

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 immuno-modulatory 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,  $\beta$ -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 amoebocyte 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<sub>2</sub> 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<sup>3</sup> cells/cm<sup>2</sup> were seeded onto 25 cm<sup>2</sup> 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<sup>6</sup>) 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<sub>2</sub> 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<sup>2</sup>) and tumor load (mm<sup>2</sup>), 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 paraffin 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<sup>^</sup>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-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, GM-CSF, MCP-1, TNF $\alpha$ , and IFN $\gamma$ ).

### 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.

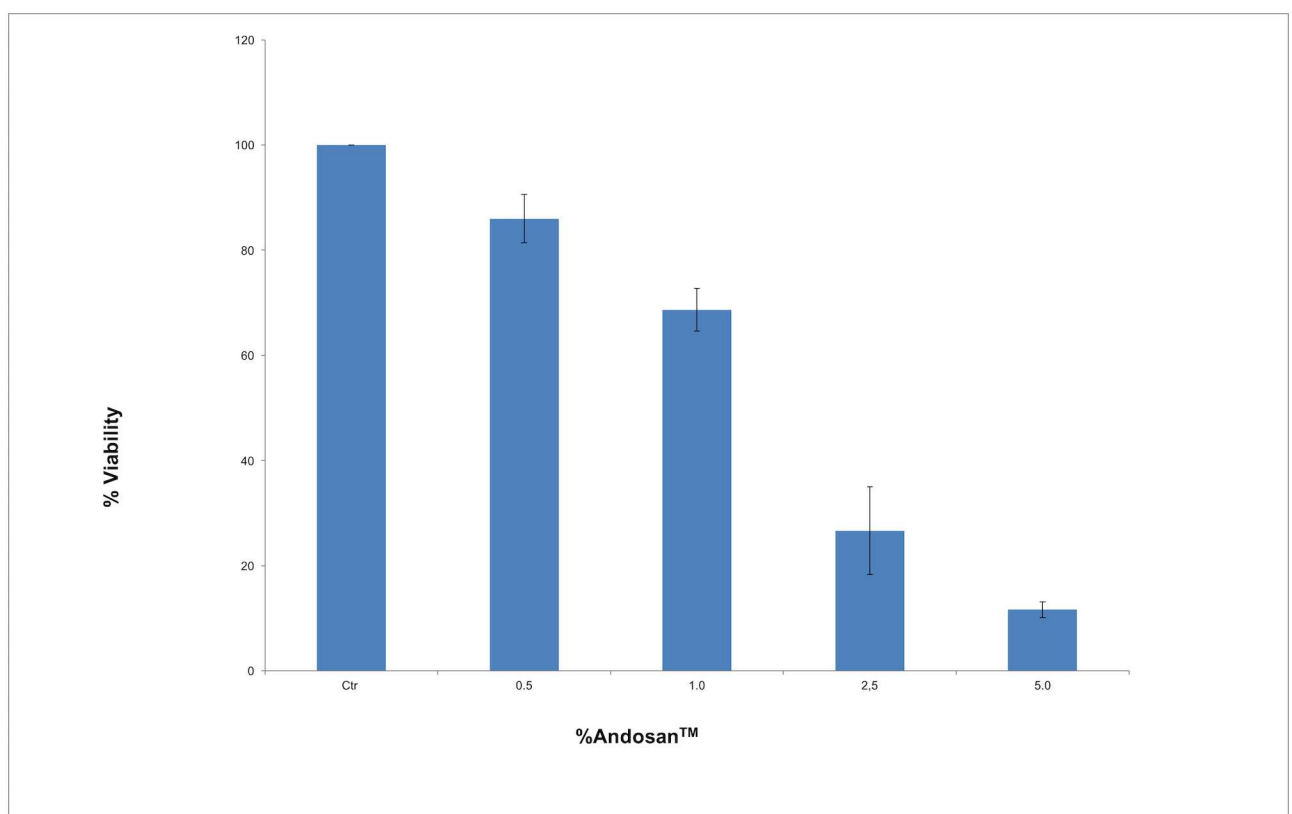
### Ethical considerations

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

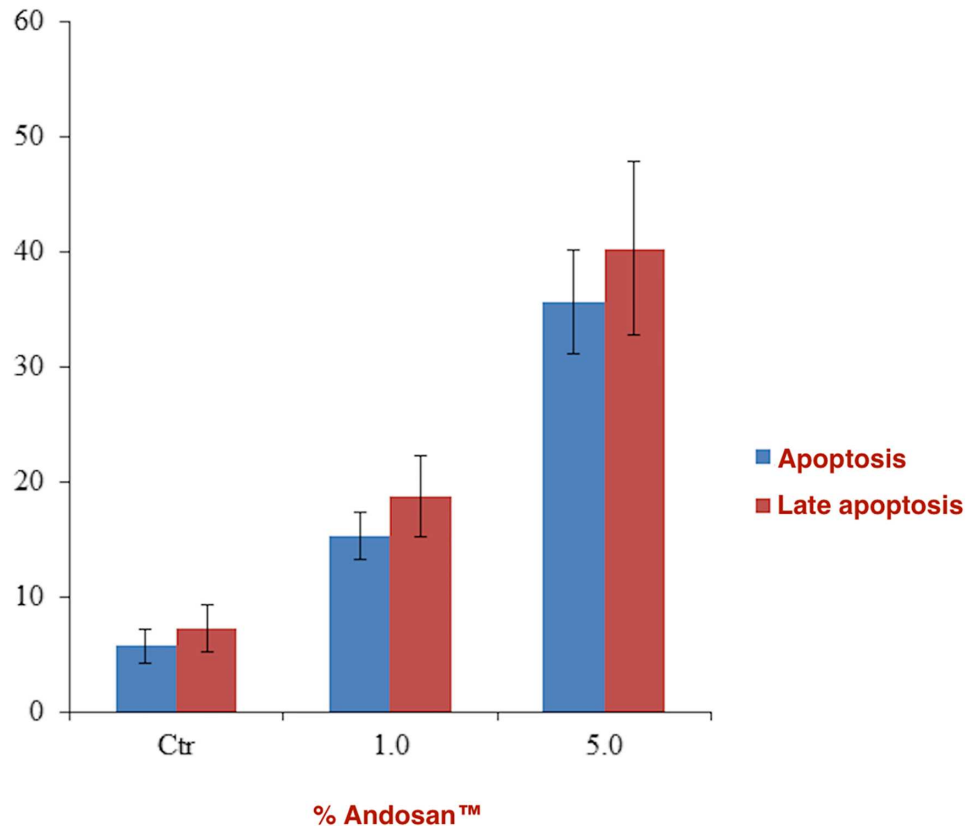
*In vitro* antitumor effect of Andosan™ was examined in cultures of Caco-2 cells after 96h. Andosan™ reduced Caco-2 cell viability in a concentration (0.5–5.0%) dependent manner (Spearman's correlation coefficient  $\rho = -0.986$ ,  $p < 0.001$  (Fig 1). Whereas the concentration of 5% of Andosan™ induced 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  $5.7\% \pm 1.5\%$  in untreated cells to  $15.3\% \pm 2.1\%$  for Andosan™ 1% and  $35.6\% \pm 4.5\%$  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  $7.3\% \pm 2.1\%$  for untreated cells to  $35.6 \pm 4.5$  for Andosan™ 1.0% and  $39.7\% \pm 7.6\%$  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  $\pm$  SD in percentage of proliferation relative Caco-2 cells cultured without Andosan™ (= 100%) and represent 3 independent experiments. Ctr: control.

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**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  $\pm$  SD and represent 3 independent experiments. Ctr: control.

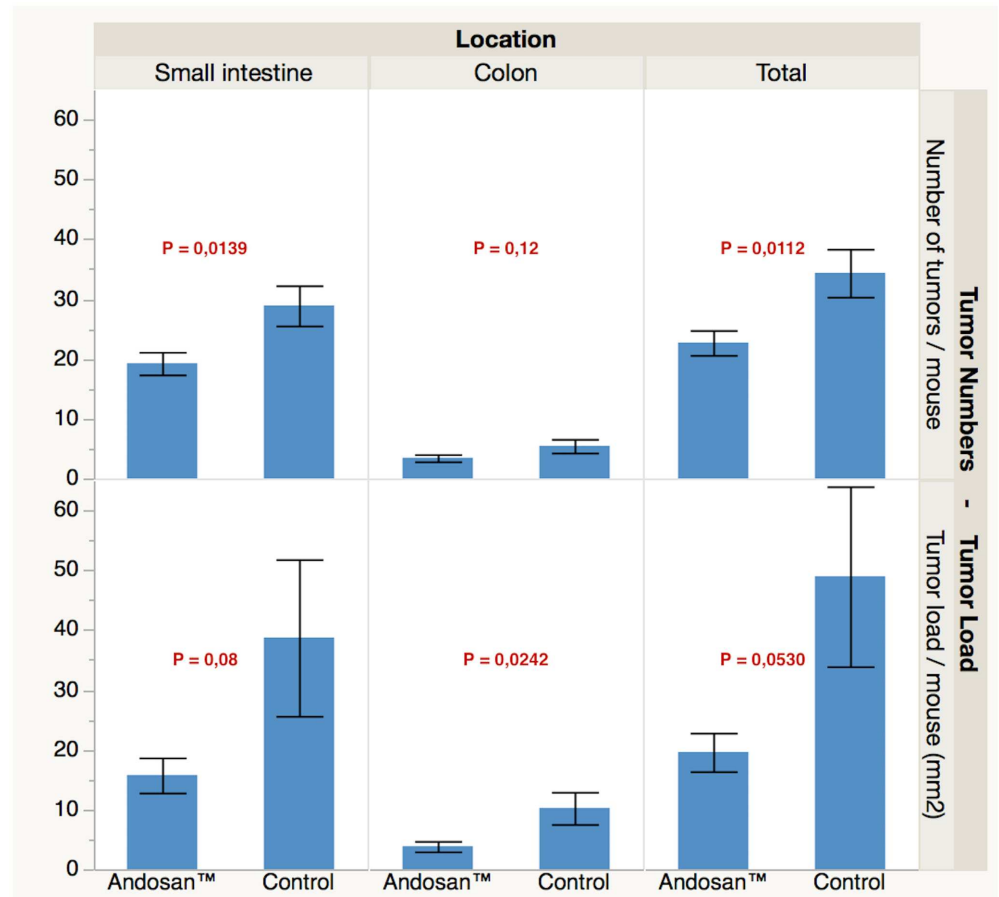
doi:10.1371/journal.pone.0167754.g002

### 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.

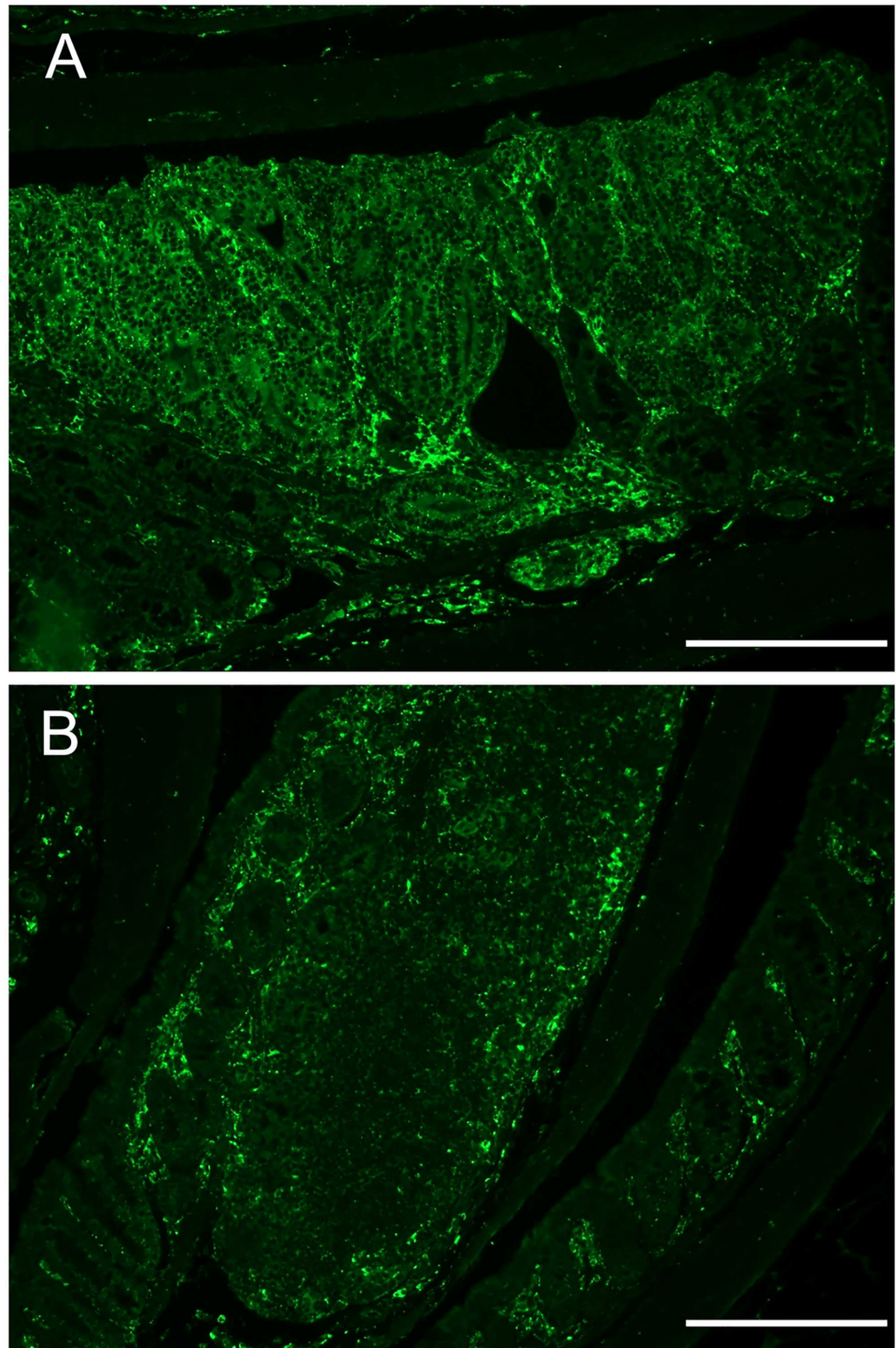
### Serum cytokine profiles in A/J Min/+ and A/J wild-type mice

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  $\mu$ m.**

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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-γ	-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

doi:10.1371/journal.pone.0167754.t001

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 exhibit 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  $\beta$ -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  $\beta$ -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  $\beta$ -glucans in murine models [52]. Similar to  $\beta$ -glucan, also other biologically active mushroom-derived molecules may be transported further by DC to lymphocytes in gut-associated lymphoid tissue (GALT), example given Peyer's 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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