

## ORIGINAL ARTICLE

## Basic and Translational Allergy Immunology

# Targeting the ICOS/ICOS-L pathway in a mouse model of established allergic asthma disrupts T follicular helper cell responses and ameliorates disease

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## Abstract

**Background:** Allergic asthma is characterized by chronic inflammation and remodelling of the airways, associated with dysregulated type 2 immune responses and allergen-specific IgE. T follicular helper cells (T<sub>FH</sub>) are crucial in T-dependent B-cell responses and have been implicated in allergic airway disease (AAD). T<sub>FH</sub>, unlike other CD4<sup>+</sup> T cells, are uniquely reliant on continuous ICOS signalling to maintain their phenotype after T-cell priming; therefore, disrupting this signal can impair T<sub>FH</sub> responses. However, the contribution of T<sub>FH</sub> to disease during chronic aero-allergen exposure and the therapeutic potential of targeting these cells have not been evaluated.

**Methods:** To establish AAD, female BALB/c mice were repeatedly exposed to house dust mite or *Alternaria alternata* three times a week for up to 5 weeks. To examine the impact of T<sub>FH</sub> on AAD, mice were allergen exposed for 5 weeks and co-administered anti-ICOS Ligand-targeted antibodies, three times a week for the last 2 weeks.

**Results:** T<sub>FH</sub> were first observed in the lung-draining lymph nodes and with further exposure were also found locally within the lungs. T<sub>FH</sub> accumulated with sustained allergen exposure, alongside germinal centre (GC) B cells. Blockade of ICOS signalling after AAD establishment successfully depleted T<sub>FH</sub> but did not affect the differentiation of other CD4<sup>+</sup> T-cell subsets. This reduced GC responses, allergen-specific IgE, inflammation, pulmonary IL-13 and airway hyper-responsiveness.

**Conclusions:** T<sub>FH</sub> are crucial in the regulation of AAD and the ICOS/ICOS-L pathway could represent a novel therapeutic target in allergic asthma.

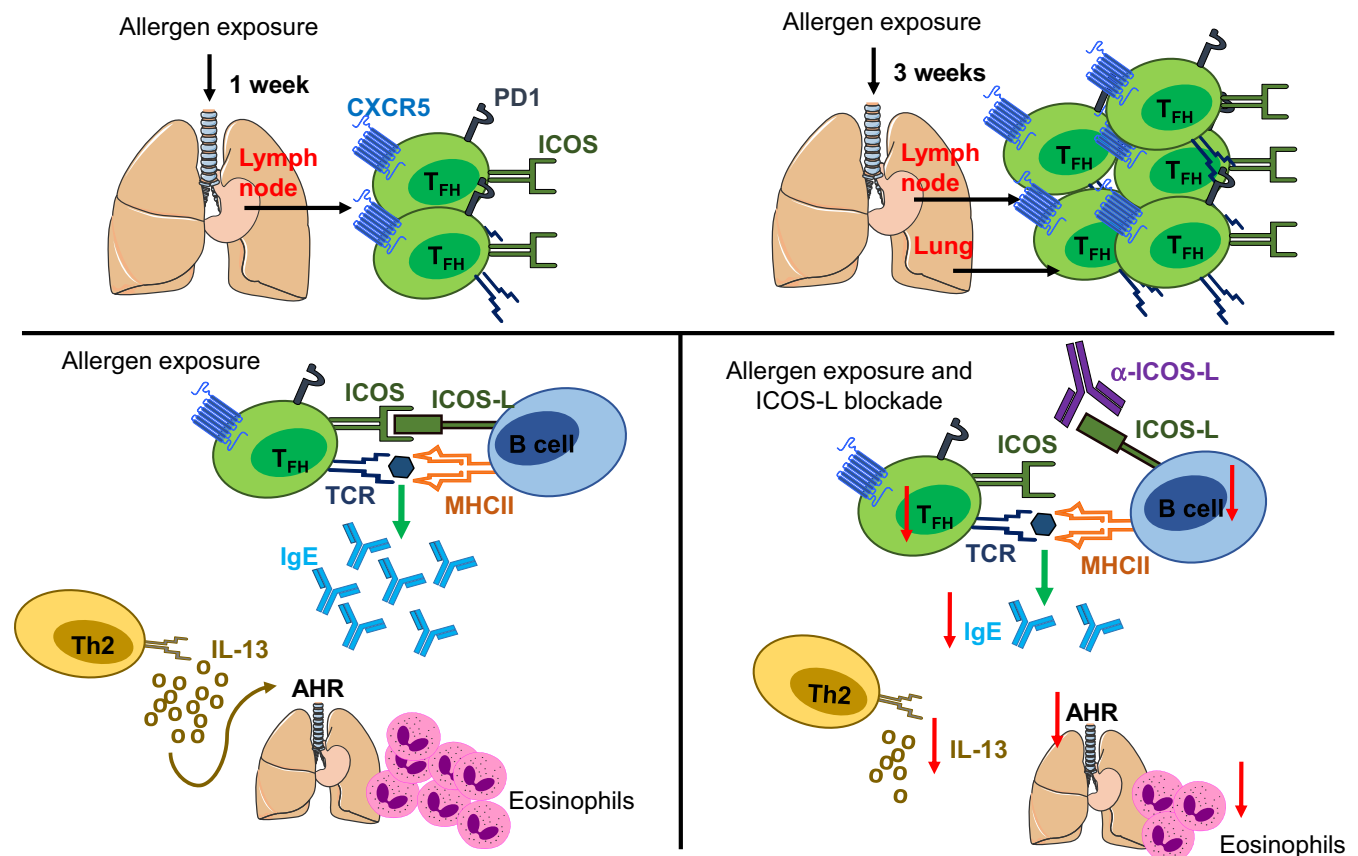
## KEYWORDS

allergic airway disease, germinal centres, ICOS, ICOS-L, T follicular helper cells

## 1 | INTRODUCTION

Allergic asthma is characterized by chronic inflammation and remodelling of the airways and dysregulated type 2 immune responses.

This results from sustained exposure to aeroallergens, including house dust mite (HDM) and fungal spores, which lead to elevated concentrations of type 2 cytokines IL-4, IL-5 and IL-13, alongside allergen-specific immunoglobulin E (IgE), eosinophilia and airway



### GRAPHICAL ABSTRACT

Chronic allergen exposure drives T follicular helper cell (TFH) formation in the lung draining lymph node after 1 week and the lung tissue after 3 weeks. TFH at both sites interact with germinal centre B cells and are critical for the production of allergen specific IgE. Allergic disease is also hallmarked by cellular inflammation and airway hyperresponsiveness driven by IL-13. Blocking ICOS and ICOS-L interactions between TFH and B cells reduces allergen specific IgE production and also dampens other hallmark symptoms of allergic airway disease.

hyper-responsiveness (AHR),<sup>1</sup> T helper 2 cells (Th2), and the type 2 cytokines they produce, have traditionally been thought of as the central drivers of the disease. It is now clear, however, that many other immune cells can produce these cytokines and play vital roles in the regulation of distinct asthma phenotypes.<sup>1</sup>

T<sub>FH</sub> are a distinct CD4<sup>+</sup> T-cell subset specialized to provide help to B cells, resulting in the production of high affinity, isotype-switched antibodies and the differentiation of B cells into memory B cells and plasma cells.<sup>2</sup> They can be identified by their expression of CXCR5, PD1, Bcl-6 and ICOS and are predominantly located within the B-cell follicles of secondary lymphoid organs (SLOs), such as lymph nodes and the spleen.<sup>2</sup> T<sub>FH</sub> differentiation is a multi-step process. First dendritic cells (DCs) present antigen and co-stimulatory signals to naive CD4<sup>+</sup> T cells within the T-cell zone of SLOs.<sup>3,4</sup> Next, pre-T<sub>FH</sub> migrate towards the T-B border<sup>5,6</sup> where antigen presentation and inducible T co-stimulator ligand (ICOS-L) co-stimulation is provided by activated B cells.<sup>7,8</sup> Fully differentiated T<sub>FH</sub> migrate into the B-cell follicle, further migrating into newly formed anatomical structures called germinal centres (GC).<sup>6</sup> Here, T<sub>FH</sub> form tight,

cognate interactions with GC B cells, providing survival and differentiation signals to the B cells in return for T-cell receptor (TCR) signalling and co-stimulation.<sup>2,5,7</sup> The co-stimulatory molecule ICOS is required for T-cell activation and is upregulated on CD4<sup>+</sup> T cells following TCR engagement.<sup>9</sup> Importantly, however, T<sub>FH</sub> uniquely require sustained ICOS/ICOS-L signalling throughout an immune response and after DC priming to maintain their phenotype, unlike other CD4<sup>+</sup> T-cell subsets.<sup>10</sup>

The role of T<sub>FH</sub> in allergic asthma appears to be complex. T<sub>FH</sub> are found in the SLOs of mice that have undergone sensitization and challenge with HDM<sup>11-13</sup> and are required for allergen-specific IgE production.<sup>14,15</sup> T<sub>FH</sub> isolated from the lung draining lymph nodes can migrate to the lungs and become Th2 cells, enhancing allergic airway disease (AAD), when injected intravenously.<sup>13</sup> In contrast, IL-21<sup>+</sup> T<sub>FH</sub> failed to generate Th2 cells when adoptively transferred into naive mice subsequently challenged with HDM, but do cause airway eosinophilia.<sup>11</sup> After helminth infection Th2 cells can become T<sub>FH</sub>,<sup>16</sup> retaining a Th2-like phenotype, and T<sub>FH</sub> themselves can obtain effector functions related to other CD4<sup>+</sup> T-cell

lineages.<sup>17</sup> Conversely, CD4<sup>+</sup> T cells lacking the T<sub>FH</sub> master transcriptional regulator Bcl6, which cannot become T<sub>FH</sub>, preferentially differentiate into lung resident Th2 cells and promote AAD.<sup>12</sup> Thus, T<sub>FH</sub> appear to be important in AAD but their exact contribution and the therapeutic potential of targeting them once disease is established are uncertain.

Here, using chronic allergen exposure models of AAD, to mimic the frequent, low dose, allergen exposures allergic asthmatics experience, T<sub>FH</sub> and GC B cells developed in the SLOs and the lung itself. Targeting the ICOS/ICOS-L pathway after AAD establishment specifically reduced the frequency of both cell types. Treatment also reduced airway eosinophilia, AHR, allergen-specific IgE and was associated with reduced concentrations of IL-13 in the lungs. This shows that blocking ICOS/ICOS-L interactions during chronic airway inflammation can target T<sub>FH</sub>-dependent immune responses and ameliorate established AAD.

## 2 | MATERIALS AND METHODS

### 2.1 | Mice

Six- to eight-week-old female BALB/c mice were purchased from Charles River Laboratories (UK) and IL-13<sup>GFP</sup> reporter mice were gifted by Professor Andrew McKenzie, University of Cambridge. Mice were housed in IVCs, and all procedures were approved by the Imperial College London Animal Welfare Ethical Review Body (AWERB) and the United Kingdom Home Office (Approval from both under project licence number 70/7463) and conducted in accordance with the Animals (Scientific Procedures) Act 1986.

### 2.2 | Induction of allergic airway disease and ICOS-L intervention

Mice were administered 25 µg HDM extract (*Dermatophagoides pteronyssinus*) or 10 µg *Alternaria alternata* (ALT) intranasally (i.n.) three times a week for up to 5 weeks (Greer Laboratories, NC, USA; Citeq, Groningen, The Netherlands). Control mice were given 25 µL PBS. In blocking experiments, from week 4 onwards, mice were co-administered 150 µg anti-ICOS-Ligand (Clone: HK5.3, BioXCell, NH, USA) or isotype control (Clone: 2A3, BioXCell, NH, USA) antibody in 200 µL PBS via intraperitoneal (i.p.) injection three times a week for 2 weeks. Mice were culled at the end of week 5. All animals were harvested 18 hours after the final allergen dose.

### 2.3 | Flow cytometry assessment

Cell suspensions were acquired as previously described<sup>18</sup> and were stained in flow cytometry buffer (PBS containing 2% foetal calf serum and 2 mmol/L EDTA). To reduce nonspecific binding, cell suspensions were incubated with antibody cocktails containing anti-CD16/32 antibody. Cells were extracellularly stained in antibody cocktails for 30 minutes at 4°C, apart from stains containing CXCR5 which were incubated at room temperature in the dark for 1 hour. For detection of intracellular cytokines, cells were incubated with

50 ng/mL phorbol myristate acetate, 500 ng/mL ionomycin and 10 µg/mL brefeldin A for 5 hours at 37°C and 5% CO<sub>2</sub>. Cells were fixed with 1% paraformaldehyde. For detection of intranuclear transcription factors, cells were fixed and permeabilized using the Foxp3/Transcriptional factor staining buffer set (eBioscience, CA, USA) according to the manufacturer's instructions. Cells were then washed and intracellularly stained at 4°C in permeabilization wash buffer (Biolegend, CA, USA). Flow cytometry data were acquired using an LSRII Fortessa (Becton Dickinson, NJ, USA) and analysed using the FlowJo 10 software (FlowJo, OR, USA). Flow cytometry antibodies are listed in Table S1.

### 2.4 | Assessment of lung function

Airway hyper-responsiveness was measured in anesthetized and tracheotomized mice in response to increasing doses of methacholine (3–100 mg/mL; Sigma-Aldrich, MO, USA) using the flexiVent system (Scireq, Montreal, Canada) as previously described.<sup>19</sup>

### 2.5 | Antibody assessment

Allergen-specific IgE and IgG1 levels were measured by coating plates with 50 µg/mL HDM then adding serially diluted serum and biotinylated IgG1 or IgE antibodies (BD Pharmingen™, Oxford, UK). Endpoint titre was calculated using baseline+2xSD based on naïve animals.

### 2.6 | Cytokine analysis

IL-13, IL-17A and IL-21 were measured using Ready Set Go Kits (eBioscience, CA, USA), Eotaxin-2 using mouse CCL24/Eotaxin-2 DuoSet ELISA and IL-5 using paired antibodies (R&D systems, Abingdon, UK). All ELISAs were performed according to manufacturer's instructions.

### 2.7 | Statistical analysis

Statistical significance was determined using the Mann-Whitney U Test and assessed using Prism 6 (GraphPad Software Inc, CA, USA). All *P* values ≤0.05 (\*) ≤0.01 (\*\*), ≤0.001 (\*\*\*) and ≤0.0001 (\*\*\*\*) were considered significant.

Methods continue in Appendix S1.

## 3 | RESULTS

### 3.1 | Repeated aero-allergen exposure generates lung- and lymphoid-resident T<sub>FH</sub>

To replicate the repeated low dose aeroallergen exposure experienced by allergic asthmatics, mice were exposed to two common aeroallergens; HDM or ALT three times a week for up to 5 weeks (Figure 1A).

T<sub>FH</sub>, defined as CXCR5<sup>+</sup>PD1<sup>+</sup>Foxp3<sup>+</sup>CD4<sup>+</sup> (Figure S1) were observed in the lung-draining mediastinal lymph nodes (mLN) of

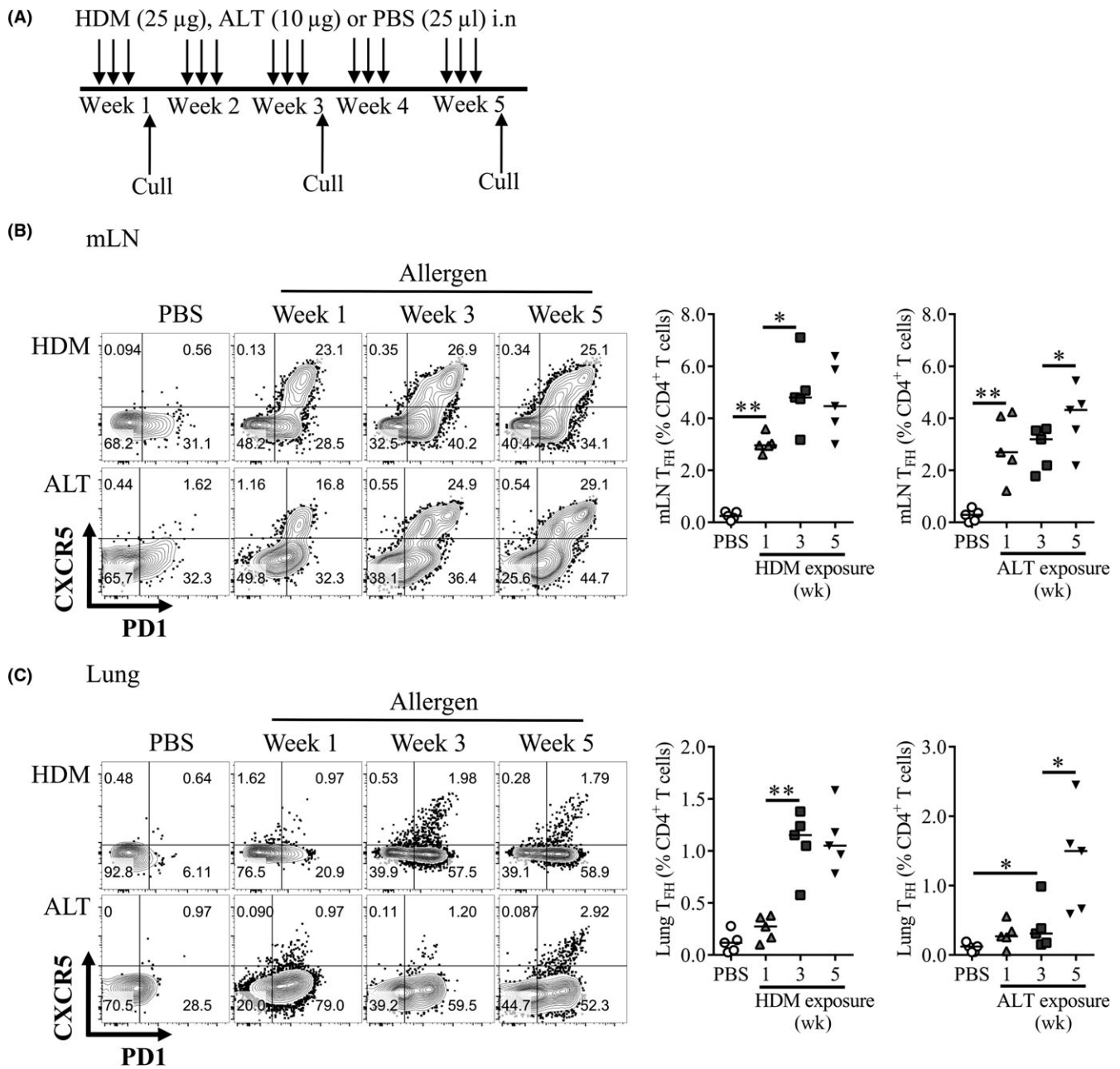


**FIGURE 1** T follicular helper cells (T<sub>FH</sub>) accumulate over time in the mediastinal lymph nodes and lung tissue. Adult female BALB/c mice were exposed to either 25  $\mu$ g house dust mite (HDM), 10  $\mu$ g *Alternaria alternata* (ALT) or 25  $\mu$ l phosphate-buffered saline (PBS), three times a week for up to 5 weeks. Flow cytometry was used to determine the frequency of T<sub>FH</sub> within cellular compartments. Representative flow plots of T<sub>FH</sub> in PBS, ALT or HDM-treated animals are displayed, pre-gated on CD4<sup>+</sup>CD3<sup>+</sup>Foxp3<sup>-</sup>CD44<sup>hi</sup>CD62L<sup>-</sup> lymphocytes. Data are quantified. T<sub>FH</sub> were defined as CXCR5<sup>+</sup>PD1<sup>+</sup>Foxp3<sup>-</sup>CD4<sup>+</sup> lymphocytes. A, Experimental set-up. B, mediastinal lymph nodes (mLN), C, lung tissue. Statistical significance was determined using a Mann-Whitney U test. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, *n* = 5 per time-point. Representative data from two independent experiments

allergen-exposed animals after 1 week but were not found in the mLNs of PBS-treated controls (Figure 1B). Continued allergen exposure further increased T<sub>FH</sub> proportions in the mLN after 3 and 5 weeks (Figure 1B). T<sub>FH</sub> were also observed in the spleen but not in the circulation of allergen-treated animals (Figure S2). Interestingly, T<sub>FH</sub> were identified in the lung tissue itself after

3 weeks of HDM inhalation and remained elevated at 5 weeks of exposure (Figure 1C). Lung T<sub>FH</sub> frequencies were also significantly increased following 3 weeks of ALT treatment and further increased after 5 weeks (Figure 1C). Therefore, prolonged allergen exposure induced both local and systemic T<sub>FH</sub> responses that increased in frequency over time.

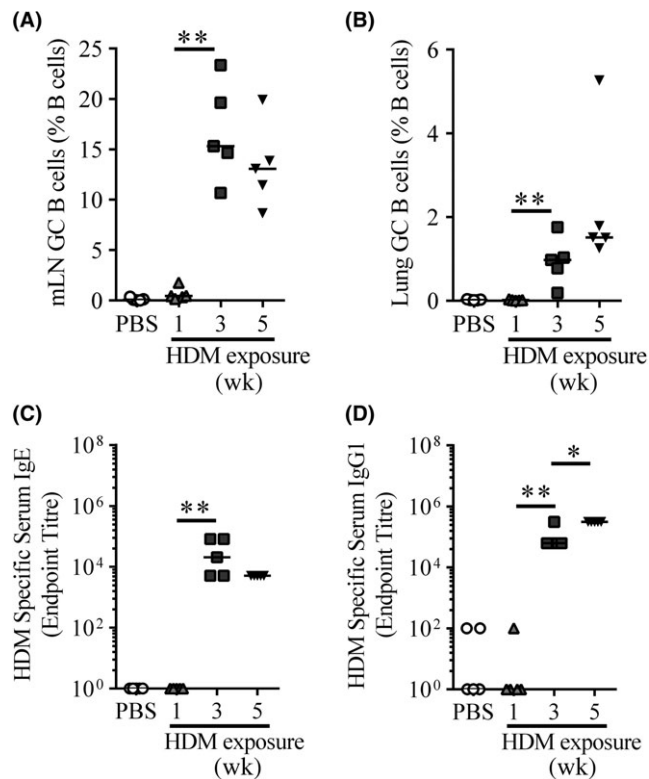


**FIGURE 2**  $T_{FH}$  induction precedes germinal centre formation and antibody production. Adult female BALB/c mice were exposed to either 25  $\mu$ g house dust mite (HDM) or 25  $\mu$ L phosphate-buffered saline (PBS), three times a week for up to 5 weeks. A-B, Flow cytometry was used to determine the frequency of germinal centre (GC) B cells in the mLN and lungs. GC B cell was defined as  $CD38^{-}GL7^{+}FAS^{+}CD19^{+}B220^{+}$  lymphocytes and quantified. A, mLN GC B cells, B, Lung GC B cells. C-D, Serum was titrated, and allergen-specific IgE and IgG1 were measured by ELISA. Endpoint titres are displayed. C, HDM-specific IgE, D, HDM-specific IgG1. Statistical significance was determined using a Mann-Whitney  $U$  test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .  $n = 5$  mice per time-point. Representative data from two independent experiments

### 3.2 | $T_{FH}$ precede the development of humoral immunity during AAD

$T_{FH}$  direct B-cell responses, driving GC formation, isotype switching, affinity maturation and B-cell differentiation.<sup>2</sup> GC B cells defined as

$CD38^{-}GL7^{+}FAS^{+}IgD^{-}IgM^{-}B220^{+}CD19^{+}$  lymphocytes (Figure S3) were absent and comparable to PBS controls in the mLN and lungs after 1 week of aero-allergen inhalation (Figure 2A and B). However, after 3 weeks of HDM exposure, GC B-cell frequencies were significantly elevated in the mLN, lungs and spleen, remaining consistently raised between weeks 3 and 5. (Figure 2A, Figure S4). Similar results were observed following ALT exposure (Figure S4).

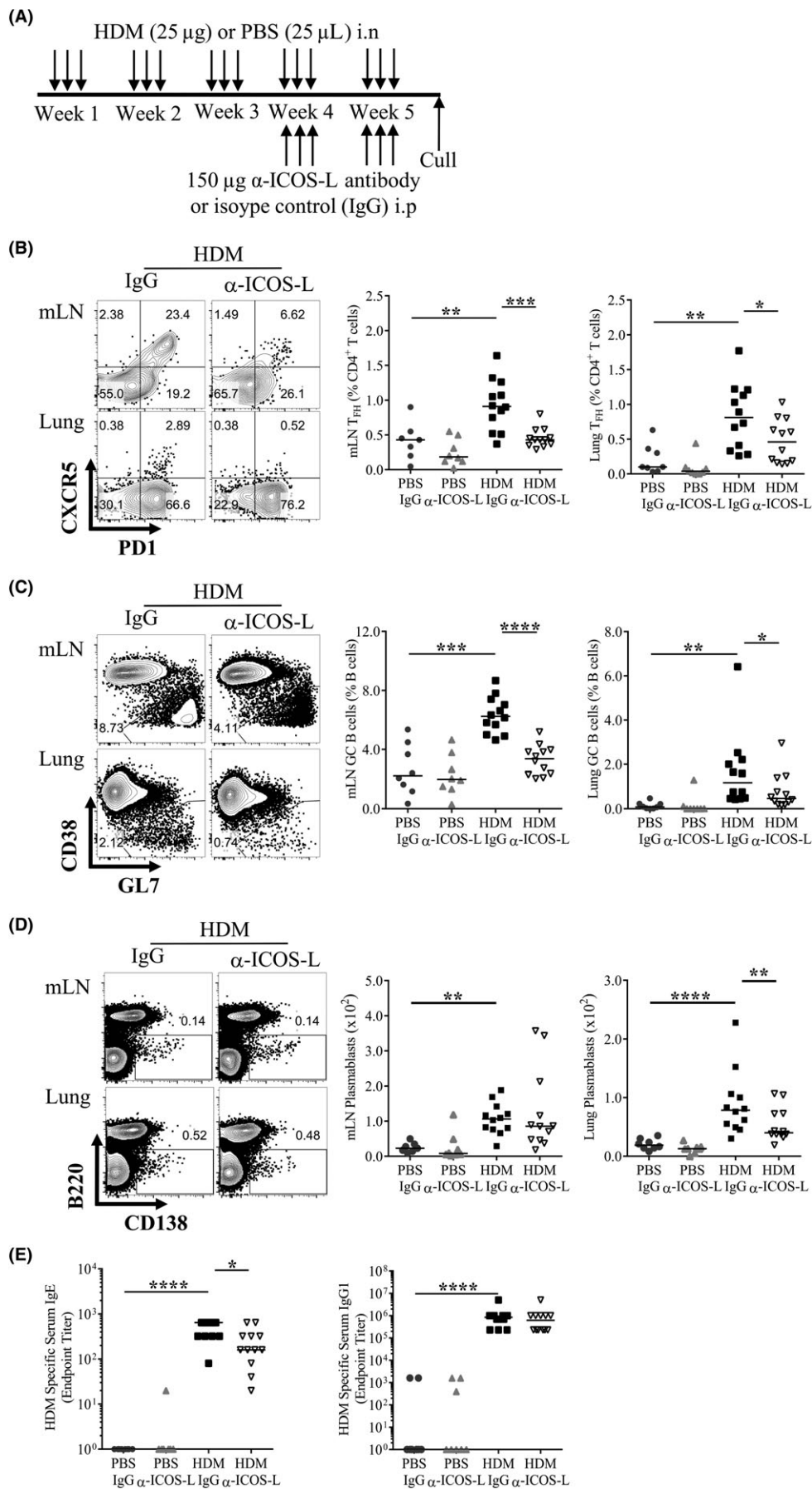
Allergen-specific antibodies, especially IgE, are a key feature of AAD. Allergen-specific IgE and IgG1 were detectable in allergen-exposed mice from 3 weeks onward (Figure 2C and D). Sustained exposure to HDM did not further alter the levels of allergen-specific IgE (Figure 2C), but did increase IgG1 (Figure 2D). These data show chronic allergen exposure to generate local and systemic GC B-cell responses, alongside allergen-specific antibody which increase over time and are preceded by  $T_{FH}$  responses.

### 3.3 | ICOS/ICOS-L interactions sustain $T_{FH}$ and GC B cells

$T_{FH}$  require sustained signalling via ICOS to maintain their phenotype and can be depleted by disrupting interactions between ICOS and ICOS-L.<sup>5,10</sup> AAD, characterized by AHR and allergic inflammation including humoral immune responses, are established after 3 weeks of allergen exposure.<sup>20,21</sup> To study the role of ICOS signalling in established AAD, allergen-treated mice were administered anti-ICOS-L antibody ( $\alpha$ -ICOS-L) or an isotype control (IgG) between weeks 3 and 5 of allergen exposure, and analysed at the end of week 5 (Figure 3A).

$\alpha$ -ICOS-L treatment substantially reduced mLN and lung  $T_{FH}$  populations after HDM exposure compared to IgG-treated animals (Figure 3B). Consistent with reduced  $T_{FH}$  responses, HDM-induced mLN and lung GC B cells were also decreased in mice treated with  $\alpha$ -ICOS-L compared to IgG controls (Figure 3C).  $\alpha$ -ICOS-L did not decrease the proportion of  $B220^{-}CD138^{+}$  plasmablasts in the mLN and lungs or the numbers of mLN plasmablasts relative to HDM and IgG-treated animals but there was a significant reduction in the number of lung plasmablasts (Figure 3D). Furthermore,  $\alpha$ -ICOS-L intervention diminished serum HDM-specific IgE but not IgG1 (Figure 3E). Alongside this,  $\alpha$ -ICOS-L reduced HDM-induced serum mast cell protease 1 (MCPT1) (Figure S5). Similar results were observed with ALT-driven AAD (Figures S6 and S7). Overall, these data show that during chronic allergen exposure,  $T_{FH}$  can be

**FIGURE 3** ICOS/ICOS-L interactions are required to sustain  $T_{FH}$  during chronic allergic airway disease (AAD). Adult female BALB/c mice were exposed (i.n.) to 25  $\mu$ g house dust mite (HDM) or 25  $\mu$ L phosphate-buffered saline (PBS), three times a week for 5 weeks. From the start of week 4, mice were also administered 150  $\mu$ g anti-ICOS-L ( $\alpha$ -ICOS-L) or isotype control (IgG) antibody (i.p) three times a week. Mice were culled at the end of week 5. A, Schematic of experimental design, B, Representative flow plots of mLN and lung  $T_{FH}$  following HDM and IgG or  $\alpha$ -ICOS-L treatment. C, Representative flow plots of mLN and lung germinal centre (GC) B cells following HDM and IgG or  $\alpha$ -ICOS-L treatment. Pre-gated on  $CD19^{+}B220^{+}$  B cells. The data are quantified for all groups. D, Representative flow plots of mLN and lung  $B220^{-}CD138^{+}$  plasmablasts following HDM and IgG or  $\alpha$ -ICOS-L treatment, pre-gated on lymphocytes. Plasmablast numbers are quantified for all groups. E, Serum was titrated, and allergen-specific antibody was measured by ELISA. Endpoint titres are displayed for IgE and IgG1. Statistical significance was determined using a Mann-Whitney  $U$  test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Data are pooled from two independent experiments,  $n = 8$  for PBS-treated groups,  $n = 12$  for HDM-treated groups



successfully depleted using  $\alpha$ -ICOS-L and this is associated with reduced GC B-cell responses, lung plasmablasts and allergen-specific IgE but not IgG1.

### 3.4 | $\alpha$ -ICOS-L treatment dampens lung inflammation and reduces AHR

Airway hyper-responsiveness and inflammation are key indicators of AAD progression.  $\alpha$ -ICOS-L administration successfully reduced aero-allergen induced cellular infiltration into the lungs compared to IgG-treated controls (Figure 4A). The total number of lung eosinophils was reduced with allergen and  $\alpha$ -ICOS-L co-administration (Figure 4B); however, the proportion of lung eosinophils were unchanged (Figure 4C). A similar trend was observed during ALT-driven AAD (Figure S8).

Allergen-treated mice showed increased AHR, with raised airway resistance and elastance, and reduced compliance compared

to PBS controls in response to increasing doses of methacholine (Figure 4D). Treatment of HDM-exposed mice with  $\alpha$ -ICOS-L resulted in reduced airway resistance and elastance and increased compliance, indicative of improved lung function compared to HDM-treated control mice (Figure 4D). This was also observed for ALT-induced AAD (Figure S6). Despite this  $\alpha$ -ICOS-L administration had no impact on HDM-induced goblet cell hyperplasia, collagen deposition or airway smooth muscle hyperplasia and hypertrophy (Figure S9A-I). Taken together the data shows that therapeutic administration of  $\alpha$ -ICOS-L after disease establishment improved airway inflammation and lung function.

### 3.5 | $\alpha$ -ICOS-L reduces overall inflammation without specifically targeting Th2 cells or ILC2s

To determine the mechanism by which  $\alpha$ -ICOS-L treatment could be impacting pulmonary inflammation and AHR, the release of

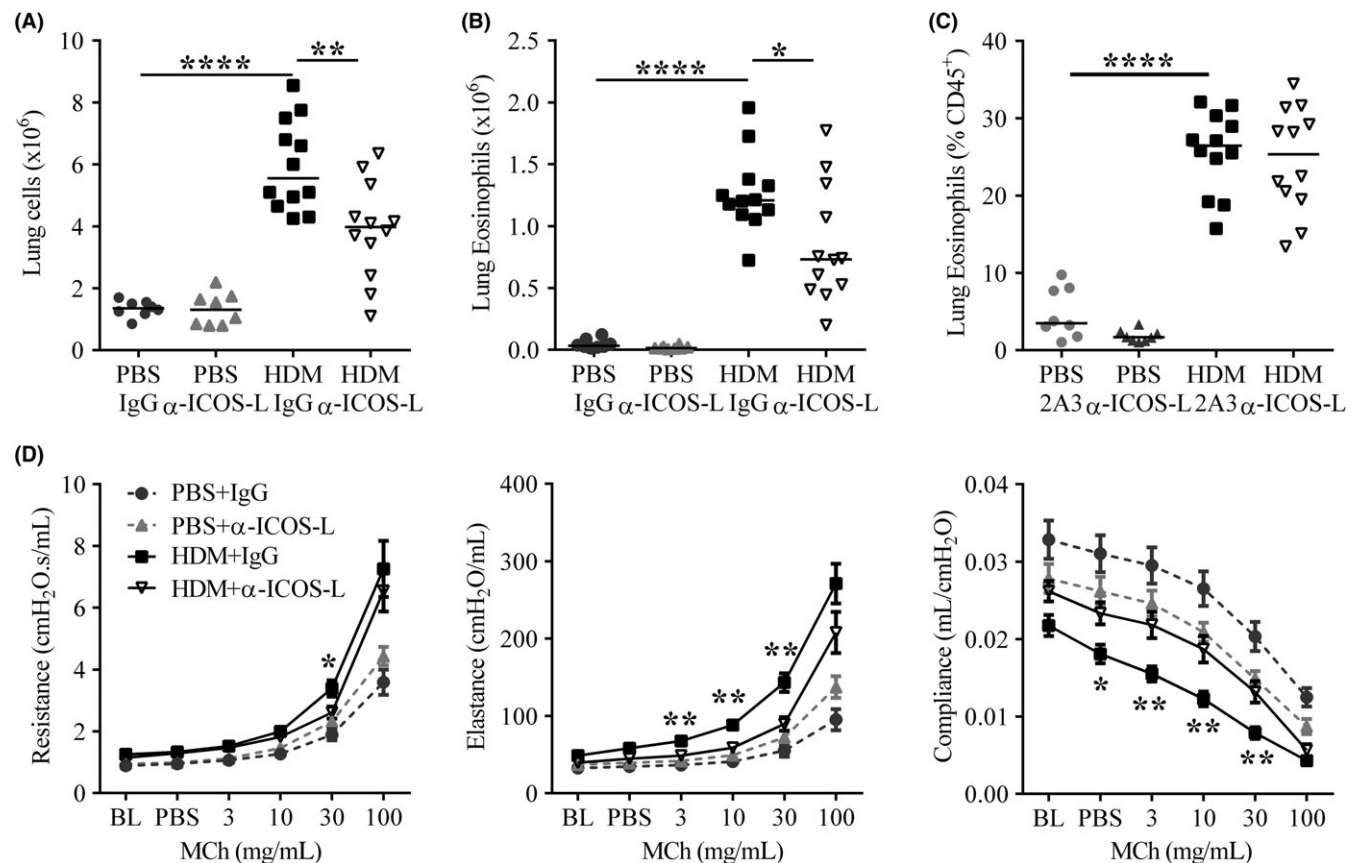


**FIGURE 4** Therapeutic ICOS-L blockade improves chronic allergic airway disease. Adult female BALB/c mice were exposed (i.n) to 25  $\mu$ g house dust mite (HDM) or 25  $\mu$ L phosphate-buffered saline (PBS), three times a week for 5 weeks. From the start of week 4, mice were also administered 150  $\mu$ g anti-ICOS-L ( $\alpha$ -ICOS-L) or isotype control (IgG) antibody (i.p) three times a week. Mice were culled at the end of week 5. A, Number of lung cells, B, Lung eosinophil numbers, C, Proportions of lung eosinophils, D, Airway hyper-responsiveness was measured by exposing mice to 0–100 mg/mL methacholine (MCh) using the flexiVent system during HDM-induced allergic airway disease. Airway resistance, elastance and compliance were measured. Curves display mean  $\pm$  SEM. Statistical significance between HDM + IgG and HDM +  $\alpha$ -ICOS-L groups was determined using a Mann-Whitney *U* test. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. Data are pooled from two independent experiments, *n* = 8 for PBS-treated groups, *n* = 12 for HDM-treated groups

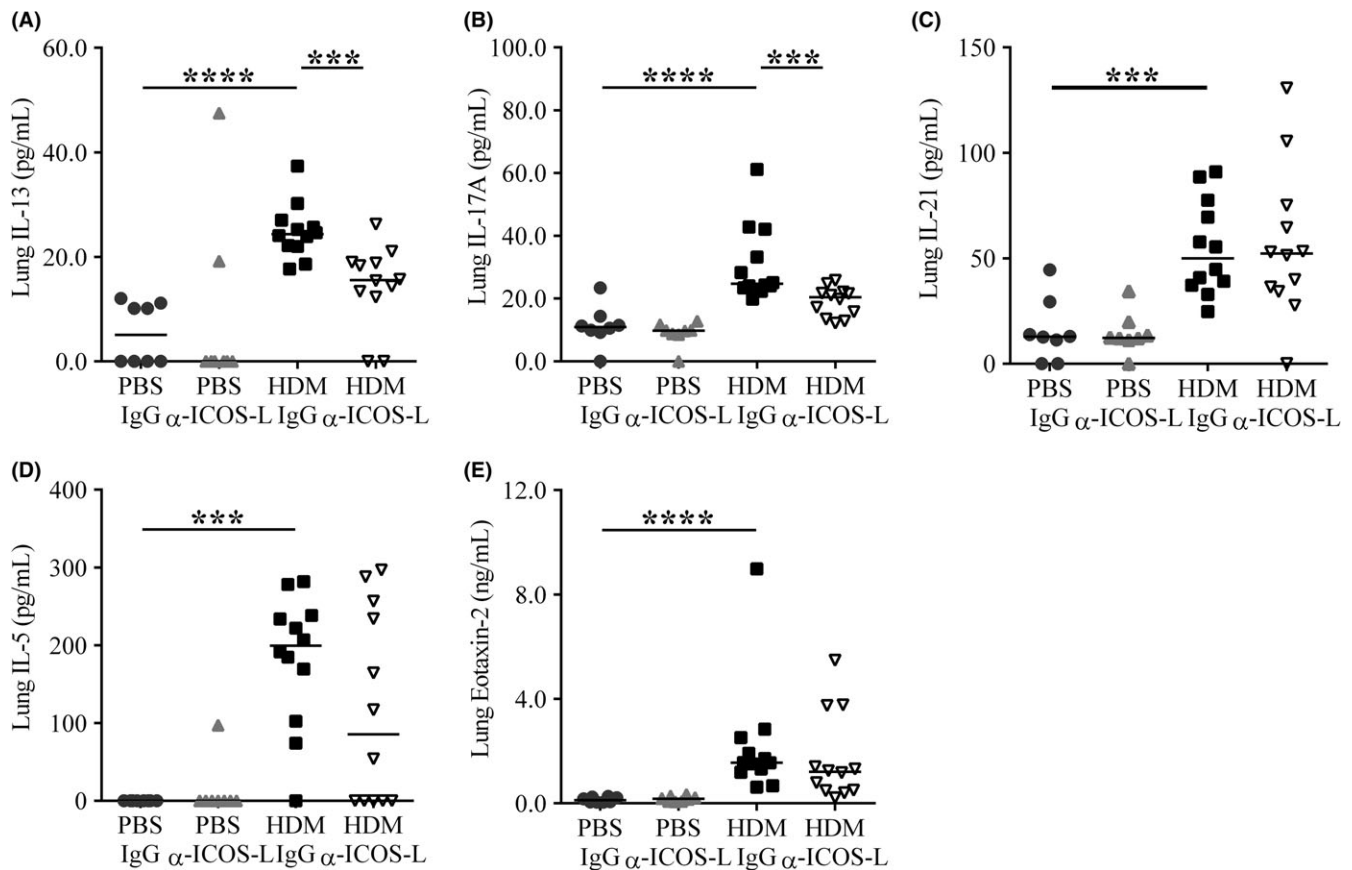


**FIGURE 5** Therapeutic ICOS-L blockade reduces pulmonary IL-13 and IL-17A. Adult female BALB/c mice were exposed (i.n) to 25  $\mu$ g house dust mite (HDM) or 25  $\mu$ L phosphate-buffered saline (PBS), three times a week for 5 weeks. From the start of week 4, mice were also administered 150  $\mu$ g anti-ICOS-L ( $\alpha$ -ICOS-L) or isotype control (IgG) antibody (i.p) three times a week. Mice were culled at the end of week 5. Pulmonary cytokines and chemokines within lung homogenate were measured by ELISA. A, IL-13, B, IL-17A, C, IL-21, D, IL-5, E, Eotaxin-2. Statistical significance was determined using a Mann-Whitney  $U$  test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Data are pooled from two independent experiments,  $n = 8$  for PBS-treated groups,  $n = 12$  for HDM-treated groups

several important soluble mediators in the lungs was measured. HDM-treated animals administered IgG displayed increased lung IL-13, IL-17A, IL-21, IL-5 and eotaxin-2 compared to PBS controls (Figure 5A-E).  $\alpha$ -ICOS-L treatment reduced lung IL-13 and IL-17A concentrations compared to IgG aeroallergen exposed mice (Figure 5A and B). IL-5 was reduced but did not reach significance, while no change was observed for Eotaxin-2 (Figure 5D and E). Interestingly, the  $T_{FH}$  effector cytokine IL-21 was unchanged after  $\alpha$ -ICOS-L treatment (Figure 5C).

As IL-13 release was reduced by ICOS-L blockade and IL-13 is central to AAD pathogenesis the cellular sources of IL-13, predominantly thought to be Th2 cells and ILC2s ( $Lin^{-}Nkp46^{-}CD45^{+}CD90.2^{+}IL-13^{+}$ ), were analysed. HDM exposure increased the proportion of IL-13 $^{+}$  CD4 $^{+}$  T cells and IL-13 $^{+}$  ILC2s in the lungs, and these were not affected by  $\alpha$ -ICOS-L intervention (Figure 6A and B). However, consistent with decreased pulmonary inflammation (Figure 4A), total numbers of IL-13 $^{+}$  CD4 $^{+}$  T cells and IL-13 $^{+}$  ILC2s showed a trend towards reduction following  $\alpha$ -ICOS-L treatment (Figure 6C).

Although ILC2s are the major ILC subset induced during HDM-driven chronic AAD,<sup>22</sup> the total ILC population was also analysed regardless of cytokine expression ( $Lin^{-}Nkp46^{-}CD45^{+}CD90.2^{+}$ ). Similarly,  $\alpha$ -ICOS-L did not alter the proportion of total ILCs but did cause a drop in ILC numbers consistent with the overall fall in pulmonary inflammation (Figure S10A). In ALT-driven AAD, IL-13 $^{+}$  CD4 $^{+}$  T cells but not ILC2s were reduced (Figure S10B-E). Collectively, these data suggest  $\alpha$ -ICOS-L treatment can therapeutically relieve established AAD by reducing pulmonary inflammation and AHR but does not appear to directly target Th2 cells or ILCs.

IL-17A $^{+}$  CD4 $^{+}$  T cells were also found to be induced following HDM and IgG exposure and again were decreased in total number, but not proportion, after  $\alpha$ -ICOS-L administration fitting with the fall in overall pulmonary inflammation (Figure S11A-C). IL-17A $^{+}$  CD4 $^{+}$  T cells were not significantly induced by ALT inhalation and were unchanged by  $\alpha$ -ICOS-L intervention (Figure S11A, D-E). IL-17A $^{+}$  ILCs were unchanged by  $\alpha$ -ICOS-L intervention (Figure S11A, G-I).

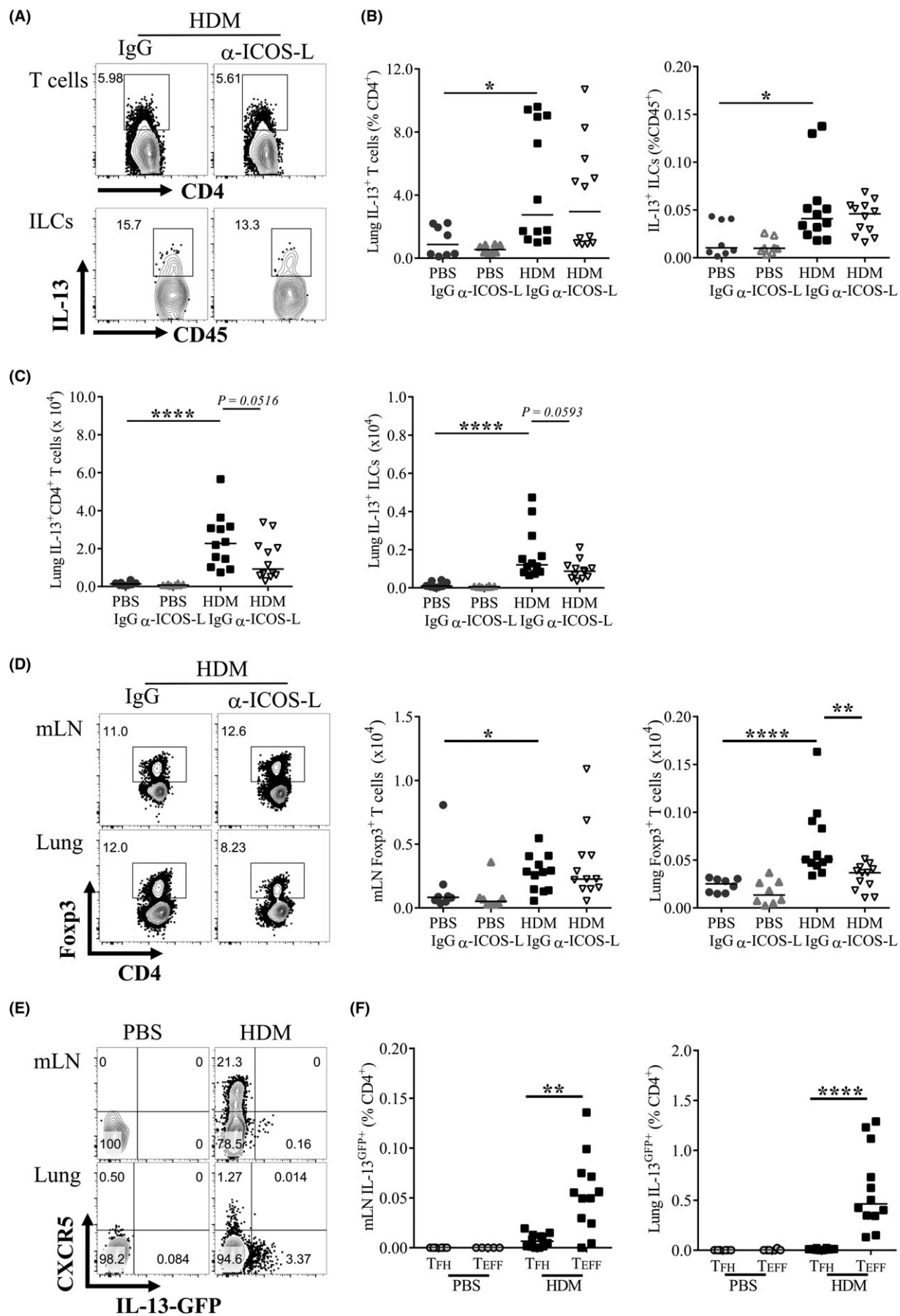


**FIGURE 6** IL-13<sup>+</sup> CD4<sup>+</sup> T cells and ILCs are not directly targeted by ICOS-L blockade. A-B, Adult female BALB/c mice were exposed to either 25 µg house dust mite (HDM) or 25 µL phosphate-buffered saline (PBS), three times a week for up to 5 weeks. From the start of week 4, mice were also administered 150 µg anti-ICOS-L (α-ICOS-L) or isotype control (IgG) antibody (i.p) three times a week. Mice were culled at the end of week 5. Flow cytometry was used to determine the frequency of lung cellular populations. A, Representative gating of IL-13<sup>+</sup> CD4<sup>+</sup> T cells and IL-13<sup>+</sup> ILC2s following HDM and IgG or α-ICOS-L treatment. Data are quantified for all groups. B, Proportions of lung IL-13<sup>+</sup> T cells and ILCs. C, Numbers of lung IL-13<sup>+</sup> T cells and ILCs. Data are pooled from two independent experiments, n = 8 for PBS-treated groups, n = 12 for HDM-treated groups. D, Representative flow cytometry of Foxp3<sup>+</sup> CD4<sup>+</sup> cells in allergen-treated mice given IgG or α-ICOS-L. Pre-gated on CD4<sup>+</sup>CD3<sup>+</sup> lymphocytes. The number of Foxp3<sup>+</sup> CD4<sup>+</sup> T cells is quantified for all groups. E-F, Adult female IL-13<sup>GFP</sup> reporter mice were exposed (i.n) to 25 µg HDM or 25 µL PBS, three times a week for 3 weeks. Flow cytometry was used to determine the frequency of IL-13<sup>GFP</sup> cells. E, Representative flow plots of CXCR5<sup>+</sup> T cells and IL-13-GFP<sup>+</sup> cells in PBS and HDM-treated animals, pre-gated on CD4<sup>+</sup>CD3<sup>+</sup>CD44<sup>hi</sup>CD62L<sup>-</sup> lymphocytes. F, Quantification of mLN and lung IL-13<sup>GFP</sup> T<sub>FH</sub> (CXCR5<sup>+</sup>PD1<sup>+</sup>CD4<sup>+</sup>CD44<sup>hi</sup>CD62L<sup>-</sup>) and T<sub>EFF</sub> cells (CXCR5<sup>-</sup>CD4<sup>+</sup>CD44<sup>hi</sup>CD62L<sup>-</sup>). Statistical significance was determined using a Mann-Whitney U test. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. Data are pooled from two independent experiments, n = 6 for PBS-treated groups, n = 12 for HDM-treated groups

Regulatory T cells (Tregs) are anti-inflammatory cells capable of suppressing AAD,<sup>23</sup> also known to express ICOS. Foxp3<sup>+</sup> Tregs in the mLNs were unaffected by α-ICOS-L intervention both by proportion and by total number compared to HDM- and IgG-treated controls (Figure 6D). Lung Tregs, however, were reduced both in proportion and total number (Figure 6D). Similar results were observed in ALT-driven AAD (Figure S12). This suggests α-ICOS-L may limit lung Foxp3<sup>+</sup> CD4<sup>+</sup> T-cell responses but despite this, the treatment still dampens hallmark features of chronic AAD.

T<sub>FH</sub> have been shown to accumulate and become dysregulated during sustained antigen exposure,<sup>24</sup> and to be capable of differentiating into Th2 cells after adoptive transfer.<sup>13</sup> Here, T<sub>FH</sub> were reduced together with both secreted IL-13 and IL-13<sup>+</sup> CD4<sup>+</sup> T cells, therefore the capacity of T<sub>FH</sub> to produce IL-13 was examined. Using IL-13<sup>GFP</sup> reporter mice, CXCR5 expression was found to be separated from IL-13<sup>GFP</sup> expression in CD4<sup>+</sup> T cells within the mLN and lungs after HDM treatment (Figure 6E and F). IL-13<sup>GFP</sup> cells were, however, readily identified within the non-T<sub>FH</sub> T effector (T<sub>EFF</sub>) population (Figure 6E and F). This suggests that T<sub>FH</sub> are not directly responsible for the increased IL-13 observed during aeroallergen-driven AAD or the reduction in IL-13 observed after α-ICOS-L intervention. Overall α-ICOS-L treatment was beneficial as a therapeutic during established AAD, targeting both pathogenic humoral immunity intervention and other key features of AAD.

## 4 | DISCUSSION

Allergic airway disease is characterized by Th2-biased lung inflammation and dysregulated humoral immunity in response to recurrent exposure to environmental aero-allergens.<sup>1</sup> Here, we examine the importance of T<sub>FH</sub> in AAD pathology and antibody-mediated immunity. Chronic allergen exposure results in the development of AAD features, including eosinophilia and IgE. In this context, T<sub>FH</sub> develop over time, both systemically and locally, and are associated with the presence of GC B cells. Therapeutic targeting of ICOS-L interrupts the T<sub>FH</sub> response, decreases humoral immunity and improves hallmark features of AAD.

T<sub>FH</sub> are initially generated in the peripheral lymph nodes, but with prolonged allergen encounter become detectable locally within the lungs. After acute infection or vaccination, T<sub>FH</sub> peak between 7 and 14 days declining as antigen availability decreases.<sup>25,26</sup> In contrast, T<sub>FH</sub> accumulate over time with repeated allergen exposure, more consistent with other chronic disease models, such as chronic LCMV infection.<sup>27,28</sup> This aligns with sustained antigen, and therefore continuous TCR stimulation, favouring T<sub>FH</sub> differentiation and resulting in more T<sub>FH</sub> in the chronic setting than acute.<sup>24,27,28</sup> Thereby, highlighting the importance of examining T<sub>FH</sub> in the context of sustained allergen to replicate the allergic asthmatic experience. Chronic allergen exposure also establishes a local lung T<sub>FH</sub> population as previously reported by others.<sup>29</sup> In this study, local T<sub>FH</sub> are accompanied by lung GC B cells indicative of local lymphoid structures. Thus mimicking the isolated lymphoid cell clusters found at increased frequencies in the lungs of asthmatics compared to healthy controls which have been implicated in pathology.<sup>30</sup>

T<sub>FH</sub> require continuous ICOS/ICOS-L signalling after priming to maintain their phenotype.<sup>10</sup> Therefore, common variable immunodeficiency patients with ICOS deficiency fail to generate T<sub>FH</sub>,<sup>31</sup> while ICOS overexpression and thus T<sub>FH</sub> accumulation occurs in *Roquin* mutant mice, which are unable to degrade ICOS.<sup>32</sup> Inhibiting ICOS/ICOS-L interactions after initial T-cell activation has been widely used in several acute models to specifically deplete T<sub>FH</sub> when antigen is limiting.<sup>5,10,24</sup> Here, we show that late therapeutic administration of α-ICOS-L during ongoing chronic inflammation, when antigen is readily available, can also sufficiently reduce both T<sub>FH</sub> resident in SLOs and tertiary lymphoid tissue.

Consistent with T<sub>FH</sub> reductions after ICOS-L blockade GC B cells in the lungs and SLOs and lung plasmablasts are also reduced. Even after this relatively short intervention HDM-specific IgE, but not IgG1, also decline. IgE's half-life is short in comparison to other immunoglobulin isotypes<sup>33</sup>; therefore, after serum transfer Der p1- and Lol p 1-specific IgE decline rapidly over a 50-day period while IgG remains stable.<sup>34</sup> Consequently, a more prolonged α-ICOS-L protocol may be required to sufficiently alter IgG1.<sup>21</sup> Nonetheless serum MCPT1, an important indicator of mast cell activation,<sup>35</sup> also decreases suggesting the observed IgE reduction to adequately

affect the inflammatory response. As  $T_{FH}$  are required for antibody generation including allergen-specific IgE,<sup>14,15</sup> and we observe reduced allergen-specific IgE even after a short intervention, this highlights the potential of transiently targeting  $T_{FH}$  to abrogate IgE-mediated clinical features not only in AAD but also in other IgE-mediated diseases.

Along with its effects on antibody-mediated immunity targeting the ICOS/ICOS-L pathway after disease establishment improves allergen-driven AHR and pulmonary inflammation. IL-13 potently induces AHR<sup>36,37</sup> and in consort HDM-induced pulmonary IL-13 is reduced after  $\alpha$ -ICOS-L treatment. Previous studies show  $T_{FH}$  to be the only  $CD4^+$  T-cell subset affected by late  $\alpha$ -ICOS-L intervention.<sup>5,10</sup> During AAD,  $T_{FH}$  depletion does not appear to be directly responsible for IL-13 reductions as AAD-induced  $T_{FH}$  do not acquire a " $T_{FH2}$ " phenotype and produce IL-13.<sup>38</sup> Instead, the predominant IL-13 source in the lungs is ILC2s and Th2 cells. ILC2s express ICOS and are dependent on ICOS/ICOS-L for their homeostatic survival and AAD initiating functions<sup>39</sup>; however, we find  $\alpha$ -ICOS-L treatment after AAD initiation to not significantly affect IL-13<sup>+</sup> ILCs, suggesting ILC2s to not require continuous ICOS signalling to mediate their function. Th2 cells decrease in number after ICOS-L blockade, along with the overall fall in gross cellular inflammation, but not by proportion, suggesting a limited role for ICOS signalling in maintaining Th2 cell differentiation. Similarly,  $\alpha$ -ICOS-L treatment slightly affects lung Treg proportions, and significantly reduces numbers after AAD. Despite the role of Tregs in suppressing AAD,<sup>23</sup>  $\alpha$ -ICOS-L treatment reduces overall disease severity, suggesting the effect on Tregs to occur as a result of reduced inflammation.

This suggests ICOS signalling after initial priming is required to maintain  $T_{FH}$ , but not ILC2 nor Th2 cells. Furthermore, this implies the dominant effect of ICOS-L blockade in AAD, beyond reducing GC activity and antibody-mediated immunity, is to dampen inflammatory immune responses. Therapeutic blockade of ICOS-L specifically reduce GC formation in the lung, and this in itself may be sufficient to limit lung inflammation. However, it remains likely that  $\alpha$ -ICOS-L alters the activity of other immune cells in vivo to reduce inflammation, although not sufficiently for us to measure an effect ex vivo in this study. Critically, irrespective of mechanism,  $\alpha$ -ICOS-L administration shows significant therapeutic potential.

Previous work blocking ICOS or ICOS-L has been done prophylactically, during disease inception<sup>40-42</sup> or prior to an exacerbation<sup>40</sup> using less clinically relevant ovalbumin-induced AAD models,<sup>40-42</sup> which are difficult to deliver clinically. Here, using a more realistic ongoing exposure model and clinically relevant aeroallergens, we show  $\alpha$ -ICOS-L to reduce disease at the height of pathology. Additionally, we show for the first time in AAD that the prevailing outcome of therapeutic ICOS blockade is not only depletion of  $T_{FH}$  and their associated GCs, but also improvements in multiple disease facets. This approach could, therefore, be complementary or advantageous to currently approved biological therapies, such as omalizumab (anti-IgE mAb), reslizumab (anti-IL-5

mAb) and mepolizumab (IL-5 antagonist), which generally favour one arm of the allergic response and thus are only effective in specific asthma endotypes.<sup>43</sup> Furthermore, blocking ICOS signalling has been proven to be safe and effective in phase I clinical trials for systemic lupus erythematosus.<sup>44</sup>

Overall, we show  $T_{FH}$  arise progressively during aero-allergen exposure, first in the lymph nodes and with prolonged exposure in the lungs themselves. Even during chronic allergen exposure,  $T_{FH}$  are reliant on ICOS-L signalling for their maintenance and this pathway can be targeted to therapeutically deplete  $T_{FH}$  and their downstream effects during chronic disease. This approach reduces inflammation and AHR and implicates  $T_{FH}$  as a pathogenic cell type during sustained allergen exposure.

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## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

## AUTHOR CONTRIBUTIONS

F.I.U, J.A.H and C.M.L designed experiments. F.I.U, J.A.H, C.J.P and S.A.W carried out the experimental work. F.I.U analysed the experimental work. F.I.U wrote the manuscript. J.A.H and C.M.L provided feedback.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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| Target        | Clone        | Supplier      | Concentration | Fluorochrome |
|---------------|--------------|---------------|---------------|--------------|
| B220          | RA3-6B2      | Biolegend     | 1:200         | BV510        |
| CD3e          | 145-2C11     | eBioscience   | 1:200         | PE-CF594     |
| CD4           | RM4-5        | Biolegend     | 1:100         | APC/CY7      |
| CD8           | 53-6.7       | Biolegend     | 1:200         | BV711        |
| CD11b         | M1/70        | Biolegend     | 1:200         | BV711        |
| CD11b         | M1/70        | eBioscience   | 1:100         | PERCP-Cy5.5  |
| CD19          | 6D5          | Biolegend     | 1:200         | BV605        |
| CD19          | 6D5          | eBioscience   | 1:100         | PERCP-Cy5.5  |
| CD38          | 90           | eBioscience   | 1:200         | Alexa700     |
| CD44          | IM7          | Biolegend     | 1:200         | PERCP-Cy5.5  |
| CD45          | 30-F11       | Biolegend     | 1:200         | FITC         |
| CD62L         | MEL-14       | Biolegend     | 1:200         | BV605        |
| CD90-2        | 30-H12       | Biolegend     | 1:100         | Alexa700     |
| CXCR5         | L138D7       | Biolegend     | 1:50          | BV421        |
| FAS           | 15A7         | eBioscience   | 1:100         | PERCP-Cy5.5  |
| Foxp3         | 11-5773-82   | eBioscience   | 1:100         | FITC         |
| GL7           | GL7          | Biolegend     | 1:100         | FITC         |
| GR1           | RB6-8C5      | eBioscience   | 1:100         | PERCP-Cy5.5  |
| Ia/Ie         | M5/114.15.2  | Biolegend     | 1:300         | BV421        |
| IFN- $\gamma$ | XMG1.2       | Biolegend     | 1:50          | BV510        |
| IgD           | 11-26c.2a    | Biolegend     | 1:100         | Pacific Blue |
| IgM           | RMM-1        | Biolegend     | 1:50          | APC/CY7      |
| IL-13         | eBio13A      | eBioscience   | 1:100         | PE/CY7       |
| IL-17A        | TC11-18H10.1 | Biolegend     | 1:100         | BV605        |
| Nkp46         | 29A1.4       | Biolegend     | 1:50          | BV421        |
| PD1           | RMP1-30      | Biolegend     | 1:200         | PE/CY7       |
| Siglec f      | E50-2440     | BD Pharmingen | 1:100         | PE           |
| TCR- $\beta$  | H57-597      | Biolegend     | 1:100         | PERCP-Cy5.5  |
| TER119        | TER119       | eBioscience   | 1:100         | PERCP-Cy5.5  |

**Table S1: Flow cytometry antibodies used in the study**

Lineage negative (Lin<sup>-</sup>) cells were defined as: CD11b<sup>-</sup>CD19<sup>-</sup>TCR- $\beta$ <sup>-</sup>TER119<sup>-</sup>GR1<sup>-</sup>CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>

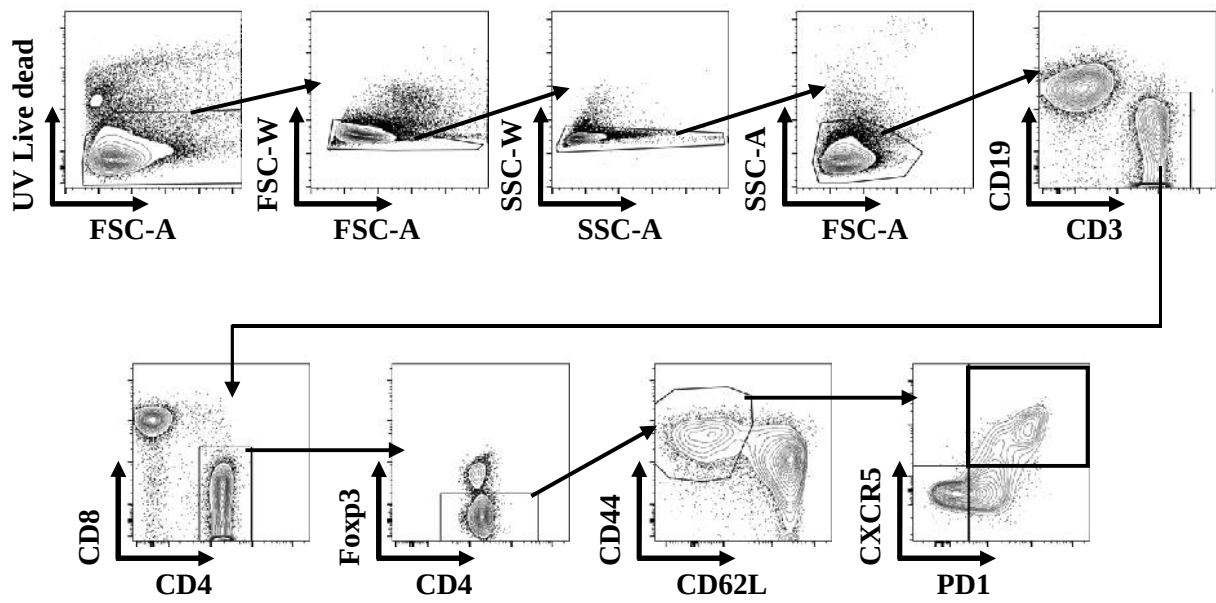


**Figure S1: T follicular helper cell gating strategy**

T follicular helper cells (T<sub>FH</sub>) were identified by flow cytometry and defined as CXCR5<sup>+</sup>PD1<sup>+</sup>Foxp3<sup>-</sup>CD44<sup>hi</sup>D62L<sup>-</sup>CD4<sup>+</sup>CD8<sup>-</sup>CD3<sup>+</sup> lymphocytes.

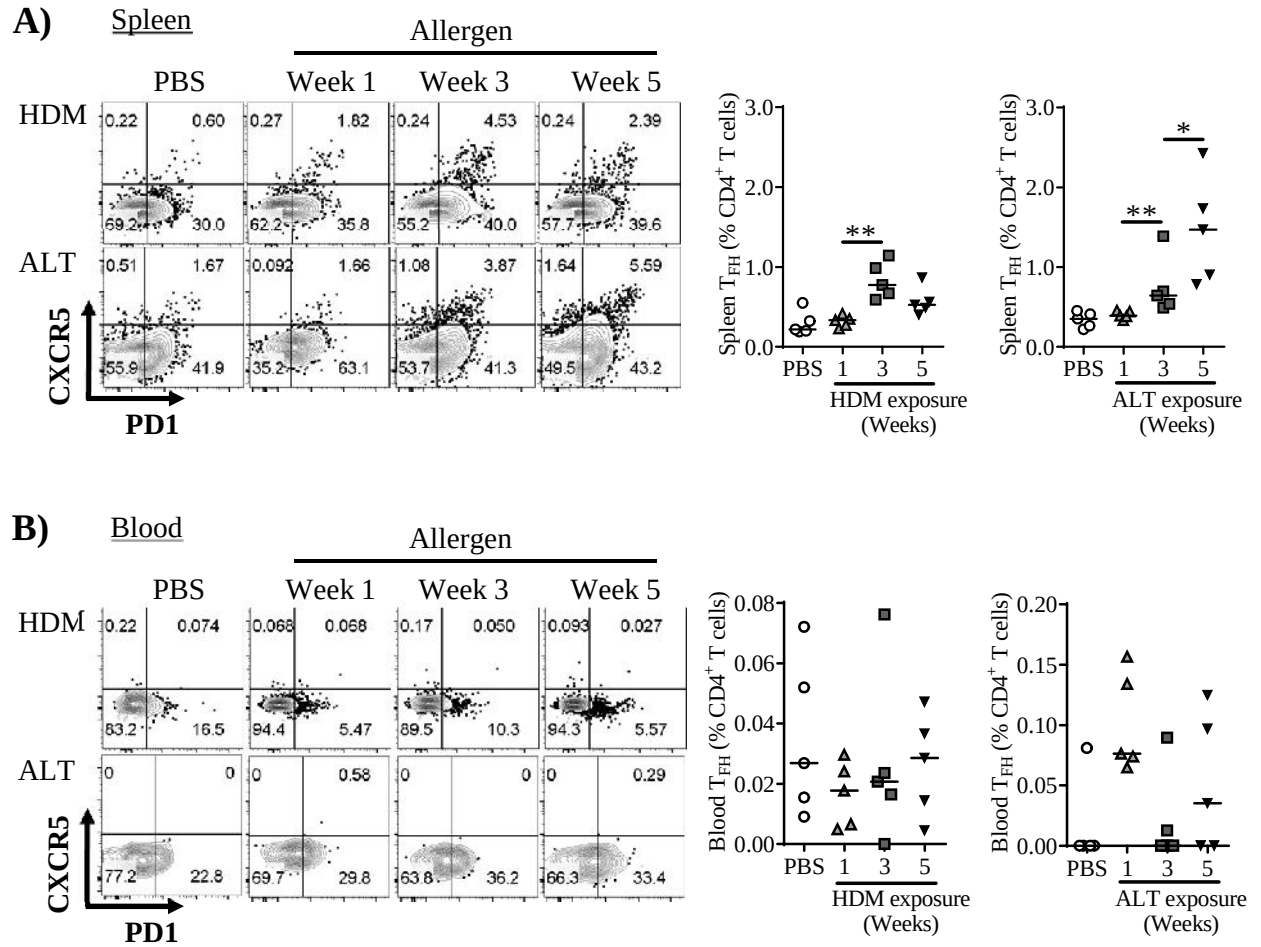


**Figure S2:  $T_{FH}$  are induced in the spleen but not in the circulation following allergen exposure.** Adult female BALB/c mice were exposed to either 25  $\mu$ g house dust mite (HDM), 10  $\mu$ g *alternaria alternata* (ALT) or 25  $\mu$ l phosphate buffered saline (PBS), 3 times a week for up to 5 weeks. Flow cytometry was used to determine the frequency of  $T_{FH}$  within cellular compartments following allergen exposure.  $T_{FH}$  were defined as CXCR5<sup>+</sup>PD1<sup>+</sup>Foxp3<sup>+</sup>CD4<sup>+</sup>. Representative flow plots of  $T_{FH}$  in PBS, ALT or HDM treated animals are displayed, pre-gated on CD4<sup>+</sup>CD3<sup>+</sup>Foxp3<sup>+</sup>CD44<sup>hi</sup>CD62L<sup>-</sup> lymphocytes. Data is quantified. **A) Spleen, B) Blood.** Statistical significance was determined using a Mann Whitney U test. \*  $P < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ,  $n = 5$  per time-point. Representative data from 2 independent experiments.

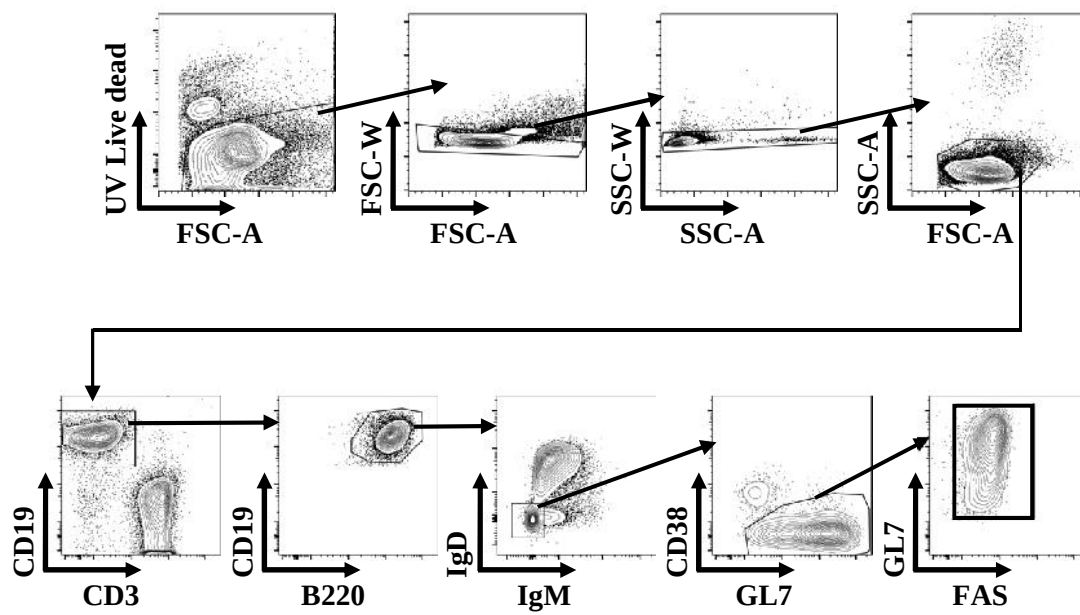


**Figure S3: Germinal centre B cell gating strategy**

Germinal centre B cells were identified by flow cytometry and defined as CD19<sup>+</sup>B220<sup>+</sup>IgD<sup>-</sup>IgM<sup>-</sup>CD38<sup>-</sup>GL7<sup>+</sup>FAS<sup>+</sup> lymphocytes.



**A)**

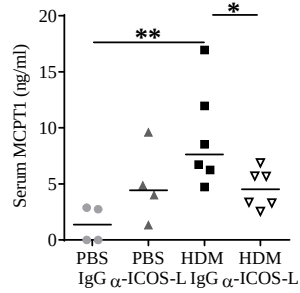


**Figure S5: ICOS/ICOS-L interactions reduces serum MCPT1.**

Adult female BALB/c mice were exposed (i.n) to 25 µg house dust mite (HDM) or 25 µl phosphate buffered saline (PBS), 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 µg anti-ICOS-L (α-ICOS-L) or isotype control (IgG) antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A)** Serum MCPT1 was measured by ELISA. Statistical significance was determined using a Mann Whitney U test. \*  $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Data is from 1 study,  $n = 4$  for PBS treated groups,  $n = 6$  for HDM treated groups.

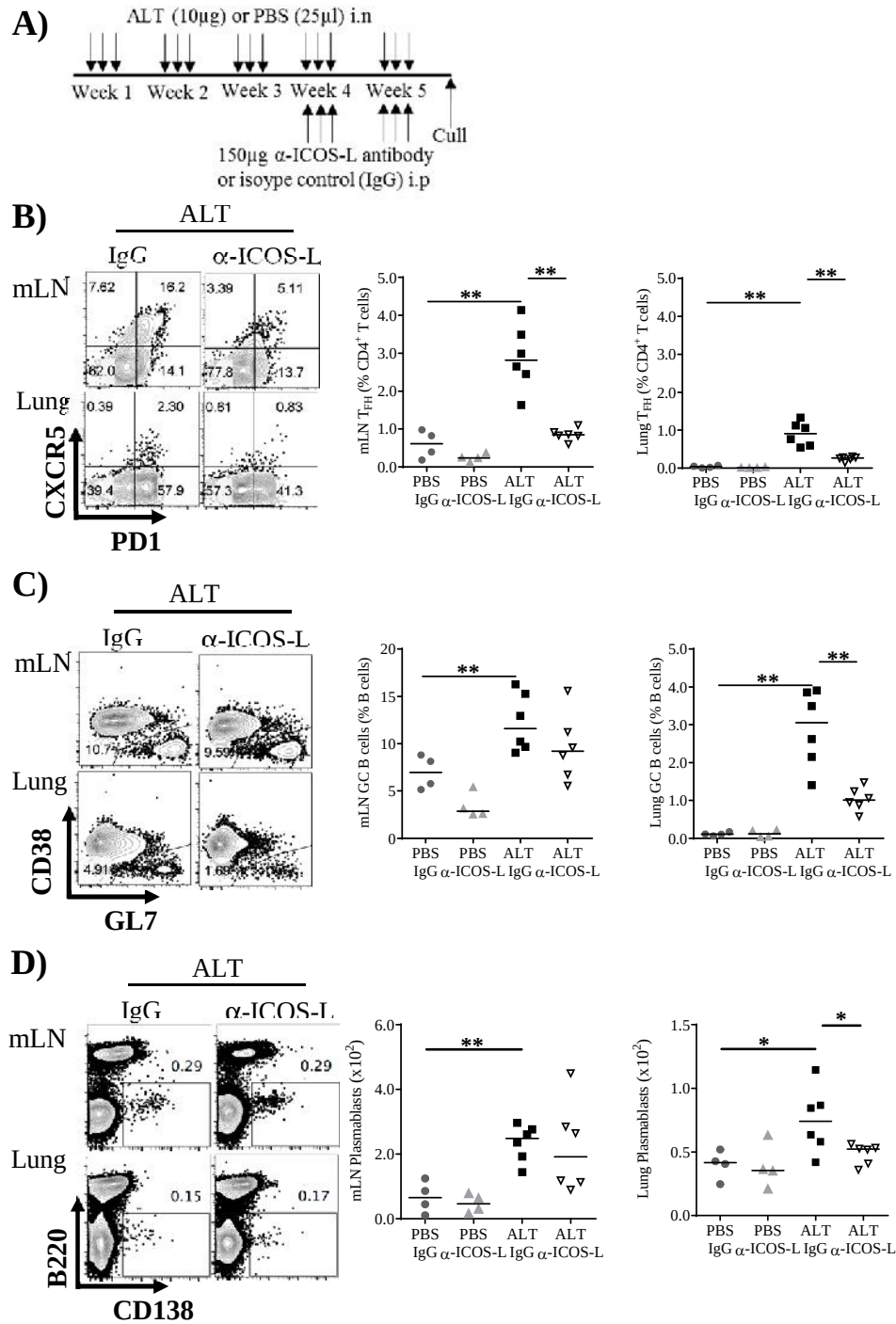
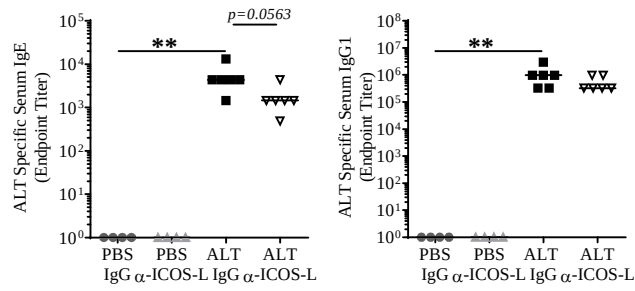
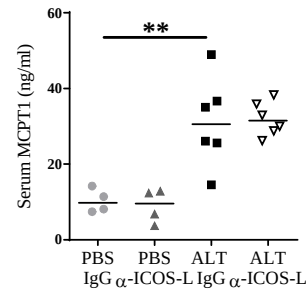


**Figure S6: ICOS signalling is required to sustain T<sub>FH</sub> during ALT driven chronic AAD.**

Adult female BALB/c mice were exposed (i.n) to 10 µg alternaria alternata (ALT) or 25 µl phosphate buffered saline (PBS), 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 µg anti-ICOS-L ( $\alpha$ -ICOS-L) or isotype control (IgG) antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A)** Schematic of experimental design, **B)** Representative flow plots of mLN and lung T<sub>FH</sub> following ALT and IgG or  $\alpha$ -ICOS-L treatment. The data is quantified for all groups. **C)** Representative flow plots of mLN and lung germinal centre (GC) B cells following ALT and IgG or  $\alpha$ -ICOS-L treatment. Pre-gated on CD19<sup>+</sup>B220<sup>+</sup> B cells. **D)** Representative flow plots of mLN and lung plasmablasts following ALT and IgG or  $\alpha$ -ICOS-L treatment. Pre-gated on lymphocytes. Plasmablast numbers are quantified for all groups. Statistical significance was determined using a Mann Whitney U test. \*  $P < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . Experiment was performed once. n=4 for PBS treated groups, n=6 for ALT treated groups.

**A)****B)**

### Figure S7: ICOS-L blockade reduces allergen specific IgE.

Adult female BALB/c mice were exposed (i.n) to 10 µg alternaria alternata (ALT) or 25 µl phosphate buffered saline (PBS), 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 µg anti-ICOS-L (α-ICOS-L) or isotype control (IgG) antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A)** Serum was titrated and allergen specific antibody was measured by ELISA. Endpoint titres are displayed for IgE and IgG1, **B)** Serum MCPT1 was determined by ELISA. Statistical significance was determined using a Mann Whitney U test. \*  $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Experiment was performed once. n=4 for PBS treated groups, n=6 for ALT treated groups.

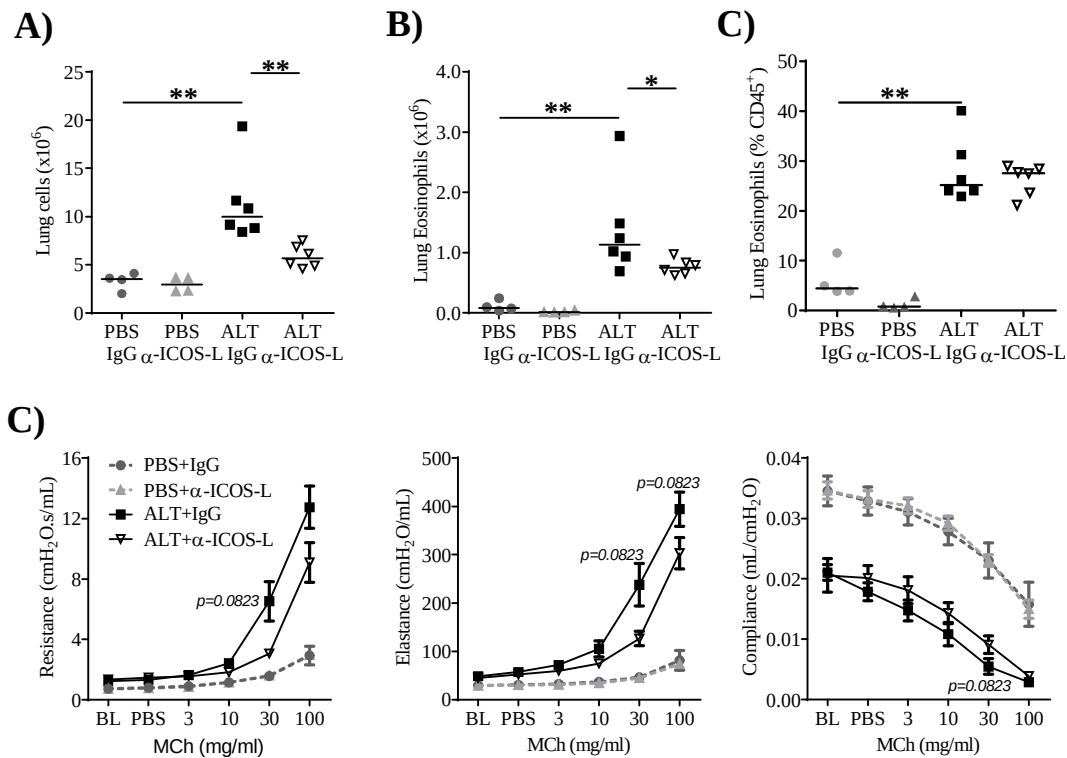


**Figure S8: Therapeutic ICOS-L blockade improves chronic allergic airway disease.**

Adult female BALB/c mice were exposed (i.n) to 10  $\mu$ g alternaria alternata (ALT) or 25  $\mu$ l phosphate buffered saline (PBS), 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150  $\mu$ g anti-ICOS-L ( $\alpha$ -ICOS-L) or isotype control (IgG) antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A)** Number of lung cells, **B)** Lung eosinophil numbers, **C)** Proportions of lung eosinophils, **D)** Airway hyperresponsiveness was measured by exposing mice to 0-100mg/ml methacholine (MCh) using the flexiVent system during ALT induced allergic airway disease. Airway resistance, elastance and compliance were measured. Curves display mean $\pm$ SEM. Statistical significance between ALT+ IgG and ALT+ $\alpha$ -ICOS-L groups was determined using a Mann Whitney U test. \*  $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . . Experiment was performed once. n=4 for PBS treated groups, n=6 for ALT treated groups.

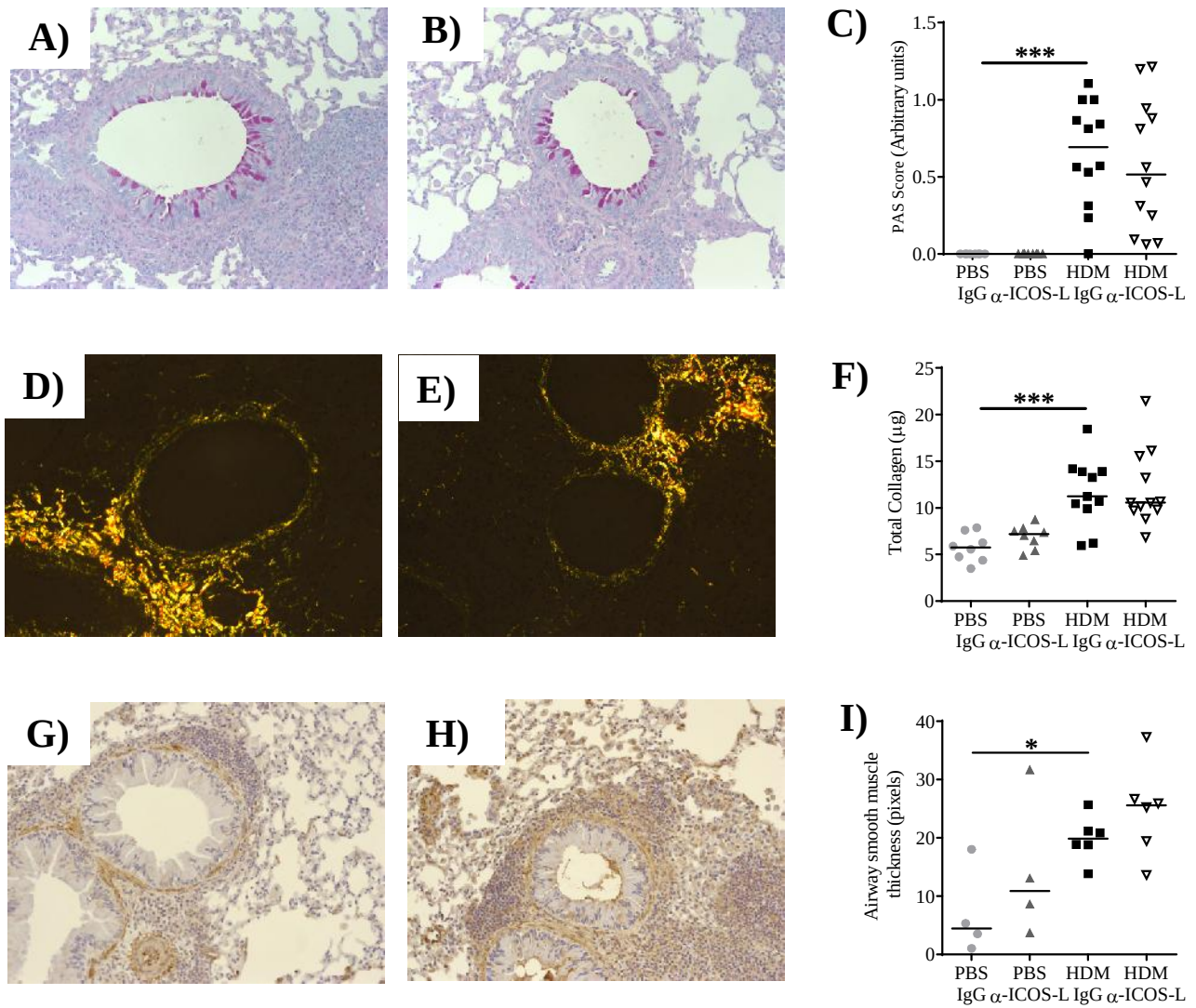


**Figure S9: Therapeutic ICOS-L blockade does not alter mucus hyper-secretion, collagen deposition or airway smooth muscle thickening.**

Adult female BALB/c mice were exposed (i.n) to 25  $\mu$ g house dust mite (HDM), 10  $\mu$ g *alternaria alternata* (ALT) or 25  $\mu$ l phosphate buffered saline (PBS), 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150  $\mu$ g anti-ICOS-L ( $\alpha$ -ICOS-L) or isotype control (IgG) antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A-B)** Representative images of Periodic Acid-Schiff (PAS) staining on paraffin embedded formalin fixed (PEFF) lung sections used to score mucus hyper-secretion. **A)** HDM+ IgG, **B)** HDM+ $\alpha$ -ICOS-L, **C)** Quantification of PAS scoring. **D-E)** Representative staining of Pico-Sirus Red on PEFF lung used to determine collagen I and III deposition. **D)** HDM+ IgG, **E)** HDM+ $\alpha$ -ICOS-L, **F)** Total collagen in the lungs determined by Sircol soluble collagen assay. **G-H)** Representative staining of airway smooth muscle cells and myofibroblasts identified by immunohistochemical staining of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). **G)** HDM+ IgG, **H)** HDM+ $\alpha$ -ICOS-L, **I)** Airway smooth muscle layer thickness was measured from  $\alpha$ -SMA stained sections. Images were taken at x20 magnification. Statistical significance was determined using a Mann Whitney U test. Data is pooled from two independent experiments, n=8 for PBS treated groups, n=12

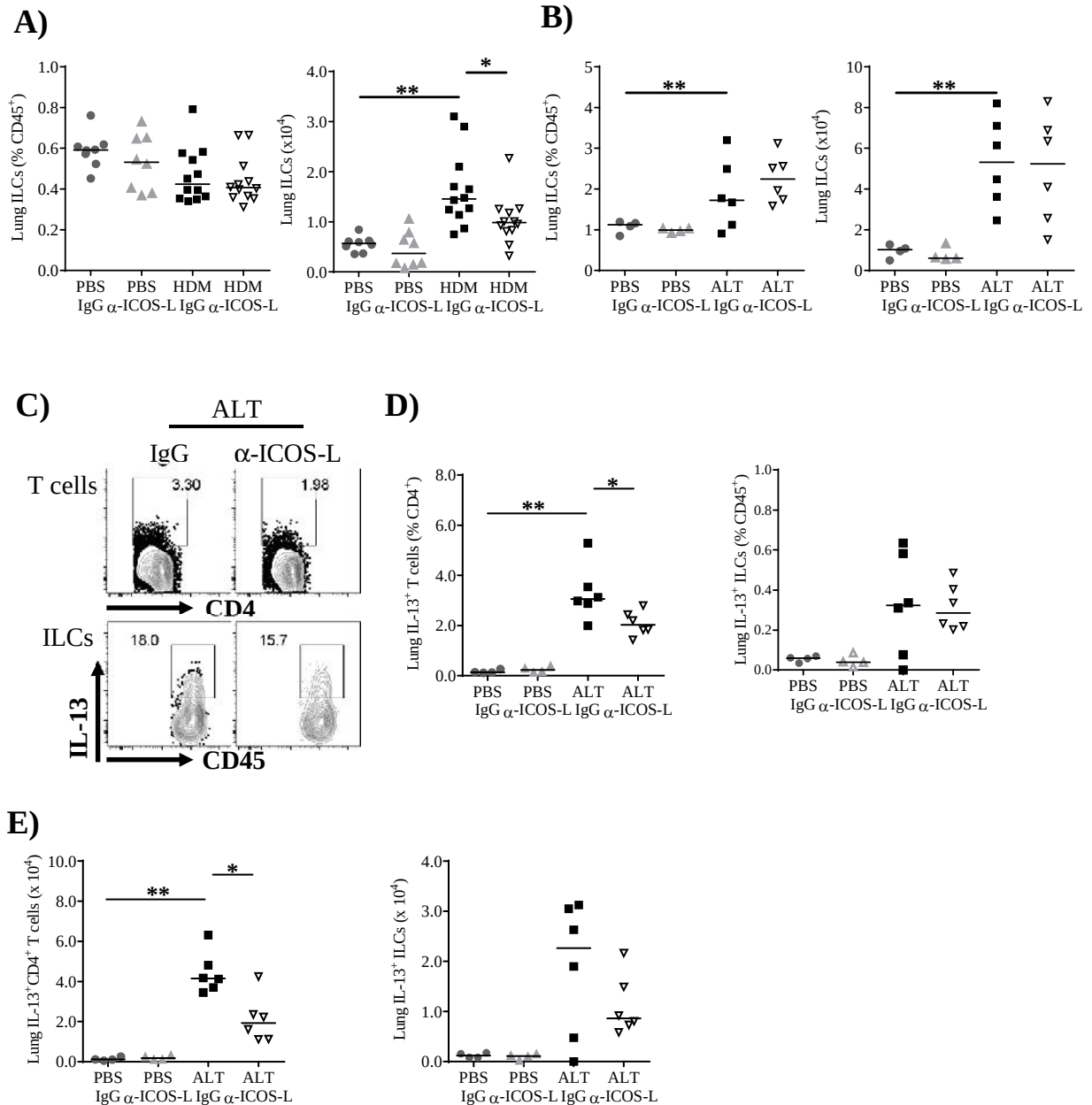


**Figure S10: IL-13<sup>+</sup> CD4<sup>+</sup> T cells are reduced by ICOS-L blockade in alternaria alternate driven allergic airway disease.**

Adult female BALB/c mice were exposed to either 25 µg House dust mite (HDM), 10 µg *alternaria alternata* (ALT) or 25 µl phosphate buffered saline (PBS), 3 times a week for up to 5 weeks. From the start of week 4 mice were also administered 150 µg anti-ICOS-L (α-ICOS-L) or isotype control (IgG) antibody (i.p) 3 times a week. Mice were culled at the end of week 5. Flow cytometry was used to determine the frequency of lung cellular populations. **A)** HDM study data – Total lung ILCs as a proportion of CD45<sup>+</sup> cells. **B-E)** Alternaria study, **B)** Total lung ILC proportions, **C)** Total number of ILCs, **D)** Representative gating of IL-13<sup>+</sup> CD4<sup>+</sup> T cells and IL-13<sup>+</sup> ILCs following HDM and IgG or α-ICOS-L treatment. Data is quantified for all groups. **E)** Proportions of lung IL-13<sup>+</sup> T cells and ILCs. **E)** Numbers of lung IL-13<sup>+</sup> T cells and ILCs. Experiment was performed once. n=4 for PBS treated groups, n=6 for ALT treated groups.

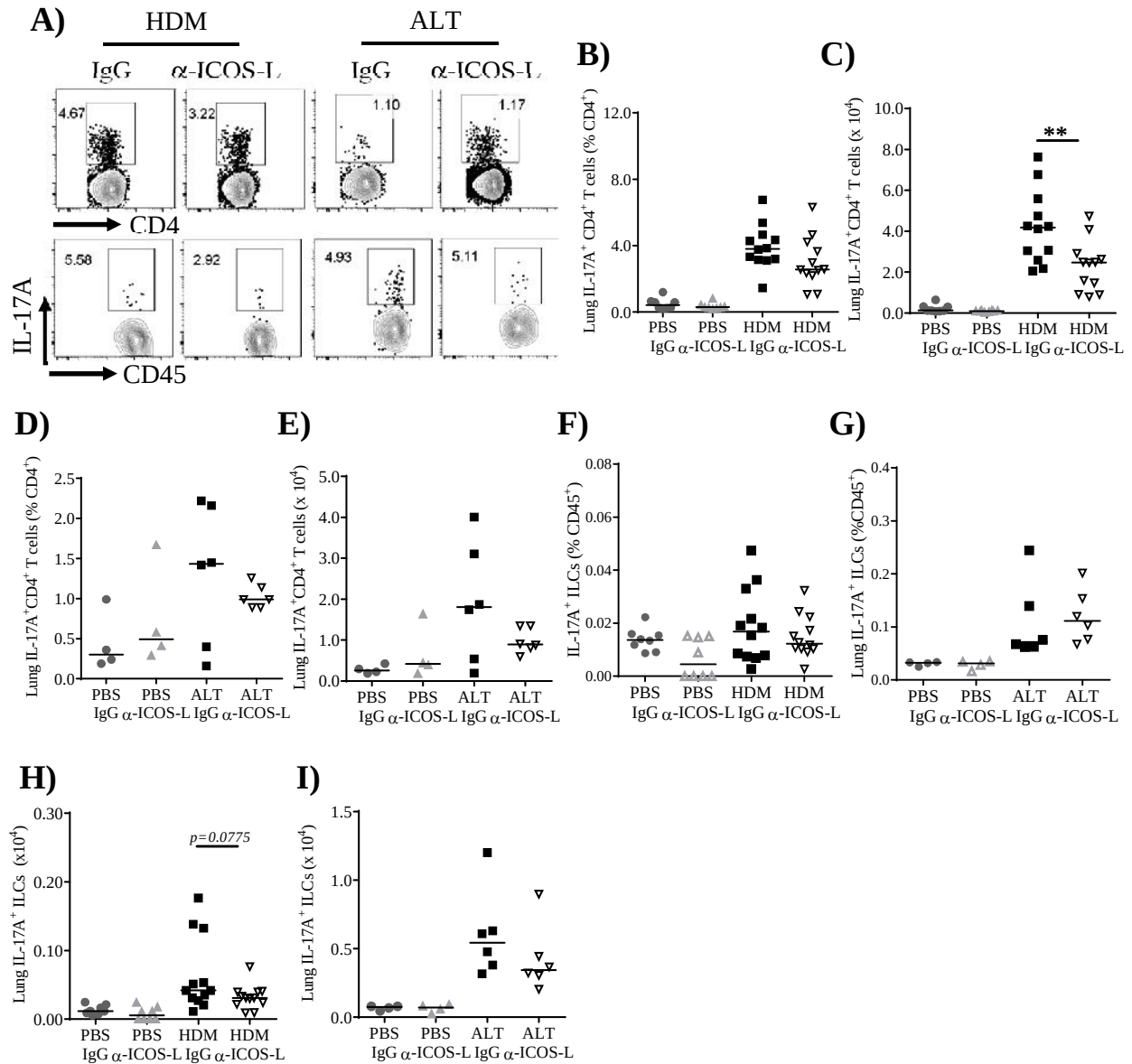


**Figure S11: IL-17A<sup>+</sup> CD4<sup>+</sup> T cells are not directly targeted by ICOS-L blockade**

Adult female BALB/c mice were exposed to either 25  $\mu$ g house dust mite (HDM), 10  $\mu$ g *alternaria alternata* (ALT) or 25  $\mu$ l phosphate buffered saline (PBS), 3 times a week for up to 5 weeks. Flow cytometry was used to determine the frequency of lung cellular populations. **A)** Representative gating of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells and IL-17A<sup>+</sup> ILCs following allergen and IgG or  $\alpha$ -ICOS-L treatment. These populations were quantified **B)** Proportions of lung IL-17A<sup>+</sup> CD4<sup>+</sup> - HDM study, **C)** Number of lung IL-17A<sup>+</sup> CD4<sup>+</sup> - HDM study, **D)** Proportions of lung IL-17A<sup>+</sup> CD4<sup>+</sup> - ALT study, **E)** Number of lung IL-17A<sup>+</sup> CD4<sup>+</sup> - ALT study, **F)** Proportions of lung IL-17A<sup>+</sup> ILCs - HDM study, **G)** Proportions of lung IL-17A<sup>+</sup> ILCs - ALT study, **H)** Number of lung IL-17A<sup>+</sup> ILCs - HDM study, **I)** Number of lung IL-17A<sup>+</sup> ILCs - ALT study. Statistical significance was determined using a Mann Whitney U test. \*  $P < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . HDM data is pooled from two independent experiments, n=8 for PBS treated groups, n=12 for HDM treated groups. ALT experiment was performed once, n=4 for PBS treated groups, n=6 for ALT treated groups.

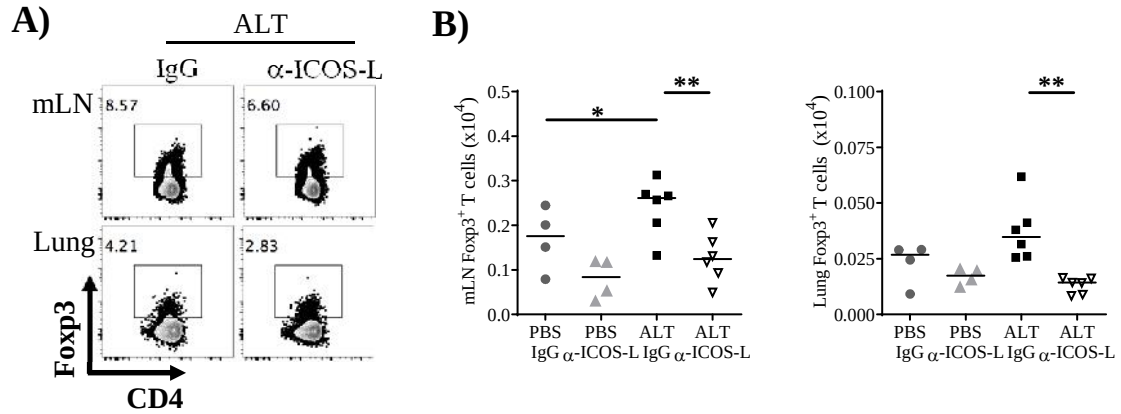


**Figure S12: Therapeutic ICOS-L blockade reduces T regulatory cells during Alternaria alternate driven allergic disease**

Adult female BALB/c mice were exposed (i.n) to 10 µg Alternaria Alternata (ALT) or 25 µl phosphate buffered saline (PBS), 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 µg anti-ICOS-L (α-ICOS-L) or isotype control (IgG) antibody (i.p) 3 times a week. Mice were culled at the end of week 5. Flow cytometry was used to determine the number of Fop3<sup>+</sup>CD4<sup>+</sup> T cells within the mLN and lungs. **A)** Representative flow cytometry of Fop3<sup>+</sup> cells in allergen treated mice given IgG or α-ICOS-L. Pre-gated on CD4<sup>+</sup>CD3<sup>+</sup> T cells. **B)** Numbers of mLN and lung Fop3<sup>+</sup>CD4<sup>+</sup> T cells were quantified. Statistical significance was determined using a Mann Whitney U test. \*  $P < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . ALT experiment was performed once, n=4 for PBS treated groups, n=6 for ALT treated groups.

## **SUPPLEMENTARY METHODS**

### ***Tissue Processing:***

Lung cells were disaggregated by incubating chopped tissue at 37 °C for 45 minutes in complete media (RPMI with 10% fetal calf serum, 2 mM L-glutamine and 100U/ml Penicillin/Streptomycin) containing 0.15 mg/ml collagenase type D (Roche Diagnostics, Burgess Hill, UK) and 25 µg/ml DNase type 1 (Roche Diagnostics). Splenic, mediastinal lymph node (mLN) and lung cells were recovered by filtering through a 100 µM nylon sieve and washing in complete media. Lung and splenic cells were then treated with red blood cell lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM Disodium EDTA) for 5 mins, washed and resuspended in complete media. Bronchoalveolar lavage (BAL) was collected by washing the airways three times with 0.4ml PBS via a tracheal cannula. BAL cells were pelleted and resuspended in 0.5ml complete media. Viable cells were counted by haemocytometer using trypan blue exclusion. Serum was acquired by collecting blood from a peripheral artery using Na-EDTA coated capillaries and centrifuging at 12,000 g for 15 mins.

### ***MCPT1 measurement:***

MCPT1 was measured using a Ready Set Go Kits (eBioscience, CA, USA) according to manufacturer' instructions.

### ***Histological assessment:***

Paraffin embedded formalin fixed lung lobes were sectioned and subjected to Periodic Acid-Schiff (PAS) and picro sirius red staining. Sections were scored and assessed as previously described<sup>1</sup>.

### ***Total Collagen:***

Total collagen was assessed in lung homogenate using the Sircol™ Soluble Collagen assay (Biocolor, Carrickfergus, UK) according to manufacturer's instructions.

***Airway smooth muscle assessment:***

Paraffin embedded formalin fixed lung lobes were sectioned and immunohistochemically stained with rabbit anti-mouse smooth muscle actin (Abcam, Cambridge, UK) as previously described<sup>2</sup>. Lung sections were imaged at x20 magnification and the size of the airway smooth muscle layer was measured. Briefly, the thickness of 10 airways were measured per mice and 10 measurements were taken per airway. The data is presented as an average. Data was blinded for analysis.

## SUPPLEMENTARY FIGURE LEGENDS

### Table S1: Flow cytometry antibodies used in the study

Lineage negative (Lin<sup>-</sup>) cells were defined as: CD11b<sup>-</sup>CD19<sup>-</sup>TCR-β<sup>-</sup>TER119<sup>-</sup>GR1<sup>-</sup>CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>

### Figure S1: T follicular helper cell gating strategy

T follicular helper cells (T<sub>FH</sub>) were identified by flow cytometry and defined as CXCR5<sup>+</sup>PD1<sup>+</sup>Foxp3<sup>-</sup>CD44<sup>hi</sup>D62L<sup>-</sup>CD4<sup>+</sup>CD8<sup>-</sup>CD3<sup>+</sup> lymphocytes.

### Figure S2: T<sub>FH</sub> are induced in the spleen but not in the circulation following allergen exposure.

Adult female BALB/c mice were exposed to either 25 μg house dust mite (HDM), 10 μg *alternaria alternata* (ALT) or 25 μl phosphate buffered saline (PBS), 3 times a week for up to 5 weeks. Flow cytometry was used to determine the frequency of T<sub>FH</sub> within cellular compartments following allergen exposure. T<sub>FH</sub> were defined as CXCR5<sup>+</sup>PD1<sup>+</sup>Foxp3<sup>-</sup>CD4<sup>+</sup>. Representative flow plots of T<sub>FH</sub> in PBS, ALT or HDM treated animals are displayed, pre-gated on CD4<sup>+</sup>CD3<sup>+</sup>Foxp3<sup>-</sup>CD44<sup>hi</sup>CD62L<sup>-</sup> lymphocytes. Data is quantified. **A)** Spleen, **B)** Blood. Statistical significance was determined using a Mann Whitney U test. \*  $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , n=5 per time-point. Representative data from 2 independent experiments.

### Figure S3: Germinal centre B cell gating strategy

Germinal centre B cells were identified by flow cytometry and defined as CD19<sup>+</sup>B220<sup>+</sup>IgD<sup>-</sup>IgM<sup>-</sup>CD38<sup>-</sup>GL7<sup>+</sup>FAS<sup>+</sup> lymphocytes.

**Figure S4: GC B cells accumulate over time following allergen exposure.**

Adult female BALB/c mice were exposed to either 25 µg house dust mite (HDM), 10 µg *alternaria alternata* (ALT) or 25 µl phosphate buffered saline (PBS), 3 times a week for up to 5 weeks. Flow cytometry was used to determine the frequency of germinal centre (GC) B cells within cellular compartments following allergen exposure. GC B cells were defined as CD38<sup>+</sup>GL7<sup>+</sup>FAS<sup>+</sup>CD19<sup>+</sup>B220<sup>+</sup> cells. Representative flow plots of GC B cells in PBS, ALT or HDM treated animals, pre-gated on CD19<sup>+</sup>B220<sup>+</sup> lymphocytes. **A)** Mediastinal lymph node (mLN), **B)** Lung, **C)** Spleen. Representative data from 2 independent experiments.

**Figure S5: ICOS/ICOS-L interactions reduces serum MCPT1.**

Adult female BALB/c mice were exposed (i.n) to 25 µg house dust mite (HDM) or 25 µl phosphate buffered saline (PBS), 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 µg anti-ICOS-L (α-ICOS-L) or isotype control (IgG) antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A)** Serum MCPT1 was measured by ELISA. Statistical significance was determined using a Mann Whitney U test. \*  $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Data is from 1 study, n=4 for PBS treated groups, n=6 for HDM treated groups.

**Figure S6: ICOS signalling is required to sustain T<sub>FH</sub> during ALT driven chronic AAD.**

Adult female BALB/c mice were exposed (i.n) to 10 µg *alternaria alternata* (ALT) or 25 µl phosphate buffered saline (PBS), 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 µg anti-ICOS-L (α-ICOS-L) or isotype control (IgG) antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A)** Schematic of experimental design, **B)** Representative flow plots of mLN and lung T<sub>FH</sub> following ALT and IgG or α-ICOS-

L treatment. The data is quantified for all groups. **C)** Representative flow plots of mLN and lung germinal centre (GC) B cells following ALT and IgG or  $\alpha$ -ICOS-L treatment. Pre-gated on CD19<sup>+</sup>B220<sup>+</sup> B cells. **D)** Representative flow plots of mLN and lung B220<sup>+</sup>CD138<sup>+</sup> plasmablasts following ALT and IgG or  $\alpha$ -ICOS-L treatment, pre-gated on lymphocytes. Plasmablast numbers are quantified for all groups. Statistical significance was determined using a Mann Whitney U test. \*  $P<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ . Experiment was performed once. n=4 for PBS treated groups, n=6 for ALT treated groups.

### **Figure S7: ICOS-L blockade reduces allergen specific IgE.**

Adult female BALB/c mice were exposed (i.n) to 10  $\mu$ g alternaria alternata (ALT) or 25  $\mu$ l phosphate buffered saline (PBS), 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150  $\mu$ g anti-ICOS-L ( $\alpha$ -ICOS-L) or isotype control (IgG) antibody (i.p) 3 times a week. Mice were culled at the end of week 5. A) Serum was titrated and allergen specific antibody was measured by ELISA. Endpoint titres are displayed for IgE and IgG1, B) Serum MCPT1 was determined by ELISA. Statistical significance was determined using a Mann Whitney U test. \*  $P<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ . Experiment was performed once. n=4 for PBS treated groups, n=6 for ALT treated groups.

### **Figure S8: Therapeutic ICOS-L blockade improves chronic allergic airway disease.**

Adult female BALB/c mice were exposed (i.n) to 10  $\mu$ g alternaria alternata (ALT) or 25  $\mu$ l phosphate buffered saline (PBS), 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150  $\mu$ g anti-ICOS-L ( $\alpha$ -ICOS-L) or isotype control (IgG) antibody (i.p) 3 times a week. Mice were culled at the end of week 5. A) Number of lung cells, B) Lung eosinophil numbers, C) Proportions of lung eosinophils, D) Airway hyperresponsiveness was measured by exposing mice to 0-100mg/ml methacholine (MCh) using the flexiVent system during ALT induced allergic airway disease. Airway resistance, elastance and compliance

were measured. Curves display mean $\pm$ SEM. Statistical significance between ALT+ IgG and ALT+ $\alpha$ -ICOS-L groups was determined using a Mann Whitney U test. \*  $P<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ . . Experiment was performed once. n=4 for PBS treated groups, n=6 for ALT treated groups.

**Figure S9: Therapeutic ICOS-L blockade does not alter mucus hyper-secretion, collagen deposition or airway smooth muscle thickening.**

Adult female BALB/c mice were exposed (i.n) to 25  $\mu$ g house dust mite (HDM), 10  $\mu$ g *alternaria alternata* (ALT) or 25  $\mu$ l phosphate buffered saline (PBS), 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150  $\mu$ g anti-ICOS-L ( $\alpha$ -ICOS-L) or isotype control (IgG) antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A-B)** Representative images of Periodic Acid-Schiff (PAS) staining on paraffin embedded formalin fixed (PEFF) lung sections used to score mucus hyper-secretion. **A)** HDM+ IgG, **B)** HDM+ $\alpha$ -ICOS-L, **C)** Quantification of PAS scoring. **D-E)** Representative staining of Picro-Sirius Red on PEFF lung used to determine collagen I and III deposition. **D)** HDM+ IgG, **E)** HDM+ $\alpha$ -ICOS-L, **F)** Total collagen in the lungs determined by Sircol soluble collagen assay. **G-H)** Representative staining of airway smooth muscle cells and myofibroblasts identified by immunohistochemical staining of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). **G)** HDM+ IgG, **H)** HDM+ $\alpha$ -ICOS-L, **I)** Airway smooth muscle layer thickness was measured from  $\alpha$ -SMA stained sections. Images were taken at x20 magnification. Statistical significance was determined using a Mann Whitney U test. Data is pooled from two independent experiments, n=8 for PBS treated groups, n=12

**Figure S10: IL-13<sup>+</sup> CD4<sup>+</sup> T cells are reduced by ICOS-L blockade in alternaria alternate driven allergic airway disease.**

Adult female BALB/c mice were exposed to either 25 µg House dust mite (HDM), 10 µg *alternaria alternata* (ALT) or 25 µl phosphate buffered saline (PBS), 3 times a week for up to 5 weeks. From the start of week 4 mice were also administered 150 µg anti-ICOS-L ( $\alpha$ -ICOS-L) or isotype control (IgG) antibody (i.p) 3 times a week. Mice were culled at the end of week 5. Flow cytometry was used to determine the frequency of lung cellular populations. **A-B)** Lung ILCs were defined as Lin<sup>-</sup>Nkp46<sup>-</sup>CD45<sup>+</sup>CD90.2<sup>+</sup> and the proportion and total number were calculated, **B)** HDM study, **C)** ALT study. **C)** Representative gating of IL-13<sup>+</sup> CD4<sup>+</sup> T cells and IL-13<sup>+</sup> ILC2s following HDM and IgG or  $\alpha$ -ICOS-L treatment. Data is quantified for all groups. **D)** Proportions of lung IL-13<sup>+</sup> T cells and ILCs. **E)** Numbers of lung IL-13<sup>+</sup> T cells and ILCs. Experiment was performed once. n=4 for PBS treated groups, n=6 for ALT treated groups.

#### **Figure S11: IL-17A<sup>+</sup> CD4<sup>+</sup> T cells are not directly targeted by ICOS-L blockade**

Adult female BALB/c mice were exposed to either 25 µg house dust mite (HDM), 10 µg *alternaria alternata* (ALT) or 25 µl phosphate buffered saline (PBS), 3 times a week for up to 5 weeks. Flow cytometry was used to determine the frequency of lung cellular populations. **A)** Representative gating of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells and IL-17A<sup>+</sup> ILCs following allergen and IgG or  $\alpha$ -ICOS-L treatment. These populations were quantified **B)** Proportions of lung IL-17A<sup>+</sup> CD4<sup>+</sup> - HDM study, **C)** Number of lung IL-17A<sup>+</sup> CD4<sup>+</sup> - HDM study, **D)** Proportions of lung IL-17A<sup>+</sup> CD4<sup>+</sup> - ALT study, **E)** Number of lung IL-17A<sup>+</sup> CD4<sup>+</sup> - ALT study, **F)** Proportions of lung IL-17A<sup>+</sup> ILCs - HDM study, **G)** Proportions of lung IL-17A<sup>+</sup> ILCs - ALT study, **H)** Number of lung IL-17A<sup>+</sup> ILCs - HDM study, **I)** Number of lung IL-17A<sup>+</sup> ILCs - ALT study, Statistical significance was determined using a Mann Whitney U test. \*  $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . HDM data is pooled from two independent experiments, n=8 for PBS treated

groups, n=12 for HDM treated groups. ALT experiment was performed once, n=4 for PBS treated groups, n=6 for ALT treated groups.

**Figure S12: Therapeutic ICOS-L blockade reduces T regulatory cells during Alternaria alternate driven allergic disease**

Adult female BALB/c mice were exposed (i.n) to 10 µg Alternaria Alternata (ALT) or 25 µl phosphate buffered saline (PBS), 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 µg anti-ICOS-L (α-ICOS-L) or isotype control (IgG) antibody (i.p) 3 times a week. Mice were culled at the end of week 5. Flow cytometry was used to determine the number of Foxp3<sup>+</sup>CD4<sup>+</sup> T cells within the mLN and lungs. **A)** Representative flow cytometry of Foxp3<sup>+</sup> cells in allergen treated mice given IgG or α-ICOS-L. Pre-gated on CD4<sup>+</sup>CD3<sup>+</sup> T cells. **B)** Numbers of mLN and lung Foxp3<sup>+</sup>CD4<sup>+</sup> T cells were quantified. Statistical significance was determined using a Mann Whitney U test. \*  $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . ALT experiment was performed once, n=4 for PBS treated groups, n=6 for ALT treated groups.

## REFERENCES

1. Saglani S, Mathie SA, Gregory LG, Bell MJ, Bush A, Lloyd CM. Pathophysiological Features of Asthma Develop in Parallel in House Dust Mite–Exposed Neonatal Mice. *American Journal of Respiratory Cell and Molecular Biology* 2009; **41**(3): 281-289.
2. Gregory LG, Mathie SA, Walker SA, Pegorier S, Jones CP, Lloyd CM. Overexpression of Smad2 drives house dust mite–mediated airway remodeling and airway hyperresponsiveness via activin and IL-25. *American journal of respiratory and critical care medicine* 2010; **182**(2): 143-154.