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Targeting Toll-like receptors by chloroquine protects mice from experimental cerebral malaria

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ABSTRACT

Excessive production of proinflammatory cytokines, elicited mostly by Th1 cells, is an important cause of cerebral malaria (CM). Dendritic cells (DCs), a critical link between innate and adaptive immune responses, rely heavily on Toll-like receptor (TLR) signaling. Using C57BL/6 mice infected with *Plasmodium berghei* ANKA (PbA) as an experimental CM model, we first confirmed that inhibition of TLR9 by suppressive oligodeoxynucleotides protected mice from CM. In addition to being a well-known antimalarial, chloroquine (CQ) has been used as an immunomodulator of endocytic TLRs because it inhibits endosomal acidification. We found that immediately before and shortly after infection by PbA, treatment with a single dose of 50 mg/kg of CQ protected mice from experimental CM. Both CQ treatments significantly inhibited expression of TLR9 and MHC-II on DCs, and reduced the number of myeloid and plasmatocytoid DCs at 3 and 5 days after infection. Consequently, activation of $CD4^+$ T cells, especially the expansion of the Th1 subsets, was dramatically inhibited in CQ treated groups, which was accompanied by a remarkable decline in the production of Th1 type proinflammatory mediators IFN-γ, TNF-α, and nitric oxide. Taken together, these results corroborated the involvement of TLR9 in CM pathogenesis and suggest that interference with the activation of this receptor is a promising strategy to prevent deleterious inflammatory response mediating pathogenesis and severity of malaria.

1. Introduction

Malaria is still an important public health problem in many countries of the tropical and subtropical regions of the world. Of the many clinical manifestations of malaria infection, cerebral malaria (CM) is the most severe form, afflicting primarily children aged 2–6 years in sub-Saharan Africa. CM appears to be mediated more by immunopathological host responses to infection than by the parasite *per se* [1,2]. Excessive serum levels of proinflammatory cytokines, particularly interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and nitric oxide (NO) have been implicated in the pathogenesis of CM in rodent and human malaria [3–5]. However, studies from several laboratories also revealed that proinflammatory cytokines such as IFN-γ are critical for successful control and resolution of malaria infection [6]. Whereas a weak proinflammatory response may lead to persistence and replication of parasites, an over-exuberant proinflammatory response may result in immunopathological consequences such as CM. Therefore, an appropriate and effective immune response to malaria infection is essential for the host to control and eliminate the malaria pathogen.

The nature of the immune response is critically dependent on the interplay between the innate and adaptive immune systems. Dendritic cells (DCs) play a central role in this interaction [7,8]. DCs activated in the spleen are able to process and present malaria antigens during infection, and provide a source of cytokines that shape up cell-mediated and humoral immunity by inducing Th1/Th2 differentiation of T cells and antibody production by B cells [9,10]. The ability of DCs to pilot both innate and adaptive immunities is highly dependent

Abbreviations: CM, cerebral malaria; CQ, chloroquine; DCs, dendritic cells; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; mDCs, myeloid dendritic cells; MHC, major histocompatibility complex; ODNs, oligodeoxynucleotides; PbA, *Plasmodium berghei* ANKA; pDCs, plasmacytoid DCs; TLRs, Toll-like receptors.

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on recognizing and signaling in response to microbial ligands that are bound by Toll-like receptors (TLRs), a family of evolutionarily conserved, signal transducing transmembrane proteins that are critical for proinflammatory cytokine production during microbial challenge [11,12]. Since their discovery in the 1990s, TLRs are implicated in the pathogenesis of multiple immune-mediated inflammatory disorders. The role of TLRs in the pathogenesis of malaria has been proposed by different studies. Although some conflicting data exist, there is a growing body of evidence demonstrating that TLRs are central mediators of proinflammatory responses in both rodent and human malaria [13–17]. Among them, TLR9 has been proposed to play an essential role in the pathogenesis of CM by recruiting immune cells into the brain [18,19]. Since excessive release of proinflammatory cytokines is an important component of the pathogenic basis of severe malaria such as CM, interference with TLR9 activation is likely to render a better clinical outcome by preventing aberrant TLR9 activation and excessive release of inflammatory mediators during CM [20].

Chloroquine (CQ) was introduced 60 years ago as an alternative antimalarial to quinine. Although it has now been mostly abandoned for treating falciparum malaria because of widespread resistance [21], CQ remains an important antimalarial drug for treating other malaria species as well as in special patient groups such as children and pregnant women [22]. In addition, CQ has been used in the treatment of diseases associated with increased secretion of proinflammatory cytokines such as lupus erythematosus [23] and protection of host from sepsis-induced mortality [24]. CQ is a well-tolerated immunomodulator and interferes with the functions of endocytic TLRs (TLR3, 7, 8, 9) by inhibiting endosomal acidification, a prerequisite for activation of several TLRs [25]. Therefore, it is likely that during treatment of malaria patients, CQ also modulates host immunity besides direct killing of the malaria parasites. Since CQ is a slow-eliminating drug and lingers in the human body for up to a month, its immunomodulatory property may affect the host immune responses against subsequent malaria infections. To test this possibility, we used the rodent experimental cerebral malaria model — C57BL/6 mice infected with *Plasmodium berghei* ANKA strain (PbA). We show that treatment with CQ before or soon after malaria infection reduced TLR9 expression on DCs and production of proinflammatory cytokines, leading to protection of mice from CM.

2. Materials and methods

2.1. Mice, parasite, and PbA infection

Female 6- to 8-week-old C57BL/6 mice were purchased from Beijing Animal Institute. Infection was initiated by intraperitoneal injection of 1×10^6 PbA parasitized red blood cells per mouse. Mice used in each experiment were matched for age and sex. Parasitemia was monitored by light microscope examination of Giemsa-stained blood smears. Parasitemia was calculated by counting the number of parasite-infected erythrocytes per 1000 erythrocytes. Mortality was checked daily. All experiments were performed in compliance with local animal ethics committee requirements.

2.2. TLR9 inhibitor and CQ treatment

Phosphorothioate-containing oligodeoxynucleotides (ODNs) including the suppressive ODN H154: 5′-CCTCAAGCTTGAGGGG-3′ and control ODN A151: 5′-TTAGGGTTAGGGTTAGGGTTAGGG-3′ were synthesized as described previously [26,27]. Suppressive ODN H154 acts specifically through TLR9, whereas the control ODN A151 does not act through TLR9 [26]. These ODNs were dissolved in physiological saline before use. One day before PbA infection, two groups of mice (H154 and A151) were injected intraperitoneally with H154 and A151, respectively, at 3 mg/kg.

CQ was purchased from Shanghai Zhongxin Pharmaceutical and dissolved in physiological saline before use. C57BL/6 mice were divided into three groups: the control group did not receive CQ treatment, while the pre-3 h and pro-6 h groups received oral administration of 50 mg/kg of CQ at 3 h before and 6 h after PbA infection, respectively.

2.3. Spleen cell cultures

Splenocyte culture was performed as previously described [28]. Briefly, mouse spleens were removed aseptically and pressed through a sterile fine-wire mesh with 10 ml RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 25 mM Hepes, 0.12% gentamicin and 2 mM glutamine. Cell suspensions were collected by centrifugation at 350 *g* for 10 min. Erythrocytes were lysed with cold 0.17 M NH_4Cl and cells were washed twice with fresh medium. The viability of cells was determined by Trypan Blue exclusion and was >90%. The final cell suspension was adjusted to 1×10^7 splenocytes/ml, and aliquots (500 μl/well) of the cell suspensions were incubated in 24-well flat-bottom tissue culture plates in triplicate for 48 h at 37 °C in a humidified 5% CO_2 incubator. Supernatant fractions were collected and stored at −80 °C for cytokine and NO analysis.

2.4. Flow cytometry analysis

To assess the subsets of DCs, spleen cells collected from mice at different times post infection were blocked with anti-CD16/32 (FcγIII/II receptor; Clone 2.4G2; BD Biosciences) and then double stained with FITC-conjugated CD11c mAb (Clone HL3; BD Biosciences), PE-conjugated anti-CD11b (Clone M1/70; BD Biosciences) or PerCP-conjugated anti-CD45R/B220 mAb (Clone RA3-6B2; BD Biosciences). To assess the expression of TLR9 of $CD11c^+$ DCs, after fixation and permeabilization, cells were incubated with biotinylated anti-TLR9 mAb (Clone 5G5; Hycult Biotech), and followed by PE-conjugated streptavidin (BioLegend, San Diego, CA, USA). The cells were then washed twice with PBS containing 1% FCS and suspended in 300 μl of PBS. Data were analyzed in a FACS calibur cytofluorometer using the CellQuest software.

2.5. Quantification of cytokines and NO production

Levels of IFN-γ and TNF-α were measured by commercial enzyme linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). To determine NO production, concentrations of NO_2^- in cell supernatants were measured by the Griess reaction [29].

2.6. Statistical analysis

Statistical significance of the differences was analyzed by *t*-test (SPSS 17.0) and the survival curve was analyzed by the Log-rank Test. A value of $P<0.05$ was considered significant.

3. Results

3.1. Inhibition of TLR9 by ODN prevents mice from experimental CM

The role of TLR9 in mediating CM has motivated interest in this system as a potential therapeutic target. A synthetic compound E446 has been found to specifically inhibit TLR9 activation, thereby preventing experimental CM in mice [20]. Here we used the rodent CM model with PbA-infected C57BL/6 mice to verify whether treatment with a TLR9-suppressive ODN prevents mice from CM. Treatment with the non-inhibitory ODN A151 did not improve the survival of the host, and all mice in the control and the A151 groups died at 8–11 days post infection (dpi) with signs characteristic of CM (Fig. 1A, $P>0.05$). In contrast, mice treated with the TLR9-selective ODN H154 clearly