Varying Effects of Different β-Glucans on the Maturation of Porcine Monocyte-Derived Dendritic Cells

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β-Glucans are well known for their immunomodulatory capacities in humans and mice. For this reason, together with the European ban on growth-promoting antibiotics, β-glucans are intensively used in pig feed. However, as shown in the present study, there is much variation in the stimulatory capacities of β-glucans from different sources. Since dendritic cells (DCs) are the first cells that are encountered after an antigen is taken up by the intestinal epithelial cell barrier, we decided to investigate the effect of two concentrations (5 and 10 μg/ml) of five commercial β-glucan preparations, differing in structure and source, on porcine monocyte-derived dendritic cells (MoDCs). Although all β-glucans gave rise to a significant reduction of the phagocytic activity of DCs, only Macrogard induced a significant phenotypic maturation. In addition to Macrogard, zymosan, another β-glucan derived from Saccharomyces cerevisiae, and curdlan also significantly improved the T-cell-stimulatory capacity of MoDCs. Most interesting, however, is the cytokine secretion profile of curdlan-stimulated MoDCs, since only curdlan induced significant higher expression levels of interleukin-1β (IL-1β), IL-6, IL-10, and IL-12/IL-23p40. Since the cytokine profile of DCs influences the outcome of the ensuing immune response and thus may prove valuable in intestinal immunity, a careful choice is necessary when β-glucans are used as dietary supplement.

Dendritic cells (DCs) are the directors of the immune system and form the messengers between the innate and the adaptive immune system. Immature DCs identify pathogens by recognizing signatures present in microbes, so-called pathogen-associated molecular patterns (PAMPs), through the expression of pattern recognition receptors (PRRs). Recognition of PAMPs by these PRRs results in the activation and maturation of DCs. This maturation process is associated with a loss of phagocytic activity and an upregulation of major histocompatibility complex (MHC) and costimulatory molecules, such as CD80, CD86, and CD40. In addition to functional and phenotypic changes, the exposure of DCs to microbial components results in the production of cytokines that modulate the T-cell polarization and their functions. Upon interaction with DCs, CD4+ T cells can differentiate into a variety of effector subsets, including Th1 and Th2 cells, the more recently identified Th17 cells, and regulatory T cells (15, 21, 43). Furthermore, DCs have been shown to trigger B-cell growth and differentiation (9, 16). DCs are thus capable of modulating the nature of immune responses, which in turn are dependent on the type of PRR that is activated. Phagocytes, such as DCs, express a wide variety of PRRs, such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors, and C-type lectin receptors on their cell surfaces (18, 28, 34). Dectin-1, a C-type lectin receptor and the most important PRR recognizing β-glucans, is expressed by various antigen-presenting cells, including immature DCs (ImDCs) and macrophages (41).

β-Glucans are one of the most abundant forms of polysaccharides found inside the cell wall of bacteria, fungi, and yeasts (23). All β-glucans are glucose polymers linked by a 1,3 linear β-glycosidic chain core and, depending on the source, they differ in length and branching structures. β-Glucans have a number of beneficial effects on the immune system, making them interesting for the development of β-glucan-based therapeutics and food supplements (6, 42). In humans, β-glucans are regularly used as prebiotic supplement to increase the stability of the gut flora, to augment innate immune responses, and to orchestrate healthy immune responses (39). In the pig industry, β-glucans are also applied as dietary supplements. However, the molecular mechanisms through which different β-glucans exert their effects are not well known (38). Nevertheless, it is important to know and understand the effect of different β-glucans on the immune system to use β-glucans efficiently in practice. In a previous study, we tested the direct effect of different β-glucan preparations on porcine monocytes, neutrophils, and lymphocytes (31). In the present study, we focused on the effects they have on DC maturation since these cells are the most important antigen-presenting cells and key players in the initiation and stimulation of the adaptive immunity.

MATERIALS AND METHODS

β-Glucans and LPS. Laminarin, curdlan, zymosan, and the β-glucan purified from Euphlaena gracilis were purchased from Sigma (Bornem, Belgium), as was lipopolysaccharide (LPS; serotype O55:B5), which served as a control. Macrogard, which is currently used as a dietary supplement in the pig industry, was kindly provided by Biotec Pharmacon ASA (Norway). A description and comparison of the carbohydrate structures, as well as the preparation and storage of these β-glucans, has been published (31). The endotoxin concentration present in each β-glucan preparation was determined by a chromogenic Limulus amebocyte lysate test (Cambrex BioScience, Inc., Walkersville, MD) and, with the
exception of curdlan (47 endotoxin units/µg of β-glucan), were consistently lower than 0.5 endotoxin units/µg of β-glucan.

Generation of monocyte-derived DCs. Porcine monocyte-derived DCs (MoDCs) were generated from peripheral blood mononuclear cells (PBMC). Briefly, peripheral blood was collected on heparin from the jugular vein of four Belgian Landrace pigs that were 8 to 12 weeks old; the PBMC were then isolated by density gradient centrifugation on a Lymphoprep (Nycoderm Pharma AS, Life Technologies, Merelbeke, Belgium). CD172a+ cells were isolated from the PBMC fraction by positive magnetic activated cell separation (Miltenyi-Biotec, Bergisch Gladbach, Germany) using anti-CD172a monoclonal antibody (MAb; 74-12-15A [24]) and anti-mouse IgG microbeads, together with LS separation columns (Miltenyi-Biotec). The obtained cells were cultured in 24-well plates at a density of 5.10^5 cells in phenol-red free Dulbecco modified Eagle medium (DMEM; Gibco, Merelbeke, Belgium) containing 10% fetal calf serum (FCS; Greiner), penicillin (100 IU/ml; Gibco), streptomycin (100 µg/ml; Gibco), recombinant porcine granulocyte-macrophage colony-stimulating factor (rGMCSF) (14), and recombinant porcine interleukin-4 (rPII-4; R&D Systems, Minneapolis, MN), followed by incubation at 37°C in a humidified atmosphere at 5% CO\textsubscript{2} to generate MoDCs as previously described (5). After 3 days, the cultures were supplemented with fresh cytokines. On day 4, cells were stimulated for 24 h with 5 or 10 µg of the different β-glucans or LPS/ml.

Phenotypy of MoDCs. The surface expression of various DC maturation markers after stimulation was assessed by flow cytometry (FACSCanto). Upon stimulation with 5 and 10 µg of the different β-glucans/ml or 1 or 10 µg of LPS/ml, the MoDCs were harvested, washed with RPMI 1640 plus 1% FCS, and labeled with a primary mouse MAb for 20 min at 4°C. The following primary MAbS were used to identify the maturation markers—anti-MHC-II MSA3 (20), anti-CD40 G28-5 (3), and a human CTLA4-muIg fusion protein (Ancell, Bayport, MN)—to detect the expression of CD80 and CD86. Cells stained with isotype-matched irrelevant MAbS were used as a negative control. After incubation, cells were washed and stained with fluorescein isothiocyanate-conjugated F(ab\textsubscript{2})\textsubscript{2} fragments of sheep anti-mouse IgG antibodies (Sigma) for another 20 min at 4°C. Next, the cells were washed, and propidium iodide was added to the cells to exclude dead cells from the flow cytometer analysis. The data were acquired on a FACSCanto flow cytometer with a minimum event count of 20,000 and analyzed using FACSDiva software (Becton Dickinson, Erembodegem, Belgium).

Antigen uptake. The phagocytic activity of β-glucan-stimulated MoDCs was evaluated with ovalbumin-dQ (ova-dQ; Invitrogen/Molecular Probes). Upon incubation with the different β-glucans (5 or 10 µg/ml) or LPS (1 or 10 µg/ml) for 24 h, the MoDCs were harvested, washed with RPMI 1640 plus 1% FCS, and incubated for 1 h with 10 µg of ova-dQ/ml at 37°C in a humidified atmosphere at 5% CO\textsubscript{2}. To analyze the background fluorescence, the uptake at 4°C was measured. The uptake of ova-dQ by stimulated MoDCs was analyzed by flow cytometry as described above.

Allogeneic mixed leukocyte reaction. Mixed leukocyte reactions were performed in 96-well round-bottom culture plates (Nunc) with CD172a-depleted cells (2 x 10^5 cells/well) as responder cells. Allogeneic MoDCs, stimulated for 24 h with the different β-glucans (5 or 10 µg/ml) or LPS (1 or 10 µg/ml), were added to the cultures as stimulator cells at a ratio of 1:30. Cocultures were performed in triplicate and maintained in DMEM, 10% FCS, 1% penicillin-streptomycin, and 50 µM 2-mercaptoethanol at 37°C in a humidified atmosphere at 5% CO\textsubscript{2}. After 5 days of culture, the cells were pulse-labeled with 1 µCi of [3H]thymyl-thymidine (Amerham ICRN, Bucks, United Kingdom) per well, and 18 h later the cells were harvested onto glass fiber filters (Perkin-Elmer Life Sciences, Brussels, Belgium). The radioactivity incorporated into the DNA was measured by using a β-scintillation counter (Perkin-Elmer). The results are presented as the mean counts per minute (cpm).

Cytokine ELISA. MoDCs were stimulated with β-glucans as mentioned above and the culture supernatant was harvested after 24 h and stored at −20°C. The concentrations of IL-1β, IL-8, IL-12p40, IL-6, tumor necrosis factor alpha (TNF-α), and IL-10 were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to the manufacturer’s recommended protocols. The cytokine concentrations were calculated using DeltaSOFT Jv 2.1.2 software (BioMetallics, Princeton, NJ) with a four-parameter curve-fitting algorithm.

Statistics. All experiments were performed with cells from four different pigs. Statistical analyses were performed using SPSS/16. One-way analysis of variance with a least-significant-difference post hoc test was performed. Levene’s test was used to assess the homogeneity of the variances. A P value of <0.05 was considered statistically significant.

RESULTS

β-Glucans enhance the upregulation of DC activation markers. Optimal antigen presentation and subsequent T-cell responses require DC maturation. This process includes the upregulation of costimulatory molecules and MHC class II (MHC-II). To investigate the effect of β-glucans on the phenotypic DC maturation, immature MoDCs were stimulated for 24 h with 5 or 10 µg of the different β-glucan preparations/ml or 1 or 10 µg of LPS/ml, and the cell surface expression of CD80/86, CD40, and MHC-II was assessed by flow cytometry. Figure 1 shows that Macrogard, at both 5 and 10 µg/ml, induced a significant upregulation of MHC-II and CD40 expression, compared to the untreated immature cells (ImDCs) (P < 0.01), whereas CD80/86 expression was significantly upregulated at a dose of 10 µg/ml (P < 0.01). Both doses of LPS (1 and 10 µg/ml) failed to induce an increased marker expression.

β-Glucan treatment downregulates the phagocytic capacity of MoDCs. To evaluate DC maturation other than analyzing the phenotype switch associated with DC activation, functional maturation of the DCs upon treatment can be assessed.
terms of phagocytosis, it is known that ImDCs can efficiently engulf antigens but, upon maturation, DCs lose this phagocytic ability. In our studies, the phagocytic capacity of MoDCs was determined through the uptake of ova-dQ was assayed by flow cytometry. Mean fluorescence intensity (MFI) values were calculated by subtracting the MFI values obtained at 4°C from those obtained at 37°C. The data are shown as the means ± the SEM for four pigs. Asterisks (*) indicate a significant difference between β-glucan-stimulated MoDCs and immature MoDCs (ImDCs) (for all β-glucans P < 0.01 except for the β-glucan from Euglena gracilis).

β-Glucan-stimulated MoDCs induce T-cell proliferation. As a result of maturation, MoDCs become potent stimulators of immune responses, resulting in an increased T-cell stimulatory capacity. In order to determine whether MoDCs stimulated with β-glucans are able to induce T-cell proliferation, β-glucan-stimulated MoDCs were cocultured with CD172a-depleted cells (Fig. 3). When stimulated at 5 μg/ml, Macrogard-, curdlan-, and zymosan-stimulated MoDCs significantly increased T-cell proliferation compared to ImDCs (P < 0.01). MoDCs stimulated with a higher dose of curdlan and zymosan (10 μg/ml) tended to induce a weaker T-cell proliferation than after stimulation with 5 μg/ml, whereas MoDCs stimulated with 10 μg of Macrogard/ml significantly increased T-cell proliferation more than after stimulation with 5 μg/ml (P < 0.01). Stimulation of MoDCs with the higher dose of LPS (10 μg/ml) also gave a significantly stronger T-cell proliferation (P < 0.001) than the lower dose (1 μg/ml) compared to the ImDCs.

β-Glucans stimulate cytokine secretion by MoDCs. Cytokine expression profile, the IL-1β, IL-6, IL-8, IL-12/IL-23p40, TNF-α, and IL-10 concentrations were determined in the culture supernatant of β-glucan-stimulated MoDCs (Fig. 4). Compared to ImDCs, stimulation with all β-glucans, except laminarin, could significantly increase TNF-α secretion. Interestingly, whereas the β-glucan from Euglena gracilis and Macrogard induced a significantly higher TNF-α production at 10 μg/ml and zymosan induced a significantly higher production at both concentrations, curdlan only induced a significant increase in TNF-α secretion at the lowest concentration (5 μg/ml) (P < 0.05). The proliferative responses of the CD172a + lymphocytes (i.e., no MoDCs were added) was less than 300 cpm.

DISCUSSION

For humans and mice, there is an extensive amount of information regarding β-glucans and their immunomodulatory effects. However, the effects are not always consistent and it is not clear whether these differences are due to the use of different β-glucan preparations or due to variation among experimental models. In the pig industry, dietary β-glucan supplementation is often used, especially during periods of stress and immune challenge such as weaning. However, the beneficial effect of β-glucan supplementation may be influenced by several factors, such as structural features and the dose of β-glucan used in the diets. In a previous study, we described the dose effect of different β-glucan preparations on porcine leukocytes (31). In the present study, we focused on the action of different β-glucans on porcine dendritic cells. DCs have not only been described to sense the luminal environment at

![FIG. 2. Analysis of the phagocytic activity of β-glucan-stimulated MoDCs.](http://cvi.asm.org/Downloaded from http://cvi.asm.org)

![FIG. 3. Analysis of the ability of β-glucan-stimulated MoDCs to enhance T-cell proliferation.](http://cvi.asm.org/Downloaded from http://cvi.asm.org)
zymosan (31). Since Macrogard-stimulated DCs are more active than MoDCs or immature MoDCs. The results of the present study demonstrate that there is a large variation in terms of DC maturation-inducing properties between the different β-glucans. All particulate commercial β-glucan preparations affect one or more characteristics typical for downstream signaling and the uptake of β-glucans by DCs (2). Recently, we demonstrated that β-glucan preparations affect one or more characteristics typical for maturation, and this is partly caused by their particulate nature. However, Macrogard, zymosan, and curdlan affect maturation more than the glucan from Euglena gracilis. Curdlan, however, also an unbranched β-(1,3)-glucan, has much higher immunostimulating capacities than the glucan Curdlan reaches the immunostimulating capacities of the glucan from Euglena gracilis, which is probably caused by the high LPS contamination (47 endotoxin units [EU]/µg). Based on the approximation that 1 EU correlates to 0.2 ng of LPS (35), we estimated the LPS contamination in curdlan to correspond to the administration of ~10 ng of LPS/µg of β-glucan, which is 100 times less than the LPS control, representing a minor but possibly stimulatory dose. We had only LPS-contaminated curdlan at our disposal, but Ferwerda et al. (10) conducted their experiments with LPS-free curdlan. In human myeloid-derived macrophages, they investigated the synergistic effect of curdlan and LPS on TNF-α and IL-10 production (10). Both TNF-α and IL-10 production was increased, and they could inhibit the synergistic effect on TNF-α production, but not IL-10 production, by a neutralizing dectin-1 antibody. These results demonstrate that in human macrophages, at least for TNF-α production, curdlan exerts its effects through dectin-1 and that curdlan in combination with LPS induces a synergistic signaling between dectin-1 and TLR-4 (10). In addition to TNF-α and IL-10, it was shown that IL-6 and IL-23 production were also mediated through dectin-1 (19, 29), while the production of all of these cytokines was enhanced after coligation of dectin-1 and TLRs (7). In our results, we demonstrated that although most of the β-glucans could induce IL-1β, TNF-α, IL-6, IL-12/IL-23p40, and IL-10 production, the highest production of all of these cytokines was found after stimulation with LPS-contaminated curdlan. In our work, we demonstrated that the combination of β-glucans with LPS and other PRR ligands gives a more accurate picture of the real situation in the intestine and should be considered when evaluating the effectiveness of a β-glucan preparation as a dietary component. However, in contrast to humans and mice and in line with earlier reports (8, 12, 25), porcine MoDCs are less responsive to LPS itself. LPS could not upregulate the expression of maturation markers and did not significantly increase the proinflammatory cytokines.
IL-1β, IL-6, and TNF-α or the Th1 cell-inducing IL-12, the chemotactrant IL-8, or the anti-inflammatory cytokine IL-10. It is well known that the spectrum of cytokines produced by DCs modulates the polarization of the T-cell response and determines the outcome of an immune response (36). We found high secretion levels of TNF-α and IL-8 in the supernatant of all β-glucan-stimulated MoDCs. TNF-α and IL-8 serve both as chemotactrants for innate and adaptive immune cells such as neutrophils and T cells, respectively, underlining the role of DCs in linking innate and adaptive immunity (11). Furthermore, we demonstrated that stimulation of MoDCs with curdian gave rise to high levels of the proinflammatory cytokines IL-6, IL-12/IL-23p40, and IL-1β, cytokines which can, together with TNF-α, direct the T cells toward a Th1 or Th17 response (37). However, it is also possible that the LPS contamination in the curdian preparation could favor regulatory T cell (Treg) responses, characterized by high IL-10 levels. To test the qualitative T-cell responses, we measured IFN-γ,

IL-17- and IFN-γ, or the anti-inflammatory cytokine IL-10. Much lower concentrations of curdlan (1 ng/ml), only IFN-γ, IL-8, or IL-10 was produced after restimulation with anti-CD3.

In summary, we have shown that β-glucans differ in structure, molecular weight, and origin and have a varying effect on the maturation of porcine MoDCs. The β-glucans derived from *Saccharomyces cerevisiae* enhance DC maturation and DC-induced T-cell proliferation. Also, curdian has a strong effect on the maturation of porcine MoDCs, which is probably caused by a costimulatory effect of the LPS contamination. These results, together with the results obtained in porcine leukocytes (31), demonstrate that different β-glucans can have varying effects on the host immune system and that a careful choice is needed when β-glucans are incorporated in the feed as a dietary supplement.

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