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Synergistic induction of CXCL10 by interferon-gamma and lymphotoxin-alpha in astrocytes: Possible role in cerebral malaria

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ABSTRACT

Cerebral malaria (CM) has a high mortality rate and incidence of neurological sequelae in survivors. Hypoxia and cytokine expression in the brain are two mechanisms thought to contribute to the pathogenesis of CM. The cytokines interferon (IFN)-γ and lymphotoxin (LT)-α and the chemokine CXCL10 are essential for the development of CM in a mouse model. Furthermore, serum IFN-γ protein levels are higher in human CM than in controls, and CXCL10 is elevated in both serum and cerebrospinal fluid in Ghanaian paediatric CM cases. Astrocytes actively participate in CNS pathologies, becoming activated in response to various stimuli including cytokines. Astrocyte activation also occurs in murine and human CM. We here determined the responsiveness of mouse and human astrocytes to IFN-γ and LT-α, with the aim of further elucidating the role of astrocytes in CM pathogenesis. Initially we confirmed that *Ifn-γ* and *Cxcl10* are expressed in the brain in murine CM, and that the increased *Cxcl10* expression is IFN-γ-dependant. IFN-γ induced CXCL10 production in human and murine astrocytes *in vitro*. The degree of induction was increased synergistically in the presence of LT-α. IFN-γ induced the expression of receptors for LT-α, while LT-α increased the expression of the receptor for IFN-γ, in the astrocytes. This cross-induction may explain the synergistic effect of the two cytokines on CXCL10 production. Expression of these receptors also was upregulated in the brain in murine CM. The results suggest that astrocytes contribute to CM pathogenesis by producing CXCL10 in response to IFN-γ and LT-α.

1. Introduction

Malaria is a devastating infectious disease causing significant mortality and morbidity. Cerebral malaria (CM), a severe complication of malaria, is associated with *Plasmodium falciparum* infection. CM results in a substantial number of fatalities even with anti-malarial drug treatment and can cause permanent neurological damage in a substantial proportion of paediatric CM survivors [1–3]. Although the pathogenesis of CM is not fully understood, central nervous system (CNS) cytokine expression is considered to be a key player, together with hypoxia caused by the sequestration of parasitised red blood cells (pRBC) in brain microvessels [4–6].

Experimental cerebral malaria (ECM) produced by *P. berghei* ANKA (PbA) infection in mice has many similarities to human CM, as reviewed elsewhere [6]. PbA infection in C57BL/6J mice results in ECM 6–7 days post inoculation (p.i.). The use of neutralising antibodies or gene knockout mice with the ECM model has revealed the role of various cytokines in the development of CM. Interferon (IFN)-γ and lymphotoxin (LT)-α are known to be essential for the development of ECM, as mice with the genes for either of the two cytokines silenced are protected from the disease [7–9]. Furthermore, the use of neutralising antibodies against murine IFN-γ resulted in a reduced incidence of ECM and prolonged the survival of otherwise ECM-susceptible mice [10]. The mice treated with the neutralising antibodies had no neurological symptoms

Abbreviations: CM, cerebral malaria; CNS, central nervous system; CXCL10, C-X-C-motif ligand 10; ECM, experimental cerebral malaria; HVEM, herpes virus entry mediator; IFN-γ, interferon-gamma; IFNγR1, interferon-gamma receptor 1; LT-α, lymphotoxin-alpha; LTβR, lymphotoxin-beta receptor; TNFR1, tumour necrosis factor receptor 1; TNFR2, tumour necrosis factor receptor 2; TNFRSF14, tumour necrosis factor receptor superfamily member 14.

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and had markedly reduced sequestration of immune cells within the brain microvessels. Patients with severe falciparum malaria, including CM, were reported to have increased serum IFN-γ levels when compared to controls [11,12], consistent with a role for IFN-γ in the pathogenesis of CM. Furthermore, IFN-γ receptor 1 (IFNγR1) gene promoter polymorphisms have been implicated in susceptibility to human CM [13].

Whereas the role of IFN-γ in CM pathogenesis has been known for decades, the role of LT-α was discovered more recently [8]. LT-α, previously known as tumour necrosis factor β, belongs to the tumour necrosis factor (TNF) superfamily. This group of proteins is involved in mediating a range of inflammatory and immune responses. LT-α has a spectrum of activities from playing a major role in the development of secondary lymph organs to regulating immune responses to certain microbes, as reviewed in [14]. Both TNF and LT-α signal through tumour necrosis factor receptor-1 and 2 (TNFR1 and TNFR2), leading to broadly similar downstream actions. In addition, LT-α can bind to herpes virus entry mediator (HVEM) with low affinity [15]. LT-α is mainly present as a homotrimer, but it can also form heterotrimers with membrane-bound LT-β and then stimulate the LT-β receptor (LTβR) [16]. Signalling through HVEM and LTβR allows LT-α to initiate different functions to those of TNF, as gene knockout mice for TNF and LT-α show different characteristics. Mice deficient in LT-α do not develop secondary lymphoid organs and Peyer's patches and fail to form the general structural organisation of the spleen [17], while mice with the TNF gene knocked out show normal secondary lymphoid organ development, but with impaired splenic structural organisation [18].

Initially it was postulated that TNF was essential for the development of CM as high serum levels of the cytokine were reported in ECM and the use of neutralising antibodies to TNF conferred protection against ECM [19]. However, the presence of high levels of circulating TNF in the absence of CM and the failure of anti-TNF therapy in paediatric CM have questioned the role of TNF in CM pathogenesis [20,21]. Subsequently, it was discovered that LT-α was the essential cytokine in the development of ECM, not TNF [8]. LT-α is believed to signal through both TNFR2 and LTβR in ECM [22], in the latter case being in the form of LT-αβ heterotrimers.

One of the key actions of IFN-γ in ECM is to stimulate the production of C-X-C-motif ligand 10 (CXCL10) [23]. This chemokine plays an essential role in CM by attracting immune cells, such as $CD8^+$ T lymphocytes, to the CNS where these cells damage the blood brain barrier (BBB) [23–27]. Neutralising antibodies to CXCL10 conferred protection against ECM and mice null for CXCR3, the receptor for CXCL10, also were protected [23,25]. Furthermore, CXCL10 is the only biomarker reported to be elevated in both the serum and cerebrospinal fluid of Ghanaian children with CM [28].

It is becoming apparent that astrocytes, the most abundant glial cell type in the CNS, are not just bystander cells, but are actively involved in determining the outcome of CNS diseases. For example, in stroke, astrocytes are thought to be the key determinant of the fate of neurons following cerebral ischemia, possibly related to their roles in glutamate uptake, ion and water homeostasis and purinergic signalling (reviewed in [29]).

Similarly, astrocytes may be playing a significant role in the pathogenesis of CM [30]. It has been proposed that they are a potential convergence point of the two major proposed mechanisms of CM pathogenesis, cytokine actions and cerebral hypoxia [31]. Astrocytes have the capacity to respond to cytokines, resulting in their activation, and astrocyte activation is evident in CM, both human and experimental [32,33]. Astrocytes, as well as brain endothelial cells, are a known source of CXCL10 in ECM [23]. Furthermore, astrocytes can be directly stimulated by IFN-γ and/or TNF *in vitro* to increase the production of CXCL10 [34]. Therefore, we hypothesised that astrocytes would respond to IFN-γ and LT-α with a synergistic, increased expression of CXCL10.

Thus, the aim of the current study was to explore, *in vitro* and *in vivo*, the potential role of astrocytes in CM pathogenesis by investigating their ability to respond to the cytokines IFN-γ and LT-α. Astrocyte activation *in vitro* was measured in terms of the production of another mediator considered requisite for CM, the chemokine CXCL10. We found that astrocytes responded synergistically to IFN-γ and LT-α in their production of CXCL10. This was likely due to the ability of each cytokine to cross-induce the expression of receptors for the other. Enhanced expression of the same receptors was demonstrated in the brains of mice undergoing CM.

2. Materials and methods

2.1. Human primary astrocytes

2.1.1. Cell culture

Human primary foetal astrocytes were isolated and grown according to previously published methods [35], and experiments were carried out according to the guidelines of the Macquarie University and University of Sydney Human Ethics Committees. Complete growth medium for the human primary astrocytes was RPMI 1640 medium (Thermo Scientific Pty. Ltd., Australia) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (Lonza Australia Pty. Ltd.) and 2% (v/v) antibiotic–antimycotic solution (10,000 U/mL penicillin, 10,000 μg/mL streptomycin and 25 μg/mL Fungizone, Life Technologies Pty. Ltd., Australia). Cells were passaged once confluent, approximately once a week, and their primary astrocytic characteristics were maintained in cell culture conditions for up to five passages. Each repeat experiment was performed on a different batch of primary cells to enhance the generalisability, reliability and accuracy of data.

2.1.2. Purity of human primary astrocyte cultures

Microglia are the major potential contaminant cell type in the human primary astrocyte cultures used in this study. Therefore, flow cytometry was used to identify the CD11b-positive microglia in each batch of human primary astrocytes before further experimentation. Mouse microglial cell lines, BV-2 or N11, were used as a positive control for the staining procedure. Cells were stained with CD11b antibody (PE-conjugated anti-mouse CD11b M1/70) diluted in FACS buffer (PBS, 1% (v/v) FBS, 5 mM EDTA and 0.05 (w/v)% sodium azide) for 45–60 min on ice in the dark while the unstained control cells from the same batch were incubated with the same volume of FACS buffer. Only the murine positive controls were blocked with an Fc block prior to the staining step (purified rat anti-mouse CD16/CD32). After the incubation, cells were washed several times and re-suspended in 500 μL of FACS buffer before being passed through a mesh to obtain single cell suspensions. Finally, samples were run on a Cytomics FC500 machine (Beckman Coulter Australia Pty Ltd., Sydney, Australia) and data were analysed using the Flow Jo software. The percentage of microglia in stained samples was compared to the corresponding unstained control samples to find the final percentage of microglial contamination in each batch, which was always less than 2%.

2.2. C8D1A murine astrocyte cell line

Preliminary experiments showed that primary neonatal mouse astrocytes responded to IFN-γ in a similar way to a mouse cell line (C8D1A) with astrocytic properties, in terms of the expression of CXCL10 and other genes regulated by this cytokine (data not shown). We therefore employed C8D1A cells, purchased from