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Investigation of the role of an evolutionarily selected autoimmunity variant in the BAFF gene in response to Malaria antigens

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Abstract

Population genetics signatures have revealed that an autoimmunity variant (BAFF-var) overexpressing soluble BAFF (sBAFF) showed significant evidence of positive selection, possibly due to adaptations to malaria infection. Interestingly, different studies have shown that mice overexpressing BAFF are protected from lethal Malaria infections.

The aim of this project is to elucidate the role of BAFF-var during the response to malaria antigens, and thus to explain the possible protective role of BAFF-var. We prepared lysates of erythrocytes infected (iRBCs) or not infected (Control, uRBCs) with *Plasmodium falciparum*, a malaria pathogen that was highly prevalent in Sardinia were BAFF-var was detected and has been positively selected. The lysates were used to stimulate PBMCs genotyped for BAFF-var. After stimulation, samples were analyzed for differential gene expression and cell profiles by flow cytometry. In PBMCs purified from BAFF-var donors and treated with iRBCs, differential levels of B- and T- cell subpopulations, immunoglobulins as well as several cytokines were observed. These variations were associated with differential gene expression in several immune-related pathways such as the NFkB2 pathway and cytokines as determined by changes in RNA and protein levels. Increased production of sBAFF was observed in patients carrying the BAFF-var allele, which leads to an increased risk of autoimmunity. Differential gene expression, levels of specific B and T cells and cytokines secretion, implicated in malaria response potentially enhanced protection against malaria. These findings describe a previously unknown mechanism by which BAFF-var can potentiate the immune system against *Plasmodium* infection.

1. Introduction

1.1. Genome-wide association studies

Genome-wide association studies (GWAS) have driven the discovery of hundreds of susceptibility loci for several human diseases and traits. Indeed, in the last years, thousands of genetic variants associated with many diseases and traits have been identified, including chronic diseases, individual response to therapies, behaviours and habits [1, 2]. Unfortunately, the known associations account for only a fraction of genetic disease risk, with the causal variant generally unidentified and the functional mechanism largely unknown.

A useful approach to dissect disease mechanisms and pathways is based on the identification of genetic associations at loci that affect both disease risk and quantitative variables. The first detected by case-control studies and the latter assessed in healthy individuals and relevant for the biology of the disease under investigation, such as circulating levels of cell populations, cytokines, lipids or glucose (**Figure 1**). The coincident associations between human multifactorial disease risk and quantitative phenotypes is a powerful approach to identify intermediate phenotypes controlling key checkpoints in disease pathogenesis that can be modulated therapeutically [3].

This approach avoids secondary effects of the disease and its therapy, and is thus more robust than standard comparisons in patients versus controls [4, 5].

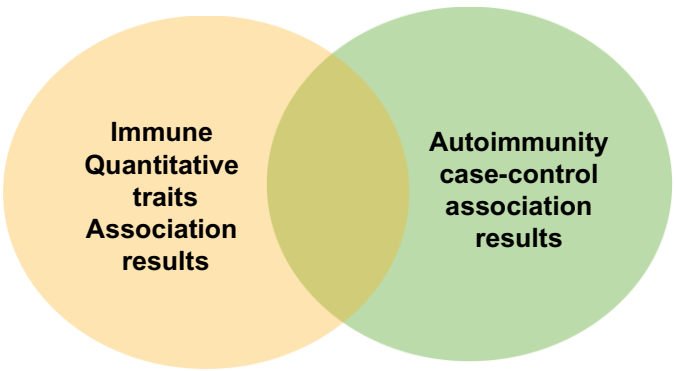

Figure 1. Searching for coincident associations between genetic variants controlling quantitative traits/endophenotypes and affecting the risk for diseases.

1.2. GWAS in Sardinia identified a new variant associated with autoimmunity

Autoimmune diseases are multifactorial disease that can be defined as a condition arising from an abnormal immune response to a normal body component. They are caused by largely unknown environmental factors in genetically susceptible individuals [6].

In the last years, GWAS have provided convincing statistical support for hundreds of independent genetic association signals associated with autoimmunity [4, 7–10]. Many of those signals are in or near genes whose products are involved in immunological processes consistent with the autoimmune nature of the diseases.

In a recent GWAS analysis in the Sardinian population, searching for coincident associations, Steri and colleagues identified a genetic variant in the TNFSF13B gene (BAFF-Var) associated with increased risk of autoimmune diseases, particularly Multiple Sclerosis (MS) and Systemic Lupus Erythematosus (SLE) [11]. TNFSF13B encodes for BAFF, a key cytokine implicated in the proliferation, differentiation and survival of B cells. Interestingly, the same variant associated with MS and SLE risk, was also significantly associated with several immune-related traits, such as an increased level of circulating soluble BAFF (sBAFF), an increased number of circulating B lymphocytes, higher levels of serum immunoglobulins M (IgM), A (IgA) and G (IgG) and decreased number of monocytes. Using ultra-high resolution genetic analysis, the association was reduced to the causal variant, an insertion/deletion that lies in the 3' untranslated region of TNFSF13B (GCTGT > A, [GCTG/-] rs200748895 and [T/A] rs374039502 in the 1000 Genomes Project-variants data set) where 'A', the minor risk-associated variant allele, created an upstream alternative polyadenylation site (APA) that generated a shorter transcript. The resulting transcript, BAFF-var mRNA, was more actively translated than the long (wild-type) BAFF-WT mRNA and led to higher production of soluble (s)BAFF. Using several approaches, it was demonstrated that the short transcript was more actively expressed partly because it lacks the binding site for the microRNA miR-15a and the RNA binding protein NF90 [11, 12].

Higher sBAFF increased circulating B cells and immunoglobulins, which in turn up-regulated humoral immunity and increased the risk of MS and SLE [11, 12].

The BAFF-var allele has higher frequency in Sardinia compared to mainland Italy and other regions in the world (26% of Sardinians carry at least one MS risk allele, in

contrast to 13.5% of individuals from mainland Italy and 3.6% in UK and Swedish populations). Indeed the presence of this allele is progressively more rare in regions proceeding from Southern to Northern Europe and is only seen occasionally and at a low level in South Asia, and absent in East Asia and Africa. Accordingly, it was assessed whether the high frequency of BAFF-var in the Sardinian population is consistent with the effects of random genetic drift or is the consequence of a genetic selection which occurred in Sardinia. To address this question, Steri and colleagues compared multiple metrics based on the differentiation of allele frequency and on haplotype conservation in 1,081 whole genomes of unrelated Sardinian and other populations, mainly Europeans from the 1000 Genomes Project Phase 3 [13]. This data analysis presented strong evidence for significant differentiation in allele frequency between Sardinians and other Europeans. Additionally, haplotypes carrying BAFF-var were significantly longer than those carrying the BAFF-WT allele. The combination of these two factors, extreme differences in allele frequency and length of the core haplotypes, suggested that BAFF-var had been positively selected, most likely as an adaptation to a selective pressure particularly prevalent in Sardinia. Due to its effects on humoral immunity, the BAFF- var allele, which promotes increased sBAFF levels, may have been selected for improved fitness against infections such as malaria, which was highly prevalent in Sardinia until it was eradicated in the 1950s [14]. This theory is supported by a report which showed that mice overexpressing human sBAFF were protected against lethal *Plasmodium yoelii* infection thereby confirming the key role that BAFF plays during malaria infections [15].

1.3. Malaria epidemiology

Malaria is one of the most prevalent infectious diseases and a global public health challenge. It is considered the fifth cause of death among infectious diseases worldwide, after respiratory infections, HIV/AIDS, diarrheal diseases, and tuberculosis, and the second cause of death in Africa after HIV/AIDS. According to the World Malaria Report 2017, an estimated 219 million cases of malaria occurred worldwide compared with 239 million cases in 2010 and 217 million cases in 2016. Most malaria cases in 2017 were in the African Region (200 million or 92%), followed by the South-East Asia with 5% of the cases and the Eastern Mediterranean Region with 2%. In Italy, for the period 2013-2017, the Istituto Superiore di Sanità has reported 3805 cases of imported malaria with only four cases in Sardinia (**Figure 2**).

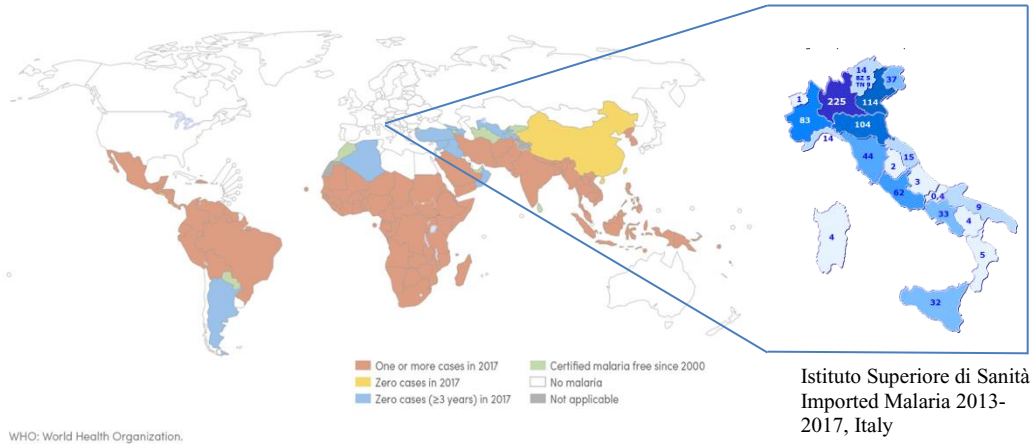

Figure 2. Map of malaria cases, in the world in 2017, and a particular overview of Italy. [16, 17]

In Sardinia, malaria was eradicated with a project called the "Sardinian Project" promoted by The International Health Division of the Rockefeller Foundation which began on November 1946. Utilizing the insecticide dichlorodiphenyltrichloroethane (DDT) all around the island, the great and intensive anti-larval campaigns destroyed 1,200,000 mosquito larval centers interrupting the transmission cycle of malaria. During 1950, for first time in its history, in Sardinia there were no new cases of malaria [14, 16, 17]

Currently malaria continues to be a serious and costly health problem worldwide. In 2017, an estimated US$ 3.1 billion were invested in malaria control and elimination globally by governments of malaria endemic countries and international partners [16].

Recently there has been much progress in the development of treatments and vaccine for malaria but only one malaria vaccine candidate (RTS,S/AS01) was approved for use in countries where malaria is endemic [19, 20]. Despite this important improvement in malaria vaccine development, much more remains to be done. Definitive treatments are still not available, and further studies are needed to discover and analyze new therapeutic targets.

1.4. *Plasmodium* life cycle

Malaria is a parasitic infection caused by *Plasmodium* parasites. The parasites are spread to people through the bites of infected female Anopheles mosquitoes, the "malaria vectors", during a blood feed. Of more than 120 *Plasmodium* species infecting mammals, birds, and reptiles, only six are known to infect humans. *Plasmodium falciparum* is responsible for the most severe disease and mortality. *Plasmodium vivax* usually produces milder disease which can become severe and recurrent; it is the most common malaria parasite outside Africa region. *Plasmodium malariae* and *Plasmodium ovale* are understudied, but the severity of illness is generally similar to uncomplicated *Plasmodium vivax*. *Plasmodium malariae* is often considered to cause chronic form of malaria. *Plasmodium knowlesi* is a primarily zoonotic infection encountered in Southeast Asia that can cause severe malaria[21, 22].

Plasmodium falciparum and *Plasmodium vivax* are the predominant species worldwide [23]. In Sardinia, both *Plasmodium vivax* and *Plasmodium falciparum* coexisted and multiple infections were common although neither strain was predominant. *P. vivax* had a higher annual incidence and represented the stable endemic nature of malaria on the island. *P. vivax* mainly affected children, and was characterized by high morbidity but low mortality rates. In contrast, *P. falciparum* had an unstable annual incidence [24].

Plasmodium parasite (spp) life cycle involves distinct stages in the insect (mosquito) and the vertebrate hosts (human). The infection in humans is initiated when sporozoites are injected with the saliva of a feeding mosquito. Sporozoites, carried by the

circulatory system to the liver, invade the hepatocytes (**Figure 3**). In the liver, the sporozoites mature into schizonts, which break and release merozoites into the blood stream. At this point, the merozoites invade erythrocytes and undergo a trophic period during which the parasite grows. The early trophozoite stage is often called "ring form" because of the morphology assumed by the *Plasmodium* (**Figure 3**). The trophozoite stage is accompanied by an active metabolism that includes the ingestion of host cytoplasm, the proteolysis of haemoglobin, and the production of hemozoin (Hz) [25–27]. At the conclusion of the trophic period, late trophozoite, multiple rounds of nuclear division without cytokinesis lead to the formation of schizont (**Figure 3**). The rupture of the schizonts in the blood stream releases merozoites, and the invasion of erythrocytes reinitiates another round of the blood-stage replicative cycle. At this stage, clinical manifestations appear [28].

A proportion of the parasites can differentiate into sexual forms known as macro- (female) or micro-gametocytes (male). Ingestion of gametocytes by the mosquito into the gut promotes the formation of male and female gametes finally generating the zygotes. The fertilized zygote becomes motile and develops into an ookinete which invades the midgut wall of the mosquito to mature into an oocyst (**Figure 3**). In the last phase of the cycle oocysts grow, break and release sporozoites that migrate to the salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle [26, 29].

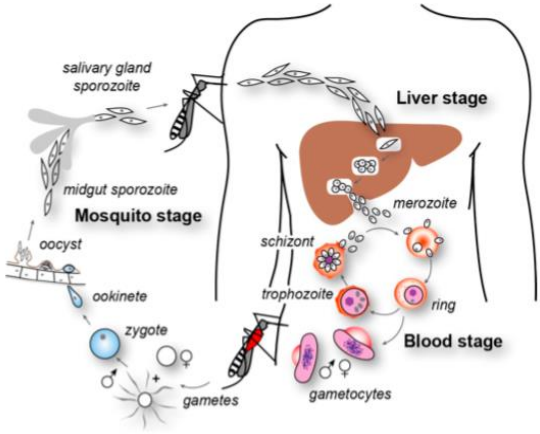

Figure 3. P. falciparum life cycle. Sexual replication in the Anopheles mosquito (definite host) and asexual replication in humans (intermediate hosts) [25].

1.5. Immunological response to *Plasmodium* infection

The immunological response to malaria infection in humans is regulated mainly through cooperation of both the innate and adaptive immune systems [30]. Considerable evidence revealed that B cells, antibodies, T cells, chemokines, cytokines and their respective receptors, all play crucial roles in the recruitment and activation of different cell types of the immune system thus modulating the complex immunological response against malaria parasites [31, 32].

B cells are essential for long-term maintenance of a protective humoral immunity to *P. falciparum,* and antibodies are a major component of the immune response during the erythrocytic stages [33].

During the pre- erythrocytic stages, CD8+ T cells play a central role in anti-malaria immunity [34]; these cells exhibit cytotoxicity to hepatocytes and are mainly involved in killing intrahepatic parasites by producing interferon-γ (IFN-γ).

Natural killer (NK) and Natural Killer T (NKT) cells are activated by IL12 to produce IFN-γ which induces the differentiation of T helper 1 (TH1) cells thus contributing to protection [35]. Additionally, the production of IFN-γ by NK cells results in maturation of Dendritic cells (DC) and in the activation of naïve CD4+ T cells [36]. CD4+ T cells have an important role in the protection against blood-stage parasites and in the activation of macrophages promoting clearance of infected red blood cells (RBCs) [37].

IFN-γ is a key player in anti-malaria immunity. It is essential for the activation of immune effector mechanisms that control pre-erythrocytic and blood-stage malaria infections but it can also exacerbate the severity of malarial disease depending on the temporal and spatial production [31, 38–40]. Components of blood-stage parasites, such as parasite-derived GPI (Glucose-6-Phosphate Isomerase), induce the production of several cytokines including IL1b, IL6 and TNFα by macrophages [41, 42]. Tumor necrosis factor alpha (TNFα) production is associated with parasite clearance and resolution of fever, but elevated levels of TNFα and IL6 have been correlated with cerebral malaria [43, 44]. Evidence also suggests that defects in the production of TGFβ and IL10, anti-inflammatory molecules, are associated with acute, severe malaria, and severe malaria anemia [45–47]. Additionally, it has been demonstrated that high ratios of IFN-γ, TNFα,

and IL12 to TGFβ or IL10 are associated with decreased risk of malaria infection but increased risk of clinical disease after infection [48].

Several studies revealed that circulating levels of various chemokines, including CCL4, CXCL4, CXCL8, and CXCL10, are significantly elevated in cerebral malaria cases compared with mild malaria cases or healthy controls [49]. In particular, the human interferon-inducible protein 10 (CXCL10) is a chemokine of the CXC family with pro-inflammatory properties [50]. CXCL10 is involved in different processes such as differentiation, activation of peripheral immune cells and regulation of cell growth. It is also known to be induced by IFN-γ and has chemotactic activity for activated TH1 lymphocytes [51–53]. $CXCL10^{-/-}$ knock out mice infected with *P. berghei* displayed significantly reduced peripheral parasitemia, suggesting that this chemokine could have a function on the development of immune responses involved in the control of parasite replication [54]. High levels of CXCL10 have also been detected in the cerebrospinal fluid of children that succumbed to cerebral malaria [55]. In line with these results, CXCL10 polymorphism associated with up regulation of CXCL10 levels resulted in increased susceptibility to cerebral malaria in human patients [56].

Complement receptor 1 (CR1) is expressed on the surface of phagocytic cells such as macrophages, B cells, neutrophils, erythrocytes and follicular dendritic cells in human. CR1 plays an important role in the clearance of circulating immune-complexes (IC) transferring them to macrophages for degradation and preventing the accumulation in the organism [57, 58]. Accumulation of circulating IC is frequently observed during several infectious diseases including malaria. In the rodent malaria model, CR1 expression on the surface of macrophages and B cells was reduced during malaria infection. Decreases in CR1 levels reduced monocyte/macrophages and B cells complement-mediated capacity to internalize IC and probably contributed to the observed accumulation of circulating IC. These results indicated that malaria induced a significant decrease of CR1 on the cell membrane of monocyte/macrophage and B cell populations leading to deficient internalization of IC by monocyte/macrophages and increased the malaria severity [59]. On the other hand, when expressed on the surface of human erythrocytes, CR1 functions as a receptor for *P. falciparum* invasion and mediates adhesion of infected erythrocytes to uninfected erythrocytes. This phenomenon is called rosetting and plays a key role in cerebral malaria [60, 61]. Polymorphisms associated with low CR1 expression on the

membranes of erythrocytes are believed to confer protection against severe malaria by reducing rosetting [62, 63].

Similar to CR1, intercellular adhesion molecule 1 (ICAM-1) also plays a key role in severe malaria and in particular, in severe cerebral malaria. It is a cell-surface receptor important for cytoadherence of *P. falciparum* [60]. ICAM-1 is present on various cell types, including lymphocytes, macrophages, and endothelial cells. Following TNFα activation, ICAM-1 allows the attachment of leukocytes to the endothelium and may permit their subsequent transmigration into peripheral tissue. *In vivo*, ICAM-1 expression is upregulated in response to a variety of inflammatory mediators including TNF and interleukin-1 (IL1). ICAM-1 expression on brain endothelium is up-regulated in severe malaria [64–66]. Nicolas Favre and colleagues demonstrated that ICAM-1 deficient mice infected by *Plasmodium berghei Anka* (PbA) did not die in the acute phase of infection. Additionally, they observed that the expression of TNF was significantly higher in ICAM-1$^{-/-}$ knockout mice than in the wild type mice [67, 68]. On the other hand, Dey and colleagues observed that the expression of ICAM-1, VCAM1, CXCL11, and CXCL2 became significantly higher with the progression of infection [69]. Together these data suggest that the up regulation of ICAM-1 is essential for malarial and cerebral malarial pathogenesis while specific reduction of ICAM-1-mediated adhesion could be an interesting drug target to reduce cerebral malaria.

Macrophage migration inhibitory factor (MIF), is a pleiotropic cytokine produced by the pituitary gland and multiple cell types, including macrophages, dendritic cells and T-cells. MIF modulates the expression of several inflammatory molecules, such as TNFα nitric oxide and cyclooxygenase 2 (COX-2) and inhibits the migration of macrophages [70]. Several studies have demonstrated that MIF plays either a protective or deleterious role in regulating the innate immune response during *Plasmodium* infection. Elevated levels of MIF protein correlate with the severity of cerebral malaria, in Malawian children [71], and placental malaria [72]. Malu and colleagues studied the role of MIF in the down-regulation of IFN-γ and up-regulation of IL4 responses at early infection, indicating that this versatile cytokine modulates adaptive responses in CD4+ T cells. These findings suggest that the MIF- enhanced IL4 responses attenuate the development of TH1 responses important in combating the malaria infection. As MIF showed different and

opposing functions during malaria infection, additional studies are needed to clarify its role [73].

The cell populations and biological molecules analyzed in this chapter perform different and important functions during *Plasmodium* infection. Unfortunately, more investigation is needed to clearly define their roles and to identify therapeutic targets in the immunological response to malaria exposure.

1.6. B cells response

During malaria infection, humoral immune responses are mostly directed to blood stage antigens, although parasitic antigens are expressed in each stage of the parasite life cycle. The first humoral response is slow to develop and ineffectively maintained. This may be partially due to the complex nature of *Plasmodium* parasites and the nature of the immune system [74]. Following B cell lymphopoiesis in the bone marrow, immature transitional B cells (TBC) are released into the circulation and migrate to the spleen where they differentiate into mature naive B cells. Following interaction with antigen-specific follicular helper T cells (TFHs) and stromal follicular dendritic cells (FDCs) naïve B cells differentiate into multiple subsets including, memory B cells (MBCs), follicular B cells (FoBs), and marginal zone B cells (MZBs) [75]. Subsequently, upon primary antigen encounter, short-lived plasma cells are secreted.

TFH and FDCs direct B cell migration and survival in the germinal center (GC) via secretion of cytokines and co-stimulatory molecules such as IL21, IL6, B cell activating factor (BAFF), CD40L and ICOS [76]. In the GC, B cells are activated and undergo a class-switch in the immunoglobulin genes and affinity selection to generate long-lived plasma cells, memory B cells (MBCs), and protective antibodies [77, 78]. Upon re-exposure to the same antigens, the memory B cells generate a secondary antibody response, faster, stronger, and characterized by high affinity. As the infection is controlled, the expanded antigen-specific B cell population contracts, leaving behind memory B cells and plasma cells. Plasma cells are long-lived, survive in the bone marrow, and continue to secrete antibodies for months or years [79, 80]. They are the main sources of the sustained antibody levels that are often seen after repeated antigen exposure, even in the absence of persistent antigen [81].

The adaptive response to an infection is a tightly controlled process in which inhibitory and pro-apoptotic receptors play an important role in regulating cell survival. Interestingly, in chronic infections like malaria [82] and autoimmune diseases [83, 84]. there is an upregulation of inhibitory and pro-apoptotic receptors on B cells and an increased frequency of a phenotypically distinct MBC subset lacking the classic memory marker CD27, IgD- CD27- B cells. The exact function of this B cell subset is not entirely understood. A phenotypically similar subset called "atypical MBC" (aMBC) has been associated with malaria exposure and, in patients with malaria, they were characterized by an exhausted or anergic phenotype. Still, the role of the anergic and/ or exhausted aMBC in chronic infection is unknown [85–89].

Several studies shown that other B cell subpopulations increase in malaria immunity. Comparing subjects exposed to malaria with those not exposed to malaria, Ubillos and colleagues found that in peripheral blood, active atypical MBCs ((aaMBC) CD19+CD10−IgD−CD21−CD27−), resting atypical MBCs ((raMBC) CD19+CD10−IgD−CD21+CD27−), active classical MBCs ((acMBC) CD19+CD10−IgD−CD21−CD27+), and plasmablast germinal center ((PCGC) CD19+IgD−CD38+), are increased in exposed individuals [90]. In line with these results, Stephens et al, showed that in rodents infected with *P. chabaudi,* the proportion of PCGC cells increased from day 0 to day 60 after infection [91].

Transitional B cells (TBC) such as $CD19^{+}CD24^{+}CD38^{+}$, are also increased in malaria infection [92]. Indeed, Sullivan et al., found that donors with greater parasite density had higher proportions of transitional B cells at the time of infection suggesting that *P. falciparum* infection led to a homeostatic expansion of transitional B cells [93]. The exact role of these B cell subpopulations in malaria immunity is still not clear and additional studies are needed to clarify their functions.

1.7. Immunoglobulins

The importance of the antibody-mediated response against *P. falciparum* parasites was shown 50 years ago in passive transfer studies. Indeed, Cohen and colleagues observed that transfer of immunoglobulin (Ig) G from immune African adults reduced the level of parasites in children [33]. Additionally, accumulating evidence suggests that IgG1 and IgG3 are protective against *P. falciparum* infection; the role for IgG2 and IgG4 in terms of protection are less clear [94–96].

During experimentally induced and naturally acquired malaria, Boyle and colleagues identified and studied IgM activity against blood-stage. Merozoite-specific IgM appears rapidly in *Plasmodium falciparum* infection and may play a significant role in naturally acquired protective immunity to malaria preventing merozoite invasion of RBCs [97].

1.8. T cells response

T lymphocytes have two important roles in malaria immunity: (i) they act as helper cells in the production of anti-plasmodium immunoglobulin, and (ii) they trigger cell-mediated mechanisms activated by macrophages and other phagocytic or cytotoxic cells [98].

After a physical interaction between the B cells and the CD4+ helper T cells, several cytokines are secreted. In the first stage of the response, IL4 and IL5 are produced by TH2 cells that regulate isotype switching thereby inducing the secretion of IgG1, IgG4 and IgE. The major role of the IgG1 and IgG3 isotypes in the Fc-y-mediated macrophage phagocytosis of infected red blood cells (RBC) makes the participation of the TH2 subset a key step in the development of the protective immune response against the *Plasmodium* blood stages [99]. In a second stage of the immunological response, phagocytic activity of human neutrophils is enhanced by IFN-γ which is secreted by activated TH1 cells. This TH1-type response triggers non-specific effector mechanisms able to kill the intra-erythrocytic plasmodia [100].

In GC, follicular T helper (TFH) cells express high levels of CXCR5 [101–103] thus helping the differentiation of naive B cells into isotype switched activated B cells, long-lived plasma cells (LLPCs) and MBCs [104]. Then TFH cells leave the GC and become memory CXCR5+CD4+ TFH cells circulating in the blood are ready to respond in case of antigen re-exposure [105–107].

1.9. Transcriptional and post-transcriptional regulation of immune responses

Cytokines and immune related pathways act as mediators of inflammation and host immune defense. Cytokine production is regulated at both transcriptional and post-transcriptional levels [108, 109].

Transcription is regulated by transcription factors (TF) that bind DNA to activate or inhibit transcription of target genes. For example, misregulation of the immune response transcriptional regulator NFkB has been linked to inflammatory and autoimmune diseases. Indeed, NFkB interacts with BRD4 (Bromodomain-containing protein 4) which is an epigenetic regulator and with P-TEFb (positive transcription elongation factor) suggesting that this ubiquitous regulator plays a role in elongation control of inflammatory genes during immune and stress responses [110–112].

Posttranscriptional regulation is regulated by noncoding RNA (ncRNA) and RNA binding proteins (RBP) interacting with *cis*-elements [113]. The presence of various RBPs and ncRNA regulating mRNAs in distinct locations enables elaborate control of cytokines under inflammatory conditions like malaria [114]. Dysregulation of cytokine mRNA decay leads to pathologies such as the development of autoimmune diseases or impaired activation of immune responses to pathogens [115].

For example the production of the cytokine BAFF was strongly regulated at post-transcriptional levels by miR-15a and by the RBP NF90. Interaction of miR-15a with *BAFF* mRNA was enhanced by NF90, suggesting a cooperative mode of action whereby NF90 binding to *BAFF* mRNA facilitated miR-15a recruitment and translational repression of *BAFF* mRNA [12]. BAFF is a vital cytokine for B cells, the regulatory paradigm described here strengthens the involvement of NF90 in controlling immunity and the production of cytokines [116, 117].

In this regard, NF90 was previously shown to influence the post-transcriptional lives of many target transcripts, particularly a subset of mRNAs including IL2, DUSP1/MKP-1 and VEGF mRNAs [116, 118–120]. NF90 was also shown to repress the translation of CCL16 and IL8 mRNAs in fibroblasts. Interestingly, NF90 can bind both DNA and RNA. In T cells, NF90 is associated with the IL2 proximal promoter to promote transcription, while post-transcriptionally, NF90 bound the 3'UTR of the IL2 mRNA to control the

nuclear export of mature IL2 mRNA to the cytoplasm and the RNA translational components [121, 122].

1.10. BAFF and Malaria

The humoral immune response plays a major role in establishing naturally-acquired immunity to malaria [33]. This immunity, unfortunately, is slow to develop and unsuccessfully maintained [123]. The complex nature of the *Plasmodium* parasite and its antigenic variation contribute to the impaired generation of a stable humoral immune memory [124, 125], still several lines of evidences indicate that the malaria parasite actively alters B-cell function [74].

A key cytokine in mediating B-cell proliferation and differentiation is the B-cell activating factor (BAFF) a cytokine that belongs to the tumor necrosis factor (TNF) family [126]. BAFF is synthesized as membrane-anchored protein by cytokine-activated myeloid cells including monocytes and dendritic cells, and subsequently cleaved to produce the soluble form, sBAFF [127]. BAFF is the natural ligand of three tumor necrosis factor receptors named BAFFR (BR3), TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor), and BCMA (B cell maturation antigen), each of which has differing binding affinities for BAFF. These receptors are expressed mainly on mature B-lymphocytes and their expression varies in dependence of B cell maturation. TACI is also found on a subset of T cells and BCMA on plasma cells, whereas BAFFR is mainly involved in positive regulation of B cell development [128]. sBAFF-BAFFR interaction is essential for B cell development from immature type-1 B cells to type-2 and mature B cells. Furthermore, this receptor binds sBAFF with a greater affinity than the other two and is more selective.

BAFF expression is mainly regulated by IFN-γ and TGFβ that in turn are increased during malaria infection [42, 129]. Membrane BAFF production and cleavage, has been demonstrated in *P. falciparum*-activated human monocytes and concomitant naïve B cell activation, *in vitro* [130]. Scholzen et al., examined BAFF induction and the activation of a B cell subset in a controlled human malaria model (CHMI). Interestingly, they show that sBAFF levels increased during CHMI and that sBAFF levels correlated with BAFF expression from monocytes and dendritic cells, as well as blood-stage parasitemia and parasite-induced IFN-γ production.

During malaria infection, the host's immune system is also activated by hemozoin (HZ), a crystalline, brown pigment that is formed and sequestered in the digestive vacuole of *Plasmodium* as a product of hemoglobin (Hb) catabolism [131]. Macrophages stimulated with hemozoin produce reactive oxygen species and mediate sBAFF expression [74].

Nduati et al., revealed that children with acute malaria exhibited high plasma sBAFF levels and elevated TACI and BCMA expression, while the BAFFR (BAFF receptor, BR3) expression level was decreased [132]. Low BAFFR expression levels may be an underlying factor for insufficient memory B cell formation in children after natural infection, since BAFFR provides B cell survival signals [131]. Finally, BAFF-overexpressing mice were protected from lethal malaria infections, indicating the important role that BAFF plays in determining the outcome of malaria infections [15].

1.11. The NF-kB pathway and BAFF

Interaction between sBAFF and BAFFR activate a signaling pathway that involves TNF receptor associated factors (TRAFs). TRAFs are trimeric intracellular proteins that bind linear TRAF-binding sequences present in several receptors including BAFFR. In the absence of sBAFF, TRAF3 binds to NFkB-inducing kinase (NIK) and, with the help of TRAF2, induces NIK degradation with a proteasome-dependent mechanism. This in turn prevents the activation of the alternative NFkB pathway. In presence of sBAFF, TRAF3 is recruited to BAFFR and degraded in a TRAF2-dependent manner. The degradation of TRAF3 results in the stabilization of NIK and activation of the alternative NF-kB pathway, which then leads to modifications in B cell survival and differentiation [126].

The signaling pathways that mediate NFkB activation can be classified into canonical and non-canonical (or alternative) pathways. The canonical pathway is a fast acting signal transduction pathway that converges to an IkB kinase (IKK) complex, composed of catalytic (IKKα and IKKβ) and regulatory (IKKγ) subunits. Upon activation, IKK phosphorylates IkBα at two N-terminal serines, triggering its ubiquitination and consequent proteasomal degradation; this induces the nuclear translocation of NFkB complexes, predominantly p50/RelA and p50/c-Rel dimers and consequent gene activation [133]. The activation of the noncanonical NFkB pathway involves different signaling molecules and leads, in a slower manner, to the predominant

activation of the p52/RelB dimer [134] (**Figure 4**).

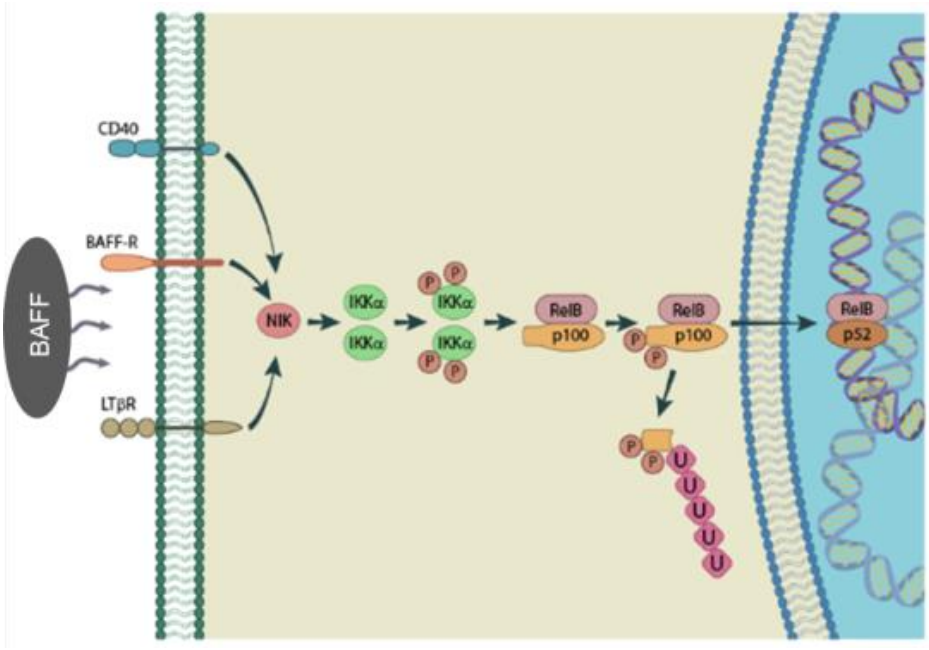

Figure 4**.** Graphic representation of the non-canonical NF-kB pathway [135].

2. Aim of the thesis

Steri and colleagues discovered an association in the TNFSF13B gene, encoding the cytokine BAFF, with two autoimmune diseases, MS and SLE. The disease-risk allele was also associated with upregulated humoral immunity through increased levels of sBAFF, B lymphocytes and immunoglobulins. The causal variant was identified: an insertion-deletion ("BAFF-var") yields the production of a shorter transcript that escapes miRNA and RNA binding protein inhibition, increasing sBAFF production that in turn up-regulates humoral immunity.

Population genetic signatures indicate that this autoimmunity variant has been evolutionarily advantageous in Sardinia, most likely due to a better fitness under infectious disease exposure such as malaria [11, 12]. Indeed, the variant has a high frequency in the Sardinian population were malaria was endemic until its eradication (1950s) and mice over-expressing BAFF are protected from lethal malaria infections [15]. Here, we propose that the up-regulated humoral immunity due to BAFF-var improves anti-malaria immunity and may prevent malaria-induced dysregulation of sBAFF production.

To test our hypothesis, we performed functional studies using *P. falciparum* lysates to treat TNFSF13B genotype-specific PBMC, and to highlight differential enrichment of host's cell populations, proteins and regulatory transcripts. In this study we tried to clarify the mechanisms underlying protection against malaria exerted by the inherited BAFF-var allele. Our results will hopefully inform about efficacy of BAFF-based therapies in the treatment of malaria infection.

3. Materials and Methods

3.1. Preparation of *P. falciparum*-infected red blood cell (RBC) lysates

P. falciparum-infected red blood cell (RBC) lysates were prepared as previously described [136]. Briefly, 3D7 *P. falciparum* cultures were maintained in fresh human O^{Rh+} erythrocytes at 3% hematocrit in RPMI 1640 medium (KD Medical) supplemented with 10% heat-inactivated ORh+human serum (Interstate Blood Bank, Memphis, Tennessee), 7.4% sodium bicarbonate (GIBCO, Invitrogen) and 25 µg/ml of gentamycin (GIBCO, invitrogen), at 37°C in the presence of a gas mixture containing 5% O2, 5% CO2 and 90% N2. Cells were passed every day and synchronized every two day in sorbitol solution. Mycoplasma free cultures of *P. falciparum* schizont infected red blood cells (iRBCs) were isolated using magnetic columns (LD MACS Separation Columns, Miltenyi Biotec). Lysates of red blood cells infected (iRBCs) and uninfected (uRBCs) with *P. falciparum* were obtained by three cycles of freeze-thaw in liquid nitrogen followed by 37°C in water bath. The infection was conducted treating human cells with a specific number of RBC lysates, infected and uninfected with P falciparum. This number is indicated as cells : lysates ratio.

3.2. Cell cultures

Primary peripheral blood mononuclear cells (PBMCs) were purified from genotyped healthy human donors, homozygotes for BAFF-WT and for BAFF-var, from the Sardinia cohort. Cells were stored in liquid nitrogen and then thawed and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% Pen/Strep in a 37 °C, 5% CO2 incubator. After 48h, PBMCs were plated in 24 multi-well dishes, incubated for 24h, then treated with iRBCs or uRBCs. The infection was conducted using 1:3 PBMC:lysates ratio. Supernatant, RNA and protein were collected 48 h later.

Raji cells were cultivated in RPMI medium containing 10% FBS, 2mM L-Glutamine and 1% Pen/Strep in a 37 °C, 5% CO2 incubator. Raji cells were plated in multi well and 24h later treated with iRBCs or uRBCs using 1:13 Raji:lysates ratio. Supernatant, RNA and protein were collected 48 h later.

3.3. FACS analysis

PBMCs cells were washed in PBS with 1% BSA (bovine serum albumin) and incubated for 30 min at RT with fluorescently labeled antibodies specific for B and T cells. Subsequently, samples were centrifuged and resuspended in Propidium Iodide (PI) solution (1 ug/ml PI and 10 ug/ml RNase A in PBS) and analyzed using BD FACS CantoII. B cell panel included the following antibodies: IgA- FITC, CD24 BV510, IgD PE, CD3 PerCP-Cy5.5, CD27 APC-H7, CD19 PE-Cy7, β7 APC, CD38 BV421 from BD Biosciences. T cell panel included the following antibodies: CXCR5 BV421, CD183 PE, CD4 APC-H7, CD3 FITC, CD196 APC, CD279 (PD-1) BV510, CD45RA PE-Cy7 from BD Biosciences. Results were analyzed using FACSDiva software (BD Bioscience Mountain View, CA, USA) and reported as mean fluorescence intensity (MFI), reflecting the levels of cell surface antigens and relative cell count with respect to hierarchically higher cell population (%).

3.4. B cells isolation

Primary B cells were isolated from PBMCs treated with lysate of *P. falciparum* (iRBCs and uRBCs). After 48h, B cells were purified using the Easy Sep negative selection system (STEMCELLS technologies). Briefly, PBMCs were resuspended in PBS media containing 2% fetal bovine serum and 1mM EDTA and Enrichment Cocktail, containing the antibody complex. After 10 minutes incubation, 50 µl of magnetics beads and recommended media were added. The samples were then placed into the magnetic support for 5 minutes to isolate the B cells. The pellet, containing B cells, was divided and lysed for RNA and protein extraction.

3.5. ELISA

Relative soluble protein levels were measured in collected supernatants of PBMCs (*BAFF-WT* or *BAFF-var)* by ELISA and Bio-Rad Bioplex. BAFF protein levels were measured using an ELISA Kit (AdipoGen). Briefly, all reagents, standard dilutions, and samples were prepared as described in the manufacturer's instructions Assay Diluent (100 µL) and 50 µL of Standard or samples were added to each well. After 3 hours of incubation, the wells were washed with washing buffer and conjugated anti-BAFF was added to each well for 1 hour. Afterwards, the plate was incubated with Substrate Solution

to develop the reaction and the plate was analyzed at 450 nm with a SUNRISE TECAN plate reader.

All the other cytokines and immunoglobulins were measured using a Bio-rad Bioplex plate. For human cytokine analysis, we used the Pro Human Cytokine 17-plex assay and for immunoglobulin analysis, we used the human IgG total isotyping assay according to the manufacturer's instructions (Bio-Rad Laboratories). Briefly, 50 µL of beads and 50 µL of Standard or samples were added to each well. After 1 hour incubation and 3 washes, 25 µL of detection antibodies were added followed by incubation for 30 minutes. Finally, Stop Solution was added to each well and the plate was read with Bio-Plex 200 instrument (Bio-Rad).

3.6. RNA isolation, reverse transcription (RT)-quantitative (q)PCR analysis

RNA was isolated from PBMCs and B cells using the TriPure isolation reagent (Roche) following the manufacturer's protocol. Briefly, samples were lysed with 500 µl TriPure and afterwards 100 µl of chloroform was added to each sample. After centrifugation, the upper layer was collected and RNA was precipitated using 500 µl of isopropanol and 1µl of Glycol Blue (Invitrogen). After centrifugation the pellet was washed in 1ml of 75% ethanol. RNA was resuspended in RNAse free water.

mRNA was reverse transcribed using First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's suggested protocol. Briefly, 400 ng of RNA was mixed with 4 µl of RT Buffer, 1 µl of dNTPs, 1 µl of Random hexamers, 0.5 µl of RiboLock RNase inhibitor, 1 µl of Reverse Transcriptase enzyme and RNase free water. The reaction tubes were incubated for 10 min at 25°C followed by 30 min at 50°C; final step was at 85°C for 5 min. The resulting cDNA was diluted 1:20 and analyzed by quantitative (q) PCR using SYBR Green mix (Kapa Biosystems) and gene specific primers listed in **Table 1**. The relative levels of RNAs were calculated by the 2-ΔCt method and β-actin levels were used for normalization.

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Table 1. ***List of primers used for qPCR.***

RT-(q)PCR primers	**Sequences (5' - 3')**
CXCL10	FW: AAACTGCCATTCTGATTTGCT RV: TTGAATGCCACTTAGAGTCAA
NF-KB2	FW: GATCGAGGTGGACCTGGTAA RV: GGGCAGTCATGTCCTTGG
MIF	FW: TCAACTATTACGACATGAACGCG RV: CTTAGGCGAAGGTGGAGTTG
CR1	FW: CCCATTGGGACATATCTGAAC RV: GCACCAGTCCAGACTGAGTTTT
I-CAM1	FW: TGTCCCCCTCAAAAGTCATC RV: GGGTCTCTATGCCCAACAAC
IL8	FW: GAGTGGACCACACTGCGCCA RV: TCCACAACCCTCTGCACCCAGT
IFNg	FW: TTTTCAGCTCTGCATCGTTTT RV: TCCGCTACATCTGAATGACCT
MIP-1B	FW: ACCGCCTGCTGCTTTTCTTA RV: CAGAGGCTGCTGGTCTCATA
GM-CSF	FW: CTGCTGCTCTTGGGCACT RV: GGATGGCATTCACATGCTC
IL10	FW: GGCACCCAGTCTGAGAACAG RV: CTTCACTCTGCTGAAGGCATC
IKK-a	FW: TGGAACAACTGTGGAACCTG RV: CCGATGCTGGTACAGACAGA
TRAF3	FW: CTAAAGCTGCACACTGACCG RV: TGTACTTGTCCTCCACGGTC
NFKB1	FW: TATGTGGGACCAGCAAAGGT RV: GCAGATCCCATCCTCACAGT

BACT	FW: CATGTACGTTGCTATCCAGGC RV: CTCCTTAATGTCACGCACGAT
U6	FW: CTCGCTTCGGCAGCACA RV: CTCGCTTCGGCAGCACA
miRNA-15	TAGCAGCACATAATGGTTTGTG

3.7. RNA sequencing

Total RNA from B cells, isolated from PBMCs after 48h treatment with iRBCs and uRBCs, were sequenced and libraries were prepared according to SMARTer® Stranded Total RNA-Seq Kit v2 (Takara Bio, Mountain View, CA). Paired-end sequencing was performed on an Illumina HighSeq 4000 instrument (Illumina Inc., San Diego, CA). For the bioinformatics analysis of RNAseq data, we performed adapter trimming of fastq files with the tool TrimGalore v0.4.5 with parameters –"illumina --paired --phred33 --clip_r1 3 --three_prime_clip_r2 3". After trimming, we used STAR to align the paired-end reads to the human reference genome (build 38) with parameter "quantMode GeneCounts". We then used Picard Tools to de-duplicate and sort the alignments. The feature-Count tools were then used to count how many reads have mapped to each human gene (using the Gencode v29 basic annotation schema). The DESeq2 algorithm was then used determine significant differences in expression (counts) between the different experimental conditions. Finally, data are match with Phenopedia database (https://phgkb.cdc.gov) from where we selected the gene of our RNA-seq that have a role in malaria.

3.8. Proteins purification and Western blot analysis

Whole-cell lysates were prepared using RIPA buffer (50 mM Tris-Cl, 1% Nonidet P-40, 0,5% sodium deoxycholate, 0,05% SDS, 1 mM EDTA, 150 mM NaCl, 1X Proteinase inhibitor) supplemented with a mammalian protease inhibitor cocktail (QIAGEN), and incubated 10 min in ice. Lysates were centrifuged at 20,000g for 15 min at 4°C and supernatant, containing proteins, were quantified using Bradford assay (Bio-Rad). Proteins were size-fractionated through 4–12% gradient polyacrylamide gels (Thermo Fisher Scientific), and transferred to a nitrocellulose membranes using Trans-Blot Turbo

RTA Transfer Kit, Nitrocellulose (Bio-Rad). Membranes were washed with TBS-T (TBS plus 0.1% Tween20) and then blocked with 5% non-fat dried milk dissolved in TBS-T. Membranes were incubated overnight with primary antibodies recognizing NFKB2 (Cell Signaling), CXCL10 (Abcam), MIF (Abcam), CR1 (Abcam), I-CAM1 (Abcam), BACT (Santa cruz), NFKB2, TRAF2, TRAF3, IKK-a (Cell Signaling), HSP90, (Santa Cruz Biotechnology), BCMA (Abcam), TACI (Abcam) and BAFF-R (Proteintech), as described in **Table 2**. Secondary antibodies conjugated with horseradish peroxidase (HRP) were used, and the signals detected by enhanced chemiluminescent reaction using SuperSignal West Femto (Thermo Scientific). Images were acquired with the Bio-Rad Universal Hood II Gel Doc System.

Table 2. ***List of antibodies and relative dilutions used for western blot.***

Primary antibody	**Dilution**
NFKB2	1:500
CXCL10	1:500
MIF	1:500
CR1	1:500
I-CAM1	1:500
TRAF3	1:500
TRAF2	1:500
IKK-a	1:500
BAFF-R	1:500
BCMA	1:500
TACI	1:500
BACT	1:1000
HSP90	1:1000

3.9. Analysis of mRNA stability by actinomycin D assays

For mRNA half-life determination, PBMCS (WT and variant) were treated for 48h with iRBCs and uRBCs, and then incubated with Actinomycin D (Act D; 5 μg/ml) to block de novo transcription. Cells were harvested at subsequent time intervals (0, 0.5, 1, 2, 4 and 6 h) of Act D treatment and total RNA was extracted and processed as described above by RT-qPCR analysis. Data from Act D assays were processed using the Prism7 software to assess mRNA decay curves.

3.10. miRNA extraction and reverse-transcription

Small RNAs were purified using miRNeasy Serum/Plasma Advanced kit (Qiagen). Supernatant (500 μl) from infected PBMCs were treated with Buffer RLP that contains guanidine thiocyanate as well as detergents to promote lysis and denature protein complex and RNases. Buffer RPP was added to precipitate proteins while supernatants containing RNA were collected. Isopropanol was added to the supernatant and samples transferred to the RNeasy UCP MinElute spin column. RNAs were washed with 80% ethanol and then resuspended in RNase free water.

miRNAs were reverse transcribed using Mir-X™ miRNA FirstStrand Synthesis (Takarabio) following the manufacturer's guidelines. RNA (3.5 μl) was mixed with 0.25 μl of RNase inhibitor, 5 μl of mRQ Buffer, 1.25 μl of mRQ enzyme. The tubes were incubated in a thermocycler for 1 h at 37°C followed by final step at 85°C for 5 min to inactivate the enzymes. The cDNA was diluted 1:10 and used for q-PCR analysis how previously described

3.11. Statistical analysis

Statistical significance was determined using two- tailed t-tests, as indicated in the Figure legends. Values were considered significant when $p < 0.05$. Tests of statistical significance were conducted using Prism 7 software (GraphPad)

4. Results

4.1. BAFF-var a new autoimmunity variant identified in Sardinians

Steri and colleagues recently identified an association between a variant in the TNFSF13B gene, encoding the cytokine BAFF and increased risk of the autoimmune diseases MS and SLE (Steri, 2017). In **Figure 5A**, it is represented the structure of TNFSF13B gene and the identified variant: an insertion-deletion in the 3'UTR, that creates an alternative polyadenylation (APA) site resulting in a shorter transcript and a high soluble BAFF (sBAFF) production, *BAFF- var* mRNA (grey).

Using supernatants of PBMCs from Sardinian donor, sBAFF was measured by enzyme-linked immunosorbent assay (ELISA). Supernatants of individual with both *BAFF-WT* alleles expressed lower sBAFF levels than individuals with both *BAFF-var* alleles (n=10) (**Figure 5B**).

BAFF-var has higher frequency in Sardinia compared to the main Italy and other regions in the world (**Figure 5C**). Accordingly, it was assessed whether the high frequency of BAFF-var in the Sardinian population is consistent with the effects of random genetic drift or if it is the consequence of selection favoring BAFF-var in Sardinia (positive selection). The statistical analyzes performed by M. Steri and M. Floris, showed that the core haplotype carrying BAFF-var is remarkably longer than haplotypes carrying variants with matched genetic features, a finding, that with the high frequency, is consistent with the hypothesis of positive selection for BAFF-var in Sardinia (**Figure 5**D).

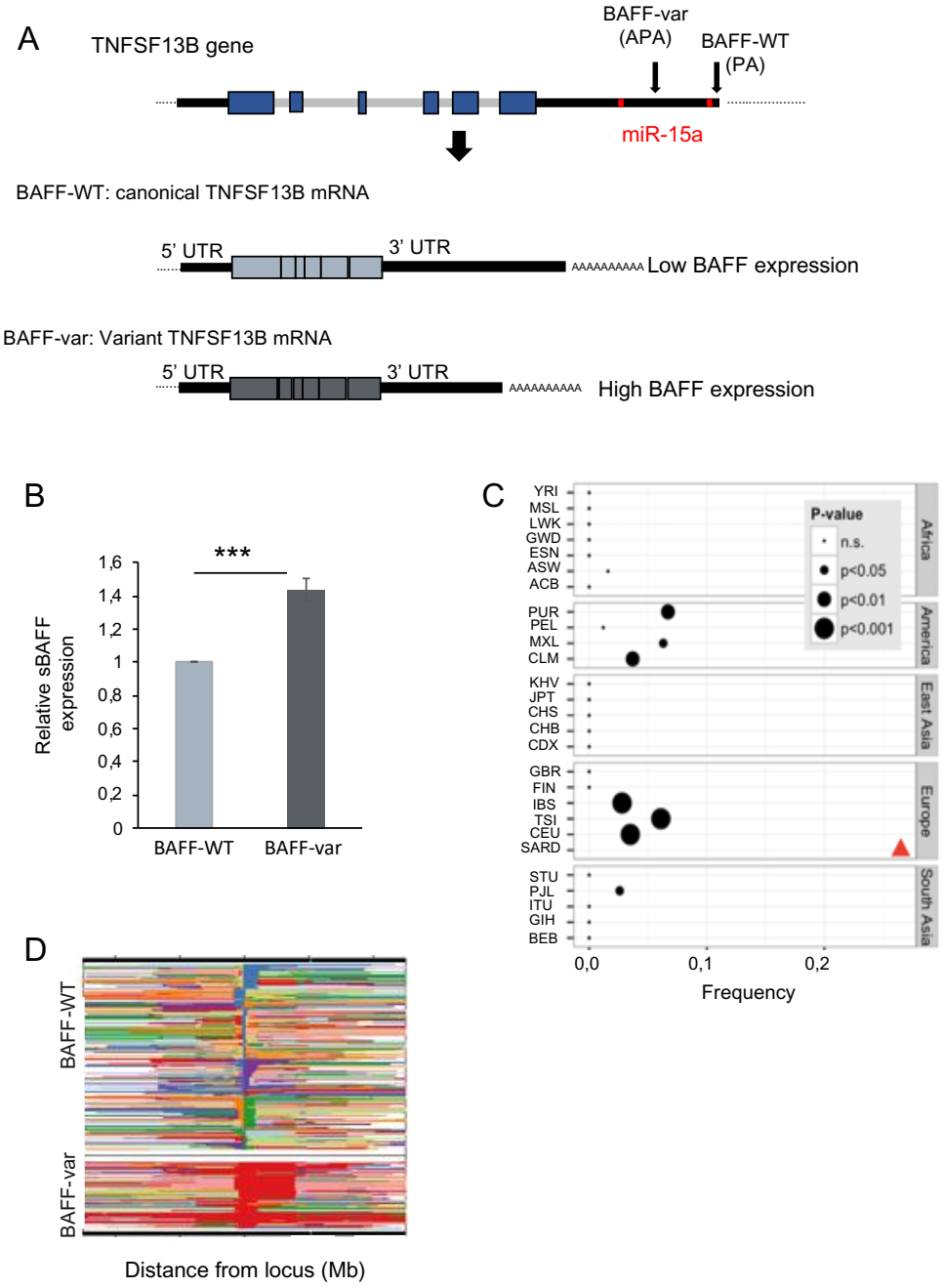

Figure 5. ***Differential regulation of BAFF from BAFF-var and BAFF-WT mRNAs.*** *(A) Top, schematic of the TNFSF13B gene, polyadenylation site (PA), and alternative polyadenylation site (APA) generated due to BAFF-var. Bottom, mRNAs generated from TNFSF13B according to polyadenylation site usage [BAFF-WT mRNA (light blue) and BAFF-var mRNA (grey)]. (B) Relative soluble BAFF proteins levels measured from cells of the Sardinia cohort by ELISA from individuals homozygous for BAFF-WT or for BAFF-var treated with uRBCs for 48h. (C) BAFF-var frequency in Sardinians (red triangle) and in 1000 Genomes Project populations (black dots). (D) BAFF-var haplotype extension. Data in B are the means and standard deviation (+SD) from at least three independent experiments. *, P < 0.05; **, P < 0.01, ***, P < 0.005.*

4.2. Effect of malaria antigens on PBMCs-WT and PBMCs-var populations and immunoglobulins

BAFF-var shows significant evidence of positive selection, possibly due to genetic adaptations to malaria infection. Thus, it was tested if changes due to BAFF-var improve anti-malaria immunity and prevent dysregulation of sBAFF induced by malaria infection. To this end, PBMCs purified from BAFF-WT and BAFF-var donors, then treated with lysates of red blood cells uninfected (uRBCs) or infected (iRBCs) with *P. falciparum*, were used to obtain the following conditions: (i) PBMC-WT infected with uRBCs, (ii) PBMC-WT infected with iRBCs, (iii) PBMC-var infected with uRBCs, (iv) PBMC-var infected with iRBCs (**Figure 6**). To highlight differential enrichment of sub-populations of B and T cells, soluble proteins and regulatory transcripts, analysis by flow-cytometry assay, Bio-Plex Multiplex Immunoassays and RNA expression quantifications were performed (**Figure 6**).

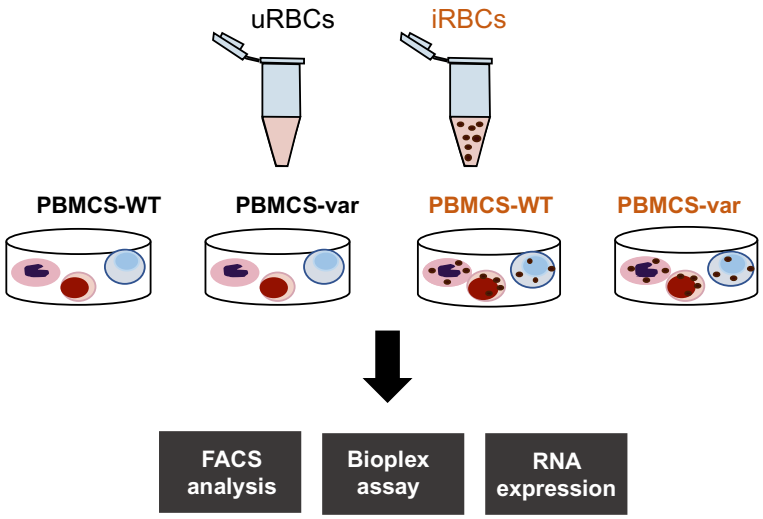

Figure 6. ***BAFF-var modulates the response to exposure to P. falciparum antigens.*** *(A) Schematic representation of PBMC cells expressing normal (BAFF-WT) or higher (BAFF-var) level of sBAFF that were treated for 48 h with lysates from red blood cells uninfected or infected with P. falciparum (uRBCs, iRBCs), as prepared by lysis of mycoplasma-free culture of RBCs at the schizont stage. After treatment, cells were prepared for FACS analysis, Bioplex assay and RNA expression.*

In general, assessing a set of markers for B and T cells by FACS, the most significant sub-populations influenced by the exposure to *P. falciparum* antigens were the memory cell populations (**Figure 7 and 8**).

In the B cell sub-populations analyzed, differences related to the presence of BAFF-var were not observed (**Figure 7A and B**), only differences related to the presence of *Plasmodium* antigens were detected. Specifically, the presence of *P. falciparum* antigens induced the expansion of IgD- CD27- and IgD- CD38+ subset with respect to B cells (**Figure 7A)** and increase of CD38 expression on number of memory B cells subsets (IgD- IgA-, IgD- CD24+, IgD- CD24- and IgD- CD27-) (**Figure 7B**).

On the other hand, in the sub-populations of T cells analyzed, differences related to both the presence of BAFF-var and antigens of *P. falciparum* (**Figures 8A and B**) were observed. The presence of *P. falciparum* antigens induced the expansion of PD1+ CXCR5+ Th2 subset with respect to helper (CD4+) T cells, and increase of CXCR5 expression on number of memory T cells subsets PD1+ CXCR5+ Th1 and PD1+ CXCR5+ Th1/Th17. Interestingly, the presence of BAFF-var significantly modulated the effect of malaria antigens in all the T cell sub-populations analyzed: PD1+ CXCR5+ Th1, PD1+ CXCR5+ Th2, PD1+ CXCR5+ Th1/Th17 considering the absolute number (**Figure 8A)**, as well as the expression level of CXCR5 on these subsets (**Figure 8B**). Most of these populations were highly expressed in BAFF-var iRBCs demonstrating that BAFF-var has an important role in the memory response to malaria infection in T cells.

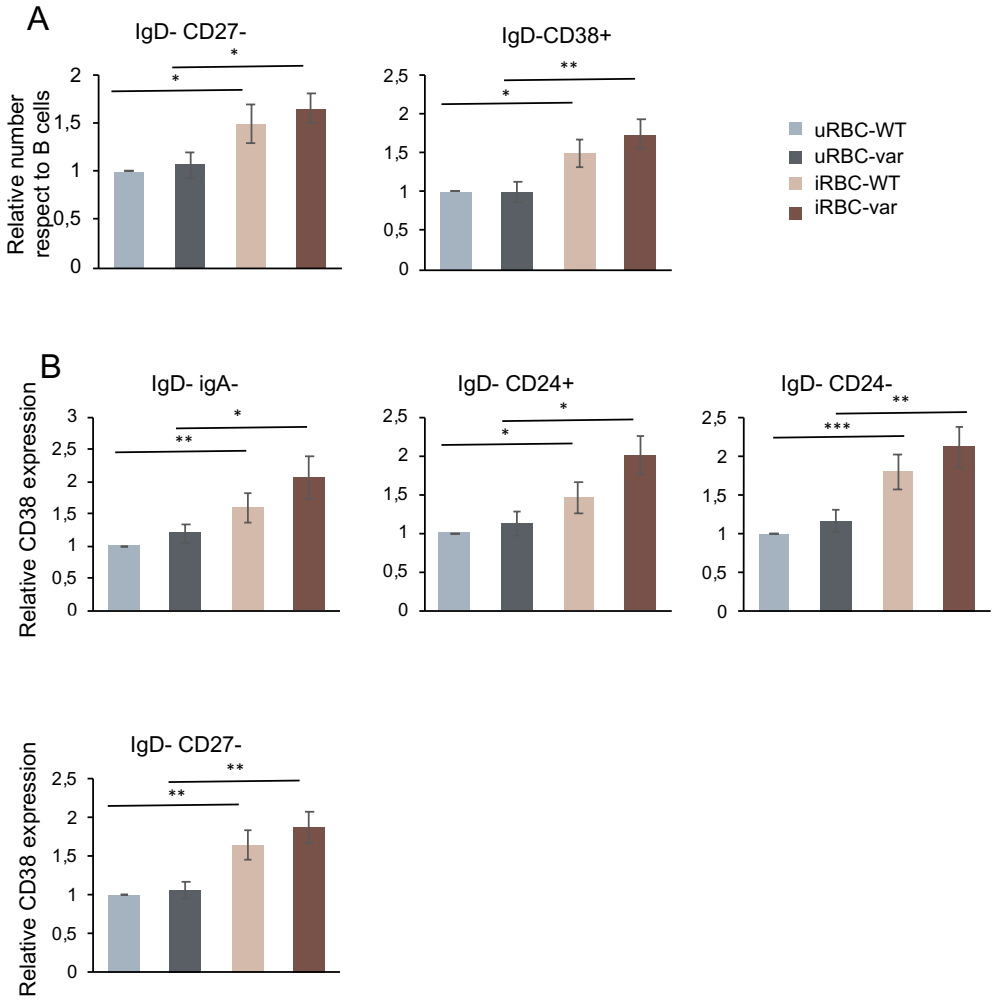

Figure 7. ***P. falciparum antigens modulates B cells production.*** *PBMC-WT (n=10) and PBMC-var (n=10) were treated for 48h with uRBCs or iRBCs. Cell were collected, treated with Propidium Iodide solution and analyzed by FACS using markers to identify B cells sub-populations. (A) Relative number with respect to B cells (%) (B) expression levels of CD38 on the indicate population. Each panel show the average of the relative expression of the populations. Data are the means and standard deviation (+SD) from at least three independent experiments. *, P < 0.05; **, P < 0.01, ***, P < 0.005.*

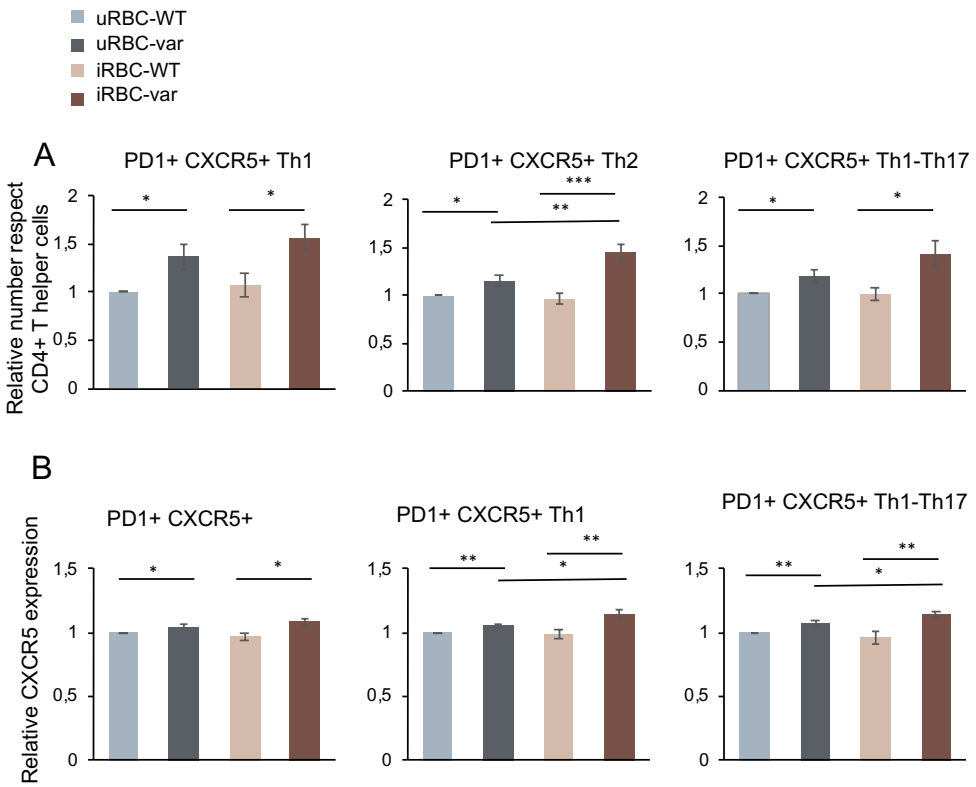

Figure 8. ***P. falciparum antigens and BAFF-var modulates T cells production.*** *PBMC-WT (n=10) and PBMC-var (n=10) were treated for 48h with uRBCs or iRBCs. Cell were collected, treated with Propidium Iodide solution and analyzed by FACS using markers specific for the T cell sub-populations indicated in the upper part of the charts. (A) Relative number with respect to T helper (CD4+) (%) (B) expression levels of CXCR5 on the indicate population. Each panel show the average of the relative expression of the populations. Data are the means and standard deviation (+SD) from at least three independent experiments. *, P < 0.05; **, P < 0.01, ***, P < 0.005.*

Antibodies that target the *P. falciparum* preventing blood-stage re-infection are key mediators of the protective immunity. Until now, most studies have largely focused on understanding IgG responses, such as IgG1 and IgG3, and IgA [95, 96]. Recently the role of IgM has been taken in consideration in malaria infection [97]. For this reason, the expression of different classes of soluble immunoglobulins including IgG1, IgG2, IgG3, IgG4, IgA, IgM were assessed by Bio-Plex multiplex assay (Bio-Rad). A differential expression related to the presence of BAFF-var in the expression of IgG1 and IgG3 in uninfected (uRBCs) samples is shown in **Figure 9** while the presence of *P. falciparum* antigens (iRBCs) significantly alters the production of IgG3 and IgM. No significant differences are observed in IgG2, IgG4 and IgA populations (**Figure 9**).

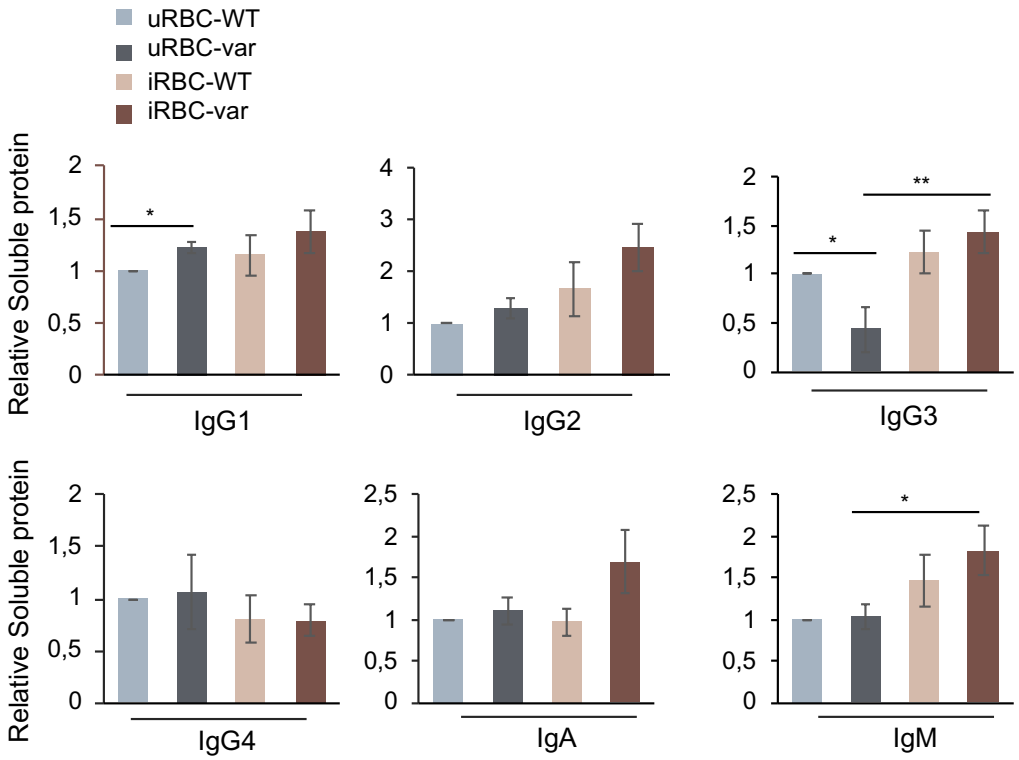

Figure 9. ***BAFF-var and P. falciparum antigens modulates immunoglobulin production.*** *(A) PBMC-WT (n=10) and PBMC-var (n=10) were treated for 48h with uRBCs or iRBCs. Supernatants were collected and the relative levels of soluble immunoglobulins expressed were quantified by Bioplex assay. Data are the means and standard deviation (+SD) from at least three independent experiments. *, P < 0.05; **, P < 0.01, ***, P < 0.005.*

4.3. Effect of *P. falciparum* antigens on cytokine production

The balance between pro- and anti-inflammatory cytokines plays a crucial role in anti-malaria immunity. Thus, the secretion of cytokines produced by PBMC-WT and PBMC-var was assessed using Bio-Plex multiplex, as described in **Figure 6**. A differential expression related to the presence of BAFF-var for IL8 and IL10 was observed (**Figure 10**). For all the other cytokines, differential expression related only to the presence of *P. falciparum* antigens is observed, as shown in **Figure 10**.

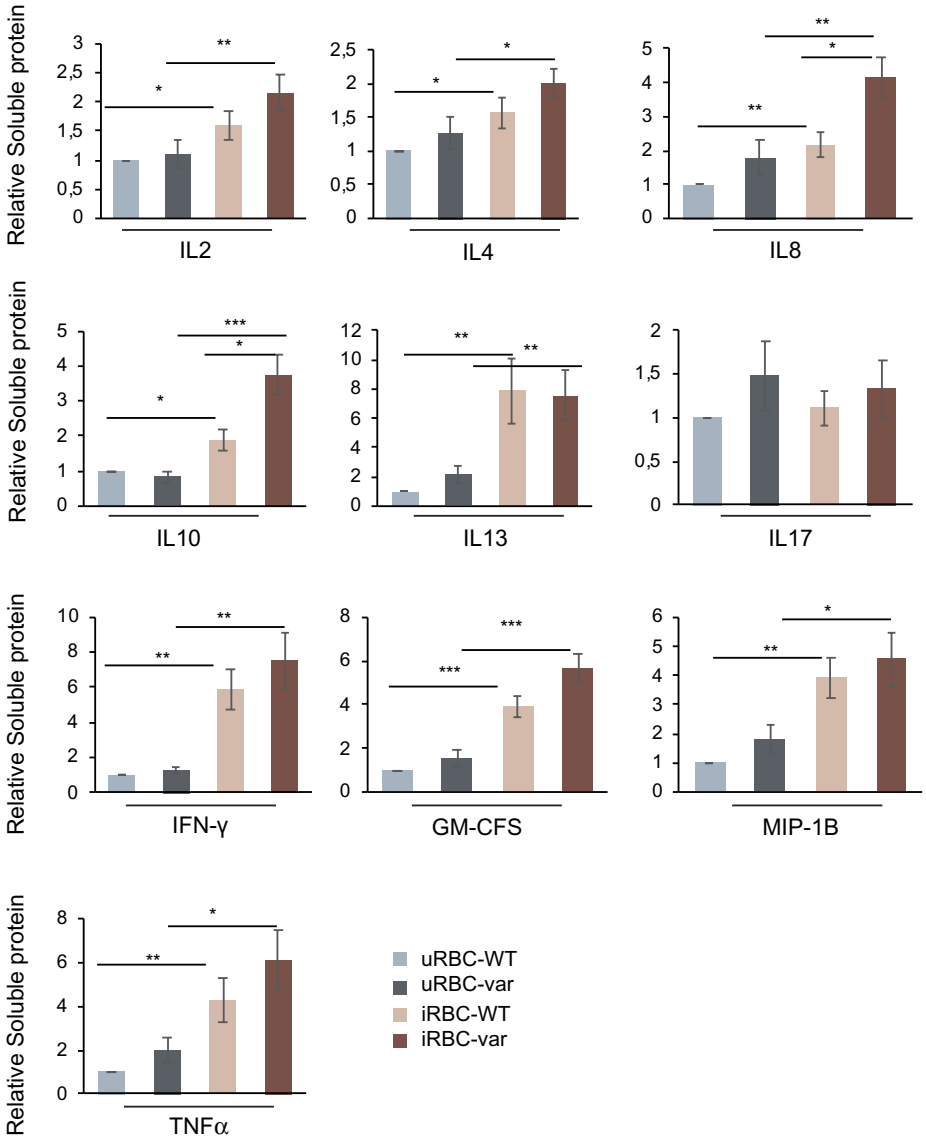

Figure 10. ***BAFF-var modulates cytokine production to exposure to P. falciparum antigens.*** *(A) PBMC-WT (n=10) and PBMC-var (n=10) were treated for 48h with uRBCs or iRBCs. Conditioned media were collected and the levels of cytokine secreted were quantified by Bioplex assay. Data are the means and standard deviation (+SD) from at least three independent experiments. *, P < 0.05; **, P < 0.01, ***, P < 0.005.*

Next, to analyze if changes in cytokine production are due to changes in RNA levels, we analyzed the expression of both mRNA and pre-mRNA cytokine transcripts in the PBMC treated samples (**Figures 11A and B**). The mRNA levels are significantly influenced by the presence of BAFF-var for *GM-CSF, IL10* and *IL8* as both pre-mRNA and mRNA levels were increased, suggesting transcriptional regulation of these transcripts. Additionally, *IL8* mRNA levels were induced in the presence of both BAFF-var and malaria antigens (PBMC-var infected with uRBCs vs PBMC-var infected with uRBCs). As the pre-mRNA levels were not significantly changed, this observation suggested post-transcriptional regulation as well (**Figures 11A and B**). The *IFN-* γ and *MIP-1b* transcripts were significantly modulated by the presence of *Plasmodium* antigens but not influenced by the presence of BAFF-var as shown in **Figure 11** (**Figures 11A and B**).

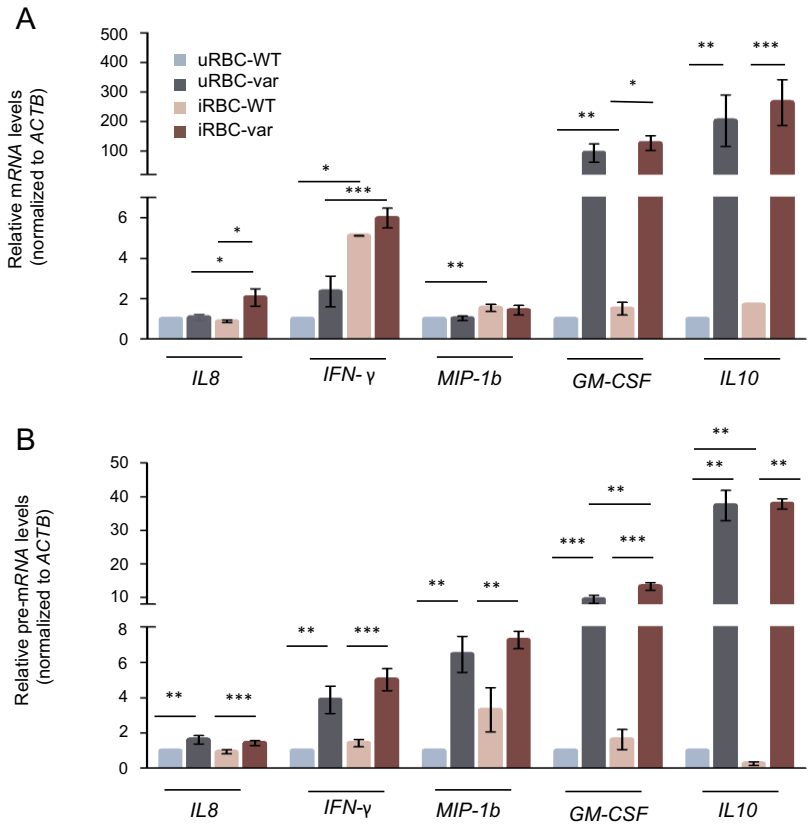

Figure 11. ***P. falciparum antigens activate cytokine expression in PBMCs.*** *Cell pellets were collected from PBMCs BAFF-WT and BAFF-var treated for 48h with iRBCs and uRBCs and used for downstream applications. (A) The levels of the indicated mRNAs (B) and pre-mRNAs were measured by RT-qPCR analysis. mRNA and pre-mRNA levels were normalized to ACTB mRNA levels. Data in A and B are the means and standard deviation (+SD) from at least three independent experiments. *, P < 0.05; **, P < 0.01, ***, P < 0.005.*

4.4. NFkB pathway investigation

BAFF is a cytokine that belongs to the tumor necrosis factor TNF ligand family and is the natural ligand of BAFFR (BR3), TACI, and BCMA. BAFFR selectively binds sBAFF with a greater affinity than the other two. Interaction between sBAFF and BAFFR activate the non-canonical NFkB signaling pathway.

To test if the non-canonical NFkB pathway could have a role in the malaria response and if the presence of BAFF-var could influence that response, the expression of mRNA and pre-mRNA transcripts encoding molecules implicated in the non-canonical NF-KB pathway were analyzed (**Figures 12A and B**). *NFkB2* and *IKK-a* mRNA levels increased in BAFF-var samples treated with iRBCs (**Figure 12A**). These changes were also reflected at the pre-mRNA levels indicating enhanced transcription (**Figure 12B**). Interestingly, in response to the presence of *P. falciparum* lysates, the *NFkB2* mRNA was significant upregulated without concomitant changes in the pre-mRNA levels inferring a post-transcriptional regulation of this expression (**Figure 12B**). Finally, *TRAF3* pre-mRNA levels showed significant increases in the BAFF-var samples which were not reflected by changes in the *TRAF3* mRNA level. The *TRAF3* mRNA changed slightly but significantly only for PBMC-var infected with uRBCs vs PBMC-var infected with uRBCs, suggesting that the presence of the variant affected the response to *P.falciparum* antigens.

To check if these changes in gene expression are specific for the non-canonical NFkB pathway, the mRNA levels of *NFkB1,* a participant in the canonical NFkB pathway, were also analyzed under these treatment conditions. No significant changes were observed in *NFkB1* mRNA levels, suggesting specific gene regulation of the non-canonical NFkB pathway (**Figure 12C**).

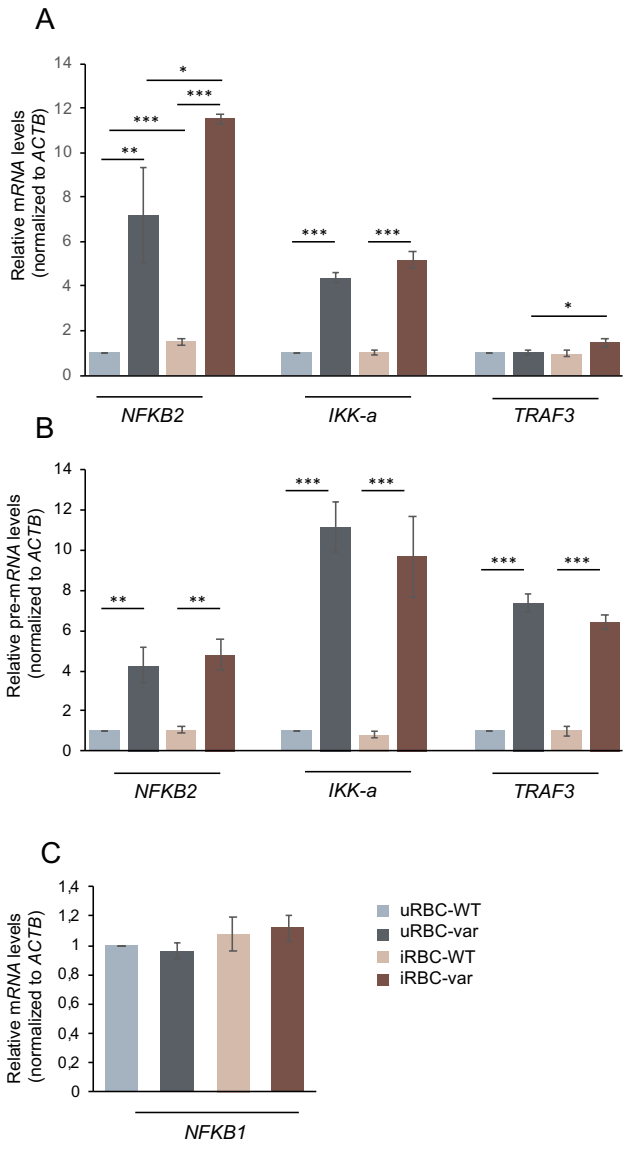

Figure 12. ***P. falciparum antigens modulate NFkB2 and NFkB1 pathway in PBMCs.*** *RNA was purified from PBMCs WT and var treated for 48h with iRBCs and uRBCs and analyzed by RT-qPCR. (A) mRNAs expression (B) and pre-mRNAs of NFkB2, IKK-a and TRAF3 were analyzed. (C) NFkB1 mRNA expression from the same samples was examined by RT-qPCR. mRNA and pre-mRNA levels were normalized to ACTB mRNA levels. Data in A, B and C are the means and standard deviation (+SD) from at least three independent experiments. *, P < 0.05; **, P < 0.01, ***, P < 0.005.*

4.5. *P. falciparum* antigens alters the transcriptome of B cells in a BAFF-var dependent manner

Humoral immunity is important in clinical disease outcome during malaria, yet to escape the host immune responses, Plasmodium parasites specifically disturb the functionality of B cell subsets. Due to the relevance of B cells in the malaria immune response we assessed the global impact of BAFF-var to malaria infection by analyzing total RNA expression patterns. To this end, we infected PBMC-WT and PBMC-var with uRBCs and iRBCs for 48h followed by isolation of B cells as described in **Material and Methods**. RNA was then extracted and sequenced; the results were validated by RT-qPCR and analyzed by western blot to determine whether changes at the RNA level were reflected by changes in protein expression (**Figure 13**).

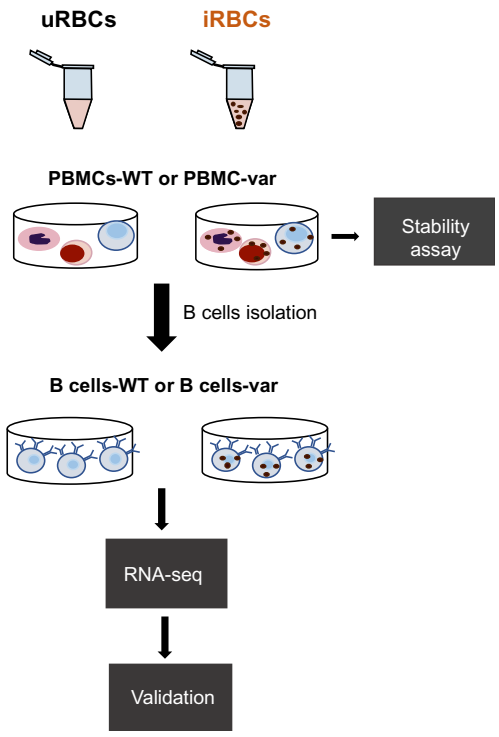

Figure 13. ***BAFF-var modulates the response in B cells to exposure to P. falciparum antigens.*** *(A) Schematic representation of PBMC cells expressing normal (BAFF-WT) or higher (BAFF-var) level of sBAFF were treated for 48 h with lysates from red blood cells uninfected or infected with P. falciparum (uRBCs, iRBCs), as prepared by lysis of mycoplasma-free culture of RBCs at the schizont stage. After 48h PBMCs were treated with actinomycin D for the stability assay. RNA extracted from B cells isolated from PBMCs were used for RNA-seq analysis and the same cells were used for mRNA, pre-mRNA and protein extraction for functional validation.*

For the analysis, total RNA levels of each condition were compared to obtain the following experimental groups: (i) iRBC-WT vs uRBC-WT (**Figure 14A, left panel**); (ii) iRBC-var vs uRBC-WT (**Figure 14A, middle panel**); (iii) iRBC-var vs uRBC-var (**Figure 14A, right panel**).

RNA sequencing analysis identified 71 genes which were upregulated (red dots) and 14 genes which were downregulated (blue dots) when comparing iRBC-WT with uRBC-WT (**Figure 14A**, **upper panel**), 165 genes upregulated and 54 genes downregulated in the iRBC-var versus uRBC-WT comparison (**Figure 14B, upper panel**), and finally 175 genes upregulated and 11 downregulated genes in iRBC-var group compared to the uRBC-var group (**Figure 14C, upper panel**).

For the RNAs most differentially abundant, relative levels were calculated for three experiment groups and plotted (**Figure 14, middle panels**). Quantification of these signals revealed that in iRBC-WT vs uRBC-WT mRNA of *ADAMTS1, FRMPD3* and *ISL2* are downregulated while *CCL4, RGS1* and *OAS3* are upregulated (**Figure 14A, middle panel**). Comparing iRBC-var vs uRBC-WT (**Figure 14B, middle panel**) we observed a downregulation of *TLR4, VGF, CR1* and *PSMD4P1* whereas *ICAM, CD69, MIF, NFkB2* and *CXCL10* are upregulated. Finally, for iRBC-var group, a downregulation of *TLR4, TGFBI,* and *TPX2* genes and upregulation of *CDC6, MX1* and *IFI6* was observed when compared to the uRBC-var expression (**Figure 14C, middle panel**).

KEGG pathway analysis of the mRNAs differentially regulated in each analysis revealed that the encoded proteins were implicated in biological processes related to infectious diseases, autoimmunity and the NFkB pathway, consistent with our previous results (**Figure 14A, B, C, lower panel**).

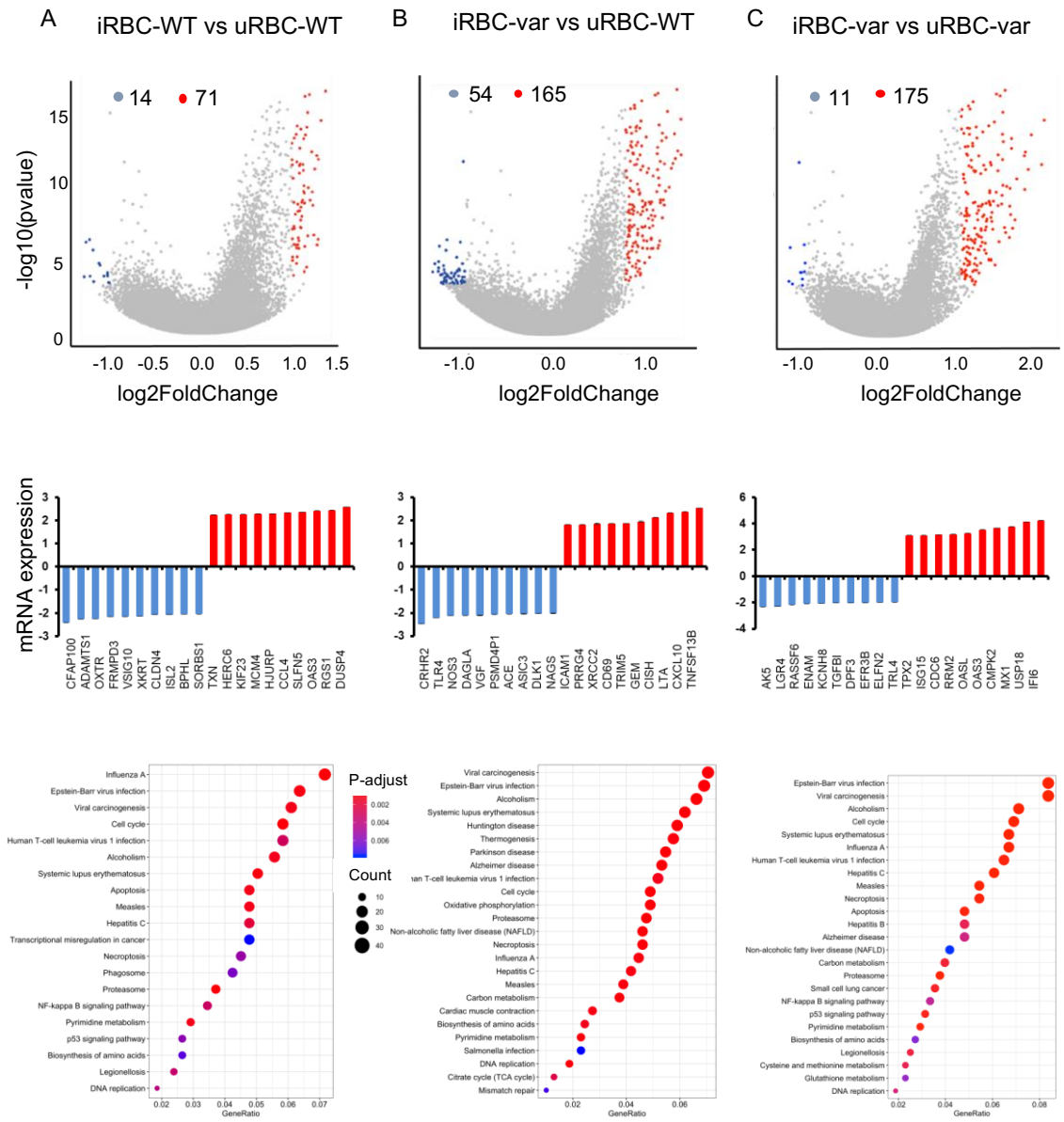

Figure 14. ***RNA-seq analysis of B cells isolated from PBMCs (WT or var) exposed to P. falciparum antigens****. (Upper) Volcano plot representation of the differential abundance of RNAs in B cells in the three different comparison groups (A-iRBC-WT vs uRBC-WT; B-iRBC-var vs uRBC-WT; C-iRBC-var vs uRBC-var), reduced (blue) and increased (red) mRNAs are highlighted. (Middle) Among the mRNAs showing differential abundance, those displaying the greatest fold increases (red) and decreases (blue) by RNA-seq analysis were plotted. (Lower) KEGG pathway enrichment analysis of the mRNAs differentially expressed in B cells from volcano plot.*

4.6. BAFF-var modulates the response to exposure to *P. falciparum* antigens

It was deeply investigated the role of some genes implicated in immune response to malaria and identified by RNA-seq analysis: *CXCL10, CR1, I-CAM1, MIF,* and *NFKB2*. Initially, levels of mRNAs and corresponding pre-mRNAs (**Figures 15A and B**), were measured as indicators of *de novo* transcription by RT-qPCR. *CXCL10* showed strong and significant upregulation in mRNA levels in samples exposed to *Plasmodium* antigens (iRBC-WT and iRBC-var compared to uRBC-WT and uRBC-var, respectively). These enhanced *CXCL10* mRNA expressed significantly exceeded changes in its pre-mRNA, suggesting strong post-transcriptional regulation. In contrast, the *CR1* mRNA expression decreased iRBC-WT compared to uRBC-WT and in uRBC-var compared to uRBC-WT while similar changes in *CR1* pre-mRNA levels indicate a transcriptional regulation of *CR1* (**Figures 15A and B**). B cells expressed higher level of *NFKB2* mRNA in response to the *P. falciparum* antigens in iRBC-var versus all the other samples (uRBC-WT, uRBC-var and iRBC-WT) suggesting a key role for BAFF-var in regulating the expression of *NFkB2* in B cells both at the basal level and in the presence of malaria antigens (**Figures 15A and B**). There were no changes in *MIF* and *ICAM-1* mRNA expression levels (**Figure 15A**).

Next, the protein levels of the selected genes were analyzed. CXCL10 protein levels were enhanced in response to both BAFF-var and *Plasmodium* antigens suggesting a regulation in the translational efficiency (**Figures 15C and D**). Under these conditions, the CR1 protein levels changed dramatically and differently when compared to its mRNA levels. Indeed, a significant upregulation of CR1 was observed in the presence of BAFF-var in both conditions (uRBCs and iRBCs). These changes suggest significant translational regulation of CR1 is induced by the presence of BAFF-var (**Figures 15C and D**).

ICAM-1 and *MIF* showed only changes in protein expression without mRNA changes, indicating a regulation of the translational efficiency of these proteins. MIF protein levels were significantly upregulated in uRBC-WT versus iRBC-WT while in cells purified from BAFF-var, there were no significant differences in MIF expression upon exposure to malaria (uRBC-var versus iRBC-var). This result indicated that the presence of increased level of sBAFF alone promoted increased MIF expression to a

threshold point which could not be further enhanced in response to *Plasmodium* antigens (**Figures 15C and D**). In contrast to the other genes analyzed, ICAM-1 protein levels were downregulated in B cells purified from BAFF-var donors,. This decreased expression was significant in the presence of *P. falciparum* antigens indicating that the increased level of sBAFF also plays a role in diminishing ICAM-1 protein expression (**Figures 15C and D**).

After activation of the noncanonical NFkB pathway, the p100 (NFkB2) full-length protein is co-translationally processed into the p52 active form such that it will translocate to the nucleus to regulate gene transcription. We analyzed both p100 and p52 by western-blot in our samples. We observed that both p100 and p52 were upregulated in B cells purified from BAFF-var donors. In addition, p52 levels increased in response to malaria antigens in a BAFF-var dependent way suggesting an upregulation due to both the presence of BAFF-var and exposure to *Plasmodium* antigens (**Figures 15C and D**).

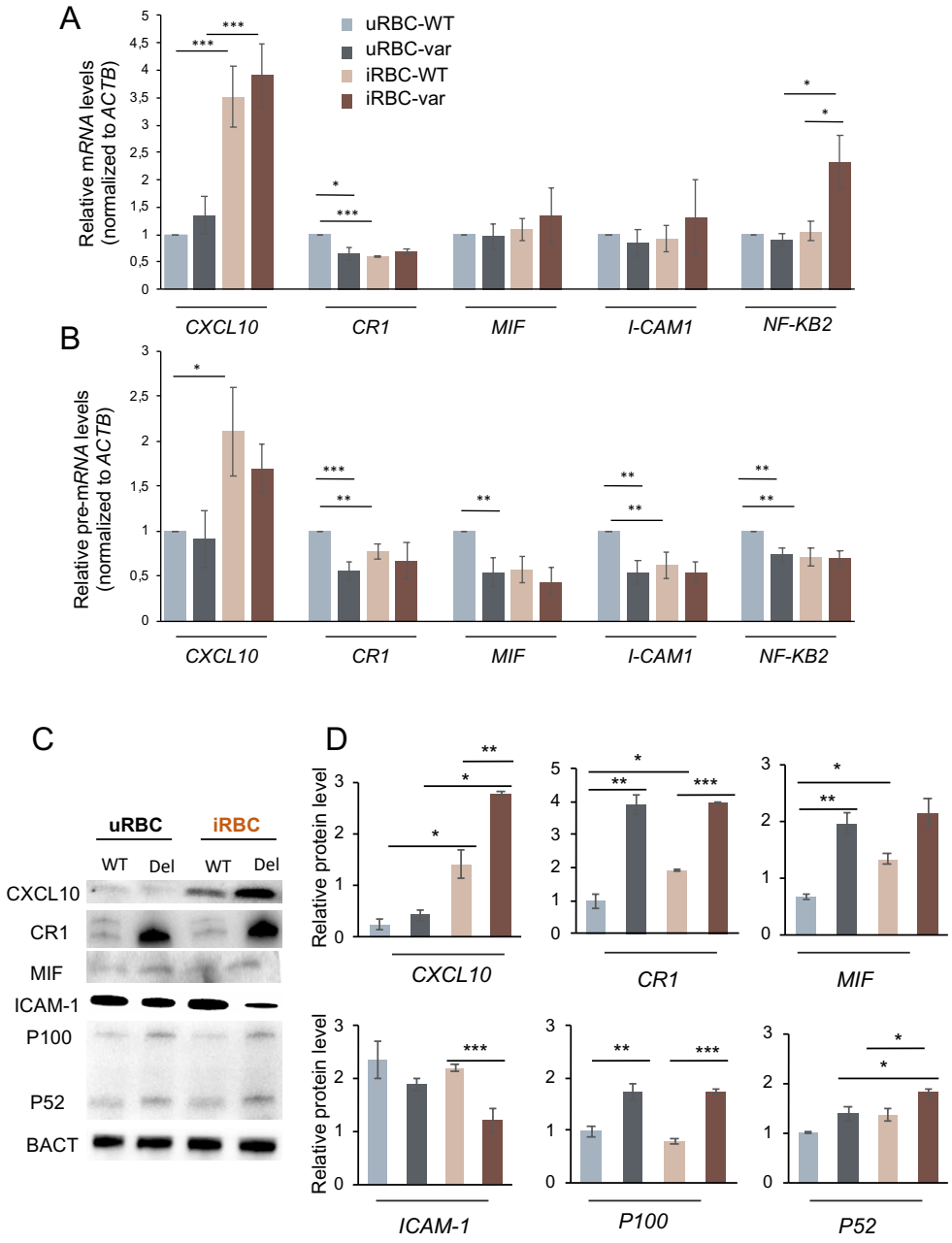

Figure 15. ***BAFF-var modulates protein, mRNA and pre-mRNA expression in response to exposure to P. falciparum antigens.*** *Protein and RNA purified from B cell isolated from PBMCs treated for 48h with iRBCs and uRBCs were analyzed by western blot and RT-qPCR. (A) RNA were collected and used for RT-qPCR to examine the expression of the indicated mRNAs (B) and pre-mRNAs. (C) Protein expression levels were assessed by western blot analysis and protein signals were quantified using ImageJ and plotted.* B-actin (BACT) *serves as a loading control. (D). Data in A, B and D are the means and standard deviation (+SD) from at least three independent experiments. *, P < 0.05; **, P < 0.01, ***, P < 0.005.*

4.7. *P. falciparum* antigens regulate NFkB2 pathway in Raji cells

Due to the relevance of NFkB2 pathway in B cell regulation after BAFF-BAFFR binding, we decided to deeply analyze it using Raji cells, a lymphoblast-like cells established by Burkitt's lymphoma that was used as experimental model. Expression of p100, p52, IKK-a, TRAF2 and TRAF3 were observed 48h after treatment with *Plasmodium* antigens. Raji cells were seeded in multiwell plates and treated with uRBC or iRBC lysates for 48 h, and proteins analyzed by western blot. Interestingly, the expression levels of p100, p52, iKK-a, and TRAF3 were increased in iRBC samples compared to uRBC. In line with the activation of the NFkB2 pathway, TRAF2 showed a downregulation in protein expression in iRBCs treated samples compared with uRBCs (**Figure 16**).

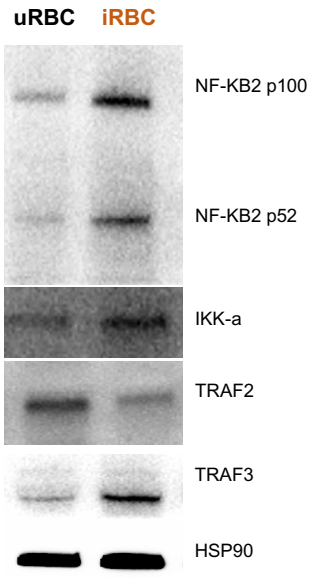

Figure 16. ***P. falciparum antigens activate NFkB2 pathway in Raji cells. (A)*** *Protein purified from Raji cells treated for 48h with uRBCs or iRBCs were analyzed by western blot*. HSP90 *serves as a loading control.*

4.8. BAFF-var and *P. falciparum* antigens modulate mRNA stability of selected genes

In isolated B cells, the mRNA levels of selected genes showed trends in steady-state levels that mirrored the changes observed in pre-mRNA levels, suggesting that BAFF-var and/or *Plasmodium* antigens might help to control their transcription (*CXCL10* and *CR1*). However, for other genes (e.g. *NFkB2* mRNAs), the changes in the pre-RNA levels did not match the observed changes in steady-state mRNA levels, suggesting that BAFF-var and/or *Plasmodium* antigens may not control their transcription, but instead may influence the stability of these mRNAs (**Figures 15A and B**).

To investigate this possibility directly, the half- lives of the selected mRNAs were analyzed by treating cells with actinomycin D (which inhibits RNA polymerase II and thus blocks *de novo* transcription). RNA was then collected at different times of actinomycin D Treatment such that the time required to reduce mRNA expression to one-half of their initial abundance was measured (t1/2; **Figures 17A, B and C**). In absence of malaria antigens (black lines), we observed a significant loss ($P < 0.05$) of the *CXCL10* mRNA in PBMC-var compared to PBMC-WT. In contrast, *NFkB2, CR1* and *MIF* mRNAs were less stable in the PBMC-WT samples compared to the PBMC-var cells (**Figures 17 A and B**). The relative stabilities of *ICAM-1* was comparable between groups.

In presence of *P. falciparum* antigens (red lines), the reduced stability of *CXCL10* and *ICAM-1* mRNAs was significantly greater ($P < 0.05$) in PBMC-var samples compared to the PBMC-WT, suggesting that the increased sBAFF expression promoted the decreased stability of these mRNAs (**Figures 17A and B**). Opposite results were observed for *MIF* and *CR1* mRNAs whereby the loss in mRNA stability was significantly greater ($P < 0.05$) in PBMC-WT compared to PBMC-var. The relative stabilities of *NFKB2* mRNAs in iRBCs treated samples was comparable between all groups (**Figures 17A and B**). The expression of *GAPDH* and *MYC* mRNA were used as negative and positive controls respectively (**Figures 17A and B**).

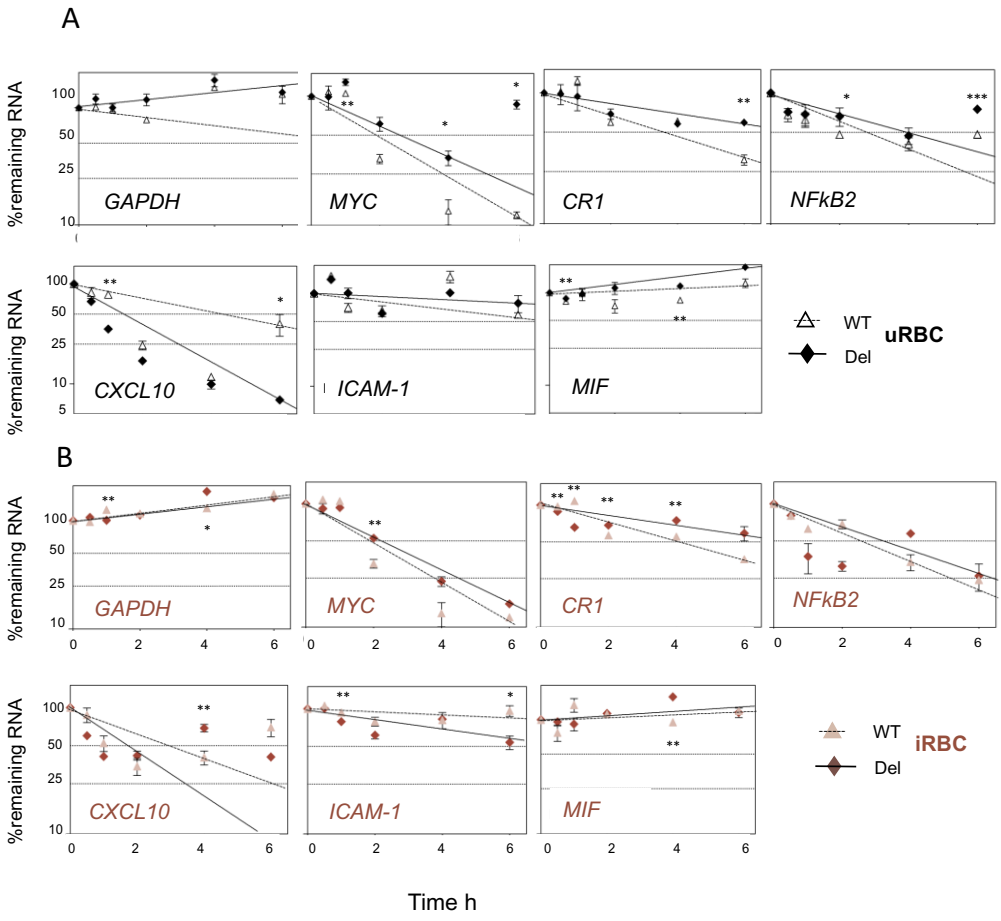

Figure 17. ***BAFF-var and P. falciparum antigens regulate the stability of selected genes.*** *PBMC cells from two different donors expressing normal (BAFF-WT) or higher (BAFF-var) levels of sBAFF were treated for 48 h with lysates from red blood cells uninfected or infected with P. falciparum (uRBCs, iRBCs), and the relative decay rates of target mRNAs were assessed by RT-qPCR analysis after treatment with actinomycin D for the indicated times (0,5, 1, 2, 4, 6). GAPDH mRNA, a stable transcript, was included as negative control, MYC as a positive control and mRNA levels were normalized to 18S rRNA levels. (A) uninfected condition (B) infected condition. Data were plotted on semi-logarithmic scales using Prism. (A,B). In (A,B) data are the means and standard deviation (+SD) from at least three independent experiments. *, P < 0.05; **, P < 0.01, ***, P < 0.005.*

4.9. *P. falciparum* antigens effects on miRNA-15 expression.

BAFF-var mRNA is more actively expressed partly because it lacks a site of repression by microRNA miR-15a, thus miR-15a selectively suppressed BAFF translation [11, 12]. Due to the relevance of this miRNA in regulating BAFF expression, we decided to analyze whether PBMC expressing BAFF-WT or BAFF-var and infected with uRBCs or iRBCs have different expression profiles for miR-15a levels. Cell lysates and supernatant were both analyzed because circulating mature miRNAs are involved in intercellular communication in cell-to-cell signaling.

To this end, the cell lysates and supernatant collected from PBMC (expressing BAFF-WT or BAFF-var) treated for 48h with uRBCs and iRBCs were used to isolate small RNAs to test for miRNA expression. Following RT-qPCR analysis, there were no significant changes in the expression of the miR-15a in any of the treatment conditions suggesting this microRNA is not implicated in the differential regulation of BAFF-WT or -var in response to *Plasmodium* antigens (**Figures 18A and B**).

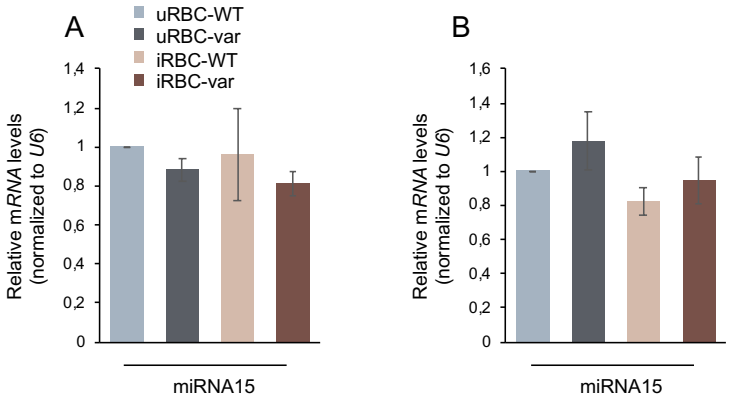

Figure 18. ***miRNA-15 are levels in response to BAFF-var and Plasmodium antigens.*** *After 48h of incubation of PBMC cells with lysates from red blood cells uninfected or infected with P. falciparum (uRBCs, iRBCs), supernatant (A) and cell pellets (B) were collected to isolate RNA. RT-qPCR was performed and relative miRNA expression obtained by normalizing to U6 expression.*

5. Conclusions and future perspectives

A recent study indicated that the overexpression of the cytokine BAFF, driven by a complex insertion–deletion variant (BAFF-Var) in the TNFSF13B gene, contributes to the increased risk of MS and SLE. Population genetic signatures indicated that this autoimmunity variant has been evolutionarily advantageous and it has been hypothesized that this was most likely due to a better fitness under malaria infection [11]. Indeed, the variant has a strikingly high frequency in Sardinia were malaria was endemic until its eradication (1950s) [14]. The malaria-driven selective pressure on BAFF-var was also supported by mice models of malaria, in which BAFF overexpression confers protection against lethal infections of *Plasmodium yoelii* [15].

The aim of this thesis was to test the hypothesis that BAFF-var was positively selected against malaria infection and clarify the mechanisms underlying such alleged protection. We thus used *P. falciparum* lysates to treat TNFSF13B genotype-specific PBMC, to assess if, in response to this exposure, there was a differential enrichment of host sub-populations, proteins and transcripts.

In line with a key role of the memory cells in malaria immunity [137], in this doctoral work I show that the most expressed B and T subpopulation responding to treatment with P. falciparum lysates were memory cells. However, in our *in vitro* setting and in the small sample set used for these functional assays we failed to show differences in B cell subpopulations attributable to BAFF-var (i.e. no significant differences were observed in samples from homozygotes for BAFF-var v/s homozygotes wild-type). This result is in contrast with Steri and colleagues who, in their GWAS in thousands of samples, found that BAFF-var was significantly associated with an increase of memory B cells (switched and unswitched) [11].

On the other hand in our experiments in vitro, levels (as %, here meant as proportion with respect to helper (CD4+) T cells) of PD1+ CXCR5+ Th1 and PD1+ CXCR5+ Th1/Th17 cells and the expression of CXCR5 (MFI) on PD1+ CXCR5+ T cells subpopulations were affected by the presence of BAFF-var but not by *Plasmodium* antigens, while levels of PD1+ CXCR5+ Th2 cells (as %) and the expression of CXCR5 (MFI) both on PD1+CXCR5+ Th1 and PD1+CXCR5+ Th1/Th17 cells showed changes due to both BAFF-var and *Plasmodium* antigens (**Figure 7, 8**).

Early findings showed that antibodies transferred from adults, previously infected with malaria, to acutely infected children could reduce blood-stage parasitemia and disease severity [33]. In the following years several studies point for a key role of immunoglobulins in anti-malaria immunity [94–97].

We thus tested if there were differences in immunoglobulin levels in supernatant of PBMC-WT or BAFF–var treated with uRBCs and iRBCs. Levels of IgG1 and IgG3 increased and decreased respectively in the supernatant in the presence of basal samples (uninfected) from BAFF-var carriers, whereas levels of IgG3 and IgM increased in response to *Plasmodium* lysates in samples of BAFF-var carriers (**Figure 9**). Interestingly, IgG3, along with IgG1, can bind the surface of infected erythrocytes and are the only two IgG subclasses able to mediate opsonization of infected red blood cells increasing protection against *P. falciparum* infection [138]. As a side note, in our in vitro assays, also IgG1 showed a non-significant trend toward an increase in the supernatant of BAFF-var carriers in the presence of plasmodium antigens.

Also the role of cytokines is fundamental for understanding the way in which innate immune response can regulate and modify the malaria outcome [42]. In our experimental conditions, the presence of BAFF-var changed mostly the protein levels of IL8 and IL10 and these effects are especially marked in samples exposed to Plasmodium antigens. At protein level, the fold induction of IL8 was than the changes observed in the mature RNA levels, indicating a significant induction of the translation efficiency due to the BAFF-var. IL8 is an inflammatory cytokine while IL10 is an anti-inflammatory cytokine playing an important role in malaria infection [42]. For all the other cytokines analyzed, the differences in expression were related only to the presence of *Plasmodium* antigens without any significant effect of the BAFF genotype (**Figure 10**).

Interactions between sBAFF and BAFFR activate the non-canonical NFkB signaling pathway. Investigating the *NFkB2* in PBMCs, we found large changes in mRNA v/s pre-mRNA levels that were due to both BAFF-var and *plasmodium* antigens suggesting a transcriptional regulation of this pathway.

To identify genes involved in the response to Malaria antigens we also performed RNA sequencing in B cells purified from PBMC treated with lysates from uRBCs and iRBCs.

Among the top differentially expressed genes, several implicated in immune response to malaria have been identified. The selected genes represented in **Figure 14** are *CXCL10, CR1, MIF, ICAM-1* and *NFKB2*. Interestingly, the same genes are also implicated in neurodegenerative diseases and autoimmune diseases [139–143].

The differential expression of *CXCL10, CR1, MIF, ICAM-1* and *NFkB2* was validated in B cells purified from PBMC treated with uRBC and iRBC lysates. *CXCL10* is a chemokines secreted in response to IFN-γ and plays different roles in monocytes/macrophages chemoattraction [144]. CXCL10 show significant upregulation due to *Plasmodium* antigens with a higher expression at the mRNA compared to the pre-mRNA level. This result suggests a potential increase in the mRNA stability as demonstrated by the RNA half-life assay in **Figure 17**. At the protein level, CXCL10 showed an upregulation due also to BAFF-var suggesting increased translational efficiency. Up regulation of CXCL10 is correlated with increased susceptibility to cerebral malaria in human patients [56].

CR1 is a member of the receptors of the complement activation (RCA) family and is expressed on the surface of phagocytic cells such as macrophages, B cells, neutrophils and follicular dendritic cells and in erythrocytes [59]. We also observed a very significant upregulation of CR1 at the protein level due to the presence of BAFF-var after treatment with both uRBC and iRBC lysates, and modest changes at the RNA level. This suggests a regulation of CR1 at the translational level induced by the presence of BAFF-var (**Figure 15**). Our results are in line with a protective role already demonstrated in literature where up regulation of CR1 increases the internalization of IC by monocyte/macrophages and decrease malaria severity.

MIF*,* macrophage migration inhibitory factor, can down-regulate IFN-γ and up-regulate IL4 responses at the early inflammation stage thereby modulating TH1 responses in malaria infection. However, in our in vitro tests, MIF shows significant changes at the protein level only in relation to BAFF-var in uninfected samples (**Figure 15**).

ICAM-*1* is present in different cell types and after TNFα activation, ICAM-1 allows the attachment of leukocytes to the endothelium and may permit their subsequent transmigration into peripheral tissue [145]. ICAM-1 deficient mice infected by *Plasmodium berghei Anka* (PbA) didn't die in the acute phase of infection suggesting that a reduction of ICAM-1 is correlated with a decrease of malaria severity [69]. In agreement

with these mouse data, in our settings ICAM-1 show a significant down-regulation at the protein level in the samples exposed to malaria lysates in the presence of BAFF-var (**Figures 15C, D**).

NFkB2 pathway plays a key role in B cells proliferation and differentiation after the binding of BAFF with its main receptor (BAFF-R) [126]. A few studies have specifically assessed the role of this NFkB non canonical pathway in malaria infection response. In our experiments, *NFKB2* shows an up regulation in mRNA level compared to pre-mRNA due to BAFF-var and to *P. falciparum* antigens, suggesting a post transcriptional regulation probably related to mRNA stability as suggested by the trend shown in **Figure 17**. At the protein level, p100 and p52*,* the two subunits constituting NFkB2*,* were upregulated in B cells purified from BAFF-var donors. Enhanced p52 expression in response to malaria antigens further suggests regulation in the p52 translation efficiency due to both the presence of BAFF-var and *Plasmodium* antigens. In Raji cells the expression of p100, p52, iKK-a, and TRAF3 were increased in iRBC samples compared to uRBC. TRAF2, in line with pathway activation show a decrease in iRBC samples **(Figure 16**).

In summary BAFF-var drives changes in gene and proteins expression of *CXCL10, CR1, MIF, ICAM-1* and *NFkB2* genes. Most of the regulation is in line with a protection against severe malaria.

Next, we have analyzed the role of miRNA-15 expression, which has been previously shown to be a key regulator of BAFF production [11, 12], also in relation to the exposure of malaria antigens. It was investigated microRNA expression in supernatant and cell lysates of PBMCs (WT / var) treated for 48h with lysates (uRBCs and iRBCs). No changes in gene expression was observed in response to plasmodium infection or related to the presence of BAFF-var (**Figure 18**). Therefore, in our experiments miRNA15 expression does not seem to be significantly affected by BAFF-var or malaria. We can conclude that our experiments, in vitro, show a regulation of T cell sub-populations, immunoglobulin, cytokine production and *CXCL10, CR1, MIF, ICAM-1, NFkB2* genes expression, in response to *Plasmodium falciparum* exposure modulated by the presence of BAFF-var as summarized in **Figure 19**.

We also analyzed different levels of regulation, both transcriptional and post-transcriptional, but additional studies are needed to validate the proper mechanism regulating gene expression.

In our recent study, it was found that NF90 can function as both a positive and a negative regulator of the transcription, mRNA stability, translation, and secretion of key immune factors implicated in the response to malaria antigens. Indeed, NF90 can regulate positively some cytokines (e.g. CCL2, CR1) and negatively other cytokines (e.g. TNF, LILRB1) [114]. This versatility is likely conferred by the ability of NF90 to bind DNA and RNA, and interact functionally with other RBPs and microRNAs. Several studies have analyzed changes in *Plasmodium* gene expression, but more studies are needed to better fully understand the changes in host cells gene expression.

Overall, our data shows that BAFF-var in the presence of *P. falciparum* antigens modulates the expression of key genes affecting the immune response to *P. falciparum* at different regulatory levels. As hypothesized, a key role of BAFF-var in increasing fitness against Malaria infection can be demonstrated by the presented results. The findings can help to identify new check-points of the molecular pathway for new therapeutic interventions.

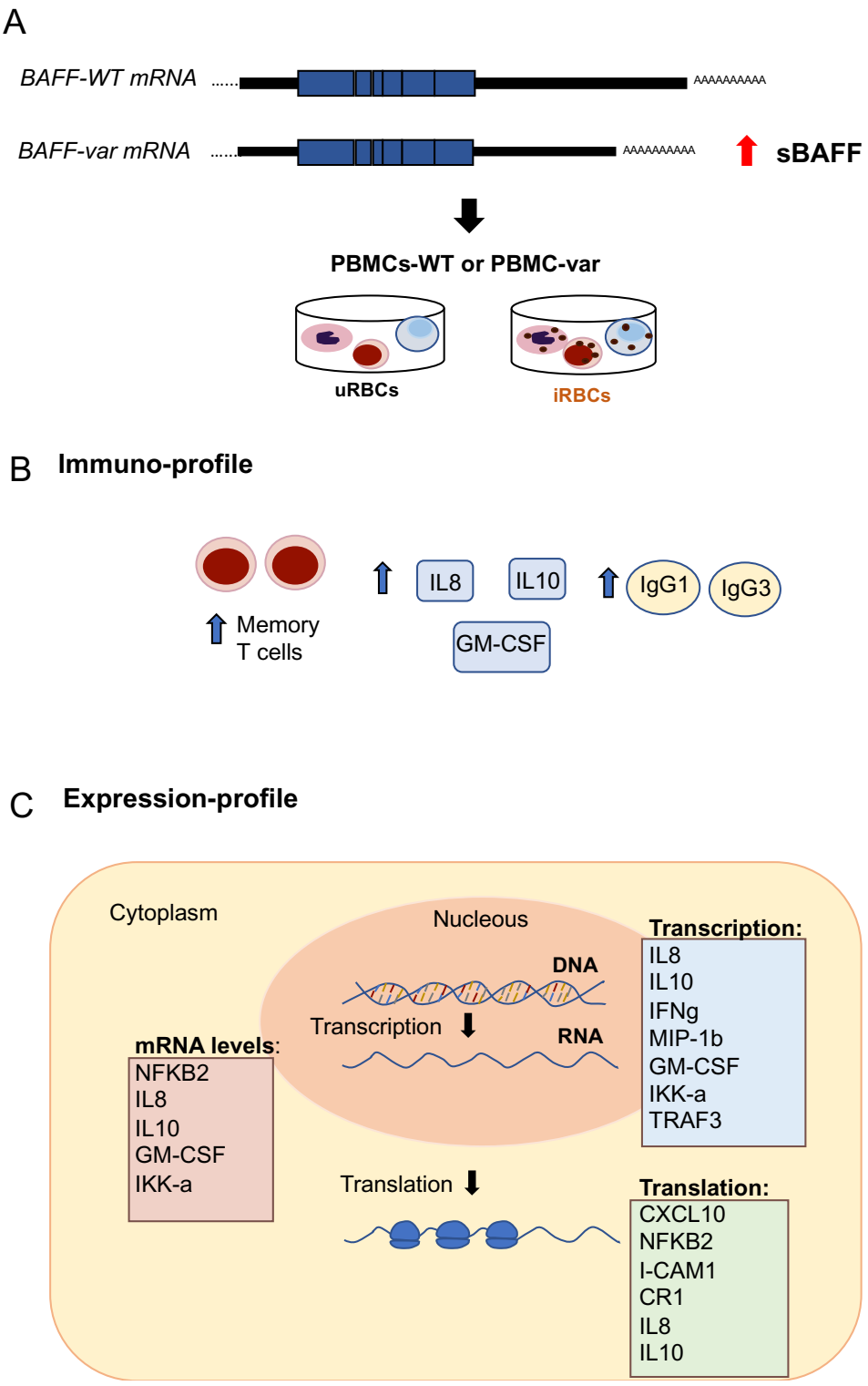

Fig.19 . ***Effects of BAFF-var in response to malaria antigens.*** *(A) Top, schematic of the TNFSF13B gene, polyadenylation site (PA), and alternative polyadenylation site (APA). (B) BAFF-var regulates the expression of subpopulation T cells, cytokine and immunoglobulins. (C) BAFF-var modulate the expression profile of molecules involved in anti-malarial immune response.*

6. Other projects in which I worked during my PhD

6.1. Cooperative translational control of polymorphic BAFF by NF90 and miR-15a

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Nucleic Acids Research, 2018, Vol. 46, No. 22

Abstract

"Polymorphisms in untranslated regions (UTRs) of disease-associated mRNAs can alter protein pro- duction. We recently identified a genetic variant in the 3′UTR of the TNFSF13B gene, encoding the cytokine BAFF (B-cell-activating factor), that generates an alternative polyadenylation site yielding a shorter, more actively translated variant, BAFF-var mRNA. Accordingly, individuals bearing the TNFSF13B variant had higher circulating BAFF and elevated risk of developing autoimmune diseases. Here, we investigated the molecular mechanisms controlling the enhanced translation of BAFF-var mRNA. We identified nuclear factor 90 (NF90, also known as ILF3) as an RNA-binding protein that bound preferentially the wild-type (BAFF-WT mRNA) but not BAFF-var mRNA in human monocytic leukemia THP- 1 cells. NF90 selectively suppressed BAFF translation by recruiting miR-15a to the 3′UTR of BAFF- WT mRNA. Our results uncover a paradigm whereby an autoimmunity-causing BAFF polymorphism pre- vents NF90-mediated recruitment of microRNAs to suppress BAFF translation, raising the levels of disease-associated BAFF".

For this project, I worked mainly on the identification of NF90 binding site in the 3'UTR of BAFF.

For the identification of the NF90 binding site in BAFF transcript we decided to finer mapping the region(s) of interaction of NF90 with *BAFF* 3′UTR. We prepared synthetic biotin-RNA fragment spanning the *BAFF* 3′UTR. After incubation with THP-1 whole cell lysates, pulldown analysis was performed using streptavidin beads followed by NF90 western blot (including the *GAPDH* 3′UTR RNA as a positive control). The result obtained revealed that NF90 associated most avidly with segment 6, a fragment which spanned the alternative polyadenylation site. Following the division of the fragment 6 into two fragments, biotinylated RNA fragment 6a and 6b, fragment 6b was found to show stronger association. Interestingly, this fragment is located immediately downstream of the APA, supporting the notion that NF90 selectively binds the *BAFF-WT* mRNA isoform (**Figure 20**).

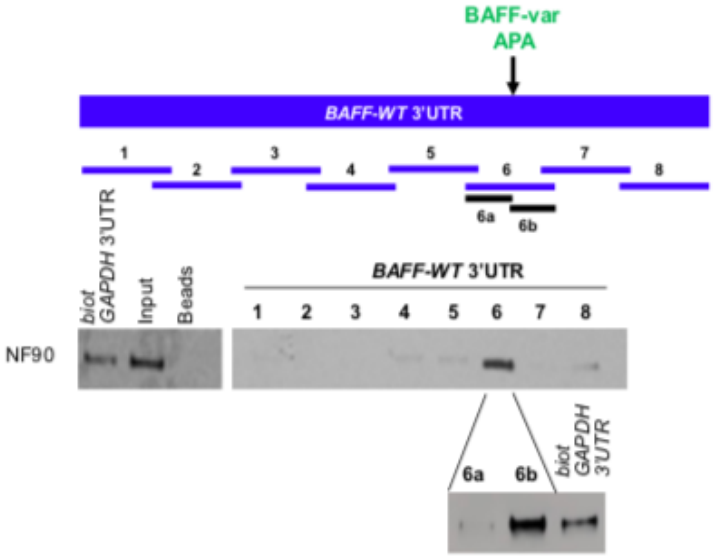

Figure 20. Identification of the region of NF90 binding site on BAFF mRNA.

6.2. NF90 regulation of immune factor expression in response to malaria antigens

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Abstract

"Nuclear factor 90 (NF90) is a dual DNA- and RNA-binding protein expressed ubiquitously in mammalian cells, including monocytes. Here, to elucidate the function of NF90 in the immune response, we analyzed systematically its influence on gene expression programs in the human monocytic cell line THP-1 expressing normal or reduced NF90 levels. RNA sequencing analysis revealed many mRNAs showing differential abundance in NF90-silenced cells, many of them encoding proteins implicated in the response to immune stimuli and malaria infection. The transcription of some of them (e.g. TNF, LILRB1, and CCL2 mRNAs) was modulated by silencing NF90. Ribonucleoprotein immunoprecipitation (RIP) analysis further revealed that a subset of these mRNAs associated directly with NF90. To understand how NF90 influenced globally the immune response to malaria infection, lysates of red blood cells infected with Plasmodium falciparum (iRBC lysates) or uninfected/mock-infected (uRBC lysates) were used to treat THP-1 cells as a surrogate of malaria infection. NF90 affected the stability of a few target mRNAs, but influenced more generally the translation and secretion of the encoded cytokines after treatment with either uRBC or iRBC lysates. Taken together, these results indicate that NF90 contributes to repressing the immune response in cells responding to P. falciparum infection and suggest that NF90 can be a therapeutic target in malaria."

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