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Chitin Is a Size-Dependent Regulator of Macrophage TNF and IL-10 Production

Carla A. Da Silva,* Cécile Chalouni,‡ Adam Williams,† Dominik Hartl,* Chun G. Lee,* and Jack A. Elias2*§

Chitin is a ubiquitous polysaccharide in fungi, insects, and parasites. We hypothesized that chitin is a size-dependent regulator of innate immunity. To test this hypothesis, we characterized the effects of chitin of different sizes on murine bronchoalveolar or peritoneal macrophages. In these studies, large chitin fragments were inert, while both intermediate-sized chitin (40–70 μm) and small chitin (SC; <40 μm, largely 2–10 μm) stimulated TNF elaboration. In contrast, only SC induced IL-10 elaboration. The effects of intermediate-sized chitin were mediated by pathways that involve TLR2, dectin-1, and NF-κB. In contrast, the effects of SC were mediated by TLR2-dependent and -independent, dectin-1-dependent pathways that involved the mannose receptor and spleen tyrosine kinase. Chitin contains size-dependent pathogen-associated molecular patterns that stimulate TLR2, dectin-1, and the mannose receptor, differentially activate NF-κB and spleen tyrosine kinase, and stimulate the production of pro- and anti-inflammatory cytokines. The Journal of Immunology, 2009, 182: 3573–3582.

Chitin is a polymer of N-acetylglucosamine, which, after cellulose, is the second most abundant polysaccharide in nature. Although it does not have a mammalian counterpart, it is found in the walls of fungi, exoskeleton of crabs, shrimp, and insects, the microfibrillar sheath of parasitic nematodes, and the lining of the digestive tracts of many insects (1–9). In these locations, chitin is used by the organism to protect it from the harsh conditions in its environment and host antiparasite/pathogen immune responses. In these settings, chitin accumulation is regulated by the balance of biosynthesis and degradation. The latter is mediated, in great extent, by chitinases which are endo-β-1,4-N-acetylglucosaminidases (5). These enzymes are produced as part of immune responses to chitin containing pathogens where they induce chitin fragmentation (5, 10). Surprisingly, although chitin and chitin fragments are produced during pathogen invasion, very little is known about their ability to regulate local inflammatory cell function and the mechanisms of the effects that have been noted have not been adequately defined.

TLRs have recently been appreciated to function as sensors of microbial and parasitic invasion that initiate innate inflammatory and immune responses against these pathogens. They mediate their responses by recognizing conserved, often times repeating structures on these pathogens called pathogen-associated molecular patterns or PAMPs (11). The specificity of TLRs for specific PAMPs, including the recognition of peptidoglycans, lipopeptides, and zymosan by TLR2 and LPS by TLR4, are now well described (11). The importance of TLR-TR interactions and of interactions between TLR and other non-TLR innate pattern recognition receptors (PRR) such as the C-type lectin dectin-1 have also been increasingly appreciated (11, 12). Chitin has a repeating molecular pattern that is analogous, in many ways, to other TLR ligands, in particular the TLR4 ligand hyaluronic acid (13, 14). Recent findings in our laboratory demonstrated that chitin is a PAMP that activates TLR2 and regulates macrophage (MO) function and acute inflammation in vitro and in vivo (15). However, the importance of chitin size in the regulation of MO function has not been adequately investigated and the mechanisms of the different responses that have been induced have not been defined.

We hypothesized that chitin is a size-dependent stimulator of MO function with modest-sized fragments inducing proinflammatory and tissue-damaging responses while small, more completely degraded, molecules induce cytokines that inhibit tissue inflammation. To test this hypothesis, we characterized the ability of chitin fragments to stimulate MO TNF and IL-10 production in vivo and in vitro and defined the different pathways that mediate these responses.

Materials and Methods

Preparation of chitin particles

Chitin fragments were generated as previously described (16). In brief, chitin powder (Sigma-Aldrich) was suspended in sterile 1× PBS (Life Technologies). Chitin of different sizes was then generated using sonication or by digestion with chitin-specific recombinant acidic mammalian chitinase (rAMCase, a gift from Dr A. Coyle, MedImmune, Gaithersburg, MD). In the former, chitin was sonicated at 25% output power three times for 10 min, chitin pellets of different sizes (big chitin (BC): 70–100 μm, largely 2–10 μm; small chitin (SC): <40 μm, largely 2–10 μm or super small chitin (SSC) fragments: <2 μm) were suspended in the desired volume of sterile PBS and autoclaved. Particle sizes and size distribution were evaluated by flow cytometry by comparing the chitin to

PI, propidium iodide; DIC, differential interference contrast; MR, mannose receptor; NBD, NEMO-binding domain.
different sized latex bead controls (0.085, 11.156, and 42.0 μm in diameter; Polysciences). Before utilization, the chitin particles were concentrated by speed vacuuming. Endotoxin levels were below the limits of detection in a Limulus amebocyte lysate assay (Sigma-Aldrich).

β-Glucanase treatment

Studies were also undertaken to determine whether β-glucanase altered the effector responses of our chitin preparations. In these experiments, chitin powder was incubated with β-glucanase or vehicle control (1U, 55°C, pH 6; Sigma-Aldrich) for 24 h. The preparations were then filtered, sized, concentrated, and assayed for endotoxin as described above.

Mice

Toll-like receptor TLR2 or TLR4 null mice on a C57BL/6 background were generated and characterized as described previously (17). Control C57BL/6 mice were obtained from The Jackson Laboratory. All mice were housed and cared for in the animal facilities at Yale University and all experiments were approved by the Institutional Animal Care and Use Committee at Yale University School of Medicine.

In vivo MO recovery

WT mice were anesthetized with i.p. ketamine hydrochloride (Hospira), IC, SC (25 μg), or vehicle control were then applied to the nares (30 μl) and instilled into their lungs. The mice were sacrificed 6 h after the single intranasal challenge and bronchoalveolar lavage fluid (BAL) was obtained as described previously (18). Each BAL sample was centrifuged and used for flow cytometry. FACS with Abs against CD11b, F4/80, Gr1, CD3, and CD4 was used to characterize these cells. More than 90–95% of these cells stained positively with F4/80 and CD11b and did not express Gr1, CD3, or CD4. Cell viability was assessed using trypan blue dye exclusion and by staining with annexin V and propidium iodide (PI).

In vitro MO culture

To obtain peritoneal MO, 10- to 15-wk-old wild-type (WT) and genetically manipulated mice received i.p. 3% thioglycollate medium (2 ml; Sigma-Aldrich). Five days later, peritoneal washings were undertaken with sterile PBS and the recovered cells were washed and plated in 6-well plates at 1.5 million cells/well. After an overnight incubation in complete medium (RPMI 1640 with l-glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml), nocodazole (10 μM; Sigma-Aldrich), fixed with 0.5 ml of ice-cold 2% paraformaldehyde, permeabilized and stained with actin dye staining Abs were from BD Biosciences. Propidium iodide (PI, 5 μg/ml unless otherwise indicated), or its vehicle control. At the desired point in time, supernatant TNF and IL-10 protein measurements were harvested BAL MO were treated with brefeldin A (2 μg/ml) and stained with CD11b, CD3, CD16/32, and PI. For intracellular cytokine staining, ex vivo-harvested BAL MO were treated with brefeldin A (2 μg/ml; Sigma-Aldrich) and annexin V-FITC (5 μg/ml; BD Biosciences) to prevent nonspecific Fc receptor binding. MO were identified by characteristic surface marker expression (F4/80⁺, CD11b⁺, CD3ε⁺, CD4⁺, Gr1⁻) (all surface staining Abs were from BD Biosciences). Propidium iodide (PI, 5 μg/ml; Sigma-Aldrich) and annexin V-FITC (5 μg/ml; BD Biosciences) were used to discriminate viable from apoptotic (annexin V⁺, PI⁻) cells and necrotic (annexin V⁺, PI⁺) leukocytes. Only viable alveolar MO were included in the analysis. For intracellular cytokine staining, ex vivo-harvested BAL MO were treated with brefeldin A (2 μg/ml; Sigma-Aldrich), fixed with 0.5 ml of ice-cold 2% paraformaldehyde, permeabilized using 0.5% saponin (Sigma-Aldrich), and stained with anti-IL-10 FITC, anti-TNF-α PE, or the appropriate isotype controls (all intracellular Abs were from eBioscience) to assess unspecified staining. Saturating concentrations of the respective Abs were used as determined by titration experiments before the study. After staining, MO were washed and after lysis of the remaining erythrocytes, cells were analyzed by flow cytometry (BD Biosciences). Ten thousand MO/samples were analyzed. Isotype controls were subtracted from the respective specific Ab expression and the results are reported as mean fluorescence intensity. Calculations were performed with CellQuest analysis software (BD Biosciences). Experiments were performed in triplicate.

Confocal evaluation of NF-κB and Syk

WT or TLR2 null 3% thioglycollate-primed peritoneal MO were cultured directly on glass coverslips in 24-well plates for 24 h. After stimulation with chitin (IC or SC, 100 μg/ml) or vehicle (1× PBS) for 20 min for NF-κB evaluations and 5 min for Syk evaluations, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin (Sigma-Aldrich) and stained with antibodies against p65 and Syk (BD Biosciences). After staining, coverslips were mounted on glass slides with DRAQ5 antifade medium (Biostatus) containing 4′,6-diamidino-2-phenylindole (DAPI). Coverslips were imaged with a confocal microscope (Zeiss LSM 710). A minimum of 100 cells per condition were analyzed. The number of p65 or Syk positive cell nuclei was determined and the results are expressed as fold change over control.

CTCGACGGCATTT-3’ and TNF-α antisense primer, 5’-CACCCATTCTCTACAGAGCAA-3’; IL-10 sense primer, 5’-AACATACCTGCTACCCGAC TCCT-3’ and IL-10 antisense primer, 5’-CTGCTTTGCTTTATTTCACA-3’; β-actin sense primer, 5’-TGAGGGAATCTGTCGGTGAAC-3’ and β-actin antisense primer, 5’-AAGAAAGAGGCTTGAAAGAGG-3’.

Flow cytometry

Thioglycollate-elicited peritoneal MO or BAL MO were suspended in FACS buffer (PBS, 2% BSA, and 2% FCS) and incubated for 30 min at room temperature with purified rat anti-mouse CD16/CD32 mAb (1 μg/10⁶ cells; mouse Fc-Block; BD Biosciences) to prevent nonspecific Fc receptor binding. MO were identified by characteristic surface marker expression (F4/80⁺, CD11b⁺, CD3ε⁺, CD4⁺, Gr1⁻) (all surface staining Abs were from BD Biosciences). Propidium iodide (PI, 5 μg/ml; Sigma-Aldrich) and annexin V-FITC (5 μg/ml; BD Biosciences) were used to discriminate viable from apoptotic (annexin V⁺, PI⁻) cells and necrotic (annexin V⁺, PI⁺) leukocytes. Only viable alveolar MO were included in the analysis. For intracellular cytokine staining, ex vivo-harvested BAL MO were treated with brefeldin A (2 μg/ml; Sigma-Aldrich), fixed with 0.5 ml of ice-cold 2% paraformaldehyde, permeablized using 0.5% saponin (Sigma-Aldrich), and stained with anti-IL-10 FITC, anti-TNF-α PE, or the appropriate isotype controls (all intracellular Abs were from eBioscience) to assess unspecified staining. Saturating concentrations of the respective Abs were used as determined by titration experiments before the study. After staining, MO were washed and after lysis of the remaining erythrocytes, cells were analyzed by flow cytometry (BD Biosciences). Ten thousand MO/samples were analyzed. Isotype controls were subtracted from the respective specific Ab expression and the results are reported as mean fluorescence intensity. Calculations were performed with CellQuest analysis software (BD Biosciences). Experiments were performed in triplicate.
Preparations were used at 100 μg/ml unless otherwise indicated. The effects of these treatments on the kinetics of TNF mRNA \((A)\) and protein \((B)\) and the dose-response relationships of these effects \((C)\) were evaluated. The effects of these treatments on the kinetics of IL-10 mRNA \((D)\) and protein \((E)\) accumulation were also evaluated. The levels of mRNA were quantified using real-time RT-PCR. Results were normalized to the levels of β-actin mRNA and expressed as a fold increase when compared with the levels in MO treated with vehicle alone. Supernatant TNF and IL-10 were assessed by ELISA. The noted values represent the mean ± SEM of four experiments performed in duplicate on MO from 12 mice. ***, \(p < 0.001\) compared with vehicle-treated cells and cells incubated with BC.

Expression of results and statistical analysis

TNF-α and IL-10 protein levels are expressed as pg/ml. In the mRNA evaluations, the ratios of the levels of mRNA encoding TNF-α or IL-10 vs β-actin were calculated for each sample and expressed as relative copy number fold increase. Results are expressed as means ± SEM. Statistical analysis was performed with the Student’s \(t\) test. Values of \(p < 0.05\) were considered significant.

Results

Chitin regulation of TNF and IL-10 production in vitro

To begin to test this hypothesis, studies were undertaken to determine whether different sized chitin molecules regulated cytokine elaboration by murine lung MO in vivo. In these experiments, 25 μg of endotoxin-free IC (40–70 μm) or SC (<40 μm, largely 2–10 μm) were given intranasally to WT C57BL/6 mice for a period of 6 h and flow cytometry was used to assess the levels of TNF and IL-10 in BAL fluid MO (>90% CD11b<sup>+</sup>F4/80<sup>+</sup>). Both IC and SC were potent stimulators of lung MO TNF protein elaboration, with IC having the strongest effect (Fig. 1A). In contrast, SC appeared to be the only size of chitin strongly inducing lung MO IL-10 protein elaboration (Fig. 1B). Thus, these studies demonstrate that chitin has size-dependent effects on murine lung MO cytokine elaboration.

Chitin regulation of TNF and IL-10 production in vitro

To understand the mechanisms by which chitin induces MO TNF and IL-10 elaboration, thioglycollate-elicited MO (>90% CD11b<sup>+</sup>F4/80<sup>+</sup>) were incubated with endotoxin-free BC (70–100 μm), IC, and 2% paraformaldehyde in PBS for NF-κB and Syk staining, respectively. They were then permeabilized with 0.2% Triton X-100 in PBS and blocked with 15% normal goat serum (Sigma-Aldrich). In the Syk evaluations, anti-FcγRIII/II (BD Pharmingen) was added to the blocking solution. NF-κB was detected using a rabbit polyclonal Ab directed against NF-κB p65 (1 μg/ml; Santa Cruz Biotechnology). Syk was detected with a rabbit polyclonal anti-phospho-ZAP70 (Tyr319)/Syk (Tyr352) (Cell Signaling Technology) (20). After incubation for 1 h, the cells were exposed for 30 min to Alexa Fluor 546-conjugated goat anti-rabbit IgG (Invitrogen) along with TO-PRO-3 iodide for nuclear staining (Molecular Probes). Coverslips were mounted in Fluoromount-G and 2% paraformaldehyde in PBS for NF-κB was detected using a rabbit polyclonal anti-phospho-ZAP70 (Tyr319)/Syk (Tyr352) (Cell Signaling Technology) (20). After incubation for 1 h, the cells were exposed for 30 min to Alexa Fluor 546-conjugated goat anti-rabbit IgG (Invitrogen) along with TO-PRO-3 iodide for nuclear staining (Molecular Probes). Control stainings used rabbit IgG. Coverslips were mounted in Fluoromount-G (Southern Biotechnology Associates). Samples were imaged using a Zeiss LSM 510 laser-scanning confocal device attached to an Axiovert 200M microscope using a water immersion, Plan-Apochromat 40 immersion objective (Zeiss) at zoom 2. TO-PRO-3 iodide and Alexa Fluor 546 were excited with laser light at wavelengths of 633 and 546 nm, respectively. Images were electronically merged using the LSM 510 software and stored as TIFF files.

Differential interference contrast (DIC) and confocal evaluation of chitin-treated peritoneal MO

IC and SC were stained with Texas Red C<sub>3</sub>-dichlorotriazine (Invitrogen) according to the instructions provided by the manufacturer. Stained chitin (100 μg/ml) or vehicle (1× PBS) was then incubated with WT 3% thioglycollate-primed peritoneal MO previously cultured directly on glass coverslips in 24-well plates for 24 h. After stimulation with stained IC or SC for 0, 10, 20, or 40 min, cells were washed five times with cold sterile 1× PBS and fixed with 4% paraformaldehyde in PBS. Samples were imaged using a Leica TCS-SP5 laser-scanning confocal microscope using an oil immersion, ×100 objective. Fluorescence and DIC images were acquired to document Texas Red-stained IC and SC in the cells and to show the whole cells. Texas Red C<sub>3</sub>-dichlorotriazine was excited with laser light at a wavelength of 633 nm.

Expression of results and statistical analysis

TNF-α and IL-10 protein levels are expressed as pg/ml. In the mRNA evaluations, the ratios of the levels of mRNA encoding TNF-α or IL-10 vs β-actin were calculated for each sample and expressed as relative copy number fold increase. Results are expressed as means ± SEM. Statistical analysis was performed with the Student’s \(t\) test. Values of \(p < 0.05\) were considered significant.

Results

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To begin to test this hypothesis, studies were undertaken to determine whether different sized chitin molecules regulated cytokine elaboration by murine lung MO in vivo. In these experiments, 25 μg of endotoxin-free IC (40–70 μm) or SC (<40 μm, largely 2–10 μm) were given intranasally to WT C57BL/6 mice for a period of 6 h and flow cytometry was used to assess the levels of TNF and IL-10 in BAL fluid MO (>90% CD11b<sup>+</sup>F4/80<sup>+</sup>). Both IC and SC were potent stimulators of lung MO TNF protein elaboration, with IC having the strongest effect (Fig. 1A). In contrast, SC appeared to be the only size of chitin strongly inducing lung MO IL-10 protein elaboration (Fig. 1B). Thus, these studies demonstrate that chitin has size-dependent effects on murine lung MO cytokine elaboration.
or SC. As observed in vivo, both IC and SC were potent stimulators of MO TNF mRNA and protein induction (Fig. 2, A–C). Interestingly, the kinetics of these responses were different with maximal TNF mRNA and protein accumulation being seen after 4 and 12 h with IC and 2 and 4 h, respectively, with SC. In all cases, these effects were dose dependent with significant TNF elaboration being seen with chitin concentrations as low as 20 μg/ml (Fig. 2C). In contrast, BC had no effect on MO TNF mRNA and protein induction (Fig. 2, A–C). This was not due to differences in particle:cell ratios because doses of BC as high as 500 μg/ml were not able

**FIGURE 3.** Specificity of the effects of chitin. Peritoneal MO were obtained from WT mice and incubated with IC, SC, SSC, or vehicle for 9 h. The chitin preparations were used at 100 μg/ml. The effects of these treatments on TNF (A and C) and IL-10 protein (B and D) were evaluated. C and D, Experiments were done using chitin pretreated with β-glucanase for 24 h. Supernatant TNF and IL-10 were evaluated by ELISA. The noted values represent the mean ± SEM of four experiments performed in duplicate on MO from 12 mice. ***, p < 0.001.

**FIGURE 4.** Roles of TLR2, dectin-1, and MR in IC regulation of MO TNF production. Peritoneal MO were obtained from TLR2 sufficient (+/+) and deficient (−/−) mice and incubated with IC or vehicle control for 9 h (A) or with Texas-Red-stained IC (TR-IC; 100 μg/ml) or vehicle control for 10 min (B). A, Experiments were done in the presence and absence of the dectin-1 blocker laminarin (1 mg/ml, 30-min preincubation), an Ab against the MR (anti-MR, 10 μg/ml, 30-min preincubation), or the actin polymerization/particle internalization blocker cytochalasin D (5 μg/ml, 30-min preincubation). Supernatant TNF was evaluated by ELISA. The noted values represent the mean ± SEM of four experiments performed in duplicate on MO from 12 mice. ***, p < 0.01 and ***, p < 0.001. B, The left panels are confocal images showing IC labeled with Texas-Red C2-dichlorotriazine (TR) and the right panels are DIC images of MO showing normal membrane morphology (DIC).
to stimulate TNF mRNA or protein (Fig. 2C). SC was also a potent stimulator of MO IL-10 mRNA and protein (Fig. 2, D and E). In contrast to SC, IC and BC did not induce MO IL-10 mRNA or protein (Fig. 2, D and E). These studies confirm the size-dependent effects of chitin on MO cytokine elaboration with both IC and SC fragments stimulating TNF elaboration and only the SC fragments stimulating MO IL-10 production.

Specificity of the effects of chitin
To ensure that the MO regulatory effects noted above were due to the chitin in our preparations, three different approaches were undertaken. In the first, we compared the MO regulatory effects of IC and SC generated by sonication or rAMCase exposure. These studies demonstrated that IC and SC had identical effects regardless of the method of preparation (data not shown). In the second, we determined whether rAMCase generated chitin fragments that were <2 μm in size and stimulated MO TNF or IL-10 production. These studies demonstrated that these very SC fragments did not stimulate MO TNF or IL-10 elaboration (Fig. 3, A and B). Lastly, studies were undertaken to determine whether β-glucanase treatment altered the ability of IC or SC to induce MO cytokine elaboration. In these studies, β-glucanase treatment did
not alter the ability of IC or SC to induce TNF and/or IL-10 (Fig. 3, C and D). When viewed in combination, these studies all support the chitin specificity of the effects that we have described.

**IC stimulation of TNF: roles of TLR, dectin-1, and the MR**

Studies were next undertaken to define the importance of TLRs, dectin-1, and the MR in the MO stimulatory effects of IC. TLR4 was the first receptor to be evaluated. These experiments demonstrated that the effects of chitin on MO cytokine elaboration were TLR4-independent because BC, IC, and SC had similar effects on mice with WT and null TLR4 loci (data not shown). In contrast, comparisons of the ability of IC to stimulate TNF elaboration by MO from WT and TLR2 null mice demonstrated that this stimulation was completely abrogated in the absence of TLR2 (Fig. 4A). In addition, the dectin-1 blocker laminarin (1 mg/ml) caused a significant, but incomplete \( (p < 0.01) \) decrease in IC stimulation of TNF production while an Ab against the MR (anti-MR) did not alter the ability of IC to stimulate MO TNF elaboration (Fig. 4A). Finally, the particle internalization and phagocytosis blockers cytochalasin D \( (5 \mu g/ml) \) and nocodazole \( (10 \mu g/ml) \) did not alter the ability of IC to stimulate MO TNF elaboration. In all cases, alterations in TNF protein production were associated with comparable alteration in mRNA accumulation (data not shown). Overall, these studies demonstrate that IC stimulates TNF production via a TLR2- and dectin-1-dependent and phagocytosis-independent mechanism.

**FIGURE 6.** Roles of TLR2, dectin-1, and MR in SC regulation of MO IL-10 production. Peritoneal MO were obtained from TLR2-sufficient (+/+ ) and -deficient (-/- ) mice and incubated with SC or vehicle control for 9 h (A and B) or with Texas Red-stained SC (TR-SC; 100 \( \mu g/ml \)) or vehicle control for 10 min (C). A and B, Experiments were done in the presence and absence of the dectin-1 blocker laminarin (1 mg/ml, 30-min preincubation), an Ab against the MR (anti-MR, 10 \( \mu g/ml \), 30-min preincubation), or the actin polymerization/particle internalization blocker cytochalasin D \( (5 \mu g/ml, \) 30-min preincubation). Supernatant TNF \( (A) \) and IL-10 \( (B) \) were evaluated by ELISA. The noted values represent the mean \( \pm \) SEM of four experiments performed in duplicate on MO from 12 mice. *, \( p < 0.05 \); **, \( p < 0.01 \); and ***, \( p < 0.001 \). C, The left panels are confocal images showing SC labeled with Texas Red C2-dichlorotriazine labeled (TR) and the right panels are DIC images of MO showing normal membrane morphology (DIC).
IC stimulation of TNF: roles of NF-κB

Studies were next undertaken to define the importance of NF-κB in the pathways that IC uses to stimulate MO TNF elaboration. In these experiments, MO were incubated with IC fragments or vehicle for 20 min, and the presence and location of p65 was assessed. In parallel evaluations, the effects of a selective NF-κB

FIGURE 7. Activation and roles of MO NF-κB and Syk in SC-induced effects. Peritoneal MO were obtained from TLR2-sufficient (+/+ ) and -deficient (−/−) mice and incubated with SC (100 μg/ml) or vehicle control for 20 min (A and B) or 9 h (C and D) for NF-κB and 5 min (E and F) or 9 h (G and H) for Syk activation assessments, respectively. A and E, NF-κB p65 and Syk were detected by confocal microscopy using a rabbit polyclonal anti-murine p65 Ab and a rabbit polyclonal anti-murine phospho-ZAP70/Syk Ab, respectively, coupled to a goat anti-rabbit Alexa Fluor 546 Ab (red). Cell nuclei were stained with TO-PRO-3 iodide (blue). Nuclear p65 translocation results in the superimposition of the red and blue stains yielding a pink color. B and F, p65 nuclear staining and Syk staining were quantitated using DeVisu analysis of a minimum of 50 cells in each incubation condition. C and D, The ability of SC to stimulate MO TNF and IL-10 production in cells treated with the NF-κB inhibitor (50 μM NBD+) or its control (NBD−) are compared. G and H, The ability of SC to stimulate MO TNF and IL-10 production in cells treated with the Syk inhibitor (piceatannol, 25 μM; +) or its control (piceatannol; −) are compared. The noted values represent the mean ± SEM of four experiments performed in duplicate on MO from 12 mice. *p < 0.05 and **p < 0.001.
IC stimulation of TNF: role of Syk

Because dectin-1 can activate Syk (20), confocal microscopy and DeVico immunofluorescent quantification were used to evaluate the phosphorylation status of Syk in cell monolayers that had been incubated for 5 min with IC or vehicle alone. In WT cells, IC increased Syk phosphorylation by 200 ± 8% (p < 0.001). In accord with the biology of Syk (22, 23), the enhanced staining localized on cellular membranes and, to a lesser degree, cellular cytoplasm (Fig. 5, D and E). These Syk regulatory effects were TLR2 independent because similar levels of Syk phosphorylation were noted in IC-treated and vehicle-treated TLR2 null cells (Fig. 5, D and E). Further confirmation of Syk activation was obtained using the Syk inhibitor piceatannol (25 μM), which caused a modest decrease in IC-induced TNF production by WT cells (Fig. 5F). In accord with our studies with laminarin, these studies demonstrate that IC stimulates MO TNF production via a pathway(s) that is only modestly dependent on Syk activation.

SC stimulation of TNF and IL-10: roles of TLR2, dectin-1, and MR

Studies were next undertaken to define the roles of TLR2, dectin-1, and the MR in the MO stimulatory effects of SC. Experiments comparing WT and TLR2 null mice demonstrated that the ability of SC to stimulate TNF production was at least partially dependent on TLR2 (Fig. 6A). Treatment with laminarin also completely abrogated and anti-MR, cytochalasin D, and nocardazole partially abrogated (p < 0.001) the TNF stimulatory effects of SC on WT MO (Fig. 6A and data not shown). Interestingly, dectin-1 played an important role in both the TLR2-dependent and-independent TNF production induced by SC because laminarin completely inhibited SC-induced TNF production by TLR2 null cells (Fig. 6A). In contrast, anti-MR did not alter, while cytochalasin D and nocardazole slightly altered (p < 0.05) the TNF production by SC-stimulated TLR2 null cells (Fig. 6A and data not shown). In all cases, alterations in TNF protein production were associated with comparable alterations in mRNA accumulation (data not shown). These studies demonstrate that SC stimulates MO TNF production via dectin-1-dependent pathways that can be TLR2 dependent and independent and can involve the MR and phagocytosis.

Experiments comparing cells from WT and TLR2 null mice also demonstrated that the ability of SC to stimulate IL-10 production was at least partially dependent on TLR2 (Fig. 6B). Laminarin also decreased SC stimulation of IL-10 production (Fig. 6B; p < 0.05). In contrast, anti-MR increased the production of IL-10 by SC-stimulated MO (Fig. 6B). Cytochalasin D and nocardazole also increased the production of IL-10 by SC-stimulated MO and confocal and DIC microscopy demonstrated that SC was taken up into the MO cytoplasm after as little as 10 min of Texas-Red C3-di-chlorotriazine-stained SC cell incubation (Fig. 6, B and C, and data not shown). Interestingly, the inhibitory effects of laminarin were still seen while the stimulatory effects of anti-MR, cytochalasin D, and nocardazole on SC-stimulated IL-10 elaboration were significantly decreased in cells from TLR2 null mice (Fig. 6B and data not shown). In all cases, alterations in IL-10 protein production were associated with comparable alterations in mRNA accumulation (data not shown). In addition, similar results were obtained when the effects of chitin were evaluated in the presence or absence of anti-MR or exogenously administered mannose (data not shown). When viewed in combination, these studies demonstrate that SC regulates MO IL-10 production via a stimulatory pathway(s) that involves dectin-1 and TLR2 and is inhibited by anti-MR and chitin phagocytosis.

SC stimulation of TNF and IL-10: role of NF-κB and Syk

In keeping with the studies described above, the roles of NF-κB and Syk kinase in the stimulatory effects of SC were also evaluated. In WT cells treated with SC, the levels of cytoplasmic and nuclear p65 (Fig. 7, A and B) were 169 ± 4% and 202 ± 4%, respectively, of those in vehicle-treated WT control cells (p < 0.001; Fig. 7, A and B). These NF-κB regulatory effects were TLR2 dependent because the levels of nuclear and cytoplasmic p65 were lower in SC-stimulated cells from TLR2 null mice vs WT control mice (Fig. 7, A and B). In accord with these modest levels of NF-κB activation, the NF-κB-inhibiting NBD peptide decreased SC-stimulated TNF production by 35 ± 1% and 55 ± 3% in WT (p < 0.01) and TLR2 null cells (p < 0.05), respectively (Fig. 7C). NBD did not alter the ability of SC to stimulate MO IL-10 production by WT or TLR2 null MO (Fig. 7D).

In contrast to our findings with NF-κB, SC was a potent activator of Syk. This was readily seen in experiments with WT cells where SC increased Syk phosphorylation by 461 ± 2% (p < 0.001; Fig. 7, E and F). Interestingly, the Syk inhibitor piceatannol inhibited SC-induced TNF production by WT cells by 87 ± 34% (p < 0.001; Fig. 7G) and abrogated SC-induced IL-10 production by WT and TLR2 null cells (p < 0.001 for both; Fig. 7H). Overall, these studies demonstrate that SC stimulates MO TNF production via pathways that are at least partially dependent on Syk kinase activation and TLR2-dependent activation of NF-κB. In contrast, SC stimulates MO IL-10 production via pathways that are not dependent on NF-κB but are impressively dependent on Syk activation.

Discussion

These studies demonstrate, that the ubiquitous polysaccharide chitin contains PAMPs that allow it to interact with, and activate, MO TLR and non-TLR innate immunity receptors. They also highlight the complexity of these interactions by demonstrating that chitin mediates its effects in a size-dependent fashion with different sized chitin fragments utilizing different combinations of TLR2, dectin-1, and, to a lesser extent, MR and phagocytosis to activate distinct signaling pathways that differentially regulate the production of TNF and IL-10. In so doing, we have demonstrated that chitin stimulates TNF production via pathways that involve TLR2, dectin-1, and NF-κB activation. In contrast, chitin stimulated IL-10 production via dectin-1-dependent and TLR2-dependent and-independent pathways that involved MR and Syk.

A striking finding in our studies is the demonstration that the size of the chitin molecule is an important determinant of its effects on MO function. Specifically, BC and SSC were inert in our experimental system, IC and SC stimulated TNF elaboration, but
only SC stimulated the anti-inflammatory cytokine IL-10. Interestingly, the stimulatory effects of IC on TNF were entirely and largely dependent on TLR2 and NF-κB, respectively. In contrast, although TLR2, dectin-1, Syk, and NF-κB were involved in SC stimulation of TNF, the contributions of dectin-1 and Syk exceeded those of TLR2 and NF-κB. In accord with these findings, dectin-1 and Syk also played major and TLR2 and NF-κB played minor roles in SC stimulation of IL-10. These findings demonstrate that different sized chitin fragments interact with different innate immune receptors to activate different intracellular signaling pathways. This is analogous to the size-dependent regulatory effects that have been attributed to the endogenous TLR4 ligand hyaluronan (13). When viewed in combination, these findings suggest that different sized chitin fragments play different roles in antiparasite/pathogen responses. First, the demonstration that large molecular weight chitin is inert in our system suggests that BC-MO interactions do not initiate the antipathogen/parasite response. On the other hand, the demonstration that IC and SC stimulate TNF elaboration can be speculated to be a mechanism by which inflammation and destruction are induced and augmented to assist in the eradication of the invading pathogen. Lastly, if one assumes that SC are generated only after the pathogen has been severely injured or killed, the finding that only SC stimulates IL-10 can be speculated to be a mechanism by which the immune and inflammatory response is down-regulated when it is no longer needed and an ongoing immune and/or inflammatory response might increase local tissue injury. IL-10 can also contribute to the development of T regulatory cells which would also limit the local pathology (20, 24). The ability of IL-10 to control the elimination of the pathogen/parasite might also allow adaptive immunity to develop (20). Thus, the size-dependent effects of chitin may contribute to the coordination and control of local immune events to ensure that pathogen elimination occurs in the absence of excessive local tissue injury.

TLR2 and dectin-1 play important roles in innate immunity, which are due, at least in part, to the ligands that these receptors bind. This is particularly true for TLR2, which binds lipoproteins, β-glucans, peptidoglycans, and lipoteichoic acid (20). Dectin-1, on the other hand, is the first example of a C-type lectin receptor whose primary ligands are carbohydrates like fungal β-glucans (25). Studies of these receptors have demonstrated that TLR2 mediates many of its effects by forming dimmers with other TLRs such as TLR1 and TLR6 (25–27). They have also highlighted the importance of TLR2-dectin-1 interactions in many of these responses and the ability of dectin-1 to function in a TLR2-dependent and -independent manner and stimulate NF-κB- and Syk-dependent pathways (25). Our studies add to our understanding of TLR2 and dectin-1 by demonstrating, for the first time, that chitin fragments are ligands for these receptors. They also demonstrate that these receptors, alone and in combination, contribute to the ability of the ligands to activate the variety of pathways that stimulate the production of TNF and IL-10. Specifically, our studies demonstrate that IC stimulates TNF via a pathway that is completely TLR2 and partially dectin-1 dependent, while SC stimulates TNF via TLR2-dependent and -independent mechanisms that both require dectin-1. They also demonstrate that SC stimulates IL-10 production via a pathway that is both TLR2 and dectin-1 dependent and a pathway that is TLR2 independent and dectin-1 dependent. In all cases, the contributions of TLR2 activation were associated with and demonstrated to be mediated by proportionate alterations in the activation of NF-κB while the contributions of dectin-1 were associated with and mediated by Syk activation. Previous studies of the effects of chitin on MO cytokine elaboration implicated the MR and MR-dependent phagocytosis processes in the pathogenesis of these responses (9). Thus, studies were undertaken to define the roles of the MR with subsequent phagocytosis and its relationship to TLR2 and dectin-1 in our experimental system. In accord with the previous reports (9), we noted that SC stimulates MO TNF production via a partially MR/phagocytosis-dependent mechanism. In contrast, MR did not play a similar role in IC stimulation of TNF and triggered a pathway that SC used to inhibit IL-10 elaboration. When viewed in combination, these studies demonstrate that the MR and phagocytosis process play highly specific roles in the MO-regulating effects of chitin. Specifically, they demonstrate that the MR is a size-dependent coreceptor for dectin-1 that participates in the stimulation of TNF and inhibition of IL-10 by SC.

Although these studies focused on in vitro and in vivo MO responses, they have a number of implications for chronic inflammatory diseases like asthma. The present concept of disease pathogenesis suggests that Th2-dominated adaptive immune responses play a key role in asthma (28). Recent studies have also raised the possibility that a chitin/chitosan-induced type 2 innate immune response may also contribute to the genesis of specific forms of this disorder (29). This comes from studies that demonstrated that the intrapulmonary administration of chitin/chitosan-coated beads elicits a type 2 innate immune response with IL-4 expression and eosinophil and basophil accumulation. These studies also suggested that leukotrienes contribute to and that acidic mammalian chitinase can inhibit this tissue response (29). However, the size of the chitin(s) inducing these responses and the role of anti-inflammatory cytokines in these responses have not been evaluated. Our findings add to these studies and provide explanations for related findings in the literature. Specifically, they demonstrate that chitin is a PAMP with a size-dependent ability to induce pro- and anti-inflammatory responses via TLR2-dependent and non-TLR receptor systems. TLR2 activation has been shown to inhibit Th2 inflammation (30) and polymorphisms of TLR2 have been noted on blood cells from patients with altered asthma susceptibility (31, 32). Our studies raise the possibility that these complex responses can be explained by exposure to or the generation of different sized chitins. SC has also been shown to decrease Th2 inflammation (9, 33). It is tempting to speculate that these effects of SC and the ability of acidic mammalian chitinase to inhibit the type 2 innate response elicited by chitin-coated beads described above are due to the generation of SC-sized fragments and the ability of these polymers to induce IL-10 elaboration by MO and possibly other cells. Lastly, it is tempting to speculate that TLR2 and/or dectin-1 polymorphisms are seen in and contribute to the onset and/or natural history of type 2 responses in diseases like asthma and those in patients with altered susceptibility to chitin-containing pathogens. Additional study will be required to test these speculations. The mechanisms that chitin uses to regulate innate and adaptive immunity and the utility of therapies that alter these pathways in the treatment of asthma and other pathogen/parasite and chronic inflammatory diseases is worthy of additional investigation.

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References


