Binding of Human Factor H–Related Protein 1 to Serum-Resistant *Borrelia burgdorferi* Is Mediated by Borrelial Complement Regulator–Acquiring Surface Proteins

Katrin Haupt, Peter Kraiczy, Reinhard Wallich, Volker Brade, Christine Skerka, and Peter F. Zipfel

1Department of Infection Biology, Leibniz Institute for Natural Product Research and Infection Biology, and 2Friedrich Schiller University, Jena, 3Institute of Immunology, University of Heidelberg, Heidelberg, and 4Institute of Medical Microbiology, University Hospital of Frankfurt, Frankfurt, Germany

**Background.** Isolates of *Borrelia burgdorferi*, the causative agent of Lyme disease, express up to 5 distinct complement regulator–acquiring surface proteins (CRASP-1, -2, -3, -4, and -5).

**Methods.** By use of ligand affinity blotting, enzyme-linked immunosorbent assay, surface plasmon resonance, and functional complement assays, we have identified factor H–related protein 1 (FHR-1) as a novel protein that binds to the bacterium via CRASP-3, -4, and -5.

**Results.** When incubated in serum, serum-resistant *Borrelia burgdorferi* strain LW2 bind FHR-1, an additional member of the human factor H protein family, and, similarly, 2 mouse FHR proteins bind to the surface. Recombinant FHR-1 binds to 3 borrelial surface proteins (CRASP-3, -4, and -5) but not to CRASP-1 and -2. A comparative analysis of the individual CRASPs revealed common as well as distinct binding profiles for the 3 human regulators. FHR-1 binds to 3 CRASPs, and factor H binds to all 5 CRASPs. In addition, factor H–like protein 1 interacts with CRASP-1 and -2 but with no other borrelial proteins.

**Conclusions.** Thus, by expressing multiple surface proteins with different binding properties, the pathogen can attach a unique combination of host complement regulators to its surface. For the pathogen, this type of surface decoration and specific acquisition of different host plasma proteins allows fine-tuning of the host immune attack.

Lyme borreliosis, the most commonly reported vectorborne infectious disease in Eurasia and the United States, is caused by bacteria of the *Borrelia burgdorferi* sensu lato complex, which includes *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* [1, 2]. Spirochetes cause local skin rash (erythema migrans), and untreated Lyme disease can result in chronic, multisystemic disorders that primarily affect the joints, the central nervous system, and the skin, thereby causing Lyme arthritis, neuroborreliosis, or acrodermatitis chronica atrophicans [1]. Upon infection, complement, which represents the first line of innate immune defense, is activated. One central strategy of *B. burgdorferi* to avoid a destructive complement attack is the binding of host fluid-phase complement regulators, such as factor H and/or factor H–like protein 1 (FHL-1) to their surface [3, 4]. Acquisition of these host proteins allows innate immune control and inhibition of the complement cascade at the level of C3 directly on the surface of the pathogen [4].

Factor H and FHL-1, the main human fluid-phase regulators of the alternative pathway of complement, are structurally related and are both encoded by the factor H gene, which is located on chromosome band 1q32 [5]. In their secreted forms, the 2 plasma proteins are exclusively composed of individual protein domains.
Figure 1. Absorption of host immune regulators from human and mouse serum. *Borrelia burgdorferi* strain LW2 bacteria were incubated in human (A and B) or mouse (C) serum or with purified proteins (D). After extensive washing, bound proteins were eluted, separated by SDS-PAGE, and identified by Western blotting using specific antibody or antiserum. Panel A shows that monoclonal antibody VIG8 reacted with the C-terminus of factor H and with factor H–related protein 1 (FHR-1). Panel B shows that anti–SCR1–4 reacted with the N-terminus of factor H and identifies factor H and factor H–like protein 1 (FHL-1). In panel C, factor H antiserum that reacts with mouse factor H and with the 2 FHR proteins present in mouse serum was used. In panel D, *Borrelia* cells were incubated with either recombinant human FHR-1, purified human factor H, or recombinant human FHL-1. Bound proteins were identified in the elute fraction with specific antiserum. The mobility of the marker proteins is indicated.

The 42-kDa FHL-1 protein consists of 7 SCRs, and the 150-kDa factor H protein includes 20 SCRs [6]. The SCRs of FHL-1 are identical to the N-terminal domain of factor H; however, the protein has a unique C-terminal extension of 4 aa [7]. Factor H and FHL-1 are members of the factor H protein family, which in humans includes 6 additional factor H–related proteins (FHR-1, -2, -3, -4A, -4B, and -5) [8]. The FHR proteins represent plasma proteins that are structurally and antigenically related to each other and to factor H and that are transcribed from separate genes. The human FHR-1 protein is composed of 5 SCR domains and exists in 2 forms. The 37-kDa FHR-1α has 1 and the 43-kDa FHR-1β has 2 carbohydrate side chains attached [9]. The 3 most C-terminal SCRs of FHR-1 (SCR3, -4, and -5) display a high homology to SCR18, -19, and -20 of factor H, suggesting similar and conserved function(s). The C-terminal SCRs of both FHR-1 and factor H bind to heparin and C3b, which is indicative of a regulatory role in complement activation [10, 11]. The precise function(s) of the FHR proteins is currently under investigation. FHR genes are conserved in evolution and have been identified in humans, chimpanzees, rats, mice, and the bony fish barred sand bass [12, 13].

Serum-resistant *B. afzelii* as well as moderately serum-resistant *B. burgdorferi* strains express surface proteins that bind the human complement regulators factor H and/or FHL-1 [14]. These borrelial lipoproteins are termed “complement regulator–acquiring surface proteins” (CRASPs). CRASPs have been divided into 2 groups: proteins that bind both factor H and FHL-1 (CRASP-1 and -2 of *B. afzelii* and *B. burgdorferi*) and proteins that exclusively bind factor H (CRASP-4 and -5 of *B. afzelii* and CRASP-3, -4, and -5 of *B. burgdorferi*) [3]. The 5 CRASPs of *B. burgdorferi* form a functionally related but structurally heterologous group of proteins. The factor H– and FHL-1–binding CRASP-1 belongs to the paralogous family gbb54...
proteins (CRASPs) and that of the size markers is indicated. was separated by SDS-PAGE, transferred to a membrane, and probed with FHR-1. The mobility of the various complement regulator–acquiring surface proteins (CRASPs) and that of the size markers is indicated.

In the present study, we have identified FHR-1 as an additional human plasma protein that binds to serum-resistant, but not serum-sensitive, B. burgdorferi strains. Serum resistance of B. burgdorferi correlates with CRASP expression, suggesting that factor H and FHL-1 attach to the microbial surface and regulate complement activation [3, 20].

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Serum-resistant B. burgdorferi strains (LW2 [skin isolate, Germany], B31, ZS7, 297, PkA-1, and N40) as well as serum-sensitive strains (PBi, PSth, G1, G2, 1/B29, and A87Sb) were grown to mid-log phase, harvested by centrifugation (5000 rpm, 8 min, 4°C), and resuspended in 300 μL of PBSA. After incubation with polyclonal antibodies (MAbs) specific for the C-terminus of FHR-1 and factor H (VIG8 and C18), cells were washed. Ali-

**Expression of recombinant FHR-1 and FHL-1.** Recombinant FHR-1 and FHL-1 were expressed in insect cells infected with recombinant baculovirus [7, 11]. Briefly, adherent Spodoptera frugiperda cells (Sf9) grown at 28°C in monolayer cultures were infected with recombinant virus at an MOI of 5.

**Construction of expression plasmids and purification of CRASPs.** Construction and expression of plasmids harboring the cspA, cspZ, and erpP genes encoding for CRASP-1, -2, and -3 was done as described elsewhere [15, 17]. CRASP-4 was amplified using primers ErpC42 (forward; 5′-GCTTTTGATTACATAATTGTTCTCGAGTATT-ACCTCTAA-3′) and ErpC3nc (reverse; 5′-GCTTTTTGATTACATAATTGTTCTCGAGTATT-ACCTCTAA-3′) of strain B31-MI [21]. The amplified DNA fragment was digested with restriction enzymes BamHI and XhoI, resulting in a gene lacking the hydrophobic leader–encoding sequences and ligated in frame into the pGEX-6P-1 vector, which includes a glutathione-S-transferase (GST) tag. Similarly, the gene for CRASP-5 was amplified using primers OspE37 (forward; 5′-GTTTTTATACTCCGGGTCTGCAAAATTCA-3′) and ErpA3nc (reverse; 5′-GTTTTTTATTCA-TATACGCGCCCTCTATAATTCTAAC-3′) and were digested with Smal. The gene lacking the hydrophobic leader sequence was ligated into expression vector pGEX-6P-1. All 5 CRASPs were expressed as GST-fusion proteins in Escherichia coli JM109 and were purified on glutathione-Sepharose, as described elsewhere [17].

**Absorption experiments.** Absorption experiments were performed as described elsewhere [14]. Briefly, B. burgdorferi cells (5 × 10⁶ cells) were grown to mid-log phase, harvested by centrifugation (5000 g for 30 min at 4°C), and resuspended in veronal buffered saline (supplemented with 1 mmol/L Mg²⁺, 0.15 mmol/L Ca²⁺, and 0.1% gelatine [pH 7.4]). B. burgdorferi strain LW2 was incubated either in normal human serum (NHS), 750 μL of mouse serum, or PBS supplemented with purified FHR-1 (2.5 μg), factor H (2.5 μg; Calbiochem), or FHL-1 (2.5 μg).

**Immunofluorescence assay.** B. burgdorferi strain LW2, which expresses all 5 CRASPs, was grown to mid-log phase, harvested, washed, and resuspended in 300 μL of PBS. Spirochetes (2 × 10⁴) were incubated with 5 μg of purified FHR-1, factor H, or FHL-1 for 1 h at room temperature. After 4 washes with PBS containing 0.2% BSA (PBSA), cells were collected and resuspended in PBSA. After incubation with polyclonal anti-serum specific for the N-terminus of factor H and FHL-1 or monoclonal antibodies (MABs) specific for the C-terminus of FHR-1 and factor H (VIG8 and C18), cells were washed. Ali-
Binding of FHR-1 to CRASPs

RESULTS

Binding of FHR proteins derived from human and mouse serum to B. burgdorferi. B. burgdorferi strain LW2 was incubated either in human or mouse serum, and bound proteins were eluted, separated by SDS-PAGE, and analyzed by Western blotting. MAb specific for the common C-terminus of FHR-1 and factor H identified FHR-1, factor H, and FHL-1 in the elute fraction (figure 1A, lane 2). An antiserum reacting with SCR1–4 identified factor H and FHL-1 (figure 1B, lane 2). Because mice represent a common reservoir for B. burgdorferi, mouse serum was used. This approach identified mouse factor H (150 kDa) and 2 murine (m) FHR-proteins, mFHR-C (100 kDa) and mFHR-E (40 kDa), in the elute fraction (figure 1C, lane 2). The results obtained with human serum were confirmed using purified FHR-1, factor H, and FHL-1. All 3 human proteins were detected in the elute fraction (figure 1D, lanes 2, 4, and 6). These experiments demonstrate binding of FHR proteins from human and mouse sources to borrelial cells.

Correlation between binding of human FHR-1 and serum resistance. To analyze whether FHR-1 binding correlates with serum resistance, serum-resistant and serum-sensitive isolates were used. FHR-1 bound to all lysates prepared from serum-resistant strains (LW2, B31, ZS7, 297, PKa-1, and N40) but not from serum-sensitive strains (PBi, P5th, G1, G2, I/B29, and...
A87Sb) (figure 2). The serum-resistant strains express several FHR-1 binding proteins, and on the basis of mobility, it can be concluded that binding occurs via CRASP-3, -4, and -5.

**Binding of human FHR-1 to intact B. burgdorferi.** Immunofluorescence microscopy was used to visualize the distribution on the surface. FHR-1 binding to intact B. burgdorferi strain LW2, which expresses all 5 CRASPs, showed a patchy pattern, indicating a clustering of the borrelial FHR-1 binding surface proteins (figure 3A). Bound factor H and FHL-1 showed a more homogenous distribution over the entire bacterial surface (figure 3B and 3C). When B. burgdorferi was incubated with human serum, the staining pattern suggested that the proteins bind simultaneously to the borrelial surface (figure 3D).

**Binding of human serum proteins FHR-1, factor H, and FHL-1 to CRASPs.** To identify which CRASP binds FHR-1, a combined ELISA and Western blot assay was used, one that allows the identification of the bound host regulators according to their mobility and reactivity. This assay showed weak binding of FHR-1α and FHR-1β to CRASP-3 and strong binding to CRASP-4 and -5 (figure 4A, top). Factor H bound strongly to CRASP-1, -2, and -5 and weakly to CRASP-3 and -4 (figure 4A, middle). In addition, FHL-1 did bind to CRASP-1 and -2 (figure 4A, bottom) but to no other CRASP. Thus, FHR-1, factor H, and FHL-1 bind to several CRASPs and show differential preference for individual CRASPs.

Binding was further assayed by ELISA to quantify and compare the intensity of binding. Purified FHR-1 bound weakly to CRASP-3 and -4 and strongly to CRASP-5. Factor H showed prominent binding to CRASP-1, -2, and -5. FHL-1 bound to CRASP-1 and -2 but no other CRASP (figure 4B).

**Surface plasmon resonance.** Having identified FHR-1 as a ligand for CRASP-3, -4, and -5, the binding profiles of FHR-1 and factor H were compared using surface plasmon resonance. FHR-1 bound to CRASP-3, as indicated by the strong association and dissociation profile (figure 5A). FHR-1 binding to CRASP-3 was more pronounced than factor H binding. When equimolar amounts of FHR-1 were used, CRASP-3 interaction was almost twice as strong as factor H interaction. Factor H dissociation was more rapid, and FHL-1 did not bind to CRASP-3. FHR-1 showed strong binding to CRASP-4 (figure 5B).
Figure 5. Surface plasmon resonance analyses. Individual complement regulator–acquiring surface proteins (CRASPs) were immobilized, and purified host immune regulators factor H–related protein 1 (FHR-1), factor H, and factor H–like protein 1 (FHL-1) were applied in fluid phase. 

A, CRASP-3. FHR-1 bound with strong intensity to CRASP-3, as indicated by the prominent association profile and the slow dissociation profile. Factor H binding was of lower intensity, and FHL-1 binding was low or absent.

B, CRASP-4. FHR-1 interacted strongly with immobilized CRASP-4, as indicated by the prominent association profile and the slow dissociation profile. Factor H binding was rather low, and FHL-1 binding was absent.

C, CRASP-5. FHR-1 bound strongly to immobilized CRASP-5, as indicated by the very prominent association profile and the slow dissociation profile. Factor H binding was of similar intensity, and FHL-1 binding was again low or absent.

5B). With surface plasmon resonance, binding of neither factor H nor FHL-1 to CRASP-4 was detectable. FHR-1 bound strongly to CRASP-5, as demonstrated by the strong association and the stability of the complex after removal of the ligand (figure 5C). Also, factor H bound to CRASP-5, and dissociation of the factor H:CRASP-5 complex was faster than that of FHR-1:CRASP-5 complex. FHL-1 did not bind to CRASP-5. These assays revealed that FHR-1 binds to 3 CRASPs and that the binding is stronger relative to that of factor H.

The affinity for the strongest associating partners—that is, FHR-1 binding to CRASP-5—was analyzed on a kinetic level. On immobilization of CRASP-5 and using FHR-1 from 0 to 15 nmol, the interaction followed a 1:1 model and reached a dissociation constant of $3 \times 10^{-9} \text{ mol/L}$, with an association rate of $3.8 \times 10^3 \text{ 1/ms}$ and a dissociation rate of $1 \times 10^{-3} \text{ 1/s}$.

When CRASP-3 and -4 were immobilized at the same concentration, the interaction was not sufficient to determine kinetic values. Therefore, the interaction between FHR-1 and each of the 3 CRASPs was compared at higher levels, allowing a relative comparison of the association and dissociation rates. FHR-1 showed the strongest association with CRASP-5, and the association with both CRASP-4 and -3 was $<3.8 \times 10^3 \text{ 1/ms}$. Similarly, the interaction between FHR-1 and CRASP-3 was the most stable, with a dissociation rate $<1 \times 10^{-3} \text{ 1/s}$.

**Competition between FHR-1 and factor H for CRASP binding.** The binding preferences of FHR-1 and factor H to CRASP-3, -4, and -5 were compared. When increasing amounts of FHR-1 and a constant concentration of factor H was used, simultaneous binding of both proteins was detected. Binding of factor H was detected with the factor H–specific MAb B22, and binding of both FHR-1 and factor H was detected with MAb VIG8. For CRASP-3, an increase in the concentration of FHR-1 resulted in decreased reactivity of the factor H–specific MAb and in increased reactivity of MAB VIG8, which reacts with both proteins (figure 6A). This pattern shows that CRASP-3 binds both proteins simultaneously. When a ratio reflecting the physiological condition was used (FHR-1:factor H ratio of 0.37:1), FHR-1 and factor H binding was comparable. Again, FHR-1 bound to CRASP-4, but factor H binding was weak (figure 6B; note the different scales of the Y-axes). Both FHR-1 and factor H also bound to CRASP-5 under the physiological setting. Increasing FHR-1 concentrations influenced factor H binding; thus, FHR-1 and factor H seem to share the same binding site(s) (figure 6C).

**Cofactor activity of factor H bound to CRASPs.** Factor H displays cofactor activity when bound to CRASP-1, -2, or 3 [15, 16]. To link binding with regulation, the cofactor activities of factor H bound to various CRASPs were compared. Prominent cofactor activity, demonstrated by the appearance of $a'68$, $a'46$, and $a'43$, was observed for factor H bound to CRASP-1 and -5 (figure 7A, lanes 1 and 5). These cleavage products
Figure 6. Binding competition. The indicated complement regulator–acquiring surface proteins (CRASPs) were immobilized on microtiter plates, and the binding of factor H–related protein 1 (FHR-1) and factor H was analyzed by ELISA. Binding of factor H was determined in the absence of FHR-1. On addition of increasing amounts of FHR-1 (molar ratios are shown), binding was visualized with antibody specific for factor H (monoclonal antibody [MAb] B22) or with MAb VIG8, which reacts with the C-terminus of both FHR-1 and factor H. A, CRASP-3. FHR-1 competed with factor H for binding, as indicated by the reduction of factor H–specific MAb B22 reactivity when FHR-1 was added. In addition, the reactivity of MAb VIG8 was increased. B, CRASP-4. The FHR-1:factor H ratio of 0.37:1 reflects the physiological condition. C, CRASP-5. Factor H bound strongly to CRASP-5, as indicated by the high absorbency. However, when FHR-1 was added, factor H binding decreased, as indicated by the reduction of factor H–specific MAb B22 reactivity, and MAb VIG8 increased. Note the different scales of the Y-axes.

appeared at lower levels when factor H was bound to CRASP-2 (lane 2) and -3 (lane 3). Thus, factor H–mediated regulatory activity does directly correlate with the binding intensity of CRASPs.

CRASP-5 binds the host complement regulator factor H, but it also binds FHR-1, which lacks complement regulatory activities. Previously, a cofactor enhancing activity was demonstrated for FHR-1 [25]. Thus, we analyzed how FHR-1 modulates the cofactor activity of CRASP-5–bound factor H. Factor H was bound in the presence of increasing amounts of FHR-1, and cofactor activity was analyzed. FHR-1, which competes with factor H for binding, decreased cofactor activity in a dose-dependent manner. Reduced cofactor activity resulting in less C3b cleavage was indicated by the intensity of the α43 band (figure 7B). Quantitative analyses of the degradation product iC3b showed that, at equal molar FHR-1:factor H ratios, degradation was reduced by 21%, and, at a 2:1 ratio, complement inhibition was reduced by 50% (data not shown). Thus, FHR-1 inhibits the regulatory functions of CRASP-5–bound factor H.

DISCUSSION

Borrelia species express different CRASPs, and CRASP surface expression is associated with serum resistance. Here, we have shown that FHR-1, an additional human plasma protein, binds to the surface of intact serum-resistant, but not serum-sensitive, B. burgdorferi strains. Recombinant FHR-1 binds to CRASP-3 (ErpP), -4 (ErpC), and -5 (ErpA). Thus, each CRASP binds at least 2 members of the factor H family: CRASP-3, -4, and -5 bind FHR-1 and factor H, and CRASP-1 and -2 bind factor H and FHL-1. This preference suggests that, on infection, B. burgdorferi acquire simultaneously 3 proteins of the factor H family and that this type of surface decoration facilitates immune escape of the pathogen and survival in the immunocompetent host.

Human FHR-1 and, similarly, mFHR-C and mFHR-E bind to borrelial strain LW2 (figure 1C) [5]. The 100-kDa mFHR-C, the 40-kDa mFHR-E, and mouse factor H bind to intact B. burgdorferi (figure 1C). Binding of mouse factor H to CRASP-orthologous Erp and OspE proteins has been shown recently [4]. Binding of human and mouse FHR proteins, which seem to be enriched on the borrelial surface (figure 1C), suggests a role for human FHRs and mFHRs in immune evasion. Currently, the biological functions of the FHRs are unknown [26, 27]. FHR-1 has a plasma concentration of ∼50 μg/mL, is a constituent of lipoprotein particles, and binds heparin and the central complement components C3b and C3d [9, 11, 22, 28–30].

Serum-resistant Borrelia strains express different numbers and combinations of CRASPs. Each CRASP binds at least 2 factor H family proteins and has a preference for 1 host regulator. CRASP-1 preferentially binds factor H, CRASP-2 preferentially binds FHL-1, CRASP-3 and -4 show a preference for
FHR-1, and CRASP-5 binds FHR-1 and factor H at similar levels. These preferences correlate with the inhibitory potential: prominent cofactor activity is observed for factor H attached to CRASP-1 and CRASP-5 (figure 7). FHR-1 lacks cofactor activity (data not shown) but competes with factor H for binding to 3 CRASPs and thereby inhibits the complement regulatory activity of factor H. When FHR-1 is present in 2-fold molar excess to factor H, complement inactivation is reduced by 50%. Thus, local expression and distribution of the 2 regulators does clearly affect complement inactivation. It has been reported previously that, in fluid phase, FHR-1 enhances cofactor activity of factor H [25]. Although both regulators bind to CRASP-5, CRASP-5–bound FHR-1 lacks complement enhancing activity but shows an inhibitory role. Because FHR-1 negatively affects the complement inhibitory activity of factor H, Borrelia species, which express multiple factor H–binding proteins, may have an advantage on contact with plasma. Thus, because FHR-1 modulates the complement regulatory activity, surface decoration with various host regulatory proteins influences C3b inactivation and immune evasion.

Figure 7. Complement regulatory activity of complement regulator–acquiring surface protein (CRASP)–bound factor H. Panel A shows that attached factor H retains cofactor activity and that this activity correlates with binding intensity. The indicated CRASPs or an unrelated control protein (p65) were immobilized on microtiter plates. Factor H, C3b, and factor I were added, and, after incubation for 15 min at 37°C, the mixture was separated by SDS-PAGE; C3b degradation fragments were then analyzed by Western blotting. The mobility of the α′ chain and the β chain of C3 and the cleavage products of the α′ chain—α′68, α′46, and α′43—are indicated. Panel B shows that factor H–related protein 1 (FHR-1) influences the cofactor activity of factor H bound to CRASP-5 in a dose-dependent manner (molar ratios are shown).
An increasing number of human pathogens are being identified that express surface molecules that bind host complement regulators of the factor H protein family (i.e., factor H, FHL-1, and FHR-1). In addition to B. burgdorferi and B. garinii, gram-positive Streptococcus pyogenes and S. agalactiae bind factor H via the bacterial surface proteins streptococcal M, Fba, and β protein [31, 32], and S. pneumoniae does so via the PspC and Hic surface proteins [33, 34]. In the gram-negative bacteria Neisseria gonorrhoeae and Hic surface proteins [33, 34]. It was recently shown that Trepomonema denticula bind FHL-1 [37]. For several proteins, the factor H and/or FHL-1 binding sides have been mapped either to the C-terminus (e.g., β protein; CRASP-1, -3, and -5; and the OspE paralogs p21, ErpA, and ErpC) or the N-terminus (e.g., M5, M6, Fba, PspC, and Hic) [27].

In summary, we have identified human FHR-1 as well as mFHR-C and mFHR-E as additional host plasma proteins that bind to the surface of borrelia. Binding of human FHR-1 is mediated by 3 CRASPs (CRASP-3 [ErpP], -4 [ErpC], and -5 [ErpA]). FHR-1 seems to be relevant for immune evasion of the pathogen, given that FHR-1 competes with factor H for binding and thus modulates the regulatory activity of factor H in complement control. Apparently by decorating its surface with distinct host proteins, the pathogen can modulate and fine-tune its immune escape during infection and in different hosts.

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References