

Therapeutic Targeting of TIRAP: Translating Molecular Insights into Anti-Inflammatory Strategies against Sepsis

Ph.D. Thesis

By
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**Mehta Family School of Biosciences and Biomedical Engineering
INDIAN INSTITUTE OF TECHNOLOGY INDORE**

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Therapeutic Targeting of TIRAP: Translating Molecular Insights into Anti-Inflammatory Strategies against Sepsis

A THESIS

*Submitted in partial fulfillment of the
requirements for the award of the degree
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by
Rajat Atre



**Mehta Family School of Biosciences and Biomedical Engineering
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INDIAN INSTITUTE OF TECHNOLOGY INDORE

I hereby certify that the work which is being presented in the thesis entitled **Therapeutic Targeting of TIRAP: Translating Molecular Insights into Anti-Inflammatory Strategies against Sepsis** in the partial fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY** and submitted in the **Mehta Family School of Biosciences and Biomedical Engineering, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from May, 2022 to November, 2025 under the supervision of Dr. Mirza S Baig, Professor, Mehta Family School of Biosciences and Biomedical Engineering, IIT Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

24-4-2026

Signature of the student with date

Rajat Atre

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

24-4-26

Signature of Thesis Supervisor with date

Prof. (Dr.) Mirza S Baig

Rajat Atre has successfully given his Ph.D. Oral Examination held on **24/04/2026**.

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Signature of Thesis Supervisor with date

Prof. (Dr.) Mirza S Baig

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-RAJAT ATRE

DEDICATED
To
ALMIGHTY
And
MY FAMILY

SYNOPSIS

Introduction

Sepsis is a life threatening condition resulting from dysregulated immune response. It is generated against an infection leading to fatal multi organ dysfunction. This chronic condition has been a significant cause for millions of deaths annually and poses prominent challenges in critical care management. [1]. The pathophysiology comprises increased vascular permeability, vasodilation, coagulopathy, exaggerated immune response giving rise to prolonged inflammation and eventually multi-organ failure **(Figure 1)** [10]. Regardless of ongoing significant efforts worldwide, sepsis remains a prominent reason for high fatalities specifically targeting the young children and elderly ones, and immunocompromised ones [1].

The U.S. Centers for disease Control and Prevention (CDC) reported the persistent sepsis burden with patient admissions around 60%, and 36% for septic shock and severe sepsis, respectively. [2]. India witnessed record 11.3 million cases, out of which 2.9 million deaths were reported. Thus, it would be essential and critical to understand the pathogenic profiles, and death rates at the regional and national levels to combat this chronic condition [3].

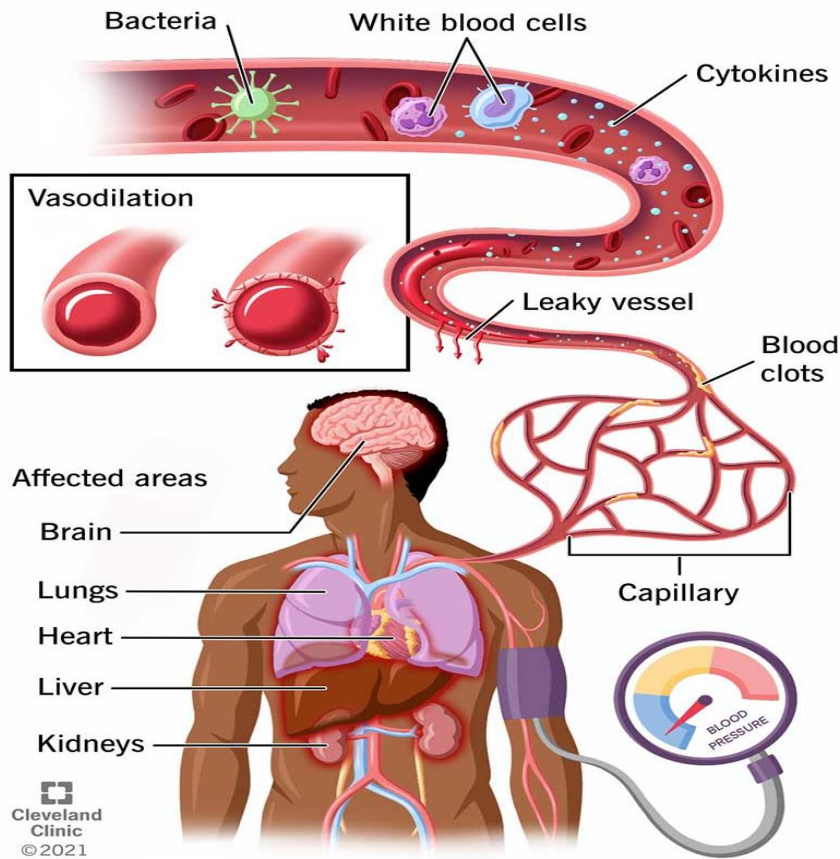


Figure 1 Pathophysiology of sepsis. *Cleveland Clinic* 2021.

Macrophage Activation and Toll-like Receptor (TLR) Signaling

Macrophages are central to the immune response during sepsis; their versatile nature is crucial for pathogen recognition, phagocytosis, and cytokine production [4]. Pathogen detection is carried out via various pattern recognition receptors (PRRs); one such major class is Toll-like receptors (TLRs) that recognize various pathogen-associated molecular patterns (PAMPs) (**Figure 2**). Among all the TLRs, TLR4 is majorly involved in Gram-negative bacteria mediated signaling through lipopolysaccharide (LPS) [5].

Adaptor proteins play a crucial role in the downstream signaling mediated by the TLRs. TIRAP (toll/interleukin-1 receptor adaptor protein) and MyD88 (myeloid differentiation primary response 88) are central to such signaling. They are responsible for the activation of MAPK, and NF- κ B pathways leading to pro-inflammatory responses (**Figure 3**). In TLR4 signaling TIRAP acts as the bridging entity between the receptor and the downstream proteins. Kinases namely, Bruton's tyrosine kinase (BTK) and protein kinase C delta (PKC δ) are mainly responsible for phosphorylation and activation of the TIRAP. TIRAP's ability to interact with various proteins gives rise to the development of a protein-protein network that ultimately amplifies the immune response [6].

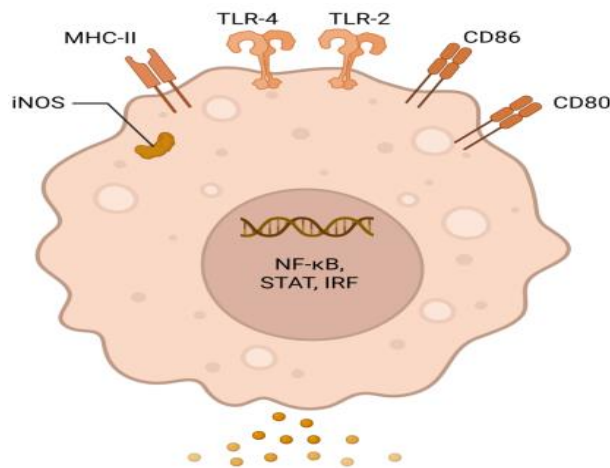


Figure 2 Macrophages and its associated receptors in inflammatory response.

TIRAP in Inflammatory Diseases

TIRAP's role in various chronic inflammatory diseases such as atherosclerosis, asthma, cancer, and arthritis has been reported. TIRAP being central to such dysregulated immune responses highlights it as a potential therapeutic strategy in multiple contexts [7].

During sepsis, TIRAP's activation leads to the production of various pro inflammatory mediators such as chemokines, cytokines, ROS, and NO that eventually develops fatal tissue injuries that goes beyond repair. Thus pharmacological inhibition of TIRAP may prove a promising drug target for such pathological conditions [8].

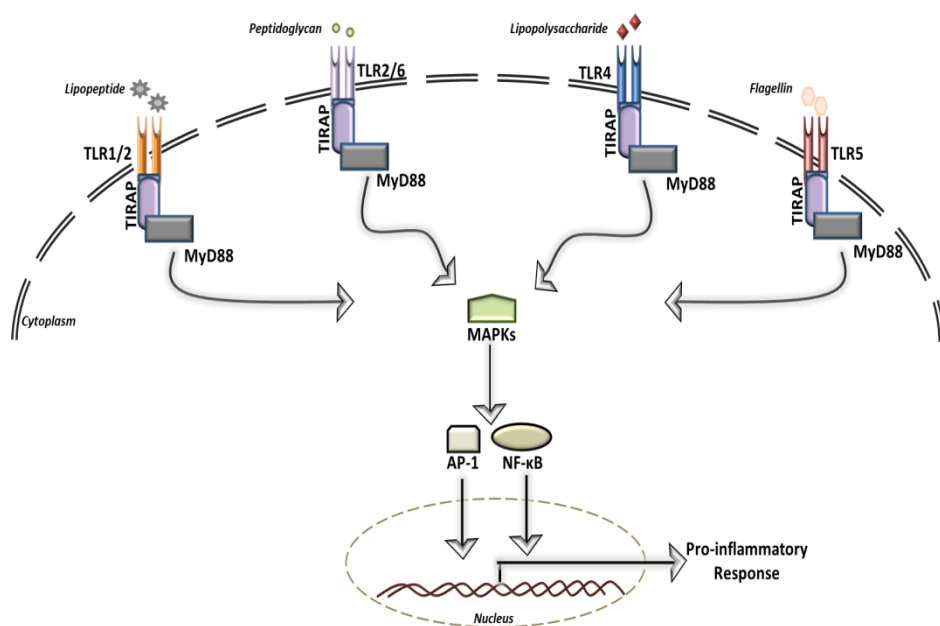


Figure 3 TIRAP dependent TLR mediated inflammatory signaling in macrophages.

Drug Repurposing and Rationale for TIRAP-Targeted Therapy

Drug discovery has always been considered a tedious process both in terms of time and cost involved. Thus, the diversion towards a new and better option was required that ultimately led us to the concept of drug repurposing. It holds an upper edge in the context that the drugs being screened are majorly FDA approved, with well characterized ADMET and pharmacokinetic profiles. This cut shorts our precious time and research work by passing these initial evaluations. It helps us to quickly translate

our research to mainstream world for therapeutic purposes [9]. The present work focuses on targeting TIRAP mediated inflammatory signaling via small molecule inhibitors as monotherapy as well as in combination to ultimately dampen the sepsis pathogenesis.

Objectives of the Study

The work focuses to assess therapeutic strategies and understanding sepsis pathophysiology via following objectives:

1. To identify the key TIRAP-mediated inflammatory signaling pathways involved in chronic inflammatory conditions.
2. To investigate novel intermediates of Dorzolamide (DZD) for their ability to inhibit TIRAP–kinase interactions and suppress macrophage-driven inflammation.
3. To develop an improved, reproducible, and ethical murine model of sepsis using enriched cecum slurry cultures.
4. To design and validate a novel combination therapy (LeDoz: Levofloxacin + DZD) that synergistically addresses infection and immune dysregulation.

Chapter-wise Thesis Organization and Summaries

Chapter 1: Introduction

It sheds light on the global health concern regarding sepsis. The pathogenesis involving hyperinflammation and immunosuppression has been discussed thoroughly. The crucial sentinels of the immune responses, the macrophages and their orchestrating ability have been discussed. It also presents the research objectives and the framework.

Chapter 2: Literature Review

The chapter begins with highlighting the importance of adaptor proteins and how vital roles they play in mediating the inflammatory cascades in immune cells. This section provides a comprehensive investigation of TIRAP biology, including its structure analysis, protein-protein interactions, including its phosphorylation by BTK and PKC δ , and its role in activating MAPKs and transcription factors. It also covers the presence of TIRAP in various pathologies and different therapeutic strategies against it such as engineered peptides, small molecules, and drug repurposing.

Chapter 3: Dorzolamide Intermediates with Anti-inflammatory Potential

This chapter discusses the original findings on the anti-inflammatory properties of DZD intermediates. The screening and binding efficiency on TIRAP at the kinase interface was demonstrated via computational docking (**Figure 4**). *In vitro* studies performed using LPS-stimulated macrophages showcased the reduced activation of TIRAP and its downstream signaling. *In vivo* studies confirmed the efficacy of intermediates specifically DRZ V in improving the survival rates of septic mice as compared to the parent drug DZD. Notably these intermediates exhibited decreased inhibition of carbonic anhydrase, thus highlighting reduced off targeting and highlighting the intermediates as promising therapeutic candidates.

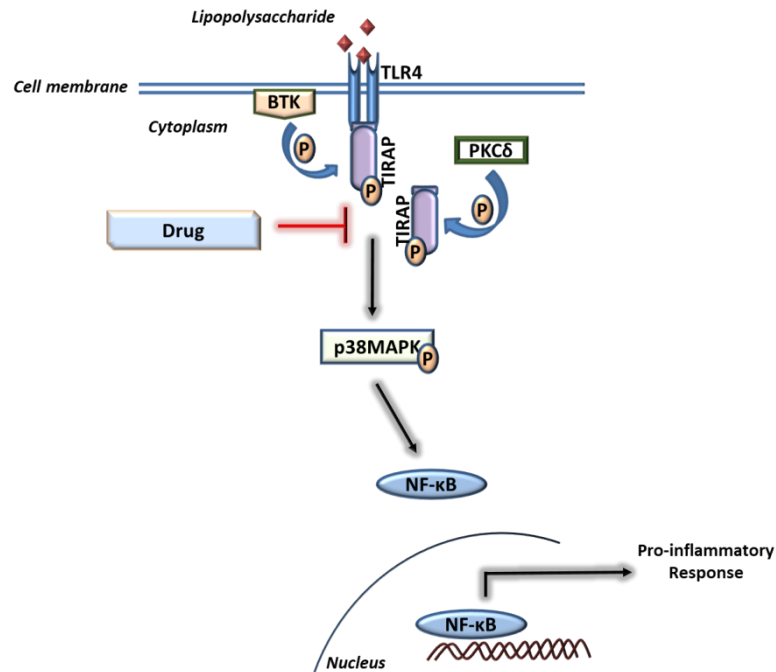


Figure 4 Illustration of TIRAP and kinases dependent signaling and a potential therapeutic approach.

Chapter 4: An Improved Mouse Model of Sepsis

This chapter showcases the understanding of the current septic models and the associated drawbacks. Herein we propose the development of an improved cecum slurry model that comprises polymicrobial culture along with gram-positive and gram-negative enriched microbiota. This model provides an edge over the existing ones by offering ethical advantages by reducing the animal sacrifice and maintaining the reproducibility for testing drug efficacy and efficiency (**Figure 5**). The experimental analysis depicted that increased microbial load correlated with reduced survival, heightened inflammatory cytokines, and organ damage, validating the model's clinical relevance.

<u>Current models</u>	<u>Parameters</u>	<u>Improved model</u>
✓	Clinical relevance	✓
✗	Reproducibility	✓
✗	Precise information on microbial composition	✓
✗	Ethically suitable as it limits the unnecessary euthanasia of animals	✓
✓	Cecal slurry polymicrobial culture can be stored at – 80 °C as glycerol stocks	✓
✗	Enrichment approach allowing to grow specific defined polymicrobial populations	✓
✓	Biochemical septic markers and cytokines up regulation	✓

Figure 5 Comparative analyses of the current sepsis models and the proposed improved sepsis mice model.

Chapter 5: LeDoz Combination Therapy

This chapter demonstrates the rational and validation of the LeDoz combination therapy (Levofloxacin + DZD) (**Figure 6**). *In silico* analysis reported Levofloxacin as a prominent TIRAP binding broad range antibiotic. The combination of these drugs showcased improved TIRAP inhibition and its downstream molecules responsible for inflammatory signaling. *In vitro* and *in vivo* studies showed reduced TIRAP activation, decreased colocalization, higher survival rates, and improved organ histology in septic mice treated with LeDoz. These synergistic effects highlight its dual antimicrobial and anti-inflammatory potential.

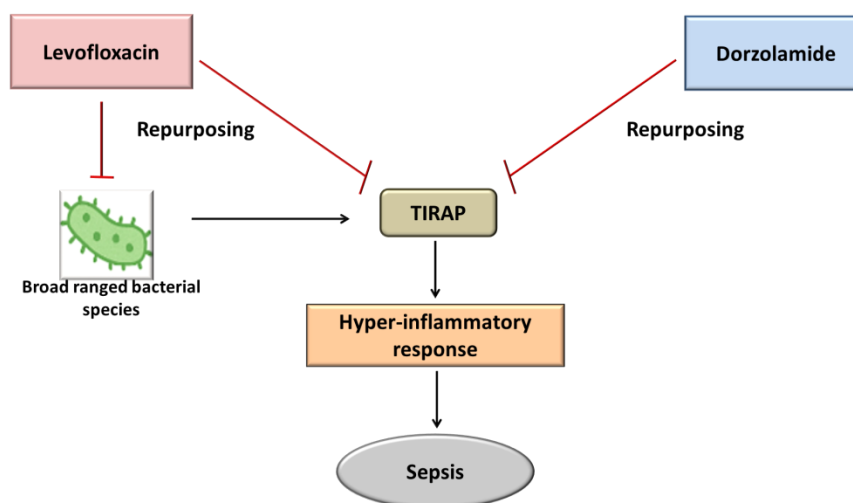


Figure 6 Representation of the proposed combination therapy targeting pathogenic load as well as the host associated inflammatory response.

Chapter 6: Summary

It sums up the findings in a short and concise way that highlights the key outcomes:

- To validate TIRAP as a therapeutic target in sepsis.
- To develop a reproducible enriched cecum slurry model with ethical advantages.
- To assess DZD intermediates and the LeDoz combination as potential therapeutic strategies.

Chapter 7: Future Prospects

The current work paves the way for future investigations that could be developed around TIRAP dependent inflammatory signaling. TIRAP's structural biology opens up the direction for studying various post translational modifications and its involvement in different chronic diseases. Further research would be required to find the way to effectively and efficiently translate these pre-clinical studies to the clinical levels and develop more precise therapeutics for sepsis.

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List of Publications

From Ph.D. Thesis Work

- **Rajat Atre**, Alexander G. Obukhov, Rahul Sharma, Faaiza Siddiqi, Fletcher A. White, Syed M. Faisal, Vivek P. Varma, Gajanan N. Darwhekar, Mirza S. Baig. Therapeutic approach targeting TIRAP mediated inflammatory response in sepsis. *Biochemical Pharmacology*, 242, 117353. (2025) (IF- 5.6) (<https://doi.org/10.1016/j.bcp.2025.117353>)
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- **Rajat Atre**, Rahul Sharma, Alexander G Obukhov, Uzma Saqib, Sadiq Umar, Gajanan N Darwhekar, Mirza S Baig. An improved mouse model of sepsis based on the cecum slurry polymicrobial intraperitoneal injection. *Life Sciences*, 345, 122584. (2024) (IF- 5.1) (<https://doi.org/10.1016/j.lfs.2024.122584>)
- **Rajat Atre**, Rahul Sharma, Gaponenko Vadim, Kundan Solanki, Khandu Wadhonkar, Neha Singh, Pramod Patidar, Rakhi Khabiya, Harshita Samaur, Sreeparna Banerjee, Mirza S. Baig, The indispensability of macrophage adaptor proteins in chronic inflammatory diseases, *International Immunopharmacology*, 119,2023,110176. (2023) (IF- 4.7) (<https://doi.org/10.1016/j.intimp.2023.110176>)

- **Rajat Atre**, Mobbassar Hassan Sk, Gias U Ahmmed, Arif Siddiquie, Mirza S Baig. Macrophages in the Crossfire: Immunometabolism, Autophagy, and Therapeutic Strategies during Sepsis. (*under review*)

Other Publications during Ph.D. Work

- Shivmuni Sarup*, **Rajat Atre***, Alexander G Obukhov, Shams Tabrez, Priyanka Yadav, Aravind Singh Kshatri, M Hassan Sk, Abdulaziz Alamri, Mohd Shahnawaz Khan, Mirza S Baig. A thioridazine-derived molecule exhibits potential anti-inflammatory activity through IKK inhibition. *Inflammopharmacology*. (2025) (IF- 5.3) (<https://doi.org/10.1007/s10787-025-01786-y>)
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- Aurelia Rughetti, Shreya Bharti, Rajkumar Savai, Spyridoula Barmpoutsis, Andreas Weigert, **Rajat Atre**, Faaiza Siddiqi, Rahul Sharma, Rakhi Khabiya, Nik Hirani, Mirza S Baig. Imperative role of adaptor proteins in macrophage toll-like receptor signaling pathways. *Future Science OA*. 2387961. (2024) (IF- 2.1) (<https://doi.org/10.1080/20565623.2024.2387961>)
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ethanolic extract of Ziziphus mauritiana lam. Fruit Pulp on various cancer cell lines. (under review)

- Pramod Patidar, **Rajat Atre**, Mirza S Baig. Toll/interleukin-1 receptor domain-containing adaptor protein (TIRAP) in Hepatic Stellate Cells: Beyond Membrane and Cytoplasmic Presence. (under review)

Patents

- Baig MS, **Atre R**, Sharma R. **A COMPOSITION FOR SEPSIS AND METHOD THEREOF**. Submitted to Indian Provisional Patent **Application No. 202321038927** dated June 06, 2023.
- Baig MS, **Atre R**. **NOVEL COMPOUND AND METHOD THEREOF TO ADDRESS THE TIRAP MEDIATED CHRONIC INFLAMMATORY SIGNALLING**. Submitted to Indian Provisional Patent **Application No. 202321075088** dated November 03, 2023.

Awards

- **Best Technology Award -2024** for the technology entitled “**A composition for sepsis and method thereof**.” Indian Institute of Technology, Indore.
- **JAPAN SAKURA SCIENCE** Exchange Program Award administered by Japan Science and Technology Agency 2024.
- **Best Oral Presentation Award** at the *In-House* Symposium organized by the Department of Biosciences and Biomedical Engineering, 2024.
- Appointed as a **Reviewer** for **International Journal of Immunology**
- **Travel Grant Award** at Annual Meeting of India BioImaging 2025 at **JSSAHER, Mysuru**.

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NOMENCLATURE

kDa- Kilo Dalton

μg/ml- Microgram per milliliter

ng/ml- Nanogram per milliliter

μM- Micro molar

U/ml- Unit per milliliter

°C- degrees Centigrade

%- Percentage

min- Minutes

h- Hour

mM- Milli molar

xg- Gravitational force

μg- Microgram

rpm- Revolutions per minute

ACRONYMS

ACCP-	American College of Chest Physician
ADMET-	Absorption distribution metabolism excretion and toxicity
ALT-	Alanine transaminase
AP-1-	Activator protein 1
AST-	Aspartate transaminase
BP-	Blood pressure
BTK-	Bruton's tyrosine kinase
CA-	Carbonic anhydrase
CASP-	Colon ascendens stent peritonitis
CDC-	Centers for Disease Control
CFTR-	Cystic fibrosis transmembrane conductance regulator
CFU-	Colony forming unit
CLP-	Cecum ligation punctures
CPCESA-	Committee for Purpose of Control and Supervision of Experiments on Animals
CRP-	C reactive protein
CS-	Cecum slurry
DAMP-	Damage associated molecular pattern
DNA-	Deoxyribonucleic acid
DZD-	Dorzolamide
ERK-	Extracellular signal regulated kinase
FiO ₂ -	Fractional inspired oxygen
H&E-	Hematoxylin and eosin
ICAM-	Intercellular adhesion molecule

ICU-	Intensive care unit
IL-6-	Interleukin 6
JAK-	Janus kinase
LB-	Lysogeny broth
LDL-	Low density lipoprotein
LeDoz-	Levofloxacin and dorzolamide
LMIC-	Low and middle income countries
LPS-	Lipopolysaccharide
LRP1-	Lipoprotein receptor related protein1
LRR-	Leucine rich repeats
MAL-	MyD88 adaptor like
MAPK-	Mitogen activating protein kinase
MD-	Molecular dynamics
MyD88-	Myeloid differentiation primary response 88
NF- κ B-	Nuclear factor kappa-B
OD-	Optical density
Ox-	Oxidized
PAMP-	Pathogen associated molecular pattern
PaO ₂ -	Partial pressure of oxygen
PDB-	Protein data bank
PKC δ -	Protein kinase C delta
PRR-	Pathogen recognition receptors
RMSD-	Root mean square deviation
RMSF-	Root mean square fluctuation
RNA-	Ribonucleic acid

RT-	Room temperature
SCCM-	Society of Critical Care Medicine
SIRS-	Systemic inflammatory response syndrome
SOCS-	Suppressor of cytokine signaling
SOFA-	Sequential organ assessment failure
STAT-	Signal transducer and activator of transcription
TDZ-	Thioridazine
TGF- β -	Transforming growth factor beta
TIR-	Toll/interleukin-1 receptor
TIRAP-	TIR domain containing adaptor protein
TLR-	Toll-like receptors
TNF α -	Tumor necrosis factor alpha
TRAF6-	Tumor necrosis factor receptor associated
TRAM-	TRIF related adaptor molecule
TRIF-	TIR domain containing adaptor inducing interferon- β
VCAM-	Vascular cell adhesion molecule
WBC-	White blood cells
WHO-	World Health Organization

Chapter 1

Introduction

Chapter 1: Introduction

1.1 Sepsis Overview

Sepsis is a critical global health burden that results from a dysregulated immune response to an infection. The imbalanced response develops the chronic inflammatory condition that results in multi organ dysfunction [1]. Severity can be understood from the statistics which highlight that over 30 million new cases and around 6 million deaths are being reported each year all over the globe [2]. Survivors are often found to have various physical complications, depicting the adverse after effects or consequences of the condition. Elderly individuals and those with weakened immune defenses face prominent vulnerability and susceptibility, positioning sepsis as an alarming concern for clinicians, and researchers [3,4]. Recognizing its devastating effect on both individuals and healthcare systems is essential for prevention, early diagnosis, and tailored therapies.

In clinical terms, sepsis can be described as a systemic inflammatory response triggered by a severe, life-threatening infection. This uncontrolled release of inflammatory mediators disrupts the body's immune balance, leading to increased vascular permeability, reduced cardiac output, and metabolic imbalance. These pathological changes can progress rapidly, resulting in tissue necrosis, multiple organ failure, hypotension, reduced tissue perfusion, and ultimately, death [5,6].

Current surveillance data from the U.S. Centers for Disease Control and Prevention (CDC) highlight the persistent burden of sepsis in hospital settings. Inpatient admissions remain notably high for septic shock (around 60%), severe sepsis (approximately 36%), sepsis caused by a known pathogen (around 31%), and sepsis of undetermined origin (roughly 27%) [7]. Furthermore, data from a large multicenter study encompassing over 90% of intensive care unit (ICU) admissions revealed

a significant increase in the proportion of patients with severe sepsis—from 7.2% to 11.1% over the study period [8]. These figures underscore the ongoing challenge of sepsis management in critical care and the urgent need for continued research and intervention strategies.

1.2 Concept

Our understanding of sepsis has undergone remarkable transformation over the decades, both in terms of medical insight and precise definition. The word *sepsis* itself is rooted in ancient Greek, meaning “decomposition” or “putrefaction.” The concept can be traced back almost 2,700 years to Homer’s epic works, and later found its way into the medical writings of Hippocrates and Galen, where it was discussed in relation to infection and decay [9].

Modern medicine’s journey to define sepsis began to take shape in the late 20th century. In 1991, Roger Bone and colleagues introduced the Systemic Inflammatory Response Syndrome (SIRS) criteria at the landmark SCCM–ACCP consensus conference, providing the first standardized diagnostic framework. For the next 25 years, this definition guided clinical practice and research around the globe [10].

As science advanced in immunology, biochemistry, and microcirculation studies, it became clear that the SIRS-based definition was too broad and often imprecise. This growing understanding helped to shape in the Sepsis-3 definition in 2016, which shifted the focus toward life-threatening organ dysfunction caused by a dysregulated host response to infection (**Figure 1.1**). The timeline from 1991 to 2016 reflects not just changes in wording, but an evolving recognition of sepsis as a complex, dynamic syndrome rather than a single, uniform disease [5,11–13].

1.2.1 Definitions

Sepsis-1 (1991)

Systemic Inflammatory Response Syndrome (SIRS): Temperature $>38^{\circ}\text{C}$ or 36°C , heart rate >90 beats/min, respiratory Rate >20 breaths/min, white blood cell (WBC) count $>12000/\text{cu mm}$ or $4000/\text{cu mm}$ or $>10\%$ immature forms (bands).

Severe Sepsis: Sepsis associated with organ dysfunction, hypoperfusion, or hypotension. Hypoperfusion and perfusion abnormalities may include but are not limited to lactic acidosis, oliguria, or acute mental status changes.

Septic Shock: Sepsis-induced hypotension persisting despite adequate fluid resuscitation, along with perfusion abnormalities such as lactic acidosis, oliguria, or acute mental status changes. Patients requiring inotropic or vasopressor agents may not initially present with hypotension at the time of assessment.

Sepsis-2 (2001)

General parameters: Fever (core temperature $>38.3^{\circ}\text{C}$); hypothermia; heart rate (90 beats/min) or >2 standard deviations (SD) above normal for age; tachypnea (respiratory rate >30 breaths/min); altered mental status; significant edema or positive fluid balance (>20 ml/kg over 24 hours); hyperglycemia (plasma glucose $>110\text{mg/dl}$ or 7.7 mM/l) in the absence of diabetes.

Inflammatory parameters: Leukocytosis (WBC $>12000/\mu\text{l}$); leukopenia (WBC $<4000/\mu\text{l}$); WBC with $>10\%$ immature forms; plasma c-reactive protein >2 standard deviation (SD) above the normal value; plasma procalcitonin >2 SD above the normal value.

Hemodynamic parameters: Arterial hypotension (systolic blood pressure < 40 mmHg in adults or $< 70\%$ Cardiac index > 3.5 l/min/m²). Organ dysfunction parameters: Arterial hypoxemia ($\text{PaO}_2/\text{FiO}_2$) < 1.5 or activated partial thromboplastin time > 60 s; ileus (absent bowel sounds); thrombocytopenia (platelet count $< 40,000$ /dl or $< 70,000$ /l). Tissue perfusion parameters: Hyperlactatemia (> 3 mmol/l); Decreased capillary refill or mottling.

Sepsis-3 (2016)

Definition: Quick Sepsis Related Organ Failure Assessment (qSOFA) ≥ 2 . Sepsis: Life-threatening organ dysfunction caused by a dysregulated host response to infection. Septic shock: A state with high mortality risk due to profound circulatory, cellular, and metabolic abnormalities.

Clinical criteria: qSOFA, based on; Altered mental status (Glasgow Coma Scale < 15); systolic blood pressure (BP) < 100 mmHg; respiratory Rate > 22 breaths/min. Suspected or documented infection with a Sequential Organ Failure Assessment (SOFA) score increase of ≥ 2 . Vasopressor requirement to maintain mean arterial pressure (MAP) ≥ 65 mmHg with lactate ≥ 2 mmol/l despite fluid resuscitation [12].

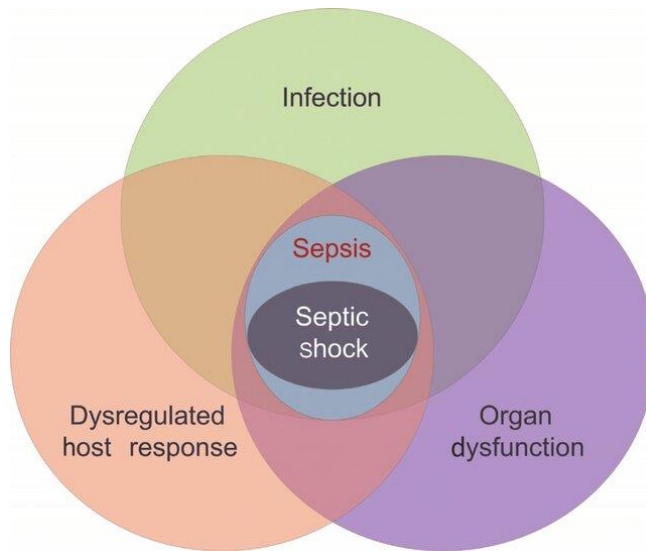


Figure 1.1 Schematic representation of sepsis as an overlapping scenario of multi-level host response [doi: 10.1097/CM9.0000000000003097].

1.3 Epidemiology

The true global burden of sepsis remains a subject of ongoing debate. Over the years, there have been vast shifts in definition, improvements in screening tools, evolving clinical management strategies, and even changes in medical coding. These parameters have greatly influenced how often sepsis is reported, sometimes leading to overestimation, and at other times underestimation. This challenge becomes even greater when assessing sepsis on a global scale, as many countries lack standardized systems for coding diagnoses.

In one landmark analysis, Fleischmann and colleagues attempted to quantify the global incidence and mortality of hospital-treated sepsis. Their review revealed a striking imbalance in that most available data originated from high-income nations, while information from low- and middle-income countries (LMICs) was scarce or entirely absent. Based largely on high-income country data, they estimated an incidence of 288 cases per 100,000 person-years and a hospital mortality rate ranging between 17% and 26% [2].

More recently, a large-scale study published in *The Lancet* in 2020 examined sepsis trends from 1990 to 2017 across global, regional, and national levels. While the findings showed a decline in age-standardized incidence and mortality that was found to be consistent with earlier reports, the total number of sepsis cases worldwide was at least double previous estimates [2,14,15]. This apparent surge was largely explained by the inclusion of data from countries with lower socioeconomic development, where sepsis rates can soar to 4,000 cases per 100,000 person-years and account for over half of all deaths [14].

These findings underscore an urgent truth: sepsis is likely a leading cause of mortality worldwide and disproportionately affects LMICs. Addressing this crisis will require a coordinated global response not only to improve prevention and treatment, but also to fill the critical gaps in epidemiological surveillance. Equally important is the need for research tailored to resource-limited settings, where current treatment guidelines may not be feasible or directly applicable [16–19].

1.4 Pathogenesis

Sepsis develops through a complex interplay between the invading pathogen and the immune cells of the affected individual [20]. Its early stages are often marked by an overwhelming inflammatory response that is generally referred to as a *cytokine storm* which is later followed by a prolonged period of immune suppression [21,22].

When an infection occurs, the innate immune system mounts a vigorous pro-inflammatory defense. For instance, gram-negative bacteria release lipopolysaccharides (LPS), potent endotoxins that engage Toll-like receptors (TLRs) on macrophages and other immune cells. This interaction triggers a cascade of signaling events leading to the release of pro-inflammatory cytokines and chemokines. However, persistent activation of TLRs can injure vascular endothelial cells, disrupt normal

coagulation pathways, and result in microvascular thrombosis (**Figure 1.2**). These processes progressively impair tissue perfusion, precipitating multi-organ dysfunction and, in severe cases, death [10,23,24].

Studies have reported that the analysis of the pathogens revealed that the top cause for sepsis pathogenesis was due to gram negative bacteria, followed by gram positive, fungal and other infectious agents [25] (**Figure 1.3**). Pathogens vary with different organ dysfunction in septic patients and post sepsis persistent organ dysfunction [44].

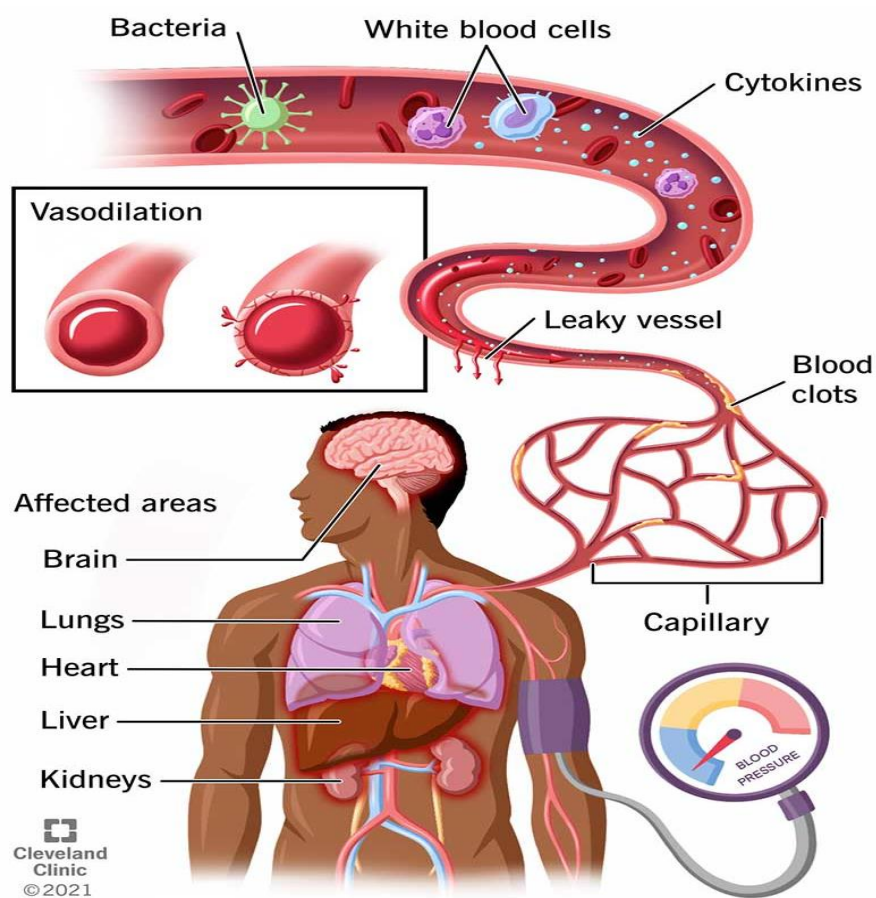


Figure 1.2 Pathophysiology of sepsis. *Cleveland Clinic 2021.*

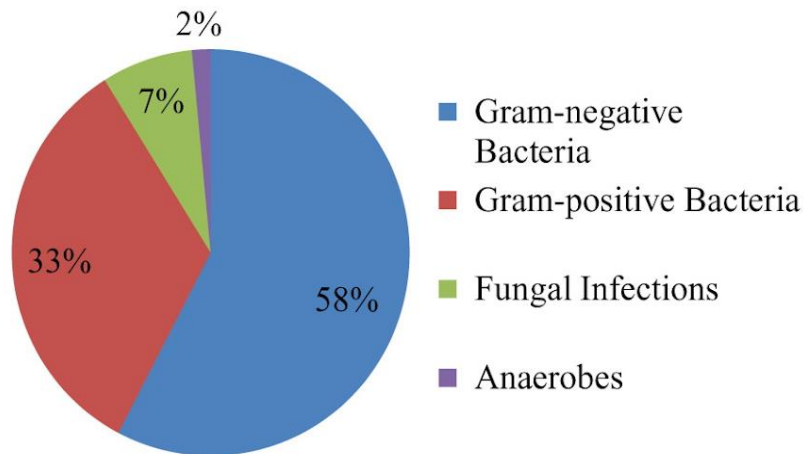


Figure 1.3 Overview of the various causative agents of sepsis [24].

1.5 Inflammation and Macrophages

Dysregulation of the immune system lies at the heart of both the onset and progression of sepsis. In its early phase, the host mounts an exaggerated inflammatory reaction, releasing large quantities of mediators that can injure not only infected tissues but also healthy host cells, including key components of the immune system. As the condition advances, this hyper inflammatory state often gives way to a phase of profound immune suppression, leaving the patient vulnerable to secondary infections and impairing recovery [26].

Studies have suggested that the multi organ dysfunction is mainly associated with uncontrolled hyper and hypo responses of various innate immune cells such as macrophages, neutrophils which makes them central for investigations in this clinical syndrome [27]. Since sepsis is an extremely complex condition that comprises both hyper & hypo activations, its diagnosis and therapeutic design become very challenging. [28–30]. Various strategies have been put forward to understand and develop treatment strategies, some of which include hemodynamics maintenance, fluid resuscitation & ventilation. However, none of these

treatment strategies achieved any significant success [31,32]. As discussed, the importance of innate immune cells, macrophages in particular have been considered central in various stages of sepsis, be it at the initiation phase or during the progression. This is due to its very nature of immunoregulation ability to maintain homeostasis. They are considered as one of the frontline defenses when the body faces pathogenic attacks. [33–38]. Studies have shown that during the course of sepsis progression, macrophages were significantly less susceptible to apoptotic cell death as compared to other immune cells such as dendritic cells or lymphocytes [39–41]. Moreover, these scouting macrophages perform every possible function during the disease development starting from polarization, cytokine production, phagocytosis of microbes, scrap and mutilated cells and organelles. All these actions are performed because of the ability of macrophages to quickly retort to the pathogenic stimuli [42]. Moreover, the differential signaling produced by various organs also directs these macrophages to polarize and act accordingly. These abilities and its important role during sepsis pathogenesis make macrophages effective and efficient therapeutic target to focus on and design appropriate strategies to make best use of them for clinical applications [43]. We have examined the relevant literature on sepsis macrophages since the development of a common definition of sepsis in order to better understand the alterations and the function of the immune system during sepsis.

1.6 Rationale and Objectives

The current work aims to focus on understanding the pathophysiology of sepsis. Sepsis is known to be caused by various pathogens, among which bacterial infections are the prominent ones. The Gram-negative species are the critical culprits behind this chronic condition. TLR4 mediated signaling plays a vital role in the recognition and initiation of the inflammatory responses. TIRAP being central to the TLR4 pathway paves the way for considering it as an appropriate target strategy for therapeutic

purposes. The strategy would be more centralized around the interactions and activity of TIRAP and its activating kinases.

Development and proposal of a completely new compound hold its own challenges. Drug discovery process is very long and wearisome and requires a lot of man power and financial assistance right from the first level continuing till the last one. And even then there is no guarantee of the success of the entire operation. So, to overcome this loophole we drew our attention towards a better and feasible approach of drug repurposing. It involves the identification and screening of the already known compounds that have been FDA approved. These compounds have already been through extensive pharmacokinetics and safety validations, saving our time and energy both in these initial procedures. It also allows us to translate our research more progressively and efficiently to the society.

Hence, to achieve these targets the current work focuses on the following key aspects:

- Analysis of novel intermediates of Dorzolamide (DZD) with enhanced therapeutic potential targeting TIRAP mediated inflammatory signaling
- Deciphering the pathophysiology of a fatal chronic inflammatory condition – Sepsis
- Designing a novel combination therapeutic strategy for sepsis

1.7 Thesis Organization

The present work has been summarized in 7 chapters to gain insight into the current thesis:

Chapter 1: It provides the base for understanding the basic definitions, concepts, and the pathophysiology of sepsis. It discusses the associated inflammation and the diverse pathogenic profile responsible for it. It also covers the importance of the macrophages in sepsis pathogenesis. The rationale behind the study and the objectives to fill those gaps along with the layout of the thesis is discussed.

Chapter 2: It covers the inflammatory signaling conducted via receptors and various adaptor proteins and highlights the biology of TIRAP and its importance in chronic signaling. TIRAP's interaction pattern and its involvement in various inflammatory, infectious, and non-infectious diseases have been discussed in detail. It also sheds light on the potential therapeutic strategies that work around TIRAP.

Chapter 3: It highlights the therapeutic potential of the dorzolamide intermediates targeting TIRAP at the common interface for dampening the downstream inflammatory signaling.

Chapter 4: Unravels the development of a standardized, reproducible, and ethically sound murine mouse model of polymicrobial sepsis using defined cecum slurry bacterial cultures, with reduced variability, ability to store and propagate defined polymicrobial or enriched (Gram-positive/Gram-negative) cultures.

Chapter 5: Depicts a novel combination of a broad-spectrum antibiotic Levofloxacin and a repurposed anti-inflammatory drug Dorzolamide to address the two distinct features of sepsis progression: killing bacteria and healing injured organs by preserving homeostasis. Furthermore, the

antibiotic used has been proposed by us to cause anti-inflammatory effects, resulting in a synergistic combination.

Chapter 6: It concludes the thesis with emphasizing the potential of small molecules to alleviate sepsis pathogenesis via TIRAP mediated signaling pathway. Elucidating the pathophysiology of sepsis via improved slurry mice model. At last demonstrating the efficacy of a combination therapy (LeDoz) that synergistically combats both infection and inflammation.

Chapter 7: It discusses the future of sepsis therapeutics that may revolve around the usage of varied combination strategies, or exploiting the post translational modification of TIRAP. It also highlights the possibility of varied TIRAP expression in comorbid conditions to provide better mechanistical and signaling aspects.

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Chapter 2

Literature Review

CHAPTER 2: Literature Review

2.1 TLRs, Adaptor Proteins, & Chronic Conditions

As discussed in the introduction, inflammation is central to sepsis, which comprises prolonged imbalanced immune response. The inflammatory signaling involves various participants from the cell surface receptors and proteins to downstream nuclear transcription factors. Adaptor proteins are one such mediator that play crucial role in such inflammatory cascades. They contain various domains that act as docking sites for other proteins to interact. Since adaptor proteins are a crucial part of the signalling pathways involved in the pathogenesis of various chronic-inflammatory diseases (**Figure 2.1**), it is necessary to have a detailed insight at a molecular level, which can provide a better understanding of the functionality of these proteins. Inhibiting the interaction between these adaptors and signalling molecules that are engaged at different phases of disease progression would enable them to be used as possible therapeutic targets for various chronic inflammatory diseases. They can be considered as an emerging area to investigate the mechanistic angle of various chronic inflammatory diseases.

The innate immune system is primarily involved in inflammation and includes cells such as fibroblasts, macrophages, dendritic cells, and mast cells, along with circulating neutrophils and monocytes [1]. These cells have specific surface receptors, known as pattern recognition receptors (PRRs), that recognize various components of microbes such as

lipoproteins, and nucleic acids, known as pathogen-associated molecular patterns (PAMPs) [2,3]. PRRs respond not only to external signals but also endogenous stimuli coming from cellular damage via damage-associated molecular patterns (DAMPs). Macrophages are present almost in every tissue and are crucial for maintaining homeostasis [4]. Macrophage polarization is one of the key steps in the inflammatory process [4]. There are two types of macrophages: classically activated (M1) and the alternatively activated (M2). Macrophages possess various kinds of surface receptors that are involved in pathogen recognition and downstream signalling [4]. Among several receptors, the scavenger receptors, mannose receptors, receptors for opsonin, and Toll-like receptors (TLRs) are the most studied [5]. TLRs mediate various signalling cascades that affect the pathogenesis of different chronic inflammatory diseases.

Toll-like receptors (TLRs) are Type I transmembrane proteins distinguished by a leucine-rich repeat (LRR) ectodomain responsible for ligand recognition, a single α -helical transmembrane segment, and a conserved cytoplasmic Toll/Interleukin-1 receptor (TIR) domain that mediates downstream signaling [6,7]. While all members of the TLR family share similar structural features, they differ in ligand specificity, patterns of expression, and in certain cases, their signaling mechanisms [8].

Their subcellular localization also varies. Some TLRs such as TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, and TLR-10 are positioned on the cell surface, where they detect a wide array of microbial cell-wall components. Examples include lipopolysaccharide (LPS) sensed via TLR-4, diacylated and triacylated lipoproteins recognized by TLR-2/TLR-1 and TLR-2/TLR-6 heterodimers, bacterial flagellin (TLR-5), as well as peptidoglycan, lipoteichoic acid from Gram-positive bacteria, and fungal products such as zymosan and dectin-1 ligands [9].

In contrast, intracellular TLRs such as TLR-3, TLR-7, TLR-8, and TLR-9 reside primarily within the endoplasmic reticulum, endosomes, or lysosomes. These receptors are specialized for detecting nucleic acids of microbial origin: TLR-3 recognizes viral double-stranded RNA, TLR-7 and TLR-8 detect viral single-stranded RNA, and TLR-9 senses unmethylated CpG DNA motifs commonly found in bacteria, viruses, and certain parasites [9].

Functionally, TLRs operate as dimers, forming either homodimers or heterodimers (for example, TLR-2/TLR-1 or TLR-2/TLR-6). Signal transduction following ligand engagement is mediated by four key adaptor proteins: myeloid differentiation primary-response 88 (MyD88), TIR domain-containing adaptor protein (TIRAP/Mal), TIR domain-containing adaptor-inducing interferon- β (TRIF), and TRIF-related adaptor molecule (TRAM). These adaptors play essential roles in determining ligand specificity and directing pathway-specific downstream responses. The

TIR domain itself is indispensable for propagating activation signals from engaged TLRs to the intracellular signaling machinery [10,11].

Intracellular signalling pathways are initiated upon the interaction of an inflammatory stimulus with its receptor. Some of the major pathways involved in this scenario are the Janus kinase (JAK)- signal transducer and activator of transcription (STAT) pathway, nuclear factor kappa-B (NF- κ B) pathway, and mitogen-activated protein kinase (MAPK) pathway [12–15]. Although these pathways are well established in their crucial role for immunomodulation, there is still a requirement for a communicator acting as a bridging entity to convey the signal from the receptor to the downstream transcription factors and enzymes to regulate the activity of proteins and gene expressions. Adaptor proteins are such bridging entities that are responsible for conducting signalling cascades in immune cells [16]. These proteins are generally not involved in any sort of enzymatic activity. Rather, they possess unique, conserved motifs and domains to carry out signal transduction [16], permitting the anchoring of upstream protein with the downstream ones [16]. Several such adaptors are involved in the pathogenesis of chronic inflammatory diseases. These protein possess specific domains, such as the Toll- interleukin-1 receptor (TIR) domain, in order to interact with receptors and other proteins for signal transduction [17,18]. Approaches such as the utilization of drugs, namely Thioridazine (TDZ) [19], and gefitinib [20], for targeting inflammatory cascades have already been accepted. Since adaptor proteins hold a vital

position in signal transduction, such proteins can also be potential therapeutic targets in various pathologies [18]. Here in we highlight the involvement of different adaptor proteins in macrophages that play central roles in various chronic inflammatory diseases (**Table 2.1**). Additionally, we provide insights into targeting these adaptor proteins for therapeutic purposes.

2.1.1 Atherosclerosis

Atherosclerosis is a chronic condition where plaque formation occurs in the walls of arteries [21]. Circulating low-density lipoproteins (LDL) play a major role in mediating the advancement of atherosclerosis by translocating into the intima region of the arteries and activating inflammation [22]. Chemokines and cytokines secreted by endothelial cells recruit immune cells to the site of inflammation [23]. Monocytes are one such cell type that can be recruited and attach to endothelial cells through different cell adhesion molecules, namely Vascular cell adhesion molecule 1 (VCAM-1), Intercellular adhesion molecule 1 (ICAM-1). This leads to the migration of monocytes to the intima of the blood vessel, where they differentiate into macrophages [24]. The macrophages engulf oxidised LDL (Ox-LDL) in the intima and are converted to foam cells, which is a hallmark of atherosclerosis [25–27]. Signalling in macrophages via adaptor proteins plays an important role in the uptake of Ox-LDL [28]. Many adaptor proteins are involved in the signalling processes of macrophages [29]. The current section aims to summarize various adaptor

proteins specifically involved in the progression of atherosclerosis that is mediated by macrophages.

Epsin- Epsins are a clathrin-coated pits associated adaptor proteins family present on the cell surface [30]. Epsin plays a major role in atherosclerosis progression [31]. The deletion of epsin in myeloid specific epsin 1 and 2 - Apolipoprotein E (ApoE) double knock-out mice (*LysM-DKO/ApoE^{-/-}*) resulted in a profound decrease in plaque size, lesion number and infiltration of immune cells into the aorta [31]. Low-density lipoprotein receptor-related protein 1 (LRP-1) is an anti-atherosclerotic protein [32]. It is found on macrophage surface and promotes efferocytosis and anti-inflammatory signalling, leading to the inhibition of atherosclerosis [32]. Degradation of LRP-1 as well as activation of epsin promotes pro-inflammatory signalling, facilitating atherosclerosis progression [31].

Myeloid differentiation primary response 88 (MyD88)- MyD88 is an adaptor protein involved in the TLR4 signalling cascade. It is activated when macrophages are stimulated by lipopolysaccharide (LPS) [29]. Binding of LPS to TLR4 initiates downstream signalling through the TIRAP/MyD88 complex and activates the NF- κ B transcription factor [29]. One study reported that *Myd88^{-/-}ApoE^{-/-}* double-null mice fed with high-fat diet showed 65% and 40% decrease in lesion formation in male and female mice, respectively, as compared to the *ApoE^{-/-}* control mice [33]. *Myd88^{-/-}ApoE^{-/-}* double-null mice depicted decreased expression of chemokines such as Monocyte chemoattractant protein (MCP)-1, 2, 3 and

chemokine (C-X-C motif) ligand 16 (CXCL16) [33]. These caused a decrease in the number of macrophages at the affected site [33], suggesting the importance of MyD88 in the activation of inflammation.

2.1.2 Cancer

Cancer is considered as one of the alarming causes of mortality over the globe as per the World health organization (WHO), with nearly one in six deaths in the year 2020 attributed to cancer. During the progression of cancer, various immune cells play functional roles that can activate or inhibit the growth of cancer cells. Among these, Tumour-associated macrophages (TAM) are abundant in the tumour microenvironment. M1 macrophage (classically activated)-derived nitric oxide, cytokines and chemokines can hamper tumour progression, while M2 (alternatively activated) macrophage-derived cytokines and growth factors, such as TGF- β , VEGF, and EGF, support cancer growth [34]. In cancer development, macrophages signalling mechanisms like the Phosphatase and tensin homolog (PTEN)/ Phosphoinositide 3 kinase (PI3K)/ Mammalian target of rapamycin (mTOR) pathway, NF- κ B, MAPK, Wnt/ β catenin, etc. can modulate polarization of macrophage and promote or suppress an immune response [35]. Different types of adaptor molecules actively participate in these signal transduction cascades and a few of them are described below.

Tumour necrosis factor (TNF) receptor associated factor (TRAF)- The involvement of TRAF in signalling pathways has been implicated in cancer pathogenesis, particularly in inflammation. In anaplastic large cell lymphoma (ALCL), the anaplastic lymphoma kinase (TRAF1-ALK) complex leads to NF- κ B activation and acts as an oncogene in TRAF1-ALK expressing cells [36]. A TRAF2-dependent proteolysis pathway in macrophages was shown to regulate pro-inflammatory TLR signalling [37], playing a role in innate immune cells during inflammation and cancer [38]. Targeting the TRAF family of proteins is likely to be a useful strategy to inhibit the progression of cancer and inflammation in macrophage signalling.

Downstream of kinase 3 (DOK-3)- As suggested by their name, these proteins act as downstream targets of protein tyrosine kinases (PTKs) and connect the kinases with their signalling effector molecules. PTKs, Bruton's tyrosine kinase (BTK), and Src family tyrosine kinases, such as Lyn, can phosphorylate DOK-3 leading to its degradation and repression of signalling pathways [39–41]. DOK-1, DOK-2, and DOK-3 deficient mice were reported to develop histiocytic sarcoma (HS), suggesting that these proteins may have a role in tumour suppression and together control the aggressiveness of HS [42].

2.1.3 Asthma

Asthma is an amalgamation of chronic inflammatory responses in the respiratory system that is characterized by goblet cell hyperplasia, and airway hyper-responsiveness (AHR), mucus hypersecretion, eosinophilic infiltration, airway remodelling. Wheezing, cough, shortness of breath, frequent sneezing etc. are some of the symptoms of asthma [43]. Processes such as efferocytosis, phagocytosis and production of anti-inflammatory cytokines that are regulated by macrophages can govern the pathophysiology of asthma. Moreover, inhibition of M2 or M1 polarization was shown to switch inflammation to more neutrophilic or eosinophilic oriented inflammation in mice, highlighting the selective recruitment of neutrophils, and eosinophils by M1 and M2 macrophages (112,113). Adaptor proteins that have been implicated in the pathophysiology of asthma are discussed below.

Signal transducing adaptor protein-2 (STAP-2) - STAP-2 is inherently expressed in macrophages and was shown to positively regulate LPS/TLR4-induced signals [46]. Overexpression of STAP-2 in Raw264.7 cells, a macrophage cell line, was shown to downregulate Macrophage colony-stimulating factor (M-CSF)-induced tyrosine phosphorylation of c-Fms and downstream signalling proteins, such as PI3K/ Ak strain transforming(Akt) and Ras/ERK [47]. STAP-2 binds both inhibitor of nuclear factor-kappa B (IkB) kinase (IKK)- α/β , and MyD88 via SH2 domain resulting in heterotrimeric MyD88, STAP-2, and IKK α/β

complex formation [48,49]. These interactions causes enhancement in MyD88- and/or IKK- α/β -dependent signals, resulting in an increase of NF- κ B activity [50]. Thus, STAP-2 can be considered as a potential therapeutic target to regulate pulmonary inflammation.

Myeloid differentiation primary response 88 (MyD88) – It has been reported that in various asthma models developed by the use of LPS or flagellin (FLA) as adjuvants, MyD88 has been centrally involved in allergic sensitization [51]. *Myd88* expression was necessary for strong eosinophilic inflammation in mouse models of asthma [51]. Signals from TLR are carried by MyD88 that ultimately modulate NF- κ B and various interferon regulatory transcription factors, which control the expression of downstream genes essential for TLR dependent cell activation. These include the expression of Major histocompatibility (MHC) molecules, cytokines, T cell costimulatory molecules, chemokines and their receptors, and many other activation associated genes critical for immune activation in response to invading pathogens [29,52].

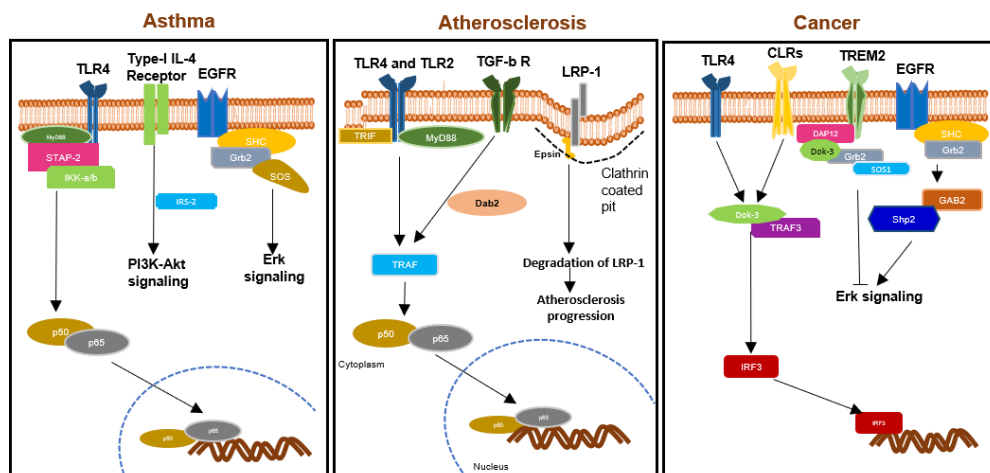


Figure 2.1 The adaptor molecules integrate signalling events critical for chronic inflammatory diseases and cancer progression.

2.1.4 Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease affecting about 1% of the population worldwide. It is primarily characterized as synovitis, which is caused by chronic inflammation in the joints, affecting the joint's lining and leading to swelling and pain, accompanied with bone erosion and joint deformity [53,54]. Macrophages are primarily involved in the innate immune response in RA, with various inflammatory cells infiltrating into the synovium along with macrophages. These macrophages differentiate into the pro-inflammatory phenotype in the synovium [55], which leads to the activation and proliferation of macrophages. In case of RA, the main signalling pathway is via TLRs, which can recognize and respond to pathogens and endogenous ligands.

Toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP)- TIRAP has drawn immense attention in recent years, because it is involved ubiquitously in the inflammatory signalling cascades [56]. The loss of NF- κ B activity in Mal/TIRAP-deficient mice was associated with decreased inflammation in a model of RA [57–59]. Both MyD88 and TIRAP can form a complex with TLRs, which mediates NF- κ B signalling and promotes the secretion of pro-inflammatory cytokines, which help in the progression of RA. There are several therapeutic strategies in

development to inhibit the secretion of pro-inflammatory cytokines via this pathway and, thus, disease progression [60].

Tumor necrosis factor receptor 1(TNFR 1)-associated death domain protein (TRADD), Receptor interacting protein (RIP) and, Tumor necrosis factor (TNF) receptor associated factor-2 (TRAF2) - The main inflammatory cytokine that play a central role in RA is TNF- α [61], which is known to mediate signalling through its intracellular receptors TNFR1 and TNFR2. During TNF- α signalling, first TNF receptor superfamily-1 associated via death domain (TRADD) binds to TNFR1 [62]. TRADD is a signal transducer adaptor protein that is mainly responsible for apoptosis. In RA, TRADD promotes the binding of additional adaptor proteins, such as Receptor Interacting Protein-1 (RIP-1) and TRAF2 [63]. RIP, which plays a major role in inflammatory gene expression and apoptosis, mediates its response via activation of IKB α kinase and nuclear translocation of NF- κ B in RA [64]. TRAF2 is a crucial protein, which can activate the JNK and c-JUN signalling cascade in RA [65]. It might be possible to inhibit the inflammatory response in RA by targeting one or more of these adaptor proteins.

2.1.5 Inflammatory Bowel Disease (IBD)

IBD is an inclusive term for Crohn's disease and ulcerative colitis identified by the gastrointestinal (GI) tract associated inflammation. Persistent diarrhoea, rectal bleeding, abdominal pain,

fatigue, bloody stool, weight loss, etc. are some of the common symptoms of IBD. Vigorous protective immune reactions are expected to develop in response to pathogenic insult, however, similar responses generated against commensal bacteria can be responsible for chronic inflammatory disorders [66]. M1 macrophages are known to hinder cell proliferation and damage tissues, while M2 macrophages support tissue repair and proliferation. The adaptor proteins described below play a vital role in manifesting these characteristic inflammatory responses.

Toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP)- TIRAP is known for its participation in the modulation of signaling pathways downstream of TLR-2 and TLR-4 via the adaptor MyD88 [57]. Tiffany et al. reported defective cytokine production as well as activation of NF- κ B and MAP kinases in response to LPS in TIRAP deficient mice. Thus, these authors concluded that TIRAP was differentially involved in TLR signalling and accounted for the downstream signalling specificity of every single TLR associated with TIRAP [57]. IL-10^(-/-) mice were shown to be predisposed to the generation of spontaneous colitis in the presence of intestinal microbiota. The same study demonstrated that PTEN deletion could disrupt the interaction between TIRAP and TLR5. These results suggest that by regulating the recruitment of TIRAP, PTEN could regulate TLR5-induced inflammatory responses in colitis [67].

Caspase Recruitment Domain Containing Protein 9 (CARD9) -

CARD9 is selectively expressed in myeloid cells, such as neutrophils and macrophages, and regulates the production of chemokines and cytokines critically involved in inflammation, ROS production, and oxidative stress [68]. Mouse models suggest that CARD9 increases the differentiation and polarization of macrophages, modulates T_H17 responses, intestinal epithelial cell restitution, and control of bacterial infection. Following Dextran sulphate sodium (DSS) administration, *Card9*-null mice developed weakened immune response and showed flawed expression of interleukins [69]. Protective functions of allelic *Card9* variants have been observed in IBD patients. These rare protective roles might give an important insight on achieving specific treatments and provide novel and improved therapeutics for IBD [70].

This emphasizes the importance of adaptors in such chronic diseases **(Figure 2.2)** and suggests that therapeutic targeting of these proteins can be a strategy for the regression of such fatal conditions.

Table 2.1 Role of Various adaptor proteins in different diseases.

S.No.	Disease	Adaptor Protein	Functional Role	Ref.
1.	Atherosclerosis	Epsin	Promotes efferocytosis and anti-inflammatory signaling.	[32]
		MyD88	Involved in TLR4 signaling cascade.	[29]
2.	Cancer	TRAF	Leads to NF-kB activation and acts as neoplastic in TRAF1-ALK expressing cells	[36]
		DOK-3	Signals downstream of the PTKs and connects the kinases with their signaling effector molecule.	[39,71,72]
3.	Asthma	STAP-2	Positively regulates LPS/TLR4-induced signaling in	[46]

			macrophages	
		MyD88	TLR signals transduced through MyD88 ultimately activate NFκB and several interferon regulatory transcription factors.	[29,52]
4.	Rheumatoid Arthritis	TIRAP	Both the MyD88 and TIRAP shows their binding to the TLR4 to form a complex which mediated the NF-KB signaling and promotes the proinflammatory cytokines secretion which helps in progression of RA.	[60]
		TRADD – RIP - TRAF2	TRADD promotes the binding of adaptor proteins such as RIP-1 and TRAF2. RIP major role in apoptosis.	[63] [64] [65]

			TRAF2 lead to activation of JNK and c-JUN signalling cascade.	
5.	Inflammatory Bowel Disease (IBD)	TIRAP	Act as bridge between TLRs (TLR-2 and TLR-4) and MyD88.	[57]
		CARD9	Regulates the production of cytokines and chemokines critically involved in inflammation, ROS production & oxidative stress.	[68]

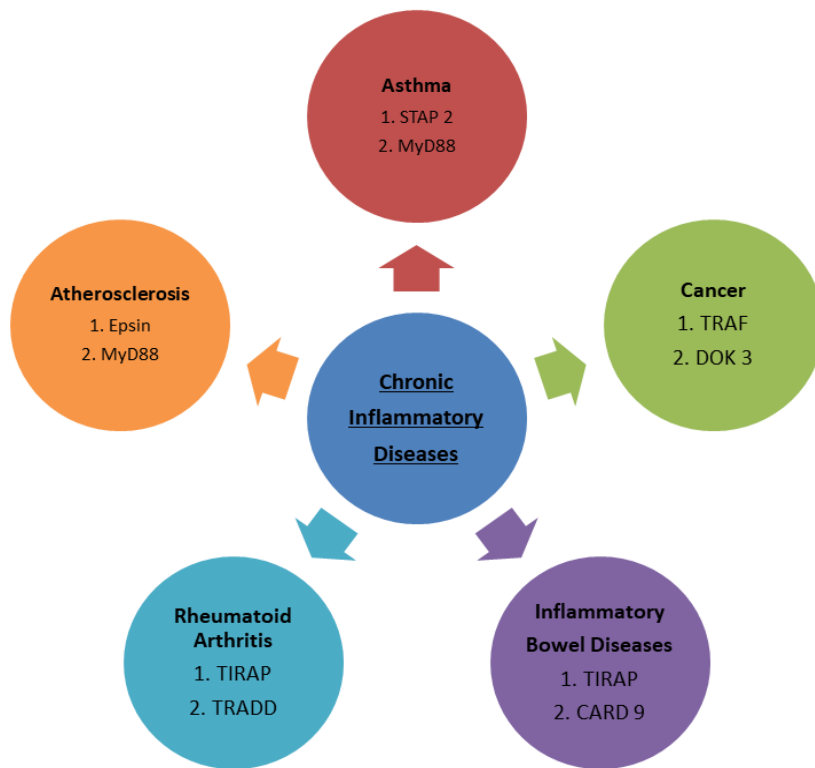


Figure 2.2 Adaptor proteins involved in macrophage signalling in different chronic inflammatory diseases.

2.2 TIRAP Overview

The Toll-interleukin-1 Receptor (TIR) domain-containing adaptor protein (TIRAP)/ MyD88 adaptor like protein (MAL) is an indispensable intracellular facilitator in immune surveillance. Its utility as a bridging entity, and precisely, its ability to interact effectively with diverse components of Toll-like Receptor (TLR)-mediated signaling, has been the subject of investigation [73]. During signal transmission, the binding mechanisms and conformational changes of TIRAP along with its versatility to interact with a variety of intracellular mediators shed light on its significance in immunological responses. A convoluted mesh of protein-protein interactions forms the foundation of TIRAP signaling, which is regulated through its TIR domain [56].

2.2.1 TIRAP structure

The inflammatory diseases involving signaling pathways governed by TIR-domain containing proteins have emerged as a key target for the development of anti-inflammatory therapeutic strategies [18]. Dimerization is one of the central phenomena to the majority of TIR domains for their functionality. These domains span over the length of about 200 amino acids and harbor a 14 residues BB loop motif. Structural studies have shown that the TIR domain of TIRAP can self-associate to form filamentous assemblies that act as a scaffold for downstream signaling complexes. Cryo-electron microscopy analyses have revealed that the TIRAP TIR domain forms proto-filaments composed of two parallel strands arranged through BB-loop-mediated head-to-tail interactions [131]. These assemblies facilitate the formation of higher-order signaling complexes, often referred to as the TIRAP signalosome, where TIRAP interacts with TLR4 and recruits MyD88 to initiate downstream signaling cascades. The cooperative assembly of these TIR-domain interactions enables efficient recruitment of IRAKs and TRAF6,

ultimately leading to activation of NF- κ B-dependent inflammatory responses. Thus, the structural organization of the TIRAP signalosome provides a mechanistic framework for understanding how individual TIR–TIR interactions are amplified into robust innate immune signaling [132,133]. TIR adaptors are known to have both heterotypic and homotypic interactions that are majorly governed by the regions in TIR that comprise evolutionary conserved residues. TIR-mediated signaling heavily relies on the function of the BB loop that can be understood by the presence of such conserved sequences that helps in signal complex assembly and stabilization [74]. TIRAP is structured into two modest domains namely an N-terminal phosphatidylinositol 4, 5-bisphosphate (PIP2) binding domain (PBD) and a C-terminal Toll/interleukin-1 receptor (TIR) domain with a total length of 221 amino acids (**Figure 2.3**). TIRAP's positioning on the membrane is very crucial to initiate the downstream signaling. Phosphatidylinositol 4-Phosphate 5-Kinase α (PIP5K α) is responsible for the generation of PIP2 that acts as a docking site for TIRAP facilitated via PBD [75,76]. TIRAP-mediated inflammatory pathways accommodate an intricate network of protein-protein interactions that involves receptors like TLR, kinases such as PKC δ , BTK and p38 MAPKs and transcription factors such as NF- κ B and AP-1 [20,56]. TIRAP's TIR domain displays structural differences in contrast to the canonic TIR domains. It comprises an extended AB loop in the oxidized state that links α A and α B that are developed due to the absence of α B helix classically situated between β B and β C strands [18]. Adaptor appointment and dimerization ability involved in TLR signaling is greatly affected by such structural reorientation that transposes the BB loop into a unique three-dimensional configuration which is central to the interaction interface. Global research efforts have prioritized disrupting TIR–TIR interactions, driving the creation of innovative therapeutic agents. These include engineered peptides, peptidomimetic compounds, and small-molecule inhibitors designed to block these critical molecular

interfaces. Such strategies aim to modulate pathological immune signaling offering potential treatments for conditions linked to dysregulated TIR-mediated inflammatory responses [18,77].

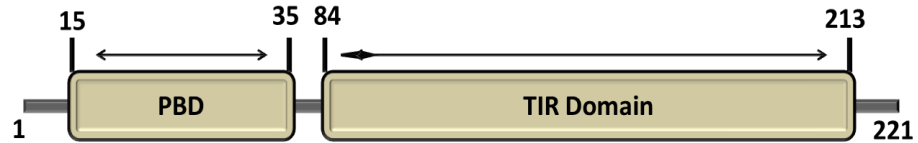


Figure 2.3 Structural organization of TIRAP

2.3 TIRAP-mediated protein-protein interactions in inflammation

TIRAP's pivotal function in mediating innate immunity makes it a key regulator of inflammation. Dysregulation of TIRAP expression or function contributes to a spectrum of pathological conditions, ranging from autoimmune and chronic inflammatory disorders to infectious diseases. Excessive TIRAP-mediated signaling is implicated in sepsis and systemic inflammation due to overproduction of pro-inflammatory cytokines such as TNF- α and IL-6. Conversely, loss-of-function mutations in TIRAP may confer protection against diseases like malaria and tuberculosis by dampening hyper-inflammatory responses [78]. TIRAP has been recently investigated to be involved not only with TLR2/4 but also with other receptors such as interferon-gamma receptor (IFNGR) and receptor for advanced glycation end-products (RAGE), highlighting its wide range involvement in immune signaling [79]. TIRAP is known to interact with a wide array of proteins such as Bruton's tyrosine kinase (BTK), protein kinase C delta (PKC δ), cytoplasmic linker protein CLIP170 and many more, which not only are the interacting partners but also involved in modulating the activity of TIRAP via phosphorylation, activation, and degradation [56,80,81]. Understanding this network of varied interactions of TIRAP would greatly help us to unravel the potential signaling as well

as therapeutic aspects in chronic inflammatory diseases (**Figure 2.4**). In this context, we have consolidated and analyzed major reported TIRAP protein–protein interactions, aiming to present a comprehensive view of the inflammatory signaling landscape in which TIRAP operates [56].

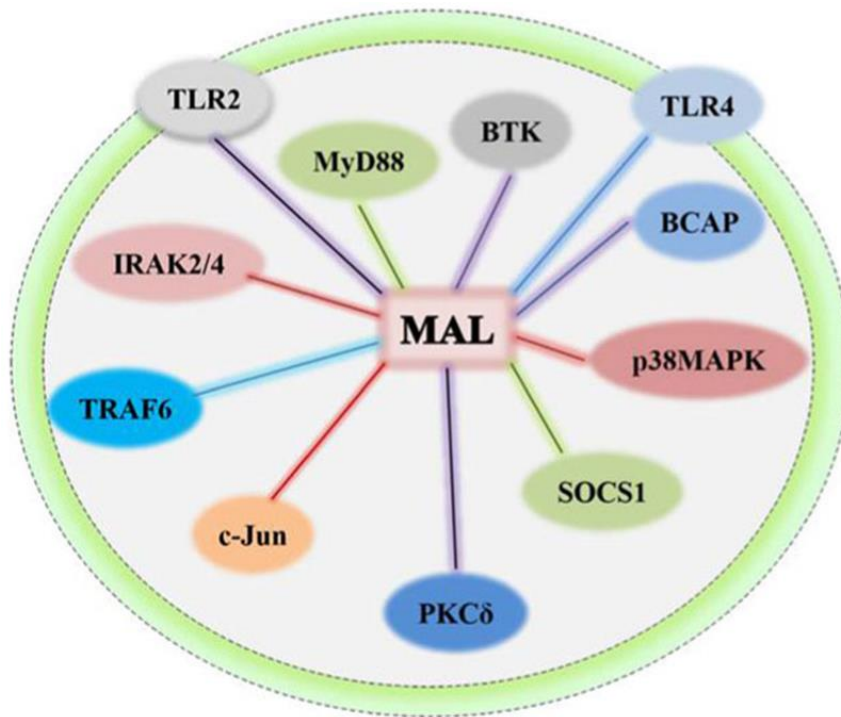


Figure 2.4 Graphical representation of major protein- protein interaction linkage of TIRAP/MAL.

2.3.1 TIRAP and Bruton’s tyrosine kinase (BTK)

TIRAP functions as a key adaptor in TLR signaling by first binding to PIP2 at the membrane, enabling engagement with activated TLRs [82]. Tyrosine phosphorylation, primarily mediated by BTK—and in part by PKCδ—at residues Y86, Y106, Y159, and Y187 is essential for TIRAP–MyD88 complex formation and activation of NF-κB and MAPK pathways [83–85]. Mutations at these sites disrupt BTK interaction and act in a dominant-negative manner. BTK activation, often driven by Src kinases, precedes this phosphorylation, while SOCS1-mediated ubiquitination

ensures rapid degradation of phospho-TIRAP, preventing prolonged signaling [86]. This tightly controlled cycle balances effective immune activation with the avoidance of chronic inflammation, though excessive BTK activity has been linked to autoimmune pathogenesis [87].

2.3.2 TIRAP and Protein Kinase C delta (PKC δ)

PKC δ has emerged as one of the most important interacting partners that regulate TIRAP's activity [80,88]. PKC δ act as an activating kinase that interacts and phosphorylates TIRAP at Y106 thereby causing its activation. The activated TIRAP then interacts with the downstream molecules such as kinases and transcription factors initiating the inflammatory response [56]. PKC δ deficiency in mice have shown to dampen the macrophage bactericidal activity, along with reduced levels of pro-inflammatory mediators such as cytokines, reactive oxygen species (ROS), and nitric oxide (NO) [89]. Its deficiency was also found to be in line with increased susceptibility to mycobacterium resulting in higher mortality [90]. Baig et al. reported a novel heterotrimeric signaling complex involving TIRAP, PKC δ , and p38 MAPK. This complex's activation was found to be responsible for the pro-inflammatory response upon LPS stimulation [56,88].

2.3.3 TIRAP and p38 mitogen-activated protein kinase (MAPK)

p38 MAPK, a prominent MAPK family member, is known to be activated upon TIRAP's interaction in LPS stimulated conditions. Its activation is responsible for the activation of the downstream transcription factors such as AP-1 and NF- κ B that ultimately results in the production of various pro-inflammatory cytokines [91–93]. The recent heterotrimeric complex formation has also shed light on the importance of such direct interactions of protein molecules during inflammatory signaling [56,88].

2.3.4 TIRAP and c-Jun

TIRAP has been known for its ability to interact with various proteins upon LPS stimulation via TLR4. The stimulation ultimately leads to the activation of the transcription factors that marks the end point of the signaling cascades with the production of inflammatory mediators. One such crucial transcription factor is c-Jun that is a key component of AP-1 unit. It is known to get phosphorylated by MAPK's certainly at the residues Ser 63 and Ser 73 resulting in its activation [94]. The inflammatory activity is governed by its ability to dimerize either with itself or with components of AP-1 unit [94–97]. The recently proposed direct interaction of TIRAP and c-Jun not only suggest its signaling importance but also sheds light on the potential therapeutic aspect. Gefitinib, a small molecule compound has been found to target this novel interaction and dampen the associated inflammatory response. The results showed that in both in vitro and in vivo studies treatment with gefitinib following LPS stimulation prominently reduced the expression of various pro-inflammatory cytokines along with increasing the survival rates of the mice [20].

2.4 TIRAP in Pathologies

TIRAP's action is highly modulated by its ability to interact with wide variety of proteins and various post-translation modifications [56]. During infections the pathogens are known to take the advantages of such modifications that help them to evade the regulatory check-points for their successful progression. *Brucella* is known to exploit such mechanisms via modulating the activities of proteins such as TcpB [98], or host proteins like CLIP170 and Triad3A [81,99]. TIRAP being central to various TLR mediated signaling makes it a promising therapeutic strategy. Studies using small molecule inhibitors or peptides have been recently reported that aim to target TIRAP differently. TIRAP's membrane localization, its interaction with various protein partners, or its activation are few major therapeutic strategies adopted for chronic conditions such as sepsis, and

autoimmune disorders [82,100,101]. TIRAP's dysregulation has been linked to different diseases ranging from infectious, and non-infectious to even cancer. As the past and ongoing efforts help us to better understand the complex nature of TIRAP's biology and its association with diseases, they open up a wide range of opportunities from therapeutic point of view for researchers and pave the way for detailed investigation in pathologies (Figure 2.5).

2.4.1 TIRAP in inflammatory diseases

Chronic inflammation has remained a global concern for a very long period of time. TIRAP has emerged as a key mediator for such chronic responses primarily via TLR pathways [56,73,102]. Abnormal regulation and modulation of TIRAP have been implicated in numerous chronic inflammatory conditions including asthma, arthritis, silicosis, and liver fibrosis.

Asthma and Airway Inflammation

Asthma is a classic example of chronic airway inflammation. TIRAP exaggerates the inflammation in response to the various pathogenic encounters or endotoxin inhalation [103]. miR-146a-5p has been found to promote M2 polarization and alleviate M1 polarization via inhibiting the TIRAP/NF- κ B pathways. It primarily focused on the NLRP3 inflammasome inhibition [103].

Silicosis

Silicosis is a chronic lung disease that is primarily caused by the exposure and inhalation of silica crystal containing dust. The condition worsens with an uncontrolled cytokine storm and cell death by apoptosis [104]. Results showed that inhibition of TLR4 mediated signaling majorly dependent on the TIRAP/MyD88 axis decreased the expression and

release of TNF- α , and other inflammatory cytokines, dampening the inflammatory cascade that drives fibrosis [104].

Radiation-Induced Liver Fibrosis

Radiation induced liver fibrosis is known to get promoted by the activity of an RNA demethylase ALKBH5 that acts on radiation induced hepatic stellate cells. A study showed that ALKBH5 promoted liver inflammation via stabilizing TIRAP mRNA through m6A demethylation [105]. As a result, the TIRAP becomes persistent and the downstream signaling is intensified. Consequently, this demonstrates the viable treatment approach focused on the epigenetic control of TIRAP [105].

Corneal Inflammation

Corneal inflammation has been associated with TLR2, TLR 4 and TLR5 mediated pathways. The results showed that in *in vivo* models TIRAP was found to be involved in TLR2/4 mediated signaling but not in TLR5 mediated signaling. TIRAP's expression was found to be in line with increased levels of cytokines and chemokines. This highlights the key role of TIRAP in keratitis induced corneal inflammation (http://rave.ohiolink.edu/etdc/view?acc_num=case1259983828).

Arthritis

TIRAP is found to be involved in mediating inflammation associated with joints [58]. Results showed that absence of TIRAP in mice reduced the recruitment of immune cells as well as provided resistance to the collagen induced-arthritis that resulted in decreased swelling cytokine release and cartilage erosion in synovial tissues [58].

2.4.2 TIRAP in infectious diseases

During infection TIRAP plays vital roles in recognition, clearance and immune activation against the pathogens via TLRs in the participating immune cells

2.4.2.1 Bacterial Infections

TIRAP in Brucellosis and Immune Evasion

TIRAP is well known for initiating inflammatory response upon pathogen encounter. Some pathogens such as *Brucella* have developed evading mechanisms to bypass this inflammatory response and thereby spread the infection and damage the host [98]. They are known to harbor a protein TcpB that specifically targets TIRAP and dampens the inflammatory response. Thus the impaired signaling opens the loop hole for pathogens progression in the body. This highlight shows that TIRAP can be manipulated under different circumstances [98].

TIRAP and *Helicobacter pylori* Infection

Helicobacter pylori infections are one of the major causes of gastric inflammation. Studies have shown that the inflammatory response mediated via TIRAP plays a prominent role in bacterial clearance [106], and regulating the mucosal immunity. In vivo studies have highlighted that TIRAP deficiency affected the release of inflammatory cytokines and impaired the pathogen clearance [106].

2.4.2.2 Viral Infections

Herpes Simplex Virus Type 1 (HSV-1)

HSV-1 results into prolonged infection in the peripheral nervous system. The mode of action involves the production of IL-15 cytokine [107]. Results showed that HSV-1 triggers the response via TLR2 mediated pathway in a TIRAP dependent manner. The disruption or impaired

recruitment of TIRAP could abolish the signaling and thereby the IL-15 response [107].

COVID-19

SARS CoV-2 infection was reported to result in higher expression of TIRAP in the patients. This was responsible for the exaggerated hyper inflammatory response [108]. Studies depicted that the increased expression of TIRAP was in line with the increased levels of inflammatory cytokines during the disease progression. This suggests the central importance of TIRAP in SARS Cov-2 pathogenesis [108,109].

2.4.3 TIRAP in cancer

Apart from the well-known roles in various chronic inflammatory disease TIRAP has also been found to play vital roles in cancer at varied stages. It contributes at different levels such as supporting the survival of cancer cells, helping in progression, or regulating the tumor microenvironment [110,111]. TIRAP when silenced resulted in reduced expression of anti-apoptotic gene Bcl-2, and transcription factor NF- κ B in U251 glioma cells. This indicates that TIRAP's presence promotes anti-apoptotic genes and AKT/NF- κ B axis that is crucial for the survival of the tumor cells [129]. Similarly, in non-small lung cancer (NSCLC) TIRAP was found to be upregulated and that enhanced the proliferation of the cancer cells. Whereas the proliferation significantly reduced when TIRAP was silenced [130]. During bladder cancer, it is found that the levels of S100A8/A9 are reportedly high. Studies showed that S100A8/A9 regulates cancer progression activity through TLR4 pathway. And it was revealed that upon TLR4 activation TIRAP was activated that was eventually responsible for the activation of TPL2. TPL2 is the main culprit behind the tumor growth, migration, and invasion. When this axis was inhibited it resulted it reduces tumor growth and invasion in *in vivo* models [112].

2.4.4 TIRAP polymorphisms and disease susceptibility

Genetic variation in TIRAP has been studied in the recent past highlighting one major single nucleotide polymorphism (SNP), rs8177374, which results in a serine-to-leucine substitution at amino acid 180 (S180L) [113]. This mutation has emerged as an interesting aspect in various pathologies.

Functional insights into the S180L Polymorphism

The S180L variation is known to affect TIRAP's ability to conduct the inflammatory response in TLR2/4 pathways. This might affect the inflammatory response completely and thereby providing an upper edge to the pathogens to evade the immune system. But that is not the case, in fact this variant in the heterozygous condition did impair the TIRAP's ability but only to abolish its over activation and unwanted prolonged response. The short term healthy acute response is still kept intact necessary for combating the pathogenic load and providing strong defense. Thus rs8177374 SNP (S180L) has proved to be a hot topic of discussion and research among the scientific community for delving into its modulatory future aspects in various chronic diseases [113,114].

2.4.5 TIRAP in non-infectious pathologies

TIRAP has already been well established when it comes to infectious and pathogenic diseases. But in the recent past studies have shown its participation in various non-infectious pathologies as well. This highlights its ubiquitous role in such wide array of pathologies.

Cardiovascular Disease and Thrombosis

TIRAP's omnipresence in various types of cells has highlighted its crucial role in different conditions. Studies have shown that TIRAP gets upregulated in platelets during myocardial infraction (MI). The major stimulant known for its activation, LPS has also been reported to get

increased possibly due to impairment in gut barriers. This increased level of LPS ultimately activates the TLR4 mediated signaling which in the TIRAP dependent manner leads to increased platelet accumulation and thrombosis establishment. Not just the endotoxins but also some DAMPs have been reported to activate this TIRAP mediated inflammatory response. HMGB1 (High mobility group box 1) activates the TLR2/TLR4 cascades resulting into the development of cardiovascular inflammation [115,116].

Neuroinflammation and Brain Injury

Subarachnoid hemorrhage (SAH), traumatic brain injury, and stroke have been associated with prolonged inflammatory responses. Studies have reported that a plant derived isoflavone named Biochanin A dampens and reduces the neuroinflammation associated with traumatic brain injury and SAH via targeting the TIRAP- NF- κ B axis [117]. TLR2/4 signaling has also been found very crucial in chronic neuroinflammatory conditions such as Alzheimer's disease (AD). Reports suggest that microglial activation is prominently increased via pathways mediated by these receptors. Thus, suggesting that TIRAP may be involved in an indirect manner [118].

Hematologic Disorders: MDS and Bone Marrow Failure

TIRAP with more and more investigation has found its role in such conditions which one could have never even thought of. Recent studies have found that in hematological conditions such as myelodysplastic syndromes (MDS), TIRAP was found to be highly upregulated which resulted in defective hematopoiesis and caused bone marrow failure [119]. TIRAP was also found to have higher levels during del 5q MDS, a subtype of MDS [120]. Another study reported upregulated expression of TIRAP in hematopoietic stem cells that caused marrow failure in in vivo models.

TIRAP was also found to promote hematopoietic stem cell exhaustion and cytopenias [121–123].

Autoimmune Diseases: Sjögren's Syndrome

Sjögren's syndrome (SS) is characterized by the presence of mucins MUC5B and MUC7 in the extracellular matrix of salivary glands. These mucins cause increased levels of pro-inflammatory cytokines. Results showed that these mucins act as ligands for TLR4 and initiate the downstream inflammatory cascades. TIRAP dependent signaling caused increased levels of IL-6, TNF- α , and interferons that were responsible for promoting this response and worsening the condition central to SS [124].

2.5 Therapeutic targeting of TIRAP

TIRAP being central to such diverse range of disease has found itself as a potential therapeutic target all over the globe. Previously, Rajpoot et al. explored the TIRAP-PKC δ axis and successfully repurposed an FDA approved compound, Dorzolamide (DZD), targeting the interface residues of PKC δ on TIRAP thereby dampening the downstream inflammatory signaling [125]. Though DZD attenuated the PKC δ mediated TIRAP activation, BTK-mediated phosphorylation remains an area to explore. In one study, molecular-docking and dynamics simulations predicted that the plant alkaloid Narciclasine binds with high affinity to the TIRAP TIR domain as well as other LPS–TLR4-pathway proteins, stabilizing the complexes and thereby suppressing pro-inflammatory signaling [126]. In another investigation, Phycocyanin treatment up-regulated miR-3150a-3p, miR-6883-3p and miR-627-5p, which depleted TIRAP transcripts and reduced cellular proliferation, establishing a post-transcriptional checkpoint on adaptor availability [127]. Interestingly, one study demonstrated that synthetic decoy peptides derived from the TIRAP TIR domain competitively interrupted TIRAP–MyD88 recruitment, abolishing downstream NF- κ B activation and highlighting the value of peptide-based

blockade of adaptor–adaptor contacts [128]. Collectively, these observations indicate that small molecules and miRNA inducers curtail TIRAP through binding or expression control, whereas peptide modalities can potentially better dismantle the protein–interaction surfaces essential for signal propagation. These findings highlight the potential of targeting this domain of TIRAP using small molecules, dual-molecule strategies, or peptide inhibitors as promising approaches to inhibit TIRAP function and disrupt downstream inflammatory signaling pathways. These strategies could pave the way for novel treatments for chronic inflammatory diseases, providing both structural insights and targeted interventions.

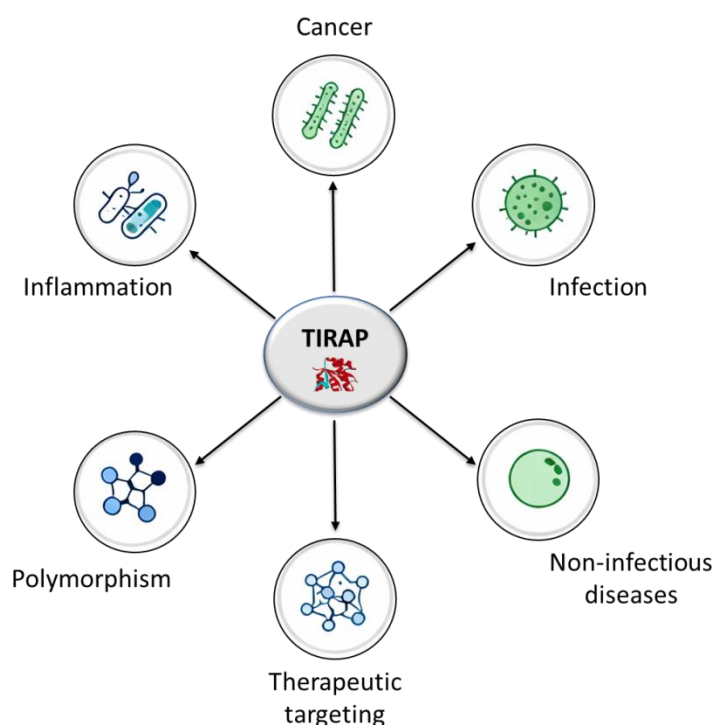


Figure 2.5 Physiological and clinical implications of TIRAP signaling. Schematic representation of TIRAP’s involvement in disease pathogenesis (infection, inflammation, cancer, and non-infectious diseases), and its potential as a drug target (therapeutic targeting).

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Chapter 3

Dorzolamide intermediates
with potential anti-
inflammatory activity

Chapter 3: Dorzolamide intermediates with potential anti-inflammatory activity

3.1 Graphical Abstract

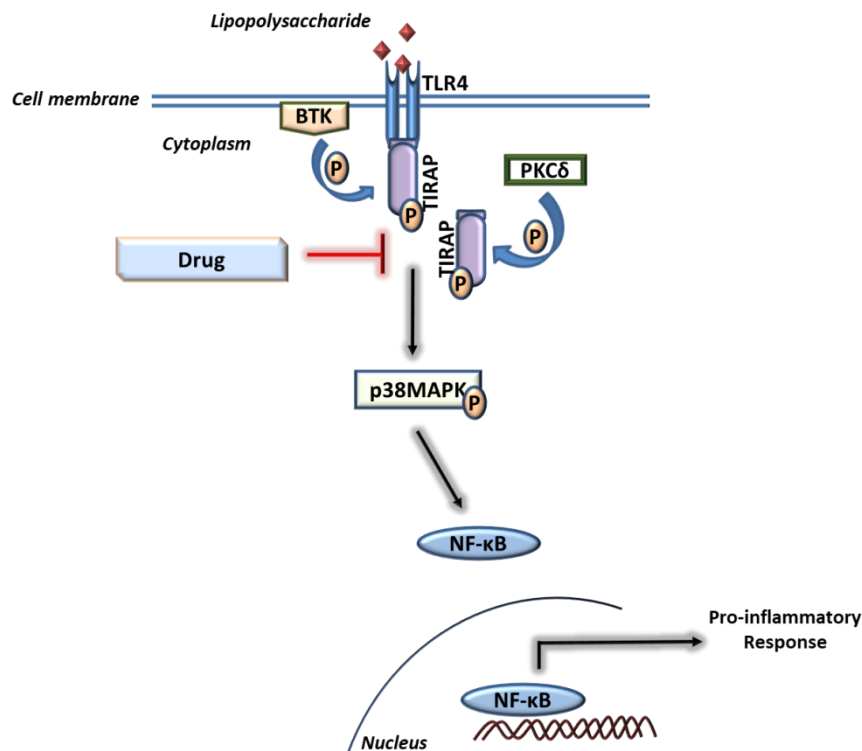


Figure 3.1 Depicting the essential interactions of TIRAP with upstream kinases PKCδ and BTK in TLR4-driven inflammatory signaling, and highlighting the potential of dorzolamide (DZD) derivatives to simultaneously inhibit these interactions at a common interface as an effective approach against chronic inflammation.

3.2 Overview

Dorzolamide (DZD), a Carbonic anhydrase (CA) inhibitor clinically used to lower intraocular pressure, exhibits anti-inflammatory effects owing to the drug's ability to inhibit the TIR domain-containing adaptor protein (TIRAP)-mediated signalling in macrophages. Here, we investigated whether DZD intermediates also demonstrate any anti-inflammatory property like DZD but with a reduced inhibition of CA. We found that several intermediates of DZD show increased binding to TIRAP at the common interface of kinases, such as Protein kinase C-delta (PKC δ) and Bruton's tyrosine kinase (BTK). Such binding results in a decreased activity of TIRAP, p38 Mitogen-activating protein kinases (MAPK), and p65, which are essential for major inflammatory signaling pathways. Remarkably, the DZD intermediates were more effective than DZD in decreasing the mRNA expression levels of pro-inflammatory cytokines in Lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. The DZD intermediates also exhibit a reduced binding energy to CA II and CA IV, highlighting their improved specificity as anti-inflammatory compounds with decreased unwanted biological effects. Furthermore, we validated the anti-inflammatory effect of the most efficient DZD intermediate, DRZ V, in a model of mouse sepsis. DRZ V-treated septic mice exhibited improved survival compared to DZD-treated septic mice. Our data indicate that the tested DZD intermediates are more effectual in dampening TIRAP-mediated inflammatory signaling as compared to DZD. Thus, DZD intermediates may be a promising option for developing novel anti-inflammatory therapeutics.

3.3 Introduction

Macrophages are essential immune cells in the host defense system that initiate, activate, and resolve inflammatory signaling. Monocytes and macrophages are involved in inducing innate immune responses against pathogens and can be found throughout the human body in resident and

circulating forms [1,2]. These phagocytic cells express unique pattern recognition receptors (PRRs), which play significant roles in first-line defence by recognizing a wide range of intra- and extracellular stimuli, such as damaged cells or Pathogen-associated molecular patterns (PAMPs), a variety of microbial components like nucleic acids and lipoproteins [3–5]. The presence of various PRRs is crucial for not only recognition of pathogens but also mediating the downstream signaling [6]. The common receptors are Toll-like receptors (TLRs), Nod-like receptors (NLRs), scavenger receptors, mannose receptors, receptors for opsonin and other types of nucleic acid sensors [1,7]. Amongst these, TLRs are the major pattern recognition receptors of the innate immune system [8–10]. Endosomal TLRs (i.e., TLR3, 7/8, and 9) identify nucleic acids of viral or bacterial origin, whereas surface-expressed TLRs (i.e., TLR1, 2, 4, 5, 6, and 10) recognize bacterial, fungal, and parasitic PAMPs [8]. When an inflammatory stimulus interacts with its receptor, specific intracellular signaling events are activated. The Mitogen-activating protein kinases (MAPKs), the nuclear factor kappa-B (NF- κ B), the janus kinase signal transducer/signal transducer and activator of transcription (JAK/STAT) pathways are some of the primary signaling cascade involved in the inflammatory conditions [11,12]. When TLR4 is activated in macrophages in response to an extracellular stimulus like lipopolysaccharide (LPS), it undergoes conformational changes in its cytoplasmic Toll/Interleukin-1 receptor (TIR) domain, which then recruits the adaptor protein TIR domain-containing adaptor protein (TIRAP) [13,14]. Interaction of TIRAP with myeloid differentiation primary response 88 (MyD88) triggers the downstream inflammatory signaling [15]. Besides MyD88, TIRAP is known to interact with multiple proteins both upstream as well as downstream [16] , making it a potential target for therapeutic purposes. TIRAP activation is primarily initiated downstream of Toll-like receptor signaling, particularly following TLR dimerization and TIR–TIR domain interactions that recruit TIRAP and promote assembly of the

downstream signaling complex. In addition to this canonical mechanism, kinases such as Bruton's tyrosine kinase (BTK) and Protein kinase C delta (PKC δ) have been reported to play important regulatory roles in modulating TIRAP activity and its downstream signaling. Thus, while BTK and PKC δ are key modulators of TIRAP-mediated responses, they represent part of a broader regulatory network controlling TIRAP activation [17]. These two kinases directly interact with TIRAP to activate a number of downstream signaling cascades that mostly involve MAPK and NF- κ B pathways [18,19]. The phosphorylation of TIRAP within its TIR domain mainly at Y86, Y106, Y159 and Y187 residues is critical for the inflammatory response [17,20,21].

Our major aim in this work was to target the common interface of TIRAP to inhibit its interaction with both BTK and PKC δ , thereby completely abrogating the downstream inflammatory signaling. Rajpoot et al., recently reported that dorzolamide (DZD), clinically used to treat increased intraocular pressure by inhibiting carbonic anhydrase (CA), is also an effective inhibitor of TIRAP - PKC δ interaction implicated in inflammatory responses [29]. Thus, DZD can be potentially repurposed for treating inflammation. However, the ability of DZD to inhibit CA activity complicates its usage as anti-inflammatory agent. Therefore, in this study, we screened the synthetic intermediates for chemical synthesis of DZD to determine whether any of them can retain anti-inflammatory activity but show no inhibitory effect against CA.

3.4 Methods

3.4.1 DZD and DZD chemical synthesis intermediates

Dorzolamide (DZD), 6-Methyl-4-oxo-5,6-dihydro-4H-thieno[2,3-b]thiopyran-2-sulfonamide (DRZ V), 5,6-Dihydro-4-hydroxy-6-methyl-4H-thieno[2,3-b]thiopyran-2-sulfonamide 7,7-Dioxide (DRZ VII), and N-Methyl-7,7-dioxido-2-sulfamoyl-5,6-dihydro-4H-thieno[2,3-b]thiopyran-

4-yl acetamide (DRZ VIII) were obtained from the Bakul Pharma Private Limited (<https://www.bakulpharma.com/intermediates.html>).

3.4.2 Murine sepsis model

All the animal experiments were approved by the Institutional Animal Ethics Committee of Acropolis Institute of Pharmaceutical Education and Research and following the policies of the Committee for Control and Supervision of Experiments on Animals, Government of India. Animal studies were conducted using wild-type Swiss albino mice. The mice used in the study were 6–8 weeks old, weighed ~25-30 g, and were kept in pathogen-free conditions with an optimal temperature of 22–25°C and relative humidity of 55–60%. A 14-hours light and 10-hours dark cycle was maintained in the animal facility. Mice had ad libitum access to feed (dry pellet) and clean water. The mouse survival study was conducted in an LPS-induced endotoxemia model. The male mice were randomly divided into five groups of 14 mice each – namely, Control, LPS, LPS + DZD, LPS + DRZ V and, LPZ + DRZ VII treatment groups. All treatments were given through the intraperitoneal (ip.) route of injection. A single lethal dose of 25 mg/kg of LPS was injected to induce sepsis. A single dose of DZD and intermediates of 25 mg/kg was administered 1 hour before LPS injection. The control group of mice only received the vehicle PBS solution. The survival rate was recorded for 72 hours. Mice lung tissue was isolated at 10 hour after treatment (as discussed earlier in the survival study, n = 3 per group), washed with sterile PBS and fixed with 4% PFA for slide preparation. The fixed lung tissue sections were dehydrated and embedded in paraffin for sectioning (5 µm thick sections). Hematoxylin and eosin (H&E) staining were then performed. Images were captured using the transmitted light microscope at 40× magnification.

3.4.3 Cell culture

The murine macrophage RAW 264.7 cell line was obtained from the National Centre for Cell Science Cell Repository (Pune, India). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with heat-inactivated 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 g/ml streptomycin (Gibco). All cells were grown in a humidified incubator with 5% CO₂ at 37°C until reaching confluency. Unless otherwise stated, cells were treated in all treatment studies with 1 µg/ml LPS (Sigma), 1 µM dorzolamide, and the intermediates at the given time points.

3.4.4 Cell viability assay

The MTT assay (Sigma) was used to evaluate the viability of cells treated with drugs. Briefly, 96-well plates with 10,000 cells per well were plated with the exponentially developing cells and left incubating for one night. Cells were then incubated for 24 hours with various doses of DZD and intermediates (0, 0.1, 0.5, 1, 5, and 10 µm/mL). Each well received 70 µL of MTT (0.5 mg/ml) after 24 hours of treatment, and the plates underwent an additional 2–4 hours of incubation at 37°C. The MTT solution was then discarded and 100 µL of DMSO was added in its place. A microplate reader was used to measure the absorbance at 570 nm [22]. The experiments were performed in triplicates.

3.4.5 Computational studies

Efficacy and safety studies are important for exploring the medicinal potential of anti-inflammatory drugs. In this study, we resorted to computational techniques to speed up the processes of identifying viable candidates for additional testing. Specifically, to predict the pharmacokinetic characteristics of the tested intermediates, we employed the following two computational tools: pkCSM [23] and ADMETlab2.0 [24].

3.4.6 Immunofluorescence

On sterile coverslips, cells were cultured until 70–75% confluency was achieved, and then they were treated with LPS, DZD & intermediates as directed. PBS (pH 7.4) was used to wash the cells twice at room temperature (RT) prior to fixation. For 15 minutes the cells were then fixed in freshly made 4% paraformaldehyde in PBS. Triton-X (Thomas Baker) of 0.01% at room temperature was used for permeabilization (10 minutes) followed by washing. Non-fat blocking milk (Bio-Rad) 5% was used for blocking purposes (1hour) on shaker. For immunostaining, the primary antibodies against TIRAP (Santa Cruz Biotechnology), PKC δ (Invitrogen), BTK (Invitrogen), or NF- κ B /p65 (Santa Cruz Biotechnology) were added at a dilution of 1:200 prepared in the TBST buffer, then at 4°C cells were incubated. Secondary antibodies Alexa Fluor 594/488 were added and provided an incubation period of 1 hour in no light area. Following TBST wash DAPI (Sigma) was used for nuclear counterstaining of cells before mounting them on coverslips. A confocal laser scanning microscope (Olympus) was utilized to study the cells, and colocalization analysis was performed using ImageJ and the JACoP plugin.

3.4.7 Quantitative reverse transcriptase PCR

Using the RNAiso Plus reagent (Takara Bio Inc.), total RNA was extracted from RAW 264.7 macrophages in accordance with the manufacturer's instructions. To determine the purity and concentration of the obtained RNA NanoDrop spectrophotometer (Thermo Scientific) was used. The cDNA Synthesis Kit (TAKARA) was used to reverse-transcribe a total of 1000 ng of mRNA for cDNA production according to the manufacturer's instructions. An Applied Biosystems' StepOnePlus Real-Time PCR System was used to carry out the real-time quantitative PCR (qPCR) utilizing the SYBR green master mix (Applied Biosystems). To quantify gene expression of GAPDH, TNF, IL-1, IL-6, IL-12, and IL-23,

the experiments were run in triplicate. The primer sequences are listed in Supplementary Table S4.1. GAPDH was used as a reference gene to standardize the relative gene expression levels of cytokine genes. The cycle threshold value was determined using the $2^{-\Delta\Delta C_t}$ technique.

3.4.8 ELISA

Serum samples were taken from each experimental mouse to measure the inflammatory response. The conventional ELISA method was used to assess pro-inflammatory cytokine concentrations.

3.4.9 Immunoblotting

RAW 264.7 cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer having protease and phosphatase inhibitors. Bradford assay dye reagent was utilized to find the protein concentration followed by resolution on 10% SDS-PAGE and blotted on nitrocellulose membrane (Bio-Rad) followed by blocking via 5% non-fat milk (Bio-Rad) and probed by primary antibodies namely TIRAP (Cell Signaling Technology), MAPK (Santa Cruz Biotechnology), NF- κ B/p65 (Santa Cruz Biotechnology), & β -Actin (Invitrogen). Following secondary antibody chemiluminescence HRP Substrate (BioRad) in a gel documentation system was used (Image Quant LAS 4000, GE Healthcare).

3.4.10 Molecular docking and virtual screening

Research Collaborator for Structural Bioscience (RCSB) platform was used to get TIRAP's crystal structure (PDB id 3UB2) and missing loop was modeled as described earlier (28). While the structure provides useful insights into the overall folding and potential interaction interfaces of TIRAP, it may not fully represent the physiological conformation of the protein in the cellular environment. The interaction pattern and residues were inspired and studied from a prior art for TIRAP's interaction with its

kinases PKC δ and BTK (28). For screening purpose the PubChem database (<https://pubChem.ncbi.nlm.nih.gov/>) was used to retrieve the compounds' structures and physicochemical data for docking. Coordinates for x, y, & z were set to 40, 36, & 34 to generate a grid around TIRAP for docking and the result output was studied via Discovery Studio Visualizer. To validate the results obtained from one platform, we used another platform, which is the Glide module of Schrodinger Suite (29). The protein–drug complexes were visualized in Discovery Studio Visualizer. The crystal structures of CAII and CAIV were obtained from the RSCB Protein Data Bank (PDB IDs 4M2U and 3FW3, respectively). Subsequently, these structures underwent preprocessing in MGL AutoDock Tools and were subjected to docking using AutoDock Vina-based QuickVina-W embedded within InstaDock. The docking procedure employed a blind search space with grid dimensions of 54, 58, and 52 Å, centered at 11.515, –1.94, and 16.03 Å in the x, y, and z directions for CAII. For CAIV, dimensions were set at 58, 56, and 54 Å, with a center at 6.963, 0.738, and 18.852 Å in the x, y, and z directions. This search space was large enough for the ligands to explore their favorable binding sites on CAII and CAIV structures.

3.4.11 Molecular dynamics simulation

Molecular dynamics (MD) simulation studies were done to validate the stability of all the docked complexes of TIRAP with dorzolamide and the derivatives. MD simulation was performed for 200 ns with GROMACS 2022.2 software [25,26]. The ligand topology was generated by Antechamber [27] and ACPYPE tools of AmberTools utility. The protein was modelled with the Amber99SB-ILDN force field, and the water molecules were represented by the TIP3P explicit model. Further, systems were solvated in a cubic box with a minimum distance of 1.0 Å between the protein and the box edges. The system was neutralized by adding required number of counterions and energy minimization was performed

for each system using the steepest descent algorithm. The system was then heated from 0 K to 300 K in the NVT ensemble for 2 ns, using the V-rescale thermostat with a coupling constant of 0.1 ps. The pressure was equilibrated at 1 bar in the NPT ensemble for 2 ns, using the Berendsen barostat with a coupling constant of 0.5 ps and a compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$. Long-range electrostatic interactions were calculated using the Particle Mesh Ewald (PME) method. The production MD simulation was run for 200 ns in the NPT ensemble, saving the coordinates every 10 ps for analysis. Generated trajectories were used for analysis of RMSD, RMSF, Radius of Gyration, and SASA values using XMGrace.

3.4.12 Statistical analysis

Statistical analysis and visualization of data was performed using GraphPad PRISM 8.0.1.244 (© 1992-2017 GraphPad Software, Inc). All data are representative of three independent experiments; all data are presented as mean $\pm$ SD. P Significance was determined using the One-way ANOVA test followed by the Tukey's multiple comparison test. **** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$. The Kaplan–Meier plot was used for survival analysis (GraphPad Prism).

3.5 Results

3.5.1 DZD intermediates attenuate the BTK-PKC δ common interface on TIRAP in virtual screening

The molecular interactions between TIRAP-BTK & TIRAP-PKC δ have been thoroughly investigated in our previous study [28]. Our initial search for clinically used compounds capable of weakening TIRAP-PKC δ interface residue interactions led us to discovering DZD as a novel inhibitor of TIRAP-PKC δ interactions [29]. Since DZD is a known inhibitor of CA, here, we sought for the compounds which would be structurally similar to DZD and can inhibit TIRAP interactions with BTK

& PKC δ but with no effects on CA activity and intraocular pressure. We focused our investigation on the synthetic intermediates that are formed during DZD chemical synthesis (**Figure 3.2**) shows the synthesis pathway for DZD (<https://www.bakulpharma.com/intermediates.html>) in which the first compound in the line is DRZ V followed by DRZ VII and DRZ VIII ultimately leading to the formation of the parent drug DZD. Initial virtual screening of the potential intermediates in respect of their ability to disrupt the TIRAP–PKC δ & TIRAP-BTK complexes was undertaken in AutoDock Vina 4.0. The PubChem database was used to obtain the atomic coordinates for these compounds. The obtained small molecules and TIRAP structure were prepared in the pdbqt format for docking in AutoDock Vina 4.0. Each molecule was allowed to dock in ten poses, which were ranked according to the docking scores in decreasing order. For binding specificity of compounds interacting at the TIRAP-BTK & TIRAP–PKC δ interface, the top pose of each molecule was selected for interaction analysis. To validate the results obtained from one platform we used another platform, the Glide module of Schrodinger suite [30]. Further, the binding site for each molecule was individually analysed in the Discovery studio Visualizer. The docked structure of intermediates with TIRAP and their interaction in 2D is depicted in (**Figure 3.3**). The binding affinity and the interacting residues of TIRAP with DZD and its intermediates are shown in (**Table 3.1**). On the basis of these parameters, three DZD intermediates were chosen for further in vitro analysis.

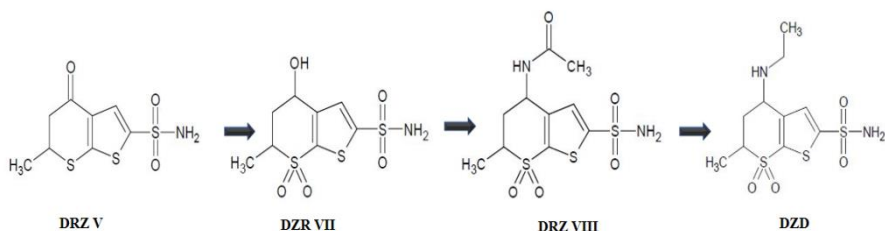


Figure 3.2 Dorzolamide (DZD) synthesis process. All DZD intermediates maintain the common sulfonamide structure.

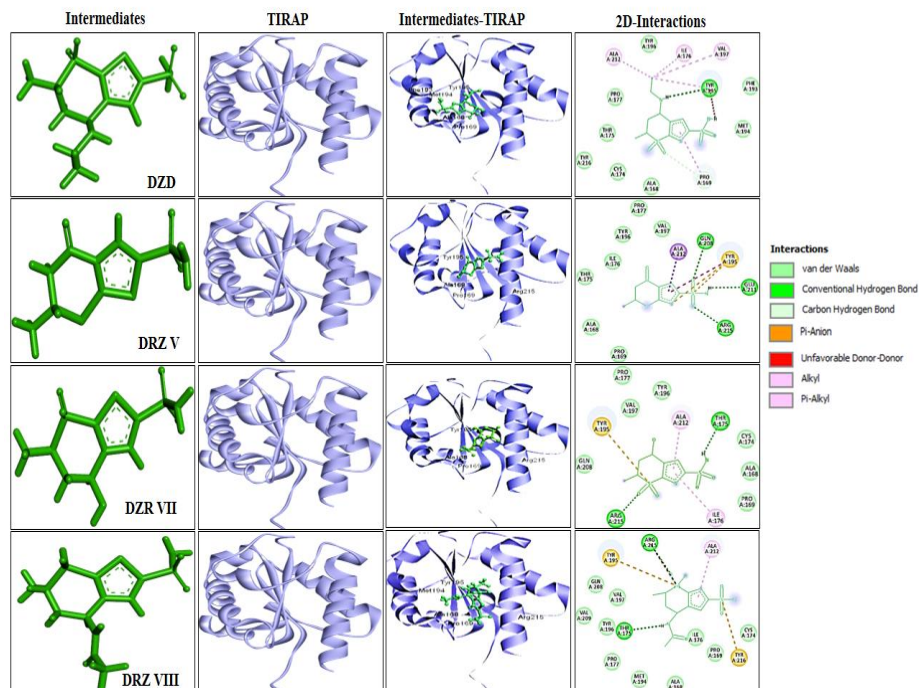


Figure 3.3 Interaction analysis of DZD intermediates targeting TIRAP at the common binding interface of BTK and PKC δ . Diagrammatic representation of docking of individual intermediates along with DZD in AutoDock Vina along with the 2D interaction diagram.

S. No.	Drug Name	Binding Energy (kcal/mol)		Interacting Residues of TIRAP
1	Dorzolamide (DZD)	Auto Dock	Schrodinger	A168, P169 , C174, T175, I176, P177, F193, M194, Y195 , Y196, V197, A212, Y216
2	DRZ V	-6.1	-4.9	A168, P169 , T175, I176, P177, Y195 , Y196, V197, Q208, E211, A212, R215
3	DRZ VII	-6.3	-5.3	A168, P169 , C174, T175, I176, P177, Y195 , Y196, V197, Q208, R215
4	DRZ VIII	-5.7	-4.6	A168, P169 , C174, T175, I176, P177, M194, Y195 , Y196, V197, Q208, V209, A212, R215, 216

Table 3.1 TIRAP interacting residues and binding energies of DZD and intermediates at the common binding site of BTK and PKC δ . Individual dock scores along with the interacting residues have been mentioned in the tabular form. Interacting residues of TIRAP with BTK and PKC δ within the 4Å region of their interaction, and the common interface residues are highlighted in bold.

3.5.2 DZD intermediates bind at TIR domain of TIRAP

To validate our docking results and to analyse the structural stability of the complexes and their movement within the hydrated environment of the active pocket of TIRAP, we performed molecular dynamics simulations. The root mean square deviations (RMSD) of backbone atoms of all complexes were calculated. A lower RMSD is indicative of a more stable conformation. It was observed that after around 125 ns, all trajectories converged at $\text{RMSD} \approx 0.3 \text{ nm}$ (3 Å) (**Figure 3.4A**). Out of all the complexes, the backbone atoms of TIRAP in complex with DRZ V had the lowest RMSD (0.303529 ± 0.0404253) and was comparable with RMSD of TIRAP with Dorzolamide, signifying a stable complex conformation. Root mean square fluctuations (RMSF) studies are helpful in surveying average atomic mobility of the protein residues throughout the course of the MD simulation run. Therefore, we next analysed RMSF

of backbone atoms for all complexes to explore the flexibility of individual residues of the TIRAP protein with respective ligands. The results of RMSF analysis (**Figure 3.4B**) illustrated that the major fluctuations (>0.3 nm) in the complexes corresponded to residues that were far from the binding pocket of the protein. It is to be noted that the active site residues of TIRAP i.e., PRO169, PHE193, MET194, and TYR195 showed considerably lesser fluctuation in TIRAP in complex with DRZ V as compared to apo protein and complexes with other ligands including Dorzolamide during the simulations run time. The low number of fluctuations of TIRAP residues in complex with DRZ V showed that the small molecule can form stable interactions with the protein during MD simulation. This analysis corresponds to the analysis of RMSD plot. The overall compactness of the protein over the course of the MD simulation was evaluated by calculating the Radius of Gyration (Rg). Rg was used to assess the degree of protein compaction and the size of the structure in relation to the apo protein. This parameter reflects the distribution of mass around the centre of gravity of the protein. When the Rg value is highest, loose packing of amino acid is indicated, and vice versa when Rg value is lowest [31]. It was observed in the Rg plot (**Figure 3.4C**) that the Rg values of all compounds remain stable between 1.35 and 1.45 nm throughout the MD run. On comparative analysis, it can be concluded that backbone and folding of TIRAP protein were consistently stable after binding to Dorzolamide and intermediates. TIRAP in complex with DRZ V had the lowest Rg, indicating that the binding induced a more compact and stable conformation. Other parameters such as solvent accessible surface area, ligand-rmsd, hydrogen (H)-bond occupancy, & H bonds have also been determined (**Supplementary figure S3.1**).

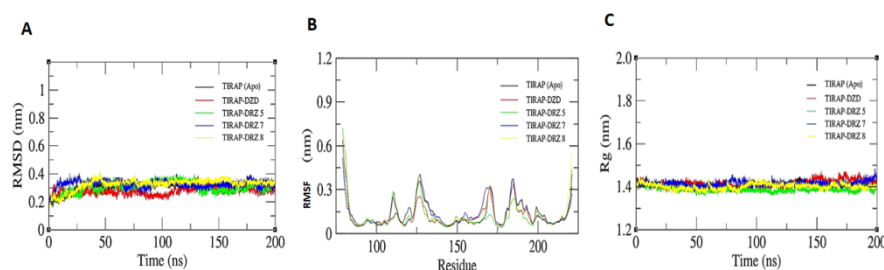


Figure 3.4 MD simulation trajectory plots of TIRAP and TIRAP in complex with DZD and intermediates. A) RMSD plot of the C-alpha backbone of TIRAP protein in complex with Dorzolamide and respective intermediates. B) RMSF plot of the C-alpha backbone of TIRAP protein in complex with Dorzolamide and intermediates. C) Rg plot of the C-alpha backbone of TIRAP protein in complex with Dorzolamide and intermediates.

3.5.3 DZD intermediates abolishes the interaction of TIRAP with its

activating kinases TIRAP is known to be responsible for mediating pro-inflammatory signaling in response to LPS stimulation in macrophages [32,33]. TIRAP activation is governed upon its phosphorylation that is mediated by two upstream kinases namely BTK and PKC δ [17,28]. In silico approaches have been used to identify the interacting interface of TIRAP responsible for its binding to the aforementioned kinases [28]. Targeting these interacting sites is a potential therapeutic approach to inhibit the TIRAP mediated signaling. Rajpoot et. al., has reported that the inhibition of TIRAP-PKC δ interaction by DZD attenuated excessive inflammation [29]. Still the BTK mediated early activation [20] remains a plausible way to continue the inflammatory response. Therefore, we next aimed at studying the interaction pattern of the kinases with TIRAP at early and late time points via immunofluorescence dual staining that is depicted in **(Figure 3.5A & B)** along with its quantification in order to completely dampen the TIRAP mediated signaling. The result shows increased colocalization of TIRAP and BTK at an early time point and TIRAP and PKC δ at the later time point in response to LPS stimulation.

The Pearson's coefficient depicts the degree of interaction or colocalization between two given proteins. Here, the increment in Pearson's coefficient between TIRAP & BTK at early time point depicts their prominent interaction which decreases gradually as seen by reduced coefficient at later time point. Initial low coefficient values indicated a weak interaction between TIRAP and PKC δ ; however, this interaction intensified at later time points, corresponding with a rise in the coefficient. This finding paves the way to proceed further with experiments to abolish these interactions and to dampen the inflammatory signaling mediated by TIRAP. This gave us insight about the temporal roles of these kinases in TIRAP activation. The kinases BTK and PKC δ are the main proteins responsible for the phosphorylation and activation of TIRAP under the influence of LPS [34,35]. Therefore, the inhibition of these interactions would effectively lead to abrogation of TIRAP mediated downstream pro-inflammatory signaling. Our results from immunofluorescence dual staining of TIRAP-BTK and TIRAP-PKC δ at time points 5 min and 60 min are shown in **(Figure 3.6)**. The interaction of TIRAP & BTK is prominent at as early as 5 minutes therefore we checked Pearson's coefficient for intermediates and DZD at an early stage. The decrease in the coefficient value highlights reduced colocalization of TIRAP & BTK **(Figure 3.6A)** where the intermediates have shown to exhibit a greater ability in abolishing the colocalization compared to DZD. Similarly, interaction between TIRAP & PKC δ is more significant at later stage therefore we checked intermediates effect on this interaction at 60 minutes. Results show the prominent reduction in coefficient value & thereby the effect of DZD intermediates on TIRAP & PKC δ interaction **(Figure 3.6B)**. Thus, highlighting the importance of these interactions and how the intermediates can effectively and efficiently result in a decreased interaction thereby reducing the colocalization of TIRAP with its activating kinases. This would help to dampen the inflammatory axis mediated by both BTK and PKC δ during chronic disease progression.

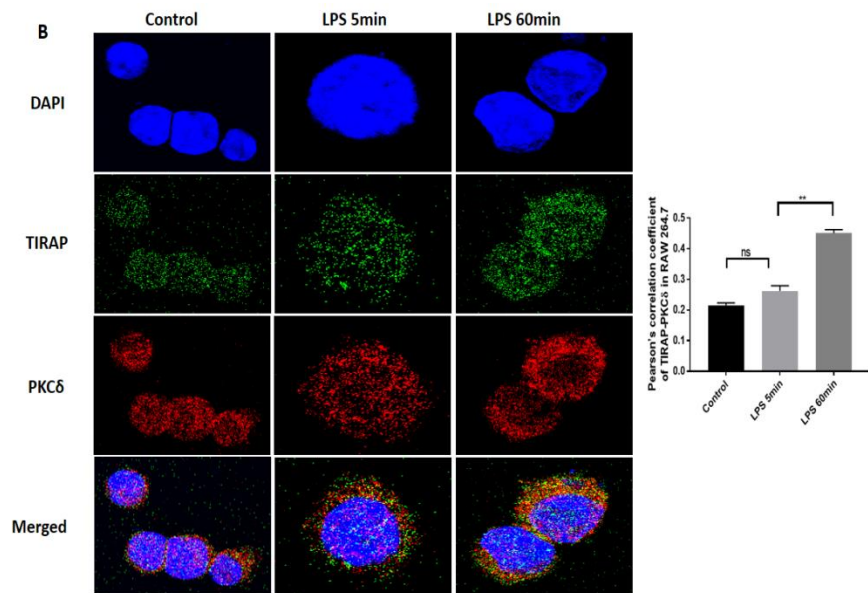
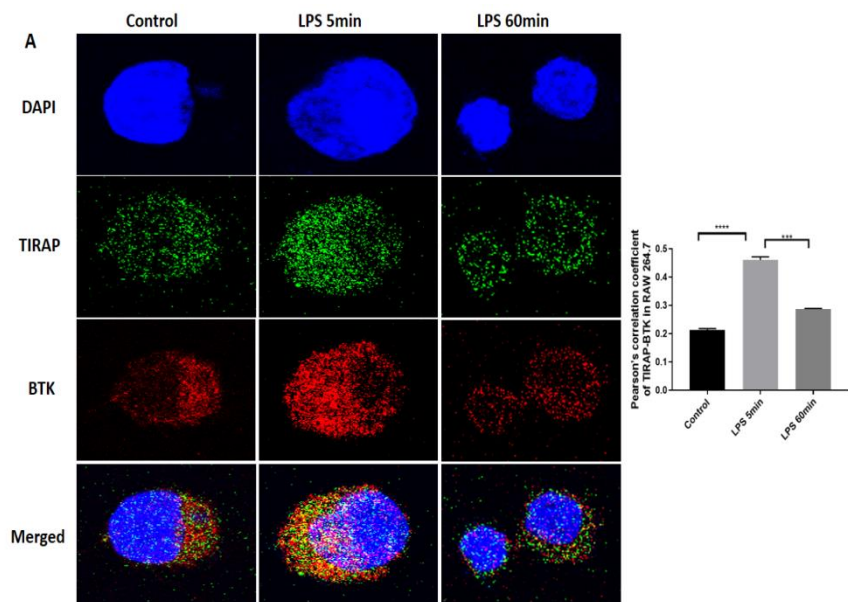


Figure 3.5A & B LPS treatment promotes the interaction of TIRAP with BTK and PKC δ in macrophages. A) RAW 264.7 macrophages were stimulated with LPS (1 μ g/ml) for 5 min and 60 min, respectively. Confocal microscopy images showing the cellular interaction of TIRAP with BTK by immunostaining with their specific primary antibodies for TIRAP, & BTK. The secondary antibodies used were Alexa Fluor 488-tagged (green) for TIRAP and Alexa Fluor 594-tagged (red) for BTK with their Pearson's correlation coefficient analysis, respectively. B) RAW 264.7 macrophages were stimulated with LPS (1 g/ml) for 5 min and 60min, respectively. Confocal microscopy images showing the cellular interaction of TIRAP with PKC δ by immunostaining with their specific primary antibodies for TIRAP, & PKC δ . The secondary antibodies used were Alexa Fluor 488-tagged (green) for TIRAP and Alexa Fluor 594-tagged (red) for PKC δ with their Pearson's correlation coefficient analysis, respectively. All data are presented as the mean $\pm$ SD, (One-way Anova test). ****P $\leq$ 0.0001; ***P $\leq$ 0.001; **P $\leq$ 0.01; *P $\leq$ 0.05.

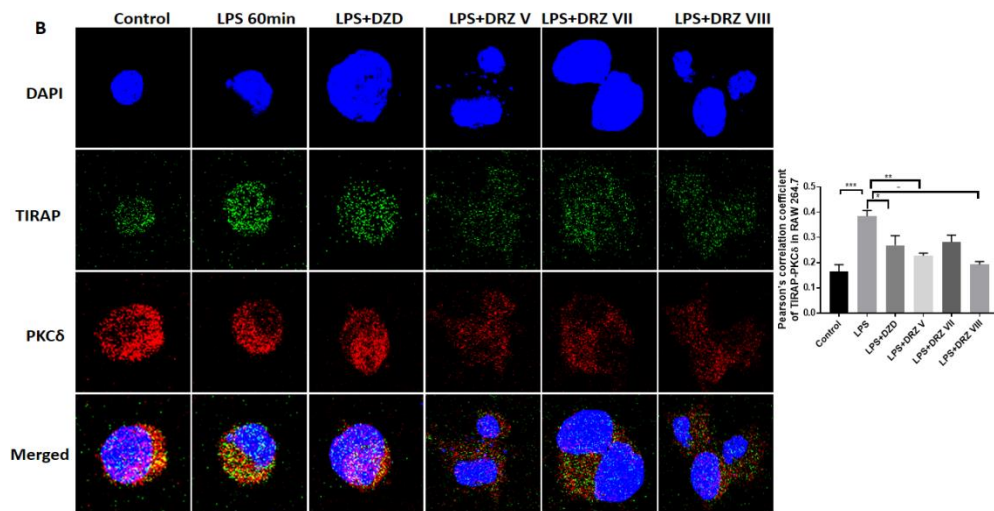
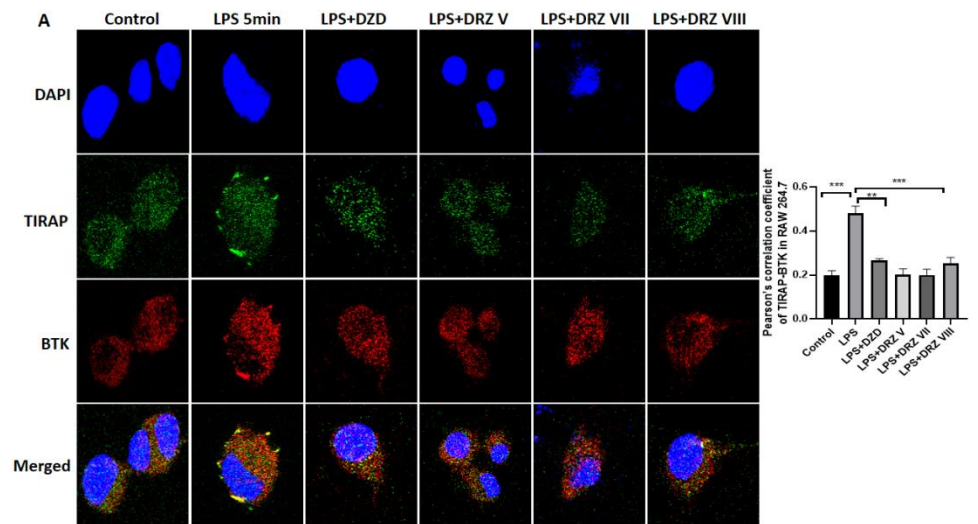


Figure 3.6A & B DZD Intermediates inhibit LPS-induced TIRAP interaction of TIRAP with BTK and PKC δ . A) Immunofluorescence staining was performed in RAW 264.7 macrophages treated with LPS alone (1 μ g/ml) for 5 min and in combination with DZD or intermediates (1 μ M) 15 min prior to LPS. TIRAP & BTK proteins were stained with specific primary antibodies. The secondary antibodies were Alexa Fluor 488-tagged (green) for TIRAP and Alexa Fluor 594-tagged (red) for BTK. Nuclei were counterstained with DAPI (blue). B) Immunofluorescence staining was performed in RAW 264.7 macrophages treated with LPS alone (1 ml) for 60min and in combination with DZD and intermediates (1 μ g/ M)15minprior to LPS. TIRAP & PKC δ proteins were stained with specific primary antibodies and then with the secondary antibody Alexa Fluor 488-tagged (green) for TIRAP and Alexa Fluor 594-tagged (red) for PKC δ . Nuclei were counterstained with DAPI (blue). All data are presented as the mean $\pm$ SD, (One-way Anova test). ****P $\leq$ 0.0001; ***P $\leq$ 0.001; **P $\leq$ 0.01; *P $\leq$ 0.05.

3.5.4 DZD Intermediates reduces the expression of the pro-inflammatory cytokines in LPS-treated macrophages

TIRAP plays a critical role in triggering inflammatory pathways and controlling pro-inflammatory cytokine production in immune cells. Quantitative RT-PCR was used to examine how the DZD intermediates affect the mRNA expression levels of the main pro-inflammatory cytokines (IL-6, IL-12, IL-23, IL-1 β and TNF- α) in macrophages. We found that the mRNA expression of all tested pro-inflammatory cytokines was increased in macrophages activated by LPS compared to control macrophages, and treatment with the DZD intermediates significantly reduced pro-inflammatory cytokine mRNA levels in LPS-activated macrophages (**Figure 3.7**) compared to LPS-induced levels. Interestingly, although LPS-induced IL-1 β and IL-6 mRNA increases in macrophages were significantly attenuated by the DZD compounds, the effect was

generally much smaller compared to IL-12, IL-23, and TNF- α group. Thus, it is possible that DZD and its intermediate exhibit a reduced strength against the inflammatory pathway in macrophages. Notably, some individual DZD intermediates were often more potent in alleviating LPS-activated inflammatory response as compared to the parent compound DZD. These suggest that the DZD intermediates may have a greater impact on the inflammatory response because they bind to TIRAP more effectively than DZD.

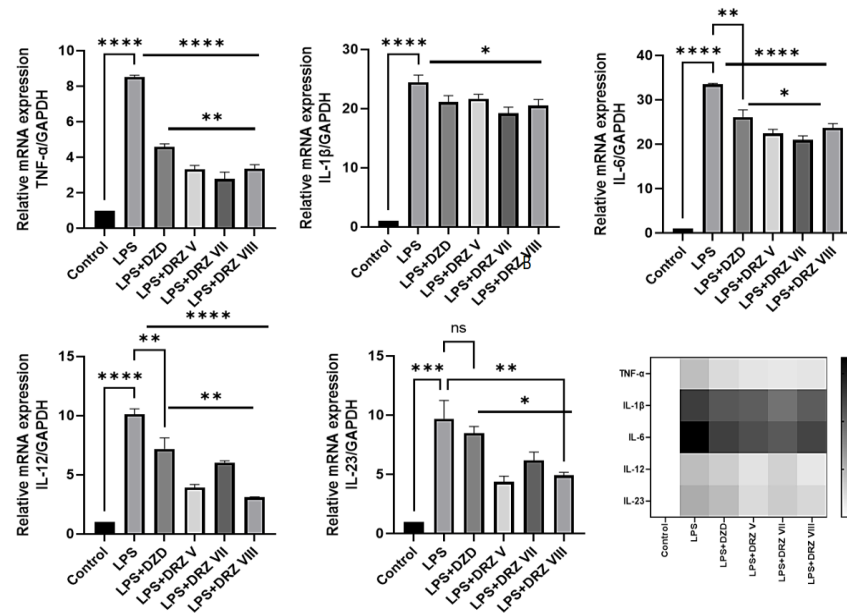


Figure 3.7 Effect of DZD intermediates on pro-inflammatory cytokine mRNA expression in LPS-stimulated RAW 264.7 cells. The total mRNA was isolated and 1 μ g from each sample was used for single-stranded cDNA preparation as per the manufacturer's instruction. Prepared cDNA was used as the template for quantitative real-time PCR, and the relative mRNA expression of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6, IL-12, and IL-23) was determined after normalizing to GAPDH's mRNA expression levels. Heat map for all pro-inflammatory cytokines in RAW 264.7 cells. All data are presented as the mean $\pm$ SD, (One-way Anova test). **** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$.

3.5.5 DZD Intermediates attenuate TIRAP-mediated NF- κ B activation via p38 MAPK

We determined that some DZD intermediates may be more effective at attenuating TIRAP interaction than DZD, as demonstrated by the immunofluorescence dual staining of TIRAP with both activators BTK and PKC δ (**Figure 3.6**). It is thus assumed that TIRAP activation would similarly be reduced. TIRAP features multiple phosphorylated tyrosine residues, with BTK and PKC δ both implicated in the phosphorylation of the tyrosine 86 (Y86) residue [17] of TIRAP. By utilising a specific general TIRAP antibody and an antibody against phosphorylated Y86 on TIRAP, we used immunoblotting approach to quantify the TIRAP protein production and its phosphorylation at Y86. The obtained immunoblotting data confirmed the drugs' ability to inhibit TIRAP phosphorylation and activation. When compared to DZD-treated cells, the Y86 phosphorylation of TIRAP was significantly reduced in the LPS-stimulated RAW 264.7 macrophages treated with DZD intermediates (**Figure 3.8A**). We hypothesized that the inhibition of TIRAP interaction with both kinases was likely the cause of the decreased Y86 phosphorylation of TIRAP in these experiments. This suggests that the DZD intermediates' anti-inflammatory action may be stronger than that of their end-product, dorzolamide. These weaker interactions of TIRAP with kinases in the presence of DZD intermediates prompted us to investigate their effects on the signaling molecules downstream of TIRAP. Previous research [36] has demonstrated the importance of phosphorylated TIRAP in activating p38 MAPK. Rajpoot et al., has also reported the inhibitory effect of DZD on p38 MAPK [29]. Thus, we set out to determine whether there are any inhibitory effects of the DZD intermediates on the activity of p38 MAPK and NF- κ B in LPS-activated macrophages. After being phosphorylated, p38 gets activated, and this leads to the activation of important transcription factor NF- κ B due to phosphorylation of its component p65 [37–39]. We found that the DZD intermediates reduced the p38 MAPK

pathway-mediated activation of NF- κ B. The production of pro-inflammatory cytokines was then triggered after the translocation of phosphorylated p65 to the nucleus. First, we used the technique of immunoblotting to examine the phosphorylation of both p38 MAPK and p65 proteins and discovered that, when compared to cells treated with DZD, their activation in mouse macrophages treated with intermediates was dramatically reduced (**Figure 3.8B**) in LPS-activated macrophages. Then, using the immunofluorescence approach, we investigated the translocation of the p65 subunit to the nuclei and discovered that it was dramatically decreased DZD intermediate-treated and LPS-stimulated macrophages as compared to LPS stimulated macrophages treated with DZD (**Figure 3.8C**). These data are consistent with the hypothesis that DZD intermediates reduce inflammation by preventing TIRAP from interacting with BTK and PKC δ kinases, which in turn limits the activation of the p38 MAPK/ NF- κ B pathway.

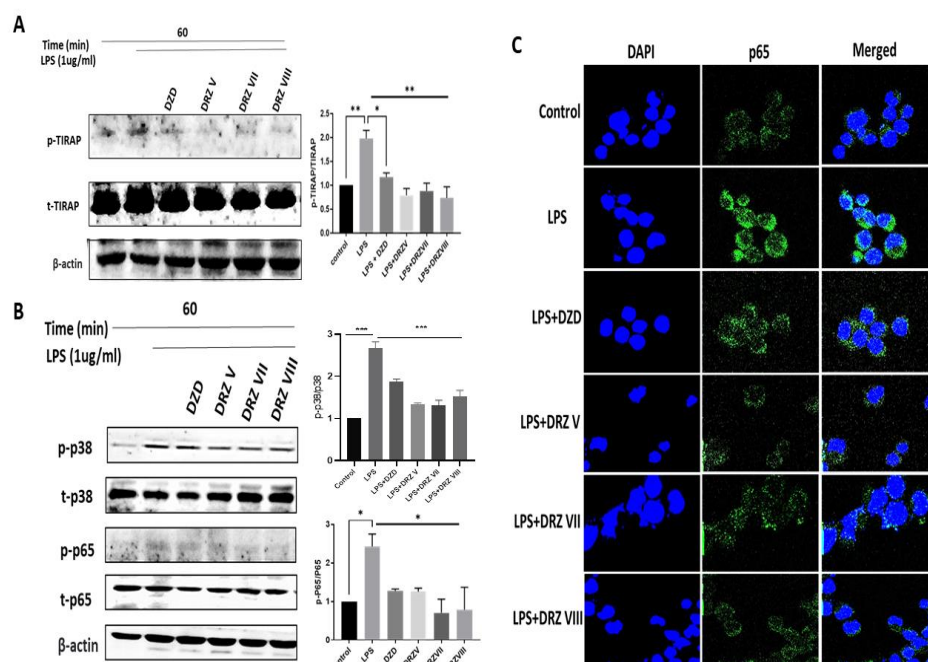


Figure 3.8 DZD Intermediates inhibit TIRAP mediated downstream signaling in LPS-stimulated RAW 264.7 cells. A) Immunoblot analysis for phosphorylated TIRAP revealed a significant down regulation of TIRAP phosphorylation that was more prominent in DZD intermediate-treated cells as compared to DZD-treated cells. B) Immunoblot analysis of p38 MAPK and NF-κB/p65 indicates the significant down regulation of p38 MAPK and NF-κB/p65 phosphorylation in DZD intermediate-treated macrophages as compared to the positive control, DZD-treated cells. C) Immunofluorescence analysis revealed a significant inhibition of nuclear translocation of NF-κB/p65 in DZD intermediate and DZD- treated cells. All data are presented as the mean $\pm$ SD, (One-way Anova test). **** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$.

3.5.6 DZD intermediates show no toxicity in cell viability assay and ADMET analysis

We next investigated the cytotoxicity of DZD and its intermediates. We employed the viability assay using RAW 264.7 cells (**Figure 3.9**). The cells underwent 24-h treatment with varied concentrations of DZD and its intermediates (i.e., 0.1, 0.5, 1, 5, 10 μ M). Only very minimal cytotoxicity

was detected at the tested concentrations with the parent drug DZD as well as all the intermediates. We then used the pkCSM server [23] and AMETlab2.0 [24] to analyse the physicochemical and ADMET properties of the intermediates. The results indicate that the drugs are safe and efficient for further processing on the basis of various parameters such as water solubility, intestinal absorption, blood-brain barrier permeability, clearance, hepatotoxicity etc. (**Table 3.2 & Supplementary figure S3.2**). This in silico approach provides us with the basic information indicating that DZD may have some hepatotoxicity, whereas hepatotoxicity of DRZV may be reduced. Comparatively, DZD depicted lesser cell viability than intermediates.

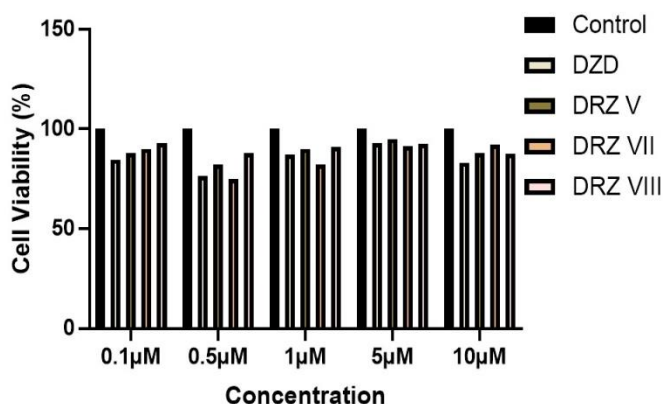


Figure 3.9 Effects of DZD and its intermediates on cell viability. The cytotoxicity of the intermediates on the viability of RAW 264.7 cells was determined using MTT analysis. The cells were treated with the indicated compounds for 24 hours at varied concentrations (i.e., 0.1, 0.5, 1, 5, 10 μM).

Parameters		Dorzolamide	DRZ V	DRZ VII	DRZ VIII
Physicochemical Properties	M.W.	324.449	263.365	297.379	338.432
	LogP	0.612	1.4625	-0.0052	0.1386
	Rotatable Bond	3	1	1	2
	Acceptor Bond	6	5	6	6
	Donor Bond	2	1	2	2
Absorption	Water Solubility	-2.819	-3.214	-2.642	-2.405
	CaCo2 Permeability	0.429	0.377	0.357	0.391
	Intestinal Absorption	79.302	93.224	80.325	86.156
	Skin Permeability	-3.415	-3.498	-3.014	-3.03
	Fraction Unbound	0.419	0.426	0.491	0.336
Distribution	BBB Permeability	-0.838	-0.536	-0.496	-0.546
	CNS Permeability	-3.144	-2.986	-3.513	-3.201
Metabolism	CYPD6 Substrate	NO	NO	NO	NO
	CYPD6 Inhibitor	NO	NO	NO	NO
Excretion	Total Clearance	0.07	0.15	0.006	-0.075
Toxicity	AMES Toxicity	NO	NO	NO	NO
	hERG Blocker	NO	NO	NO	NO
	Skin Sensitization	NO	NO	NO	NO
	Hepatotoxicity	YES	NO	YES	YES

Table 3.2 Physicochemical and ADMET analysis of DZD and its intermediates. Prediction of pharmacokinetic properties of ADMET (absorption, distribution, metabolism, excretion, and toxicity) of DZD and its intermediates through computational tool pkCSM.

3.5.7 Molecular docking studies suggest DRZ V and DRZ VII has less affinity with CA II and CA IV but not DRZ VIII

DZD is a well-known drug which is clinically used to treat glaucoma by reducing intraocular pressure via inhibition of CA. Repurposing of such drug as an anti-inflammatory agent is complicated because of its possible effects on intraocular pressure. To investigate the effect of the DZD intermediates on CA, we docked them to CA II and CA IV structures and compared binding energy to that of DZD. **Figure 3.10** shows that DRZ V and DRZ VII bind with CA II and CA IV with a lower affinity as compared to DZD.

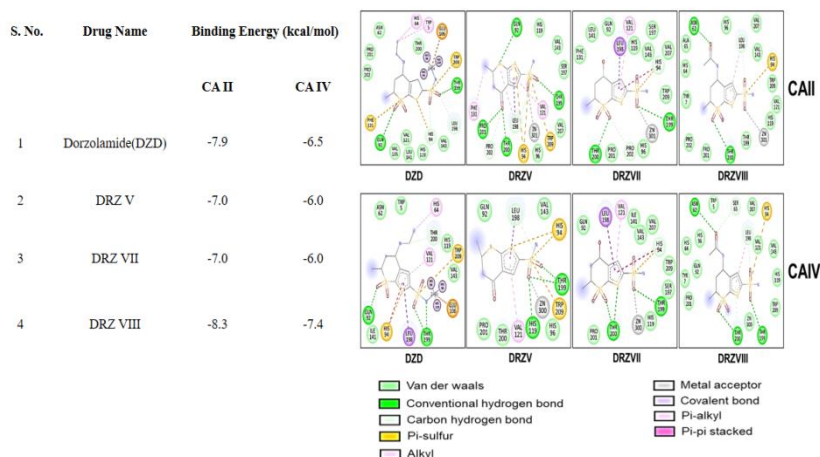


Figure 3.10 Computational analysis of interactions of DZD Intermediates with either CA II (4M2U) or CA IV (3FW3). Individual dock scores along with the interacting residues are shown.

3.5.8 DRZ V treatment efficiently protected mice from septic injury and mortality

Since DRZ V and DRZ VII exhibited stronger anti-inflammatory effects compared to DZD in the aforementioned in vitro investigations, we were especially interested to determine if they can reduce septic injury and death in a murine model. In order to compare the efficacy of the intermediates to DZD, 5 groups of 14 mice each were established, with Group A being the control, Group B being LPS-induced septic mice, Group C being DZD-treated septic mice, Group D being DRZ V treated septic mice, and Group E being DRZ VII treated septic mice. The drugs were administered as a single dosage one hour prior to injecting LPS. All injections were given intraperitoneally, and each group was monitored for 72 hours afterward. More than 60% of the mice died in the control group of septic mice after 36 hours, but only (37%), (19%), and (28%) of the mice perished in Groups C, D, and E, respectively, during 36 hours post LPS-treatment. 72 hours of observation revealed that roughly (82%) of the septic mice died, compared to (55%) of the mice in Group C, (19%) of the animals in Group D, and (37%) of the mice in Group E (Fig.9a). DZD

treatment increased survival rates in the septic mouse population by around (45%), whereas treatment with drugs DRZ VII and DRZ V increased survival rates by (63%) and (81%), respectively (**Figure 3.11A**). Then, at 10 hours after infection, we took tissue samples from 3 mice from each group and performed H&E staining to see the infiltration of immune cells causing inflammation and the damage on the alveolar septum in order to compare the anti-inflammatory effect of the intermediates on the most affected organ, which are the lungs. The tissue section images were then observed at 40x magnification using a light microscope. The lungs of infected mice showed alveolar haemorrhages, thickening of the alveolar septum, deformed epithelial and endothelial cell structure, increased infiltration of inflammatory cells, and pulmonary edema, as well as other symptoms. However, in the DZD treated group there was a decreased immune cells number, intact epithelial lining as well as there was less damaged septum, and epithelial and endothelial cell structure (**Figure 3.11B**). In contrast to it, those treated with DZD intermediates had much fewer damaged tissues and immune cell infiltration. Thus, the intermediates produce far more potent anti-inflammatory properties than DZD (**Figure 3.11B**). To further investigate the effect of the drugs on pro-inflammatory cytokines level, we isolated the serum from the different groups and observed low cytokine levels (**Figure 3.11C**). This shows that the DZD intermediates were more effective at reducing the mortality, inflammation, and tissue damage in the septic mice.

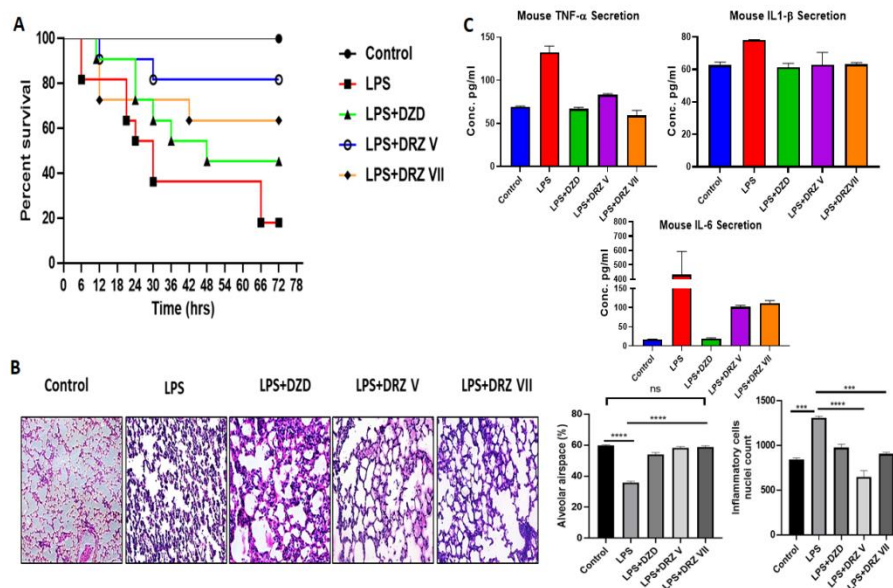


Figure 3.11 Treatment with DZD intermediates protects mice from septic damage and death more effectively as compared to DZD. A) Kaplan–Meier survival curves of the mice (n = 14, each group) treated with LPS alone (25 mg/ kg, i.p.) or in combination with DZD and intermediates (25 mg/kg, i.p.) 1 hour prior to LPS treatment. In the control group, all mice received the vehicle DMSO/phosphate-buffered saline only. The mortality of mice was recorded every 6 hours consecutively for 3 days (72 hours). B) The histomorphological study from the lung tissue section was performed to access the LPS-induced lung damage and the effect on the lung from the administration of DZD intermediates. The (B-i) Control, (B-ii) LPS, (B-iii) LPS + DZD, (B-iv) LPS + DRZ V and (B-v) LPS + DRZ VII groups were treated as described for (A) for 10 hours (n = 3 per group) and sacrificed for lung tissue isolation and fixed in 10% formalin for preparation of 5 μ m thick sections on a glass slide. The tissue sections were stained in hematoxylin and eosin and imaged under a light microscope at 40x magnification. Quantification of percentage of alveolar airspace, and inflammatory cells nuclei count from the lung tissue sections are shown in the right panel. C) Serum cytokines were checked to see the effects of intermediates on the production of pro inflammatory cytokines.

All data are presented as the mean $\pm$ SD, (One-way Anova test).
**** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$.

3.6 Discussion

Inflammation is an important biological response to tissue damage or harmful external pathogenic encounters. However, a protracted and unchecked inflammatory response may cause a homeostasis imbalance and a number of illnesses. Pathogen recognition via PRRs presented on the macrophages leads to the initiation of the inflammatory response [40]. Toll-like receptors (TLRs) are one of the major receptors that recognize microbial wall components and activate a variety of signaling pathways, such as those that encourage the production of pro-inflammatory cytokines [41]. Stimulation of TLR4/2 signaling by their classical ligands LPS/lipoproteins leads to subsequent recruitment and activation of the cytoplasmic adaptor proteins like TIRAP and MyD88 for further activation of downstream transcription factors and hence expression of cytokines [13,42–44]. Owing to its prominent role in TLR4/2 signaling, TIRAP has been a protein of interest for several studies. The TIR domain of TIRAP is a targeted area for its phosphorylation and activation in response to LPS via TLR4/2 [45]. TIRAP is known to participate in various protein-protein interactions under different inflammatory conditions. Therefore, exploring the interactions that directly influence TIRAP is essential [16]. Interactions with two major kinases have been shown to be involved in the activation of the TIR containing TIRAP. These kinases are Bruton's tyrosine kinase (BTK) and Protein kinase C delta (PKC δ) [17]. The phosphorylation of TIRAP on its TIR domain via these kinases has been found to be responsible for its activation and thereby its downstream inflammatory signaling [17].

The phosphorylation of TIRAP, namely at Y86, Y106, Y159, and Y187, in its TIR domain mediates its activation [17,20,21]. The significance of PKC δ in the activation of TIRAP, which results in a downstream

inflammatory response, as well as the competitive interaction between BTK and PKC with TIRAP for its activation, have both been highlighted in a few earlier investigations, including ours [17–19]. Rajpoot et al., have recently reported the inhibition of the TIRAP-PKC δ interaction via a repurposed anti-inflammatory agent DZD that has shown to prominently dampen the inflammatory response [29]. This inhibition opens the space for us to further investigate the possibility to target not only PKC δ but also BTK in order to completely abolish the activation of TIRAP and associated downstream inflammatory signaling. Since anti-inflammatory properties of DZD have already been reported proving its efficiency and effectiveness, we were interested to investigate compounds sharing structural similarities to DZD. Interestingly, from one of the pharmaceutical companies (<https://www.bakulpharma.com/index.html>) we found that during the synthesis of DZD there is the occurrence of few intermediates that undergo certain chemical and structural modifications having the same skeletal structure to finally yield the parent drug molecule DZD (**Figure 3.2**). We wanted to investigate how these differences in side chains would affect the ability of the compound. Moreover, the success in identifying better efficiency of an intermediate than its parent compound would eventually lead to shift focus on synthesizing just the intermediate rather than DZD, and it would be beneficial to reduce the production cost of the active compound, time, and manpower. Even a small efficient and cost-effective tactic in the drug discovery can result in a huge effect on the socio economic and healthcare sectors.

In the current study, we screened the DZD intermediates against our novel target i.e., TIRAP's common interface of BTK & PKC δ interaction. One of the previous studies [28] has highlighted the common TIRAP residues via in silico approach that formed the base of our study. This in silico approach gave us the promising results showing targeted binding of intermediates on TIRAP (**Figure 3.3**). Notably, DZD intermediates exhibited a better binding efficiency compared to the parent drug DZD as

depicted by the dock scores obtained from two different platforms (**Table 3.1**). These data prompted us to further investigate the stability of DZD intermediate binding with TIRAP, using 200 ns molecular dynamics simulations of the interaction dynamic between DZD or its intermediates with TIRAP (**Figure 3.4**). We validated the interaction of the kinases BTK and PKC δ individually with TIRAP via the immunofluorescence approach in LPS-stimulated RAW 264.7 cells (**Figure 3.5A & B**). The results showed that at the early time point, TIRAP appeared to have a stronger interaction with BTK as compared to PKC δ in LPS-stimulated cells. Whereas at later time points, it was PKC δ that showed increased interaction with TIRAP compared to BTK. This result highlights the temporal role of these kinases. BTK acting at an early stage of the inflammation whereas PKC δ interacts with TIRAP responsible for later stage of the inflammation. This highlights the need to target both these interactions in order to completely abrogate the downstream inflammatory cascade. Our immunofluorescence dual staining data demonstrate that the DZD intermediates were more effective in dampening the interaction of BTK & TIRAP than DZD (**Figure 3.6A**). Similarly, the intermediates were seen to reduce the interaction of PKC δ & TIRAP with greater efficiency as compared to the parent drug (**Figure 3.6B**). To determine how the DZD intermediates affect the mRNA expression levels of the most important pro-inflammatory cytokines (IL-6, IL-12, IL-23, IL-1 β and TNF- α) in macrophages, quantitative RT-PCR was used. It's interesting to note that we found that intermediates considerably reduce the expression of cytokines in LPS-stimulated RAW 264.7 cells as compared to DZD (**Figure 3.7**). In the presence of DZD and intermediates, we examined the phosphorylation of Y86 in TIRAP. The Y86 phosphorylation of TIRAP in the LPS-stimulated cells treated with the intermediates was dramatically decreased when compared to DZD-treated cells (**Figure 3.8A**). This suggested that the reduced Y86 phosphorylation of TIRAP was most likely caused by the suppression of TIRAP interaction with both kinases

as depicted by immunofluorescence dual staining. We then investigated the downstream signaling of TIRAP which is critical for increasing the expression of pro-inflammatory cytokines. In TIRAP-mediated macrophage inflammatory signaling, MAPK activation plays a significant role and is primarily responsible for activating transcription factors [19,36]. We discovered that, in comparison to macrophages activated by LPS, DZD intermediate-treated RAW 264.7 macrophages exhibit a substantial reduction in p38 MAPK (**Figure 3.8B**). Transcription factors, particularly AP-1 and NF- κ B, are activated downstream of activated p38 MAPK [37–39]. Therefore, we examined the impact of DZD and intermediates on the phosphorylation as well as the nuclear translocation of NF- κ B/p65 using immunoblotting and immunofluorescence, and found that both of these processes are significantly inhibited in intermediate-treated RAW 264.7 macrophages as compared to DZD alone (**Figure 3.8B & C**). We found (**Figure 4.9**) that neither the parent drug DZD nor any of the intermediates displayed any significant cytotoxicity at any concentration indicating that it may be safe to use them *in vivo*.

One well-known medication on the market for the treatment of glaucoma is DZD. Its putative activity in glaucoma patients raises concerns about its repurposing as an anti-inflammatory drug. We docked the screened DZD intermediates against CA II and CA IV (Carbonic anhydrase) and compared them with DZD to see what impact the intermediates had on the glaucoma-causing enzymes. We found that, as compared to DZD, most intermediates except DRZ VIII bind with decreased affinity to CA II and CA IV (**Figure 3.10**). Hence, for *in vivo* experiments we selected DRZ V & DRZ VII. Our results indicated that in LPS-induced septic mice intermediates significantly increased the survival as compared to DZD treated mice (**Figure 3.11A**). Notably, DRZ V proved to be highly efficient in decreasing the mortality followed by DRZ VII. Similar effective and prominent protective effects of the intermediates were observed in the lung tissues of the septic mice (**Figure 3.11B**). Results

obtained from the serum analysis suggested that proinflammatory cytokines were down regulated in the drug-treated septic mice group **(Figure .11C)**. Herein we propose that identified DZD intermediates may be better and more cost-effective anti-inflammatory agents than the parent drug in decreasing the TIRAP mediated inflammatory signalling.

3.7 References

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Chapter 4

An improved mouse model of
sepsis based on
intraperitoneal injections of
the enriched culture of cecum
slurry

Chapter 4: An improved mouse model of sepsis based on intraperitoneal injections of the enriched culture of cecum slurry

4.1 Graphical Abstract

<u>Current models</u>	<u>Parameters</u>	<u>Improved model</u>
☒	Clinical relevance	☒
☐	Reproducibility	☒
☐	Precise information on microbial composition	☒
☐	Ethically suitable as it limits the unnecessary euthanasia of animals	☒
☒	Cecal slurry polymicrobial culture can be stored at – 80 °C as glycerol stocks	☒
☐	Enrichment approach allowing to grow specific defined polymicrobial populations	☒
☒	Biochemical septic markers and cytokines up regulation	☒

Figure 4.1 Schematic representations of the current models of sepsis in comparison with the proposed improved cecum slurry sepsis mice model.

4.2 Overview

Sepsis is a life-threatening clinical syndrome comprising multi-organ dysfunctions caused by a disproportionate body immune response. There are several animal sepsis models which are based on cecum ligation, cecal puncture, and cecum slurry injection. The major limitation of all current sepsis models is the high variability owing to the variable degree of ligation, puncture and inconsistent microbial composition used for sepsis initiation. The primary objective of this work is to demonstrate the feasibility of a standardized method for sepsis development. The cecal slurry bacterial culture was developed and preserved in glycerol stocks. Antibiotics aztreonam and vancomycin were used for generating several defined, enriched cecal slurry bacterial cultures. Mice survival was assessed until 48h post injection, and the tissue samples were collected after 10h from sepsis initiation. The results indicate that increasing polymicrobial load resulted in lower survival rates and was associated with the higher number of infiltrating immune cells and necrosis. H&E (Haematoxylin & Eosin) staining & serum markers revealed that septic mice exhibited increased inflammation and significant damage to the liver and kidneys. The defined gram-negative and gram-positive specific cecal slurry bacterial cultures were developed and their efficiency in inducing sepsis was characterized. Enriched cecal slurry bacterial cultures can be stored in glycerol stocks at -80°C . This has an ethical advantage of avoiding unnecessary animal euthanasia for each experiment and provides a standardization capability of sepsis development.

4.3 Introduction

Sepsis, a life-threatening condition resulting from a dysregulated immune response to infection, remains a formidable challenge in the landscape of healthcare, contributing to alarming mortality rates [1]. Despite on-going efforts to combat sepsis, its pervasive impact continues to be a global concern, with over 30 million people diagnosed annually, leading to

approximately 6 million deaths [2]. Survivors often grapple with long-lasting complications, and the elderly, particularly those with weakened immune systems, are disproportionately affected [3,4]. Despite decades of dedicated research, the complete understanding of sepsis pathophysiology remains elusive, and existing in vivo models, particularly murine models, face inherent limitations [5].

Murine models have played a pivotal role in advancing our understanding of sepsis progression, although the debate persists regarding their ability to faithfully replicate the complexities of human sepsis [6–9]. Model of endotoxemia using Lipopolysaccharide (LPS) injection have been used to investigate the sepsis physiology [10]. It is one of the easiest models in which purified LPS is intraperitoneally injected into mice. Since, LPS represents just a component of gram-negative bacteria, it is difficult to have any conclusive remarks in terms of its outcomes for a polymicrobial disease condition. Among the various induction methods employed, the gold standard, Cecal Ligation Puncture (CLP), encounters challenges related to reproducibility due to the influence of multiple factors on infection severity [11]. Another widely used approach, Colon Ascendens Stent Peritonitis (CASP), a model of abdominal sepsis induced by the leakage of the colon contents into the abdominal cavity, lacks precise information on microbial composition, introducing an element of uncertainty [12,13]. The Cecum Slurry (CS) method, involving the euthanasia of mice for cecal content, also results in high variability in microbial populations, affecting the reproducibility of experimental outcomes [7,14–17].

In response to these challenges, this study introduces an upgraded and improved method for reproducible sepsis induction. This innovative approach centres on the intraperitoneal injection of a standardized polymicrobial population derived from cecum slurry which was expanded by culturing in LB media and could be long-term stored frozen for a future

use [18]. Importantly, the proposed method further presents an enrichment approach, allowing for the selective growth of either Gram-positive or Gram-negative bacterial cultures [19]. This feature adds an extra layer of control and standardization to sepsis induction, addressing a key limitation of existing models. It should be noted that the proposed novel sepsis model is not aimed at modifying the existing sepsis methods. Models such as CLP, CASP, CS, etc. hold their own advantages which should not be overlooked. These older models involve a systemic inflammatory response caused by an autologous infection, induced by own cecal or colon bacteria of each individual animal, mirroring the characteristics of clinical sepsis. Conversely, we propose a different approach that provides a more ethical, technically efficient, and very reproducible sepsis model. Importantly, this new sepsis model is easily scalable for antisepsis drug testing in the pharmaceutical industry setting where standardization is essential.

4.4 Methods

4.4.1 Animal studies

4–5-month-old male and female Swiss albino mice in the ratio of 3:2, each weighing 30 g, were obtained from the College of Veterinary Science and Animal husbandry, Mhow, Indore, Madhya Pradesh, India. All mice were kept at 25°-28°C and a 14:10 h of light and dark cycle with 24x7 available drinking water and chow. Mice were acclimated for 7 days prior to experiment. This animal study was approved by the Institutional Animal Ethics Committee (IAEC) of Acropolis Institute of Pharmaceutical Education and Research and conducted in accordance with the policies of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India.

4.4.2 Preparation of cecum slurry

Five (3 male + 2 female) 4–5-month-old mice were used for initial cecal slurry collection. They were first euthanized by cervical dislocation under

anaesthesia (Chloroform) and the whole cecum was dissected with sterile forceps and scissors. By using forceps and spatula, the entire cecal content was collected in a sterile petri dish and the whole content was then resuspended in 5 ml sterile 1X PBS by continuous pipetting.

4.4.3 Culture and stock preparation

1ml of cecal slurry resuspended in 1X PBS was inoculated in 50 ml Luria Broth (HIMEDIA M575-500G) and was cultured overnight at 37°C on a shaker (220 RPM). The culture was then filtered (70 µm mesh strainer) to remove slurry particles. 1:50 part of that 1° culture was then inoculated in 50 ml LB media (2° culture). By utilizing this inoculum volume, the OD reached to 1 after 3 hours of incubation. OD was measured using UV spectrophotometer (PerkinElmer UV/VIS Spectrometer Lambda 35) at 600 nm. Different volumes of the culture media were then used for preparing injections with different bacterial concentration and the remaining media was used for preparing cryovials of 50% glycerol stock which were then stored at -80°C. For enrichment of cultures with a particular type of bacteria, the following specific antibiotics (20 µgml⁻¹) were used: Aztreonam to kill Gram negative bacteria, and Vancomycin to kill Gram positive bacteria. We added 5 ml LB media in each of five 15 ml tubes and supplemented the media with respective antibiotics (20 µgml⁻¹) and labelled the tubes as follows: tube A (Blank), tube B (LB + Inoculum), tube C (LB + Aztreonam + Inoculum), tube D (LB + Vancomycin + Inoculum), and tube E (LB + Levofloxacin + Inoculum). The media containing specific antibiotic was inoculated with 1:50 part of primary culture (1.5 OD) and incubated at 37 °C for 3 hours on an orbital shaker at 220 revolutions per minute (RPM). The tubes were then placed on ice and OD was measured using an UV spectrophotometer at 600 nm to determine the growth pattern of bacteria under different conditions. Antibiotics were added to the secondary culture. To check the growth rate of bacteria in these antibiotics rich media, OD was measured at 600 nm.

To validate the growth rate, spreading with primary culture was additionally done on agar plates containing Luria Agar (HIMEDIA M557-500G) and respective antibiotics. These were then observed after overnight incubation at 37°C. Gram staining (see below) was performed to confirm the type of microbiota in polymicrobial population. This enrichment culture method was replicated with both fresh cecal slurry and the 50% glycerol stock stored for 2 months at - 80° C.

4.4.4 Preparing samples for injections

Injection samples were prepared for four groups of 12 mice each, with different bacterial concentrations that were 2×10^9 CFUml⁻¹, 1×10^9 CFUml⁻¹, 0.5×10^9 CFUml⁻¹, and 0.1×10^9 CFUml⁻¹ per mice, by taking 2ml, 1ml, 0.5ml, and 0.1ml of culture media (1 OD) per mouse, respectively. It has been reported that an 1OD bacterial culture has a bacterial concentration of about 10^9 CFU in 1 ml of culture media [20]. The media was then centrifuged at 6000 xg at 4°C and then the pellet containing bacteria was resuspended in sterile 1X PBS and injected at 500 µl per mouse.

4.4.5 Serum isolation

Animal was euthanized by cervical dislocation under anaesthesia (Chloroform), and whole blood was collected by cardiac puncture. Whole blood samples were allowed to coagulate for 30 minutes at room temperature and then were centrifuged at 7,000 RPM for 25 minutes at 4° C to separate the serum from the clot. The isolated serum samples were then stored in -80°C.

4.4.6 Cytokine analysis

ELISA kits (R&D systems) were used to measure cytokines levels, following the manufacturer's instructions. IL-6, TNF- α , and IL-1 β levels were measured from exudates obtained from various groups.

4.4.7 Measurement of biochemical parameters

Serum levels of C-reactive protein (CRP), bilirubin, alanine transaminase (ALT), aspartate transaminase (AST), urea, and creatinine were analysed by commercial detection kits from R&D systems following the manufacturer's instructions.

4.4.8 H&E staining

Haematoxylin and eosin stain was used for staining nuclei (blue) and cytoplasm/extracellular space (pink). The tissue samples preserved in 4% formaldehyde were used for mounting and H&E staining. The prepared slides were then observed using an Invitrogen EVOS M5000 Cell Imaging System, and the images were taken at 40X. The alveolar space and the infiltrating immune cells were counted by using ImageJ.

4.4.9 Gram staining

To validate the enriched culture, whether it is gram positive or negative bacteria, a simple gram staining technique was performed by using gram staining kit (HIMEDIA-K001-1KT, HiMedia Laboratories, LLC), where crystal violet was used to stain gram positive while safranin was used to stain gram negative bacteria. The culture media was used immediately after measuring the CFU.

4.4.10 Mortality analysis

Mice were injected with different polymicrobial concentrations and were monitored for up to 48 hours post injection. The Kaplan-Meier curve was used for graphical presentation, where Y-axis represented percent survival. The tissue samples of the lungs, liver and kidneys were collected 10 hours post injection in animals from two groups (2 mice/group). These were preserved in 4% formaldehyde and were analysed after H&E staining. Each experiment was performed twice, and the survival curve was constructed by the data acquired in each group.

4.4.11 ImageJ analysis

ImageJ allows users to measure various parameters in images, such as intensity, size, shape, and texture. It also provides tools for performing statistical analyses on the image data. Here, ImageJ was used to analyse the H&E-stained tissue section images. The cell number was measured by first splitting the images into appropriate channels and then setting up a threshold for an appropriate size of cells. The values were then analysed and plotted in GraphPad PRISM 7.0.4.216 (GraphPad Software. Inc).

4.4.12 Statistical analysis

Statistical analysis and visualization of data was performed using GraphPad PRISM 7.0.4.216 (GraphPad Software. Inc). All data points are representative of two independent experiments; all data are presented as mean $\pm$ SD. Significance was determined using the One-way ANOVA test. **** $P \leq 0.00001$; *** $P \leq 0.0001$; ** $P \leq 0.001$; * $P \leq 0.01$.

4.5 Results

4.5.1 Effect of different bacterial concentrations on survival of mice

To determine the effect of bacterial concentrations on the mortality of mice presenting with experimental sepsis, we divided 60 mice into 5 groups (A, B, C, D, and E), with each group containing 12 mice. The mice were intraperitoneally injected with 2×10^9 CFUml⁻¹ (group A), 1×10^9 CFUml⁻¹ (group B), 0.5×10^9 CFUml⁻¹ (group C), or 0.1×10^9 CFUml⁻¹ (group D) bacterial suspension, respectively, whereas the group E was kept as control (saline) (**Figure 4.2**). Mice then were monitored after every 2 h. We found 80% mortality at 6 hours in group A, 50% mortality at 6 h in group B, 50% mortality at 16 h in group C, and only 20% mortality at 20 h in group D. 100% mortality was observed in group A with the maximum bacterial concentration after 16 h post injection. Conversely, in the group D with the minimum concentration only 20%

mortality was observed at 20 h post injection, and all survivor mice showed fast recovery (**Figure 4.2**). When closely inspected, all mice in group A, B, and C developed white eyes and loss of vision after 12 h, indicating severe sepsis condition [21]. The mouse eyes were unchanged in group D, which indicates the insufficient polymicrobial concentration to cause severe sepsis. The whole experiment was replicated using the same glycerol stock of the polymicrobial cultures to validate the results.

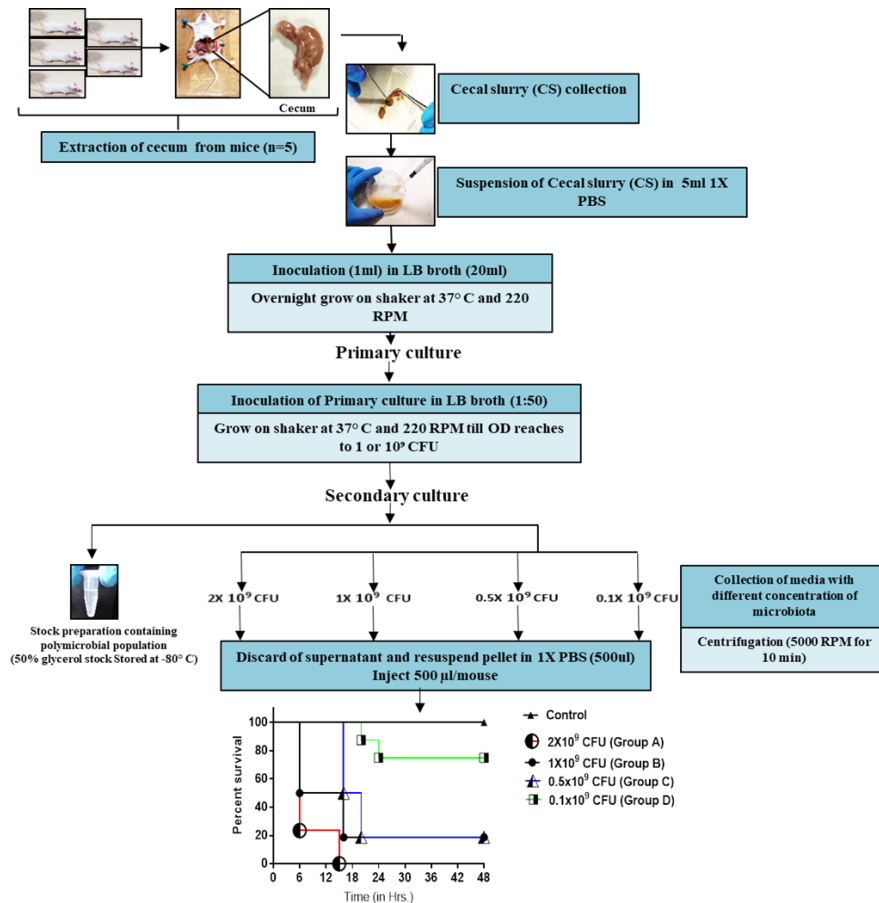


Figure 4.2 Protocol for sepsis induced by injections of the cecal slurry bacterial culture and the survival curve in mice treated with 2×10^9 CFU/ml

¹, 1×10^9 CFUml⁻¹, 0.5×10^9 CFUml⁻¹, and 0.1×10^9 CFUml⁻¹ (12 mice per group). The cecal slurry bacterial culture stock was stored at -80° C.

4.5.2 Effect of different bacterial concentration on different organs of mice

To see the effect of different bacterial concentrations, we collected tissue samples of the lungs, liver and kidneys in group C and D to compare the organ damage and to quantify inflammation between the maximum tolerated concentration (group C) and the insufficient polymicrobial concentration (group D) to cause sepsis. All the samples were collected 10 h after injection. We dissected two mice from each group and collected the indicated organs in 4% formaldehyde after rinsing with 1X PBS. The samples were then sectioned and subjected to H&E staining. We compared the H&E-stained lung tissue of Group D mice with the untreated one (**Figure 4.3A**) and observed more infiltration of immune cells (depicted by black arrows) and an increase in necrosis (depicted by red arrows) in the septum, indicating inflammation and tissue damage. Consistently, the group C's lung tissue had more infiltrated immune cells than the one from the group D. We also quantified the number of cells infiltrating into the alveolar septum (**Figure 4.3B**) and the vacant space between the septum (**Figure 4.3E**) by using ImageJ bundled with 64-bit Java 8 in 536x389 pixels images. We found that the degree of immune cell infiltration corresponded well with the polymicrobial concentration, and the vacant space was decreased in accordance with it, clearly indicating that sepsis was more significant with increasing inflammation. The alveoli were damaged more in group C compared to group D, further confirming that the severity of sepsis increased in group C. We next compared the liver tissue (**Figure 4.3A**). We found more immune cells as well as larger necrosis affected areas in group C than in group D, again indicating that inflammation and tissue damage was increased with high polymicrobial concentration. We observed a similar pattern in the kidneys (**Figure**

4.3A). We also counted the number of infiltrating immune cells and the percent of necrotic area in the liver (**Figure 4.3C and F**) and kidney (**Figure 4.3D and G**) and found that the number and necrotic area increased. In short, all the three organs had increased inflammation and exhibited the signs of multi-organ damage that likely led to sepsis and then further to septic shock, resulting in animal death.

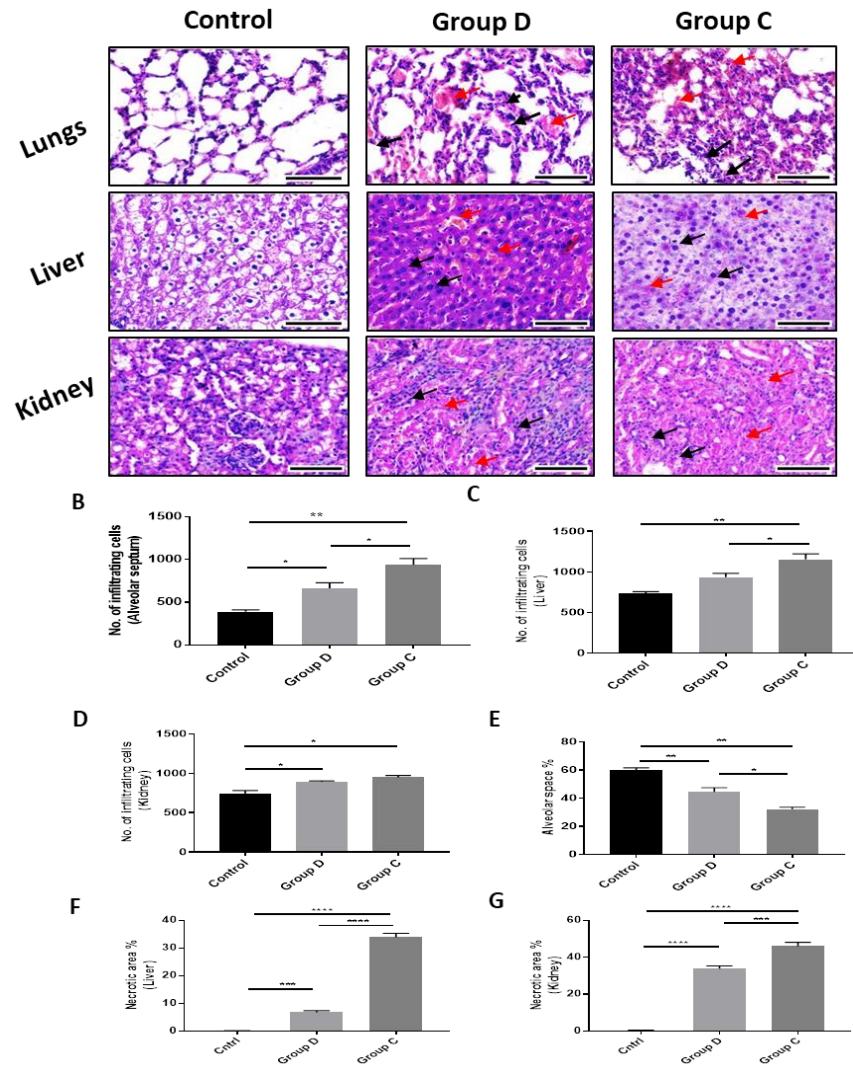


Figure 4.3 Effect of cecal slurry polymicrobial culture concentration on mice. (A) H&E-stained sections of the lung, liver and kidney tissue at 40X (75 μ m scale bars). Samples were collected from control mice and mice

treated with either 0.5×10^9 CFUml⁻¹ or 0.1×10^9 CFUml⁻¹ of polymicrobial cultures (black arrow and red arrow depicts infiltration of immune cells and necrotic area, respectively). (B)-(D) shown are the numbers of immune cells infiltrating in the alveolar septum, liver, and kidney. (E) Shown are the alveolar space percentages between the alveolar septum. (F)-(G) are showing the necrotic area in terms of percentage in the liver and kidney, respectively. The whole experiment was replicated with the 50% glycerol stock of polymicrobial cultures. All data are representative of three independent experiments; all data are presented as mean $\pm$ SD. P-values significance was determined using One-way ANOVA test. ****P $\leq$ 0.0001; ***P $\leq$ 0.001; **P $\leq$ 0.01; *P $\leq$ 0.05.

4.5.3 Effect of sepsis induced by the enriched cecal slurry bacterial culture on pro-inflammatory cytokines, liver enzymes levels and biological metabolites related to the kidney function in mice

To further characterize the extent of inflammation and tissue damage in septic mice, we determined the serum levels of pro-inflammatory cytokines, liver enzymes, and metabolites related to the kidney function. The levels of pro-inflammatory cytokines were measured by using the cytokines' ELISA kit. We focused on three major pro-inflammatory cytokines, namely: TNF- α , IL-1 β , and IL-6. To induce sepsis, mice were injected with the cecal slurry bacterial culture at 0.5×10^9 CFUml⁻¹, whereas the control mice were injected with saline. We observed that the serum pro-inflammatory cytokine levels were significantly elevated in the septic mouse group compared to the control mouse group (**Figure 4.4A, B, and C**), indicating increased systemic inflammation. Consistently, serum levels of CRP, a marker of systemic inflammation, were also elevated in septic mice compared to control mice (**Figure 4.4H**). We also found that the serum levels of the liver enzymes, AST and ALT, were elevated in septic mice relative to control mice (**Figure 4.4F,G**), further confirming the presence of liver damage which was already documented

above. In agreement, serum bilirubin levels were also increased (**Figure 4.4I**). Serum urea and creatinine levels were significantly elevated in septic mice compared to control mice (**Figure 4.4D, E**), indicating the presence of kidney damage in septic mice. All these data confirm that our novel approach for sepsis development is very effective for inducing significant inflammatory septic conditions leading to liver and kidney damage.

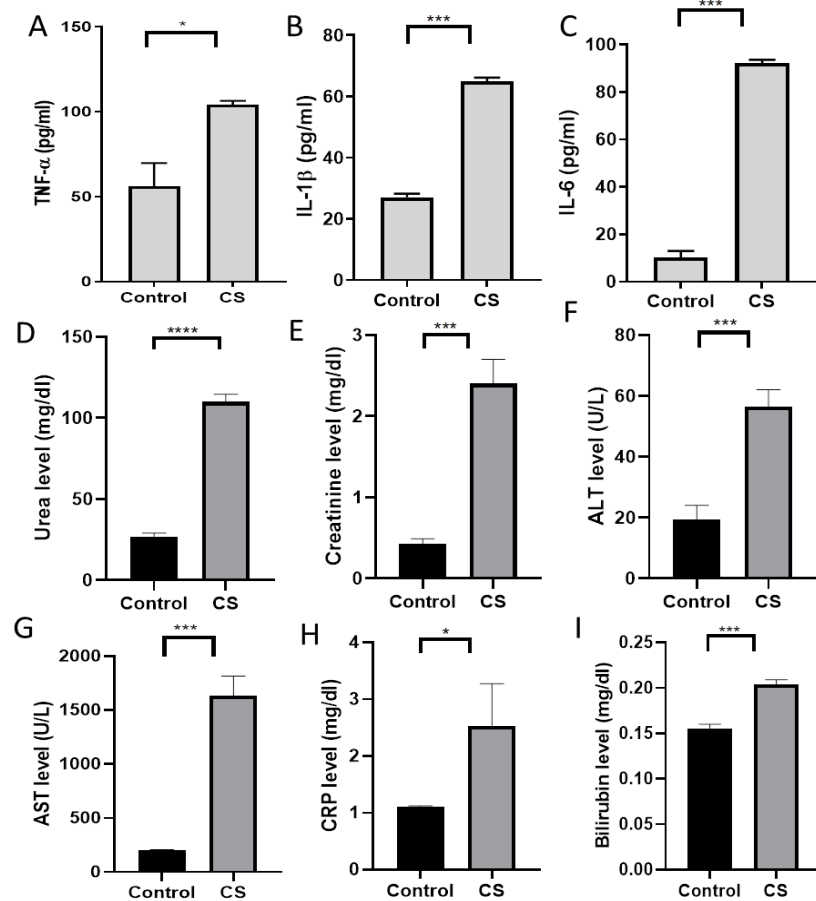


Figure 4.4 Comparisons of pro- inflammatory cytokines, liver enzymes, and other clinically relevant biological metabolites in the serum of septic and control mice. (A-C) The comparisons of pro-inflammatory cytokine levels in the serum of septic and control mice. (D-I) Shown are the

comparisons of serum levels for Urea, Creatinine, ALT, AST, CRP, and Bilirubin in control vs. septic ALT: Alanine Transaminase, AST: Aspartate Transaminase, CRP: C Reactive Protein. All data are presented as mean $\pm$ SD. P-values significance was determined using t-test. **** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$.

4.5.4 Inducing gram-negative and gram-positive sepsis by enriching bacterial cultures

The above experiments were performed to induce polymicrobial sepsis. We next used the same approach but introduced the enriched bacterial cultures to induce gram-negative or gram-positive bacterial sepsis (**Figure 4.5**). We employed two antibiotics, aztreonam and vancomycin, which can selectively target gram-negative or gram-positive bacteria, respectively. Additionally, we used a broad-spectrum antibiotic levofloxacin to determine if there was any growth other than bacteria. The OD of culture media containing no antibiotics was 0.75 while aztreonam, vancomycin and levofloxacin enriched cultures had a value of 0.41, 0.7, and 0.05 OD, respectively (**Figure 4.6A**), indicating that the broad-spectrum antibiotic levofloxacin can kill all type of bacteria and that there were no fungi in the cecal slurry. Notably, the gram-positive bacteria grew at lower rate than the gram-negative bacteria. For further validation, we also performed spreading of polymicrobial culture on agar plates with no antibiotics (**Figure 4.6C-I**), aztreonam (**Figure 4.6C-II**), vancomycin (**Figure 4.6C-III**), or levofloxacin (**Figure 4.6C-IV**). The results showed the same pattern of growth as observed by measuring the OD values at 600 nm. To confirm the action of respective antibiotics, we next performed gram staining by taking 20 μ l of suspension from each tube and making smears on slides. We utilized the HIMEDIA-K001-1KT kit for gram staining and analysed the stained smears using the microscope (EVOS M5000 Invitrogen) at 40X. In cecal slurry bacterial cultures, there was growth of all type of bacteria that can be visualized by violet and pink stains (**Figure**

4.6B-I). There were more gram-positive bacilli bacteria in tube C identified by the crystal violet (**Figure 4.6B-II**) and further the strings of gram-positive bacilli, which cannot be formed by gram negative bacilli (https://tmedweb.tulane.edu/pharmwiki/doku.php/bacteria_101_cell_walls_gram_staining_common_pathogens), can also be observed. Accordingly, there were more gram-negative cocci and bacilli in Tube D (**Figure 4.6B-III**). As expected, there was no growth of any microbial population in Tube E (**Figure 4.6B-IV**). For future experiments, the enriched culture can be stored at -80°C as 50% glycerol stocks or can be used for inducing gram-negative or gram-positive sepsis by using the respective cultures and injecting the mice with the desired bacterial concentrations. Furthermore, the cultures may be propagated to amplify specific bacterial population.

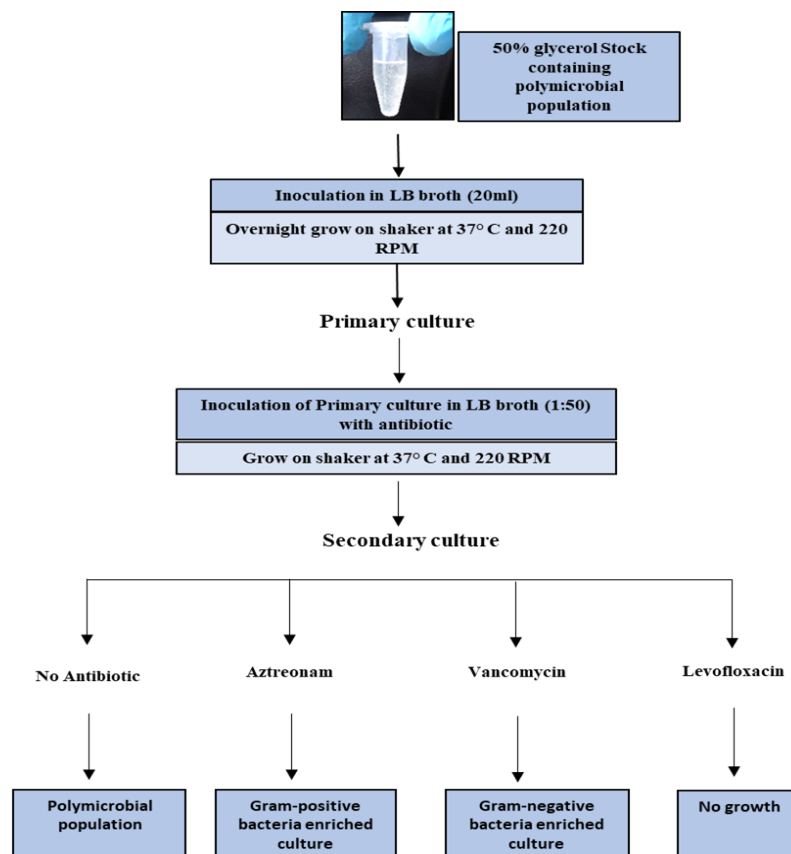


Figure 4.5 Enrichment protocol - Glycerol stock was cultured with respective antibiotics to generate specific bacterial populations.

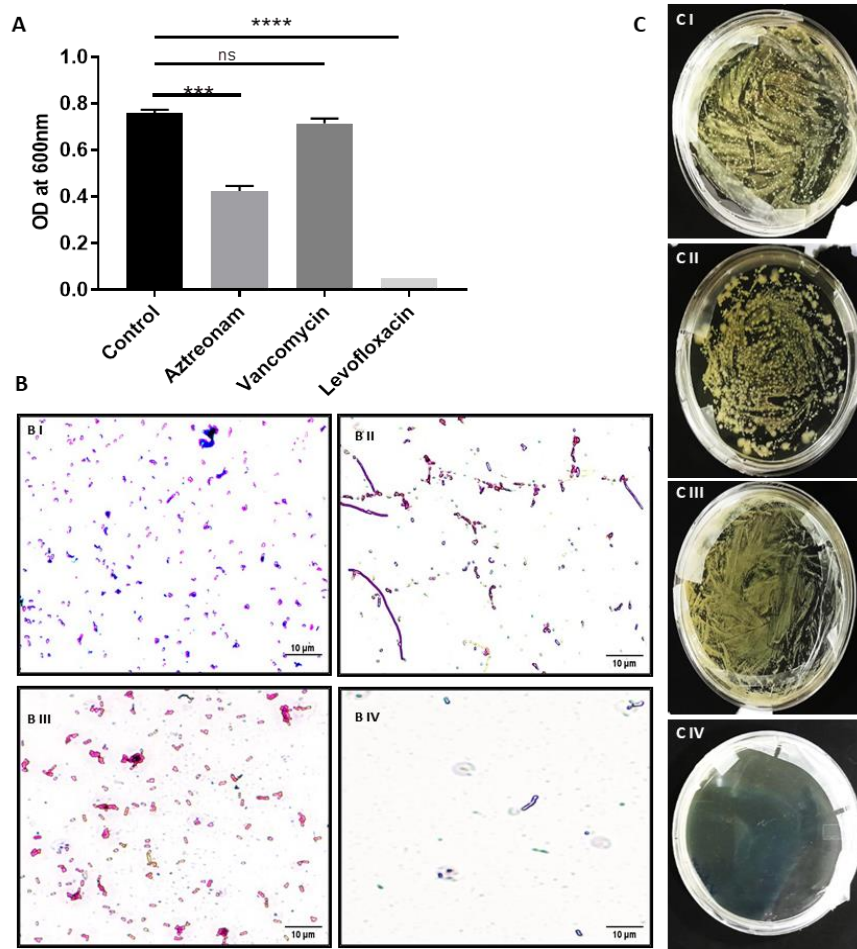


Figure 4.6 Results showing culturing cecal slurry polymicrobiota in media enriched with antibiotics (20 μ g/ml) targeting gram -ve and +ve bacteria. (A) The bar graph depicts the growth of bacteria in different conditions where Aztreonam, vancomycin, and levofloxacin targets gram -ve bacteria, gram +ve and both, respectively. Levofloxacin was used to see whether there are any other microbes beside bacteria in the cecal slurry. The OD was taken after 3 hrs. and the error bar shows the OD value after 3.30 hrs. (B) The gram-stained images where (I) represents the culture without antibiotics, (II) represents aztreonam, (III) vancomycin and (IV) levofloxacin treated culture. (C) Growth pattern with different antibiotics. Same amount of LB culture media was spread on LA with (I) no antibiotics, (II) aztreonam, (III) vancomycin and (IV) levofloxacin. All

data are represented as mean $\pm$ SD. P Significance was determined using One-way ANOVA test. **** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$.

4.6 Discussion

Sepsis is a common clinical condition that is associated with a high morbidity and mortality throughout the globe [4]. This life-threatening syndrome may occur due to the unceasing exaggerated inflammatory response along with an immunosuppressed state [11,22–24]. Though much advancement has been made in the direction of finding concrete solutions to treat sepsis, it still remains one of the most serious clinical problems. It is very important to understand the pathophysiology of the disease to design effective therapeutic strategies against this condition. Considering the complexity and the involvement of various organs in the biology of sepsis, it is the immediate requirement to develop better therapies against this multi organ dysfunction condition.

Animal sepsis models are widely used to study the pathophysiology of sepsis. However, it is crucial to identify the right model which can be reproducible and capable of mirroring both cellular and molecular aspects of sepsis, such as observed in humans. Several approaches have been developed in the past to study sepsis. These include the CLP [25], CASP [5], CS [7,14–17] methods and few others.

The CLP approach is considered as the “Gold standard” to develop and replicate the human condition of polymicrobial infection in animals [25]. Despite its wide acceptance, it has several shortcomings. This model is complicated due to high variability in terms of size and shape of cecum that ultimately affects sepsis severity. Similarly, such models as CASP [12,13] and CS [7,14–17] that are also aimed to mirror sepsis conditions recapitulating the human state have limitations. These models require the euthanization of additional animals for every single new experiment.

Furthermore, these approaches do not provide a sufficient insight on the microbial flora composition that is delivered into the abdominal cavity from the cecum, bringing the uncertainty factor in the induction of sepsis model. In the CS model, the slurry is prepared fresh every time [7,14–17], thus adding a high amount of variability every time the experiment is performed. Though a new method was recently developed where storage option has been proposed, making an alternative to animal euthanize for each experiment, the approach still lacks the reproducibility of sepsis conditions among different experiments and again lacks information on the composition of microbial population. Hence, a better and improved approach is required to tackle the obstacles being faced in the development of sepsis models.

Herein we propose a novel and efficient method for inducing polymicrobial sepsis in animals. Our protocol is based on the intraperitoneal injections of defined bacterial cultures with known polymicrobial population. In our experiments, we compared five animal groups, including a control group. Each treatment group received varying doses of polymicrobial bacterial load. Before animal injection, the polymicrobial cultures were grown in the standard LB medium that is a standardizable approach, allowing sepsis induction with varied titrated inoculum dosages in order to develop the diseased state with varying severity as per the requirement. The results from the survival curves depict that the groups injected with higher CFU had early mortality and the mortality rate decreased with the decreases in CFU. This can prove to be helpful in order to regulate and plan the experiments to better understand the biology and various factors involved at varying stages of sepsis.

Other methods required the cecum slurry to be freshly prepared for every new experiment; new advancements have made it possible to store frozen cecum slurry suspensions [18]. In our study, we make a step forward and propose the storage of the artificially propagated polymicrobial culture

derived from cecal slurry that can be stored at -80°C in 50% glycerol for a very long period. In our experiments, similar results were obtained in replicate experiments using the frozen bacterial stocks indicating that the frozen bacteria preserved viability.

As sepsis is characterized by multiorgan dysfunctions, we dissected different organs, which are supposedly affected by sepsis, to see the effect of the varied microbial population used for sepsis induction. The lungs, liver, and kidneys were analysed for infiltration of immune cells to compare the extent of inflammation using H&E-staining. The significant increases in the levels of pro inflammatory cytokines (TNF- α , IL-1 β , and IL-6) and CRP suggested that the administered polymicrobial culture resulted in the development of inflammatory septic conditions. Conversely, the increased levels of AST, ALT, bilirubin, creatinine, and urea in the septic mice indicated the presence of significant damage to the liver and kidneys. The severity of sepsis correlated with the survival results, confirming our ability to successfully monitor disease progression. Importantly, our approach provides ethically benefits as it does not require euthanizing animals for every new experiment.

In this study, we also focused on overcoming the inconsistency of previously proposed methods to standardize the bacterial inoculum [18]. We put forward a culture enrichment strategy that permits us to grow a specific population i.e.; gram-negative or gram-positive bacteria. Uniquely, we used special antibiotics to harvest these bacterial species' specific cultures and validated the results via gram staining. This enrichment method could give the researchers an effective and efficient way to generate specific cultures in order to investigate various disease models which require a defined microbial population. Moreover, without the usage of any special antibiotics one can just grow a polymicrobial bacterial population from the same initial bacterial culture stored at -80° C as glycerol stocks. Thus, avoiding the variability associated with previous

approaches which utilized undefined cecum slurry preparations for sepsis induction. In sum, we propose an enriched cecal slurry bacterial culture sepsis model that provides an important benefit of standardizing the data in the sepsis research field. Although the current sepsis models are important to mirror clinical settings, our standardized sepsis model allows the researchers from different laboratories effectively and quantitatively compare their results. Our defined cecal slurry polymicrobial culture can be stored at -80°C as glycerol stocks for prolonged durations and later propagated as needed for distribution to all laboratories working in the sepsis field. The major advantages of our protocol are as follows: 1) ability to standardize the bacterial inoculum, 2) ethically suitable as it limits the unnecessary euthanasia of animals, 3) effective and technically sound method, and 4) enrichment approach allowing to grow specific defined polymicrobial populations. These advantages definitely help further our understanding of the pathophysiology of sepsis and may contribute to saving lives of affected patients.

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Chapter 5

Inhibiting TIRAP-Mediated Inflammatory Signaling: A promising Therapeutic Strategy against Sepsis

Chapter 5: Inhibiting TIRAP-Mediated Inflammatory Signaling: A promising Therapeutic Strategy against Sepsis

5.1 Graphical Abstract

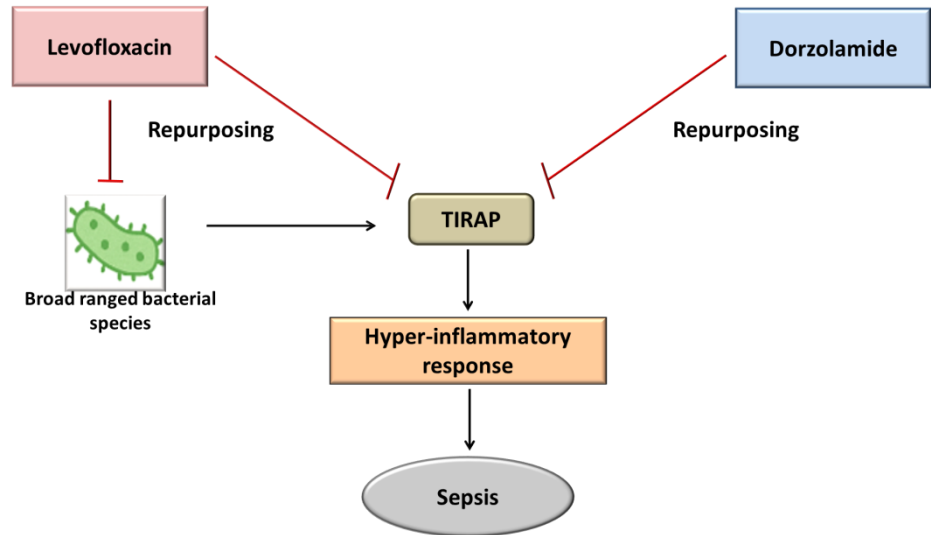


Figure 5.1 The diagram shows the key elements of the proposed LeDoz combination therapy for treating sepsis. Levofloxacin and Dorzolamide are the two constituents of “LeDoz.” Both drugs inhibit TIRAP activity involved in the pathogenesis of sepsis. Additionally, Levofloxacin is a broad-spectrum antibiotic.

5.2 Overview

Timely therapeutic targeting of the dysregulated immune response in sepsis is essential to restore immune homeostasis and prevent progression to organ dysfunction. We investigated the efficacy of a combination treatment with levofloxacin and dorzolamide (LeDoz), to address these two separate aspects of sepsis. We used an in-silico approach to screen FDA-approved antibiotics targeting Toll/interleukin-1 Receptor (TIR) domain-containing adaptor protein (TIRAP). The cecum slurry (CS) septic mouse model was subsequently utilized to validate the candidates. Survival of mice, tissue mRNA expression and morphology, cytokine levels, and other biochemical markers were monitored. Virtual screening identified a broad-spectrum antibiotic levofloxacin as a candidate. The in vivo studies confirmed that the combination of levofloxacin and dorzolamide increased the survival rate of septic mice. Hematoxylin and eosin (H&E) tissue staining, cytokine levels, as well as immunofluorescence dual staining and serum biomarkers all showed the reestablishment of homeostatic conditions in the LeDoz-treated group of septic mice. In vitro analyses also confirmed the ability of LeDoz to attenuate TIRAP-mediated inflammatory signaling. The combination of levofloxacin and dorzolamide exhibits both an antibacterial effect and a strong potential of synergistically reducing chronic inflammation in the host by inhibiting the activation of TIRAP.

5.3 Introduction

Sepsis is a multi-organ dysfunction fatal condition that develops due to a dysregulated and dysfunctional host's immune response [1]. Sepsis continues to be a global issue despite continuous attempts to battle it; around 30 million people are diagnosed with sepsis each year, resulting in over 6 million fatalities [2,3]. With the ongoing efforts and strategies, a reduction in the mortality rates has been observed globally, yet sepsis remains the cause of death for over 25% of patients [4]. A subcategory of

sepsis, known as septic shock, identified by metabolic, cellular and circulatory dysfunctionalities, still accounts for approximately 60% hospital mortality rates [4]. Despite ongoing research, the multifactorial and dynamic nature of sepsis continues to pose challenges in fully elucidating its pathophysiology. The murine models have played a central role for better understating the underlying mechanisms [5,6]. To date, there is no efficient treatment for sepsis, hence it is necessary that patients with early septic symptoms are identified and taken care of with appropriate therapeutic strategies at the earliest time from the onset of disease, which comprise the induction of suitable antibiotics, source control measures, and resuscitation with intravenous fluids and vasoactive drugs, if indicated [7]. Though few attempts have been made in this direction, researchers have failed to move beyond clinical trials because of insufficient biological activity of the tested treatments [7]. For example, since the TNF α levels are markedly elevated during the first hours after sepsis induction in animal models, an anti-TNF α monoclonal antibody was evaluated in clinical trials to manage sepsis. However, no significant improvement was observed in septic patients enrolled in the clinical trial possibly due to the fact that the TNF α levels were not monitored in the enrolled patients, and these levels could be too low compared to those observed in animal models [7]. The failures may also be related to the complex epidemiology of sepsis. Indeed, around 60 to 70% of septic patients were found to have different types of pathogenic microorganisms in their blood cultures [7–9]. Furthermore, a study revealed that targeting the pathogenic organisms at the site of infection was associated with high mortality of patients [10].

The major currently available treatments for sepsis are built around the use of antibodies that target pro-inflammatory cytokines. However, none of these approaches targets the fundamental elements that drive illness progression, the signaling remains unexplored. In this study, we sought a

combination therapy, a method of treating an ailment by combining two or more treatments to produce a more effective therapeutic impact than would be gained by utilizing each treatment or medication alone. It was discovered that gram-negative bacteria are the top cause of death among all infections that cause sepsis [11]. Although mortality caused by gram-positive bacteria has also been reported [11], our main focus remained gram-negative bacteria-mediated signaling. In this study, we chose a combination therapy, which combines an antibiotic with an anti-inflammatory drug. The target of our anti-inflammatory drug is Toll/interleukin-1 Receptor (TIR) domain-containing adaptor protein (TIRAP), which is activated when bacterial lipopolysaccharide (LPS) binding triggers toll-like receptor 4 (TLR4) signaling. TIRAP activation ultimately results in the nuclear translocation of various pro-inflammatory transcription factors such as activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). TIRAP has been already suggested as a potential therapeutic target [12–17]. It is well known that TIRAP activation is primarily triggered by the actions of two kinases: Protein kinase C δ (PKC δ) and Bruton's tyrosine kinase (BTK) [18]. After TIRAP interacts with BTK and PKC δ , the TIRAP's TIR domain gets phosphorylated at Y86, Y106, Y159, and Y187 tyrosines [18–20] and then a downstream signaling cascade that mostly transduces via the NF- κ B and mitogen-activated protein kinase (MAPK) pathways is activated [21–23].

The main objective of the present study is to use a broad-spectrum antibiotic, Levofloxacin, and a repurposed anti-inflammatory drug, Dorzolamide, which reduce sepsis progression by killing bacteria and healing injured organs via reestablishing homeostasis. Furthermore, we found that the drug combination exhibits potent anti-inflammatory activity, resulting in a synergistic effect.

5.4 Materials and methods

5.4.1 Cell culture

The RAW 264.7 murine macrophage cell line was obtained from the cell bank of the National Centre for Cell Science located in Pune, India. RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (Gibco, UK) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, UK), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, UK) in a humidified incubator at 37°C with 5% CO₂ until they reached the maximum confluence. In all treatment groups, unless otherwise specified, cells were treated at the indicated time points with 1 µM dorzolamide, 1 µM levofloxacin, and 1 µg/ml LPS (Sigma, MO, USA).

5.4.2 Immunoblotting

Cells were harvested & washed with PBS 3x and then lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with phosphatase & protease inhibitors. The protein concentration was determined using the Bradford assay dye reagent (Bio-Rad). Equivalent protein amounts were separated by SDS-PAGE, resolved, and transferred to nitrocellulose membranes (Bio-Rad, USA). Nitrocellulose membranes were blocked in 5% nonfat milk (Bio-Rad, USA) for 1 hour at room temperature and then probed with the primary antibody at either 4°C overnight or 4 hours at room temperature. After incubation with the primary antibody, the membranes were washed three times in the TBST solution before being exposed to an HRP-conjugated secondary antibody for an hour at room temperature. The blots were then incubated with the chemiluminescence HRP Substrate (Bio-Rad, USA) and imaged using a gel documentation system (Image Quant LAS 4000, GE Healthcare, Uppsala, Sweden) following a second TBST wash. ImageJ (National Institutes of Health; doi.org/10.1038/nmeth.2089) was used to determine band intensities. The antibodies used against mouse were as follows:

TIRAP (Cell Signaling Technology, USA), p-TIRAP (Invitrogen, USA), p38 α MAPK (Santa Cruz Biotechnology, CA, USA), p-p38 α MAPK (Santa Cruz Biotechnology, CA, USA), NF- κ B/p65 (Santa Cruz Biotechnology, CA, USA), p-NF- κ B/p65 (Cell Signaling Technology, USA), β -Actin (Invitrogen, USA), & secondary HRP conjugated antibody.

5.4.3 Quantitative reverse transcriptase PCR

RNA was extracted from cells using the RNAiso Plus reagent (Takara Bio Inc., Shiga, Japan). Nanodrop spectrophotometer was used to check the purity and concentration of isolated RNAs. cDNAs were prepared using the cDNA Synthesis Kit (TAKARA, Japan), per the manufacturer's instructions. The SYBR green master mix (Applied Biosystems, MA, USA) was utilized for the real-time quantitative PCR (qPCR) to determine the expression levels of the following mouse genes: *GAPDH*, *IL-1 β* , *TNF- α* , *IL-6*, *IL-12*, and *IL-23*. The expression levels of cytokine genes were normalized to that of GAPDH as a reference gene. The $2^{-\Delta\Delta C_t}$ approach was employed to analyze the cycle threshold value in relation to relative cytokine expression.

5.4.4 Serum isolation

Mice were sacrificed under anesthesia as described below. Blood was collected via cardiac puncture. Blood samples were incubated at room temperature for around 30-45 minutes and then were centrifuged for 25 min at a speed of 7000 RPM. Supernatants were collected and stored for further usage at -20°C.

5.4.5 Cytokine analysis

Inflammatory cytokines (TNF- α , & IL-1 β) concentrations in blood serum isolated from different mouse groups were determined using ELISA kits (R&D systems) according to the manufacturer's instructions.

5.4.6 Measurement of biochemical markers

The concentrations of various biochemical markers present in serum such as bilirubin, aspartate transaminase (AST), C - reactive protein (CRP), alanine transaminase (ALT), urea, and creatinine were determined by respective kits following the manufacturer's instructions.

5.4.7 Molecular docking & virtual screening

The crystal structure of TIRAP (PDB Id 3UB2) was obtained from the Research Collaborator for Structural Bioscience (RCSB) database, and then checked for any missing area to be modeled [24]. The common interacting residues of PKC δ and BTK with TIRAP were obtained from our previous study [24]. FDA approved drug library was analyzed using the Autodock Vina tool to identify the molecules that can dock to TIRAP's residues involved in PKC δ and BTK interactions [25]. A grid was designed with x, y, and z equivalent to 40, 36, and 34 casing TIRAP entirely. The results obtained were evaluated using Discovery Studio Visualizer [26]. Visualizing tools such as Discovery Studio Visualizer [26] and Chimera [27] were used to depict the complexes.

5.4.8 Molecular Dynamics Simulation

Molecular Dynamics (MD) simulations were conducted to ascertain the stability of the complex resulting from the binding of Levofloxacin to the TIRAP protein using GROMACS version 2022 [28,29]. The amber99sb-ildn force field was employed and the TIP3P water model was used for solvation. Ligand topology was generated using Antechamber and ACPYPE tools within the AmberTools suite [30]. To maintain electrostatic balance, four sodium ions were introduced. The Steepest Descents algorithm was utilised for energy minimisation which successfully converged in $F_{max} < 1000$. The system's pressure and temperature conditions were established through sequential NVT (constant Number of particles, Volume, and Temperature) and NPT (constant

Number of particles, Pressure, and Temperature) equilibrations for 2 nanoseconds each. The system was immersed in a cubic solvation box, ensuring a minimum 1.0 Å separation between the protein and the box boundary. The MD production run was carried out for 200 nanoseconds at a temperature of 300K and pressure at 1.0 bar. The coordinates were saved every 10 picoseconds for subsequent analysis. Trajectory data were analyzed to evaluate Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), Radius of Gyration, and Solvent Accessible Surface Area (SASA) metrics.

5.4.9 H&E staining

Hematoxylin is a basic dye that stains the acidic components of cells, such as the nucleus and some cytoplasmic structures, blue to purple. Eosin is an acidic dye that stains the basic components of cells, such as cytoplasm and extracellular matrix, pink to red. 4% paraformaldehyde was utilized for preserving tissues that were used for the embedding in paraffin, mounting and staining. After viewing the prepared slides under a microscope equipped with an Invitrogen EVOS M5000 Cell Imaging System, images were captured at a magnification of 40X. ImageJ was then used to count the infiltrating immune cells in the tissues.

5.4.10 Murine model

All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) of Acropolis Institute of Pharmaceutical Education and Research (AIPER) and conducted in accordance with the policies of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Government of India; animal protocol #AIPER/IAEC/2023/03). Swiss albino mice (4–5-month-old; ~25 g) were used for the in vivo studies. The mice were kept in a hygienic environment with a relative humidity of 55–60% and a temperature of 22–25°C. A light and dark cycle of 14:10 hours was maintained in the animal room. The

mice had ad libitum access to clean water and dry pellet food. To investigate the synergistic therapeutic effect of the combination of levofloxacin and dzd (LeDoz) in Cecum Slurry (CS) - injected mice, animals were randomly subdividing in five groups with 12 mice (male) in each group, namely control, CS, CS + levofloxacin, CS + dzd, and CS + LeDoz. An intraperitoneal injection route was used to inject 0.5×10^9 CFUml⁻¹ of CS to develop septic condition. A one-time dose of levofloxacin, dzd, and LeDoz of 7.1 mg/kg and 25 mg/kg respectively was given intravenously 1 h prior to injecting CS while the untreated control/sham group received vehicle PBS and/or DMSO solution. The survival was observed for 96 h. The tissues were isolated at 10 h after treatment for histomorphological studies (n=3).

5.4.11 Immunohistochemistry

Tissues were isolated from the treated mice of different groups and fixed in 4% paraformaldehyde for 1h. The tissue was imbedded in paraffin, and the obtained paraffin blocks were cut into 5 µm thick sections which were deparaffinized using xylene and ethanol. Sections were rinsed with water and subjected to the heat-induced epitope retrieval procedure by incubating them at a temperature of 90 °C in Tris-EDTA buffer for 10 minutes to activate the antigen epitopes. Sections were next blocked in 5% BSA (MP Biomedicals, USA) for 1h and then incubated with primary antibody against the protein of interest overnight at 4 °C. After 2x washes, sections were incubated with the secondary antibodies for 1h at room temperature. Nuclear counterstaining was done using DAPI (Thermo Fisher Scientific, USA) according to manufacturer's instructions. Stained tissues were analyzed using an Olympus confocal laser scanning microscope equipped with a 100X objective.

5.4.12 ImageJ analysis

ImageJ was used to analyze the H&E-stained tissue section images, enabling quantification of key features such as cell number. To do this, the images were first split into appropriate color channels, followed by applying a threshold to isolate cells based on their size. This allowed for accurate identification and counting of individual cells. The resulting data were then compiled and statistically analyzed using GraphPad Prism (version 7.0.4.216, GraphPad Software Inc.), allowing us to visualize and interpret the results effectively.

5.4.13 Statistical analysis

The data was analyzed and plotted using GraphPad PRISM 7.0.4.216 (© 1992-2017 GraphPad Software, Inc). All data are representative of three independent experiments and presented as mean $\pm$ SD. The P values were determined using the One-way ANOVA test. Data sets were considered significantly different if the p value was less than 0.05.

5.5 Results

5.5.1 Virtual screening data indicate that Levofloxacin and DZD of LeDoz bind to adjacent structural elements within the BTK-PKC δ common interface on TIRAP

Our initial search for clinically used compounds capable to weaken TIRAP-PKC δ interface residue interactions led us to discovering DZD as an inhibitor of TIRAP-PKC δ interactions [31]. In this study, we screened an antibiotic compound library against TIRAP, and a broad-range antibiotic levofloxacin was found to have a high binding affinity to the BTK-PKC δ common interface on TIRAP. Figures show the structures of the TIRAP's interacting domain with its activating kinases BTK and PKC δ (**Figure 5.2A, B & D**). Molecular docking of both drugs together on TIRAP showed that levofloxacin and DZD interact with non-

overlapping segments on the TIR domain of TIRAP that is responsible for its interaction with BTK and PKC δ (**Figure 5.2C & D**). This result further supports our hypothesis that the LeDoz drug combination may synergistically inhibit TIRAP activation and thereby TIRAP-mediated downstream inflammatory signaling in sepsis.

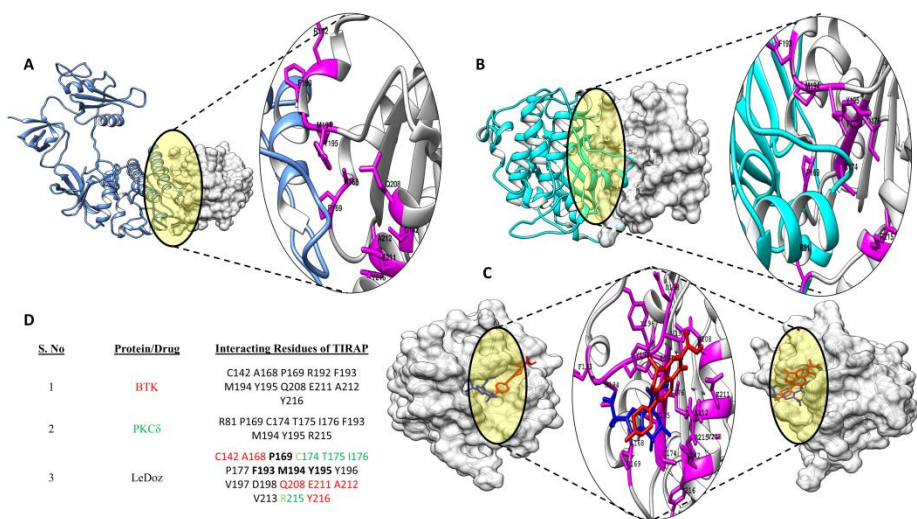


Figure 5.2 In silico interaction analysis of TIRAP with its kinases and LeDoz. (A & B) The structures of TIRAP's interface with BTK and PKC δ along with its zoomed views. (C) Representation and analysis of interface of TIRAP with LeDoz (levofloxacin and DZD) from the Chimera tool. (D) Shown are the critical interface residues of TIRAP involved in interactions with BTK, PKC δ , and LeDoz, respectively. Red highlights TIRAP's interacting residues with BTK, whereas green highlights TIRAP's interacting residues with PKC δ . The residues in bold are the common interface for both BTK and PKC δ .

5.5.2 LeDoz inhibited TIRAP mediated downstream signaling in LPS stimulated macrophages

TIRAP is well known to be phosphorylated and activated upon LPS stimulation. We expect that it would be also efficient in imparting its effect against TIRAP phosphorylation. Results showed that LPS stimulated RAW macrophage cells when treated with levofloxacin and DZD alone, phosphorylation of TIRAP Y86 was reduced in comparison to LPS treated cells. LeDoz showing the synergistic effect inhibited the phosphorylation even more prominently than the cells treated with the drugs individually (**Figure 5.3A**). The same has been quantified as well that too depicts the evident phosphorylation inhibition of TIRAP by LeDoz. The role of TIRAP in the activation of p38 MAPK and subsequently the phosphorylation of the transcription factor NF- κ B has been reported in our earlier studies. Thus we wanted to check whether LeDoz could impart its synergistic effect on the same TIRAP mediated downstream signaling or not. Results showed that cells treated with individual drugs stimulated with LPS did decrease the phosphorylation of p38 MAPK and NF- κ B but cells treated with LeDoz has significant reduction in phosphorylation as compared to their individual effect. The same has been quantified as well that too depicts the evident phosphorylation inhibition (**Figure 5.3A**).

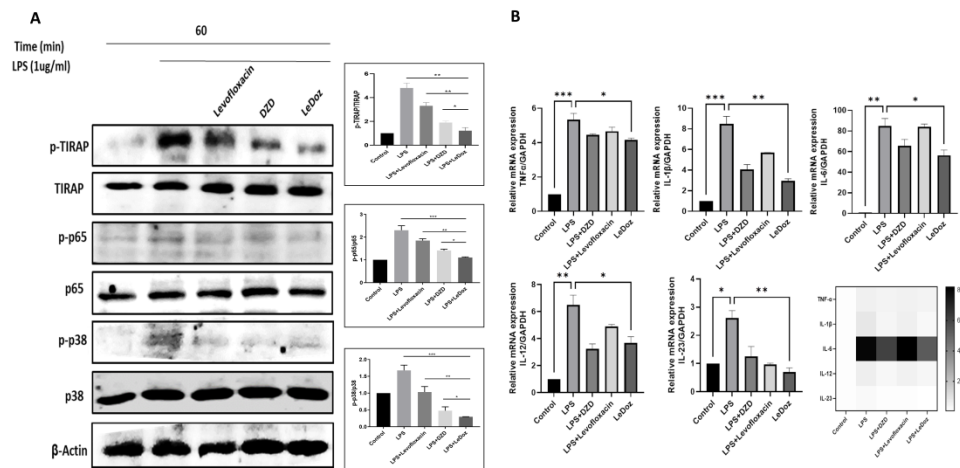


Figure 5.3 LeDoz inhibits TIRAP mediated downstream signaling and expression of pro-inflammatory cytokines expression. A and B) Immunoblot analysis of TIRAP, p38 MAPK, NF-κB/p65. C) Relative mRNA expression of TNF-α, IL-1β, IL-6, IL-12, IL-23, and heat map for all cytokines in RAW 264.7 cells, respectively. All data are presented as the mean ± SD, ****P < 0.0001 (One-way Anova test).

5.5.3 LeDoz reduced the pro-inflammatory cytokines mRNA expression in LPS stimulated macrophages

Our results have depicted that LeDoz evidently inhibited the phosphorylation of TIRAP as well as the kinases and transcription factors involved in the downstream signaling (**Figure 5.3A**). We assume that this inhibitory effect would also be reflected in differential expression of pro-inflammatory cytokines. We observed that LPS stimulated cells when treated with drugs alone, reduced the pro-inflammatory cytokines (IL-6, IL-12, IL-23, IL-1β and TNF-α) expression compared to positive control only LPS treated cells (**Figure 5.3B**). The synergistic effect of both these drugs as LeDoz was clearly more prominent in the cytokine expression

reduction. This suggests that LeDoz may have a greater impact because it binds to TIRAP more effectively than levofloxacin and dzd alone.

5.5.4 LeDoz protected mice from septic injury and mortality

We first set out to determine whether a combination treatment with LeDoz exhibits any additional benefit compared to individually used dorzolamide or levofloxacin to treat the polymicrobial population-induced sepsis in mice. We established five groups of 12 mice each that were untreated mice, cecal slurry-induced septic mice, dorzolamide-treated septic mice, levofloxacin-treated septic mice group, or the LeDoz-treated septic mice. All groups were observed for 96 hours. We found that all mice in the septic mouse group died after 44 hours. In the dorzolamide treated group, we observed 84% mortality, whereas in the levofloxacin treated group, 67% mortality was observed after 44 hours. Conversely, in the LeDoz group, 50% mortality was observed after 44 hours (**Figure 5.4A**). At 96 hours, there was about approximately 40% and 20% more surviving animals in the LeDoz group compared to the individually treated groups and 50% more survival in the LeDoz group compared to the septic mice group, indicating that the LeDoz combination treatment exhibited a better efficacy in treating sepsis than the therapies with individual drugs.

We next determined whether the LeDoz treatment alleviates organ damage observed in the septic mice. We focused on the major organs, such as the lungs, liver, and kidneys. These organs were collected, fixed, sectioned and then stained with H&E staining. We established that the tissues of CS-treated septic mice had increased infiltration of inflammatory cells depicted by the black arrows, pulmonary edema, distorted epithelial, thickening of the alveolar septum, high congestion depicted by green arrows, & increased necrotic area depicted by red arrows (**Figure 5.4B**). The LeDoz treated group had intact epithelial lining, a reduced damage to the septum, and black arrows show fewer infiltrating immune cells in the tissue (**Figure 5.4B**). The histomorphology of LeDoz-treated group tissues

resembled that of the control group mice (**Figure 5.4B**). We quantified the histomorphological images of the tissues for the vacant space between the alveolar septum (**Figure 5.4C**) and number of cells infiltrating the tissue (**Figure 5.4D, E**) via ImageJ with 64-bit Java 8. We found that due to inflammation there was more congestion in the lungs. This resulted in decrease in the vacant space between the alveolar septum in the septic group as compared to the treated ones. The treated groups resembled the control group in terms of higher alveolar space (**Figure 5.4C**). We also found that the numbers of infiltrating immune cells were in accordance with septic condition, and the degree of infiltration of these cells decreased in the treated groups and was the least in the combination treatment just slightly higher than the control (**Figure 5.4D, E**).

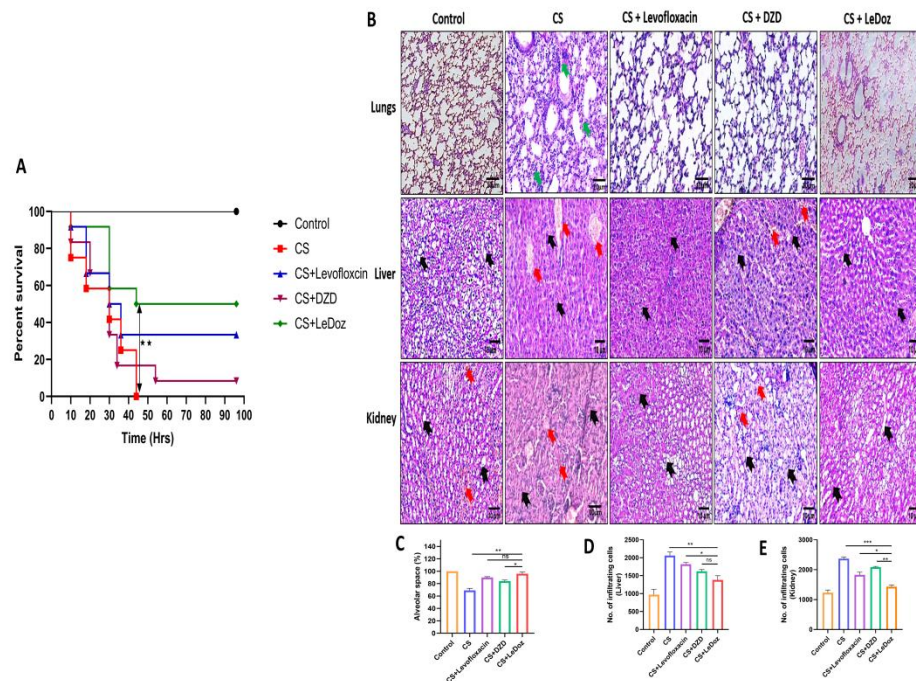


Figure 5.4 Treatment with LeDoz combination therapy shields mice from septic damage and death more effectively as compared to individual drugs. A) Kaplan–Meier survival curves of the mice (n = 12, each group) treated with CS alone (0.5×10^9) or in combination with DZD (25 mg/kg) and levofloxacin (7.1 mg/kg) 1 h prior to CS treatment. In the control group,

all mice received the vehicle DMSO/phosphate-buffered saline only. The mortality of mice was recorded for 96 h. B) The histomorphological study from the lung, liver & kidney tissue section was performed to assess the CS-induced tissue damage and the protective effect on the tissues from the administration of LeDoz and individual drugs. Different groups were treated as described in (A) for 10 h (n = 3 per group) and then sacrificed for tissue collection. The fixed tissue sections were stained with hematoxylin and eosin and imaged under a light microscope at 40x magnification. C), D), & E) Quantification of percentage of inflammatory cells nuclei count from the tissue sections, are shown respectively. All data are presented as the mean $\pm$ SD, ****P < 0.0001 (One-way Anova test).

5.5.5 LeDoz reduced pro-inflammatory cytokines levels in septic mice

Pro-inflammatory cytokine levels are commonly elevated in serum of septic mice, contributing to tissue damage associated with sepsis. Therefore, we asked whether LeDoz can modulate the level of pro-inflammatory cytokines in blood serum. Blood samples were collected by cardiac puncture in mice from all five experimental groups: the control group, the cecal slurry-induced septic group, the dzd-treated septic mouse group, the levofloxacin-treated mouse group, and the LeDoz-treated mouse group. Serum was isolated and cytokine levels were determined using commercially available ELISA kits. We found that the levels of IL-1 β and TNF- α were significantly decreased in the LeDoz-treated group of cecal slurry-induced septic mice compared to the control CS groups (**Figure 5.5A, B & C**). Conversely, IL-6 levels did not differ significantly. We also determined the effect of LeDoz on the mRNA expression level of pro-inflammatory cytokines in different tissues. We found that treatments with levofloxacin or dzd alone inhibited the mRNA expression of pro-inflammatory cytokines in all the three mouse tissues (lungs, liver, and kidneys) of CS-induced/solo drug treated groups compared to tissues from

mice of the CS treatment group. Remarkably, we found that the LeDoz combination treatment imparted a significant synergistic inhibitory effect on mRNA expression as compared to the CS group in the presence of the individual drugs (**Figure 5.5C, D, & E**).

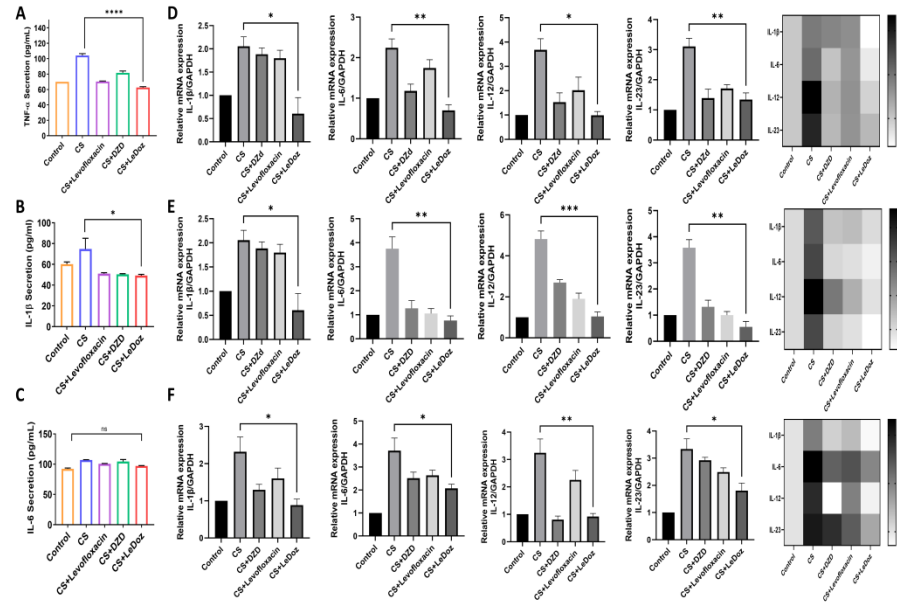


Figure 5.5 LeDoz treatment reduces NF-kB mediated transcriptional activation of pro-inflammatory cytokine expression in different tissues. The levels of serum cytokines were determined to assess the effects of LeDoz on the production of pro-inflammatory cytokines (A) TNF- α , (B) IL-1 β and (C) IL-6 respectively. Shown are the quantitative reverse transcriptase PCR data for the relative mRNA expression of all cytokines and the heat map (D-F) in mice lung, liver & kidney tissues at 10 h as indicated. All data are presented as the mean $\pm$ SD, **** P < 0.0001 (One-way Anova test).

5.5.6 LeDoz treatment improves the metabolite profile in blood of septic mice

We next determined whether LeDoz treatment affects various factors such as urea, CRP, ALT, AST, bilirubin, & creatinine in serum isolated from different groups of septic mice. We observed a significant decrease in the levels of urea, creatinine, ALT, & CRP in the LeDoz-treated mouse group as compared to the CS and individual drug-treated mouse groups (**Figure 5.6A-C, & F**). Conversely, no prominent reduction in the levels of AST & bilirubin was detected (**Figure 5.6D & E**).

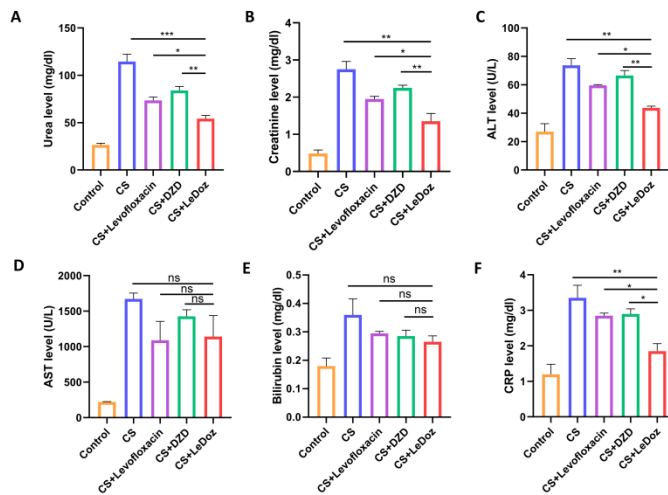
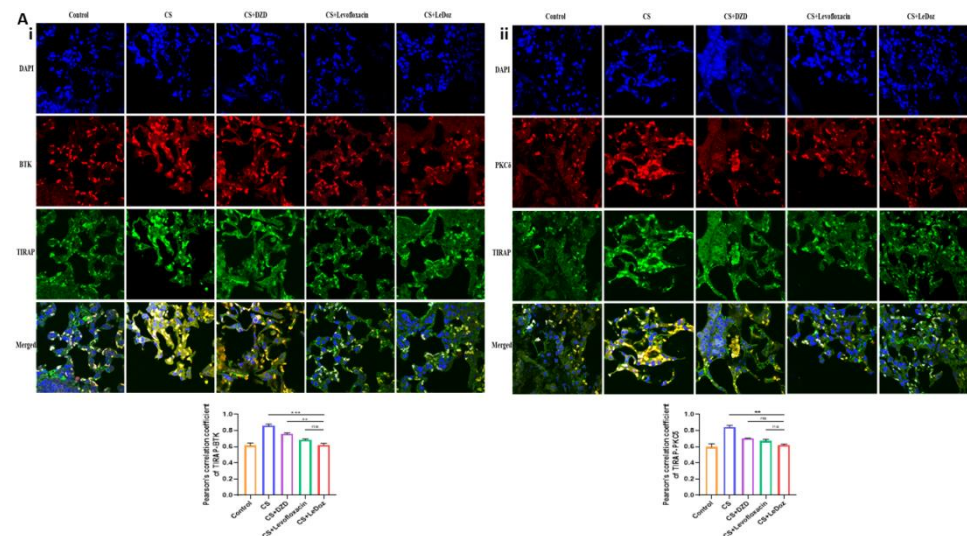


Figure 5.6 Effect of LeDoz on clinically relevant liver enzymes and other biological metabolites in the serum of septic mice. (A–F) Shown are the comparisons of levels for urea, creatinine, ALT, AST, bilirubin, & CRP in the serum of control, septic, and drug-treated septic mice (n = 3 per group). ALT: alanine transaminase, AST: aspartate transaminase, CRP: C reactive protein. All data are presented as the mean $\pm$ SD, ****P < 0.0001 (One-way Anova test).

5.5.7 LeDoz treatment decreases TIRAP colocalization with BTK and PKC δ in septic mouse tissues

We next investigated the colocalization between TIRAP & BTK as well as TIRAP & PKC δ via immunofluorescence dual staining **Figure 5.7** in the lung, liver, and kidney tissues isolated from mice of control and drug-treated groups. We observed that in the mice treated with CS the colocalization between TIRAP and its activating kinases was significantly higher as compared to that of the control group. Treatment with levofloxacin and dzd alone reduced TIRAP's colocalization with the kinases that can be clearly seen via the Pearson's correlation coefficient that indicates the degree of co-localization of two proteins (**Figure 5.7**). Mice treated with LeDoz showed prominent synergistic effects in reducing the co-localization of TIRAP with BTK as well as PKC δ as shown in **Figure 5.7A, B, & C** for lungs, liver, & kidney tissue sections, respectively. The results indicate that LeDoz treatment synergistically inhibits TIRAP activation and thereby dampens the TIRAP mediated inflammatory response in septic mice.



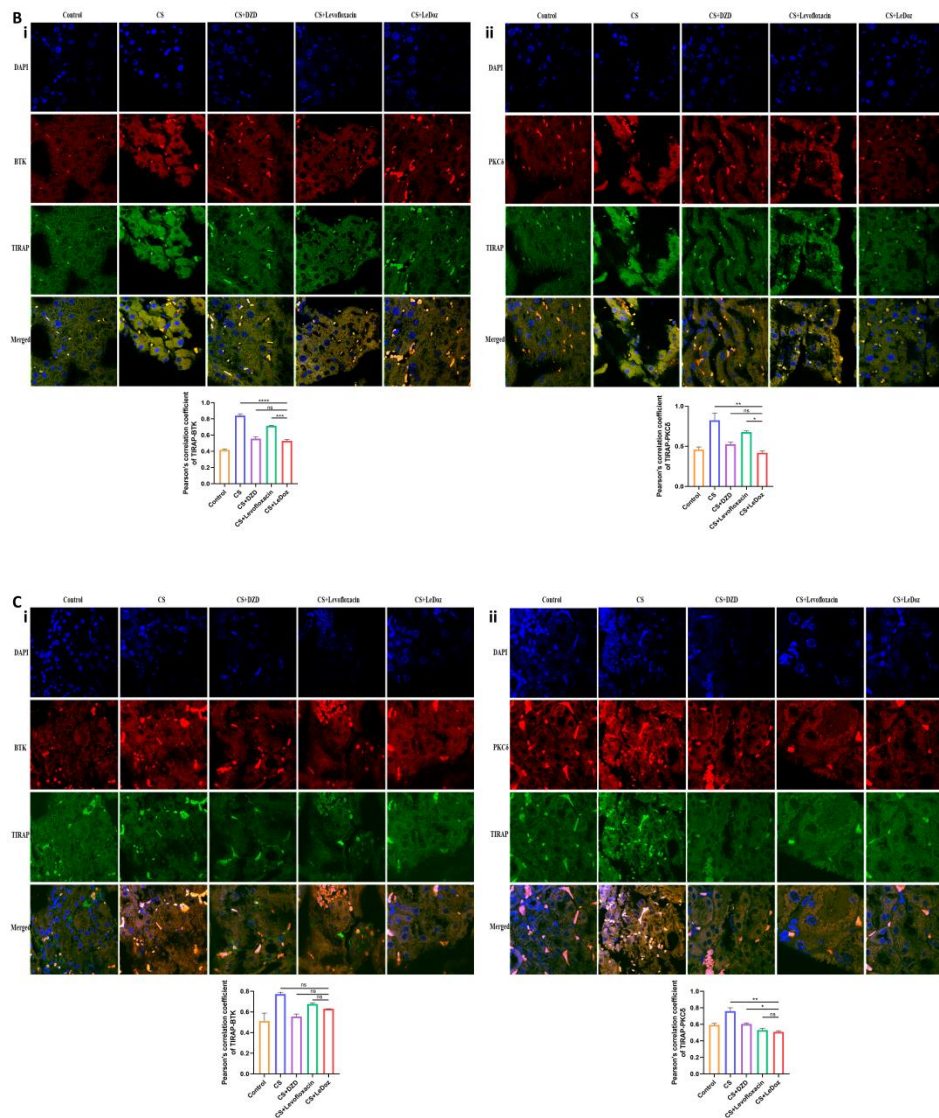


Figure 5.7 LeDoz inhibits CS-induced TIRAP interaction with BTK and PKCδ in different tissues. Immunofluorescence dual staining was performed in (Ai, ii) Lung, (Bi, ii) Liver, and (Ci, ii) Kidney tissues from mice treated with CS alone (0.5×10^9 , i.p.) and in combination with DZD (25 mg/kg, i.p.) and levofloxacin (7.1 mg/kg, i.v.) 1 h prior to CS treatment. TIRAP was stained with Alexa Fluor 488-tagged (green) and BTK and PKCδ with Alexa Fluor 594-tagged (red) secondary antibodies. Nuclei were counterstained with DAPI (blue). These immunofluorescence data indicate that TIRAP colocalization with PKCδ and BTK was

significantly decreased in LeDoz-treated mouse tissues. Scale bar = 20 μ m. All data are presented as the mean $\pm$ SD, ****P < 0.0001 (One-way Anova test).

5.6 Discussion

Sepsis is a fatal organ dysfunction syndrome that results from an uncontrolled and dysregulated host immunological response to any infection or damage [1]. It was reported that about 20% of deaths worldwide were related to sepsis [32,33]. This life threatening condition has affected not only adults but also children, with over 2.9 million pediatric cases being reported globally [32,33]. One of the main reasons for the high mortality of septic patients is the delayed diagnosis and inappropriate treatment of the condition, allowing the septic patients to deteriorate [34,34,35]. There are currently several strategies for sepsis treatment that can be broadly subcategorized as the pathogen-directed treatment, such as intravenous immunoglobulin [36], bacteriophages [37], targeted monoclonal antibodies [38,39], and host-directed therapeutics that includes selepressin [40] and Toll-Like Receptor Ligand Binders [34,41,42]. Current clinical management of sepsis primarily relies on early administration of broad-spectrum antibiotics, such as tazobactam, meropenem or carbapenems, to control the underlying infection [43]. While effective at reducing microbial load, these treatments do not address the excessive and dysregulated host immune response that contributes significantly to organ failure and mortality. In recent years, preclinical studies have explored various immunomodulatory strategies aimed at targeting innate immune pathways—particularly TLR signaling and its adaptors like TIRAP. Molecules such as TAK-242 (a TLR4 inhibitor) [44], TIP1 peptides [45], decoy peptides [46] and other TIR-targeting compounds [12,47] have shown promising results in animal models by reducing cytokine storms and improving survival. However, these interventions often lack antimicrobial action and face translational hurdles

due to safety, specificity, or poor pharmacokinetics. Still, the concern remains that the stated approaches are short of getting overall control over all together. Thus, there is an urgent need to develop new strategies to tackle both arms of sepsis pathogenesis i.e., countering the infectious pathogenic entities in the body and suppressing the prolonged, uncontrolled hyper-inflammatory response. To address this clinical problem, we chose a novel approach, which combines an antibiotic, uniquely exhibiting anti-inflammatory activity, and a potent anti-inflammatory drug. In this study, we used repurposed anti-inflammatory medication DZD and a broad-spectrum antibiotic levofloxacin. DZD has previously been adapted to target TIRAP and prevent its activation in our earlier research [31]. Levofloxacin has the ability to eradicate a wide variety of bacterial species and, additionally, as we show herein, decreases inflammation in the host by inhibiting TIRAP-mediated inflammatory signaling events. TIRAP has been known to serve as an important adapter protein in the TLR4-mediated inflammatory signaling cascade. The TIRAP signaling initiates with its phosphorylation and thereby activation mainly by two of its interacting kinases BTK and PKC δ . This activated TIRAP then interacts with downstream kinases such as p38 MAPK that eventually leads to translocation of the transcription factors into the nucleus. The primary position of TIRAP in the signaling pathway makes it an effective focus for anti-inflammatory therapies.

We began from screening the FDA approved compound library of antibiotics to find those that may interact with the common interface of PKC δ & BTK on TIRAP [24]. Based on the specific binding and high dock scores in screening results, we identified a broad-spectrum antibiotic levofloxacin. Levofloxacin is a well-known antibiotic that targets the bacterial protein DNA Gyrase to hamper the DNA replication process of bacteria and thereby causing their death [48]. Molecular docking shows the structure of the binding pocket of TIRAP with individually docked

levofloxacin and DZD. We obtained encouraging results from this in silico method, predicting that levofloxacin can bind to TIRAP's common interface with PKC δ and BTK, suggesting that this antibiotic may exhibit anti-inflammatory activity besides its antibacterial effects. These findings led us to use 200 ns molecular dynamics simulations to model the dynamic interaction between levofloxacin and TIRAP to further explore the stability of levofloxacin binding with TIRAP. Based on various parameters, including RMSD, RMSF, SASA, on Rg, and H-bonds, the results showed the presence of stable binding upon the interaction of levofloxacin with TIRAP (**Supplementary figure S5.1**). These results suggested that levofloxacin, apart from being an antibiotic, can also target host protein TIRAP involved in inflammatory signaling.

Next, we investigated whether the binding pattern and the efficiency of DZD and levofloxacin can be modeled using the molecular docking procedure while docking the drugs together to mimic the synergistic effect of LeDoz. We found that Levofloxacin and DZD interacted with non-overlapping segment within the binding pocket on TIR domain of TIRAP that was central to its interaction with its activating kinases PKC δ & BTK (**Figure 5.2A, B & D**) as compared to the binding of individual drugs. This result strongly supports our hypothesis that these two drugs should come together to impart a synergistic effect. We then decided to investigate whether levofloxacin in combination with a known anti-inflammatory drug DZD may exhibit a synergistic effect. We then looked for the effect of LeDoz on TIRAP and its downstream mediated signaling in LPS stimulated macrophages (**Figure 5.3**). The results showed that LeDoz evidently inhibited the TIRAP phosphorylation at Y86 as compared to drugs and LPS alone treated cells (**Figure 5.3A**). Similarly, we found that LeDoz imparted its synergistic effects by reducing the phosphorylation of p38MAPK and transcription factor p65 NF-kB (**Figure 5.3A**). Since TIRAP and its signaling were affected by the synergistic action of LeDoz, we next set to ascertain whether this inhibition can affect

pro-inflammatory cytokine expression. Indeed, we found that LeDoz was clearly more prominent in the cytokine expression reduction compared to the individual drugs (**Figure 5.3B**).

To validate our combination approach, we used a CS induced septic mice model for the *in vivo* studies. A total of five groups (n=12) were established namely control, CS-induced septic group, CS-induced septic groups treated with dzd, levofloxacin, & LeDoz respectively. Firstly, we determined the effectiveness of the combination through survival analysis. We observed all the groups for 96 hours and found that all mice in the septic group died after 44 hours. Conversely, there was about 50% more survival in the LeDoz group compared to the septic mouse group, indicating that the combination therapy with LeDoz has a better efficiency in treating sepsis than using these drugs individually (**Figure 5.4A**). To assess the potential of the combined drugs in preventing the sepsis-associated organ damage, we performed H&E staining of three major organ tissues isolated from mice of all experimental groups, such as the lungs, liver, and kidneys (**Figure 5.4B**). The tissues of CS-treated mice had increased infiltration of inflammatory cells, thickening of the alveolar septum, and high congestion. On the other hand, the LeDoz-treated group had fewer infiltrating immune cells, intact epithelial lining, less damaged septum that was resembling that of the control group mice. The anti-inflammatory effect of DZD has been observed earlier in the endotoxemia model [31]. In our study, DZD alone did not show any prominent effect due to the presence of polymicrobial species in CS. It is not a natural target of the drug since it acts against LPS induced inflammatory signaling. LPS represents a part of gram-negative bacteria and not the entire pathogen. Levofloxacin alone showed higher efficiency in comparison to DZD because of its well-known activity of targeting bacterial pathogenic load. But when the two drugs were given in combination as LeDoz the synergistic effect of the drugs was prominent. We then assessed the common endpoint of any inflammatory signaling,

the levels of serum cytokines. We observed differences in the IL-1 β and TNF- α serum cytokine levels isolated from mice with different groups, but no effect on IL-6 levels (**Figure 5.5A, B & C**).

We next determined the effect of the drugs on tissue mRNA expression levels. Tissues from LeDoz-treated mice showed a reduced cytokines' RNA expression in comparison to drugs alone in CS-treated mice (**Figure 5.5D, E, & F**). Similarly, LeDoz effectively and significantly reduced the levels of various serum biochemical parameters such as urea, CRP, ALT, & creatinine (**Figure 5.6**) in comparison to the individual drugs. Then, we determined whether the activation of our protein of interest, TIRAP, is affected by LeDoz. Therefore, we performed immunofluorescence dual staining in mice tissues from all tested groups. We observed that in the mice treated with CS the interaction between TIRAP and its activating kinases was significantly increased as compared to that of the control group. Treatment with levofloxacin and dzd alone reduced TIRAP's interaction with the kinases. Conversely, mice treated with LeDoz showed prominent synergistic effects in reducing the co-localization of TIRAP with both BTK as well as PKC δ (**Figure 5.7A, B, & C**). The observed data clearly indicate that LeDoz inhibits TIRAP-mediated inflammatory response by exerting synergistic effects against it.

5.7 Conclusion

We demonstrated herein that it is possible to use a combination therapy approach to treat sepsis and the associated organ dysfunctions. Uniquely, we identified an antibiotic, levofloxacin, with a significant anti-inflammatory activity and demonstrated that it can effectively eliminate pathogens decreasing the dysregulated immune response in our septic mouse model. Furthermore, we found that the combination of this antibiotic and DZD has a unique synergistic inhibitory effect while targeting the TIRAP-mediated inflammatory response. TIRAP, an adapter molecule, is a key player in the TLR-mediated signaling that is activated

by bacteria which are shown to be the leading cause of death in septic patients [11]. Importantly, both levofloxacin and DZD are FDA approved drugs and safe for clinical use. Thus, repurposing them for sepsis treatment, considering their strong synergistic impact, provides a quick path from the laboratory's wet bench to bedside in clinics. We fully recognize that our current study has certain limitations that must be addressed to better translate our findings into a clinical context. Most notably, the LeDoz combination was administered prophylactically—prior to the induction of sepsis—which does not reflect typical clinical scenarios where treatment is initiated after symptom onset. Although this approach allowed us to evaluate the mechanistic potential of the therapy under controlled conditions, future studies will be designed to assess its therapeutic efficacy when administered at clinically relevant time points. Additionally, our work utilized a single murine model of sepsis (cecal slurry), and further validation in other well-established models, such as cecal ligation and puncture (CLP) or endotoxin-induced sepsis, will be essential to confirm the broader applicability of our findings. We also acknowledge the lack of pharmacokinetic data and the absence of long-term outcome analysis, such as immunosuppression or post-sepsis relapse. We are fully committed to addressing these points and will incorporate these considerations into the design of our future investigations to ensure both scientific rigor and translational relevance. Our combination approach can be used as a stepping stone for developing new efficient combination therapies for sepsis that may include complex multifunctional beneficial agents with a goal to reduce deaths from sepsis.

5.8 References

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Chapter 6

Summary

Chapter 6: Summary

Sepsis owing to its complex nature still remains a difficult challenge in the medical community. The pathophysiology itself is what makes it so challenging to manage. The uncontrolled pathogenic load and the resulting dysregulated host immune response are the key reasons for its rapid widespread. The traditional approaches consist of the usage of monoclonal antibody, antibiotics, supportive care, and fluid resuscitation. Despite all these efforts sepsis still remains at the top for claiming millions of lives. One area that has not been much explored is the host targeted strategy. This paved the way for the current research work.

We, investigate the role of TLR4 dependent TIRAP mediated pro-inflammatory immune signaling. TIRAP holding the top most position in the signaling cascade makes it an attractive and suitable target to explore. Not only has this but TIRAP's ability to interact with various proteins also made it an approachable target. TIRAP is known to get phosphorylated and activated by the activity of two kinases. They comprise Protein Kinase C δ (PKC δ) and Bruton's Tyrosine Kinase (BTK). An in depth analysis of TIRAP's such interactions and its structural biology helped us to form a basic understanding of its working pattern.

We investigated the therapeutic efficiency of DZD intermediates. Wherein we studied that how effectively these compounds were able to bind TIRAP at the common interface of both the kinases. The result showed that these intermediates were able to inhibit TIRAP phosphorylation thereby its activation. This dampened the downstream signaling involving the molecules such as MAPK and NF- κ B. *In vivo* studies also confirmed that intermediates specifically DRZ V significantly reduced the septic damage caused by LPS as compared to the parent drug DZD. These findings shed light on the potential therapeutic targeting of TIRAP in chronic inflammatory conditions.

Sepsis being the central theme of the thesis, led us to investigate and form a stronger base for elucidating its pathophysiology. The current sepsis models include CLP, CASP, and LPS induced endotoxemia. These models are considered as gold standard in the scientific community. There's no denying regarding their potential and advantages they bring in, but there is always some room for improvement available. Herein we propose an improved cecum slurry based septic model that provides various advantages and an upper edge over the current approaches. It comprises the culturing of the cecal contents and preparing different concentrations based on the required colony forming units (CFUs). Along with this the culture could also be stored as glycerol stocks for future usage. This provides an efficient reproducibility and avoids the chances of variability among the different experimental sets. The polymicrobial culture could also be enriched for specific gram-positive or gram-negative populations. The results provided the validation for the proposed model as the higher pathogenic load correlated with decreased survival, increased levels of serum cytokines and various biochemical markers, and distorted tissue histomorphology.

The results from the prior understanding led us to the development of a novel combination therapy for sepsis. We propose the use of a broad range antibiotic levofloxacin and an anti-inflammatory drug DZD to impart synergistic effects. The rationale behind this strategy was to target both the pathogenic load and its infection along with the dysregulated host immune response. The LeDoz combination was found to effectively bind and target TIRAP covering a broader range of residues crucial for its binding with the kinases. In vitro studies showed that LeDoz inhibited TIRAP phosphorylation more prominently as compared to the drugs individually. LeDoz also reduced the activation of downstream molecules such as "MPAK" and transcription factor NF- κ B significantly. In vivo experiments using the improved cecal slurry sepsis model we found that LeDoz prominently reduced the mortality as compared to the effect of

individual drugs. Serum and tissue cytokines were also analyzed to assess the potency of the combination. Immunohistochemistry (IHC) of different organs from all the different groups showed that the synergistic effect was due to the reduced colocalization of TIRAP with its activating kinases. Thus, the current results open up a wide possibility of such combination approaches.

Still, there remains certain open hangs in the current work which could be definitely addressed in the future. Primary limitation of the present study is that direct experimental evidence demonstrating a physical interaction between the compounds and TIRAP is limited. While our biological and computational analyses suggest that the compounds modulate TIRAP-dependent signaling, these observations do not conclusively confirm direct binding. It is also possible that the compounds influence upstream or parallel signaling pathways that indirectly affect TIRAP activity. Future studies could address this by performing direct binding and biophysical assays, such as surface plasmon resonance, isothermal titration calorimetry, or mutational analysis of the proposed interaction region. Such experiments would help to more clearly establish whether the compounds directly interact with TIRAP and further define their precise mechanism of action.

At last with the concluding remarks, the work done in this thesis incorporates the deeper understanding of TIRAP mediated inflammatory signaling along with the development of potential therapeutics. It includes the identification of DZD intermediates as capable small molecule inhibitors. The proposal of an improved cecum slurry based sepsis model sheds light on the better comprehension of its pathophysiology along with reproducibility and ethical advancements. Lastly, the development of the combination therapy LeDoz imparting the synergistic effects tackles both the infection and the exaggerated immune response.

Chapter 7

Future Prospects

Chapter 7: Future Prospects

Sepsis remains one of the most complex and critical global health threats, demanding rapid intervention to prevent its devastating systemic effects. The current work has focused on the varied aspects of sepsis pathology i.e. the clearance of pathogenic load and modulating the host immune response via TIRAP regulation. Along with it the efforts were also put in place to propose an improved sepsis model with ethical advantages and enrichment protocol. This formed the base for future endless possibilities to be explored in the direction of both therapeutics as well as elucidating the signaling mechanisms in sepsis.

- TIRAP phosphorylation is considered to be one of the major post translational modifications that regulate its activity under various pathogenic conditions. A detailed insight into TIRAP's structure revealed the presence of various cysteine residues which could be prominent sites for another crucial post translational modifications, nitrosylation or glutathionation. It would be of great importance to investigate that how nitrosylation would modulate TIRAP's activity in terms of activation, degradation, and interaction with other proteins.
- Sepsis is comprised of both the hyperinflammation and immunosuppression stages. So, it would be really interesting to explore the possibility of proposing the combination approach that could target both the initial hyper-inflammatory phase via TIRAP inhibition as well as the later immunosuppressed phase.
- Sepsis is characterized by multi-organ dysfunction conditions. The major organs affected include kidney injury, cardiac dysfunction and many more. It would be exciting to investigate the organ specific effects of the current proposed therapeutic approaches to accelerate these pre-clinical studies to the clinical levels.

- Though much research have been focused on the involvement of Gram-negative species mediated septic infection, the contribution of Gram-positive species is also crucial. Herein, we propose the therapeutics that could focus on the infections caused by both these groups to completely abrogate the downstream inflammatory responses.
- Sepsis has generally accompanied with different secondary infections and chronic diseases. Obesity, alcoholic liver fibrosis, and diabetes are the major associations that are known to deteriorate patient's health. Since TIRAP has been studied in sepsis in the current work, it would be of great interest to investigate the heterogeneity of TIRAP expression in such comorbid conditions. It would help to understand the varying nature of TIRAP across different conditions and develop more effective and efficient therapeutics.

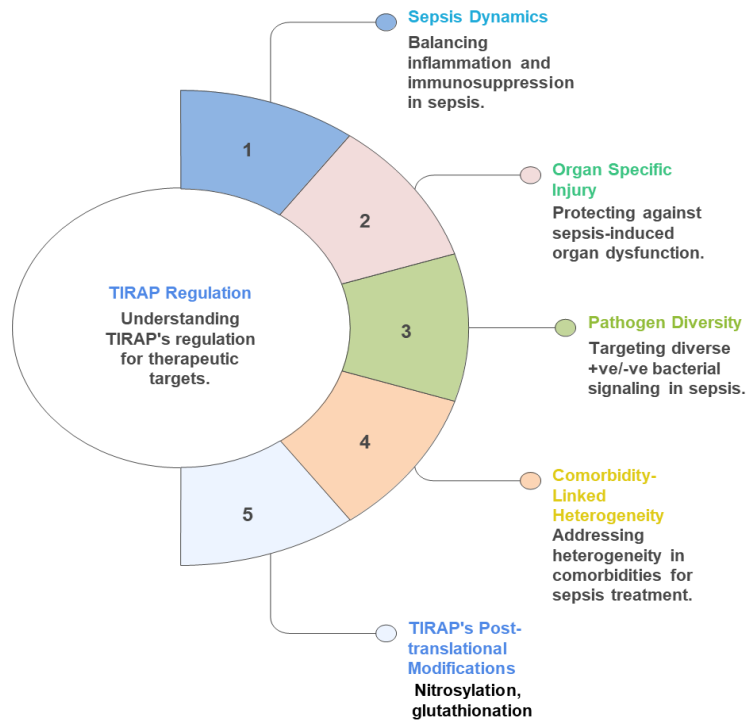
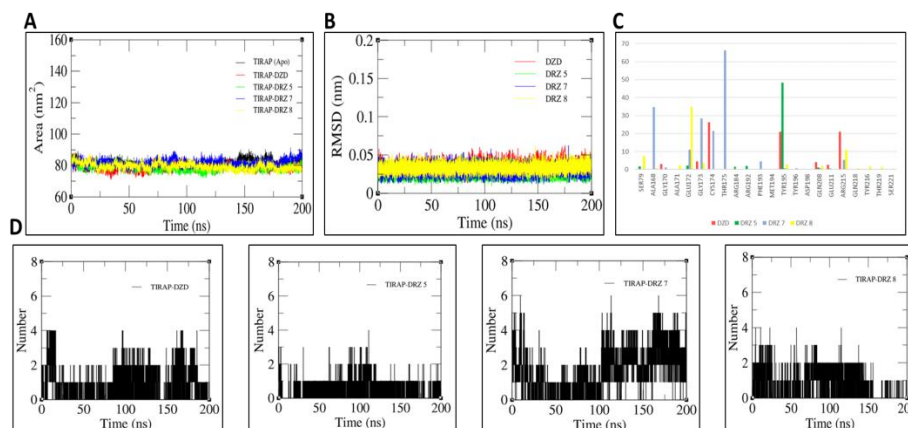


Figure 7.1 Potential future directions from the current research work.

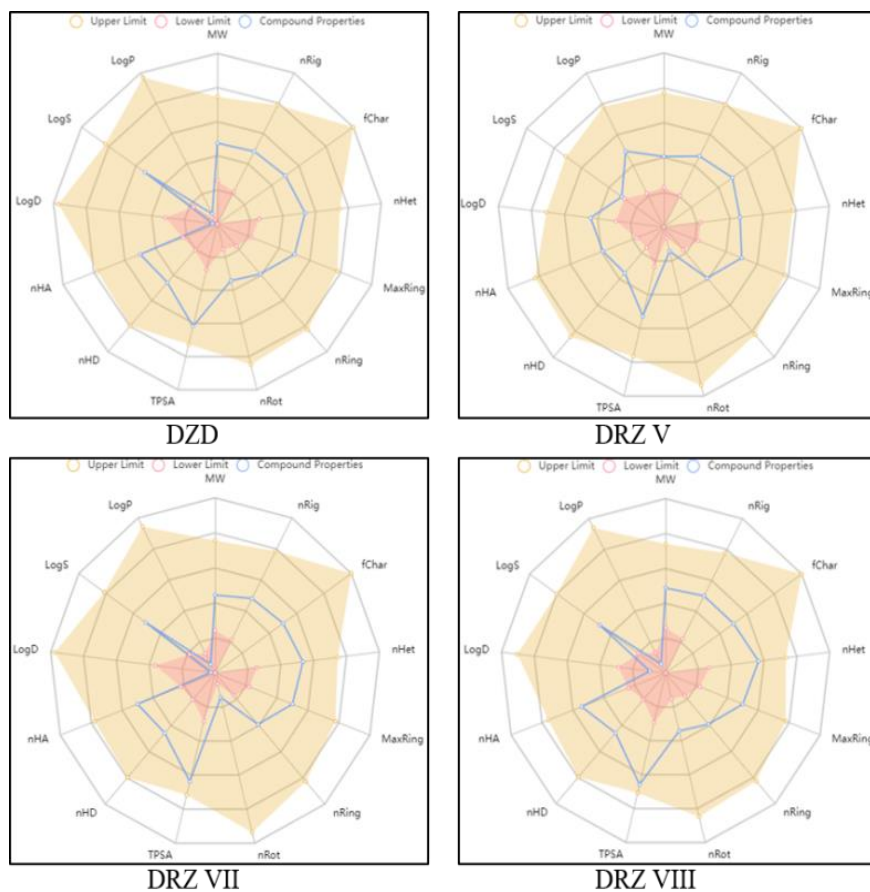
APPENDIX

Appendix- A: Supplementary Figures

1. Supplementary Figures from Chapter 3: Dorzolamide intermediates with potential anti-inflammatory activity

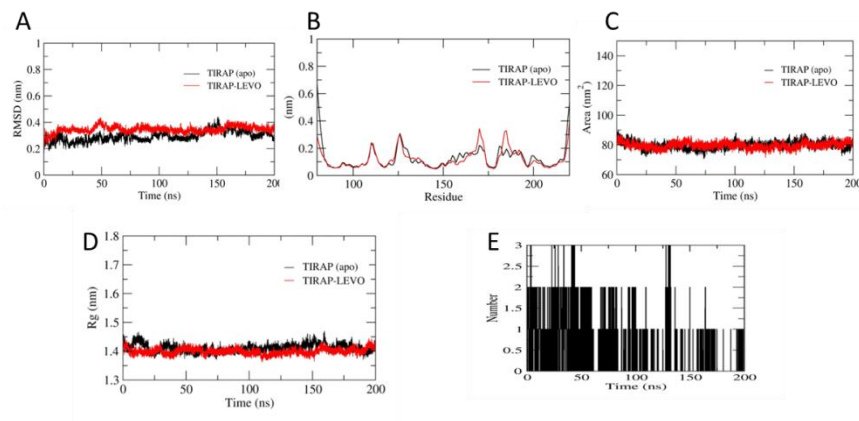


Supplementary Figure S3.1 MD Simulation Trajectory Plots of TIRAP (Apo) and TIRAP in complex with Dorzolamide and intermediates. A) SASA plot of TIRAP protein in complex with Dorzolamide and intermediates. B) RMSD of ligands (Dorzolamide and intermediates) in complex with TIRAP. C) Hydrogen Bond Occupancy. D) Hydrogen Bonds formed between TIRAP and ligands (Dorzolamide and intermediates).



Supplementary Figure S3.2 Physicochemical and ADMET analysis of DZD and its intermediates. Prediction of pharmacokinetic properties of ADMET (absorption, distribution, metabolism, excretion, and toxicity) of DZD and its intermediates through computational tool admetlab2.0.

2. Supplementary Figures from Chapter 5: Inhibiting TIRAP-Mediated Inflammatory Signaling: A promising Therapeutic Strategy against Sepsis



Supplementary Figure S5.1 MD Simulation Trajectory Plots of TIRAP (apo) and TIRAP in complex with levofloxacin. A) RMSD plot of the backbone of TIRAP protein in complex with Levofloxacin. B) RMSF plot of the individual residues of TIRAP protein in complex with Levofloxacin. C) SASA plot of TIRAP protein in complex with levofloxacin. D) Rg plot of the backbone of the TIRAP protein in complex with levofloxacin. E) Hydrogen bond formation between TIRAP and levofloxacin.

Appendix-B: Supplementary Tables

S No.	Primer Name	Sequence
1	mTNFa-F	5'-GCCTCTTCTCATTCCTGCTTG-3'
2	mTNFa-R	5'-CTGATGAGAGGGAGGCCATT-3'
3	mIL1b-F	5' TGGACCTTCCAGGATGAGGACA 3'
4	mIL1b-R	5' GTTCATCTCGGAGCCTGTAGTG 3'
5	mIL6-F	5'- CGCTATGAAGTTCCTCTCTGC -3'
6	mIL6-R	5'- CTAGGTTTGCCGAGTAGATCTC -3'
7	mIL12p40- F	5'- TCGCAGCAAAGCAAGATGTG -3'
8	mIL12p40-R	5'- GAGCAGCAGATGTGAGTGGC -3'
9	mGAPDH-F	5'- AATGTGTCCGTCGTGGATCT -3'
10	mGAPDH-R	5'- CCCTGTTGCTGTAGCCGTAT -3'
11	mIL23-F	5'- TGCTGGATTGCAGAGCAGTAA-3'
12	mIL23-R	5'- GCATGCAGAGATTCCGAGAGA-3'

Supplementary Table S3.1 Primer sequences