Immune Netw. 2024 Aug;24(4):e24
https://doi.org/10.4110/in.2024.24.e24
pISSN 1598-2629·eISSN 2092-6685

IMMUNE NETWORK

Brief Communication

Complement C5a Receptor Signaling in Macrophages Enhances Trained Immunity Through mTOR Pathway Activation

Eun-Hyeon Shim [1,2,3,†], **Sae-Hae Kim** [4,†], **Doo-Jin Kim** [5], **Yong-Suk Jang** [1,4,*]

[1]Innovative Research and Education Center for Integrated Bioactive Materials and the Department of Bioactive Material Sciences, Jeonbuk National University, Jeonju 54896, Korea
[2]Department of Bioscience, University of Science and Technology (UST), Daejeon 34113, Korea
[3]Infectious Disease Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 34141, Korea
[4]Department of Molecular Biology and The Institute for Molecular Biology and Genetics, Jeonbuk National University, Jeonju 54896, Korea
[5]Department of Medicine, College of Medicine and Medical Research Institute, Chungbuk National University, Cheongju 28644, Korea

OPEN ACCESS

Received: Jan 9, 2024
Revised: May 28, 2024
Accepted: May 31, 2024
Published online: Jun 5, 2024

***Correspondence to**
Yong-Suk Jang
Department of Molecular Biology and The Institute for Molecular Biology and Genetics, Jeonbuk National University, 567 Baekje-daero, Deokjin-gu, Jeonju 54896, Korea.
Email: yongsuk@jbnu.ac.kr

†Eun-Hyeon Shim and Sae-Hae Kim contributed equally to this work.

Copyright © 2024. The Korean Association of Immunologists
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (https://creativecommons.org/licenses/by-nc/4.0/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

ORCID iDs
Eun-Hyeon Shim
https://orcid.org/0000-0003-3538-473X
Sae-Hae Kim
https://orcid.org/0000-0003-1235-735X
Doo-Jin Kim
https://orcid.org/0000-0003-4934-4931
Yong-Suk Jang
https://orcid.org/0000-0002-1675-4224

Conflict of Interest
The authors declare no potential conflicts of interest.

ABSTRACT

Complement C5a receptor (C5aR) signaling in immune cells has various functions, inducing inflammatory or anti-inflammatory responses based on the type of ligand present. The Co1 peptide (SFHQLPARSRPLP) has been reported to activate C5aR signaling in dendritic cells. We investigated the effect of C5aR signaling via the Co1 peptide on macrophages. In peritoneal macrophages, the interaction between C5aR and the Co1 peptide activated the mTOR pathway, resulting in the production of pro-inflammatory cytokines. Considering the close associations of mTOR signaling with IL-6 and TNF-α in macrophage training, our findings indicate that the Co1 peptide amplifies β-glucan-induced trained immunity. Overall, this research highlights a previously underappreciated aspect of C5aR signaling in trained immunity, and posits that the Co1 peptide is a potentially effective immunomodulator for enhancing trained immunity.

Keywords: Adjuvants, immunogenic; C5a receptor; Immunomodulator; Inflammation; Trained immunity

INTRODUCTION

The complement system is integral to the host defense mechanism, contributing significantly to pathogen clearance and facilitating the connection between innate and adaptive immunity (1). Key outcomes of complement activation include the formation of the membrane attack complex, which directly targets pathogens, and the production of anaphylatoxins (C3a and C5a). These anaphylatoxins interact with their respective receptors, C3a receptor (C3aR) and C5a receptor (C5aR), to promote the generation of proinflammatory cytokines and the chemotactic recruitment of various immune cells (2). Notably, C5a-C5aR signaling has variable effects based on the cell type and ligands involved (3). For instance, while C5a reduces IL-6 and TNF expression in LPS-stimulated macrophages, it enhances anti-

Generated by Xmlinkpress

Abbreviations

BCG, Bacillus Calmette-Guérin; C3aR, C3a receptor; C5aR, C5a receptor; DPBS, Dulbecco's PBS; RPS6, ribosomal protein S6; TIbV, trained immunity-based vaccine.

Author Contributions

Conceptualization: Kim SH, Jang YS; Data curation: Jang YS; Formal analysis: Jang YS; Funding acquisition: Kim SH, Jang YS; Investigation: Shim EH, Kim SH; Methodology: Kim SH; Project administration: Jang YS; Supervision: Kim DJ, Jang YS; Validation: Shim EH, Kim SH, Kim DJ, Jang YS; Visualization: Shim EH, Kim SH; Writing - original draft: Shim EH, Kim SH, Kim DJ; Writing - review & editing: Kim SH, Kim DJ, Jang YS.

inflammatory cytokine production in monocytes (4,5). In addition, C5a-licensed renal macrophages play a crucial role in protecting against systemic *Candida* infections by inhibiting the mTOR complex 1 signaling triggered by *Candida*. Conversely, C5a-C5aR signaling in alveolar macrophages induces an apoptotic response through the degradation of BCL-2 (6,7).

The mTOR signaling pathway in monocytes/macrophages plays a pivotal role in macrophage training. This phenomenon is closely associated with the development of trained immunity *in vivo*, where innate immune cells develop immunological memory (8). For instance, priming macrophages with β-glucan from *Candida albicans* activates the mTOR pathway, leading to epigenetic reprogramming and heightened responsiveness, such as increased IL-6 and TNF-α expression upon subsequent stimulation (9). The concept of macrophage training aligns with observations in vaccine-induced trained immunity. For example, the Bacillus Calmette-Guérin (BCG) vaccine offers broad protection against various pathogens by enhancing trained immunity in different immune compartments, including hematopoietic progenitors and mucosal areas (10,11). This concept is being explored for the development of trained immunity-based vaccines (TIbVs) and epigenetic adjuvants, particularly to boost antiviral immunity during pandemics (12).

In a previous study, we identified the Co1 peptide (SFHQLPARSPLP), which interacts with C5aR using a phage display library, and demonstrated that activation of C5aR by the Co1 peptide triggers ROS and chemokine production in monocyte-derived dendritic cells in Peyer's patches (13-15). However, the specific effects of C5aR-Co1 signaling on macrophages have not been fully explored. This study aims to elucidate how the Co1 peptide induces macrophage training through C5aR signaling-mediated activation of the mTOR pathway. Our results suggest a potential role for the Co1 peptide as an epigenetic adjuvant, expanding its use in immunomodulation and vaccine development.

MATERIALS AND METHODS

Experimental materials and cells

All chemicals and laboratory wares used in this study were acquired from Sigma-Aldrich (St. Louis, MO, USA) and SPL Life Sciences (Pocheon, Korea), unless noted otherwise. The murine macrophage cell line RAW 264.7 (30 passages) was sourced from the Korean Cell Line Bank (Seoul, Korea). These cells (<40 passages) were cultured in DMEM (Welgene, Gyeongsan, Korea) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT, USA) and incubated at 37°C in a 5% CO_2 atmosphere.

Arrays

RAW 264.7 cells were either treated with the C5aR antagonist W54011 (10 μM; R&D Systems, Minneapolis, MN, USA) or left untreated, followed by stimulation with Co1 peptide (1 μM or 22 μM; Peptron, Yusung, Korea) for 15 min. After treatment, the cells were washed, and total proteins were extracted from lysates homogenized in lysis buffer containing both protease and phosphatase inhibitor cocktails. The levels of total protein were determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Phosphorylation arrays were conducted using the Human/Mouse AKT Pathway Phosphorylation Array C1 (Raybiotech, Peachtree Corners, GA, USA) as per the manufacturer's instructions. Mean pixel intensity for these arrays was quantified using ImageJ software. Briefly, background signals were subtracted from experimental spot signals,

and the data were normalized to the positive control spot signals on the control array. The following formula was used to normalize signal intensity:

$$X(N2)=X2\times P1\div P2$$

P1, mean signal density of positive control spots on the control array; P2, mean signal density of positive control spots on the experimental array; X2, signal intensity of spot X on the experimental array; X(N2), normalized signal intensity of spot X on the experimental array.

Western blotting analysis

RAW 264.7 cells were pretreated for 1 h with or without rapamycin (20 nM) and then stimulated with Co1 peptide (1 μM or 22 μM) for 15 min. Total protein concentration in the cell lysates was determined using the Pierce™ BCA Protein Assay Kit. Following this, 20 μg total protein samples were subjected to SDS-PAGE and transferred onto polyvinylidene fluoride membranes. These membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 and then incubated with the specified primary Abs (Cell Signaling Technology, Danvers, MA, USA). This was followed by incubation with HRP-conjugated anti-mouse Abs (Cell Signaling Technology).

In vitro-trained immunity model using peritoneal macrophages

To isolate peritoneal macrophages, initially, 5 mL of medium (5% FBS/Dulbecco's PBS [DPBS]) were injected into the peritoneal cavity; this injection was followed by gentle abdominal massage to dislodge attached peritoneal cells into the medium. The medium was collected by aspiration. Red blood cells were lysed with ACK solution (Thermo Fisher Scientific), and macrophages were enriched using a Macrophage Enrichment Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) in accordance with the manufacturer's instructions. Enriched peritoneal macrophages were incubated for 2 h, and non-adherent cells were removed by gentle washing with DPBS. The remaining cells were treated with the indicated factors for 24 h, then washed and incubated in culture medium for 3 days. On day 4, the cells were primed with IFN-γ (25 ng/mL; R&D Systems) for 12 h. Subsequently, the cells were stimulated with LPS (1 μg/mL, LPS-EK Ultrapure; InvivoGen, San Diego, CA, USA) for 4 h, and supernatants were collected for the measurement of cytokine concentrations (16).

In vivo-trained immunity mouse model

Specific pathogen-free female BALB/c mice, 8-wk-old, were acquired from Koatech Laboratory Animal Center (Pyeongtaek, Korea). The mice were housed and cared for in accordance with the guidelines of the Animal Center of Jeonbuk National University. Ethical approval for all experimental procedures was granted by the Institutional Animal Care and Use Committee of Jeonbuk National University (approval No. NON2023–216). For the training phase, BALB/c mice were intraperitoneally primed and boosted with either β-glucan (1 mg; InvivoGen) or Co1 peptide (100 μg), followed by a challenge with LPS (InvivoGen) on day 7 (16).

Cytokine assay

Cytokine concentrations in culture supernatant and serum samples were quantified using the LEGENDplex™ Mouse Macrophage/Microglia Panel (BioLegend, San Diego, CA, USA) and BD™ Cytometric Bead Array, in accordance with the manufacturer's instructions.

Statistical analysis

Prism 10 (GraphPad Software, Boston, MA, USA) was used for statistical analysis. One-way ANOVA was used to determine statistical significance among different groups. Differences between groups were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

C5aR-Co1 peptide signaling activates Akt/mTOR signaling pathway in macrophages

Previous research has demonstrated that C5aR-C5a signaling influences the mTOR pathway in renal macrophages during *Candida* infection (6). To investigate the potential modulation of mTOR signaling in macrophages by C5aR signaling induced by its ligand, the Co1 peptide, we analyzed the phosphorylation of serine/threonine kinase Akt and mTOR pathway-related proteins in RAW264.7 cells after Co1 peptide treatment (**Fig. 1**). Further, to establish the phosphorylation as a result of C5aR-mediated signaling, we compared the phosphorylation pattern following the blockage of C5aR signaling using a C5aR antagonist (W54011) (**Fig. 1A**). Co1 peptide stimulation induced phosphorylation of Akt and mTOR, leading to subsequent phosphorylation of p70S6K (ribosomal protein S6 kinase) and enhancing protein synthesis through ribosomal protein S6 (RPS6) phosphorylation. The increase in phosphorylation was reduced upon C5aR signaling inhibition, affirming that Akt/mTOR activation is contingent

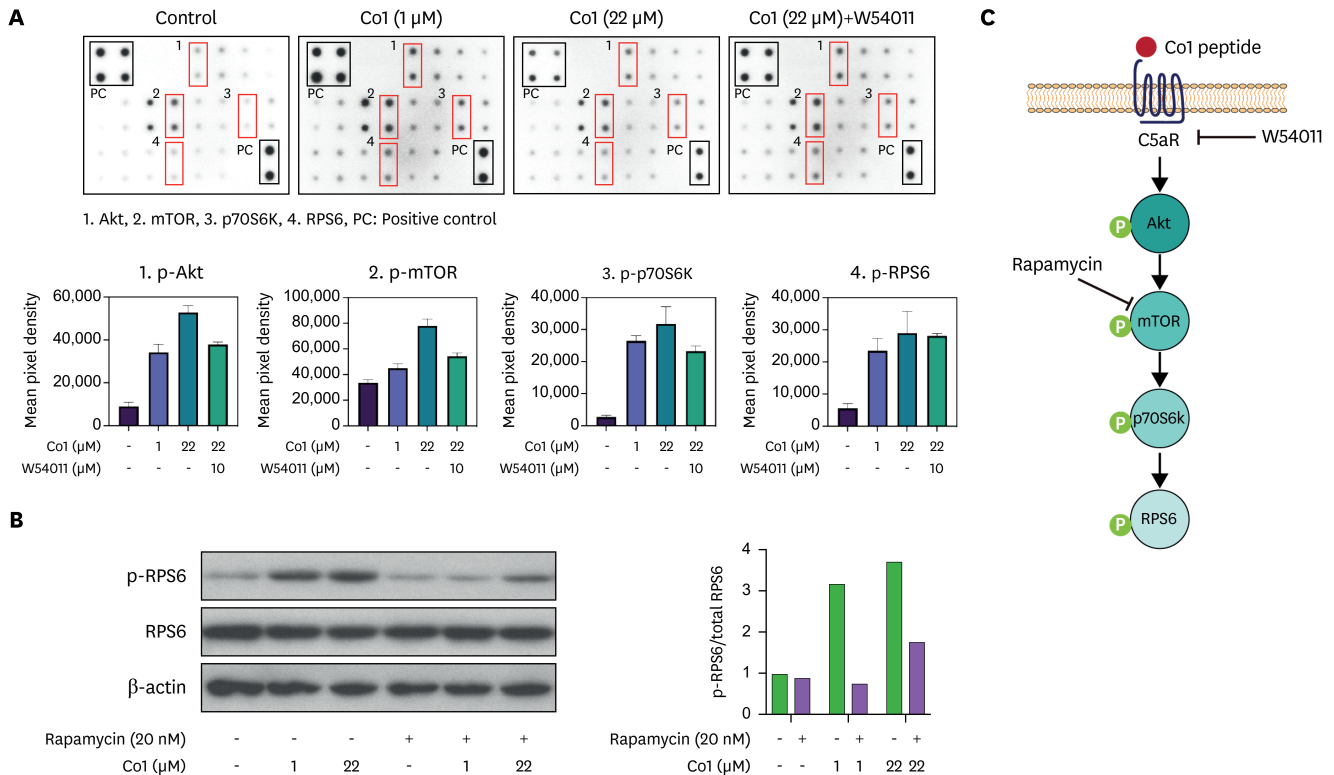

Figure 1. Activation of Akt/mTOR signaling pathway in macrophages by C5aR-Co1 peptide signaling. (A) RAW264.7 cells were treated with each indicated molecule for 15 min. The panel displays array data and densitometry results for p-AKT, p-mTOR, p-P70S6, and p-RPS6 in RAW264.7 cell lysates. Bar graph shows the mean of replicated spots in the array for indicated molecules. Data are representative of three independent experiments. (B) RAW264.7 cells were preincubated with or without rapamycin for 1 h, followed by treatment with each indicated molecule. Bar graph shows the level of p-S6 normalized to total S6 protein. Data are representative of three independent experiments. (C) The schematic shows our hypothesis that C5aR-Co1 peptide signaling activates the mTOR signaling pathway.

IMMUNE NETWORK

on C5aR-Co1 peptide signaling. However, as shown in the bottom panel of **Fig. 1B**, this inhibition of phosphorylation by W54011 was absent in RPS6. To corroborate that C5aR-Co1 peptide signaling activates the mTOR pathway, we evaluated RPS6 phosphorylation levels under conditions with mTOR signaling inhibited by rapamycin. Consistent with results for the other mTOR signaling proteins upon W54011 antagonist treatment, Co1-induced RPS6 phosphorylation was abolished by rapamycin (**Fig. 1B**). Thus, our data demonstrate that C5aR-Co1 peptide signaling initiates the mTOR signaling pathway in macrophages, as depicted in **Fig. 1C**.

C5aR-Co1 peptide signaling induces *in vitro* peritoneal macrophage training via the mTOR pathway

The canonical mTOR signaling pathway is implicated in macrophage training, leading to the production of pro-inflammatory cytokines (17). To determine whether C5aR-Co1 signaling induces macrophage training via mTOR signaling, we analyzed cytokine expression in peritoneal macrophages trained with β-glucan, C5a, or the Co1 peptide, as previously described (**Fig. 2A**) (16). Activation of C5aR signaling by C5a (100 nM) or Co1 peptides (7.3 μM) resulted in the upregulation of pro-inflammatory cytokines (IL-6 and TNF-α), compared with the PBS group (**Fig. 2**). Moreover, this enhancement was inhibited by rapamycin (**Fig. 2**). Therefore, we hypothesized that C5aR-Co1 peptide signaling may facilitate macrophage training, as depicted in **Fig. 2B**.

C5aR-Co1 peptide signaling enhances *in vivo* β-glucan-induced macrophage training

To investigate the function of C5aR-Co1 peptide in an *in vivo* training model, mice were trained with the indicated factors, and subsequently challenged with LPS, as previously described (16). Upon LPS challenge, the serum levels of IL-6 and TNF-α were elevated in mice trained with β-glucan, indicative of trained immunity (16). Although repeated training with Co1 peptide alone did not lead to increased serum levels of IL-6 and TNF-α, sequential training with β-glucan followed by Co1 peptide resulted in elevated cytokine levels, comparable to those in the group trained twice with β-glucan (**Fig. 3**). This suggests that C5aR-Co1 peptide signaling can augment β-glucan-mediated macrophage training.

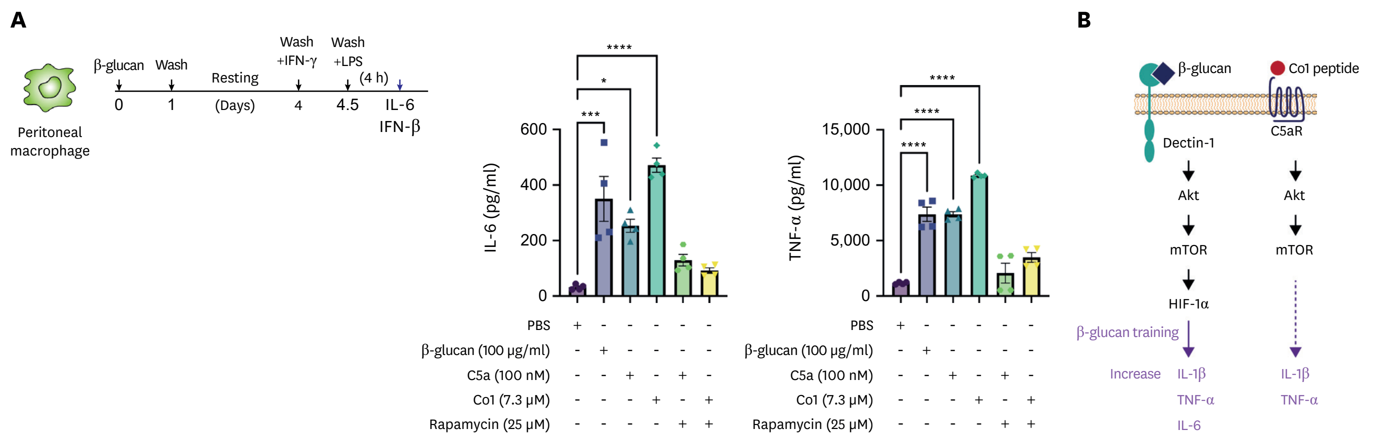

Figure 2. C5aR-Co1 peptide signaling induces *in vitro* peritoneal macrophage training via the mTOR pathway. (A) Schematic diagram of the *in vitro* model strategy for inducing trained immunity. Bar graph shows the levels of the indicated cytokines in supernatants. Data are presented as means ± standard errors; *p*-values were analyzed by ordinary one-way ANOVA in Prism; Data are representative of three independent experiments. (B) Schematic diagram of the β-glucan training pathway and our hypothesis that C5aR-Co1 peptide signaling induces cytokines via the mTOR signaling pathway.
*$p<0.05$, **$p<0.01$, ***$p<0.0005$, ****$p<0.0001$.

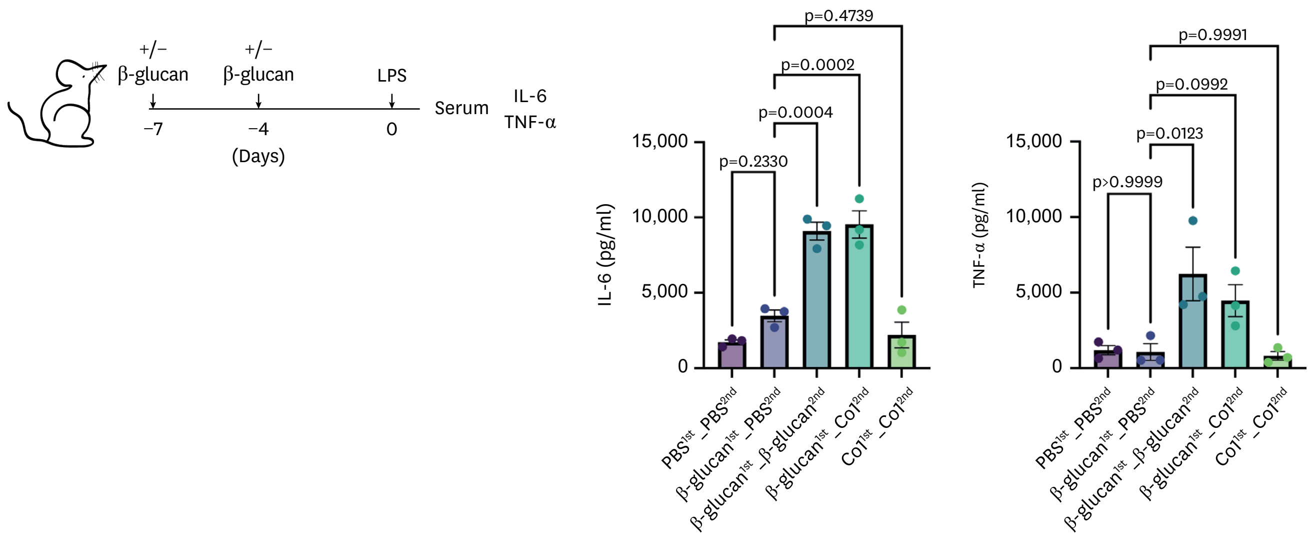

Figure 3. Enhancement of β-glucan-induced macrophage training by C5aR-Co1 signaling. Schematic diagram illustrates the *in vivo* model strategy for inducing trained immunity. Serum was collected 60 min after LPS challenge. Bar graph shows the levels of the indicated cytokines in serum. Data are presented as means ± standard errors; p-values were analyzed by ordinary one-way ANOVA in Prism.

C5a receptor signaling has varied effects on immune cells, inducing inflammatory or anti-inflammatory responses depending on the ligand type. Activation of C5aR with C5a promotes inflammation and chemotaxis, whereas interaction with chemotaxis inhibitory proteins of *Staphylococcus aureus* (CHIPS) impedes phagocytic cell recruitment (18). This study focuses on the role of C5aR-Co1 peptide signaling in macrophage training and trained immunity.

TIbVs

In contrasting with traditional vaccines that trigger Ag-specific adaptive immune responses, TIbVs enhance resistance to a wide range of pathogens (19). A recent study reported that a 'protein-free vaccine' consisting of aluminum hydroxide, monophosphoryl lipid A, and fungal mannan can protect against nosocomial pathogens such as methicillin-resistant *Staphylococcus aureus* without inducing Ag-specific immunity (20). BCG vaccination also confers protection against lethal influenza virus and SARS-CoV-2 challenges through MyD88 signaling activation (21). Agents capable of inducing trained immunity could serve as preventive measures against various pathogens and/or as immune adjuvants when combined with specific Ags (22). Notably, our study suggests that the Co1 peptide can train innate immunity, highlighting its potential as an anti-infectious agent or adjuvant for eliciting trained immunity.

ACKNOWLEDGEMENTS

This research was supported by the Basic Science Research Program (2019R1A2C2004711 to Yong-Suk Jang and 2021R1I1A1A01059681 to Sae-Hae Kim) and (2017R1A6A1A03015876 to Yong-Suk Jang) of the National Research Foundation (NRF), funded by the Korean Ministry of Science and ICT and the Korean Ministry of Education, respectively. Dr. Yong-Suk Jang was supported by the "Research Base Construction Fund Support Program" funded by Jeonbuk National University in 2024. Eun-Hyeon Shim was supported by the BK21 FOUR program in the Department of Bioactive Material Sciences. Cytological studies were performed using the

instruments installed in the Center for University-Wide Research Facilities (CURF) at Jeonbuk National University.

REFERENCES

1. Walport MJ. Complement. First of two parts. *N Engl J Med* 2001;344:1058-1066. PUBMED | CROSSREF
2. Pandey S, Maharana J, Li XX, Woodruff TM, Shukla AK. Emerging insights into the structure and function of complement C5a receptors. *Trends Biochem Sci* 2020;45:693-705. PUBMED | CROSSREF
3. Mastellos DC, Hajishengallis G, Lambris JD. A guide to complement biology, pathology and therapeutic opportunity. *Nat Rev Immunol* 2024;24:118-141. PUBMED | CROSSREF
4. Seow V, Lim J, Iyer A, Suen JY, Ariffin JK, Hohenhaus DM, Sweet MJ, Fairlie DP. Inflammatory responses induced by lipopolysaccharide are amplified in primary human monocytes but suppressed in macrophages by complement protein C5a. *J Immunol* 2013;191:4308-4316. PUBMED | CROSSREF
5. Yuk JM, Kim JK, Kim IS, Jo EK. TNF in human tuberculosis: a double-edged sword. *Immune Netw* 2024;24:e4. PUBMED | CROSSREF
6. Desai JV, Kumar D, Freiwald T, Chauss D, Johnson MD, Abers MS, Steinbrink JM, Perfect JR, Alexander B, Matzaraki V, et al. C5a-licensed phagocytes drive sterilizing immunity during systemic fungal infection. *Cell* 2023;186:2802-2822.e22. PUBMED | CROSSREF
7. Sun L, Guo RF, Gao H, Sarma JV, Zetoune FS, Ward PA. Attenuation of IgG immune complex-induced acute lung injury by silencing C5aR in lung epithelial cells. *FASEB J* 2009;23:3808-3818. PUBMED | CROSSREF
8. Horng T. mTOR trains heightened macrophage responses. *Trends Immunol* 2015;36:1-2. PUBMED | CROSSREF
9. Cheng SC, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, Giamarellos-Bourboulis EJ, Martens JH, Rao NA, Aghajanirefah A, et al. mTOR- and HIF-1α-mediated aerobic glycolysis as metabolic basis for trained immunity. *Science* 2014;345:1250684. PUBMED | CROSSREF
10. Cirovic B, de Bree LC, Groh L, Blok BA, Chan J, van der Velden WJ, Bremmers ME, van Crevel R, Händler K, Picelli S, et al. BCG vaccination in humans elicits trained immunity via the hematopoietic progenitor compartment. *Cell Host Microbe* 2020;28:322-334.e5. PUBMED | CROSSREF
11. Jeyanathan M, Vaseghi-Shanjani M, Afkhami S, Grondin JA, Kang A, D'Agostino MR, Yao Y, Jain S, Zganiacz A, Kroezen Z, et al. Parenteral BCG vaccine induces lung-resident memory macrophages and trained immunity via the gut-lung axis. *Nat Immunol* 2022;23:1687-1702. PUBMED | CROSSREF
12. Pulendran B, S Arunachalam P, O'Hagan DT. Emerging concepts in the science of vaccine adjuvants. *Nat Rev Drug Discov* 2021;20:454-475. PUBMED | CROSSREF
13. Kim SH, Shim EH, Kim DJ, Jang YS. $C5aR^+$ dendritic cells fine-tune the Peyer's patch microenvironment to induce antigen-specific $CD8^+$ T cells. *NPJ Vaccines* 2023;8:120. PUBMED | CROSSREF
14. Kim SH, Seo KW, Kim J, Lee KY, Jang YS. The M cell-targeting ligand promotes antigen delivery and induces antigen-specific immune responses in mucosal vaccination. *J Immunol* 2010;185:5787-5795. PUBMED | CROSSREF
15. Kim SH, Cho BH, Kim KS, Jang YS. Complement C5a promotes antigen cross-presentation by Peyer's patch monocyte-derived dendritic cells and drives a protective $CD8^+$ T cell response. *Cell Reports* 2021;35:108995. PUBMED | CROSSREF
16. Saz-Leal P, Del Fresno C, Brandi P, Martínez-Cano S, Dungan OM, Chisholm JD, Kerr WG, Sancho D. Targeting SHIP-1 in myeloid cells enhances trained immunity and boosts response to infection. *Cell Reports* 2018;25:1118-1126. PUBMED | CROSSREF
17. Ochando J, Mulder WJ, Madsen JC, Netea MG, Duivenvoorden R. Trained immunity - basic concepts and contributions to immunopathology. *Nat Rev Nephrol* 2023;19:23-37. PUBMED | CROSSREF
18. de Haas CJ, Veldkamp KE, Peschel A, Weerkamp F, Van Wamel WJ, Heezius EC, Poppelier MJ, Van Kessel KP, van Strijp JA. Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. *J Exp Med* 2004;199:687-695. PUBMED | CROSSREF
19. Sánchez-Ramón S, Conejero L, Netea MG, Sancho D, Palomares Ó, Subiza JL. Trained immunity-based vaccines: a new paradigm for the development of broad-spectrum anti-infectious formulations. *Front Immunol* 2018;9:2936. PUBMED | CROSSREF
20. Yan J, Nielsen TB, Lu P, Talyansky Y, Slarve M, Reza H, Novakovic B, Netea MG, Keller AE, Warren T, et al. A protein-free vaccine stimulates innate immunity and protects against nosocomial pathogens. *Sci Transl Med* 2023;15:eadf9556. PUBMED | CROSSREF

21. Lee A, Floyd K, Wu S, Fang Z, Tan TK, Froggatt HM, Powers JM, Leist SR, Gully KL, Hubbard ML, et al. BCG vaccination stimulates integrated organ immunity by feedback of the adaptive immune response to imprint prolonged innate antiviral resistance. *Nat Immunol* 2024;25:41-53. PUBMED | CROSSREF
22. Scheid A, Borriello F, Pietrasanta C, Christou H, Diray-Arce J, Pettengill MA, Joshi S, Li N, Bergelson I, Kollmann T, et al. Adjuvant effect of Bacille Calmette-Guerin on hepatitis B vaccine immunogenicity in the preterm and term newborn. *Front Immunol* 2018;9:29. PUBMED | CROSSREF