Escape from Immune Surveillance by *Capnocytophaga canimorsus*

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*Capnocytophaga canimorsus*, a commensal bacterium from dogs' mouths, can cause septicemia or meningitis in humans through bites or scratches. Here, we describe and characterize the inflammatory response of human and mouse macrophages on *C. canimorsus* infection. Macrophages infected with 10 different strains failed to release tumor necrosis factor (TNF)-α and interleukin (IL)-1α. Macrophages infected with live and heat-killed (HK) *C. canimorsus* 5 (*Cc5*), a strain isolated from a patient with fatal septicemia, did not release IL-6, IL-8, interferon-γ, macrophage inflammatory protein–1β, and nitric oxide (NO). This absence of a proinflammatory response was characterized by the inability of Toll-like receptor (TLR) 4 to respond to *Cc5*. Moreover, live but not HK *Cc5* blocked the release of TNF-α and NO induced by HK *Yersinia enterocolitica*. In addition, live *Cc5* down-regulated the expression of TLR4 and dephosphorylated p38 mitogen-activated protein kinase.

These results highlight passive and active mechanisms of immune evasion by *C. canimorsus*, which may explain its capacity to escape from the host immune system.

Millions of people are bitten by dogs every year, but only 5%–10% of dog bites become infected. The overall mortality of such infections is around 6 deaths out of 100 million population annually in the United States [1, 2]. However, since 1976, there have been numerous case reports describing sepsis or meningitis after dog bites. Bacteria isolated from these dramatic cases were found to belong to a new species, which was later called *Capnocytophaga canimorsus* [3].

The genus *Capnocytophaga* contains 7 species, all of them encountered in the oral cavity of humans or domestic animals. But *C. canimorsus*, commonly found in dogs, is the only one associated with severe human infections [4]. The genus *Capnocytophaga* belongs to the class of Flavobacteria. Flavobacteria in turn belong to the Bacteroidetes phylum, which includes various species of *Bacteroides* and *Porphyromonas gingivalis* [5, 6]. In the bacterial evolutionary tree, the Bacteroidetes phylum is very remote from proteobacteria and the common human pathogens. Although substantial knowledge on the metabolism and genetics of *Bacteroides* species exists, much less is known about Flavobacteria and especially about *Capnocytophaga* species.

More than 160 cases of severe infections with *C. canimorsus* have been reported in the literature. Patients infected with *C. canimorsus* have been bitten, scratched, or sometimes simply licked by a dog or occasionally by a cat. Symptoms usually appear after 2–3 days, and patients are generally admitted to the hospital several days after the exposure, with symptoms of sepsis or meningitis. Mortality is as high as 30% for septicemia, but it is only around 5% for meningitis [7]. Many of these infections involve patients who have had a splenectomy, are alcoholic, or are immunocompromised, but a significant number involve healthy people with no obvious risk factors [8–11]. *C. canimorsus* is thus an emerging pathogen, and cannot be considered simply as an opportunistisic bacterium. Little is known about the pathogenesis of infection with *C. canimorsus*. It has been reported to multiply in J774.1 mouse macrophages and to be cytotoxic, presumably by secreting a toxin [12]. More studies have been devoted to *C. ochracea*,
which is an agent of periodontal disease and has been reported to degrade lactoferrin [13], IgG [14], and IgA [15] and to produce an immunosuppressive factor [16]. In addition, the lipopolysaccharide (LPS) from C. ochracea has been shown to be an antagonist for human Toll-like receptor (TLR) 4 [17].

The overall clinical evolution of C. canimorsus infections suggests that the bacterium elicits little inflammation, at least in the early stages of the infection, which would allow time for multiplication, up to a stage at which it causes general sepsis and a deadly shock. In some cases, the patient even died of a secondary aspergillosis, suggesting that C. canimorsus might have induced some immunodeficiency (J. B. le Polain, personal communication). In this article, we test this hypothesis, and we show that indeed C. canimorsus does not elicit inflammation and that at least 2 different mechanisms contribute to this effect.

MATERIALS AND METHODS

Bacterial strains and identification. The strains of C. canimorsus are listed in table 1. The identity of the clinical isolates was confirmed by 16S rRNA sequencing using primers 27F and 1100R [20]. Alignment analysis of 16S rRNA was performed using T-COFFEE (available at: http://www.EMBnet.org) [21]. Wild-type (wt) Yersinia enterocolitica Wild-type Y. enterocolitica HOPEMT, were used as control bacteria [22].

Bacterial growth condition. C. canimorsus was routinely grown on Heart Infusion Agar (Difco) supplemented with 5% sheep blood (Oxoid) for 2 days at 37°C in an incubator containing 5% CO₂. Y. enterocolitica was pregrown overnight with continuous shaking (120 rpm) in brain-heart infusion at 22°C. Before infection, the type III secretion system of Y. enterocolitica was induced as described elsewhere [23].

Cell culture and infection. Murine macrophage J774.1 cells (ATCC TIB-67), human monocytic THP-1 cells (ATCC TIB-20), and HEK293 cells (ATCC CRL-1573) were cultured as recommended by the American Type Culture Collection. Isolation and maintenance of bone marrow–derived macrophages (BMDMs; provided by B. Combaluzier) from C57/Bl6 mice has been described elsewhere [24]. Human monocytes were isolated from peripheral-blood mononuclear cells using Ficoll-Paque density gradient centrifugation and were further purified using anti-CD14 microbeads (Miltenyi Biotec). Unless otherwise indicated, cells were seeded in medium without antibiotics at a density of 10⁵ cells/cm² 15 h before infection. Infection was performed at 37°C at an MOI of 20. When specified, bacteria were killed by the addition of ceftazidime (final concentration of 10 μg/mL). For priming of J774.1 cells, cells were pretreated with interferon (IFN)−γ (500 U/mL; BD Pharmingen) for 48 h before infections. Increased respiratory burst in IFN−γ−primed J774.1 cells was measured using 3′-(p-aminophenyl) fluorescein, as recommended by the manufacturer (Molecular Probes).

Video microscopy. J774.1 cells were placed under a Leica DMIRE2 microscope equipped with a thermostated (37°C) and CO₂-equilibrated chamber. Three-hour video sequences were recorded using a digital camera (Hamamatsu Photonics) and OpenLab software (version 3.1.2). Sequences were converted to QuickTime format (Apple).

Cytokine assay. Cell-free supernatants were assayed for cytokine and chemokine production using commercial ELISA kits (BD Pharmingen) or the Bio-Plex mouse and human cytokine multiarray system (Bio-Rad Lab).

Nitric oxide (NO) assay. NO production was estimated as the amount of nitrite released in the culture medium, by use of modified Griess reagent (Sigma).

Lactate dehydrogenase (LDH) release assay. LDH release determinations were performed using the CytoTox 96 cytotoxicity assay kit (Promega).

NF-κB assay. Human TLR2, TLR4, TLR5, MD-2, and CD14 expression plasmids (provided by M. F. Smith, C. J. Kirschning, and K. Miyake) have been described elsewhere [25]. Ligands for TLRs were obtained from Invivogen. HEK293 cells were transfected in 12-well plates using Fugene 6 (Roche). Each transfection contained 100 ng of TLR, 100 ng of MD-2, 100 ng of CD14, 500 ng of pELAM-Luc, 100 ng of pCMV−β-galactosidase, and 3 μL of Fugene 6. Transfections were cultured for 24 h, and cells were stimulated as indicated for an additional 24 h. NF-κB activity was determined by measuring the luciferase activity present in cell extracts. Luciferase values were normalized for differences in transfection efficiency on the basis of β-galactosidase activity in the same extracts and were expressed as fold induction values relative to the unstimulated empty vector control.

Immunoblotting. For each experimental condition, 3 × 10⁶ J774.1 cells were harvested by centrifugation at 20,000 g for 5 min and resuspended in 100 μL of cell lysis buffer (20 mmol/L Tris, 100 mmol/L NaCl, 0.2% [vol/vol] Triton X-100, and 10 mmol/L EDTA [pH 7.4]). Lysates were centrifuged at 20,000 g for 5 min and diluted in 5× SDS/PAGE sample buffer. Samples were boiled for 5 min, and proteins were separated on 12% SDS/PAGE gel. Anti-p38 mitogen-activated protein kinase (MAPK) and anti–phospho-p38 MAPK were purchased from Cell Signaling Technology.

Reverse-transcription polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted using the RNeasy Kit (Qia-gen). RT of RNA was performed with 1 μg of RNA, RT buffer, 0.4 mmol/L each dNTP, 40 U of RNaseOUT (Invitrogen), 8 mmol/L DTT, 500 ng of oligo(dT) primer, and 10 U of SuperScript II reverse transcriptase (Invitrogen) at 42°C for 1 h. PCR primer sequences are listed in table 2. The reaction mixtures were subjected to 30 (β-actin, TLR2, and TLR4), 45

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Table 1. *Capnocytophaga canimorsus* (Cc) strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Collection no.</th>
<th>Identity, %</th>
<th>Biological origin</th>
<th>Year of isolation</th>
<th>History and geographical origin</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>Cc2</td>
<td></td>
<td>96.2</td>
<td>Human fatal septicemia after dog bite</td>
<td>1989</td>
<td>G. Wauters &amp; M. Delmée, Univ. Clinics St. Luc, Brussels, Belgium</td>
<td>[9]</td>
</tr>
<tr>
<td>Cc3</td>
<td></td>
<td>99.0</td>
<td>Human septicemia</td>
<td>1990</td>
<td>G. Wauters &amp; M. Delmée → St. Jan Hospital, Brugge, Belgium</td>
<td>[18]</td>
</tr>
<tr>
<td>Cc5</td>
<td></td>
<td>100</td>
<td>Human fatal septicemia after dog bite</td>
<td>1995</td>
<td>G. Wauters &amp; M. Delmée → Clinic of Libramont, Libramont, Belgium</td>
<td>...</td>
</tr>
<tr>
<td>Cc7</td>
<td>BCCM/LMG 11510, CCUG 12569, CDC A3626</td>
<td>99.6</td>
<td>Human septicemia</td>
<td>1998</td>
<td>G. Wauters &amp; M. Delmée → KUL, Leuven, Belgium</td>
<td>...</td>
</tr>
<tr>
<td>Cc9</td>
<td>BCCM/LMG 11541, CCUG 17234, ATCC 35978</td>
<td>100</td>
<td>Human septicemia</td>
<td>1965</td>
<td>BCCM/LMG → CCUG → R. Weaver, CDC, Atlanta, Georgia → Virginia</td>
<td>[19]</td>
</tr>
<tr>
<td>Cc10</td>
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<td>100</td>
<td>Human septicemia</td>
<td>...</td>
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<td>[19]</td>
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<tr>
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<td>BCCM/LMG 11551, MCCM 01373</td>
<td>99.1</td>
<td>Human septicemia</td>
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<td>BCCM/LMG → MCCM → A. von Graevenitz, Univ. Zürich, Zürich, Switzerland</td>
<td>...</td>
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<tr>
<td>Cc12</td>
<td>ATCC 35979, CDC 7120</td>
<td>100</td>
<td>Human septicemia</td>
<td>...</td>
<td>ATCC → R. Weaver, CDC, Atlanta, Georgia → California Health Dept. → San Antonio Community Hospital, San Antonio, Texas</td>
<td>...</td>
</tr>
<tr>
<td>Cc13</td>
<td></td>
<td>99.4</td>
<td>Healthy dog’s (Jackie) saliva</td>
<td>2005</td>
<td>Direct isolation, Basel, Switzerland</td>
<td>Present study</td>
</tr>
<tr>
<td>Cc14</td>
<td></td>
<td>100</td>
<td>Healthy dog’s (Pouchka) saliva</td>
<td>2005</td>
<td>Direct isolation, Basel, Switzerland</td>
<td>Present study</td>
</tr>
</tbody>
</table>

**NOTE.** ATCC, American Type Culture Collection; BCCM/LMG, Belgian Co-ordinated Collection of Microorganisms; CCUG, Culture Collection University of Göteborg; CDC, Centers for Disease Control and Prevention; KUL, Katholieke Universiteit Leuven; MCCM, Medical Culture Collection Marburg.  
* Determined by comparing the 16S rRNA sequence of each strain to a reference strain (Cc12; ATCC 35979). Minimal length of 16S rRNA sequence analyzed is 300 bp.
(interleukin [IL]–1 receptor–associated kinase M [IRAK-M]), and 35 (myeloid differentiation factor 88 [MyD88] and suppressor of cytokine signaling 1 [SOCS-1]) cycles of amplification. **Statistical analysis.** Results are median or mean ± SD values. Quantitative data were compared by use of the Mann-Whitney U test. Differences were considered to be significant when P < .05.

**RESULTS**

**Replication and survival of C. canimorsus in the presence of J774.1 mouse macrophages.** Macrophages are one of the primary mammalian defense lines against bacterial pathogens. They not only recognize, engulf, and kill microorganisms, but they also mobilize the antimicrobial host defense by secreting chemokines and cytokines. We first investigated whether *C. canimorsus* can survive in the presence of mouse macrophages. We analyzed *C. canimorsus*-infected J774.1 macrophages by video microscopy and observed that bacteria interacting with macrophages were not internalized (figure 1A). In agreement with these observations, viable count experiments showed that *C. canimorsus* survive and multiply in the presence of J774.1 cells (figure 1B). Furthermore, we found that the presence of macrophages in the cell culture medium enhanced bacterial growth by ~100-fold at 24 h after infection. Addition of cytochalasin D had little effect on the bacterial growth rate, confirming that there was an insignificant level of phagocytosis. We also monitored cytotoxicity for macrophages by measuring LDH release. As controls, we included wt (E40 wt) and multieffector knockout (ΔHOPEMT) *Y. enterocolitica* strains. Interestingly, the growth of *C. canimorsus* did not induce cytotoxicity (figure 1C). Macrophages infected with *C. canimorsus* remained unaltered, adherent, and viable, as examined by trypan blue dye exclusion (data not shown). As expected, cytotoxicity was observed by wt *Y. enterocolitica* [26] and by ΔHOPEMT *Y. enterocolitica* [27]. Hence, *C. canimorsus* seems to replicate in the presence of macrophages without inducing cytotoxicity.

**Absence of potent proinflammatory response in mouse macrophages.** To determine whether *C. canimorsus* infection induces secretion of proinflammatory cytokines, J774.1 cells were infected with 10 different strains of *C. canimorsus* at a 20:1 bacteria/macrophase ratio, and culture supernatants from 24 h after infection were analyzed for the release of tumor necrosis factor (TNF–α) and IL-1α. As shown in figure 2A, *C. canimorsus* induced only negligible amounts of TNF–α and IL-1α, similar to those of mock-infected control cells and those induced by E40 wt, which prevents inflammation by injecting *Y. enterocolitica* [28] into cells. As expected, the multieffector knockout *Y. enterocolitica* ΔHOPEMT strain effectively induced secretion of TNF–α and IL-1α [30]. Further analysis was pursued with *C. canimorsus* 5 (ΔG5; provided by M. Delmée, University of Louvain, Belgium), a strain isolated from a patient with fatal septicemia (table 1). Production of proinflammatory IL-6 and anti-inflammatory IL-10 by cells infected with both live and heat-killed (HK) C5 was only marginal, compared with that by mock-infected control cells (figure 2A). To test whether this lack of cytokine release was due to a low dose of bacteria, macrophages were exposed to C5 or ΔHOPEMT for 2 h at various bacteria/macrophase ratios, and supernatants from 24-h cultures were tested for TNF–α production. Although we observed some dose dependency in the response of macrophages to C5, the amount of TNF–α released was significantly less than that release by ΔHOPEMT-infected cells.

Priming of macrophages with IFN–γ has been shown to increase cellular responsiveness to inflammatory stimuli [31, 32]. To test whether primed macrophages exhibit an enhanced response to *C. canimorsus*, J774.1 cells were prestimulated with IFN–γ for 48 h, infected, and analyzed for cytokines, as described above. Surprisingly, we could not detect a significant release of IL-1α, IL-6, or TNF–α in activated J774.1 cells infected with either live or HK C5 (figure 2B).

We next examined the production of IL-6, IFN–γ, and TNF–α in *C. canimorsus*-infected BMDMs 24 h after infection. We
observed relatively moderate levels of IL-6, IFN-γ, and TNF-α on infection with either live or HK Cc5 (figure 2C). Although levels of cytokines released on infection with Cc5 were somewhat higher than those released by infected J774.1 cells, they were significantly lower than those released by BMDMs infected with HK Y. enterocolitica.

**No induction of a proinflammatory response in human monocytes by C. canimorsus infection.** To avoid any bias due to host species specificity, human monocytic THP-1 cells were infected, and culture supernatants from 6 h after infection were analyzed for the release of IL-1β, IL-6, IL-8, IFN-γ, TNF-α, and macrophage inflammatory protein-1β. Consistent with the weak immune responses of mouse macrophages, neither live nor HK Cc5 induced detectable proinflammatory cytokine and chemokine production (figure 3A). Infection with live or HK Cc5 of human monocytes isolated from peripheral blood also resulted in low levels of IL-8 and TNF-α (figure 3B). These results together demonstrate that not only mouse but also human macrophages fail to trigger robust immune response to *C. canimorsus* infection.
Figure 2. Absence of tumor necrosis factor (TNF–α), interleukin (IL–1α), IL-6, and IL-10 release by mouse macrophages infected with Capnocytophaga canimorsus. A, J774.1 cells, either mock infected or infected (MOI of 20) for 24 h with the indicated strains. To determine the dose response of TNF–α release, the indicated strains were used to infect macrophages at the indicated MOIs. Bacteria were killed with ceftazidime after 2 h, and supernatants from 24 h after infection were analyzed for TNF–α production. B, J774.1 cells, prestimulated with interferon (IFN)–γ (500 U/mL) for 48 h and either mock-infected or infected (MOI of 20) for 24 h with the indicated strains. C, Bone marrow–derived macrophages (BMDMs), infected with the indicated strains (MOI of 20). Cell culture supernatants were collected 24 h after infection, and levels of cytokine production were analyzed by ELISA. The results are representative of 5 independent experiments. Error bars represent the SD values. HK, heat killed; wt, wild type.
Figure 3. Cytokine, chemokine, and nitric oxide (NO) production during *Capnocytophaga canimorsus* infection. A, THP-1 cells, either mock infected or infected (MOI of 20) for 6 h with the indicated strains. Levels of interleukin (IL)–1β, IL-6, IL-8, interferon (IFN)–γ, tumor necrosis factor (TNF)–α, and macrophage inflammatory protein (MIP)–1β were analyzed by ELISA. B, Human monocytes, either mock infected or infected (MOI of 20) for 5 h with the indicated strains. Levels of IL-8 and TNF-α were measured by ELISA. C, J774.1 and THP-1 cells and human monocytes, either mock infected or infected (MOI of 20) with the indicated strains for 24 h, 6 h, and 5 h, respectively. Cell culture supernatants were collected, and the levels of NO were determined using Griess reagent. Data are the mean for triplicates from 5 independent experiments. Error bars represent the SD values. HK, heat killed; wt, wild type.
Lack of detectable NO production by *C. canimorsus* in mouse and human macrophages. NO is one of the primary mediators of the host cell defense against many intracellular and extracellular bacteria. We, therefore, assessed NO production by *C. canimorsus*-infected J774.1, THP-1, and human monocytes. Cells were infected as described above, and the amount of NO was measured as nitrite production. Although J774.1, THP-1, and human monocytes were capable of NO production in response to HK or ΔHOPEMT *Y. enterocolitica*, infection with live or HK Cc5 did not result in any detectable NO production (figure 3C).

Lack of interaction of Cc5 with TLR4 and TLR5. TLRs are a family of cell surface molecules that participate in innate immune response by recognizing pathogen-associated molecular patterns. Recognition and subsequent stimulation of TLRs trigger signaling events that result in the production of proinflammatory cytokines [33]. We next investigated whether TLRs are capable of recognizing *C. canimorsus*. To determine which, if any, TLRs may play a role in the response of macrophages to *C. canimorsus*, we examined the *C. canimorsus*-induced NF-κB response of HEK293 cells transfected with TLR2, TLR4, or TLR5. In the transfection experiment shown in figure 4, HEK293 cells were cotransfected with the NF-κB–Luc reporter, pCMV–β-galactosidase plasmids, and either empty pcDNA3.1 vector or expression plasmids for TLR2, TLR4 (with CD14 and MD-2 expression plasmids), or TLR5. Cultures were stimulated with either known purified TLR ligands (Pam3CSK4 for TLR2, *Escherichia coli* LPS for TLR4, and *Salmonella thyphimurium* flagellin for TLR5) and live or HK Cc5 for 24 h and assayed for luciferase activity.

Cells transfected with TLR4 or TLR5 were unable to activate NF-κB in response to *C. canimorsus* infection (figure 4A).
transfected TLR4 and TLR5 were indeed functional, as demonstrated by the ability of the cells to activate NF-κB in response to *E. coli* LPS or flagellin, respectively. We next tested whether *C. canimorsus* can act as an antagonist of TLR4. To this end, we stimulated transfected cells simultaneously with *E. coli* LPS and live or HK C5. Live but not HK C5 prevented *E. coli* LPS–induced TLR4 stimulation (figure 4A). This suggests that *C. canimorsus* can indeed inhibit NF-κB activation induced by TLR4, but this abrogation is not mediated by the antagonistic action of its LPS.

TLR2-transfected HEK293 cells were capable of activating NF-κB in response to live or HK C5 (figure 4B). To compare the cytokine-inducing capacity of C5 with that of a TLR2 agonist (Pam3CSK4), we infected J774.1 cells with either live or HK C5 and Pam3CSK4 (1 or 10 µg/mL) for 24 h and analyzed the release of IL-6. As shown in figure 4B, the level of IL-6 release was significantly lower in J774.1 cells infected with either C5 or HK C5 than with Pam3CSK4. Thus, whereas C5 (or HK C5) could lead to NF-κB activation via TLR2, this activation did not lead to a potent inflammatory response by macrophages.

**Inhibition by C5 of proinflammatory response induced by HK *Y. enterocolitica***. To further analyze the antagonistic action of C5 on TLR4 activation, we examined whether C5 can block the proinflammatory response induced by HK *Y. enterocolitica*. J774.1 cells were incubated with live or HK C5 (MOI of 20) in the presence or absence of HK *Y. enterocolitica*, and the supernatants from 24 h after infection were tested for TNF-α and NO release. The results shown in figure 5A clearly demonstrate that there was no TNF-α or NO in the culture supernatants when live but not HK C5 were added together with HK *Y. enterocolitica*. This inhibitory effect could also be observed when J774.1 cells were preinfected with live C5 for 24 h and then stimulated with HK *Y. enterocolitica*. Live C5, however, could not inhibit TNF-α and NO release if J774.1 cells were prestimulated with HK *Y. enterocolitica* (data not shown). Addition of cytochalasin D (5 µg/mL) during infection had no effect on the ability of C5 to suppress TNF-α and NO release, showing that internalization of *C. canimorsus* is not necessary for this action (figure 5B). Release of several other cytokines and chemokines, such as IL-3, IL-5, IL-6, IL-17, and RANTES (data not shown), was also inhibited by live C5, suggesting that the antagonistic effect of live C5 is global.

**Regulation of TLR4 and p38 MAPK by C5**. To determine whether C5 can modulate the expression level of key molecules involved in the production of a proinflammatory response, we analyzed the mRNA level of TLR2, TLR4, MyD88, IRAK-M, and SOCS-1 in J774.1 cells infected with live or HK C5 in the presence or absence of HK *Y. enterocolitica* for 24 h by RT-PCR (figure 5C). We found no significant change in the level of TLR2, MyD88, IRAK-M, and SOCS-1 on infection with either live or HK C5, suggesting that C5 does not directly regulate the expression of negative regulators, such as IRAK-M and SOCS-1, nor of MyD88 and TLR2. In contrast, mRNA expression of TLR4 infected with live *C. canimorsus* was reduced. Down-regulation in TLR4 mRNA level was not the result of *C. canimorsus* LPS–induced tolerance, because HK C5 did not have an effect. Finally, we assessed the activation of p38 MAPK, one of the prominent targets of the TLR signaling cascade. The activation state of p38 MAPK was analyzed using antibody specific for its phosphorylated form. Infection of J774.1 cells with live C5 for 24 h resulted in complete dephosphorylation of p38, whereas HK C5 had no effect. C5 infection did not alter the level of total p38 (figure 5D). It is interesting to note that suppression of p38 phosphorylation by live C5 coincided with the inhibition of inflammatory responses as well as the down-regulation of TLR4.

**DISCUSSION**

The clinical overview of *C. canimorsus* infections hints at the ability of the bacterium to avoid the immune system, at least in the early stages of the infection. To this end, we characterized the immune response from mouse and human macrophages on *C. canimorsus* infection. We found that *C. canimorsus* does not activate signals leading to the release of proinflammatory cytokines, chemokines, and NO. This confirms and extends a previous report that normal whole blood produces lower levels of IL-1β and IL-6 in response to *C. canimorsus* than in response to other gram-negative bacteria [34]. Both mouse and human macrophages were unable to produce potent proinflammatory responses on infection with either live or HK *C. canimorsus*. Our data thus suggest relatively silent entry of *C. canimorsus* into the host, where it replicates and survives in the presence of macrophages. We did not observe any cytotoxicity by the 10 strains of *C. canimorsus* for macrophages. This result is at odds with the data from Fischer et al. [12], especially because the strain used in this previous study was included in our experiments [12]. At this time, we have no explanation for this discrepancy.

Why are macrophages unable to mediate proinflammatory responses against *C. canimorsus*? We found that one of the immune sensors of invading pathogens, TLR4, is unable to activate NF-κB on encountering either live or HK *C. canimorsus*. This suggests that the lipid A moiety of *C. canimorsus* LPS may be different from that of the enteric bacteria, for example, in its acylation pattern [35]. Further investigation on the structure of *C. canimorsus* LPS is under way, to determine its structure and to characterize its endotoxic activity. Although *C. canimorsus* does not react with TLR4, it interacts with TLR2. Activation of TLR2 by *C. canimorsus*, however, does not seem to result in a potent proinflammatory response. Further investigation of the functional role of TLR2 activation, as well as identification of the TLR2 ligand from *C. canimorsus*, is needed.
Figure 5. Inhibition of heat-killed (HK) Yersinia enterocolitica–induced proinflammatory response by Capnocytophaga canimorsus 5 (Cc5). A, J774.1 cells, infected with the indicated strains (MOI of 20) together with or without HK E40 (MOI of 20) for 24 h. Culture supernatants were analyzed for the release of nitric oxide and tumor necrosis factor (TNF)–α. B, J774.1 cells, infected as in panel A, in the presence of cytochalasin D (CytD) at 5 μg/mL. C, J774.1 cells, amplified by polymerase chain reaction (PCR). After infection of J774.1 cells with the indicated strains (MOI of 20) for 24 h, total RNA was extracted, reverse transcribed, and amplified by PCR. The PCR products were separated in 1.2% agarose gels. D, J774.1 cells, infected with the indicated strains (MOI of 20) for 24 h. After infection, cell lysates were prepared and separated on a 12% SDS-PAGE gel. Immunoblotting was performed using an antibody specific for the phosphorylated form of p38 (p-p38) and total p38. IRAK-M, interleukin-1 receptor–associated kinase M; MyD88, myeloid differentiation factor 88; SOCS-1, suppressor of cytokine signaling 1.
We further provide evidence that NO, TNF-α, and other proinflammatory cytokines are absent from the culture supernatant of macrophages when stimulated by HK Y. enterocolitica in the presence of live C. canimorsus. Live C5 could degrade and/or prevent the release of NO and cytokines produced by macrophages through interference with the proinflammatory-signaling cascade. We favor the second interpretation for 2 reasons. First, it would be unlikely that C5 would degrade NO and all tested cytokines. Second, infection of live C5 does not do. Down-regulation of p38 MAPK activation correlates well with its critical role in cytokine production, phagocytosis, and regulation of CD14, TLR2, and TLR4 expression [36–40]. Clearly, this active anti-inflammatory response of C. canimorsus raises a number of questions that need to be addressed: what signaling pathways are targeted and by what kind of toxin or effector? It is striking to note that other pathogens, such as Ehrlichia chaffeensis [39], Francisella tularensis [41], Listeria monocytogenes [42], and Leishmania donovani [43], also down-regulate the inflammatory cascade through p38 inhibition. Whether these targets are modulated by common mechanisms or by similar effectors is yet to be determined.

C. canimorsus is a commensal of dogs' and cats' mouths. Recent observations have shown that commensal bacteria often do not trigger an inflammatory response but instead, in some recent observations have shown that commensal bacteria often do not trigger an inflammatory response but instead, in some

\[ \text{C. canimorsus} \]

that the passive mechanism involves the escape of TLR4 detection in a previously healthy man following a dog bite. Int J Clin Pract 1998; 52:205.


Frandsen EV, Kjeldsen M, Kilian M. Inhibition of Prevotella and Capno-

Acknowledgments

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References


5. Hayashi F, Okada M, Zhong X, Miura K. PCR detection of Capsy-

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cells may be viewed as regulatory quenchers of the inflammatory cascade. We favor the second interpretation for 2 reasons. First, it would be unlikely that C5 would degrade NO and all tested cytokines. Second, infection of live C5 does not do. Down-regulation of p38 MAPK activation correlates well with its critical role in cytokine production, phagocytosis, and regulation of CD14, TLR2, and TLR4 expression [36–40]. Clearly, this active anti-inflammatory response of C. canimorsus raises a number of questions that need to be addressed: what signaling pathways are targeted and by what kind of toxin or effector? It is striking to note that other pathogens, such as Ehrlichia chaffeensis [39], Francisella tularensis [41], Listeria monocytogenes [42], and Leishmania donovani [43], also down-regulate the inflammatory cascade through p38 inhibition. Whether these targets are modulated by common mechanisms or by similar effectors is yet to be determined.

C. canimorsus is a commensal of dogs' and cats' mouths. Recent observations have shown that commensal bacteria often do not trigger an inflammatory response but instead, in some cases, have the capacity to actively turn it down. Hence, commensals may be viewed as regulatory quenchers of the inflammation, preventing the progression from the acute to the chronic state [44]. In conclusion, C. canimorsus possesses both passive and active mechanisms of immune evasion. It is likely that the passive mechanism involves the escape of TLR4 detection through a unique LPS structure. Although it remains unclear how C. canimorsus antagonizes TLR4 activation by the active mechanism, it seems to target critical players that are also manipulated by other pathogens. A better understanding of this mechanism could lead not only to the prevention of these dramatic infections but also to a better understanding of the regulation of the innate immune system.

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References


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We further provide evidence that NO, TNF-α, and other proinflammatory cytokines are absent from the culture supernatant of macrophages when stimulated by HK Y. enterocolitica in the presence of live C. canimorsus. Live C5 could degrade and/or prevent the release of NO and cytokines produced by macrophages through interference with the proinflammatory-signaling cascade. We favor the second interpretation for 2 reasons. First, it would be unlikely that C5 would degrade NO and all tested cytokines. Second, infection of live C5 does not do. Down-regulation of p38 MAPK activation correlates well with its critical role in cytokine production, phagocytosis, and regulation of CD14, TLR2, and TLR4 expression [36–40]. Clearly, this active anti-inflammatory response of C. canimorsus raises a number of questions that need to be addressed: what signaling pathways are targeted and by what kind of toxin or effector? It is striking to note that other pathogens, such as Ehrlichia chaffeensis [39], Francisella tularensis [41], Listeria monocytogenes [42], and Leishmania donovani [43], also down-regulate the inflammatory cascade through p38 inhibition. Whether these targets are modulated by common mechanisms or by similar effectors is yet to be determined.

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