients receiving ASCT and 27.0 months for patients not receiving ASCT. For patients ≤ 60 years receiving ASCT, median OS increased from 68.3 months in period 1 (n=99) to 86.4 months in period 2 (n=101). However, the difference failed to reach statistical significance (p=0.22). The median OS only increased from 57.3 months to 61.2 months between the two periods for patients 61-65 years receiving ASCT (p=0.87)(n.s). A negative impact on age on survival after ASCT was also found in the previous Nordic study (146) and in a comprehensive analysis of patients included in studies conducted by Intergroup Francais de Myelome (178). The median survival after start of second-line therapy increased between the two periods for patients ≤60 years (34.5 months vs. 46.5 months). This difference is statistically significant (p=0.015)). For patients 61-65 years there was no substantial difference in median survival after start of second-line therapy between the two periods.

Reaching complete response (CR) was shown to be a major prognostic factor, both for OS and for time to new treatment, for both age groups. This was most clearly demonstrated for patients ≤60 years, where median OS was 133.7 months for patients reaching CR, both periods together (Table 3). The prognostic importance of treatment response after ASCT has been shown in several previous publications (179,180). The importance of achieving CR after ASCT for survival was also shown in a recent meta-analysis, which included 24 clinical studies (181). Time to new treatment remained approximately the same in both age groups in both study periods. This indicates that the net difference in overall survival was primarily due to the effect of second-line treatment. In patients ≤60 years the use of second ASCT increased from 16.8% to 41.7% between the two periods, while in patients 61-65 years salvage ASCT was given in only 3.2% and 10.2% of the patients, respectively, in the two periods. Furthermore, the use of the novel drugs lenalidomide, pomalidomide, bortezomib and carfilzomib, as well as the number of treatment lines after progression, increased for both groups but were higher in patients ≤ 60 years compared to patients 61-65 years.

Our study indicates that the prognosis for MM patients receiving ASCT has considerably improved since the previous Nordic study (146), which showed an OS of 63 months in patients ≤60 years and 50 months for patients 61-65. The improvement seems to be due to an active attitude to salvage ASCT and the introduction of novel drugs in relapse treatment.

The results presented in this study are in the same range as those reported from Malmø, Sweden, for the period 2000-2005, based on data from the Swedish Cancer Registry (169). They are also comparable to the results from a publication from the Swedish Myeloma Registry, comprising 4904 patients, diagnosed in the period 2008-2015, where the median OS years was found to be 93.6 months for patients ≤ 60 years and 64.8 months for patients 60-70 years (170).

In conclusion, this investigation shows that recent progress in MM treatment is reflected in real- world clinical results for Norwegian patients.

Discussion

The four first papers in this thesis may be regarded as pilot studies, exploring biological effects of the mushroom product Andosan™. Paper 1 and 2 concern immunomodulatory effects. In paper 1, a stimulatory effect of Andosan™ on surface markers on MDDC associated with activation and antigen presentation was shown. This result is in line with a previous study, showing that Andosan™ increased the production of pro-inflammatory cytokines in MDDC in culture (22). However, these results are in contrast with results from an *in vivo* study, showing that intake of Andosan™ had predominantly anti-inflammatory effects in healthy volunteers (25). On the background of the pro- inflammatory changes accompanying MM, the treatment effect obtained with IMiDs in this disease, and the anti-inflammatory effects of Andosan™ in healthy volunteers, it was decided to conduct a clinical study, exploring the effect of Andosan™ as adjuvant treatment in patients undergoing ASCT for MM. This investigations showed changes in a number of immunologic parameters in patients receiving Andosan™, in part indicating an anti-inflammatory effect, but it is unknown whether these effects had a clinical significance (Paper 2).

Paper 3 concerns *in vitro* studies, exploring cytotoxic effects of Andosan™ on human malignant hematological cells. These experiments showed that this mushroom product has

a dose-related cytotoxic effect on human leukemia cell lines and myeloma cell lines, including primary myeloma cells from the Norwegian Myeloma Biobank. For three myeloma cell lines, it was shown that this effect was related to cell cycle arrest. In a further study, presented in paper 4, it was showed that Andosan™ also had a cytotoxic effect on human epithelial colorectal adenocarcinoma cells, connected with apoptosis.

In conclusion, a dose- dependent cytotoxic effect of Andosan™ was found in several different malignant cell lines *in vitro.* On the background of the cytotoxic effect of Andosan™ in colorectal adenocarcinoma cells, it was decided to investigate the possible preventive effects of Andosan™ against the development of intestinal tumors in a mouse strain which has a genetic defect predisposing to such tumors (Paper 4). This investigation showed that in animals receiving drinking water with addition of Andosan™, the number of intestinal tumors developing per animal were significantly lower than in animals receiving regular drinking water. Thus, a protective effect against intestinal tumors was found.

The possible mechanisms of action of Andosan™ are at present unclear. Both *Agaricus blazei* Murill and the two other mushrooms, contained in Andosan™, *Hericeus erinaceus* and *Grifola frondosa*, are rich in polysaccharides and β-glucan in raw form. Several previous investigations on the biological effect of AbM have used extracts from the mushroom with a documented high content of β-glucans (7-11). It had therefore previously been assumed that the immunomodulating effect, documented with Andosan™ in early investigations, could be attributed to β-glucans. However, a recent investigation, using ethanol fractionation, showed that the total content of polysaccharides in this product is low, only about 2%, corresponding to 0,09% β-glucan (79).

On this background, the content of carbohydrates in Andosan™ was in February 2019 analyzed by the commercial laboratory VITAS- Analytical Services, Oslo, Norway, by three different methods:

1. Measurement of free monosaccharides: 10 mL of Andosan was evaporized and derivatized before analysis by gas chromatography-mass spectrometry (GC-MS).
2. Measurement of total monosaccharides after methanolysis: 10mL Andosan™ was evaporized, methanolysed, re- evaporized, derivatized, and analyzed by GC-MS.

3. Measurement of glucose after enzymatic degradation of β- glucan to glucose: 10 mL of Andosan™ was evaporized, hydrolyzed with lichenase and β- glucosidase, re-evaporized, derivatized and analyzed by GC-MS.

The results are shown in the table below (Unpublished results).

Quantitative values													
	Batch	Batch	Exp	Xylose	Arabinose	Ribose	Rhamnose	Mannose	Galactose	Glucose	Glucuronic	Galacturonic	beta-glucan
Test type		number	Date								acid	acid	
				µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL
Free monosaccharides	1	012014B	NOV-2015	2,0	3,7	0,4	<0.2	<0.2	0,5	1,1	<0.5	<0.5	n.a
	2	012017A	DEC-2018	2,3	5,5	0,5	<0.2	<0.2	0,5	0,5	<0.5	<0.5	n.a.
	3	022017A	AUG-2019	7,0	4,3	5,0	0,3	0,1	2,1	0,6	0,7	0,9	n.a
Methanolysis	1	012014B	NOV-2016	23,4	14,1	0,4	2,7	1,7	8,6	11,7	<0.5	<0.5	n.a
	2	012017A	DEC-2019	112,7	69,5	2,4	8,7	2,2	19,7	7,4	<0.5	<0.5	n.a.
	3	022017A	AUG-2020	61,0	37,4	1,9	3,8	2,1	14,0	6,9	<0.5	<0.5	n.a
Enzymatic hydrolysis	1	012014B	NOV-2017	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2,5	n.a.	n.a.	1,2
	2	012017A	DEC-2020	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2,4	n.a.	n.a.	1,7
	3	022017A	AUG-2021	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1,4	n.a.	n.a.	0,8

Relative values												
	Batch	Batch	Exp	Xylose	Arabinose	Ribose	Rhamnose	Mannose	Galactose	Glucose	Glucuronic	Galacturonic
Test type		number	Date								acid	acid
				%	%	%	%	%	%	%	%	%
Free monosaccharides	1	012014B	NOV-2015	25,6	47,7	5,7	n.a.	n.a.	6,4	14,5	n.a.	n.a.
	2	012017A	DEC-2018	25,0	58,4	5,2	n.a.	n.a.	5,7	5,7	n.a.	n.a.
	3	022017A	AUG-2019	33,2	20,5	23,8	1,6	0,6	10,0	2,7	3,2	4,4
Methanolysis	1	012014B	NOV-2016	37,4	22,6	0,6	4,2	2,7	13,8	18,7	n.a.	n.a.
	2	012017A	DEC-2019	50,7	31,2	1,1	3,9	1,0	8,9	3,3	n.a.	n.a.
	3	022017A	AUG-2020	48,0	29,4	1,5	3,0	1,6	11,0	5,4	n.a.	n.a.

Comments: With method 2, a considerable higher amount of monosaccharides is found, compared to method 1, which indicates that the different sugars are present in the form of polysaccharides. The enzymatic hydrolysis in method 3 shows that β-glucan is present, but in small quantity (~1µg/mL).

In conclusion, these analyzes, based on GC-MS, confirm that polysaccharides and β- glucans are present in Andosan™, but in low quantities. It is therefore uncertain to what degree the immunological effects of Andosan™ might be attributed to these compounds. However, as already mentioned, a high molecular weight extract, containing 10% glucose (presumably including β-glucans) from Andosan™, was found to inhibit legumain activity in macrophages (79), indicating that glucose and β- glucans in this product may exert biological effects, in spite of their low quantity.

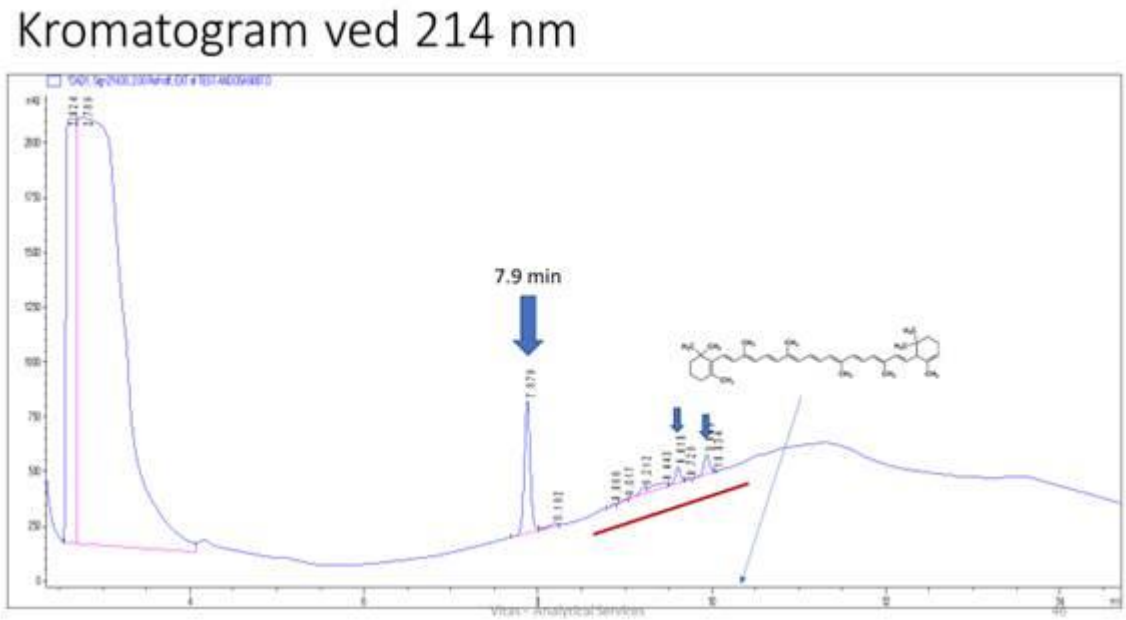
In addition to β-glucan, which is a constitutional part of the mushrooms in the product, Andosan™ has also been found to contain a number of bioactive metabolites, in particular blazein, ergosterol, agarol and agaritine. The sterol blazein was been shown to induce apoptosisin human lung cancer cells and human stomach cancer cells (67). Ergosterol from

AbM was reported to have a tumoricidal effect in human sarcoma, xenografted in mice, by inhibition of neovascularization (68). The ergosterol derivative agarol from AbM was shown to exert a cytotoxic effect on human cell by increasing ROS and by stimulating apoptosis (69). Agaritine, which is a phenylhydrazine derivate isolated from AbM and from *Agaricus bisporus,* has been shown to induce apoptosis in leukemic cells (70). In some publications it has been claimed that agaritine has carcinogenic effects, but this has not been confirmed by other reports (72). Also, extracts of *Hericium erinaceus* (~15% of Andosan™ content), containing β-glucan and several GC-MS identifiable compounds, have been shown to induced tumor regression in a mouse model due to induction of NK and macrophage activation and inhibition of angiogenesis (182). Moreover, polysaccharides of *Grifola frondosa* (~3% of Andosan™ content) exhibited antitumor activity in mouse models (183,184) due to DC maturation, followed by induction of a tumor-antigen specific T cell response.

Further perspectives: An analysis of Andosan™ by VITAS-Analytical Services, Oslo, performed in October 2018, has focused on the content of carotenoids, using high pressure liquid chromatography with diode array detector (HPLC-DAD). By examination in the 3D UV spectra, several large peaks, indicating lipids of high concentration, were discovered. One peak, eluting at 7.9 minutes with UV max at 214 nm (A) and multiple peaks, eluting from 8.1 to 11.2 minutes, with UV max from 260-280 nm (B), were found. (See figure below).

A preliminary assessment indicates that these peaks probably represent steroids or esters of steroids, like, for instance, triterpenoids. These compounds, which have potentially active structures and are being present in high concentrations, would be interesting to

identify in further studies.



A retrospective study, based in a search in the patient registry in OUH, on MM in patients ≤65 years receiving ASCT in the period 2001-2010, showed a marked increase in survival compared to the result of a Nordic study, conducted in the period 1994-1997. This improvement is linked to the introduction of novel drugs, IMiDs and proteasome inhibitors. Because of the recommendation of ASCT as first line treatment for MM patients ≤ 65 years in the guidelines, issued by the Norwegian Health Directorate, it is likely, that the majority of these patients are referred to the regional university hospital for this treatment. It is therefore considered, that this investigation has a population-based character. The study shows that recent progress in MM treatment is reflected in the survival of Norwegian patients.

Conclusions

This thesis presents evidences of immunomodulating and cytotoxic and antitumor effects of Andosan™ *in vitro* and in an animal model. Effects on immunological parameters were also found when this products was given in addition to ASCT to MM patients in a clinical study, These immunomodulatory effects together with the antitumor effect observed on primary myeloma cells ex vivo, could have contributed to a clinical improvement in the MM patients, which the suggested by a trend to longer OS in the Andosan™ group relative to

the placebo group. The mechanism of action of Andosan™ is unkown. However, GS-MC analyses of Andosan™ indicate content of steroids and ester-steroids in the extract, which have known anti-inflammatory effects and could partly explain the observed effects. Further studies can contribute to clarify whether Andosan™ may play a role as complementary treatment in defined cancer diseases.

Errata

Page 12- Figure 1.

The following should be added to the figure legend: “Open Access article- reprinting permitted”.

Page 13. Figure 2.

The following should be added to the figure legend: ”Reprinted from www. amazon.com (commercial advertisement).”

Page 15-16. Figure 3.

The following should be added to the figure legend: ” Reprinted from www.facebook.com (homepage of *Agaricus blazei* Murill).”

Page 17. Figure 4.

The following should be added to the figure legend: “Open Access article- reprinting permitted.”

Page 23-24. Figure 5.

The following should be added to the figure legend: “ Open Access article- reprinting permitted”.

Page 31. Figure 7.

The following should be added to the figure legend: “ Open Access article- reprinting permitted”.

Page 33-34. Figure 8.

The following should be added to the figure legend: “ Reprinted with permission from the publisher”.

Page 35. Figure 9.

The following should be added to the figure legend: “ Reprinted with permission from the publisher”.

Page 40. Figure 8

The figure should be renamed to figure 10.

The second last sentence in the text before the figure should be rewritten to: " ..Also, an increased frequency of the proliferation marker Ki67 is noted in the nuclei in the tumors (figure 10)..". The following should be added to the figure legend: " Reprinted with permission from the publisher".

Page 42. **Pathogenesis**

The last sentence in the paragraph should be rewritten to: ".. The interaction between plasma cells and the bone marrow is illustrated in Figure 11".

Page 43. Figure 8.

The figure should be renamed to Figure 11.

Page 54.

The sentence on top of the page " See figure below" should be changed to " See Figure 1." The figure should be named Figure 1, which should be inserted before the figure legend: " Cytotoxic effect on Andosan^{TM} on the myeloma cell lines…" .The following should be added to the figure legend: "Unpublished figure".

Page 59-60. Figure 1.

The following should be added to the legend: "Unpublished figure".

Page 64

Further perspectives.

The last sentence in the paragraph ("See figure below") should be rewritten to :" See Figure 1".

The figure below should receive the designation " Figure 1", followed by the legend: "For explication- see text above". The following should be added to the legend:" Unpublished figure".

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Stimulation of human monocytic cells by the medicinal mushroom *Agaricus blazei* Murill induces expression of cell surface markers associated with activation and antigen presentation

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Abstract

Agaricus blazei Murill (AbM) is an edible *Bacidiomycota phyllum* mushroom used in traditional medicine as a remedy against a wide range of diseases, including infection and cancer. It is rich in β-glucans and antitumor protein-glucan complexes, which have been shown to have stimulating effects on cells involved in innate immunity, such as monocytes, NK cells and dendritic cells. The present report shows that stimulation of monocyte-derived dendritic cells (MDDC) with an AbM-based extract, AndoSan™, induces modulation of expression of cell surface markers. Incubation of MDDC with 10% of AndoSan™ resulted in upregulation of CD1a, CD14, CD40, CD80, CD83 and CD86, *de novo* expression of CD69 and downregulation of CD11c expression. The modulation was time dependent. Our investigation shows the ability of AndoSan™ to immunomodulate and activate MDDC *in vitro*, and agrees with the recent findings of increased production of cytokines and chemokines in AndoSan™-stimulated MDDC cultures. Furthermore, it is demonstrated that AbM can stimulate human promonocytic THP-1 cells via TLR2 and thus induce activation and migration of transcription factor NF-κB from cytosol to the nucleus. It is therefore probable that binding to TLR2 is a significant mechanism behind AndoSan™ activation of MDDC.

Keywords: *Agaricus blazei* Murill, monocyte-derived dendritic cells (MDDC), cell surface markers, CD1, CD11, CD14, CD40, CD69, CD80, CD86

Introduction

Mushrooms are rich in β-glucans, which are a broad class of bioactive polysaccharides with strong immunomodulating properties, found in the cell wall of yeast, mushrooms and fungi [1]. Generally, immunostimulation by medicinal mushrooms occurs via antigen-presenting cells (APC) in the innate immune system, i.e. monocytes, macrophages and dendritic cells (DC). The cellular response is triggered by the detection of conserved microbial derived molecules, named pathogen-associated molecular patterns (PAMP), by pattern recognition receptors (PRR) on immune cells [2] such as Toll-like receptors (TLR) [3], which are situated on the surface of immune cells. The binding of PAMP to PRR results in release of proinflammatory and Th1 cytokines [4]. Toll-like receptors 1, 2, 4, and 6 are transmembrane receptors for bacteria and fungi expressed on the surface of monocytes and monocyte derived DC (MDDC) [5,6]. β-glucans from mushrooms and LPS from Gram-negative bacteria are classical ligands for Toll- like receptors 2 and 4 [7,8]. The stimulation of PRR induces activation and translocation of the transcription factor NF-kB from the cytosol to the nucleus. This translocation is important for the initiation of innate immunity and inflammation [9]. After PAMP- PRR binding, the APC engulf and degrade invading pathogens, and this process further stimulates innate and adaptive immunity through the secretion of cytokines and chemokines. This activation process also leads to presentation of processed antigen to naïve T cells, transforming them into Th1, Th2 or T regulatory cells [10].

The effects of medicinal mushrooms have been the subject of a large number of preclinical and clinical studies [11]. In particular, the potential role of medicinal mushrooms as adjuvant therapy for solid tumors and acute leukemia has been extensively investigated [12-15]. The edible *Bacidiomycota* mushroom *Agaricus* blazei Murill (AbM) grows naturally in an area near São Paulo, Brazil, where it has been used in traditional medicine against a variety of diseases, including infection and cancer [16]. Spores of AbM were taken to Japan in the mid-60s for commercial cultivation and research. AbM is rich in antitumor protein-glucan complexes [17], and β-glucans [18], which are potent stimulators of macrophages [19,20], granulocytes [21], natural killer (NK) cells [22] and DC [23]. The immunomodulatory properties and health effects of AbM have recently been reviewed [24].

DC are "directors" of the immune system and link together innate and adaptive immunity. They are primarily responsible for sensitization of naïve T cells to protein antigen *in vivo* [25]. Upon stimulation DC and other immune cells release signal

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substances such as cytokines and growth factors and express activation markers on the cell surface. DC are defined by their constitutional expression of the following cell surface markers: CD1a (LFA-1), the maturation marker CD83 [26] and CD205 –a general DC marker [27]. DC kill microbes, stimulate Th cells and regulate B cell Ig production [28]. AbM has been shown by others and us to have stimulatory effects *in vitro* on the production of proinflammatory cytokines in immune cells, including monocytic cells and MDDC [29-31]. The main purpose of the present study was to examine the modulatory effect of AbM on cell surface markers in MDDC. Our group has previously demonstrated that AbM stimulation results in activation and translocation from the cytosol to the nucleus of the transcription factor NF-κB through binding of AbM to TLR2 in promonocytic THP-1 cells. A similar mechanism has earlier been demonstrated by binding of LPS to TLR4 [32]. In the present study the role of TLR2 and TRL4 in the stimulation of promonocytic THP-1 cells by AbM was further examined.

Materials and methods

Cells

Monocyte derived dendritic cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats from blood donors at the Blood Bank of Oslo University Hospital,Ulleval, using a modified version of Sallusto's procedure. Shortly, PBMC were isolated by centrifugation of a buffy coat through a Lymphoprep® layer (Axis-shield PcC AS), and washed in Hank's Balanced Salt Solution (HBSS) before resuspension in RPMI 1640 containing 10% AB Rh^+ serum. The cells were incubated in air with 5% CO_2 at 37°C for 60-90 min in Nuncleon™ Surface cell culture bottles for adherence of monocytes, before washing off of loose cells with HBSS and continued cultivation in RPMI 1640 supplemented with streptomycin/penicillin, Glutamine-L (20 mM), GM-CSF (800 U/ml) and IL-4 (500 U/ml) (Fisher Scientific, fisher.no@thermofisher.com). Cell culture medium was changed on day 3 and also on day 6, when the cultures were shown to contain MDDC as determined by flow cytometry assay of DC-specific surface markers. During further experiments with the cells, IL-4 and GM-CSF was added to the medium to prevent the cells from reverting to monocytes [33].

Promonocytic cells

Cells of the human promonocytic cell line THP-1 were purchased from ATCC (Middlesex, UK). The cells were maintained in RPMI 1640 with 10mM HEPES, 50 mM β-mercaptoethanol, 1 mM sodium pyruvate, 2.5 mg/ml D-glucose, penicillin/streptomycin, 0.7 mM L-glutamine and 10% FCS. The cells were split every 3-4 days and kept at 5% saturated atmosphere at 37^{0}C.

Reagents

The commercial mushroom extract AndoSan™ and the pure AbM extract contained therein were both obtained from ACE Co Ltd., Gifu-ken, Japan, via Immunopharma AS, Oslo, Norway. According to the producer, the AndoSan™ extract is constituted of 82% AbM, 15% *Hericium erinaceum* (He) and 3% *Grifola frondosa* (Gf), all *Basidiomycetes* mushrooms. The LPS content of AndoSan™ was found to be <0.5 pg/ml [36]. *E. coli* LPS 055:GB5 (Cat. No. L6529) and phosphate-buffered saline (PBS) were purchased from from Sigma-Aldrich, St.Louis, MO, USA.

Experiments

The MDDCs were stimulated with the AbM-based extract AndoSan™ or with PBS (negative controls) for 24h and 48h and the expression of a range of cell surface markers associated with MDDC maturation, activation or function (see below), were examined by flow cytometry. MDDCs were also stimulated with LPS 1.5 µg/l (positive controls) and the expression of the cell surface markers CD69 and C86 were examined by flow cytometry after 24h.

Flow cytometry analysis was performed with a Becton Dickins FACSCalibur Canto II flow cytometer and the software, CellQuest (BD Bioscience, San Jose, CA, USA). MDDC cells were seeded into 6-well plates (Nuncleon) with AbM extract AndoSan™ in concentrations of 0% (=10% PBS control) and 10 % for 24h and 48h. Cells were stained by incubation for 15-20 minutes with FITC- or PE-labeled antibodies, including isotype controls, before washing with PBS, resuspension in a PBS-EDTA-BSA-glucose buffer, and examination in the flow cytometer.

The following FITC- or PE-labeled mouse monoclonal Abs were used in flow cytometry analyses. CD1a (cat.no. 555806), CD11b (347557), CD11c (IM1760), CD45/CD14 BD Leucogate™ (342408), CD40 (555588), CD69 (555531), CD80 (557227), CD83 (556910), CD86 (555657), and appropriate IgG1,k (555748) and IgG2b, k (555742) isotype controls, were all purchased from Beckman Coulter Co., Marseille, France.

THP-1 cells were seeded in 6 well plates at a concentration of $0.7x10^6$ cells/ml and incubated with 10% of AbM or PBS for 1h. Then $0.5x10^6$ cells were stained with TLR2 (cat.nr. MCA2152PE, AbD Serotec) or TLR4 (cat.nr. MCA2061PE, AbD Serotec) and unstained cells were used as control. The samples were analysed on a Becton Dickinson FACSCalibur flow cytometer.

Western Blotting was performed in THP-1 cells stimulated with 10% AbM for 1h. Nuclear extract kit (cat. Nr. 40010, Active Motif) was used to isolate nuclear and cytoplasmatic extract from the THP-1 cells. The protein extracts were applied to the wells in a 12-15% acrylamide gel and separated by electrophoresis. The Precision Plus Kaleidoscope Standards (cat.nr. 161-0375, BioRad) were used to detect protein size, and NF-κB control cell extract (cat.nr. 9242, Cell Signaling) was used as a positive control (not shown in blot). TransBlot SD semi Dry Transfer cells from Bio Rad was used to transfer the proteins to an Immuno-Blot polyvinyliden difluorid membrane (cat.nr. 162-0177 BioRad). The blot was blocked over night in

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a 5% solution of dried milk before further incubation with primary and secondary antibodies. NF-κBp65 (22B4) Rabbit mAb (cat. nr. 4764 Cell Signaling) was used to detect the levels of the house keeping protein α-tubulin (11H10). Anti-rabbit HRP-linked antibody (cat.nr. 2125, Cell Signaling) were used as a secondary antibody. The blot was incubated in Immuno-star HRP substrate (cat.nr. 170-5040 BioRad) before blot images were taken in a Molecular Imager ChemiDocXRS+ system. All experiments were performed minimum twice.

Presentation of data

Levels of surface markers on MDDC as determined by mean fluorescence intensity , are shown in flow cytometry diagrams. Negative controls were either cells stimulated with AndoSan™ but stained with appropriate fluorochrome-labeled isotype control, or cells stimulate with PBS and stained with the proper labeled antibody to the cell surface marker examined. Positive controls were cells stimulated with LPS and stained with proper labelled Ab to marker.

Results of the stimulation experiment with AbM on the human promonocytic cell line THP-1 are presented as protein bands in Western Blotting.

Results

Stimulation of MDDCs with AndoSan™ 10% for 24h resulted in neoexpression of CD69 (**Figure 1**), a strong upregulation of CD1a, CD14, CD40, CD80, CD83 and CD86 (**Figures 2A-2F**) and a downregulated expression of CD11c (**Figure 3**). No changes in these markers were found in MDDC incubated with PBS. Stimulation with 0.5 µg/ml of *E.coli* LPS for 24h led to a similar degree of upregulation of CD86 (**Figure 2F**) and neoexpression of CD69 (not shown) as seen with AbM. For most of the upregulated markers there was a further increase of the expression after 48h incubation (data not shown). No changes in these markers were seen after PBS stimulation.

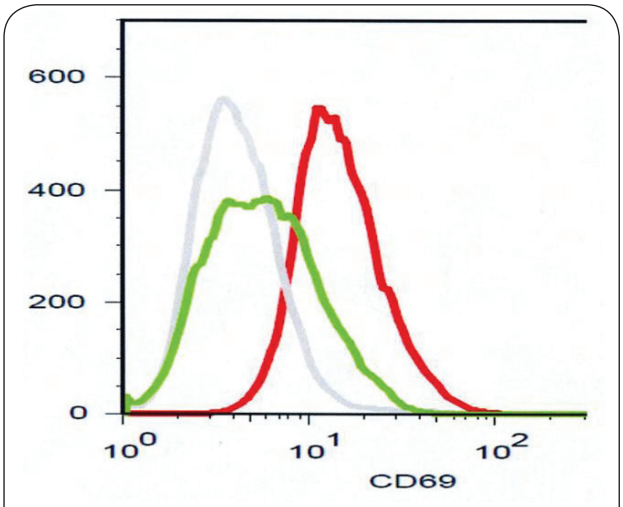

Figure 1. Neoexpression of CD69 on MDDC after incubation with AndoSan™.
Flow cytometry examination showing neoexpression of CD69 on MDDC incubated with AndoSan™ for 24h (red curve) compared to incubation with PBS (control) (green curve). The cells were stained with mouse monoclonal FITC/PE- labelled antibodies or isotype control (gray curve).

Western blotting performed after incubation of 10% AbM with promonocytic THP-1 cells showed that NF-kB p-65 was absent from the nucleus before incubation but present in the nucleus 1h after incubation (**Figure 4**). Control for the mRNA formation in the cells was the housekeeping gene product, a-tubulin. Addition of anti-TLR2, but not anti-TLR4, antibodies prior to incubation with AbM inhibited the translocation of NF-kB p-65 from cytosol to the nucleus, but did not affect expression of α-tubulin.

Discussion

The main purpose of the study was to investigate if the AbM-based extract, AndoSan™ activates MDDC as seen from modulation of cell surface marker expression. We detected neoexpression of CD69 (**Figure 1**) and increased expression of CD1a, CD14, CD40, CD80, CD83 and CD86 (**Figures 2A-2E**) after stimulation with AndoSan™ compared to incubation with PBS. Interestingly, stimulation with this mushroom extract induced a similar upregulation of CD69 (associated with activation) and CD86 (associated with antigen-presentation) as did stimulation with *E.coli* LPS. These findings are in line with the results from a previous investigation, where stimulation with AndoSan™ on MDCC was found to increase production of cytokines in a similar manner as stimulation with LPS, and, in some cases, in even higher quantities than did stimulation with LPS [**34**]. The β (or leukocyte)- integrins, i.e. the CD11/CD18 complex, including CD11b and CD11c receptors are expressed on both macrophages and MDDC and play a role in cell-to cell adhesion [**35**]. These antigens are rapidly upregulated after activation and promote strong attachment of leukocytes to the vascular endothelium and subsequent transendothelial migration. We found that both CD11b and CD11c were expressed on MDDC before stimulation with AbM. Previously, we have reported that incubation of whole blood with AndoSan™ led to upregulation of CD11b on monocytes and granulocytes accompanied by a reciprocal decrease in CD62L due to shedding [**36**]. In contrast, stimulation of MDDC with AndoSan™ resulted in a strong down-regulation of CD11c (**Figure 3**). This was most probably not due to increased expression and subsequent shedding of this antigen. Rather, it could be caused by binding of AbM sugar moieties to a similar lectin site of CD11c as shown for CD11b [**3**] and subsequent hiding of the antigen epitope for detection by its fluorescence-labeled antibody when examined in a flow cytometry. However, this contrasts with the virtually unchanged signal for CD11b and the increased CD11c levels reported after stimulation of MDDC with another β-glucan-rich mushroom, *Ganoderma lucidum* [**37**]. Possibly, the reduced CD11c expression secondary to AbM stimulation might play

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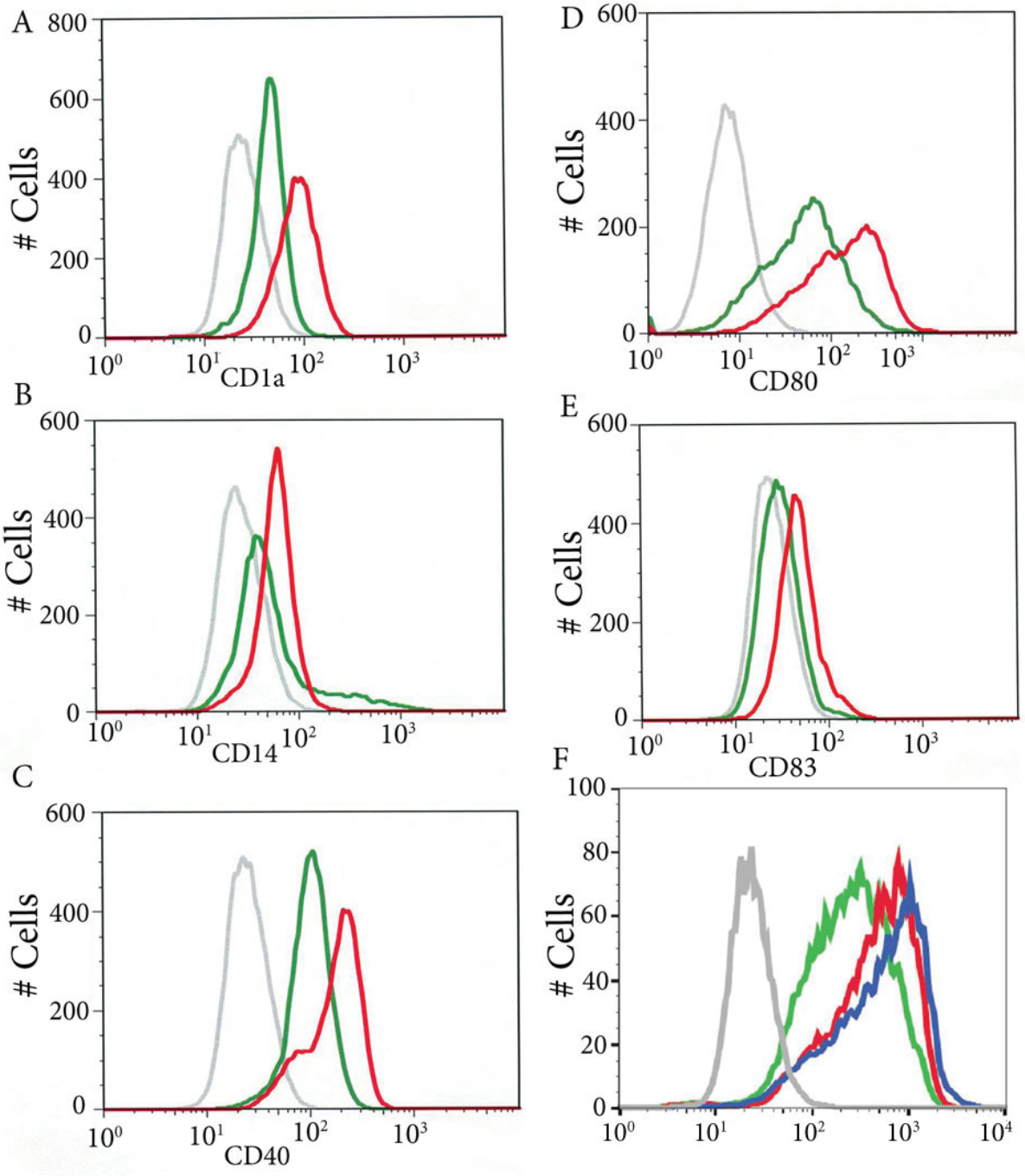

Figure 2. Upregulation of several cell surface markers on MDDC after incubation with AndoSan™.
Flow cytometry examination showing upregulation of CD1a, CD14, CD40, CD80, CD83 and CD86 in MDDC incubated with AndoSan™ for 24h (red curve) compared to incubation with PBS (control) (green curve). The PBS controls show that the cells were true MDDC. The cells were stained with mouse monoclonal FITC/PE-labelled antibodies or isotype control (gray curve). In Figure 2F (CD86) there is in addition shown expression of the surface marker after LPS stimulation (blue curve). NB: Flow cytometry findings after stimulation of AndoSan™ on MDCC for the markers CD1a, CD14 and CD80 have been published previously [**36**]. Permission to publish the modified curves in the present article has been granted by the copyright holder Elsevier LTD.

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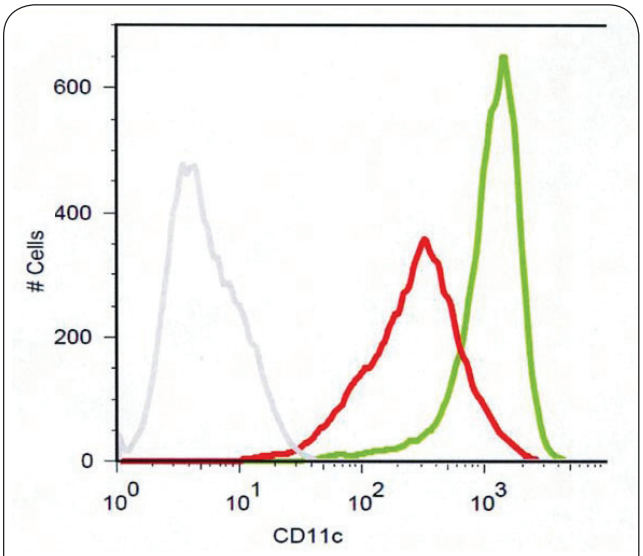

Figure 3. Downregulation of CD11c on MDDC after incubation with AndoSan™.
Flow cytometry examination showing down regulation of CD11c on MDDC incubated with AndoSan™ for 24h (red curve) compared to incubation with PBS (control) (green curve). The cells were stained with mouse monoclonal FITC/PE- labelled antibodies or isotype control (gray curve).

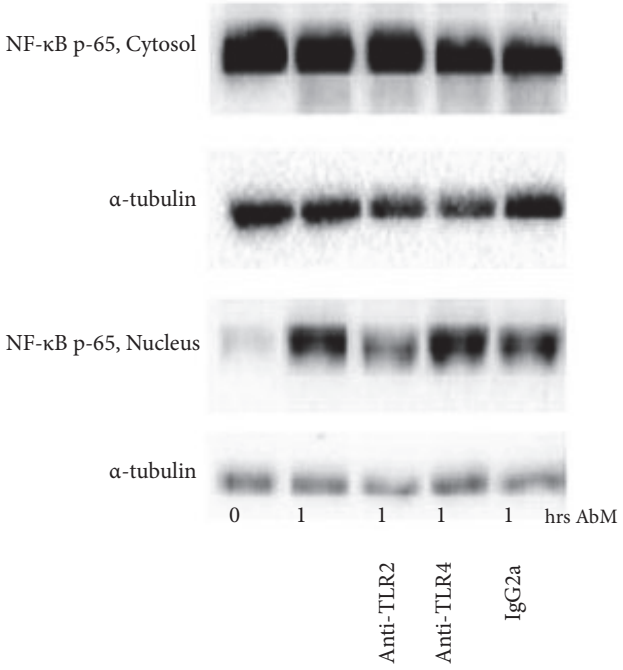

Figure 4. Activation of nuclear transcription factor NF-κB.
As shown by its migration from cytosol to the nucleus after stimulation with AbM for 1h of human promonocytic THP-1 cells. Western blotting was performed on cytoplasmic and nuclear fractions from THP-1 cells stimulated with 10% of AbM for 1h in the absence or presence of either anti-TLR2, anti-TLR4 or IgG2a (isotype control) antibodies. NF-κB control cell extract was used as a positive control (not shown) for NF-κB, and α-tubulin was a control product of this house-keeping gene for protein levels. The lower panels show translocation of NF-kB from cytosol to nucleus in the THP-1 cells. This translocation was inhibited by preincubation with anti-TLR2 but not anti-TLR4 antibody, showing that the NF-kB translocation was mainly induced via TLR2.

a role in reducing MDDC transendothelial migration *in vivo*. If so, this might promote a signal for the MDDC to remain and function in the blood vessels.

We have previously shown in transfected HEK239 cells that both AbM and AndoSan™ induced activation of NF-κB via TRL2. We also found in promonocytic THP-1 cells, which bear both TLR2 and TLR4, that AbM stimulation induced rapid activation and cytoplasma-to-nucleus translocation of transcription factor NF-κB [**32**]. The second aim of the study was to investigate further the mechanism of stimulation in promonocytic THP-1 cells, and is was demonstrated that AbM stimulation of these cells is mediated via TLR2 but not via TRL4.

CD69, which is seen on activated T, B and NK cells, was found to be expressed *de novo* on AbM-stimulated MDDC (**Figure 1**). Together with the upregulation of CD86 this may suggest a possible antigen presentation enhancing function for AbM on MDDC, which may explain some of the properties reported for AbM in the defense against infections and cancer. A similar upregulation for CD86 has been observed after stimulation of MDDC with β-glucan from *Ganoderma lucidum,* although the stimulation in that case occurred via TLR4 [**38**]. In addition, upregulation of CD14 was also noted on AbM stimulated MDDC. The CD14 molecule is associated with TRL4 binding of LPS [**39**]. An increased CD14 expression could therefore indicate an enhanced ability of these cells to kill Gram-negative bacteria. This may be a possible explanation of the protective effect reported earlier on AndoSan™ against Gram-negative sepsis in a mouse model [**40**].

Conclusions

The mushroom extract AndoSan™ upregulated CD1a, CD14, CD40, CD80, CD83 and CD86, neoexpressed CD69, and down-regulated CD11c in MDDC. The upregulation of these markers shows that AndoSan™ indeed activates MDDC *in vitro*. Moreover, upregulation of CD86 together suggests an increased antigen-presenting property of the stimulated MDDC. The results are in line with data from a previous study demonstrating that stimulation of MDDC by AbM leads to increased production of proinflammatory, chemotactic and Th1-type cytokines. The results of a Western blot investigation on AbM-stimulated THP-1 promonocytic cells indicate that a significant part of the action of AbM on MDCCs *in vitro* may be due to stimulation via TLR2.

Competing interests

Jon-Magnus Tangen and Anne Merethe Tryggestad declare that they have no competing interests. Geir Hetland is a stockholder in the company Immunopharma A/S which imports AndoSan™.

Authors' contributions

Authors' contributions	JMTA	AMAT	GH
Research concept and design	--	--	✓
Collection and/or assembly of data	--	✓	✓
Data analysis and interpretation	✓	--	✓
Writing the article	✓	✓	✓
Critical revision of the article	--	--	--
Final approval of article	✓	✓	✓
Statistical analysis	--	--	--

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Clinical Study

Immunomodulatory Effects of the *Agaricus blazei* Murrill-Based Mushroom Extract AndoSan in Patients with Multiple Myeloma Undergoing High Dose Chemotherapy and Autologous Stem Cell Transplantation: A Randomized, Double Blinded Clinical Study

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Forty patients with multiple myeloma scheduled to undergo high dose chemotherapy with autologous stem cell support were randomized in a double blinded fashion to receive adjuvant treatment with the mushroom extract AndoSan, containing 82% of *Agaricus blazei* Murrill (19 patients) or placebo (21 patients). Intake of the study product started on the day of stem cell mobilizing chemotherapy and continued until the end of aplasia after high dose chemotherapy, a period of about seven weeks. Thirty-three patients were evaluable for all study endpoints, while all 40 included patients were evaluable for survival endpoints. In the leukapheresis product harvested after stem cell mobilisation, increased percentages of Treg cells and plasmacytoid dendritic cells were found in patients receiving AndoSan. Also, in this group, a significant increase of serum levels of IL-1ra, IL-5, and IL-7 at the end of treatment was found. Whole genome microarray showed increased expression of immunoglobulin genes, Killer Immunoglobulin Receptor (KIR) genes, and HLA genes in the *Agaricus* group. Furthermore, AndoSan displayed a concentration dependent antiproliferative effect on mouse myeloma cells *in vitro*. There were no statistically significant differences in treatment response, overall survival, and time to new treatment. The study was registered with Clinicaltrials.gov NCT00970021.

1. Introduction

Multiple myeloma is a neoplastic disorder caused by malignant transformation of plasma cells. The main clinical features are bone marrow failure, lytic bone lesions, and renal insufficiency. The age adjusted incidence of multiple myeloma is 4/100 000 and median age at diagnosis is 70 years [1]. High dose chemotherapy with autologous stem cell support is standard first line treatment for patients below the age of 65–70 years, taking into consideration patients' general condition and comorbidity [2]. The majority responds well to the initial treatment, but all patients eventually relapse. In recent

years, the introduction of three new drugs, thalidomide, bortezomib, and lenalidomide, has significantly improved the treatment results, but multiple myeloma remains an incurable disease. In patients below 65 years of age, 10 years survival is currently estimated to about 30% [3].

Agaricus blazeii Murrill (AbM) is an edible Basidiomycetes mushroom, which grows naturally in Piedade outside Sao Paulo in Brazil. It is widely used locally as a food supplement and as treatment for various medical conditions [4]. In the 1960s, AbM was brought to Japan for industrial cultivation and this became the starting point for scientific research. The fruiting body of AbM is rich in β-glucans, which is a potent stimulant of the innate immune system. β-Glucan from AbM has been shown to have an antitumor effect both *in vitro* (fibrosarcoma [5], ovarian cancer [6], hepatocarcinoma [7], and leukemia cells [8]) and in animal models (fibrosarcoma [9], multiple myeloma [10], and lung cancer [11]). It has therefore been assumed that the medicinal effect of AbM is mainly due to the immunostimulatory effect of β-glucans [12]. Ovarian cancer patients receiving AbM in addition to chemotherapy were found to have an improved quality of life and a higher NK-cell activity than patients receiving only chemotherapy [13]. Our group has previously performed a number of *in vitro*, preclinical, and clinical studies using the commercial Japanese product, AndoSan, which contains 82% of AbM together with two other mushrooms, *Grifola frondosa* and *Hericium erinaceus*. An increased production of cytokines and chemokines was demonstrated after incubation with AndoSan in cultures with human monocytes, umbilical vein endothelial cells, and monocyte derived dendritic cells (MDDC) [14, 15]. Incubation with AndoSan in MDDC cultures was shown to increase the expression of cell surface markers associated with activation and antigen presentation [16, 17]. Furthermore, in an mRNA assay on monocytic cells incubated with AndoSan, there was an upregulation of genes related to immune function, in particular in genes connected with proinflammatory cytokines [18]. In contrast, in patients with chronic hepatitis C receiving treatment with AndoSan, genes involved in apoptosis and cell proliferation were found to be upregulated but not genes related to immune function [19]. Oral intake of AndoSan was found to have an immunosuppressive effect both in healthy volunteers [20] and in patients with inflammatory bowel disease [21]. The reason for the difference between *in vitro* and *in vivo* effect on immune function may possibly be due to differential absorption from the gut of substances with immunomodulating effects [20]. An extended search in the Medline and PubMed databases failed to show any reports of toxic effects of AbM. Also, herb interaction studies with an *Agaricus* extract, later named AndoSan, demonstrated a very low inhibition of cytochrome P-450 metabolism (less than that for green tea), making clinically relevant adverse effects unlikely [22].

Based on the data cited above and *in vitro* experiments included in this report, showing an antiproliferative effect of AndoSan on mouse myeloma cells, we decided to investigate immunomodulating and clinical effects of AndoSan given as adjuvant therapy to patients with multiple myeloma scheduled to undergo high dose chemotherapy with autologous stem cell support.

2. Materials and Methods

2.1. Study Design. Patients with newly diagnosed multiple myeloma who had completed induction treatment and were scheduled to undergo stem cell mobilisation followed by high dose chemotherapy with autologous stem cell support at Oslo University Hospital, Norway, were eligible for the study. The patients received written and oral information about the study by the treating physician. Upon written consent, patients were randomized in a double blinded fashion to receive either AndoSan or placebo orally, 60 mL daily, starting from the day of stem cell mobilisation and continuing until the end of aplasia after high dose chemotherapy, a period of approximately seven weeks. Randomisation was performed by a study nurse drawing an envelope from a preprepared stack, containing a number from 1 to 50, in a random fashion. Each number corresponded to an allocated treatment (*Agaricus* or placebo), which was determined beforehand by a flip of a coin and known only to the study nurse. The study product (*Agaricus* or placebo) was prepared by the study nurse in identical dark glass bottles, identified only by the patient's study number. Thus, the content of the bottles was known to the study nurse but was blinded to the patients and the rest of the hospital staff.

The primary end points were (1) changes in serum levels of cytokines, chemokines, and growth factors in peripheral blood, (2) differences in expression levels of genes involved in immune activation by whole genome assay, both measured on the day of inclusion and at the end of intake of the study product, and (3) differences in the stem cell harvest product of a number of mononuclear cell subsets associated with the immune system. All biological samples were kept at -20°C and analyzed together at the end of the study. The blinding was unravelled after all laboratory tests had been performed. The secondary end points were (1) clinical response to treatment, (2) time in neutropenia, (3) days with body temperature above 38.0°C, (4) days with i.v. antibiotics after stem cell infusion, (5) time to new treatment, (6) overall survival, and (7) quality of life. The basis for the sample size ($n = 40$) was the results of two earlier studies showing significant changes in the levels of cytokines in healthy volunteers ($n = 14$) [20] and in patients with ulcerative colitis ($n = 10$) and Crohn's disease ($n = 11$) [21] after intake of AndoSan. The data were collected by the principal investigator and stored at a research file at the server of Oslo University Hospital. The study was approved by the Regional Committee for Medical and Health Research Ethics (REC South East). The Norwegian Medicines Agency was notified of the study according to national regulations.

2.2. Study Product. The commercial mushroom extract AndoSan, produced by the company ACE Ltd., Japan, and distributed by Immunopharma AS, Norway, was used as source of AbM. The extract contains the following Basidiomycetes mushrooms: 82.4% of AbM, 14.7% of *Hericium*

erinaceus, and 2.9% of *Grifola frondosa*. AndoSan is registered as a food product in Japan, EU, and Norway.

Placebo was water with added color.

2.3. Chemotherapy. Stem cell mobilisation was induced by cyclophosphamide 2 g/m^2 i.v and G-CSF. Stem cell harvesting by leukapheresis was started when the CD34+ cells in peripheral blood were >20 × 10^9/L, that is, on days +10–+12 after stem cell mobilizing chemotherapy. Aliquots of the leukapheresis product were kept frozen and analysed by flow cytometry at the end of the study. High dose chemotherapy was melphalan 200 mg/m^2 i.v. on day +2, followed by reinfusion of autologous stem cells on day 0 [23].

2.4. Proliferation Assay. A ^{3}H-thymidine incorporation assay was performed on MOPC315.BM cells, that is, mouse myeloma cells [24]. The cells were suspended in RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Diegem, Belgium) and 1% Penicillin/Streptomycin (Sigma-Aldrich), plated in 96-well plates (5×10^4/100 μL/well) and cultured for 24 hours at 37°C and 5% CO_2 in the presence of different AndoSan concentrations. For the last 16 hours of culture, 0.17 μCi of [Methyl-^{3}H] thymidine (Perkin Elmer, Zaventem, Belgium) was added to each well. DNA was harvested on Multiscreen Harvest Plates (Millipore, Carrightwohill, Cork, Ireland) using Filter Mate Harvester (Perkin Elmer). Plates were dried for 3-4 hours before adding 25 μL/well of Microscint O (Perkin Elmer) followed by radioactivity measurement (c.p.m.) with TopCount NXT Microplate Scintillation Counter (Perkin Elmer).

2.5. Quantitation of Cytokine Levels. The following cytokine, chemokine, and growth factor serum levels were measured at the day of inclusion and 1–3 days after the end of the intake of AndoSan, using multiplex bead-based sandwich immunoassay technology (Bio-Rad Laboratories AB, Sundbyberg, Sweden), strictly following the manufacturers instruction: IL-1β, IL-1ra, IL-4, IL-5, IL-6, IL-7, IL-8, IL-13, Eotaxin, G-CSF, IFN-γ, IP 10, MCAF, MP1-α, MP1-β, PDGF, RANTES, and TNF-α.

2.6. Identification of T-Cell Subsets, NK-Cells, and Dendritic Cell Subsets in the Leukapheresis Product. The leukapheresis products were suspended in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum and were analyzed using flow cytometry. Eight-colour analyses were performed for the identification of the lymphocyte subsets with the following monoclonal antibodies: CD2, CD3, CD4, CD5, CD7, CD16, CD25, CD56, CD45, CD45RA, CD45RO, CD9, CD127, CCR7, and HLA-DR antigens. All antibodies were purchased from BD Biosciences (San José, CA, USA) except for anti-CD8, anti-CD56, and anti-CD127 which were purchased from Beckman Coulter (Brea, CA, USA); anti-CCR7 was purchased from R&D systems (MN, USA) and anti HLA-DR from Biolegend (San Diego, CA, USA). The Blood Dendritic Cell Enumeration Kit (Miltenyi Biotech GmbH, Bergish Gladbach, Germany) was used according to the supplier's protocol to determine plasmacytoid dendritic cells, type 1 and type 2 myeloid dendritic cells. Flow cytometry analysis was performed on the LSR II instrument (BD Biosciences). Data analysis was performed using the Flow-Jo software (Tree Star, Ashland, OR, USA).

2.7. Gene Expression Studies. Bone marrow aspirate for gene expression studies was taken on the day of inclusion and 1–3 days after end of intake of the study product. Microarray analyses were performed using the Affymetrix GeneChip Human Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA, USA), which contains more than 28,000 gene transcripts. 150 ng of total RNA was subjected to Ambion WT Expression Kit (Ambion/Life Technologies, Carlsbad, CA, U.S.) and GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA) following the manufacturers' protocols for whole genome gene expression analysis.

For comparison of gene expressions, a two-way ANOVA model was used. All raw intensities of microarray datasets were quantile normalized [25]. Genes with low expression variation, that is, maximum to minimum intensity less than twofold difference, were excluded leaving 7564 genes. Subsequently, a pair-wise Fisher's linear discriminant analyse [26] was used to select the top two percentages of the most differently expressed genes (i.e., 152 genes) between control and the *Agaricus* group. The selected genes were classified into four clusters by using K-means clustering algorithm [25]. In addition, microarray data were analysed through the use of Ingenuity Pathway Analysis (http://www.ingenuity.com/).

2.8. Clinical Data

2.8.1. Classification. The patients were classified at inclusion according to the international staging system for multiple myeloma [27].

Treatment response was assessed by changes in the serum level of the M-component from start of induction until three months after high dose chemotherapy according to the international uniform response criteria [28].

Time to regeneration of neutrophils was the time between infusion of stem cells and the first day of a stable neutrophil count of $0.5 \cdot 10^9$/L or above.

Time to new treatment was the time between inclusion and start of second line chemotherapy following progression or follow-up as of July 1, 2014.

Overall survival was the time between inclusion and follow-up as of July 1, 2014, or death.

2.8.2. Quality of Life. Health related quality of life was measured at start of the study and three months after end of aplasia using the QLQ-C 30 questionnaire validated for multiple myeloma [29].

2.9. Statistics. Differences between changes in serum levels of cytokines, chemokines, and growth factor before and after treatment in the two treatment groups were calculated by the Independent Samples t-test on the IBM SPSS Statistics 21 program. The same program was used for calculation of differences in cell surface expression of leukocyte antigens

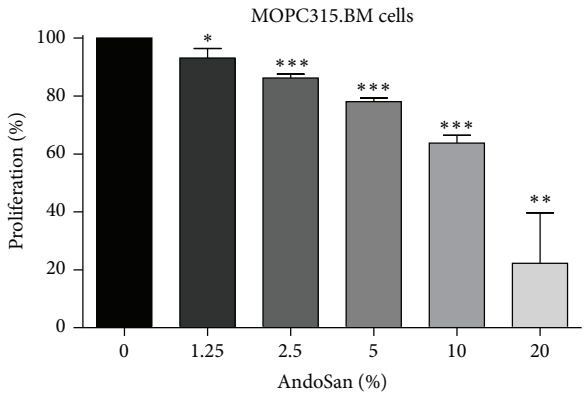

Figure 1: Effect of AndoSan on the proliferation of a murine multiple myeloma cell line *in vitro*. Proliferation of MOPC315.BM cells was assessed by ^{3}H-labelled thymidine incorporation in the presence of different AndoSan concentrations (1.25%–20%). Results are expressed in percentage of proliferation (mean ± SD) relative to MOPC315.BM cells cultured without AndoSan (= 100%) and represent 3 independent experiments. Within each experiment, proliferation was assessed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (unpaired Student's *t*-test).

in the leukapheresis product. The survival analyses were performed by the Kaplan-Meier test. Statistical methods used in gene expression analyses are reported under that section.

3. Results and Discussion

3.1. Myeloma Proliferation Assay. AndoSan significantly inhibited the proliferation of MOPC315.BM murine myeloma cells *in vitro* at a concentration of 1%. The observed inhibition was dose dependent (Figure 1).

3.2. Patient Number. From beginning of August 2009 until end of November 2010, 44 consecutive patients were invited to participate in the study, and 40 patients accepted. Nineteen were randomized to the *Agaricus* group and 21 to the placebo group. Three patients in the *Agaricus* group and four patients in the placebo group later withdrew from the study. Consequently, the total number of patients completing the study was 33, 16 in the *Agaricus* group and 17 in the placebo group. All included patients were evaluated for treatment response and survival, except for two patients with nonsecretory disease in each group who could not be evaluated for treatment response. Patients' characteristics are shown in Table 1. The inclusion was stopped according to schedule as the stipulated sample size had been reached.

3.3. Cytokines, Chemokines, and Leucocyte Growth Factors. A significant increase in serum levels of IL-1ra, IL-5, and IL-7 from inclusion until the end of intake of the study product was observed in the *Agaricus* group. No significant differences were seen in any of the other cytokines, chemokines, and growth factors (Table 2).

3.4. Cell Surface Markers in the Leukapheresis Product. Significantly higher percentages of Treg cells (CD4+, CD127d+,

Table 1: The distribution of the patients in the two study groups. The clinical stage at inclusion according to the International Staging System [27] is indicated.

(a)

Agaricus ($n = 19$)		
M/F	Age	Stage
M	65	II
M	56	I
M	36	I
F	61	I
M	63	I
F	65	II
M	59	I
F	64	II
M	56	III
M	46	III
M	62	II
M	66	I
M	65	I
F	48	II
F	49	I
M	64	I
M*	59	II
F*	59	II
F*	59	II

*Patients who withdrew from the study.
M/F = 12/7; mean age = 57,4; I = 9, II = 8, and III = 2.

(b)

Placebo ($n = 21$)		
M/F	Age	Stage
M	56	II
M	42	II
M	55	III
M	61	III
M	61	II
M	62	II
F	44	III
M	60	II
M	56	II
F	62	I
M	58	II
F	56	II
M	52	II
M	61	I
M	55	I
M	52	II
M	51	I
M*	63	I
M*	63	II
F*	54	III
F*	52	III

*Patients who withdrew from the study.
M/F = 12/6; mean age = 56,6; I = 5, II = 11, and III = 5.

Table 2: Mean serum levels and range are shown for cytokines, chemokines, and growth factors (in pg/mL) at inclusion and at the end of intake of study product for the *Agaricus* ($n = 16$) and the placebo group ($n = 17$). The statistical relationship of the differences of the means of the two groups is shown in the column "A/P."

	Agaricus			Placebo			A/P
	Start Mean	End Mean	Difference	Start Mean	End Mean	Difference	*P* value
IL-1ra	53.21	84.51	31.30	94.79	69.46	−25.33	$P = 0.02$
(Range)	(7.21–125.52)	(21.9–268.37)		(21.69–268.37)	(6.10–167.65)		
IL-4	1.57	2.01	0.44	1.82	1.84	0.02	n.s.
(Range)	(0.39–3.31)	(0.75–4.17)		(0.54–5.27)	(0.42–4.97)		
IL-5	1.66	2.89	1.23	2.75	2.21	−0.54	$P = 0.05$
(Range)	(0.02–4.89)	(0.19–9.37)		(0.36–8.29)	(0.43–5.60)		
IL-6	4.65	7.71	3.07	9.55	10.39	0.84	n.s.
(Range)	(0.03–11.32)	(0.88–18.40)		(2.29–28.67)	(2.60–32.82)		
IL-7	4.77	6.91	2.12	6.52	6.12	−0.40	$P = 0.05$
(Range)	(1.37–8.30)	(2.61–14.06)		(2.09–10.41)	(2.34–13.18)		
IL-8	12.59	15.14	2.54	13.49	15.41	1.91	n.s.
(Range)	(4.13–27.89)	(6.82–48.34)		(5.74–26.00)	(6.10–38.79)		
IL-13	5.58	5.98	0.40	6.78	9.27	2.49	n.s.
(Range)	(0.87–20.94)	(0.02–13.45)		(0.87–12.48)	(0.87–39.33)		
Eotaxine	93.11	105.31	12.20	162.33	112.39	−49.94	n.s.
(Range)	(0.11–247.02)	(16.59–229.25)		(23.02–600.07)	(11.00–368.73)		
G-CSF	19.06	21.94	2.89	22.34	19.54	−2.79	n.s.
(Range)	(5.60–41.66)	(9.63–38.64)		(9.99–34.41)	(7.82–33.92)		
gammaIFN	55.41	75.36	19.96	65.69	62.06	−3.62	n.s.
(Range)	(3.24–179.72)	(17.25–161.35)		(13.32–205.88)	(1.64–156.12)		
IP10	3198.89	3647.61	448.71	3230.03	3677.51	447.40	n.s.
(Range)	(1047.70–8564.00)	(391.37–12899.26)		(562.41–9314.66)	(277.79–13636.42)		
MCAF	63.73	43.46	−19.27	50.17	43.98	−6.20	n.s.
(Range)	(8.14–236.81)	(13.89–97.58)		(15.37–90.94)	(19.88–134.84)		
MIPa	50.46	42.48	−7.98	62.87	46.69	−16.16	n.s.
(Range)	(28.88–71.61)	(21.56–84.55)		(17.66–238.61)	(21.73–79.61)		
PDGF	426.31	450.75	24.44	454.23	*158.52*	−295.71	n.s.
(Range)	(75.90–1327.12)	(19.06–3841.38)		(45.84–1632.87)	(20.99–309.16)		
RANTES	10217.89	7497.97	−2719.93	6496.38	4672.52	−1823.86	n.s.
	(1846.39–30848.81)	(1595.65–29043.80)		(644.32–23964.69)	(119.20–10435.40)		
TNF alpha	6.29	12.35	6.07	10.52	12.05	1.53	n.s.
	(0.97–21.66)	(0.22–42.11)		(0.97–41.87)	(0.22–50.85)		

and CD25+) and plasmacytoid dendritic cells (CD303+) were noted in the *Agaricus* group compared to the placebo group (Table 3).

3.5. Gene Expression Studies. Gene expression studies were performed for eight patients in the *Agaricus* group and six patients in the placebo group at the time of inclusion and at the end of study. The selected differentially expressed genes were grouped in four clusters using *K*-means clustering algorithm. In cluster three are located several immunoglobulin related genes (IGKC, IgHV4-31, and IGKC) and genes related to Natural Killer cells, that is, Killer Immunoglobulin Receptors (KIR2DL3 and KIR2DL4). A low level of expression was noted for these genes in the control group and a high level of expression was shown in the *Agaricus* group (Figure 3). The Ingenuity system for phenotype-specific clustering of genes demonstrated upregulation of endosomatic HLA genes (Figure 4) and the plasma membrane CD86 gene (not shown) in the *Agaricus* group relative to the placebo group. Furthermore, this analysis showed a downregulation of IL-7 and CCL2 (MCP-1) genes in the *Agaricus* group relative to placebo group, whereas expression of IL-5 gene was unaltered (data not shown).

The records from the gene expression studies are registered in GEO (record number GSE 60869).

3.6. Treatment Responses. Treatment response could not be formally evaluated in two patients in each group because of

Table 3: T-lymphocyte subsets and dendritic cell subsets.

	Agaricus Mean	(*n* = 16) Range	Placebo Mean	(=17) Range	*P* value
% of T-lymphocytes					
CD3+ T cells	86.2	(58.9–94.2)	86.7	(71.7–95.3)	n.s.
CD4+ T cells	50.8	(20.0–80.4)	51.8	(7.10–73.6)	n.s.
CD8+ T cells	40.4	(10.9–67.9)	39.6	(15.7–83.3)	n.s.
% of CD4+ T cells					
Naive (CD45RA+/CD27+)	24.7	(5.5–64.0)	29	(8.8–51.4)	n.s.
Central memory (CD45RA−/CD27+)	40.8	(20.3–72.3)	46.7	(30.0–73.5)	n.s.
Effector memory (CD45RA−/CD27−)	19.2	(3.1–37.2)	21.8	(5.5–38.8)	n.s
Terminally differentiated memory (CD45RA+/CD27−)	6	(0.3–38.5)	3.1	(0.2–9.4)	n.s
T reg = (CD4+/CD127d+/Cd25+)	11.8	(4.5–18.2)	9	(4.0–17.8)	$P = 0.04$
HLA-DR+ CD4+	29.6	(13.4–55.7)	26.6	(7.9–55.0)	n.s
% of CD8+ T cells					
Naive (CD45RA+/CD27+)	27.8	(2.4–69.9)	31.4	(5.9–62.5)	n.s
Central memory (CD45RA−/CD27+)	17.3	(1.5–32.8)	20.1	(3.2–43.5)	n.s.
Effector memory (CD45RA−/CD27−)	22.9	(4.4–51.7)	16.8	(4.6–49.3)	n.s.
Terminally differentiated memory (CD45RA+/CD27−)	32.8	(1.4–55.7)	31.7	(5.4–79.0)	n.s
HLA-DR+ CD8+	39.1	(7.5–71.6)	36.2	(5.9–69.3)	n.s
Others					
%NK cells (CD2 or CD7+ CD3−)	8.4	(0.9–35.8)	6.7	(1.7–26.7)	n.s.
% CD56b+	9.3	(0.7–35.5)	10.7	(0.1–32.7)	n.s.
% CD56b+ CD16+	70.6	(42.2–94.0)	69.4	(39.9–95.5)	n.s.
%CD56−CD16+	6.4	(0.1–34.1)	2.6	(0.1–15.3)	n.s.
%CD94+	65.5	(25.9–96.4)	60.8	(22.1–84.7)	n.s.
% of all cells except for CD14+ monocytes and CD19+ cells					
BDCA1 (CD1c+)	0.6	(1.0–1.3)	0.6	(0.1–2.4)	n.s.
BDCA2 (CD303)	1.1	(0.2–2.3)	0.7	(0.1–1.4)	$P = 0.04$
BDCA3 (CD141)	0.1	(0.04–0.4)	0.1	(0.03–0.3)	n.s.

The respective cell populations are given as frequencies of the cellpopulation to which it is a subset: T-cells and NK-cells as percentage of total lymphocytes; the major T-cell subsets (including CD4 positive, CD8 positive, and CD4/CD8 double negative or double positive); and NK cell subsets (including CD94 positive, CD94 positive, CD56 bright positive, CD56 positive, Cd16 positive, and CD16 positive) as percentages of total T-cells and NK-cells, respectively: naive, central memory, effector memory, and terminally differentiated memory T-cells as well as CD4 positive T regulatory T-cells of CD4 and CD8 positive T-cells, respectively. The dendritic cell populations are determined within total cells excluding the CD14 positive and CD19 positive cells.

nonsecretory disease. At inclusion, in the *Agaricus* group, 8/17 patients had reached at least partial remission after induction treatment, while the corresponding figure in the placebo group was 10/19. At the end of study, 16/17 patients in the Agaricus group and 18/19 patients in the placebo group had reached at least partial response.

Median time to regeneration of neutrophils was 14.2 days in the *Agaricus* group and 13.9 in the placebo group (n.s.).

Days with temperature above 38.0°C were 3.5 in the *Agaricus* group and 3.6 in the placebo group (n.s).

Days with i.v. antibiotics were 8.6 in the Agaricus group and 10.0 in the placebo group (n.s).

Health related quality of life assessment revealed no differences between the study groups (data not shown).

3.7. Survival. At follow-up, 11/19 patients in the *Agaricus* group and 16/21 patients in the placebo group had started new treatment. In the placebo group, both patients with nonsecretory disease had started new treatment, whereas one of the two patients with nonsecretory disease in the *Agaricus*

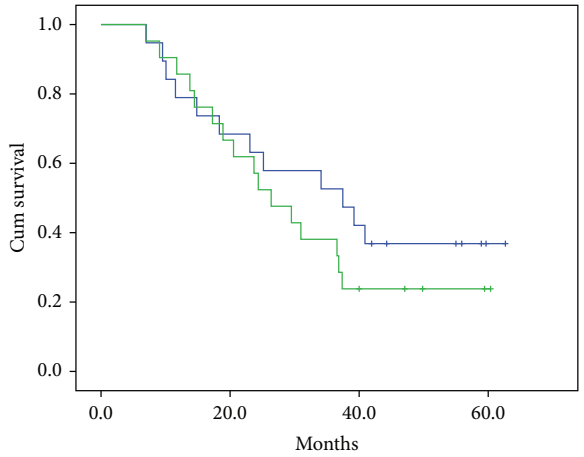

Figure 2: Time to new treatment. Mean time to new treatment in the *Agaricus* group (n = 19) was 37.3 months (upper (blue) curve) and in the placebo group (n = 21) 31.4 months (lower (green) curve) (P = 0.47 (n.s)).

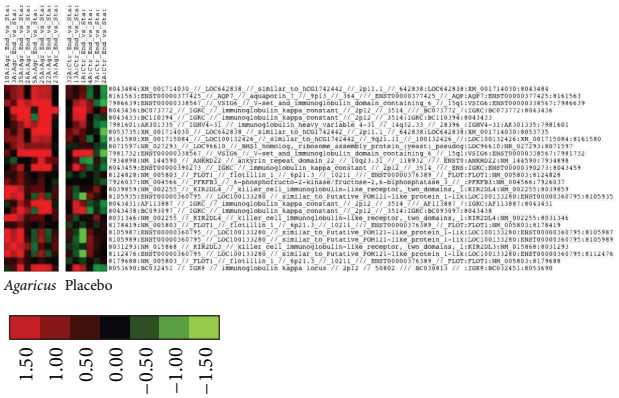

Figure 3: Gene expression analysis. K-means clustering algorithm. Cluster three. Several immunoglobulin related genes (IGKC, IgHV4-31, and IGKC) and genes related to Natural Killer cells, Killer Immunoglobulin Receptors (KIR2DL3 and KIR2DL4), are grouped together. These genes are more highly expressed in the *Agaricus* group (left column).

group had not. Mean time to new treatment was 37.3 months in the *Agaricus* group and 31.4 months in the placebo group (P = 0.49). Median observation time was 29.5 months (Figure 2) (n = 40).

At the same time point, 13/19 patients in the *Agaricus* group and 11/21 patients in the placebo group were alive. Mean overall survival was 50.7 months in the *Agaricus* group and 47.4 months in the placebo group (P = 0.93). Median observation time was 48.0 months (n = 40).

3.8. Discussion. A significant increase of IL-1ra, IL-5, and IL-7 serum levels was found in the *Agaricus* group compared to placebo, albeit the genes for IL-5 and IL-7 were found to be downregulated or unaltered, respectively, in the gene expression analysis. Also, the gene for the proinflammatory chemokine CCL2 (MCP-1) was found to be downregulated in the *Agaricus* relative to the placebo group.

IL-1ra is a natural inhibitor of the proinflammatory cytokine IL-1β, which serves a modulator for a variety of immune responses [30]. In particular, recombinant IL-1ra (Anakinra) is used in the treatment of rheumatoid arthritis and a variety of other autoimmune diseases [31, 32]. Elevated levels of IL-1ra have been documented in several types of cancer [33] including multiple myeloma, where it is associated with an improved prognosis [34]. IL-1ra counteracts IL-1β, which stimulates the production of IL-6 by bone marrow stroma cells. IL-6 is an important growth factor in multiple myeloma [35]. In a clinical trial, treatment with recombinant IL-1ra in patients with smoldering or indolent multiple myeloma was associated with a decreased myeloma proliferative rate [36]. On this background, the elevated levels of IL-1ra found in patients treated with AndoSan in our study may indicate a positive treatment effect of this product. This is also in line with the finding of reduced expression of the gene for the proinflammatory chemokine CCL2 (MCP-1).

The main role of *IL-5* is to stimulate the production of eosinophils [37]. It has recently been shown that eosinophils can stimulate the growth of malignant plasma cells [38]. The elevated levels of IL-5 found in the AndoSan group may therefore be interpreted as a negative factor, although there was an unaltered level of expression of the IL-5 gene. No clinical study on the role of IL-5 in multiple myeloma has to our knowledge been published.

IL-7 is a strong stimulator of both B-lymphocytes and T-lymphocytes [39]. In patients with multiple myeloma treated with high dose of melphalan with stem cell support, a gradual rise in plasma levels of IL-6, IL-7, and IL-15 was noted in aplasia, peaking on day +10 after infusion of hematopoietic stem cells [40]. It has been suggested that these cytokines may stimulate the proliferation of T cells in the autograft, among them also specific antimyeloma T cells [40]. In this perspective, the elevated IL-7 levels found in the AndoSan group may be interpreted as a positive treatment effect.

In the harvested stem cell product, increased percentages of regulatory T cells and plasmacytoid dendritic cells were found.

Regulatory T cells (Tregs) constitute a subpopulation of T cells, which modulates the immune system, maintains tolerance to self-antigens, and counteracts autoimmune disease [41]. Elevated levels of Tregs in peripheral blood have been found in both solid tumors and hematological malignancies [42, 43], including multiple myeloma, in which Tregs play a role in reducing immunosurveillance [44]. Excess of Tregs might result from the influence of inflammatory cytokines produced by tumor cells and tumor infiltrating lymphocytes [44]. In a clinical study on multiple myeloma, levels of Tregs were found to increase with increasing disease activity. Furthermore, high levels of Tregs were found to reflect a lower progression-free survival and total survival in patients treated with conventional chemotherapy. However, a predictive value for the levels of Tregs was not found in patients treated with high dose chemotherapy and stem cell support in this study [45]. In another study [46], the balance between suppressive Tregs and proinflammatory Th-17 cells was found to show

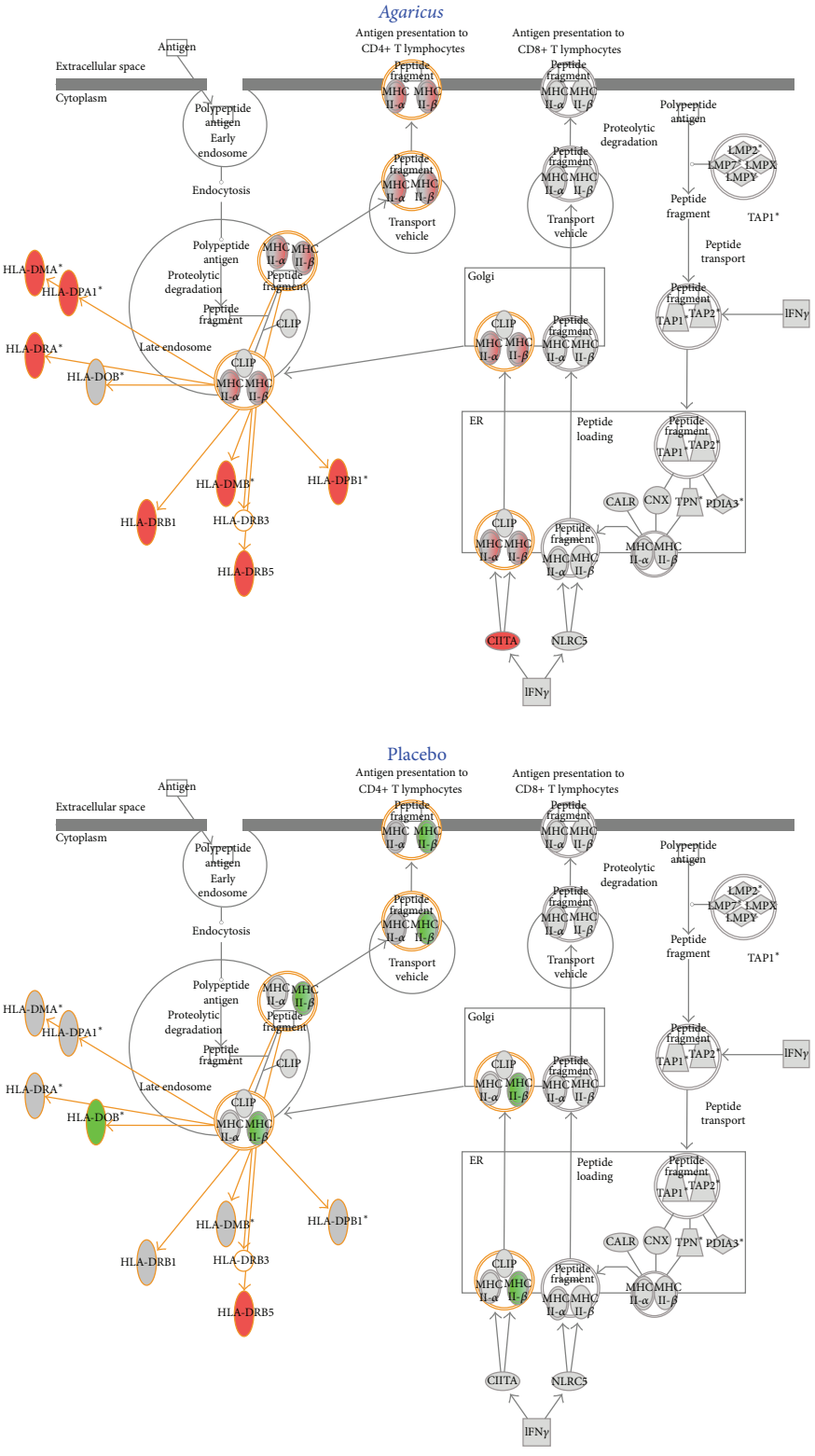

Figure 4: Ingenuity Pathway Analysis showing upregulation of genes in the HLA presentation pathway (symbols in red) in the *Agaricus* group and downregulation of HLA genes (symbols in green) in the placebo group.

a prognostic value for survival. In the present study, an increased portion of Tregs in the leukapheresis product was found in the *Agaricus* group compared to placebo. This may be interpreted as an immunosuppressive factor with negative impact on prognosis.

Plasmacytoid dendritic cells *(pDC)* stimulate both the innate and the adaptive immune system and induce tolerance. pDC levels are lower in multiple myeloma compared to control, the lowest levels being found in the clinically most advanced cases [47]. In the present study, proportions of pDCs in the leukapheresis product were higher in the patients who received AndoSan compared to control, which may suggest that AndoSan has a stimulatory effect, that is, a positive treatment effect, in these patients.

Concerning the genetic analysis, an interesting pattern was revealed by K-means clustering algorithm. In cluster three, a number of immunoglobulin related genes (i.e., IGKC, IgHV4-31, and IGKC) and genes related to Natural Killer cells, Killer Immunoglobulin Receptors (i.e., KIR2DL3 and KIR2DL4), were grouped together. These genes had a low level of expression in the control group but were highly expressed in the *Agaricus* group suggesting an immunomodulatory effect of AndoSan (Figure 3). Furthermore, using the Ingenuity analysis system, an upregulation of HLA genes (Figure 4) and of the CD86 gene was found. In a previous study, we have found an upregulation of CD86, CD83, and CD80 on dendritic cells cultivated in the presence of AndoSan [15]. In the proliferation assay, a dose dependent inhibitory effect of AndoSan towards mouse myeloma cells was found, starting at 1%. This indicates that AndoSan may also have a direct antiproliferative effect on myeloma cells, which may be clinically significant. In earlier studies an inhibitory/tumoricidal effect of *Agaricus* has been reported in fibrosarcoma [5, 9] and ovarian cancer [6] and in human hepatocarcinoma [7] and leukemic cells [8].

A major difficulty in interpreting the results of this study is the fact that the composition of AbM and thus its presumed mechanism of action is at present unclear. Originally, it has been claimed from the producer that AndoSan contained 89% of carbohydrates, of which β-glucan constituted 28% [20]. However, a pharmacological analysis, which became publicly available after the completion of our study, showed that the net carbohydrate content in this product is considerably lower, that is, only 2%, corresponding to 0.09% of β-glucan. According to this analysis, the majority of the carbohydrates in this product consist of mono- and oligosaccharides [48]. The main mechanism of action of AndoSan may therefore be linked to other substances than β-glucans.

4. Conclusion

The study showed evidence of a number of immunomodulating effects of AndoSan, used as adjuvant therapy to high dose of melphalan with autologous stem cell support in patients with multiple myeloma, which possibly may have a clinical significance. However, the results must be interpreted with caution because of the restricted sample size of the study. No statistically significant clinical impact of AndoSan was detected, although trends for a longer median time to next treatment (37.5 months versus 31.2 months) and a shorter period of i.v. antibiotics (8.6 days versus 10.0 days) were noted in the *Agaricus* group. Further investigations of the effect of AndoSan in multiple myeloma in larger patient populations with a sample size large enough to detect clinical differences are needed in order to clarify whether AndoSan may have a role in the treatment of this disease.

Disclosure

Geir Hetland is a cofounder and shareholder of Immunopharma AS.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Cytotoxic Effect on Human Myeloma Cells and Leukemic Cells by the *Agaricus blazei* Murill Based Mushroom Extract, Andosan™

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Agaricus blazei Murill is an edible mushroom of the Basidiomycetes family, which has been found to contain a number of compounds with antitumor properties, such as proteoglycans and ergosterol. In the present investigation, we show that the commercial mushroom product Andosan, which contains 82.4% *Agaricus blazei* Murill, together with medicinal mushrooms *Hericium erinaceus* (14.7%) and *Grifola frondosa* (2.9%), has a cytotoxic effect on primary myeloma cells, other myeloma cell lines, and leukemia cell lines *in vitro.* Although the exact content and hence the mechanisms of action of the Andosan extract are unknown, we have found in this investigation indications of cell cycle arrest when myeloma cell lines are cultivated with Andosan. This may be one of the possible explanations for the cytotoxic effects of Andosan.

1. Background

Agaricus blazei Murill (AbM) is an edible mushroom of the Basidiomycetes family, which grows naturally in the Piedade, coastal rainforest, area in Brazil. Besides being a popular food ingredient, AbM is also used by the local population as a remedy against several diseases, in particular against infection and cancer [1]. After commercial cultivation was started in 1965, AbM has been the subject of extensive scientific investigations, which have revealed strong immunomodulating and antitumor effects [2]. A major part of this research has been conducted on extracts from the mushroom's fruiting body. This part of the mushroom is rich in polysaccharides, in particular β-glucans, which have been shown to have strong immunomodulating properties, acting mainly through the stimulation of the innate immune system [3]. The mycelium of AbM has been less well investigated and few details are known concerning its biochemical composition. In the present investigation, a commercial mushroom extract, Andosan, containing 82.4% of *Agaricus blazei Murill,* extracted from the mycelium of the mushroom, has been used. This product also contains two other Basidiomycetes mushrooms, *Hericium erinaceus* (14.7%) and *Grifola frondosa* (2.9%). Antitumor properties have also been attributed to the two latter mushrooms [4, 5]. A recent independent investigation has shown that Andosan, in contrast to extracts from the fruiting body of AbM, contains only a very low amount of polysaccharides (2% of carbohydrates in dry weight, corresponding to 0.009% β-glucan per mL) [6]. On this background, it seems doubtful that the biological effects, which have been observed with Andosan (see below), may be attributed exclusively to the effect of β-glucans

or other polysaccharides. An immunomodulating effect of Andosan has been reported in several investigations [7, 8]. A proinflammatory effect has been found *in vitro* in human monocytes, human vein endothelial cells [9], and monocyte derived dendritic cells [10]. However, a predominant anti-inflammatory effect was found *in vivo* in healthy volunteers who ingested Andosan for 12 days [11]. In addition, it has been shown that this product ameliorates the skewed Th1/Th2 balance by increasing the Th1 response [7], which is known to have anti-infection and antitumor activities [12]. This effect has been shown to be mediated by small molecules (<12.5 kD) [13], which may easily be taken up from the gut into the blood circulation. Several reports have been published regarding antitumor effects of AbM, the majority using extracts from the fruiting body. It has been shown that β-glucans from the fruiting body of AbM have strong tumoricidal effects in preclinical models. The mechanisms involved include enhanced systemic immunity, antioxidant effect, and direct cytotoxic effect by induction of apoptosis [14, 15]. A number of compounds extracted from the fruiting body of the mushroom have been found to be involved in the cytotoxic effects, such as ergosterol [16], the ergosterol derivative agarol [17], agaritine [18], proteoglycans, and other polysaccharides [19, 20]. Reports concerning antitumor effects of extracts from the mycelium of AbM are scarce. A polysaccharide complex from the mycelium of AbM has been shown to have activity against Ehrlich ascites tumor and sarcoma in a mouse model [21]. Furthermore, it has been reported that an ethanol-soluble fraction of Andosan inhibits the activity of the tumor-associated protease, legumain, which may indirectly indicate an antitumor effect [6]. Also, a tumoricidal effect of Andosan has been found by our group in a mouse cancer colon model, as well as a cytotoxic effect correlating with apoptosis, in a human cancer colon cell line [22]. In humans, the use of an AbM extract from the fruiting body as an adjuvant to conventional chemotherapy was found to improve quality of life and increase NK cell activity in patients with gynecological cancer [23].

Multiple myeloma is a malignant disease caused by transformation and clonal expansion of bone marrow plasma cells. The main clinical features are lytic lesions caused by local growth of malignant plasma cells in bones and renal insufficiency due to deposition of paraproteins produced by myeloma cells [24]. Most patients respond well to initial therapy, but relapse occurs in virtually all cases [25]. Our group has previously reported immunomodulating effects of Andosan used in addition to high-dose chemotherapy in patients with multiple myeloma. Furthermore, in the same report, we also documented a direct cytotoxic effect of Andosan on a mouse myeloma cell line [26].

Leukemias constitute the largest group of hematologic malignancies. Despite improvements in therapy, leukemia remains a deadly disease for many patients, especially in the older age group [27]. Research for new treatment principles in order to improve the therapy for multiple myeloma and leukemia is therefore needed.

On this background, we decided to investigate the possible cytotoxic effects of Andosan *in vitro* on primary myeloma cells and human myeloma and leukemic cell lines.

2. Materials and Methods

2.1. Andosan™. The mushroom extract Andosan was provided by the company Immunopharma AS (organization number 994924273), Oslo, Norway. This commercial product contains extracts from the mushrooms *Agaricus blazei* Murill (mycelium) (82.4%), *Hericium erinaceus* (14.7%), and *Grifola frondosa* (2.9%) and is produced by the company ACE Co. Ltd., Gifu-ken, Japan. The production process comprises fermentation and heat sterilization (commercial information). The lipopolysaccharide (LPS) content was found to be <0.5 pg/mL using the Limulus amebocyte lysate test (COA-MATIC Chromo LAL; Chromogenix, Falmouth, MA, USA). The mushroom extract was stored at 4°C in sterile conditions in dark bottles until use.

2.2. Myeloma Cell Lines: Proliferation Assay. The human myeloma cell lines RPMI-8226 and U226 were obtained from the American Tissue Culture Collection (ATCC) (Rockville, MD, USA). INA-6 cells were a kind gift from Dr. Renate Burger, University Medical Center Schleswig-Holstein, Kiel, Germany. The cells were passaged twice a week using media containing 20%–10% fetal calf serum in RPMI-1640 (Sigma-Aldrich, Schnelldorf, Germany) containing L-glutamine (100 μg/mL) and gentamicin (20 μg/mL). For INA-6 cells, recombinant human interleukin-6 (Biosource, Camarillo, CA, USA) 1 ng/mL was added to the media. The cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. The CellTiter-Glo assay (Promega, Madison, WI, USA), which measures the cells' ATP content, was used to estimate the relative rates of cell proliferation according to the manufacturer's instructions. In short, 10,000 cells were seeded in a total of 100 μL in white opaque 96-well plates and cultured with Andosan at the concentrations 0.5%, 1%, 2%, and 4% or control (PBS) for 72 h. The assay reagent was added to the wells and the plates were mixed for 2 min using a microplate shaker. Then, the plates were left at room temperature for 10 min before luminescence was detected using a Victor 1420 multilabel counter (PerkinElmer Inc., Waltham, MA, USA). The measures were performed in duplicate and the results were noted as levels of ATP synthesis and converted to per cent of controls.

2.3. Cell Cycle Analysis. For cell cycle analysis by flow cytometry, 1×10^6 cells (controls and treated myeloma cells) were washed with PBS and fixed by slow addition of 2 mL 100% ice-cold methanol on a mixer and stored at −20°C until analysis. Cell cycle analysis was performed according to Vindeløv et al. [28] (modified) and analyzed on a FACSAria cell sorter (Becton Dickenson, San Jose, CA). Methanol fixed cells were centrifuged (500 g/5 min at 4°C) and the pellet was washed with 1 mL PBS (500 g/5 min at 4°C). The washed cell pellet was resuspended in 200 μL of ice-cold solution A, 200 μL of solution B, and finally 200 μL of solution C. For each solution added, the cells were gently vortexed and incubated for 10 min in the dark. All solutions contained a base of 1 mg/mL trisodium citrate, 1 μl/mL Nonidet P-40, 522 μg/mL spermine, 51 μg/ml Trizma HCl, and 9.5 μg/ml Trizma base. In addition, solution A contained 30.0 μg/mL trypsin, solution B

contained 0.1 mg/mL RNase and 0.5 mg/mL trypsin inhibitor, and solution C contained 0.28 mg/mL propidium iodide (PI) and 1.16 mg/mL spermine. Cell nuclei were kept on ice in the dark until being analyzed by flow cytometry. 10,000 nuclei were recorded. Aggregated nuclei were excluded in a dot plot displaying a pulse width of PI (PI-w linear scale) and pulse height of PI (PI-h linear scale). Single nuclei were displayed in a histogram of pulse area of PI (filter 616/23). Flow cytometric analysis was performed with FACSDiva software version 6.0 (Becton Dickenson, San Jose, CA). The cell lines and myeloma cell lines INA-6, RPMI-8266, and U226 were cultured with the addition of Andosan 10% or with PBS (controls). The results were noted as per cent of cells in cell cycle phases sub-G1, G1, S, and G2, respectively. For each cell line, flow cytometric analysis was performed five times and the mean values were noted.

2.4. Leukemia Cell Lines: Cell Proliferation Assay. The human leukemic cell lines KG1a, HL 60, and Meg 01 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany. The cells were cultured in RPMI-1640 medium (ATCC 30-2001) supplemented with 10% fetal bovine serum (ATCC, cat. number 3020) and 1% antibiotic mix (Sigma A5955) and maintained in a humidified atmosphere with 5% CO_2 at 37°C. The media were changed twice a week. For the cytotoxic assay, the cells were seeded in 24-well plates at a density of 7.5×10^4 cells/mL and treated with various concentrations of Andosan (5.0% and 10.0%), or a matched concentration of PBS as a control, for 96 hrs. The total number and percent viable cells were counted by NucleoCounter using the NucleoCassette kit (ChemoMetec, Allerød, Denmark) according to the manufacturer's manual. For controls and each concentration of Andosan, the mean of five parallel measurements was noted. The results were converted to per cent of the number of viable cells in controls (100%).

2.5. Primary Myeloma Cells: Cell Proliferation Assay. Primary CD138+ myeloma cells from ten patients were isolated from bone marrow specimens included in the Norwegian Myeloma Biobank using RoboSep automated cell separator and human CD138 Positive Selection Kit (Stem Cell Technologies, Grenoble, France). The study was approved by the Regional Ethics Committee (approval 2016/828/REK Midt) and all patients gave informed consent. To determine the effect of Andosan on the viability of primary myeloma cells, 5000 cells were seeded per well in 96-well plates. The cells were cultivated in 2% heat-inactivated human serum (HS; Blood Bank, St. Olav's University Hospital, Trondheim, Norway) in RPMI and 2 ng/mL interleukin-6 (IL-6) (Gibco, Thermo Fisher, Waltham, MA, USA). Andosan was added to a final volume of 200 μL per well at the concentrations 0.5%, 1%, 2%, and 4%. All samples were run in duplicate. The effect on cell viability was determined by counting the number of viable myeloma cells in each well after 72 h incubation using ScanR automated image acquisition and analysis (Olympus, Hamburg, Germany) as previously described [29]. The measures were performed in duplicate and the results were converted to per cent of the number of viable cells in controls (100%).

2.6. Peripheral Blood Mononuclear Cells. Peripheral blood mononuclear cells from three healthy blood donors were grown in the RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (ATCC, cat. number 30-2020) and 1% Antibiotic Antimycotic Solution (Sigma, A5955), and maintained in a humidified atmosphere with 5% CO_2 at 37°C. The media were changed twice a week. For cytotoxicity assay, the cells were seeded in 24-well plates at a density of 7.5×10^4 cells/mL with various concentrations of Andosan (0.5%, 1%, 5%, and 10%) or PBS (control) for 72 hrs. The total number and percent viable cells were counted by NucleoCounter using the NucleoCassette kit (ChemoMetec, Allerød, Denmark), according to the manufacturer's manual.

Statistics. The differences between the means of the percent of viable cells in controls (100%) and the means of the percent of viable cells in cultures with Andosan 4% (myeloma cell lines and primary myeloma cells) or Andosan 10% (leukemia cell lines and peripheral blood mononuclear cells) were calculated by the paired samples t-test using the IBM SPSS 23 statistical computer program. p values below 0.05 were considered statistically significant. In primary myeloma cells and myeloma cell lines, the correlations between Andosan concentration and viability of the cells were calculated by Pearson's product moment correlation. For cell cycle analysis, a comparison of the percentage of cells in sub-G1 phase and in G1 phase of the cell cycle in cells cultured with Andosan and cells cultured with PBS (controls) was made with the Bonferroni method.

3. Results

3.1. Primary Myeloma Cells. The results from two patients were excluded from the analysis because of initial low cell viability (20% and 13%, resp.). The results from the remaining eight patients were considered to be evaluable. In seven patients, a dose-related inhibitory effect of Andosan was noted (correlation coefficient: −0.71 to −0.99), with a reduction of viable myeloma cells from 19.5% to 82.4% in cultures with 4% Andosan compared to controls. In contrast, in one patient (number 244), the number of viable cells increased during culture with Andosan, although there was no correlation (correlation coefficient: 0.06) (Table 1). Comparison of the means of controls versus the means of cell cultures with Andosan 4% showed a statistically significant difference ($p = 0.01$).

3.2. Myeloma Cell Lines. In the myeloma cell lines RPMI-8226, U226, and INA-6, a dose-related inhibitory effect of Andosan was found (correlation coefficient: −0.94), expressed as per cent reduction of ATP content compared to controls. Comparison of the means of the controls versus the means of cells cultured with Andosan 4% showed a statistically significant difference ($p = 0.02$) (Table 2). Furthermore, in a cell cycle study, the percentage of cells was higher in sub-G1 phase ($p < 0.002$) and lower in the G1 phase

Table 1: Cytotoxic effect of Andosan on myeloma cells from 8 patients. The numbers of viable cells after 72 hrs of culture were noted and converted to per cent of controls (100%). Mean: mean of duplicates; SE: standard error. Comparison of means of controls versus means of cultures with Andosan 4% showed a statistically significant difference ($p = 0.01$).

Patient number	Control	Andosan 0.5%	Andosan 1.0%	Andosan 2.0%	Andosan 4.0%
139					
Mean	100	93.69	99.12	97.61	80.47
SE	4.04	1.35	1.11	2.11	0.44
244					
Mean	100	117.91	122.5	114.15	110.22
SE	1.33	2.07	2.1	4.19	2.13
969					
Mean	100	65.73	62.3	55.42	52.37
SE	1.95	2.18	2.22	2.66	2.39
185					
Mean	100	86.6	83.14	73.71	52.28
SE	0.88	1.38	0.66	1.82	0.77
409					
Mean	100	69.24	55.75	50.6	17.66
SE	1.5	2.05	2.05	2.05	2.18
715					
Mean	100	90.35	86.8	84.12	73.11
SE	1.54	1.73	2.72	0.92	1.21
2925					
Mean	100	91.81	90.92	80.78	54.27
SE	0.87	3.4	2.19	1.88	1.88
355					
Mean	100	82.22	85.21	78.34	70.77
SE	2.17	2.64	2.39	3.15	2.95

Table 2: Cytotoxic effect of Andosan on myeloma cell lines. The effects are measured as levels of DNA synthesis and converted to per cent of controls (100%). Mean: mean of duplicate experiments. Comparison of the means of controls compared to means of cell lines cultured with Andosan showed a statistically significant difference ($p = 0.02$). SE: standard error.

Cell type	Control	Andosan 0.5%	Andosan 1%	Andosan 2%	Andosan 4%
INA-6					
Mean	100	93.9	71.67	66.06	49.46
SE	0.24	1.89	2.15	1.27	1.31
RPMI-8221					
Mean	100	94.88	88.53	77.21	66.12
SE	1.92	1.95	1.07	2.07	2.22
U226					
Mean	100	96.92	87.13	79.7	69.27
SE	0.93	1.24	1.95	2.07	2.22

($p < 0.001$) in cells cultured with Andosan 10% (paired t-test) compared to controls, which is suggestive of cell cycle arrest in these cells (Figure 1).

3.3. Leukemia Cell Lines. In leukemia cell lines, a comparison of the means of the controls versus the means of the cells cultured with Andosan 10% showed a significant statistical difference ($p = 0.02$) (Table 3).

3.4. Peripheral Blood Mononuclear Cells. A comparison of the means of the controls and the means of the cells cultured with Andosan 10% showed no statistical difference (Table 4).

4. Discussion

This study shows a predominantly dose-related cytotoxic effect of Andosan on primary myeloma cells and human

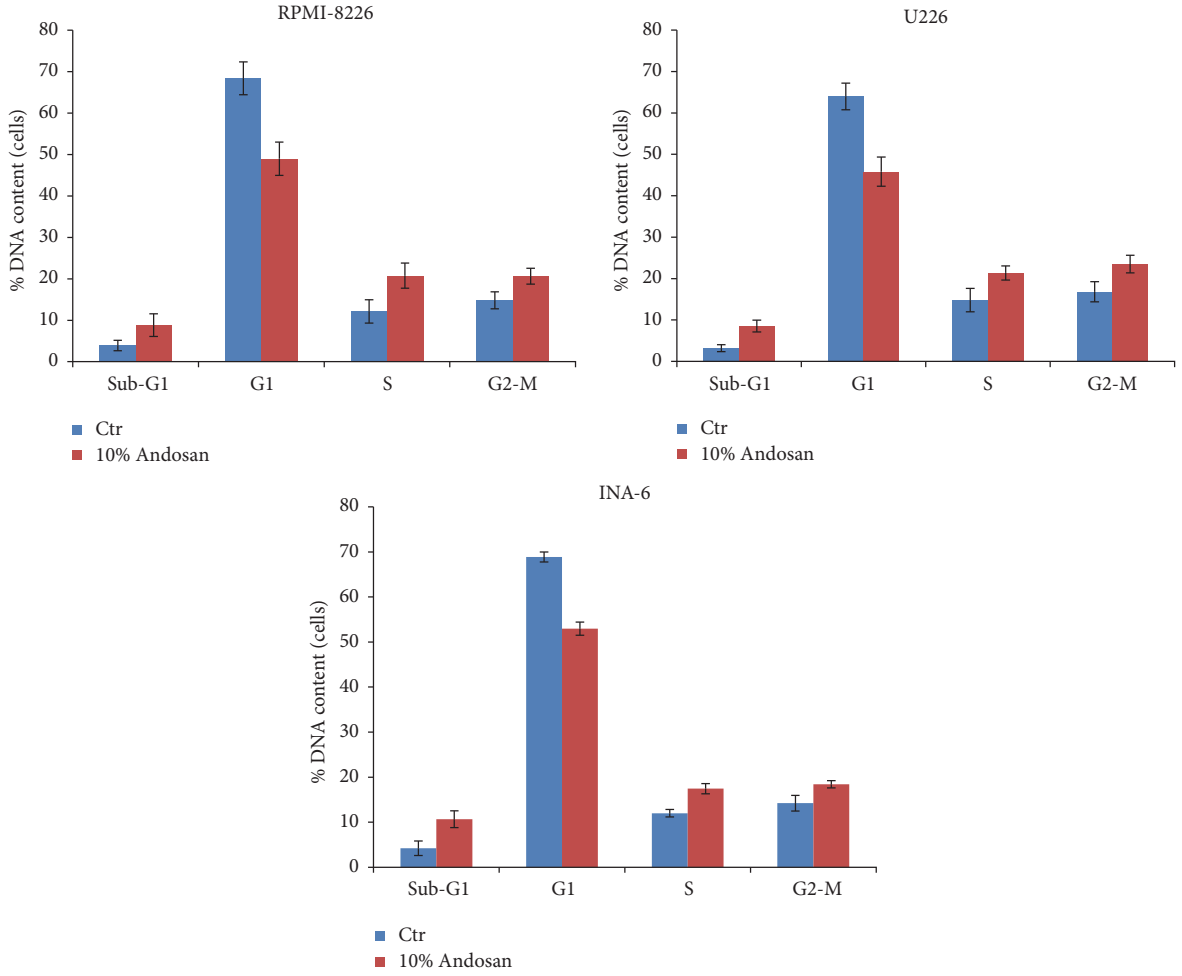

Figure 1: Cell cycle analysis. The figure shows the percentage of cells cultured with either Andosan 10% or PBS (controls) in different cell cycle phases (sub-G1 phase, G1 phase, S-phase, and G2 phase). The percentage of cells was higher in the sub-G1 phase ($p < 0.002$) and lower in the G1 phase ($p < 0.001$) in cells cultured with Andosan 10% compared to controls (paired t-test), which is suggestive of cell cycle arrest in these cells.

Table 3: Cytotoxic effect of Andosan on leukemia cell lines. Mean: mean of five experiments; SE: standard error. Comparison of the means of control with means of cells cultured with Andosan 10% showed a statistically significant difference ($p = 0.02$).

	Controls	Andosan 5%	Andosan 10%
HL 60			
Mean	100	83.83	76.16
SE	0	2.6	3.13
Kgla			
Mean	100	84.81	74.6
SE	0	1.92	2.3
Meg			
Mean	100	92	83
SE	0	3.16	4.1

myeloma and leukemic cell lines *in vitro*. These results are in line with previous reports of cytotoxic effects of different compounds (β- glucans, proteoglycans, ergosterol, and agaritine) extracted from AbM preparations from the fruiting body, on different malignant tumors, both *in vitro* and in animal models [14–20]. In particular, it has been shown that an extract of the fruiting body of *Agaricus blazei* Murill had an antitumor effect in a mouse myeloma model, when given together with a marine phospholipid [30]. In the case of Andosan, which is a commercial mushroom extract where the exact content is not known, a firm conclusion regarding the mechanism behind the cytotoxic effects is not possible. However, it is remarkable that indications of cell cycle arrest were found when the myeloma cell lines RPMI-8226, U226, and INA-6 were cultivated with Andosan. We and others [19, 20, 22] have previously found that AbM extracts can have cytotoxic effects on tumor cells by induction of apoptosis. Also, it has been documented that an ethanol-soluble fraction of Andosan inhibits the tumor-associated protease legumain in the murine macrophage-like cell line RAW 264.7 [6]. This may indirectly indicate an antitumor

Table 4: Effect of Andosan on peripheral blood mononuclear cells. Mean: mean of 5 experiments; SE: standard error. Comparison of the means of controls versus means of Andosan 10% showed no statistical difference (p = ns).

	Controls	Andosan 0.5%	Andosan 1%	Andosan 5%	Andosan 10%
Mean	100	97.70	97.02	97.00	95.22
SE	0.97	0.96	1.07	0.62	1.39

effect of this fraction, as legumain is secreted by a number of malignant cells [31]. In fact, in the mouse model for colon cancer, Andosan did also induce reduced expression of legumain in the intestinal wall [6]. Moreover, it increased levels of Th1 cytokine IL-12 in addition to proinflammatory cytokines [22]. The latter is in contrast to what we have usually observed in humans consuming Andosan. However, it agrees with our previous *in vitro* finding of Andosan-induced NF_K-B activation via stimulation of TLR2 in dendritic cells [32]. Furthermore, the possibility of a synergistic effect between the three mushrooms contained in Andosan—*Agaricus blazei* Murill, *Grifola frondosa*, and *Hericium erinaceus*—may also be taken into consideration. Importantly, Andosan did not have a toxic effect neither on human peripheral mononuclear cells (PMNC) nor on normal human hematopoietic stem cells (G. Hetland et al., unpublished results).

The cytotoxic effects of Andosan found in this investigation on primary myeloma cells and myeloma cell lines are particularly interesting in light of the previously reported immunomodulating effects mostly associated with antitumor properties, when this product was used as an adjuvant treatment in myeloma patients undergoing high-dose chemotherapy [26]. It therefore seems plausible that the mushroom extract may have both cytotoxic and immunomodulating antitumor mechanisms of action in myeloma. Further investigations are needed in order to clarify whether Andosan may have a role in the treatment of multiple myeloma.

Data Access

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

Ethical Approval

The study on primary CD138+ myeloma cells from ten patients included in the Norwegian Myeloma Biobank was approved by the Regional Ethics Committee (approval 2016/828/REK Midt).

Consent

All patients provided informed consent.

Disclosure

The authors received no special funding for this work, except for Andosan, which was provided free of charge from the company Immunopharma R&D.

Conflicts of Interest

Author Geir Hetland has patent applications and financial interests as a shareholder in Immunopharma AS of Norway, commercializing material (Andosan) pertinent to this article. The other authors have no conflicts of interest to declare.

Authors' Contributions

The concept of the study was made by Jon-Magnus Tangen, Toril Holien, and Geir Hetland. Lab analyses were performed by Toril Holien, Mohamed Reza Mirlashari, and Kristine Misund. The study was administered by Jon-Magnus Tangen and Geir Hetland. The data were compiled and analyzed by Jon-Magnus Tangen, Toril Holien, and Geir Hetland. The original draft of the manuscript was made by Jon-Magnus Tangen. Editing and rewriting were performed by Jon-Magnus Tangen, Toril Holien, Mohamed Reza Mirlashari, Kristine Misund, and Geir Hetland.

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IV

PLOS ONE

RESEARCH ARTICLE

The *Agaricus blazei*-Based Mushroom Extract, Andosan™, Protects against Intestinal Tumorigenesis in the A/J Min/+ Mouse

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Abstract

Background

The novel A/J Min/+ mouse, which is a model for human Familial Adenomatous Polyposis (FAP), develops spontaneously multiple adenocarcinomas in the colon as well as in the small intestine. *Agaricus blazei* Murill (AbM) is an edible *Basidiomycetes* mushroom that has been used in traditional medicine against cancer and other diseases. The mushroom contains immunomodulating β-glucans and is shown to have antitumor effects in murine cancer models. Andosan™ is a water extract based on AbM (82%), but it also contains the medicinal *Basidiomycetes* mushrooms *Hericeum erinaceus* and *Grifola frondosa*.

Methods and findings

Tap water with 10% Andosan™ was provided as the only drinking water for 15 or 22 weeks to A/J Min/+ mice and A/J wild-type mice (one single-nucleotide polymorphism (SNP) difference), which then were exsanguinated and their intestines preserved in formaldehyde and the serum frozen. The intestines were examined blindly by microscopy and also stained for the tumor-associated protease, legumain. Serum cytokines (pro- and anti-inflammatory, Th1-, Th2 -and Th17 type) were measured by Luminex multiplex analysis. Andosan™ treated A/J Min/+ mice had a significantly lower number of adenocarcinomas in the intestines, as well as a 60% significantly reduced intestinal tumor load (number of tumors x size) compared to control. There was also reduced legumain expression in intestines from Andosan™ treated animals. Moreover, Andosan™ had a significant cytotoxic effect correlating with apoptosis on the human cancer colon cell line, Caco-2, in vitro. When examining serum from both A/J Min/+ and wild type mice, there was a significant increase in anti-tumor Th1 type and pro-inflammatory cytokines in the Andosan™ treated mice.

material (AndoSan™) pertinent to this article: i) WO2005065063 A2, Appl. No.:10/585600, NO- and PCT-filed Jan 2004 and Jan 2005, respectively, by Inventor Hetland Geir, and ii) NO20090003383, Appl. No.: NO20090003383 20091119, by Inventor Hetland Geir filed by Applicant Immunopharma AS in Nov 2009. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

Conclusions

The results from this mouse model for colorectal cancer shows significant protection of orally administered Andosan™ against development of intestinal cancer. This is supported by the finding of less legumain in intestines of Andosan™ treated mice and increased systemic Th1 cytokine response. The mechanism is probably both immuno-modulatory and growth inhibition of tumor cells by induction of apoptosis.

Introduction

Agaricus blazei Murill (AbM) is an edible medicinal mushroom of the family, *Basidiomycetes*, and a close relative to *Agaricus bisporus*, the champignon. AbM was first described in 1893 and it is also known as *Agaricus subrufesence*, *A*. *rufotegulis* and recently as *A*. *brasiliensis* because of its origin in a rain forest area near Piedade, Brazil [1]. According to legend, the frequency of geriatric diseases, including cancer, was lower there than in neighboring areas due to the local high intake of AbM as food in Piedade. Since the 1960-ies Japanese researchers have detected immuno-modulating and antitumor properties of AbM in studies in the mouse [2,3]. *Hericium erinaceus* and *Grifola frondosa* are two other edible *Basidiomycetes* mushrooms used in traditional Eastern medicine that have similar antitumor effects [4,5].

In collaboration with Shinshu Agricultural University, Nagano, Japan, a strain of AbM was chosen that had optimal properties both with regard to assumed health effects and cultivation ability. It was mixed with 15% of *H*. *erinaceus* and 3% of *G*. *frondosa* to obtain a more potent product. In 2004 this AbM-based mixed *Basidiomycetes* mushroom extract was found to be the only one among other Japanese AbM extracts tested blindly in a pneumococcal sepsis mouse model at the Norwegian Institute of Public Health, Oslo, that significantly reduced bacteremia and increased the animals' survival rate [6]. It was later trade-marked Andosan™ and chosen for further studies, which showed that it also protected against Gram-negative sepsis [7] and allergy [8] in other mouse models. These effects together with the antitumor property of the mushrooms contained in Andosan™, is probably foremost due to the immuno-modulatory relative shift induced by the mushroom, from a Th2 to a predominant Th1 response [9, 8]. In human studies we have found that Andosan™ induced increased expression of genes related to cancer defence in peripheral blood leukocytes (cell signaling and cycling, and transcriptional regulation) [10], and it also proved to have anti-inflammatory properties in IBD patients and healthy individuals without any pathological findings in blood samples or clinical side effects [11, 12]. In a recent placebo-controlled clinical study, Andosan™ was given orally as adjuvant therapy for patients with multiple myeloma undergoing high-dose chemotherapy and bone marrow transplantation and found to have immunomodulatory and anti-inflammatory effects [13]. Clinically, trends for a longer median time to next treatment and shorter antibiotics use were noted in the mushroom extract group. The assumed role of AbM in immune system modulation and disease control is reviewed in Hetland et al 2011 [14].

Previously, β-glucan polysaccharide [3] and ergosterol containing lipid [15] isolated from AbM, have been shown to have in vivo antitumor activity in transplantable tumor-bearing mouse models. Later, chemically induced carcinogenesis has been used as experimental tumor models in rodents for both hepatocarcinogenesis and colon carcinogenesis studies [16,17] on possible beneficial effects of AbM extracts. The former study demonstrated hepatoprotective effect of orally administered *A*. *blazei* extract [16], and the latter [17] showed tendency to

reduced dysplastic aberrant crypt formation in colon but no difference in colon tumor incidence after *A. blazei* ingestion [17].

Colorectal cancer is the 4th most frequent type of cancer in Western societies, but the 2nd deadliest after lung cancer [18]. There is increasing evidence for a link between inflammation and colorectal cancer [19]. Approximately 14% of colon cancer has a familial background, such as for the inflammatory bowel disease, ulcerative colitis, and 1% is caused by familial adenomatous polyposis (FAP) [18]. The multiple intestinal neoplasia (Min/+) mouse is frequently used as model for human FAP and colorectal cancer because it is heterozygous for a mutation in the tumor suppressor gene APC, which leads to the formation of numerous intestinal adenomas [20,21]. Complete somatic inactivation of APC in discrete crypts of the intestinal epithelium appears to be the initial event of the tumorigenesis in Min/+ mice, human FAP and in the majority of sporadic colorectal cancer in humans [22]. In contrast to human FAP, conventional C57 BL/6J Min/+ mice develop tumors predominantly in the small intestine [23,24,25,26]. Therefore, the novel Min/+ mouse on the A/J genetic background provides a better model for colon cancer because these mice spontaneously develop numerous colonic adenomas that eventually progress to carcinomas in old individuals [27].

Legumain is a tumor-associated proteolytic enzyme (asparaginyl endopeptidase) that is expressed in kidney, placenta, and spleen [28] and important for normal kidney function [29]. High levels of the protease legumain have been detected in solid tumors and associated with increased tumor invasion and metastasis [30,31]. The protease has been detected on tumor cell surface and in tumor microenvironment [32], where it has been shown to destroy extracellular matrix by degrading its major component, fibronectin [33]. Moreover, legumain is found on tumor associated macrophages, which are important for tumor development and metastasis [34]. Legumain is highly expressed in colorectal cancer cell lines and associated with poor outcome in colon cancer [35]. Recently, we have shown that Andosan™ reduced the activity of legumain in rat macrophages [36].

In the present study we have investigated whether the AbM-based Andosan™ extract had any influence on the development of adenomatous tumors in small intestines and colon/rectum of A/J *Min*/+ mice when added to the drink water, and whether it affected intestinal expression of the tumor-associated and metastasis-promoting protease, legumain. Moreover, we measured serum cytokine levels in the animals and examined putative cytotoxic effect of the mushroom extract on the human colon cancer cell line, Caco-2.

Materials and Methods

Reagents and cell line

Andosan™ is a mixed *Basidiomycetes* mushroom water extract of the mycelium of AbM (82.4%), *Hericeum erinaceus* (14.7%) and *Grifola frondosa* (2.9%) produced and heat-(124˚C for 1 hour) sterilized by ACE Co Ltd, Gifu-ken, Japan, and GMP-certified by Meiji Co. Ltd, Japan, and contained and stored in sterile dark-glassed bottles at room temperature until use. The extract with a final concentration of 340 g/l was imported to Norway as food (mushroom juice) and provided by the company Immunopharma AS (organization no. 994924273), Oslo, Norway. The LPS content of Andosan™ was found be <0.5 pg / ml, using the Limulus amebocyte lysate test (COAMATIC Chromo-LAL; Chromogenix, Falmouth, MA, USA). The results from tests for heavy metals were conformable with Japanese regulations for health foods and potential radioactivity in the extract was not detected.

Caco-2 cells cultures and *in vitro* cytotoxicity

The human epithelial colorectal adenocarcinoma cells (Caco-2) were obtained from American Type Culture Collection (ATCC® HTB-37™, no:HTB-37) and were grown in Eagle's Minimum Essential Medium (EMEM, cat no. 30–2003, ATCC, USA) supplemented with 10% fetal bovine serum (FBS, cat no. 30–2020, ATCC, USA) and 1% antibiotic mix (Sigma Aldrich, cat. no. A5955) and maintained in a humidified atmosphere with 5% CO_2 at 37˚C in an incubator (Forma Series II Water Jacket 3111, Thermo Scientific) and the media were changed twice a week. Cytotoxicity experiments were conducted using cells with less than 5 passages. For cytotoxicity assay, 5 x 10^3cells/cm^2 were seeded onto 25 cm^2 culture flasks and allowed to attach overnight. The medium was then replaced with new medium containing various concentrations of Andosan (0.5, 1.0, 2.5 and 5.0%) or PBS (control). After 96 hours cells were washed 3 times with PBS (Sigma, D8537) and the cells were harvested by using Trypsin-EDTA solution (ATCC, cat. no. 30–210). The total number and percent viable cells were counted by NucleoCounter using the NucleoCassette kit (Chemometec, Allerød, Denmark) according to the manufacture's manual.

Quantification of cellular apoptosis by flow cytometry

Following the examination of the effect of Andosan™ on cell growth, the effect of Andosan™ on cell apoptosis was determined and the percentage of apoptotic cells was quantified using the annexin V. The annexin V-binding assays were performed according to manufacturer's protocol (BD PharMingen). Briefly, cultured cells (1×10^6) were collected, washed twice with cold PBS and resuspended in 1 ml binding buffer. Then 5 μl of Fluorescein isothiocyanate (FITC)-conjugated annexin V (Cat. No. 556419, BD Pharminogen, San Jose, CA, USA) and 7-aminoactinomycin (7-AAD, cat, No. 51-68981E, BD Pharminogen, San Jose, CA, USA). were added to 100 μl of cell suspension and incubated for 15 minutes at room temperature in the dark. Finally, 400 μl of binding buffer (Cat. No. 556454, BD Biosciences, USA) was added to samples and analyzed by using the Gallios flow cytometer as soon as possible (within 1 hour).

Mouse breeding, experimental design, and scoring of intestinal lesions

This study was carried out in strict accordance with the laws and regulations for animal experiments in Norway and was approved by the National Experimental Animal Board in Norway. All mice (originally purchased from The Jackson Laboratory, Bar Harbor, ME) were bred at the animal facility of Norwegian Institute of Public Health. The *Min*/+ trait from C57BL/6J *Min*/+ mice was transferred to A/J mice by backcrossing for more than 12 generations.

For the purpose of this experiment, A/J *Min*/+ males/females were mated with A/J +/+ (wild type) females/males, with one or two females and one male housed in each disposable plastic cage (Innovive, San Diego, CA, USA) on aspen chip bedding in paper bags for enrichment (Nestpack, Datesand, Manchester, UK). The animal room was maintained on a 12-hour light/dark cycle, with controlled humidity (55 ± 5%) and temperature (20–24˚C). Tap water and feed was available *ad libitum*. When 3–4 weeks old, the *Min*/+ and wild type pups were identified by an allele-specific PCR-assay, as described previously [37], individually ear punched, and each litter was separated in male and female cages. In total, A/J *Min*/+ mice (N = 46) and wild type mice (N = 30) from 28 litters were included. All animals were fed a standard rodent diet RM1 (Special Diet Services, Witham, UK) for the duration of the study. The communal tap water was routinely monitored for microbiological quality and ion content, supplied to the mice in disposable plastic bottles (Innovive). The cages were randomly divided in two groups, either receiving were given 10% of Andosan™ *Agaricus* bM extract in tap water,

or tap water only (controls), for 15 or 22 weeks and killed with CO_2 and exsanguinated for serum (1900G, 10min, 15˚C) that was frozen (-80˚C).

The small intestine and the colon were removed and rinsed in ice-cold PBS before they were longitudinally incised and fixed flat between wet (PBS) filter papers for a minimum of 24 hours in 10% neutral buffered formalin. After a 5 second stain in 0.2% methylene blue (George T. Gurr Ltd., London, United Kingdom) dissolved in 10% neutrally buffered formalin. The intestinal surface was examined by transillumination in the inverse light microscope equipped with an eyepiece graticule to determine the number (#), size (mm^2) and tumor load (mm^2), defined as the sum of the area of all tumors (# tumors x size). A colonic tumor was defined as a lesion with >30 aberrant crypts.

Legumain immunofluorescence staining of intestines

Swiss-rolls were prepared [27] by longitudinal opening of the intestine followed by rolling up along the same axis with the lumen facing inwards and embedding the rolls into parrafin blocks. Immunofluorescence staining was performed on deparaffinized sections of swiss-rolled intestines from the A/J Min/+ mice using a primary antibody against legumain (R&D Systems; AF2199) and secondary antibody conjugated with Alexa 488 (Jackson ImmunoResearch). Stained sections were analyzed by fluorescent microscopy (Olympus IX-81) using the corresponding software (Cell^P).

Cytokine measurement

Serum cytokine measurements were performed using the multiplex bead-based sandwich immunoassay Bio-Plex xMAP technology (Bio-Rad, Austin, TX, USA) with a Luminex IS 100 instrument (Bio-Rad, Hercules, CA, USA) and Bio-Plex Manager (version 6.0.1) software for analysis of 12 different cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, GM-CSF, MCP-1, TNFα, and IFNγ).

Statistical analysis

Cytokine values and tumor numbers were dependent variables in ANCOVAs (JMP pro, SAS Institute, Cary, NC, USA) where the effects of genotype (*Min* or wildtype) and treatment group (Andosan or water) were evaluated together with age of the pups and sex as covariates. Using the minimum AIC criterion, genotype, age and sex was removed from the model and only the effect of treatment is reported.

The random effect of litter within treatment (N = 28) was also evaluated [38] and found significant, but not taken into account because of the high number of litters within each treatment group would increase the risk of type II error for the effect of treatment.

Transformations of data did not improve the heteroscedasticity of some data, and non-parametric tests resulted in only minor changes in p-values. Thus, we used the same analysis for all responses. No correction of p-values is done for the number of tests applied (e.g., Bonferroni), even if some of the responses are correlated.

Ethical considerations

The investigation was approved by the local ethical committee, implementing national laws for animal studies.

Results

Cytotoxic effect of Andosan™ in vitro on human cancer coli cell line

In vitro antitumor effect of Andosan™ was examined in cultures of Caco-2 cells after 96h. Andosan™ reduced Caco-2 cell viability in a concentration (0.5–5.0%) dependent manner (Spearman's correlation coefficient rho ρ = -0.986, $p<0.001$ (Fig 1). Whereas the concentration of 5% of Andosan™ induced killing of near 90% of the Caco-2 cells (two-tailed t-test, $p<0.001$, even 0.5% of Andosan™ had a significant, albeit low (14%), cytotoxic effect (two-tailed t-test, $p = 0.006$). We next assessed whether the growth inhibitory effect of Andosan™ on Caco-2 cells was correlated with increased apoptosis. After treatment with 1 and 5.0% for 96 h, cellular apoptosis was measured by flow cytometry using the annexin V/7-AAD dual cell staining. Treatment with Andosan™ 1.0% and 5.0% for 96 hours increased the population of early apoptotic cells (7-AAD-negative and annexin V-positive cells) from 5.7% ± 1.5% in untreated cells to 15.3% ±2.1 for Andosan™ 1% and 35.6% ± 4.5% for Andosan™ 5.0% treated cells ($p<0.01$) (Fig 2). The population of late apoptotic cells (7-AAD-positive and annexin V positive cells) increased from 7.3% ± 2.1% for untreated cells to 35.6 ± 4.5 for Andosan™ 1.0% and 39.7% ± 7.6% for Andosan™ 5.0% treated cells ($p<0.01$). This result suggests that Andosan™ induced growth inhibition of Caco-2 cells, at least in part, by induction of apoptosis.

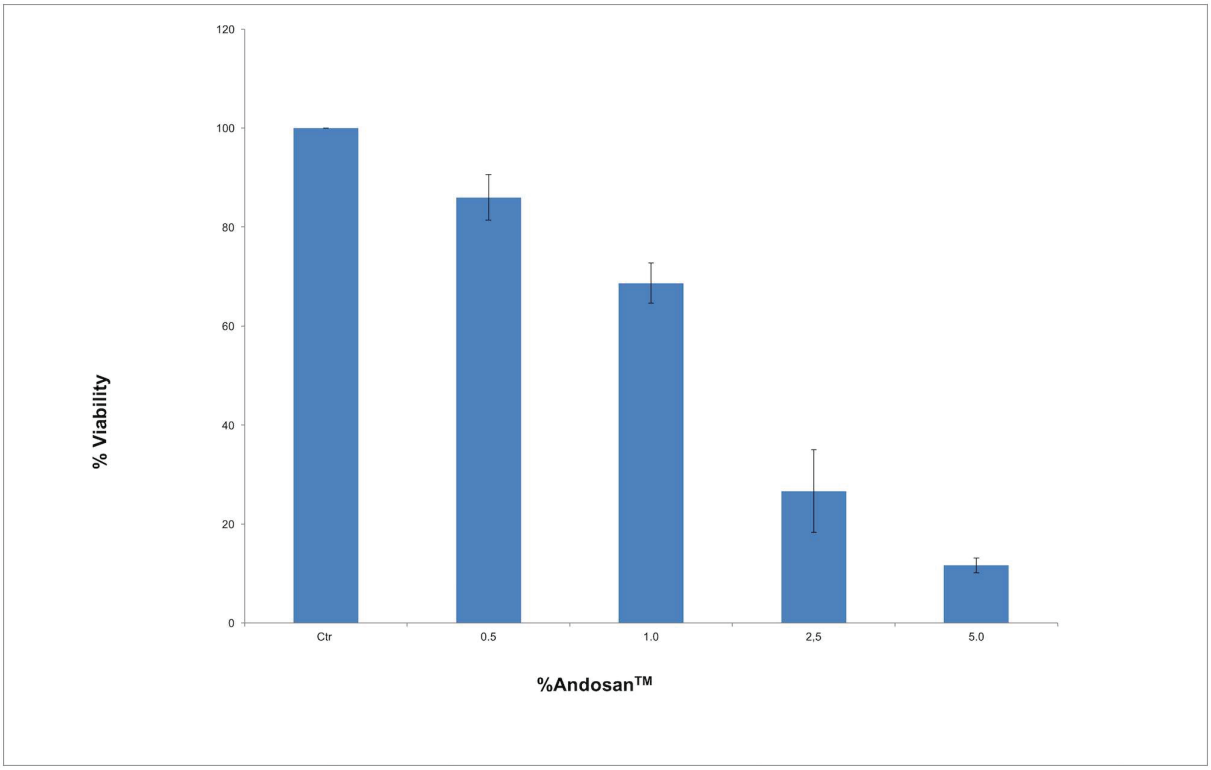

Fig 1. Effect of Andosan™ on the proliferation of Caco-2 cell line *in vitro*. Various concentrations of Andosan™ (0.5%–5.0%) were added to the culture medium and viability was assessed after 96 h. Results are expressed as mean ± SD in percentage of proliferation relative Caco-2 cells cultured without Andosan™ (= 100%) and represent 3 independent experiments. Ctr: control.

doi:10.1371/journal.pone.0167754.g001

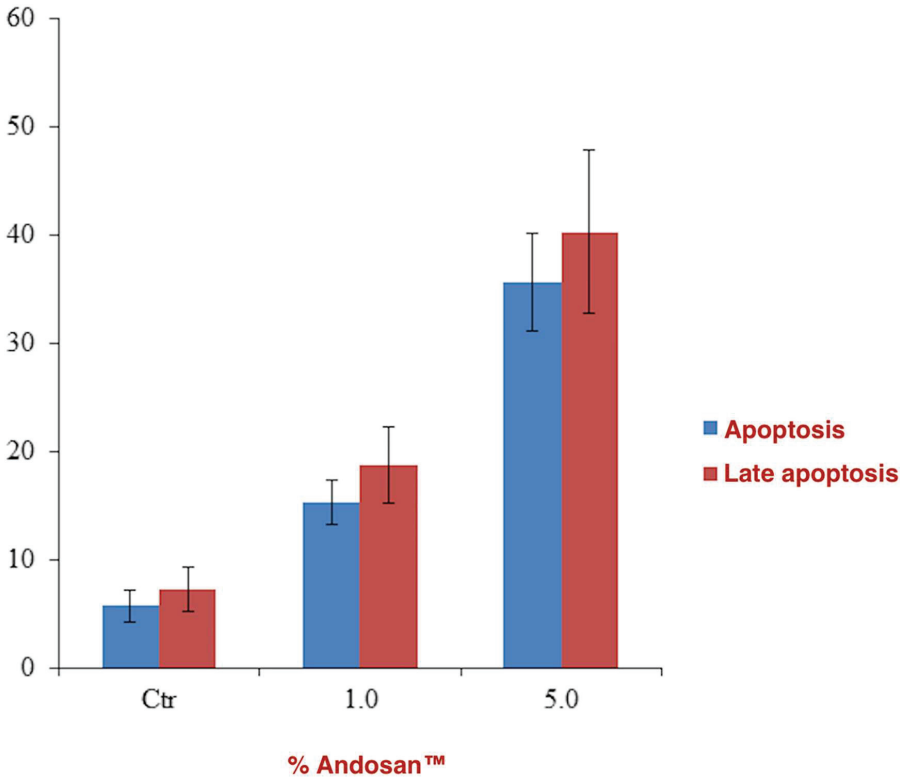

Fig 2. Andosan™ induces apoptosis in Caco-2 cells. Caco-2 cells were treated for 96 h with 1.0% or 5.0% Andosan™. Binding of annexin V was used as marker for apoptosis and 7-AAD as marker for late apoptosis (necrosis). The results are expressed as mean ± SD and represent 3 independent experiments. Ctr: control.

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Antitumor effect of Andosan™ in A/J *Min/+* mice

In A/J *Min/+* mice given 10% of Andosan™ in the drink (tap) water for 22 weeks, in average 23 tumors/mouse were found in their intestines by microscopy of the formalin preserved tissue. This was statistically significantly ($p = 0.021$) fewer tumors than the average 34 tumors/mouse counted in intestines of such mice given only ordinary drink (tap) water (Fig 3). The microscopy was done in a blinded fashion in such a way that the treatment group of the individual mouse examined, was unknown to the pathologist. When the size of the tumors was noted and multiplied with number of tumors, there was an approximately 60% significant reduction in the tumor load in both small intestines ($p<0.001$) and colon/rectum ($p = 0.024$) of the Andosan™ treated mice relative to the tumor load in the control animals (Fig 3). Similar but less pronounced findings were done in intestines of animals treated for 15 weeks with or without Andosan™ (data not shown). Also intestines of wild type mice were examined and found not to contain tumors. Since there was no difference in body weight or cecum weight between the groups (not shown), Andosan™ in drink water did not affect water or feed intake or intestinal bacterial load and should therefore not bias the results.

Effect on legumain expression in intestines

In swiss-roll sections of intestines immunofluorescently stained for legumain a strong expression of this metastasis-promoting protease was seen in the spontaneous tumors of untreated

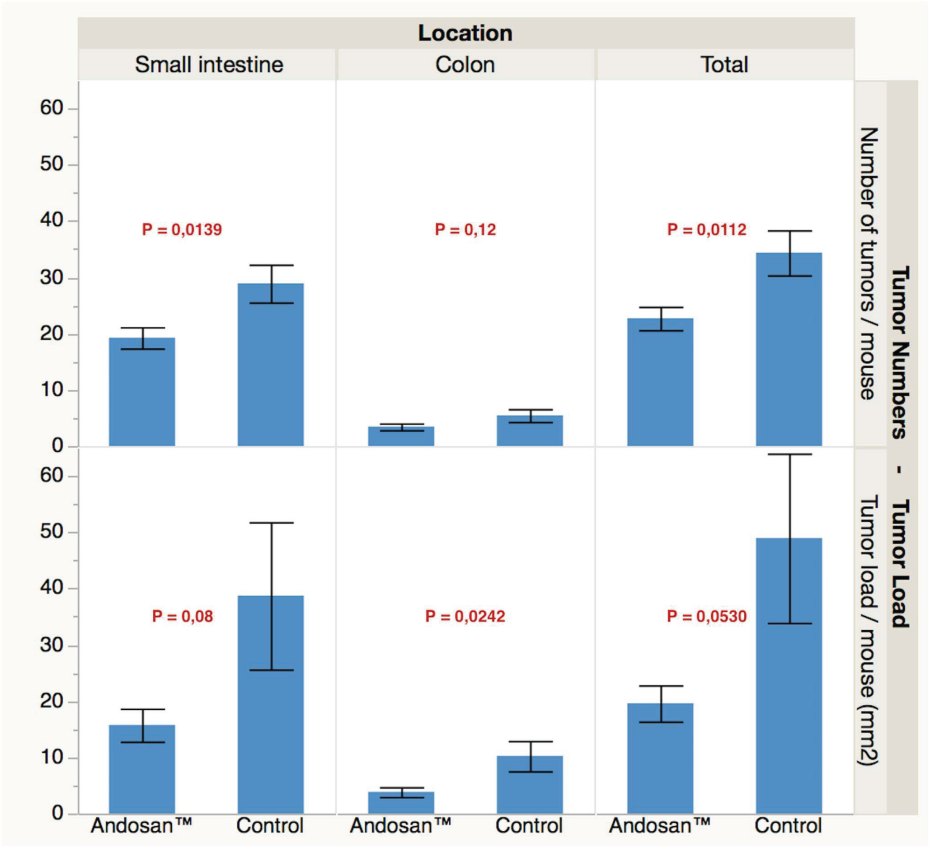

Fig 3. The effect of Andosan™ on the development of tumors in small intestine and colon in A/J Min/+ mice. Andosan™ (10%) was added or not (control) in drink water to A/J Min/+ mice (n = 46) for 22 weeks, when the animals were killed and their intestines were examined by microscopy. Both the number of tumors (top panel) and the tumor load (# tumors x size) was lower in the Andosan™ relative to the control group.

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animals. Furthermore, overall less expression was observed in the intestine from the Andosan™ treated compared with the untreated (Fig 4) animals. However, because no tumors could be identified in sections from the treated animal, a putative difference in legumain expression within tumors could not be shown.

Serum cytokine profiles in A/J Min/+ and A/J wild-type mice

Sera of both A/J Min/+ mice and wild-type controls that had been sacrificed by exsanguination, were subjected to Luminex multi(12-)plex analysis for Th1, Th2, Th17, pro- and anti-inflammatory cytokines. For Andosan™-treated compared with untreated animals, there was a significant increase in Th1 type cytokine IL-12p70 and in pro-inflammatory cytokines IL-1β, MCP-1 and TNFα (Table 1). However, Th2 and Th17 type cytokine responses were not affected.

Discussion

Our data show that the mixed *Basidiomycetes* mushroom extract Andosan™ given orally, protects against development of intestinal cancer in the colorectal cancer model, A/J *Min*/+ mice.

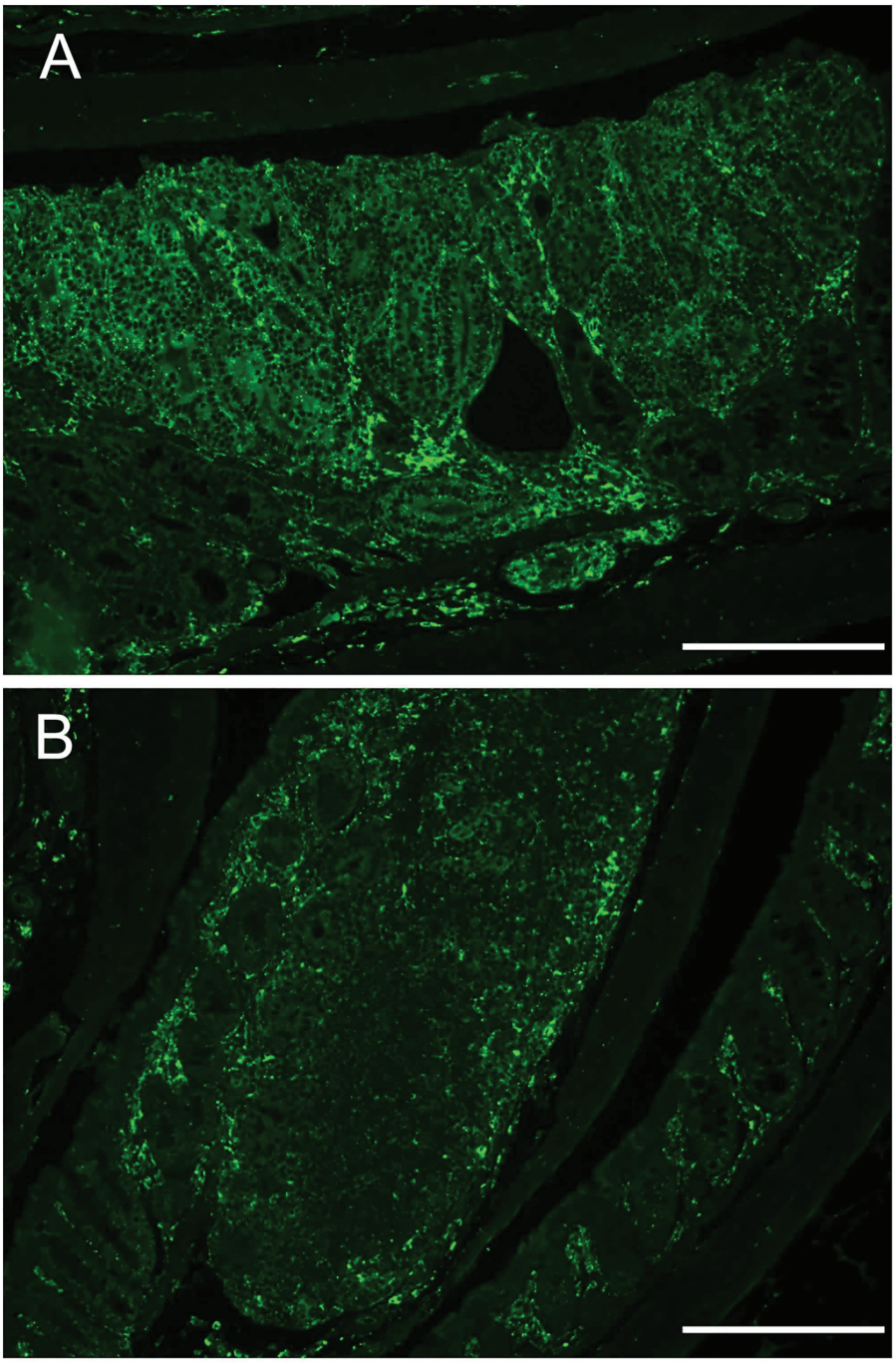

Fig 4. Representative sections showing overall higher expression of legumain (diffuse yellowish fluorescence staining) in the untreated (A) versus the Andosan™-treated (B) intestine. Notably, legumain expression was higher in tumor tissue seen in untreated animals. Scale bars represent 200 μm.

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Table 1. The Effect of Treatment with Andosan on Cytokine Values and Tumor Counts.

Response	Estimate*	P-value
IL-1β	36,34	***0,0013***
IL-2	2,14	0,52
IL-4	-2,10	0,66
IL-5	-3,19	0,44
IL-6	-4,58	0,41
IL-10	8,48	*0,06*
IL-17A	-5,28	0,83
GM-CSF	14,59	***0,0039***
IFN-y	-0,55	0,94
MCP-1	51,25	*0,03*
TNFα	92,34	***0,0013***
IL-12p70	46,70	***0,0018***
Number of small intestinal tumors	-4,83	***0,0139***
Number of colon tumors	-1,00	0,12
Total tumor number	-5,83	***0,0112***
Tumor load small intestine	-11,49	0,08
Tumor load colon	-3,20	***0,0242***
Total tumor load	-14,68	***0,0530***

* Positive values = Andosan treated group means are higher

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This is supported by the finding of i) Lower levels of the tumor-associated protease, legumain, in intestines of the Andosan™ treated A/J *Min*/+ mice, ii) Higher serum levels of Th1 type cytokine, IL-12p70 in the treated A/J *Min*/+ and wild-type mice, and iii) Strong, dose-dependent cytotoxic effect induced in vitro by Andosan™ on the human cancer colon cell line, Caco-2. Previously, another AbM extract has been shown to have cytotoxic effect on yet another human cancer colon cell line, HT-29, in addition to eight other human cancer cell lines [17]. Recently, we have found anti-proliferative effect of Andosan™ on a murine myeloma cell line [13]. Of the two additional mushrooms contained in Andosan™, *Hericium erinaceus*, which comprises ~15% of the mixed mushroom extract, has been shown to specifically inhibit metastasis of colon cancer in a transplanted mouse model [4]. Also the other *Basidiomycetes* mushroom, *Grifola frondosa*, comprising ~3% of Andosan™, has antitumor effects in mice [5, 39]. Moreover, AbM extracts have been shown to exibit apoptotic effect on leukemia cells [40], which was the mechanism of death of Caco-2 cells incubated with Andosan™ as well.

Within tumors, legumain is produced by several cell types, including tumor-associated macrophages. While this metastasis-promoting protease was observed to be highly expressed in the spontaneous tumors and overall less expressed in the intestine of Andosan™-treated animals, we were not able to identify tumors in the examined gut section from the latter. Thus, a direct coupling of reduced legumain content to reduced tumor load and effect of Andosan™ on legumain expression within tumors, remains elusive in the examined intestines. Recently, it has been found that both Andosan™ as such and the polar high molecular weight fraction of it, inhibited production of legumain by a rat macrophage cell line (RAW264.7) as well as the activation of legumain proform [36]. It has been shown both that mice lacking legumain (asparaginyl endopeptidase) have normal levels of the major cytokines [41] and that cytokines do not alter legumain expression in epithelial cells [42]. Hence, the currently increased pro-

inflammatory cytokines and others in serum should not be influenced by reduced intestinal (epithelial) legumain expression and vice versa in the Andosan™ treated mice.

This and other immuno-modulating effects such as increased cytokine production [43, 44], upregulation of adhesion molecules on leukocytes [45] and dendritic cell activation [46], are brought about by AbM—and probably also He and Gf—stimulation of monocytes, granulocytes and NK cells via TLR2, and probably dectin-1 and CD11b/18 [47,48]. In vitro Andosan™ stimulation of monocytic cells has shown increased expression of genes related to immune function [49], including the gene for IL-23 in the IL-12 family. This is in line with the current finding of an Andosan™-induced increase in the Th1 cytokine IL-12p70, which we have observed previously ex vivo in spleen cells harvested from Andosan™ treated Balb/c mice in the allergy model [8]. In vivo, oral intake of Andosan™ in a few patients with chronic HCV infection increased the expression in peripheral mononuclear leukocytes of genes related to tumor defense[10]. Probably β-glucans and other substances in mushrooms such as AbM act as danger signals and engage first the innate immune system, which then skews the adaptive immune system towards a Th1 type immune response. When studying the intestines by surface examination after administration or not of Andosan™, besides the tumors, no signs of necrosis or abnormal morphology were observed. This indicates that the mechanism of action for the Andosan™ effect in vivo in A/J *Min*/+ mice is foremost protection against tumor development (tumorigenesis) in the intestines—possibly through indirect anti-tumor Th1 response of Andosan™—and to a lesser degree a direct cytotoxic effect on tumor cells (Fig 1). The animal study was terminated at 22 weeks of age for ethical reasons before any death from intestinal cancer occurred. The intestinal adenomas become adenocarcinomas in A/J *Min*/+ mice at 30 weeks of age [27], where after the animals commence to die of cancer.

In the in vivo situation, the substances in Andosan™ will interact with the intestinal microbiota, which as a result may produce other biologically active metabolites that may affect the host. Since cereal β-glucans are found to alter gastrointestinal microbiota in pigs [50] and can ameliorate diseases through improvement of gut microbiota [51], such polysaccharides and other ingredients in Andosan™ may also have influenced the composition and activity of microbiota in the A/J *Min*/+ mice in our experiments. Furthermore, substances in the mushroom extract may be absorbed across the intestinal wall by microfold (M) cells and DC, as shown for β-glucans in murine models [52]. Similar to β-glucan, also other biologically active mushroom-derived molecules may be transported further by DC to lymphocytes in gut-associated lymphoid tissue (GALT), example given Peyers' patches, but also circulated in blood in the rodents [53].

The present observation in the mice of an increased pro-inflammatory response is in contrast to our findings with Andosan™ in humans. Previously, we have found reduced levels of pro-inflammatory serum cytokines after intake of Andosan™ in patients with the inflammatory bowel diseases, Crohn's disease and ulcerative colitis (UC) [11]–the latter of which predisposing for colon cancer—and in patients with multiple myeloma [13]. In a very recent larger, placebo-controlled clinical study in UC patients we did also observe improved clinical effects of Andosan™ after 3 weeks [54]. Since the same dose of Andosan™ (60 ml/day) had also been used in the clinical multiple myeloma study for 7 weeks without side-effects [13], the extract is considered safe. Hence, to determine whether Andosan™ could be a safe, add-on treatment for patients with colorectal cancer, a clinical trial should be performed in such patients with relapse after surgery or who are on palliative treatment only.

Supporting Information

S1 Text. Hetland et al. Andosan, animal and apoptosis Raw Data.
(XLSX)

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RESEARCH ARTICLE Open Access

Improved outcome in patients following autologous stem cell transplantation for multiple myeloma in south eastern Norway 2001–2010: a retrospective, population based analysis

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Abstract

Background: With the advent of novel drugs improved overall survival in patients with multiple myeloma, including patients who received up-front autologous stem cell transplantation (ASCT), has been reported from several centers. Here we report on overall survival in a population-based cohort of patients receiving ASCT as first line treatment and in whom novel agents were an option for second and later lines of treatment.

Methods: Patients with multiple myeloma ≤ 65 years of age who were considered for ASCT from 01.01.2001–31.06. 2005 (period 1) and from 01.07.2005 until 31.12.2009 (period 2) at Oslo University Hospital (OUH) were identified. Relevant data were collected from the patients' medical records.

Results: Altogether, 293/355 patients received ASCT. In all, median OS was 82.9 months in patients ≤ 60 years of age and 59.0 months in patients 61–65 years. For patients ≤ 60 years of age median OS increased from 70. 6 months to 87.7 months ($p = 0.22$) and median survival after start of second line therapy increased from 34. 5 months to 46.5 months ($p = 0.015$) between the two periods. For patients 61–65 years of age median OS increased from 57.3 months to 61.2 months ($p = 0.87$) and median survival after start of second line therapy was practically unchanged (32.6 months vs. 33.1 months ($p = 0.97$) between the periods. In patients ≤ 60 years of age salvage ASCT was used in 34% of the patients while in patients 61–65 years of age salvage ASCT was used in 7.3% of the patients. The use of salvage ASCT and novel drugs, as well as the number of treatment lines, were higher in patients ≤ 60 years of age and increased during the study period.

Conclusion: In patients ≤ 60 years of age an increased median OS of 17 months between the two periods were noted, but the difference failed to reach statistical significance. However, a statistically significant difference in median survival of 12 months after start of second line therapy was found in this age group, which may be explained by a more active second line treatment. In patients 61–65 years only a slight increase of survival, not statistically significant, was noted between the periods.

Keywords: Multiple myeloma, Autologous stem cell transplantation, Overall survival, Novel drugs

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Background

Multiple myeloma is a neoplastic disorder caused by malignant transformation of plasma cells. The main clinical features are lytic bone lesions, bone marrow failure, and renal failure [1]. The incidence of multiple myeloma in Norway for the period 2001–2005 was 9.7/100000 for males and 6.5/100000 for females. Median age at diagnosis was 71.2 years for males and 69.3 years for females (Cancer Registry of Norway). The majority of patients respond to chemotherapy, but virtually all patients relapse and median survival with conventional chemotherapy was around three years [2]. In the 1990s autologous stem cell transplantation (ASCT) was introduced as first line treatment for patients below 65 years of age. The superiority of ASCT over conventional chemotherapy was first demonstrated in the French IFM study, which showed a median overall survival (OS) and a median event free survival of 57 and 44 months, respectively, for patients receiving ASCT, compared to 44 and 18 months, respectively, for patients receiving conventional chemotherapy [3]. These results were later confirmed by a British study [4]. Since the late 1990s ASCT has been standard first line treatment for myeloma patients < 65 years of age, based both on clinical efficacy [5] and effect on quality of life [6]. Between 1994 and 2000 the Nordic Myeloma Study Group conducted two population based studies on the clinical impact of ASCT, which in patients ≤ 60 years of age showed a median OS of 63 months in the ASCT group versus 39 months in a historical control group [7] and in patients 61–65 years of age showed a median OS of 50 months in the ASCT group versus 27 months in historical controls [8]. Since the late 1990s several new drugs have been introduced in the treatment of multiple myeloma, starting with the immunomodulatory drug (IMiD) thalidomide in 1999 [9], followed by the proteasome inhibitor bortezomib in 2003 [10] and the second generation IMiD, lenalidomide, in 2005 [11]. Late in the study period, new drugs such as the third generation IMiD pomalidomide [12], the second generation proteasome inhibitor carfilzomid [13] and the alkylator bendamustin [14] became available. In the context of ASCT the novel drugs are nowadays being used both as induction and consolidation treatment, as well as in first relapse or later lines of therapy. Their positive impact on survival in patients receiving ASCT has been shown in several multicenter clinical trials (reviewed in [15]). Also, several recent population-based studies have shown a steady improvement of survival in multiple myeloma in general, particularly in patients ≤ 60 years of age [16, 17].

On this background, we performed a population-based analysis of the treatment results in multiple myeloma in patients 65 years of age or younger in the South-East Health Region of Norway diagnosed in a nine-year period starting from 2001. According to the national Norwegian treatment guidelines ASCT was the preferred first line treatment for multiple myeloma in patients ≤ 65 years of age in this period. These patients were accordingly referred to the regional treatment centres for ASCT, with very few exceptions.

Methods

A search was made in the patient administrative system at Oslo University Hospital (OUH) to identify patients ≤ 65 years of age who had been referred to the hospital with a diagnosis of multiple myeloma (C90.0 in the ICD 10 diagnostic system), and considered for ASCT in the period 01.01.2001–31.12.2009. The patient records of the selected patients were reviewed. OUH is the regional reference centre for the South-East Health Region of Norway (population: 2.9 million). Follow-up data were collected from patients' records at OUH and referring hospitals.

Treatment

Induction treatment

Induction treatment in the period was either vincristine, doxorubicin and dexamethasone (VAD) or cyclophosphamide and dexamethasone (Cy/Dex) [18], with the exception of two patients who received bortezomib/dexamethasone.

Stem cell mobilizing therapy

Cyclophosphamide 2000 mg/m^2 i.v. and G-CSF from day 4.

High dose therapy

Melphalan 200 mg/m^2 (140 mg/m^2 in case of creatinine clearance < 30 ml/min/m^2). In five patients tandem transplantation was performed.

Consolidation

Between October 2005 and April 2009 eligible and consenting patients were randomized between no consolidation or consolidation with bortezomib [19]. Also, in the beginning of the study period consolidation with α-interferon was given. This treatment was part of the protocol in the previous Nordic ASCT studies [7, 8] but was later gradually abandoned in routine clinical practice, mainly because of it's negative impact on quality of life. Furthermore, from 01.08.2009 until 31.11.2010 eligible and consenting patients were included in a clinical study and randomized to receive adjuvant treatment with the medicinal mushroom product Andosan™, which mainly contains the edible *Basidiomycetes* mushroom *Agaricus blazei* Murill, or with placebo [20].

Patients not receiving ASCT were treated at the discretion of the responsible physician (i.e. not by protocol).

Second line treatment was provided at the discretion of the responsible physician (i.e. not by protocol).

Patients were considered candidates for a second ASCT if the time from first ASCT to second line treatment was > 12 month.

Diagnosis, response evaluation, disease progression

Diagnostics and response evaluation were based on the criteria applied by the Nordic Myeloma Study Group in previous ASCT studies [21], with some minor modifications:

The diagnosis of multiple myeloma was accepted if criteria A + C, A + D or B + C + D of the following was accepted: (A) serum monoclonal component (M-protein) concentration of immunoglobulin IgG > 30 g/l, IgA > 20 g/l, the presence of M-protein IgD or IgE regardless of concentration or Bence-Jones proteinuria > 1 g/l. (B) M-protein in serum or urine at lower concentration than described under A; (C) at least 10% plasma cells in bone marrow aspirate or biopsy verified plasmacytoma of bone or soft tissue; and (D) osteolytic bone lesions.

Indication for ASCT

Only patients fulfilling criteria for treatment-demanding multiple myeloma were considered for ASCT or alternative chemotherapy.

Treatment response

Complete response (CR) was defined as the disappearance of M-protein from serum and urine in agarose gel electrophoresis. Partial response (PR) was defined as at least 50% reduction of the initial serum M-protein concentration and a reduction of Bence-Jones protein to < 0.2 g/L. Minor response (MR) was defined as a 25% to 50% reduction of the initial serum M-protein concentration and a reduction of Bence-Jones protein by at least 50% but exceeding 0.2 g/L. The best response achieved at any time after ASCT was registered in the study. Progressive disease (PD) was defined as an increase of the M- component by ≥ 25%. Stable disease (SD) was defined as neither fulfilling any response criteria nor criteria for progressive disease.

Classification

The patients were grouped according to the Durie and Salmon classification [22] and also according to the ISS classification [23] in cases where serum β2-microglobulin and serum albumin at diagnosis were available.

Survival

Total survival was the time between the date of diagnosis and follow-up (01.05.2017) or death. For patients who were lost to follow-up total survival was the time from the date of diagnosis until last control.

Time to next treatment was the time from the date of diagnosis until start of second line treatment, or follow-up. This parameter is based on the clinical decision by the responsible physician to start treatment and not on the fulfillment of formal criteria for disease progression.

Survival after start of second line treatment was the time from start of second line treatment until follow-up or death.

Statistics

Statistics was performed by the IBM SSPC 23 computer program. Survival analyses were performed by the Kaplan-Meier method. The median values and 95% confidence interval (CI) are indicated. Differences in survival were calculated by the Log Rank and Wilcoxon tests. Comparisons of the number of treatment regimens used in various time periods were done by the Independent samples t-test.

Results

A search in the patient administration system of OUH identified a total of 623 patients ≤ 65 years of age with a diagnosis of multiple myeloma (C 90.0 in the ICD 10 diagnostic system) between 01.01.2001 and 31.12.2009. After review 268 patients were excluded (monoclonal gammopathy of uncertain significance, solitary myeloma, multiple myeloma with no treatment indication, primary plasma cell leukemia, treatment started before 01.01.2001, AL-amyloidosis, other types of hematologic malignancies, patients not residing in the South-East Health Region of Norway). A total of 355 treatment-demanding patients with multiple myeloma were included in the study, 293 patients received ASCT and 62 patients received other types of treatment. Three patients of foreign origin, who returned to their countries after ASCT, were censored for survival at the last control in Norway. No other patients were lost to follow-up. For patients offered ASCT, type of M-component and clinical stage at diagnosis are presented in Table 1 and treatment details and treatment response are presented in Table 2. The reasons for not giving ASCT were: comorbidity (31 patients), insufficient stem cell harvest (11 patients), complications to induction treatment (10 patients), disease progression (5 patients), earlier chemotherapy for other type of cancer (1 patient), no consent (4 patients).

Survival

Overall survival patients ≤60 years of age

In the study period 233 patients started treatment for multiple myeloma, 212 (91%) patients received ASCT and 21 (9%) patients received other treatments. Two patients refused ASCT and 19 patients did not receive ASCT because of comorbidity or for other clinical

Table 1 Patient chararcteristics-patients receiving ASCT

	Patients ≤ 60 years of age	Patients 61–65 years of age
	N (%)	*N* (%)
M-Component		
IgG κ	76 (36,1)	31 (37,5)
IgG λ	24 (11,9)	11 (13,8)
IgA κ	27 (12,0)	13 15,9)
IgA λ	12 (6,6)	7 (8,3)
light chain κ	42 (19,1)	10 (13,2)
light chain λ	16 (7,3)	5 (6,3)
non secretory	13 (6,0)	2 (2,5)
biclonal	2 (1,0)	0 (0)
no information	0	2 (2,5)
Stage Durie&Salmon		
IA	55 (26,4)	22 (27,1)
IB	5 (2,4)	2 (2,4)
IIA	82 (38,9)	33 (41,0)
IIB	16 (7,4)	4 (5,0)
IIIA	40 (18,5)	17 (21,2)
IIIB	14 (6,4)	3 (3,3)
Stage ISS		
ISS I	65 (31,0)	21 (26,3)
ISS II	40 (18,5)	18 (22,6)
ISS III	39 (18,1)	19 (23,8)
No information*)	68 (32,4)	23 (27,3)

*)β-globulin missing

Table 2 Treatment characteristics- Patients receiving SCT

	Patients ≥ 60 years of age	Patients 61–65 years of age
	N (%)	*N* (%)
Induction treatment		
VAD	90 (42,6)	32 (39,5)
Cy/Dex	120 (56,5)	49 (60,5)
Vel/Dex	2 (0,9)	0
Consolidation		
IFN	38 (17,9)	10 (12,3)
Bortezomib	25 (11,9)	7 (8,6)
No consolidation	149 (70,2)	64 (79,1)
Treatment response		
Progressive disease	1 (0,6)	3 (3,6)
Stable disease	7 (3,2)	1 (1,2)
Minimal response	9 (4,2)	2 (2,4)
Partial response	102 (48,1)	49 (59.0)
Complete response	57 (27,2)	19 (22,9)
Not evaluable	36 (16,7)	9 (10,9)

VAD vincristin- adriamycin-dexamethasone, *Cy/Dex* cyclophosphamide-dexamethasone, *Vel /Dex* Bortezomib-dexamethasone, *IFN* α- Interferon

reasons. Median OS for all patients was 75.4 months (95% CI 63.5–87.3), 82.9 months (95% CI 70.8–95.0) for patients receiving ASCT and 27.0 months (95% CI 17.0–37.1) for patients not receiving ASCT ($p < 0.0001$). For patients receiving ASCT and starting therapy between 01.01.2001 and 31.06.2005 ($n = 99$) median OS was 70.6 months (95% CI 53.2–88.1), while median OS was 87.7 months (95%CI 75.2–100.1) for patients receiving ASCT and starting therapy between 01.07.2005 and 31.12.2010 ($n = 113$). Thus, the median overall survival increased by 17 months between these two periods (Fig. 1). However, this difference was not statistically significant ($p = 0.22$).

Patients 61–65 years of age

In the study period 122 patients in this age group started treatment, 81 (66%) patients received ASCT and 41 (34%) patients received other treatments. Two patients refused ASCT and 39 patients did not receive ASCT because of comorbidity or for other clinical reasons.

Medan OS for all patients in this age group was 46.0 months (95% CI 37.5–54.5). For patients receiving ASCT median OS was 59.0 months (95% CI 43.0–76.6) and for the other patients median OS was 31.6 months (95% CI 15.1–48.1) ($p < 0.0001$). For the patients starting treatment in the period 01.01.2001–31.06.2005 and receiving ASCT, median OS was 57.3 months (95% CI 45.2–69.4) ($n = 32$), while median OS was 61.2 months (95% CI 29.7–92.7) for patients receiving ASCT and starting treatment in the period 01.01.2005–31.12.2009 ($n = 49$). Thus, only a small improvement of overall survival with approximately 4 months between these two periods for patients receiving ASCT was noted ($p = 0.87$) (Fig. 2).

Second line therapy

Patients ≤60 years of age

Second line therapy was performed in 187/212 patients ≤ 60 years of age who received ASCT as first line therapy (88%). Mean age at start of second line therapy was 57.3 years. Of the 25 patients who did not receive second line therapy two patients died within 90 days of ASCT (early death) and six patients died from non-myeloma disease. The remaining 17 patients were alive without disease progression at follow-up. In total, 64 patients (34.2%) received salvage ASCT. Their median age was 56.5 years. Median time to second line therapy was 36.1 months (95% CI 33.3–38.8) in patients who started treatment between 01.01.2001 and 31.06.2005 ($n = 89$) and 33.5 months (95% CI 27.3–39.4) in patients who started treatment between 01.07.2005 and 31.12.2009 ($n = 98$). Thus, the time to new treatment practically did not change during

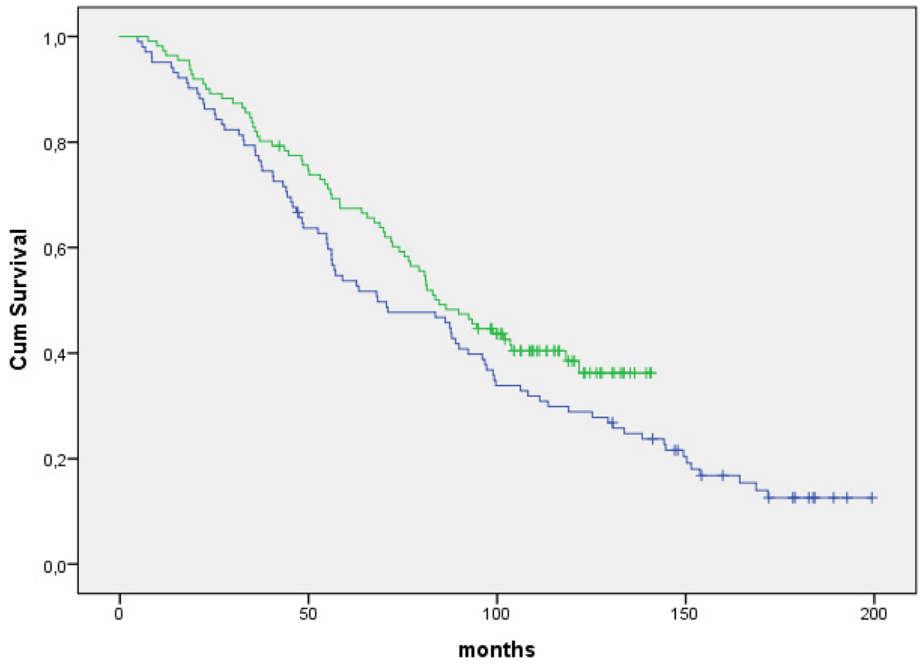

Fig. 1 Overall survival in patients ≤ 60 years of age receiving ASCT. Blue curve: Patients who started treatment in the period 01.01.2001–31.06.2005 ($n = 99$). Median overall survival: 70.6 months (95% CI 53.2–88.1). Green curve: Patients who started treatment in the period 01.07.2005–31.12.2009 ($n = 113$). Median overall survival: 87.7 months (95% CI 75.2–100.1). $P = 0.21$ (ns)

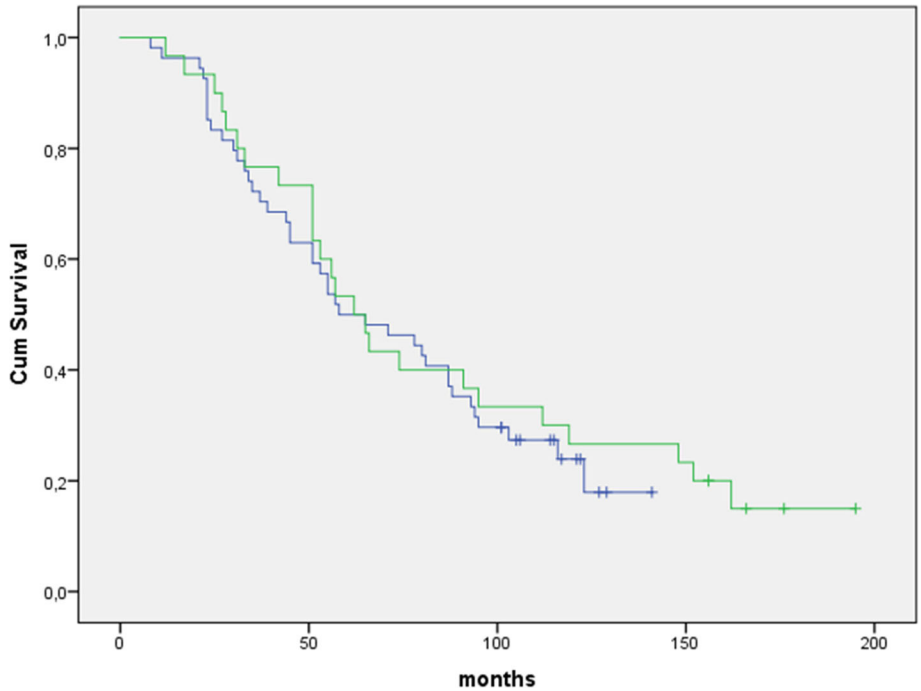

Fig. 2 Overall survival in patients 61–65 years of age receiving ASCT. Blue curve: Patients who started treatment in the period 01.01.2001–31.06.2005 ($n = 32$). Median overall survival = 57.3 months. Green curve = Patients who started treatment in the period 01.07.2005–31.12.2009 ($n = 49$). Median overall survival = 61.2 months. ($p = 0,87$(ns))

the study period. For patients starting treatment in the first period median survival after start of second line therapy was 34.5 months (95% CI 23.6–45.3), while for patients who started treatment in the second period median survival after start of second line therapy was 46.5 months (95% CI 36.8–56.2) ($p = 0.015$).

Figure 3 shows the percentage of patients given different second line treatment regimens in the two periods. In the second period there was a significant increase of the use of salvage ASCT, from 18.8% (17/90 patients) to 41.7% (47/97 patients) of the patients) ($p < 0.0001$), an increase in the use of bortezomib (from 61.4 to 76.0%) ($p = 0.1$), lenalidomide (from 31.7 to 57.4%) ($p < 0.001$) and pomalidomide (from 5.7 to 14.8%) ($p = 0.02$), as well as a decreased used of melphalan from 48,1% to 31,9% ($p = 0,01$) and thalidomide from 48,0% to 36,3% ($p = 0,1$), compared to the first period.

The median number of lines of therapy given beyond first line was 2.96 in the first period and 3.63 in the second period ($p = 0.002$).

Patients 61–65 years of age

Among the 81 patients who received ASCT, second line therapy was started in 65 patients (80%). Mean age at start of second line therapy was 65.5 years. Of the 16 patients who did not receive second line treatment two patients died within 90 days from ASCT (early death) and six patients died from non-myeloma related causes. The remaining eight patients were alive without disease progression at follow-up. Six patients received salvage ASCT (9.2%). Median time to second line therapy was 35.7 months (95% CI 24.0–47.9) in patients who started treatment between 01.01.2001 and 31.06.2005 ($n = 32$) and 37.3 months (95% CI 20.7–53.9) in patients who started treatment between 01.07.2005 and 31.12.2009 ($n = 49$) ($p = 0,82$). Median survival after start of second line therapy was 32.6 months (95% CI 21.7–42.9)($n = 31$) in the first group and 33.1 months (95% CI 19.3–46.9) in the second group ($n = 49$) ($p = 0,97$).

Figure 4 shows the percentage of patients given different second line treatment regimens in the two periods in this age group. Salvage ASCT was used in 9.2% (6/65) of the patients, and there was an increased use of salvage ASCT from 3.7 to 13.1% between the study periods (1/27 patients vs. 5/38 patients). Furthermore, a decrease in the use of melphalan/prednisolone (from 64.5 to 30.6%) ($p = 0,01$) and thalidomide (from 64.5 to 44.9%) ($p = 0,09$) as well as an increased use of bortezomib (from 48.4 to 59.2%) ($p = 0,15$) and lenalidomide (from 25.8 to 44.9%) ($p = 0,02$), was noted between the study periods.

The median number of lines of therapy delivered beyond first line was 2.84 in the first period and 3.00 in the second period ($p = 0,71$).

Influence of CR versus non CR after ASCT on survival parameters

In patients < 60 years CR was reached in 57/176 (27.2%) of the patients evaluable for response, while response was not evaluable in 36 patients. Statistically significant increased OS and time to second line therapy was found in CR patients compared to non CR patients in both treatment periods (Table 3). In patients 61–65 years CR was reached in 19/72 of the patients evaluable for response, while response was not evaluable in 9 patients. Also in this age group a trend for a survival advantage in CR patients was found (Table 4). However, the results

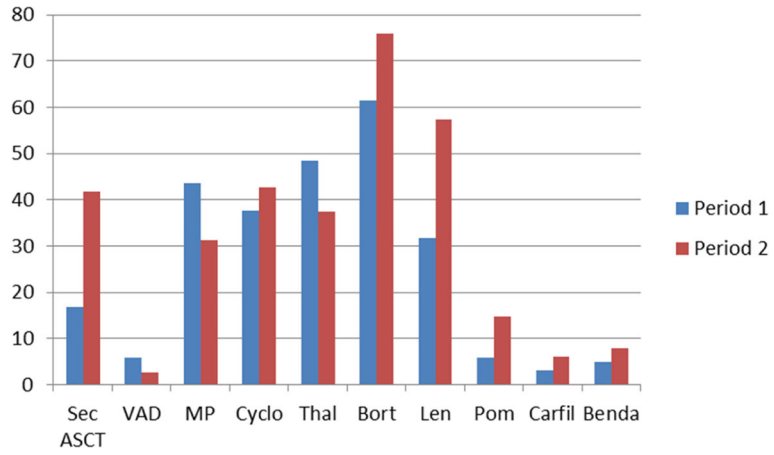

Fig. 3 Second and later lines of treatment. Patients ≤ 60 years of age. Percentage of patients given different treatments in the two periods. Period 1 = 01.01.2001–31.06.2005 Period 2 = 01.07.2005–31.12.2009. Sec ASCT = Salvage ASCT, VAD = Vincristin-Adriamycine-Dexamethasone, MP = Melphalan –Prednisolone, Cyclo = Cyclophosphamide, Thal = Thalidomide, Bort = Bortezomib, Len = Lenalidomide, Pom = Pomalidomide, Carfi = Carfilzomide, Benda = Bendamustine

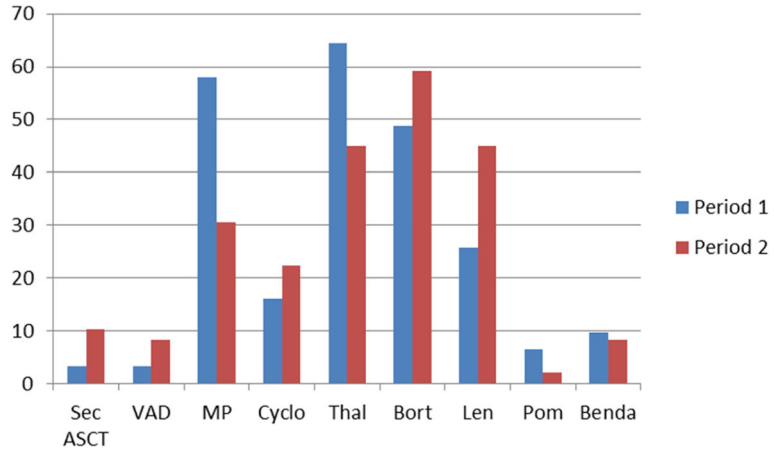

Fig. 4 Second and later lines of treatment. Patients 61–65 years of age. Percentage of patients given different treatments in the two periods. Period 1 = 01.01.2001–31.06.2005 Period 2 = 01.01.2005–31.12.2009. Sec ASCT = Salvage ASCT, VAD = Vincristin-Adriamycine- dexamethasone, MP = Melphalan- Prednisolone, Cyclo = Cyclophosphamide, Thal = Thalidomide, Pom = Pomalidomide, Benda = Bendamustin

must be interpreted with caution because of the low number of patients in this age group.

Discussion

In this population based retrospective study of the outcome of ASCT in clinical practice a median OS of 82.9 months was found in patients ≤ 60 years of age and 59.0 months in patients 61–65 years of age. This represents an improved outcome compared to previous Nordic studies, conducted in the periods 1994–1997 and 1998–2000, respectively, which showed a median overall survival of 63 months in patients ≤ 60 years of age [7] and 50 months for patients 61–65 years of age [8]. Furthermore, our findings in this population-based study indicate an improved survival during the study period as median OS increased from 70.6 months to 87.7 months for patients ≤ 60 years of age. However, the difference failed to reach statistical significance, which may be explained by the heterogenicity of this population based patient material, which results in larger confidence intervals than usually encountered in prospective clinical studies with strict inclusion criteria. In patients 61–65 years of age, median OS only increased from 57.3 months to 61.2 months between the periods. An inferior survival in the age group 61–65 years of age compared to patients ≤ 60 years of age was previously reported in a Nordic study [8], and recently a similar result was found in a comprehensive analysis of 2316 patients 61–65 years of age included in studies conducted by the Intergroup Francais de Myelome [24]. Time to new treatment remained approximately the same in both age groups and in both study periods. This indicates that the net difference noted in overall survival was due to differences in the results of salvage therapy following relapse. In the younger age group median OS after start of salvage therapy increased from 33,5 months to 46,5 months between the two periods ($p = 0.015$), whereas OS practically did not change in the higher age group (32.6 months vs 33.1 months). In patients ≤ 60 years of age 34.3% of the patients received salvage ASCT and the use of salvage ASCT increased significantly between the two periods, from 16.8 to 41.7%. In the higher age group the use of salvage ASCT increased only from 3.7 to 13.1% between the two periods. The differences in the use of salvage ASCT

Table 3 Survival parameters - CR versus non CR after ASCT. Patients ≤ 60 years. Number of patients evaluable for treatment response = 176

	Median Overall Survival (Months) (95% CI)			Median Time to New Treatment (Months) (95% CI)		
	CR[1]	Non CR[2]	*P*- value	CR	Non CR	*P*-value
Period 1[3] (*N* = 69)	92.3 (33.1–151.6)	56.1 (39.1–73.1)	*P* = 0.04	45.4 (7.0–110.3)	36.6 (31.3–42.0)	*P* = 0.001
Period 2[4] (*N* = 107)	Not reached[a]	81.0 (76.1–113.3)	*P* = 0.001	49.7 (8.4–91.0)	29.4 (25.6–33.2)	*P* = 0.001
Period 1 + 2 (*N* = 176)	133.7 (80.6–186.9)	79.3 (62.2–96.3)	*P* = 0.001	49.7 (19.2–80.1)	31.3 (25.2–37.3)	*P* = 0.001

[a]Mean overall survival = 112.3 months (95% CI 99.7–124.4)
[1]Number of CR patients: Period 1: 24/69 Period 2: 33/107
[2]Number of non CR patients Period 1: 45/69 Period 2: 74/107
[3]Period 1 = 01.01.2001–31.06.2005
[4]Period 2 = 01.07.2005–31.12.2009

Table 4 Survival parameters - CR versus non CR after ASCT. Patients 61–65 years. Number of patients evaluable for treatment response = 72

	Median Overall Survival (Months) (95%CI)			Median Time to New Treatment (Months) (95%CI)		
	CR[1]	Non CR[2]	*P*- value	CR	Non CR	*P*-value
Period 1[3] (*N* = 32)	59.8 (41.0–78.5)	52.1 (8.4–95.8)	*P* = 0.8 (ns)	28.7 (5.0.-51.4)	35.7 (14.9–56.5)	*P* = 0.8 (ns)
Period 2[4] (*N* = 40)	75.0 (46.3–102.6)	50.1 (42.1–68.1)	*P* = 0.2 (ns)	56.4 (32.4–80.9)	29.9 (16.4–39.3)	*P* = 0.02
Period 1 + 2 (*N* = 72)	68.5 (51.7–85.4)	52.3 (48.1–56.1)	*P* = 0.4	43.9 (28.7–59.0)	31.3 (24.3–37.8)	*P* = 0.04

[1]Number of CR patients: Period 1: 6/32 Period 2: 13/40 Total 19
[2]Number of non CR patients Period 1: 26/32 Period 2: 27/40 Total 53
[3]Period 1 = 01.01.2001–31.06.2005
[4]Period 2 = 01.07.2005–31.12.2009

may be explained by the differences in age at start of second line therapy (mean age was 57.3 years in patients ≤ 60 years of age and 65.5 years in patients 61–65 years of age). In both groups an increased use of bortezomib, lenalidomide and pomalidomide was noted in the second period. However, the use of novel drugs was generally higher in patients ≤ 60 years of age than in the higher age group. Furthermore, in younger patients the number of treatment lines after progression increased between the two periods. This may be interpreted as a more active approach to treatment after progression in younger patients, both at OUH and at the other hospitals in the region, where a majority of the patients were followed after their first ASCT. Furthermore, our study shows a clear survival advantage of patients reaching CR after ASCT, versus non CR patients. This result is in line with previous reports from other population based studies [25, 26].

Conclusion

The results in this study confirms other population based reports of increased survival in recent years in patients receiving ASCT [25, 27] and shows that patients with multiple myeloma in Norway benefit from improved treatment in routine clinical practice. The improvement is most pronounced in patients ≤ 60 years of age, which may be explained by an increased use of salvage ASCT and novel drugs; in other words a more active approach to treatment at progression in this age group.

Abbreviations

ASCT: High dose chemotherapy with autologous stem cell support; CR: Complete response; IMiD: Immunomodulatory drug; MR: Minor response; OS: Overall survival; OUH: Oslo University Hospital; PD: Progressive disease; PR: Partial response; SD: Stable disease

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Availability of data and materials

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

Authors' contributions

Research concept and design: JMT, FHS, TG-D, GET. Collection and assembly of data: JMT, ES, KA, CDV. Patient recruitement: NG, TG-D. Data analysis and interpretation: JMT, FHS, GET. Writing the article: JMT, FHS, GET. Critical revision of the article: JMT, FHS, GET,TG-D, NG. Final revision of the article: JMT, FHS, TG-D, NG, ES, KA, CDV, GET. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The search in the patient administrative system, compiling of the clinical data and their subsequent statistical analysis were approved by the Data Protection Officer at Oslo University Hospital, with reference to the Norwegian Patient Record Act § 6, cf. the Health Personel Act §26. In short, these acts state that a Norwegian health institution may create internal patient databases for quality control of the treatment, provided that specific guidelines to assure the security of the data are followed. For the creation of such databases patient consent or consent from the Ethical Comitee is not required. The data may be subject to publication if completely anonymized.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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