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# *Helicobacter pylori*'s Evasion of the Immune System Could Establish an Inflammatory Environment That Potentially Induces the Development of Coronary Artery Disease

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**ABSTRACT**

***Background:*** *Helicobacter pylori* is responsible for one of the most common human infections and is a major risk factor for stomach ulcer disease and gastric cancer. *H. pylori* infection has been reported to be associated with generation and development of coronary artery disease (CAD). Moreover, diabetic patients positive for *H. pylori* infection showed a higher prevalence of CAD compared to *H. pylori*-negative patients. The main association between *H. pylori* infection and CAD seems to be generation of chronic low-grade inflammation.
***Objectives:*** The current study aimed toinvestigate *H. pylori*'s capability to induce low-grade inflammation in the host; therefore *H. pylori* was compared to Escherichia coli *E. coli* in its ability to activate neutrophils. Furthermore, *H. pylori*'s capability to induce apoptosis in peripheral blood lymphocytes was studied.
***Materials and Methods:*** Peripheral blood neutrophils were treated with bacterial cells and the expression of the integrin CD11b that is critical for neutrophils adhesion, migration, and immune functions was assessed by flow cytometry. Additionally, peripheral blood lymphocytes were treated with *H. pylori* or *E. coli* then bacterial-induced apoptosis was examined by Annexin-V and Propidium Iodide (PI) staining.
***Results:*** The obtained data showed that CD11b expression on cells treated with *H. pylori* was significantly lower than cells treated with *E. coli*. Furthermore, *H. pylori* induced apoptosis in lymphocytes significantly more than *E. coli*. Conclusions: Diminished neutrophilic activation along with enhanced lymphocytic apoptosis could explain enhanced predisposition to CAD through induced chronic low-grade inflammation.

*Keywords: Helicobacter pylori*; Neutrophils; Lymphocytes; Apoptosis; Antigens CD11b; Phagocytosis



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Implication for health policy/practice/research/medical education:
*Helicobacter pylori* is responsible for one of the most common human infections and is a major risk factor for stomach ulcer disease and gastric cancer. *H. pylori* infection has been reported to be associated with generation and development of coronary artery disease (CAD). Moreover, diabetic patients positive for *H. pylori* infection showed a higher prevalence of CAD compared to H. pylori-negative patients. The main association between *H. pylori* infection and CAD seems to be the generation of chronic low-grade inflammation. To understand *H. pylori*`s capability to induce low-grade inflammation in the host better, *H. pylori* was compared to Escherichia coli E. coliin its ability to activate neutrophils. Furthermore, we studied *H. pylori*`s capability to induce apoptosis in peripheral blood lymphocytes was studied.

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## 1. Background

Helicobacter *pylori* is a Gram-negative helical-shaped bacterium responsible for stomach ulcer and gastric cancer (1). *H. pylori* infection has been associated with various extra gastric diseases such as, cardiovascular disease, diabetes, lung disease, and neurological disorders (2). *H. pylori*'s high global infection rate and its association with major human health complications put the bacteria in the most wanted list of researchers around the globe. Coronary artery disease (CAD) is the most common type of heart disease and it is the leading cause of human death worldwide (3).

High titers of *H. pylori*-specific IgG antibodies have been shown in cases with CAD (4). Elevated *H. pylori*-specific IgG antibodies were in parallel with development and progression of CAD (4). Also, CAD is one of the common extra-gastric manifestations of *H. pylori* infection. *H. pylori* has been suggested to be involved in the pathogenesis of CAD through colonization in endothelial cells, changes in lipid profile, increased coagulation and platelet aggregation, enhanced LDL-oxidation, and activation of inflammatory responses. Moreover, promotion of a low-grade systemic inflammation, and induction of molecular mimicry mechanisms have been linked to *H. pylori* infections (5, 6).

Vascular damage induced by *H. pylori* due to autoimmune mechanisms partly explains occurrence and destabilization of coronary atherosclerotic plaques (7). *H. pylori* infection can also increase the risk factors for ischemic coronary events by inducing Diabetes Mellitus, insulin resistance, micro albuminuria and metabolic syndrome (8, 9). The possibility of modifying clinical history of CAD through eradication of this pathogen is remained to be determined. More importantly, it is not known why *H. pylori* infections are most associated to CAD compared to any other Gram-negative bacterial infection.

*H. pylori* is able to evade the immune system thorough multiple mechanisms. In fact, it has been shown that *H. pylori* is less recognized by the innate immune system compared to most of the other Gram-negative bacteria (10). This will result in persistent infection and consequently the increased risk for CAD. The main advantage of *H. pylori* to other Gram-negative bacteria to evade the innate immune response is not completely understood. Neutophils are the most important cellular components of the innate immunity (11) and neutrophil loss could result in severe immunodeficiency. Phagocytosis of the pathogens is one of neutrophils major functions to fight the infection.

A mature neutrophil migrates from the blood towards the tissues, following a chemotactic gradient to a pathogen. Neutrophil functions are regulated by 2 inegrins, including CD11b/CD18. Circulating neutrophils express low amount of CD11b/CD18 in an inactive conformation. Activation and up-regulation of CD11b/CD18 mediates neutrophil adhesion, migration, and accumulation at the sites of inflammation. Neutrophil activating protein (HPNAP) is one of the major virulence factors produced by *H. pylori*. HPNAP is known to enhance neutrophil's adhesion to endothelial cells and also induce reactive oxygen species (ROS) in them.

## 2. Objectives

The current study showed that *H. pylori* can suppress the neutrophil activation by inhibiting the activation and up-regulation of CD11b on the surface of these cells which was in contrast with the function of HPNAP and elucidated a new mechanism for *H. pylori* evasion of the innate immune response. Furthermore, the possible inhibitory effects of *H. pylori* on the main cells of the adaptive immunity, namely T and B-lymphocytes were studied. The results showed a more pro-apoptotic function by *H. pylori* treatment on T-cells compared to *E. coli*. Hereby, an inhibitory function by *H. pylori* on the major cells of the innate and adaptive immune system to further evade the immune system to establish a lifelong infection in human population was shown.

## 3. Materials and Methods

### 3.1. Bacterial Strain and Culture

*H. pylori* was isolated from urease-positive samples of gastric antral biopsies. Bacterial cells grown on microaerophilic condition (85% N2, 10% CO2, 5% O2) in 37°C on *H. pylori* specific media consisted of Brucella agar (Merk, Darmstadt, Germany) supplemented with yeast extract, meat extract, ferrous sulfate, 10% (v/v) fresh sheep blood, Amphotricine B (1.6mg/L) (Fungizone, Gibco), Vancomycin (6mg/L) (Sigma-Aldrich, Steinheim, Germany) and Trimpetoprime (5mg/L) (Sigma-Aldrich, Steinheim, Germany(.

Grown bacteria were characterized by colony morphology, microscopic evaluation after Gram staining, rapid urease test, oxidase and catalase test 48-72 h post-culture. Bacterial cells were re-suspended in phosphate buffer saline (PBS), and enumerated using spectrophotometer. A multiplicity of infection (MOI) of 100 was used for all blood cell treatments (108 bacterial cells/ 106 cells). *E. coli* (PTCC 1335) was cultured on nutrient agar and nutrient broth.

### 3.2. Whole Blood Treatment with H. pylori and E. coli

Whole blood was collected from healthy donors in heparinized tubes by venipuncture of the forearm vein. First, leukocytes were counted and adjusted to a concentration of 106/100 l in PBS and then transferred into test tubes.

For bacterial treatment 108 live bacterial cells or PBS (negative control) were added to each tube. Test tubes were incubated at a thermo mixer machine for 10 min at 370C to allow proper mixing of the cells. Afterwards, test tubes were incubated at 370C and 5% CO2 for an additional 3h. The treatment groups were shown in *Table 1*. All experimental groups were in duplicates. After 3h, cells were stained for surface CD11b with FITC-conjugated antibody or isotope control antibody (SeroTec,UK)as shown in *Table 1*.

**Table 1.** Table of Different Experimental Groups

| Treatment | Staining Protocol |
|---|---|
| **PBS** | No staining |
| **PBS** | FITC-conjugated murine anti-human IgG1 monoclonal antibody |
| **PBS** | FITC-conjugated murine anti-human CD11b monoclonal antibody |
| ***H. pylori* ($10^8$bacteria per $10^6$cells)** | FITC-conjugated murine anti-human IgG1 monoclonal antibody |
| ***H. pylori* ($10^8$bacteria per $10^6$cells)** | FITC-conjugated murine anti-human CD11b monoclonal antibody |
| ***E. coli* ($10^8$bacteria per $10^6$cells)** | FITC-conjugated murine anti-human IgG1 monoclonal antibody |
| ***E. coli* ($10^8$bacteria per $10^6$cells)** | FITC-conjugated murine anti-human CD11b monoclonal antibody |

Ab staining was performed for 20min in dark. Cells were washed two times and red blood cell (RBC) lysis was carried out using a commercial RBC lysis solution (DAKO, S3350). Neutrophil CD11b expression was analyzed by using flow cytometer (Partech, Germany). Neutrophil population was initially gated based on their typical forward-scatter and side-scatter characteristics *Figure 1.* Mean fluorescence intensity (MFI) of the CD11b expressing neutrophils was calculated using WindowsTMFlowMax software (Partec, Germany).

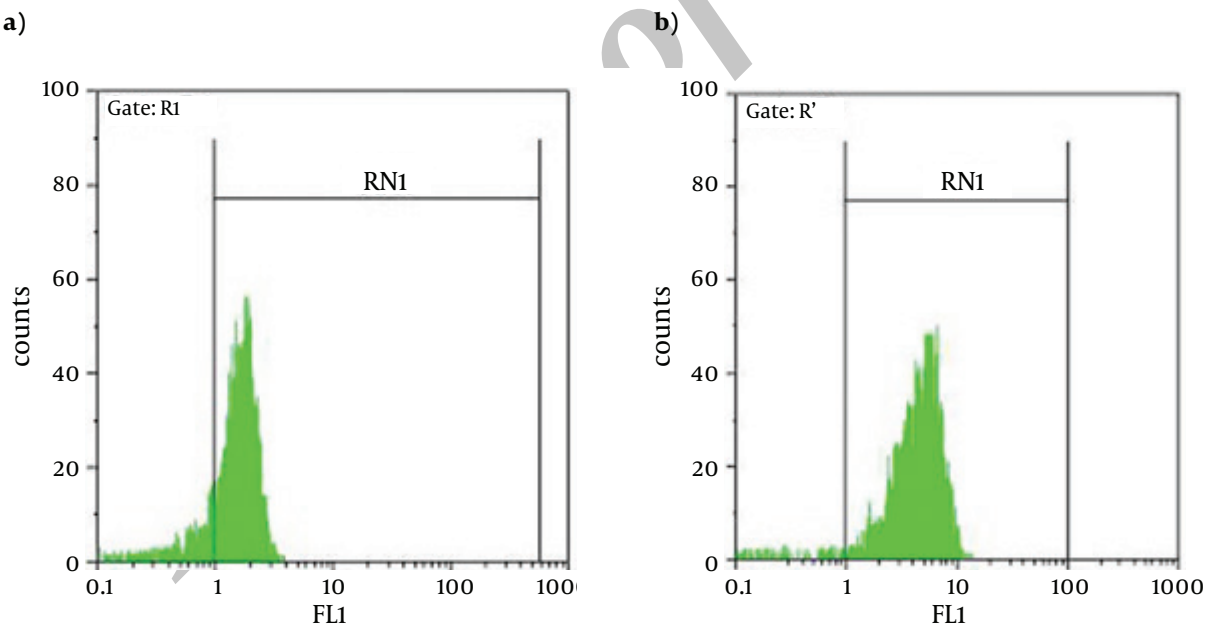


**Figure 1.** Neutrophil Population Based on the Forward and Side-Scatter Characteristics

### *3.3. Isolation of Peripheral Blood Mononuclear Cells and Bacterial Treatment*

PBMCs were obtained from healthy donors using His opaque 1077 (Sigma-Deisenhofer, Germany) according to manufacturer's protocol. PBMCs were counted using a hem cytometer and diluted in RPMI-1640 (Sigma, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS). PBMCs were co-cultured with Bacterial cells for 24h at 370C. Doxorubicin was used as a positive control for apoptosis induction in lymphocytes ( *Table 2* ). Bacterial induced-apoptosis in lymphocytes was examined by using annexing V apoptosis detection kit (BD Pharming) according to the manufacturer's protocol. Results were analyzed using WindowsTMFlowMax software (Partec, Germany).

**Table 2.** Studying of Apoptosis Treatment Groups

| Negative Control | No Treatment |
|---|---|
| ***H. pylori*** | $10^8$bacteria per $10^6$cells |
| ***E. coli*** | $10^8$bacteria per $10^6$cells |
| **Positive control** | Doxorubicin |

### 3.4. Statistical Analysis

Statistical comparisons were performed using Student's t-test with the aid of GraphPad Prism software (GraphPad Prism Software, Inc., La Jolla, CA,USA).

## 4. Results

*H. pylori* reduces neutrophil activation by decreasing the extent of CD11b expression on the surface of these cells.It was previously shown that *H. pylori* induces less TNF- mRNA expression on PBMCs compared to *E. coli* (12). To further extent the current research on *H. pylori*'s immune-evasive mechanisms, which could potentially result in higher incidence of CAD in patients infected with *H. pylori*, the surface expression of CD11b on neutrophils ( *Figure 2* ) treated with either *H. pylori* or *E. coli* were compared.

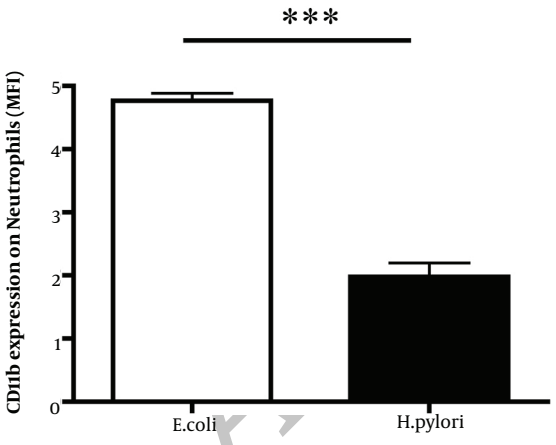


**Figure 2.** Cd11b Expression (Mfi) on the Surface of Neutrophils Treated With a) *H. pylori* and b) *E. coli*

Neutrophils in whole blood were treated with clinical strains of *H. pylori* or *E. coli* in a 100 MOI. Then blood cells were stained for surface CD11b expression, followed by RBC lysis and flow cytometry analysis. Neutrophils treated with *H. pylori* showed significant reduced levels of CD11b expression on their surface compared to the neutrophils treated with *E. coli* ( *Figure 3* ), suggesting that *H. pylori* could induce a sub-optimal activation of neutrophils in order to establish a life-long chronic infection, which could potentially increase the chance of CAD in the infected individuals.On one hand, the current study and other groups showed that *H. pylori*'s lipopolysaccharide (LPS) is less stimulatory to PBMCs compared to *E. coli* and other Gram negative bacteria.

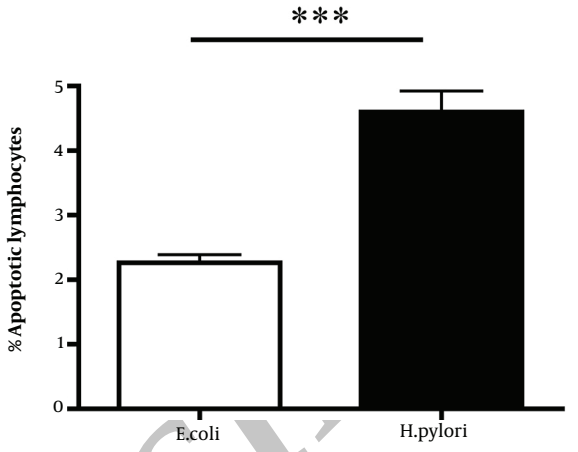


**Figure 3.** Comparison of CD11b Expression on the SurfaceOf Neutrophils Treated With *H. pylori* Or *E. coli*

On the other hand, CD11b has proved to play an important role in neutrophil's phagocytosis (13). In order to find a role for bacterial non-structural genes in reduced activation of neutrophils, wild-type *H. pylori* and iron-uptake deficient mutant were compared in their ability to resist neutrophils phagocytic activity. The current research preliminary studies using the iron-uptake mutant strains of *H. pylori* suggest that iron-uptake capacity of bacterial cells could suppress neutrophil's phagocytosis and activation (unpublished data). The findings show a new mechanism of *H. pylori*'s resistance to cellular immunity, which could result in enhanced chronic inflammation in CAD patients. *H. pylori* induces stronger apoptosis in lymphocytes compared to *E. coli*.

Lymphocytes play an important role in adaptive immunity and induced lymphocyte apoptosis could potentially help to establish bacterial infections in the patients. *H. pylori* and *E. coli* in their ability to induce apoptosis on PBMCs were compared. PBMCs was isolated from peripheral blood of healthy donors using Histopaque 1077. Isolated PBMCs were then co-cultured with either *H. pylori or E. coli* in RPMI-1640 supplemented with 10% FBS for 24h.

Doxorubicin-treated cells were used as positive controls for apoptosis. When lymphocytes were stained with PI and FITC-labeled annexing V, apoptotic cells were identified as PI-negative and annexing V-positive cells by FACS analysis. The results showed significant increase of apoptosis among PBMCs co-cultured with *H. pylori* compared to the cells co-cultured with *E. coli* ( *Figure 4* ). These results provide a potential immune-evasive mechanism of *H. pylori* infection in humans.

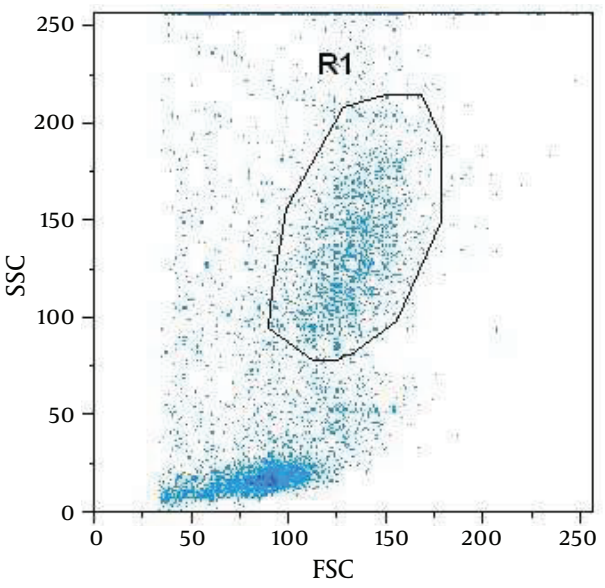


**Figure 4.** Comparison of Induced Apoptosis in Lymphocytes Treated with *H. pylori* and *E. coli* From Triplicate Tests

## 5. Discussion

*H. pylori* infection is closely related to major gastric diseases, such as gastric ulcer and gastric cancer. It is increasingly evident that *H. pylori* infection might enhance the occurrence of CAD in the infected individuals. CAD is the most common heart disease in the world. Considering the prevalence of *H. pylori* infection in the world and its potential role in increasing the chance of CAD attracts attentions to the underlying mechanisms of this relationship. *H. pylori* can establish a lifelong chronic infection in the host, which provides a chronic inflammatory environment necessary for the development of CAD in the patients.

In the present study, it was reported that *H. pylori* induces a suboptimal activation of neutrophils that could potentially suppress the mounted innate immune response and help to establish a chronic inflammatory environment in the periphery, necessary for CAD development. The role of soluble antigens of *H. pylori* in the activation of CD11b on the surface of neutrophils had been reported before (14). In the current study, the intact bacterial cells were used and similar results were found. Furthermore, for the first time, a role for non-structural genes of the bacteria in the suppression of neutrophil activation was observed.

The unpublished results show a direct link between iron-uptake genes in the bacteria and host neutrophil activation and function. Neutrophil functions are modulated by the highly abundant integrin CD11b/CD18, which is normally present in an inactive conformation in circulating neutrophils. Proper activation and up-regulation of CD11b/CD18 on the surface of neutrophils, warrants neutrophil normal function. Here it was shown that *H. pylori* interferes with normal neutrophil functions as a result of *H. pylori*'s iron-uptake genes function (unpublished data). *H. pylori* is capable of inducing apoptosis in various cells of the immune system (15).

Here, it was shown that apoptosis was significantly higher among *H. pylori* ¬treated lymphocytes than lymphocytes treated with *E. coli*. The current research results support the findings from Lewis *et al*. (15) that showed *H. pylori* induces apoptosis in B- and T-cell lines. This could potentially help the bacteria to establish a chronic infection, which could result in chronic inflammation necessary for CAD. Diminished neutrophilic activation along with enhanced lymphocytic apoptosis could explain enhanced predisposition to CAD through induced chronic low-grade inflammation.

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## Financial Disclosure

The authors declare that they have no competing financial interests.

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## Authors' Contribution

Saman Maleki-Vareki was MSc student and conducted all the experiments and helped in manuscript preparation. Hamid Zarkesh-Esfahani was the supervisor and helped with designing the experiments, analyzing the data and manuscript preparation. Mohaddeseh Behjati helped in analyzing the data and manuscript preparation.

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