

**BACKGROUND:** Human and rodent leukocytes express high levels of the glucocorticoid-inducible protein annexin 1 (ANXA1) (previously referred to as lipocortin 1). Neutrophils and monocytes have abundant ANXA1 levels.

**Aim:** We have investigated, for the first time, ANXA1 ultrastructural expression in rat eosinophils and compared it with that of extravasated neutrophils. The effect of inflammation (carrageenin peritonitis) was also monitored.

**Methods:** Electron microscopy was used to define the sub-cellular localisation of ANXA1 in rat eosinophils and neutrophils extravasated in the mesenteric tissue. A pair of antibodies raised against the ANXA1 N-terminus (i.e. able to recognise intact ANXA1, termed LCPS1) or the whole protein (termed LCS3) was used to perform the ultrastructural analysis.

**Results:** The majority of ANXA1 was localised in the eosinophil cytosol (~60%) and nucleus (30–40%), whereas a small percentage was found on the plasma membrane (< 10%). Within the cytosol, the protein was equally distributed in the matrix and in the granules, including those containing the typical crystalloid. The two anti-ANXA1 antibodies gave similar results, with the exception that LCPS1 gave a lower degree of immunoreactivity in the plasma membrane. Inflammation (i.e. carrageenin injection) produced a modest increase in eosinophil-associated ANXA1 reactivity (significant only in the cytoplasm compartment). Extravasated neutrophils, used for comparative purposes, displayed a much higher degree of immunoreactivity for the protein.

**Conclusion:** We describe for the first time ANXA1 distribution in rat eosinophil by ultrastructural analysis, and report a different protein mobilisation from extravasated neutrophils, at least in this acute model of peritonitis.

**Key words:** Neutrophil, Lipocortin 1, Ultrastructure, Carrageenin, Peritonitis

## Annexin 1 localisation in tissue eosinophils as detected by electron microscopy

Sonia M. Oliani<sup>1,2</sup>, Amilcar S. Damazo<sup>2</sup> and Mauro Perretti<sup>3,CA</sup>

<sup>1</sup>Department of Biology, IBILCE – UNESP, São José do Rio Preto, SP, Brazil; <sup>2</sup>Department of Morphology, UNIFESP – EPM, São Paulo, SP, Brazil; and <sup>3</sup>The William Harvey Research Institute, Bart's and The London, Queen Mary School of Medicine and Dentistry, Charterhouse Square, London EC1M 6BQ, UK

<sup>CA</sup> Corresponding Author  
Tel: +44 20 7882 6065  
Fax: +44 20 7882 6076  
E-mail: M.Perretti@qmul.ac.uk

## Introduction

Migration of eosinophils into specific tissue sites is a hallmark of allergic diseases such as asthma and allergic rhinitis. Eosinophil recruitment can contribute to the perpetuation of the condition by releasing tissue-damaging agents such as major basic protein, eosinophil peroxidase, lipid mediators and multi-potent cytokines.<sup>1</sup> Therefore, it is important to have a better understanding of the mechanisms involved in the selective migration of eosinophils from the blood to allergic tissues and the further activation of these cells in the tissue in response to specific stimuli.<sup>2</sup>

Glucocorticoid hormones are now used as first-line drugs for the treatment of asthma, and have wide applications in the therapeutic control of other allergic

and inflammatory diseases. Inhibition of cytokine-stimulated eosinophil survival is one of the important actions of glucocorticoids.<sup>3</sup> One of the mechanisms responsible for glucocorticoid therapeutic efficacy is increased expression of the anti-inflammatory protein annexin 1 (ANXA1).<sup>4</sup> Few studies have addressed the link between steroids and ANXA1 production from resident lung cells.<sup>5–8</sup> Similarly, even less studies have focused on ANXA1 and the eosinophil. In a murine model of an allergic air-pouch we found that endogenous ANXA1 did not mediate the anti-inflammatory effect of dexamethasone,<sup>9</sup> and a similar finding was obtained with respect to chemokine-induced eosinophil trafficking in the skin.<sup>10</sup> It is unclear whether these data generated in murine systems may be translated to other species, including man.

A similar paucity of data exists with regard to ANXA1 expression/localisation and the eosinophil polymorphonuclear leukocyte. The study of Das *et al.*<sup>9</sup> reported that mouse eosinophils contained ANXA1, as determined with a flow cytometric assay. A more recent study reported ANXA1 expression on the cell surface of human eosinophils and its modulation by incubation with fluticasone propionate.<sup>11</sup>

The majority of ultrastructural studies performed so far on ANXA1 have focused on the neutrophil. In resting human neutrophils, large amounts of the protein (between 2 and 4% of total cytosolic proteins) are in the cytoplasm.<sup>12,13</sup> A large proportion of the intracellular protein co-localises with the gelatinase granules, as determined by confocal and electron microscopy analyses.<sup>14</sup> This pattern of ultrastructural distribution explains easily the rapid mobilisation of ANXA1 seen *in vitro* and *in vivo* when the neutrophil adheres to an endothelial monolayer.<sup>15,16</sup>

The present study was prompted to address the basic question of ANXA1 expression in the eosinophil, using a rat model of peritonitis.

## Materials and methods

### Animals

Male Sprague-Dawley rats (200–250 g body weight; Bantin and Kingman, Hull, UK) were maintained on a standard chow pellet diet with tap water *ad libitum*. Animals were housed at four animals per cage in a room with controlled lighting (lights on 08:00–20:00 h) and temperature (21–23°C). Rats were not used before 2–3 days after their arrival. Experimental work was performed according to Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act 1986).

### Model of inflammation

Experimental peritonitis was induced in rats ( $n = 5$ ) by the intraperitoneal (i.p.) injection of 1.5 mg/kg of carrageenin (type lambda; Sigma Chemical Co., Poole, Dorset, UK) in phosphate-buffered saline (PBS) as recently described.<sup>16,17</sup> Sham animals were treated with PBS alone ( $n = 5$ ). In all cases, rats were sacrificed 4 h later. Animals were anaesthetised by injection with sodium pentobarbital (50 mg/kg) and perfused through the heart left ventricle with sterile saline (30–50 ml per rat) for 30–60 sec. This was followed by a slow infusion of 100 ml of cold paraformaldehyde (2% in PBS) lasting for 5–10 min.

### Fixation, processing, and embedding for immunocytochemistry (electron microscopy)

After perfusion, fragments of the mesentery were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde,

0.1% sodium cacodylate buffer (pH 7.4) for 24 h at 4°C. They were then washed in sodium cacodylate, dehydrated through a graded series of ethanol, and embedded in LR Gold (London Resin Co., Reading, UK).<sup>16</sup> Sections (0.5  $\mu\text{m}$  thick) were stained with Toluidine blue for light microscope analysis and subsequent electron microscopy. For electron microscopy, sections (approximately 90 nm thick) were cut on an ultramicrotome (Reichert Ultracut, Leica, Austria) and placed on nickel grids for immunogold labelling.

### Post-embedding immunogold labelling

To detect ANXA1 in the tissues, a recently established immunogold staining procedure was used.<sup>16</sup> Sections of the mesenteric tissues were prepared for electron microscopy by standard methods. Briefly, mesentery was stained with uranyl acetate (2% w/v in distilled water), dehydrated through increasing concentrations of ethanol (70–100%) and embedded in LR Gold resin. Ultrathin sections were prepared and incubated with the following reagents at room temperature: (a) 0.1 M PBS containing 0.1% egg albumin (PBEA); (b) 2.5% normal rabbit serum in PBEA for 1 h; (c) two different anti-ANXA1 antibodies diluted 1:300 in PBEA: LCS3 and LCPS, a sheep polyclonal antibody raised against the N-terminal peptide of human ANXA1 (peptide Ac2-26),<sup>18</sup> with non-immune sheep serum used as the control of the reaction (final dilutions of 1:300 in PBEA); (d) after five washes (3 min each) in PBEA, with a donkey anti-sheep IgG (Fc fragment specific) antibody (1:50 in PBEA) conjugated to 15-nm colloidal gold (British Biocell, Cardiff, UK). After 1 h at 4°C, sections were extensively washed in PBEA and then in distilled water. Ultrathin sections were stained with uranyl acetate and lead citrate before examination on a Jeol 1200 EX II electron microscope, Jeol USA Inc., Peabody, MA.

### Data handling and statistical analysis

Immunocytochemical analysis of eosinophil infiltrated in the perivascular connective tissue was performed with randomly photographed sections.<sup>16</sup> The area of each eosinophil compartment (membrane, cytosol, granule and nucleus) was determined with a point-counting morphometric method using a square test grid with 8.7 mm spacing.<sup>17</sup> The density of immunogold (number of gold particles/ $\mu\text{m}^2$ ) was calculated and expressed for each cell compartment. Values are reported as the mean  $\pm$  SEM of the number of electron micrographs.

Statistical differences between means were determined by analysis of variance followed, if significant, by the Bonferroni test. A probability values less than 0.05 was taken as significant.

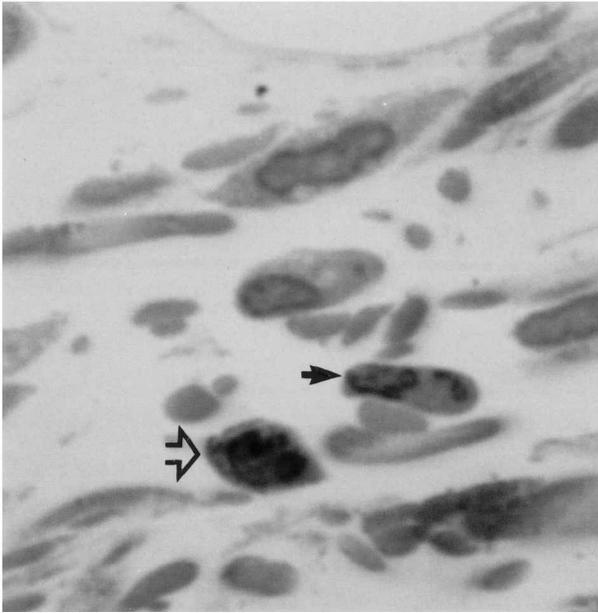


FIG. 1. Representative micrograph of the inflamed mesentery 4 h post-carrageenin injection. Carrageenin was injected i.p. to rats, and the mesenteric tissue collected 4 h later. Eosinophils can be seen localised both intravascularly as well as in the extravascular tissue (open arrow). An extravascular neutrophil is indicated (closed arrow). Sections ( $0.5\ \mu\text{m}$ ) were stained with May-Grünwald and Giemsa ( $\times 1500$ ).

## Results

### Carrageenin peritonitis

In line with several studies, including our recent ones,<sup>16,17</sup> carrageenin peritonitis is characterised by a rapid (4 h) and intense extravasation of blood-borne leukocytes into the mesenteric tissue, most of which are neutrophils (Fig. 1). However, we could also detect a small percentage of eosinophils in the tissue (as well as a certain number of resident macrophages) (Fig. 1).

### Ultrastructural analysis for ANXA1 in extravasated eosinophils

The immunocytochemistry reaction with LCPS1, an antibody against the peptide N-terminal of annexin 1, showed the presence of gold particles on the nucleus and the cytosol of the eosinophils. Table 1 presents the quantitative data for the group injected with PBS, with a total of  $(\sim 7 \pm 0.4) \times 10^{12}$  and  $(9 \pm 0.8) \times 10^{12}$  of gold particles/ $\mu\text{m}^2$  in the nucleus and the cytosol, respectively. Injection of carrageenin modestly elevated the ANXA1 immunoreactivity with a significant increase only in the cytoplasmic compartment (Table 1). Figure 2A illustrates the staining obtained with LCPS1, and hence due to the intact protein. Figure 2B shows the result produced with LCS3. Table 1 shows that there was not much difference between the two antibodies in terms of total immunoreactivity.

In analogy to our previous study,<sup>16</sup> the extravasated neutrophils presented a much higher degree of ANXA1 immunoreactivity (Fig. 2C).

A higher magnification of peritoneal eosinophil clarifies this aspect further (Figs 3 and 4). The reaction with LCS3 gave clearer results with apparent clusters of ANXA1 immunoreactivity on the cytoplasmic granules that contained the crystalloid (Fig. 3). Sites for expression of the protein in the cytosol and cytoplasmic vacuoles were also evident. Figure 4A shows that intact ANXA1 was present in the granule as visualised with LCPS1, whereas Fig. 4B confirms the specificity of the staining produced, with essentially an absence of gold particles following incubation with a control non-immune sheep serum.

## Discussion

The present study reports for the first time the localisation of ANX-1 in the eosinophil *in vivo* as seen at the ultrastructural level. So far, the problem of ANX-1 distribution in leukocytes has been predominantly

**Table 1.** Distribution of ANXA1 immunoreactivity in rat eosinophils in basal and inflammatory conditions

Treatment, staining antibody	ANXA1 immunoreactivity (gold particles/ $\mu\text{m}^2$ )				
	Nucleus	Cytosol	Granules	Membrane	Total
PBS, LCPS1 antibody	$7.2 \pm 0.4$	$6.2 \pm 0.6$	$3.9 \pm 0.5$	$0.5 \pm 0.3$	$16.4 \pm 0.8$
Carrageenin, LCPS1 antibody	$9.7 \pm 1.1$	$8.2 \pm 0.8^*$	$5.1 \pm 0.6$	$0.9 \pm 0.2$	$21.5 \pm 0.9^*$
Carrageenin, LCS3 antibody	$10.9 \pm 0.9$	$8.4 \pm 0.9^*$	$5.2 \pm 1.0$	$1.9 \pm 0.4^*$	$24.8 \pm 2.1^*$

Rats were treated with PBS (5 ml/kg i.p.) or carrageenin (1.5 mg/kg i.p.) 4 h prior to removal of the mesenteric tissue. Tissues were processed as described in Materials and methods, and stained with either LCPS1 (a sheep serum raised against the ANXA1 N-terminus region, hence it recognises intact ANXA1) or with LCS3 (a sheep serum raised against the entire protein, hence it recognises all ANXA1 species, including cleaved forms that may be present). The number of gold particles/ $\mu\text{m}^2$  of cell area refers to the following cellular compartments: the nucleus, the plasma membrane, the cytoplasmic matrix (cytosol) and the granule (clearly identified by the presence of the crystalloid). The total number is also reported. Some tissue sections were also stained with a non-immune sheep serum, producing essentially no gold particle staining (see Fig. 4B for a representative micrograph). Data are presented as the mean  $\pm$  SEM of 10 distinct eosinophils examined from the micrographs of the tissue sections produced from three different rats.

\*  $p < 0.05$  versus the corresponding PBS group value.

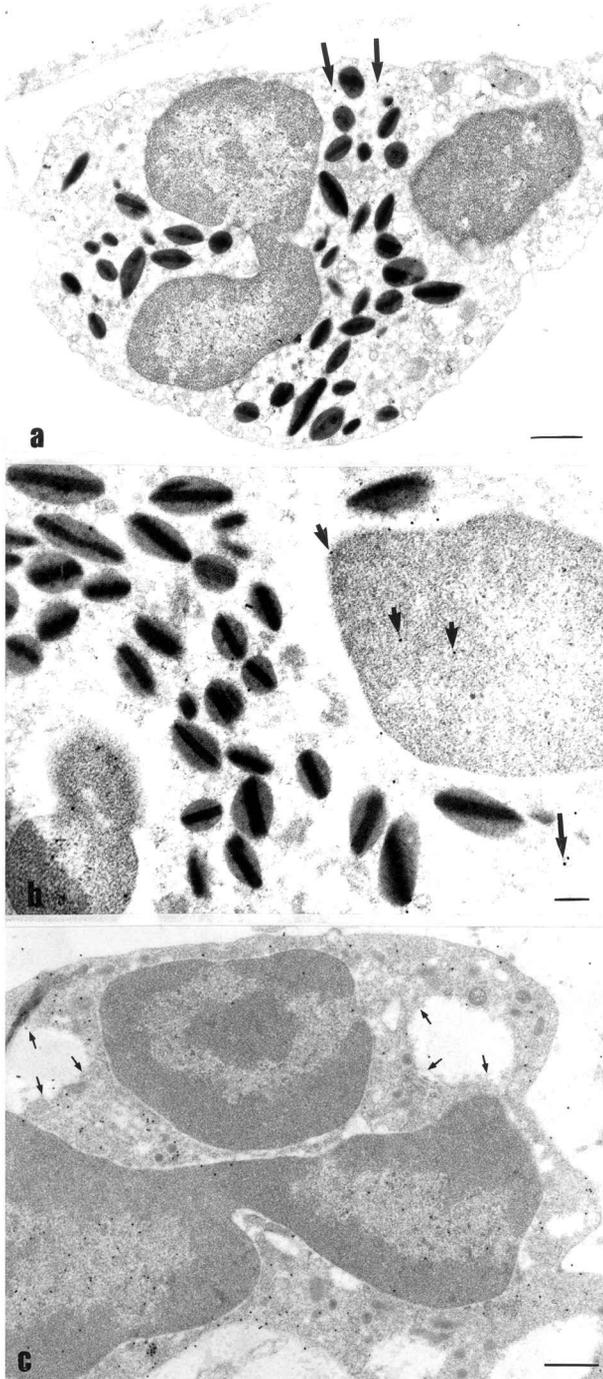


FIG. 2. Electron micrographs showing ANXA1 immunogold in extravascular eosinophils as detected by LCPS1 and LCS3 antisera. Treatment was as in Fig. 1. Sections were stained with a polyclonal sheep serum raised against the specific ANXA1 N-terminus (termed LCPS1) or with a polyclonal sheep serum raised against full-length human ANXA1 (termed LCS3). (A) LCPS1 staining for ANXA1 shows a significant proportion in the cytosol (arrows) and in granules. (B) Similar but more intense immunostaining with LCS3, with arrows highlighting gold particles in the cytosol and nucleus. (C) Extravasated neutrophils were greatly activated as indicated by the presence of large vacuoles in the cytosol (arrows). LCS3 produced an intense degree of immunoreactivity both in the cytosol and the nucleus. Bars: (A) and (C) 0.5  $\mu\text{m}$ , (B) 0.2  $\mu\text{m}$ .

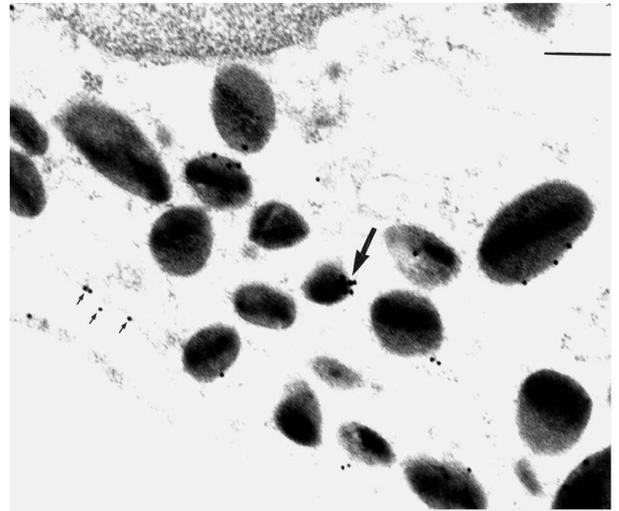


FIG. 3. ANXA1 protein expression in rat mesenteric eosinophils: LCS3 immunostaining. Treatment was as in Fig. 2, and endogenous ANXA1 immunoreactivity in an extravasated eosinophil was detected with LCS3. Gold particles are clearly evident both in granules (large arrow) as well as in cytoplasmic vacuoles (small arrows). Bar: 0.5  $\mu\text{m}$ .

addressed in neutrophils, mast cells and macrophage-like cells.<sup>14,16,17,19,20</sup>

There is a vacuum in ANXA1 biology that is the expression and function that the protein may exert in the eosinophil polymorphonuclear leukocyte. As stated in the Introduction, both exogenous and endogenous ANXA1 did not seem to play a functional role in murine models of eosinophil extravasation. This may be a species-specific phenomenon or it may be characteristic of the skin microcirculation (since the two studies examined employed skin models of allergic inflammation).<sup>9,10</sup> In fact, there are indications that ANXA1 may have functional roles in human systems during asthma and other pathologies characterised by eosinophil influx.<sup>6,8,11</sup>

ANXA1 is a protein that lacks signal peptide,<sup>21</sup> yet it is found in the extracellular medium, certainly in inflammatory conditions.<sup>22</sup> Studies in the past 5 years have proposed that it is the extravasating neutrophil that brings ANXA1 into the inflamed tissue.<sup>16,22</sup> *In vitro*, adherent neutrophils release ANXA1<sup>15</sup> as the result of a controlled process of exocytosis,<sup>14,16</sup> probably linked to the specific localisation (in cytosolic granules) of the protein in this cell type. It is therefore possible that ANXA1 may play functional roles in eosinophils distinct from those that it plays in the neutrophil, and the initial reason for this may be simply linked to its localisation, hence its susceptibility to be mobilised during the process of extravasation.

In the present study we could demonstrate ANXA1 expression and distribution in the rat eosinophil. In analogy to other cell types, ANXA1 was found in the nucleus, the cytoplasm and also in close contact with

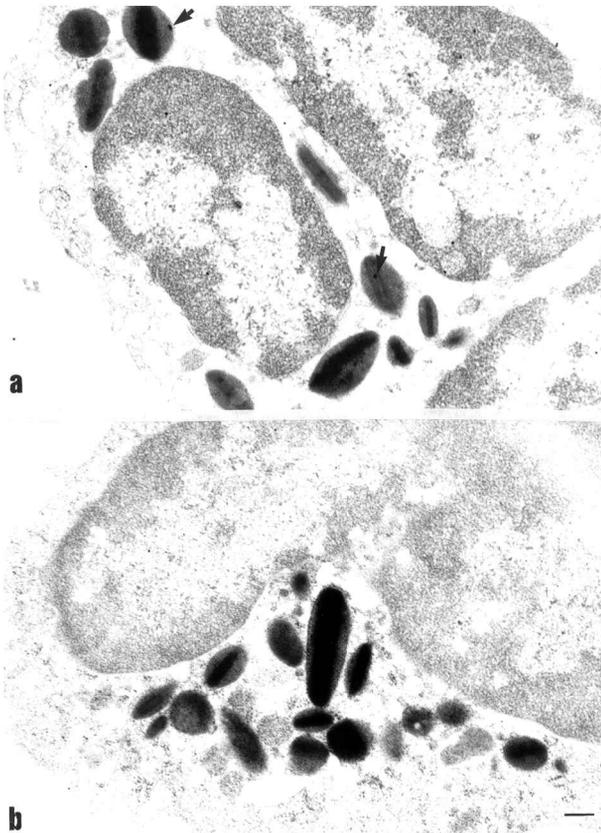


FIG. 4. Intact ANXA1 in rat mesenteric eosinophils: LCPS1 immunocytochemistry. Untreated rats were used for this staining. (A) Eosinophil view showing gold particles associated in the nucleus and specific granules (arrows). (B) Control section incubated with non-immune sheep serum. No gold labeling was observed. Bars: 0.2  $\mu$ m.

the plasma membrane. This distribution is generally in line with observations made in other cells, such as neutrophils, mast cells and macrophages. A partial nuclear localisation for ANXA1 was initially reported in endothelial cells<sup>23</sup> and then seen also in neutrophils and mast cells.<sup>16,17</sup> The function of nuclear ANXA1 is presently obscure. Several groups have reported the existence of at least three sub-cellular pools of the protein.<sup>21,24,25</sup> One pool is cytosolic, the second is strongly associated with the plasma membrane and can only be solubilised by zwitterionic detergents<sup>26</sup> (ANXA1 being an integral membrane protein), and the third pool is found loosely attached on the external leaflet of the plasma membrane (ANXA1 being a peripheral membrane protein, easily recovered by washing cells with ion chelators). Our electron microscopy analysis confirms this distribution of the protein in rat eosinophils, and sheds some light on its exact localisation in the cytoplasm. The majority of the protein (~60%) was found in the cytoplasmic matrix, whereas a lower degree was associated with the granules. Interestingly, apparent clusters of ANXA1 were seen in some of the characteristic eosinophil granules that contained the crys-

talloid. This initial analysis shows a marked difference from the neutrophil, in which there is much more ANXA1 immunoreactivity (see reference 16 for rat neutrophils), and there is a larger granular portion.<sup>16,27</sup>

During the process of extravasation, leukocyte adhesion brings about a whole series of adhesion molecules and other proteins on the cell surface, through a process of controlled exocytosis.<sup>28,29</sup> Cytoplasmic granules and/or vesicles fuse with the plasma membrane to increase the amounts of pro-inflammatory (adhesion molecules) and anti-inflammatory (ANXA1) mediators within that microenvironment. Regarding ANXA1, this model is valid for human<sup>15</sup> and rodent neutrophils;<sup>16,30</sup> however, it may be not true for the eosinophil. In essence, the lower degree of ANXA1 granular association demonstrated in rat eosinophil, as well as its lack of modulation by the inflammatory reaction (at variance from mast cells, for instance<sup>17</sup>), may suggest that a minor amount of the protein may be externalised upon eosinophil adhesion to the endothelium; hence, ANXA1 may have modest effects on the process of eosinophil trans-endothelial passage.

Based on analogy to the neutrophil and macrophage, eosinophil ANXA1 may modulate processes of exocytosis<sup>31,32</sup> or phagocytosis.<sup>20</sup> Analysis of the inhibitory effects on eosinophil soluble lipid mediator generation<sup>11</sup> or radical species formation<sup>33</sup> that endogenous ANXA1 may play could be the next step in an attempt to associate a functional role to the distribution presently reported. Similarly, ANXA1 localisation and function in the eosinophil may change after glucocorticoid treatment, and it may also be related to the tissue origin of the eosinophil (e.g. skin versus airways or peritoneum). In conclusion, this is the first ultrastructural study that has investigated ANXA1 distribution in the eosinophil. Future studies will address the potential functional role(s) for ANXA1 in this cell type.

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