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Anti-complement activity of the *Ixodes scapularis* salivary protein Salp20

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ABSTRACT

Complement, a major component of innate immunity, presents a rapid and robust defense of the intravascular space. While regulatory proteins protect host cells from complement attack, when these measures fail, unrestrained complement activation may trigger self-tissue injury, leading to pathologic conditions. Of the three complement activation pathways, the alternative pathway (AP) in particular has been implicated in numerous disease and injury states. Consequently, the AP components represent attractive targets for therapeutic intervention. The common hard-bodied ticks from the family Ixodidae derive nourishment from the blood of their mammalian hosts. During its blood meal the tick is exposed to host immune effectors, including the complement system. In defense, the tick produces salivary proteins that can inhibit host immune functions. The Salp20 salivary protein of *Ixodes scapularis* inhibits the host AP pathway by binding properdin and dissociating C3bBbP, the active C3 convertase. In these studies we examined Salp20 activity in various complement-mediated pathologies. Our results indicate that Salp20 can inhibit AP-dependent pathogenesis in the mouse. Its efficacy may be part in due to synergic effects it provides with the endogenous AP regulator, factor H. While Salp20 itself would be expected to be highly immunogenic and therefore inappropriate for therapeutic use, its emergence speaks for the potential development of a non-immunogenic Salp20 mimic that replicates its anti-properdin activity.

1. Introduction

Complement (C), a major component of innate immunity, presents a rapid and robust defense of the intravascular space. Complement marks infectious agents for immune clearance or lysis, promotes the local inflammatory response, facilitates T cell lineage commitment, and directs B cell activation and antibody production (Ricklin et al., 2010; Fearon and Locksley, 1996; Carroll, 2004; Kemper and Atkinson, 2007). Complement is also a principal cause of tissue damage: C-related disease and injury can be traced to inappropriate complement activation (humoral autoimmunity) (Chen et al., 2010) or to inadequate complement regulation (atypical hemolytic uremic syndrome, age-related macular degeneration) (Liszewski and Atkinson, 2015). Therapeutic agents designed to modulate complement activity have begun to emerge in the clinical setting (Ricklin and Lambris, 2007, 2013).

There are three complement activation pathways: the classical pathway responds to antibody:antigen complexes, the lectin pathway responds to microbial surfaces, and the alternative pathway activates spontaneously at low level (Ricklin et al., 2010). Each pathway results in the assembly of the C3 convertases, the enzymes that catalyze the cleavage of C3, forming the C3a and C3b fragments. Nascent C3b can bind covalently to a nearby target surface where it presents a new site for convertase assembly as part of a positive feedback loop (the amplification loop) that drives the rapid and robust complement response. Surface-bound C3 convertases can associate with additional C3b, forming a C5 convertase. C5 convertase cleaves C5 to yield C5a and C5b. C5a, as well as C3a, promotes inflammatory reactions. C5b initiates the complement terminal pathway (TP), leading to the assembly of the membrane attack complex (MAC), membrane perturbation, and cell lysis. Host regulatory proteins protect self-tissues from harmful complement activity (Liszewski and Atkinson, 2015).

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Of the three complement activation pathways, the alternative pathway (AP) in particular has been implicated in numerous disease and injury states (Liszewski and Atkinson, 2015; Holers, 2008). The AP C3 convertase is assembled on a target in a multi-step process that begins with the covalent attachment of nascent C3b to the surface followed by association of C3b with factor B (FB), and cleavage of C3bB by factor D (FD) at a single FB site, forming an active but unstable C3 convertase (T1/2 ~90 s; (Medicus et al., 1976)), C3bBb. An additional protein, properdin (P), binds to C3bBb, rendering the convertase 5–10-fold more stable (Fearon and Austen, 1975).

The common hard-bodied *Ixodes* ticks derive nourishment from the blood of their mammalian hosts. During its blood meal, which can last more than 5 days, the tick is exposed to host immune effectors, including the fluid phase complement components. In its own defense, the tick has evolved salivary proteins that can inhibit host immune functions (Das et al., 2001; Gillespie et al., 2000; Nuttall et al., 2000; Wikel, 1999). The *Ixodes scapularis* proteins Salp20 and Isac, and the closely related *Ixodes ricinus* proteins IRAC I and II and IXAC-B1-5, inhibit the complement AP (Valenzuela et al., 2000; Lawrie et al., 1999; Lawrie et al., 2005; Tyson et al., 2007) by binding to properdin, blocking its activity, and displacing it from the C3bB and C3bBb complexes (Tyson et al., 2008; Couvreur et al., 2008).

Properdin (P) is an attractive target for therapeutic inhibition since it is AP-specific, and known properdin-deficient individuals, nearly all males as properdin is encoded by the X chromosome (Goundis et al., 1989), are well except for increased susceptibility to meningococcal disease (Sjoholm et al., 1988; Densen, 1989), a condition that can be addressed by vaccination (Densen et al., 1987). Here we examine Salp20 activity in various AP-mediated animal models. Our results indicate that Salp20 can inhibit AP-dependent pathogenic processes in the mouse. Its efficacy may be part in due to synergic effects it shares with the endogenous AP regulator, factor H.

2. Materials and methods

2.1. Proteins and sera

Purified complement proteins and pooled normal human serum were purchased from CompTech. The Salp20 protein used in this investigation was a recombinant protein that carries a V5 epitope and a 6x Histidine tag fused to the carboxy terminus of the Salp20 sequence (S20NS). The protein was produced in High Five cells and purified as previously described (Tyson et al., 2007). Activity was confirmed by C3b deposition assay (Barilla-LaBarca et al., 2002) and/or by hemolytic assay (below). Recombinant factor B D254G (Hourcade et al., 1999) was prepared and quantified as previously described (Hourcade and Mitchell, 2011). Briefly, human 293T kidney cells were transfected in serum-free medium with factor B D254G cDNA cloned in pSG5 (Stratagene) expression vector. Supernatants containing FB protein were treated with benzamidine-Sepharose (Amersham Biosciences cat#17-5123-10) to remove nonspecific proteases, dialyzed and stored in phosphate buffer supplemented with 25 mM NaCl. Recombinant factor B was quantified by ELISA and examined by Western blotting. A negative control supernatant was prepared with the expression vector incorporating the FB coding sequence in reverse orientation.

2.2. Sheep erythrocyte AP convertase assay

Antibody-sensitized sheep erythrocytes (CompTech cat#B200) were opsonized with C3b as previously described (Hourcade et al., 1995) and stored at a final concentration of 10^8 cells per mL at 4 °C for up to a week. For each reaction, 100 μL C3b-opsonized cells were treated with the stabilizing D254G FB variant (50–400 pg, as indicated), FD (5 ng), and properdin (50 ng) in 10 mM MgEGTA buffer (total volume 250 μL) for 30 min at 30 °C. The convertases formed were detected by treating the cells with 2.5% guinea pig serum in 40 mM EDTA buffer for 60 min at 37 °C on an orbital shaker at 190 rpm to permit the assembly of the membrane attack complex and subsequent cell lysis. The resulting reaction mixtures were centrifuged at 2000 rpm for 5 min and the OD_{414} of the supernatants were determined. Negative control (blank) reactions were performed in the absence of FB and 100% lysis was determined with reactions performed with distilled water. The fraction of cells lysed (y) = (OD reaction–OD blank)/(OD 100% lysis–OD blank). Average lytic sites per cell (Z value) were calculated assuming "one-hit" kinetics: $Z = [1 - \ln(y)]$ (Borsos et al., 1961; Whaley, 1985). In some cases, cells were treated with Salp20 and/or FH along with FB, FD and properdin to determine the effects of the regulators on net convertase formation. The inhibitory effects of Salp20 were expressed as mean ± SD derived from the Z values obtained with 300 pg FB in three separate experiments. The values used to quantify the additive effects of Salp20 and FH were derived from the Z values obtained with 200 ng FB in three separate experiments.

2.3. LPS-dependent AP activation assay

C57BL/6J mice (The Jackson Laboartory) were administered i.p. with various concentrations of Salp20. Plasma samples were collected at various time points (2–4 h) for an LPS-dependent AP activation assay (Sfyroera et al., 2005; Kimura et al., 2008; Miwa et al., 2013). Briefly, plasma was diluted (1:10 in Mg^{2+}-EGTA GVB^{2+} buffer), applied to LPS-coated ELISA plates (2 μg/well), and incubated at 37 °C for 1 h. After washing, goat anti-mouse C3 Ab (1:4000 dilution, MP Cappel, cat#55463) was added followed by HRP-conjugated anti-goat IgG (1:2000 dilution; Jackson ImmunoResearch), after which substrate reagent (cat#DY999, R&D Systems) was added for 10 min. The reaction was stopped with 1 M H_2SO_4 and the OD of samples measured at 450 nm.

To assess the AP activity in the OVA-induced asthma model, mice were sacrificed on day 17 (see below) and their lungs lavaged 3 times with 1 mL of sterile PBS. Samples were cleared by centrifugation at 14,000 rpm for 20 min at 4 °C. Cleared samples were diluted at 1:2 in Mg^{2+}-EGTA GVB^{2+} buffer prior to being applied to LPS-coated plates. Post lavage, lungs were harvested and homogenized in 2 mL of cold PBS. Homogenates were cleared twice with centrifugation at 14,000 rpm × 20 min at 4 °C. Samples were normalized by protein concentration based on absorbance at 280 nm and diluted 1:2 in Mg^{2+}-EGTA GVB^{2+} buffer prior to being applied to LPS-coated plates. AP activity was measured as above.

2.4. OVA-induced asthma model

Adult male C57BL/6 mice (8–12 weeks) were immunized i.p. on day 0 and day 7 with 20 μg OVA suspended in 2.25 mg of aluminum hydroxide (total volume 100 μL). On days 14–16 mice were challenged intranasally (i.n.) with 30 μg OVA in PBS (total volume 30 μL) following anesthesia with isoflurane. Mice were sacrificed on day 17 and their bronchoalveolar lavage fluid (BALF) and lungs were harvested for analysis and histology. In some experiments mice were also administered heat inactivated or active Salp20 (9 μg i.n.) concomitantly with OVA (total volume 45 μL).

IL-13 level in the BALF was measured with an ELISA kit (cat#DY413, R&D Systems). For C3a ELISA, BALF (100 μL) was applied to plates coated with anti-mouse C3a monoclonal antibody (4 μg/mL, cat#558250, BD Pharmingen) and incubated for 2 h at RT, followed by biotinylated anti-mouse C3a monoclonal antibody (250 ng/mL, cat#558251, BD Pharmingen). After washing and incubation with streptavidin-peroxidase (400 ng/mL, cat#890803, R&D Systems), 100 μL of peroxide-chromogen solution (cat#DY999,