The role of chemokines and their receptors during protist parasite infections

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SUMMARY

Protists are a diverse collection of eukaryotic organisms that account for a significant global infection burden. Often, the immune responses mounted against these parasites cause excessive inflammation and therefore pathology in the host. Elucidating the mechanisms of both protective and harmful immune responses is complex, and often relies of the use of animal models. In any immune response, leucocyte trafficking to the site of infection, or inflammation, is paramount, and this involves the production of chemokines, small chemotactic cytokines of approximately 8–10 kDa in size, which bind to specific chemokine receptors to induce leucocyte movement. Herein, the scientific literature investigating the role of chemokines in the propagation of immune responses against key protist infections will be reviewed, focussing on *Plasmodium* species, *Toxoplasma gondii, Leishmania* species and *Cryptosporidium* species. Interestingly, many studies find that chemokines can in fact, promote parasite survival in the host, by drawing in leucocytes for spread and further replication. Recent developments in drug targeting against chemokine receptors highlights the need for further understanding of the role played by these proteins and their receptors in many different diseases.

Key words: Chemokine, chemokine receptor, protist, malaria, toxoplasmosis, leishmaniasis, cryptosporidiosis.

INTRODUCTION

Parasitic diseases are a global problem for both humans and livestock, causing significant rates of morbidity and mortality, as well as huge economic losses, the full extent of which is often difficult to accurately define. A complete understanding of the nuances of host–pathogen interactions is vital to the process of identifying potential drug or vaccine targets.

The pathology and life cycle of many protist parasitic infections, including malaria and toxoplasmosis is dependent upon the host initiation of an effective innate and adaptive immune response (Esche *et al.* 2005). The trafficking of host leucocytes to the site of infection is highly dependent upon the coordinated and specific production of proteins by the host cells. These small proteins, called chemokines, orchestrate the movement of immune cells, and propagate the inflammatory response, therefore playing crucial roles in the defence against parasites. In addition to this, many parasites can manipulate the immune response to its own benefit, through using host leucocytes for replication and further spread (McGovern and Wilson, 2013).

In this review, the role that chemokines play in the delicate interaction between host and protist parasites will be explored. Of particular interest are the causative parasites of malaria, toxoplasmosis, leishmaniasis and cryptosporidiosis, all diseases which are of significant relevance to human health.

WHAT ARE CHEMOKINES?

Chemokines are a complex superfamily of small extracellular signalling proteins (~8–10 kDa), expressed by nucleated cells (Graves and Jiang, 1995; McColl, 2002; Niu *et al.* 2011). Members of the chemokine superfamily contain 20–90% sequence homology (Rostene *et al.* 2007) and are divided into four main subfamilies, determined by the arrangement of conserved cysteine residues within the chemokine structure as illustrated within Fig. 1. Chemokine-dependant migration is critical for initiating an effective inflammatory immune response, with chemokine production induced in response to pro-inflammatory cytokines during infection (Graves and Jiang, 1995; Wong and Fish, 2003). While chemokine production is strongly associated with inflammation and leucocyte recruitment, their role extends to various homeostatic and developmental functions, which are achieved through their constitutive production (Sallusto *et al.* 1999; Raz and Mahabaleshwar, 2009). In addition, some chemokines mainly the CC chemokines CCL20 and CCL28 and the CXC chemokines CXCL6, 9, 10, 11 and 14 are not known to exhibit antimicrobial activity against many Gram-positive and -negative bacteria as well as some fungi as reviewed by Yung and Murphy (2012).

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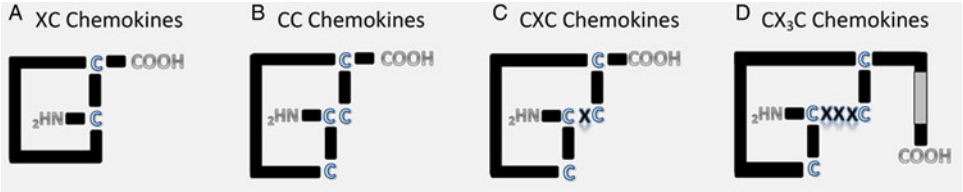

Fig. 1. **Chemokine superfamily classification.** Chemokines are classified based on the arrangement of conserved cysteine motifs (shown in hollow lettering below) in the amino terminus. (A) XC chemokines only contains two conserved cysteine residues. There are only two members of this family in humans (XCL1, XCL2), with both binding the XCR1 receptor. (B) The CC chemokines are defined by having two of the first four conserved cysteine residues adjacent to each other. There are at least 28 known members of this group in humans. (C) The CXC chemokines are so called due to the presence of an amino acid separating the first two of the conserved cysteines. In humans, 17 CXC chemokines have been described. (D) The CX_3C chemokine group contains one member, CX_3CL1, which binds to the CX_3CR1 receptor, and is defined by the presence of three amino acids separating the first two cysteine residues, as well as a mucin-like domain (indicated by grey band).

Chemokines exert their effect on cells through binding chemokine receptors. Chemokine receptors can be described as being either G protein-coupled receptors (GPCR) or atypical chemokine receptors (ACKRs) (Bachelerie *et al*. 2014). These two types differ mainly through the presence of the conserved DRYLAIV motif at the end of transmembrane domain 3 in classical chemokine receptors, but not in ACKRs (Ulvmar *et al*. 2011). There are four types of typical, or classical, chemokine receptors: CCR, XCR, CXCR and CX_3CR, with their classification being based on the type of chemokine ligands with which they interact (Rajagopalan and Rajarathnam, 2006). Chemokine and chemokine receptor interactions are highly complex, exhibiting a great deal of cross-binding and redundancy. These relationships, and the differences between human and mouse chemokine families have previously been reviewed and summarized (Zlotnik and Yoshie, 2012).

In addition to these 'classical' chemokine receptors, ACKRs have also been identified and well characterized (Nibbs and Graham, 2013). These are structurally similar to the typical GPCRs, but lack the ability to couple G-proteins (Ulvmar *et al*. 2011). To date, four ACKRs have been described and these have significant effects on immune regulation (Nibbs and Graham, 2013). ACKRs bind multiple chemokines, as shown in Table 1, with ACKR2, 3 and 4 all known to have the ability to act as a chemokine scavenger through internalizing and destroying ligands (Luker *et al*. 2010; Comerford and McColl, 2011; Hoffmann *et al*. 2012; Nibbs and Graham, 2013; Watts *et al*. 2013). While ACKR1 can also internalize ligands, it does not destroy them, but aids the process of transcytosis (Rot, 2005; Zhao *et al*. 2011; Nibbs and Graham, 2013).

Table 1. Atypical chemokine receptors (ACKRs) and known ligands in humans

ACKR	Former name(s)	Ligands
ACKR1	DARC	CCL2, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL17 CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, CXCL11
ACKR2	D6, CCBP2	CCL2, 3, 4, 5, 7, 8, 11, 13, 14, 17, 22, 3L1
ACKR3	CXCR7, RDC1	CXCL11, CXCL12
ACKR4	CCRL1, CCXCKR, CCR11	CCL19, CCL21, CCL25, CXCL13

ROLE OF CHEMOKINES IN HOST–PROTIST INTERACTIONS

The generation of chemokine and chemokine receptor knockout mice has been critical for our understanding of the role of individual ligands and receptors in the development and pathology of a number of diseases and infections (Rothenberg, 2000). These techniques have allowed for both the *in vitro* and *in vivo* monitoring of the host response to parasitic infection. Alteration of the chemokine system often demonstrates its critical role through increases in parasite load, exacerbated symptoms, increased mortality, and decreased immune cell migration. Table 2 demonstrated the breadth of studies utilizing chemokine and chemokine receptor knockout mice to better understand their role in the control and pathogenesis of various protist parasite infections.

While it is recognized that chemokine production by infected host and bystander cells serve to facilitate a protective immune response, it has also been found that many infections, including the protists detailed herein, can facilitate their own spread throughout the body by inducing chemokine production, which will draw in leucocytes to the site of infection, thereby providing additional cells to be infected. This concept of using host cell to spread is known as the Trojan horse hypothesis and has been shown for *Plasmodium* spp. (Wykes and Horne-Debets,

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Table 2. Chemokine/chemokine receptor knockout mice used in parasite studies

Parasite	Knockout model	Reference
Cryptosporidium parvum	CCR2$^{-/-}$	de Sablet *et al*. (2016)
	CCR5$^{-/-}$	Campbell *et al*. (2002)
		Lacroix-Lamandé *et al*. (2008)
	CXCR3$^{-/-}$	Lantier *et al*. (2013)
Leishmania donovani	CCL3$^{-/-}$	Sato *et al*. (1999)
	CCR2$^{-/-}$	Sato *et al*. (1999)
	CCR5$^{-/-}$	Sato *et al*. (1999)
Leishmania major	CCR1$^{-/-}$	Rodriguez-Sosa *et al*. (2003)
	CCR2$^{-/-}$	Sato *et al*. (2000)
Plasmodium spp.	CCL2$^{-/-}$	Pattaradilokrat *et al*. (2014)
	CXCL4$^{-/-}$	Srivastava *et al*. (2008)
	CXCL9$^{-/-}$	Campanella *et al*. (2008)
	CXCL10$^{-/-}$	Campanella *et al*. (2008)
		Nie *et al*. (2009)
	CCR2$^{-/-}$	Belnoue *et al*. (2003*a*)
		Sponaas *et al*. (2009)
		Weidanz *et al*. (2010)
	CCR5$^{-/-}$	Belnoue *et al*. (2003*b*)
		Nitcheu *et al*. (2003)
	CXCR3$^{-/-}$	Srivastava *et al*. (2008)
		Campanella *et al*. (2008)
Toxoplasma gondii	CCR1$^{-/-}$	Khan *et al*. (2001)
		Schulthess *et al*. (2012)
	CCR2$^{-/-}$	Benevides *et al*. (2008)
		Dunay *et al*. (2008)
		Egan *et al*. (2009)
		Dunay *et al*. (2010)
	CCR5$^{-/-}$	Luangsay *et al*. (2003)
		Khan *et al*. (2006)
		Ibrahim *et al*. (2014)
	CCR7$^{-/-}$	Noor *et al*. (2010)
	CXCR2$^{-/-}$	Del Rio *et al*. (2001)
	CXCR3$^{-/-}$	Cohen *et al*. (2013)

2012), *Toxoplasma gondii* (Da Gama *et al*. 2004; Lambert *et al*. 2009; Elsheikha and Khan, 2010; Sanecka and Frickel, 2012; Coombes *et al*. 2013) and *Leishmania* spp. (Laskay *et al*. 2003; van Zandbergen *et al*. 2004; Shapira and Zinoviev, 2011) as discussed.

Malaria

Plasmodium is a genus of intracellular apicomplexan protists, which are the causative agent of malaria. While over 100 species of *Plasmodium* have been identified (Tuteja, 2007), only five of these cause malaria in humans. These species are *Plasmodium falciparum, Plasmodium ovale, Plasmodium malariae, Plasmodium knowlesi* and *Plasmodium vivax* (Kantele and Jokiranta, 2011); with *P. falciparum* infection strongly associated with higher disease severity and mortality.

The life cycle of *Plasmodium* spp. is complex, with infection acquired by humans through sporozoite transmission from infected female *Anopheles* mosquitoes during feeding. Sporozoites migrate to and replicate within hepatocytes for ~7–10 days, resulting in merozoite release into the bloodstream (Tuteja, 2007). The importance of hepatocytes to the *Plasmodium* life cycle is still not completely elucidated; however, a recent review by Kaushansky and Kappe (2015) summarizes key molecular mechanisms thought to support the specific infection of hepatocytes, and the environment provided by hepatocytes, which permits the parasite to transform into the merozoite stage (Kaushansky and Kappe, 2015). Once released, merozoites infect and asexually replicate within red blood cells, with some merozoites undergoing sexual differentiation (Tuteja, 2007). These gametocytes are then transmitted to mosquitoes during feeding, with fertilization occurring within the mosquito's gut. The discovery that the blood stage of *Plasmodium* spp. can infect cells other than erythrocytes, namely plasmacytoid dendritic cells (DCs) (Wykes *et al*. 2011; Wykes and Horne-Debets, 2012), has led researchers to believe that this parasite can adopt the Trojan-horse style of survival and dissemination.

The host immune response to the malarial parasite is a key factor in the development and outcome of the infection. Indeed, as with many infections, the host immune response can control parasitaemia; however, excessive inflammation often leads to

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infection-associated pathology. Few stages of the *Plasmodium* spp. life cycle are extracellular, making it much easier for the parasite to evade the host immune system. Upon initial infection, immunity is induced, with subsequent exposures leading to induction of protective mechanisms in the host, such as antibodies against each stage of infection, cytokine production, induction of cytotoxic T cells (Schofield *et al*. 1987) and Th1 responses (Torre *et al*. 2002; Perez-Mazliah and Langhorne, 2014). Interferon-gamma (IFN-γ) has been identified as a key cytokine in the response against malaria infection (Artavanis-Tsakonas and Riley, 2002; McCall and Sauerwein, 2010). Indeed, it has been found that IFN-γ and interleukin (IL)-12 production occurs in a Toll like receptor (TLR)-9 and MyD88-dependant manner, with these cytokines then further enhancing the expression of TLRs by host immune cells (Franklin *et al*. 2009). This is one mechanism thought to contribute to the excessive pro-inflammatory response responsible for the symptoms of the infection.

With such a profound immune reaction to the presence of the parasite, the role of chemokines and their receptors in the promotion of inflammation during malarial infection has been explored. A particular focus of many studies has been the development and pathogenesis of cerebral malaria (CM), a complication of *P. falciparum* infection in humans which results in the obstruction of small blood vessel in the brain with infected erythrocytes, and is often fatal (Hora *et al*. 2016). Clinically, CM is characterized by seizures and loss of consciousness, due to encephalopathy (Newton *et al*. 2000).

One receptor which has been extensively studied in the context of CM is CCR5. CCR5 is expressed by monocytes (Combadiere *et al*. 1996; Ubogu *et al*. 2006), activated T cells (Dragic *et al*. 1996; Ubogu *et al*. 2006; Jiang *et al*. 2009), DCs (Lee *et al*. 1999) and natural killer (NK) (Khan *et al*. 2006) cells, with ligands including CCL3, 4, 5, 7, 8, 13, 14 and 16, and as will be discussed, has been identified as playing key roles in the pathogenesis of several protist infections. CCR5-deficient mice exhibit substantially reduced development of CM in comparison with their wild-type counterparts, with wild-type mice exhibiting higher numbers of CD8+ T cells and both serum levels and splenocyte production of the pro-inflammatory Th1 cytokine tumour necrosis factor alpha (TNF-α) also increased (Belnoue *et al*. 2003*b*).

Other chemokine receptors have also been implicated in the pathogenesis of malaria. CCR2 has been shown in murine experimental models to play little role in the development of CM (Belnoue *et al*. 2003*a*). $CCR2^{-/-}$ mice typically exhibit the inability of monocytes to traffic out of the bone marrow (Serbina and Pamer, 2006; Tsou *et al*. 2007; Jia *et al*. 2008; Shi *et al*. 2011; Shi and Pamer, 2011). During experimental *Plasmodium chabaudi* infection, $CCR2^{-/-}$ mice exhibit prolonged phases of acute parasitaemia when compared to their wild-type counterparts (Sponaas *et al*. 2009). CCL2, a known CCR2 ligand, is a chemoattractant for monocytes and macrophages, and an experimental murine model of malaria utilizing the *Plasmodium yoelli nigeriensis* strain has shown that production (or overproduction) of this chemokine can contribute to immunopathology and host mortality (Pattaradilokrat *et al*. 2014).

Another chemokine receptor of importance during CM in humans and mice is that of CXCR3 and its ligands CXCL4, CXCL9 and CXCL10. Indeed, CXCL10 has been highlighted as a potential diagnostic and prognostic biomarker for CM in humans (Armah *et al*. 2007; Jain *et al*. 2008; Lucchi *et al*. 2011; Wilson *et al*. 2011). In an experimental murine model, using the *P. berghei* strain, both CXCL9 and CXCL10 were found in the brain at significantly high levels, with infected $CXCR3^{-/-}$ mice exhibiting protection against development of CM (Campanella *et al*. 2008). Subsequent analysis confirmed that these mice had significantly fewer T cells in the brain compared with their wild-type counterparts. Furthermore, CXCL4, a platelet derived chemokine, is elevated in the brains of mice infected with *P. berghei*, with platelets becoming activated early on in infection and CXCL4 contributing to the development of CM through destruction of the blood–brain barrier (Srivastava *et al*. 2008). Histological examination of brains from infected $CXCL4^{-/-}$ and $CXCR3^{-/-}$ mice show less cerebral inflammation and damage than their wild-type counterparts, driven by fewer CD4+ and CD8+ T cells in the brain (Srivastava *et al*. 2008).

Toxoplasmosis

Toxoplasma gondii is another Apicomplexan protist, first identified by Nicolle and Manceaux in 1908. This is an intracellular pathogen, which can infect most warm-blooded animals (Dubey, 1996). The infection has a high prevalence in humans, with previous studies identifying an overall seroprevalence of 22·5% for anti-*Toxoplasma* antibodies in the USA (Jones *et al*. 2001). Despite this high prevalence, many individuals are unaware of their infection status, as a competent immune system has the ability to asymptomatically control this infection (Paspalaki *et al*. 2001). The infection often occurs as a result of ingesting *T. gondii* bradyzoites (slow, dormant cysts), which may be present in the undercooked meat of an infected animal (Dubey, 2009). In recent times, the ability of *T. gondii* to be transmitted in the environment and through water sources has been recognized (Jones and Dubey, 2010). Once ingested, the parasite has the ability to cross the intestinal epithelial barrier and replicate within cells of the lamina propria (Speer and Dubey,

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1998). From here, tachyzoites (the fast, replicating stage) can disseminate to various tissues throughout the body (Barragan and Sibley, 2002), such as brain and muscle tissue, where the parasite can exist dormant for long periods of time as cysts (Dubey *et al*. 1998).

Toxoplasmosis has the potential to be particularly detrimental in immunocompromised individuals; often causing encephalitis and multiple organ failure (Paspalaki *et al*. 2001). The infection may also occur through congenital transmission; with the severity of symptoms ranging from retinochoroiditis, to hydrocephalus and abortion of the developing foetus (Gilbert *et al*. 2006). The severity of the symptoms is largely dependent on the gestational age of transmission, with early transmission associated with a disease phenotype of a higher severity (Pinard *et al*. 2003).

The development of immunity to *T. gondii* is complex, with most leucocytes involved in some manner (Miller *et al*. 2009; Munoz *et al*. 2011; Coombes and Hunter, 2015). The chemokine system has a critical role in the response to the initial stages of *T. gondii* infection, allowing establishment of an effective innate immune response to the parasite. For example, knockout studies have demonstrated that CCR5 is essential for the recruitment of NK cells and host survival during infection (Khan *et al*. 2006). The $CCR5^{-/-}$ models have identified that without CCR5-mediated NK cell recruitment; there is a reduction in the CCR5 ligands, CCL3, CCL4 and CCL5, as well as reduced IFN-γ production in spleen, lung, liver and small intestine. Together, infected $CCR5^{-/-}$ mice did not exhibit the typical tissue damage as a consequence of excessive inflammation; however, they did have an increased parasite burden (Khan *et al*. 2006).

Interestingly, the parasite can also influence the host immune response through secretion of immunomodulatory proteins, such as Cyclophilin 18 (TgCyp18) and the dense granule protein GRA24. TgCyp18 binds to CCR5 (Aliberti *et al*. 2003), as illustrated in Fig. 2A, and acts to mimic the known CCR5-binding chemokines CCL3, CCL4 and CCL5 (Aliberti *et al*. 2003; Ibrahim *et al*. 2010). Through stimulating IL-12, TNF-α and nitric oxide production by DCs and macrophages, TgCyp18 can promote Th1 type responses in a CCR5-dependent manner (Aliberti *et al*. 2003; Ibrahim *et al*. 2009). As illustrated in Fig. 2B, in addition to the production of pro-inflammatory cytokines, immune cell recruitment is further promoted through host cell chemokine production, including CCL2, 3, 4 and 5 in response to the parasite. Recruitment of inflammatory monocytes, DCs and NK cells, and induction of Th1 and CD8+ cytotoxic T cells, targets infected cells for destruction, but provides new potential host cells for the parasite.

CCL2 is produced in response to exposure of intestinal cells to the tachyzoite form of the parasite (Rachinel *et al*. 2004; Gopal *et al*. 2011), in particular to the surface protein SAG1 (Brenier-Pinchart *et al*. 2006). In addition, the *T. gondii* dense granule protein GRA24 has been shown to induce CCL2 production by infected host cells, in an infection model utilizing the type II strain *Pru* (Braun *et al*. 2013). CCL3, CCL4 and CCL5 are also produced by intestinal cells (Gopal *et al*. 2011) and blood leucocytes (Bliss *et al*. 1999) in response to *T. gondii* tachyzoites. It seems paradoxical that this parasite deliberately triggers the recruitment of cells designed to destroy it, however as previously mentioned, and illustrated in Fig. 2B, this parasite adopts the Trojan-horse method of dissemination to establish infection by attracting further cells to infect (Da Gama *et al*. 2004; Lambert *et al*. 2009; Elsheikha and Khan, 2010; Sanecka and Frickel, 2012; Coombes *et al*. 2013).

Other chemokine receptor knockout mice have been utilized in murine models of *T. gondii* infection, as summarized in Table 2, to demonstrate the role of certain cell types in the pathogenesis or control of infection. For example, $CCR1^{-/-}$ mice have defects in neutrophil trafficking, and so use of these mice allowed researchers to dissect the neutrophilic response during early phases of *T. gondii* infection (Khan *et al*. 2001). In addition, the necessity of CCR1 and its ligand CCL3 in driving the recruitment of inflammatory monocytes to the intestine during *T. gondii*-induced ileitis has been demonstrated through the use of $CCR1^{-/-}$ mice (Schulthess *et al*. 2012). Moreover, studies using mice deficient in either CCR2 or its ligand CCL2, clearly demonstrated the importance of early monocyte recruitment to the intestine during *T. gondii* infection, with knockout mice unable to control parasite replication (Dunay *et al*. 2008). Subsequent studies by the same group confirmed this important role of monocytes, but showed that neutrophil depletion had no impact on the ability to control infection, but indeed may actually contribute to pathology (Dunay *et al*. 2010).

As with many parasites studies, knowledge has been gained through the use of animal models, but this always leaves the question as to relevance for human infection. While studies using human cells are limited, it has been found that infection of human epithelial and fibroblast with the highly virulent RH strain of *T. gondii* results in production of the monocyte and neutrophil chemoattractants CCL2, CXCL1 and CXCL8 (Denney *et al*. 1999). Importantly, host cell invasion and lysis was necessary for this response, with stimulation with *T. gondii* lysates not sufficient to induce the response (Denney *et al*. 1999). More recently, studies examining the impact of neuronal *T. gondii* infection showed similar results, with human bone marrow endothelial cells and microglial cells showed significant expression of CCL2 and CXCL1 after 8 h of

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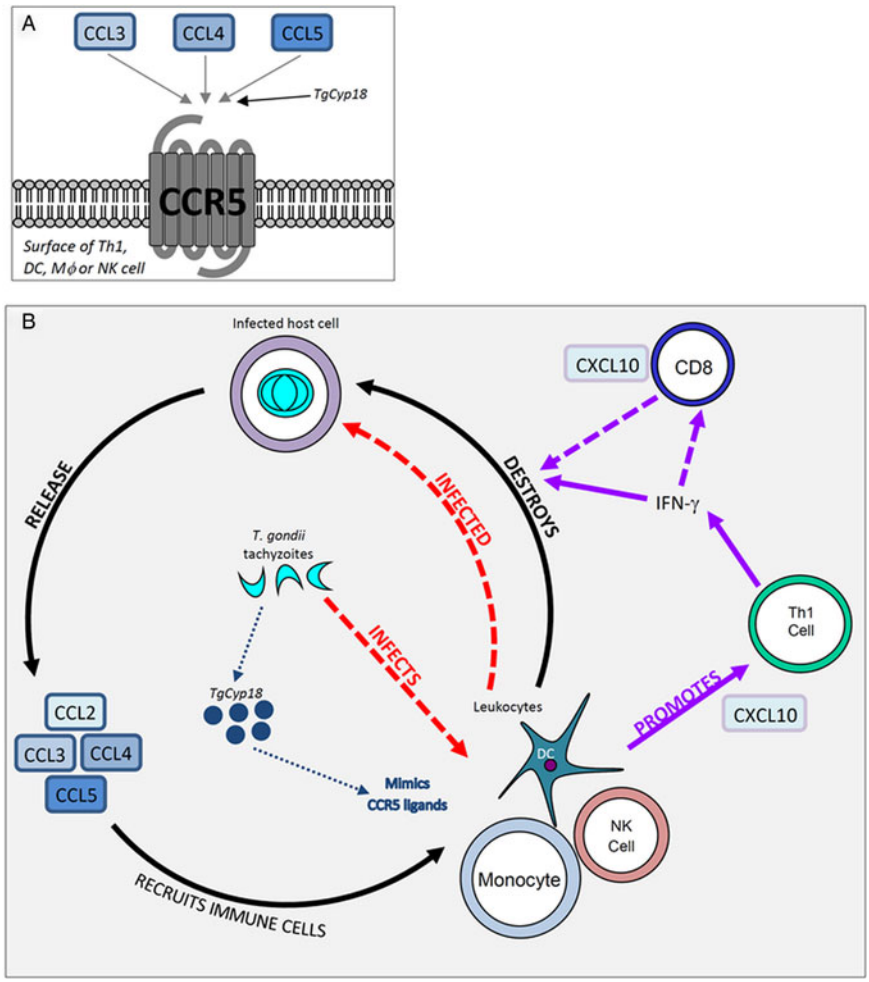

Fig. 2. Leucocyte trafficking during *T. gondii* infection. (A) Known CCR5 ligands. (B) Infection of host immune cells results in the production of a number of inflammatory chemokines, which serve to recruit more immune cells. Promotion of a Th1-type response, with the involvement of CD8+ cytotoxic T cells is critical for the destruction of infected host cells.

infection with either the RH or PRU strains, whereas infection of a neuroblastoma cell line only stimulated production of CXCL1 after 24 h of infection (Mammari *et al*. 2014). CXCL8 was produced by all cell types at much earlier time-points post-infection, indicating a key role in initiating immune responses (Mammari *et al*. 2014).

Cryptosporidiosis

The causative agents of cryptosporidiosis, *Cryptosporidium*, such as *Plasmodium* and *T. gondii* are Apicomplexan protists. The main species of *Cryptosporidium*, which causes gastroenteritis in humans are *Cryptosporidium parvum* and *Cryptosporidium hominus* and are mainly transmitted via the oral–fecal route (Tzipori and Ward, 2002). Cryptosporidiosis is usually a short-term infection, but it can cause severe disease in those who are immunocompromised and in the young, and it can persist in the lower gastrointestinal tract for up to 5 weeks. Cryptosporidiosis is one of the leading causes of diarrhoeal disease in developing countries. This parasite is highly resistant to chlorine (Korich *et al*. 1990), therefore meaning it presents a particular challenge to remove it from water systems, which is the main route of transmission.

Unlike *Plasmodium* and *Leishmania*, *Cryptosporidium* does not require a vector, and unlike *T. gondii*, it is minimally invasive for humans. Controlling *Cryptosporidium* infection is highly dependent on immune cell recruitment. Indeed, parasite infection is restricted to the intestinal epithelia and in addition, in neonates (Auray *et al*. 2007; Lantier *et al*. 2013) and in the immunodeficient host, there is a low representation of immune cells in the intestinal mucosa (Steege *et al*., 1997) as well as chemokines (Lantier *et al*. 2013). Several papers have reviewed the immune response to this parasite and like many of the other parasites discussed, IFN-γ, IL-12 and the induction of the Th1-type response is key to immune control of parasite (Borad and Ward, 2010; Petry *et al*. 2010; McDonald *et al*. 2013).

Of the CC chemokines, both CCL5 and CCL20 have been shown to play crucial roles in mediating immune responses against the parasite in models of

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both human and mouse infection, respectively. CCL5 is chemotactic for Th1T cells (Kawai *et al*. 1999) and monocytes (Schall *et al*. 1990), and has been shown in a human intestinal cell line model to be produced, along with CXCL8 and TGF-β, in response to *C. parvum* infection (Laurent *et al*. 1997; Maillot *et al*. 2000) to draw in cells associated with aiding recovery. The mucosal-tissue associated chemokine, CCL20, which is known to exhibit antimicrobial activity (Yang *et al*. 2003) is downregulated in a neonatal mouse model of *C. parvum* infection, resulting in increased parasite burden (Guesdon *et al*. 2015).

Due to immunocompetent adults being fairly resistant to infection, little work has been done utilizing chemokine receptor knockout mice; indeed, in a study using adult $CCR5^{-/-}$ mice, attempts to establish the infection were not successful (Campbell *et al*. 2002). Despite this, the role of CCR5 in neonatal infection has been explored. Expression of mRNA for CCR5 and its ligands (CCL3, 4, 5) increases in the intestine of neonates from 4 to 6 days post-infection (Lacroix-Lamandé *et al*. 2008; Lantier *et al*. 2013), suggesting a key role for CCR5 in the control of neonatal infection; however, $CCR5^{-/-}$ neonates showed a higher parasite burden only at the initial stages of infection, and recovered as well as the wild-type controls (Lacroix-Lamandé *et al*. 2008). The authors hypothesized that the redundancy in the chemokine system prevented the absence of CCR5 from inhibiting cell recruitment. Indeed, the authors noted that $CCR5^{-/-}$ mice has double the amount of CCR2 mRNA (Lacroix-Lamandé *et al*. 2008). CCR5 is an important chemokine receptor involved in monocyte trafficking, as is CCR2. Almost no inflammatory monocytes are found in the intestine of neonatal mice; however a recent study by (de Sablet *et al*. 2016) has shown that upon infection with *C. parvum* this increases significantly. Infected neonatal $CCR2^{-/-}$ mice have, as expected, very few inflammatory monocytes in their intestine; however it has also been found that these mice differ from their wild-type counterparts in the permeability of the intestinal wall (de Sablet *et al*. 2016). $CCR2^{-/-}$ mice did not lose epithelial barrier function to the same extent as wild-type mice, indicating that monocytes may contribute to this during *C. parvum* infection in neonates (de Sablet *et al*. 2016).

Development and initiation of a successful immune response is highly dependent on the recruitment and functioning of professional antigen presenting cells such as DCs. As with the monocytic populations, neonatal intestines have few DCs, but upon infection with *C. parvum*, this number increases (Lantier *et al*. 2013). CXCL10 is a ligand for CXCR3 (Loetscher *et al*. 1996), and attracts T cells, B cells, NK cells, DCs and macrophages (Sallusto *et al*. 1998; Liu *et al*. 2011). To analyse the role of CXCL10 and its receptor CXCR3 in neonatal infection, $CXCR3^{-/-}$ neonates were infected and it was found that DCs were not recruited to the intestine and that these mice did not control infection as well as their wild-type counterparts (Lantier *et al*. 2013).

As previously mentioned, cryptosporidiosis is also a severe and life-threatening disease for the immunocompromised, for example AIDS patients. The severity of disease in these individuals highlights the necessity for a functional CD4+ T cell response to the parasite. CXCL10 has also been studied in the context of *C. parvum* infection as a complication of AIDS. In jejunal biopsies from AIDS patients with, and without *C. parvum* infection, CXCL10 was found in high levels in those patients with the dual infection, and this correlated with parasite burden (Wang *et al*. 2007). CXCL10 was localized to epithelial cells, and has been hypothesized to play a key role in the resolution of *C. parvum* infection in AIDS patients (Wang *et al*. 2007).

Leishmaniasis

Leishmania species are protists, which are members of the Sarcomastigophora phylum. *Leishmania* species exist in two forms; the intracellular amastigote form, and the promastigote form. The life cycle of the parasite involves amastigote and promastigote morphologies (Wheeler *et al*. 2011), with extracellular promastigotes colonizing the sandfly midgut, while the intracellular amastigotes are associated with the mammalian host (Bates, 2007). On initial infection of the host, promastigotes are engulfed by phagocytes and reside within the phagolysosome, where the parasite has the ability to inhibit phagolysosome biogenesis though its surface glycolipid lipophosphoglycan (Moradin and Descoteaux, 2012). The parasite then differentiates into amastigotes within macrophages, replicating within the phagolysosome, before being released to infect cells in various tissues around the body (Moradin and Descoteaux, 2012). The host can then transmit the parasite to uninfected sandflies, where it is believed that the sandfly ingests amastigote-infected macrophages during feeding (Dostálová and Volf, 2012).

Leishmaniasises can be described as visceral, cutaneous or mucocutaneous, causing 20 000–30 000 deaths annually (World Health Organization figures, 2016). Visceral leishmaniasis is the most severe form of the disease, causing enlargement of the spleen and liver, most commonly caused by the *Leishmania chagasi* and *Leishmania donovani* strains. Cutaneous leishmaniasis is disfiguring to those infected, and is caused by the *Leishmania amazonensis, Leishmania major* and *Leishmania mexicana* strains, whereas mucocutaneous leishmaniasis is mostly associated with the *Leishmania brasiliensis* strain. A common feature of these infections is the extensive inflammatory reaction (Nylen and Gautam, 2010; Oliveira *et al*. 2014; Melo *et al*. 2015; Rodrigues *et al*. 2015).

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The immune response elicited against *Leishmania* spp. is complex, particularly as the parasite preferentially infects phagocytic cells, including DCs, macrophages and neutrophils. While the importance of granulocytes for the control of *Leishmania* infections is clear, studies have shown this parasite can actually evade the killing mechanisms of phagocytes, and use these cells to hide and spread infection (Laskay *et al*. 2003; van Zandbergen *et al*. 2004; Shapira and Zinoviev, 2011). Resistance to leishmaniasis requires induction of a Th1-type response, with both IFN-γ and IL-12 crucial for this response (Kemp *et al*. 1994; Kurtzhals *et al*. 1994; Ghalib *et al*. 1995; Alexander *et al*. 1999). A number of studies and reviews have examined the expression of chemokines, and their role in mediating immunity against *Leishmania* spp. (Teixeira *et al*. 2006; Oghumu *et al*. 2010). Again, with examples for *L. donovani* and *L. major* included in Table 2, chemokine receptor knockout mice have been instrumental in dissecting the pathways of inflammation involved in developing immunity to infection, along with development of tissue pathology.

In a murine model of visceral leishmaniasis, utilizing *L. donovani*, CCL2, CCL3 and CXCL10 were found to be produced in the first week of infection by the liver, at a point when inflammation is being initiated (Cotterell *et al*. 1999). By comparing wild-type BALB/c mice with *SCID* mice, the authors found that early T-cell-independent chemokine expression does not mediate the inflammation associated with development of the characteristic granulomas found in the livers of infected mice (Cotterell *et al*. 1999). *Leishmania donovani*-infected $CCL3^{-/-}$ mice have shown that this chemokine is not essential for control of the parasite; however, spleen cells from mice infected for 8 weeks produce significantly higher levels of IFN-γ than the wild-type controls, with CD4+ T cells the main source of this cytokine within the spleen cell mixture (Sato *et al*. 1999).

The role of CCL3 has also been examined in a murine model of cutaneous leishmaniasis, using *L. major* as the infective parasite (Steigerwald and Moll, 2005). DCs exposed to *L. major* were found to reduce expression of the receptors CCR2 and CCR5, as well as their ability to respond to CCL2 and CCL3; however, these cells upregulated expression of the receptor CCR7, and were therefore more responsive to its ligand CCL21 (Steigerwald and Moll, 2005). The authors of this particular study postulate that in response to the parasite, the immune system modulates the recruitment of DCs to the secondary lymphoid organs for antigen presentation. Indeed, a more recent study has shown that the lymph node hypertrophy that occurs during *in vivo* murine *L. major* infection, is due to bystander DCs expressing elevated levels of CCR7 in a TLR-9-dependent manner, leading to enhanced immune cell recruitment during the first weeks post-infection (Carvalho *et al*. 2012). Additionally, studies looking at the hours immediately after exposure to *L. major* promastigotes demonstrated immediate and transient upregulation of CCL2 and CXCL1 production by murine macrophages (Racoosin and Beverley, 1997).

These studies, amongst many others, demonstrate that chemokines are induced at early and late stages of infection. However, these are in murine models of infection. What is known about chemokines in human leishmaniasis? Lesions from humans suffering from different degrees of cutaneous leishmaniasis (localized, intermediate and diffuse) showed elevated CCL2, CCL3, CCL4, CCL8 and CXCL10 in localized lesions and CCL3 in diffuse lesions (Ritter *et al*. 1996; Díaz *et al*. 2013). Indeed, incubation of human blood mononuclear cells with *L. major* results in production of CCL2 and CXCL8 (Badolato *et al*. 1996).

Concluding remarks

The chemokine, and chemokine receptor system is complex, with slight variations between humans and mice, for example, human CCL13 has no mouse equivalent, and while mouse CCL8 binds CCR8, human CCL8 does not, suggesting that human and mouse CCL8 are not equivalent chemokines (Zlotnik and Yoshie, 2012). This highlights the need for careful translation of research findings from animal model to human disease. This review has focused on four protists, responsible for a significant amount of disease burden globally, and not only highlights the complexity of the chemokine system, but clearly demonstrates gaps in our understanding of the immune response to these organisms. The interesting paradox with chemokines is that these proteins draw in the cells that can facilitate resistance, but in some cases, may actually facilitate infection spread through providing more cells for the parasite to infect, allowing the parasites to adopt a 'Trojan-horse' style of deception from the host immune system. Therefore, there is a necessity for controlled balance between immunity for host protection, and immunity contributing to disease pathology.

The parasites discussed within this article are all members of the Protista kingdom; however, the diseases they cause in humans are quite different, mainly due to the specificities of each life cycle. Common to all is the importance of a Th1-type immune response for control of parasite replication, and with that, the inflammation-induced pathologies driven by excessive inflammation. Studies utilizing chemokine and chemokine-receptor knockout mice have been crucial in elucidating the immune pathways driven by these infections. For example, as highlighted previously, studies have been conducted with all of the protists discussed using $CCR5^{-/-}$ mice

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(Table 2), giving insight into the role of CCR5 expressing cells (monocytes, activated T cells, DCs, NK cells) in protective immune responses and development of pathology.

In recent times, the development and investigation of the use of chemokine receptor agonists, and antagonists for disease therapy (Castellani *et al*. 2007; Mohit and Rafati, 2012) has been an area of investigation in the field of chemokine biology; however much of this work focuses on the use these new therapies as cancer treatments (Ruffini *et al*. 2007; Wu *et al*. 2009) and could be extended to infectious disease. This, along with the recognition that chemokines may also prove useful as potential biomarkers for infectious diseases, for example, as that described for CXCL10 in CM, highlights the need for research focus in this area.

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