

Synthetic di-sulfated iduronic acid attenuates asthmatic response by blocking T-cell recruitment to inflammatory sites

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Identification of carbohydrate sequences that determine affinity to specific chemokines is a critical step for strategies to interfere with chemokine-mediated leukocyte trafficking. Here, we first characterized the development of allergic asthma in Tie2-dependent and inducible *Ext1*-knockout (Tie2-*Ext1*^{KO}) mice. We showed that heparan sulfate is essential for leukocyte recruitment in the peribronchial region and bronchoalveolar lavage fluid (BALF), and is crucial for induction of airway hyperresponsiveness. Our glycan microarray showed a unique affinity profile of chemokine CCL20 to substructures of heparin and heparin-like oligo/di/monosaccharides. Among them, we identified a synthetic and not naturally occurring monosaccharide, 2,4-*O*-di-sulfated iduronic acid (Di-S-IdoA), as a potential inhibitor for CCL20–heparan sulfate interaction. Mice injected with Di-S-IdoA via tail vein or nasal inhalation showed attenuated leukocyte recruitment into inflammatory sites and BALF. These results demonstrate a critical role of chemokine–heparan sulfate interaction in the asthma development and Di-S-IdoA as a potential drug for asthma treatment.

sulfated monosaccharide | lymphocyte recruitment

Asthma is a common allergic disease characterized by chronic airway inflammation, mucus hypersecretion, and airway hyperreactivity to inhaled allergens (1). Despite the importance of T lymphocytes in adaptive immunity and host defense, their accumulation in airway in allergic asthma causes Th2-mediated pulmonary inflammation. The asthmatic inflammatory response is orchestrated by T-cell trafficking network among lung, blood circulation, secondary lymphoid organ, and peripheral tissue (2). Of note, the significant increase of T cells in the airway in asthma is mostly due to T-cell recruitment from regional lymph nodes rather than their proliferation at the inflamed site (3). Therefore, a therapeutic approach that shuts off the trafficking pathway of pathogenic T cells should significantly inhibit the Th2-mediated inflammation in allergic asthma.

It is well known that the destination of T-cell trafficking pathway is tightly restricted by the profile of chemokines, lipid chemoattractants, and T-cell chemokine receptors. As a part of immune surveillance, naïve T cells and central memory T cells constantly access secondary lymphoid organs from blood circulation via specialized high endothelial venules (HEVs). The interaction between T cells and HEV cells includes in a stepwise manner (4, 5), L-selectin–dependent tethering and rolling, activation, firm arrest, and transendothelial migration. Besides 6-sulfo sialyl Lewis X as a L-selectin ligand, HEVs constitutively express chemokine CCL21 and CCL19 and attract T cells that express its cognate receptor CCR7 (5). In contrast to this homeostatic homing, circulating T cells interact with inflamed blood vessels in lung after asthmatic exposure to an inhaled allergen. Among numerous combinations of chemokines and their receptors, there is considerable evidence that CCL20 and its

cognate receptor CCR6 may contribute to the pathogenesis of asthma (6). CCL20–CCR6 plays a key role in the recruitment of Th17 (7) cells and Th2 cells (8). Indeed, CCL20 is highly enriched on inflammatory epithelium (9) and CCR6 is expressed on memory T cells infiltrated in the lung during allergic inflammation (7). In addition, CCR6-deficient mice have decreased airway responsiveness, and reduced recruitment of eosinophils into lung (10, 11). These findings suggest that CCL20–CCR6 axis is a putative target for the treatment of asthma.

Cumulative evidence in vivo and in vitro indicates that chemokines cannot be functionally active in HEVs and inflamed sites without their interaction with heparan sulfate (12). Heparan sulfate protects chemokines from proteolysis, immobilizes them on the endothelium surface and produces chemokine gradients in the vasculature. Heparan sulfate is composed of repeating disaccharide units of uronic acid [glucuronic acid (GlcA) or iduronic acid (IdoA)] and *N*-acetylglucosamine (GlcNAc) carbohydrates. Some of GlcA (or IdoA) carbohydrates are subsequently *O*-sulfated, and GlcNAc carbohydrates are partially modified with *N*-deacetylation and *N*-sulfation (13). Previous reports have indicated that the sulfation patterns in heparan sulfate are more restricted than expected (14), and the sulfation

Significance

Asthmatic inflammation is orchestrated by T-lymphocyte cell trafficking network within lungs, blood circulation, secondary lymphoid organ, and peripheral tissue. Here, we demonstrated that T cell and following eosinophil recruitment was substantially reduced in our recently generated mouse model, where heparan sulfate synthase exostosin-1 (*Ext1*) is knockout in an inducible manner. Moreover, we discovered that even a monosaccharide, 2,4-disulfated iduronic acid (Di-S-IdoA), bound to chemokine CCL20 and significantly inhibited CCL20 binding to heparan sulfate and to endothelial cell surface. We found that Di-S-IdoA attenuated asthmatic reaction, measured by T cell, eosinophil, and CCL20 recruitment in asthmatic mice. These findings show for the first time (to our knowledge) that sulfate monosaccharide can be developed into a potent therapeutic agent for treating asthma.

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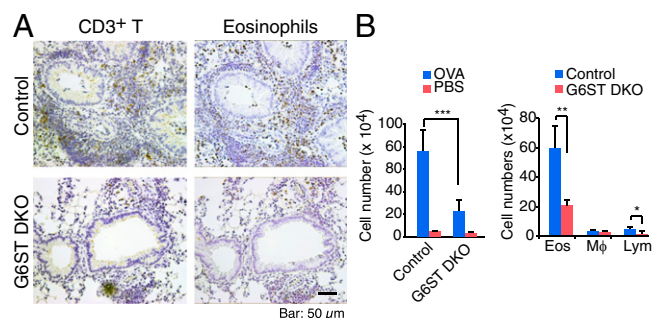


Fig. 2. Reduced OVA-induced airway inflammation in L-selectin ligand-deficient mice. (A) Immunohistochemical staining of control or GlcNAc6ST-1/2 double-knockout (G6ST DKO) mice challenged with OVA. (B) Number of infiltrating cells in BALF recovered from control or G6ST DKO mice. Quantification of total BALF cells (Left) and each cell type (Right) are shown. Error bars indicate SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Notably, CCL20 exhibited high affinity to disaccharide **10** and even monosaccharide **11**. Observed fluorescent intensities of those carbohydrates were comparable to 5-kDa natural heparin.

A Unique and Unnatural Monosaccharide Di-S-IdoA Inhibits Heparan Sulfate–CCL20 Interaction. Monosaccharide **11** is Di-S-IdoA, which contains two axial sulfate groups. Because Di-S-IdoA is an un-

natural synthetic monosaccharide that has never been isolated from a natural source, we were prompted to further characterize its interaction with CCL20 in more detail. The data from surface plasmon resonance (SPR) showed that CCL20 binds to immobilized Di-S-IdoA in the micromolar range ($K_D = 2.9 \times 10^{-6}$ M) (Fig. 4A). We next analyzed the inhibitory effect of Di-S-IdoA. Interaction between immobilized hexasaccharides **1** and CCL20 was blocked by Di-S-IdoA in a concentration-dependent manner and 500 μ M Di-S-IdoA inhibited at a comparable level of 5,000 μ M heparin (Fig. 4B), whereas nonsulfated IdoA showed no such inhibitory activity at 5 mM. We next synthesized Di-S-IdoA with aminopentyl linker (Figs. S1 and S2). Interestingly, in the cultured F2 cell, which is known to express endogenous heparan sulfate, Di-S-IdoA also interfered with the CCL20–heparan sulfate interactions in a dose-dependent manner (Fig. 4C). These results suggest that Di-S-IdoA is an effective as a functional inhibitor of CCL20 chemokine activity. To next study the specificity of Di-S-IdoA, the inhibitory effect of Di-S-IdoA on the bindings between the various proteins and endothelial cells was assayed. It is known that CCL21 (23, 24), IL-8 (23, 25), L-selectin (26, 27), and complement component 5a (C5a) (28, 29) are involved both in the binding to heparin/heparan sulfate in vitro and in the asthma pathogenesis. The result showed that Di-S-IdoA did not block the attachment of CCL21 to mouse endothelial F2 cells, whereas heparin efficiently blocked (Fig. 4D). Di-S-IdoA significantly blocked the binding of L-selectin

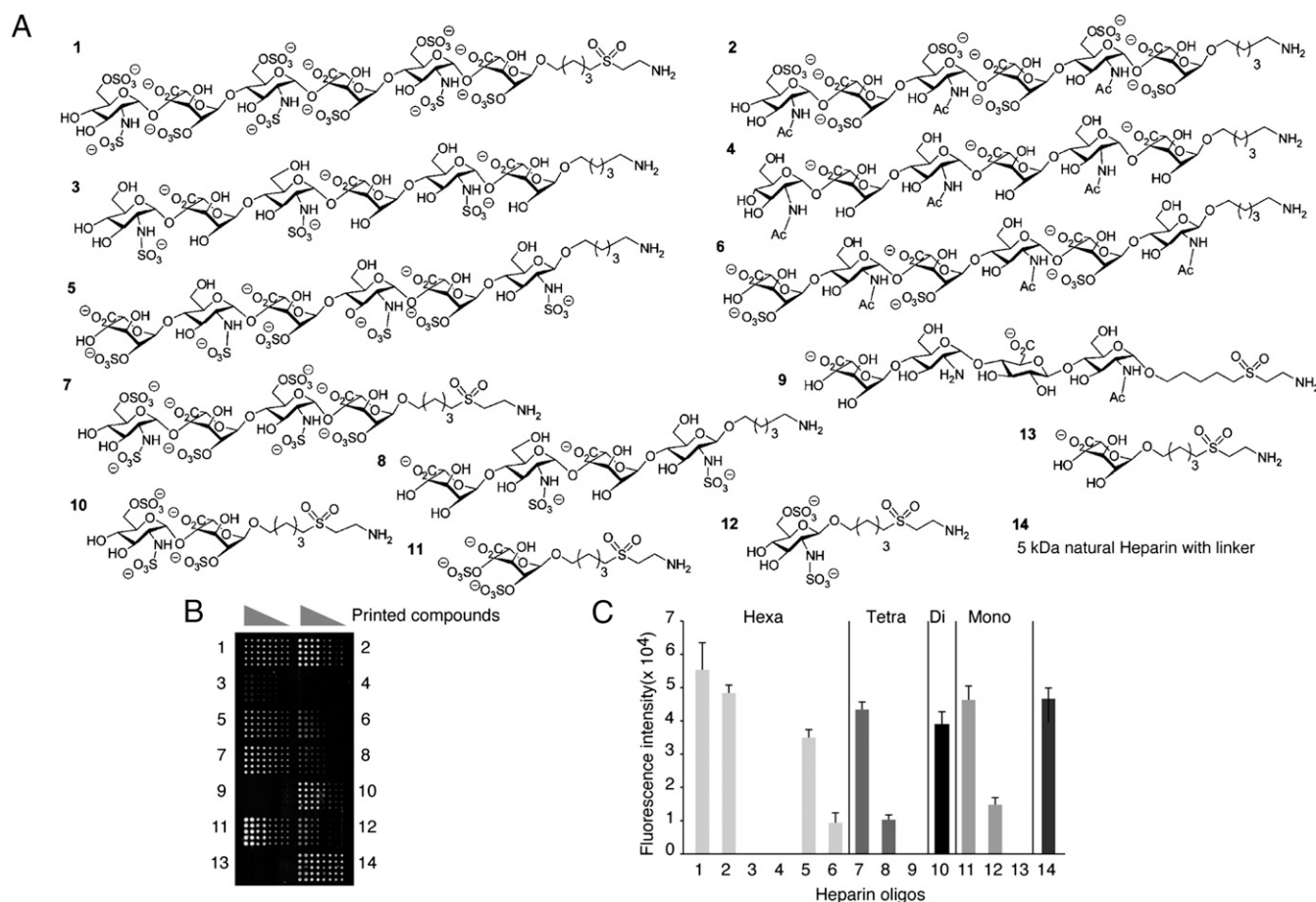


Fig. 3. Binding of CCL20 to heparin-like oligosaccharides on a glycan microarray. (A) Compounds tested for glycan microarray. (B) Binding of recombinant human CCL20 to heparin oligosaccharide-like glycans on a microarray. Numbers 1–14 donate different glycan structures. Each sugar was printed on the slide at four different concentrations ranging from 1,000, 250, 63, and 16 μ M in 10 replicas. (C) Quantification of the binding of CCL20 to the heparin oligosaccharide-like glycans shown in B ($n = 10$; concentration of printed compounds, 250 μ M).

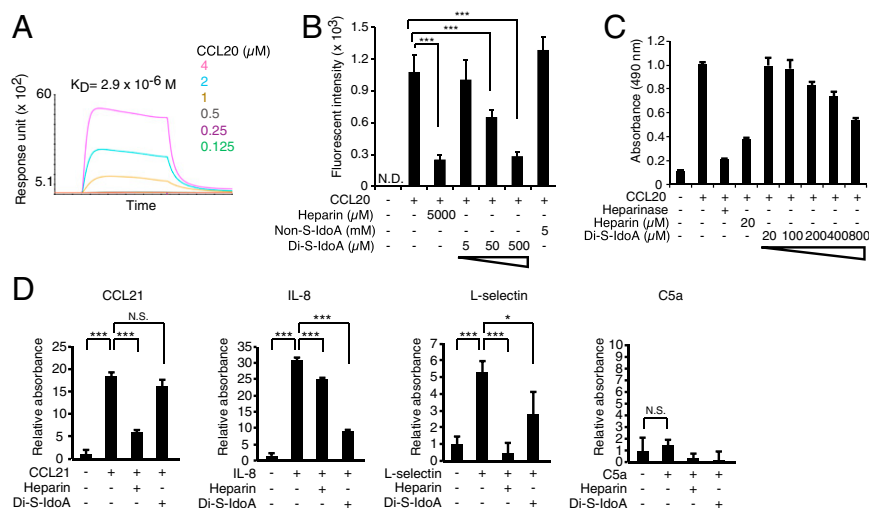


Fig. 4. An affinity of unnatural 2,4-di-sulfated Iduronic acid (Di-S-IdoA) to CCL20 and its inhibitory effect on CCL20-heparan sulfate interaction. (A) Binding kinetics of CCL20 to immobilized Di-S-IdoA (monosaccharide 11) by SPR. (B) Inhibition assay with heparin oligosaccharide-immobilized plate. Binding of CCL20 to heparin-coated plate was assayed in the presence of soluble heparin, nonsulfated IdoA (Non-S-IdoA), and various concentration of Di-S-IdoA ($n = 30$). (C) Inhibitory activity of Di-S-IdoA in the binding of CCL20 to F2 endothelial cells. Heparinase treatment preceded the addition of the cells. The data are representative of two experiments with similar results. (D) Inhibitory activity of Di-S-IdoA in the binding of various proteins to F2 endothelial cells. Di-S-IdoA (800 μ M) or heparin (20 μ M) was used for the inhibition. Error bars indicate SD. * P < 0.05 and *** P < 0.001.

to F2 cells. However, Di-S-IdoA showed even higher inhibitory effect than heparin in IL-8 binding. In this experimental model, C5a did not show any bindings to F2 cells. Those results indicate Di-S-IdoA has unique binding preferences distinct from heparin.

Inhibitory Effect of Di-S-IdoA in OVA-Challenged WT Mice. To test the possibility of Di-S-IdoA as an inhibitor of CCL20 activity in vivo, we injected either PBS or Di-S-IdoA into tail vein right before the reexposure of OVA in WT mice. Pretreatment of Di-S-IdoA decreased the mucus secretion and recruitment of CD3⁺ T cells in the lung tissues (Fig. 5A). We also observed a reduced total leukocyte number in Di-S-IdoA-treated and neutralizing anti-CCL20 antibody-treated mice but not non-sulfated IdoA (Non-S-IdoA)-treated mice (Fig. 5B). These results suggest that i.v. administered Di-S-IdoA can access at the inflamed postcapillary venule, where it inhibits the activity of chemoattractants.

Because CCL20 has been demonstrated to be mainly synthesized from airway epithelial cells, we hypothesized that administration of Di-S-IdoA through intranasal inhalation would interfere the localization and presentation on the vascular endothelial cell surface in inflamed lung. To test the accessibility of Di-S-IdoA through the lung alveoli, we administered Di-S-IdoA by inhalation before OVA challenge in WT mice. Indeed, we found robust expression of CCL20 on the vascular endothelial cell wall in the OVA-challenged WT mice. In contrast, there was little expression of CCL20 in the Di-S-IdoA-pretreated mice, although heparan sulfate is present on vascular surface (Fig. 5C). These data suggest that CCL20 presentation on the endothelial cells at the lung inflammatory site was blocked by Di-S-IdoA administration. Indeed, the number of total leukocytes in BALF was dramatically decreased in 100 μ g of Di-S-IdoA treatment (P < 0.05) compared with saline-treated mice (Fig. 5D). Blood tests from the WT mice treated with Di-S-IdoA showed no side effect in liver function, kidney function, and blood cell count (Table S1). Taken together, our results demonstrated that inhalation administration of Di-S-IdoA was effective in reducing airway inflammation in allergen-challenged WT mice.

Discussion

Despite extensive research efforts in the past decades, the prevalence and severity of asthma are still increasing in the developed countries. The pathology of allergic asthma is directly

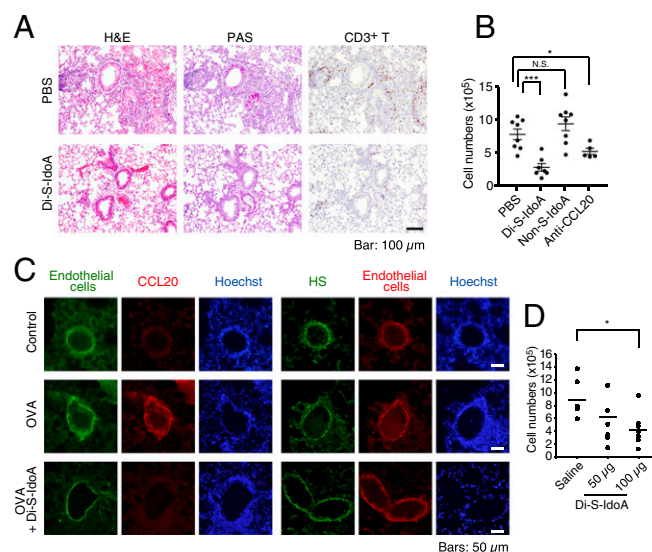


Fig. 5. Di-S-IdoA administration attenuated OVA-induced airway inflammation. (A) Histology and immunohistochemical staining of lungs from mice receiving either PBS or Di-S-IdoA. (B) Quantification of infiltrating T cells in the airway of mice receiving PBS, Di-S-IdoA, Non-S-IdoA, or neutralizing anti-CCL20 antibody ($n = 5-8$ mice per group). Result are shown as mean $\pm$ SEM. * P < 0.05 and *** P < 0.001. (C) Immunofluorescent staining of CCL20, heparan sulfate, and endothelial cells in the lung from pulmonary Di-S-IdoA-inhaled mice. Images are representatives from mice treated with PBS or Di-S-IdoA (100 μ g), respectively. Note that the primary antibody against CCL20 was injected through the tail vein before the collection of lung tissue. (D) Pulmonary administration of Di-S-IdoA decreases OVA-induced airway inflammation. Leukocyte infiltration into BALF following inhalation pretreatment with either saline or different concentration of Di-S-IdoA (50 and 100 μ g) ($n = 5-7$ mice per group). * P < 0.05.

linked to the consequence of long-term chronic Th2-mediated inflammation in the lung at the site of continuous exposure to allergens (30). Previous report has showed that a partial loss of *N*-sulfate in heparan sulfate contributes to attenuation of airway inflammation (16). However, the role of the main chain of heparan sulfate in the pathogenesis of asthma had not been addressed because of the embryonic lethality of homozygous *Ext1*^{-/-} mouse. In this study, we used our established inducible gene knockout system to circumvent this lethality (20). In contrast to WT mice, Th2-mediated allergic inflammation in the lung in OVA-challenged Tie2-*Ext1*^{IKO} mice was abolished as assessed by eosinophil recruitment to the lung, and eosinophil, macrophage, and lymphocyte infiltration into BALF. Moreover, Tie2-*Ext1*^{IKO} mice showed lower airway hyperresponsiveness to MCh than control mice did, indicating that GlcA/IdoA-GlcNAc repeat of endothelial heparan sulfate is not only essential for lymphocyte, eosinophil, and macrophage recruitment, but also for airway responsiveness a cardinal feature of asthma. Importantly, we have shown that the proliferation and rolling ability of lymphocytes in Tie2-*Ext1*^{IKO} mice do not change during homing (20). In another report, lymphocyte-specific deletion of heparan sulfate has little effects on their differentiation (31). Our results also showed that selectin ligand-deficient G6ST DKO mice had decreased lymphocyte recruitment into the lung. These results not only reemphasize the importance of T-cell trafficking in the pathogenesis of asthma, but also indicate the potential importance of blocking of this T-cell trafficking pathway as an effective therapeutic strategy for asthma.

Among the asthma-associated chemokines, the role of CCL20 in asthma is only beginning to be understood and a large part of its physiological significance remains to be elucidated. Given that affinity between chemokine and heparan sulfate also varies depending on type of chemokines (18), we hypothesized there are specific oligosaccharide patterns preferable for binding to CCL20. In this study, a glycan microarray identified unnatural and synthetic monosaccharide Di-S-IdoA, which has two axial O-sulfations at C2 and C4 positions, as a potent inhibitor of CCL20. This finding was surprising because previously seven other chemokines including CCL21 did not show high affinities for Di-S-IdoA as equivalent to sulfated hexasaccharides (18). In addition, the data that nonsulfated IdoA did not show any binding to CCL20 suggests that two sulfate groups are important to form a pool of negative charge on one side and this ionic charge contributed to interaction with CCL20. Furthermore, inhalation as well as i.v. administration of this compound significantly decreased lymphocyte recruitment into the allergen-challenged lung inflammatory site, suggesting that CCL20-mediated Th17 and Th2 cell recruitments in early phase is critical for following disease development including eosinophil, macrophage recruitments.

Heparin, a soluble analog of heparan sulfate, and its derivatives have an antiinflammatory activity as well as an anticoagulant effect. A role for heparin in improving symptoms in inflammatory disease was demonstrated in neonatally immunized rabbits (32), naturally sensitized sheep (33), and asthma patients in clinical trials (34, 35). The mechanism of its antiinflammatory effect is likely due to the interference with the chemokine retention on vascular heparan sulfate, blockage of interleukins, selectins, and complement components. In this regard, a therapeutic approach using heparin-derived low-molecular-weight saccharides would be beneficial (12). However, so far, X-ray crystallography of natural heparin revealed that a disaccharide unit of heparin is not sufficient and at least tetrasaccharide is necessary to inhibit activity of chemokine RANTES *in vivo* (36). In this context, our result that a monosaccharide with unusual sulfation pattern can interfere with the chemokine-heparan sulfate interaction is a novel finding. Taken together, our findings strongly suggest that Di-S-IdoA binds to CCL20, resulting in inhibiting CCL20 binding to heparan sulfate and to the cellular receptor, CCR6.

As a whole, our results have demonstrated an essential role of endothelial heparan sulfate in T-cell trafficking into lung and thus in the pathogenesis of allergic asthma. We identified a novel monosaccharide Di-S-IdoA as a potent inhibitor of chemokine CCL20. With the establishment of this new tool for CCL20, we determined the effect of Di-S-IdoA in lymphocyte recruitment into the allergen-challenged lung inflammation site. Pulmonary inhalation drug delivery of this compound may help to attenuate asthmatic symptoms by suppressing chemokine-mediated inflammatory responses, mucus production, and airway responsiveness.

Materials and Methods

Reagents and Animals. Tie2-*Ext1*^{IKO} mice were generated and maintained as reported (20). To induce the deletion of endothelial heparan sulfate, mice of 4-wk age were treated with doxycycline for 3 consecutive weeks before experiments. All protocols for animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Sanford-Burnham Medical Research Institute, and were conducted in accordance with National Institutes of Health guidelines. OVA was purchased from Sigma, heparan sulfate antibody 3G10 from US Biological, recombinant human CCL20 from R&D and Shenandoah Biotech, recombinant human CCL21 from Sino Biological, recombinant human IL-8 from Cell Sciences, recombinant mouse L-selectin from Life Technologies, recombinant human C5a from BioVision, eosinophil anti-MBP antibody (Dr. James Lee, Mayo Clinic, Scottsdale, AZ) (37), anti-CD3 ϵ antibody from Santa Cruz, anti-macrophage inflammatory protein 3 α (CCL20) antibody from Abcam, neutralizing anti-CCL20 antibody and anti-CCL21 antibody from R&D, anti-His antibody from Clontech, and anti-von Willebrand factor antibody from Dako.

OVA-Induced Model of Asthma in Mice. For sensitization, mice were intraperitoneally administered a suspension containing 50 μ g of OVA and 0.5 mg of aluminum hydroxide (Sigma) in 200 μ L of PBS on day 0 and day 12. On days 23, 25, and 27, the mice were briefly anesthetized with isoflurane and challenged intranasally with 20 μ g of OVA in 50 μ L of PBS through the nose. For studies with a therapeutic intervention, 100 μ g of Di-S-IdoA or Non-S-IdoA, or 50 μ g of neutralizing anti-CCL20 antibody was administered either i.v. or inhalationally 30 min before each OVA challenge. Twenty-four hours after the last OVA challenge, the mice were killed and the lung bronchoalveolar lavage was performed by infusion of 1 mL of 0.1% PBS through the trachea with Surflash IV catheters (Terumo). The lung lavage was subjected to leukocyte counting by a cytometer, and each subpopulation was analyzed by cytochrome followed by a stain with Hema-3 (Fisher).

Immunohistochemistry. Mouse lung lobes were fixed with 4% (wt/vol) PFA followed by paraffin embedding and histological examination by H&E staining and PAS staining. For evaluation of immune infiltration into lung inflammatory sites, tissues were stained by anti-CD3 ϵ antibody and anti-MBP antibody described above.

Airway Responsiveness to MCh. Airway responsiveness to MCh was assayed 24 h after the final OVA challenge in anesthetized, intubated, and ventilated mice as previously described (38). Intubated and ventilated mice were anesthetized intraperitoneally. The dynamic airway resistance was determined in mice exposed to nebulized PBS or MCh at 0, 3, 24, and 48 mg/mL.

Glycan Microarray. Microarrays were fabricated as previously reported (39). Microarray slide was incubated with 100 μ L (5 μ g) of rhCCL20 (R&D Systems) in HBS-N containing 0.01% Tween 20 for 1 h at room temperature under mild shaking, washed three times with HBS-N, and dried by centrifugation. The slide was then reacted with 100 μ L (2 μ g) of anti-hCCL20 goat IgG (R&D Systems) for 1 h, followed by incubation with anti-goat Alexa Fluor 594 antibody (Invitrogen) for 1 h. Slides were scanned with a GenePix 4300A microarray scanner and analyzed by GenePix Pro software. Binding of CCL20 to immobilized heparin-like oligosaccharide 1 in the presence of Non-S-IdoA and Di-S-IdoA was observed in the same manner. The amino group of each monosaccharide sample was acylated before use by treatment with a solution of acetic anhydride-triethylamine-methanol (2:3:5, vol/vol/vol) followed by condensation and lyophilization. The inhibition solution contained 5 mM 5-kDa heparin (Santa Cruz Biotechnology), 5 mM Non-S-IdoA, or 5, 50, and 500 μ M Di-S-IdoA.

Immunohistochemistry. For heparan sulfate staining, tissue sections were treated with heparinase at 37 °C for 1 h before incubation with anti-

heparan sulfate 3G10 antibody. Alexa Fluor 568 anti-mouse IgG2b was used for secondary reaction. Vascular endothelial cells were visualized by anti-von Willebrand factor antibody (1:100 dilution). Lung endothelial CCL20 could not be visualized by the general fluorescent staining protocol. Instead, localization of blood vessel CCL20 was examined by i.v. injection of anti-CCL20 antibody (25 μ g per mouse) through mouse tail vein. Thirty minutes after the injection, lungs were collected and frozen in OCT compound (Tissue Tek). Frozen tissue sections were then incubated with Alexa Fluor 568 anti-rabbit IgG (Invitrogen).

To avoid the cross-reaction in double staining with CCL20, sections were blocked with anti-rabbit IgG antibody and protein A. Then the sections were incubated with anti-von Willebrand factor antibody, followed by incubation with FITC-anti-rabbit IgG-Fc antibody (Jackson ImmunoResearch).

Statistical Analyses. All data are showed as means $\pm$ SD. Unpaired two-tailed Student *t* test was used for statistical analyses. We considered *P* values of less than 0.05 as statistically significant. Degrees of statistical significance are presented as **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

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