

Chitin particles induce size-dependent but carbohydrate-independent innate eosinophilia

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ABSTRACT

Murine M ϕ that phagocytose CMP develop into M1; this response depends on the size and the chemical composition of the particles. In contrast, recent studies concluded that chitin particles induce M2 and eosinophil migration, promoting acquired Th2 immune responses against chitin-containing microbes or allergens. This study examined whether these apparently inconsistent responses to chitin could be induced by variation in the size and chemical composition of the chitin particles. We compared the responses of M ϕ with CMP, LCB, and Sephadex G-100 beads (>40 μ m). Beads were given i.p. to WT mice and to mice deficient in a CRTH2, a receptor for the eosinophil chemoattractant PGD₂. In contrast to the M1 activation induced by CMP, i.p. administration of LCB or Sephadex beads induced within 24 h a CRTH2-dependent peritoneal eosinophilia, as well as CRTH2-independent activation of peritoneal M ϕ that expressed Arg I, an M2 phenotype. LCB-induced M ϕ exhibited elevated Arg I and a surface MR, reduced surface TLR2 levels, and no change in the levels of CHI3L1 or IL-10 production. Our results indicate that the effects of chitin in vivo are highly dependent on particle size and that large, nonphagocytosable beads, independent of their chemical composition, induce innate eosinophilia and activate M ϕ expressing several M2, but not M1, phenotypes. *J. Leukoc. Biol.* 90: 000–000; 2011.

Abbreviations: Arg I=arginase I, CHI3L1=chitinase 3-like 1, chitosan-MP=chitosan-microparticle(s), CMP=chitin microparticles at 1–10 μ m, CRTH2=chemoattractant receptor-homologous molecule expressed on Th2 cells, DP=D-prostanoid receptor, FSC=forward-scatter, HK-BCG=heat-killed *Mycobacterium bovis* bacillus Calmette-Guerin, LCB=large chitin beads at >50 μ m, LTB₄=leukotriene B₄, M ϕ =macrophage(s), M1=classically activated macrophage, M2=alternatively activated macrophage, MFI=mean fluorescent intensity, MR=mannose receptor, NSB=nonspecific binding, PC=peritoneal cell(s), SIGN-R1=specific ICAM-3-grabbing nonintegrin-related 1, TP=thromboxane A₂ receptor

Introduction

Allergic asthma and related Th2-mediated inflammatory disorders have become increasingly common in recent years, but mechanisms inducing the immune responses and the means to control them have remained poorly understood. The eosinophilia and M2, observed in allergic inflammatory sites, have the potential to control extracellular parasite growth, but they also contribute to up-regulation of humoral and allergic immunity [1, 2]. In contrast, M1 by TLR ligands promotes innate and acquired cell-mediated immune responses, resulting in elimination of intracellular pathogens and down-regulation of allergic responses [2, 3].

As chitin, a naturally occurring β -(1-4)-poly-N-acetyl D-glucosamine, can activate M1, it has been implicated as an antiallergic agent, but its exact immunoregulatory roles remain uncertain and controversial [4–7]. We originally determined that CMP, as “mimetic microbes” and “antiallergic agents,” induce M1 activation with production of IL-12, IL-18, and TNF- α through a phagocytosis-mediated complex process rather than through a receptor-mediated transmembrane signal [6–10]. Phagocytosis of control particles, such as polystyrene and chitosan (de-acetylated polyglucosamine), does not activate M ϕ , indicating that the “chitin” chemical structure is required for M1 activation [9]. Further, M1 activation does not occur in response to soluble chitin or nonphagocytosable LCB or when phagocytosis of CMP is inhibited by cytochalasin D (actin polymerization inhibitor) [8, 11]. Thus, major features of M1 activation by CMP include phagocytosis, activation of MAPK, and TLR2-dependent Th1 cytokine production [7, 8, 11–13].

In sharp contrast, Reese et al. [5] recently demonstrated that intranasal or i.p. administration of chitin particles results in infiltration of eosinophils to the local sites and activation of

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local M ϕ with high Arg I, an M2 phenotype. They hypothesized that innate immune responses induced by chitin particles support the development of Th2-mediated allergy caused by chitin-containing allergens and microorganisms, for example, helminths, fungi, and crustaceans [5]. Despite reference to our previous study of CMP as a Th1 adjuvant [6], the report failed to provide the nature and characteristics of chitin particles that were used in the study [5]; it was thus impossible to identify variables that might explain the obviously controversial M ϕ responses to chitin.

M1 and M2 use different and competitive arginine metabolic pathways. M2 degrade L-arginine by the arginine hydrolytic enzyme Arg I, whereas the M1 metabolize arginine with iNOS [14]. El Kasmi et al. [15] found that *Mycobacterium tuberculosis*, a Th1 adjuvant, induces Arg I in mouse M ϕ through the TLR pathway, suggesting Arg I expression by M1 as well as M2. In addition, M2 express increased levels of lectins, such as intelectin, chitinases, and chitinase-like proteins, and several MRs [14, 16, 17]. Of the cytokines involved in M ϕ polarization, the immunosuppressive cytokine IL-10 is produced by M2 and suppresses Th1 cytokine production by local M ϕ , cell-mediated immunity against intracellular bacteria, and apoptosis of infected M ϕ [14, 18, 19]. Although other TLR ligands (e.g., HK-BCG, CpG, and LPS) increase IL-10 production, neither CMP nor LCB induce elevated IL-10 production in our in vitro M ϕ studies [11, 12].

The chitin particles used by Reese et al. [5] were obtained from New England Biolabs (Ipswich, MA, USA). As we show here, they fall into the class of LCB (see Fig. 1). The data from Reese et al. [5] demonstrated that LCB-induced eosinophil recruitment was independent of mast cell activation but mediated by LCB-activated M ϕ producing the eicosanoid LTB₄, which attracted eosinophils through the LTB₄ receptor (BLT1) [5]. It is well established that mucosal or i.p. administration of Sephadex beads (cross-linked dextran) at similar sizes (>40 μ m) induces local eosinophilia, probably through mast cell activation [5, 20–22]. CRTH2 is also expressed on eosinophils. Importantly, it is a receptor for the eosinophil chemoattractant PGD₂ and has long been implicated in mediation of allergic disease via mast cell release of PGD₂ that attracts CRTH2⁺ eosinophil infiltration [23–25].

In the present study, we compared our original CMP and LCB along with positive and negative controls, including Sephadex G-100 beads, HK-BCG, chitosan-MP, and saline as vehicle control, all of which were given i.p. in experimental mice. We determined (i) whether particle-induced peritoneal eosinophilia is dependent on particle size or carbohydrate composition, (ii) whether chitin particle-induced eosinophil migration and appearance of high Arg I-positive M ϕ in the peritoneum are dependent on CRTH2, and (iii) whether chitin particle-induced IL-10 production by M ϕ is also size-dependent.

MATERIALS AND METHODS

Reagents and antibodies

CMP and chitosan-MP were prepared as described previously [8, 11, 26]. The microparticle preparations were sized cytometrically using 1 and 10

μ m latex beads (Sigma-Aldrich, St. Louis, MO, USA) as standards. LCB and Sephadex G-100 beads were purchased from New England Biolabs and GE Healthcare Biosciences (Pittsburgh, PA, USA), respectively. CpG-ODN (5' TCC ATG ACG TTC CTG ACG TT 3'; unmethylated) with phosphorothioate backbone was purchased from TriLink (Sorrento Mesa, CA, USA). HK-BCG was prepared as described previously [27].

Mice

Nonpregnant, female C57BL/6 and Balb/c mice, 8–14 weeks old, were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Homozygous CRTH2^{-/-} breeders with C57BL/6 or Balb/c backgrounds were described previously [28] and bred at Florida Atlantic University (Boca Raton, FL, USA). Mice were maintained in barrier-filtered cages and fed Purina laboratory chow and tap water ad libitum. Experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of Florida Atlantic University.

Peritoneal M ϕ preparation following in vivo treatment with CMP, LCB, chitosan-MP, HK-BCG, or saline

Groups of mice (four to five/group) received 1 mg CMP, 1 mg LCB, 1 mg chitosan-MP, 1 mg HK-BCG, or 0.1 ml saline i.p. on Day 0. In some groups, mice were given 3 mg/kg Ramatroban (Cayman Chemical, Ann Arbor, MI, USA), a CRTH2 antagonist, on the day before and the day of i.p. particle injection. Peritoneal lavage with 5 ml saline was performed 1 day later [29]. Nucleated PC were counted with a Coulter counter (Model Z1, Beckman Coulter, Hialeah, FL, USA). Differential cell counts were performed manually on cytopsin preparations (Shandon Southern Instruments, Sewickley, PA, USA), stained with Diff-Quik. To enrich plastic-adherent M ϕ , following a wash with serum-free RPMI 1640, 2 $\times$ 10⁶ PC/ml were suspended in RPMI 1640 plus 5% heat-inactivated FBS and incubated in culture dishes for 2 h. Nonadherent cells (lymphocytes) were removed by washing with media. For IL-10 production, peritoneal adherent cells (approximately 10⁶ cells/ml) were stimulated with CpG (1 μ g/ml) or saline for 24 h at 37°C.

RAW264.7 cell culture

RAW 264.7 cells (American Type Culture Collection, Manassas, VA, USA) were grown and maintained in RPMI 1640 containing 5% FBS as described previously [11, 12].

Western blot analysis

Peritoneal M ϕ , prepared as described above, were washed three times with cold saline. Washed cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 4 mM EDTA, 0.1% SDS, 1:500 protease inhibitor cocktail, 1% Nonidet P-40, 0.1% sodium deoxycholate, 10 mM NaF, and 2 mM Na₃VO₄). Debris was eliminated by centrifugation (10 min, 10,000 g), and protein concentration in the lysate was measured by bicinchoninic acid assay (Pierce, Rockford, IL, USA) with BSA as standard. Equal amounts (2–5 μ g) of protein from each sample were separated by SDS-PAGE. Proteins were then transferred to a PVDF membrane (Millipore, Bedford, MA, USA), which was blocked with 10% nonfat dry milk and incubated with antibodies [anti-Arg I, 1:200 (BD Transduction Laboratories, San Diego, CA, USA), at 22°C and at 4°C for anti-CHI3L1, 1:5000 [30], and anti-iNOS, 1:4000 (Upstate, Temecula, CA, USA); anti-COX-2, 1:800 (Cayman Chemical); and anti- β actin, 1:5000 (Sigma-Aldrich), for a constitutively expressed protein control] in 7.5% nonfat dry milk overnight. Following incubation with peroxidase-conjugated goat anti-rabbit IgG for CHI3L1 or donkey anti-mouse IgG for Arg I and β -actin (both 1:10,000; Jackson ImmunoResearch, West Grove, PA, USA), stained proteins were detected by chemiluminescence (ECL Plus, Amersham, Piscataway, NJ, USA) following the manufacturer's instructions.

Measurement of mediators

Peritoneal lavage with 1 ml saline was performed for IL-10, TNF- α , and nitrite measurement. IL-10 and TNF- α levels in culture supernatants and

peritoneal lavage were measured by two-site ELISA specific for the respective cytokines (BD Biosciences, San Diego, CA, USA). Nitrite as an indicator of NO was measured by the Griess reagent [27].

Cytometric detection of surface antigens

Standard methodology was used for the direct and indirect immunofluorescent characterization of peritoneal leukocytes. In brief, 10^6 PC were incubated with Fc Block (BD PharMingen, Franklin Lakes, NJ, USA) in 50 μ l PBS for 5 min to block NSB of antibodies to cellular FcRs. Subsequently, optimal concentrations of PE-conjugated F4/80 antibody and other single fluorochrome-conjugated antibodies recognizing surface antigens (TLR2, MR, CD205, SIGN-R1, Dectin-1), purchased from BioLegend (San Diego, CA, USA) and BD PharMingen, were added in 50 μ l FACS buffer for dual staining. After 30 min incubation on ice, the cells were washed three times with FACS buffer and fixed in 200 μ l 1% paraformaldehyde. Fluorescence of stained cells was quantified with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using the FlowJo program (Tree Star, Ashland, OR, USA). M ϕ fractions were electronically gated by FSC and side-scatter and positive expression of F4/80.

Confocal microscopy of intracellular CMP

M ϕ that phagocytosed CMP were fixed in 4% paraformaldehyde in PBS, pH 7.5, for 40 min [11, 12]. The fixed cells were permeabilized with 1% Triton X-100 in PBS for 15 min and incubated in blocking buffer consisting of PBS with 10% FBS 2 h at 22°C prior to incubation with FITC-chitin-binding protein (1:500, bacterial chitinase, NSB) and anti-CHI3L1, 1:1000, in blocking buffer overnight at 22°C. Subsequently, cells were washed with PBS three times and incubated with Cy3-conjugated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch) for 1 h at 22°C. After washing three times, nuclei were stained with DAPI (Vector Laboratories, Burlingame, CA, USA) at 1.5 μ g/ml. Cells were examined with a laser-scanning confocal microscope (LSM700, Zeiss, Thornwood, NY, USA).

IL-10 mRNA detection by RT-PCR

IL-10 expression was analyzed by RT-PCR as described previously [12]. Briefly, after stimulation of RAW 264.7 cells (5×10^5 /ml) with CpG or saline for 6 h or 24 h in the presence or absence of CMP, RNA extraction with TRizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcription with SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen) with oligo-(dT) primer were performed. PCR primers used in this study were: IL-10 (forward: 5'-GGT TGC CAA GCC TTA TCG GA-3', reverse: 5'-ACC TGC TCC ACT GCC TTG CT-3') and GAPDH as a constitutively expressed control (forward: 5'-TTC ACC ACC ATG GAG AAG GC-3', reverse: 5'-GGC ATG GAC TGT GGT CAT GA-3').

Endotoxin removal

Endotoxin was removed from soluble materials for culture and i.p. injection by filtration and sterilization through a 0.22- μ m Zetapore membrane (AMF Cuno, Cuno, Meriden, CT) [31]. The final preparations were monitored for endotoxin by the *Limulus* amoebocyte assay (Sigma-Aldrich) [10].

Statistics

Differences between mean values were analyzed by Student's *t* test with Statcel software. *P* < 0.05 was considered statistically significant.

RESULTS

Sizes of CMP and LCB

Fig. 1A shows that over 96% of CMP used in this study were at 1–10 μ m diameter. **Fig. 1B** shows that the New England Biolabs chitin particles (LCB) and the Sephadex G-100 beads had diameters over 40 μ m, and the majority of LCB was slightly bigger than the Sepha-

dex beads. **Fig. 1C** and **D** shows that control RAW264.7 M ϕ expressed CHI3L1 constitutively in the cytosol and nuclear region. After CMP were phagocytosed, intracellular CMP were detected by FITC-bacterial chitinase (**Fig. 1D**). Our previous studies have demonstrated that phagocytosis of CMP is sufficient for M1 activation, which does not occur following phagocytosis of latex beads or micro-particles composed of chitosan (deacetylated chitin) [8, 9, 11].

Size-dependent particle induction of eosinophilia

To examine the effects of particle size and composition, mice were given i.p. injections of 1 mg LCB, 1 mg Sephadex beads, 1 mg CMP, 1 mg HK-BCG, or saline vehicle alone. Eosinophils in the peritoneal fluid were counted, and the results are shown in **Fig. 2**. These results indicate that eosinophilia occurred only in response to LCB and Sephadex beads. In contrast, the smaller phagocytosable CMP and HK-BCG did not induce eosinophil migration into the peritoneal cavity (**Fig. 2**).

CRTH2, a PGD₂ receptor, has long been implicated as a mediator of Th2-activated up-regulation of allergic diseases, including attraction of eosinophil migration [23–25]. To test the contribution of CRTH2, eosinophilia was assessed in CRTH2^{-/-} and WT (Balb/c) mice given i.p. injection of LCB or Sephadex beads. As seen in **Fig. 3A**, the eosinophilic response was significantly lower in CRTH2^{-/-} mice than in WT mice. LCB-induced eosinophilia was also reduced significantly by treatment with the CRTH2 antagonist Ramatroban before i.p. administration of LCB in C57BL/6 mice (**Fig. 3B**), further confirming the role of CRTH2. The LCB-induced eosinophilia and the role of CRTH2 were observed in Balb/c and C57BL/6 mice, but the magnitude of the response was strain dependent. Our results indicate that large, nonphagocytosable beads induce local eosinophilia in a manner that is independent of the specific carbohydrate composition of the particles.

Size-dependent induction of M1 or M2 phenotypes

As shown in **Fig. 4**, the levels of the 35- and ~40-kDa isoforms of Arg I were slightly increased in peritoneal (Arg I^{low} M ϕ) from WT mice given i.p. CMP or HK-BCG relative to those from WT mice treated with saline. According to El Kasmi et al. [15], the induction of Arg I^{low} in M ϕ by CMP or HK-BCG would be TLR dependent but STAT6 independent. It is therefore particularly important to note that the treatment of mice with LCB or Sephadex G-100 resulted in M ϕ expressing greater levels of the Arg I isoforms (Arg I^{high} M ϕ) than in those from CMP- or HK-BCG-treated mice (**Fig. 4A** and **C**). Whereas eosinophilia was dependent on CRTH2, the LCB-induced Arg I^{high} expression was independent of CRTH2 (**Fig. 4**).

Constitutive levels of CHI3L1 were detected in peritoneal M ϕ from mice treated with saline and unchanged by the treatments with CMP or LCB (**Fig. 4**).

Selected surface antigens expressed by F4/80⁺ peritoneal M ϕ were determined cytometrically. As shown in **Fig. 5**, TLR2, CHI3L1, MR, CD205, Dectin-1 (α β -glucan receptor), and SIGN-R1 were expressed by peritoneal M ϕ isolated from saline-treated mice. M ϕ activated by i.p. CMP exhibited increased Dectin-1 MFI and slightly increased MFIs for TLR2 and CHI3L1, but MFIs for MR, CD205, and SIGN-R1 were decreased. In contrast,

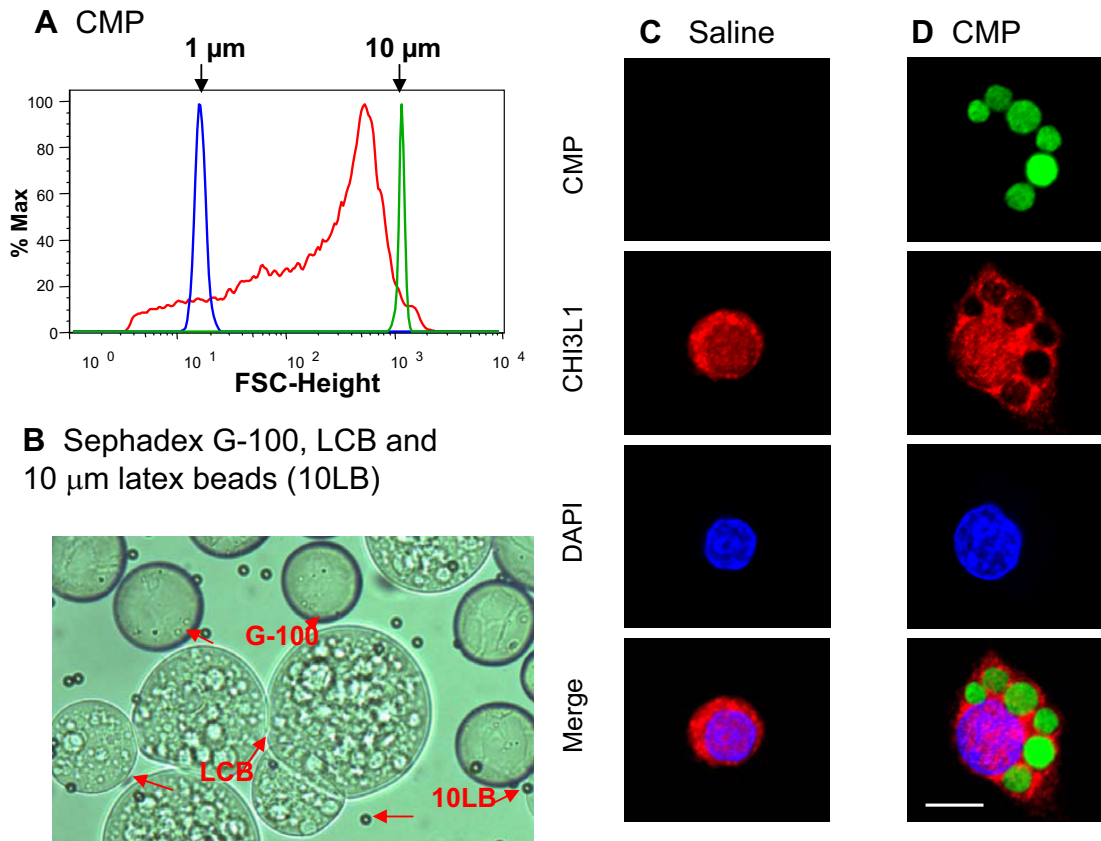


Figure 1. Chitin particle sizes. (A) CMP size distribution (red) determined by FACS. References are 1 (blue) and 10 (green) μm latex beads. (B) Light microscopy of a mixture of LCB, Sephadex G-100 (G-100), and 10 μm latex beads (10LB). (C and D) Confocal microscopy of RAW264.7 M ϕ with saline (C) or after in vitro phagocytosis of CMP (D), stained with FITC-bacterial chitinase (CMP; green), Cy3-anti-CHI3L1 (red) antibody, and DAPI (blue). Original bar = 10 μm .

M ϕ activated by LCB exhibited decreased MFIs for TLR2, CHI3L1, CD205, and SIGN-R1 and slightly increased MR MFI (Fig. 5). Dectin-1 expression by peritoneal M ϕ in response to LCB was biphasic with predominantly decreased MFI relative to saline control cells, whereas for other M ϕ , the Dectin-1 MFI was increased relative to controls (Fig. 5).

We observed previously that neither CMP nor LCB induced IL-10 production in vitro [11]. To determine whether i.p. administration of these particles induces local production of IL-10 and inflammatory TNF- α and nitrite, these levels were measured in peritoneal lavage. Fig. 6 shows that neither CMP nor larger particles (LCB and Sephadex G-100) induced local IL-10 production, although CMP induced TNF- α and nitrite. As expected, HK-BCG, the positive control, increased all three mediators (Fig. 6).

IL-10 production ex vivo by peritoneal M ϕ

We also investigated whether peritoneal M ϕ , activated by i.p. CMP, LCB, or chitosan-MP, produce IL-10 when further stimulated ex vivo with CpG. As shown in Fig. 7, peritoneal M ϕ elicited by CMP had reduced IL-10 production. However, pretreatment of mice with LCB or chitosan-MP had no effect on CpG-elicited IL-10 production (Fig. 7). These results suggest that CMP reduces IL-10 levels under inflammatory conditions that characteristically exhibit elevated IL-10.

In vitro effects of chitin particles on M ϕ activation

RAW264.7 M ϕ , as a model of tissue M ϕ isolated from normal mice, have been used frequently to study M1 activation by

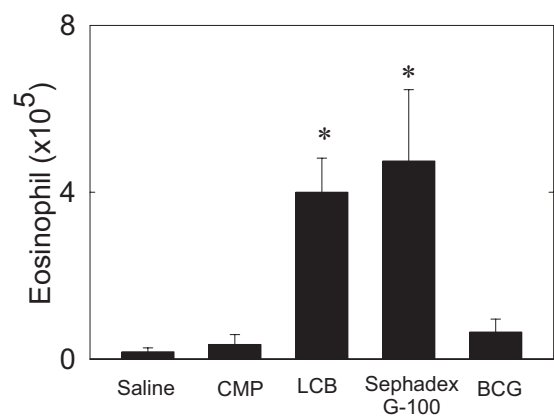


Figure 2. i.p. administration of chitin particles induces local eosinophilia in a size-dependent manner. Groups of C57BL/6 mice were given 1 mg LCB, 1 mg CMP, 1 mg Sephadex G-100 beads, 1 mg HK-BCG, or saline i.p. One day later, peritoneal lavage was performed, and eosinophils were counted. Mean $\pm$ SE; $n = 4$; * $P < 0.001$ compared with the saline control group.

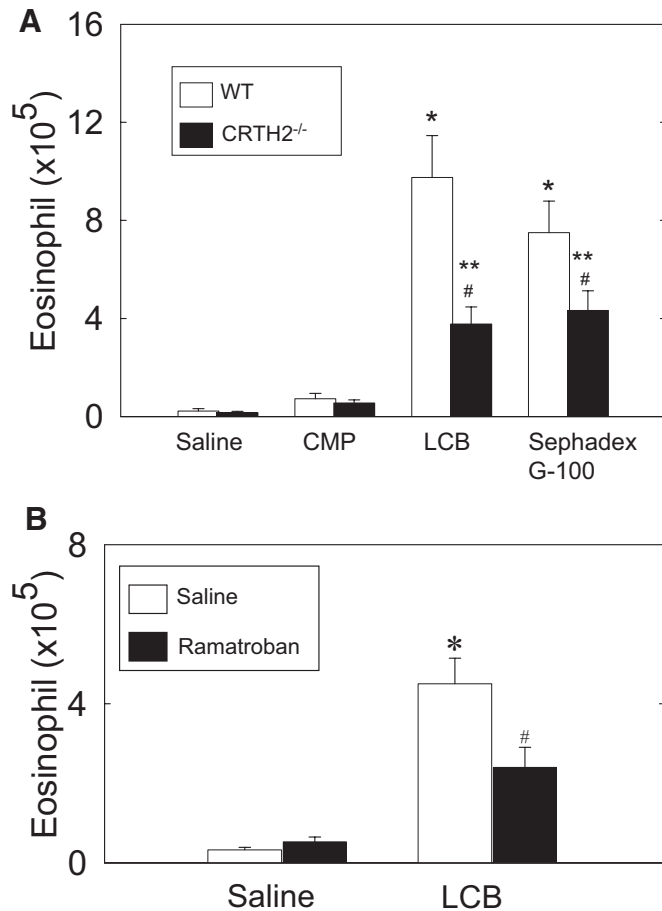


Figure 3. CRTH2-dependent eosinophil migration induced by nonphagocytosable particles. (A) Groups of Balb/c (WT) and CRTH2^{-/-} mice were given 1 mg LCB, Sephadex G-100, CMP, or saline i.p. One day later, peritoneal lavage was performed, and eosinophils were counted. Mean $\pm$ SE; $n = 4$; * $P < 0.001$ and ** $P < 0.01$ compared with the saline control, respectively; # $P < 0.01$ compared with the corresponding WT group. (B) Groups of C57BL/6 received 3 mg/kg Ramatroban orally twice on Days -1 and 0, 1 h before i.p. administration of 1 mg LCB or saline. Mean $\pm$ SE; $n = 4$. * $P < 0.01$ compared with the saline/saline and saline/LCB; # $P < 0.05$ compared with saline/LCB and Ramatroban/LCB.

CMP [11, 12]. LCB-activated RAW264.7 M ϕ (M2) was reported to attract LCB₄-dependent eosinophil migration in vitro [5]. To further examine the effects of LCB on M ϕ , RAW cells were stimulated with LCB, CMP, and control particles. Fig. 8 shows that LCB did not induce iNOS or COX-2, whereas CMP induced these M1 phenotypes. It should be noted that the level of iNOS in peritoneal M ϕ in mice given i.p. CMP (data not shown) was much lower than that of CMP-activated RAW cells (Fig. 8). In this regard, although Arg I was detected in RAW cells that were treated with a mixture of IL-4, IL-13, and cholera toxin, we were unable to detect Arg I bands in RAW cells following any of the treatments listed (data not shown). These results suggest that the expressions of LCB-induced Arg I and CMP-induced iNOS are significantly distinct between in vitro and in vivo conditions of M ϕ activation,

which would be analogous to HK-BCG-induced COX-2 [27, 32]. On the other hand, CHI3L1 expression was unchanged by any of the treatments in vivo (Fig. 4) or in vitro (Fig. 8).

CMP reduces in vitro CpG-induced IL-10 production

To characterize further the inhibitory effect of CMP on CpG-stimulated IL-10 production, RAW cells were stimulated with CpG accompanied by pre- or post-treatment with CMP. As shown in Fig. 9, IL-10 production was inhibited, not only by pretreatment with CMP but also by post-treatment delayed 6 h. When the culture supernatants isolated from RAW cell cultures treated with CMP were added to CpG-stimulated RAW cell culture, IL-10 production was not reduced (data not shown), suggesting that the suppression of IL-10 production by CMP is probably not associated with an endocrine- or paracrine-soluble mediator. However, CMP did not alter CpG-induced IL-10 mRNA levels at 6 h and 24 h, suggesting post-transcriptional regulation of IL-10 production by CMP (Fig. 10).

DISCUSSION

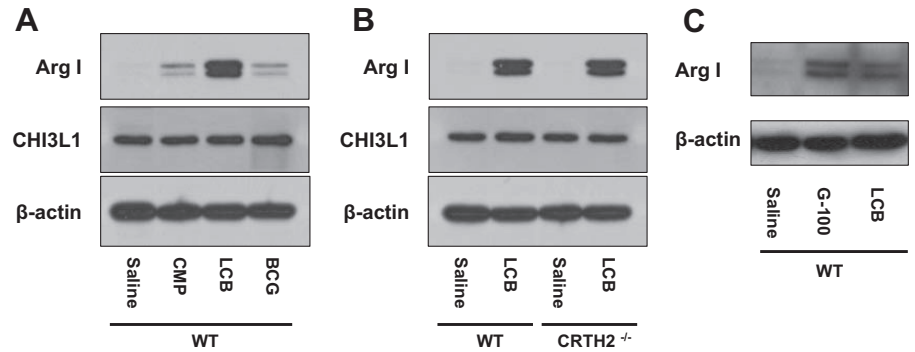
The results presented here indicate that LCB, but not CMP, induce eosinophil migration within 24 h after i.p. administration, and i.p. administration of Sephadex G-100 beads (Figs. 2 and 3), Sepharose (agarose) beads (galactose polymer, 40–145 μ m; Sigma-Aldrich; data not shown), or LCB, which were prepared as a byproduct of CMP preparation (data not shown), also induced peritoneal eosinophilia. i.v. or intratracheally administered Sephadex beads are known to induce lung eosinophilia and granuloma formation in experimental animals [20, 33–35]. The pathophysiological changes induced by Sephadex beads are associated with nonspecifically increased Th2 cytokines such as IL-4, IL-5, IL-13, and eotaxin in the lungs [36] and airway hyper-responsiveness [37]. Taken together, our results are consistent with the conclusion that exposure to nonphagocytosable carbohydrate particles induces local eosinophilia in a manner independent of chemical composition.

The results also indicate that i.p. LCB, but not CMP, induce local M ϕ activation with increased expression of Arg I, MR, and Dectin-1, in addition to the innate eosinophilia. Therefore, chitin particle-induced Arg I demonstrated by Reese et al. [5] and the additional M2 phenotypes, including found in inflammatory zone 1 or resistin-like molecule α , MR, and chitinase 3-like 3, reported by Satoh et al. [38], would be a result of the nonphagocytosable size of their particles.

TLR2 and MR have been identified previously as binding proteins essential for phagocytosis of CMP, resulting in M1 activation in vivo and in vitro [8, 13] (Figs. 5 and 6). In contrast, when M ϕ are treated in vitro with LCB, neither M1 nor M2 phenotypes are induced [11, 12] (Fig. 8). It is therefore suggested that LCB-induced Arg I^{high} M ϕ activation in vivo requires additional factors that may include additional cells, humoral factors, or both. We hypothesize that nonphagocytosable particles activate local mast cells in TLR-dependent and -independent manners [39, 40].

PGD₂ acts via the GPCRs, DP, and CRTH2 [24]. The expression patterns and signaling pathways for DP and CRTH2 are different, suggesting that they have distinct roles in allergic

Figure 4. i.p. LCB enhances Arg I expression but not CHI3L1. (A) Groups of WT (C57BL/6) were given 1 mg LCB, 1 mg CMP, 1 mg Sephadex G-100 beads, 1 mg HK-BCG, or saline i.p. (B) Groups of WT and CRTH2^{-/-} mice were given 1 mg LCB or saline i.p. (C) Groups of WT mice were given 1 mg Sephadex G-100, 1 mg LCB, or saline i.p. One day later, peritoneal lavage was performed, M ϕ were isolated, and their cellular proteins were extracted. Arg I, CHI3L1, and β -actin levels were determined by Western blot as indicated in Materials and Methods. Results are representative of two separate experiments.



responses [24, 41]. It appears that signals via DP promote eosinophil survival, whereas signals via CRTH2 mediate shape change, chemotaxis, and degranulation of eosinophils [24, 42, 43]. In human nasal mucosa, CRTH2 is expressed on eosinophils and a subset of T cells [44]. Eosinophil infiltration into mucosal sites appears to be regulated by CRTH2, but not DP [45]. Our studies clearly indicate that eosinophilia induced by LCB is, at least in part, dependent on CRTH2, whereas Arg I^{high} M ϕ activation is CRTH2 independent.

The reduction of eosinophilia in the CRTH2^{-/-} mice and in mice treated with Ramatroban is consistent with the hypothesis that the eosinophilia induced by LCB and Sephadex particles was mediated, at least in part, by CRTH2, although our data do not permit us to eliminate the possibility that the inhibition by Ramatroban was a result of its inhibition of TP. However, our working model is supported further by several recent reports that other more specific inhibitors of CRTH2 (i.e., with little to no antagonism of TP) prevent eosinophilia in murine models of inflammation and/or that specific TP inhibitors had no effect [46–48]. Together, our results and these reports provide compelling support for the hypothesis that CRTH2⁺ eosinophils are chemotactically guided to PGD₂.

The available data suggest that the PGD₂ may be derived from mast cells in response to LCB. Murine peritoneal M ϕ , elicited by i.p. administration of elicitors (e.g., thioglycollate, Sephadex beads, or bacterial components including HK-BCG), have been widely used for M ϕ studies; peritoneal mast cell activation by these elicitors would contribute to a five- to ten-fold increase in monocyte-infiltrated peritoneal M ϕ [22, 49–51]. These exudated M ϕ release significantly lower concentrations of PGs and LTs than untreated, resident peritoneal M ϕ [29, 32, 52]. Although peritoneal M ϕ isolated from normal mice (C57BL/6) released ~5 pg LTB₄ and 8 pg PGD₂/10⁶ M ϕ , stimulated with 1 μ M A23187 calcium ionophore for 2 h at 37°C, release of these eicosanoids from peritoneal M ϕ was not stimulated by i.p. CMP or LCB (data not shown). However, in vitro exposure of RAW264.7 cells with CMP—but not LCB—enhanced their release of PGE₂ [11, 12] and LTB₄/PGD₂ (data not shown). Therefore, PC other than M ϕ are more likely sources of the endogenous PGD₂ and LTB₄, driving eosinophilic responses to peritoneal LCB or Sephadex beads [22, 53]. As known producers of PGD₂ and LTB₄, mast cells likely contribute to the LCB-induced eosinophilia.

M ϕ from immunocompromised populations (infected, aged, neonates, tumor-bearers, atherosclerotics, and diabetics) fre-

quently express high levels of IL-10 [54–61], which potentially impact host defense and reduce the efficacy of immunotherapy. Recent studies have demonstrated that M ϕ , rather than T cells, are the major source of IL-10 in tuberculosis, and M ϕ -derived IL-10 triggers aspects of M2 activation, including enhanced Arg I and promotion of *M. tuberculosis* reactivation [62, 63]. However, our results show that compared with M ϕ from control mice, some peritoneal M ϕ from mice i.p. treated with LCB or CMP express higher levels of Dectin-1 (Fig. 5). Although Dectin-1 is known to augment yeast zymosan-stimulated IL-10 production [64], neither CMP-induced nor LCB-induced M ϕ activation in vivo resulted in increased IL-10 production. We found that our CMP bound TLR2, MR, and CHI3L1 but not Dectin-1 during CMP phagocytosis (data not shown).

A provocative finding from our studies is that ongoing IL-10 production by M ϕ in vitro can be down-regulated post-transcriptionally when M ϕ phagocytose CMP but not chitosan-MP (Fig. 9). IL-10 production by M ϕ may be regulated transcriptionally [65, 66] and post-transcriptionally [67, 68]. Ma et al. [65] have shown that activation of transcription factor specificity protein 1 via p38 induces transcription of the IL-10 gene in LPS-stimulated THP-1 cells. In addition, Németh et al. [68] have shown that the 3'-untranslated region of IL-10 mRNA plays a repressive role in IL-10 translation, indicating translational regulation.

In apparent contrast to our results, Da Silva et al. [69] found that chitin particles at <40 μ m (largely 2–10 μ m), but not at 40–70 μ m or 70–100 μ m, induce IL-10 production by thioglycollate-elicited peritoneal M ϕ in a manner dependent on TLR2/Dectin-1 but independent of phagocytosis. Furthermore, in research on a mouse asthma model, this group reported that instillation of nonphagocytosable chitin particles at 40–70 μ m induced TNF- α production in thioglycollate-elicited peritoneal M ϕ in vitro [69], pulmonary inflammation with neutrophil but not eosinophil infiltration [13], and a Th2 adjuvant activity for allergen sensitization [70]. Recent reports often lack reliable cytometric size distributions of chitin particles that were used. This has somewhat impaired the ability to replicate experiments. If the reference latex beads do not distinguish phagocytosable versus nonphagocytosable particles (1–10 μ m vs. particles >50 μ m), the possibility that the preparation included phagocytosable and nonphagocytosable particles in an unknown ratio cannot be ruled out.

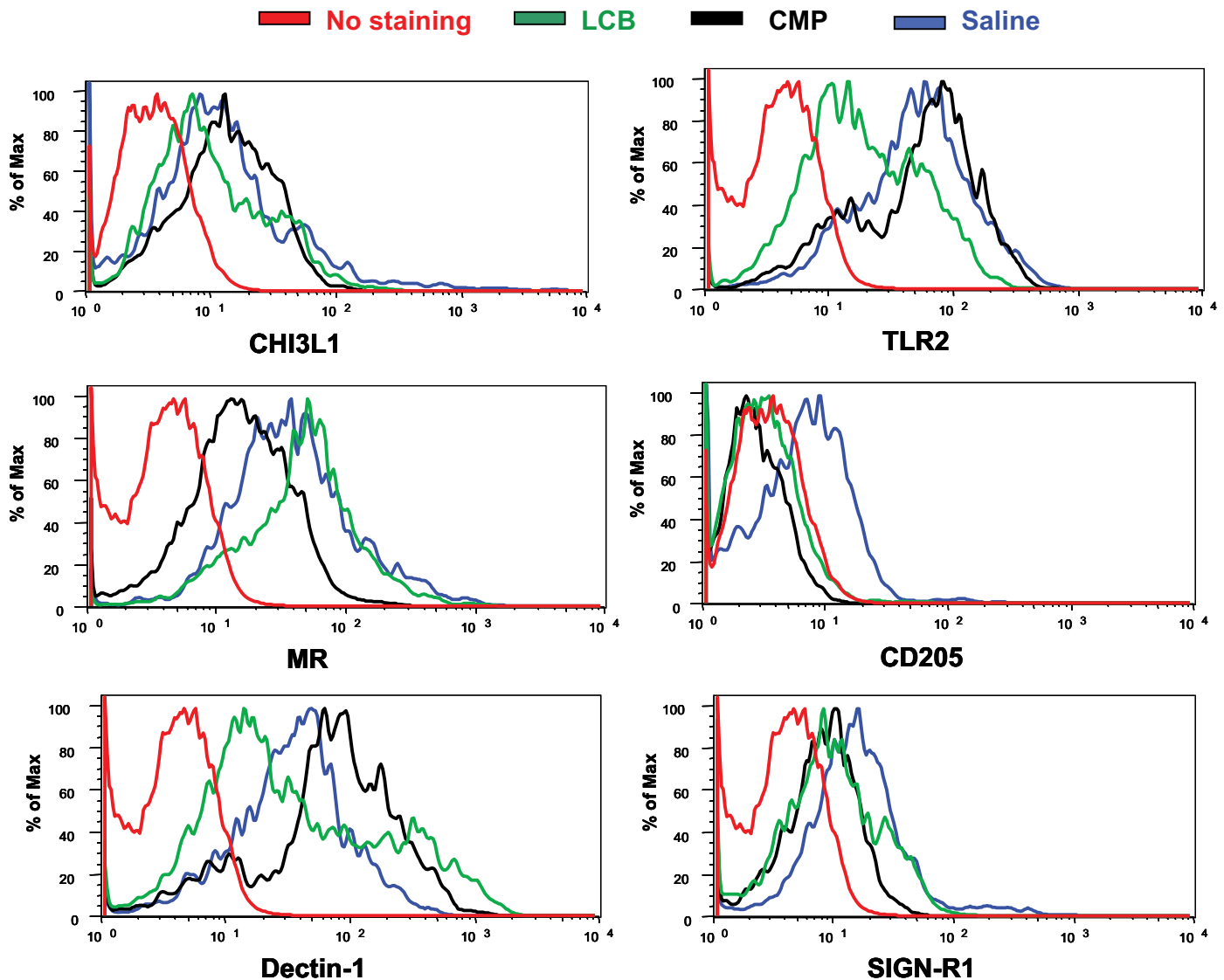


Figure 5. Selected surface antigens in peritoneal M ϕ from mice given i.p. LCB or CMP. C57BL/6 mice were given LCB, CMP, or saline i.p. One day later, PC were isolated, stained with fluorochrome-conjugated mAb listed, and analyzed by flow cytometry. For FACS analysis, single peritoneal M ϕ were electronically gated by FSC and side-scatter and further gated on F4/80⁺ cell fractions.

Mizoguchi [30] demonstrated that CHI3L1 (also known as YKL-40, breast regression protein 39, or human cartilage glycoprotein 39), which binds chitin but lacks enzymatic activity to lyse microbial cell walls, is expressed primarily by epithelial cells and M ϕ of inflamed intestines in dextran sulfate-induced colitis. CHI3L1, which can be up-regulated after proinflammatory cytokine stimulation, enhances the adhesion and internalization of commensal microbes into colonic epithelial cells, where the intracellular microbes act as “surrogate pathogens” to stimulate a chronic inflammatory response [30]. Th1 and Th2 cytokines enhance CHI3L1 production in vitro [30]. Studies of CHI3L1^{-/-} mice by Lee et al. [71] demonstrated that CHI3L1 induces M2 in vitro and regulates lung eosinophilia in mice with allergen-induced Th2 responses. In the current study, peritoneal M ϕ and RAW264.7 cells constitutively express

CHI3L1. The total cellular CHI3L1 levels are unchanged by the treatments with CMP or LCB (Fig. 4), although the surface CHI3L1 level seems to be decreased or increased slightly in peritoneal M ϕ treated in vivo with LCB or CMP, respectively (Fig. 5). We also found that pretreatment of RAW264.7 cells with the neutralizing anti-CHI3L1 prior to CMP exposure did not affect the stimulation of TNF- α (data not shown). It seems unlikely that CHI3L1 regulates “acute/innate” M ϕ activation by CMP, LCB, or mycobacteria or probably eosinophil migration in our models.

In conclusion, the recent apparent contradictory reports [5, 38, 70] indicate that chitin particle-induced innate immunity supporting Th2/allergic responses appears to be a consequence of using much larger, nonphagocytosable LCB. Our data also demonstrate that administration of nonantigenic car-

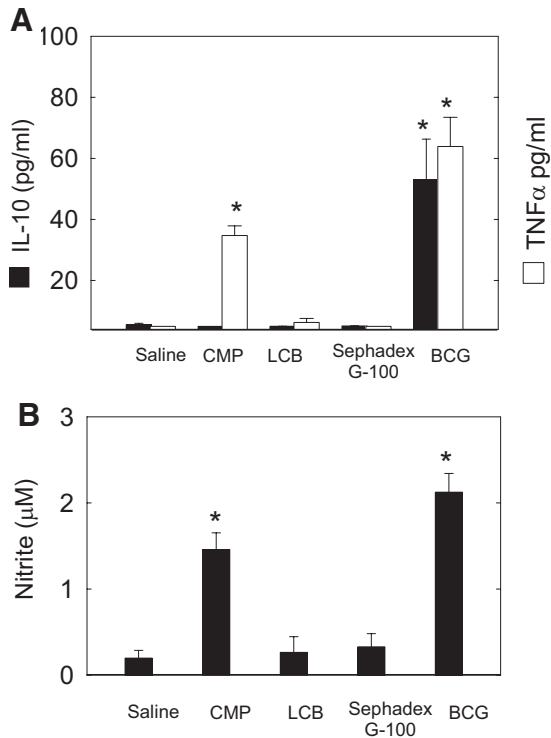


Figure 6. TNF- α , nitrite, and IL-10 levels detected in peritoneal lavage fluid. Groups of C57BL/6 mice were given CMP, LCB, Sephadex G-100, HK-BCG, or saline i.p. One day later, peritoneal cavities were washed with 1 ml saline. The supernatants were isolated from PC by centrifugation. IL-10 and TNF- α levels (A) in the supernatants were measured by ELISA and nitrite levels (B) by the Griess reagent. Mean $\pm$ SE; $n = 4$. * $P < 0.01$ compared with the cytokine levels of peritoneal lavage fluid isolated from mice given saline i.p.

bohydrate beads ($>40 \mu\text{m}$) other than LCB is also potentially acting as a Th2 adjuvant. The data presented here clearly demonstrate distinct responses to CMP and LCB, and CMP

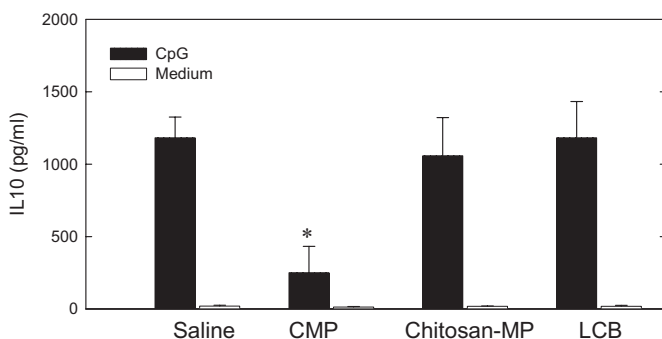


Figure 7. Priming effects of CMP administered i.p. on ex vivo IL-10 production elicited from peritoneal M ϕ . Peritoneal M ϕ were isolated from C57BL/6 mice, which previously received i.p. CMP, chitosan-MP, LCB, or saline as control. Isolated peritoneal M ϕ were stimulated further with CpG or saline in vitro at 37°C for 24 h. Mean $\pm$ SE; $n = 3$. * $P < 0.01$ compared with cells isolated from saline control mice and stimulated with CpG. The data shown are representative of two independent experiments.

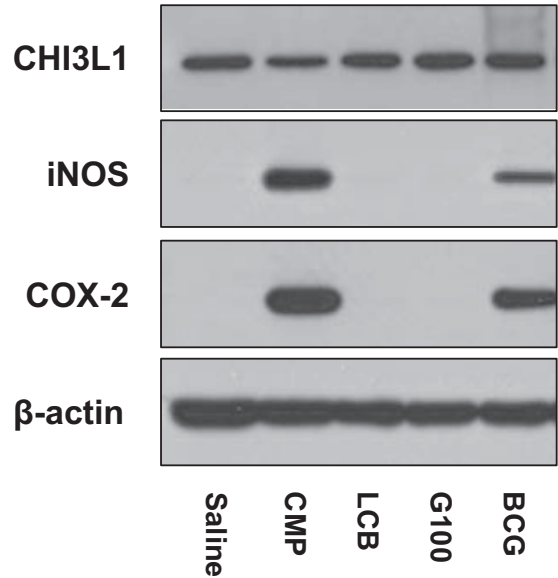


Figure 8. CHI3L1, iNOS, and COX-2 in RAW264.7 cells exposed to LCB in vitro. M ϕ were incubated with 100 $\mu\text{g}/\text{ml}$ LCB, CMP, Sephadex G-100, 50 $\mu\text{g}/\text{ml}$ HK-BCG or saline. After 24 h, proteins were extracted. CHI3L1, iNOS, COX-2, and β -actin levels were determined by Western blot, as indicated in Materials and Methods. Results are representative of two separate experiments.

induces well-defined markers for M1 activation and LCB-inducing Arg I, which is usually, but not always, a phenotype of M2 activation. Finally, although we have confirmed features of cellular responses to CMP in these studies and find its properties suitable for a Th1 adjuvant, the results also emphasize the importance of careful quality control in the production of phagocytosable particles without introducing undesirable effects on Th2 responses with eosinophilia.

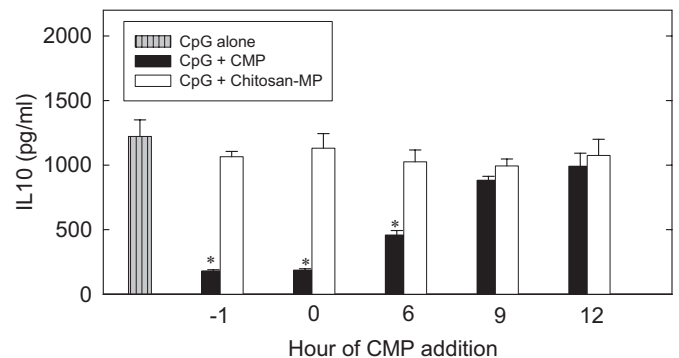


Figure 9. Effects of the addition of CMP at selected time-points on CpG-induced in vitro IL-10 production by RAW264.7 cells. CMP were added to cultures of M ϕ at the indicated time-points; at 0 h, CpG was added to the cultures. Culture supernatants were harvested at 24 h. As comparison controls, 100 $\mu\text{g}/\text{ml}$ chitosan-MP were added for CMP. Saline alone, CMP alone, and chitosan-MP alone all had $<50 \text{ pg}/\text{ml}$ IL-10 (data not shown). Mean $\pm$ SD; $n = 3$. * $P < 0.01$ compared with cells stimulated by CpG alone.

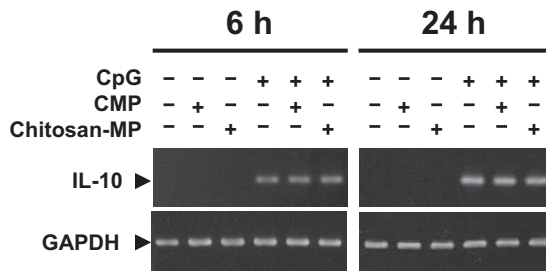


Figure 10. Effects of in vitro addition of CMP on the levels of CpG-induced IL-10 mRNA. RAW264.7 cells were stimulated with CpG in the presence of 100 μ g/ml CMP or chitosan-MP for 6 h and 24 h. RNA extraction and RT-PCR were done as described in Materials and Methods. mRNA levels of IL-10 and GAPDH were shown. The data shown are representative of three independent experiments.

AUTHORSHIP

M.K., A.N., and Y.S. wrote the paper and designed experiments; M.K., A.N., T.S., and E.G. performed experiments; M.K., A.N., E.M., Y.M., M.N.S., M.N., C.K.D., and R.A.H. analyzed data; M.N., E.M., and Y.M. contributed reagents; M.K., M.N., E.M., M.N.S., R.A.H., C.K.D., and Y.S. edited the manuscript; and Y.S. conceived of the research. All authors revised and approved the manuscript.

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KEY WORDS:

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