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The role of complement in innate, adaptive and eosinophil-dependent immunity to the nematode *Nippostrongylus brasiliensis*

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Abstract

Complement may be important for immunity to infection with parasitic helminths, by promoting the recruitment of leukocytes to infected tissues and by modulating the function of cytotoxic effector leukocytes. However, the importance of complement *in vivo* during helminth infection is poorly understood. In this study, mice lacking classical (C1q-deficient), alternative (factor B-deficient) or all pathways of complement activation (C3-deficient) were used to assess the role of complement in immunity to the nematode *Nippostrongylus brasiliensis*. Double-mutant complement-deficient/IL-5 transgenic (Tg) mice were used to determine if complement is required for the strong eosinophil-dependent resistance to this parasite. Complement activation on larvae (C3 deposition), extracellular eosinophil peroxidase activity, larval aggregation and eosinophil recruitment to the skin 30 min post-injection (p.i.) of larvae were reduced in factor B-deficient mice. Inhibition of the C5a receptor with the antagonist PMX53 impaired eosinophil and neutrophil recruitment to the skin. C3 deposition on larvae was minimal by 150 min p.i. and at this time cell adherence, larval aggregation, eosinophil recruitment and degranulation were complement-independent. Factor B and C3 deficiency were associated with higher lung larval burdens in primary infections. Complement-deficient/IL-5 Tg mice were highly resistant to *N. brasiliensis*, suggesting that eosinophils can limit infection in a complement-independent manner. Potent secondary immunity was similarly complement-independent. In conclusion, although the alternative pathway is important for parasite recognition and leukocyte recruitment early in *N. brasiliensis* infections, the parasite soon becomes resistant to complement and other factors can compensate to promote eosinophil-dependent immunity.

Keywords: Complement; Helminth; Eosinophil; Alternative pathway; *Nippostrongylus brasiliensis*

1. Introduction

The complement system is a fundamental feature of innate and adaptive immune responses to infection with many pathogens. *In vitro* complement activation on the surface of helminths is well established and, while it may not cause direct damage to the parasites, complement may be important in aiding leukocyte-mediated immunity. Complement activation causes generation of C3a and C5a that act as chemotactic factors for leukocytes. The C3 cleavage products C3b and iC3b, deposited on the surface of the parasite can facilitate leukocyte adherence (Gasque, 2004). Eosinophils are one of the major effector leukocytes contributing to resistance against helminths (Butterworth, 1984; David et al., 1980; Rainbird et al., 1998). Basal eosinophil numbers in peripheral blood and tissues are typically low in naïve animals, however during helminth infection eosinophilia can develop (Rothenberg, 1998). Transgenic (Tg) mice that over-express IL-5 (IL-5 Tg) display constitutive eosinophilia and are highly resistant to infection with *Nippostrongylus brasiliensis*

Abbreviations: C5aR, C5a receptor; EPO, eosinophil peroxidase; i.p., intra-peritoneally; L3, infective third-stage *N. brasiliensis* larvae; MPBS, mouse-osmolality PBS; MQ, Milli-Q; NMS, normal mouse serum; p.i., post-injection; s.c., sub-cutaneously

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(Dent et al., 1997), *Strongyloides stercoralis* (Herbert et al., 2000) and *Angiostrongylus costaricensis* (Sugaya et al., 2002), with drastically reduced worm burdens relative to infections in WT mice. Importantly, the mechanism of how eosinophils provide resistance to helminth infection is unknown. Rapid recruitment of these cells to sites of infection, adherence to the surface of the parasite and release of cytotoxic granule proteins that can inflict damage may all be critical (Butterworth, 1984; Daly et al., 1999; David et al., 1980; MacKenzie et al., 1981; Specht et al., 2006). Activation of the complement system may facilitate some or all of these processes, though little is known about the importance of complement *in vivo* during helminth infection. However, complement is important for eosinophil adherence and killing of some helminths *in vitro* (Butterworth, 1984; Rainbird et al., 1998; Shin et al., 2001).

Complement activation is initiated by three distinct pathways (Gasque, 2004). The classical pathway is principally dependent on C1q recognition of antibody bound to the surface of a pathogen, but C1q can bind to other molecules or bind directly to pathogens. The lectin pathway is similar to the classical pathway, but is C1q-independent, and is activated by lectin recognition of pathogen-associated molecular patterns. The alternative complement pathway is initiated via spontaneous hydrolysis of C3 by a factor B-dependent C3-convertase. The C3 molecule is central to all three pathways. *In vitro* studies have demonstrated that the relative importance of the three pathways of complement activation differ depending on the species of helminth and on the host. The classical pathway predominates when antibody is present (Shaio et al., 1990), while the alternative (Giacomin et al., 2005; Yates et al., 1985) and lectin (Gruden-Movsesijan et al., 2003) pathways predominate in serum from naïve animals. We have previously demonstrated the critical role of the alternative pathway for mediating C3 deposition and eosinophil adherence to the infective-stage larvae (L3) of *N. brasiliensis in vitro*, though the lectin pathway played a role for more mature stages of the parasite (Giacomin et al., 2005). The mechanism of complement activation *in vivo* on any species of helminth has not been determined. This has previously been difficult to characterise, in part because some species of helminth cease to bind complement after a period of time within the host (Dessein et al., 1981; Stankiewicz et al., 1989).

We have for the first time examined the role of the different pathways of complement activation in primary and secondary resistance of mice to the nematode *N. brasiliensis*. We studied immune responses at the site of initial infection (the skin) and the kinetics of parasite migration to the lung and the gut in mice genetically deficient in all pathways of complement activation (C3-deficient, $C3^{-/-}$), the classical complement pathway (C1q-deficient, $C1qa^{-/-}$) or the alternative complement pathway (factor B-deficient, $Bf^{-/-}$). Complement-deficient mice also expressing an IL-5 transgene were used to assess the role of complement in facilitating the strong eosinophil-dependent resistance of IL-5 Tg mice to this parasite. The role of the anaphylotoxin C5a was assessed using a C5a receptor (C5aR) inhibitor. Given our previous *in vitro* observations (Giacomin et al., 2005), we hypothesized that absence of the alternative complement pathway would impair immune responses to *N. brasiliensis* infection.

2. Materials and methods

2.1. Parasitological techniques

Techniques for *N. brasiliensis* maintenance and preparation for experiments were as previously described (Daly et al., 1999; Giacomin et al., 2005). Mice were injected sub-cutaneously (s.c.) or intra-skin air pouch with 100 μL of larval suspension containing approximately 500 L3 suspended in mouse osmolality PBS (MPBS) (Sheridan and Finlay-Jones, 1977).

2.2. Animals and sera

C57BL/6 male and female WT, heterozygous IL-5 Tg (Tg5C2) (Dent, 2002) carrying approximately 50 copies of the IL-5 transgene, $C1qa^{-/-}$ (Botto et al., 1998), $Bf^{-/-}$ (Matsumoto et al., 1997) and $C3^{-/-}$ mice (Wessels et al., 1995) were used. Double-mutant C1q-, factor B- or C3-deficient mice that were also IL-5 Tg were generated by crossing each complement-deficient mouse strain with IL-5 Tg mice. IL-5 Tg offspring were selected by screening for blood eosinophilia and then crossed with the homozygous knockout line. Double-mutant $C1qa^{-/-}$, $Bf^{-/-}$ or $C3^{-/-}$/IL-5 $Tg^{+/-}$ progeny were selected by PCR diagnosis of respective complement genes and by screening for blood eosinophilia. Normal mouse serum (NMS) was obtained from WT C57BL/6 mice by cardiac puncture under pentobarbitone anaesthesia. Mice were bred at the University of Adelaide and handled according to institutional animal ethics guidelines.

2.3. Photography and enumeration of skin-stage larvae

Representative photographs of samples of larvae recovered from air pouches were prepared using a dissecting microscope or Olympus BH-2 microscope and an Olympus C-35AD-4 camera. Total numbers of larvae recovered from air pouches were counted using a dissecting microscope after vigorous pipetting to disaggregate larvae.

2.4. Analysis of C3 deposition in vivo and ex vivo

Larvae recovered from air pouches were disaggregated by pipetting and washed 3× with 10 mL MPBS. Approximately 50 larvae/100 μL MPBS were incubated at 37 °C/5% CO_2 for 1 h with either 25 μL MPBS (to detect *in vivo* C3 deposition) or in 20% NMS (to examine *ex vivo* C3 deposition). Larvae were washed with PBS containing 0.05% Tween-20 (PBST) and resuspended in a final volume of 100 μL. C3 was detected by incubation with 50 μL of 1:50 (v/v) dilution of FITC-conjugated anti-mouse C3 Ab (ICN/Cappel, Aurora, OH, USA) for 1 h at room temperature. Larvae were washed as described above and resuspended in a minimal volume of PBS. Suspensions of parasites were transferred to a glass slide and examined under UV illumination with an Olympus BH-2 microscope at 10× objec-