# Recombinant expression and studies on outer membrane protein from *Salmonella enterica* Typhimurium and their role in aberrant AID expression in B-cells

**M.Sc. Thesis**


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**INDIAN INSTITUTE OF TECHNOLOGY INDORE**


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# Recombinant expression and studies on outer membrane protein from *Salmonella enterica* Typhimurium and their role in aberrant AID expression in B-cells

**A THESIS**

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

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# INDIAN INSTITUTE OF TECHNOLOGY INDORE

## CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **Recombinant expression and studies on outer membrane protein from *Salmonella enterica* Typhimurium and their role in aberrant AID expression in B-cells** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT 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 July 2023 to May 2025 under the supervision of Professor Prashant Kodgire.

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.

22 May 2025

**Signature of the student with date**
**(DEVEISH NIGAM)**

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

P.V. Kodgire

Signature of the Supervisor of
M.Sc. thesis (with date)
**(Prof. Prashant Kodgire)**

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DEVEISH NIGAM has successfully given his/her M.Sc. Oral Examination held on **06 May 2025.**

P.V. Kodgire

Signature(s) of Supervisor(s) of MSc thesis
Date: 22/05/2025

P.V. Kodgire

Convener, DPGC
Date: 22/05/2025

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- **Deveish Nigam**

# DEDICATION

This thesis is wholeheartedly dedicated to those who have supported me mentally and have been my motivation for working hard.
Those who have embered my flames of passion for learning and continuously improving myself.

# Abstract

Outer membrane proteins (OMPs) in gram-negative bacteria confer the ability to interact with the host, evade the immune system, and resist antibiotics. This study focused on YchP, a putative invasin protein of *Salmonella enterica* serovar Typhimurium, which allows the bacteria to adhere to and invade the host and potentially has a vital role in altering the host's immune response. We expressed, purified, and refolded YchP from a genetically modified *Escherichia coli* Rosetta strain. To explore how YchP affects the immune system, we introduced it to Raji human B-cells. We aimed to examine the effect of YchP in the production and expression of activation-induced cytidine deaminase (AID). This critical enzyme helps B-cells generate diverse antibodies through somatic hypermutation (SHM) and class-switch recombination (CSR). We evaluated the apparent change in protein AID levels and some regulatory genes of AID after stimulation with YchP based on the results of western blot from the stimulated Raji human B-cells. Our results suggested that AID protein levels decreased significantly. These results indicate that YchP may suppress AID activity, which could help bacteria evade the immune system by weakening antibody diversity. These experiments shed light on *Salmonella's* potential immune evasion strategy to protect itself. Further exploration is needed to understand how YchP triggers this response and what it means for bacterial infections and immune defense.

**Keywords**: *Salmonella enterica* Typhimurium; YchP; Activation-induced cytidine deaminase (AID); Invasin.

# LIST OF PUBLICATIONS

**1)** Recombinant expression and studies on YchP from *Salmonella enterica* Typhimurium and its role in down-regulating AID expression and thereby affecting class switching in B-cells. Deveish Nigam, Rahul Chaudhari, and Prashant Kodgire*. (Under preparation)

## Other publication:

**1)** Engineering OMVs carrying OmpA from *S.* Typhimurium for targeted modulation of human B-cell function through AID expression and class switch recombination. Rahul Chaudhari, Mallar Dasgupta, Deveish Nigam, and Prashant Kodgire*
Submitted, Microbial Pathogenesis

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# ACRONYMS

**CDC** - Centers for Disease Control and Prevention

**T3SS** - Type III Secretion System

**SPI** - *Salmonella* pathogenicity island

**OMP** - Outer Membrane Protein

**OMV** – Outer Membrane Vesicle

**RNI** - Reactive Nitrogen Intermediate

**ROS** – Reactive Oxygen Species

**IAT** - Inverse Autotransporter

**MAMP** - Microorganism-associated molecular pattern

**PRR** - pattern recognition receptor

**TLR** - Toll-like receptor

**NLR** - Nod-like receptor

**RLR** - Retinoic acid-inducible gene-I (RIG-I)-like receptor

**CLR** - C-type lectin receptor

**LPS** - Lipopolysaccharide

**ALR** - Absent in melanoma-2 (AIM2)-like receptor

**MyD88** - Myeloid differentiation primary-response protein 88

**TIRAP** - TIR-domain-containing adaptor protein

**TRIF** - TIR-domain-containing adaptor protein inducing interferon-β

**TRAM** - TRIF-related adaptor molecule

**APC** – Antigen Presenting Cell

**BCR** – B-cell receptor

**IRAK-1 -** Interleukin-1 receptor-associated kinase

**MAPK -** Mitogen-activated protein kinase

**AID -** Activation-induced cytidine deaminase

**V(D)J** – Variable, Diversity, and Joining

**SHM** – Somatic Hypermutation

**CSR** – Class-switch recombination

**NES** – Nuclear export signal

**UNG -** Uracil-(N)-glycosylase

**BER** – Base excision repair

**MMR** – Mismatch repair

**O.D.** – Optical Density

**Ni NTA -** Nickel nitriloacetic acid

**LDAO/DDAO -** Lauryldimethylamine oxide/ Dodecyldimethylamine oxide

**DAPI -** 4′,6-diamidino-2-phenylindole

**GAPDH -** Glyceraldehyde-3-phosphate dehydrogenase

# Chapter 1

# Introduction

Members of the genus *Salmonella* are characterised as rod-shaped bacilli. It is a gram-negative, flagellated pathogen belonging to the Enterobacteriaceae family. *Salmonella* are mesophiles that grow optimally at 35-43 °C and can survive in low oxygen conditions, making them facultative anaerobes [1]. The genus is comprised mainly of two distinct species, *Salmonella bongori* and *Salmonella enterica*. Based on the presence of antigens, they are divided into more than 50 serotypes containing O antigen (somatic), a part of the lipopolysaccharide, and more than 2400 serotypes containing H antigen (flagellar), which can switch from phase 1 to phase 2 helping them to evade the immune system, and a Vi antigen which is a superficial antigen overlying the O antigen seen only in a few serovars [2, 3]. These serotypes can be categorized as typhoidal and non-typhoidal. Typhoidal includes *Salmonella* paratyphi and *Salmonella* typhi, which cause typhoid fever or paratyphoid and are characterised by high fever, abdominal pain, and systemic symptoms. They require prompt medical treatment. The non-typhoidal, which typically causes acute diarrhoea and gastroenteritis, has symptoms such as diarrhoea, abdominal cramps, fever, and vomiting. Salmonellosis, a common food poisoning, is caused by the *Salmonella* species. It presents with a range of symptoms, including diarrhoea (sometimes bloody), vomiting, headaches, abdominal cramps, and fever with or without dehydration. The typhoid and paratyphoid fevers, collectively known as enteric fever, are specific types of Salmonellosis. These fevers are characterised by prolonged high fever, abdominal pain, and systemic symptoms.

Understanding these symptoms is crucial for early diagnosis and prompt medical treatment. *Salmonella* is found in contaminated water and the guts of animals, and as a result, is responsible for the spread of the bacteria through contaminated milk, eggs, meat, and other products.

*Salmonella* can be transmitted from animal to animal through the fecal-oral route and inhabit the intestines. Upon entering the intestines, they colonize, invade, and proliferate in the intestinal epithelium cells and the lymphoid follicles of the ileum and colon region. *Salmonella* pathogenicity island (SPI) is crucial for the host-pathogen interaction and the Type III Secretion System (T3SS). T3SS is responsible for the needle complex formation, which envelopes the bacteria and, upon interaction, protrudes from the inner membrane of the bacteria to the host cell membrane to deliver the effectors that modulate the cellular functions [4, 5]. These effectors stimulate the host cells to modify their actin cytoskeleton, resulting in outward extension of the cell membrane to engulf the bacteria, resembling the process of phagocytosis [6]. Invasion by the bacteria induces pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, TNF-2, IFN-γ, MCP-1, and GM-CSF, to be secreted, causing an acute inflammatory response and may be responsible for intestinal damage [3].

The global impact of *Salmonella* infections is significant, with reports from the Centers for Disease Control and Prevention (CDC) indicating ~150 million illnesses and ~60,000 deaths worldwide yearly [7]. Non-typhoidal *Salmonella enterica* serovar Typhimurium infection is endemic to Southeast Asian countries, including Thailand, Malaysia, Malawi, Cambodia, Indonesia, Myanmar, Singapore, India, the Philippines, and sub-Saharan Africa [8]. Approximately 20% of the Indian population is affected by non-typhoidal *Salmonellae* [9].

With no vaccines, the most effective method for countering *Salmonella* is antibiotics, thanks to the antibiotic revolution brought by Alexander Fleming, who accidentally discovered penicillin and contributed to the discovery of multiple other antibiotics. Traditional antibiotics like ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole have been used for the treatment of typhoid fever [10, 11]. Since then, *Salmonella* has evolved and developed several multidrug-resistant strains [12]. Bacteria are

flagged as multidrug-resistant if they have resistance against three or more classes of antibiotics, according to the World Health Organization (WHO) [13]. Many strains of *Salmonella* developed antibiotic resistance patterns such as ASSuT (ampicillin, streptomycin, sulfonamides, and tetracycline) and ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) [14, 15]. The development of multidrug-resistant strains can result from the interplay of many factors, including Outer Membrane Proteins (OMPs), Outer Membrane Vesicles (OMVs), toxins, formation of biofilms, misuse of antibiotics, horizontal gene transfer, etc.

### 1.1. Outer Membrane Proteins (OMPs)

One main reason for such resistance development is the presence of OMPs in gram-negative bacteria. Gram-negative bacteria have a comparatively thinner peptidoglycan layer than gram-positive bacteria. However, it has an advantage due to an outer membrane containing Lipopolysaccharides and proteins. These proteins are responsible for structural integrity, pathogenesis, adhesion, signaling, host-pathogen interactions, immune evasion, and transport of molecules, including antimicrobial peptides and nutrients. These amphipathic proteins have both hydrophobic domains, which interact with the membrane, and hydrophilic domains, which face either the cytosol or the cell exterior. They are rich in β-sheets, which majorly form the hydrophobic domain, forming a β-barrel embedded in the outer membrane. Bacterial OMPs are formed in the cytosol and transported to the outer membrane after the N-terminal signal peptide removal in the periplasm [16]. The proteins are folded and assembled through the Skp/DegP or SurA pathway [17, 18].

Many OMPs of *Salmonella*, like OmpA, OmpC, and OmpF, have already been investigated and studied in detail for the study of antibiotic resistance, with the loss of these OMPs impairing the organism's resistance

to the antibiotics [19, 20]. OmpA renders protection to bacteria from Reactive Nitrogen Intermediates (RNIs) and β-lactams (e.g., ceftazidime, meropenem) [21, 22]. PagN contributes to adherence to the mammalian cells and mediates high-level invasion [23]. OmpD restrains the bacterial proliferation in macrophages, helping in preventing host overactivation (ROS formation) [24]. Studies have shown that decreased expression of OmpC/OmpF limits antibiotic entry [25, 26]. OmpV mediates the adhesion and invasion of the bacteria, thus regulating the virulence of the *Salmonella* [27].

YchP is another OMP of *Salmonella* that has not yet been studied in detail. YchP is a putative invasion protein of *Salmonella enterica* serovar Typhimurium, belonging to the inverse autotransporter (IAT) family of proteins based on sequence similarity, that plays a role in evasion. YchP comprises 474 amino acid sequences and has an approximate molecular weight of 52 kDa. The predicted model of YchP by SIGNAL P 6.0 shows a 40 amino acid-long N-terminal signal peptide with the initial 17 amino acids having a higher probability for the outer membrane. Data from NETSurf P 3.0 shows that the secondary structure of YchP consists of 5 α-helixes and 22 β-sheets, and the tertiary structure in UniProt (I.D. A0A0F6B263) shows that only 16 out of 22 β-sheets are responsible for forming the β-barrel. The C-terminal domain protrudes out to the extracellular matrix and allows for interaction with the environment, facilitating adherence to the host.

### 1.2. Interactions with Immune Cells

Microorganism-associated molecular patterns (MAMPs), including DNA, RNA, lipoproteins, LPS, and peptidoglycan, offer an engagement site for the interaction of microbes with the pattern recognition receptors (PRRs) of an immune cell and allow the induction of pro-inflammatory signaling cascade [28]. A cell expresses multiple different PRRs on its cell surface,

including Toll-like receptors (TLRs), Nod-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs), and Absent in melanoma-2 (AIM2)-like receptors (ALRs) [29]. Among these, TLRs are membrane-bound receptors and exist as transmembrane proteins with a leucine-rich repeat (LRR) motif at the ectodomain [30]. There are multiple types of TLRs present specific to their ligands, such as TLR2 for Lipopolysaccharide (LPS); TLR5 for Flagellin; TLR6 and TLR4 for lipoprotein, lipoteichoic acid, and others; and many more for different MAMPs. Binding the ligand to the LRR of TLRs causes dimerization and commences the downstream signaling through Toll/IL-1R (TIR) domain adapters, MyD88 (myeloid differentiation primary-response protein 88), TIRAP (TIR-domain-containing adaptor protein), TRIF (TIR-domain-containing adaptor protein inducing interferon-β), and TRAM (TRIF-related adaptor molecule) [31]. TLRs have a major role in mediating immune responses in the gut [30].

The most advanced immune response is generated through the adaptive immune system, B-cells, and T-cells. B-cells are the key regulators of the immune system, functioning as antigen-presenting cells (APCs), secreting cytokines, and providing antibodies. B-cells have different surface receptors, B-cell receptors (BCR), MHC class I and II, and other accessory receptors to recognize the antigen and present it to different immune cells, and produce antibodies against. B-cells can be activated in either T-cell-independent or T-cell-dependent manners. After the activation, BCRs disappear.

In monocytes, OmpV is recognized by the TLR1/2 heterodimer. In macrophages and intestinal epithelial cells, both TLR1/2 and TLR2/6. Downstream signaling involves MyD88, IRAK-1, MAPK, and transcription factors NF-κB and AP-1 [27]. In a study by Nicholas Arpaia et al, they concluded that TLR signaling is required for *Salmonella typhimurium* virulence, because of the induction of SPI-2 genes, which result in

replication and virulence of the microbe through TLR2-dependent phagosome acidification [32]. NF-κB promotes remodeling of the actin cytoskeleton and upregulates the transcription of certain genes, including c-MYC, PAX5, and Activation-induced cytidine deaminase (AID) in B-lymphocytes [33, 34, 35].

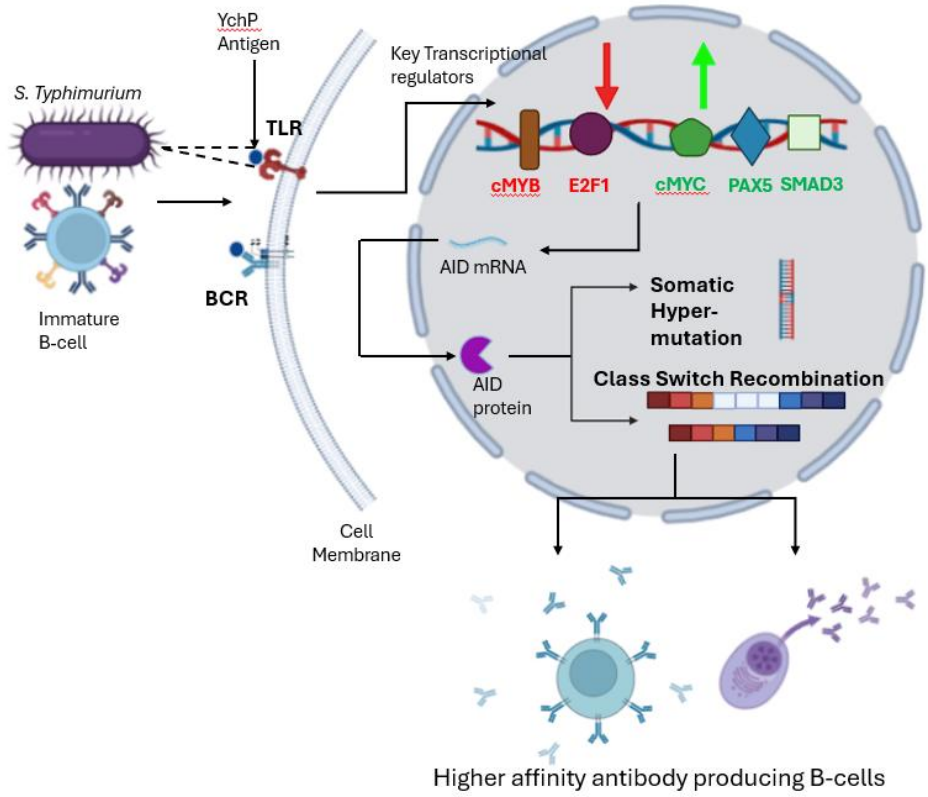


**Figure 1.1:** Schematic overview of the signaling cascade induced by the interaction between YchP, an OMP, and TLR, activating regulators of AID, hence in turn upregulating or downregulating the expression of AID and regulators of AID.

### 1.3. Activation-induced cytidine deaminase (AID)

We are living in a battle of survival against the microorganisms. As they evolve further to counter our medications, we also must evolve our methods for human preservation. Various substances and microbes can elicit an

immune response. Most of the defense is done by our body's innate immune system, which includes anatomical protection, physiological protection, inflammatory response, and phagocytic response. Innate response, though fast and non-specific, is not able to provide protection when pathogens get past it. Here comes the role of the adaptive immune system, which can recognize all the foreign particles and generate a specific response to each of them. The adaptive immune system consists of two immunities: Humoral, which includes our B-lymphocytes, and cell-mediated, which includes our T-lymphocytes.

We are naturally gifted with an ever-evolving weapon against any microorganism we can encounter: our antibodies. We encounter various microbes during our everyday lives, some of which are harmless and some harmful, yet we can prevent most infections. This is because of the ability of our B-cells to produce diverse antibodies; they are capable of producing multiple different types of antibodies with different affinities for the same microbe, even before it encounters the antigens. One reason for such diversity is the combinations that can be created from 11,000 heavy chains and 320 light chains, giving us about $3.5 \times 10^6$ variants. This is known as combinatorial diversification [36, 37]. Further, antibody diversity is again generated due to multiple mechanisms: Insertion and deletion of random nucleotides, Junctional diversification, Somatic hypermutation (SHM), and V(D)J recombination.

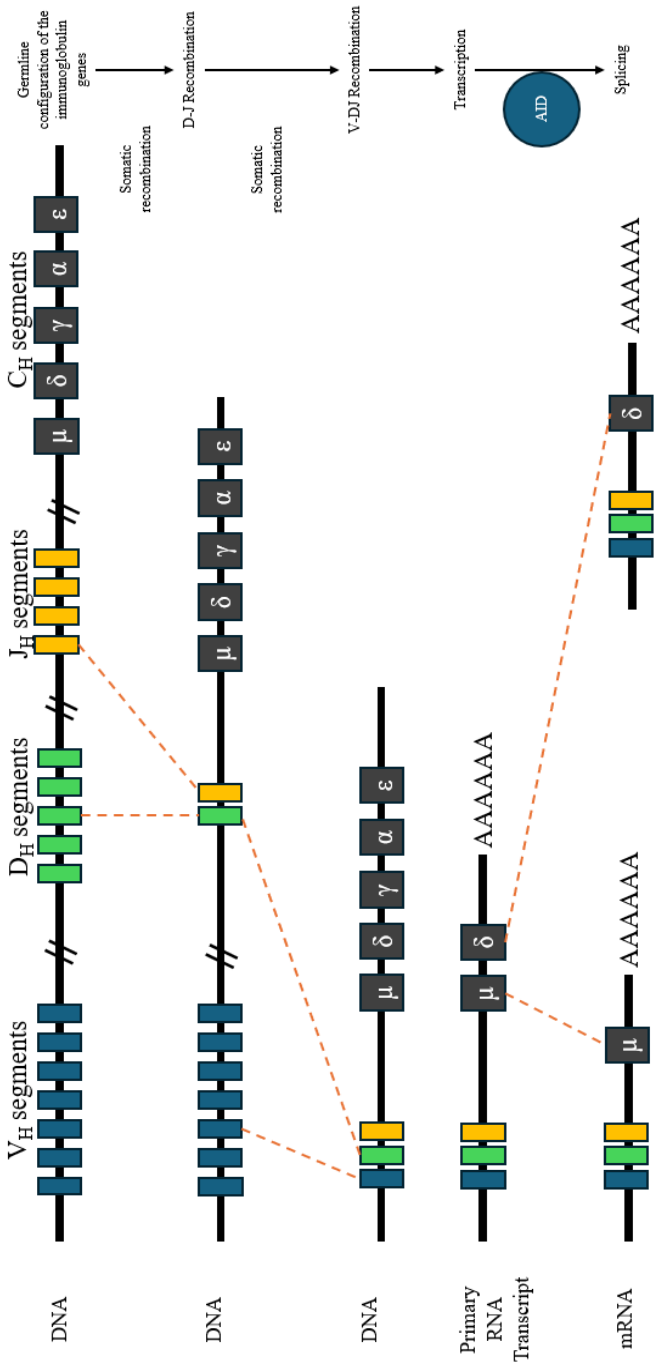


**Figure 1.2:** A representative image describing the mechanism of V(D)J recombination for the variable heavy chain in the Ig locus of the B-cells to produce antibodies with different affinities.

All these mechanisms are regulated by a primary protein, AID. AID belongs to the APOBEC family encoded by the gene *aicda*, located on chromosome 12 in humans [38, 39]. This protein is produced explicitly in

B-cells and in minor amounts in T-cells. AID is directed to the immunoglobulin genes and induces point mutations on a single strand by deaminating cytosine to uracil, activating the DNA mismatch repair mechanism, base excision repair, or DNA replication. Here, the repair mechanism creates a mutation that leads to the variation. AID is responsible for inducing SHM and plays an important role in Class-Switch recombination (CSR) for the production of the antibody with different heavy chains, IgG, IgM, IgA, IgD, and IgE. CSR is the deletion and recombination between the switch regions present around genes of different heavy chains [40].

Because of the mutational ability of AID, it is under strict and controlled expression through Transcriptional, post-transcriptional, and translational level modifications. Activation of NF-κB activates several activators of AID, such as SMAD3, STAT6, PAX5, c-MYC, etc. Incidentally, the induction of the downstream signal by TLRs activates c-MYC and PAX5. Aberrant regulation in AID can cause autoimmune diseases upon increased expression and immunodeficiency upon decreased expression, as the antibodies' diversity declines. AID possesses a substantial nuclear export signal (NES), which exports AID to the cytoplasmic space and aids in regulating the effects of AID as well. Nuclear AID is significantly less stable, with a half-life of only 2.5 hours, than cytoplasmic AID, which has a half-life of approximately 8 hours [41].

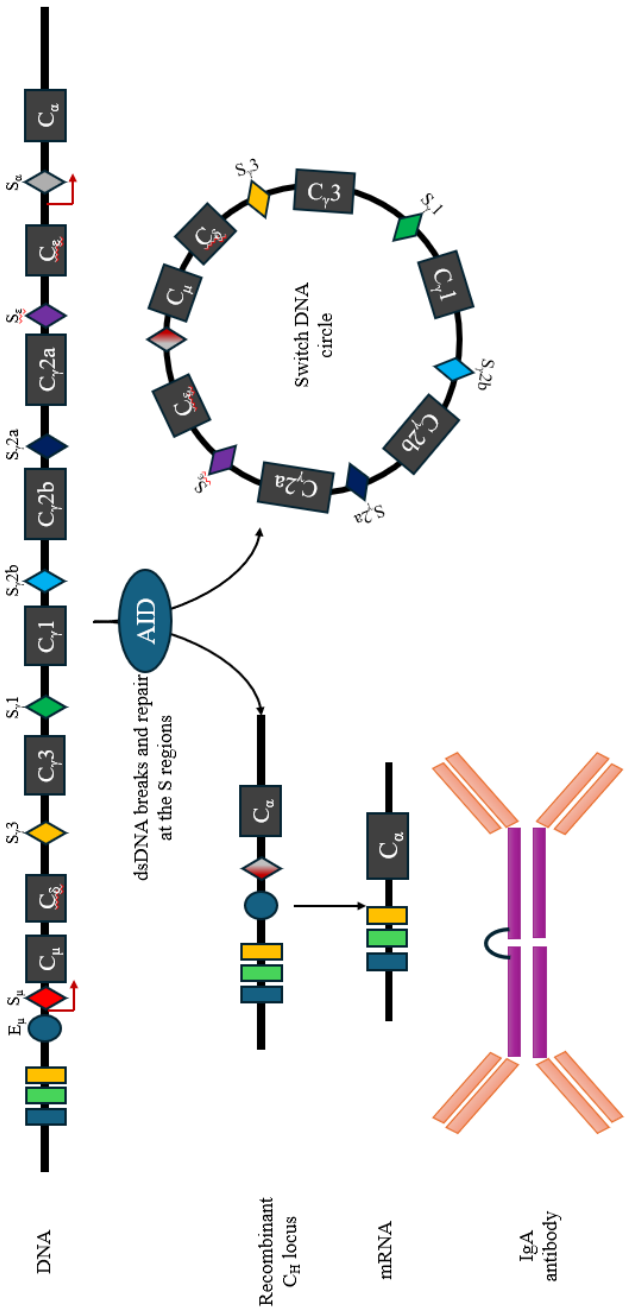


**Figure 1.3:** A representative image describing the mechanism of CSR for the constant heavy chain in the Ig locus of the B-cells to produce antibodies with different $F_{ab}$ regions and different properties depending on the type of chain expressed.

### 1.3.1. Somatic Hypermutation (SHM):

SHM is one of the primary reasons why our B-cells can produce such diverse antibodies with different affinities to the antigen and provides for affinity maturation. AID is the primary contributor to this process by

actively inducing mutations at the Ig locus at a rate of ~$10^{-3}$ base pairs per generation [42]. AID in the G1 phase of the cell cycle preferentially targets the WRCY motif in the locus deaminating Cytosines (C) into deoxy-Uracil (U) [43, 44]. This mutation is recognized by the base excision repair (BER) pathway, and either one of the BER glycosylases, UNG, TDG, SMUG1, and MDB4, is recruited. Uracil-(N)-glycosylase (UNG) excises the base and creates an abasic site, with APEX1 or APEX2 (in germinal center B-cells) creating an incision besides the abasic site, and subsequently POLB fills the gap and ligates through LIG1 or LIG3 [42, 45, 46].

Additionally, U-G mismatches can be repaired by the Mismatch Repair (MMR) pathway, where MSH2/MSH6 recognizes the mismatches and initiates the repair pathway [47]. Endonuclease complex PMS2 and MLH1 make an incision 5' of the mismatch, allowing the formation of a nick to facilitate the activity of EXO1, an exonuclease, to create a single-stranded gap. Replicative polymerases then fill this gap [48, 49, 50].

### 1.3.2. Class-Switch Recombination (CSR):

Naïve or immature B-cells are only able to produce IgM and IgD antibodies, and to produce various antibody classes. AID helps in recombining the Heavy constant regions. There are five classes given to antibodies based on the expressed gene for the constant heavy chain of the antibodies, namely IgM, IgD, IgA, IgG, and IgE. CSR is achieved by splicing and rejoining the DNA between the two S regions of 1 and 10 kb in length, situated upstream of all the constant heavy genes, except for Cδ [51, 52]. CSR is explained by the 'cut and paste' mechanism. The S-regions are G-rich with a high density of WGCW motif. At the G1 phase, like SHM, AID results in the deamination of cytosines to uracil, initiating BER and MMR repair pathways. The DSBs are recombined predominantly by non-homologous end joining (NHEJ) [53, 54, 55].

Here in this study, we aim to identify the response of the B-lymphocytes upon stimulation with the YchP protein expressed in *Salmonella.* We identified the change in the expression of AID at the transcriptional and translational levels through RT-qPCR and western blot. We also studied the effect of YchP stimulation on the regulatory genes of AID, such as for promoters we checked the expression of PAX5, c-MYC, STAT6, and SMAD3, and for repressors we selected E2F1 and c-MYB. Depending on the upregulation or downregulation of AID expression, we could predict the potential of YchP as either a vaccine candidate or an immune evasion mechanism used by *Salmonella.* We also investigated the interaction of YchP with the TLR2 receptor present on the B-cell surface.

# Chapter 2

# Objectives

1. Stimulation study of Raji human B-cells with the purified YchP protein to determine the aberrant AID expression at the transcript and translational level.

# Chapter 3

# Materials and Methods

## 3.1. Materials:

**3.1.1. Strains:** Rosetta *E. coli* (DE3) with recombined pET43a vector of YchP for expression and Raji human B-cells.

**3.1.2. Chemicals:** Luria Bertani broth (HiMedia), Luria Bertani agar (HiMedia), Ampicillin (HiMedia), Tris buffer, NaCl, Imidazole (Sigma Aldrich), Lysozyme, Urea, Triton X-100, β-mercaptoethanol, Bradford reagent (HiMedia), PMSF (HiMedia), LDAO/DDAO (SRL), Bio-Rad resin or Ni-NTA beads, Glycerol, Ammonium persulphate, N,N,N′,N′ - Tetramethylethylenediamine (TEMED), Sodium Dodecyl Sulphate, Acrylamide, bis-Acrylamide, Stained and unstained Protein ladder (Bio-Rad), Glycine, Nuclease free water, TRIzol reagent (ThermoFisher), Protease Inhibitor cocktail, Chloroform, Ethanol, Imidazole, EDTA, Fetal Bovine Serum (FBS), DMSO, RPMI 1640 media, Penicillin-Streptomycin, Tween 20, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Nuclease Free Water, Anti-TLR2 antibody, Anti-His mouse IgG monoclonal antibody, Anti-AID mouse IgG monoclonal antibody, Anti-PAX5 rabbit IgG monoclonal antibody, Anti-c-MYC rabbit monoclonal antibody, Anti-TLR2 rabbit monoclonal antibody, HRP conjugated Anti-mouse rabbit IgG monoclonal antibody, HRP conjugated Anti-rabbit goat IgG monoclonal antibody, nitrocellulose membrane (Amersham, GE Healthcare), 3,3',5,5'-Tetramethylbenzidine (TMB), Luminol, DAPI (4′,6-diamidino-2-phenylindole), Goat anti-mouse Alexa Fluor 488 (Invitrogen, Cat. A-11001), and Goat anti-rabbit Alexa Fluor 594 (Invitrogen, Cat. A-11012).

**3.1.3. Instruments:** Centrifuge, Ni-NTA column, Water bath, Orbital shaker, mini dry bath, SDS-PAGE apparatus, Western blot unit, Biosafety cabinet class 2, AKTA system, Incubator shaker, dialysis bags, magnetic stirrer, Rota spin, microscope, Nanodrop, Chemidoc, Image quant LAS 4000 Gel Doc system (GE Healthcare),.

## 3.2. Methods:

### 3.2.1. Recombinant protein expression, solubilization, and purification.

Transformed *Escherichia coli* Rosetta (BL21(DE3)) cells were inoculated in 5 mL Luria-Bertani broth as primary culture and were grown in an incubator at 220 rpm, 37 °C for 14-16 h in the presence of ampicillin (100 µg/mL). 1% of the volume of the secondary media was inoculated in 800 mL of LB broth. After reaching the Optical Density (O.D.) of ~ 0.6 at 600 nm, they were induced with 0.1 mM isopropyl-β-d-1-thiogalactopyranoside (IPTG) (Hi-Media). Allowed the induction to continue for 4 h at 37 °C, 220 rpm. The induced cells were pelleted and resuspended in 50 mL buffer containing 20 mM Tris-Cl, pH 8.0, NaCl 500 mM, 0.1 mM PMSF (Hi-Media), and 10% glycerol at 6,000 rpm, 10 min, 4 °C. Resuspended cells with lysozyme in a water bath at 30 °C for about 25 min. Subsequently, cell lysate was prepared by sonication at 65% amplitude, the pulse of 2-s on and 2-s off cycle for at least 45 min and centrifuged the resultant at 14000 rpm for 40 min at 4 °C for separation of supernatant and pellet which were analyzed on 12% SDS-PAGE gels to check the expression of the desired outer membrane protein. The cell pellet was washed 3 times with 10 mL of wash solution containing (20 mM Tris-Cl, 500 mM NaCl, and 1% Triton X-100).

For solubilization, the induced pellet was resuspended with 10 mL solubilization buffer of pH 8.0 containing 20 mM Tris-Cl pH 8.0, 500 mM

NaCl, and 8 M Urea and incubated at room temperature overnight, and centrifuged at 12,500 rpm at 10 °C for 20 min to separate the supernatant and insoluble pellet. The solubilized supernatant was subjected to purification.

Purification was done by gravity column. 2 mL of Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Ni-NTA, Bio-Rad, Cat. 1560133) was introduced into the column, washed with three column volumes of autoclaved distilled water. Equilibrated the column with 30 mL of equilibration buffer containing 20 mM Tris-Cl pH 8.0, 500 mM NaCl, 8 M Urea, 5 mM imidazole, 5 mM β-mercaptoethanol, and 1% Triton X-100. Then, the solubilized protein was incubated with the charged and equilibrated beads for 2 h to ensure proper binding of the protein and the beads, and then passed through the gravity column. The flow-through, washes, and elutions were collected in a fresh Falcon tube. Washes were provided with Tris buffer containing 20 mM Tris-Cl pH 8.0, 500 mM NaCl, 8 M Urea, 5 mM β-mercaptoethanol, and 1% Triton X-100 and imidazole with varying concentrations. Two washes were provided for YchP with 30 mL of washing buffer (20 mM Tris-Cl pH 8.0, 500 mM NaCl, 8 M Urea with 60 mM imidazole, and 90 mM imidazole). Three protein elutes with 20 mM Tris-Cl pH 8.0, 500 mM NaCl, 8 M Urea with 150 mM imidazole, 300 mM imidazole, and 500 mM imidazole. Stripping of the beads was done with 0.1 M EDTA to clean the beads for reuse, and the purification quality was checked by loading fractions of each purification step on a 12% SDS PAGE gel.

### 3.2.2. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS PAGE).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a standard method for separating proteins by size using an electric field. SDS, an anionic surfactant, denatures proteins by breaking non-covalent bonds and coats them with a negative charge proportional to their molecular weight. In discontinuous SDS-PAGE, proteins first pass through a stacking gel at pH 6.8, where glycine, with a low net negative charge, creates a 'pushing' effect that equalizes protein migration. Next, in the resolving gel at pH 8.8, both proteins and glycine acquire a more substantial negative charge, allowing the proteins to separate by size, with glycine leading the migration front. The running buffer contains 30.3 g Tris base, 144 g glycine, and 10 g SDS (for 1 L of 10X solution), which maintains the system's conductivity. When the external electric field is applied, the proteins migrate towards the positive electrode, the anode, at different speeds based on their respective molecular weights, with smaller proteins travelling faster and covering a more significant distance than larger proteins. The gel is then stained in the Staining solution containing Coomassie Blue dye R250, acetic acid, and methanol at the end of the SDS PAGE electrophoresis. We can also estimate the protein concentration based on the size and intensity of the bands in the gel. For induced and uninduced samples, pellet and supernatant were prepared using 6X loading dye with 10 µL of sample and 5 µL of dye and heating at 95 °C for 5 min. Samples were loaded onto the gel, which was run in the 1X running buffer at 90 V for 2 h. The gel was stained for 6 h and destained for the required period by changing the destaining solution after every hour, and the gel was analyzed.

### 3.2.3. Refolding, buffer exchange, and concentration of purified protein.

The eluted fraction of purified YchP were combined and 5-fold diluted into 10 mL of buffer containing (20 mM Tris-Cl, 300 mM NaCl, 1 mL Glycerol, and 50 mg LDAO), and incubated overnight with constant stirring using Rota spin at 4 °C. Diluted protein was then further proceeded for buffer exchange with dialysis buffer containing 20 mM Tris-Cl, 200 mM NaCl, 10% glycerol, 20 mM imidazole, 5 mM EDTA, and 0.1% LDAO and concentrated using a 10-kDa cutoff concentrator (Amicon Ultracentrifugal filter, UFC901008). Centrifuged at 3500 x g, 4 °C until the volume of the protein in the concentrator reached 10-fold less than the original loaded protein. Collected the concentrated protein in an autoclaved microcentrifuge tube and stored it at 4 °C.

### 3.2.4. Size exclusion chromatography for studying oligomerization.

Size exclusion chromatography (SEC) or Gel filtration chromatography separates proteins based on their shape and size. It works on the principle of sieving molecules through and around the inert beads. The inert beads are uncharged and have no specific affinity; they contain tiny uniform pores where the smaller-sized proteins get trapped while the larger proteins, unable to fit in the pores, pass around the beads. The largest proteins are eluted first, and gradually, the smallest follow. It is also used for studying dimer, trimer, or oligomer formation, helping us determine the protein's absolute molecular weight and dimer, trimer, or oligomer state. A Superdex 75 increase 10/300 GL column with a flow rate of 0.400 mL/min has been chosen. The column was cleaned using autoclaved, bath-sonicated distilled water, and it was equilibrated using a buffer that included 500 mM NaCl and 50 mM Tris-Cl pH 8.0. A 500 µL of the refolded and concentrated YchP protein sample was manually inserted into the column, allowing the AKTA system to generate the chromatogram.

### 3.2.5. Raji human B-cell Culturing.

A new batch of Raji human B-cells was thawed from the liquid nitrogen storage at -195 °C. These cells were cultured in 100 mm plates with RPMI 1640 media supplemented with 10% FBS and 1% Pen-Strep solution (100 U/mL penicillin and 100 µg/mL streptomycin) and kept the cells at 5% CO2 concentration, 37 °C in the incubator. The cells were observed the next day for their growth, and the media was changed to eliminate any contamination from the DMSO added before for cryopreservation. Cells were passaged when they reached a confluency of ~80%. Cell morphology and contamination were routinely checked using a phase-contrast microscope.

### 3.2.6. MTT assay and Trypan Blue assay for cytotoxicity test

Seeded a 24-well plate with a certain number of Raji human B-cells and stimulated them with varying concentrations (0, 0.625, 1.25, 2.5, 5, 10, 20, and 40 µg/mL) of the purified YchP protein in duplicates, allowed the stimulation to run for 24 h and kept the plate in a $CO_2$ incubator. Prepared the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution in 1X PBS. After the incubation, the cells were observed, and the media was aspirated from the wells after centrifuging the plate at 1,000 x g, 4 °C for 5 min. Equal volumes of MTT solution and serum-free media were added to each well, allowing the cells to be incubated at 37 °C for 4 h. Later, MTT solvent (Isopropanol) was added, and the plate was wrapped in silver foil and placed over an orbital shaker for about 30 min. The OD of the wells was taken at 590 nm to estimate the cytotoxicity of the protein. This gives us the protein concentration at which 50% of the cells are viable.

Similar to the MTT assay samples, Raji human B-cells were stimulated with the same concentrations of the purified YchP protein for 24 h in a 60 mm petri plate each and were kept in the incubator. The next day,

10 µL of the culture from each Petri plate was extracted in a new MCT and mixed with 30 µL of Trypan Blue to create a 1/4 dilution. These were individually loaded onto the haemocytometer and manually counted for dead and live cells in replicates. The values obtained from both MTT and Trypan blue assays were used to calculate the IC50 value and concentrations for further studies.

### 3.2.7. Co-localization assay

To check for the interaction of the YchP protein with the TLR2 receptor of the cells, we cultured Raji human B-cells, $5*10^6$ cells. These cells were then washed with 1X PBS and fixed with 4% paraformaldehyde for 20 min. Subsequently, the cells were permeabilized using PBS-T (supplemented with 0.1% Triton-X). Blocking of the cells were done through PBS-T supplemented with 5% BSA for 30 min and then incubated with the primary antibodies: anti-TLR2 and anti-His, each diluted at 1:500. Subsequently, the cells were washed three times with PBS-T to remove the primary antibodies, then these cells were treated with secondary antibodies, Goat anti-mouse Alexa Fluor 488 (Invitrogen, Cat. A-11001) diluted to 1:200 and Goat anti-rabbit Alexa Fluor 594 (Invitrogen, Cat. A-11012) diluted to 1:400 in PBS-T with 2% BSA. An Olympus confocal laser scanning microscope at a magnification of 60X was used to capture the images.

### 3.2.8. Raji human B-cell stimulation.

About $8*10^7$ cells were seeded in 90 mm plates and stimulated with the 0, 0.625, 1.25, and 2.5 µg/mL concentrations of the purified and endotoxin-free protein. These plates were incubated for different time

intervals: 0, 4, 8, and 24 h for protein estimation. At the selected time slots, the cells were pelleted.

### 3.2.9. Bradford assay for protein estimation and Western Blotting.

Stimulated Raji human B-cells were pelleted and washed with 1X PBS buffer at 1800 rpm for 5 min at 4 °C. The pellet was resuspended in REPA buffer and sonicated at 65% amplitude, 2 on/2 off cycle for 22 s, and later stored at -20 °C after centrifuging them at 10000 rpm for 5 min at 4 °C. The protein samples collected were diluted appropriately for the Bradford assay procedure. Using the data from the Bradford assay, the appropriate volume was calculated to load into SDS-PAGE for similar concentrations across different samples.

Western Blotting is a very sensitive technique to detect the target protein in the sample using the antigenic properties of the proteins in different species. The proteins from the gel are transferred to a nitrocellulose membrane or nylon membrane under the influence of an electric field. We loaded our gel with the nitrocellulose membrane into the blotting sandwich and placed the cassette in the transfer tank with the freshly made transfer buffer at 18 V with an ice pack overnight. On the next day, the membrane was washed in TBST three times and allowed to be incubated with the blocking solution of 5% skim milk (SM) powder in TBST for 1 hour to block the vacant spots of the membrane, preventing non-specific binding of the antibodies. The primary antibodies are raised against the target protein and allowed to interact with the bands on the membrane and the specific protein due to the epitope-paratope reaction overnight at 4 °C. The membrane is rinsed 3-5 times with TBST to remove the remaining primary antibodies. A secondary antibody fluorescently or enzymatically tagged against the Fc region of the primary antibody is raised and allowed to incubate with the membrane for 1 h; the secondary antibodies will be specific to the constant

region of the primary antibodies and interact with them. The appropriate substrate or fluorescent signal is provided, which for us was the combination of 3,3',5,5'-Tetramethylbenzidine (TMB) and luminol in equal ratio, and the location of the specific protein is visualized using a Chemidoc CCD camera-based imager to capture the chemiluminescent signals.

# Chapter 4

# Result

## 4.1. Expression of YchP protein.

The recombinant Rosetta strain carrying the vector pET43a with YchP protein was inoculated and induced with 0.1 mM isopropyl-β-d-1-thiogalactopyranoside (IPTG). Allowed the culture to grow appropriately, harvested the cells through centrifugation, and washed the pellet with the lysis buffer containing 20 mM Tris-Cl pH 8.0, NaCl 500 mM, 0.1 mM PMSF, and 10% glycerol. The cell pellet was treated with lysozyme in a water bath at 37 °C for 25 min. Sonicated the solution and separated the cell pellet and supernatant by centrifugation, and the samples were analyzed on 12% SDS-PAGE for the induction of the desired protein.

YchP, having a molecular weight of ~52 kDa, was observed as a prominent band in the cell pellet, as seen in **Lane 4** of **Figure 4.1**, while no expression can be seen in the uninduced samples.

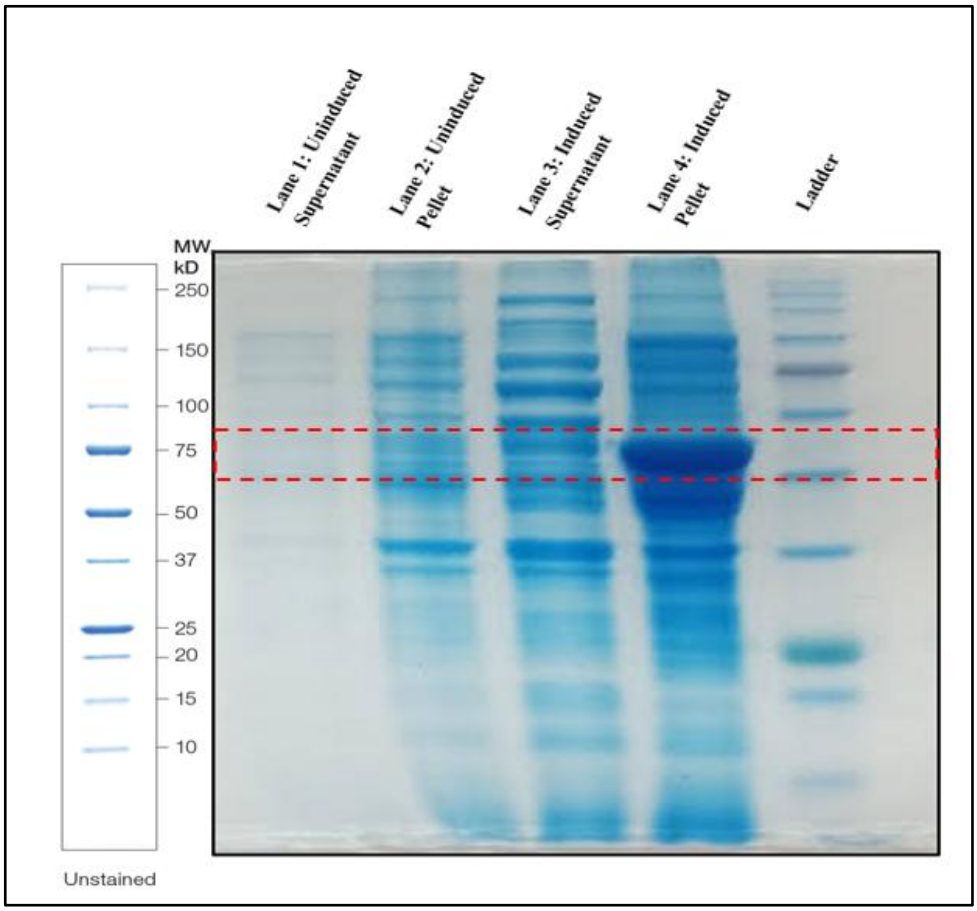


**Figure 4.1:** Expression gel of the YchP protein showing a thick, prominent band at ~52 kDa. **Lane 1:** Uninduced pellet, **Lane 2:** Uninduced supernatant, **Lane 3:** Induced pellet, **Lane 4:** Induced supernatant, and **Lane 5:** Unstained Protein ladder.

## 4.2. Solubilization and purification of YchP protein.

The cell pellet was solubilized in 10 mL of solubilization buffer containing 20 mM Tris-Cl, pH 8.0, 500 mM NaCl, and 8 M Urea and incubated with the solubilization buffer overnight at room temperature on an orbital shaker. We centrifuged the solubilization buffer with the cell pellet at 12,500 rpm, 20 min, 10 °C. The supernatant was incubated with Ni-NTA beads over an orbital shaker at room temperature for 1 h. Then, the protein was eluted with different concentrations of imidazole 50, 90,

150, 300, and 500 mM. We took the 150 mM and 300 mM imidazole fractions **(Lane 6 and 7 in Figure 4.2)**, which had the least non-specific bands and a thick band representing YchP protein elution on 12% SDS-PAGE.

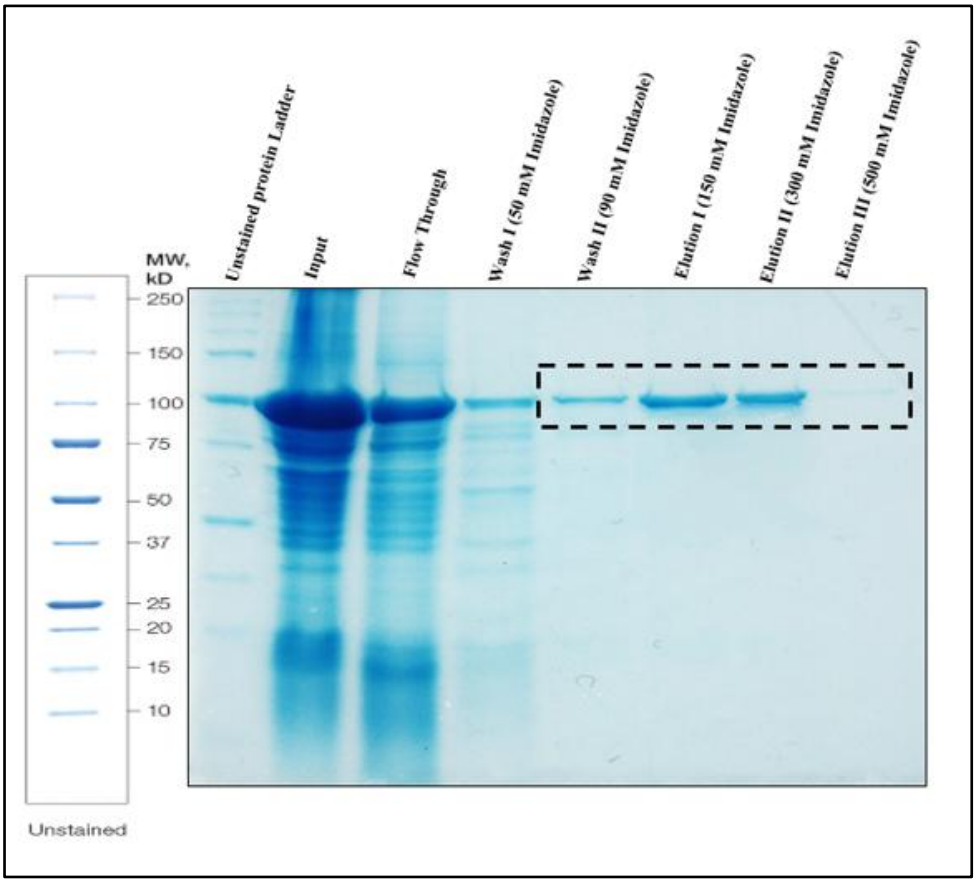


**Figure 4.2:** Purification gel for the solubilized YchP protein purified through the Ni-NTA column. **Lane 1:** Ladder, **Lane 2:** Input fraction, **Lane 3:** Flow through, **Lane 4:** Wash I (50 mM Imidazole), **Lane 5:** Wash II (90 mM Imidazole), **Lane 6:** Elution I (150 mM Imidazole), **Lane 7:** Elution II (300 mM Imidazole), and **Lane 8:** Elution III (500 mM Imidazole), respectively.

### 4.3. Buffer exchange, refolding, and concentration.

Buffer exchange was performed using a dialysis solution of 50 mM Tris-Cl, 300 mM NaCl, 5% glycerol, 30 mM imidazole, and 0.5 M EDTA. The dialyzed protein was refolded and added to a refolding buffer containing 20 mM Tris-Cl pH 8, 300 mM NaCl, and 0.1% LDAO, incubated the sample on a Rota spin at 4 °C overnight, and concentrated with an Amicon with a 10 kDa cut off.

### 4.4. Size exclusion chromatography.

SEC is performed using a Superdex 75 increase 10/300 GL column with a 0.400 mL/min flow rate. YchP showed a peak at 10 mL. A 12% SDS-PAGE was run to analyse the specificity of the elute from the column, which showed us a single band (**Figure 4.3**).

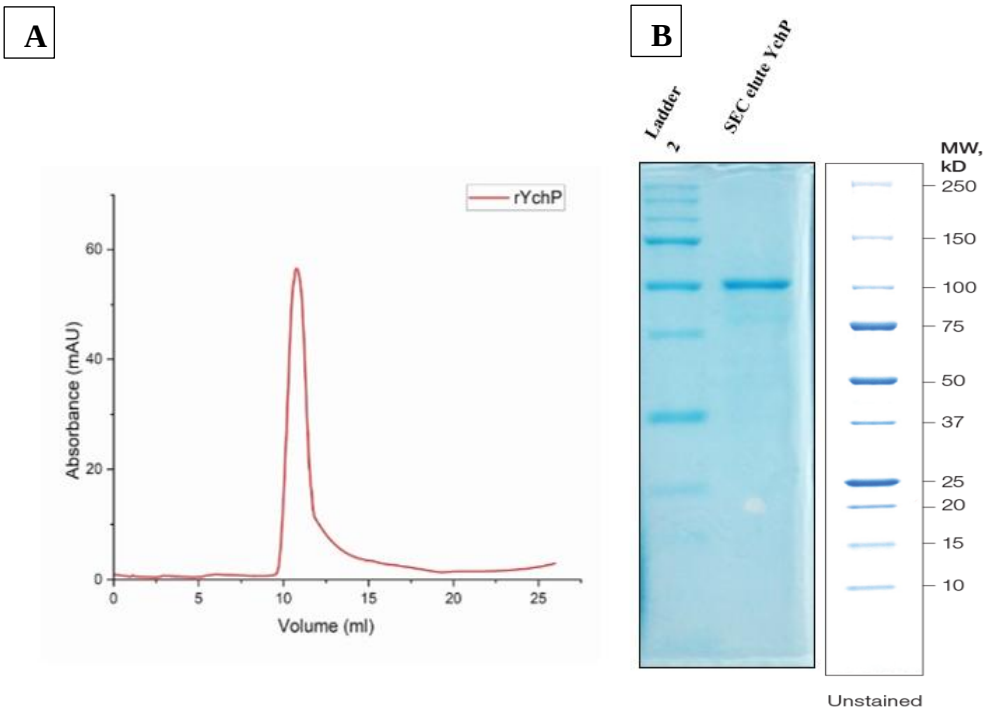


**Figure 4.3:** Analysis after Size exclusion chromatography. **A)** Chromatograph plotted from the AKTA size exclusion chromatography. **B)** 12% SDS-PAGE ran for the SEC eluted protein. **Lane 1:** Unstained Protein ladder, **Lane 2:** SEC eluted YchP.

### 4.5. Cytotoxicity assay.

MTT assay was performed, and after the addition of MTT solvent (isopropanol), the plate was allowed to rest for 10 min before taking the reading for dissolving the formazan crystals thoroughly. We also performed a Trypan blue assay with the same concentration of YchP. We counted the cells using a haemocytometer and generated a graph to demonstrate the relationship between cell viability and YchP concentrations. From **Figures 4.4 A** and **4.4 B,** the Half-maximal inhibitory concentration **(**IC50) value was found to be 5 µg/mL. We chose 5 µg/mL based on OD, cell count, and morphological data. The cellular morphology, **Figure 4.5 A-D,** exhibited dense clustering and a healthy cell appearance, indicating active proliferation. In contrast, the images in **Figure 4.5 E-H** revealed decreased cell density and disrupted morphology, signifying cytotoxic effects.

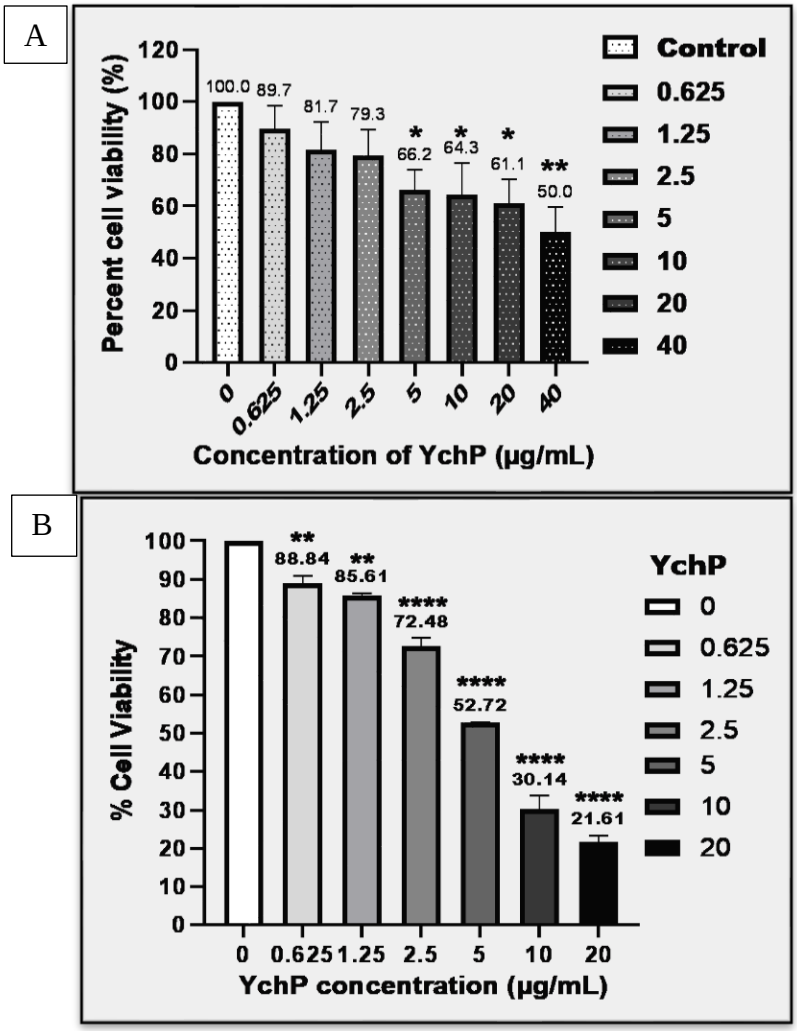


**Figure 4.4:** Graphical representation of the concentration of YchP used in the stimulation of Raji human B-cells and the cell viability percentage for analysing the cytotoxicity of the protein. **A)** MTT assay **B)** Trypan Blue exclusion assay

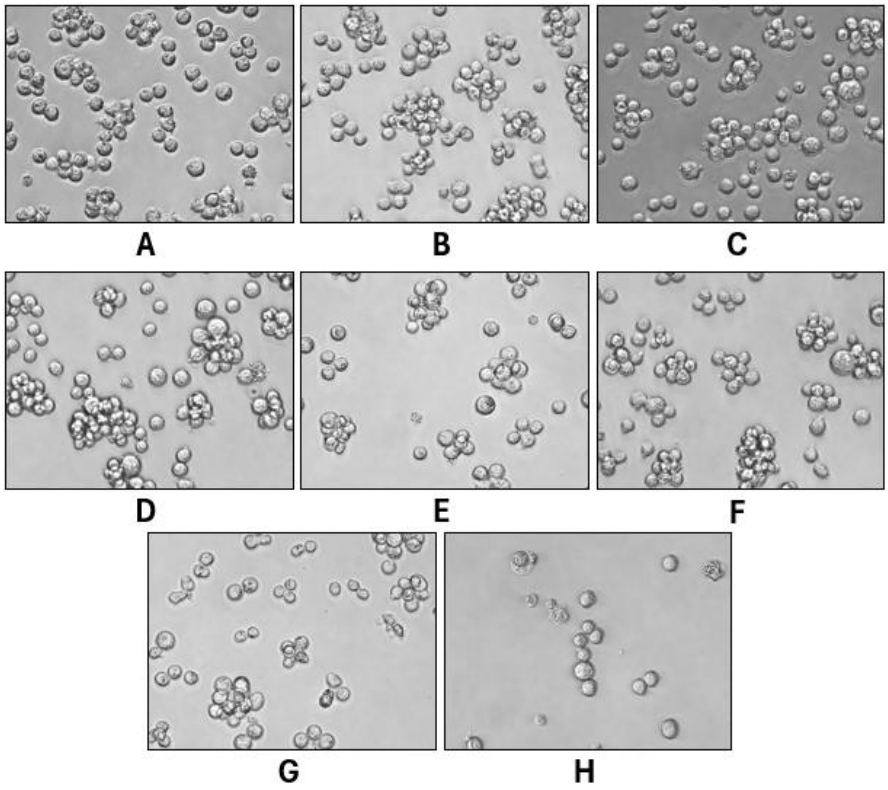


**Figure 4.5:** Raji human B-cells stimulated with various concentrations of purified YchP protein and observed under a microscope. **A)** 0 µg/mL (control) **B)** 0.625 µg/mL **C)** 1.25 µg/mL **D)** 2.5 µg/mL **E)** 5 µg/mL **F) 1**0 µg/mL **G)** 20 µg/mL **H) 4**0 µg/mL

## 4.6. Co-localisation of YchP.

Co-localisation studies were conducted using fluorescence microscopy to investigate the potential interaction between the YchP protein and TLR2 receptors. Raji human B-cells were stimulated for 24 h with purified YchP protein and subsequently stained with DAPI (4′,6-diamidino-2-phenylindole) to visualise nuclear morphology. Fluorophore-conjugated antibodies targeting YchP and TLR2 were used: Alexa Fluor 488 (excitation at 495 nm) for YchP and Alexa Fluor 594 (excitation at 590 nm) for TLR2. Each fluorophore was excited independently at its optimal excitation

wavelength, and emission signals were recorded accordingly. DAPI was excited at 358 nm to mark nuclei. The resulting images were processed and merged using appropriate imaging software to assess spatial overlap. In **Figure 4.6**, merged fluorescence images demonstrate significant co-localisation of Alexa Fluor 488 and Alexa Fluor 594 signals, indicating a potential interaction or close spatial proximity between YchP and TLR2 on the cell membrane.

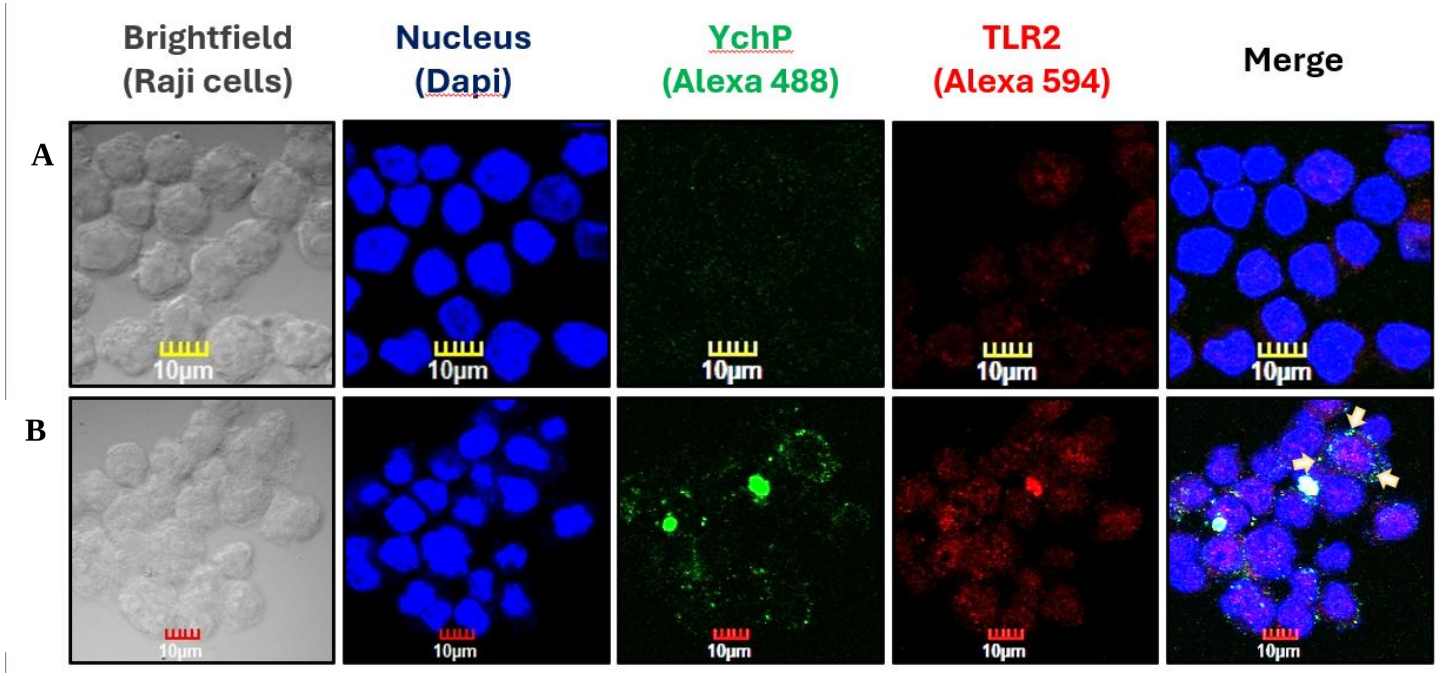


**Figure 4.6:** Co-localisation study of YchP and TLR2. A) Raji human B-cells (Tris buffer-control), and B) YchP-stimulated Raji human B-cells for 24 hours, stained with DAPI and treated with primary and secondary antibodies. Green fluorescence and red fluorescence represent the location of YchP and TLR2, respectively. Merged images were generated with arrows pointing to the site of high concentrations of overlap of fluorescence.

## 4.7. AID regulation.

Mature B-cells spontaneously express high AID levels upon being stimulated with the foreign antigen. Further, AID leads to the production of higher-affinity antibodies against the antigen through Class-switching and Somatic hypermutation. Upon stimulation with the different concentrations of YchP protein and at various time intervals, we found a transient increase in the AID levels at 4 h, followed by a decreasing trend in the levels observed from the western blot in **Figures 4.7** and **4.8**.

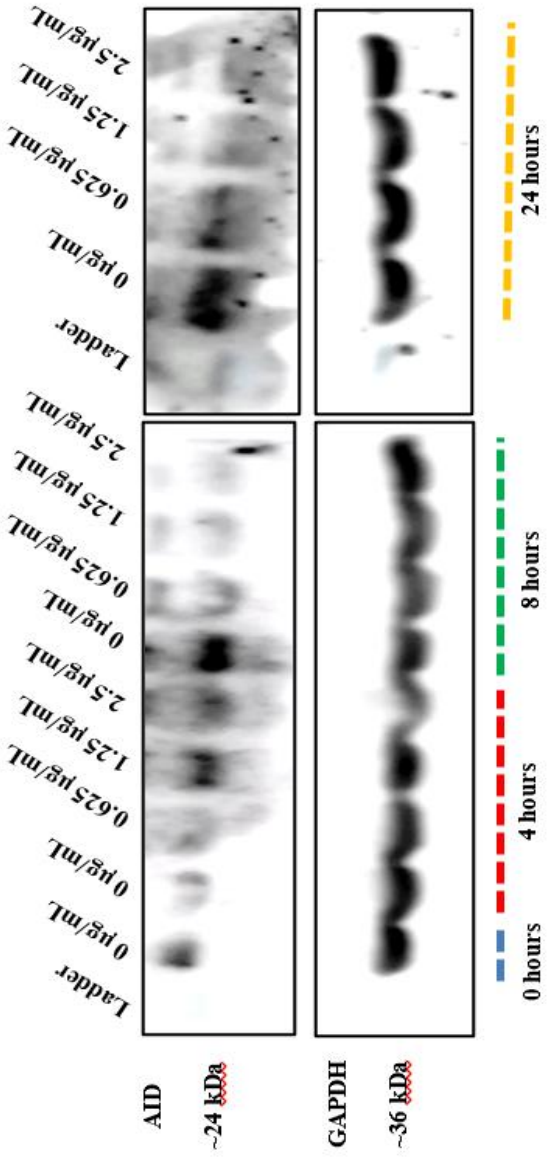


**Figure 4.7:** Analysis of Western blot of the extracted protein samples from the Raji human B-cells, checked for the expression of AID protein and used GAPDH as control.

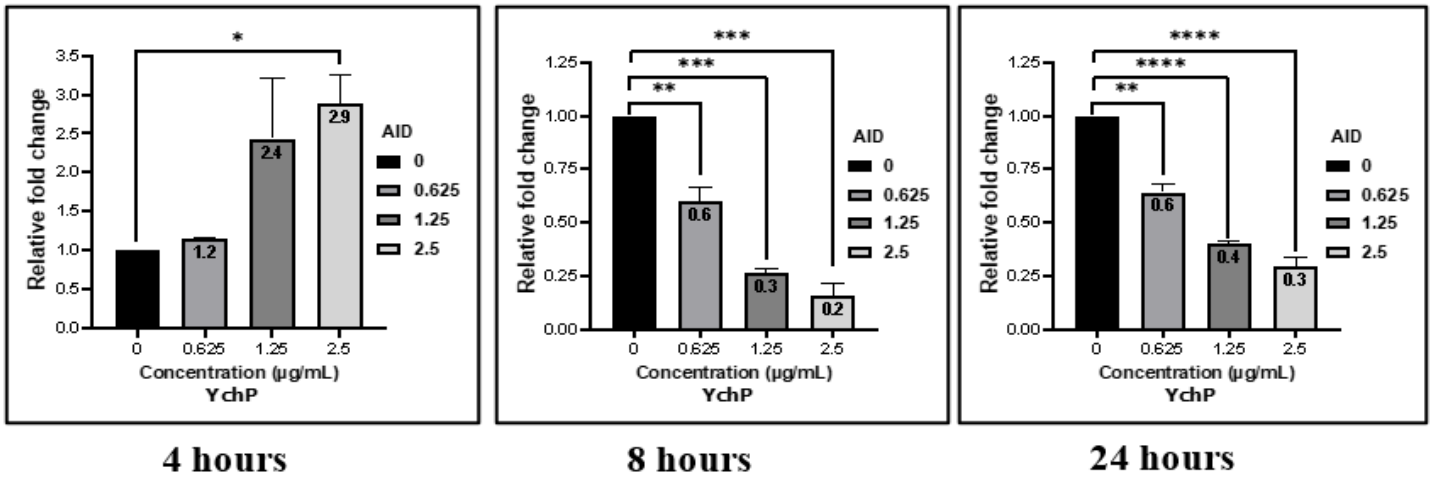


**Figure 4.8:** Graphical representations of intensities from the western blot to assess the change in the fold of the AID protein upon stimulation with the purified protein, YchP.

### 4.8. AID transcription factor regulation.

AID is strongly regulated in the cell at the transcriptional, post-transcriptional, and post-translational levels since it can cause off-target mutations leading to cancer development and changes in expression patterns. There are several positive AID regulators, including NF-κB, PAX5, c-MYC, SMAD3, and STAT6, and several negative regulators, including c-MYB and E2F1. Here, we treated the cells with varying concentrations of YchP protein and extracted the protein at different stimulation time intervals of 0, 4, 8, and 24 h. We also examined the protein levels of some of these transcription factors, PAX5 and c-MYC, to infer their contribution to the AID expression.

Protein analysis of PAX5 and c-MYC was observed to increase initially with increasing concentration till 4 h and then decrease drastically upon the increase of time of stimulation in **Figures 4.10** and **4.11**. Further study through qRT-PCR needs to be done to evaluate and confirm the change in the transcript level of transcription factors.

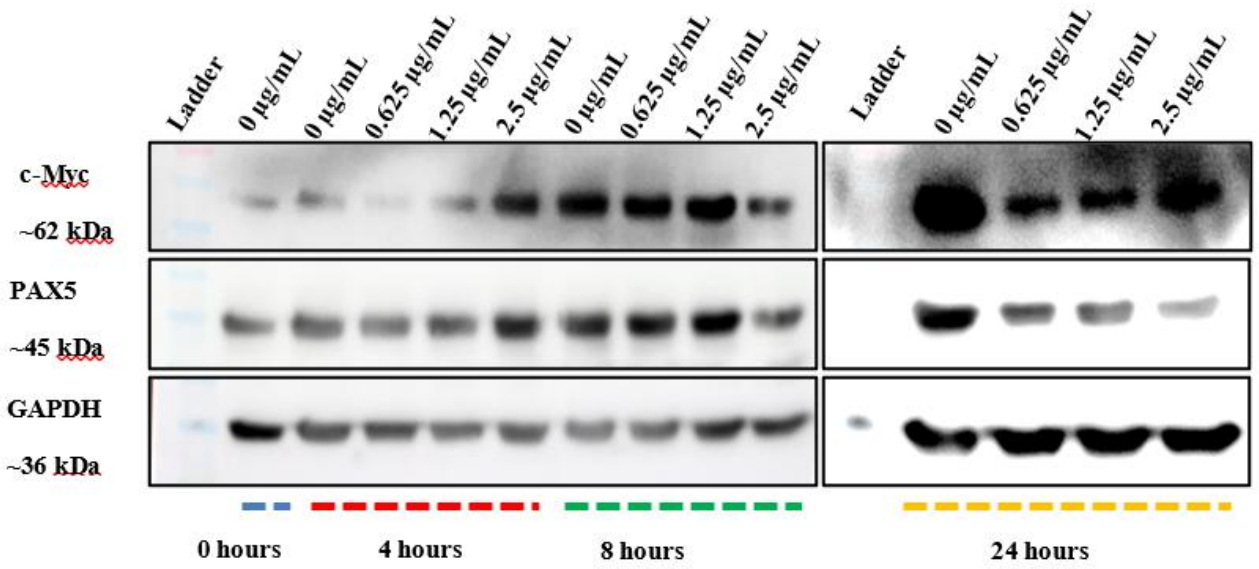


**Figure 4.9:** Analysis of Western blot of the extracted protein samples from the Raji human B-cells, checked for the expression of AID regulator proteins, including c-MYC, and PAX5, and used GAPDH as control.

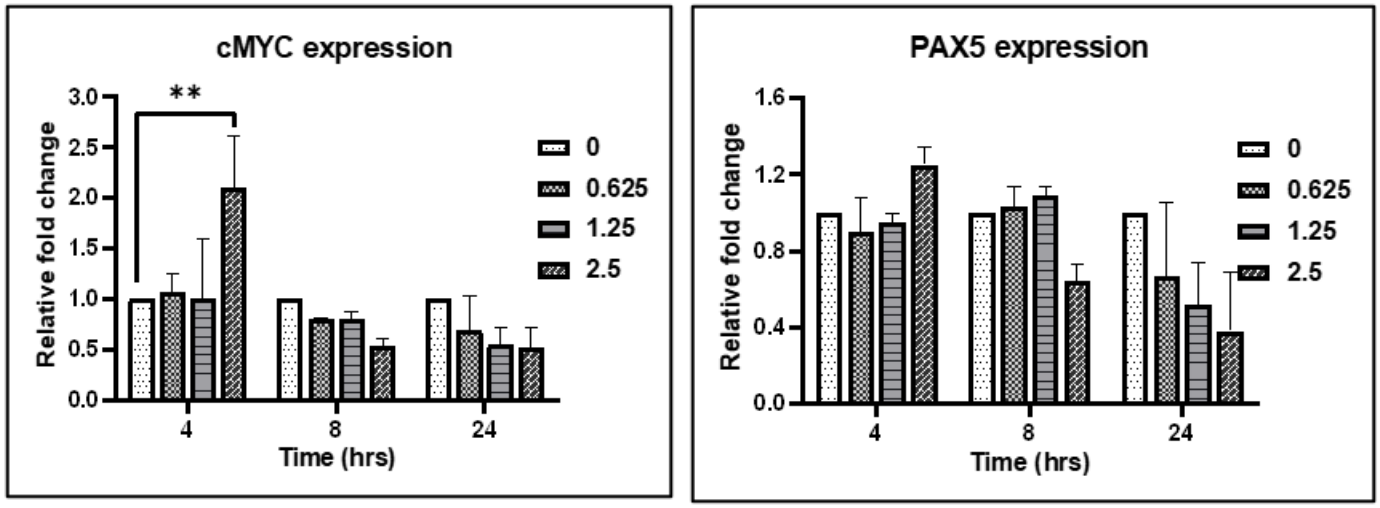


**Figure 4.10:** Graphical representation of intensities from the western blot to assess the change in fold of the AID regulator proteins (c-MYC and PAX5) upon stimulation with the purified protein, YchP.

# Chapter 5

# Discussion

*Salmonella* is one of the four global causes of diarrheal diseases, with a global burden of 510,000 cases and 62,000 deaths in 2021, as reported by the Institute for Health Metrics and Evaluation (IHME). In a study by Minelva R Nanton et al., showed that B-lymphocytes play a crucial role in fighting against the *Salmonella* infection [56]. Upon encountering an antigen, naïve B-cells mature and start producing antibodies. The mature B-cells undergo some genetic modifications, such as V(D)J recombination, SHM, and CSR, for the production of higher-affinity antibodies against the pathogens. These antigenic molecules responsible for this could be any of the MAMPs, including DNA, RNA, lipoproteins, LPS, flagellin, and toxins. Bacterial OMPs are a family of highly conserved proteins. They are responsible for various roles, such as structural integrity, pathogenesis, adhesion, signaling, host-pathogen interactions, immune evasion, and transport of molecules, including antimicrobial peptides and nutrients. Many OMPs have been studied for their antigenicity and role in immune system activation in the hopes of generating potential vaccines against the pathogens. Certain OMPs have been shown to enhance the expression of AID protein in B-cells, making them potential candidates for vaccine development. For example, OmpA has been studied for its ability to induce AID expression. In contrast, some OMPs provide immune evasion strategies for bacteria; specifically, *H. pylori*'s HomA and HomB have been found to repress AID expression. [57, 58]. A study on OmpA of *Shigella flexneri* 2a shows a positive impact on the expression of antibodies and T-helper cell 1 (Th1) immune response in mouse models, suggesting a potential role in vaccine development [59].

Similar to other studies, we were curious to understand the impact of YchP, a putative invasin protein expressed on the surface of *Salmonella*, on the immune system. YchP is present on the surface of the bacteria,

allowing for the interaction with the host and promoting internalization by the host cell. Here, we expressed and purified the YchP protein from the recombinant *E. coli*. Stimulated Raji human B-cells with the purified fraction of the protein and observed for the alternate expression of the AID transcript and translational product. Raji human B-cells are derived from a patient with Burkitt lymphoma. Raji cells have been used in cancer research, anti-cancer drug development, and investigating the immune response. Raji B-cells originated from the germinal center B cells and exhibit high recombination activity, closely mimicking the natural mechanisms of antibody diversification in human B cells.

Initially, we also performed a co-localization assay to show the interaction of YchP with TLR2 receptors present on the surface of B-cells. Upon stimulation of Raji human B-cells, at protein levels, AID showed an overall decreasing trend in expression as the time and concentration of stimulated protein were increased.

AID is regulated by several transcription factors, such as NFκB, SMAD3, cMYC, and STAT6. Furthermore, we analysed the expression of proteins of known activators of AID, including PAX5 and c-MYC.The expression profile for c-MYC and PAX5 was observed through western blot, which showed a decrease in the expression of both proteins. Further study is needed to evaluate and justify the data received from the qRT-PCR.

In summary, YchP of *Salmonella* present on the outer surface of the bacteria interacts with the TLR2 receptor and modulates its downstream cell signaling. We reported that YchP protein allows *Salmonella* with a potential to evade the immune system by decreasing the expression of AID protein, leading to reduced antibody diversity as SHM and CSR are dependent on the production of AID.

# Chapter 6

# Conclusion and future work

*Salmonella enterica* Typhimurium is one of the main causative agents responsible for causing diarrhoeal diseases. In this work, we have successfully expressed and purified the YchP, a putative invasin OMP of *Salmonella,* which allows for the interaction of the bacteria with the host cells. Invasin proteins allow the bacteria to adhere to the host cells and induce internalization. We stimulated Raji human B-cells with the purified antigen and observed the AID protein expression. AID protein is responsible for producing higher-affinity antibodies by inducing mutations at the genomic level at specific sites.

We observed a decrease in AID expression through western blot with increasing antigen (YchP) concentration and increasing periods. With the decreasing trend in AID, we also observed the expression of two of the transcription activators of AID, c-MYC and PAX5. Both c-MYC and PAX5 followed a similar trend of decreasing expression with increasing concentration and time. From the received data, we can conclude that YchP enables the bacteria to invade the immune system and replicate inside the host. Since YchP is responsible for immune evasion, we can potentially develop monoclonal antibodies or different non-toxic materials to neutralize the antigen to prevent *Salmonella enterica* Typhimurium from infecting and causing disease.

Our data is limited to the translational level of AID and some of the transcription factors. Further research at the transcript level through qRT-PCR of the AID and transcription factors could further elucidate details on the effects of the YchP protein on the host cells.

# APPENDIX-A

>ENA|ACY88601|ACY88601.1 *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. 14028S hypothetical protein

ATGTCATTCCTCTTATCTTTTGTTGTAGAAGTTGCCGACACGTTGAGCCGTATTGTTTTT
CGTTCATTCTCACTCTCGCTTCTCTTGCTGGCTGCCAGCGGTACGATCCGTGCGCAGGCG
CAAGATCCTTTCACGCAAAACCGTCTGCCGGATTTAGGCATGATGCCGGAATCCCACGAA
GGGGAAAAGCATTTTGCCGAGATGGCCAAAGCGTTTGGCGAAGCCAGCATGAAAAATAAC
GATCTGGATACTGGTGAACAGGCGCGGCAATTCGCTTTCGGACAGGTACGCGATGTGGTC
AGCGAGCAGGTTAACCAGCAGCTTGAAAGCTGGCTATCAGCCTGGGGCAGCGCCAGCGTG
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GGACTGGTAAGCAACATTGGCGTAGGACAGCGCTGGGCGCAGGATGGCTGGCTTCTTGGC
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CAGGTGCGCAGCGTGCACGGTATTCGTCATTTGAGCTGGCAGGGCGATACACAGGCATTA
AGTTTGACGGCAGGAACGGACACCCGCAGTACCGAGGGCTGGACAATCATTATGCCGGCC
TGGGATCACCGCGAAGGCGCGGCAAATCGCTGGCGTTTATCGGTGGTGGTTGAGGATGAA
AAAGGTCAGCGTGTCTCTTCCAATGAGATCACGCTCGCGCTGACTGAACCGTTTATCACG
ATGCCGGACGATAATCCGCATTGGCAACCGTTCCAGGAGCAATAA

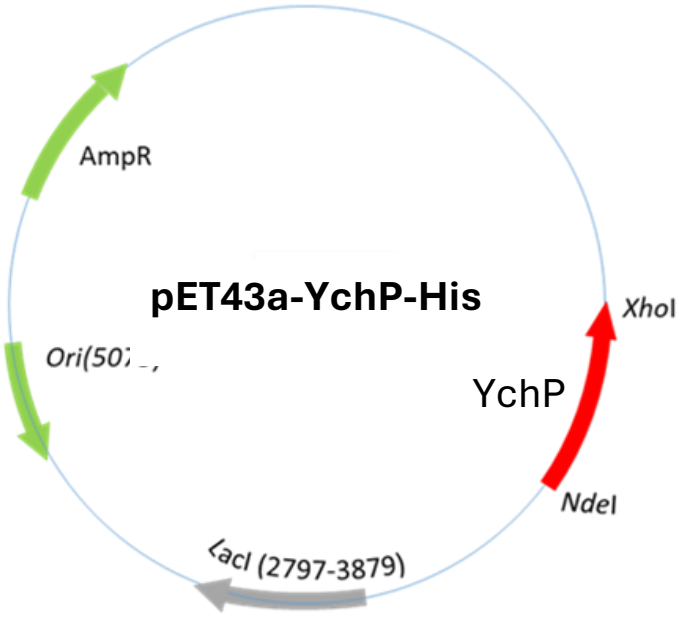
# APPENDIX-B

>ACY88601.1 hypothetical protein STM14_2138 [*Salmonella enterica* subsp. *enterica* serovar Typhimurium str. 14028S]

MSFLLSFVVEVADTLSRIVFRSFSLSLLLLAASGTIRAQAQDPFTQNRLPDLGMMPESHE
GEKHFAEMAKAFGEASMKNNDLDTGEQARQFAFGQVRDVVSEQVNQQLESWLSAWGSASV
DINVDNEGHFNGSRGSWFIPLQDKQRYLTWSQLGLTQQTDGLVSNIGVGQRWAQDGWLLG
YNTFYDNLLDENLQRAGFGAEAWGEYLRLSANYYQPFADWQTHTATLEQRMARGYDINAQ
MRLPFYQHINTSVSLEQYFGDSVDLFDSGTGYHNPVALKLGLNYTPVPLLTMTAQHKQGE
SGVSQNNLGLTLNYRFGVPLKKQLAASEVAQSQSLRGSRYDTPQRNSLPTMEYRQRKTLT
VFLATPPWDLTPGETVALKLQVRSVHGIRHLSWQGDTQALSLTAGTDTRSTEGWTIIMPA
WDHREGAANRWRLSVVVEDEKGQRVSSNEITLALTEPFITMPDDNPHWQPFQEQ

# APPENDIX-C

Schematic vector map of pET43a-YchP-His



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