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Resolving the Mutually Exclusive Immune Responses of Chitosan with Nanomechanics and Immunological Assays

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Multifaceted functions displayed by both pro- and anti-inflammatory properties of chitosan hinder its effective development as an immunomodulatory agent. Herein, the contributions of the bending stiffness of chitosan with regard to its immune regulatory properties toward inflammation are investigated. The anti-inflammatory properties of chitosan molecular weight (MW) with a shorter (≈1 kDa) or longer (≈15 kDa) than the persistent length (L_p) are compared using immunological assays and nanomechanics-based experiments on the surface forces apparatus (SFA). Interestingly, 1 kDa chitosan significantly enhances the generation of anti-inflammatory regulatory T cells (Tregs) through the Dectin-1-dependent pattern recognition receptor (PRR) on antigen-presenting cells. SFA analyses also show a similar trend of interaction forces between chitosan and diverse PRRs depending on their MW. The results obtained in the immunological and nanomechanical experiments are consistent and imply that the binding features of PRRs vary depending on the MW of chitosan, which may alter immune activity. In accordance, in vivo administration of only 1 kDa represses inflammatory responses and suppresses the progression of experimental colitis. This study elucidates a previously unexplored bending stiffness-dependent immune regulatory property of chitosan and suggests the applicability of low MW (rod-like) chitosan as a pharmaceutical ingredient to treat diverse inflammatory disorders.

1. Introduction

Previous studies suggest that chitosan, chitin and their derivatives may be applicable as potential immune modulators. Interestingly, however, numerous studies published to date have reported that chitosan and chitin have both anti- and pro-inflammatory (immune-stimulatory) activities. The factors involved in these contradictory immunological activities of chitosan, chitin and their derivatives remain unclear. Chitosan and its derivatives are known to activate macrophages, dendritic cells (DCs) and granulocytes by inducing pro-inflammatory molecules and cytokines such as IFN-γ, TNF-α, and IL-1β.[1–3] In contrast, other reports showed that chitosan and its derivatives could suppress inflammation through secretion of the anti-inflammatory cytokines IL-10 and TGF-β.[4–6] Furthermore, chitosan activates the MyD88 (MAPK), Ap-1, and NF-κB pathways related to both pro- and anti-inflammatory responses by activating or attenuating these pathways.[1,6,7] The factors and underlying mechanism involved in the opposing functions of chitosan, chitin and their

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derivatives in immune responses remain unknown. There are several explanations for the conflicting immune activities of chitosan, such as various chitin origins, different degrees of acetylation, and tertiary structures depending on the manufacturing and purification process, and the size depending on the molecular weight.[8–12] Among the explanations, we hypothesize that these mutually exclusive immune responses of chitosan may be attributed to the size of these molecules, as immune cells recognize them through pattern recognition receptors (PRRs) on innate and adaptive immune cells.

Innate and adaptive immunity work together to respond to exposure to diverse antigens through the mucosal or systemic route. Innate immune cells respond immediately and stimulate adaptive immunity for prolonged immune responses. Therefore, for a comprehensive understanding of a material's immune activity, research covering both innate and adaptive immune responses is necessary. However, most immunological experiments with chitosan have focused on innate immune cells, such as macrophages, DCs, and granulocytes, and their secretion of cytokines, which are relatively transient events.[3,13] Furthermore, the long-term organismal contributions of innate immune cells to host physiology after exposure to chitosan, however, largely remain unknown. T cells are pivotal adaptive immune cells, and studies identifying immune modulatory effects on T cell differentiation and functions can provide a broad understanding of the interplay between innate and adaptive immunity contributing to prolonged immune responses. However, most studies investigating chitosan-mediated immune responses have thus far focused on innate immune cells only. Specifically, most pro-inflammatory studies of chitosan have been conducted on innate immune cells, and anti-inflammatory studies have also been conducted on innate immune cells and in experimental animal models of immune diseases (Table S1, Supporting Information). Although a handful of studies have addressed conventional T helper cell-mediated immunity, to our knowledge, none of the studies investigated any possible Treg-inducing properties of chitosan.[14]

Chitosan is naturally extracted from a variety of natural sources and undergoes different manufacturing processes, resulting in considerable differences in the composition of the final purified product, which may result in variability in its effects on measured immune activities across experiments. One possible reason for such variability in chitosan's effect on the immune system is proposed to be due to enzymatic cleavage of the glycosidic bond in chitosan.[10,15] For example, chitin- or chitosan-coated beads were directly administered to the lungs of mice, and innate effector cells, such as eosinophils and basophils, which induce allergies, accumulated. However, the presence of acidic mammalian chitinase (AMCase), an enzyme that digests chitin by cleaving its glycosidic bonds, prevented the accumulation of chitin-induced innate effector cells.[9] Another reason is attributed to the differences in MW and particle size of the molecules in aqueous solutions that are used for experiments. For example, the effect of chitin on macrophages is known to differ based on chitin particle size. Intermediate chitin fragments (40–70 μm) stimulate the secretion of pro-inflammatory mediators such as TNF-α and IL-17, but small chitin fragments (<40 μm) were found to induce both TNF-α and IL-10.[10,16] These results suggest a definitive correlation between the MW of chitosan particles and its outcome on mutually exclusive immune responses.

A key parameter most affected by the physical properties of the polymer according to the changes in the MW is the bending stiffness of the polymer, which significantly changes around the persistent length (L_p) as a reference point. When a polymer chain is shorter than the L_p of the polymer, the polymer behaves like a rigid rod. When the chain is longer than L_p, the polymer behaves like a flexible thread (very short hair feels stiff, but long hair feels less stiff, which is an example of the change in bending stiffness of hair with L_p as the reference point.). Indeed, the bioactivities, water solubility, and bending stiffness of chitosan show many differences based on the L_p of chitosan (≈9 nm, 3.5 kDa) as a reference point.[17,18] Therefore, a study requires in-depth multidisciplinary studies employing immunological as well as biophysical and mechanical approaches depending on the MW to understand the precise immune modulatory functions of chitosan in health and disease.

In this study, we investigated the probable effects of the molecular weight of chitosan on promoting anti-inflammatory responses by facilitating the induction of $CD4^+Foxp3^+$ regulatory T (Treg) cells, a key immune cell population required for establishing systemic and tissue-based immunological tolerance.[19] Herein, we treated naïve T cells with chitosans of different MWs and determined the PRRs on antigen-presenting cells that affect Treg differentiation. We also employed various knockout mouse strains to identify the origin of previously reported conflicting immune activities. Traditional studies showing the interaction between chitosan and PRRs have examined only immunological phenomena, but in this study, in addition to in vitro and in vivo immunological assays, we also carried out direct interaction force measurement assays between chitosans and PRRs using the surface forces apparatus (SFA). Interaction forces between chitosans and PRRs were successfully measured at the molecular level, and the findings were correlated between these interaction energies and immunological responses. Subsequently, functionally relevant chitosan of appropriate MWs was administered to an inflammatory bowel disease animal model to confirm the applicability of chitosans as immune modulating materials. The combination of in vitro, in vivo, and nanomechanical studies in this study provides a new perspective on the utility of size-based fractionation of chitosan as a therapeutic module against inflammatory diseases.

2. Results

2.1. Characterization of Chitosan

Chitosan used in immunological activity studies is mostly low molecular weight (LMW) chitosan (<15 kDa) (Table S1, Supporting Information). As stated in the Introduction, there could be differences in immune activities due to polymer rigidity according to MW. Therefore, ≈1 kDa (below the persistence length, rod-like chain) and ≈15 kDa (above the persistence length, thread-like chain) were selected with the persistence length of chitosan ($L_p \approx 9$ nm) as a reference point.[17] Both chitosans were characterized for their MW and distribution, charge density, water solubility, and endotoxin contamination (**Table 1**). Their MW and polydispersity index (Mw/Mn; PDI) were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and a multiple angle light scattering

Table 1. Characterization of 1 and 15 kDa chitosan.

Molecular weight	Degree of acetylation [%]	Degree of polymerization	M_n [g mol^{-1}]	M_w [g mol^{-1}]	PDI	Endotoxin [EU mL^{-1}]
1 kDa	2.6	≈6	942.89×10^3	1061.57	1.13	7.4 ± 0.23 (0.6[a])
15 kDa	15	≈111	15.94×10^3	17.23×10^3	1.21	0.14 ± 0.01

[a] Endotoxin level after endotoxin removal.

detector (SEC-MALS) (Table S2, Figures S2 and S3, Supporting Information). 1 kDa chitosan has a number average MW (Mn) of 942.89×10^3 g mol^{-1} and a weight average (Mw) of 1061.57 g mol^{-1}, so the PDI is 1.13. 15 kDa chitosan has the number average MW (Mn) 15.94×10^3 g mol^{-1}, and the weight average (Mw) 17.23×10^3 g mol^{-1}, so the PDI of 15 kDa is 1.21. The degree of chitosan acetylation (DA) was confirmed using ^{1}H-NMR. The level of DA was calculated to be 2.6% for 1 kDa chitosan and 15% for 15 kDa chitosan. The degree of chitosan polymerization was ≈6 (1 kDa chitosan) and ≈111 (15 kDa chitosan), so the single chitosan chain had ≈0.16 (1 kDa chitosan) and ≈16.78 (15 kDa) acetyl groups (Figure S1, Supporting Information). The water solubility of 15 kDa chitosan is 0.7 μg mL^{-1}, whereas that of 1 kDa chitosan is more than 1 g mL^{-1}. Endotoxin contamination was confirmed through the LAL test. The endotoxin level at 15 kDa was as low as 0.14 ± 0.01 EU mL^{-1}, while 1 kDa chitosan was measured as high as 7.4 ± 0.23 EU mL^{-1} (Table 1).

In consideration of the influences of the DA and endotoxin contamination on immune activities, 1 kDa chitosan was acetylated with acetic anhydride to make the acetyl group content 14.5% similar to 15 kDa (Figure S4a, Supporting Information). From the FTIR data, it was confirmed that the DA was increased because the acetyl groups were attached to the amine groups, not the hydroxyl groups, through the fact that the C–O (1235 cm^{-1}) and C═O vibration (1740 cm^{-1}) peaks had no change (Figure S4b, Supporting Information).[20] Using an endotoxin removal kit, the endotoxin level of 1 kDa chitosan was lowered to 0.6 EU mL^{-1}.

2.2. Naïve CD4$^+$ T Cell Differentiation with Chitosan of Different MWs

Most studies investigating chitosan-mediated immune responses have thus far focused on innate immune cells (Table S1, Supporting Information). Except for a few reports on T helper cells, such studies addressing the effects of chitin and chitosan on adaptive immunity are markedly limited. We addressed this question by testing the Treg-inducing properties of 1 and 15 kDa chitosans. Chitosans of both MWs at 500, 1000, and 2500 μg mL^{-1} were used to treat primary mouse DCs for 12 h. Then, mature DCs and naïve CD4$^+$ T cells were co-cultured under suboptimal Treg induction conditions for 3 d, and markers of Treg and T cell viability were analyzed by flow cytometry.

To verify the experimental conditions with TGF-β, naïve T cell differentiation was confirmed by treating 1000 to 3500 μg mL^{-1} 1 and 15 kDa chitosans in the absence of TGF-β. The cell viability of T cells treated with 1 kDa chitosan for the entire concentration range tested was maintained at 80%–90%, whereas among cells treated with 15 kDa chitosan, 50% of the live cells died while processing 2000 μg mL^{-1} chitosan, and 80% of cells died upon treatment with 3000 μg mL^{-1} chitosan. As expected, chitosan was unable to induce Treg cell differentiation in the absence of TGF-β, confirming that TGF-β is essential for inducing Treg cell differentiation from DCs treated with chitosan (Figure S8, Supporting Information).

Both the groups treated with 1 kDa and 15 kDa chitosan displayed cell viability similar to that of the control group (**Figure 1**a,b). Treg cell differentiation was analyzed by detecting the expression of Foxp3$^+$, an essential lineage determining factor of Treg cells, within the CD4$^+$ T cell compartment (CD4$^+$Foxp3$^+$ cells). The frequencies of CD4$^+$Foxp3$^+$ Treg cells among cells treated with either group of chitosans at 500 μg mL^{-1} were 1.61% (1 kDa chitosan) and 0.9% (15 kDa chitosan), which were the same values observed for the control group (1.05%). However, upon treatment with 1000 μg mL^{-1} chitosan, CD4$^+$Foxp3$^+$ T cells (9.03%) were slightly increased among cells treated with 1 kDa chitosan. At a 2500 μg mL^{-1} dose, the proportion of CD4$^+$Foxp3$^+$ T cells increased up to 59.8% among cells treated with 1 kDa chitosan, but among cells treated with 15 kDa chitosan, 2.9% were CD4$^+$Foxp3$^+$ T cells. The 1 kDa chitosan from 500 to 2500 μg mL^{-1} increased Treg cell differentiation by 37-fold, but 15 kDa chitosan at the above concentrations had no significant effect on Foxp3 expression levels (Figure 1c,d). The dose-dependency of the effect of 1 kDa chitosan on Treg cell differentiation was measured by increasing the treatment dose among eight groups from 1000 to 3500 μg mL^{-1}. Foxp3 expression was gradually increased with increasing chitosan doses up to 2000 μg mL^{-1}, at which point the same level of expression was observed (Figure S9, Supporting Information).

2.3. Immunological and Nanomechanical Experiments Showing the Interaction between Chitosan and Pattern Recognition Receptors

Unlike 15 kDa chitosan, 1 kDa chitosan was found to be capable of inducing Treg cells in a DC-dependent manner, suggesting a probable size-dependent molecular interaction between chitosans and PRRs on the DC surface. To test this possibility, we identified the PRRs that affect Treg cell differentiation in the presence of chitosans (immunological correlation) and investigated how the molecular interaction forces between chitosans and receptors varied depending on their MW (mechanical correlation). To examine the immunological correlation, DCs from Dectin-1 (encoded by *Clec7a*) knockout (KO) mice, an anti-mannose receptor antibody, and a MyD88 inhibitor that can block every Toll-like receptor (TLR) except TLR3, were used to block Dectin-1, mannose receptor (MR), and TLRs, respectively. Interestingly, we

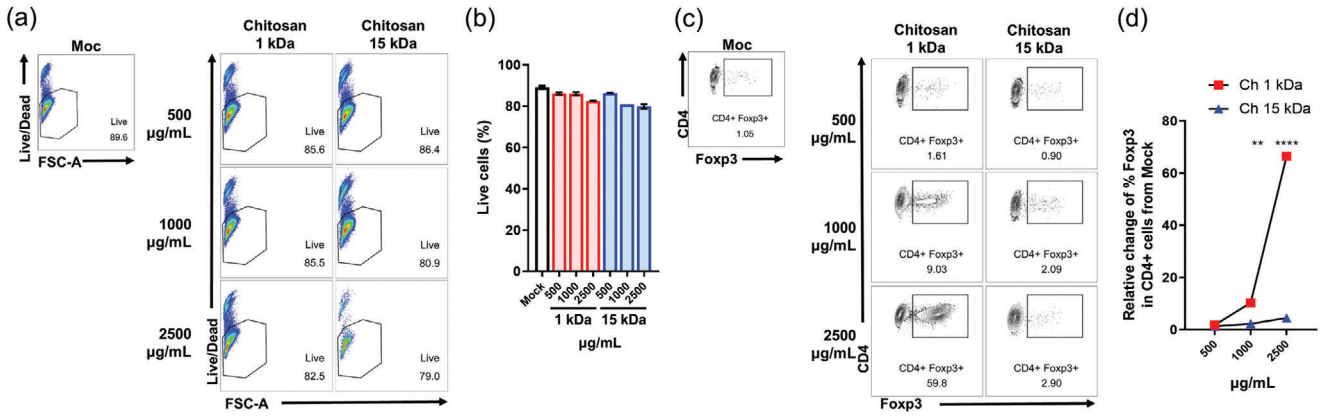

Figure 1. Comparison between Treg cell-inducing activity of different MW species of chitosan. Chitosans of the indicated molecular weight were co-cultured with CD11c$^+$ splenic DCs for 12–14 h, followed by co-culture with CD4$^+$ naïve T cells for 72 h. a) Flow cytometry plots showing cell viability. Dead cells were live/dead positive, and live cells were live/dead negative. b) The percentage of gated cells is shown as a bar graph. c) Contour plots of the flow cytometry data showing Foxp3 expression in total CD4$^+$ T cells. d) The relative change in the percentage of Foxp3$^+$ cells among mock cells is shown as a dot graph according to the concentrations. $^{**}p < 0.01$, $^{****}p < 0.0001$ (Student's *t*-test).

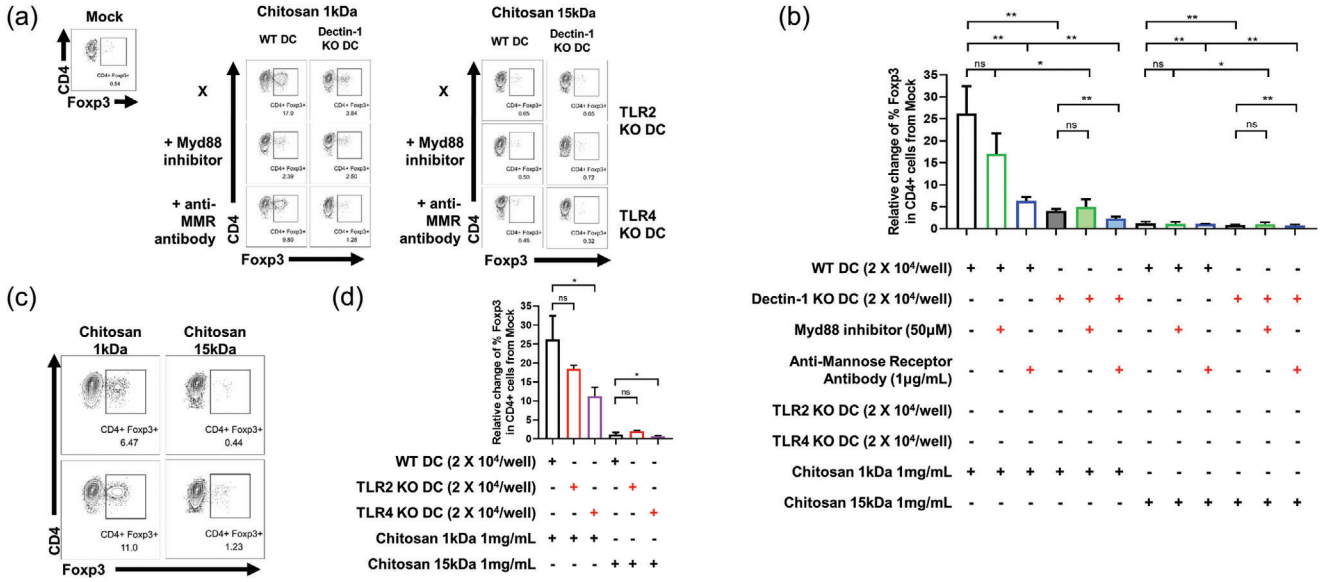

Figure 2. Comparison of Treg cell-inducing activities of two different MW varieties of chitosan with respect to various PRRs. Chitosans were co-cultured with WT, Dectin-1-KO, TLR2-KO, and TLR4-KO CD11c$^+$ splenic DCs for 12–14 h, followed by co-culture with naïve CD4$^+$ T cells for 72 h. Inhibitors or blocking antibodies were administered before co-culturing DCs with chitosans. a,c) Contour plots of flow cytometry showing Foxp3 expression in total CD4$^+$ T cells. b,d) The relative proportion of Foxp3$^+$ cells with respect to mock-treated cells is shown as a bar graph. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (Student's *t*-test).

observed that the Treg-inducing capability of Dectin-1-deficient DCs upon treatment with 1 kDa chitosan was reduced to almost sevenfold compared to that of WT DCs. On the other hand, treatment with anti-mannose receptor antibody and MyD88 inhibitor decreased Treg induction by 4-fold and 1.5-fold, respectively. Next, an anti-MR antibody and MyD88 inhibitor were used to treat DCs from Dectin-1-KO mice to examine the synergistic activity of the two receptors. When both Dectin-1 and MR were blocked, the reduction in Treg induction was more pronounced compared to conditions where only Dectin-1 was blocked. On the other hand, when MyD88 was blocked on Dectin-1-deficient DCs, the treatment did not demonstrate any further decrease in Treg induction (**Figure 2**a,b). As MyD88 blockade affected Treg induction upon 1 kDa chitosan treatment, we next examined the role of TLR2 and TLR4 in 1 kDa chitosan-mediated Treg induction, as these receptors are upstream of MyD88 and are known to bind chitosan.[10,21,22] We observed that Treg induction was 2.6-fold lower upon TLR4 blockade and 1.5-fold lower upon TLR2 blockade than upon treatment of wild-type mice with 1 kDa chitosan (Figure 2c,d). Taken together, when 1 kDa chitosan was used to treat the mice, dectin-1 was dominantly involved, but TLRs were marginally or not involved in Treg induction. The expression levels of molecules involved in Treg differentiation induction were confirmed to be decreased in the following order:

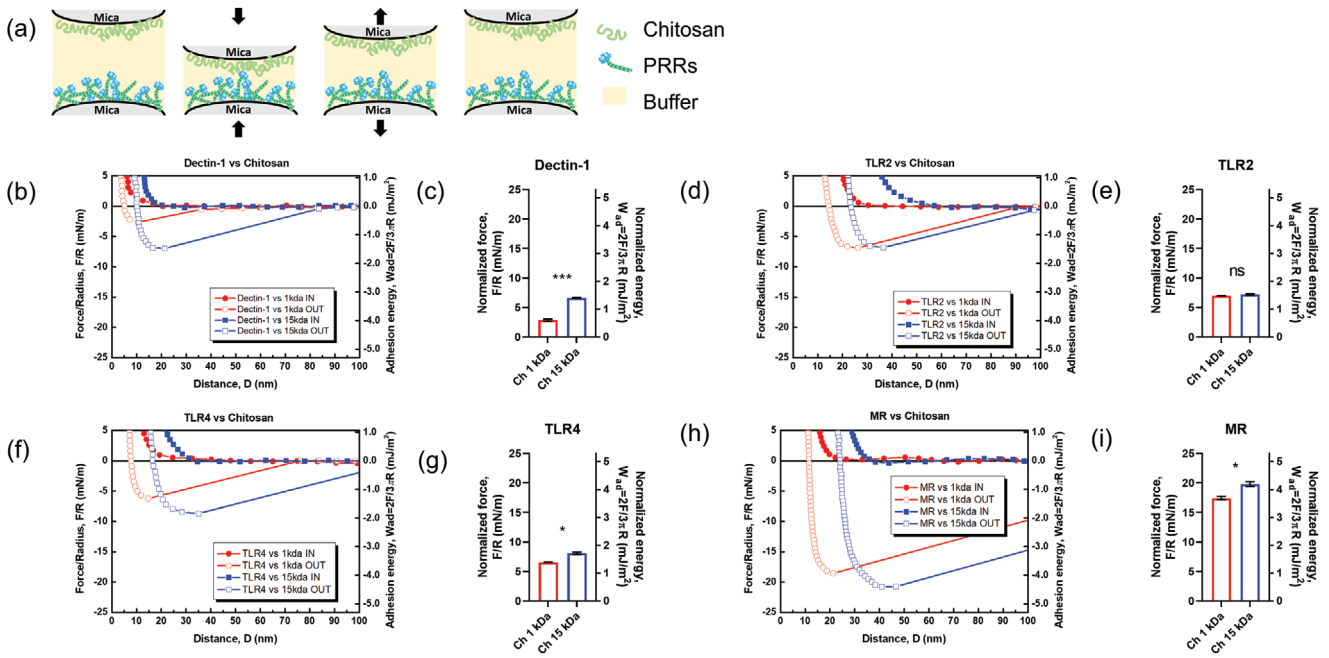

Figure 3. SFA analysis of chitosan and PRRs. a) Schematic illustration of the SFA analysis using chitosan and PRRs. Force–distance curves for b) dectin-1 versus chitosan, d) TLR2 versus chitosan, f) TLR4 versus chitosan, and h) mannose receptor versus chitosan. Normalized interaction forces and energies as c) dectin-1 versus chitosan, e) TLR2 versus chitosan, g) TLR4 versus chitosan, and i) mannose receptor versus chitosan. Red graph; 1 kDa chitosan, blue graph; 15 kDa chitosan. Filled circle; 1 kDa chitosan move in curve, empty circle; 1 kDa chitosan move out curve, filled square; 15 kDa chitosan move in curve, empty square; 15 kDa chitosan move out curve. $*p < 0.05$, $***p < 0.001$, ns: not significant. (Student's *t*-test).

Dectin-1, MR, TLR4, and TLR2. In stark contrast to the 1 kDa chitosan-treated groups, treatment with 15 kDa chitosan had no effect in any of the experimental conditions tested (Figure 2).

To gain an understanding of the mechanical binding between chitosan and PRRs, the interaction forces between PRRs and chitosan were measured by a SFA, which allows quantification of interaction energies between biological molecules. The SFA technique has been used to measure normal/frictional forces between two opposing surfaces in aqueous media, e.g., electrostatic forces, hydrogen bonding, hydrophobic interactions, cation–π interactions, van der Waals forces, and specific biological interactions, including sugar–lectin interactions.[23,24] The interaction force when two surfaces coated with chitosans (sugar) and PRRs (lectins) were approached and receded was measured using SFA to quantify the interaction energy between chitosan and PRRs. Dectin-1, TLR2, TLR4, and MR were chosen as the PRRs. The interaction forces were measured with a contact time of 10 min, and the interactions of 1 and 15 kDa chitosans with individual receptors were compared in buffer (20×10^{-3} M HEPES, 50×10^{-3} M $NaNO_3$, 3×10^{-3} M $Ca(NO_3)^2$ pH 7.4). The interactions of Dectin-1 and 1 and 15 kDa chitosans were $W_{ad} \approx 0.605$ and 1.408 mJ m^{-2}, respectively (**Figure 3**b,c). The interactions of TLR2 with 1 and 15 kDa chitosans were W_{ad} 1.487 and ≈1.531 mJ m^{-2}, respectively (Figure 3d,e). The values of TLR4 with 1 and 15 kDa chitosans were $W_{ad} \approx 1.396$ and 1.1714 ± 0.002 mJ m^{-2}, respectively (Figure 3f,g). The W_{ad} for interaction of the MR with 1 kDa chitosan was $\approx 3.698 \pm 0.012$ mJ m^{-2}, while that with 15 kDa chitosan was 4.202 ± 0.018 mJ m^{-2} (Figure 3h,i). To confirm that the interaction energies changed depending on the molecular weight of chitosan, the differences in interaction energies for each receptor and 1 or 15 kDa chitosan were calculated. The difference in the interaction energies between Dectin-1 and each chitosan was greatest at ≈0.803 mJ m^{-2}, while that between TLR2 and each chitosan was 0.059 mJ m^{-2}, which was the lowest difference between the interaction energies with two chitosans at the two MWs. The differences in interaction energies for TLR4 and MR were ≈0.318 and $\approx 0.504 \pm 0.001$ mJ m^{-2}, respectively. These results confirmed that the difference in interaction force with chitosan of different MWs decreased in the following order: Dectin-1, MR, TLR4, and TLR2.

2.4. Anti-Inflammatory Effect of Chitosan in a DSS-Induced Colitis Animal Model

Our data, based on immunological and SFA-based experiments, strongly demonstrated that LMW chitosan has anti-inflammatory effects on the immune system. To further show the potential role of LMW chitosan as an immune suppressor, in vivo experiments were conducted to confirm that chronic inflammatory diseases could be prevented by specifically applying 1 kDa chitosans to a DSS-induced colitis model. Chitosan with an MW of 1 or 15 kDa was administered to mice by daily feeding, and 1% DSS was administered from the seventh day of chitosan administration to induce experimental colitis. Weight loss and colon length were measured, and the frequencies of the Treg population and secreted cytokines in the intestine were analyzed by flow cytometry after sacrifice of the mice at the end of the experiment.

In the group treated with 1% DSS, the body weight of the mice decreased from the eighth day, and on the 13th day, the body

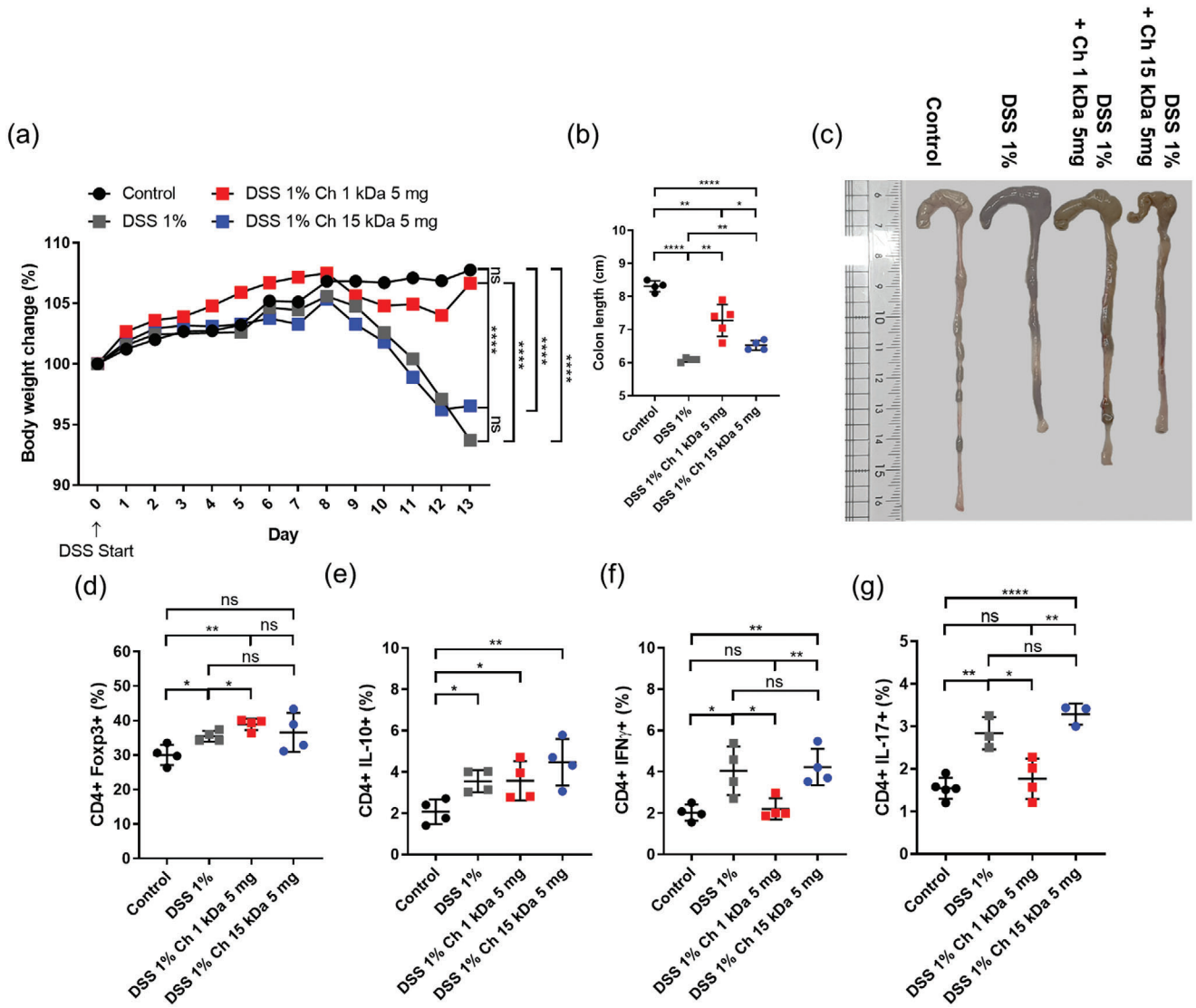

Figure 4. Comparison between the anti-inflammatory activities of 1 and 15 kDa chitosan on DSS-induced colitis progression. a) Changes in body weight of mice induced with colitis with 1% DSS. b) Colon length at the endpoint of the experiment. c) Representative organs (cecum to colon) of animals in each group. d–g) Analyses of Foxp3$^+$ Treg populations and cytokine-producing cells (IL-10, IFN- γ, and IL-17) from colon LP CD4$^+$ T cells. Graphs with error bars show the means ± SEMs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns: not significant. (Student's *t*-test).

weight was 10% less than that of the control group and decreased to less than 95% of the body weight compared to the first DSS feeding day. In the groups where 1% DSS feeding was supplemented with 5 mg of 1 or 15 kDa chitosans, there was no significant difference in weight compared to that of the control group treated with 1 kDa chitosan, and weight loss was clearly reduced compared to that of the group treated with only 1% DSS. On the other hand, 15 kDa chitosan administration was not found to be prophylactically beneficial (**Figure 4**a). In concert with these results, while treatment with 1 kDa chitosan prevented typical DSS-mediated shortening of the colon, no such effect was observed in the 15 kDa chitosan-treated animals (Figure 4b,c).

The effect of the above treatments on the Treg population and cytokine secretion in the colonic lamina propria (LP) was also examined. Treg cells were found to be significantly increased in the group administered 1 kDa chitosan compared to the control group and the group administered 1% DSS. Furthermore, 1 kDa chitosan treatment specifically resulted in an efficient reduction in the pro-inflammatory cytokines IFN-γ and IL-17. The level of IL-10, a major anti-inflammatory cytokine, however, remained unaffected (Figure 4d–g). These findings confirm that 1 kDa chitosan prevented DSS-induced colitis, but 15 kDa chitosan did not.

3. Discussion

It is important to know which type of chitosan has appropriate immune activity for its specific applicability as an immune modulator. Immune modulators in autoimmune diseases should mitigate the inflammatory response without immunodeficiency, which can cause infection.[25] The biological properties of

chitosan vary depending on its MW.[8] In this study, the immune activity was evaluated using 1 kDa shorter than L_p of chitosan (L_p ≈ 9 nm, 3.5 kDa) or 15 kDa longer than L_p where the polymer rigidity and water solubility of chitosan are being to change.[26] Experiments were conducted to determine whether 1 and 15 kDa chitosan have anti-inflammatory activity in terms of adaptive immune cells. The effects of chitin and chitosan on the expression or activation of innate immune cells have already been studied extensively, yet those of adaptive immune cells have hardly been explored. As studies that confirmed immune activity in T cells were sufficiently informed about the immune activity of substances, we focused on adaptive immune cells without innate immune cell.[27] Naïve T cells can differentiate into different types of T cells and for in vitro and in vivo T cell induction and for that, T cell receptor (TCR) signaling, costimulatory molecules, and cytokines are essential. Chitosan plays a key role in regulating costimulatory molecules and cytokines through DC activation. We focused on the anti-inflammatory activity of chitosan, in this case adding TGF-β, known as an essential cytokine for Treg cell differentiation. The transcription factor Foxp3, the master regulator of Treg cells, was detected to determine the anti-inflammatory activity of adaptive immune cells.

As this study examines the difference in immune activity according to the molecular weight of chitosan, we purified chitosan before the experiment and performed a control experiment regarding DA (Figure S4, Supporting Information). When the DA of 1 kDa chitosan was acetylated to a level similar to that of 15 kDa and endotoxin was removed, there was no significant difference from the expression pattern of Foxp3$^+$ of untreated 1 kDa chitosan. It appears that DA and endotoxin do not significantly participate in Treg differentiation (Figure S5, Supporting Information). Then, we compared Treg differentiation in 1 kDa chitosan and 15 kDa chitosan. An increase in Foxp3 expression indicated an increase in Treg cell differentiation when 1 kDa chitosan was applied. In contrast, 15 kDa chitosan had no effect on inducing Treg cell differentiation. (Figure 1). In the case of 1 kDa chitosan, it showed an anti-inflammatory response by inducing Treg cell differentiation, whereas 15 kDa chitosan did not. As this difference in immune activity may be due to solubility in physiological conditions, 15 kDa chitosan DA was acetylated to 41.9 to make it soluble in physiological conditions, and then Treg differentiation induction was examined (Figure S7, Supporting Information).[36] Interestingly, acetylated soluble 15 kDa chitosan cannot induce Foxp3 expression in all concentration groups as like insoluble 15 kDa chitosan (DA 15).

The interaction of chitosans with PRRs is hypothesized to change according to the MW of chitosan, and this pattern may cause various immune activities of chitosans. Chitin, an acetylated form of chitosan, binds with proteins for various purposes in several species.[28,29] In particular, it is a well-known pattern-associated molecular pattern (PAMP) and component of the fungal cell wall in fungi, and the binding of PAMPs to PRRs on immune cells is a critical step in initiating immune signaling pathways.[30,31] Chitin and chitosan have been studied with Toll-like receptors (TLR2, TLR4) and C-type lectin (Dectin-1, mannose receptor), receptors that may influence immune responses.[16,21,29,31] These receptors also depend on the size of chitin and whether the immune response is pro- or anti-inflammatory. Alternatively, the immune response of the same receptor may vary depending on chitin size.[10,22] Likewise, most studies related to this issue have been conducted to identify cytokine secretion after PRRs on the surface of macrophages or DCs. Herein, Treg cell differentiation upon treatment with 1 and 15 kDa chitosans was examined when PRRs were inhibited. After 1 kDa chitosan treatment, Treg cell differentiation was induced through the Dectin-1- and MR-dependent pathways, of which the Dectin-1 pathway was dominant but the TLR pathway was marginal or independent. Then, Treg cell differentiation induction increased in the following order: dectin-1, MR, TLR4, and TLR2. When both Dectin-1 and MR receptors were blocked, minimum Treg differentiation was observed, confirming that several receptors act together. In the case of 15 kDa chitosan, it showed no specific immune activity because there was no increase or decrease in Treg cell differentiation when the receptors were blocked (Figure 2).

Additionally, measurement of the molecular-level interaction between chitosan and PRRs using SFA and immune experiments were conducted to provide a new perspective and an in-depth understanding of the various immune activities of chitosan dependent on MW. Research to measure the interaction energies between PRRs and ligands using SFA and the correlation of the measured forces based on immune activity has not yet been carried out.[23,32] The differences in interaction force between the binding of 1 and 15 kDa chitosans to the same receptor followed the order Dectin-1 > MR > TLR4 > TLR2. In the case of TLR2, there was no difference in interaction energies between its binding to 1 and 15 kDa chitosan (Figure 3). The above SFA results are consistent with the PRRs inhibition tendency data by using immune assays, which showed that the receptors dominated Treg cell differentiation with the application of 1 kDa chitosan (in the order dectin-1 > MR > TLR4 > TLR2).

Immune activity according to the size of ligand binding to PRRs has rarely been conducted, but there are studies about TLR2 and dectin-1 related to ligand size. When the size of chitin was less than 5-mers, it was able to bind to TLR2 but did not secrete IL-6. However, when it was longer than 6-mer, IL-6 was secreted.[22] In the case of dectin-1, when the size of β-1,3-glucan is 6-mer, it binds weakly or absents with dectin-1, but when it is longer than 25-mer, it has strong binding.[33] It seems that each receptor requires a certain level of binding strength with ligands, which may be due to bending stiffness depending on the molecular weight of ligands. The results obtained in the immunological and nanomechanical experiments are consistent and imply that the binding features of PRRs vary depending on the MW of chitosan, which may alter immune activity. In addition, while the above studies showed only the presence and strength of the binding force between the ligand and receptor, this study is meaningful in that it actually measures the molecular binding force and numerically presents the interaction force.

The DSS-induced colitis mouse model was selected as an inflammatory disease model to verify the preventative effect of the anti-inflammatory function of chitosan in vivo. In studies where chitosan was administered to a DSS-induced colitis mouse model, the severity of symptoms, cytokine secretion, and related pathways were identified, but the impact of adaptive immunity was not analyzed; for instance, the number of Treg cells in the gut and the level of cytokines secreted from T cells were not examined.[4,12] Chitosan with an MW of 1 kDa was

orally administered to a DSS-induced colitis model to confirm its effectiveness in preventing inflammatory diseases. Intestinal Treg cells were increased, pro-inflammatory cytokine secretion was decreased, and anti-inflammatory cytokine secretion was increased. Furthermore, the typical symptoms of colitis were prevented by the anti-inflammatory responses induced by 1 kDa chitosan. However, 15 kDa chitosan did not induce an anti-inflammatory response or relieve colitis symptoms (Figure 4).

In summary, 1 and 15 kDa chitosan were confirmed to have different immune activities, and the reason for the difference was studied in terms of the interaction of these chitosans with PRRs. In vitro and in vivo, 1 kDa chitosan had an anti-inflammatory effect, but 15 kDa chitosan did not induce specific immune activity. PRRs showed a large difference in interaction energies according to the chitosan MW in the following order (Dectin-1 > MR > TLR4 > TLR2); these PRRs were dominantly involved in Treg cell differentiation in the presence of 1 kDa chitosan. The binding of each PRR varied according to the MW, which may impact immune activity.

4. Conclusion

Many studies on the application of chitosan as an immune modulatory component in food, drugs or biomedicines have been conducted, but conflicting experimental results have been reported due to the lack of a well-controlled and holistic study. The rod-like LMW chitosan used in this study (≈1 kDa) was thoroughly characterized and confirmed to have an anti-inflammatory response in vitro and in vivo. Immunosuppressive responses were observed with only a water-soluble LMW variety of chitosan, and the origin of this immunosuppression was shown to be correlated with the extent of interaction of chitosan with PRRs. In addition, nanomechanical measurements between the chitosan and PRRs obtained using the SFA technique were consistent with immunological studies. This suggests a new perspective in selecting chitosan that considers appropriate immune activity according to the application purpose. In the case of immunosuppressant drugs, the immune system weakens the destruction of immune cells, which increases the risk of infection. Chitosan at 1 kDa, which had an anti-inflammatory effect, did not affect cell viability even when the dose was increased. Our study is the first to demonstrate that water-soluble 1 kDa chitosan may be applicable as an immune modulator that suppresses the inflammatory response without degrading the immune system in various biomedical and pharmaceutical fields.

5. Experimental Section

Mice: All mice were supplied and managed in the Specific Pathogen-Free facility of POSTECH Biotech Center. All the experimental procedures and numbers of mice were approved by the POSTECH Institutional Animal Care and Use Committee. (Approval No. POSTECH-2020-0021)

Primary Cell Preparation and Culture: Total splenocytes were collected from the spleens of C57BL/6 mice. The spleen was mechanically dispersed into single cell suspensions with a 100 µm cell strainer. Red blood cells (RBCs) in the spleen were removed by RBC lysis buffer (Biolegend, San Diego, CA). $CD11c^+$ splenic dendritic cells (spDCs) from C57BL/6, Dectin-1 KO, TLR2 KO, and TLR4 KO mice were obtained from total splenocytes with anti-CD11c magnetic beads (Miltenyi Biotec, Auburn, CA) with a magnetic column according to the manufacturer's manual. $CD4^+CD25^-CD44^-$ naïve T cells from C57BL/6 mice were obtained using the negative isolation method. After a single-cell suspension of the peripheral lymph nodes (pLNs) or spleen was obtained, biotinylated antibodies for surface markers of diverse cell types except CD4 (CD8a, CD11b, CD11c, CD19, CD24, CD25, CD44, CD49b, B220, TER119, TCRγ/δ, Biolegend, San Diego, CA) were added. Streptavidin magnetic beads and an EasySep magnet were used to remove other cells and obtain $CD4^+$ naïve T cells by the manufacturer's manual (Stem Cell Technologies, Vancouver, Canada). These cells were cultured in T cell media, which consisted of RPMI medium (10×10^{-3} M HEPEs, 10% fetal bovine serum, 3×10^{-3} M L-glutamine, 100 U mL^{-1} streptomycin, 100 U mL^{-1} penicillin, nonessential amino acids, sodium pyruvate, 50×10^{-6} M 2-β-mercaptomethanol).

DC and T Cell Co-culture for Foxp3$^+$ Treg Induction: $CD11c^+$ splenic DCs (2×10^4 cells) were co-cultured with chitosan in 96-well cell culture plates for 12–14 h. GM-CSF (10 ng mL^{-1}, Peprotech, Cranbury, NJ) was added for stimulation. Chitosan was washed out by centrifugation, and the remaining DCs were co-cultured with $CD4^+$ $CD25^-$ $CD44^-$ naïve T cells (2×10^5 cells) for 72 h. IL-2 (100 U mL^{-1}, Frederick National Laboratory for Cancer Research, Frederick, MD), anti-CD3 antibody (0.1 µg mL^{-1}, BioXcell, Lebanon, PA), and TGFβ (0.1 ng mL^{-1}, Miltenyi Biotec, Auburn, CA) were added for regulatory T cell induction. Anti-MMR antibody (1 µg mL^{-1}, Bio-Rad, Hercules, CA) and Myd88 inhibitors (50×10^{-6} M, Invivogen, San Diego, CA) were added and incubated for 2 h before starting co-culture with chitosan. After completion of co-culture, the cells were harvested for flow cytometry.

Flow Cytometry: To distinguish live cells, cultured total cells were stained with fixable viability dye (FVD, Thermo, Waltham, MA) for 30 min at 4 °C. To detect surface markers, the cells were stained with a mixture of suitable antibodies for 20 min at 4 °C or room temperature. Anti-CD4 was used for $CD4^+$ T cells (clone RM4-5, Biolegend). To detect intracellular transcription factors, cells were fixed after staining for surface markers by the fixation/permeabilization method (Thermo) for 2 h at 4 °C. The cells were stained with anti-Foxp3 (clone FJK-16s, Thermo) for 30 min at 4 °C. Cells were analyzed with an LSRfortessa or CantoII flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ). Data were evaluated with FlowJo (BD). To detect intracellular cytokines in colon T cells, cells were stimulated with PMA/ionomycin for 4–6 h. Golgistop (BD) was added to restrict the secretion of cytokines from the cells during stimulation. The staining of dead cells and surface molecules was performed as described above. Cells were fixed and permeabilized with the same method and stained with anti-IFN-gamma (clone XMG1.2, Thermo), anti-IL-10 (clone JES5-16E3, Biolegend), and anti-IL-17 (TC11-18H10.1, Biolegend) antibodies.

Chitosan Acetylation and Endotoxin Removal: 1 kDa chitosan was dissolved in 2% acetic acid and diluted with methanol at a 1:2 volume ratio. Acetic anhydride was added by calculating the equivalent ratio (0.2 eq) for 5 h, dialysis and freeze drying. Methanolic KOH (0.5×10^{-3} M) was added to dried chitosan, stirred for 5 h, and centrifuged at 7000×*g* with methanol twice. Precipitates were collected and dialysis was performed for 2 d. Acetylated chitosan was lyophilized and used in the experiment. Agarose resin with polymyxin B sulfate was used for endotoxin removal, and the manufacturer's manual was followed for removal (ToxinClean Endotoxin Removal kit, bioneer, Daejeon, Republic of Korea).

Chitosan Characterization: A 500 MHz proton nuclear magnetic resonance spectrometer (Avance III Ascend 500, Bruker, Germany) was used to measure the DA of chitosan. 1 kDa (Kitto Life Co., Pyeongtaek, Republic of Korea) and 15 kDa (Polysciences Inc., Warrington, PA) chitosan (5 mg) were dissolved in DCl/D_2O (1 mL, 1% v/v) and transferred to NMR tubes. ^1H NMR spectra were acquired with 64 transients. The calculation of the chitosan DA was performed from the area of the δ (ppm) signals in the N-acetyl proton peak and from the integration of the GlcN H2 and H2-H6 peaks in the ^1H NMR [Equation (1)][34]

$$\%\ DA = \left(\frac{2 \times A_{CH3}}{A_{H2-H6}}\right) \times 100 \quad (1)$$

Chitosan molecular weight and PDI were measured by the MALDI-TOF MS and SEC-MALS system. MALDI-TOF MS (Autoflex speed LRF,

Bruker, Billerica, MA) was used to 1 kDa chitosan. A 1 kDa chitosan solution (50% methanol) and a matrix of 2,5-dihydroxybenzoic acid solution (30% ethanol) were mixed at a ratio of 1:1. Laser desorption ionization mass spectrometry (LDI-MS) was obtained with a 355 nm Smartbeam-11 laser. The power of the laser was set slightly above the ionization threshold to acquire good resolution and signal-to-noise ratios, and the source accelerating potential was managed at +20 kV. MALDI-TOF MS: m/z DP 3–5 $[M+Na]^+$ calculated as $n \times 161.16$ (C6H11O4N) + 1.00 (H) + 17.00 (OH) + 22.99 (Na) 524.48, 684.64, 846.8, measured 524.58, 686.03, 246.93. DP 5–11 $[M\text{-}H_2O+Na]^+$ calculated for 828.8, 989.96, 1151.12, 1312.28, 1473.44, 1634.6, 1795.76, measured 828.49, 989.94, 1150, 1311.45, 1472.91, 1634.36, 1795.81. 15 kDa chitosan molecular weight and PDI were measured by the SEC-MALS system. A size-exclusion chromatography column (TSK-gel-GMPWXL, Tosoh Corporation, Tokyo, Japan) was connected to a MALS detector (DAWN HELEOS II, Wyatt Technologies, CA, USA) and a differential refractive index detector (Optilab T-rEX, Wyatt Technologies). A 15 kDa chitosan solution (5 mg mL^{-1} in 100 $\times 10^{-3}$ M acetic acid, 150 $\times 10^{-3}$ M $NaNO_3$) was injected (100 µL). The column was pre-equilibrated with buffer (100 $\times 10^{-3}$ M acetic acid, 150 $\times 10^{-3}$ $NaNO_3$) at a flow rate of 0.5 mL min^{-1}. Weight-averaged molar masses were calculated using ASTRA software (V6, Wyatt Technology).

Aacetylation of chitosan was characterized by FTIR spectroscopy obtained using a Fourier transform infrared (FTIR) spectrometer (Nicolet iS50, Thermo Scientific, USA), with a resolution of 4 cm^{-1} and an accumulation of 32 scans between 4000 and 400 cm^{-1} in ATR mode. The characteristic peaks at 1646, 1584, and 1370 cm^{-1} correspond to the amide I and amide II, and stretching vibration of $-CH_3$. Absorption peak of C–O (1235 cm^{-1}) and C=O vibration (1740 cm^{-1}) are not shown. Endotoxins of chitosan and endotoxin-removed chitosan were detected by the manufacturer's manual (PYROGEN Plus Gel Clot LAL Assay, Lonza, Basel, Switzerland).

Chitosan–PRRs Interaction Force Measurement: The nanomechanical properties of chitosan and PRRs were measured in aqueous solutions with a SFA 2000 (SurForce LLC, Goleta, CA) system. Two cylindrical glass disks were glued with backside silver-coated muscovite mica (Grade #1, S&J Trading, Floral Park, NY) through epoxy glue (EPON 1004 F, Exxon Chemicals, Irving, TX). Chitosan solution (70 µL, 10 $\times 10^{-6}$ M in 150 $\times 10^{-3}$ M acetic acid, pH 3.0) and PRR solution (70 µL, 0.5 $\times 10^{-6}$ M in 20 $\times 10^{-3}$ M HEPES, 3 $\times 10^{-3}$ M $Ca(NO_3)^2$, 50 $\times 10^{-3}$ M $NaNO_3$, pH 7.4) were dropped on the surface of each mica and incubated for 1 h in a chamber saturated with deionized water vapor. The surfaces were then thoroughly rinsed with 150 $\times 10^{-3}$ M acetic acid and DW to remove unbound chitosan and PRRs.[23,35] The two disks were loaded onto the SFA chamber in a crossed-cylinder geometry. A buffer droplet (50 µL, 20 $\times 10^{-3}$ M HEPES, 3 $\times 10^{-3}$ M $Ca(NO_3)^2$, 50 $\times 10^{-3}$ M $NaNO_3$ at pH 7.4) was injected into the two facing mica surfaces. The measured force, *F*, is correlated to the interaction energy per unit area *W* of two planar surfaces using the Derjaguin approximation [Equation (2)]

$$F = 1.5\pi RW \quad (2)$$

The interactions between two surfaces were measured at intervals of ≈2 s, 10 min, and 60 min at RT.

DSS-Induced Colitis Mouse Model Studies: Mice were fed chitosan by oral administration 7 d prior to the start of colitis induction. One percent DSS in drinking water was supplied for a week, and weight change compared to the original weight at Day 0 (first day of chitosan feeding) was measured. The 1% DSS drinking water was changed every other day. When the average weight loss reached 90% of the initial weight, the mice were sacrificed, and their colons were isolated. Colon length was measured before the isolation of colon LP T cells. To isolate T cells in the colon LP, fat and Peyer's patches were removed from the colon, and the lumen was opened. Tissues were incubated in buffer containing 10 $\times 10^{-3}$ M EDTA for 20 min with shaking to remove epithelial cells. The supernatant was removed with mesh, and the remaining tissues were washed with PBS twice and digested by collagenase (Roche) and DNase (Roche) for 45 min with shaking. Tissues were removed by mesh, and the supernatant was centrifuged to obtain the cells as pellets. (1500 rpm, 5 min, 4 °C) The pellets were suspended in double layers of 40% and 75% Percoll (GE Healthcare) and centrifuged (2000 rpm, 20 min, RT without deceleration). Cells between two layers were harvested and used for flow cytometry analysis.

Statistical Analysis: All statistical analyses were performed with GraphPad Prism software (San Diego, CA).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

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