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Chitin and its derivative chitosan differently activate porcine innate immune cells

Nidhal Guendouz, Bert Devriendt *

Laboratory of Immunology, Department of Translational Physiology, Infectiology and Public Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, Merelbeke 9820, Belgium

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ABSTRACT

In livestock species such as pigs, antibiotics are frequently used during weaning, a period of increased susceptibility to infections. However, the emergence and spread of antimicrobial resistance necessitate alternative strategies to improve animal health. Immune potentiating polysaccharides, including chitin, chitosan, and their derivatives, show promise as functional feed ingredients, yet their direct effects on porcine innate immune cells remain unclear. Here, ex vivo porcine primary neutrophils, monocytes, and monocyte-derived macrophages were used to assess the immunomodulatory properties of structurally distinct chitin-based polymers. In peripheral blood mononuclear cells, neither chitin nor chitosan induced significant secretion of IL-1β, TNF-α, or IL-6, suggesting that only specific subpopulations mediate these effects. Following fractionation, cytokine production was restricted to the $CD14^+$ monocyte compartment, where high concentrations of chitosan elicited strong pro-inflammatory responses. To further investigate innate immune programming, trained immunity assays were performed. In monocytes, chitin did not induce training or tolerance, whereas low-molecular-weight, highly deacetylated chitosan promoted a trained phenotype, evidenced by enhanced cytokine production upon lipopolysaccharide restimulation. In contrast, in monocyte-derived macrophages, chitin pretreatment induced a tolerance-like phenotype, characterized by reduced cytokine responses following subsequent stimulation, while chitosan had no significant effect.

These findings demonstrate that structural differences in chitin-based polymers determine the magnitude and direction of innate immune responses, supporting their targeted use as functional immunomodulators to enhance health and resilience in livestock.

1. Introduction

In livestock production, antibiotics have long been used not only for therapeutic purposes but also as growth promotors and disease prevention (Cremonesi et al., 2024). This prophylactic use of antibiotics instigated the emergence and spread of antimicrobial resistance and as such preventive use of antibiotics in livestock production has been banned in the EU from 2006. Nevertheless, global use of antibiotics in livestock species is still high, especially during critical periods of life such as the weaning period in piglets (EFSA, 2025; Van Boeckel et al., 2019). The weaning period marks a transition from maternal milk to solid feed, and this dietary shift, combined with environmental and social stressors, renders piglets highly susceptible to intestinal pathogens, including enterotoxigenic *Escherichia coli* (ETEC) (Dierick et al., 2022). This vulnerability often leads to metaphylactic antibiotic use, further fuelling a global antimicrobial crisis. As a result, global efforts to combat antimicrobial resistance (AMR) have been strengthened through the 2015 WHO Global Action Plan and the 2016 United Nations Political Declaration, both later endorsed by the World Organisation for Animal Health (OIE) and the Food and Agriculture Organization (FAO) and further consolidated by the European Union's One Health Action Plan in 2017.

Vaccination and passive immunity are key strategies to protect animals from infectious diseases and to reduce use of antibiotics (Hoelzer et al., 2018). Vaccination trains the immune system for future pathogen encounters, whereas passive immunity provides immediate, short-term protection through the transfer of antibodies from mother to offspring (Butler et al., 2017). Although effective, vaccine-induced protection can vary with factors such as timing, pathogen exposure, and animal condition. Moreover, active immunity requires time to

* Corresponding author.
E-mail address: b.devriendt@ugent.be (B. Devriendt).

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develop, which may leave animals vulnerable during early stages, and vaccines are typically pathogen-specific, with multivalent formulations still offering only limited breadth of protection. Together, these limitations emphasize the need for complementary approaches. One promising strategy is the use of nutraceuticals or functional feed ingredients, bioactive dietary compounds that provide health benefits beyond basic nutrition (Gupta et al., 2019). Within this broader group, immune-stimulating feed additives are specifically included in diets to modulate the immune system by enhancing innate or adaptive responses and improving resilience to infection (EFSA, 2018). Incorporating such feed ingredients could strengthen host defences and help reduce antibiotic use. A typical example of immune potentiating feed additives are β-glucans from yeast or fungi (Hermans et al., 2021). These β-glucans can activate porcine innate immune cells and provide protection against ETEC infection (Sonck et al., 2010; Stuyven et al., 2009; Hermans et al., 2021). They exert their immune stimulating effects via their binding to complement receptor 3 and dectin−1 expressed by porcine innate immune cells (Baert et al., 2015). This receptor interaction triggers trained innate immunity, a phenomenon which involves metabolic and epigenetic reprogramming of innate immune cells, resulting in enhanced responses upon later challenges. Currently, β-glucan-mediated trained innate immunity has not yet been shown for porcine innate immune cells (Byrne et al., 2021). In contrast to trained innate immunity, some molecules, including endotoxins, can trigger tolerance. The latter is characterized by a state of hypo-responsiveness with diminished cytokine production upon restimulation (Divangahi et al., 2021). Trained innate immunity and tolerance are in fact opposing programs of innate immune memory. They share underlying epigenetic and metabolic mechanisms, but their biological effects diverge according to whether the desired outcome is amplified defence or controlled dampening of inflammation (Lajqi et al., 2023).

Another immune potentiating molecule that has been tested as nutraceutical is chitin, a linear polysaccharide composed of N-acetylglucosamine units linked by β(1→4) glycosidic bonds, forming long chains that provide structural support in fungal cell walls as well as crustacean and arthropod exoskeletons (Izadi et al., 2025; Elieh Ali Komi et al., 2018; Da Silva et al., 2008). Chitosan, derived from the partial deacetylation of chitin, has also attracted considerable interest as a functional feed additive (Rahman et al., 2024; Confederat et al., 2021; Guan et al., 2019). The potential of chitosan to promote immune responses or to exhibit anti-inflammatory effects, depends on the specific cell type and dosage (Ghattas et al., 2025; Yeh et al., 2016). It acts as a pro-inflammatory stimulus, particularly on mouse macrophages triggering the production of cytokines and chemokines, and upregulating TLR4 expression (Bian et al., 2024; Zhang et al., 2014), thereby boosting innate immunity against pathogens. Chitin and its derivative chitosan illustrate structure–function–dependent immune activity, demonstrating that their immunomodulatory effects are not solely mediated through specific receptors (Bueter et al., 2013). In particular, highly deacetylated chitosan (C100) induces mitochondrial oxidative stress, functioning as a potent Th1-skewing vaccine adjuvant (Turley Joanna et al., 2021). Chitosan also exhibits immunomodulatory properties, stimulating the secretion of various cytokines. Larger chitin particles (40–70 μm) tend to be pro-inflammatory, while small particles (1–10 μm) may induce tolerance (Lee et al., 2008). A recent hypothesis suggests that highly deacetylated chitosan may penetrate immune cells through membrane disruption or endocytic uptake, bypassing surface receptor-mediated recognition (He et al., 2021).

Chitosan can modulate porcine immunity in vivo via both systemic and gut-associated pathways. Dietary chitosan oligosaccharides have been reported to benefit humoral immune function in sows and their progeny, reflected by improved reproductive outcomes and signs of enhanced immune status (Fang et al., 2025). In parallel, accumulating data indicate that chitosan oligosaccharides also act on intestinal immune signalling by supporting gut development, attenuating intestinal inflammatory responses, and boosting antioxidant defences, thereby contributing to mucosal immune equilibrium (Lan et al., 2024). Collectively, these observations indicate that chitosan exerts not only local effects within the intestine but also broader immunomodulatory actions, underscoring its potential as a nutritional strategy to strengthen immune resilience in livestock. In pigs, these molecules have also been evaluated for their immunomodulating capacity. One study demonstrated that chitosan nanoparticles loaded with inactivated swine influenza A virus (SwIAV) vaccine enhanced mucosal and systemic immune responses in pigs, reducing viral shedding upon challenge infection with heterologous swine influenza A virus strains (Dhakal et al., 2018). Dietary supplementation with 250 mg/kg chitosan oligosaccharide (COS) in early weaned piglets increased serum IgA, IgG, and IgM levels while enhancing cytokine production, demonstrating immune enhancement through undefined mechanisms (Liu et al., 2008). Additionally, previous studies indicated that supplementation with chitosan oligosaccharide (COS; MW 2.47 kDa, degree of deacetylation 90.2%) modulates microbial composition in the gut by elevating beneficial bacterial levels and reducing pathogenic populations, thereby supporting improved growth performance in pigs (Yang et al., 2012; Liu et al., 2008). Moreover, combined maternal and post-weaning COS reported benefits to piglet intestinal health, short-chain fatty acids, and metabolism-related gene expression (Fang T et al., 2025). Studies above shows that chitin/chitosan have beneficial effects on gut health/animal performance and can influence the immune system. However, evidence is indirect and it is unknown which innate immune cells are activated in pig. Therefore, the present study aimed to elucidate the response of porcine innate immune cells towards chitin and its derivative chitosan.

2. Materials and methods

2.1. Chitin and chitosan derivatives

Chitin (C7170), chitosan with low molecular weight (448869) and chitosan with a degree of deacetylation of 75% (C3646) were purchased from Sigma Aldrich and diluted in HBSS without calcium and magnesium (Thermo Fisher, 14175095) to a final concentration of 10 mg/mL. Upon sonication (amplification 20%, time: 2 min, pulse: 01/01), these products were stored at 4°C for maximum 6 months.

2.2. Endotoxin quantification

Endotoxin levels in chitin and chitosan preparations were quantified using the GenScript ToxinSensor chromogenic LAL assay (L00350C) according to the manufacturer's instructions. Trace amounts of endotoxin were detected: chitin = 0.365 EU/mg, chitosan LMW = 0.181 EU/mg, chitosan DDA75 = 0.537 EU/mg.

2.3. Particle size distribution analysis

Chitin, chitosan DDA75 and chitosan LMW were prepared as described. Images were taken using light microscopy (Leica, Wetzlar, Germany) and the particle size distribution was analysed using Fiji (ImageJ version 1.54p). Particle projected areas were converted to equivalent circular diameters (ECD) using $D_{eq} = 2\sqrt{A/\pi}$, where A equals the particle area in μm^2. Particle counting was performed from multiple randomly selected microscopy fields until representative and stable particle size distribution profiles were obtained for each sample and the obtained distributions were consistent across independently analysed microscopy fields (Figure S1). Minimum ECD, maximum ECD, mean ECD as well as D10, D50 and D90 values are reported in supplementary Table S1.

2.4. Isolation of porcine neutrophils and PBMCs

Neutrophils and peripheral blood mononuclear cells (PBMCs) were

isolated as previously described (Ma et al., 2024; Baert et al., 2015). Briefly, peripheral blood from the jugular vein was collected from seven 10–24 weeks old piglets.Neutrophils were separated by density gradient centrifugation on a discontinuous Percoll gradient (68% and 75%) (Cytiva), while PBMCs were separated on Lymphoprep density gradient (Axis-Sheild, Dundee, UK). Upon collection of the interphase, neutrophils were resuspended at a density of 2×10^5 cells/well, while PMBCs were resuspended at 2.5×10^6 cells/well in complete RPMI medium containing 10% fetal calf serum (FCS) (Greiner), 1% penicillin/streptomycin (Gibco), 1% non-essential amino acids (NEAA) (Gibco) and 1% sodium pyruvate (Gibco).

2.5. Cytotoxicity assay

The cytotoxicity of the tested compounds was evaluated at concentrations of 10 mg/mL, 2.5 mg/mL, and 1 mg/mL using fluorescence microscopy. Cell viability was assessed by staining with propidium iodide (Thermo Fisher, 5 μg/mL), enabling the visualization of membrane-compromised cells. Triton X−100 (0.5%) was included as a positive control to confirm assay sensitivity. Microscopic analysis revealed no significant increase in PI-positive cells at any of the tested concentrations, indicating that the compounds were not cytotoxic under these conditions (Figure S2).

2.6. Quantification of reactive oxygen species (ROS)

Neutrophils (2×10^5 cells/well) or PBMCs (2×10^6 cells/well) were seeded in a flat-bottomed white 96-well microplate and cultured for 2 h at 37°C, 5% CO_2 in RPMI without phenol red (Gibco). The culture medium was then removed and replaced with luminol buffer (100 μg/mL) for background chemiluminescence measurements. Chitin, Chitosan low molecular weight and chitosan with a degree of deacetylation of 75% were then added at different concentrations (0 mg/mL, 0.001 mg/mL, 0.01 mg/mL, 0.1 mg/mL, 1 mg/mL, 2.5 mg/mL, 10 mg/mL) to the cells. Phorbol 12-myristate 13-acetate (PMA) (50 μg/mL) was used as a positive control. Chemiluminescence was measured for 2 h using a Luminoskan.

2.7. Isolation of primary porcine monocytes

Monocytes were isolated from the PBMC fraction using CD14 + Magnetic Activated Cell Sorting (MACS) as previously described (Hermans et al., 2021). Briefly, PBMCs were incubated with an anti-CD14 monoclonal antibody (MIL−2, in house production) for 40 min on ice in MACs buffer (1% FCS in PBS-EDTA). Cells were then washed with MACS buffer (PBS−1mM EDTA + 1% FCS) and incubated with anti-mouse IgG microbeads (Miltenyi) for 20 min on ice. Using LS columns, the CD14 + monocytes were then isolated. The purity of the isolated monocyte population and the CD14- cell fraction was confirmed by evaluating CD14 expression by flow cytometry (Cytoflex, Beckman Coulter).

2.8. Generation of monocyte-derived macrophages

Macrophages were generated from CD14 + monocytes using M-CSF-mediated differentiation. Briefly, monocytes were seeded in a 24-well plate at a density of 1.2×10^6 cells/well in DMEM (Gibco) supplemented with 3 ng/mL M-CSF (R&D systems), 10% FCS, 1% penicillin/streptomycin, 1% NEAA and 1% Na-Pyruvate. The monocytes were then incubated for 7 days at 37°C, 5% CO_2, 90% humidity. The medium was changed every 2–3 days. Upon differentiation, the cells were washed with PBS and the cells were incubated in PBS−6 mM EDTA for 20 min at 4°C. Next, cells were collected by vigorously pipetting. Differentiation of the monocytes into macrophages was evaluated by measuring the expression of the cell surface marker CD169 using flow cytometry (Baert et al., 2015). Briefly, cells were stained with a mouse anti-pig CD169 monoclonal antibody (41D3, IgG1, 1:100; kindly provided by prof. Hans Nauwynck, Laboratory of Virology, Ghent University) or staining medium for 40 min at 4°C, washed with PBS−1% FCS and incubated with a goat anti-mouse IgG1 AlexaFluor 647 secondary antibody (10 μg/mL, Invitrogen) for 20 min at 4°C. Sytox blue (1:1000, Thermo Fisher) was used to exclude dead cells. The data were acquired on a flow cytometer (Cytoflex) with a minimum event count of 10,000 in the live, singlet cell gate.

2.9. Trained innate immunity in monocytes and macrophages

Monocytes and monocyte-derived macrophages were stimulated for 24 h with chitin, chitosan LMW and chitosan with DDA 75% at 10 μg/mL, β-glucan (MacroGard, 50 μg/mL), MPLA (Sigma Aldricht, 1 μg/mL) or medium alone. Supernatants were collected and stored at −80°C for subsequent cytokine analysis. Fresh medium was added and the cells were allowed to rest for 7 days, while changing the medium every 3 days. On day 7, cells were re-stimulated with 5 μg/mL lipopolysaccharide (LPS) (O111: B4, Sigma) for 24 h at 37°C, 5% CO_2. Unstimulated cells serving as a negative control at day 1, were maintained in culture for the full 7 days before LPS stimulation on day 7.

2.10. Cytokine ELISAs

The cell-free supernatant was collected and the concentrations of TNF-α, IL−1β, IL−6 and IL−10 were measured using commercially available ELISA kits (Duoset, R&D systems) according to the manufacturer's instructions. Absorbance values were measured using a Tecan Spark (Tecan Group Ltd., Switzerland; VVI−13512R). Concentrations were calculated using DeltaSoft software (version 2.1.2).

2.11. Data analysis

All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Data distribution was assessed for normality using the Shapiro–Wilk test, and homogeneity of variances was evaluated using Levene's test. Depending on the outcome of these tests, comparisons between groups were performed using One-Way ANOVA or non-parametric tests such as Friedman's test or Wilcoxon's test, as specified in the figure legends. A p-value < 0.05 was considered significant. Each dot shown in the graphs represents data obtained from an individual animal.

3. Results

3.1. Chitin and chitosan induce ROS production in a dose-dependent manner in porcine monocytes and neutrophils

To assess the capacity of chitin and chitosan to elicit reactive oxygen species (ROS) production by porcine innate immune cells, both monocytes and neutrophils were incubated with varying concentrations of chitin, low molecular weight (LMW) chitosan and chitosan with a degree of deacetylation of 75% (DDA75). As shown in Fig. 1b, chitin, chitosan LMW and chitosan DDA75 induced ROS production in neutrophils at the highest tested concentration (10 mg/mL) as compared to the negative control, whereas lower concentrations were not able to trigger ROS production by porcine neutrophils. In porcine monocytes, only chitin at the highest concentration induced a significant increase in ROS levels compared with the untreated control, whereas chitosan LMW and chitosan DDA75 did not elicit significant changes (Fig. 1b). In neutrophils, chitin induced less ROS production as compared to chitosan LMW ($p = 0.005$) and DDA75 ($p = 0.007$). Phorbol 12-myristate 13-acetate (PMA) served as a positive control, inducing strong ROS production in monocytes and neutrophils.

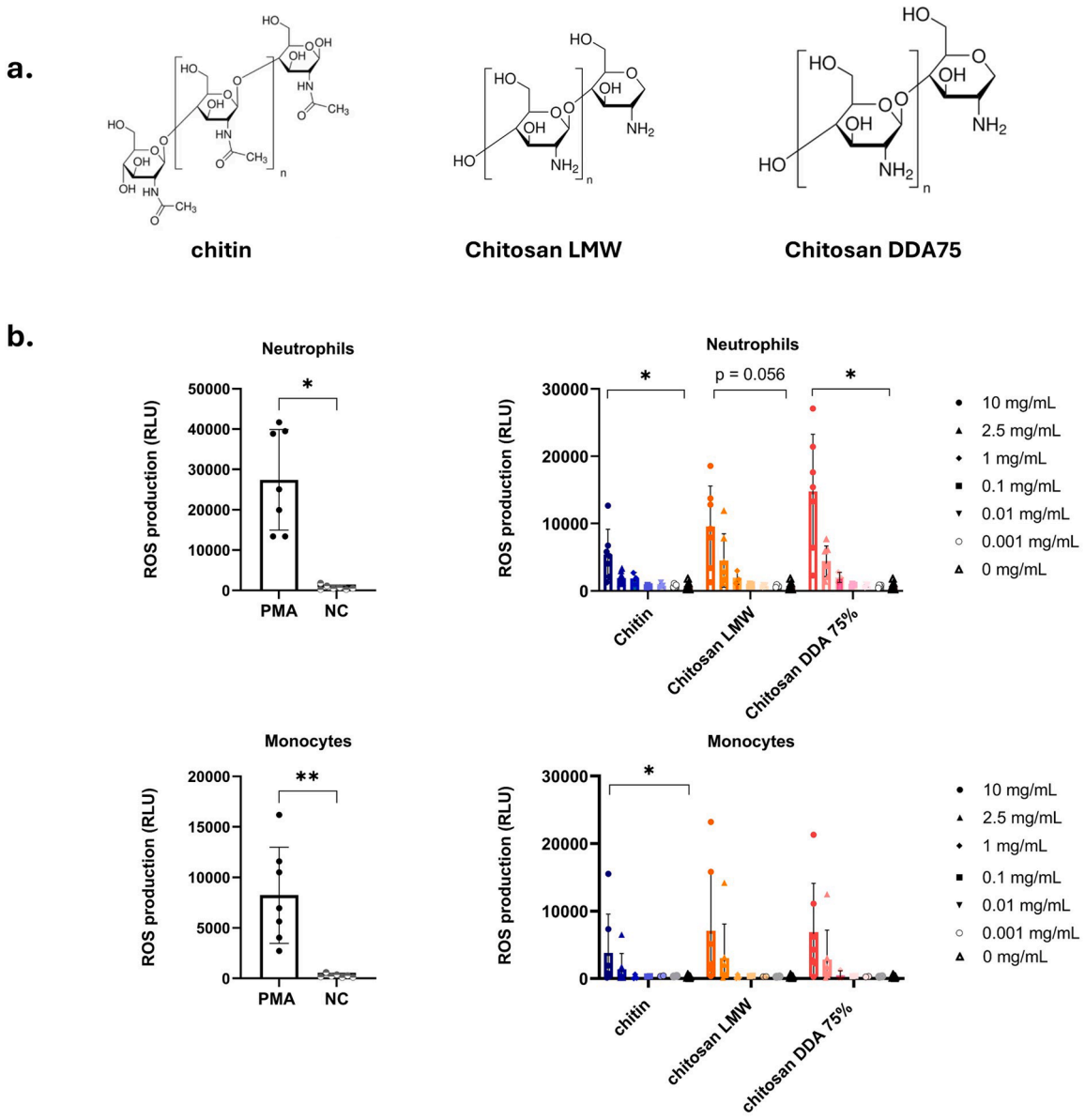

Fig. 1. Chitin and chitosan molecules induce a dose-dependent ROS production by both neutrophils and monocytes. (a) Chemical structures of chitin, chitosan low molecular weight, and chitosan with a 75% degree of deacetylation (DDA75). (b) Neutrophils (2×10^5) or monocytes (2×10^5) were incubated with chitin, chitosan low molecular weight (LMW) or chitosan with a 75% degree of deacetylation (DDA) at different concentrations (0 mg/mL, 0.001 mg/mL, 0.01 mg/mL, 0.1 mg/mL, 1 mg/mL, 2.5 mg/mL, 10 mg/mL) or PMA (50 μg/mL) for 2 h at 37°C and their ROS production was measured via a chemiluminescence assay. n = 4–7 individual animals. The error bars represent the ± SD. The data were analyzed using a nonparametric Friedman's test or T-test. *, $P < 0.05$; **, $P < 0.01$.

3.2. Chitin and chitosan derivatives do not stimulate pro-inflammatory cytokine secretion in PBMCs

To investigate the pro-inflammatory potential of chitin and its chitosan derivates, peripheral blood mononuclear cells (PBMCs) were stimulated with varying concentrations of chitin, chitosan LMW or chitosan DDA75 for 24 h. TNF-α, IL1-β and IL−6 cytokine levels in the culture supernatants were subsequently measured by ELISA. Fig. 2 shows that the positive control (LPS stimulation) triggered the secretion of IL−1β, TNF-α and IL−6 by PBMCs as compared to the untreated control. In contrast, chitin, chitosan LMW and chitosan DDA75 did not trigger TNF-α and IL−6 secretion by PBMCs at the tested concentration. Chitosan LMW and DDA75 however induced IL−1β secretion by PBMCs at 1 mg/mL, while chitin was unable to induce this.

3.3. Monocytes respond to chitin and chitosan derivatives with cytokine secretion

While PBMCs did not respond to chitin, chitosan LMW or chitosan DDA75 with the secretion of pro-inflammatory cytokines, these responses might have been masked by the heterogenous nature of PBMCs. To unmask these responses, CD14 + cells were isolated from the PBMCs and both CD14 + and CD14- cells were stimulated with chitin, chitosan LMW and chitosan DDA75. Fig. 3 shows that the positive control LPS only elicited IL−1β and TNF-α responses in CD14 + cells (Fig. 3a). Likewise, only the CD14 + cells responded to chitin, chitosan LMW and chitosan DDA75 with increased secretion of IL−1β and TNF-α as compared to the control cells. Together, these results show that chitin and chitosan derivatives distinctly modulate cytokine production in PBMC subsets, with $CD14^+$ monocytes displaying markedly higher responsiveness to chitin and the tested chitosan derivatives.

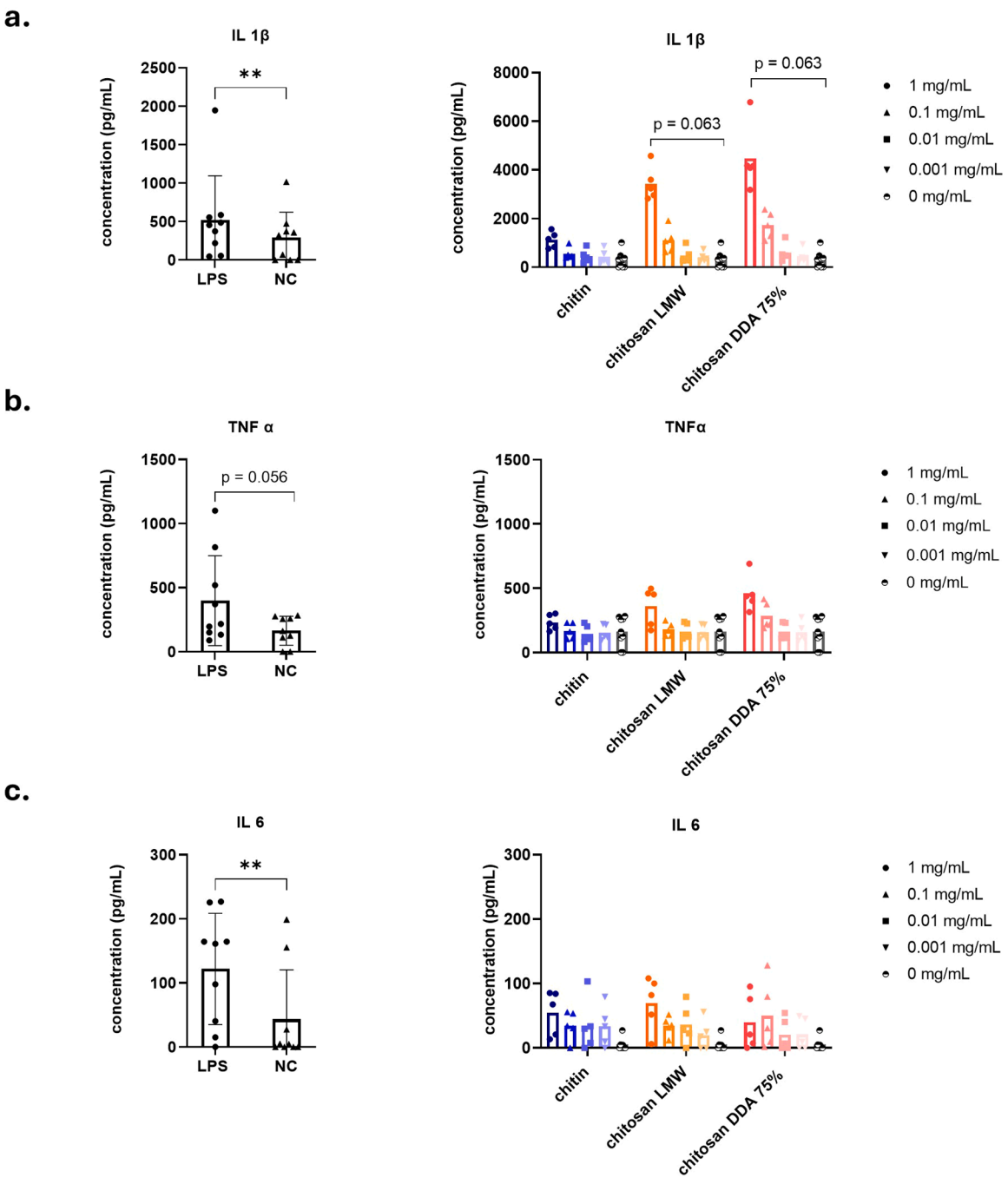

Fig. 2. Chitin and chitosan stimulation does not induce pro-inflammatory cytokine secretion by PBMCs. PBMCs (2.5×10^6 cells/well) were stimulated with chitin, chitosan low molecular weight (LMW) and chitosan with degree of 75% deacetylation (DDA) at different concentrations (1 mg/mL, 0.1 mg/mL, 0.01 mg/mL, 0.001 mg/mL), LPS (5 μg/mL) or unstimulated cells (negative control) (0 mg/mL) for 24 h. The supernatant was then tested for cytokine secretion using ELISA. n = 5–9 individual animals. The data were analyzed using a non-parametric test. *, $P < 0.05$; **, $P < 0.01$.

3.4. Chitosan induced trained innate immunity in porcine monocytes

The results above show that porcine monocytes respond to chitin and chitosan stimulation. To understand whether chitin and chitosan can affect the response of these cells towards a second stimulus, a phenomenon known as trained innate immunity (Netea et al., 2020), porcine monocytes were primed on day 1 with chitin, chitosan LMW or chitosan with DDA75 and restimulated with LPS following a resting period of seven days (Fig. 4a). To avoid robust stimulation of the monocytes, which might result in exhaustion, the concentration of chitin and the chitosan derivatives was lowered to 10 μg/mL. As expected, chitin, chitosan LMW and chitosan DD75 did not trigger IL1β and TNFα secretion at day 1 as compared to the negative control cells (NC1 and NC2) (Fig. 4b,c) and responses remained absent at day 3 during the resting period. In contrast, LPS stimulation on day 7 induced markedly higher TNFα secretion levels in chitosan LMW and chitosan DDA75-primed monocytes as compared to unprimed cells that were stimulated with LPS (NC1). Chitin did not induce these increased TNFα secretion levels. In addition, this training effect of chitosan was absent for IL1β secretion. Together, these data indicate that chitosan LMW and DDA75 triggered trained innate immunity in porcine monocytes.

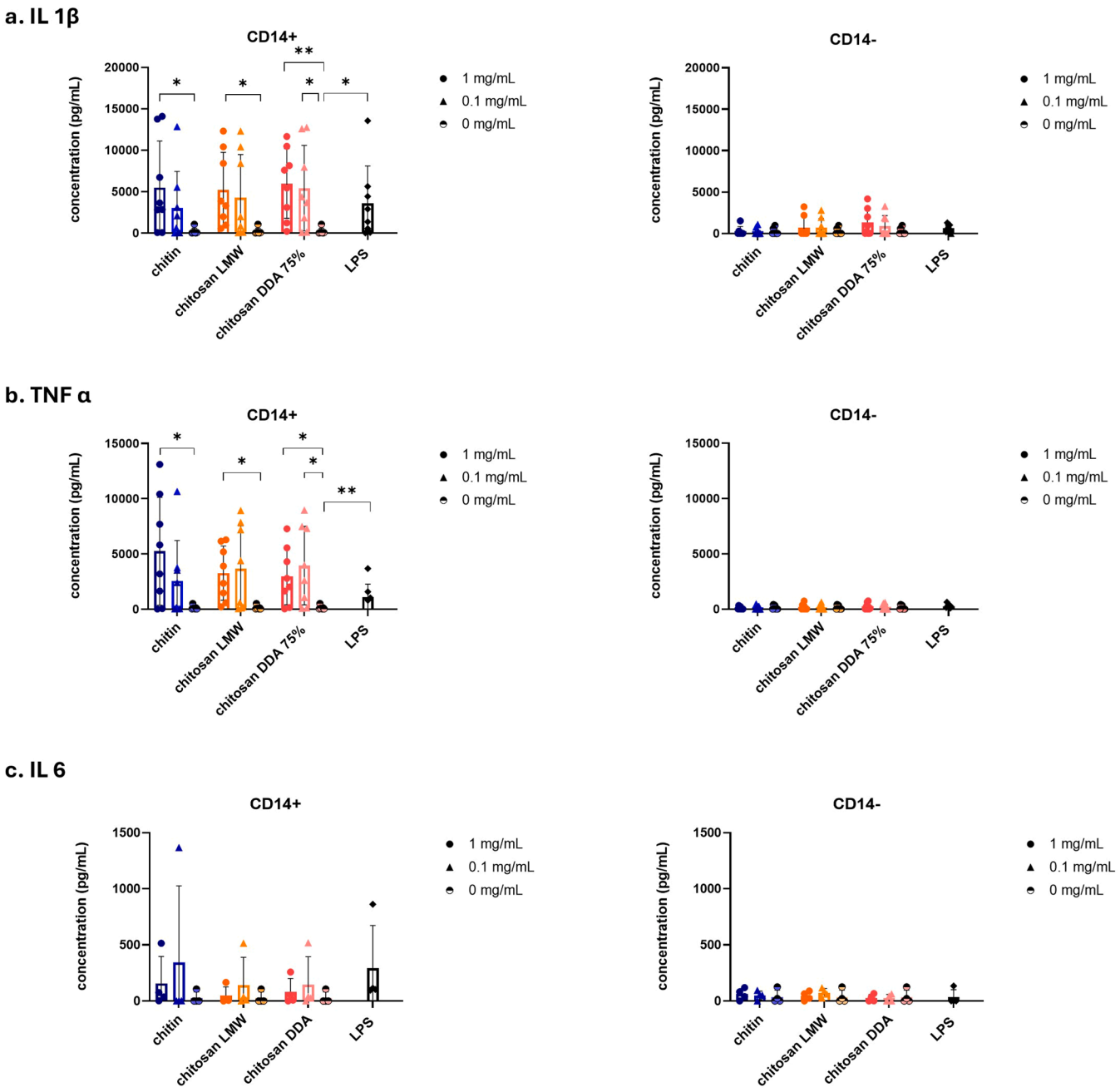

Fig. 3. Chitin and chitosan elicit TNF-α and IL−1β secretion by CD14 + monocytes. Monocytes (CD14 +) or CD14- cells (1.2 $\times 10^6$ cells/well) were stimulated with chitin, chitosan low molecular weight (LMW) and chitosan with a degree of deacetylation (DDA) 75% at different concentrations (1 mg/mL, 0.1 mg/mL), LPS (5 μg/mL) or not stimulated (0 μg/mL) for 24 h. Points represent individual animals with bars indicating the mean ± SD. The data were analyzed using a Friedman test or ONE-way Anova test. n = 4–8 individual animals. *, $P < 0.05$; **, $P < 0.01$.

3.5. Chitin induces tolerance in macrophages, whereas priming with chitosan did not alter LPS-induced cytokine production by macrophages

Monocytes are not the only innate immune cells that can be trained. Also macrophages display trained innate immune responses (Saeed et al., 2014). To evaluate whether chitin and chitosan influence trained innate immune responses in porcine macrophages, monocytes were differentiated into macrophages (suppl. Fig. 1) and exposed to chitin, chitosan LMW and chitosan DDA75 on day 1 (Fig. 5a). As a control, we used β-glucans. Fig. 5b,c shows that β-glucans triggered IL−1β and TNFα secretion by macrophages as compared to the negative controls, while chitin and the chitosan derivatives were unable to induce these cytokine responses. These β-glucan-induced cytokine responses remained elevated at day 3 during the resting period. LPS stimulation of unprimed macrophages at day 7 induced the secretion of IL−1β and TNFα as compared to the negative control cells (NC2). While macrophages primed with chitosan LMW or DDA75 secreted similar cytokine levels as compared to unprimed cells upon LPS stimulation, macrophages that were primed with chitin secreted lower IL−1β and TNFα levels compared to the LPS-stimulated unprimed cells (NC1). These data indicate that chitin induced tolerance in porcine monocyte-derived macrophages, whereas chitosan LMW and chitosan DDA75 did not alter the response of macrophages to a subsequent LPS stimulation.

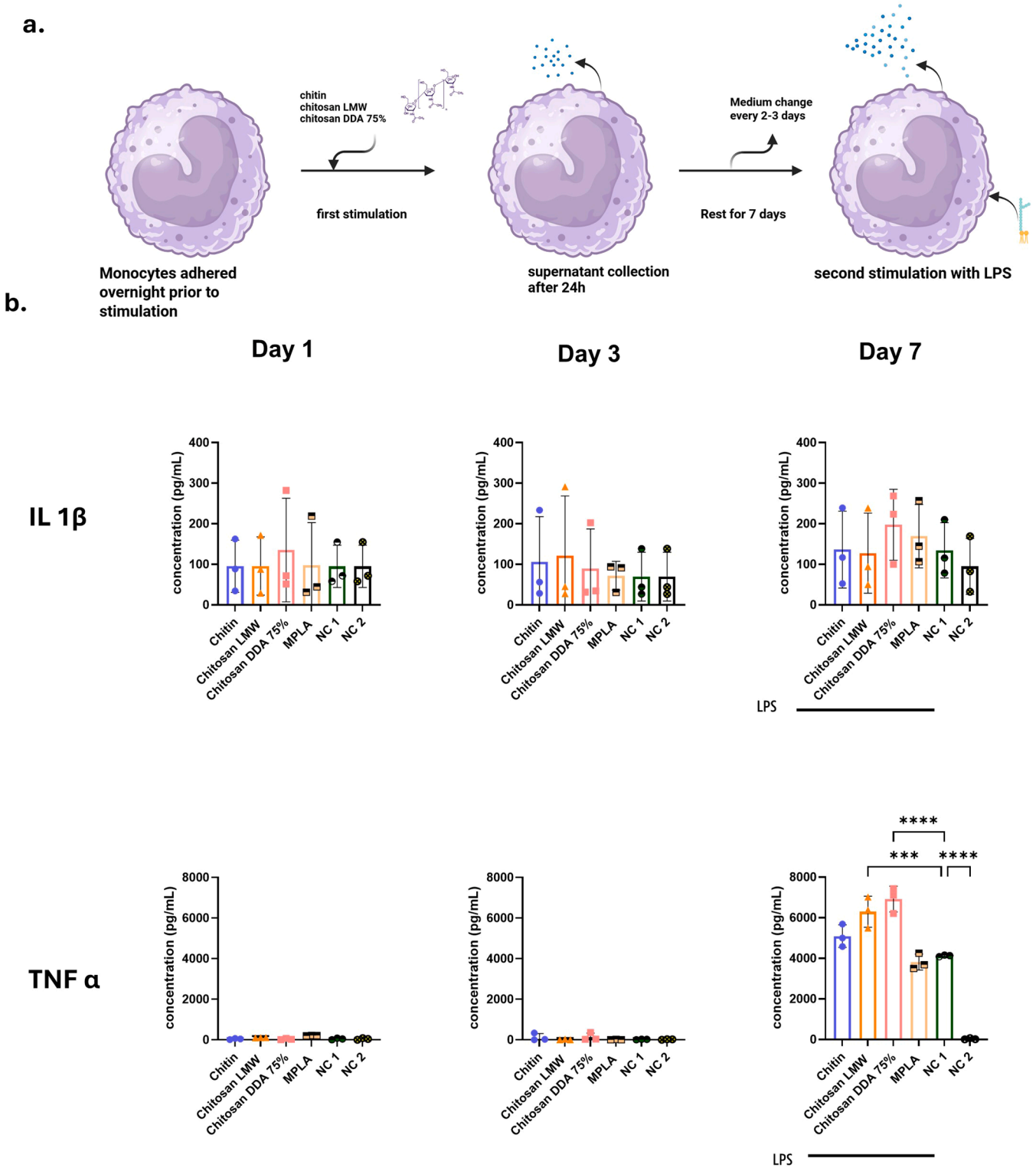

Fig. 4. Chitosan induces trained innate immunity in porcine monocytes. Porcine monocytes were isolated and seeded at a density of 1.2×10^6 cells per condition. On day 1, cells were primed with chitin, chitosan low molecular weight (LMW), or chitosan with a degree of deacetylation of 75% (DDA) at a concentration of 10 μg/mL or with MPLA (1 μg/mL) as a positive control. Unstimulated cells (NC2) served as control. n = 3 individual animals. Culture supernatants were collected to assess cytokine production following primary stimulation. Monocytes were then washed and allowed to rest until day 7 while changing the medium every 2–3 days, after which they were restimulated with lipopolysaccharide (LPS; 5 μg/mL). Supernatants collected were analyzed by ELISA for IL−1β and TNF-α concentrations. Data are presented as individual animals with bars indicating mean ± SD. Statistical significance was determined by one way ANOVA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). NC1 = cells stimulated after 7 days with only LPS.

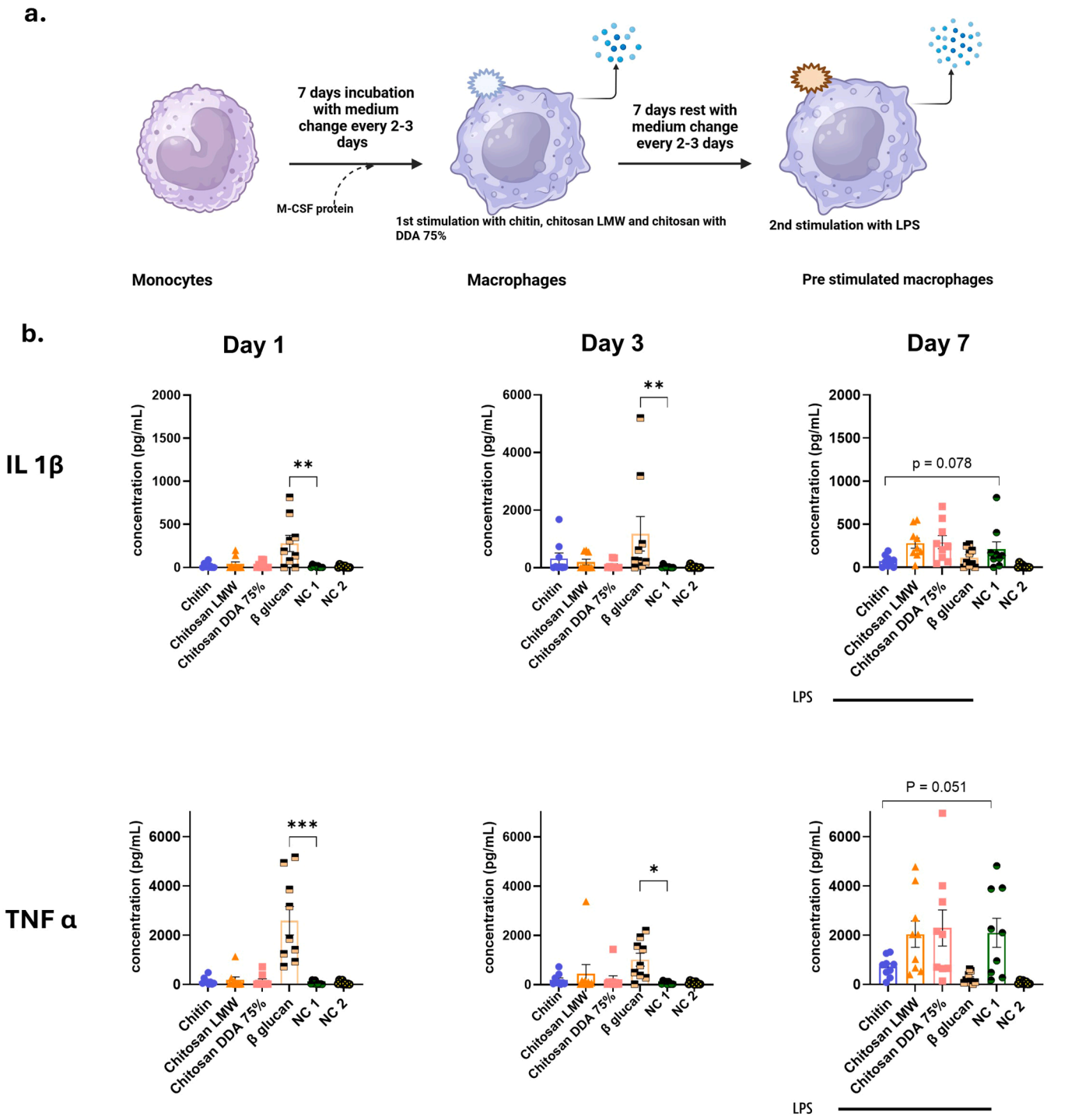

Fig. 5. Chitin, but not chitosan derivatives, induce tolerance features in monocyte derived macrophages. Monocyte-derived macrophages (1×10^6) were primed with chitin, chitosan low molecular weight (LMW), chitosan with a degree of deacetylation of 75% (DDA) (10 μg/mL), β glucan (50 μg/mL) or not stimulated (negative control 0 μg/mL) for 24 h. The supernatant was collected, the cells were washed with warm medium then kept in culture for 7 days, while changing the medium every 3 days. At day 7, the cells were restimulated with LPS (5 μg/mL) and the supernatant was analyzed for cytokine secretion using ELISA. n = 9 individual animals. The error bars represent the mean ± SD. The data were analyzed with a non-parametric Friedman test or a T-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). NC1 and NC2 are untreated monocytes-derived macrophages, NC1 = cells + LPS, NC2 = cells + medium.

4. Discussion

Although in vivo studies have shown that chitin and chitosan can activate immune cells, this evidence is not limited to pigs, as similar effects have also been reported in other animal models (Muzzarelli, 2010). In pigs, however, several studies suggest that these compounds may modulate immune responses and gut health but, it remains unclear whether this activation occurs through direct or indirect mechanisms. Therefore, we investigated whether these polysaccharides could activate primary porcine immune innate cells. Here, we addressed the effect of chitin, chitosan low molecular weight (LMW) and chitosan with degree of deacetylation of 75% (DDA75) on porcine neutrophils, monocytes and macrophages. Our findings show that these innate immune cell types respond differently to chitin and chitosan derivatives. Chitosan

LMW and DDA75 consistently acted as the strongest activators of reactive oxygen species (ROS) production and cytokine secretion, whereas chitin elicited more moderate responses in these cells. Furthermore, chitosan derivatives induced trained innate immunity in monocytes, whereas chitin triggered tolerance in macrophages. These results underscore how structure and acetylation affect innate sensing and downstream activation of innate immune cells.

Both monocytes and neutrophils increase ROS production in response to chitin and chitosan derivatives. LMW and DDA75 chitosan showing the greatest potency, consistent with a higher cationic charge and improved cell interaction associated with increased deacetylation (Huang et al., 2004). Once inside the cell, chitosan is thought to escape endosomes through the proton sponge effect, a process in which this polymer absorbs protons pumped in by vacuolar ATPase. As the endosomal pH shifts towards chitosan's pKa, it becomes increasingly protonated, driving osmotic swelling. This expansion can ultimately rupture the endosomal membrane and release of the polymer into the cytoplasm (Bueter et al., 2014). The cytosolic presence of chitosan in turn activates macrophages and neutrophils, where higher concentrations drive stronger oxidative metabolism and inflammatory signalling (Lee et al., 2008). Enhanced solubility and charge associated with a higher DDA likely increases uptake of chitosan by immune cells, potentially facilitating their activation (Wagener et al., 2025). In addition to the degree of deacetylation, particle size of chitin also affects how strongly it activates the immune system. For instance, chitin of intermediate particle size (40–70 μm) most effectively stimulated myeloid cells, leading to increased production of ROS and pro-inflammatory cytokines (Da Silva et al., 2009). Overall, the physicochemical properties of chitin and chitosan can modulate how it interacts with immune cells. For chitosan, this immune cell activation depends on its degree of deacetylation and its ability to promote endosomal escape.

PBMCs and monocytes secreted the pro-inflammatory cytokines TNF-α, IL−1β, and IL−6 in response to chitosan LMW and chitosan DDA75, consistent with observations that a higher degree of deacetylation enhances cytokine production by human PBMCs (Wagener et al., 2025). Chitin on the other hand evoked consistently lower cytokine levels in these assays. Together, these observations suggested that chitosan exposure may condition monocytes toward an increased inflammatory responsiveness, while chitin induces an anti-inflammatory response. Previous studies have reported that chitin and chitosan can trigger a pro-inflammatory or an anti-inflammatory profile, characterized by increased IL−10 production alongside a concomitant reduction in pro-inflammatory cytokine secretion. For instance, chitosan induced the polarisation of alternatively activated macrophages associated with an increased release of several chemokines (MCP−1, IP−10, MIP−1β) (Fong et al., 2015). Similarly, other studies reported that chitosan with a DDA of 80% at concentrations equal or lower than 100 μg/mL triggers IL−1ra without inflammasome activation, while higher doses (500 μg/mL) induced IL−1β and CCL5/RANTES secretion. These responses reflect distinct underlying mechanisms, with IL−1β secretion indicating inflammasome activation, whereas CCL5/RANTES production occurs independently of inflammasome activation and is instead driven by NF-κB–mediated transcriptional pathways (Guzmán-Morales et al., 2011; Oliveira et al., 2012). Moreover, high DDA chitosan scaffolds (95%) promoted M2 macrophage polarisation and decreased pro-inflammatory cytokines, whereas 85% DDA scaffolds favoured M1 polarization and increased inflammation (Vasconcelos et al., 2013). Additional evidence indicates that LMW chitosan (3–50 kDa) increases the production of M1-associated mediators including TNF-α, IL−6, IFN-γ, and iNOS (Wu et al., 2015), supporting its role in pro-inflammatory activation. Together, these reports highlight that the effects of chitin and chitosan on immune cells are highly context dependent and influenced by factors such as their physicochemical properties (particle size, degree of deacetylation) and concentration as well as the cell type (Liu et al., 2008; Wagener et al., 2014). This may explain the attenuated inflammatory responses induced by chitin observed in the present study.

In addition to promoting immune cell activation, chitosan LMW and DDA75 stimulated monocytes increased their secretion of TNF-α but not IL−1β upon LPS restimulation, indicating their potential to trigger trained innate immunity. This cytokine-specific effect might indicate different regulatory pathways. Trained innate immunity is known to involve glycolytic rewiring, mitochondrial remodelling and epigenetic changes (Netea et al., 2016; Ferreira et al., 2024). These processes may explain the LPS-induced cytokine-selective pattern observed in chitosan LMW and DDA75 trained monocytes. TNF-α is mainly regulated at the transcriptional level, whereas IL−1β also requires inflammasome-dependent processing (Ford et al., 2025), which may account for the absence of trained innate immunity for the IL−1β responses. In contrast, chitin stimulated monocyte-derived macrophages attenuated responses to a second LPS stimulation indicating tolerance. Trained immunity is known to involve glycolytic rewiring, mitochondrial remodelling and epigenetic changes (Stothers et al., 2021). These pathways possibly underlie the chitosan-driven training phenotypes in monocytes. A previous study implied the potential of chitosan to induce innate immune memory in a human microglia cell line. Cells stimulated with LPS and chitosan produced more pro-inflammatory cytokine transcript levels upon a second stimulation with the same molecules (Thu et al., 2024). However, the combined stimulation of the cells with LPS and chitosan during the first and second treatment impedes to draw firm conclusions on the abiltiy of chitosan to trigger trained innate immunity. In addition, this effect appears to be cell type- and structure-dependent, as in our study chitosan did not induce a similar training in monocyte-derived macrophages. This discrepancy may reflect differences in cell lineage, tissue origin, or the physicochemical properties of the chitosan used, reinforcing the concept that chitosan-driven immune modulation is governed by both structural characteristics and cellular context. Chitin has been shown to induce IL10 secretion via mannose receptor-mediated uptake and NOD2/TLR9 signalling in murine bronchoalveolar or peritoneal macrophages (Da Silva et al., 2009) and can dampen LPS-driven inflammation in vivo, providing a mechanistic rationale for tolerance-like outcomes after chitin priming. The trained innate immunity inducing properties of chitosan LMW and DDA75 support the use of chitosan as immunopotentiator in preventive therapies such as vaccines or functional feed. Oppositely, chitin's ability to dampen LPS driven inflammation points to its suitability in applications requiring controlled immune engagement to attenuate inflammation.

While our experiments focused on immune cells, it is worth noting that chitin and chitosan can also affect epithelial cells, which form the first line of defence at mucosal surfaces. LYSMD3 has been identified as a mammalian pattern recognition receptor in human lung epithelial cells that directly binds chitin (He et al., 2021). Recognition of chitin by LYSMD3 activates NF-κB and induces the production of IL−6, CXCL−8, and the alarmin IL−33 in lung epithelial cells. In addition to purified chitin, *Alternaria* extract, which contains chitin, also activated lung epithelial cells in LYSMD3-dependent manner and contributed to type 2-skewed inflammatory responses, highlighting the role of this pattern recognition receptor in mediating epithelial sensing of environmental chitin (He et al., 2021). Whether LYSMD3 is present in other species and epithelial cells lining other mucosal surfaces such as the intestine, remains to be investigated.

This study has some limitations. The primary immune cells may not completely represent the complexity of immune responses in living animals, where tissue and whole-body factors also play a role (Mestas and Hughes, 2004). In addition, only a single concentration (10 μg/mL) was evaluated in the trained innate immunity experiments, and different concentrations may induce distinct immunological outcomes. Previous studies have shown that the immunomodulatory effects of innate immune stimuli are dose-dependent (Ifrim et al., 2014), highlighting the need for future studies investigating broader concentration ranges. Furthermore, only a small set of particle sizes, molecular weights, and

deacetylation levels were tested, and these mixtures are heterogeneous in terms of structure. Using well-characterized chitin and chitosan molecules with defined structural characteristics could help to further unravel the underlying structure-function relationships that govern their immune-modulating effects in terms of trained innate immunity and tolerance (Da Silva et al., 2009; Wagener et al., 2014). Finally, while our results suggest that chitin and chitosan can modulate immune cell activation and induce tolerance or trained innate immunity, the specific molecular mechanisms remain largely unknown. Further research should address whether chitosan triggers metabolic reprogramming and epigenetic modifications that are known to underlie trained immunity. The involvement of pattern recognition receptors, such as LYSMD3, as well as downstream signalling pathways, including NF-κB activation, was assumed based on previous literature rather than directly tested in our experiments (Mohyuddin et al., 2021; Li et al., 2025). Future studies incorporating receptor-blocking approaches, pathway-specific inhibitors, and analyses of metabolic and epigenetic changes will be essential to confirm the mechanisms driving the observed immune modulation and to clarify how structural features of chitin and chitosan contribute to these effects.

In conclusion, chitin and chitosan derivatives differentially shape activation of porcine innate immune cells in a structure dependent manner. While chitin induces tolerance, chitosan with a lower molecular weight and degree of acetylation favours trained innate immunity. This offers evidence for their application as functional feed additives in pig production to boost immune resilience and decrease antibiotic use during vulnerable periods like weaning.

Ethics statement

All animal procedures were performed in accordance with relevant guidelines for the care and use of animals in research. Blood samples were collected from healthy pigs under protocols approved by the Ethical (EC2023–22), ensuring minimal stress and discomfort to the animals.

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CRediT authorship contribution statement

Nidhal Guendouz: Writing – original draft, Methodology, Formal analysis. **Bert Devriendt:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Data curation.

Conflict of interest

The authors declare no competing interests.

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Author contributions

N.G., designed and carried out the experiments, performed data analysis and drafted the manuscript, B.D., conceived the presented idea, designed the experiments, supervised the project, provided critical feedback and drafted the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.molimm.2026.06.002.

Data availability

Data will be made available on request.

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