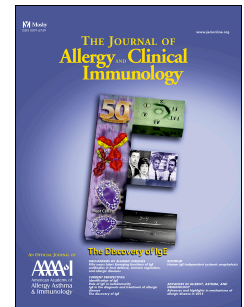


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Eosinophil degranulation products drive a pro-inflammatory fibroblast phenotype

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Capsule summary: In this study, we found that eosinophil-derived degranulation products stimulate bronchial fibroblasts to release chemotactic factors that promote neutrophil migration. This finding suggests that eosinophil-fibroblast interactions may promote airway neutrophilia in severe asthma.

Key words: Severe asthma, eosinophils, neutrophils, bronchial fibroblasts, IL3, IgG, degranulation, chemotaxis, IL8, IL6.

Abbreviations: CXCL1, chemokine (C-X-C motif) ligand 1; CXCL8, chemokine (C-X-C motif) ligand 8; EDN, eosinophil-derived neurotoxin; EPX, eosinophil peroxidase; ES, eosinophil supernatants; HA-IgG, heat-aggregated human serum immunoglobulin; HBF, human bronchial fibroblasts; ICAM1, intercellular adhesion molecule 1; IgG, immunoglobulin; IL3, interleukin 3; IL3-HAIgG, IL3 primed for 20 h and seeded on HA-IgG for 6 h; IL5, interleukin 5; IL6, interleukin 6; IL8, interleukin 8; rhIL3-HAIgG, recombinant human IL3 plus soluble HA-IgG.

To the Editor:

Severe asthma is characterized by persistent airflow obstruction and ongoing symptoms despite intense corticosteroid treatment. Asthma severity is associated with airway inflammation and increased numbers of neutrophils and eosinophils^{1, 2}. While neutrophilia can be driven by type-17 immune responses, the mechanisms that lead to recruitment and persistence of neutrophils in asthmatic airways remain unknown. Eosinophils contribute to asthma pathobiology by releasing granule proteins and additional factors including cytokines and chemokines that can activate other cell types, such as bronchial fibroblasts^{3, 4}. While bronchial fibroblasts contribute to airway remodeling in asthma by depositing extracellular matrix proteins, their interactions with eosinophils are not well-characterized⁴. We recently performed RNA sequencing on parenchymal lung fibroblasts stimulated with activated-eosinophil conditioned media⁵. Pathway analysis revealed a gene expression profile enriched in genes involved in neutrophil-recruitment and activation⁵. In the present study, we sought to expand upon these investigations using *ex vivo* cell culture models relevant to asthma pathogenesis. Specifically, our goals were to determine the effect of eosinophil-degranulation products on primary human bronchial fibroblasts (HBF) and neutrophil recruitment.

A recent report found the presence of immunoglobulin G (IgG) complexed with eosinophil peroxidase (EPX) in eosinophilic asthma⁶, suggesting that this can stimulate eosinophil degranulation. We have developed a model of aggregated IgG-induced eosinophil degranulation by priming human circulating eosinophils with interleukin 3 (IL3) for 20h, followed by plating on heat-aggregated human serum IgG (HAiGg) for 6h (IL3-HAiGg condition) or cultured without IgG (IL3 condition)^{5, 7}. In the current study, we obtained circulating blood eosinophils from allergic subjects (**Table E1**), activated them using the above approach, and collected eosinophil supernatants (ES) to measure the concentration of eosinophil-derived neurotoxin (EDN), a marker of degranulation (**Fig. E1**). There was an approximate 10-fold increase in EDN released following IL3-HAiGg treatment compared to IL3 alone (**Fig. E1**), indicating strong degranulation on complexed IgG. ES was then collected from activated eosinophils (IL3-HAiGg ES), mixed 1:1 with serum-free media, and used to stimulate primary cultures of HBF from donors without allergy or asthma.

In the first set of experiments, four different HBF lines were stimulated with IL3-HAiGg ES from four different eosinophil donors. Eosinophil medium alone, medium with 1 ng/ml IL3 and 0.5 µg/ml HAiGg (rhIL3-HAiGg), and ES from IL3-treated eosinophils (IL3 ES) were used as controls⁵. After 24h of incubation, we observed a significant increase in the expression of four pro-neutrophilic genes, *CXCL8*, *IL6*, *CXCL1*, and *ICAM1* by quantitative PCR in HBF treated with IL3-HAiGg ES compared to controls (**Fig. 1A-D**)^{5, 8}. This was consistent with our previous findings in parenchymal lung fibroblasts⁵. We then assessed HBF secretion of the pro-neutrophilic chemokines IL8 and IL6 after 72h by ELISA. In collected supernatants, there was a significant increase in release of both IL8 and IL6 by HBF stimulated with IL3-HAiGg ES compared to control conditions (**Fig. 1E-F**). Notably, IL8 and IL6 concentrations in the IL3-HAiGg ES alone were low (**Fig. 1E-F**), indicating that HBF are the predominant source of IL8 and IL6 in this system. Interestingly, the induced production of IL8 by fibroblasts was not

strongly associated with levels of either EDN ($r=0.37$, $p=0.29$, $n=10$) or IL8 ($r=0.36$, $p=0.30$, $n=10$) in the IL3-HAIG ES. This suggests that other eosinophilic mediator(s) besides granule proteins or IL8 stimulate IL8 production by HBF.

Consequently, we sought to determine whether HBF-conditioned media would modify neutrophil chemotaxis. Again, we utilized IL3-HAIG ES from four different donors to activate HBF from four different donors for 72h. HBF-conditioned media was then collected and used as a stimulus for a transwell-based neutrophil chemotaxis assay. Controls included HBF growth medium (negative control), IL3-HAIG ES alone, and HBF basal medium supplemented with recombinant IL8 (positive control). There was a significant increase in neutrophil chemotaxis in response to conditioned media from IL3-HAIG ES stimulated-HBF compared to controls (**Fig. 2A**). Importantly, we found a clear correlation between IL8 released in the HBF-conditioned media and neutrophil chemotaxis ($r=0.94$, $p<0.0001$, $n=12$, **Fig. 2B**), indicating that IL8 is a marker for chemotactic activity generated by these media. Importantly, IL3-HAIG ES alone did not induce neutrophil movement above baseline, suggesting that the fibroblast-derived soluble factors are critical for neutrophil recruitment. Altogether, these data show that HBF exposed to IL3-HAIG ES release *de novo* soluble factors that efficiently induce neutrophil chemotaxis.

In summary, this study utilized a physiologically relevant *ex vivo* model of eosinophil degranulation to explore the role of eosinophil degranulation products on the release of neutrophilic mediators by primary HBF. We have observed an amplification of neutrophil-recruitment by eosinophil-fibroblast interaction, which has not been reported previously. HBF have a known role in matrix deposition in asthma, but there is little information on how fibroblasts modify airway inflammatory responses. Inflammatory signaling from parenchymal or vascular lung fibroblasts is not without precedent in pulmonary disorders⁹, and our work suggests that HBF participate in the inflammatory response through neutrophil recruitment.

Although we utilized *ex vivo* cell culture models, a strength of this study is the use of primary cultures of human cells from physiologically relevant sources. Eosinophils were obtained from allergic donors, HBF from airway biopsies, and neutrophils from human whole blood samples. Eosinophil priming and degranulation was modeled using prolonged IL3-exposure followed by plating on HAIG⁷, which is meant to recapitulate the increased IL3 levels found in bronchoalveolar lavage fluid after an *in vivo* allergen challenge⁷, followed by exposure to high IgG levels that are present in airways of asthmatic patients⁶. Eosinophil stimulation using this model results in stronger degranulation and amplified release of pro-inflammatory and pro-fibrotic mediators compared to plating on HAIG alone, IL5 priming and plating on HAIG, or IL3 stimulation alone^{5, 7}. Thus, this experimental design appears to be relevant to the *in vivo* biology that drives asthma pathology.

In conclusion, using an *ex vivo* model of eosinophil degranulation, we have found that HBF release IL6 and IL8, and promote neutrophil migration. These findings suggest that the interplay between eosinophil-derived soluble mediators and HBF may be essential for recruiting neutrophils to the airway *in vivo*, thereby promoting persistent airway inflammation and remodeling in severe asthma.

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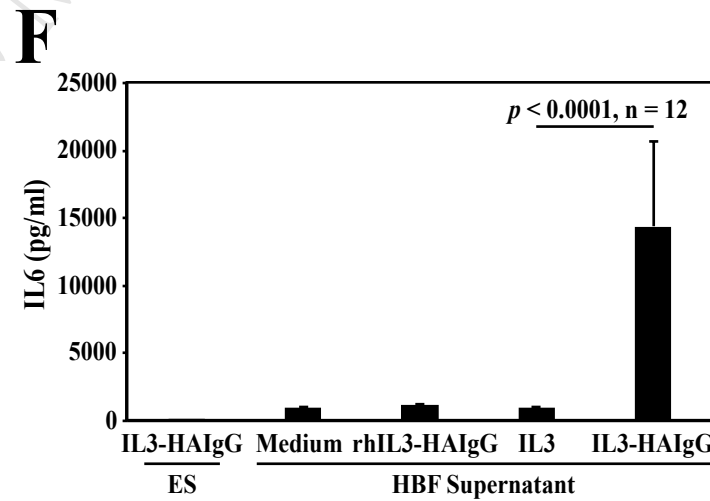
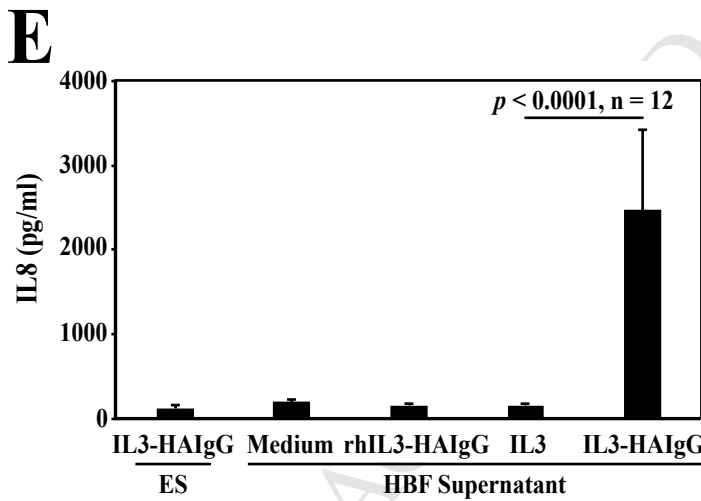
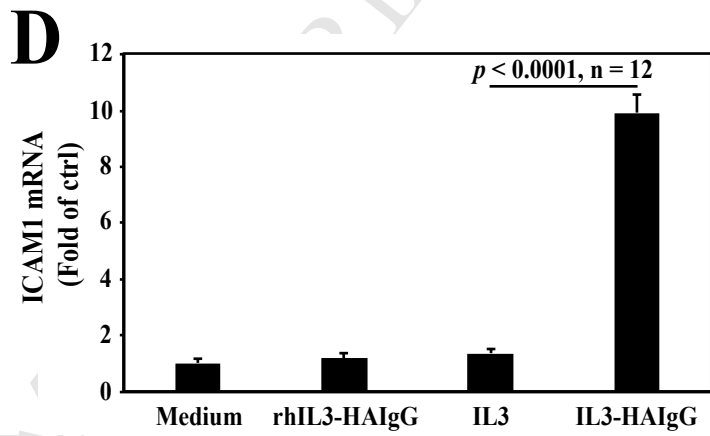
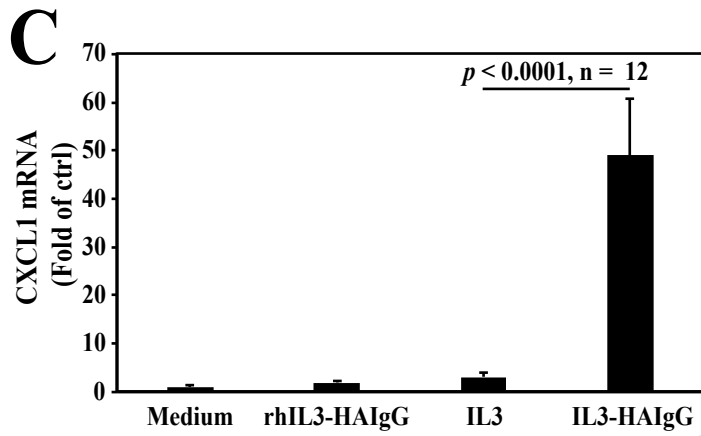
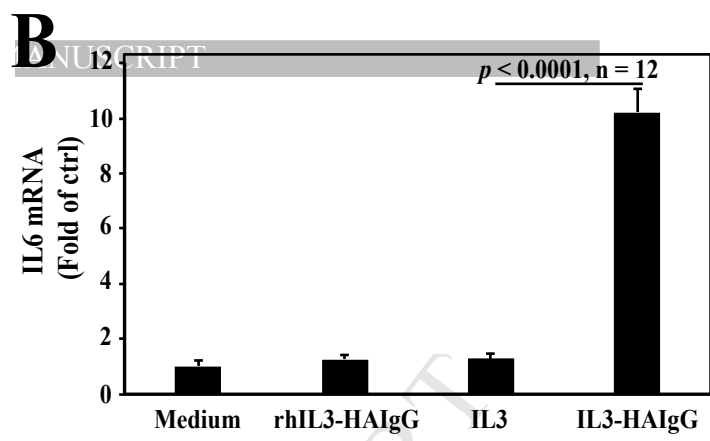
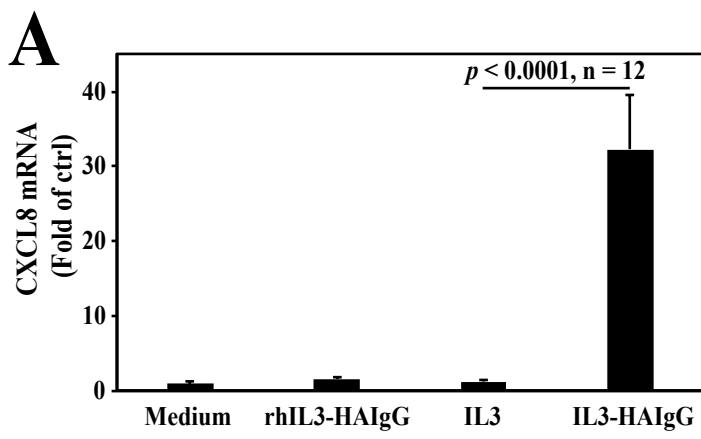
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Figure Legends:

Figure 1. HBF stimulated by IL3-HAIG-activated eosinophils express chemokines important for neutrophil migration. HBF stimulated with IL3-HAIG ES, IL3 ES, rhIL3-HAIG, or growth medium for 24h (A-D) or 72h (E, F). mRNA levels of CXCL8 (A), IL6 (B), CXCL1 (C) and ICAM1 (D) in HBF. IL8 (E) and IL6 (F) ELISA of supernatant from HBF or from IL3-HAIG ES alone.

Figure 2. Supernatants from IL3-HAIG ES-stimulated HBF induce increased neutrophil chemotaxis. (A) Transwell neutrophil migration in response to indicated HBF supernatants, IL3-HAIG ES, 30 ng/ml IL8, or growth medium. (B) Correlation of transwell neutrophil migration to IL8 content in IL3-HAIG ES- stimulated HBF supernatants.

Figure E1. Degranulation of eosinophils by IL3 and IL3-HAIG treatment. Peripheral blood eosinophils were primed with IL3 (4 ng/ml) for 20 h. Subsequently, eosinophils were plated on heat-aggregated human serum IgG (HAIG) for 6h (IL3-HAIG condition) or were cultured without IgG for 6h (IL3 condition). Total cellular EDN released in the supernatant was measured by ELISA using eosinophils from eleven donors.



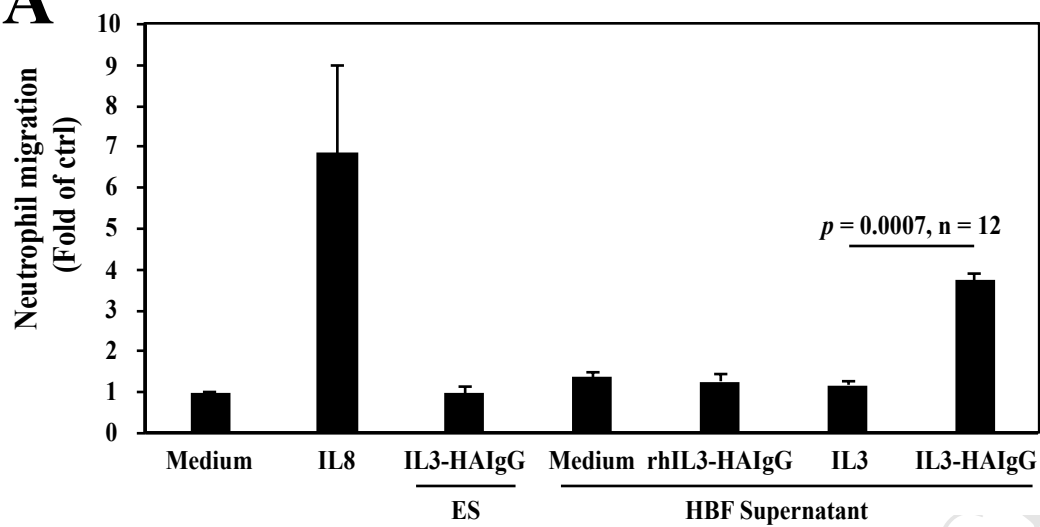
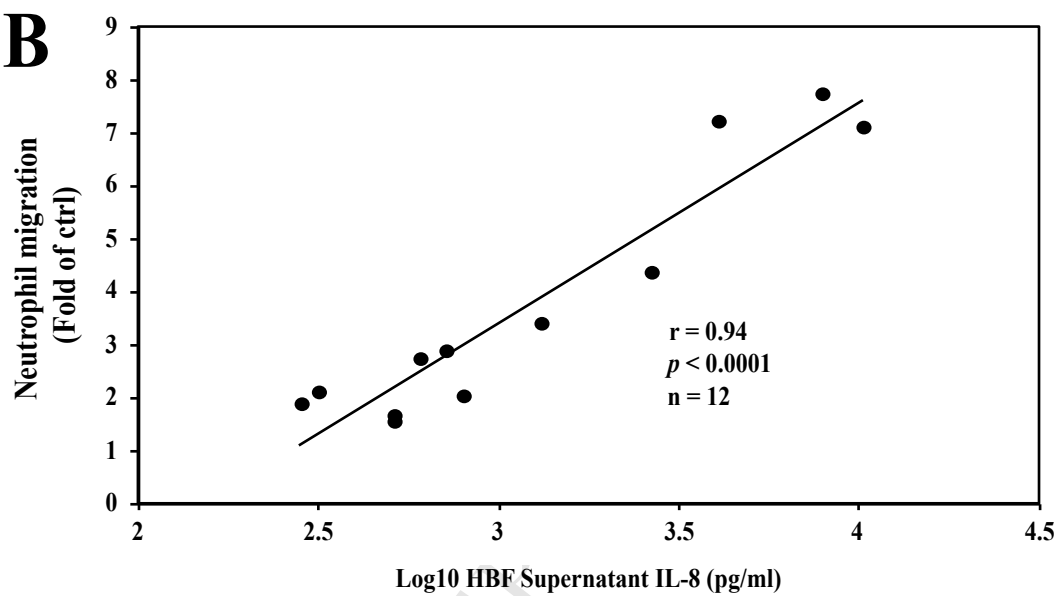
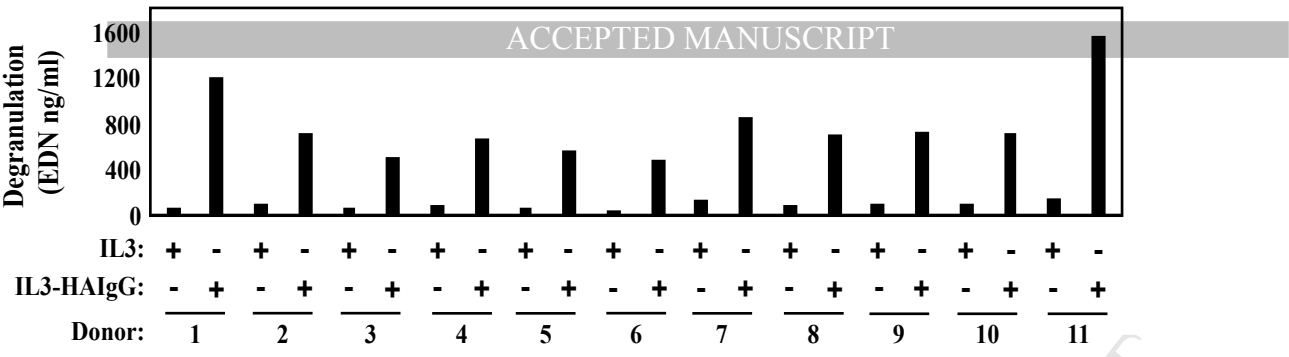
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Table E1: Blood eosinophil and neutrophil donors' characteristics

Donor #	Cell used	Sex	Age	Asthma	Rhinitis	Allergy
1	Eos	Male	45	Yes	No	Yes
2	Eos	Female	30	Yes	Yes	Yes
3	Eos	Male	49	No	Yes	Yes
4	Eos	Female	54	Yes	Yes	Yes
5	Eos	Female	51	No	Yes	Yes
6	Eos	Female	23	No	Yes	Yes
7	Eos	Male	49	Yes	Yes	Yes
8	Eos	Female	30	No	Yes	Yes
9	Eos	Male	41	Yes	Yes	Yes
10	Eos/Neut	Male	25	No	Yes	Yes
11	Eos/Neut	Female	52	Yes	Yes	Yes
12	Neut	Male	21	Yes	Yes	Yes

Eos, eosinophils; Neut, neutrophils



Methods

Subjects and cell preparation

All of the study protocols were approved by the University of Wisconsin-Madison Health Sciences Institutional Review Board. Informed consent was obtained from subjects prior to participation.

Blood eosinophils and neutrophils were from donors with known allergy, rhinitis and/or asthma and were purified as previously described¹. Briefly, heparinized blood was centrifuged through Percoll (1.090 g/ml) and the red blood cells (RBC) were lysed from the granulocyte-containing pellet. This polymorphonuclear leukocyte fraction, which consist of approximately 92% neutrophils with 5% eosinophils, was used for neutrophil studies. Eosinophils were purified by negative selection using anti-CD16, anti-CD3, anti-CD14 and anti-glycophorinA immunogenic beads (Mylteni, Bergisch Gladbach, Germany) leaving behind eosinophils with purity and survival of >99%.

Human bronchial fibroblasts (HBF) were obtained from healthy non-atopic non-smoker adults. Bronchial biopsy specimens were obtained via bronchoscopy and de-identified. Histologic assessment of the specimen was performed to ensure normal bronchial tissue architecture. HBF were isolated as previously described². Briefly, tissue specimen were digested at 4 °C in fibroblast growth medium (FGM Bulletkit, cc-3132 Lonza, Basel, Switzerland) containing collagenase H (1 mg/ml) and then cultured in FGM Bulletkit medium which included 2% fetal bovine serum (FBS), human recombinant insulin (CC-4021J, Lonza), recombinant human fibroblast growth factor-B (CC-4065J, Lonza) and gentamycin sulfate amphotericin B (GA1000, CC4081J, Lonza). Expanded populations of fibroblasts were sub-cultured every several days, resulting in the development of a homogenous fibroblast population. All primary culture experiments were performed on HBF from passages 2-4 and maintained on tissue culture plastic.

Preparation of eosinophil-conditioned media

As previously described^{1,3}, peripheral blood eosinophils were cultured at 1×10^6 /ml in medium, termed RPMI 10% FBS (consisting of RPMI 1640 with L-glutamine and 25 mM HEPES (Corning, Corning, NY), 10% FBS (Gibco, Thermo Fisher Scientific, Verona, WI, USA) with antibiotic/antimycotic (Gibco), 2mM L-glutamine (Gibco) and 100 mg/ml ciprofloxacin-HCL) with IL3 (4 ng/ml, R&D Systems Inc., Minneapolis, MN, USA) for 20 h. After 20 h stimulation with IL3, eosinophils were washed and suspended at 1×10^6 /ml in fresh medium (no cytokine), and 1 ml was added to a 24-well plate, which had been coated overnight with heat-aggregated human serum immunoglobulin G (HAIGG, 10 μ g/ml; 500 μ l/well; Sigma, I-2511) and saturated with 0.1 % gelatin for 30 min at 37 °C. Heat aggregation of human IgG was performed in PBS for 30 min at 63 °C. IL3-preactivated eosinophils were also seeded on uncoated (no HAIGG) wells, as the negative control for eosinophil degranulation. After a 6 h incubation, the two types of supernatant fluids (conditioned media: IL3 and IL3 on HA-IgG (IL3-HAIGG)) were harvested and stored at -80 °C, before analysis by ELISA for EDN, IL8, and IL6 release, and before addition on cultured HBF.

Reverse transcription quantitative real time PCR

Real time PCR was done as described previously¹. Briefly, total RNA was harvested and subjected to random-primer reverse transcription using the Superscript III system (Invitrogen/Life Technologies, Grand Island, NY, USA). Real time PCR analysis was performed using iTaq SYBR Green supermix (172-5121, Bio-Rad, Hercules, CA, USA) or Taqman (for *CXCL1* only; Applied Biosystems, Foster City, CA, USA) in ABI 7500 multicolor real time PCR detection system (Applied Biosystems). Standard curves were performed and efficiencies determined for each set of PCR primer pairs used with the iTaq SYBR Green method, including the housekeeping gene, GUSB. Efficiencies were: 93% (GUSB), 101% (IL8), 95% (IL6) and 96% (ICAM1). Primer sequences were: GUSB Forward (CAGGACCTGCGCACAAGAG), GUSB Reverse (AGCGTGTGACCCCATTC), IL8 Forward (CTTGGCAGCCTTCCTGATTT), IL8 Reverse (TTCTTTAGCACTCCTTGGCAAAA), IL6 Forward (TGCAGATGAGTACAAAAGTCCTGAT), IL6 Reverse (GTGGTTATTGCATCTAGATTCTTTGC), ICAM1 Forward (GCCAGGAGACACTGCAGACA) and ICAM1 Reverse (TGGCTTCGTCAGAATCACGTT).

ELISA

IL8 and IL6 proteins were measured utilizing “in-house” sandwich ELISA as previously described⁴. Coating antibodies included mouse monoclonal anti-human IL8 (clone G265-5, BD Biosciences, San Jose, CA) and mouse monoclonal anti-human IL6 (clone 6708, R&D Systems Inc.). Detection antibodies included biotinylated mouse monoclonal anti-human IL8 (clone G265-8, BD Biosciences) and biotinylated polyclonal goat anti-human IL6 (R&D Systems, Inc.). The ELISA assay sensitivities were <3 pg/ml for IL8 and <6 pg/ml for IL6 (to correct for ELISA IL8 and ELISA IL6). Human EDN was measured using an ELISA kit (MBL, Woburn, MA) with minimum detection limit of 0.62 ng/ml¹.

Neutrophil migration assay

Neutrophil migration assay was performed as previously⁵. Briefly, transwell chambers with 3 μ m pore size (Corning, Corning, NY, USA) were coated with 200 μ l of plasma fibronectin at 10 μ g/ml. Chambers were incubated at 4 °C overnight, rinsed with PBS and allowed to dry for at least 2 h. Supernatants were obtained from HBF treated with supernatants from either IL3-HAIGG, IL3-activated eosinophils, or directly with rhIL3 and IgG (rhIL3-HAIGG), or medium alone. These HBF-derived supernatants were diluted in equal volume of RPMI 10% FBS media and 600 μ l added to the bottom of the chamber. Additional controls included undiluted RPMI 10% FBS media, undiluted RPMI 10% FBS + 30 ng/ml IL8, and IL3IgG-activated eosinophil supernatants diluted in equal volume of RPMI 10% FBS (without fibroblast stimulation). Peripheral blood neutrophils were derived as above and 2×10^6 cells/ml were maintained in RPMI 10% FBS at 37 °C for two hours before plating 200 μ l (4×10^5) into the transwell inserts. The number of neutrophils that had migrated into the chamber was counted 30 min following plating into transwells by utilizing trypan blue exclusion and a hemocytometer. The number of neutrophils migrating into the negative control media (RPMI 10% FBS) at 30 min post-plating was considered the control and all other values were represented as the fold of that control.

Statistical analyses

Outcome measures were compared between different treatments using linear mixed-effect analysis of variance (ANOVA) models with fixed effects for treatment and random effects for donor to account for within-donor correlation. Tukey's procedure was used to control the overall significance level for multiple comparisons between treatments. Measurements were log-transformed where appropriate for approximate normality and homogeneity of variance. Relationships between continuous measures were examined using the Pearson correlation of log-transformed values.

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