IMMUNOMODULATORY EFFECT OF *Christia vespertilionis* LEAF EXTRACT (CVLE) ON B CELL ACTIVATING FACTOR (BAFF) - MEDIATED MONOCYTE PROLIFERATION

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AMDI	Advanced Medical & Dental Institute
SD	Standard deviation
BAFF	B cell activating factor
APRIL	A proliferation inducing ligand
BAFF-R	B cell activating factor receptor
TACI	Transmembrane Activator and CAML Interactor
BCMA	B-cell maturation antigen
MZ B cell	Marginal zone B cell
FO B cell	Follicular B cell
TNF	Tumour Necrosis Factor
TNFSF	TNF Superfamily
SLE	Systemic lupus erythematosus
RA	Rheumatoid arthritis
pSS	Primary Sjogren's syndrome
MM	Multiple myeloma
CV	Christia vespertilionis
CVLE	Christia vespertilionis leaf extract
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate (PtdIns (3,4,5) P3)
Akt	Protein kinase B
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
TNF-α	Tumour necrosis factor-alpha
IFN-γ	Interferon-gamma
IL	Interleukin
Ig	Immunoglobulin
RF	Rheumatoid factors
PBMC	Peripheral blood mononuclear cells
DC	Dendritic cell
BCR	B cell receptor
TEMED	Tetramethylethylenediamine

LIST OF APPENDICES

KESAN IMUNOMODULASI EKSTRAK *Christia vespertilionis* (CVLE) TERHADAP PERCAMBAHAN SEL MONOSIT AKIBAT FAKTOR PENGAKTIFAN SEL B (BAFF)

ABSTRAK

Ekspresi aberan faktor pengaktifan sel B telah dikait rapat dengan pelbagai gangguan imunologi, terutamanya penyakit autoimun dan kanser limfoid. Antara pelbagai gangguan imunologi, tahap BAFF yang tinggi telah dilaporkan secara konsisten dalam kalangan pesakit lupus eritematosus sistemik (SLE), menjadikannya calon yang sesuai untuk antagonisme terapeutik. Walaupun terdapat beberapa perubatan sintetik yang menyasarkan BAFF, rawatan tersebut memberi kesan sampingan buruk kepada sebilangan besar pesakit. Oleh itu, pendekatan alternatif menggunakan produk semula jadi dari tumbuhan tradisional mejadi fokus utama bagi para penyelidik pada masa ini. *Christia vespertilionis* (CV) atau lebih dikenali sebagai Daun Rerama, merupakan salah satu tumbuhan tradisional yang mempunyai pelbagai sifat perubatan, namun kesan imunomodulasinya masih belum dilaporkan. Oleh hal yang demikian, kajian ini dijalankan untuk menilai kesan imunomodulasi ekstrak daun CV berair (CVLE) terhadap percambahan sel monosit, khususnya melalui pengawalan mekanisme BAFF. Assai kebolehlangsungan sel menggunakan CCK-8 telah dijalankan untuk menentukan kepekatan CVLE yang tidak toksik kepada sel normal dan sel imun. Kesan percambahan sel telah dinilai untuk 12, 24, 48 dan 72 jam pada sel monosit THP-1. Kesan imunomodulasi melalui pengawalan mekanisme berkaitan dengan BAFF telah ditentukan menggunakan analisis western blot, manakala ELISA telah digunakan untuk menentukan rembesan sitokin pro-radang (TNF-α dan IFN-γ) oleh monosit THP-1 yang telah dirangsang menggunakan BAFF. Hasil daripada kajian

semasa menunjukkan bahawa kepekatan CVLE melebihi 1 mg/ml telah memberi kesan toksik kepada sel normal NIH/3T3-L1. Seterusnya, esktrak Daun Rerama (1 mg/ml) telah mengurangkan percambahan sel monosit THP-1 yang dirangsang menggunakan BAFF pada 72 jam, berkemungkinan melalui pengawalan mekanisme BAFF/PI3K, Ekspresi protein subunit pengawal selia PI3K p85α menurun dalam sel monosit yang dirangsang menggunakan BAFF. Selain itu, CVLE juga telah mengurangkan rembesan pro-radang (TNF-α dan IFN-γ) oleh monosit THP-1 yang dirangsang menggunakan BAFF. Secara keseluruhannya, CVLE menunjukkan potensi sifat imunomodulasi dengan mengurangkan percambahan sel monosit, berkemungkinan melalui mekanisme BAFF/PI3K/Akt dan rembesan sitokin pro-radang, TNF-α dan IFN-γ. Hasil daripada kajian ini menunjukkan bahawa CVLE mempunyai potensi sebagai perubatan terapeutik semula jadi bagi rawatan penyakit yang berkaitan dengan BAFF, terutamanya penyakit lupus eritematosus sistemik.

IMMUNOMODULATORY EFFECT OF *Christia vespertilionis* LEAF EXTRACT (CVLE) ON B CELL ACTIVATING FACTOR (BAFF) - MEDIATED MONOCYTE PROLIFERATION

ABSTRACT

Aberrant expression of B cell activating factor (BAFF) has been strongly implicated in a range of immunological disorders, particularly autoimmune diseases and lymphoid malignancies. Among the various disorders influenced by BAFF dysregulation, systemic lupus erythematosus (SLE) is particularly notable, as elevated levels of BAFF have been consistently reported in patients with SLE, thereby positioning BAFF as a promising target for therapeutic intervention. While synthetic BAFF antagonists are currently used for treatment, a substantial percentage of patients do not respond well to these treatments, justifying the need for alternative approaches with natural products from traditional/medicinal plants. *Christia vespertilionis* (CV), commonly known as the butterfly wing plant demonstrated various medicinal properties, though its immunomodulatory effects remain largely unexplored. Therefore, this study evaluates the potential immunomodulatory effects of aqueous CV leaf extract (CVLE) on BAFF-mediated monocyte proliferation. Cell viability assay using CCK-8 was conducted to determine the non-toxic working concentrations of aqueous CVLE (0.125 – 1 mg/ml) on both normal and immune cell lines. The proliferative effects of aqueous CVLE were assessed on immune cells, namely, THP-1 monocytes at 12, 24, 48 and 72 hours. Western blotting analysis determined the impact of aqueous CVLE on BAFF-mediated signalling, while ELISA was used to determine the impact of CVLE on secretion of pro-inflammatory cytokine (TNF-α and IFN-γ) in THP-1 monocytes when stimulated with BAFF. In the present study,

aqueous CVLE concentrations > 1 mg/ml was observed to be toxic on normal NIH/3T3-L1 fibroblasts. Interestingly, treatment with 1 mg/ml of aqueous CVLE suppressed proliferation of BAFF-stimulated THP-1 monocytes. Next, BAFF-stimulated THP-1 monocytes showed decreased protein expression of the PI3K p85α regulatory subunit, potentially through downregulation of BAFF-induced PI3K/Akt signalling pathway. Treatment with 1 mg/ml of CVLE also was shown to reduce TNF-α and IFN-γ secretion in BAFF-stimulated THP-1 monocytes, suggesting its potential to regulate pro-inflammatory cytokine release, associated with immune dysregulation. In conclusion, CVLE exhibits potential immunomodulatory properties by reducing monocyte proliferation and pro-inflammatory cytokine secretion through BAFF-mediated signalling in THP-1 monocytes. These findings suggest that CVLE may be a potential natural therapeutic target for managing BAFF-related immunological disorders, especially SLE.

CHAPTER 1

INTRODUCTION

1.1 Research Background

The immune system is a sophisticated defence network comprised of cells, tissues, and organs that operate in concert to protect the body against infectious agents such as microbes or viruses, thereby maintaining immunity (Balasubramaniam et al., 2024). Immunity is achieved when the immune system recognises and tolerates substances that belong to the body or host (self) while rejecting non-self-substances, including foreign particles or pathogens (Balasubramaniam et al., 2024). Establishing tolerance through central and peripheral tolerance, subsequently achieving immunity with the aid of innate and adaptive immunity are crucial in the maintenance of balanced immune homeostasis (Balasubramaniam et al., 2024; Gremese et al., 2020). However, the loss of immunological tolerance due to malfunctions or imbalances in any of these systems often leads to chronic inflammation, which can contribute to the development of both autoimmunity and cancer, ultimately compromising immune function (Franks & Slansky, 2012; Zhao et al., 2021). The present study focuses primarily on pathogenesis of autoimmune disease, specifically, Systemic Lupus Erythematosus (SLE).

SLE is a prototypical autoimmune disease that affects multiple tissues and organs, including the skin, joints, kidneys, central nervous system, and cardiovascular system (Catalina et al., 2020). SLE is characterised by the production of pathogenic autoantibodies directed against nucleic acids and their binding proteins, reflecting a global loss of immune tolerance (Choi et al., 2012). The primary clinical manifestations observed in SLE patients include inflammation, immune complex deposition, vasculitis and vasculopathy (Mok & Lau, 2003). Lupus nephritis, a severe

consequence of SLE, is a significant driver of morbidity and mortality among SLE patients (Mohan & Putterman, 2015). According to a comprehensive systematic analysis of 112 studies representing 20% of countries worldwide, 43.7 per 100,000 individuals, i.e. approximately 3.41 million people, are affected by SLE globally, with 0.4 million new cases diagnosed annually (Blanco, 2024; Tian et al., 2023). In Malaysia, 69.05 per 100,000 individuals are affected by SLE, and the mortality risk remains three times greater than that of the general population, despite advancements in SLE management and treatment (Elsisi et al., 2024).

At the systemic level, aberrant activation of innate and adaptive immune system, characterised by release of pro- and anti-inflammatory cytokines, and production of pathogenic autoantibodies, particularly, B cells, contribute to tissue or end-organ injury in SLE patients, respectively (Choi et al., 2012; Mohan & Putterman, 2015). Cytokine networks play an important role in the pathogenesis of SLE, with B cell activating factor (BAFF) being one of the most dysregulated cytokines in this disease (Mok & Lau, 2003; Vincent et al., 2014). Also known as B lymphocyte stimulator (BLyS), BAFF belongs to the Tumour Necrosis Factor (TNF) superfamily of ligands and receptors (TNFSF). It is a survival factor essential for the maturation, differentiation and survival of B cells that takes place in the periphery (Balasubramaniam & Mokhtar, 2024; Mackay & Browning, 2002; Schneider et al., 1999). BAFF shares approximately 48% sequence homology and 33% amino acid identity with another TNFSF cytokine called a proliferation inducing ligand (APRIL) (Balasubramaniam & Mokhtar, 2024; Hahne et al., 1998). BAFF is primarily synthesised as a type II transmembrane protein but can be cleaved by furin protease to form a soluble homotrimer (BAFF trimer or BAFF 3-mer), which can self-assemble into multimers of 20 trimers (BAFF 60-mer or BAFF multimer) (Balasubramaniam &

Mokhtar, 2024; Liu & Davidson, 2011b). BAFF binds to three receptors, namely, B cell maturation antigen (BCMA/TNFRSF17), calcium modulator and cyclophilin ligand interactor (TACI/TNFRSF13B) and BAFF receptor (BAFF-R/BR3/TNFRSF13C), which are expressed at different stages of B cell maturation and differentiation (Balasubramaniam & Mokhtar, 2024; Liu & Davidson, 2011b). Initially, BAFF and its receptors were thought to be expressed or secreted by haematopoietic cells, including monocytes, macrophages, neutrophils and dendritic cells (DCs), which are major producers of soluble BAFF in the immune system. However, further studies revealed that non-haematopoietic cells such as adipocytes, keratinocytes, neurites, osteoclasts, and podocytes also express or secrete BAFF and its receptors. These cells are significantly involved in inflammatory responses and disease manifestations, suggesting a broader role for BAFF beyond haematopoietic system (Balasubramaniam & Mokhtar, 2024; Mackay et al., 2003; Mackay & Browning, 2002; MacKay & Schneider, 2009).

Despite binding to three receptors, binding of BAFF to BAFF-R exhibits the highest specificity and affinity. In fact, studies using *in vitro* cell lines and *in vivo* murine models, where BAFF or BAFF-R is overexpressed or absent, demonstrated that BAFF/BAFF-R axis is solely responsible for peripheral B cell development, especially, beyond the transitional 1 (T1) stage. Dysregulation in BAFF/BAFF-R signalling in murine models leads to lupus-like symptoms, underscoring its critical role in immune homeostasis and its link to immunological disorders (Balasubramaniam & Mokhtar, 2024; Batten et al., 2000; Groom et al., 2002). Under normal physiological conditions, BAFF levels are tightly regulated, providing just enough support for B cells to survive many weeks. B cells in a diverse repertoire receive minimal death signals through their B cell receptor (BCR), meaning they are not strongly activated

by the antigens and exhibit a low dependence on BAFF for their maintenance (Kalled, 2005). Competition between healthy and autoreactive B cells with limited BAFF ensures that only non- or weakly autoreactive B cells survive, preventing the persistence of harmful self-reactive B cells (Kalled, 2005; MacKay & Schneider, 2009). However, in autoimmune diseases like SLE and B cell malignancies such as non-Hodgkin's lymphoma (NHL), aberrant BAFF expression disrupts this balance. Increased BAFF availability per cell allows strongly autoreactive B cells with a higher dependence on BAFF to counteract BCR-induced death signals subsequently enabling them to escape deletion (Kalled, 2005; Lesley et al., 2004; Thien et al., 2004). Consequently, these self-reactive B cells survive and proliferate, breaking immune tolerance and driving autoimmunity (Kalled, 2005; MacKay & Schneider, 2009). Additionally, BAFF supports the uncontrolled growth of malignant B cells, contributing to cancer development (Mackay et al., 2003). Therefore, targeting BAFF through immunotherapy is a promising strategy for managing both autoimmune disease and B cell cancers (Liu & Davidson, 2011b).

Besides B cells, monocytes are also active contributors to SLE pathogenesis, through production and regulation of BAFF (Li et al., 2010; Vincent et al., 2014). As mentioned previously, monocytes are among the major sources of soluble BAFF and express all three BAFF system receptors (BCMA, TACI and BAFF-R) (Chang et al., 2006; Kampa et al., 2020; Vincent et al., 2014). Elevated BAFF levels not only promote B cell survival and differentiation but also monocyte activation by triggering inflammatory and survival pathways and driving pro-inflammatory cytokine secretion (Chang et al., 2006). In SLE, circulating monocytes showed increased BAFF expression which is further upregulated upon stimulation by interferons, particularly IFN-α/β/γ (Kerfoot et al., 2008; Nikoleri et al., 2024; Vincent et al., 2014),

highlighting the interplay between innate immune activation and BAFF regulation. In murine models of lupus, monocyte-derived BAFF, along with BAFF derived from other innate immune cells, plays a pivotal role in driving B cell maturation and survival (Kerfoot et al., 2008). These findings suggest that monocyte-derived BAFF amplifies autoimmune responses in SLE by reinforcing B cell activation, while inflammatory cytokines further augment BAFF production in monocytes, creating a self-perpetuating cycle of immune dysregulation and chronic inflammation (Giordano et al., 2023). Although there is growing evidence supporting the role of BAFF in monocytes, these studies remain limited, and deeper mechanistic research is needed to fully elucidate the role of monocytes in BAFF-mediated signalling in SLE.

Current treatment options available for SLE include corticosteroids, immunosuppressive agents, antimalarial drugs, non-steroidal anti-inflammatory drugs (NSAIDs), systemic lymph node irradiation therapy, and plasma treatment (Pasdaran et al., 2023). More recently, biologics targeting B cells (B-cell immunomodulating therapies) through BAFF/BAFF-R blockade have emerged as promising immunomodulatory drug candidates for SLE treatment (Durcan & Petri, 2016; La Cava, 2010). These biologics, including belimumab, blisibimod, tabalumab, BAFF-Trap, ianalumab, and briobacept, target the BAFF/BAFF-R axis due to its critical role in autoimmune disease pathophysiology (Balasubramaniam & Mokhtar, 2024). Among these, belimumab, is the only biologic currently licensed for lupus and has shown particular efficacy in SLE patients with active disease and higher serological activity (Basta et al., 2020). However, the outcomes of B cell targeted therapies through BAFF/BAFF-R blockade have been mixed, showing both promise and limitations in clinical trials. Nevertheless, immunosuppressives agents like mycophenolate mofetil, azathioprine and methotrexate remain critical for managing

organ specific disease manifestations, but their efficacies are limited, particularly in renal disease (Durcan & Petri, 2016). Also, the complexity and heterogeneity of SLE contribute to suboptimal responses to standard therapies in a substantial proportion of patients, leading to persistent inflammation, organ damage, and functional deterioration, subsequently affecting the quality of life of SLE patients (La Cava, 2010; Mok, 2017). Given these challenges, it is crucial to adopt a holistic approach to SLE drug development. Thus, exploring natural compounds derived from traditional/medicinal plants as immunomodulators offers a potential pathway to creating safer and more efficient therapies with less severe adverse effects for immunological disorders including autoimmunity and cancer (Balkrishna et al., 2020).

Herbal treatments have been in use for millennia to cure a variety of ailments, however, there is still an ongoing debate on the origins of plant-based medications with pre-historical evidence documenting the use of plants for medicinal purposes as early as 60,000 years ago (Giannenas et al., 2020; I. Su, 2019). According to World Health Organisation (WHO), a medicinal plant is defined as a plant, which, in one or more of its organs contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis (Karunamoorthi et al., 2012; WHO, 2000; WHO, 2013). The WHO has accounted about 60% of the world's population relying on traditional medicine while 80% of the population in the developing countries depend almost entirely on traditional medical practices, in particular herbal remedies, for their primary healthcare. This is because the use of plants for therapeutic purposes is more preferable since plant materials are more affordable and accessible than synthetic drugs in these countries. At the moment, approximately 35,000 – 70,000 plant species are used worldwide for medicinal purposes, with approximately 6500 and 7000 plants identified in Southeast and South

Asian countries, respectively (Dapar et al., 2020). Besides, medicinal/traditional plants have anti-asthmatic, anti-arrhythmic, anti-inflammatory, hepatoprotective, hypercholesteraemic, antifungal, cardiotonic, diuretic and other medicinal properties. They also have an antagonistic effect on oxidative stressors, decreasing the formation of various free radicals which are associated with many diseases. These properties are contributed by the presence of phytochemicals or bioactive compounds that are proven to regulate the cellular immune system. Collectively, immunomodulators from traditional/medicinal plants or herbs, boosts physical and chemical health, strengthens the body's defensive mechanism and subsequently increases longevity (Balasubramaniam et al., 2024).

One example of traditional plant known for its medicinal properties is *Christia vespertilionis* (CV). CV is an herbaceous plant found in most Southeast Asian countries and Brazil (Murugesu et al., 2020). The plant is found abundantly and is locally known as "Daun Rama-rama" (Malay) in Malaysia or butterfly wing plant (English) due to its unique trifoliate leaf shape. It has been traditionally used to treat snake bites, tuberculosis, bronchitis, inflamed tonsils, colds and muscle weaknesses. Moreover, it also improves poor blood circulation and promotes healing in respiratory and skin related disorders (Ibrahim et al., 2022; Murugesu et al., 2020). Topical applications of crushed CV leaves on affected body parts was used to treat bone fractures and scabies. Anti-cancer (Lee et al., 2020; Yasin et al., 2020), anti-plasmodial (Nguyen-Pouplin et al., 2007), antimalarial (Nguyen-Pouplin et al., 2007; Upadhyay et al., 2013; Yasin et al., 2020), anti-proliferative (Hofer et al., 2013), antioxidant (Murugesu et al., 2020) and anti-inflammatory properties of CV was observed due to the presence of various phytocompounds such as polyphenols, alkaloids, and terpenoids.

1.2 Problem statement of the proposed study

Despite a long history of use in traditional medicine and growing evidence of its diverse pharmacological properties, little is known about the potential role of aqueous CV leaf extract (CVLE) in modulating immune function, particularly in autoimmune diseases. Although recent studies have examined the anti-cancer properties of CV, particularly its role in inducing apoptosis (Hofer et al., 2013), yet the immunomodulatory impact of CVLE on BAFF-mediated signalling has not been investigated. BAFF is a critical survival factor that not only drives monocytes activation but also B cell persistence and contributes to autoimmunity (Chang et al., 2006; Vincent et al., 2014). This study therefore aims to determine the effects of CVLE on BAFF-mediated signalling in monocytes and B cells, both of which play pivotal roles in SLE pathogenesis. The findings of this present study may offer novel insights into the therapeutic potential of CV, particularly in targeting BAFF and its receptors in autoimmune disorders.

1.3 Scope and delimitations

While this study was originally designed to investigate the immunomodulatory effects of aqueous CVLE on B cells, significant disruption during experimental phase affected the direction of research. The Ramos B cell line, which was intended as the primary model for BAFF-induced proliferation, encountered serious technical issues. As a result, the intended B cell experiments could not be completed using the full range of planned treatment conditions. The study focus was therefore redirected towards monocytes (THP-1), where BAFF-mediated PI3K/Akt signalling was further explored. Preliminary data from the initial Ramos cell trials were retained (Refer to Appendix) to provide exploratory insight but were interpreted with caution due to the

limitations in replicates and standardisation. Another challenge was the scarcity of literature specifically addressing the role of BAFF in monocytes within the context of SLE. While several studies have reported on BAFF biology in general, few have focused on its monocyte-specific functions in this disease, making direct comparison with existing findings difficult.

1.4 Research Objectives

The general objective of this study is to determine the effect of aqueous CVLE on BAFF-mediated monocyte proliferation and cytokine secretion. The specific objectives of this study were as follows:

1. To determine the effect of aqueous CVLE on the viability and BAFF-mediated proliferation of THP-1 monocytes as compared to NIH/3T3-L1 fibroblasts.
2. To identify the effect of CVLE on BAFF signalling in THP-1 monocytes.
3. To examine the effect of CVLE on BAFF-mediated pro-inflammatory cytokine secretion in THP-1 monocytes.

1.5 Hypothesis of the study

This study hypothesises that the aqueous CVLE exerts immunomodulatory effects on human monocytes by interfering with BAFF-mediated signalling pathways. Specifically, it is proposed that CVLE treatment will reduce the proliferation of THP-1 monocytes and attenuate pro-inflammatory cytokine secretion, specifically TNF-α and IFN-γ, potentially through the downregulation of PI3K/Akt signalling cascade. By targeting this pathway, CVLE may offer a natural therapeutic strategy for modulating immune responses in conditions characterised by aberrant BAFF expression.

CHAPTER 2

LITERATURE REVIEW

2.1 The association between inflammation, autoimmunity and cancer

2.1.1 Inflammation

Inflammation is an essential biological mechanism that protects the body from harmful or noxious stimuli such as injury, infections, pathogens, damaged cells, and toxic compounds (Chen et al., 2017). Although initially thought to be involved in host defence against pathogens, inflammatory mechanisms are equally important for repair, regeneration and restoration of tissue homeostasis (Greten & Grivennikov, 2019). Thus, the primary function of inflammation is to serve as a protective mechanism by rapidly destroying or isolating underlying source of disturbance, removing damaged tissue and then restoring disrupted tissue homeostasis (Ashley et al., 2012). Inflammation can be classified as acute or chronic inflammation, depending on the time and amplitude of the stimulus (Qu et al., 2018).

Acute inflammation is a transient response that typically involves the buildup of immune cells such as neutrophils at the site of injury or infection leading to redness, swelling, heat and pain. A successful acute inflammatory response is characterised by the elimination of infection agents followed by the resolution and repair phase, which is mediated mainly by tissue-resident and recruited macrophage (Medzhitov, 2008). However, when inflammation becomes chronic, it persists over a longer period of time, even in the absence of an ongoing infection or injury. The beginning of chronic inflammation is often characterised by the replacement of neutrophils or continuous recruitment of immune cells such as macrophages, and lymphocytes, which leads to tissue damage (Ashley et al., 2012; Nathan & Ding, 2010). Chronic inflammation is often driven by various factors, including infection, tissue injury, genetic

predispositions, autoimmune reactions and environmental triggers such as smoking, obesity and pollution (Furman et al., 2019). For instance, obesity is associated with a state of low-grade, chronic inflammation due to the release of pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α) and Interleukin (IL)-6, from adipose tissue, contributing to systemic inflammatory responses (Gregor & Hotamisligil, 2011).

At the molecular level, pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), play a significant role in recognising pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs) during inflammation. Once activated, PRR initiates a signalling cascade that leads to the production of pro-inflammatory cytokines like TNF-α, IL-1 and IL-6, which amplify the inflammatory response (Takeuchi & Akira, 2010). These cytokines recruit more immune cells to the site of infection or injury, perpetuating the inflammatory response.

2.1.2 Inflammation leading to autoimmunity

While inflammation is essential for defence and repair, chronic and dysregulated inflammation can disrupt immune tolerance and lead to autoimmunity. Autoimmunity arises when the immune system mistakenly recognises self-antigens as foreign and mounts immune response against the body's own tissues (Romagnani, 2006).

Both genetic predispositions, including human leukocyte antigen (HLA) variants, gene polymorphisms, and mutations in immune regulatory genes, and environmental factors such as infections, smoking, hormonal influences, toxic chemical exposure and chronic stress contribute to onset of autoimmunity. Once triggered, chronic inflammation perpetuates the autoimmune response, leading to tissue damage (Rosenblum et al., 2015; Yasmeen et al., 2024). This persistent inflammation, driven by both innate and adaptive immune systems, promotes the survival and activation of

autoreactive immune cells through release of inflammatory cytokines, ultimately breaking tolerance and resulting sustained autoimmunity (Anka et al., 2022; Rider et al., 2016; Yasmeen et al., 2024).

Inflammatory autoimmune conditions such as SLE, rheumatoid arthritis (RA), and multiple sclerosis, are driven by chronic inflammation that failed to resolve which involve both innate and adaptive immune cells. For instance, in RA, activated macrophages and DCs produce high levels of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, which drive the inflammatory response (Firestein, 2003). This pro-inflammatory environment also activates autoreactive B cells, producing autoantibodies that form immune complexes and contribute to tissue damage (Liu & Davidson, 2011a).

B cells play a central role in autoimmunity by producing autoantibodies and presenting antigens to T cells, which further drives the autoimmune response. Chronic inflammation contributes to dysregulated B cell survival by allowing autoreactive B cells to survive instead of being eliminated. Inflammatory cytokines including BAFF further support their persistence, overriding central and peripheral immune tolerance mechanisms such as clonal deletion, anergy and receptor editing (Bonasia et al., 2021; Liu & Davidson, 2011a; Vincent et al., 2014). This continuous activation of B cells in these conditions not only sustains the autoimmune response but also contributes to a pro-inflammatory environment that may set the stage for further complications, such as cancer.

2.2 SLE disease progression

2.2.1 Interaction between monocytes and B cells in SLE disease pathogenesis

SLE is characterised by dysregulated immune responses involving both innate and adaptive immunity. Monocytes, as part of the innate immune system, contribute to SLE progression through cytokine secretion, antigen presentation, and direct stimulation of B cells, thereby facilitating the breakdown of self-tolerance (Dörner & Lipsky, 2016).

Monocytes are heterogenous population, with distinct subsets contributing differently to host defense and inflammation. In humans, they are classified into three subsets based on CD14 (LPS co-receptor) and CD16 [Fragment crystallizable γ receptor III (FcγRIII)] expression, namely, $CD14^{++}CD16^{-}$ classical, $CD14^{++}CD16^{+}$ intermediate and $CD14^{low}CD16^{++}$ non-classical monocytes (Hirose et al., 2019). The classical subset, which corresponds to $Ly6C^{hi}$ monocytes in mice, plays a central role in acute inflammation by migrating to inflamed tissues and differentiating into monocyte-derived dendritic cells and macrophages. Non-classical monocytes, equivalent to $Ly6C^{lo}$ cells in mice, instead patrol the vascular endothelium and facilitate tissue repair, partly by recruiting neutrophils when necessary (Nomura et al., 2022).

Notably, monocytes in SLE exhibit an aberrant activation patterns, often displaying increased expression of TLRs, particularly TLR7 and TLR9, which recognise nucleic acids from self-antigens (Lövgren et al., 2004). Upon activation, monocytes produce pro-inflammatory cytokines such as IFN-α, IFN-β, IFN-γ, IL-6 and BAFF (Giltiay et al., 2012). These changes not only perpetuate systemic inflammation but also modulate T and B cell responses, creating a feed-forward loop of immune activation. In lupus nephritis, specific subsets of monocytes such as the patrolling or the non-classical monocytes have been shown to infiltrate the kidney early in disease,

mediating tissue damage independently of circulating autoantibodies (Kuriakose et al., 2019).

Additionally, monocytes contribute to B cell activation through antigen presentation. In SLE, defective clearance of apoptotic cells leads to the accumulation of nuclear autoantigens, which are engulfed by monocytes and presented to B cells via major histocompatibility complex (MHC) class II molecules. These interactions not only stimulate B cell proliferation but also drive the differentiation of plasma cells that secrete pathogenic autoantibodies, such as anti-dsDNA antibodies (Muñoz et al., 2010). Furthermore, monocyte-derived DCs can further amplify B cell responses by providing costimulatory signals through CD80/CD86 and secreting cytokines that skew B cells toward an inflammatory phenotype (Lee et al., 2020).

2.2.2 The role of cytokines in SLE pathogenesis

As mentioned in the previous section, monocytes play diverse roles, including phagocytosis, antigen presentation and cytokine secretion (Hirose et al., 2019). Notably, dysregulated cytokine production by monocytes promotes abnormal activation of autoreactive B and T cells, positioning cytokines as key mediators linking innate and adaptive immunity. This imbalance not only signals ongoing immune activation but also contributes to tissue damage, autoantibody production and establishment of pro-inflammatory environment, ultimately driving chronic progression of autoimmune diseases such as SLE.

Genetic and transcriptional studies highlight upregulated IRF family members, especially interferon regulatory factors (IRF)-5 and IRF3, driving increased basal production of key inflammatory cytokines such as IFN-α, TNF-α and IL-6 (Byrne et al., 2012). Furthermore, a more recent scRNA-seq study confirmed elevated monocyte counts in the peripheral blood of SLE patients, with the overexpression of IRF1 and a

surge of pro-inflammatory mediators including IFN-γ, IL-1β, IL-6 and IL-8 (Wang et al., 2025). Nevertheless, the present study emphasises the role of pro-inflammatory cytokines, specifically TNF-α and IFN-γ, as these cytokines are not only highly relevant to SLE pathogenesis but also have the potential to induce BAFF expression (Chang et al., 2006; Hodge et al., 2014). Both TNF-α and IFN-γ possess immunoregulatory and pro-inflammatory functions across a range of cell types (Postal & Appenzeller, 2011; J. Zhang, 2007); however, in SLE patients, their activity skews predominantly toward a pro-inflammatory phenotype (W. Liu et al., 2022; Zhu et al., 2010).

In Medical Research Laboratory lymphoproliferation mice (MRL/*lpr*) lupus mice, increased TNF expression in both the serum and kidney tissue is linked to greater severity of inflammatory organ damage. The elevated TNF-α is associated specifically to renal disease (Postal & Appenzeller, 2011; Yokoyama et al., 1995). Conversely, reduced TNF-α production in New Zealand White (NZB/W) mice has been linked to the development of severe manifestations, such as nephritis, indicating that TNF-α deficiency is a key contributor to lupus-like autoimmunity in this strain (Postal & Appenzeller, 2011). In human SLE, by contrast, high TNF-α levels consistently correlate with increased disease activity and organ involvement, including lupus nephritis (Aringer & Smolen, 2005; Ghorbaninezhad et al., 2022).

IFN-γ, although classically associated with T helper 1 (Th1) cells, is also produced by activated monocytes and NK cells in SLE. It primes antigen-presenting cells, upregulates MHC class II expression, and promotes a pro-inflammatory feedback loop that sustains autoreactive T- and B-cell responses (Pollard et al., 2013; Theofilopoulos et al., 2001). IFN-γ also influences germinal center dynamics, fostering the differentiation of autoreactive B cells into plasma cells and amplifying autoantibody production (Lee et al., 2019).

Monocyte-derived cytokines such as TNF-α and IFN-γ not only drive inflammatory responses and activate autoreactive lymphocytes in SLE but also enhance BAFF expression, fostering B-cell survival and autoantibody production. Given BAFF's pivotal role in bridging innate and adaptive immune dysregulation, the following section examines its biology, regulation, and contribution to SLE pathogenesis.

2.3 Introduction to BAFF

Cytokines from the TNF family are critical regulators of both physiological and pathological immune responses. The discovery of TNF as a mediator of inflammation approximately 30 years ago marked the beginning of the extensive research into the TNF superfamily (TNFSF) and its receptors (TNFRSF) (Croft & Siegel, 2017). The first evidence suggesting TNF as a mediator of inflammation came from its ability to induce tumour necrosis and systemic inflammatory responses in animal models. These early studies revealed that TNF is a potent cytokine produced by immune cells in response to infection or injury, capable of driving inflammation, tissue damage and immune activation (Beutler et al., 1985; Carswell et al., 1975; Tracey et al., 1986). To date, 19 structurally related cytokines belonging to the TNFSF and their corresponding receptors have been identified, each playing vital roles in regulating immune responses include cellular survival, cellular proliferation, cellular differentiation and effector functions across diverse immune cell types (Sonar & Lal, 2015; Ward-Kavanagh et al., 2016). Among these, BAFF stands out as a key player in B cell survival, activation, differentiation and homeostasis (Schneider et al., 1999).

BAFF was independently discovered by several research groups and has been referred to by various names, including BLyS, Tumour Necrosis Factor Ligand Superfamily Member 13B (TNFSF13B), TNF- and APOL-related leukocyte expressed ligand (TALL-1), and TNF homolog activates apoptosis, nuclear factor-κB and c- Jun NH2 terminal kinase (THANK) (Balasubramaniam & Mokhtar, 2024; Yang et al., 2014). It shares approximately 50% sequence homology with APRIL (a proliferation-inducing ligand), another cytokine from the TNF ligand superfamily, also known as TNF-related death ligand-1 (TRDL-1), TALL-2, TNFSF13A, and CD256. The BAFF/APRIL system interacts with three receptors: BCMA, TACI, and BAFF-R.

These receptors exhibit selective binding affinities whereby TACI binds both BAFF and APRIL with equal affinity, BCMA preferentially binds APRIL, and BAFF-R specifically binds BAFF with high selectivity and affinity (Balasubramaniam & Mokhtar, 2024).

Initially, BAFF and its receptors were thought to be expressed primarily by haematopoietic cell lineages with monocytes, macrophages, neutrophils, and DCs being the major producers of soluble BAFF in the immune system. In haematopoietic cells, BAFF/BAFF-R influences functions of monocytes/macrophages, DCs, and T cells while being primary responsible for overall B cell survival. However, recent evidence have revealed that BAFF and its receptors are also expressed by non-haematopoietic cells, including adipocytes, keratinocytes, neurites, osteoclasts, and renal cells such as glomerular mesangial cells and podocytes (Balasubramaniam & Mokhtar, 2024). In non-haematopoietic cells, BAFF/BAFF-R contributes to tissue-specific functions and disease processes, such as adipose tissue inflammation (Alexaki et al., 2009; Kim et al., 2009), skin barrier function (Matsushita et al., 2007), neuroinflammation (Bakhuraysah et al., 2021; Tada et al., 2013), bone resorption (Hemingway et al., 2011; L. Wang et al., 2021), and kidney injury (Zheng et al., 2015). This broad expression pattern underscores the multifaceted roles of BAFF/BAFF-R axis in both immune and non-immune tissues.

Importantly, *in vitro* and *in vivo* studies have demonstrated that the BAFF/BAFF-R axis plays a central role in B cell maturation and differentiation (Batten et al., 2000; Groom et al., 2002; Thompson et al., 2000). Emerging evidence indicates that BAFF also influences monocyte activation, promotes their differentiation into macrophages and modulates cytokine secretion, highlighting its broader immunomodulatory role in bridging the innate and adaptive arms of the immune system

(Chang et al., 2006). Dysregulation of this axis has been implicated in the pathogenesis of autoimmune diseases such as SLE, RA, as well as B cell malignancies, including B cell lymphomas (Mackay & Cancro, 2006; Mackay & Leung, 2006; Tangye et al., 2006).

2.3.1 BAFF variants

The *baff* gene is located on the distal arm of the human chromosome 13 (13q32-q34) with a size of 39 kb. The human *baff* gene contains six exons and five introns. Exon 1 encodes for transmembrane and flanking region, exon 2 encodes for the furin-processing site, while exon 3 to 6 encodes for the TNF homology domain responsible for ligand-receptor interaction. The schematic representation of *baff* gene is illustrated in **Figure 2.1.**

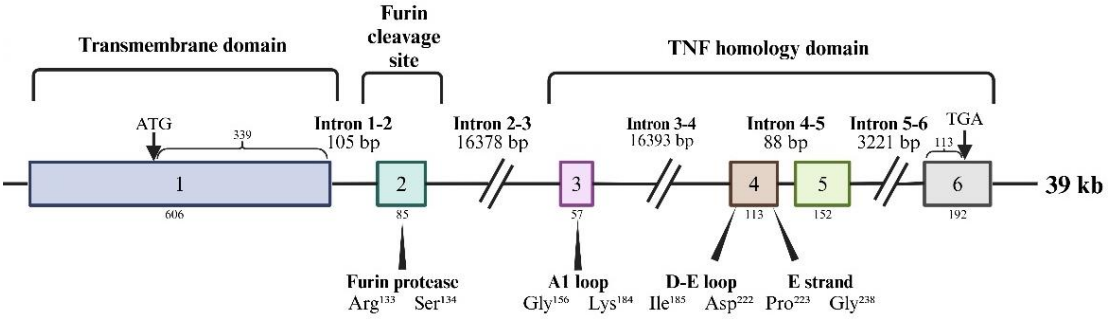

Figure 2.1 **Schematic representation of *baff* gene.** Exons are represented as boxes. The size of each exon (bp) is indicated below it, and the size of each intron are also indicated (thin line). Adapted from (Lahiri et al., 2012). Created with Biorender.com. Bp: base pairs, kb: kilobase

Splicing or mutations in the gene leads to transcript variants. Primary transcript of encoded by human *baff* gene contains 2576 base pairs with an open reading frame of 858 bp. Alternative splicing or mutations in the *baff* gene led to BAFF variants such as, ψBAFF, ΔBAFF and Δ4BAFF. The schematic representation of BAFF variants are illustrated in **Figure 2.2.**

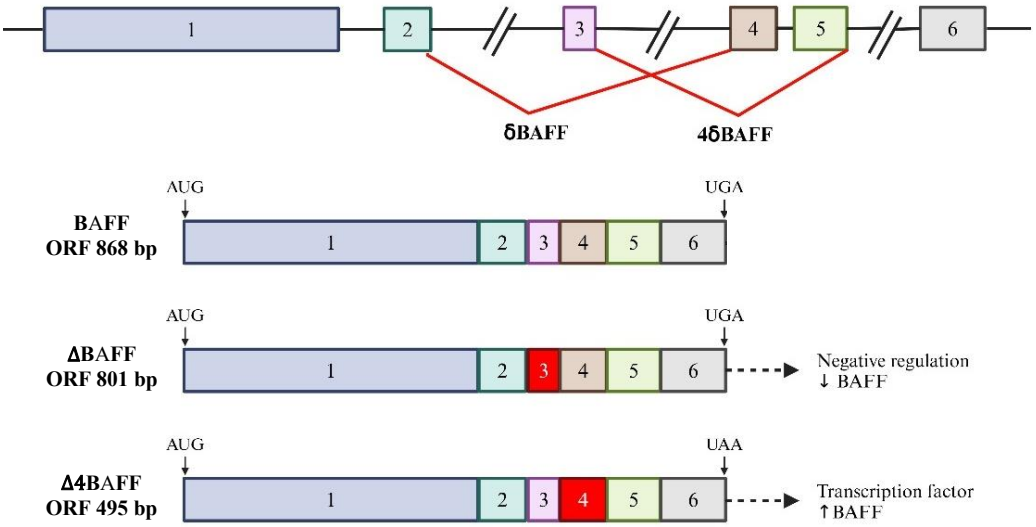

Figure 2.2 **Schematic representation of BAFF transcripts.** Colour of boxes correspond to exons (bp) of genes described in Figure 2.1. The number of base pairs (bp) in the open reading frame is indicated next to each transcript. Adapted from (Lahiri et al., 2012). Created with BioRender.com

ψBAFF is the longest variant of BAFF which was identified in human cell lines such as HL-60 (acute promyelocytic leukaemia) and U937 (diffuse histiocytic lymphoma). However, sequencing analysis of this variant have indicated that the variant is non-functional due to the incomplete splicing of introns which results in premature stop codons (Alturaiki, 2020; Daridon et al., 2008).

ΔBAFF (801 bp), a shorter variant, is generated by the in-frame deletion of exon 3, results in the loss of 57 nucleotides and 19 amino acids in the A-A1 loop region. This deletion removes residues Ile^{156} to Lys^{184} and substitutes Gly^{185} with arginine, creating a new N-linked glycosylation site at Asn^{155}. The A-A1 loop is critical for receptor binding, trimerization, and signalling activation in full-length BAFF. Its absence in ΔBAFF renders the variant non-functional, allowing it to act as a negative regulator by competitively suppressing the BAFF signalling. ΔBAFF forms disulphide-bonded heteromultimers with full-length BAFF, suppressing its dissociation from the cell membrane and dampening downstream signalling (Gavin et al., 2003; Thompson et al., 2016). Studies in BAFF transgenic mice have demonstrated that ΔBAFF negatively

regulates both humoral (B cell survival, maturation, and antibody production) and cellular (T cell and DC responses) immunity and even in later stages of B cell development (Gavin et al., 2005).

The recently discovered Δ4BAFF (495 bp) lacks exon 4 and 114 nucleotides, resulting in the deletion of 38 amino acids from the central region of the BAFF protein, including parts of the D-E loop and E strand, which are responsible for receptor binding, trimerization and signalling activation. Unlike ΔBAFF, Δ4BAFF functions as a transcription factor that enhances BAFF production in autoimmune diseases and cancer (Le Pottier et al., 2010; Thompson et al., 2016). Furthermore, Δ4BAFF has been observed in B cells from patients with chronic lymphocytic leukaemia (CLL), synoviocytes from RA patients, and epithelial cells from primary Sjogren's syndrome (pSS) patients (Le Pottier et al., 2012). This variant is primarily localised in the endoplasmic reticulum but can also be found on the nuclear membrane of transfected cells (Lahiri et al., 2012).

2.3.2 BAFF protein

BAFF protein is a type II transmembrane protein that lacks a cleavable signal peptide at the N-terminus, a characteristic feature of TNF ligand family. It consists of 285 amino acids with a molecular weight of 31.2 kDa. Notably, >95% of BAFF proteins collected from supernatants are processed into smaller 18 kDa fragments that are readily detectable in cell extracts. This processing is mediated by furin convertase enzyme, which cleaves BAFF into its soluble form. Soluble BAFF can exist as trimers (BAFF 3-mer) or further assemble into a highly-ordered 60-mer complex composed of 20 BAFF 3-mers (Balasubramaniam & Mokhtar, 2024). The 60-mer complex is particularly potent in activating the BAFF receptors such as BAFF-R, TACI and BCMA, due to its ability to cluster receptors and amplify downstream signalling

pathways like NF-κB and PI3K/Akt pathways. This multimeric organisation is critical for BAFF to promote B cell survival, maturation, and immune homeostasis (Balasubramaniam & Mokhtar, 2024; Bossen & Schneider, 2006; Schneider et al., 1999). Structural differences and functional roles of BAFF in B cell biology are illustrated in **Figure 2.3**.

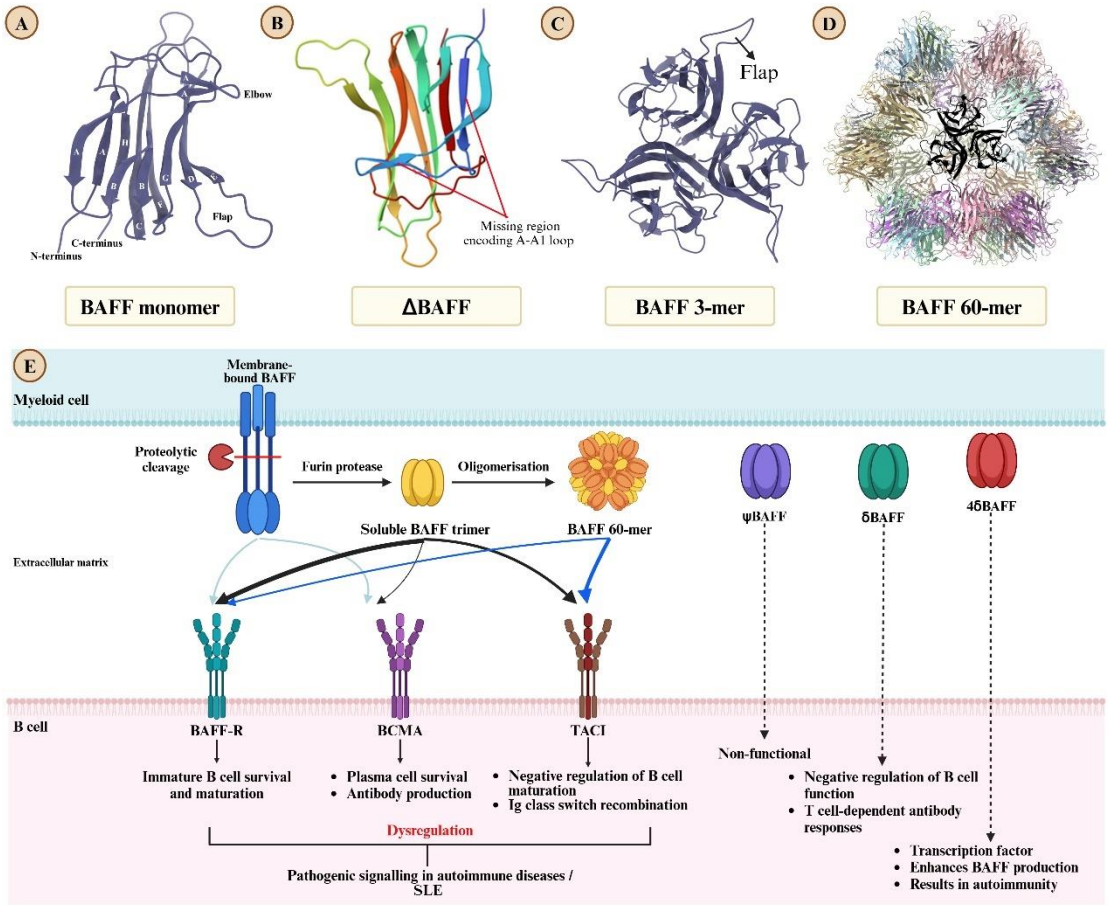

Figure 2.3 **Structural forms and functional roles of BAFF in B cell biology.** (A) BAFF monomer in the ribbon representation. Flap D-E loop is implicated in 60-mer formation. (B) BAFF monomer indicating the missing sequence encoded by exon 2 resulting in ΔBAFF. (C) BAFF trimer. (D) BAFF 60-mer, with one trimer highlighted in black at the centre of the structure. (E) Myeloid cells are major producers of BAFF. BAFF exists in both membrane-bound and soluble forms, with the latter generated through furin protease-mediated proteolytic cleavage as indicated by dashed lines. The soluble BAFF trimer can further oligomerise into the highly active 60-mer form. BAFF signals through three distinct receptors, with each receptor (BAFF-R, TACI, and BCMA) mediating specific functions. Arrow thickness indicates the relative binding affinities of different forms of BAFF. BAFF-R promotes immature B cells survival and maturation, BCMA supports plasma cell survival and antibody production, while TACI negatively regulates B cell maturation and mediated Ig class switch recombination. Dysregulation of BAFF signalling contributes to pathogenic outcomes, which ultimately results in immunological disorders including SLE. Models were drawn with

ChimeraX based on coordinate files IOQE and IOTZ. Adapted from Lahiri et al., (2012) and Bossen & Schneider, (2006). Created with BioRender.com.
ΔBAFF: delta BAFF, BAFF 3-mer: BAFF trimer, SLE: systemic lupus erythematosus,

2.3.3 BAFF-specific receptor, BAFF-R

BAFF-R is the recently discovered receptor in the BAFF/APRIL system that is specific to the ligand BAFF (Thompson et al., 2016). It was discovered following the earlier identification of TACI and BCMA, which can bind both BAFF and APRIL (Mackay et al., 2003). In humans, the *baff-r* gene is located on chromosome 22 at a locus 22q13.1-13.31 (Thompson et al., 2016). The primary transcript of human BAFF-R gene contains 4779 bp, while the coding region contains 555 bp which spans over 3 exons (Sevdali et al., 2021). The first exon encodes for ligand-binding domain, the second exon encodes for the transmembrane domain and flanking region, while the third exon encodes for intracellular domain. Schematic representation of BAFF-R gene, its protein structure, and ligand-receptor interaction is depicted in **Figure 2.4**. Apart from the cell membrane, BAFF-R also can be found in other parts of the cells such as cytoplasm, nuclear envelope and nucleoplasm (Yang et al., 2014).

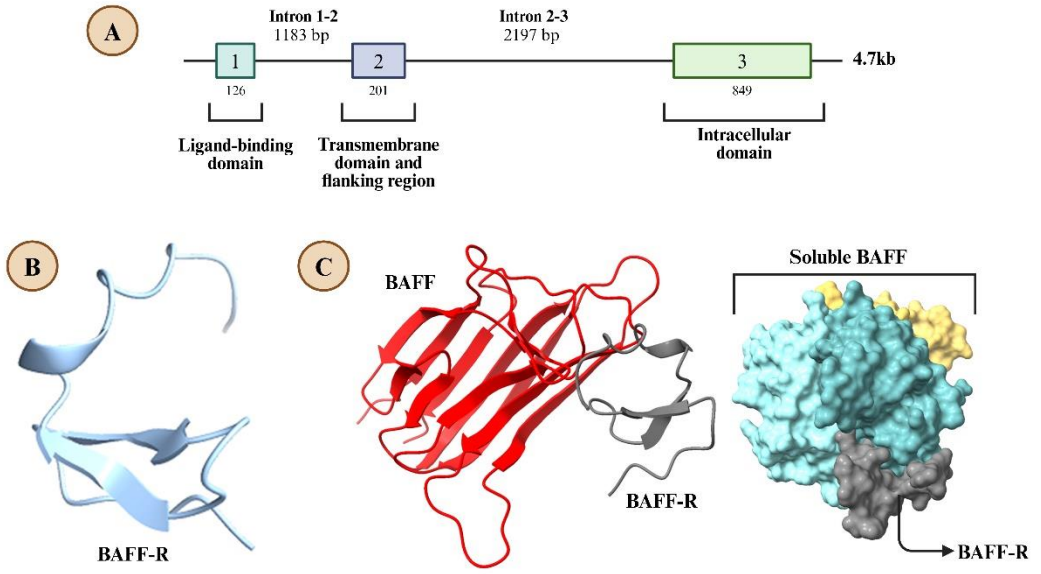

Figure 2.4 **BAFF-R gene, protein structure and ligand-receptor interaction.** (A) Schematic representation of *baff-r* gene. (B) BAFF-R protein structure in the ribbon

representation (C) Binding of BAFF-R to BAFF monomer in ribbon representation and binding of BAFF-R to soluble BAFF trimer in surface representation. Models were drawn with ChimeraX based on coordinate files 1OQE and 1OTZ. Created with BioRender.com.

As mentioned earlier, BAFF and its related cytokine APRIL bind to three receptors, namely, BAFF-R, TACI and BCMA. BAFF-R which binds specifically to BAFF with high affinity, was discovered in BAFF-binding BJAB cell line which lacked the expression of BCMA and TACI (Schneider, 2005). The specificity and differences in binding affinity among these receptors are determined by structural features, including a conserved β-hairpin structure and a variable motif known as DXL motif (Mackay & Gommerman, 2015; Yang et al., 2014). Structural differences between BAFF receptors are shown in **Figure 2.5**. BAFF-R contains a single loop in its variable motif, while TACI and BCMA adopt two differentially oriented helix-loop-helix motifs. While the β-hairpin structure alone in BAFF-R is sufficient for BAFF binding, interaction of BAFF-R with APRIL requires additional structural elements such as specific hydrophobic residues in the hairpin structure. The absence of aromatic residues in BAFF-R and a hydrophobic residue (Leu38), along with its unique loop structure, prohibits APRIL from binding to BAFF-R ensuring the specificity of BAFF/BAFF-R interactions (Balasubramaniam & Mokhtar, 2024; Bossen & Schneider, 2006; Yang et al., 2014).