

## Role of Airway Epithelium in Engulfing Apoptotic Eosinophils

Faris Q Alenzi DIC, Ph.D.

Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud University, AlKharaj 11942, Saudi Arabia

### Abstract

**Introduction:** This study aims to investigate which recognition pathways are important in engulfing apoptotic eosinophils.

**Methods:** Here, two epithelial cell were selected namely (large airway bronchial epithelial cells) LAECs and A549. The inhibition assay was examined by resting and dexamethasone-stimulated epithelial cells. Confocal microscopy confirmed the engulfment of apoptotic eosinophils.

**Results:** Macrophages and LAECs recognized and phagocytosed apoptotic eosinophils. Dexamethasone and IL-1 increased the capacity of LAECs to engulf apoptotic eosinophils. More interestingly, inhibiting monoclonal antibodies (Mabs) abolished the uptake of apoptotic cells by LAECs.

**Conclusion:** On the basis of the above findings, the LAECs is capable of recognizing and engulfing apoptotic eosinophils and that enhanced by interleukin-1 (IL-1 $\alpha$ ) and dexamethasone.

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### Introduction

Eosinophils are non-dividing, bone marrow-derived, fully differentiated end cells. They are approximately 10-15 $\mu$ m in diameter and are morphologically distinguishable by their bilobed nucleus and distinct granules of varying sizes. Eosinophil half-life in the circulation is 13-18 hours<sup>1</sup>. In healthy individuals, the majority of eosinophils are found in tissues, primarily in the gut, where they survive for several days to weeks. Their presence in the skin or the airway in significant numbers is usually associated with diseases such as Asthma<sup>2</sup>. Much of the inflammation in asthma is thought to be a consequence of the inappropriate accumulation of eosinophils and the subsequent release of their potent armory of mediators, including cytotoxic granule proteins, lipid mediators, cytokines, and chemokines<sup>3</sup>. Apoptosis is a complex, tightly regulated and active cellular process and 2 commonly referred pathways referred to as the intrinsic

and extrinsic pathways. Eosinophils have been convincingly shown to undergo apoptosis when cultured in vitro<sup>4-5</sup>.

Apoptotic cell removal from the tissues is thought to be dependent on their death by apoptosis, followed by their recognition and phagocytosis by macrophages<sup>6</sup> resident bronchial epithelial cells<sup>7</sup>, type II alveolar adenocarcinoma epithelial cell line A549 smooth muscle cells<sup>8</sup> lung fibroblasts<sup>9</sup> human liver Kupffer cells<sup>10</sup> or dendritic cells<sup>11</sup>.

In this regard, Stern et al<sup>6</sup> have demonstrated that monocyte-derived human macrophages recognize and ingest apoptotic eosinophils via integrin-and sugar/lectin-dependent mechanisms. Walsh et al and other groups<sup>12-14</sup> demonstrated that human small airway bronchial epithelial cells (SAECs) and human alveolar epithelial cells (A549) ingest apoptotic peripheral blood eosinophils (PBE), but not freshly isolated viable eosinophils, in a specific lectin or  $\alpha$ v $\beta$ 3, CD36 membrane receptor-mediated fashion.

The relatively restricted numbers of purified eosinophils available from normal or mildly allergic donors often limits the scope of studies on their biology. The EoL-1 leukaemic cell line has been established for some years and when differentiated using dibutyryl cyclic adenosine monophosphate (dbcAMP), displays many eosinophilic features including comparable membrane receptor phenotype together with expression and secretion of two eosinophil-specific granule proteins, eosinophilic cationic protein (ECP) and major basic protein (MPB). Differentiated EoL-1 have been used to elucidate the factors controlling apoptosis in eosinophils including mAb-dependent ligation of Fas<sup>15</sup> and treatment with glucocorticoids under culture conditions that utilise low serum-containing media<sup>16</sup>. This study aimed to examine the usefulness of apoptotic differentiated EoL-1 as phagocytic targets for human large airway bronchial epithelial cells (LAECs) and the alveolar epithelial cell line A549 and to investigate which recognition pathways are important in that process. Confocal microscopy was used to examine phagocytosis of apoptotic

differentiated EoL-1 and ascertain f-actin involvement in phagocytic cup formation by LAEC and A549 cells. The function of the recognition receptors involved in phagocytosis of apoptotic EoL-1 by LAEC or A549 were also examined.

## Materials & Methods

### Cultured epithelial cells

The Type II alveolar cell line, A549, which was obtained from the ECACC, Salisbury, Wiltshire, UK was cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), penicillin (100?g/ml), streptomycin (100?g/ml), and 2mM L-glutamine. Normal human bronchial epithelial cells (also referred to as large airway) (NHBE or LAEC, respectively) were commercially obtained from Clonetics Inc., MD, USA, a subsidiary of Biowhittaker and grown in their specialised small airway growth medium (SAGM). SAGM consisted of small airway basal medium (SABM) supplemented with gentamicin sulphate and amphotericin-B (0.5ml), bovine pituitary extract (2ml; ~7.5mg/ml), hydrocortisone (0.5ml; 0.5mg/ml), human recombinant epidermal cell growth factor [0.5ml; 0.5?g/ml in bovine serum albumin (BSA) solution], epinephrine (0.5; 0.5mg/ml), transferrin (0.5ml; 10mg/ml), insulin (0.5ml; 5mg/ml), retinoic acid (0.5ml; 0.1?g/ml in HBSS), triiodothyronine (0.5ml; 6.51?g/ml) and fatty acid free BSA (5ml; 50mg/ml). Although human LAEC can be maintained in culture for five to six passages using the supplier's serum-free medium<sup>17</sup>, all experiments presented here were performed with cells which had been passaged a maximum of 3 times to ensure no loss of phenotype. These studies used LAEC from at least 7 different donors and their identity as epithelial cells was guaranteed by the supplier. However, we further confirmed the epithelial identity of SAEC or LAEC by their cobblestone morphology and uniform positive immunostaining with mAb to the epithelial marker cytokeratin peptide 19 and CD9, CD44 or ICAM-1. Cultures were split at 80-90% confluence by trypsin digestion and sub-cultured in the same specialised medium.

### Cultured EoL-1 cells

The human eosinophilic leukaemia cell line, EoL-1, which was obtained from the ECACC, Salisbury, Wiltshire, UK was cultured and grown in RPMI 1640 medium supplemented with FCS (10%), penicillin (100?g/ml), streptomycin (100?g/ml), and 2mM L-glutamine at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere. EoL-1 cells were maintained in culture at 0.5-2.0x10<sup>6</sup> cells/ml; spilt 1:2 to 1:3 every 3-4 days; and seeded out at 0.5x10<sup>6</sup> cells/ml.

### Culturing and differentiation of EoL-1 cells

EoL-1 cells were differentiated by culturing with either 0.1mM dbcAMP for 9 days with cell number re-adjustment to 5x10<sup>5</sup>/ml every 3 days in 75 cm<sup>2</sup> culture flasks or 0.5mM butyric acid for 7 days. Cell viability was determined by trypan blue dye exclusion. Viability remained > 90%. For interaction/uptake studies, the differentiated EoL-1 cells were cultured in suspension at a density of 1x10<sup>5</sup> cells/ml and transferred to RPMI media containing 0.05% FCS. The cells were treated with 10<sup>-6</sup>M dexamethasone for 96 hours to induce apoptosis. All apoptotic cells used in the interaction/uptake experiments were greater than 70% as judged by a combination of annexin V-FITC and propidium iodide (PI).

### Apoptosis Induction In Differentiated EoL-1 cells

A total of 0.1mL of differentiated EoL-1 cells (2x10<sup>5</sup>) was incubated for 5, 10, 20, 24, or 48 hours in 96-well flexible flat-bottomed plates (Becton Dickinson) alone or with experimental dexamethasone.

### Differentiated apoptotic EoL-1 Staining

Differentiated apoptotic EoL-1 were washed twice with warmed medium and resuspended gently in prewarmed (37°C) CellTracker orange CMTMR reagent-containing (10iM) medium. The cells were incubated for 30 min under growth conditions and then washed before incubation for another 30 min in fresh prewarmed medium to ensure complete modification of the probe.

### Engulfment of apoptotic differentiated EoL-1 by epithelial cells

This was a modification of a previously established assay by Erwig et al<sup>18</sup>. Epithelial cells were trypsinised, seeded into 24-well plates (1x10<sup>5</sup> cells/well) and allowed to divide to confluence. 24 h prior to interaction monolayers in certain experiments were stimulated with cytokines (10<sup>-10</sup>M) or dexamethasone (10<sup>-6</sup>M). Washed apoptotic differentiated EoL-1 (3x10<sup>5</sup>/well) was added to resting, cytokine or steroid stimulated epithelial monolayers for 60 min at 37°C and 5% CO<sub>2</sub> humidified air, which preliminary experiments established as the optimum time-point in our lab. After stringent washing with PBS/0.02M EDTA buffer, to remove non-ingested eosinophils and fixation with 2% glutaraldehyde in PBS, differentiated EoL-1 cells were visualised under a fluorescent microscope (Nikon, Eclipse, TE-2000U). A blinded investigator counted at least 200 macrophages or epithelial cells in randomly selected fields, and the proportion that had ingested one or more eosinophils

was expressed as a percentage. We examined at least 10 visual fields at x200 magnification. All slides were blinded counted by two pathologists. The mean intra-observer coefficient of variation for repeat counts was less than 4%. Interaction assays used confluent monolayers, which were assessed by visual inspection. The cobblestone appearance was also indicative of the epithelial nature of the cells. Images were taken with an Olympus 1X70 and were manipulated in Microsoft PowerPoint. (Original magnification x200)

### **Antibodies**

mAb/pAb to  $\alpha$ 5,  $\beta$ 5, PS receptor,  $\alpha$ 3, CD36 or isotype-matched controls were applied to the monolayers at a final concentration of 10<sup>5</sup>g/ml in a volume of 400 $\mu$ l culture medium. After 30 mins the monolayers were washed with 0.1% in PBS/BSA and 400 $\mu$ l fresh medium containing 3x10<sup>5</sup> apoptotic differentiated EoL-1 added as per interaction assay protocol.

### **Inhibition Assay**

For these experiments, epithelial cells grown in 24 well plates were pre-incubated for 30 min at 37°C, 5% CO<sub>2</sub> humidified air with the inhibitory factors prior to the interaction assay. Both untreated and treated monolayers were washed prior to use with 0.1% PBS/BSA. Stock solutions were prepared when required and not stored for longer than 1 month at 4°C. Glucose, mannose and galactose had a MW of 180.2. Thus, 225.1 mg/5ml sterile PBS yielded an isotonic stock solution of 250mM. Glucosamine and galactosamine had a MW of 215.6 and so 269.5 mg/5ml sterile PBS resulted in 250mM stock solution. N-acetyl-glucosamine had a MW of 221.1 and thus 286.375mg was added to 5ml sterile PBS to achieve a 250mM solution. 205.25mg of fucose, with a MW of 164.2, was added to 5ml sterile PBS yielding a 250mM stock solution. Aged differentiated EoL-1 and epithelial cells were pre-incubated with the tetrapeptides or sugars prior to their use in the interaction assay using methods derived from Savill et al (19). The tetrapeptides RGDS or RGEs were used at a final concentration of 2mM and monosaccharides at a final concentration of 25mM in 400 $\mu$ l of fresh culture medium. Exposure of both apoptotic differentiated EoL-1 and epithelial cells to these agents was performed to prevent integrin or lectin involvement by either cell type with the other. Post exposure to inhibitory agents the monolayer and the apoptotic differentiated EoL-1 were washed in 0.1% PBS/BSA and the cells permitted to interact in 400 $\mu$ l of fresh growth medium.

### **Confocal microscopy**

A549 or LAEC were grown in 8 well chamber slides (Nunc, Napierville, IL). Cells were cultured to confluence and then allowed to interact with apoptotic differentiated EoL-1 for 60 min as per the normal interaction assay criteria. Apoptotic cells were pre-labelled with 1 $\mu$ M CellTracker Orange<sup>®</sup> for 15 min at 37°C prior to the interaction assay and washed twice with PBS before addition to the epithelial monolayers. After stringent washing monolayers were fixed with 4% paraformaldehyde. F-actin was visualised by staining with Oregon Green<sup>®</sup> phalloidin. Briefly, fixed monolayers were washed twice with 0.05% Triton<sup>®</sup> X-100 in PBS before 20 min incubation with 1%BSA in PBS to prevent non-specific background staining. Monolayers were washed with PBS and 200 $\mu$ l of staining solution (800 $\mu$ l 0.05% Triton<sup>®</sup> X-100 in PBS + 8 $\mu$ l Oregon Green<sup>®</sup> phalloidin) was added to each well for 15 min at room temperature in darkness. Monolayers were then washed 3 times with PBS before treatment with ProLong<sup>®</sup> Antifade kit. Samples were viewed using a Zeiss Axioplan 2/LSM 510 META confocal microscope and images produced using Zeiss LSM Data Server software.

### **Determination of apoptotic cells by Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL) in Dex-treated EoL-1 cells:**

The cells were pelleted at 1800 rpm for 5 min then resuspended in Dulbecco's MEM (Gibco) at a concentration of 2x10<sup>5</sup>/ml and 100  $\mu$ l volumes cytopun on to cleaned microscope slides at 450 rpm for 10 min (Shandon Cytospin 2;Shandon, Pittsburgh, PA, USA). Slides were air dried overnight, rehydrated in TBS for 15 min at room temperature and dried. The cells were covered by a 5 ml droplet of protein K diluted 1:100 in 10mM Tris (pH 8), incubated 5 min at room temperature then dipped three times into TBS and dried. The specimen was covered with 100  $\mu$ l of supplied equilibration buffer and incubated for 30 min at RT. Excess buffer was poured off and freshly prepared TdT labelling mixture (3  $\mu$ l TdT enzyme in 57  $\mu$ l TdT labelling reaction mix (Frag EL:Calbiochem, Nottingham, UK) was layered on to the cells. The slide was incubated at 37°C in humidified chamber for 1.5 hour then washed x3 in TBS at room temperature. A coverslip was applied over mounting medium (Frag EL) and sealed with nail varnish to prevent evaporation. At least 500 cells from randomly selected fields were scored by fluorescent light microscopy (494 nm). Viable cells stained blue whilst apoptotic cells appeared as small fragmented bodies staining bright green.

### Statistical analysis

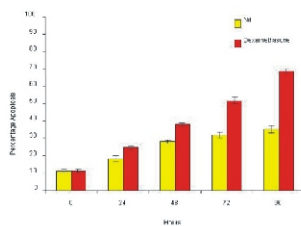
All data are presented as mean  $\pm$  SEM and where n is given this represents the number of experiments. Statistical analysis was by the unpaired two-tailed Student's *t*-test, where a *p* value of  $<0.05$  was considered statistically significant.

## Results

### Dexamethasone-induced apoptosis in EoL-1 by low serum conditions

Initial studies using differentiated EoL-1 cells cultured in 10% FCS demonstrated that under these high serum conditions the cells were relatively resistant to dexamethasone-induced apoptosis. However, by lowering the serum concentration to 0.05% found that dexamethasone treatment of differentiated EoL-1 cells induced their apoptosis. As shown in **Figure-1** treatment of differentiated EoL-1 cells under these conditions with  $10^{-6}$  M dexamethasone caused in a **trend** increase in apoptosis between 24 and 96 hours compared with untreated cells.

Figure-1



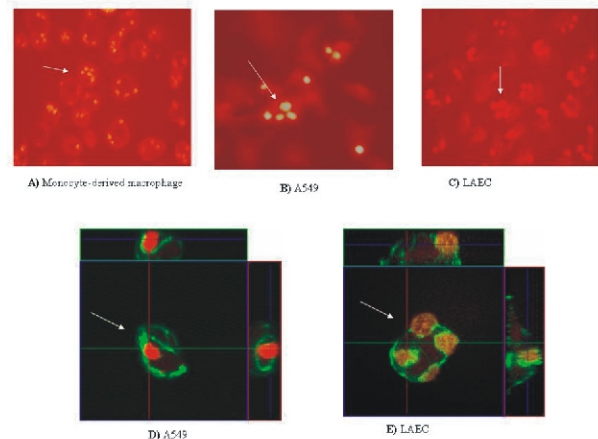
**Figure-1:- Differentiated EoL-1 were cultured in the presence of  $10^{-6}$  M dexamethasone and assessed for apoptosis induction after 0, 24, 48, 72, and 96 hours. Percentage of dexamethasone-induced apoptosis in differentiated EoL-1 under low serum concentration (0.05%). Each bar represents the mean  $\pm$  SEM of five experiments.**

### LAEC and A549 epithelial cells ingest apoptotic differentiated EoL-1

Figure-2 demonstrates phagocytosis of apoptotic EoL-1 by human monocyte derived macrophages (A) A549 (B), or LAEC (C) photomicrograph. EoL-1 were labeled with CellTracker orange and can clearly be seen within the phagocytes. Non-ingested or adherent aged differentiated EoL-1 were removed by vigorous washing with PBS/EDTA. To further confirm ingestion of a representative experiment that clearly demonstrates uptake of apoptotic differentiated EoL-1 by unstimulated LAEC, and A549. Several monocyte derived macrophages, LAEC, and A549 can be seen to have ingested apoptotic differentiated EoL-1 visualized as

orange-staining bodies. We observed no evidence of either interaction with or ingestion of non-apoptotic differentiated EoL-1 by either resting or dexamethasone-stimulated LAEC or A549. Any adhesive interaction between the freshly prepared differentiated EoL-1 and the resting or dexamethasone-stimulated LAEC or A549 would have been disrupted by a combination of the stringent washes together with the use of 0.02 mol/L EDTA in the washing buffer. Confocal microscopy permitted detailed 3D images of F-actin stained epithelial cells ingesting apoptotic differentiated EoL-1 to be obtained. **Figure 2D** depicts a representative A549 ingesting an apoptotic differentiated EoL-1, which is centralized on the cell surface. Cross-sections of the A549, shown on the sides of the image, reveal the EoL-1 to be in a tightly fitting 'pit' on the A549 surface surrounded by a dense concentration of F-actin. **Figure 2E** also highlights this phenomenon but in addition captures a LAEC in the process of ingesting several apoptotic EoL-1. Orthogonal views showing the cell in cross-section, revealed phagocytic cup formation and F-actin rich membrane extensions covering the cells. The image also shows complete membrane coverage of an apoptotic cell (right hand side orthogonal view).

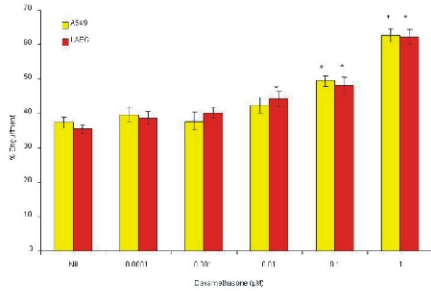
Figure-2



**Figure-2:- Representative photomicrographs of basal ingestion of differentiated apoptotic EoL-1 by monocyte derived macrophages (A), A549 (B) and LAEC (C) using florescent microscopy. Apoptotic differentiated EoL-1 were distinguished via CellTracker orange staining and are clearly visible within the macrophages and epithelial cells. Confocal images micrograph obtained using a Zeiss Axioplan 2/LSM 510 META microscope, showing phagocytosis of apoptotic differentiated EoL-1 by airway bronchial epithelial cells. (D) An apoptotic EoL-1 (red) is clearly visible in the center of the A549 epithelial cells, which has been stained for F-actin using Oregon green phalloidin. A dense green (F-actin rich) annulus is**

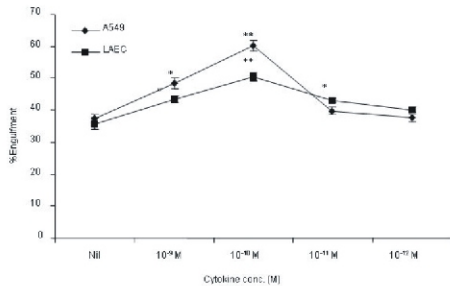
also evident surrounding the EoL-1. (E) Large airway epithelial cell is shown ingesting multiple apoptotic differentiated EoL-1. Orthogonal views generated using Zeiss LSM Data Server software shows distinct engulfment of the apoptotic cell into the cytoplasm of airway epithelial cells. (Original magnification 400x)

Figure-3



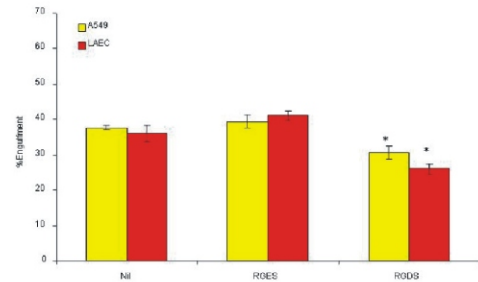
**Figure-3:** The effect of increasing concentrations of dexamethasone on airway epithelial cells engulfment of apoptotic differentiated EoL-1. Each bar represents the mean ± SEM of four (A549) or four (LAEC) experiments. (\*P<0.05).

Figure-4



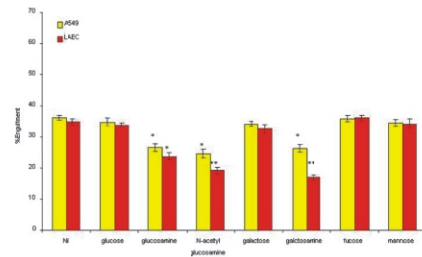
**Figure-4:** The effect of increasing concentrations of IL-1α on airway epithelial cells engulfment of apoptotic differentiated EoL-1. Each point represents the mean ± SEM of four (A549) or four (LAEC) experiments. (\*P<0.05, \*\*P<0.005).

Figure-5



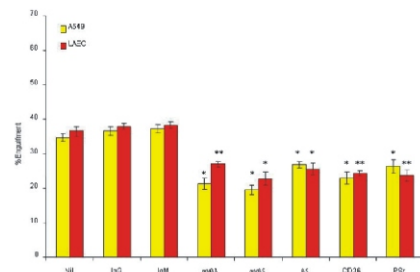
**Figure-5:** RGDS blockade of integrin interactions on differentiated EoL-1 and airway epithelial cells highlighted the involvement of integrins in the phagocytic process. Untreated cells and those exposed to RGES control tetrapeptide showed no inhibition of phagocytosis. Data represents the mean ± SEM of four (A549) or four (LAEC) experiments. (\*P<0.05).

Figure-6



**Figure-6:** Parent sugars had no inhibitory effect on phagocytosis of apoptotic differentiated EoL-1 by the airway epithelial cell types. Addition of amino sugars, which are the targets for lectin binding, resulted in significant decreases. Data represents from four (A549) or four (LAEC) experiments. (\*P<0.05, \*\*P<0.005).

Figure-7



**Figure-7:** Involvement of specific integrins was clearly evident by the decreases in phagocytosis observed with mAb treatment relative to isotype-matched controls and untreated cells. Values are mean ± SEM of four (A549) or four (LAEC) experiments. (\*P<0.05, \*\*P<0.005).

### Dose-response of stimulation of LAEC and A549 epithelial cells with dexamethasone or IL-1 $\alpha$

We next assessed the effect on uptake of aged EoL-1 by LAEC or A549 cells after stimulation for 24 hours with dexamethasone or IL-1 $\alpha$ . Figure-3 & 4 show the effect on the uptake of apoptotic differentiated EoL-1 by resting LAEC or A549 after prestimulation with increasing concentration of dexamethasone or IL-1 $\alpha$ . The optimal dexamethasone concentration was  $10^{-6}$  mol/L for both epithelial cell types, and this enhanced the capacity of LAEC or A549 cells to ingest multiples of apoptotic differentiated EoL-1 compared with untreated epithelial cells in a dose-dependent manner. The maximal increase in the number of A549 or LAEC ingesting apoptotic differentiated EoL-1 was seen with IL-1 $\alpha$  at a concentration of  $10^{-10}$  mol/L, and this represented an approximate 60% or 50% of the numbers of stimulated A549 or LAEC, respectively, capable of ingesting apoptotic differentiated EoL-1.

### Receptors involved in apoptotic differentiated EoL-1 recognition and engulfment by LAEC or A549

We also investigated which receptors were responsible for the recognition of apoptotic differentiated EoL-1 by the epithelial cells LAEC or A549. Incubation of apoptotic differentiated EoL-1 with the tetrapeptide RGDS significantly inhibited their uptake by both LAEC and A549, whereas the control tetrapeptide RGEK had no measurable effect on ingestion of apoptotic differentiated EoL-1 (Figure-5). Preincubation of apoptotic differentiated EoL-1 with the amino sugars glucoseamine, n-acetyl glucosamine, and galactosamine significantly inhibited apoptotic differentiated EoL-1 by LAEC or A549. In contrast, the parent sugars glucose, galactose, mannose, and fucose had no measurable inhibitory effect (Figure-6). These findings provided evidence that ingestion of apoptotic differentiated EoL-1 by LAEC or A549 is dependent on an RGD-dependent signal and that a membrane receptor molecule of the integrin family is involved. The role of  $\alpha$ v $\beta$ 3,  $\alpha$ v $\beta$ 5,  $\alpha$ 5, CD36, and PSR in recognition of apoptotic differentiated EoL-1 by LAEC or A549 was examined using our standard inhibition assay. Using immunostaining and flow cytometry,  $\alpha$ v $\beta$ 3,  $\alpha$ v $\beta$ 5, PSR and CD36 were expressed at moderate to high levels in both LAEC and A549, while the subunit  $\alpha$ 5 was found to be expressed in A549 epithelial cell. All mAbs, but not isotype-matched controls, significantly inhibited uptake of apoptotic differentiated EoL-1 by both LAEC and A549. Moreover, the anti-PSR mAb significantly decreased apoptotic differentiated EoL-

1 uptake by LAEC or A549 (Figure- 7). No additive effect was observed when the mAbs to either  $\alpha$ v $\beta$ 3,  $\alpha$ v $\beta$ 5,  $\alpha$ 5, CD36 or PSR were used in combination with either LAEC or A549.

### Discussion

In the present study, I have demonstrated that apoptotic differentiated eosinophilic cell line EoL-1 were recognized and ingested by the human LAEC and A549 via specific recognition mechanisms and that this process is up regulated by the pro-inflammatory cytokine IL-1 $\alpha$  and the glucocorticoid dexamethasone. The airway epithelium is primarily made up of ciliated, nonciliated, and basal cells. Epithelial cell damage resulting in ciliary dysfunction and loss is a major feature of asthma pathogenesis and is thought to be an important contributor to the development of airway hyperresponsiveness. Walsh's group previously reported that the SAEC and the type II adenocarcinoma-derived cell line A549 have been well characterized as being representative and capable of recognition and engulfment apoptotic peripheral human eosinophils<sup>12-13</sup>. Treatment of EoL-1 cells with dexamethasone caused in a marked increase in apoptosis compared with untreated cells, suggesting that the differentiated EoL-1 apoptotic response to glucocorticoids was similar to primary human eosinophils<sup>20-21</sup>. More recently, these findings were extended to show that SAEC, LAEC and A549 have similar capacity and receptor-usage in the recognition and engulfment of apoptotic eosinophils. Interestingly, either resting or cytokine stimulation SAEC, LAEC, or A549 did not recognise or ingest apoptotic neutrophils<sup>14</sup>.

Glucocorticoid therapy for the treatment of asthma reduces inflammation in the airways by inhibiting cytokine production and stimulating PBE clearance<sup>22</sup>. High concentrations of glucocorticoids have been shown to induce eosinophil apoptosis in vitro<sup>23-24</sup>. Chauhan et al<sup>16</sup> demonstrated that treatment of undifferentiated EoL-1 cells with dexamethasone under low serum-containing media induced an apoptotic pathway that was inhibited by IL-5. Here, it was confirmed that differentiated EoL-1 are sensitive to apoptosis induction by glucocorticoids treatment only under low serum-containing media. Our results revealed that EoL-1 cells are centralized on the A549 surface surrounded by a dense concentration of F-actin. This is indicative of phagocytic cup formation by the phagocyte and explains why these cells were not removed by washing buffer.

Confocal microscopy was used to confirm that apoptotic differentiated EoL-1 engulfment by LAEC or A549. Moreover, the observation that stimulation of LAEC or A549 with IL-1 $\alpha$  enhances their phagocytic capacity for apoptotic differentiated EoL-1 is interesting as the same cytokine is thought to promote eosinophil accumulation via enhanced eotaxin production by the airway epithelium<sup>25</sup>. However, since inflammation is normally a helpful and self-limiting response it would make sense that the cytokines concerned in the accumulation of eosinophils would also prepare resident cells to remove apoptotic eosinophils, a process similar to that described for clearance of apoptotic neutrophils by cytokine-stimulated monocyte-derived macrophages<sup>26</sup>. The ability of glucocorticoids to resolve eosinophilic inflammation may involve acceleration of eosinophil apoptosis<sup>24,27</sup> and their subsequent recognition and engulfment by alveolar macrophages<sup>28</sup>. Treatment of LAEC and A549 with the glucocorticoid dexamethasone enhanced the numbers of cells engulfing apoptotic differentiated EoL-1. This finding was in line with work by Liu et al<sup>29</sup> that established the potentiation of nonphlogistic clearance of apoptotic neutrophils and eosinophils by inflammatory macrophages following exposure to glucocorticoids but not non-glucocorticoid steroids. The response to dexamethasone was dose dependent for LAEC and A549 both in terms of the numbers of their cells phagocytosing apoptotic differentiated EoL-1 and the numbers of those cells taking up multiple apoptotic meals.

The mechanisms by which apoptotic cells are recognised appear to vary according to the cell type responsible for their engulfment. Involvement of lectin and integrin in the recognition of apoptotic cells have been described in several studies:

- 1) an uncharacterised lectin-dependent interaction<sup>30</sup>;
- 2) CD36 and  $\alpha$ v $\beta$ 3 involvement in macrophage recognition of apoptotic eosinophils or neutrophils<sup>31-32</sup>;
- 3)  $\alpha$ v $\beta$ 5 has been implicated in the recognition of apoptotic cells by immature dendritic cells<sup>33</sup>; and rod outer segments by retinal pigmented epithelium<sup>34</sup>;
- 4) a stereo-specific recognition of phosphatidylserine that is expressed on the of the apoptotic cell after the loss of membrane asymmetry<sup>35-36</sup>; and
- 5) macrophage scavenger receptors<sup>37-38</sup>.

In the present study, we found that the amino sugars glucoseamine, n-acetyl glucosamine, and galactosamine significantly inhibited uptake of aged human apoptotic differentiated EoL-1 by LAEC or A549. The tetrapeptide RGDS was used to blockade integrin interactions

between differentiated EoL-1 and airway epithelium as it contained the ubiquitously recognised RGD motif for integrins. Incubation of apoptotic differentiated EoL-1 with the tetrapeptide RGDS significantly inhibited their uptake by LAEC or A549, while the control tetrapeptide RGEK had no measurable effect on ingestion of apoptotic differentiated EoL-1. Monoclonal antibodies to  $\alpha$ v $\beta$ 3,  $\alpha$ v $\beta$ 5,  $\beta$ 5, and CD36 inhibited the phagocytic process and highlighted their specific roles in the process. These findings are similar to the previous work with apoptotic human PBE<sup>12,14</sup>. Furthermore, flow cytometry indicated that  $\alpha$ v $\beta$ 3,  $\alpha$ v $\beta$ 5 and CD36 were expressed at moderate to high levels in both LAEC and A549, while, the subunit  $\beta$ 5 was found to be expressed in A549 epithelial cell.

The exposure of phosphatidylserine (PS) in the outer leaflet of the plasma membrane is one of the most striking changes on the surface of apoptotic cells. We observed inhibition of uptake of apoptotic differentiated EoL-1 following mAb-dependent blockade of the putative phosphatidylserine receptor (PSR). We have extended previous findings for PSR in phagocyte-apoptotic cell interactions<sup>39-41</sup> to show a role for this receptor in epithelial cell recognition of apoptotic differentiated EoL-1. This receptor appears to be responsible for inducing the release of a key antiinflammatory, antiimmunogenic molecule, TGF- $\beta$ .

Macrophages have been shown to be the most important and efficient cells in the recognition and engulfment of apoptotic cells. These professional phagocytes have the ability to ingest multiples of apoptotic cells which, combined with rapid digestion of their apoptotic "meal", probably explains much of their efficiency<sup>42-43</sup>. Several nonprofessional phagocytic cell types have been established to recognise and ingest apoptotic cells<sup>8-11</sup>. Moreover, it has been shown that dendritic cells recognise and ingest apoptotic cells via the scavenger receptor CD36<sup>44</sup>. Another study has demonstrated that human liver kupffer cells phagocytose apoptotic lymphocytes via lectin-dependent recognition of increased expression of n-acetylgalactosamine, D-galactose, and mannose residues<sup>10</sup>. Hall et al<sup>9</sup> have shown that human fibroblasts, including those derived from the lung, recognize and ingest apoptotic neutrophils and that this involves the participation of the vitronectin receptor and a mannose-/fucose-specific lectin. However, although LAEC or A549 also utilize the vitronectin receptor in recognition of apoptotic differentiated EoL-1, a role for the mannose-/fucose-specific lectin was not observed

as neither sugar had any inhibitory effect on recognition or engulfment.

In summary, it was demonstrated that large airway epithelial cell and A549 alveolar epithelial cells are capable of recognizing and engulfing apoptotic differentiated EoL-1 and that this process can be enhanced by stimulation of LAEC or A549 cell with the

proinflammatory cytokines IL-1 $\alpha$  and dexamethasone. The membrane receptors involved in LAEC recognition of apoptotic human differentiated EoL-1 appear similar to those reported for macrophages and small airway epithelial cell. Thus, EoL-1 should provide a useful tool to further study the mechanisms and function outcomes of the phagocytic removal of apoptotic eosinophils by airway epithelial cells.

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