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The C3-C3aR axis modulates trained immunity in alveolar macrophages

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eLife Assessment

This study explores how complement protein C3 and its signalling may modulate immune training in alveolar macrophages. The findings are an **important** contribution to the field of trained immunity. The findings are **convincingly** supported by in vivo and ex vivo experiments, encompassing both pharmacological and genetic-based approaches.

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Abstract

Complement protein C3 is crucial for immune responses in mucosal sites such as the lung, where it aids in microbe elimination and enhances inflammation. While trained immunity – enhanced secondary responses of innate immune cells after prior exposure – is well-studied, the role of the complement system in trained immune responses remains unclear. We investigated the role of C3 in trained immunity and found that alveolar macrophage *C3* and *C3aR1* expression increased in humans after an intranasal exposure to a training stimulus. *In vivo*, trained wild-type mice showed significantly elevated pro-inflammatory cytokines and increased C3a levels upon a second stimulus. *Ex vivo*, trained C3-deficient alveolar macrophages (AMs) displayed reduced chemokine and cytokine output as well as impaired phagocytosis and reactive oxygen species (ROS) production compared to wildtype AMs. Real-time confocal microscopy of live, intact mouse alveoli revealed that AMs internalize C3 rapidly after alveolar microinstillation, as compared to C3a. Correspondingly, the blunted cytokine output was restored by exogenous C3 but not by C3a. Inhibiting C3aR, both pharmacologically and with a genetic C3aR knockout, prevented this restoration, indicating the necessity of C3aR engagement. Mechanistically, trained WT AMs demonstrated enhanced glycolytic activity compared to C3-deficient AMs – a defect corrected by exogenous C3 in a C3aR-dependent manner. These findings reveal that C3 modulates trained immunity in AMs through C3aR signaling and highlight a novel role for C3 in trained immunity.

Introduction

The complement system is a crucial part of immunity, consisting of proteolytic enzymes that generate fragments which enhance antibody binding and help induce phagocytosis (Sahu et al., 2022). A central component of this system is the C3 protein, which is cleaved into its constituent components C3a and C3b upon activation (Kulkarni et al., 2018). C3b tags pathogens for phagocytosis and plays a vital role in the formation of the C3 convertase, which cleaves C5 and eventually forms the membrane attack complex that lyses targeted cells (Janssen et al., 2006). Meanwhile, C3a acts as an anaphylatoxin, binding to its cognate C3a receptor (C3aR) on and within cells, and modulates inflammatory responses (Kildsgaard et al., 2000). C3 activation has also been shown to favor an increase in glycolysis, suggesting a plausible mechanism for enhanced inflammatory activity (Friščić et al., 2021; S. Kang et al., 2024). Moreover, C3 is present at low levels in the bronchoalveolar lavage (BAL) fluid of uninjured mice as well as humans, indicative of localized production (Bolger et al., 2007). This local C3 production increases during injury, affecting mucosal responses to infection (Bolger et al., 2007). Additionally, the conversion of C3 to a conformationally altered C3(H_2O) moiety increases during inflammation (Elvington et al., 2019). This C3(H_2O) form is that which is internalized by various cell types, playing a key role in modulating survival and effector immune responses (Elvington et al., 2017; Kulkarni et al., 2019). Thus, local C3 activity plays an important role in modulating mucosal immune responses (Kulkarni et al., 2024).

"Trained immunity" refers to cells such as innate immune cells (i.e., monocytes and macrophages) exhibiting robust, enhanced inflammatory responses upon secondary stimulation after a prior insult (Quintin et al., 2012; Saeed et al., 2014). This form of "memory" is considered broadly antigen nonspecific, yet lasts several months after the initial stimulus (Netea et al., 2020). Underlying the induction of trained immunity are epigenetic and metabolic reconfigurations occurring after an initial stimulus, which prime transcriptional machinery for rapid activation after engaging secondary insults (Bekkering et al., 2014; Fanucchi et al., 2021). For instance, the bacterial and fungal cell wall component 1,3-D-β-glucan, binds the dectin-1 pattern recognition receptor, which induces metabolic changes favoring persistent glycolytic activity (Cheng et al., 2014; Earhart et al., 2023). This, in turn, promotes shifts in epigenetic modifications such as histone acetylation and methylation favoring strong pro-inflammatory gene expression upon restimulation, and can be long-lasting (Fok et al., 2019; Tercan et al., 2021). While knowledge of trained immunity is primarily systemic, there is growing evidence for site-specific effects such as in alveolar macrophages (AMs) (Chakraborty et al., 2023; Zahalka et al., 2022). Little is currently known about how the complement system affects trained immunity. Here, we sought to investigate whether the C3 protein – a key component of immune responses at mucosal sites such as the respiratory system – affects trained immunity in AMs and determine whether any effect is mediated by C3aR activity.

Results

To assess the relevance of C3 in response to a training stimulus, we interrogated a publicly available dataset of bronchoalveolar lavage specimens from human volunteers who underwent aerosolized Bacillus Calmette Guerin (BCG) administration (Marshall et al., 2025). Both the mean expression as well as the percentage of AMs expressing *C3* and *C3aR1* increased at day 2 post-BCG exposure, and remained elevated at day 7, as compared to their expression in AMs of volunteers who received saline (Fig. 1A-C). *C3* expression was highest in the activated alveolar macrophage subset (Supplementary Fig. S1A-C). To investigate the role of C3 in pulmonary immune cell-trained immunity, we inoculated C57BL/6J wild-type (WT) and B6.129S4-*C3*^{tm1Crr}/J C3 knockout (C3KO) mice intranasally with heat-killed *Pseudomonas aeruginosa* (HKPA) for training or vehicle control (PBS, untrained). After 14 days, lipopolysaccharide (LPS) from *Escherichia coli* was also administered intranasally to both mouse strains to induce secondary stimulation for 24 h, followed by euthanasia and bronchoalveolar lavage (BAL) as previously described (Fig. 1D) (Sahu et al., 2023). BAL proinflammatory chemokines and cytokines (CXCL1, CXCL2, IL-6, and

TNFα) were quantified using ELISAs. Additionally, C3a, which is generated when C3 is activated and cleaved, was also measured using an ELISA specific to its neo-epitope. CXCL1, CXCL2, IL-6, and TNFα were all significantly elevated in trained versus untrained WT BAL (Fig. 1E). Levels of C3a were increased in trained versus untrained WT BAL, indicating enhanced C3 activation is part of the trained immune response (Fig. 1F). The enhancement in BAL IL-6 and TNFα with training was blunted in C3-deficient mice compared to WT mice (Fig. 1G). Of note, no difference was observed in BAL protein, neutrophils and cytokines (i.e., CXCL1, TNFα) at day 14 (prior to LPS exposure) in C3-deficient compared to wild-type mice, regardless of prior HKPA (training) exposure (Fig.S1D-G), suggesting the resolution of acute inflammation. These results suggest C3 is required for trained immunity *in vivo*, and that the observed responses to the secondary challenge are not confounded by persistent inflammation from the initial exposure.

To examine how C3 affects trained immunity specifically in tissue-resident phagocytes, we set up an *ex vivo* culture system using primary alveolar macrophages (AM) from C3-deficient and wildtype mice (Gorki et al., 2022). Like the *in vivo* experiments (Fig. 1), we used HKPA to induce trained immunity in cultured AMs, followed days later by LPS challenge and analysis of cytokine secretion in the presence or absence of C3 deficiency (Fig. 2A). Supporting our *in vivo* results, training by HKPA was sufficient to augment LPS-induced proinflammatory cytokine production from primary AMs (Fig. 2B). However, C3-deficient AMs demonstrated blunted LPS-induced proinflammatory cytokine production post-training, as measured by IL-6 and TNFα secretion (Fig. 2C) as well as impaired phagocytosis and reactive oxygen species (ROS) production (Fig. S2A-C). Employing heat-killed *Candida albicans* (HKCA) in place of HKPA produced similar results (Fig. 2D-G). To assess if the in vivo training specifically influenced AMs, we also trained the mice in vivo using HKPA (using PBS as a control for 14 days, then harvested AM, and provided the second stimulus (LPS) ex vivo. In vivo trained AM from C3-deficient demonstrated a blunted cytokine response to LPS ex vivo compared to AM from wildtype mice (Fig. S2D), These data suggest a specific role for C3 in AM immune training, consistent with our *in vivo* observations.

In addition to synthesizing C3, multiple cell types internalize C3 as C3(H_2O) in vitro, which promotes cell survival and effector responses (Elvington et al., 2017; Kulkarni et al., 2019). To assess if C3 uptake by AMs can overcome the defect in responses from C3-deficient AMs, we first interrogated if mouse AMs internalize C3 in vivo. Labeled C3a was separately used as a control. Real-time confocal microscopy of live, intact mouse alveoli (Fig. 3A&B) revealed that $CD11c^+$ AMs internalize fluorescently labeled C3 rapidly after alveolar microinstillation, as compared to C3a (Fig.3C-G). Incubation of human precision-cut lung slices with labeled C3 revealed that human AM also internalize C3 in situ, thereby, validating our observations from the intact mouse lung using human lung tissues (Fig. S3).

To mechanistically validate the role of C3 uptake in the training of AMs, we cultured C3-deficient AMs with exogenous C3 at a dose that leads to the cellular uptake of C3(H_2O) (15 μg/mL, Fig 4A) (Elvington et al., 2017; Kulkarni et al., 2019). As a control, we incubated cells with labeled C3a, which is not internalized rapidly (Mogilenko et al., 2022). As measured by IL-6 and TNFα after a secondary stimulus, exogenous C3 protein restored the trained responses by AM to WT levels (Fig. 4B). In contrast, these responses were not restored to WT levels by exogenous C3a, which stays outside the cell (Fig. 4C).

Upon internalization, C3 is cleaved to C3a (Elvington et al., 2017). Intracellular C3a binds to C3aR and polarizes cytokine production in $CD4^+$ T cells (Liszewski et al., 2013). To investigate whether a similar pathway could be important to C3-mediated trained immunity, we pre-treated AMs with a cell-permeable C3aR antagonist (SB290157) before immune training (Fig. 4D). C3aR antagonism blunted LPS-induced cytokine production in both WT and C3-deficient AMs that had previously been rescued with exogenous C3 (Fig. 4E). Importantly, genetic C3aR deficiency phenocopied the blunting of cytokine production seen in C3-deficient cells. Specifically, this defect in cytokine production post-training could not be rescued by exogenous C3 in C3aR-deficient AMs (Fig. 4F). Altogether, these findings support a causal role for C3 in AM trained immunity via the C3aR.

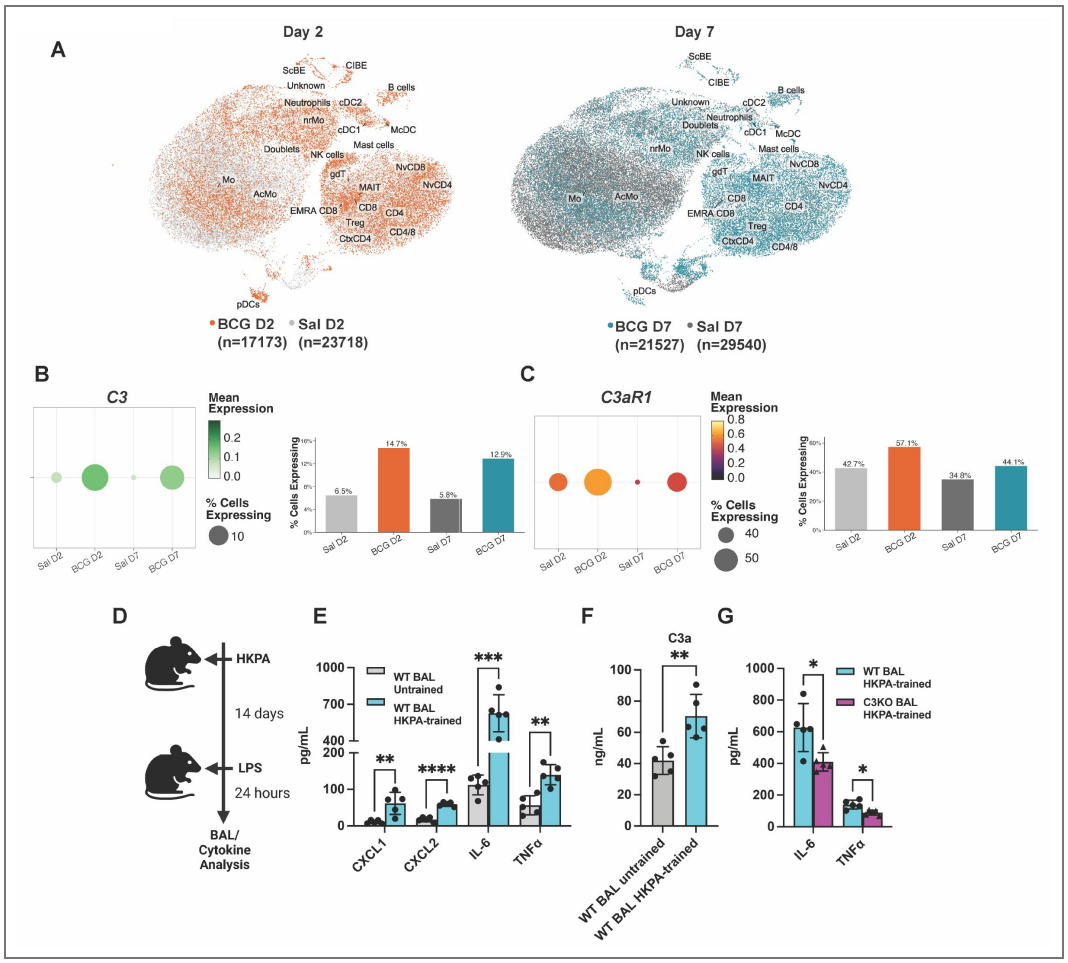

Figure 1. C3 deficiency predisposes to impaired pulmonary trained immunity.

(A) UMAP (Uniform Manifold Approximation and Projection) plots showing the identity of each cell cluster in human bronchoalveolar lavage (BAL) from BCG- and saline (Sal)-treated donors at Day 2 (BCG n=17,173; Sal n=23,71S) and Day 7 (BCG n=21,527; Sal n=29,540) post-vaccination. N=3 volunteers in each group. Cell clusters include: macrophage (Mo), activated macrophage (AcMo), non-resident macrophage (nrMo), **NK** cells, γ δ T cells, plasmacytoid DC (pDC), conventional DC (cDC1, cDC2), migratory DC (McDC), B cells, MAIT cells, CD4, CDS, CD4/S, cytotoxic-like CDS T cells (CtxCD4), terminally differentiated effector memory CD45RA-re-expressing CDS T cells (EMRA CDS), naÏfve T cells (NvCD4, NvCDS), T regulatory cells (Treg), neutrophils, mast cells, doublets, ciliated bronchial epithelial cells (GIBE), secretory bronchial epithelial cells (ScBE), and unidentified cells (Unknown). Data from Marshall et al, 2025. (B) Dot plot and bar chart showing C3 expression across all human alveolar macrophages (Mcp; Mo, AcMo, nrMo pooled) across conditions. Dot size reflects the percentage of cells expressing C3; dot color reflects mean normalized expression. Bar chart shows percentage of C3-expressing M φ per condition. (C) As in (B), for C3AR1. BCG vaccination increases the proportion of C3AR1-expressing alveolar macrophages at both timepoints relative to saline controls. (D) Schematic representing the training of mice via the intranasal route with heat-killed Pseudomonas aeruginosa (HKPA) and subsequent restimulation with lipopolysaccharide (LPS), followed by BAL and cytokine analysis. Created with *BioRender*. (E) *WT* untrained compared against WT-trained BAL levels of CXCL1, CXCL2, IL-6, and TNF α. Comparison of BAL C3a levels, similar to (E). (G) WT-trained versus C3-deficient (C3KO)-trained BAL concentrations of IL-6 and TNFα. WT-trained levels derived from (E) for comparison with C3KO-trained mice. Data were compared with two-sided unpaired t-tests with (E,G) or without (F) Holm-Sidak correction for multiple hypothesis testing. Each point represents a measurement from one mouse with at least n=4 in each group, mean± SD shown. *p < 0.05, **p < 0.01, ***p < 0.001.

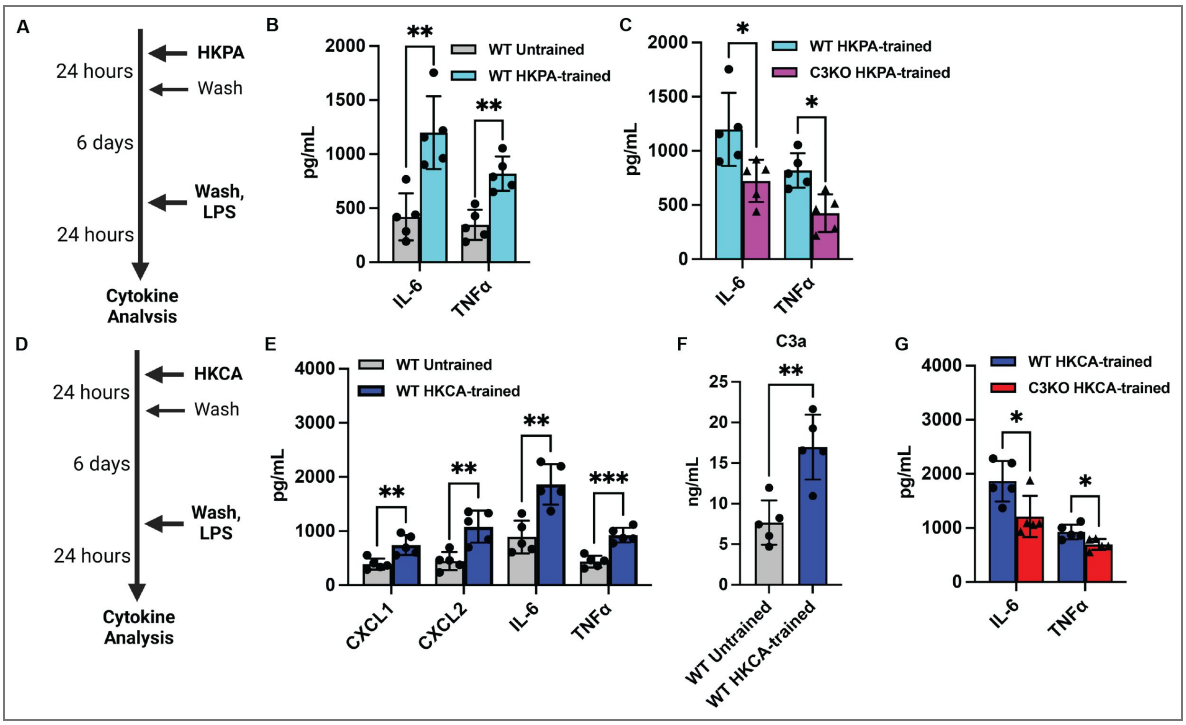

Figure 2. C3 deficiency results in impaired trained immune responses in ex vivo alveolar macrophages (AMs).

(A) Schematic representing in vitro training of AMs with HKPA, with later stimulation by LPS and subsequent cytokine analysis of the supernatants. Created with *BioRender*. (B-C) Effects of HKPA-induced training in vitro on IL-6 and TNFα in supernatant from (B) WT AM, and (C) their comparison with C3KO-trained AMs. (D) Schematic representing in vitro training of AMs with HKCA, with subsequent restimulation by LPS and cytokines in supernatant. Created with *BioRender*. (E) Effects of heat-killed Candida albicans (HKCA)-induced training in vitro on cytokines in supernatant from WT AM. (F) Comparison of C3a levels post-HKCA training, similar to (B). (G) Comparison of IL-6 and TNFα post-HKCA training in WT versus C3KO AMs. WT-trained levels derived from (D) for comparison with C3KO-trained AMs. Data were compared with two-sided unpaired t-tests with (B,C,E,G) or without (F) Holm-Šidák correction for multiple hypothesis testing. Each point is a technical replicate from pooling AMs from at least n=4 mice in each group, with mean ± SD shown. Each experiment repeated twice. *p < 0.05, **p < 0.01, ***p < 0.001.

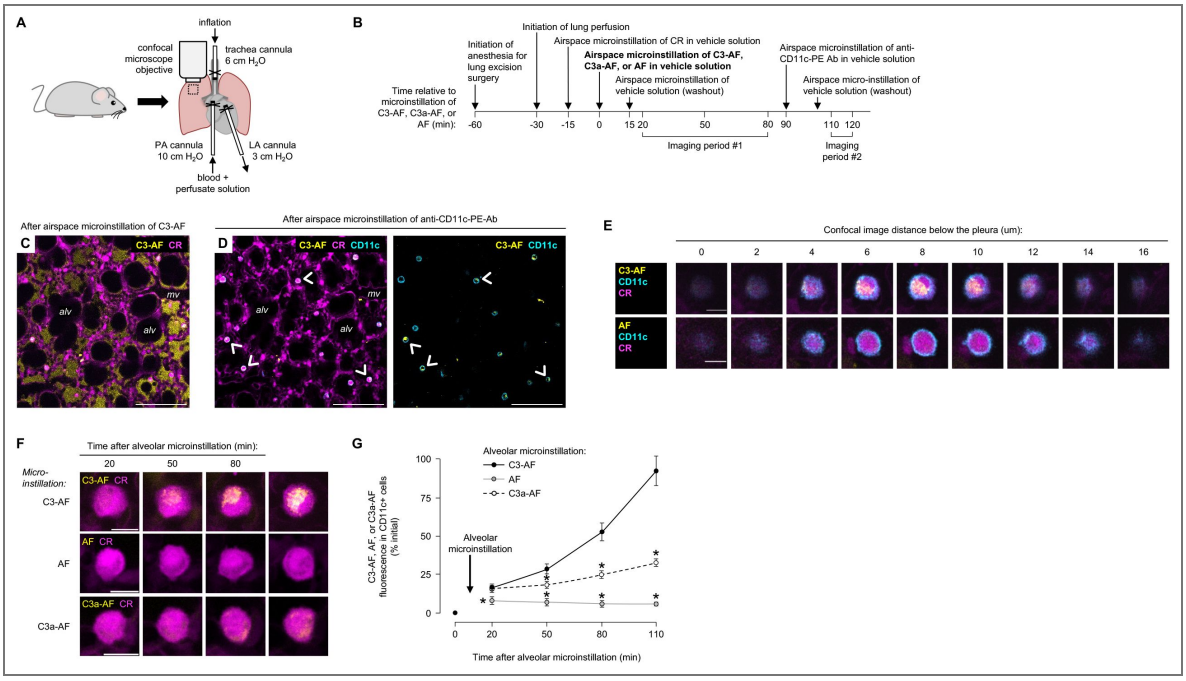

Figure 3. Alveolar macrophages take up C3 from airspaces of live alveoli in situ.

Cartoons in A-B show the experimental design of the confocal imaging studies shown in C-G. As indicated in A-B, we microinstilled alveolar airspaces of live, intact, perfused mouse lungs sequentially with: cell-permeant calcein red-orange dye (*CR*); Alexa Fluor 647 (*AF*), AF-tagged C3 (*C3-AF*), or AF-tagged C3a (*C3a-AF*); and phycoerythrin (*PE*)-tagged anti-CD11c Ab. The confocal image in C shows C3-AF fluorescence (*yellow*) in alveolar airspaces and CR fluorescence (*magenta*) in airspace-facing cells, including the alveolar epithelium and alveolar macrophages. *alv*, example airspace; *mv*, microvessel. Confocal images in D show the same alveoli, but CD11c fluorescence (*cyan*) now marks CD11c+ cells. *Arrowheads* point out example CD11c+ cells with intracellular C3-AF fluorescence. High power confocal images of CD11c+ cells (E-F) and group data (G) show C3-AF accumulated in cytosols of CD11c+ cells over time. In G, *circles* indicate mean ± SEM fluorescence in all of the CD11c+ cells present in imaging fields of at least 30 alveoli; $n = 4$ microinstillations in 2 lungs per group; $*p < 0.05$ versus C3-AF by ANOVA with post hoc Tukey testing. C3-AF, C3a-AF, and AF fluorescence in airspaces was normalized to C3-AF, C3a-AF, and AF fluorescence in glass micropipettes. Scale bars: 100 (C-D) and 10 (E-F) μm.

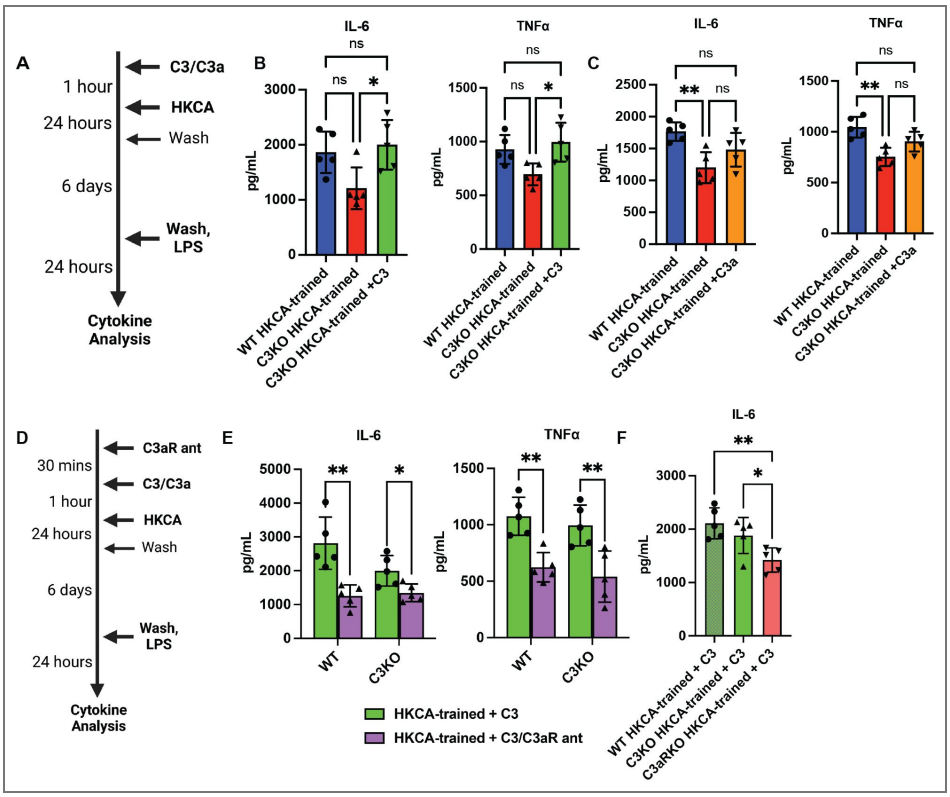

Figure 4. C3 uptake enhances trained immune responses in ex vivo alveolar macrophages (AMs) via the C3a receptor (C3aR).

(A) Schematic representing in vitro training of AMs with HKCA, with pre-treatment of C3 or C3a prior to induction of training, and later stimulation by LPS and subsequent cytokine analysis of the supernatants. Created with *BioRender*. (B) Effects of adding C3 prior to training on IL-6 and TNFα levels from C3KO AMs and their comparison with WT-trained AMs. (C)Effects of adding C3a prior to training, similar to (B).(D) Schematic representing addition of the C3aR antagonist prior to C3 treatment and in vitro training of AMs with HKCA, with later stimulation by LPS and subsequent cytokine analysis of the supernatants. Created with *BioRender*. (E) Effects of C3aR antagonism on IL-6 and TNFα levels from trained WT and C3KO AMs treated with exogenous C3. (F) Comparison of IL-6 levels post-HK-CA-training in C3aR-deficient (C3aRKO), C3KO and WT AMs treated with exogenous C3. Data were compared using one way ANOVA with Dunnett's post hoc tests (B,C,F) or two-sided unpaired t-testing with Holm-Šidák correction for multiple testing (E). Each point is a technical replicate made by pooling AMs from at least n=4 mice in each group, with mean ± SD shown, and each experiment was repeated twice. *p < 0.05, **p < 0.01.

To explore transcriptomic changes relevant to how C3 influences trained immune responses, we performed bulk RNASeq comparing trained WT to trained C3KO AMs. C3-deficiency led to differential expression of not only innate immune genes but also several genes involved in metabolism post-training, including those relevant to glycolysis such as *gnpda1* and *aldoc* (Fig. 5A, Table S1). These metabolism-linked genes are significant because prior studies show that enhanced glycolytic metabolism is critical for trained immunity (Cheng et al., 2014). Therefore, we investigated whether the absence of C3 affects glycolytic flux in AMs (Fig. 5B). As measured by extracellular acidification rate, untrained C3-deficient AMs had comparable basal and maximum glycolytic flux compared to WT but failed to augment glycolysis upon training with HKCA (Fig. 5C). We next tested the ability of exogenous C3 to rescue the induction of training-associated glycolysis in C3-deficient AMs (Fig. 5D). Exogenous C3 returned glycolysis induction to the level of trained WT cells, suggesting intracellular processing of C3 is important for this effect (Fig. 5E). Like cytokines (Fig. 4), inhibiting C3aR prevented exogenous C3 from rescuing glycolysis induction during immune training (Fig. 5E).

Discussion

Our data suggests C3 is upregulated after an initial inhalational exposure, and is required for effective reprogramming of AMs. In its absence, AMs demonstrate blunted immune and metabolic responses to a subsequent stimulus, which are rescued by exogenous C3 and are C3aR-dependent. Of note, an intracellular C3aR has been reported by several groups (Liszewski et al., 2013; Quell et al., 2017; Zha et al., 2019). We show that C3 is rapidly internalized by AMs, similar to other immune and non-immune cell types (Elvington et al., 2017; Ishii et al., 2021; Kulkarni et al., 2019), whereas C3a in itself is not internalized. We propose that in AMs, the internalization of C3(H_2O) provides an intracellular source of C3a (first suggested in (Elvington et al., 2017)), which potentially engages with the C3aR to result in reprogramming. While our data suggests that exogenously added C3a does not significantly affect AM reprogramming in our model system, we cannot rule out that other C3-mediated paracrine signaling mechanisms may also be at play. Regardless, our data indicate that C3 makes a mechanistic contribution to trained immunity in AMs and implicate engagement of the C3aR as part of the pathway. We also acknowledge that other immune cells such as dendritic cells and neutrophils, and non-immune cells such as epithelial cells, endothelial cells and fibroblasts can also be involved in immune training (Bigot et al., 2025; Friščić et al., 2021; Moorlag et al., 2020).

Strengths of our study include *in vitro* and *in vivo* approaches to inducing trained immunity, orthogonal endpoints to quantify immune training (cytokine and ROS production, phagocytosis and metabolic readouts), and mechanistic gain- and loss-of-function experimental approaches. Future work involves identifying the downstream effectors of C3 and C3aR in the AM training process and their connection to immunometabolism. We also need to establish whether C3a-C3aR-mediated AM reprogramming can lead to altered disease outcomes. Systemically administered β-glucan has been shown to induce peripheral trained immunity and aggravate lung injury (Prevel et al., 2025) similar to disease in models of periodontitis and arthritis (Haacke et al., 2025). However, training with β-glucan also reduces bleomycin-induced lung fibrosis (Y.-Y. Kang et al., 2024). Hence, we aim to optimize relevant intrapulmonary exposures to assess how trained immune responses are modulated by the C3a-C3aR axis, while also clarifying the extent to which central C3-C3aR-mediated training alters AM responses. The results may have clinical implications since trained immunity is linked to vaccine effectiveness (for example, with respect to the Bacillus Calmette-Guérin vaccine) and overall immune resilience (Arts et al., 2018; Kleinnijenhuis et al., 2015). Moreover, humans with C3 deficiency demonstrate impaired responses to vaccination (Kim et al., 2018; Pekkarinen et al., 2015). It is tempting to speculate that local augmentation of C3 and/or C3aR activity – could enhance vaccine effectiveness for hard-to-vaccinate pathogens like *Mycobacterium tuberculosis*.

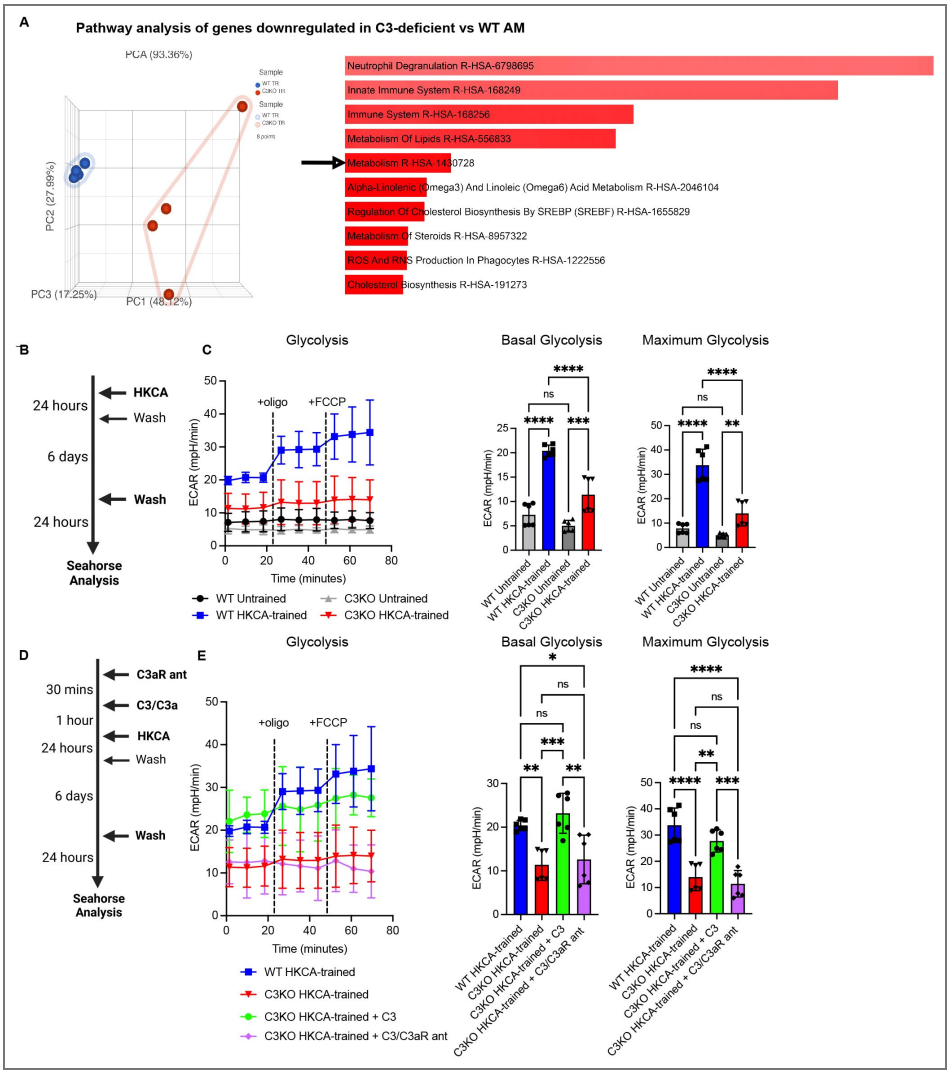

Figure 5. C3-C3aR axis is required for glycolysis as a part of trained immune responses in alveolar macrophages (AMs).

(A) Principal component analysis (PCA, left) and EnrichR analysis of 391 genes (right, Table S1) downregulated in HKCA-trained C3KO vs WT AM by filtering genes (FDR step up ≤0.05). Arrow shows metabolism gene set in EnrichR; bars ranked by p-value. (B) Schematic representing in vitro training of AMs with HKCA. Created with *BioRender*. (C) Extracellular acidification rate (ECAR) from Seahorse analysis representing full glycolytic activity, and basal and maximum glycolysis in untrained and HKCA-trained WT and C3KO AMs. (D) Schematic representing addition of the C3aR antagonist (SB290157) prior to C3 treatment and in vitro training of AMs with HKCA. Created with *BioRender*. (E) Seahorse analysis in the presence and absence of exogenous C3 supplementation and C3aR antagonism. Each point is a technical replicate of pooled AMs from at least n=4 mice in each group, with mean ± SD shown. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 when analyzed using one way ANOVA with Dunnett's post hoc tests.

Materials and methods

Details including mouse strains, design, models of trained immunity, readouts, and statistical analysis are described as per the ARRIVE guidelines and included in Supplementary Material.

Data availability

RNA sequence data were deposited in GEO. Sequencing data have been deposited in GEO under the accession code GSE281001 at *https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE281001*. All data generated or analyzed during this study are included in the manuscript and supporting files; source data files have been provided for all figures.

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Additional files

Supplementary Material

Supplementary Figures

Table S1

Note

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Peer reviews

Reviewer #1 (Public review):

[Editors' note: The revised manuscript addressed the concerns of both reviewers, who have concluded that the manuscript is convincing and important. The manuscript can move towards the Version of Record.]

Summary:

This study is built on the emerging knowledge of trained immunity, where innate immune cells exhibit enhanced inflammatory responses upon challenged by a prior insult. Trained immunity is now a very fast-evolving field and has been explored in diverse disease conditions and immune cell types. Earhart and the team approached the topic from a novel angle and was the first to explore a potential link to the complement system.

The study focused on the central complement protein C3 and investigated how its signalling may modulate immune training in alveolar macrophages. The authors first performed in vivo experiments in C57BL mouse models to observe the presence of enhanced inflammation and C3a in BAL fluid following immune training. These changes were then compared with those from C3-deficient mice, which confirmed the involvement of C3a. This trained immunity was further validated in ex vivo experiments using primary alveolar macrophage, which was blunted in C3-deficiency, and, intriguingly, rescued by adding exogenous C3 protein, but not C3a. The genetic-based findings were supported by pharmacological experiments using the C3aR antagonist SB290157. Mechanistically, transcriptomic analyses suggested the involvement of metabolism-linked, particularly glycolytic, genes, which was in agreement with an upregulation of glycolytic flux in WT but not C3-deficient macrophages.

Collectively, these data suggest that C3, possible through engaging with C3aR, contributes to trained immunity in alveolar macrophages.

Strengths:

The conclusions reached were well supported by in vivo and ex vivo experiments, encompassing both genetic-knockout animal models and pharmacological tools.

The transcriptomic and cell metabolism studies provided valuable mechanistic insights.

Weaknesses:

For the in vivo experiments, the histopathological and other inflammatory markers (Fig 1.) were not directly linked to alveolar macrophages by experimental evidence. Other innate immune cells (e.g. dendritic cells, neutrophils) and endothelial cells could also be involved in immune training and contribute to the pathological outcomes. These cells were not examined or mentioned in the study.

For the ex vivo experiments assessing immune training in alveolar macrophages, only the release of selected inflammatory factors were measured. Macrophage activities constitute multiple aspects (e.g. phagocytosis, ROS production, microbe killing), which should also be considered to better depict the effect of trained immunity.

The proposed mechanism of C3 getting cleaved intracellularly then binding to lysosomal C3aR need to be further supported by experimental evidence.

There was an absence of any validation in human-based models.

Comments on the revised version.

The revised manuscript now encompasses a much wider scope and stronger evidence.

The authors have included the re-analysis of a recently published dataset of human volunteers who received aerosolized BCG exposure compared to saline. Although not proven causality, this data helped strengthen the human relevance of the findings presented in this research and directly rationalized the decision to focus on Ams. The persistence of elevated C3/C3aR1 expression to day 7 further supports the idea that complement-associated reprogramming is not merely an acute inflammatory phenomenon. Whilst it may be outside of the scope of this current study, it would be helpful to clarify in future studies whether other complement components (C5, factor B, factor D) were also modulated in the dataset, to contextualize whether the response is uniquely centered on C3/C3aR1 or part of a broader complement activation program.

The authors have also expanded the functional characterization of trained alveolar macrophages by including phagocytosis and ROS generation measurements. It is intriguing that HKPA training did not markedly alter the phagocytosis and ROS production by alveolar macrophages relative to the control group, however, C3 deficiency significantly dampened these responses in both trained and untrained groups. This reduction is in congruence with the cytokine release data, but there could be other factors involved.

I appreciate the careful revision and much more expansive mechanistic interpretation regarding intracellular C3aR, and that further studies are underway to better understand the cell type-specific, subcellular localization of C3a-C3aR in alveolar macrophages.

Overall, the revised data interpretation and discussion significantly improved in balance and contextualization of the findings.

https://doi.org/10.7554/eLife.104977.2.sa2

Reviewer #2 (Public review):

Earhart et al. investigated the role of the complement system in trained innate immunity (TII) in alveolar macrophages (AM). They used a WT and C3 knockout murine model primed with locally administered heat-killed P. aeruginosa (HKPA). Additionally, they employed ex vivo AM training models using C3 knockout mice, where reconstitution of C3 and blockade of C3R were performed. The study concluded that the C3-C3R axis is essential for inducing TII in macrophages in the ex vivo model. The manuscript is well-written and easy to follow.

Comments on revised version.

My concerns have been addressed, and the provided data is convincing supporting the manuscript's claims.

https://doi.org/10.7554/eLife.104977.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *This study is built on the emerging knowledge of trained immunity, where innate immune cells exhibit enhanced inflammatory responses upon being challenged by a*

> *prior insult. Trained immunity is now a very fast-evolving field and has been explored in diverse disease conditions and immune cell types. Earhart and the team approached the topic from a novel angle and were the first to explore a potential link to the complement system.*
>
> *The study focused on the central complement protein C3 and investigated how its signalling may modulate immune training in alveolar macrophages. The authors first performed in vivo experiments in C57BL mouse models to observe the presence of enhanced inflammation and C3a in BAL fluid following immune training. These changes were then compared with those from C3-deficient mice, which confirmed the involvement of C3a. This trained immunity was further validated in ex vivo experiments using primary alveolar macrophage, which was blunted in C3-deficiency, and, intriguingly, rescued by adding exogenous C3 protein, but not C3a. The genetic-based findings were supported by pharmacological experiments using the C3aR antagonist SB290157. Mechanistically, transcriptomic analyses suggested the involvement of metabolism-linked, particularly glycolytic, genes, which was in agreement with an upregulation of glycolytic flux in WT but not C3-deficient macrophages.*
>
> *Collectively, these data suggest that C3, possibly through engaging with C3aR, contributes to trained immunity in alveolar macrophages.*
>
> *Strengths:*
>
> *The conclusions reached were well supported by in vivo and ex vivo experiments, encompassing both genetic-knockout animal models and pharmacological tools.*

The transcriptomic and cell metabolism studies provided valuable mechanistic insights.

We thank the reviewers for acknowledging the importance of the work.

> *Weaknesses:*
>
> *For the in vivo experiments, the histopathological and other inflammatory markers (Figure 1) were not directly linked to alveolar macrophages by experimental evidence. Other innate immune cells (eg. dendritic cells, neutrophils) and endothelial cells could also be involved in immune training and contribute to the pathological outcomes. These cells were not examined or mentioned in the study.*

We agree with the suggestions from Reviewer 1 that other cell types such as dendritic cells, neutrophils, and endothelial cells can also be involved in immune training. As the focus of this study was on alveolar macrophages, we specifically focused on these cell types. However,

(1) We have re-analyzed a recently published dataset of human volunteers who received aerosolized BCG exposure compared to saline. We observe that by Day 7, aerosolized BCG exposure alters the expression of *C3* and *C3aR1* in alveolar macrophages in the human bronchoalveolar lavage (BAL) fluid, compared to saline. We have included this new analysis in a revised Figure 1 to clarify why our focus is on investigating the C3-C3aR1 axis in alveolar macrophages.

(2) We have conducted new experiments where we train the mice *in vivo*, collect the alveolar macrophages, and then provide the second stimulus *ex vivo*. We observe a similar phenotype in the *in vivo* trained, *ex vivo* stimulated alveolar macrophages. We have included this new data in a new Figure S2.

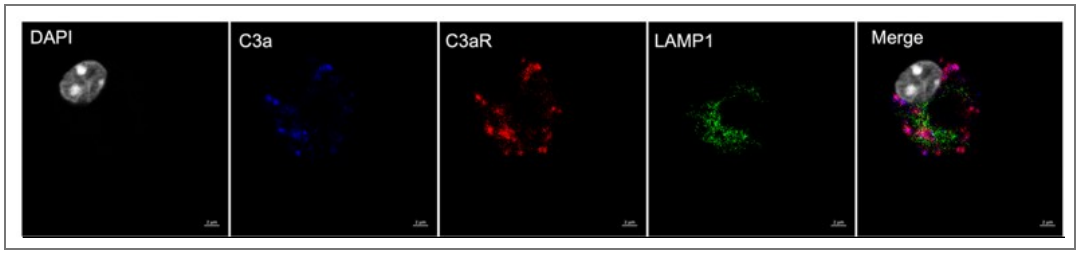
(3) We have updated our Discussion to state "However, we also acknowledge that other immune cells such as dendritic cells and neutrophils, and non-immune cells such as epithelial cells, endothelial cells and fibroblasts can also be involved in immune training (Bigot et al., 2025; Friščić et al., 2021; Moorlag et al., 2020)."

> *(2) For the ex vivo experiments assessing immune training in alveolar macrophages, only the release of selected inflammatory factors were measured. Macrophage activities constitute multiple aspects (e.g. phagocytosis, ROS production, microbe killing), which should also be considered to better depict the effect of trained immunity.*

We agree with the reviewer and have conducted additional experiments to assess immune responses influenced by training in alveolar macrophages. Specifically, we show that in addition to impairing the release of proinflammatory cytokines such as TNFα and IL-6, C3-deficient alveolar macrophages exhibit significantly lower phagocytosis and ROS production compared to WT alveolar macrophages post-training with heat-killed *Pseudomonas aeruginosa*. Results from these additional experiments have been included in new Figure S2.

> *(3) The proposed mechanism of C3 getting cleaved intracellularly and then binding to lysosomal C3aR needs to be further supported by experimental evidence.*

The mechanism of C3 being cleaved intracellularly involves serine protease-dependent cleavage of C3 to C3a and has been experimentally demonstrated previously (Liszewski et al. Immunity 2013; Elvington et al. J Clin Invest 2017). A prior report demonstrated that intracellular C3a interacted with a lysosomal C3aR to promote $CD4^+$ T cell survival (Liszewski et al. Immunity 2013). Based on the reviewer's suggestions, we performed confocal microscopy on alveolar macrophages. Although we clearly observed intracellular colocalization of C3a (using a monoclonal antibody to the neo-epitope) with C3aR, we observed only some colocalization with LAMP1, a lysosomal marker (see Author response image 1). Hence, we will refrain from making comments on how C3 binds to lysosomal C3aR intracellularly in alveolar macrophages, as this may be cell type-specific or stimulation-specific. We have now revised the sentence in the manuscript to remove any references to lysosomal C3aR and now state – "Upon internalization, C3 is cleaved to C3a (Elvington et al., 2017), binds to C3aR, and affects cytokine production in $CD4^+$ T cells (Liszewski et al., 2013)". We have not incorporated the Author response image 1 in the main manuscript as we would like to explore this further to precisely define the subcellular localization of C3a-C3aR in alveolar macrophages, but have provided it for the reviewer to explain the basis of the rewording in the revision.



Author response image 1. C3a-C3aR colocalization in mouse *ex vivo* cultured alveolar macrophages (mexAM). mexAMs were harvested and cultured as per the protocol from Gorki et al. (2022). Cells were incubated in a Millicell EZ Slide 8-well glass chamber slide overnight to allow for adherence, then fixed, permeabilized, and incubated with anti-C3a conjugated to AF555 (blue, Hycult HM1072), anti-C3aR conjugated to AF647 (red, Hycult HM1123), and anti-LAMP1 (green, Cell Signaling 99437) overnight at 4°C. Slides were washed 3X in PBS (5 min each) and mounted overnight at 4°C in ProLong Diamond Antifade Mountant with DAPI (white). Images were acquired on a Zeiss LSM 880 confocal microscope at 63X. At least 6 cells per condition imaged. Experiments were conducted in duplicate (technical replicates) and repeated (for biological replicates). Scale bar, 2 μm.

> *(4) There was an absence of any validation in human-based models.*

We acknowledge that the observations need to be validated in human-based models. The focus of our manuscript is on training in alveolar macrophages. Unfortunately, we do not have access to an adequate representation of human alveolar macrophages for our *ex vivo* testing to account for individual-level variation in immune responses. We anticipate this work will form the basis of these future studies. In the interim, we re-analyzed a recently published publicly available dataset of human BAL specimens from human volunteers who underwent aerosolized BCG administration (Marshall et al. Nat Comm 2025). We observe an increase in *C3* and *C3aR1* expression at Day 2, which persists through Day 7 post-training with aerosolized BCG compared to aerosolized saline specifically in human alveolar macrophages. We have included this data in Revised Figure 1. We also validated C3 uptake in alveolar macrophages using precision-cut lung slices from human donors. We have included this additional data in new Supplementary Figure 3.

> ***Reviewer #2 (Public review):***
>
> *Earhart et al. investigated the role of the complement system in trained innate immunity (TII) in alveolar macrophages (AM). They used a WT and C3 knockout murine model primed with locally administered heat-killed P. aeruginosa (HKPA). Additionally, they employed ex vivo AM training models using C3 knockout mice, where reconstitution of C3 and blockade of C3R were performed. The study concluded that the C3-C3R axis is essential for inducing TII in macrophages in the ex vivo model. The manuscript is well-written and easy to follow. However, I have the following major concerns.*
>
> *(1) The secondary challenge to assess the reprogramming of innate cells in the BAL was conducted 14 days after the initial exposure to HKPA. However, no evidence is provided to confirm that homeostasis was re-established following the primary exposure. Demonstrating the resolution of acute inflammation is essential to ensure that the observed responses to the secondary challenge are not confounded by persistent inflammation from the initial exposure.*

We thank the reviewer for giving us an opportunity to clarify this point. We have now included additional data from the bronchoalveolar lavage fluid of these mice to show that the levels of protein leaked into the BAL, levels of proinflammatory cytokines (e.g., TNFα, CXCL1) and the neutrophils (all relevant to the acute phase of inflammation) were similar between the untreated and treated wildtype mice. This new data has been included in Figure S1.

> *(2) In Figure 1D, cytokine production by BAL cells from WT and C3KO mice after HKPA exposure and LPS challenge is shown. However, it is unclear whether the reduced response in trained C3KO mice is due to a defect in trained immunity or an intrinsic inability of C3KO cells to respond to LPS. To clarify this, the response of trained C3KO cells should also be compared to untrained C3KO controls after the LPS challenge. This comparison is necessary to determine if the reduction is specifically related to innate immune memory or a broader impairment in LPS responsiveness. Such control should be included in all ex vivo training and LPS stimulation experiments as well.*

We thank the reviewers for their suggestions. We have conducted additional experiments and we observe no significant differences in the BAL cytokine levels between the wildtype and C3-deficient mice post-training in the absence of infection. This new data has been included in Supplementary Figure S1.

Additionally, we came across several manuscripts, including a recent one in eLife as a part of this Series (Gu et al. Elife 2021; Zahalka et al. Mucosal Immunol 2022; Prevel et al Elife 2025) that have done *in vivo* training followed by an *ex vivo* challenge. Hence, we have conducted new experiments to compare the response of *in vivo* HKPA-trained wildtype (WT) and C3-deficient (C3KO) alveolar macrophages compared to untrained AMs after an *ex vivo* LPS challenge. This new data has been included in Figure S2.

> *(3) The data presented provide evidence of alterations in the functional and metabolic activities of innate cells in the lung, indicating the induction of innate immune memory in a C3-C3R axis-dependent pathway. However, it remains to be established whether such changes can lead to altered disease outcomes. Therefore, the impact of these changes should be demonstrated, for instance, through an infection model to support the claim made in the study that C3 modulates trained immunity in AMs through C3aR signalling.*

We acknowledge this is a Limitation of our manuscript. As this is a Short Report, we focused on how C3, via the C3aR, affects the reprogramming of alveolar macrophages. Recent work demonstrated that systemically administered β-glucan induces peripheral trained immunity and aggravates lung injury (Prével et al., 2025), similar to disease in models of periodontitis and arthritis (Haacke et al., 2025). However, training with β-glucan also reduces bleomycin-induced lung fibrosis (Kang et al., 2024). Hence, our ongoing work involves optimizing relevant intrapulmonary exposures to assess how trained immune responses are modulated by the C3a-C3aR axis. We have included the Reviewer's critique in our revised Discussion as a limitation, while referencing the abovementioned manuscripts.

> *(4) Figure 3, panels B and C - stats should be shown for comparing WT-HKPA-trained and C3KO HKPA-trained.*

These suggestions have been incorporated into Revised Fig 3B and 3C (now Figure 4).

> *(5) In Figure 4, where the proper untrained C3KO is included, the data presented in Figure 4C show an increase in basal and maximum glycolysis in trained C3KO compared to their untrained control counterparts. Statistical analysis should be provided for this comparison. Based on these data, it appears that metabolic reprogramming occurs even in the absence of C3. Furthermore, C3KO cells intrinsically exhibit reduced glycolytic capacity compared to WT. These observations challenge the conclusions made in the manuscript. Therefore, without the proper control (untrained C3KO) included in all experimental approaches, it is impossible to draw an evidence-based conclusion that the C3-C3R axis plays a role in the induction of innate immune memory.*

We have included the statistical comparisons for all the groups in Figure 4C (now Figure 5), as suggested by the reviewer. The data suggests that C3-deficient (C3KO) alveolar macrophages have a blunted metabolic response to training, as compared to C3-sufficient (WT) alveolar

macrophages. However, the C3KO cells do not have reduced glycolytic capacity compared to WT in the absence of training. The blunted response in trained C3KO AMs is rescued by exogenous C3, but is then reversed by C3aR antagonism. We have also provided new data/analyses with proper controls (untrained C3KO) in the other Figures (for example, in Figures S1, S2 and 3B&C (now Figure 4)). Taken together, the data would suggest that the effects of C3 in AM reprogramming are C3aR-dependent.

> *(6) The Results and Discussion sections should be separated, and the results should be thoroughly analyzed in the context of published literature. Separating these sections will allow for a clearer presentation of findings and ensure that the discussion provides a comprehensive interpretation of the data.*

We thank the reviewer for this suggestion. The manuscript has been submitted as a Brief Report, and hence, we adhered to the instructions to authors for this format. However, we have added an additional section towards the end of the manuscript based on the Reviewer's suggestion.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *It is intriguing that whilst there was a significant elevation of C3a in the cell culture medium of alveolar macrophages, the addition of exogenous C3a failed to rescue the phenotypes of C3-deficient alveolar macrophages. Please help discuss this.*

Alveolar macrophages secrete both C3 and proteases, which can cleave C3 to C3a in the supernatant. However, we used the addition of exogenous C3a to compare it to the addition of full-length C3. C3 is internalized by multiple cell types, including alveolar macrophages (as demonstrated in new Figure 3 of our Revised Manuscript) as C3(H_2O) in comparison to C3a. Hence, we propose that the internalization of C3(H_2O) provides an intracellular source of C3a (previously reported in Elvington et al. J Clin Invest 2017), which engages with the C3aR to result in alveolar macrophage reprogramming. In comparison, incubating cells with C3a does not exert similar effects. We have included these comments in a separate section towards the end of the manuscript.

> *Please provide details for the statement "cell-permeable C3aR antagonist (SB290157)" (Figure 3E). Could a paracrine-based mechanism also be at play?*

SB290157 does not act selectively on the cell surface, but rather, can also enter cells. Our data, along with previously published reports (Quell et al. J Immunol 2017; Zha et al. Cancer Immunol Res 2019), suggest that C3aR may be intracellular in AMs. However, SB290157 can also block any receptor that may be present on the surface. For this reason, we used exogenous C3a as a way to interrogate surface C3aR signaling, and did not observe significant changes in AM reprogramming with exogenous C3a. However, as this is an indirect approach, we cannot completely rule out a paracrine-based mechanism and have included this limitation in the Discussion section of the revised manuscript.

> *For Figure 3, please also provide the statistical analysis results for WT versus C3KO HKCA-trained cells. The statistical tests described in the legend for Figure 3D seem to apply to Figure 3E. Please check the labels.*

These suggestions have been incorporated into Revised Figure 3 (now Figure 4).

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