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ANATOMICAL PATHOLOGY

Induction of peripheral lymph node addressin in human nasal mucosa with eosinophilic chronic rhinosinusitis

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Summary

Eosinophilic chronic rhinosinusitis (ECRS) is characterised by formation of nasal polyps with prominent eosinophilic infiltration; however, how eosinophils are recruited in this pathological setting remains unclear. In the present study, we carried out quantitative immunohistochemical analysis of nasal polyps associated with ECRS (*n*=30) and non-ECRS (*n*=30) to evaluate expression of an L-selectin ligand peripheral lymph node addressin (PNAd) on vascular endothelial cells. We found that PNAd was induced primarily on the luminal surface of venular vessels present in nasal mucosa in both ECRS and non-ECRS, while the number of PNAd-expressing vessels in ECRS significantly exceeded that seen in non-ECRS. Moreover, the number of eosinophils attached to the luminal surface of PNAd-expressing vessels in ECRS was significantly greater than that in non-ECRS, while the number of neutrophils and lymphocytes attached did not differ significantly between conditions. Furthermore, eosinophils, which express cell surface L-selectin, adhered to PNAd-expressing Chinese hamster ovary (CHO) cells in a calcium-dependent manner, and that adhesion was significantly inhibited by pretreatment of eosinophils with DREG-56, an anti-human L-selectin monoclonal antibody. These findings combined suggest that interaction between L-selectin and PNAd plays at least a partial role in eosinophil recruitment in human nasal mucosa with ECRS.

Key words: Eosinophilic chronic rhinosinusitis (ECRS); eosinophil recruitment; L-selectin; peripheral lymph node addressin (PNAd); sialyl 6-sulfo Lewis X.

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INTRODUCTION

Chronic rhinosinusitis (CRS), one of the most common chronic otorhinolaryngological diseases, is characterised by chronic mucosal inflammation of the nasal cavity and paranasal sinuses with clinical symptoms, including nasal discharge, nasal congestion and hyposmia lasting longer than 12 weeks.[1] CRS is divided into two subtypes, namely, CRS with nasal polyps (CRSwNP) and CRS sans (without) nasal polyps (CRSsNP). CRSwNP, particularly cases seen in Western countries, shows pronounced eosinophil infiltration; however, the aetiopathogenesis of CRSwNP is heterogeneous and the degree of eosinophil infiltration differs substantially from case to case. Indeed, neutrophil infiltration is dominant in CRSwNP traditionally in Japan, and this type of CRSwNP exhibits a positive response to endoscopic sinus surgery (ESS) and low-dose, long-term erythromycin treatment (macrolide therapy). However, since the 2000s, the number of CRSwNP cases refractory to and recurring immediately after ESS has increased in Japan,[2] possibly due to westernisation of eating habits and environments.[3] Pathological examination indicates that these refractory CRSwNP cases show prominent eosinophil infiltration.[2] Thus, refractory CRSwNP with prominent eosinophil infiltration is designated eosinophilic CRS (ECRS).[2]

It was not until 2015 that clinical criteria for ECRS diagnosis were proposed by the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis (JESREC).[3] To make a clinical diagnosis of ECRS, the JESREC scoring system assessed unilateral or bilateral disease, the presence of nasal polyps, blood eosinophilia and dominant shadow of ethmoid sinuses in computed tomography (CT) scans.[3] However, definitive ECRS diagnosis is possible only after histopathological examination and is defined as occurring when the number of eosinophils infiltrating nasal mucosa exceeds 70 per high power field (at ×200 magnification).[3] It is noteworthy that mucosa-infiltrating eosinophils originate from the circulation. Thus, a battery of cell adhesion molecules expressed on circulating eosinophils likely interacts with cognate cell adhesion molecules on vascular endothelial cells before cells infiltrate sites of mucosal inflammation.

In chronic inflammatory states, vessels morphologically similar to high endothelial venules (HEVs) in secondary lymphoid organs are induced in non-lymphoid tissues, and these vessels are implicated in lymphocyte recruitment from the circulation to those sites.[4,5] For the initial step of lymphocyte recruitment, L-selectin expressed on circulating lymphocytes interacts weakly, similar to hook-and-loop fasteners (Velcro), with peripheral lymph node addressin (PNAd), a set of sulfated sialomucins recognised by MECA-79 monoclonal antibody[6] and expressed on the luminal surface of HEV-like vessels, resulting in lymphocyte 'rolling' along the endothelium. This 'rolling' is followed by lymphocyte chemokine-dependent activation, integrin-mediated firm adhesion to vascular endothelium, and transmigration across blood vessels.[5,7]

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We previously demonstrated induction of PNAd-expressing HEV-like vessels in chronic inflammatory conditions, including chronic *Helicobacter pylori* gastritis,[8–10] ulcerative colitis,[11,12] autoimmune pancreatitis,[13] and chronic prostatitis associated with benign prostatic hyperplasia.[14] In these studies, we also demonstrated that the number of PNAd-expressing HEV-like vessels is positively correlated with the degree of chronic inflammation. Based on these observations we hypothesised that PNAd is induced on vessels in human nasal mucosa with ECRS and could be recognised by L-selectin expressed on eosinophils, contributing to eosinophil recruitment in ECRS. Indeed, it is reported that eosinophils employ L-selectin for 'rolling' along cytokine-stimulated endothelial cells *in vivo*.[15] However, whether PNAd is induced on vascular endothelial cells in allergic inflammation, including ECRS, remained unclear.

Here, we carried out quantitative histopathological analysis of human nasal mucosa and found that PNAd-expressing vessels were preferentially induced in ECRS.

MATERIALS AND METHODS

Tissue samples

Formalin fixed, paraffin embedded tissue blocks of surgical specimens of nasal polyps associated with ECRS (n=30) and those associated with non-ECRS (n=30) were retrieved from the pathology archives at University of Fukui Hospital and its affiliated hospitals. Histopathological diagnosis of ECRS was made according to JESREC criteria.[3] Analysis of human nasal polyp tissues was approved by the Ethics Committee of the Faculty of Medical Sciences, University of Fukui (reference number 20150041, approved 4 October 2015).

Immunohistochemistry

Monoclonal antibodies used as primary antibodies were: QBEND10 (mouse IgG; Immunotech, France) directed against human CD34, which is a marker of vascular endothelial cells and functions as a scaffold protein for sialyl 6-sulfo Lewis X-capped *O*-glycans, the carbohydrate moiety of PNAd;[16] and MECA-79 (rat IgM; BD Pharmingen, USA), recognising 6-sulfo *N*-acetyllactosamine attached to extended core 1 *O*-glycans, which overlaps with sialyl 6-sulfo Lewis X and constitutes PNAd.[6,17]

Immunohistochemical staining for CD34 was carried out using the Histofine system (Nichirei, Japan) as per the manufacturer's protocol. That for MECA-79 was conducted using an indirect method. Briefly, tissue sections were deparaffinised in xylene and rehydrated in ethanol, and after quenching endogenous peroxidase activity with 0.3% H_2O_2 methanol solution for 30 min, antigens were retrieved by autoclaving sections in 10 mM Tris/HCl buffer (pH 8.0) containing 1 mM EDTA at 105°C for 15 min. After blocking non-specific protein binding with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 30 min, sections were incubated with either QBEND10 or MECA-79 for 60 min. After TBS washing, sections incubated with QBEND10 were treated with horseradish peroxidase (HRP)-conjugated Histofine Simple Stain (Nichirei), while those incubated with MECA-79 were treated with HRP-conjugated goat anti-rat IgM (Jackson ImmunoResearch, USA) for 60 min. After TBS washing, the colour reaction was developed with 3,3′-diaminobenzidine containing 0.02% H_2O_2 for 5 min. Sections were briefly counterstained with haematoxylin.

Quantification of PNAd-expressing vessels

We first determined the number of CD34-positive or PNAd-positive vessels in the entire area of tissue specimens under a microscope at ×200 magnification. We then divided the number of PNAd-positive vessels by the number of CD34-positive vessels in order to calculate the percentage of PNAd-expressing vessels ($PNAd^+/CD34^+$ vessel ratio), as described previously.[10]

Quantification of leukocyte subpopulations attached to PNAd-expressing vessels

Using MECA-79-immunostained tissue sections, we determined the number of cells in respective leukocyte subpopulations (eosinophils, neutrophils or lymphocytes) attached to the luminal surface of PNAd-expressing vessels in the entire area of each specimen. The mean number of cells in each subpopulation attached per 100 PNAd-expressing vessels was calculated as described.[14]

Isolation and purification of human eosinophils

Eosinophils were isolated from healthy volunteers by density centrifugation using Polymorphprep (Axis-Shield, Norway) followed by negative selection with magnetic-activated cell sorting (MACS) using an Eosinophil Isolation Kit, human (Miltenyi Biotec, Germany) according to the manufacturer's instructions. Eosinophil purity of >98%, as assessed by eosin staining, was routinely obtained. L-selectin expression on eosinophils was confirmed by fluorescence-activated cell sorting (FACS) analysis with an anti-human L-selectin monoclonal antibody DREG-56 (mouse IgG; BD Pharmingen).[18]

Eosinophil adhesion assay on Chinese hamster ovary (CHO) cells stably expressing PNAd

A CHO cell line stably expressing human CD34 decorated with sialyl 6-sulfo Lewis X attached to both extended core 1 and core 2-branched *O*-glycans, which can function as PNAd, was previously established.[9] Using this line, we conducted an eosinophil adhesion assay essentially as described.[9,19] Briefly, 5×10^4 CHO cells were seeded in individual wells of Lab-Tech 4-well chamber slides (Nalge Nunc International, USA) 24 hours prior to assay; 1×10^6 human eosinophils isolated above were suspended in 500 µL RPMI 1640 supplemented with 20 mM HEPES (pH 7.0), layered onto CHO cells in each well, and incubated at 4°C for 30 min on a rotating shaker. For inhibition experiments, eosinophils were pretreated at 4°C for 10 min with 10 µg/mL of DREG-56 monoclonal antibody, which blocks L-selectin function.[18] Non-adherent cells were removed by rinsing three times with 1 mL/well of RPMI 1640, with or without 1 mM EDTA. After fixation with phosphate-buffered saline (PBS) containing 1% glutaraldehyde, slides were observed using a microscope with Nomarski differential interference optics. The number of CHO cells and adherent eosinophils in five fields at ×200 magnification was determined, and the number of adherent eosinophils per 100 CHO cells was calculated.

Statistical analysis

Data are reported as means with standard deviation (SD). Differences among groups were statistically analysed by Student's t-test or one-way analysis of variance (ANOVA), where appropriate, using GraphPad Prism 7 software (GraphPad Software, USA). p values less than 0.05 were considered significant.

RESULTS

Induction of PNAd on vessels in human nasal mucosa with ECRS

While the number and composition of inflammatory cells differed between ECRS and non-ECRS, nasal polyps associated with both types of CRS shared histological similarities: namely, both were composed of covering respiratory epithelium and a loose oedematous stroma containing large numbers of venular vessels, and inflammatory cells were distributed around these vessels (Fig. 1, top panels). Thus we performed immunohistochemical staining for PNAd, which participates in 'rolling' of lymphocytes and other leukocyte subpopulations, including eosinophils.[15] In parallel, we conducted immunostaining for CD34 to identify all vessels in a tissue section. As shown in Fig. 1, all vessels were CD34-positive, and some of those were also PNAd-positive. After analysing a total of 60 nasal polyps associated with either ECRS (n=30) or non-ECRS (n=30), we found that the percentage of PNAd-expressing vessels in ECRS (24.6 ± 11.1%) was significantly greater than that in non-ECRS (10.8 ± 5.8%) (p<0.001) (Fig. 2).

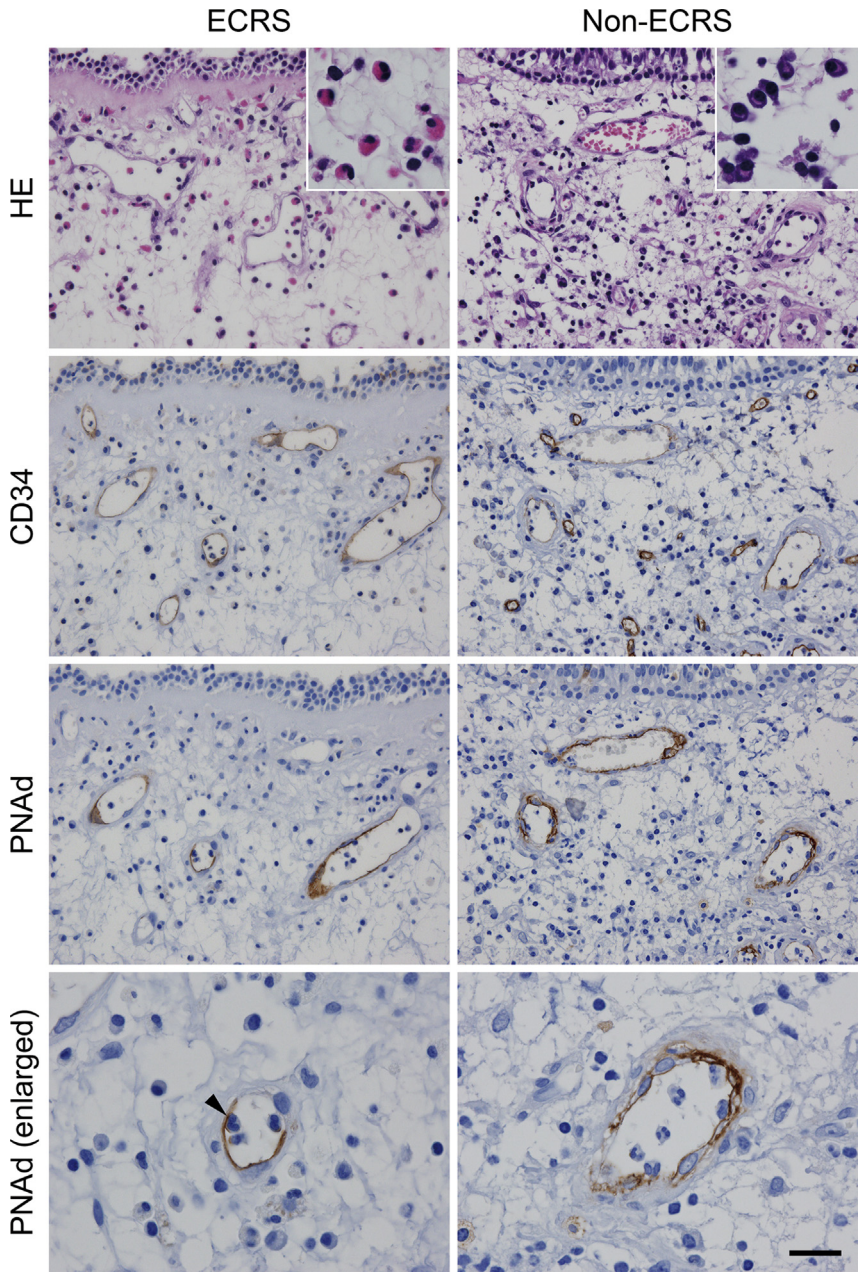

Fig. 1 PNAd induction in human nasal mucosa with ECRS (left panels) and non-ECRS (right panels). Serial tissue sections were stained with haematoxylin and eosin (HE) and immunostained for CD34 and PNAd. Both types of nasal polyps are composed of covering respiratory epithelium and oedematous stroma containing venular vessels and inflammatory cells. Eosinophils are the chief inflammatory cells in ECRS, while lymphocytes and plasma cells are predominant in non-ECRS (insets in top panels). PNAd is expressed on a fraction of CD34-positive vessels in both types of CRS. Panels in the bottom row are enlarged from those in the third row; arrowhead indicates eosinophil attached to the luminal surface of PNAd-expressing vessels in ECRS. Bar = 40 μm for bottom panels and insets in top panels, and 100 μm for the rest.

Close association of eosinophils with PNAd-expressing vessels in ECRS

We then determined the number of cells in each leukocyte subpopulation attached to the luminal surface of PNAd-expressing vessels in ECRS and non-ECRS. As shown in Fig. 3, the dominant leukocyte subpopulation attached was neutrophils, followed by lymphocytes, in both ECRS and non-ECRS, and the number of cells from both leukocyte subpopulations attached to PNAd-expressing vessels did not differ between two types of CRS (neutrophils 8.4 ± 8.2 vs 12.0 ± 13.1 per 100 PNAd-expressing vessels; lymphocytes 6.7 ± 5.8 vs 9.4 ± 9.7). On the other hand, while their absolute number was less than that of neutrophils or lymphocytes, the number of eosinophils attached to the luminal surface of PNAd-expressing vessels in ECRS was greater than that seen in non-ECRS (3.6 ± 3.4 vs 0.5 ± 0.8) with high statistical significance ($p<0.001$) (see also Fig. 1, bottom panels).

L-selectin-mediated eosinophil adhesion to PNAd-expressing Chinese hamster ovary (CHO) cells

The above findings suggest that circulating eosinophils begin ‘rolling’ along venular endothelium via interaction between L-selectin and PNAd, an activity required for subsequent eosinophil chemokine-dependent activation,

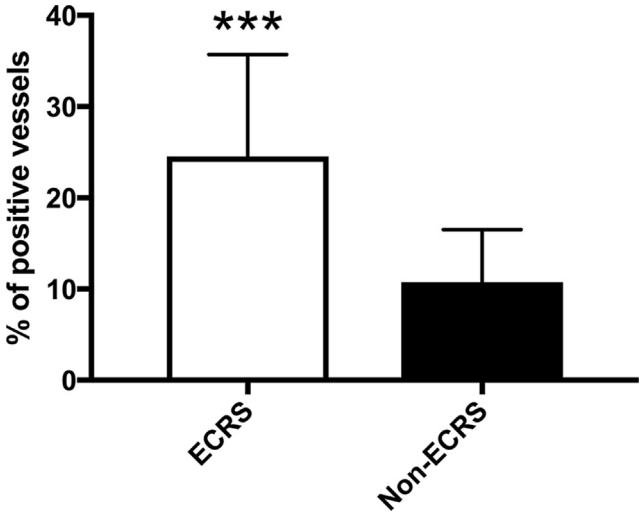

Fig. 2 Quantification of PNAd-expressing vessels in nasal mucosa with ECRS and non-ECRS. The percentage of PNAd-positive vessels among CD34-positive vessels is shown for ECRS (open bar) and non-ECRS (closed bar). Data are presented as means with SD. ***, $p<0.001$.

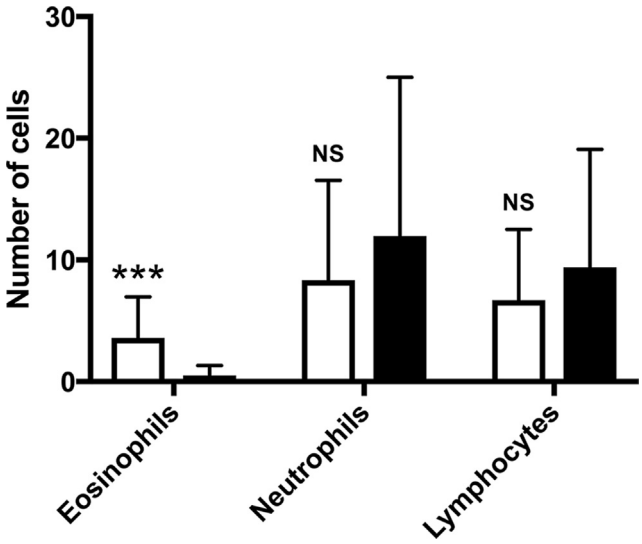

Fig. 3 The number of leukocyte subpopulations (eosinophils, neutrophils and lymphocytes) attached per 100 PNAd-expressing vessels in ECRS (open bars) and non-ECRS (closed bars). Data are presented as means with SD. ***, $p<0.001$; NS, not significant.

integrin-mediated firm adhesion to vascular endothelium, and transmigration across vessels. To determine whether eosinophils employ L-selectin for PNAd binding, we first confirmed expression of L-selectin on the eosinophil cell surface by FACS analysis (Fig. 4). We then conducted an eosinophil adhesion assay on CHO cells stably-expressing PNAd, namely, CD34 decorated with sialyl 6-sulfo Lewis X attached to both extended core 1 and core 2-branched *O*-glycans. As shown in Fig. 5 and 6, eosinophils robustly adhered to PNAd-expressing CHO cells (80.1 ± 10.1 per 100 PNAd-expressing CHO cells), and that adhesion was abolished in the presence of EDTA (6.2 ± 3.4), indicating that adhesion is calcium-dependent. Moreover, pretreatment of eosinophils with DREG-56 monoclonal antibody significantly decreased the number of adherent eosinophils (43.4 ± 6.6). These findings collectively indicate that eosinophil adhesion to PNAd-expressing CHO cells is at least in part mediated by L-selectin.

DISCUSSION

Here we demonstrate that PNAd is induced on vessels in human nasal mucosa with ECRS, and that eosinophils are attached to the luminal surface of these vessels. We also demonstrate that eosinophils adhere to PNAd-expressing CHO cells calcium-dependently and that such adhesion is inhibited by anti-L-selectin antibody. These findings combined suggest that PNAd-expressing vessels play at least a partial role in eosinophil recruitment in ECRS.

Several glycosyltransferases and sulfotransferases are known to function in biosynthesis of the PNAd carbohydrate moiety, which is sialyl 6-sulfo Lewis X attached to extended core 1 *O*-glycans. Among them, core 1 extending β1,3-*N*-acetylglucosaminyltransferase is a unique enzyme that adds an *N*-acetylglucosamine (GlcNAc) residue to the non-reducing galactose (Gal) in core 1 moieties through a β1,3-linkage to form extended core 1 structures.[17] However, *N*-acetylglucosamine-6-*O*-sulfotransferase 1 (GlcNAc6ST-1)[20–22] and GlcNAc6ST-2[23,24] are critical for sulfation of non-reducing GlcNAc at the C6-position in HEVs[25,26] and HEV-like vessels.[11,12] As previously proposed by Drayton *et al.*, lymphotoxin αβ produced by T cells increases mRNA and protein levels of GlcNAc6ST-2 (and likely GlcNAc6ST-1) in endothelial cells, and this enzyme then modifies oligosaccharide side chains of the core protein (such as CD34) in the Golgi apparatus, giving rise to PNAd on HEV-like vessels.[27] It is tempting to speculate that similar mechanisms operate in PNAd induction in human nasal mucosa with ECRS.

In the present study, we found that the number of eosinophils attached to the luminal surface of PNAd-expressing vessels in ECRS was significantly greater than that seen in non-ECRS, while the number of neutrophils and lymphocytes attached in both types of CRS did not differ significantly. These findings suggest the existence of a mechanism favouring eosinophil adhesion to PNAd-expressing vessels in ECRS. Several molecules reportedly function in selective eosinophil adhesion. Among them, α4β1 and α4β7 integrins, which are expressed on eosinophils but not neutrophils, likely play a partial role in selective eosinophil adhesion to endothelial cells through interaction with cytokine-inducible endothelial counter-receptors vascular cell adhesion molecule 1 (VCAM-1) and mucosal addressin cell adhesion molecule 1 (MAdCAM-1), respectively.[28] On the other hand, chemokine receptor 3 (CCR3), which is expressed on eosinophils, plays a pivotal role in selective eosinophil chemotaxis and transendothelial migration through interaction with eotaxin and related chemokines.[29] Indeed, Saito *et al.* demonstrated that nasal polyp extract-induced eosinophil migration was completely inhibited by a CCR3 antagonist.[30] We recently employed whole-transcriptome analysis with next-generation sequencing to reveal that expression of transient receptor potential cation channel, subfamily

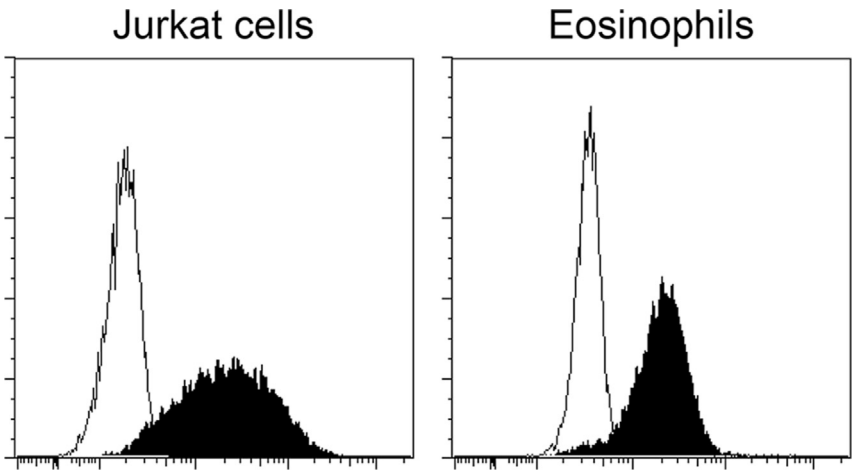

Fig. 4 Expression of L-selectin on human eosinophils. Jurkat cells (left, positive controls) and human eosinophils (right) were stained with DREG-56 and subjected to FACS analysis. White peaks represent negative controls established in the absence of primary antibody. X- and Y-axes indicate fluorescence intensity and number of events, respectively.

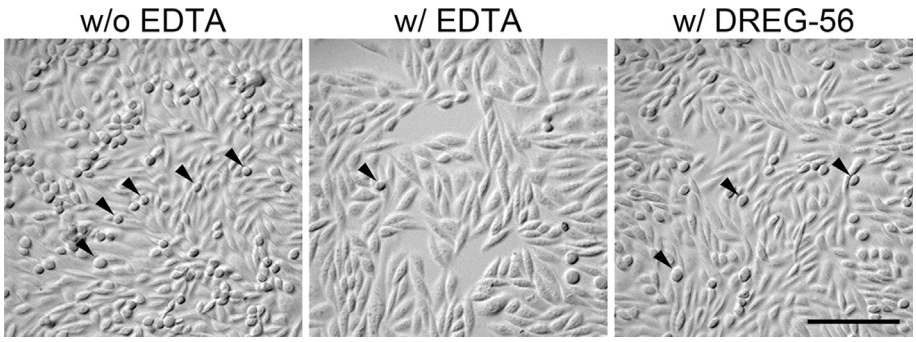

Fig. 5 Eosinophil adhesion to PNAd-expressing CHO cells in the absence (w/o EDTA) or presence (w/ EDTA) of EDTA, as observed by Nomarski differential interface optics. Eosinophil adhesion (arrowheads) is substantially decreased following pretreatment with an anti-L-selectin monoclonal antibody DREG-56 (w/ DREG-56). Bar = 100 μm.

V, member 3 (TRPV3),[31] cystatin SN (CST1)[32] and periostin (POSTN)[33] increases in ECRS. These factors may also function in preferential eosinophil recruitment in ECRS.

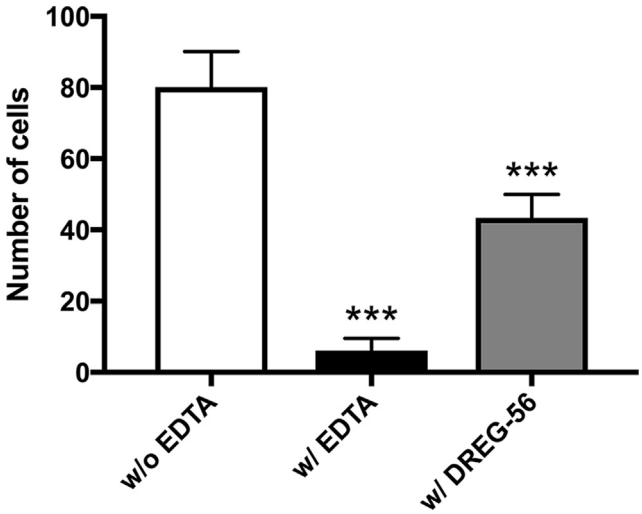

Fig. 6 Quantification of data shown in Fig. 5. The number of eosinophils adhering per 100 CHO cells was determined by examining five fields at ×200 magnification. Robust eosinophil adhesion to CHO cells (w/o EDTA; open bar) is significantly inhibited by treatment with EDTA (w/ EDTA; closed bar) or DREG-56 (w/ DREG-56; grey bar). ***, $p<0.001$.

In our adhesion assay, eosinophil adhesion to PNAd-expressing CHO cells significantly decreased when eosinophils were pretreated with anti-L-selectin antibody DREG-56; however, relatively large numbers of eosinophils remained adherent when compared to conditions including EDTA. Thus DREG-56 levels used in the assay may not have been sufficient to completely block eosinophil adhesion to PNAd-expressing CHO cells. Alternatively, other calcium-dependent carbohydrate-binding proteins, including galectins and sialic acid-binding immunoglobulin-like lectins (Siglecs), expressed on eosinophils may interact with PNAd and/or its intermediates, contributing to residual eosinophil adhesion.[34–36]

It is widely believed that manipulating leukocyte recruitment to inflammatory sites can ameliorate disease activity or severity,[37] leading to use of anti-adhesion therapies to antagonise leukocyte recruitment. Rosen *et al.* reported that intravenous administration of MECA-79 to allergic sheep significantly diminished airway responses to inhaled allergens and blocked accumulation of all classes of leukocytes in bronchoalveolar lavage fluid.[37] We previously reported that the number of MECA-79-positive HEV-like vessels induced in chronic inflammatory diseases including chronic *Helicobacter pylori* gastritis,[8] ulcerative colitis[11,12] and chronic prostatitis associated with benign prostatic hyperplasia[14] is positively correlated with severity of chronic inflammation. These observations combined with findings reported here

indicate that MECA-79 could be a potential therapeutic tool in management of ECRS. Further studies are required to support this hypothesis.

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