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Transcriptional Regulatory Patterns of Activation-Induced Cytidine Deaminase (AID) Expression in the Context of *Plasmodium Falciparum* Infection

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DECLARATION

I, Reuben Ayivor-Djanie, declare that the work presented in this thesis is my own original work, except where specified that the information has been derived from other sources and have been appropriately referenced. This thesis does not include any work which has been submitted to this institution or to any other institution for the award of a degree.

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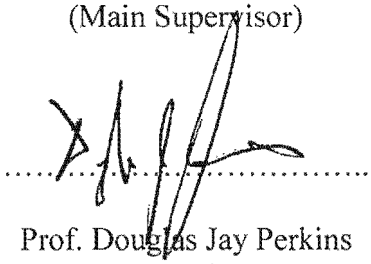

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ABSTRACT

Activation-Induced cytidine Deaminase (AID) initiates two important immunoglobulin gene modification processes in B lymphocytes; somatic hypermutation (SHM) and Class Switch Recommendation (CSR). Despite this important function, the aberrant expression of AID is associated with a number of cancers. Although the regulation of AID is strict and complex, there is evidence that *Plasmodium falciparum* infections can lead to a deregulated expression of the enzyme. It is not known how infection with the malaria parasite mediate this deregulated expression and activity. The aim of this work was to investigate the transcriptional regulatory patterns of AID and transcriptional factors (TFs) associated with AID expression. First, the transcriptional regulatory patterns of AID expression in *P. falciparum*-exposed geminal center B-cells was investigated in tonsillar mononuclear cells (MNCs) obtained from six (6) children from a malaria holoendemic region (Ghana) and one (1) child from a malaria free region (New Mexico, USA). Total MNCs were sorted into four distinct sub-populations; naïve B lymphocytes (NB), germinal center B lymphocytes (GC), memory B lymphocytes (MB) and plasmablasts (PB). The expression patterns of AID and its alternatively spliced variants, as well as the AID related TFs, Pax5, HoxC4, Bcl6, Bach2, Irf8, Prdm-1, XBP-1, miR181b and miR155 were investigated in the B-cell sub-populations by qRT-PCR. The Ghanaian tonsils had GC B-cell frequencies ranging from 14% to 51%, and GC B cells accounted for only 7% of the total MNCs in the New Mexican tonsil. While the expression of positive AID TFs Pax5, HoxC4, Bcl6, Bach2 and Irf8 were significantly upregulated ($p < 0.0001$), negative regulatory TFs of AID, Prdm-1 and XBP-1 were significantly downregulated ($p < 0.0001$) in GC B cells compared to PBs. Together with the full-length AID mRNA transcript (AIDFL), AID-ΔE4, AID-ΔE4, AID-ΔE4a, and AID-ivs3 were upregulated ($p < 0.0001$) in GC B cells compared to NB, MB and PBs. The expression of miR181b

was down-regulated in all the B-cell subsets, but miR155 was significantly ($p < 0.0001$) down-regulated in both MB and GC B lymphocytes. The expression patterns of AID and related TFs were investigated in children with asymptomatic *P. falciparum* infection. In asymptomatic infected children, significantly ($p = 0.00197$) higher levels of AID transcripts were observed compared to uninfected children, independent of the EBV infection status. The expression of Bcl6, Pax5 Prdm-1 and Irf4 were also higher in children with asymptomatic infection compared to uninfected children, but the expression of XBP-1 and Irf8 did not vary significantly ($p > 0.05$). Lastly, the expression patterns of AID and related TFs were assayed in total white blood cells (WBCs) isolated from whole blood of children between ages 0 -3 years with either severe malaria anemia (SMA), uncomplicated malaria (Non-SMA) or febrile but aparasitemic (AP). Elevated AID transcripts levels were observed in children with SMA, which was not associated with Hb levels (Pearson's *R*, *p* value: AP = -0.36, 0.35, non-SMA = 0.26, 0.16 and SMA = 0.09, 0.62), fever, or body temperature (Pearson's coefficient, *R, p:* AP = 0.29, 0.36, Non-SMA = 0.20, 0.24 and SMA = 0.16, 0.38). The levels of AID transcripts correlated with parasite loads in children with SMA (Pearson's $R = 0.41$, $p = 0.018$), but not with titers of IgG (Pearson's coefficient, *R, p:* Non-SMA = 0.1946, 0.6022 and SMA = -0.0414, 0.1903) and IgM (Pearson's coefficient, *R, p:* Non-SMA = -0.1241, 0.6022 and SMA = 0.26, 0.1903) to EBV viral capsid antigen (VCA). While Irf8 and XBP-1 levels were higher in *P. falciparum* infected children, irrespective of the SMA status ($p < 0.01$), Pax5 transcript levels were higher ($p < 0.01$) in SMA children than non-SMA children. The expression levels of Prdm-1 and Irf4 were not different ($p > 0.05$) among SMA, non-SMA and Aparasitemic children. The expression of miR181b was lowest in SMA children, and miR155 levels were not different in all three groups. All four splice variants of AID were significantly higher in SMA children than non-SMA and aparasitemic children. In conclusion, enhanced AID transcription in

GC B lymphocytes from Ghanaian tonsils was associated with elevated levels of Pax5, HoxC4, Bcl6, Bach2 and Irf8, as well as the down-regulation of miR155 and the expression of AID alternative splice variants. *P. falciparum* infection is associated with increased levels of AID and Pax5 transcripts in both asymptomatic and SMA children. This study is the first to document the effect of asymptomatic and acute *P. falciparum* infection on the transcriptional regulatory patterns of AID.

DEDICATION

To
God Almighty

Agnes Naadei Penelope Djanie

My wife,
Maame Efua Debuwaa,

and my two daughters
Aseye Ayivor-Djanie
Senanu Ayivor-Djanie

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3′ UTR	3′ untranslated regions
AID	Activation-Induced cytidine deaminase
ALL	Acute Lymphoblastic Leukemia
APOBEC-1	apolipoprotein-B mRNA-editing cytidine deaminases, catalytic polypeptide-1
APRIL	a proliferation-inducing ligand
ATL	Adult T-cell Leukemia
Bach2	BTB domain and CNC Homolog 2
Bcl6	B-cell lymphoma 6
BCR	B-cell receptor
bHLH	basic helix-loop-helix
Blimp-1	B lymphocyte-induced maturation protein 1
BSA	Bovine serum albumin
cDNA	Complementary Deoxyribonucleic Acid
CLL	Chronic Lymphocytic Leukemia
CRE	Cis-Regulatory Elements
CSR	Class Switch Recommendation
DLBCL	Diffused Large B cell Lymphomas
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dsRNA	Double stranded Ribonucleic Acid
eBL	Endermic Burkitt's Lymphoma
EBNA-1	Epstein-Barr virus nuclear antigen-1

EBV	Epstein-Barr Virus
EENT	Ear Eye Nose and Throat Clinic
FBS	Fetal Bovine Serum
GALTs	gut-associated lymphoid tissues
GC	Germinal center B lymphocytes
IFN-γ	Interferon- gamma
IgH	Immunoglobulin Heavy chain
IgL	Immunoglobulin Light chain
IL4	Interluekine-4
IL5	Interluekine-5
Irf4	Interferon Regulatory Factor-4
Irf8	Interferon Regulatory Factor-\8
LMP2A	EBV latent membrane protein-2A
LPS	Lipopolysaccharide
MAMPS	Microbe-Associated Molecular Patterns
MB	Memory B lymphocytes
MNC	Mononuclear cells
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response 88
NB	Naïve B lymphocytes
*P.f.*HRP-II	Plasmodium falciparum Histidine Rich Protein-II
Pax5	Paired Box Protein 5
PB	Plasmablasts

PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
*Pf*Hz	Hemozoin produced by *Plasmodium falciparum*
Prdm-1	beta-inlterferon positive-regulatory domain 1 binding factor
PRRs	pattern recognition receptors
RDT	Rapid Diagnostic Test
RNA	Ribonucleic Acid
RT-qPCR	Reverse Transcription quantitative Polymerase Chain reaction
SHM	Somatic Hypermutation
SMA	Severe Malaria Anemia
TGF-β	Tumor Growth factor-beta
TLR	Toll-like Receptors
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF-R	Tumor Necrosis Factor Receptor
TRAFs	TNF-R associated factors
V(D)J	variable, diversity and joining gene segments of Immunoglobulin gene
VCA	Viral Capsid Antigen
WACCBIP	West African Center for Cell Biology for Infectious Pathogens
XBP-1	X-box binding protein 1

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

The discovery of Activation-Induced cytidine deaminase (AID), and its biological functions has enlightened our understanding of B-cell humoral responses over the last two decades. The first report of the enzyme and its function in murine B-lymphoma cells was published in 1999 by a group in Japan. In this work, AID was identified as a novel member of the apolipoprotein-B mRNA-editing cytidine deaminases, catalytic polypeptide-1 (APOBEC-1) (Muramatsu et al., 1999). Although AID shares about 34% amino acid homology with members of the APOBEC-1 family, it is unique. AID neither binds to nor edits apoB mRNA. Today, it is known that AID binds to and creates point mutations in both single and double-stranded DNA, especially in germinal centers (GC) B lymphocytes undergoing somatic hypermutation (SHM) and class switch recombination (CSR) in secondary lymphoid tissues during antibody maturation (Muramatsu et al., 1999).

The biological importance of AID in the adaptive immune response is clinically significant in two ways. On one hand, AID is essential for the diversification of the immunoglobulin repertoire by initiating SHM and CSR in both human and murine B lymphocytes undergoing antigen maturation (Muramatsu et al., 2000, Revy et al., 2000). Mutations in the AID gene that render the AID protein defective in its functions have been shown to lead to Severe forms of immune deficiencies and a general lack of high-affinity antibodies (Revy et al., 2000). In addition, AID also plays a very important role in the programming of pluripotent cells as shown in its role in the epigenetic remodeling and genetic variation in somatic cells (Chahwan et al., 2010). On the other hand, the aberrant expression and activity of the enzyme is also associated with numerous cancers of B and

non-B cell origins (Kumar et al., 2014). The mutational activities of AID have been implicated to be the cause of genetic lesions seen in many cancers. Therefore, while it plays essential roles in producing highly specific and effective antibodies that fight pathogens, AID can also promote deleterious effects (Kumar et al., 2014).

Expectedly, for a very important and mutagenic enzyme, the regulation of the expression and activity of AID is not only rigid, but complex as well (Stavnezer, 2011). The transcription of the AID gene is regulated by the binding of at least 19 activating and repressive transcriptional factors in a B-cell stage-specific manner (Tran et al., 2010, Crouch et al., 2007, Park et al., 2009). At the posttranscriptional level, the abundance of the mature mRNA is regulated by microRNAs (Teng et al., 2008, de Yebenes et al., 2008) and alternative splicing (Wu et al., 2008). The translated protein is regulated by a complex mechanism of shuttling between the cytosol and the nucleus, phosphorylation at specific amino acids that can activate or deactivate the enzyme, and a very short nuclear half-life (Stavnezer, 2011). Despite these multistep regulatory checkpoints, the expression and activity of AID is reported to be deregulated in response to *P. falciparum* infection in both humans and mice (Torgbor et al., 2014). A typical example of how *P. falciparum* infection leads an undesired activity of AID is seen in the pathogenesis of the African (endemic) form of Burkitt's lymphoma (eBL) (Thorley-Lawson et al., 2016).

Since the discovery of the pediatric cancer in the 1960s, infections with Epstein-Barr Virus (EBV) and *Plasmodium falciparum* malaria have been considered risk factors for the occurrence of the cancer (Dalldorf et al., 1964, Burkitt, 1958). On one hand, the pathogenesis of eBL has been linked to infection with EBV because the genome of the virus has been detected in almost all eBL tumors (Epstein et al., 1964, zur Hausen et al., 1970), and the virus is known to immortalize its host B lymphocytes by shutting off apoptotic signals (Miller, 1982).

The contribution of *P. falciparum* malaria to eBL is however unclear. There are two theories that seek to explain how *P. falciparum* malaria and EBV infection cooperate to cause the cancer. One school of thought suggests that since *P. falciparu*m induces the polyclonal activation and expansion of B lymphocytes, which activates the lytic replication of EBV (Donati et al., 2004), it potentiates the risk of the characteristic translocation and deregulation of *c-myc* as seen in eBL (Rochford et al., 2005). The other proponents argue that *P. falciparum* infection impairs EBV-specific T-cell immunity and enhances the replication of the EBV, thereby, increasing the chances of the *c-myc* translocation (Moormann et al., 2011). Although these theories are not mutually exclusive, they both propose that *P. falciparum* infection creates the permissive environment for EBV replication and infection of naïve B lymphocytes (NBs). This increases the risk for developing the genetic lesion that propagated the cancer, since the infected NBs are driven through the GC reaction to generate memory B lymphocytes where the virus establishes latency.

Beyond the permissive environment it may create for replication of EBV, there is evidence to support a school of thought that infections with *P. falciparum* play an important part in the events that lead to the cancer. A recent case-control study involving 862 children suspected to have eBL (80.9% diagnosed) and 3000 population controls in three malaria endemic regions in eastern Africa, revealed that eBL was positively associated with in-patient treatment for malaria, at least 12 months prior to enrolment in the study (Peprah et al., 2019). They also found a positive association with the occurrence of eBL in children who have lived (or live) in areas with high incidence of *P. falciparum* (Peprah et al., 2019). This is the first study to report the treatment for febrile *P. falciparum* malaria and the occurrence of eBL, and it provides compelling evidence for the involvement of the parasite beyond epidemiological coincidence.

1.2 Problem Statement

While it is unclear how the parasite contributes directly to the development of the genetic lesions that cause eBL and other malignancies, infection with *P. falciparum* has a profound effect on B-cell mediated immunity. Infection with the parasite is characterized by hyperglobulinemia (Abele et al., 1965), B-cell overactivation (Greenwood and Vick, 1975a, Donati et al., 2004), production of different autoantibodies (Kataaha et al., 1984) and a dysregulated immune response (Illingworth et al., 2013, Asito et al., 2008). The genetic modifications associated with these processes predispose B cells to genomic instability, via the activity of AID. In fact there is evidence to show that exposure to *P. falciparum* antigens induces high expression of AID in the germinal centers of B lymphocytes, *in-vitro* and *ex-vivo* (Torgbor et al., 2014). This induction of AID expression is postulated to be the molecular link between infections with the malaria parasite and the pathogenesis of eBL for example (Thorley-Lawson et al., 2016). In other related studies, chronic infection of mice with *Plasmodium chabaudi*, has been demonstrated to lead to the abnormal expression of AID and the occurrence of AID-specific mutations in multiple non-Ig loci (Yamane et al., 2011) and different lymphomas with post GC phenotypes (Robbiani et al., 2015).

Together, these studies suggest that *P. falciparum* infection can contribute to genomic instability in B and non-B lymphocytes via a deregulated expression of AID. Given the complex and multi-faceted regulatory mechanisms controlling the expression and activity of AID (Stavnezer, 2011), it is yet to be shown how a pathogen like the malaria parasite directly induces the deregulated expression of the enzyme. It has been reported that GC B cells isolated from children in malaria endemic regions have higher levels of AID transcripts compared to GC B cells from malaria free regions (Torgbor et al., 2014). There is currently no explanation on how infection with the *P. falciparum* affects the expression of transcription factors and microRNAs that regulate the

transcription of AID and the abundance of its transcripts in B cells.

1.3 Hypothesis

To provide proof that exposure to *P. falciparum* predisposes B lymphocytes to the genomic instability via the expression of AID, this work hypothesized that **infection with *P. falciparum* potentiates a deregulated transcription of AID.**

1.4 Aims and Objectives

The main aim of this study was to **"investigate the transcriptional regulatory patterns of** AID **expression in the context of *P. falciparum* infection in B lymphocytes".**

1.4.1 Specific Aim One

To investigate the transcriptional regulatory patterns of* AID *expression in Plasmodium falciparum exposed and non-exposed Tonsillar B-lymphocytes.

Hypothesis

Exposure to *P. falciparum* induces a deregulated AID transcriptional regulatory program in Geminal center B cells.

Rationale

The germinal centers of peripheral lymphoid tissues are the main sites for antigen-specific SHM and CSR (MacLennan and Gray, 1986) and they provide the primary source for highly specific antibody-producing B lymphocytes needed to fight invading pathogens (MacLennan, 1994). As important as the geminal center reaction is, it is however the origin of the majority of B-cell lymphomas as well (Kuppers et al., 1999, Stevenson et al., 1998). Recent studies show that infection with *P. falciparum* increases the throughput of geminal center B cells, with a deregulated

AID expression and might contribute to the events that lead to the chromosomal lesion that leads to eBL (Torgbor et al., 2014, Thorley-Lawson et al., 2016). Although the general transcriptional regulatory programs for AID expression in pre-GC, GC and post-GC B lymphocytes have been studied, there is currently no evidence on how *P. falciparum* infection affects the expression of the transcription factors that are known to activate or repress the transcription of the AID gene. To understand the transcriptional regulatory patterns specific to B-cell sub-populations in secondary lymphoid tissues, this study sought to:

I. Obtain and isolate total mononuclear cells (MNCs) from Tonsillar tissue of children (up to 16 years) undergoing routine tonsillectomies in a malaria endemic region.
II. Sort total lymphocytes into distinct B-cell sub-populations [Naïve B lymphocytes (NBs), Memory B lymphocytes (MBs), Germinal Center B lymphocytes (GCs) and Plasmablasts (PBs)] by flow cytometry.
III. Assay for mRNA levels of AID and its related transcriptional factors by qPCR in sub-populations of Tonsillar B lymphocytes.
IV. Assay for levels of miR-155 and miR-181b in Tonsillar B-cell sub-populations
V. Investigate the regulation of AID by alternate mRNA splicing.

1.4.2 Aim Two

To investigate the transcriptional regulatory patterns of* AID *expression in Asymptomatic Plasmodium falciparum infected children.

Hypothesis

Prolonged asymptomatic infection with *P. falciparum* induces a deregulated AID transcriptional regulatory program in circulating B cells.

Rationale

Chronic exposure to *P. falciparum* antigens has been proposed to predispose children to eBL (Burkitt, 1958). The fact that *P. falciparum* infection can induce polyclonal activation of memory B lymphocytes (Donati et al., 2004) and impair EBV specific T-cell immunity (Moormann et al., 2007) only indicates indirect causality. The finding that *P. falciparum* antigens can induce the aberrant expression of AID (Torgbor et al., 2014) with consequent off-target activity (Robbiani et al., 2009), suggests a direct upstream molecular involvement of the parasite in the event that may lead to the cancer.

Although the expression of AID has been shown to be induced in peripheral blood of children with non-febrile *P. falciparum* infection (Wilmore et al., 2015), not much is known about the regulation of the transcription of AID in response to protracted asymptomatic *P. falciparum* infection. Since children older than five years are known to tolerate the parasite more than those between ages 1-5 (Bloland et al., 1999a), this study sought to understand the transcriptional regulatory patterns in children between ages 6-16 with asymptomatic *P. falciparum* infection. Specifically, this study sought to:

I. Determine the *P. falciparum* and EBV infection status of primary school children in a malaria endemic region.

II. Assay for mRNA levels of AID and related transcription factors in PBMCs of asymptomatic *P. falciparum* infected children.

III. Determine the levels of AID mRNA splice variants in PBMCs of asymptomatic *P. falciparum* infected and non-infected children.

1.4.3 Aim Three

To investigate the transcriptional regulatory patterns of* AID *expression in children with clinical symptoms of* Severe *Malaria Anemia.

Hypothesis

Acute infection with *P. falciparum* induces a deregulated AID transcriptional regulatory program in circulating B cells.

Rationale

There are no data on the expression of AID in children with acute symptoms of *P. falciparum* infections. Inferences can be drawn from the fact that hemozoin (*PfHz*), the metabolic by-product of heme in *P. falciparum*-infected RBCs, can induce the expression of AID (Torgbor et al., 2014). Since *PfHz* and heme breakdown products are a characteristic feature in Severe malaria anemia (Awandare et al., 2007, Perkins et al., 2011), this part of the study investigated the transcriptional patterns of the expression of AID and its related transcription factors in children from zero to 36 months with Severe malaria anemia. Specifically, this work sought to:

I. assay for the *ex-vivo* levels of AID mRNA and related transcription factors in total white blood cells (WBCs) from whole blood of children with uncomplicated and Severe malaria anemia.

II. assay for the levels of miR-155 and miR-181b in PBMCs of children with uncomplicated and Severe malaria anemia

III. determine the levels of AID mRNA splice variants in PBMCs of children with uncomplicated and Severe malaria anemia.

1.5 Significance of Study

It is known that *P. falciparum* is a potent inducer of AID activity in B cells as part of the antibody production process (Torgbor et al., 2014). Despite the rigid regulatory mechanisms controlling the expression and activity of AID, *P. falciparum* infection is reported to result in a dysregulated expression of the enzyme thereby promoting genomic instability in B cells (Robbiani et al., 2015). It is not clear how infection with the parasite results in this deregulated expression. The current study explores the transcriptional regulatory programs of AID expression in geminal centers of persons from malaria endemic regions and in children with asymptomatic and symptomatic infections and provides insights into how infection with the parasite contribute to the deregulated transcription of AID.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 AID is Required for Effective Antibody Response

Vertebrates have the ability to fight a broad range of invading pathogens in a specific and highly effective manner, and at the same time develop long term memory in most cases. This ability is possible due to the broad diversity and highly specific response of adaptive immunity. The recombination of the variable (V), diversity (D) and joining (J), V(D)J segments in the immunoglobulin heavy (H) chain loci and the V and J gene segments in the immunoglobulin gamma (Igλ) or immunoglobulin kapa (Igκ) light chain loci, equips B lymphocytes with the primary repertoire of genes for antibody production (Xu et al., 2007). Mature naïve B lymphocytes with rearranged V(D)J gene segments typically express IgM and/or IgD in circulation. Although the affinities of these expressed antibodies are low, they are enough to provide the flexibility needed for naïve B lymphocytes to interact with almost any possible antigen (Orthwein and Di Noia, 2012). High affinity antibodies with high specificity are however required to neutralize and clear most antigens. In addition to V(D)J rearrangements, B lymphocytes are equipped with additional mechanisms to further enhance the specificity of the antibodies they express (Klein and Dalla-Favera, 2008).

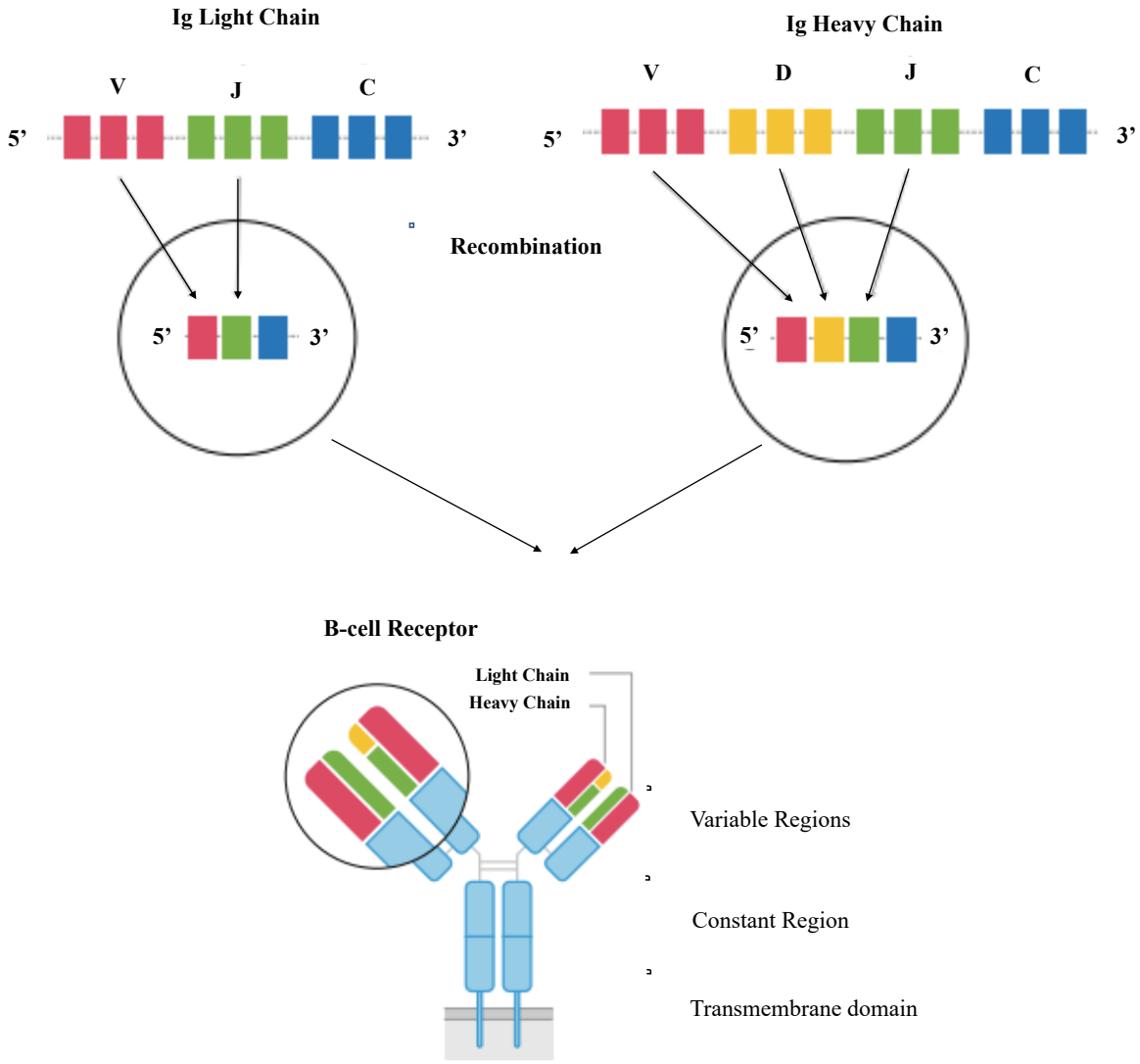

Figure 2.1: V(D)J Recombination of germline Ig heavy and light chains
Antibodies comprise light and heavy chains, each of which is further subdivided into constant and variable regions. Rearrangement of the genes that make up the variable region allows the many different antibody specificities to be generated. Germline DNA contains many different variable (*V*), diversity (*D*) (in the case of the heavy chain only) and joining (J) gene segments (not all segments are shown). Adapted from (Rajewsky, 1998)

When naïve B cells encounter an antigen, they are driven to migrate to the lymphoid tissues, where they are driven to undergo the GC reaction, involving two main Ig gene diversification processes; Somatic Hypermutation (SHM) and Class Switch Recombination (CSR) (Klein and Dalla-Favera, 2008). During SHM, the heavy (IgH) and light (IgL) chains of the immunoglobulin gene are diversified through point mutations in the variable regions of IgH and IgL. This, under normal physiological conditions, leads to antibodies with improved antigen recognition and binding (Figure 2.1). These B lymphocytes are then selected by antigen presenting cells and T-cells, resulting in affinity maturation of the antibody response (Orthwein and Di Noia, 2012). While SHM proceeds, exons of the constant region of the IgH that encode for IgM and IgD are switched for exons encoding IgG, IgE or IgA isotypes through CSR, resulting in the production of antibodies with conserved specificity and enhanced biological properties (Orthwein and Di Noia, 2012). AID is essential for both SHM and CSR to proceed (Revy et al., 2000, Muramatsu et al., 2000). B lymphocytes that have successfully gone through SHM and CSR may differentiate into plasma cells for the production of antibodies with varied biological activities and binding affinities against pathogens and self-antigens.

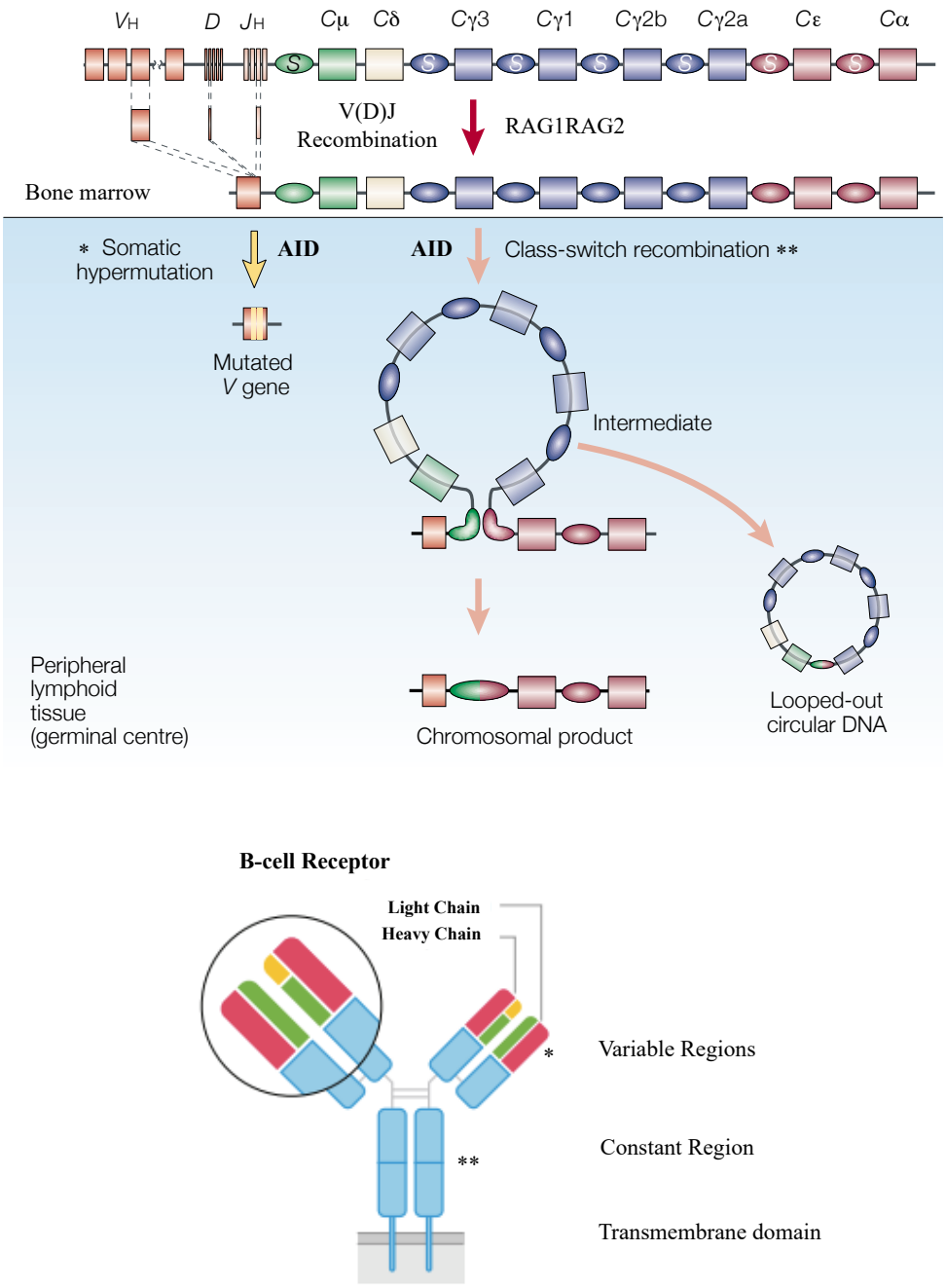

Figure 2.2 AID-mediated SHM and CSR in B lymphocytes

*SHM: AID makes point mutations in the Variable (V) exon of the IgH chain. **CSR: AID mediates the rearrangement of the constant (C) region of the IgH chain resulting in the deletion of the *S*μ (IgM) region in exchange for other *S* regions. Adapted from (Kinoshita and Honjo, 2001)

Functionally, AID deaminate dexocytidine (dC) residues to deoxeuridine (dU) in single-stranded and supercoiled DNA (Xu et al., 2005, Conticello, 2008). This deamination occurs during the transcription of the V(D)J gene segment in the case of SHM and the formation of the R-loop structures within transcribed regions of the switch region repeats. The dU:dG mismatch is then repaired by base excision mediated by either Uracil-DNA glycosylases. Point mutations in the variable segment of the Ig gene leads to SHM while mutations in the constant region of the Ig gene results in double-stranded breaks and the switch of one switch exon for another in CSR (Rada et al., 2004), (Figure 2.2).

2.2 Signal Transducing Pathways that induce AID Expression

2.2.1 CD40:CD40L Engagement

The expression of AID is largely specific to B lymphocytes and stage-specific. Its expression can be induced by T-cell dependent or independent stimuli. T cell-dependent induction is achieved through the CD40:CD40L (CD154) engagement, whiles T cell-independent induction is achieved by microbe-associated molecular patterns (MAMPS), and the crosslinking of the B cell receptors (BCR) (Zan and Casali, 2013). These primary stimuli induce the expression of AID, peaking at about 48 hours, through the activation of canonical and non-canonical pathways of NF-κB signaling (Pone et al., 2012). Binding of CD154 to its receptor (CD40) on B lymphocytes is required for the activation the tumor necrosis factor receptor (TNF-R). TNF-Rs relays activation signals to TNF-R associated factors (TRAFs), which, in turn activate NF-κB (Bishop and Hostager, 2003, Graham et al., 2010). The activation of NF-κB can be achieved in one of two ways; by the cooperation between IκB kinases (IKKs) and TRAF6 (canonical NF-κB) and the TRAF2/3 complex (non-canonical NF-κB) (Graham et al., 2010). The activated p65/p50 canonical and p52/RelB non-canonical NF-κB heterodimers then translocate to the nucleus (Oeckinghaus et

al., 2011, Sun, 2011), (Figure 2.3) . These heterodimers, work in synergy to activate the AID promoter. The activation of the AID promoter leads to the binding of one or more transcription factors (Stat6, C/EBP, Smad3/4, Myb, Pax5, E2A, E2f and BATF) to other regulatory regions, offering additional regulatory layers for the expression of the AID gene (Tran et al., 2010), (Figure 2.3).

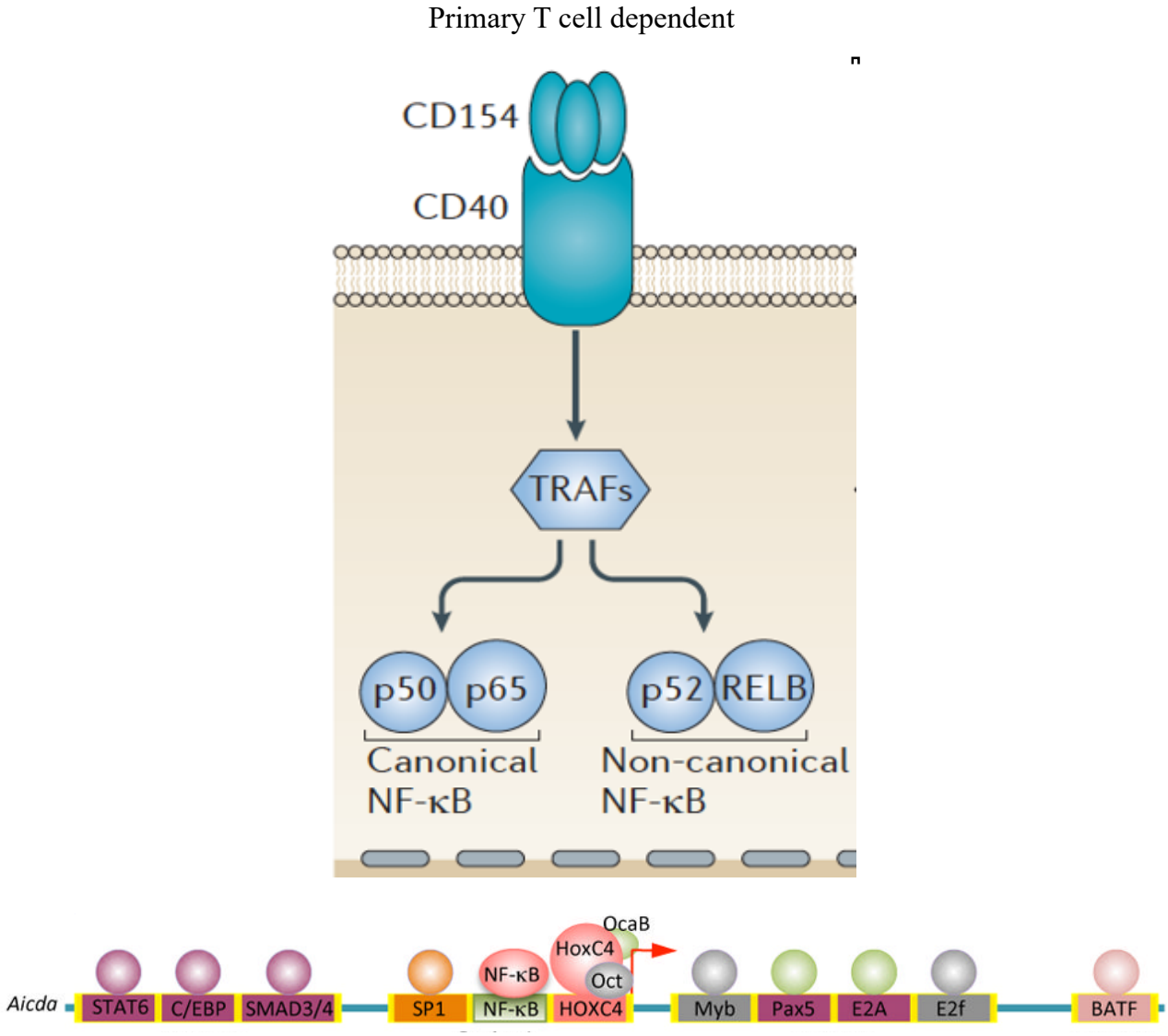

Figure 2.3: Activation of AID transcription via CD40 engagement
Adapted from(Zan and Casali, 2013); (Krebs et al., 2014)

In addition to CD40:CD154 engagement, IL-4 and TGF-β can serve as secondary stimuli for AID induction in a T cell-dependent manner. IL4 and TGF-β can also induce CSR in humans by inducing transcription of switch segments of the Ig targeted for recombination. IL-4 elicits B lymphocytes to produce and secret IgG1 and IgE, for the binding and clearance of extracellular pathogens (Zan and Casali, 2013). TGF-β on the other hand induces the switch to IgA in response to commensal microorganism in the GIT and respiratory tract. Together with IL4 and TGF-β, BAFF and APRIL enhances the expression of AID. While BAFF induces the transcription of AID through the activation of the p38MAP kinase/CREB and JNK/AP-1 pathways after binding to BCMA (Kim et al., 2011), it has been shown that APRIL directly activates the transcription of HoxC4 in an NF-κB dependent manner. HoxC4, in turn, binds the promoter of the AID gene and activates its transcription (Park et al., 2013), (Figure 2.4).

Secondary CSR-inducing Stimuli

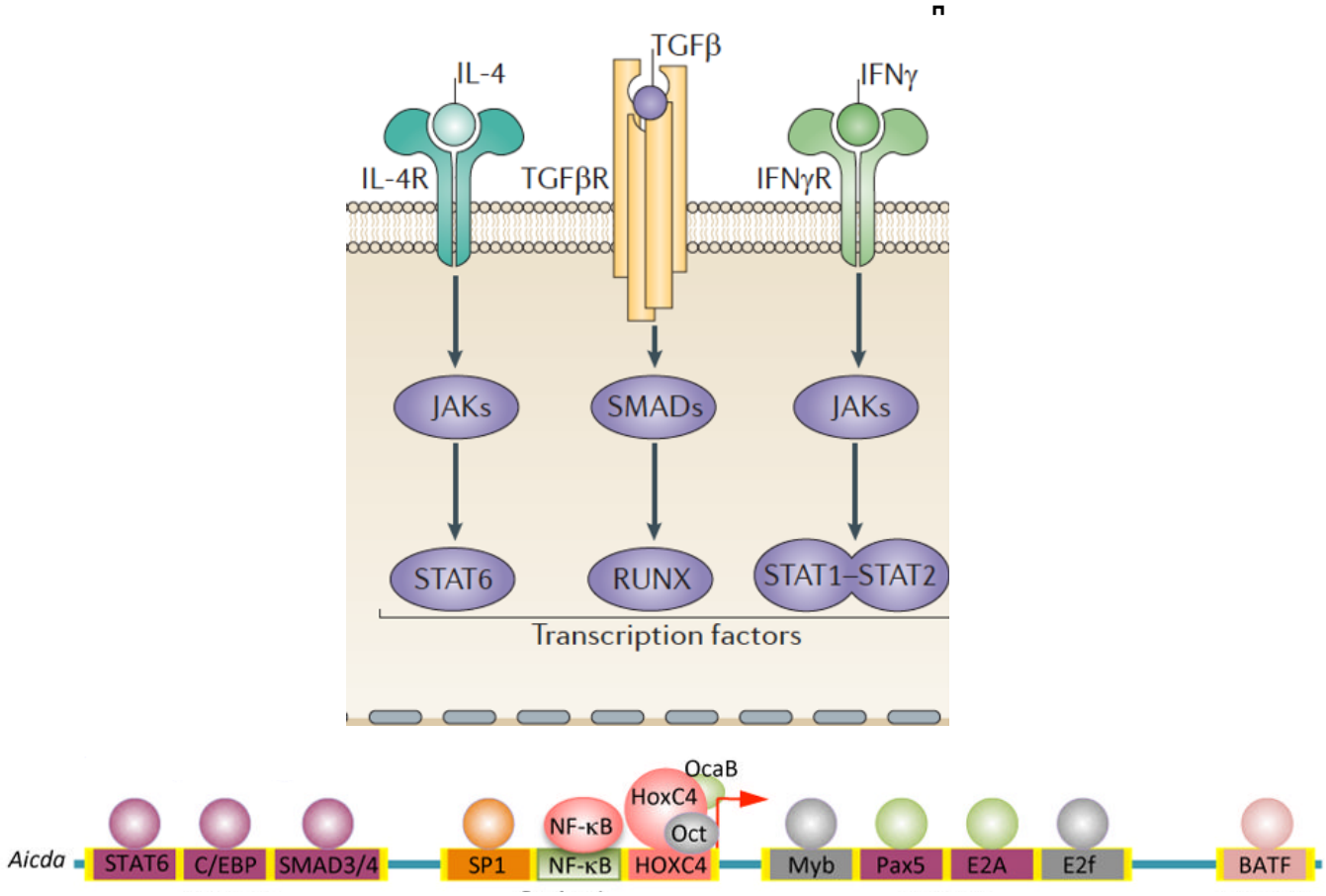

Figure 2.4: Activation of AID transcription by secondary stimuli
Adapted from(Zan and Casali, 2013); (Krebs et al., 2014)

2.2.2 Crosslinking of the B-cell Receptor

The crosslinking of the B-cell receptor (BCR) is essential for the development of early-stage B lymphocytes and activation and differentiation of mature B lymphocytes during antibody maturation. Co-stimulating B lymphocytes with CD154 and anti-IgM have been demonstrated to lead to the differentiation of B lymphocytes into post-germinal center phenotypes with hypermutated and class switched Ig genes in humans (Zan et al., 1999). Although signals from activated BCR is important for the T-dependent GC formation, the crosslinking of the BCR alone may not be enough to induce the expression of AID in gut-associated lymphoid tissues (GALTs), as has been demonstrated in mice that do not express BCR. Although these mice lack the expression of the IgH, the EBV latent membrane protein-2A (LMP2A) is able to mimic BCR signaling, thereby, promoting the development and survival of the B lymphocytes (Casola et al., 2004). These mice are not able to form germinal centers after exposure to T-dependent antigens, but they can develop germinal centers in GALTs, including the Payer's patches, with normal expression of AID, SHM and CSR (Casola et al., 2004). Therefore, activation of BCR may regulate AID expression by either promoting the transcription of AID via signals from CD40 or by inhibiting the action of CD30 during the initiation of AID induction (Xu et al., 2007).

2.2.3 Engagement of Toll-like Receptors (TLRs)

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that recognize and bind microbe-associated molecular patterns such as, LPS, double-stranded RNA, bacterial CpG DNA and some sugar-protein and lipid-protein complexes (Trinchieri and Sher, 2007). The stimulation of TLRs serve as signals for the activation of human B lymphocytes as they are involved in the direction of the adaptive immune response in a T-dependent manner. Engagement of TLR4 and

TLR9 has been shown to be an upstream event for the induction of AID expression in mice and humans, respectively. In murine B lymphocytes, TLR4 works in synergy with cytokines such as IL-4, IL-5, IFN-γ, TGF-β and LPS to induce proliferation and immunoglobulin class switch in primary B lymphocytes *in vitro* (Trinchieri and Sher, 2007). Lipopolysaccharide (LPS) alone only induces a weak AID expression, but in synergy with IL-4 and/or BCR crosslinking, it can induce effective expression of AID (Schrader et al., 2005, Muramatsu et al., 1999). Viral particles from the human papillomavirus type 16 (HPV16) have been reported to directly induce Ig class switch through TLR4-MyD88 (Yang et al., 2005). The interaction of both TLR4 and MyD88 is essential to the induction of AID expression and Ig class switch as suggested by the lack of AID transcripts in functionally mutated TLR4 and decreased AID transcripts and IgG levels in myD88$^{-/-}$ B lymphocytes. The HPV16 can also work in synergy with the activation of the CD40 signaling pathway to enhance the induction of AID expression via separate CD40-mediated Traf2/3 signaling pathways and TLR4-MyD88-Traf6 transduction pathways, via the activation of NF-κB (Yang et al., 2005), (Figure 2.5).

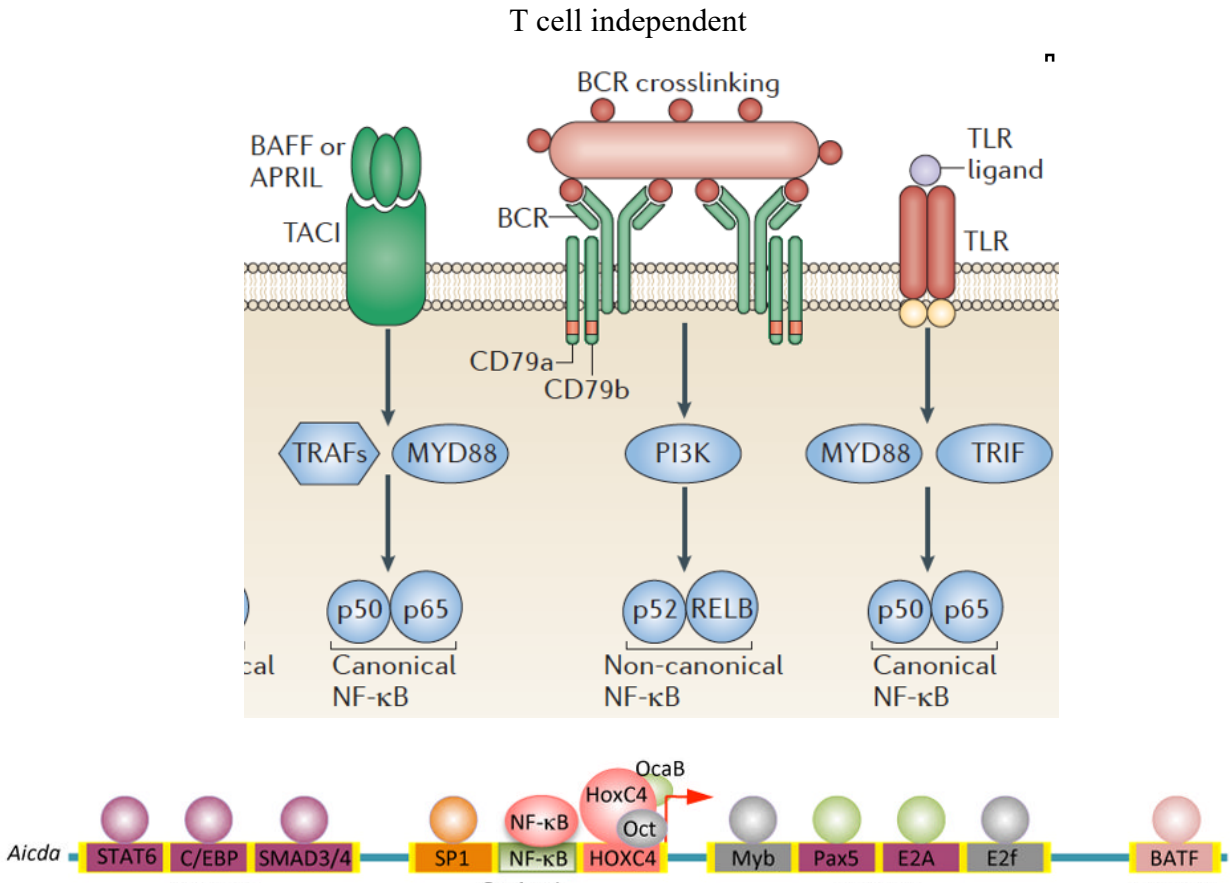

Figure 2.5: Activation of AID transcription by BCR and TLR engagement
Adapted from (Zan and Casali, 2013) and (Krebs et al., 2014)

In human and murine B lymphocytes challenged with bacterial CpG-DNA, the induction of AID expression can be mediated through the intracellular TLR9 (Krieg, 2002). The expression of TLR9 is up-regulated in B lymphocytes stimulated with anti-CD40 monoclonal antibodies or by BCR crosslinking agents (Bernasconi et al., 2003). CpG DNA enhances the expression and signaling of TLR9 resulting in the increased proliferation of primary B lymphocytes in response to CD154 stimulation. Concomitantly, the expression of AID is enhanced in these cells, as well as increased frequency of CSR. This suggests that TLR9 plays an important role in mediating the up-regulation

AID and expression, leading to CSR, following induction by CD40 and BRC signals (Ruprecht and Lanzavecchia, 2006).

2.3 AID **Deficiency and Aberrant Expression Results in** Severe **Pathologies**

As expected from the crucial role AID plays in the diversification of the antibody repertoire, mutations that leads to the loss of function or deficiency in AID activity causes hyper-IgM syndrome, in which individuals cannot expressed other immunoglobulin isotypes apart from IgM. (Orthwein and Di Noia, 2012). In addition, AID is essential to the self-tolerance of B lymphocytes in both mice and human (Kuraoka and Kelsoe, 2011).

While it is true that limiting levels of AID either limits or completely abrogates the processes of CSR and SHM, its overexpression has also been shown to result in high frequencies of CSR and SHM, sometimes resulting in deleterious genomic lesions (Orthwein and Di Noia, 2012). Recent studies suggest that higher levels of AID increase the risk of developing certain cancers. The oncogenic ability of AID is a result of its off target mutagenic activity at non-Ig loci in normal B and non-B lymphocytes. An increased risk of the *IgH-cMyc* translocations and AID-specific mutations at non-Ig loci in normal B lymphocytes has been reported to be associated with increased AID expression (Klein et al., 2011, Robbiani et al., 2009). For an important mutagenic protein such as AID, a fine balance between expression and regulation is necessary for maintenance of genomic stability.

2.3.1 Role of AID in lymphoid cancers

The off target and aberrant activity of AID can lead to cancers in B and non-B lymphocytes alike. The double stranded chromosomal breaks in *c-myc* and *IgH* loci that leads to the c-myc/IgH translocation, as seen in Burkitt's lymphoma and murine plasmacytomas, has been reported to be mediated by AID (Robbiani and Nussenzweig, 2013). Across the genome of activated B lymphocytes, AID can induce double-stranded DNA breaks in un-transcribed non-Ig loci as well (Staszewski et al., 2011). In addition, AID-mediated chromosomal double stranded breaks at WR*C*Y or WR*C* (W = A/T, R = A/G, Y = C/T) motifs have also been reported (Greisman et al., 2012). In addition to translocation involving the Ig locus, AID induces DNA DSBs in other non-Ig loci, which results in one form of cancer or the other. For instance, DNA DSBs in genes such as BCL6 and Irf4 can lead to chromosomal translocations associated with diffused large B cell lymphomas (DLBCL), and multiple myelomas in mature B lymphocytes (Robbiani et al., 2009, Jankovic et al., 2010).

In mice with transplanted bone marrow, aberrant expression of AID has been shown to promote the genesis of B-cell lymphomas and leukemia in a cell lineage-dependent manner (Komeno et al., 2010). Aberrant expression of AID, can be induced by the cancer promoting BCR-ABL1 kinase in pre-B acute lymphoblastic leukemia (ALL) and B lymphoid chronic myelogenous leukemia (CML) (Feldhahn et al., 2007, Klemm et al., 2009, Iacobucci et al., 2010). In BCR-ABL1 ALL, AID accelerates the clonal selection by suppressing regulation by tumor suppressor genes [(Rhoh, Cdkn1a (p21), and Blnk (SLP65)], increasing SHM and genetic instability (Gruber et al., 2010). Furthermore, elevated expression of AID promotes chromosomal lesions and is associated with the progression and survival of chronic lymphocytic leukemia (CLL) (Palacios et al., 2010).

There is evidence to suggest that AID mediated mutations can cause cancer in T cells as AID is expressed in CD4+ T cells. In an attempt to determine the mechanism accounting for the presence of somatic mutations in various genes during leukemogenesis in adult T cell leukemia (ATL) cells, Ishikawa and colleagues reported that the AID protein was expressed in lymph nodes and skin lesions. In addition, they found that AID is highly induced in cells infected with the T cell leukemia virus type 1 (HTLV-1) compared to un-infected T cells and PBMCs. They associated this aberrantly expression of AID to Tax, which is able to activate the transcription of AID via the NF-κB signaling (Ishikawa et al., 2011).

2.3.2 Role of AID in non-lymphoid cancers

The role of AID in non-lymphoid carcinogenesis is largely as a genotoxic factor. Its expression and dysregulation are induced by inflammation and microbial infection in non-lymphoid cells, proving a link between chronic inflammation and enhanced susceptibility to somatic mutations mediated by AID. AID has been implicated in the mutagenesis that causes cholangiocarcinogenesis during bile duct formation, where it causes mutations in cancer-related genes such as *c-myc* and *p53* (Komori et al., 2008). Moreover, almost 93% of human tissue with cholangiocarcinoma (CC) have significantly more AID mRNA transcripts that normal liver tissue (Komori et al., 2008). Similarly, while AID expression induced by pro-inflammatory cytokines induces mutations in TP53 in cells derived from the colon, its endogenous protein is enhanced in the mucosa of ulcerative colitis patients and colitis-associated colorectal cancer (Endo et al., 2008).

In mice, the mutagenic activities of AID that cause cancers in multiple organs have been demonstrated, linking inflammation to cancers both in humans and mice (Morisawa et al., 2008). Cancers such as Barret's esophageal adenocarcinoma (Morita et al., 2011), a subset of human lung (Shinmura et al., 2011) cancers and dysplasia-carcinoma (Shinmura et al., 2011), have been reported to be pathophysiological consequences of the mutagenic activity of AID. The persistence of *Helocobacter pylori* in gastric epithelial cells causes prolonged inflammation, which in turn induces the aberrant expression of AID, resulting in the accumulation of deletions in chromosomal loci (Matsumoto et al., 2007). In gastric cancer for instance it has been shown that AID, induced by *H. pylori* infection targets the tumor suppressor CDKN2b-CDNK2a locus (Matsumoto et al., 2010).

2.4 Transcriptional Regulation of AID Expression

To maintain the genomic stability of B and non-B lymphocytes, the expression and enzymatic activity of AID needs to be tightly regulated (Pasqualucci et al., 2008, Hasham et al., 2010). The regulation of AID expression and activity is multilayered and complex. In the first instance, the transcription of the AID gene that encodes AID is regulated by the binding of at least 19 different repressive and activating transcription factors (Stavnezer, 2011, Tran et al., 2010). In addition to the full-length mRNA, AID is also regulated by alternative splicing into four different known variants (Wu et al., 2008). Studies have shown that each B-cell expresses a specific splice variant and only the full-length mRNA has been reported to be functional since other variants do not have the required structure (van Maldegem et al., 2010) and may affect the efficiency of CSR in mice (Sala et al., 2015).

In addition to alternate splicing, microRNAs (miR) are used as an additional post-transcriptional regulatory mechanism. The abundance of the mature AID mRNA is regulated at least two confirmed microRNAs, miR-155 and miR-181b, which bind the the 3' UTR of the mature AID mRNA and target it for degradation (Teng et al., 2008). After translation of the mature mRNA, the activity and abundance of the protein is regulated by nuclear-cytosolic shuttling (Ito et al., 2004), phosphorylation (Pasqualucci et al., 2006), and stabilization by heat-shock proteins in the cytosol (Orthwein et al., 2010).

2.4.1 Structural Regulatory Elements of the AID gene

The structure of the AID gene offers an additional regulatory mechanism for its transcription. There are four distinct and conserved regulatory regions (Regions I, II, III and IV), arranged in parallel into cis-regulatory elements (CRE). These CRE regions bind to different transcription factors that either up-regulate or repress the transcription of the gene (Tran et al., 2010). Region-I contains the AID promoter with binding sites for NF-κB, HoxC4/Oct and the specificity proteins 1 or 3 (Sp1/Sp3) transcription factors. CRE IV lies about 9kb before of the AID promoter and contains domains for Stat6 and Smad3/4 docking. In addition, Pax5 and the transcription factor 3 (TCF3/E2A/E47) both have binding sights in region II. BATF has binding sites in region III located about 17Kb downstream of the promoter (Tran et al., 2010, Ise et al., 2011), (Figure 2.6).

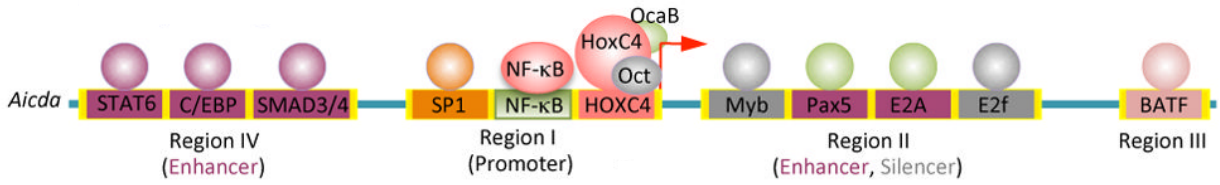

Figure 2.6: Structural regulatory elements of the AID gene
Adapted from (Zan and Casali, 2013)

To counteract the action of transcription inducers, the AID gene has four putative but conserved repressive CREs found in Region II. Two of these have been proposed to have binding domains for Myb, which can induce or suppress transcription depending on the DNA targets. In addition, the E2F family of transcription factors have also been proposed to have binding sites in other CREs, and hence can activate or repress transcription. Moreover a 350bp CT-rich sequence has been proposed to act as a silencer of transcription (Tran et al., 2010). In resting NBs and MBs, Myb and E2F are essential regulators of AID transcription, although this silencing effect can be dampened by stimuli that induce immunoglobulin class switching and some cytokines in B lymphocytes. Moreover, the activation of AID transcription mediated by Pax5 and E2A has been shown to be antagonized by the inhibitory E-box protein Id2 in switching B lymphocytes, leading to effective suppression of AID expression (Xu et al., 2007).

2.4.2 Transcriptional Regulation of AID Expression

2.4.2.1 NF-κB and HoxC4 Activation and the Induction of AID Transcription

The nuclear factor kappa-light-chain-enhancer of activated **B** cells (NF-κB) and the homoebox protein Hox-C4 (HoxC4) are the two principal factors that directly induce the transcription of the AID gene. NF-κB is activated via one of two stimuli. Stimuli that engage either CD40, TLR and BCR, induce the canonical NF-κB activity pathway, while those engaging TLR and TACI induce the non-canonical pathway, although signals that engage CD40, TLRs or TACI alone, have been shown to induce AID expression on their own (Pone et al., 2012, He et al., 2004). The activation of the NF-κB pathway is relevant to the induction of AID transcription in two ways. First, its activation results in the recruitment of NF-κB subunits to the promoter and enhancer regions of the AID gene. In the non-canonical pathway, p52 is recruited and it binds to the AID promoter (Park et al., 2009) while the canonical pathways recruits the NF-κB p65 subunit to an upstream enhancer element (Tran et al., 2010). Secondly, the kinetics of NF-κB induction and transcription of AID mirror each other. It takes from 48 to 60 hours after stimulation for the expression of AID to reach its peak, mirroring the expression dynamics of the non-canonical pathway of NF-κB (Pone et al., 2012). While the non-canonical pathway mediates a prolonged AID expression, the canonical pathway is rapid and transient (Smale, 2011). Therefore, signals that induce both pathways of NF-κB mediated gene expression are likely to results in a rapid and sustained expression of AID.

The challenge with linking NF-κB activation with AID expression is that it is expressed in copious amounts. Hence, there must be additional genes it activates that are specific to B cell lineage and differentiation, to link its activation to AID regulation. Other transcription factors have been shown

to regulate AID expression (Tran et al., 2010). Chief among these is HoxC4, the only member of the Hox gene family that is preferentially expressed in murine and human B lymphocytes. HoxC4 expression is induced by AID-inducing stimuli, CD154 $(CD40L)^{+}$IL4 in human B lymphocytes and $CD154^{+}IL4^{+}$ TGF-β in murine B lymphocytes (Meazza et al., 1995).

Park and colleagues have demonstrated that HoxC4 directly binds to the conserved AID promoter core, and induces AID transcription. Deficiency in HoxC4 leads to Severe impairment of SHM and reduces the efficiency of CSR in $HoxC4^{-/-}$ B lymphocytes, although both can be reversed with re-expression of AID (Park et al., 2009). The function of HoxC4 has been well characterized. Stimulating HoxC4-deficient mice with CD154 and IL4 does not result in significant reduction in SHM and CSR, both of which can be restored with enforced AID expression (Boulet and Capecchi, 1996). Although the binding of HoxC4 to DNA is weak, it interacts with the homeodomain transcription factors Oct1 and/or Oct2, and the co-activator OcaB to form a complex with either HoxC4, Oct1/2 or OcaB, all of which forms part of protein complexes that enhance the transcription of AID (Qin et al., 1998).

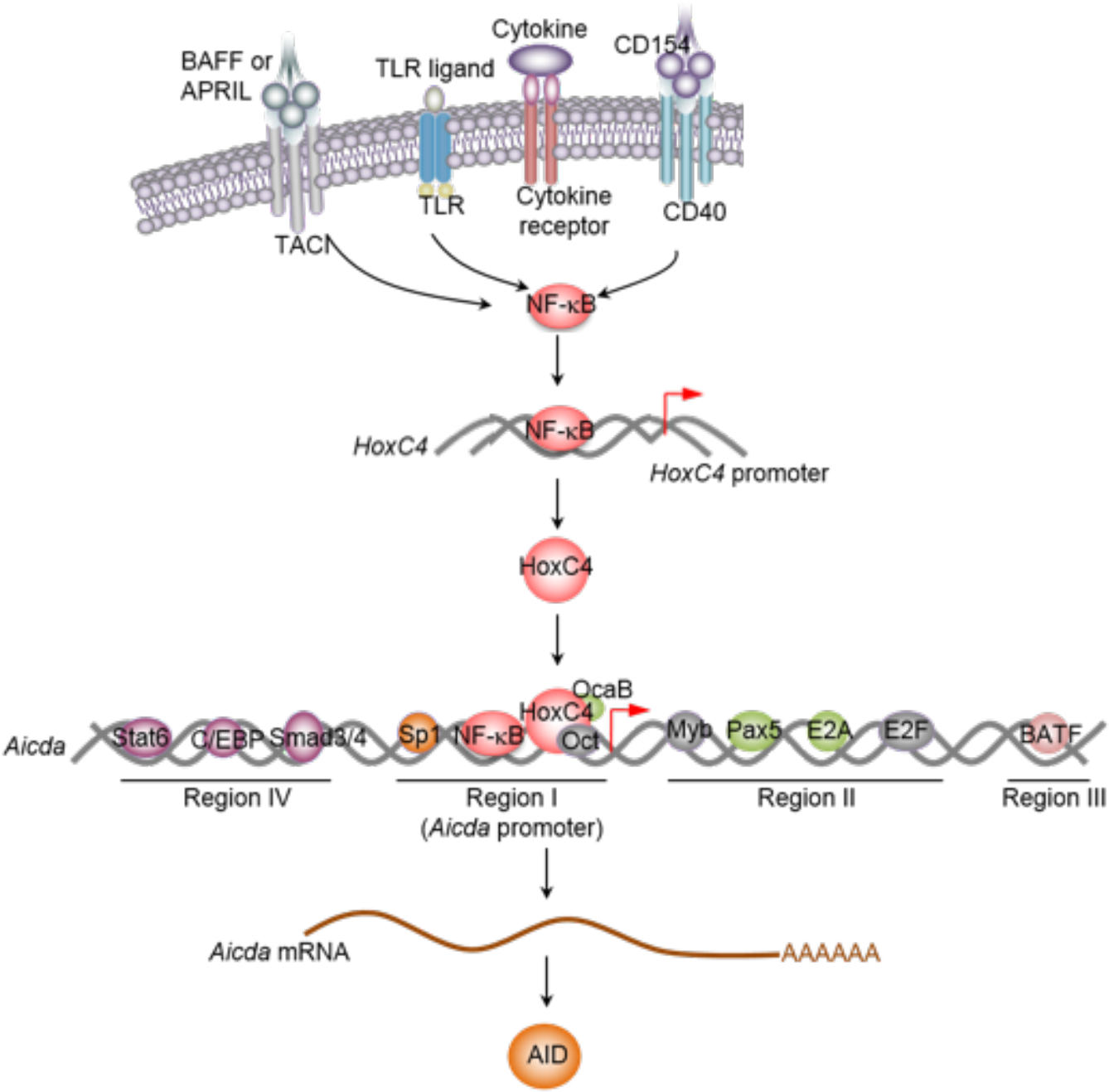

Figure 2.7: NF-κB mediated activation of AID transcription
Adopted from (Zan and Casali, 2013)

2.4.2.2 E47 Enhances AID transcription

The *e2a* gene, encodes two proteins of the basic helix-loop-helix (bHLH) protein family, E12 and E47. These proteins are known to bind to DNA at enhancer sequence sites (E-box), and are essential for B cell activation, maturation and development. Thus far, the role of E47 in regulating AID expression is quite understood, albeit in mice (Xu et al., 2007). Centroblasts of murine germinal centers are the highest expressors of E47. Its expression is however moderately reduced in centrocytes, and drastically reduced in murine plasma and memory B lymphocytes. This pattern mirrors that of AID and key activators of AID transcription, Pax5 and HoxC4, suggesting that E47 is part of the enhanceosome that activate AID expression (Xu et al., 2007). Coincidentally, binding of DNA by E47 and the expression of AID are both induced by the same stimuli, CD40, LPS, IL4 and BCR crosslinking, *in vitro* (Quong et al., 1999). E47 binds to two E-box sites in the conserved Region II in the first intron of the AID gene and transactivates the AID promoter. In addition, E47 can bind to another E-box site in the AID promoter, close to the binding sites for Pax5 and HoxC4, and lead to a synergistic induction of the transcription of AID (Sayegh et al., 2003).

The transactivating properties of E47 are antagonized by transcription factors of the "inhibitor of DNA-binding" protein (Id) family. Typically, Id1, Id2, Id3 and Id4 do not possess structural binding domains required for direct interaction with DNA. Hence by forming heterodimers with E-box binding proteins such as E12 and E47, they prevent binding to their target DNAs and inhibiting the transactivation of their target genes (Murre, 2005). The predominantly expressed Id proteins in mature B lymphocytes are Id2 and Id3. Although Id3 deficiency has been linked to sub-optimal CSR, which can be reversed by enforced expression of AID in murine splenic B lymphocytes (Sayegh et al., 2003), its expression is induced by the crosslinking of the BCR, and not by CD40L, IL4 or LPS. In addition, the expression of Id3 is essential to the induction of

proliferation in response to BCR crosslinking in B lymphocytes, but not CD40, IL4 or LPS-activated B-cell proliferation. Hence, the suppression of AID expression offered by Id3 does not depend on cell proliferation, implying that Id3 is not involved in the down-regulation of AID expression during the GC reaction (Pan et al., 1999).

Conversely, the AID-related transcriptional inhibitory activity Id proteins points more to Id2 than Id3. In plasma cells where AID expression is down-regulated, Blimp1 represses not only the expression of the *e2a*, with concomitant decreased expression of E47, but the expression of Id3 as well (Shaffer et al., 2002). Blimp1 also suppresses Bcl6 expression. Bcl6 suppresses the transcription of the *Id2* gene, resulting in the up-regulation of Id2 expression in plasma cells (Shaffer et al., 2000). Moreover, stimulating $Id2^{-/-}$ B lymphocytes with LPS+IL4 induces a five-fold expression of AID transcripts relative to wild type B lymphocytes, implying that Id2 may act by down-regulating the expression of AID (Gonda et al., 2003). An enforced expression of Pax5 is enough to overcome the AID-related inhibitory effect of Id2, suggesting that in centroblasts, in the presence of Pax5 and E2A, the potential inhibition of AID expression by Id2 is suppressed (Xu et al., 2007).

2.4.2.3 Pax5 and AID expression

The paired protein box 5 (Pax5) is another transcription factor known to regulate the transcription of the AID gene. It is essential in maintaining the identity of B lymphocytes throughout the B-cell ontogeny. It plays a dual transcriptional regulatory role in B lymphocytes, a function that results from its transcription-activation and transcription-repression domains (Cobaleda et al., 2007). Pax5 expression and activity is significantly up-regulated in murine and human GC B lymphocytes undergoing CD40 and/or IL4-induced SHM (Lin et al., 2002, Merluzzi et al., 2004). The kinetics

of the expression of Pax5 mirrors that of AID, peaking after 2 days and declining after 4 days post stimulation with LPS (Schrader et al., 2005). More compelling data show that when the expression of Pax5 is enforced in murine pro-B lymphocytes, the expression of the otherwise silent AID is induced. The same group later reported that Pax5 regulates the expression of AID by binding directly or indirectly to the AID promoter (Gonda et al., 2003).

2.4.2.4 Role of Irf4 and Irf8 in the Regulation of AID Expression

Two interferon (IFN) regulatory factors (Irf), Irf4 and Irf8, regulate the expression of AID. They possess a characteristic DNA-binding domain made of five conserved tryptophan repeats, with which they can weakly bind to IFN-inducible genes. Apart from binding to DNA, Irf4 and Irf8 can bind to each other, as well as other proteins, including Stat6 and E47 as part of their transcriptional regulatory programs in proliferating and differentiating B and T cells (Taniguchi et al., 2001). Functionally, both Irf4 and Irf8 are preferentially recruited to form components of protein complexes that interact with DNA. The Irf4 has been shown to form a complex with the Pu.1 protein, while Irf8 complexes with SpiB, both of which can bind to Irf specific ETS component elements (Escalante et al., 2002).

Although expressed by other cells, Irf4 and Irf8 are predominantly expressed by B and T cells. Irf4 initiates the AID transcription in pre-GC B lymphocytes. While it is highly expressed in immature B lymphocytes of murine bone marrow and maximally expressed in plasma cells, centroblasts in the GC are low expressers of Irf4 (Cattoretti et al., 2006). When $Irf4^{-/-}$ murine GC B lymphocytes are challenged with either CD154 or LPS in the presence of IL4, there is suppressed AID expression, coupled with impaired differentiation into plasma cells. Irf4 enhances the

expression of AID in one of two ways; by activating trans-factors such as Pax5 or by down-regulation the expression of transcriptional repressors of AID expression, such as Id2 (Klein et al., 2006).

The induction pathways and functions of Irf4 and Irf8 seem to overlap as suggested by Lu and colleagues who have shown that murine bone marrow pre-B lymphocytes that are double deficient for Irf4 and Irf8 ($Irf4^{-/-}$ and $Irf8^{-/-}$) fail to transition to B lymphocytes, while those deficient in either Irf4 or Irf8 can mature into B lymphocytes. They however show that the expression pattern of Irf8 is slightly different from that of Irf4. It is maximally expressed in the dark zone GC B lymphocytes, down-regulated in circulating B lymphocytes and moderately expressed in bone marrow B lymphocytes (Lu et al., 2003). As displayed by the expression and function of Irf8 in the light zone GC B lymphocytes, deficiency in Irf8 results in a marked reduction of Bcl6 and AID transcripts, and its expression is negatively regulated by Blimp1 in plasma cells (Shaffer et al., 2002).

2.4.2.5 Blimp-1 is a negative regulator of AID expression

The B lymphocyte-induced maturation protein 1 (Blimp-1), encoded by the beta-interferon positive-regulatory domain 1 binding factor (Prdm-1) gene, is considered a master regulator of plasmacytoid differentiation of B lymphocytes. It inhibits a range of transcription factors that are known to enhance the expression of AID (Shaffer et al., 2002). In $Prdm\text{-}1^{-/-}$ cells stimulated with LPS alone or together with IL4, an increased CSR is reported, suggesting that Blimp1 is a negative regulator of AID expression (Omori et al., 2006). Blimp1-mediated AID inhibition occurs via three mechanisms; either via direct suppression by binding and inactivating the AID promoter, repression of Pax5, E2A, Irf4, or by up-regulating Id2 expression. This repression of AID by

Blimp-1 cannot be rescued by enforced Pax5 re-expression of Pax5, because Blimp1 inactivates the AID promoter via its putative binding site (Shaffer et al., 2002).

Indirectly, the Blimp1-mediated repression of AID expression is achieved either through the up-regulation of Id2 or Irf4 expression. As discussed earlier, Id2 inhibits the expression of Pax5 and E2A. The repression of AID expression by Blimp1 through Irf4 is somehow convoluted. In centroblasts, Irf4 indirectly induces AID by up-regulating intermediate *tans*-activators of AID transcription by suppressing Id2. The repressive function of Blimp1 is however powerful enough to override these activities of Irf4. Moreover, because Irf4 also up-regulates the expression of Blimp1, there seem to be a negative feedback inhibition of Irf4 mediated AID induction by Blimp1 (Klein et al., 2006, Sciammas et al., 2006).

2.4.2.6 Bach2 and Bcl6 Up-regulate AID expression by suppressing Blimp-1

In GC B lymphocytes, the Blimp1-mediated down-regulation of AID is relieved by the action of the BTB domain and CNC Homolog 2 (Bach2) protein (Ochiai et al., 2006). This is confirmed by the evidence that stimulating BACH2 deficient ($Bach^{-/-}$) B lymphocytes with LPS significantly reduces the abundance of AID transcripts with concomitant reduction of CSR. However, this defect in AID transcription and CSR could be rescued with enforced AID expression, corroborating the thought that BACH2 is essential for both SHM and CSR by regulating other important genes involved in the process (Muto et al., 2004). It is however not clear how BACH2 is down-regulated in plasma cells to allow for the AID repression mediated by Blimp1.

The B-cell lymphoma 6 (Bcl6) protein negatively regulates Blimp-1 expression in B lymphocytes transitioning between the germinal center reaction and plasmacytoid differentiation (Niu et al.,

1998). Although Pax5 also suppresses Blimp-1 expression, its expression is down-regulated in B lymphocytes transitioning to plasma or memory cells, therefore, Bcl6 is the major negative regulator of Blimp-1 induction in B lymphocytes at the transitional stages. Bcl6 is regulated at the post-transcriptional/post-translational level in response to BCR crosslinking as demonstrated in Ramos B lymphocytes, known to undergo spontaneous somatically hypermutate (Niu et al., 1998).

2.4.3 MicroRNAs and AID Expression

MicroRNAs (miRNAs) are short (~22 nucleotides) non-coding RNAs, many of which play important roles in regulating gene expression. Mammals express thousands of microRNAs. These miRNAs together control the expression of many protein coding genes. Functionally, miRNAs bind to evolutionarily conserved sequences within the 3′ untranslated regions (3′ UTR) of their target mRNAs. This binding may either result in the degradation of the target mRNA and/or inhibition of translation, thereby negatively regulating protein expression at the post-transcriptional level (O'Connell et al., 2010).

In the development and differentiation of B lymphocytes, miRNAs play important roles. In cells actively undergoing CSR, and SHM and differentiation, microRNAs have been reported to be important regulators of genes that are essential to these processes (Li et al., 2013). Typically, miR-155, miR-181b and miR-361, have been shown to suppress the expression of AID, and miR-30a and miR-125b have been reported to be involved in the silencing of Blimp-1, by binding to conserved miRNA sites in the 3'UTRs of AID and Prdm-1 mRNA, and by causing their degradation (White et al., 2014).

The role of miR-155 in regulating the expression of AID in the germinal center reaction has been studied quite extensively. It is coded for by the *MIRN155* gene in humans. Its deregulated expression has been reported to be associated with various cancers (Eis et al., 2005). The oncogenic nature of miR-155 is two-sided. Its overexpression is a significant risk for the generation of high-grade B-cell neoplasm, possibly by increasing the proliferation of pre-B lymphocytes in murine bone marrow and spleen (Costinean et al., 2006). On the other hand, in cancers such as Burkitt's lymphoma, the mature form of miR-155 is lowly expressed or absent (Kluiver et al., 2005, Kluiver et al., 2006).

The precise contribution of miR-155 to lymphomagenesis is not known, but the microRNA is an essential modulator of the immune response whose expression is turned-on by stimuli that induce immunoglobulin class switching, together with AID in GC B lymphocytes. In addition, miR-155 may be involved in the cellular mechanisms involved in stabilizing microRNAs, as its deficiency in B lymphocytes leads to the degradation and deregulated expression of hundreds of microRNAs, including those that are targets of miR-155. This deficiency has been reported to result in a defective GC reaction and impaired antibody response *in vivo* (Thai et al., 2007). Dorsett and colleagues (2008) have reported that the down-regulation of AID mRNA levels caused by miR-155 in $AICD^{+/-}$ B lymphocytes is associated with a down-regulated class switching and a reduced frequency of c-Myc/IgH translocations (Dorsett et al., 2008). In addition, disrupting the binding site of miR-155 in the mature AID mRNA increases the levels and nuclear half-life of the mature AID mRNA and protein, respectively, with concomitant rise in CSR and increased occurrence of the c-Myc/IgH translocation (Teng et al., 2008, Dorsett et al., 2008).

In germinal center B lymphocytes, the repressive activity of miR-155 with regards to AID expression has to be down-regulated. This is achieved primarily by the action of Bcl6, a positive regulator of AID expression. In B lymphocytes undergoing the germinal center reaction, Bcl6 is responsible for the repression of miR-155 and miR-361 expression. By binding the second intron in and within a 300bp sequence 5' of the transcription start site of the miR-155 host gene (*MIR155HG*), Bcl6 represses the transcription of miR-155 (Basso et al., 2012).

The 3'UTR of the AID mRNA has multiple binding sites for miR-181b and the miR has been reported to be a negative regulator of AID expression in B lymphocytes. Using miR retroviral vectors transduced into primary B lymphocytes, De Yébenes and colleagues have shown that miR-181b induces up to a 70% decrease in CSR (de Yebenes et al., 2008). They further show that the expression of miR-181b was independent of cell proliferation, but on the activation status of B lymphocytes, as miR-181b expression is down-regulated in B lymphocytes stimulated by LPS and IL-4. They conclude that miR-181b is essential to the regulation of CSR in resting, but not activated B lymphocytes, possibly via the down-regulation of AID expression (de Yebenes et al., 2008). It is not yet clear how the expression of miR-181b is regulated. The current understanding is that its expression is highest in B lymphocytes that are not undergoing CSR and SHM, implying that it plays a role in preventing the diversification of Ig gene in naïve, resting and terminally differentiated B lymphocytes.

2.4.4 Regulation of AID by Alternate mRNA Splicing

In addition to transcriptional and post-transcriptional regulation by transcription factors and microRNAs, recent studies have reported the existence of alternatively spliced AID mRNA, suggesting an additional regulatory mechanism for the expression of AID (Wu et al., 2008, Iacobucci et al., 2010). The full-length AID mRNA (AID-FL), along with the alternatively spliced variants AID-ΔE4, AID-ΔE4a, AID-ivs3, and AID-ΔE3E4 have been detected in both tumors and normal B lymphocytes (Albesiano et al., 2003, McCarthy et al., 2003). Interestingly, exons 1 and 2 are preserved in all 4 splice variants, conserving their nuclear localization sequence (NLS) and the N-terminus required for SHM. AID-ΔE4a has part of exon 4 spliced off, but still retains the nuclear export sequence (NES) domain and the CSR specific domain, close to the C-terminus. The splice variant AID-ΔE4 has exon 4 completely spliced and a translational stop codon at the 3' end of exon 3, while and AID-ΔE3E4 has both exon 3 and 4 spiced off. Although the AID-ivs3 splice variant retains all five exons, there is an intervening sequence inserted between exons 3 and 4, containing a premature stop codon (Figure 2.8).

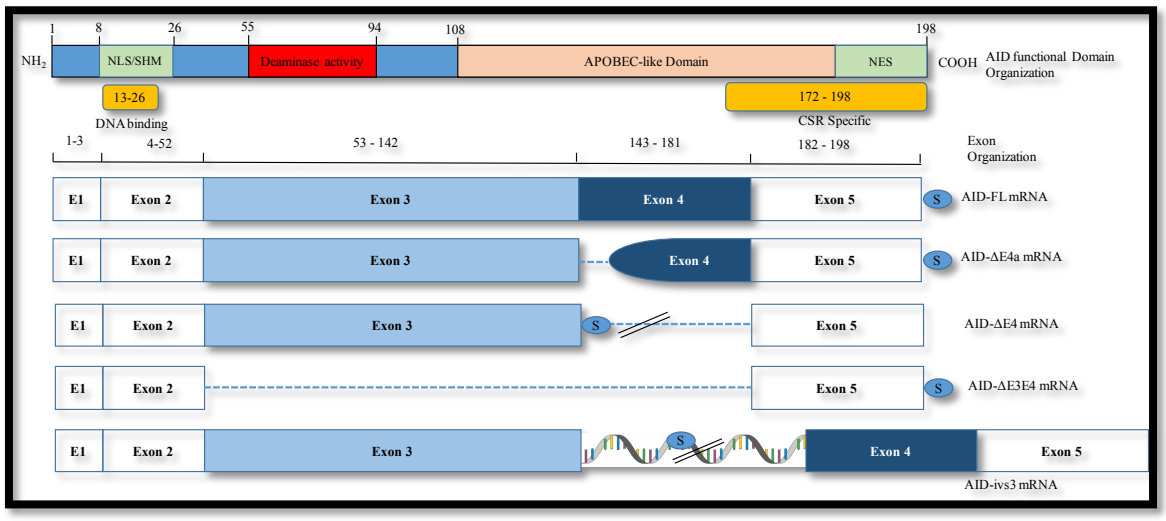

Figure 2.8 Functional Domain and Exon organization of AID and its mRNA splice variants. Adopted from (Zan and Casali, 2013) and (Wu et al., 2008)

The splicing of portions of the AID mRNA has consequences for the functions of the translated AID protein. When AID-ΔE4, AID-ΔE4a, and AID-ivs3 are cloned and transfected into cell lines, they show altered subcellular localization patterns as a result of the silenced nuclear export sequence at the C-terminus (Wu et al., 2008). The deamination activity of these splice variants is still a subject of conflicting debate.

While some studies have reported that AID-ΔE4 and AID-ΔE4a have increased mutational activity by reverting an inactive GFP reporter gene (McCarthy et al., 2003), other studies also report that the splice variants are biochemically inactive (van Maldegem et al., 2010). It is however agreed that these splice variants cannot mediate CSR (Wu et al., 2008). So far, all four splice variants have been detected in B lymphocytes derived from both murine and human GCs (Wu et al., 2008) as well as chronic lymphocytic leukemia (CLL) cells (McCarthy et al., 2003). In CLL, the level of AID is positively correlated to the severity of disease (Patten et al., 2012) and since the splice variants have been reported to have altered mutational activity, it has been suggested that the splice variants could be a regulatory mechanism of moderating the levels of functional AID-FL protein or even contribute to off-target activity (Wu et al., 2008).

In summary, the transcriptional and post-transcriptional regulation of AID is three-fold. First, the induction of AID transcription involves a complex layer of primary and secondary stimuli that activate the NF-κB signaling pathway. This leads to the activation of different transcription factors, with different binding domains in the AID gene, which in turn, can lead to induction or suppression of AID transcription. After transcription, miRNAs such as miR-155 and miR181b regulate the abundance of the mature mRNA, while alternative mRNA splicing is employed to regulate the

activity of the enzyme (Figure 2.9). Although there are other regulatory layers for AID expression, the current study focuses on the transcriptional and post-transcriptional regulatory pattern of AID expression.

2.5 *P. falciparum* and the expression of AID

Plasmodium falciparum is one of the parasites that grows and multiplies in restricted niches of their hosts (Soulard et al., 2015). As a result, the parasite can limit contact with the host's immune system, thereby colonizing the host and transmitting itself for a long time. This chronic infection results in a dysregulated immune system with overactivated B cells and the secretion of autoantibodies (Adu et al., 1982, McGregor et al., 1956). Early studies suggested that antigens associated with *P. falciparum* infection acted as potent activators of B cells, leading to increased levels of antibodies (Greenwood and Vick, 1975b, Greenwood et al., 1979), which is a prominent feature of human malaria (Abele et al., 1965). The mechanism underlying this polyclonal activation of B cells remains partly understood, because the identity of the antigens responsible for this activation are still unknown. It has been shown that the cysteine-rich interdomain region (CIDR1-α) of the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), expressed on the surface of *P. falciparum*-infected red blood cells, can interact with and induce B-cell activation and proliferation (Donati et al., 2004). Later, the same group demonstrated that the interaction between CIDR1-α and B cells latently infected with EBV, results in the reactivation of the virus, thereby increasing the EBV^+ positive B-cell number (Chêne et al., 2007).

One of the important biological features of *P. falciparum* infection is the exceptional polymorphic antigenic variation exhibited by the parasite, demonstrated in the expression of *Pf*EMP-1 (Scherf et al., 2008). Notably, the parasite is able to switch between 60 different antigenic forms of *Pf*EMP-

1 as part of its immune evasion strategies (Scherf et al., 2008). In addition to enhancing the immune evasion strategies of the parasite, the diversity of antigens accounts for major clinical consequences as shown in the ability of P*f*EMP-1 to mediate different cellular interaction and pathologies by infected erythrocytes (Claessens et al., 2014, Lavstsen et al., 2012). The variety of antigens expressed by the parasite could account in part to the activation of several clones of B cells.

The polyclonal activation of B lymphocytes by *P. falciparum* antigens has direct implications for the expression of AID. Work done by Robbiani and colleagues demonstrate that *Plasmodium* infection in mice promotes AID-mediated genomic instability and the development of AID-dependent lymphomas of B cells origins (Robbiani et al., 2015). In their work, they report that although the malaria parasite may not be an absolute requirement for the development of lymphomas in murine B cells, the infection favors mature B cell cancers through the protracted expression of AID in GC B cells (Robbiani et al., 2015). This work supports the theory that malaria infection increases the throughput of B cells through the geminal centers of secondary lymphoid tissues (Torgbor et al., 2014). Using primary human tissue, Torgbor and colleagues demonstrated that that chronic infection with *P. falciparum* malaria have high frequencies of GC B cells that express elevated levels AID expression (Torgbor et al., 2014). Together, these studies demonstrate the potency of *P. falciparum* antigens to induce the expression of AID in geminal centers of lymphoid tissues. The question of how the expression of AID is regulated in response to stimulating *P. falciparum* antigens still remains unclear.

2.6 Summary of Literature

The literature reviewed here indicate that AID plays an important role in the biology of the immune response, as well as the pathobiology of several neoplasms. The documented cellular mechanisms for the regulation of AID is rigid and complex. Figure 2.9 summarizes the documented regulatory mechanisms at the transcriptional and post-transcriptional levels of AID expression. Despite this tight regulatory mechanism, infection with *P. falciparum* is reported to result in a deregulated AID expression and activity. Information on how infection with the parasite can circumvent these regulatory mechanisms is scarce. Firstly, the roles of transcription factors known to have an effect on the expression of the enzyme is well documented, but little is known about how the activity of these transcription factors is affected by infections with the parasite. There is also scare data regarding how infection with the parasite affects the expression microRNAs that regulate the abundance of AID mRNA. Alternate mRNA splice variants of AID have been reported and some data is available of which of these variants has biological activity. However, it is not known how infection with malaria affects the expression of these splice variants.

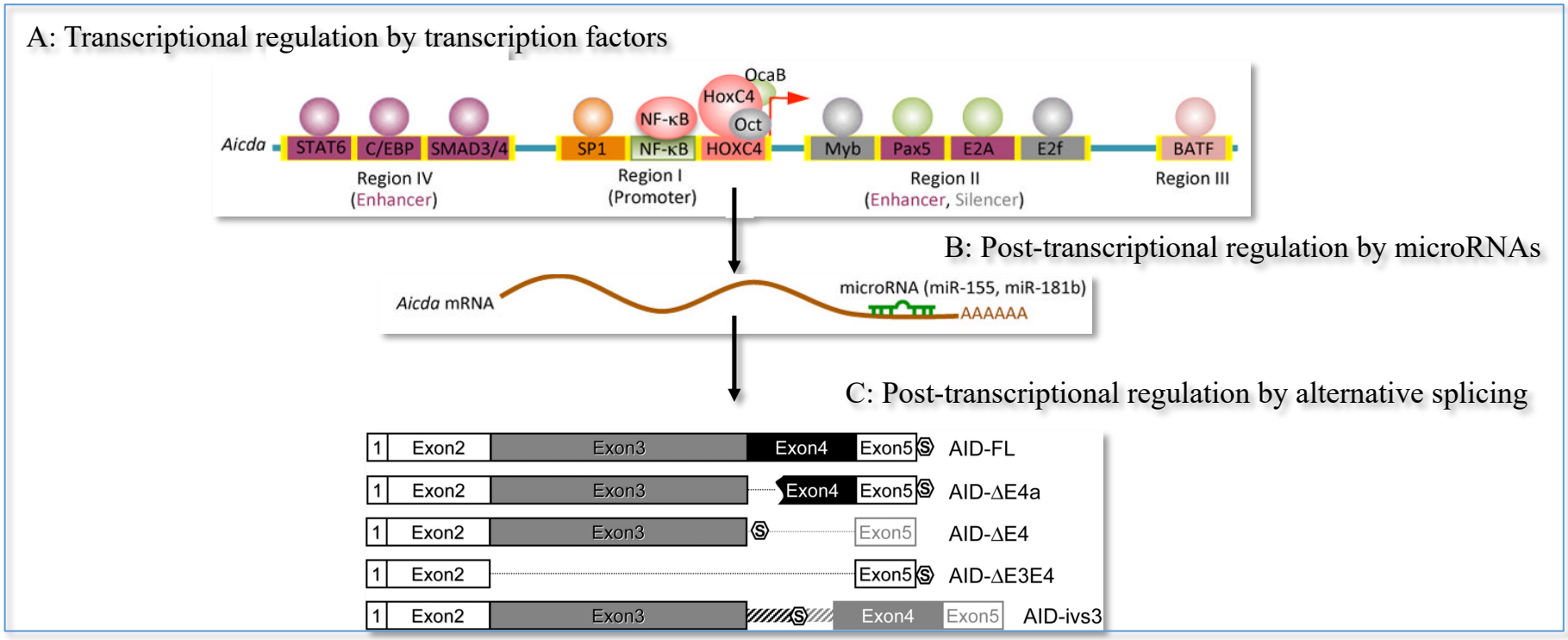

Figure 2.9: Summary of transcriptional and post-transcriptional regulation of AID.
Adopted from (Zan and Casali, 2013) and (Wu et al., 2008)

CHAPTER THREE

3.0 METHODS

3.1 Study Design, Participants and Study Sites

Three types of samples were used for this work. Tonsillar tissue from children between ages 6 to 16, undergoing routine tonsillectomies at the EENT Department of the Cape Coast Teaching Hospital (CCTH) was used to achieve the objectives for specific aim one (1).

To satisfy aim two (2), peripheral blood samples were taken from healthy primary school children in the Ho West District of the Volta Region.

For aim three (3), archived RNA samples with matched plasma from children with fever, with or without *P. falciparum* infection were obtained from the laboratories of Dr. Douglas J. Perkins, at the Center for Global Health, Department of Internal Medicine, Health Sciences, University of New Mexico. Children with *P. falciparum* infection were later classified into those with (SMA) without severe anemia (non-SMA).

3.1.1 Collection of Tissue Samples

3.1.1.1 Ethical Statement and Exclusion Criteria

The collection of Tonsillar tissue was approved by the Institutional Review Board of the Cape Coast Teaching Hospital (CCTH). Tonsillar tissue samples were collected only after informed parental consent and accent from children had been obtained. As HIV infection is a confounding factor for this study, children with known clinical history of HIV infection were excluded from the study. Cape Coast is the Regional capital of the Central Region of Ghana. The city lies 05°05' N, 01° 15' W, and is considered an urban setting in the Coastal savannah region on the Gulf of Guinea. Although the transmission of malaria in Cape Coast is seasonal, with a high prevalence in the rainy

infection as detected by both microscopy and PCR does not vary across both rainy and dry seasons (Ayanful-Torgby et al., 2018).

3.1.2 Sampling of healthy Asymptomatic *P. falciparum* infected children

3.1.2.1 Ethical Statement, Inclusion and Exclusion criteria

Sampling of blood from primary school children in the Ho West District of the Volta region of Ghana was approved by the Institutional Review Board of the Ghana Health Service (GHS-ERC: 29/11/15), as part of a bigger study to evaluate the prevalence of polyparasitic infection among primary school children (Ages 6-14) in the Volta Region (Orish et al., 2019). Blood samples from children with other parasitic infections as diagnosed in stool and urine was excluded from this work.

3.1.2.2 Study Site – Dzolokpuita, Ho West

Dzolokpuita is the district capital of the Ho West district of the Volta Region of Ghana and lies in a region that has been described previously (Dinko et al., 2016). The district lies between latitudes 6.33º32”N and 6.93º63”N and longitudes 0.17º45” E and 0.53º39”E. Although the district is located in the mountainous middle belt of the Region, close to the highest points in the country, Dzolokpuita lies in the valleys between the highlands of Amedjofe and Taviefe. There are two rainfall regimes, the major season in April-July and a minor season in September-November.

3.1.3 RNA and Plasma Samples from Children with SMA

Archived RNA samples extracted from 81 PBMCs, with matched plasma (65) was retrieved from the laboratories of Dr. Perkins at the Center for Global health, Internal Medicine, University of New Mexico. These are day-one samples taken from children recruited from the pediatric ward of the Siaya County Referral Hospital in the Siaya County, Nyanza Province in Western Kenya between 2009 and 2012. The area lies 1,140 to 1,430m above sea level with an annual rainfall between 800 and 2000 mm and temperatures ranging between 15ºC and 30ºC (Ong'echa et al., 2006). This region is known to be holoendemic for *P. falciparum* malaria transmission (Bloland et al., 1999b), with peak transmission between April and August (Beier et al., 1994).

In selecting samples for this study, samples from children with bacteremia, as diagnosed by blood culture, and HIV as diagnosed by serology and PCR, were excluded. Those with fever, with or without *P. falciparum* infection as diagnosed by microscopy and PCR were selected for the current study. The samples were grouped into three (3) main categories. Those with febrile illness, but no evidence of *P. falciparum* infection were grouped together as Aparasitemic (AP). Those with *P. falciparum* infection were further grouped into two main groups. Samples with hemoglobin (Hb) levels below 6.0 g/dL were classified as having Severe Malaria Anemia (SMA), and those with Hb levels above 6.0 g/dL were classified as non-SMA samples (Ong'echa et al., 2006).

3.2 Processing of Tonsillar Tissue and Isolation of Mononuclear Cells (MNCs)

3.2.1 Lysis of Tonsillar tissue for DNA extraction

The tissues were transported from the Cape Coast Regional Hospital in cold PBS supplemented with 0.5% BSA (PBSA) to the Cell Biology and Immunology (CBI) laboratories at the West African Center for Cell Biology for Infectious Pathogens (WACCBIP), University of Ghana, for processing. Processing of tissues and isolation of total MNCs started 4-5 hours post-surgery. Tissues were thoroughly washed in PBSA to remove blood and cut open with tweezers. A 25mg tissue sample was weighed in a 1.5ml Eppendorf tube and incubated in 180µL of QIAamp DNA Blood Mini Kits lysis buffer (Buffer ATL), with 20µL of proteinase K at 56^{o}C, with intermittent shaking, until tissue was completely lysed. The lysate was stored at -80^{o}C for DNA extraction later.

3.2.2 Isolation of Total Tonsillar Mononuclear cells (MNCs)

Isolation of total MNCs was performed as described by (Torgbor et al., 2014) with modifications. Briefly, the tissue was homogenized in a closed tissue grinder (Fischerbrand®, USA) in cold PBSA. Tissue homogenate was sieved into 50 ml falcon tubes through a 0.4micron cell strainer to eliminate connective tissue and debris. The filtrate was centrifuged at 1800rpm for 8 minutes and the supernatant was discarded. The pellets were pooled and re-suspended in fresh PBSA to the 50ml mark. Approximately 25 ml of the cell suspension was layered onto 20 mls of Lymphoprep™ (Axis Shield PoC AS, Norway) and centrifuged at 1800rpm for 30 minutes at room temperature with no brakes. Total MNCs were collected at the interphase (buffy coat) into new 50 ml falcon tubes and the volume was adjusted to 50 ml with PBSA. An aliquot was taken for cell counting and determination of viability using trypan blue exclusion. The buffy coats were washed twice in

PBSA and cryopreserved in fetal bovine serum (FBS) (Sigma, St. Louis, USA) supplemented with 10% dimethyl sulfoxide (DMSO).

3.2.3 Extraction of DNA from Tonsillar tissue

Total DNA was purified from the tonsil lysate using the QIAamp DNA Blood Mini Kit. Frozen Tonsillar tissue lysate (section 3.2.1) was thawed at room temperature and mixed well by vortexing. Exactly 200 µL of Buffer AL was added to tissue lysate and mixed well. The mixture was incubated at 70ºC for 10 minutes. The mixture was briefly centrifuged. Later, 200 µL of absolute ethanol was added and mixed. The mixture was carefully applied to the QIAmp Mini spin column, placed over a 2mL collection tube. The column was capped and centrifuged for 6000 x g for 1 minute. The column was place into a new collection tube and the flow though was discarded. 500 µL of Buffer AW1 was added to the column and spun at 6000 x g for 1 minute. Exactly 500 µL of Buffer AW2 was added directly to the column and centrifuged at 12000 x g for 3 minutes. The column was placed in a new collection tube and centrifuged at 12000 x g for 1 minute. To elute the DNA, the column was transferred to a clean nuclease free 1.5 mL Eppendorf tube and 200 µL of nuclease free deionized-water was carefully added directly to the column and incubated at room temperature for 1 minute, followed by centrifugation at 6000g for 1 minute. The eluted DNA was stored at -20ºC for later use.

3.2.4 Extracellular staining for flow cytometric sorting

Cryopreserved Tonsillar MNCS were quick-thawed at 37^{o}C and washed in warmed staining buffer (PBS supplemented with 0.1% NaN_3 and 0.5% BSA) and stained immediately without *ex vivo* culture or stimulation. Briefly, about $4x10^7$ cells were incubated with 2 µL of 5µg/mL each of the following fluorophore conjugated antibodies on ice for 30 minutes; 7AAD viability stain (Biolegend, CA, USA), fluorescein isothiocyanate conjugated anti-human CD3 (FITC-CD3, clone SK7, Biolegend, CA, USA), pacific blue conjugated anti-human CD19 (PB-CD19, clone SJ25C1, Biolegend, CA, USA), pacific blue conjugated anti-human CD20 (PB-CD20, clone 2H7, Biolegend, CA, USA), allophycocyan conjugated anti-human IgD (APC-IgD, clone IA6-2, Biolegend, CA, USA) and phycoerythrin conjugated anti-human CD38 (PE-CD38, clone HIT2, Biolegend, CA, USA). The cells were then washed twice in staining buffer, resuspended in 500 µL staining buffer and kept on ice until sorting.

3.2.5 Multiparametric Flow cytometric sorting of tonsil MNCs

Tonsil MNCs were sorted on the sy3200 Cell Sorter (Sony Biotechnology Inc, USA) at the flow cytometry core facility, Health Sciences, University of New Mexico. Single-stained and unstained controls were used to set compensation using the WinList software using unstained and single-stained control. Immediately before acquisition, the cells were filtered through a 2.0µm filter (Cell Strainer, BD Biosciences, USA) to remove cell clumps. Before the actual sorting, a total of 200,000 events per tube were acquired to define the gating regions and strategy.

3.3 Sampling and Processing of Venous blood from Healthy Primary School Children

3.3.1 RDT, Blood Smear and filter paper spots

Venous blood (3 mL) was collected from children between ages 6-14 years at the Dzolokpuita Evangelical Presbyterian Primary School into tubes with anticoagulant (EDTA) during the minor rain season in November 2016. A drop of blood was used for a spot test of *P. falciparum* infection with the ONE STEP Malaria *P.f.*HRP-II Antigen Rapid Diagnostic Test (RDT) (#05FK58, Standard Diagnostics Inc. Korea). The RDT results were read after 15minutes and recorded. To test for *P. falciparum* infection by microscopy, a thick smear was also prepared on a clean, well labeled frosted end slide on the spot and allowed to dry. These slides were kept in a slide box, with a desiccant, until staining for light microscopy. Too blood spots per sample were made on a Whatman Protein Saver^{TM} 903 filter paper (GE Healthcare BioSciences Corp.PA, USA). The spots were allowed to dry and kept in a box with a desiccant for later use. The Remaining blood was kept on ice in a box and transported to the Volta Regional Hospital in Ho for polymorphonuclear cell (PBMC) separation.

3.3.2 Isolation of Plasma and PBMCs from whole blood

Purification of total WBCs from whole blood was carried out at the Laboratories of the Volta Regional Hospital 2-3 hours post blood collection. For plasma separation, blood was spun at 1800rpm for 10minutes. About 400 µL of plasma was pipetted into new 1.5 mL Eppendorf tubes and kept at -80^{o}C for later use. The remaining whole blood (about 2.5mL) was diluted 1:1 with 1X PBS and carefully layered on 3 mL of room temperature-warmed Lymphoprep^{TM} (Axis Shield PoC AS, Oslo, Norway), in a 15 mL falcon tube. The tube was then centrifuged at 1800 rpm for 30minutes with no breaks. The upper layer was aspirated and the buffy coat was pipetted into a

new 15 ml tube. The buffy coat was washed twice by adding and mixing with 5 mL of cold 1X PBS and centrifugation at 1800rpm for 10 minutes. The cells were then cryopreserved in fetal bovine serum (FBS) with 10% dimethyl sulfoxide (DMSO) at -80^{o}C, and transported later to Accra for storage in liquid nitrogen.

3.3.3 DNA Extraction for Dry Blood Spots

Total DNA was extracted from the dry blood spots on filter paper as described by (Dinko et al., 2016). Briefly, 2 pieces of dry blood spots from each sample were cut out of the protein cards with a paper punch and aseptically transferred into a new 1.5mL Eppendorf tube. To avoid cross contamination, the punch was flamed and cleaned in between punches. Dry RBCs and cells were lysed by adding 1mL of freshly prepared 0.5% saponin in PBS to completely soak the card. The tubes were incubated at room temperature overnight. The tubes were then centrifuged at 4000xg for 2 minutes and the supernatant was discarded. The card was washed repeatedly with 1mL PBS until the disappearance of the reddish colour from heme. 50µl of 20% chelex100 was suspension in 100µL nuclease free water (Sigma, UK) and added to each tube. The content of the tube was boiled in a wet heat-block for 20 minutes, followed by centrifugation at 4000rpm for 2minutes. The supernatant containing the DNA was carefully transferred into a new tube, and stored at -20^{o}C for later use.

3.3.4 Light Microscopy for diagnosis of *P. falciparum* infection

Air-dried thick films and methanol (96-100%)-fixed thin films were stained with Geimsa (10%) for ten minutes and allowed to dry. The thick films were examined under the oil emersion objective on a light microscope for the presence of the malaria parasite, while thin films were used to differentiate between *Plasmodium* species. A slide was declared negative after 100 fields have been examined with no parasite found. The slides were independently re-examined by an experienced microscopist and the results were recorded as positive if both reading matched.

3.4 RNA Extraction

Total RNA was extracted from cells lysed in trizol reagent (Invitrogen) using the Direct-zol RNA extraction kit (Zymo Research, USA). Briefly, cell lysates were kept at room temperature until completely thawed and mixed well by vortexing. An equal volume ethanol (95-100%) was added to the cell lysate and mix thoroughly. The mixture was then transferred into Zymo-SpinTM IIICG Column in a collection tube and centrifuged at 12000g for 30 seconds. The column was transferred into a new collection tube and the flow-through was discarded. Exactly 400 µL of RNA Wash Buffer was added to the column and centrifuged at 12000g for 30 seconds. To eliminate contaminating DNA, each sample was treated with a DNase-1, in column, according to the manufacturer's instructions. A DNase mix (5 µL DNase I + 75 µL DNA Digestion Buffer) was prepared in an RNase-free Eppendorf tube, and added directly to the column. The column was incubated at room temperate for 15 minutes. Exactly 400 µL of Direct-zolTM RNA PreWash was added to the column and centrifuged at 12000g for 30 seconds. The flow-through was discarded and the step was repeated one more time. The RNA was washed by adding 700 µL RNA Wash Buffer to the column and centrifuged for 2 minutes to ensure complete removal of the wash buffer.

The column carefully transferred into a new RNase-free tube. Total RNA was eluted by adding 100 µL of DNase/RNase-Free Water directly to the column matrix, incubating for 5 minutes and centrifuging at 12000g for 30 seconds. The RNA concentrations and purity were determined with the NanodropOne. RNA dilutions of 3ng/µL were prepared from the stock RNA samples for cDNA synthesis.

3.5 Complementary DNA synthesis and miR cDNA library generation

3.5.1 First Strand cDNA synthesis

30ng of total RNA extracted from PBMCs of primary school children was converted to first strand cDNA using the iScript^{TM} cDNA Synthesis Kit (Biorad, CA, USA) following the manufacturer's instructions. For cDNA synthesis reaction mix, 4 µL of 5X iScript Reaction Mix, 1µL of iScript Reverse Transcriptase and 5µL of Nuclease free water was mixed for each reaction. 10µL of 3.0ng/µL RNA reverse transcribed for each sample. The reaction was incubated for 5 minutes at 25^{o}C, 46^{o}C for 20 minutes, and 95^{o}C for 1 min, on the ProFlex PCR System (Thermo Fisher Scientific). The cDNA was diluted 5 times and used as templates to subsequent PCRs. First strand cDNA from archived RNA extracted from PBMCs of a cohort of sick febrile children with or without malaria and Tonsillar B-cell subsets were synthesized using the RevertAID First Strand cDNA Synthesis kit (Thermo Scientific) following the manufacturer's instructions. The cDNA synthesis master mix was prepared as shown in table 3.1 below. Exactly 10µL of RNA was reverse transcribed with this mix by incubating at 25^{o}C for 5 minutes followed by 42^{o}C for 60 minutes and 70^{o}C for 5 minutes.

Table 3.1 cDNA Synthesis Master Mix

Component	Volume/reaction (µL)
5X Reaction Buffer	4
RiboLock RNase Inhibitor	1
10 mM dNTP Mix	2
RevertAID M-MuLV Reverse Transcriptase	1
Nuclease free Water	2
Total	**10**

3.5.2 miR cDNA library preparation

cDNA libraries for microRNAs was prepared using the TaqMan® Advanced miR cDNA Synthesis Kit, following the manufacturer's instruction.

3.5.2.1 Poly(A) tailing of microRNAs

RNA samples and reagents were thawed on ice and gently mixed by vortexing. The reagents were centrifuged briefly to eliminate air bubble. The poly(A) tailing reaction mix was prepared by mixing 0.5 µL of 10X Poly(A) Buffer, 0.5µL ATP, 0.3 µL Poly(A) enzyme and 1.7 µL RNase-free water. The reaction mix was thoroughly mixed and centrifuged to eliminate air bubbles. A total of 3µL of master mix was incubated with 2µL of 3.0ng/µL RNA sample in a 1.5mL Eppendorf tube. The tube was closed tightly and incubated in the ProFlex PCR System (Thermo Fisher Scientific) for 45 minutes at 37ºC followed by 10 minutes at 65ºC. The reaction mix was transferred to ice immediately or incubated at 4ºC until needed. This product was used for the adaptor ligation step.

3.5.2.2 Adaptor Ligation

The master mix for ligating an adaptor to the 3'end of the poly(A) tailed miRs was made by mixing 3 µL of 5X DNA ligase Buffer, 4.5µL of 50% PEG, 0.6 µL of 25X ligation Adaptor, 1.5 µL RNA ligase and 0.4 µL RNase-free water, for each reaction. The ligation reaction mix was vortexed and centrifuged to eliminate air bubbles. The ligation reaction mix (10 µL) was added directly to the poly(A) tailed miR and mixed well by vortexing. The tubes were closed tightly and incubated for 60 minutes at 16^{o}C and transferred onto ice immediately.

3.5.2.3 Reverse Transcription Reaction

The RT mix was prepared by mixing 6 µL of 5X RT Buffer, 1.2 µL of 25mM dNTP mix, 1.5 µL of 20X Universal RT Primer, 3 µL of 10X RT enzyme mix and 3.3 µL of RNase-free water for each sample. The mix was vortexed and centrifuged to eliminate air bubbles and 15 µL was transferred to the poly(A) tailed, adaptor ligated miRs. This was mixed well and centrifuged briefly and incubated in the ProFlex PCR System (Thermo Fisher Scientific) for 15 minutes at 42^{o}C followed by 5 minutes at 85^{o}C and transferred onto ice immediately.

3.5.2.4 miR cDNA library Preparation

Sufficient miR-Amp reaction mix was prepared by mixing 25µL of 2X miR-Amp Master mis, 2.5µL 20X miR-Amp Primer Mix and 17.5µL RNase-free water. This was mixed by vortexing and centrifuged to eliminate air bubbles. Exactly 45µL of this mix was transferred into a new 1.5mL Eppendorf tube. 5 µL of RT reaction product was added and vortexed briefly. The tubes were centrifuged and incubated in the ProFlex PCR System (Thermo Fisher Scientific) at 95^{o}C for

1minute followed by 14cycles of 95ºC for 3seconds and 60ºC for 30seconds. The reaction was stopped at 99oC for 10 minutes and the product was stored at -20ºC for further use.

3.6 Polymerase Chain Reactions (PCR)

3.6.1 PCR for detection of *P. falciparum* in blood and Tonsillar tissue

To detect the presence of *Plasmodium* species in tonsillar tissues and whole blood from healthy primary school children, a nested PCR approach was used. In the first round of PCR, *Plasmodium* genus specific primers (**rPLU6F**: 5'-TTAAAATTGTTGCAGTTAAAACG-3' and **rPLU5R**; 5'-CCTGTTGTTGCCTTAAACTTC-3') was used to amplify a segment of the 18s ribosomal RNA (rRNA) shared by *P. falciparum*, *P. malariae, P. ovale and P. vivax*. A total of 5µL of DNA template was amplified with the *Taq* DNA Polymerase with Standard *Taq* Buffer (#M0273, New England Biolabs Inc. Ipswich, MA, USA) was used for the reaction with the master mix setup as show in table 3.1 below. The PCR was run on the ProFlex PCR System (Thermo Fisher Scientific). Cycling conditions were as follows: initial denaturation at 95ºC for 2 minutes, 35 cycles of 95ºC for 15seconds, 58ºC for 60seconds, and 68ºC for 90seconds followed by a final extension step at 68ºC for 5minutes. The amplicons were placed on ice right after the reaction or incubated at 4ºC and transferred to -20ºC for use later.

Table 3. 2 Reaction mix for *Plasmodium sp*. PCR

Reaction components	Volume (µL)
10X Standard *Taq* Buffer	2.5
10mM dNTPs	0.5
10 µM rPLU6F	0.5
10 µM rPLU5R	0.5
Taq DNA Polymerase	0.125
Nuclease free water	15.875
Template DNA	5
Total	**25**

A multiplex nested PCR was used for the simultaneous detection of four species of the malaria parasite (*P. falciparum, P. malariae, P. ovale and P. vivax*). Primer sets found within the region flanked by rPLU6 and rPLU5 and specific for each species was used to amplify the product of the first PCR reaction. The primer sets are presented in table 3.3 below.

Table 3. 3 Primer sets for *Plasmodium species* detection

Target	Primer Name	Sequence (5'- 3')
P. falciparum	rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT
	rFAL2	ACACAATGAACTCAATCATGACTACCCGTC
P. malariae	rMAL1	ATAACATAGTTGTACGTTAAGAATAACCGC
	rMAL2	AAAATTCCCATGCATAAAAAATTATACAAA
P. ovale	rOVA1	ATCTCCTTTACTTTTTGTACTGGAGA
	rOVA2	GGAAAAGGACACTATAATGTATCCTAATA
P. vivax	rVIV1	CGCTTCTAGCTTAATCCACATAACTGATAC
	rVIV2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA

Two microliters (2 µL) of the round 1 PCR amplicon was used as template for the nested PCR reaction. The master mix was prepared the same way as in the first round PCR, but with 0.5µL of 10µM of the primers listed in table 3.3 above. Nuclease-free water was added to make up a 23µL master mix per reaction. The amplification product served as the DNA template for the NEST 2 amplifications. The cycling conditions for the nested reaction were the same as the first round. Amplification product of the nested reaction was resolved in a 2% agarose gel electrophoresis with ethidium bromide staining and visualized under UV illumination in the Amasham gel imager (ThermoScientific, USA).

3.6.2 Nested PCR for detection of AID mRNA splice variants

Nested PCR was used to identify AID mRNA splice variants expressed in Tonsillar B-cell subsets and PBMCs for healthy and sick children. Primers and conditions used for this PCR was as described by (Wu et al., 2008), with modifications, using the Go*Taq* DNA polymerase and the Go*Taq* Green Master Mix (Promega Corporation, Madison, WI, USA). The forward primer P1 (5'-AGGCAAGAAGACACTCTGGACACC-3') and the reverse primer P2 (5'-GTGACATTCCTGGAAGTTGC-3') were used for the nest-1 reaction. Nest-1 amplicon (2 µL) was used as template for the nest-2 reaction using NP1 (5'-GACAGCCTCTTGATGAACCGG-3') and NP2 (5'-TCAAAGTCCCAAAGTACGAAATGC-3') as forward and reverse primers respectively. The cycling conditions were the same for both reaction; initial denaturation at 95°C for 2minutes followed by 35 cycles of 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. Final extension was at 72°C for 5 minutes. The amplicon of nest-2 (10 µL) was resolved on a 1.5% agarose gel with ethidium bromide staining and visualized under UV illumination. The remaining amplicon was stored at -20°C for later use.

3.7 Identification of AID mRNA splice variants

The splice variants expressed in the GC B lymphocytes from one (1) Ghanaian tonsil, and an age-matched New Mexican tonsil were cloned into a pGEM®-T vector for sequencing.

3.7.1 Purification of PCR product

PCR products from AID mRNA splice variant nested PCR reaction were cleaned and concentrated with the DNA Clean & Concentrator™ (Zymo Research, Corp., CA, USA), following the manufacturer's instructions. Exactly 30 µL of PCR product (3.6.2) was placed into a clean 1.5mL Eppendorf tube and 150µL of DNA binding buffer was added and mixed well by vortexing. The mixture was then transferred into the Zymo-Spin™ Column in a collection tube and centrifuged. The column bound DNA was washed twice with 200µL of DNA wash buffer by centrifuging at 12000xg for 30seconds. The column was centrifuged at 12000g for 2 minutes to remove traces of wash buffer and the DNA was eluted with 25µL of nuclease-free water. The DNA concentration was then quantified on the nanodropOne.

3.7.2 Poly-A tail Ligation of PCR Amplicon

To ensure the elimination of blunt ends in the purified PCR product, the amplicons were A-tailed by incubating with GoTaq Polymerase at 72°C for 20 minutes in the presence of dNTPs and APT. The pGEMT®-T vector reagents were thawed on ice and briefly centrifuged to collect the content at the bottom of the tube. The ligation reaction was prepared in a 0.5 mL Eppendorf tube by mixing 5µL of 2X rapid ligation buffer containing T4 DNA ligase, 1µL of 50ng pGEMT®-T vector and 2µL of nuclease free water. A total of 8µL of ligation mix was added to 2 µL of A-tailed PCR product, mixed well and incubated at 4°C overnight.

3.7.3 Transformation of JM109 Competent Cells

The ligation product was used to transform JM109 High Efficiency Competent Cells (Promega Corporation, Madison, WI, USA). Isopropyl β- d-1-thiogalactopyranoside (**IPTG**) (40 µL), 40 µL of 5-Bromo-4-Chloro-3-Indolyl-**beta**-D-Galactoside (X-gal) stock solution and 40 µL of 100mg/mL carbenicillin was spread on top of an LB/carbenicillin plate with a spreader and allowed to dry at 37ºC for 30 minutes. The JM109 high efficiency competent cells were thawed on ice. Exactly 2 µL of ligation reaction was added to 25 µL of JM109 High Efficiency Competent Cells on ice. The cells were mixed well by flicking the bottom of the tube and placed on ice for 20 minutes, followed by incubation for 50 seconds at 42ºC in a water heating block without shaking and returned to ice for 2 minutes. Exactly 350µL of room temperature-warmed SOC medium was added to the cells with the ligation reactions and incubated for 1.5 hours at 37ºC with shaking at 150 rmp. A 150 µL aliquot of the transformed cells were plated onto the LB/carbenicillin plates and incubated overnight at 37ºC. Distinct white colonies were picked unto new LB/carbenicillin plates with number-marked grids and incubated overnight. The colonies were then picked into new Eppendorf tubes and lysed in 50 µL of nuclease free water.

3.7.4 Colony PCR

AID nested primer sets NP1 and NP2 were used to amplify AID in the lysed transformed cells. 10 µL of the PCR product was resolved on a 1.5% agarose gel and products from colonies with single bands were selected and purified for sanger sequencing. Sequences were aligned with reference AID spice variants.

3.8 Quantitative RT-PCR

3.8.1 RT-qPCR for AID, transcription factors and miRs

The Taqman Fast Advanced Master Mix (Thermo Scientific) was used together with TaqMan Gene Expression Assays to quantify the levels of AID and related transcription factors and microRNAs in PBMCs, Tonsillar B-cell subsets. For the quantification of AID and its related transcription factors, the following assays were used; AID (Hs00221068_m1), XBP1(Hs00964360_m1), IRF4(Hs00180031_m1), IR8(Hs00175238_m1), HOXC4(Hs00538088_m1), BACH2(HS00935338_M1) ACTB(Hs99999903_m1), MKI67(Hs01032443_m1), BCL6(Hs00277037_m1), PAX5(Hs00277134_m1) and PRDM-1(Hs00153357_m1). For the quantification of miRs the TaqMan miR Assays hsa-miR-155-5p (483064_mir), hsa-miR-181b-5p (478583_mir) were used. Briefly, 5 µL of 2X TaqMan® Fast Advanced Master Mix, 0.5 µL of Gene expression Assay and 2 µL of 10µg/mL Bovine Serum Albumin (BSA) was mixed for each reaction. Exactly 2.5 µL of cDNA prepared as described in section 3.5 was added in triplicates, and run on the QuantStudio-5 PCR system using the already programmed fast run option. The run data was exported to Microsoft excel and the gene expression levels were calculated. The fold expression levels of AID and related transcription factors were normalized to the expression of β-actin, while the expression of miR181b and miR155 were normalized to the expression of miR191, using the $2^{-\Delta CT}$ method. In tonsillar B-cell subsets, the expression of AID was expressed as fold differences relative to the β-actin normalized expression in naïve B lymphocytes.

3.8.2 RT-qPCR for AID splice variants

The levels of AID splice variants in Tonsillar B-cell subsets were determined by RT-qPCR using Power SYBR® Green PCR Master Mix (Applied Biosystems, USA) and splice variant specific primers described by (Zaprazna et al., 2019). Briefly, the SYBR Green PCR Master Mix was kept at 25ºC until completely thawed and mixed. For a 10 µL reaction, 5 µL of SYBR Green Master Mix was mixed with 0.3 µL each of forward and reverse primers and 2.4 µL of cDNA template per sample. The real time PCR was run with the following cycling conditions: 95ºC for 10 minutes, followed by 45 cycles of 95ºC for 15 seconds and 60ºC for 60 minutes. The relative levels of each splice variant were normalized to the expression of β-actin.

3.9 Determination of EBV infection status in plasma samples

3.9.1 Detection of EBNA-1 IgM and IgG

Detectable IgM and IgG to the Epstein-Barr virus nuclear antigen-1 (EBNA-1) and the viral capsid antigen (VCA) were used to assay for prior exposure to EBV in healthy primary school children and children with Severe malaria anemia respectively. Semi-quantitative levels EBNA-1 IgG and IgM levels were determined using the ELISA-VIDITEST anti-EBNA-1 EBV IgM, and IgM kits (ODZ001, Vidia). The microwells were allowed to warm to room temperature. Exactly 100 µL of plasma samples diluted 1:100 were aliquoted into appropriate wells and incubated for 30 minutes at room temperature. The liquid was aspirated and the wells washed by adding 250 µL of wash buffer to each well. The wash buffer was aspirated from the wells and excess wash buffer was dried by gently tapping the wells on a dry, clean tissue paper. Exactly 100 µL of anti-human IgG Px-conjugate was added to each well and incubated for 60 minutes at room temperature. The liquid was aspirated and the wells were washed three times with wash buffer. Exactly 100 µL of TMB

was added to each well and incubated for 10 minutes at room temperature in the dark. The reaction was stopped by adding 100 µL of stop solution and the absorbance was measured at 450 nm. To determine which sample was positive or negative, a cut-off OD was determined by multiplying the absorbance of the Standard with the correction factor provided by the manufacturer. Semi-quantitative levels were determined by dividing the sample absorbance by the cut-off value.

3.9.2 Detection of EBV VCA IgG and IgM

Since EBNA-1 is a latent protein expressed by EBV in B lymphocytes, antibodies against it are acquired much later in life. In children under 3 years who are having their prime exposure to the virus, antibodies to the viral capsid antigen is one of the earliest antibodies. The EBV-VCA IgG (MBS580104) and IgM (MBS580103) kit (MyBiosource, USA) were used to determine the EBV infection status of children under three years with or without SMA. The manufacturer's instruction was followed without modifications. The wells were placed in holders and the positive and negative controls were added. 100µl of 1:20 diluted samples were added to each well and incubated at room temperature for 20 minutes. The liquid was aspirated and the wells were washed three times with 300µl of wash buffer. Exactly 100µl of enzyme conjugate was added to each well and incubated at room temperature for 20 minutes. The liquid was aspirated and the wells were washed. 100µl of TMB was added and incubated for 10 minutes at room temperature. The reaction was stopped with 100µl of stop solution and the absorbance was read at 450nm immediately. The cut-off value was calculated by multiplying the absorbance of the Calibrator with the Calibrator factor provided by the manufacturer. The antibody index (titer) was determined by dividing the OD of each sample by the cut-off value.

3.10 Data and Statistical Analysis

Raw data obtained from experiments were processed in Microsoft Excel 2018 and transferred to GraphPad prism 7.0a (GraphPad Software, Inc.) and the RStudio Version 1.2.1335 (R Foundation for Statistical Computing) for further analysis.

For specific aim one, Kruskal-Wallis test was performed in GraphPad to compare categorical variables between Tonsillar B-cell subsets, and MegaX was used to perform multiple sequence alignment for AID mRNA splice variants. For specific aim two, Chi squares were used to compute statistical differences in percentages for EBV seropositivity asymptomatic and un-infected children while Mann-Whitney test was used to compare categorical data between children with and without *P. falciparum* infection.

Chi squares was used to compare differences in percentages for EBV seropositivity among aparasitemic, non-SMA and SMA children while Mann-Whitney test was used to compare categorical data between children with and without SMA. Pearson's correlation test was used in RStudio to determine correlation between dependent (SMA phenotypes) and independent variables (temperature, fever, Hb and parasite loads) in the SMA study. Statistical significance was set at *p*-values less than 0.05. Stars were used to indicate the level of statistical significance between groups; * = $p < 0.05$, ** = $p<0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, *ns* = no statistical significance observed.

CHAPTER FOUR

4.0 RESULTS

4.1 Transcriptional regulatory patterns of AID expression in Tonsillar B lymphocytes of children exposed to malaria

Although the expression of AID has been reported in cells not actively undergoing CSR and SHM, the geminal center (GC) in secondary lymphoid tissues is the site for the most important function of AID in response to infections. In order to understand the general transcriptional regulatory patterns of AID expression in secondary lymphoid tissues that have been exposed to *P. falciparum* malaria, the transcript levels of AID and related positive or negative transcriptional factors was measured in B-cell subsets from tonsillar tissues from Ghanaian children.

4.1.1 Characteristics of Tonsillar B-cell subsets

Mononuclear cells isolated from tonsillar tissue of six (6) children exposed to malaria and one (1) malaria naïve child were sorted by flow cytometry into four (4) B-cell subsets as described by Lee and colleagues (2016), with modifications. Lymphocytes were gated from a forward-side scatter (FSC) plot (Figure 4.1A), and single cells were gated for in an FSC Area vs. FSC Height plot (Figure 4.1B). Live lymphocytes were selected as 7AAD negative (Figure 4.1C) and B lymphocytes were identified as $CD3^-$, $CD19^+$ and/or $CD20^+$ (Figure 4.1 D). Four B-cell subsets, Naïve B lymphocytes (NB), Germinal center B lymphocytes (GC), Memory B lymphocytes (MB) and Plasmablasts (PB), were sorted based on the expression levels of IgD and CD38 (Lee et al., 2016), (Figure 4.1E).

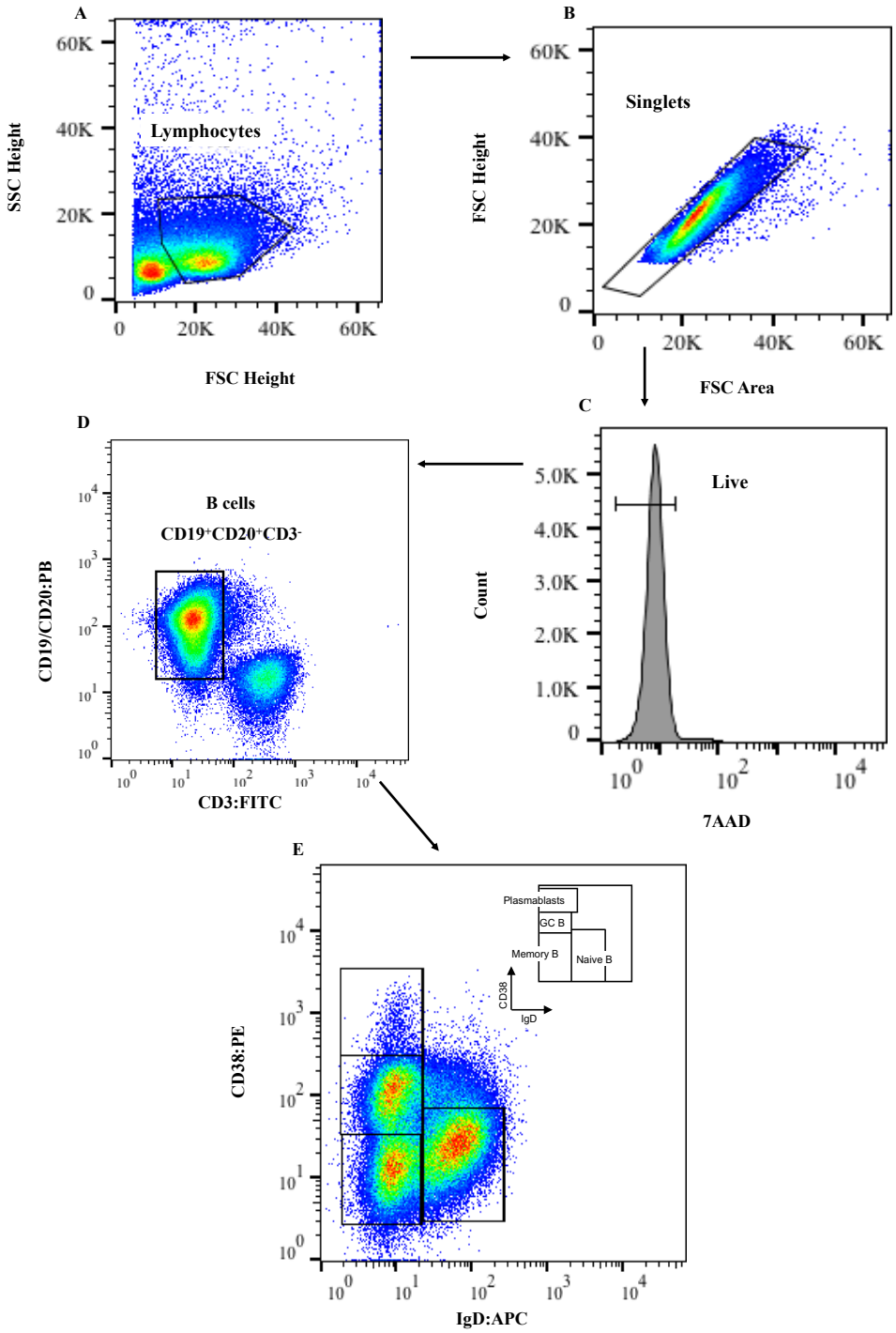

Figure 4.1 Gating strategy for sorting of Tonsillar B-cell subsets

Lymphocytes were gated among total tonsillar mononuclear cells **(A).** From singlet lymphocytes **(B)**, live cells **(C)** was gated for $CD3^{-}CD19^{+}$and/$CD20^{+}$ B lymphocytes **(D). E**: B-cell subsets were defined by expression of CD38 and IgD. NB:$IgD^{+}CD38^{-}$, MB:$IgD^{-}CD38^{-}$, GC:$IgD^{-}CD38^{+}$ and PB: $IgD^{-}CD38^{++}$.

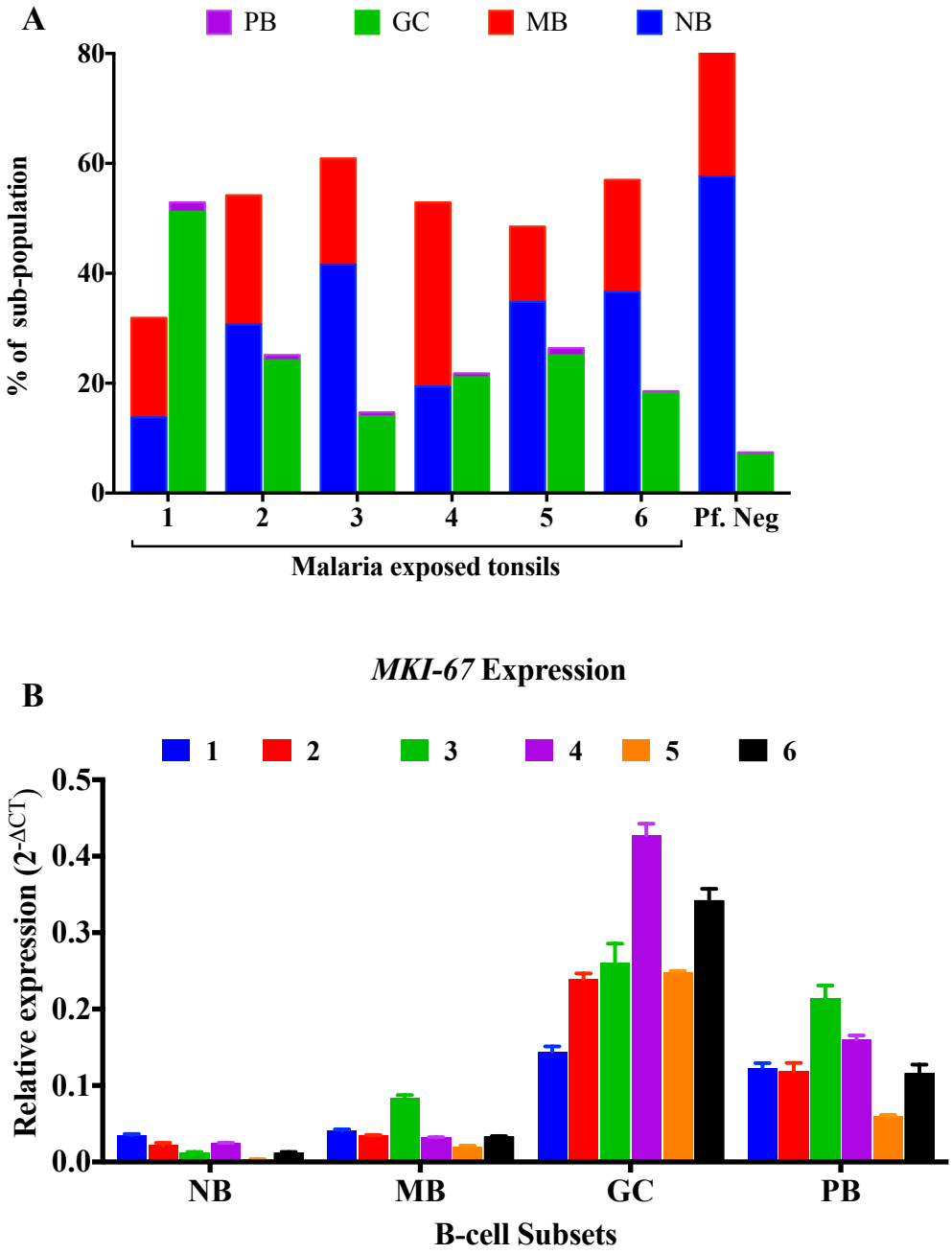

Figure 4.2 Characteristics of sorted Tonsillar B lymphocytes

A: Proportions of B-cell subsets in Ghanaian Tonsillar MNCs, *Pf.* Neg is malaria non-exposed Tonsillar tissue from a malaria free region (New Mexico, USA). **B**: Fold expression levels of MKI-67 mRNA in B-cell subsets. Each bar represents the mean of technical triplicate PCR runs from all six tonsils.

The frequencies of NB lymphocytes ranged between 13.8% to 41.6% while GC, MB and PB lymphocytes ranged from 14% - 51%, 18.1% - 33.5%, and 0.36% - 1.69% respectively in the Ghanaian tonsils (Figure 4.2A). The malaria naïve tonsil (*Pf*. Neg), had the highest proportion of naïve B lymphocytes (57.6%) and the least proportion of germinal center B lymphocytes (7%), confirming previous evidence that tonsils from malaria endemic regions have higher proportions of GC cells (Torgbor et al., 2014).

The cell cycle antigen mKI67, a maker of cell proliferation, has been used to characterize GC B lymphocytes and plasmablasts previously (Allen et al., 2007). GC B lymphocytes proliferate during SHM and CSR, while plasmablasts proliferate in the process of producing antibodies. mKI67 transcripts were therefore used to characterize the sorted B-cell subsets. Expectedly, there were significantly higher mKI67 levels in the GCs and PBs versus NB and MB lymphocytes in all six Ghanaian tonsils (Figure 4.2B).

4.1.2 Expression patterns of AID, Prdm-1, XBP-1, Irf4 and Irf8

The relative expression levels of transcription factors associated with the transcription of AID in B lymphocytes transitioning between the geminal center to memory or plasma cells were measured by RT-qPCR. The levels of mRNA of target genes were normalized to the expression of β-actin and fold changes were calculated relative to their normalized expression in naïve B lymphocytes. Prdm-1 which codes for Blimp-1 is the key regulator of plasmacytoid differentiation and the regulation of AID expression in plasma cells. Expectedly, Prdm-1 was found to be significantly up-regulated in PB lymphocytes versus MB lymphocytes ($p < 0.0001$) and GC B lymphocytes ($p = 0.0042$) in all six tonsils with mean fold-changes ranging from 3 to 77 (Figure 4.3A). Although

Blimp-1 and the X-box binding protein 1 (XBP-1) are thought to work together in regulating both the expression of AID and plasmacytoid differentiation, the transcript levels of XBP-1 in GC B lymphocytes and PB lymphocytes were not different. The levels were however significantly lower in MB lymphocytes when compared to both GCs and PBs ($p < 0.05$) from the six tonsils analyzed (Figure 4.3B).

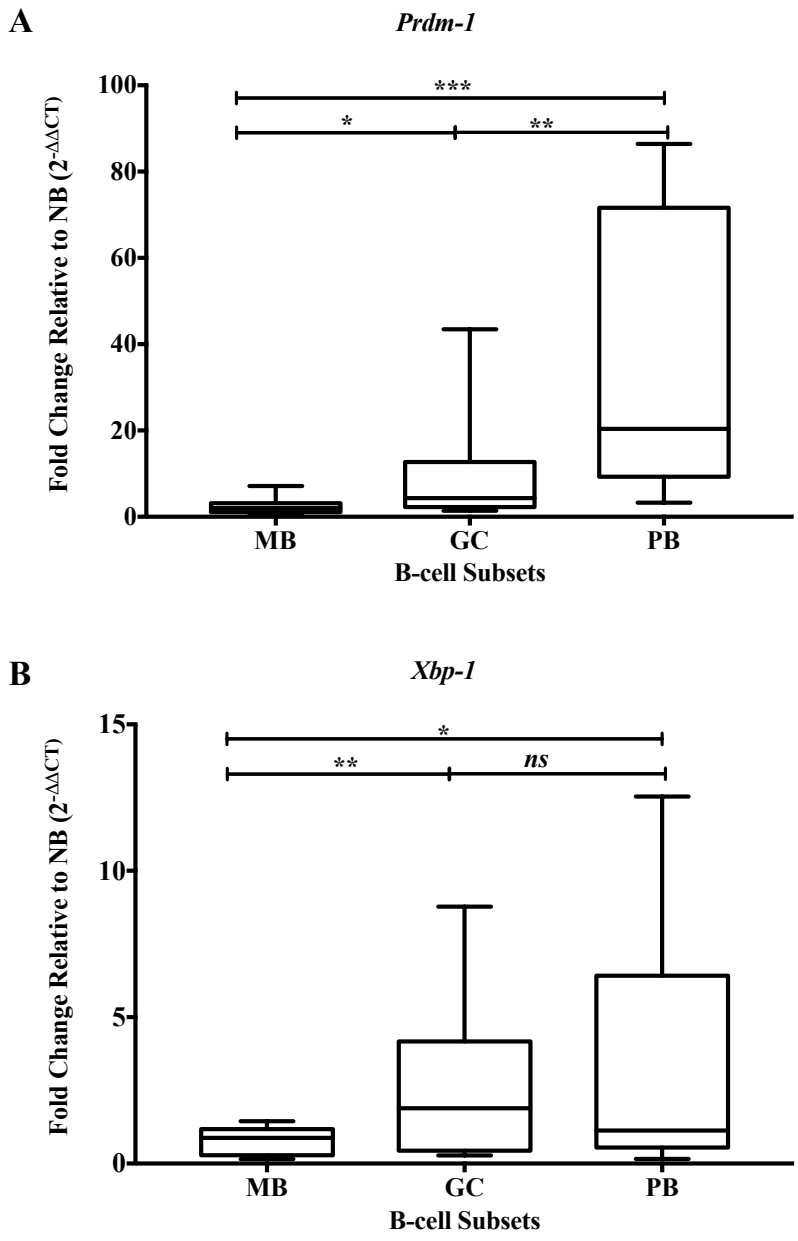

Figure 4.3 Expression of Prdm-1 and XBP-1 in malaria exposed Tonsillar B lymphocytes
Fold change in the expression of Prdm-1(A), XBP-1(B) are presented as plots showing the median with range (minimum to maximum) of 6 samples run in technical triplicates. The levels of all genes were normalized to β-actin expression and are expressed as fold changes relative NB. Stars represent level of statistical significance as tested by unpaired student's t-test between B-cell subsets. * = $p<0.05$, ** = $p<0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, *ns* = no statistical significance observed.

The expression of Irf4 and Irf8 followed a slightly different pattern. On one hand, the expression of Irf4 was generally the same in all four B-cell subsets, as reflected in the mean 1-fold change in MB, PB and GC B lymphocytes in all 6 tonsils analyzed (Figure 4.4A). The expression pattern of Irf8 was consistent with what has been reported previously: expression was highest in GC B lymphocytes followed by MB lymphocytes and down-regulated in PB lymphocytes (Figure 4.4B).

To determine if there are differences in the expression of Prdm-1, XBP-1, Irf4 and Irf8 in Tonsillar B-cell subsets that have not been exposed to malaria, these transcription factors were quantified in B-cell subsets sorted from a malaria naïve tonsil (NM) and compared to a malaria exposed (GH) tonsil of the same age. While there were equal levels of Prdm-1 transcripts in both the GH and NM GC B lymphocytes, significantly more ($p = 0.003$) and lower ($p < 0.0001$) transcripts were measured in the GH PB and MB lymphocytes, respectively (Figure 4.5A). Levels of XBP-1 transcripts were 10-fold higher in NM GC B lymphocytes ($p = 0.0007$) and MB lymphocytes ($p = 0.0005$) than measured in the GH GC B and MB lymphocytes.

However, in PB lymphocytes, the levels were significantly lower in NM PB lymphocytes than GH PB lymphocytes (Figure 4.5B). While Irf4 transcript levels remained relatively equivalent among MB, PB and GC B lymphocytes in the GH tonsils, its levels were highest in the NM NB lymphocytes and moderately high in GC B lymphocytes but down-regulated in PB lymphocytes. Comparing GH and NM B cell subsets, Irf4 transcripts were significantly higher in NM MB lymphocytes ($p = 0.0260$) but 88-fold and 2.5-fold lower in both PB ($p = 0.0018$) and GC B lymphocytes ($p = 0.0015$) respectively (Figure 4.5C). While the expression of Irf8 was up-regulated in MB and GC of both GH an NM tonsils, the levels of the transcription factor was

significantly higher in NM MB lymphocytes compared to GH MB lymphocytes (p = 0.00117). The transcript levels were however not different in GC B lymphocytes from both donors. Although the levels of Irf8 was lowest in PB of both tonsils, the GH PB lymphocytes expressed significantly higher compared to NM PBs (Figure 4.5D)

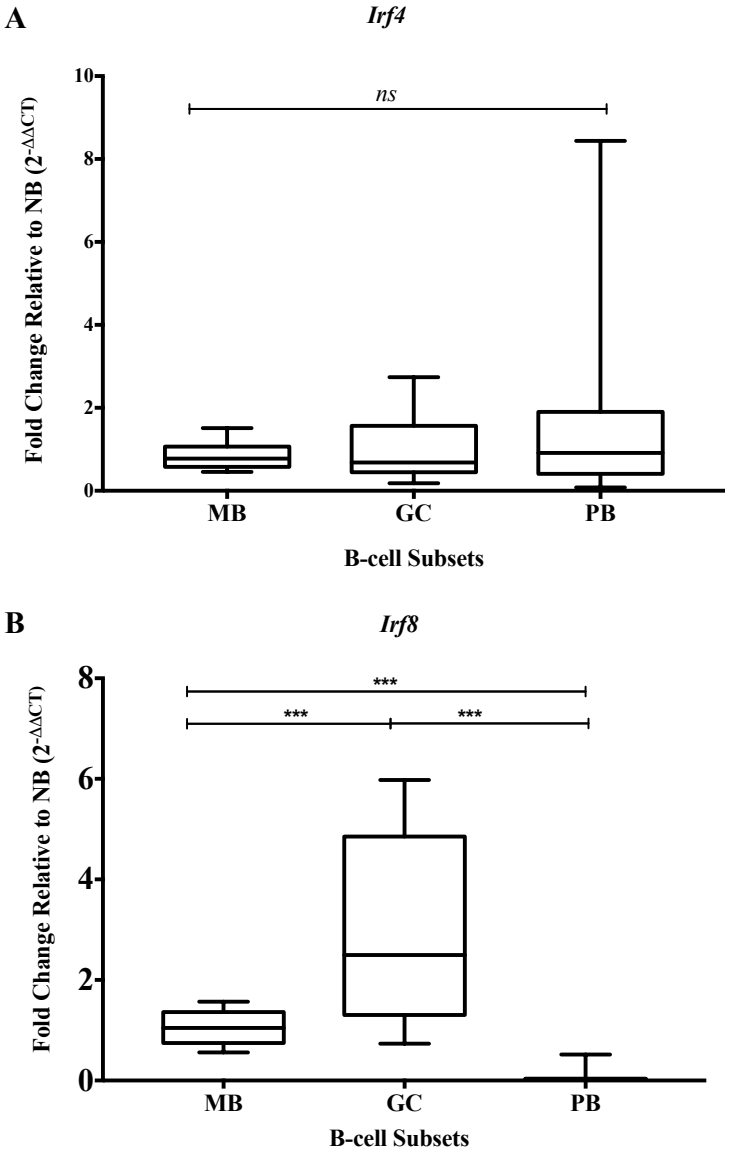

Figure 4.4 Expression of Irf4 and Irf8 in malaria exposed Tonsillar B lymphocytes
Fold change in the expression of Irf4(A) and Irf8(B) are presented as box plots showing the median with range (minimum to maximum) of 6 samples run in technical triplicates. The levels of all genes were normalized to β-actin expression and are expressed as fold changes relative to NB. Stars represent level of statistical significance as tested by Mann-Whitney test between B-cell subsets. * = p<0.05, ** = p<0.01, *** =p < 0.001, **** = p < 0.0001, *ns* = no statistical significance observed.

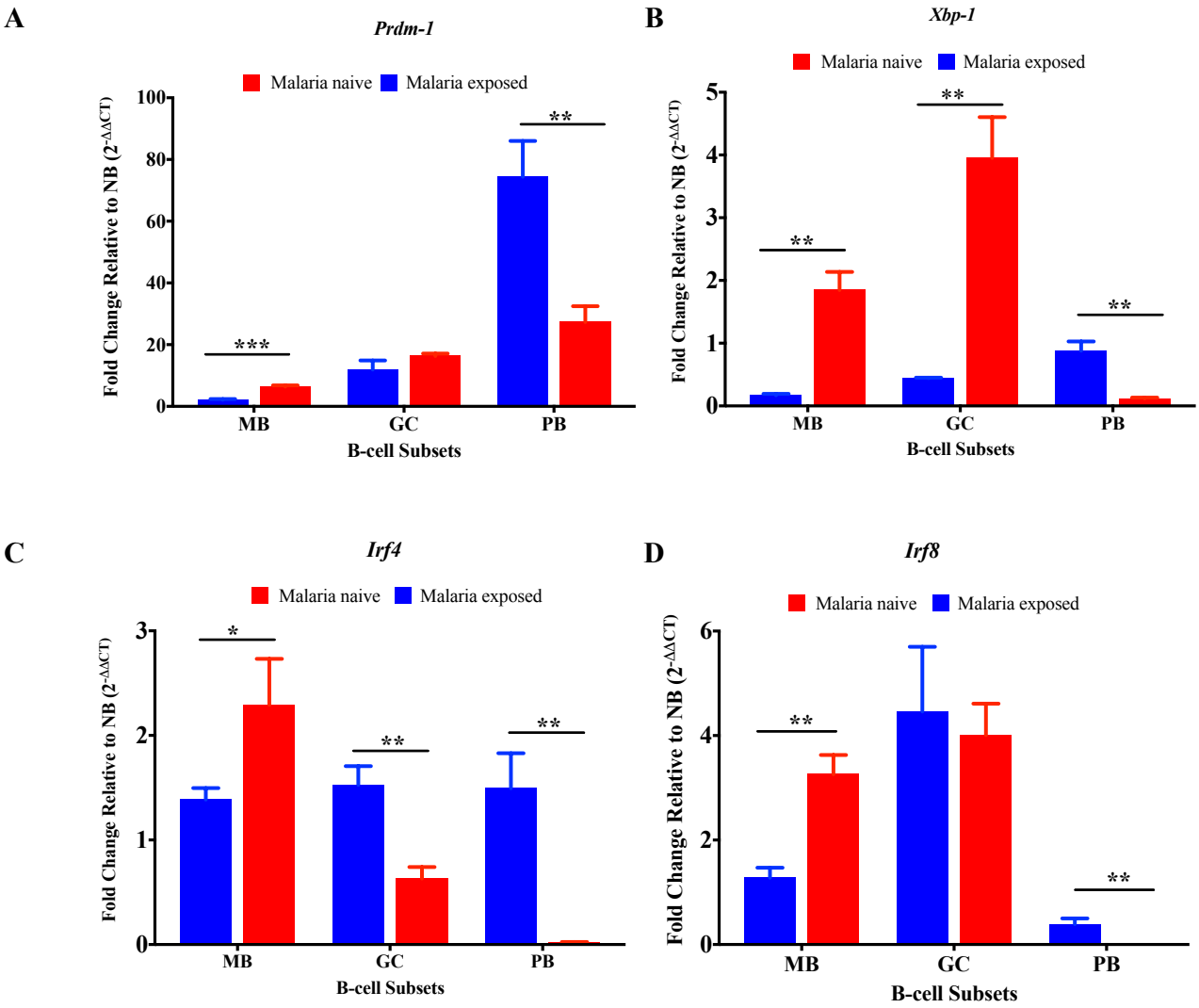

Figure 4.5 Patterns of Prdm-1, XBP-1, Irf4 and Irf8 expression in malaria exposed and naïve Tonsillar B lymphocytes: The expression levels of target genes are compared in age-matched malaria exposed and naïve Tonsillar B-cell subsets. Fold changes in the expression of Prdm-1(A), XBP-1(B), Irf4(C) and Irf8(D) are presented as bar graphs showing the mean of technical triplicates RT-qPCR runs. Error bars are standard errors of the mean. The levels of all genes were normalized to β-actin expression and are expressed as fold changes relative NB. Stars represent level of statistical significance by Mann-Whitney test between B-cell subsets. * = $p<0.05$, **= $p<0.01$, *** =$p < 0.001$, **** = $p < 0.0001$, *ns* = no statistical significance observed.

4.1.3 Expression of Pax5, HoxC4, Bcl6 and Bach2 in Tonsillar B cell subsets

The transcription factors Pax5, Bcl6, Bach2 and HoxC4 are all positive regulators of AID expression. While Pax5 and HoxC4 bind directly to promoter regions of the AID gene, Bcl6 and Bach2 up-regulate the transcription of AID by down-regulation negative regulators of AID expression in GC B lymphocytes undergoing SHM and CSR. The levels of Pax5 and HoxC4 are presented in Figure 4.6A and B, and levels of Bcl6 and Bach2 are presented in Figure 4.7 A and B. In general, transcripts for these genes were highly expressed in GC B lymphocytes in all six tonsils tested and lowest in PB lymphocytes. Comparing the age-matched Tonsillar B cell subsets, the transcript levels of Pax5 (Figure 4.8A), BCL6 (Figure 4.8C) and Bach2 (Figure 4.8D) were comparable in both GH and NM GC B and MB lymphocytes. In PBs the expression of Pax5, Bcl6 and Bach2 are down-regulated in both GH and NM PBs, but significantly lower ($p<0.05$) in NM PB lymphocytes (Figure 4.8 A, C and D). Expression of HoxC4 was significantly higher ($p < 0.05$) in all three B cell subsets of the GH tonsils compared to NM B tonsils (Figure 4.8B).

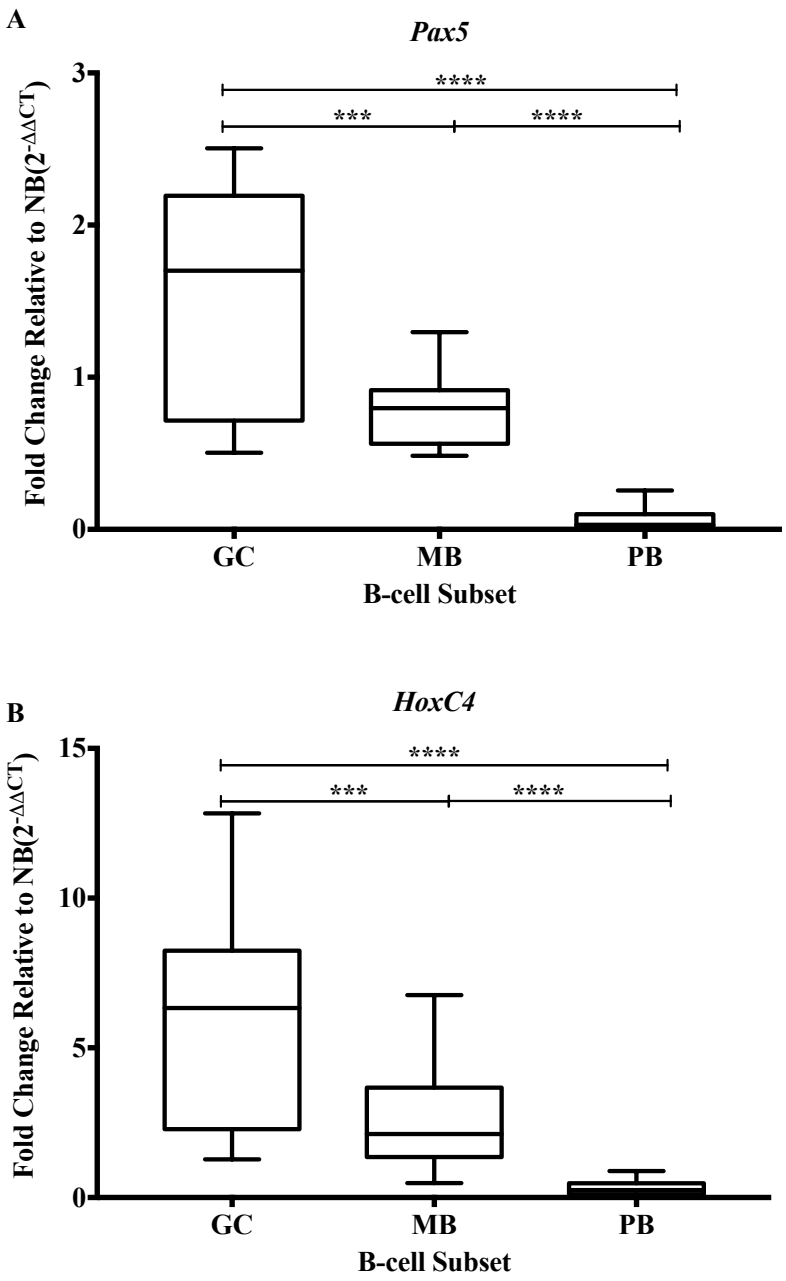

Figure 4.6 Expression of Pax5, HoxC4, in malaria exposed Tonsillar B-cell subsets

Fold change in the expression of Pax5(A), HoxC4(B), are presented as box plots showing the median with range (minimum – maximum) of 6 samples run in technical triplicates. The levels of all genes were normalized to β-actin expression and are expressed as fold changes relative to NB. Stars represent level of statistical significance as tested by unpaired student's t-test between B-cell subsets. * = $p<0.05$, ** = $p<0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, *ns* = no statistical significance observed.

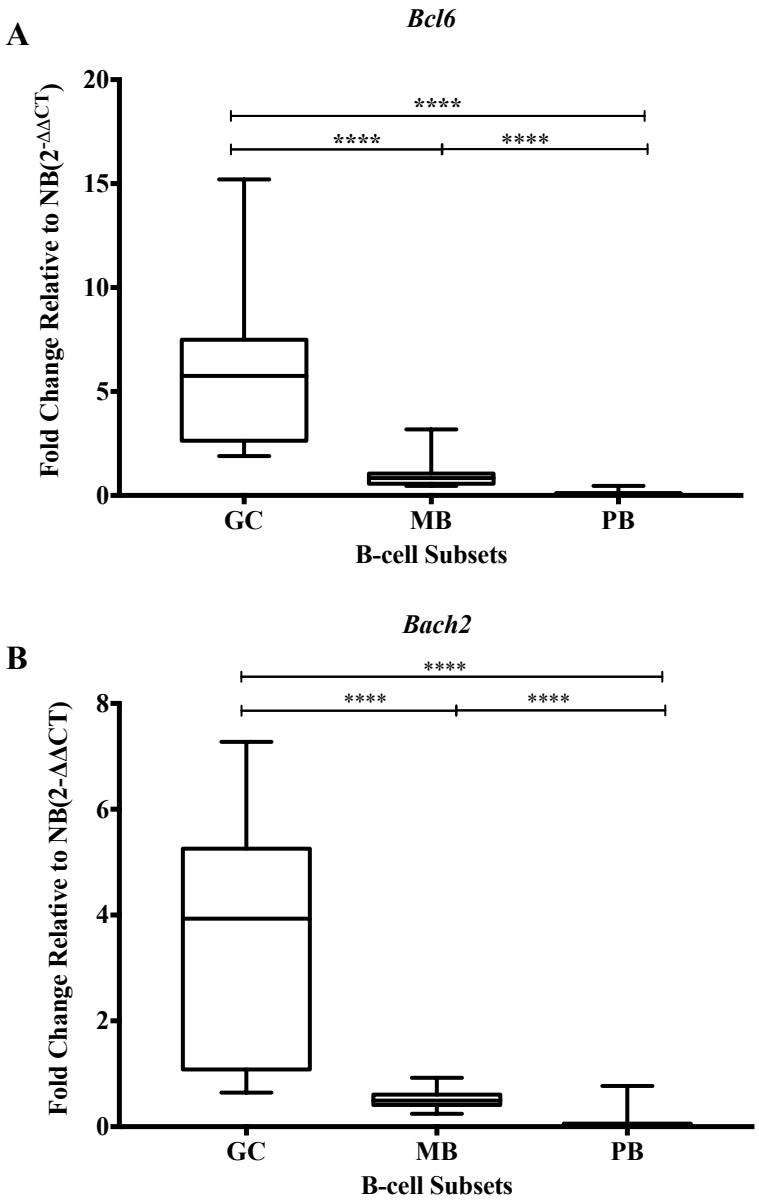

Figure 4.7 Expression of Bcl6 and Bach2 in malaria exposed Tonsillar B-cell subsets
Fold change in the expression of Bacl6(A) and Bach2(B) are presented as box plots showing the median with range (minimum-maximum) of 6 samples run in technical triplicates. The levels of all genes were normalized to β-actin expression and are expressed as fold changes relative to NB. Stars represent level of statistical significance as tested by unpaired student's t-test between B-cell subsets. * = $p<0.05$, ** = $p<0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, *ns* = no statistical significance observed.

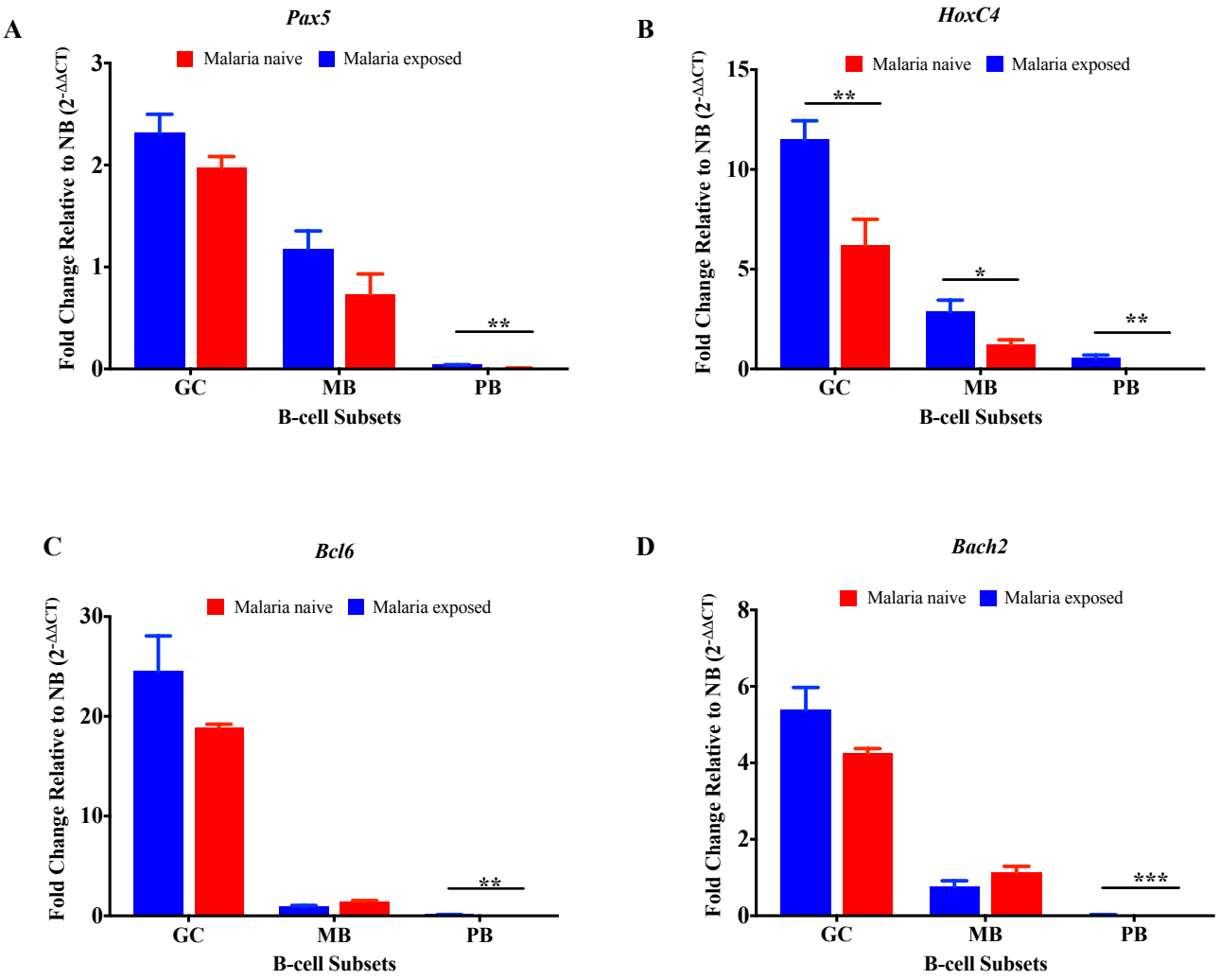

Figure 4.8 Expression of Pax5, HoxC4, Bcl6 and Bach2 in Tonsillar B lymphocytes
The expression levels of target genes are compared in age-matched malaria exposed and naïve. Fold change in the expression of Pax5(A), HoxC4(B), Bacl6(C) and Bach2(D) are presented as bar graphs showing the mean of technical triplicates RT-qPCR runs. Error bars are standard errors of the mean. The levels of all genes were normalized to β-actin expression and are expressed as fold changes relative to naïve B lymphocytes (NB). Stars represent level of statistical significance as tested by unpaired student's t-test between B-cell subsets. * = $p<0.05$, ** = $p<0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, *ns* = no statistical significance observed.

4.1.4 AID transcript levels in Tonsillar B-cell subsets

To understand how the expression of the above transcription factors are related to AID expression in Tonsillar B cell subsets, the transcripts levels of AID was measured by RT-qPCR in the sorted B lymphocytes. Expectedly, the levels of AID transcripts were highest in the GC B lymphocytes of all the Ghanaian tonsils. Figure 4.9A shows the mean levels of AID mRNA in all six Ghanaian tonsils analyzed. Although the levels on AID transcripts were higher in the B-cell subsets of a GH tonsil when compared to an age-matched NM tonsil (Figure 4.9B), the fold-changes relative to their respective NB lymphocytes remained the same in MB, PB and GC B lymphocytes, suggesting that the changes in the transcription of the AID gene relative to NB lymphocytes is not different.

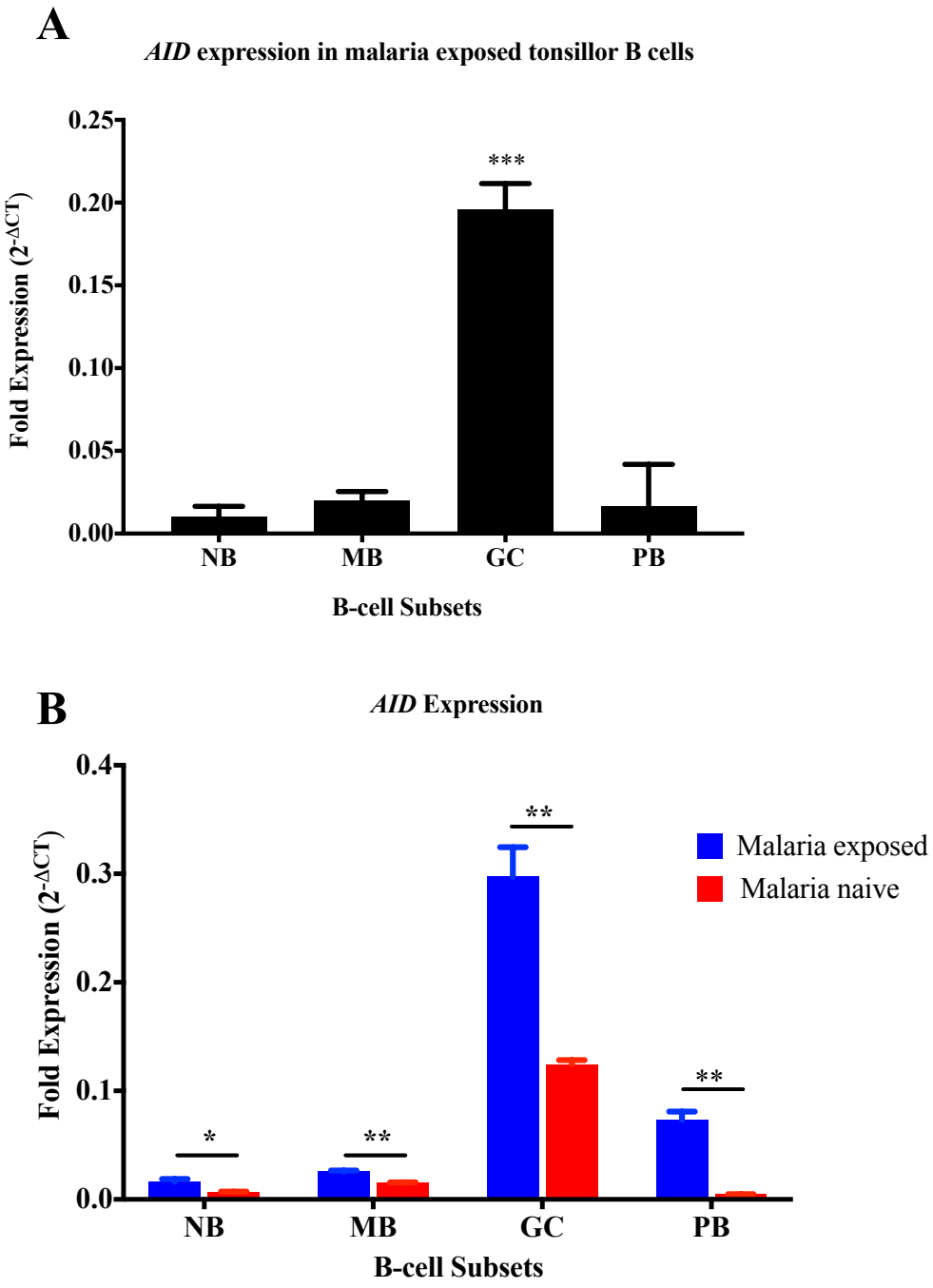

Figure 4.9 Levels of AID mRNA transcript in Tonsillar B-cell subsets.
A: The expression of AID relative to β-actin presented as the mean expression from B-cell subsets from 6 Ghanaian tonsils. **B:** Comparison of AID transcripts levels in age-matched malaria exposed and naïve tonsils. * = $p<0.05$, ** = $p<0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, *ns* = no statistical significance observed.

4.1.5 AID mRNA Splice variants in Tonsillar B lymphocytes

Nested PCR using primers that detect the full-length AID transcript (AIDFL) and all four reported splice variants (Wu et al., 2008) revealed at least three bands corresponding to AIDFL(~594 base pairs), AIDΔE4a (~564 base pairs) and AIDΔE3E4 (~207 base pairs) by resolution on a 1.5% agarose gel (Figure 4.10A). To determine the identity of the PCR products from the GC B lymphocytes of age matched GH and NM tonsils, the amplicons were cloned into a pGEMT® vector and used to transform JM109 Competent Cells for sequencing. From multiple sequence alignments of the DNA from JM109 colonies, only colonies with AID-FL (594 bp), AID-ΔE4 (478 bp), AID-ΔE3E4 (207 bp) have been identified in the GH GC B lymphocytes thus far. In the NM GC cells only one variant AID-ΔE4 (478 bp) has been detected in addition to AID-FL (594 bp). Multiple sequence alignments of these sequences show that the sequences of these variants are highly conserved as there was little variation between the observed sequences and the reference sequences. Figure 4.10B shows a representative alignment of the AID-FL from the GH and NM GC B lymphocytes in comparison to sequences obtained from AID-FL amplicon from the Burkitt' lymphoma cell line Raji.

To determine which of these splice variants are expressed in the B cell subsets, variant-specific primers (Zaprazna et al., 2019) were used to amplify distinct regions of each primer using RT-qPCR. Figure 4.11A illustrates the location of the targets of primer sets for each variant. In Burkitt's lymphoma cell lines Raji and Ramos, AID-FL is the predominant transcript expressed. Lower transcripts of AID-ΔE4a and AIDivs3 were detected in both Raji and Ramos, but AID-ΔE4 was detected in only Ramos (Figure 4.11B).

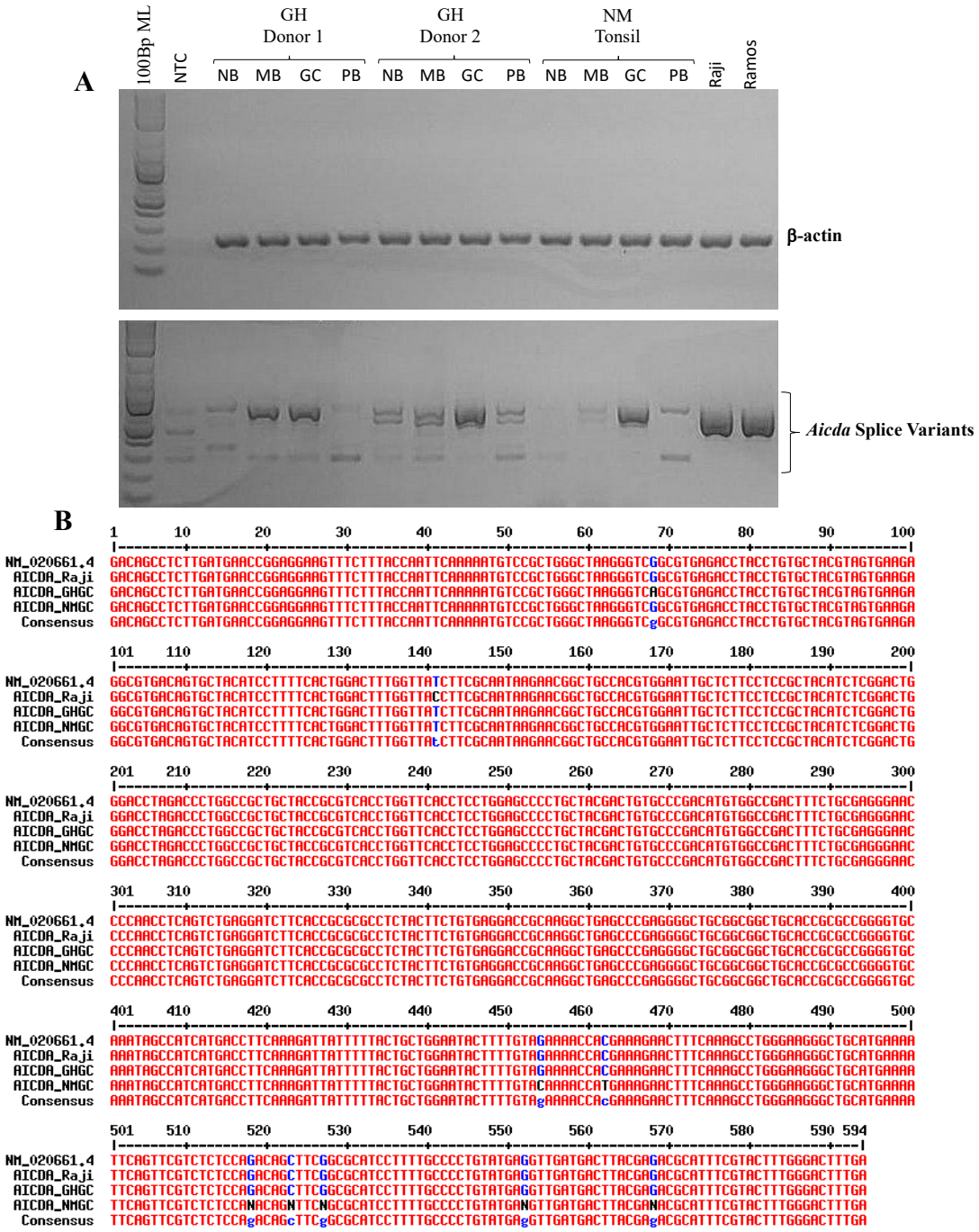

Figure 4.10 Identification of AID mRNA splice variants in Tonsillar B-cell subsets

A: A representative agarose gel image showing the different AID splice variants in B-cell subsets from malaria exposed (GH) and one malaria naïve (NM) tonsils. β-actin was amplified as loading control. **B:** Representative multiple sequence alignment of AIDFL sequenced from PCR product of GC B lymphocytes from malaria exposed and naïve tonsils.

In the Tonsillar B-cell subsets, however, all four variants were detected. AID-FL was predominantly expressed in the GC B lymphocytes from all six tonsils analyzed with significant differences between MB and PB lymphocytes (Figure 4.11C). Similarly, AID-ΔE4a was highly expressed in the GC B lymphocytes, with significant difference among PB, MB and GC cells. Although transcripts of AID-ΔE4 were detected in all B-cell subsets, their levels were not different across the three B-cell subsets. Equal levels of AIDΔE3E4 were detected in MB and GC B lymphocytes which were both higher than the levels detected in PB lymphocytes. Significant levels of AIDivs3 was detected in GC B lymphocytes compared to MB and PB lymphocytes (Figure 4.11C).

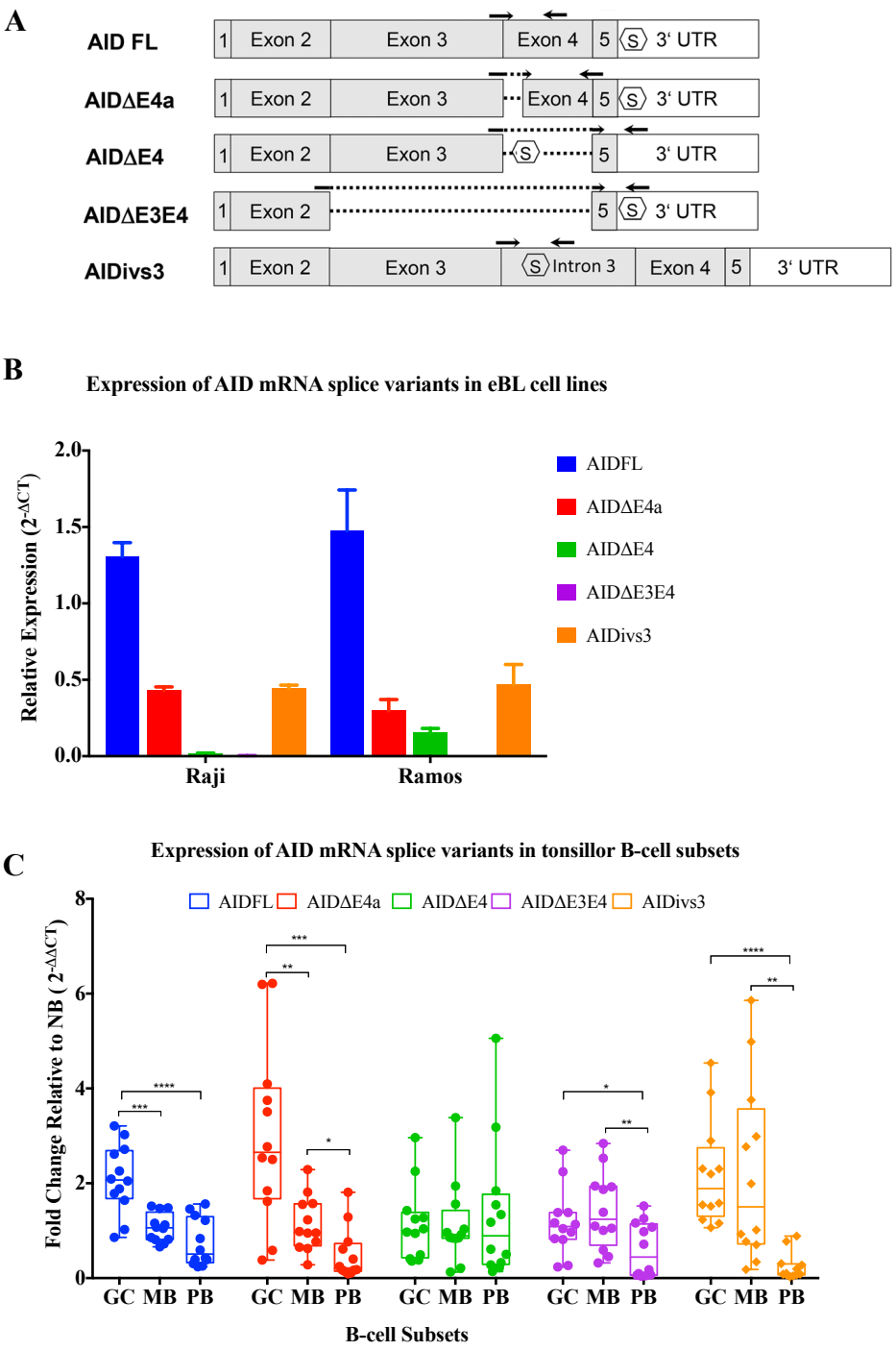

Figure 4.11 Transcript levels of AID splice variants in Tonsillar B-cell subsets.
A: Illustration of primers for amplifying AID splice variants. **B:** Expression of AID splice variants in two Burkitt's lymphoma cell line (Raji and Ramos). **C:** Fold change in AID splice variant expression levels in Ghanaian tonsils. * = p<0.05, ** = p<0.01, *** = p < 0.001, **** = p < 0.0001, *ns* = no statistical significance observed.

4.1.6 miR155 and miR181b expression in Tonsillar B-cell subsets

miR155 and miR181b are key post-transcriptional regulators of AID expression. The expression levels of these microRNAs were measured in Tonsillar B cell subsets and are presented as relative expression levels to the expression of miR191. The expression of miR181b was down-regulated in the B-cell subsets analyzed, with mean relative levels ranging from 0.1 in memory B lymphocytes to 0.8 in plasmablasts (Figure 4.12A). The differences in the mean expression was significant among the B cell subsets ($p<0.0001$). In the GC of secondary lymphoid tissues, miR155 is important to the regulation of AID mRNA levels. Relative to the expression of miR191, the expression of miR155 was down-regulated in MBs and GCs, but up-regulated in NBs cells and PBs (Figure 4.12B).

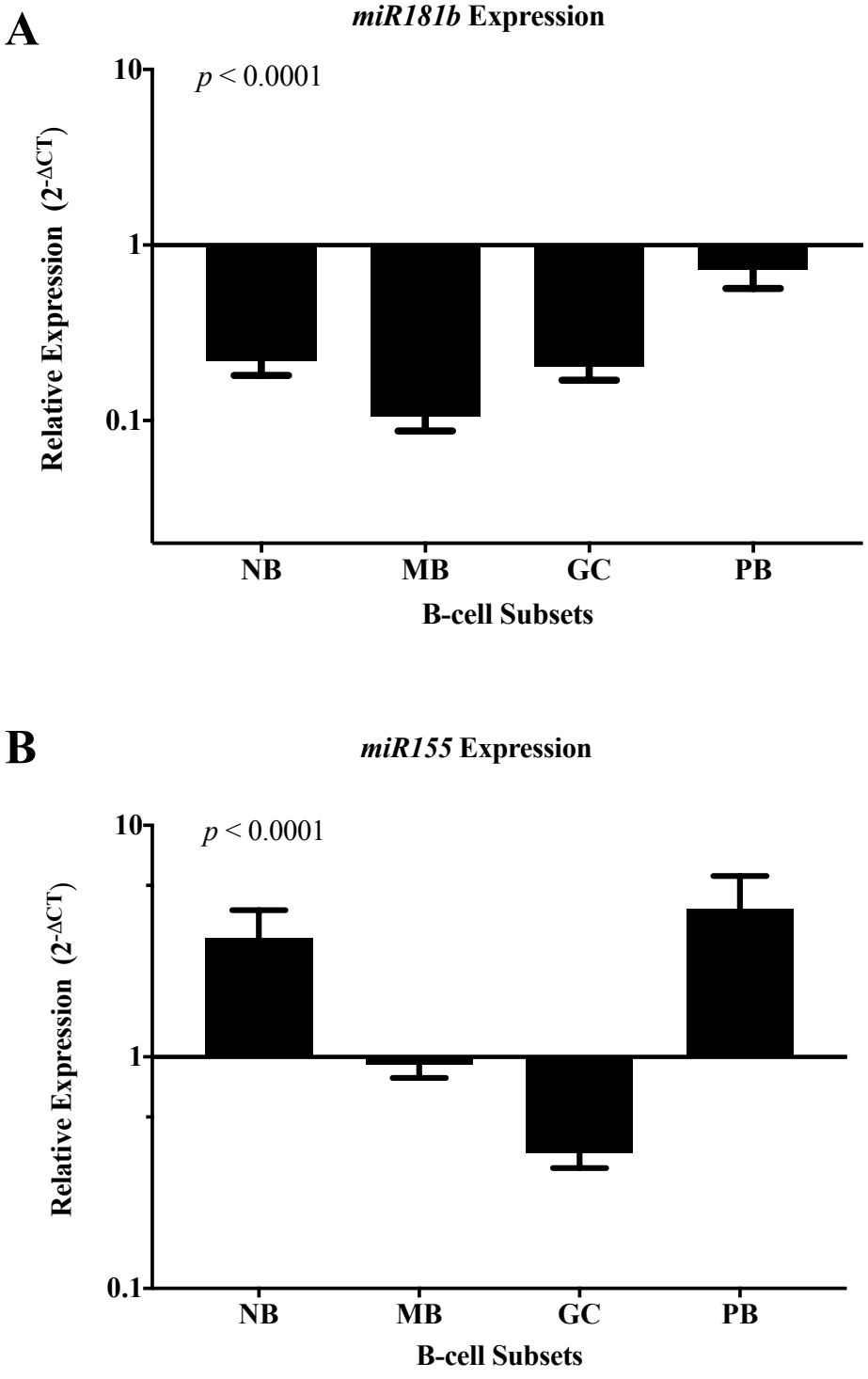

Figure 4.12 Expression of miR155 and miR181b in Tonsillar B-cell subsets
Expression levels of miR181b (A) and miR155 (B), normalized to the expression of miR191 in all six malaria exposed tonsils. Kruskal Wallis test was used to establish statistical differences among B cell subsets.

4.2 Transcriptional regulatory patterns of AID expression in asymptomatic *P. falciparum*-infected children

Prolonged infections with *P. falciparum* and EBV have been linked to the risk of incidence of endemic Burkitt's lymphoma. However, it is not clear what role the malaria parasite plays in the genesis of the cancer, especially in healthy children with asymptomatic infections. This part of the study presents results linking the expression patterns of AID and related transcription factors in PBMCs of otherwise healthy Ghanaian children infected with *P. falciparum*.

4.2.1 Demographic and laboratory characteristics of participants

Venous blood from 88 non-febrile (temperature </= 37ºC) children was obtained from primary school children aged from 5 to 16 years. Out of the 88, 54 (61.6%) were female and 34(38.6%) were males. The participants were grouped into categories based on the diagnosis of *P. falciparum* infection by RDT and confirmed by PCR. Those who tested positive for both PCR and RDT were classified as asymptomatic (n = 19), and those who were double negative for PCR and RDT were classified as *P. falciparum* negative (69). There was no significant difference in the mean ages of the participants in both groups (p = 0.2126). In addition, there was no significant difference in any of the erythrocyte indices measured between asymptomatic and *P. falciparum* negative individuals (Table 4.1). Among the leukocyte indices measured, white blood cell (WBC) count and lymphocyte counts were the same between the two groups, but the asymptomatic infected children had higher granulocyte (p = 0.0020) and monocyte counts (p = 0.0468). Again, the asymptomatic children had reduced platelet counts (p = 0.0119) while the mean platelet volume (MPV) in the asymptomatic individual was higher than the non-infected children (p = 0.0023). The prevalence of EBV seropositivity to EBNA-1 IgG and IgM was 93% among those without *P. falciparum*

infection while all (100%) the asymptomatic infected children were seropositive for EBNA-1 IgG (Table 4.1).

Table 4.1 Demographic and laboratory characteristic of healthy primary school children

Characteristics	***Pf.* Negative**	**Asymptomatic**	***P***	***n***
Age (years)	9.56(0.184, *n*= 69)	10.32(0.697, *n*=19)	0.2126	88
Female Gender, no. (%)*	44, (50.3)	10, (52.6)	0.8460	88
Erythrocyte indices				
Hemoglobin (g/dL)	11.34(0.099)	11.31(0.3069)	0.9215	88
Hematocrit (%)	33.17(0.291)	33.36(0.8933)	0.8315	88
RBC($x10^{12}$/L)	4.281(0.042)	4.376(0.117)	0.4792	88
MCV (fl)	77.99(0.4707)	76.59(1.474)	0.3549	88
MCH (pg)	25.59(0.178)	25.88(0.5644)	0.2138	88
MCHC (g/L)	35.94(1.789)	33.86(0.2147)	0.7024	88
RDW (%)	13.8(0.0889)	13.76(0.2174)	0.08756	88
Leucocyte indices				
WBC ($x10^9$/L)	5.871(70.1253)	5.974(0.3665)	0.7970	88
Granulocytes ($x10^9$/L)	1.328(0.06729)	2.321(0.7513)	**0.0020**	88
Lymphocytes ($x10^9$/L)	3.711(0.0772)	3.342(0.1950)	0.1303	88
Monocytes ($x10^9$/L)	0.830(0.0293)	1.021(0.1056)	**0.0468**	88
Platelet indices				
Platelet counts ($x10^9$/L)	294.200(6.621)	256.80(11.2300)	**0.0119**	88
MPV (fl)	9.997(0.0765)	14.970(0.4.9480)	**0.0023**	88
EBV Infection				
EBNA-1 Positive, no (%)*	82, (93)	19 (100)	0.2605	

Data is presented as mean (standard deviation). Unpaired students **t** test was conducted for differences in mean between groups. Statically significant values ($p < 0.05$) are presented in bold. *Chi squares were used to estimate statistical differences between Gender and EBV exposure in the two groups. **RBC**, red blood cell count; **MCV**, mean corpuscular volume; **MCH**, mean cell hemoglobin; **MCHC**, mean cell hemoglobin concentration; **RDW**, red cell distribution width; **WBC**, white blood cell count; **MPV**, mean platelet volume.

4.2.2 EBV and *P. falciparum* infection results in elevated AID transcripts

The level of AID transcripts was compared between asymptomatic (n = 19) and *P. falciparum* uninfected children (n = 69). There were higher AID transcripts in the *P. falciparum* infected children than the *Pf.* Negative children ($p = 0.0004$). To eliminate possible confounding biases with the disparity in numbers, the level of AID transcripts in age-matched RDT^-PCR^- children and RDT+PCR+ children were compared and there was a significant expression ($p = 0.00197$) of AID in the *P. falciparum* infected children (Figure 4.13A). Next, the contribution of EBV and *P. falciparum* to the levels of AID transcripts were measured. Interestingly, among RDT^-PCR^- children with prior exposure to EBV as expressed by the positivity of EBNA-1 IgG and/or IgM did not have a significant effect on the levels of AID measured (Figure 4.13B). Hence the conclusion that infections with both EBV and *P. falciparum* is associated with elevated levels of AID transcripts in healthy children.

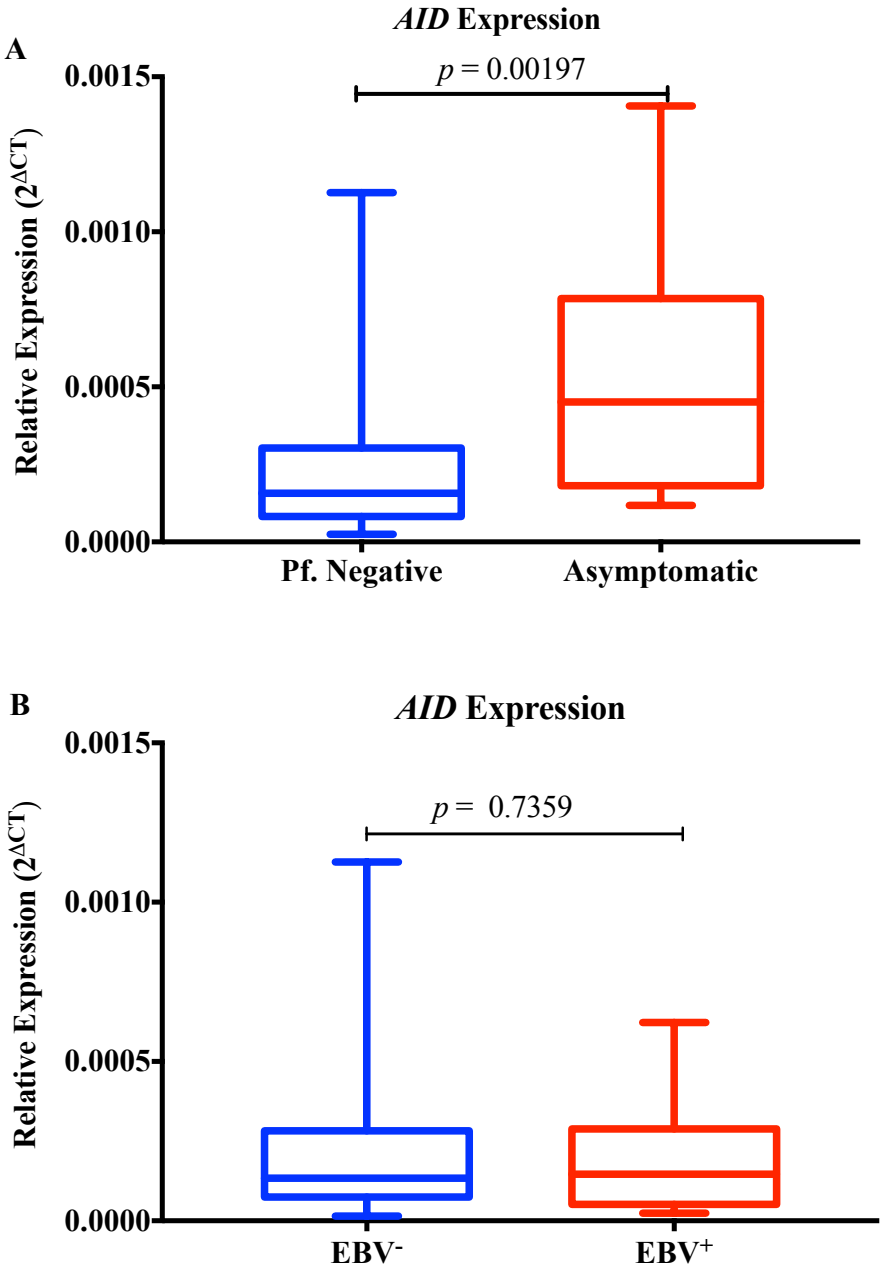

Figure 4.13 AID expression in asymptomatic *P. falciparum* infected children
A: β-actin normalized AID transcript levels in age-matched asymptomatic P. falciparum infected and non-infected children. **B:** β-actin normalized AID levels in children with detectable and non-detectable EBV EBNA-1 antibodies. Each box shows the range (minimum to maximum) with the median. Mann-Whitney test was used to estimate statistical significance at 95% confidence interval. * = $p<0.05$, ** = $p<0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, *ns* = no statistical significance observed.

4.2.3 Expression patterns of AID associated transcription factors

To determine the effect of chronic *P. falciparum* infection on the expression of transcription factors known to regulate the expression of AID, the levels of Irf8, Irf4, Prdm-1, XBP-1, Pax5 and Bcl6 were measured by RT-qPCR from age- and gender-matched children within the two groups. Among the transcription factors that were measurable, the expression of XBP-1 was not different between the uninfected and asymptomatic children. Prdm-1 and Irf4 on the other hand were significantly up-regulated ($p < 0.05$) in asymptomatic children than P. falciparum uninfected children (Figure 4.14).

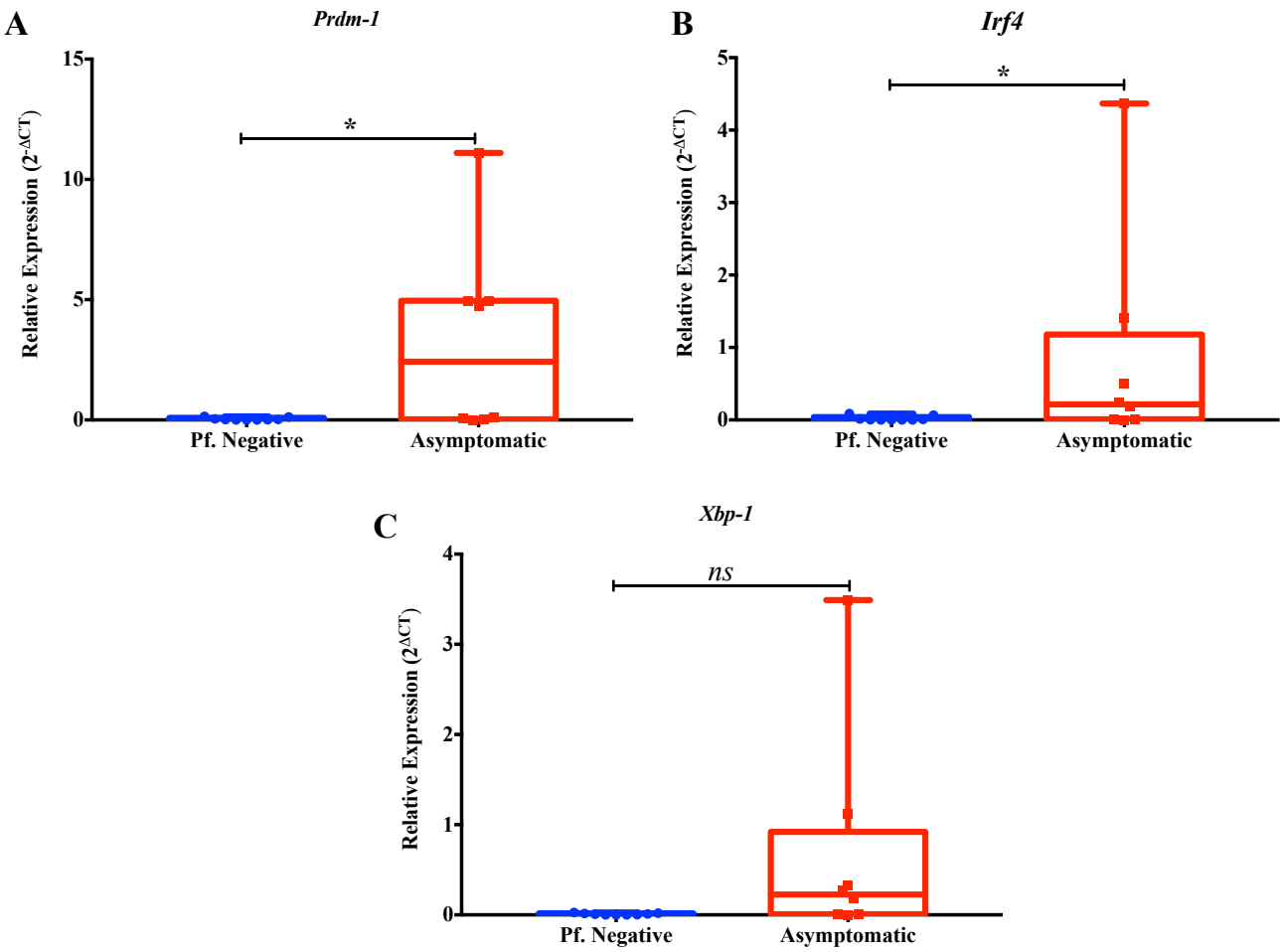

Figure 4.14 Expression Prdm-1, Irf4 and XBP-1 in asymptomatic *P. falciparum*-infected children: Expression Prdm-1 (A), Irf4 (B), XBP-1 (C) in age and gender matched healthy children with or without *P. falciparum* infection. Boxes represent summery of range (minimum – maximum) with median. Significance was determined by Mann-Whitney test. * = $p<0.05$, ** = $p<0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, *ns* = no statistical significance observed.

The expression of Irf8 and XBP-1 were not significantly different between asymptomatic and uninfected children. The expression of Prdm-1, Irf4, Pax5 and Bcl6 were significantly up-regulated in asymptomatic children than P. falciparum uninfected children ($p < 0.05$) (Figure 4.14).

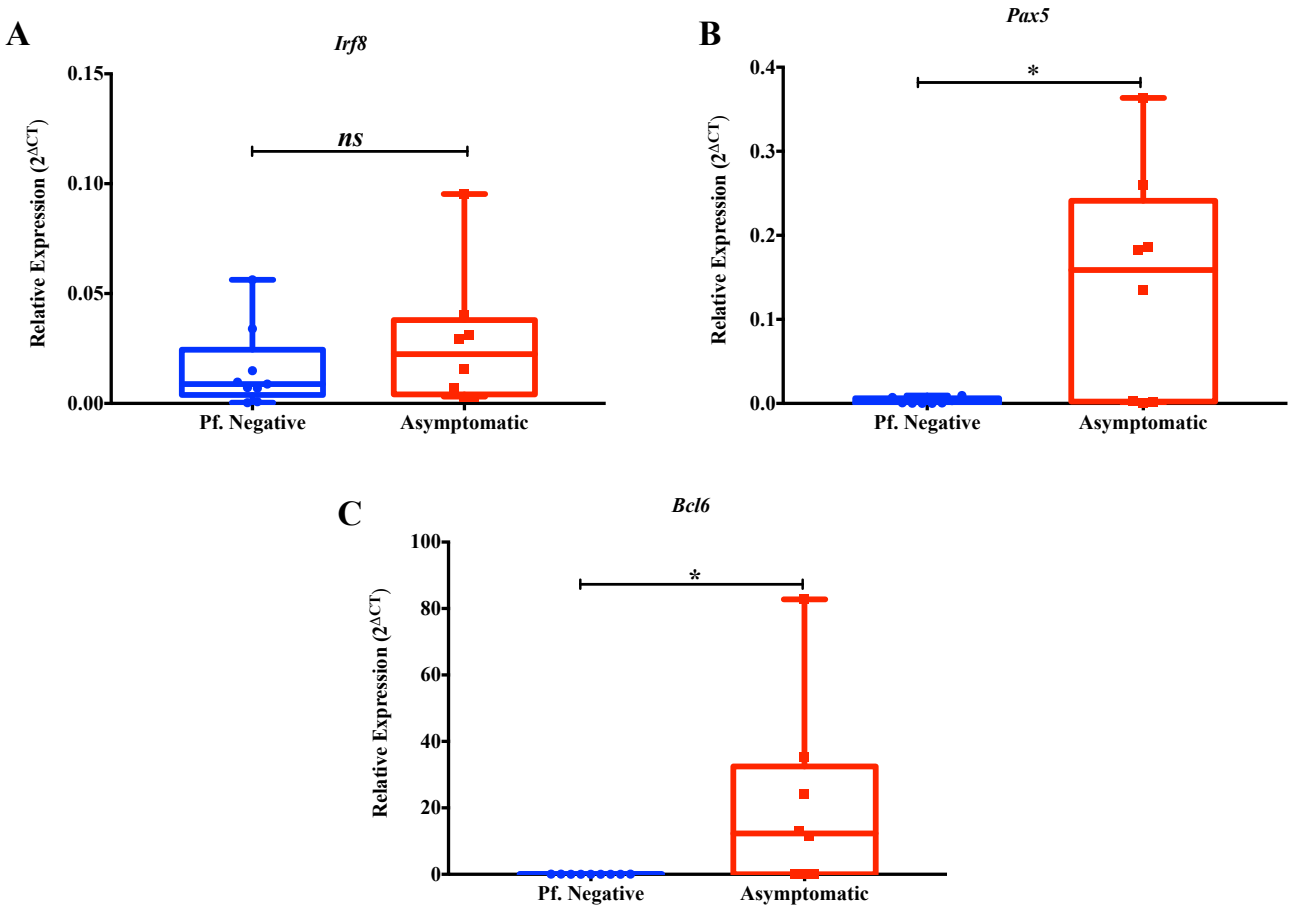

Figure 4.15 Expression Irf8, Pax5 and Bcl6 in asymptomatic *P. falciparum*-infected children
Expression Irf8 (A), Pax5 (B) and Bcl6 (C) in age and gender-matched healthy children with or without *P. falciparum* infection. Boxes represent summery of range (min – max) with median. Significance was determined by Mann-Whitney test. * = $p<0.05$, ** = $p<0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, *ns* = no statistical significance observed.

4.2.4 Expression of AID splice variants in asymptomatic *P. falciparum*-infected children

The levels of AIDFL and the reported splice variants were measured and normalized to the expression of GAPDH in age-matched children with and without *P. falciparum* infection. In agreement with earlier results, the level AIDFL was significantly higher ($p < 0.05$) in asymptomatic infected children (Figure 4.16). The mean relative expression of the other variants was very low and there were no differences between *Pf.* negative and asymptomatic children.

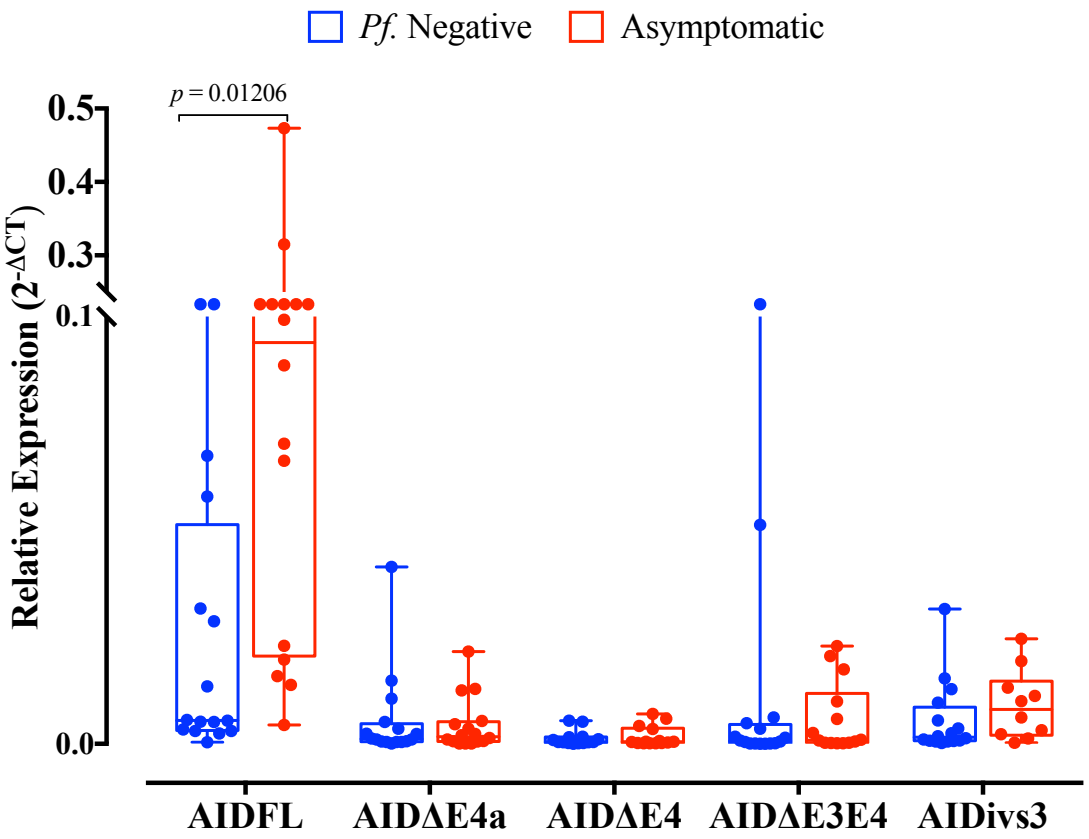

Figure 4.16 Expression of AID splice variants in *Pf.* Asymptomatic children
Levels of AID mRNA splice variants are normalized to the expression of GAPDH. Plots represent range (min – max) with median. Mann Whitney u test was used to determine statistical differences. * = $p<0.05$, ** = $p<0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, *ns* = no statistical significance observed.

4.3 Transcriptional regulatory patterns of AID expression in children with clinical symptoms of Severe Malaria Anemia.

This part of the study aimed to understand how acute illness in children prone to malaria affects AID expression and transcriptional regulation. Using RNA and plasma samples from a cohort of children taken between 2009 and 2012, this part of the study presents the results on the expression of AID and related transcription factors as well as miR181b and mir155 in febrile children, with or without *P. falciparum* infection.

4.3.1 Demographic and Clinical Characteristics of participants

Archived samples from 81 children aged between 2.87 and 36.37 months, presenting at hospital with acute febrile illness, negative for HIV and bacteremia, were selected for this study. Out of these 81 subjects, 12 were negative for *P. falciparum* infection by microscopy and PCR, and were classified as aparasitemic (AP). The remaining 69 with detectable parasite loads were stratified into two categories; Severe malaria anemia, SMA (Hb < 6.0 g/dL, $n = 35$) and non-SMA (Hb > 6.0 g/dL, $n = 34$). The mean ages of three groups were the same as was the parasite density between SMA and non-SMA categories (Table 4.1).

Since the categories were created based on the Hb levels, HB, hematocrit and RBC counts were different across the three groups. Together, those infected with *P. falciparum* had significantly lower Hb compared to the AP group ($p = 0.0019$). The Hb levels between the non-SMA and AP categories were, however, comparable. Hematocrit and RBC count followed a similar trend (Table 4.1). Other red blood cell indices such as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were comparable among the three categories. Among the leukocyte indices, white blood cell counts (WBC), granulocyte counts, lymphocyte counts and monocytes counts were comparable.

Thrombocytopenia was observed in the SMA and non-SMA groups but not in the AP group, as indicated by the low platelet counts (Plt) and mean platelet volume (MPV) in the *P. falciparum* infected individuals.

Table 4.2 Demographic and laboratory characteristics of samples

Characteristics	**AP**	**Non-SMA**	**SMA**	***P***	***n***
Demographic indices					
Age (months)	16.77(19.92)	11.63(7.33)	11.77(17.27)	0.187	81
Parasitemia (/µL) *	-	25632(66 470)	20777(41115)	0.9477	69
Erythrocyte indices					
Hemoglobin (g/dL)	9.8(4.73)	7.7(2)	5.25(0.35)	**<0.0001**	81
Hematocrit (%)	31.45(11.57)	25.2(8.00)	17.95(3.2)	**<0.0001**	81
RBC(x10^{12}/L)	4.695(1.11)	4.07(1.39)	2.445(0.46)	**<0.0001**	81
MCV (fl)	69.65(13.87)	68.(13.2)	71.25(12.45)	0.1003	81
MCH (pg)	20.8(6.12)	20.7(4.4)	21.65(3.38)	0.2726	81
MCHC (g/L)	30.15(3.57)	30.1(2.6)	29(4.28)	0.6824	81
RDW (%)	20.2(6.6)	19.7(5.1)	20.2(6.31)	0.4575	81
Reticulocyte Count (%)					
Leucocyte indices					
WBC (x10^9/L)	12.85(4.825)	10.6(7.4)	14.55(10.55)	0.4337	81
Granulocytes (x10^9/L)	4.85(2.65)	5(4.6)	5.7(4.95)	0.5172	81
Lymphocytes (x10^9/L)	7.45(5.55)	5.6(5.3)	6.35(6.125)	0.5587	81
Monocytes (x10^9/L)	0.8(0.975)	0.9(0.9)	1.1(1.175)	0.0795	81
Platelet indices					
Platelet counts (x10^9/L)	295(251.3)	140(84)	138(82)	**0.0001**	81
MPV (fl)	6.75(1.08)	7.6(1.3)	7.85(0.68)	**0.0086**	81

Unless indicated, the median (interquartile range) is presented here. Kruskal-Wallis test was conducted for differences in medians among three groups. *Mann-Whitney test was used for differences in parasite density between Non-SMA and SMA. Statistically significant values ($p < 0.05$) are presented in bold. **AP**, Aparasitemic; **SMA**, Severe malaria anemia; **RBC**, red blood cell count; **MCV**, mean corpuscular volume; **MCH**, mean cell hemoglobin; **MCHC**, mean cell hemoglobin concentration; **RDW**, red cell distribution width; **WBC**, white blood cell count; **MPV**, mean platelet volume.

4.3.2 AID Expression in Severe Malaria Anemia

4.3.2.1 Fever and Hb levels are not associated with elevated AID transcript levels.

The aberrant expression of AID is associated with inflammation and fever. To understand how fever relates to the abundance of AID transcripts in children with Severe malaria anemia, the level of AID transcripts in AP, SMA and non-SMA were compared to the reported number of days the children have had fever. The length of reported fever was grouped into three main categories, those with no fever, those with fever up to three days (1-3 days) and those with fever between 4 and 14 (4-14) days, prior to the hospital visit. Children with SMA (Hb < 6.0 g/dL) had higher AID transcripts than those with non-SMA ($p < 0.001$) and those without P. falciparum infection, AP, ($p = 0.001$) (Figure 4.17A). Among the selected samples, only children with SMA could be grouped into all three categories of reported fever. Although children with reported fever had slightly more AID transcripts than those with no reported fever, the differences were not statistically significant ($p = 0.4776$) among these sub groups, (Figure 4.17B).

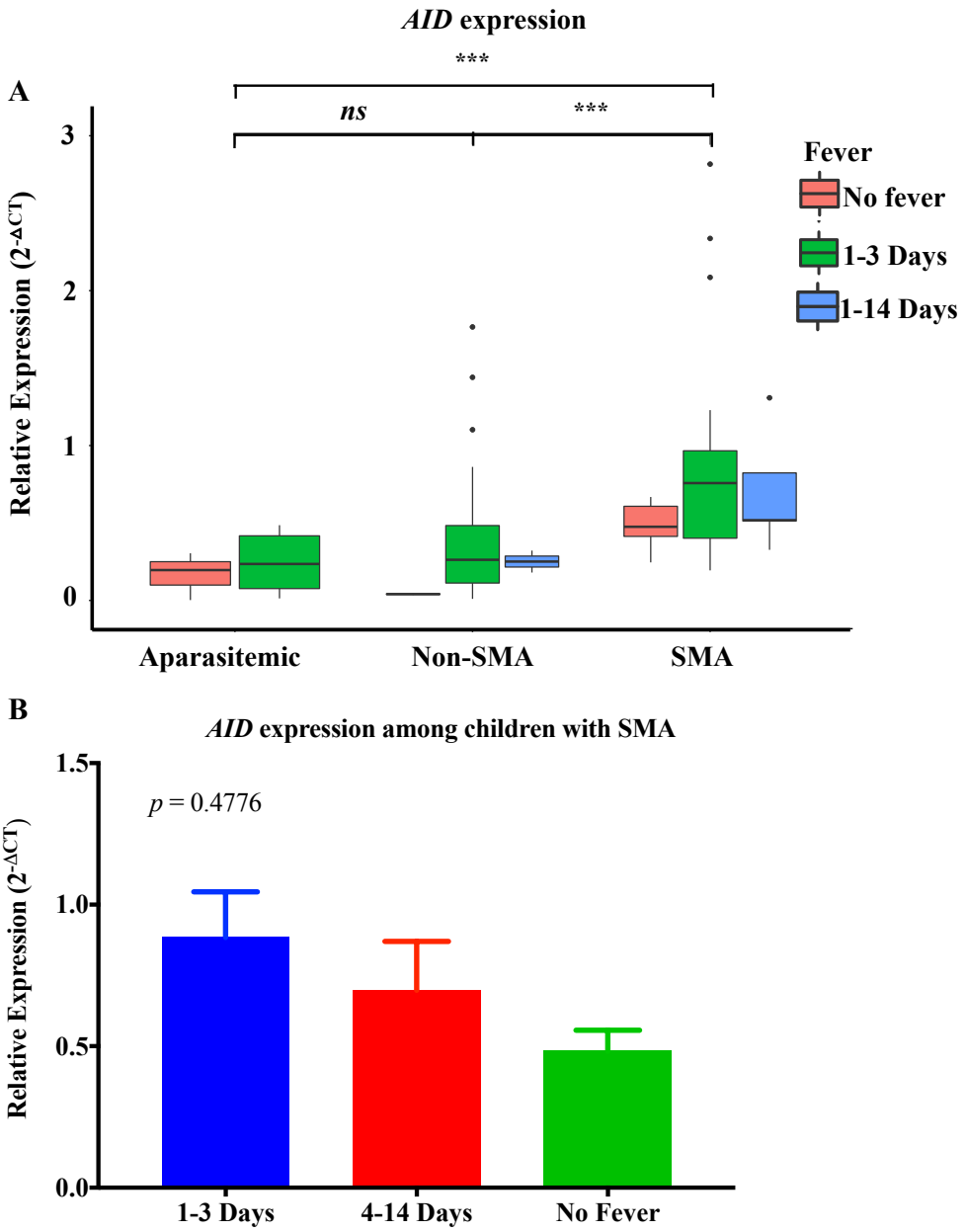

Figure 4.17 Expression of AID in children with SMA

A: β-actin normalized levels of AID transcripts in children with or without Severe malaria anemia. Reported fever is classified into three groups, based on the length of febrile episode. **B:** Fold AID expression in children with SMA, separated into the length of febrile episode expressed as means with standard error of mean. Kruskal Wallis test was used to evaluate the differences among the three groups. * = p<0.05, ** = p<0.01, *** =p < 0.001, **** = p < 0.0001, *ns* = no statistical significance observed.

To confirm this finding, body temperatures of the children were used for correlation analysis between body temperature and AID transcripts levels. Correlation analysis revealed a weak and insignificant correlation between body temperature and AID transcript levels, (Pearson's coefficient, *R, p:* AP = 0.29, 0.36, Non-SMA = 0.20, 0.24 and SMA = 0.16, 0.38). It can be concluded, that since the strength of the relationship was the same across the three groups, (p =/> 0.05), fever could not have accounted for the difference in AID transcripts observed (Figure 4.18A). There was no significant positive correlation between Hb levels and AID levels in children infected with *P. falciparum*, whether classified as AP, SMA or non-SMA (Pearson's R, p value: AP = -0.36, 0.35, non-SMA = 0.26, 0.16 and SMA = 0.09, 0.62) (Figure 4.18B).

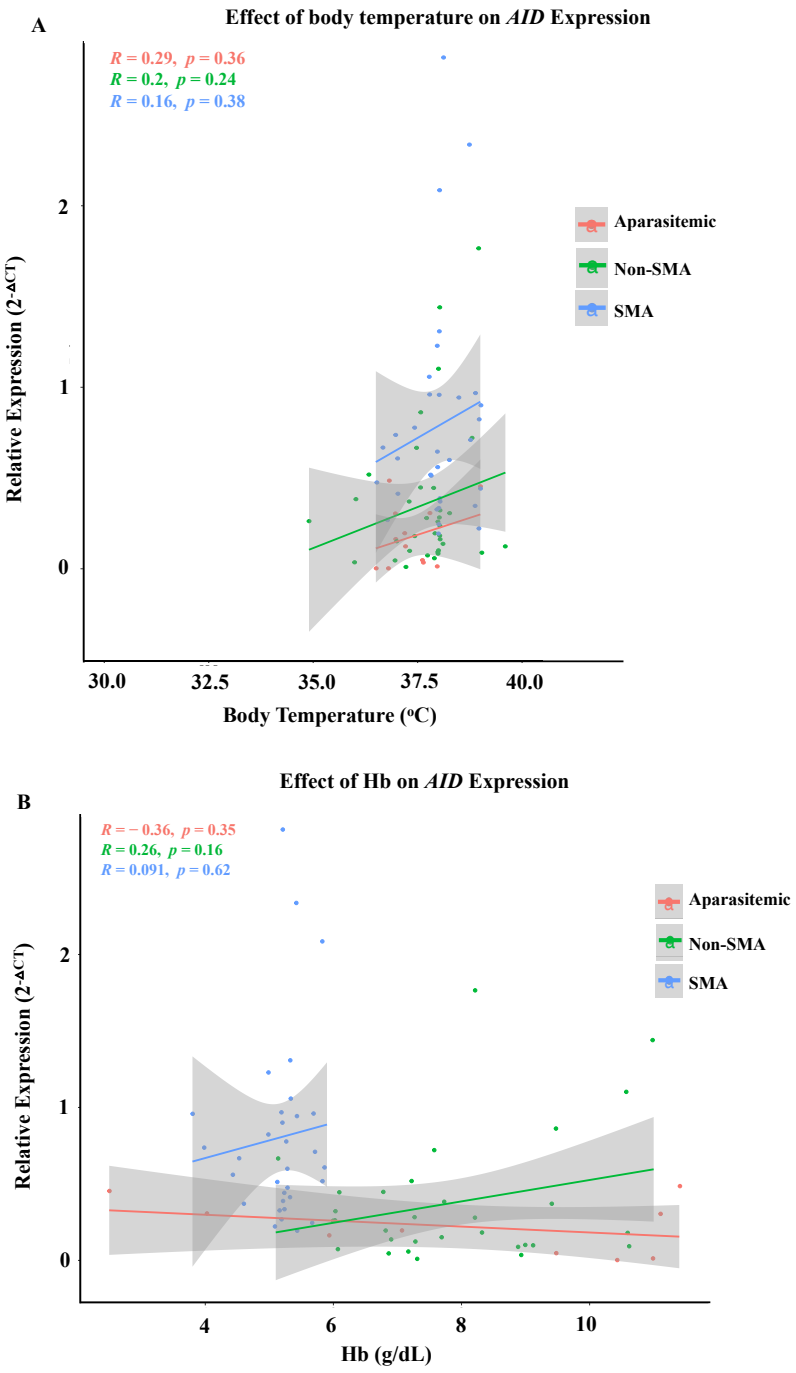

Figure 4.18 Correlation between fever, Hb and AID expression
A: Correlation of body temperature and AID expression levels among aparasitemic, non-SMA and SMA groups, showing Pearson's correlation coefficient *R* and *p* values. **B:** Correlation of Hb level and AID expression among aparasitemic, non-SMA and SMA children.

4.2.2.2 *P. falciparum* load is associated with elevated AID transcript in children with SMA

Next, the levels of AID were compared with parasite density among the two groups of children with *P. falciparum* infection. Although parasite load was not associated with the severity of anemia observed in the participants (MW test, $p = 0.9477$) (Table 4.2), there was a significant positive correlation (Pearson's $R = 0.41$, $p = 0.018$) between parasite load and AID transcripts among the children with SMA. On the other hand, the level of AID was not affected by increasing parasite loads in the children with Hb > 6.0 g/dL (Figure 4.19A). The data here suggests that SMA and parasite loads are associated with AID up-regulation.

The synergistic cooperation between *P. falciparum* and Epstein-Barr virus (EBV) has been reported to contribute to a dysregulated AID expression. To understand how co-infections with *P. falciparum* and EBV affect the levels of AID transcripts, the EBV VCA IgG and IgM levels were measured and samples were classified based on *P. falciparum* and EBV infection. Results from the 65 plasma samples tested for antibodies against the viral capsid antigen (AP =11, non-SMA = 24 and SMA = 30) suggest that all the children, irrespective of *P. falciparum* infection status have been seroconverted to EBV. Therefore, the EBV VCA IgG and IgM titers were correlated with the levels of AID transcripts in children who have been exposed to the virus and were infected with *P. falciparum*. All the children infected with *P. falciparum* (SMA and non-SMA) were positive for EBV CVA specific IgG and IgM, suggesting a possible reactivation of the viral lytic cycle and/or reinfection of new B lymphocytes. There was no significant correlation between EBV VCA IgG and IgM titers and AID transcript levels (Figure 4.19 B-E)

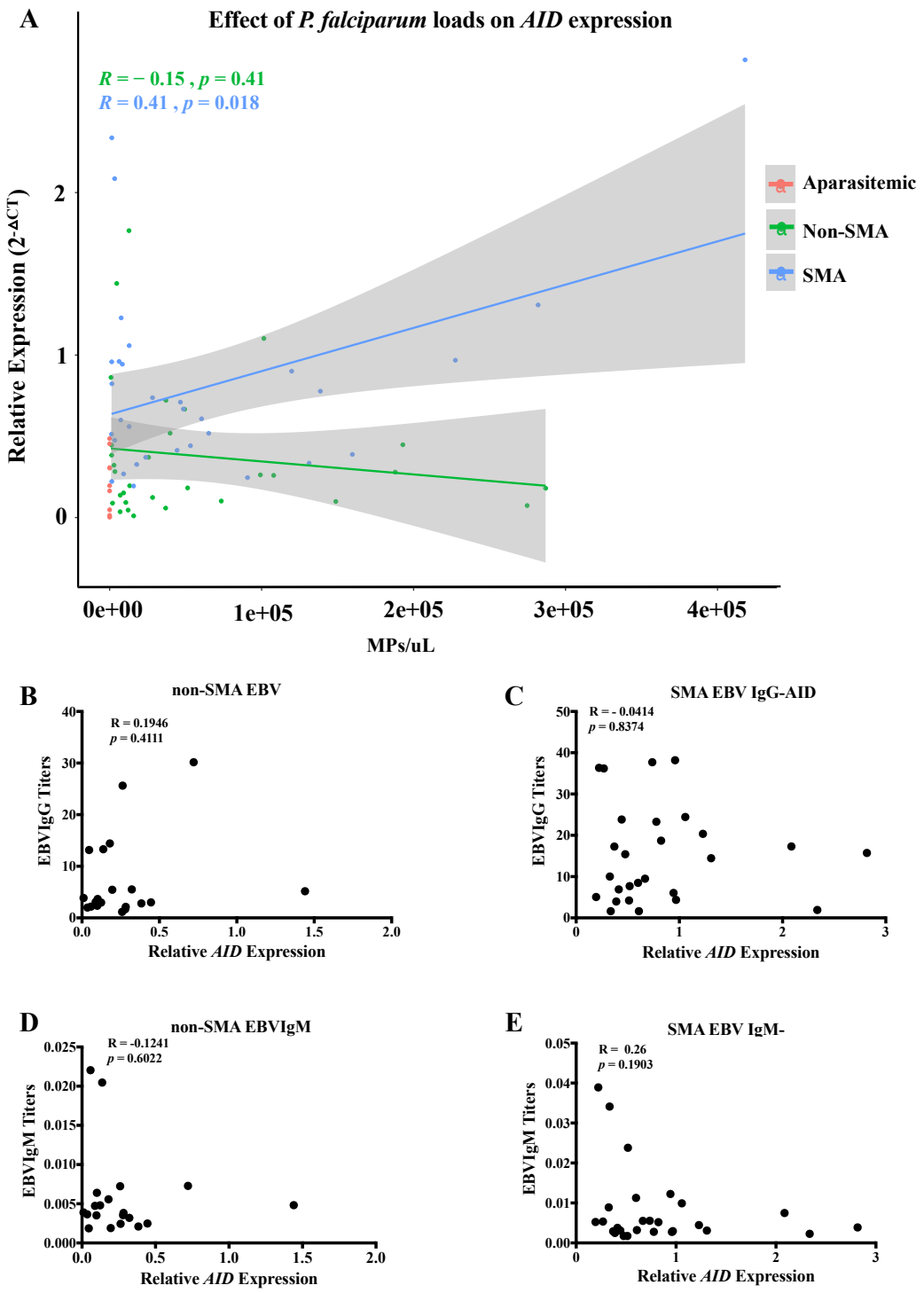

Figure 4.19 *P. falciparum*, EBV infection and AID transcript levels in SMA children
A: Correlation analysis of *P. falciparum* density and AID transcripts among aparasitemic, non-SMA and SMA. Correlation analysis of EBV CVA IgG and IgM in non-SMA (B and D) and SMA (C and E) children. Pearson's correlation coefficient and *p* values are shown on each plot.

4.3.3 Expression patterns of AID associated transcription factors in children with SMA

Five transcription factors (Prdm-1, Irf4, XBP-1, Irf8 *and* Pax5) were detectable by qPCR in the samples analyzed. The expression levels of Prdm-1 and Irf4 were similar among aparasitemic and *P. falciparum* infected children, with or without SMA (Figure 4.20 A and B). On the other hand, while the expression of XBP-1 was the same between non-SMA and SMA children, their levels were higher ($p < 0.01$) when compared to aparasitemic children (Figure 4.20C).

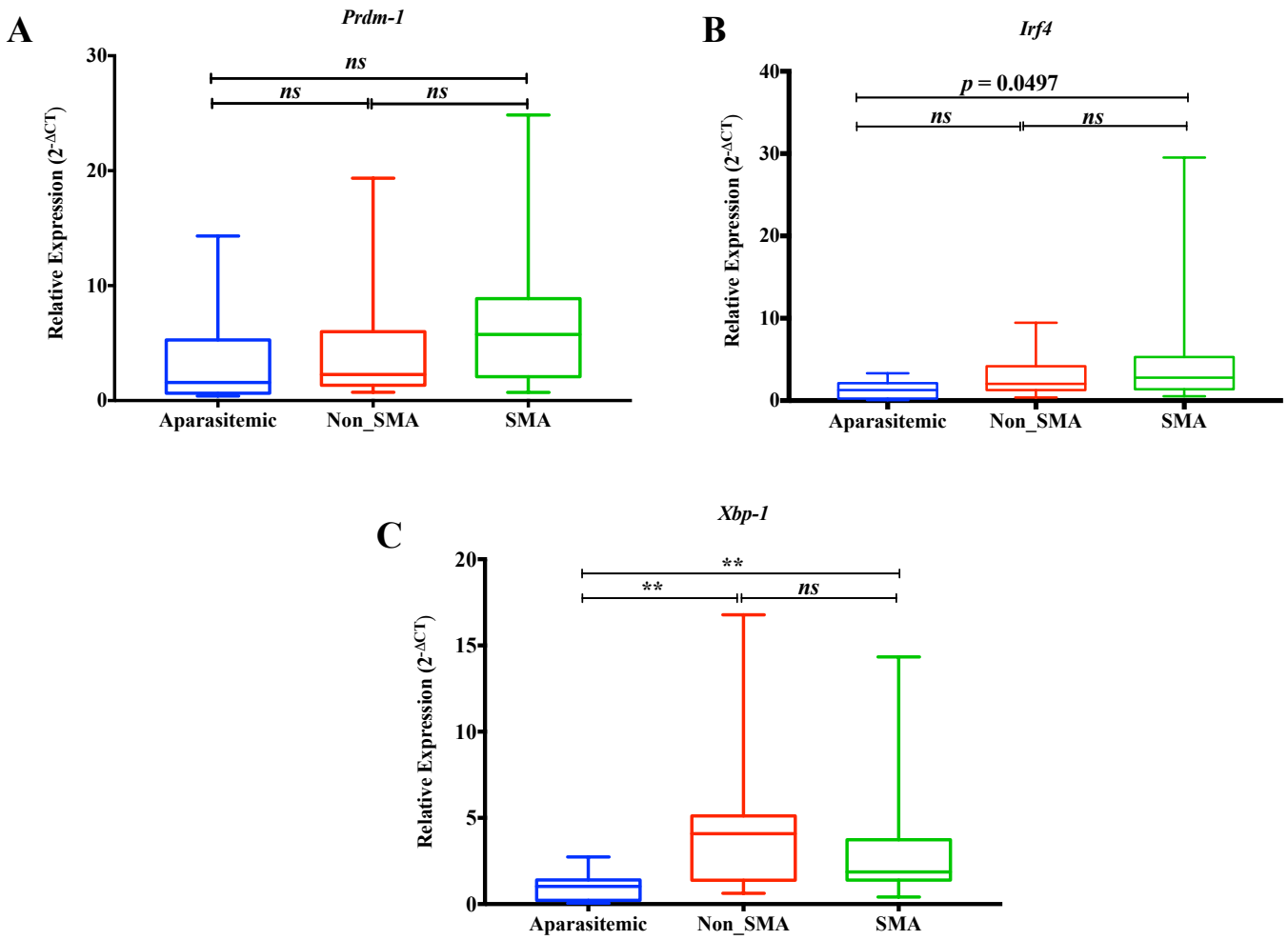

Figure 4.20 Expression patterns of Prdm-1, Irf4 and XBP-1 in children with SMA

Expression patterns of Prdm-1(A), Irf4 (B) and XBP-1 (C) in SMA and non-SMA children. Boxes represent median with range (minimum – maximum). Statistical differences among groups were determined by Kruskal Wallis test and in between two groups by Mann-Whitney test. * = $p<0.05$, ** = $p<0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, *ns* = no statistical significance observed.

Similar to the expression of XBP-1, the levels of Irf8 transcripts was not different between SMA and non-SMA children, but significantly elevated (p <0.01) when both groups are independently compared to aparasitemic children (Figure 4.21A). The expression pattern of Pax5 was somewhat different from the aforementioned. While its expression was not different between the aparasitemic and non-SMA groups, Pax5 levels were higher in the SMA group when compared to both the non-SMA and aparasitemic groups (p < 0.001), (Figure 4.21B).

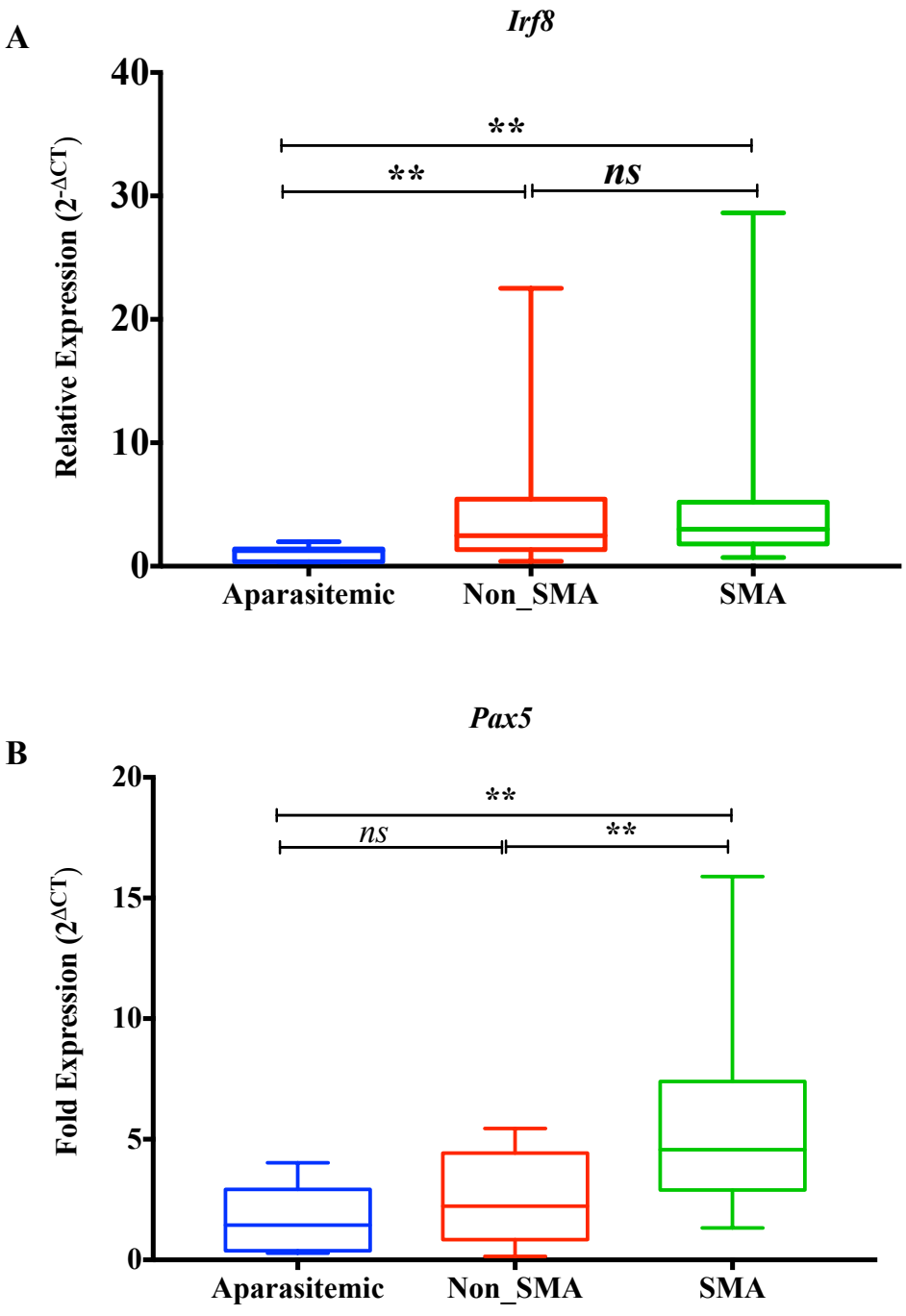

Figure 4.21 Expression of Irf8 and Pax5 in children with SMA

Boxes represent median with range (minimum – maximum). Differences among groups were determined Kruskal Wallis test and in between two groups by Mann-Whitney test. * = p<0.05, ** = p<0.01, *** =p < 0.001, **** = p < 0.0001, *ns* = no statistical significance observed.

4.3.4 Patterns of miR181b and miR155 Expression in Severe Malaria

miR181b and miR155 play important roles in the post-transcriptional regulation of AID expression. The levels of these microRNAs in aparasitemic, non-SMA and SMA were determined by RT-qPCR. Although there was a downward trend in the expression of miR155, the levels were not significantly different among the three groups and between groups (Figure 4.22A). miR181b on the other hand is down-regulated in SMA children versus aparasitemic children ($p < 0.05$) but not different from non-SMA children (Figure 4.22B).

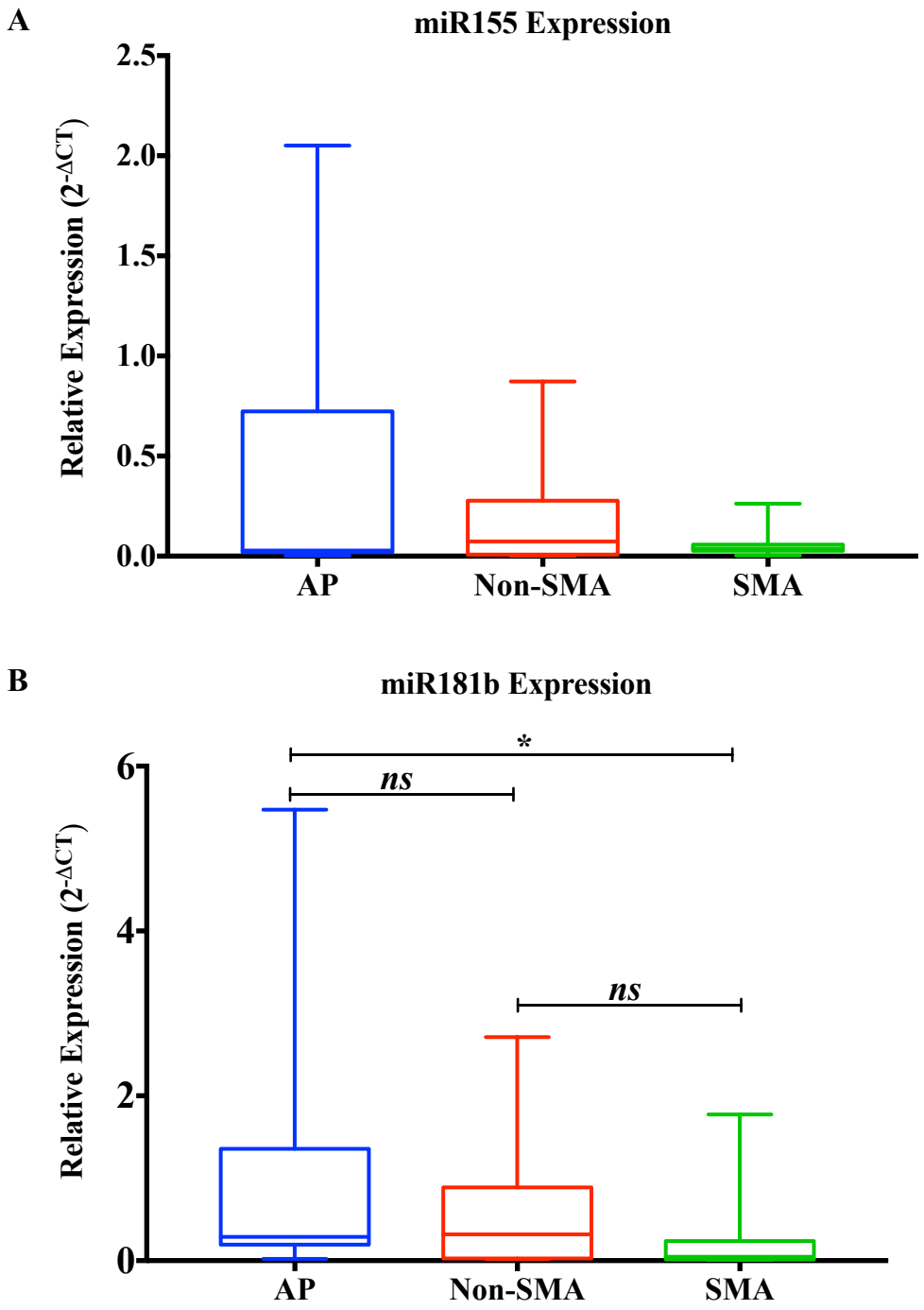

Figure 4.22 Expression patterns of miR181b and miR155 in SMA

Expression of miR181b (A) and miR155 (B) in children with/without SMA. The Expression levels of miR181b and miR155 were normalized to the expression of miR191 and are presented as relative expression levels. Boxes represent median with range (minimum – maximum). * = p<0.05, ** = p<0.01, *** = p < 0.001, **** = p < 0.0001, *ns* = no statistical significance observed.

4.3.5 Expression of AID Splice variants in Children with SMA

The expression levels of AID mRNA splice variants were measured and normalized to the expression of GAPDH in the WBCs of aparasitemic, non-SMA and SMA children. In addition to the full-length variant of AID (AIDFL) all the reported alternatively spliced mRNAs were detected in WBCs of aparasitemic, non-SMA and SMA children investigated in this study (Figure 4.23). With the exception of AIDivs3, which had the same expression level between aparasitemic and SMA children, the expression of all variants were significantly expressed in children with SMA ($p < 0.05$).

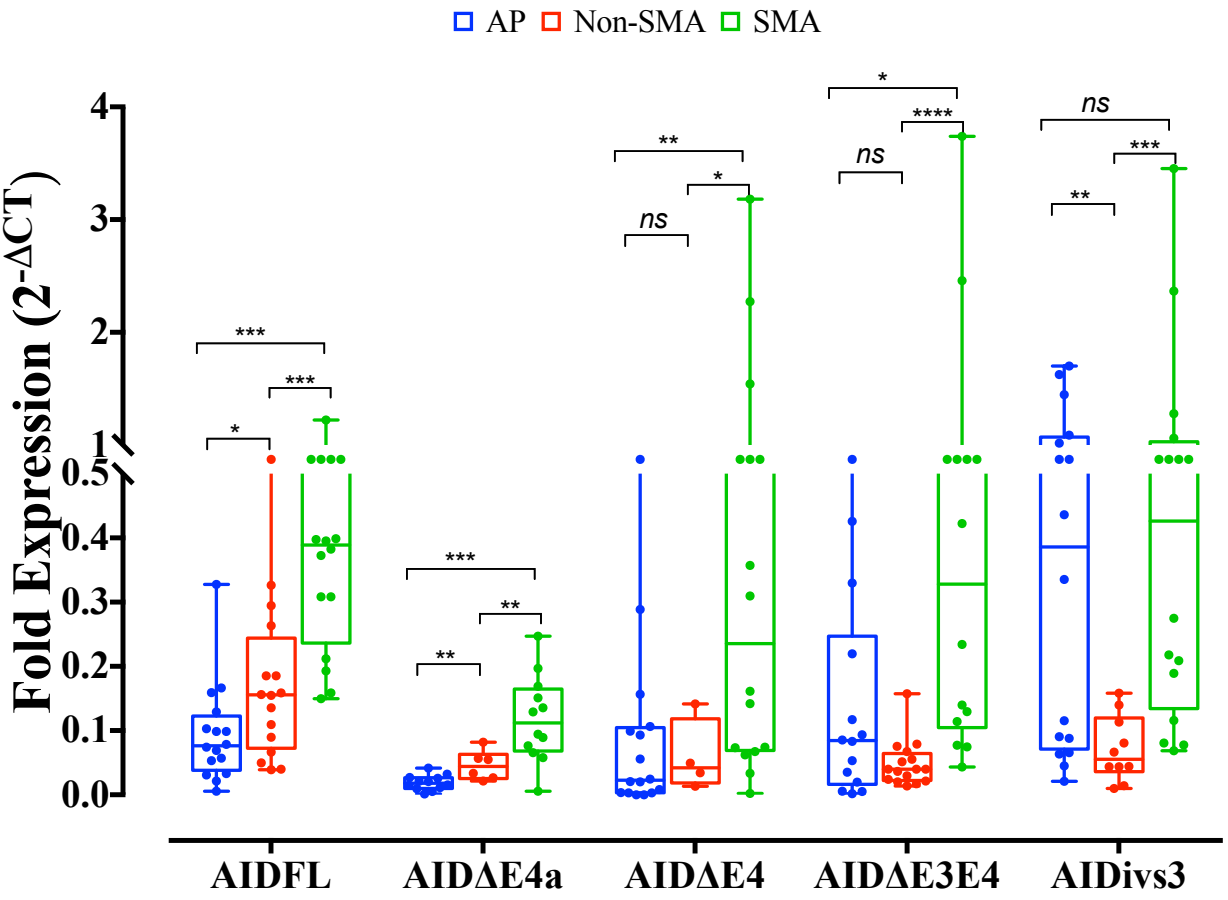

Figure 4.23 Expression of AID splice variants in children with SMA

Levels of AID mRNA splice variants are normalized to the expression of GAPDH. Plots represent range of range (min – max) with median. Mann-Whitney u test was used to determine statistical differences between two groups and Kruskal Wallis test for statistical difference among AP, non-SMA and SMA. * = $p<0.05$, ** = $p<0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, *ns* = no statistical significance observed.

CHAPTER FIVE

5.0 DISCUSSION

The aim of this study was to investigate the expression patterns and regulatory proteins associated with the activation or repression of AID. In a bid to understand how the transcription of AID is regulated in healthy tonsils, section 5.1 discusses the general trends of AID expression and the transcriptional programs that regulate AID in Tonsillar B-cell subsets. Chronic exposure to *P. falciparum* antigens is hypothesized to contribute to prolonged stimulation of B lymphocytes to undergo the GC reaction, thereby increasing the risk of aberrant AID activity. Section 5.2 discusses the trends in AID expression and the transcriptional regulatory programs associated with AID activity in different age groups of asymptomatic and symptomatic *P. falciparum* infected children.

5.1 Transcriptional Regulatory patterns of AID expression in Ghanaian Tonsils

Tonsillar tissue from children without Burkitt's lymphoma was used to understand the patterns of expression of AID, in the context of exposure to stimulating *P. falciparum* antigens in BL-free germinal centers. The Ghanaian tonsils analyzed had a mean of 25.6% of B lymphocytes with GC phenotype. This high-throughput of B lymphocytes in the germinal center has been shown to be characteristic of tonsils obtained from malaria endemic regions, and the proportions of GC B lymphocytes observed in this study fall within the range observed in a previous study (Torgbor et al., 2014). The germinal center B lymphocytes were also characterized by the significant up-regulation of the positive activators of AID expression; Pax5, Bcl6, Irf8, HoxC4 and Bach2. On the other hand, transcription factors known to regulate the transition of GC B lymphocytes into plasma cells, Prdm-1 (Blimp-1) and XBP-1 were highly expressed in plasmablasts but not in GC B lymphocytes. The expression of Irf4 however was the same from naïve B lymphocytes to

memory B lymphocytes and plasmablasts. Expectedly, the expression of AID was highest in GC B lymphocytes. The levels of AID transcripts were higher than observed in both EBV positive (Raji) and negative (Ramos) Burkitt's lymphoma cell lines.

Pax5 is one of the transcription factors that directly activate the expression of AID. It is known to bind directly to the promoter of AID and activate its transcription, as well as repress the expression of Blimp1. Together with other proteins, Pax5 recruits AID to key sequences in the immunoglobulin heavy (IgH) chain locus (Grundstrom et al., 2018). Its expression in the GC of Ghanaian tonsils together with the elevated expression of AID in the Ghanaian tonsils, is therefore not surprising. Similarly, the expression of HoxC4 enhances the transcription of AID directly. It forms part of the complex that binds to the promoter of AID and activates its expression. Therefore, the up-regulation of AID and Pax5 is expected.

Bcl6 and Bach2 enhance the transcription of AID indirectly. The expression of Bcl6 in the germinal center B lymphocytes enhances the expression AID by acting as a rheostat factor responsible for suppressing apoptotic and cell-cycle arrest responses associated with the toxic effect of the high genetic modifications that occurs in cells undergoing SHM and CSR (Ranuncolo et al., 2007). Therefore, its up-regulation, is most often coupled with the expression and activity of AID. Secondly, Bcl6 enhances the expression of AID by binding to miR155 and prevents the microRNA from binding to AID, and targeting it for degradation. The expression of Bach2 has also been shown to up-regulate the transcription of AID, although the mechanism is still not clear. It has been proposed that Bach2 enhances the expression of AID indirectly by up-regulating the expression of transcription factors such as BATF (Ise et al., 2011), and *Blc6* (Alinikula et al., 2011). In addition, Bach2 represses the expression of Blimp-1. Since Blimp1 is the main inhibitor of AID transcription and activity, its repression by Bach2 allows for the transcription of AID,

contributing to the elevated levels of AID transcripts in the GC B lymphocytes.

Irf8 was also up-regulated in the GC B lymphocytes of Ghanaian tonsils. It has previously been reported that Irf8 is highly expressed in centroblasts, B lymphocytes undergoing SHM, and plays an important role in the transcriptional regulation of the germinal center reaction, including the direct regulation of AID expression (Lu et al., 2003). The up-regulation of AID in the Ghanaian tonsil B lymphocytes is therefore not surprising. On the other hand, there were no changes in the expression Irf4 from naïve B lymphocytes to plasma and memory B lymphocytes. This finding varies slightly from what has been reported previously, as Irf4 expression is absent in centroblasts, up-regulated in only a subset of centrocytes and expressed highly in plasma cells (Falini et al., 2000). Although it is not clear how Irf4 is associated with the regulation of AID expression, some studies have shown that its deletion impairs AID expression and class-switch recombination (Klein et al., 2006). The finding from this part of the study suggests that for the most part, the transcription of AID is independent of Irf4 expression in the germinal centers of Ghanaian tonsils. The high expression of Prdm-1 and XBP-1 in plasmablasts is consistent with what is known (Turner et al., 1994). Prdm-1 is considered the most important regulator of plasmacytoid B cell differentiation, and is known to act after B lymphocytes have been induced to differentiate into plasma cells by the transcription factor XBP-1 (Turner et al., 1994). Their up-regulation in plasmablasts was also not surprising.

MicroR181b and miR155 are two of the most characterized microRNAs known to directly regulate the abundance of mature AID mRNA (Kaga et al., 2015) in the periphery and lymphoid tissues, respectively . Relative to the expression of miR191, the expression of miR181b was down-regulated in the B-cell subsets from all the tonsils analyzed. This suggests that the microRNA-mediated post-transcriptional regulation of AID expression in the germinal centers of secondary

tissues may not be associated with the expression of miR181. It is still not clear how the expression of miR181b is down-regulated in germinal centers of lymphoid tissues, but the AID-directed post-transcriptional function of miR155 is suppressed by Bcl6. The transcription factor positively regulates AID expression by binding and clearing miR155 in germinal centers of lymphoid tissues (Basso et al., 2012). Therefore, the up-regulation of Bcl6 could account for the down-regulation of miR155 observed in the germinal center B lymphocytes. In plasmablasts where the expression of Bcl6 was low, miR155 was highly expressed.

Alternative mRNA splicing is another mechanism associated with the regulation of AID expression (Wu et al., 2008). In addition to the full-length AID transcript, all four (4) known AID splice variants (AIDΔE4a, AIDΔE4, AIDΔE3E4 and AIDiv3) were observed in the Ghanaian Tonsillar B-cell subsets. This study was successful in cloning and identifying two of the splice variants together with the full-length AID transcript from GC B lymphocytes from a Ghanaian tonsil. Sequence analysis of these variants reveals that the sequences are highly conserved with close to 100% homology to reference sequences in the NCBI database. In addition to AIDFL, all the four splice variants were quantifiable in Tonsillar B-cell subsets, suggesting that the splice variants are expressed in all stages of B-cell development in the Ghanaian tonsil.

Previous studies suggest that these splice variants do not enhance the efficiency of CSR in mice (Sala et al., 2015), probably because their proteins lack the structural support at the active site (van Maldegem et al., 2010) and are not present in measurable quantities (Rebhandl et al., 2014). However, at least one recent study has associated the expression of these splice variants with trisomy 12 in chronic lymphocytic leukemia in humans, suggesting that we still do not fully understand the roles of the AID splice variants in the germinal center reaction (Zaprazna et al., 2019). Hence, the expression of all four variants in the Ghanaian tonsils could either contribute to

mechanisms aimed at limiting the unwanted activity of AIDFL.

The importance of AID in the germinal centers of secondary lymphoid tissues is undisputed. Apart from creating the conducive site for SHM and CSR, the germinal center provide the environment for the specialization of B lymphocytes into memory and plasma cells (Berek et al., 1991). The important role of the GC reaction comes with its own challenges, and a major one is provided by the aberrant activity of AID. Many lymphomas of B lymphocytes originate from the GC of lymphoid tissues, implicating the GC as the site for deleterious events that start these cancers. (MacLennan and Gray, 1986, Stevenson et al., 1998). Apart from the lymphoblastic and mantle-cell lymphomas, B-cell lymphomas classified to be from the non-Hodgkin family, possess a somatically hypermutated variable region of the Ig gene (Stevenson et al., 1998). This suggests that cells that started these cancers were either undergoing or have transited the GC reaction. That these lymphomas are characterized by chromosomal translocation and patterns of aberrant somatic hypermutations (Kuppers and Dalla-Favera, 2001), implicates AID and the geminal center reaction. Since these genetic lesions represent errors associated with the malfunction in the diversification of the immunoglobulin repertoire, AID can be implicated as a central contributor to the pathogenesis of these cancers. In this study, the constitutive up-regulation of all transcription factors that directly or indirectly activate the transcription of AID can be considered as a risk for B cell instability. This is the first study to provide evidence of the expression patterns of AID and transcription factors involved in the transcriptional regulatory programs in humans, in relation to *P. falciparum* infection.

In order to understand the differences in the regulation of AID transcription in *P. falciparum* exposed GC B lymphocytes, analysis of one Ghanaian and one New Mexican Tonsillar GC B lymphocytes revealed modest differences in the expression patterns of AID. However, since only

one tonsil from each site was compared, this data can only be considered preliminary. This is the first attempt to characterize the expression patterns of AID and related transcription factors GC B lymphocytes from a malaria endemic region. While this study confirms the finding of other studies, this study also provides additional understanding on what accounts for the elevated AID transcripts in GC B lymphocytes of *P. falciparum* exposed tonsils.

5.2 Transcriptional Regulatory Patterns of AID expression in *P. falciparum* infected children

The patterns of AID expression and related transcription factors were measured in the blood of children with *P. falciparum* infection. Two categories of samples were used for this part of the study. WBC samples from children with asymptomatic *P. falciparum* infection was used to investigate the patterns of transcriptional regulation of AID in chronically infected individuals. The rationale was that these children have had adequate exposure to the malaria parasite, and hence, can tolerate infections with the parasite longer than children below five years. The effect of prolonged infections with *P. falciparum* on the expression of AID was studied in these children, in order to understand how the transcription of AID is regulated in children with active, but asymptomatic infections. Secondly, apart from pregnant women, children below age 5 years are most susceptible to the Severest forms of malaria disease.

Currently, there is little data linking chronic and acute *P. falciparum* infection to the expression and regulation of AID. However, previous studies on the etiology and pathologies of *P. falciparum* malaria suggest that infections with the parasite have an effect on the B compartment of the immune system. Severe malaria anemia (SMA) is one of the complicated pathologies associated with *P. falciparum* infections in children. Among other factors, one of the key signatures of SMA

is reduced Hb levels due to the lysis of *P. falciparum*-infected or uninfected RBCs (Dondorp et al., 1999), or the inhibition of erythropoiesis by the *P. falciparum* metabolic end product of heme, hemozoin (*PfHz*) (Awandare et al., 2007, Perkins et al., 2011).

PfHz has been shown to be a potent stimulator of the immune response to *P. falciparum*, and induction of AID expression. First, *PfHz* can stimulate AID expression through the engagement of TLR-9. After release into the blood from iRBCs, *PfHz* is phagocytosed by circulating monocytes, neutrophil and macrophages. This mechanism of clearing *PfHz* from the circulation has been reported to stimulate the innate immune response to *P. falciparum* through the engagement of TLRs (Shio et al., 2010), and also promotes the production of various mediators of inflammation. These inflammatory cytokines have been proposed to promote a dysregulated innate immune response, as has been shown in the ability of purified hemozoin (*pHz*) to cause the "shaking chills" and body temperature rise associated with malaria (Brown, 1912, Perkins et al., 2011).

In a quest to reduce parasite loads and restore the hindered erythropoiesis occasioned by the accumulation of phagocytosed *PfHz*, interleukin-12 (IL-12), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) are released (Stevenson et al., 1995). The possible damage caused by these pro-inflammatory mediators can be averted by the equal and timely release of transforming growth factor-β (TGF-β) and interleukin-4 (IL-4) (Clark et al., 2006). Since the discovery that TNF-α is associated to malaria disease (Clark, 1978), elevated levels of the cytokine have been reported to be associated with the morbidity and mortality in individuals infected with *P. falciparum* (Grau et al., 1989, Kwiatkowski et al., 1990). It has been shown that TNF-α plays an important role in reducing parasite loads and replication (Kern et al., 1989, Kwiatkowski et al., 1989).

Of relevance to the current study, TNF-α, TGF-β, and IL-4 are all secondary stimuli that can induce the transcription of AID resulting in CSR (Zan and Casali, 2013). There is evidence to show that TNF-α can regulate the expression of AID via transcription. Recently, Duan and colleagues (2016) have shown that dose-dependent treatment of cells with TNF-α results in the up-regulation of AID transcription and expression. This induction, they found, was mediated through NF-κB signaling and was reduced when TNF-α signaling is blocked with antibodies (Duan et al., 2016). In the early stages of the immune response to malaria infection in children, IFN-γ plays key roles in protection against *P. falciparum* malaria (D'Ombrain et al., 2007). In fact, the ability of individuals from the Fulani tribe to produce IFN-γ, compared to individuals from other ethnic groups from West Africa, confer a significant tolerance for *P. falciparum* infection (McCall et al., 2010). *PfHz* also induces the production of IL-4, which in turn, has a direct effect on T cell dependent expression of AID, as the cytokine is crucial for BCR activation. Expectedly, *P. falciparum* is associated with the increased production of IL-4. In a recent study, high levels of IL-4 were found in children infected with *P. falciparum* (Elhussein et al., 2015).

In addition to secondary stimuli provided by cytokines, proteins from the parasite can directly induce activation of B lymphocytes. Firstly, the *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP-1) has the ability to activate different clones of B lymphocytes, which results in the production of cytokines and antibodies (Donati et al., 2004). In addition to inducing cytokine production, *PfHz* is an agonist for TLR9 (Parroche et al., 2007), and can activate the myeloid differentiation factor 88 (MyD88). The expression of AID is one of the downstream effects of TLR9 activation, therefore, these B-cell activators from the malaria parasite may induce AID

expression in chronically infected children. The elevated levels of AID transcripts observed in asymptomatic and acute *P. falciparum* infected children was therefore expected.

EBV and *P. falciparum* infection are known to synergistically up-regulate the expression of AID, *in vitro*. The effect of EBV infection on the expression of AID has only been shown *in vitro*. Firstly, the virus can induce the expression of AID, *in vitro* through the binding of the EBV nuclear antigen 3C (EBNA3C) to conserved regulatory elements located close to the AID transcription start site (Kalchschmidt et al., 2016). In addition, the EBV-encoded latent membrane protein-1 (LMP-1) also induces the expression of AID through the up-regulation of Egr-1, which can enhance the expression of AID (Kim et al., 2013). EBNAs and LMP-1 are proteins associated with EBV latent infection of memory B lymphocytes. Since the numbers of EBV infected cells are maintained at very low frequency by EBV-specific CD8+ T cells, the contribution of these proteins alone to the increased AID expression may be negligible. One critical condition required for the reactivation of EBV and infection of new cells is infection with *P. falciparum*. Although there is scarce information on the direct effect of EBV infection on the expression of AID *in vivo*, a recent study suggests that the expression of AID in the blood of children living in malaria endemic regions is associated to the intensity of *P. falciparum* transmission, in EBV positive children (Wilmore et al., 2015). Therefore, although *P. falciparum* infection can suppress EBV-specific $CD8^+$ activity and reactivate EBV to its lytic form, the effect of the dual infection on AID up-regulation depends on the parasite more than the virus. This is evidenced from the fact the expression of AID in *P. falciparum* negative children was the same irrespective of the EBV exposure status.

Prolonged and untreated *P. falciparum* infections is characterized with polyclonal B-cell activation and hyperglobulinemia (Abele et al., 1965, Donati et al., 2004). The main transcription factors that mediate the differentiation of B lymphocytes into antibody secreting cells are the B lymphocyte-induced maturation (Blimp-1), X-box binding protein-1 (XBP-1) and Irf4 (Lin et al., 2003, Shapiro-Shelef and Calame, 2005, Mittrucker et al., 1997). Although the expression of XBP-1 is associated with the development of plasmablasts, its absence or down-regulation does not affect the production of antibody secreting cells (Taubenheim et al., 2012). The differentiation of plasmablasts into antibody secreting B lymphocytes has been shown to depend on the expression of Irf4 (Mittrucker et al., 1997) and Blimp-1 (Shapiro-Shelef et al., 2003). The relationship between Irf4 and Blimp-1 in the process of plasmablasts differentiation is one of cooperation. The process is initiated by Irf4 by activating the Prdm-1 gene that codes for Blimp-1 (Sciammas et al., 2006, Kwon et al., 2009).

Pax5 and Bcl6 are inhibitors of XBP-1 and Blimp1 respectively, and hence play crucial roles in the inhibition of plasma cell differentiation in GC B lymphocytes. In this study, the expression of Prdm-1, Irf4, Pax5 and Blc6 was significantly elevated while XBP-1 and Irf8 levels were the same in asymptomatic and uninfected children. While the expression of Prdm-1 and Irf4 could be responsible for maintaining antibody production by terminally differentiated plasma cell, the expression of Pax5 and Bcl6 could account for the elevated AID transcripts observed in asymptomatic *P. falciparum* infected children. In children with symptomatic malaria, the levels of Prdm-1 and Irf4 were higher than observed in asymptomatic and uninfected children. However, the levels were not different when compared among the AP, Non-SMA and SMA groups. Given that these children had fever, the higher levels explain the activation of immunoglobulin secretion,

hence the levels of Prdm-1 and Irf4 are not different. Conversely, the expression of XBP-1 and Irf8 were significantly elevated in acute *P. falciparum* infected children compared to those with aparasitemia. Again, the expression of Pax5 was associated with acute *P. falciparum* infection as well as SMA. Together, Pax5 and Irf8 could account for the significantly elevated AID transcripts measured in children with febrile *P. falciparum* infection and Severe malaria anemia.

There is little information on the expression of miR181b and miR155, in the context of *P. falciparum* infection. A recent study has reported of the microRNA profiles in whole blood of adults with imported *P. falciparum* malaria (Li et al., 2018). Although they did not report any finding regarding miR-155 and miR181b, they discovered the down-regulation of miR181a in the whole blood of adults with *P. falciparum* malaria. In the current study, there was a general low level of miR155 and miR181b expression in both children with asymptomatic and acute *P. falciparum* infections, although these differences were not statistically significant. The results presented here suggest that increased AID transcript levels may be independent on the expression and activity of these two microRNAs.

The presence and abundance of alternatively spliced AID mRNA variants are yet to be studied in relation to infections with *P. falciparum*. Findings in the study suggests that apart from the full-length mRNA of AID, the other four known splice variants are not differentially expressed in asymptomatic *P. falciparum* infected children. However, under acute *P. falciparum* infection, the levels of all four splice variants are enhanced. This is the first study to associate chronic and acute *P. falciparum* infections with the expression of alternatively spliced AID mRNA.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Transcriptional regulatory patterns of AID expression in Plasmodium falciparum exposed and non-exposed Tonsillar B-lymphocytes

This study confirmed reports by other groups of high numbers of GC B lymphocytes in tonsils from malaria endemic regions. The GC B lymphocytes were also 'high expressors' of AID. The elevated levels of AID in the GC B lymphocytes correlated with elevated levels of Irf8, Pax5, HoxC4, Bach2 and Bcl6 transcripts, all of which are positive regulators of AID expression. On the other hand, negative regulators of AID expression Prdm-1 and XBP-1 were down-regulated in GC B lymphocytes and highly expressed in plasmablasts. The study was able to identify and quantify alternatively spliced mRNA variants of AID in GC B lymphocytes from Ghanaian tonsils. In addition to AIDFL, the variants AIDΔE4a, AIDΔE3E4 and AIDivs3 are significantly expressed in GC B lymphocytes, compared to memory B lymphocytes and plasmablasts. The levels of miR181b and miR155 were also measured in the Tonsillar B cell subsets. While miR181b was down-regulated in all four B cell subsets, miR155 was up-regulated in naïve B lymphocytes and plasmablasts but down-regulated in GC and memory B lymphocytes, suggesting that miR181b may not be involved in the regulation of AID transcription in the GC. The elevated levels of AID transcripts in GC B cells of *P. falciparum* exposed tonsil, are therefore associated with the elevated expression of positive regulators of AID transcription (Irf8, Pax5, HoxC4, Bach2 and Bcl6) and the downregulation of negative regulators of AID transcription (Blimp-1/Prdm-1 and XBP-1). The abundance of AID mRNA transcripts in *P. falciparum* exposed GC B cells is also associated with the downregulation of miR181b and miR155. In addition, the high expression of AIDΔE4a,

AIDΔE3E4 and AIDivs3 may contribute to the deregulation of AID expression in *P. falciparum* exposed GC B cells.

***Transcriptional regulatory patterns of* AID *expression in Asymptomatic Plasmodium falciparum infected children*.**

The study also investigated the expression patterns of AID and related transcriptional factors in children with asymptomatic *P. falciparum* infection. The expression of AID was significantly higher in children with asymptomatic *P. falciparum* infection than uninfected children. Infection with EBV alone was not associated with the increased expression of AID, as shown by the same level of AID transcripts in EBV^+ and EBV^- children without *P. falciparum* infection. Of the detectible positive regulators of AID transcription measured, Bcl6 and Pax5 were up-regulated in asymptomatic infected children, as were negative regulators Prdm-1 and Irf4. The expression of XBP-1 and Irf8 were not different between the *P. falciparum* uninfected and infected children. All four alternative splice variants of AID were detected in both asymptomatic and uninfected children, albeit at very low and not at significantly different levels.

***Transcriptional regulatory patterns of* AID *expression in children with clinical symptoms of* Severe *Malaria Anemia*.**

Beyond the transcriptional regulation of AID in normal Tonsillar tissue and chronic *P. falciparum* infected children, this study investigated the regulatory patterns of AID in children with severe malaria anemia. The levels of AID transcripts were elevated in children with SMA compared to children with non-SMA and aparasitemic children. Levels of AID transcripts were not associated with reported fever, body temperature or Hb level. However, higher levels of AID correlated with

increasing malaria parasite loads. Similar to the observation in asymptomatic children, the level of AID transcripts did not depend on the level of exposure to EBV as measured in IgG and IgM antibodies to the viral capsid antigen. Expression of miR181b was significantly higher in SMA than non-SMA and aparasitemic children, while miR155 was not differentially expressed among the three groups. In addition to the AIDFL, all four splice variants of AID were highly expressed in SMA children compared to non-SMA and aparasitemic children.

This work demonstrates the expression patterns of AID and related transcriptional factors, as well as miR-181b and miR155 in tonsils from a malaria endemic region. The current work is also the first to document the expression patterns of AID and its alternatively spliced variants and AID-related transcription factors, as well as miR181b and miR155 expression GC B cells of malaria exposed children and in children with asymptomatic and acute *P. falciparum* infection.

6.2 Limitations of the study

In investigating the transcriptional regulation of AID in Tonsillar B lymphocytes, this work used Tonsillar tissue from a malaria endemic region only. A comparison with the appropriate number of malaria non-exposed Tonsillar tissue could therefore not be made. In addition, this work measured the level of transcripts of AID and its related transcription factors. The interpretation of the results therefore is limited to the patterns and levels of expression. A cross-sectional method was used in investigating the effect of acute malaria on AID and transcription factor expression. This limits the interpretation of the results to expression patterns at a single time point.

6.3 Recommendations for future work

B cell subsets from additional tonsillar tissue would need to be analyzed to increase the statistical interpretation of this work. In addition, there is the need to obtain tonsillar tissue from malaria-free regions to serve as negative controls. The levels of mRNA transcripts may vary with protein levels. Hence, the presence and amounts of the proteins assayed for in this work would need to be determined. A longitudinal study would be needed to investigate the expression levels of AID in children with acute infection, during treatment and some weeks after resolution of clinical symptoms.

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APPENDIX

Appendix I: Multiple sequence alignment for AIDΔE4 (478 bp)

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AY536517.1   GACAGCCTCTTGATGAACCGGAGGAAGTTTCTTTACCAATTCAAAAATGTCCGCTGGGCT 60
AICDA-GHGC   GACAGCCTCTTGATGAACCGGAGGAAGTTTCTTTACCAATTCAAAAATGTCCGCTGGGCT 60
AICDA-NMGC   GACAGCCTCTTGATGAACCGGAGGAAGTTTCTTTACCAATTCAAAAATGTCCGCTGGGCT 60
AICDA-RAMOS  GACAGCCTCTTGATGAACCGGAGGAAGTTTCTTTACCAATTCAAAAATGTCCGCTGGGCT 60
             ************************************************************

AY536517.1   AAGGGTCGGCGTGAGACCTACCTGTGCTACGTAGTGAAGAGGCGTGACAGTGCTACATCC 120
AICDA-GHGC   AAGGGTCGGCGTGAGACCTACCTGTGCTACGTAGTGAAGAGGCGTGACAGTGCTACATCC 120
AICDA-NMGC   AAGGGTCGGCGTGAGACCTACCTGTGCTACGTAGTGAAGAGGCGTGACAGTGCTACATCC 120
AICDA-RAMOS  AAGGGTCGGCGTGAGACCTACCTGTGCTACGTAGTGAAGAGGCGTGACAGTGCTACATCC 120
             ************************************************************

AY536517.1   TTTTCACTGGACTTTGGTTATCTTCGCAATAAGAACGGCTGCCACGTGGAATTGCTCTTC 180
AICDA-GHGC   TTTTCACTGGACTTTGGTTATCTTCGCAATAAGAACGGCTGCCACGTGGAATTGCTCTTC 180
AICDA-NMGC   TTTTCACTGGACTTTGGTTATCTTCGCAATAAGAACGGCTGCCACGTGGAATTGCTCTTC 180
AICDA-RAMOS  TTTTCACTGGACTTTGGTTATCTTCGCAATAAGAACGGCTGCCACGTGGAATTGCTCTTC 180
             ************************************************************
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AY536517.1   CTCCGCTACATCTCGGACTGGGACCTAGACCCTGGCCGCTGCTACCGCGTCACCTGGTTC 240
AICDA-GHGC   CTCCGCTACATCTCGGACTGGGACCTAGACCCTGGCCGCTGCTACCGCGTCACCTGGTTC 240
AICDA-NMGC   CTCCGCTACATCTCGGACTGGGACCTAGACCCTGGCCGCTGCTACCGCGTCACCTGGTTC 240
AICDA-RAMOS  CTCCGCTACATCTCGGACTGGGACCTAGACCCTGGCCGCTGCTACCGCGTCACCTGGTTC 240
             ************************************************************

AY536517.1   ACCTCCTGGAGCCCCTGCTACGACTGTGCCCGACATGTGGCCGACTTTCTGCGAGGGAAC 300
AICDA-GHGC   ACCTCCTGGAGCCCCTGCTACGACTGTGCCCGACATGTGGCCGACTTTCTGCGAGGGAAC 300
AICDA-NMGC   ACCTCCTGGAGCCCCTGCTACGACTGTGCCCGACATGTGGCCGACTTTCTGCGAGGGAAC 300
AICDA-RAMOS  ACCTCCTGGAGCCCCTGCTACGACTGTGCCCGACATGTGGCCGACTTTCTGCGAGGGAAC 300
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AY536517.1   CCCAACCTCAGTCTGAGGATCTTCACCGCGCGCCTCTACTTCTGTGAGGACCGCAAGGCT 360
AICDA-GHGC   CCCAACCTCAGTCTGAGGATCTTCACCGCGCGCCTCTACTTCTGTGAGGACCGCAAGGCT 360
AICDA-NMGC   CCCAACCTCAGTCTGAGGATCTTCACCGCGCGCCTCTACTTCTGTGAGGACCGCAAGGCT 360
AICDA-RAMOS  CCCAACCTCAGTCTGAGGATCTTCACCGCGCGCCTCTACTTCTGTGAGGACCGCAAGGCT 360
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AY536517.1   GAGCCCGAGGGGCTGCGGCGGCTGCACCGCGCCGGGGTGCAAATAGCCATCATGACCTTC 420
AICDA-GHGC   GAGCCCGAGGGGCTGCGGCGGCTGCACCGCGCCGGGGTGCAAATAGCCATCATGACCTTC 420
AICDA-NMGC   GAGCCCGAGGGGCTGCGGCGGCTGCACCGCGCCGGGGTGCAAATAGCCATCATGACCTTC 420
AICDA-RAMOS  GAGCCCGAGGGGCTGCGGCGGCTGCACCGCGCCGGGGTGCAAATAGCCACCATGACCTTC 420
             ********************************************** **********

AY536517.1   AAAGCCCCTGTATGAGGTTGATGACTTACGAGACGCATTTCGTACTTTGGGACTTTGA 478
AICDA-GHGC   AAAGCCCCTGTATGAGGTTGATGACTTACGAGACGCATTTCGTACTTTGGGACTTTGA 478
AICDA-NMGC   AAAGCCCCCGTATGAGGTTGATGACTTACGAGACGCATTTCGTACTTTGGGACTTTGA 478
AICDA-RAMOS  AAAGCCCCTGTATGAGGTTGATGACTTACGAGACGCATTTCGTACTTTGGGACTTTGA 478
             ******** **************************************************
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Appendix II: Multiple sequence alignment for AID-ΔE3E4 (207 bp)

```
AY534975.1   GACAGCCTCTTGATGAACCGGAGGAAGTTTCTTTACCAATTCAAAAATGTCCGCTGGGCT 60
AICDA-GHGC   GACAGCCTCTTGATGAACCGGAGGAAGTTTCTTTACCAATTCAAAAATGTCCGCTGGGCT 60
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AY534975.1   AAGGGTCGGCGTGAGACCTACCTGTGCTACGTAGTGAAGAGGCGTGACAGTGCTACATCC 120
AICDA-GHGC   AAGGGTCGGCGTGAGACCTACCTGTGCTACGTAGTGAAGAGGCGTGACAGTGCTACATCC 120
             ************************************************************

AY534975.1   TTTTCACTGGACTTTGGTTATCTTCGCAATAAGCCCCTGTATGAGGTTGATGACTTACGA 180
AICDA-GHGC   TTTTCACTGGACTTTGGTTATCTTCGCAATAAGCCCCTGTATGAGGTTGATGACTTACGA 180
             ************************************************************

AY534975.1   GACGCATTTCGTACTTTGGGACTTTGA 207
AICDA-GHGC   GACGCATTTCGTACTTTGGGACTTTGA 207
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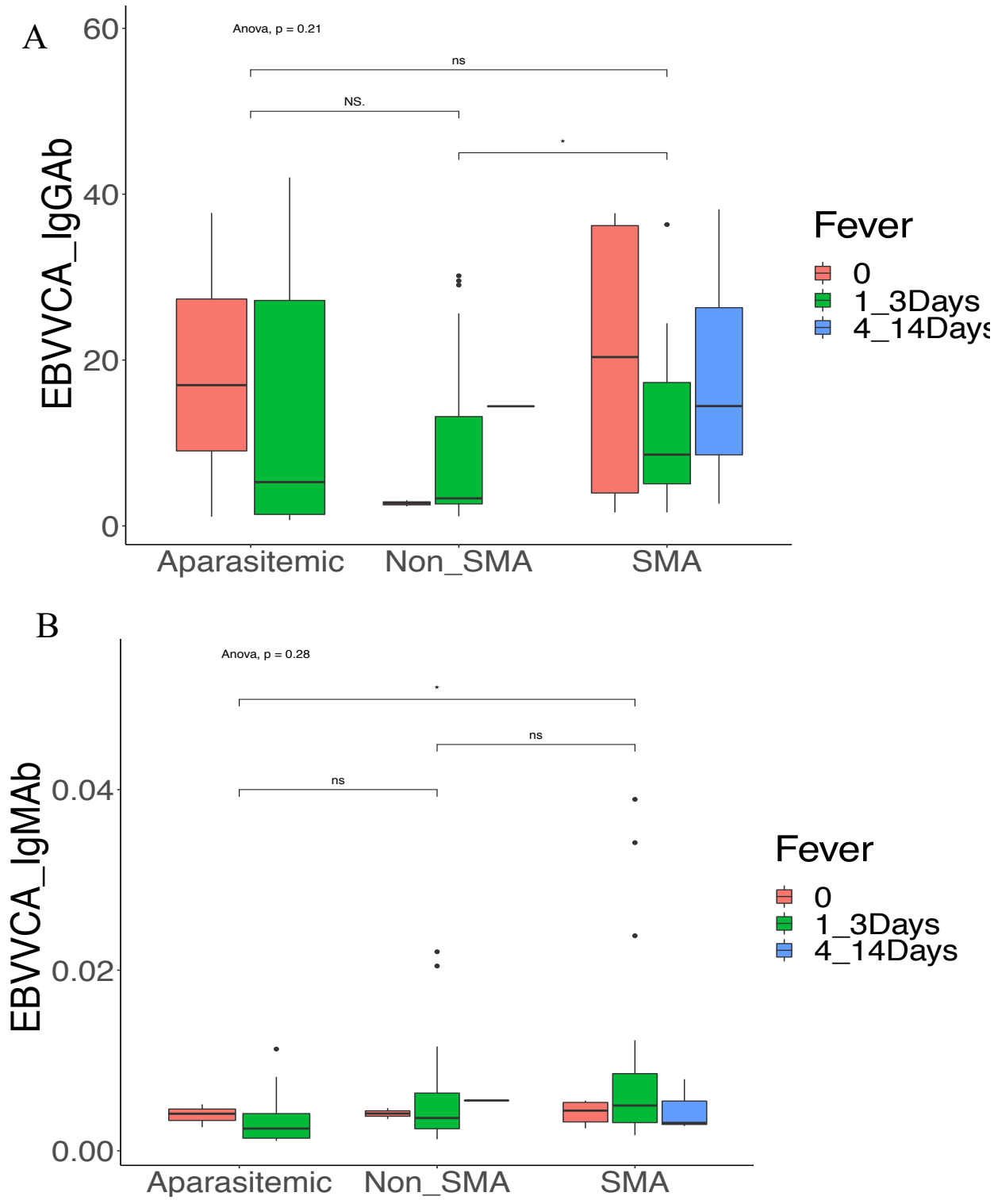
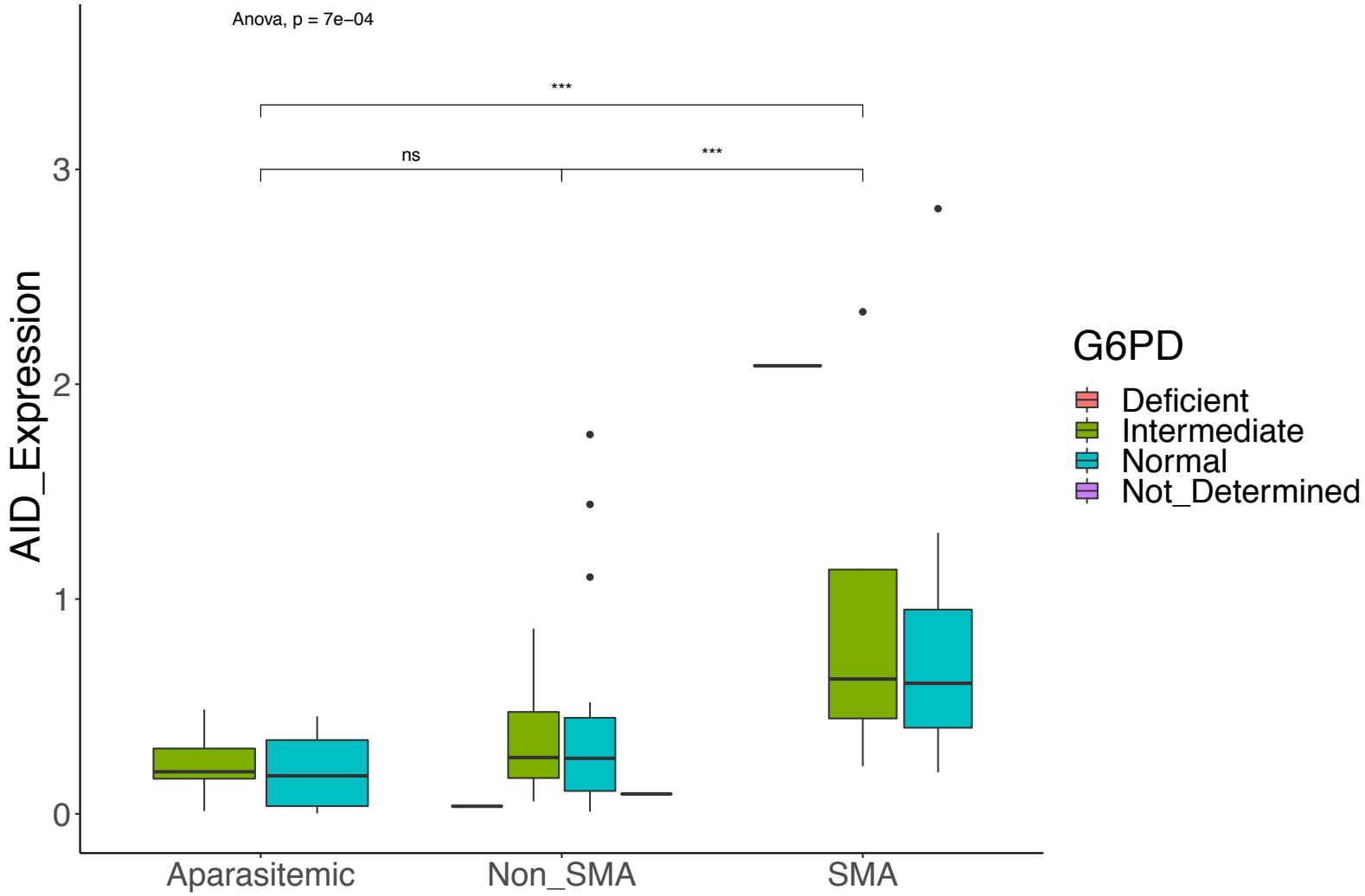
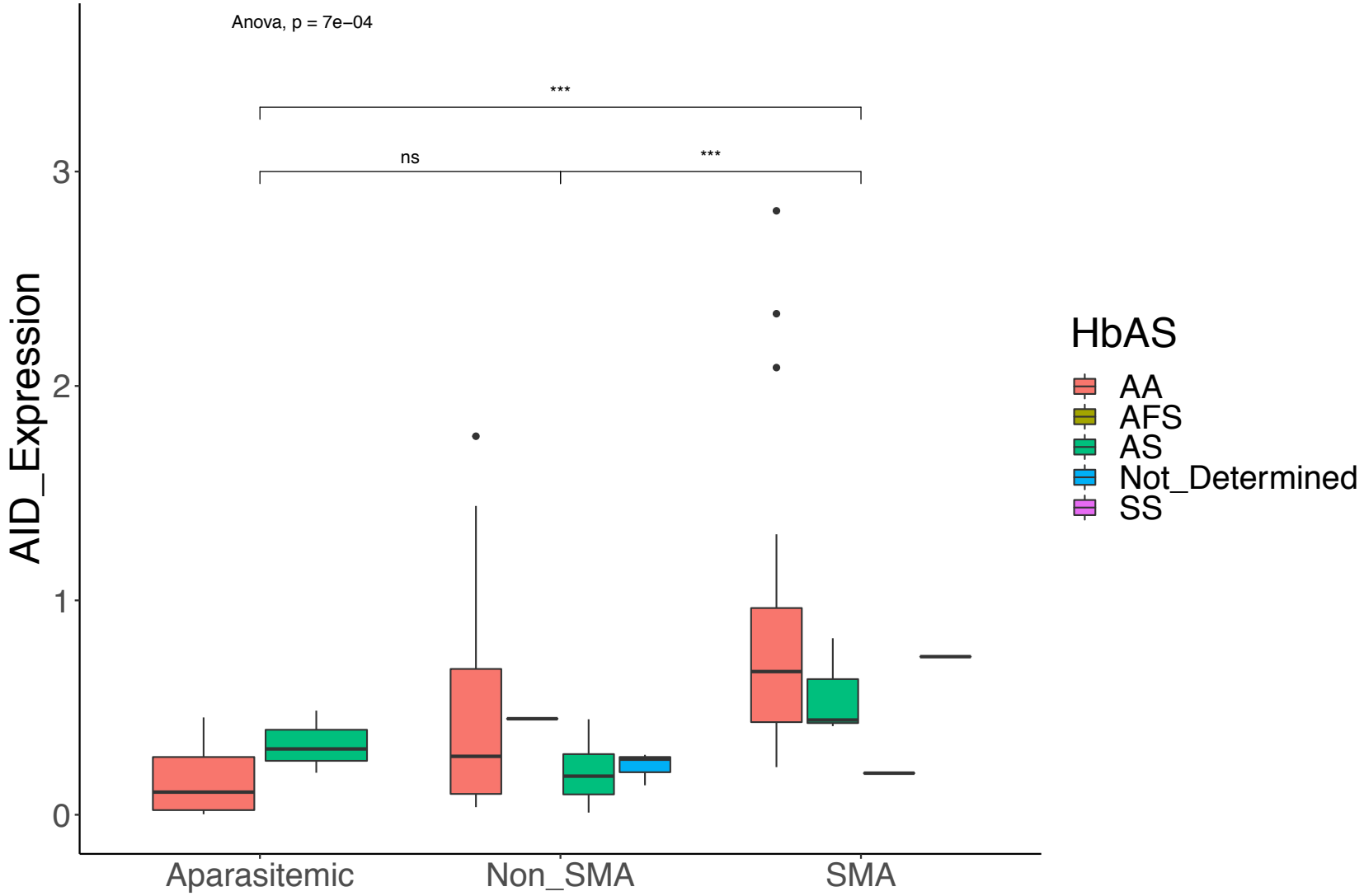


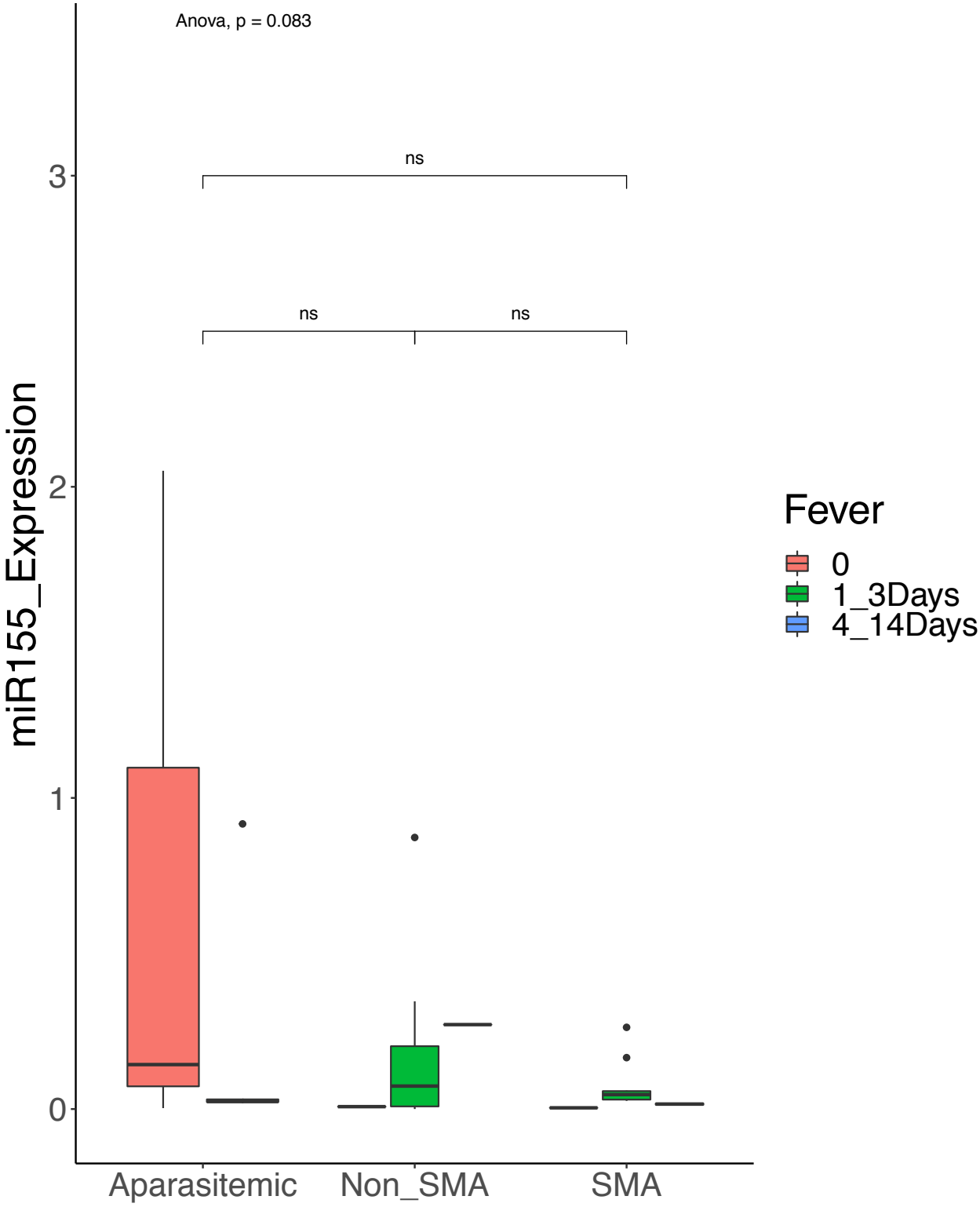
Appendix III: EBV VCA IgG (A) and IgM (B) antibody titers in children with SMA



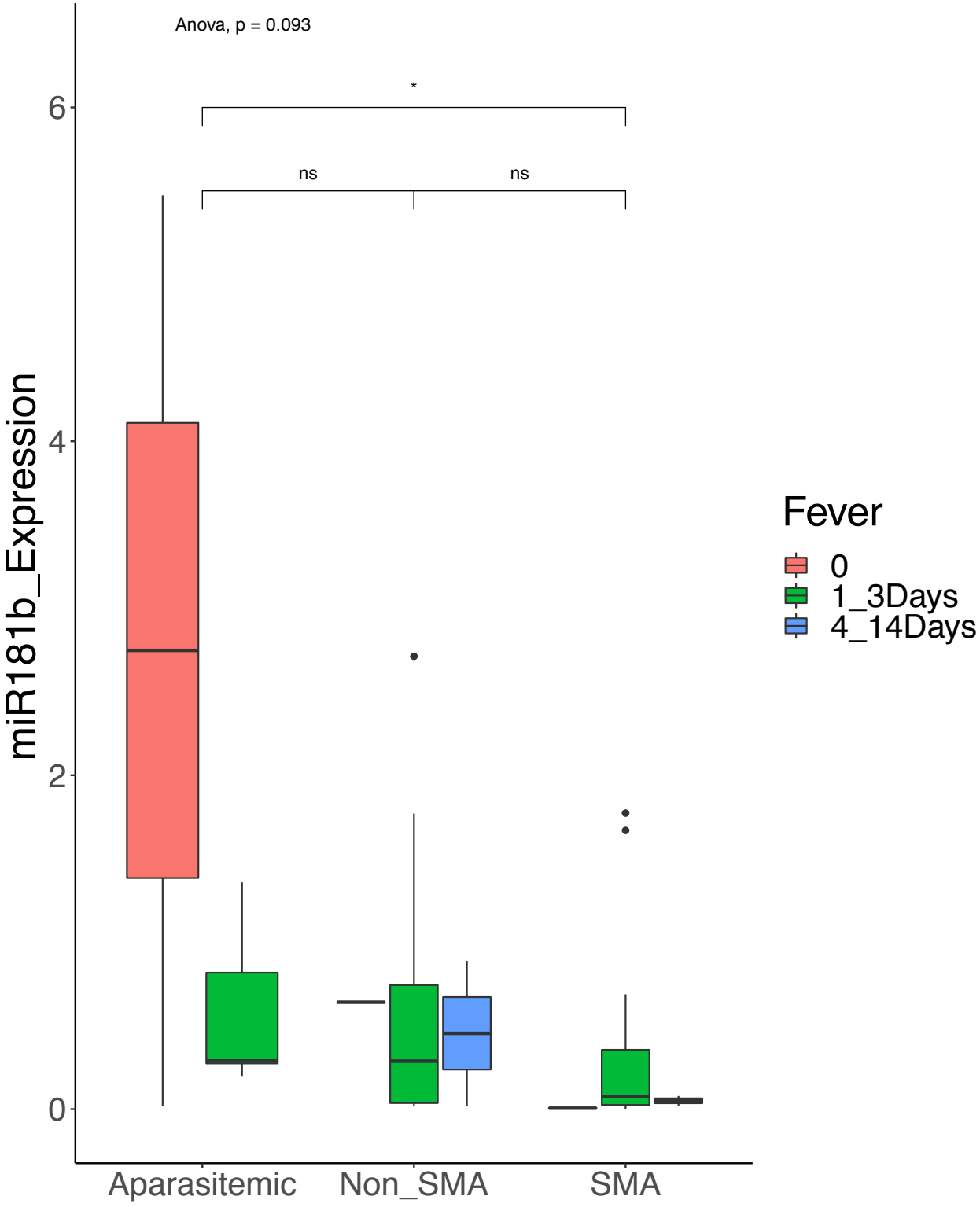
Appendix IV: G6PD deficiency and AID expression in children with SMA



Appendix V: Hb abnormalities and AID expression in children with SMA



Appendix VI: miR-155 expression and fever in children with SMA



Appendix VII: miR-181b expression and fever in children with SMA

Appendix VIII: Enrollment form for tonsil collection

ENROLLMENT FORM

DATE: ____/_____/_____ *(Day / month / year)* **Study ID**_____

1. Personal Details

1.1 Name of Participant: ……

1.2 Father's name: …….

1.3 Mother's name: ……

1.4 Study ID. No: ……

1.5 Date of Birth ……/……../…… *(Day / month / year)*

1.6 Age (yrs) ……

1.7 Was weighing card or birth certificate available? Yes=1 No=2 ☐

1.8 Sex: Male=1 Female=2 ☐

1.9 Informed consent obtained: Yes= 1 No=2 ☐

1.10 Informed consent given by: Parent=1 Guardian=2 Participant=3 ☐

1.11 Community of residence ……..

1.12 Duration of residence in the community ……Yrs .

2. History of illness

2.1 When was the last time the participant was ill? *Please indicate*……

2.2 What was the suspected illness? .....

2.3 Was the participant taken to Health centre/hospital? Yes=1 No=2 ☐

2.4 Has the participant taken any anti-malaria drug in the last two weeks? Yes=1 No=2 ☐

2.5 Did the participant take any traditional or herbal medication? Yes=1 No= 2 ☐

2.6 Indicate which of the following the participant felt or showed? *Please indicate Yes or No*

2.6.1 Fever Yes=1 No=2 ☐

2.6.2 Headache Yes=1 No=2 ☐

2.6.3 Nausea/Vomiting Yes=1 No=2 ☐

2.6.4 Chills/Rigor Yes=1 No=2 ☐

2.6.5 Joint pains Yes=1 No=2 ☐

2.6.6 Diarrhoea Yes=1 No=2 ☐

2.6.7 Convulsions Yes=1 No=2 ☐

☐

2.6.8 Jaundice/Deep yellow eyes Yes=1 No=2

2.6.9 *Please specify any other symptoms*……

2.7 How many times in a year has participant been diagnosed with malaria (approx)....……..

2.8 Body temperature (axillary) at visit: ……°C

2.9 Weight in **Kg**…….

2.10 Height in **cm**……..

2.11 a) Sickle cell trait status known? Yes=1 No=2

b) If known, what is the genotype? Normal(AA)=1 Carrier(AS)=2 Sickling(SS)=3 *Others=4*

2.12 Does the participant regularly sleep under a bednet? Yes=1 No=2

3. Socio-Economic Background

3.1 Mother's educational background:

None=0 Primary=1 Middle=2 JSS=3 Vocation=4 Secondary=5 Tertiary=6

3.2 Mother's occupation: Housewife=1 Farmer=2 Teacher=3 Trader=4 Other = 5

Other please specify……

3.3 Father's educational background:

None=0 Primary=1 Middle=2 JSS=3 Vocation=4 Secondary=5 Tertiary=6

3.4 Father's occupation: Farmer=1 Teacher=2 Trader=3 Other = 4

Other please specify……

3.5 How many children do you have in your household? *Please indicate*: 1 2 3 4 5 ≥ 6

3.6 How many siblings does the child have? *Please indicate*: 1 2 3 4 5 ≥ 6

3.7 Indicate which of the following owned by either mother or father or guardian

3.7.1 Cement block house Yes=1 No=2

3.7.2 Thatched house Yes=1 No=2

3.7.3 Car(s) Yes=1 No=2

3.7.4 T.V set Yes=1 No=2

3.7.5 Radio set Yes=1 No=2

3.7.6 Farm Yes=1 No=2

3.7.7 Fridge Yes=1 No=2

4.1 Name of interviewer: ……..

Signature/code: …….

Appendix IX: PARENTAL CONSENT FORM FOR CHILDREN (UP TO 16 YEARS)

Title: Regulation of the Expression of Activation-Induced cytidine Deaminase (AID**) in the Context of *Plasmodium falciparum* Infection**
(Study to find out how the production AID is controlled in white blood cells that fight against the malaria germ)

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General Information About the Study

We are inviting you and your child/ward to participate in a research study intended to investigate how the malaria germ may cause a particular type of cancer in children. This cancer occurs in a group of white blood cells that help the body to fight infections. Although there is scientific evidence that the malaria germ may cause this cancer in mice, we do not understand how the malaria germ can do this in humans. Therefore, we want to investigate how the malaria germ affects the production and control of a particular molecule that has been implicated to initiate events that start the cancer. We would need the group of white blood cells from humans to be able to achieve our aim. The surgery your child has been scheduled to undergo would remove a pair of tissue under the tongue that contains a lot of these cells, which we can use for this study. We are, therefore, recruiting children who are to undergo a surgery to remove their tonsils at the Cape Coast Teaching Hospital EENT Clinic. Since the cancer occurs primarily in those whose bodies are still learning how to fight against malaria, the study will involve children between 2-16 years old. Should you agree to take part in this study, we will take the tissue sample and isolate the white blood cells. These cells will be stored and later exposed to the malaria germ. We would then evaluate the effects this exposure has on these cells. We will also take about a teaspoonful (2-3ml)

of blood from your child at the time of surgery. From this blood, we will test for the presence of the malaria germ and the body's ability fight a common childhood virus (EBV) that is also implicated in the development of the cancer. Because HIV infection is a confounding factor for this study, we would screen the blood for HIV in order to exclude HIV positive samples. The plasma from the blood (which contains the body's defense entities or antibodies) will also be stored to be used to assess the body's ability to fight infections. **The samples will be taken to Department of Biochemistry, Cell and Molecular Biology of the University of Ghana, Legon, so that we can use some special machines to test them. Some may also be taken to the University of New Mexico in the US for more advanced testing using machines that are not available in Ghana. Some of these tests are very difficult and may take a long time, so we may keep the samples for up to five years**.

Possible Risks and Discomforts
Beyond the risks and discomforts with the surgery, there are no additional risks and discomforts associated with this study.

Possible Benefits
Your child will have standard intensive clinical management but derive no other direct benefit from the study. The results from the tests, as part of this study, would be made available to your Doctor, who is part of this study.

Confidentiality
Your child will not be identified by name in any publication, meeting, abstract, or report derived from the study results or information collected. No information on your child's genes or heredity will be attributed to him/her. Records of patient names and study numbers will be stored in both a locked file and secured computer files, and accessible only to key investigators. Individual laboratory results will be made available to clinical personnel involved in the care of patients, the patients themselves, and other caregivers of the patient. All information in paper form will be destroyed after the appropriate holding period.

Compensation
There will be no compensation for enrolling into the study.

Voluntary Participation and Right to Leave the Research
The participation of you and your child is voluntary, you may withdraw at any time during the study, and access to health care for your child will not depend on your participation.

Your rights as a Participant
All the procedures and tests described in this study, as well as subsequent amendments, will be reviewed and approved by the Cape Coast Teaching Hospital Ethical Review Committee before the commencement of the study. If you have any questions about your rights as a research participant, you can contact _____ of Cape Coast Teaching Hospital Ethical Review Committee on _____.

Appendix X: VOLUNTEER AGREEMENT

The above document, which describes the benefits, risks and procedures for the research titled, ***Regulation of the Expression of Activation-Induced cytidine Deaminase (***AID***) in the Context of Plasmodium falciparum Infection,*** has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to allow my child/ward to participate as a volunteer.

Name of child/volunteer:_____

_____ _____

Date Name and signature or thumbprint of parent or guardian

If parents/guardians cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the parent/guardian of the child/volunteer. All questions were answered and the parent/guardian has agreed to allow his/her child take part in the research.

_____ _____

Date Name and signature of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

_____ _____

Date Name and Signature of Person Who obtained consent

Appendix XI: CHILD ASSENT FORM

Note: This form should be used only for children who are 12-16 years old.

My name is ……..…….... and I am part of a team from……..…… We are conducting a research study entitled ***Regulation of the Expression of Activation-Induced cytidine Deaminase (***AID***) in the Context of Plasmodium falciparum Infection.*** We are trying to understand how the malaria germ may cause a type of cancer in children.

If you agree to be in this study, you will be asked a few questions about how you are feeling and the tissue that would be removed during your surgery would be given to us. We would also take about a teaspoon of your blood for laboratory tests, as part of the study. If you do not agree to be part of the study, you would still go through the surgery but the tissue would be discarded.

Your participation in this study will result in *a better understanding of how the malaria germ causes cancer in children of your age.*

Apart from the discomfort associated with the surgery, there would be no added pain if you decide to join the study.

You can stop participating at any time if you feel uncomfortable. No one will be angry with you if you do not want to participate.

Your information will be kept confidential. No one will be able to know how you responded to the questions and your information will be anonymous.

You may ask me any questions about this study. Or you can also contact the leader of this study, who is **Mr. Reuben Ayivor-Djanie** from the West African Centre for Cell Biology of Infectious Pathogens, at the University of Ghana. You can call him at any time *on his mobile phone at* **0244545878**.

Please talk about this study with your parents before you decide whether or not to participate. We have l also asked permission from your parents to enrol you in the study. Even if your parents say "yes" you can still decide not to participate.

By signing (thumbprinting) below, it means that you understand and know the issues concerning this research study. If you do not want to participate in this study, please do not sign this form. You and your parents will be given a copy of this form after you have signed it.

This assent form which describes the benefits, risks and procedures for the research titled, ***Regulation of the Expression of Activation-Induced cytidine Deaminase (***AID***) in the Context of Plasmodium falciparum Infection,*** has been read and/or explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate.

Child's Name:…… **Interviewer's Name:……**

Child's Signature/Thumbprint:…… **Interviewer's Signature……..**

Date: **……** Date: **……**