Title

Functional differences in airway dendritic cells determine susceptibility to IgE-sensitisation

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Inhaled allergen uptake and IgE-sensitisation

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ABSTRACT

Respiratory IgE-sensitisation to innocuous antigens increases the risk for developing diseases such as allergic asthma. Dendritic cells (DC) residing in the airways orchestrate the immune response following antigen exposure and their ability to sample and present antigens to naïve T cells in airway draining lymph nodes contributes to allergen-specific IgE-sensitisation. In order to characterise inhaled antigen capture and presentation by DC subtypes *in vivo*, we used an adjuvant-free respiratory sensitisation model using two genetically distinct rat strains, one of which is naturally resistant and the other naturally susceptible to allergic sensitisation. Upon multiple exposures to ovalbumin (OVA), the susceptible strain developed OVA-specific IgE and airway inflammation whereas the resistant strain did not. Using fluorescently tagged OVA and flow cytometry, we demonstrated significant differences in antigen uptake efficiency and presentation associated with either IgE-sensitisation or resistance to allergen exposures in respective strains. We further identified $CD4^+$ conventional DC (cDC) as the subset involved in airway antigen sampling in both strains, however $CD4^+$ cDC in the susceptible strain were less efficient in OVA sampling and displayed increased MHC-II expression compared to the resistant strain. This was associated with generation of an exaggerated Th2 response and a deficiency of airway regulatory T cells in the susceptible strain. These data suggest that subsets of cDC are able to induce either sensitisation or resistance to inhaled antigens as determined by genetic background, which may provide an underlying basis for genetically determined susceptibility to respiratory allergic sensitisation and IgE production in susceptible individuals.

INTRODUCTION

Induction of antigen-specific IgE to inhaled non-pathogenic proteins (IgE-sensitisation) is known to increase the risk for development of allergic rhinitis and allergic asthma, the

prevalence of which has increased significantly over the last decades and continues to increase (1). In healthy individuals, the airway mucosal immune system is highly efficient in preventing IgE-sensitisation through the induction of antigen specific tolerance to the wide variety of innocuous aeroallergens that are inhaled daily (2-4). However, in susceptible individuals, these regulatory mechanisms are defective, leading to the development of elevated levels of systemic allergen specific IgE, a response mediated by activation of pro-allergic T helper type 2 (Th2) cells that also home to respiratory tissue and initiate allergic airways inflammatory responses following aeroallergen re-exposure. To date however, the mechanisms that lead to initial IgE-sensitisation or tolerance processes, and the factors that govern efficiency of these mechanisms *in vivo,* are not well understood.

Allergic respiratory sensitisation is defined by Th2 cell activation and the production of pro-allergic type 2 cytokines including IL-4, -5, -9 and -13 (5). These cytokines are produced in the lymph nodes by activated allergen-specific Th2 cells, and in the airways by migratory Th2 cells and by resident non-allergen specific group 2 innate lymphoid cells that have been activated by IL-33 expressing epithelial cells (6). The type 2 cytokines, together with cognate signals from allergen-specific Th2 cells, stimulate B cells in lymphoid follicles to differentiate and produce allergen specific IgE, which then binds to Fcε receptors expressed on several immune cell populations in the airway mucosa including mast cells and airway dendritic cells (DC). Re-exposure to inhaled allergen subsequently leads to mast cell degranulation, along with DC activation and effector/memory Th2 cell recruitment into airway tissue and induction of local airways inflammation. On the other hand, prevention of IgE-sensitisation and allergic inflammation in resistant individuals can be achieved through an array of processes including shut down of DC antigen capture and presentation (7), prevention of DC maturation (8),

persistent IL-10 production by DC (9), and induction of T regulatory (Treg) cells that restrict T cell activation as well as further DC activation (4, 10, 11).

Dendritic cells have been implicated in the process of IgE sensitisation and airway inflammation through their ability to induce and direct T cell responses (12), and are a key target for therapeutic prevention of IgE sensitisation. Airway conventional DC (cDC) seed the airways as "immature" DC, defined as expressing relatively low levels of MHC Class II (MHC-II) and co-stimulatory molecules, combined with highly efficient mechanisms for capture of inhaled proteins (13, 14). In the airways, cDC have a short half-life of less than 24 h during homeostasis and even less during inflammatory challenge, with rapid and constitutive migration to airway draining lymph nodes (ADLN) (15-17). In humans and rodents, cDC and plasmacytoid DC (pDC) make up the two main classes of DC in the airways (18). In rats, cDC express MHC-II and CD11b, with varied expression of CD4, CD172 and CD103 (19) whereas pDC express MHC-II, CD4 and CD172 but lack expression of CD11b and CD103 (20).

Antigen-bearing cDC migrate to ADLN, where they play key roles in instructing the activation and differentiation of naïve antigen-specific $CD4^+$ T cells, which in the respiratory system has been shown to default to a Th2 biased response under homeostatic conditions (2, 21). Previous reports from our group and others have identified cDC as the subset that most efficiently samples inhaled antigens in rats (22, 23) and appear able to induce both tolerance and sensitisation depending on stimulation (24, 25). Conventional DC have also been described to induce both sensitisation and tolerance in mice (26, 27) and this balance is orchestrated through expression of co-stimulatory molecules such as MHC-II, CD40 and CD86 as well as through cytokines such as IL-10 and IL-12 (28). Co-stimulatory molecules

106 on DC are induced through exposure to pathogen responsive signals, such as thymic stromal
107 lymphopoietin and IL-33 from airway epithelial cells (29) or directly through pathogen
108 derived components such as LPS which are detected by TLR and other innate immune
109 receptors on DC (12, 30, 31). Exposure to an innocuous substance in the absence of these
110 stimulatory signals results in limited DC maturation/activation and antigen presentation, that
111 tends to promote tolerance and maintain homeostasis (32). In addition to cDC, pDC have
112 been reported to also be involved in tolerance induction upon exposure to non-pathogenic
113 material in mice (33, 34) however, their role in human and rat sensitisation and tolerance is
114 less clear.
115
116 We have previously shown, using rat models of adjuvant-induced allergic airways disease that
117 genetic background influences the immune response to repeated allergen exposures (35) and
118 that prevention of airway symptoms is also dependent on efficient allergen sampling by cDC
119 in the airways (22). However, how these factors influence "natural" adjuvant-free
120 sensitisation following exposure to inhaled protein allergens remains unclear. In this study,
121 we used rat strains with different genetic susceptibility to IgE-sensitisation (PVG – resistant;
122 BN – susceptible) to determine if genetic background influences DC phenotype, antigen
123 uptake and the subsequent $CD4^+$ T cell responses that underlie susceptibility or resistance to
124 IgE-sensitisation and allergic airways inflammation. By comparing initial immune response to
125 inhaled allergen in naïve rats under adjuvant-free conditions, we demonstrate a significant
126 genetically-determined difference in the ability of airway $CD4^+$ cDC to sample, process and
127 present inhaled allergens between resistant and susceptible rat strains, which has a profound
128 effect on the ensuing $CD4^+$ T cell response and susceptibility or resistance to IgE-
129 sensitisation.
130

RESULTS

Genetically susceptible BN rats develop IgE-sensitisation and allergic airways inflammation to inhaled OVA during natural exposure regimes

To analyse the cellular events associated with susceptibility to IgE-sensitisation following aeroallergen inhalation, we compared the PVG and BN rat strains, which are respectively resistant versus highly susceptible to IgE-sensitisation and allergic inflammation. Using these strains, we have previously shown that sensitisation with OVA aluminium hydroxide (AlOH) induces high versus low titres of OVA specific IgE in BN versus PVG rats, and additionally, repeated exposures of OVA aerosols induces chronic versus self-resolving allergic airways disease in BN compared to PVG (22). In the present study, we focused on the responsiveness of these two rat strains to *de novo* aeroallergen exposure in the absence of any OVA pre-sensitisation. To determine the natural immune response to inhaled protein in naïve animals from both strains, we administered OVA or PBS intransally daily for 10 days. Following this exposure regimen, elevated OVA-specific IgE titres were observed in the BN strain, with no detectable IgE in the OVA exposed PVG strain or in PBS exposed control animals from either strain after 10 days (Fig. 1A). Both strains did, however, develop an antibody-mediated, IgG2a dominated, immune response to OVA and titres were generally higher in the susceptible BN strain (Fig. S1A). Ten daily intranasal OVA exposures did not affect the total cell number recovered from bronchoalveolar lavage (BAL) from either of the two strains (Fig. 1B) indicating no overt airways inflammation in either strain. However, if OVA exposures were extended to 21 days in total, the susceptible BN strain displayed an increase in total recovered BAL cells compared to the resistant PVG strain or PBS treated controls (Fig. 1C). After 21 daily exposures, the OVA specific IgE titres in the susceptible BN and resistant PVG strain remained positive and negative respectively (Fig. S1B) and both strains still showed elevated titres of other OVA specific antibody isoforms (Fig. S1C). In regards to number of

specific cell types in BAL after OVA exposure, a significant increase in total lymphocyte numbers was observed in the resistant strain after 10 OVA exposures, which was not observed in the susceptible strain (Fig. 1D). After 21 exposures, increases in total numbers of neutrophils, eosinophils and lymphocytes were observed in the susceptible BN strain compared to PBS control with no differences to PBS control detected in the resistant PVG strain (Fig. 1E). The cellular compositions of BAL after 10 and 21 exposures are displayed in Fig. S1D-E. Together this data demonstrate that 10 OVA exposures induced OVA specific IgE-sensitisation in the susceptible BN strain, which resulted in allergic airways inflammation if exposures continued to 21 daily exposures, whereas the PVG strain remained resistant to IgE-sensitisation and airways inflammation for up to 21 daily OVA exposures.

Aeroallergen uptake by airway DC is decreased in IgE-susceptible BN rats

Airway DC are central to tailoring the $CD4^+$ T cell response to inhaled allergen exposure, and efficient allergen uptake by airway DC is crucial for protection against inflammatory airways disease in allergen-sensitised rats (22). We therefore hypothesised that the susceptibility of naïve BN rats to respiratory IgE-sensitisation by intranasal OVA exposure may be related to the efficiency of aeroallergen uptake by airway DC. We used confocal microscopy and flow cytometry to analyse distribution and numbers of airway DC in trachea and lung of naïve BN and PVG rats. In the trachea, MHC-II expression and morphology were used to identify airway DC, which were distributed throughout the airways (Fig 2A) with no quantitative differences between the two rat strains as judged by flow cytometry (Fig 2B). However, the susceptible BN strain displayed higher proportion of MHC-II^+ cells in peripheral lung compared to the resistant strain (Fig. 2B). We next quantified the frequency of MHC-II^+/$CD11b^+$ cDC in trachea and lung by flow cytometry, which were further subdivided into $CD4^+$/$CD172^+$ ($CD4^+$) and $CD4^-$/$CD172^-$ ($CD4^-$) cDC, as well as MHC-II^+/$CD11b^-$/$CD4^+$

pDC as previously described for rats (19, 20, 24, 36, 37) (Fig. S2A). We also included MHC-II$^+$/CD11b$^+$ cells that were CD172$^+$/CD4$^-$, since this subset also displayed the ability to take up OVA (see below) but did not coincide with a previously described rat DC subset neither did this cell type fall within the monocyte subsets defined by CD43/His48 expression (38) (data not shown). The DC subsets were also sorted and analysed by light microscopy, which showed typical dendritic cell characteristics (Fig. S2B). Surface expression of DC markers was quantified on all subsets in both trachea and ADLN (Fig. S2C). Of note, large differences were found in expression levels of several markers between both tissues and between the two strains. To compare the relative proportions of each DC subset in respiratory tissues we used principal component analysis to group each tissue for both strains based on the ratio of DC subset as percentage of MHC-II$^+$ cells (Fig. 2C). This analysis showed that each strain generally grouped together for each tissue and limited difference in DC proportions between the strains was observed in ADLN, partial separation between strains appeared for DC proportions within the lungs, and the trachea separated substantially from the other tissues and also separated clearly between the two strains. Indeed, in the trachea, the resistant PVG strain displayed higher number of CD4$^+$ cDC compared to the susceptible BN strain (Fig 2D) and higher proportion of CD172$^+$/CD4$^-$ cDC in the lungs from the susceptible strain (Fig. S3A). We reasoned that different numbers of DC at baseline may affect the overall ability to sample and present antigens and thus induction of appropriate T cell activation and differentiation programs.

To investigate the antigen capture efficiency and onwards migration of DC in each strain, we administered fluorescently tagged OVA to naïve rats and compared total number (trachea) or proportion (lung digests) of OVA$^+$ DC at 2 h and 24 h after administration, representing peak time points for antigen capture and migration respectively (Fig. 2E-F). Two hours after

exposure in the trachea the number of OVA^+ DC were significantly higher in the resistant PVG strain compared to the susceptible BN strain (Fig. 2E). After 24 h, significantly fewer OVA^+ DC remained in the trachea of the resistant PVG strain, whereas no significant changes in the susceptible BN strain were observed (Fig. 2E). Antigen uptake was also higher in the peripheral lungs of the resistant PVG strain compared to the susceptible BN strain (Fig. 2F), however there were no differences in the proportion of OVA^+ cells, between the two time points suggesting that OVA may be retained in the lungs for at least 24 h after intranasal exposure as previously observed (39).

The above data suggest that inhaled OVA is largely transported out of the trachea within 24 h, particularly in the resistant strain but retained for at least 24 h in peripheral lungs. This is consistent with our previous rat and mouse studies showing a more rapid turnover rate for trachea DC as compared to peripheral lung DC (39). In this previous study, antigen sampled in the trachea appeared in the ADLN within 24 h post OVA exposure. For this reason, we focused our subsequent analyses on tracheal DC subsets. To identify the specific DC subset(s) involved in OVA uptake, we analysed OVA^+ tracheal DC at 2 h post intranasal OVA administration in each strain (Fig. 2G, Fig S3B-C). We observed OVA uptake across several subsets in both strains with significantly more uptake in $CD4^+$ cDC and $CD172^+/CD4^-$ cDC in the resistant PVG strain compared to the susceptible BN strain. The $CD4^+$ cDCs also carried more OVA in the resistant strain as judged by OVA MFI in OVA^+ cells (Fig. 2H). When analysed as total number of OVA^+ cells, significantly more $OVA^+/CD4^+$ cDC were found in the resistant strain, and this subset made up the major OVA sampling subset in both strains (Fig. 2I). Twenty-four hours post OVA administration, the majority of OVA^+ cDC disappeared from the trachea in the resistant PVG strain, resulting in no difference in numbers of OVA^+ DC subsets between the strains at this time point (Fig. 2J). To determine if the $CD4^+$

cDC were activated in response to OVA uptake, and if this differed between the susceptible and resistant rat strains, we analysed CD86 and MHC-II expression on OVA^+ and OVA^- $CD4^+$ cDC 2 h after OVA exposure. In OVA-exposed animals, only $OVA^+/CD4^+$, but not $OVA^-/CD4^+$ cDC, displayed upregulation of CD86 (Fig. 2K) but not MHC-II (data not shown) compared to unexposed DC in respective strain, with no detectable difference between the strains. Airway DCs can become activated through TLR signalling and we therefore also compared TLR expression in sorted $CD4^+$ cDC but only observed minor differences in TLR1 and 8 expression between the two strains (Fig S3D). Together, these data suggest that $CD4^+$ cDC are the major subset involved in OVA sampling in the trachea in both rat strains, but the resistant PVG strain displays more efficient allergen sampling and migration within DC subsets compared to the susceptible BN strain. Interestingly, the initial response of airway DC to OVA uptake appears comparable in both strains, at least in regards to expression of MHC-II and CD86 on $OVA^+/CD4^+$ cDC.

OVA-carrying DC in the ADLN of IgE-susceptible rats display a mature phenotype.

During primary exposure to inhaled proteins, a major role for airway DC is to carry and present antigen to naïve T cells in the ADLN. Depending on the state of DC maturation and activation, different effector T cell responses are then induced (32, 40). We therefore analysed the number of DC and OVA^+ DC subsets in ADLN at 2 h and 24 h post OVA exposure applying the same gating as previously shown (Fig S2A). In ADLN of naïve animals, we did not observe any significant differences in numbers of DC between the two strains (Fig. 3A). However, two hours post OVA administration, significantly more $OVA^+/CD4^+$ cDC were observed in the ADLN in the resistant strain compared to the susceptible strain (Fig. 3B). After 24 h, the proportion of OVA^+ DC was comparable in both strains (Fig. 3C). Maturation of antigen presenting DC is a key step in tailoring the adaptive immune response where

antigen presentation by immature DC has been shown to induce Treg and potentially plays a role in maintain homeostasis whereas induction by mature DC induces an antigen specific inflammatory response. To analyse DC maturation in both strains we compared proportion of MHC-II^{hi} (mature) and MHC-II^{lo} (immature) OVA^{+}/CD4^{+} cDC (Fig 3D) in both strains. We discovered that OVA^{+}/CD4^{+} cDC arriving in ADLN 2 h were mainly MHC-II^{lo} immature DC in both strains after 1-4 exposures (Fig 3E). However, at 24 h post OVA exposure, the susceptible strain displayed significantly higher proportion of MHC-II^{hi}/OVA^{+}/CD4^{+} mature cDC compared to the resistant strain where a balanced immature/mature ratio was observed after 1-4 exposures (Fig 3F). To test if these OVA^{+}/CD4^{+} cDC from both strains were able to induce an OVA specific T cell response we sorted CD4^{+} cDC from ADLN after one OVA exposure *in vivo* and incubated these cells with purified OVA responsive T cells from respective strain. We observed that CD4^{+} cDC exposed to OVA in the susceptible BN strain induced significantly less T cell proliferation compared the resistant PVG strain (Fig 3G). However, once supplemented with OVA *in vitro*, CD4^{+} cDC from the susceptible strain were able to induce efficient OVA specific T cell proliferation (Fig 3H), suggesting that the functional ability of CD4^{+} cDC from the susceptible strain to induce a T cell response is restricted by the limited amount of OVA uptake *in vivo*. Together these data suggest that, consistent with other studies (32), low-level antigen delivery by mature CD4^{+} cDCs may favour an inflammatory immune response resulting in airway inflammation in the susceptible strain.

Repeated OVA exposures induces airway Tregs in IgE-resistant rats

As shown above, antigen presentation in the IgE-resistant and -susceptible rat strains differed in (i) how rapidly OVA^{+} DC migrate to ADLN, (ii) OVA carrying DC numbers, (iii) maturation status of OVA^{+} DC and (iv) the ability of these DCs to induce T cell proliferation

281 *in vitro*. We therefore hypothesised that these differences would be reflected in $CD4^+$ T cell
282 activation and subset differentiation in ADLN *in vivo*. To track the T cell response we
283 analysed T cell numbers and proportions in ADLN after 1, 4 and 10 OVA exposures (Fig.
284 4A-F and Fig S4A-E). A total of 10 daily OVA exposures significantly increased total
285 cellularity in the ADLN of the susceptible strain (Fig S4A). This included T cell numbers
286 (Fig. 4B) including $CD4^+$ (Fig 4C) but not $CD8^+$ (Fig 4D) T cells, as well as
287 $CD4^+/CD25^+/FoxP3^+$ Tregs (Fig 4E) and a trend towards increased $CD4^+/CD25^+/FoxP3^-$ T
288 effector cells (Fig. 4F). However, only minor changes in cell proportions were observed (Fig
289 S4B-E).
290
291 To test if the increased cellularity was due to increased proliferation we compared proportion
292 of Ki67 positive $CD4^+$ T cells, and surprisingly observed that the resistant PVG strain
293 displayed a significant increase in proportion of $Ki67^+$ cells in response to OVA exposure
294 (Fig 4G). Comparing Ki67 expression in $CD4^+$ Tregs and T effector cells, only $CD4^+$ Treg
295 increased proportion of $Ki67^+$ cells in the resistant strain (data not shown). This suggests that
296 increased ADLN cellularity in the susceptible strain may not be due to increased rates of
297 proliferation within a specific subset but rather cell accumulation as a result of reduced
298 migration and/or increased cell survival.
299
300 To compare induction of OVA-specific T cells that can respond to OVA re-challenge, ADLN
301 digests from animals exposed to four OVA exposures were labelled with CFSE or Violet cell
302 tracer and re-stimulated with OVA *in vitro*. Only in the susceptible BN strain did OVA re-
303 stimulation induce significant T cell proliferation (Fig. 4H) in line with Fig 3G. Comparing
304 the $CD4^+$ Treg and T effector response in the two strains, significantly more $CD4^+$ T effector
305 cells responded in the susceptible strain compared to the resistant strain (Fig. 4I). Out of the

306 responding $CD4^+$ cells, significantly more cells produced IL-4 in the susceptible BN strain
307 compared the resistant PVG strain. In the resistant strain, OVA-responding cells instead
308 produced IFN-γ (Fig 4J). Together, these data suggest that OVA-exposure induced expansion
309 and accumulation of OVA-specific $CD4^+$ T cells including $CD4^+$ Tregs and Th2 effector cells
310 in the ADLN of the susceptible BN strain, whereas in the resistant PVG strain, OVA
311 exposures favoured a $CD4^+$ Treg and limited Th1 response. This further aligns with the
312 induction of Th2-dependent IgE in the susceptible strain, and absence of IgE induction in the
313 resistant strain.
314
315 Following exposure to inhaled antigen, $CD4^+$ T cells are activated in the ADLN and
316 differentiate into effector subsets (Th1, Th2, Th17 and Treg) prior to migrating back into
317 respiratory tissues. OVA exposure induced increased $CD4^+$ T cell proliferation in the resistant
318 PVG strain and ADLN expansion and accumulation in the susceptible strain. We therefore
319 investigated if this lead to a deficient airway response in the susceptible BN strain. In the
320 airways, we did not detect any difference in number of $CD3^+$ (Fig 5A) or $CD4^+$ T cells in
321 response to OVA exposure compared to PBS control in either strain, although the resistant
322 strain displayed higher baseline numbers of $CD4^+$ T cells (Fig 5B). We did observe that 10
323 OVA exposures resulted in significantly more airway $CD8^+$ T cells in the resistant strain
324 compared to the susceptible strain although this was not different compared to PBS control in
325 the resistant strain (Fig 5C). In line with the increased $CD4^+$ Treg proliferation in the ADLN
326 of the resistant strain, we also observed significantly more $CD4^+$ Tregs in the airways in
327 response to OVA-exposure (Fig 5D). Of note, this difference was also evident in naïve
328 animals. At the same time, we did not detect any significant difference in number of $CD4^+$ T
329 effector cells between the two strains (Fig 5E). We have previously observed that a deficient
330 uptake of antigen is associated with a deficient $CD4^+$ Treg response in sensitised rats from the

susceptible strain (22). Although the mechanism remain unclear, by increasing the OVA exposure dose in the susceptible strain we observed an increased number of $CD4^+$ Tregs in the airways (Fig 5F), suggesting that antigen dose is tightly associated with airway $CD4^+$ Treg induction and we are currently investigating this mechanism further. In summary limited-OVA presentation by mature $CD4^+$ cDCs in the susceptible strain is associated with Th2 induction, allergic sensitisation and airway inflammation as well as insufficient induction of $CD4^+$ airway Tregs.

DISCUSSION

Although allergic sensitisation to aeroallergens affects almost 50% of most populations in developed countries, the immunological processes governing susceptibility to development of IgE-mediated inflammatory airways disease to innocuous antigens are not clear. In the current study we took advantage of two well-described genetically different rat strains: IgE-resistant PVG rats and IgE-susceptible BN rats, the latter which are susceptible to OVA specific IgE-sensitisation and airway inflammation upon repeated intranasal OVA exposures (41). Using these rat strains, our aim was to identify differences in DC airway subsets and their ability to capture, transport and present inhaled antigen to determine if this is associated with susceptibility or resistance to IgE-sensitisation and airways inflammation. We focused our studies on the initial early phase of primary antigen exposure that occurs in naïve individuals before establishment of IgE production and development of airways inflammation. We confirmed that airways inflammation develops following sensitisation and prolonged exposure in the genetically susceptible BN rat strain, whereas the IgE-resistant PVG rat strain remains asymptomatic under similar allergen exposure regimes. Using this model, we discovered that allergen-specific IgE-sensitisation was associated with significant differences in allergen sampling capacity and altered maturation of OVA carrying DC from airways to

ADLN, with larger number of $CD4^+$ cDC capturing and transporting OVA to ADLN in the resistant compared to the susceptible strain. Multiple daily OVA exposures also induced exaggerated $CD4^+$ Th2 cell expansion in the ADLN in susceptible BN rats, whereas the response in the resistant strain instead favoured generation of a Th1 response and induction of $CD4^+$ airways Tregs, aligning this study with previous work from our group showing that airway Tregs are crucial for preventing airway inflammation (4).

Continuous antigen exposure eventually leads to Th1 or Th2 derived Tregs in healthy individuals in order to restrict chronic airways inflammation (42). Given the symptomatic inflammatory response observed in BN rats, this conversion appears deficient in this strain. Initial T cell responses to antigen are dependent on DC-T cell interactions and are orchestrated through increased expression of MHC-II and co-stimulatory molecules such as CD40, CD80 and CD86, and through the production of cytokines such as IL-12 and IL-10 (28). We found fewer OVA^+ cDC in the ADLN of susceptible BN rats after intranasal OVA exposure compared to resistant PVG rats and although they carried comparable amounts of OVA as to the OVA^+ cDC in resistant PVG rats (data not shown), the OVA^+ cDC in the susceptible strain expressed a more mature phenotype and higher MHC-II levels. This is all likely to be significant, as the number of antigen-bearing DC, their maturation status and the amount of antigen they process and present, all play a significant role in activation of naïve $CD4^+$ T cells and subsequent differentiation into effector T helper subsets (43, 44). We hypothesise that in resistant PVG rats, naïve OVA-specific $CD4^+$ T cells entering the ADLN after intranasal OVA exposure encounter a mix of immature and mature OVA^+ DC whereas in the susceptible BN strain, such $CD4^+$ T cells instead encounter a more limited number of OVA^+ DC that almost exclusively express a mature phenotype with increased MHC-II expression. Dendritic cell maturation is orchestrated through complex pathways in response

to environmental stimuli, which presumably was identical in both strains of rats in this study. However differences in genetic background may influence how environmental stimuli are detected and interpreted. Many environmental stimulants are detected by TLRs on airway epithelial cells, and/or directly by airway DC (45) and we did observe that $CD4^+$ cDC expressed slightly higher levels of TLR1 and 8 in the susceptible strain which may influence the activation threshold to certain environmental stimuli in this strain.

In addition to DC maturation, data from mouse models using OVA-specific DO11.10 transgenic T cells also suggest that the DC to T cell ratio significantly influence T cell responses. In the study, high DC:T cell ratios (similar to situation in the ADLN of PVG rats) favoured regulatory IL-10 production, whereas low DC to T cell ratios (akin to the ADLN of BN rats) instead favoured pro-allergic, IL-4 producing Th2 cell induction (9). Similarly, low antigen doses (and thus low levels of antigen presentation) were shown to induce IL-4 and IL-10 producing Th2 cells whereas high antigen doses, together with IL-12 induced IL-10 producing Th1 cells (46). Thus, we speculate that the low OVA^+ DC to $CD4^+$ T cell ratio in IgE-susceptible BN strain may partly explain the exaggerated IL-4 producing Th2 response leading to IgE sensitisation in this strain.

There has been substantial discussion in the literature regarding discrete immunogenic versus tolerogenic DC subsets, particularly deriving from mouse studies. In mice, findings suggesting that $CD103^+$ cDC induce respiratory tolerance (27) and $CD103^+$ cDC are also involved in presenting apoptotic antigen from the airways (47). The other main cDC subset, $CD11b^+$ cDC, appear to be involved in inducing Th2 immunity in response to house dust mite (26). Plasmacytoid DC have also been implicated to induce and mediate tolerance (34) in mice although this remains a topic of discussion (27). In rats, DC subsets are not as well

studied as in mice and are defined differently, instead relying on expression of CD11b, CD4 and CD172. In rats, previous work suggest that $CD4^+$ cDC can induce both $CD4^+$ Th1/Th2 as well as $CD8^+$ T cell proliferation (24) whereas $CD4^-$ cDC mainly induce Th1 proliferation (24) and may also be involved in tolerance induction and presentation of apoptotic material (48, 49), possibly aligning them with $CD103^+$ cDC in mice. Of note is that $CD4^-$ cDC in rats express high levels of CD103 in trachea but not in ADLN (Fig. S2C). Interestingly, both $CD4^+$ and $CD4^-$ cDC in rats have also been observed to produce IL-10 in response to TLR activation (25), a pathway which has been proposed to favour induction of Tregs in mice (9). In our setting, $CD4^+$ cDC are the major antigen presenting subset able to both induce and prevent airway inflammation, possibly conditional on the number of antigen carrying DC as well as their maturation and/or activation level (50, 51). This aligns with previous findings showing that $CD4^+$ cDC are involved in resolving airway inflammation in sensitised rats (22) as well as able to stimulate immune activation (24). Although $CD4^-$ cDC are reported as having tolerance-inducing features, prevention of airway symptoms in the resistant strain appear to be independent on $CD4^-$ cDC mediated antigen uptake and presentation in our setting although these cells may also be able to induce non-inflammatory responses under altered conditions or to other antigens.

We have previously reported that efficient allergen uptake by airway DC is directly related to the ability to induce functional $CD4^+$ Tregs in sensitised rats (22). We further reported that the ability to sample allergen was not inherently deficient in DC from the susceptible strain and *in vitro* experiments showed comparable ability of DC from the susceptible and resistant strain to take up antigen and activate antigen specific T cells (22). Together with the findings in the current study, this suggests that the local microenvironment *in vivo* prevents efficient antigen uptake in the susceptible strain and thus also contributes to IgE sensitisation. The exaggerated

expansion of T cells in ADLN in response to repeated OVA exposure in the susceptible BN strain but not in the PVG strain suggests that the susceptible strain may be deficient in cellular control or migration mechanisms. Repeated OVA exposures induced accumulation of $CD4^+$ T cells, including $CD4^+$ Tregs and $CD4^+$ T effector cells in the ADLN of the susceptible BN strain. However, T cell proportions were maintained and cell proliferation was more pronounced in the resistant strain. Previous functional comparison of Treg cells from OVA-AlOH sensitised and OVA aerosol challenged rats from the resistant and susceptible strain suggests that the susceptible strain mounts Treg responses that are inefficient in migrating to the airways, possibly due to lower CCR4 expression. Lower expression of IL-10 may also affect their ability to efficiently control Th2 activation and proliferation as observed *in vitro* (22). Allergen dose during re-challenge was also shown to be associated with induction of $CD4^+$ airway Tregs and this association was also confirmed using naïve animals in this study. Given the importance of sufficient antigen dose for induction of $CD4^+$ airway Tregs, the observed accumulation and expansion of $CD4^+$ Tregs in the ADLN was unexpected and suggests that antigen dose may be mainly involved in inducing Treg migration but the exact mechanism remain to be clarified. One consequence of failed Treg migration in the susceptible strain is lower baseline levels of $CD4^+$ airway Tregs and this may in turn influence the DC activation and maturation threshold (52), which affect the risk of IgE sensitisation and subsequently contributes to fuelling the inflammatory and IgE-promoting airway environment observed in the susceptible strain.

In summary, by comparing the *in vivo* response to exposure of an innocuous aeroantigen in two rat strains with natural genetic resistance or susceptibility to respiratory IgE-sensitisation, we have identified significant differences in airway DC subset composition and capacity to sample and deliver inhaled antigen to the ADLN between the resistant and susceptible strains.

Furthermore, resistance to airway symptoms in this setting was associated with a limited T cell response and induction of $CD4^+$ airway Tregs. Our data also suggest that although discrete DC subsets may favour a particular T cell response, this is a highly dynamic process dependent on DC antigen sampling, maturation, migration and activation capacity. Given that these factors differ substantially depending on genetic background in rodent models suggests large variations within humans and need to be considered in future studies.

METHODS

Animal model and tissue processing

Seven to nine weeks old Piebald Virol Glaxo (PVG) and Brown Norway (BN) male rats, bred at the Telethon Kids Institute, were exposed daily per intranasal administration to 100-1000 μg OVA grade V (Sigma-Aldrich, NSW, Australia) or PBS for up to 21 days during light isoflourane induced anaesthesia. For the last exposure before organ harvest, 100 μg Alexa Fluor 594 labelled OVA (Thermo Fisher, NSW, Australia) in PBS was used 2 or 24 h prior to organ harvest. Blood for serum generation was collected prior to the animals were humanely euthanized by an intraperitoneal Lethabarb injection (Virbac, NSW, Australia). Airway draining lymph nodes, trachea and distal PBS-perfused lung were dissected out, mechanically disintegrated and digested with 0.75 mg/ml (ADLN) or 1.5 mg/ml (trachea and lung) collagenase-IV (Worthington, NJ, USA) and 0.1 mg/ml DNase-I (Sigma-Aldrich) in 140 mM NaCl, 5 mM KCl, 10 mM Na_2HPO_4, 5 mM NaH_2PO_4 and 10 mM glucose (GKN buffer) supplemented with 10% FCS for 30 min (ADLN) or 90 min (trachea and lung) at 37 ºC, mixed thoroughly to extract cells and filtered through a nylon membrane into a single cell suspension before stained for flow cytometry as per below. The local animal ethics committee approved all animal experiments, which adhere to the national guidelines for the use of research animals in Australia.

OVA specific antibody titres.

OVA specific IgE serum titres were analysed using passive cutaneous anaphylaxis as previously described (53). OVA specific IgG was analysed using a modified rat Ig isotyping ELISA (eBioscience/Thermo Fisher). Serum was diluted in PBS and incubated on OVA-coated Maxisorp plates (Nunc, Denmark), washed and incubated with mouse anti-rat isotype-specific antibodies (eBioscience) followed by detection of mouse IgG using HRP-linked sheep anti-mouse (Amersham/GE, NSW, Australia) and developed according to manufacturers instructions.

Bronchoalveolar lavages

Bronchoalveolar lavages were collected from freshly euthanized animals by twice flushing the airways with 9 ml GKN buffer. Samples were kept on ice before erythrocytes were lysed in 17 mM Tris-HCl, 0.14 M NH_4Cl at pH 7.2, remaining cells were washed and counted before 75 000 cells were spun onto a super frost microscopy slide and stained with Eosin-Azure B (Perth Scientific, WA, Australia) as per manufacturers instructions. Differential count was performed blinded on at least 300 cells per sample.

DC tissue distribution

Whole trachea from naïve animals were excised as per above and fixed in 70% ethanol overnight. Samples were immunostained overnight using mouse anti-MHC-II monoclonal antibody directly conjugated with APC (BD, NSW, Australia) and rabbit anti-cytokeratin antibody followed by detection overnight using a goat anti-rabbit-Oregon Green antibody (Thermo Fisher). Samples were embedded using Vectashield mounting medium and analyzed

using a Leica TCS SP2 AOBS Multiphoton Confocal microscope (Leica Microsystems, NSW, Australia).

Flow Cytometry and cell sort

Freshly isolated cells ($1x10^6$) were stained with either a dendritic cell panel: RT1B/MHC-II-PerCP (#557016, BD), CD11b-V450 (#562108, BD), CD172-PE (#552298, BD), CD103-Biotin (#MCA 1029B, Serotec/Bio-Rad, NSW, Australia), CD45RA-APC-Cy7 (#561624, BD), CD86-FITC (#555018, BD) and CD4-APC (#550057, BD) followed by Strep-V500 (#561419, BD); or T cell panel: CD3-FITC (#557354, BD), CD4-PE-Cy7 (#201516 Biolegend), CD8-PerCP (#558824, BD), CD25-Biotin (#559981, BD), CD45RA-APC-Cy7 (BD) followed by Strep-V450 (#560797, BD), fixation, permeabilisation and intracellular staining using FoxP3 staining solution (eBioscience) with FoxP3-PE (#12-5773-82, eBioscience), IL10-APC (#562156, BD) and Ki67-AF700 (#561277, BD) at 4 ºC before acquisition on a LSR Fortessa (BD) and analysed using FlowJo 10 (FlowJo LLC, OR, USA). For cell sorting, cells were isolated from lungs, airways or ADLN as above and stained with DC panel or with CD3 and CD4 for T cell purification prior to sorting using FACSAria (BD). Sorted cells were used for *in vitro* cultures or spun onto on super frost slides, stained with Eosin-Azure B and analysed with a BX53 microscope (Olympus, VIC, Australia).

In vitro cultures

For in vitro recall response analysis, PVG and BN rats were administered 4 intranasal OVA exposures as above, rested for 7 days before ADLNs were harvested and processed as above. Single cell suspensions were stored in liquid nitrogen until use. Before culture, cells were thawed, stained with 2.5 μM CFSE or 5 μM Cell Tracer Violet (both from Thermo Fisher), washed and resuspended in RPMI (Thermo Fisher) supplemented with 10% FCS, 300 μg/ml

L-glutamine, 20 μg/ml gentamycin and 50 μM 2-mercaptoethanol prior to plating 0.5-1x10^6 cells/200 μl in U-bottom-plates and culturing with 0-50 μg/ml OVA for 48-72 h at 37 ºC and 5% CO_2. For cytokine detection cells were cultured with 1x Brefeldin A (Biolegend) for the final 4 h. For analysis, cultured cells were stained with RT1B-PerCP, CD4-PE Cy7, CD25-BV421 (#565608, BD) and intracellular FoxP3-PE or RT1B-PerCP, CD4-PE Cy7 and intracellular IL4-PE (#555082, BD) and IFNγ-FITC (#559498, BD) as above and analysed by flow cytometry. For co-cultures, $CD4^+$ cDCs were sorted as above from pooled ADLN digests and co-cultured with sorted $CD4^+$ T cells from ADLN of animals sensitised through a 0.5 ml intraperitoneal injection of 0.2 mg/ml OVA in AlOH and cultured for 66 h with or without 50 μg/ml OVA. T cell proliferation was assessed by ^{3}H-thymidine incorporation during the final 18 h and analysed as previously (22).

Statistics

All experimental groups consisted of at least 3 animals from two or more independent experiments, for detailed animal numbers in each experimental group, see Table S1. All animals were randomly assigned into experimental groups and no animals were excluded during the study, all flow cytometry analysis was performed blinded. Statistical significance of difference was calculated between dependent groups using two-way ANOVA followed by Fishers LSD post-test or using student t-test for comparison of two independent groups. Principal component analysis of DC proportions was calculated using JMP 13 (SAS, NSW, Australia), all other statistics was calculated using Prism 7.0 (GraphPad, CA, USA).

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Supplementary information is available at *Immunology & Cell Biology's* website

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1 OVA exposure induces antigen specific IgE followed by airway immune cell infiltration in the susceptible BN rat strain. A Titres of OVA specific IgE as measured through passive cutaneous anaphylaxis after 10 daily OVA or PBS exposures in resistant or susceptible rat strains. **B-C** Total cell number in bronchoalveolar lavages after 10 (**B**) and 21 (**C**) daily exposures compared to naïve (**B**) or PBS (**C**) control in resistant or susceptible rat strains. **D-E** Total cell number of indicated cell type in BAL from resistant or susceptible strains after 10 (**D**) and 21 (**E**) daily exposures compared to naïve (**D**) or PBS control (**E**). Data are displayed as individual data points. Box and whiskers indicate mean of n = 4 - 11 ± 95% CI of at least 2 independent experiments. Statistical significance of differences was assessed using two way ANOVA followed by Fishers LSD post test and displayed as $^{\#}$/*: $p<0.05$, $^{\#\#}$/**: $p<0.01$, $^{\#\#\#}$/***: $p<0.001$ where * indicate comparison as depicted and $^{\#}$ indicates comparison to intra strain control.

Figure 2 OVA uptake occurs mainly in trachea and is more efficient in the resistant PVG strain compared to the susceptible BN strain. A Distribution of MHC-II^{+} cells in trachea of the susceptible and resistant strains as viewed from the airway lumen. **B** Proportion of MHC-II^{+} cell in tissue digests in trachea and lung from the resistant and susceptible strains. **C** Principal component analysis using proportion of DC subsets to all MHC-II^{+} cells in trachea, lungs and ADLN in resistant and susceptible rats. **D** Number of DC subsets in trachea from resistant and susceptible rats. **E-F** Number or proportion of cell gate of OVA^{+} DC 2 h or 24 h post-intranasal administration of OVA-AF596 in trachea (**E**) and lungs (**F**) in resistant and susceptible rats. **G** Proportion of OVA^{+} cells in each respective DC subset in trachea 2 h post OVA administration in resistant and susceptible rats. **H** Quantity of OVA carried by OVA^{+} DC in the trachea 2 h after OVA exposure in the resistant and susceptible strain. **I-J**

752 Number of OVA^+ cells in trachea 2 h (**I**) or 24 h (**J**) post OVA administration in resistant and
753 susceptible rats. **K** Expression of CD86 in OVA^+ and OVA^- $CD4^+$ cDC 2 h post OVA
754 administration in trachea of resistant and susceptible rats normalised to unexposed $CD4^+$
755 cDC. Data are displayed as representative image of two independent experiments (**A**) or as
756 mean of n = 3 - 8 ± SD of at least 2 independent experiments. Statistical significance of
757 differences was assessed using two way ANOVA followed by Fishers LSD post test and
758 displayed as n.s: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.
759
760 **Figure 3 OVA presenting DC display a mature profile in the susceptible strain. A**
761 Number of DC subsets in ADLN in resistant and susceptible rats. **B-C** Number of OVA^+ DC
762 subsets 2 h (**B**) and 24 h (**C**) post OVA-AF596 administration in resistant and susceptible rats.
763 **D** Gating strategy to identify MHC-II^{hi} and MHC-II^{lo} $OVA^+/CD4^+$ cDC in ADLN post OVA
764 exposure in the resistant and susceptible strain. **E-F** Proportion of MHC-II^{hi} and MHC-II^{lo}
765 within $OVA^+/CD4^+$ cDC 2 h (**F**) and 24 h (**G**) post OVA exposure in the resistant and
766 susceptible strain. **H** Proliferation of OVA specific $CD4^+$ T cells as judged by ^3H thymidine
767 incorporation after 84 h co-culture with airway $CD4^+$ cDC exposed to OVA *in vivo* (prior to
768 isolation) or *in vitro* from both strains. Data are displayed as mean of n = 3 - 8 ± SD of at least
769 2 independent experiments. Statistical significance of differences was assessed using two way
770 ANOVA followed by Fishers LSD post test and displayed as n.s: not significant, *: $p<0.05$,
771 **: $p<0.01$, ***: $p<0.001$. In **D**, representative plots are shown from respective strain.
772
773 **Figure 4 OVA exposure induces exaggerated ADLN expansion in the susceptible strain**
774 **with a Th2 dominated response. A** Gating strategy of $CD3^+$ cells to identify, $CD8^+$, $CD4^+$,
775 $CD4^+$ Treg and T effector cells. **B-F** Total cell numbers of $CD3^+$ (**B**), $CD3^+/CD4^+$ (**C**),
776 $CD3^+/CD8^+$ (**D**), $CD4^+$ Treg (**E**) and $CD4^+$ T effector cells (**F**) in ADLN in OVA or PBS

exposed resistant (R) and susceptible (S) rats after 0-10 OVA exposures. **G** Proportion of $Ki67^+/CD4^+$ T cells in ADLN of OVA or PBS exposed resistant and susceptible rats. **H-J** T cell proliferation (**H**), as measured by CFSE or Cell tracer dilution, to *in vitro* OVA re-exposure of total ADLN digests exposed *in vivo* to 4 OVA exposures followed by 7 days incubation from resistant and susceptible as well as proliferation of Treg and Teffector cells in each strain (**I**). **J** IL-4 or IFN-γ production in OVA responding $CD4^+$ cells from each strain after 48 h of OVA stimulation *in vitro*. Data are displayed as mean of n = 3 - 8 ± SD of at least 2 independent experiments. Statistical significance of differences was assessed using two way ANOVA followed by Fishers LSD post test and displayed as n.s: not significant, $^{\#/*}$: $p<0.05$, $^{\#\#/**}$: $p<0.01$, ***: $p<0.001$. * indicates comparison as depicted and $^{\#}$ indicates comparison to intra strain PBS control.

Figure 5 OVA exposure dose is associated with induction of $CD4^+$ airway Tregs. A-E Total cell numbers of $CD3^+$ (**A**), $CD3^+/CD4^+$ (**B**), $CD3^+/CD8^+$ (**C**), $CD4^+$ Treg (**D**) and $CD4^+$ T effector cells (**E**) in trachea of OVA or PBS exposed resistant and susceptible rats after 0-10 daily exposures. **F** Number of $CD4^+$ Tregs in naïve or rats exposed to 4 exposures of 100 µg (1x) or 1000 µg (10x) OVA. Data are displayed as mean of n = 3 - 8 ± SD of at least 2 independent experiments. Statistical significance of differences was assessed using two way ANOVA followed by Fishers LSD post test and displayed as n.s: not significant, $^{\#/*}$: $p<0.05$, **: $p<0.01$, $^{\#\#\#/***}$: $p<0.001$. * indicates comparison as depicted and $^{\#}$ indicates comparison to intra strain PBS or naïve control.

Fig 1 (Leffler et. al)

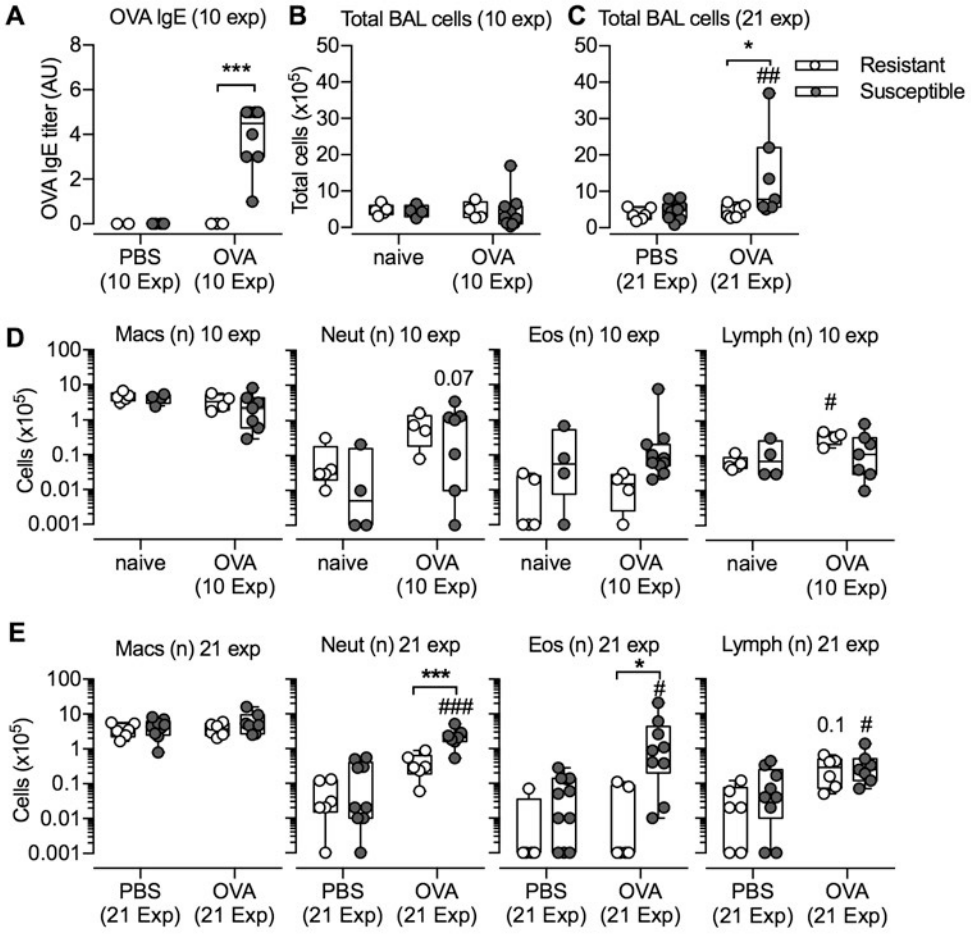


Fig 2 (Leffler et. al)

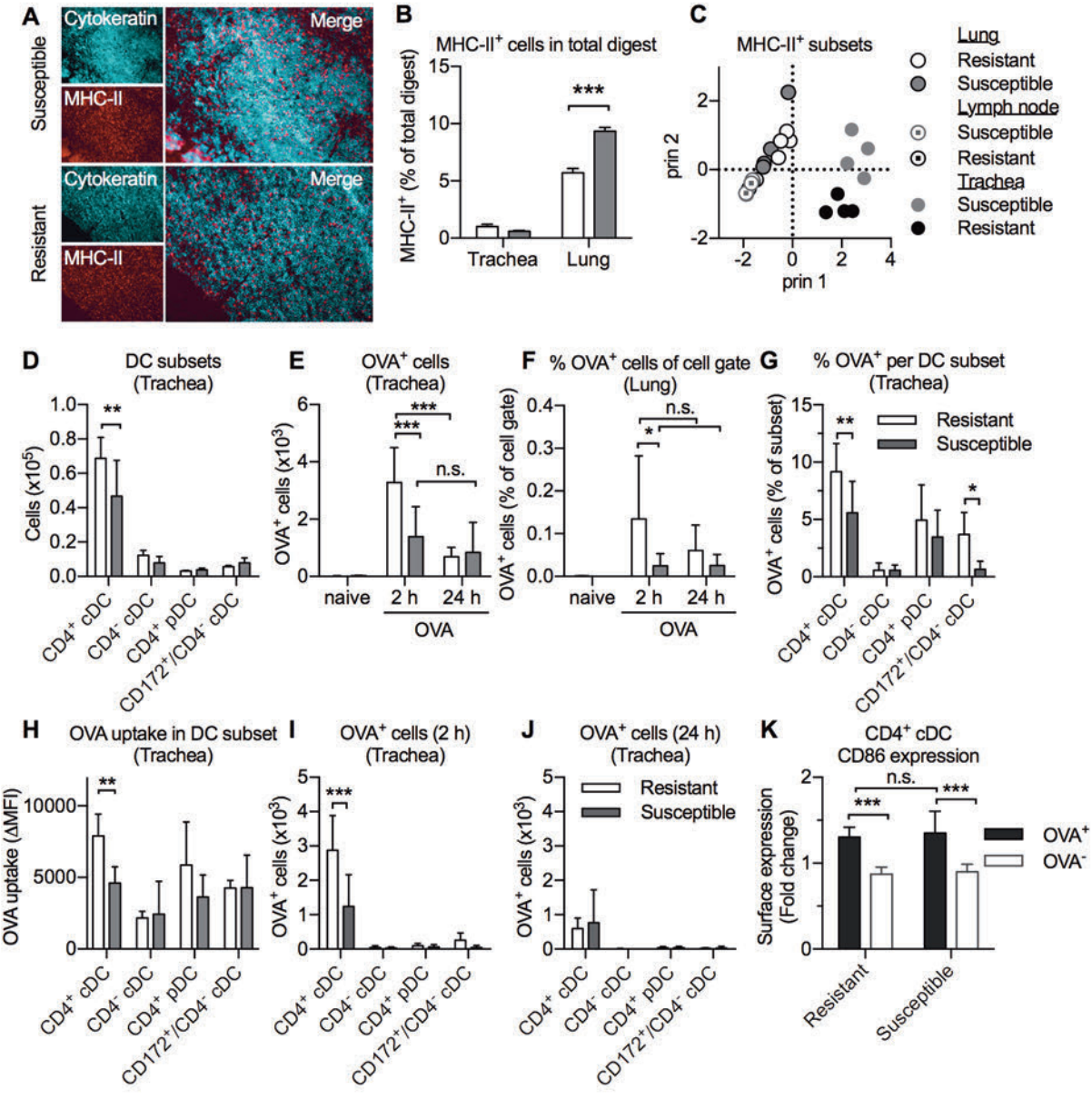


Fig 3 (Leffler et. al)

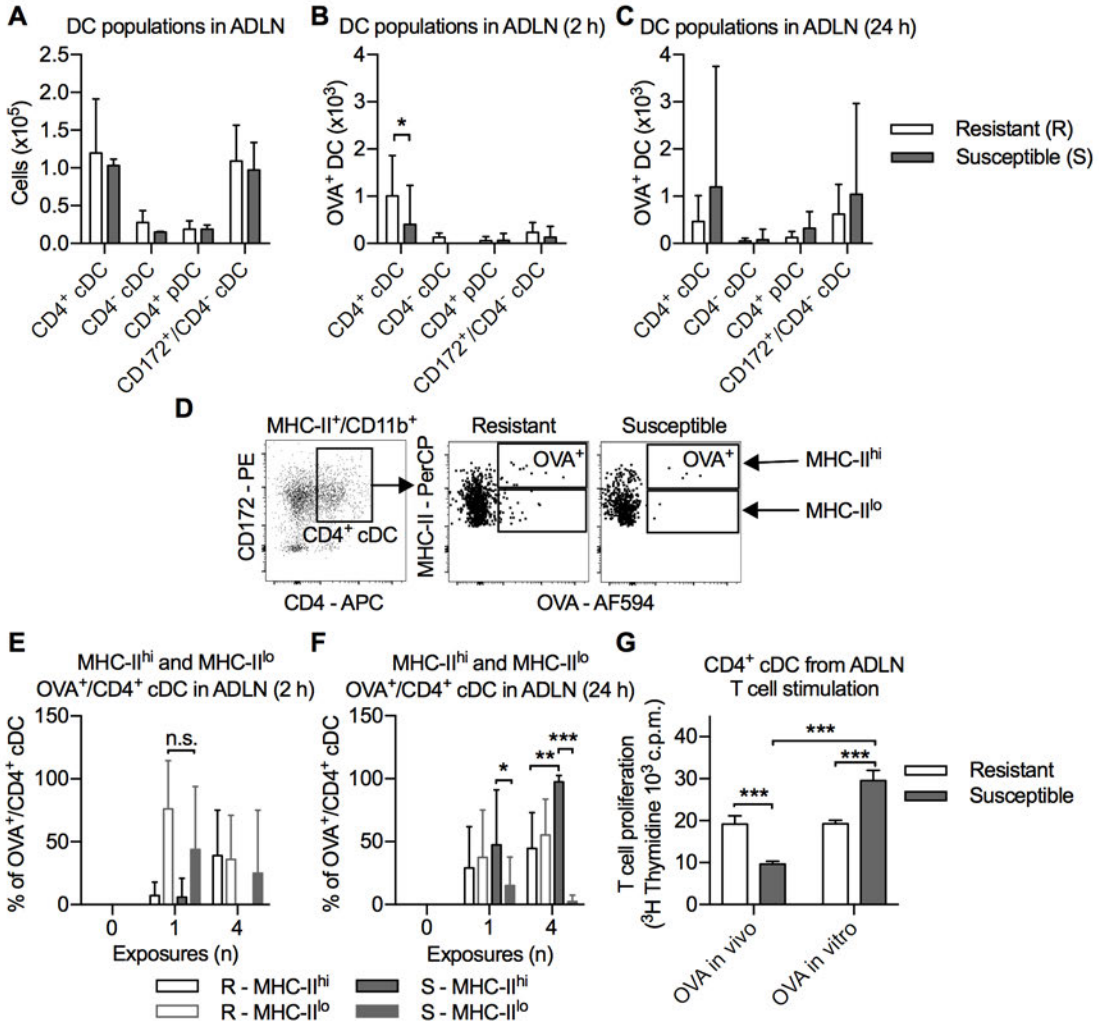


Fig 4 (Leffler et. al)

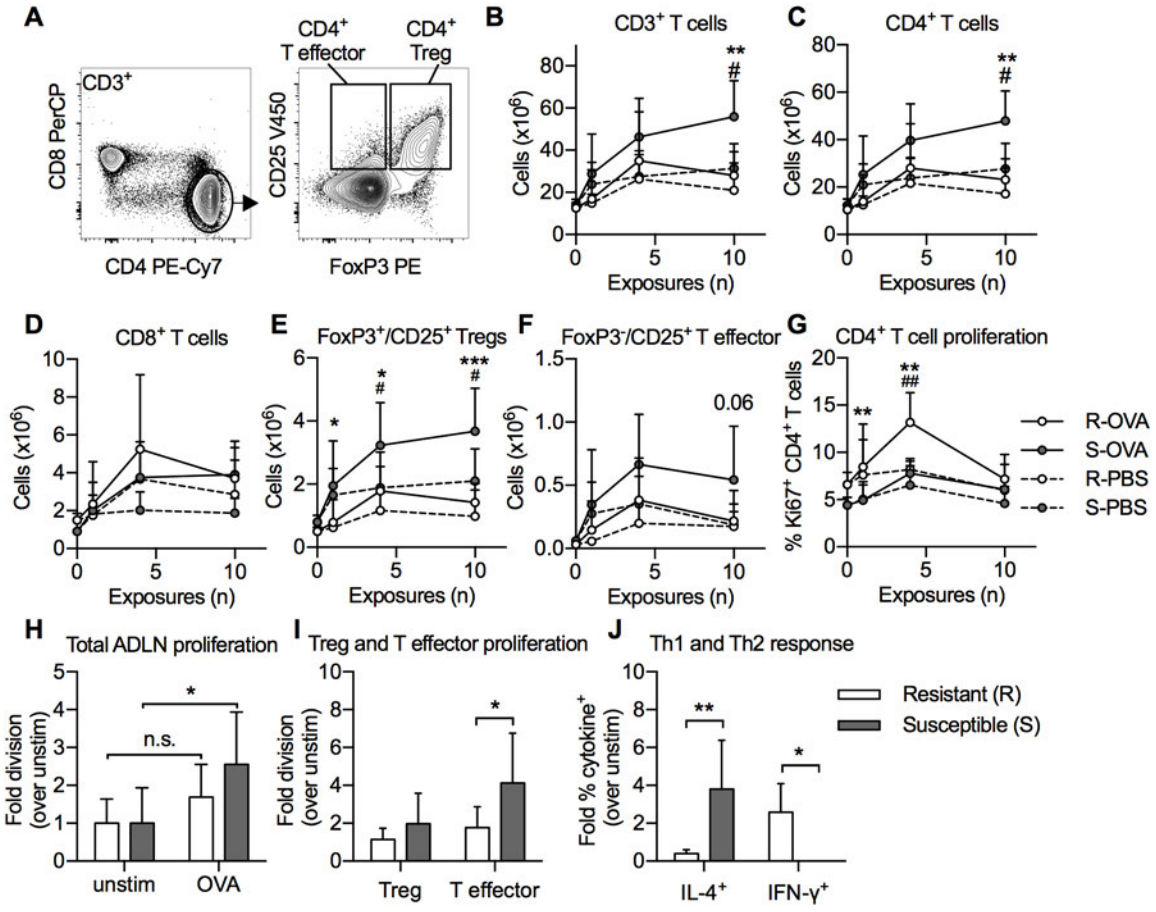
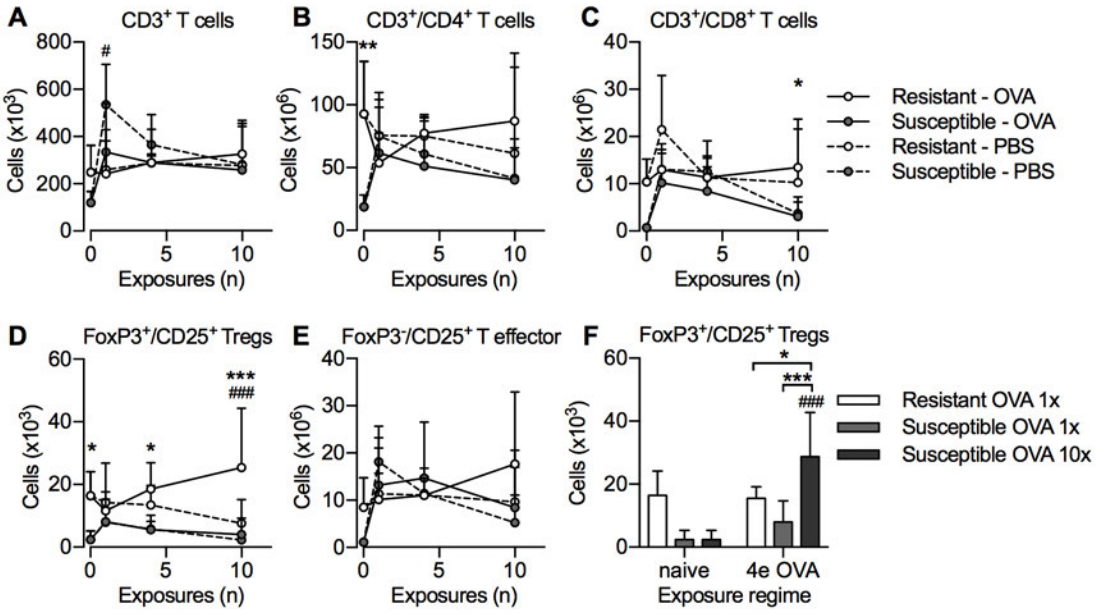


Fig 5 (Leffler et. al)



Supplementary Figure Legends

Figure S1 OVA exposure induces antigen response in both strains with airway eosinophilia in the susceptible strain. A-C Serum titres of OVA specific Ig isoforms after 10 OVA exposures (**A**) as well as OVA specific IgE (**B**) and Ig isoforms (**C**) after 21 exposure in resistant and susceptible rats. OVA specific Ig isoforms were determined by ELISA and OVA specific IgE by passive cutaneous anaphylaxis. **D-E** Proportion of immune cells in BAL after 10 (**D**) and 21 (**E**) exposures compared to naïve (**D**) or PBS (**E**) control in resistant or susceptible rat strains. Data are displayed as individual data points. Box and whiskers indicate mean of n = 4 - 11 ± 95% CI of at least 2 independent experiments. Statistical significance of differences was assessed using two way ANOVA followed by Fishers LSD post test and displayed as $^{\#}$/*: $p<0.05$, $^{\#\#}$/**: $p<0.01$, $^{\#\#\#}$/***: $p<0.001$ where * indicate comparison as depicted and $^{\#}$ indicates comparison to intra strain control.

Figure S2 DC subsets can be detected using flow cytometry in rat airway tissues but differ in phenotype between tissues and strains. A Gating strategy to detect $CD172^{+}/CD4^{-}$ cDC, $CD4^{+}$ cDC, $CD4^{-}$ cDC and $CD4^{+}$ pDC in lung from resistant rat strain using flow cytometry. **B** Identified DC subsets were sorted and stained with Eosin-Azure B and analysed at 400x magnification. **C** Expression of MHC-II, CD4, CD172, CD11b and CD103 on each DC subset in both trachea and ADLN from naïve susceptible (S) and resistant (R) rats. Data are displayed as mean ± SD of n = 4 from at least 2 independent experiments. Statistical significance of differences was assessed using two way ANOVA followed by Fishers LSD post test and displayed as *: $p<0.05$, ** $p<0.01$.

Figure S3 OVA uptake in DC subsets in susceptible and resistant strains. A Proportion of DC subsets of $MHC\text{-}II^{+}$ cells in lung digests from the resistant and susceptible strain**. B-C**

OVA uptake in trachea of susceptible (**B**) and resistant (**C**) rats 2 h post OVA-AF594 or PBS exposure in each DC subset. **D** Expression of TLRs in $CD4^+$ cDC sorted from naïve airways in each strain. Expression was determined by reverse transcription quantitative PCR using QuantiTect kit (Qiagen) and primers as previously described (1). Data are displayed as mean ± SD from n = 3 - 4 of at least 2 independent experiments. Statistical significance of differences was assessed using two way ANOVA followed by Fishers LSD post test (**A**) or students t-test for independent comparisons of expression data (**D**) and displayed as *: $p<0.05$, ** $p<0.01$. For **B-C** representative plots, displaying OVA-uptake for each strain, are shown for 1 out of 6 animals and compared to representative PBS controls. Numbers indicate percentage of OVA^+ cells in respective subset, strain and treatment.

Figure S4 OVA exposure induces exaggerated ADLN expansion but maintain T cell proportions in both strains. A Total cell numbers of ADLN in OVA or PBS exposed resistant (R) and susceptible (S) rats. **B-E** Proportion of $CD4^+$ (**B**) or $CD8^+$ (**C**) cells as proportion of $CD3^+$ T cells or $CD4^+$ Treg (**D**) or $CD4^+$ T effector (**E**) as proportion of $CD4^+$ T cells in ADLN of OVA or PBS exposed resistant and susceptible rats after 0-10 exposures. Data are displayed as mean ± SD from n = 3 - 8 collected over at least 2 independent experiments. Statistical significance of differences was assessed using two way ANOVA followed by Fishers LSD post test and displayed as n.s: not significant, $^{\#}$: $p<0.05$, $^{\#\#}$/**: $p<0.01$. * indicates comparison as depicted and $^{\#}$ indicates comparison to intra strain PBS control.

References

1. Strickland DH, Thomas JA, Mok D, Blank F, McKenna KL, Larcombe AN, et al. Defective aeroallergen surveillance by airway mucosal dendritic cells as a determinant of risk for persistent airways hyper-responsiveness in experimental asthma. Mucosal Immunol. 2012;5(3):332-41.

Fig S1 (Leffler et. al)

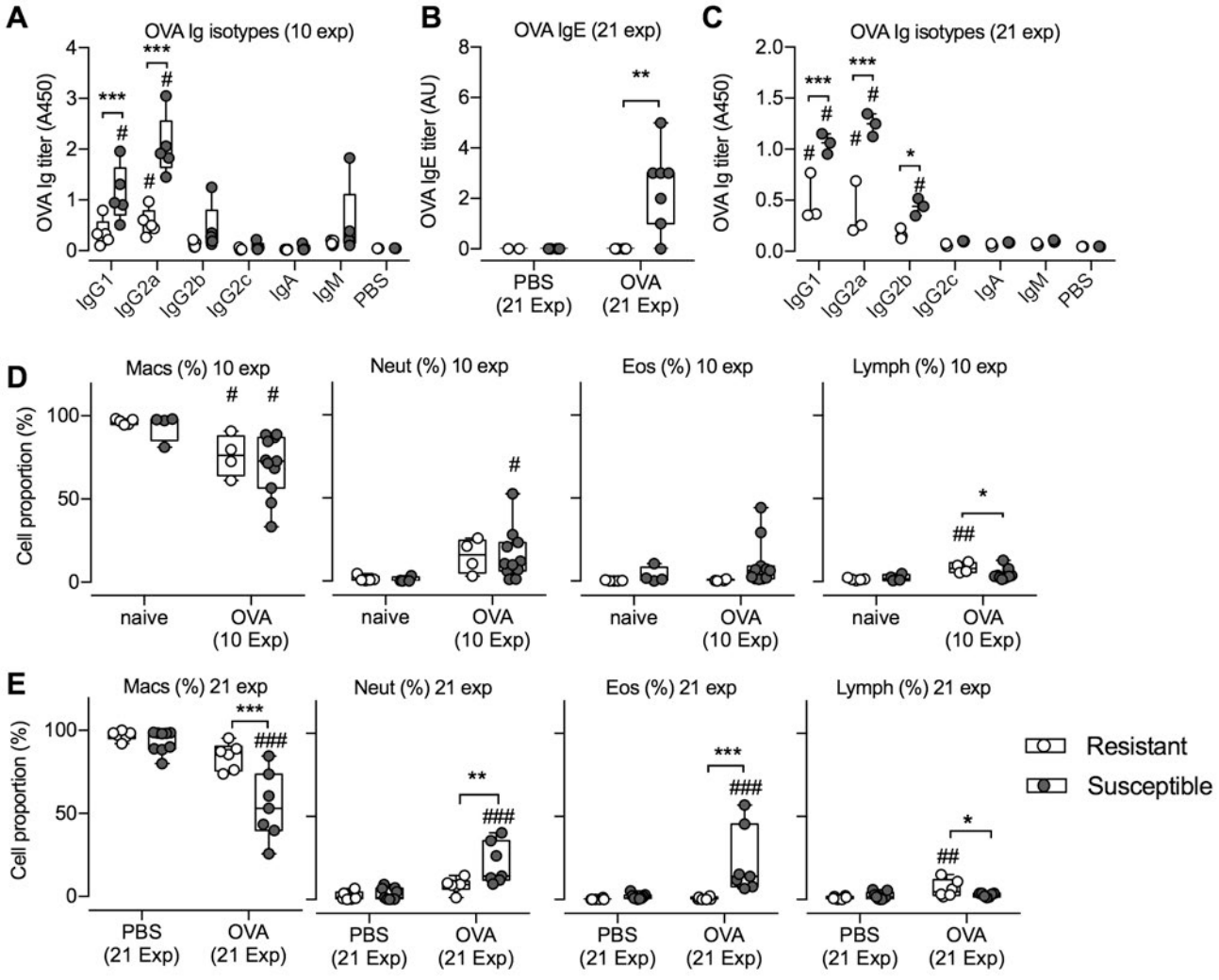


Fig S2 (Leffler et. al)

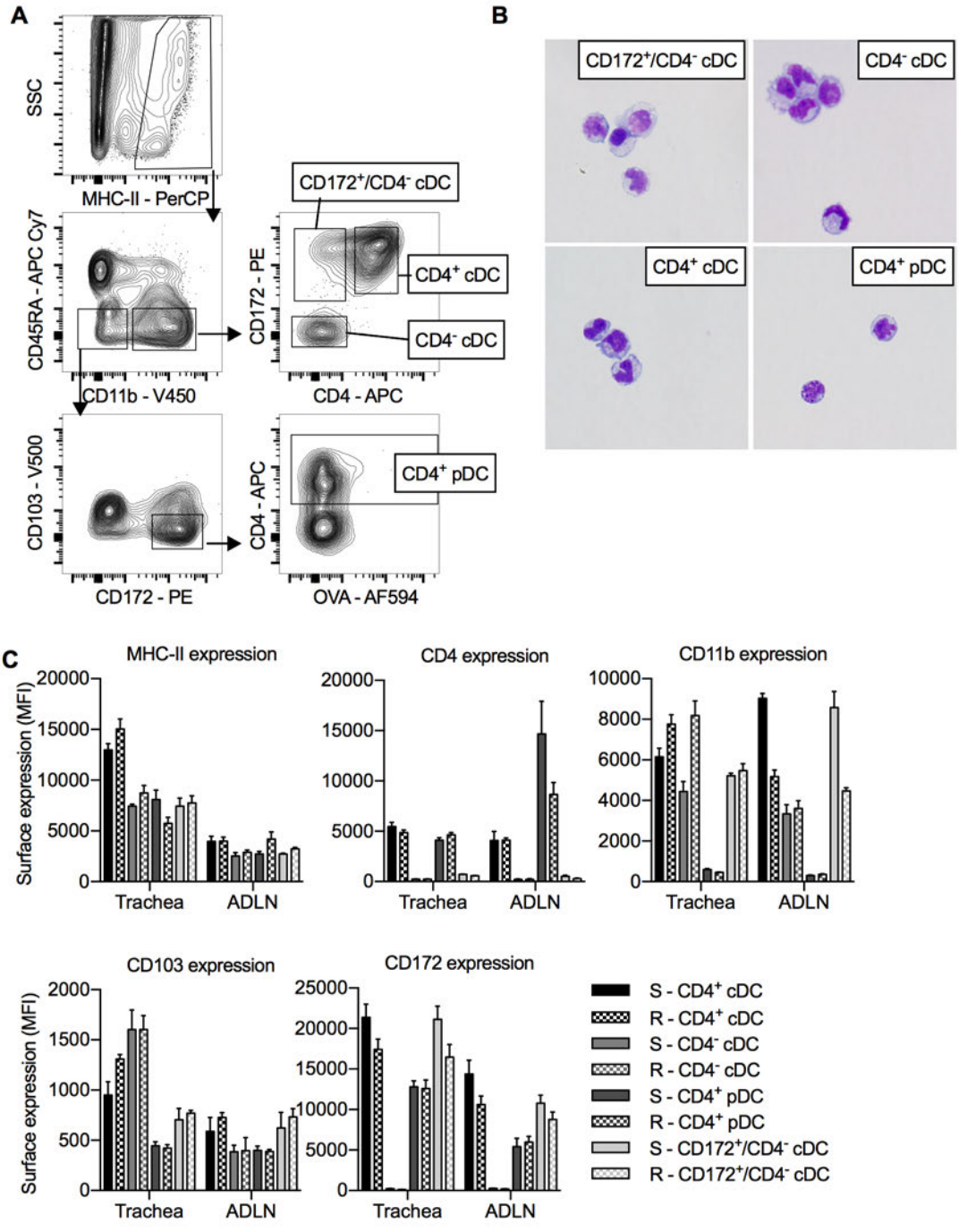


Fig S3 (Leffler et. al)

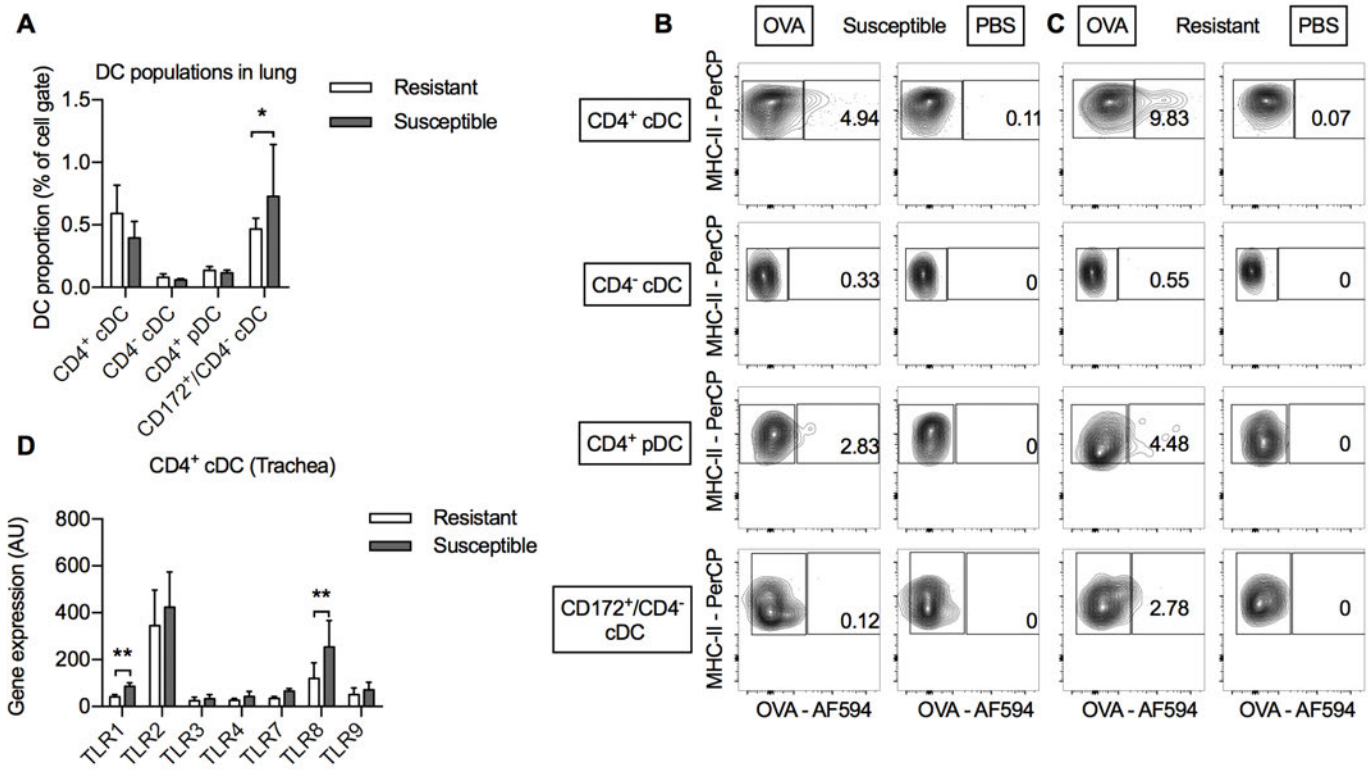


Fig S4 (Leffler et. al)

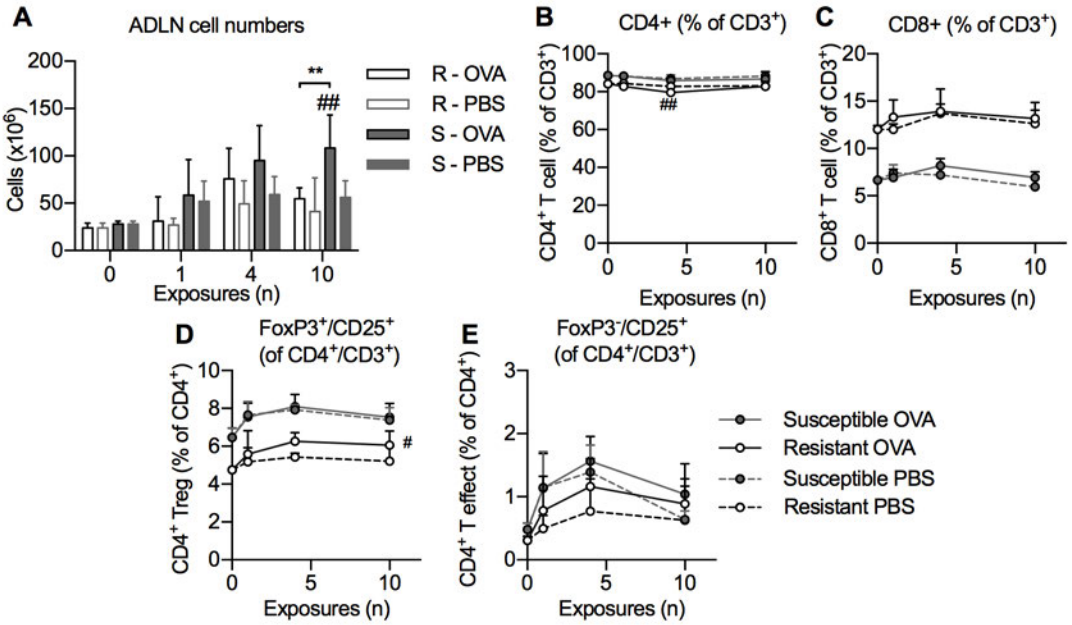


Table S1 Number of animals included in each experimental group for flow cytometry analysis. All experimental groups were collected at minimum two independent times.

Exposures	PVG 2 h OVA	PVG 24 h OVA	PVG 24 h PBS	BN 2 h OVA	BN 24 h OVA	BN 24 h PBS
0	4			4		
1	6	6	3	6	8	4
4	4	4	4	4	4	4
10	6	6	8	4	4	4