doi: 10.1111/j.1365-2222.2011.03855.x
Clinical & Experimental Allergy, **41**, 1793–1803

ORIGINAL ARTICLE **Experimental Models of Allergic Disease**

Annexin-1-deficient mice exhibit spontaneous airway hyperresponsiveness and exacerbated allergen-specific antibody responses in a mouse model of asthma

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Clinical & Experimental Allergy

Summary

Background Glucocorticoids are the mainstream drugs used in the treatment and control of inflammatory diseases such as asthma. Annexin-1 (ANXA1) is an anti-inflammatory protein which has been described as an endogenous protein responsible for some anti-inflammatory glucocorticoid effects. Previous studies have identified its importance in other immune diseases such as rheumatoid arthritis and cystic fibrosis. ANXA1-deficient ($^{-/-}$) mice are Th2 biased, and ANXA1 N-terminus peptide exhibits anti-inflammatory activity in a rat model of pulmonary inflammation.

Objective ANXA1 protein is found in bronchoalveolar lavage fluid from asthmatics. However, the function of ANXA1 in the pathological development of allergy or asthma is unclear. Thus, in this study we intended to examine the effect of ANXA1 deficiency on allergen-specific antibody responses and airway responses to methacholine (Mch).

Methods ANXA1$^{-/-}$ mice were sensitized with ovalbumin (OVA) and challenged with aerosolized OVA. Airway resistance, lung compliance and enhanced pause (PenH) were measured in naïve, sensitized and saline or allergen-challenged wild-type (WT) and ANXA1$^{-/-}$ mice. Total and allergen-specific antibodies were measured in the serum.

Results We show that allergen-specific and total IgE, IgG2a and IgG2b levels were significantly higher in ANXA1$^{-/-}$ mice. Furthermore, naïve ANXA1$^{-/-}$ mice displayed higher airway hypersensitivity to inhaled Mch, and significant differences were also observed in allergen-sensitized and allergen-challenged ANXA1$^{-/-}$ mice compared with WT mice.

Conclusions In conclusion, ANXA1$^{-/-}$ mice possess multiple features characteristic to allergic asthma, such as airway hyperresponsiveness and enhanced antibody responses, suggesting that ANXA1 plays a critical regulatory role in the development of asthma.

Clinical Relevance We postulate that ANXA1 is an important regulatory factor in the development of allergic disease and dysregulation of its expression can lead to pathological changes which may affect disease progression.

Keywords airway hyperresponsiveness, allergen-specific antibody production, asthma
Submitted 07 December 2010; revised 14 July 2011; accepted 28 July 2011

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Cite this as: F. S. P. Ng, K. Y. Wong, S. P. Guan, F. B. Mustafa, T. S. Kajiji, P. Bist, S. K. Biswas, W. S. F. Wong and L. H. K. Lim, *Clinical & Experimental Allergy*, 2011 (41) 1793–1803.

Introduction

Allergy is becoming an increasingly common condition in the developed world, and it is important in its understanding to identify genes and proteins which are critical in the development of the allergic phenotype. Dysfunctions in expression or regulation of these proteins could render a higher susceptibility to allergy, and could be clinically useful biomarkers in diagnosis or disease severity. Airway inflammation, reversible airway obstruction and airway hyperresponsiveness (AHR) are characteristic features of human asthma.

Glucocorticoids [e.g. fluticasone propionate (Flovent®, GlaxoSmithKline, UK) and budesonide (Pulmicort®, AstraZeneca, UK)] are by far, the most effective for anti-inflammatory therapy of asthma and other inflammatory diseases. This is due to the fact that they inhibit the expression of many pro-inflammatory genes and can also induce anti-inflammatory genes, such as annexin-1 (ANXA1) [1]. Indeed, ANXA1 is thought to function by mimicking the anti-inflammatory actions of glucocorticoids (GC) [2], the mainstream drugs used in the treatment and prevention of allergy. In a positive feedback loop, ANXA1 itself modulates GC-induced adrenocorticotropic hormone secretion from the anterior pituitary gland [3, 4], suggesting a control of production between ANXA1 and GCs.

ANXA1 is abundantly secreted in respiratory fluid, and has been reported to be up-regulated in asthmatic bronchial lavage fluid [5]. Furthermore, alveolar macrophages express ANXA1, and GCs enhance ANXA1 expression [6]. Degradation of ANXA1 is thought to result in chronic inflammation and unresolved inflammatory conditions, seen in smokers [7] and cystic fibrosis [8], where a cleaved form (33 kDa) of ANXA1 is expressed instead of intact (37 kDa) ANXA1. This suggests that ANXA1 can be functionally impaired during chronic inflammation, and low levels of intact ANXA1 or increased levels of the cleaved ANXA1 could be a marker or predictor of disease.

ANXA1 was originally defined as a potent inhibitor of phospholipase A2, and has subsequently been shown to act as a stress protein and has roles in proliferation, differentiation and apoptosis [9]. ANXA1 is anti-migratory and induces adherent cell detachment from the endothelium [10, 11]. ANXA1 is released upon cellular adhesion onto the endothelium, and binds to its putative receptor, the f-Met-Leu-Phe (FMLP) receptors, FPR (formyl peptide receptor) and FPRL1 (FMLP receptor like). ANXA1-deficient mice ($ANXA1^{-/-}$) were previously shown to be insensitive to GC *in vivo* [12]. Furthermore, $ANXA1^{-/-}$ mice exhibited more inflammation than wild-type (WT) mice, and $ANXA1^{-/-}$ neutrophils exhibited increased transmigration and activation [13]. More importantly, ANXA1 was found to skew T helper cell polarization to a Th1 phenotype [14]. The ANXA1 peptide inhibits neutrophil activation and influx into the peritoneal cavity in response to zymosan [15] and other stimuli and has recently been shown to inhibit allergic inflammation in the rat [16]. In addition, recent studies have implicated that $ANXA1^{-/-}$ mice are biased towards a Th2 phenotype [17]. However, the endogenous function of ANXA1 in the development of allergic disease such as allergic asthma is unclear.

Thus, in this study, we investigated the role of ANXA1 in a mouse model of ovalbumin (OVA)-induced asthma using $ANXA1^{-/-}$ mice, focusing on two main characteristics of allergic asthma, namely allergen-specific antibody production and AHR.

Materials and methods

Mice

BALB/C mice (6–8 weeks, female) were obtained from Laboratory Animal Centre, Singapore. Annexin 1 null ($ANXA1^{-/-}$) mice on BALB/C background (backcrossed over 10 generations) [12] were a kind gift from Professor Roderick Flower and were bred in pathogen-free conditions in our animal facility. All animal work was approved by the Institutional Animal Care and Use Committee and followed National Advisory Committee for Laboratory Animals Research (NACLAR) Guidelines (Guidelines on the Care and Use of Animals for Scientific Purposes). The BALB/C strain of mice was used as the WT control.

Sensitization and airway challenge

Mice were sensitized with an intraperitoneal injection of 20 μg of OVA (grade V; Sigma, St. Louis, MO, USA) emulsified in 2 mg of aluminium hydroxide in a total volume of 100 μL on days 1 and 14. On days 28, 29 and 30, mice were placed in a Plexiglas chamber and challenged either with saline or 1% aerosolized OVA in saline for 30 min using an ultrasonic nebulizer (De Vilbiss, Somerset, PA, USA).

Determination of enhanced pause

Airway responsiveness to inhaled methacholine (Mch) in conscious, spontaneously breathing animals was additionally assessed by recording respiratory pressure curves (PenH) by whole body plethysmography (Buxco Research System, Wilmington, NC, USA). Aerosolized phosphate-buffered saline (PBS) or Mch in doubling concentrations (5–40 mg/mL) was nebulized through an inlet of the main chamber for 12 s, and readings (minute volume, tidal volume, breathing frequency and enhanced pause or PenH) were recorded for 5 min following each nebulization. Baseline PenH values did not differ significantly between any of the groups. PenH was expressed as a% increase above PBS challenge baseline values. The log of the dose of Mch required for a 200% increase of airway resistance over the baseline (log PC200; in mg/mL) was calculated for each mouse to represent the degree of airway resistance. Lower log PC200 values represent greater airway resistance.

Measurement of AHR (resistance and compliance)

Mice were anaesthetized and tracheotomy was performed as described [18]. Mice were intubated with a

cannula that is connected to a multiport that leads to the pneumotach, ventilator and nebulizer within the FinePointe Series RC Sites (Buxco Research System). The system was calibrated for air flow and air pressure. The mouse was ventilated at a fixed breathing rate of 140 breaths/min and the lung resistance (R_l) and dynamic compliance (C_{dyn}) in response to increasing concentrations of nebulized Mch (0.5– 8.0 mg/mL) were recorded using FinePointe software (Buxco Research System).

Bronchoalveolar lavage

Two millilitres of warmed PBS (1 mL ×2) was used to obtain the bronchoalveolar lavage (BAL). The first lavage was centrifuged at 500 g for 10 min. The supernatant was separated from the cell pellet and stored at −80°C for subsequent cytokine analysis by ELISA. The cell pellet was pooled with the subsequent BAL and a total cell count was performed using Turks stain (0.01% crystal violet in 3% acetic acid [19]). Differential counts were performed on May–Grumwald/Giemsa-stained cytospins and determined by counting approximately 200 cells for each sample. The investigator counting the cells was blinded to the treatment group assignment of each section.

Histological analysis

Lungs were fixed in 10% neutral formalin, paraffinized, cut into 4-μm sections and stained with haematoxylin and eosin (H&E) for examining cell infiltration. Semi-quantitative analysis for inflammation was performed in a blinded fashion and scored: 0, normal; 1, few cells; 2, a ring of inflammatory cells one cell layer deep; 3, a ring of inflammatory cells two to four cells deep; 4, a ring of inflammatory cells of four cells deep.

Measurements of OVA-specific antibodies

OVA-specific antibody production was measured using a home-made ELISA. Microtitre plates were coated with OVA (10 μg/mL) in 0.1 M $NaHCO_3$ at 4°C overnight. The plates were washed with PBS/0.05% Tween-20 and blocked with PBS/10% fetal calf serum (FCS). Serum samples were diluted in PBS/3% FCS and incubated in the wells for 2 h. Serial dilutions of a standardized reference serum, obtained from pooling batches of OVA-sensitized mice serum were used as a reference standard. After washing, biotinylated anti-IgE or anti-IgG subclass antibodies (Biolegend, San Diego, CA, USA) were added to the well and incubated for 1 h. The plates were washed with PBS/Tween followed by the addition of avidin-alkaline phosphatase. The plates were washed with before addition of the phosphatase substrate. The plates were allowed to develop for 30 min. The plates were read in an ELISA plate reader at an OD of 405 nm.

Cytokine and mediator measurements

BAL cytokines and total serum IgE was measured by capture ELISA assays with reagents from Biolegend (SAn Diego, CA, USA). A biotinylated rat anti-mouse IgE was used to detect captured IgE. A standard curve for total IgE was generated using a purified mouse IgE standard. Eosinophil peroxidase (EPO) was measured by an *o*-phenylenediamine method, as described previously [19]. IL-4 cytokine production was measured using an ELISA kit from R&D Systems (Minneapolis, MN, USA).

Statistical analysis

For parametric data, differences between groups were analysed with Student's *t*-test; for non-parametric data, differences between groups were analysed with the Wilcoxon rank sum test. To compare WT vs. ANXA1$^{-/-}$ treatment groups, a two-way ANOVA was performed with Bonferroni's *post hoc* tests to compare between groups. Results were expressed as means ± SEM, and unless otherwise stated, were considered statistically significant at $P < 0.05$.

Results

The murine model of allergen-induced airway inflammation and AHR is useful as it mimics many of the features of human asthma, including airway hyperreactivity, production of allergen-specific IgE and leucocyte activation and infiltration, particularly eosinophils and lymphocytes. We sensitized ANXA1$^{-/-}$ and WT BALB/C mice with OVA, and challenged them either with aerosolized saline as a control (OVA/SAL) or 1% OVA (OVA/OVA) using a 3-day challenge protocol.

OVA sensitization was very effective, inducing a time-dependent increase in both total and OVA-specific IgE in both WT and ANXA1$^{-/-}$ mice. However, the serum obtained from ANXA1$^{-/-}$ mice contained significantly higher levels (33.4-fold; $P = 0.0021$) of OVA-specific IgE when compared with WT mice by day 28 of immunization (Table 1). Even at day 14 of sensitization, a higher level of OVA-specific IgE was observed in ANXA1$^{-/-}$ mice. A significant increase in OVA-specific and total IgE (Figs 1a and b) was observed in the ANXA1$^{-/-}$-challenged group, respectively, when compared with the WT OVA-challenged mice on day 32. Similarly, ANXA1$^{-/-}$ OVA/SAL and OVA/OVA mice produced higher OVA-specific IgG2a and IgG2b when compared with the WT groups of similar treatment (Figs 1c and d).

Table 1. Time responses of ovalbumin (OVA)-specific IgE production in wild-type (WT) and annexin-1-deficient (ANXA1$^{-/-}$) mice

OVA-specific IgE (ELISA units/mL)	Day 1	Day 14	Day 21	Day 28
WT	0.9 ± 0.05	5 ± 0.5	14.9 ± 1.9	16.1 ± 2.2
ANXA1$^{-/-}$	0.9 ± 0.04	187.4 ± 79.2	328.5 ± 118.2**	539 ± 69.9**

Mice were sensitized with OVA on days 1 and 14. OVA-specific IgE was measured in serum using ELISA with OVA as the capture. Data are normalized to a standardized reference serum and are expressed as mean ± SEM of at least five mice per group.
**$P < 0.01$ vs. WT mice on the same day of treatment.

We next determined the effect of the loss in ANXA1 on AHR to bronchoconstricting agents, a characteristic phenotype of asthma. Airway hyperreactivity was first measured using whole body barometric plethysmography as measured by the non-invasive PenH method [18]. We found that in the absence of immunological sensitization and challenge (naïve), ANXA1$^{-/-}$ mice exhibited a higher level of basal airway reactivity compared with WT mice when challenged with 40 mg/mL of Mch (Fig. 2a), analysed using a two-way ANOVA. PC200 values were calculated, corresponding to the concentration of Mch which induced a 200% increase in PenH. The PC200 value of naïve ANXA1$^{-/-}$ mice was 9.7 ± 1.2 mg/mL compared with 21.1 ± 4.5 mg/mL in naïve WT mice. This indicates that mice lacking ANXA1 developed spontaneous airway hyperreactivity suggesting that there was intrinsic hypersensitivity in the airways of ANXA1$^{-/-}$ mice irrespective of allergen sensitization or challenge. We predicted that ANXA1$^{-/-}$ mice would be more sensitive to Mch when sensitized and challenged with OVA, compared with their WT counterparts (Fig. 2b). As a result of sample variability, two-way ANOVA showed no statistical differences in the Mch concentration response for WT and ANXA1$^{-/-}$ mice of the same treatment. However, a significant increase in PenH was observed in saline as well as OVA-challenged ANXA1$^{-/-}$ mice compared with WT mice of the same treatment when stimulated with 40 mg/mL of Mch. PC200 (PenH) values of sensitized and saline-challenged ANXA1$^{-/-}$ mice were compared with WT saline-challenged mice, and it was found that the PC200 of ANXA1$^{-/-}$ OVA/SAL mice was significantly lower than OVA/SAL WT mice (Fig. 2c, $P = 0.03$). Allergen challenge significantly lowered the PC200 level in WT but not in ANXA1$^{-/-}$ mice further. This demonstrates that ANXA1$^{-/-}$ mice exhibited higher airway reactivity, once again not dependent on allergen sensitization or challenge, but on the deletion of ANXA1.

To confirm the PenH results, as PenH is not a reliable readout of AHR [20], R_l and C_{dyn} was measured in response to increasing concentrations of aerosolized

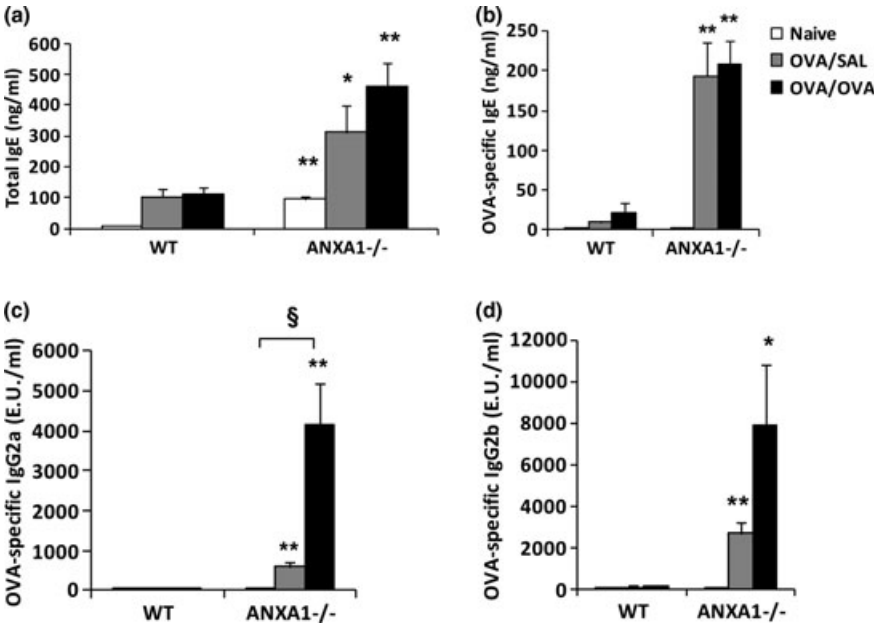

Fig. 1. Enhanced total and ovalbumin (OVA)-specific antibody production in annexin-1 (ANXA1)-deficient ($^{-/-}$) mice. Mice were sensitized with OVA on days 1 and 14 and challenged with 1% OVA in saline through inhalation on day 28 for 3 days. Total IgE (a) and OVA-specific IgE (b), IgG2a (c) and IgG2b (d) were measured in the serum of naïve, sensitized (OVA/SAL) and challenged (OVA/OVA) mice. Data are expressed as mean ± SEM from 5 to 10 mice per group. *$P < 0.05$, **$P < 0.01$ between wild-type and ANXA1$^{-/-}$ mice of the same treatment group. §$P < 0.05$ vs. ANXA1$^{-/-}$ OVA/SAL. SAL indicates saline.

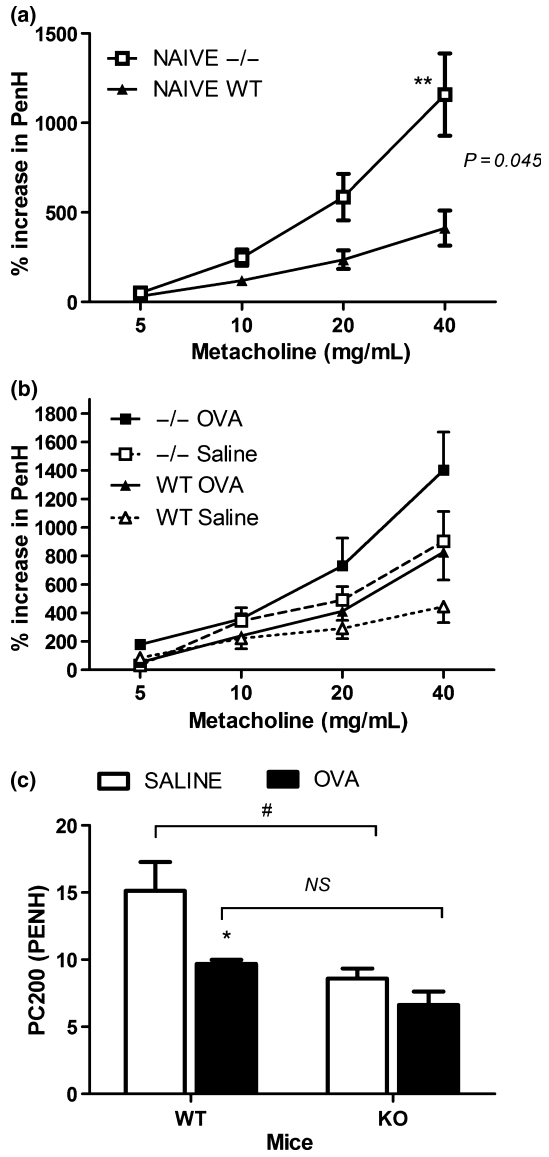

Fig. 2. A loss in annexin-1 (ANXA1) results in increased enhanced pause (PenH). Enhanced pause was recorded in (a) naïve or (b) saline or ovalbumin (OVA)-sensitized and challenged wild-type (WT) and ANXA1$^{-/-}$-deficient mice using PenH with a non-invasive whole body plethysmograph system before and after the indicated concentrations of aerosolized methacholine. Analyses were performed 24 h after the last saline or OVA aerosol challenge. Increases in PenH are related to an increase in airway resistance. Results are reported as maximal fold increase of PenH relative to baseline. Data are expressed as mean ± SEM of six mice per group. Numbers indicate *P*-values of the two-way ANOVA between WT and ANXA1$^{-/-}$ mice of the same treatment group. $^{*}P < 0.05$, $^{**}P < 0.01$ between WT and ANXA1$^{-/-}$ mice of the same treatment group at the same concentration of methacholine. (c) PC200 values corresponding to the concentration (mg/mL) of methacholine which induced a 200% increase in PenH was calculated. Data are expressed as mean ± SEM of six mice per group. $^{*}P < 0.05$ vs. OVA/SAL of same genotype; $^{\#}P < 0.05$ between WT and ANXA1$^{-/-}$ OVA/SAL mice. SAL indicates saline.

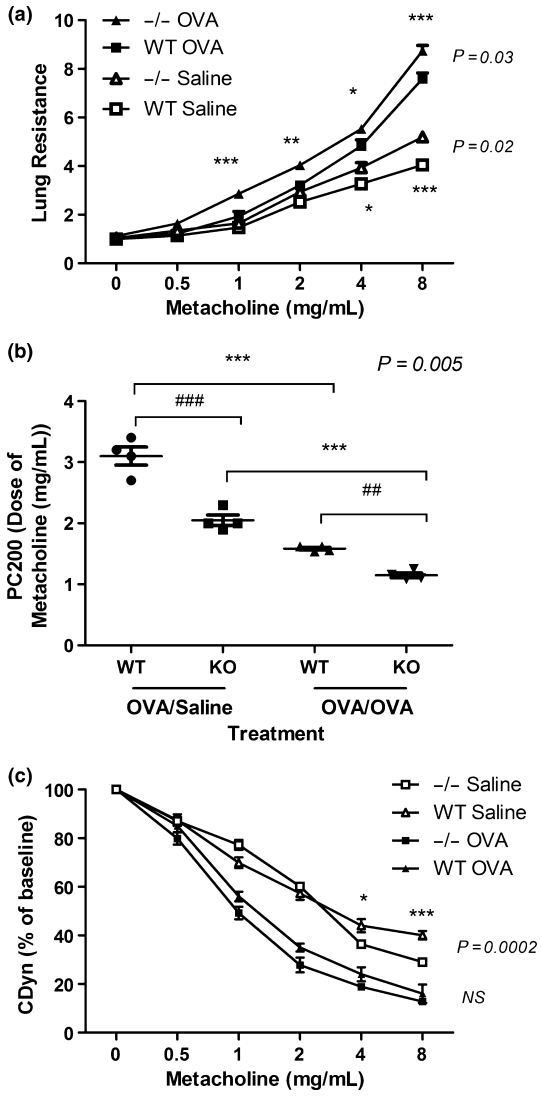

Fig. 3. Annexin-1-deficient (ANXA1$^{-/-}$) mice exhibit higher airway hyperresponsiveness. (a, b) Lung resistance (R_l) and (c) dynamic compliance (C_{dyn}) were measured in mechanically ventilated mice in response to aerosolized methacholine 24 h after the last saline aerosol or ovalbumin (OVA) aerosol in wild-type (WT) and ANXA1$^{-/-}$ mice. (a) R_l is expressed as raw values while (c) C_{dyn} is expressed as raw percentage change from the baseline level. Data are expressed as mean ± SEM of four mice per group. Numbers indicate *P*-values of the two-way ANOVA between WT and ANXA1$^{-/-}$ mice of the same treatment group. $^{***}P < 0.001$, $^{**}P < 0.01$ between WT and ANXA1$^{-/-}$ mice of the same treatment group at the same concentration of methacholine. (b) PC200 corresponding to the concentration (mg/mL) of methacholine which induced a 200% increase in R_l was calculated. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. OVA/SAL of same genotype; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ between WT and ANXA1$^{-/-}$ mice of the same treatment group. SAL indicates saline.

Mch in mechanically ventilated mice [21]. R_l in both WT and ANXA1$^{-/-}$ mice was markedly increased in OVA/OVA group as compared with the OVA/SAL group (Fig. 3a). Furthermore, it was observed that ANXA1$^{-/-}$ mice showed a significant enhancement in R_l, in both OVA-sensitized and OVA-challenged groups, when compared with WT mice of the same treatment

(two-way ANOVA, $P = 0.02$ and 0.03, respectively), with significant differences observed at lower concentrations in the OVA/OVA group. PC200 (R_l) values were calculated for each treatment group and it was determined by two-way ANOVA that PC200 (R_l) values were significantly different between both WT and ANXA1$^{-/-}$ OVA/SAL mice as well as WT and ANXA1$^{-/-}$ OVA/OVA-challenged mice. Interestingly, OVA challenge significantly lowered the PC200 level in WT as well as ANXA1$^{-/-}$ mice, but to a smaller extent. C_{dyn} in WT and ANXA1$^{-/-}$ mice was reduced substantially after OVA challenge (Fig. 3c). As there was some variability in baseline C_{dyn} measurements between the groups, the C_{dyn} data are presented as percentage of the baseline. The intrinsic role of ANXA1 in C_{dyn} was not as significant as for R_l, as marked differences were only observed in OVA/SAL ANXA1$^{-/-}$ vs. WT-treated groups (two-way ANOVA, $P = 0.0002$) but not the OVA/OVA group. Significant reductions in C_{dyn} were observed in OVA/SAL ANXA1$^{-/-}$ mice at the highest concentrations of Mch. These results confirm that ANXA1 is involved intrinsically in the reactivity of the airways to bronchoconstricting agents such as Mch, as this hyperreactivity is exhibited in naïve as well as saline-challenged mice.

To determine if the loss of ANXA1 could result in enhanced inflammation after OVA sensitization and challenge in the lung, cells infiltrating the BAL were counted and differentials were determined in WT and ANXA1$^{-/-}$ mice. Total cell counts in the BAL of OVA/SAL or OVA/OVA WT and ANXA1 were not significantly different. OVA sensitization and challenge induced the recruitment of eosinophils in both WT and ANXA1$^{-/-}$ mice (Fig. 4a), and a significant increase in eosinophil infiltration in the BALF of OVA-challenged ANXA1$^{-/-}$ mice was observed ($P = 0.049$; Fig. 4a). EPO levels in the BAL and lungs were analysed to quantify eosinophil degranulation and recruitment, respectively (Figs 4b and c). OVA challenge induced higher EPO levels in the BAL of both WT and ANXA1$^{-/-}$ mice, which was significant in ANXA1$^{-/-}$ but not WT mice, due to variability. No significant difference in BAL EPO levels were evident between WT and ANXA1$^{-/-}$ OVA/OVA mice. Higher levels of EPO were found in the lungs after OVA challenge in both the WT and ANXA1$^{-/-}$ mice (Fig. 4c), with higher levels of EPO in lungs of ANXA1$^{-/-}$ OVA-challenged mice, when compared with the WT mice. Representative photomicrographs of WT and ANXA1$^{-/-}$ OVA/SAL and OVA/OVA lungs are shown in Fig. 4d. H&E staining of formalin-fixed lungs of WT and ANXA1$^{-/-}$ mice challenged with saline exhibited normal lung architecture. Challenge with OVA induced significant leucocyte infiltration in the perivascular and peribronchial spaces in both WT and ANXA1$^{-/-}$ mice. Interestingly, quantification of the

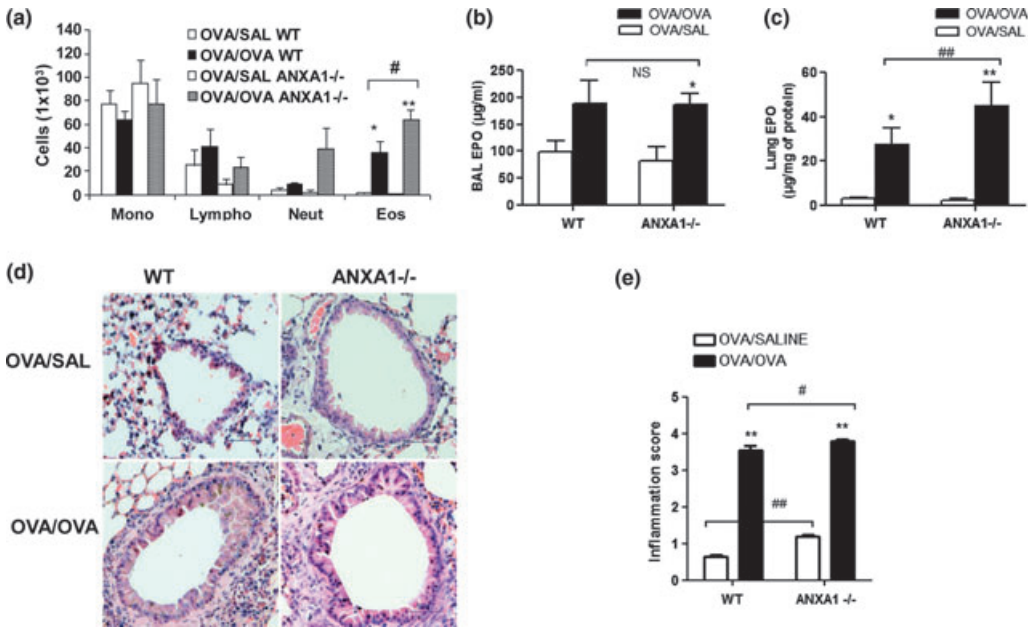

Fig. 4. Eosinophil infiltration after allergen challenge. (a) Differential cell counts were obtained from bronchoalveolar lavage (BAL) fluids 24 h after the last saline or ovalbumin (OVA) aerosol challenge. Data are expressed as mean ± SEM of six to ten mice per group. (b) BAL and (c) lungs from sensitized (OVA/SAL) and challenged (OVA/OVA) mice were analysed for eosinophil peroxidase content. (d, e) Lungs were fixed in 10% formalin and embedded in paraffin before sections were cut at 4 µm and stained with haematoxylin and eosin. Magnification: ×200. Data are representative of four to six mice per treatment group. *$P < 0.05$, **$P < 0.01$ vs. OVA/SAL of same genotype; $^{\#}P < 0.05$ between wild-type and ANXA1$^{-/-}$ OVA/OVA-treated mice. SAL indicates saline.

histological sections demonstrated a higher inflammation score in the ANXA1$^{-/-}$ mice, both saline and OVA-challenged (Fig. 4e).

D'Acquisto et al. [17] described previously that ANXA1$^{-/-}$ mice were more Th2 biased. Therefore, IL-4 expression was analysed in the lungs of WT and ANXA1$^{-/-}$ mice using quantitative PCR. IL-4 gene expression was significantly higher in ANXA1$^{-/-}$ mice than that in normal mice when compared with OVA/SAL mice (Fig. 5a). We next examined IL-4 production in BAL after OVA challenge in both WT and ANXA1$^{-/-}$ mice. ANXA1$^{-/-}$ mice produced a significantly higher level of IL-4 (Fig. 5b) after OVA challenge. This indicates that ANXA1 may regulate IL-4 expression and production during allergen challenge. These data, and other published results [17] suggest that ANXA1$^{-/-}$ mice are more biased towards a Th2 phenotype. The mechanisms behind this phenotypic switch were thought to involve the mitogen activated protein kinase (MAPK) known as extracellular signal-related kinase (ERK), as ANXA1$^{-/-}$ T cells were shown to have defective ERK activity. Thus, in our study, we analysed the level of phospho-ERK in WT and ANXA1$^{-/-}$ lungs after sensitization and challenge. Challenge of WT mice with OVA induced ERK phosphorylation, particularly ERK2 (Fig. 5c). However, OVA challenge did not induce an increase in ERK phosphorylation in ANXA1$^{-/-}$ mice. ERK is phosphorylated by MAPK/ERK kinase (MAPKK or MEK), which itself is phosphorylated by activated Ras/Raf. To examine if ANXA1 could also regulate MEK signalling after OVA challenge, we analysed phosphorylated and total MEK levels in OVA/SAL and OVA/OVA WT and ANXA1$^{-/-}$ lung homogenates. Similar to ERK phosphorylation, MEK phosphorylation was increased after OVA challenge in the WT but not the ANXA1$^{-/-}$ lungs. As ANXA1 is thought to activate nuclear factor (NF)-κB to exert its effects on the adaptive immune response [14, 17], we examined levels of phosphorylated and total inhibitor of kB in lung homogenates following sensitization and challenge with OVA in both WT and ANXA1$^{-/-}$ mice using western blotting. Once again, no increase in phospho-IκB was observed in OVA-challenged ANXA1$^{-/-}$ mice, while levels of phospho-IκB were induced in WT OVA/OVA

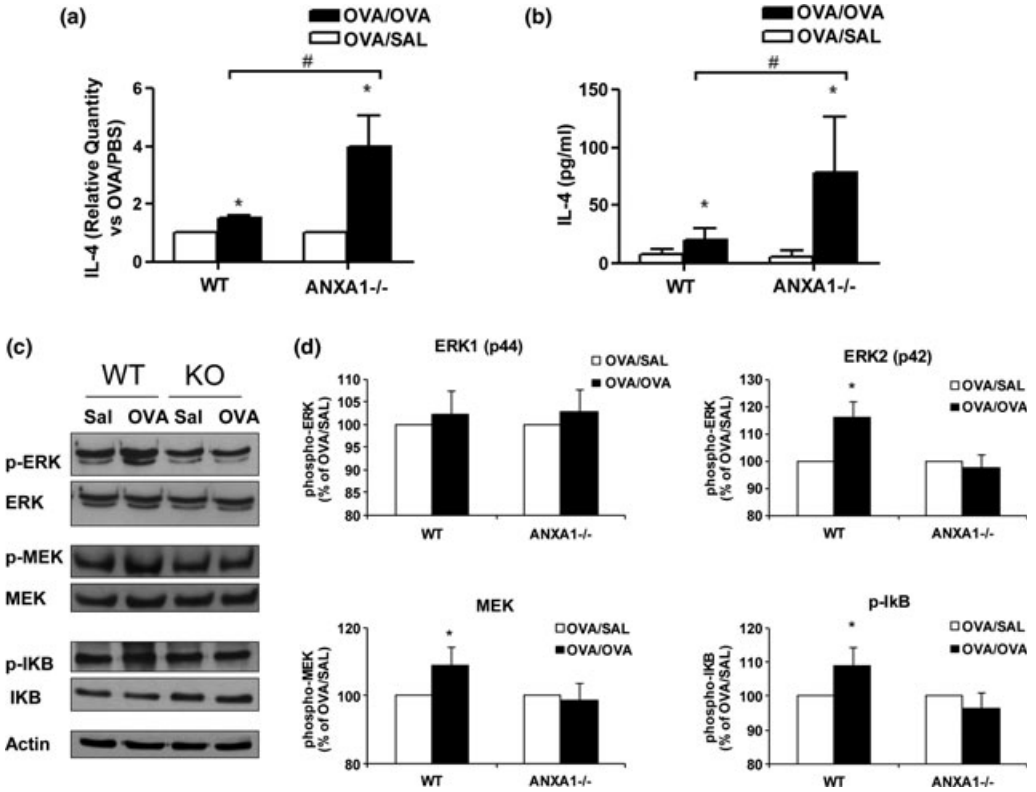

Fig. 5. Annexin-1-deficient (ANXA1$^{-/-}$) mice exhibit higher IL-4 expression and impaired lung ERK and nuclear factor (NF)-κB activity. (a, b) IL-4 expression and production was measured using real-time PCR and ELISA. Data are expressed as mean ± SEM of at least five mice per group. *$P < 0.05$, **$P < 0.01$ vs. OVA/SAL of same genotype; $^{\#}P < 0.05$ between wild-type and ANXA1$^{-/-}$ OVA/OVA-treated mice. (c) Lungs from sensitized (OVA/SAL) and challenged (OVA/OVA) mice were lysed and blotted for the indicated proteins using western blotting. Actin was used as a loading control for each blot. Data shown is representative of two to three experiments. (d) Graphical representation of the western blotting densitometry. Results are expressed as percentages of phospho vs. total protein normalized against actin and presented as histograms of mean ± SEM of $n = 3$. *$P < 0.05$ vs. OVA/SAL of same genotype. OVA indicates ovalbumin and SAL indicates saline.

mice, compared with saline-challenged mice. Additional western blots were performed on two other WT and ANXA1$^{-/-}$ mice for phosphorylated and total ERK to show reproducibility (Fig. S1). Once again, ANXA1$^{-/-}$ mice exhibited suppressed levels of ERK phosphorylation before and after OVA challenge, confirming the results that ANXA1 regulates ERK phosphorylation. Figure 5d shows graphical representations of the results of western blotting. As illustrated, significant increases in phosphorylated ERK2 (p42), MEK and IkB, but not ERK1 (p44) were observed in lungs of WT OVA/OVA mice. However, these increases were not observed in ANXA1$^{-/-}$ OVA/OVA mice.

These signalling results were confirmed using intracellular flow cytometry of cells obtained from BALF. Different populations of cells (lymphocytes, monocytes and granulocytes) were gated using forward- and side-scatter plots (Fig. 6a). Interestingly, ERK was found to be differentially expressed within the populations, with lowest expression in lymphocytes and highest in granulocytes (Fig. 6b). A shift in phosphorylated ERK vs. total ERK was observed in OVA-challenged WT in all leucocyte

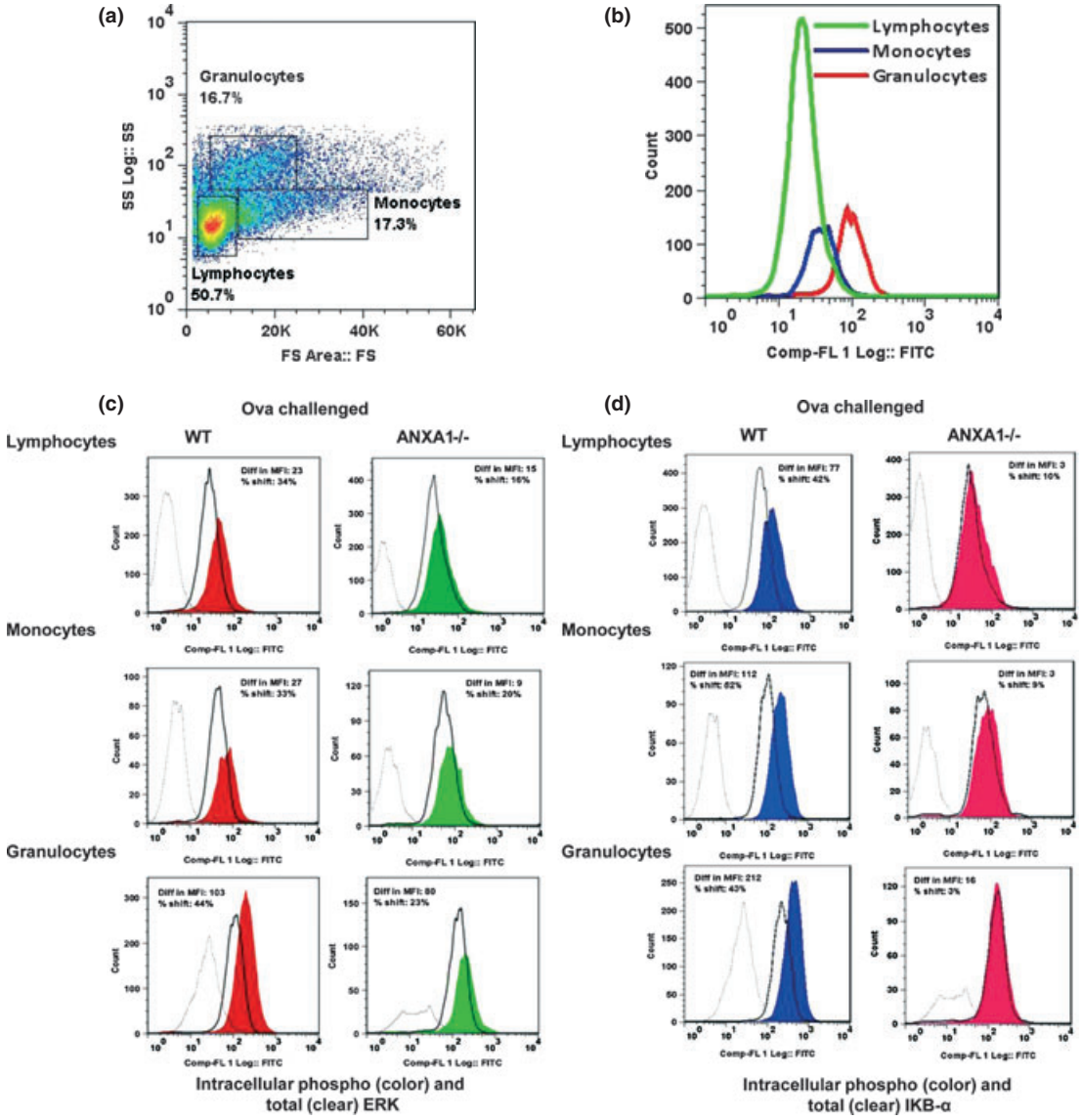

Fig. 6. Intracellular flow cytometric analysis of phosphorylated ERK and IkB levels in bronchoalveolar lavage (BAL) cells of ovalbumin (OVA)-challenged mice. Cells were obtained from BAL fluid and analysed for phosphorylated and total ERK and IkB using intracellular flow cytometry. (a) Leucocyte subsets were individually gated using forward- and side-scatter plots. (b) Intracellular staining of total ERK in the three leucocyte subsets. Levels of intracellular phosphorylated (coloured histograms) vs. total (clear histograms) (c) ERK and (d) IkB in lymphocytes, monocytes and granulocytes comparing wild-type and annexin-1-deficient OVA/OVA mice. Numbers in the histograms represent the calculated difference in the mean fluorescence intensity compared with the total protein and the percentage shift of the histogram to the right. Results shown are representative of three separate experiments with similar results.

subsets. However, smaller shifts in phosphorylated ERK vs. total ERK were observed in ANXA1$^{-/-}$ OVA-challenged mice (Fig. 6c). Confirmatory intracellular flow cytometry was also performed for phosphorylated and total IkB. A shift in phosphorylated IkB vs. total IkB was observed in all OVA-challenged WT BAL leucocyte subsets, which was not observed in all ANXA1$^{-/-}$ OVA-challenged leucocytes (Fig. 6d). These data confirm that ANXA1$^{-/-}$ mice have defective lung ERK and NF-κB activation after OVA challenge.

Discussion

The major findings of this study are that AHR and allergen-dependent antibody responses are significantly enhanced in ANXA1$^{-/-}$ mice. Importantly, the airway hyperreactivity observed in ANXA1$^{-/-}$ is not related to OVA sensitization and challenge, but essentially inherent in these ANXA1$^{-/-}$ mice. As the development of AHR is one of the hallmarks of asthma, these results demonstrate potential for a critical role of ANXA1 in protection against the development of asthma. AHR was accompanied by marked augmentation of OVA-specific Ig, increased IL-4 production and eosinophilia.

As even naïve mice exhibited AHR to bronchoconstricting agents, it may be possible that ANXA1 may affect the function and differentiation of smooth muscle cells. ANXA1 expression is up-regulated during smooth muscle cell differentiation and silencing ANXA1 inhibits its differentiation [22]. ANXA1 is associated with actin microfilaments, is involved in actin reorganization [23] and has recently been described as a new functional linker between actin filaments and phagosomes during phagocytosis [24]. These studies suggest that ANXA1 may be involved in the intrinsic physiology of cell structure and differentiation, which may explain the inherent hypersensitivity of ANXA1$^{-/-}$ airways to bronchoconstricting agents.

Spontaneous airway hyperreactivity has also been described in T-bet-deficient mice, where in the absence of allergen exposure, T-bet deficiency induces an asthmatic phenotype, with spontaneous airway reactivity [25]. T-bet, a Th1-specific T-box transcription factor is thought to transactivate the IFN-γ in Th1 cells, and can redirect fully polarized Th2 cells into Th1 cells. Logistically, silencing T-bet would induce higher Th2 polarization and thus induce an asthma-like phenotype. Indeed, D'Acquisto et al. [17] showed previously that CD3-stimulated Th0 cells obtained from ANXA1$^{-/-}$ mice expressed lower levels of T-bet and higher levels of GATA-3, the Th2 transcription factor, compared with control cells. Consistent with our study, higher levels of IL-4 production was observed in ANXA1$^{-/-}$ mice. Similarly, exogenous administration of the ANXA1 N-terminus peptide has been shown to inhibit human T cell responses to the house dust mite allergen (DerP) [26], demonstrating the importance of ANXA1 in the regulation of Th2-dependent responses.

Another interesting finding in the present study is the significant production of not only specific IgE, but other Ig during allergen sensitization in ANXA1$^{-/-}$ mice. As IgE levels are another important hall mark of allergy, this indicates that ANXA1 is important in the development of the allergic response. It is important to note that the high level of IgE may be unrelated to the exacerbated AHR observed in ANXA1$^{-/-}$ mice, as naïve, non-immunized ANXA1$^{-/-}$ mice also exhibited enhanced AHR. The enhanced production of IgE and other Ig during allergen sensitization may be induced by high levels of IL-4 produced, as IL-4 drives the Th2 immune response and together with CD40L can induce B cell class-switching from IgM to IgE. The serum level of OVA-specific IgE was already enhanced in the ANXA1$^{-/-}$ mice 7 days after the first immunization, which suggests a marked increase in the Th2-type immune response induced by OVA. However, the level of specific IgG2a was also higher in immunized ANXA1$^{-/-}$ mice serum before and after OVA challenge. This observation that both IgE and IgG2a can be regulated similarly was reported in other studies [27, 28], suggesting that Th1- and Th2-type antibody responses are not mutually counteractive. These data suggest that ANXA1 may have a general suppressive effect on allergen-specific antibody production. ANXA1 is not expressed in CD19/20^{+} B lymphocytes [29], while in hairy cell leukaemia, a specific B cell leukaemia, ANXA1 expression is specifically up-regulated and is used as a simple diagnostic assay for hairy cell leukaemia [30]. The functional role of ANXA1 in B cell function has not been reported previously; thus, it is uncertain if ANXA1 affects B cell development or direct activation.

We found that OVA-induced inflammation was only minimally affected by ANXA1 deficiency, which was surprising as multiple studies have shown ANXA1 to have functional anti-inflammatory activity [10, 13, 15, 16]. However, many of these studies were focused on the effect of ANXA1 on neutrophil activation and recruitment, both exogenously [10, 15] and endogenously [12] in response to acute stimuli such as zymosan and carrageenin. Previous reports have suggested that ANXA1 may not [31, 32] be involved in eosinophil recruitment. However, local treatment of ANXA1 peptide Ac2-26 in a rat pleurisy model inhibited plasma protein leakage, eotaxin generation and accumulation of both neutrophils and eosinophils in the lung [16]. However, ANXA1 did not directly inhibit eosinophil chemotaxis *in vitro*. From the study by Bandeiro-Melo et al. [16], we can surmise that ANXA1 may inhibit eotaxin and other cytokine production and thus inhibit eosinophil recruitment indirectly. In addition, in the

present study, while lung EPO levels were significantly different between WT and ANXA1$^{-/-}$ mice, levels of BAL EPO were not. BAL EPO represents eosinophil degranulation, as readings are taken from the cell-free lavage fluid, while lung EPO represents eosinophil quantity or recruitment to the lung. This data indicates that ANXA1 does not inhibit eosinophil degranulation, yet has minimal effects on eosinophil recruitment.

Endogenous ANXA1 can be released upon cellular adhesion onto the endothelium upon cell activation [11], and binds to its receptors, the FMLP receptors, FPR and the lipoxin A4 receptor FPRL1 (FMLP receptor like, or ALXR1 [33]) to exert its anti-inflammatory effects. A recent study demonstrated that lipoxin A4 was capable of inhibiting AHR and pulmonary inflammation [34] in a mouse model of asthma. As ANXA1 was also reported to act via ALXR1 [35, 36], it may be the mechanism through which ANXA1 modulates AHR. Previous studies have implicated that ANXA1-induced FPR-mediated signalling is ERK and NF-κB mediated, and cells from ANXA1$^{-/-}$ mice exhibit lower NF-κB activity [17, 37]. Indeed, we have shown that our ANXA1$^{-/-}$ mice have defective ERK and NF-κB activation in lungs and cells after sensitization and challenge. Furthermore, we have recently shown that ANXA1 can functionally regulate NF-κB activity in breast cancer cells, which modulates metastatic capacity [38].

In conclusion, our study adds proof to the proposition that ANXA1 may play a role in limiting the pathogenesis of asthma, and is critical in the development of certain asthma characteristics such as allergen-specific antibody production and airway hyperreactivity.

Acknowledgements

This work was supported by grants to LHKL from the National Medical Research Council of Singapore (NMRC-0966/2005) and the BioMedical Research Council of Singapore (BMRC 07/1/21/19/50) as well as grant to WSFW from BioMedical Research Council of Singapore (BMRC 06/1/21/19/443). We would like to thank Professor Roderick Flower from the William Harvey Research Institute for the ANXA1-deficient mice, Dr Chua Kaw Yan (Department of Pediatrics) from the Yong Loo Lin School of Medicine, National University of Singapore, for use of the Buxco system.

Conflict of interest: The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. ERK phosphorylation lower in ANXA1$^{-/-}$ (KO) lungs.

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