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Curdlan-mediated regulation of human phagocyte-specific chitotriosidase

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ABSTRACT

Human phagocyte-specific chitotriosidase is part of innate immunity and shows anti-fungal activity towards chitin-containing fungi. We investigated the effect of stimulation of the C-type lectin receptor dectin-1 by β-1,3-glucan (curdlan) on chitotriosidase expression and release by human phagocytes. We observed that curdlan triggers chitotriosidase release from human neutrophils. In addition, we show that curdlan impairs chitotriosidase induction in monocytes. Finally, curdlan temporarily induces chitotriosidase in enzyme-expressing monocyte-derived macrophages, followed by reduction of chitotriosidase expression after prolonged stimulation. These data on regulation of phagocyte-specific chitotriosidase following curdlan recognition support an important role of chitotriosidase in the elimination of chitin-containing pathogens.

1. Introduction

Human phagocyte-specific chitotriosidase contributes to innate immune responses towards chitin-containing fungi, both in vitro and in vivo [1–3]. This enzyme has first been described in Gaucher disease patients and serves as a critical biomarker to monitor disease onset, development and therapeutic efficacy [4,5]. It has been purified from a Gaucher patient spleen and cloned from a human macrophage (mϕ) cDNA library [6,7]. A close relative is acidic mammalian chitinase (AMCase), which has been linked to asthma [8,9].

Chitinases belong to family 18 of glycosyl hydrolases and hydrolyze chitin, the linear polymer of *N*-acetylglucosamine, the second most abundant polysaccharide in nature. Chitin serves as an indispensable structural component in a variety of organisms, including fungi and arthropods [5,10–13]. Chitin induces the accumulation of IL4-expressing innate immune cells in mice and alternative activation of mϕs, with concomitant production of leukotriene B4, which is essential for proper immune cell recruitment [14].

In mice, chitin particles of a certain size are recognized by mϕs through toll-like receptor (TLR)-2, the C-type lectin receptor dectin-1 and to a lesser extent by the mannose receptor [15,16]. In addition, chitin can be recognized by the FIBCD1 gene product, which is expressed apically by enterocytes [17]. A secreted C-type lectin, RegIIIγ (HIP/PAP in man), is present in Paneth cell secretory granules in the mouse and recognizes chitin as well [18].

We have previously shown that peptidoglycan triggers release of chitotriosidase from neutrophils and that activation of nucleotide-binding oligomerization domain (NOD)-2 in human mϕs temporarily induced this chitinase [19].

In addition to chitin, also β-glucan is a major structural component of the fungal cell wall. Dendritic cell-associated C-type lectin-1 (dectin-1) is involved in the innate immune recognition of this carbohydrate [20]. Thus far it is not known if stimulation of dectin-1 using β-1,3-glucan (curdlan) regulates chitotriosidase secretion by human neutrophils, or influences regulation of expression by macrophages. We therefore studied the effect of dectin-1-derived signals on human phagocytic chitotriosidase expression and release. First, enzyme release from neutrophils stimulated with curdlan was studied. Second, it was addressed how curdlan affects induction of chitotriosidase expression by monocytes. Finally, the effect of curdlan on mϕs, which already express the enzyme, was determined.

2. Materials and methods

2.1. Isolation of human phagocytes and culture conditions

Monocytes and neutrophils were isolated and cultured as described [2,19]. Monocytes were cultured in RPMI/10%HS for 7 days

Abbreviations: Curdlan, β-1,3-glucan; dectin-1, dendritic cell-associated C-type lectin-1; TLR, toll-like receptor

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in the presence or absence of curdlan from *Alcaligenes faecalis* (Sigma). Chitotriosidase activity (nmol/ml*h) was determined in cell-free culture supernatants, using fluorescent 4-MU-triacylchitotrioside (Sigma) [2]. Alternatively, monocytes were matured for 7 days in culture medium alone towards chitotriosidase expressing mϕs, which were washed and further cultured in medium or stimulated with curdlan. Next, chitotriosidase activity was determined in cell-free supernatants at indicated time points and gene expression in response to dectin-1 triggering was analysed (see Section 2.2 below).

Neutrophils were seeded at 4 million cells in a final volume of 200 μl RPMI/0.5% BSA and stimulated with curdlan for 90 min. Activities of chitotriosidase were measured in cell-free neutrophil supernatants. LY294002 was used to inhibit PI3kinase, SB302580 to inhibit p38 MAP kinase and PD98059 to inhibit p42/44 MAP kinase or ERK 1/2 (all from Sigma, used at 50 μM).

2.2. Quantitative PCR

Total RNA was extracted using the nucleospin II extraction kit (Macherey-Nagel, Düren, Germany) and concentrations were measured with the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). 500 ng of RNA per sample was transcribed into first strand cDNA using Superscript II reverse transcriptase (Invitrogen). cDNA was diluted 20 times prior to gene-specific analysis by real-time PCR applying the iCycler MyiQTM system (Biorad Laboratories, Veenendaal, The Netherlands). Target genes were normalized to ribosomal phosphoprotein 36B4. The following gene-specific primers were used: CCR7 forward primer 5′-tgaggtcacggacgattacat-3′; reverse primer 5′-gtaggcccacgaaacaaatgat-3′; TNFα forward primer 5′-ggcgtggagctgagagata-3′; reverse primer 5′-cagccttggcccttgaaga-3′; MIP1β/CCL4 forward primer 5′-gcgtgactgtcctgtctctcc-3′; reverse primer 5′-ccacaaagttgcgaggaagc-3′; IL10 forward primer 5′-tgccttcagcagagtgaagactt-3′; reverse primer 5′-tcctccagcaaggactcccttta-3′; CCL18 forward primer 5′-cccagctcactctgaccact-3′; reverse primer 5′-gtggaatctgcc aggaggta-3′; CD163 forward primer 5′-gcacatcactcagaatttcaatgg-3′; reverse primer 5′-acatagatcatgcatctgtcatttg-3′; 36B4 forward primer 5′-tcgacaatggcagcatctac-3′; reverse primer 5′-atccgtctccacagacaagg-3′. Data were analysed using the delta C_t method [21]. Statistical analysis was performed using the Student's *t* test (GraphPad Software, San Diego, CA). A probability value of $P < 0.05$ was considered to be significant.

2.3. Western blot analysis

Aliquots of cell-free culture supernatants were analysed for chitotriosidase protein. Following SDS–PAGE, proteins were transferred to nitro-cellulose (Fischer Scientific, Pittsburgh, USA) and chitotriosidase expression was assessed using a rabbit anti-chitotriosidase polyclonal as described previously [19] and for detection secondary IRDYE conjugates were used (Westburg).

3. Results

3.1. Curdlan triggers chitotriosidase release by human neutrophils

In the circulation neutrophils are the chitotriosidase-containing cells that can contribute to first line defence against chitin-containing pathogens. It has been demonstrated that human neutrophils

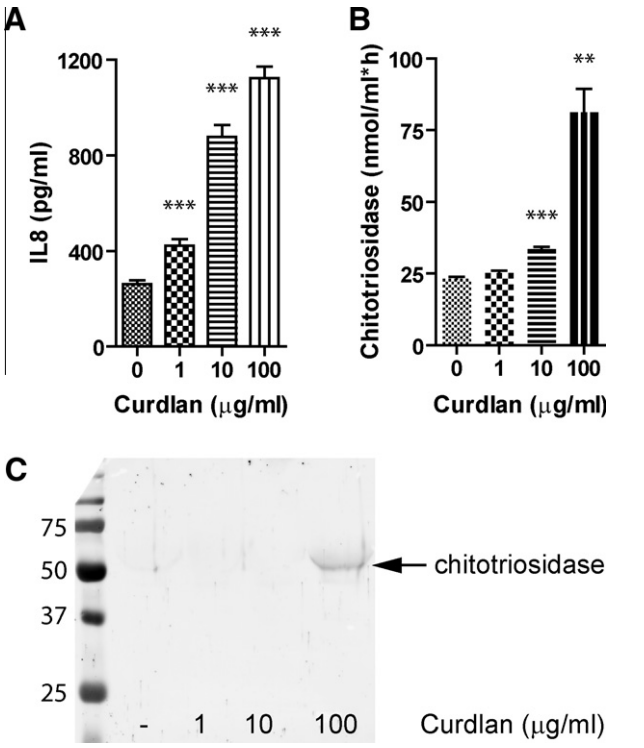

Fig. 1. IL8 and chitotriosidase release from human neutrophils stimulated with curdlan. (A) IL8 release after 24 h of stimulation. (B) Chitotriosidase activity released after 90 min of stimulation. (C) Western blot analysis of neutrophil supernatants, following 90 min stimulation with medium or curdlan. On the *y*-axis mean values ±S.D. are depicted; $n = 4$, $^{**}P < 0.01$; $^{***}P < 0.001$.

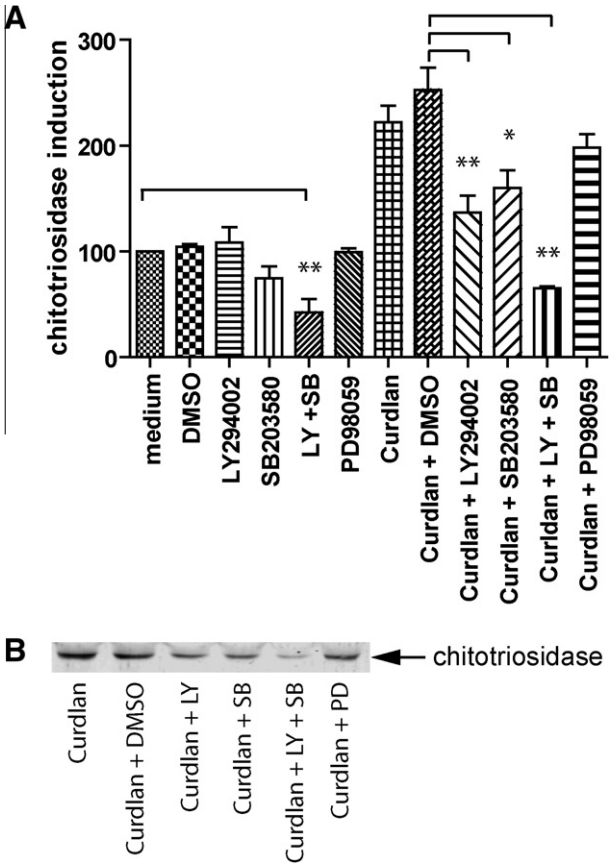

Fig. 2. Signalling pathways involved in curdlan-induced release of chitotriosidase from human neutrophils. (A) Inhibition of PI3 kinase and p38 MAP kinase reduces release of chitotriosidase. On the *y*-axis mean chitotriosidase activity compared to medium ±S.D. is depicted; $n = 4$, $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Western blot analysis of chitotriosidase protein released in neutrophil supernatants.

release the enzyme from their specific granules both in vitro and in vivo [2,19]. In this way chitotriosidase contributes to chitonolytic innate anti-fungal responses. We studied whether dectin-1 stimulation, using curdlan, triggered chitotriosidase release. First, functionality of curdlan was tested. Neutrophils were stimulated for 24 h with curdlan and we found a dose-dependent increase of IL8 release (Fig. 1A). Next, we measured chitotriosidase activity in cell-free culture supernatants after 90 min of stimulation with curdlan. A significant increase in enzyme activity was observed, which was paralleled by increased amounts of secreted protein as detected by western blot (Fig. 1B and C). We have excluded the possibility that cell death caused this induction of chitotriosidase release from neutrophils. Next we addressed the signalling cascades involved in this curdlan-mediated release. Curdlan did not trigger an increase of the intracellular Ca^{2+} concentration (data not shown). Previously we have demonstrated that chitotriosidase release induced with peptidoglycan was inhibited by the p38 MAP kinase inhibitor SB203580 and the PI3 kinase inhibitor LY294002 [19]. Here we observed that chitotriosidase release induced by curdlan, used at 100 μg/ml, was also reduced by about 50% by each of these inhibitors, while their combined use completely reduced release (Fig. 2A), suggesting that both kinases are involved in curdlan-induced chitinase release. Inhibition of ERK 1/2, using PD98059, gave no significant inhibition, suggesting that this kinase is not essential for the curdlan-mediated release of chitotriosidase. By western blot we confirmed release of chitotriosidase at the level of protein (Fig. 2B).

3.2. Curdlan impairs induction of chitotriosidase in human monocytes

Human chitotriosidase was discovered as the mϕ-specific chitinase [4–7]. We first studied the effect of curdlan on chitotriosidase induction by human monocytes. Fresh monocytes do not express the enzyme and require maturation towards mϕs for induction. Monocytes were matured in the presence of different concentrations of curdlan. We observed a potent inhibitory effect on the induction of chitotrioisidase expression. When compared to medium-exposed monocytes, chitotriosidase expression was virtually absent both at the level of RNA and activity (Fig. 3A and B). In part, this reduced expression could be explained by the fact that cell viability of stimulated monocytes was impaired after 7 days at the highest concentrations used (Fig. 3C). However, at a concentration of 1 μg/ml, curdlan did not affect viability, but still chitotriosidase was reduced to a large extend. By microscopy we observed that the

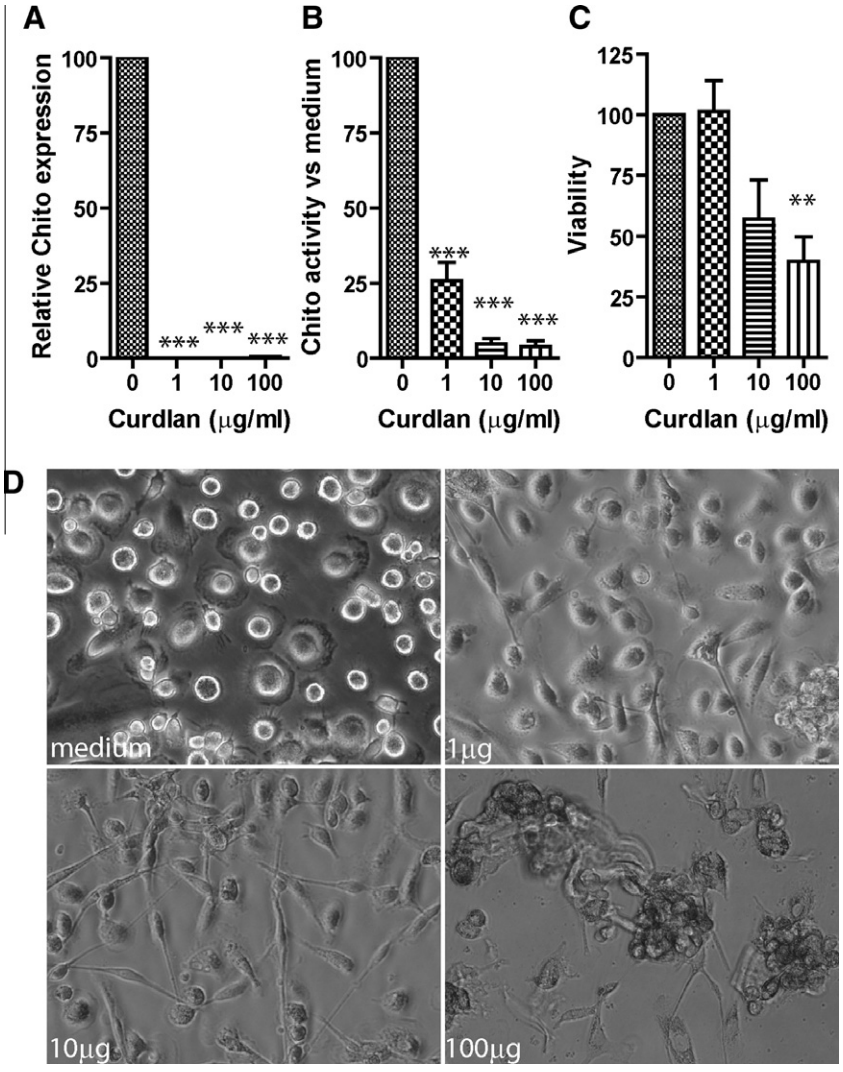

Fig. 3. Stimulation of human monocytes with curdlan inhibits induction of chitotriosidase synthesis. (A) Real time PCR analysis of chitotriosidase expression. (B) Chitotriosidase activity. (C) Cell viability. On the *y*-axis chitotriosidase activities are depicted compared to culture in medium alone ±S.D. $n = 5$, $^{*}P < 0.05$; $^{***}P < 0.001$. (D) Respective images of mϕ stimulated for 7 days with medium, or curdlan (1, 10, or 100 μg/ml).

morphology of the cells also differed in the presence of curdlan. The round shape of the control mϕs became more protruded as is depicted in Fig. 3D.

3.3. Curdlan temporarily induces chitotriosidase in human chitinase-expressing mϕs

We demonstrated that the induction of chitotriosidase is inhibited when monocytes are stimulated with curdlan, already at the concentration when viability is not affected. Next, it was addressed whether curdlan affected chitotriosidase expression in mϕs. To answer this question matured mϕs, cultured for 7 days to allow chitotriosidase induction, were washed and stimulated for 4 or 48 h with curdlan. First functionality of the stimulation was determined. By quantitative real-time PCR analysis we observed a rapid induction of inflammatory genes such as TNFα, CCR7 and MIP-1β (Fig. 4A). Anti-inflammatory IL10 was also highly induced after 4 h, followed by induction of the IL10-responsive genes CCL18 and CD163 after 48 h (Fig. 4B). Next we analysed chitotriosidase activity in cell-free culture supernatants and found a minor, but significant, induction in the presence of curdlan after 4 h of stimulation (Fig. 4C, left panel). Prolonged stimulation (48 h) showed a reduction when compared to stimulation with medium only (Fig. 4C, right panel). At the level of RNA, comparable effects on expression were noted (data not shown). Furthermore, we did not observe cell death of macrophages after 48 h of stimulation with curdlan, which might have contributed to the reduction in activity. Total loss of enzyme activity was not observed after 48 h.

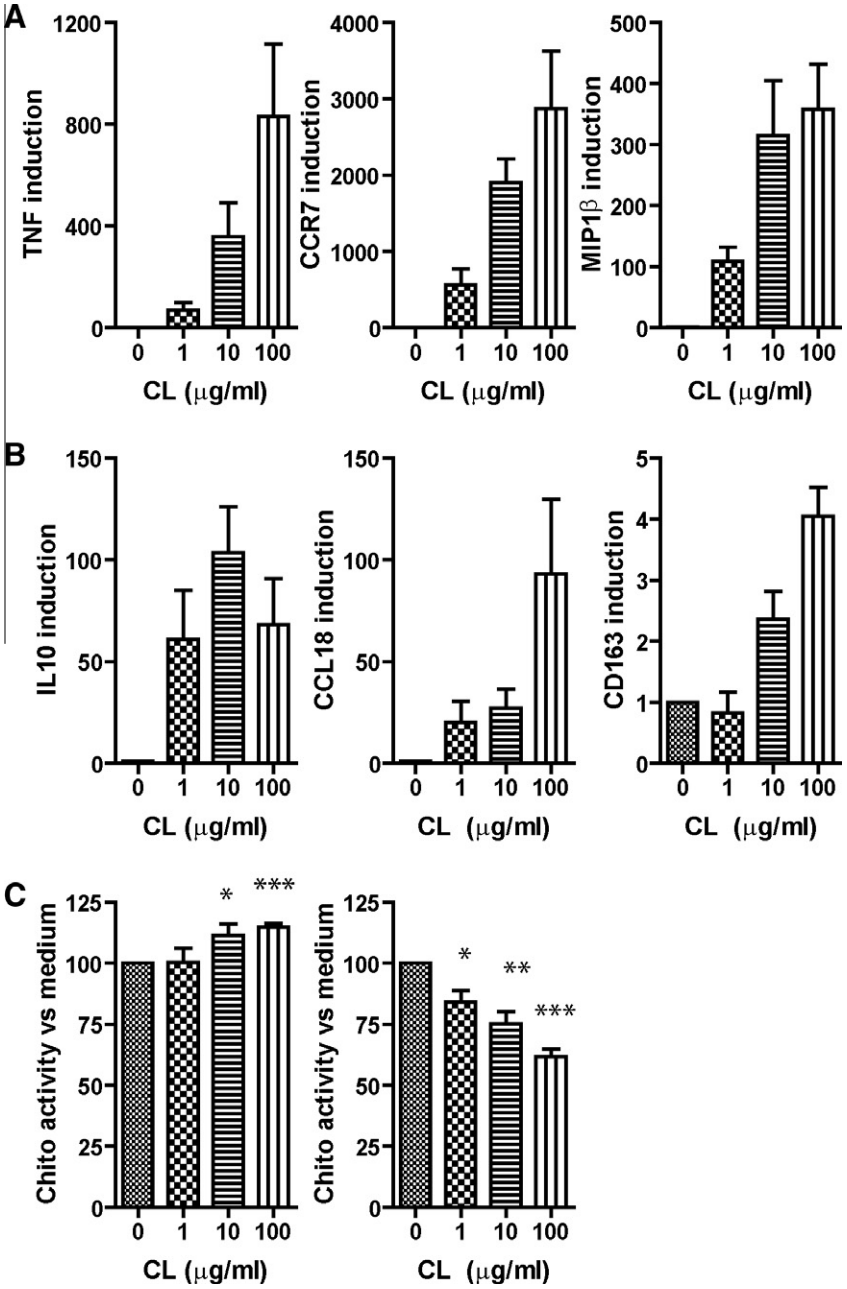

Fig. 4. Mϕ phenotype- and chitotriosidase expression in response to curdlan. (A) Real-time PCR analysis of inflammatory gene expression of TNF, CCR7 and MIP1β after 4 h of stimulation ($n = 3$). (B) Real time PCR analysis of anti-inflammatory gene expression of IL10 after 4 h of stimulation and CCL18 and CD163 after 48 h ($n = 3$). (C) Analysis of chitotriosidase activity after 4 and 48 h of stimulation. On *y*-axis activity is depicted compared to culture in medium alone as mean ±S.D. $n = 5$, $^{*}P < 0.05$; $^{**}P<0.01$; $^{***}P < 0.001$.

4. Discussion

Chitotriosidase has recently been implicated in anti-fungal responses and as such can be viewed as component of innate immunity [1–3]. Enhanced chitotriosidase activity is for instance observed in sera following bacterial, fungal, viral and protozoan parasite infections [4,22–24]. Moreover, a higher risk for Gram negative bacterial infection is observed in children treated for AML, which have a defective chitotriosidase allele [25].

Our investigations on stimulation of human phagocytes with the dectin-1 agonist curdlan can be summarized as follows. First, human neutrophils release chitotriosidase from their specific granules after stimulation with curdlan. Second, induction of chitotriosidase is strongly reduced following stimulation of monocytes with curdlan. Third, curdlan induces a pro-inflammatory response in monocyte-derived macrophages, which is characterized by the acute induction of TNFα, CCR7 and MIP1β. In parallel, an anti-inflammatory program is activated characterized by the rapid induction of IL10, followed by the induction of the IL10-dependent genes CCL18 and CD163. Fourth, chitotriosidase expressing human monocyte-derived macrophages show a short term minor induction of the chitinase, followed by a reduction after prolonged stimulation. The findings reported here regarding the effects of curdlan on chitotriosidase in human phagocytes are to our knowledge novel and add to previous studies allowing for more efficient chitinolytic responses.

In the circulation, neutrophils are abundantly present. They can be recruited to wounds, a potential pathogen entry site, and to other sites with infections. At these sites immediate chitinolytic anti-pathogenic responses can be mounted as for instance GM-CSF, TLR ligands and curdlan induce rapid release of chitotriosidase form specific granules of neutrophils [2,19]. Thus far we have not been able to detect AMCase activity in chitotriosidase-deficient Gaucher patient sera, nor did we detect AMCase expression in control, or Gaucher spleen (unpublished observations). Furthermore, human monocyte-derived macrophages expressing high levels of chitotriosidase do not express AMCase and in the presence of the Th2 cytokine IL4 chitinase activity is virtually absent [2,8]. Lastly, AMCase activity was undetectable in human neutrophils and macrophages from chitotriosidase-deficient individuals. This suggests that in man chitotriosidase is the sole chitinase involved in phagocyte-mediated innate immunity. Recombinant human chitotriosidase has been shown to target several fungi in vitro and in vivo, encompassing *Aspergillus niger*, *Aspergillus fumigatus*, *Candida albicans* and *Cryptococcus neoformans* [1–3]. Such fungi are for instance recognized by TLRs and dectin-1, which have been shown to trigger chitotriosidase release, thus allowing for chitin degradation in the fungal cell wall [20,26].

The contribution of monocytes to acute chitotriosidase-mediated anti-pathogenic responses can be neglected. Induction of the chitinase takes fairly long and stimulation with curdlan, TLR ligands and the intracellular nucleotide-binding oligomerization domain ligand muramyl dipeptide, severely hampers induction of the enzyme [19].

Stimulation of chitotriosidase expressing mϕs with curdlan revealed a rapid minor induction of activity, followed by a 40% reduction after 48 h of stimulation. Possibly the curdlan-mediated induction of IL10 causes this reduction of chitotriosidase, as it has been shown earlier that IL10 negatively regulates its expression [27]. Importantly, chitotriosidase activity is far from eradicated, still granting efficient chitinase responses. Combining the latter with the neutrophil-mediated chitotriosidase responses, this grants a very efficient chitinolytic activity against chitin-containing pathogens, including fungi.

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