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Intranasal immunization with the 54 kDa hemagglutinin pili protein of Streptococcus pneumoniae that increase the expression of β-defensin-2

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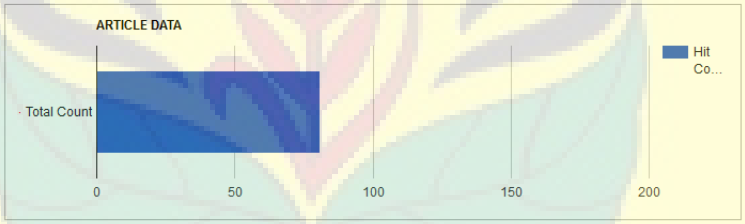
ABSTRACT

Introduction: This study aimed to determine the ability of intranasal immunization with 54 kDa pili proteins of Streptococcus pneumoniae to improve the expression of β-defensin-2 through the upregulation of interleukin (IL)-17A and IL-22 expression. **Materials and Methods:** Purified 54 kDa pili proteins were used as an antigen in immunized Wistar rats intranasally. Wistar strain (female) mice, aged 12–16 weeks, were divided into two groups. Control, Group 1 treatment with 40 mL of phosphate-buffered saline (PBS) containing adjuvants alone, namely, 2 μg cholera toxin B, and Group 2 with 40 μL of PBS containing the combined antigen-adjuvant mucosal immune responses identified from nasal washing inspection of Wistar rats concentrated on IL-17A, IL-22, and β-defensin-2 indicators. **Results:** The results presented here indicate that mice immunized with combined antigen-adjuvant therapy had higher levels of IL-17A, IL-22, and β-defensin-2 than the other groups. Analysis of variance testing demonstrated that there were significant differences between mice immunized with a combined antigen-adjuvant regime compared to the other groups. Pearson correlation testing indicated there is a strong positive relationship between concentrations of IL-17A and β-defensin 2, and a weakly positive correlation between IL-22 levels and β-defensin 2. As well, between IL-17A and IL-22 versus β-defensin 2, there was a moderately positive relationship. **Conclusion:** The intranasal immun… hemagglutinin pili proteins of S. pneumoniae enhanced the expression of β-defensin-2

KEYWORDS

Intranasal immunization Streptococcus pneumoniae pili protein β-defensin-2



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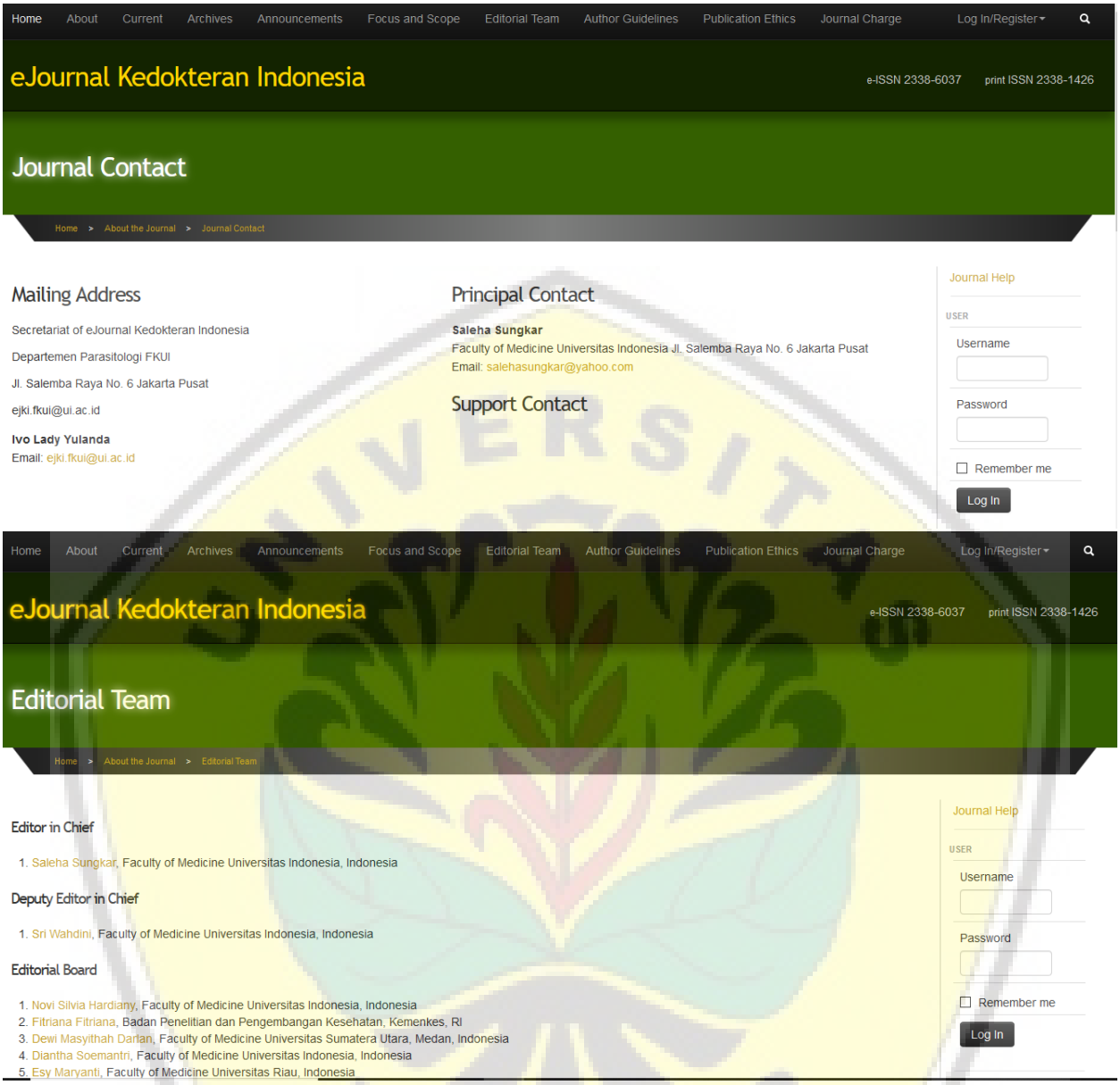

Research Article

Intranasal immunization with the 54 kDa hemagglutinin pili protein of *Streptococcus pneumoniae* that increase the expression of β-defensin-2

Diana Chusna Mufida[1]*, Dini Agustina[1], Yunita Armiyanti[2], Kusworini Handono[3], Sumarno Reto Prawiro[4], Sanarto Santoso[4]

ABSTRACT

Introduction: This study aimed to determine the ability of intranasal immunization with 54 kDa pili proteins of *Streptococcus pneumoniae* to improve the expression of β-defensin-2 through the upregulation of interleukin (IL)-17A and IL-22 expression. **Materials and Methods:** Purified 54 kDa pili proteins were used as an antigen in immunized Wistar rats intranasally. Wistar strain (female) mice, aged 12–16 weeks, were divided into two groups. Control, Group 1 treatment with 40 mL of phosphate-buffered saline (PBS) containing adjuvants alone, namely, 2 µg cholera toxin B, and Group 2 with 40 µL of PBS containing the combined antigen-adjuvant mucosal immune responses identified from nasal washing inspection of Wistar rats concentrated on IL-17A, IL-22, and β-defensin-2 indicators. **Results:** The results presented here indicate that mice immunized with combined antigen-adjuvant therapy had higher levels of IL-17A, IL-22, and β-defensin-2 than the other groups. Analysis of variance testing demonstrated that there were significant differences between mice immunized with a combined antigen-adjuvant regime compared to the other groups. Pearson correlation testing indicated there is a strong positive relationship between concentrations of IL-17A and β-defensin 2, and a weakly positive correlation between IL-22 levels and β-defensin 2. As well, between IL-17A and IL-22 versus β-defensin 2, there was a moderately positive relationship. **Conclusion:** The intranasal immunization with 54 kDa hemagglutinin pili proteins of *S. pneumoniae* enhanced the expression of β-defensin-2.

KEY WORDS: Intranasal immunization, *Streptococcus pneumoniae* pili protein, β-defensin-2

INTRODUCTION

Streptococcus pneumoniae, or pneumococcus, is the leading cause of respiratory tract infections, sinusitis, community-acquired pneumonia, bacteremia, otitis media, and invasive diseases, like meningitis.[1-3] Pneumococcal pneumonia is a lung infection with morbidity and mortality rates that are exceptionally high in developing countries.[3,4]

S. pneumoniae has a variety of virulence factors, namely, a polysaccharide capsule, pili, surface proteins, and toxins. Virulence factors are very important and include those that are antiphagocytic as well as those that influence adhesion, invasion, transport of iron and other heavy metals, defense against oxidative stress, pneumolysin production, and biofilm formation.[5]

S. pneumoniae features two types of pili that act as adhesins. Type 1 pili serve to strengthen and facilitate colonization and biofilm formation of microcolonies.[6] Previous studies have shown that *S. pneumoniae* pili contain a protein that acts as a hemagglutinin, a 54 kDa protein. Hemagglutinin is associated with adhesion, as has been demonstrated in *Shigella dysenteriae*, so it is believed that hemagglutinin can act as an indicator of adhesion during early initiation of infection.[7,8] As such, adhesin and pili are the main targets for fighting infection and pneumococcal infections, meaning the function and immunogenicity of pneumococcal pili

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and adhesin are relevant to the development of a new generation of pneumococcal vaccines that would be more efficacious.[9]

Vaccination or active immunization may also be administered intranasally. Providing different antigen formulations intranasally has shown promise in enhancing the immune response and eliminating pathogens at the sites of infection.[10] Intranasal immunization, specifically mucosal immunization, may protect the lungs against upper respiratory tract infections and a variety of other pathogens. Nasal mucosa is an appropriate place for this sort of vaccine not only because the cavum nasi is the entry of pathogenic respiratory tract bacteria but also based on the mucosal immune system stimulation of the respiratory tract by nasopharyngeal-associated lymphoid tissue.[11]

Intranasal immunization increases the expression of interleukin (IL)-17A as demonstrated by Zygmunt *et al*. (2009) – intranasal immunization increases CCR6 expression on the surface of CD4 + T-cells.[12] CCR6 is a biomarker for T-helper 17 (Th17).[13] Th17 produces some cytokines, including IL-17A and IL-22,[14] which both act synergistically to induce respiratory epithelial cells to produce antimicrobial substances, like β-defensin-2.[15] Against this backdrop, the purpose of this study was to determine the ability of intranasal immunization with 54 kDa pili proteins from *S. pneumoniae* to stimulate the production of β-defensin-2.

MATERIALS AND METHODS

Pili Isolation

Bacteria were cultured in biphasic TCG-Brain heart Infusion(BHI) media enriched with lamb's blood and transferred to a 100 mL tube, to which was added trichloroacetic acid to a concentration of 3%. The tube was then shaken for 30 min and left at room temperature for 1 h, followed by centrifugation at 4°C at 6000 rpm for 30 min. As much as 3 g of deposited bacteria was suspended with 6 mL of phosphate-buffered saline (PBS) (pH 7.4). Bacterial pili were then cut using a bacterial pili cutter with a speed of 5000 rpm at 4°C for 30 s, and this was repeated up to 4 times. Subsequently, centrifugation took place at a speed of 12,000 rpm at 4°C for 15 min. The supernatant containing the pili proteins was stored at − 20°C.[16]

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) of Pili Proteins from *S. pneumoniae*

To determine the molecular weight of pili proteins, SDS-PAGE was carried out. The protein samples were heated (100°C) for 5 min in a buffer containing 62.5 mM Tris-HCl(pH6.8),10%glycerol,and0.001%bromophenol blue with 5% (v/v) mercaptoethanol. The separating gel concentration was 12.5% with a 3% stacking gel and the voltage employed was 125 mV and 400 mA for 60 min. Furthermore, the gel was stained using coomassie brilliant blue for 30 min.[16]

Pili Protein Purification

Pili protein purification was conducted with electroelution. The 54 kDa pili protein band resulting from electrophoresis was cut from the gel. This band was placed on a cellulose membrane and run with electrophoresis buffer. Electroelution using horizontal electrophoresis with a voltage of 25 volts for 120 min was performed. What resulted from the electroelution was next dialyzed using PBS twice for 24 h at 4°C. The purified were next stored at − 20°C.

Immunization with 54 kDa Pili Protein

Wistar strain (female) mice, aged 12–16 weeks, were divided into three groups, with each group consisting of six animals. Control mice were immunized intranasally with PBS by as much as 40 μL, Group 1 treatment animals were immunized with 40 mL of PBS containing adjuvants alone, namely, 2 μg cholera toxin B (CTB), and Group 2 mice were immunized with 40 μL of PBS containing the combined antigen-adjuvant. Immunizations were administered intranasally at 20 mL per nostril. All mice were immunized on days 0, 7, and 14. One week following the last immunization, nasal rinse isolation was performed.[17,18] The Brawijaya University Ethic Committee has approved this study (698/ H25 1. 11/KE/2015).

Nasal Rinse Isolation and Vaginal Washings

One week after the last immunization, the animals were anesthetized with ether, after which the animal was sacrificed by decapitation. Nasal rinse isolations were performed on a retrograde basis by inserting 1–2 mL of sterile saline through the trachea and droplets of saline solution through the nostrils were accumulated into a sterile Eppendorf tube. The vagina was washed by flushing it with 1.5–2 mL of saline through a sterile pipette. The resulting wash fluid was accumulated into a new Eppendorf tube and stored in the refrigerator.[19]

Measuring the Concentrations of IL-17A, IL-22, and β-defensin-2

The nasal rinse concentration of IL-22, IL-17 A, β-defensin-2, and vaginal rinse β-defensin-2 was measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit for IL-22, IL-17A, and β-defensin-2 by the procedure plant (bioassay technology [BT]-laboratory E1473Ra no paint, paint Biolegend No. 437 907, and BT-laboratory paint No. E1334Ra). Samples were loaded into the wells to which 10 mL of antibodies for IL-17A, IL-22 or β-defensin-2 were added. After that, 50 μL of streptavidin-horseradish peroxidase was added, after

which the entire mixture was shaken and incubated for 1 h at 37°C. After 1 h, the plate was washed with a washing solution, and 50 mL of substrate solution A and 50 mL of substrate solution B were added, and the mixture was again incubated for 10 min. The reaction was then quenched with 50 mL of stop solution. Color expression was measured by optical density with an ELISA reader through wavelength at 450 nm.

The data generated based on IL-17A, IL-22, and β-defensin 2 concentrations are presented as mean ± standard deviation. The differences between groups were assessed with analysis of variance (ANOVA) ($P > 0.05$), and to determine the relationship between the levels of IL-17A and IL-22 with β-defensin-2 concentrations, Pearson correlation testing ($P < 0.05$) was carried out.

RESULTS

S. pneumoniae was cultured on TCG-BHI biphasic medium enriched with 5% lamb blood. After two periods of incubation 24 h each, the bacteria were harvested and the pili cut until the color of the supernatant was the same as PBS. To determine the profile of the pili proteins, the resulting solution was placed on SDS-PAGE and stained with coomassie blue afterward. SDS-PAGE revealed that the dominant pili proteins had a molecular weight of 67, 54, 25, and 11 kDa [Figure 1]. Proteins drank with a molecular weight of 54 kDa were excised from the gel and purified by electroelution dialysis. Purification employed an antigen to immunize the experimental animals (in this case, rats).

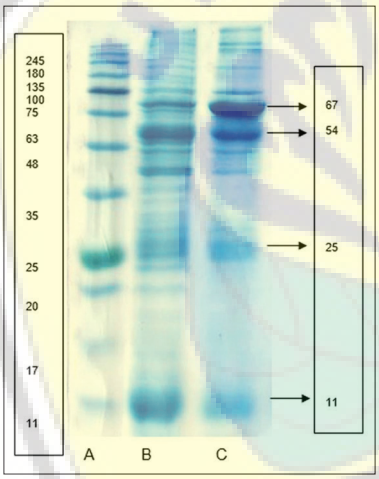

Figure 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis pili proteins of *Streptococcus pneumoniae* with coomassie blue staining. (A) A protein marker, (B) whole bacteria, and (C) pili

The Wistar mice were immunized intranasally with different loads of various vaccines, and the nasal rinse was isolated 1 week after the second booster. The nasal rinse was then measured for levels of the cytokines, IL-22 and IL-17A, and β-defensin-2 with ELISA. The vaginal wash was utilized to exclusively measure the concentration of β-defensin-2. The overall results of the ELISA are presented in Figure 2a and b.

IL-22 [Figure 2a and b] was found in nasal rinses from Wistar rats at the highest concentration. IL-17A was observed in experimental animals immunized with the antigen (protein pili; 54 kDa) plus the adjuvant. One-way ANOVA statistical testing yielded that IL-22 was significantly different concerning a concentration between Wistar rats that were immunized with the antigen plus the adjuvant and the other groups ($P = 0.02$; $P < 0.05$). One-way ANOVA statistical testing also provided evidence that IL-17A in nasal rinses was also significantly different between Wistar rats immunized with the antigen and adjuvant versus control rats and those immunized with the adjuvant alone ($P = 0.00$).

ELISA showed that the concentration of β-defensin-2 in the nasal rinses and vaginal washings was the highest in rats immunized with the antigen plus the adjuvant [Figure 3]. One-way ANOVA analysis of β defensin-2 of the nasal rinses and vaginal washings

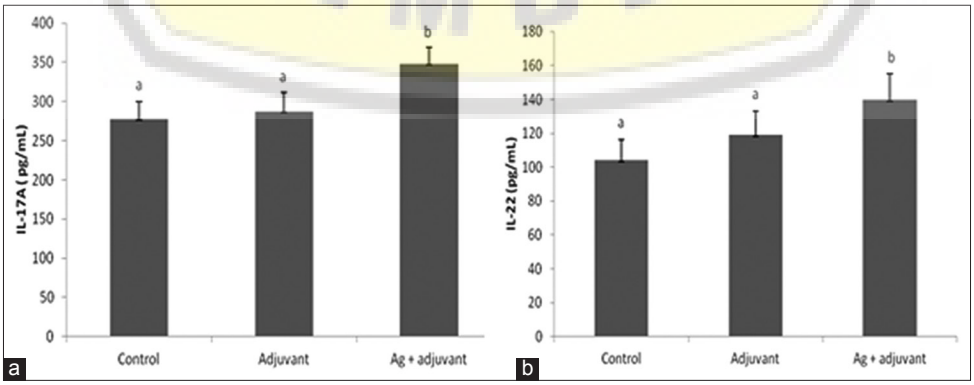

Figure 2: Enzyme-linked immunosorbent assay results of Wistar rats that had been immunized with pili proteins 54 kDa. (a) The concentration of interleukin (IL)-22 nasal rinses of control and treatment of mice, (b) the concentration of IL-17A nasal rinses of control and treatment mice

showed that there were significant differences between rats immunized with the antigen plus the adjuvant compared to control mice and immunized with the adjuvant alone ($P = 0.001$); the same was the case for β-defensin-2 in nasal rinses and vaginal rinses ($P = 0019$).

To determine the relationship between IL-22 and IL-17A with the concentration of β-defensin-2 in nasal rinses and vaginal washings, correlation testing was performed. The correlation test findings uncovered that concentrations of IL-22 and β-defensin-2 in nasal rinses were not statistically significant ($P = 0.06$; $P < 0.05$), and there was a positive and moderately strong correlation ($r = 0.451$). The case was different for IL-17A and β-defensin-2 in nasal rinses – there was a statistically significant relationship ($P = 0.00$) with a strong positive correlation ($r = 0758$). The correlation between IL-22 and IL-17A in nasal rinses with β-defensin-2 concentrations in vaginal rinses showed that IL-22 and IL-17A correlated statistically significantly ($P = 0.016$) with a moderately positive correlation with IL-22 ($r = 0.558$) and IL-17A ($r = 0560$).

DISCUSSION

Differentiation of naïve T-cells into Th17 antigens can be induced by immunization. This differentiation can be detected by an increase in cytokines expressed by Th17 cells, among others, such as IL-17, IL-17F, IL-22, IL-26, and CCL-20. In work presented here, there was increased expression of IL-22 and IL-17A [Figure 2a and b], so it can be established that intranasal immunization with pili protein (54 kDa) from *S. pneumoniae* with CTB adjuvant is capable of bringing about differentiation of naïve T-cells into Th17 antigens. The ability of Th17 to express cytokines varies depending on the Th17 subset. As an example, mature Th17 cells express IL-17A and IL-17F, but not express IL-22.[20] In addition to the factors above, elevated expression of IL-17A and IL-22 was also dependent on the route of immunization. Intranasal immunizations activate Th17 cells in the lung and nasal cavities > 10%.[12]

Expression of IL-17A and IL-22 in Wistar rats immunized with the antigen plus adjuvant was in a high yield compared to the other experimental groups. These findings resemble other studies conducted by Lundgren *et al*. (2012), where increased levels of IL-17A and IL-22 in peripheral blood mononuclear cells exposed to whole cells of *S. pneumoniae* antigens, pneumolysoids, and proteins necessary for cell separation in group β streptococcus were observed.[21]

Expression of IL-17A is influenced by external and internal factors, the latter being those found in the local environment. One of the external stimuli that trigger infectious pathogens are the associated molecular patterns, which consists of molecules derived from extracellular pathogens, such as bacteria and fungi, that induce differentiation of naive T-cells into Th17. Internal factors critical for Th17 differentiation are predominantly TGF-β, IL-6, and IL-21.[20]

IL-17A and IL-22 can be generated by adaptive immune cells (Th17) and innate immune cells, such as natural killer T cells, $CD117^+$ $CD127^+$ leukocyte lymphoid tissue inducer, $NKp46^+$ mucosal cells, γδ human T-cells, and granulocytes. IL-17A is a multifactorial cytokine, which gives rise to inflammation through increasing the production of inflammatory cytokines, chemokines, matrix remodeling proteins, adhesion molecules, antimicrobial molecules, and acute phase reactants. The main function of IL-17A in bacterial infections is a proinflammatory factor in recruiting neutrophils into the area of inflammation, bringing about the expression of G-CSE to improve granulopoiesis and control the accumulation and activity of neutrophils during inflammation with anti-apoptotic debilitating inflammatory cytokines. Besides this, IL-17A induces the expression of β-defensin-2, an S1000 protein, and various chemokines, among others, CXCL5, CXCL9, CCL3, and CCL-20, in the epithelium of the airways.[17,20,22]

The ability of IL-17A to stimulate the expression of human β-defensin-2 (HBD-2) in the respiratory epithelium has been investigated *in vitro* within the human tracheobronchial network. It was shown that IL-17A brings about the expression of HBD-2 more powerfully (×75) compared to other cytokines, specifically IL-1α, IL-1β, IL-6, and tumor necrosis factor-α (TNFα) (5–20×). The ability of IL-17A to increases the expression of β-defensin-2 functions in a dose-dependent manner based on the respiratory tract and time. Even small concentrations of IL-17A (1 ng/mL) to carry out the stimulation were observed to do so significantly.[23] These findings are in line with our study – there is a strongly positive correlation between the concentration of IL-17A and the expression of β-defensin-2 in nasal rinses.

IL-17A has been reported to induce the production of the proinflammatory cytokine, IL-1β, which is a major activator of the expression of HBD-2 in cultured lung epithelial A549 cells with mononuclear phagocytes. The IL-1β promoter increases the activity of HBD-2 at a concentration of 100 pg/mL, and there is peak activation at 1 ng/mL. IL-1β ultimately elevates HBD-2 concentrations through NFκB activation.[24] Another study reported that IL-17A increases the expression of HBD-2 directly through the JAK and NFκB signaling pathways.[23] Ultimately,

it seems that that IL-17A brings about the expression of β-defensin-2 through NFκB directly or indirectly through cytokine IL1β.

IL-22 is often produced together with IL-17A. IL-22 functions in non-immune cells, particularly those of the epithelial variety and others, such as cells from the skin, intestines, lungs, and kidneys. Similar to IL-17A, IL-22 induces antimicrobial peptides, acute phase reactants, and matrix metalloproteinase, but unique to IL-22 is that it mediates re-epithelialization and inhibition of differentiation of keratinocytes.[25-27] IL-22 has been noted to have an essential function in host defenses against experimental pneumonia infection. The IL-22 expression is regulated by IL-23 *in vivo* but features prominently in regulating the production of IL-6 and CCL3 in the lungs. Furthermore, IL-22 inhibits the colonization by *S. pneumoniae* in the nasopharyngeal region through a mechanism of inducing β-defensin-2 in the respiratory tract. In addition to producing antimicrobial β-defensin-2, IL-17A, and IL-22 synergistically bring about the production of S1000 protein.[15,21,28] The synergy between IL-17A and IL-22 is expected as a result of the convergence of the STAT3 signaling pathway along with NF-κB, which is induced by IL-22 and IL-17A receptors, respectively. The specific mechanism surrounding how IL-22 activates the STAT3 signaling pathways through convergence with ACT1-P13K-NF-κB to cooperate with IL-17 and produce antimicrobial peptides is still unclear. However, there is a possibility that this takes place at the level of the downstream kinase signaling pathway involving IL-22R and IL-17R, including a protein kinase activated by the mitogen as well as jun N-terminal kinase.[29]

Intranasal immunization with *S. pneumoniae* pili hemagglutinin protein (54 kDa) triggers the expression of β-defensin-2. Intranasal immunization of Wistar rats using the antigen plus the adjuvant in this study yielded the highest demonstrable expression of β-defensin-2 versus the other groups [Figure 3]. It also illustrates that the CTB adjuvant employed did not further upregulate expression but served as an immunostimulatory molecule.

β-defensins are essential antimicrobial defenses against pathogenic microbes on the skin and gastrointestinal tract mucosa, as well as the mucosa in respiration and reproduction. There are six classes of β-defensins in humans, but in the respiratory tract, just β-defensins 1–4 have been observed. β-defensin-1 is constantly expressed in the epithelium, but β-defensin 2, β-defensin-3, and β-defensin-4 are expressed through induction by various pathogens, such as bacteria, viruses, and fungi.[30]

HBD-2 has a high degree of activity as a Gram-negative bactericidal agent, able to target *Escherichia coli*,

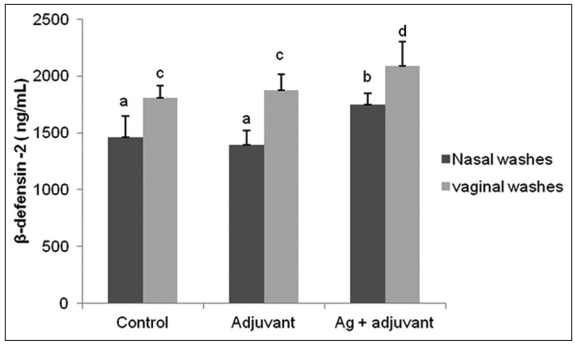

Figure 3: The concentration of β-defensin-2 of nasal and vaginal rinse of control and treatment Wistar rats

Pseudomonas aeruginosa (LD_{90}: 10 μg/mL) and the fungus *Candida albicans* (LD_{90}: 25 μg/mL). In Gram-positive bacteria, β-defensin-2 functions as a bacteriostatic against *Staphylococcus aureus* at a dose of >100 μg/mL, so it more potent against Gram-negative bacteria than bacteria that is Gram-positive.[31,32] The mechanism of β -defensin-2 as an antimicrobial is through several means: (a) The formation of holes in the membranes of microorganisms resulting in cell content leakage; (b) activating enzymes and pathways for the degradation of bacterial walls; (c) damaging the cell wall; (d) preventing attachment of bacteria to the host cell epithelium; and (e) the bacterial membrane depolarization.[31]

Besides its antimicrobial function, β-defensin-2 has selective chemotactic activity in cells expressing CCR6, specifically immature dendritic cells, and CD4CD45RO memory cells. β-defensin-2 also causes the differentiation of naïve DC into mature DC and induces DC to produce cytokines, in particular, IL-1, TNF, IL6, and IL-12, and chemokines in the form of IL-8, MDC, IP-10, and MIPI-α, and upregulates CCR7.[33]

High concentrations of β-defensin-2 in the nasal and vaginal rinses in Wistar rats immunized intranasal with the antigen plus adjuvant, when compared to the other experimental groups, showed that intranasal immunization could induce immune systems locally, or the nasal and vaginal mucosa and colorectal regions.[34] The mechanism underlying the increased expression of β-defensin-2 in mucosal immunization from a remote location is not yet clear, though it is understood to be based on B-cells homing in on the mucosal immune system to produce immunoglobulin A (IgA) antibodies.

CONCLUSION

The study demonstrated that intranasal immunization using *S. pneumoniae* pili proteins (54 kDa) were capable of increasing the expression of IL-22, IL-17A, and β-defensin-2 as reflected in nasal rinses

and vaginal washings. Therefore, *S. pneumoniae* pili proteins (54 kDa) are immunogenic and can stimulate mucosal immune responses in rats. Further work must be performed investigating humoral mucosal immune responses focusing on respiratory tract indicators, secretory IgA, and their expression.

What is known about this topic?

- *S. pneumoniae* pili contain a protein that acts as a hemagglutinin, a 54 kDa protein
- Adhesin and pili are the main targets for fighting infection and pneumococcal infections, meaning the function and immunogenicity of pneumococcal pili and adhesin are relevant to the development of a new generation of pneumococcal vaccines
- Th17 produces some cytokines, including IL-17A and IL-22, which both act synergistically to induce respiratory epithelial cells to produce antimicrobial substances, like β-defensin-2.

What this study adds?

- Intranasal immunization using *S. pneumoniae* pili proteins (54 kDa) was capable of increasing the expression of IL-22, IL-17A, and β-defensin-2 as reflected in nasal rinses and vaginal washings
- *S. pneumoniae* pili proteins (54 kDa) are immunogenic and can stimulate mucosal immune responses in rats.

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AUTHORS' CONTRIBUTIONS

Conception and design: DCM, analysis and interpretation of the data: DCM, DA, drafting of article: DCM, critical revision of the article for important intellectual content: YA, KH, SRP, SS, final approval of the article: KH, SRP, SS, administrative, technical, or logistic support: YA, collection and assembly of data: DCM.

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