Core 2 oligosaccharides mediate eosinophil and neutrophil peritoneal but not lung recruitment

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WE HAVE INVESTIGATED the importance of cell-surface serine- and/or threonine-linked oligosaccharide adhesion molecules synthesized by the Golgi enzyme core 2 β-1,6-N-acetylgalactosaminyltransferase (C2GlcNAcT) in mediating eosinophil trafficking to the lung in studies utilizing C2GlcNAcT-I-deficient mice. The number of bronchoalveolar eosinophils, the number of lung eosinophils, and airway responsiveness to methacholine were not significantly different in C2GlcNAcT-I-deficient compared with wild-type mice sensitized and challenged by inhalation with ovalbumin. C2GlcNAcT-I-deficient mice do not demonstrate defects in neutrophil trafficking to the lung in response to lipopolysaccharide (LPS). In contrast, ragweed-sensitized C2GlcNAcT-I-deficient mice exhibit significantly reduced eosinophil trafficking to the peritoneal cavity in response to ragweed peritoneal challenge. C2GlcNAcT-I-deficient mice also have significantly reduced neutrophil trafficking to the peritoneal cavity in response to LPS challenge. Overall, these studies demonstrate an important role for serine/threonine-linked oligosaccharides synthesized by the Golgi enzyme C2GlcNAcT-I in eosinophil and neutrophil trafficking to the peritoneum but not for eosinophil or neutrophil trafficking to the lung.

Much evidence has accumulated indicating that sia-lated and/or fucosylated lactosaminoglycans such as sLe\(^x\) are involved in leukocyte (especially neutrophil) trafficking (30, 33). sLe\(^x\) is expressed by neutrophils and can serve as a ligand for the endothelial-expressed selectin adhesion molecules (20, 29, 30). The importance of sLe\(^x\) to neutrophil trafficking is suggested from a rare immunodeficiency syndrome, leukocyte adhesion deficiency II, in which the absence of sLe\(^x\) from the cell surface of neutrophils, resulting from an inherited metabolic disease affecting the metabolism of fucose (a component of sLe\(^x\)), is associated with increased peripheral blood neutrophil counts, impaired neutrophil binding to E- and P-selectin in vitro, impaired rolling of neutrophils on endothelium in vivo, and recurrent bacterial infection (14, 21, 31). However, not all studies demonstrate that sLe\(^x\) is essential for leukocyte...

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binding to vascular E- and P-selectins, as demonstrated in studies in which a variant of the HL-60 cell line that lacks sLe\textsuperscript{x} expression binds well to E- and P-selectin (32).

Previous studies of C2GlcNAcT-I-deficient mice demonstrate that these mice have significant impairments in O-glycan and sLe\textsuperscript{x} synthesis and significant impairments in neutrophil adhesion to the endothelial adhesion molecules P-selectin and E-selectin in flow chamber studies in vitro (9). In addition, C2GlcNAcT-I-deficient mice demonstrate a significant impairment in neutrophil recruitment to the peritoneum after thioglycollate stimulation in vivo (80% inhibition; see Ref. 9). Taken together, these observations suggest an important role for the O-glycan adhesion ligands synthesized by the enzyme C2GlcNAcT-I in neutrophil adhesion to P-selectin and to E-selectin. In vivo studies have demonstrated significant impairments in leukocyte rolling on endothelium in the cremaster muscle of C2GlcNAcT-I-deficient mice (25). This impaired rolling of leukocytes deficient in C2GlcNAcT-I is mainly the result of impaired binding of P-selectin glycoprotein ligand-1 (PSGL-1) to P-selectin with lesser contributions from E-selectin (25). Eosinophils, like neutrophils, roll efficiently on P-selectin (3, 6, 28, 34, 35). However, in contrast to neutrophils, eosinophils roll less efficiently on E-selectin (26), suggesting that P-selectin is more important than E-selectin to eosinophil recruitment (8, 19). Based on previous studies with C2GlcNAcT-I-deficient neutrophils (9), we hypothesized that O-glycans synthesized by C2GlcNAcT-I might also be important in the eosinophil interaction with endothelial P-selectin. We have therefore investigated the importance of O-glycans to eosinophil recruitment to the lung and peritoneum in C2GlcNAcT-I-deficient and wild-type mice.

**MATERIALS AND METHODS**

C2GlcNAcT-I-deficient mice. Female C2GlcNAcT-I-deficient and control C57BL/6 wild-type mice were kindly provided by Dr. Jamey Marth (University of California San Diego, San Diego, CA; see Ref. 9) and were used when they reached 8–10 wk of age. C2GlcNAcT-I-deficient mice exhibit no significant organ abnormalities (9). All animal experimental protocols were approved by the University of California, San Diego, animal subjects committee.

Mouse model of eosinophilic pulmonary inflammation. Pulmonary eosinophilia in mice was induced as previously described in this laboratory (2, 5). In brief, C57BL/6 mice were sensitized by an intraperitoneal injection of 50 μg ovalbumin (Ova)/1 mg alhydrogel (Aldrich Chemical, Milwaukee, WI) in 0.9% sterile saline on days 0 and 12. Nonsensitized mice received 1 mg of alhydrogel in 0.9% saline. On day 24, mice (n = 4/group) were exposed three times for 30 min (at 30-min intervals) to an aerosol of Ova (10 mg/ml) in 0.9% saline (nonsensitized control mice received saline only). The aerosolized Ova protocol was repeated on days 26, 28, and 30. After the last aeroallergen challenge (3 h), mice were killed by CO\textsubscript{2} asphyxiation.

Bronchoalveolar lavage eosinophils. Bronchoalveolar lavage (BAL) cells from mice were recovered by lavage with 1 ml of PBS via a tracheal catheter (2, 5). The resulting BAL cells were immediately separated from BAL fluid by centrifugation (700 g for 5 min). A PBS dilution of the recovered BAL cells was added to trypan blue, and the viability and total number of BAL white blood cells were counted with a hemocytometer. Differential leukocyte counts were performed after brief acetone fixation and staining of the BAL cells with May-Grünwald-Giemsa stains. The percentage of eosinophils, neutrophils, and mononuclear cells present on each slide was assessed by counting a minimum of 300 cells in random high-power fields using a light microscope (×40 magnification).

Lung tissue eosinophils. Lung tissues embedded in optimum cutting temperature compound in 10 × 50 × 50-mm tissue wells were cryosectioned at 10 μm and acetone fixed on poly-l-lysine-coated slides. Total eosinophil numbers were...
enumerated by detection of eosinophil peroxidase using diaminobenzidine (DAB) staining and microscopic examination, as described in this laboratory (2, 5). Slides were incubated at room temperature for 1 min in the presence of cyanide buffer (10 mM potassium cyanide, pH 6), rinsed in PBS, and incubated for 10 min with the peroxidase substrate DAB (Vector Laboratories, Burlingame, CA). Slides were subsequently washed in PBS, counterstained with hematoxylin, air-dried, and examined by light microscopy (×40 magnification). Five random fields were selected, and eosinophils were counted to determine total eosinophil number per microscope field.

Peripheral blood eosinophils. Blood was collected from the carotid artery, and red blood cells were lysed using a 1:10 solution of 100 mM potassium carbonate and 1.5 M ammonium chloride (2). The remaining cells were cytospun (3 min at 500 rpm) on microscope slides and air-dried. Eosinophil counts were performed as described above.

Bone marrow eosinophils. Bone marrow cells were flushed from femurs with 1 ml PBS and cytopsin on microscope slides, and separate slides were stained with Wright-Giemsa and examined by light microscopy (×40 magnification). The number of the plethysmograph, and pressure differences between the chest and the reference chamber are recorded. In the plethysmograph, mice were exposed for 3 min to nebulized PBS and subsequently to increasing concentrations of nebulized methacholine (MCh; Sigma, St. Louis, MO) in PBS using an Aeronec ultrasonic nebulizer (Devilbiss). After each nebulization, recordings were taken for 3 min. The enhanced pause (P_{enh}) was used to monitor airway responsiveness. P_{enh} correlates closely with pulmonary resistance measured by conventional two-chamber plethysmography in ventilated mice (11). P_{enh} values measured during each 3-min sequence are expressed for each MCh concentration (3–24 mg/ml).

RT-PCR: C2GlcNAcT-I expression by neutrophils and eosinophils. Previous studies have demonstrated that mouse neutrophils express C2GlcNAcT-I (9). To determine whether wild-type mouse eosinophils express C2GlcNAcT-I, mouse eosinophils and neutrophils were purified from the peripheral blood of interleukin-5 transgenic mice as previously described (3, 2). Total cellular RNA was isolated from mouse eosinophils (purity >95%) and mouse neutrophils (purity >95%) using Trizol reagent (Life Technologies, Gaithersburg, MD) following the manufacturer’s instructions. The cell pellet was suspended in 500 μl of Trizol reagent and allowed to stand at room temperature for 5 min by lysis. Chloroform (100 μl) was added to each tube for 15 s, and samples were centrifuged at 12,000 rpm for 15 min at 4°C. The clear aqueous supernatant was transferred to a fresh tube, and 250 μl isopropanol were added overnight at 4°C. Samples were centrifuged at 12,000 rpm for 15 min at 4°C. The RNA was washed with 500 μl of 75% ethanol, air-dried, and dissolved in diethylpyrocarbonate-treated water.

For the synthesis of the first-strand cDNA, 1–5 μg of total RNA were mixed with 500 ng of oligonucleotide and 12 μl of sterile distilled water, heated to 70°C for 10 min, and then quick-chilled on ice. Four microliters of 5× first-strand buffer, 2 μl of 0.1 M dithiothreitol, and 1 μl of 10 mM dNTP mix were added and incubated at 42°C for 2 min. Superscript II (200 units) was added, and the mixture was incubated at 42°C for 50 min. The reverse transcriptase reaction was terminated by the incubation of the reaction tube samples at 70°C for 15 min, and the first-strand cDNA was cooled to 4°C. RT-PCR was carried out using mouse C2 GlcNAcT specific primers (C2 GlcNAcT-S: 5’-GCATCTAACCTGGAACATC; C2 GlcNAcT-AS: 5’-GCCGCTTCTTGAATTGCACTG; see Ref. 23). PCR amplification was carried out with a 50-μl reaction volume consisting of a PCR buffer containing 1.5 mM MgCl_2, 10 mM of each dNTP, 50 pM of each primer, and 1 unit of Taq DNA polymerase (all reagents were obtained from GIBCO-BRL). The reaction mixture was denatured at 95°C for 30 s, annealed at 50°C for 30 s, and extended at 72°C for 40 s. The PCR was amplified for 30 cycles, followed by an extension step of 8 min at 72°C to extend the partially amplified products. The PCR products (620 bp) were analyzed by electrophoresis on 1.5% agarose gel, and the products were visualized by staining with ethidium bromide. PCR products for eosinophil or neutrophil genes were normalized to housekeeping genes (L32) before C2GlcNAcT-I mRNAs were quantified by densitometry.

Mouse model of peritoneal neutrophil recruitment. The techniques used for ragweed immunization and challenge are similar to those previously described in this laboratory (3). C. elegans C2GlcNAcT-I-deficient or wild-type control mice were immunized by a series of five injections of a 1:1,000 dilution of a ragweed pollen extract (Holliester-Stier, Spokane, WA); 0.1 ml is injected subcutaneously on days 0 and 1, and 0.2 ml is injected subcutaneously on days 6, 8, and 14. A control group of ragweed-immunized mice (challenged with PBS diluent) and nonimmunized mice (prepared by subcutaneous injections of isotonic saline instead of the ragweed pollen extract) followed the same immunization schedule. Four mice were included in each group of mice studied. The mice were challenged on day 20 by intraperitoneal injection of 0.2 ml of the ragweed allergen (or control PBS diluent). In previous studies, we have examined the kinetics of eosinophil recruitment in the peritoneal cavity and demonstrated maximal recruitment at 24–48 h postragweed peritoneal challenge (3). Therefore, 48 h after intraperitoneal allergen challenge (day 22), the mice were killed by cervical dislocation. Two milliliters of PBS containing 6 U/ml of heparin were injected intraperitoneally, the abdomen was massaged, and the peritoneal fluid was collected after the peritoneum was opened. An appropriate PBS dilution of the recovered peritoneal fluid was added to trypan blue, and the viability and total number of white blood cells were counted with a hemocytometer. Differential leukocyte counts were performed after brief acetone fixation and staining of the peritoneal cells with May-Grünwald-Giemsa stains. The percentage of eosinophils present on each slide was assessed by counting a minimum of 300 cells in random high-power fields using a light microscope (×40 magnification)
control PBS, for 30 min. Mice were killed 24 h after completion of the nebulization. BAL cells were recovered by lavage via a tracheal catheter in 1 ml of PBS and processed to enumerate the number of BAL neutrophils (Wright Giemsa stain).

Statistics. Results in the different groups of mice were compared by ANOVA followed by posttesting using a statistical software package (In Stat, San Diego, CA), as previously described (4). P values of <0.05 were considered statistically significant. All results are given as means ± SD.

RESULTS

Effect of C2GlcNAcT-I deficiency on eosinophilic airway inflammation. Sensitization and Ova allergen challenge of wild-type mice (n = 3 experiments with 4 mice/group in each experiment; 12 mice total/group) induced a significant BAL eosinophilia (10.88 ± 4.16 × 10⁵ BAL eosinophils/ml) compared with mice that were not sensitized or challenged with Ova (0.0017 ± 0.0006 × 10⁵ BAL eosinophils/ml; P < 0.001) or compared with mice immunized with Ova and challenged with PBS diluent (0.0019 ± 0.0010 × 10⁵ BAL eosinophils/ml; P < 0.001).

Eosinophil recruitment to the lung was similar in C2GlcNAcT-I-deficient compared with wild-type mice challenged with Ova in both BAL (wild-type mice 10.88 ± 4.16 × 10⁵ vs. C2GlcNAcT-I-deficient 12.09 ± 4.61 × 10⁵ eosinophils/ml; n = 3; P = not significant (NS); Fig. 2) and lung (wild-type mice 22.5 ± 15.3 vs. C2GlcNAcT-I-deficient 24.0 ± 15.7 eosinophils/high-power field; n = 3; P = NS; Fig. 3).

Effect of C2GlcNAcT-I deficiency on blood and bone marrow eosinophilia. Previous studies have demonstrated an increase in circulating neutrophils with no associated increase in bone marrow progenitor frequencies in C2GlcNAcT-I-deficient mice (9). In this study, we have investigated eosinophil levels in blood and bone marrow. The percentage of eosinophils in blood and bone marrow was similar in C2GlcNAcT-I-deficient compared with wild-type mice challenged with Ova in both blood (wild-type mice 12.5 ± 6.4 vs. C2GlcNAcT-I-deficient mice 13.4 ± 9.1% eosinophils; n = 3; P = ND; Fig. 4) and bone marrow (wild-type mice 16.3 ± 5.2 vs. C2GlcNAcT-I-deficient mice 11.7 ± 1.1% eosinophils; n = 3; P = NS; Fig. 4). The absolute number of blood eosinophils was also similar in wild-type mice (6.6 ± 4.4 × 10⁵ eosinophils/μl) compared with C2GlcNAcT-I-deficient mice (7.3 ± 2.9 × 10² eosinophils/μl; n = 3; P = NS).

Effect of C2GlcNAcT-I deficiency on airway hyperreactivity to MCh. Airway responsiveness to MCh was significantly increased in both wild-type and C2GlcNAcT-I-deficient mice after Ova sensitization and Ova challenge (Fig. 5). There was no significant difference in MCh responsiveness in wild-type compared with C2GlcNAcT-I-deficient mice (Fig. 5). Ova-sensitized mice that did not undergo Ova challenge or mice challenged with Ova without sensitization showed a minimal change in P_{eh} in response to MCh (data not shown).

RT-PCR: C2GlcNAcT-I expression by neutrophils and eosinophils. To determine whether eosinophils, like previous studies in neutrophils (9), expressed
C2GlcNAcT-I, we isolated mRNA from mouse eosinophils and neutrophils and converted it to cDNA. PCR studies demonstrate that mouse eosinophils, like neutrophils, express approximately equivalent levels of C2GlcNAcT-I (Fig. 6).

Effect of C2GlcNAcT-I deficiency on peritoneal eosinophil inflammation. Intrapерitoneal administration of ragweed in ragweed-sensitized mice induced significant recruitment of eosinophils in the peritoneal cavity (postragweed 7.6 $\pm$ 2.5 vs. preragweed 1.2 $\pm$ 0.7 x $10^4$ eosinophils/ml; n = 3; P < 0.05). There was a significant 86% inhibition of eosinophil recruitment in the peritoneal cavity of C2GlcNAcT-I-deficient compared with wild-type mice challenged with ragweed (n = 3; P < 0.05; Fig. 7).

Effect of C2GlcNAcT-I deficiency on peritoneal neutrophil inflammation. Intrapерitoneal administration of LPS induced significant recruitment of neutrophils in the peritoneal cavity (post-LPS 4.5 $\pm$ 2.1 vs. pre-LPS 0.4 $\pm$ 0.3 x $10^4$ neutrophils/ml; n = 3; P < 0.01). There was a significant 82% inhibition of neutrophil recruitment in the peritoneal cavity of C2GlcNAcT-I-deficient compared with wild-type mice challenged with LPS (n = 3; P < 0.05; Fig. 8).

DISCUSSION

In this study, we have shown important differences in requirements for O-glycan synthesis in mediating leukocyte recruitment to the peritoneum compared with the pulmonary microcirculation. This study confirms an important role for O-glycans in neutrophil recruitment to the systemic mesenteric circulation after LPS challenge, as has previously been demonstrated for neutrophil recruitment after thioglycollate administration (9). Both studies, using different stimuli for neutrophil recruitment, demonstrated an ~80% reduction in neutrophil recruitment to the peritoneal cavity. We also demonstrated an important role for...
O-glycans in eosinophil recruitment to the peritoneal cavity after ragweed allergen challenge. In contrast to the important role of O-glycans in mediating neutrophil and eosinophil recruitment in the peritoneal circulation, leukocyte recruitment (neutrophil and eosinophil) in the pulmonary vascular bed is not impaired, suggesting important differences in the requirements for O-glycans in the systemic and pulmonary vascular beds. Intravital microscopy studies of neutrophil rolling in the mesenteric circulation in vivo have demonstrated that neutrophil rolling is dependent on engagement of neutrophil selectins (L-selectin and PSGL-1; see Refs. 10, 12, 27, 31) and endothelial selectins (predominantly P-selectin with strengthening by E-selectin; see Refs. 15, 17, 36). C2GlcNAcT-I-deficient neutrophils demonstrate significant impairments in adhesion to the endothelial adhesion molecules P-selectin and E-selectin in in vitro flow chamber studies (9). In vivo studies have demonstrated that impairments in rolling of leukocytes deficient in C2GlcNAcT-I are mainly the result of impaired binding of PSGL-1 to P-selectin with lesser contributions from E-selectin (25). Taken together, previous in vitro (9) and current in vivo studies suggest that C2GlcNAcT-I-deficient neutrophils are deficient in ligands (\(s\)Le\(^{x}\)) that bind to mesenteric and cremaster endothelial adhesion receptors (predominantly P- and E-selectin) important to neutrophil recruitment to the peritoneal cavity and cremaster.

In contrast to the importance of C2GlcNAcT-I to leukocyte (eosinophil and neutrophil) recruitment to the peritoneum, leukocyte recruitment to the lung is not dependent on adhesion ligands synthesized by C2GlcNAcT-I. This is evident from studies of either eosinophil or neutrophil recruitment to the lung, which is not impaired in C2GlcNAcT-I-deficient compared with wild-type mice. Leukocytes derived from C2GlcNAcT-I-deficient mice have reduced levels of adhesion ligands (\(s\)Le\(^{x}\)) synthesized by C2GlcNAcT-I but express similar levels of PSGL-1, L-selectin, and CD11a, CD11b, and CD18 compared with wild-type mice (9). Lymphocyte number, lymphocyte homing, and lymph node morphology are normal in C2GlcNAcT-I-deficient mice (9).

The Ova-challenged C2GlcNAcT-I-deficient mice do not exhibit an increase in the percentage of peripheral blood eosinophils compared with wild-type mice, as might be anticipated if C2GlcNAcT-I-deficient eosinophils exhibited impairments in their trafficking into tissues. Because C2GlcNAcT-I-deficient mice challenged with Ova develop significant airway eosinophilic inflammation, it is not surprising that these mice develop similar levels of airway responsiveness to MCh compared with wild-type mice. Thus the development of BAL, lung, and bone marrow eosinophilia and the development of airway responsiveness to MCh all underscore the absence of differences in eosinophil trafficking to the lung in C2GlcNAcT-I-deficient compared with wild-type mice.

Our studies of C2GlcNAcT-I-deficient mice suggest important differences in the requirements for adhesion ligands synthesized by C2GlcNAcT-I in mediating leukocyte recruitment to the peritoneum compared with the lung. There are several potential explanations for the differences in leukocyte requirements for adhesion ligands in different vascular beds. One explanation is that the paradigm of leukocyte rolling and firm adhesion applies to the high-pressure systemic circulation but not to the low-pressure pulmonary circulation where leukocytes may not require selectins to decelerate and initiate adhesion to endothelium. In support of this explanation are studies demonstrating adhesion molecule-independent recruitment of neutrophils to the lung (18), suggesting that the paradigm of neutrophil rolling and firm adhesion does not apply to the pulmonary vascular bed. In this regard, neutrophil recruitment to the lung is independent of selectins in several mouse models of neutrophilic inflammation (i.e., streptococcus pneumonia; see Ref. 18) but is partially dependent on P-selectin in other models of neutrophil recruitment to the lung (13). However, studies with eosinophils suggest that adhesion molecules, including selectins, are required for eosinophil trafficking to the lung (5). In this regard, several laboratories, including our own laboratory, have demonstrated an important role for the endothelial selectin P-selectin in mediating eosinophil recruitment to the lung in vivo in studies with P-selectin-deficient mice (5, 7). Thus, although this study suggests important differences in requirements for adhesion ligands synthesized by C2GlcNAcT-I in mediating leukocyte recruitment to the peritoneum compared with the lung, it does not address whether adhesion molecules synthesized by non-C2GlcNAcT-I pathways support the rolling and adhesion cascade of either neutrophils or eosinophils in the pulmonary circulation. Improved methods to directly visualize leukocytes in the pulmonary microcirculation in vivo, as can be accomplished in the peritoneal microcirculation, are needed to address this question.

In summary, this study demonstrates the importance of O-glycans linked to the peptide (serine/threonine) backbone of leukocyte cell surface proteins in...
mediating neutrophil and eosinophil recruitment in the peritoneal microcirculation (9). In contrast, leukocyte (neutrophil and eosinophil) recruitment to the lung is not dependent on O-glycans synthesized by the Golgi enzyme C2GlcNAcT-I. These studies suggest that targeting of O-glycans would not inhibit eosinophilic inflammation in the lung associated with asthma.

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REFERENCES


