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IL-5-stimulated eosinophils adherent to periostin undergo stereotypic morphological changes and ADAM8-dependent migration

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Summary

Background—IL-5 causes suspended eosinophils to polarize with filamentous (F)-actin and granules at one pole and the nucleus in a specialized uropod, the “nucleopod”, which is capped with P-selectin glycoprotein ligand-1 (PSGL-1). IL-5 enhances eosinophil adhesion and migration on periostin, an extracellular matrix protein upregulated in asthma by type 2 immunity mediators.

Objective—Determine how the polarized morphology evolves to foster migration of IL-5-stimulated eosinophils on a surface coated with periostin.

Methods—Blood eosinophils adhering to adsorbed periostin were imaged at different time points by fluorescent microscopy, and migration of eosinophils on periostin was assayed.

Results—After 10 min in the presence of IL-5, adherent eosinophils were polarized with PSGL-1 at the nucleopod tip and F-actin distributed diffusely at the opposite end. After 30–60 min, the nucleopod had dissipated such that PSGL-1 was localized in a crescent or ring away from the cell periphery, and F-actin was found in podosome-like structures. The periostin layer, detected with monoclonal antibody Stiny-1, shown here to recognize the FAS1 4 module, was cleared in wide areas around adherent eosinophils. Clearance was attenuated by metalloproteinase inhibitors or antibodies to disintegrin metalloproteinase 8 (ADAM8), a major eosinophil metalloproteinase, previously implicated in asthma pathogenesis. ADAM8 was not found in podosome-like structures, which are associated with proteolytic activity in other cell types. Instead, immunoblotting demonstrated proteoforms of ADAM8 that lack the cytoplasmic tail in the supernatant. Anti-ADAM8 inhibited migration of IL-5-stimulated eosinophils on periostin.

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Contributions

MWJ and DFM conceived and designed the study. MWJ and MK carried out experimental eosinophil assays and collected data. MWJ, MK, and CLN conducted data and image analyses. VB and DSA produced recombinant proteins. VB did antibody mapping. MWJ, VB, and DFM interpreted data and participated in manuscript preparation.

Conflict of interest

MWJ received a fee for consulting from Guidepoint Global, a fee from Genentech for speaking, and funds for research from Hoffmann-La Roche; and is an advisory board member for Genentech. The other authors have no conflicts of interest.

Conclusions and Clinical Relevance—Migrating IL-5-activated eosinophils on periostin exhibit loss of nucleopodal features and appearance of prominent podosomes along with clearance of the Stiny-1 periostin epitope. Migration and epitope clearance are both attenuated by inhibitors of ADAM8. We propose, therefore, that eosinophils remodel and migrate on periostin-rich extracellular matrix in the asthmatic airway in an ADAM8-dependent manner, making ADAM8 a possible therapeutic target.

Keywords

eosinophils; IL-5; periostin; podosomes; ADAM8; migration; Stiny-1; FAS1 modules

Introduction

Asthma is frequently characterized by airway inflammation rich in eosinophils [1]. Airway eosinophilia is associated with exacerbations [1–3] and likely plays a role in airway remodeling [1, 4, 5]. Recruitment of eosinophils from the circulation requires that blood eosinophils become activated, leading to their arrest on activated endothelium, extravasation, and continued movement through the bronchial tissue by interaction with the extracellular matrix (ECM) [6–9].

The type 2 immunity cytokine interleukin (IL)-5 [10] causes eosinophils in suspension to polarize with cortical filamentous actin (F-actin) and granules moving to one pole and the nucleus to the opposite pole into a specialized uropod, the “nucleopod” [11]. The nucleopod is enriched in cell-surface receptors including P-selectin glycoprotein ligand-1 (PSGL-1) and activated $\alpha_M\beta_2$ integrin [11]. IL-5 enhances the adhesion and migration, mediated by $\alpha_M\beta_2$ integrin, of eosinophils on periostin [12–14], an ECM protein [15] that is upregulated by type 2 cytokines such as IL-13 in the asthmatic airway, particularly in patients with asthma identified as T helper cell (Th) 2 immunity-high or type 2-high subjects [3, 16, 17]. A higher level of serum periostin in asthma is associated with a higher number of sputum and tissue eosinophils and higher degree of airway inflammation, as reported by fraction of exhaled nitric oxide (FENO) [18–20]. Administration of anti-IL-13 or anti-IL-4 receptor α causes an increase in blood eosinophil count [3, 21, 22], consistent with a scenario in which IL-13- or IL-4-induced factors, including periostin, promote eosinophil trafficking.

We asked if and how the polarized morphology of activated eosinophils observed in suspension evolves when cells adhere to periostin in the presence of IL-5. We now report that eosinophils transition from a morphology similar to that in suspension to a more spread morphology in which the dominant adhesive structures are podosomes containing F-actin, gelsolin, and actin-related protein (Arp)-3. In addition, we report that the cells modify the periostin substrate and migrate on periostin in a manner involving disintegrin metalloproteinase-8 (ADAM8), which has been implicated in asthma in several human and mouse studies [23–30], including in an analysis of copy number variation [31].

Methods

Eosinophils

Eosinophils were obtained and purified from heparinized blood of donors ($n = 34$) with allergy and/or asthma by negative selection using a cocktail of anti-CD16, anti-CD14, anti-CD3, and anti-glycophorin beads as before [11, 12, 32]. The purity and viability of eosinophils were 98%. The studies were approved by the University of Wisconsin-Madison Health Sciences Institutional Review Board. Informed written consent was obtained from each subject before participation.

Periostin and ADAM8

The shortest carboxy (C)-terminal splice variant of human periostin, i.e., lacking sequences encoded by differentially spliced exons 17, 18, 19, and 21 (PN0, UniProt identifier No. Q15063–7), was cloned into pAcGP67.coco (hereafter pCOCO-PN0), expressed in insect cells using a baculovirus system, and purified as previously described [12, 33, 34]. The complementary DNA (cDNA) sequences corresponding to periostin FAS1 1–2 (residues P97–L365 relative to M1 of PN0), FAS1 2 (G234–L365), FAS1 2–3 (G234–I492), FAS1 3–4 (D368–L628), and FAS1 3 module-C terminus (D368–Q721) were amplified by polymerase chain reaction (PCR). The periostin FAS1 3-C terminus cDNA sequence was amplified from the pCOCO-PN0 plasmid, thus lacking alternative exons 17, 18, 19, and 21. Primers were designed to obtain PCR amplicons with 5′ *KpnI* and 3′ *NheI* recognition sites. These sites were utilized for insert ligation into the pET-ELMER vector, which contains sequences for a 6xHis tag and thrombin cleavage site 5′ of the *KpnI* restriction site [35]. After sequence verification, plasmids were transformed into BL21(DE3) competent *Escherichia coli*. The 6xHis-tagged recombinant proteins were expressed and purified as described [35], except that proteins were eluted from NiNTA agarose (Qiagen, Valencia, CA, USA) using pH 7.0 elution buffer containing 300 mM imidazole, which was removed through dialysis to obtain purified samples. PN0 was dialyzed into phosphate-buffered saline (PBS), pH 7.4; periostin FAS1 2 was dialyzed into 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.5; and the rest of the protein samples were dialyzed into 1 mM acetic acid, pH 4.5. PN0 (produced in insect cells) contains a 6xHis tag followed by a thrombin cleavage site at the C-terminal end of the protein, whereas all other constructs (produced in bacteria) contain these two features at their amino (N) termini. As an alternative to insect cell-expressed PN0, human periostin, containing the alternatively spliced sequences, and expressed as a secreted protein by mouse NSO myeloma cells, was purchased from R&D Systems (Minneapolis, MN, USA) as before [12]. Recombinant human ADAM8, encompassing amino acids No. 158–497, which consists of part of the prodomain and the entire catalytic and disintegrin domains, expressed by mouse NSO cells, was from R&D Systems.

Antibodies and other reagents

Mouse anti-periostin clone Stiny-1 [34] and anti-P-selectin glycoprotein ligand (PSGL)-1 KPL-1 [32] monoclonal antibodies (mAbs), and goat anti-disintegrin metalloproteinase (ADAM) 8 ectodomain polyclonal antibody [36] were as before. Anti-gelsolin clone 2 mAb and rhodamine-phalloidin were from BD Transduction Laboratories (San Diego, CA, USA). Rabbit anti-ADAM8 cytoplasmic domain and anti-actin-related protein (Arp)-3 polyclonal

antibodies, mouse anti-cortactin mAb 4F11, and the ADAM inhibitor tumor necrosis factor- α processing inhibitor (TAPI)-1 were from EMD Millipore (Temecula, CA, USA). TAPI-1 was dissolved at 10 mM in dimethyl sulfoxide (DMSO) and was used at 25 μ M as described [37]. Recombinant human interleukin (IL)-5 was from R&D Systems. Alexa Fluor 488-conjugated anti-mouse and anti-rabbit F(ab')₂ fragments were from Cell Signaling Technology (Danvers, MA, USA). Alexa Fluor 488-conjugated anti-goat F(ab')₂ fragment and 4',6-diamidino-2-phenylindole (DAPI) were from Life Technologies Corporation (Eugene, OR, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse and peroxidase-conjugated anti-goat and anti-rabbit secondary antibodies were from Jackson Laboratories (West Grove, PA, USA). Isotype controls mouse IgG₁ and mouse IgG_{2b} were from BioLegend (San Diego, CA, USA), rabbit IgG and goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and rabbit mAb IgG was from Cell Signaling. BB94 (batimastat) was from Tocris Bioscience (Bristol, UK). BB94 was dissolved at 10 mM in DMSO and was used at 50 μ M [38].

Fluorescence microscopy

Fluorescent microscopy staining of adherent cells was performed as before [12, 36], with the following modifications. After eosinophils were resuspended at 6×10^5 /ml in Roswell Park Memorial Institute (RPMI) with 0.2% bovine serum albumin, they were equilibrated for 1 h at 37°C [11]. Eosinophils (500 μ l, i.e., 3×10^5 cells) were allowed to adhere in the presence or absence of IL-5 (10 ng/ml) for 10, 30, or 60 min to coverslips coated with periostin (produced by insect cells, coated at 5 μ g/ml). Primary antibodies were used at 10 μ g/ml to stain antigens in adherent cells after fixation as before [36], except that anti-Arp-3 was used at 1:100. Alexa-conjugated secondary antibodies were used at 1:1000 [11]. Nuclei were stained for 5 min with DAPI diluted in PBS according to the manufacturer's instructions, and then cells were washed once with PBS. Coverslips were mounted with Immumount (Thermo Scientific, Pittsburgh, PA, USA). Representative eosinophils were viewed [36] and photographed [12, 32] as before, i.e., at least 200 cells were observed on each coverslip. Eosinophils were quantified on whether they were associated or not with clearance of the substrate layer as described [36]. In addition, cleared area, cell circumference, cell area, and peripheral F-actin and PSGL-1 staining were quantified using the Fiji version of ImageJ (<http://fiji.sc/Fiji>). Confocal microscopy was performed as before [11] or with an A1R+ microscope (Nikon Instruments, Melville, NY), using a 60 $\times$ or 100 $\times$ oil immersion objective with 1.0 airy unit pinhole and 0.3 μ m z stack thickness. Images were acquired and exported using NIS-Elements Advanced Research (AR) software (Nikon Instruments). Images of staining with isotype controls were acquired and processed using settings identical to those with the relevant specific antibodies.

SDS-PAGE

Periostin constructs (1 μ g) were run under reducing conditions (5% β -mercaptoethanol) on 10% SDS-PAGE. Gel was stained using Gelcode Blue Stain Reagent (Thermo Scientific, Madison, WI, USA).

Immunoblotting

Periostin constructs (4 pmol) were run on an SDS-PAGE gel as described above. Eosinophils (20×10^6) were resuspended in 2 ml RPMI (i.e., at 10×10^6 /ml) without added albumin, and equilibrated for 1 h at 37°C. Then, 1 ml suspension (10×10^6 cells) was incubated with IL-5 (10 ng/ml) and 1 ml was incubated without IL-5 for 1 h. Cells were centrifuged for 10 min at $260 \times g$ (1,200 rpm in a Sorvall Technospin R centrifuge, Du Pont, Wilmington, DE, USA). Supernatants were carefully aspirated (leaving 50 μ l), precipitated in glass tubes with 80% acetone at -20°C overnight, and centrifuged for 10 min at 4°C at $8,000 \times g$ (8,500 rpm in an SS-34 rotor, Sorvall RC-5B centrifuge, Du Pont). Cell pellets and supernatant precipitates were resuspended in 25 μ l PBS, then dissolved by adding 50 μ l 4% SDS, 4 M urea, 5% glycerol, 62.5 mM Tris, pH 6.8 with bromophenol blue, i.e., to a total volume of 75 μ l (corresponding to 10×10^6 cells). Samples (15 μ l per lane) were run under non-reducing conditions on 8% SDS-PAGE. Thus, each 15 μ l (each lane of) cell or supernatant sample contained material originating from 2×10^6 cells. Immunoblotting was performed by transfer to polyvinylidene difluoride (PVDF) membranes using Bio-Rad Trans-Blot Turbo mini PVDF Transfer Pack and Bio-Rad Trans-Blot Turbo Blotting System (Bio-Rad, Hercules, CA, USA) (for periostin constructs) or as described [34] (for cell and supernatant samples), incubation with primary antibodies at 0.5 μ g/ml, and detection of bands by peroxidase-conjugated secondary antibodies at 1:20,000 and enhanced chemiluminescence (SuperSignal™ West Pico Chemiluminescent Substrate, Thermo Scientific, Madison, WI, USA, or Perkin Elmer, Waltham, MA, USA, respectively). Specificity of the secondary antibodies was assessed by omitting the primary antibodies. Images were processed using the BioSpectrum 810 imaging system and VisionWorks LS software (UVP, Upland, CA, USA).

Cell motility assay

Cell motility was assessed as before [12] with the following modifications. Wells were coated with 10 μ g/ml periostin from R&D, blocked with fetal bovine serum (FBS). Then 1 μ m-diameter Polybeads were added to the wells [12]. After eosinophils were resuspended at 2×10^6 /ml in RPMI with 20% FBS, they were allowed to “rest” for 1 h at 37°C. Eosinophils were then diluted 1:100 to 20,000/ml in RPMI-20% FBS and 50 μ l (i.e., 1,000 cells) was added to each well (containing a bead layer in 50 μ l RPMI) in the presence of IL-5 (10 ng/ml final concentration) and incubated for 20 h at 37°C. Wells were viewed in an Eclipse Ti inverted microscope (Nikon Instruments). Images were acquired and exported using NIS-Elements AR. Results were quantified using Fiji.

Statistical analysis

Paired *t*-test was used to compare data between two groups of treatments of cells from the same donors. Unpaired *t*-test was used to compare data between two groups of fields or cells. One-way ANOVA with Tukey’s multiple comparisons test was used to compare data among several time points. *P* 0.05 was considered significant. Analyses were performed using Prism (GraphPad Inc., San Diego, CA, USA).

Results

Upon adhesion to periostin in the presence of IL-5, eosinophils transition from a polarized morphology similar to that in suspension to become spread and display podosomes

To learn how the morphology of eosinophils evolves upon adhesion to periostin, purified human blood eosinophils adhering to adsorbed recombinant human periostin in the presence or absence of IL-5 (10 ng/ml) were fixed and imaged at different time points by fluorescent microscopy. After 10 min in the presence of IL-5, eosinophils adherent to periostin were polarized with PSGL-1 gathered tightly over the nucleopod tip (Fig. 1A), similar to that in suspended cells stimulated with IL-5 for 5–10 min [11], or more diffusely in a crescent at the nuclear pole of the cell (Fig. 1B). F-actin was localized with labeled phalloidin at the opposite end of the cell (Fig. 1A and B), as in suspended cells [11]. After 60 min with IL-5, eosinophils were more spread, the nucleus was more centralized, PSGL-1 was in a ring around the nucleus away from the cell periphery, and F-actin was localized outside of the ring of PSGL-1 in punctate structures (Fig. 1D and H). After 30 min, the cells had a mixed or intermediate appearance with some dots or puncta of F-actin and larger crescent of PSGL-1 (Fig. 1C). In the absence of IL-5, adherent eosinophils were rare (consistent with our earlier finding that in the absence of IL-5 about 5–10% of cells attach, whereas IL-5 increases the number of attached cells, most often about four-six-fold to about 25–40% [12]) and not polarized, with a homogeneous distribution of PSGL-1 over the cell surface, no spreading, and no puncta positive for F-actin (Fig. 1E–G). Orthogonal views demonstrated that the puncta of IL-5-treated eosinophils were just above the surface of the periostin-coated coverslip. Fiji software was used to quantify the morphological changes, including cell spreading and distributions of F-actin and PSGL-1, upon adhesion to periostin in the presence of IL-5 (Fig. 1I–L). This quantification confirmed the visual impression that cells had greater circumference (Fig. 1I) and area (Fig. 1J) after adhering for 30–60 min compared to at 10 min and that F-actin (Fig. 1K) and PSGL-1 (Fig. 1L) covered part of the periphery at 10 min but no longer localized at the cell periphery after 30–60 min.

The morphology of the punctate structures in eosinophils adhered to periostin in the presence of IL-5, as noted briefly previously [12], characterizes podosomes, dynamic adhesive contacts distinct from classical focal adhesions and present in many cell types, including cancer cells, macrophages, osteoclasts, dendritic cells, and vascular smooth muscle cells. Podosomes in these cell types contain multiple cytoskeletal and other proteins, including gelsolin, the Arp-2/3 complex, and cortactin [39]. Gelsolin (Fig. 2A and C) and Arp-3 (Fig. 2D and F) colocalized with F-actin in the punctate structures in eosinophils. Cortactin did not localize to podosomes, but instead was found at the cell periphery (Fig. 2G and H). These staining results indicate that although the punctate structures in eosinophils adherent to periostin have many of the features of podosomes, they are not identical in composition to podosomes in other cell types.

Periostin is cleared around adherent eosinophils by a mechanism attenuated by metalloproteinase inhibitors

We found previously that phorbol myristate acetate (PMA), which does not induce nucleopod formation in eosinophils [11], increases podosome formation in eosinophils

adhering to VCAM-1 and that eosinophils clear the VCAM-1 substrate [36]. To learn if eosinophils adhere to periostin after stimulation with a physiological agonist, IL-5, also modify adhesive ligand, we stained for the substrate-bound periostin, using anti-periostin mAb Stiny-1. Cleared areas were present in the coating after adhesion of eosinophils for only 10 min (Fig. 3A). The layer was cleared progressively around adherent eosinophils, so that a greater area appeared to be cleared after 60 min (Fig. 3B–D). Image analysis demonstrated that the cleared area in the absence of IL-5 was $96 \pm 34 \mu\text{m}^2/\text{cell}$ (mean $\pm$ standard error of the mean [SEM]) and in the presence of IL-5 $660 \pm 150 \mu\text{m}^2/\text{cell}$ ($P < 0.001$, $n = 28$ cells). However, it can be noted that the proportion of cells associated with clearance was similar in the presence or absence of IL-5 (Fig. 3F). Since both a higher number of cells adhere in the presence of IL-5 [12] and the cleared area per cell is greater with IL-5, as just mentioned, it can be concluded that the overall area of periostin clearance is greater in the presence of IL-5. Clearance was attenuated by the metalloproteinase inhibitor BB94 (batimastat) (Fig. 3E and F), indicating that at least one member of the superfamily of metalloproteinases [40] is involved in the eosinophil-mediated periostin degradation.

MAB Stiny-1 maps to the FAS1 4 module of periostin

To localize the Stiny-1 epitope, we immunoblotted periostin constructs containing different modules of periostin (Fig. 4A). Stiny-1 bound only to constructs containing the FAS1 4 module (Fig. 4B). Thus, the clearance detected by immunofluorescence microscopy with Stiny-1 represents modification of at least the FAS1 4 module.

Periostin modification is attenuated by anti-ADAM8

In a recently published quantitative proteomic dataset of purified (unstimulated) blood eosinophils pooled from three individuals [41], eight metalloproteinases were detected in the following order of abundance: ADAM (disintegrin metalloproteinase) 8 (100% relative abundance), MMP (matrix metalloproteinase) 25 (58%), ADAM10 (17%), ADAM19 (3.8%), MMP9 (2.1%), ADAM17 (0.85%), MMP8 (0.41%), and ADAM28 (0.24%) [41]. Consistent with the proteomics data, ADAM8 and MMP25 were by far the most abundant metalloproteinase transcripts in a recent RNA Seq analysis of human blood eosinophils [42]. Eosinophils express relatively high levels of cell-surface ADAM8 at baseline, as assayed by flow cytometry [36]. For these reasons, we examined the role of ADAMs, especially ADAM8, in eosinophil-mediated periostin clearance, by using the ADAM inhibitor TAPI-1 [37] and polyclonal anti-ectodomain ADAM8 antibodies. TAPI-1 and anti-ADAM8 attenuated periostin Stiny-1 epitope clearance (Fig. 5A–F).

ADAM8 detected by immunofluorescence

We previously demonstrated co-localization of ADAM8 with podosomes in PMA-stimulated VCAM-1-adherent eosinophils associated with wide-spread clearance of VCAM-1 [36] and therefore looked for similar co-localization in IL-5-stimulated eosinophils adherent to periostin. ADAM8 (Fig. 6B) was detected by immunofluorescent microscopy in eosinophils adherent to periostin, either in the presence (Fig. 6B) or absence (Fig. 6C) of IL-5. Two different anti-ADAM8 antibodies were used. Polyclonal anti-ADAM8 ectodomain and anti-cytoplasmic domain antibodies (Fig. 6A) stained eosinophils in a specific manner compared

to the relevant controls (Fig. 6B). Staining was of larger punctate structures and in a reticular pattern surrounding the nucleus in IL-5-stimulated cells (Fig. 6B) and of a central area some peripheral sites in non-stimulated cells (Fig. 6C).

When compared to F-actin in IL-5-stimulated eosinophils, punctate ADAM8 was detected in the same confocal microscopic slices, i.e., at the same level, as were the podosome-like structures (Fig. 6B) and therefore in close contact with the cell-substrate interface. However, punctate ADAM8 was localized more centrally whereas the podosome-like structures were more peripheral, and there was no overlap between the two (Fig. 6B). The reticular ADAM8 staining in IL-5-stimulated cells was central and at higher planes (not shown). When compared to F-actin in unstimulated cells, ADAM8 was mostly localized more centrally whereas F-actin was mostly cortical (Fig. 6C).

ADAM8 proteoforms in eosinophils and eosinophil supernatant detected by immunoblotting

Since the periostin Stiny-1 epitope was cleared in a relatively large area, a soluble rather than transmembrane form of ADAM8 is likely to be responsible. One route via which ADAM8 could be released to the supernatant is via proteolysis at the plasma membrane [40, 43] of the canonical 824-residue isoform 1 of ADAM8 (UniProt No. P78325-1) (Fig. 6A). To investigate whether soluble ADAM8 can be released from the cells, we performed immunoblotting of cells and released proteins after incubating suspended eosinophils for 1 h with IL-5. Lysates and supernatants contained a major band of ~75 kDa and a minor band of ~95 kDa both of which reacted with the anti-ectodomain and anti-cytoplasmic domain antibodies, more strongly in the lysate than in the supernatant (Fig. 6E). A similar pattern was found when IL-5 was omitted during the one-hour incubation. Comparing this pattern to that found in COS-7 cells transfected with the canonical isoform of ADAM8 [43], the 95- and 75-kDa bands likely represent full-length canonical ADAM8 and canonical ADAM8 lacking the prodomain, respectively. In the supernatant, bands of smaller size, most prominently ~60 kDa, were detected that reacted with the anti-ectodomain but not with the anti-cytoplasmic domain antibody (arrowheads, Fig. 6E). The 60 kDa band is of a size compatible with canonical ADAM8 lacking the pro-, transmembrane and cytoplasmic domains. Control lanes with recombinant human ADAM8 encompassing part of the prodomain and the entire catalytic and disintegrin domains confirmed that such a fragment containing a portion of the extracellular segment of ADAM8 reacted with the anti-ectodomain but not with the anti-cytoplasmic domain antibody (Fig. 6D). In summary, the results indicate that soluble forms of ADAM8 are released from eosinophils over a one-hour incubation period and compatible with the observation that anti-ADAM8 attenuates clearance of the periostin Stiny-1 epitope over relatively large areas.

ADAM8-mediated eosinophil migration on periostin

Because ADAM8 appeared to be involved in modification of the periostin layer by adherent eosinophils, we asked whether ADAM8 is also involved in the previously demonstrated migration of eosinophils on periostin, which is enhanced approximately ten-fold by IL-5 [36]. As before, IL-5-stimulated eosinophils displayed random motility on periostin, clearing long tracks in a “lawn” of beads in periostin-coated wells (Fig. 7A). In the presence of anti-

ADAM8 ectodomain antibodies, tracks were fewer and shorter (Fig. 7B). Quantification of motility as cleared area of tracks using Fiji software demonstrated a significant inhibitory effect of anti-ADAM8 on eosinophil migration on periostin compared to control IgG (Fig. 7C).

Discussion

Upon adherence to periostin, IL-5-stimulated eosinophils transitioned from a polarized morphology similar to that adopted by stimulated eosinophils in suspension to a spread morphology dominated by F-actin-rich punctate structures, which we conclude are podosomes by virtue of their appearance and content of gelsolin and Arp-3. Podosomes are dynamic adhesive contacts found in cancer cells and several normal cell types and distinct from classical focal adhesions of fibroblasts [39]. Podosomes in tumor cells are associated with local loss and degradation of the ECM ligand, e.g., fibronectin and collagen, immediately under the podosome [39].

The Stiny-1 mAb epitope, which we map here to the FAS1 4 module of periostin, was lost from the periostin layer over large areas in the vicinity of eosinophils. The wide-spread loss contrasts to discrete clearings of ECM molecules under individual podosomes of cancer cells. The wide-spread clearance was attenuated by metalloproteinase or ADAM inhibitors, or by anti-ADAM8 antibodies, indicating that the process is mediated by the metalloproteinase ADAM8. The canonical isoform of ADAM8 is a transmembrane protein with expected primary structure of an ADAM family member [40]. In addition, ADAM8 is annotated as also existing as alternatively spliced 742-residue isoform 3 (UniProt No. P-78325-3), which lacks the transmembrane domain and cytoplasmic tail and has the potential to be secreted. ADAM8 is known to be expressed at a relatively high level on the eosinophil surface at baseline, as assessed by flow cytometry [36]. We previously found co-localization of ADAM8 and podosomes in eosinophils stimulated by PMA and adhering to VCAM-1, which is also cleared around adherent cells [36]. It should be noted that eosinophils stimulated by PMA do not polarize and do not form nucleopods [11]. In eosinophils stimulated to adhere to periostin by IL-5, however, ADAM8 was not found in the podosome-like structures. Instead, antibodies to both extracellular and intracellular domains stained punctate structures that were more central than the podosome-like structures and in a central reticular pattern that probably represents the biosynthetic pool of ADAM8 in endoplasmic reticulum. In unstimulated eosinophils adherent to periostin, ADAM8 was also localized in a central area. Immunoblotting of the supernatant of suspended eosinophils incubated for 1 h (with or without IL-5) revealed ADAM8 bands that lack the cytoplasmic domain. Although these bands most likely are shed parts of the canonical proteoform, one or more may represent secretion of the alternatively spliced soluble isoform 3. Further studies using proteoform-specific antibodies are needed to investigate these possibilities. Thus, purified blood eosinophils appear to shed and/or secrete forms of ADAM8 *in vitro*. Soluble ADAM8 is found in serum of healthy human individuals at about 200–300 pg/ml and can be increased in disease states, e.g., in some eosinophilic pneumonias [44]. The contributions of blood or tissue eosinophils to the circulating pool of ADAM8 remain to be elucidated.

ADAM8 is induced or upregulated after antigen challenge in mice, or in lung or sputum in human asthma or severe asthma [23–27]. Further, copy number variation analyses of multiple populations placed ADAM8 in the top three of 61 implicated “asthma genes” [31]. Studies with ADAM8-deficient mice in models mimicking asthma have yielded conflicting results with ADAM8 implicated as either promoting or regulating eosinophil recruitment, airway responsiveness and remodeling [28–30, 45, 46]. However, a recent article demonstrated that a peptide inhibitor of ADAM8 decreased airway eosinophil numbers, tissue remodeling, and type 2 cytokines, and attenuated airway hyperresponsiveness in mice [47], supporting pro-eosinophil recruitment and pro-asthma roles for ADAM8.

Interestingly, the contrary results regarding ADAM8 and mouse asthma models are somewhat similar to what was found in murine knock-outs of periostin [15, 48–53]. Several studies on periostin-deficient mice, or with an anti-periostin mAb in wild type mice, indicate that periostin promotes allergic inflammation, eosinophil recruitment, generation of type 2 cytokines, and airway hyperresponsiveness [15, 48, 51–53]; whereas other mouse studies indicate that periostin does not influence leukocyte recruitment but suppresses type 2 responses, airway remodeling and responsiveness [15, 49, 50]. There may be several possible explanations for the discrepancies in effects on airway remodeling and responsiveness in the mouse studies, but overall those studies indicate that periostin is involved in and may promote and/or regulate airway inflammation and remodeling [15]. The human studies on the adhesive and pro-migratory capacity of periostin and the association between periostin and airway eosinophilia and inflammation [12, 13, 18, 20], as well as the majority of the mouse studies support a scenario in which periostin promotes eosinophil recruitment to the airway. Thus, on balance the evidence seems to point to pro-eosinophil recruitment roles for both periostin and ADAM8.

In conclusion, upon adhesion to periostin, IL-5-stimulated eosinophils undergo morphological change characterized by dissipation of the nucleopod, formation of podosome-like structures, and the ability to modify adsorbed periostin and migrate on periostin. The latter processes are mediated, at least in part, by ADAM8. Rather than the highly spatially restrictive proteolysis associated with the classic podosome, modification of periostin is wide-spread and likely due to ADAM8 shed or secreted by eosinophils. Our previous *in vitro* migration data indicate that intermediate concentrations of coated periostin promotes migration of IL-5-stimulated eosinophils and that higher concentrations of periostin inhibit migration [12]. Thus, we envision that *in vivo* eosinophils use ADAM8 to partially degrade periostin deposited in response to type 2 immunity mediators and that such degradation paves the way for the eosinophil during movement through tissue, in a manner similar to metalloproteinase-mediated cancer cell invasion. ADAM8-mediated remodeling of matrix and eosinophil recruitment likely play significant roles in the asthmatic airway, and thus ADAM8 may be a possible therapeutic target in asthma.

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Fig. 1.

Alterations in the morphology of eosinophils upon adhesion to periostin. (A-H) Localization of PSGL-1 (green), F-actin (red), and nucleus (blue) in eosinophils adherent to periostin in the presence (A-D, H) or absence (E-G) of IL-5. Cells were allowed to adhere for 10 (A, B, and E), 30 (C and F), or 60 (D, G, and H) min before fixation and staining. (H) Confocal image, 0.5 μm slice + xz and yz planes. Cells were analyzed by immunofluorescent staining using mAb to PSGL-1 and FITC- or Alexa Fluor 488-conjugated secondary antibody. F-actin was stained with rhodamine-phalloidin and nuclei with DAPI. Arrows, punctate F-actin-containing structures (podosomes). Bar, 10 μm . (I-L) Quantitation of changes in morphology and F-actin and PSGL-1 localization in eosinophils adherent to periostin in the

presence of IL-5 for 10, 30 or 60 min, using the Fiji version of ImageJ (<http://fiji.sc/Fiji>): (I) cell circumference, (J) cell area, (K) peripheral F-actin staining as percentage of circumference, and (L) peripheral PSGL-1 staining as percentage of circumference (mean $\pm$ SEM, $n = 30$ cells at each time point, $*P = 0.05$, $***P = 0.001$ versus 10 min).

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Fig. 2.

Gelsolin and Arp-3 colocalize with F-actin in podosomes in IL-5-stimulated eosinophils adherent to periostin, whereas cortactin does not. Localization of gelsolin (green, A and C), Arp-3 (green, D and F), cortactin (green, G and H), F-actin (red, B, C, E, F, and H), and nucleus (blue, C and G) in eosinophils adherent to periostin for 1 h in the presence of IL-5. (G and H) Confocal images, 0.3 μ m slices close to the substrate level. Cells were analyzed by immunofluorescent staining using mAb to gelsolin or cortactin, or polyclonal antibody to

Arp-3, and Alexa Fluor 488-conjugated secondary antibody. F-actin was stained with rhodamine-phalloidin and nuclei with DAPI. Arrows, podosomes. Bars, 10 μ m.

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Fig. 3.

The Stiny-1 periostin epitope is cleared around adherent eosinophils in a manner attenuated by the metalloproteinase inhibitor BB94. (A-C) Clearance of the periostin substrate layer by eosinophils adherent for 10 (A) or 60 (B, C) min in the presence of IL-5. (C) Confocal image, volume view. (D, E) Clearance at 60 min by adherent eosinophils not treated with IL-5 and incubated with DMSO vehicle (1:200) (D) or DMSO and 50 μ M BB94 (E). The periostin layer was visualized with mAb Stiny-1 and Alexa Fluor 488-conjugated secondary antibody (green). F-actin was stained with rhodamine-phalloidin (red, A-C) and nuclei with

DAPI (blue). Arrowheads, areas of periostin clearance. Arrows, podosomes. Bar, 10 μm . (F) Quantification of cells associated with clearance as described [36] (mean $\pm$ standard error of the mean [SEM], $n = 3$ donors, $*P = 0.05$ versus control without BB94).

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Fig. 4.

Stiny-1 mAb binds to the FAS1 4 module of periostin. (A) SDS-PAGE Gelcode Blue staining of PN0 and periostin FAS1 modules (reduced, 1 μ g/well). (B) Western blot of PN0 and periostin FAS1 modules (4 pmol/well) using Stiny-1 mAb. PN0, shortest C-terminus splice variant of periostin lacking exons 17, 18, 19, and 21 (80 kDa); 1–2, periostin FAS1 1 to FAS1 2 (32 kDa); 2, periostin FAS1 2 (16 kDa); 2–3, periostin FAS1 2 to FAS1 3 (30 kDa); 3–4, periostin FAS1 3 to FAS1 4 (31 kDa); 3-C, periostin FAS1 3 to the C-terminus (42 kDa). Molecular size markers (kDa) on the left in (A) and (B).

Fig. 5.

Stiny-1 periostin epitope clearance is attenuated by the ADAM inhibitor TAPI-1 or by anti-ADAM8 ectodomain antibodies. Clearance from the periostin substrate layer by IL-5-treated eosinophils adherent to periostin for 1 h in presence of DMSO vehicle (A), DMSO and 25 μ M TAPI-1 (B), non-immune control (ctrl) goat IgG (C), or 10 μ g/ml anti-ADAM8 ectodomain polyclonal antibodies (D). The periostin layer was visualized with mAb Stiny-1 to periostin and Alexa Fluor 488-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). Arrowheads, areas of periostin clearance. Bar, 10 μ m. (E)

Quantitation of cells associated with clearance as described [36] (mean $\pm$ SEM, $n = 3$ donors, $*P = 0.05$ versus DMSO or IgG control). (F) Quantification of clearance as percentage of field area using Fiji (mean $\pm$ SEM, $n = 4$ fields for each treatment, $*P = 0.05$ versus IgG control).

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Fig. 6.

Immunofluorescence microscopy and immunoblotting indicate that ADAM8 in adherent eosinophils does not localize to podosomes and that ADAM8 is shed to the medium. (A) Detection of regions recognized by anti-ectodomain and anti-cytoplasmic domain polyclonal antibodies on a schematic modified from [54] of the canonical form or isoform 1 of ADAM8 (UniProt identifier No. P78325–1). Black box, signal peptide; PRO, prodomain; CATA, catalytic (metalloproteinase) domain; DIS, disintegrin domain; CR, cysteine-rich domain; EGF, epidermal growth factor-like domain; TM, transmembrane segment; IC, intracellular or

cytoplasmic domain. (B) Localization of ADAM8 (green) and F-actin (red) in eosinophils adherent to periostin for 1 h in the presence of IL-5. Confocal images, 0.3 μ m slices close to the substrate level. (C) Localization of ADAM8 (green) and F-actin (red) in eosinophils adherent to periostin for 1 h in the absence of IL-5. Confocal images, 0.3 μ m slices. Cells in (B) and (C) were analyzed by immunofluorescent staining using polyclonal anti-ectodomain or cytoplasmic domain antibodies, or control goat or rabbit IgG and Alexa Fluor 488-conjugated secondary antibody. F-actin was stained with rhodamine-phalloidin and nuclei with DAPI (blue). Bar, 10 μ m. (D) Recombinant human ADAM8 encompassing amino acids No. 158–497 (predicted molecular mass 38 kDa, 3 pmol/well) immunoblotted with anti-ADAM8 ectodomain or cytoplasmic domain antibody under non-reducing conditions. (E) Cell lysates (lanes labeled C) and supernatants (lanes labeled S) of eosinophils incubated in suspension in the presence of IL-5 for 1 h, immunoblotted with anti-ADAM8 ectodomain or cytoplasmic domain antibody under non-reducing conditions. Arrowheads point to ~60–65 kDa bands in the supernatant reactive with anti-ectodomain but not with anti-cytoplasmic domain antibodies. A similar pattern was found when IL-5 was omitted during the one-hour incubation (not shown, figure shows the lanes from a single blot with the samples from IL-5-stimulated cells from a representative donor). Arrows, molecular size (kDa) markers.

Fig. 7.

Eosinophil motility on periostin is inhibited by anti-ADAM8. Paths of eosinophils treated with IL-5 migrating in the presence of non-immune control (ctrl) goat IgG (A) or 10 μ g/ml anti-ADAM8 ectodomain polyclonal antibody (B) were revealed by perturbation of a monolayer of 1- μ m diameter latex beads. Wells were photographed after 20 h. Bar, 100 μ m. (c) Morphometric analysis of track areas using Fiji (mean $\pm$ SEM, $n = 3$ donors, ** $P < 0.01$ versus goat IgG control).