ANTI-rFILF POLYCLONAL ANTIBODIES ENHANCE MACROPHAGE PHAGOCYTIC ACTIVITY BY OPSONIZING *Acinetobacter baumannii*

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ABSTRACT

This study aimed to assess the efficacy of polyclonal antibodies (pAbs) against the *Acinetobacter baumannii* fimbrial protein FilF by opsonizing bacterial cells and enhancing macrophage activity. The rFilF was expressed, purified, and used to immunize BALB/c mice. Serum samples were collected to measure pAb titers. Bacterial cells were opsonized by pAbs and incubated with RAW264.7 macrophages. Phagocytic activity was assessed using flow cytometry. Results demonstrated that pAbs anti-rFilF significantly increased macrophage phagocytosis of *A. baumannii*. These findings suggest that immunization with rFilF induces opsonizing antibodies which can enhance macrophage-mediated bacterial clearance.

Keywords: Antibody*; Acinetobacter baumannii*; Opsonophagocytosis; Phagocyte; Opsonization.

1 INTRODUCTION

The rise of multidrug-resistant (MDR) microorganisms represents a significant threat to global public health. Projections estimate that by 2050, more than 300 million premature deaths will be caused by MDR infections. [1-2] This year, the World Health Organization (WHO) has identified carbapenem-resistant *Acinetobacter baumannii* (CRAb) as critical priority for antibiotic development. This opportunistic pathogen predominantly affects immunocompromised patients in hospitals, causing a range of infections such as pneumonia, bacteremia and urinary tract infections. [3]

Several emerging therapies have been researched to control *A. baumannii* infections such as anti-virulence strategies, microbiome modulation, probiotics, bacteriophages, drug repurposing, immunomodulation and prophylactic vaccination. [4] Despite no licensed vaccine, subunit vaccines, particularly those targeting antigenic proteins represents promising candidates to develop specific immune response. Fimbriae proteins are filamentous structures on the bacterial surface and perform a crucial role in adhesion to cell host, colonization and biofilm formation. These proteins are vital *A. baumannii* virulence factors, facilitating the initial stages of infection and contributing to the persistence of pathogens within the host. [5-7] FilF is a fimbrial protein highly conserved among different strains, making it a favorable target for the development of antibodies and vaccines. [6-7] We previously reported that polyclonal antibodies (pAbs) against the recombinant form of FilF (rFilF) could inhibit biofilm formation of CRAb clinical isolates. Biofilm formation is a major virulence factor that contributes to *A. baumannii* persistent infections and biotic and abiotic colonization. This reduction in biofilm formation indicated a potential mechanism by which these antibodies could neutralize the pathogen and aid in infection control. [8]

After design and express antigenic recombinant proteins, it is important to understand the immune mechanisms. Besides neutralization, antibodies can mediate other effector functions, including complement activation, cytotoxicity and opsonization. By opsonizing, or marking pathogens, for phagocytosis, antibodies can facilitate their recognition and destruction by phagocytic cells, contributing significantly to the immune defense against infections. Commercial vaccines like those for pneumococcus, meningococcus, and *Haemophilus influenzae* type b employ opsonophagocytic mechanisms to enhance body's immune response. [9-11] Therefore, this study aims to investigate if anti-rFilF pAb opsonization facilitate macrophage phagocytic activity against *A. baumannii* strains, providing a mechanism insight into the immune response elicited by rFilF-based vaccines.

2 MATERIAL & METHODS

Recombinant FilF protein was produced as described by Pereira et al. (2024). [8] The *filf* gene was chemically synthesized and cloned into the recombinant plasmid pET-28a-FilF, which was transformed into *Escherichia coli* BL21 (DE3) Star. The expression of the protein was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by protein purification using nickel affinity chromatography with HisTrap™ Sepharose column (Cytiva, USA). The purified protein was quantified and analyzed by SDS-PAGE to confirm its identity. To assess the antigenicity of rFilF, native antibodies presented in the serum of *A. baumannii*-infected patients were used in Western blot analysis.

According to Singh et al. (2016), the production of polyclonal antibodies (pAbs) was conducted.[6] Three 6-8-week-old BALB/c mice were immunized intraperitoneally with 50 µg of rFilF. The first dose (day 0) included the antigen with Complete Freund's Adjuvant (Sigma-Aldrich, Co., USA), while subsequent doses (day included the antigen with Incomplete Freund's Adjuvant (Sigma-Aldrich, Co., USA). As control, three mice were immunized with phosphate-buffered saline (PBS 1x) at the same time points. The doses were administered at a 1:1 ratio of antigen to adjuvant. Blood samples were collected via submandibular vein puncture at all time points. Five doses were administered on days 0, 14, 21, 28 and 35, and on the 42nd day after the first immunization, the mice were euthanized by total blood collection through cardiac puncture after anesthesia with isoflurane. The production of antibodies was monitored using indirect Enzyme-Linked Immunosorbent Assay (ELISA) to assess the seroconversion of immunized animals.

The opsonophagocytosis assay was conducted using murine RAW264.7 macrophages according to Luo et al. (2012).[12] One day before the assay, RAW264.7 macrophages were plated in 24-well microplates and cultured in RPMI 1640 medium supplemented with L-glutamine, 10% fetal bovine serum (SFB), and 1% antibiotic-antimycotic solution (overnight, 37ºC, 5% CO_2). Opsonophagocytosis assays were performed with both standard strain of *A. baumannii* ATCC®19606™ and a CRAb clinical isolate, The strains were adjusted to a McFarland 0.5 scale, and the bacterial suspension was stained with Hoescht (H33342, Sigma-Aldrich, Co., USA) for 15 minutes at 37ºC. After washing the cells and eluting the *pellet* in RPMI 1640 medium, the bacteria was incubated with antibodies (1 hour, 80 RPM, 37ºC). The antibodies from immune serum (immunized mice), and not-immune serum (animals that receive only PBS 1x) were heated at 56ºC to inactivate complement system proteins. After, the antibody-treated bacterial suspension was incubed in microplate with adhered RAW264.7 macrophages in a ratio of 20:1 (bacteria per macrophage) for 1 hour at 37ºC and in an atmosphere of 5% CO_2. Then the cells were trypsinized and the well contents were recovered and resuspended in 1x PBS with 4% formaldehyde. We assess the macrophage viability and the cell structures in Romanowsky stain for visualization under an optical microscope.

For evaluation of the results, the recovered cell contents were analyzed by flow cytometry using the Attune ® Acoustic Focusing Flow Cytometer (Applied Biosystems, UK) equipped with ultraviolet light (UV 405 nm) and photomultiplier filter VL1 (450/40 nm). The intensity of phagocytosis and the percentage of phagocytized cells were measured. Over 2.500 cells samples were selected, and these were separated into subsets according to cytometric characteristics such as size and complexity, determining fluorescence intensity. Cellular debris was discarded based on forward scatter vs. side scatter and negative fluorescence of H33342, aiming to reduce potential errors.

3 RESULTS & DISCUSSION

After assessing the rFilF antigenicity, it was used to immunize BALB/c mice. Seroconversion could be observed through ELISA assay one week after the first immunization, and the production of IgG antibodies remained increasing until the end of the experiment. Through the assay of antibody-mediated phagocytosis, it was possible to observe that the opsonizing activity of anti-rFilF antibodies induced a higher intensity of phagocytosis. Macrophage exposed to bacterial suspensions from both strains pre-opsonized by anti-rFilF pAbs showed a higher percentagem of phagocytosed cells, with a 20% increase compared to untreated cells (Figure 1). This assay confirms that na immunogenic mechanism of immunization of rFilF is the production of opsonizing antibodies that facilitate antigen presentation to phagocytes, such as macrophages, which act as the first line of defense in the immune system.

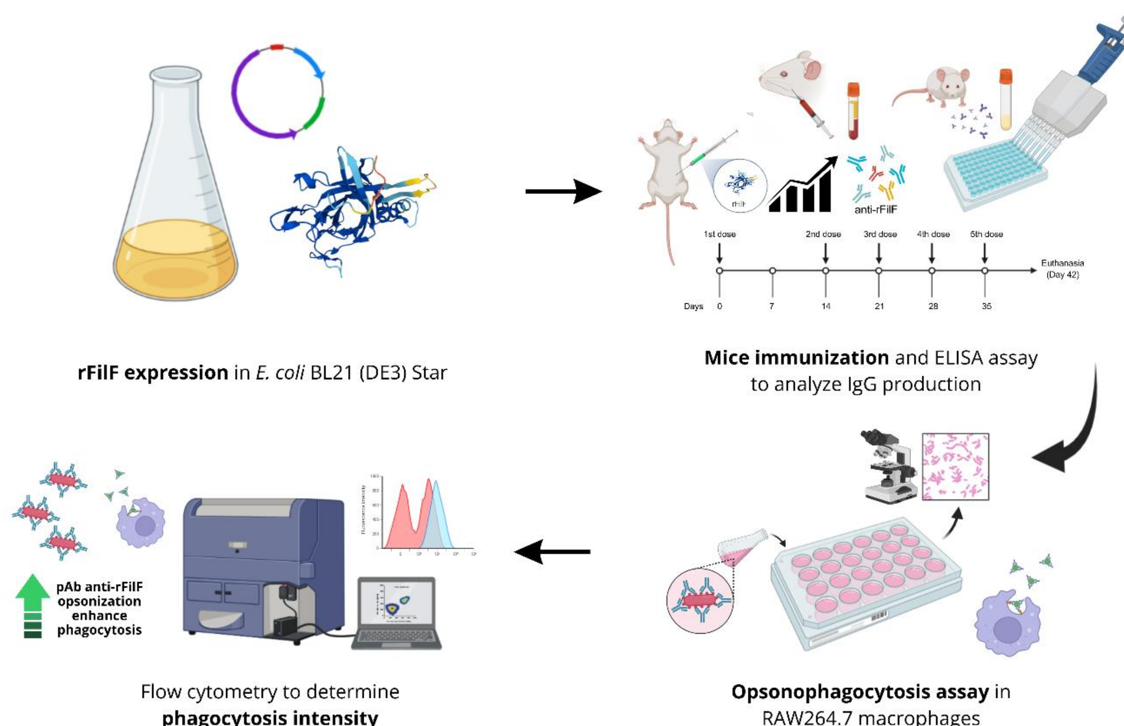

Figura 1: Flowchart summarizing this study

Studies have shown that other recombinant proteins involved in *A. baumannii* virulence can induce strong antibody response in animal models, and that could contribute to enhance phagocytic activity by immune cells.[6,12,13] The production of antibodies that boost phagocytosis is a vital indicator of the immune system's capability to recognize and combat infections.[12,13] This support our findings that immunization with rFilF can improve the immune system against *A. baumannii*.

4 CONCLUSION

The anti-rFilF pAbs effectively opsonized both bacterial strain of A. baumannii, resulting in improved phagocytic performance of RAW264.7 macrophages. Therefore, it can be estimated that immunization with rFilF would lead to opsonizing antibodies production and that could highly increase the phagocytic activity by macrophages as an immunoprotective mechanism.

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