Role of Hcp, a type 6 secretion system effector, of Aeromonas hydrophila in modulating activation of host immune cells

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Recently, we reported that the type 6 secretion system (T6SS) of Aeromonas hydrophila SSU plays an important role in bacterial virulence in a mouse model, and immunization of animals with the T6SS effector haemolysin co-regulated protein (Hcp) protected them against lethal infections with wild-type bacteria. Additionally, we showed that the mutant bacteria deleted for the vasH gene within the T6SS gene cluster did not express the hcp gene, while the vasK mutant could express and translocate Hcp, but was unable to secrete it into the extracellular milieu. Both of these A. hydrophila SSU mutants were readily phagocyted by murine macrophages, pointing to the possible role of the secreted form of Hcp in the evasion of the host innate immunity. By using the ΔvasH mutant of A. hydrophila, our in vitro data showed that the addition of exogenous recombinant Hcp (rHcp) reduced bacterial uptake by macrophages. These results were substantiated by increased bacterial virulence when rHcp was added along with the ΔvasH mutant in a septicaemic mouse model of infection. Analysis of the cytokine profiling in the intraperitoneal lavage as well as activation of host cells after 4 h of infection with the ΔvasH mutant supplemented with rHcp indicated that this T6SS effector inhibited production of pro-inflammatory cytokines and induced immunosuppressive cytokines, such as interleukin-10 and transforming growth factor-β, which could circumvent macrophage activation and maturation. This mechanism of innate immune evasion by Hcp possibly inhibited the recruitment of cellular immune components, which allowed bacterial multiplication and dissemination in animals, thereby leading to their mortality.

INTRODUCTION

Aeromonas hydrophila is among the most common species associated with wound and soft tissue infections, gastroenteritis and septicemia in the host (Chopra & Houston, 1999; Janda & Abbott, 1998). Our laboratory group and others have characterized several virulence factors from Aeromonas species, which are secreted via the type 2 and type 3 secretion systems (Carvalho-Castro et al., 2010; Chopra et al., 2006; Sha et al., 2005, 2007; Sierra et al., 2007; Tan et al., 2008; Vilches et al., 2009). Our most recent studies are focused on the type 6 secretion system (T6SS) and its effectors from diarrhoeal isolate SSU of A. hydrophila (Suarez et al., 2008, 2010). Although the T6SS is highly conserved in Proteobacteria, the general mechanism as to how this system operates remains poorly understood (Das & Chaudhuri, 2003).

A. hydrophila SSU has one chromosomally located T6SS gene cluster which is regulated by the σ54 activator encoded by the virulence-associated gene, vasH (Suarez et al., 2008). We reported that the ΔvasH mutant of A. hydrophila SSU was unable to express genes encoding haemolysin-coregulated protein (Hcp) and its effectors from diarrhoeal isolate SSU of A. hydrophila (Suarez et al., 2008, 2010). Although the T6SS is highly conserved in Proteobacteria, the general mechanism as to how this system operates remains poorly understood (Das & Chaudhuri, 2003).

A supplementary figure, showing the effect of Hcp and rHcp on bacterial growth and murine macrophages, is available with the online version of this paper.
2008). Importantly, both Hcp and VgrG proteins represent a hallmark of the T6SS secreted proteins in all of the bacteria that possess this system (Bingle et al., 2008; Cascales, 2008; Filloux et al., 2008; Pukatzki et al., 2009). Although the role of VgrG1 in bacterial virulence was convincingly demonstrated recently by us in A. hydrophila SSU (Suarez et al., 2010) and V. cholerae (Ma et al., 2009; Ma & Mekalanos, 2010), the mechanism of Hcp in modulating the organism’s virulence is poorly understood.

Innate immunity is the first line of host defence against the challenged organisms, and pattern recognition receptors sense different microbial ligands (known as pathogen-associated molecular patterns, PAMPs) (Barton & Medzhitov, 2003; Janeway & Medzhitov, 2002; Medzhitov & Janeway, 1999; Taylor et al., 2005), resulting in the triggering of signalling cascades that determine the host immune response by modulating maturation, activation and recruitment of cellular effectors [e.g. neutrophils, macrophages, dendritic cells (DCs) and natural killer cells] (Henneke & Golenbock, 2004; Hume, 2006; Medzhitov & Janeway, 1999; Pflüdemann et al., 2006).

Phagocytosis is crucial for both innate and adaptive immunity (Coombes et al., 2004; Henneke & Golenbock, 2004; Medzhitov & Janeway, 1999), and macrophages and DCs are professional antigen-presenting cells that act as tissue sentinels and are able to present antigens to naïve T-cells (Gordon & Taylor, 2005; Hume, 2006, 2008). Bacteria have developed different mechanisms to avoid innate immunity ranging from their ability to avoid recognition by toll-like receptor-4 (Ernst et al., 1999; Hajjar et al., 2002; Kawasaki et al., 2004), altering antigenicity of surface molecules to avoid phagocytosis (Seifert, 1996), interfering with mitogen-activated protein kinase signalling cascades (Park et al., 2002; Sweet et al., 2007; Thieffs et al., 2006), modulating actin polymerization and apoptosis (Abrahams & Hensel, 2006; Pujol & Bliska, 2005; Rückdeschel et al., 2002; Viboud & Bliska, 2005), manipulating phagosomal trafficking and maturation (Dramsi & Cossart, 2002; Sturgill-Koszycki et al., 1994; Uchiya et al., 1999), and inducing the production of immunosuppressive cytokines, particularly interleukin (IL)-10. The latter mechanism avoids activation of macrophages, maturation of DCs and recruitment of granulocytes (McGuirk et al., 2002; Sing et al., 2002; Zuaney-Amorim et al., 2002).

Previously, we demonstrated that A. hydrophila SSU ΔvasH and ΔvasK mutants were easily phagocytosed by murine RAW 264.7 macrophages compared with the phagocytosis of wild-type (WT) bacteria (Suarez et al., 2008). Further, we showed that the ΔvasH mutant strain did not express the gene encoding Hcp and that the ΔvasK mutant was able to produce Hcp, but was unable to secrete it (Suarez et al., 2008). Hence, we hypothesized that the secreted form of Hcp could be playing a role in inhibition of phagocytosis of A. hydrophila SSU by macrophages. Here, we report that Hcp indeed played a role in modulating the innate immunity by inhibiting the phagocytosis of A. hydrophila SSU, thus allowing its multiplication and spread to different organs of the host. Our results show that Hcp is able to bind to macrophages and induce the production of IL-10 and transforming growth factor (TGF)-β, affecting the activation and maturation of macrophages, and, consequently, the recruitment of other cellular immune components needed to clear bacterial infection.

**METHODS**

**Cell line and bacterial strains.** RAW 264.7, a murine macrophage cell line, was maintained in Dulbecco’s modified eagle medium with high glucose (DMEM) (Invitrogen), supplemented with 10% fetal bovine serum (FBS) under standard cell culture growth conditions.

*A. hydrophila* SSU Δact (act encodes the type 2 secretion system-secreted cytotoxic enterotoxin) (Xu et al., 1998) and Δact/ΔvasH isogenic mutant strains were developed in the laboratory as described previously (Suarez et al., 2008). These mutants were grown in LB medium supplemented with kanamycin (50 μg ml⁻¹) for *A. hydrophila* Δact, and kanamycin, streptomycin (40 μg ml⁻¹) and spectinomycin (50 μg ml⁻¹) for the *A. hydrophila* Δact/ΔvasH mutant.

**Recombinant Hcp protein.** The hcp (hcp2 residing within the T6SS cluster) gene was cloned in the pET-30a vector for the production of recombinant protein as reported previously (Suarez et al., 2008). rHcp containing a 6 × His tag was purified by nickel affinity chromatography, dialysed against PBS, and then passed through a polymyxin column (Bio-Rad) to remove any residual lipopolysaccharide (LPS). The pass-through fraction was filtered by using a 0.2 μm filter, and the protein concentration was quantified by using the Bradford assay (Bio-Rad). The removal of LPS from rHcp was verified by the Limulus amebocyte lysate assay (Pyrosate-Dial Medical Supply), and the purity of rHcp was verified by Coomassie Blue staining of the gel.

**Cell viability.** To determine host cell viability (RAW 264.7 cells treated with 10 μg rHcp ml⁻¹ for different time periods), we performed the 7-amino actinomycin D (7-AAD) (Becton Dickinson) and the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) (Chemicon) assays, as described previously (Sierra et al., 2007).

**Conditioned medium.** To obtain conditioned medium containing Hcp, we grew the Δact mutant strain of *A. hydrophila* SSU (1 × 10⁶ c.f.u.) in 3 ml DMEM supplemented with 0.5 % FBS at 37 °C. After 2 h, the bacteria were removed by centrifugation, and the supernatant was passed through a 0.2 μm membrane filter. The resulting medium was tested by Western blot analysis for the presence of Hcp by using specific antibodies, and used immediately in the phagocytosis assay.

**Western blot analysis.** Western blot analysis was performed to detect Hcp in the conditioned medium collected after infection of RAW 264.7 cells with various strains of *A. hydrophila*. Briefly, tissue culture supernatant was filtered by using a 0.2 μm filter to remove bacteria; proteins in the supernatant were separated by using 15% SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane which was blocked with 5% skimmed milk +1% BSA overnight at 4 °C. Mouse–anti-Hcp serum was used at a dilution of 1 : 2000 for 1 h, and horseradish peroxidase-conjugated, goat–anti-mouse antibodies were used at a dilution of 1 : 5000 for 45 min. The blot was then developed by using ECL-Western blotting detection reagent (GE Healthcare).

**Phagocytosis assay.** Briefly, 1.5 × 10⁶ RAW 264.7 cells suspended in 1 ml DMEM supplemented with 0.5 % FBS were split into aliquots in polypropylene tubes (12 × 75 mm). Different bacterial strains were added at an m.o.i. of 10 for 30 min. Then, the cell suspension was
centrifuged at 250 g for 5 min and the supernatant was removed. The pellet was resuspended in 500 μl of DMEM plus 0.5 % FBS (above) and 250 μg gentamicin ml⁻¹ (CellGro) to kill extracellular bacteria. After 2 h, the macrophages were washed three times with 1 ml DMEM to remove dead bacteria and gentamicin. The host cells were lysed in 500 μl sterile water, and the cell lysates were plated on LB agar plates containing kanamycin (50 μg ml⁻¹) to determine c.f.u. (Suarez et al., 2008). We examined complete lysis of the host cells under the microscope before plating the cell lysates.

**Animal survival.** Groups of 10 Swiss Webster female mice (Charles River) were challenged via the intraperitoneal (i.p.) route with a sublethal dose of *A. hydrophila Δact/ΔvasH* mutant, either alone or in combination with different amounts of rHcp (1–10 μg). As a control, a group of mice was challenged with either the highest dose of rHcp (10 μg) or the Δact parental strain. The survival of mice was followed for 16 days post-infection.

**Bacterial spread.** Mice were infected via the i.p. route with a sublethal dose of *A. hydrophila Δact/ΔvasH* mutant alone or in combination with rHcp (10 μg). The animals were euthanized after 48 h, and sections of liver, spleen and lungs were homogenized and used to determine bacterial burden (Agar 48 h, and sections of liver, spleen and lungs were homogenized and used to determine bacterial burden (Agar et al., 2009).

**Intraperitoneal lavage.** After 4 h of challenge of mice via the i.p. route with a sublethal dose of *A. hydrophila Δact/ΔvasH* mutant alone or in combination with rHcp, the animals were euthanized, and the peritoneal cavity was flushed with 1.5 ml sterile Hanks’ solution (Invitrogen). The lavage was centrifuged and centrifuged at 250 g for 5 min. The supernatant was used for evaluating various cytokines/chemokines by the multiplex bead array, and the pellets containing host cells were used to determine their phenotypes by flow cytometry (see below).

**Hcp-binding assay.** The whole cell population from the intraperitoneal lavage of mice was incubated with rHcp (10 μg ml⁻¹) for 1 h. Then, the cells were incubated for 30 min with anti-mouse CD16/ CD32 antibodies (Becton Dickinson). Next, the cells were dispensed into different tubes and incubated with pre-immune mouse serum (1:100) as an isotype control, or with the mouse anti-Hcp serum (1:100) for 1 h. Subsequently, the host cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse antibodies (Invitrogen) for 45 min, followed by incubations with PE-Cy5-conjugated anti-mouse F4/80, PE-conjugated anti-mouse Gr-1 antibodies, and their respective isotype controls. Between steps, the cells were washed twice with chilled Hanks’ solution, and all the incubations were performed on ice. The cells were acquired in a FACScanto (Becton Dickinson) flow cytometer and analysed by using FACSdiva software (Becton Dickinson).

**Quantification of Hcp produced by the Δact mutant of *A. hydrophila SSU*.** To determine the amount of Hcp produced by the parental *A. hydrophila* strain in the mouse peritoneum after 4 h of infection, we infected animals via the i.p. route with *A. hydrophila* parental strain in the mouse peritoneum after 4 h of infection, indicating physiological relevance of the rHcp doses used in this study (Fig. 1c). Our positive and negative controls in this assay included macrophages infected with the Δact and Δact/ΔvasH strains levels by a multiplex bead array (Millipore) following the manufacturer’s instructions. The data were acquired and analysed in a Bioplex 200 system which uses Bioplex manager software v.5.0 (Bio-Rad).

**Flow cytometry.** Cell pellets collected after intraperitoneal lavages of mice (see Intraperitoneal lavage) were stained with a panel of antibodies conjugated with FITC (anti-CD11c, anti-CD69), PE (anti-Gr-1, anti-MHC-class II) and PE-Cy5 (anti-F4/80) for flow cytometry (eBiosciences). The cells were incubated with antibodies for 1 h and then washed twice with 1 ml PBS and fixed with 2 % parafomaldehyde. The cells were acquired in a FACScan and analysed by FACSDiva software (Becton Dickinson). Prior to incubation with antibodies, receptors Fcγ III/II were blocked using anti-CD16/CD32 antibodies (BD Pharmingen) for 30 min.

**Statistical analysis.** The data were analysed by one-way ANOVA and Tukey’s post-test with GraphPad Prism version 4 software, and the animal survival curves were analysed by the Logrank test. At least three independent experiments were performed to represent biological replicates, unless otherwise stated.

**RESULTS**

**Hcp inhibits the phagocytosis of *A. hydrophila SSU Δact/ΔvasH* mutant in vitro**

Since the secretion of Hcp was affected in both the ΔvasH and ΔvasK mutant strains (Suarez et al., 2008), we hypothesized that the secreted form of Hcp could be playing a role in the bacterial inhibition of phagocytosis. To test this, we used conditioned medium from the Δact parental strain of *A. hydrophila* which contained Hcp (Fig. 1a) in the phagocytosis assay. Consequently, RAW 264.7 murine macrophages were infected with the Δact/ΔvasH mutant in the conditioned medium, thus allowing complementation of the mutant with exogenous Hcp protein. We noted that the ability of the *A. hydrophila Δact/ΔvasH* mutant to be phagocytosed by macrophages was reduced in the presence of Hcp (Fig. 1b). To confirm that this effect was indeed due to Hcp and not to other secreted bacterial proteins, we performed a phagocytosis assay by using the Δact/ΔvasH mutant in conjunction with different concentrations of purified rHcp. We found that phagocytosis of the mutant by macrophages was inhibited by rHcp in a dose-dependent fashion, with statistically significant differences observed at rHcp doses of 5.6 μg and greater (Fig. 1c) compared with mutant bacteria (Δact/ΔvasH) alone without rHcp.

To determine whether the doses of rHcp necessary to inhibit phagocytosis in our in vitro experiments were physiologically relevant, we measured the amounts of Hcp produced by the parental Δact mutant strain of *A. hydrophila* in the mouse peritoneum and we found 8–10 μg Hcp after 4 h of infection, indicating physiological relevance of the rHcp doses used in this study (Fig. 1c).
of A. hydrophila, respectively. As expected, the former showed minimal phagocytosis, while the latter exhibited much higher levels of phagocytosis by macrophages. These data clearly indicate that the secreted Hcp plays a role in bacterial phagocytosis. We confirmed that the difference in phagocytosis between the parental Δact strain of A. hydrophila and that of the Δact/ΔvasH mutant was not due to differences in their growth rates (Supplementary Fig. S1a, available with the online version of this paper) or to toxic effects of rHcp on RAW 264.7 cells for at least up to 6 h, as determined by MTT and 7-AAD assays (Supplementary Fig. S1b, c), and as well as by microscopic visualization of host cells (data not shown). The purity of rHcp was evaluated by Coomassie-Blue-staining of the gel (Fig. 1c, inset). In addition, we tested rHcp for any residual LPS and found less than 1 endotoxin unit in 10 μg of the recombinant protein, which has no influence on macrophage activation.

Hcp decreases the survival rate of mice infected with the Δact/ΔvasH mutant of A. hydrophila SSU

Since the secreted form of Hcp decreased bacterial phagocytosis, and we showed earlier that mice infected via the i.p. route with the ΔvasH mutant had a better survival rate compared with mice infected with the WT strain (Suarez et al., 2008), we hypothesized that bacteria producing Hcp would have a better chance of evading the innate immunity and causing systemic effects. Hence, we challenged mice with a sublethal dose of the Δact/ΔvasH mutant together with rHcp at different concentrations and monitored deaths for 16 days. We found that the addition of rHcp decreased the survival rates of mice after infection with the Δact/ΔvasH mutant, with 100% of the mice dying with 5 and 10 μg of rHcp (Fig. 2). These data indicated complementation of the above mutant with exogenous rHcp in terms of bacterial virulence.

Hcp enhances the spread of the Δact/ΔvasH mutant of A. hydrophila SSU in a mouse model

Since our data showed that the presence of rHcp increased the death rate of mice infected with sublethal doses of the Δact/ΔvasH mutant, we evaluated the bacterial load in mouse organs after 48 h of infection in the presence of rHcp. Our results indicated that rHcp allowed the mutant bacteria to spread more efficiently to different organs (lungs, livers and spleens) possibly resulting in the animals’ death due to a systemic effect (Fig. 3). These bacterial numbers in different organs of mice infected with the Δact/ΔvasH mutant in the presence of rHcp were very similar to their numbers reported recently from mouse organs after infection with the Δact parental strain of A. hydrophila (Sierra et al., 2010). Our results supported the prediction that Hcp acted in innate immunity by avoiding bacterial clearance in the peritoneal cavity by phagocytosis, thus allowing organisms to multiply and spread to various organs.
Hcp binds to intraperitoneal immune cells of mice

Our earlier study showed that Hcp present in the conditioned medium of the WT strain of A. hydrophila SSU was able to bind RAW 264.7 macrophages (Suarez et al., 2008). To confirm that Hcp also binds to primary macrophages, we collected whole cell populations from the peritoneal cavity of naïve mice and, after incubating them with rHcp ex vivo, we performed a multicoloured flow cytometry analysis by using antibodies against Hcp, F4/80 and Gr-1. Fig. 4(a) shows the forward scatter versus the side scatter plot from the whole cell population isolated after the lavage. For analysis, we evaluated rHcp binding to cells gated on F4/80 (macrophages) (Fig. 4b) and on Gr-1 (granulocytes) (Fig. 4c). As can be seen, rHcp was able to bind macrophages (Fig. 4d) and granulocytes (Fig. 4e), although the differential shift of the curve for granulocytes indicated that Hcp binding to this particular cell type was much less compared with its isotype control and that for the macrophages.

Hcp modulates the expression of activation markers on intraperitoneal immune cells

Since our data indicated that rHcp binds to cells involved in the innate immune response, we analysed, by flow cytometry, the status of these cells after 4 h of infection with the Δact/ΔvasH mutant of A. hydrophila given along with two different doses of rHcp. Specifically, we analysed changes in the percentage of macrophages (F4/80), granulocytes (Gr-1) and DCs (CD11c). In addition, we examined the expression of CD69 (an early activation marker) and the major histocompatibility complex (MHC) class II. As controls, we used lavages from uninfected (given only PBS) mice, infected with the Δact/ΔvasH mutant alone along with PBS, and injected via the i.p. route with rHcp alone.

We noted a significant increase in the percentage of Gr-1-positive cells in the peritoneal cavity of animals infected with the Δact/ΔvasH mutant of A. hydrophila when compared with the uninfected mice (Fig. 5a). However, this difference was independent of the presence of rHcp. Additionally, our results showed no significant changes in the percentage of macrophages and DCs in the peritoneal cavity after 4 h of infection, although we noted marginal increases in CD11c-positive cells (not statistically significant) related to the increasing concentrations of rHcp (Fig. 5a).

We then analysed the expression of CD69 and MHC class II in the total cell population as well as in cells gated for F4/80, Gr-1 and CD11c after cells were infected with the Δact/ΔvasH mutant and given rHcp. As shown in Fig. 5(b), the percentage of CD69 in F4/80- and Gr-1-positive cells
decreased, and these decreases were dependent on the dose of rHcp used. Likewise, the percentage of MHC class II-positive cells in F4/80 cells decreased after mice were infected with the $\Delta act/\Delta vasH$ mutant and given the higher dose of rHcp. However, the expression of MHC-class II showed an increase in CD11c cells (with 10 µg rHcp), although the data were not statistically significant (Fig. 5c). Likewise, injection of rHcp alone did not significantly change the percentage of CD69 and MHC class II in any of the cell types analysed compared with the uninfected group of mice.

**Hcp modifies cytokine/chemokine production profiles induced by the A. hydrophila SSU $\Delta act/\Delta vasH$ mutant**

Since rHcp modulates the expression of activation markers on macrophages, granulocytes and DCs, we analysed cytokine/chemokine patterns induced by the $\Delta act/\Delta vasH$ mutant in the peritoneal cavity after 4 h of infection (Table 1 and Fig. 6). We found that infection of mice with the mutant induced the production of a wide range of pro- and anti-inflammatory cytokines/chemokines and growth factors. However, the addition of rHcp, along with the $\Delta act/\Delta vasH$ mutant, during infection significantly modified the levels of some of these cytokines/chemokines. Thus, the concentrations of IL-2, IL-10, IL-15, IL-12p70, macrophage inflammatory protein (MIP)-1β, MIP-2, granulocyte colony-stimulating factor (G-CSF), IL-6, and keratinocyte-derived chemokine (KC) were increased by adding rHcp; however, the production of gamma interferon (IFN-γ), IL-1α and macrophage colony-stimulating factor (M-CSF) was inhibited (Table 1 and Fig. 6). On the other hand, intraperitoneal lavages from mice injected with rHcp alone showed low or non-detectable levels of cytokines/chemokines (Table 1).

**Hcp induces an alternative pathway of macrophage activation**

Activation of macrophages is an important mechanism in innate immunity against foreign invading organisms. There are two types of macrophage activation. M1 or classical activation is mainly a pro-inflammatory or T helper (Th)1-like response focused on removal and clearing of the infection or debris produced by an injury, and M2 or...
Fig. 5. rHcp modulates the levels of activation markers on intraperitoneal innate immune cells of mice. Flow cytometric analysis of intraperitoneal cells recovered from lavages 4 h after infection with 3×10^7 c.f.u. A. hydrophila SSU Δact/ΔvasH mutant in the presence of different doses of rHcp (1 and 10 μg). The PBS group represents animals challenged with only bacteria and PBS in place of rHcp. (a) Percentage of positive cells for F4/80 (macrophages), Gr-1 (granulocytes) and CD11c (DCs). (b) and (c) Percentage of positive cells expressing CD69 (b) and MHC class II (c) for the total population or for cells gated for F4/80, Gr-1 or CD11c. Statistically significant differences were measured by the one-way ANOVA test followed by Tukey’s test. *P<0.05; **P<0.01. Error bars indicate sd.

alternative activation is focused more on tissue remodelling and wound healing. The latter is characterized by a low infiltration of cellular components and a Th2-like cytokine phenotype. Our data indicated that the presence of rHcp during infection with the Δact/ΔvasH mutant could modulate an alternative pathway of macrophage activation, which is supported by decreases in some pro-inflammatory cytokine levels, such as IFN-γ and IL-1α, and increases in anti-inflammatory cytokines, such as TGF-β, IL-6, IL-9 and IL-10 (Table 1 and Fig. 6). These results were also supported by the low levels of M-CSF and tumour necrosis factor (TNF)-α induced by infection with the Δact/ΔvasH mutant in mice, which remained unaltered in the presence of rHcp (Table 1 and Fig. 6). Taken together, these results correlated with the minimal macrophage recruitment in the peritoneal cavity of mice after infection with the Δact/ΔvasH mutant of A. hydrophila SSU (Fig. 5a, F4/80 panel).

DISCUSSION

In this report, we described the role of the secreted form of Hcp in modulating the innate host immune response, specifically by inhibiting phagocytosis by macrophages. It has been reported that some bacterial proteins are able to interfere with phagocytosis by inducing host cell apoptosis, especially via caspase-1 activation (Abrahams & Hensel, 2006; Diacovich & Gorvel, 2010). We also showed that episomal expression of the hcp gene in epithelial cells induced apoptosis (Suarez et al., 2008); however, there are no available data to show whether extracellular Hcp has any effect on the viability of eukaryotic cells. Based on 7-AAD and MTT assays, we did not detect any significant toxicity in macrophages which could be associated with rHcp until up to 24 h of incubation, except for a small increase in mitochondrial activity (measured by MTT) detected after 2 h of incubation. We believe this initial increase was due to the activation of macrophages by rHcp. Also, we found an increase in the percentage of 7-AAD-positive cells (~6% of the total population) as well as a decrease in the mitochondrial activity at 24 h that could be related to cell death after stimulation with rHcp. Since our experiments did not require incubation times longer than 4 h, we consider that the inhibition of phagocytosis in vitro due to Hcp present in the medium was independent of any toxic effects of Hcp on macrophages. Additionally, growth curves of the A. hydrophila Δact and Δact/ΔvasH mutant strains were similar, indicating that differences in phagocytosis of the A. hydrophila Δact/ΔvasH mutant compared with that of its parental strain were not related to any differences in growth rates caused by the deletion of the vasH gene.

There are several examples of bacterial proteins that target phagocytosis at different levels in order to establish an infection. Our results showed that the secretion of Hcp into the extracellular medium played an important role in inhibiting innate immunity mediated by macrophages in vitro. Similarly, the presence of rHcp during infection increased bacterial virulence and allowed bacterial spread to different mouse organs after infection with the A. hydrophila Δact/ΔvasH mutant. These data indicated that the increased virulence of this mutant in the presence of Hcp was associated with the inhibition of phagocytosis.

Macrophages, immature DCs and granulocytes are the main components of innate immunity and the first line of defence against invading organisms. Previously, we reported that the secreted form of Hcp binds RAW 264.7 macrophages (Suarez et al., 2008). In this report, we extended this observation and tested the binding of rHcp to primary intraperitoneal cells. We found that rHcp bound mainly to macrophages, although some binding to granulocytes was
also detected. We speculate that differences in Hcp binding between macrophages and granulocytes could be related to differences in the Hcp-binding receptor(s) on these two cell types. Additionally, we tested binding of rHcp to human epithelial cell lines, such as HT-29 and HeLa. We found that rHcp bound to HeLa and HT-29 cells at levels that were five and nine times lower, respectively, compared with RAW cells (data not shown). Together, these data suggest that macrophages are the major cell target for Hcp.

Analysis of cytokine/chemokine levels in peritoneal lavages showed increases, dependent on the dose of rHcp used, in those involved in granulocyte/neutrophil recruitment and maturation, such as G-CSF, KC and MIP-2. These data may suggest that Hcp does not affect the recruitment of granulocytes in the peritoneal cavity of mice after infection with the Δact/ΔvasH mutant; however, reduction in CD69 levels on the surface of granulocytes in an rHcp dose-dependent manner indicates that this T6SS effector could have an effect on granulocyte activation.

The percentage of DCs (CD11c) was somewhat increased in mice infected with the Δact/ΔvasH mutant in the presence of rHcp. Importantly, these cells also showed

### Table 1. Cytokine/chemokine levels in the peritoneal lavages of mice

Concentrations are in pg ml⁻¹; mean ± SD. ND, Not detected.

| Cytokine/chemokine* | A. hydrophila SSU Δact/ΔvasH mutant | rHcp (10 μg) |
|---------------------|-------------------------------------|-------------|
|                     | + PBS                                | + rHcp (10 μg) |
| Eotaxin             | 1018 ± 38                           | 1088 ± 35   | 120 ± 38 |
| G-CSF               | 126849 ± 18670                      | 184895 ± 11335† | 434 ± 108 |
| GM-CSF              | 128 ± 12                            | 142 ± 24    | 13 ± 2  |
| IFN-γ               | 414 ± 70                            | 183 ± 59§   | ND      |
| IL-1α               | 276 ± 11                            | 240 ± 14†   | 18 ± 5  |
| IL-1β               | 811 ± 68                            | 872 ± 90    | ND      |
| IL-2                | 12 ± 3                              | 27 ± 12‡    | ND      |
| IL-3                | ND                                  | ND         | ND      |
| IL-4                | ND                                  | ND         | ND      |
| IL-5                | 129 ± 22                            | 100 ± 18    | ND      |
| IL-6                | 62608 ± 11044                       | 118731 ± 22410† | 398 ± 105 |
| IL-7                | ND                                  | ND         | ND      |
| IL-9                | 141 ± 11                            | 209 ± 6‡    | 49 ± 5  |
| IL-10               | 109 ± 32                            | 192 ± 32†   | ND      |
| IL-12p40            | ND                                  | ND         | ND      |
| IL-12p70            | 123 ± 3                             | 133 ± 3†    | 8 ± 2   |
| IL-13               | ND                                  | ND         | ND      |
| IL-15               | 14 ± 1                              | 20 ± 0.5§   | ND      |
| IL-17               | 1332 ± 112                          | 1774 ± 259  | ND      |
| IP-10               | 413 ± 33                            | 395 ± 19    | 198 ± 73 |
| KC                  | 19104 ± 4320                        | 36500 ± 6353† | 47 ± 27  |
| LIF                 | ND                                  | ND         | ND      |
| LIX                 | 560 ± 55                            | 568 ± 11    | 38 ± 10  |
| MCP-1               | 16108 ± 1562                        | 17860 ± 711 | 200 ± 73 |
| M-CSF               | 12 ± 2                              | 7 ± 2‡      | ND      |
| MIG                 | 2180 ± 173                          | 1919 ± 96   | 55 ± 23  |
| MIP-1α              | 401 ± 29                            | 481 ± 18    | 86 ± 7  |
| MIP-1β              | 526 ± 76                            | 734 ± 61†   | 60 ± 17  |
| MIP-2               | 6288 ± 935                          | 10754 ± 633† | 20 ± 5  |
| RANTES              | 207 ± 15                            | 238 ± 17    | 14 ± 3  |
| TNF-α               | 16 ± 2                              | 22 ± 3      | ND      |
| VEGF                | ND                                  | ND         | ND      |

*GM-CSF, granulocyte-macrophage colony-stimulating factor; LIF, leukaemia inhibitory factor; LIX, LPX-inducible CXC chemokine; MCP, monocyte-chemoattractant protein; MIG, monocyte induced by IFN-γ; VEGF, vascular endothelial growth factor.

†P<0.05 compared with + PBS.
‡P<0.01 compared with + PBS.
§P<0.001 compared with + PBS.
Macrophage activation is an important step in the modulation of immune responses, and a subdivision of alternative activation (M2) called ‘deactivation’ is mediated by the production of immunosuppressive cytokines IL-10 and TGF-β, downregulation of MHC class II molecules and pro-inflammatory cytokines/chemokines, low levels of IL-4 and IL-13 production, and finally low levels of production of IL-12p70 and IFN-γ. In this context, the role of Hcp as a putative Th1 cytokine, inducing the maturation of DCs, has been studied.

We previously reported that immunization of mice with rHcp conferred protection against future infections with lethal doses of WT A. hydrophila SSU (Suarez et al., 2008). Overall, the results reported in this study highlighted the importance of Hcp in early stages of A. hydrophila infection. Therefore, adaptive immunity mediated by antibodies could neutralize the effect that Hcp has on inhibition of phagocytosis and, thus, enhance bacterial clearing by opsonization-mediated phagocytosis.

We also have characterized other A. hydrophila SSU toxins with enzymic activities that could impair phagocytosis. For example, VgrG1, a T6SS effector protein, has actin-ADP-ribosylation activity (Suarez et al., 2010), and the type 3 secretion system effector protein AexU, has ADP-ribosyltransferase and Rho–GAP activities (Sha et al., 2007; Sierra et al., 2007, 2010). Overall, A. hydrophila SSU has developed multiple mechanisms to circumvent innate immunity in order to establish an infection, and the role for each of these mechanisms in the disease process may depend on the host environment as well as the stage of infection.

In summary, Hcp binds to macrophages and induces the production of immunosuppressive cytokines IL-10 and TGF-β which results in impaired recruitment and inhibition of phagocytosis. This is the first report, to our knowledge, that implicates a T6SS effector protein in the regulation of macrophage function.
knowledge, that highlights how T6SS effector Hcp modulates the activation of macrophages to cause systemic infection in a mouse model. We focused our study on only one isolate of *A. hydrophila*. Whether other species of *Aeromonas* harbouring the T6SS behave similarly needs to be further elucidated.

**ACKNOWLEDGEMENTS**

This work was supported by the grants from NIH/NIAID (AI041611) and the Environmental Protection Agency. J.C.S. and G.S. were supported by J.W. McLaughlin Endowment Pre-doctoral Fellowships. J.C.S. was also supported by the Vale–Asche fellowship, University of Texas Medical Branch at Galveston. We thank Mark Griffin for his proficiency in flow cytometry acquisition data. We thank Ms Mardelle Susman for editorial assistance.

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Edited by: H. Hilbi