

## REVIEW ARTICLE

The role of chemokines in severe malaria:  
more than meets the eyeLISA J. IOANNIDIS<sup>1</sup>, CATHERINE Q. NIE<sup>2</sup> and DIANA S. HANSEN<sup>1\*</sup><sup>1</sup> *The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3052, Australia*<sup>2</sup> *Burnet Institute, 85 Commercial Road, Melbourne, Victoria 3004, Australia*

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## SUMMARY

*Plasmodium falciparum* malaria is responsible for over 250 million clinical cases every year worldwide. Severe malaria cases might present with a range of disease syndromes including acute respiratory distress, metabolic acidosis, hypoglycaemia, renal failure, anaemia, pulmonary oedema, cerebral malaria (CM) and placental malaria (PM) in pregnant women. Two main determinants of severe malaria have been identified: sequestration of parasitized red blood cells and strong pro-inflammatory responses. Increasing evidence from human studies and malaria infection animal models revealed the presence of host leucocytes at the site of parasite sequestration in brain blood vessels as well as placental tissue in complicated malaria cases. These observations suggested that apart from secreting cytokines, leucocytes might also contribute to disease by migrating to the site of parasite sequestration thereby exacerbating organ-specific inflammation. This evidence attracted substantial interest in identifying trafficking pathways by which inflammatory leucocytes are recruited to target organs during severe malaria syndromes. Chemo-attractant cytokines or chemokines are the key regulators of leucocyte trafficking and their potential contribution to disease has recently received considerable attention. This review summarizes the main findings to date, investigating the role of chemokines in severe malaria and the implication of these responses for the induction of pathogenesis and immunity to infection.

Key words: chemokines, severe malaria, pathogenesis.

## INTRODUCTION

Malaria is one the most serious infectious diseases of humans with over 250 million clinical cases every year worldwide. The infection is transmitted to humans by *Anopheles* mosquitoes that are infected with parasites of the genus *Plasmodium*. Most cases of severe disease are caused by *Plasmodium falciparum*, which is endemic in sub-Saharan Africa and throughout the tropics. The blood stage of the parasite is largely responsible for malaria-associated pathology (Miller *et al.* 2002). The fatalities are associated with a range of various disease syndromes including acute respiratory distress, metabolic acidosis, hypoglycaemia, renal failure, anaemia, pulmonary oedema and cerebral malaria (CM) (White and Ho, 1992). This disease syndrome is the most severe complication resulting from *P. falciparum* infection and accounts for nearly 1 million deaths every year (Murray *et al.* 2012). Children under the age of five are particularly susceptible to this condition, which is characterized by convulsions, seizures and coma. Similar to young

children, pregnant women (particularly primigravid women) are at an increased risk of infection and might develop placental malaria (PM). This serious complication has been found to be associated with adverse pregnancy outcomes including premature labour, intrauterine growth retardation and low birth weight delivery, and is responsible for more than 75 000 infant deaths each year (McGregor, 1984; Steketee *et al.* 2001; Desai *et al.* 2007).

Mature forms of blood-stage malaria express parasitic proteins on the surface of the infected erythrocyte (such as *P. falciparum* Erythrocyte Membrane Protein 1), which allows them to bind to endothelial cells, sequester in vascular beds and avoid clearance in the spleen. Although the precise mechanisms leading to severe malaria syndromes are not completely understood, it is accepted that sequestration of parasitized red blood cells (pRBC) is a major determinant of disease development. Parasite sequestration is thought to induce obstructions in blood flow resulting in hypoxia and haemorrhages (Miller *et al.* 2002) that appear to be associated with the development of organ-specific syndromes such as CM and PM. A large body of literature indicates that in addition to parasite sequestration, inflammatory responses mediated by cytokines such as TNF

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(Molyneux *et al.* 1993), IFN- $\gamma$  and IL-1 $\beta$  (Pongponratn *et al.* 2003) are associated with disease severity in human malaria.

Much useful evidence on the inflammatory mechanisms contributing to the induction of severe malaria has been provided by the *Plasmodium berghei* ANKA model. This rodent infection has many features in common with human disease and is thus the best available model for certain aspects of clinical malaria (Schofield and Grau, 2005; Hansen, 2012). Like in humans, *P. berghei*-ANKA pRBC have been found to accumulate in brains of susceptible mice during infection. Moreover, recent evidence revealed that *P. berghei*-ANKA pRBC adhere to brain microvascular endothelial cells in a VCAM-1-dependent fashion (El-Assaad *et al.* 2013). A large body of work demonstrated that host immune responses elicited during *P. berghei* ANKA infection result in detrimental inflammation and contribute to cerebral disease induction. Host responses mediated by inflammatory cytokines such as TNF (Grau *et al.* 1987), LT- $\alpha$  (Engwerda *et al.* 2002), IFN- $\gamma$  (Grau *et al.* 1989) and effector cells such as CD4<sup>+</sup> (Grau *et al.* 1986; Yanez *et al.* 1996; Villegas-Mendez *et al.* 2012) and CD8<sup>+</sup> T cells (Belnoue *et al.* 2002; Nitcheu *et al.* 2003), NKT cells (Hansen *et al.* 2003) and NK cells (Hansen *et al.* 2007; Ryg-Cornejo *et al.* 2013) have been shown to contribute to severe malaria in this model.

In addition to pRBC, human post-mortem studies revealed the presence of leucocytes and platelets within the brain microvasculature in a substantial proportion of CM cases (Porta *et al.* 1993; Patnaik *et al.* 1994; Grau *et al.* 2003; Hunt and Grau, 2003; Taylor *et al.* 2004). Interestingly, sequestered monocytes and macrophages were found to be more abundant in CM paediatric patients than in severe malarial anaemia (SMA) patients (Coltel *et al.* 2004). Similarly, although the sequestration of pRBC in the placenta appears to be responsible for initiation of pathology, histological evidence revealed the infiltration of various leucocyte populations including monocytes, macrophages, T cells and granulocytes (Ordi *et al.* 2001). An important intervillitis occurs in PM cases with evident sequestration of both pRBC and leucocytes preventing efficient blood flow across the placenta. Moreover, mononuclear intervillous inflammatory infiltration has been found to be associated with low birth weight and preterm delivery (Ordi *et al.* 1998, 2001).

In experimental mouse models, several leucocyte populations including macrophages, neutrophils, T cells, NK cells and platelets have been found in brain blood vessels of CM-affected mice during infection (Grau *et al.* 1987, 1993; Ma *et al.* 1996; Belnoue *et al.* 2002; Nitcheu *et al.* 2003; Hansen *et al.* 2007; Lundie *et al.* 2008). From these populations, CD8<sup>+</sup> T cells comprise a high proportion of the brain-sequestered leucocyte pool of

*P. berghei*-ANKA-infected mice and appear to mediate CM via a perforin-dependent mechanism (Belnoue *et al.* 2002; Nitcheu *et al.* 2003). Moreover, infection of  $\beta_2$ -microglobulin<sup>-/-</sup> mice as well as antibody depletion studies demonstrated that CD8<sup>+</sup> T cells contribute to the induction of experimental cerebral malaria (ECM) (Yanez *et al.* 1996).

The increasing body of evidence illustrating the presence of sequestered host cells together with infected erythrocytes in organs such as the brain or placenta during infection suggested that in addition to secreting cytokines and mediating systemic responses, leucocytes might also contribute to disease by migrating to the site of parasite sequestration, thereby exacerbating organ-specific inflammation. These observations attracted substantial interest in identifying trafficking pathways by which leucocytes are recruited to target organs during severe malaria syndromes and the potential contribution of chemokines to disease. In this review we have summarized the main findings to date investigating the role of chemokines during severe malaria including experimental infection in murine models as well as human field studies. The implications of those findings for the induction of pathogenesis and immunity to malaria are discussed.

#### THE ROLE OF CHEMOKINES IN HOMOEOSTASIS AND INFLAMMATION

Chemoattractant cytokines or chemokines are key regulators of leucocyte trafficking. Chemokine-guided movement allows leucocyte migration to various lymphoid tissues and deployment of immune cells to peripheral sites of pathogen challenge and inflammation. Chemokines are a superfamily of low molecular weight polypeptides of about 8–14 kDa, which bind and signal through seven-transmembrane spanning G-protein coupled receptors (GPCRs). To date, nearly 50 chemokines have been identified (Table 1) as well as numerous chemokine-binding proteins that do not induce cell-signalling events (Murphy, 2002). Different chemokines share a significant degree of sequence homology and are subdivided into CC, CXC, CX<sub>3</sub>C and C subfamilies based on the relative positions of conserved cysteine residues near the N-terminus (Luster, 1998).

Chemokines and chemokine receptors are essential in two distinct processes of leucocyte migration. The first is the directional migration and positioning of leucocytes within lymphoid organs as well as in peripheral tissues. In this context, cells encounter chemoattractant signals in a complex spatial and temporal pattern, in which cells follow a gradient of increasing concentration towards the source of the chemokine (reviewed in Rot and von Andrian (2004)). The second trafficking process mediated by chemokines involves the arrest of migrating leucocytes on the vascular endothelium followed by extravasation

Table 1. Chemokines and chemokine receptors

Cell type	Receptor	Chemokine <sup>a</sup>
Eosinophil Monocyte Activated T cell Dendritic cell	CCR1	<b>MCP-3 (CCL7), MCP-4 (CCL13), MIP-1<math>\alpha</math> (CCL3), RANTES (CCL5), HCC-1 (CCL14)</b>
Basophil Monocyte Activated T cell Dendritic cell NK cell	CCR2	<b>MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13), MCP-5 (CCL12)</b>
Eosinophil Basophil Dendritic cell Activated T cell Resting T cell	CCR3	<b>Eotaxin-1 (CCL11), Eotaxin-2 (CCL24), MCP-3 (CCL7), MCP-4 (CCL13), RANTES (CCL5), PARC (CCL18)</b>
Activated T cell Dendritic cell	CCR4	<b>TARC (CCL17), MDC (CCL22)</b>
Monocyte Activated T cell Dendritic cell NK cell	CCR5	<b>MIP-1<math>\alpha</math> (CCL3), MIP-1<math>\beta</math> (CCL4), RANTES (CCL5), HCC-1 (CCL14)</b>
Dendritic cell	CCR6	<i>MIP-3<math>\alpha</math> (CCL20)</i>
Activated T cell Resting T cell	CCR7	<b><i>MIP-3<math>\beta</math> (CCL19), SLC (CCL21)</i></b>
Monocyte Activated T cell Resting T cell	CCR8	<b>I-309 (CCL1)</b>
Resting T cell Monocyte Dendritic cell	CCR9	<b>TECK (CCL25)</b>
Monocyte activated T cell NK cell	CX <sub>3</sub> CR1	<b>Fractalkine (CX3CL1)</b>
Neutrophil	CXCR1	<b>IL-8 (CXCL8), GCP-2 (CXCL6)</b>
Neutrophil	CXCR2	<b>IL-8 (CXCL8), GCP-2 (CXCL6), GRO-<math>\alpha</math> (CXCL1), GRO-<math>\beta</math> (CXCL2), GRO-<math>\gamma</math> (CXCL3), ENA-78 (CXCL5), NAP-2 (CXCL7)</b>
Activated T cell NK cell	CXCR3	<b>IP-10 (CXCL10), MIG (CXCL9), I-TAC (CXCL11)</b>
Monocyte Resting T cell Dendritic cell	CXCR4	<b>SDF-1 (CXCL12)</b>
NK cell Activated T cell Dendritic cell	XCR1	<i>Lymphotactin (XCL1), SCM-1<math>\beta</math> (XCL2)</i>

<sup>a</sup> Chemokines are identified with both common names and systematic names in parenthesis. Bold font identifies inflammatory/inducible chemokines. Homeostatic chemokines are shown in italic font. Bold and italic font denotes chemokines belonging to both classifications.

from the blood vessel into lymphoid and inflamed tissues. In this process, chemokines are immobilized by sulphated sugars of the glycosaminoglycan (GAG) family on the luminal surface of vascular endothelial cells, which is essential for optimal adhesion of leucocytes to the endothelium (Rot, 1992; Tanaka *et al.* 1993).

Functionally, chemokines can be divided into two broad categories: constitutively expressed chemokines that maintain homeostatic functions, and inducible chemokines that are usually upregulated

in response to infection and/or inflammation (Sallusto *et al.* 2000; Moser *et al.* 2004). This distinction can be somewhat arbitrary as some chemokines fall into both categories depending on the biological context (Table 1). Inflammatory chemokines are produced by several cell types including stromal, endothelial and epithelial cells as well as leucocytes. Constitutive chemokines are produced in the thymus and lymphoid tissues and regulate homeostatic functions of the immune system such as maintaining steady-state leucocyte homing and

Table 2. Association between chemokines and the outcome of human malaria infections

Chemokine	Production during infection	Association	Reference
CXCL8	Increased in the serum and plasma of patients with severe malaria	Serum CXCL8 levels were shown to correlate with parasite density	(Burgmann <i>et al.</i> 1995; Lopera-Messa <i>et al.</i> 2012)
	Increased in the CSF of children with CM	CSF CXCL8 levels were independently predictive of CM mortality	(Armah <i>et al.</i> 2007; John <i>et al.</i> 2008a,b)
	Increased in the placentae of women with PM	Placental CXCL8 levels were associated with placental monocyte infiltration and adverse pregnancy outcomes	(Abrams <i>et al.</i> 2003)
CXCL9	Increased in the plasma of patients with severe malaria	Serum CXCL9 levels were shown to correlate with parasite density	(Ayimba <i>et al.</i> 2011)
	Increased in the placentae and peripheral blood of women with PM	Placental CXCL9 levels were negatively associated with low birth weight	(Muehlenbachs <i>et al.</i> 2007; Dong <i>et al.</i> 2012)
CCL5 (RANTES)	Reduced in the serum of patients with severe malaria	Reduced RANTES levels were associated with suppression of erythropoiesis and thrombocytopenia	(Ochiel <i>et al.</i> 2005; Were <i>et al.</i> 2006)
	Increased mRNA expression in brain samples from CM patients	Not determined	(Sarfo <i>et al.</i> 2004)
CCL4	Increased in the plasma of children with CM	Not determined	(Jain <i>et al.</i> 2008)
	Increased in the CSF of children with CM	CSF CCL4 levels were independently predictive of CM mortality	(Armah <i>et al.</i> 2007)
	Increased in the placentae of women with PM	Placental plasma CCL4 levels were associated with low birth weight	(Abrams <i>et al.</i> 2003; Muehlenbachs <i>et al.</i> 2007)
CXCL10	Increased in the serum and plasma of children with CM	Circulating IP-10 levels were independently associated with CM mortality	(Armah <i>et al.</i> 2007; Jain <i>et al.</i> 2008; Wilson <i>et al.</i> 2011)
	Increased in the CSF of children with CM	Not determined	(Armah <i>et al.</i> 2007)
	Increased in the placentae and peripheral blood of women with PM	Not determined	(Muehlenbachs <i>et al.</i> 2007; Boström <i>et al.</i> 2012)
CCL2	Increased in the placentae and peripheral blood of women with PM	Placental CCL2 levels were associated with placental monocyte infiltration	(Abrams <i>et al.</i> 2003; Dong <i>et al.</i> 2012)
CCL3	Increased in the placentae and peripheral blood of women with PM	Placental and peripheral blood CCL3 levels were associated with placental monocyte infiltration	(Abrams <i>et al.</i> 2003)
CXCL13	Increased in the placentae and peripheral blood of women with PM	Placental CXCL13 levels negatively correlated with low birth weight	(Muehlenbachs <i>et al.</i> 2007; Dong <i>et al.</i> 2012)
CXCL16	Increased in the placentae of women with PM	Not determined	(Muehlenbachs <i>et al.</i> 2007)

cell compartmentalization within lymphoid organs (Yoshie *et al.* 1997). Leucocytes have the capacity to switch the expression of receptors from constitutive to inflammatory chemokines, which allows for different migratory patterns. For example, upon antigen-specific activation and differentiation into effector function, T cells upregulate a repertoire of chemokine receptors and adhesion molecules, which facilitates their exit from secondary lymphoid organs and migration to peripheral inflamed tissues (Xie *et al.* 1999; Weninger *et al.* 2001).

Different effector cell subsets have been shown to express distinct chemokine receptor patterns, allowing them specific migration patterns to exert their function. Polarization of CD4<sup>+</sup> T cells to T<sub>H</sub>1 and T<sub>H</sub>2 subsets is associated with the upregulation of a distinct set of chemokines and their chemokine receptors, which is influenced by the cytokine

milieu during priming (Sallusto *et al.* 1998; Bromley *et al.* 2008). Typically, T<sub>H</sub>1 cells are predominantly characterized by the expression of CXCR3, CCR5 and CXCR6, whereas T<sub>H</sub>2 cells express CCR3, CCR4 and CCR8. However, the association of chemokine receptors with T-helper phenotypes *in vivo* shows a much more complex profile (Annunziato *et al.* 1999; Kim *et al.* 2001), suggesting that CD4<sup>+</sup> T cell subsets cannot always be accurately identified solely on the basis of their chemokine receptor expression. Moreover, increasing evidence suggests that the chemokine receptor repertoire expressed on activated T cells shows a degree of dynamic plasticity, whereby several factors including strength of antigenic signals, tissue-specific imprinting by priming dendritic cells or cytokine milieu during priming can influence the trafficking phenotype of T cells (reviewed in Mora and von Andrian (2006)).

## ASSOCIATION OF CHEMOKINES WITH HUMAN SEVERE MALARIA SYNDROMES

Several inflammatory chemokines have been found to be associated with severe malaria syndromes in case-control studies (Table 2). Increased levels of CXCL8 and CXCL9 as well as reduced levels of CCL5 (also known as RANTES) have been observed in severe malaria patients (Burgmann *et al.* 1995; Ochiel *et al.* 2005; Ayimba *et al.* 2011; Lopera-Mesa *et al.* 2012). Moreover, CXCL8 and CXCL9 levels have been shown to correlate with parasite density (Burgmann *et al.* 1995; Ayimba *et al.* 2011) suggesting that increased parasitic loads might induce these responses. In support of that concept, *in vitro* studies demonstrated that *P. falciparum*-infected erythrocytes are able to induce CCL2, CCL20, CXCL1, CXCL2, CXCL6 and CXCL8 production by human endothelial cells (Viebig *et al.* 2005; Chakravorty *et al.* 2007; Tripathi *et al.* 2009).

Severe malarial anaemia is one of the most common clinical manifestations of disease in children (Marsh *et al.* 1995). Both erythropoietic suppression (Kurtzhals *et al.* 1997) and RBC destruction (Looareesuwan *et al.* 1991) are thought to contribute to the development of SMA. Reduced circulating levels of CCL5 have been found to be associated with SMA in children (Ochiel *et al.* 2005; Were *et al.* 2006). Moreover, reduced CCL5 production was associated with the suppression of erythropoiesis and malaria-induced thrombocytopenia (Were *et al.* 2006), suggesting that CCL5 may be involved in the regulation of the erythropoietic response during malaria infection. It is thought that monocytic-acquisition of *P. falciparum* hemozoin (*pfHz*) may in part contribute to CCL5 suppression during SMA, as children with high levels of intramonocytic *pfHz* have been shown to have lower circulating CCL5 levels than children with only low levels of intramonocytic *pfHz* (Were *et al.* 2009). This is further supported by the observation that CCL5 production in IFN- $\gamma$ -stimulated PBMCs from malaria-infected children decreased with increasing levels of intramonocytic *pfHz* (Were *et al.* 2009).

Although reduced circulating levels of CCL5 have been shown to be associated with CM (John *et al.* 2006), increased transcription of this chemokine has been observed in brain samples from CM patients (Sarfo *et al.* 2004). Circulating levels of CCL4 (also known as MIP-1 $\beta$ ), CXCL10 (also known as IP-10), CXCL4 and CXCL8 have been found to be significantly elevated in CM cases compared with mild malaria cases or healthy control individuals (John *et al.* 2006, 2008b; Armah *et al.* 2007; Jain *et al.* 2008; Wilson *et al.* 2011). Amongst these chemokines, CXCL10 appears to be the most accurate predictor of CM mortality (Jain *et al.* 2008; Wilson

*et al.* 2011). Moreover, cerebrospinal fluid levels of CXCL10, CXCL8 and CCL4 have been found to be significantly higher in children with CM compared with children with SMA and non-malaria-infected individuals (Armah *et al.* 2007; John *et al.* 2008a).

## CHEMOKINES AND HUMAN PLACENTAL MALARIA

Most of the available information to date on the role of chemokines in the pathogenesis of severe malaria in humans has been obtained from PM studies. During this serious pregnancy complication, parasite sequestration appears to induce secretion of pro-inflammatory cytokines and chemokines as well as the recruitment of macrophages and monocytes to the intervillous space (Rogerson *et al.* 2003a,b). Consistent with these observations, increased levels of CCL2 (also known as MCP-1), CCL3 (also known as MIP-1 $\alpha$ ), CCL4, CXCL8, CXCL9, CXCL13 and CXCL16 have been observed in the placentae of women with PM (Abrams *et al.* 2003; Chaisavaneeyakorn *et al.* 2003; Muehlenbachs *et al.* 2007; Dong *et al.* 2012). Reduced levels of CCL5 and increased levels of CXCL10, CXCL9 and CCL2 have also been observed in the peripheral blood of women with PM (Bostrom *et al.* 2012; Dong *et al.* 2012). However, only placental CCL4, CXCL8, CXCL9 and CXCL13 have been associated with adverse pregnancy outcomes such as low birth weight deliveries (Abrams *et al.* 2003; Muehlenbachs *et al.* 2007; Dong *et al.* 2012).

The  $\beta$ -chemokines CCL2, CCL3 and CCL4 are chemo-attractants for monocytes (Rollins, 1997), while CXCL8 is thought to promote monocyte adhesion (Gerszten *et al.* 1999). Thus, production of these chemokines in the placenta during infection may promote monocyte infiltration of the intervillous space. Consistent with this idea, it has been reported that placental CCL2, CCL3 and CXCL8 levels are associated with placental monocyte infiltration (Abrams *et al.* 2003). The intravillous infiltrate primarily consists of monocytes and macrophages. Small numbers of B cells, T cells and granulocytes have also been observed in this infiltrate (Ordi *et al.* 2001). Although it is unclear how these cells migrate to the intervillous space during infection, it is possible that recruitment of B and T cells may be mediated by CXCL13 and CXCL9 respectively.

Both maternal and fetal cells appear to contribute to chemokine production in the placenta during PM. Histopathological analysis of malaria-infected placentae has shown that maternal macrophages and fetal stromal cells contribute to CCL3, CCL4 and CXCL13 production in this organ (Abrams *et al.* 2003; Muehlenbachs *et al.* 2007), while CCL2, CCL3, CCL4 and CXCL10 have been found in

Table 3. Effect of genetic deletion or neutralization of chemokines/chemokine receptors on the outcome of malaria infection in rodent models

Chemokine/ chemokine receptor	Parasite	Mouse strain	Effect on disease	Reference
CCR2	<i>P. berghei</i> -ANKA	CCR2 <sup>-/-</sup>	CCR2 <sup>-/-</sup> mice were fully susceptible to CM	(Belnoue <i>et al.</i> 2003a)
	<i>P. chabaudi adami</i>	CCR2 <sup>-/-</sup>	No effect	(Weidnatz <i>et al.</i> 2010)
	<i>P. chabaudi chabaudi</i> AS	CCR2 <sup>-/-</sup>	Parasite clearance was delayed and monocyte recruitment to the spleen was reduced in CCR2 <sup>-/-</sup> mice	(Sponaas <i>et al.</i> 2009)
CCR5	<i>P. berghei</i> -ANKA	CCR5 <sup>-/-</sup>	CCR5 <sup>-/-</sup> mice were shown to be 80% resistant to CM	(Belnoue <i>et al.</i> 2003b)
	<i>P. berghei</i> -ANKA	CCR5 <sup>-/-</sup>	CCR5 <sup>-/-</sup> mice only showed delayed onset of cerebral disease	(Nitcheu <i>et al.</i> 2003)
CCL3 CCL4 CCL5	<i>P. berghei</i> ANKA/ <i>P. yoelii yoelii</i> 17X clone 1.1		<i>P. berghei</i> ANKA-mediated CM was inhibited by the co-infection <i>P. yoelii yoelii</i> 17X clone 1.1. Protection was associated with reduced CCL3, CCL4 and CCL5 levels in the brain	(Clark and Phillips, 2011)
CXCL9	<i>P. berghei</i> -ANKA	CXCL9 <sup>-/-</sup>	CXCL9 <sup>-/-</sup> mice were partially protected from CM	(Campanella <i>et al.</i> 2008)
CXCL10	<i>P. berghei</i> -ANKA	CXCL10 <sup>-/-</sup>	CXCL10 <sup>-/-</sup> mice had reduced cerebral intravascular inflammation and peripheral parasitaemia, and were protected from CM	(Nie <i>et al.</i> 2009)
	<i>P. berghei</i> -ANKA	C57BL/6	CXCL10 neutralization during infection reduced cerebral intravascular inflammation and peripheral parasitaemia, and protected mice from CM	(Nie <i>et al.</i> 2009)
	<i>P. berghei</i> -ANKA	C57BL/6	Pharmacological inhibition of CXCL10 in combination with anti-malarial therapy protected mice from CM	(Wilson <i>et al.</i> 2013)
CXCL4	<i>P. berghei</i> -ANKA	PAF4 <sup>-/-</sup>	PAF4 <sup>-/-</sup> mice were partially protected from CM and showed reduced T cell infiltration into the brain during infection	(Srivastava <i>et al.</i> 2008)
CXCR4	<i>P. chabaudi</i> CR	C57BL/6	CXCR4 blockade resulted in increased recrudescent parasitaemias	(Garnica <i>et al.</i> 2002)

intervillous blood mononuclear cells isolated from malaria-infected placentae (Suguitan *et al.* 2003). In addition, *in vitro* differentiated syncytiotrophoblasts have been shown to produce CCL3, CCL4 and CXCL8 in response to stimulation with malaria hemozoin (Lucchi *et al.* 2008, 2011), which is highly abundant in the placenta during PM (Galbraith *et al.* 1980).

#### THE ROLE OF CHEMOKINES IN SEVERE DISEASE AND IMMUNITY: LESSONS FROM MOUSE INFECTION MODELS

The initial observations supporting a role for inflammatory chemokines in the aetiology of ECM were provided by gene expression studies. Microarray analysis revealed that the expression of CCR5, CXCR3 and CCR1 and several of their chemokines including CXCL9, CXCL10, CCL2, CCL3 and CCL9 significantly increases in response to infection in CM-affected mice (Sexton *et al.* 2004; Hansen *et al.* 2005). CCL5, CCR1, CCR3 and CCR5 mRNA expression was also detected in brains of Swiss

Webster mice and appeared to contribute to the inflammatory response that results in cellular degradation in the cerebellum during *Plasmodium yoelii* (17XL) infection (Sarfo *et al.* 2005). Further chemokine gene expression studies in brains of ECM-susceptible mice confirmed that CXCL10, CXCL9 and CCL5 and to lesser extent CCL2, CCL7, CCL3, CCL4 and CXCL2 are upregulated during infection with *P. berghei* ANKA (Hanum *et al.* 2003; Campanella *et al.* 2008; Miu *et al.* 2008; Van den Steen *et al.* 2008). Interestingly, the expression of CCL2, CCL5, CCL4, CXCL9 and CXCL10 is induced by either IFN- $\gamma$  or TNF, which is consistent with the important role of these pro-inflammatory cytokines in ECM pathogenesis.

To study the mechanism of leucocyte recruitment to the brain during malaria infection several studies analysed the chemokine receptor usage of brain-sequestered leucocytes from infected mice. CD8<sup>+</sup> T cells isolated from the spleen and brain of malaria-infected mice significantly upregulated CCR5, CCR2, CXCR4 and CXCR3 expression (Nitcheu *et al.* 2003), initially suggesting that trafficking via these chemokine receptors could be involved in the

recruitment of inflammatory cells during severe disease. However, further studies indicated that CCR2 deficient mice (Table 3) are fully susceptible to ECM (Belnoue *et al.* 2003a). Although there has been conflicting evidence on the role of CCR5 in ECM with CCR5<sup>-/-</sup> mice reported to be either 80% resistant to *P. berghei* ANKA-mediated CM (Belnoue *et al.* 2003b) or to display only a delayed onset of cerebral disease symptoms (Nitcheu *et al.* 2003), mixed-*Plasmodium* species infection experiments support a role for this trafficking pathway in CM. *Plasmodium berghei* ANKA-induced CM is inhibited by the co-infection with the non-virulent *P. yoelii yoelii* 17X clone 1.1 (Table 3). Protection was found to be associated with reduced accumulation of CD8<sup>+</sup> T cells in the brain vasculature as well as reduced CCL3, CCL4 and CCL5 levels in the brain (Clark and Phillips, 2011).

Further phenotypic characterization of leucocytes isolated from brain blood vessels of malaria-infected mice by flow cytometry revealed that 80–90% of NK cells and T cells expressed CXCR3, indicating that the expression of this chemokine receptor is strongly associated with lymphocyte trafficking during CM (Hansen *et al.* 2007). Moreover, *in vitro* chemotaxis assays revealed that whereas T cells from naive mice were unable to migrate in response to the CXCR3-ligand CXCL10, T cells from *P. berghei* ANKA-infected mice showed a 3-fold increase in their CXCL10-mediated chemotaxis (Hansen *et al.* 2007), indicating that during malaria, T cells acquire the capacity to migrate in response to this chemokine. This appears to be a feature specific of CM-susceptible mouse strains, as T cells from malaria-infected CM-resistant BALB/c animals were found to be unable to respond to CXCL10 chemotactic stimulus (Van den Steen *et al.* 2008). Together, this evidence supports a role for CXCR3-mediated trafficking in the migration of pathogenic T cells to the brain during ECM. In agreement, it has been found that 70–80% of CXCR3<sup>-/-</sup> mice are resistant to *P. berghei*-mediated CM (Campanella *et al.* 2008; Miu *et al.* 2008).

The relative involvement of the 3 CXCR3 chemokines (CXCL9, CXCL10 and CXCL11) in the recruitment of inflammatory leucocytes to the brain of malaria-infected mice has been extensively investigated (Table 3). As CXCL11 is not normally expressed in CM-susceptible C57BL/6 mice (usually used in ECM experiments) a role for this chemokine in this model is not expected. CXCL9<sup>-/-</sup> mice were found to be partially protected from *P. berghei*-ANKA-mediated CM (Campanella *et al.* 2008). However, whether the increased survival rates to malaria infection in the absence of this chemokine result from reduced leucocyte recruitment to the brain and/or a differential induction of immune response to infection has not been examined.

The role of the CXCR3 chemokine CXCL10 has been studied in more detail. CXCL10<sup>-/-</sup> mice or mice receiving anti-CXCL10 neutralizing antibodies over the course of *P. berghei*-ANKA infection were found to have reduced cerebral intravascular inflammation and were protected against fatality (Nie *et al.* 2009). Moreover, pharmacological inhibition of CXCL10 together with anti-malarial therapy also protects infected animals from CM-mediated mortality (Wilson *et al.* 2013). Interestingly, CXCL10 blockade during infection resulted in significantly reduced peripheral parasitaemia, suggesting that the absence of this chemokine has a beneficial effect for the development of parasite-specific responses involved in the control of parasite replication (Nie *et al.* 2009). Since *Plasmodium* spp. are blood-borne parasites, the spleen constitutes a key site in the initiation of immune responses and control of parasite replication (Looareesuwan *et al.* 1993; Sayles *et al.* 1993; Yap and Stevenson, 1994; Chotivanich *et al.* 2002). Moreover, it has been proposed that the spleen is the site of initial induction of inflammatory cells that migrate to the site of parasite sequestration in target organs (Renia *et al.* 2006). Consistent with that concept, the increased resistance to infection observed in the absence of CXCL10-mediated cell trafficking was found to be associated with a preferential accumulation and subsequent expansion of parasite-specific CD4<sup>+</sup> T cells in spleens of infected animals (Nie *et al.* 2009). Overall these findings are consistent with two concepts: (1) trafficking pathways might control the balance between pathogenic organ-specific inflammation and spleen-mediated protective immunity and (2) inflammatory processes that occur during infection are not only detrimental for their involvement in severe disease but can also compromise the induction of anti-parasite immunity by inducing leucocyte migration away from the spleen.

The cellular sources responsible for the secretion of CXCR3 chemokines during ECM have not been extensively investigated, with only a few studies focusing primarily on brain tissue. Gene expression analysis (Miu *et al.* 2008) as well as immunohistochemistry approaches (Campanella *et al.* 2008) revealed that CXCL9 is predominantly expressed by endothelial cells and surrounding microglial cells. There is conflicting evidence on the cellular sources of CXCL10 during infection. Whereas CXCL10 RNA was found to be upregulated in brain endothelial cells and astrocytes clustering around IP-10 positive microvessels (Miu *et al.* 2008), immunohistochemistry studies showed CXCL10 staining mainly in neurons throughout the brain parenchyma and only occasionally on endothelial cells, but not on astrocytes (Campanella *et al.* 2008). Whereas the endothelial and glial localization of CXCL9 and CXCL10 producing cells is consistent with a role for these chemokines in the recruitment of inflammatory leucocytes to the site of parasite sequestration

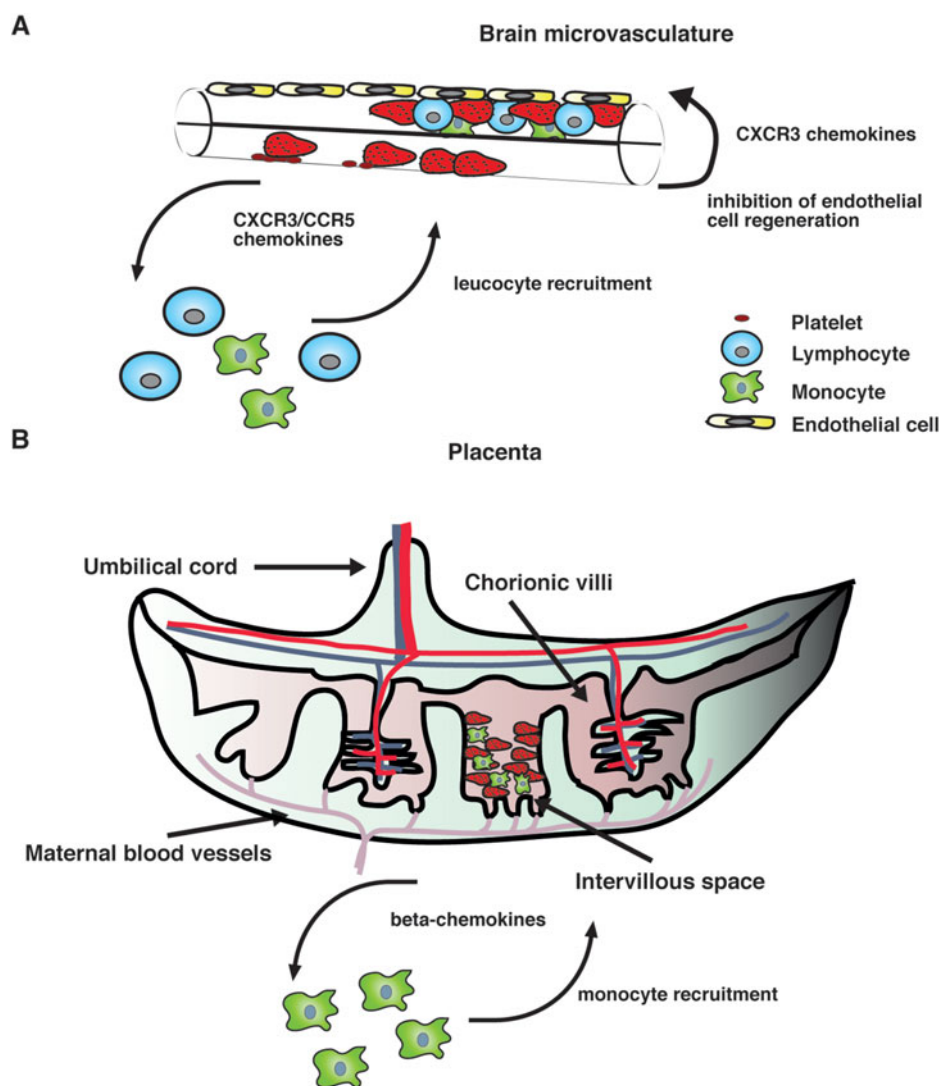


Fig. 1. A hypothetical model of action of chemokines in human severe malaria syndromes. (A) After binding to the brain microvasculature, sequestered pRBC induce activation of vascular endothelial cells, which results in the release of inflammatory cytokines as well as CXCR3 and CCR5 binding chemokines. It is possible that local production of these chemokines stimulates the accumulation of CXCR3<sup>+</sup> and CCR5<sup>+</sup> leucocytes. In addition, some CXCR3 chemokines such as CXCL10 that have angiostatic activity could inhibit endothelial cell regeneration of the brain microvasculature, thereby compromising the integrity of the blood–brain barrier. (B) In the placenta, both maternal and fetal cells might contribute to the production of  $\beta$ -chemokines in response to infection. These mediators stimulate the recruitment of monocytes and macrophages to the intervillous space, which appears to be associated with adverse pregnancy outcomes.

(intravascular infiltration without extravasation into the brain parenchyma), further research is required to determine the effect that CXCL10 upregulation might have in neuronal tissue and the effector signals responsible for this response during infection.

In addition to CXCL9 and CXCL10, the platelet-derived chemokine, platelet factor 4 (PF4 or CXCL4), which binds CXCR3B, a spliced variant of the chemokine receptor CXCR3, was found to be induced in the brain during *P. berghei*-ANKA infection (Srivastava *et al.* 2008). Furthermore, PF4<sup>-/-</sup> mice showed reduced ECM mortality and T cell infiltration into the brain during infection (Srivastava *et al.* 2008). Thus CXCL4 might contribute to the recruitment of T cells to the brain in ECM

pathogenesis. In addition, CXCL4 was also found to enhance monocyte activation in response to *P. berghei*-ANKA infection (Srivastava *et al.* 2010). The precise contribution of this response to disease severity requires further investigation.

Chemokines are not only mediators of cell trafficking during inflammation but are also involved in the recruitment of immune cells to secondary lymphoid organs whereby they enhance the development of protective responses to infection. A few studies have addressed the role that chemokines play in the control of parasite densities using rodent malaria infections. CCR2 is a receptor involved in the recruitment of various cells including activated T cells, NK cells, dendritic cells and monocytes. Whereas genetic



deficiency of this chemokine does not appear to affect parasite growth during *P. berghei*-ANKA (Belnoue *et al.* 2003a) or *Plasmodium chabaudi adami* infection (Weidanz *et al.* 2010), parasite clearance was found to be delayed in CCR2<sup>-/-</sup> mice infected with *Plasmodium chabaudi chabaudi* AS (Sponaas *et al.* 2009). CCR2 is involved in the recruitment of bone marrow-derived inflammatory monocytes in response to infection (Serbina *et al.* 2008). The reduced control of parasitaemia observed in CCR2<sup>-/-</sup> mice correlated with significantly lower numbers of inflammatory monocytes in the spleen of these animals, suggesting these cells play a role in controlling blood-stage malaria. Monocytes as well as dendritic cells are also capable of migrating in response to CXCL12, which recognizes the CXCR4 receptor. CXCR4 blockade during *Plasmodium chabaudi* CR infection has also been shown to result in increased recrudescence parasitaemias (Garnica *et al.* 2002), however the precise mechanism by which this trafficking pathway modulates parasite density in this model requires further investigation.

#### CONCLUDING REMARKS

Over the past few decades, most of the work on soluble factors associated with the development of severe malaria has mainly focused on the analysis of cytokine responses both in human studies and rodent infection models. The role of chemokines in severe disease induction has only recently started to become appreciated and receive more attention. Cytokines are primarily secreted by immune cells and their main function is modulation of immune responses. Unlike cytokines, chemokines are secreted by a broad range of cellular sources in lymphoid organs and/or inflamed tissue and they appear to control a range of diverse processes including leucocyte trafficking, angiogenesis and tissue remodelling. It is then possible that the cellular sources of chemokines produced in response to malaria differ in inflamed tissue and lymphoid organs and that they control different processes. Further research is needed to fully appreciate the contribution of these chemokine-mediated processes to severe malaria and the precise mechanisms by which inflammatory chemokines exacerbate disease.

Our current knowledge on human PM supports the idea that the intravillous infiltrate that accompanies parasite sequestration mainly consists of monocytes and macrophages. Consistent with this observation, elevated levels of monocyte-attracting  $\beta$ -chemokines such as CCL2 and CCL3 have been found in infected placentae and their production appears to be associated with cellular infiltration (Abrams *et al.* 2003), strongly suggesting that chemokine secretion in the placenta during infection recruits monocytes to the intervillous space (Fig. 1).

On the other hand, mainly CXCR3 and CCR5 binding chemokines have been found to be associated with CM severity in humans (Armah *et al.* 2007). Although the precise mechanism by which these chemokines contribute to human disease remains unclear, it is possible that their local production in the brain during infection might stimulate the accumulation of CXCR3<sup>+</sup> and CCR5<sup>+</sup> leucocytes (Fig. 1). Consistent with this hypothesis, increased CXCR3 and CCR5 mRNA levels have been observed in brain samples from children that have succumbed to *P. falciparum*-mediated CM (Sarfo *et al.* 2004). In addition to their chemotactic function, some inflammatory chemokines such as CXCL10 have been shown to exert angiostatic activity (Angiolillo *et al.* 1995; Luster *et al.* 1995; Romagnani *et al.* 2001). It is then possible that locally produced CXCL10 may inhibit endothelial cell regeneration of the brain microvasculature, thereby compromising the integrity of the blood–brain barrier (Fig. 1). In support of this view, the angiogenic factor VEGF has been found to be protective against CM-associated mortality (Armah *et al.* 2007; Jain *et al.* 2008).

Although rodent malaria infection models are not perfect, CXCR3 and CCR5 chemokines (such as CXCL10 and CCL4) that were identified as biomarkers of CM in humans (Armah *et al.* 2007) have also been found to participate in ECM induction in mice (Campanella *et al.* 2008; Nie *et al.* 2009). Thus animal models appear to provide a fast and cost-effective resource to investigate chemokine-mediated mechanisms of disease and for assessment of potential therapeutic interventions (Nie *et al.* 2009; Wilson *et al.* 2013). Moreover, good mouse models of SMA and PM have been developed (Evans *et al.* 2006; Neres *et al.* 2008; Rodrigues-Duarte *et al.* 2012) and are now available to explore the involvement of chemokines in these disease syndromes.

Current views in the field propose that the same inflammatory responses that contribute to severe malaria might be also involved in the control of parasitaemia. This concept has discouraged the use of anti-cytokine treatments (van Hensbroek *et al.* 1996) as adjunctive therapy for complicated malaria cases as it might reduce inflammation but also result in immunosuppression, which could be detrimental for the control of infection. Anti-chemokine therapies (Nie *et al.* 2009) are emerging as potential safe therapeutic alternatives to improve outcomes of severe malaria cases during treatment with anti-malarial drugs, as they alleviate organ-specific inflammation without inducing a generalized immunosuppression of the host.

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