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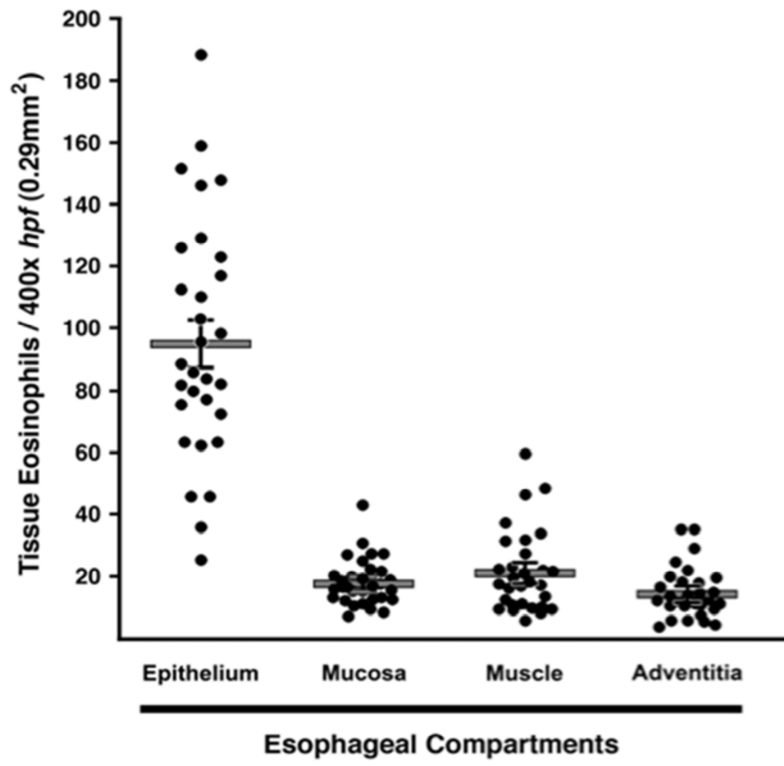

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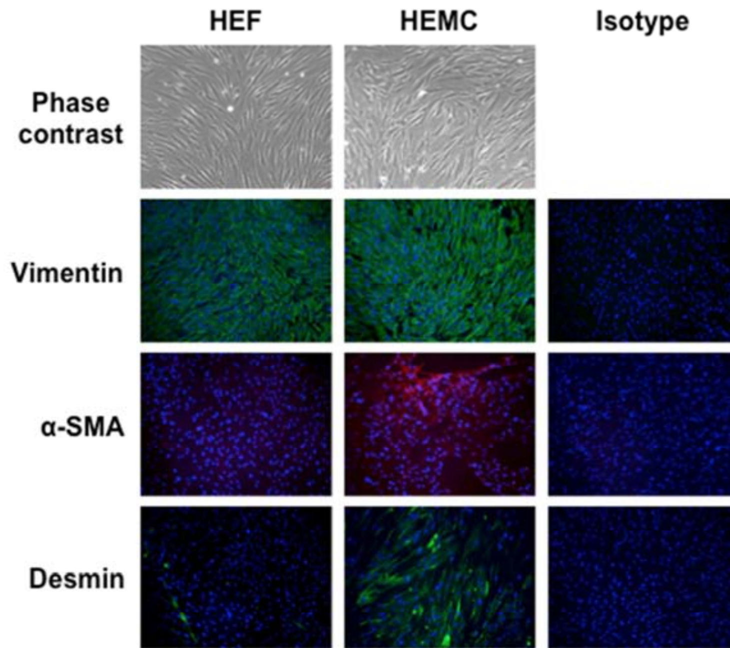

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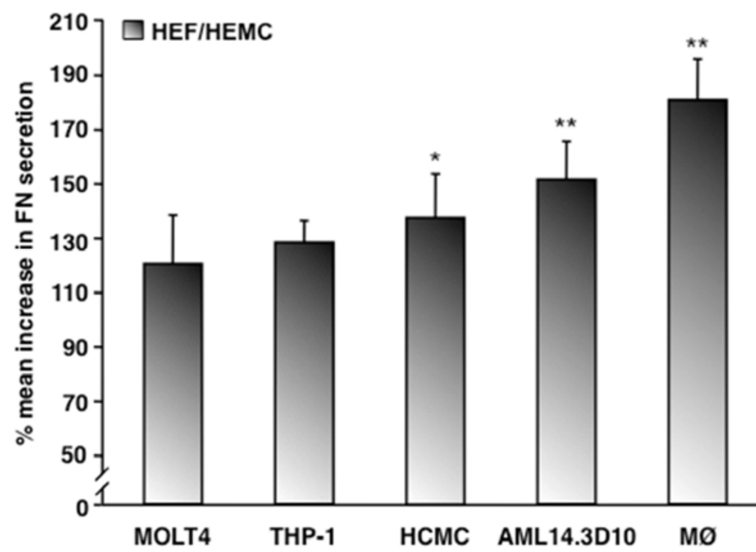

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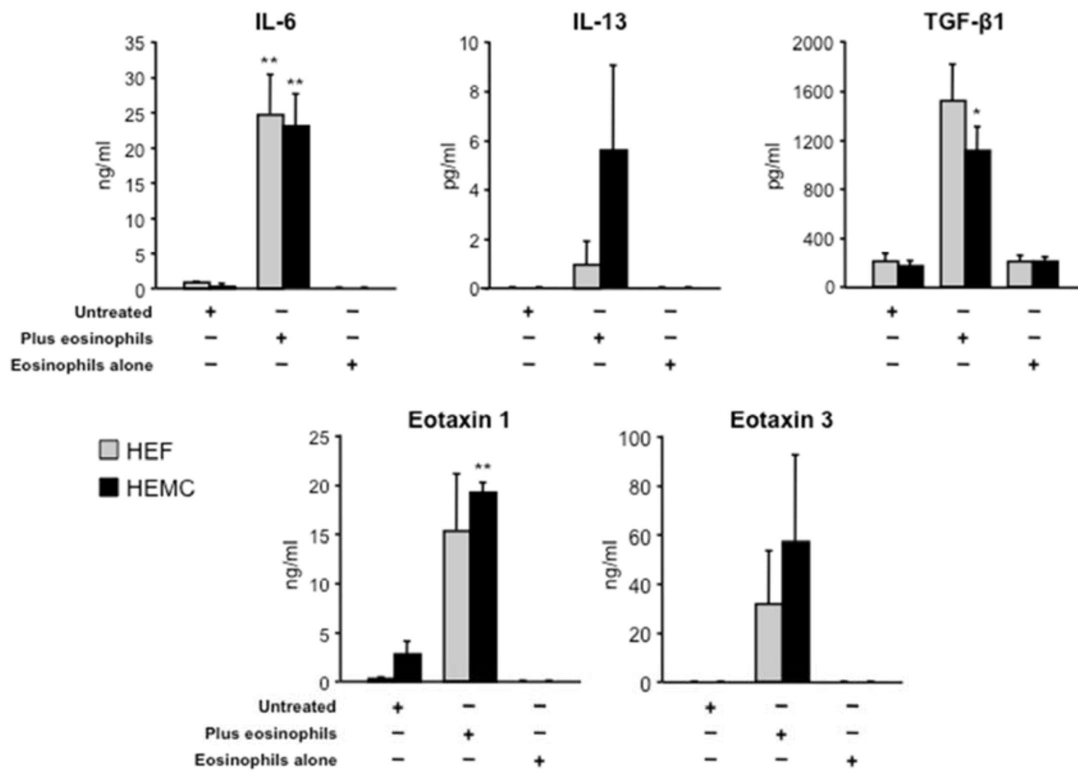

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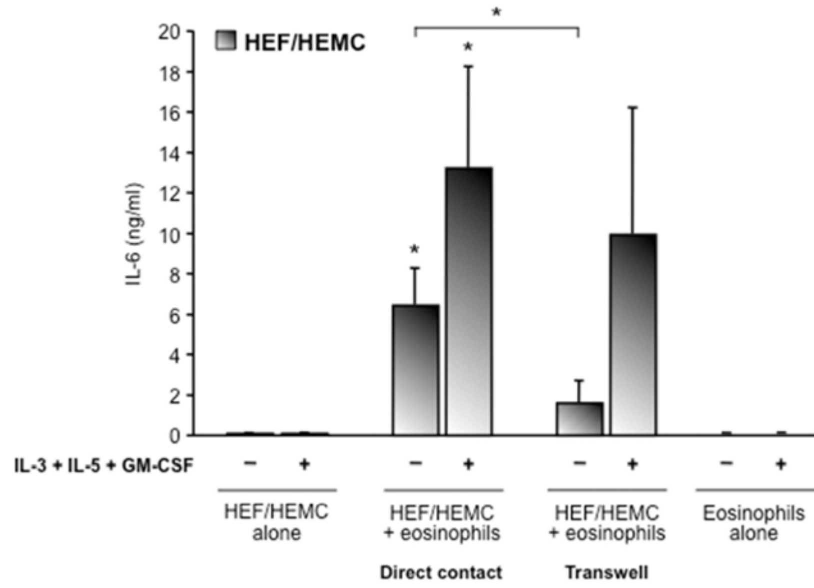

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SUPPLEMENTAL MATERIALS AND METHODS

Full thickness eosinophilic esophagitis specimen

The full thickness esophageal specimen was surgically derived from a 56-year old man with chronic dysphagia and intermittent food impaction. Symptoms of heartburn, but not dysphagia, resolved while taking proton pump inhibition. An upper endoscopy demonstrated rings, longitudinal furrows and white exudates throughout the entire esophagus. Proximal esophageal biopsies showed 40 eosinophils per high power field (eos/HPF) while distal esophageal biopsies showed 50 eos/HPF during long-term proton pump inhibition (PPI). A 1 cm ulcerated nodule was identified at the esophagogastric junction with a biopsy being consistent with esophageal adenocarcinoma. The patient did not receive chemotherapy and underwent gastro-esophagectomy with mediastinal lymph node dissection and creation of esophagogastrostomy. For our study we used the specimen of the proximal, carcinoma-negative margin, located in the resected portion of the mid-esophagus. On time of resection patient was on PPI but not corticosteroid therapy.

Biopsy organ culture

Organ culture for the evaluation of cytokine production was performed as previously reported [1]. Mucosal tissue was placed on a cell culture insert filter (pore size 0.4 µm; Becton Dickinson, Franklin Lakes, NJ) of a 6-well plate containing 1.3 ml of RPMI 1640 (BioWhittaker, Rockland, ME) with 50 U/ml penicillin, 50µg/ml streptomycin and HEPES buffer (Bio-Whittaker) in the lower chamber. The culture was performed in 5% CO_2 at 37^0C overnight, after which the undernatants were recovered and kept at -80^0C until assayed for cytokine content by enzyme-linked immunosorbent assay (ELISA) or the Luminex multiplex platform (Luminex, Austin, TX, USA).

Eosinophil peroxidase and hematoxilin & eosin staining

Serial sections from formalin-fixed and paraffin embedded slides derived from the full thickness esophageal specimen as well as the cohorts of esophageal patient biopsies were stained with (1) Hematoxylin and Eosin (H&E) for general histopathological assessments and (2) immunohistochemistry using a mouse anti-eosinophil peroxidase monoclonal antibody (EPX-mAb) to evaluate eosinophil tissue infiltration and the release of EPX (i.e., eosinophil degranulation) as previously described [2].

Numerical algorithm for the diagnosis of EoE

A previously described algorithm using EPX-mAb-based immunohistochemistry was used to distinguish EoE from control patients [2]. This scoring system is based on a scale from 0 to 50, the extremes of which are representative of the esophagi of control subjects (score 0) and severe EoE patients (score 50), respectively. Histopathologic scoring was performed by three patient/medical history blinded investigators also blinded to each other's scoring. Briefly, diagnostic scoring of patients using EPX-mAb-based immunohistochemistry consists of evaluating the following five staining parameters per subject: (1) Reproducibility, expressed as percent of all biopsies with significant eosinophil infiltration and/or degranulation; (2) Patchiness, the percent area of the maximally affected biopsy showing significant eosinophil infiltration and /or degranulation; (3) Degranulation - the level of extracellular EPX release observed in the maximally affected biopsy and extent of degranulation in multiple tissue biopsies; (4) Peak number of intact eosinophils in a single 400X high powered foci (0.29mm^2); (5) Mean average number of intact eosinophils in five random 400x high powered foci (0.29mm^2). Total EPX staining algorithmic score represents the sum of values derived from each of these five parameters. As noted above, the scoring system scale ranges from 0 - 50. Scores less than 5 are representative of the esophagi of control subjects, scores 5 - 35 are representative of the esophagi of GERD subjects, and EoE patients have scores of 36 -50.

The eosinophil tissue infiltration of the epithelium, mucosa, muscle and adventitia of full thickness esophageal specimen were evaluated by three patient/medical history-blinded investigators assessing the number of eosinophils in each compartment as the mean average counts of ten high powered fields (400X (0.29mm^2)) evenly distributed within a given compartment around the lumen of the esophageal specimen.

Cell Lines (HEF, HEMC, AML14.3D10)

Cell isolation and cultures of primary esophageal fibroblasts and muscle cells derived from human esophagectomy specimens (See Materials and Methods) were performed from the histologically normal margin as previously described [1]. The esophageal tissues were washed and the mucosa was separated from the underlying muscle layers by sharp dissection. Mucosal fragments were placed in Hank's balances salt solution containing 50 U/ml of penicillin and 50 mg/ml of streptomycin (BioWhittaker) for 1h at room temperature, cut in small fragments, and cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 4 mmol/l of L-glutamine, 50 U/ml of penicillin, 50 mg/ml of streptomycin and HEPES buffer as described previously [3]. Mucosal fragments gave rise to an outgrowth of cells with typical fibroblast morphology (3-4 days), they were termed human esophageal fibroblasts (HEF) and by 2-3 weeks confluent monolayers were obtained, which maintained a typical fibroblast morphology.

The muscle tissue was minced into small fragments that were incubated overnight in Hank's balanced salt solution containing antibiotics, 2.5% HEPES, collagenase type II (31 U/ml) and deoxyribonuclease type I (3228 U/mg, Worthington Biochemical Corp, Lakewood, NJ). After filtering the digested tissue, the resulting cell suspension was washed, plated and described for HEFs. After 1-2 weeks clusters of cells were observed and these were grown similar to HEF, giving rise to spindle-like cell monolayers. These cells were termed human esophageal muscle cells

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(HEMC). Cells were maintained in the same culture medium and sub-cultured twice weekly. Cells between passages 3-9 were used. No differences were noted in regard to morphology, marker expression, growth pattern, life span as well as ECM or cytokine secretion among lines from individual subjects, regardless of diagnosis or treatment.

The human eosinophil myelocyte cell line AML14.3D10 [4] corresponds to mature eosinophils and is the most representative human eosinophil cell line for in vitro studies, and was a kind gift of Dr. Fred Hsieh, Department of Pathobiology, Cleveland Clinic, Cleveland, USA. It was cultured in RPMI 1640 media supplemented with 8% FBS, 2mmol/l sodium pyruvate, 1 mmol/l L-glutamine, 50μmol/l b-mercaptoethanol and 1% penicillin/streptomycin and cells were sub-cultured twice weekly. MOLT-4 were cultured as previously described [5]. PBT were isolated and cultured as previously described [6]. Mature human mast cells were kindly provided by Dr. Fred Hsieh, Dept. of Pathobiology, Cleveland Clinic, and developed from umbilical cord blood as described previously [7]. Cells were seeded at 10^6 per ml and cultured in the presence of 100 ng/ml stem cell factor, 50 ng/ml IL-6, and 10 ng/ml IL-10 (R & D Systems) in RPM1 1640-based media. Nonadherent cells were passaged every week into media containing fresh cytokines. Once cells reached maturity, defined by >95% toluidine-blue positivity and positive immunostaining for both tryptase and chymase, they were used for experiments. The human monocytic cell line THP-1 was a kind gift from Dr. Carol de la Motte, Dept of Pathobiology, Cleveland Clinic. Cells were cultured in RPMI 1640 media supplemented with 10% FBS, 1 mmol/l L-glutamine, 1% penicillin/streptomycin, 1% 1M HEPES, 1% non-essential amino acids, 50μmol/l b-mercaptoethanol and 2mmol/l sodium pyruvate and cells were sub-cultured twice weekly. Primary human monocytes were derived from healthy blood donors via elutriation through the Cleveland Clinic CTSC core facility and used immediately after isolation in the adhesion assays.

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Immunofluorescence

Twenty thousand HEF or HEMC/well were seeded onto glass 8-well chamber slides (Nunc, Naperville, IL, USA). After culture under various conditions, slides were rinsed in PBS and fixed with either ice-cold acetone or 4% paraformaldehyde at room temperature (RT) for 10 minutes. Before application of the primary antibody, fixed cells were blocked with 3% FBS in PBS. All primary and secondary antibodies were diluted in 1% FBS. For detection of mesenchymal markers the following antibodies and concentrations were used: mouse anti-human a-smooth muscle actin (Sigma) at 1:50 dilution; mouse anti-human desmin (Abcam, Cambridge, MA, USA) at a 1:100 dilution; mouse anti-human vimentin (BD Bioscience, San Jose, CA) at 1:100 dilution. The respective antibody isotypes (Sigma and Santa Cruz) were used as controls for the primary antibody. After 2-hour incubation with the primary antibody at room temperature, slides were rinsed three times with PBS and the AlexaFluor 488 or AlexaFluor 594 antibody (Molecular Probes, Eugene, OR, USA) was added at a dilution 1:500 for 1 hour at 37^{0}C. For nuclear counterstaining Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) was used to counterstain nuclei. Slides were analyzed using an Olympus microscope and ImagePro software (Bethesda, MD, USA).

Cell culture, co-culture and induction of cytokine and matrix production

40.000 HEF and HEMC cells were plated in 24-well cluster plates, incubated with serum-free medium for 24h and stimulated with interleukin (IL)-4 (5-40 ng/ml; Peprotech, Rocky Hill, NJ, USA), IL-6 (5-40 ng/ml; Peprotech), IL-13 (5-40 ng/ml; Peprotech), TGF-β1 (0.01-1 ng/ml; Peprotech) or serum-free medium for 72h. After determining optimal stimulatory conditions cytokines were used at the following concentrations: IL-4, 20 ng/ml; IL-6, 20 ng/ml; IL-13, 20 ng/ml; TGF-β1, 5 ng/ml.

For co-culture experiments AML14.3D10 were pre-incubated for 24h with or without the presence

of optimal stimulatory doses IL-3 (10 ng/ml, Peprotech), IL-5 (20 ng/ml, Peprotech) or GM-CSF (20 ng/ml, Peprotech), and then added to the HEF and HEMC cultures at ratios from 1:10 to 10:1 with or without the presence of the same cytokines for 72h in 50% HEF and 50% AML14.3D10 culture medium. For transwell experiments transwell plates (Costar, Corning Inc., Corning, NY) with 0.4μm pores were used. HEF and HEMC were cultured as described above in 24-well plates. Eosinophils were resting or activated for 24h prior to co-culture and the activating cytokines IL-3, IL-5 and GM-CSF remained present throughout the rest of the experiments. Transwell inserts containing eosinophils in ratios from 1:10 to 10:1 were added to the wells and the culture medium below the membrane was collected. This approach has been described previously [8].

Cell sonicates were produced after thoroughly washing the cells with PBS. Cells were sonicated with 3-4 five-second bursts (Heat Systems-Ultrasonics Inc., Plainview, NY). The resulting lysate was centrifuged at 10000 rpm for 10 mins at 4°C and the supernatants stored at -80^{0}C until use.

At the end of all the above-described experiments culture supernatants or undernatants were harvested and centrifuged at 300g for 5 minutes to remove any residual eosinophils.

ECM content in the culture medium was measured by FN ELISA (Millipore, Chicago, IL, USA) or aminoterminal propeptide of collagen I (PINP) radio-immunoassay (RIA; Orion Diagnostics, Espoo, Finland). Cytokines were measured using the Luminex multiplex platform (Luminex, Austin, TX, USA) following manufacturers protocols, except for TSLP, which was measured using a Quantikine ELISA kit (R&D Systems, Minneapolis, MN).

Adhesion assay

The adhesion assay was performed according to a previously described method [9]. Briefly, 40.000 HEFs or HEMCs were plated on 24-well cluster plates (Costar) and serumdeprived overnight. Cells were incubated with various mediators or serumfree medium for 72h. Subsequently, the wells were

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thoroughly rinsed with Hank's balanced salt solution and the HEF and HEMC monolayers were overlaid with 1 ml of medium containing 10^6 calcein-labeled AML14.3D10 eosinophils. After 1 hour of HEF/HEMC–eosinophil coculture at 37°C, the wells were gently rinsed 4 times with calcium- and magnesium containing phosphate-buffered saline (PBS) to remove all non-adherent eosinophils. The calcein-labeled eosinophils were automatically counted by microscopic examination of 9 random fields for each well using an Olympus microscope and ImagePro software (Bethesda, MD, USA).

Evaluation of ICAM-1 and VCAM-1 surface expression

Surface expression of HEF or HEMC adhesion molecules was detected using flow-cytometry. HEF and HEMC were cultured in $25cm^2$ culture flasks and incubated with various mediators for 72h. After 72h cells were harvested by a brief treatment with 0.25% trypsin–EDTA and washed once in 2% FBS plus 0.2% sodium azide in D-PBS. This treatment does not alter the expression level of ICAM-1 or VCAM-1 compared with that of untreated cells [10]. Cells were resuspended in 100μl PBS with 5% FBS and 1μl of primary antibody (mouse anti-human ICAM-1, Novocastra, Newcastle, England; mouse anti-human VCAM-1, R&D) for 30 minutes at 4^0C. After washing them twice cells were resuspended in 100μl PBS with 5% FBS and 1μl of secondary antibody (goat anti-mouse IgG AF488, Invitrogen, Eugene, OR, USA) was added for 30 mins at 4^0C. HEF and HEMC were washed, fixed with 0.5% ultrapure formaldehyde and analyzed with FACScan (Becton Dickinson).

Immunoblotting

Protein extraction was performed using a lysis buffer containing 50 mM TRIS pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% Na-deoxycholate and 1% protease and phosphatase inhibitor cocktail (Sigma). The concentration of proteins in each lysate was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's recommendations.

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Immunoblotting was performed as previously described [11]. Equivalent amounts of proteins (20 μg) were fractionated on a 6 or 8% Tris-glycine gel and electrotransferred to a PVDF membrane (Millipore, Billerica, MA). Nonspecific binding was blocked by incubation with 5% milk in 0.1% Tween 20/Tris-buffered saline (Fisher Scientific, Hanover Park, IL) for 30 min., followed by overnight incubation at 4°C with the primary antibody(s). Rabbit anti-human G3PDH (Trevigen, Gaithersburg, MD, USA) at 1:500 dilution, mouse anti-human fibronectin (Becton & Dickinson) at 1:1000 dilution, rabbit anti-human p38 MAPK (Cell signaling) at 1:1000 dilution, rabbit anti-human phospho-p38 MAPK (Cell signaling) at 1:1000 dilution, rabbit anti-human SMAD2 (Cell signaling) at 1:1000 dilution, rabbit anti-human phospho SMAD2 (Cell signaling) at 1:1000 dilution, rabbit anti-human SMAD3 (Cell signaling) at 1:1000 dilution, rabbit anti-human phospho-SMAD3 (Cell signaling) at 1:1000 dilution. Membranes were washed 6 times with 0.1% Tween 20/Tris-buffered saline, incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Sigma), washed again, and incubated with the chemiluminescent substrate (Super Signal; Pierce, Rockford, IL, USA) for 5 minutes, after which they were exposed to film (Kodak). Significant variability in gel loading was assessed by calculating the degree of variability among the different bands of GAPDH expression in the same gel.

Data analysis

Statistical analyses between 2 groups were performed using the paired or unpaired t test and differences between multiple groups were tested using analysis of variance (ANOVA). Results are expressed as the mean +/- SEM. Pearson's chi-square test was used for gender. The sensitivity, specificity, positive and negative predictive values were estimated to assess the validity of each marker in diagnosing EoE. In addition, Receiver Operating Characteristic (ROC) curves were constructed and the areas under the curves (AUC) with their corresponding 95% confidence intervals were estimated. This was done for each cytokine separately, for eosinophil numbers and

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histologic features and the EoE histologic score. SAS version 9.1 software (The SAS Institute, Cary, NC) and R version 2.4.1 software (The R Institute for Statistical Computing Vienna, Austria) were used for all analyses. A $P<0.05$ was considered statistically significant.

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SUPPLEMENTAL RESULTS

Correlation between mediators and EoE histology

We investigated a potential correlation between the levels of the secreted mediators with typical histologic EoE features, namely eosinophil number and degranulation, microabscesses and surface layering. Levels of IL-5, IL-6, IL-13 and eotaxin-1 positively and significantly correlated with all the above-mentioned histologic features, while TGF-β1 levels correlated only with the number of eosinophils (Supplemental Table 3).

To assess the potential discriminatory capacity of cytokine levels for a diagnosis of EoE compared to controls area under the receiver operating curves (AUCs) were constructed revealing values between 0.719 and 0.859 (Supplemental Table 4). Thus, the ability of mediators secreted in EoE tissue was inferior compared to that of classical histologic features and a previously described combined histologic score [2].

Morphologic and phenotypic characterization of human esophageal mesenchymal cells

Phase microscopy and fluorescence immunohistochemistry were performed to characterize HEF and HEMC. All HEF and HEMC were positive for vimentin and the majority (>80%) of HEMC expressed detectable levels of α-SMA and desmin, while these proteins were only occasionally found (<5%) in HEF (Supplemental Fig.2).

Lack of production of TSLP by human esophageal mesenchymal cells

The possible production of thymic stromal lymphopoietin (TLSP) by HEF and HEMC was investigated in view of recent evidence showing the involvement of this cytokine in EoE [12]. Under unstimulated conditions both HEF (n=10) and HEMC (n=8) produced nominal amounts of TSLP at the

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threshold of ELISA sensitivity, and these levels did not increase after stimulation with TGF-β1 (HEF: 28.3 and 28.3 pg/ml, respectively; HEMC: 34.7 and 33.1 pg/ml, respectively) or IL-1β, IL-4, IL-6 and IL-13 (data not shown).

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: Density of eosinophils in all layers of a surgically resected full thickness EoE specimen

Eosinophil density was highest in the epithelium, but they were also abundant in the submucosa, muscle and adventitia. Counts represent 30 individual high power fields per tissue layer.

Supplemental Figure 2: Characterization of HEF and HEMC

After isolation HEF and HEMC were plated on glass slides and assessed for morphology and the typical mesenchymal markers vimentin, α-smooth muscle actin (α-SMA) and desmin by brightfield and fluorescence microscopy. All HEF and HEMC were strongly positive for vimentin; only a small fraction of HEF stained positive for α-SMA and desmin, while the vast majority of HEMC expressed both of these markers. 100X magnification. Figure representative of 6 experiments.

Supplemental Figure 3: Secretion of FN by HEF and HEMC in response to co-culture with different human immune cells

HEF and HEMC were co-cultured for 72h in direct contact with the human T-cell line MOLT4, the human monocytic cell line THP-1, human cord blood derived mast cells (HCMC), the AML14.3D10 cells or primary human monocytes. HEF and HEMC increased secretion of FN upon direct contact with HCMC, AML14.3D10 and monocytes. N=3 each for HEF/HEMC. *$p<0.05$, **$p<0.01$ compared to untreated HEF/HEMC.

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Supplemental Figure 4: Secretion of cytokines by HEF and HEMC in response to co-culture with human eosinophils

HEF and HEMC were co-cultured for 72 with AML14.3D10. Co-culture of both cell types increased secretion of IL-6, TGF-β1 and eotaxin 1; IL-13 and eotaxin 3 were only detected upon co-culture of HEF/HEMC and eosinophils. N=5 for HEF and HEMC. *$p<0.05$, **$p<0.01$.

Supplemental Figure 5: Secretion of IL-6 by HEF and HEMC in response to co-culture with human eosinophils.

HEF and HEMC were co-cultured for 72 h with AML14.3D10 with and without eosinophil activation by IL-3, IL-5 and GM-CSF. Eosinophils in contact with HEF/HEMC increased their secretion of IL-6; eosinophil activation enhanced the IL-6 by HEF and HEMC, and inhibition of direct contact by a transwell system reduced IL-6 secretion. N=4 for HEF/HEMC combined. *$p<0.05$ compared to no eosinophils unless otherwise indicated.

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Supplemental Table 1

Active production of cytokines in eosinophilic esophagitis mucosal biopsy specimens

Cytokine*	EoE#	Control#	P value
IL-5	6.9 [1; 16]	0 [0; 0]	<0.001
IL-6	1280 [566; 2572]	410 [153; 798]	<0.002
IL-13	5 [0; 33]	0 [0; 0]	<0.001
Eotaxin-1	13 [1; 39]	0 [0; 11]	<0.001
TGF-β1	113 [43; 187]	57 [41; 67]	0.006

* Cytokine values are expressed as medians [25th percentile; 75th percentile] of fg/ml/mg of total protein.

N=18 for EoE and n=21 for control for IL-5, IL-6, IL-13, and eotaxin-1; n=14 for EoE and n= 17 for control for TGF-β1.

IL: interleukin; TGF-β1: transforming growth factor-β1

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Supplemental Table 2

Demographic and clinical characteristics of patients that provided esophageal biopsies*

	EoE (n=18)	**Control** (n=21)	**P value**
Variable			
Age at biopsy	36.4±10.9	40.7±132.	0.28[a]
Male gender	11 (61.1)	5 (23.8)	0.018[c]
Race			0.99[c]
White	18 (100)	19 (90.5)	
Black	0 (0.0)	1 (4.8)	
Asian	0 (0.0)	1 (4.8)	
Atopic status (eczema, hay fever, rhinitis, asthma)	14 (77.8)	11 (52.3)	0.099[b]
Medications at time of endoscopy			
None	6 (33.3)	3 (14.2)	0.26[c]
Inhaled or nasal steroids	3 (16.7)	6 (28.5)	0.46[c]
Bronchodilators	3 (16.7)	6 (28.5)	0.46[c]
Non-steroidal anti-inflammatory drugs	0 (0.0)	2 (9.5)	
Other	6 (33.3)	9 (42.8)	0.54[b]
Swallowed steroids	0 (0.0)	0 (0.0)	
Systemic steroids	0 (0.0)	0 (0.0)	
Proton pump inhibitors	7 (38.9)	12 (57.1)	0.26[b]
Previous dilations	4 (22.2)	1 (4.8)	0.16[c]
Symptoms at time of endoscopy			0.009[c]
Dysphagia	15 (83.5)	9 (42.9)	
Heartburn	0 (0.0)	6 (28.5)	
Chest pain	4 (22.2)	2 (9.5)	
Abdominal pain	1 (5.6)	6 (28.6)	
None	1 (5.6)	0 (0.0)	
Endoscopic findings			
Normal	0 (0.0)	14 (66.7)	
EoE	18 (100)	0 (0.0)	
Other (hiatal hernia, gastritis, Schatzki's ring)	0 (0.0)	7 (33.3)	

* Endoscopic esophageal biopsies are not full thickness and mostly reflect what is in the epithelial layer.
Values represent mean±SD or (%)

P values: [a], ANOVA; [b], Pearson's chi square test; [c], Fisher exact test.

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Supplemental Table 3

Correlation between the levels of the secreted mediators with typical histologic EoE features in the biopsy specimens

Cytokine[#]	**Eosinophil number***	**rho**	**Eosinophil degranulation***	**Micro-abscesses***	**Surface layering***
IL-5	<0.001	0.78	<0.001	<0.001	<0.001
IL-6	<0.001	0.58	<0.004	<0.004	<0.001
IL-13	<0.001	0.6	<0.001	<0.001	<0.001
Eotaxin-1	<0.001	0.48	<0.05	<0.01	<0.01
TGF-β1	<0.05	0.53	NS**	NS	NS

[#] N=18 for EoE and n=21 for control for IL-5, IL-6, IL-13, and eotaxin-1; n=14 for EoE and n= 17 for control for TGF-β1.

IL: interleukin; TGF-β1: transforming growth factor-β1.

* P value; ** Not significant; Spearman correlation coefficient for cytokine concentrations versus eosinophil number .

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Supplemental Table 4

Discriminatory capacity of cytokine levels and histologic features for a diagnosis of EoE compared to controls

Discriminatory parameter	AUC (95% CI)
IL-5	0.859 (0.751-0.966)
IL-6	0.786 (0.668-0.904)
IL-13	0.781 (0.655-0.908)
Eotaxin-1	0.790 (0.670-0.910)
TGF-b1	0.719 (0.469-0.969)
Peak eosinophils (H&E stain)	0.986 (0.962-1.000)
Peak eosinophils (EPX stain)	0.991 (0.969-1.000)
Degranulated eosinophils	0.938 (0.866-1.000)
Microabscesses	0.806 (0.690-0.921)
Surface layering	0.899 (0.805-0.994)
Combined histologic score	1.000 (1.000-1.000)

Values derived from the results of Table 3

IL: interleukin; TGF-β1: transforming growth factor-β1; EPX: eosinophil peroxidase; AUC: area under the receiver operating curve; CI: confidence interval

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Author names in bold designate shared co-first authors.

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