ORM (yeast)-like protein isoform 3 (ORMDL3) has recently been identified as a candidate gene for susceptibility to asthma; however, the mechanisms by which it contributes to asthma pathogenesis are not well understood. Here we demonstrate a functional role for ORMDL3 in eosinophils in the context of allergic inflammation. Eosinophils recruited to the airways of allergen-challenged mice express ORMDL3. ORMDL3 expression in bone marrow eosinophils is localized in the endoplasmic reticulum and is induced by interleukin-3 and eotaxin-1. Overexpression of ORMDL3 in eosinophils causes increased rolling, distinct cytoskeletal rearrangement, extracellular signal-regulated kinase (1/2) phosphorylation and nuclear translocation of nuclear factor kappa B. Knockdown of ORMDL3 significantly inhibits activation-induced cell shape changes, adhesion and recruitment to sites of inflammation in vivo, combined with reduced expression of CD49d and CD18. In addition, ORMDL3 regulates interleukin-3-induced expression of CD48 and CD48-mediated eosinophil degranulation. These studies show that ORMDL3 regulates eosinophil trafficking, recruitment and degranulation, further elucidating a role for this molecule in allergic asthma and potentially other eosinophilic disorders.
Several genome-wide association studies provide strong evidence that polymorphisms in the region including ORM (yeast)-like protein isoform 3 (ORMDL3) on chromosome 17q21 contribute to childhood as well as adult-onset asthma in ethnically diverse populations1–8. The human ORMDL genes, which include ORMDL1 on chromosome 2, ORMDL2 on chromosome 12 and ORMDL3 on chromosome 17, are expressed ubiquitously in adult and fetal tissues, including the lung, with human ORMDL3 gene demonstrating 96% homology with mouse ORMDL3 (ref. 9). ORMDL3 gene encodes an endoplasmic reticulum (ER) trans-membrane protein that binds to sarco-ER Ca\(^{2+}\) pump regulating ER-mediated Ca\(^{2+}\) signalling and cellular stress by facilitating the unfolded-protein response in transfected HEK293 cells10. In addition, ORMDL3 functions as a mediator of sphingolipid homeostasis in yeast and changes in gene expression or mutations in its phosphorylation sites cause dysregulation of Ca\(^{2+}\) homeostasis and sphingolipid metabolism11. Although genetic studies have clearly demonstrated that the ORMDL3 locus is a risk factor for asthma, the function(s) of ORMDL3 in mediating allergic airway inflammation is only now beginning to emerge. A recent study showed that ORMDL3 is an inducible, ER-expressed protein in bronchial epithelial cells where it regulates expression of metalloproteases and chemokines and that ORMDL3 may be linked to asthma via an ER unfolded-protein response pathway12.

This study also demonstrated that mRNA for ORMDL3 is expressed by mouse bone marrow (BM)-derived eosinophils at significantly higher levels compared with ORMDL1 and ORMDL2 mRNA and that ORMDL3 protein is induced in bronchoalveolar lavage fluid (BALF) eosinophils of mice after allergen challenge. However, the function of this protein at a cellular level in leukocytes is not known. Interestingly, a recent case-controlled study in Korean children indicated a link between ORMDL3 gene variants and eosinophilic inflammation13. Eosinophils are the predominant inflammatory leukocytes that infiltrate airways and promote inflammation during allergic asthma14. Herein, we asked whether ORMDL3 has a role in regulating eosinophil trafficking, migration and recruitment as well as activation-dependent degranulation, which are critical events that support the development and maintenance of tissue inflammation during allergic asthma and other eosinophil-driven disorders. Our studies show that ORMDL3 has a role in promoting eosinophil trafficking and activation via regulation of integrins (CD49d and CD18) and CD48.

**Results**

**Allergen challenge induces expression of ORMDL3 in the lungs.** Given the identification of ORMDL3 as a candidate gene associated with asthma, we first examined whether ORMDL3 expression was evident in murine lungs exposed to various allergens by immunohistology with a polyclonal antibody against human ORMDL3 that is known to react with mouse ORMDL3. Ability of this antibody to bind ORMDL3 was established by western blot analysis of bacterial lysates expressing recombinant human ORMDL3 (His-tagged) or recombinant mouse ORMDL3 (GST-tagged), which showed bands of ~17kDa for His-ORMDL3 and ~43kDa for GST-ORMDL3 fusion protein, respectively (Supplementary Fig. S1a), corresponding to the molecular weight of ORMDL3 (ref. 12). In lung sections, relative to control mice, where ORMDL3 expression was noted mostly in airway epithelial cells and endothelial cells by immunohistology (Fig. 1a, middle panel), mice challenged with an extract of *Alternaria alternata* demonstrated ORMDL3 expression associated not only with airway epithelial cells and endothelial cells but also with inflammatory cells recruited to the allergic airways (Fig. 1a, right panel). Western blot analysis of lung tissue lysates with this antibody further indicated increased expression of ORMDL3 in the lungs after exposure to *Alternaria* extract (Supplementary Fig. S1b). Exposure to other allergens such as ovalbumin (chronic exposure) or cockroach antigen (acute exposure) also showed ORMDL3 expression in leukocytes in the lungs of mice, although the level of expression is variable and likely to be allergen-specific (Supplementary Fig. S2a). As eosinophils are the major inflammatory cells recruited to the airways in response to *Alternaria* challenge (Supplementary Fig. S2b), we next examined whether eosinophils in the lung tissue of *Alternaria*-exposed mice express ORMDL3 by staining sequential lung sections for expression of ORMDL3 and eosinophil-specific major basic protein (MBP) with antibodies against ORMDL3 and MBP, respectively (Fig. 1b). Areas with several MBP-positive cells also stained positive for ORMDL3. In addition, dual immunofluorescence staining of BALF cells from *Alternaria*-exposed mice with these antibodies confirmed that MBP-positive cells (eosinophils) recruited to the airways express ORMDL3 (Fig. 1c).

Expression of ORMDL3 by eosinophils was further validated in BM-derived murine eosinophils by RT–PCR (see Supplementary Table S1 for primer details) and western blot analysis, which demonstrated a major protein band of molecular weight ~17 kDa (Fig. 1d). Consistent with the pattern indicated for this antibody by the manufacturer, additional protein bands in the molecular weight range of ~45–70 kDa were noted. Owing to the significant homology between ORMDL proteins 1, 2 and 3 (>80%)9, one cannot unequivocally rule out that the antibody does not react with ORMDL1 or 2. However, previous studies indicating that ORMDL3 mRNA expression is induced in the lungs of mice challenged with interleukin (IL)-4 or IL-13 (Th2 cytokines expressed in response allergen challenge) and that BM-derived eosinophils express predominantly ORMDL3 mRNA relative to ORMDL1 and 2 (ref. 12) support our demonstration of ORMDL3 protein expression in *Alternaria*-challenged murine lungs and eosinophils with this antibody.

Previous studies indicate that ORMDL3 is an ER membrane protein in transfected HEK293 (ref. 9,10) and A549 epithelial cells12. We examined sub-cellular localization of ORMDL3 in murine eosinophils by immunofluorescence staining. Expression was restricted largely to the cytoplasm (Fig. 1e). To further elucidate this, eosinophils were transfected with ORMDL3-GFP (green fluorescent protein). RT–PCR with specific primers demonstrated increased ORMDL3 expression and confocal microscopy showed a fluorescent (GFP) signal localized to the cytoplasm in transfected eosinophils (Fig. 1f). ORMDL3-GFP-transfected eosinophils (Fig. 1g, left panel) stained with ER-Tracker Red (Fig. 1g, middle panel) showed that GFP-tagged ORMDL3 expression overlapped with ER-Tracker Red staining (Fig. 1g, right panel) indicating that ORMDL3 expression in eosinophils is predominantly confined to the ER, consistent with previous findings in epithelial cells.

**ORMDL3 is induced by IL-3 and participates in cell activation.** As exposure to allergens induces ORMDL3 expression in the lungs of mice, we evaluated whether expression of ORMDL3 at a cellular level in murine eosinophils is regulated by IL-3, IL-5, eotaxin-1 and RANTES that promote allergic asthma14. RT–PCR and qPCR indicated that IL-3 and eotaxin-1 markedly induce ORMDL3 expression, whereas IL-5 and RANTES had no effect (Fig. 2a, top and bottom panel). Western blot analysis of eosinophil lysates demonstrated increased ORMDL3 protein expression after exposure to IL-3 (Fig. 2b), confirming its ability to upregulate ORMDL3 expression. In human eosinophils, IL-3 has been shown to induce rapid activation of extracellular signal-regulated kinase (ERK; 1/2) but not p38 (ref. 15). Likewise,
ORMDL3, when overexpressed, can regulate NF-κB translocation of NF-κB (Fig. 2d). Nuclear translocation and activation of NF-κB in eosinophils is known to induce expression of multiple proteins (such as cytokines and adhesion molecules) resulting in further cell activation. Exposure of eosinophils to eotaxin-1 induces cytoskeletal rearrangement, which enables cells to undergo morphological changes that are essential for cell motility. As eotaxin-1 upregulated ORMDL3 expression, we examined whether increased ORMDL3 expression in eosinophils may alter the actin cytoskeleton based on phalloidin staining. ORMDL3-GFP-transfected eosinophils exhibited striking changes in F-actin cytoskeleton based on phalloidin staining. Although control-GFP-transfected cells demonstrated a distinct pattern of phalloidin binding localized to the cell periphery/margin, ORMDL3-GFP-transfected cells exhibited irregular phalloidin binding all over the cell (Fig. 2e). The important role of the cytoskeleton in promoting activation-
dependent cell shape changes required for cell motility\(^{22,23}\) together with the ability of ORMDL3 to alter the F-actin cytoskeleton when overexpressed, prompted us to investigate whether ORMDL3 has a role in regulating eosinophil trafficking. Overexpression of ORMDL3 in eosinophils resulted in significantly increased eosinophil rolling on recombinant murine (rm) VCAM-1 under conditions of flow compared with cells transfected with control-GFP (Fig. 2f).

**ORMDL3 regulates integrin expression and cell adhesion.** The role of ORMDL3 in eosinophil trafficking was further validated by knockdown studies using ORMDL3-specific small interfering RNA (siRNA). Treatment of eosinophils with ORMDL3-siRNA resulted in a marked reduction in ORMDL3 mRNA expression by RT–PCR and protein expression by western blot analysis as well as immunofluorescence staining (Fig. 3a) without significantly affecting cell viability or proliferation relative to cells treated with control-siRNA (Supplementary Table S2). Interestingly, knockdown of ORMDL3 had no effect on eosinophil rolling (Supplementary Fig. S3a). This could be due to redundant eosinophil-expressed adhesion molecules such as galectin-3 that can promote eosinophil rolling on VCAM-1 (ref. 24). However, a significant reduction in adhesion of ORMDL3-siRNA-treated eosinophils to rm VCAM-1 and rm ICAM-1 was noted compared with control-siRNA-treated cells (Fig. 3b). Confocal microscopy studies after phalloidin staining revealed that control-siRNA-treated eosinophils adherent to VCAM-1 and ICAM-1 exhibited cell spreading with distinct cell polarization (leading edge formation and development of uropodia and filopodia) (Fig. 3c, middle panel) similar to untreated cells (Fig. 3c, left panel), whereas knockdown of ORMDL3 resulted in not only a reduced number of adherent cells but also limited cell spreading/polarization (Fig. 3c, right panel). Quantitation of these changes in cell morphology revealed that a significantly larger percentage of ORMDL3-siRNA-treated eosinophils adhered to VCAM-1 and ICAM-1 do not spread (retain a compact round cell body without leading edges) compared with control-siRNA-treated or untreated eosinophils (Fig. 3d) correlating with the number of adherent cells in adhesion assays (Fig. 3b).

As knockdown of ORMDL3 in eosinophils resulted in reduced cell adhesion and inhibition of activation-dependent (that is, adherent on VCAM-1 and ICAM-1) directed cell shape changes, we examined whether ORMDL3 regulates expression of adhesion molecules that are known to mediate eosinophil trafficking (rolling and adhesion). Knockdown of ORMDL3 in eosinophils resulted in decreased mRNA levels of z4 and B2, counter receptors for VCAM-1 and ICAM-1, respectively (Fig. 3e). Consistent with decreased mRNA levels of z4 and B2, immunostaining of ORMDL3-silenced eosinophils adherent on VCAM-1 and ICAM-1 demonstrated decreased surface expression of z4 and
Mac-1 (αM), respectively (Fig. 3f, left panels) and control-siRNA-treated (Fig. 3f, middle panels) eosinophils. Conversely, eosinophils overexpressing ORMDL3 demonstrated increased expression of α4 and β2 by RT–PCR (Supplementary Fig. S3b). mRNA levels of αM and αL as well as that of β1 remained unaffected in ORMDL3-silenced eosinophils (Supplementary Fig. S3c), suggesting that the reduced surface expression of Mac-1 is most likely due to decreased expression of the β2 subunit by these cells.

**ORMDL3 regulates migration and CD48-mediated degranulation.** Cellular events such as adhesion and migration are known to be regulated by [Ca\(^{2+}\)](i) levels through integrin activation and cytoskeletal changes. Previous studies have demonstrated a role for ORMDL3 in regulating ER-mediated Ca\(^{2+}\) homeostasis. As our data suggest that ORMDL3 is involved in regulating cytoskeletal changes and adhesion of eosinophils, we examined whether [Ca\(^{2+}\)](i) levels in control-siRNA-treated cells were significantly higher than in ORMDL3-transfected eosinophils (left panels) are shown as controls. Scale bar, 20 μm. (d) Quantitation of changes in morphology of adherent eosinophils treated with control-siRNA or ORMDL3-siRNA or left untreated as in (c). The number of adherent cells in each field was counted and the number of cells in the field that do not spread and/or polarize (retain a round compact cell body without leading edges) were identified and expressed as a percentage of the total number of adhered cells. Five fields were counted for each cover-slip. Adherent control-siRNA-treated (middle panels) and untreated (media alone) eosinophils (left panels) are shown as controls. Scale bar, 20 μm. (d) Quantitation of changes in morphology of adherent eosinophils treated with control-siRNA, ORMDL3-siRNA or left untreated as in (c). The number of adherent cells in each field was counted and the number of cells in the field that do not spread and/or polarize (retain a round compact cell body without leading edges) were identified and expressed as a percentage of the total number of adhered cells. Five fields were counted for each cover-slip. (e) Expression of α4 and β2 integrins in eosinophils treated with control-siRNA or ORMDL3-siRNA by RT–PCR. Expression of β-actin is shown as the internal control. (f) Expression of integrin α4 and Mac-1 (αM) by untreated (left panels), control-siRNA-treated (middle panels) and ORMDL3-siRNA-treated (right panels) eosinophils adherent on VCAM-1 and ICAM-1, respectively, by immunofluorescence staining and confocal microscopy. Scale bar, 50 μm. Data shown in (a), (c), (e) and (f) are representative of two to three independent experiments with eosinophils from different mice. Combined data (mean ± s.e.m.) of three experiments in triplicate in (b) and two experiments in duplicate in (d) are shown. *P < 0.01 in (b) and (d) compared with control-siRNA. Statistical significance was determined by unpaired two-tailed Student’s t-test.

Figure 3 | ORMDL3-mediated eosinophil adhesion and regulation of integrin receptors α4 and β2. (a) Expression of ORMDL3 in BM-derived eosinophils transfected with control-siRNA or ORMDL3-siRNA by RT–PCR (top left) as well as western blot (bottom) and immunofluorescence staining (top right) with antibodies against ORMDL3. Scale bar, 50 μm. (b) Adhesion of eosinophils treated with control-siRNA or ORMDL3-siRNA to rm VCAM-1 and rm ICAM-1 under static conditions. (c) Changes in morphology of ORMDL3-siRNA-transfected eosinophils adherent on VCAM-1- or ICAM-1-coated cover-slips evaluated by confocal microscopy after phalloidin staining (right panels). Adherent control-siRNA-treated (middle panels) and untreated (media alone) eosinophils (left panels) are shown as controls. Scale bar, 20 μm. (d) Quantitation of changes in morphology of adherent eosinophils treated with control-siRNA, ORMDL3-siRNA or left untreated as in (c). The number of adherent cells in each field was counted and the number of cells in the field that do not spread and/or polarize (retain a round compact cell body without leading edges) were identified and expressed as a percentage of the total number of adhered cells. Five fields were counted for each cover-slip. (e) Expression of α4 and β2 integrins in eosinophils treated with control-siRNA or ORMDL3-siRNA by RT–PCR. Expression of β-actin is shown as the internal control. (f) Expression of integrin α4 and Mac-1 (αM) by untreated (left panels), control-siRNA-treated (middle panels) and ORMDL3-siRNA-treated (right panels) eosinophils adherent on VCAM-1 and ICAM-1, respectively, by immunofluorescence staining and confocal microscopy. Scale bar, 50 μm. Data shown in (a), (c), (e) and (f) are representative of two to three independent experiments with eosinophils from different mice. Combined data (mean ± s.e.m.) of three experiments in triplicate in (b) and two experiments in duplicate in (d) are shown. *P < 0.01 in (b) and (d) compared with control-siRNA. Statistical significance was determined by unpaired two-tailed Student’s t-test.
ORMDL3-silenced eosinophils (Fig. 4a), and similar to levels in untreated cells (Supplementary Fig. S4a). This is in contrast to previous studies where knockdown of ORMDL3 in HEK293 and Jurkat T cells had no significant effect on basal cytosolic Ca$^{2+}$ levels$^{10,27}$. This difference in the effect of ORMDL3 knockdown on basal [Ca$^{2+}$], between our study and the previous studies may be due to the different cell types investigated (primary cells (eosinophils) versus transformed epithelial and T cell lines). Along with decreased agonist (eotaxin-1)-induced eosinophils versus transformed epithelial and T cell (Fig. 4b), Conversely, eosinophils overexpressing ORMDL3 demonstrated increased migration towards eotaxin-1 compared with cells transfected with control-GFP, although statistical significance was not achieved (Supplementary Fig. S4b). The ability of ORMDL3 to regulate eosinophil migration was further validated in vivo where, in comparison with infused carboxyfluorescein succinimidyl ester (CFSE)-labelled control-siRNA-treated eosinophils, a reduced number (three-fold reduction) of CFSE-labelled ORMDL3-siRNA-treated eosinophils were found to recruit to the inflamed peritoneum of mice exposed to thioglycollate (Fig. 4c,d).

Our observation that IL-3 induces ORMDL3 expression by eosinophils together with previous studies demonstrating that IL-3 can induce expression of CD48, a GPI-anchored protein involved in cellular activation, costimulation and adhesion of eosinophils$^{28}$, prompted us to investigate whether ORMDL3 regulates CD48 expression. Relative to unstimulated (no IL-3) murine eosinophils treated with control-siRNA, ORMDL3-silenced cells exhibited reduced CD48 mRNA expression by RT–PCR (Fig. 4e, left) as well as protein expression by western blot analysis (Fig. 4e, centre) and immunofluorescence staining (Fig. 4e, right). Although exposure to IL-3 induced CD48 expression in eosinophils (Supplementary Fig. S4c), there was a marked inhibition of IL-3-induced CD48 mRNA expression in ORMDL3-silenced eosinophils compared with untreated and control-siRNA-treated cells (Fig. 4f). Given that CD48 cross-linking on human eosinophils results in activation-dependent cell degranulation$^{28}$, we examined whether ORMDL3 regulates CD48-induced degranulation of murine eosinophils (Fig. 4g).

**Figure 4 | ORMDL3-dependent eosinophil migration and CD48-mediated degranulation.** (a) Basal and eotaxin-1 (100 nM)-induced [Ca$^{2+}$], levels in eosinophils treated with control-siRNA (a total of 598 cells) or ORMDL3-siRNA (a total of 870 cells) by digital video fluorescence imaging after loading with Fura-2 AM. (b) Chemotaxis of untreated, control-siRNA-treated and ORMDL3-siRNA-treated eosinophils in response to eotaxin-1 (100 nM) in vitro. (c) Recruitment of infused CFSE-labelled control-siRNA- or ORMDL3-siRNA-treated eosinophils to the peritoneum in a mouse model of thioglycollate (TG)-induced inflammation by flow cytometry. (d) Dot plots from a representative experiment showing recruitment of CFSE-labelled untreated, control-siRNA-treated and ORMDL3-siRNA-treated eosinophils to the peritoneum of a TG-exposed mouse. The experiment was repeated three times with similar results (n = 3 mice per condition). (e) Expression of CD48 in eosinophils treated with control-siRNA or ORMDL3-siRNA by RT-PCR (left), western blot analysis (middle) and immunofluorescence staining (right). Scale bar, 50 μm. (f) CD48 expression after IL-3 treatment in control-siRNA-treated, ORMDL3-siRNA-treated and untreated eosinophils by RT-PCR. Expression of β-actin is shown as the internal control in panels (e,f). (g) EPO release by untreated, control-siRNA-treated and ORMDL3-siRNA-treated eosinophils after activation with anti-CD48 by colorimetric assay expressed as per cent change in absorbance relative to treatment with control IgG. In the panels (a,b,g), combined data (mean ± s.e.m.) of three experiments in duplicate or triplicate and in (c), combined data (mean ± s.d.) of two experiments are shown. Data shown in (e) and (f) are representative of two to three independent experiments with eosinophils from different mice. $^{*}$P < 0.01 in the panels (a,g) and < 0.05 in (b) compared with control-siRNA. Statistical significance was determined by unpaired two-tailed Student’s t-test.
Activation of control eosinophils (treated with media containing transfection reagent without any siRNA) with anti-CD48 mAbs induced cellular degranulation as measured by eosinophil peroxidase (EPO) release. Activation with anti-CD48 after ORMDL3 knockdown resulted in a five-fold reduction in degranulation compared with control-siRNA-treated eosinophils. These results indicate that ORMDL3 is not only required for IL-3-induced expression of CD48 but also for activation-dependent (CD48-mediated) degranulation of eosinophils.

Discussion

Considering Th2 cytokines and chemokines exert various immunomodulatory effects on eosinophils, it is interesting that IL-3, but not IL-5, induces ORMDL3 gene expression. Although the receptor for IL-3 and IL-5 share a β-chain our results suggest that the selective IL-3-induced upregulation of ORMDL3 is potentially via a IL-3R z-chain-specific mechanism and independent of common β-chain-mediated signalling in eosinophils. Likewise, induction of ORMDL3 gene expression by eotaxin-1, but not RANTES, although both chemokines can bind to CCR3 (refs 30,31), is suggestive of selective signalling mechanisms that potentially regulate ORMDL3 expression in eosinophils. In human eosinophils, eotaxin-1 is known to induce rapid activation of ERK, which is necessary for eotaxin-induced cytoskeletal rearrangement, chemotaxis and degranulation. Overexpression of ORMDL3 in murine eosinophils was associated with increased levels of phosphorylated ERK (1/2), but not p38, which suggests association of specific MAP kinase signalling pathways with ORMDL3. Further, increased ORMDL3 expression was found to result in distinct cytoskeletal changes. Rearrangement of the cytoskeleton in response to a stimulus enables changes in cell shape/morphology (spreading and polarization), which are essential for cell trafficking (rolling and adhesion) and migration. Indeed, eosinophils overexpressing ORMDL3 demonstrated increased rolling on VCAM-1 and migration.

\[\text{[Ca}^{2+}\text{]}_i\text{ acts as a biologically important second messenger after}\]

stimulation with cytokines and chemokines. ORMDL3-silenced eosinophils had reduced basal and agonist-induced \([\text{Ca}^{2+}]_i\text{ levels relative to control cells. This function of ORMDL3 in regulating}\ [\text{Ca}^{2+}]_i\text{ can impact various functional responses in eosinophils that are driven by changes in } [\text{Ca}^{2+}]_i\text{, such as cytoskeletal reorganization and cell shape change/morphology, as well as modulation of integrin receptors, cell adhesion, migration and degranulation}\text{, which were all affected in the present study in ORMDL3-silenced eosinophils. Specifically, ORMDL3 knockdown studies resulted in reduced surface expression of } \text{α4 and Mac-1 along with decreased adhesion to VCAM-1 and ICAM-1 in eosinophils. Conversely overexpression of ORMDL3 resulted in increased } \text{α4 and β2 mRNA expression. This could potentially be through NF-κB activation and translocation as previous studies have shown that } \text{α4 expression in monocytes-derived dendritic cells}\text{ is dependent on NF-κB activation/nuclear translocation. Further, with cell surface expression of CCR3 (receptor for eotaxin-1) remaining unaffected after ORMDL3 knockdown (Supplementary Fig. 5d), decreased migration of these cells suggests that signalling via ORMDL3 might be essential for eotaxin-1-induced eosinophil migration and recruitment to sites of inflammation in vivo.}\n
Induction of CD48 in eosinophils by IL-3 is β-chain-independent similar to IL-3-induced upregulation of ORMDL3 described here. ORMDL3 appears to have a role in regulating CD48. Not only IL-3-induced expression of CD48 but also CD48-mediated eosinophil degranulation (EPO release) is inhibited in ORMDL3-silenced eosinophils. We postulate that ORMDL3 regulates CD48 via NF-κB activation in eosinophils based on the following lines of evidence. First, CD48 is known to be a target gene for NF-κB. Second, IL-3 has been shown to induce NF-κB activation in eosinophils. Third, in our studies, we show that IL-3 induces ORMDL3 as well as CD48, with expression of the latter being dependent on ORMDL3 and finally, overexpression of ORMDL3 is associated with NF-κB activation.

In summary, we demonstrate for the first time a functional role for ORMDL3 in regulating eosinophil trafficking, recruitment and degranulation, which may promote airway eosinophilia and inflammation in allergic asthma. Further, our studies suggest that blockade of ORMDL3 may serve as a potential means to ameliorate eosinophilic inflammation during allergic asthma and other eosinophilic disorders.

Methods

Mousetail of allergic airway inflammation. C57BL/6 mice (male, 6–8 weeks) were challenged with 50 μg of A. alternata extract (Greer Laboratories Inc.) in 50 μl of PBS or PBS alone (control) intranasally on days 0, 3 and 6 under anaesthesia. Mice were killed 18–24 h after the last challenge and evaluated for development of inflammation based on cellular infiltration in BALF and lung tissue. In addition, lung tissue was harvested from C57BL/6 mice exposed to chronic challenge with ovalbumin or acute challenge with cockroach antigen. All animal studies were performed following standards and procedures approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Immunohistology. Expression of ORMDL3 in paraffin-embedded lung tissue sections was evaluated using rabbit anti-ORMDL3 (0.5 μg ml⁻¹) for Alternaria-challenged [31] and mouse anti-CD48 (Santa Cruz Biotechnology, Inc., 1.25 μg ml⁻¹) or control IgG followed by rabbit anti-rabbit secondary antibodies and avidin-biotin–HRP complex followed by peroxidase AEC substrate kit (both from Vector Laboratories) was used for detection. Lung tissue eosinophils were evaluated by staining for eosinophil-specific MBP with rat mAbs against murine MBP (2 μg ml⁻¹), provided by Dr J. Lee, Mayo Clinics Arizona, Scottsdale, AZ.17,23,24 Slices were examined under a Nikon Microphot EPI-FL microscope and images were captured with an Olympus DP71 camera.

Immunofluorescence staining and confocal microscopy. BALF cells from Alternaria-exposed mice were stained for CD48, CD18, CD11b and Mac-1 by staining for eosinophil-specific MBP with rat mAbs against murine MBP (2 μg ml⁻¹), provided by Dr J. Lee, Mayo Clinics Arizona, Scottsdale, AZ.24 Slices were examined under a Nikon Microphot EPI-FL microscope and images were captured with an Olympus DP71 camera.

Murine eosinophils. Murine eosinophils were cultures from BM of C57BL/6 mice. Cells between 12–14 days of culture differentiated based on Hema 3 staining and verified for expression of both MBP (by confocal microscopy with rat mAb against murine MBP at 2.5 μg ml⁻¹) and Siglec-F (by flow cytometry with PE-conjugated rat anti-mouse Siglec-F at 5 μg ml⁻¹) were used in studies. See Supplementary Methods for detailed culture conditions.

RT-PCR and quantitative real-time RT-PCR. Primers specific for murine ORMDL3 (5′-CACACGGTTGATGAAAGCT-3′ and 3′-TTTGCCTTGTCTGGAGATCT-5′) were designed using Primer3 (v. 0.4.0, available online at http://frodo.wi.mit.edu/primers3/). Expression of CD48, CD18 (B2), CD49d (α4), CD11a (αL), CD11b (αM), CD29 (β1) and β-actin were examined using previously published primer sequences (Supplementary Table S1). All primers were synthesized by Integrated DNA Technologies. PCR products were analysed by agarose gel (1.2%) electrophoresis. qPCR was carried out in a iQ5 multicolour real-time PCR detection System (Bio-Rad) using the same primer pair.

Supplementary Methods for detailed culture conditions.
for ORMDL3 described above under the following conditions: initial denaturation at 95 °C for 2 min followed by 44 cycles of 95 °C for 15 s and 64 °C for 1 min. The relative amount of mRNA for each sample was calculated based on its threshold cycle, Ct, suggested by the software (IQ5 Optical System software) in comparison to the Ct of the housekeeping gene β-actin27. Results were expressed as fold change (2^ΔΔCt) in expression after subtraction of internal β-actin control.

**Western blot analysis.** Murine BM-derived eosinophil lysates were analysed using antibodies against i) ORMDL3 (0.16 µg ml^-1), ii) CD48 (0.2 µg ml^-1), iii) ERK (1/2) (0.01 µg ml^-1), iv) phospho-ERK (1/2), (0.12 µg ml^-1), v) p38 (0.026 µg ml^-1), vi) phospho-p38 (0.3 µg ml^-1) (antibodies for (iii)-(vi) were from Cell Signaling Technology) and vii) β-actin (BD Transduction Laboratories, 0.06 or 0.12 µg ml^-1). The total number of adherent cells was counted and the number of cells in the field that do not spread (retain a compact round cell body without leading edges) were identified and expressed as a percentage of the total number of adhered cells. Five fields were counted for each sample.

**Expression of recombinant ORMDL3.** Full-length cDNA encoding the human ORMDL3 gene (Thermo Scientific Open Biosystems) was cloned into pET-28a vector containing a His tag, while full-length cDNA encoding the mouse ORMDL3 gene (Thermo Scientific Open Biosystems) was cloned into pEGX-6p-2 vector containing a GST tag. The resulting expression plasmids of the human or mouse ORMDL3 gene were transformed into E. coli Rosetta (DE3) pLysS cells. IPTG (1 µM)-induced recombinant bacteria were lysed in RIPA buffer containing protease and phosphatase inhibitors were examined for ORMDL3 expression as described for BM eosinophils. Expression of β-actin was monitored as an internal control in all cases and detection was carried out using secondary antibodies appropriate for the detection system used. See Supplementary Methods for experimental details. Representative full-length western blots are shown in the Supplementary Information (Supplementary Figs S5,S6).

**Overexpression and knockdown of ORMDL3.** Full-length cDNA for ORMDL3 was cloned into pEGFP-N1 using standard cloning procedures. BM-derived mouse eosinophils were transfected with pEGFP-N1 containing cDNA for ORMDL3 (ORMDL3-GFP) or with the empty vector (control-GFP) using Trans IT-2020 DNA transfection reagent (Mirus Bio LLC) as recommended by the manufacturer. Transfected cells cytotoxicentricated onto glass slides, fixed, mounted in DAPI were evaluated by confocal microscopy and by RT–PCR.

**Assessment of cytoskeleton.** Eosinophils were cytotoxicentricated onto glass slides, air dried, permeabilized with 4% paraformaldehyde, cytotoxicentricated onto glass slides and evaluated by confocal microscopy.

**Induction of ORMDL3 and CD48 expression.** BM-derived eosinophils (1 × 10^6 per ml) were suspended in culture medium alone or medium containing IL-5, IL-3 (both from PeproTech), RANTES-1 (R&D Systems, all at 100 ng ml^-1) or eotaxin-1 (PeproTech, 100 mM) for 2 h at 37 °C and 5% CO2 after which cells were analysed for ORMDL3 mRNA expression by RT–PCR and qPCR. To evaluate CD48 mRNA expression, eosinophils were treated with IL-5 as described above. For protein expression, eosinophils were treated with IL-3 for 12 h and then examined for ORMDL3 and CD48 by western blot analysis followed by densitometry using ImageJ (for ORMDL3).

**In vitro flow chamber assay.** Rolling of BM-derived eosinophils (1–2 × 10^7 per ml) on rm VCAM-1 coated cover slips under conditions of flow (1 ml min^-1), wall shear stress ~ 1.0–2.0 dynes cm^-2) was evaluated in an in vitro parallel plate flow chamber39. Interaction of the infused eosinophils with VCAM-1 was observed using a Leitz Wetzlar inverted microscope and images were recorded for subsequent offline analysis to determine the number of cells demonstrating multiple discrete interruptions and flow slowly (rolling) relative to non-interacting cells. Results were expressed as the number of rolling cells per min.

**Cell morphology and adhesion molecule expression.** Eosinophils were allowed to adhere to rm VCAM-1 or ICAM-1-coated cover slips for 30 min. After washing with PBS to remove non-adherent cells, adhered cells were fixed with 4% paraformaldehyde, permeabilized and stained with Alexa Fluor 488 Phalloidin (Invitrogen) and visualized by confocal microscopy39. To quantitate differences in cell morphology, the number of adherent cells in a given field of each cover-slip was counted and the number of cells in the field that do not spread (retain a compact round cell body without leading edges) were identified and expressed as a percentage of the total number of adhered cells. Five fields were counted for each sample.

**Cell activation and EPO release assay.** Eosinophils (2 × 10^6 per 200 µl) were incubated with anti-mouse CD48 mAb (BioLegend) or control hamster IgG (BioLegend) at 37 °C for 2 h in culture media containing transfection reagent without any siRNA after which EPO supernatants were determined by colorimetric assay. Results are expressed as percentage of control relative to treatment with control IgG after subtraction of background.

**Statistical analysis.** Data are presented as mean ± s.e.m. unless otherwise stated. Statistical significance between two groups was determined using a two-tail unpaired Student’s t-test. A P-value < 0.05 was considered as significant.

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42. Author contributions

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43. Additional information

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