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

Elizabeth A. Kelly[1], **Stephane Esnault**[1], **Sean H. Johnson**[1], **Lin Ying Liu**[1], **James S. Malter**[2], **Mandy E. Burnham**[3], and **Nizar N. Jarjour**[1]

[1]Department of Medicine, Allergy, Pulmonary, and Critical Care Medicine Division, University of Texas Southwestern Medical Center, Dallas, TX

[2]Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX

[3]Department of Biomolecular Chemistry, University of Wisconsin School of Medicine and Public Health, Madison, WI 53792, USA

Abstract

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 TGF-β superfamily member. TNFα (TNF) induces activin A by other cell types and is often present at the site of allergic inflammation along with the eosinophil activating common β (βc) chain-signaling cytokines (IL-5, IL-3, GM-CSF). Previously, we established that the combination of TNF plus a βc chain-signaling cytokine synergistically induces eosinophil synthesis of the remodeling enzyme MMP-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 ERK MAP kinases. *In vivo*, following segmental airway allergen challenge of subjects with mild allergic asthma, activin A mRNA was upregulated in airway eosinophils compared to circulating eosinophils, and *ex vivo*, circulating eosinophils tended to release 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 post-transcriptional control of activin A mRNA. We propose that, an environment rich in IL-3+TNF will lead to eosinophil–derived activin A, which plays an important role in regulating inflammation and/or fibrosis.

Corresponding author: Elizabeth A. Kelly, Ph.D., Allergy, Pulmonary and Critical Care Medicine Division, Department of Medicine, University of Wisconsin School of Medicine and Public Health, 600 Highland Avenue, K4/928 CSC, Madison, WI 53792-9988, USA. Phone: 608-263-3253, FAX: 608-263-3746, eak@medicine.wisc.edu.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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Introduction

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,[4] promote macrophage differentiation to a type 1 or type 2 phenotype,[6, 7] affect dendritic cell differentiation,[8] and induce a regulatory T cell population.[1] Activin A also induces proliferation of fibroblasts[9, 4] and airway smooth muscle[10] and generation of extracellular matrix.[9, 4] 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 obstruction pulmonary disease,[13] and fibrotic diseases of the lung[14].

TNF-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 LPS, 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 β (βc) chain-signaling cytokines (IL-5, IL-3, GM-CSF) to enhance eosinophil synthesis of the profibrotic enzyme MMP-9.[16] TNF is elevated in patients with asthma,[17, 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 to other βc chain-signaling cytokines in inducing or regulating 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 eosinophils[27] 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 Th2-type airway inflammation that can lead to fibrosis.[28–30] Moreover, eosinophils have the potential to attract and activate Th1, Th2, Th17 cells[31–35] 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 since 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 similar manner. Our objectives were to determine if the combination of TNF plus a βc chain-signaling cytokine induces eosinophil synthesis of activin A and if, like 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.[35]

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 (IFN-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 supernates for 72 h (Figure 1b).

The selective induction of activin A by IL-3+TNF was not due to changes in eosinophil survival. While 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 (Figure 1c and 1d).

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 compared to 499 pg/ml for highly purified eosinophils plus 3% neutrophil or 10 pg/ml 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 2-fold increase in *INHBA* mRNA compared to 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 post-transcriptional regulation, possibly through mRNA stabilization. The decay rates of *INHBA* mRNA were determined after the addition of a transcription inhibitor, 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

2-fold greater when eosinophils were stimulated with IL-3+TNF compared to 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 to GM-CSF+TNF may contribute to the prolonged versus transient accumulation of *INHBA* mRNA (Fig. 2A) and may explain the abundant versus negligible protein release (Figure 1) in response to IL-3+TNF versus GM-CSF+TNF.

MAP kinases and NF-κB are required for eosinophil generation of activin A

In eosinophils, IL-3+TNF activates MAP kinases, 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/ERK inhibition, approximately 60% by the NF-κB inhibitor, but was not affected by blockade of the JNK pathway (Figure 3).

MAP kinases 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 MAP kinases 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 MAP kinase inhibitors could indicate that MAP kinases 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 a MAP kinase-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% 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, only minimal changes were seen in *INHBA* mRNA accumulation at 4 h.

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

Since simultaneous treatment of eosinophils with p38 MAPK and MAPK/ERK inhibitors abrogated 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 if 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 to 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 ± SD), respectively. There was also an airway allergen-induced rise in circulating eosinophils. Total numbers of circulating eosinophils increased from 288 ± 81 to 590 ± 222 per mm^3 in response to challenge. *INHBA* mRNA levels were significantly greater in BAL compared to circulating eosinophils (Figure 5). Airway allergen challenge may “prime” circulating eosinophils for activin A generation. Compared to 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 (Figure 1S in Supplementary online material). 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 due to recent allergen exposure. When data were analyzed without this “outlier”, medians with quartiles were 214 (158, 266) versus 97 (54–146) and the p value was 0.05.

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 post-transcriptional 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,[40, 11, 1, 9] 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 and colleagues 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.[11] The discrepancies between the Kariyawasam study and ours could be due in part to the type and timing of allergen challenge. Compared to the inhalation challenge used in the Kariyawasam study, we administered allergen by bronchoscopy into a specific bronchopulmonary segment. Segmental challenge leads to robust, but localized eosinophilic airway inflammation 48 h after challenge.[41] 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.[15] 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 supernates 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 βc 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 to GM-CSF+TNF, IL-3+TNF activation of eosinophils induced a prolonged and heightened accumulation of mRNA with a 2-fold 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 post-transcriptional regulation of activin A by mRNA stabilization leads to increase 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 MAP kinase 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-kB binding[3] 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 to 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 down-regulation of IFN-γ-induced expression of indoleamine 2,3-dioxygenase mRNA,[43] induction of eosinophil MMP-9,[16] 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).[44] Unlike IL-3, GM-CSF and IL-5 induced transient activation of p90S6K and RPS6, and rapid dephosphorylation of RPS6, which was phosphatase 1-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 down-stream 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 to 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 bronchoalveolar lavage (BAL) fluid 48 h after segmental bronchoprovocation with allergen (SBP-Ag) in subjects with allergic asthma. Subjects for the SBP-Ag study included 5 males and 3 females between the age of 19 and 36 who had mild-allergic asthma (positive skin-prick test, FEV_1 93.8 ± 9.3 % of predicted (mean ± SD), and a methacholine PC_{20} of 1.4 (0.4, 5.0) mg/ml (geometric mean with 1st and 3rd quartiles).

Detailed methods for bronchoscopy, SBP-Ag, BAL, and eosinophil isolation have been described previously.[45]

Eosinophil purification

Circulating eosinophils were purified from heparinized blood. The granulocytes were obtained after centrifugation of HBSS-diluted blood over Percoll (1.090 g/ml), RBCs were lysed by water (25 sec) followed by 10X concentrated HBSS. Eosinophils were negatively selected from the granulocyte population utilizing anti-CD16, anti-CD3, and anti-CD14 immunomagnetic beads (AutoMac system, Miltenyi Biotec 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 blot analysis of activin A in neutrophils.

Cell culture

Eosinophils were cultured at $1–2\times10^6$ cells/ml in RPMI-1640 containing 10% FBS and 1% penicillin/streptomycin and stimulated with 10 ng/ml of βc chain-signaling cytokine (IL-3, IL-5, GM-CSF), a Th1 cytokine (IFN-γ), or a Th2 cytokine (IL-4) alone or in combination with 10 ng/ml of TNF for up to 72 h as indicated. Pharmacological inhibitors purchased from Calbiochem (La Jolla, CA, USA) were used to study NF-κB and the MAP kinase pathways. For studies of activin A protein, eosinophils were preincubated for 1 h with inhibitor and then stimulated with IL-3±TNF as indicated. Due to 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 MAP kinase 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-κB 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.

ELISA

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

Immunoprecipitation

Eosinophil or neutrophil cell pellets (from 15 million cells) were lysed in buffer containing 10mM Tris HCl (pH 7.48), 0.1 mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 14mM $Na_4P_2O_7$, 2mM Na_3VO_4, 0.1% Triton X-100, 0.1% SDS, mammalian cocktail protease inhibitors (Sigma-Aldrich Corp, St. Louis, MO, USA) and 1mM PMSF.[46] The suspension was sonicated five times with 5 second 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 x g/10 min/4 °C). The lysate was incubated at 4°C for

24–30 h with protein G-coupled magnetic beads (EMD Millipore Corp, Billerica, MA, USA) coated with polyclonal goat anti-human activin A antibody (R&D Systems). The beads were collected with a magnet and resuspended in 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 gel.

Western blotting

SDS-PAGE gels (13.5%) were run with a constant current of 30 mA and a voltage ceiling of 160V, and then transferred to a PVDF membrane for 1h at 100V. 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) and a secondary HRP-conjugated goat anti-mouse ab (Life Technologies, Grand Island, NY, USA). Imaging was then performed with SuperSignal™ West Femto chemiluminescent substrate (Life Technologies) on an Alpha Innotech FluoroChem™ HD imager.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Activin A is comprised of two inhibin βA subunits encoded by the *INHBA* gene.[3] 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 ($2^{-\Delta\Delta Ct}$) as described previously.[26]

mRNA decay

The transcription inhibitor 5,6-dichloro-1-beta-ribofuranosyl benzimidazole (DRB, 25 μg/ml) was added to eosinophil cultures 4.5 h after addition of stimuli (cytokines at 10 ng/ml) 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 % of *INHBA* mRNA remaining compared to 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 % inhibition, eosinophil % viability and % survival—were compared across experimental conditions and time points using linear mixed effect models, with fixed effect terms for condition, or for condition, time, and condition-by-time interaction, as appropriate, and a random effect term for subject to account for within-subject correlation. Multiple pairwise comparisons were conducted only in the presence of a significant ANOVA F-test using Fisher's protected least significant difference method. Activin A concentrations and INHBA mRNA fold-changes were log-transformed for analysis. Analyses were conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). A two-sided p-value less than 0.05 was regarded as statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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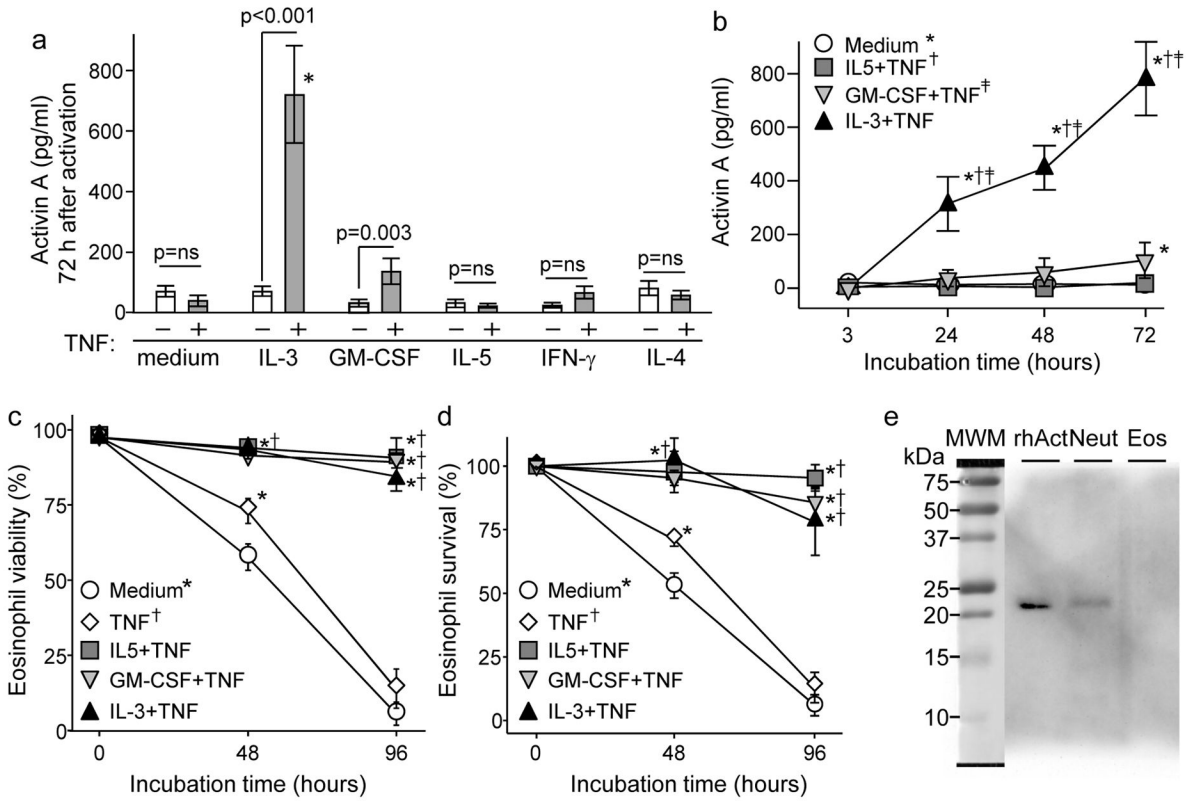

Figure 1.

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 supernatant fluid from eosinophils (2×10^6 cells/ml) stimulated for 72 h with indicated cytokines (10 ng/ml) without (white bars) or with (gray bars) TNF (10 ng/ml). Data are represented as mean±SEM 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) than 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±SEM of experiments on eosinophil preparations from 5 subjects. *p<0.05 versus medium, $^{\dagger}p$<0.05 versus stimulation with IL-5+TNF, $^{\ddagger}p$<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 to viable cells plated at T0. Data are represented as mean±SEM of experiments on eosinophil preparations from 3 subjects *p<0.05 versus medium, $^{\dagger}p$<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 3 subjects.

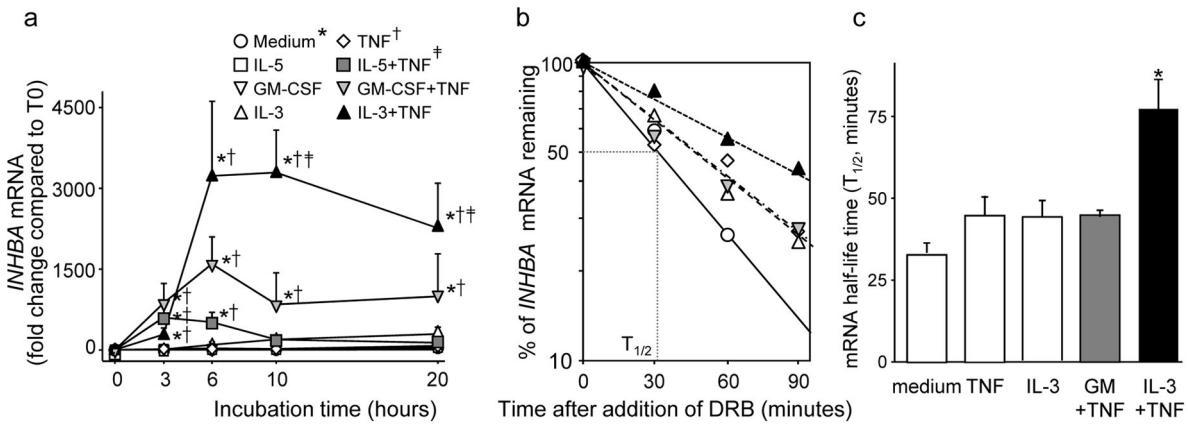

Figure 2.

Kinetics and stabilization of eosinophil activin A mRNA (*INHBA*) induced by βc 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), IL-3+TNF (black up triangles),. Levels of *INHBA* mRNA (encoding the inhibin βA subunits of activin A) were determined by RT-qPCR, normalized to *GUSB*, and expressed as fold change ($2^{-\Delta\Delta}$Ct) from 0 h. Data are mean±SEM of experiments on eosinophil preparations from 3 subjects. *p<0.05 versus medium, †p<0.05 versus TNF, and ‡p<0.05 vs 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 % mRNA remaining compared to T0. Data are represented as the mean of experiments on eosinophil preparations from 3–5 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. (d) Calculated half-life time: The bar graph depicts the calculated half-life time ($t_{1/2}$) for each experiment expressed as mean ±SEM. *p<0.05 for IL-3+TNF activation versus resting (medium alone) or cells stimulated with other cytokines.

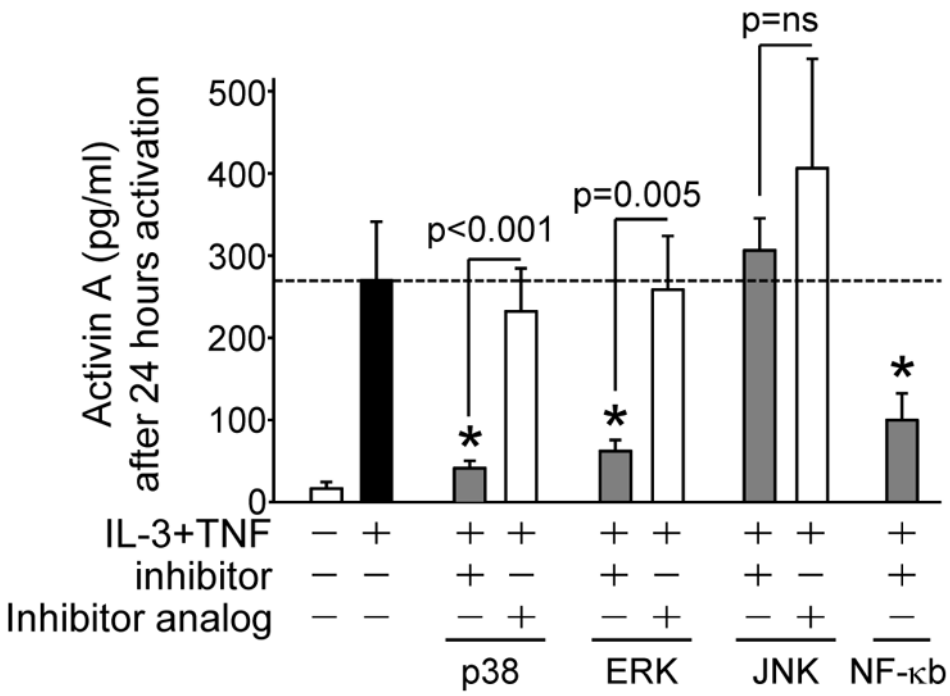

Figure 3.

Effect of MAP kinase and NF-κB inhibitors on IL-3+TNF-induced eosinophil activin A. Eosinophils were preincubated for 1 h with the p38 MAPK inhibitor SB203580 or its inactive analog SB202474, the MAPK/ERK kinase inhibitor U0126 or its inactive analog U0124, the JNK inhibitor II or its inactive analog, or the NF-κB inhibitor BAY 11-7082 (no analog available), and then they were stimulated with IL-3+TNF for 24 h. Concentrations of activin A were measured in cell culture supernatant fluid by ELISA. Data are represented as mean±SEM of eosinophil preparations from 7 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).

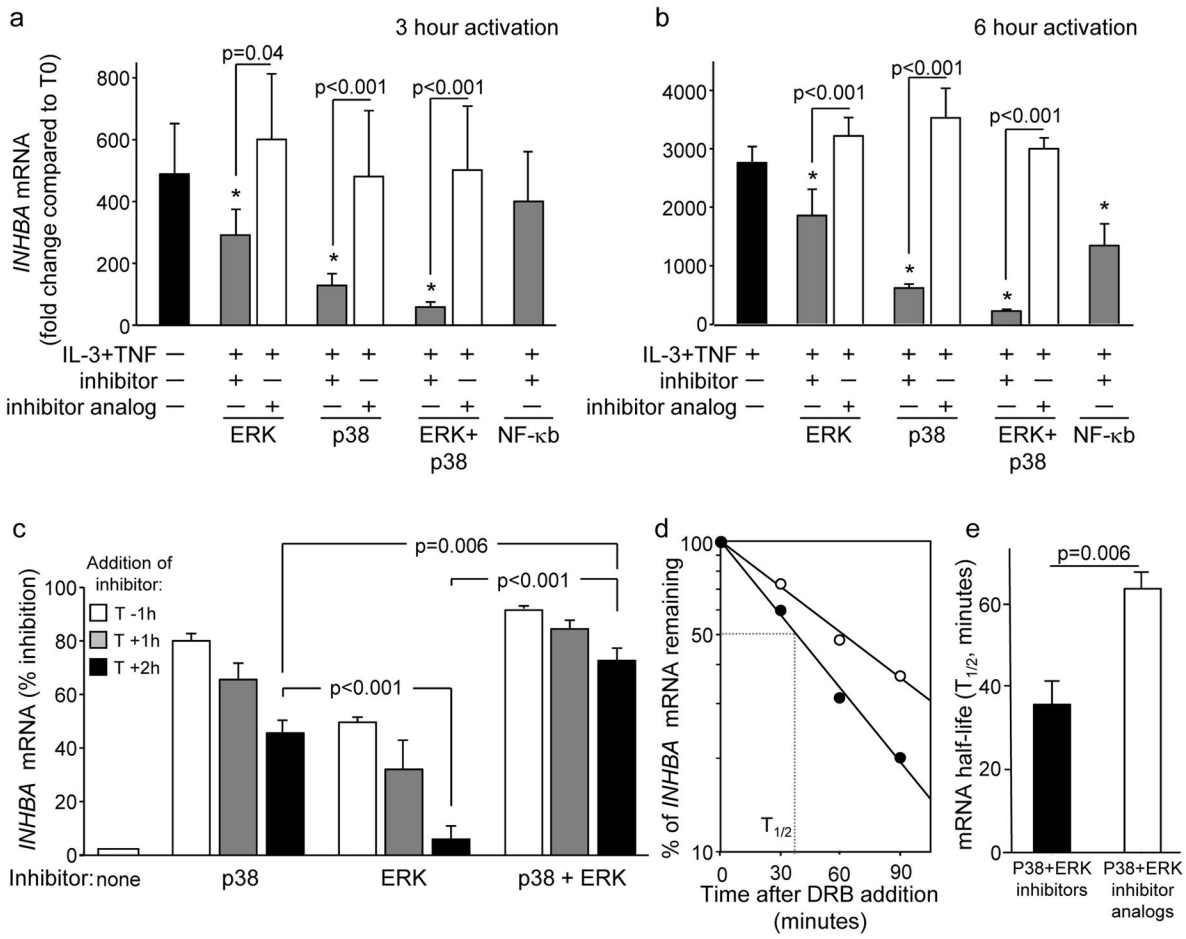

Figure 4.

Effect of MAP kinase and NF-κB inhibitors on activin A mRNA (*INHBA*) accumulation after eosinophil stimulation with IL-3+TNF. **(a)** Eosinophils were preincubated for 30 min with the p38 MAPK inhibitor SB203580 or its inactive analog SB202474, the MAPK/ERK kinase inhibitor U0126 or its inactive analog U0124, or the NF-κB inhibitor BAY 11-7082 (no analog available) as indicated, IL-3+TNF was added (T=0 h). Cells were harvested (**a)** 3 h or **(b)** 6 h after addition of IL3+TNF. *INHBA* mRNA was quantified by RT-qPCR. Data were normalized to *GUSB* and expressed as fold change ($2^{-\Delta\Delta}$Ct) from T=0 h. Data are an average of experiments on eosinophil preparations from 5 subjects. The p values for specific inhibitor versus its analog are indicated on the graph.*p<0.05 versus IL-3+TNF without inhibitor (black bar). **(c)** Eosinophils were stimulated for a total of 4 h with IL-3+TNF. The p38 MAP kinase inhibitor SB203580 or the MEK kinase inhibitor U0126 or their analogs were added singly or together 30 min before (white bars) or 1 (gray bars) and 2 h (black bars) after activation. *INHBA* mRNA was quantified by RT-qPCR. Data were normalized to *GUSB* and fold change from resting eosinophils was determined. Results are expressed as % inhibition compared to eosinophils cultured with IL-3+TNF in the presence of respective inactive analogs using the formula: [1-(fold *INHBA* mRNA increase with inhibitor(s)/fold *INHBA* mRNA increase with analogs) x 100]. Data are presented as mean±SEM of eosinophil preparations from 3 subjects. **(d)** mRNA decay curves: eosinophils were

pretreated for 30 min with the p38 MAP kinase inhibitor SB203580 in combination with the MEK kinase inhibitor U0126 (black circles) or a combination of the respective inactive analogs SB202474 and U0124 (white circles). Eosinophils were stimulated with IL-3+TNF for 4.5 h before addition of the transcription inhibitor DRB and cells were harvested at T=0, 30, 60, and 90 min. *INHBA* mRNA was quantified by RT-qPCR. Data were normalized to *GUSB* and expressed as the % of mRNA remaining compared to T=0h. The half-life time ($t_{1/2}$) of *INHBA* mRNA in eosinophils treated with inhibitors is depicted graphically by the line crossing the 50% remaining point. (e) Calculated half-life time: the half-life of *INHBA* mRNA calculated for each experiment and expressed as mean±SEM of experiments on eosinophil preparations from 3 subjects.

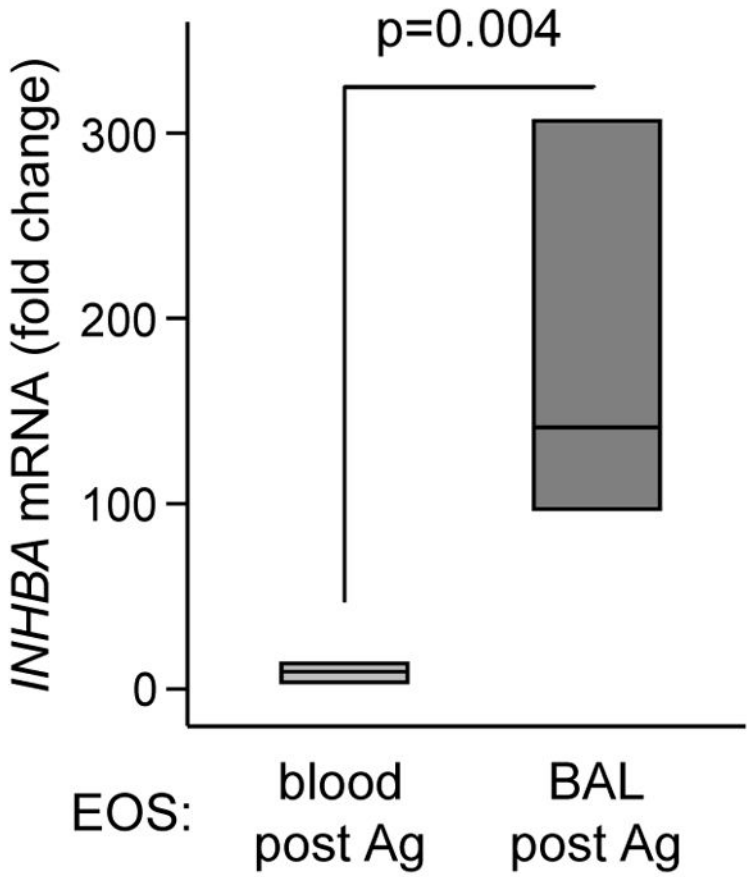

Figure 5.

Evidence of *in vivo* expression of activin A by human eosinophils Steady-state levels of *INHBA* mRNA were determined by RT-qPCR in eosinophil preparations with purity ≥99%. Eosinophils are from blood and BAL of 7 subjects who underwent segmental allergen challenge 48 h earlier. Data were normalized to *GUSB* and expressed as fold change ($2^{-\Delta\Delta Ct}$) compared to a single blood eosinophil sample from a control subject who did not undergo SBP-Ag.