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Chitin Elicits CCL2 from Airway Epithelial Cells and Induces CCR2-Dependent Innate Allergic Inflammation in the Lung

René M. Roy,^{*,†} Marcel Wüthrich,^{*} and Bruce S. Klein^{*,‡,§}

Chitin exposure in the lung induces eosinophilia and alternative activation of macrophages and is correlated with allergic airway disease. However, the mechanism underlying chitin-induced polarization of macrophages is poorly understood. In this paper, we show that chitin induces alternative activation of macrophages in vivo but does not do so directly in vitro. We further show that airway epithelial cells bind chitin in vitro and produce CCL2 in response to chitin both in vitro and in vivo. Supernatants of chitin-exposed epithelial cells promoted alternative activation of macrophages in vitro, whereas Ab neutralization of CCL2 in the supernate abolished the alternative activation of macrophages. CCL2 acted redundantly in vivo, but mice lacking the CCL2 receptor, CCR2, showed impaired alternative activation of macrophages in response to chitin, as measured by arginase I, CCL17, and CCL22 expression. Furthermore, CCR2 knockout mice exposed to chitin had diminished reactive oxygen species products in the lung, blunted eosinophil and monocyte recruitment, and impaired eosinophil functions as measured by expression of CCL5, IL-13, and CCL11. Thus, airway epithelial cells secrete CCL2 in response to chitin and CCR2 signaling mediates chitin-induced alternative activation of macrophages and allergic inflammation in vivo. *The Journal of Immunology*, 2012, 189: 2545–2552.

Exposure to fungal- and insect-related Ags early in life are a significant risk factor in the development of allergy and asthma (1). Besides intrinsic protease activity present in many allergens, the additional presence of pathogen-associated molecular patterns such as LPS and β -glucan contributes to the allergenicity of inhaled environmental allergens (2, 3). Common to the cell wall of fungi and the exoskeleton of insects and crustaceans is the polysaccharide chitin, a linear chain of β -1,4-*N*-acetylglucosamine sugars. Chitin is among the most abundant polymers on our planet and is recognized by, and elicits responses from, organisms across the kingdoms of life. As an example, *N*-acetylglucosamine oligosaccharides trigger antifungal immune responses in plants via specific chitin receptors that share homology with mammalian chitinases and chitinase-like proteins (4, 5). In humans, elevated chitin exposure in the workplace and at home is correlated with asthma and other allergic diseases (6–8). Furthermore, murine models have demonstrated that exposure to chitin particles results in innate allergic inflammation characterized by alternatively activated macrophages (M2) and eosinophilia (9–11). Thus, chitin represents a potential allergy promoting pathogen-associated molecular pattern with a significant public health impact.

Among airway immune cells, macrophages are “ambidextrous” cells capable of initiating or suppressing inflammatory responses (12). In response to environmental or microbial exposures, macrophages are polarized into phenotypically distinct activation states: classical (M1) or M2. M2 antagonize proinflammatory Th1 responses and are associated with the development of Th2-associated inflammation central to the pathogenesis of allergy and asthma. Although chitin exposure induces M2 polarization in vivo (9, 10), macrophages exposed to chitin in vitro fail to acquire an M2 phenotype and instead secrete TNF- α (13–16). Thus, the disparity of polarization between macrophages exposed to chitin in vitro and in vivo suggests that an intermediary is required for chitin-induced M2 polarization in vivo.

Airway epithelial cells are the initial point of contact for inhaled allergens and coordinate with pulmonary dendritic cells (DC) to induce Th2 responses central to the pathogenesis of asthma. In a model of house dust mite-induced asthma, activation of epithelial cells was required for the subsequent development of allergic responses in the airway (2). In this study, we investigated the intermediary cells and products that facilitate chitin-induced M2 polarization and allergic airway inflammation. We demonstrate that airway epithelial cells produce CCL2 (MCP-1) in response to chitin and that the CCL2 receptor CCR2 is required for chitin-induced M2 polarization and allergic inflammation in vivo.

Materials and Methods

Mice

CCR2 knockout (KO) (stock number 004999) and CCL2KO (stock number 004434) mice, aged 5–8 wk, were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 wild-type (WT) mice (strain code 01C55), aged 5–8 wk, were obtained from National Cancer Institute (Frederick, MD). For all experiments, five mice were used per group. Mice were housed and cared for according guidelines from the University of Wisconsin Animal Care and Use Committee, who approved this work.

Reagents and cell culture

Chitin purified from crab shells was purchased from Sigma-Aldrich (C9752). AMJ2-C11 murine macrophages (CRL-2456) and LA-4 murine lung epithelial cells (CCL-196) were obtained from American Type Culture Collection. AMJ2-C11 cells were maintained in RPMI 1640 medium with

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Abbreviations used in this article: ArgI, arginase I; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; DC, dendritic cell; DCF, 2'-7'-dichlorofluorescein diacetate; KO, knockout; M1, classically activated macrophage; M2, alternatively activated macrophage; NOS2, NO synthase 2; WT, wild-type.

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10% heat-inactivated FBS and 1% penicillin/streptomycin (complete RPMI 1640 medium). LA-4 cells were maintained in F-12 Ham's media with 15% FBS and 1% penicillin/streptomycin. Anti-CCL2-neutralizing Ab (MAB479) and mouse cytokine Ab array (ARY006) were purchased from R&D Systems.

Chitin purification

Ground chitin particles were dissolved in 12.5 M HCl and incubated for 30 min at 40°C with frequent agitation. The solution was transferred to a cooled beaker and slowly neutralized with ice-cold NaOH. The insoluble fraction was collected and washed in H₂O three times followed by a wash in ethanol. The purified chitin particles were then dried in a speedvac before storage at -20°C. Before use, chitin particles were resuspended in endotoxin-free PBS, sonicated, and then filtered through a 10- μ m nylon filter. Protein was <2% by mass as determined by bicinchoninic acid assay. Endotoxin levels as measured by Pyrogen Plus assay (Lonza) were <0.03 EU/ml.

Binding assays

For chitin binding assays only, chitin particles < 10 μ m were labeled with FITC as described previously (17). For all other exposures, unlabeled chitin particles were used. AMJ2-C11 macrophages or LA-4 lung epithelial cells were incubated with chitin particles over varying doses and time intervals. Cell samples were washed three times, fixed with 0.5% paraformaldehyde, and analyzed by fluorescence microscopy. All binding assays were performed at 4°C. Binding index is calculated as number of particles/100 cells.

Administration of chitin

Mice were anesthetized via an i.p. injection of etomidate. The anesthetized mouse was then suspended from their front incisors and intubated using a BioLite Intubation System (18). Chitin particles were suspended at indicated concentrations in 20 μ l PBS and administered via the intubation tube into the airway.

Bronchoalveolar lavage

At varied intervals after chitin administration, mice were anesthetized with isoflurane and euthanized by exsanguination. One millimeter of PBS/2 mM EDTA was administered via intratracheal catheter to lavage the airway. Bronchoalveolar lavage fluid (BALF) was placed on ice prior to centrifugation at 300 \times g for 5 min. Cell-free supernatants were frozen at -20°C for later analysis. The BALF cell pellet was resuspended in PBS/1% BSA for analysis by flow cytometry or lysed for RNA extraction. For isolation of alveolar macrophages, the BALF cell pellet was resuspended in PBS/1% BSA, incubated with anti-mouse CD11c-biotin Ab (BD Biosciences) on ice for 30 min, washed, and incubated with streptavidin-coated magnetic beads (BD Biosciences). For chemokine secretion experiments, isolated CD11c⁺ cells were resuspended in RPMI 1640 medium with 10% FBS and 1% penicillin/streptomycin and incubated overnight in 24-well plates at 37°C.

Mouse airway epithelial cell isolation

Airway epithelial cells were isolated as previously described (19) with slight modifications. The pulmonary vasculature was perfused with 5 ml PBS via the right ventricle, and Liberase (Roche) was instilled via a catheter placed in the trachea, followed by 1 ml 1% low melt agarose (ISCBioExpress). The animal was placed on ice to harden the agarose prior to removing the lungs, which were then placed in 2 ml Liberase solution for 1 h at room temperature. Lungs were then teased apart with forceps in DMEM with 1% penicillin/streptomycin (HyClone) and shaken for 10 min at 200 rpm. The resulting suspension was filtered through a 40- μ m filter and centrifuged at 250 \times g for 10 min. After centrifugation, the cells were stained with anti-mouse CD45-biotin Ab (eBioscience) on ice for 30 min, washed, and incubated with streptavidin-coated magnetic beads (BD Biosciences) for CD45⁺ cell depletion. The CD45-depleted suspension was stained with anti-mouse CD326-PE Ab (eBioscience) on ice for 20 min, washed, and incubated with anti-PE-coated magnetic beads (BD Biosciences). The CD326⁺ cells were resuspended in DMEM with 10% FBS and 1% penicillin/streptomycin and incubated overnight in 6-well plates at 37°C. A portion of the cells was analyzed by flow cytometry to assess purity.

Measurement of chemokines

Following overnight incubation at 37°C, cell-free supernatants were collected and stored at -20°C for later analysis by ELISA. ELISA kits for CCL2, CCL17, CCL22, and Ym1 were obtained from R&D Systems.

Flow cytometry

Lung cell suspensions were prepared by mincing lungs through a 70- μ m filter using a 3-ml syringe plunger. The resulting homogenate was digested with Liberase/DNAse I, and the RBCs were lysed with ammonium chloride/potassium bicarbonate buffer. All samples were blocked with anti-mouse CD16/32 Ab prior to staining with fluorochrome-conjugated Abs. Events were gated on forward light scatter/side scatter parameters to exclude debris and on live cells based on Violet Fixable Live-Dead stain (Molecular Probes). Fluorochrome-conjugated Abs used were Mac-3-FITC, Siglec F-PE, CD90.2-PerCP-Cy5.5, CD11b-PECy7, Ly6C-allophycocyanin, CD11c-Alexa700, and Ly6G-allophycocyanin-Cy7. Abs were obtained from BD Biosciences, eBioscience, and BioLegend. Cells were collected for analysis on a BD Biosciences LSRII cytometer and data analyzed with FloJo software (Tree Star).

Real-time PCR

Total RNA was isolated from lung homogenates or isolated cell populations using a Qiagen RNeasy mini kit, and cDNA was prepared using the Bio-Rad iScript cDNA synthesis kit. cDNA was amplified using Bio-Rad SSoFast EvaGreen Supermix in a BioRad MyIQ Real-Time PCR detection system. Primers were designed using Primer-BLAST (20). Relative transcript quantity was calculated using the comparative cycle threshold method with β -actin transcript as the control transcript (21).

Reactive oxygen species measurement

The presence of reactive oxygen species (ROS) in cell-free BALF was assessed using 2'-7'-dichlorofluorescein diacetate (DCF), which becomes fluorescent after exposure to peroxidase and hydrogen peroxide (22). Eighty microliters of BALF or PBS was added to 120 μ l DCF/HBSS in 96-well black opaque plates. After a 20-min incubation in the dark, fluorescence intensity above an empty control 96-well black plate was measured using a Bio-Rad Versadoc5000MP imaging system.

Statistics

The means of fold change in transcript, chemokine protein content, or numbers of leukocytes were compared with control using an unpaired *t* test with a two-tailed *p* < 0.05 considered statistically significant. For experiments where percent reduction versus WT mice were reported, a one-sample *t* test was used to compare values to a hypothetical value of 0 (i.e., no change) with *p* < 0.05 considered statistically significant. All statistical analysis was performed using Prism software (GraphPad). In all figures, error bars represent the SD of the data.

Results

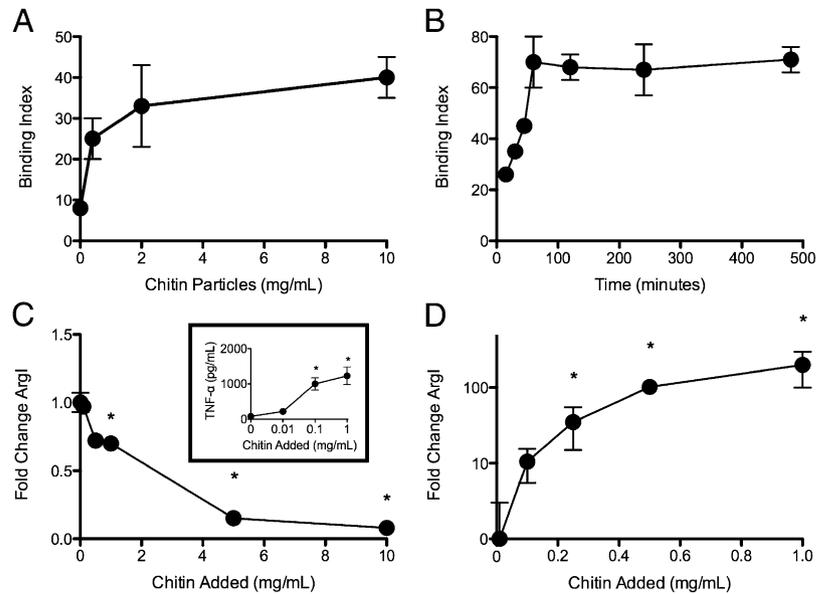
Interaction of chitin with macrophages in vitro and in vivo

To understand the direct effect of chitin exposure on macrophages, we investigated the binding of chitin particles to macrophages and the activation profile of the cells after they were exposed to chitin particles in vitro. AMJ2-C11 macrophages bound FITC-labeled chitin particles in a dose- and time-dependent saturable manner (Fig. 1A, 1B). Because FITC may alter surface binding properties of particles, we confirmed our findings with unlabeled chitin particles that were stained after binding with the chitin selective dye Uvitex 2B. To assess the activation state of macrophages exposed to chitin, we measured expression of arginase I (ArgI), a canonical product of the M2 phenotype (23, 24). Chitin particles failed to induce ArgI expression in AMJ2-C11 macrophages but did activate the cells and induce production of TNF- α , a marker of classical activation in vitro (Fig. 1C). Chitin also failed to induce ArgI expression in alveolar macrophages in vitro (data not shown). Because the context of chitin exposure likely affects the activation state of macrophages, we also evaluated the activation of macrophages in vivo. Accordingly, ArgI expression was strongly induced in the lung following intratracheal exposure to chitin particles (Fig. 1D). Thus, chitin promotes disparate macrophage activation states in vitro and in vivo and the cells exhibit an M2 phenotype only in vivo.

Chitin binds to airway epithelial cells and elicits CCL2 in vitro

Because of the apparent dichotomy of macrophage activation observed above, we hypothesized that chitin was M2 indirectly

FIGURE 1. Macrophages bind chitin and become M2 polarized *in vivo* but not *in vitro*. Dose-dependent (A) and time-dependent (B) binding of chitin particles by AMJ2-C11 macrophages. Binding was performed at 4°C *in vitro* ($n = 3$). Chitin-induced ArgI expression (C) and release of TNF- α (inset) *in vitro* ($n = 3$) and *in vivo* ($n = 3$) (D). * $p < 0.05$ versus no chitin control.



in vivo. Epithelial cells form a barrier to particles and high m.w. molecules in the airway and are activated by chitin-containing allergens such as house dust mite (2, 3). To test whether chitin interacts with airway epithelial cells, we exposed LA-4 murine epithelial cells to FITC-labeled chitin particles *in vitro*. LA-4 cells bound FITC-labeled chitin particles in a dose- and time-dependent saturable manner (Fig. 2A, 2B). Unlabeled chitin particles bound to LA-4 epithelial cells in a similar fashion. To determine whether LA-4 cells were activated by exposure to chitin particles, we used a proteomic array to investigate chitin-induced release of cytokine and chemokine into the cell supernate. Exposure to chitin particles induced a dose-dependent increase in CCL2 secretion but did not induce any of the other 39 targets in the array (Fig. 2C). Notably, chitin exposure to LA-4 epithelial cells failed to induce IL-4 or IL-13 production, cytokines that promote the alternative activation of macrophages. In addition, CCL7, which shares a receptor with CCL2, was also not produced by chitin-exposed epithelial cells *in vitro*. We confirmed the dose-dependent increase in CCL2 production by chitin-exposed epithelial cells by measuring CCL2

in epithelial cell supernatants in amounts ranging up to 300 pg/ml (Fig. 2D).

Chitin exposed airway epithelial cells M2 in a CCL2-dependent manner in vitro

To further investigate whether chitin-exposed epithelial cells could promote the alternative activation of macrophages, we incubated AMJ2-C11 macrophages in supernatants from chitin-exposed LA-4 cells. Supernatants from chitin-exposed epithelial cells resulted in a chitin dose-dependent increase in ArgI expression in the macrophages (Fig. 3A). Given our observation that chitin induces CCL2 secretion from epithelial cells *in vitro*, and prior work that demonstrated an impairment in alternative activation of macrophages in CCL2-deficient animals (25, 26), we investigated whether CCL2 in epithelial cell supernatant is required for alternative activation of macrophages *in vitro*. We used a CCL2-neutralizing Ab to block the product in chitin-exposed epithelial cell supernatants added to the macrophages. In the presence of neutralizing CCL2 Ab, chitin-exposed epithelial cell supernatant lost the ability to induce ArgI

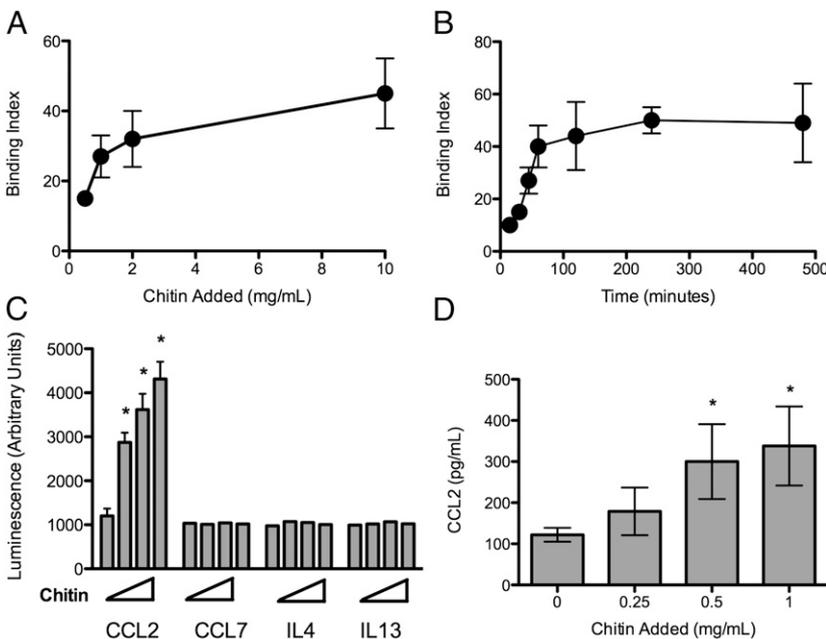


FIGURE 2. Airway epithelial cells bind chitin and produce CCL2 following chitin exposure. Dose-dependent (A) and time-dependent (B) binding of chitin particles to LA-4 epithelial cells. Binding was performed at 4°C *in vitro* ($n = 3$). (C) Chitin induced CCL2, CCL7, IL-4, and IL-13 production ($n = 3$). Chitin added in amounts of 0, 0.25, 0.5, and 1.0 mg/ml is represented by the crescendo. (D) CCL2 production by LA-4 cells 16 h following chitin exposure ($n = 3$). * $p < 0.05$ versus no chitin control.

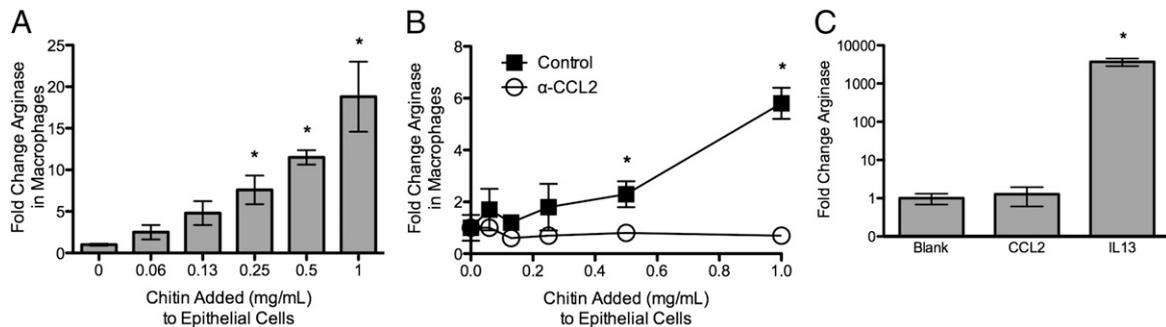


FIGURE 3. Epithelial cell CCL2-dependent M2 polarization in vitro. **(A)** ArgI expression in macrophages cultured in chitin-exposed epithelial cell supernatant for 16 h ($n = 5$, $*p < 0.05$ versus no chitin added). **(B)** Experiment done in the presence of 100 $\mu\text{g/ml}$ CCL2 neutralizing or isotype control Ab ($n = 3$, $*p < 0.05$). **(C)** Effect of recombinant CCL2 or IL-13 (50 ng/ml) on AMJ2-C11 macrophage ArgI expression in vitro ($n = 3$, $*p < 0.05$ versus PBS).

expression in macrophages (Fig. 3B). However, unlike recombinant IL-13, recombinant CCL2 alone was not sufficient to induce ArgI expression in macrophages (Fig. 3C). Therefore, CCL2 is necessary but not sufficient to mediate ArgI expression in macrophages exposed to chitin-stimulated epithelial cells in vitro.

Chitin induces CCL2 from airway epithelial cells in vivo

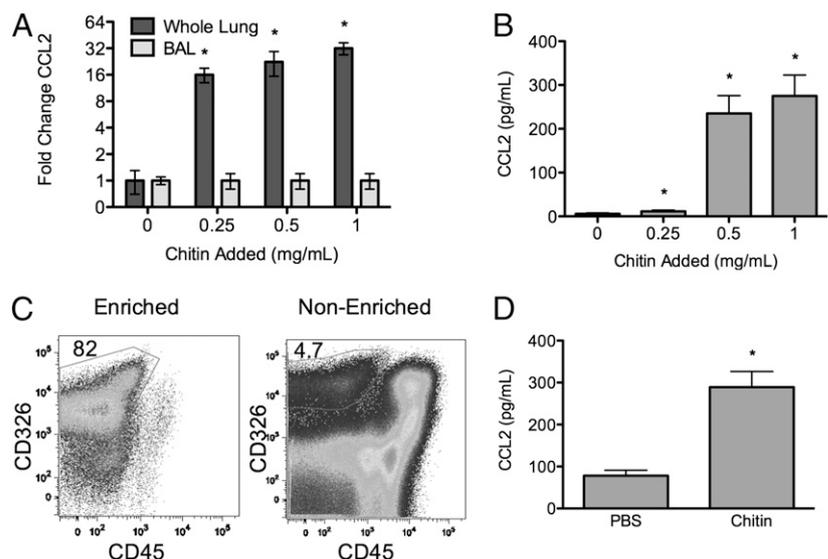
Next, we investigated whether CCL2 is produced by lung epithelial cells after exposure to chitin particles in vivo. Following chitin administration into the airway, we found a dose-dependent increase in CCL2 expression in whole-lung homogenates (Fig. 4A). Although CCL2 protein in cell-free BALF was increased in a dose-dependent fashion by chitin exposure (Fig. 4B), we found no increase in CCL2 in the BALF cell pellet (Fig. 4A), suggesting a lung parenchymal source of CCL2. To investigate whether lung epithelial cells produce CCL2 in response to chitin exposure, we isolated these cells from chitin- and PBS control-exposed lungs. We achieved a ~ 20 -fold enrichment of epithelial cells from whole-lung homogenates by depleting hematopoietic cells (CD45^+) and selecting for cells expressing the epithelial marker CD326 (EPCAM) (Fig. 4C) (27). Following overnight culture of cells ex vivo, the CD45^+ cell populations from chitin-exposed mice did not increase CCL2 production. In contrast, CD326^+ enriched epithelial cells from chitin-exposed mice secreted significantly more CCL2 than PBS control-exposed mice (Fig. 4D). Hence, CD326^+ epithelial cells represent a source of CCL2 in vivo following chitin exposure in the airway.

Chitin-induced alternative activation of macrophages is CCR2 dependent in vivo

To see whether CCL2 plays a role in chitin-induced alternative activation of macrophages in vivo, we analyzed the expression kinetics of CCL2 and the alternative activation marker ArgI in the lung. CCL2 is expressed as early as 2 h following exposure to chitin, whereas ArgI is not expressed until 16 h (Fig. 5A), temporally supporting a role for CCL2 in the induction of ArgI. To determine whether CCL2 is necessary for ArgI expression in the lung, we administered chitin to the airways of CCL2KO mice. We found no impairment in Arg I expression. However, we observed that CCL2KO mice had a significant compensatory increase in CCL7 expression in response to chitin (Fig. 5B).

Because CCL7 signals through the same receptor as CCL2—that is, CCR2 (28, 29)—and contributes to CCR2-mediated activity in parallel with CCL2 (30), we investigated whether CCR2 is required for chitin-induced ArgI expression in vivo. Whole-lung homogenates showed significantly delayed and reduced ArgI expression after chitin exposure in CCR2KO mice compared with WT mice (Fig. 5C). To address whether the defect in M2 polarization in chitin-exposed CCR2KO mice was due to an increase in M1 polarization, we measured NO synthase 2 (NOS2) expression in whole-lung homogenates. Both WT and CCR2KO mice failed to demonstrate an increase in the M1 polarization marker NOS2 following exposure to chitin (Fig. 5D). To determine whether the CCR2-dependent reduction in ArgI expression occurred in alveolar macrophages and therefore reflected an impaired M2 phe-

FIGURE 4. Chitin induces epithelial cell CCL2 secretion in vivo. Chitin induced CCL2 transcript expression in whole-lung homogenate and BAL **(A)** and CCL2 protein level in BAL ($n = 4$, $*p < 0.05$) **(B)**. **(C)** To verify an epithelial source of CCL2, CD326^+ cells were isolated from chitin- or PBS-exposed lungs. The purity of cell separation was assessed by flow cytometry. **(D)** Chitin-exposed CD326^+ cells cultured overnight ex vivo following isolation produce CCL2 ($n = 3$, $*p < 0.05$ versus PBS).



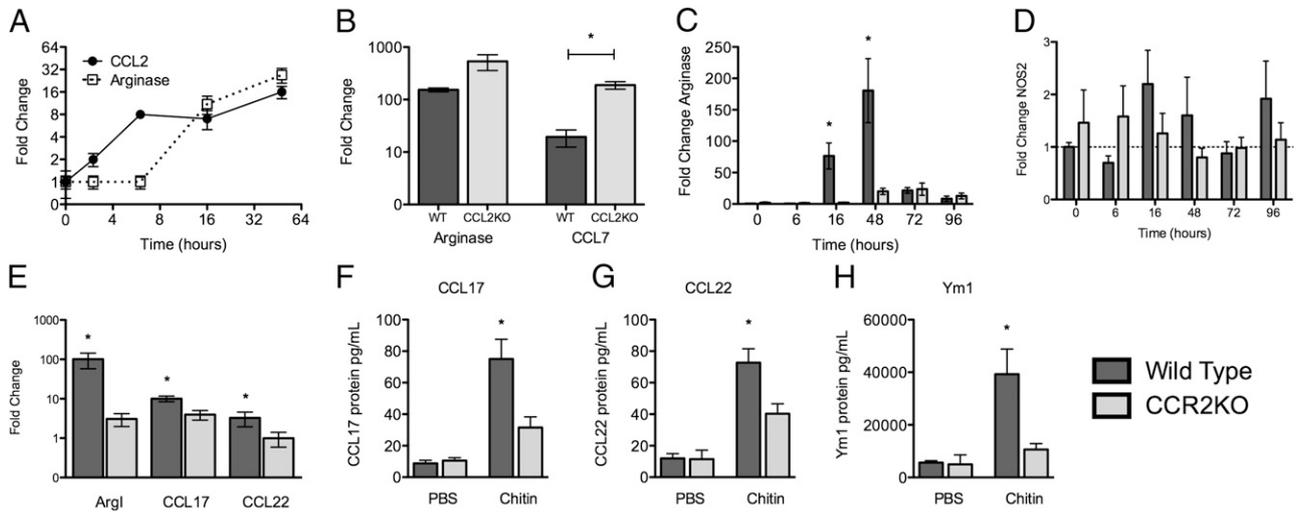


FIGURE 5. Chitin induces CCR2-dependent M2 polarization in vivo. **(A)** Kinetics of Arg1 and CCL2 expression following chitin exposure in vivo ($n = 3$). **(B)** Arg1 and CCL7 expression in WT and CCR2KO mice ($n = 2$, $*p < 0.05$). **(C)** Chitin induction of Arg1 expression in whole lung in WT and CCR2KO mice ($n = 3$, $*p < 0.05$, WT versus CCR2KO). **(D)** Chitin induction of NOS2 expression in whole lung in WT and CCR2KO mice ($n = 3$, $*p < 0.05$, WT versus CCR2KO). **(E)** Chitin-induction of Arg1, CCL17, and CCL22 expression in CD11c⁺ cells from BALF of WT and CCR2KO mice ($n = 2$, $*p < 0.05$, WT versus CCR2KO). **(F–H)** Chitin induced CCL17, CCL22, and Ym1 protein from CD11c⁺ cells ($n = 2$, $*p < 0.05$, WT versus CCR2KO).

nototype, we isolated and analyzed CD11c⁺ cells from bronchoalveolar lavage (BAL). Isolated CD11c⁺ cells were >95% CD11b^{lo}/Mac-3⁺, indicating their identity as macrophages rather than DC. CD11c⁺ cells from CCR2KO mice had significantly lower levels of Arg1 expression than did WT mice (Fig. 5E). To substantiate a defect in M2 polarization in CCR2KO mice, we also measured the expression of other M2 signature chemokines in isolated CD11c⁺ cells. Both CCL17 and CCL22 were expressed at significantly lower levels in CD11c⁺ cells from CCR2KO mice compared with WT mice (Fig. 5E). Similarly, CD11c⁺ cells isolated from the BALF of chitin-exposed CCR2KO mice secreted less CCL17, CCL22, and Ym1 following overnight incubation ex vivo than did CD11c⁺ cells isolated from the BAL of chitin-exposed WT mice (Fig. 5F–H). Therefore, CCR2 signaling is required for chitin-induced M2 polarization in the lung.

Chitin exposure elicits neutrophilic and eosinophilic inflammation and recruits monocytes in a CCR2-dependent manner

To characterize and compare the allergic inflammatory response in the lungs of WT and CCR2KO mice, we quantified leukocyte subsets in whole-lung homogenates following chitin exposure. Overall, cell numbers, influx of CD11c⁺/Mac3⁺/CD11b^{lo} macrophages, Ly6G^{hi} neutrophils, and Thy-1⁺ T cells were all unaffected in CCR2KO mice (Fig. 6A, 6B, 6D, 6F). However, recruitment of CD11b⁺/Ly6C^{hi} monocytes was dependent on CCR2 in response to chitin exposure (Fig. 6E). Furthermore, chitin-induced recruitment of SiglecF⁺/CD11c[−] eosinophils was significantly reduced in CCR2KO mice (Fig. 6C). Thus, CCR2 was linked functionally to the recruitment of eosinophils into the lung following exposure to chitin.

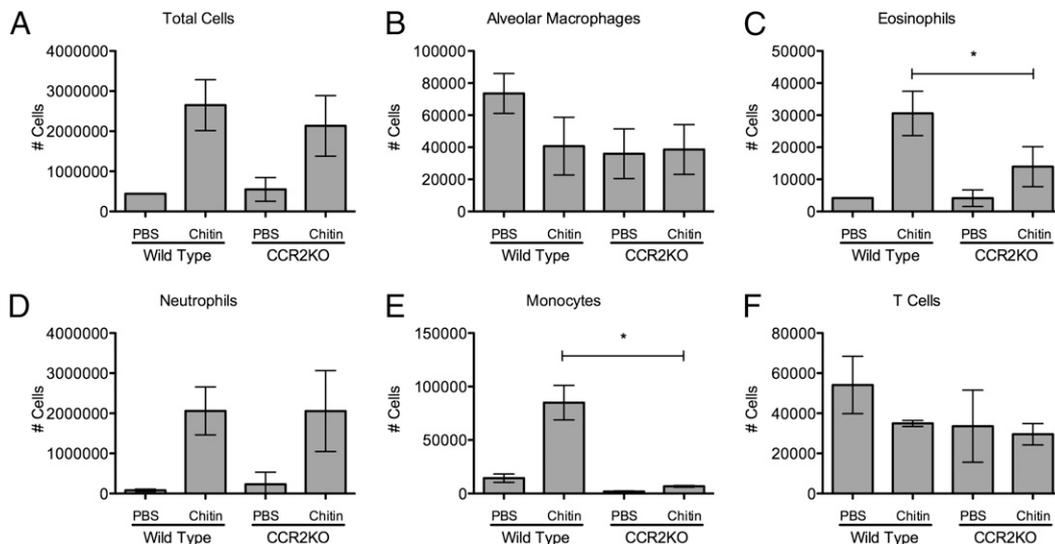


FIGURE 6. Chitin-induced eosinophil and monocyte recruitment is CCR2 dependent. Cell numbers in leukocyte subsets in whole-lung homogenate 48 h after chitin exposure in WT and CCR2KO mice. **(A)** Total live cells. **(B)** CD11c⁺/CD11b^{lo}/Mac3⁺ alveolar macrophages. **(C)** Siglec F⁺/CD11c[−]/CD11b⁺ eosinophils. **(D)** Ly6G⁺/SSC^{hi}/CD11b⁺ neutrophils. **(E)** CD11b⁺/Ly6C^{hi}/Ly6G[−] monocytes. **(F)** Thy1⁺ T cells. $n = 3$, $*p < 0.05$ WT versus control.

Eosinophils have an activation defect in chitin-exposed CCR2-deficient mice

Because we found alterations in the numbers of eosinophils and other innate immune cells recruited to the lung, we investigated whether eosinophil function and other inflammatory parameters in CCR2KO mice were otherwise altered in their response to chitin exposure. Exposure to allergens increases the production of ROS in the airway (31, 32); however, the role of CCR2 in allergen or chitin-induced ROS production remains undefined. BAL obtained from chitin-exposed mice converted significantly more of the redox-sensitive reduced 2'-7'-dichlorofluorescein diacetate dye to fluorescent DCF in WT mice as compared with CCR2KO mice (Fig. 7A). To further investigate eosinophil function during innate allergic inflammation in chitin-exposed mice, we sorted these cells by FACS and analyzed the expression of products associated with eosinophil activation (33). Sorted SiglecF⁺/CD11c⁻ eosinophils from whole-lung homogenates of chitin-exposed animals showed that CCR2KO mice had 60–80% less expression of CCL5, IL-13, and CCL11 than similarly exposed WT mice (Fig. 7B) and also had reduced surface expression of the activation marker CD69 (Fig. 7C). Thus, CCR2 is required for recruitment, activation, and function of allergic innate immune cells such as eosinophils after chitin exposure in vivo.

Discussion

Our work provides new insight into the cellular mechanisms that drive M2 polarization during innate allergic inflammation in response to chitin. Our study suggests that macrophages are not M2 polarized directly on exposure to chitin in the lung. Instead, we demonstrate that chitin stimulates CCL2 production from airway epithelial cells, and we establish a pivotal role for CCR2 in chitin-induced macrophage polarization and innate allergic inflammation in the airway. Isolated CD326⁺ cells from chitin-exposed lungs and LA-4 epithelial cells exposed to chitin in vitro produced elevated levels of CCL2. Compared with WT mice, CCR2KO mice exposed to chitin demonstrated reduced expression of M2 markers (ArgI, CCL17, and CCL22), eosinophil recruitment, and eosinophil activation in the lung. Thus, we propose that the respiratory epithelium modulates M2 polarization of macrophages upon chitin exposure.

Airway epithelial cells are among the earliest cells exposed to inhaled substances and actively collaborate with immune cells to mount innate and adaptive responses. Because chitin is associated with many inhaled allergens, we hypothesize that chitin recognition by airway epithelial cells may promote an epithelial proallergy program to otherwise innocuous agents via secretion of CCL2. Multiple lines of evidence underscore the association of CCL2 with asthma and allergic airway disease. Elevated CCL2 levels are

present in BALF obtained from individuals with asthma (34), and CCL2 is elicited upon airway allergen challenge in humans (35). In animal models, OVA/alum sensitization and challenge provokes increased CCL2 expression (36). Similarly, chitin-associated cockroach (37) or *Aspergillus fumigatus* Ags (38) also provoke CCL2 production in animal asthma models. Our findings suggest that epithelial cells may be an important source of CCL2 during exposure to chitin-containing allergens, thereby promoting M2 polarization.

Consistent with a role for airway epithelial cell CCL2 in allergic responses, chitin exposure promotes M2 polarization in vivo via an indirect mechanism (10) that is dependent on CCR2. We propose that airway epithelial cell-derived CCL2 is a key chemokine in promoting M2 polarization and innate allergic inflammation in response to chitin. Indeed, CCL2 expression precedes ArgI expression in chitin-exposed lungs. Following chitin exposure, Ab neutralization of CCL2 in chitin-exposed epithelial cell supernatants also inhibits M2 polarization in vitro. Airway epithelial cells are an important source of CCL2 in viral infections associated with asthma exacerbations (39, 40). Our findings that CCL2 promotes M2 polarization in a setting of chitin-induced allergic inflammation are consistent with previous reports demonstrating a role for CCL2 in tumor (41)- and thermal injury (25)-associated M2 polarization.

We observed that epithelial cell CCL2 is necessary for M2 polarization in response to chitin in vitro; however, CCL2 was dispensable for M2 polarization in vivo. The upregulation of the CCR2 ligand, CCL7, in CCL2-deficient mice following chitin exposure may obscure the necessary role of CCL2 in chitin-induced M2 polarization we observed in vitro. To address this issue, we investigated the host response to chitin in a CCR2-deficient mouse. In the absence of CCR2, chitin exposure failed to elicit M2 in the lung. Our results reflect the established central role of CCR2 in mediating the effects of both CCL2 and CCL7 in vivo. For example, in a model of infection with the fungal pathogen *Histoplasma capsulatum*, CCR2KO mice demonstrated increased susceptibility to the fungus and an increased fungal burden in the lung (42). Neutralization of either CCL2 or CCL7 was not sufficient to increase the fungal burden; however, neutralization of both chemokines did. Similarly, CCR2KO mice are more susceptible to *Listeria monocytogenes* infection, whereas CCL2KO or CCL7KO mice demonstrate an intermediate susceptibility phenotype (30). Moreover, M2 polarization canonically depends on IL-4R α and its ligands IL-4 and IL-13. Although neither CCL7 nor IL-4 or IL-13 were detected following epithelial cell exposure to chitin in vitro, all three products are present in the lung following chitin exposure in vivo. Our work does not preclude a role for IL-4 in chitin-induced innate allergic inflammation. However, future studies should address whether the CCL2–CCR2 axis and IL-4R α

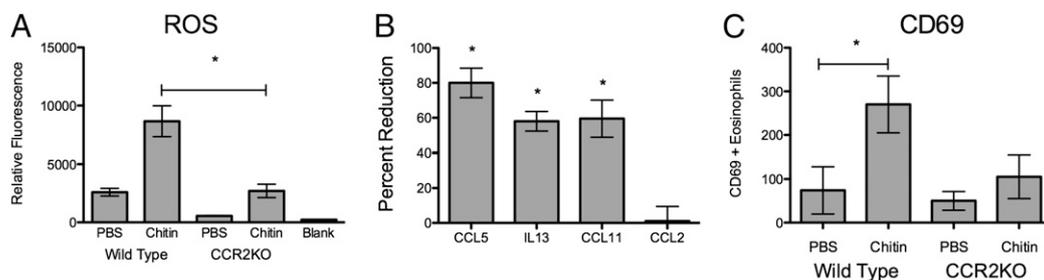


FIGURE 7. Chitin-induced ROS production eosinophil activation is CCR2 dependent. **(A)** ROS levels in BALF from WT and CCR2KO animals 48 h after chitin exposure ($n = 3$, $*p < 0.05$, WT versus CCR2KO). Siglec F⁺/CD11c⁻ lung eosinophils were sorted by FACS from CCR2KO mice versus WT mice 48 h after chitin exposure. **(B)** Percent reduction of eosinophil CCL5, IL-13, and CCL11 expression in CCR2KO versus WT mice ($n = 3$, $*p < 0.05$). **(C)** Number of CD69⁺ eosinophils isolated from mouse lungs 48 h following exposure ($n = 3$, $*p < 0.05$ versus PBS).

signaling represent linear, convergent, or parallel signaling pathways in chitin-initiated M2 polarization.

CCR2KO mice also demonstrate reduced eosinophil recruitment and activation in response to chitin, further suggesting a role for CCR2 in eosinophilic inflammation. Murine eosinophils contain CCR2 mRNA (43) and may express low levels of CCR2 protein on their cell surface (44). However, this is unlikely to explain the reduction in eosinophil recruitment in CCR2KO mice because eosinophils do not migrate in response to CCL2 (45) and migrate toward CCL7 via a CCR3-dependent mechanism (46). Chitin-induced eosinophil recruitment is abrogated in mice lacking the receptor for leukotriene B₄ (9), suggesting a link between eosinophil recruitment and leukotriene signaling following chitin exposure. Because chitin fails to activate eosinophils directly (47), the defect in activation and recruitment in CCR2KO mice must be linked to upstream factors. M2 macrophages elicited by chitin exposure are potent sources of leukotriene B₄ (9). Thus, the reduction in chitin-induced eosinophil recruitment in CCR2KO mice may be due to the reduction in M2 polarization.

CCR2 is required for egress (48) and homing (49) of monocytes to sites of inflammation. We show a near-complete reduction of Ly6C^{hi} monocytes recruited to the lung following chitin exposure in CCR2KO mice. Ly6C^{hi} monocytes may differentiate into CD11b⁺ inflammatory DCs, which accumulate in the allergic airway and are critical mediators of Th2 adaptive immune response in the airway (50). Recently, CCR2⁺Ly6C^{hi} monocytes were reported to recruit eosinophils to colonic mucosa via CCL11 in an experimental colitis model (51). The partnering of Ly6C^{hi} monocytes with M2 macrophages to recruit and/or activate eosinophils could represent an intriguing interaction to therapeutically target to reduce eosinophilic inflammation during asthma and allergy.

In conclusion, we show that airway epithelial cells secrete CCL2 and that CCR2 signaling is required for events leading to M2 polarization and recruitment and activation of eosinophils upon chitin exposure. The findings clarify the mechanisms that drive alternative activation of macrophages in response to chitin and identify possible targets of therapeutic intervention in the setting of chitin induced innate allergic inflammation.

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Disclosures

The authors have no financial conflicts of interest.

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