



Contents lists available at SciVerse ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

The development of a sensitive and specific ELISA for mouse eosinophil peroxidase: Assessment of eosinophil degranulation *ex vivo* and in models of human diseaseSergei I. Ochkur^a, John Dongil Kim^{a,b}, Cheryl A. Protheroe^c, Dana Colbert^c, Redwan Moqbel^d, Paige Lacy^b, James J. Lee^{a,*}, Nancy A. Lee^{c,*}^a Division of Pulmonary Medicine, Department of Biochemistry and Molecular Biology, Mayo Clinic Arizona, Scottsdale, AZ, United States^b Pulmonary Research Group, Department of Medicine, University of Alberta, Edmonton, AB, Canada^c Division of Hematology/Oncology, Department of Biochemistry and Molecular Biology, Mayo Clinic Arizona, Scottsdale, AZ, United States^d Department of Immunology, University of Manitoba, Winnipeg, MB, Canada

ARTICLE INFO

Article history:

Received 7 September 2011

Received in revised form 3 October 2011

Accepted 5 October 2011

Available online xxxx

Keywords:

EPX

Eosinophilia

Granule proteins

Allergic inflammation

ABSTRACT

Mouse models of eosinophilic disorders are often part of preclinical studies investigating the underlying biological mechanisms of disease pathology. The presence of extracellular eosinophil granule proteins in affected tissues is a well established and specific marker of eosinophil activation in both patients and mouse models of human disease. Unfortunately, assessments of granule proteins in the mouse have been limited by the availability of specific antibodies and a reliance on assays of released enzymatic activities that are often neither sensitive nor eosinophil specific. The ability to detect immunologically and quantify the presence of a mouse eosinophil granule protein in biological fluids and/or tissue extracts was achieved by the generation of monoclonal antibodies specific for eosinophil peroxidase (EPX). This strategy identified unique pairs of antibodies with high avidity to the target protein and led to the development of a unique sandwich ELISA for the detection of EPX. Full factorial design was used to develop this ELISA, generating an assay that is eosinophil-specific and nearly 10 times more sensitive than traditional OPD-based detection methods of peroxidase activity. The added sensitivity afforded by this novel assay was used to detect and quantify eosinophil degranulation in several settings, including bronchoalveolar fluid from OVA sensitized/challenged mice (an animal model of asthma), serum samples derived from peripheral blood recovered from the tail vasculature, and from purified mouse eosinophils stimulated *ex vivo* with platelet activating factor (PAF) and PAF + ionomycin. This ability to assess mouse eosinophil degranulation represents a specific, sensitive, and reproducible assay that fulfills a critical need in studies of eosinophil-associated pathologies in mice.

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1. Introduction

In both mice and humans, eosinophils generally comprise 1–3% of circulating peripheral blood leukocytes with absolute

counts below 300 cells/mm³ of blood. However, elevations in the numbers of eosinophils, and/or their activation leading to the release of granule proteins (*i.e.*, degranulation), are linked with a variety of inflammatory disease states. Indeed, the list of diseases and their associated mouse models is significant, including allergic diseases of the skin (*e.g.*, atopic dermatitis; human (Leiferman, 1989 vs. mouse (Herz et al., 1998)), the lung (*e.g.*, asthma; human (Lacoste et al., 1993) vs. mouse (Kips et al., 2003)), and the gastrointestinal tract (*e.g.*, eosinophil esophagitis; human (Rothenberg et al., 2001) vs. mouse

* Corresponding authors at: Department of Biochemistry and Molecular Biology, Mayo Clinic Collaborative Research Building, Mayo Clinic Arizona, 13400 E. Shea Blvd., Scottsdale, AZ 85259, United States. Tel.: +1 480 301 7183; fax: +1 480 301 7017.

E-mail addresses: jjlee@mayo.edu (J.J. Lee), nlee@mayo.edu (N.A. Lee).

(Mishra et al., 2001)), as well as autoimmune neurologic disorders (e.g., multiple sclerosis; human (Correale and Fiol, 2004) vs. mouse (Gladue et al., 1996)), cancer (human (Samoszuk, 1997) vs. mouse (Cormier et al., 2006)), transplantation rejection (human (Goldman et al., 2001) vs. mouse (Le Moine et al., 1999)), and infection with parasitic (human (Klion and Nutman, 2004) vs. mouse (Behm and Ovington, 2000; Fabre et al., 2009)) and fungal (human (Schubert, 2006) vs. mouse (Kobayashi et al., 2009)) pathogens. Evaluations of human subjects with eosinophil-mediated diseases often include assessments of eosinophil degranulation (i.e., release of granule proteins), which are commonly performed tasks utilizing commercially available reagents (see for example Jang and Choi, 2000). However, similar evaluations in mouse models of human disease are far from commonplace and are often assumed not to be even possible (see for example Stelts et al., 1998). This perception results from two logistical issues: (i) antibodies specific for mouse eosinophils and/or eosinophils granule proteins are limited in number and (ii) many investigators have noted a significantly attenuated degranulation response in mouse vs. human eosinophils (see for example Persson and Erjefalt, 1999). Consequently, to date only histological assessments (see for example Lee et al., 2004), single-dimensional immunoblot assays (Mould et al., 2000; Ochkur et al., 2007), and non-specific enzymatic assays (Strath et al., 1985) have been reported as assessments of mouse eosinophil degranulation. That is, there are currently no available eosinophil-specific assays using an ELISA format capable of assessing mouse eosinophil numbers or levels of degranulation in fluid samples. This lack of a sensitive high throughput assay has severely limited the ability to evaluate available mouse models and, in turn, limited the utility of the mouse as a model of eosinophil-mediated human diseases.

We describe in this report the development and utility of an easy to perform eosinophil-specific ELISA based on a pair of eosinophil peroxidase (EPX)-specific monoclonal antibodies we have generated by sensitizing eosinophil peroxidase knockout mice with purified mouse EPX. The EPX-based ELISA that was developed functions in high-throughput formats, representing a sensitive and quantitative assay for the detection of eosinophils and, more importantly, eosinophil degranulation. The studies presented also demonstrate that this ELISA affords a 10-fold increase in sensitivity relative to the widely used OPD-based peroxidase activity assay. Thus, EPX-based ELISA solves the logistical problems faced by investigators using the mouse as a model system of human disease – the availability of an easy to perform high throughput assay of biological fluid samples from mice that is both eosinophil-specific and sensitive enough to quantify the lower levels of eosinophil degranulation observed in this animal.

2. Materials and methods

2.1. Mice

Wild type and transgenic/gene knockout mice (6–12 weeks of age) on C57BL/6 background were used in experiments. The following transgenic and gene knockout animals were used as part of these studies: (i) transgenic line of mice (*I5*) constitutively over-expressing IL-5 from T

cells (NJ.1638 (Lee et al., 1997)); (ii) double transgenic mouse model of severe asthma (*I5/E2*) constitutively over-expressing IL-5 from T cells and human eotaxin-2 from Clara cells (Ochkur et al., 2007); (iii) transgenic mice congenitally devoid of eosinophils (*PHIL* (Lee et al., 2004)); (iv) triple transgenic mice over-expressing IL-5 and human eotaxin 2 on an eosinophil-less background (*I5/E2/PHIL* (Ochkur et al., 2007)); (v) eosinophil peroxidase deficient gene knockout mice (*EPX*^{−/−} (Denzler et al., 2001)); and (vi) compound double transgenic mice expressing IL-5 from T cells and human eotaxin-2 from Clara cells that are also devoid of eosinophil peroxidase (*I5/E2/EPX*^{−/−}). Mice were maintained in ventilated micro-isolator cages housed in the specific pathogen-free animal facility at the Mayo Clinic Arizona. Protocols and studies involving animals were performed in accordance with National Institutes of Health and Mayo Foundation institutional guidelines.

2.2. Antibodies

EPX specific monoclonal antibodies were generated through the immunization of EPX knockout mice (*EPX*^{−/−} (Denzler et al., 2001)), with purified EPX protein derived from eosinophils recovered from hyper-eosinophilic IL-5 transgenic mouse (NJ.1638 (Lee et al., 1997)). The production and screening of EPX monoclonal antibodies was performed as previously described (Protheroe et al., 2009). Briefly, peripheral blood eosinophils were isolated from NJ.1638 mice and eosinophil secondary granules were recovered as a source for the purification of eosinophil peroxidase. *EPX*^{−/−} mice were repeatedly sensitized by injections with 25 µg of purified EPX with RIBI™ adjuvant (RIBI ImmunoChem Research Inc., Hamilton, MT). Mice were screened for high antibody titers and corresponding spleens were harvested to generate antibody secreting hybridomas (Myeloma Fusion Partner, P3X63-Ag8.653; ATCC, Manassas, VA). Approximately 2000 total hybridomas were generated and subsequently assessed for use in an ELISA format, yielding seven monoclonal antibodies that were capable of binding cell-free EPX in this format. Additional pair-wise binding assays to EPX showed that each antibody pairing among these seven displayed equal binding to EPX relative to the binding observed when using individual antibodies alone (data not shown). Two monoclonal antibodies were randomly selected for use in the development of an EPX sandwich ELISA assay (clone MM25-429.1.1 as the capture antibody and clone MM25-82.2.1 as the detection antibody). The detection antibody (MM25-82.2.1) was biotinylated using the EZ-Link NHS-LC-Biotin kit (Pierce, Cat # 21336). The efficiency and batch to batch reproducibility of this process were remarkably high, generating biotin-labeled EPX-specific detection antibody with 8–12 molecules of biotin per molecule of immunoglobulin. The purification of EPX, the generation of specific mouse monoclonal antibodies, and the subsequent identification of an antibody pair for use in an EPX-specific ELISA are schematically summarized in Fig. 1.

2.3. Eosinophil standards

Mouse eosinophil extracts were prepared for use as defined samples in the creation of assay standard curves. Briefly, peripheral blood eosinophils were purified from NJ.1638

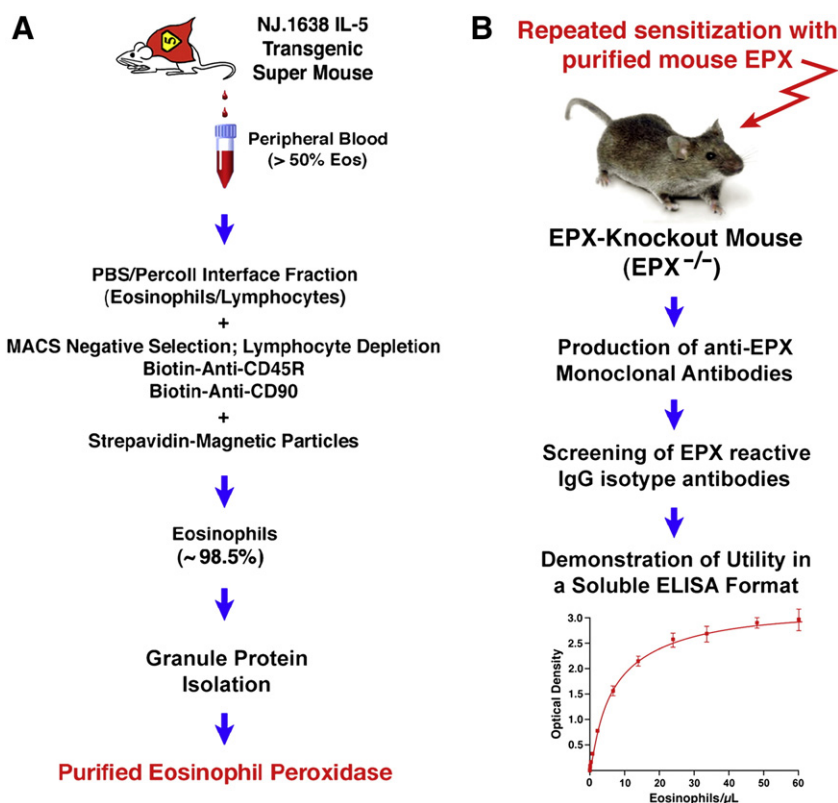


Fig. 1. The generation of mouse anti-EPX monoclonal antibodies and the development of an EPX-specific sandwich ELISA. (A) Eosinophil peroxidase is purified using mouse peripheral blood eosinophils from NJ.1638 transgenic mice constitutively over-expressing the eosinophilopoietic cytokine IL-5 (Lee et al., 1997). The strategy used a combination of physical methodologies to pre-select for eosinophils from blood (Percoll gradient centrifugation) and magnetic bead separation using cell surface specific markers (MACS). The details of this cell isolation strategy, as well as the purification of eosinophil peroxidase, are described in a previous study (Protheroe et al., 2009). (B) EPX-specific monoclonal antibodies with utilities in an ELISA format were generated by the sensitization of EPX knockout (EPX^{-/-}) with purified EPX. The generation and screening of EPX-specific monoclonal antibodies were described earlier (Protheroe et al., 2009). Antibodies useful in a soluble sandwich ELISA format were identified on the basis of pair-wise comparisons of avidity to EPX relative to the binding of the individual monoclonal antibodies alone.

mice, yielding cell populations with >98% purity (Borchers et al., 2002). The recovered eosinophils (suspended in 1× PBS) were centrifuged at 10,000 × g for 10 min (4 °C). This cell pellet was initially suspended in 1× PBS at a concentration of 60,000 eosinophils/μL, after which 4 volumes (i.e., 80% of the total suspended volume) of a solution containing 0.22% hexadecyltrimethylammonium bromide (Sigma-Aldrich, Cat # H-5882)/0.3 M sucrose were added and the tubes mixed thoroughly (~1 min) on a vortex. Typically, 100 μL aliquots of eosinophil extract, now at a concentration equivalent of 12,000 eosinophils/μL, were prepared, flash frozen in liquid nitrogen, and stored at -80 °C until use. Individual tubes of this eosinophil extract was insensitive to cycles of freeze-thaw (10 total were tested) and without loss of signal in this ELISA format. Serial dilutions of this eosinophil extract were used for assay optimization, standard curves, and quality controls in the described experiments.

2.4. ELISA detection system

We developed an ELISA using a non-peroxidase based detection system to rule out any interference from the enzymatic activity of eosinophil peroxidase. An alkaline phosphatase

detection system was chosen as an alternative in combination with BluePhos substrate from KPL (Gaithersburg, MD, USA). This substrate provides exceptionally high signal intensity per unit of enzyme activity. The assay developed utilized the KPL pre-optimized ELISA reagent system that includes: Coating Solution Concentrate 10× (KPL, Cat # 50-84-00), 10% BSA Diluent/Blocking Solution Kit (KPL, Cat # 50-61-00), Wash Solution Concentrate 20× (KPL, Cat # 50-63-00), and BluePhos Micro-well Phosphatase Substrate System (KPL, Cat # 50-88-00), APstop Solution (KPL, Cat # 50-89-00). The other reagents utilized in the development of this ELISA assay include, Streptavidin-Alkaline Phosphatase (Strep-AP) from RD (R&D Systems, Minneapolis, MN, Cat # AR001) and Trizma hydrochloride buffer solution (Sigma-Aldrich, Cat # T2319-1L), which was used to prepare Streptavidin-AP Diluent. Solid phase 96 well Nunc-Immuno Plates with MaxiSorp surface (Thermo Scientific-Nunc, Cat # 439454) were used as the support structure to perform this ELISA as a high throughput assay.

2.5. ELISA protocol

The basic Sandwich/Capture ELISA protocol was created as per the manufacturer's instructions (i.e., KPL – www.KPL).

com). This initial protocol was used as a starting point for the development of the final optimized assay:

1. A micro-titer plate is pre-treated with 2 µg/ml anti-EPX monoclonal antibody MM25-429.1.1 (capture antibody) in 100 µl of Coating Solution at 4 °C overnight.
2. Coated wells of the micro-titer plate are cleared of unbound antibody with three cycles of rinsing using Wash Solution. Potential areas of non-specific binding in each well of the plate are blocked by a 30 min room temperature pre-incubation with 300 µl of Blocking Solution.
3. Following the incubation with Blocking Solution, 100 µl of sample (and/or standard) is added and the plate is incubated at room temperature without shaking for 1.5 h.
4. The wells of the micro-titer plate are cleared of unbound target antigen with three cycles of rinsing using Wash Solution. 100 µl of biotylated anti-EPX monoclonal antibody MM25-82.2.1 (detection antibody) is added to each well at a final concentration of 0.8 µg/ml and the plate is incubated at room temperature for 1.5 h.
5. The wells of the micro-titer plate are cleared of unbound detection antibody with three cycles of rinsing using Wash Solution. 100 µl of Strep-AP (diluted 1/500 in 1% BSA, 0.05% Tween 20, 0.025 M Tris, 0.5 M NaCl (pH 7.4)) are added to each well and the plate is incubated at room temperature for 20 min.
6. The wells of the micro-titer plate are cleared of unbound Strep-AP with three cycles of rinsing using Wash Solution. 100 µl of BluePhos substrate are added to each well and the plate is incubated at 37 °C for 1 h with gentle rotation.
7. The colorimetric reaction is terminated with the addition of 100 µl of Stop Solution. Absorbance of individual wells of the plate is determined at a wavelength of 610–630 nm with a BioTek µQuant Microplate Spectrophotometer with KC4™ Data Analysis Software from Bio-Tek (Winooski, VT).

2.6. Eosinophil stimulation ex vivo

Mouse peripheral blood eosinophils were collected and purified from NJ.1638 mice as previously described (Borchers et al., 2002). These cells were counted and re-suspended in Phenol Red-free RPMI at 10^6 cells/ml. 200 µl aliquots (2×10^5 cells) of this suspension were incubated for 6 h in an atmosphere of 5% CO₂ and 95% humidity to assess the release of EPX in the cultured supernatant following exposure to 50 ng/ml PAF-C18 (Alexis Biochemicals ALX-301-008) or 50 ng/ml PAF-C18 plus 1 µM

ionomycin (Sigma-Aldrich, Cat # I0634); DMSO (Sigma-Aldrich, Cat # D5879) alone was used as a vehicle control. Following incubation, the cells were centrifuged at $1300 \times g$ for 5 min. The recovered supernatants were re-spun at $13,000 \times g$ for 5 min generating final cell and organelle-free supernatants that were stored at –80 °C until used.

2.7. Ovalbumin sensitization/aerosol challenge model of allergic respiratory inflammation

Wild type mice were subjected to an established OVA sensitization/respiratory challenge protocol as previously described (Lee et al., 2004). Briefly, mice were sensitized on day 0 and day 14 by intraperitoneal injection (i.p.) of 20 µg OVA (grade V; Sigma-Aldrich, Cat # A-5503) and 2.25 mg of Imject® Alum (Pierce, Cat # 77161) in 100 µl of 1× PBS. These OVA sensitized animals were subsequently challenged on days 24, 25, and 26 of the protocol with an aerosol generated from a 1% (w/v) OVA solution in sterile saline using a collision nebulizer; control animals were challenged with an aerosol generated using saline alone. On protocol day 28, the mice were euthanized and bronchoalveolar lavage fluid (BAL) was collected as previously described (Lee et al., 2004). BAL fluid was flash-frozen in liquid nitrogen and stored at –80 °C until used.

2.8. Preparation of peripheral blood serum

Peripheral blood (200–400 µl) derived from tail bleeds of mice was collected directly in microcentrifuge tubes and allowed to clot on ice (i.e., 4 °C) during a 30 min incubation. Following this incubation, serum was derived from these samples by high-speed centrifugation ($10,000 \times g$) at 4 °C for 10 min. The serum samples collected were and flash-frozen in liquid nitrogen and stored at –80 °C until used.

2.9. Eosinophil peroxidase enzymatic activity assay

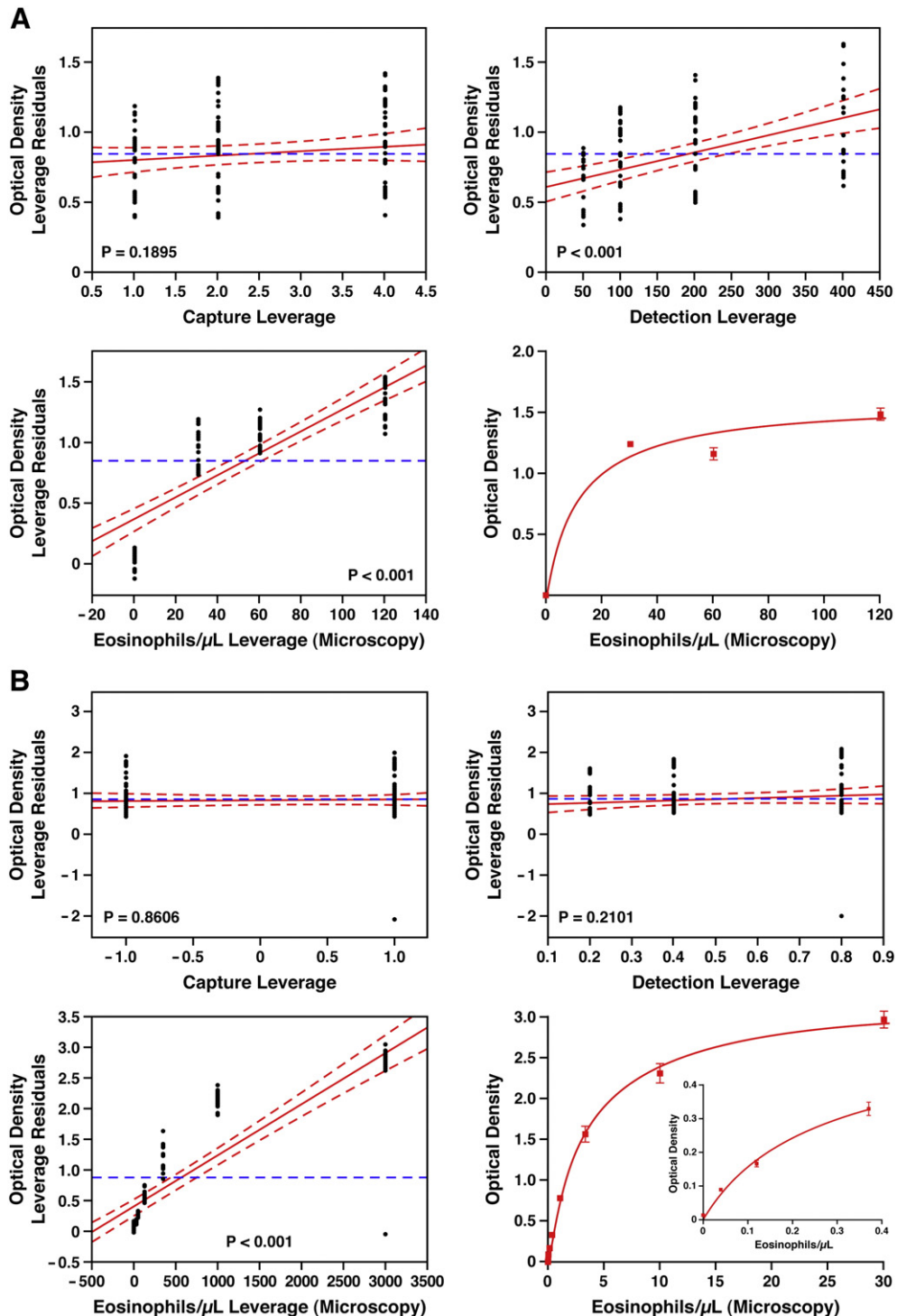
Eosinophil peroxidase activity was assessed in a micro-titer plate as a 125 µl final volume assay. Specifically, in each well 75 µl of OPD-substrate solution (50 mM Tris-HCl, pH 8, 0.1% Triton X-100 (Sigma-Aldrich, Cat # T9284), 8.8 mM H₂O₂ (Sigma-Aldrich, Cat # H1009), 6 mM KBr (Sigma-Aldrich, Cat # P0838), and 10 mM OPD (o-phenylenediamine, Sigma-Aldrich, Cat # P8412)) were combined with 50 µl of the sample to be assayed and incubated at 37 °C for 30 min; negative con-

Fig. 2. Full factorial analysis of the selected EPX-specific antibody pair was employed to optimize the sensitivity (signal to noise ratio) of the EPX-specific sandwich ELISA. (A) Leverage plots and ELISA results using initial assay conditions. The comparative factorial assays used to assess the functionality of the EPX-based ELISA assay are summarized in the leverage plots. Actual values in the leverage plots (●) are presented with horizontal blue dashed lines in each plot representing the overall average. Predicted values derived from the fitted curves are shown as solid red lines with 95% CI (confidence interval) as flanking red dashed lines. The distance from each data point to the line of fit is the error or residual for that point. The distance from each data point to the horizontal blue dashed lines is what the error would be if you removed the effects this point has in the fitted curve model. As a result, the strength of the effect of varying each parameter is shown by how the line of fit is suspended away from the horizontal by the data points. The fitted curves each correlated well with the observed data – about 70% of total variation in the OD accounted for by fitting the regression line. If the 95% confidence curves cross the horizontal reference line (dashed blue line), then the effect is significant; if the curves do not cross, variations of this parameter failed to achieve statistical significance (at the 5% level). The standard curve derived from the ELISA under the initial assay conditions is shown by optical density as a function of input eosinophils/µl derived from a manual cell differential (Microscopy). (B) Subsequent optimization experiments and the resulting final EPX-based ELISA assay. The leverage plots show that the assay modifications instituted based on the response observed using the initial conditions resulted in an ELISA assay that did not display significant improvement of signal by changing the Capture or Detection antibodies over the concentrations examined whereas the signal (i.e., optical density) varied only as a function of input eosinophils/µl (microscopy). The standard curve derived from the ELISA using the final conditions is shown by optical density as a function of input eosinophils/µl derived from a manual cell differential (microscopy). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

trol samples also contained 10 mM of the peroxidase inhibitor resorcinol (1,3-Benzenediol, Sigma-Aldrich, Cat # R-5645). Following incubation, 50 μ L of 2 N H_2SO_4 was added to stop the reactions and the absorbance of each sample was measured at 490 nm using a BioTek μ Quant Microplate Spectrophotometer with KC4™ Data Analysis Software from Bio-Tek (Winooski, VT).

2.9.1. Statistical analysis

GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, CA) was used for plotting and basic analysis. JMP (SAS Institute, Cary, NC) was used as a design of experiment statistical platform. Data are expressed as the mean $\pm$ SEM. Statistical analysis for comparisons between groups was performed pair-wise using a



Student's *T* test. Differences between mean values were considered significant when $p < 0.01$.

3. Results

3.1. The development and subsequent optimization of an EPX-specific sandwich ELISA

A full-factorial approach was taken in the development of an EPX-based ELISA (Fig. 2). Specifically, the initial optimization of capture and detection EPX antibody concentrations was designed as a full ($3 \times 4 \times 4$) factorial experiment (Fig. 2(A)) centered on initial ELISA conditions (2 $\mu\text{g}/\text{ml}$ capture antibody (coating the plates overnight, ~ 15 h) and 0.2 $\mu\text{g}/\text{ml}$ of biotinylated detection antibody) that generated the dose response curve in the lower right-hand panel. Capture antibody (factor 1) was varied on 3 levels 1, 2, and 3 $\mu\text{g}/\text{ml}$. Detection antibody (factor 2) had 4 levels 0.05, 0.10, 0.20, 0.40 $\mu\text{g}/\text{ml}$ and eosinophil extract (factor 3) was assessed over a 0, 30, 60, and 120 eosinophils/ μl range derived from a manual cell differential (Microscopy). These factorial analyses showed that variation of the capture antibody had little effect on the assay's signal, whereas the concentration of detection antibody and the eosinophil extract each had significant effects. The amount of noise (*i.e.*, non-specific background) was the same at different antibody concentrations (data not shown); therefore, the goal of the experiments was simply to maximize the specific signal (*i.e.*, optical density reading). In a similar fashion, we also conducted experiments that optimized time of plate coating with the capture antibody, the temperature of enzymatic (alkaline phosphatase) reaction, and the narrowing of the spectral scan for maximal absorbance of the developed plates from the company's (KPL) recommended range for BluePhos from 595–650 nm to 610–630 nm (data not shown). Based on these initial results of assay responsiveness, a second round of optimization was performed in a $2 \times 3 \times 8$ factorial format (Fig. 2(B)) where capture antibody was assessed at 1 and 2 $\mu\text{g}/\text{ml}$, detection antibody at 0.2, 0.4, 0.8 $\mu\text{g}/\text{ml}$, and the mouse eosinophil extract in the range of 0, 0.04, 0.12, 0.37, 1.11, 3.33, 10, and 30 eosinophils/ μl . The standard curve derived from the ELISA using the optimized and final conditions (2 $\mu\text{g}/\text{ml}$ capture antibody

(coating the plates for 2 days, 30–40 h) and 0.8 $\mu\text{g}/\text{ml}$ of biotinylated detection antibody) showed that this final assay has a limit of detection (*i.e.*, measureable signal three standard deviations above background) of 0.03 eosinophils/ μl .

3.2. The EPX-based ELISA is uniquely EPX specific and is also a quantitative tool allowing for the assessment of eosinophil numbers even in mixed leukocyte populations

The optimized EPX-based ELISA was compared relative to manual cell counting and differential analyses as a means to count eosinophils in “buffy coats” (*i.e.*, total white blood cell fractions) from the blood of mice (Fig. 3). The groups of mice chosen allowed us to assess the utility of the assay as a means of counting eosinophils from mixed populations of leukocytes derived from wild type (1–3% eosinophils), *PHIL* (0% eosinophils), and *I5/E2* cytokine/chemokine double transgenic (30–40% eosinophils) mice. In each case, assessments of the same cell samples (over the entire range of eosinophil concentrations examined) revealed that the EPX-based ELISA quantitatively reproduced the number of eosinophils determined by traditional counting methods (Microscopy). As expected the assessments diverged when examining the eosinophil numbers in the blood of eosinophil peroxidase knockout (*EPX*^{−/−}) and compound double transgenic/knockout (*I5/E2/EPX*^{−/−}) mice (Fig. 3). That is, while manual cell counts and differentials accurately counted the number of eosinophils present, the EPX-based assay yielded no signal due to the lack of EPX in these mice. However, this lack of signal is significant as it demonstrates that the EPX-based ELISA is absolutely EPX specific, showing no cross-reactivity with other peroxidases and, in turn, no other cell types.

3.3. The enhanced sensitivity of the EPX-based ELISA allows for the detection and quantification of eosinophil degranulation using ex vivo cell culture assays

Eosinophils were isolated from the blood of transgenic mice as described in the Materials and methods and stimulated in culture to elicit degranulation (Fig. 4). A direct pairwise comparison of induced release of EPX was performed assessing cell-free EPX in the culture media with an estab-

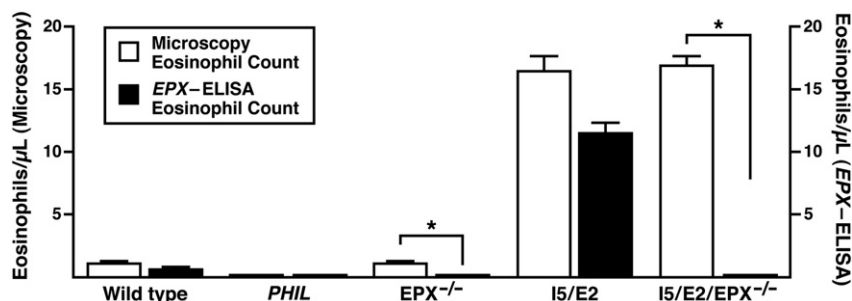


Fig. 3. EPX-based ELISA is a specific, sensitive, and quantitative method to identify eosinophils. Buffy coats (white blood cells fractions) were collected from low speed spins of peripheral blood of wild type mice, animals congenitally devoid of eosinophils (*PHIL* (Lee et al., 2004)), gene knockout mice deficient of eosinophil peroxidase (*EPX*^{−/−} (Denzler et al., 2001)), hyper-eosinophilic (peripheral blood counts $> 100,000$ cells/ μl and $> 30\%$ eosinophils) double transgenic mice constitutively expressing IL-5 and human eotaxin 2 from mature T cells and Clara cells, respectively (*I5/E2* (Ochkur et al., 2007)), and eosinophil-less double transgenic mice (*I5/E2/EPX*^{−/−}). The white blood cell concentration of each sample was determined manually with a hemacytometer and adjusted to 40 leukocytes/ μl . Cytospin slide preparations of each sample were stained (Diff-Quick (Fisher)) and then subjected to cell differential assessment to determine the number of eosinophils/ μl (Microscopy). In addition, the number of eosinophils in each sample was determined by ELISA with the number of eosinophils/ μl calculated from a standard curve using known numbers of eosinophils (*EPX*-ELISA). * $p < 0.05$.

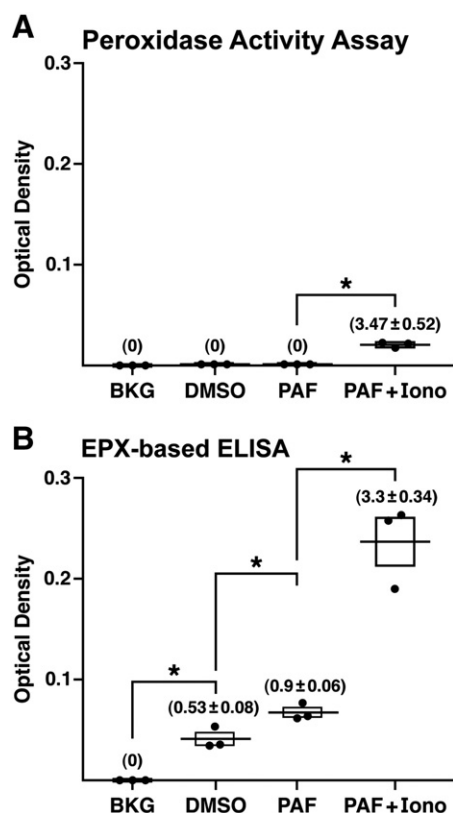


Fig. 4. EPX-based ELISA displays a 10-fold increase in sensitivity relative to assessment of peroxidase activity in *ex vivo* assessments of eosinophil degranulation. Scatter plots of cell-free EPX released by stimulating eosinophils with the secretagogues platelet activating factor (PAF) or PAF and Ionomycin (PAF+Iono) are presented in a side-by-side comparison using (A) the OPD colorimetric peroxidase activity assay and (B) the EPX-based ELISA. The absorbance of DMSO containing media prior to incubation with eosinophils was measured (optical density at 450 nm for the OPD colorimetric enzymatic activity assay and optical density at 630 nm for the EPX-based ELISA assay) to assess the assay's background (BKG). Supernatants from eosinophils cultured in DMSO containing media alone served as a vehicle control for secretagogue-induced EPX release (DMSO). The resulting absolute values of eosinophils/ μ l corresponding to each optical density assessment were derived from assay-specific standard curves and presented as numbers in parenthesis above each group. The data are presented as means $\pm$ SEM. * $p < 0.05$.

lished and widely used enzymatic peroxidase activity assay (Fig. 4(A)) vs. the EPX-based ELISA (Fig. 4(B)). These data showed that relative to background control (BKG), the limited sensitivity offered by the OPD enzymatic activity assay prevented the detection of EPX from eosinophils cultured with the secretagogue platelet activating factor (PAF). Moreover, this enzymatic assay was able only to provide a small, but measurable, signal above background after the eosinophils were cultured with both PAF and Ionomycin (PAF+Iono). In contrast, the EPX-based ELISA displayed a nearly 10-fold increase in assay responsiveness relative to the OPD enzymatic activity assay following stimulation with PAF+Iono (0.23 vs. 0.02 optical density units, respectively). This significantly enhanced sensitivity now allows for the detection (*i.e.*, relative to background control (BKG)) of eosinophil degranulation resulting from stimulation of eosinophils with PAF alone. Interestingly, the EPX-based ELISA was also capable of detecting and quantifying

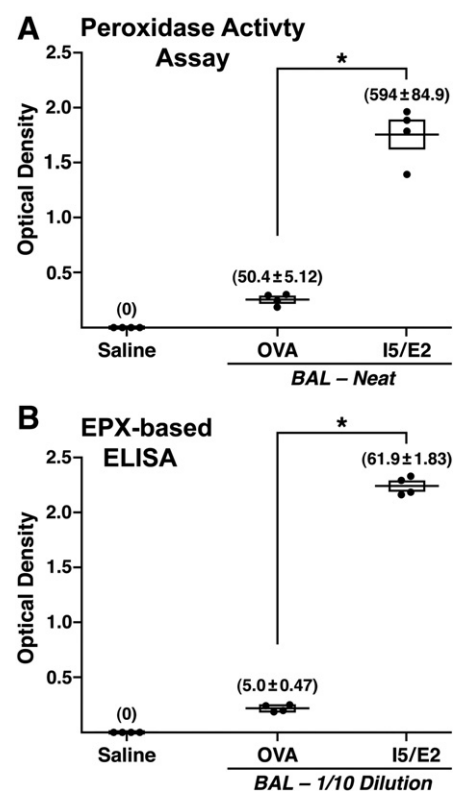


Fig. 5. EPX-based ELISA provides a specific and uniquely sensitive assessment of the eosinophil degranulation occurring in the airway lumen of mice. Airway luminal assessments of cell-free EPX were presented as scatter plots in a side-by-side comparison using (A) the OPD colorimetric peroxidase activity assay and (B) the EPX-based ELISA. Airway EPX levels in bronchoalveolar lavage fluid (BAL) were measured in ovalbumin sensitized/aerosol challenged wild type mice (OVA; $\sim 1\text{--}2 \times 10^6$ total BAL eosinophils/ml); OVA sensitized/saline challenged animals served as allergen provocation controls (Saline; $< 10^2$ total BAL eosinophils/ml). Airway EPX levels in the hyper-eosinophilic double transgenic model of severe asthma were measured and presented for comparison (I5/E2; $\sim 1\text{--}2 \times 10^7$ total BAL eosinophils/ml). As indicated, it was necessary to dilute the BAL fluid (1:10) prior to assessment using the EPX-based ELISA to normalize the signal (optical density) relative to the response produced by the OPD colorimetric peroxidase activity assay. The resulting absolute values of eosinophils/ μ l corresponding to each optical density assessment were derived from assay-specific standard curves and presented as numbers in parenthesis above each group. The data are presented as means $\pm$ SEM. * $p < 0.05$.

degranulation from eosinophils cultured without stimulation (*i.e.*, media with vehicle (DMSO)).

3.4. The EPX-based ELISA provides a sensitive and quantitative assay for the detection of eosinophil degranulation using fluid samples derived from mouse models of human disease

The utility of the EPX-based ELISA relative to the less sensitive OPD-peroxidase activity assay was demonstrated in side-by-side comparisons of cell-free EPX assessments performed on bronchoalveolar lavage fluid (BAL) recovered from mouse models of respiratory disease (Fig. 5). Specifically, cell-free EPX levels were measured in the airways of ovalbumin sensitized/ovalbumin aerosol-challenged (OVA) wild type mice (ovalbumin sensitized and saline-challenged mice were used as controls (Saline)) as well as an allergen naïve double

transgenic model of severe asthma (I5/E2 (Ochkur et al., 2007)). These data showed that while both the OPD-peroxidase enzymatic assay (Fig. 5(A)) and the EPX-based ELISA (Fig. 5(B)) were capable of quantifying the release of EPX in the airways of OVA aero-challenged mice (OVA), the EPX-based ELISA again displayed a 10-fold increase in sensitivity, displaying the same signal (optical density units) with BAL fluid diluted 1 to 10. This same signal enhancement was observed at the higher range of the scale with the EPX-based ELISA again displaying a 10-fold enhanced detection of cell-free EPX in the BAL from the double transgenic model of severe asthma (I5/E2).

3.5. The sensitive and quantitative characters of the EPX-ELISA allow for the assessments of even low levels of EPX in serum derived from peripheral blood of unmanipulated wild type mice

The EPX-based ELISA assessments of *ex vivo* cultured eosinophils described above suggested that even “resting” blood eosinophils displayed a detectable level of continual release of EPX. As a result, the EPX-based ELISA was used to determine whether the blood sera of unmanipulated wild type mice with 1–3% eosinophils (~8000 leukocytes/mm³ of blood) also displayed low levels of released EPX (Fig. 6). These data showed that as expected, knockout mice devoid of eosinophil peroxidase (I5/E2/EPX^{-/-}) displayed no evidence of circulating serum EPX. In contrast, the ELISA assay was easily capable of detecting significant levels of cell-free EPX in the sera of the double transgenic severe asthma mice (I5/E2). More significantly, low levels of cell-free EPX were also detected in the sera of unmanipulated wild type mice.

4. Discussion

The creation, and now availability, of an EPX-based ELISA in the mouse to detect and quantify eosinophil degranulation provides logistical solutions to issues surrounding studies of the role(s) of eosinophils in this animal. These issues include

technical/strategic advances as well as scientific observations relevant to our understanding of mouse eosinophil effector function(s):

4.1 Technical/strategic advances:

- 4.1.1 The previously available assays assessing eosinophil degranulation are based on enzymatic activities (*i.e.*, peroxidase or ribonuclease activities) that are not absolutely eosinophil-specific. In contrast, the EPX-based ELISA is an assay that is independent of enzymatic activity (even the lack thereof). Moreover, EPX-based ELISA assessments from wild type vs. EPX^{-/-} mice demonstrated that the assay is target protein specific.
- 4.1.2 The ELISA developed is an easy to use protocol taking advantage of commonly available disposable plasticware and reagents. In addition, this ELISA was shown to function as a high throughput assay executable in micro-titer plate venues.
- 4.1.3 The utility of the assay is highlighted by the increased sensitivity it affords relative to established assays that universally detect cell-free EPX by assessing the peroxidase activity associated with this secondary granule protein (more often than not as a colorimetric enzymatic assay). Direct comparisons showed that the EPX-based ELISA is 10-fold more sensitive than measurements made via these peroxidase enzymatic assays. This increased sensitivity promotes a lower threshold of detection in a given sample and, in turn, the quantification of this granule protein even in small and/or dilute samples. An additional contributing factor to both the utility and sensitivity of the EPX-based ELISA is that unlike the peroxidase enzymatic assays, the ELISA doesn't require the presence of intact and enzymatically active protein. That is, even partially degraded samples with limited peroxidase activity nonetheless may contain epitope-

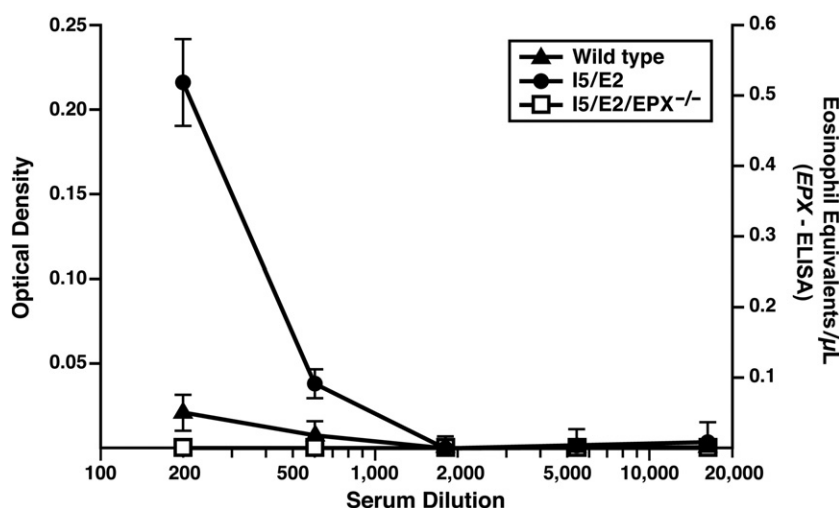


Fig. 6. EPX-based ELISA of serum provides a sensitive and quantitative ability to assess levels of cell-free eosinophil peroxidase in the blood of mice. Serial dilutions of serum samples (using saline) from wild type, hypereosinophilic double transgenic (I5/E2), and eosinophil peroxidase deficient compound double transgenic-knockout (I5/E2/EPX^{-/-}) mice were assessed for eosinophil peroxidase using the EPX-based ELISA. Optical density derived from assessments at each serum dilution is plotted together with the resulting absolute values of Eosinophil-Equivalents/μl corresponding to optical density assessments derived from assay-specific standard curves.

bearing fragments that are sufficient for the detection/quantification of EPX by the ELISA.

4.2 Scientific observations relevant to mouse eosinophil effector function(s):

- 4.2.1 Although from a technical standpoint we have shown that the EPX-based ELISA is target-protein (i.e., EPX) specific, the question of whether EPX itself is absolutely eosinophil-specific is still unresolved. The data presented, however, addresses this issue. EPX-based ELISA of extracts derived from peripheral white blood cell fractions of wild type vs. eosinophil-less *PHIL* mice showed that in the absence of eosinophils EPX was not detectable from the remaining white blood cells. These data suggest that eosinophils are the only significant source of EPX among the various peripheral leukocyte subtypes in circulation and thus represent a valid target of an eosinophil-specific assay.
- 4.2.2 Relative to human samples, eosinophil degranulation from mouse sources has been difficult to assess because of the limited capacity of mouse eosinophils to execute this effector function (Stelts et al., 1998; Persson and Erjefält, 1999; Malm-Erjefält et al., 2001). However, the 10-fold enhancement in sensitivity of the EPX-based ELISA vs. the established enzymatic activity assay for EPX demonstrated that while low on an absolute scale, EPX release by mouse eosinophils in *ex vivo* assays of degranulation was measureable and could be quantified. Significantly, these *ex vivo* data showed that even in the absence of stimuli to promote degranulation, “resting” peripheral blood eosinophils were nonetheless sources of released EPX. A similar observation was noted *in vivo* where even otherwise healthy unmanipulated wild type animals displayed a low, but detectable, level of serum EPX. Although this release of EPX in both the *ex vivo* and *in vivo* studies may have occurred as a consequence of sample preparation, these results nonetheless suggest that the release of EPX, and presumably the other granule proteins, is not restricted to responses directly linked to one or more specific stimuli. Instead, peripheral blood eosinophils appear to undergo a continual piecemeal release of EPX while in circulation; the functionality, if any, of this steady-state circulating cell-free EPX remains unknown.
- 4.2.3 The apparent inability of mouse eosinophils to undergo significant degranulation relative to their human counterparts has been a frustrating aspect of working with mouse models of disease, particularly mouse models of asthma where eosinophil granule protein release is an easily measureable event in the airways of patients (see for example Filley et al., 1982; Erjefält et al., 1999). The increased sensitivity of EPX-based ELISA, together with the eosinophil specificity of the assay, presents a solution to this issue. Specifically, our data showed that like human subjects eosinophil degranulation in BAL fluid from aero-allergen challenged mice is now an easily measureable endpoint assessment that is also quantifiable.

The significance of this EPX-based ELISA will extend beyond studies of mouse models of human disease. Sequence comparisons of the mouse EPX gene with its human orthologue demonstrated that the encoded mammalian peroxidases derived from these two genes are >86% identical at the amino acid level (Horton et al., 1996). Thus, when the issue of cross-reactivity was examined, the mouse anti-mouse EPX monoclonal antibodies used to develop this EPX-based ELISA recognized epitopes in mouse EPX that are identical to epitopes in human EPX. This cross-reactivity has allowed us to develop an equally specific, sensitive, and quantitative ELISA recognizing human EPX. The availability of this assay has permitted direct side-by-side comparisons of mouse vs. human degranulation as well as the assessment of biological fluid samples from patients as a diagnostic tool in disease management (manuscript in preparation).

Acknowledgments

The authors wish to thank the members of Lee Laboratories as well as colleagues within the greater eosinophil community for insightful discussions and critical comments that directly led to the development of the EPX-based ELISA and, in turn, the preparation of this manuscript. We also wish to acknowledge the invaluable assistance of the Mayo Clinic Arizona Statistical support group (Amylou Dueck, PhD, Yu-Hui J. Chang, and Joseph Hentz), our staff medical graphic artist (Marv Ruona), and the excellent administrative support provided to Lee Laboratories by Linda Mardel and Shirley (“Charlie”) Kern. The Mayo Foundation and grants from the United States National Institutes of Health [NAL (HL058723) and J.J.L. (HL065228, RR0109709)], the American Heart Association [NAL (05556392) and J.J.L. (0855703)], the Canadian Institutes of Health Research [RM (MOP89748)], and the Lung Association of Alberta [JDK] were the sources of funding used in the performance of studies as well as data analysis. These funding sources had no involvement in study design, data collection (including analysis and interpretation), the writing of the manuscript, or the decision to submit for publication.

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