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Exploring Berberine as a Novel Therapeutic Agent Against Staphylococcus aureus Infections: Targeting Inflammation and Antibiotic Resistance

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Exploring Berberine as a Novel Therapeutic Agent Against *Staphylococcus aureus* Infections: Targeting Inflammation and Antibiotic Resistance

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Abbreviations

- **AKT** - Protein Kinase B
- **APC** - Antigen-Presenting Cell
- **ARDS** - Acute Respiratory Distress Syndrome
- **ATCC** - American Type Culture Collection
- **BBR** - Berberine
- **CA-MRSA** - Community-Associated Methicillin-Resistant *Staphylococcus aureus*
- **CASP** - Caspase
- **CCL** - Chemokine (C-C motif) Ligand
- **CDK** - Cyclin-Dependent Kinase
- **CHIPS** - Chemotaxis Inhibitory Protein of Staphylococci
- **CLEC** - C-Type Lectin
- **COX** - Cyclooxygenase
- **CREB** - cAMP Response Element-Binding Protein
- **CTLD** - C-Type Lectin Domain
- **CXCL** - Chemokine (C-X-C motif) Ligand
- **CXCR** - CXC Chemokine Receptor
- **DAMP** - Damage-Associated Molecular Pattern
- **DC** - Dendritic Cell
- **ELISA** - Enzyme-Linked Immunosorbent Assay
- **HA-MRSA** - Hospital-Associated Methicillin-Resistant *Staphylococcus aureus*
- **HIF** - Hypoxia-Inducible Factor
- **HKSA** - Heat-Killed *Staphylococcus aureus*
- **IFN** - Interferon
- **IkB** - Inhibitor of kappa B
- **IL** - Interleukin
- **JNK** - c-Jun N-terminal Kinase
- **LPS** - Lipopolysaccharide
- **MAPK** - Mitogen-Activated Protein Kinase
- **MHC** - Major Histocompatibility Complex
- **MOI** - Multiplicity of Infection
- **MRSA** - Methicillin-Resistant *Staphylococcus aureus*
- **NADPH** - Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
- **NETs** - Neutrophil Extracellular Traps
- **NFkB** - Nuclear Factor kappa B
- **NK** - Natural Killer
- **NLR** - NOD-Like Receptor
- **NOX** - NADPH Oxidase
- **PAMP** - Pathogen-Associated Molecular Pattern
- **PBP2a** - Penicillin-Binding Protein 2a
- **PGD2** - Prostaglandin D2
- **PPR** - Pattern Recognition Receptor
- **PSGL** - P-Selectin Glycoprotein Ligand

- **PTGS2** - Prostaglandin-Endoperoxide Synthase 2 (also known as COX-2)
- **PVL** - Panton-Valentine Leukocidin
- **ROS** - Reactive Oxygen Species
- **SCC-mec** - Staphylococcal Cassette Chromosome mec
- **SpA** - Staphylococcal Protein A
- **TCM** - T Central Memory (T cells)
- **TCR** - T Cell Receptor
- **Th** - T Helper (cells)
- **TLR** - Toll-Like Receptor
- **TNF** - Tumor Necrosis Factor
- **TNFR** - Tumor Necrosis Factor Receptor
- **Treg** - Regulatory T (cells)
- **VRE** - Vancomycin-Resistant Enterococci
- **VRSA** - Vancomycin-Resistant *Staphylococcus aureus*

Abstract

Staphylococcus aureus is a versatile Gram-positive bacterium commonly found on human skin. While often harmless, it can cause various infections when the skin is compromised, ranging from minor issues to life-threatening conditions in immunocompromised individuals.
The bacterium's ability to resist antibiotics, particularly methicillin, underscores its clinical challenge. *Staphylococcus aureus* can also acquire vancomycin resistance. It evades immune responses through multiple mechanisms, including capsule formation, interference with immune proteins like staphylococcal protein A, and secretion of toxins such as Panton-Valentine Leukocidin. Understanding these mechanisms is crucial for developing effective strategies against *Staphylococcus aureus* infections.

The immune response to *Staphylococcus aureus* infections is orchestrated through a complex interplay of innate and adaptive immune mechanisms. Upon encountering *Staphylococcus aureus*, innate immune cells such as neutrophils, monocytes/macrophages, dendritic cells, and natural killer cells are mobilized to the infection site. Neutrophils play a crucial role in the initial defense by phagocytosing bacteria and releasing antimicrobial peptides and reactive oxygen species through an oxidative burst. Monocytes differentiate into macrophages that engulf bacteria and secrete cytokines and chemokines to recruit and activate other immune cells. Dendritic cells process and present *Staphylococcus aureus* antigens to T cells, initiating adaptive immune responses. T cells, including CD4+ and CD8+ subsets, differentiate into various effector and memory cells to coordinate long-term immune defense. B cells produce antibodies that neutralize toxins and facilitate bacterial clearance. The inflammatory cytokines IL-1β, IL-6, TNF-α, and IFN-γ orchestrate immune responses, regulating inflammation and coordinating immune cell communication. These immune components form a robust defense

system against *Staphylococcus aureus,* although the bacterium's ability to evade immune detection poses challenges in treatment and vaccine development.

In this study, berberine, a natural compound derived from plants like goldenseal and barberry, was explored for its potential as an inhibitor of *Staphylococcus aureus* mediated inflammation. Known for its broad antimicrobial properties and therapeutic benefits in traditional medicine systems such as Traditional Chinese Medicine and Ayurveda, berberine has been extensively studied for its ability to combat various infections and inflammatory conditions without causing liver damage. It acts by disrupting bacterial cell membranes, inhibiting biofilm formation, and reducing virulence factors, making it a promising candidate against antibiotic-resistant strains like methicillin-resistant *Staphylococcus aureus*. Furthermore, berberine exhibits potent anti-inflammatory effects by modulating cytokine production and inflammatory pathways, which could mitigate excessive inflammation during *Staphylococcus aureus* infections.

We hypothesized that berberine can significantly inhibit TNF-α production, gene expression involved in inflammatory pathways, and reactive-oxygen species production in response to *Staphylococcus aureus* stimulation in both mouse macrophage and human monocyte cell lines. The results demonstrated that berberine effectively inhibited TNF-α production in RAW 264.7 mouse macrophage and U937 human monocyte cell lines when stimulated with heat-killed *Staphylococcus aureus* (ATCC strain #33591). This inhibition was dose-dependent, with 20 μg/mL of berberine suppressing over 90% of TNF-α production compared to controls ($p < 0.001$), highlighting its robust anti-inflammatory activity without inducing cytotoxic effects.

Moreover, when tested against clinical isolates of *Staphylococcus aureus* (EC01-EC04) isolated from the skin of eczema patients, berberine at concentrations ranging from 2.5 to 20 μg/mL

consistently inhibited TNF-α production by over 90% ($p < 0.001$) in U937 cells, reinforcing its potential clinical relevance. Berberine also significantly downregulated key genes involved in inflammatory pathways in U937 cells and inhibited reactive oxygen species production in both RAW 264.7 and U937 cells at 20 μg/mL ($p < 0.01$), indicating its capacity to reduce oxidative stress associated with bacterial infections.

Overall, these results suggest that berberine possesses dual therapeutic actions against *Staphylococcus aureus* infections by suppressing inflammatory responses and oxidative stress. This study provides a strong foundation for further exploration of berberine's clinical applications, potentially as an adjunct therapy alongside conventional antibiotics, addressing critical clinical needs in infectious and inflammatory diseases.

1. Introduction

1.1. *Staphylococcus aureus*

1.1.1. Overview of *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, facultative anaerobe commonly found on the skin, of approximately 30% of people, especially in warm, humid areas, where it usually exists as a commensal organism without causing any symptoms in about 30% (Chen et al., 2022). However, when the skin barrier is compromised*, S. aureus* can exploit this opportunity to colonize the area and turn pathogenic. The range of infections caused by *S. aureus* varies widely, from minor skin infections to chronic wounds and even severe, life-threatening diseases. If the immune system fails to eliminate the initial infection, S. aureus can spread to other tissues (Chen et al., 2022; Goldmann & Medina, 2018), potentially leading to sepsis and death. This makes *S. aureus* infections particularly concerning for individuals who are immunocompromised (Luo et al., 2024), diabetic, elderly, pediatric, or have chronic skin conditions.

1.1.2. Antibiotic Resistance in *S. aureus*: A Member of the ESKAPE Pathogens

The *S. aureus* species show extensive genetic variation and is associated with increasing bacterial resistance to antibiotics due to its plasticity and rapid co-evolutionary (Chen et al., 2022) adaptability. Nearly 18,000 different strains of *S. aureus* have been identified worldwide through molecular typing. *S. aureus* is included in a group of bacteria known as the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species) (Luo et al., 2024) involved in infections that are characterized by multidrug resistance. Of these, some *S. aureus* strains have developed mechanisms of resistance to the most antimicrobial drugs commonly used to treat

Gram-positive infections including beta-lactams, glycopeptides, and oxazolidinones (Chen et al., 2022).

1.1.3. Emergence and Evolution of Methicillin-resistant *Staphylococcus aureus*

Methicillin-resistant *S. aureus* (MRSA) is among the most formidable pathogens in contemporary medicine. The initial MRSA strains emerged in the 1960s (Mlynarczyk-Bonikowska et al., 2022), distinguished by their resistance to all β-lactam antibiotics available at that time. This resistance arose through the independent acquisition of the staphylococcal cassette chromosome mec (SCCmec) complex by various multidrug-resistant strains of S. aureus (Lee et al., 2018; Stogios & Savchenko, 2020).

1.1.3.1. SCCmec Types and Resistance Mechanisms

There are 12 known types of SCCmec (I–XII), classified based on their cassette chromosome recombinase (ccr) complex and mec complex class. Types I, II, and III are larger elements that include genes conferring resistance to multiple antibiotics, predominantly found in hospital-associated MRSA (HA-MRSA). In contrast, smaller elements like types IV and V are typically associated with community-associated MRSA (CA-MRSA) and are also present in some HA-MRSA clones. Over time, the distinction between HA-MRSA and CA-MRSA has become less clear (Lee et al., 2018).

All SCCmec types contain the mecA gene, except for type XI, which carries mecC. The mecA gene encodes penicillin-binding protein 2a (PBP2a) (Lee et al., 2018; Mlynarczyk-Bonikowska et al., 2022), a peptidoglycan transpeptidase with low affinity for most β-lactam antibiotics (Lee et al., 2018). In the presence of β-lactams, which inhibit the native penicillin-binding proteins of *S. aureus*, PBP2a can continue peptidoglycan biosynthesis. Additionally, a variant of mecA, known as mecC, has been identified in various animal and human isolates, encoding a similar protein

called PBP2aLGA (Lee et al., 2018). In 2018, plasmid-borne methicillin resistance based on mecB (Lee et al., 2018) was discovered, although its resistance mechanism remains unclear.

1.1.3.2. Regulation of mecA and Resistance Phenotype

The expression of mecA is regulated by the genes mecI, mecR1, and mecR2, as well as blaZ, blaI, and blaRI. Moreover, numerous auxiliary or fem genes play a crucial role in shaping the resistance phenotype (Lee et al., 2018). Research suggests that the level of mecA transcription does not directly correlate with the degree of methicillin resistance. For instance, the stringent stress response, triggered by various stress conditions such as the antibiotic mupirocin, can enhance PBP2a activity without affecting mecA transcription. Additionally, inactivation of the VraS protein, part of a two-component regulatory system, can lead to mecA transcription without increasing PBP2a activity. The chaperone foldase protein PrsA also influences methicillin resistance by modifying the levels of properly folded PBP2a in the membrane, independently of mecA transcription (Lee et al., 2018).

1.1.4. Acquired Vancomycin Resistance

Vancomycin resistance in vancomycin-resistant enterococci (VRE) is mediated by the vanA operon (Mlynarczyk-Bonikowska et al., 2022; Stogios & Savchenko, 2020; Vo et al., 2024), which produces a modified peptidoglycan precursor that vancomycin cannot effectively bind, thereby diminishing the antibiotic's efficacy. This operon is carried by the transposon Tn1546 on conjugative plasmids, facilitating its spread among enterococci and leading to outbreaks of VRE infections. The mobility of vanA also raises the risk of transferring vancomycin resistance to other Gram-positive bacteria, including *S. aureus* (Vo et al., 2024).

The acquisition of vanA by *S. aureus* through mechanisms such as conjugation, transposition, and plasmid integration (Mlynarczyk-Bonikowska et al., 2022; Stogios & Savchenko, 2020) presents a significant clinical and epidemiological threat. Despite the relatively low number of vancomycin-resistant *S. aureus* (VRSA) cases, the potential for widespread dissemination of resistance genes remains high due to genetic exchange via conjugation and the presence of recombination hotspots in the *S. aureus* genome (Vo et al., 2024).

This underscores the importance of monitoring methicillin sensitivity in *S. aureus* strains from patients co-infected with VRE. The findings emphasize the need for strengthened surveillance and comprehensive laboratory monitoring to manage the risk of VRSA (Vo et al., 2024) and the spread of multidrug-resistant genes. A deeper understanding of the mechanisms behind vanA acquisition and integration will be crucial in developing strategies to mitigate the spread of vancomycin resistance in *S. aureus*.

1.1.5. Factors That Protect from Host Immune System

1.1.5.1. Capsule

The most effective strategy for a bacterium to evade phagocytosis is by masking its antigenic or immunogenic cell wall proteins with a polysaccharide capsule. About 75% of clinical *S. aureus* isolates have either a capsule or microcapsule, mainly featuring capsular polysaccharide 5 (CP5) or capsular polysaccharide 8 (CP8) (de Jong et al., 2019). When grown under conditions that favor capsule production, these bacteria show resistance to opsonophagocytosis and subsequent killing. However, the *S. aureus* capsule does not completely prevent the deposition of complement components on the bacterial surface or the binding of specific antibodies (de Jong et

al., 2019). In contrast, other bacteria, like Streptococcus pneumoniae, produce a much thicker capsule (Goldmann & Medina, 2018).

1.1.5.2. SpA

S. aureus uses staphylococcal protein A (SpA) to disrupt protective humoral immune responses by polyclonally activating B cells. Most *S. aureus* strains express SpA, which binds to immunoglobulins, targeting both the Fcγ and Fab regions of IgG and IgM (de Jong et al., 2019; Goldmann & Medina, 2018). Binding to the Fcγ region inhibits the killing of *S. aureus*, while binding to the Fab region activates and expands B cells, especially those of the VH3-family idiotypes. This superantigenic effect limits the host's response to other *S. aureus* factors (Goldmann & Medina, 2018; Rossi et al., 2014).

SpA forms a complex with human Fab via a surface on B-cell antigen receptors, inducing VH3-biased plasmablast responses against SpA and inhibiting responses to other *S. aureus* factors (Goldmann & Medina, 2018; Rossi et al., 2014). In murine B cells, SpA binding down- regulates receptors, leading to cell death and impeding memory generation. To counteract SpA's effects, a non-toxigenic SpA variant, SpAKKAA, was developed. This variant elicited neutralizing antibodies against SpA, promoting immune responses to other staphylococcal antigens and conferring protective immunity against *S. aureus*, suggesting SpAKKAA as a potential vaccine candidate (Goldmann & Medina, 2018).

1.1.5.3. Countering Oxygen Dependent Killing

S. aureus is named for its golden staphyloxanthin "jacket," which acts as an antioxidant, protecting the bacterium from hydrogen peroxide and singlet oxygen. Mutants lacking

staphyloxanthin demonstrate reduced survival in laboratory and animal studies. The genes crtM and crtN are essential for staphyloxanthin production, making CrtM inhibitors a potential therapeutic target for this virulence factor (Goldmann & Medina, 2018).

S. aureus also produces superoxide dismutases, sodA and sodM, which convert superoxide radicals into less harmful substances. Knockout strains that lack these enzymes show decreased virulence in mice. While enzymes like KatA degrade hydrogen peroxide produced by neutrophils (Vorobjeva & Chernyak, 2020), their exact role in virulence remains debated. Another enzyme, AhpC, detoxifies internally produced hydrogen peroxide and aids in nasal colonization (Goldmann & Medina, 2018).

To counteract nitric oxide stress from immune cells, *S. aureus* uses flavohemoglobin to scavenge nitric oxide and l-lactate dehydrogenase to maintain redox balance by producing l-lactate. These adaptations highlight strategies of *S. aureus* to evade immune responses (Goldmann & Medina, 2018) and thrive within the host.

1.1.5.4. Neutrophil Extracellular Trap Evasion

The secreted nuclease Nuc from *S. aureus* plays a crucial role in the breakdown of neutrophil extracellular traps (NETs). Both in vitro and in vivo studies indicate that an isogenic strain of S. *aureus* lacking Nuc has a diminished capacity to degrade NETs compared to the wild-type strain. This impairment leads to slower clearance of bacteria in the lungs and increased mortality following intranasal infection in vivo. As a result, Nuc enables staphylococci to evade entrapment by extracellular fibers, thus evading destruction by antimicrobial peptides and proteases. Additionally, Nuc, in collaboration with secreted adenosine synthase, converts NETs

into deoxyadenosine, which induces caspase-3-mediated cell death in immune cells (de Jong et al., 2019).

1.1.5.5. Superantigens

Superantigens represent a significant group of secreted virulence factors (Goldmann & Medina, 2018) produced by *S. aureus*. These proteins induce nonspecific activation of T lymphocytes by binding directly to major histocompatibility complex (MHC) class II molecules and T-cell receptors (TCRs) outside the antigen-binding cleft. This interaction triggers an uncontrolled release of cytokines (Bestebroer et al., 2007), leading to an exaggerated immune response that can result in severe inflammation.

Antigen-presenting cells (APCs), such as macrophages and dendritic cells, typically play a vital role in immune responses by engulfing and processing bacteria or antigens for presentation to antigen-specific T lymphocytes via MHC molecules in endocytic compartments. However, *S. aureus* superantigens bypass this conventional process by directly interacting with MHC class II molecules and TCRs, activating a substantial proportion—up to 20%—of T cells (Bestebroer et al., 2007). Notable superantigens associated with human diseases include toxic shock syndrome toxin 1 (TSST-1) and staphylococcal enterotoxins (Ses), which can lead to toxic shock syndrome (TSS) characterized by severe inflammation and tissue damage (Bestebroer et al., 2007) due to the excessive release of cytokines like interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor α (TNF-α).

Additionally, *S. aureus* secretes other virulence factors such as chemotaxis inhibitory protein (CHIPS) and superantigen-like proteins such as SSL5 and SSL7 (Bestebroer et al., 2007), which

contribute to immune evasion. CHIPS hinders neutrophil responses by binding to receptors critical for neutrophil activation and migration. SSL5 inhibits neutrophil extravasation into inflamed tissues by disrupting the rolling of leukocytes on vascular endothelium, a process facilitated by P-selectin glycoprotein ligand-1 (PSGL-1) (Bestebroer et al., 2007). These interactions illustrate sophisticated mechanisms through which *S. aureus* evades the host immune system, enhancing its pathogenicity.

1.1.5.6. Panton-Valentine Leukocidin

Panton-Valentine Leukocidin (PVL) is a toxin that forms pores in neutrophil membranes, leading to cell lysis (Goldmann & Medina, 2018). While research suggests PVL plays a role in the disease process, its impact on clinical outcomes remains unclear. Globally, PVL has been associated with community-onset MRSA, although not all CA MRSA strains carry PVL genes, particularly in Australia (Luo et al., 2024). Historical data suggest that current MRSA strains may be traced back to a highly virulent PVL-producing clone from the 1950s and 1960s (Jhelum et al., 2024; Lee et al., 2018). Despite PVL's known ability to cause dermonecrosis in rabbits and its leukocidal properties, there is limited experimental evidence demonstrating that PVL directly causes invasive disease in animal models (Jhelum et al., 2024).

The prevalence and genetic diversity of community-onset staphylococcal disease vary significantly by region. In the USA, the PVL-producing MRSA clone ST8/USA300 is endemic and primarily responsible for these infections, which have shifted from being community-acquired to healthcare-associated. In contrast, Europe exhibits rare occurrences of USA300, with community-onset MRSA displaying greater diversity, including many PVL-positive methicillin-sensitive strains. In specific European regions like Greece, PVL-positive MRSA is prevalent in

both community and hospital settings. Australia demonstrates distinct patterns where both MRSA and methicillin-sensitive *S. aureus* (MSSA) clones carry PVL genes, but USA300 is infrequent. Despite regional disparities, clinical presentations of community-onset staphylococcal disease such as abscesses and skin infections are globally similar, with some cases progressing to severe conditions like necrotizing pneumonia, sepsis, and musculoskeletal disease (Jhelum et al., 2024).

There exists an association between PVL genes and invasive disease, suggesting PVL serves as a marker for severe infections, prompting certain countries to implement public health measures targeting PVL-positive cases. While aggressive strategies such as screening and decolonization may be warranted if PVL-positive disease is rare and severe, evidence supporting their effectiveness in community-onset cases is limited. Decolonization efforts in intensive-care units have shown cost-effectiveness and health benefits, yet randomized trials have provided scant evidence on reducing recurrence of community-onset *S. aureus* skin infections, and no studies have specifically evaluated the cost-effectiveness of controlling PVL-positive infections (Shallcross et al., 2013).

1.2. *Immune response in association with S. aureus infections*

Upon pathogen invasion, the host's immune response involves the production and release of cytokines, which are proteins mainly produced by white blood cells in response to pathogens. These cytokines are crucial for defending against infections, and their absence can be harmful. However, excessive production can lead to severe side effects, including multiple organ dysfunction and death (Fournier & Philpott, 2005).

1.2.1. Innate Immunity

1.2.1.1. Neutrophils

Neutrophils, constituting about 60% of leukocytes in the bloodstream, are essential for the initial defense against pathogens and play a critical role in controlling *S. aureus* infections. Individuals with neutrophil deficiencies exhibit heightened susceptibility to *S. aureus*, underscoring the importance of these cells. Neutrophils rapidly migrate to infection sites, where they clear pathogens and debris, and interact with various immune cells to influence inflammatory responses. Recent studies also highlight the significant involvement of liver Kupffer cells in *S. aureus* infection, responsible for pathogen clearance in the liver, acting as a critical initial barrier to prevent the spread of infection to other organs (Leliefeld et al., 2015).

At inflammation sites, neutrophils engage with lymphocytes and antigen-presenting cells (APCs) through direct cell contact or mediators like proteases, cytokines, and reactive oxygen species (ROS). This interaction not only helps clear infections but also orchestrates adaptive immunity. Neutrophils can travel to draining lymph nodes (LNs), where they influence early T-cell responses and interact with B-cells to modulate humoral responses (Leliefeld et al., 2015; Riaz & Sohn, 2023). Despite their critical role, *S. aureus* has developed numerous strategies to evade neutrophil defenses, including hindering extravasation, activation, chemotaxis, phagocytosis, and opsonization, as well as directly killing neutrophils through cytolytic toxins or inducing apoptosis. These evasion tactics present significant challenges in developing an effective vaccine against *S. aureus* (Spaan et al., 2013).

Furthermore, neutrophils, previously considered a homogeneous population, exhibit multiple phenotypes influenced by differentiation programs in the bone marrow or signals from inflammatory tissues. This heterogeneity plays a role in various diseases, including cancer and

infections. Neutrophils' ability to affect antigen presentation by APCs or function as APCs themselves adds complexity to their role in immune regulation. Their dynamic interactions with lymphocytes and other immune cells at inflammation sites and in LNs are pivotal in modulating both innate and adaptive immune responses (Soler-Rodriguez et al., 2000), highlighting their multifaceted contributions to immune defense and regulation.

1.2.1.2. Monocytes and Macrophages

Monocytes and macrophages are crucial elements of the innate immune system, especially important in bacterial sepsis. These cells produce inflammatory cytokines like TNF-α, IL-1β, and IL-6, which are key players in the onset of septic shock. IL-10 acts as an anti-inflammatory mediator, inhibiting these proinflammatory cytokines. Additionally, IL-8 attracts neutrophils to infection sites, while MCP-1 and MIP-1 attract monocytes (Pidwill et al., 2020).

Infections caused by Gram-positive bacteria, such as *S. aureus*, induce systemic cytokine responses that peak around 50-75 hours after infection onset, in contrast to the rapid 1–5-hour response seen in Gram-negative infections. During sepsis, TNF-α, IL-1β, and IL-6 are primarily produced by blood mononuclear cells and tissue macrophages, with increased levels of IL-12 as well. IL-8 production is partially stimulated by TNF-α, while IFN-γ production is influenced by IL-12 and IL-8. Although Gram-positive infections generally induce lower cytokine levels than Gram-negative infections, both types result in similar shock patterns in the host (Fournier & Philpott, 2005).

Macrophages and neutrophils, derived from myeloid precursor cells, serve as professional phagocytes essential to innate immunity. They share core functions such as sensing and migrating towards infection sites to engulf pathogens. Macrophages, however, also function as APCs, initiating adaptive immune responses by presenting antigens to T cells. Unlike neutrophils, which respond quickly at infection sites, monocytes (which differentiate into macrophages) arrive later. Macrophages are long-lived and strategically positioned throughout tissues, contributing to tissue development, homeostasis, and immune surveillance.

Tissue-resident macrophages come from diverse developmental origins: some arise from circulating monocytes, while others originate from embryonic precursors that colonize tissues early in development. These macrophage populations exhibit distinct transcriptional profiles tailored to their tissue locations while maintaining fundamental roles in phagocytosis and immune regulation. Examples include Kupffer cells in the liver and Langerhans cells in the skin, each fulfilling unique roles specific to their respective tissues (Pidwill et al., 2020).

During *S. aureus* infections, macrophages play a pivotal role in the initial immune response by engulfing bacteria. However, some *S. aureus* bacteria may survive intracellularly, leading to abscess formation and dissemination to distant sites. This interaction between macrophages and *S. aureus* involves complex mechanisms including pathogen recognition and cytokine signaling mediated by receptors such as scavenger receptors, complement receptors, and Fc receptors (Fournier & Philpott, 2005).

1.2.1.3. Dendritic Cells

Dendritic cells (DCs) are pivotal in activating adaptive immunity, strategically located at epithelial surfaces where they process and present antigens to T cells via MHC molecules. Recent studies highlight the crucial role of DCs in regulating anti-staphylococcal T cell responses, though our understanding of DC interactions with *S. aureus*, particularly in human models, remains limited (Balraadjsing et al., 2019; Karauzum & Datta, 2017).

In mouse models of *S. aureus* bloodstream infection, DCs contribute to controlling the infection by producing interleukin-12 (IL-12), which promotes Th1 cell responses. Depletion of DCs significantly impairs bacterial clearance from the lungs and kidneys. *S. aureus* has evolved mechanisms to evade or modulate DC and T cell responses, such as deploying superantigens to non-specifically stimulate T cells and DCs, leading to excessive pro-inflammatory responses that can result in shock and death. Moreover, *S. aureus* can evade phagocytic killing and persist within various phagocytic cells, including mouse DCs (Karauzum & Datta, 2017).

1.2.1.4. Natural Killer Cells

Natural killer (NK) cells were initially recognized as large granular lymphocytes with the primary function of spontaneously killing tumor cells. Further research has revealed them to be a distinct lineage of lymphocytes capable not only of cytotoxicity but also of cytokine production. Over time, NK cells have evolved sophisticated mechanisms to regulate cytolytic activity, preventing unnecessary tissue damage. Recent studies have emphasized understanding how NK cells differentiate between target cells and healthy ‘self’ cells, introducing the concept of dynamic equilibrium. This equilibrium is maintained by a complex interplay of activating and

inhibitory receptors on the NK cell surface, which determine whether NK cells will be activated to eliminate target cells (Vivier et al., 2008).

NK cell maturation involves a process called NK cell 'education,' where they recognize self-molecules, such as MHC class I molecules, under normal conditions. This process engages inhibitory receptors like Ly49 in mice and KIRs in humans, underscoring the adaptive nature of NK cells. This adaptability allows NK cells to modulate their responses dynamically in different environmental contexts (Genardi et al., 2020; Vivier et al., 2008).

Regarding natural killer T (NKT) cells, especially type II NKT cells, they play a crucial role in the immune response against *S. aureus* infection. Both invariant (iNKT) and type II NKT cells are activated during *S. aureus* bacteremia, but only type II NKT cells are essential for reducing bacterial load and limiting neutrophil infiltration in the liver and kidneys. Type II NKT cells recognize polar lipids derived from *S. aureus*, leading to IFN-γ production through TCR-CD1d engagement and TLR-mediated activation. Adoptive transfer experiments with type II NKT cells from transgenic mice have confirmed their ability to reduce *S. aureus* burden in the spleen, correlating with IFN-γ production. Additionally, an expansion of CD161+CD4+ T cells, including type II NKT cells, has been observed in *S. aureus* bacteremic patients, highlighting the clinical relevance of these findings (Genardi et al., 2020).

1.2.2. Adaptive Immunity

1.2.2.1. T cells

T cells, originating from the thymus, possess unique receptors (TCRs) that recognize peptides presented by MHC molecules on antigen-presenting cells (APCs). Evidence shows that T cells

are involved in *S. aureus* infections, as indicated by detectable T cell responses in humans and the bacteria's influence on T cells through superantigens. However, T cells are not essential for protection against *S. aureus* in mice, and humans with T cell deficiencies rarely suffer from *S. aureus* infections.

Most T cells are either CD4+ or CD8+. CD8+ T cells target intracellular pathogens, which is less relevant for the primarily extracellular *S. aureus*. CD4+ T cells, on the other hand, differentiate into various subsets based on the cytokine environment, contributing to immune memory and responses. These subsets, including Th1, Th2, and Th17 cells, play distinct roles in *S. aureus* infections.

Th1 cells, activated by IL-12 and expressing IFN-γ, can be both protective and harmful in *S. aureus* infections. They enhance bacterial clearance but can also cause immunopathology. Th2 cells, driven by IL-4, are involved in fighting extracellular parasites but can exacerbate conditions like atopic dermatitis, which is often associated with *S. aureus*.

Th17 cells, characterized by IL-17 production, enhance epithelial barrier functions and recruit neutrophils, providing protection against mucocutaneous *S. aureus* infections. However, their exact role in human staphylococcal immunity remains unclear, although deficiencies in Th17 pathways are linked to increased susceptibility to infections.

Regulatory T cells (Tregs) help maintain immune balance by suppressing inflammation. *S. aureus* may exploit Tregs to diminish effective T cell responses during chronic infections. Maintaining the right balance of Treg activity is crucial for controlling infection without causing excessive inflammation (Karauzum & Datta, 2017).

1.2.2.2. B cells

B cells are essential for secreting antibodies (immunoglobulins) that neutralize toxins and promote pathogen clearance through phagocytosis. The critical role of antibodies in defending against infections is evident in their ability to enhance immune responses against various viruses and encapsulated bacteria, especially in patients with impaired B cell maturation. This vulnerability can be effectively managed with periodic administration of pooled donor immunoglobulins.

Recent studies suggest that antibodies may not be indispensable for defense against *S. aureus* infections in individuals lacking B cells or antibodies, as their susceptibility appears comparable to those with intact immune systems. However, research indicates that primary cutaneous *S. aureus* infections can elicit antibody-mediated protection against future infections under certain circumstances. Antibodies facilitate efficient phagocytosis by opsonizing bacterial targets via their Fab segments and interacting with phagocytes through their Fc regions. IgM, due to its polymeric structure, is particularly effective in this process.

In Gram-positive bacterial infections like those caused by *S. aureus*, antibodies on bacterial surfaces activate the classical complement pathway, leading to the deposition of C3b on bacterial surfaces. This activation involves C3 convertases from the lectin, classical, or alternative pathways. Additionally, complement factors such as C5a, produced during the C3-C3b interaction, serve as chemoattractants for immune cells, playing a crucial role in immune responses during systemic *S. aureus* infections (Karauzum & Datta, 2017).

1.2.3. Pattern Recognition Receptors

1.2.3.1. Toll-like Receptors

Toll-like receptors (TLRs) are pivotal receptors in the innate immune system, crucial for sensing pathogen-associated molecular patterns (PAMPs) from pathogens and damage-associated molecular patterns (DAMPs) from damaged cells (Kawasaki & Kawai, 2014). They are expressed widely across immune and non-immune cells, initiating immune responses upon activation. TLR activation leads to the production of cytokines and interferons essential for combating infections, though dysregulation can cause harmful inflammatory responses (Kawasaki & Kawai, 2014).

In humans, TLR1-TLR10 recognize diverse PAMPs through their leucine-rich repeat (LRR) domains, facilitating interactions with bacterial lipoproteins, nucleic acids, and other microbial components. Ligand binding induces TLR dimerization, crucial for downstream signaling via adaptor proteins like MyD88 and TRIF, activating NF-κB and MAP kinase pathways to promote immune gene expression (Kawasaki & Kawai, 2014; Wicherska-Pawłowska et al., 2021).

S. aureus, a pathogen detected by multiple TLRs, triggers robust immune responses including the release of proinflammatory mediators and antimicrobial peptides. However, *S. aureus* employs several evasion strategies against TLR2 activation. These include inhibiting heterodimer formation (e.g., SSL3 protein), mimicking TLR signaling domains (e.g., TirS), and activating inhibitory receptors (e.g., PIR-B). These mechanisms allow *S. aureus* to evade immune detection and promote infection, highlighting the intricate interplay between pathogen evasion strategies and host immune response modulation. Understanding these interactions is crucial for developing targeted therapies against staphylococcal infections

(Askarian et al., 2018).

1.2.3.2. NOD-like Receptors

NOD-like receptors (NLRs) are vital elements of the innate immune system, serving as intracellular sensors that detect microbial infections and cellular stress. Upon recognizing specific PAMPs or DAMPs, NLRs initiate various signaling pathways, including NF-κB and mitogen-activated protein kinase (MAPK) pathways, and can form inflammasomes. Inflammasomes are multiprotein complexes that activate caspase-1, which leads to the maturation and secretion of pro-inflammatory cytokines such as IL-1β and IL-18, and induces pyroptosis, a form of programmed cell death (Almeida-da-Silva et al., 2023; Franchi et al., 2009).

In the context of *S. aureus*, NLRs are essential in orchestrating an immune response. NLRs like NOD2 recognize muramyl dipeptide, a component of bacterial cell walls, triggering inflammatory pathways and activating the body's defense mechanisms against the infection. Activation of NLRs by *S. aureus* can lead to the formation of inflammasomes, promoting cytokine secretion and recruiting immune cells to the infection site (Franchi et al., 2009). This contributes to the inflammatory response aimed at controlling and eliminating the pathogen.

1.2.3.3. C-type Lectin Receptors

The C-type lectin receptors (CLR) superfamily, which consists of a diverse group of PRRs, has been categorized into 17 sub-groups based on their similarities in evolutionary history and structural features. A key feature of CLEC receptors is their possession of a C- type

lectin-like domain (CTLD), which typically requires calcium ions to efficiently bind glycans. However, some receptors within the C-type lectin family, known as C-type lectin-like receptors (CTLRs), have a CTLD but do not rely on calcium ions for ligand recognition. This distinction between CLECs and CTLRs can sometimes be unclear, leading to varying classifications over time as our knowledge advances.

Because CTLRs do not depend on calcium ions, they can recognize a wide range of ligands, including lipids and proteins, in addition to carbohydrates, through their unique carbohydrate-binding domains (CBD). While CLECs primarily recognize carbohydrates, CTLRs broaden their ability to detect other types of molecules beyond these traditional targets. Both CLECs and CTLRs act as critical PRRs on immune cells derived from the myeloid lineage, detecting various PAMPs to initiate signaling pathways that stimulate immune responses. Some receptors are classified as collectins or selectins, while others have distinct roles or exhibit specific patterns of expression that make classification challenging (Scur et al., 2023).

In the context of microbial infections, such as those caused by *S. aureus*, these receptors play a crucial role in detecting bacterial components and triggering immune responses. *S. aureus* is a significant human pathogen that colonizes mucosal surfaces and causes serious infections like bacteremia, pneumonia, and skin infections. During infection, the bacterium adjusts the expression of its virulence genes, complicating treatment due to antibiotic resistance and inducing severe inflammatory reactions. *S. aureus* employs various strategies to evade immune detection, including interference with PRR recognition, inhibition of complement activation, and hindrance of neutrophil recruitment. Understanding these evasion tactics is crucial for developing effective therapies against staphylococcal infections and managing the

associated inflammatory complications (Scur et al., 2023; Wicherska-Pawłowska et al., 2021).

1.2.4. Kinase Signal Transducers

1.2.4.1. Mitogen-activated Protein Kinase 1

Mitogen-activated protein kinase 1 (MAPK1), also known as ERK2, plays a pivotal role in the MAPK signaling cascades, which convert extracellular signals into cellular responses. These cascades involve a sequence of phosphorylation events carried out by three types of kinases: MAPK kinase kinases (MAPKKKs), MAPK kinases (MAPKKs), and MAPKs. In mammalian cells, at least 14 MAPKKKs, 7 MAPKKs, and 12 MAPKs have been identified.

MAPK pathways, including the classical ERK pathway, relay and integrate signals from various stimuli, triggering responses such as cellular proliferation, differentiation, development, inflammatory responses, and apoptosis. The ERK pathway is activated through receptor tyrosine kinases (RTKs) and involves key proteins such as Grb2, Sos, Ras, and a kinase cascade comprising c-Raf-1 (MAPKKK), MEK1/2 (MAPKK), and ERK1/2 (MAPKs).

Once activated, ERK1/2 translocates to the nucleus, where it phosphorylates transcription factors such as Elk-1, c-Fos, and CREB. This phosphorylation alters gene expression to promote growth and mitosis. ERK signaling is crucial for cell cycle progression, particularly the transition from G1 to S phase, by regulating cyclin D1 expression and the activity of cyclin-dependent kinases (Cdks).

In addition to its role in cell proliferation, ERK1/2 is involved in inflammatory responses by phosphorylating substrates like RSK, cytosolic phospholipase A2, and microtubule-associated

proteins. These phosphorylation events influence the assembly of cytosolic microtubules and the mitotic spindle, which are critical for cell division and inflammatory responses. ERK signaling can also be activated by GPCRs and cytokine receptors through mechanisms involving scaffold proteins like integrins and β-arrestin. Crosstalk between ERK and other MAPKs, such as JNK and p38, further modulates cellular responses to inflammation and stress. For example, VEGF-induced ERK activation leads to JNK activation, which is essential for cell proliferation and the G1/S phase transition.

The ERK pathway regulates cell cycle regulators like Cdk2 and Cyclin E, influencing their nuclear translocation and activity. ERK can also promote the degradation of cell cycle inhibitors such as p27^kip1 via the ubiquitin-proteasome pathway, thus promoting cell cycle progression and proliferation.

MAPK1 (ERK2) is central to the MAPK signaling cascades that govern inflammation and cellular responses. Through its complex interactions and regulatory mechanisms, ERK2 modulates processes ranging from cell proliferation and differentiation to inflammatory responses, underscoring its importance in both normal physiology and pathological conditions (Zhang & Liu, 2002).

1.2.4.2. Mitogen-activated Protein Kinase 3

Mitogen-activated protein kinase 3 (MAPK3), also known as ERK1, is a crucial player in the MAPK signaling pathways, which convert extracellular signals into a variety of cellular responses. These pathways involve a series of phosphorylation events carried out by three levels of kinases: MAPKKKs, MAPKKs, and MAPKs. In mammals, a wide array of MAPKKKs, MAPKKs, and MAPKs have been identified, highlighting the complexity and adaptability of

these signaling networks.

MAPK pathways, including the classical ERK pathway, transmit and amplify signals from various stimuli, resulting in responses such as cellular proliferation, differentiation, development, inflammatory responses, and apoptosis. The ERK pathway is initiated through receptor tyrosine kinases (RTKs) and involves key intermediates like Grb2, Sos, Ras, and a kinase cascade consisting of c-Raf-1 (MAPKKK), MEK1/2 (MAPKK), and ERK1/2 (MAPKs).

Once activated, ERK1 translocates to the nucleus, where it phosphorylates transcription factors such as Elk-1, c-Fos, and CREB. This leads to changes in gene expression that promote growth and cell cycle progression. This pathway is essential for cell cycle regulation, particularly the transition from G1 to S phase, by modulating the expression and activity of cyclin D1 and Cdks.

ERK1 also plays a significant role in inflammatory responses by phosphorylating substrates like RSK, cytosolic phospholipase A2, and microtubule-associated proteins. These phosphorylation events affect the assembly of cytosolic microtubules and the mitotic spindle, which are crucial for cell division and inflammatory processes.

ERK signaling can be initiated by GPCRs and cytokine receptors through mechanisms involving scaffold proteins such as integrins and β-arrestin. Crosstalk between ERK and other MAPKs, including JNK and p38, further refines cellular responses to inflammation and stress. For instance, VEGF-induced ERK activation leads to JNK activation, which is essential for cell proliferation and G1/S phase transition.

The ERK pathway is pivotal in controlling cell cycle regulators such as Cdk2 and Cyclin E,

influencing their nuclear translocation and activity. ERK can also regulate the degradation of cell cycle inhibitors like p27^kip1 via the ubiquitin-proteasome pathway, thereby promoting cell cycle progression and proliferation.

MAPK3 (ERK1) is integral to the MAPK signaling cascades that regulate inflammation and cellular responses. Through its intricate interactions and regulatory mechanisms, ERK1 influences processes ranging from cell proliferation and differentiation to inflammatory responses, underscoring its vital role in both normal physiology and disease states (Zhang & Liu, 2002).

1.2.4.3. Mitogen-activated Protein Kinase 14

Mitogen-activated protein kinase 14 (MAPK14), also known as p38α, is a critical component of the MAPK signaling pathways that mediate cellular responses to stress and inflammation. MAPK14 belongs to the p38 MAPK family, which includes several isoforms activated by environmental stress, pro-inflammatory cytokines, and other stimuli.

MAPK14 operates within the MAPK cascades, which include three tiers of kinases: MAPKKKs, MAPKKs, and MAPKs. This signaling pathway begins with the activation of MAPKKKs, which phosphorylate and activate MAPKKs, leading to the activation of MAPK14. This cascade ensures the transmission, amplification, and integration of signals from various stimuli, resulting in appropriate cellular responses, including inflammation, apoptosis, cell differentiation, and stress responses.

MAPK14 is activated by dual phosphorylation on a Thr-Gly-Tyr motif within its activation loop. This phosphorylation is primarily mediated by upstream kinases such as MKK3, MKK6, and, in

some contexts, MKK4. Activated MAPK14 then phosphorylates various substrates, including transcription factors (e.g., ATF-2, Sap-1a), protein kinases (e.g., MAPKAP kinase-2/3), and other signaling molecules that modulate cellular processes.

MAPK14 plays a pivotal role in the inflammatory response by regulating the production of pro-inflammatory cytokines such as TNF-α and IL-1. It achieves this by activating transcription factors that increase the expression of these cytokines. MAPK14 also influences the stability and translation of cytokine mRNAs, further amplifying the inflammatory response. Additionally, MAPK14 regulates the activity of cytosolic phospholipase A2, which is involved in producing inflammatory mediators like prostaglandins and leukotrienes.

MAPK14 is activated by various stressors, including UV radiation, heat shock, osmotic stress, and oxidative stress. Upon activation, MAPK14 induces the expression of genes that help the cell cope with stress, including those involved in DNA repair, cell cycle arrest, and apoptosis. By phosphorylating transcription factors such as p53, MAPK14 can promote the expression of pro-apoptotic genes, thereby facilitating programmed cell death in response to irreparable cellular damage.

MAPK14 interacts with other MAPK pathways, including the ERK and JNK pathways, allowing for integrated cellular responses to complex stimuli. This crosstalk ensures that signals from different sources are coordinated and appropriately modulated. The regulation of MAPK14 activity is tightly controlled by various feedback mechanisms, including the expression of phosphatases that dephosphorylate and inactivate MAPK14, thereby preventing excessive or prolonged activation.

MAPK14 also influences cell cycle regulation, particularly at the G1/S and G2/M transitions. It can induce cell cycle arrest by modulating the activity of Cdks and the expression of cyclins and other cell cycle regulators. This function is crucial for maintaining genomic integrity and preventing the proliferation of damaged cells.

MAPK14 (p38α) is a central player in the MAPK signaling pathways, crucial for mediating responses to inflammation, stress, and other stimuli. Through its regulation of cytokine production, stress responses, and cell cycle checkpoints, MAPK14 ensures appropriate cellular reactions to environmental and physiological challenges. Its role in these processes highlights its importance in both normal cellular function and the pathogenesis of various diseases, including inflammatory disorders and cancer (Zhang & Liu, 2002).

1.2.4.4. Cyclin-dependent Kinases

Cyclin-dependent kinases (CDKs), traditionally recognized for their roles in regulating the cell cycle and RNA polymerase II during gene transcription, are now known to have emerging functions in modulating inflammatory responses. CDKs 1, 2, 4, 6, 7, and 9, along with their regulatory partners (cyclins, INK, and CIP/KIP proteins), are understood to influence inflammation independently of cell cycle control.

In differentiated immune cells, CDKs regulate inflammatory gene expression through interactions with chromatin-associated transcription factors such as NF-κB, STAT, and AP-1. These interactions can be both kinase-dependent and independent, underscoring the multifaceted role of CDKs in inflammation.

Therapeutically targeting CDKs could impact cell migration, immune cell activation, neoangiogenesis, and inflammation, extending beyond merely inhibiting cell proliferation. This broadens the potential for CDK inhibitors in treating inflammatory diseases and cancer-related inflammation.

Recent studies have elucidated that nuclear CDKs, particularly CDK6, support the expression of inflammatory mediators by associating with transcription factors during the G1 phase of the cell cycle. For instance, cytokine-induced CDK6 recruits to chromatin and interacts with NF-κB, STAT, and AP-1 families to promote the expression of genes like VEGF-A and p16INK4A. Interestingly, CDK6's role in recruiting the NF-κB subunit p65 is largely independent of its kinase activity, indicating a non-catalytic function in gene regulation.

The involvement of CDKs in the expression of proinflammatory genes opens avenues for therapeutic interventions aimed at controlling inflammation, whether in chronic inflammatory diseases or tumor environments. CDKs thus play crucial roles beyond cell cycle regulation, directly influencing inflammatory responses and offering new targets for anti-inflammatory therapies (Schmitz & Kracht, 2016).

1.2.4.5. AKT1

Akt1 is pivotal in promoting acute inflammation by regulating vascular permeability, thereby inducing edema and facilitating the extravasation of leukocytes. Studies using Akt1-deficient mice demonstrated markedly reduced inflammation compared to wild-type counterparts, characterized by decreased infiltration of neutrophils and monocytes. Importantly, these effects were attributed to Akt1 expression within the host vasculature rather than in circulating cells. In vitro experiments further underscored the importance of the Akt1-endothelial nitric oxide

synthase pathway and VE-cadherin in controlling histamine-induced alterations in junctional permeability (Di Lorenzo et al., 2009).

1.2.5. CASPASE 3

Cells respond to changes in their environment by adjusting gene expression, particularly in response to specific extracellular signals. This process involves synthesizing and releasing cytokines, receptors, and enzymes that shape the microenvironment. Gene expression is initiated at the transcriptional level, which is context-dependent and influenced by the array and sequence of incoming signals. Thus, transcription serves as a critical regulatory pathway determining cellular outcomes, including cytokine production. This interaction highlights the significant communication between caspases and cytokines at the transcriptional level.

Caspases, including Caspase 3 (CASP3), target a diverse range of substrates that extend beyond apoptosis. These substrates include cytokines, repair proteins, RNA-binding proteins, structural proteins, signaling molecules, cell cycle regulators, and transcription factors, indicating that caspases have broader roles in cellular functions, including transcriptional regulation and inflammation.

TNF receptors can trigger both apoptotic pathways (via caspase activation) and survival pathways (through kinases and transcription factors such as AP-1 and NF-κB). NF-κB, known for its anti-apoptotic functions, plays a crucial role in cell survival by protecting cells from apoptosis. For example, macrophages require continuous NF-κB activation to maintain viability and mitochondrial function. Inhibiting NF-κB pathways can increase caspase activity, promoting apoptosis, whereas active NF-κB supports the expression of anti-apoptotic proteins like TRAF-1, TRAF-2, cIAPs, and members of the Bcl-2 family.

There is significant crosstalk between apoptotic and survival signaling pathways. For instance, c-FLIP, an inhibitor of caspase-8, activates NF-κB and Erk pathways, enhancing cell survival through increased c-FLIP expression. Conversely, caspases can dampen survival pathways by cleaving proteins such as RIP and TRAF-1, which are critical for NF-κB activation. Caspase-8, for instance, cleaves RIP and TRAF-1, inhibiting NF-κB induction and promoting apoptosis.

CASP3 plays a role in inactivating survival signaling molecules and transcription factors. It cleaves the epidermal growth factor receptor (EGFR), disrupting anti-apoptotic signaling pathways and affecting downstream survival mechanisms. CASP3 also truncates I-κBα, an inhibitor of NF-κB, thereby enhancing NF-κB suppression and promoting apoptosis. Moreover, CASP3 can cleave the p65 subunit of NF-κB, illustrating its role in modulating inflammatory responses.

CASP3 is also involved in regulating the cell cycle and differentiation by cleaving cell cycle inhibitors like Wee1 and p27Kip1, which impact cell proliferation. CASP3 activity is observed in both dividing and migrating cells, indicating its influence on essential cellular processes such as cell division and movement. This demonstrates that caspases, including CASP3, not only execute apoptosis but also regulate critical cellular processes that maintain genomic stability and progression through the cell cycle.

Overall, CASP3 plays a complex role in inflammation by modulating signaling pathways and transcription factors. It inactivates proteins involved in survival signaling, influences NF-κB and other transcription factors, and contributes to cytokine maturation and regulation of the cell cycle. This multifaceted role underscores the importance of CASP3 beyond apoptosis,

significantly impacting inflammatory responses and maintaining cellular homeostasis ("The Role of Caspases in Modulation of Cytokines and Other Molecules in Apoptosis and Inflammation").

1.2.6. Transcription Factors

1.2.6.1. Nuclear Factor-κB

Nuclear factor-κB (NF-κB) is a family of inducible transcription factors crucial for regulating numerous genes involved in immune and inflammatory responses (Iliopoulos et al., 2009). This family includes five members: NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. These proteins mediate gene transcription by binding to the κB enhancer element as hetero- or homodimers. Typically, NF-κB proteins are kept inactive in the cytoplasm by inhibitory proteins, such as the IκB family, with IκBα being the most studied. NF-κB1 and NF-κB2 precursors, p105 and p100, also function similarly to IκB (Liu et al., 2017).

NF-κB activation occurs through two main pathways: the canonical and noncanonical pathways. Both are crucial for immune and inflammatory regulation but differ in their mechanisms. The canonical pathway is triggered by various stimuli, including cytokine receptors, PRRs, TNF receptors, and T- and B-cell receptors. Activation involves the IκB kinase (IKK) complex, which phosphorylates IκBα, leading to its degradation and the nuclear translocation of NF-κB dimers (Iliopoulos et al., 2009) like p50/RelA and p50/c-Rel. The IKK complex consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NEMO (IKKγ). Various stimuli can activate IKK, causing IκBα degradation and subsequent NF- κB activation (Liu et al., 2017).

The noncanonical NF-κB pathway responds to specific stimuli, like certain TNF receptor family members (e.g., LTβR, BAFFR, CD40, RANK). This pathway does not involve IκBα degradation but rather the processing of p100, mediated by NF-κB-inducing kinase (NIK) and IKKα, leading to the generation of p52 and the formation of the p52/RelB complex (Iliopoulos et al., 2009). The canonical NF-κB pathway is involved in a wide range of immune responses, while the noncanonical pathway supplements the canonical pathway for specific adaptive immune functions.

NF-κB is a key regulator of inflammatory responses, controlling the expression of pro-inflammatory genes in innate immune cells and influencing the activation, differentiation, and function of inflammatory T cells. It also regulates inflammasomes, which are protein complexes activated in response to microbial or PAMPs and DAMPs. Chronic inflammatory diseases often involve deregulated NF-κB activation (Liu et al., 2017).

Inflammation, a protective response to infection and tissue damage, involves vasodilation and immune cell recruitment. While normally beneficial, uncontrolled inflammation can cause significant tissue damage and contribute to chronic diseases. NF-κB plays a central role in both innate and adaptive immune responses by inducing the transcription of pro-inflammatory cytokines and chemokines, particularly in macrophages, dendritic cells, and neutrophils.

In macrophages, NF-κB activation leads to the production of cytokines and chemokines, facilitating the differentiation of macrophages into pro-inflammatory (M1) or anti-inflammatory (M2) states. M1 macrophages produce cytokines like IL-1, IL-6, IL-12, and TNF-α, promoting Th1 and Th17 cell differentiation, while M2 macrophages secrete IL-10 and IL-13, aiding in

inflammation resolution and wound healing. TLR signals regulate macrophage polarization, with TLR4 ligands such as LPS promoting M1 differentiation (Liu et al., 2017).

In adaptive immunity, NF-κB is essential for naive T cell activation via TCR signaling, influencing the differentiation of effector T cells into Th1, Th2, Th17, and Tfh cells. These effector T cells secrete distinct cytokines and mediate various immune responses. NF-κB regulates T cell differentiation through both cytokine production in innate immune cells and intrinsic T cell mechanisms. For instance, c-Rel and RelA are crucial for Th1 and Th17 differentiation by inducing lineage-specific transcription factors and cytokines (Karauzum & Datta, 2017).

Inflammasomes, which include NLRP3, ASC, and pro-caspase 1, play a critical role in immune responses by activating caspase 1 and processing pro-IL-1β and pro-IL-18 into active cytokines. NF-κB is vital for inflammasome priming by inducing the transcription of NLRP3 and pro-IL-1β. However, NF-κB signaling can also regulate inflammasome activity via autophagy, suggesting a complex role in balancing pro-inflammatory and anti-inflammatory functions (Liu et al., 2017).

Overall, both canonical and noncanonical NF-κB pathways are integral to the regulation of immune responses, inflammation, and the maintenance of immune homeostasis (Liu et al., 2017), making them significant targets for therapeutic strategies against inflammatory diseases.

1.2.6.2. cAMP Response Element-binding Protein

cAMP response element-binding protein (CREB) is a key transcription factor involved in various cellular processes, including proliferation, survival, and differentiation. Activated by growth factors and inflammatory

signals, CREB initiates the transcription of genes with a cAMP-responsive element. Notably, CREB regulates several immune-related genes such as IL-2, IL-6, IL-10, and TNF-α.

Phosphorylated CREB can inhibit NF-κB activation by preventing CREB binding protein (CBP) from associating with the NF-κB complex, thereby reducing proinflammatory responses. In monocytes and macrophages, CREB provides an anti-apoptotic survival signal, enhancing their longevity and function. In T and B lymphocytes, CREB promotes proliferation, survival, and regulation of immune responses, including Th1, Th2, and Th17 responses. CREB is also essential for the generation and maintenance of regulatory T cells (Tregs).

CREB activation involves phosphorylation at serine 133 by various kinases, such as PKA, PKC, CaMKs, and pp90 RSK. This phosphorylation allows CREB to interact with CBP or p300, initiating the transcription of CREB-responsive genes. These interactions facilitate chromatin remodeling and the recruitment of transcription machinery (Wen et al., 2010), thereby regulating numerous cellular processes.

CREB influences the expression of genes involved in cell cycle regulation, glucose homeostasis, and synaptic plasticity, which are crucial for proliferation and differentiation. It also regulates immune-related genes and responses, including cytokine production and TLR signaling pathways. CREB's modulation of the NF-κB pathway impacts inflammatory responses (Liu et al., 2017) through interactions with CBP/p300 and competition for binding sites (Wen et al., 2010).

Moreover, CREB activation promotes anti-apoptotic signals, enhancing the survival of immune cells like macrophages, which is vital for robust immune responses against pathogens.

In T cells, CREB activation is involved in proliferation, cytokine production, and Treg function. Different signaling pathways, including those mediated by PKA, PKC, and MAPKs, lead to CREB phosphorylation, which is essential for T cell activation and function (Karauzum & Datta, 2017). In B cells, CREB phosphorylation, triggered by BCR stimulation and involving PKCδ and pp90 RSK, promotes B cell activation and proliferation (Wen et al., 2010).

CREB is crucial for the generation and maintenance of Tregs, which are important for immune tolerance and the prevention of autoimmune responses. CREB stabilizes FoxP3 expression through a TGF-β-dependent mechanism involving the demethylation of specific regions in the FoxP3 locus (Wen et al., 2010), thus promoting Treg development and function.

Overall, CREB is a multifaceted transcription factor playing significant roles in cellular and immune processes. It regulates inflammation, cell survival, and proliferation, and is vital for the proper functioning of T and B cells and the maintenance of immune homeostasis.

1.2.6.3. Hypoxia-inducible Factor

The cellular response to hypoxia is mediated by the hypoxia-inducible factor (HIF) family of transcription factors, which are crucial for metabolic adaptations under low oxygen conditions. HIF expression in immune cells can be triggered not only by hypoxia but also by other stress factors such as inflammation and infection. HIF regulates various aspects of immune function, influencing processes from phagocyte activity to T cell differentiation. In immune cells, HIF stabilization can occur independently of oxygen levels, often induced by factors like bacterial pathogens, LPS, and inflammatory cytokines like TNF-α. This stabilization leads to changes in metabolism and function, affecting inflammatory responses and immune cell survival (Malkov et al., 2021).

HIF-1α and HIF-2α are expressed in various immune cell types, including macrophages, neutrophils, dendritic cells, and lymphocytes. Their expression patterns are influenced by different stimuli and can impact immune cell function in distinct ways. For instance, HIF-1α is crucial for promoting inflammation and enhancing antimicrobial functions in myeloid cells like macrophages and neutrophils. In contrast, HIF-2α is involved in regulating inflammatory responses in myeloid cells and promoting T cell (McGettrick & O'Neill, 2020) differentiation (McGettrick & O'Neill, 2020).

Moreover, the hypoxia pathway and HIF significantly modulate immune cell metabolism, particularly glycolysis, which is essential for immune cell activation and effector functions. This metabolic reprogramming influences immune cell differentiation and function, affecting outcomes in inflammatory diseases and infections.

Additionally, HIF's role in T cell development and differentiation, particularly in Th17 and Treg cell lineages, underscores its importance in shaping adaptive immune responses. HIF activity in CD8+ T cells also impact their effector functions and migratory capacity, with implications for host protection and immunopathology (Malkov et al., 2021).

Overall, the interplay between hypoxia, inflammation, and HIF signaling in immune cells highlights the intricate mechanisms underlying immune responses and opens avenues for therapeutic interventions in inflammatory disorders and infections.

1.2.7. Cytokines

1.2.7.1. Tumor Necrosis Factor

TNF was initially identified as a serum factor capable of causing the hemorrhagic necrosis of tumors in patients following acute bacterial infections. However, TNF's role extends far beyond its anticancer properties; it's crucial in inflammation and immune system signaling.

After its discovery, researchers managed to purify, sequence, and clone the genes encoding TNF and its receptors in humans and mice. This led to studies that revealed TNF's broader biological functions, showing that it wasn't just a potential anticancer agent but a key player in regulating immune responses (Webster & Vucic, 2020).

TNF operates through two main receptors: TNFR1 and TNFR2. TNFR1, which is widely expressed, binds both the membrane-bound and soluble forms of TNF, activating pathways like NF-κB and MAPK. These pathways are crucial for cell survival, inflammation, and immune responses. TNFR2, mainly found in immune and endothelial cells, binds more effectively to membrane-bound TNF and also activates NF-κB and MAPK pathways, but it doesn't induce cell death due to its lack of a death domain.

The regulation of TNF signaling is tightly controlled through phosphorylation and ubiquitination events. These modifications govern the formation and activity of intracellular signaling complexes associated with TNF receptors. Disruption in these regulatory mechanisms can lead to severe inflammatory diseases.

TNF's role in inflammation was further emphasized when it was identified as cachectin, the protein responsible for endotoxin-induced wasting disease (cachexia) in mice. This connection

underscores TNF's impact on inflammatory processes. Depending on the cellular context and regulatory signals, TNF can switch from promoting cell survival to initiating cell death pathways, such as apoptosis and necroptosis (Parameswaran & Patial, 2010).

In summary, TNF is a pivotal cytokine in regulating immune responses and inflammation. Although initially discovered for its tumor necrosis capabilities, TNF is now recognized for its extensive role in signaling pathways that mediate cell survival, death, and inflammatory responses. The necessity for tight regulation of TNF activity is underscored by its involvement in severe inflammatory conditions, highlighting its dual role as a critical regulator and a potential contributor to pathological inflammation.

1.2.7.2. Interleukin-1β

Human Interleukin-1β (IL-1β) was first purified in 1977, with its importance in biology growing significantly after its cDNA was cloned in 1984. Clinical trials have shown that using monoclonal antibodies to neutralize IL-1β can reduce diseases related to inflammation, such as atherosclerosis and cancer progression. IL-1β is produced in response to microbial products through TLR ligands and by IL-1 itself, both in vivo and in vitro. The IL-1β precursor accumulates in the cytosol until it is processed by the NLRP3 inflammasome and caspase-1 into an active cytokine. This processing and secretion can be blocked by specific caspase-1 inhibitors (Dinarello, 2018).

In the context of *S. aureus* infection, IL-1β is crucial for the inflammatory response. *S. aureus* has developed various strategies to evade neutrophil defenses, such as hindering neutrophil extravasation, activation, chemotaxis, and phagocytosis, and directly killing neutrophils with

cytolytic toxins or inducing apoptosis, which complicates vaccine development. During *S. aureus* infection, IL-1β helps control the infection, with liver Kupffer cells playing a significant role in pathogen clearance, serving as a crucial initial barrier to bacteremia and the spread of infection to other organs.

Processed IL-1β is released from cells through multiple pathways, including exocytosis, membrane microvesicles, transporters, or pyroptosis, a caspase-1-dependent form of cell death. The intracellular increase in calcium and phospholipase C activity are necessary for mature IL-1β secretion. While caspase-1 is the primary enzyme for processing IL-1β, other proteases like proteinase-3, elastase, matrix metalloprotease 9, granzyme A, and mast cell chymase can also process the IL-1β precursor extracellularly. IL-1β's role in inflammation includes not only initiating the immune response but also creating a feedback loop where its presence further stimulates IL-1β production, amplifying the inflammatory response. Studies on IL-1β-deficient mice show varied responses to inflammatory challenges, with reduced acute phase responses and inflammation in some models, but heightened febrile responses to stimuli like LPS, IL-1β, or IL-1α in others, highlighting IL-1β's central role in managing inflammation and immune responses during S. aureus infections (Ren & Torres, 2009).

1.2.7.3. Interleukin-6

Interleukin-6 (IL-6) is a multifunctional cytokine involved in numerous biological processes, such as immune responses, inflammation, hematopoiesis, and embryonic development. It plays critical roles in both acute and chronic inflammation, with dysregulated IL-6 signaling contributing to chronic inflammatory diseases, autoimmune conditions, and the cytokine storm

observed in COVID-19. Key players in inflammation include non-immune and immune cells, cytokines like IL-1β, IL-6, and TNFα, and transcription factors like NF-κB and STAT3. Notably, the IL-6 amplifier (IL-6 Amp) enhances an inflammatory cascade by synergistically activating NF-κB and STAT3, creating a positive feedback loop that leads to uncontrolled inflammation in various diseases.

Discovered in 1986, IL-6 is part of the IL-6 family of cytokines and is implicated in a wide range of physiological and pathological processes. Its expression is induced by stimuli such as TLR ligands, IL-1, TNFα, ROS, and zinc, and it is subject to post-transcriptional regulation. The IL-6 receptor (IL-6R) complex consists of IL-6Rα and gp130, with gp130 being a common component among IL-6 family cytokines. IL-6 signaling occurs through both classic and trans-signaling pathways, expanding its target cell range and contributing to chronic inflammation. The signal transduction pathways activated by IL-6 include JAK family tyrosine kinases and STAT transcription factors, which regulate gene expression crucial for cell survival, proliferation, inflammation, and tumor development. Negative regulators, such as SOCS3 and TRAF5, modulate IL-6 signaling to maintain homeostasis and prevent excessive inflammation (Hirano, 2021). Understanding the complex mechanisms of IL-6 signaling provides insights into the pathogenesis of inflammatory diseases and offers potential therapeutic targets for intervention.

1.2.7.4. Interferon-gamma

Interferon-gamma (IFNγ) is a pivotal cytokine in both innate and adaptive immunity, predominantly produced by activated CD4+ and CD8+ T cells as well as NK cells. While critical for host defense, excessive release of IFNγ is associated with chronic inflammatory and

autoimmune diseases.

Knockout models have underscored IFNγ's involvement in pathological processes linked to chronic immune activation. However, emerging evidence indicates that IFNγ also has anti-inflammatory functions. It promotes the production of anti-inflammatory molecules like IL-1 receptor antagonist and IL-18 binding protein, modulates the production of pro-inflammatory cytokines, induces apoptosis, and disrupts signal transduction through suppressors of cytokine signaling.

Beyond its pro-inflammatory effects, such as upregulating IL-12, IL-15, TNFα, and other pro-inflammatory mediators, and activating NF-κB, IFNγ can also exert protective anti-inflammatory properties. Clinical trials in conditions like rheumatoid arthritis, lupus nephritis, and insulin-dependent diabetes mellitus highlight IFNγ's dual role, where it can mitigate disease progression under certain conditions (Muhl & Pfeilschifter, 2003). This dual nature of IFNγ in inflammation emphasizes its complex role, balancing both pro-inflammatory and anti-inflammatory activities.

1.2.7.5. Interleukin-24

The induction of Interleukin-24 (IL-24) by MRSA infection in keratinocytes is crucial for promoting type 2 immune responses, which are central to the pathophysiology of atopic dermatitis (AD). In murine models, deleting IL-24 in keratinocytes leads to reduced tissue levels of IL-4 and IgE, resulting in improved skin barrier function and less itching. Conversely, administering recombinant IL-24 worsens type 2 immune reactions in AD models, highlighting its role in promoting allergic inflammation. IL-24 stimulates IL-33 production in keratinocytes via IL- 20Rb receptor complexes, further driving type 2 immune responses and contributing to AD-like symptoms. This positions IL-24 as a potential therapeutic target for AD management,

offering an alternative with potentially fewer systemic side effects.

Beyond AD, IL-24 also affects systemic allergic responses, as evidenced by reduced circulating IgE levels in IL-24-deficient mice, suggesting its involvement in allergic inflammation in distant organs. Additionally, IL-24 levels are significantly influenced by *S. aureus* exposure, indicating a specific adaptive response of keratinocytes to this pathogen. IL-24's role in intercellular communication establishes crucial pathways linking microbiota, skin cell adaptation, and immune alterations, which are vital for developing new therapeutic strategies for AD associated with microbial colonization (Qian et al., 2024).

1.2.8. Chemokines

1.2.8.1. CXCL8 (Interleukin-8)

Interleukin-8 (IL-8), a potent neutrophil chemotactic factor, plays crucial roles in both pathological and physiological contexts. Through its receptors, CXCR1 and CXCR2, IL-8 initiates a series of phosphorylation events that lead to neutrophil chemotaxis and activation. Dysregulated IL-8 signaling at the CXCR1/2 axis can result in immunopathology, producing an activated, prothrombotic neutrophil phenotype characterized by degranulation and NET formation.

Targeting this axis can reduce neutrophil activation and subsequent inflammatory responses (de Jong et al., 2019). Clinically, IL-8 is a valuable biomarker for diagnosing neonatal sepsis, demonstrating significant sensitivity and specificity. Additionally, IL-8 plays a key role in acute respiratory distress syndrome (ARDS), with its levels being associated with mortality and poor outcomes in various ARDS etiologies, including sepsis, pneumonia, trauma, and transfusion-related lung injury (Cesta et al., 2021).

1.2.8.2. C-C Chemokine 2 (MCP-1)

C-C chemokine 2 (CCL2), also known as monocyte chemoattractant protein 1 (MCP-1), plays a crucial role in inflammation during *S. aureus* infection by facilitating the recruitment and activation of monocytes. These cells are pivotal in the immune response against *S. aureus*, as they migrate to the infection site and contribute to the inflammatory process essential for combating the pathogen. CCL2 acts by attracting monocytes to the infected tissues, thereby aiding in their infiltration and subsequent participation in immune defenses (Carson et al., 2017).

The expression of CCL2 is induced in response to various inflammatory stimuli, including microbial infections like *S. aureus*. This chemokine interacts primarily with the CCR2 receptor on monocytes, directing them towards the site of infection. Experimental studies manipulating CCL2 signaling have demonstrated significant impacts on immune responses in models of *S. aureus* infection, highlighting its critical role in orchestrating the inflammatory cascade necessary for effective host defense (Sipprell et al., 2023).

Moreover, while CCL2's involvement in monocyte chemotaxis during inflammation is well-established, its influence on macrophage polarization remains an area of ongoing research. CCL2 production is commonly associated with TH2/M2 responses, but it also plays roles in TH1/M1 inflammatory conditions, indicating its versatile function in modulating immune responses tailored to specific pathogens like *S. aureus*.

1.2.8.3. Macrophage Inflammatory Protein-2

Macrophage Inflammatory Protein-2 (CXCL2) is pivotal for recruiting neutrophils by binding to CXCR2 receptors on their surfaces. Released by macrophages, CXCL1 and CXCL2 play a crucial role in orchestrating the initial phase of neutrophil recruitment to tissues. Upon entering tissues, neutrophils initiate antibacterial activities through the secretion of proteolytic enzymes

and ROS. This process is mediated via the activation of the protein kinase C (PKC)/NADPH pathway downstream of CXCL2 interaction with CXCR2. However, excessive neutrophil recruitment can lead to tissue damage due to heightened oxidative stress (G. Wang et al., 2021).

In the context of *S. aureus* infection, CXCL2 (also known as MIP2) is indispensable for recruiting neutrophils to the infection site. This recruitment is vital for the formation of neutrophil abscesses, which are crucial for eliminating the bacterial infection. Neutrophil recruitment begins with the recognition of pathogens through PRRs of the innate immune system, followed by the induction of pro-inflammatory responses. CXCL2, along with chemokines like CXCL1, CXCL5, and CXCL8, attracts neutrophils by interacting with CXC-chemokine receptor 2 (CXCR2) expressed on neutrophils. Additionally, adhesion molecules such as P-selectin, E-selectin, ICAM1 on endothelial cells, and L-selectin and LFA1 on neutrophils facilitate neutrophil rolling, adhesion, and diapedesis, thereby enhancing the immune response against *S. aureus* infection (Miller & Cho, 2011).

1.2.9. Reactive Oxygen Species

ROS are integral to cellular function and contribute significantly to disease development, acting as crucial signaling molecules generated during enzymatic reactions within cells. Depending on the cellular context, ROS can support normal physiological functions or initiate detrimental responses associated with metabolic dysfunction and inflammation. Diseases like atherosclerosis, diabetes, and stroke often exhibit dysregulated ROS levels, emphasizing the importance of understanding ROS signaling in disease pathogenesis.

NADPH oxidases (NOX), pivotal components of the innate immune system, initiate the respiratory burst crucial for microbial elimination. NOX1, NOX2, and NOX4, primarily expressed in the vascular system, participate in inflammation-induced vascular damage. Neutrophils and macrophages utilize NOX2 to produce ROS, aiding in microbial clearance through the oxidative burst, where superoxide radicals convert to hydrogen peroxide to facilitate antimicrobial actions. Additionally, ROS influence antigen presentation, T cell activation, and cytokine release, regulating inflammatory responses. Dysregulated TNF-NOX2 signaling perpetuates chronic inflammation and tissue injury (Forrester et al., 2018).

Mitochondrial ROS (MtROS) also play a vital role in inflammation by influencing cytokine release, activating inflammasomes, and modulating NF-κB signaling. MtROS contributes to macrophage activation and NLRP3 inflammasome activation, shaping inflammatory responses. Furthermore, crosstalk between NF-κB and Nrf2 pathways modulates antioxidant defenses and inflammatory reactions. The master regulator TNF-α governs cellular processes such as survival and apoptosis through ROS-mediated pathways, significantly impacting inflammation and disease progression. A comprehensive grasp of these ROS-mediated mechanisms is crucial for developing effective therapies targeting inflammatory and metabolic disorders (Checa & Aran, 2020).

1.2.10. NETosis – Release of Extracellular Traps

Neutrophil extracellular traps (NETs) are pivotal in inflammation and infection by entrapping and eliminating microbes. When activated, neutrophils release NETs composed of chromatin and granule proteins, effectively capturing pathogens. This mechanism is crucial for microbial clearance, particularly at sites of inflammation. NETs are induced by various stimuli, including

cytokines, bacterial components like LPS, and pathogens such as *S. aureus*. The formation of NETs depends on the production of ROS by NOX.

During infection with *S. aureus*, NETs play a particularly potent role in host defense against the pathogen. Research indicates that *S. aureus* triggers robust NET formation, which aids in trapping and killing the bacteria. Studies using NOX inhibitors and neutrophils from patients with chronic granulomatous disease (CGD), which lack functional NOX, underscore the crucial role of ROS in initiating NET formation.

Moreover, NETs are implicated in various inflammatory conditions, as they are abundant at inflammatory sites, such as in human appendicitis and streptococcal infections. Their impact extends beyond microbial killing to influencing the immune response, as they contain antimicrobial peptides and enzymes that contribute to inflammation. Overall, NETs are vital mediators of the innate immune response, playing a crucial role in inflammation and the host's defense against microbial infections (Fuchs et al., 2007).

1.2.11. Prostaglandin-Endoperoxide Synthase 2 (COX2)

Recent research has emphasized the significant role of prostaglandin D2 (PGD2) in the inflammatory response of macrophages during *S. aureus* infection, particularly highlighting the involvement of PRRs such as TLR2 and NLR pyrin domain-containing 3 (NLRP3). Upon *S. aureus* infection, there is a notable increase in PGD2 synthesis in macrophages, which is dependent on the presence of TLR2 and NLRP3.

When these receptors are absent, there is impaired production of PGD2, underscoring their critical role in regulating inflammatory responses.

PGD2 functions in modulating both pro-inflammatory and anti-inflammatory cytokine secretion and enhances macrophage phagocytosis. Interestingly, treatment with exogenous PGD2 has been observed to decrease the secretion of pro-inflammatory cytokines and chemokines, as well as anti-inflammatory cytokines, while not significantly impacting macrophage phagocytic activity.

These findings indicate that PGD2 plays a multifaceted role in the inflammatory response to *S. aureus* infection, suggesting its potential as a therapeutic target. Furthermore, the differential effects of endogenous versus exogenous PGD2 on the expression of TLR2 and NLRP3 highlight its complex and significant role in immune regulation during infection (Bao et al., 2024).

1.3. Introduction to Berberine as Potential Inhibitor of *S. aureus* Growth and Virulence

1.3.1. Chemical Properties and Sources of Berberine

Berberine (BBR) is a non-basic, quaternary benzylisoquinoline alkaloid widely used in Traditional Chinese Medicine (TCM) and Ayurveda (Och et al., 2020). Known for treating abdominal disorders, detoxification, and its antibacterial and antipyretic properties, berberine has garnered interest for its potential anti-cancer and metabolic disorder benefits. It functions as an alkaloid antibiotic and botanical anti-fungal agent, found in plants like goldenseal, barberry, and Oregon grape. Berberine acts as an antilipemic, hypoglycemic, antioxidant, and antineoplastic agent by inhibiting various enzymes, including aldehyde reductase and acetylcholinesterase, and

is used to treat obesity, diabetes, hyperlipidemia, heart failure, and infections without causing liver damage (Zou et al., 2017).

BBR is particularly valued in pharmacology and medicinal chemistry for synthesizing various bioactive derivatives. The primary natural source is the Berberis genus in the Berberidaceae family, which has extensive therapeutic applications in traditional medicine. Berberis species have been used for over 3000 years in TCM to treat conditions like inflammation, infectious diseases, diabetes, and constipation. In Ayurveda, these plants treat infections of the ear, eye, and mouth, heal wounds, cure hemorrhoids, aid digestion, and treat dysentery, uterine, and vaginal disorders. They also help reduce obesity and act as antidotes for scorpion stings and snakebites (Neag et al., 2018).

1.3.2. Previous Studies on Berberine's Anti-Inflammatory Effects

BBR exhibits significant anti-inflammatory properties by reducing proinflammatory cytokines such as TNF-α, IL-13, IL-6, IL-8, and IFN-γ. It counters TNF-α-mediated barrier defects in cell models through pathways involving tyrosine kinase, pAkt, and NF-κB. Studies have shown that BBR can repair cytokine-induced intestinal epithelial damage and inhibit IL-8 production, aiding mucosal healing in conditions like colitis.

Additionally, BBR inhibits the transcription factors AP-1 and NF-κB at concentrations of 10^-4 mol/L or higher, reducing LPS-induced MCP-1/CCL2 production. It activates AMP- activated protein kinase (AMPK) in macrophages, leading to decreased expression of TNF-α, IL- 1β, IL-6, MCP-1, iNOS, and COX-2. This effect is nullified by AMPK inhibitors, indicating that BBR's anti-inflammatory actions are mediated via AMPK activation. Furthermore, BBR inhibits COX-2 expression and reduces prostaglandin E2 (PGE2) levels, enhancing its anti-inflammatory

effects (Och et al., 2020).

1.4. Addressing the Challenges of Antibiotic-Resistant *S. aureus*: Potential of Berberine as a Therapeutic Agent

Current research on *S. aureus* infections faces significant challenges and gaps that need to be addressed. One of the primary concerns is the rise of antibiotic-resistant strains, particularly MRSA, which limits treatment options and complicates patient care. There is a pressing need for the development of new antibiotics effective against these resistant strains, but the pipeline for novel antibiotics remains insufficient.

Another critical issue is the formation of *S. aureus* biofilms, which pose a substantial obstacle to treatment efficacy. Biofilms create a protective environment that enhances bacterial resistance to both antibiotics and the immune system, leading to persistent infections that are difficult to eradicate. Finding strategies to disrupt or prevent biofilm formation is crucial for improving outcomes in *S. aureus* infections.

In this context, small molecules like berberine have emerged as potential candidates for inhibiting the growth and virulence of *S. aureus*. Berberine, derived from plants, has shown antimicrobial properties against various pathogens, including *S. aureus*. Studies indicate that berberine can disrupt bacterial cell membranes, inhibit biofilm formation, and reduce bacterial virulence factors. Moreover, berberine possesses anti-inflammatory properties, which may help mitigate the excessive inflammatory response triggered by *S. aureus* infections.

However, gaps in research remain regarding the optimal use of BBR. It's essential to conduct more studies to understand the specific molecular mechanisms through which BBR acts against *S. aureus* and to evaluate its effectiveness in clinical settings. Additionally, investigating BBR's potential in combination therapies with existing antibiotics could open new avenues for combating antibiotic-resistant *S. aureus* infections.

Addressing these research gaps requires a multidisciplinary approach, from basic research to clinical trials, to enhance our understanding and treatment of *S. aureus* infections, particularly in the face of rising antibiotic resistance. We hypothesized that BBR inhibits TNF-α, a key regulator of inflammation, in response to infection with laboratory *S. aureus* strain and clinical strains of *S. aureus* isolated from the skin of patients with severe eczema undergoing steroid withdrawal. Additionally, we hypothesized that BBR's anti-inflammatory effects would reduce ROS generation and the expression of genes involved in primary inflammatory pathways as described by Wang et al (Z. Z. Wang et al., 2021).

2. Specific Aims

Aim 1: Determine berberine inhibitory effect on TNF-α production in mouse macrophage cell line RAW 264.7 and human monocyte cell line U937

To determine whether BBR inhibits TNF-α production in both mouse macrophage cell line RAW 264.7 and human monocyte cell line U937.

This will be addressed by using cell lines which will be each be cultured and stimulated with ATCC strain (#33551) HKSA (MOI 2:1) in the presence of BBR at doses of (2.5, 5, 10, 20) μg/mL for 24 hrs. Levels of TNF-α in the culture supernatants will be determined by ELISA.

In addition, *S. aureus* heat-killed clinical isolates cultured from the skin of severe eczema patients will be used (MOI 2:1) to stimulate human monocyte cell line U937 cultured in the presence of BBR at doses of (2.5, 5, 10, 20) μg/mL for 24 hrs. Levels of TNF-α in the culture supernatants will be determined by ELISA.

Aim 2: Determine berberine inhibitory effect on relative gene expression involved in inflammatory pathways in the human monocyte cell line U937

To show the effect of BBR on the gene expression associated with the TNF AGE-RAGE, and infection inflammatory pathways.

Human monocyte cell line U937 will be cultured in the presence of BBR at 20 μg/mL and stimulated with ATCC strain (#33551) HKSA (MOI 2:1) for 24 hrs. Cells will then be harvested and gene expression analysis of kinase signal transducers MAPK1, MAPK3, and

AKT1; inflammatory cytokines TNF-α, IL-1β, and IL-6; and CASP3 and PTGS2 performed by qRT-PCR.

Aim 3: Determine berberine inhibitory effect on intracellular ROS production in the mouse macrophage cell line RAW 264.7 and the human monocyte cell line U937

To show the inhibitory effects of BBR on the intracellular ROS production in response to stimulation by heat-killed *S. aureus*.

Mouse macrophage cell line RAW 264.7 and human monocyte cell line U937 will each be cultured and pre-treated with BBR at 20 μg/mL and stimulated with ATCC strain (#33551) HKSA (MOI 2:1) for 24 hrs. Cells will then be washed and ROS production will be determined using the DCFDA/ H2DCFDA-Cellular ROS assay kit following the manufacture's instructions.

3. Methods

3.1. Preparation of Berberine

BBR compound was obtained from Xi'an SaiYang Biotechnology, LLC(China). HPLC fingerprint of berberine compound was performed on a Waters 2690 HPLC system coupled with a 2996 PDA detector (Waters, Milford, MA) for quality control.

3.2. Isolation and Identification of *S. aureus* from Eczema Patients

The skin swab was taken from topical steroid withdrawal syndrome patients (TSW) (N=8) visiting the Integrated Medicine Clinic at Westchester Medical Center (WMC). The swab was rolled on to a Mannitol Salt Agar (MSA)(Thermofisher Scientific, MA), covering the entire surface of the plate and incubated in a CO2 incubator at 37 °C for 24 hrs. Single isolated colonies were obtained and these colonies were further streaked on to a Nutrient agar (NA) (Thermofisher Scientific, MA) plate for 24 hrs. 2-3 isolated single colonies were emulsified on DNA/RNA shield collection tube (Zymo Research Corporation, CA) and were used for sequencing analysis to identify Staphylococcus strains. Four different strains were identified, and they were coded as EC01, EC02, EC03, EC04 respectively.

3.3. Bacteria Preparation and Cell Stimulation

S. aureus (#33591, ATCC, VA) and all clinical strains (EC01-EC04) were streaked onto a nutrient agar plate and incubated at 35-37 °C 24 hrs. Several isolated single colonies were picked and inoculated on a nutrient broth and incubated for 4-hrs. at 37°C shaker at 225rpm. The turbidity was adjusted to obtain 0.5 MacFarland standard ($1.2x10^8$ CFU/mL) by measuring O.D. values at 625nm using a spectrophotometer. The O.D. value was adjusted to 0.08-0.1, equivalent to 0.5 MacFarland standard. The bacteria were heat-killed by incubating at 65° C for 1 hr. The

multiplicity of infectivity (MOI) was determined based on the number of cells used for different experimental conditions and bacterial suspension was added to respective experimental wells.

3.4. Cell Culture, TNF-α Measurement and Cell Viability Assessment

Murine macrophage cells (RAW264.7 cells) (ATCC, VA) were cultured in growth medium containing DMEM (Gibco, NY) supplemented with 10% fetal bovine serum (FBS) (BioWest, FL) and 1% penicillin/streptomycin (Corning, VA). Cells were allowed to grow for 2-3 days until 75% confluency was observed. RAW264.7 cells were plated onto a 48-well culture plate at a concentration of 2×10^5 cells/mL and allowed to attach to the surface of the plate for 1-2 hrs. BBR at different concentrations (2.5, 5, 10, 20 µg/mL) were added to the respective wells and finally the cells were stimulated with heat-killed Staphylococcus aureus (HKSA) at multiplicity of infection (MOI) 2:1. The cells containing different concentration of BBR and HKSA were incubated for 24-hrs after which cell culture supernatant was harvested and TNF-α levels were determined by ELISA as per manufacturer's instructions. Cell viability was evaluated using trypan blue exclusion. Similarly, human monocytes (U937 cells, ATCC, VA) were cultured in ATCC-formulated RPMI-1640 (Gibco, NY) medium containing 10% FBS and 1% penicillin/streptomycin. Cells were allowed to grow for 2-3 days and after 75% confluence was obtained, cells were used for experiment. Briefly, cell concentration was adjusted to 2 x 10^6/ mL 250µL of this was plated on a 48-well cell culture plate. 250 µL of BBR at different concentrations (2.5, 5, 10, 20 µg/mL) were added to each well to make a total volume of 500µL per well. The BBR-treated cells were then stimulated with ATCC and clinical strain HKSA at MOI 2:1. The cells were incubated for 24-hrs after which cell supernatant were harvested and TNF- α levels were determined by ELISA following the manufacturer's protocol. Cell cytotoxicity was evaluated by Trypan blue exclusion.

3.5. qRT-PCR Analysis

U937 cells ($2x10^6$ /mL) were added in a 6-well plate along with BBR (20µg/mL) followed by stimulation with ATCC strain HKSA at MOI 2:1. Cells were incubated at 37°C in 5% CO2 for 24 hrs. The cell pellet was harvested, and RNA was extracted by adding RLT buffer following the extraction protocol of the All-prep DNA/RNA Mini Kit (QIAGEN, MD) according to the manufacturer's instructions. Total RNA was subjected to reverse transcription using Revert Aid RT Kit (Thermofisher Scientific, MA) and expression levels were determined by qRT-PCR using SYBR® Green PCR Master Mix (Themofisher Scientific, MA). The human primer sequence in the study are listed in (Table 1).

Gene	Forward (5' – 3')	Reverse (5' – 3')
TNFα	CTCTTCTGCCTGCTGCACTTTG	ATGGGCTACAGGCTTGTCACTC
IL6	ATGAACTCCTTCTCCACAAGCG	GAAGAGCCCTCAGGCTGGACT
MAPK1	ACACCAACCTCTCGTACATCGG	TGGCAGTAGGTCTGGTGCTCAA
AKT1	TGGACTACCTGCACTCGGAGA	GTGCCGCAAAAGGTCTTCATGG
CCND1	ATGTTCGTGGCCTCTAAGATGA	CAGGTTCCACTTGAGCTTGTTC
IL1β	CCACAGACCTTCCAGGAGAATG	GTGCAGTTCAGTGATCGTACAGG
MAPK3	AAGGGGTCAAAGGTGGAACC	TGCCTTGAT GACGCCGTATT
PPARγ	AGAGCCTTCCAACTCCCTCA	CAAGGCATTTCTGAAACCGA
PTGS2	CGGTGAAACTCTGGCTAGACAG	GCAAACCGTAGATGCTCAGGGA
CASP3	GGAAGCGAATCAATGGACTCTGG	GCATCGACATCTGTACCAGACC

Table 1: Human primers used for qRT-PCR

3.6. Reactive Oxygen Species

Intracellular ROS levels were measured using the DCFDA/ H2DCFDA-Cellular ROS assay kit (Abcam, MA) as per the manufacturers protocol. Briefly, 25000 RAW264.7 cells were serum starved for 24 hrs. and seeded on a dark, clear bottom 96-well microplate overnight to allow the cells to adhere. After overnight incubation. The cells were washed with PBS twice and pre-treated with BBR (20µg/ml) for 30 mins after which the cells were stimulated with HKSA (MOI, 2:1). 24-hours later, DCFDA (20µM) was overlayed on top of the treated cells for 45 mins at 37^{o}

C in the dark and the plate was read in an end point setting using a fluorescent reader (with excitation and emission wavelength of 485 and 535 nm, respectively. In a similar experiment, U937 cells were cultured with BBR at different concentrations and stimulated with HKSA at MOI 2:1. The cells were incubated overnight at 37° C after which cells were washed twice with PBS. ROS levels were measured as described previously. Briefly, DCFDA (20μM) was overlayed on top of the treated cells for 45 mins at 37°C in the dark and the plate was read in an endpoint setting using a fluorescent reader (with excitation and emission wavelength of 485 and 535 nm, respectively.

3.7. Statistical Analysis

All statistical analyses were conducted using GraphPad Prism 9 (San Diego, CA). One-way ANOVA (analysis of variance) was employed, followed by Bonferroni correction for all pairwise comparisons. For skewed data, differences between groups were assessed using one-way ANOVA on ranks followed by Dunn's test. P-value calculations were two-tailed, with a p-value < 0.05 considered statistically significant.

4. Results

4.1. BBR Inhibition of TNF-α Production in Mouse Macrophage Cell Line RAW 264.7 and Human Monocyte Cell Line U937

To determine the effect of BBR on TNF-α production, BBR was administered at various concentrations (2.5, 5, 10, and 20 μg/mL) to mouse macrophage cell line RAW 264.7 and human monocyte cell line U937, followed by stimulation with ATCC strain *S. aureus* (HKSA ATCC #33591) at an MOI of 2:1. The results demonstrated a dose-dependent inhibition of TNF-α production, with 20 μg/mL of BBR reducing TNF-α levels by more than 90% ($p < 0.001$) compared to the untreated control (Figure 1A), and this inhibition was not associated with any

cell cytotoxicity (Figure 1B).

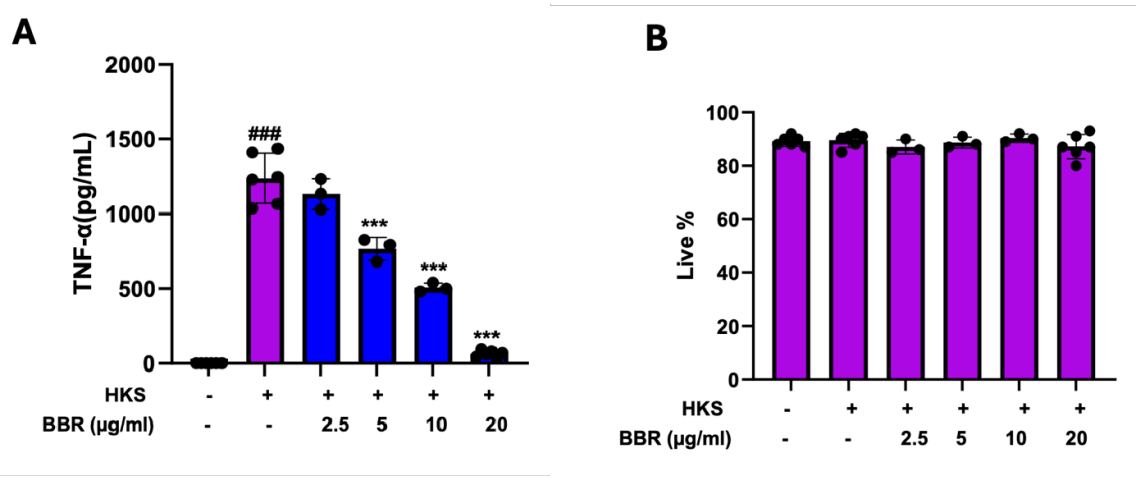


Figure 1: Dose dependent effect of BBR on ATCC strain heat killed *S. aureus* in RAW 264.7 cell line. RAW 264.7 cells were cultured in presence and absence of ATCC strain (#33591) HKSA (MOI, 1:2) and BBR at different concentrations (2.5g/ml, 5 µg/ml, 10 µg /ml and 20 µg/ml) respectively for 24 hrs. (A) BBR pre-treatment showed a dose dependent inhibition of TNF-α levels following HKSA stimulation. (B) Cell viability measured by Trypan blue exclusion showed no cell cytotoxicity across all groups. Differences between groups were assessed using one-way ANOVA on ranks followed by Dunn's test. P-value calculations were two-tailed, with a p-value < 0.05 considered statistically significant. Data represents mean ± SD. (N=3-4 replicates; $^{###}p<0.001$ vs untreated; ***$p<0.001$ vs HKSA).

Similarly, U937 cells treated with BBR at different concentrations (2.5, 5, 10, and 20 µg/mL) and stimulated with HKSA (ATCC #33591) exhibited comparable results, showing significant inhibition of TNF-α production (Figures 2A and 2B).

Overall, these findings from two different in vitro cell lines indicate that BBR effectively inhibits TNF-α production without inducing cytotoxicity, highlighting its potential as an anti-inflammatory agent.

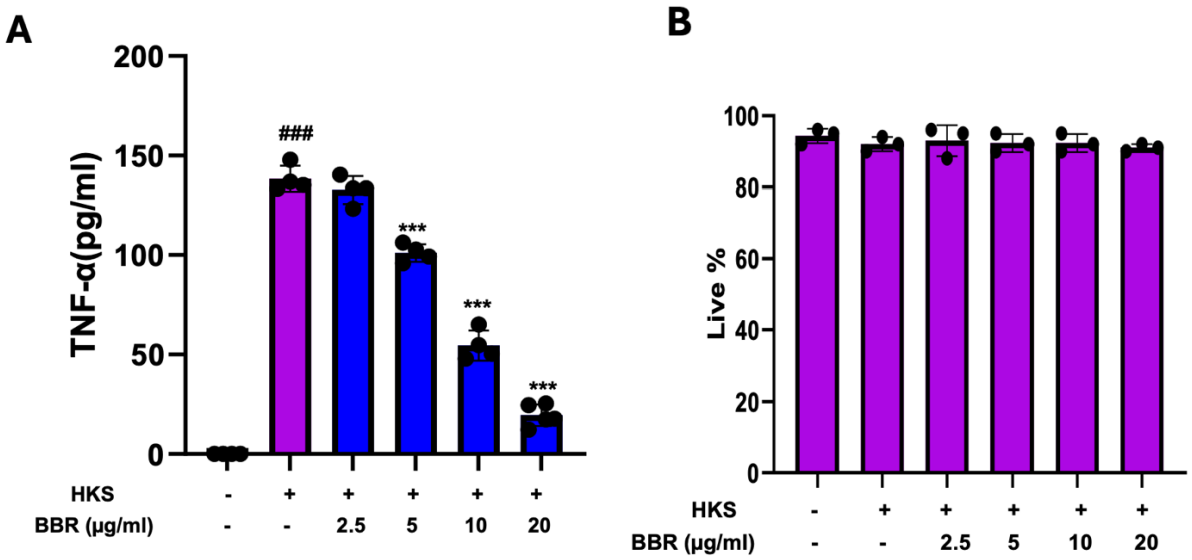


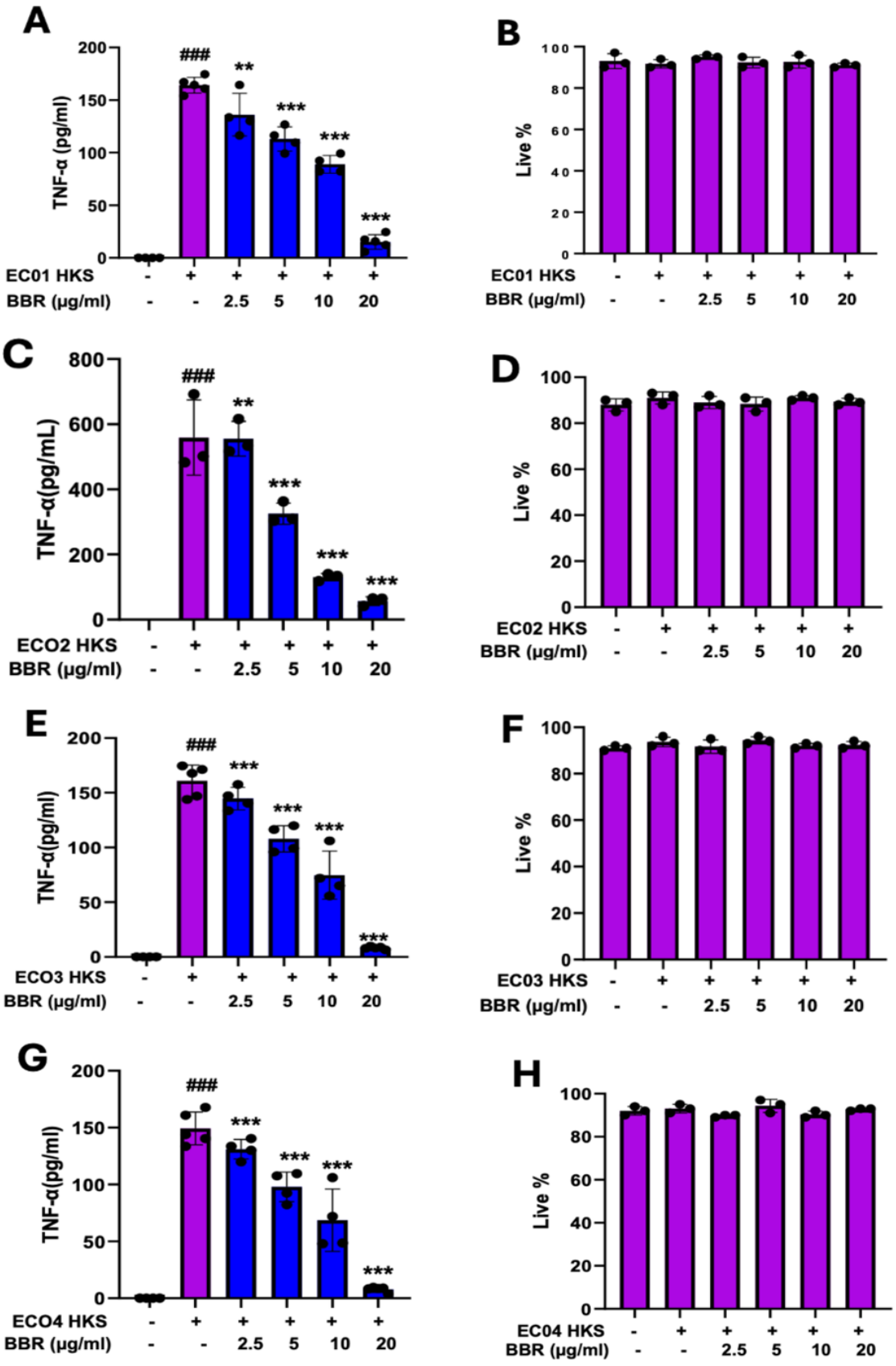
Figure 2: Dose dependent effect of BBR on ATCC strain heat killed *S. aureus* on U937 cell line. U937 cells were cultured in presence and absence of ATCC strain (#33591) HKSA (MOI 1:2) and BBR at different concentrations (2.5g/ml, 5 µg/ml, 10 µg /ml and 20 µg/ml) respectively. (A) TNF-α levels measured by ELISA showed a dose dependent reduction in presence of BBR. (B) Cell viability measured by trypan blue exclusion showed no cell cytotoxicity across all groups. Differences between groups were assessed using one-way ANOVA on ranks followed by Dunn's test. P-value calculations were two-tailed, with a p-value < 0.05 considered statistically significant. Data represents mean ± SD. (N=3-4 replicates; $^{###}p<0.001$ vs untreated; ***$p<0.001$ vs HKSA).

4.2. BBR Inhibited TNF-α Production Following Clinical Isolates of *S. aureus* Stimulation in Human Monocyte Cell Line U937

To determine the effect of BBR on TNF-α production, U937 cells were pre-treated with BBR at

varying concentrations (2.5, 5, 10, and 20 μg/mL) and subsequently stimulated with different clinical strains of HKSA (EC01, EC02, EC03, EC04) at a multiplicity of infection (MOI) of 2:1. Consistent results were observed across all clinical strains, with the 20 μg/mL concentration of BBR inhibiting more than 90% of TNF-α production ($p < 0.001$) compared to the untreated control, without any associated cytotoxicity (Figure 3A-H).

These findings suggest that the clinical strains isolated from human eczema skin are capable of stimulating TNF-α production in U937 cells, and that BBR treatment effectively reverses this stimulation. Additionally, while the strains exhibit variability in TNF-α production levels following stimulation, BBR consistently reduces TNF-α production across all strains tested.


A
TNF-α (pg/ml)
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BBR (μg/ml) - - 2.5 5 10 20
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TNF-α(pg/mL)
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ECO2 HKS - + + + + +
BBR (μg/ml) - - 2.5 5 10 20
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EC04 HKS - + + + + +
BBR (μg/ml) - - 2.5 5 10 20

Figure 3A-3H: Dose-dependent effect of BBR on clinical isolate (EC01, EC02, EC03, EC04) of heat-killed *S. aureus* in U937 cell lines. U937 cells were cultured in the presence and absence of clinical isolates of HKSA ($1x10^6$ CFU/mL) and BBR at different concentrations. BBR treatment (2.5g/ml, 5 µg/ml, 10 µg /ml and 20 µg/ml) respectively showed (A) EC01, (C) EC02, (E) ECO3, (G) ECO4 a dose-dependent inhibition of TNF-α production and (B) EC01, (D) EC02, (F) EC03, (H) EC04, without any cell cytotoxicity in presence of heat-killed clinical *S. aureus* isolates. TNFα levels were measured by ELISA and cell cytotoxicity was evaluated by trypan blue exclusion. Differences between groups were assessed using one-way ANOVA on ranks followed by Dunn's test. P-value calculations were two-tailed, with a p-value < 0.05 considered statistically significant. Data represents mean ± SD. (N=3-4 replicates; $^{###}p<0.001$ vs untreated; ** $p<0.01$; ***$p<0.001$ vs HKSA).

4.3. BBR Inhibits Genes Associated with TNF-α, AGE-RAGE, and Inflammatory Signaling Pathways

To determine the mechanism of action of BBR on TNF-α inhibition, we studied key target molecules from previously identified highly relevant pathways associated with BBR (Z. Z. Wang et al., 2021). We selected the pathways that were highly relevant to inflammation: TNF- α, AGE-RAGE and Infection inflammatory pathways (Figure 4). The genes associated with these pathways were analyzed and we found nine genes common between these pathways: TNF, IL1B, IL6, AKT1, MAPK1, MAPK3, CASP3, PTGS2, MAPK14, respectively (Figure 4). CCND1 was common in AGE-RAGE and Inflammatory pathways, and CREB1 was common in TNF-α and Inflammatory pathways respectively (Figure 4). CDK6 and HIF1A were specific to Inflammatory pathways (Figure 4). In order to validate if BBR had an effect on the genes associated with these pathways, we performed a qRT-PCR to study expression analysis (Figure 5A-5H). We found significant inhibition of expression of (5A) TNF-α ($p<0.001$); (5B) IL-1β ($p<0.001$); (5C) IL-6 ($p<0.001$); (5D) AKT; (5E) MAPK1 ($p<0.05$; (5G) CASP3 ($p<0.01$); and (5H) PTGS2 ($p<0.001$)) respectively in presence of BBR (20 µg/mL) as compared to untreated control. There was no significant difference in the expression levels of (5F) MAPK3 between

BBR-treated and untreated groups. Overall, these results show that BBR has an anti-inflammatory effect, and its anti-TNF activity is associated with inhibition of expression of key genes and targets associated with these highly relevant inflammatory pathways.

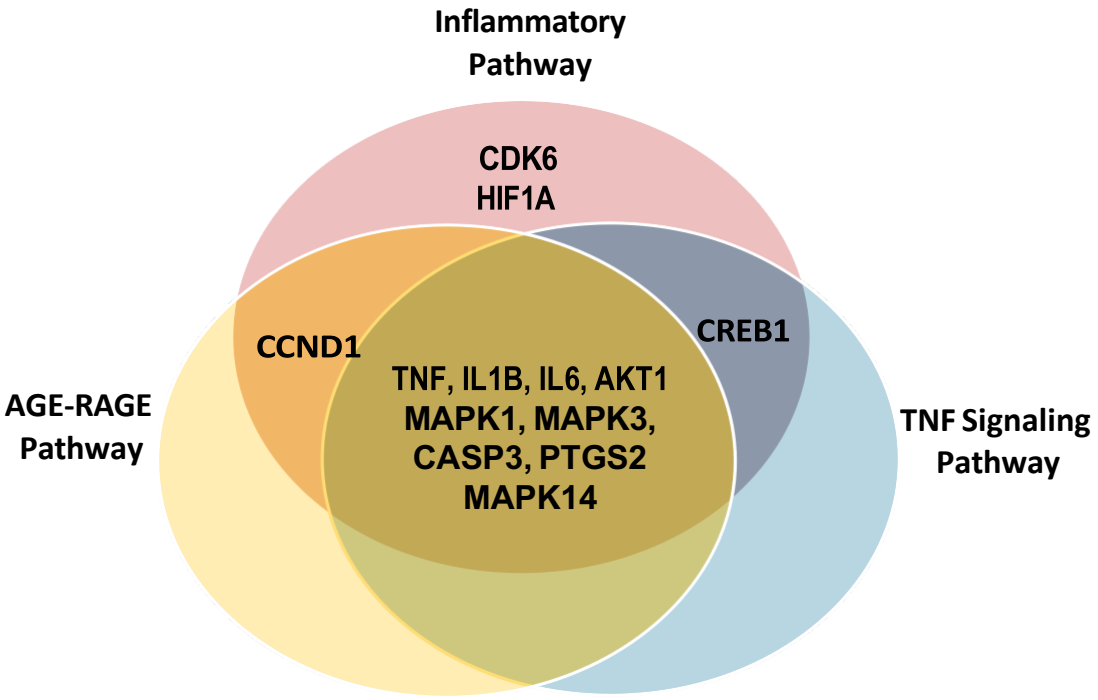
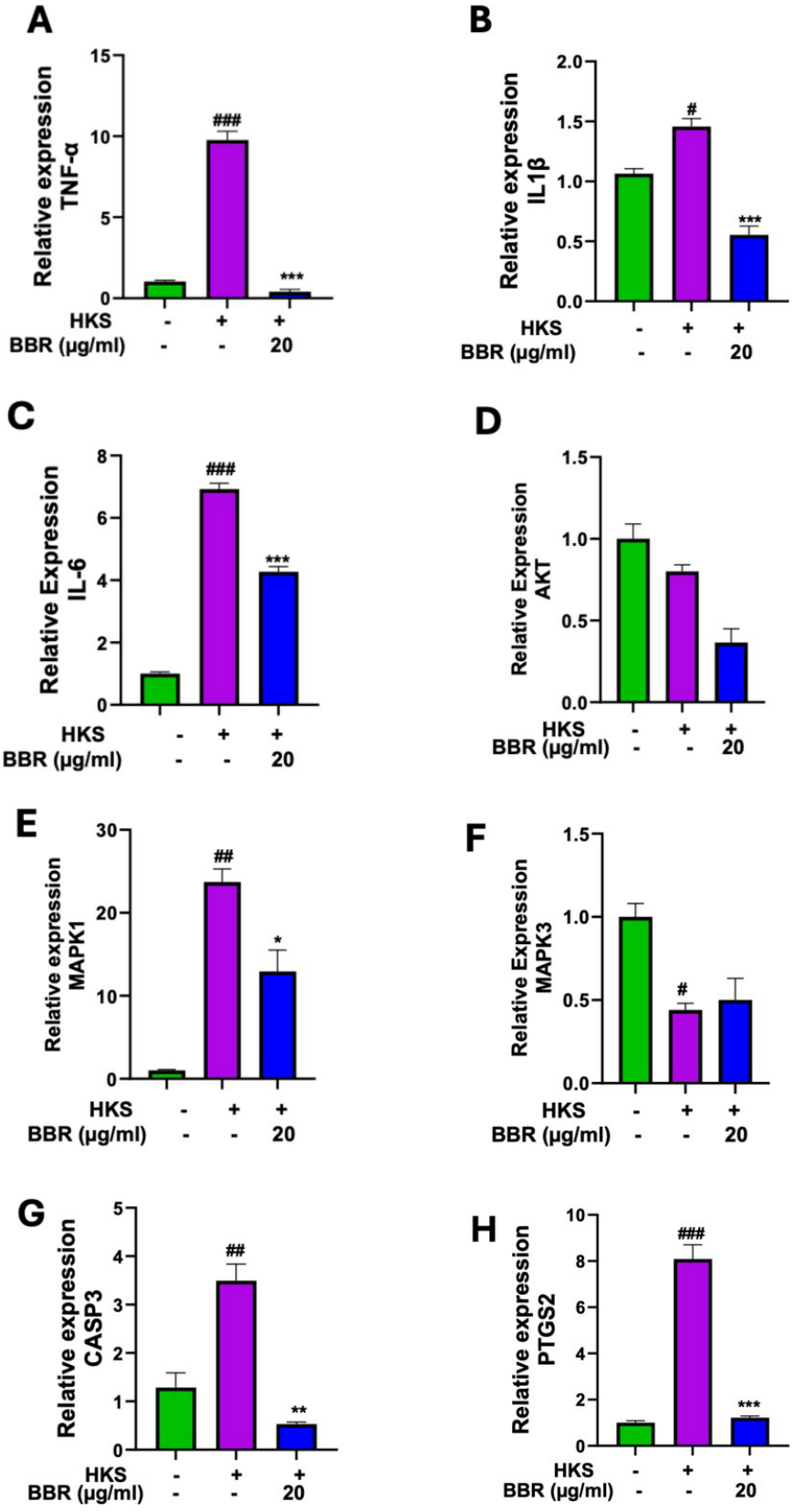


Figure 4: Venn diagram of common genes among the TNF, AGE-RAGE, and Infection Inflammatory pathways. Venn diagram showing common genes among the TNF, AGE-RAGE, and Infection Inflammatory pathways obtained from previously published database (Z. Z. Wang et al., 2021). The most dominant pathways were TNF signaling pathway, AGE-RAGE pathway and Inflammatory pathway. The genes common in all three pathways were TNF, IL1B, IL6, AKT1, MAPK1, MAPK3, CASP3, PTGS2, and MAPK14.


A
Relative expression TNF-α
15
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***
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BBR (μg/ml) - - 20
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Relative expression IL1β
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1.5
1.0
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0.0
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HKS - + +
BBR (μg/ml) - - 20
C
Relative Expression IL-6
8
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4
2
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HKS - + +
BBR (μg/ml) - - 20
D
Relative Expression AKT
1.5
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HKS - + +
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E
Relative expression MAPK1
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Relative Expression MAPK3
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HKS - + +
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Relative expression PTGS2
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HKS - + +
BBR (μg/ml) - - 20

Figure 5: Effect of BBR on gene expression associated with TNF AGE-RAGE inflammatory pathways U937 cells were cultured in the presence of BBR (20µg/mL) and ATCC (#33591) HKSA (MOI, 2:1) for 24 hrs. Cells were harvested and gene expression analysis was performed was performed using qRT-PCR. (A) There was a significant decrease in expression of (A) TNF-α. (B) IL-1ᵦ, (C) IL-6, (D) AKT, (E) MAPK1, (G) CASP3, (H) PTGS2, and interestingly the was no significant difference with the relative expression of (F) MAPK3 in presence of BBR. Differences between groups were assessed using one-way ANOVA on ranks followed by Dunn's test. P-value calculations were two-tailed, with a p-value < 0.05 considered statistically significant. Data represents mean ± SD. (n=triplicate culture. $^{###}p<0.001$; $^{##}p<0.01$; $^{#}p<0.05$ vs untreated; ***p<0.001; **p<0.01; *p<0.05 vs HKSA)

4.4. BBR Inhibited ROS Production Following HKSA Stimulation

Since the most potent effect in TNF-α inhibition was observed with BBR (20 µg/mL), we further evaluated the effect of BBR in ROS production by RAW and U937 cells. Our results show that BBR significantly inhibited ATCC (#33591) HKSA stimulated ROS production by RAW cells (Figure 6, p<0.01) and U937 cells (Figure 7, p<0.01) respectively.

Overall, these results highlighted the in-vitro protective effect of BBR in preventing detrimental effects of ROS stimulated by *S. aureus*.

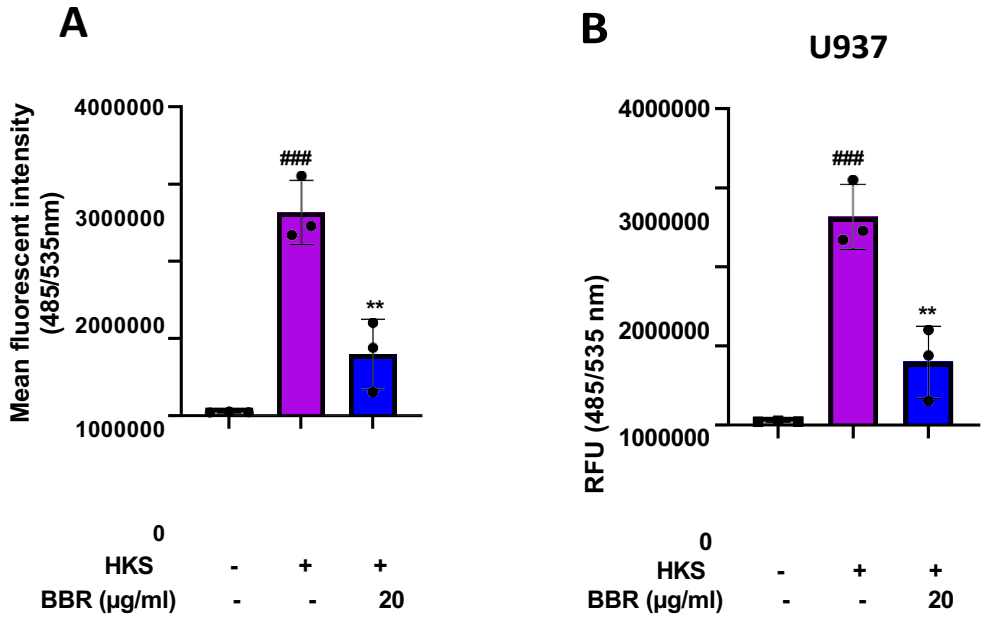

Figure 6: Effect of BBR on ROS production in HKSA stimulated RAW 264.7 and U937 cell lines: Intracellular ROS levels were measured using the DCFDA/ H2DCFDA-Cellular ROS

assay kit (Abcam, MA) as per the manufacturer's instructions. Both

(A) RAW 264.7 and (B) U937 cells pre-treated with BBR (20 μg/ml) showed reduced ROS production as compared to untreated cells stimulated with ATCC (#33591) HKSA (MOI, 1:2). Differences between groups were assessed using one-way ANOVA on ranks followed by Dunn’s test. P-value calculations were two-tailed, with a p-value < 0.05 considered statistically significant. Data represents mean± SD (n=triplicate culture; $^{###}p<0.001$ vs untreated; ***$p<0.001$ vs HKSA).

5. Discussion:

5.1. Major Findings and Importance

In this study, we found that BBR significantly inhibits TNF-α production in both the mouse macrophage cell line RAW 264.7 and the human monocyte cell line U937 after stimulation with HKSA strains. These results are important because they suggest a new therapeutic approach for managing *S. aureus* infections, especially given the increasing antibiotic resistance and the persistent nature of biofilm-associated infections.

Previous research has demonstrated that berberine has anti-inflammatory properties, such as inhibiting key cytokines and transcription factors like NF-κB and AP-1 (Och et al., 2020; Z. Z. Wang et al., 2021). Our study builds on this by showing that BBR not only reduces TNF-α but also decreases the expression of other inflammatory genes like IL-1β, IL-6, and PTGS2, and lowers ROS production in response to HKSA (Figure 7). This broad inhibition of inflammatory responses emphasizes BBR's potential as a comprehensive anti-inflammatory agent.

5.2. Novelty and Use of Monocytes

A novel aspect of our research is the use of the human monocyte cell line U937 to study the inflammatory cascade, particularly focusing on the AGE-RAGE pathway. This pathway plays a crucial role in chronic inflammation. We found that BBR effectively inhibits TNF-α production in monocytes stimulated with both a commercially available *S. aureus* strain and clinical isolates cultured from the skin of eczema patients undergoing steroid withdrawal.

The consistent inhibition of TNF-α production by BBR across different *S. aureus* strains highlights its potential as a therapeutic option for various *S. aureus*-induced infections. This

finding is particularly relevant for eczema patients who frequently experience *S. aureus* infections and require new treatments due to the limitations of current therapies.

5.3. Mechanistic Insights

Our study sheds light on how BBR exerts its anti-inflammatory effects. By inhibiting the expression of key inflammatory genes such as TNF, IL-1β, IL-6, CASP3, and PTGS2, BBR's anti-TNF-α activity appears to be mediated through the downregulation of these critical pathways (Figure 7). This partially explains how BBR reduces TNF-α in monocytes stimulated with HKSA, leading to a decreased overall inflammatory response.

5.4. Limitations

However, our study has some limitations. We did not investigate other downstream signals like additional cytokines and chemokines, which are essential for a complete understanding of BBR's anti-inflammatory effects. Additionally, we focused only on gene expression and did not measure the protein levels of these inflammatory mediators. Future research should address these limitations by including protein-level analyses and examining a broader range of inflammatory signals.

5.5. Conclusion

In conclusion, our study demonstrated that BBR significantly reduces TNF-α production in both the mouse macrophage cell line RAW 264.7 and the human monocyte cell line U937 after stimulation with both a commercially available HKSA strain as well as clinically isolated strain

of HKSA. This inhibition was dose-dependent, with the highest concentration (20μg/mL) achieving over 90% reduction compared to controls, without inducing cytotoxic effects.

Furthermore, BBR effectively decreased the expression of other key inflammatory genes such as IL-1β, IL-6, PTGS2, and CASP3, reinforcing its role in mitigating the inflammatory response. The study also highlighted BBR's ability to reduce ROS production, which is critical in managing oxidative stress during *S. aureus* infections.

Our investigation into the human monocyte cell line U937's response to BBR, particularly focusing on the AGE-RAGE pathway, adds an additional perspective to the understanding of chronic inflammation management. The consistent inhibition of TNF-α production by BBR across different *S. aureus* strains, including those from eczema patients undergoing steroid withdrawal, underscores its potential as a versatile therapeutic option. This finding is especially relevant for managing *S. aureus* infections in patients with chronic conditions like eczema, who often face limited treatment options due to antibiotic resistance.

The broad inhibition of inflammatory responses and reduction in ROS production demonstrated by BBRin our study position it as a promising therapeutic candidate for managing *S. aureus*-induced infections. Future exploration of berberine in combination with existing antibiotics could provide a synergistic approach, enhancing the efficacy of current treatments and potentially overcoming the challenges posed by antibiotic-resistant *S. aureus* strains. This research paves the way for innovative treatment strategies aimed at improving outcomes for patients suffering from inflammatory conditions and persistent bacterial infections.

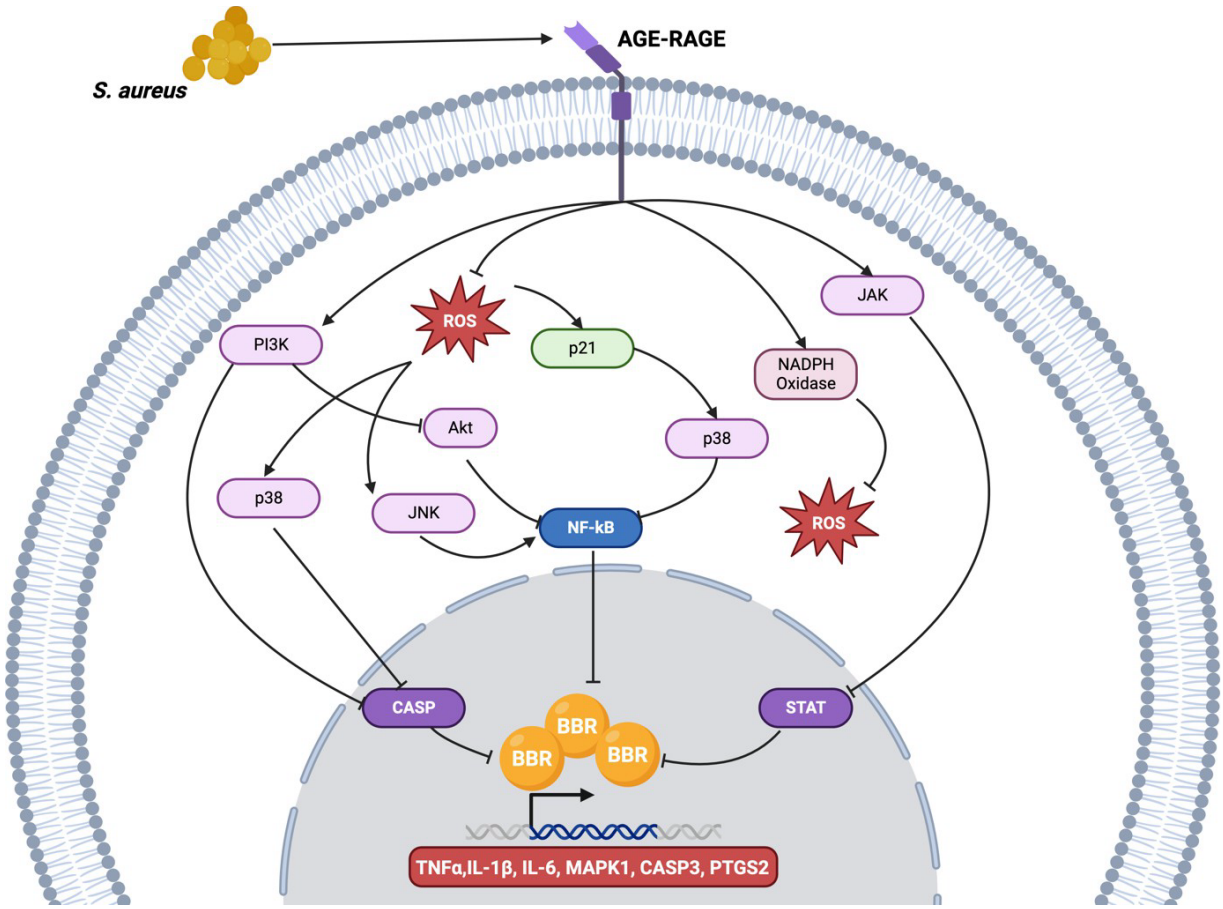

Figure 7: Hypothetical inhibition pathway. BBR-treated U937 human monocyte cells stimulated with heat-killed *S. aureus* resulted in a significant decrease in the relative gene expression of TNF-α, IL-1β, IL-6, MAPK1, CASP3, and PTGS2. This is possibly due to the inhibition of the translocation of CASP3 and NF_KB and the subsequent reduction of cytosolic inflammatory proteins. Additionally, with BBR treatment, the production of ROSwas significantly decreased further reducing the translocation of NF_KB to the nucleus inhibiting the upregulation of the pro-inflammatory cytokines and proteins: TNF-α, IL-1β, IL-6, MAPK1, CASP3, and PTGS2, limiting the positive signaling feedback loop from the additional cytokine production. Created with BioRender.com

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