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Cadherin-related family member 3 upregulates the effector functions of eosinophils

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To the Editor,

Acute respiratory infections including rhinovirus (RV) infections are a major cause of asthma exacerbations.¹ Recent studies suggest that eosinophils play important roles in the development of asthma exacerbation.² Not only neutrophils but also eosinophils increase in asthmatic airways during viral infection,³ suggesting that eosinophils are indeed recruited to and activated in the airways during virus-related asthma exacerbations.

Cadherin-related family member 3 (CDHR3), a member of the cadherin superfamily, is a transmembrane protein with six extracellular cadherin domains. However, the biological function of CDHR3 is still unknown. Recently, Bønnelykke et al. reported that a coding single nucleotide polymorphism (SNP) (rs6967330; C₅₂₉Y) in CDHR3 is associated with severe exacerbations of childhood asthma.⁴ Furthermore, we reported that CDHR3 is a receptor for rhinovirus C (RV-C) species,⁵ which is more virulent and more likely to cause exacerbations of childhood asthma as compared with RV-B.^{1, 6} CDHR3 rs6967330; C₅₂₉Y increases the expression of CDHR3 protein on the cell surface^{4, 5} resulting in increased RV-C binding and progeny yields.⁵ Moreover, this SNP is associated with increased RV-C illnesses in vivo.⁷ Therefore, CDHR3-Y₅₂₉ variant is likely to promote the development of asthma exacerbations by increasing susceptibility to RV-C illnesses. CDHR3 is mainly expressed on the apical surface of ciliated epithelial cells, but the natural ligands and function are unknown.

In this study, we examined whether CDHR3 binds to and activates eosinophils. We first examined

the effect of CDHR3 on eosinophil adhesion. Eosinophils were obtained from healthy volunteers, and their adhesion to CDHR3 or intercellular adhesion molecule (ICAM)-1 was measured using residual eosinophil peroxidase (EPO) assays. Compared to control or ICAM-1, CDHR3 induced significantly higher spontaneous adhesion of eosinophils (Figure 1A). More than 10 $\mu\text{g/ml}$ of CDHR3 augmented eosinophil adhesion, and 100 $\mu\text{g/ml}$ of CDHR3 induced a maximal response (Figure 1B). IL-5 further enhanced eosinophil adhesion to CDHR3 (Figure 1B). Eosinophil adhesion to CDHR3 was inhibited by anti- αM integrin or anti- β2 integrin mAb, but not by anti- α4 integrin mAb (Figure S1), suggesting that eosinophils can adhere to CDHR3 via $\alpha\text{M}\beta\text{2}$ integrin. The inhibition of adhesion by anti- αM integrin or anti- β2 integrin mAb could be due to steric hindrance. CDHR3-induced eosinophil adhesion of asthmatic patients was higher than that of healthy volunteers (Figure 1C).

We next examined the effect of CDHR3 on superoxide anion (O_2^-) generation or release of eosinophil-derived neurotoxin (EDN) by eosinophils. Compared to medium control, CDHR3 induced significantly greater eosinophil O_2^- generation, to a level similar to that induced by IL-5 (Figure 1D). Eosinophil O_2^- generation induced by CDHR3 was suppressed by anti- αM integrin or anti- β2 integrin mAb but not by anti- α4 integrin mAb (Figure S1). In addition, CDHR3 induced significantly higher EDN release (Figure 1E) compared to control. Anti- αM integrin or anti- β2 integrin mAb, but not anti- α4 integrin mAb, suppressed EDN release induced by CDHR3 (Figure S1).

We next tested whether CDHR3 C₅₂₉Y mutation affects eosinophil functions. Eosinophils adhesion to plates coated with the wild-type CDHR3-C₅₂₉ or the CDHR3-Y₅₂₉ variant protein were similar (Figure S2). Eosinophil O_2^- generation by CDHR3 variant protein did not differ from that by wild-type CDHR3 protein (Figure S2). Therefore, we transfected HeLa cells with these CDHR3 variants, and then examined effects of CDHR3 C₅₂₉Y mutation on eosinophil adhesion and functions.

We previously demonstrated that expression of CDHR3-Y₅₂₉ variant after transfection of HeLa-H1 cells lead to enhanced surface expression of protein as compared to CDHR3-C₅₂₉,⁵ whereas levels of overall cellular expression of CDHR3 were similar,⁵ and confirmed these finding in this study (data not shown). Along with increased CDHR3 expression on the cell surface, transfection with CDHR3-Y₅₂₉ also increased eosinophil adhesion compared to CDHR3-C₅₂₉ or the negative control (Figure 2A). Similar results were obtained when we used fluorescently-labeled eosinophils (Figure 2B, 2C) or performed major basic protein (MBP) staining (Figure S3). Furthermore, CDHR3-Y₅₂₉ transfection induced greater eosinophil O₂⁻ generation (Figure 2D) and EDN release (data not shown).

In this study, we found that CDHR3 activated eosinophil function such as adhesion, O₂⁻ generation and degranulation. This was true for plate-bound CDHR3 (Figure 1) and for CDHR3 expressed in transfected HeLa cells (Figure 2). However, the effects of genetic variation at rs6967330 on eosinophil adhesion and function depended on whether the proteins were plate bound (Figure S2; no difference) or expressed in HeLa cells (Figure 2; Y₅₂₉ induced increased eosinophil activation). These findings suggest that the difference in eosinophil adhesion was due to the level of surface expression of CDHR3 protein, but not due to the different ability of the CDHR3 variants to induce eosinophil adhesion. The plate-bound experiments demonstrate that if the display of CDHR3 protein on a surface is similar, the effects on eosinophil adhesion are also similar.

Given the close association between eosinophilic inflammation and asthma exacerbation,² CDHR3-Y₅₂₉ could promote eosinophil adhesion and proinflammatory functions linked to asthma exacerbations. Recent studies suggested that CDHR3-Y₅₂₉ increases the risk for developing chronic rhinosinusitis or severe adult asthma with early onset, suggesting that CDHR3 variant may contribute to eosinophilic inflammation in chronic rhinosinusitis as well as bronchial asthma.

Whether CDHR3 expression is increased in the airway of patients with asthma is incompletely understood. For example, Bai et al. reported that CDHR3 mRNA expression in airway epithelial cells cultured at air-liquid interface was slightly lower in cells obtained from donors with asthma.⁸ Furthermore, Everman et al. reported that CDHR3 is exclusively expressed on ciliated cells, and that expression is greatest in cells undergoing ciliation as compared to mature ciliated cells,⁹ suggesting that eosinophil adhesion to ciliated cells could be greatest in airways with active cell differentiation or repair. In addition, Jones et al. reported that CDHR3 protein expression of bronchial epithelial cells increased in atopic asthma as compared with that in non-atopic controls.¹⁰ Additional studies are needed to test for cell surface expression of CDHR3 in vivo with regard to CDHR3 genotype at rs6967330 and asthma status.

We found that CDHR3-induced eosinophil adhesion of asthmatic patients was higher than that of healthy volunteers (Figure 1C), although the mechanism is unknown. In this study, eosinophil adhesion to CDHR3 in allergic asthma did not differ from that in non-allergic asthma (data not shown). Furthermore, in allergic asthma, the titer of specific IgE or total IgE did not affect the degree of eosinophil adhesion (data not shown), suggesting that IgE-mediated mechanisms are unlikely to be involved.

One limitation of this study is that the experiments were performed in vitro and these findings need to be confirmed in nontransformed airway epithelial cells and in clinical studies. Another limitation is that we tested for interactions between CDHR3 and a limited number of integrins. It is possible that other integrins in addition to α M integrin or β 2 integrin may interact with CDHR3.

In conclusion, CDHR3 upregulated eosinophil functions such as adhesion, O_2^- generation and EDN release. Furthermore, eosinophil activation by CDHR3 was at least partially mediated through α M β 2

integrin. These findings suggest that genetic variation in CDHR3 can modify the risk for developing asthma by activating eosinophils, as well as by increasing the risk of RV-C induced illnesses.

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CONFLICTS OF INTEREST

J.E.G received consultant's fees from Regeneron, Ena Therapeutics and Meissa Vaccines Inc. The rest of the authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

K.N. participated in the direction of the study, performed the experiments, analyzed the data, and wrote the manuscript. T.S. performed the experiments. Y.A.B. participated in the direction of the study, analyzed the data, and edited the manuscript. T.N. performed the experiments. T.K. and T.S. participated in the data analyses. S.U. performed the experiments and analyzed the data. J.E.G. and M.N. participated in the direction of the study, analyzed the data, and edited the manuscript. All authors read and approved the final manuscript.

Keywords

asthma, eosinophils, virus

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SUPPORTING INFORMATION

Additional supporting information may be found in the Supporting Information section at the end of the article

Figure legends

FIGURE 1. Effect of CDHR3 on eosinophil functions. (A and B) Effect of CDHR3 on eosinophil adhesion. A, Eosinophils (1×10^5 cells/ml) collected from non-allergic donors were incubated in CDHR3 or ICAM-1-coated plates (10 μ g/ml) or in medium (HBSS/gel)-coated plates, and their adhesion was measured. As for IL-5, eosinophils (1×10^5 cells/ml) were incubated with IL-5 (100 pM) in medium-coated plates and their adhesion was measured. Data are shown as means $\pm$ SEM of 6 experiments using cells from different donors. * $P < 0.05$ vs. spontaneous adhesion (Spon). # $P < 0.05$

vs. ICAM-1. B, Eosinophils (1×10^5 cells/ml) collected from non-allergic donors were incubated with or without IL-5 (100 pM), and their adhesion to various concentrations of rh-CDHR3 (0.1-100 $\mu\text{g/mL}$) was measured ($n = 6$). * $P < 0.05$ and *** $P < 0.001$ vs. spontaneous adhesion (Spon). # $P < 0.05$ and ## $P < 0.01$ vs. buffer (without IL-5). C, Effect of asthma on CDHR3-induced eosinophil adhesion. Eosinophils (1×10^5 cells/ml) collected from healthy volunteers or asthmatic patients were incubated in CDHR3-coated plates (1 or 10 $\mu\text{g/mL}$) and their adhesion was measured ($n = 6$). * $P < 0.05$ and ** $P < 0.01$ vs. spontaneous adhesion (Spon). ## $P < 0.01$ vs. healthy volunteers. D, Effect of CDHR3 on eosinophil O_2^- generation. Eosinophils (1×10^6 cells/ml) from non-allergic donors were incubated in CDHR3 or ICAM-1-coated plates (10 $\mu\text{g/mL}$) or in medium-coated plates. As for IL-5, eosinophils (1×10^6 cells/ml) were incubated with IL-5 (100 pM) in medium-coated plates. The generation of eosinophil O_2^- was examined ($n = 6$). Maximum value of eosinophil O_2^- generation over the next 240 min were shown. * $P < 0.05$ and ** $P < 0.01$ vs. spontaneous O_2^- generation (Spon). ## $P < 0.01$ vs. ICAM-1. E, Effect of CDHR3 on EDN release. Eosinophils (1×10^6 cells/ml) from non-allergic donors were incubated in CDHR3 or ICAM-1-coated plates (10 $\mu\text{g/mL}$) or in medium-coated plates for the 240 min. As for IL-5, eosinophils (1×10^6 cells/ml) were incubated with IL-5 (100 pM) in medium-coated plates for the 240 min. Levels of EDN in cell-free supernatants were quantified using ELISA ($n = 6$). ** $P < 0.01$ vs. spontaneous EDN release (Spon). # $P < 0.05$ vs. ICAM-1.

FIGURE 2. Effect of CDHR3 C₅₂₉Y gene mutation on eosinophil functions. A, Effect of CDHR3 C₅₂₉Y gene mutation on eosinophil adhesion to HeLa cells transfected with plasmid DNA. HeLa cells were transfected with plasmids encoding wild-type CDHR3 (C₅₂₉) or CDHR3 variant (Y₅₂₉) or

lipofectamine only (Lipo), and eosinophil adhesion to transfected cells was measured ($n = 6$). * $P < 0.05$ and ** $P < 0.01$ vs. Lipo. ## $P < 0.01$ vs. C₅₂₉. B, Fluorescently-labeled eosinophil adhesion to transfected HeLa cells. HeLa cells were transfected with plasmids encoding wild-type CDHR3 (C₅₂₉) or CDHR3 variant (Y₅₂₉) or lipofectamine only (Lipo). Eosinophils were labeled with calcein-AM and then incubated with the transfected HeLa cells for 20 min. After the plates were washed, cells were observed under a fluorescence microscope. Representative figures of 3 independent experiments with different donors were shown. Scale bar, 100 μm . C, Fluorescently-labeled eosinophil adhesion to transfected HeLa cells at higher magnification. Scale bar, 20 μm . D, Effect of CDHR3 C₅₂₉Y gene mutation on eosinophil O₂⁻ generation. HeLa cells were transfected with plasmids encoding wild-type CDHR3 (C₅₂₉) or CDHR3 variant (Y₅₂₉) or lipofectamine only (Lipo), and eosinophils were incubated with transfected HeLa cells for 240 min. The generation of eosinophil O₂⁻ was examined ($n = 6$). The maximum value of eosinophil O₂⁻ generation was shown. * $P < 0.05$ and ** $P < 0.01$ vs. Lipo. # $P < 0.05$ vs. C₅₂₉.

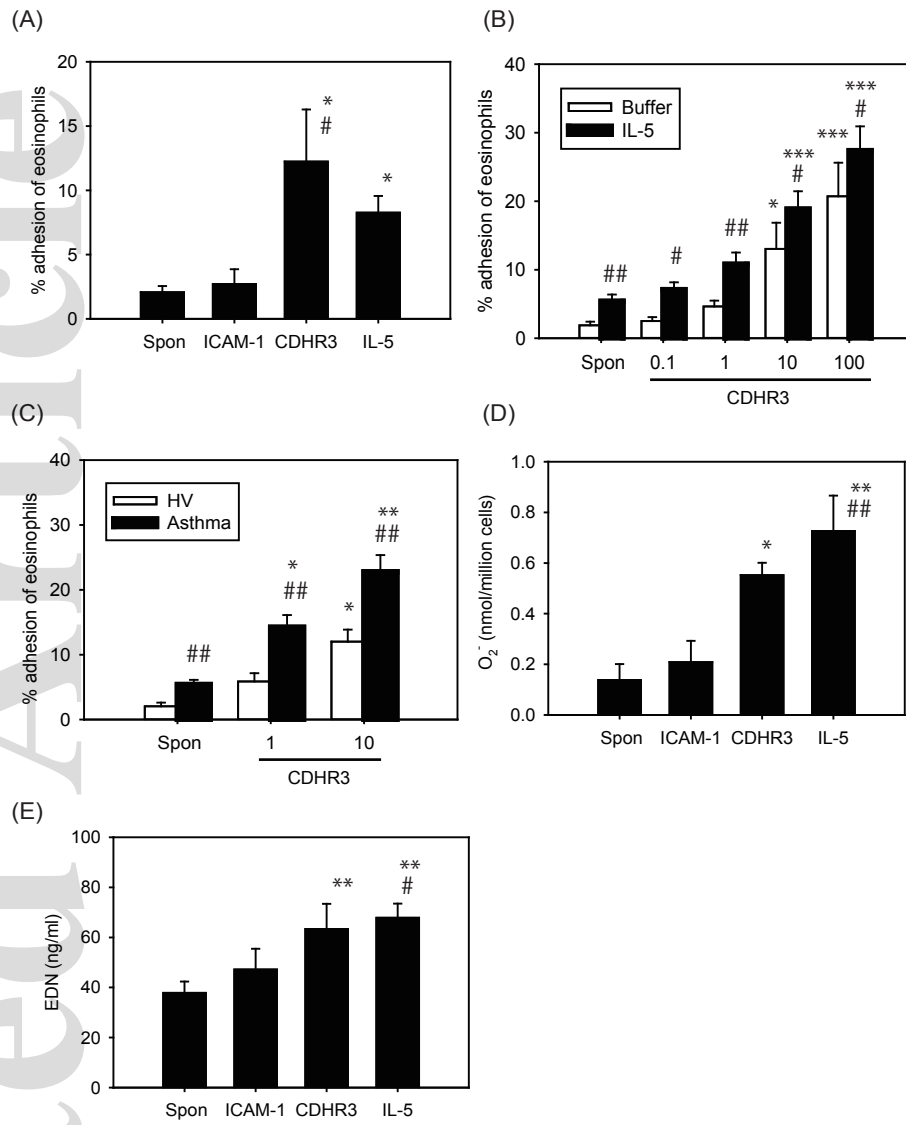


Figure 1

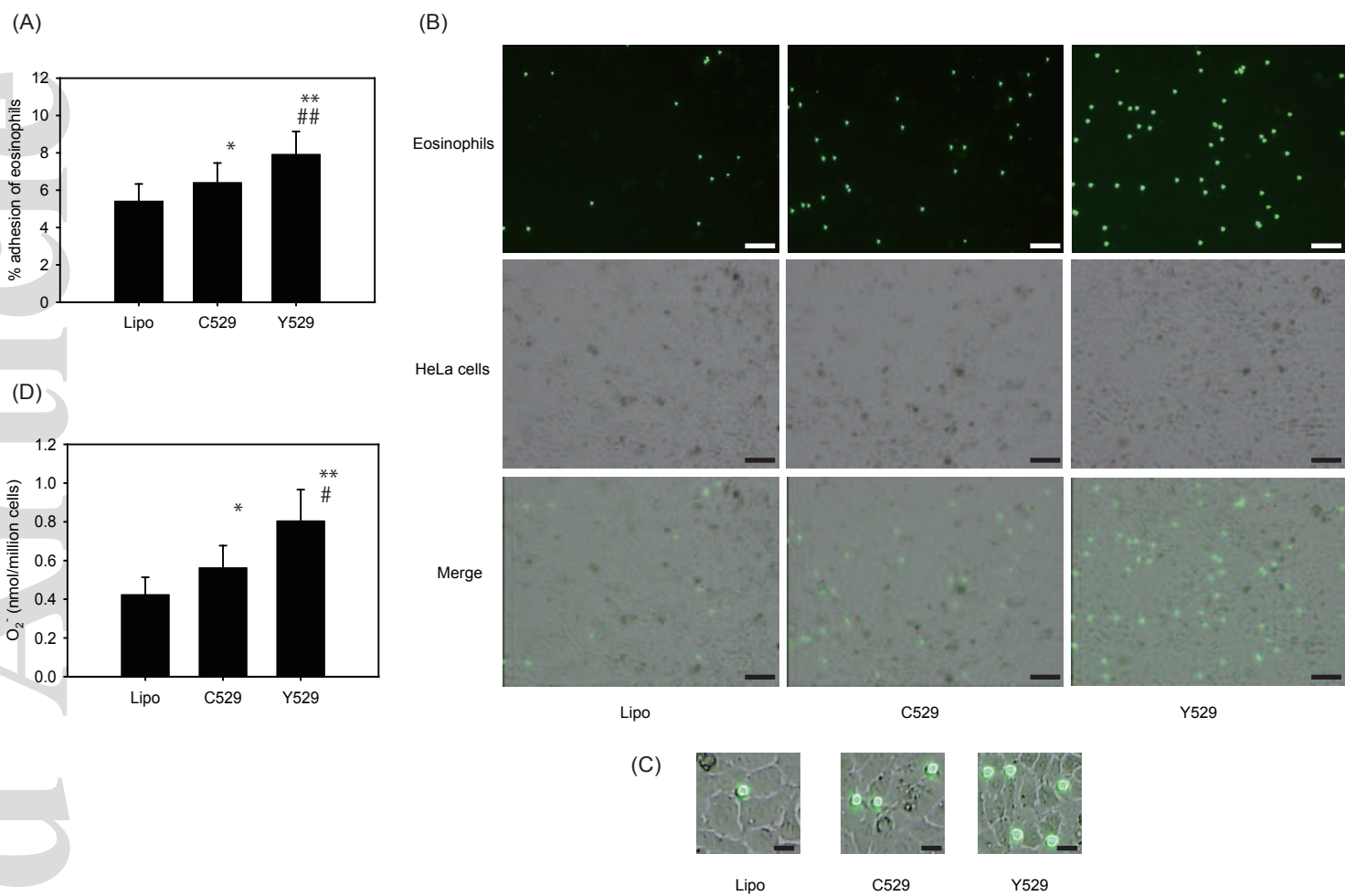


Figure 2

Online Supporting Information

Cadherin-related family member 3 upregulates the effector functions of eosinophils

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MATERIALS AND METHODS

Preparation of eosinophils and cadherin-related family member 3 (CDHR3) protein

Eosinophils were isolated from peripheral blood collected from non-allergic healthy volunteers with a peripheral blood differential eosinophil count of < 5%. Healthy volunteers were defined as subjects with no history of allergic disease such as asthma or rhinitis and no symptoms such as coughing or sneezing associated with allergic disease. In selected experiments, we collected eosinophils from patients with well-controlled (Asthma Control Test > 20), mild or moderate asthma (Global Initiative for Asthma; step 2 or 3, n = 6 (allergic 4, non-allergic 2), blood eosinophil counts; $388 \pm 115/\mu\text{l}$). They had no asthma exacerbations and did not take any systemic corticosteroids during the 3 months before collecting blood. The numbers of males and females, ranging in age, were comparable among the donors. This study was approved by the Ethical Committee of Saitama Medical University, and written informed consent was obtained before collection of each blood sample. Eosinophils were isolated by the combination of density gradient centrifugation and negative selection using immunomagnetic beads, as previously described.¹⁻⁵ Briefly, 10 ml of dextran were added to 40 ml of heparinized blood, and erythrocytes were removed as sediment. The system-Histopaque® (Sigma-Aldrich, St. Louis, MO) was used for isolation

according to the manufacturer's instructions. Separated lymphocytes were removed, 2 ml of sterile distilled water and 2 ml of 1.8% salt solution were added, followed by 20 ml of the sterilized PBS, and the neutrophils were separated by centrifuge. The remaining cells were washed, then incubated with anti-CD16 Ab-coated magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 30 min at 4 °C, and finally filtrated with a column containing steel wool placed in a magnetic field (Miltenyi Biotech). Eosinophils, which passed through the column, were resuspended in Hank's balanced salt solution (HBSS) supplemented with gelatin to a final concentration of 0.1% (HBSS/gel). Over 98% of the cells were eosinophils, as determined by morphological criteria using May-Grünwald-Giemsa staining or by major basic protein (MBP) staining. Eosinophil viability was > 99%, as determined by Trypan blue dye exclusion. Recombinant human (rh) proteins corresponding to extracellular domains 1-6 (positions 20-713) of wild-type (C₅₂₉) or variant (Y₅₂₉) CDHR3 were obtained from GenScript (Piscataway, NJ).

Eosinophil adhesion

Eosinophil adhesion to rh-CDHR3 or rh-intercellular adhesion molecule (ICAM)-1-coated plates was assessed based on the residual eosinophil peroxidase (EPO)

activity of adherent eosinophils, as previously described.¹⁻⁵ ICAM-1 is an adhesion molecule that can activate eosinophil functions,⁶ and a cellular receptor for the majority of RV-A (major) and all of RV-B serotypes.⁷ That is why we used ICAM-1 as a control in this study. Briefly, eosinophils (100 μ l of 1×10^5 cells/ml in HBSS/gel) were incubated in the presence or absence of IL-5 (100 pM; R&D Systems, Minneapolis, MN) in rh-CDHR3 (10 μ g/ml) or rh-ICAM-1-coated plates (10 μ g/ml; R&D Systems) at 37 °C for 20 min. Corresponding control wells were coated with HBSS/gel. The plates were washed with HBSS and 100 μ l of HBSS/gel was added to the wells. Standards that were comprised of 100 μ l of serially diluted cell suspensions (1×10^3 , 3×10^3 , 1×10^4 , 3×10^4 , and 1×10^5 cells/ml) were added to the empty wells. The EPO substrate (1 mM o-phenylenediamine, 1 mM H₂O₂, and 0.1% Triton X-100 in Tris buffer, pH 8.0) was then added to all wells, and the plates were incubated for 30 min at room temperature. The reaction was stopped by adding 20 μ l of 4 M H₂SO₄, and absorbance was measured at 490 nm. In some experiments, eosinophils were preincubated with 3 μ g/ml of mouse IgG1 (clone MOPC-21; Becton Dickinson, Franklin Lakes, NJ), anti- α M integrin monoclonal (m) Ab (clone 2LPM19c; Pierce, Rockford, IL), anti- β 2 integrin mAb (clone L130; Becton Dickinson), or anti- α 4 integrin mAb (clone HP2/1; Cosmo Bio Co. Ltd, Tokyo, Japan) for 15 min before addition

to the wells. Each experiment was performed in quadruplicate using eosinophils from a single donor, and the percentage eosinophil adhesion was determined from mean values that were calculated from log dose response curves. Generally, eosinophil adhesion started at 20 min after incubation, peaked at 30 min and lasted for 60 min in our system.³ In a preliminary study, similar kinetic of eosinophil adhesion to CDHR3 was observed (data not shown). CDHR3-induced eosinophil adhesion at 30 min was slightly higher than that at 20 min, however the difference was not significant (data not shown). Therefore, we presented the data of eosinophil adhesion at 20 min in this study.

In selected experiments, we evaluated eosinophil adhesion using fluorescently-labeled eosinophils. Eosinophils were labeled with calcein-AM (Cell Counting Kit-F; Dojindo Molecular Technologies, Tokyo, Japan) for 30 min and then washed with HBSS to remove fluorescence in the medium. Then, labeled-eosinophils (100 μ l of 1×10^5 cells/ml) were incubated in the plates with coated-protein or transfected-cells at 37 °C for 20 min. After the plates were washed with HBSS, we observed eosinophil adhesion under a fluorescence microscope (Biozero BZ-8000; Keyence, Osaka, Japan). Eosinophil viability after incubation was > 97%, as determined by Trypan blue dye exclusion.

Eosinophil superoxide anion (O_2^-) generation

Eosinophil O_2^- generation was measured in 96-well ELISA plates based on the superoxide dismutase (SOD)-inhibitable reduction of cytochrome C as previously described.²⁻⁴ We initially added SOD (0.2 mg/ml in HBSS/gel; 20 μ l) to SOD control wells and then HBSS/gel to all wells of rh-CDHR3 or rh-ICAM-1-coated plates (10 μ g/ml each) to bring the final volume to 100 μ l. The eosinophil density was adjusted to 1.25×10^6 cells/ml of HBSS/gel mixed in a 4:1 ratio with cytochrome C (12 mg/ml of HBSS/gel), and 100 μ l of eosinophil suspension was added to all wells. After adding IL-5 (100 pM) to the eosinophils, the absorbance of the cell suspensions in the wells was measured at 550 nm in an Immuno-Mini (NJ-2300; Japan Intermed Co., Tokyo, Japan), followed by repeated measurements over the next 240 min. In some experiments, suspended eosinophils were preincubated with 3 μ g/ml of mouse IgG1, anti- α M integrin mAb, anti- β 2 integrin mAb, or anti- α 4 integrin mAb for 15 min before addition to the wells. Each reaction was evaluated in duplicate against the control reaction in wells containing 20 μ g/ml of SOD. The results were adjusted for a 1 ml reaction volume, and O_2^- generation was calculated at an extinction coefficient of $21.1 \text{ mM}^{-1}\text{cm}^{-1}$, as nanomoles of cytochrome C reduced per 1.0×10^6 cells/ml minus SOD control. The maximum value observed over the incubation time

was determined for evaluation of the effects on eosinophil O_2^- generation. Cell viability, as determined by Trypan blue exclusion at the end of each experiment, remained at 94% after a 240 min incubation.

Eosinophil degranulation

Eosinophils (1×10^6 cells/ml) were incubated in 96-well plates for the 240 min and were then immediately centrifuged (1500 rpm) at 4 °C for 10 min. Recovered cell-free supernatants were measured for eosinophil-derived neurotoxin (EDN), as described previously.²⁻⁴ EDN concentrations were quantified using ELISA kits (Medical and Biological Laboratory Co Ltd, Nagoya, Japan).

Transfection with plasmids encoding wild-type CDHR3 (C₅₂₉) or CDHR3 variant (Y₅₂₉)

Transfection of HeLa cells with plasmids encoding wild-type CDHR3 (C₅₂₉) or CDHR3 variant (Y₅₂₉) were performed as described previously.⁸ We selected HeLa cells in this study because they do not naturally express CDHR3, can be efficiently transfected with plasmid DNA, and we had successfully transfected HeLa cells with plasmids encoding

wild-type CDHR3 and CDHR3 variant in another study.⁸ Plasmid DNAs were prepared by Plasmid Maxi kit (Qiagen, Valencia, CA) and transfected into monolayers of HeLa cells in 96-well plate using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. We have already shown that expression of CDHR3 Y₅₂₉ variant after transfection of HeLa-H1 cells lead to enhanced surface expression of protein as compared to CDHR3 C₅₂₉,⁸ whereas levels of overall cellular expression of CDHR3 were similar.⁸ We also confirmed the same results in this study (data not shown). Eosinophil adhesion or eosinophil O₂⁻ generation when incubated with transfected HeLa cells was performed as described above.

MBP staining

Cells were fixed in 4% paraformaldehyde/PBS and permeabilized by 0.5% Triton X/PBS. The cells were then incubated with mouse anti-human MBP Ab (Bio Rad, Richmond, CA), and followed by Alexa Fluor 488 conjugated goat anti-mouse IgG (Invitrogen). Nuclear staining was performed using Hoechst 33342 (Invitrogen). The cells were observed under a fluorescence microscope.

Statistical analysis

Values are expressed as means $\pm$ SEM. Results were compared using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test when differences were significant, or a paired t-test for analysis of the differences between two groups.

Values of $P < 0.05$ were considered statistically significant.

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Figure legends

FIGURE S1. Effect of anti-integrin-mAbs on eosinophil activation induced by CDHR3. A, Effect of anti-integrin-mAbs on eosinophil adhesion to CDHR3. Eosinophils from non-allergic donors were pre-incubated with mouse IgG1, anti- α M integrin mAb, anti- β 2 integrin mAb or anti- α 4 integrin mAb (3 μ g/ml, respectively) for 15 min. The adhesion of the eosinophils to rh-CDHR3 was then assessed (n = 6). ** $P < 0.01$ vs. spontaneous adhesion (Spon). # $P < 0.05$ and ## $P < 0.01$ vs. mouse IgG1. B, Effect of anti-integrin-mAbs on eosinophil O_2^- generation induced by CDHR3. Eosinophils were pre-incubated with mouse IgG1, anti- β 2 integrin mAb, anti- α M integrin mAb or anti- α 4 integrin mAb (3 μ g/ml, respectively) for 15 min, and then incubated in CDHR3-coated plates (10 μ g/ml) for 240 min. The generation of eosinophil O_2^- was then examined (n = 6). The maximum value of eosinophil O_2^- generation were shown.* $P < 0.05$ vs. spontaneous O_2^- generation (Spon). ## $P < 0.01$ vs. mouse IgG1. C, Effect of anti-integrin-mAbs on EDN release induced by CDHR3. Eosinophils were pre-incubated with mouse IgG1, anti- β 2 integrin mAb, anti- α M integrin mAb or anti- α 4 integrin mAb (3 μ g/ml, respectively) for 15 min, and incubated for the 240 min with CDHR3. Levels of EDN in supernatants were quantified (n = 6). * $P < 0.05$ vs. spontaneous EDN release (Spon). # $P <$

0.05 vs. mouse IgG1.

FIGURE S2. Effect of CDHR3 C₅₂₉Y protein mutation on eosinophil functions. A, Effect of CDHR3 C₅₂₉Y protein mutation on eosinophil adhesion to CDHR3. Eosinophils (1×10^5 cells/ml) were collected from non-allergic donors, and their adhesion to various concentrations of wild-type CDHR3 (C₅₂₉) or CDHR3 variant (Y₅₂₉) protein (0.1-100 μ g/ml, each) was measured (n = 6). B, Effect of CDHR3 C₅₂₉Y protein mutation on eosinophil O₂⁻ generation. Eosinophils (1×10^6 cells/ml) from non-allergic donors were incubated in wild-type CDHR3 (C₅₂₉) or CDHR3 variant (Y₅₂₉) protein-coated plates (10 μ g/ml) for 240 min. The generation of eosinophil O₂⁻ was examined (n = 6). The maximum value of eosinophil O₂⁻ generation was shown.

FIGURE S3. MBP staining. A. Purity of eosinophils. After eosinophils were isolated, the cells were fixed, permeabilized, and then incubated with mouse anti-human MBP Ab and followed by Alexa Fluor 488 (green) conjugated anti-mouse IgG. May-Grünwald-Giemsa staining was also performed in the same sample. MBP staining cells were observed under a fluorescence microscope. Scale bar, 20 μ m. B. Effect of CDHR3-mutant transfection on

eosinophils adhesion to transfected cells. HeLa cells were transfected with plasmids encoding wild-type CDHR3 (C529) or CDHR3 variant (Y529) or lipofectamine only (Lipo), and eosinophils were incubated with transfected cells for 20 min. The cells were washed, fixed, permeabilized, and then incubated with mouse anti-human MBP Ab and followed by Alexa Fluor 488 (green) conjugated anti-mouse IgG. Nuclear staining was performed using Hoechst 33342 (blue). Representative figures of 3 independent experiments with different donors were shown. Scale bar, 100 μ m. C, Eosinophils adhesion to transfected cells at higher magnification. Scale bar, 20 μ m.

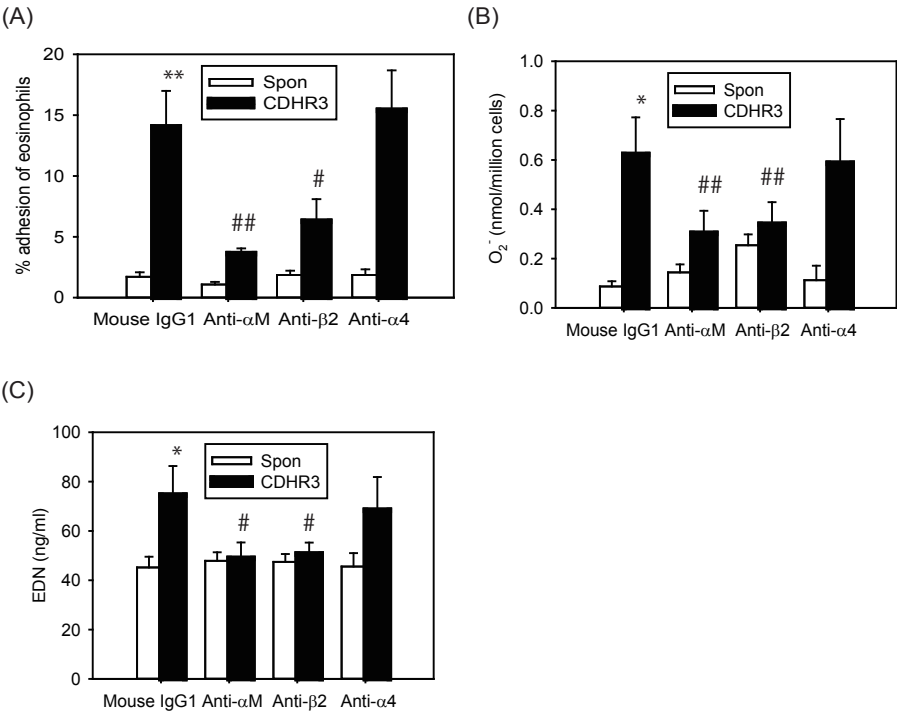
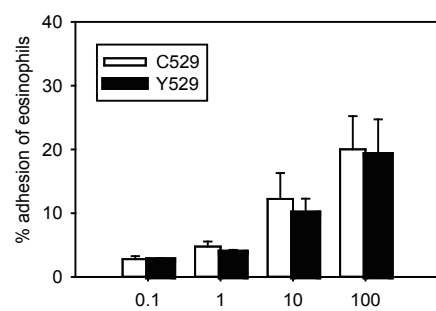


Figure S1

(A)



(B)

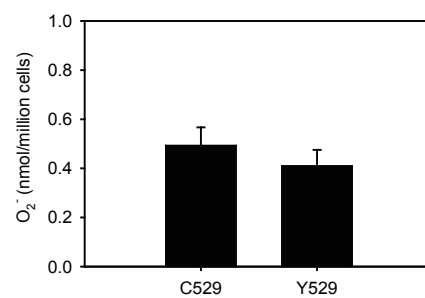
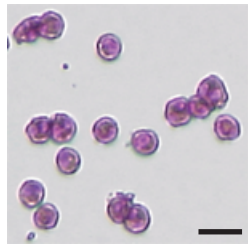
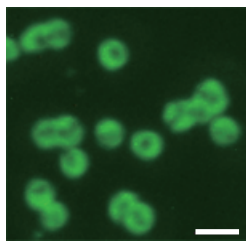


Figure S2

(A)

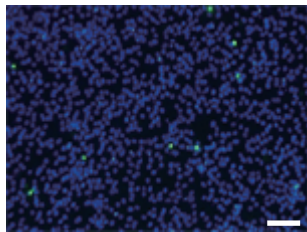


May-Grünwald-Giemsa

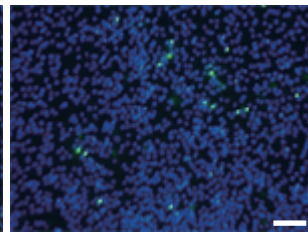


MBP

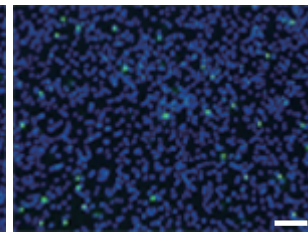
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Lipo

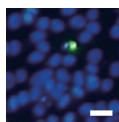


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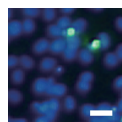


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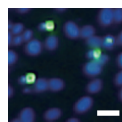
(C)



Lipo



C529



Y529

Figure S3