Supplemental On-Line Material

MATERIALS AND METHODS

Mice

IL-5 transgenic mice (NJ.1638 [1]) were used as the source of peripheral blood eosinophils and, in turn, eosinophil secondary granules from which eosinophil peroxidase was purified. In addition, gene knockout mice deficient of eosinophil peroxidase were used in the production of anti-EPX monoclonal antibodies. The mice were maintained in ventilated micro-isolator cages housed in the specific pathogen-free (SPF) animal facility at Mayo Clinic Arizona. Protocols and studies involving animals were carried out in accordance with NIH and Mayo Foundation institutional guidelines.

Isolation of mouse eosinophils and eosinophil secondary granules

Eosinophils and, in turn, eosinophil secondary granules were purified from peripheral blood and spleens harvested from four (4) month old IL-5 transgenic mice. Specifically, 1-1.2ml of peripheral blood (>100,000 eosinophils/mm^3, representing 50% of total white blood cells) were collected (20U/ml heparin was added to prevent clotting) from mice via cardiac puncture and stored on ice until use. Following resection, spleens were diced into small sections and sheared through 18, 20 and 22 gauge needles in PBS containing 20U/ml heparin and filtered through a 40µm Nylon mesh (Cat. No. 35-2340, Becton Dickinson, Franklin Lakes, NJ).

Peripheral blood and disassociated splenocytes were pooled and the red blood cells lysed in ice-cold distilled water for 20 seconds prior to low speed centrifugation (1000x g, 10 minutes at 4°C) to collect intact cells. This lysis cycle was repeated as necessary to remove all evidence of red blood cell contamination (usually 2-3 times). The final preparation of leukocytes was resuspended in the RPMI 1640/5% FCS and total cell counts were acquired using a hemocytometer; cell differentials were performed from cytospin preparations counting >300 cells. The recovered leukocytes were overlaid onto Percoll E as described previously and the buffy coats from these discontinuous gradients were harvested [2]. These buffy coat cells were washed with PBS/2% BSA, incubated with antibodies recognizing CD90 and CD45R for 15 min at 4°C (10µl antibody/10^7 cells (Miltenyi Biotec, Inc., Auburn, CA)), and eosinophils were purified to >95% homogeneity using deletion chromatography on a MACS immunomagnetic separation column as per the manufacture's instructions (Miltenyi Biotec, Inc.). Purified eosinophils recovered following MACS were lysed at 37°C for 30 minutes by incubation in 0.5ml of 0.25M sucrose containing heparin (10,000 Units/10^8 cells) and DNase I (100 Units/10^8 Cells). Eosinophil secondary granules were subsequently harvested by high-speed centrifugation of these lysed leukocytes (12,000 x g at 4°C for 20 minutes).

Purification of Eosinophil Peroxidase

Granules derived from ~10^9 purified eosinophils were resuspended in 10^{-4}M HCl and the granules disrupted by probe sonication using a Branson 450 sonifier (Branson Ultrasonics, Danbury, CT) at a constant duty cycle setting using a pulse of 30 seconds. The pH of the resulting sonicate was assessed with pH paper and 5-10µl of 10^{-2}M HCl

was added prior to repeating the sonication of the granules. If necessary, this process was repeated a third time or until the pH of the granule lysate reached 3.5. This acidified suspension was centrifuged at 8,000 x g (20 minutes at 4°C) to remove any acid insoluble proteins/debris. The supernatant from this centrifugation represents the collective sum of the cationic eosinophil secondary granule proteins (ESGPs) from which eosinophil peroxidase was purified.

Protein concentration of the ESGPs was determined by BCA assay (Pierce Endogen, Inc.) and an aliquot of the ESGPs (20 mg) was salt precipitated (4°C for 1 hour) by the addition of ammonium sulphate ($(NH_4)_2SO_4$) to a concentration of 10%. Eosinophil peroxidase and other precipitated proteins were recovered by centrifugation (5000 x g for 20 minutes at 4°C). These ammonium sulphate precipitated proteins were resuspended in 0.025M sodium acetate (NaOAc) pH 5.0 in preparation for size selection using Sepharose G-50 chromatography. EPX and several contaminating proteins within the void volume of the G-50 column were subsequently subjected to HPLC using a CM preparative column. EPX (~70kDa) was uniquely recovered (as judged by silver-stained PAGE) by elution from this column using an increasing gradient of NaCl in 0.025M NaOAc pH 5.0.

Monoclonal antibody production and screening

Monoclonal antibodies recognizing mouse eosinophil peroxidase were generated by repeated sensitization of eosinophil peroxidase knockout mice ($EPX^{-/-}$ [3]) using established methods and/or protocols [4]. Briefly, $EPX^{-/-}$ mice (C57BL/6J background)

were immunized (by intraperitoneal injection) a total four times at 2-4 week intervals with 25µg of purified mouse EPX (injection emulsified in RIBI™ adjuvant (RIBI ImmunoChem Research Inc., Hamilton, MT)). Antibody titers were assessed from blood recovered from the tail vasculature. Immunized mice received a final intravenous injection of 25µg of purified EPX 3-4 days prior to recovery of spleens and generation of antibody secreting hybridomas (Myeloma Fusion Partner (P3X63-Ag8.653, ATCC, Manassas, VA).

Potential hybridomas resulting from the immunization of $EPX^{-/-}$ mice with purified mouse eosinophil peroxidase were screened using a step-wise strategy (~2000 total hybridomas): **(i)** An initial ELISA-based screen selected for IgG-secreting cells (470 IgG^{+} hybridomas); **(ii)** Using purified eosinophil peroxidase and purified eosinophil major basic protein (MBP), all IgG positive hybridomas were subsequently screened by ELISA for reactivity to EPX and the lack of a response to MBP (130 $IgG^{+}/EPX^{+}/MBP^{-}$ hybridomas); **(iii)** Randomly selected hybridomas were cloned by limiting serial dilution (20 hybridomas); **(iv)** These mouse monoclonal anti-mouse eosinophil peroxidase antibodies were finally screened on the basis of their abilities to function in mouse sample-based assays such as immunohistochemistry, western blots, and ELISA, and then on their abilities to detect human eosinophil peroxidase in both immunohistochemical and ELISA formats (seven (7) total hybridomas achieved these criteria). Hybridoma MM25-82.2.1 was identified on the basis of its robust responses in the assays outlined above and was selected for the studies presented.

Tissue preparation and slide production

Esophageal biopsies were fixed in 10% formalin and embedded in paraffin. Four (4) micron thick sections were obtained for traditional histopathology (e.g., staining with hematoxylin-eosin (H&E)) and immunohistochemistry using **EPX-mAb**. H&E sections were analyzed for numbers of inflammatory cells including eosinophils and neutrophils in up to 10 high-power fields, eosinophilic micro-abscesses (aggregates of ≥4 eosinophils), presence or absence of intercellular edema, basal zone hyperplasia >20% of the epithelial thickness, lamina propria papillae elongation to > 2/3 of the epithelial height, lamina propria eosinophils, and lamina propria fibrosis. Eosinophils were counted in the areas where they appeared most numerous. For all cases of histological EoE, accompanying clinical data, and gastric and duodenal biopsies when available were reviewed to exclude eosinophilic gastroenteritis and other inflammatory bowel diseases.

Immunohistochemistry Protocol

Infiltrating intact eosinophils and evidence of eosinophil degranulation (i.e., the presence of free cytoplasmic granules and/or extracellular matrix deposition of ESGPs) were assessed by immunohistochemistry using the mouse monoclonal anti-eosinophil peroxidase antibody (**EPX-mAb**) noted above (MM25-82.2.1). Immunocytochemical staining was performed with Dako detection/visualization reagents purchased from Dako Cytomation (Carpinteria, CA). Positive control slides (eosinophil-containing sections from patients identified by traditional pathological assessments) and negative control slides (both antibody isotype controls and negative tissue control sections) were

included as part of the processing of each group of slides examined. It is noteworthy that this staining protocol was designed specifically for use in typical academic/hospital histology units, requiring no specialized equipment or technical insights beyond those currently available in these settings. Tissue sections were dried in an oven at 65°C for 60 minutes prior to deparaffinization, rehydration, and target retrieval. This extended drying time is necessary to assure maximum adherence of esophageal tissue to the slides. Subsequent to this drying step, the tissue sections were deparaffinized in xylene (three (3) changes in fresh xylene for 5 minutes each) prior to rehydration in a descending series of ethanol/H_2O slide baths. Antigen retrieval [5] was performed on the rehydrated sections at 125°C for 30-60 seconds at a pressure of 17-23psi using a Decloaking Chamber (as per the manufacturer's instructions (Biocare Medical (Concord, CA), Cat No. DC2002)) and 1x Dako Target Retrieval Solution (Dako, Cat. No. S1699). Following antigen retrieval, the slides were rinsed with deionized water and incubated at room temperature in Dako Cytomation Proteinase K (Dako, Cat No. S3020) for 5 minutes (~200µl/slide). Proteinase K digested slides were subsequently rinsed (three (3) times for 5 minutes each) with 1x Dako Wash Buffer (Dako Cytomation, Cat. No. S3006) prior to blocking the slides in preparation of antibody staining with a 10 minute incubation (~200µl/slide) in Dako Dual Endogenous Blocking Solution (Dako Cytomation, Cat No. S2003). The blocked slides were rinsed (one (1) time for 5 minutes) with 1x Dako Wash Buffer. These slides were then incubated (40 minutes) with **EPX-mAb** at a concentration of 10µg/ml. Specifically, **EPX-mAb** (1mg/ml) was diluted 1:100 (~200µl/slide) with Dako antibody diluent with background reducing components (Dako Cytomation, Cat. No. S3022). Negative control slides were stained with IgG2a

mouse antibodies (10µg/ml) derived from antigen-naive wild type mice (Dako Cytomation, Cat. No. X0943) diluted again with Dako antibody diluent with background reducing components. The antibody-stained slides were rinsed (three (3) times for 5 minutes each) with 1x Dako Wash Buffer prior to application of a secondary visualizing biotinylated antibody/streptavidin-alkaline phosphatase (AP) conjugate (Dako Cytomation LSAB 2 System - (AP), Cat. No. K0674) as per the manufacturer's instructions. Specific **EPX-mAb** based staining was visualized with a 10 minute incubation using Permanent Red substrate-chromogen (Dako Cytomation, Cat. No. K0695) followed by a single rinse with distilled water. Permanent Red visualized slides were counter-stained (approximately one (1) minute) with methyl green (Dako Cytomation Ready-to-use Methyl Green, Cat. No. S196230), rinsed in free flowing deionized water, and air dried. Stained slides were dipped once quickly in xylene and cover-slipped with Consul mount/xylene mounting media (Shandon Cat. No. 9990441 (Thermo Scientific, Pittsburgh, PA)) prior to photomicroscopy using a Zeiss Axiophot microscope and a AxioCam MRc5 digital camera.

Eosinophil peroxidase monoclonal antibody production

Monoclonal antibodies reactive to mouse eosinophil peroxidase (EPX) were generated as part of ongoing studies evaluating the role(s) of eosinophils in various disease settings (e.g., asthma and other allergic disorders) as well as assessments of mouse models of human diseases. EPX was purified from peripheral blood and spleen-derived eosinophils of IL-5 transgenic mice (NJ.1638 [1]) as described in the Materials and Methods and outlined in **Supplemental Figure 1 (S-Figure 1)**. Typically, several

hundred micrograms of purified EPX were recovered from the blood/spleen of ten (10) transgenic animals.

Previous attempts to generate mouse monoclonal and/or rabbit polyclonal antibodies with a high degree of reactivity/specificity to either human or mouse EPX that are also useful in a variety of detection platforms (e.g., IHC, western blots, etc.) have been problematic (our unpublished observations); most likely, in part, because of the extraordinary sequence identity that these proteins display among mammalian species (e.g., >94% amino acid identity between mouse and human EPX [6]). We employed the novel experimental strategy of sensitizing knockout mice devoid of eosinophil peroxidase with purified EPX as a means of increasing the immunogenicity of this protein and maximizing the number of available epitopes. This strategy resulted in several hundred antibody-producing hybridomas that met two basic selection criteria: They secreted monoclonal antibodies that displayed reactivity to mouse EPX in a single antibody ELISA format and the monoclonal antibodies were of the IgG subtype. These antibodies were further screened in a stepwise strategy for their utility in detection formats with samples derived from mouse models of disease and subsequently, their applicability in assays utilizing clinical samples derived from patients. Thus, the several hundred hybridomas that were initially generated and preliminarily characterized were further selected to a group of seven (7) monoclonal antibodies on the basis of their utility in multiple detection formats in the mouse including IHC, western blots, and ELISA and their ability to display cross-reactivity with human eosinophil peroxidase in similar assays. A single mouse monoclonal antibody from this selected group (**EPX-**

mAb; MM25-82.2.1) was chosen for further study and used for the histological studies of this report.

Validation of eosinophil-specificity in clinical biopsies

Although our screening strategy generated mouse anti-mouse eosinophil peroxidase monoclonal antibodies with cross-reactivity to human EPX, this strategy did not address questions of eosinophil-specificity in clinical samples or the sensitivity of immunohistochemical assays using this monoclonal antibody. The specificity and sensitivity of an **EPX-mAb** based immunohistochemical assay for human tissue samples were determined through the assessment of bone marrow biopsies derived from patients (processed via several different fixatives and post-biopsy methods (e.g., plus or minus decalcification)) that presented with unique marrow disorders (e.g., lymphoma, idiopathic eosinophilic syndrome, mastocytosis, and myeloproliferative disease (data not shown)). **Supplemental Figure 2 (S-Figure 2)** presents a representative assessment of the marrow from patients with yet another of these marrow disorders, myelodysplastic syndrome. Marrow clot biopsies from myelodysplastic syndrome patients were fixed with formalin prior to paraffin embedding and the generation of 4μm sections. Marrow sections were stained for histological assessments followed serially by **EPX-mAb** based immunohistochemistry. A representative H&E stained section is presented in **S-Figure 2(A)**. This section displays the multitude of leukocyte subtypes characteristic of these biopsies, including megakaryocytes, neutrophils, eosinophils (arrowheads), mast cells, and mononuclear leukocytes such as lymphocytes and monocytes. Subsequent to the staining and photographing of this H&E section, the coverslip was removed following a brief immersion in xylene and the *H&E* removed through the decloaking process associated with antigen retrieval. The destained slide was then subjected to **EPX-mAb** based

immunohistochemistry and the region of the slide previously examined by H&E staining was once again photographed (**S-Figure 2(B)**). This strategy revealed that ***(i)*** Eosinophils identified by H&E staining also stained positive following **EPX-mAb** based immunohistochemistry (e.g., compare the leukocytes with arrowheads in panels **A** *vs.* **B**); ***(ii)*** Positive staining by **EPX-mAb** immunohistochemistry was limited only to eosinophils with no detectable signal derived from any of the other cell types present in the section; ***(iii)*** The **EPX-mAb** based immunohistochemical assay displayed a significant level of sensitivity with the identification of eosinophils possible with a 1:100 dilution of a 1mg/ml **EPX-mAb** stock (i.e., final working concentration of 10µg/ml).

DEVELOPMENT OF A NUMERICAL ALGORITHM FOR THE DIAGNOSIS OF EoE

EPX-mAb based immunohistochemistry of mid-proximal biopsies (>7cm from the esophageal-gastric junction) from EoE patients (**Group I**), GERD patients (**Group II**), and "normal" control subjects (**Group III**) allowed for the identification of several **EPX-mAb** based histopathological markers that correlated with disease pathologies. Beyond the simple appearance of these markers in EoE patients (and to some extent GERD), quantitative differences were also observed suggesting a hierarchy whereby some markers were more likely to be associated with disease than others. In an attempt to stratify esophageal patients, a scoring system was developed with which to identify subjects with EoE (**Supplemental Table 2 (S-Table 2)**). The resolution of the scoring strategy developed was designed low enough to negate/minimize observer to observer variability. Specifically, the observed magnitude of the diagnostic markers examined was scored on a scale of 1 to 4; each marker was further assigned a 1 to 4 priority factor (see detailed descriptions in **Supplemental On-Line Material**) based on the frequency by which they were observed in EoE patients diagnosed by traditional pathological assessments (i.e., previously established diagnostic guidelines). These assessments have been incorporated into a quantitative algorithm that permits a histological scoring strategy for patient diagnosis. The scoring system developed is based on a scale of 0 to 50, the extremes of which are representative of the esophagi of control subjects and severe EoE patients, respectively. The histopathologic scoring of the slides was performed independently by two research lab-based staff and two hospital/clinic-based colleagues, including a senior pathologist with a specialty in GI

diseases.

EPX-mAb Diagnostic Marker 1: Presence or Absence of Infiltrating Tissue Eosinophils

Similar to traditional histopathologic assessments, evidence of eosinophils infiltrating the epithelial areas of esophageal biopsies using **EPX-mAb** based immunohistochemistry **(Figure 1)** was consistently observed among the EoE patients we have examined. Our quantitative assessments of EoE patients identified two parameters that correlated with disease as determined by the current guidelines. The maximal number of eosinophils in a single focus within the available biopsies from a patient and the average number of eosinophils/40x *hpf* as determined from 5 selected areas within the available biopsies.

(A). *Maximum in a single focus:* Patient biopsies were scanned at low (5x, 20mm^2 field of view) power to identify foci associated with elevated numbers of accumulating eosinophils. Selected areas were chosen and the number of intact eosinophils[1]/40x *hpf* was determined. The sample was assigned a "maximum in a single focus score" based on the number of intact eosinophils present as follows: **0** = <2; **1** = 2-5; **2** = 6-10; **3** = 11-14; **4** = ≥15. Similar to traditional histopathologic assessments, any patient that displayed ≥15 eosinophils/40x *hpf* using **EPX-mAb** based immunohistochemistry was in fact an EoE patient. However, GERD patients also potentially displayed this marker, prompting us to

[1] An intact eosinophil is either an **EPX**$^+$ leukocyte or an **EPX**$^+$ cellular fragment associated with a morphologically identifiable eosinophilic nucleus.

differentially weight the importance of this parameter by assigning a priority factor of three (3) and thus create an EPX "Intact eosinophils single focus" score range of 0-12.

(B). *Average of five 40x (i.e., hpf) fields:* A total of five designated[2] 40x fields were examined and the number of intact eosinophils present determined. The average number of intact eosinophils present in five fields was determined and an "average of five designated fields" score based on these counts was determined: **0** = <2; **1** = 2-5; **2** = 6-10; **3** = 11-14; **4** = ≥15. This parameter displayed a one-to-one correlation with an EoE diagnosis, prompting us to assign a priority factor of four (4), the highest among the parameters examined. This yielded an EPX "Average intact eosinophil" score range of 0-16.

EPX-mAb Diagnostic Marker 2: Evidence of Eosinophil Degranulation

A unique observation from our studies of EoE patients using **EPX-mAb** based immunohistochemistry was that degranulation was common (**Figure 2**). The importance of this observation is hard to overestimate because this parameter was independent of identifying intact infiltrating tissue eosinophils. That is, **EPX-mAb** based immunohistochemistry detects not only intact eosinophils but also eosinophil degranulation in areas with nominal numbers of intact eosinophils. Moreover, our

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[2] Designated fields are defined as microscopic fields at the center of the five largest biopsies available from a given patient. In cases with less than five available biopsies, designated fields are defined as microscopic fields at the center of the available biopsies with additional fields included as needed to a total of five. These additional fields are taken from the largest available biopsy, starting first in the upper left quadrant and successively moving in a clockwise fashion to the other quadrants of the biopsy, as needed. It is noteworthy that these designations of fields is

examination of EoE vs. GERD patients showed that extensive degranulation in the biopsies was always associated with EoE, whereas GERD patients displayed lower levels of degranulation. This has led to the identification of two parameters that were used as part of our histopathologic scoring algorithm.

(A). *Level of degranulation in the maximally affected biopsy*[3]: The level of degranulation observed within the maximally affected patient biopsy was determined by scanning 40x *hpf* **(Supplemental Figure 3 (S-Figure 3))** using the following scale: **0** = No identifiable[4] eosinophil infiltration or degranulation observed at high (40x, 0.29mm^2 field of view) power in any of the available biopsies; **1** = The presence of >3 intact eosinophils (i.e., eosinophilic microabscesses) in at least a single 40x *hpf* with no evidence of extracellular release of EPX; **2** = EPX extracellular release is evident in at least a single 40x *hpf* but is limited to areas surrounding >3 otherwise intact eosinophils; **3** = The presence of enucleated eosinophils (i.e., cytoplasmic fragments), the presence of free granules (i.e., EPX-containing secondary granules not associated with fragmented eosinophils, and/or nominal evidence of EPX extracellular matrix deposition in at least a single 40x *hpf*; **4** = Biopsies containing at least a single 40x *hpf* that displayed robust eosinophil degranulation (i.e., release of EPX) characterized by the presence of free secondary granules and extensive (>50%

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independent of identifying areas of localized concentrations of infiltrating eosinophils and thus are likely to yield average scores that, if anything, may underestimate the density of eosinophils in affected areas.

[3] The maximally affected biopsy is subjectively defined by scanning the available patient biopsies at low (5x, 20mm^2 field of view) power, identifying the one with the greatest percent area displaying a significant eosinophil infiltration and/or degranulation.

of the field) extracellular matrix deposition of EPX. The correlation of this parameter with EoE was not absolute as not all patients with a diagnosis via traditional pathologic assessments displayed the maximal level (i.e., level 4) and some GERD patients also displayed the maximal level of this marker. This prompted us to differentially weight the importance of this parameter by assigning a priority factor of two (2) and thus create an EPX "Degranulation in the maximally affected biopsy" score range of 0-8.

(B). *Eosinophil degranulation in multiple biopsies*: Eosinophil degranulation associated with a given patient was further evaluated on the basis of whether multiple (i.e., ≥2) biopsies display at least a single 40x *hpf* with eosinophil degranulation characterized as level 3 or 4. If yes, the degranulation EPX score was increased by two (2) points. If the answer was no, additional degranulation points were not assigned. The inclusion of these additional degranulation points as part of our biopsy evaluations derives from our assessments of EoE patients showing that eosinophil degranulation within multiple biopsies occurred more frequently in EoE (>90% of all cases examined) and thus provided a slightly greater predictive value of this disease relative to degranulation occurring in a single biopsy.

EPX-mAb Diagnostic Marker 3: The Extent of Eosinophil Infiltration and/or Eosinophil Degranulation in the Maximally Affected Biopsy

[4] Identifiable eosinophil infiltration or degranulation is defined as >3 intact eosinophils in at least a single 40x *hpf* and/or >10% of any single 40x *hpf* displaying evidence of extracellular deposition of **EPX**.

EPX-mAb immunohistochemistry allowed for the assessments at low (5x, 20mm^2 field of view) power of entire sets of biopsies (**Supplemental Figure 4 (S-Figure 4)**). This ability facilitated the identification and preliminary characterization of the maximally affected biopsy among the available tissue fragments. Significantly, our preliminary assessments of the maximally affected biopsies from EoE patients demonstrated that the extent to which this biopsy displayed significant eosinophil infiltration and/or degranulation was nominally predicative of disease. This parameter ("Patchiness") was incorporated in the histopathologic scoring algorithm on the basis of the percent area of the maximally-affected biopsy displaying significant eosinophil infiltration and/or degranulation using the following scale: **0** = <10%; **1** = 10-24%; **2** = 25-49%; **3** = 50-74%; **4** = 75-100%. Because the maximal level of "Patchiness" (i.e., level 4) was common in EoE (i.e., >85% of all cases examined), and was never observed in GERD patients, this parameter was predictive of disease. This prompted us to assign a priority factor of two (2), yielding an EPX "Patchiness" score range of 0-8.

EPX-mAb Diagnostic Marker 4: The Extent of Eosinophil Infiltration and/or Eosinophil Degranulation among the Available Patient Biopsies

The ability to scan and identify quickly/efficiently eosinophils and/or degranulation among multiple available biopsies from individual EoE patients provided yet another parameter that appeared to correlate with disease. Specifically, examination at low (5x, 20mm^2 field of view) power of the available biopsies from patients following **EPX-mAb** immunohistochemistry (**Supplemental Figure 4 (S-Figure 4)**) often showed that only a fraction of the available biopsies displayed significant eosinophil infiltration and/or

eosinophil degranulation, thus representing yet another parameter that correlated with the probability of an EoE diagnosis. This parameter ("Reproducibility") was incorporated in the histopathologic scoring algorithm as percentage of the available patient biopsies that display significant eosinophil infiltration and/or degranulation using the following scale:

0 = <1%; **1** = 1-24%; **2** = 25-49%; **3** = 50-74%; **4** = 75-100%. Similar to the limited extent by which the parameter "Patchiness" was associated with a differential diagnosis of EoE, the parameter "Reproducibility" was common (i.e., >80% of all cases examined) in both EoE and GERD. Accordingly this parameter was assign a minimal priority factor of one (1), yielding an EPX "Reproducibility" score range of 0-4.

This **EPX-mAb** based immunohistochemistry scoring system was applied to EoE, GERD, and control subjects (see *Human Subjects*, **Materials and Methods**). The results from application of this scoring system were summarized in **Figure 3** and **Supplemental Table 3** (**S-Table 3**).

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1 (S-Figure 1)

A schematic review of the protein purification strategy of mouse eosinophil peroxidase. Blood/spleen eosinophils from the IL-5 transgenic line of mice NJ.1638 were the source material for the purification of eosinophil peroxidase. The strategy (described in detail in the Materials and Methods) uses both physical means of eosinophil isolation (discontinuous Percoll E gradient centrifugation) as well as cell surface marker selection (MACS) to achieve the isolation of sufficient numbers of eosinophils that also displayed a purity of >95% (contaminating leukocytes include neutrophils and monocytes). The subsequent purification of eosinophil peroxidase occurred in a three step approach: ***(i)*** Cell lysis, physical separation/isolation of secondary granules, and the disruption/sonication of granules to isolate acid soluble proteins; ***(ii)*** Separation of acid soluble proteins by size selection chromatography; ***(iii)*** Purification of eosinophil peroxidase by preparative HPLC. The photomicrographs provided include a representative peripheral blood film from NJ.1638 mice and a cytospin of the resulting purified eosinophil population used to isolate eosinophil peroxidase. In both cases, the slides were stained with Wright-Giemsa (Diff-Quik, Fisher (Dade Behring Inc. (Newark, DE)) and coverslipped prior to photo-documentation. Purified secondary granules were fixed and subjected to electron microscopy as previously described [7]. The electron photomicrograph (original magnification, 512,500x) is representative of the isolated granule fraction containing predominantly membrane-bound secondary granules with a distinct electron microscopic morphology: major basic

protein (MBP)-derived electron-dense cores surrounded by an electron-translucent matrix area that contains the remaining abundant acid-soluble granule proteins (i.e., eosinophil peroxidase and the eosinophil associated ribonucleases).

Supplemental Figure 2 (S-Figure 2)

Immunohistochemistry of human bone marrow biopsies shows that the mouse anti-mouse eosinophil peroxidase monoclonal antibody MM25-82.2.1 is eosinophil-specific displaying no reactivity towards other human leukocytes. A bone marrow biopsy from a patient with myelodysplastic syndrome was initially subjected to hematoxylin-eosin (H&E) staining and a selected field was photographed allowing for a cell differential. After photography, the coverslip was removed (brief incubation in xylene) and the hematological stains removed as part of the antigen retrieval process). **EPX-mAb** based immunohistochemistry was performed on the destained slide and the exact location of the previously photographed H&E field was determined and photographed for comparison. This strategy demonstrated that among all the marrow-derived leukocytes only eosinophils were identified by staining with **EPX-mAb** (corresponding arrowheads in both photographs identify a subset of these eosinophils). Scale bar = 20μm.

Supplemental Figure 3 (S-Figure 3)

Eosinophilic esophagitis patients display quantitatively different levels of eosinophil degranulation that correlates with disease severity. Representative photomicrographs at high (40x, 0.29mm^2 field of view) power of the four described

levels of eosinophil degranulation within esophageal biopsies. (**A**) Degranulation Level 1: The presence of >3 intact eosinophils with no extracellular release of EPX; (**B**) Degranulation Level 2: Release of EPX is evident (i.e., degranulation is observed) but limited to areas surrounding >3 intact eosinophils; (**C**) Degranulation Level 3: Detection of eosinophil cytoplasmic fragments and free granules, including limited areas of extracellular matrix deposition; (**D**) Degranulation Level 4: Extensive EPX extracellular matrix deposition and detection of free granules in >50% of at least a single microscopic field. Scale bar = 50μm.

Supplemental Figure 4 (S-Figure 4)

EPX-mAb based immunohistochemistry demonstrated that often only a fraction of available biopsies displayed either focal areas of eosinophil accumulation and/or areas of eosinophil degranulation. A representative low (5x, 20mm^2 field of view) power photomicrograph is shown that contains nine (9) available biopsies from an EoE patient, highlighting the often observed heterogeneity encountered with these biopsies. Scale bar = 100μm.

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S-Table 1. Clinical and Endoscopic Assessments as well as Clinicopathologic Diagnoses of Esophageal Study Patients

Patient Group (Cohort)		Patient Number	Age (Years)	Clinical Symptoms	Endoscopic Observations	Clinicopathologic Diagnosis
I	(Adult)	1	53	Dys, Imp, Ref	Ery	EoE
		2	37	Imp, Ref	HH, Ery	EoE
		3	72	Dys, Imp, Vom	HH, Ring	EoE
		4	38	Imp, Ref	Unremarkable	EoE
		5	63	Dys, Imp, Ref	Ery	EoE
		6	50	Dys, Imp, Ref	Ery	EoE
		7	38	Dys	Unremarkable	EoE
		8	25	Dys, Ref, Vom, CP	Unremarkable	EoE
		9	26	Dys, Imp, Ref, AP	Ring, HH	EoE
		10	53	Dys, Imp, Ref, Vom	Ring, Fur, Ery	EoE
		11	54	Dys	Ring, Ery	EoE
		12	28	Dys	Str, HH, Ery	EoE
		13	53	Dys, Imp, Vom	Ring, Fur, HH, Ery	EoE
		14	47	Dys, Imp, Ref	Ring, Fur, Ery	EoE
		15	75	Dys, Imp, Ref, CP	Ring, HH, Ery	EoE
		16	32	Dys, Imp, Ref	Unremarkable	EoE
		17	72	Dys, Imp, Ref	Ery	EoE
		18	47	Dys, Imp, Ref, CP	Ery	EoE
		19	54	Dys, Ref, AP	Ring, HH, Ery	EoE
	(Pediatric)	20	2	Ref, Vom	Fur, Plq	EoE
		21	2	Vom, Feed	Fur	EoE
		22	10	Dys, Ref	Fur, Plq	EoE
		23	9	Vom	Ring, Plq	EoE
		24	2	AP, Vom, Feed	Unremarkable	EoE
		25	2	Feed, Vom, Dys	Unremarkable	EoE
		26	2	FTT	Plq	EoE
II	(Adult)	27	68	Ref	HH, Ery	GERD
		28	45	Dys, Ref	HH, Ery	GERD
		29	58	Ref, Vom	HH, Ery	GERD
		30	80	Ref	HH	GERD
	(Pediatric)	31	3	Ref, Vom	Unremarkable	GERD
		32	18	AP, Ref	Ery	GERD
		33	1	Vom	Unremarkable	GERD
III	(Adult)	34	47	Ref	Ring, HH	Control
		35	52	Dys, Ref, Vom	Unremarkable	Control
		36	57	AP	Unremarkable	Control
		37	51	AP	Unremarkable	Control
		38	64	Dys, Imp, Vom	Unremarkable	Control
		39	78	Dys, Vom	Unremarkable	Control
	(Pediatric)	40	0.67	Vom	Unremarkable	Control
		41	2	Feed	Unremarkable	Control
		42	9	Ref, AP	Unremarkable	Control
		43	16	AP	Unremarkable	Control
IV	(Adult)	44	44	Dys, Imp, Vom	Ring, Fur, Ery	Indeterminate
		45	38	Dys, Imp, Ref	Ring, Str, HH	Indeterminate
		46	40	Dys, Ref	Ery	Indeterminate
		47	29	Imp	Ring, Ery	Indeterminate
	(Pediatric)	48	17	AP	Unremarkable	Indeterminate
		49	4	Feed	Unremarkable	Indeterminate
		50	3	Vom, Ref	Unremarkable	Indeterminate
		51	15	Vom	Unremarkable	Indeterminate
		52	7	AP	Unremarkable	Indeterminate
		53	13	Dys, Imp	Ery	Indeterminate
		54	3	AP	Unremarkable	Indeterminate
		55	13	AP, FTT	Fur	Indeterminate

Key of Clinical and Endoscopic Patient Assessments

AP = Abdominal Pain
CP = Chest Pain
Dys = Dysphagia
Ery = Erythema
Feed = Feeding Intolerance
Fur = Furrowing
FTT = Failure to Thrive
HH = Hiatal Hernia
Imp = Impaction
Ring = Ring Structures
Ref = Reflux
Str = Strictures
Vom = Vomiting
Plq = Plaque

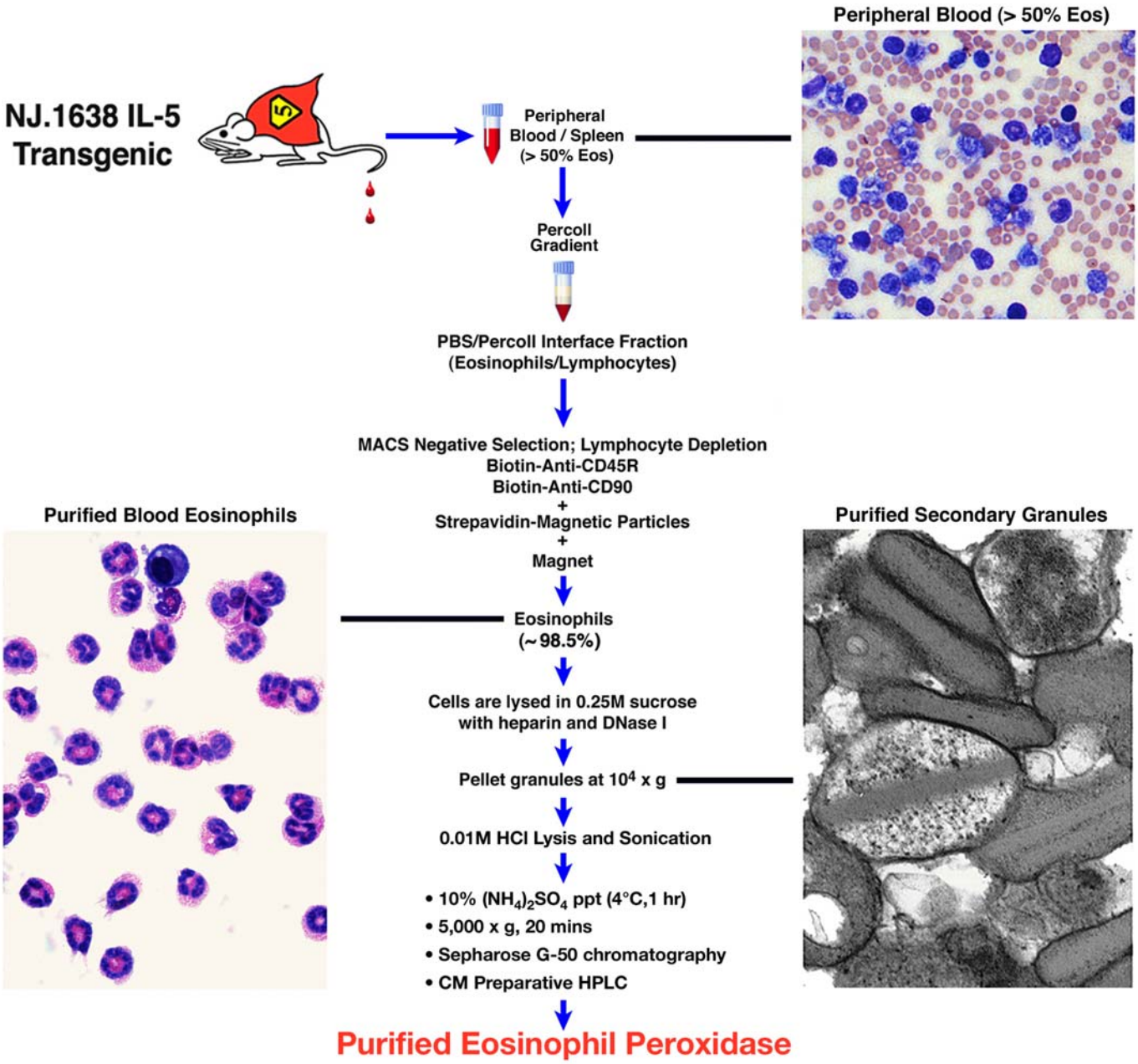
S-Table 2. Worksheet for calculation of EPX-mAb based Immunohistochemistry Diagnostic Scoring

EPX-mAb Staining Parameter	Numerical Score					Priority Factor	EPX-mAb Staining Parameter Score
Reproducibility	Percent of all biopsies with signficant eosinophil infiltration and/or degranulation					**1**	
	0 (<1%)	**1** (1-24%)	**2** (25-49%)	**3** (50-74%)	**4** (75-100%)		
Patchiness	Percent area of the maximumly affected biopsy showing significant eosinophil infiltration and/or degranulation					**2**	
	0 (<10%)	**1** (10-24%)	**2** (25-49%)	**3** (50-74%)	**4** (75-100%)		
Degranulation	**Part 1** - Level of degranulation observed in maximumly affected biopsy					**2**	
	0	**1**	**2**	**3**	**4**		
	Part 2 - Extent of Level 3 or4 degranulationn either ≤ 1 or ≥2 biopsies					**1**	
	0 (≤1 biopsy)		**2 (≥2 biopsies)**				
Eosinophil Infiltrate: Maximum Single Focus	Number of intact eosinophils - Peak value in a single 40x hpf					**3**	
	0 (<2)	**1** (2-5)	**2** (6-10)	**3** (11-14)	**4** (≥15)		
Eosinophil Infiltrate: Average of Five Designated Foci	Number of intact eosinophils - Peak value in an average of five (5) 40x hpfs					**4**	
	0 (<2)	**1** (2-5)	**2** (6-10)	**3** (11-14)	**4** (≥15)		
Total EPX-mAb based Immunohistochemistry Score							
Scoring Scale: 0 - 50							
<5 ≡ Control	**5 - 35 ≡ GERD**					**36 - 50 ≡ EoE**	

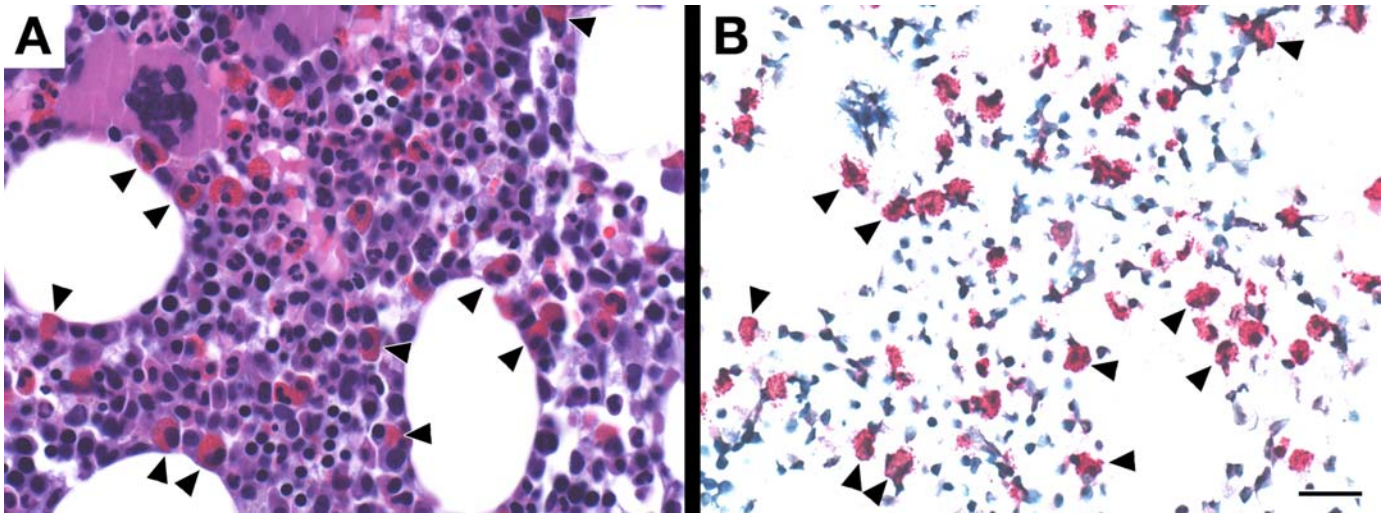
S-Table 3. Intra/Inter observer-blinded assessments of patients using EPX-mAb based immunohistochemistry

Patient Group (Cohort)		Patient Number	Traditional Pathology Diagnosis	EPX-mAb Staining Scores						Total EPX Score (A+B+C+D+E)	EPX-based Diagnosis
				Reproducibility (A)	Patchiness (B)	Degranulation (C)		Eosinophil Infiltrate: Maximum Single Focus (D)	Eosinophil Infiltrate: Average of Five Designated Foci (E)		
						Part 1	Part 2				
I	(Adult)	1	EoE	4	8	8	2	12	8	42	EoE
		2	EoE	4	8	8	2	12	8	42	EoE
		3	EoE	4	8	8	2	12	12	46	EoE
		4	EoE	4	6	6	2	12	16	46	EoE
		5	EoE	4	8	8	2	12	16	50	EoE
		6	EoE	3	6	6	0	12	12	39	EoE
		7	EoE	4	8	6	2	12	16	48	EoE
		8	EoE	4	8	8	2	12	12	46	EoE
		9	EoE	4	8	6	2	12	12	44	EoE
		10	EoE	2	6	6	0	12	16	42	EoE
		11	EoE	4	8	8	2	12	16	50	EoE
		12	EoE	4	6	8	2	12	16	48	EoE
		13	EoE	4	8	8	2	12	8	42	EoE
		14	EoE	4	8	6	2	12	16	48	EoE
		15	EoE	4	8	8	2	12	16	50	EoE
		16	EoE	4	8	8	2	12	16	50	EoE
		17	EoE	4	8	8	2	12	16	50	EoE
		18	EoE	4	8	8	2	12	16	50	EoE
		19	EoE	4	8	6	2	12	16	48	EoE
	(Pediatric)	20	EoE	4	8	8	2	12	16	50	EoE
		21	EoE	4	8	8	2	12	16	50	EoE
		22	EoE	4	8	6	2	12	8	40	EoE
		23	EoE	4	8	8	2	12	16	50	EoE
		24	EoE	4	6	6	2	12	8	38	EoE
		25	EoE	4	8	8	2	12	16	46	EoE
		26	EoE	4	8	6	2	12	16	50	EoE
II	(Adult)	27	GERD	3	0	4	0	9	4	20	GERD
		28	GERD	2	2	4	0	3	4	15	GERD
		29	GERD	3	6	6	2	12	4	33	GERD
		30	GERD	3	2	6	2	3	4	20	GERD
	(Pediatric)	31	GERD	3	2	4	0	6	4	19	GERD
		32	GERD	0	0	0	0	0	0	0	Control
		33	GERD	3	0	6	0	3	0	12	GERD
III	(Adult)	34	Control	0	0	0	0	0	0	0	Control
		35	Control	0	0	0	0	0	0	0	Control
		36	Control	0	0	0	0	0	0	0	Control
		37	Control	0	0	0	0	0	0	0	Control
		38	Control	0	0	0	0	0	0	0	Control
		39	Control	0	0	0	0	0	0	0	Control
	(Pediatric)	40	Control	0	0	0	0	0	0	0	Control
		41	Control	0	0	0	0	3	0	3	Control
		42	Control	0	0	0	0	0	0	0	Control
		43	Control	0	0	0	0	3	0	3	Control
IV	(Adult)	44	Indeterminate	4	8	8	2	9	8	39	EoE
		45	Indeterminate	3	4	6	2	6	4	25	GERD
		46	Indeterminate	4	8	8	2	9	8	39	EoE
		47	Indeterminate	4	8	8	2	9	8	39	EoE
	(Pediatric)	48	Indeterminate	4	6	6	2	12	12	42	EoE
		49	Indeterminate	4	4	4	0	6	4	22	GERD
		50	Indeterminate	4	8	6	2	12	16	48	EoE
		51	Indeterminate	4	8	8	2	12	16	50	EoE
		52	Indeterminate	4	8	8	2	12	16	50	EoE
		53	Indeterminate	4	4	8	2	12	16	46	EoE
		54	Indeterminate	2	0	4	0	6	4	16	GERD
		55	Indeterminate	4	6	8	2	12	12	44	EoE

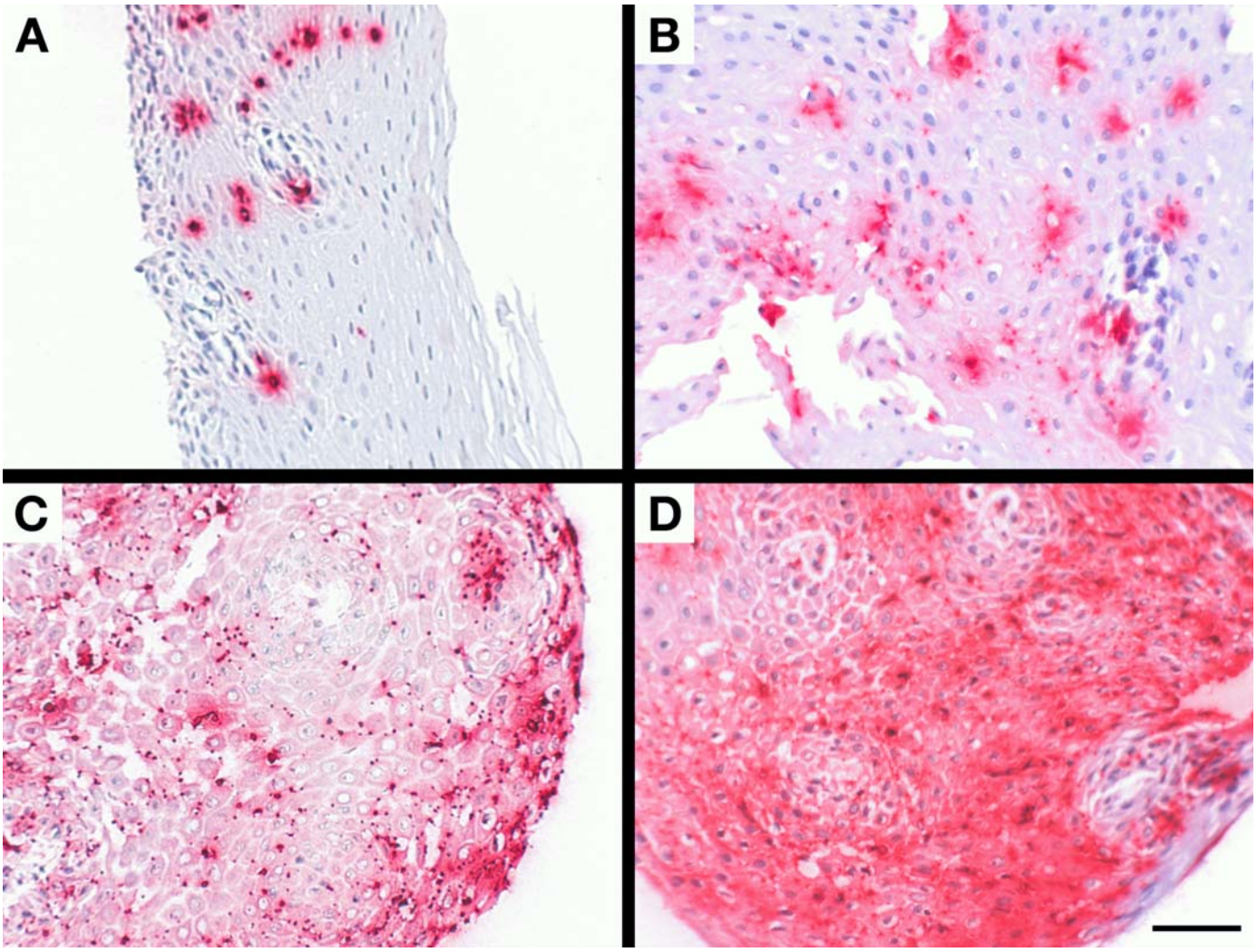
SUPPLEMENTAL FIGURE 1 (S-Figure 1)



SUPPLEMENTAL FIGURE 2 (S-Figure 2)



SUPPLEMENTAL FIGURE 3 (S-Figure 3)



SUPPLEMENTAL FIGURE 4 (S-Figure 4)