The Helicobacter pylori HopQ outermembrane protein inhibits immune cell activities

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ABSTRACT

We previously showed that the colorectal cancer colonizing bacterium Fusobacterium nucleatum protects tumors from immune cell attack via binding of the fusobacterial Fap2 outer-membrane protein to TIGIT, a checkpoint inhibitory receptor expressed on T cells and NK cells. Helicobacter pylori, the causative agent for peptic ulcer disease, is associated with the development of gastric adenocarcinoma and MALT lymphoma. The HopQ outer-membrane adhesin of H. pylori was recently shown to bind carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) including CEACAM1, an inhibitory receptor expressed mainly by activated T and NK cells. Here we investigated the possibility that similar to Fap2, HopQ can also inhibit immune cell activities by interacting with CEACAM1. We used several approaches to confirm that HopQ indeed interacts with CEACAM1, and show that CEACAM1-mediated activation by HopQ, may inhibit NK and T cell functions.

Introduction

Already in 1951 endocarditis or bacteremia with Streptococcus bovis (currently called Streptococcus gallolyticus) was suggested as an indicator for the presence of colon cancer.1,2 The precise pathophysiology linking Streptococcus gallolyticus (S. gallolyticus) with colon cancer remains obscured, although a recent study suggests that S. gallolyticus increase tumor cell proliferation by activating the Wnt/β-catenin signaling pathway.3 In 1994, H. pylori was recognized as a type I carcinogen. Today, it is considered the most common etiologic agent of infection-related cancers, which represent 5.5% of the global cancer burden.4,5 Epidemiological evidence as well as experimental data indicate that the presence of H. pylori is associated with gastric adenocarcinoma and with gastric mucosa-associated lymphoid tissue (MALT) lymphoma.6 The presence of H. pylori in stomach mucosa is crucial for the chronic inflammation which leads to gastric cancer development.6 With this respect, the cytotoxin-associated gene A (CagA) protein of H. pylori, which is delivered into gastric epithelial cells via bacterial type IV secretion, is an oncoprotein that can induce malignant neoplasms in mammals.7

Recently, the outer membrane protein HopQ was shown to be involved in helicobacter-mediated pathogenicity. It was demonstrated that HopQ interacts with various members of the CEACAM family expressed on the gastric epithelium specifically during gastritis and gastric cancer.8–10 It was further demonstrated that this interaction facilitates the translocation of the CagA protein into the gastric cells.8,11 CEACAM1 serves also as an inhibitory receptor on various immune cell subsets.12,13 It was shown in the past that binding of Opa (Opacity-associated proteins) proteins of pathogenic Neisseria strains to CEACAM1 impairs normal maturation of immature dendritic cells, suppresses lymphocyte responses to activating stimuli, and also hinders phagocytic engulfment of the bacteria.13 The killing activity of practically all immune cells is controlled by inhibitory and activating receptors. We showed previously that the bacterium Fusobacterium nucleatum (F. nucleatum) that is enriched in colorectal cancer may contribute to tumor progression by binding to the inhibitory receptor TIGIT. We demonstrated that the fusobacterial Fap2 outer surface protein interacts with TIGIT and activates it to inhibit the activity of NK and T cells.14

Here, we demonstrate that the H. pylori may protect tumors from immune cell attack via the interaction of its HopQ outer surface protein with CEACAM1 expressed on the immune cells.

Results

Helicobacter pylori clinical isolates directly interact with human CEACAM1

We showed in our previous study that F. nucleatum can protect tumors from attack due to an interaction between the fusobacterial Fap2 outer-surface protein with the inhibitory receptor TIGIT.14
Since *H. pylori* and *S. gallolyticus* (formerly named *S. bovis*) are also linked with tumor development we wondered whether these two bacteria might also interact with immune cell receptors. To investigate this, we used a reporter system that we had previously generated\cite{14,15} in which murine thymoma BW cells are transfected with chimeric proteins in which the extracellular portion of inhibitory or activating receptor is fused to the mouse zeta chain. In this reporter system mouse IL-2 is secreted if the receptor is bound and triggered by specific ligands. We used BW cells expressing either human immune inhibitory receptors (CEACAM1, KIR2DL1 and TIGIT, Figure 1(a) upper panels) or human activating receptors (NKp44, NKp30, NKp46 and CD16, Figure 1(a) lower panels), and incubated them with three strains of *H. pylori* ((HP), Figure 1(b)), or with three *S. gallolyticus* (SG) strains isolated from peripheral blood of patients with proven colorectal cancer (Figure 1(c)).

As can be seen in Figure 1(b), mouse IL-2 was detected upon incubation of all 3 *H. pylori* strains with BW/CEACAM1 cells, indicating that *H. pylori* binds CEACAM1. The CEACAM1 activation was specific since other BW reporters were not activated (Figure 1(b)). Interestingly, the three *S. gallolyticus* strains isolated from patients with colorectal cancer did not activate any of the BW reporters (Figure 1(c)).

To corroborate these findings, we labeled *H. pylori* W0508 (Figure 2), *H. pylori* P12 (Figure 2 and Supp. Figure 1), and *H. pylori* W1354 (Supp. Figure 1) with FITC and examined their binding to the CEACAM1-negative parental BW cells and the human EBV transformed B cell line 721.221. We also tested the binding of the FITC-labeled *H. pylori* strains to the same cells transfected to express CEACAM1 (Figure 2, Supp. Figure 1). We observed some *H. pylori* binding to the parental CEACAM1-negative BW and 721.221 cells (Figures 2(b), and (d), and Supp. Figure 1), indicating that *H. pylori* binds to cells also in the absence of CEACAM1. However, increased binding of the three *H. pylori* tested strains to the CEACAM1-expressing cells was observed in all bacteria to target ratios (Figure 2(b–c)),

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**Figure 1.** *H. pylori* activates CEACAM1.

(a) Flow cytometry of BW cells expressing human NK cell inhibitory receptors: CEACAM1, KIR2DL1, TIGIT or human activating receptors: NKp44, NKp30, NKp46 and CD16, all fused to mouse zeta chain. The various BW cells were stained with the antibodies against the receptors indicated above the histograms. The black empty histograms represent the specific staining, and the grey filled histograms represent the control staining with the secondary antibody only. (b-c) The various BWs were incubated with the *H. pylori* strains W0508, W1354 (1354) and HP-P12 (b) or with *S. gallolyticus* isolates SG1, SG2 and SG3 (c), at bacteria to BW ratio of 100:1. The presence of mouse IL-2 in the supernatants was determined by ELISA 48hr later (represented by pg/ml). The figure shows one representative experiment out of four performed. The error bars are derived from triplicates. **** p < 0.0001 repeated measures one way ANOVA (compared with other groups).
for strain W0508, Figure 2(d) and Supp. Figure 1(b) for P12, and Supp. Figure 1(a) for W1354). Thus, we concluded that \textit{H. pylori} directly binds human CEACAM1.

\textbf{The \textit{Helicobacter pylori} HopQ protein interacts with CEACAM1}

Koniger et al.\textsuperscript{8} reported by using other methods than those used here, that members of the CEACAM family, including CEACAM1 bind \textit{H. pylori}. HopQ was found to mediate this binding.\textsuperscript{8,11} To validate these observations, we generated a CEACAM1-Ig fusion protein in which the extracellular domain of CEACAM1 is fused to the Fc portion of human IgG1. As a control, we used an NKp30-Ig fusion protein (Control-Ig) that was prepared in a similar manner. As can be seen in Figure 3(a), a specific HopQ-dependent binding of CEACAM1-Ig was observed to the WT \textit{H. pylori} P12 (Figure 3(a), top, left panel). No binding was observed to the HopQ-deficient mutant of \textit{H. pylori} p12 (P12ΔhopQ, Figure 3(a), bottom, left panel) or when the NKp30-Ig control was used (Figure 3(a) right panels). We further observed that whereas FITC-labeled \textit{H. pylori} p12 binds preferably to BW CEACAM1 positive cells (Figure 2(d), and Supp. Figure 1(b)), the FITC-labeled P12ΔhopQ lacks specific binding to BW CEACAM1 (Supp. Figure 1(c)). Both the WT \textit{H. pylori} p12 and the P12ΔhopQ possess weak binding to the parental CEACAM1-negative BW cells (Figure 2(d), and Supp. Figure 1(b–c)).

To further corroborate these results, we used again the BW/CEACAM1 reporter system. We verified that CEACAM1 is expressed on the BW cells (Figure 3(b)), and incubated WT \textit{H. pylori} P12 and P12ΔhopQ with BW/CEACAM1 at various bacteria to cells ratios. Importantly, while the parental \textit{H. pylori} P12 strain was found to activate the BW/CEACAM1 reporter system in a dose dependent manner, P12ΔhopQ \textit{H. pylori} failed to do so (Figure 3(c)). In order to demonstrate direct binding of CEACAM1 to HopQ, we used far-western blotting. Parental \textit{H. pylori} P12 and its HopQ deficient mutant (P12ΔhopQ) were subjected to SDS-PAGE without boiling (N), or after
denaturation by boiling (D), blotted to a nitrocellulose membrane and interacted with CEACAM1-Ig. While clear binding to a protein with molecular mass of approximately 70 kDa (matching the size of HopQ) was observed in the boiled P12 sample and in the native P12 sample, CEACAM1 binding was not detected to samples prepared from P12ΔhopQ (Figure 3(d)).

**Helicobacter pylori inhibits T and NK cell function via HopQ**

Because CEACAM1 also functions as an inhibitory receptor in a variety of immune cells including T and NK cells, we hypothesized that the HopQ binding to CEACAM1 might inhibit immune cell activities, similarly to the binding of fusobacterial Fap2 to TIGIT. To test this, we isolated CD4+ T cells from peripheral blood of healthy donors and cultured them with anti-CD3 mAb (data not shown). We then used the bulk CD8 + T cells expressing CEACAM1 to perform a re-directed killing assays and monitored killing by staining for CD107a. Previous studies showed that the presence of CD107a+ cells correlate well with cytotoxic activity for CD8⁺ T/NK cells. We incubated p815 cells with anti-CD3 mAb as before, then pre-coated the p815 cells with or without P12 or P12ΔhopQ H. pylori. Then we cocultured the p815 cells with CD8 + T cells expressing CEACAM1 and stained the CD8 + T cells for CD107a. As shown in Figure 4(e), anti-CD3 mAb indeed induced marked redirected killing of the p815 cells by the CD8+, CEACAM1+ T cells. In the presence of the WT H. pylori P12, this IFN-γ secretion was inhibited (Figure 4(c)). This inhibition was significantly less pronounced when the P12ΔhopQ H. pylori was used (Figure 4(c)). No significant IFN-γ secretion was observed with CEACAM1-positive CD4 cells only (Figure 4(c)), or when the CD4 cells incubated with p815 pre-coated or not with P12 or P12ΔhopQ H. pylori in the absence of anti CD3 mAb (data not shown).

Lastly, we also investigated whether H. pylori can inhibit T or NK cell cytotoxicity in a HopQ dependent manner. We isolated CD8 + T cells from peripheral blood of healthy donors (Figure 4(d)) and activated them with IL-2 (the CD8 + T cells expressing CEACAM1 are shown in Figure 4(d)). We then used the bulk CD8 + T cells expressing CEACAM1 to perform a re-directed killing assays and monitored killing by staining for CD107a. Previous studies showed that the presence of CD107a⁺ cells correlate well with cytotoxic activity for CD8⁺ T/NK cells. We incubated p815 cells with anti-CD3 mAb as before, then pre-coated the p815 cells with or without P12 or P12ΔhopQ H. pylori. Then we cocultured the p815 cells with CD8 + T cells expressing CEACAM1 and stained the CD8 + T cells for CD107a. As shown in Figure 4(e), anti-CD3 mAb indeed induced marked redirected killing of the p815 cells by the CD8+, CEACAM1+ T cells. In the presence of the WT H. pylori P12, this redirected killing was significantly inhibited (Figure 4(e)). In contrast, in the presence of the P12ΔhopQ H. pylori no inhibition was observed (Figure 4(e)).

Finally, we asked whether H. pylori can inhibit NK cell cytotoxicity in a HopQ dependent manner. As in T cells, CEACAM1 is also expressed on NK cells only following activation and is mainly expressed on the CD16-negative
We isolated CD16-negative NK cells, and activated them as described in "Materials and Methods". Indeed, following activation, most of the CD16-negative NK cells expressed CEACAM1 (Figure 4(f)). We then performed CD107a assay using 721.221 cells as a target cells, and NK cells that expressed CEACAM1 as effectors.

In the presence of the WT H. pylori and bulk NK cells that express CEACAM1, killing was significantly inhibited (Figure 4(g)). In contrast, when the killing was performed in the presence of P12ΔhopQ H. pylori no inhibition was observed (Figure 4(g)). As control for all of these experiments we incubated the target cells with CagA-deficient H. pylori, which behaved similarly to WT H. pylori. Taken together, these results indicate that binding of the HopQ protein of H. pylori to CEACAM1 may inhibits immune cell activities.

**Discussion**

After discovering that the colon cancer associated bacterium, *F. nucleatum* inhibits antitumor immunity, we wanted to examine whether this represents a single case or a more general phenomenon. We therefore investigated whether additional cancer-associated bacteria such as *S. galolyticus* and *H. pylori*, the two...
bacterial species most associated with cancer in humans, are also capable of inhibiting immune cell activities. Indeed, we found that *H. pylori* suppresses immune cells activity by binding to CEACAM1.

It was reported that HopQ binds to various members of the CEACAM family. The HopQ binding to CEACAM1 mediates attachment and facilitates the translocation of the CagA protein into the gastric cells. Importantly, we show that the interaction of HopQ with CEACAM1 expressed on immune cells may inhibit immune cell activities. Thus, using one bacterial protein, HopQ, and one cellular protein, CEACAM1, *H. pylori* infection might lead both to transformation and, at the same time, might protect developing tumors from immune cell attack. Interestingly, similarly to HopQ, Fap2 that interacts with the inhibitory receptor TIGIT also performs both cell-adhesion and immunosuppression functions.

Accumulating evidence suggest that both *H. pylori* and its human host utilize several strategies to suppress innate and adaptive immune response in an attempt to avoid pathology (for review see). The fact that *H. pylori* can be found in more than half of the human population, yet approximately 85% of *H. pylori* infections are mild and asymptomatic, might suggest for a peaceful co-existence of this bacteria with its human host. The HopQ–CEACAM1 interactions might contribute to such co-existence.

Gastric cancer progress from intraepithelial tumor without invasion of the lamina propria (carcinoma in situ) to a more aggressive tumor infiltrating the gastric wall (muscilaris mucosa, the submucosa and then the muscularis propria). *H. pylori* was identified mainly within the gastric surface mucus and crypts and not in the depth of the infiltrating tumor. Therefore, we suggest that the main immunomodulatory effect of this bacteria on immune cell (CEACAM1 activation by HopQ) occurs in the early stages of tumor development/progression.

The TIGIT and CEACAM1 inhibitory receptors are newly-investigated immune checkpoint inhibitors. A key challenge in the oncology field is to understand why certain patients respond to checkpoint inhibitors, while others are not. The ability of *F. nucleatum* and *H. pylori* to manipulate TIGIT and CEACAM1, respectively, might explain at least part of this patients’ heterogeneity.

### Materials and methods

**Primary human NK cells, T cells, cell lines, fusion proteins and antibodies**

Primary human NK cells were isolated from PBLs of healthy donors, using the EasySep human NK cell enrichment kit (StemCells Technologies). NK cell purity was 100% as determined by being positive for CD56 and NKP46 and negative for CD3 expression. CD16-negative NK cells were isolated using the human APC selection kit (STEMCELL Technologies). Isolated NK cells negative for CD16 were activated with IL-2. Primary human CD4 + and CD8 + cells were isolated from PBLs of healthy donors. Activated NK and T cells were generated by culturing isolated NK and T cells together with irradiated feeder cells (2.5 × 10⁴ allogeneic PBMCs from two donors and 5 × 10⁵ RPMI8866 cells in each well) and 20μg/ml PHA (Roche, Rehovot, Israel). Both PBMCs and RPMI 8866 cells were irradiated in 6000 rad before seeding in 96-well U-bottom plates, in the presence of IL-2. CEACAM1 expression was detected around two weeks later. The cultures were maintained in DMEM:F-12 Nutrient Mix (Sigma Aldrich, Rehovot, Israel; 70:30), 10% human serum (Sigma Aldrich), 2 mM glutamine (Biological Industries; BI, Beit-Haemek, Israel), 1 mM sodium pyruvate (BI), 1× nonessential amino acids (BI), 100 U/ml penicillin (BI), 0.1 mg/ml streptomycin (BI) and 500 U/ml rhIL-2 (Peprotech, Rehovot, Israel). A week later, irradiated feeder cells (2.5 × 10⁴ allogeneic PBMC from two donors and 5 × 10³ RPMI8866 cells in each well) were added.

The following cell lines were used: the human EBV transformed 721.221 cells, the mouse thymoma BW cells (BW5147. G.1.4 ATCC “TIB-48” Mus musculus thymus) and the murine mastocytoma p815 cell line. The generation of the various CEACAM1 transfectants: BW/CEACAM1, 721.221/CEACAM1 was previously described. All cells, except human primary NK, CD8 +, and CD4 + T cells were grown in RPMI medium supplemented with 10% FCS. Human NK, CD8 +, and CD4 + T cells were grown in the presence of 10% human sera, supplemented with human IL-2. The generation of CEACAM1-Ig, and the Control-Ig fusion proteins was previously described. The following commercial antibodies were used anti-human NKP46 (clone 9E2), anti-NKp30 (clone p30-15), anti NKP44 (clone p44-8), anti CD16 (clone B73.1), anti TIGIT (clone MBSA43), anti CEACAM1 (clone ASL-32), anti KIR2DL1 (clone HP-MA4), anti CD56 (clone HCD56), anti CD8 (clone SK1), and anti CD4 (clone A161A1), all from Biolegend.

The study was approved by the local Helsinki committee and complies with the Declaration of Helsinki (number HMO-0030–12).

**Bacterial strains and growth conditions**

*H. pylori* strains W0508 and W1354 were isolated from gastric ulcers at the clinical microbiology and laboratory of the Hadassah-Hebrew University Medical Center, Ein Kerem, Jerusalem, Israel. Both isolates, *H. pylori* P12 and P12ΔhopQ, were grown on chocolate, or on Wilkins-Chalgren blood agar plates (Novamed, Israel), under microaerobic conditions in jars using the Oxoid CampyGen atmosphere generating system. The generation of an isogenic HopQ mutant by replacement of the entire gene by a chloramphenicol resistance cassette was described before.

*S. gallolyticus* strains SG1, SG2 and SG3 were isolated from peripheral blood of patients with colorectal cancer at the clinical microbiology and laboratory of the Hadassah-Hebrew University Medical Center, Ein Kerem, Jerusalem, Israel, and were grown in brain-heart infusion broth (BHI) (Difco, MD, USA), at 37°C in an atmosphere enriched with 5% CO₂.
**BW assays**

The various bacteria were placed in 96 well plates and incubated for one hour at 37°C in medium containing RPMI supplemented with 10% FCS and penicillin-streptomycin. Subsequently, cells (50,000 of the appropriate BW or BW transfectants) were added and incubated together with the bacteria for 48 hours at 37°C, 5% CO₂. The final cell to bacteria ratio was 1:100 or as indicated. Next, supernatants were collected and the presence of mouse IL-2 in the supernatants was determined using standard ELISA assay.

**FITC labelling of bacteria and binding to cells**

*H. pylori* (~10⁹ CFU/ml) was labelled with fluorescein isothiocyanate (FITC) (0.1 mg/ml in PBS; Sigma-Aldrich) for 30 min at room temperature and washed three times in PBS. FITC labelled bacteria were incubated with various cells at various bacteria to cell ratios for 30 minutes at 4°C. Cells were washed and bacterium binding was detected using flow cytometry.

**Far western blotting**

P12 and P12ΔhopQ were grown for 8–10 days. Colonies were scraped from the chocolate or blood agar plate using a QuadLoop (Miniplast, Israel). One loop full of each bacterium was resuspended in PBS in an Eppendorf tube, washed with 1 ml PBS and sedimented at 13400 rpm for 3 minutes. The pellets were resuspended with 100 µl of Laemmli Sample Buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) or native buffer (lacking β-mercaptoethanol). The denaturative samples were boiled at 95°C for 10 minutes. The samples were centrifuged at 13400 rpm for 3 minutes, the supernatant subjected to SDS PAGE (7%) and then transferred to a nitrocellulose membrane as described before. The membrane was blocked in 5% skim milk for 2 h at room temperature and incubated overnight with CEACAM1-Ig (2 µg/ml), at 4°C. The membrane was washed three times (0.05% Tween 20 in PBS) and incubated with HRP-conjugated α – human IgG (company) diluted 1:5,000 in blocking solution for 1 hour in room temperature. After 3 washes (0.05% Tween 20 in PBS), proteins were detected using the ECL western blotting detection substrate.

**Killing and cytokine secretion assays**

For the redirected cytokine or killing assay with T cells, 25000/well of NK cells were pre-coated with anti-CD3 mAb and bacteria (at bacteria to target ratio of 100:1). For NK cell killing assay, 25000/well of NK cells were incubated with 721.221 cells that were pre-coated with bacteria (at bacteria to target ratio of 100:1) in a E:T ratio of 1:1. For both the redirected and direct killing assays, cells were incubated in the presence of 0.1µg allopurinol-conjugated CD107a mAb (Biotest, Ness Ziona, Israel) for 2 h at 37°C. CD107a levels on the effectors cells were determined by flow cytometry.

**Statistical analysis**

GraphPad Prism software version 6.0 was used for statistical analysis. Statistical tests used are indicated in the figure legends.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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