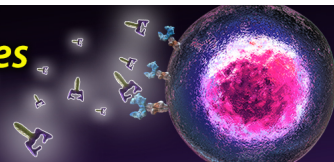




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J Immunol 2016; 196:2021-2030; Prepublished online 29
January 2016;
doi: 10.4049/jimmunol.1500747
<http://www.jimmunol.org/content/196/5/2021>

**Supplementary
Material** [http://www.jimmunol.org/content/suppl/2016/01/29/jimmunol.150074
7.DCSupplemental](http://www.jimmunol.org/content/suppl/2016/01/29/jimmunol.1500747.DCSupplemental)

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Clusterin Modulates Allergic Airway Inflammation by Attenuating CCL20-Mediated Dendritic Cell Recruitment

Gyong Hwa Hong,^{*,†} Hyouk-Soo Kwon,[‡] Keun-Ai Moon,[†] So Young Park,[‡] Sunjoo Park,[†] Kyoung Young Lee,[†] Eun Hee Ha,^{*,†} Tae-Bum Kim,[‡] Hee-Bom Moon,[‡] Heung Kyu Lee,^{*,§} and You Sook Cho[‡]

Recruitment and activation of dendritic cells (DCs) in the lungs are critical for Th2 responses in asthma, and CCL20 secreted from bronchial epithelial cells (BECs) is known to influence the recruitment of DCs. Because asthma is a disease that is closely associated with oxidative stress, we hypothesized that clusterin, an oxidative stress regulatory molecule, may have a role in the development of allergic airway inflammation. The aim of this study was to examine whether clusterin regulates CCL20 production from the BECs and the subsequent DC recruitment in the lungs. To verify the idea, clusterin knockout ($Clu^{-/-}$), clusterin heterogeneous ($Clu^{+/-}$), and wild-type mice were exposed intranasally to house dust mite (HDM) extract to induce allergic airway inflammation. We found that the total number of immune cells in bronchoalveolar lavage fluid and the lung was increased in $Clu^{-/-}$ and $Clu^{+/-}$ mice. Of these immune cells, inflammatory DCs ($CD11b^{+}CD11c^{+}$) and $Ly6C^{high}$ monocyte populations in the lung were significantly increased, which was accompanied by increased levels of various chemokines, including CCL20 in bronchoalveolar lavage fluid, and increased oxidative stress markers in the lung. Moreover, HDM-stimulated human BECs with either up- or downregulated clusterin expression showed that CCL20 secretion was negatively associated with clusterin expression. Interestingly, clusterin also reduced the level of intracellular reactive oxygen species, which is related to induction of CCL20 expression after HDM stimulation. Thus, the antioxidant property of clusterin is suggested to regulate the expression of CCL20 in BECs and the subsequent recruitment of inflammatory DCs in the airway. *The Journal of Immunology*, 2016, 196: 2021–2030.

Asthma is a chronic inflammatory airway disease characterized by Th2-dominant immune reactions. Exposure to environmental allergens, such as house dust mite (HDM), is one of the critical factors that causes asthmatic airway inflammation. Bronchial epithelial cells (BECs) are the frontline physical barrier first encountered by inhaled allergens and are critical immune cells that play a key role in producing proinflammatory cytokines and chemokines that activate and recruit immune cells into the lungs (1, 2). In fact, BECs were suggested to play an important role in mounting the immune reactions of asthmatic airways through recruitment of dendritic cells (DCs) in response to allergen exposure (3–5).

The ubiquitously expressed secretory glycoprotein clusterin is found in several types of lining epithelial cells. Studies of clusterin have focused primarily on its role in protecting against heat and other stresses. Therefore, it has been classified as a chaperone protein (6, 7) and stress-inducible biomarker (8, 9). Because many stress-induced transcription factors, including heat shock transcription factor-1 and activator protein-1, can recognize the conserved clusterin promoter (10, 11), it is particularly sensitive to minute environmental changes, including oxidative stress, heat, and radiation (12). In fact, clusterin shows antioxidative and antiapoptotic properties, and its expression indicates a state of increased oxidative injury. The results of our previous study showed that increased clusterin levels in PBMCs obtained from asthmatic patients clearly correlated with increased oxidative stress status (13). However, the precise role of clusterin in asthmatic BECs, which are the first cells to encounter various allergens, remains to be clarified.

CCL20 is a well-known chemokine that is primarily expressed by epithelial cells, such as BECs, intestinal epithelial cells, and keratinocytes (14). It functions in the recruitment of inflammatory cells by binding to CCR6 expressed on DCs, neutrophils, and memory T lymphocytes. Particularly, DCs, which are critically linked to initiation of immunity to Ags, are recruited to certain sites through CCL20–CCR6 interaction. Recently, CCL20 was reported to be upregulated in various inflammatory diseases, such as allergic airway disease (15, 16), rheumatoid arthritis (17), and inflammatory bowel disease (18).

HDM extracts can activate various receptors expressed on BECs and induce inflammatory cytokines and chemokines, including CCL20. Although the exact mechanism underlying CCL20 induction by HDM remains to be clarified, it was reported recently that oxidative stress regulates HDM-induced epithelial cell activation, which leads to CCL20 production (19–21). In addition,

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Received for publication April 2, 2015. Accepted for publication January 3, 2016.

This work was supported by the Korean Health Technology Research and Development Project (Grants H113C1962 and H114C2628) of the Ministry of Health and Welfare, Republic of Korea.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; BEC, bronchial epithelial cell; DC, dendritic cell; HDM, house dust mite; HNE, hydroxynonenal; MDA, malondialdehyde; NAC, N-acetylcysteine; pDC, plasmacytoid DC; rCLU, recombinant clusterin; ROS, reactive oxygen species; siRNA, small interfering RNA; WT, wild-type.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1500747

recent studies associated elevated oxidative stress with the initiation of inflammatory signaling cascades and pathogenesis of asthma (22–24).

Taken together, it can be assumed from the existing evidence that CCL20 production by BECs may be regulated by clusterin through the modulation of the oxidative stress milieu, which, in turn, controls allergic inflammation. In our current study, we hypothesized that clusterin may negatively regulate CCL20 production in response to HDM exposure by reducing intracellular oxidative stress.

Materials and Methods

Generation of murine models of HDM-induced asthma

All mice were bred in a specific pathogen-free animal facility. Clusterin-knockout mice ($Clu^{-/-}$) were purchased from the Jackson Laboratory (Bar Harbor, ME), and their wild-type (WT) littermates were used for wild-type control. Animal study protocols were approved by the Institutional Animal Care and Use Committee (Asan Medical Center).

To generate the HDM-induced asthma model, we sensitized mice by intranasal installation with 30 μ g HDM extract (*Dermatophagoides pteronyssinus*; Yonsei University, Seoul, Korea) for five consecutive days for 6 wk. The control group received no treatment. Bronchoalveolar lavage fluid (BALF), lymph nodes, and lung tissues were obtained from WT, $Clu^{+/+}$, and $Clu^{-/-}$ mice in HDM-stimulated and control settings 24 h after the last immunization. Histopathologic results were compared among the groups.

BALF analysis

A total of 2 ml BALF was obtained after tracheostomy by lavage using PBS. Cells were collected by centrifugation at $400 \times g$ for 10 min at 4°C, and the pellets were resuspended in PBS. First, the number of total cells was counted, and differential cell counts were determined after cytocentrifugation (StatSpin CytoFuge 12; Iris, Norwood, MA) at $400 \times g$ for 5 min at room temperature, followed by a Diff-Quik stain (Sysmex, Kobe, Japan) and fixation with a synthetic mounting medium (Histomount; Ted Pella, Redding, CA). At least 300 cells were counted in each preparation to measure the number of monocytes, eosinophils, neutrophils, and lymphocytes in the BALF. In addition, the concentrations of various cytokines, such as CCL2, CCL11, IL-8, CCL5, IP-10, IL-4, IL-5, IL-13, and CCL20, in BALF were measured using ELISA.

Histopathological evaluation

The lungs were perfused with 5 ml PBS through the right ventricle and fixed with 10% neutral buffered formalin. Fixed lungs were embedded in paraffin and sectioned at 4 μ m. To examine the magnitude of inflammation around the bronchial and vascular area, lung sections were stained with H&E. To quantify the inflammation in the lung, more than seven sections of the bronchus of each animal were randomly selected and given scores ranging from 0 to 3, based on the level of peribronchial and perivascular inflammation. The values were assigned according to the following parameters: 0, no inflammation; 1, occasional inflammatory cells; 2, most bronchi or vessels surrounded by a thin layer of inflammatory cells; and 3, most bronchi or vessels surrounded by a thick layer of inflammatory cells.

Flow cytometry analysis of total lung cells

To obtain total lung cells, the lungs were perfused with 5 ml PBS through the right ventricle and minced using sterile blades in RPMI 1640 medium with 1% penicillin/streptomycin. Collagenase and DNase I were added to the minced lung tissues. After incubation for 1 h at 37°C, lung cells were filtered through a 70- μ m strainer. Isolated lung cells were stained with Abs to mouse CD103, MHC class II, NK1.1, CD45, CD3, CD19, BST2, B220, Siglec F, Ly6c, Ly6G, CD11c, CD11b, Gr-1, and F4/80 that were conjugated to FITC, PE, PerCP-Cy 5.5, PE-Cy7, allophycocyanin, allophycocyanin-Cy7, or Pacific Blue (all from eBioscience, San Jose, CA). The stained cells were analyzed using a FACSCanto (BD Bioscience, San Jose, CA) and FlowJo software (TreeStar, Ashland, OR).

Immunoblot analysis for measuring reactive oxygen species from mice lungs

Mice lung tissues were lysed on ice in lysis buffer (Cell Signaling, Danvers, MA) containing protease inhibitors for 20 min and then centrifuged at $18,000 \times g$ for 10 min at 4°C. The protein was obtained, separated using SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. To

detect a footprint molecule indicating exposure to reactive oxygen species (ROS), anti-4-hydroxyl-2-nonenal (HNE; Alpha Diagnostic, San Antonio, TX) Abs were used. Anti- β -actin (Bioworld, St. Louis Park, MN) was used as a housekeeping molecule. Anti-rabbit, -goat, and -mouse secondary Abs were purchased from Bethyl Laboratories (Montgomery, AL). These protein bands were detected by ECL solution (GenDEPOT, Barker, TX). The intensity of the resulting bands was densitometrically analyzed using ImageJ.

Malondialdehyde assay

Frozen lung tissues from mice were homogenized in lysis buffer. The homogenates were incubated for 30 min on ice and centrifuged. Supernatants were collected for total malondialdehyde (MDA) measurements. The reaction mixture contained 100 μ l lung supernatants, 100 μ l 8.1% SDS, 200 μ l 20% acetic acid (pH 3.5), and 200 μ l 0.8% thiobarbituric acid, and it was incubated for 60 min at 95°C. The mixture was centrifuged briefly to separate the phases, and the absorbance of the upper phase was measured at 532 nm. Absorbance was converted to μ M MDA from a standard curve generated with 1,1,3,3-tetramethoxypropane.

Cell culture and stimulation with LPS and HDM

To evaluate the role of clusterin in BECs, the human bronchial cell line BEAS-2B was purchased from the American Type Culture Collection (Manassas, VA) and cultured in LHC-9 medium (Life Technologies, Carlsbad, CA). Cells were grown on tissue-culture plates coated with collagen (Welgene). Cells were seeded at a density of 2×10^5 cells/well in six-well plates and stimulated for 24 h with LPS (Sigma-Aldrich, St. Louis, MO) at concentrations of 0.1–10 μ g/ml and with HDM extracts at concentrations of 1–10 μ g/ml.

Modulation of clusterin expression

For target gene overexpression, BEAS-2B cells were transfected with pcDNA 5/FRT/TO vector or adenovirus vector containing clusterin and cultured for 24 h. Empty vector or D-galactosidase adenovirus was used as a control. Transfection was performed with 1 μ g vector and Lipofectamine 2000 reagent, according to the manufacturer's protocol using Opti-MEM media (both from Invitrogen, Carlsbad, CA). Cells were used for experiments 24 h after transfection.

For target gene-silencing experiments, small interfering RNA (siRNA) targeting human clusterin and nontargeting control siRNA were purchased from GE Dharmacon (Lafayette, CO). Transfection was performed with 20 nM siRNA and RNAiMAX reagent (Invitrogen), according to the manufacturer's protocol using Opti-MEM media. Twenty-four hours after transfection, the media were changed, and cells were used for experiment.

ELISA

The concentrations of all chemical mediators in this study (i.e., IL-4, IL-5, IL-13, CCL2, CCL5, CCL11, CCL20, IP-10, and clusterin), were measured in each in vitro experiment using DuoSet ELISA development kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Measurement of intracellular ROS

Clusterin-overexpressing BEAS-2B cells were incubated with HDM and labeled with 5 μ M 2',7'-dichlorofluorescein (Invitrogen) for 30 min at 37°C. 2',7'-Dichlorofluorescein fluorescence was measured with excitation and emission settings of 495 and 525 nm, respectively, using a Wallac 1420 Victor2 multilabel plate reader (PerkinElmer, Boston, MA).

Statistical analysis

Results are presented as the mean $\pm$ SEM. Two-way ANOVA was used to determine differences among multiple groups, with post hoc comparisons performed using the Bonferroni method. For comparisons between two groups, the Student *t* test was performed (GraphPad Prism; GraphPad, La Jolla, CA). The *p* values < 0.05 were considered statistically significant.

Results

Clusterin deletion in mice increases recruitment of inflammatory cells to the lung in HDM-induced asthma model

First, the clusterin level was measured in homogenized lung tissues, and we confirmed that clusterin was not expressed in $Clu^{-/-}$

mice. We also detected lower clusterin expression in heterozygous ($Clu^{+/-}$) mice compared with WT mice (Fig. 1A). We then generated an HDM-induced asthma murine model, as described in Fig. 1B. In both $Clu^{-/-}$ and $Clu^{+/-}$ asthmatic mice, increased infiltration of inflammatory cells around the peribronchial and perivascular area was observed in the histopathologic examination of the lung (Fig. 1C, 1D). In addition, a higher number of inflammatory cells was also found in BALF from $Clu^{-/-}$ and $Clu^{+/-}$ mice. Also, there was increased infiltration of immune cells, including macrophages and eosinophils in the BALF of both $Clu^{-/-}$ and $Clu^{+/-}$ mice (Fig. 1E). These results were similar to those from a

conventional acute asthma model using HDM and i.p. sensitization with alum (Supplemental Fig. 1).

Clusterin deletion in mice increases IL-4 and chemokine levels in BALF

First, Th2-type cytokines were measured in the BALF from mice of the HDM-induced asthma model. The levels of IL-5 and IL-13 were not significantly changed in clusterin-deficient mice compared with WT asthmatic mice (data not shown), whereas IL-4 was increased in $Clu^{-/-}$ asthmatic mice (Fig. 2A). We next measured chemokine levels, including CCL2, CCL5, CCL11, CCL20,

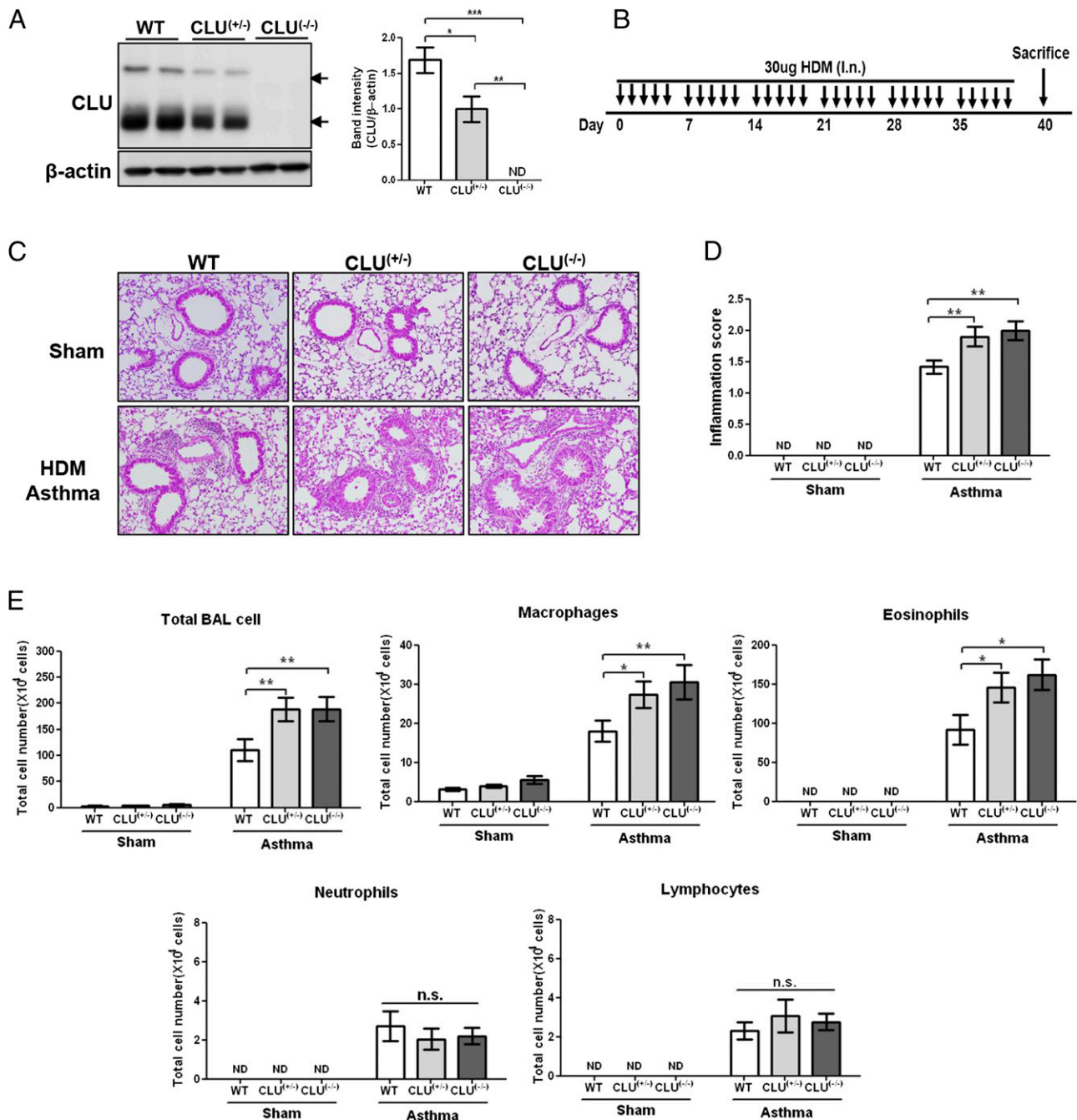


FIGURE 1. Clusterin deletion increases recruitment of inflammatory cells to the lung in HDM-induced mouse model of asthma. (A) Clusterin expression in lung tissues from WT, $Clu^{+/-}$, and $Clu^{-/-}$ mice. Clusterin precursor (upper arrow) and clusterin α (lower arrow) were detected by Western blotting (left panel), and their expression levels were normalized to that of β -actin (right panel). (B) Protocol for the HDM-induced asthma model. (C) Histology of lung sections after H&E staining (original magnification $\times 200$). (D) Statistical analysis of the inflammation score. (E) Total cell number and differential cell analysis of BALF in each group. Similar results were obtained from three independent experiments ($n \geq 8$ /group). * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. ND, not detected; n.s., not significant.

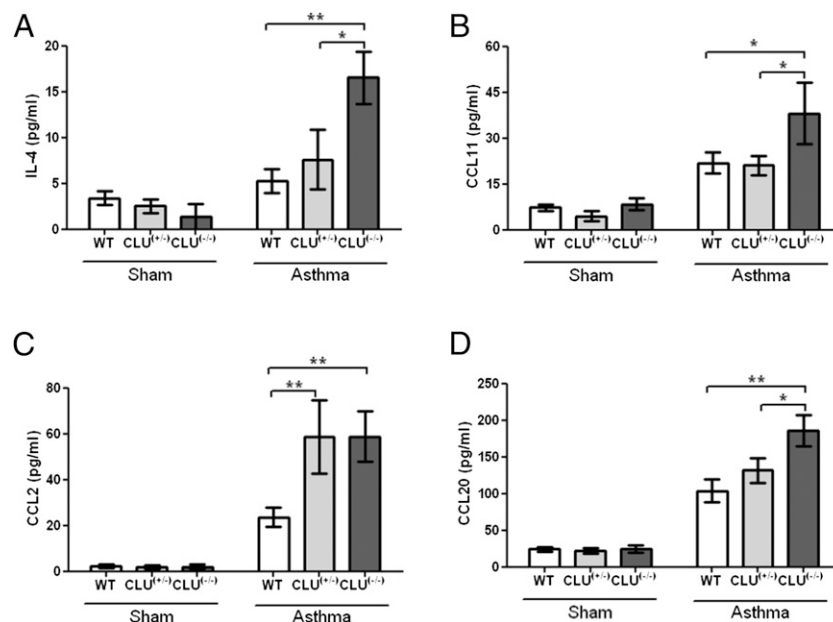


FIGURE 2. The effect of clusterin on IL-4 and chemokine production in HDM-induced mouse model of asthma. Levels of IL-4 (**A**), CCL11 (**B**), CCL2 (**C**), and CCL20 (**D**) in BALF. Similar results were obtained from three independent experiments ($n \geq 8/\text{group}$). $*p < 0.05$, $**p < 0.001$.

IL-8, and IP-10, in BALF produced by activated BECs. CCL20, CCL2, and CCL11 levels were higher in the BALF of Clu^{-/-} and Clu^{+/-} mice than in that of WT mice (Fig. 2B–D). There were no significant differences in the level of IL-8 (data not shown). IP-10 and CCL5 were not detected in this model.

Clusterin deletion in mice alters DC homing into the lung

Lung DCs can be subdivided into several groups based on their surface markers: CD103⁺ conventional DCs, CD11b⁺ conventional DCs, and plasmacytoid DCs (pDCs) (25, 26). Because more chemokines that can recruit DCs were produced in Clu^{+/-} and Clu^{-/-} mice, we also evaluated immune cell populations in the lung using flow cytometry to identify cell composition in the lung. First, recruitment of each DC subset was examined as described in Fig. 3A. pDCs were identified by nonexpression of CD3, CD19, NK1.1, and expression of B220 and BST2 among MHCII⁺CD11c⁺ cells, whereas CD103 or CD11b was used for identifying CD103⁺ DCs or CD11b⁺ DCs. An increase in the frequency of CD103⁺ CD11b⁺ DCs and a decrease in the frequency and total cell number of CD103⁺ CD11b⁻ DCs were observed in Clu^{+/-} and Clu^{-/-} mice (Fig. 3B, 3D, Supplemental Fig. 2A). In contrast, there was no significant difference in the frequency of pDCs in the lung between the groups (Fig. 3C, 3D).

Clusterin deletion in mice increases recruitment of immune cells, including eosinophils and Ly6C^{high} monocytes

Recent studies suggested that resident alveolar macrophages display an intrinsic ability to promote regulatory T cells to maintain tolerance in the steady-state (27–29), whereas recruited monocytes promote allergic lung inflammation (30). Thus, we checked alveolar macrophages and recruited monocyte populations in this model, as described in Fig. 4A. Alveolar macrophages were significantly reduced in all asthmatic mice; however, there was no difference between the groups (Fig. 4B, Supplemental Fig. 2B). Interestingly, a higher frequency of monocytes was observed in Clu^{-/-} mice, even in the steady-state. Furthermore, recruited Ly6C^{high} monocytes were increased in Clu^{-/-} asthmatic mice (Fig. 4C). We also analyzed neutrophils and eosinophils. Similarly to the BALF analysis, neutrophil frequency in lung showed no significant difference between the groups in this HDM-induced asthma model (Supplemental Fig. 3). In contrast, after gating

out the neutrophils, increased eosinophils were detected in the clusterin-deficient condition. These results were similar to the pattern of immune cells in BALF (Fig. 4B, 4C).

Clusterin deletion in mice increases oxidative stress-induced lipid peroxidation in the lung

Because clusterin was reported to protect cells from oxidative stress (8), we tested whether the absence of clusterin could cause the oxidative stress-induced lung tissue damage. To examine whether clusterin regulates oxidative stress in vivo after HDM exposure, we measured the levels of MDA and HNE, which are biomarkers of tissue injury and oxidative stress, respectively (31). Lung tissues were obtained from unsensitized female mice at 6–7 wk of age, and each lipid peroxidation marker was measured. Notably, MDA and HNE levels gradually increased with clusterin deficiency (Fig. 5). In addition, HNE expression was remarkably increased in the lungs compared with the conventional acute asthma model using HDM and i.p. sensitization with alum (Supplemental Fig. 1D). These results demonstrate that oxidative stress is regulated by clusterin.

HDM and LPS promote secretion of CCL20, but not clusterin, from human BECs

Next, the role of clusterin was investigated using BECs. First, we measured mRNA expression of CCL2, CCL20, and CCL11 because these chemokines showed meaningful changes in vivo. However, only CCL20 mRNA expression was increased significantly in BEAS-2B after stimulation with 10 μg HDM (Supplemental Fig. 4). Therefore, the level of CCL20 secretion from human BECs was measured when the cells were exposed to HDM and LPS. As shown in Fig. 6A and 6B, HDM and LPS induced CCL20 secretion in a dose-dependent manner. We next measured the level of clusterin expression in activated BEAS-2B cells upon LPS and HDM stimulation. In contrast, clusterin expression was significantly decreased in LPS- and HDM-stimulated BEAS-2B cells (Fig. 6C, 6D).

Clusterin negatively regulates CCL20 secretion in activated BEAS-2B cells when they are exposed to HDM

We investigated whether the magnitude of clusterin expression could modulate CCL20 release. First, intracellular clusterin overexpression was induced using an adenovirus-transfection

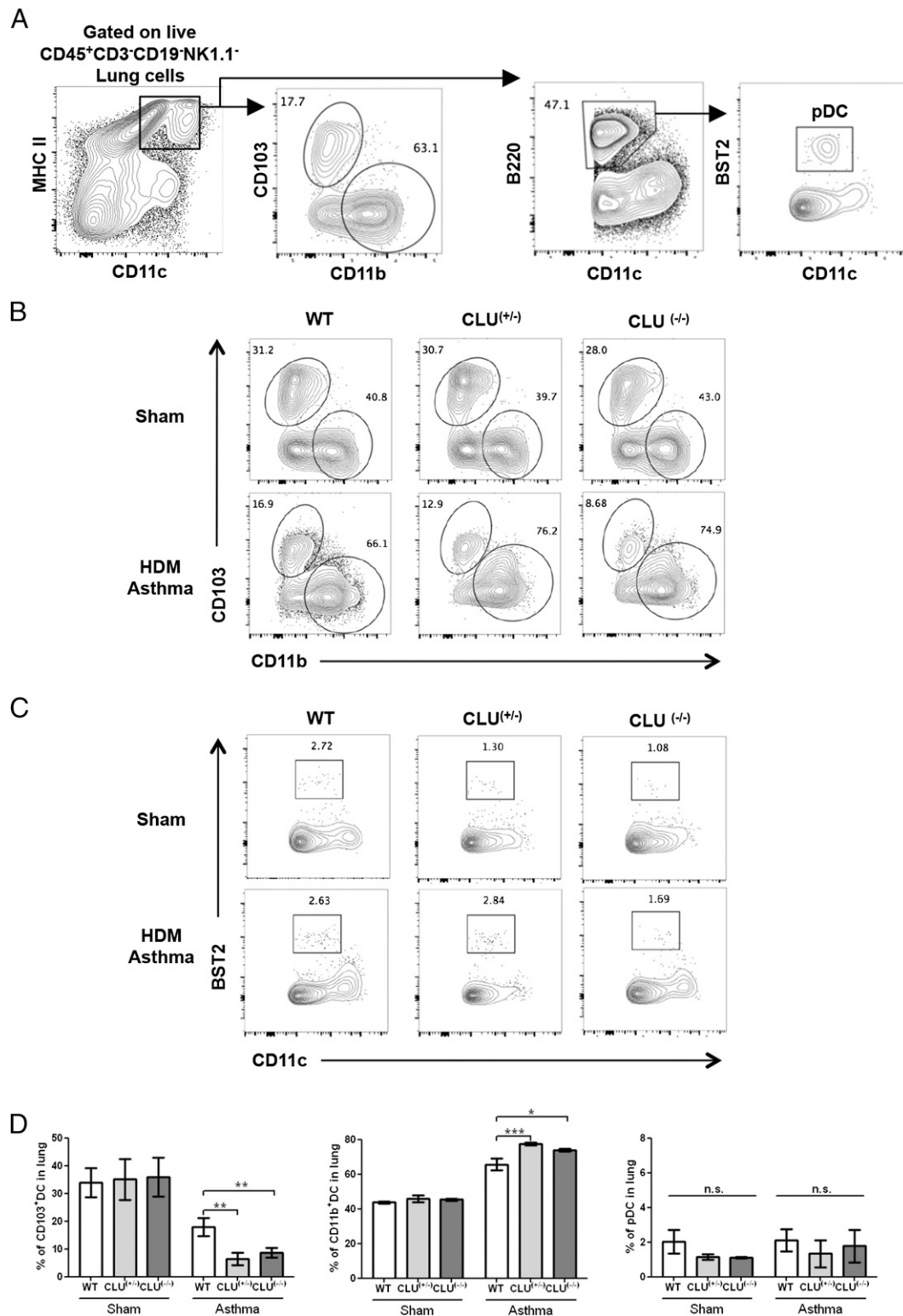


FIGURE 3. Clusterin deletion alters DC homing into the lung. **(A)** Gating strategy for DCs in the lung. **(B)** Representative dot plots show the CD103⁺ DC population within the *left gates* and CD11b⁺ DC population within the *right gates* among lung DCs. **(C)** Representative dot plots show the BST2⁺ CD11c^{int} pDCs within the gates among lung DCs. **(D)** The frequency of DC subsets in lung. Similar results were obtained from three independent experiments ($n \geq 8$ /group). * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. n.s., not significant.

system. CCL20 levels dramatically decreased in clusterin-overexpressing cells (Fig. 7A). Next, we investigated whether extracellular treatment with recombinant clusterin (rCLU) could inhibit CCL20 production in BEAS-2B cells exposed to HDM. We found decreased CCL20 secretion in rCLU-treated cells

(Fig. 7B). Then, to examine the effect of the suppression of clusterin expression in BEAS-2B cells, the cells were transfected with clusterin siRNA or control siRNA. Downregulated clusterin gene expression was confirmed at the mRNA level after siRNA transfection. Robust secretion of CCL20 from HDM-stimulated

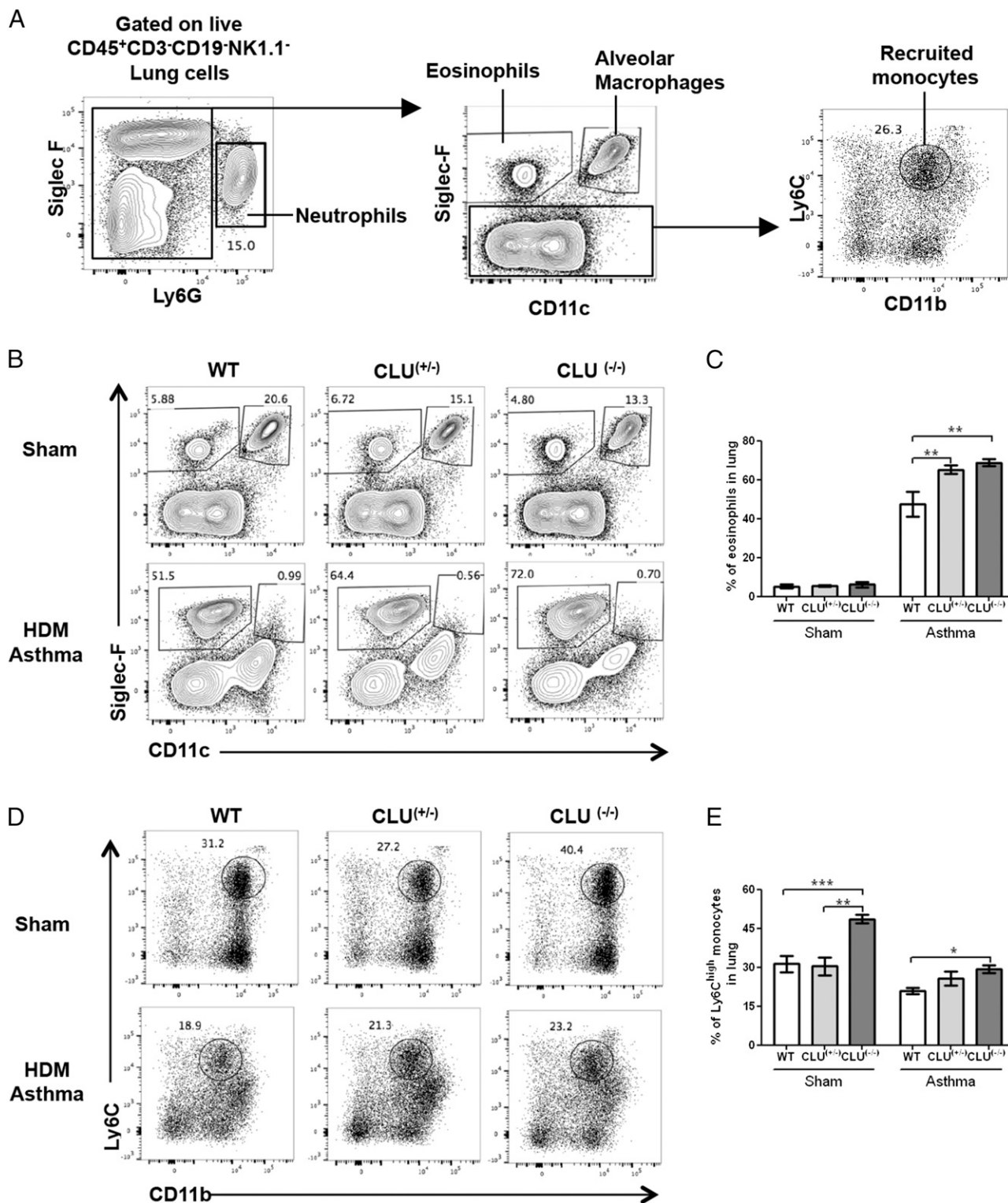


FIGURE 4. Clusterin deletion increases the recruitment of Ly6C^{high} monocytic cells and eosinophils. **(A)** Gating strategy for neutrophils, eosinophils, alveolar macrophages, and monocytes. **(B)** Representative dot-plots show the eosinophils population (left gate) and SiglecF⁺CD11c⁺ alveolar macrophages population (right gate) in lung of mice challenged with HDM. **(C)** The frequency of eosinophils in lung. Dot plot **(D)** and the frequency **(E)** showing the gate Ly6C^{high} monocyte population in lung of mice challenged with HDM. Similar results were obtained from three independent experiments ($n \geq 8$ /group). * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

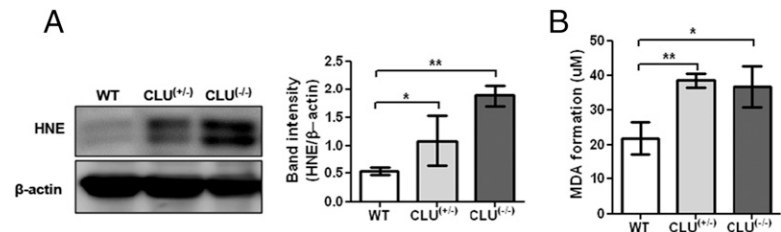
BEAS-2B cells was seen when clusterin was suppressed (Fig. 7C).

Clusterin downregulates HDM-induced ROS generation

Because clusterin is a highly sensitive biosensor of increased oxidative stress, we evaluated its effect on the regulation of intracellular

ROS levels. First, we found that *N*-acetylcysteine (NAC), a well-known antioxidant, significantly reduced HDM-induced CCL20 secretion in BEAS-2B cells (Fig. 8A). After HDM treatment, the intracellular ROS level in BEAS-2B cells was increased for up to 15 min before normalizing after 60 min (Fig. 8B).

FIGURE 5. Oxidative stress in unsensitized mouse lungs. **(A)** 4-HNE-modified proteins were detected (*left panel*), and HNE expression was normalized to β -actin expression (*right panel*). **(B)** MDA formation in the lung. $n \geq 4$ for each group. * $p < 0.05$, ** $p < 0.001$.



Next, the intracellular ROS level was measured in clusterin-overexpressing conditions after HDM stimulation in BEAS-2B cells. As shown in Fig. 8C, dramatically decreased intracellular ROS was detected in clusterin-overexpressing conditions. Then, to investigate whether secreted clusterin can also regulate oxidative stress, untransfected BEAS-2B cells were treated with culture supernatants from BEAS-2B cells overexpressing clusterin. Supernatant-treated cells had reduced intracellular ROS (Fig. 8D). This observation confirms that the secreted clusterin regulates oxidative stress against HDM.

Discussion

In the present study, we found that clusterin is negatively regulated by CCL20 production and that clusterin could be a regulatory molecule with an anti-inflammatory effect in the asthmatic airway that is exerted through reduced recruitment of DCs and decreased oxidative stress biomarkers in the lung. It also appears that BECs are critically involved in this process by regulating the production of CCL20 when the cells are exposed to Ags and stimulants under various microenvironmental conditions.

Asthma is a complex and heterogeneous disease. Although asthma cannot be linked to a single pathogenic mechanism because of the complexity of the disease, one of its invariable characteristics is sustained allergic airway inflammation (32, 33). The inflammation is probably initiated by inappropriate immune responses to certain Ags or irritants in the airway and persists as a result of abnormalities in immune homeostasis. A myriad of genetic and environmental factors, including dysfunctional immune cells and airway constitutional cells, increased ROS in the

microenvironment, and exposure to certain Ags, is suggested to be critical for the development and persistence of allergic inflammation (22, 34, 35).

Clusterin, which is abundantly and widely distributed in our bodies, is a stress-inducible protein that protects cells from variable cellular stress. In particular, clusterin is a highly sensitive cellular biosensor of oxidative stress (8, 9). Increased generation of oxidative stress was reported in both the serum and exhaled breath of asthmatic patients compared with healthy controls. Indeed, clusterin from asthmatic patients clearly correlated with increased oxidative stress status (13). Moreover, clusterin is reported to exhibit intra- and extracellular interactions with inflammation-associated molecules, such as complement factors, $\text{I}\kappa\text{B}\alpha$, and $\text{TGF-}\beta$, suggesting that it plays an important role in modulating conditions related to inflammation and immune responses (36–38). Therefore, it is presumed that an improper regulation of clusterin would be critically linked to the development and persistence of airway inflammation.

In the present study, we show that there is a potential anti-inflammatory role for clusterin in allergic airway inflammation. Our histopathologic findings and BALF cell counts indicated higher immune cell recruitment in asthmatic mice lacking clusterin. In addition, the Th2 cytokine, IL-4, was significantly increased in $\text{Clu}^{-/-}$ mice, based on the analysis of cytokines from BALF. Moreover, as expected, expression of CCL2 and CCL11 as well as CCL20 was increased in the BALF of $\text{Clu}^{-/-}$ mice.

Lung DCs can be subdivided into several groups based on their surface markers in the steady-state: CD103^+ DCs, CD11b^+ DCs, and pDCs (25, 26). The CD11b^+ DC population expands with the

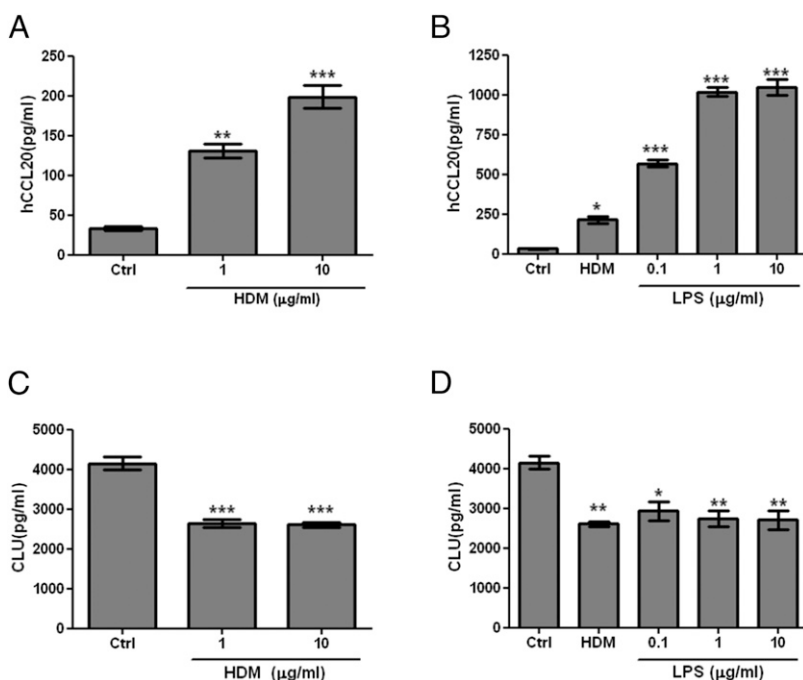
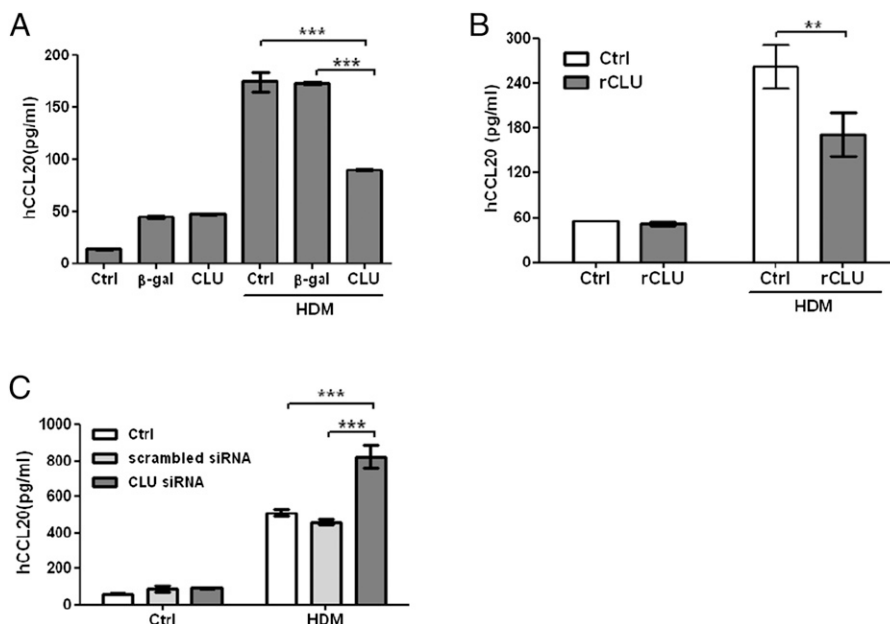


FIGURE 6. CCL20 and clusterin release from activated BEAS-2B cells. BEAS-2B cells were treated with various doses of HDM or LPS (1–10 $\mu\text{g/ml}$). CCL20 (**A** and **B**) and clusterin (**C** and **D**) secretion was measured in the supernatants. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

FIGURE 7. Effect of clusterin on CCL20 secretion. BEAS-2B cells were transfected with adenovirus carrying the gene for clusterin or β -galactosidase. (A) Clusterin-overexpressing BEAS-2B cells were stimulated with HDM for 24 h, and CCL20 levels in the supernatants were measured. (B) CCL20 levels were measured in normal BEAS-2B cells exposed or not to HDM with rCLU. (C) BEAS-2B cells were transfected with clusterin siRNA and stimulated with HDM for 24 h. CCL20 levels in the supernatants were measured. Similar results were obtained from two independent experiments. $**p < 0.001$, $***p < 0.0001$.



recruitment of inflammatory DCs (39–41). Interestingly, the CD11b⁺ DC population increased dramatically in response to HDM sensitization in $Clu^{-/-}$ mice, whereas there was a significant decrease in the frequency of CD103⁺ DCs, which are the predominant DC population in the steady-state, in $Clu^{+/-}$ and $Clu^{-/-}$ mice. Because these immune cells were reported to be recruited into the inflammatory site expressing chemokines, the increased CCL20, CCL2, and CCL11 levels could be involved with enhancing homing of inflammatory DCs and eosinophils to the lungs. In addition, the populations of Ly6C^{high} monocytes and eosinophils in $Clu^{+/-}$ and $Clu^{-/-}$ mice were increased compared with those seen in WT mice. Interestingly, it was reported that these chemokines can lead to the accumulation of CD11b⁺ DCs, as well as Ly6C^{high} inflammatory monocytes, which can differentiate into lung CD11b⁺ DCs during ongoing inflammation (42). Therefore, it is conceivable that increased Ly6C^{high} monocytes contributed to the increased CD11b⁺ DCs in the current study.

BECs produce various cytokines and initiate immune responses in asthmatic airways. CCL20 is one of the principal chemokines expressed by activated BECs (19–21), and HDM has been regarded as a specific stimulus for CCL20 secretion from the airway epithelium, because the production of CCL20 was substantial compared to the production of other stimuli-treated cells (43). In our present analyses, we showed that intracellular clusterin negatively regulates the secretion of CCL20 from BECs. In fact, BECs with downregulated clusterin released higher levels of CCL20 after HDM treatment. In contrast, overexpression of clusterin decreased CCL20 production compared with activated normal BECs. We also investigated whether extracellular treatment with rCLU could inhibit CCL20 production in BECs exposed to HDM. We found decreased CCL20 secretion, indicating that intracellular and extracellular clusterin inhibited allergen-induced CCL20 production by BECs. Recent studies showed that the CCL20-dependent chemotactic response of DCs plays an important role in the initiation of

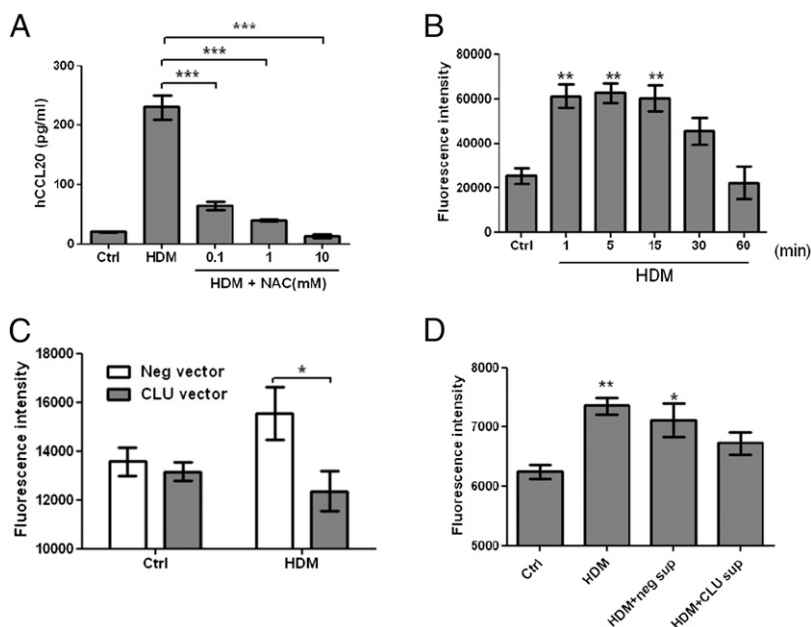


FIGURE 8. Effect of intracellular ROS regulation on HDM-induced CCL20 production in BEAS-2B cells. (A) BEAS-2B cells were stimulated with HDM after NAC pretreatment. CCL20 levels were measured after 24 h of HDM stimulation. $***p < 0.0001$, versus HDM treatment. (B) HDM-induced intracellular ROS generation was measured in BEAS-2B cells. $**p < 0.001$, versus control. (C) Intracellular ROS levels were measured in clusterin-overexpressing BEAS-2B cells exposed to HDM for 5 min. $*p < 0.05$. (D) Untransfected BEAS-2B cells were treated with culture supernatants from BEAS-2B cells that were transfected with clusterin gene-containing vector or empty vector. Intracellular ROS levels were measured after exposure to HDM for 5 min. Similar results were obtained from three independent experiments. $*p < 0.05$, $**p < 0.001$, versus control.

immune responses (44, 45): CCR6 deficiency attenuated allergic pulmonary inflammation in a cockroach Ag model (46). Taken together, the results of the current study indicate that clusterin regulates recruitment of inflammatory immune cells into the bronchial epithelium and allergic airway inflammation by modulating CCL20 expression in BECs.

Nevertheless, it is largely unknown how clusterin modulates CCL20 release in BECs. Because clusterin is a highly sensitive biosensor of increased oxidative stress, and intracellular ROS generation is considered to influence CCL20 release, we evaluated the effect of clusterin on the regulation of intracellular ROS levels and CCL20 production. We found that HDM stimulation of BECs instantly (<1 min) increased the generation of intracellular ROS. This result is consistent with previous findings that many stimuli initiate intracellular signaling through a rapid increase in intracellular ROS. In addition, in our study, upregulation of clusterin reduced both intracellular ROS levels and CCL20 production, and similar results were obtained with NAC-pretreated BECs. Moreover, downregulated clusterin expression promoted oxidative stress in lung tissues, which could be associated with further aggravation of airway inflammation in asthmatic *Clu*^{-/-} mice. These results suggest that the antioxidant function of clusterin may be closely related to the underlying mechanism of CCL20 production in BECs after exposure to allergens.

In summary, our current findings indicate that clusterin may be useful in the regulation of allergic inflammation in the initiation stage by attenuating the CCL20-mediated homing of DCs. Thus, our results suggest that clusterin may be a novel therapeutic target molecule for the regulation of allergic airway inflammation.

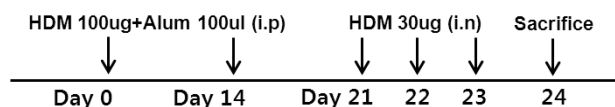
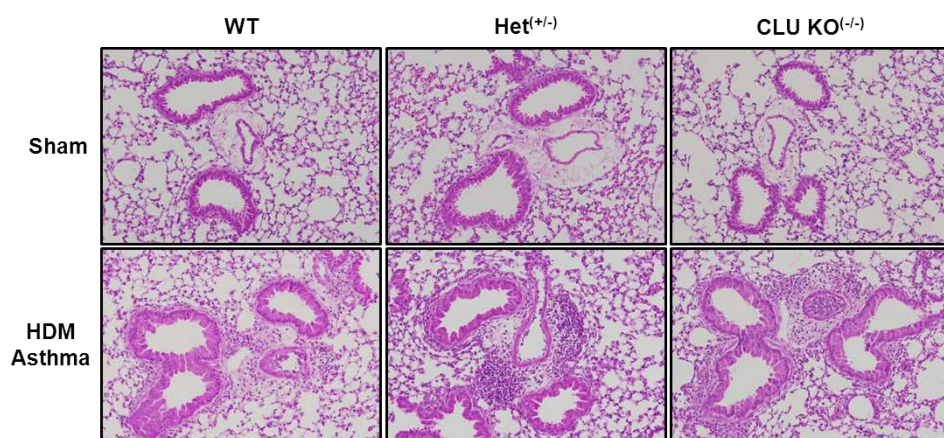
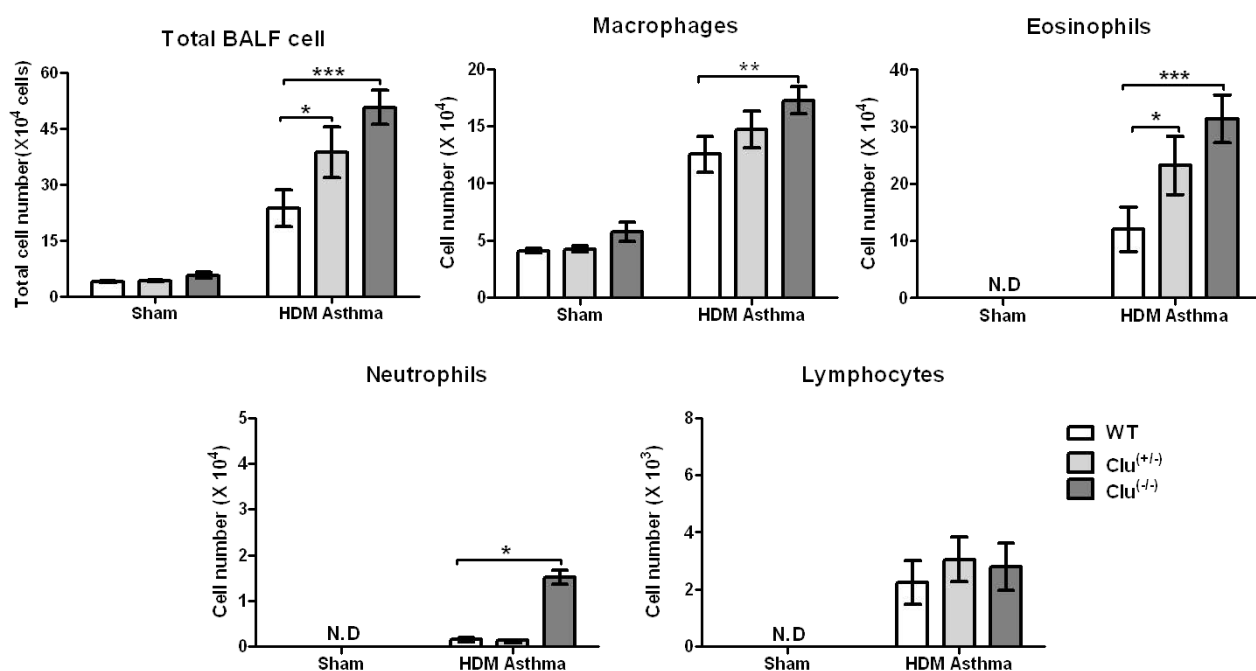
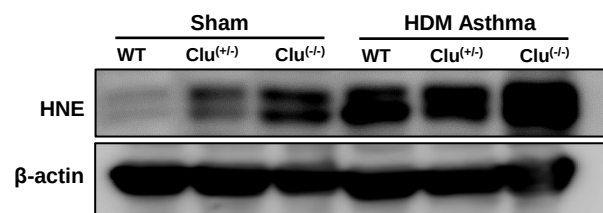
Disclosures

The authors have no financial conflicts of interest.

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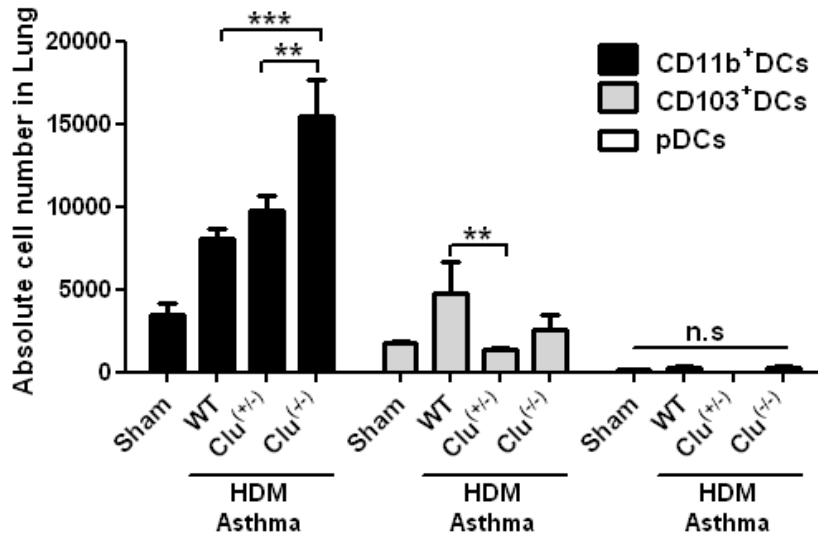
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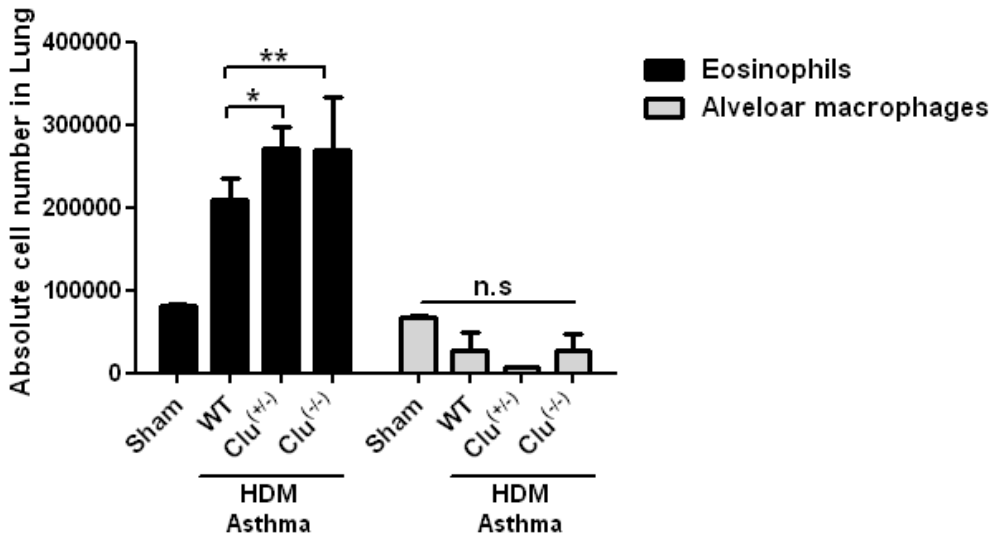
A.**B.****C.****D.**

Supplemental figure 1. Clusterin deletion increases recruitment of inflammatory cells to the lung in HDM-induced conventional asthma model. **(A)** Protocol for the HDM-induced conventional asthma model. **(B)** Histology of lung sections after H&E staining (original magnification: $\times 200$). **(C)** Total cell number and differential cell analysis of BALF in each group. Similar results were obtained from two independent experiments. **(D)** 4-hydroxynonenal (4-HNE)-modified proteins were detected using Western blotting WT, wild-type mice; Clu^(+/-), clusterin heterozygous mice; Clu^(-/-), clusterin knockout mice. *p < 0.05, **p < 0.01, ***p < 0.0001

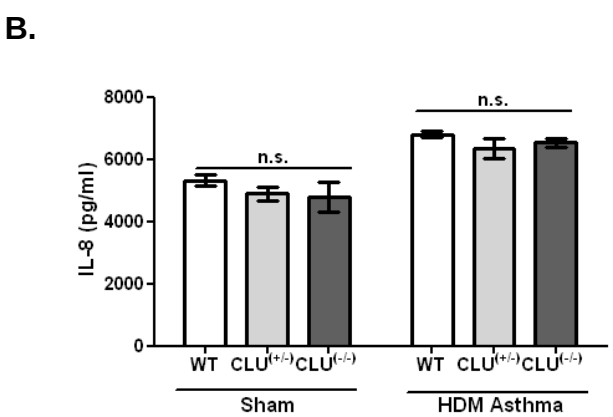
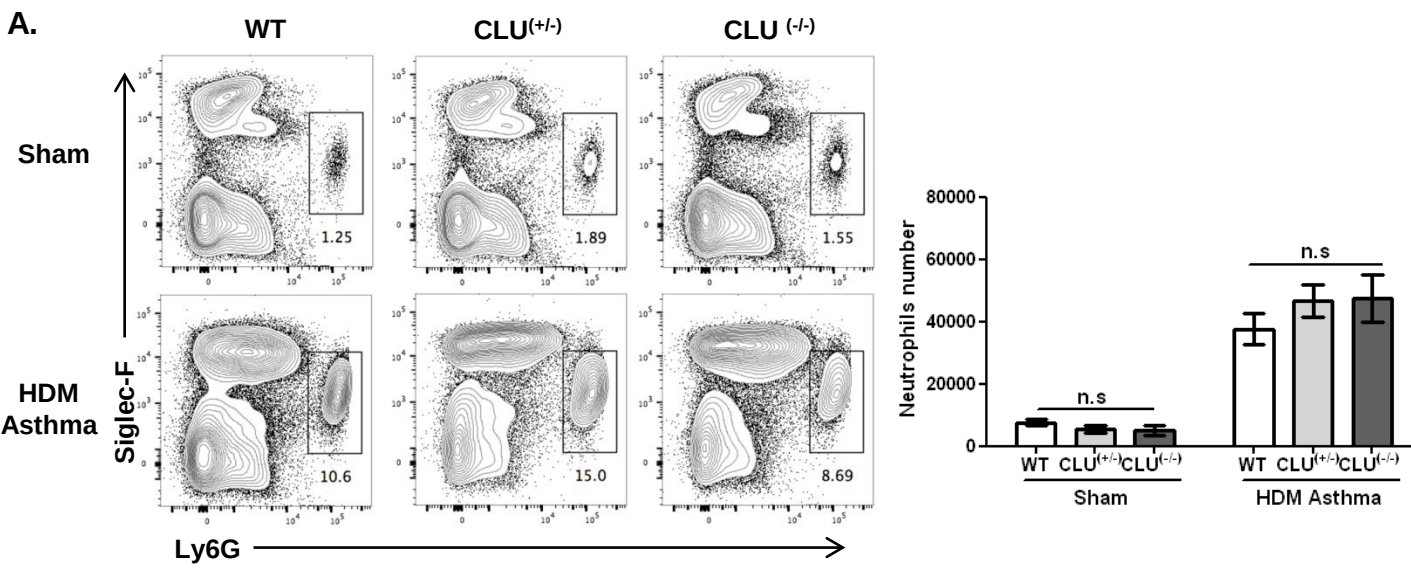
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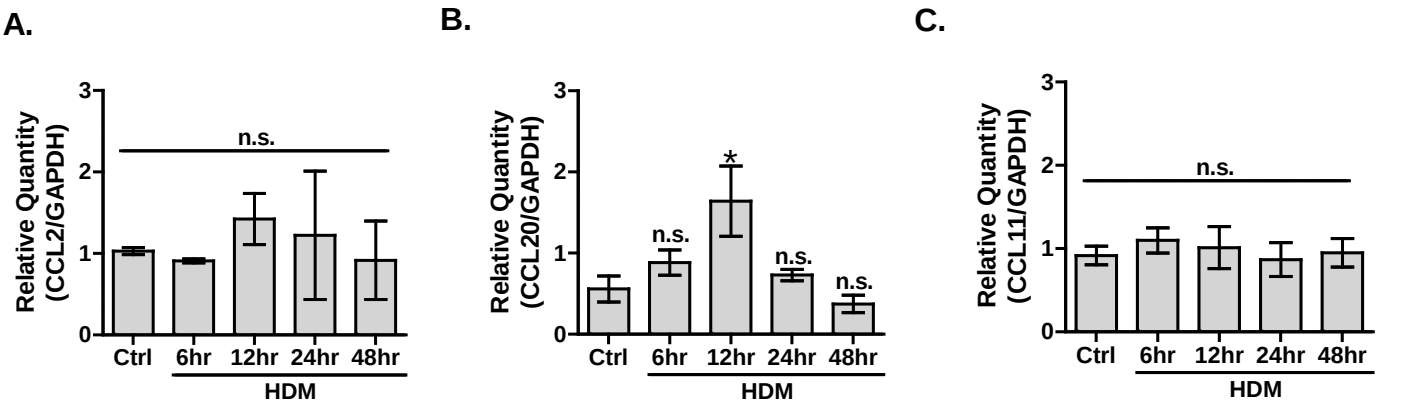
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Supplemental figure 2. The absolute number of inflammatory cells in the mouse lungs of repeated intranasal HDM challenge. **(A)** The absolute number of CD11b⁺ DCs, CD103⁺ DCs and pDCs. **(B)** The absolute number of eosinophils and alveolar macrophages. The absolute cell number was calculated by multiplying the flow cytometric frequency of the cell population by the total number of cells obtained from the lung. Similar results were obtained from three independent experiments. $n \geq 8$ for each group. WT, wild-type mice; Clu^{+/+}, clusterin heterozygous mice; Clu^{-/-}, clusterin knockout mice. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$



Supplemental figure 3. . Clusterin deletion does not affect the recruitment of neutrophils to the lung. **(A)** Dot blot and the absolute cell number showing neutrophils in the mouse lungs of repeated intranasal HDM challenge. **(B)** IL-8 levels in BALF. Similar results were obtained from three independent experiments. $n \geq 8$ for every group. n.s., not significant.



Supplemental figure 4. Chemokine levels in HDM-treated BEAS-2B. Time course of CCL2 (A), CCL20 (B), CCL11 (C) mRNA expressions from HDM-exposed BEAS-2B were determined by RT-PCR. * $p < 0.05$ versus control. n.s., not significant.