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Inhaled *Acinetobacter lwoffii* exposure promotes lung PD-L1^{+} neutrophils and dampens viral-induced type 2 immunity

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Introduction

Wheeze is a common childhood respiratory symptom triggered by seasonal viral infections. Approximately 30–50% of children are diagnosed with at least one wheezing episode in the first three years of life.[1] Wheezing disorders resolve in most children, but approximately 30% of children under 5 years continue to have recurrent wheezing episodes triggered by viral infections and an increased risk of developing asthma in childhood.[2,3]

Accumulating evidence from numerous epidemiological studies has shown the protective effect of the farming environment in reducing the risk of childhood allergies and asthma[4,5]. Exposure to farmyard microbes has been identified as being important in mediating this protective effect.[6,7] Experimental studies have demonstrated intranasal administration of inactivated cowshed-derived bacteria strains, *Lactococcus lactis, Acinetobacter lwoffii* (*A.lwoffii*)[8] or *Staphyloccus sciuri*[9] can protect from allergen-induced airway inflammation in adult mice. We have shown protection from house dust mite (HDM)-induced airways disease in early life following inhaled, lyophilised *A.lwoffii* in neonatal mice.[10] Epidemiological studies suggest farm environments also protect from wheezing independent of allergen sensitisation[11], while children with the risk allele in the 17q21 locus, common to both viral wheeze and asthma, are protected if they are brought up on farms.[12] We hypothesised that exposure to inhaled farmyard microbes would protect against viral-induced pulmonary pathology that is associated with wheezing. Using a previously established neonatal murine model of recurrent RSV infection,[13] we investigated the effect of inhaled, lyophilised *A.lwoffii* exposure on viral-induced immune responses and the molecular mechanisms mediating immune protection.

Materials and methods

Animals, viral infection and bacterial exposure

Neonatal BALB/c mice were bred in-house and exposed to bacteria and/or viral infection followed by published procedures.[10,13] Further details are provided in the Online Repository.

Cell recovery

WT BALB/c neonatal mice received a euthanising dose of sodium pentobarbital intraperitoneally (i.p.) followed by exsanguination via subclavian vein. Bronchoalveolar lavage (BAL) was performed by cannulating the trachea and flushing the lungs with sterile PBS three times with methods detailed in previous study[14] and detailed in Online Repository.

Flow cytometry

Surface and intracellular stains were performed. Protocols for staining, antibodies used for surface and intracellular staining and gating strategies for myeloid cells and innate lymphoid cells are detailed in Online Repository.

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Protein measurements

Homogenisation of lung tissues were performed, measurement of IFNγ, IL-13, IL-17A and IL-10 was undertaken as per the manufacturer's instructions (Table S2). IL-5 from BAL and lung supernatant was determined by an in-house ELISA detailed in Online Repository.

Quantitative RT-PCR

Quantitative RT-PCR of selected genes of interest was performed and the viral load *in vivo* was assessed according to published methods.[15]

Pulmonary neutrophil bulk RNA sequencing and analysis

Methods of neutrophil enrichment and bulk RNA sequencing and analysis is detailed in in Online Repository. The RNAseq data provided in this manuscript will be available from the corresponding author upon reasonable request.

Statistical analysis

All results were expressed as mean and standard error of the mean (SEM). Data were analysed using GraphPad Prism 9 software. Mann-Whitney or Kruskal Wallis with post hoc Dunn's multiple comparisons test on non-parametric data were performed to detect differences between groups. Statistical significance was accepted when $P < 0.05$.

Results

Early-life continuous exposure to lyophilised, inhaled A.lwoffii dampens type-2 immunity induced by secondary RSV infection in neonatal mice

To determine whether inhaled *A.lwoffii* exposure would reduce viral-induced type 2 immunity, neonatal BALB/c mice were administered lyophilized and inactivated *A.lwoffii* intranasally (i.n.) every other day from postnatal day 3 (PND3) to PND19, in conjunction with primary and secondary RSV infections (indicated as ReRSV) at PND5 and PND15 (Fig. 1a).[10,13] While no significant impact on growth rate (Fig. S1a) was observed, a significant reduction in viral load (as measured by RSV L gene) was observed at PND20 in RSV infected neonatal mice following *A.lwoffii* exposure compared to infection alone (Fig. 1b). Overall pulmonary immune responses, characterised by $CD45^+$ leukocytes, were equally elevated in the lungs of neonatal mice that received *A.lwoffii* exposure alone, RSV infection alone and a combination of *A.lwoffii* and RSV infection (Fig. 1c). Further phenotyping of various immune cell subsets by flow cytometry (Fig. S1b) revealed a significant increase in the proportions and numbers of $Ly6C^+CD11b^+$ inflammatory macrophages and monocytes (IMM), $CD64^+F4/80^+CD11b^+CD11c^-SiglecF^-$ interstitial macrophages and $Ly\text{-}6G^+CD11b^+$ neutrophils in lungs and airways (Fig. 1d and e, Fig. S1c) of neonatal mice that had *A.lwoffii* exposure. In contrast, RSV infection promoted immune responses with increased proportions and numbers of $CD4^+$ and $CD8^+$ T cells (Fig. 1d and f), alongside eosinophils (Fig. 1g). Early life exposure to a farming environment is associated with lower asthma prevalence[16] with children living on traditional farms having lower circulating eosinophils than those who do not.[15] Consistently continued exposure to *A.lwoffii* concomitant with RSV infection resulted in reduced pulmonary eosinophils and eosinophil to neutrophil ratio in lungs and airways compared to recurrent RSV infection alone (Fig. 1g), alongside reduced RSV-induced mucus hypersecretion (Fig. 1h and i). Overall, continuous exposure to inhaled farmyard microbes reduced RSV-induced eosinophilia and mucus hypersecretion in neonatal mice.

Exposure to A.lwoffii reduced Th1 and Th2 responses in RSV infected neonatal mice

To determine whether *A.lwoffii* exposure modulated T cell responses generated during RSV infection, pulmonary cytokines and intracellular cytokine production by $CD4^+$ T cells were analysed (Fig. 2a–j). Exposure to inhaled *A.lwoffii* significantly dampened both RSV-induced type 1 cytokine IFNγ (Fig. 2a,b) and type 2 cytokines IL-5 and IL-13 (Fig. 2c–f) in the lungs of the neonatal mice. *A.lwoffii* exposure, with or without RSV infection, preferentially increased IL-17A (Fig. 2g), a pleotropic cytokine that is important for the mobilisation of neutrophils,[17] concomitant with increased $IL\text{-}17^+CD4^+$ T cells in these groups (Fig. 2h). While comparable concentrations of IL-10 in the lungs of neonatal mice from all groups were seen (Fig. 2i), $IL\text{-}10^+CD4^+$ T cell numbers were elevated during all treatment conditions compared to PBS controls (Fig. 2j). Similar findings were also observed with $CD4^+$ T cells in the airways (Fig. S2a). Following *ex vivo* stimulation with RSV peptides, *A.lwoffii* suppressed virus-specific $IL\text{-}5^+$and $IL\text{-}13^+$, but not $IFN\gamma^+CD4^+$ T cells and increased $IL\text{-}10^+CD4^+$ T cells (Fig. 2k), suggesting specific immune modulation of virus-specific Th2 inflammation following *in vivo* treatment with *A.lwoffii*. In addition, *A.lwoffii* exposure suppressed viral-induced expansion of polyclonal $IFN\gamma^+$, $IL\text{-}10^+$ and $IL\text{-}13^+$ $CD8^+$ T cells (Fig. 2l and Fig. S2b; RSV-specific $IFN\gamma^+CD8^+$ and $IL\text{-}10^+CD8$ T cells were however not affected (Fig. S2c).

Since innate lymphoid cells (ILC) are also known to facilitate antiviral type 1 immune responses[18] or may potentiate viral-induced type 2 responses,[19] various ILC subsets were analysed. Notably, $Tbet^+$ type 1 innate lymphoid cells (ILC1) and natural killer cells (NK) increased in response to RSV infection (Fig. S2d and Fig. S2e). However, neither their number nor their IFNγ production were affected by *A.lwoffii* exposure (Fig. S2d and Fig. S2e). *A.lwoffii* exposure increased total $GATA3^+$ activated type 2 innate lymphoid cells (ILC2) compared to untreated mice (Fig. S2f). However, analysis of intracellular cytokine production revealed no differences in the number of $IL\text{-}5^+$ ILC2s or $IL\text{-}13^+$ ILC2s in infected animals, with or without *A.lwoffii* exposure (Fig. 2m and Fig. S2g). Overall, exposure to *A.lwoffii* dampened type 1 and type 2 immunity induced by early life RSV infection, predominantly via effects on $CD4^+$ T cells.

Continous A.lwoffii exposure is required for maximal suppression of RSV-induced type 2 immune response in early life

Epidemiological studies suggest that early, frequent and long-term exposure to farmyard dust is necessary for maximal protection from asthma,[20,21] while the priming of primary T cell responses to infection determines their subsequent development upon reinfection. We therefore tested the effect of continuous exposure to inhaled *A.lwoffii* throughout the primary and the secondary RSV infection against shorter periods of exposure during the initial RSV infection, to investigate if *A.lwoffii* exposure during the first RSV infection would be sufficient to limit their type 2 responses post secondary infection. To this end, RSV-infected BALB/c neonatal mice received 2 (2x) or 4 (4x) doses of *A.lwoffii* every other day from PND3 to PND5 or PND10. This was compared to continuous *A.lwoffii* exposure (x8) from PND3 to PND19 (Fig. 3a). A partial reduction in the number of pulmonary $IL\text{-}5^+CD4^+$ and $IL\text{-}13^+CD4^+$ T cells was observed in infected mice with 2x or 4x *A.lwoffii*, compared to RSV infection alone (Fig. 3b). Continuous (8x) *A.lwoffii* elicited further reduction in pulmonary Th2 cells (Fig. 3b), although graded reduction in eosinophils, *Il5, Il13* and *muc5ac* expression was less clear (Fig. 3c, d and e). This suggests the suppressive effect of inhaled *A.lwoffii* on type 2 inflammation is not limited to the period of T cell priming during primary RSV infection, but requires continuous exposure to be most effective.

Dendritic cells (DC) are vital to prime T cell responses, however in neonatal mice that exhibited reduced type 2 responses with 4x *A.lwoffii* and 8x *A.lwoffii*, lower DC numbers were observed with 2x or 4x *A.lwoffii*

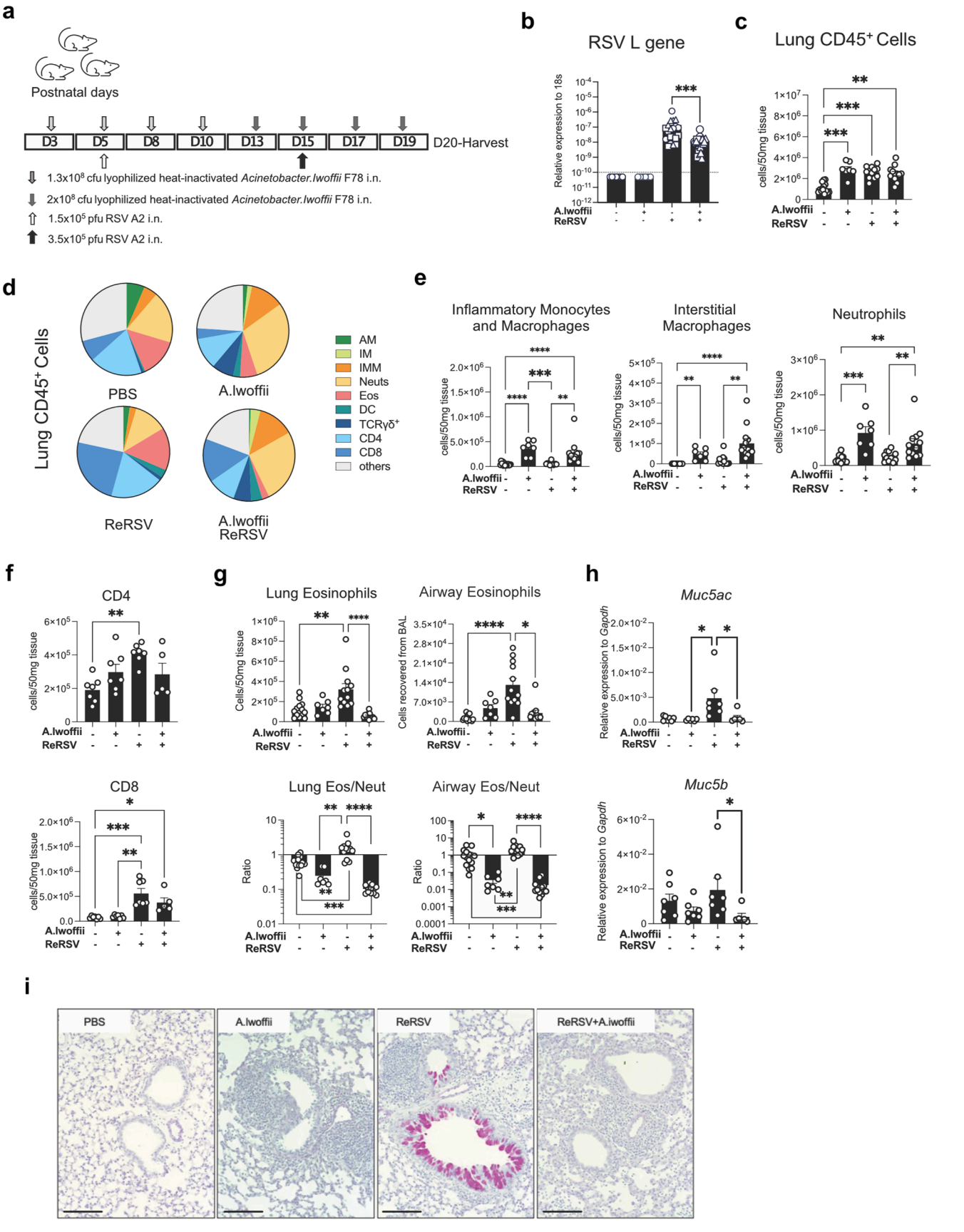

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Fig. 1. Continuous exposure to inhaled lyophilised inactivated farmyard microbes *A.lwoffii* dampened neonatal viral-induced lung diseases. (a) Neonatal BALB/c mice received intranasal delivery of lyophilised inactivated *A.lwoffii* from PND3 to PND19 every other day in conjunction with primary and secondary RSV infection at PND5 and PND15, at indicated doses respectively. Pulmonary immune responses and inflammation were analysed 5 days post recurrent infection (5dpri) at PND20. (b) Expression of RSV L gene relative to *18 s* housekeeping gene from lung. Data pooled from three independent experiments indicated by circles, squares and triangles respectively. (c) Number of total $CD45^+$ leukocytes in the lung enumerated by flow cytometry. (d) Proportions of various immune cell populations in lung. (e) $CD11b^+Ly6C^+$ inflammatory monocytes and macrophages, interstitial macrophages and neutrophils in the lung enumerated by flow cytometry. (f) Number of $CD4^+$ T cells, $CD8^+$ T cells in the lung enumerated by flow cytometry. (g) Number of eosinophils and eosinophil to neutrophil ratio in lungs and airways. (h) Expression of mucus related genes *Muc5ac* and *Muc5b* relative to *Gapdh* in the lung. (i) Representative image of Periodic Acid-Schiff (PAS) staining on lung tissue sections acquired by Leica Microscope at x200 magnification. Scale bar represents 100 μm. Non-parametric Mann-Whitney tests performed in (b). Kruskal-Wallis with post-hoc Dunn's tests performed in (e–h). Statistical significance indicated by *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$0.001 < P < 0.0001$, ****$0.0001 < P < 0.00001$.

exposures, while higher DC numbers were observed with continuous exposure, when compared with RSV infection alone (Fig. 3f). In contrast to DCs, lung neutrophil numbers were low in RSV-infected animals and those with short *A.lwoffii* exposures, but were found to be significantly increased in infected mice that received 8 doses of *A.lwoffii* continuously (Fig. 3g). This suggests that frequent and continuous *A.lwoffii* exposure, but not RSV infection, sustained an elevated number of neutrophils in the lung. Neutrophils are potent and immediate responders to microbial exposures, critical for the clearance of pathogenic bacteria to prevent infectious diseases. Excessive mobilisation and accumulation of neutrophils, however, may play a pathological role in chronic diseases. Increased circulating neutrophils which had a specific phenotype characterised by reduced expression of CXCR4 and CD11b were reported in Amish children from traditional farming environments who were protected from asthma.[6] In line with the data from children, neonatal mice exposed to *A.lwoffii* also had enhanced circulating neutrophils (Fig. 3h), which unlike those found after RSV infection, expressed lower CXCR4 and CD11b (Fig. 3i).

Early life exposure to A.lwoffii induced neutrophils with a distinct phenotype compared to RSV infection or neutrophil chemoattractant

To further understand phenotypic differences with regards to maturation and activation in the neutrophils induced by different exposures in early life, various surface markers were compared 24 h after inhalation of *A.lwoffii* and/or primary RSV infection and/or neutrophil chemoattractant KC (CXCL1), in 5 day old neonatal BALB/c mice (Fig. 4a). KC was used to compare the neutrophils induced by *A. lwoffii* and RSV to those found in a model of neutrophil recruitment to a sterile environment.[22,23] Significant changes in neutrophil abundance were not observed in the periphery, however, recruitment of neutrophils to the airways was readily detected following all exposures, with variability in the degree of neutrophil mobilisation (Fig. 4b). *A.lwoffii* or a combination of both *A.lwoffii* and RSV induced most neutrophils in the lungs and airways, compared to KC, RSV, or KC with RSV (Fig. 4b). Neutrophils induced by inhaled *A.lwoffii* also exhibited a different phenotype to those induced by RSV or KC (Fig. 4c, Fig. S3a). KC-induced neutrophils are distinct in their low expression of CD64, the neutrophil activation marker,[23] across all anatomical locations. The activation state of neutrophils induced by KC and RSV mirrors those induced by RSV alone, with high expressions of CD64, CD49d, TLR4 at all sites and high expressions of CD62L, CD11b, CXCR4, CXCR2 and CD101 in the blood. In contrast, *A.lwoffii*-induced neutrophils, with or without RSV infection, displayed a reduction in the expression of CD101, TLR4, CD49d across all tissue types, and reduced expressions of CXCR4 and CXCR2 in the blood and lung (Fig. 4c and Fig. S3a). Collectively, there was a distinct expression profile and phenotype of *A.lwoffii*-induced neutrophils compared to RSV-induced or KC-induced neutrophils.

To delineate the phenotypic differences of neutrophils associated with acute response to secondary RSV infection or continuous *A.lwoffii* exposure, neonatal mice were sacrificed at PND16, which was approximately 18 h post secondary RSV infection (Fig. 4d and Fig. S4a). Neutrophil frequencies were increased in the lungs and airways of animals treated with *A.lwoffii* or RSV alone, with the highest increase observed in combined treatment (Fig. S4b). Flow cytometric analysis of surface marker expression on neutrophils showed RSV-specific upregulation of CD64 in the lung and airway (Fig. S4c). The reduction of CXCR4 and CD62L expression on neutrophils was significant when comparing *A.lwoffii* or *A.lwoffii* with RSV group with PBS, but not between RSV-infected animals with PBS control (Fig. S4c). Overall, continuous *A. lwoffii* exposure induced neutrophils with a different phenotype compared to RSV infection (Fig. 4e).

Differential gene expression and altered gene responses following A.lwoffii exposure or RSV infection

Having identified distinct neutrophil phenotypes induced by *A. lwoffii* compared to RSV infection, and to understand the transcriptional differences in the respective neutrophils, we performed bulk RNA-seq on enriched and sorted neutrophils (DAPI-stained and $Ly6G^+$) from the lung 24hrs after continuous *A.lwoffii* exposure, secondary RSV infection or *A.lwoffii* concomitant with RSV infection (Fig. 5a). Intravenous labelling of neutrophils revealed that almost all neutrophils from PBS control mice were intravascular (Fig. S5a), consistent with previous reports.[24] *A.lwoffii* exposure or viral infection meanwhile promoted extravasation of neutrophils into the pulmonary parenchyma and airways (Fig. S5a). Principle component analysis (PCA) showed that neutrophils isolated from lung vasculature of PBS-treated mice separated from those isolated from challenged mice (Fig. S5b), demonstrating that steady state intravascular lung neutrophils are transcriptionally distinct to neutrophils from infected animals. While *A.lwoffii*-induced neutrophils clustered away from the RSV-induced neutrophils, neutrophils from mice treated with *A.lwoffii* and RSV did not cluster distinctly from *A.lwoffii* or RSV alone (Fig. S5b). Gene set enrichment analysis (GSEA)[25] of pre-ranked differentially expressed genes (adjusted P value < 0.05, absolute Log2(FC) > 2.5) from paired comparisons amongst four treatment conditions, i.e. PBS, *A.lwoffii* only, RSV only, *A.lwoffii* and RSV (Fig. S5c) demonstrated shared and distinct hallmark pathways that are regulated by different treatments (Fig. 5b). RSV infection significantly upregulated IFNα and IFNγ responses while numerous pathways including NK-kB mediated TNFα signalling, protein secretion, oxidative phosphorylation were downregulated. In contrast, *A.lwoffii*-induced neutrophils, with or without RSV, were more transcriptionally active and upregulate many hallmarks inflammatory and metabolic activities (Fig. 5b).

Analysis of commonly upregulated genes using Venn diagrm revealed 112 genes uniquely expressed by RSV infection and 2621 genes uniquely expressed by *A.lwoffii*. 40 genes were uniquely upregulated by combined *A.lwoffii* exposure and RSV infection (Fig. 5c), these genes include interferon-stimulated genes, neutrophil surface markers and activation genes such as *Ly6c2, Ly6a, Lgals3bp,* and genes involved in aging such as Exog and in NETosis such as *Mlkl, Ccnb1* (Supplementary Table S4). We focused on the 377 genes that were commonly upregulated by *A.lwoffii* only and *A.lwoffii* with RSV, but not RSV only (Fig. 5c). K-means clustering analysis was performed on these 377 genes to identify clusters with similar expression profiles and potentially common to the same pathway (Fig. 5d). Notably, genes involved in antimicrobial and proinflammatory responses (*Il1a, Il1b* and *Il23a*),

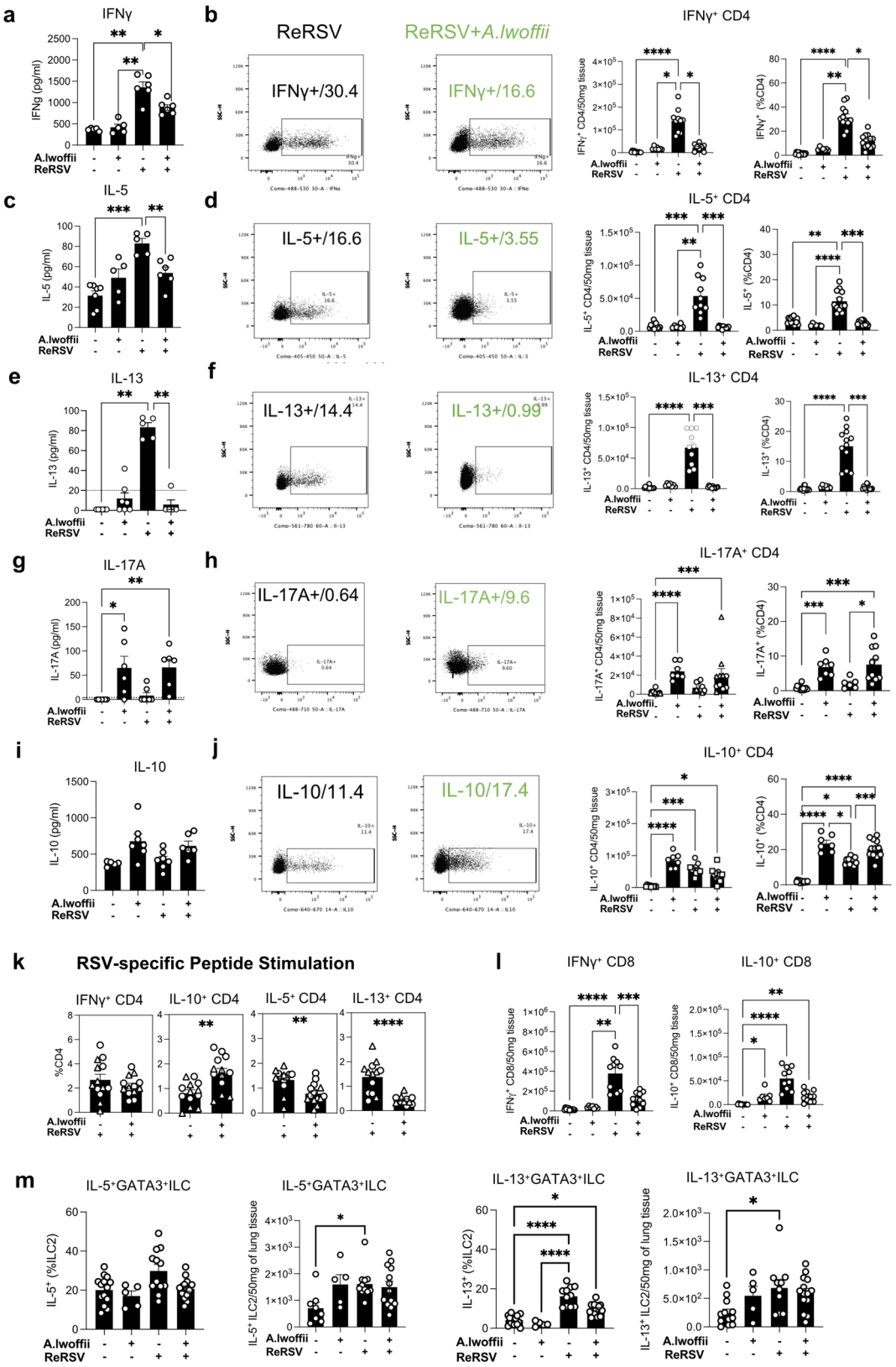

Fig. 2. Exposure to *A.lwoffii* reduced viral-induced type 2 inflammation in neonatal mice. Protein levels of IFNγ (a), IL-5 (c), IL-13 (e), IL-17A (g) and IL-10 (i) in lung homogenates measured by ELISA. Representative flow cytometry plots, numbers and percentages of $CD4^+$ T cells producing intracellular cytokine, including IFNγ (b), IL-5 (d), IL-13 (f), IL-17A (h) and IL-10 (j) following *ex vivo* stimulation of PMA and ionomycin in the presence of Brefeldin A for 4 h. (k) Cytokine production from $CD4^+$ T cells following 5 h *ex vivo* RSV-specific peptide stimulation. Circles and triangles represent data from two independent and repeated experiments. Following *ex vivo* stimulation of PMA and ionomycin in the presence of Brefeldin A for 4 h, IFNγ and IL-10 production by $CD8^+$ T cells (l), IL-5 and IL-13 production by activated $GATA3^+$ ILC2s (m) were measured. Kruskal-Wallis with post-hoc Dunn's tests performed in (a–J), (l) and (m). Non-parametric Mann-Whitney tests performed in (k). Statistical significance indicated by *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$0.001 < P < 0.0001$, ****$0.0001 < P < 0.00001$.

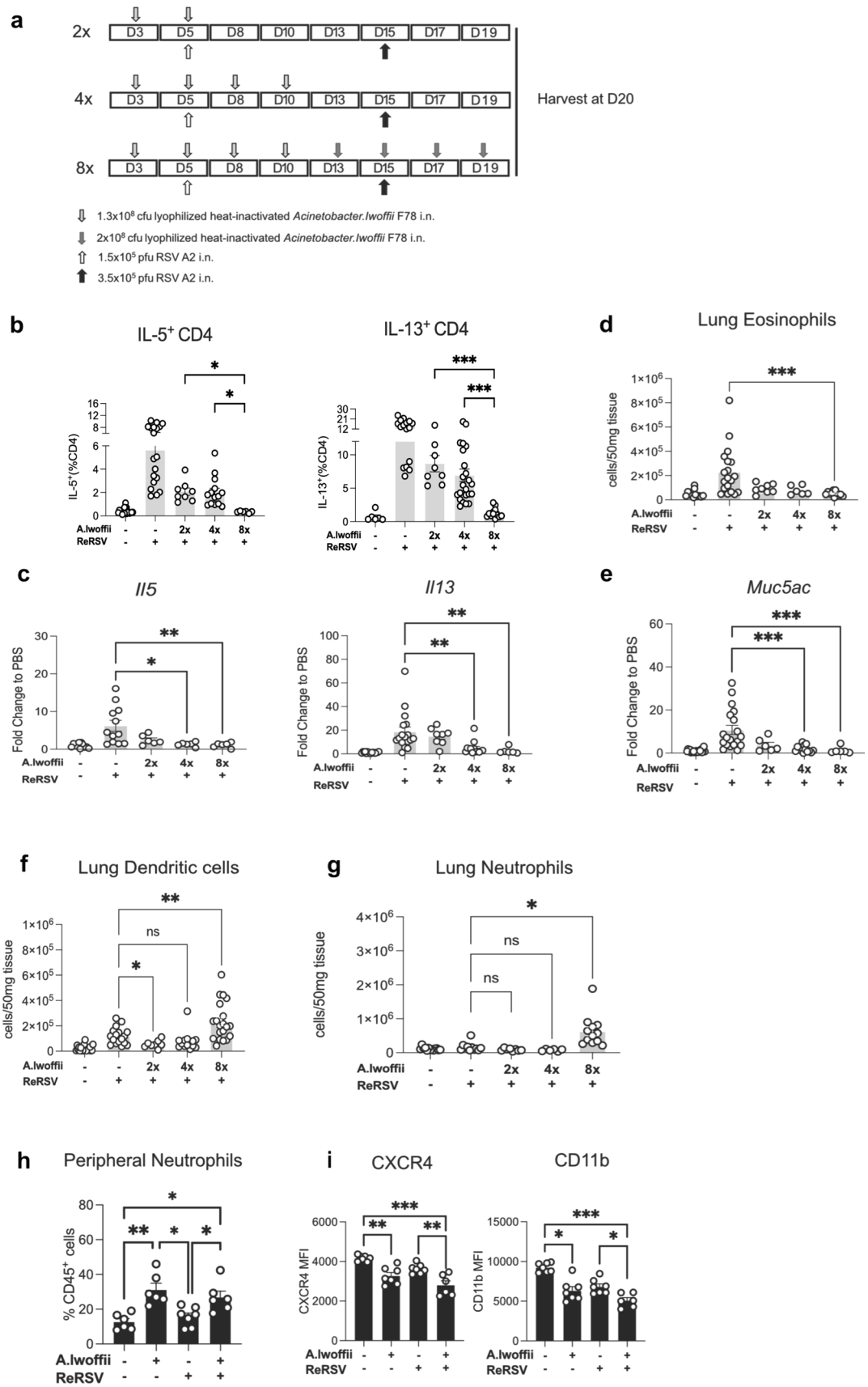

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Fig. 3. Early *A.lwoffii* exposure during DC priming of T cells was not sufficient to achieve maximal type 2 suppression. (a) Neonatal BALB/c mice received intranasal delivery of lyophilised inactivated *A.lwoffii* from PND3 to PND5 (2x), PND3 to PND10 (4x) or PND3 to PN19 (8x) every other day in conjunction with recurrent RSV infection at PND5 and PND15. Pulmonary immune response and inflammation were analysed 5 days post recurrent RSV infection (5dpri). (b) IL-5 and IL-13 production by $CD4^+$ T cells following 4 h *ex vivo* PMA and ionomycin stimulation. (c) Expression of *Il5* and *Il13* genes in the lung. (d) Number of lung eosinophils. (e) Gene expression of *Muc5ac* in the lung.(f) Number of total $MHCII^+CD11c^+$ DCs in the lung. (g) Number of neutrophils in the lung. In the model of continuous *A.lwoffii* exposure (8x), (h) proportion of peripheral neutrophils. (i) Surface expression of CXCR4 and CD11b on circulating neutrophils. Kruskal-Wallis tests with Dunn's multiple comparisons tests among 2x, 4x and 8x *A.lwoffii* in (b). Non-parametric Mann-Whitney tests performed between RSV infected animals exposed to various doses of *A.lwoffii* to recurrent RSV infection alone in (c-g). Statistical significance indicated by $*0.01 < P < 0.05$,$**0.001 < P < 0.01$, $***0.001 < P < 0.0001$, $****0.0001 < P < 0.00001$.

granulocyte maturation and recruitment (*Csf3* and *Ccl4*) were found in cluster 1, which was most highly upregulated in *A.lwoffii*-induced neutrophils, but relatively reduced in mice with concomitant recurrent RSV infection and *A.lwoffii* exposure (Fig. 5d). In cluster 2, genes involved in immunoregulation such as *Il10* and *Cd274*, which encodes PD-L1, were observed to be equally elevated in *A.lwoffii*-induced neutrophils and neutrophils induced by *A.lwoffii* exposure and RSV infection combined (Fig. 5d and e). PD-L1 is the most widely expressed ligand for PD-1, a co-inhibitory receptor found on T cells which can potently inhibit T cell activation and function.[26] Recently PD-L1 expression on neutrophils has been reported in various diseases such as systemic lupus erythematosus (SLE), tuberculosis and injury-induced infections,[27–29] and neutrophils that express *Cd274* are reported in two hubs in an integrated map of global neutrophil compartments in mice called NeuMap, one hub that shares signature genes with infection and another one with immunosuppression.[30] Collectively, these data suggest that lung neutrophils undergo differential transcriptional activity under the various challenges, RSV infection inducing type I and III interferon pathways and continuous *A.lwoffii* exposure upregulating anti-microbial and immunoregulatory genes, in addition to promoting type I and III interferon pathways. Furthermore, the combined treatment of RSV and *A.lwoffii* was able to sustain the expression of immunoregulatory genes, while reducing genes with proinflammatory properties.

Exposure to inhaled A.lwoffii induced PD-L1$^+$ pulmonary neutrophils in neonatal mice

Given PD-L1 can be a potent inhibitor of T cell activation and was uniquely differentially expressed by *A.lwoffii* independent of RSV infection, we investigated PD-L1 expression on pulmonary and circulating neutrophils by flow cytometry, in addition to other phenotypic markers. Six distinct populations were determined following FlowSom clustering analysis of surface markers CD101, CXCR4, PD-L1, CD62L, CXCR2 and CD64 on circulating and tissue resident neutrophils from the four treatment conditions (PBS, *A.lwoffii* only, RSV only, *A.lwoffii* and RSV) (Fig. 6a,b). Amongst them, population 2 (Pop2) and 5 comprised neutrophils in the lung, while Pop0, 1, 3 and 4 constituted circulating neutrophils (Fig. 6c). Dissecting population subsets with respect to treatment revealed dynamic changes in neutrophils from lung and blood (Fig. 6d,e). In the blood, a dominant Pop4 of CXCR4 and CD101 expressing neutrophils was observed in PBS-treated animals (Fig. 6a,d). A graded reduction in Pop4 was observed with RSV infection alone, *A.lwoffii* alone, with the most reduction observed in mice receiving both *A.lwoffii* and RSV challenge (Fig. 6d). Instead, an increased frequency of Pop0, Pop1 was observed following combined *A.lwoffii* and RSV challenge or *A.lwoffii* alone respectively (Fig. 6d). Both populations exhibited lower CXCR4 and CD101 expression but differed in CD62L expression (Fig. 6a). Interestingly, the lung neutrophil populations, Pop2 and Pop5, were distinct from the blood neutrophils by expressing higher PD-L1(Fig. 6a). Between the two populations, Pop2 expressed higher PD-L1, but lower CXCR4 and CD101 compared to Pop5 (Fig. 6a). *A.lwoffii* treatment, with or without RSV, induced more Pop2 compared to RSV treatment (Fig. 6e), indicating that *A.lwoffii* exposure recruited immature neutrophils with immunoregulatory potential to the site of viral infection.

Late A.lwoffii intervention partially suppressed IL-13$^+$CD4$^+$ T cells and promoted emergence of CD101$^-$PD-L1$^+$ neutrophils

Given the ability of neutrophils to rapidly respond to stimuli, and following the identification of PD-L1$^+$ neutrophils distinctly induced by *A.lwoffii* exposure, we determined whether intervention with *A.lwoffii* after the first RSV infection (Late) was sufficient to promote PD-L1$^+$ neutrophils and dampen Th2 responses. To this end, neonatal BALB/c mice were exposed to inhaled *A.lwoffii* from PND13 to PND19, during the second RSV infection (Fig. 7a). *A.lwoffii* exposure promoted neutrophils in the lung, in particular the PD-L1$^+$ subsets (Fig. 7b). Notably, in contrast to a prominent expansion of PD-L1$^+$ DCs, IMMs and eosinophils following secondary RSV infection, a significant increase in the proportion and number of total PD-L1$^+$ neutrophils was observed after exposure to *A.lwoffii* and *A.lwoffii* with ~~Re~~RSV (Fig. S6a). Furthermore, lung neutrophils expressed PD-L1 constitutively (Fig. 7c and Fig. S6b), in contrast to DCs which upregulated PD-L1 expression upon viral infection or microbial exposure (Fig. S6c). Notably, the number of total DCs and the mean expression of PD-L1 on DCs were found to be equally elevated by A.*lwoffii*, RSV, or *A.lwoffii* and RSV combined, although RSV infection favoured higher PD-L1 expression on $CD103^+CD11b^-$ type 1 conventional DCs (cDC1s), while *A.lwoffii* and combined *A.lwoffii* and RSV promoted higher numbers of type 2 conventional DCs and monocytes derived DCs (moDCs) and increased PD-L1 expression on cDC2s (Fig. S6d). Comparing various phenotypic markers of PD-L1^{hi} versus PD-L1^{low} neutrophils showed that PD-L1^{hi} neutrophils had higher expression of CD64 and CD11b, lower expression of CD62L compared to their PD-L1^{low} counterparts in all treatment conditions (Fig. 7d). However, major differences in the expression of CXCR4 and CD101 were independent of PD-L1 and were reduced on both PD-L1^{low} and PD-L1^{hi} neutrophils following *A.lwoffii* exposure (Fig. 7d). Additionally, significantly more $CD101^-$ PD-L1^{hi} neutrophils were induced after *A.lwoffii* exposure either alone, or together with RSV infection, whereas $CD101^+$PD-L1^{low} neutrophil numbers remained comparable (Fig. 7e). In conjunction, late *A.lwoffii* intervention partially reduced eosinophil number and eosinophil to neutrophil ratio in the airways of RSV infected neonatal mice (Fig. 7f), along with reductions in IL-13 protein level and $IL\text{-}13^+CD4^+$ and $IL\text{-}5^+CD4^+$ T cell numbers in the lung (Fig. 7g). The suppressive effect of late *A.lwoffii* intervention on $IL\text{-}13^+$ $CD4^+$ T cells was RSV-specific (Fig. S6e). In $CD8^+$ T cells, however, late *A.lwoffii* intervention resulted in the suppression of by-stander $CD8^+$ T cell activation associated with RSV infection only, demonstrated by reduced $IFN\gamma^+CD8^+$ T cells following *ex vivo* stimulation with PMA and ionomycin, but not with RSV peptides (Fig. S6f). Proportions of activated RSV-specific ($CD44^+$ $Tetramer^+$) $CD8^+$ T cells, in accordance, remained unaffected by *A.lwoffii* intervention (Fig. S6g). Collectively, while viral load remained unchanged following intervention (Fig. 7h), mucus hypersecretion associated with type 2 immune responses was reduced, along with a significant reduction in *Muc5ac* expression (Fig. 7i,j). These data indicated that late *A.lwoffii* exposure ameliorated viral-induced type 2 immunity in neonatal mice, with concomitant induction of $CD101^-$PD-L1$^+$ neutrophils.

A preliminary understanding of the role of PD-L1 on neutrophils during secondary RSV infection and late *A.lwoffii* exposure was investigated by intranasal delivery of anti-PD-L1 antibody at a previously established dose,[31] in neonatal mice three times per week starting from

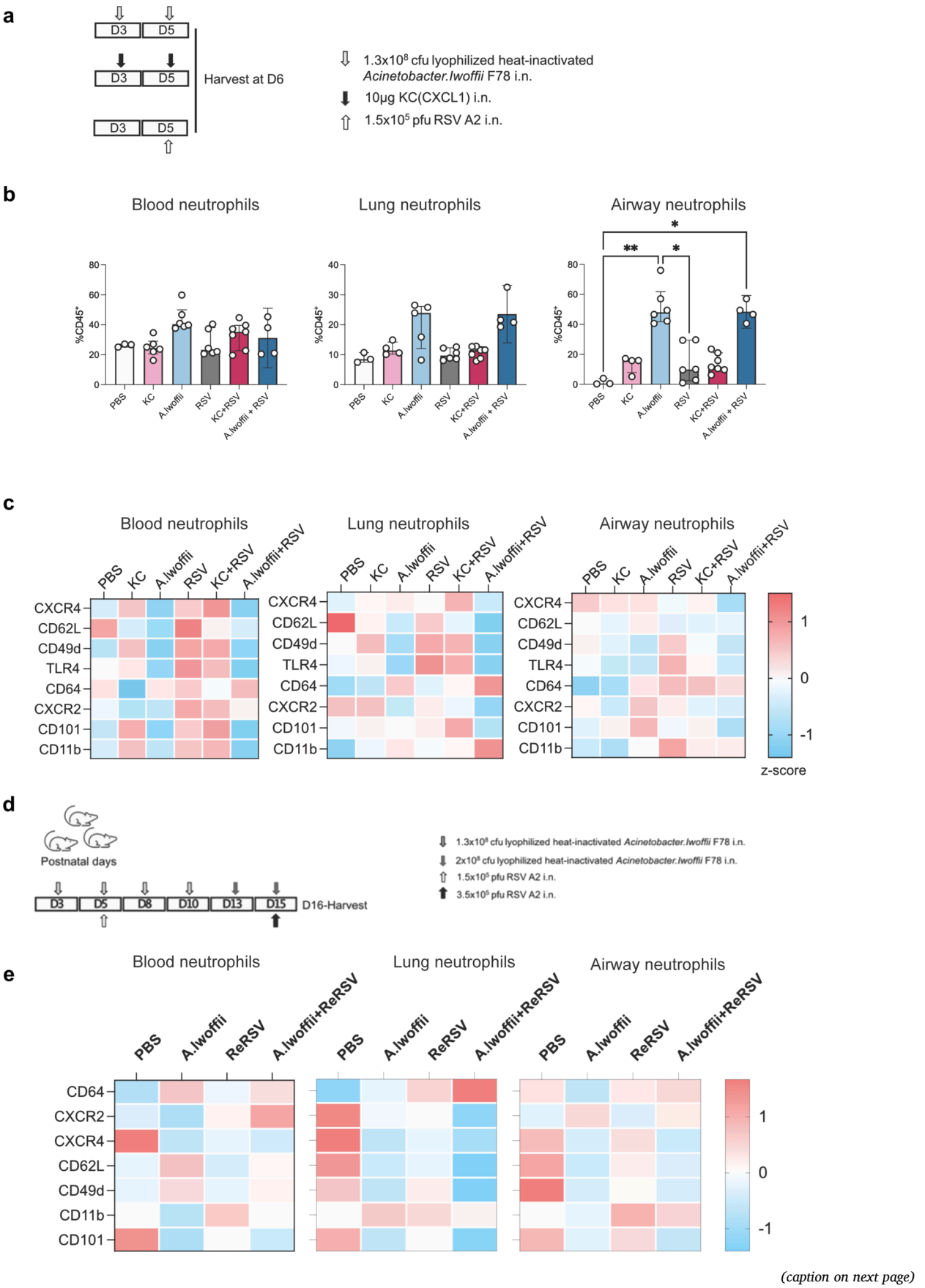

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Fig. 4. Early-life exposure to *A.lwoffii* induced neutrophils with a distinct immunophenotype compared to RSV infection or neutrophil chemoattractant. (a) Neonatal BALB/c mice received intranasal delivery of lyophilised *A.lwoffii*, KC (CXCL1) on PND 3 and PND5, RSV infection on PND5, a combination of *A.lwoffii* and RSV, or a combination of KC and RSV. Neutrophil phenotype was analysed 24 h after the last challenge (PND6). (b) Proportions of neutrophils in the periphery, lung and airway. (c) Expression of various surface markers on neutrophils from the blood, lung and airway. (d) Neonatal BALB/c mice received intranasal delivery of lyophilised inactivated *A.lwoffii* from PND3 to PND19 every other day in conjunction with primary and secondary RSV infection at PND5 and PND15, at indicated dose respectively. On the day of combined treatment, RSV was inoculated 6 h after *A.lwoffii* exposure. Neutrophil phenotype was analysed 24 h after the last dose of *A.lwoffi* exposure (PND16). (e) Expression of various surface markers on neutrophils from the blood, lung and airway. Kruskal-Wallis with post-hoc Dunn's tests performed in (b). Relevant statistical significance indicated by $*0.01 < P < 0.05$,$**0.001 < P < 0.01$.

PDN12, prior to the first *A.lwoffii* exposure (Fig. S7a). Intranasal delivery of anti-PD-L1 antibody significantly increased the number of IFNγ^{+}CD4^{+}, IL-5^{+}CD4^{+} T cells in the airways of mice infected with RSV, with a small increase in the number of IL-13^{+} and IL-10^{+}CD4^{+} T cells, compared to isotype control (Fig. S7b). This suggests that blocking PD-L1 enhances T cell responses to secondary RSV infection. In mice that received both *A.lwoffii* treatment and RSV infection, however, no effect of anti-PD-L1 administration on T cell cytokine production was observed compared to isotype controls (Fig. S7b). While anti-PD-L1 administration achieved reduction of PD-L1 expression on neutrophils, eosinophils, IMMs and DCs in the airways and lungs of RSV-infected animals, the level of PD-L1 expression was moderately affected on these myeloid cells in the airways of mice treated with *A.lwoffii* but not in their lungs (Fig. S7d), perhaps explaining the minimal impact observed in overall immune responses. Additionally intranasal delivery of anti-PD-L1 antibody reduced the CD101^{+} PD-L1^{+} neutrophils in the lungs of RSV-infected animals (Fig. S7e), likely due to the presence of the blocking antibody on the surface of the neutrophils. Collectively, this confirmed the expression of PD-L1 on airway and lung neutrophils, and intranasal blockade of PD-L1 increased Th2 responses to RSV infection.

Discussion

Recurrent respiratory infections in early life, predominantly caused by viruses, represent a significant global health burden, and can impact the long-term health of an infant throughout childhood and beyond. Using an early-life recurrent RSV infection model in neonatal mice, we have demonstrated that continuous exposure to inhaled lyophilized and inactivated farmyard bacteria *A.lwoffii* F78 ameliorates viral-induced airway disease with features characterised by eosinophilia, mucus hypersecretion, and Th2 responses. The beneficial effect was most potent when exposure to ~~inhaled~~ *A.lwoffii* was provided continuously throughout the course of recurrent viral infection, but therapeutic intervention also resulted in improvement. The protective effects of *A.lwoffii* correlated with the frequency of neutrophils in the airways, lungs and circulation, with the induction of PD-L1, the co-inhibitory ligand for T-cell activation, upregulated on pulmonary neutrophils induced by *A.lwoffii*. Our findings highlight the heterogeneity of neutrophils and the complex roles they may play in modulating T cell responses to respiratory viral infections in early life, and in facilitating the protective effects of environmental bacteria in the context of wheezing disorders in early life.

Emerging evidence suggests that neutrophils are heterogeneous[30] and likely to play diverse roles in the context of infection and allergy.[32–34] The present study demonstrates that the protective effect of continuous exposure to lyophilized inactivated *A.lwoffii* was accompanied by a concomitant induction of neutrophils, and their sustained recruitment to the lungs. While recurrent RSV infection also promoted transient neutrophilia within 24 h, the neutrophils induced by *A.lwoffii* exhibited reduced expression of CXCR4 and CD11b compared to those induced by RSV infection, both in the circulation and lungs. This aligns with the reported observation of increased circulating neutrophils with reduced expression of CXCR4 and CD11b in Amish children, who are protected from asthma by frequent livestock exposures compared to children from the Hutterite community with modernised farming practices.[6] We also observed that at 24 h following primary RSV infection in early life, neutrophils expressed higher CXCR4 and CD49d compared to those induced by two doses of inhaled *A.lwoffii*. The CXCR4^{hi} CD49d^{hi} neutrophils have been previously described as a population of pro-allergic neutrophils induced by low-dose LPS in animal models of allergen induced airways disease, and allergen models of exacerbation induced by influenza virus or exposure to ozone[35]. Mechanisms by which these "pro-allergic" neutrophils potentiate type 2 immune responses have been shown to be mediated through the release of neutrophil extracellular traps NETs[35], or by acting as professional antigen presenting cells to directly interact with effector T cells.[36] Additionally, in the mouse model of Sendai virus-induced airway hyperreactivity, expression of CD49d^{+} on neutrophils has been shown to be dependent on type I interferon signalling and promote type 2 immunity via interaction with FcεRI expressing dendritic cells in the lung.[37] Together with these studies, the present study supports the heterogeneity of neutrophils influenced by different microbial signals that is associated with different disease outcomes.

We have identified and characterised, by bulk RNAseq and flow cytometry, a population of PD-L1^{+} neutrophils induced by continuous *A.lwoffii* exposure. PD-L1 expression on neutrophils associates with T cell suppression to facilitate tissue specific roles[38,39]. These neutrophils have been meticulously described in cancer[40], severe COVID19 patients[41] and recently, during sepsis[42] and in the integrated global map of neutrophils in mice[30] We have further demonstrated that that neutrophil activation, reflected by shedding of CD62L is associated with PD-L1 expression and blocking PD-L1 prevented reduction of CD62L in RSV-induced neutrophils. In contrast, expression of maturation marker CD101 and CXCR4, a marker for aged neutrophils,[43] occurs independently of PD-L1. *A.lwoffii* exposure promoted the emergence of PD-L1^{+} immature neutrophils characterised by reduced expression of CD101 and abundance of genes associated with granule formation and protein synthesis. This suggests that following exposure to inhaled *A.lwoffii*, neutrophils emerge prematurely from the bone marrow with an immunoregulatory phenotype.

Epidemiological studies have indicated that frequent and long-term exposure to farmyard dust is necessary for maximal protection from asthma.[20,21] This has been recapitulated experimentally in the present study with different doses of *A.lwoffii* exposure. It is important to highlight that although prophylactic benefit of *A.lwoffii* could be achieved via early intervention, the exposure must be long term to be most effective, suggesting that DC-associated immunomodulation at T cell priming is limited and *A.lwoffii*-induced innate responses present at the mucosal site is involved in fine-tuning the adaptive T cell immunity. We demonstrated the emergence of CD101^{-}PD-L1^{+} neutrophils in the lung in parallel with a dampened effect of type 2 immune responses via therapeutic *A.lwoffii* intervention during recurrent RSV infection. The immunosuppressive property in modulating T-cell proliferation and activity has been observed in neutrophils, particularly in cancer and pathogen-mediated systemic inflammation.[44] The PD-L1^{+} neutrophils are capable of suppressing CD4^{+} T cell proliferation in the lymph node via direct interactions with PD1 ligand.[39] Our preliminary experiment with intranasal administration of anti-PD-L1 during secondary RSV infection increased the number of IFNγ^{+}CD4^{+} T cells and IL-5^{+}CD4^{+} T cells in the airways, directly proving the involvement of PD-L1 in the modulation of T cell response during viral infections. However, we acknowledge PD-L1 is expressed on many myeloid cells, not just neutrophils, therefore the effects of anti-PD-L1 antibody cannot be ascribed solely to neutrophils.

The protective effect of bacteria exposure against RSV infection-

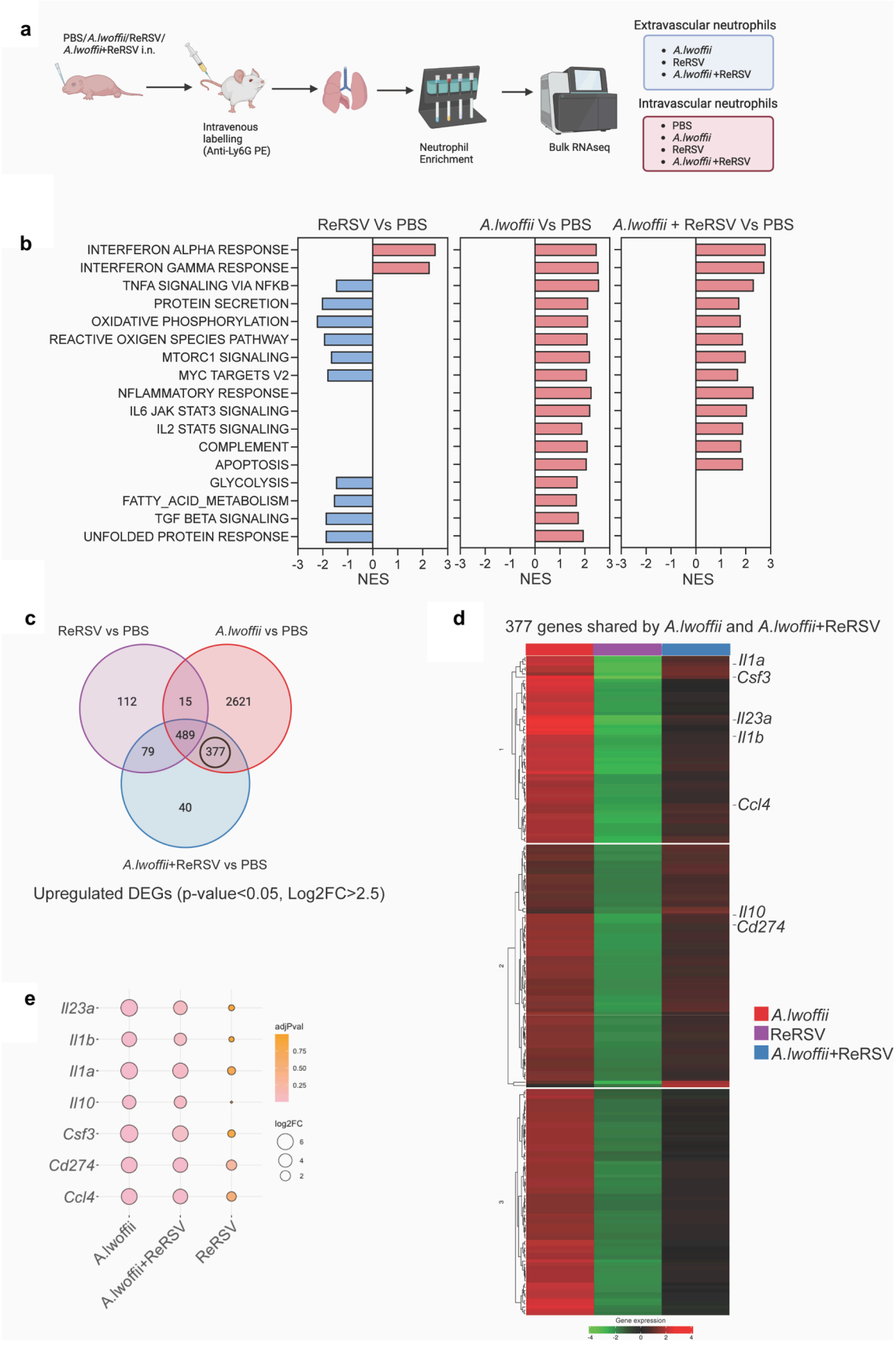

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Fig. 5. Differential gene expression and altered gene responses following *A.lwoffii* exposure or RSV infection. (a) Neonatal BALB/c mice received intranasal delivery of lyophilised inactivated *A.lwoffii* from PND3 to PND16 every other day in conjunction with primary and secondary RSV infection at PND5 and PND15, at indicated doses respectively. On the day of combined treatment, RSV was inoculated 6 h after *A.lwoffii* exposure. 24 h after the last dose of *A.lwoffi* exposure (PND16), lung neutrophils with i.v. anti-Ly6G labelling were enriched and isolated and sorted for bulk RNA sequencing. Downstream analysis included both the extravascular and intravascular neutrophils from the same treatment conditions. (b) Enrichment of various hallmark pathways induced by RSV (ReRSV), *A.lwoffii* or RSV and *A.lwoffii* by gene-set enrichment analysis (GSEA). (c) Venn diagram of differentially upregulated genes (p-value < 0.05, Log2FC > 2.5) from paired comparisons of ReRSV Vs PBS, *A.lwoffii* Vs PBS, *A.lwoffii* and ReRSV Vs PBS. 377 genes that were commonly shared by *A.lwoffii* and *A.lwoffii* with ReRSV were encircled. (d) Heatmap of the commonly shared 377 genes by k-means clustering. (e) Expression of selected genes from (d).

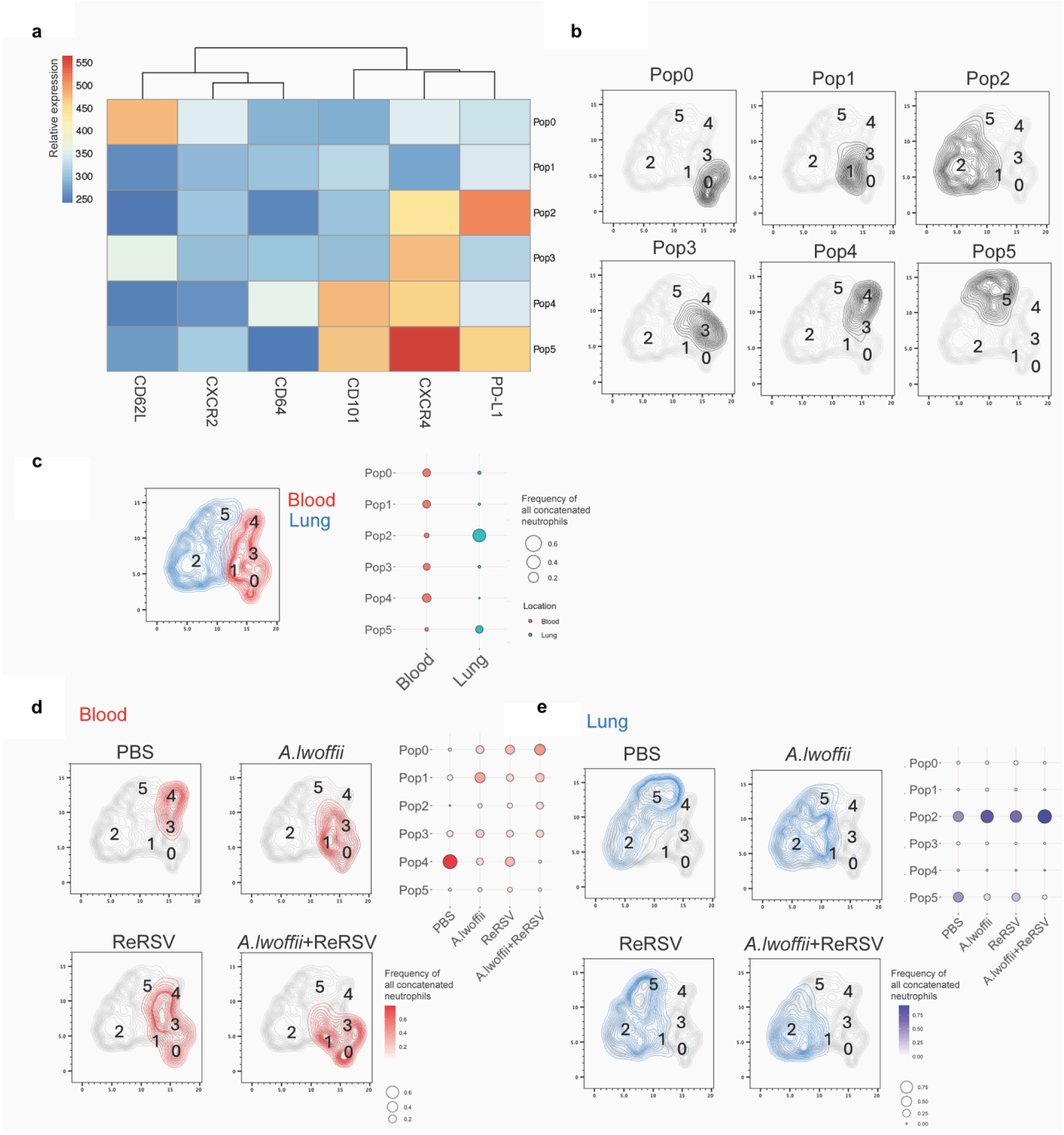

Fig. 6. Exposure to inhaled *A.lwoffii* promoted PD-L1^{+} pulmonary neutrophils in neonatal mice. At 24 h post recurrent RSV infection in the model of continuous *A. lwoffii* exposure, neutrophil phenotype was analysed by flow cytometry. (a) FlowSom clustering analysis were generated from concatenated blood and lung neutrophils in all treatment conditions. (b) Uniform Manifold Approximation and Projection (UMAP) of various neutrophil populations generated from FlowSom analysis. (c) UMAP and proportions of neutrophil populations in lung and blood. (d) UMAP and proportions of neutrophil populations in respective treatment conditions in blood.(e) UMAP and proportions of neutrophil populations in respective treatment conditions in lung.

induced type 2 responses has been previously demonstrated in adult mice by oral supplementation of live *Lactobacillus johnsonii*, mediated by

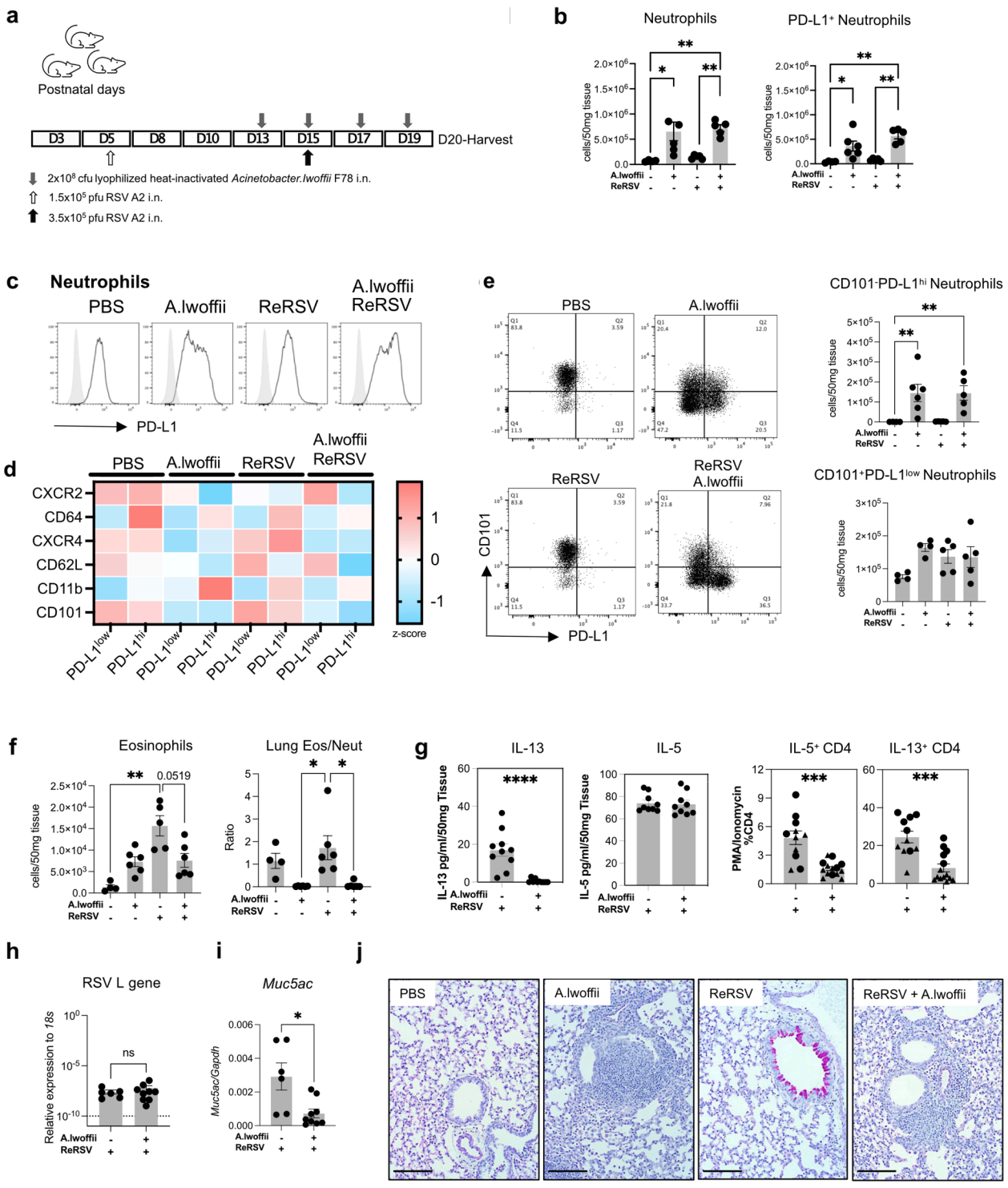

Fig. 7. Therapeutic intervention with *A.lwoffii* modulates type 2 immunity by facilitating increased PD-L1 neutrophils. (a) Neonatal BALB/c mice received intranasal delivery of lyophilised inactivated *A.lwoffii* from PND13 to PND19 every other day in conjunction with primary and secondary RSV infections at PND5 and PND15. Pulmonary immune responses and inflammation were analysed 5 days post recurrent infection (5dpri). (b) Number of neutrophils and PD-L1^{+} neutrophils in the lung. (c) Representative histogram of PD-L1 expression on neutrophils. (d) Expression of neutrophil phenotypic markers between PD-L1^{hi} and PD-L1^{low} populations. (e) Representative flow plot of CD101 and PD-L1 expression of neutrophils. Number of CD101^{-}PD-L1^{hi} neutrophil and CD101^{+}PD-L1^{low} neutrophils. (f) Number of eosinophils and eosinophil to neutrophil ratio in lung. (g) Protein levels of IL5, IL-13 measured in lung homogenates and cytokine production by CD4 T cells following *ex vivo* PMA/Ionomycin stimulation. (h) RSV L gene expression in the lung. (i) Expression of mucus related gene *Muc5ac* in the lung. (j) Representative image of Periodic Acid-Schiff (PAS) staining on lung tissue sections acquired by Leica Microscope at x200 magnification. Scale bar represents 100 μm. Kruskal-Wallis with post-hoc Dunn's tests performed in (b), (e) and (f) and non-parametric Mann-Whitney tests performed in (h) and (i). Statistical significance indicated by *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$0.001 < P < 0.0001$, ****$0.0001 < P < 0.00001$.

immunomodulatory metabolites[45]. This mechanism is likely to be distinct to the present study, in which we administered inactivated bacterial lysate via intranasal exposures to directly induce immune responses in the lung mucosa. Although the presence of bacterial pathogen associated molecular patterns (PAMPs), such as LPS, in the bacterial lysate has been shown to suppress type 2 immune responses induced by allergens such as house dust mite in mice,[35,46] intranasal exposure of LPS before RSV infection was reported to exacerbate inflammation.[47] This is in contrast to the protective effect of intranasal exposures of OM-85, an inactivated bacterial lysate of 21 identified human respiratory strains, that attenuated lung inflammation and reduced viral burden when administered multiple times prior to RSV infection in mice.[48] However, the effect of OM-85 on type 2 immune responses induced by repeated RSV infections in early life has not been explored.

Overall, we have demonstrated that continuous exposure to inhaled lyophilized and inactivated farmyard bacteria *A.lwoffii* F78 induced PD-L1^{+} immature neutrophils in the lung associated with a significant reduction in eosinophilia, mucus hypersecretion and Th2 responses that were induced by viral (RSV)-induced infection in early life. The distinct phenotype of *A.lwoffii* induced neutrophils, compared to RSV-induced neutrophils contributes to protection from viral-induced type-2 pathology.

CRediT authorship contribution statement

Kunyuan Tian: Writing – original draft, Methodology, Investigation, Formal analysis. **Lucia Labeur-Iurman:** Investigation. **Simone A. Walker:** Investigation. **Dimple Zope:** Investigation. **Erika von Mutius:** Writing – review & editing, Resources. **James A. Harker:** Writing – review & editing, Supervision. **Clare M. Lloyd:** Writing – review & editing, Supervision, Conceptualization. **Sejal Saglani:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mucimm.2026.02.007.

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