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TLRs innate immunereceptors and *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) CIDR1α-driven human polyclonal B-cell activation

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ABSTRACT

Chronic malaria severely affects the immune system and causes polyclonal B-cell activation, as evidenced by the presence of hypergammaglobulinemia, elevated levels of autoantibodies, loss of B-cell memory and the frequent occurrence of Burkitt's lymphomas (BL) in children living in malaria endemic areas.

Previous studies have shown that the cysteine-rich interdomain region 1α (CIDR1α) of the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) of the FCR3S1.2 strain, subsequently named CIDR1α, interacts with B cells partially through the binding to the B-cell receptor (BCR). This interaction leads to an activated phenotype, increased survival, and a low degree of proliferation. CIDR1α preferentially activates the memory B-cell compartment, therefore PfEMP1 is considered to act as a polyclonal B-cell activator and its role in memory maintenance has been suggested.

In this report, we extend the analysis of the PfEMP1–CIDR1α B-cell interaction and demonstrate that PfEMP1–CIDR1α increases the expression of TLR7 and TLR10 mRNA transcripts and sensitizes B cells to TLR9 signalling via the MyD88 adaptor molecule. Furthermore, despite its ability to bind to surface Igs, PfEMP1–CIDR1α-induced B-cell activation does not seem to proceed through the BCR, since it does not induce Lyn and/or phospho-tyrosine mediated signalling pathways. Rather PfEMP1–CIDR1α induces the phosphorylation of downstream kinases, such as ERK1/2, p38 and IKBα, in human B cells. These findings indicate that PfEMP1–CIDR1α induces a persistent activation of B cells, which in turn can contribute to the exhaustion and impairment of B-cell functions during chronic malaria infection.

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1. Introduction

Infection with *Plasmodium falciparum* is still a major health problem worldwide, causing about 225 million new malaria cases each year, according to the WHO malaria report 2010. Malaria severely affects the immune system, in particular the B-cell compartment, as indicated by the presence of hypergammaglobulinemia, elevated autoantibody titres, and the frequent occurrence of Burkitt's lymphoma in children living in malaria holoendemic regions (Abele et al., 1965; Adu et al., 1982; McGregor et al., 1956; Greenwood and Vick, 1975; Banic et al., 1991; Bates and Bedu-Addo, 1997). The mechanisms leading to this B-cell disregulation are not fully understood.

A variety of malarial proteins that might affect B-cell functions are expressed at the surface of the parasitized red-blood cells (pRBCs). Attention has been focussed on the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family, a highly polymorphic and modular family of proteins composed of Duffy binding-like (DBL) and cysteine-rich interdomain regions (CIDR) (Su et al., 1995; Chen et al., 2000; Flick et al., 2001). Previous studies have shown that the CIDR1α of PfEMP1 from the FCR3S1.2 strain binds to CD36, PECAM-1/CD31, and to the Fab- and Fc-fragments of immunoglobulins (Ig) from various classes (IgG, IgM) and different species (Chen et al., 1998; Donati et al., 2004). Furthermore, CIDR1α binds to and directly activates purified human B cells from non immune donors inducing activation, proliferation, increased survival and antibody secretion. These characteristics led to the definition of PfEMP1–CIDR1α as a polyclonal B-cell activator (Donati et al., 2004, 2006).

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At present, little is known about the intracellular mechanisms triggered by the binding of PfEMP1–CIDR1α to B cells. Earlier characterization and comparison of the gene-expression profile induced by PfEMP1–CIDR1α and by anti-Ig activation of human B cells demonstrated a difference in the signatures imposed by these stimuli (Donati et al., 2006). The results suggested that the PfEMP1–CIDR1α-induced activation involves receptors other than Igs or concomitantly through Igs with additional receptors, which would lead to the activation of different signalling pathways (Donati et al., 2006).

The B-cell receptor (BCR) found on mature B cells is a multiprotein complex consisting of an antigen binding subunit, the membrane Ig (mIg), and a signalling subunit. The latter is a disulfide-linked heterodimer comprising the Igα and Igβ proteins, each containing a single immunoreceptor tyrosine-based activation motif (ITAM) within their cytoplasmic tail. Following BCR cross-linking, the *Src*-family protein tyrosine kinase (PTK) Lyn phosphorylates the ITAMs favouring the induction of several kinase cascades, such as mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), IkB kinase (IKK) complex, and phosphatidylinositol 3-kinase (PI3K)/Akt (Campbell, 1999; Dal Porto et al., 2004).

In addition to the specific immune response mediated by BCR engagement, Toll-like receptors (TLRs) are known to play a key role in B-cell interaction with pathogens (Bernasconi et al., 2002; Trinchieri and Sher, 2007). To date ten different TLRs have been identified in humans. TLRs are pattern recognition receptors (PRRs) that recognize a wide range of microbial motifs at the cell surface (TLRs 1, 2, 4, 5, 6 and 10) or within endosomes (TLRs 3, 7, 8 and 9), leading to the activation of innate immune responses (Takeda et al., 2003). All TLRs, except TLR3, use the downstream adaptor molecule myeloid differentiation factor 88 (MyD88), whereas TLR3 signals via the TIR domain-containing adaptor-inducing IFN-γ (TRIF); TLR4 recruits both (MyD88 and TRIF). Upon activation, MyD88 initiates signalling cascades that promote NF-kB and activator protein 1 (AP-1) activation leading to subsequent inflammatory responses (Akira and Takeda, 2004).

TLRs are highly expressed on antigen-presenting cells, such macrophages and dendritic cells (DCs), and promote their recruitment and maturation. Consequently, TLRs indirectly control T- and B-cell responses (Reis e Sousa, 2006; Bourke et al., 2002). On human B cells, TLRs (TLR2, 6, 7, 9 and 10) are expressed almost exclusively in the memory subset (Ruprecht and Lanzavecchia, 2006; Lanzavecchia and Sallusto, 2007). Although their relevance in the formation of Ab responses remains controversial, a role for TLRs in regulating human B-cell immunity is widely accepted (Ruprecht and Lanzavecchia, 2006; Lanzavecchia and Sallusto, 2007; Bernasconi et al., 2003; Poeck et al., 2003; Hornung et al., 2002; Bekeredjian-Ding et al., 2005).

Recent findings in a murine model point to the occurrence of a possible synergy between TLRs and other immune mechanisms in the host responsiveness to different antigens (Trinchieri and Sher, 2007). Noteworthy, the internalization of the BCR–antigen complex induces re-localization of TLR9 from endosomes to the same autophagosome-like compartments as the antigen-bound internalized BCR, which leads to a synergistic signalling through NF-kB and MAPKs phosphorylation (Chaturvedi et al., 2008).

To further understand the mechanisms involved in the PfEMP1–CIDR1α induced B-cell activation we studied the effect of PfEMP1–CIDR1α on TLRs, their expression and TLRs-related signalling pathways. Our results provide evidence that the PfEMP1–CIDR1α-mediated B-cell interaction results in an increased expression of TLR7 and TLR10 mRNA, and an enhanced TLR9-driven signalling via MyD88. The signalling induced by PfEMP1–CIDR1α does not seem to involve the BCR structure, since Lyn and other major BCR-triggered phosphorylation pathways are not activated. On the other hand, PfEMP1–CIDR1α triggers the phosphorylation of two members of the MAPK family: ERK1/2 and p38MAPK, and also of the inhibitor of kBα (IkBα). The results suggest that PfEMP1–CIDR1α-induced B-cell activation is exerted through these downstream kinases and may involve recruitment of nuclear factor-kB (NF-kB).

Taken together, PfEMP1–CIDR1α – induced B-cell activation seems to involve at least two distinct signalling pathways. The results of these multiple stimulation outcomes may play an important role in the aberrant B-cell activation that is characteristic of malaria infections.

2. Materials and methods

2.1. Production of recombinant Ags

PfEMP1–CIDR1α, from the cloned strain FCR3S1.2$_{var1}$, was cloned in the pGEX-4T plasmid (Amersham Biosciences, Uppsala, Sweden) and expressed in *Escherichia coli* (BL21) as previously described (Chen et al., 2000). The PfEMP1–CIDR1α-GST fusion protein, referred to as PfEMP1–CIDR1α, was expressed and purified according to the manufacturer's instructions. GST produced by the empty vector was used as control and is referred to as GST. The purity was determined by SDS-PAGE and Western blot, as described (Chen et al., 1998).

2.2. Cell isolation and cell cultures

Buffy coats from peripheral venous blood of healthy individuals who had not been previously exposed to malaria were obtained from the blood bank of the Karolinska Hospital. Mononuclear cells were isolated by centrifugation over Lymphoprep (Nycomed Pharma, Zurich, Switzerland). CD19$^+$ B cells were purified by positive selection using an AutoMACS sorter (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction. In all the experiments more than 94% of recovered cells were CD19 positive as revealed by FACS analysis.

Purified B cells were resuspended in RPMI 1640 supplemented with 10% foetal calf serum (FCS) (GIBCO, Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/mL of penicillin and 2 mM glutamine, plated into 24-well plates (2×10^6 cells/well) in a final volume of 1 mL and cultured for up to 16 h at 37 °C in 5% CO_2, in either medium alone or medium containing anti-Ig F(ab′)$_2$ (Jackson ImunoResearch Laboratories), anti-human CD40 mAb S2C6 (Mabtech, Stockholm, Sweden), phosphorothioate-backbone modified CpG ODN 2006 (CpG) (Invitrogen), Imiquimod-R837 (Invivogen, San Diego, CA, USA), GST or PfEMP1–CIDR1α at final concentrations of 10 μg/mL, 1 μg/mL, 2.5 μg/mL, 1 μg/mL, 50 μg/mL and 100 μg/mL, respectively.

2.3. Proliferation assays

To assess cellular proliferation, purified B cells were plated into round-bottomed 96-well plates (5×10^4 cells/well) in a final volume of 200 μL. Cultures were seeded in triplicate in the presence of the same concentration of stimulants as above. A mixture of Phorbol myristate acetate (PMA) (5 ng/mL) plus ionomycin (500 ng/mL) (Sigma–Aldrich, Taufkirchen, Germany) and Pansorbin (1:4000) (Calbiochem, San Diego, CA, USA) was used as positive control. To understand the effects of coincubation with both stimuli, 1 μg/mL Imiquimod-R837 (Invivogen) was added to PfEMP1–CIDR1α containing cultures at the initiation of the culture and after 12 h of incubation.

Cultures were incubated for 72 h and pulsed with 1 μCi of [^{3}H]thymidine (Amersham Biosciences) during the last 12 h of the incubation period. Cells were harvested onto a fiberglass filter, and

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