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The effect of liposome encapsulation on the pharmacokinetics of recombinant secretory leukocyte protease inhibitor (rSLPI) therapy after local delivery to a guinea pig asthma model.

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Title: The effect of liposome encapsulation on the pharmacokinetics and activity of recombinant Secretory Leukocyte Protease Inhibitor (rSLPI) therapy after local delivery to a guinea pig asthma model.

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Abstract

Purpose: Inhaled recombinant Secretory Leukocyte Protease Inhibitor (rSLPI) has shown potential for the treatment of inflammatory lung conditions. Rapid inactivation of rSLPI by cathepsin L (Cat L) and rapid clearance from the lungs has limited clinical efficacy to-date. Encapsulation of rSLPI within 1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine]:Cholesterol liposomes (DOPS-rSLPI) protects rSLPI against Cat L inactivation *in vitro*. The aim of this study was to determine the effect of liposomes on rSLPI pharmacokinetics and activity *in vitro* and after local delivery to the airways *in vivo.* *Methods:* Transport of DOPS-rSLPI and free-rSLPI across a polarised air-liquid epithelial monolayer was measured. An asthma guinea pig model was administered either DOPS-rSLPI liposomes or free-rSLPI by intratracheal instillation. *Results:* Apparent permeability (P_{app}) of free-rSLPI was significantly higher at 4.9 $x10^{-6}$cm/s than for DOPS-rSLPI, P_{app} of 2.05 $x10^{-7}$cm/s. *In vivo* studies confirmed this result. Plasma rSLPI concentrations were highest in free-rSLPI treated animals compared with those treated with DOPS-rSLPI, there also appeared to be a trend for higher intracellular rSLPI content in animals dosed with DOPS-rSLPI compared to free-rSLPI. Eosinophil influx was recorded as a measure of inflammation. Pre-dosing with either free-rSLPI or DOPS-rSLPI prevented inflammatory response to antigen challenge to levels comparable to control animals. *Conclusion:* Encapsulation of rSLPI in DOPS:Chol liposomes improves stability, reduces clearance and increases residence time in the lungs after local delivery.

Keywords: rSLPI, liposome, lung, intratracheal instillation, asthma, guinea pig

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Abbreviations

AHR	Allergic hyperresponsiveness
AIC	Air interface culture
AMs	Alveolar macrophages
Anti-NE	Anti-neutrophil elastase
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
Cat L	Cathepsin L
Chol	Cholesterol
EAR	Early allergic response
Free-rSLPI	rSLPI alone
DOPS	1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine]
DOPS-rSLPI	rSLPI encapsulated in DOPS:Chol liposomes
i.t.	Intratracheal instillation
LAR	Late allergic response
NE	Neutrophil elastase
Ova	Ovalbumin
P_{app}	Apparent permeability coefficient
rSLPI	Recombinant secretory leukocyte protease inhibitor
SLPI	Secretory leukocyte protease inhibitor
TEER	Trans-epithelial electrical resistance

Introduction

Local drug delivery to the lungs is an effective means of treating a range of pulmonary diseases such as asthma, cystic fibrosis, bronchitis and emphysema [Suarez, 2000 #110]. SLPI is an 11.75kDa protein found intracellularly in the serous cells of submucosal tracheal and bronchial glands and in non-ciliated cells of the bronchus and bronchial epithelium. It is known to protect the lungs from excessive tissue damage caused by leukocyte proteases during inflammation (Yang et al., 2005). Its abundance in the upper respiratory tract suggests that the primary role of SLPI is to provide an anti-Neutrophil Elastase (anti-NE) shield for the tracheobronchial tree (Fryksmark et al., 1982, Mooren et al., 1983). SLPI has also been found to possess anti-bacterial, anti-viral and anti-inflammatory activity including the ability to reduce nuclear factor-κB (NF-κB) activation intracellularly (Greene et al., 2004, Jin et al., 1997, Lentsch et al., 1999, McNeely et al., 1995, Taggart et al., 2002, Zhang et al., 1997). rSLPI is therefore a highly promising therapeutic for inflammatory lung disease.

Delivery of rSLPI directly to the lungs by inhalation increases targeting to its site of action and has shown an increased half-life over intravenous administration (Bergenfeldt et al., 1990, Gast et al., 1990, Stolk et al., 1995). rSLPI's therapeutic activity has been demonstrated previously in animal asthma models (Wright et al., 1999) and in human studies (McElvaney et al., 1993, McElvaney et al., 1992). The success of inhaled rSLPI therapy has been limited, however, by rapid clearance and extensive degradation by proteases, particularly cathepsins. *In vivo* human studies involving the delivery of rSLPI

locally to the lung indicated that rSLPI does not accumulate on the respiratory surface and instead moves rapidly from the epithelial surface to the interstitium of the lung after inhalation (Vogelmeier et al., 1996). It therefore requires repeated dosages every 12 hours in order to maintain therapeutic effectiveness (McElvaney et al., 1993).

We have shown in an earlier study that encapsulation of rSLPI within1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine]:Cholesterol (DOPS:Chol) liposomes protects rSLPI against Cat L inactivation *in vitro* (Gibbons et al., 2008). This delivery system is biocompatible and bioactive *in vitro*, is easily aerosolised and retains its protective properties post-nebulisation (Gibbons et al., 2008). Liposomes have advantages over other potential vehicles for lung targeting, including sustained release delivery of their cargo in the lungs, increased drug residence time in the lungs (Couvreur et al., 1991, McCullough and Juliano, 1979, Taylor et al., 1989), improved stability of the drug both *in vitro* and *in vivo*, biocompatibility (Niven and Schreier, 1990), local targeting providing increased potency and reduced toxicity (Gavalda et al., 2005a, Gavalda et al., 2005b, Griffiths et al., 1999, Letsou et al., 1999). Also, the high loading capacity of liposomes and low excipient to drug ratio of lipid based carriers results in lower excipient accumulation in the lungs after repeated administration compared to polymer based carriers (Bhavane et al., 2003). The application of liposomes for the delivery of peptides or proteins to the lungs, however, has yet to be fully explored.

In this paper the effect of liposome encapsulation on the pharmacokinetics and activity of rSLPI after local delivery to the airways was assessed *in vitro* and *in vivo. In vitro*

transport studies of free-rSLPI (rSLPI alone, non-encapsulated) and DOPS-rSLPI (rSLPI encapsulated in DOPS:Chol [7:3] liposomes) across Calu-3 airway epithelial monolayers grown under air interface culture (AIC) conditions were used to predict the effect of liposome encapsulation on rSLPI transport *in vivo* and to determine the value of this cell model for the prediction of formulation effects on protein transport rates in the lungs.

An active model of asthma by Ova sensitisation in guinea pigs was used to determine if rSLPI encapsulation in DOPS-rSLPI liposomes has a significant effect on rSLPI's activity and/or its pharmacokinetics after local administration to the lungs. Ovalbumin (Ova)-sensitised animals exposed to an Ova aerosol display the staggered inflammatory reactions associated with the asthma condition (Toward and Broadley, 2004). This model has previously been used to assess the effect of rSLPI delivered via inhalation (Wright et al., 1999). Asthma inflammation is associated with increased infiltration of inflammatory cells and the concurrent expression and release of inflammatory agents, which regulate processes of inflammation (Barnes, 2000, O'Byrne P and Postma, 1999). Of the inflammatory cell influx that occurs in the late allergic response (LAR) phase, eosinophils are of particular interest in asthma pathophysiology since they are the predominant inflammatory cell type detected in the airways of asthmatic patients (Frigas and Gleich, 1986). This asthma model was used to assess the degree to which free-rSLPI and DOPS-rSLPI regulated eosinophil influx in the LAR phase of the asthmatic response. Also, pharmacokinetic analysis was carried out by means of a single point assay of rSLPI in plasma, bronchoalveolar lavage fluid (BALF) and cell lysate samples.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), Ham's F12, Penicillin/Streptomycin (Bio-Science®); Foetal Calf Serum (FCS) (Sigma); Transwell plate (Costar); Trypsin/EDTA (1X) Liquid (Gibco); Calu-3 cells (American Type Culture Collection®, Manassas, VA, USA); 1,2- Cholesterol and 1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine] (DOPS) (Avanti Polar–Lipids® Inc.); Recombinant human rSLPI was a gift from Amgen® (Thousand Oaks, CA, USA); Human Sputum Leukocyte Elastase (Elastin Products Company®, Missouri, USA); Rabbit anti-SLPI polyclonal IgG and goat anti-rabbit IgG-HRP antibody (Santa Cruz Biotechnology® Inc.); I-block (Applied Biosystems); Super-Signal West Pico Chemiluminescent S (Medical Supply Company®, Dublin, Ireland); Human Neutrophil Elastase (NE) (Elastin Products Co.); Vydac narrow bore C18 column (#218TP5205), (Vydac, Hesperia, CA); Male Hartley guinea pigs (Hilltop Lab Animals Inc. Hilltop Drive, Scottdale, PA); IA-1C MicroSprayer (Penn-Century Inc, Philadelphia, PA); Harvard Rodent Ventilator, Model 683 (Harvard Apparatus, Suthnatick, MA), Human rSLPI ELISA (Quantikine), Cathepsin L, N-(Methoxysuccinyl)-Ala-Ala-Pro-Val 4-nitroanilide and all other reagents were obtained from Sigma-Aldrich® (Tallaght, Dublin, Ireland).

Methods

Liposome preparation and characterisation

Dioleoylphosphatidylserine:Cholesterol (DOPS:Chol) liposomes were prepared by the conventional thin film hydration procedure. Briefly DOPS was mixed with Cholesterol at a ratio of 7:3 and dissolved in Chloroform:Methanol (2:1). The 7:3 ratio for DOPS:Chol was based on previously published studies (Gibbons et al, 2008) wherein we determined this lipid mix provided adequate liposomal stability for our application. Solvent was removed by evaporation using a rotary evaporator at approximately 100rpm. rSLPI was incorporated into the formulation in a rehydrating buffer (Phosphate Buffered Saline (PBS), pH 7.4). 6ml batches of DOPS-rSLPI were prepared for in vivo studies composed of DOPS (5.67mg/ml), Chol (1.145mg/ml) and rSLPI (0.33mg/ml). Size reduction of the liposome suspension was achieved by extrusion using a mini-extruder (Avanti Polar-Lipids Inc.) through 200nm pore size polycarbonate membranes. Non-encapsulated protein was removed by centrifugation at 45 000 rpm at 4ºC for 40 minutes. The supernatant was removed and the pellet washed with PBS and re-centrifuged. This step was repeated for a further two washes. When required 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine:Cholesterol (DOPC:Chol) 7:3 liposomes were prepared by the conventional thin film hydration procedure as described above. For high content analysis, L-α-Phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) was added to fluorescently label liposome preparations.

The size distribution of the liposomes was determined by dynamic light scattering (DLS) (HPPS®, Malvern Instruments) and zeta potential of the liposomes was analysed using

laser Doppler electrophoresis (LDE) (Zetasizer Nano ZS®, Malvern Instruments). The encapsulation efficiency of rSLPI in DOPS:Chol liposomes was determined by reverse phase-HPLC (RP-HPLC). Briefly, liposomes were disrupted via 0.5% Triton X and loaded onto a Vydac narrow bore C_{18} column (#218TP5205, Vydac®, Hesperia, CA) for RP-HPLC analysis using a slightly modified procedure previously described (Gibbons et al., 2008). Gradient elution occurred over 40 minutes using a mobile phase of water and acetonitrile with 0.1% trifluoroacetic acid and rSLPI. Area under the curve was analysed at 214nm. The supernatant samples (non-encapsulated rSLPI) were also analysed by RP-HPLC. %EE was defined as the rSLPI encapsulated in liposomes as a percentage of loading dose. The Stewart assay was used to determine the concentration of phospholipid present in the liposomal formulations. Briefly 2ml chloroform and 2ml ferrothiocyanate reagent and 0.1ml of liposome sample were vortexed vigorously for 1 minute. The resultant mixture was centrifuged at 1,000rpm for 5min and the lower chloroform layer removed by glass pipette and measured at 485nm. Based on a standard curve for the appropriate lipid, the concentration of phospholipid present in the sample was calculated.

rSLPI stability and in vitro activity

Western blot analysis was carried out to ensure that the molecular weight of rSLPI had not been altered during the formulation steps. Samples and standards containing 125ng rSLPI were electrophoresed on 15% polyacrylamide gel and blotted onto nitrocellulose. After blocking in I-block®, rSLPI was detected using affinity purified rabbit anti-SLPI polyclonal IgG (1:1000 in I-block) for 1h followed by incubation with goat anti-rabbit

IgG-HRP antibody (1:7500) for 1h. Development was carried out using SuperSignal West Pico® chemiluminescent substrate kit (Taggart et al., 2001).

Activity of rSLPI was assayed by measuring its inhibition of human neutrophil elastase (NE) activity on the substrate N-methoxy-succinyl-Pro-Ala-Ala-Val-*p*-nitroanilide. rSLPI was incubated with NE at room temperature for 5min. Upon addition of the substrate, the change in absorbance (ΔAbs) at 405nm was measured from T_0 to T_{5min} (McElvaney et al., 1992).

Cell Culture

The Calu-3 cell line is a bronchial epithelial cell line isolated from an adenocarcinoma of the lung, as established by Fogh et al (Fogh et al., 1977). Cells were grown in 150cm^2 flasks at 37ºC in a humidified atmosphere containing 5% CO_2 with media replenished approximately every second day. Media consisted of a 1:1 mixture of Ham's F12:DMEM containing 10% foetal bovine serum (FBS), 100µg/ml penicillin G and 100µg/ml streptomycin sulphate. For transport studies 0.5 x10^6 cells/cm^2 cells were seeded onto filter supports (12 well Transwell plate, Costar®) and grown at an air-liquid interface.

A human monocyte-like cell line (THP-1) was cultured in T25, vented flasks. Culture medium was free from antibiotics and consisted of RPMI-1640 with 10% Foetal bovine serum. Cells were seeded at a density of 2.5 x 10^^{5} cells / ml and allowed to grow to a confluence of 1 x 10^^{6} cells / ml before splitting or plating the cells. Cells grew in suspension and were centrifuged at 200xg for 10 minutes when changing medium or seeding cells. To differentiate cells into adherent macrophage like cells 100nM PMA was used for 72 hours at 37°C / 5% CO_2.

Measurement of Trans-epithelial Electrical Resistance (TEER)

TEER values were measured using the EVOM voltohmeter (World Precision Instruments, USA) fitted with STX 'chopstick' electrodes. TEER was expressed as resistance (Ω) by unit area (cm^2). Readings above 700Ω.cm^2 were considered representative of a confluent monolayer. Readings were taken prior to addition of supplemental media. The resistance of unseeded wells was measured as the control

resistance. TEER values were measured before addition of free-rSLPI (10µg/ml) and DOPS-rSLPI (10µg rSLPI per ml) and at defined intervals during the incubation time period of 0, 2, 6, 24 and 30 hours. The TEER for each well was calculated using the following equation: $\mathbf{T}_{\text{Cell Monolayer}} = (\mathbf{T}_{\text{Test}} - \mathbf{T}_{\text{Control}})$, where

$T_{\text{Cell Monolayer}}$ = Resistance of the cell layer alone ($\Omega.cm^2$),

T_{Test} = Resistance of the cell layer and filter ($\Omega.cm^2$)

T_{Control} = Resistance of the filter alone ($\Omega.cm^2$).

Transport studies of free-rSLPI and DOPS-rSLPI Across a Calu-3 Monolayer

rSLPI transport across airway mucosa was assessed using Calu-3 monolayers, cultured as described, 8 days after seeding. rSLPI (10µg rSLPI in 100µl Krebs® solution) was added to each Transwell® containing confluent Calu-3 monolayers as either free-rSLPI or rSLPI encapsulated in DOPS-rSLPI liposomes. Samples were removed from the basolateral chamber and replaced with an equivalent volume of Krebs® solution at 5, 30, 60, 120, 240 and 420 minutes and apical samples were taken at 420 minutes. Basolateral and apical samples were assayed for rSLPI concentration by ELISA (Quantikine®). Apparent permeability rate (Papp) was used to assess the transport of rSLPI across the cell monolayer. Results were expressed as the apparent permeability coefficient (P_{app}) of rSLPI. P_{app} is calculated using the following equation:

$P_{app} = dQ/dt \times 1/A.C_0$

Where,

dQ/dt = Rate of appearance of mass in the basolateral chamber (mg/s)

A = Surface area of the filter (cm^2), which is $1.12cm^2$

C_0 = Initial concentration of drug in the apical chamber (mg/ml).

High content analysis of liposome uptake into THP-1 cells

To quantify the number of liposomes per THP-1 cell a high-content screening system was developed with an INCell 1000 lyser (GE). Various size ranges were evaluated in 48-well plates with 0.5ml of PMA treated THP-1 cells at a density of 1x10^5 cells / ml per well. Cells were differentiated with 100mM PMA for 72 hours, followed by replacement with fresh media and were incubated for 2 hours with rhodamine-labelled liposomes of different sizes, washed and analysed at 37°C. Cells were then fixed in 4% paraformaldehyde and stained for F-actin with 200µl of Phalloidin-TRITC (5µg/ml) and for nucleus using 100µl of Hoechst (10µg/ml). Dual object analysis was carried out on the INCell 1000 to quantify the rate of cell uptake. Cells were identified as objects with both a defined radius of cell shape and also a minimum surface area of the nucleus. Positive objects for both these characteristics were then scanned for liposomes of a defined size range. Images were analysed using the dual area object analysis algorithm (GE) to quantify the number of liposomes uptake (Table 1). The number of liposomes per cell is quantified by the software first identifying cells; it does so by using two organelle markers, the nucleus and the cytoplasm. In this study the nucleus was fluorescently labelled with Hoechst (blue) 360nm / 460nm (nucleus), and with Phalloidin-FITC (green) 535nm / 620nm (cytoplasm). A threshold for area intensity for the respective dyes is employed in the identification of defined cells. Once identified, the software can use a size exclusion algorithm to count the number of fluorescently- labelled particles within the cell; the software can be programmed to count only those fluorescent

particles within a defined size range. The software will only count particles that are within the defined region, i.e. within the cell, co-localised with the intracellular cytoplasmic phalloidin stain.

Guinea Pig Asthma Model

In vivo studies involving the guinea pig asthma model were carried out according to an IACUC approved protocol (Web ID 8135). Male Hartley guinea pigs (400-600g) were actively sensitized by intra-peritoneal (i.p.) injection of 0.1mg ovalbumin [Ova] in 100mg aluminium hydroxide gel (Smith and Broadley, 2007, Wright et al., 1999). Control animals, which would not be sensitised to Ova, were given a sterile saline i.p. injection instead of ovalbumin. Guinea pigs were anaesthetised using an anaesthetic cocktail comprised of ketamine (50mg/kg) and xylazine (5mg/kg) delivered subcutaneously (s.c.). When required for i.t. instillation, light anaesthesia was used to ensure that the animals did not suffer respiratory depression.

A previous study by Wright et al demonstrated the ability of a single 5mg/kg dose of rSLPI to inhibit airway hyperresponsiveness (AHR) when administered up to 48 hours prior to Ova challenge (Wright et al., 1999). In another study carried out by the same group, rSLPI was administered over four days as once daily doses of 3mg with the final dose administered 30 minutes prior to antigen challenge providing 48 and 100% inhibition of peak early and late phase bronchoconstriction respectively. In our study, a single dose containing 2.5mg rSLPI per kg (or the equivalent volume of PBS/ DOPS-Blank) was administered intratracheally and guinea pigs were challenged 24 hours later with Ova. We also studied a three-day prophylactic dosing regime where guinea pigs

received once daily doses of 1mg rSLPI/kg body weight. For all groups examined, guinea pigs were sensitised with 100µg Ova by i.p. injection, or saline injected for control animals on day one. Fourteen days later the animals were dosed by intratracheal instillation. An IA-1C MicroSprayer® (Penn-Century Inc, Philadelphia, PA) was used to deliver a plume of liquid aerosol directly to the lungs of the guinea pigs via an intratracheal catheter. Two 1ml boluses of air were administered through the MicroSprayer® cannula directly after each instillation. This ensured that the air passage was clear for ventilation. After each dose the animals were held in an upright position on a heating pad for 10 minutes before laying them at an angle in the prone position until fully recovered from the anaesthetic. Intratracheal instillation of therapeutic or blank samples were administered according to one of the following dosage regimes:

i) Single Prophylacticly Dosed Guinea Pigs

On day 14 of the study, guinea pigs were administered one dose of the following by i.t. instillation: i) free-rSLPI (2.5mg rSLPI/kg), ii) DOPS-rSLPI (2.5mg rSLPI/kg), iii) DOPS-Blank or iv) PBS (pH 7.4). On day 15 of the study, guinea pigs were Ova challenged by intratracheal instillation, followed 24 hours later (day 16 of the study) by euthanasia and collection of plasma, BALF and cell samples for ELISA analysis. A late allergic response (LAR) to Ova challenge was also examined using cell differentiation analysis to measure the influx of inflammatory cells into the airways as symptomatic of the asthmatic response to Ova.

ii) Three-Day Prophylacticly Dosed Guinea Pigs

On day 14 of the study, guinea pigs were initiated on a three-day prophylactic dosage regime, whereby animals received once-daily i.t. instillations of either i) rSLPI (1mg/kg body weight), ii) DOPS-rSLPI (1mg rSLPI per kg), iii) DOPS-Blank or iv) PBS (pH 7.4) for three days. On the 3rd day of dosing (day 16 of the study), animals were euthanised 2 hours after the final dose and plasma, and BAL cell samples were collected for ELISA analysis. Collection of BALF proved impossible in this dosage regime due to pneumothorax. BAL cell samples could still be collected and assayed despite pneumothorax, as the concentration assayed was normalised to that in one million cells, and therefore not reliant on BAL volume.

rSLPI distribution

Plasma samples were obtained by cardiac puncture after the animal was euthanised. 7ml was withdrawn from the cardiac puncture and 100µl Heparin added. The sample was placed at an angle for 5 minutes then mixed by stirring and centrifuged at 12,000 rpm for 10 minutes. The supernatant was removed and stored at -80ºC. Samples were thawed and diluted a minimum of 20 fold with Calibrator Diluent RD5T from the SLPI ELISA kit (Human SLPI ELISA, Quantikine®, R&D Systems).

Bronchoalveolar lavage (BAL) was carried out to retrieve airway cells and BALF. Directly after euthanasia of the animal, 5ml PBS (pH 7.4) was passed into the lungs using a 5ml syringe and withdrawn 1 minute later. The lungs were flushed three times with 5ml PBS. BALF was pooled and centrifuged immediately at 500 relative centrifugal force

(r.c.f.) for 5 minutes. The BALF supernatant was removed and stored at -80ºC until required for ELISA analysis (Quantikine®).

Cell pellets from BAL were made up to a volume of 1ml with PBS. The total cell count (cells/ml) present was calculated 1:1 with Trypan blue using a Neubauer haemocytometer (Fisher Scientific International Inc.). For the intracellular assay of rSLPI, cell pellets were thawed and centrifuged at 1,100rpm to obtain a cell lysate and to remove cellular debris. Dilutions of the cell lysate were made in Calibrator Diluent RD5T. Concentrations assayed in the cell lysate samples were normalised to the amount present in one million cells. rSLPI assay was carried out using the Human SLPI ELISA kit (Quantikine®).

Inflammatory Cell Influx

Eosinophils are the predominant inflammatory cell type in asthmatic airways and as such were a useful marker in this study for airway inflammation in the late phase asthmatic response and airway hyperresponsiveness that occurs between 6 and 24 hours after Ova challenge (Danahay and Broadley, 1997, Frigas and Gleich, 1986). Inflammatory cell influx was tested in guinea pigs where a single prophylactic dose of test or blank sample was administered and the influx of eosinophils into the lungs 24 hours after Ova challenge assessed as a marker of inflammation.

An aliquot of the cell pellet was spun down on a microscope coverslip at 1,100rpm for 7 minutes. A minimum of 500 cells was counted and the proportion of eosinophils, monocytes, neutrophils and basophils present was calculated (Smith and Broadley, 2007,

Smith and Johnson, 2005). The cells were fixed with acetic alcohol (3% acetic acid in 95% methanol) for 1 minute before rinsing the fixative off gently with distilled water. The slides were placed on a rack and covered with 1ml of Leishman's stain (0.15%w/v in 100% methanol), for approximately 20 seconds. 2ml of a pH 6.8 buffer was added to the coverslip and the two solutions were mixed by tipping the coverslip up and down for 7 minutes. This was then removed by rinsing with distilled water followed by treating of the coverslip with buffer (pH 6.8) for a further 2 minutes. The buffer was rinsed off with distilled water and the coverslip allowed to dry and mounted on a microscope slide. The slides were examined and cells counted using a light microscope using a 100 magnification lens. Neutrophils, eosinophils, monocytes and basophils were counted if present. Results were expressed as the percentage eosinophil cells present as a percentage of the total cell count.

Statistical Analysis

Results were expressed as mean ± standard deviation. Where appropriate the unpaired t-test was used to determine the significance of results. A one-way analysis of variance (ANOVA) was used to determine the significance of results obtained for percentage inflammatory cell influx. In all cases, a probability value of less than 0.05 was considered to be significant.

Results

Liposome encapsulation of rSLPI

rSLPI was encapsulated in DOPS:Chol liposomes with an average encapsulation efficiency of 74.1 ±2.97%. Post-extrusion, these liposomal systems were found to be 153.6 ±2.47nm in size and had a ζ-potential of -58.8 ± 1.46 mV". The stability of rSLPI after encapsulation was confirmed by RP-HPLC and western blot analysis and the retention of anti-protease activity was assessed by its ability to inhibit neutrophil elastase (NE) activity *in vitro*. No degradation of rSLPI was evident with encapsulated rSLPI retaining 92.6 ±10.1% of its anti-NE activity compared to rSLPI prior to encapsulation. In vitro release studies using a dialysis method indicated that the liposomes provided a degree of controlled release. 73% of free rSLPI was released within 4 hours (240mins), with a three-fold reduction in the amount of rSLPI released from the rSLPI-liposomes at each time point sampled compared with the free rSLPI controls.

In vitro rSLPI Transport Across Calu-3 Airway Epithelial Cell Monolayers

In vitro transport studies were conducted to assess the effect of liposome encapsulation on rSLPI transport across an air liquid interface using Calu-3 airway epithelial cell monolayers. This was carried out as a preliminary *in vitro* study to predict the rate of systemic absorption of free-rSLPI compared to rSLPI encapsulated in DOPS-rSLPI *in vivo*. Firstly a TEER analysis study was carried out to assess the effect of free-rSLPI and DOPS-rSLPI incubation on Calu-3 cell monolayer integrity.

TEER values were recorded over a 30 hour incubation of Calu-3 cells with free-rSLPI and DOPS-rSLPI. TEER values were taken at defined time points over the 30 hour incubation. There was no significant difference in the integrity of the monolayer between control cells (incubated alone) and cells incubated with free-rSLPI or DOPS-rSLPI.

Cells were incubated with free-rSLPI (10µg rSLPI/ml) and DOPS-rSLPI (10µg rSLPI/ml) for 4 hours. TEER values were taken before and after completion of the study to ensure that the cell monolayer remained uncompromised throughout the study. Samples were removed from transwells at defined time points. ELISA (Quantikine, R&D Systems®) was used to quantify rSLPI concentration in the samples removed. The calculated apparent permeability (P_{app}) for free-rSLPI was $4.9x10^{-6}$cm/s while P_{app} for DOPS-rSLPI was calculated to be $2.0x10^{-7}$cm/s. Transport across the monolayer therefore occurred at a faster rate for free-rSLPI than rSLPI encapsulated in DOPS-rSLPI liposomes (Figure 1).

***In vivo* study**

An asthma guinea pig model was used to fully elucidate the effect of encapsulation on the anti-inflammatory activity of rSLPI. The study was carried out as outlined previously. Two weeks after the initial ovalbumin challenge animals were anaesthetised and dosed by intratracheal (i.t.) instillation using either a single prophylactic dose or a three-day prophylactic dose regime.

In the single prophylactic dose regime, animals were administered either 2.5mg/kg free-rSLPI or rSLPI encapsulated in liposomes (DOPS-rSLPI). Control animals were administered an equivalent volume of blank liposomes (DOPS-Blank) or phosphate buffered saline (pH 7.4) in place of the test substance. Plasma and BALF samples were collected 48 hours after the single prophylactic dose (day 16). All biological fluid samples were analysed using a highly sensitive ELISA kit that can detect rSLPI in picogram concentrations.

Plasma samples from the study animals were obtained by cardiac puncture. Plasma levels of rSLPI in animals assayed 48 hours after the single prophylactic dose (2.5mg rSLPI/kg by i.t.) showed significantly higher plasma concentrations for guinea pigs dosed with free-rSLPI of 12.3 ±3.07ng/ml compared to those dosed with DOPS-rSLPI (0.52 ±0.85ng/ml) ($p < 0.001$) (Figure 2).

rSLPI was still detectable in bronchoalveolar lavage fluid (BALF) 48 hours post-instillation for animals treated with free-rSLPI (single i.t. dose, 2.5mg rSLPI/kg). Significantly higher concentrations of rSLPI were observed in the BALF for free-rSLPI treated animals of 7.58 ±1.52ng rSLPI, compared to BALF concentrations assayed in animals treated with DOPS-rSLPI (3.22 ±1.73ng rSLPI) ($p < 0.02$) (Figure 3).

Cell lysates of BALF cells taken from animals 48 hours after the single prophylactic dose were also assayed for rSLPI concentration. Although not statistically significant, there appeared to be a trend towards higher rSLPI intracellular concentrations in animals

treated with DOPS-rSLPI 48 hours after dosing, with intracellular rSLPI concentrations of 0.267 ±0.20µg/10^6cells compared to 0.10 ±0.029µg/10^6cells for those treated with free-rSLPI (Figure 4).

In the three-day prophylacticly dosed regime, guinea pigs were administered once daily i.t. instillations for three days of either 1mg/kg free-rSLPI or rSLPI encapsulated in liposomes (DOPS-rSLPI). Control animals were administered an equivalent volume of blank liposomes (DOPS-Blank) or phosphate buffered saline (pH 7.4) in place of the test substance. On the 3rd day of dosing (day 16 of the study), animals were euthanised 2 hours after the third and final dose. Plasma, and cell samples were assayed for rSLPI content using ELISA analysis.

Plasma samples from the three-day prophylacticly dosed animals were obtained by cardiac puncture. Plasma levels of rSLPI in animals assayed 2 hours after final dosing showed a significantly higher plasma rSLPI concentration for animals dosed with free rSLPI (1mg/kg; n=2) of 418.3 ±63.71ng/ml rSLPI compared to 30.1 ±3.66ng/ml rSLPI for animals dosed with DOPS-rSLPI (1mg rSLPI/kg; n=2) ($p < 0.02$) suggesting that rSLPI from the DOPS-rSLPI liposome formulation is not cleared as rapidly to the systemic circulation as free-rSLPI (Controls [DOPS-Empty or PBS] n=6).

Cell lysates of BALF cells taken from these animals 2 hours after dosing (1mg rSLPI/kg once daily for three days) were also assayed for rSLPI concentration and demonstrated significantly higher rSLPI concentrations in the cell lysate of animals treated with DOPS-

rSLPI at 5.15 ±0.086µg/10^6cells (n=2) compared to animals treated with free rSLPI (0.22 ± 0.098µg/10^6cells; n=2) ($p < 0.0005$). Intracellular rSLPI concentration for animals treated with free rSLPI and DOPS-rSLPI were statistically higher than for control animals (Controls [DOPS-Empty or PBS] n=9).

Inflammatory Cell Infiltration

Inflammatory cell infiltration into the airways, specifically eosinophil influx, is indicative of local lung inflammation and is known to occur between 6-24 hours after early allergic response (EAR). The percentage cell type present in the airways was expressed as a percentage of total cells present. A minimum of 500 cells was counted after cell differentiation using Leishman's stain (Figure 5 & 6). Control animals that received Ova challenge only, displayed a significantly higher level of eosinophil influx compared to all other groups of animals ($p < 0.001$) (Figure 6). A significantly lower level of eosinophils was observed in the BAL of animals prophylacticly treated with a single prophylactic dose of free-rSLPI and DOPS-rSLPI. The eosinophil levels in these treated animals were statistically similar to that for unchallenged animals ($p > 0.05$) (Figure 6). There was no statistical difference in neutrophil count between the animal groups.

After euthanasia of guinea pigs, various organs were assessed for the incidence of toxicity induced by free-rSLPI or DOPS-rSLPI treatment. The liver, gall bladder and lungs were all assessed for toxicity. No abnormalities, which would suggest toxicity, were observed in the organs of any test group.

Discussion

Our previous work demonstrated highly efficient encapsulation of rSLPI within 1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine]:Cholesterol (DOPS:Chol) liposomes, which protect rSLPI against Cat L inactivation *in vitro* (Gibbons et al., 2008). In this paper the effect of liposome encapsulation on the pharmacokinetics and activity of rSLPI after local delivery to the airways was assessed *in vitro* and *in vivo.*

An *in vitro* transport study was conducted using Calu-3 airway epithelial cells grown to confluency at an air-liquid interface to form intact monolayers capable of mimicking the mucosal cell layer in the bronchial region of the airways (Shen et al., 1994). The Calu-3 cells line has previously been used as a means of potentially predicting the transport of materials across the airway epithelium *in vivo* (Fiegel et al., 2003, Foster et al., 2001). In our study a significant difference was observed in apparent permeabilities of free-rSLPI and DOPS-rSLPI across the Calu-3 monolayer indicating that transport occurred at a much faster rate for free-rSLPI compared to that for rSLPI encapsulated in DOPS-rSLPI liposomes (Figure 1). This rapid transport of free-rSLPI across the airway epithelial cells could explain the rapid clearance of the inhaled protein previously seen in *in vivo* studies (McElvaney et al., 1993, Vogelmeier et al., 1996). The encapsulation of rSLPI within this liposome carrier therefore appears to retard transport across the epithelium, which could signify increased rSLPI residence time within the lungs when encapsulated in DOPS-rSLPI liposomes compared to free-rSLPI.

To determine the effect that encapsulation of rSLPI in liposomes has on its *in vivo* pharmacodynamics, *in vivo* studies were carried out using a guinea pig asthma model. *In vivo* studies using rSLPI therapy have previously been carried out in sheep, mouse and guinea pig asthmatic models (Wright et al., 1999). In studies by Wright et al using the sheep model, it was observed that the prophylactic dosage regime of 3mg daily for four days provided inhibitory activity equivalent to that achieved by a single 100mg aerosol dose of rSLPI. Using a different animal model to test the effect of rSLPI on hyperresponsiveness, Wright et al administered intratracheal doses of rSLPI (1mg/kg) to guinea pigs at daily intervals for three days with the final dose administered one hour before antigen challenge. It was observed that this inhibited the development of hyperresponsiveness induced by histamine bronchoprovocation with a lower ED_{50} <0.05mg/kg compared to a single dose of rSLPI given one hour before antigen challenge displayed an ED_{50} of 0.56mg/kg. In a murine model treated with rSLPI 30 minutes prior to antigen challenge it was found that rSLPI treatment inhibited eosinophil, lymphocyte and neutrophil influx decreased by almost 100%.

In our study, Ova-sensitised guinea pigs were dosed by intratracheal (i.t.) instillation with either a single prophylactic dose of free-rSLPI (2.5mg rSLPI/kg), or DOPS-rSLPI liposomes (2.5mg rSLPI/kg) where samples were collected from guinea pigs 48 hours after euthanasia, or, a three-day prophylactic dose regime of once daily doses of either free-rSLPI (1mg rSLPI/kg), or DOPS-rSLPI (1mg rSLPI/kg) where samples were collected 2 hours after the final dose. The high rSLPI concentration seen in plasma samples at 2 hours post i.t. instillation of free-rSLPI indicates that rSLPI is rapidly

cleared from the lungs into the systemic circulation while DOPS-rSLPI is not cleared so readily and may remain in the lungs. A significantly higher concentration of rSLPI was also observed in the plasma of animals treated with a single dose of free-rSLPI compared to animals treated with DOPS-rSLPI 48 hours after treatment suggesting the retention of liposome-encapsulated rSLPI within the lungs and corroborates the high apparent permeability of free-rSLPI observed in the *in vitro* transport study.

Other studies investigating the clearance of rSLPI from the lungs using various different *in vivo* models (rats, sheep and human) have also demonstrated that free-rSLPI is rapidly cleared from the lungs to the extent that a once daily dose would not be sufficient to maintain therapeutic rSLPI levels within the lungs (Gast et al., 1990, McElvaney et al., 1993, Vogelmeier et al., 1990). In a study where rSLPI was nebulised into the lungs of sheep, Vogelmeier et al found the half-life of rSLPI in the epithelial lining fluid of the lungs to be 12 hours (Vogelmeier et al., 1990). When rSLPI was administered intratracheally to the lungs it disappeared from the lungs with a half-life of 4 to 5 hours. In that study rSLPI was absorbed systemically from the lungs with a resulting maximal plasma level of about 2µg/ml approximately one to two hours after administration (Gast et al., 1990) which also corroborates the data found in our study. Many studies have documented the ability of liposomes to provide sustained release for their encapsulated material (McCullough and Juliano, 1979, Murry and Blaney, 2000, Taylor et al., 1989). The trend for rapid systemic clearance of free-rSLPI relative to DOPS-rSLPI treated animals up to 48 hours after administration indicates that encapsulating rSLPI in the liposome carrier promotes a longer residence time within the lungs.

Despite the rapid absorption of free-rSLPI from the lungs into the systemic circulation (Figure 2) 2 hours and 48 hours after administration, free-rSLPI was still detectable in the BALF in trace concentrations (approximately 8ng/ml) (Figure 3). This residual presence of rSLPI in the BALF may be due to its biphasic clearance from the lungs. In biphasic clearance, materials that deposit in certain regions of the lungs are rapidly cleared, while those that deposit in other areas, typically non-ciliated regions, are cleared at a slower rate (Smith et al., 1989, Stolk et al., 1995).

The relative lack of DOPS-rSLPI in the BALF 48 hours after administration (Figure 3) leads to the following question; if rSLPI was not in the systemic circulation nor in the BALF of DOPS-rSLPI treated animals, then where was it? Intracellular rSLPI concentrations in BAL cells were assayed. BAL cells were collected from the three-day prophylacticly dosed guinea pigs 2 hours after the final dose and assayed for rSLPI concentration. An extremely significant increase in intracellular rSLPI concentrations was observed for DOPS-rSLPI treated animals versus free-rSLPI treated animals ($p<0.05$). BAL cells were also collected from animals 48 hours after a single prophylactic dose, and although not statistically significant, there appeared to be a trend for higher intracellular rSLPI concentrations in animals treated with DOPS-rSLPI compared to those treated with free-rSLPI 48 hours post-administration (Figure 4). The variability between samples in the single dose study was almost certainly due to the 48 hour gap between dosing and sampling. The majority of cells present in the BALF of free-rSLPI and DOPS-rSLPI treated animals were monocytes (Figure 5) and it can therefore be postulated that a large proportion of the encapsulated DOPS-rSLPI was targeted to

monocytes in the lungs. Uptake facilitated by the DOPS liposomes that was indicated by our in vivo data (Fig 4a) was confirmed by studies to assess the impact of liposome composition on uptake by monocytes in vitro (Fig 4b). These studies confirmed a significant increase in monocyte uptake of DOPS liposomes compared to DOPC liposomes at a range of concentrations. Qualitative and quantitative data from HCA studies confirmed that DOPS liposomes were capable of efficiently targeting monocytes, a property that was independent of size (Fig 4b). This technique allowed individual liposomes per cell to be counted, thereby removing the subjectivity of microscopy studies.

It is already known that phosphatidylserine (PS) lipids enhance macrophage uptake (Chiu et al., 2001, Martin et al., 1995) and PS expression on the surface of cells has long been recognised in cell biology to signal apoptosis and to trigger the phagocytosis of apoptotic cells by macrophages (Bratton and Henson, 2008, Fadok et al., 1992, Martin et al., 1995). Further studies have reported extensive uptake of DOPS liposomes by macrophages and demonstrated enhanced alveolar macrophage uptake of negatively charged liposomes deposited in the lungs of mice (de Haan et al., 1996). Along with its extracellular antiprotease activity, rSLPI also exhibits intracellular activity in alveolar macrophages as it is here that it exerts an anti-inflammatory activity by binding to nuclear factor (NF)-kappa B binding sites (Taggart et al., 2005). The encapsulation of rSLPI within liposomes that are specifically taken up AMs can therefore be regarded as a targeted carrier system. Liposome delivery to AMs has been successfully applied to the pulmonary delivery of cytotoxic antimicrobial agents such as amphotericin B (Brajtburg et al., 1990, Janknegt,

1996, Janknegt et al., 1992, Lopez-Berestein et al., 1983) and to enhance the efficacy of other antimicrobial agents targeting AMs such as ciprofloxacin (Conley et al., 1997, Wong et al., 2003).

Early mast cell responses as well as late leukocyte activation following allergen-bronchoprovocation significantly increase protease load in the airways (Fahy et al., 1995, Wenzel et al., 1988). This increase in proteolytic burden results in the inflammation and airway remodelling associated with asthma, as with other inflammatory lung diseases such as COPD, emphysema and cystic fibrosis. rSLPI provides a broad spectrum inhibitory activity against mast cell and leukocyte serine proteases and has previously been shown to prevent antigen-induced airway responses such as early and late phase responses, development of airway hyperresponsiveness, inflammatory cell influx, mucociliary dysfunction and airway inflammation in animal models of asthma (Barrios et al., 1998, Forteza et al., 2001, Wright et al., 1999). Studies have previously shown that rSLPI inhibits inflammatory cell recruitment (Murata et al., 2003, Sehnert et al., 2004, Wright et al., 1999) and so a reduction in inflammatory cell influx to the lungs was used in our study as a marker for free-rSLPI and DOPS-rSLPI activity *in vivo*.

A significant reduction in eosinophil infiltration was observed for both free-rSLPI and DOPS-rSLPI treated animals. Indeed the eosinophil levels of those treated with free-rSLPI and DOPS-rSLPI were the same as those seen in unchallenged (non-asthmatic) animals (Figure 6). Other studies examining the effect of prophylactic treatment with rSLPI on eosinophil influx have also demonstrated its ability to limit inflammatory cell

influx (Murata et al., 2003, Sehnert et al., 2004, Wright et al., 1999) but the controlled and targeted release properties exhibited by this liposome delivery system may allow DOPS-rSLPI to elicit its therapeutic effects for a longer period of time beyond the 24 hours post-challenge tested in this study.

Conclusions:

Overall, the results of this study suggest that DOPS-rSLPI reduced the rate of clearance of rSLPI into the systemic circulation, increased lung residence-time and preferentially targeted alveolar macrophages, post-inhalation. This pharmacokinetic effect may offer a sustained pharmacodynamic activity over time. The observed *in vitro-in vivo* correlation for DOPS-rSLPI supports the use of Calu-3 monolayers for predicting the effect of excipients on protein transport in the lungs *in vivo*. In the broader context this work adds to knowledge in the field of liposomes for inhalation and particularly to their application for protein delivery and targeting. Prudent lipid selection can produce stable, protective delivery systems for inhalation with the ability to protect proteins, reduce clearance to the systemic circulation and potentially to target specific sites of action such as alveolar macrophages *in vivo*.

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Figure Legends:

Figure 1: Cumulative transport of free-rSLPI and rSLPI encapsulated in DOPS-rSLPI liposomes across a Calu-3 monolayer over 4 hours (n=4 ± S.D.)

Figure 2: rSLPI plasma concentrations in single prophylacticly dosed guinea pigs. Plasma was collected 48 hours after a single 2.5mg rSLPI/kg dose by intratracheal instillation (free-rSLPI n=3; DOPS-rSLPI n=5; Control [DOPS-Empty or PBS] n= 6; ±S.D.)

Figure 3: rSLPI concentration present in bronchoalveolar lavage fluid (BALF) of single prophylacticly dosed guinea pigs. BALF was collected 48 hours after a single 2.5mg rSLPI/kg dose by intratracheal instillation. (free-rSLPI n=3; DOPS-rSLPI n=5; Control [DOPS-Empty or PBS] n=3; ±S.D.)

Figure 4: a) rSLPI concentration in BALF cells taken from single prophylacticly dosed guinea pigs. Cell lysates were collected 48 hours after a single 2.5mg rSLPI/kg dose by intratracheal administration (i.t.). (free-rSLPI n=3; DOPS-rSLPI n=5; Control [DOPS-Empty or PBS] n=9; ±S.D.) b) Uptake of neutral (DOPC) and anionic (DOPS) liposomes by differentiated THP-1 cells determined by high content analysis (HCA). Cells were incubated for 2 hours with rhodamine-labelled liposomes of different sizes, washed and analysed. Data represented as means ± SEM (n = 9).

Figure 5: Images taken of Leishman stained cells present in the BAL of guinea pigs after a single prophylactic dose of either i) control unchallenged animal, ii) Ova challenged only, iii) treated

with free-rSLPI (2.5mg rSLPI/kg) and Ova challenged or iv) treated with DOPS-rSLPI (2.5mg rSLPI/kg) and Ova challenged.

Figure 6: Percentage cell type present in the airways expressed as a percentage of total cells present. A minimum of 500 cells was counted after cell differentiation using Leishman's stain (Ova + DOPS-rSLPI n=5 ±S.D.; Ova + free-rSLPI n=3 ± S.D.; Ova challenged only n=3 ±S.D.; Control unchallenged animals n= 4±S.D.)

References

BARNES, P. J. (2000) Chronic obstructive pulmonary disease. *N Engl J Med,* 343**,** 269-80.

BARRIOS, V. E., MIDDLETON, S. C., KASHEM, M. A., HAVILL, A. M., TOOMBS, C. F. & WRIGHT, C. D. (1998) Tryptase mediates hyperresponsiveness in isolated guinea pig bronchi. *Life Sci,* 63**,** 2295-303.

BERGENFELDT, M., BJORK, P. & OHLSSON, K. (1990) The elimination of secretory leukocyte protease inhibitor (SLPI) after intravenous injection in dog and man. *Scand J Clin Lab Invest,* 50**,** 729-37.

BHAVANE, R., KARATHANASIS, E. & ANNAPRAGADA, A. V. (2003) Agglomerated vesicle technology: a new class of particles for controlled and modulated pulmonary drug delivery. *J Control Release,* 93**,** 15-28.

BRAJTBURG, J., POWDERLY, W. G., KOBAYASHI, G. S. & MEDOFF, G. (1990) Amphotericin B: delivery systems. *Antimicrob Agents Chemother,* 34**,** 381-4.

BRATTON, D. L. & HENSON, P. M. (2008) Apoptotic cell recognition: will the real phosphatidylserine receptor(s) please stand up? *Curr Biol,* 18**,** R76-9.

CHIU, G. N., BALLY, M. B. & MAYER, L. D. (2001) Selective protein interactions with phosphatidylserine containing liposomes alter the steric stabilization properties of poly(ethylene glycol). *Biochim Biophys Acta,* 1510**,** 56-69.

CONLEY, J., YANG, H., WILSON, T., BLASETTI, K., DI NINNO, V., SCHNELL, G. & WONG, J. P. (1997) Aerosol delivery of liposome-encapsulated ciprofloxacin: aerosol characterization and efficacy against Francisella tularensis infection in mice. *Antimicrob Agents Chemother,* 41**,** 1288-92.

COUVREUR, P., FATTAL, E. & ANDREMONT, A. (1991) Liposomes and nanoparticles in the treatment of intracellular bacterial infections. *Pharm Res,* 8**,** 1079-86.

DANAHAY, H. & BROADLEY, K. J. (1997) Effects of inhibitors of phosphodiesterase, on antigen-induced bronchial hyperreactivity in conscious sensitized guinea-pigs and airway leukocyte infiltration. *Br J Pharmacol,* 120**,** 289-97.

DE HAAN, A., GROEN, G., PROP, J., VAN ROOIJEN, N. & WILSCHUT, J. (1996) Mucosal immunoadjuvant activity of liposomes: role of alveolar macrophages. *Immunology,* 89**,** 488-93.

FADOK, V. A., VOELKER, D. R., CAMPBELL, P. A., COHEN, J. J., BRATTON, D. L. & HENSON, P. M. (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol,* 148**,** 2207-16.

FAHY, J. V., KIM, K. W., LIU, J. & BOUSHEY, H. A. (1995) Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin Immunol,* 95**,** 843-52.

FIEGEL, J., EHRHARDT, C., SCHAEFER, U. F., LEHR, C. M. & HANES, J. (2003) Large porous particle impingement on lung epithelial cell monolayers--toward improved particle characterization in the lung. *Pharm Res,* 20**,** 788-96.

FOGH, J., FOGH, J. M. & ORFEO, T. (1977) One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst,* 59**,** 221-6.

FORTEZA, R. M., AHMED, A., LEE, T. & ABRAHAM, W. M. (2001) Secretory leukocyte protease inhibitor, but not alpha-1 protease inhibitor, blocks tryptase-induced bronchoconstriction. *Pulm Pharmacol Ther,* 14**,** 107-10.

FOSTER, K. A., YAZDANIAN, M. & AUDUS, K. L. (2001) Microparticulate uptake mechanisms of in-vitro cell culture models of the respiratory epithelium. *J Pharm Pharmacol,* 53**,** 57-66.

FRIGAS, E. & GLEICH, G. J. (1986) The eosinophil and the pathophysiology of asthma. *J Allergy Clin Immunol,* 77**,** 527-37.

FRYKSMARK, U., OHLSSON, K., POLLING, A. & TEGNER, H. (1982) Distribution of antileukoprotease in upper respiratory mucosa. *Ann Otol Rhinol Laryngol,* 91**,** 268-71.

GAST, A., ANDERSON, W., PROBST, A., NICK, H., THOMPSON, R. C., EISENBERG, S. P. & SCHNEBLI, H. (1990) Pharmacokinetics and distribution of recombinant secretory leukocyte proteinase inhibitor in rats. *Am Rev Respir Dis,* 141**,** 889-94.

GAVALDA, J., MARTIN, M. T., LOPEZ, P., GOMIS, X., RAMIREZ, J. L., RODRIGUEZ, D., LEN, O., PUIGFEL, Y., RUIZ, I. & PAHISSA, A. (2005a) Efficacy of nebulized liposomal amphotericin B in treatment of experimental pulmonary aspergillosis. *Antimicrob Agents Chemother,* 49**,** 3028-30.

GAVALDA, J., MARTIN, T., LOPEZ, P., GOMIS, X., RAMIREZ, J. L., RODRIGUEZ, D., LEN, O., PUIGFEL, Y., RUIZ, I. & PAHISSA, A. (2005b) Efficacy of high loading doses of liposomal amphotericin B in the treatment of experimental invasive pulmonary aspergillosis. *Clin Microbiol Infect,* 11**,** 999-1004.

GIBBONS, A. M., MCELVANEY, N. G., TAGGART, C. C. & CRYAN, S. A. (2008) Delivery of rSLPI in a liposomal carrier for inhalation provides protection against cathepsin L degradation. *J Microencapsul***,** 1-10.

GREENE, C. M., MCELVANEY, N. G., O'NEILL, S. J. & TAGGART, C. C. (2004) Secretory leucoprotease inhibitor impairs Toll-like receptor 2- and 4-mediated responses in monocytic cells. *Infect Immun,* 72**,** 3684-7.

GRIFFITHS, G. D., PHILLIPS, G. J. & BAILEY, S. C. (1999) Comparison of the quality of protection elicited by toxoid and peptide liposomal vaccine formulations against ricin as assessed by markers of inflammation. *Vaccine,* 17**,** 2562-8.

JANKNEGT, R. (1996) Liposomal formulations of cytotoxic drugs. *Support Care Cancer,* 4**,** 298-304.

JANKNEGT, R., DE MARIE, S., BAKKER-WOUDENBERG, I. A. & CROMMELIN, D. J. (1992) Liposomal and lipid formulations of amphotericin B. Clinical pharmacokinetics. *Clin Pharmacokinet,* 23**,** 279-91.

JIN, F. Y., NATHAN, C., RADZIOCH, D. & DING, A. (1997) Secretory leukocyte protease inhibitor: a macrophage product induced by and antagonistic to bacterial lipopolysaccharide. *Cell,* 88**,** 417-26.

LENTSCH, A. B., JORDAN, J. A., CZERMAK, B. J., DIEHL, K. M., YOUNKIN, E. M., SARMA, V. & WARD, P. A. (1999) Inhibition of NF-kappaB activation and augmentation of IkappaBbeta by secretory leukocyte protease inhibitor during lung inflammation. *Am J Pathol,* 154**,** 239-47.

LETSOU, G. V., SAFI, H. J., REARDON, M. J., ERGENOGLU, M., LI, Z., KLONARIS, C. N., BALDWIN, J. C., GILBERT, B. E. & WALDREP, J. C.

(1999) Pharmacokinetics of liposomal aerosolized cyclosporine A for pulmonary immunosuppression. *Ann Thorac Surg,* 68**,** 2044-8.

LOPEZ-BERESTEIN, G., MEHTA, R., HOPFER, R. L., MILLS, K., KASI, L., MEHTA, K., FAINSTEIN, V., LUNA, M., HERSH, E. M. & JULIANO, R. (1983) Treatment and prophylaxis of disseminated infection due to Candida albicans in mice with liposome-encapsulated amphotericin B. *J Infect Dis,* 147**,** 939-45.

MARTIN, S. J., REUTELINGSPERGER, C. P., MCGAHON, A. J., RADER, J. A., VAN SCHIE, R. C., LAFACE, D. M. & GREEN, D. R. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med,* 182**,** 1545-56.

MCCULLOUGH, H. N. & JULIANO, R. L. (1979) Organ-selective action of an antitumor drug: pharmacologic studies of liposome-encapsulated beta-cytosine arabinoside administered via the respiratory system of the rat. *J Natl Cancer Inst,* 63**,** 727-31.

MCELVANEY, N. G., DOUJAIJI, B., MOAN, M. J., BURNHAM, M. R., WU, M. C. & CRYSTAL, R. G. (1993) Pharmacokinetics of recombinant secretory leukoprotease inhibitor aerosolized to normals and individuals with cystic fibrosis. *Am Rev Respir Dis,* 148**,** 1056-60.

MCELVANEY, N. G., NAKAMURA, H., BIRRER, P., HEBERT, C. A., WONG, W. L., ALPHONSO, M., BAKER, J. B., CATALANO, M. A. & CRYSTAL, R. G. (1992) Modulation of airway inflammation in cystic fibrosis. In vivo suppression of interleukin-8 levels on the respiratory epithelial surface by aerosolization of recombinant secretory leukoprotease inhibitor. *J Clin Invest,* 90**,** 1296-301.

MCNEELY, T. B., DEALY, M., DRIPPS, D. J., ORENSTEIN, J. M., EISENBERG, S. P. & WAHL, S. M. (1995) Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. *J Clin Invest,* 96**,** 456-64.

MOOREN, H. W., KRAMPS, J. A., FRANKEN, C., MEIJER, C. J. & DIJKMAN, J. A. (1983) Localisation of a low-molecular-weight bronchial protease inhibitor in the peripheral human lung. *Thorax,* 38**,** 180-3.

MURATA, E., SHARMIN, S., SHIOTA, H., SHIOTA, M., YANO, M. & KIDO, H. (2003) The effect of topically applied secretory leukocyte protease inhibitor on the eosinophil response in the late phase of allergic conjunctivitis. *Curr Eye Res,* 26**,** 271-6.

MURRY, D. J. & BLANEY, S. M. (2000) Clinical pharmacology of encapsulated sustained-release cytarabine. *Ann Pharmacother,* 34**,** 1173-8.

NIVEN, R. W. & SCHREIER, H. (1990) Nebulization of liposomes. I. Effects of lipid composition. *Pharm Res,* 7**,** 1127-33.

O'BYRNE P, M. & POSTMA, D. S. (1999) The many faces of airway inflammation. Asthma and chronic obstructive pulmonary disease. Asthma Research Group. *Am J Respir Crit Care Med,* 159**,** S41-63.

SEHNERT, B., CAVCIC, A., BOHM, B., KALDEN, J. R., NANDAKUMAR, K. S., HOLMDAHL, R. & BURKHARDT, H. (2004) Antileukoproteinase: modulation

of neutrophil function and therapeutic effects on anti-type II collagen antibody-induced arthritis. *Arthritis Rheum,* 50**,** 2347-59.
SHEN, B. Q., FINKBEINER, W. E., WINE, J. J., MRSNY, R. J. & WIDDICOMBE, J. H. (1994) Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl- secretion. *Am J Physiol,* 266**,** L493-501.
SMITH, N. & BROADLEY, K. J. (2007) Optimisation of the sensitisation conditions for an ovalbumin challenge model of asthma. *Int Immunopharmacol,* 7**,** 183-90.
SMITH, N. & JOHNSON, F. J. (2005) Early- and late-phase bronchoconstriction, airway hyper-reactivity and cell influx into the lungs, after 5'-adenosine monophosphate inhalation: comparison with ovalbumin. *Clin Exp Allergy,* 35**,** 522-30.
SMITH, R. M., TRABER, L. D., TRABER, D. L. & SPRAGG, R. G. (1989) Pulmonary deposition and clearance of aerosolized alpha-1-proteinase inhibitor administered to dogs and to sheep. *J Clin Invest,* 84**,** 1145-54.
STOLK, J., CAMPS, J., FEITSMA, H. I., HERMANS, J., DIJKMAN, J. H. & PAUWELS, E. K. (1995) Pulmonary deposition and disappearance of aerosolised secretory leucocyte protease inhibitor. *Thorax,* 50**,** 645-50.
TAGGART, C. C., CRYAN, S. A., WELDON, S., GIBBONS, A., GREENE, C. M., KELLY, E., LOW, T. B., O'NEILL S, J. & MCELVANEY, N. G. (2005) Secretory leucoprotease inhibitor binds to NF-kappaB binding sites in monocytes and inhibits p65 binding. *J Exp Med,* 202**,** 1659-68.
TAGGART, C. C., GREENE, C. M., MCELVANEY, N. G. & O'NEILL, S. (2002) Secretory leucoprotease inhibitor prevents lipopolysaccharide-induced IkappaBalpha degradation without affecting phosphorylation or ubiquitination. *J Biol Chem,* 277**,** 33648-53.
TAGGART, C. C., LOWE, G. J., GREENE, C. M., MULGREW, A. T., O'NEILL, S. J., LEVINE, R. L. & MCELVANEY, N. G. (2001) Cathepsin B, L, and S cleave and inactivate secretory leucoprotease inhibitor. *J Biol Chem,* 276**,** 33345-52.
TAYLOR, K. M., TAYLOR, G., KELLAWAY, I. W. & STEVENS, J. (1989) The influence of liposomal encapsulation on sodium cromoglycate pharmacokinetics in man. *Pharm Res,* 6**,** 633-6.
TOWARD, T. J. & BROADLEY, K. J. (2004) Early and late bronchoconstrictions, airway hyper-reactivity, leucocyte influx and lung histamine and nitric oxide after inhaled antigen: effects of dexamethasone and rolipram. *Clin Exp Allergy,* 34**,** 91-102.
VOGELMEIER, C., BUHL, R., HOYT, R. F., WILSON, E., FELLS, G. A., HUBBARD, R. C., SCHNEBLI, H. P., THOMPSON, R. C. & CRYSTAL, R. G. (1990) Aerosolization of recombinant SLPI to augment antineutrophil elastase protection of pulmonary epithelium. *J Appl Physiol,* 69**,** 1843-8.
VOGELMEIER, C., GILLISSEN, A. & BUHL, R. (1996) Use of secretory leukoprotease inhibitor to augment lung antineutrophil elastase activity. *Chest,* 110**,** 261S-266S.
WENZEL, S. E., FOWLER, A. A., 3RD & SCHWARTZ, L. B. (1988) Activation of pulmonary mast cells by bronchoalveolar allergen challenge. In vivo release of histamine and tryptase in atopic subjects with and without asthma. *Am Rev Respir Dis,* 137**,** 1002-8.

WONG, J. P., YANG, H., BLASETTI, K. L., SCHNELL, G., CONLEY, J. & SCHOFIELD, L. N. (2003) Liposome delivery of ciprofloxacin against intracellular Francisella tularensis infection. *J Control Release,* 92**,** 265-73.

WRIGHT, C. D., HAVILL, A. M., MIDDLETON, S. C., KASHEM, M. A., LEE, P. A., DRIPPS, D. J., O'RIORDAN, T. G., BEVILACQUA, M. P. & ABRAHAM, W. M.-. (1999) Secretory leukocyte protease inhibitor prevents allergen-induced pulmonary responses in animal models of asthma. *J Pharmacol Exp Ther,* 289**,** 1007-14.

YANG, J., ZHU, J., SUN, D. & DING, A. (2005) Suppression of macrophage responses to bacterial lipopolysaccharide (LPS) by secretory leukocyte protease inhibitor (SLPI) is independent of its anti-protease function. *Biochim Biophys Acta,* 1745**,** 310-7.

ZHANG, Y., DEWITT, D. L., MCNEELY, T. B., WAHL, S. M. & WAHL, L. M. (1997) Secretory leukocyte protease inhibitor suppresses the production of monocyte prostaglandin H synthase-2, prostaglandin E2, and matrix metalloproteinases. *J Clin Invest,* 99**,** 894-900.

Figure 1:

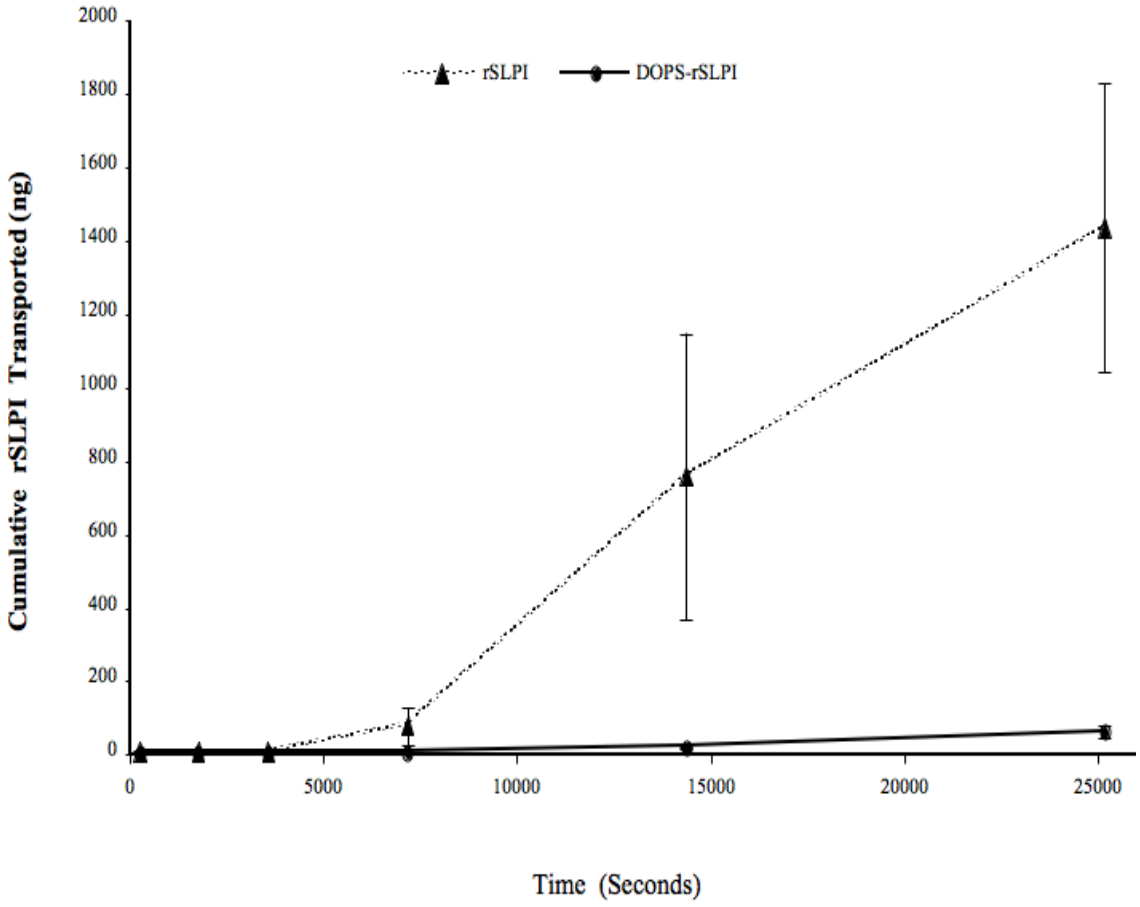

rSLPI
DOPS-rSLPI
Cumulative rSLPI Transported (ng)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
5000
10000
15000
20000
25000
Time (Seconds)

Figure 2:

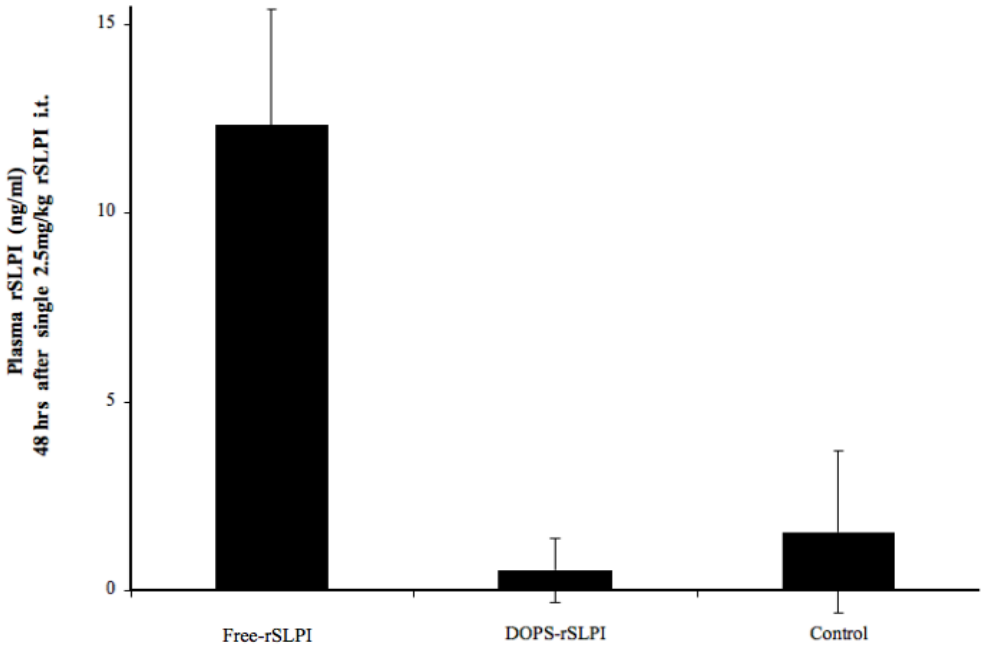

Plasma rSLPI (ng/ml)
48 hrs after single 2.5mg/kg rSLPI i.t.
15
10
5
0
Free-rSLPI
DOPS-rSLPI
Control

Figure 3:

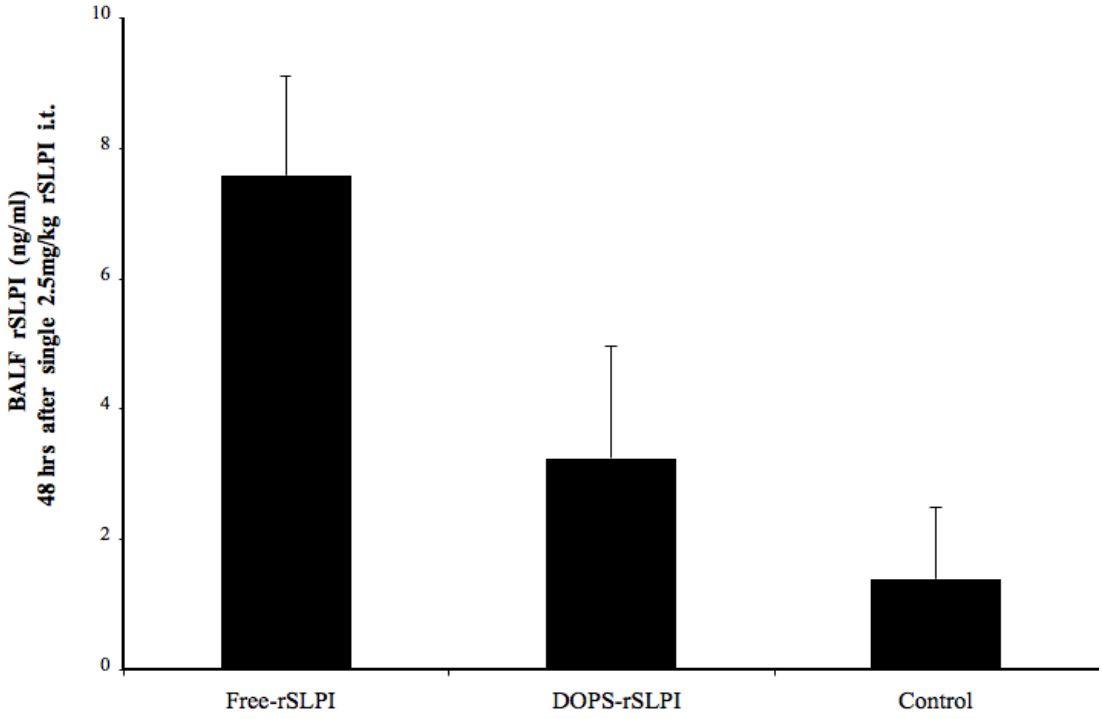
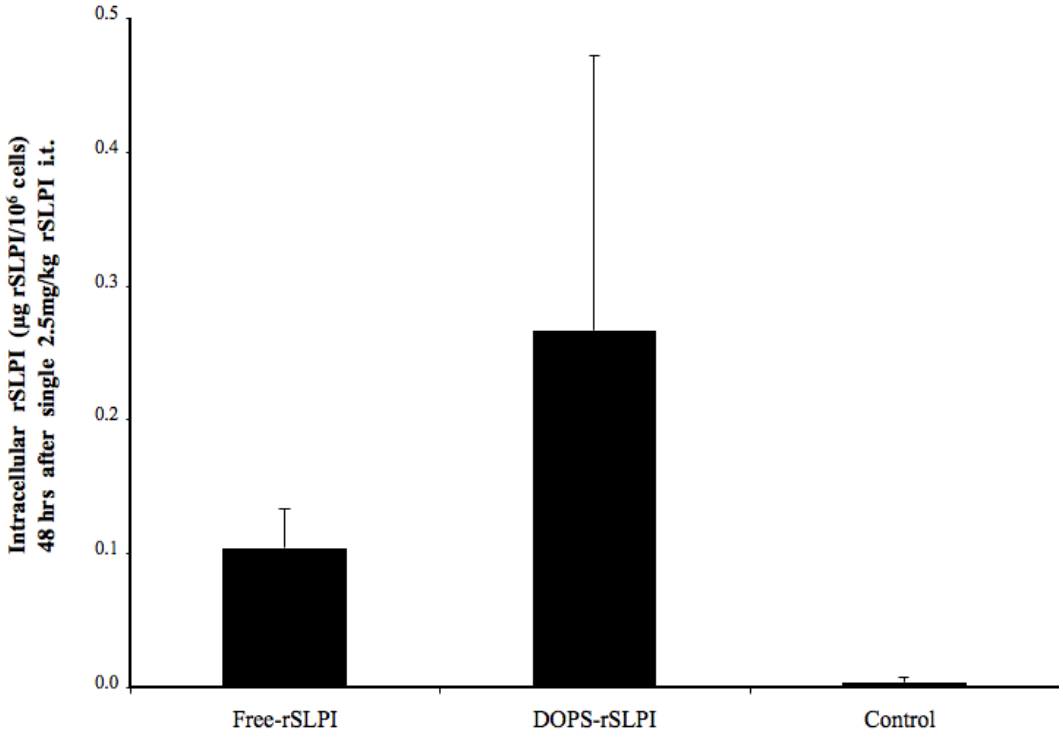

BALF rSLPI (ng/ml)
48 hrs after single 2.5mg/kg rSLPI i.t.
10
8
6
4
2
0
Free-rSLPI
DOPS-rSLPI
Control

Figure 4: a)


Intracellular rSLPI (µg rSLPI/10⁶ cells)
48 hrs after single 2.5mg/kg rSLPI i.t.
0.5
0.4
0.3
0.2
0.1
0.0
Free-rSLPI
DOPS-rSLPI
Control

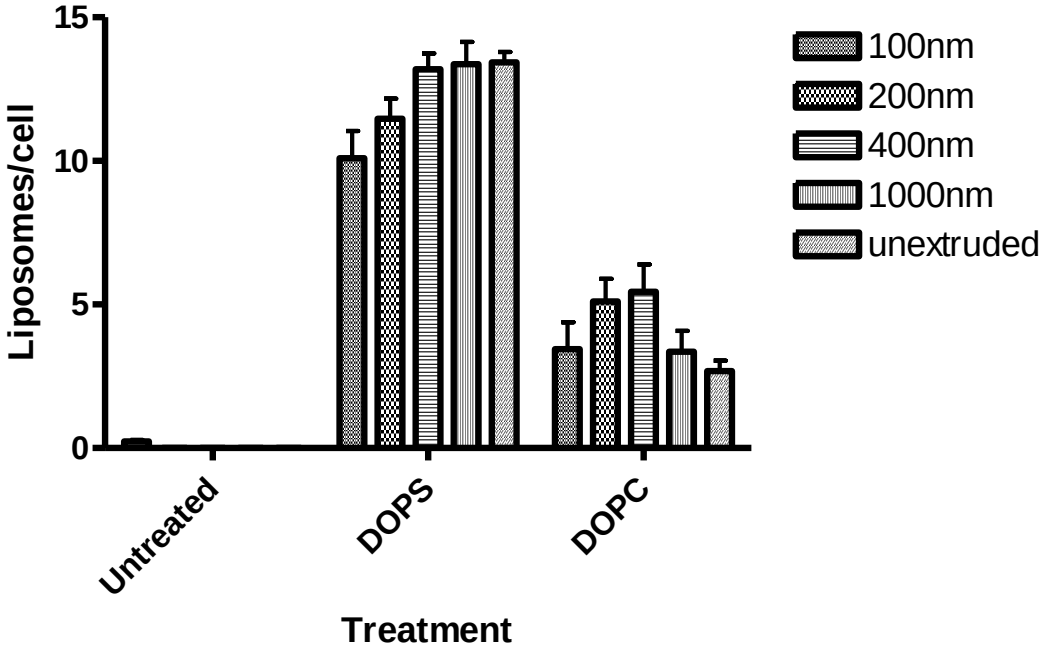

Liposomes/cell
15
10
5
0
Untreated
DOPS
DOPC
Treatment
100nm
200nm
400nm
1000nm
unextruded

Figure 5

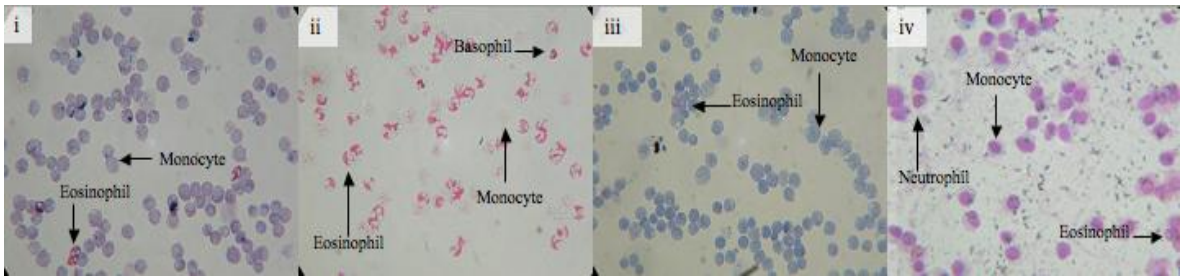
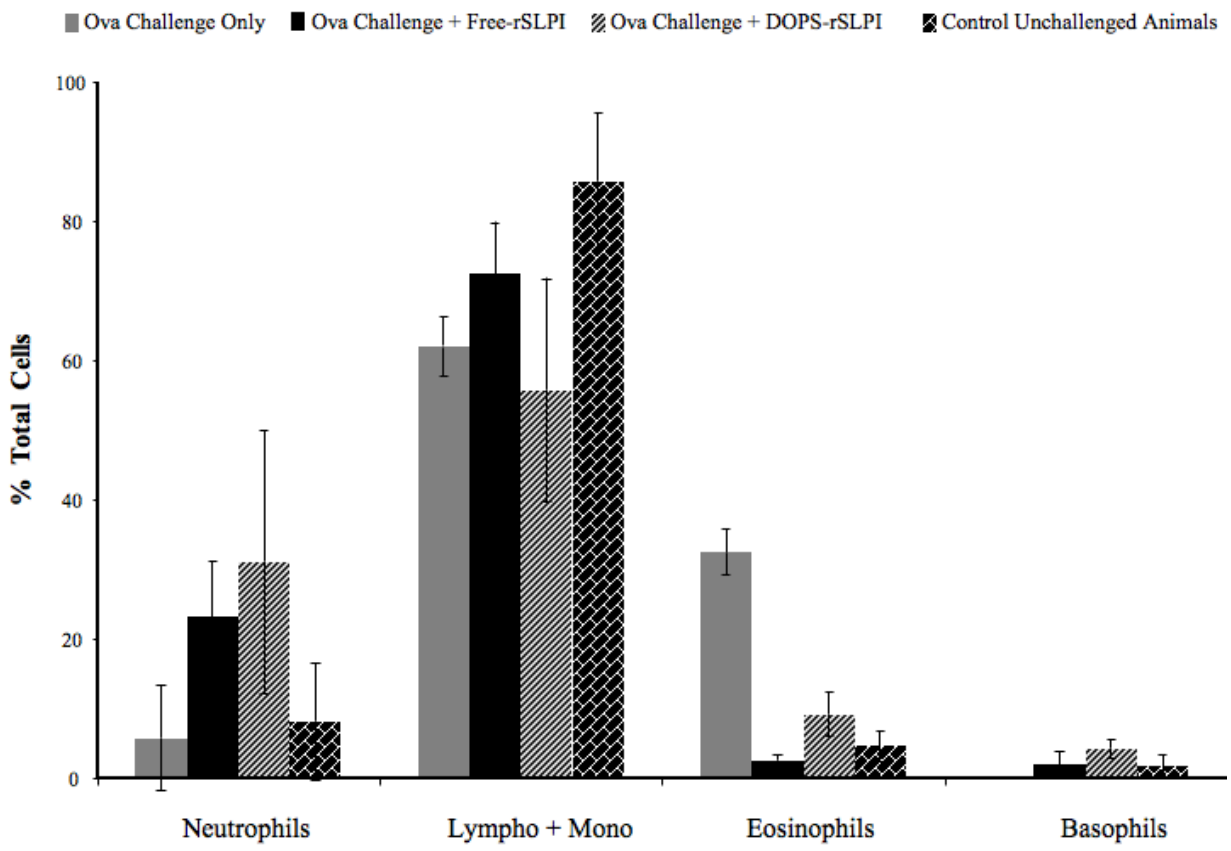

i
Monocyte
Eosinophil
ii
Basophil
Monocyte
Eosinophil
iii
Monocyte
Eosinophil
iv
Monocyte
Neutrophil
Eosinophil

Figure 6


Ova Challenge Only
Ova Challenge + Free-rSLPI
Ova Challenge + DOPS-rSLPI
Control Unchallenged Animals
% Total Cells
100
80
60
40
20
0
Neutrophils
Lympho + Mono
Eosinophils
Basophils