Human eosinophil activin A synthesis and mRNA stabilization are induced by the combination of IL-3 plus TNF

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Eosinophils contribute to immune regulation and wound healing/fibrosis in various diseases, including asthma. Growing appreciation for the role of activin A in such processes led us to hypothesize that eosinophils are a source of this transforming growth factor-ß superfamily member. Tumor necrosis factor-α (TNF) induces activin A by other cell types and is often present at the site of allergic inflammation along with the eosinophil-activating common ß (ßc) chain-signaling cytokines (interleukin (IL)-5, IL-3, granulocyte-macrophages colony-stimulating factor (GM-CSF)). Previously, we established that the combination of TNF plus a ßc chain-signaling cytokine synergistically induces eosinophil synthesis of the remodeling enzyme matrix metalloproteinase-9. Therefore, eosinophils were stimulated ex vivo by these cytokines and in vivo through an allergen-induced airway inflammatory response. In contrast to IL-5+TNF or GM-CSF+TNF, the combination of IL-3+TNF synergistically induced activin A synthesis and release by human blood eosinophils. IL-3+TNF enhanced activin A mRNA stability, which required sustained signaling of pathways downstream of p38 and extracellular signal–regulated kinase mitogen-activated protein kinases. In vivo, following segmental airway allergen challenge of subjects with mild allergic asthma, activin A mRNA was upregulated in airway eosinophils compared with circulating eosinophils, and ex vivo, circulating eosinophils tended to release more activin A in response to IL-3+TNF. These data provide evidence that eosinophils release activin A and that this function is enhanced when eosinophils are present in an allergen-induced inflammatory environment. Moreover, these data provide the first evidence for posttranscriptional control of activin A mRNA. We propose that an environment rich in IL-3+TNF will lead to eosinophil–derived activin A, which has an important role in regulating inflammation and/or fibrosis.

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Activin A was initially described as an endocrine factor promoting pituitary follicle-stimulating hormone synthesis and release; however, it is emerging as a critical component of inflammation, immunoregulation and fibrosis.1–5 Activin A can regulate monocyte/macrophage production of proinflammatory factors,6 promote macrophage differentiation to a type 1 or type 2 phenotype,6,7 affect dendritic cell differentiation8 and induce a regulatory T cell population.1 Activin A also induces proliferation of fibroblast4,9 and generation of extracellular matrix.4,9 Thus activin A and the cells that produce it may provide a link between inflammation and fibrosis. Activin A expression is associated with inflammation and/or fibrosis in lung diseases, including asthma,11 acute respiratory distress syndrome,12 chronic obstructive pulmonary disease13 and fibrotic diseases of the lung.14

Tumor necrosis factor-α (TNF) induces activin A production by monocyte/macrophages and stromal cells.2,3 Furthermore, neutrophils release preformed activin A after exposure to high concentrations of TNF, but not in response to typical neutrophil activators such as lipopolysaccharide, interleukin (IL)-8 or N-formyl-Met-Leu-Phe.15 TNF is a well-known activator of eosinophils and, as we have previously demonstrated, can interact synergistically with common ß (ßc) chain-signaling cytokines (IL-5, IL-3, granulocyte-macrophages colony-stimulating factor (GM-CSF)) to enhance eosinophil synthesis of the profibrotic enzyme matrix metalloproteinase (MMP)-9.16 TNF is elevated in patients with asthma17,18 and in mice, inhibition of TNF reduces antigen-induced eosinophilic airway inflammation.19,20

We and others have highlighted the important role of IL-3 compared with other ßc chain-signaling cytokines in inducing or regulating the expression of eosinophil cell surface molecules.21–26 Whether IL-3 activates eosinophils in an atopic environment is not known; however, the observation that blocking IL-5 is only partially effective in eliminating tissue eosinophils27 raises the possibility that
other eosinophil-active cytokines, such as IL-3, contribute to the presence and activation of tissue eosinophils.

Eosinophils are often associated with T helper type 2 (Th2)-type airway inflammation that can lead to fibrosis.26–30 Moreover, eosinophils have the potential to attract and activate Th1, Th2 and Th17 cells31–33 and can influence fibrosis by releasing profibrotic factors.28 Considering these established eosinophil functions and the overlapping properties of activin A, we hypothesized that eosinophils are a source of activin A. Because TNF is a potent inducer of activin A in several other cell types, and as TNF synergizes with βc chain-signaling cytokines to induce eosinophil synthesis of the remodeling factor MMP-9, we hypothesized that eosinophils generate activin A in a similar manner. Our objectives were to determine whether the combination of TNF plus a βc chain-signaling cytokine induces eosinophil synthesis of activin A and whether, similar to MMP-9, eosinophil synthesis of activin A is controlled by mRNA stabilization. Preliminary data from four subjects have been reported previously in a book chapter.36

RESULTS

IL-3+TNF induces eosinophil activin A protein

Human blood eosinophils were used to investigate the conditions under which activin A is produced and regulated. A significant and synergistic upregulation of eosinophil activin A release during the 72-h culture was induced by the combination of IL-3+TNF but not by TNF alone or in combination with the other βc chain-signaling cytokines (GM-CSF or IL-5), Th1 (interferon-γ) or Th2 (IL-4) cytokines (Figure 1a). IL-3+TNF-induced activin A was detectable 24 h after stimulation and continued to accumulate in culture supernatants for 72 h (Figure 1b).

The selective induction of activin A by IL-3+TNF was not due to changes in eosinophil survival. Although viability and survival were significantly decreased at 48 and 96 h in eosinophils cultured in medium or TNF alone, there was no significant reduction when eosinophils were in the presence of IL-3+TNF, GM-CSF+TNF or IL-5+TNF (Figures 1c and d).

Because neutrophils are a known source of activin A,15 neutrophil ‘add-back’ experiments were performed to address the possibility that a small number of ‘contaminating’ neutrophils (typically < 3% of a purified eosinophil preparation) are responsible for the activin A protein in supernatants of IL-3+TNF-stimulated eosinophils. Highly purified (>99%) eosinophils, purified eosinophils plus addition of neutrophils to equal 3% of the total population and that small number of neutrophils without eosinophils were stimulated with IL-3+TNF. The average amount of activin A in 72-h culture supernatants from highly purified eosinophils was 405 pg ml⁻¹ compared with 499 pg ml⁻¹ for highly purified eosinophils plus 3% neutrophil or 10 pg ml⁻¹ for neutrophils without eosinophils (n = 2).

Although eosinophils are known to store and rapidly release preformed cytokines,37 there was little spontaneous (medium) or rapid (within 3 h) release of activin A (Figure 1b), implying de novo synthesis rather than release of preformed protein. Providing further evidence that eosinophils do not store preformed activin A, activin A was evident in lysates of freshly isolated neutrophils but was not detected in lysates of freshly isolated eosinophils (Figure 1e).

IL-3+TNF induces rapid accumulation and stabilization of eosinophil activin mRNA

Cytokines used alone had no significant effect on activin A mRNA (INHBA) level (Figure 2a). GM-CSF+TNF or IL-5+TNF induced a transient rise in INHBA mRNA that peaked between 3 and 6 h (Figure 2a). In contrast, IL-3+TNF had a prolonged effect. At 6 h, IL-3+TNF elicited a twofold increase in INHBA mRNA compared with GM-CSF+TNF or IL-5+TNF, and INHBA mRNA levels remained elevated for 20 h.

The rapid and abundant accumulation of INHBA mRNA between 3 and 6 h raised the possibility of IL-3+TNF-induced posttranscriptional regulation, possibly through mRNA stabilization. The decay rates of INHBA mRNA were determined after the addition of a transcription inhibitor 5,6-dichloro-1-beta-ribofuranosyl benzimidazole (DRB) to eosinophils that had been activated with IL-3+TNF for 4.5 h. As calculated using the decay curves (Figure 2b), the half-life of INHBA mRNA was nearly twofold greater when eosinophils were stimulated with IL-3+TNF compared with either cytokine alone or the combination of GM-CSF+TNF (Figure 2c). Importantly, the enhanced stabilization of INHBA mRNA induced by IL-3+TNF compared with GM-CSF+TNF may contribute to the prolonged versus transient accumulation of INHBA mRNA (Figure 2a) and may explain the abundant versus negligible protein release (Figure 1) in response to IL-3+TNF versus GM-CSF+TNF.

Mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB are required for eosinophil generation of activin A

In eosinophils, IL-3+TNF activates MAPKs, as well as NF-κB.16 Thus pharmacological inhibitors were used to determine signaling events that contribute to IL-3+TNF-induced activin A. IL-3+TNF-induced activin A was reduced 75% by p38 MAPK or MAPK/extracellular signal–regulated kinase (ERK) inhibition, approximately 60% by the NF-κB inhibitor, but was not affected by blockade of the JNK (c-Jun N-terminal kinase) pathway (Figure 3).

MAPKs are required for both the early (0–3 h) and delayed/sustained (3–6 h) stage of INHBA mRNA accumulation

The dichotomy between the early (0–3 h) but transient rise in INHBA induced by GM-CSF+TNF or IL-5+TNF and delayed/sustained (3–6 h) mRNA accumulation induced by IL-3+TNF suggests that INHBA gene expression is controlled at multiple levels over time. To determine the requirement of the MAPKs and NF-κB in the early and the delayed stage of INHBA mRNA accumulation, eosinophils were pretreated with pharmacological inhibitors, IL-3+TNF was added and INHBA mRNA levels were determined 3 and 6 h later. Expression of INHBA mRNA at both time points was significantly reduced by inhibition of p38 MAPK or MAPK/ERK alone and nearly abolished by simultaneous inhibition of p38 MAPK and MAPK/ERK pathways (Figure 4a). In contrast, inhibition of NF-κB had little effect on early INHBA mRNA expression at 3 h but partially reduced INHBA mRNA accumulation 6 h after eosinophil stimulation (Figure 4b).

Prolonged MAPK activation is required for eosinophil INHBA mRNA accumulation

The reduced accumulation of eosinophil INHBA mRNA 6 h after activation in the presence of MAPK inhibitors could indicate that MAPKs are required for a prolonged period or that accumulation of INHBA mRNA at 6 h (delayed stage) is simply dependent on the early stage in an MAPK-independent manner. To determine whether prolonged activation of p38 MAPK and MAPK/ERK is required, inhibitors were added 1 h before or 1 or 2 h after eosinophil stimulation with IL-3+TNF, and INHBA mRNA was assessed at 4 h. Simultaneous inhibition of MAPK/ERK and p38 MAPK at any time point decreased INHBA mRNA expression by ≥ 75% (Figure 4c). When present at the initiation of the culture or added 1 or 2 h after stimulation, the p38 MAPK inhibitor alone resulted in a ≥ 50%
Figure 1 Synergistic induction of eosinophil activin A release by IL-3+TNF but not GM-CSF+TNF or IL-5+TNF. (a) Concentrations of activin A in the supernatant fluid from eosinophils (2 x 10^6 cells ml^(-1)) stimulated for 72 h with the indicated cytokines (10 ng ml^(-1)) without (white bars) or with (gray bars) TNF (10 ng ml^(-1)). Data are represented as mean±s.e.m. of experiments on eosinophil preparations from 5–12 individual subjects with 12 paired samples for medium only, IL-5+TNF, GM-CSF+TNF and IL-3+TNF. IL-3+TNF stimulation is significantly greater than (<0.05) all other stimulations. (b) Kinetics of activin A generation by eosinophils cultured for 3, 24, 48 or 72 h with medium alone (white circles), IL-5+TNF (dark gray squares), GM-CSF+TNF (gray down triangles) or IL-3+TNF (black up triangles). Data are represented as mean±s.e.m. of experiments on eosinophil preparations from five subjects. *P<0.05 versus medium, †P<0.05 versus stimulation with IL-5+TNF, ‡P<0.05 versus stimulation with GM-CSF+TNF. (c and d) Viability and survival were assessed by exclusion of trypan blue. Viability (c) is expressed as the percentage of viable cells and survival (d) as the percentage of viable cells compared with viable cells plated at T0. Data are represented as mean±s.e.m. of experiments on eosinophil preparations from three subjects. *P<0.05 versus medium, †P<0.05 versus TNF. (e) Immunoprecipitation (15 million cells) and immunoblotting of neutrophil or eosinophil lysates prepared from freshly isolated cells. Activin A standard was loaded at 1.5 ng. Markers were run on the same gel but photographed separately. Blot is a representative example of experiments on eosinophil preparations from three subjects.

Figure 2 Kinetics and stabilization of eosinophil activin A mRNA (INHBA) induced by 8c chain-signaling cytokines with or without TNF. (a) Kinetics: eosinophils were cultured for 0, 3, 6, 10 and 20 h with medium (white circles), IL-5 (white squares), GM-CSF (white down triangles), IL-3 (white up triangles), TNF (white diamonds), IL-5+TNF (dark gray squares) or GM-CSF+TNF (light gray down triangles) and IL-3+TNF (black up triangles). Levels of INHBA mRNA (encoding the inhibin 8A subunits of activin A) were determined by RT-qPCR, normalized to GUSB, and expressed as fold change (2^ΔΔCt) from 0 h. Data are mean±s.e.m. of experiments on eosinophil preparations from three subjects. *P<0.05 versus medium, †P<0.05 versus TNF and ‡P<0.05 versus IL-5+TNF at corresponding time point. (b) mRNA decay curves and calculated half-life time: eosinophils were cultured for 4.5 h with medium alone (white circles), IL-3 (white up triangles), TNF (white diamonds), IL-3+TNF (black up triangles) or GM-CSF+TNF (gray down triangles). After addition of DRB, cells were harvested at T=0, 30, 60 and 90 min and INHBA mRNA was quantified by RT-qPCR. Data were normalized to GUSB and expressed as the percentage of mRNA remaining compared with T0. Data are represented as the mean of experiments on eosinophil preparations from 3 to 5 subjects. The half-life time (T_{1/2}) of INHBA mRNA in resting eosinophils is depicted graphically by the line crossing the 50% remaining point. (c) Calculated half-life time. The bar graph depicts the calculated half-life time (T_{1/2}) for each experiment expressed as mean±s.e.m. *P<0.05 for IL-3+TNF activation versus resting (medium alone) or cells stimulated with other cytokines.
reduction in accumulation of INHBA mRNA at 4 h, suggesting that continuous activation of the p38 MAPK pathway is necessary for optimal INHBA mRNA synthesis over time. Conversely, if addition of the MAPK/ERK inhibitor was delayed until 2 h after eosinophil stimulation with IL-3+TNF for 24 h, concentrations of activin A were measured in cell culture supernatant fluid by enzyme-linked immunosorbent assay. Data are represented as mean ± s.e.m. of eosinophil preparations from seven subjects. The P-values for specific inhibitor versus its analog are indicated on the graph.*P<0.05 versus IL-3+TNF alone (black bar and dotted line).

p38 MAPK and MAPK/ERK contribute to eosinophil INHBA mRNA stabilization

As simultaneous treatment of eosinophils with p38 MAPK and MAPK/ERK inhibitors abrogated the expression of INHBA mRNA, their effect on INHBA mRNA stabilization was assessed. Eosinophils were pretreated with inhibitors followed by stimulation with IL-3+TNF for 4.5 h. Based on the mRNA decay curves from 0 to 90 min after transcription inhibition with DRB (Figure 4d), the combination of p38 MAPK and MAPK/ERK inhibitors versus their inactive analogs significantly reduced INHBA mRNA stabilization (Figure 4e).

In vivo, eosinophils are a potential source of activin A under allergic conditions

To determine whether eosinophils are a potential source of activin A in vivo under allergic conditions, we used segmental airway allergen challenge to induce a strong eosinophil response that would allow for purification of eosinophils from the airway that could then be compared with circulating eosinophils from the same individual. The percentage of bronchoalveolar lavage (BAL) eosinophils before and 48 h after Ag were 0.6±0.3% and 73.6±4.6% (mean±s.d.), respectively. There was also an airway allergen-induced rise in circulating eosinophils. Total numbers of circulating eosinophils increased from 288±81 to 590±222 mm−3 in response to challenge. INHBA mRNA levels were significantly greater in BAL compared with circulating eosinophils (Figure 5). Airway allergen challenge may ‘prime’ circulating eosinophils for activin A generation. Compared with eosinophils from individuals who did not undergo airway allergen challenge, circulating eosinophils obtained after challenge tended to release more activin A when stimulated ex vivo with IL-3+TNF (Supplementary Figure S1). Medians with quartiles were 214 (158, 266) versus 110 (61–181) with a P-value of 0.06. Interestingly, a large amount of activin A was released from eosinophils obtained from a control subject (gray diamond) who had allergic rhinitis and atopic dermatitis and was symptomatic on the day of the study owing to recent allergen exposure. When data were analyzed without this ‘outlier’, medians with quartiles were 214 (158, 266) versus 97 (34–146) and the P-value was 0.05 (Supplementary Figure S1).

DISCUSSION

We have established, based on ex vivo experiments, that human eosinophils can synthesize and release activin A upon stimulation with the combination of IL-3+TNF. In addition, we determined that IL-3+TNF synergistically stabilized INHBA mRNA through mechanisms involving the p38 MAPK and MAPK/ERK signaling pathways. Furthermore, prolonged p38 MAPK activation was required for activin A synthesis. These data provide the first evidence for posttranscriptional control of INHBA mRNA and a novel understanding of the mechanisms that regulate activin A synthesis.

Utilizing blood and airway eosinophils obtained after airway allergen challenge in volunteers with allergic asthma, we provided in vivo evidence that eosinophils are a potential source of activin A. We postulate that the greater steady-state level of activin mRNA in airway versus circulating eosinophils is due to eosinophil activation by local mediators present after allergen challenge and/or integrin-mediated signaling as eosinophils migrate though the tissues.38 The atopic environment also increased the propensity of circulating eosinophils to release activin A in response to IL-3+TNF, suggesting in vivo priming.39 To our knowledge, synthesis and/or release of activin A by human eosinophils have not been previously reported.

Although activin A has been observed in the airways of asthmatics and in mouse models of antigen-induced airway inflammation,1,9,11,40 in previous studies its association with eosinophils was not detected. In eosinophil-deficient mice, IL-13 administration led to increased concentrations of activin A in BAL fluid,40 demonstrating that eosinophils are not required for airway activin A in this animal model. In a study of atopic asthmatics who underwent an inhaled allergen challenge, Kariyawasam et al.11 found no increase in the number of activin-positive cells in endobronchial biopsies obtained at 24 h, and immunostaining identified neutrophils as the predominant source of activin A at that time point. The discrepancies between the study by Kariyawasam et al.11 and ours could be due in part to the type and timing of allergen challenge. Compared with the inhalation challenge used in the study by Kariyawasam et al.,11 we administered allergen by bronchoscopy into a specific bronchopulmonary segment. Segmental challenge leads to robust but localized eosinophilic airway inflammation 48 h after challenge.31 In contrast to neutrophils,15 we showed that eosinophils do not appear to store activin A. This could also explain why immunostaining of endobronchial biopsies identified the source of activin as neutrophils, but not eosinophils. Ex vivo studies have shown that neutrophils store and, following stimulation with TNF, rapidly (within hours) release activin A.13 We confirm the presence of activin A in neutrophil lysates; however, activin A was not detected in eosinophil lysates and was not rapidly released into culture supernatants after IL-3+TNF stimulation. Furthermore, the combination of IL-3+TNF induced de novo synthesis and subsequent sustained release of activin A during the 72-h culture.

Cooperative interaction of signaling events downstream of TNF and the Fcε chain-signaling cytokine receptors led to different patterns of INHBA mRNA accumulation and protein release. GM-CSF+TNF or
IL-5+TNF induced a transient increase in mRNA that peaked around 3 h and resulted in very little protein release. We postulate that the transient nature of INHBA mRNA is due to its rapid decay. Compared with GM-CSF+TNF, IL-5+TNF activation of eosinophils induced a prolonged and heightened accumulation of mRNA with a twofold greater increase in INHBA mRNA stability. A modest increase in mRNA stability can have a significant impact on protein production by enhancing translation efficiency. Thus, a 2-fold rise in mRNA half-life can result in a 10-fold increase in protein.42 The associations among enhanced mRNA accumulation, mRNA stability and protein production strongly suggest that posttranscriptional regulation of activin A by mRNA stabilization leads to increased translation of protein. Direct proof, however, will require studies to identify and mutate the sequences that destabilize activin A mRNA.

Optimal generation of IL-3+TNF-induced activin A required pathways mediated by p38 MAPK and ERK, and to a lesser extent, NF-κB. The initial expression of INHBA mRNA was dependent on p38 MAPK and ERK with little contribution from NF-κB. However, both of the kinases and NF-κB contributed to some extent to the later (3–6 h) stage of INHBA mRNA accumulation. The involvement of NF-κB and the observation that INHBA does not contain consensus sequences for NF-κB binding41 may indicate induction of a second signal that has not yet been described. Stabilization of INHBA mRNA required activation of ERK and p38 MAPK. The p38 MAPK inhibitor further decreased mRNA accumulation compared with ERK inhibition, suggesting that p38 MAPK is a candidate for future studies to determine how signaling affects trans-elements involved in INHBA mRNA stabilization or destabilization.

IL-5, GM-CSF and IL-3 signal through the βc component of their respective receptors and have overlapping biological functions, yet IL-3 was unique in its ability to synergize with TNF for induction of eosinophil activin A. Accumulating evidence suggests that IL-3 influences various eosinophil functions, including downregulation of interferon-γ-induced expression of indoleamine 2,3-dioxygenase mRNA,43 induction of eosinophil MMP-916 and regulation of several eosinophil cell surface proteins.21–26. The mechanisms underlying the
selectivity of IL-3 over GM-CSF or IL-5 are not completely understood. We have recently demonstrated that, in contrast to IL-5 and GM-CSF, IL-3 selectively induces translation of the eosinophil surface protein, semaphorin 7A. The increase in translation is due to prolonged activation of 90-kDa ribosomal S6 kinase (p90S6K) and ribosomal protein S6 (RPS6). Unlike IL-3, GM-CSF and IL-5 induced transient activation of p90S6K and RPS6 and rapid dephosphorylation of RPS6, which was phosphatase I-dependent. Although the mechanism intrinsic for IL-3+TNF-induced activin A are likely different from IL-3-induced semaphorin A, it is reasonable to postulate that eosinophil activation with GM-CSF+TNF or IL-5+TNF is limited by induction of an inhibitory factor. Future studies are warranted to determine where the signaling pathways of IL-3/GM-CSF/IL-5 might diverge, the effect of TNF on these pathways and the positive and negative effects that respective downstream targets have on protein translation.

In conclusion, we have demonstrated that eosinophils are a source of activin A and can be activated ex vivo by synergistic signals induced through IL-3 and TNF. Increased activin A mRNA in airway, compared with circulating eosinophils and the enhanced propensity of circulating eosinophils obtained after allergen challenge to release activin A following ex vivo stimulation with IL-3+TNF indicate that an atopic environment contributes to eosinophil generation of activin A in vivo. We speculate that IL-3 and TNF present in airways during allergic inflammation induce eosinophil release of activin A with potential contributions to immune regulation and tissue repair/remodeling.

METHODS

Human subjects

The University of Wisconsin-Madison Health Sciences Human Subjects Committee approved the study protocols, and informed written consent was obtained from each subject prior to participation. For ex vivo mechanistic studies, blood eosinophils were obtained from allergic subjects. To confirm in vivo expression of activin A, eosinophils were obtained from the circulation and BAL fluid 48 h after segmental bronchoprovocation with allergen (SBP-Ag) in subjects with allergic asthma. Subjects for the SBP-Ag study included five males and three females between the age of 19 and 36 years who had mild allergic asthma (positive skin-prick test, FEV₁ (forced expiratory volume in 1 s) 93.8 ± 9.3% of predicted (mean ± s.d.) and a methacholine PC₂₀ of 1.4 (0.4, 5.0) mg ml⁻¹ (geometric mean with first and third quartiles). Detailed methods for bronchoscopy, SBP-Ag, BAL and eosinophil isolation have been described previously. Eosinophil purification

Circulating eosinophils were purified from heparinized blood. The granulocytes were obtained after centrifugation of HBSS (Hank’s Balanced Salt Solution)-diluted blood over Percoll (1.090 g ml⁻¹), red blood cells were lysed by water (25 s) followed by 10× concentrated HBSS. Eosinophils were negatively selected from the granulocyte population utilizing anti-CD16, anti-CD3 and anti-CD14 immunomagnetic beads (AutoMac system, Miltenyi Biotech Inc., Auburn, CA, USA) to deplete neutrophils, T cells and monocytes, respectively. The eosinophil purity was >99%. The positively selected fraction was used for western blotting analysis of activin A in neutrophils.

Cell culture

Eosinophils were cultured at 1–2×10⁶ cells ml⁻¹ in RPMI-1640 containing 10% fetal bovine serum and 1% penicillin/streptomycin and stimulated with 10 ng ml⁻¹ of Flc chain-signaling cytokine (IL-3, IL-5, GM-CSF), a Th1 cytokine (interferon-γ) or a Th2 cytokine (IL-4) alone or in combination with 10 ng ml⁻¹ of TNF for up to 72 h as indicated. Pharmacological inhibitors purchased from Calbiochem (La Jolla, CA, USA) were used to study NF-kB and MAPK pathways. For studies of activin A protein, eosinophils were preincubated for 1 h with inhibitor and then stimulated with IL-3-gTNF as indicated. Because of variability among eosinophils from different donors, three concentrations of the pharmacological inhibitors and an equivalent amount of corresponding analogs were used for each donor. The concentration of inhibitor that suppressed accumulation of activin A protein and caused the least toxicity as measured by eosinophil survival is reported. Inhibitors included the p38 MAPK inhibitor SB203580 or its inactive analog SB202474 (0.5, 1 and 2 μM), the MEK kinase inhibitor U0126 or its inactive analog U0124 (2, 5 and 10 μM), the JNK inhibitor II or its inactive analog II (5, 10 and 20 μM) or the NF-kB inhibitor BAY 11–7082 (1, 2 and 4 μM). Based on the protein studies, the most appropriate inhibitor concentrations were used for mRNA analysis. U0126 and its analog were used at 2 μM, and SB203580 and its analog were used at 0.5 μM.

Enzyme-linked immunosorbent assay

Concentrations of activin A were determined using the R&D Systems DuoSet antibodies (Minneapolis, MN, USA).

Immunoprecipitation

Eosinophil or neutrophil cell pellets (from 15 million cells) were lysed in buffer containing 10 mM Tris HCl (pH 7.48), 0.1 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 14 mM Na₃PO₄, 2 mM NaI, 0.1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), mammalian cocktail protease inhibitors (Sigma-Aldrich Corp., St Louis, MO, USA) and 1 mM PMSF. The suspension was sonicated five times with 5-s pulses (output setting 0.5, Sonicator 3000, Misonix, Farmingdale, NY, USA), repeatedly passed through a syringe (28 gauge needle) and clarified by centrifugation (12 000 g min⁻¹). To precipitate endogenous activin A, cells were incubated with 2 μM primary mouse monoclonal anti-human activin A antibody (clone 69403, R&D Systems) for 1 h at 4 °C for 24–30 h with protein G-coupled magnetic beads (EMD Millipore Corp, Billerica, MA, USA) to deplete neutrophils, T cells and monocytes, respectively.

The wells were washed 10 times with 20 μl of non-reducing lithium dodecyl sulfate sample buffer (Thermo Fisher Scientific, Grand Island, NY, USA). Samples were heated to 90 °C for 5 min and loaded onto a 13.5% SDS-PAGE (polyacrylamide gel electrophoresis) gel.

Western blotting

SDS-PAGE gels (15%) were run with a constant current of 30 mA and a voltage ceiling of 160 V and then transferred to a polyvinylidene difluoride membrane for 1 h at 100 V. Membranes were blocked with 5% nonfat dry milk in TRIS-buffered saline with 0.1% Tween 20. Protein was detected with a primary mouse monoclonal anti-human activin A ab (clone 69403, R&D Systems).
Real-time quantitative PCR (RT-qPCR)

Activin A is comprised of two inhibin βA subunits encoded by the INHBA gene.2 mRNA levels of INHBA and the reference gene β-glucuronidase (GUSB) were analyzed by RT-qPCR and fold-change was calculated using the comparative cycle threshold (Ct) method (2−ΔΔCt) as described previously.26

mRNA decay

The transcription inhibitor DRB (25 μg·mL−1) was added to eosinophil cultures 4.5 h after addition of stimuli (cytokines at 10 ng·mL−1), and eosinophils were harvested 30, 60 and 90 min thereafter. INHBA mRNA levels present immediately before addition of DRB (T = 0 h) were set to 100%. The percentage of INHBA mRNA remaining compared with T = 0 h was presented for each time point after the addition of DRB. The half-life of mRNA was defined as the time required to attain a 50% reduction of mRNA after DRB addition.47

Statistical analysis

The outcome measurements—activin A concentration, INHBA mRNA fold change and the percentage of inhibition, eosinophil percentage viability and the fold changes were log-transformed for analysis. Analyses were conducted using a subject correlation. Multiple pairwise comparisons were conducted only in the presence of a significant change in the comparison of interest.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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35 Esnault S, Kelly EA, Nettensbom LM, Cook EB, Serogy CM, Jarjour NN. Human eosinophils release IL-18 and increase expression of IL-17A in activated CD4(+) T lymphocytes. Clin Exp Allergy 2012; 42: 1756–1764.

36 Kelly EAB. Potential role of eosinophils and tumor necrosis factor alpha in tissue remodeling. In: Lee JJ, Rosenberg HF (eds). Eosinophils in Health and Disease. Elsevier: Oxford, UK, 2013, pp 404–411.

37 Spencer LA, Bonjour K, Melo RC, Weller PF. Eosinophil secretion of granule-derived cytokines. Front Immunol 2014; 5: 496.

38 Johansson MW, Mosher DF. Integrin activation states and eosinophil recruitment in asthma. Front Pharmacol 2013; 4: 33.

39 Luijk B, Lindemans CA, Kanten D, van der HR, Bertics P, Lammers JW et al. Gradual increase in priming of human eosinophils during extravasation from peripheral blood to the airways in response to allergen challenge. J Allergy Clin Immunol 2005; 115: 997–1003.

40 Hardy CL, Lemasurier JS, Olsson F, Dang T, Yao J, Yang M et al. IL-13 regulates secretion of the TGF-ß superfamily cytokine activin A in allergic airway inflammation. Am J Respir Cell Mol Biol 2009; 42: 667–675.

41 Caihoun WJ, Jarjour NN, Gleich GJ, Stevens CA, Busse WW. Increased airway inflammation with segmental versus aerosol antigen challenge. Am Rev Respir Dis 1993; 147: 1465–1471.

42 Rajagopalan LE, Malter JS. Turnover and translation of in vitro synthesized messenger RNAs in transfected, normal cells. J Biol Chem 1996; 271: 19871–19876.

43 Odemuyiwa SO, Ghahary A, Li Y, Puttagunta L, Lee JE, Musat-Marcu S et al. Cutting edge: human eosinophils regulate T cell subset selection through indoleamine 2,3-dioxygenase. J Immunol 2004; 173: 5909–5913.

44 Esnault S, Kelly EA, Shen ZJ, Johansson MW, Malter JS, Jarjour NN. IL-3 maintains activation of the p90S6K/RPS6 pathway and increases translation in human eosinophils. J Immunol 2015; 195: 2529–2539.

45 Liu LY, Sedgwick JB, Bates ME, Vrtis RF, Gern JE, Kita H et al. Decreased expression of membrane IL-5R alpha on human eosinophils: I. Loss of membrane IL-5 alpha on eosinophils and increased soluble IL-5R alpha in the airway after antigen challenge. J Immunol 2002; 169: 6452–6458.

46 Spencer LA, Szela CT, Perez SAC, Kirchhoffer CL, Neves JS, Radke AL et al. Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially. J Leukoc Biol 2009; 85: 117–123.

47 Esnault S, Shen ZJ, Whitesel E, Malter JS. The peptidyl-prolyl isomerase Pin1 regulates granulocyte-macrophage colony-stimulating factor mRNA stability in T lymphocytes. J Immunol 2006; 177: 6999–7006.

The Supplementary Information that accompanies this paper is available on the Immunology and Cell Biology website (http://www.nature.com/icb)