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Regulation of Eosinophil Recruitment and Allergic Airway Inflammation by Tropomyosin Receptor Kinase A

Mythili Dileepan, Xiao Na Ge,[1] Idil Bastan,[2] Yana G. Greenberg, Yuying Liang, P. Sriramarao,[3] and Savita P. Rao

Eosinophilia is a hallmark of allergic airway inflammation (AAI). Identifying key molecules and specific signaling pathways that regulate eosinophilic inflammation is critical for development of novel therapeutics. Tropomycin receptor kinase A (TrkA) is the high-affinity receptor for nerve growth factor. AAI is associated with increased expression of TrkA by eosinophils; however, the functional role of TrkA in regulating eosinophil recruitment and contributing to AAI is poorly understood. This study identifies, to our knowledge, a novel mechanism of eotaxin-mediated activation of TrkA and its role in regulating eosinophil recruitment by using a chemical-genetic approach to specifically inhibit TrkA kinase activity with 1-NM-PP1 in TrkA^{F592A}–knock-in (TrkA-KI) eosinophils. Blockade of TrkA by 1-NM-PP1 enhanced eosinophil spreading on VCAM-1 but inhibited eotaxin-1 (CCL11)–mediated eosinophil migration, calcium flux, cell polarization, and ERK1/2 activation, suggesting that TrkA is an important player in the signaling pathway activated by eotaxin-1 during eosinophil migration. Further, blockade of matrix metalloprotease with BB-94 inhibited eotaxin-1–induced TrkA activation and eosinophil migration, additively with 1-NM-PP1, indicating a role for matrix metalloproteases in TrkA activation. TrkA inhibition in *Alternaria alternata*–challenged TrkA-KI mice markedly inhibited eosinophilia and attenuated various features of AAI. These findings are indicative of a distinctive eotaxin-mediated TrkA-dependent signaling pathway, which, in addition to other TrkA-activating mediators, contributes to eosinophil recruitment during AAI and suggests that targeting the TrkA signaling pathway to inhibit eosinophil recruitment may serve as a therapeutic strategy for management of eosinophilic inflammation in allergic airway disease, including asthma. ***The Journal of Immunology*****, 2020, 204: 000–000.**

Tropomycin receptor kinase A (TrkA), also known as neurotrophic tyrosine kinase receptor type 1 (NTRK1), is a member of the receptor tyrosine kinase (RTK) family and the high-affinity receptor for nerve growth factor (NGF) (1). NGF/TrkA signaling plays a significant role in regulating neuronal survival, development, and function (2) and has been extensively studied in the context of pain management (3, 4) as well as tumor development (5, 6). Previous studies have also indicated a role for NGF and TrkA signaling in allergic airway inflammation (AAI). AAI is an inflammatory disease of the airways characterized by increased recruitment of inflammatory cells (especially eosinophils), elevated levels of Th2 cytokines, proinflammatory chemokines, and growth factors that, together, contribute to the overall disease pathogenesis (7). TrkA is expressed in many cell types in the lungs, such as inflammatory cells, including eosinophils, bronchial and alveolar epithelial cells, smooth muscle cells, and vascular endothelial cells (8). Elevated NGF levels have been detected in the bronchoalveolar lavage fluid (BALF) and serum of patients with AAI and asthma (9). Evidence from animal models of AAI supports an important role for NGF and TrkA signaling in allergic asthma [reviewed in Ref. (10)]. Recent studies have also shown that TrkA/NTRK1 is a transcriptional target of the Th2 cytokine IL-13 in human epithelial cells and is strongly upregulated in patients with eosinophilic esophagitis, conferring increased responsiveness to NGF, and is thus likely to contribute to allergic inflammation (11).

Although it is clear that NGF/TrkA signaling plays a role in AAI, the causal relationship between NGF/TrkA signaling and allergic asthma or the mechanism involved remains unclear because of the lack of a specific method thus far to interrogate the NGF/TrkA signaling pathway. Both NGF and TrkA gene knockout in mice leads to a lethal phenotype because of defective neuron development (12, 13). TrkA inhibitors, like other RTK inhibitors, have limited drug specificity. TrkA kinase inhibitors used in previous studies also inhibit other kinases of the RTK family (14). The most commonly used TrkA inhibitor K252a blocks the activities of other kinases, such as myosin L chain kinase and protein kinase C (PKC) (15). Anti-NGF Ab or NGF–small interfering RNA cannot

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Abbreviations used in this article: AAI, allergic airway inflammation; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; BM, bone marrow; $[Ca^{2+}]_i$, intracellular calcium; CCR3, CCR type 3; CRA, cockroach allergen; GPCR, G-protein–coupled receptor; IHC, immunohistochemical staining; MBP, major basic protein; MMP, matrix metalloprotease; NGF, nerve growth factor; 1-NM-PP1, 1-tert-butyl-3-(naphthalen-1-ylmethyl)-1H-pyrazolo[3,4-d] pyrimidin-4-amine; PAS, periodic acid–Schiff; PFA, paraformaldehyde; R_L, pulmonary resistance; RTK, receptor tyrosine kinase; α-SMA, α-smooth muscle actin; t-ERK, total ERK; TrkA, tropomycin receptor kinase A; TrkA-KI, TrkA^{F592A}–knock-in; t-TrkA, total TrkA; WT, wild-type.

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elucidate the functional importance of the TrkA kinase activity. Further, anti-NGF Ab cannot distinguish the functional roles of its two receptors TrkA and p75NTR nor completely block the activation of the receptors if they are activated through cross-talk with other receptors (e.g., G-protein–coupled receptors [GPCRs]) (16–18).

Eotaxin-1 (CCL11) is the predominant chemokine contributing to airway eosinophil recruitment and exerts its activity on eosinophils via the GPCR CCR type 3 (CCR3) (19). In this study, we have delineated a functional role for TrkA signaling in regulating eotaxin-1–induced eosinophil recruitment and the development of AAI in mice by using a combined genetic-chemical approach to specifically control the TrkA kinase activity in vitro and in vivo. An F592A mutation within the ATP-binding pocket in the kinase subdomain V allows the selective and reversible inhibition of TrkA by an ATP analogue, such as 1-tert-butyl-3-(naphthalen-1-ylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (1-NM-PP1) (20). Using the $TrkA^{F592A}$–knock-in (TrkA-KI) mouse strain (21) in an *Alternaria alternata*–induced AAI model, we have established that TrkA signaling plays an important role in AAI development and uncovered a distinct signaling mechanism wherein TrkA mediates the effects of eotaxin on cellular recruitment.

Materials and Methods

Mouse model of AAI

A TrkA-KI mouse strain ($TrkA^{F592A}$) wherein the ATP-binding pocket in the kinase subdomain V is mutated by replacing the phenylalanine residue with alanine, resulting in the generation of an ATP analogue–sensitive kinase model [which undergoes specific inhibition by an ATP analogue, such as 1-NM-PP1 (21)], was used for in vivo studies. TrkA-KI mice were obtained from Dr. Ye (Department of Pathology and Laboratory Medicine, Emory University School of Medicine; Atlanta, GA) and subsequently bred and maintained at the University of Minnesota. TrkA-KI mice (8–12 wk, male and female) were challenged with 25 μg of *A. alternata* extract (Greer Laboratories) in 50 μl of PBS on days 0, 3, and 6 or with PBS (control) as described previously (22). Allergen-exposed mice were divided into three groups: one group received no treatment; the second group received TrkA kinase inhibitor 1-NM-PP1 (Axon Medchem) in DMSO/PBS i.m. at a dosage of 10 mg/kg/d on days 0, 3, and 6; and the third group received the same dosage on days 0, 3, 4, 5, and 6. In pilot studies, DMSO/PBS was not found to exert nonspecific pathological effects in the lung (Supplemental Figs. 1, 2); thus, allergen-exposed mice were administered DMSO/PBS as a vehicle only in select experiments. Wild-type (WT) mice (Charles River Laboratories) were bred in house for culture of bone marrow (BM)–derived eosinophils. All studies involving mice were performed following standards and procedures approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Sample collection and analysis

Mice were sacrificed 24 h after the last allergen challenge. Total number of cells in the BALF was counted in a hemocytometer, and differential cell counts in the BALF and blood were determined from cytocentrifuged samples based on morphologic and histologic criteria after staining with Hema 3 System (Thermo Fisher Scientific). BALF supernatants were stored at −80°C for later analysis. Right lungs were snap-frozen and left lungs were first perfused with 4% paraformaldehyde (PFA), fixed in 4% PFA, and paraffin embedded. Lung tissue harvested from WT C57BL/6 mice challenged with *A. alternata* (22), OVA (23), or cockroach allergen (CRA) (24) and paraffin embedded in a similar manner was used in some studies.

Lung histology

Paraffin-embedded lung tissue sections (4 μm) were stained with H&E (Leica Biosystems) to determine cellular infiltration. After Ag retrieval, expression of total and phosphorylated TrkA (t-TrkA and p-TrkA, respectively) in lung tissue sections was detected by immunofluorescence staining using rabbit polyclonal Abs against t-TrkA (no. 2505S, at 2 μg/ml; Cell Signaling Technology) and rabbit mAb against p-TrkA (no. 4168S, detects TrkA phosphorylated at Tyr785, at 2 μg/ml; Cell Signaling Technology), respectively. Rabbit IgG was used as a control for both Abs. Bound Abs were detected using rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). To examine p-TrkA expression by lung tissue eosinophils, sections were dual-stained with mAb against p-TrkA and rat mAb against eosinophil-specific major basic protein (MBP; 5 μg/ml) (25), followed by FITC-conjugated anti-rat and rhodamine-conjugated anti-rabbit secondary Abs (Jackson ImmunoResearch Laboratories). DAPI was used to visualize nuclei, and slides were examined by confocal microscopy.

For all immunohistochemical staining (IHC) analyses, tissue sections were subjected to Ag retrieval followed by the quenching of endogenous peroxidase activity prior to staining with specific Abs. VECTASTAIN ABC Kits containing biotinylated secondary Abs and avidin-biotin HRP complex (Vector Laboratories) were used along with the Peroxidase AEC Substrate Kit (Vector Laboratories) for detection. Evaluation and quantitation of lung tissue eosinophil infiltration were performed by IHC for MBP with rat mAb against murine MBP (2 μg/ml), as described previously (25). Expression of α-smooth muscle actin (α-SMA) was assessed using an mAb against α-SMA (2 μg/ml; Sigma-Aldrich), and the area of the α-SMA in peribronchial smooth muscle layer was quantitated from captured images (four to five airways per mouse) using ImageJ Image Analysis Software, as described previously (24). Results were expressed as α-SMA–positive area (square micrometer) per millimeter of basement membrane length. Rat IgG (for MBP) and mouse IgG (for α-SMA) were used as control Abs. Airway mucus accumulation was evaluated by staining lung sections with periodic acid–Schiff (PAS) reagent (Sigma-Aldrich). The PAS-positive area (mucus secretion) in airways of relatively similar size (three to seven airways per mouse) was quantitated by ImageJ analysis and expressed as PAS-positive area (square micrometer) per 100 μm of basement membrane length (24). Stained slides were examined using a Nikon Microphot EPI-FL Microscope (Nikon Instruments) and images were captured with an Olympus DP71 Camera.

Measurement of airway hyperresponsiveness

Airway hyperresponsiveness (AHR) was measured in anesthetized allergen-challenged TrkA-KI mice treated with 1-NM-PP1 (10 mg/kg/d on days 0, 3, 4, 5, and 6) or DMSO/PBS (as vehicle control) using the FinePointe Resistance and Compliance System (Buxco), as described previously (26). Allergen-challenged mice without any treatment (1-NM-PP1 or DMSO) and naive mice treated with DMSO/PBS or with 1-NM-PP1 served as additional control groups. Changes in pulmonary resistance (R_L) in response to saline followed by increasing concentrations of inhaled methacholine (3–50 mg/ml) were continuously monitored and expressed as mean R_L values for each dose.

Measurement of lung cytokines, chemokines, and IgE

Th1 (IL-2, IFN-γ)/Th2 (IL-4, IL-5, IL-13) cytokine and TNF-α levels in supernatants of lung tissue lysates were determined using Cytometric Bead Array Flex Set Kits (BD Biosciences) with a FACScan flow cytometer equipped with CellQuest Pro (BD Biosciences) for data acquisition and FlowJo Software (Tree Star) for analysis. Eotaxin-1 and eotaxin-2 in the BALF and IL-33 in lung lysates were measured using ELISA Kits (R&D Systems). IgE level in the BALF was measured using a mouse IgE ELISA Kit (BioLegend). All kits were used as per the manufacturer's recommendation.

Culture of murine eosinophils

Eosinophils were routinely cultured from BM of naive male or female TrkA-KI or WT mice, as described previously (26, 27). Cells between day 12 and 18 of culture were used in studies after assessing purity with Hema 3 (i.e., differentiation of granulocyte populations based on pink, granular cytoplasm and bilobed purple nuclei) and expression of eosinophil-specific MBP and Siglec-F by immunofluorescent staining and flow cytometry, respectively.

Immunofluorescent staining and actin polymerization

BM-derived eosinophils (2×10^5) were treated with eotaxin-1 (PeproTech) at 100 nM (or PBS) for 5 min at 37°C, cytocentrifuged on to glass slides, fixed (4% PFA in PBS, 20 min at room temperature), and blocked with goat serum in TBS for 30 min. Cells were then incubated overnight at 4°C with polyclonal Abs against t-TrkA (5 μg/ml), rabbit mAb against p-TrkA (2 μg/ml; both from Cell Signaling Technology), or rabbit mAb against Cdc42 (2 μg/ml; Abcam) followed by Rhodamine Red-X affinity-purified goat anti-rabbit IgG (15 μg/ml, 1 h at room temperature; Jackson ImmunoResearch Laboratories). Cells treated with rabbit IgG served as the negative control. In some experiments, cells were first incubated with 1-NM-PP1 (10 μM, 15 min), BB-94, a pan matrix metalloproteases (MMPs) inhibitor (10 μM, 10 min; Sigma-Aldrich), or DMSO as the

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vehicle control at 37°C and then exposed to eotaxin-1 for an additional 5 min before staining, as described above. Eosinophils treated with Z-VAD-FMK (R&D Systems), a pan-caspase inhibitor, PMSF (Sigma-Aldrich), an inhibitor of serine proteases (both at 10 μM), or appropriate vehicle controls (DMSO or isopropyl alcohol, respectively) before exposure to eotaxin-1 served as negative controls. Cells were examined by confocal microscopy after counterstaining with DAPI using a FLUOVIEW FV1000/BX61–Confocal Laser Scanning Biological Microscope (Olympus) equipped with an UPlanSApo Lens (original magnification of ×20 per 0.85 [oil]) and a PlanApo N Lens (original magnification of ×60 per 1.42 [oil]), at ambient temperature. FV10-ASW 2.0 Software (Olympus) was used for image acquisition.

Actin polymerization in eotaxin-treated eosinophils was evaluated as described (23). Briefly, 2×10^5 eosinophils seeded onto poly-L-lysine–coated coverslips were incubated with 10 μM 1-NM-PP1 or DMSO for 20 min and then exposed to eotaxin-1 (100 nM, 2 or 10 min) or left untreated. At the end of incubation, cells were fixed and permeabilized with 4% PFA containing 0.1% saponin, incubated with Alexa Fluor 488 Phalloidin (20–30 min in the dark; Thermo Fisher Scientific), counterstained with DAPI, and evaluated by confocal microscopy.

Adhesion and migration assays

Role of TrkA in regulating eosinophil adhesion to VCAM-1 and eotaxin-1–induced migration was evaluated as described previously (23). In the adhesion assays, the number of adherent cells per field in randomly selected fields (~20 fields per coverslip at ×400 original magnification) was manually determined. Next, the number of adherent cells exhibiting spreading was counted and expressed as a percentage of the total number of adherent cells in the field. In the case of migration, migrated cells in a fixed number of randomly selected nonoverlapping fields were counted using an Olympus CK2 Inverted Microscope (×200 original magnification) and expressed as percent migration relative to the migration of vehicle (DMSO)-treated cells.

Cell surface receptor expression

Cell surface adhesion molecule expression (CD49d, CD11a, CD11b, L-selectin, and CD18) in cells treated with 1-NM-PP1 (10 μM) or DMSO was examined by flow cytometry as described previously (23). All Abs were used at 10 μg/ml. To examine whether TrkA regulates basal and/or agonist-induced expression of CCR3 (the receptor for eotaxin-1), eosinophils were first treated with 1-NM-PP1 or DMSO and then exposed to eotaxin-1 (100 nM) or PBS (control) for 5 min at room temperature before flow cytometry, using FITC-conjugated rat anti-mouse CCR3 (2.5 μg/ml; R&D Systems) with FITC-conjugated rat IgG2a as the isotype control.

Calcium imaging assay

Role of TrkA in regulating eotaxin-1–induced changes in intracellular calcium ($[Ca^{2+}]_i$) concentration was evaluated by real-time digital video fluorescence imaging, using Fura-2 AM (Sigma-Aldrich) as described previously (24). Basal $[Ca^{2+}]_i$ was first measured for 2 min after which eosinophils were treated with 1-NM-PP1 or vehicle and evaluated for an additional 3 min. Cells were then stimulated with eotaxin-1 and monitored for 2 min to assess agonist-induced $[Ca^{2+}]_i$ responses. Cells were finally exposed to 2 μM ionomycin, free acid (BioVision), as a positive control and monitored for an additional 3 min. The ratio of fluorescence emissions at 340 and 380 nm was determined at each time point, which is directly reflective of the amount of $[Ca^{2+}]_i$.

Western blot analysis

WT BM-derived murine eosinophils (18 d culture, $7–10 \times 10^6$ cells per treatment) were treated with eotaxin-1 (100 nM) or NGF [100 ng/ml, based on previous studies using human eosinophils (28)] for 5 min. Cells were collected by centrifugation and cell pellets were resuspended in a radioimmunoprecipitation assay buffer containing 1 mM orthovanadate as well as protease and phosphatase inhibitors. Cell suspensions were subjected to three freeze-thaw cycles followed by three cycles of sonication (5–7 s each) on ice with 2 min intervals. Cell lysates were centrifuged and total protein in the supernatants was quantified using the BCA Protein Assay Kit (Thermo Fisher Scientific). Expression of p-TrkA and t-TrkA in cell lysates (30–60 μg per lane) was evaluated by SDS-PAGE (8%) under reduced conditions followed by immunoblotting with mAb against p-TrkA (sc-8058, 1 μg/ml; Santa Cruz Biotechnology) in the presence or absence of a p-TrkA mAb blocking peptide (5× concentration of primary Ab; Santa Cruz Biotechnology) or polyclonal Abs against t-TrkA (0.15 μg/ml; Cell Signaling Technology). Membranes were incubated with the primary Ab overnight at 4°C and then exposed to HRP-conjugated goat anti-mouse or anti-rabbit IgG (0.16 μg/ml; Jackson ImmunoResearch Laboratories). Expression levels of actin were monitored as an internal control using HRP-conjugated mAb against actin (66 ng/ml; Santa Cruz Biotechnology).

For detection of t-ERK(1/2) and p-ERK(1/2), TrkA-KI BM-derived eosinophils were first treated with 10 μM 1-NM-PP1 or DMSO followed by eotaxin-1 (100 nM) for an additional 5 min. Cell lysates (20 μg per lane) were electrophoresed (10% SDS-PAGE) and subjected to immunoblotting using rabbit mAb against t-ERK(1/2) (0.04 μg/ml) and rabbit polyclonal Abs against p-ERK(1/2) (0.088 μg/ml) (both from Cell Signaling Technology), respectively. Bound Abs were detected using HRP-conjugated goat anti-rabbit IgG (0.16 μg/ml; Jackson ImmunoResearch Laboratories). Protein bands were detected using Western Bright ECL (Advansta) and visualized on x-ray films. Intensity of detected bands was quantified using ImageJ Image Analysis Software. Expression level of p-TrkA and t-TrkA was normalized against actin, whereas the expression level of p-ERK(1/2) was normalized against that of t-ERK(1/2).

Statistical analysis

All data are presented as mean ± SEM unless otherwise stated. Representative or combined data of experiments performed at least three times are shown for in vitro studies. Statistical significance between two groups was determined using a two-tail unpaired Student *t* test. Statistical significance between multiple groups was determined by one-way ANOVA followed by Tukey post hoc analysis. Statistical analysis of AHR studies was performed by two-way ANOVA followed by Tukey post hoc analysis. Differences were considered significant at $p < 0.05$.

Results

Exposure to allergens leads to activation of TrkA in the lungs

Previous studies have indicated a role for NGF/TrkA signaling in allergic asthma [reviewed in Ref. (10)]; however, the causal relationship and the underlying mechanism(s) involved are not well understood. To understand the significance and role of TrkA signaling in promoting allergic asthma, we first investigated whether allergen exposure leads to activation of TrkA in the lung. Lung tissue of mice exposed to *A. alternata*, an airborne fungal allergen known to induce and/or exacerbate allergic asthma in humans (29), was analyzed for expression of p-TrkA by immunofluorescent staining (Fig. 1A). Epithelial cells lining the airways as well as inflammatory cells recruited to the lungs were positive for expression of p-TrkA. Epithelial cells lining the airways of control mice showed minimal p-TrkA staining. Activation of TrkA in airway epithelial cells and recruited inflammatory cells was also noted in the lungs of mice exposed to other allergens such as OVA or CRA (Fig. 1B). We next examined whether TrkA is activated in eosinophils, the predominant inflammatory cells recruited to allergic airways. Dual immunofluorescence staining of lung sections from *A. alternata*–challenged WT mice for expression of MBP, an eosinophil-specific granule protein, and p-TrkA showed that several eosinophils (based on MBP expression) in the peribronchial region were positive for expression of p-TrkA (Fig. 1C, white arrows), thus demonstrating that TrkA in eosinophils is activated under inflammatory conditions. In addition, cells positive for p-TrkA but not MBP were also detectable (Fig. 1C, yellow arrows), indicating that TrkA is also activated in other inflammatory cells recruited to the inflamed lungs.

Eotaxin-1 activates TrkA in eosinophils

Eotaxin-1 is a major chemoattractant for eosinophils during allergic inflammation (19). Because eotaxin-1 levels are elevated in the lungs of *A. alternata*–challenged mice (30) and TrkA in eosinophils in the inflamed lungs of *A. alternata*–challenged mice is activated (Fig. 1C), we examined whether eotaxin-1 can activate TrkA in eosinophils and thereby regulate eosinophil recruitment. Eosinophils cultured from the BM of WT mice were treated with eotaxin-1 and examined for activation of TrkA by immunofluorescence staining (i.e., p-TrkA expression). Relative to untreated cells, eosinophils exposed to eotaxin-1 exhibited increased

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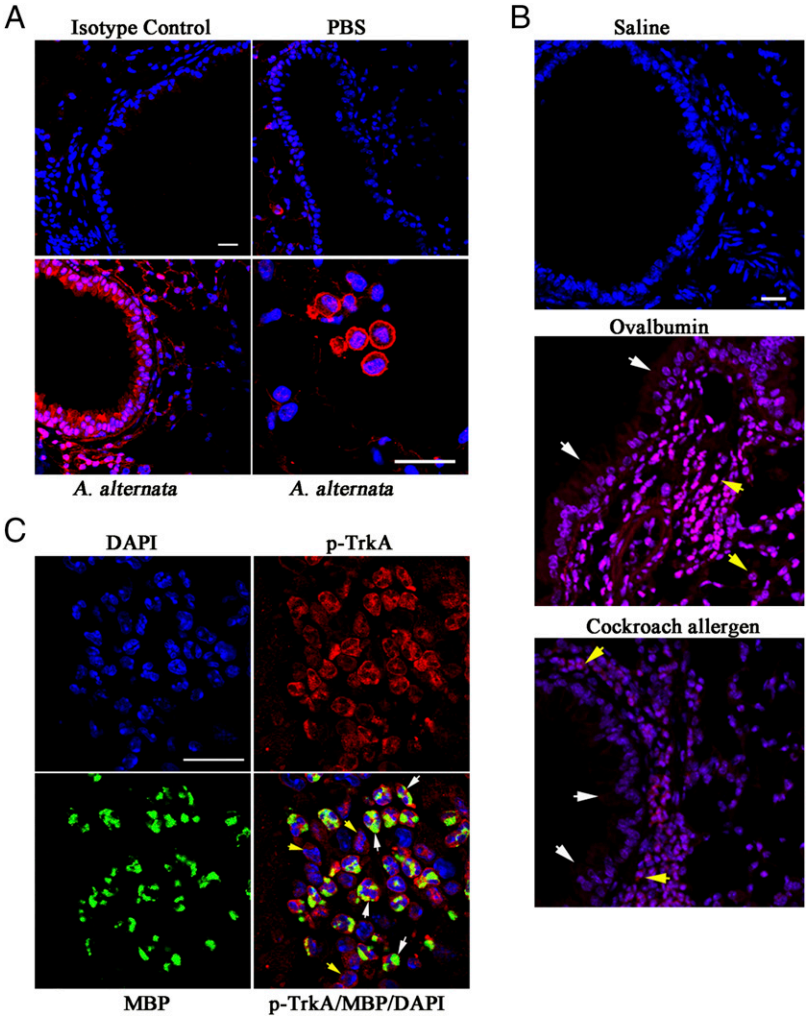

FIGURE 1. Allergen-exposure leads to activation of TrkA in the lungs. (**A**) Expression of p-TrkA in the airway epithelium (lower left panel) and inflammatory cells (lower right panel) of WT mice exposed to *A. alternata* challenge or PBS (control, upper right panel) after IHC with anti–p-TrkA. IHC of lung sections from allergen-challenged mice with a control Ab (upper left panel) is shown as a negative control. (**B**) Expression of p-TrkA in the airway epithelium (white arrows) and inflammatory cells (yellow arrows) of WT mice exposed to saline alone or challenged with OVA or CRA after IHC staining with anti–p-TrkA. (**C**) Dual-immunostaining of lung sections from *A. alternata*–exposed WT mice with Abs against eosinophil-specific MBP (green) and p-TrkA (red). Yellow arrows, cells positive for p-TrkA only; white arrows, cells positive for p-TrkA and MBP. Scale bars, 20 μm. Data representative of three mice per group are shown in (A)–(C).

expression of p-TrkA (Fig. 2A), similar to that noted with NGF, a well-known ligand of TrkA (Supplemental Fig. 3A). This finding was confirmed by Western blot analysis. WT eosinophils showed two immunoreactive bands with the molecular mass of ~85 kDa and ~60 kDa with p-TrkA Ab, which increased in intensity after eotaxin-1 treatment. The expression level of t-TrkA (~110 kDa), in contrast, remained similar in the two groups (Fig. 2B, upper and lower panels).

Because of the limited drug specificity of known TrkA inhibitors, we used a genetic-chemical approach to further examine the role of eotaxin-1–induced TrkA activation. In these studies, eosinophils cultured from BM of TrkA-KI mice that have a point mutation in the ATP-binding pocket, allowing specific inhibition of TrkA kinase activity by an ATP analogue such as 1-NM-PP1, (21) were used. Treatment with eotaxin-1 or NGF activated TrkA in TrkA-KI eosinophils exhibiting two p-TrkA mAb immunoreactive bands of ~85 kDa and ~60 kDa by Western blot analysis, similar to that observed in WT eosinophils (Supplemental Fig. 3B). Incubation of eotaxin-1–treated eosinophil lysates with the primary Ab in the presence of a p-TrkA Ab–blocking peptide markedly decreased the intensity of the 85- and 60-kDa bands, demonstrating the specificity of the bands detected by the Ab (Supplemental Fig. 3C). Additionally, immunofluorescence staining revealed that relative to untreated cells, TrkA-KI eosinophils exhibit increased expression of p-TrkA when exposed to eotaxin-1, thus confirming that eotaxin-1 activates TrkA in eosinophils and demonstrating that the point mutation in TrkA in TrkA-KI eosinophils does not alter its ability to undergo phosphorylation (Fig. 2C, lower panels). Expression of t-TrkA was similar in control and eotaxin-1–treated cells (Fig. 2C, upper panels). Further, treatment of TrkA-KI eosinophils with 1-NM-PP1 prior to eotaxin-1 inhibited TrkA activation compared with cells pretreated with DMSO, the vehicle control, based on reduced expression of p-TrkA by immunofluorescence staining (Fig. 2D). Next, because metalloproteases have been shown to play a role in the activation of TrkA through cleavage in neuronal cells (31), we examined whether TrkA activation in eosinophils is mediated by metalloproteases. Eosinophils were treated with the potent broad-spectrum MMP inhibitor BB-94 before exposure to eotaxin-1 and analyzed for p-TrkA expression by immunofluorescence staining. Cells pretreated with BB-94 showed markedly decreased p-TrkA expression after eotaxin-1 treatment compared with cells treated with vehicle control (Fig. 2E). Further, on Western blots, decreased generation of the ~85 and ~60 kDa p-TrkA immunoreactive bands was noted in eosinophils pretreated with BB-94 relative to vehicle before eotaxin-1 exposure (Supplemental Fig. 4A). In contrast, cells pretreatment with a pan-caspase inhibitor Z-VAD-FMK or PMSF did not inhibit eotaxin-1–induced TrkA activation (Supplemental Fig. 4B). These findings indicate that activation of TrkA by eotaxin-1 involves MMPs.

TrkA regulates eosinophil adhesion and migration

Given the role of TrkA in migration of neuronal (32) and nonneuronal cells (18, 33), we examined whether TrkA signaling

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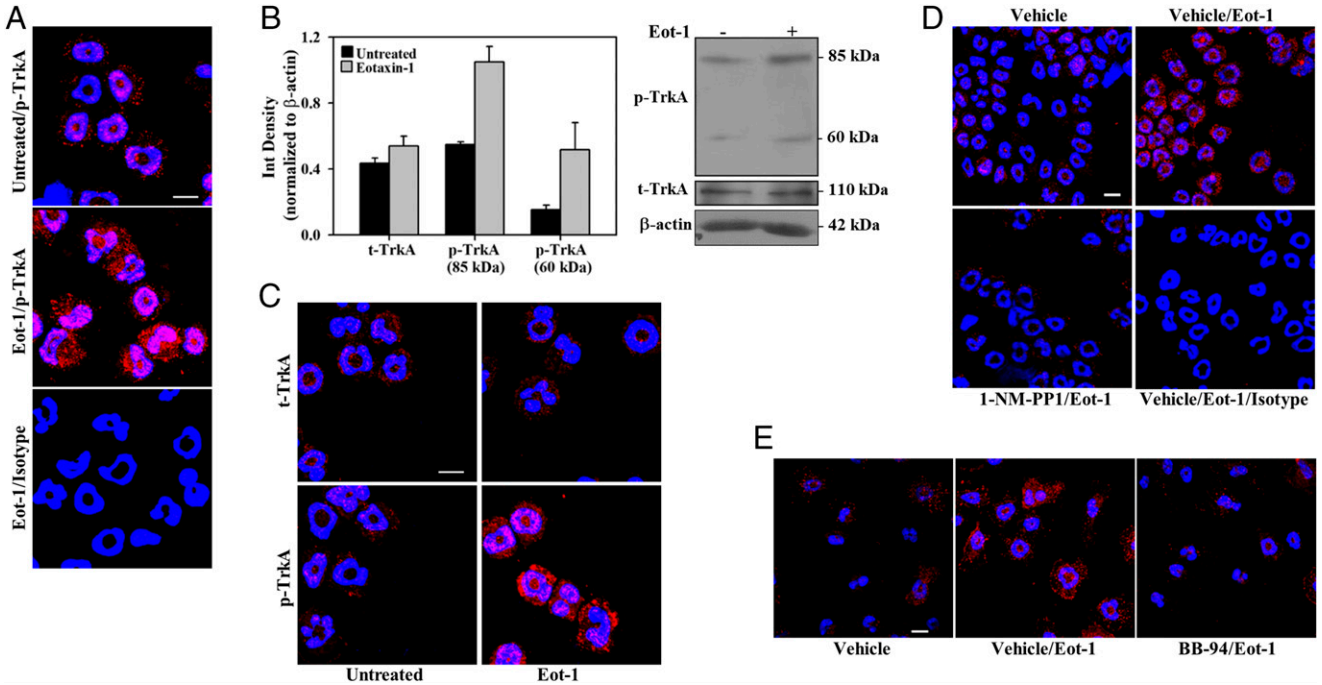

FIGURE 2. Activation of TrkA by eotaxin-1 in murine eosinophils is MMP-mediated. (**A**) Expression of p-TrkA in eotaxin-1–treated and control (untreated) BM eosinophils from WT mice after immunofluorescence staining with anti–p-TrkA. (**B**) Expression of p- and t-TrkA in eotaxin-1–treated (5 min) and control WT BM eosinophils by Western blot analysis followed by densitometric analysis of protein bands. Combined data (mean ± STD) of two out of four experiments with similar results are shown. A representative blot is shown below graph. (**C**) p- and t-TrkA expression in eosinophils from TrkA-KI mice after exposure to eotaxin-1 by immunofluorescence staining. (**D**) Expression of p-TrkA in TrkA-KI eosinophils pretreated with 1-NM-PP1 (10 μM) or vehicle (DMSO) and then exposed to eotaxin-1 (100 nM). (**E**) Effect of the broad-spectrum MMP inhibitor BB-94 versus vehicle on eotaxin-1–induced activation of TrkA by immunofluorescence staining. Scale bars, 10 μm. Representative data of experiments repeated at least three times with similar results using eosinophils from different mice are shown in (A)–(E).

is involved in eotaxin-1–induced eosinophil migration. Because the endothelial adhesion molecule VCAM-1 supports eosinophil trafficking (rolling and stable adhesion) in vivo (34) prior to transmigration, we first evaluated the ability of TrkA-KI eosinophils pretreated with 1-NM-PP1 or DMSO to adhere to VCAM-1. No statistically significant difference was noted between 1-NM-PP1– and vehicle-treated cells in the number of adherent cells on VCAM-1 (Fig. 3A, left panel). Whereas majority of the adherent control cells retained a compact round cell body with F-actin localized at the cell periphery, most of the adherent 1-NM-PP1–treated cells exhibited increased actin polymerization and augmented spreading (Fig. 3A, right panel and Fig. 3B). To determine whether the increased spreading noted in 1-NM-PP1–treated cells was due to changes in expression of cell surface adhesion molecules, 1-NM-PP1– or vehicle-treated cells were analyzed for expression of CD49d, CD11a, CD11b, L-selectin, or CD18 (Fig. 3C). No difference in expression of these molecules was noted between the two groups. In addition to altered cell spreading on VCAM-1, treatment with 1-NM-PP1 strongly inhibited migration of TrkA-KI eosinophils toward eotaxin-1 compared with DMSO-treated cells (Fig. 3D). Because MMPs play a role in eotaxin-1–induced activation of TrkA (Fig. 2E), we examined the effect of BB-94 independently and in combination with 1-NM-PP1 on eotaxin-1–induced migration of TrkA-KI eosinophils. Although both 1-NM-PP1 and BB-94 significantly inhibited migration independently, an additive inhibitory effect was noted when 1-NM-PP1 and BB-94 were used in combination, which was significantly higher than 1-NM-PP1 alone. Expression level of the eotaxin-1 receptor CCR3 was unaffected by 1-NM-PP1 treatment at baseline and in the presence of eotaxin-1 (Fig. 3E), suggesting that the level of expression of CCR3 is not regulated by TrkA.

Blockade of TrkA activation inhibits eotaxin-1–induced calcium flux, cytoskeletal changes, and ERK1/2 activation

We examined the effect of 1-NM-PP1 on signaling events downstream of CCR3. Eotaxin-1 is known to induce calcium flux in eosinophils (35). Baseline (unstimulated) $[Ca^{2+}]_i$ levels were similar in 1-NM-PP1– and DMSO-treated eosinophils by real-time digital video fluorescence imaging (Fig. 4A). In response to eotaxin-1, DMSO-treated eosinophils demonstrated a surge in free $[Ca^{2+}]_i$ within a few seconds, followed by a decline. In contrast, $[Ca^{2+}]_i$ levels in 1-NM-PP1–treated cells remained similar to baseline levels even after eotaxin-1 treatment. Both groups of cells demonstrated a similar response to ionomycin, indicating that Ca^{2+} release, as such, is not affected and that it is the eotaxin-1–induced Ca^{2+} response that is abrogated by TrkA inactivation. Ca^{2+} signaling in response to cell activation is known to play a role in cell migration through cytoskeletal reorganization (36). Kinetics of cytoskeletal changes in TrkA-KI eosinophils in response to cell activation (i.e., eotaxin-1) was examined by confocal microscopy based on phalloidin binding (indicative of F-actin polymerization). In the absence of eotaxin-1, vehicle-treated cells exhibited a diffused pattern of F-actin in the cytoplasm. Upon exposure to eotaxin-1 for 2 min, most cells showed increased phalloidin staining at one end of the cell along with formation of distinct lamellipodia and filopodia. After 10 min, most cells continued to show increased phalloidin binding at one end of the cell but were of irregular shape. In contrast, 1-NM-PP1–treated cells showed increased baseline phalloidin binding mostly around the nucleus and were more spread out even in the absence of

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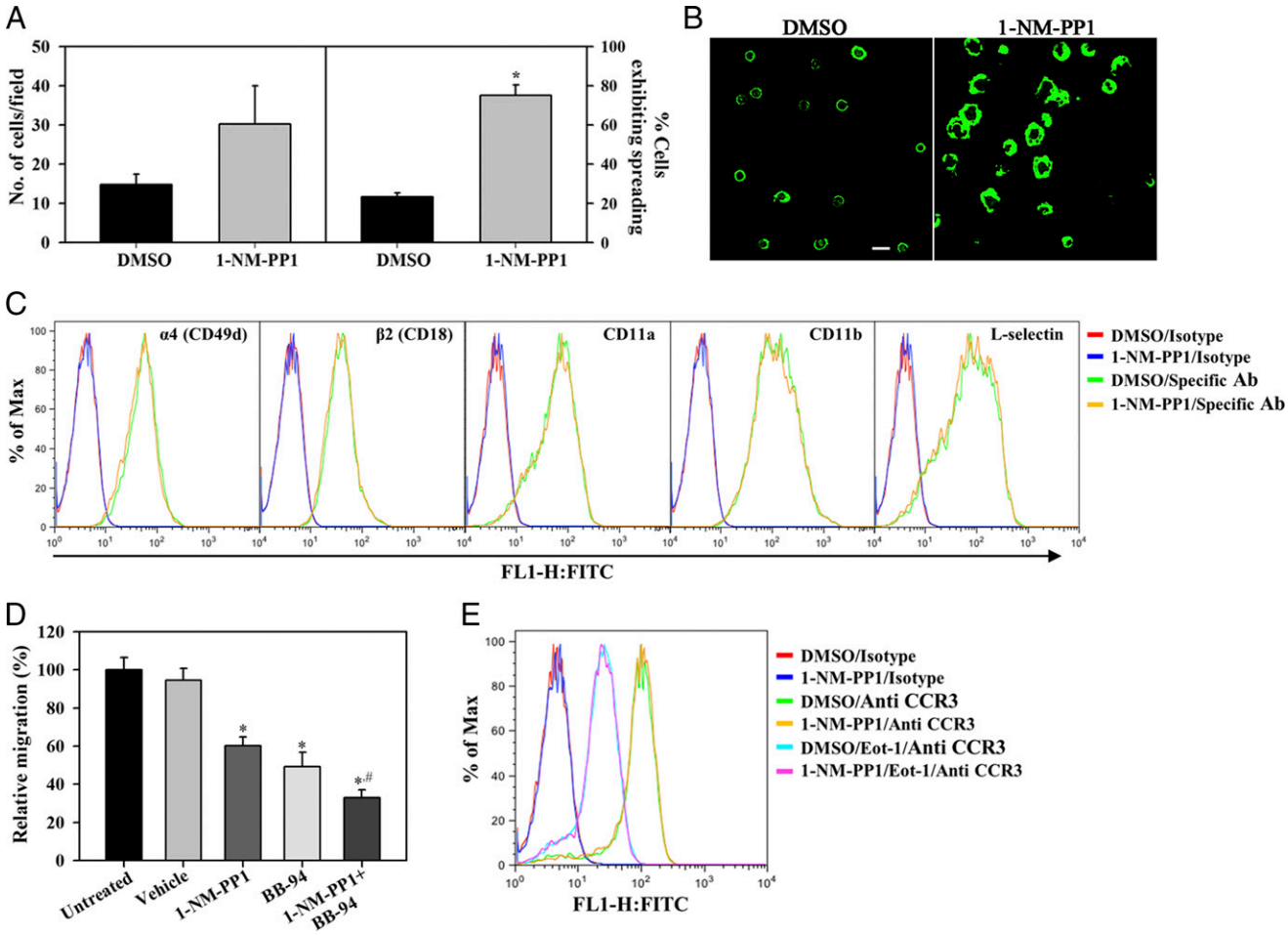

FIGURE 3. Activation of TrkA promotes eosinophil migration. (**A** and **B**) Adhesion and morphological change (cell spreading) in TrkA-KI eosinophils adherent on rmVCAM-1–coated coverslips after treatment with 1-NM-PP1 (10 μM, 20 min). Scale bar, 20 μm. (**C**) Effect of 1-NM-PP1 on expression of cell adhesion molecules in TrkA-KI eosinophils by flow cytometry. (**D**) In vitro migration of TrkA-KI eosinophils toward eotaxin-1 (100 nM) across membranes in transwell chambers after pretreatment with 1-NM-PP1 and BB-94 (both at 10 μM) independently as well as in combination or with vehicle. (**E**) Effect of 1-NM-PP1 on CCR3 expression in TrkA-KI eosinophils with or without exposure to eotaxin-1 (5 min) by flow cytometry. Combined data (mean ± SEM) of three-four experiments in duplicate are shown in (A) and (D). Representative data of three independent experiments with eosinophils from three different mice are shown in (B), (C), and (E). $^{*}p < 0.01$ in (A) and (D) for comparison with vehicle-treated cells, $^{\#}p < 0.05$ in (D) for comparison with 1-NM-PP1–treated cells.

eotaxin-1. After 2 min of exposure to eotaxin-1, cells continued to exhibit increased phalloidin binding around the nucleus and at the periphery but with irregular spreading lacking clear lamellipodia and filopodia formation and acquiring a multipolar shape. After 10 min of exposure to eotaxin-1, most of the 1-NM-PP1–treated cells showed diffused phalloidin binding and less spreading relative to non–eotaxin-treated cells (Fig. 4B). Thus, blockade of TrkA activation with 1-NM-PP1 appears to result in elevated F-actin levels and irregular kinetics of F-actin polymerization/depolymerization resulting in altered cytoskeletal changes relative to control cells upon stimulation with eotaxin-1.

The Rho family of small GTPases (Rac1, RhoA, and Cdc42) plays an important role in cellular actin organization and cytoskeletal changes (37), with Cdc42 being involved in regulating cell polarity (38). Changes in expression of Cdc42 in 1-NM-PP1–treated TrkA-KI eosinophils after exposure to eotaxin-1 (2 min) were examined by immunofluorescence staining. A larger number of vehicle-treated cells showed Cdc42 expression localized at one end of the cell at the cell periphery relative to cells pretreated with 1-NM-PP1 in which Cdc42 expression was mostly noted in the center of the cell with minimal staining at the cell periphery (Fig. 4C, left and right panels). These findings suggest that blockade of TrkA activation interferes with rearrangement/redistribution of Cdc42. Finally, activation of ERK1/2, an important signaling event in eotaxin-1–exposed eosinophils that is required for cell migration in vitro (39) and recruitment in vivo (40), was strongly inhibited in TrkA-KI eosinophils treated with 1-NM-PP1 relative to vehicle-treated cells (Fig. 4D). Overall, these data indicate that TrkA signaling is essential for eotaxin-induced migration of eosinophils.

1-NM-PP1 inhibits allergen-induced cellular airway inflammation

To evaluate the role of TrkA in regulating airway eosinophil recruitment in vivo and determine whether specific blockade of TrkA signaling can inhibit the development of airway inflammation, we used an experimental model of allergic asthma wherein TrkA-KI mice were challenged with *A. alternata* (22) and administered with 1-NM-PP1 or left untreated as outlined in (Fig. 5A). Analysis by immunofluorescence staining demonstrated marked TrkA activation in the airway epithelium and inflammatory cells of lung sections from *A. alternata*–challenged TrkA-KI mice, whereas only minimal activation of TrkA was noted in the lungs of control mice. Activation of TrkA was markedly inhibited in

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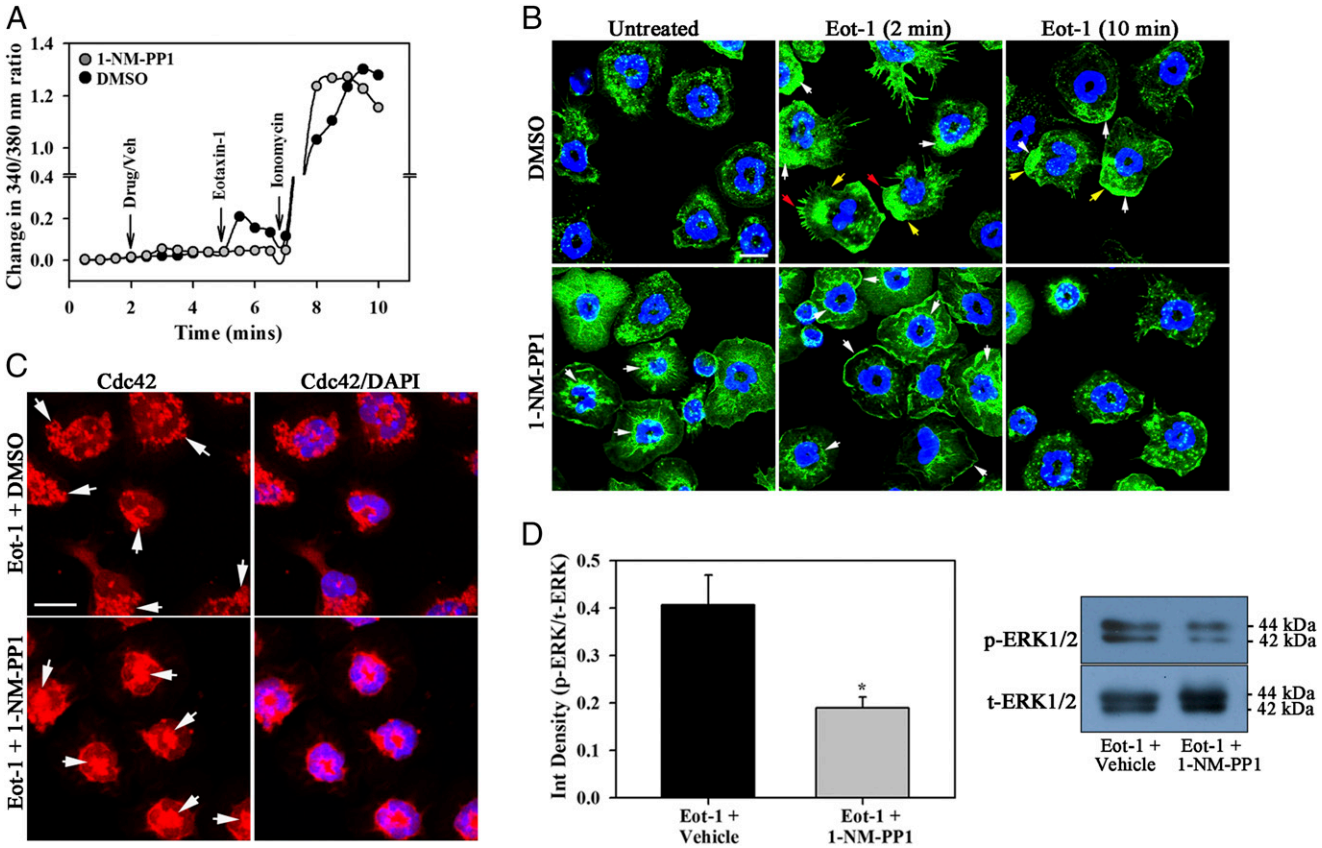

FIGURE 4. Blockade of TrkA activation inhibits eotaxin-1–induced calcium flux, cytoskeletal changes and ERK(1/2) activation in eosinophils. (**A**) $[Ca^{2+}]_i$ levels in eosinophils treated with 10 μM 1-NM-PP1 or vehicle followed by eotaxin-1 (100 nM) and then 2 μM ionomycin (positive control) at the indicated time points by digital video fluorescence imaging using fura-2 AM; >200 cells were analyzed for each treatment. (**B**) FITC-Phalloidin staining of TrkA-KI eosinophils adherent on poly-L-lysine, treated with 1-NM-PP1 (20 min) or vehicle, and exposed to eotaxin-1 for the indicated time points. White arrows, regions of increased phalloidin binding; red arrows, filopodia; yellow arrows, lamellipodia. (**C**) Immunofluorescence staining for Cdc42 expression in adherent TrkA-KI eosinophils treated with 1-NM-PP1 (or vehicle) and then eotaxin-1 as in (B). Arrows indicate Cdc42 expression. (**D**) t-ERK and p-ERK(1/2) expression in eotaxin-1–treated TrkA-KI eosinophils after pretreatment with 1-NM-PP1 or vehicle by densitometric analysis of protein bands after Western blot analysis. A representative blot is shown. Scale bars, 10 μm. Combined data (mean ± SEM) of four experiments are shown in (A) and (D), and data representative of three independent experiments with eosinophils from three different mice are shown in (B) and (C). $*p < 0.05$ in (D) for comparison with vehicle-treated cells.

allergen-exposed TrkA-KI mice treated with three or five doses of 1-NM-PP1 (Alt+1-NM-PP1-3 and Alt+1-NM-PP1-5, respectively) compared with untreated counterparts. t-TrkA expression level in the airway epithelium and inflammatory cells was similar in the lungs of all groups of mice (Fig. 5B). Thus, TrkA-KI mice exhibit activation of TrkA in the lungs in a manner similar to WT mice in response to *A. alternata* challenge (Fig. 1A), and 1-NM-PP1 specifically inhibits this activation. Additionally, whereas untreated allergen-challenged TrkA-KI mice showed significantly increased recruitment of inflammatory cells in the lung tissue based on H&E staining and in the BALF compared with control mice, allergen-challenged TrkA-KI mice administered with 1-NM-PP1 at three or five doses exhibited markedly reduced recruitment of inflammatory cells in the peribronchial area of the lungs (Fig. 5C) and in the BALF (Fig. 5D). Further, in the Alt+1-NM-PP1-5 group, a significantly lower number of eosinophils and neutrophils were present in the BALF relative to untreated mice (Fig. 5E). The number of macrophages and lymphocytes in the BALF were also lower in this group, although the reduction was not statistically significant. The effect of the blockade of TrkA on lung tissue eosinophilia was examined by IHC for the expression of eosinophil-specific MBP. As expected, allergen-challenged TrkA-KI mice showed significantly increased infiltration of lung tissue with eosinophils compared with control mice. Although administration of three doses of 1-NM-PP1 did not have an effect on lung tissue eosinophils, the number of eosinophils in the lung tissue of Alt+1-NM-PP1-5 group was significantly lower than in the untreated *A. alternata*–challenged group (Fig. 5F, 5G). These findings indicate that TrkA signaling plays a significant role in the recruitment of inflammatory cells, specifically eosinophils, to allergic airways, and inhibiting TrkA activation in vivo prevents eosinophil recruitment to allergen-exposed lungs. Eosinophil counts in the peripheral blood of allergen-challenged TrkA-KI mice administered with 1-NM-PP1 were also lower compared with untreated mice (data not shown). Because eotaxin-1 signaling is affected in the presence of 1-NM-PP1, recruitment of generated eosinophils from the BM milieu is also likely to be affected, resulting in low numbers in the circulation.

Effect of 1-NM-PP1 on allergen-induced Th2 cytokines, eotaxins, and IgE

Lung tissue from allergen-challenged TrkA-KI mice with or without 1-NM-PP1 treatment was evaluated for expression of IL-2, IFN-γ, IL-4, IL-5, IL-13, and TNF-α. Allergen challenge significantly induced the level of IL-5 and IL-13 in the lungs. The level of IL-4, in contrast, was only marginally higher than in the control group. Compared with untreated allergen-challenged mice, IL-5 levels were significantly lower in mice treated with 1-NM-PP1 at three or five doses. Although IL-13 levels were also substantially

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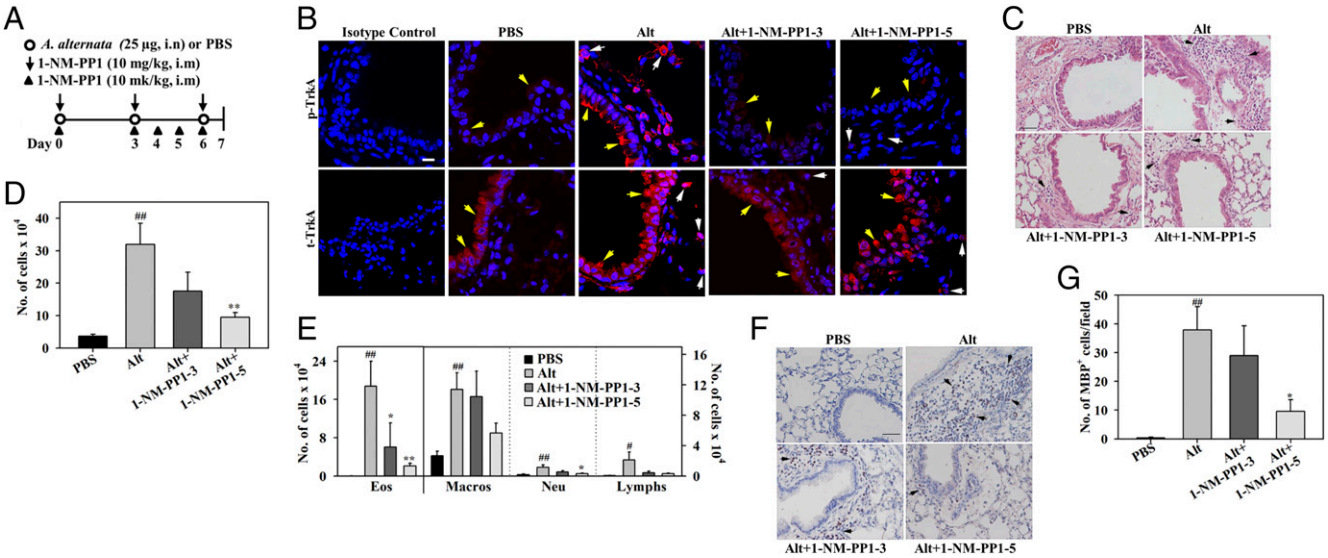

FIGURE 5. TrkA kinase inhibition attenuates allergen-induced airway cellular inflammation. (**A**) Outline of protocol for treatment of *A. alternata*–challenged TrkA-KI mice with 1-NM-PP1. (**B**) t- and p-TrkA expression in lung sections from PBS (control) and *A. alternata*–challenged TrkA-KI mice with or without 1-NM-PP1 treatment. Airway epithelium: yellow arrows; inflammatory cells: white arrows. (**C**) H&E staining of lung sections from mice described in (B). (**D** and **E**) Total cells and differential cell counts in BALF of mice described in (B). (**F** and **G**) Infiltrated eosinophils in lung tissue of mice described in (B) detected by IHC for MBP (stained dark brown, black arrows) and quantitation of MBP-positive cells, respectively. A representative image for each group is shown in (B), (C), and (F). Scale bars, 10 μm in (B) and 50 μm in (C) and (F). Combined data (mean ± SEM) of mice from at least three independent experiments are shown in (D), (E), and (G) [n = 7–12 mice per group in (D), 6–10 mice per group in (E), and 5–7 mice per group in (G)]. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for comparison with control group, $^{*}p < 0.05$, $^{**}p < 0.01$ for comparison with untreated *A. alternata*–challenged group. i.n.; intranasal.

lower in both 1-NM-PP1–treated groups, a statistically significant reduction was noted only in allergen-challenged mice that received five doses of 1-NM-PP1. Although TNF-α expression was not induced by *A. alternata* challenge, the level of this cytokine in 1-NM-PP1–treated mice (three or five doses) tended to be lower than in untreated mice (Fig. 6A). IL-2 and IFN-γ levels were below detection limit. In addition to reducing levels of Th2 cytokines, administration of 1-NM-PP1 at three or five doses significantly inhibited IL-33 levels in the lungs of *A. alternata*–challenged mice (Fig. 6B).

Eotaxin-1 and -2 are predominant chemokines responsible for recruitment of eosinophils to allergic airways (19). Because eosinophil infiltration in the lungs of 1-NM-PP1–treated allergen-challenged mice was significantly reduced, we examined whether this effect was due to reduced levels of eotaxins in the lungs of these mice. Expression of eotaxin-1 and -2 in the BALF from *A. alternata*–challenged mice with or without 1-NM-PP1 treatment was measured by ELISA. Eotaxin-1 levels in the allergen-challenged mice tended to be higher than in control mice and marginally reduced by 1-NM-PP1 treatment (not statistically significant) (Fig. 6C). Eotaxin-2 levels, in contrast, were significantly higher after allergen challenge. Importantly, blockade of TrkA activation with 1-NM-PP1 at three or five doses significantly reduced the expression of allergen-induced eotaxin-2 levels (Fig. 6C). Because elevated IgE levels are a distinguishing feature of allergic inflammation, we examined the effect of 1-NM-PP1 treatment on allergen-induced IgE levels in the BALF by ELISA. Consistent with the allergic disease phenotype, IgE levels in *A. alternata*–challenged mice were higher than in control mice. A trend in reduction of allergen-induced IgE levels was observed in *A. alternata*–challenged mice treated with five doses of 1-NM-PP1 (Fig. 6D).

1-NM-PP1 attenuates allergen-induced airway structural changes

Allergen exposure leads to structural changes in the airways, such as increased mucus production and airway smooth muscle hyperplasia/hypertrophy, which in turn can contribute to airflow obstruction and AHR (41). We examined whether blockade of TrkA activation alters *A. alternata*–induced AHR. *A. alternata*–challenged mice treated with 1-NM-PP1 at five doses showed improved airway function with a significant reduction in R_L to inhaled methacholine at 50 mg/ml compared with vehicle-treated mice (Fig. 7A). Administration of 1-NM-PP1 to naive mice did not exert any nonspecific effects on R_L to inhaled methacholine, which was similar to that observed in PBS-exposed mice. Based on PAS staining and immunohistology for expression of α-SMA (a regulator of smooth muscle contraction), *A. alternata*–challenged mice exhibited significantly increased airway mucus secretion and smooth muscle thickening, respectively, compared with control mice. Blockade of TrkA activation in the allergen-challenged mice with three or five doses of 1-NM-PP1 significantly reduced allergen-induced airway mucus secretion (Fig. 7B, 7C) and smooth muscle mass (Fig. 7D, 7E) relative to untreated mice. These studies suggest that activation of TrkA promotes structural changes and the development of AHR in allergic airways, and inhibition of this signaling pathway attenuates the development of these changes along with improved airway function.

Discussion

Eosinophilia is the hallmark of airway inflammation in asthma (42). Eosinophils are a source of various cytokines, chemokines, growth factors, and highly cytotoxic cationic granule proteins that are secreted upon activation (43) and known to exert various proinflammatory effects (reviewed in Ref. 44). Thus, identifying key players involved in selective eosinophil recruitment and the specific pathways promoting airway eosinophilic inflammation is critical for the identification of novel therapeutic targets. Previous studies indicate a role for TrkA in experimental models of AAI, albeit in the context of NGF-mediated effects. Studies using blocking Abs or small interfering RNA specific for NGF have

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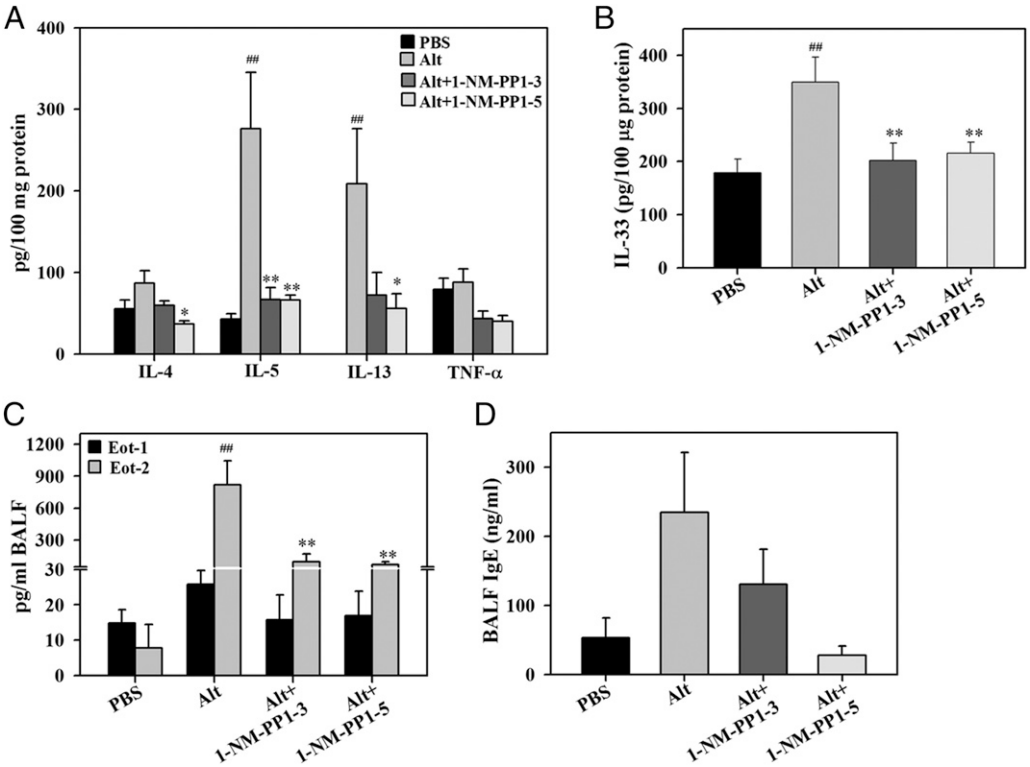

FIGURE 6. Effect of TrkA kinase inhibition on allergen-induced lung cytokines, chemokines, and IgE. (**A**) Th2 cytokine and TNF-α levels in lung lysates from control and *A. alternata*–challenged TrkA-KI mice with or without 1-NM-PP1 treatment. (**B**) IL-33 levels in lung lysates from mice described in (A). (**C**) Eotaxin-1 and -2 levels in BALF of mice described in (A). (**D**) Total IgE levels in BALF of mice described in (A). Combined data (mean ± SEM) of $n = 6$–10 mice for control and allergen-challenged groups and 4–10 mice for 1-NM-PP1–treated groups in (A)–(D) are shown. $^{\#\#}p < 0.01$ for comparison with control group, $^{*}p < 0.05$, $^{**}p < 0.01$ for comparison with untreated *A. alternata*–challenged group.

demonstrated the contributory role of NGF (reviewed in Ref. 10) and TrkA (45) in the development of experimental allergic asthma, but the importance of TrkA receptor signaling or its functional mechanism by targeted inactivation of TrkA kinase activity was not established. In the current study, we have used a combined genetic-chemical approach [TrkA-KI mouse strain (21)] to specifically control the TrkA kinase activity and established a distinct role for TrkA signaling in eosinophil recruitment in vitro and in vivo.

Various cells in the lung, including eosinophils, bronchial and alveolar epithelial cells, smooth muscle cells, and vascular endothelial cells, express TrkA (8). In the current study, we found that exposure to not only the fungal allergen *A. alternata* but also allergens such as OVA and CRA leads to activation of TrkA in the airway epithelium and in inflammatory cells recruited to the lungs of WT mice. Although TrkA activation and signaling mediated by NGF is extensively characterized in neuronal cells (46), its activation in other cell types, specifically eosinophils, is less investigated. TrkA is expressed by human peripheral blood eosinophils (28), and its expression on BALF eosinophils is upregulated after allergen challenge (47). Along these lines, in the current study, eosinophils recruited to the inflamed lungs of *A. alternata*–exposed mice exhibited activation of TrkA. Eotaxin-1, the predominant chemokine contributing to airway eosinophil recruitment, is elevated in the lungs of humans with allergic asthma (48) and in animal models of AAI (49), including *A. alternata*–induced models (30). In the current study, WT BM-derived eosinophils exposed to eotaxin-1 showed rapid (5 min) activation of TrkA by immunofluorescence staining, a finding that has hitherto not been reported. Additionally, on Western blots, whereas expression level of t-TrkA remained unaltered after eotaxin-1 treatment, showing a single protein band of ~110 kDa, increased intensity of two bands of molecular mass of ~85 and ~60 kDa immunoreactive for p-TrkA (confirmed by Ab-blocking peptide studies) was noted. This observation was confirmed in eosinophils from TrkA-KI mice wherein 1-NM-PP1, an ATP analogue that selectively inhibits TrkA activation in these mice, prevented eotaxin-1–induced activation of TrkA in immunofluorescence staining studies. Overall, these findings identify eotaxin-1 as a new player involved in TrkA activation.

TrkA signaling initiated by direct binding of ligands such as NGF involves auto-phosphorylation of specific tyrosine residues located intracellularly in the kinase domain of the receptor, which then creates docking sites for effector proteins that initiate activation of various intracellular signaling pathways (1). However, studies have shown that TrkA can be transactivated by ligands of the GPCR family of transmembrane receptors even in the absence of NGF via second messengers or receptor colocalization (16–18). In this context, studies have suggested a role for MMPs, specifically MMP-9, in inducing activation of TrkA upon agonist/ligand binding to associated GPCRs (reviewed in Ref. 50). Because eotaxin-1 exerts its activity on eosinophils via the GPCR CCR3 (19), we investigated whether MMPs play a role in eotaxin-1–induced activation of TrkA. In eosinophils pretreated with a pan-MMP inhibitor (BB-94), but not with a pan-caspase inhibitor or PMSF, eotaxin-1 failed to activate TrkA, suggesting that eotaxin-1–induced activation of TrkA is MMP-dependent. Eotaxin-1 has been shown to induce expression of MMPs in eosinophils (51) and other cell types (52, 53). In neuronal cells, MMPs can cleave the ectodomain of TrkA, generating cell-bound, tyrosine phosphorylated, signaling-competent fragments (31). Furthermore, TrkA has many predicted MMP cleavage sites in the extracellular and intracellular regions

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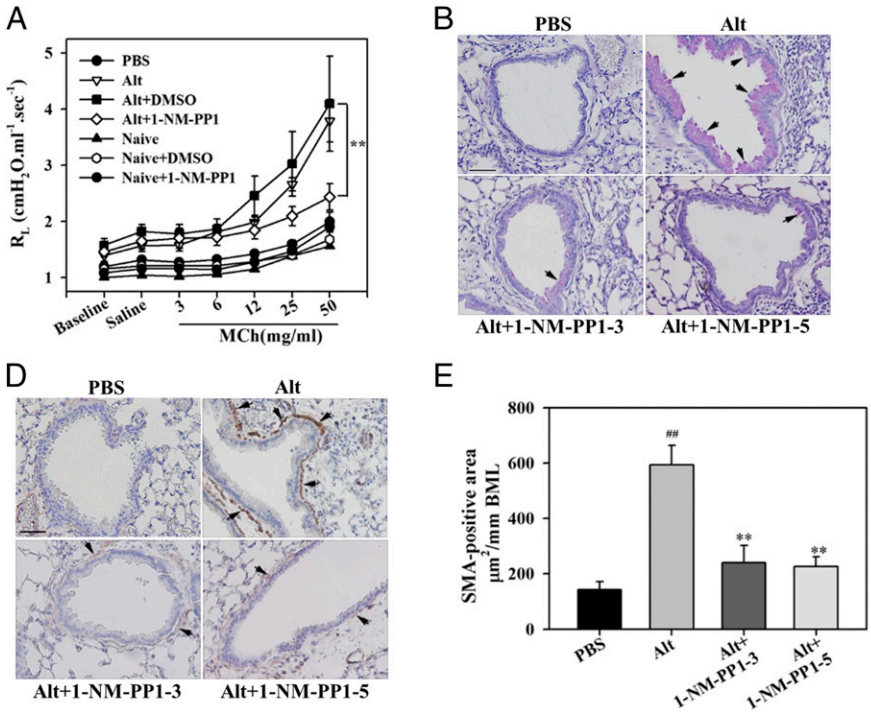

FIGURE 7. TrkA kinase inhibition attenuates allergen-induced AHR, mucus secretion, and smooth muscle thickening. (**A**) Measurement of AHR following exposure to increasing concentrations of aerosolized methacholine (MCh) in mechanically ventilated control and *A. alternata*–challenged TrkA-KI mice that were untreated or treated with 1-NM-PP1 (five doses) or vehicle. (**B** and **C**) Airway mucus secretion in control and *A. alternata*–challenged TrkA-KI mice with or without 1-NM-PP1 treatment assessed by PAS staining (stained dark-pink, black arrows) and quantitation of PAS-positive area, respectively. (**D** and **E**) Airway smooth muscle thickening in mice described in (B) assessed by IHC for α-SMA expression (stained brown, black arrows) and quantitation of α-SMA-positive area, respectively. Representative image for each group is shown in (B) and (D). Scale bars, 50 μm. Combined data (mean ± SEM) of n = 5–7 mice per group in (A) and 6–7 mice per group in (C) and (E) are shown. $^{\#\#}p < 0.01$ for comparison with control group, $^{**}p < 0.01$ for comparison with untreated *A. alternata*–challenged group.

based on CleavPredict (http://cleavpredict.sanfordburnham.org), a platform for substrate cleavage prediction for MMPs (54). Our observation of two p-TrkA immunoreactive protein bands in eotaxin-1–treated cells suggestive of cleaved fragments together with blockade of TrkA activation by an inhibitor of MMPs, indicates that eotaxin-1–induced activation of TrkA in eosinophils is likely to be mediated by MMPs.

Studies have shown that TrkA signaling is involved in regulating NGF-driven migration of neuronal and nonneuronal cells (32, 33) as well as non–NGF-mediated migration via GPCR transactivation (18). Adhesion to the vascular endothelium and chemoattractant-induced migration are key steps involved in eosinophil trafficking and recruitment to sites of inflammation (55). In the current study, blockade of TrkA signaling with 1-NM-PP1 did not alter the number of eosinophils that adhered to VCAM-1 in a statistically significant manner but induced striking changes in cell shape. A large number of 1-NM-PP1–treated cells demonstrating exaggerated spreading and increased actin polymerization, although expression level of several cell surface adhesion molecules, including CD49d (the counter receptor for VCAM-1), remained unaffected. Despite the enhanced spreading on VCAM-1, inhibition of TrkA activation significantly reduced eotaxin-1–induced migration. Additionally, MMP inhibitor BB-94 independently, and additively with 1-NM-PP1, reduced eosinophil migration, further supporting a role for MMPs in eotaxin-1–induced activation of TrkA. Expression level of CCR3 in the absence or presence of eotaxin-1 remained unaltered by blockade of TrkA activation. Thus, inactivation of TrkA signaling appears to inhibit migration by impeding cell motility via regulation of signaling events downstream of CCR3 rather than by enhanced adhesion or receptor expression level.

Eotaxin-1 induces a calcium flux in eosinophils, and impaired Ca^{2+} signaling in eosinophils in response to eotaxin-1 is associated with altered actin polymerization and reduced migration (24, 26). In the current study, inhibition of TrkA signaling specifically blocked eotaxin-1–induced Ca^{2+} flux. Studies have previously shown that TrkA participates in NGF-induced Ca^{2+} signaling in other cell types (56). Cell polarization and migration are coordinated by Ca^{2+} signaling via regulation of integrin activation and modulation of actin dynamics (i.e., coordinated polymerization and depolymerization of F-actin) through activation of signaling molecules that interact with actin (PKC and calmodulin-dependent kinases) (36). In support of this, along with inhibition of agonist-induced $[Ca^{2+}]_i$, blockade of TrkA signaling with 1-NM-PP1 resulted in altered F-actin polymerization and failure of cells to form distinct lamellipodia and filopodia. Given the role played by the Rho family of small GTPases in regulating the actin cytoskeleton (37) and specifically that of Cdc42 in triggering filopodia formation and steering cell polarity during migration (38, 57), we examined whether TrkA regulates Cdc42 expression. Consistent with the inability of 1-NM-PP1–treated eosinophils to form clear lamellipodia and filopodia after activation with eotaxin-1, translocation of Cdc42 to the leading edge was abrogated in these cells in contrast to control cells with Cdc42 expression largely localized to the peri-nuclear region or dispersed all over the cell. Another signaling pathway activated by CCR3 stimulation in human and mouse eosinophils is the ERK(1/2) MAPK pathway (24, 58). ERK(1/2) activation is an important signaling event necessary for eotaxin-1–induced shape change and migration of eosinophils (39, 59). Further, in the context of NGF stimulation, TrkA is known to engage ERK(1/2) downstream to promote various events during neuronal development (1). In our

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study, inhibition of TrkA signaling significantly reduced activation of ERK(1/2) in response to eotaxin-1. Taken together, these studies suggest that TrkA signaling participates in eosinophil migration by regulating the actin cytoskeleton and cell polarization via engagement of other intracellular signaling molecules such as $[Ca^{2+}]_i$, Cdc42 and ERK(1/2).

Once recruited to allergic airways, eosinophils play a critical role in the development and maintenance of allergic asthma by participating in events such as Ag presentation and release of stored proinflammatory mediators (cytokines, chemokines, reactive oxygen species, lipid mediators, granule proteins) (60). Therefore, strategies to inhibit airway eosinophil recruitment and suppress Th2 cytokines to mitigate asthma symptoms remain the focus of active research (61). Consistent with our findings that blockade of TrkA signaling inhibits eosinophil migration in vitro, administration of 1-NM-PP1 to TrkA-KI mice significantly inhibited recruitment of inflammatory cells, specifically eosinophils, to the airways and in the lung tissue in a model of *A. alternata*–induced AAI. Along with the reduced eosinophilia, 1-NM-PP1–treated mice displayed significantly lower levels of allergen-induced Th2 cytokines (IL-5, IL-4, and IL-13), IL-33, IgE, and eotaxin-2 in the lungs. In addition to eosinophils, lymphocyte recruitment to the lungs was lower in 1-NM-PP1–treated mice, which is likely to be in part responsible for the decreased levels of these Th2 cytokines. Because TrkA is expressed by many cell types in the lung in addition to eosinophils (e.g., bronchial and alveolar epithelial cells, smooth muscle cells, and vascular endothelial cells), in vivo administration of 1-NM-PP1 can target TrkA signaling that is ligand-induced (via mediators such as NGF) or by transactivation (via mediators such as eotaxin-1) in all these cell types. In the current study, exposure of mice to *A. alternata* resulted in activation of TrkA in airway epithelial cells, which was markedly inhibited by 1-NM-PP1. Airway epithelial cells contribute to the development of AAI through release of various proinflammatory mediators including IL-33 (62), a Th2 phenotype–promoting and –maintaining cytokine (through IL-5 and IL-13 production by Th2 cells and innate lymphoid cells) (63), and eotaxin-2 (in response to IL-4 and IL-13) (64), both of which were significantly inhibited in *A. alternata*–challenged mice treated with 1-NM-PP1. Reduced levels of IL-33 in 1-NM-PP1–treated *A. alternata*–challenged mice may additionally be responsible for lower Th2 cytokine levels in these mice. Studies have also shown that eotaxin-2 cooperates with IL-5 in regulating recruitment of eosinophils, which in turn can serve as an important source of IL-13 to induce AHR (65). Thus, blockade of TrkA signaling in vivo not only inhibits eosinophilic inflammation by impeding eosinophil motility and decreased eotaxin-2 levels but also results in a muted Th2 response.

AHR is a characteristic feature of allergic asthma. Increased airway smooth muscle mass and mucus hypersecretion, which are recognized features in allergic asthma, are known to contribute to the development of AHR (66, 67). In the current study, *A. alternata*–challenged mice treated with 1-NM-PP1 had significantly decreased AHR associated with reduced airway smooth muscle mass and mucus secretion relative to untreated allergen-challenged mice. Studies have shown that activation of TrkA signaling promotes human airway smooth muscle cell proliferation (68) and smooth muscle cells can be a source of eotaxins during asthma (69). Mediators such as granule proteins (e.g., MBP, eosinophil peroxidase), cytokines (e.g., IL-13), and leukotrienes released by recruited eosinophils upon activation are known to directly or indirectly (via activation of mast cells and basophils) cause AHR and induce mucus hypersecretion (reviewed in Ref. 70). Thus, in addition to eosinophils, specific blockade of TrkA signaling during allergic asthma can target other cell types, such as smooth muscle cells and epithelial cells, resulting in attenuation of various features of airway inflammation.

In summary, we have established the functional role of TrkA signaling in AAI in a disease model by demonstrating that specific inactivation of TrkA signaling significantly diminishes the cardinal features of asthma such as airway eosinophilia, Th2 cytokine and eosinophil-specific chemokine levels, structural changes in the airways, and AHR. Importantly, to our knowledge, our studies have uncovered a novel mechanism of TrkA activation and function in eotaxin-mediated signaling and migration of eosinophils. Together these studies further advance current knowledge of the role of TrkA signaling in asthma pathogenesis and suggest that manipulation of the TrkA signaling pathway may serve as a therapeutic target in the management of allergic airway disease.

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Disclosures

The authors have no financial conflicts of interest.

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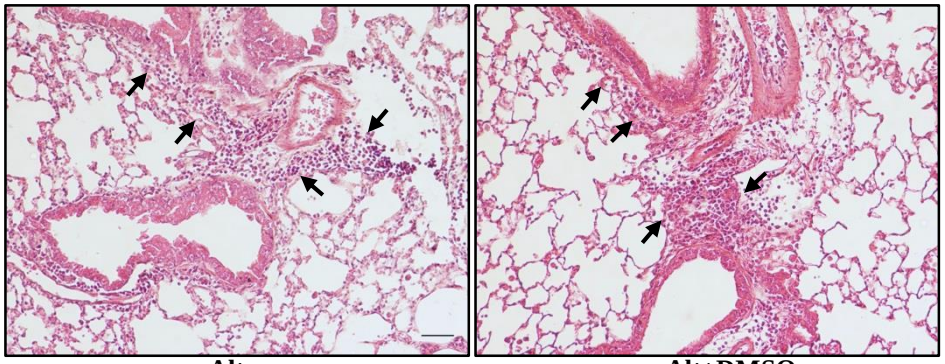
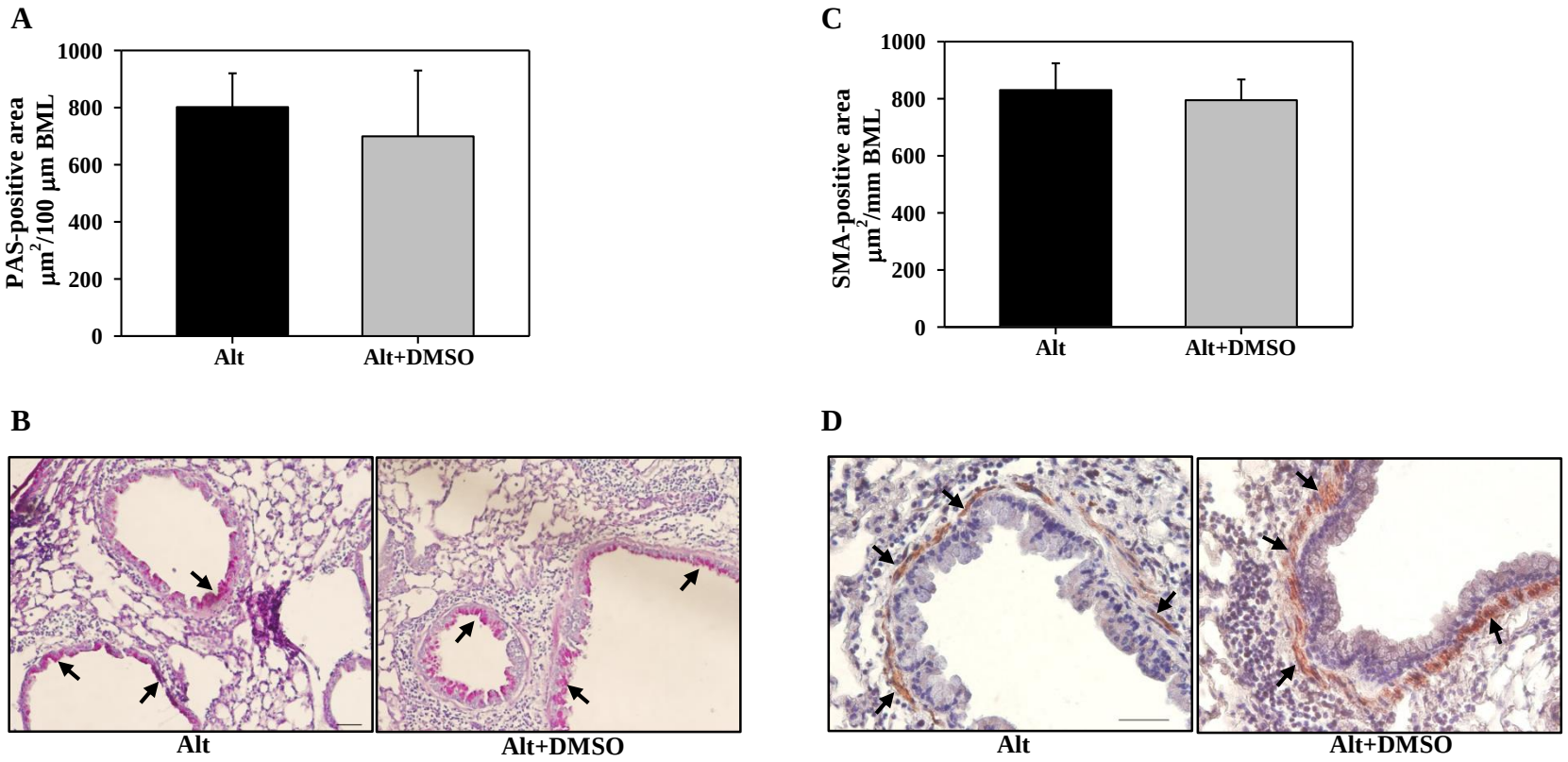
Supplementary Figures



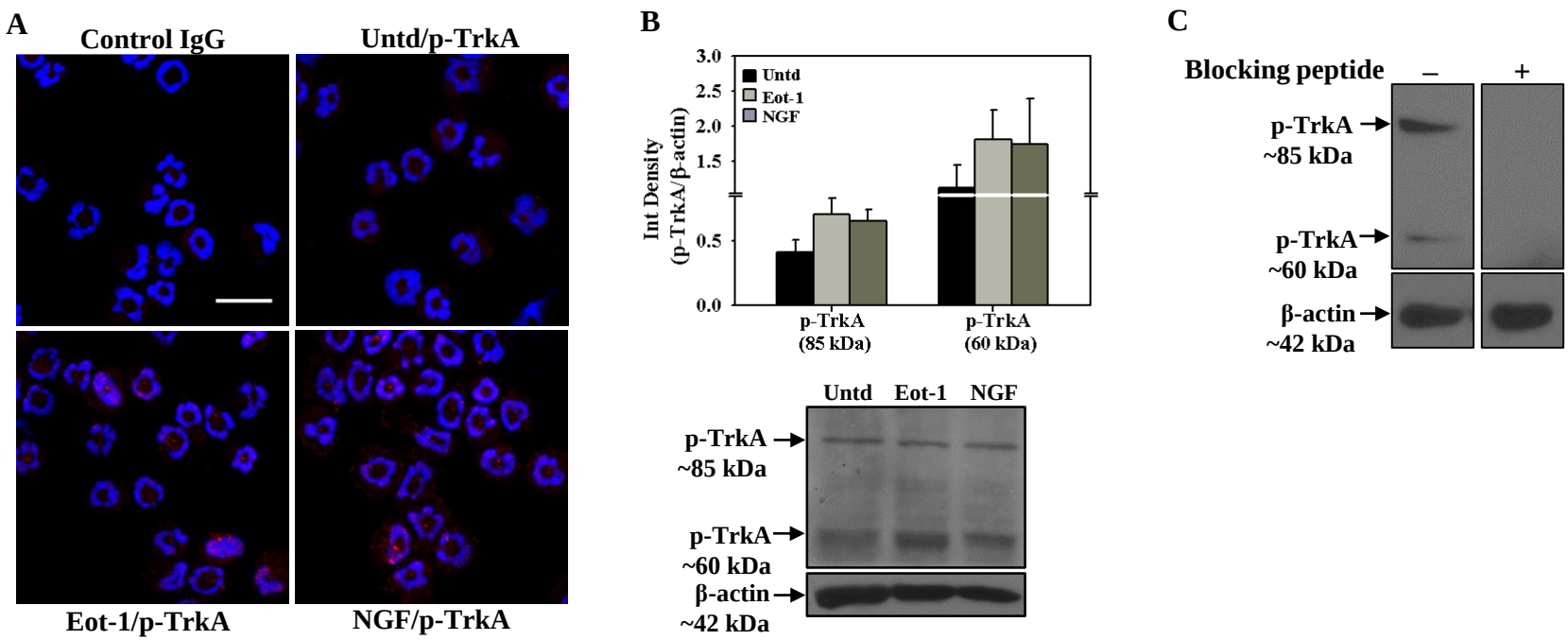
Supplemental Fig. 1 Airway cellular infiltration in *A. alternata*-challenged TrkA-KI mice is not altered by vehicle (DMSO/PBS). TrkA-KI mice were challenged with *A. alternata* as described in the main manuscript. One group of the *A. alternata*-challenged mice were administered DMSO/PBS (vehicle for 1-NM-PP1) on days 0, 3, 4, 5 and 6. Lung sections from both groups were stained with H & E to evaluate allergen-induced cellular infiltration. Data representative of four mice/group is shown. Scale bar, 50 µm. Arrows indicate inflammatory cells.

Supplemental Fig. 1



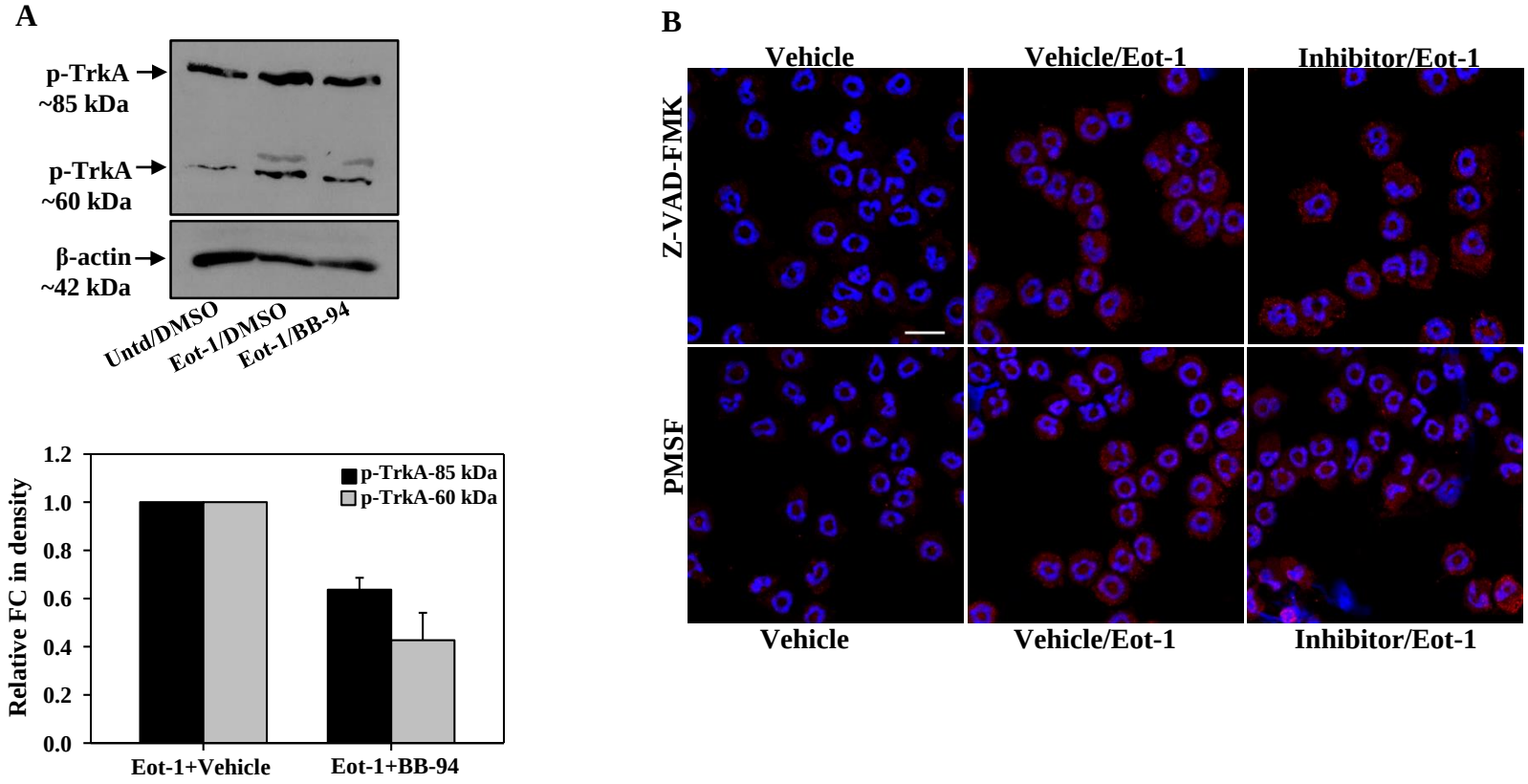
Supplemental Fig. 2 Airway mucus secretion and smooth muscle thickness in *A. alternata*-challenged TrkA-KI mice are not altered by vehicle. TrkA-KI mice challenged with *A. alternata* and administered DMSO/PBS (vehicle for 1-NM-PP1) on days 0, 3, 4, 5 and 6, or left untreated were examined for (A and B) airway mucus secretion and (C and D) smooth muscle hyperplasia as described in the main manuscript. Quantitative data (4-6 mice/group) and representative images are shown. Scale bar, 50 µm. Arrows highlight positive staining.

Supplemental Fig. 2



Supplemental Fig. 3 Activation of TrkA by eotaxin in TrkA-KI eosinophils and specificity of p-TrkA mAb. (A) p-TrkA expression in eotaxin-1 (Eot-1)- or NGF-treated WT eosinophils by immunofluorescence staining with p-TrkA mAb. Scale bar, 20 μm. Untd; Untreated. (B) TrkA-KI eosinophils were treated with eotaxin-1 or NGF and analyzed by Western blot with p-TrkA mAb as described in the main manuscript. Densitometry data and a representative image (below graph) are shown. (C) Eotaxin-1-treated TrkA-KI eosinophils were analyzed by Western blots with p-TrkA mAb in the absence and presence of a blocking peptide specific for the antibody. Membranes containing equal amounts of the sample in duplicate lanes were cut. One lane was incubated with p-TrkA mAb without blocking peptide while the other lane was incubated with p-TrkA mAb after pre-incubation with the blocking peptide. Expression of β-actin is shown as the internal control. In B (upper panel), combined data (Mean ± SEM) and in A and C, representative data of three independent experiments with eosinophils from different mice are shown.

Supplemental Fig. 3



Supplemental Fig. 4 Effect of protease inhibitors on TrkA activation by eotaxin-1. (A) Effect of BB-94 (pan MMP inhibitor) on p-TrkA activation in eotaxin-1-treated WT eosinophils by Western blot analysis with p-TrkA mAb. Untd; Untreated. Densitometric data is shown below blot. (B) p-TrkA expression in eotaxin-1 (Eot-1)-exposed TrkA KI eosinophils pre-treated with a pan caspase inhibitor (Z-VAD-FMK), serine protease inhibitor (PMSF) or respective vehicle control (DMSO for Z-VAD-FMK, isopropyl alcohol for PMSF) by immunofluorescence staining with p-TrKA mAb. Scale bar, 20 μm. In A, representative data and in B (lower panel), combined data (Mean ± SEM), of three independent experiments with eosinophils from three different mice is shown.

Supplemental Fig. 4