

# Morphologic, cytochemical, and ultrastructural features of gray eosinophils in nine cats

Emma Holmes<sup>1</sup>  | Rose Raskin<sup>2</sup>  | Paul McGill<sup>3</sup> | Balázs Szladovits<sup>1</sup> 

<sup>1</sup>Pathobiology and Population Sciences, Royal Veterinary College, University of London, Hertfordshire, UK

<sup>2</sup>Department of Comparative Pathobiology, College of Veterinary Medicine, Purdue University, West Lafayette, IN, USA

<sup>3</sup>Bioimaging, GlaxoSmithKline, David Jack Centre, Research and Development, Ware, UK

## Correspondence

Emma Holmes, Pathobiology and Population Sciences, Royal Veterinary College, University of London, Hawkshead Lane, Hatfield, Hertfordshire, AL9 7TA, UK.  
Email: eholmes@rvc.ac.uk

## Abstract

**Background:** Gray eosinophils, resembling those in sighthound dog breeds, have not previously been reported in cats.

**Objectives:** We aimed to provide a morphologic, cytochemical, and ultrastructural description of gray eosinophils in cats.

**Methods:** Blood films examined as part of routine hematology profiles in cats from May 2015 to July 2018 were evaluated for the presence of gray eosinophils. When identified with modified Wright stain, cells were morphologically assessed and additionally stained with Diff-Quik, ALP, Luna, and Luxol fast blue stains and compared with feline controls. Two cases were prepared for transmission electron microscopy (TEM) and compared with a feline control.

**Results:** Gray eosinophils were identified in 9 of 2641 cats during the study period. Compared with typical feline eosinophils, these cells contained abundant round granules instead of the characteristic rod-shaped specific granules. These granules lacked the characteristic intense pink/red staining with Romanowsky stains and did not stain with ALP, Luna, or Luxol fast blue stains. On TEM, the classical electron-dense core of these granules was replaced by a core with fragmented or amorphous internal material. Typical eosinophils were not identified in any cat in which gray eosinophils were identified.

**Conclusions:** The distinct morphologic, cytochemical, and ultrastructural changes in gray feline eosinophils might be associated with a reduction or lack of major basic protein (MBP) in specific granule cores. Similar to canine gray eosinophils, accurate recognition of these cells is essential to prevent their misclassification as toxic neutrophils.

## KEYWORDS

major basic protein, secondary granule, specific granule

Gray or “vacuolated” eosinophils (GE) are commonly reported in the Greyhound and other sighthound dog breeds, and occasionally in nonsighthound dog breeds, but they have not been reported in the cat.<sup>1,2</sup> In the dog, the term “gray eosinophil” reflects the presence of variably sized, unstained granules resembling clear vacuoles.<sup>2</sup>

Ultrastructural characterization of canine gray eosinophils shows a decrease, not only in the overall secondary granule size but

also in the dense matrix and outer rim width, resembling changes observed in people with eosinophil peroxidase deficiency.<sup>3</sup> Functional abnormalities have not yet been found, and their distinctive cytologic appearance is thought to be due to a difference in granule staining properties, which appears more prominent with aqueous Romanowsky stains such as Diff-Quik.<sup>1,2</sup> Accurate recognition of these cells is important in peripheral blood film examinations

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where these cells can be mistaken for toxic neutrophils, especially in sighthounds, which might also have a concurrently lower WBC or neutrophil count compared with other canine breeds.<sup>4</sup> Inaccurate classification (underestimation) by hematology analyzers has also been reported, although for dogs with eosinophil concentrations within reference intervals, this is unlikely to be clinically significant.<sup>1</sup>

Following identification of suspected gray eosinophils in a routine feline hematology sample from the initial case, the aim of this project was to morphologically, cytochemically, and ultrastructurally describe these cells, not previously reported in cats, and determine their clinical significance.

Ethical approval for the project was obtained (URN 2019 1928-2). A total of 4257 blood films from 2641 cases, stained with modified Wright stain (automated stainer; Hema-tek 2000, Bayer) from routine feline hematology profiles were evaluated between May 2015 and July 2018 for the presence of gray eosinophils. When gray eosinophils were identified, additional blood smears were made from EDTA blood for further cytochemical staining and analysis. Two cases and a feline control were selected for transmission electron microscopy (TEM) evaluation.

Blood films from cats identified with gray eosinophils and feline controls were stained with an aqueous Romanowsky stain and cytochemical stains for alkaline phosphatase (ALP), Luna, and Luxol fast blue as previously described.<sup>5</sup> For TEM analyses, buffy coat preparations from two cats with gray eosinophils and one control cat were fixed with 3% glutaraldehyde (Sigma-Aldrich). In brief, samples were postfixed in 1% osmium tetroxide and, following dehydration in ascending ethanol concentrations, were infiltrated with and embedded in Agar 100 resin (Agar Scientific Ltd). The samples were then cured for at least 20 hrs at 60°C. Subsequently, ultrathin sections (90 nm) were cut and then stained with 0.5% uranyl acetate and 3% lead citrate. The sections were evaluated in a transmission electron microscope (H7500, Hitachi) at an accelerating voltage of 80kV. Gray eosinophils were identified based on size, shape, and granule structure, after exclusion of neutrophils and basophils and visually compared with feline control eosinophils.

Over the 3-year study period, gray eosinophils were identified in a total of nine (of 2641) cats presenting to the Queen Mother Hospital for small animals (QMHA) at the Royal Veterinary College (RVC). Cats were comprised of five males (four neutered; one intact)

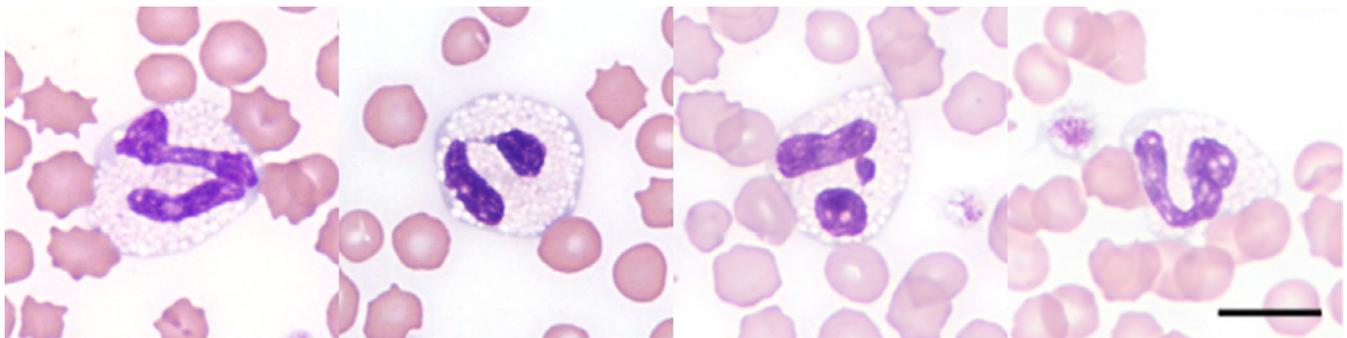
and four females (one neutered; three intact) with ages that ranged from 2 months to 12.5 years. Domestic shorthair (4/9) cats were most commonly represented, with British shorthair (BSH) cats accounting for one-third of cases (3/9). There was also one Ragdoll (1/9) and one domestic longhair (1/9) cat. Presenting clinical signs were related to the underlying clinical diseases, which included congenital extrahepatic portosystemic shunts (3/9), neoplasia (2/9; lymphoma and hemangiosarcoma), gastrointestinal disease (IBD; 1/9), peripheral vestibular disease (1/9), and two cases lacking a definitive diagnosis. Hematologic changes were mild in the majority of cases and included mild lymphopenia (3/9) attributed to a stress/steroid response, microcytosis (2/9) associated with portosystemic shunts (PSS), mild thrombocytopenia (1/9), or were unremarkable (2/9). The remaining cat had a mild to moderate Heinz body hemolytic anemia.

For both the methanolic and aqueous Romanowsky stains, the same eosinophil morphology was identified in 100% of the gray feline eosinophils, which was repeatable across follow-up samples in two cases. These cells measured approx. 9-10  $\mu\text{m}$  in diameter, had a band to segmented nucleus (average two to three lobes) with clumped mature chromatin. Cells had pale blue/gray cytoplasm that contained abundant small (approx. 0.2-0.5  $\mu\text{m}$  diameter), round, non-staining granules (Figure 1). Eosinophils with a typical feline eosinophil morphology were not identified in any of these cases. Granules also lacked Luna and Luxol fast blue staining (Figure 2). Eosinophil concentrations in all nine cats were within the reference interval ( $0-1.5 \times 10^9/\text{L}$ ) and ranged from  $0.09$  to  $0.79 \times 10^9/\text{L}$ .

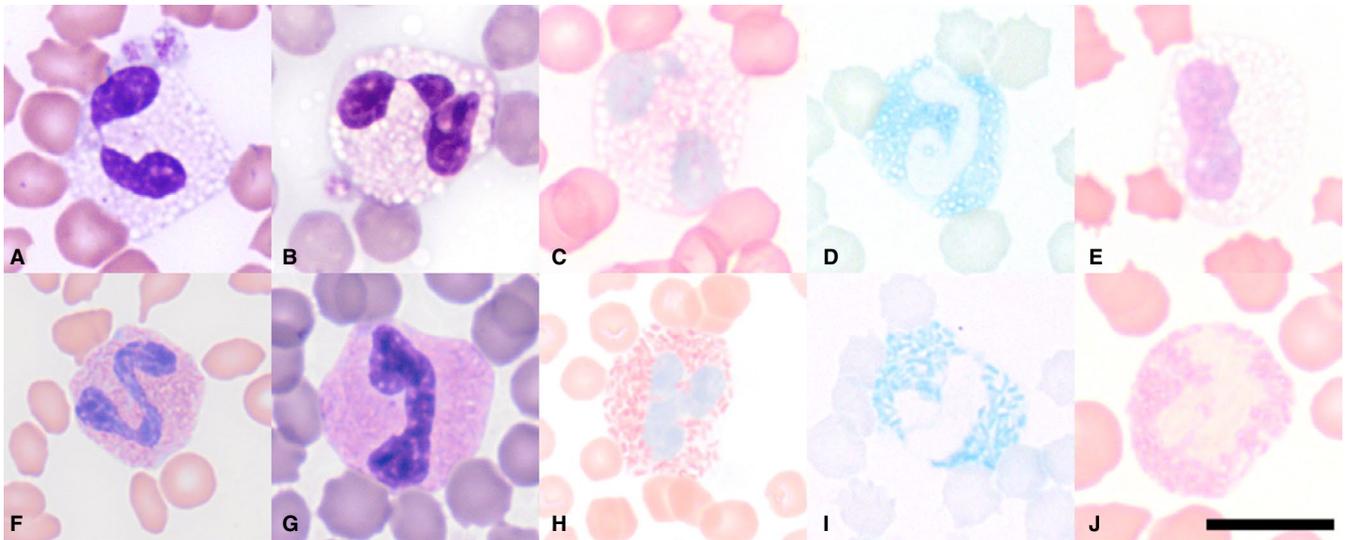
TEM analysis of the gray eosinophils identified ultrastructural abnormalities of the specific granules (Figure 3). Compared with the feline control eosinophils, the characteristic electron-dense core of specific granules was not present in either cat with gray eosinophils. Instead, granules had a fragmented core with a homogenous peripheral cortex and thin limiting membrane. Neutrophil and basophil ultrastructure were similar to controls.

This study describes the morphologic, cytochemical, and ultrastructural features of specific granules in a population of cats with gray eosinophils, not previously described in this species.

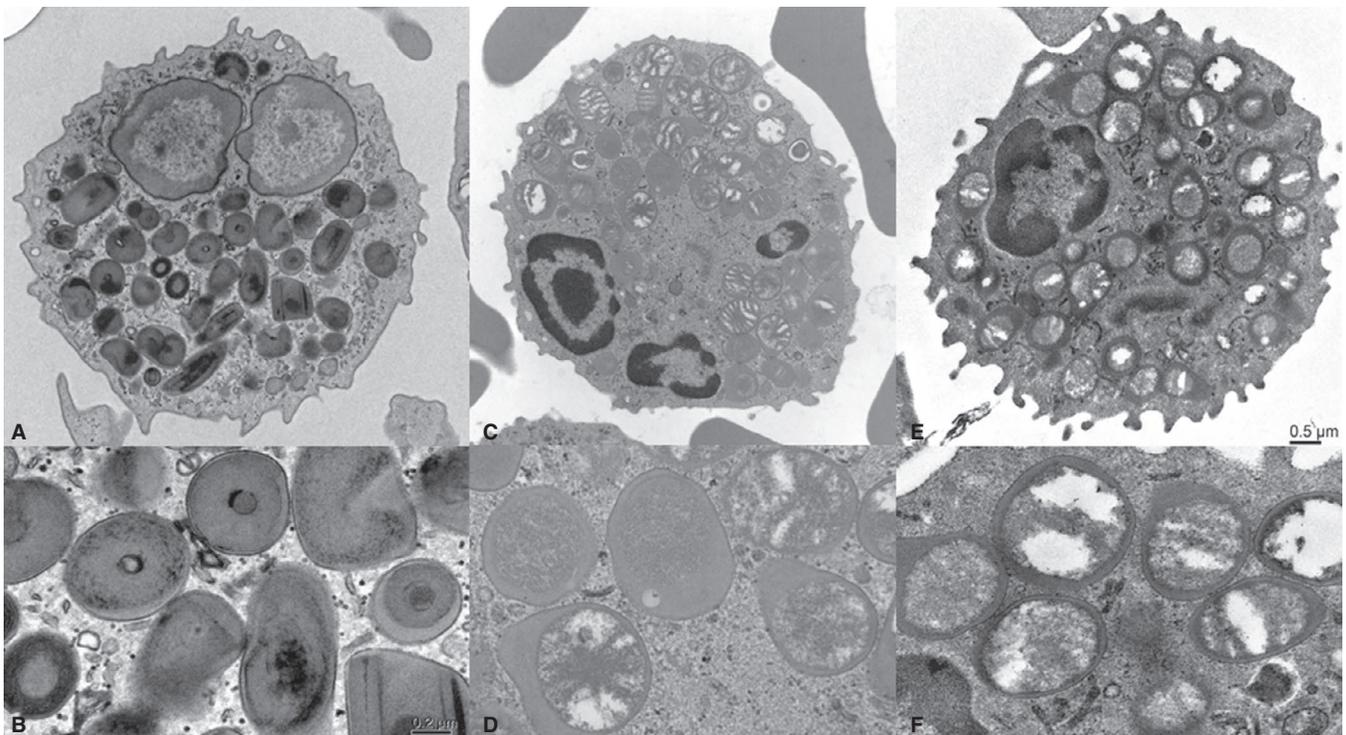
Eosinophils in most species are identified on light microscopy by the "eosin-loving" properties of their granules, the characteristic staining pattern a result of the high cationic protein content. The most numerous and cationic of these proteins is the major basic



**FIGURE 1** Photomicrographs showing a population of granulocytes with small, round, nonstaining granules, and pale blue cytoplasm; peripheral blood smear; modified Wright stain; Bar = 10  $\mu\text{m}$



**FIGURE 2** Photomicrographs of A-E: feline gray eosinophils, F-J: feline control eosinophils,  $\times 100$  objective. Nonstaining clear, round granules with methanolic Romanowsky (modified Wright stain; A), aqueous Romanowsky stains (Diff-Quik; B). Granules remain negative with Luna (C), Luxol fast blue (D), and alkaline phosphatase (BCIP-NBT) staining (E). Classic rod-shaped, red/pink staining granules in control eosinophils with a methanolic Romanowsky (modified Wright stain; F) and aqueous Romanowsky stain (Diff-Quik; G). Control eosinophils granules show intense red staining with Luna (H) and mid-blue staining with Luxol fast blue (I). Alkaline phosphatase (BCIP-NBT) staining of control feline eosinophils demonstrated stain uptake by granules (J). Bar = 10  $\mu$ m



**FIGURE 3** Transmission electron micrographs of control (A-B) and gray (C-F) eosinophils in the cat. Control feline eosinophil with characteristic specific granules containing a central electron-dense core  $\times 15\,000$  (A); higher magnification of specific granules in control cat  $\times 60\,000$  (B); Gray eosinophil granules with fragmented pattern and absence of electron-dense core in two cats  $\times 15\,000$  (C and E); higher magnification of abnormal specific granules, lacking characteristic electron-dense core  $\times 60\,000$  (D and F)

protein (MBP), which forms the crystalline lattice structure of the core of eosinophil (specific/secondary) granules. It is this core that gives the eosinophil its unique ultrastructural feature.<sup>6-9</sup>

Eosinophil-specific granule abnormalities are occasionally described in the literature and are noted in association with eosinophil peroxidase (EPO) deficiency (Presentey's anomaly). This

is characterized in people and mice by a reduction in electron-lucent matrix volume with maintenance of electron-dense granule cores.<sup>10</sup> While this is suspected to be the abnormality present in canine gray eosinophils, an EPO deficiency would not explain the striking ultrastructural changes noted in the cats of this study.<sup>3</sup> Eosinophil secondary granules in both MBP-1 knockout mice (MBP-1<sup>-/-</sup>) and cystatin F-deficient mice (CF null) have a staining pattern and ultrastructural appearance with similarities to those detected in the eosinophil secondary granules in the cats of this study. MBP-1<sup>-/-</sup> mice eosinophil secondary granules lack the characteristic pink/red staining with Wright-Giemsa, and at the ultrastructural level, the electron-dense core is absent. This indicates that the core structure in mice eosinophils is dependent on the presence of MBP. MBP is also significantly reduced in cystatin F-deficient mice (CF null) compared with wild-type (WT) counterparts, and accordingly, they have eosinophil secondary granules with an extensive electron-lucent periphery and cores with internal material which is often amorphous or lucent. Given the similar changes to the electron-dense cores within eosinophil secondary granules in the cats of this study, a complete lack of or significant decrease in the MBP content in the eosinophil secondary granules is considered likely.

Luna staining is commonly used for histologic identification of eosinophils in many species and is described as highly specific.<sup>11</sup> Biebrich scarlet, the chromatophore used within the Luna staining protocol, has a high affinity for basic proteins and readily highlights eosinophil cytoplasmic granules as a result of their basic nature.<sup>6,12</sup> The suspected lack or marked reduction in MBP content in the granules of these cats likely accounts for the negative Luna staining compared with control cats. To identify a reduction or lack of MBP in these cats, further work will focus on the validation and optimization of techniques for the use of an anti-major basic protein antibody to stain for MBP, with genetic analysis and sequencing of the proteoglycan 2 (PRG2) gene in these cats to compare with control cats.

Other differentials considered for the atypical findings on the ultrastructural analysis included granule deterioration due to eosinophil activation and granule content release or granule deterioration due to improper fixation. Three different mechanisms of granule content release are recognized in eosinophils: exocytosis, piecemeal degranulation (PMD), and eosinophil cytolysis (ECL). Granule proteins could be secreted by classical exocytosis, in which a single granule fuses with the plasma membrane, or compound exocytosis, in which multiple granules fuse together before fusing with the plasma membrane to release their contents. Specific granule components are released through membrane-bound secretory vesicles in PMD with empty granule chambers retained within the cell. In ECL, there is a loss of cell cytoplasm, with rupture of the cell membrane and release of membrane-bound specific granules and chromatolysis of the cell nucleus.<sup>9,13,14</sup> Partially empty specific granules or free extracellular specific granules with signs of cell chromatolysis were not identified on TEM analysis of feline gray eosinophils, suggesting that granule release is not the cause for the atypical granule

appearance. An improper fixation method also appears unlikely, given the repeatability of the findings and the structural integrity of other leukocytes. Eosinophil nuclei were intact without evidence of fragmentation or pyknosis, and specific granule limiting membranes remained well preserved.

The significance of gray eosinophils in cats is not fully understood. Functional abnormalities were not suspected, given that affected cats did not appear to have clinical signs associated with eosinophil dysfunction or deficiency. However, if the lack of MBP is confirmed, functional abnormalities, such as reduced parasite immunity similar to CF null mice, could be considered.<sup>15</sup>

In summary, in this study, we described the morphologic, cytochemical, and ultrastructural features of gray eosinophils in a series of cats. While not currently expected to be of clinical concern, in contrast to gray eosinophils in sighthounds, cells exhibit distinct cytochemical and ultrastructural changes. The absence of the characteristic electron-dense core in specific granules is proposed as the cause of the features identified, with a decrease in or complete lack of MBP content suspected. Similarly, to sighthounds, accurate recognition of these cells is important to prevent their classification as toxic neutrophils and an association with inflammation.

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#### DISCLOSURE

The authors have indicated that they have no affiliations or financial involvement with any organization or entity with a financial interest in, or in financial competition with, the subject matter or materials discussed in this article.

#### ORCID

Emma Holmes  <https://orcid.org/0000-0002-2673-8105>

Rose Raskin  <https://orcid.org/0000-0002-2445-9786>

Balázs Szladovits  <https://orcid.org/0000-0002-1926-3455>

#### REFERENCES

- Giori L, Gironi S, Scarpa P, Anselmi A, Gualtieri M, Paltrinieri S. Grey eosinophils in sighthounds: frequency in 3 breeds and comparison of eosinophil counts determined manually and with 2 hematology analyzers. *Vet Clin Pathol*, 2011;40(4):475-483.
- Iazbik MC, Couto CG. Morphologic characterization of specific granules in Greyhound eosinophils. *Vet Clin Pathol*, 2005;34(2):140-143.
- Berghoff N, Kemner SM, Young KM, Scott MA. Ultrastructural abnormalities of Greyhound grey eosinophils. Proceedings of the 49th Annual ASVCP meeting. Atlanta, Georgia, Nov 8-12 2014, E13-E14.
- Zaldívar-López S, Marín LM, Iazbik MC, Westendorf-Stingle N, Hensley S, Couto CG. Clinical pathology of Greyhounds and other sighthounds. *Vet Clin Pathol*, 2011;40(4):414-425.
- Luna LC. *Manual of histological staining methods of the armed forces institute of pathology* (3rd ed.). New York: McGraw-Hill Book Co. 1968; pp. 111-112.

6. Denzler KL, Farmer SC, Crosby JR, et al. Eosinophil major basic protein-1 does not contribute to allergen-induced airway pathologies in mouse models of asthma. *J Immunol.* 2000;165(5509-5517).
7. Jones DG. The eosinophil. *J Comp Path.* 1993;108:317-335.
8. Presentey B, Jerushalmy Z, Ben-Bassat M, Perk K. Genesis, ultrastructure and cytochemical study of the cat eosinophil. *Anatomical Rec.* 1980;196:119-127.
9. Spencer LA, Bonjour K, Melo RCN, Weller PF. Eosinophil secretion of granule-derived cytokines. *Front Immunol.* 2014;5:496.
10. Denzler KL, Borchers MT, Crosby JR, et al. Extensive eosinophil degranulation and peroxidase-mediated oxidation of airway proteins do not occur in a mouse ovalbumin-challenge model of pulmonary inflammation. *J Immunol.* 2001;167(1672-1682).
11. Gomes P, Torres SMF, Plager DA, Jessen CR, Lee JJ. Comparison of three staining methods to identify eosinophils in formalin-fixed canine skin. *Vet Dermatol.* 2013;24:323-328.
12. Eversole RR, Mackenzie CD, Beuving LJ. A photoreactive fluorescent marker for identifying eosinophils and their cytoplasmic granules in tissues. *J Histochem Cytochem.* 2003;51(2):253-257.
13. Ward JM, Wright JF, Wharran GH. Ultrastructure of granulocytes in the peripheral blood of the cat. *J Ultrastructure Res.* 1972;39:389-396.
14. Young KM, Meadows RL. Eosinophils and their disorders. In DJ Weiss & KJ Wardrop, editors. *Schalm's Veterinary Haematology* (6th edition, Chapter 43). Wiley-Blackwell, 2010:281-289.
15. Matthews SP, McMillan SJ, Colbert JD, Lawrence RA, Watts C. Cystatin F ensures eosinophil survival by regulating granule biogenesis. *Immunity.* 2016;44:795-806.

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