Immunology and Inflammation

The C3-C3aR axis modulates trained immunity in alveolar macrophages

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Alexander P Earhart, Rafael Aponte Alburquerque, Marick Starick, Aasritha Nallapu, Lorena Garnica, Ayse Naz Ozanturk, Rahul Kumar Maurya, Xiaobo Wu, Jeffrey A Haspel, Hrishikesh S Kulkarni

Division of Pulmonary and Critical Care Medicine, John T. Milliken Department of Medicine, Washington University School of Medicine, St Louis, United States • Division of Rheumatology, John T. Milliken Department of Medicine, Washington University School of Medicine, St Louis, United States • Department of Medicine, University of California, Los Angeles David Geffen School of Medicine, Los Angeles, United States

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 data presented is mainly **solid**, but **incomplete** in parts.

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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 *in vivo*, trained wild-type mice showed significantly elevated pro-inflammatory cytokines and increased C3a levels upon a second stimulus, whereas C3-deficient mice exhibited a blunted cytokine response and heightened evidence of lung injury. *Ex vivo*, C3-deficient alveolar macrophages (AMs) displayed reduced chemokine and cytokine output after training, which 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, affecting cytokine production and metabolic reprogramming, 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 (Frišćić et al., 2021; 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).

A new concept of immune "memory" has recently emerged, whereby innate immune cells, particularly monocytes and macrophages, exhibit robust, enhanced inflammatory responses upon secondary stimulation after a prior insult (Quintin et al., 2012; Saeed et al., 2014). This form of "memory" is termed trained immunity and 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 and discussion

To investigate the role of C3 in pulmonary immune cell-trained immunity, we first 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. 1A**) (Sahu et al., 2023). BAL proinflammatory chemokines and cytokines (CXCL1, CXCL2, IL-6, and TNFα), total protein content (a marker of alveolar-capillary barrier disruption) and RAGE (a marker of epithelial injury), 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. 1B**). Levels of C3a were increased in trained versus untrained WT BAL, indicating enhanced C3 activation is part of the trained immune response (**Fig. 1C**). The enhancement in BAL IL-6 and TNFα with training was blunted in C3-deficient mice compared to WT mice (**Fig. 1D**). In contrast to the blunted training response, BAL total protein and RAGE were elevated in trained C3KO versus WT mice post-LPS challenge, and alveolar damage was worse on histopathology in C3-deficient compared to WT mice (**Figs. 1E, F**). These results suggest C3 is important for trained immunity *in vivo*.

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, HKPA was sufficient to induce trained immune responses from primary alveolar macrophages (**Fig. 2B**). However, C3-deficient AMs had blunted trained immune responses as measured by IL-6 and TNFα secretion (**Fig. 2C**). Employing heat-killed *Candida albicans* (HKCA) in place of HKPA produced similar results (**Figs. 2D-G**). These data suggest a specific role for C3 in AM immune training, consistent with our *in vivo* observations.

To mechanistically validate the role of C3 or its products in trained immunity, 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 2A**) (Elvington et al., 2017; Kulkarni et al., 2019). As a control, we incubated cells with C3a, which is not internalized (**Fig. 3A**) (Mogilenko et al., 2022). As measured by IL-6 and TNFα, exogenous C3 protein restored AM trained immunity to WT levels (**Fig. 3B**). In contrast, trained immunity was not restored by exogenous C3a, which stays outside the cell (**Fig. 3C**).

Upon internalization, C3 is cleaved to C3a (Elvington et al., 2017). Intracellular C3a binds to C3aR in lysosomes, and affects cytokine production in $CD4^+$ T cells (Liszewski et al., 2013). To investigate whether a similar pathway could be important to trained immunity, we pre-treated AMs with a cell-permeable C3aR antagonist (SB290157) before immune training (**Fig. 3D**). C3aR antagonism blunted AM trained immunity in both WT and C3KO AMs that had previously been rescued with exogenous C3 (**Fig. 3E**). Importantly, genetic C3aR deficiency phenocopied the blunting of AM trained immune responses seen in C3-deficient cells (**Fig. 3F**). Altogether, these findings support a causal role for C3 in AM trained immunity via its intracellular binding of C3aR.

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, including those relevant to glycolysis such as *gnpda1* and *aldoc* (**Fig. 4A**, 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. 4B**). 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. 4C**). We next tested the ability of exogenous C3 to rescue the induction of training-associated glycolysis in C3-deficient AMs (**Fig. 4D**). Exogenous C3 returned glycolysis induction to the level of trained WT cells, suggesting intracellular processing of C3 is important for this effect (**Fig. 4E**). Like cytokines (**Fig. 3**), inhibiting C3aR prevented exogenous C3 from rescuing glycolysis induction during immune training (**Fig. 4E**).

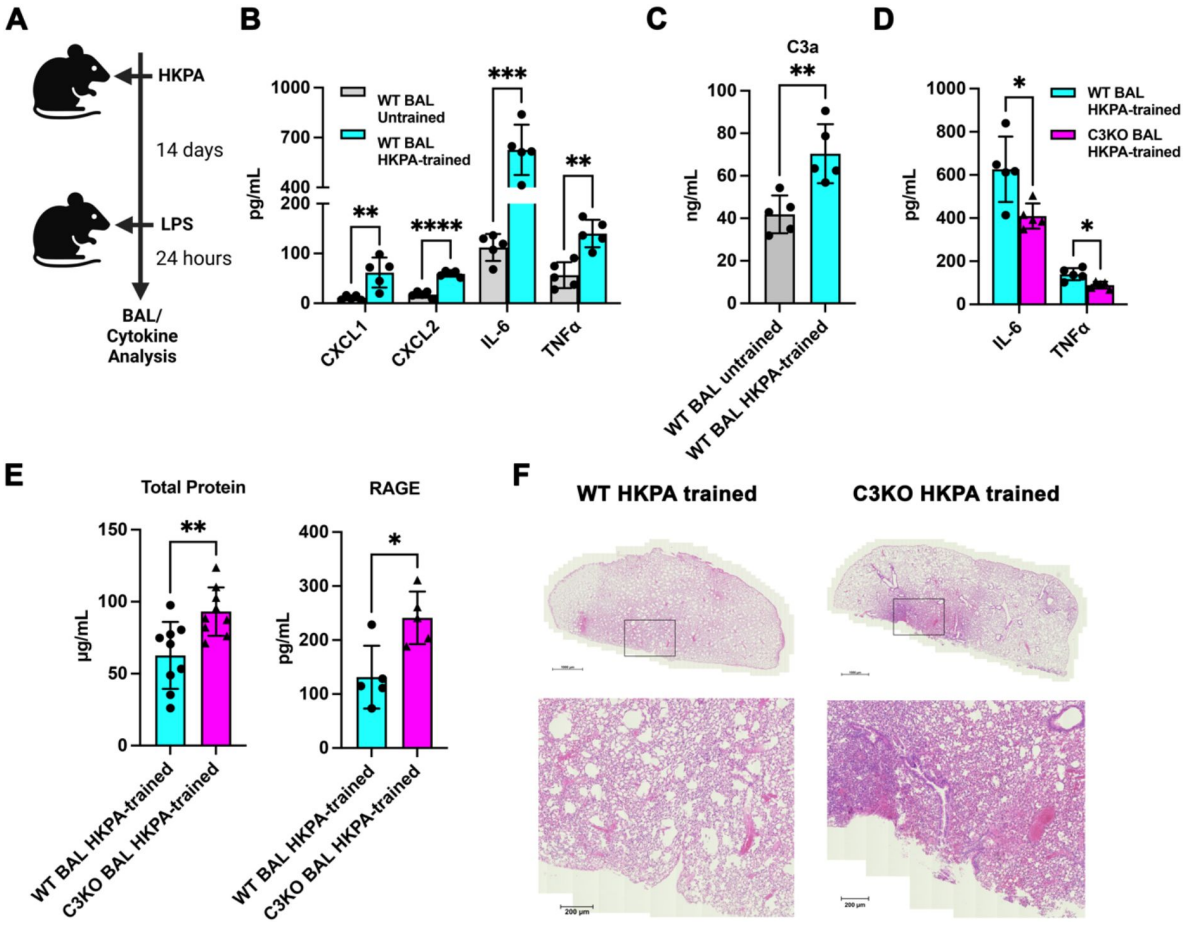

Figure 1.

C3 deficiency predisposes to impaired pulmonary trained immunity and diminished protection from lung injury.

(A) Schematic representing the training of mice via the intranasal route with heat-killed *Pseudomonas aeruginosa* (HKPA) and subsequent restimulation with lipopolysaccharide (LPS), followed by bronchoalveolar lavage (BAL) and cytokine analysis. Created with *BioRender*.

(B) WT untrained compared against WT-trained BAL levels of CXCL1, CXCL2, IL-6, and TNFα.

(C) Comparison of BAL C3a levels, similar to (B).

(D) WT-trained versus C3-deficient (C3KO)-trained BAL concentrations of IL-6 and TNFα. WT-trained levels derived from (B) for comparison with C3KO-trained mice.

(E) Concentrations of protein and RAGE from WT-trained versus C3KO-trained mice.

(F) Representative histopathological slides of lungs showing increased tissue damage in C3KO-trained versus WT-trained mice (N=5 in each group), high power view from selected area.

Data were compared with two-sided unpaired t-tests with (B, D) or without (C, E) Holm-Šidák correction for multiple hypothesis testing. Each point represents a measurement from one mouse, n = 5-10 for each group with mean ± SD shown. *p < 0.05, **p < 0.01, ***p < 0.001.

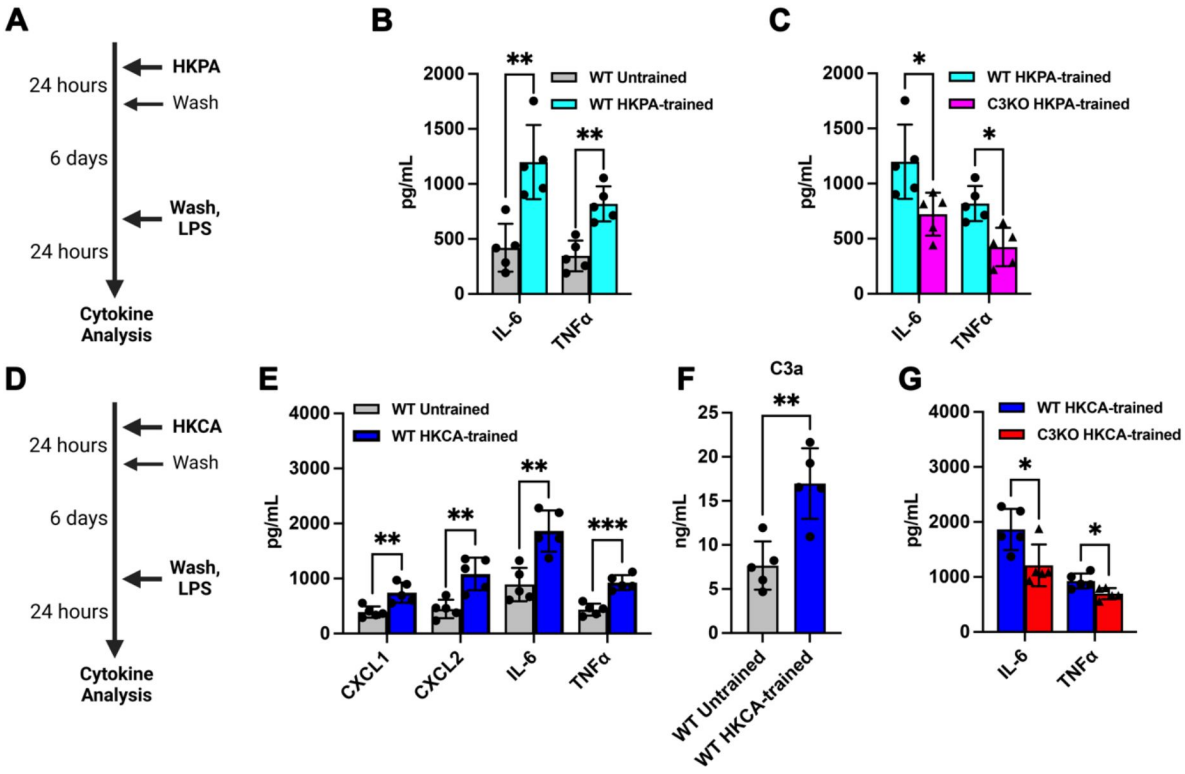

Figure 2

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 cytokine analysis of the supernatants. Created with *BioRender*.
(E) Effects of heat-killed *Candida albicans* (HKCA)-induced training *in vitro* on CXCL1, CXCL2, IL-6 and TNFα 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 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, ***p < 0.001.

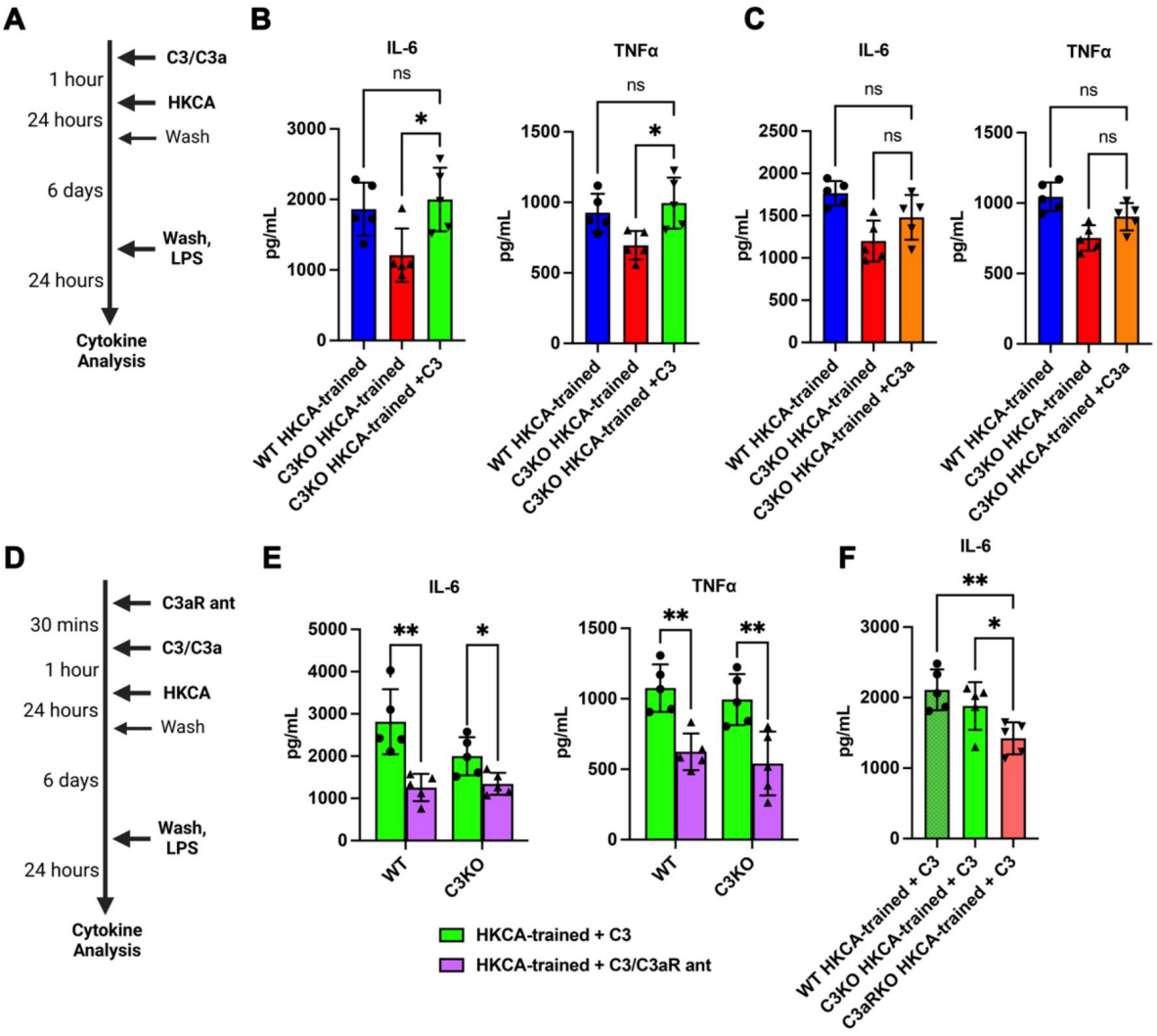

Figure 3.

C3 uptake enhances trained immune responses in ex vivo alveolar macrophages via the C3a receptor (C3aRs).

(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-HKCA-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 (D). 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.

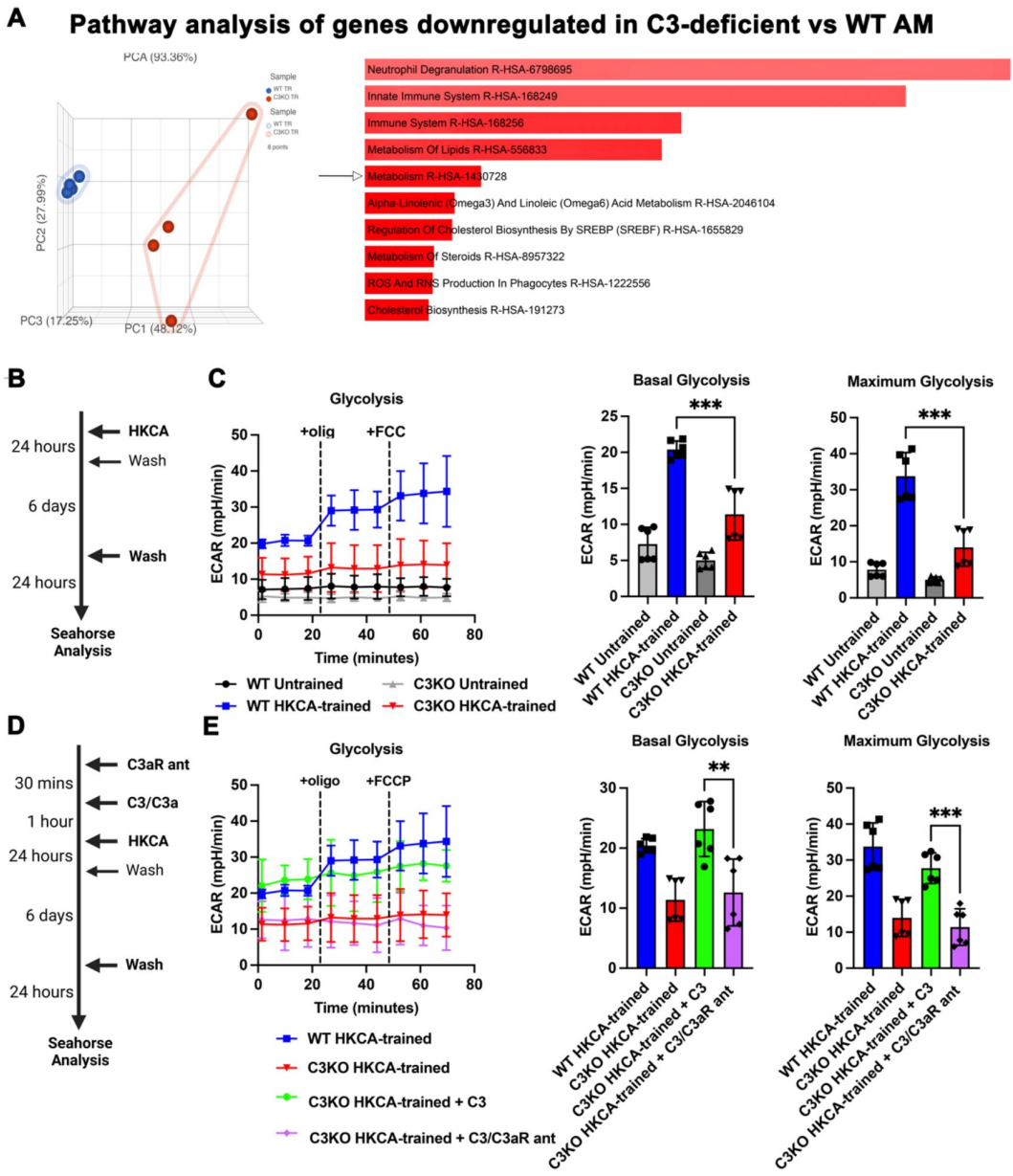

Figure 4.

C3-C3aR axis is required for glycolysis as a part of trained immune responses in alveolar macrophages.

(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 using an unpaired t-test.

Collectively, these data indicate that C3 makes a mechanistic contribution to trained immunity in AMs and implicate engagement of the C3aR as part of the pathway. Strengths of our study include *in vitro* and *in vivo* approaches to inducing trained immunity, orthogonal endpoints to quantify immune training (cytokine and metabolic), 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. The results may have clinical implications since trained immunity is linked to vaccine effectiveness in some cases (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*.

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

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*.

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Additional information

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Contributions

A.P.E. was involved in conceptual development, methodology, investigation, statistical analysis, writing – original draft, writing – review and editing; and funding acquisition; R.A., M.S., A.N., L.G., A.N.O., R.K.M., X.W. and J.H. were involved in the methodology, investigation, and writing – review and editing; H.S.K. was involved in conceptual development, methodology, investigation, statistical analysis, writing – original draft, writing – review and editing; supervision, and funding acquisition.

Additional files

Supplementary Material

Table S1

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Author information

Alexander P Earhart
Division of Pulmonary and Critical Care Medicine, John T. Milliken Department of Medicine, Washington University School of Medicine, St Louis, United States
ORCID iD: 0000-0002-8015-6026

For correspondence: alexander.earhart@wustl.edu

Rafael Aponte Alburquerque
Division of Pulmonary and Critical Care Medicine, John T. Milliken Department of Medicine, Washington University School of Medicine, St Louis, United States
ORCID iD: 0000-0002-4771-2801

Marick Starick
Division of Pulmonary and Critical Care Medicine, John T. Milliken Department of Medicine, Washington University School of Medicine, St Louis, United States
ORCID iD: 0000-0003-4227-7852

Aasritha Nallapu
Division of Pulmonary and Critical Care Medicine, John T. Milliken Department of Medicine, Washington University School of Medicine, St Louis, United States
ORCID iD: 0009-0000-1189-8547

Lorena Garnica
Division of Pulmonary and Critical Care Medicine, John T. Milliken Department of Medicine, Washington University School of Medicine, St Louis, United States
ORCID iD: 0009-0003-7644-6535

Ayse Naz Ozanturk
Division of Pulmonary and Critical Care Medicine, John T. Milliken Department of Medicine, Washington University School of Medicine, St Louis, United States

ORCID iD: 0000-0002-0187-3642

Rahul Kumar Maurya
Division of Pulmonary and Critical Care Medicine, John T. Milliken Department of Medicine, Washington University School of Medicine, St Louis, United States
ORCID iD: 0009-0007-6996-5459

Xiaobo Wu
Division of Rheumatology, John T. Milliken Department of Medicine, Washington University School of Medicine, St Louis, United States

Jeffrey A Haspel
Division of Pulmonary and Critical Care Medicine, John T. Milliken Department of Medicine, Washington University School of Medicine, St Louis, United States
ORCID iD: 0000-0001-5839-0265

Hrishikesh S Kulkarni
Division of Pulmonary and Critical Care Medicine, John T. Milliken Department of Medicine, Washington University School of Medicine, St Louis, United States, Department of Medicine, University of California, Los Angeles David Geffen School of Medicine, Los Angeles, United States
ORCID iD: 0000-0003-4830-5084

For correspondence: hskulkarni@mednet.ucla.edu

Editors

Reviewing Editor
Boris Novakovic
Murdoch Childrens Research Institute, Melbourne, Australia

Senior Editor
Satyajit Rath
Indian Institute of Science Education and Research (IISER), Pune, India

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.

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.

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 and then binding to lysosomal C3aR needs to be further supported by experimental evidence.

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

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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.

(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.

(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.

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

(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.

(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.

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Author response:

We thank both reviewers for their suggestions on improving our manuscript, which is focused on demonstrating that the C3a-C3aR axis modulates trained immune responses in alveolar macrophages. The Short Report format precludes separating the Results and Discussion sections. However, we will work towards a clearer presentation of findings and providing a more comprehensive interpretation of the data in the Revision, by addressing the points brought up by both Reviewers.

We agree with the suggestions from Reviewer 1 that (1) other cell types such as dendritic cells, neutrophils, and endothelial cells can also be involved in immune training, and (2) macrophages have other activities beyond releasing inflammatory cytokines, and will clarify both these points in the Revision. The mechanism of C3 being cleaved intracellularly and binding to lysosomal C3aR involves cathepsin-dependent cleavage of C3 to C3a and has been experimentally proven (Liszewski et al. Immunity 2013). However, we will clarify this mechanism in the revision. We also acknowledge that the observations need to be validated in human-based models. Currently, 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. However, we anticipate this work will form the basis of these future studies.

We also appreciate Reviewer 2's suggestions regarding demonstrating the resolution of acute inflammation after the initial exposure to heat-killed *Pseudomonas*. We will address this critique by performing additional experiments, which will be included in the Revision. We also agree that the responses of trained C3-deficient cells should be compared to untrained

C3-deficient controls after the LPS challenge. We will include this data in the Revision, in addition to the requested data for Figures 3 and 4. We would like to clarify that we do not observe baseline differences between untrained C3-sufficient (wildtype) and C3-deficient alveolar macrophages, even in their glycolytic capacity, and thus, anticipate that our revised data will strengthen the conclusions from the original manuscript.

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