Apolipoprotein E controls the development of monocyte-derived alveolar macrophages upon pulmonary inflammatory adaptation

Apolipoprotein E controls Dectin-1-dependent development of monocyte-derived alveolar macrophages upon pulmonary β-glucan-induced inflammatory adaptation

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Summary

The lung is constantly exposed to the outside world and optimal adaptation of immune responses is crucial for efficient pathogen clearance. However, mechanisms that lead to lung-associated macrophages' functional and developmental adaptation remain elusive. To reveal such mechanisms, we developed a reductionist model of environmental intranasal β-glucan exposure, allowing for the detailed interrogation of molecular mechanisms of pulmonary macrophage adaptation. Employing single-cell transcriptomics, high dimensional imaging, and flow cytometric characterization paired with *in vivo* and *ex vivo* challenge models, we reveal that pulmonary low-grade inflammation results in the development of Apolipoprotein E (ApoE) -dependent monocyte-derived alveolar macrophages ($ApoE^+CD11b^+$ AM). $ApoE^+CD11b^+$ AMs expressed high levels of CD11b, ApoE, Gpnmb, and Ccl6, were glycolytic, highly phagocytic, and produced large amounts of interleukin 6 upon restimulation. Functional differences were cell intrinsic and myeloid cell-specific ApoE ablation inhibited $Ly6c^+$ monocyte to $ApoE^+CD11b^+$ AM differentiation dependent on M-CSF secretion, promoting $ApoE^+CD11b^+$ AM cell death and thus impeding $ApoE^+CD11b^+$ AM maintenance. *In vivo*, β-glucan-elicited $ApoE^+CD11b^+$ AMs limited the bacterial burden of *Legionella pneumophilia* post-infection and improved the disease outcome *in vivo* and *ex vivo* in a murine lung fibrosis model. Collectively these data identify $ApoE^+CD11b^+$ AMs generated upon environmental cues, under the control of ApoE signaling, as an essential determinant for lung adaptation enhancing tissue resilience.

Introduction

The lung is exposed to a variety of immunostimulatory agents shaping the its' immune responses [1]. How such environmental non-pathological immune activation is controlled at the cellular and molecular level is poorly understood. β-glucans are integral components of environmental pathogenic and non-pathogenic fungi and are proposed as immune modulators [2]. Ambient concentrations of β-glucan oscillate over the course of the year and, in combination with pathogen exposure, correlate with allergic rhinitis increases [3]. Recognition of β-glucan by Dectin-1 modulates systemic immune responses through a process termed innate immune memory [4], characterized by increased cytokine responses of monocytes, upon a secondary heterologous stimulus, facilitated by a metabolic and epigenetic rewiring, allowing a more efficient first line immune response [4-9]. Lung-specific mechanism of β-glucan, the organ where it is most often recognized, and how it acts as a non-genetic modifier of immune responses to subsequent disease, remain unknown.

Alveolar space resident immune cells are the body's respiratory first line of defence, ensuring efficient immune response induction against airborne pathogens, while regulating immune activation to ensure intact lung function [10]. Alveolar macrophages (AM) and monocytes constitute the major mononuclear phagocytes (MPs) found during homeostasis in humans and mice within the alveolus. Alveolar AMs and monocytes express high amounts of Dectin-1 and are highly plastic [11]. Dectin-1 signalling critically depends on spleen tyrosine kinase (Syk) which upon activation triggers phospholipase C gamma 2 (PLCγ2) dependent calcium release, downstream nuclear factor of activated T-cells (NFAT) and extracellular signal-regulated kinase (ERK) activation. This cascade leads to production of interleukin (IL) 2 and 10. Furthermore Syk activates CARD9 leading to nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling and the release of tumor necrosis factor alpha (TNFα) and IL-6. Additionally, Dectin-1 ligation directly induces reactive oxygen species (ROS) production via activation of phosphoinositide 3 kinase (PI3K) [12,13]. Murine homeostatic AMs are embryonically derived with only minor contribution of adult bone marrow (BM) homeostasis [14,15]. Pulmonary viral infection or radiation was demonstrated induce differentiation of $Ly6c^+$ monocytes into long-lived monocyte-derived alveolar macrophages (MoAM) [16-18]. During viral infection MoAM derived IL-6 is crucial for the defence against subsequent *Streptococcus pneumoniae* infection [16]. Finally, viral-induced environmental adaptation affects resident AMs-dependent CD8+ T-cells rewiring, inducing efficient bacterial clearance [19].

Chronic lung diseases, like asthma or pulmonary fibrosis, hinge on environmental factors. Thus, understanding environmental immune adaption in broncho-alveolar MPs is crucial for insights into cellular, functional, and molecular consequences. [20,21-24].

To investigate this we developed a reductionist model of a single low dose intranasal β-glucan exposure. Using single-cell transcriptomic, functional *in vivo* and *ex vivo* analysis of cellular development function, this model allowed us to dissect acute and chronic the molecular adaptations of macrophages upon environmental cues. We show that a single intranasal β-glucan exposure induces developmentally and functionally modified Apolipoprotein E (ApoE)$^{+}$CD11b^{+} MoAMs, detected up to 21 days post β-glucan exposure. ApoE^{+}CD11b^{+} MoAMs are glycolytic, highly phagocytic and release, upon activation, high amounts of IL-6. Functional changes are cell-intrinsic and upon subsequent infection with *Legionella pneumophila* or a challenge by bleomycin-induced fibrosis lead to improved *in vivo* outcomes. Molecularly, ApoE^{+}CD11b^{+} MoAM are controlled by the Dectin-1 - Card9 pathway, whereas maintenance of ApoE^{+}CD11b^{+} MoAMs depends on paracrine ApoE and macrophage colony-stimulating factor (M-CSF). Taken together, we identify ApoE as a crucial checkpoint for low-grade inflammation-associated M-CSF controlled monocyte-to-macrophage differentiation triggered by the Dectin-1 - Card9 pathway within the immune adapted microenvironment of lung.

Results

Intranasal β-glucan induces ApoE$^+$CD11b$^+$ alveolar macrophages.

The lung is constantly exposed to pollutants, sterile and non-sterile pathogens, and components thereof. Our understanding of the cellular and molecular mechanisms of immune adaptation to these environmental cues is limited. To investigate this, we developed a simplified model involving a single low-dose intranasal exposure to β-glucan particles (200 µg), mimicking environmental exposure [25]. To assess the impact of this stimulation and investigate its lasting effects, we examined bronchoalveolar lavage fluid (BALF) resident macrophages (CD45$^+$Lin$^-$SSC^{int-hi}) of C57BL/6 mice seven days after intranasal PBS or β-glucan treatment using single-cell transcriptomics (**Figure 1A, S1A-C**). Dimensionality reduction using uniform manifold approximation and projection analysis (UMAP) and unsupervised clustering (Louvain) revealed five distinct transcriptional clusters within the BALF (**Figure 1A, B**). Here, high expression of alveolar macrophage (AM) signature genes Siglecf and Itgax identified all investigated cells as AMs (**Figure S1D, E**) [26]. Further analysis identified Cluster 0, 1 as subsets of resident AMs expressing Ear2, Wfdc21 and Hmox1, respectively [26,27]. Cluster 2 (proliferating AM) expressed genes linked to proliferation such as Top2a, Mki67, and Birc5 [28]. Cluster 3 (ApoE$^+$ AMs), expressed genes associated with a lipid-associated inflammatory monocyte-derived macrophage (MoMac) phenotype, including Apoe, Cd63, Spp1, Gpnmb, and Trem2 [16,29,30]. Cluster 4 (ISG$^+$ AMs), was characterized by expression of interferon-stimulated genes (ISG), such as Ifit2, Ifit3, Ifi204, and Isg15 (**Figure 1B**). To determine which cluster was associated with β-glucan-induced environmental adaptation, relative contribution of each stimulatory condition to individual clusters was examined (**Figure 1C-E, S1F**). Here ApoE$^+$ AMs were only present within the BALF of β-glucan exposed mice seven days prior, concomitant with a reduction in proliferating AMs. Previous studies suggested that CD11b expression on stimulated AMs serves marks enhanced inflammatory potential [31,32]. Therefore, we employed co-detection by indexing (CODEX)-enabled high-dimensional imaging to characterize the phenotype of ApoE$^+$ AMs at the protein level in the lung and in BALF seven days post-β-glucan stimulation (**Figure 1F, S1G**) [33]. ApoE$^+$ AMs co-expressed the classical AM markers, CD11c and SiglecF, but also expressed significant amounts of CD11b, ApoE, and GPNMB proteins (**Figure 1F, S1G**). Furthermore, to confirm overlap of ApoE mRNA expression and CD11b protein expression, we measured ApoE mRNA levels using PrimeFlow. ApoE mRNA signals were detectable only in BALF CD11b$^+$ AMs isolated from mice stimulated with β-glucan seven days prior (**Figure 1G**). Consequently, we refer to this AM subpopulation as ApoE$^+$CD11b$^+$ AMs. To investigate the β-glucan-induced cellular dynamics of ApoE$^+$CD11b$^+$ AMs we monitored the BALF from day 0 to day 21 post-β-glucan exposure using flow cytometry (**Figure 1H, I, S1H, I**). This analysis

revealed that, in line with the single-cell transcriptomic data, ApoE^{+}CD11b^{+} AMs peaked at day seven post-β-glucan inoculation and gradually declined until day 21 (**Figure 1H, I**). In agreement with this, we observed an overall increase in total AMs peaking at day seven post-β-glucan exposure (**Figure S1J, K**). Generation of ApoE^{+}CD11b^{+} AMs was associated with a transient influx of neutrophils and eosinophils on days 1 and 3 (**Figure S1L, M**). To confirm the macrophage identity of ApoE^{+}CD11b^{+} AMs, SiglecF expression on CD11b^{-} AMs, ApoE^{+}CD11b^{+} Ams, and Ly6c^{+} monocytes was assessed. Both CD11b^{-} and ApoE^{+}CD11b^{+} AMs expressed high levels of SiglecF, whereas Ly6c^{+} monocytes did not, further establishing ApoE^{+}CD11b^{+} AMs as part of the AM compartment (**Figure S1N**). Finally, to understand abundance ApoE^{+}CD11b^{+} AMs in total lung single-cell suspensions, we quantified ApoE^{+}CD11b^{+} AMs using flow cytometry (**Figure S1O**). ApoE^{+}CD11b^{+} AMs were also present in full lung suspensions, displaying similar quantity and marker profile as in BALF. In summary, intranasal β-glucan induces ApoE^{+}CD11b^{+} alveolar macrophages as a cellular response to environmental stimulation.

ApoE^{+}CD11b^{+} AMs are derived from monocytes and depend on CCR2.

Both acute and chronic inflammation induce the recruitment of MoMacs into the bronchoalveolar space [16,31,34]. To understand whether β-glucan-induced environmental adaptation induces a new resident alveolar macrophage cell state or results in the recruitment and differentiation of Ly6c^{+} monocytes into ApoE^{+}CD11b^{+} AMs, we tracked the influx of Ly6c^{+} monocytes into the BALF using flow cytometry. Post-β-glucan stimulation, Ly6c^{+} monocytes were recruited to the bronchoalveolar space, peaking three days after stimulation, remaining elevated on day 7, and gradually declining from day 14 onwards (**Figure 2A, S2A**). To connect these findings with the emergence of ApoE^{+}CD11b^{+} AMs, we utilized the Ms4a3^{creRosa26}tdTomato mice, enabling genetic tracing of the BM-derived granulocyte-macrophage progenitors (GMP).

Genetic lineage tracing revealed that 86% ± 7.8% of ApoE^{+}CD11b^{+} AMs were labelled with tdTomato, indicating BM GMP lineage origin (**Figure 2B, C**). CODEX imaging showed co-expression of CD11b and tdTomato in SiglecF^{+}CD11c^{+} AMs within tissue sections from mice stimulated with β-glucan seven days prior, supporting their GMP and monocyte origin (**Figure 2D**). To determine if Ly6c^{+} monocytes are systemically mobilized and recruited to the lung from the BM following β-glucan stimulation, we assessed the abundance of Ly6c^{+} monocytes, cMOP and GMP in the blood and BM, respectively (**Figure 2E, F, S2B-G**). This revealed a reduction in Ly6c^{+} monocytes in the blood, accompanied by a compensatory increase in BM Ly6c^{+} monocytes seven days after β-glucan stimulation. To confirm the monocytic BM origin of ApoE^{+}CD11b^{+} AMs, we utilized CCR2-deficient

mice, in which the recruitment of Ly6c$^+$ monocytes into peripheral tissues is impaired [35]. To investigate dependence of ApoE$^+$CD11b$^+$ AMs on CCR2, we intranasally inoculated control and CCR2-deficient mice with β-glucan and analysed the BALF seven days later using flow cytometry (**Figure 2G, S2H**). This analysis revealed a significant reduction in ApoE$^+$CD11b$^+$ AMs in CCR2-deficient mice following β-glucan inoculation. In summary, these results collectively demonstrate that ApoE$^+$CD11b$^+$ AMs induced by β-glucan exposure originate from BM Ly6c$^+$ monocytes in a CCR2-dependent manner.

ApoE$^+$CD11b$^+$ AMs exhibit an elevated release of interleukin-6 (IL-6).

MoMacs have been linked to heightened inflammatory responses following high-grade inflammatory and infectious events, such as influenza A infection [16]. Specifically, the increased production of IL-6 is a hallmark feature of functionally modified MoMacs during acute inflammation. However, it remains unclear whether BALF-resident mononuclear cells functionally adapt to local low-grade inflammation. To address this, we investigated the functional profile of AMs seven days after exposure to β-glucan. We isolated BALF AMs, and subsequently re-stimulated them with PBS or lipopolysaccharide (LPS) for 24 hours *in vitro*. Analysis of IL-6 release in the supernatants using ELISA revealed that *in vivo* β-glucan pre-exposed macrophages released significantly higher amounts of IL-6 upon LPS re-stimulation, compared to PBS-pre-treated counterparts (**Figure 3A**). To identify the cellular source responsible of increased IL-6 production, we purified BALF-resident CD11b$^-$ and ApoE$^+$CD11b$^+$ AMs of intranasal β-glucan treated mice seven days earlier and restimulated them with LPS (**Figure 3B**). Only ApoE$^+$CD11b$^+$ AMs released comparable amounts of IL-6 to those observed in complete BALF AM preparations (**Figure 3A**). Subsequently, intracellular flow cytometric analysis demonstrated a significant increase in IL-6$^+$CD11b$^+$ AMs following β-glucan exposure compared to PBS-exposed controls (**Figure 3C, S3A**). Our data indicated that the generation of ApoE$^+$CD11b$^+$ AMs in response to β-glucan exposure depends on CCR2 (**Figure 2G**). To confirm that the increased IL-6 observed in *ex vivo* restimulated AMs can be directly attributed to CCR2-dependent ApoE$^+$CD11b$^+$ AMs, we exposed CCR2-deficient and control mice to β-glucan. Seven days later, we enriched BALF AMs were enriched, restimulated and restimulated with LPS for 24 hours *in vitro* (**Figure 3D**). This analysis revealed that the elevated IL-6 levels observed in β-glucan-exposed BALF AMs are CCR2-dependent, providing evidence that ApoE$^+$CD11b$^+$ AMs are the primary source of increased IL-6 during β-glucan-induced environmental adaptation. Finally, to causally establish whether the enhanced IL-6 production is an intrinsic cellular feature of ApoE$^+$CD11b$^+$ AMs, we transferred CD45.2$^+$ BALF resident AMs into naïve CD45.1$^+$ mice five days after β-glucan-induced environmental adaptation. Two days later, we restimulated BALF AMs

with LPS *in vitro* (**Figure 3E, S3B, C**). Transfer of β-glucan-experienced BALF AMs led to an increased IL-6 production upon *in vitro* restimulation of AMs within the recipient mouse. These findings causally establish the intrinsic β-glucan-induced functional change in $ApoE^{+}CD11b^{+}$ AMs.

β-glucan aids lung bacterial defense and experimental fibrosis recovery.

Previous studies associated altered cytokine responses following systemic β-glucan stimulation with increased glycolysis in MPs[7,8]. To investigate this in our system, we measured glycolysis. This revealed a significant increase in glycolysis, glycolytic capacity, and reserve seven days after β-glucan exposure in BALF AMs (**Figure 4A, B, S4A, B**). In addition, induction of glycolysis in MPs is associated with enhanced phagocytosis[36,37]. Thus, we assessed BALF AM phagocytosis of *Staphylococcus aureus-coated* particles *in vitro* isolated from mice adapted to PBS or β-glucan seven days prior. This demonstrated a significant increase in phagocytic activity in BALF AMs isolated from β-glucan-adapted mice but not PBS-adapted (**Figure 4C, D, S4C, D**). This prompted us to investigate the *in vivo* functional impact of intranasal β-glucan adaptation in response to acute bacterial challenge. We infected C57BL/6 mice, β-glucan-adapted or PBS-adapted seven days earlier, with *Legionella pneumophila* and analysed the BALF's bacterial burden and cellular composition two days post-infection[38]. β-glucan-adapted mice exhibited a significant reduction in bacteria detected in BALFs, along with an increased count of proinflammatory macrophages associated with bacterial clearance (**Figure 4E, F, S4E**).

Furthermore, we examined whether β-glucan adaptation had effects beyond the modulation of acute bacterial infection using bleomycin-induced experimental lung fibrosis. β-glucan-adapted mice showed significantly higher survival rates, lower disease burden, and reduced weight loss over a 14-day observation period post-bleomycin inoculation (**Figure S4F-I**). Moreover, the pro-resolution associated effectors, IL-4 and IL-33, were enhanced on day 3 post-bleomycin inoculation, while on day 14 post-bleomycin TSLP decreased in β-glucan adapted mice (**Figure S4J-L**). No difference in lung fibrotic area was observed (**Figure S4M, N**). These findings highlight the substantial regulatory role of $ApoE^{+}CD11b^{+}$ AMs elicited by environmentally adaptation in the control and severity of acute and chronic inflammation. To elucidate the direct effect of $ApoE^{+}CD11b^{+}$ AMs on the development of lung fibrosis, we generated bronchoalveolar lung organoids (BALOs) containing myofibroblasts [39]. We treated them with transforming growth factor-beta (TGF-β) to induce a fibrotic response. BALF AMs isolated from β-glucan or PBS-adapted mice were added to day 21 BALOs 24 h before TGF-β pro-fibrotic stimulation and co-cultured for 48 hours. Adding TGF-β to BALOs led to the increased production of fibroblast smooth muscle actin (SMA), a hallmark of lung fibrosis. We quantified SMA production in β-glucan or PBS-

adapted AMs-supplemented fibrotic BALOs. We observed a significant reduction in SMA production when β-glucan-adapted AMs were added, while the addition of PBS-adapted AMs showed no effect (**Figure 4G, H, S4O**). In conclusion, functional *in vivo* and *in vitro* data establish ApoE^{+}CD11b^{+} AMs as crucial environmentally induced modulators of lung inflammation, providing valuable insights into the molecular mechanisms underlying their role in mitigating fibrosis.

β-glucan induces ApoE^{+}CD11b^{+} AMs via Dectin-1/Card9.

β-glucan is recognized by various receptors, including CR3, Dectin-1 and CD5 [40-42]. Dectin 1 is most prominently on MPs. To understand how Dectin 1 regulates ApoE^{+}CD11b^{+} AMs, we flow cytometrically profiled its' expression on BALF macrophages **(Figure 5A)**. This revealed that homeostatic Dectin-1 expression is largely confined to resident AMs with only a small fraction of monocytes expressing Dectin-1. To assess the role of Dectin-1 for development of ApoE^{+}CD11b^{+} AMs, we intranasally inoculated control or Dectin1$^{-/-}$ mice with β-glucan and flow cytometrically analysed BALF resident immune cells seven days later. This revealed that generation of ApoE^{+}CD11b^{+} AMs is dependent on Dectin-1 expression, whereas initial inflammatory recruitment of Ly6c^{+} monocytes to the BALF is not **(Figure 5B, C)**. Next, to understand whether immune cell-intrinsic or stromal cell recognition via Dectin-1 is critical for the development of ApoE^{+}CD11b^{+} AMs and thus environmental adaptation, we transferred Dectin1$^{-/-}$ or control (CD45.2^{+}) BM into lethally irradiated CD45.1^{+} control mice and analysed their BALF seven days post environmental adaption by β-glucan. Here, generation of ApoE^{+}CD11b^{+} AMs was entirely dependent on hematopoietic expression of Dectin-1 **(Figure 5D, S5A, B)**. CARD9 mediates activation of Nfκb by Dectin-1 [12,13,43]. To investigate if ApoE^{+}CD11b^{+} AMs require CARD9 for their development we treated lethally irradiated mice reconstituted with Card9$^{-/-}$ or control BM with β-glucan or PBS and flow cytometrically analysed the BALF seven days later. This revealed that development of ApoE^{+}CD11b^{+} AMs depends on Dectin-1-elicited Card9-dependent signalling **(Figure 5E, S5C, D)**. Next, we investigated whether the loss of ApoE^{+}CD11b^{+} AMs by abrogating Dectin-1 or Card9 signalling leads to a loss of increased IL-6 secretion upon *in vitro* LPS restimulation in BALF macrophages. In line with the data obtained in the CCR2$^{-/-}$ mouse model, enhanced IL-6 secretion was abolished in the absence of Dectin-1 or Card9 signalling and thus can be attributed to ApoE^{+}CD11b^{+} AMs **(Figure 5F, G)**.

β-glucan triggers ApoE^{+}CD11b^{+} AM differentiation via myeloid ApoE.

ApoE is expressed in various MoMac populations associated with different low-grade or chronic inflammatory diseases but its role in monocyte-to-macrophage differentiation and maintenance remains unexplored [16,29]. During β-glucan-induced environmental adaptation, ApoE was highly expressed in ApoE^{+}CD11b^{+} AMs and detectable at the protein level as early as one day after intranasal β-glucan stimulation, coinciding with BALF Ly6c^{+} monocyte recruitment (**Figure 6A, B, 2A**). To elucidate the role of ApoE in the environmental adaptation of the lung MP repertoire, we intranasally inoculated ApoE^{flox}LysM^{Cre} mice, which lack ApoE expression within the myeloid lineage, with β-glucan. Next, we flow cytometrically analysed the composition of the BALF MP compartment seven days later. β-glucan-stimulated ApoE^{flox}LysM^{Cre} mice did not exhibit increased numbers of BALF Ly6c^{+} monocytes and, as a consequence, failed to generate ApoE^{+}CD11b^{+} AMs (**Figure 6C, D, S6A, B**). Next we examined whether loss of myeloid ApoE influences the observed feedback on blood and BM Ly6c^{+} monocytes. Myeloid ApoE deficiency abrogated the decrease in blood monocytes and the compensatory increase in BM Ly6c^{+} monocytes observed in control mice. Myeloid ApoE deficiency showed no effect on cMOPs or GMPs seven days after β-glucan stimulation (**Figure S6C-F**). Subsequently, we investigated whether ApoE controls the generation of ApoE^{+}CD11b^{+} AMs through paracrine or autocrine signalling, as both modes have been described previously [44,45]. Here, we generated mixed BM chimeras with a 50:50 ratio of wildtype (CD45.1^{+}) and ApoE^{flox}LysM^{Cre} (CD45.2^{+}) cells or congenic mixed wildtype (CD45.1^{+}) : wildtype (CD45.2^{+}) control chimeras. After reconstitution, we intranasally stimulated them with β-glucan and flow cytometrically analysed their BALF MP repertoire seven days later. This revealed that both WT/WT and WT/ApoE^{flox}LysM^{Cre} chimeras efficiently generated ApoE^{+}CD11b^{+} AMs seven days after β-glucan exposure, supporting a paracrine signalling mode (**Figure 6E, S6G, H**). Next, we examined the contribution of ApoE-deficient CD45.2^{+} cells to the pool of total ApoE^{+}CD11b^{+} AMs. We found that both ApoE-proficient (CD45.1) and deficient (CD45.2) cells equally contributed to the pool of β-glucan stimulated ApoE^{+}CD11b^{+} AMs (**Figure 6F**). This demonstrate that a paracrine myeloid cell-derived source of ApoE is sufficient to rescue the generation of ApoE^{+}CD11b^{+} AMs during environmental adaptation in the lung.

Myeloid-derived Apolipoprotein E controls survival of ApoE^{+}CD11b^{+} AMs by regulation of cholesterol storage and M-CSF secretion

To elucidate ApoE's role in the differentiation and survival of ApoE^{+}CD11b^{+} AM, we conducted experiments to determine whether myeloid-derived ApoE influences the initial commitment of Ly6c^{+} monocytes to or the maintenance and survival of MoMacs. We used control and ApoE^{flox}LysM^{Cre} mice three days after intranasal β-

glucan exposure. On day three post β-glucan exposure similar numbers of BALF Ly6c^{+} monocytes were present in control and ApoE^{flox}LysM^{Cre} mice. This suggests that ApoE does not regulate the initial commitment to the macrophage lineage or the recruitment of monocytic precursors to the BALF. However, our results established a critical time window during which ApoE is essential for monocyte-to-macrophage differentiation following β-glucan inoculation (**Figure 7A, B, S7A, B**). To investigate potential molecular dysregulation caused by the absence of ApoE in differentiating macrophages, we assessed intracellular cholesterol content and distribution of BALF ApoE^{+}CD11b^{+} AMs three days after β-glucan stimulation. This revealed that ApoE-deficient CD11b^{+} AMs have increased intracellular cholesterol, as indicated by filipin staining (**Figure 7C, D**). Previous data linked dysregulated ApoE signalling to cholesterol accumulation in the endoplasmic reticulum and a reduction in protein synthesis[46]. To test these mechanisms in our experimental system of monocyte-to-macrophage differentiation, we analysed the co-localization of BODIPY-cholesterol with the endoplasmic reticulum-associated protein Calreticulin and early or late endosome markers (EEA1 and LAMP1) using confocal microscopy. This revealed that, in ApoE-deficient differentiating macrophages three days after β-glucan exposure, BODIPY-cholesterol co-localizes with calreticulin, accumulating at the endoplasmic reticulum (**Figure 7E, F**). M-CSF-releasing monocytes differentiating into macrophages has been described to be crucial for lung monocyte-to macrophage differentiation [47]. To assess whether aberrant cholesterol accumulation affects the M-CSF-M-CSFR macrophage survival circuit, we monitored BALF and lung tissue intra- and extracellular M-CSF secretion. Here, ApoE-deficiency resulted in a reduction of both BALF and lung tissue extracellular and intracellular production of M-CSF on day one and three after β-glucan exposure (**Figure 7G, H, S7C-E**), leading to an increase in TUNEL^{+} ApoE-deficient CD11b^{+} AMs in ApoE^{flox}LysM^{Cre} mice (**Figure 7I**). To determine if the loss of M-CSF plays a crucial molecular role in the loss of differentiating monocytes to macrophages following β-glucan-induced environmental adaptation, we employed antibody-mediated M-CSF receptor (CSF-1R) blockade on days 0 and 3 after β-glucan stimulation and analysed treated and control animals seven days later. Mice treated with anti-CSF-1R-blocking antibody exhibited significantly lower numbers of ApoE^{+}CD11b^{+} AMs seven days after β-glucan inoculation, underscoring the importance of M-CSF in the monocyte-to-macrophage differentiation process following β-glucan stimulation (**Figure 7J, S7F-I**). Taken together this suggests ApoE as a central regulator of pulmonary monocyte-to-macrophage differentiation and survival via the M-CSF signalling axis upon β-glucan-induced environmental adaptation.

Discussion

Within our modern-day environment, the lung is constantly exposed to a plethora of sterile immunostimulatory components. However, the developmental, functional and molecular consequences for lung-resident macrophages are incompletely understood. Here, we show that a single non-pathologic intranasal β-glucan stimulus induces the development of MoAM, which highly express CD11b and ApoE and are characterised by their superior IL-6 production capacity in response to secondary LPS stimulation. Additionally, $ApoE^{+}CD11b^{+}$ AMs are glycolytic, highly phagocytic, and modify the outcome of a secondary bacterial infection and of a chronic fibrotic response *in vivo*. Molecularly this is instructed by Dectin-1 mediated recognition of β-glucan and its signalling adapter adaptor protein CARD9. Further anaylsis revealed a crucial role of ApoE for the maintenance of BALF-resident $ApoE^{+}CD11b^{+}$ AMs via the control of macrophage-derived M-CSF. This reveals that ApoE is a crucial checkpoint for monocyte to macrophage differentiation in the face of environmental adaptation and couples cellular cholesterol metabolism to differentiation and function.

Prior studies examined the development of MoAMs during viral, bacterial and fungal infections, radiation or bleomycin-induced fibrosis [16,18,38,48-51]. However, how broncho-alveolar macrophages adapt their transcriptome, metabolism and function to ambient immune-stimulatory components beyond the effects of the acute recognition of such stimuli remains poorly understood [52]. Here, we show that although the initial pulmonary inflammation evoked by β-glucan is minimal, functionally modified MoAMs arise from $Ly6c^{+}$ monocytes within the BALF, a process prior affiliated to e.g. viral or bacterial infection. This validates the model as suitable for low-grade environmentally induced inflammation. $ApoE^{+}CD11b^{+}$ AMs are induced for up to 21 days post β-glucan stimulation leading to functional modification of the macrophage repertoire in the lung, demonstrating the importance of low-grade inflammatory sterile insults in shaping the overall immune competence of the lung-resident macrophage repertoire.

β-glucan, a cell wall component of many pathological and non-pathological fungi, can be found within ambient air [25]. During fungal infection, monocytes and MoMacs are major antifungal effectors, via reactive oxygen species and the activation of antifungal neutrophils, in mice and man [50,53,54]. Furthermore, during the later stage of fungal pathogenesis, monocyte descendants are important for induction of $CD4^{+}$ T-cell responses [51].

Recently studies evaluated the role of β-glucan for the induction of innate immune training systemically but did not examine its effects at the level of the tissue [4,55]. Systemically β-glucan-induced functional modulations were accompanied by the induction of glycolysis and the enhanced release of the pro-inflammatory cytokines IL-6 and TNFα in circulating $Ly6^{+}$ monocytes similar to β-glucan-induced functional adaptation in the lung. Systemic β-

glucan administration expands BM GMP / MPP like progenitors, resulting in enhanced pathogen clearance or tissue maladaptation [56-58]. We show that pulmonary β-glucan administration only minimally affects BM progenitors and that generation of ApoE$^+$CD11b$^+$ AMs induces cMOP expansion and recruitment of CCR2-dependent Ly6c$^+$ monocytes to the lung. Dectin-1 recognizes β-glucan upon challenge; Dectin-1 downstream signaling is heterogenous and determines the functional output. We show that tissue adaptation induces Dectin-1 CARD9-dependent signalling circuits, in concordance with increased IL-6 levels, most probably via activation of Nfκb signalling. Finally, β-glucan-induced macrophages upregulate ApoE, a protein demonstrated to be part of various disease-specific MoMac gene signatures, e.g. during influenza infection, lung fibrosis, or obesity [16,29,48]. Its functional role in MoMac development was not investigated. In hematopoietic stem cells ApoE was shown to inhibit proliferation and subsequent progenitor maturation by controlling sensitivity towards granulocyte macrophage stimulating factor and IL-3 [59]. We show that myeloid-specific deletion of ApoE leads to the accumulation of cholesterol at the endoplasmatic reticulum, loss of M-CSF production and increased cell death ultimately inhibiting development of long-lived ApoE$^+$CD11b$^+$ AMs upon intranasal β-glucan challenge. ApoE$^+$ MoMac are also found in white adipose tissues during obesity, a condition conferring training-like feature to MPs or during influenza-induced lung inflammation supporting the crucial role of ApoE for the development of functionally adapted macrophages during inflammation [29,60]. Other work has established functional Nfκb-responsive elements within the ApoE gene and CARD9 directly activates Nfκb thus linking activation of inflammatory Nfκb responses to the induction of inflammatory adaptation in MoMacs.

Collectively we provide evidence that a single non-pathological environmental stimulation via the Dectin-1 – CARD9 axis generates inflammation-experienced MoMacs, which modify subsequent pulmonary acute and chronic inflammation under the control of ApoE, thus molecularly linking macrophage inflammatory amplitude to lung resilience and disease susceptibility.

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Author contributions statement

Conceptualization: H.T., D.A.B., J.H., N.K., A.S.; Formal Analysis and Investigation: H.T., D.A.B., J.H., N.K., J.S-S., J.Y., K.B., A.L.A., C.O.S., F.P., M.K., L.V., Y.X., M.G., L.B., W.T., C.C.B., S.S., A.I.V.A., A.H.G., J.D., A.A., S.C., K.H., G.B., D.L.W., F.G., J.R., M.B., J.L.S.; Writing: H.T., D.A.B., J.H., A.S.; Supervision: A.S..

Competing interests Statement

The authors declare no competing interests.

Figure Legends

Figure 1 Intranasal β-glucan exposure generates environmentally adapted ApoE^{+}CD11b^{+} alveolar macrophages within the broncho-alveolar space.

(A-E) Single-cell RNA sequencing of the broncho-alveolar lavage fluid (BALF) of male 8-12 weeks old C57BL/6J (WT) mice after intranasal stimulation with 200 µg β-glucan or PBS (n=10202 cells). Seven days after exposure, cells of three mice per condition were harvested and sorted for SSC^{hi}, Lin^{-} (B220, CD19, CD3ε, Nk1.1,

Ter-119), DRAQ7$^-$ singlets (n=3 mice, one independent experiment). (A) Uniform-manifold approximation and projection (UMAP) analysis of both conditions combined shows five different clusters. (B) Heatmap of top ten highly expressed genes for each of the five clusters. (C, D) UMAP from (A) separated by PBS (C, n= 4845 cells) or β-glucan (D, n= 5357 cells) condition. (E) Percentage of contribution of the five annotated clusters to overall cells split by conditions. (F) 5µm frozen section of the left lobe of the lung of a *Ms4a3-cre*^{Rosa26TOMATO} mouse 7 days after β-glucan exposure stained with a 17-plex CODEX antibody panel. Overlay image shows the markers used to identify AM populations. Single stainings of these markers are shown in grayscale. Filled arrows indicate ApoE$^+$CD11b$^+$ AMs, whereas open arrows CD11b$^-$ AMs. Scale bar represents 100 µm in the large image and 10 µm for the enlargements (n= one representative mouse of two independent experiments). (G) Detection of *ApoE* mRNA expression in the AM subsets of the BALF 7 days after intranasal PBS or β-glucan exposure in WT mice by Prime Flow (n= 3 mice pooled per group, one out of two independent experiments shown). (H, I) Flow cytometric quantification of absolute numbers (H) or frequency (I) of ApoE$^+$CD11b$^+$ AM (CD45$^+$SiglecF$^+$CD64$^+$CD11c$^+$CD11b$^+$) in the BALF in a time course from 1 to 21 days post β-glucan stimulation of WT mice (n= 9-10 mice, two independent experiments). Data in (H, I) are depicted as mean ± SD, ordinary one-way ANOVA with Tukey's multiple comparisons.

Figure 2 ApoE$^+$CD11b$^+$ AMs are monocyte-derived and CCR2-dependent.

(A) Flow cytometric quantification of absolute Ly6c$^+$ monocyte (CD45$^+$Ly6g$^-$SiglecF$^-$CD64^{int}CD11b$^+$Ly6c$^+$) numbers in the BALF 1 to 21 days post β-glucan stimulation of WT mice (n=9-10 mice, two independent experiments). (B, C) Flow cytometric analysis of BALF from *Ms4a3-cre*^{Rosa26TOMATO} mice seven days after intranasal PBS or β-glucan stimulation (n=8, two independent experiments). (B) Percentage of tdTomato$^+$ labelling in CD11b$^-$ and ApoE$^+$CD11b$^+$ AM and (C) proportion of tdTomato$^+$ labelling in CD11b$^-$ and ApoE$^+$CD11b$^+$ AM compared to monocytes (CD45$^+$SiglecF$^-$Ly6g$^-$CD11b$^+$F4/80$^+$). (D) CODEX multiplexed immunostaining of the left lobe of a *Ms4a3-cre*^{Rosa26TOMATO} mouse 7 days after β-glucan exposure (enlargement from Fig. 1F). Filled arrowheads indicate ApoE$^+$CD11b$^+$ AMs, whereas empty arrowheads indicate CD11b$^-$ AMs. tdTomato reporter signals are represented in red. Scale bar represents 50 µm. (E, F) Absolute counts of Ly6c$^+$ monocytes in the blood (E) or in the BM (F) of WT mice seven days after PBS or β-glucan by flow cytometry (n = 6 mice, two individual experiments). (G) Flow cytometric quantification of absolute ApoE$^+$CD11b$^+$ AM numbers in the BALF seven days after β-glucan exposure in WT or CCR2$^{-/-}$ mice (n=7-8 mice, two individual

experiments). Data are depicted as mean ± SD, (A, G) ordinary one-way ANOVA with Tukey's multiple comparisons, (B, E, F) unpaired two-tailed student's *t*-test).

Figure 3 ApoE^{+}CD11b^{+} AMs show increased release of interleukin 6 and induction of glycolysis.

(A-C) BALF cells were harvested from WT or *Ms4a3-cre^{ROSA26TOMATO}* mice seven days after intranasal exposure with PBS or β-glucan and subsequently restimulated *in vitro* with LPS for 18h. (A) Quantification of IL-6 protein levels by ELISA in the cell culture supernatant 24 h after LPS restimulation of WT mice (n=13-15 mice, three individual experiments). (B) CD11b^{+} Ms4a3^{+} AM and CD11b^{-} Ms4a3^{-} AM were sorted from the pooled BALF of PBS or β-glucan stimulated *Ms4a3-cre^{ROSA26TOMATO}* mice and seeded with 0.2x10^{5} cells per well prior to LPS restimulation (n= 9 mice for PBS, n= 31 mice for β-glucan, one dot represents the pooled supernatant of two technical replicate wells, here minimum 9 data points per group, two individual experiments). (C) Percentage of IL-6^{+} cells among ApoE^{+}CD11b^{+} AM after restimulation with LPS for 6-8 h followed by intracellular staining and flow cytometric analysis (n=6-7 mice, two individual experiments). (D) Quantification of IL-6 protein levels by ELISA in the cell culture supernatant 24 h after restimulation with LPS of WT and CCR2$^{-/-}$ mice (n=9-10 mice, two individual experiments). (E) BALFs of PBS or β-glucan experienced CD45.2 WT mice were harvested and pooled five days after stimulation. 2x10^{5} cells in 35 µl were intratracheally transferred into CD45.1 mice. Quantification of IL-6 protein levels by ELISA in the cell culture supernatant after restimulation with LPS 48 h post transfer (n=7-8 mice, two individual experiments). Data are depicted as mean ± SD, (A-E) ordinary one-way ANOVA with Tukey's multiple comparisons.

Figure 4 Environmental adaptation induced by β-glucan significantly improve pulmonary bacterial clearance and outcome of bleomycin-induced fibrosis

(A, B) Extracellular acidification rate (A) and glycolysis (B) in BALF cells seven days after PBS or β-glucan stimulation of WT mice measured by Seahorse (n=6 mice, one independent experiment). (C, D) AM cells from day seven PBS or β-glucan experienced WT mice were selected by adherence and subsequently treated with 2.5 µm pHrodo *S.aureus* bioparticles (n=3 mice pooled per condition, technical replicates: 6 control wells, 12-19 treated wells per group, one out of two independent experiments shown). (C) Representative curve of absolute phagocytosis^{+} AM numbers over the time course of 7 h (here shown as mean ±SD of all technical replicates). (D) Absolute numbers of phagocytosis^{+} AM 2 h after adding the pHrodo *S.aureus* bioparticles. (E, F) C57BL/6J WT mice were intranasally stimulated with PBS or β-glucan followed by intratracheal infection with 5x10^{6} CFU

Legionella pneumophilia at day seven post primary stimulation and analysis at day nine (n=9-10 mice, two independent experiments). Quantification of bacterial load in BALF (E) and absolute numbers of $ApoE^+CD11b^+$ AM by flow cytometry nine days post primary stimulation (F). (G, H) Representative confocal images (G) and SMA area quantification (H) of BALOs co-cultured with PBS- or β-glucan-experienced AM 48 h after induction of fibrosis via TGF-β. Seven days after stimulation, $2.5x10^4$ AM of PBS or β-glucan experienced WT mice were co-cultured with day 21 lung BALOs for 24 h. AM-organoid co-cultures were subsequently treated with 1.05 ng/ml TGF-β for 48 h prior to fixation and antibody staining. Myofibroblasts were stained for α-SMA (n=6-8 organoids per condition from two replicate wells, one out of two independent experiments shown). Scale bars in (G) represent 50 µm. Data are depicted as mean ± SD, (B, E, F) unpaired two-tailed student's *t*-test, (D) ordinary one-way ANOVA with Tukey's multiple comparisons, (H) Two-tailed Mann-Whitney test.

Figure 5 Generation of $ApoE^+CD11b^+$ AMs by β-glucan is dependent on the Dectin-1 – Card9 signalling axis.

(A) Percentage of monocyte and macrophage populations contributing to $Dectin\text{-}1^+$ cells in the WT mouse lung pregated on $CD45^+Lin^-Ly6g^-CD64^+$ cells (n=11 mice, two independent experiments) by flow cytometry. (B, C) Absolute $ApoE^+CD11b^+$ AM (B) and $Ly6c^+$ monocyte (C) numbers in the BALF seven days after PBS or β-glucan exposure in WT or $Dectin1^{-/-}$ mice (n=5-9, two independent experiments) by flow cytometry. (D, E) Absolute $ApoE^+CD11b^+$ AM numbers in the BALF seven days after PBS or β-glucan exposure in $Dectin1^{-/-}$ (D, n=4-10 mice, two independent experiments) or $CARD9^{-/-}$ (E, n=8-9 mice, two independent experiments) BM chimeras by flow cytometry. (F) Quantification of IL-6 protein levels by ELISA in the cell culture supernatant 24 h after LPS restimulation of WT and $Dectin1^{-/-}$ mice (n=7-10 mice, two independent experiments). (G) Quantification of IL-6 protein levels by ELISA in the cell culture supernatant 24 h after LPS restimulation of WT and $CARD9^{-/-}$ mice (n=9-10 mice, independent experiments). Data are depicted as mean ± SD, (B-G) ordinary one-way ANOVA with Tukey's multiple comparisons.

Figure 6 Paracrine myeloid-derived Apolipoprotein E controls $ApoE^+CD11b^+$ AM differentiation upon β-glucan-induced environmental adaptation.

(A) Violin plot of *ApoE* RNA expression levels in the BALF seven days after β-glucan exposure by scRNA seq. (B) WT mice were stimulated with β-glucan and BALF was harvested at different time points. Plot shows ApoE protein levels in the BALF measured by ELISA (n=4-5 mice, one independent experiment). (C, D) Absolute

numbers of ApoE^{+}CD11b^{+} AM (C) and Ly6c^{+} monocytes (D) seven days after intranasal β-glucan exposure of control or ApoE^{flox}LysM^{Cre} mice by flow cytometry (n=8-10 mice, three independent experiments). (E, F) Lethally irradiated CD45.1^{+}/CD45.2^{+} male mice were reconstituted with 1.5x10^{6} CD45.1^{+} mixed with CD45.2^{+} BM cells (WT/WT) or with CD45.1^{+} mixed with ApoE^{flox}LysM^{Cre} CD45.2^{+} BM cells (WT/ ApoE^{flox}LysM^{Cre}) for 12 weeks and subsequently intranasally stimulated with PBS or β-glucan (n=8-9 mice, two independent experiments). Flow cytometric quantification of ApoE^{+}CD11b^{+} AM numbers (E) and contribution of donor cells (CD45.1^{+} or CD45.2^{+}) to the ApoE^{+}CD11b^{+} AM pool (F) seven days after exposure. Data are depicted as mean ± SD, (B-F) ordinary one-way ANOVA with Tukey's multiple comparisons.

Figure 7 Myeloid-derived Apolipoprotein E controls survival of ApoE^{+}CD11b^{+} AMs by regulation of cholesterol storage and M-CSF secretion.

(A-B) Absolute numbers of ApoE^{+}CD11b^{+} AM (A) and Ly6c^{+} monocytes (B) three days after intranasal β-glucan exposure in control or ApoE^{flox}LysM^{Cre} mice (n=7-8, two independent experiments) by flow cytometry. (C, D) BALF of PBS or β-glucan stimulated control or ApoE^{flox}LysM^{Cre} mice was harvested three days after exposure, seeded and fixed after 2 h. Filipin staining was performed followed by immunofluorescence analysis. Representative images are shown in (C). Scale bars represent 5 µm. Plot in (D) shows mean filipin signal intensities of individual ApoE^{+}CD11b^{+} AM in the different conditions (n=3 mice per condition, two independent experiments). (E, F) Three days post stimulation of PBS or β-glucan stimulated control or ApoE^{flox}LysM^{Cre} mice, AM from the BALF were selected by adherence and afterwards incubated with 0.5 µM BODIPY-Cholesterol overnight. Cells were fixed for 15 min the next day and immunofluorescence was performed. (E) Representative confocal images, the scale bar represents 5 µm. (F) Quantification of overlapping signals of BODIPY-Cholesterol and the organelle markers Calreticulin (ER), EEA1 (endosomes) and LAMP1 (lysosomes) (n=2 mice per condition, two independent experiments). (G) BALF of PBS or β-glucan stimulated control or ApoE^{flox}LysM^{Cre} mice was harvested 24 h after exposure and seeded. Cells were fixed after 2h and immunostained to detect SiglecF, CD11b, and M-CSF. Plot shows mean M-CSF signal intensities of individual ApoE^{+}CD11b^{+} AMs (n=3 mice per condition, two independent experiments). (H) Quantification of M-CSF protein levels in the BALF one day after β-glucan exposure in control or ApoE^{flox}LysM^{Cre} mice (n=6 mice, two independent experiments) measured by ELISA. (I) BALF of PBS or β-glucan stimulated control or ApoE^{flox}LysM^{Cre} mice was harvested three days after exposure, seeded and fixed after 2 h. TUNEL staining was performed, followed by conventional immunofluorescence to detect SiglecF and CD11b. Plot shows mean TUNEL signal intensities of individual

ApoE^{+}CD11b^{+} AM (n=3 mice per condition, two independent experiments). (J) Absolute numbers of ApoE^{+}CD11b^{+} AM in the BALF of WT mice seven days after intranasal β-glucan treatment together with 500 µg of CSF-1R antibody or the respective isotype control and follow-up treatment 12 h and 3 days later (n=8-9 mice, two independent experiments). Data are depicted as mean ± SD, (A, B, G-I) ordinary one-way ANOVA with Tukey's multiple comparisons, (D, F) Two-tailed Mann-Whitney test, (J) unpaired two-tailed student's *t*-test.

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Methods

Animal studies and mouse models

All mice used in this study were bred in the animal facility of the LIMES Institute, University of Bonn, Germany or Center for Translational Cancer Research, Klinikum rechts der Isar, Technical University of Munich, Germany. Animals were housed in IVC mice cages under conventional conditions (12 h/12 h light/dark cycle, 22°C), with ad libitum access to food and water. All experiments were performed using C57BL/6J (WT) mice, which also served as controls for CCR2, Dectin-1, CARD9 knockout mice. For $ApoE^{flox}LysM^{Cre/+}$ mice, Cre-negative $ApoE^{flox}LysM^{+/+}$ littermates were used as controls. Eight to twelve weeks old male mice were used for experiments. All experiments were approved by government of North Rhine-Westphalia (84-02.04.2017.A347, 81-02.04.2020.A454).

Intranasal stimulation

Mice were anesthetized by intraperitoneal injection of ketamine/xylazine and intranasally inoculated with endotoxin-free 1x PBS (EMD Millipore) or 200 µg β-glucan from *Candida albicans.* BALF was collected one, three, seven, 14 or 21 days after inoculation. For cell analysis, the lung was flushed 3x with 1 ml cold 1x PBS with 10 mM EDTA. Afterwards, the fluid was centrifuged for 5 min at 365 g at 4°C and the supernatant was discarded. For cytokine and chemokine assessment, the lung was flushed 3x with the same 1 ml of cold 1x PBS with 10 mM EDTA. Afterwards, the supernatant without cells was frozen in liquid nitrogen until analysis. Supernatants were thawn on ice and centrifuged for 5 min at 10 000 rpm at 4°C to remove debris. M-CSF (R&D Systems) and ApoE (Abcam) protein levels were measured by ELISA according to manufacturer's protocols.

Macrophage transfer

C57BL/6J CD45.2 wildtype donor mice were intranasally stimulated with PBS or β-glucan as described above. At day five, BAL fluid was harvested and cells of the same condition were pooled. After centrifugation, the supernatant was discarded and cells were resuspended in LPS-free 1x PBS. Afterwards, $2x10^5$ donor cells in the volume of 35 µl were intratracheally transferred into CD45.1 recipient mice. BALF was harvested 48 h post transfer and either analyzed by flow cytometry or used for *ex vivo* restimulation with LPS (see below).

In vivo CSF-1 receptor blockade

Before *in vivo* application, the amount of the anti-CSF-1R or the isotype control antibody was calculated per cohort. To reduce the application volume, a Concentrator Plus vacuum centrifuge (Eppendorf) was used in V-AQ mode to evaporate excess liquid to yield a final product in 1/4 of the starting volume. C57BL/6J wildtype mice were intranasally stimulated with β-glucan mixed with 500 µg anti-CSF-1R antibody (BioLegend) or 500 µg isotype control (BioLegend). 12 h and three days after the initial treatment, intranasal application of 500 µg anti-CSF-1R antibody or the respective isotype control was repeated before analysis at day seven.

Pulmonary fibrosis and *Legionella pneumophila* infection

Mice were intranasally stimulated with β-glucan or PBS seven days before induction of pulmonary fibrosis or infection by *Legionella pneumophila.* For fibrosis induction, *Streptomyces verticillus* bleomycin (Sigma-Aldrich, 0.75 mg/kg body weight) was administered by intranasal installation. Body weight and health status were scored on a daily basis. Analysis was performed three or 14 days after bleomycin application. For bacterial infections, intratracheal application of *Legionella pneumophila* ($5x10^6$ CFU/mouse) was performed and mice were sacrificed two days after induction. For bacterial load determination, BALF supernatant was plated in duplicates on CYE-plates and grown for 3-4 days at 37°C in a non-CO_2 incubator.

Bone marrow Chimeras

BM chimeras were generated by multiple intraperitoneal busulfan (Sigma-Aldrich) injections or irradiation of recipient mice with 10 Gy. Afterwards, 5 x 10^6 (busulfan-treated mice) or 1.5 $x10^6$ (irradiated mice) freshly isolated BM cells from the donor animals were intravenously injected into the recipients. Peripheral blood chimerism was assessed 28 days after reconstitution by flow cytometry. BM chimeras were used in experiments after eight – twelve weeks of reconstitution.

Flow cytometry and cell sorting

For flow cytometry, cells of the broncho-alveolar space were harvested by flushing the lungs with 3x 1 ml ice cold 1x PBS containing 10 mM EDTA. After centrifugation with 365 g for 5 min at 4°C, cell pellets were resuspended in antibody mix and stained for 35 min at 4°C. After washing with FACS buffer (1x PBS, 2 mM EDTA, 0.5% BSA (SERVA)), life/death stain was performed using DRAQ7 (BioLegend, 1:1000 in FACS buffer) for 5 min at RT. Red blood cell lysis was performed only if necessary. For the lung tissue, the more segmented lobe was minced and enzymatically digested for 45 min at 37°C in HBSS (PAN Biotech) supplemented with 10%

FCS (Sigma-Aldrich), 0.2 mg/ml collagenase IV (Sigma-Aldrich) and 0.05 mg/ml DNase I (Sigma-Aldrich). Afterwards, the tissue pieces were homogenized with a 19G syringe and filtered through a 70 µm strainer. Red blood cell lysis was performed once for 5 min at RT prior to life/death stain and acquisition. In case of blood analysis, blood was collected in 1x PBS with 10 mM EDTA and stained in antibody mix for 35 min at 4°C. Red blood cell lysis was performed twice for 5 min at RT prior to life/death stain and acquisition. For BM cells, femur and tibia were flushed with 1x PBS, stained with antibody mix for 1 h and red blood cell lysis and life/death stain were subsequently performed. Cells were washed and resuspended in FACS buffer and recorded using FACS Symphony A5 (Becton Dickinson). FACS data were analyzed using FlowJo v10.8.1 (Becton Dickinson).

Cell sorting was performed using an ARIA III (Becton Dickinson) instrument. Briefly, cells from the lung lavage were antibody stained followed by life/death stain. Cell sorting was performed using a 100 µm nozzle into cooled 1.5 ml reaction tubes containing FACS buffer.

Monoclonal anti-mouse antibodies, including anti-CD45R (clone RA3-6B2, BioLegend, 1:400), anti-CD117 (clone 2B8, BioLegend, 1:200), anti-CD11b (clone M1/70, BioLegend, 1:200), anti-CD11c (clone N418, BioLegend, 1:200), anti-CD11c (clone N418, BioLegend, 1:200), anti-CD135 (clone A2F10, BD Bioscience, 1:200), anti-CD192 (clone SA203G11, BioLegend or clone 475301, BD Bioscience, 1:200), anti-CD19 (clone 6D5, BioLegend, 1:200), anti-CD150 (clone 475301, BioLegend, 1:200), anti- CD3 (clone 17A2, BioLegend, 1:200), anti-CD131 (clone JORO 50, BD Bioscience, 1:200), anti-CD45 (clone I3/2.3, BioLegend or 30-F11, BioLegend/BD Bioscience, 1:200), anti-CD45.1 (clone A20, BD Bioscience, 1:200), anti-CD45.2 (clone 104, BD Bioscience, 1:200), anti-CD16/32 (clone 2.4G2, BDHorizon or clone 93, BioLegend, 1:100), anti-CD206 (clone C068C2, BioLegend, 1:200), anti-CD48 (clone HM48-1, BioLegend, 1:100), anti-CD90 (clone 53-2.1, BioLegend, 1:200), anti-CD64, (clone X54-5/7.1, BioLegend, 1:100), anti-CX3CR1 (clone SA011F11, BioLegend, 1:100), anti-F4/80 (clone BM8, BioLegend, 1:100), anti-IL-6 (clone MP5-20F3, BD Biosciences, 1:100), anti-Ly6C (clone HK1.4, BioLegend, 1:200), anti-Ly6G (clone 1A8, BioLegend/BD Bioscience, 1:200), anti-MERTK (clone 2B10C42, BioLegend, 1:200), anti-MHC2 (clone M5/114.15.2 , BioLegend/BD Bioscience, 1:200), anti-NK-1.1 (clone PK136, BioLegend, 1:200), anti-Ly-6A/E (clone D7, Thermo Fisher Scientific, 1:200), anti-Siglec F (clone E50-2440, BD Biosciences, 1:200), anti-mouse TCR beta chain (clone H57-597, BioLegend, 1:400), anti-TER-119, (clone TER-119, BioLegend, 1:200), anti-CD34 (clone SA376A4, BioLegend, 1:100), anti-CD335 (clone 29A1.4, BioLegend, 1:200), anti-CD115 (clone AFS98, BioLegend or clone T38-320, BD Bioscience, 1:100), anti-CD24 (clone M1/69, BioLegend/BD Bioscience, 1:100), anti-Sca-1 (clone D7, BioLegend, 1:100), anti-CD43 (clone S7, BD Bioscience, 1:200) were used for flow cytometry or cell sorting.

PrimeFlow RNA detection by flow cytometry

BALFs of three stimulated wildtype mice were pooled, centrifuged at 365 g for 5 min at 4°C and resuspended in antibody mix for surface staining. Samples were transferred to 1.5 ml microcentrifuge tubes provided by the PrimeFlow RNA Assay Kit (ThermoFisher Scientific) and stained for 35 min at 4° C. Afterwards, life/death staining was performed using Zombie NIR Fixable Viability dye (1:1000 in PBS, BioLegend) for 10 min at RT. In the further steps, samples were handled according to manufacturer's instructions.

***Ex vivo* stimulation and assessment of cytokine production**

BALF of stimulated mice was collected, centrifuged at 365 g for 5 min at 4°C and resuspended in 1 ml RPMI1640 (PAN Biotech) supplemented with 10% FCS (Sigma-Aldrich), 2 mM GlutaMAX (Gibco), 1% MEM non-essential amino acids (Sigma Aldrich), 1 mM Na-Pyruvate (Gibco), 50 U/ml Pen-Strep (Gibco) and 0.1% β-Mercaptoethanol. Cells were counted and seeded with 0.2 x10^5 cells per well. After 2 h resting in 500 µl medium at 37°C and 5% CO_2, medium was exchanged to wash away non-adherent cells. Remaining macrophages were subsequently stimulated with 10 ng/ml LPS (Sigma-Aldrich) in a final volume of 500 µl. For intracellular cytokine stain, cells were restimulated for 4 h, then 2.5 µg Brefeldin A (BioLegend) and 2 nM Monensin (BioLegend) were added to each well and incubated for further 2 h. Cells were harvested in 1x PBS using a cell scraper followed by staining of surface markers by antibodies for 30 min at 4°C. Cells were washed and stained with Zombie NIR fixable viability dye (1:1000 in PBS, BioLegend) for 15 min. Afterwards, cells were permeabilized using the Cytofix/Cytoperm kit (Becton Dickinson, adapted from manufacturer's protocol). In brief, cells were resuspended in 200 µl Cytofix/Cytoperm solution per tube and incubated for 20 min at 4°C. Cells were washed twice with Perm/Wash and intracellularly stained by 100 µl Perm/Wash containing IL-6 (MP5-20F3; 1:100) antibody or the corresponding isotype control for 30 min at 4°C. Cells were washed twice with Perm/Wash and resuspended in 1x PBS before acquisition. For cytokine assessment from the supernatant, cell culture supernatant was harvested 24 h after LPS and snap frozen for further analysis. Supernatants were thawn on ice and centrifuged for 5 min at 10 000 rpm at 4°C to remove debris. IL-6 (ThermoFisher) protein levels were measured by ELISA. For multiplex cytokine and chemokine analysis, a customized 18-plex Procartaplex kit (ThermoFisher) was used according to manufacturer's protocols and run on Luminex FLEXMAP 3D (ThermoFisher) device.

828 ***In vitro* phagocytosis assay**

829 BALF cells of PBS or β-glucan inoculated mice were seeded with 0.2 x10^5 cells per well in a 96 well plate and
830 selected by adherence as before. Medium was exchanged to 100 µl medium or 100 µl medium containing 2.5 µg
831 pHrodo *S. aureus* bioparticles (Sartorius) per well. Phagocytosis was monitored every 10 min for 7 h in total using
832 the microscopy-based approach of the Incucyte instrument (Sartorius). Analysis was performed using the Incucyte
833 basic analyzer software in standard mode with two channels (phase and orange).

834

835 **Extracellular flux analysis**

836 BALF of two mice was pooled and 0.5 – 1x10^5 cells were plated in a 96 well Seahorse plate (Agilent) in Seahorse
837 XF base medium (Agilent) supplemented with 5% L-Glutamine (Sigma-Aldrich), 10% FCS (Sigma-Aldrich) and
838 50 U/ml Pen/Strep (Gibco) for 2 h at 37°C and 5% CO_2. Before acquisition, cells were washed and incubated in
839 FCS and Glucose free Seahorse XF base medium with 5% L-Glutamine (Sigma-Aldrich) and 50 U/ml Pen/Strep
840 (Gibco). During the run, 100 mM Glucose (Sigma-Aldrich) solution was injected into port A leading to a final
841 glucose concentration of 10 mM per well. This was followed by injection of 10 µM Oligomycin A (Sigma-
842 Aldrich, final concentration 1 µm) solution and 500 mM 2-Desoxyglucose (Sigma-Aldrich, final concentration
843 50 mM). Glycolysis, glycolytic capacity and glycolytic reserve were calculated using the Agilent Wave software.
844 After the Seahorse assay, cell numbers per well were determined for normalization using the CyQUANT NF Cell
845 Quantification Assay (Thermo-Fisher) and a TECAN plate reader.

846

847 **Lung organoid generation, fibrosis induction by TGF-β and immunofluorescence staining**

848 Organoid cultures were prepared as previously described and cultured at 37°C with 5% CO_2 [39]. In brief, lung
849 single cell suspensions were prepared from adult wildtype mice and $CD31^+CD45^+CD16/32^+$ cells were depleted
850 by antibody-coupled magnetic beads. From the $CD31^-CD45^-CD16/32^-$ negative fraction, $EpCAM^{high}CD24^{low}Sca\text{-}1^+$
851 BASC and $EpCAM^-Sca\text{-}1^+$ rMC were isolated by FACS. 5x10^3 BASC and 1.8x10^4 rMC were pooled and
852 mixed with growth factor-reduced Matrigel (Corning) (1:1) and seeded on 12 mm cell culture inserts in a 24-well
853 plate. α-MEM medium (ThermoFisher) supplemented with 10% FCS (ThermoFisher), 50 U/ml Pen/Strep
854 (ThermoFisher), 1x Insulin-Transferrin-Selenium (ThermoFisher) and 2 µg/ml heparin (Stemcell Technologies)
855 was added to the wells to obtain an air-liquid interface. For the co-culture, alveolar macrophages were obtained
856 from the BALF of wildtype mice seven days after PBS or β-glucan inoculation. Subsequently, 2.5x10^4 AM were
857 seeded on top of the Matrigel layer of the day 21 organoid cultures. 24 h later, the organoid-AM co-cultures were

858 treated with medium containing either PBS or 1.05 ng/ml TGF-β (Miltenyi Biotech) to induce fibrosis. After 48
859 h of TGF-β treatment, cultures were fixed in 4% PFA, permeabilized and blocked overnight with 1x PBS
860 containing 0.5% Triton-X-100 (ThermoScientific) and 5% donkey serum (PAN Biotech) (blocking buffer).
861 Primary and secondary antibodies were incubated overnight in blocking buffer. The samples were cleared by
862 glycerol-fructose clearing as recently described [1].
863
864 **Histology**
865 Mice were anesthetized followed by trans-cardial perfusion with 10 ml ice cold 1xPBS containing 10 mM EDTA
866 using the lung-heart circulation. Lungs were removed and fixed in 4% PFA overnight at 4°C (for paraffin-
867 embedded tissue) or infiltrated with 1 ml 50% OCT (in 1xPBS), removed, and fixed for 6 h in 1.3% PFA at 4°C.
868 For paraffin sections lungs were dehydrated and paraffin embedded. For frozen sections, after fixation lungs were
869 dehydrated in 10, 20, and 30% sucrose (in 1x PBS) for 24 h at 4°C. After dehydration, the left lobe was separated
870 and embedded in OCT. Sections of 5 µm were prepared for immunohistochemistry.
871
872 **Immunofluorescence and histology staining**
873 Coverslips containing frozen tissue sections were left drying on drierite beads for 5min and subsequently fixed
874 on ice-cold acetone for 10 min. Afterwards, sections were washed twice and permeabilized with 0.2% Triton X-
875 100 for 20min at RT. Afterwards, sections were washed twice with 1x PBS and photobleached as described before
876 [2]. Following photobleaching, sections were blocked in 3% BSA for 1 h at RT. After blocking, primary antibodies
877 were added and left incubating overnight at 4°C. Sections were then washed three times with 1x PBS and
878 secondary antibodies and nuclear staining were subsequently added and left incubating for 1 h at RT. Samples
879 were washed as before and coverslips were mounted using mounting medium. Fluorescently labeled primary
880 antibodies were added after washing the secondary antibodies and left incubating 2 h at RT. For
881 immunofluorescence of cultured cells no acetone fixation and photobleaching were performed.
882 Histological evaluation of lung fibrosis was performed by Picrosirius red and Masson's trichrome stainings of
883 two consecutive paraffin-embedded 5 µm tissue sections as previously described [3,4].
884
885 **Filipin, TUNEL, BODIPY-Cholesterol staining**
886 5-8x10^4 BALF cells were seeded in complete RPMI medium in a 24-well plate containing sterile glass coverslips.
887 Cells were left adhering for 3 h at 37°C. For filipin and TUNEL stainings, cells were washed with 1x PBS and

subsequently fixed with 4% PFA for 30 min. For filipin staining, after washing away the fixative, cells were incubated in 100 mM glycine for 10 min at RT, and subsequently blocked with 3% BSA supplemented with 50 µg/ml filipin (Sigma-Aldrich) for 2 h at RT. Cells were washed three times with 1x PBS and immunostained as indicated above, but DRAQ5 (ThermoFisher Scientific) was used as a counterstain. For TUNEL staining, manufacturer's instructions were followed and immunofluorescence was performed after TUNEL (ThermoFisher Scientific). For BODIPY-cholesterol staining, BALF cells were seeded in 8-well chamber slides, left as before, and incubated overnight in complete RPMI1640 medium supplemented with 0.5 µM BODIPY-Cholesterol (Biomol). Cells were washed three times with 1x PBS, fixed, permeabilized and immunostained as described above.

Imaging

Images of the Picrosirus Red and Trichrome stainings were acquired using the OLYMPUS Slideview VS200 (Evident Corporation). Sections were analyzed at a 20-fold magnification. Images of immunofluorescence of tissue sections and cultured cells were acquired using a Zeiss LSM 880 Airyscan system using a 60x oil immersion objective (NA) with a z-spacing of 500 nm. Images were acquired using the 405, 488, 561 and 640 nm laser lines. During acquisition, nuclei showing the prototypical shape of neutrophils or eosinophils were excluded.

Image analysis

To quantify signal intensities from different markers from individual BALF cells, images were analyzed with a customized pipeline in CellProfiler. Briefly, Hoechst or DRAQ5 signals were used to segment the cells. A second primary detection step was added to create a mask of all SiglecF$^+$ objects. This mask was subsequently merged onto the nuclei mask and only overlapping objects were further analyzed. A secondary object detection step was incorporated to distinguish between ApoE$^+$CD11b$^+$ and CD11b$^-$ cells and create a mask of alveolar macrophages. Analysis of immunofluorescence of tissue sections was performed in QuPath [5]. Nuclear signals were used to identify all objects using a radius of 2µm. For each channel, an object classifier was created to set the detection threshold based on the mean signal intensity. Subsequently, these classifiers were combined to identify alveolar macrophages. Individual cell mean intensities were exported. To measure the area of fibrosis and the Ashcroft score, scoring was performed in four different areas of each slide as previously described [6,7]. Quantification of fibrotic area from total tissue area was performed with ImageJ. The Ashcroft score was quantified using scores ranging from 0 to 8 by two independent investigators, which were blinded to the treatments. To score fibrosis in

cultured organoids, images were imported into QuPath and the area of the organoid was annotated using DAPI as a reference. A pixel classifier to detect SMA positive areas was trained using the control samples (i.e. untreated and TGF-β treated). 5-10 exemplary regions of SMA and background spots were selected. The trained model was applied to analyze all the images. The total SMA^+ area of each organoid was quantified. To quantify the percentage of co-localization and overlapping area of BODIPY-Cholesterol with cellular organelles, full z-stacks of single $ApoE^+CD11b^+$ and $CD11b^-$ AMs were uploaded to Fiji and analyzed using the JACoP plugin, as described before [8]. Thresholds were established using 5 randomly selected images from each condition and then applied in all the images.

CODEX multiplexed imaging and analysis

Fresh frozen sections of the left lobe of the lung of 8-week *Ms4a3-cre*^{Rosa26TOMATO} mouse seven days after intranasal PBS or β-glucan were prepared and stained following manufacturer's instructions. Briefly, sections were fixed in ice-cold acetone for 10 min. Afterwards, samples were rehydrated and permeabilized for 20 min with 0.2% Triton-X100. Sections were photobleached twice for 1 h as indicated before [8]. After photobleaching, samples were equilibrated for 30 min in staining buffer (Akoya Biosciences), and subsequently stained with a 17-plex CODEX antibody panel overnight at 4°C. After staining, samples were washed in staining buffer, fixed in ice-cold methanol and washed. A final fixation step with BS3 crosslinker (Sigma Aldrich) was performed. Specimens were stored in CODEX storage buffer (Akoya Biosciences) at 4°C for a maximum of one week before imaging. BALF cells from wildtype mice were seeded on CODEX coverslips after harvesting seven days after intranasal stimulation. 2 h after seeding, cells were fixed with 4% PFA for 20 min, washed, and stored in PBS at 4°C until CODEX staining. Except for the initial drying step, the same staining protocol and CODEX panel as for the lung sections were used.

Antibody detection was performed in a multicycle experiment with the corresponding fluorescently-labeled reporters, following manufacturer's instructions. Images were acquired with a Zeiss Axio Observer widefield microscope (Carl Zeiss AG, Jena, Germany) using a 20x air objective (NA 0.85) and a z-spacing of 1.5 µm. The 405, 488, 568, 647 nm fluorescent channels were used. After acquisition, images were exported using the CODEX Instrument Manager (CIM, Akoya Biosciences) and processed with the CODEX Processor v1.7 (Akoya Biosciences). Cells were segmented using DAPI signals and ATPase I membrane staining to define the cell borders. Cell classification to detect alveolar macrophages and other MPs was performed in CODEX MAV (Akoya Biosciences), following a similar gating scheme as the one used for flow cytometry.

Preparation of Seq-Well arrays and libraries

Seq-Well arrays and libraries were generated as previously described [9]. Briefly, arrays were generated by pouring PDMS master mix into master molds and then functionalized by plasma treatment, washing with acetone, incubation with 0.2% chitosan solution and subsequent incubation in PGA buffer under vacuum pressure. For library generation, $1.1x10^5$ barcoded mRNA-capture beads in Bead Loading Buffer were loaded onto the array. $2\text{-}3x10^4$ BALF cells in RPMI1640 medium (Gibco) with 10% FCS (Sigma-Aldrich) were loaded and rocked for 10 min. The loaded arrays were washed, sealed by polycarbonate membranes under mild vaccum, incubated for 30 min at 37°C in Agilent clamps (Agilent) and then incubated in a guanidinium-based lysis buffer for 20 min. After incubation in hybridization buffer, the mRNA capture beads were washed from arrays and collected. Reverse transcription was performed on the bead pellet using a Maxima Reverse Transcriptase reaction (ThermoFisher) for 30 min at RT followed by 90 min incubation at 52°C before stopping the reaction with TE buffer supplemented with 0.01% Tween-20. Excess primers were digested by exonuclease ExoI (New England Biolabs). Beads were counted and the reverse transcribed cDNA libraries were amplified in a PCR reaction. After PCR, $2\text{-}4x10^4$ beads were pooled and cleaned using AMPure XP beads (Beckman Coulter). The library integrity was assessed using a High Sensitivity D5000 assay (Agilent) for Tapestation 4200 (Agilent).

Sequencing

The cDNA libraries (1 ng) were tagmented with the prepared single-loaded Tn5 transposase mixed with pre-annealed linker oligonucleotides and afterwards cleaned using MinElute PCR kit (Qiagen) following the manufacturer's instructions. The Illumina indices (Illumina) were added to the tagmented product by PCR and subsequently cleaned by AMPure XP beads (Beckman Coulter). The final library quality was assessed using a High Sensitivity DNA5000 assay (Agilent) and quantified using the Qubit high-sensitivity dsDNA assay (ThermoFisher). Seq-Well libraries were equimolarly pooled and clustered at 1.4 pM concentration with 10% PhiX using High Output v2.1 chemistry (Illumina) on a NextSeq500 system (Illumina). Sequencing was performed paired-end as followed: custom Drop-Seq Read 1 primer for 21 cycles, 8 cycles for the i7 index and 61 cycles for Read 2. Single-cell data were demultiplexed using bcl2fastq2 (v2.20) (Illumina). Fastq files were loaded into a snakemake-based data pre-processing pipeline (version 0.31, available at https://github.com/Hoohm/dropSeqPipe) [10].

scRNAseq data analysis

Sequencing reads were mapped to the mouse reference genome mm10 using STAR alignment from the Drop-seq pipeline (v2.0.0) as previously described [10]. Next, we assess the quality of our libraries and excluded cells with low quality (<500 genes per cell), doublets (>3000 genes per cell), or dead cells (>10% of mitochondrial content). All genes expressed in less than five cells were filtered out.

Cell clustering analysis was performed using the Seurat package (v4.1.1) according to instructions [11]. In brief, the expression data was log normalized with a scale factor of 10.000. After scaling, PCA was performed using the top 2000 variable genes for a linear dimensional reduction. The first 10 PCA components were used to cluster cells by the Louvain algorithm. To obtain an optimal cluster resolution, we set the resolution parameter in the FindClusters function as 0.25 to generate 5 major clusters, which were visualized after non-linear dimensional reduction with UMAP. Differentially expressed genes (DEGs) in each cluster were identified by using the default Wilcoxon rank sum test in the FindAllMarkers function, and were defined with logfc.threshold >0.25 and min.pct >0.25.

Statistics

Statistical analysis and comparison was performed using Prism 10 (GraphPad). Data are shown as mean ± SD. Statistical significance was assessed by student's t-test (unpaired) or ordinary one-way ANOVA with Tukey's multiple comparisons test. Survival of animals is displayed in Kaplan-Meier survival curves. A P value < 0.05 was considered as statistically significant, exact P values are displayed in the figures. Mice were randomly allocated to the control or treatment groups by the investigator. Mice numbers are indicated as "n" in the figure legends, as well as the number of independent experiments.

Data Availability

The scRNA-seq raw reads and processed data were submitted to the NCBI GEO database accession number GSE211575. All code used for data visualization of the scRNA-seq data can be found at https://github.com/SchlitzerLab/Trained_immunity_2022.

Code Availability

All code used for data visualization of the scRNA-seq data can be found at https://github.com/SchlitzerLab/Trained_immunity_2022.

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Supplemental Figure LegendsSupplemental Figure 1 to Figure 1

(A-F) ScRNA sequencing of the BALF seven days after intranasal stimulation with PBS or β-glucan in wt mice. (A) Violin plot of feature gene counts per cluster. (B) Violin plot of UMI counts per cluster. (C) Violin plot of mitochondrial gene percentage per cluster. (D, E) Violin plot of *Siglecf* (D) and *Itgax* (E) RNA expression levels. (F) Percentage of contribution of condition, PBS or β-glucan, to the five clusters. (G) AM of PBS or β-glucan treated WT mice were selected by adherence from the BALF 7 days after exposure and subsequently stained with a 17-plex CODEX antibody panel. Overlay image shows the markers used to identify AM populations. Filled arrows indicate $ApoE^{+}CD11b^{+}$ AMs, whereas open arrows $CD11b^{-}$ AMs. Scale bar represents 10 µm (n= 2 mice pooled, two independent experiments). (H) Flow cytometry gating strategy pre-gated on Lin^{-} (Ly6g, B220, CD19, CD3ε, Nk1.1, Ter-119) and $CD45^{+}$ cells to define $ApoE^{+}CD11b^{+}$ AM in the BALF after intranasal PBS or β-glucan stimulation. (I) Flow cytometric quantification of $ApoE^{+}CD11b^{+}$ AM numbers as frequency among AM in the BALF in a time course from 1 to 21 days post β-glucan stimulation of wt mice (n= 9-10 mice, two independent experiments). (J, K) Flow cytometric quantification of absolute numbers (J) or frequency (K) of AM ($CD45^{+}SiglecF^{+}CD64^{+}CD11c^{+}$) among $CD45^{+}Lin^{-}$ cells in the BALF in a time course from 1 to 21 days post β-glucan stimulation of wt mice (n= 9-10 mice, two independent experiments). (L, M) Flow cytometric quantification of frequency of neutrophils (L) and eosinophils (M) among $CD45^{+}Lin^{-}$ cells in the BALF in a time course from 1 to 21 days post β-glucan stimulation of wt mice (n= 9-10 mice, two independent experiments). (N) Mean fluorescence Intensity (MFI) of SiglecF on indicated cell subsets in lung seven days post stimulation with β-glucan (n=4 mice, two independent experiments). (O) UMAP representation of intranasally stimulated lungs with PBS or β-glucan seven days after stimulation (pooled n=3 mice per condition). Data are depicted as mean ± SD, (I-N) ordinary one-way ANOVA with Tukey's multiple comparisons.

Supplemental Figure 2 to Figure 2

(A) Quantification of frequency of $Ly6c^{+}$ monocytes among $CD45^{+}Lin^{-}$ cells in the BALF in a time course from 1 to 21 days post β-glucan stimulation of WT mice by flow cytometry (n= 9-10 mice, two independent experiments). (B, C) Percentage of $Ly6c^{+}$ monocytes among $CD45^{+}Lin^{-}$ cells in the blood (B) or in the BM (C) of WT mice seven days after PBS or β-glucan by flow cytometry (n = 6 mice, two individual experiments). (D-G) Frequency among $CD45^{+}Lin^{-}$ cells and absolute numbers of cMOP (D, E) and GMP (F, G) in the BM of WT mice seven days after PBS or β-glucan by flow cytometry (n = 6 mice, two individual experiments). (H) Quantification of $ApoE^{+}CD11b^{+}$ AM frequency among $CD45^{+}Lin^{-}$ cells in the BALF seven days after β-glucan

exposure in WT or CCR2$^{-/-}$ mice (n=7-8 mice, two individual experiments) by flow cytometry. Data are depicted as mean ± SD, (A, H) ordinary one-way ANOVA with Tukey's multiple comparisons, (B-G) unpaired two-tailed student's *t*-test.

Supplemental Figure 3 to Figure 3

(A) BALF cells were harvested from WT mice seven days after intranasal exposure with PBS or β-glucan and subsequently restimulated *in vitro* with 10 ng/ml LPS. Representative flow cytometry dot plots of IL-6^{+} ApoE^{+}CD11b^{+} AM after restimulation with LPS for 6-8 h followed by intracellular staining gated on ApoE^{+}CD11b^{+} AM (n=6 mice, two independent experiments). (B) Experimental setup of macrophage transfer. PBS or β-glucan experienced CD45.2^{+} WT macrophages were harvested five days after stimulation and intratracheally transferred into CD45.1^{+} mice. Readout per flow cytometry or *in vitro* restimulation 48 h after transfer. (C) Representative flow cytometry plot of the BALF of CD45.1^{+} recipient mice 48 h post transfer of β-glucan experienced CD45.2^{+} donor macrophages.

Supplemental Figure 4 to Figure 4

(A, B) Measurement of glycolytic reserve (A) and glycolytic capacity (B) in BALF cells seven days after PBS or β-glucan stimulation by extracellular flux analysis (n=6 mice, one independent experiment). (C, D) AM cells from day seven PBS or β-glucan experienced WT mice were selected by adherence and subsequently treated with 2.5 µm pHrodo *S.aureus* bioparticles (n=3 mice pooled per condition, technical replicates: 6 control wells, 12-19 treated wells per group, one out of two independent experiments shown). (C) Total integrated intensity (OCU x µm²/image) of the phagocytosis signal in AM over the time course of 7 h (here shown as mean ±SD of all technical replicates). (D) Quantification of the total integrated intensity in phagocytosis^{+} AM 2 h after adding the pHrodo *S.aureus* bioparticles. (E) Experimental setup of an acute secondary bacterial infection. Intranasal PBS or β-glucan stimulation at day zero was followed by intratracheal inoculation with *Legionella pneumophila* at day seven and sacrifice two days after infection. (F) Experimental setup of a chronic secondary fibrosis challenge. Intranasal PBS or β-glucan stimulation at day 0 was followed by intratracheal application of *Streptomyces verticillus* bleomycin at day seven and sacrifice three or 14 days after fibrosis induction. (G-I) Health assessment of mice after pretreatment with PBS or β-glucan (day 0) prior to fibrosis induction (day 7). (G) Kaplan-Meier plot of survival (n=35 mice, two independent experiments). (H) Disease burden (general health appearance, spontaneous behavior, body weight) (n=14 mice, one representative experiment from two independent experiments shown).

(I) Body weight as % change based on the initial weight at day 0 (n=14 mice, one representative experiment from two independent experiments shown). (J-L) Quantification of IL-4 (J), IL-33 (K) and TSLP (L) protein levels 3 or 14 days after bleomycin treatment (n=6-7 mice, one independent experiment). (M, N) Quantification of the fibrotic area as percent of the total lung tissue area (M) and the fold change of Ashcroft score (N) by histology 14 days after bleomycin treatment (n=8-11 mice, two independent experiments). (O) Representative confocal images of lung BALOs co-cultured with PBS- or β-glucan-experienced AM. As a control to TGF-β, AM-BALO co-cultures were treated with medium only. After fixation, co-cultures were stained for α-SMA (n=6-8 organoids per condition from two replicate wells, one out of two independent experiments shown). Scale bars represent 50 µm. Data are depicted as mean ± SD, (A-B, L-N) unpaired two-tailed student's *t*-test, (D) ordinary one-way ANOVA with Tukey's multiple comparisons, (G) Mantel-Cox test, (H, I) ordinary two-way ANOVA with Šídák's multiple comparisons.

Supplemental Figure 5 to Figure 5

(A, B) Frequency of $ApoE^{+}CD11b^{+}$ AM among $CD45^{+}Lin^{-}$ cells in the BALF (A) seven days after PBS or β-glucan exposure in $Dectin1^{-/-}$ BM chimeras and the respective blood chimerism for $Ly6c^{+}$ monocytes (B) assessed by flow cytometry (n=4-10 mice, two independent experiments). (C, D) Frequency of $ApoE^{+}CD11b^{+}$ AM among $CD45^{+}Lin^{-}$ cells in the BALF (C) seven days after PBS or β-glucan exposure in $CARD9^{-/-}$ BM chimeras and the respective blood chimerism for $Ly6c^{+}$ monocytes (D) assessed by flow cytometry (n=8-9 mice, two independent experiments). Data are depicted as mean ± SD, (A, C) ordinary one-way ANOVA with Tukey's multiple comparisons.

Supplemental Figure 6 to Figure 6

(A, B) Frequency of $ApoE^{+}CD11b^{+}$ AM (A) and $Ly6c^{+}$ monocytes (B) among $CD45^{+}Lin^{-}$ cells seven days after intranasal β-glucan exposure of control or $ApoE^{flox}LysM^{Cre}$ mice by flow cytometry (n=8-10 mice, three independent experiments). (C-F) Absolute numbers of $Ly6c^{+}$ monocytes in the blood (C) or BM (D), cMOP (E) and GMP (F) in the BM of control or $ApoE^{flox}LysM^{Cre}$ mice seven days after intranasal β-glucan exposure quantified by flow cytometry (n=6-8 mice, two independent experiments). (G, H) Lethally irradiated $CD45.1^{+}/CD45.2^{+}$ male mice were reconstituted with $1.5x10^{6}$ $CD45.1^{+}$ mixed with $CD45.2^{+}$ BM cells (WT/WT) or with $CD45.1^{+}$ mixed with $ApoE^{flox}LysM^{Cre}$ $CD45.2^{+}$ BM cells (WT/ $ApoE^{flox}LysM^{Cre}$) for 12 weeks and subsequently intranasally stimulated with PBS or β-glucan (n=8-9 mice, two independent experiments). Flow

cytometric quantification of $ApoE^{+}CD11b^{+}$ AM frequency (G) among $CD45^{+}Lin^{-}$ cells and chimerism of $Ly6c^{+}$ monocytes in blood, BALF and BM (H). Bars in (H) are further subdivided into $CD45.1^{+}$ or $CD45.2^{+}$ to display contribution of transferred mixed BM donors. Data are depicted as mean ± SD, (A-G) ordinary one-way ANOVA with Tukey's multiple comparisons.

Supplemental Figure 7 to Figure 7

(A) Frequency of $ApoE^{+}CD11b^{+}$ AM (A) and $Ly6c^{+}$ monocytes (B) among $CD45^{+}Lin^{-}$ cells three days after intranasal β-glucan exposure in control or $ApoE^{flox}LysM^{Cre}$ mice (n=7-8, two independent experiments) by flow cytometry. (C) Quantification of M-CSF protein levels in the BALF in a time course from one to 21 days post β-glucan stimulation of WT mice (n=5 mice, one independent experiment). (D, E) Lungs from WT mice were harvested 24 h after β-glucan stimulation, fixed, and frozen in OCT. 5 µm sections were prepared and stained with antibodies to identify AMs and visualize M-CSF production. Representative images are shown in (D). Arrows indicate $ApoE^{+}CD11b^{+}$ AMs. Seven regions of the same size from samples in (D) were acquired and analyzed as explained in Materials and Methods. Plot in (E) shows the M-CSF mean signal intensities of $CD11b^{-}$ and $ApoE^{+}CD11b^{+}$ AMs. Each dot represents an individual cell. (F) Experimental setup of *in vivo* CSF-1R blockade. WT mice were intranasally stimulated with β-glucan mixed with an anti-CSF-1R antibody or the respective isotype control and repeatedly treated 12 h and 3 days after initial stimulation. (G- I) Frequency of $ApoE^{+}CD11b^{+}$ AM (G) among $CD45^{+}Lin^{-}$ cells and absolute numbers of AM (H) and $Ly6c^{+}$ high monocytes (I) in the BALF of WT mice seven days after intranasal β-glucan treatment together with 500 µg of CSF-1R antibody or the respective isotype control (n=8-9 mice, two independent experiments). Data are depicted as mean ± SD, (A-C) ordinary one-way ANOVA with Tukey's multiple comparisons, (E) Two-tailed Mann-Whitney test, (G-I) unpaired two-tailed student's *t*-test.