

Immunomodulatory Effects of *Helicobacter pylori* on Pro and Anti-inflammatory Cytokines Production in Peripheral Whole Blood Cells Culture

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Recent documentations about sepsis as a systemic inflammatory response and inefficiency of some antimicrobial treatments in immunological disorders outline that any intervention modifying the immune response with therapeutic intent, will be rational. *Helicobacter Pylori (H.pylori)*, with major role in etiopathogenesis of duodenum peptic ulcer and gastric lymphoma, exerts its immunomodulatory properties with long time intestinal colonization and protection against several certain inflammatory disorders. Due to the unclearness of this pathogen immunomodulatory effects on whole blood cells culture, the aim of this study is evaluation of *H.pylori* whole body immunomodulatory effects on whole blood cells culture. In this study, after *H.pylori* isolation from a pathological positive biopsy specimen, it was cultured in Brucella Blood Agar. Then, bacteria was co-cultured with whole blood cells of 7 persons in RPMI-1640 for 22h with CO₂ incubation. At last, TNF-alpha and TGF-beta concentrations of supernatants were assayed by quantitative ELISA. Finally, non-parametric tests were measured out for data analysis. Our results showed upregulation of TGF-beta and TNF-alpha secretion, exposure to *H.pylori* (p value 0.017 and 0.018, respectively) compared to their untreated culture. These data show that immune response of *H.pylori* may promote pro and anti-inflammatory responses. It seems that clinical applications of cytokines secretion, may be a promising window for further investigation leading to improvement of immune related diseases.

Key words: Immunomodulation, *Helicobacter pylori*, Whole blood cells, Cytokine.

According to the previous studies, it is demonstrated that infectious diseases are one of the major challenges for Health System in which, alteration in the soluble mediators function fails to induce an adequate immune response and is

accompanied by hypo/hyper activity of immune responses. In addition, some problems as: reappearance of invasive pathogens and changing nature of infectious disease manifestation, add more considerations up for scientific researchers and physicians. Accordingly, in light of these problems, sepsis has been continued to be a worldwide dilemma with the prevalence of high mortality¹.

Also, indiscriminately usage of antibiotics

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has led to antibiotics resistance, multi drug resistant strains reappearance and chronicity of the diseases, recently. Overall, these issues, visualize several severe gaps in clinical trials which led scientists to look for novel directions toward immunobiology targeting¹. Therefore, it is expected that an optimistic insight and supportive point of view on other therapeutic strategies should be created. This is where, it is rational to interface with some elements contributing to the re-regulation of the dysregulated immune responses.

The term “Immunomodulation” refers to any intervention modifying the immune response with therapeutic intent. “Immunomodulators” are considered as active substances in immunotherapy based therapeutic methods. These agents are approved by the International Organization for Food and Drug Administration (FDA) with fewer side effects and less potential for drug resistance advantages. There are a wide variety of immunomodulators from natural and organic compounds to synthetic and recombinant ones²⁻⁴.

In this strategy, activity of the host immune system, changes through the dynamic regulation of signaling molecules such as: antibodies, soluble receptors, bio drugs, hormones, acute or chronic stress, adhesion molecules, neurotransmitter peptides, cytokines and even bacterial extract (or cellular membrane fractions of bacteria). Generally, these changes include in immunostimulation, immunosuppression and immunological tolerance induction assisting to adjustment of the desired immunity level on the circumstances¹⁻³.

Growing number of scientific investigations and documentations indicate that certain biological agents, specially a well-known group of microorganisms, also exert immunomodulatory properties by modulating leukocyte function and circulating concentrations of proinflammatory cytokines as extracellular (glyco) protein key regulators and messengers of the immune system^{5,6}.

Helicobacter Pylori (*H.pylori*), a spiral gram negative bacterial pathogen with long term colonization in the antrum and duodenum, is associated with gastric atrophy, duodenum peptic ulcers, gastrointestinal adenocarcinoma and Mucosal Associated Lymphoid Tissue (MALT) lymphoma⁷⁻¹¹. In addition of several bacterial

virulence factors role for its pathogenicity, host immune system responses help to gastric damage development. Immunopathology of *H.pylori* mainly depends on Th1 cells and their secreted cytokines. It is demonstrated that *H.pylori* confers protection against some extra gastric diseases such as: Asthma, Allergy, atopic diseases and chronic inflammatory disorders. This bacterium, presents its immunomodulatory properties with deviation of adaptive immune system toward immune tolerance induction through CD4⁺ CD25⁺ Regulatory T cells, reprogramming of CD103⁺ Dendritic cells to tolerogenicity phenotype and associated interleukin-18 (IL-18)⁸⁻²⁰.

Anyway, our knowledge of the immunological communications about protection against pathogens is superficial. Subsequently, evaluation of immunopathophysiology of the pathogens, outstanding about cytokines activity and their receptors, cytokines cascades involved in microbial-host immune responses interactions, and recruitment of an appropriate immunomodulator to immunologic phase of diseases should be considered to accelerate the realization of overcoming to diseases rate^{2,3}. It seems that clinical applications of shifts in cytokines secretion due to the immunomodulators usage, may be a potentiated window for elimination of undesirable immunological reactions leading to improvement of immune levels for whom suffering from such immune related disorders.

Since that, there has been no study on whole blood cells with whole body of *H.pylori* in order to assess immunomodulation, the purpose of this research is conducting an *in vitro* study evaluating immunomodulatory properties of *H.pylori* on peripheral whole blood cells culture by cytokines assay.

MATERIALS AND METHODS

Ethical considerations

This study accessed ethic's approval from the Zanjan University of Medical Sciences Research Ethic's Committee (no. ZUMS.REC. 1394.143). Among the medical faculty students, seven young healthy (*H.pylori*-non infected, without chronic inflammatory diseases history) volunteer women (range 20-25 years old) were recruited for the purpose of this study. First, by

giving them explanations about moral confidence and biosafety, oral consent was reached and then, a written informed consent was achieved.

Study design

RPMI-1640 (complete medium) preparation

To the RPMI-1640 as incomplete serum free cell culture medium ((Gibco. KBC Company) With L-Glutamine and NaHCO_3), penicillin-streptomycin solution 1% (100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin) (Sigma-Aldrich) was added, which supplemented with 10% (vol/vol) heat inactivated (56°C for 30 min) Fetal Bovine Serum (FBS) (Gibco). This, was designated as a complete homogenous cell culture medium following gently inverting.

Immunomodulatory compound preparation

Bacterial strain culture

First, from a pure bacterial culture of an approved pathological positive biopsy endoscopy specimen (CagA^+ , VacA^+) belonged to a peptic ulcer patient, isolation of *H. pylori* was done. After transferring this bacterial culture petri dish into an equipped designated microbiology laboratory in a jar with gas pack C (Merck.KGaA. Germany), a loop of bacteria introduced into a Brain Heart Infusion (BHI) broth culture (Merck. Germany) as an enrichment/activator medium and incubated for 24 hours at 37°C in a microaerophilic condition.

Subsequently, Brucella Blood Agar (supplemented with 10% defibrinated sheep's blood (Provided by ROSHD, Med Biotech, IRAN), 10 $\mu\text{g/L}$ vancomycin (DANA Pharmaceutical Company, IRAN), 5 $\mu\text{g/L}$ trimethoprim (Amin Pharmaceutical Company, IRAN), 2.5 IU/L polymixin B and Brucella Agar (Merck. Germany)) was considered as a reference culture medium. A further streak cultured plate of bacteria was prepared and incubated for 5 days at 37°C at a candle jar to ensure bacterial characteristics (Main stock cultures were kept at -20°C with 18% glycerol in BHI broth).

Then, colonies with tiny, round, raised, flat, transparent features and 0.5-1 millimeter in diameter, were observed and microbial tests such as Gram staining for purity and bacterial morphology assessments were done.

Ultimately, the nature of bacterial strain was confirmed through positive results for biochemical tests such as tube Urease (Urea Agar culture), slide Catalase and oxidase.

Here, viable counts of cultured bacterium (Colony Forming Unit) was measured at Optical Density ($\text{OD}_{600\text{nm}}$) $1.3 \pm 0.1 \times 10^9$ ($\text{OD}_{600\text{nm}}$ at exponential growth phase of bacteria is equivalent to 3.4×10^9 after 28h incubation).

Bacterial solution preparation

It should be noted that concentrations of whole bacteria used overall this experiment, were reached in our previous trials in order to induce immunomodulatory effects as follows:

Here, confirmed colonies were scrapped from Brucella Blood Agar surface and transferred into BHI broth. Next, 1.5 ml of 24 hours harvested *H.pylori* in BHI broth media was collected into an eppendorf microtube and centrifuged (during log growth phase of bacteria). Supernatant fluid was removed and remained pellets were once suspended in 1.5 ml of 0.01M ice-cold sterile PBS (Phosphate Buffered Saline, $\text{pH}=7.28$) (MERCK. Germany). The eppendorf microtube was again centrifuged. Next to discarding supernatant, 1.5 ml of RPMI-1640 was added which followed by gently mixing to reach homogenous bacterial solution. Concentration was determined by colony forming unit (CFU) counting.

Whole blood cells preparation

To substructure TNF-alpha and TGF-beta expression manifestation in the response to antigenic stimulation (immunomodulator), human peripheral whole blood cells were chosen for our experiment's end.

Participants (blood donors)

Median age and BMI of participants were shown in table2. All donors had antigens A on their erythrocytes surface (blood group A).

Blood collection

Participants were pleased to come to the laboratory. Blood samples were withdrawn by an authorized supervisor with sterile gauge needles. Antecubital venous blood (7 ml) was collected into vacutainer blood collection tubes containing sodium heparin (Caspian Tamin Co. Iran).

Blood analysis

Blood samples which were collected into heparinized tubes, first inverted several times to mix properly with anticoagulant agent. Then, they were delivered to hematology ward for hematological analysis immediately to achieve the best unbiased results by an automated hematology analyzer (Nihan Koden. Cell Tac E. Japan). Each

sample was measured twice and the average value was recorded for all donors (Table 2).

Study procedure

After preparation of reagents, procedure of study was defined as below:

Whole blood cells culture stimulation

After confirmation about study design, first of all, stimulant was reconstituted by bringing it to room temperature up. As shown in Table 1, required volumes of RPMI-1640 were added into the culture wells of a 48 flat bottomed wells sterile-polystyrene cell culture plate (Jet Biofil. Mainland) (1.55 ml and 0.19-0.29 ml, Max and working well volume respectively) followed by 0.25 ml of heparinized whole blood cells (final estimation: 2×10^6 cells per ml). Next, followed by adding the appropriate amount of bacteria solution (Table 1) in aseptic condition under biological safety cabinet (Laminar flow class II). Then, ingredient of each well was gently mixed by several times aspiration and ejection.

Incubation conditions of treated whole blood cells

Thereafter, antigen stimulated specimens (table1), were incubated for 22h at 37°C and 5% CO₂ atmosphere in a 90% humidified chamber (Time of incubation was set). After cultivation, and cell counting, viability assay was done for human whole blood cells.

Supernatant collection

After 22h incubation, supernatants of all samples were transferred into 1.5 ml microcentrifuge eppendorf tubes and spun for 5 min at 5000 rpm at room temperature (Sigma). Subsequently, supernatant phase of fluid for all samples, were collected into another new labelled eppendorf tubes and stored temporarily at -20°C freezer.

Measurement of cytokine secretion

First, supernatant fluids were removed from freezer and kept at room temperature for thermal equilibrium. Results of this study, were documented with scales of secreted cytokines (TGF-beta and TNF-alpha) profile of homogenated human peripheral whole blood cells stimulated by pathogenic isolate of *H.pylori* through an Enzyme Linked Immunosorbent Assay (ELISA) method. All assays were run in triplicate for all wells containing samples and the average scores were registered by a blind technologist.

ELISA for cytokine assay

TGF-beta1

The assay was performed in order to manufacturer's instruction commercial kit for quantitative detection of TGF-beta1 cytokine (eBioscience. Bender Med System GmbH. Austria. REF BMS249/4/) in cell culture supernatants which was estimated as below:

Briefly, after pretreatment of samples with assay buffer 1x, 1N HCl and 1N NaOH, 100 µl of assay buffer 1x was added to blank well. 100 µl of each standard solutions was added to standard wells. Then, 60 µl of assay buffer 1x and 40 µl of vortexed pretreated samples were added to sample wells. After incubating for 2h at room temperature and 400 rpm (set on a microplate thermoshaker), the human anti TGF-beta1 coating antibodies which are adsorbed onto microwells, bind to human TGF-beta present in the samples and standards.

Next to washing, 100 µl of Biotin-Conjugated anti human TGF-beta detecting antibody was added to each microwell strips and incubated as clarified recently in order to bind to complex which was made in previous step.

After washing, unbound biotin conjugates were removed and 100 µl diluted Streptavidin- Horse Radish Peroxidase (HRP) was added to all wells and incubated as said before, to bind to 3-fraction complex which was created in previous step.

After last washing for removing unbound Streptavidin-HRP, 100 µl of Tetra Methyl Benzidine (TMB) Substrate (as chromogene), was added to each well to associate to last 4-fraction complex. It was incubated for about 30 minutes at room temperature for color development without exposure to direct intense light. Color intensity is proportional to the HRP activity.

After that, as soon as the highest standard was reached to dark blue color and an OD of 0.90-0.95 for standard solution was recorded, enzyme-substrate reaction should be terminated by pipetting 100 µl of stop solution (Sulfuric Acid) into each well. This is where, changing pH of the medium by stop solution seems essential for inhibition of enzymatic reaction and stabilizing color development to reach an accurate intensity measurement. Absorbance of each well was read immediately on an ELISA MICROPLATE READER

(Garni Company) using 450 nm (primary) and 620 nm (reference) wave lengths. Cytokines concentrations were calculated with a standard curve.

TNF-alpha

The main investigation procedure of TNF-alpha assay was done similarly to TGF-beta exception for sampling step (samples don't any need to pretreatment and only should be diluted by adding sample diluent buffer) in order to manufacturer's instruction (eBioscience. DX. Bender Med Systems GmbH. Austria. REF BMS223/4CE).

Two kits for mentioned cytokines were human specific with sensitivity of 8.6 ± 2 pg/ml and 2.3 ± 2 pg/ml for TGF-beta and TNF-alpha, respectively.

Statistical Analysis of data

The Statistical Package for the Social

Sciences (SPSS) version 16.0 computer software (SPSS Inc., USA) was used for the statistical analysis of acquired data. Non-parametric statistical tests (Freidmann's and Mann Whitney) and descriptive tests were applied for information and data of the experiment. Results were expressed as Mean plus minus Standard Deviation (SD). Statistical significance was considered at p value < 0.05 .

RESULTS

Hematological parameters of blood samples

Table 2 indicates the Mean and SD scores recorded for hematological analysis of blood samples. All of these hematological parameters scores fell into the normal ranges.

Cytokines production by *H.pylori* stimulated whole blood cells culture

Table 1. Consumed volumes of RPMI-1640 and immunostimulants solution for each well

Well	RPMI-1640 (Medium/Dilutor) (µl)	Diluted bacterial solution (adjuvant) (µl)	Whole blood (target) (µl)
1*	1000	-	-
2*	750	-	250
3	725	25	250

Final volume for each well was considered 1000µl.
1* and 2* were blank for culture medium and blood cells, respectively

Table 2. Descriptive Statistics results of hematological parameters recorded for blood samples at a glance

Parameters	Age	BMI	HCT	WBC	RBC	PLT	NEU	HGB	LYMPH
Mean ±SD	22.42± 2.14	23.89± 1.53	42.12± 0.15	4.60± 0.24	4.77± 0.10	172± 40.0	2.46± 30.2	13.97± 0.3	2.02±

RBC= Red Blood Cells WBC= White Blood Cells , LYMPH= Lymphocyte, NEUT= Neutrophil, HCT= Hematocrit, PLT= Platelet, HGB= Hemoglobin, BMI= Body Mass Index

Table 3. TGF-beta cytokine production (pg/ml) in response to *H.pylori* stimulated whole blood cells culture

Well	RPMI-1640(1)	RPMI+whole blood(2)	RPMI+whole blood+ <i>H.pylori</i> (3)
Mean±SD	blank	25.28±2.87	43±1.73

Table 4. TNF-alpha cytokine production (pg/ml) in response to *H.pylori* whole blood cells culture

Well	RPMI-1640(1)	RPMI+whole blood(2)	RPMI+whole blood+ <i>H.pylori</i> (3)
Mean±SD		97±2.16	157.14±2.96

TGF-beta cytokine production by *H.pylori* stimulated whole blood cells culture Table 3, shows the average concentrations of the TGF-beta cytokines produced in the *H.pylori* stimulated whole blood cells culture meanwhile the nature of the wells were clarified after data statistical calculation.

As shown in Table 3, *H.pylori* significantly upraised TGF-beta cytokines secretion by whole blood cells after 22h incubation (well no.3) compared to control well (well no.2) (p value=0.017).

TNF-alpha cytokine production by *H.pylori* stimulated whole blood cells culture

Table 4, shows the average concentrations of the TNF-alpha cytokines produced in the *H.pylori* stimulated whole blood cells culture meanwhile the nature of the wells were clarified after data statistical calculation.

As shown in Table 4, *H.pylori* significantly upraised TNF-alpha cytokine production by whole blood cells after 22h incubation (well no.3) compared to control well (well no.2) (p value=0.018).

DISCUSSION

H.pylori is a bacterium with colonization in gastric mucosa, has tight association to several adverse clinical sequelae like peptic ulcers, gastric cancers (metaplasia and dysplasia of gastric mucosa) induction, non- Hodgkin lymphoma, bacteremia (as a rare complication) and amazing ability for co-existence with humans for decades with subsequent evasion of chronic gastric inflammation²¹⁻²⁵. Persistence and severity of *H.pylori* infection, depends on bacterial components, host associated factors (such as susceptibility, immune responses and genetic factors) and environmental factors that determine bacterium switching to commensalism or pathogenicity variations^{2, 21, 23-26}.

This prolonged interaction between bacterium and host immune system, makes it as a feasible infectious agent for triggering autoimmunity which is characterized with loss of tolerance to self-antigens. There are strong documentations on *H.pylori* infection and it's association with development of many extra gastric chronic autoimmune diseases such as: urticaria,

psoriasis, Schoenline Henoch Purpura (SHP), vasculitis, scleroderma, typhoid fever, chronic Hepatitis C, Coronary Artery Diseases (CADs), type 1 diabetes, chronic autoimmune thyroiditis (Hashimoto's) and Graves' Diseases. Infection with *H.pylori* elicits a significant immunomodulation^{23, 27-34}.

In addition, it is demonstrated that *H.pylori* chronic infection may also be beneficial to host immune system for conferring protection against allergy, rhinitis, asthma, atopic dermatitis (Eczema) and Inflammatory Bowel Diseases (IBD) which is highly associated with suppressive Treg cells function^{9, 12-14, 18, 20, 24, 35-47}.

Inducible Treg cells are considered to immune tolerance maintenance through induction of anergy and deletion of autoreactive T cells and believed to be generated by tolerogenic semi mature Dendritic Cells (DCs) which are highlighted for us with converting naïve T cells into Foxp3 Treg via antigen presentation in the absence of co-stimulatory molecules. Reprogramming of *H.pylori* experienced DCs to tolerogenic phenomenon, requires inflammasome and caspase-1 activation, secretion of IL-18, and a TGF-beta dependent manner^{23, 24, 36, 48}.

Several studies investigated the role of declining in *H.pylori* infection prevalence at increased risk of celiac diseases^{16, 49}. Others, suggest earlier eradication of *H.pylori* for alteration in immunopathophysiology of neurodegenerative disorders including Multiple Sclerosis (MS), peripheral neuropathies, migraine, Parkinson's Disease (PD), Alzheimer's Disease (AD), epilepsy, Crohn's diseases, and Ophthalmological disorders as: glaucoma and non-arteritic anterior ischemic optic neuropathy, even others such as Idiopathic Thrombocytopenic Purpura (ITP), eosinophilic esophagitis and HIV⁵⁰⁻⁶⁰.

These already mentioned, had been clinical studies based on *H.pylori* immunomodulation properties. So far, whole body of *H.pylori* effects on whole blood cells in order to immunomodulation assessment has remained unclear. Overall, in the present study aimed to investigation of *H.pylori* immunomodulatory impact on peripheral whole blood cells culture, our results corroborated that *H.pylori* may trigger a strong modulation and upregulation on both TGF-beta and TNF-alpha cytokines secretion in whole

blood cells compared to control (p value= 0.017, 0.018, respectively). The amounts of secreted TNF-alpha by whole blood cells after exposure with *H.pylori* were more than TGF-beta. So, it seems that *H.pylori* can induce Th1 and Th2 responses which promote pro and anti-inflammatory actions. There are no such as this study and our results are in accordance with some partially similar previous studies. *M.Wiese et.al* in their study aimed to investigation of Immunomodulatory effects of live *Lactobacillus Planatarum* and *Helicobacter Pylori* on some cytokines and cell surface markers induction in whole blood culture, showed that both, *H. pylori* alone and in combination with *L. plantarum*, had a strong modulatory effect on the synthesis of IL-10 and an inhibitory effect on IFN-gamma secretion⁶¹.

Jay Luther et.al showed that *H pylori* DNA has the ability to downregulate pro-inflammatory responses from DC and this may partly explain the inverse association between *H pylori* and IBD with lower systemic type 1 IFN⁶².

Frank Meyer et.al indicated that Th1 polarization of the gastric immune response may be due in part to the direct effects of multiple different *H. pylori* components that enhance IFN-gamma and IL-12 production while inhibiting both IL-2 production and cell proliferation that may be necessary for Th2 responses⁶³.

Songhua Zhang et.al observed that antigen-specific functional differences between the T cell repertoires of *H. pylori* positive and -negative subjects for a set of 90 immunoinformatic-predicted T-cell epitopes from seven genetically diverse *H. pylori* strains. Cytokine ELISA measurements performed using in vitro PBMC culture supernatants, demonstrated significantly higher levels of TNF-alpha, IL-2, IL-4, IL-6, IL-10, and TGF-beta1 in the *H. pylori*-infected subjects, whereas IL-17A expression was not related to the subjects *H. pylori*-infection status²².

Benedetta Romi et.al for finding if and how *H. pylori* could induce and modulate human T cell activation, studied direct interaction of live, but not killed bacteria with purified CD3+ T lymphocytes on lymphocytes freshly isolated from peripheral blood of *H. pylori*-negative donors. The results showed activation of CD3+ T lymphocytes, predominantly gamma-delta T cells, upregulation

of CD69 and cytokines such as TNF-alpha and IFN-gamma favouring to inflammatory microenvironment leading to incompletely clearance of pathogen, local inflammation and eventually to the gastric and duodenal diseases⁶⁴.

In conclusion, to attribute and generalize results of this study to clinical manifestations, it should be noticed that there is a complex immune response to *H.pylori*. In addition, this pathogen has ability to induce Th1 and Th2 immune responses. Induction of TNF-alpha production as a multifunctional cytokine has a key role in apoptosis, cell survival and antitumor activity as well as in inflammation and immunity. Upregulation of TGF-beta secretion, stimulates Th2 responses differentiation, Th1-mediated autoimmune disease reduction, and appears immunological tolerance leading to Treg cell proliferation, multi-organ inflammation and autoantibodies development diminution.

CONCLUSION

According to results of our study, *H. pylori* has a special modulatory impact on immune system responses with pro and anti-inflammatory cytokine induction.

Immune related anomalies have been still remained as a most common concerns for health care system. It appears that consideration of time for immune intervention, accurate understanding on immunopathophysiology of infectious related immune disorders, predomination of pro and/or anti-inflammatory cytokines and conditions of immune microenvironment which are pivotal to fulfill immunomodulation.

This study refers to an optimistic point of view provided to further investigation on clinical applications of alteration in cytokines secretion due to the immunomodulators usage. This may be a promising window in order to elimination of undesirable immunological reactions and improvement of immune related diseases.

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