Stimulation of human monocyctic 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 phylum 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, AndoSan⁶⁴, induces modulation of expression of cell surface markers. Incubation of MDDC with 10% of AndoSan⁶⁴ 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 AndoSan⁶⁴ to immunomodulate and activate MDDC in vitro, and agrees with the recent findings of increased production of cytokines and chemokines in AndoSan⁶⁴-stimulated MDDC cultures. Furthermore, it is demonstrated that AbM can stimulate human promonocyctic 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 AndoSan⁶⁴ 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-κB 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...
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 TLR4 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 fromuffy 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 auffy 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% CO₂ 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°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 Dickinson 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 0.7x10⁶ cells/ml and incubated with 10% of AbM or PBS for 1h. Then 0.5x10⁶ 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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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.

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, α-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 a role in the immune response.
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.
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.

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 AS 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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