

Increased NF- κ B Activity in HCT116 Colorectal Cancer Cell Line Harboring TLR4 Asp299Gly Variant

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ABSTRACT

Toll-Like Receptor 4 (TLR4), considered one of the most important TLR, recognizes lipopolysaccharide of gram-negative bacteria. Recognition of ligands by TLRs induces signaling pathways resulting in activation of transcription factors such as NF- κ B which are involved in the expression of inflammatory cytokines and chemokines. To prevent an inappropriate immune response, a complex network of molecules negatively regulates TLRs and their associated signaling pathways.

Two cosegregating single nucleotide polymorphisms of the human TLR4 gene, namely Asp299Gly and Thr399Ile, have been associated with hyporesponsiveness to inhaled LPS. The purpose of this study was to determine the impact of TLR4 gene variant on NF- κ B activity in colorectal cancer cell line. HCT116 cells were transfected with wild-type and mutants Flag-CMV1-TLR4 expression vectors. Western blot analysis was performed to evaluate selected molecules involved in TLR4 signaling. NF- κ B activity was assessed by dual-luciferase reporter assay and cytokine profiles were evaluated by ELISA and Cytometric Bead Array method.

Results showed that the activity of pNF- κ B was higher in cells harboring TLR4 D299G compared to the other cells. However, the activity of pAKT, pERK1 and pIRAK was higher in wild-type. The results of cytokine measurements showed about four fold higher level of IL-8 in cells with wild-type TLR4.

This study suggest that TLR4 Asp299Gly gene variant has an impact on TLR4 signaling and potentially on intestinal homeostasis due to impaired control signals at the epithelial cell level which may lead to chronic intestinal inflammation and interrupted intestinal homeostasis and may eventually lead to colorectal cancer.

Keywords: Colorectal cancer; Polymorphism; Toll like receptors

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INTRODUCTION

Cancers are a number of chronic diseases that are caused by defective genome surveillance and signal-

transduction mechanisms. Chronic inflammation and infections have been implicated in the initiation and development of several human cancers including those of the stomach, liver, colon, and urinary bladder.^{1,2} Infectious organisms affect the host through pattern-recognition receptors (PRRs), most commonly those that belong to the Toll-like receptor (TLR) family. TLR engagement activates numerous signal-transduction pathways and most importantly the NF- κ B pathway.³ These factors are involved in the expression of inflammatory cytokines and chemokines. However, the activation of NF- κ B through the stimulated TLRs in local chronic inflammation may serve as an initiator and give the infected or injured cells a second chance to evolve into cancer cells and proliferate out of control. To prevent an inappropriate or an overactive immune response, a complex network of molecules negatively regulates TLRs and their associated signaling pathways.⁴ Intestinal epithelial cells (IEC) must "tolerate" the commensal flora which is constantly present in the lumen and maintain mucosal homeostasis by controlled inflammatory responses, but IEC must also readily "sense" danger signals of potential harmful pathogens, so that appropriate immune responses of the lamina propria are activated. When tissue homeostasis is chronically perturbed, interactions between innate and adaptive immune cells can be disturbed. Evidence is emerging that the essential mechanisms of intestinal epithelial tolerance versus intolerance towards luminal bacterial ligands are distinctly mediated through Toll-like receptors (TLRs).⁵ TLR4, considered one of the most important TLRs, recognizes LPS of Gram-negative bacteria.⁶ The ability of certain individuals to respond properly to TLR ligands may be impaired by single nucleotide polymorphism (SNPs) within TLR genes.⁷⁻⁹

Two cosegregating single nucleotide polymorphisms (SNPs) of the human TLR4 gene, Asp299Gly or D299G (rs4986790) and Thr399Ile or T399I (rs4986791), have been correlated with a hyporesponsiveness to inhaled lipopolysaccharide (LPS).¹⁰ Disruption of TLR4 signaling could create an inappropriate innate and adaptive immune response necessary to remove pathogens which would result in a more severe inflammation.^{11,12} It is speculated that the presence of TLR4 mutations impact the gut homeostasis and results in impairment of TLRs activation which may lead to inflammation and cancer. The purpose of this study was to determine the impact

of variations of TLR4 gene on signal transduction and also cytokine profiles secreted from colorectal cancer cell line.

MATERIAL AND METHODS

Cell Culture and Reagents

Colorectal carcinoma Cell lines which were used in this study include, HT-29 (ATCC, HTB-38), CaCo2 (ATCC, HTB-37) and HCT116 (ATCC, CCL-247) from American tissue culture collection (ATCC). Peripheral blood mononuclear cells (PBMC) were used as a positive control for *TLR4* expression. The Cells were maintained in RPMI medium supplemented with 10% fetal bovine serum and 0.6% Penicillin-Streptomycin at 37°C in a 5% CO₂ atmosphere (All cell culture reagents were purchased from Invitrogen, Carlsbad, CA, USA).

Expression of TLR4 on Human Colorectal Carcinoma Cell Lines by Flow cytometry

Human colorectal carcinoma cell lines included HT-29, CaCo2, and HCT116 were evaluated for TLR4 Expression. According to manufacture instruction, One μ g anti-human TLR4 (eBioscience) antibody was added against the surface TLR4, incubated for 30 min on ice in the dark. One ml PBS/0.5% BSA was added, centrifuged at 200g, 4°C for 10 min, the supernatant discarded and the above steps were repeated for secondary Ab, mouse PE-conjugated anti human IgG Ab, cells were then analyzed by flow cytometry using the CellQuest software (BD Biosciences).

Transfection

Early passage of HCT116 cells were plated at a density of 3×10^4 cells/well in 6-well plates 24 hours before transfection. Transient transfection was performed by using Turbofect in vitro transfection reagent from Fermentas. Flag-tagged human TLR4 wild-type and mutants (Asp299Gly and Thr399Ile) expression vectors were kindly provided as a gift by Dr. Stefanie and Dr. Rallabhandi (University of Maryland). The DNA plasmid pmax GFP (from Amax) was used as a positive control for transfection.

Western Blots (WB) Analysis

Early passage of HCT116 cells were plated at a density of 3×10^4 cells/well in 6-well plates twenty-four hours before transfection. Transfection was performed

with wild-type Flag-CMV1-TLR4 and D299G, T399I mutant expression vectors. The cells were incubated for a further 48 hours post-transfection. Transfected cells were treated with 1µg/ml LPS at variant time. Supernatants of all treatments were collected to measure cytokines production. Western blot analysis was performed by standard methods. Briefly, Cells were lysed with ice-cold lysis buffer. Protein concentration of the extracts was measured by using the Bradford method (Bradford, 1976) according to manufacturer's instructions. Ten micrograms of the protein was loaded onto 12% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, then blotted with primary antibodies (Table 1) followed by secondary antibody conjugated to peroxidase. The membrane was immersed in substrate solution ChemiGLOW Chemiluminescent substrate (Alpha Innotech Corporation, USA) for 5 minutes in dark then was visualized using Alpha Innotech FluorChem® 5500 Imaging Systems.

Dual-luciferase Reporter Assay

Signal NFκB Reporter Assay Kit (SABiosciences) was used to assess the activity of NF-κB in LPS-treated

HCT116 cells with wild-type and mutants *TLR4*. Early passage of HCT116 cells were plated at a density of 3×10^4 cells/well in 24-well plates 24 hours before transfection. The confluency on the day of transfection was 60-80%. Cells were co-transfected with the mixture of 600 ng wild-type Flag-CMV1-*TLR4* or D299G or T399I mutant expression vectors and 600 ng of Signal Reporter in 300µl media using Turbofect transfection reagent (Fermentas). Kit contains Negative control which is a mixture of non-inducible firefly luciferase reporter and constitutively expressing Renilla construct. The cells were stimulated with LPS (*Escherichia coli* 0111:B4, Sigma) 48 h after transfection for different times. NF-κB luciferase activities in cell lysates were measured using Dual Luciferase Reporter Assay System (Promega) and a model GloMax™ 96 Microplate Luminometer (Promega) according to the manufacturer's instructions. All transfection experiments were done in triplicate wells and repeated separately two times.

Table 1. Primary antibodies used for Western Blot

Antibody	Colon	Source	Dilution
Phospho-NF-κB p65 (Ser536)	Rabbit Polyclonal	Cell Signaling Technology	1:2000
NF-κB p65	Rabbit Polyclonal	Cell Signaling Technology	1:1000
Phospho-IκBα (Ser32/36)	Mouse monoclonal	Cell Signaling Technology	1:1000
IκBα (112B2)	Mouse monoclonal	Cell Signaling Technology	1:1000
p-IRAK(ser376)	Rabbit Polyclonal	Cell Signaling Technology	1:1000
IRAK	Rabbit Polyclonal	Cell Signaling Technology	1:1000
Phospho-p44/42 MAPK (Erk 1/2)	Mouse monoclonal	Cell Signaling Technology	1:1000
p44/42 MAPK (Erk1/2)	Mouse monoclonal	Cell Signaling Technology	1:1000
Phospho-AKT (Thr308)	Rabbit Polyclonal	Santa cruz biotechnology, inc	1:1000
AKT	Rabbit Polyclonal	Santa cruz biotechnology, inc	1:1000

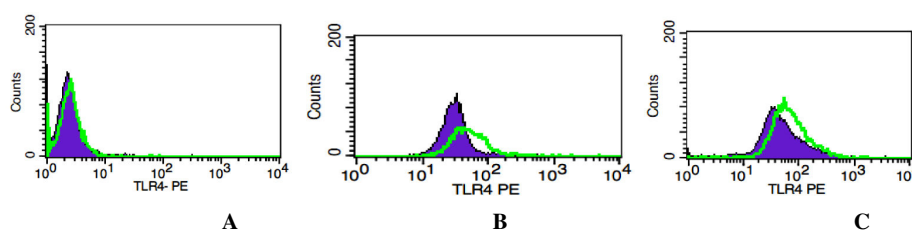


Figure 1. FACS analysis of *TLR4* expression on the surface of untransfected and untreated colorectal cancer cell lines. A: HCT116, B: HT29 and C: CaCo2. Blue regions represent isotype controls and green regions represent *TLR4* Ab. *TLR4* was detected on HT29 and CaCo2 cell lines at low levels but not detected on the HCT116 cell line.

Cytokine Profiles

Cells transfected with wild-type and mutants TLR4 were treated with one $\mu\text{g/ml}$ LPS (*Escherichia coli* 0111:B4, Sigma) at various times. The culture supernatant was removed and frozen at -80°C until use. ELISA kit for TGF- β and IL-17 were purchased from Invitrogen (Carlsbad, CA, USA). Cytometric bead array (CBA) for IL-6, IL-10, IL-8, VEGF and IFN γ analysis were purchased from CBA (BD Biosciences, San Diego, CA, USA). The measurement of these cytokines was done according to the manufacturer's instructions. Samples were analyzed on a BD FACSCalibur flow cytometer using FCAP Array

software and raw data were analyzed by BDFACSCanto II software. Standard curves for each cytokine was generated by using the reference cytokine concentrations supplied by the manufacturers.

Statistical Analysis

Statistical analyses were conducted using the ANOVA general linear models procedure (GLM) of SAS software (SAS Institute, 2005). When ANOVA revealed significant effects, means were separated by Duncan's multiple range tests. Data with $p < 0.05$ were considered to be significant.

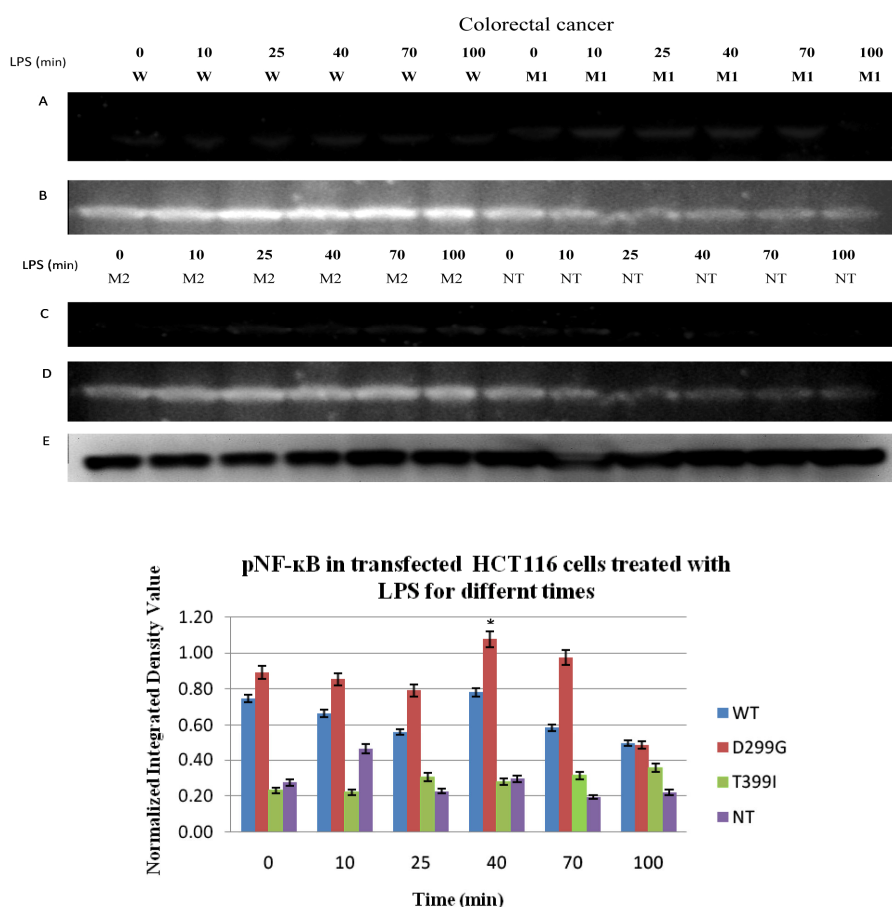


Figure 2. Western blot analysis of pNF- κ B activity in transfected HCT 116 cell line with *TLR4* wild-type (W) and D299G (M1) T399I (M2) Mutants. NT represents cells treated only with transfection reagent. The cells were treated with $1\mu\text{g/ml}$ LPS at indicated times. A: pNF- κ B, B: NF- κ B, C: pNF- κ B, D: NF- κ B, E: Tubulin. The band intensities were measured using AlphaEase software and the phosphorylated protein level was normalized against the total form of kinase level. Data are mean \pm SE of three separate experiments, * $p < 0.05$.

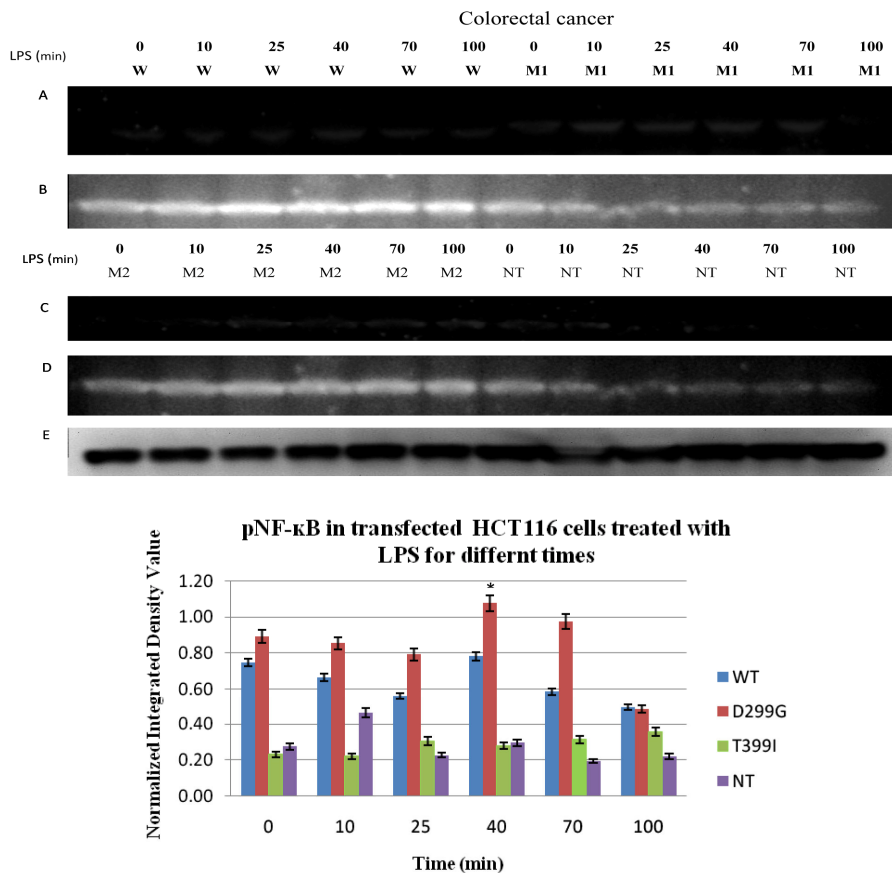


Figure 2. Western blot analysis of pNF-κB activity in transfected HCT 116 cell line with *TLR4* wild-type (W) and D299G (M1) T399I (M2) Mutants. NT represents cells treated only with transfection reagent. The cells were treated with 1μg/ml LPS at indicated times. A: pNF-κB, B: NF-κB, C:pNF-κB, D:NF-κB, E: Tubulin. The band intensities were measured using AlphaEase software and the phosphorylated protein level was normalized against the total form of kinase level. Data are mean ± SE of three separate experiments,* $p < 0.05$.

RESULTS

Expression of *TLR4* on Human Colorectal Cancer Cell Lines by Flow Cytometry

The expression of *TLR4* on HCT116, HT29 and CaCo2 cell lines was analysed by flow cytometry. HT29 and CaCo2 cell lines expressed *TLR4* at low level. *TLR4* was not detected on HCT116 cell line (Figure 1).

Since HCT116 cell line did not express *TLR4*, it was selected for transfection with wild-type (WT) and mutant *TLR4* plasmids to evaluate the impact of variants of *TLR4* on the NF-κB activity and molecules involved in cell signaling.

WB Analysis of Molecules Involved in *TLR4* Signaling

The molecules which are involved in *TLR4* signaling include; NF-κB p65, IκBα, AKT, ERK-1 and IRAK were analyzed by WB to compare the response of *TLR4* variants to LPS.

WB Analysis of pNF-κB and pIκB

pNF-κB p65 was evaluated in HCT116 cells transfected with *TLR4* WT and mutants in response to 1 μg/ml LPS over 10 to 100 mins (Figure 2).

As Figure 2 shows the level of pNF-κB in cells transfected with D299G *TLR4* genotype was significantly higher compared to the other cells ($p < 0.05$). After stimulation with LPS the level of pNF-

κ B in D299G and WT was increased at 40 min and then decreased.

Measuring the degree of I κ B phosphorylation as a surrogate marker of pNF- κ B activation showed maximal activation of I κ B phosphorylation by 10 min after LPS stimulation, and decreased thereafter in D299G cells. In wild-type and T399I cells I κ B phosphorylation reached its maximal activation by 70 min post stimulation and then decreased. Although the kinetics of phosphorylation remained the same, the level of I κ B activation was lower in wild-type compared to the mutants.

Expression of p-AKT, P-ERK1 and p-IRAK in Cells with Different *TLR4* Genotypes

The level of pAKT in WT was higher than D299G genotype and significantly increased in response to

LPS time course (Figure 3). pAKT was not detected in the T399I and non-transfected cells.

pERK1 in wild-type was higher than mutants and non-transfected cells. The expression of this protein was highest in wild-type and D299G mutant at about two hours LPS stimulation. pERK in non-transfected cells was decreased in response to the LPS time course but was increased in transfected cells (Figure 4).

The level of pIRAK protein in wild-type genotype was higher compared to the other cells and it was decreased in response to the LPS in a time course fashion. The expression of this protein was decreased also in the cells with D299G genotype in response to LPS time course; however, in T399I and non-transfected cells no changes were observed (Figure 5).

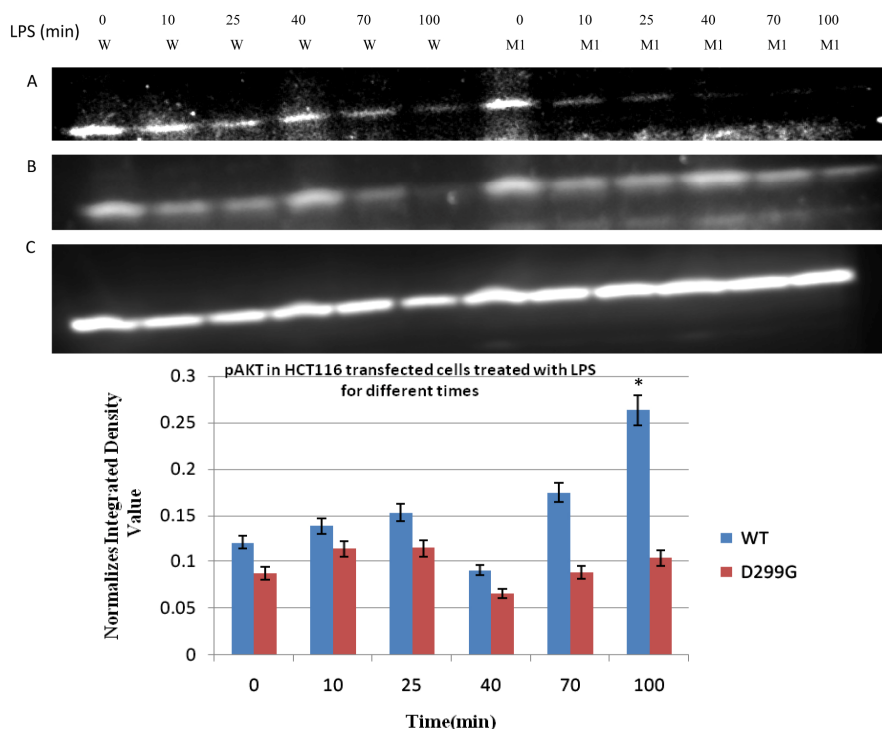


Figure 3. Western blot analysis of AKT protein in transfected HCT 116 cell line with *TLR4* wild-type (W) and D299G (M1). The cells treated with 1 μ g/ml LPS for indicated times. A: p-AKT, B: AKT, C: Tubulin. The band intensities were measured using AlphaEase software, and the phosphorylated protein level was normalized against the total form of protein. Data are mean \pm SE of three separate experiments, * $p < 0.05$ (Compared with D299G).

Colorectal Cancer

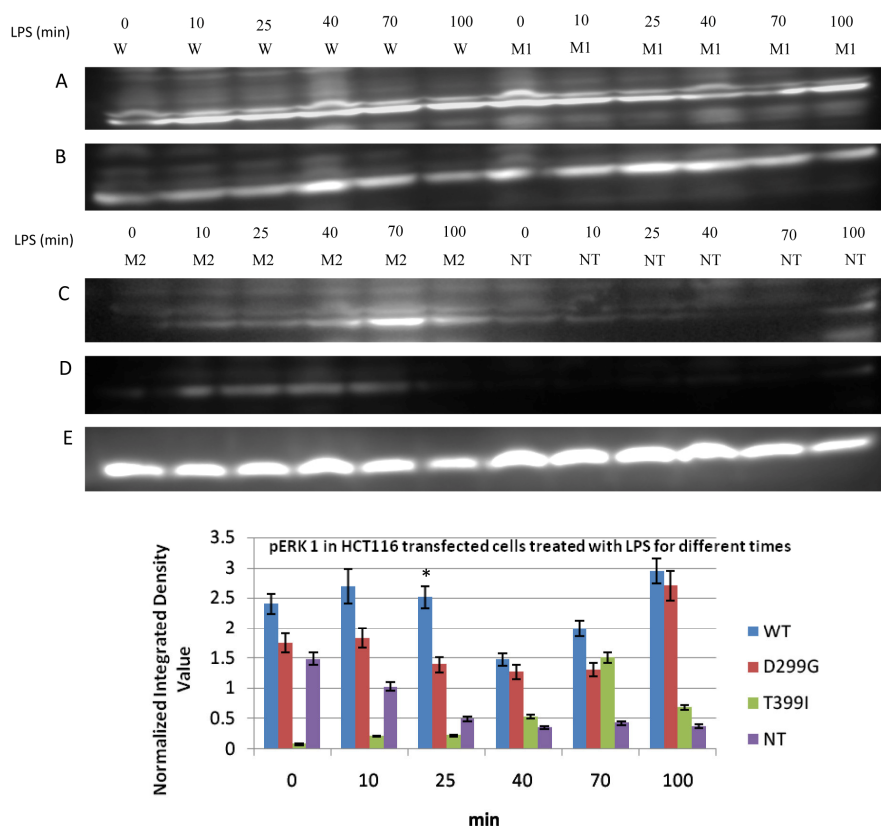


Figure 4. Western blot analysis of p-ERK1 protein in transfected HCT116 cell line with *TLR4* wild-type (W), D299G (M1) and T399I (M2) Mutants. The cells treated with 1µg/ml LPS for indicated times. A: p-ERK1, B: ERK1, C: p-ERK1 D: ERK1, E: Tubulin. The band intensities were measured using AlphaEase software, and the phosphorylated protein level was normalized against the total form of protein. Data are mean±SE of three separate experiments, * $p < 0.05$ (Compared with other cell types).

Dual Luciferase

HCT116 cells were transiently co-transfected with a pNF-κB promoter-regulated luciferase reporter plasmid and *TLR4* expression vectors; 48 hours after transfection were stimulated with LPS (1µg/ml) at various time points. pNF-κB luciferase activities in cell lysates were measured using the Dual Luciferase Reporter Assay System.

The results were consistent with Western blot analysis as the level of pNF-κB in transfected cells with the D299G *TLR4* genotype was higher compared to the wild-type and T399I genotypes. Wild-type genotype had lower pNF-κB activity compared to the other genotypes. With the D299G *TLR4* genotype, pNF-κB was increased in response to LPS in a time course experiment. However, in wild-type genotype, no

significant changes were observed in response to LPS (Figure 6).

Cytokine Profiles

Transfected cells with wild-type and mutants *TLR4* were treated with one µg/ml LPS for different times. The culture supernatant was removed and frozen at -80°C until use. TGF-β and IL-17 were quantified by ELISA. IL-6, IL-10, IL-8, VEGF and IFNγ were evaluated by Cytometric bead array (CBA).

Figure 7 shows that the HCT116 cells produced IL-17 at a low level. Secreted IL-17 in wild-type *TLR4* genotype was slightly decreased in response to the LPS. However, in D299G and T399I *TLR4* genotypes it was increased at two hours after LPS exposure.

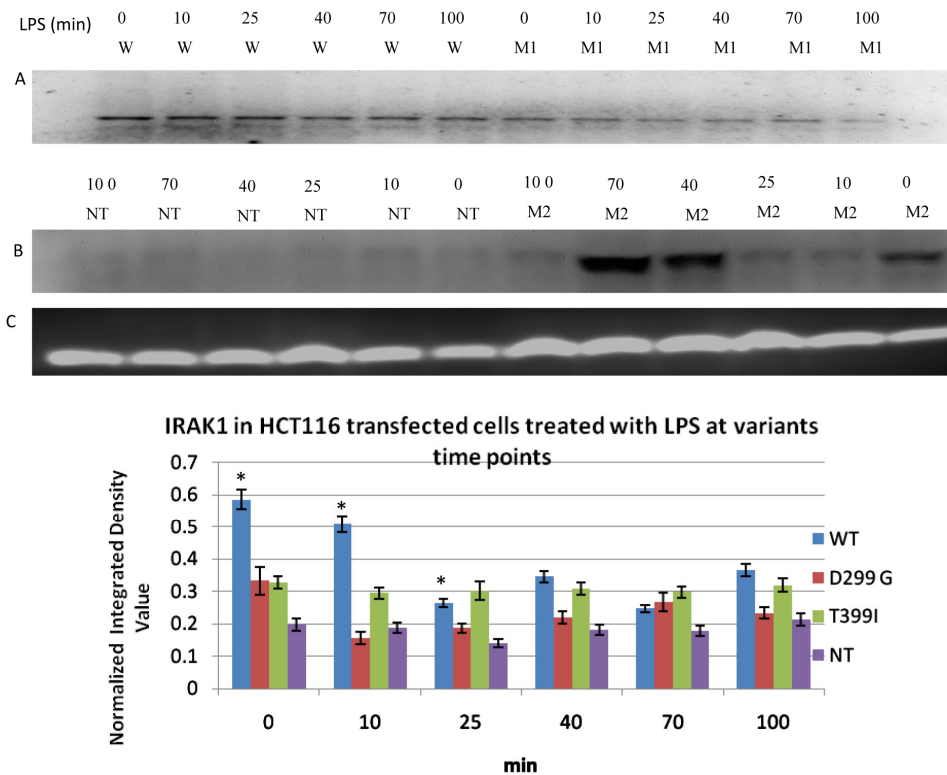


Figure 5. Western blot analysis of p-IRAK protein in transfected HCT116 cell line with *TLR4* wild-type (W), D299G (M1) and T399I (M2) Mutants. The cells treated with 1µg/ml LPS for indicated times. A: p-IRAK, B: p-IRAK, C: Tubulin. The band intensities were measured using AlphaEase software, and the phosphorylated protein level was normalized against the tubulin. . Data are mean±SE of three separate experiments,* $p < 0.05$ (Compared with other cell types).

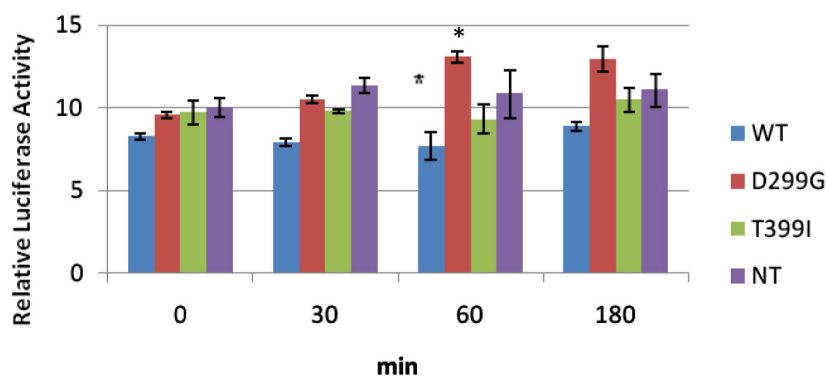


Figure 6. HCT116 cells were cotransfected with the mixture of luciferase reporter plasmid and *TLR4* expression vectors. Wild-type (WT), D299G and T399I genotypes. Cells were stimulated with one µg/ml LPS 48 h after transfection. PNF-κB luciferase activities in cell lysates were measured using Dual Luciferase Reporter Assay System. Data are normalized by dividing Firefly luciferase activity with that of Renilla luciferase. The relative values are presented as fold decrease over indicated control. All transfection experiments were done in triplicate wells and repeated separately three times. Data are mean ±SE of three separate experiments,* $p < 0.05$ (Compared with other cell types).

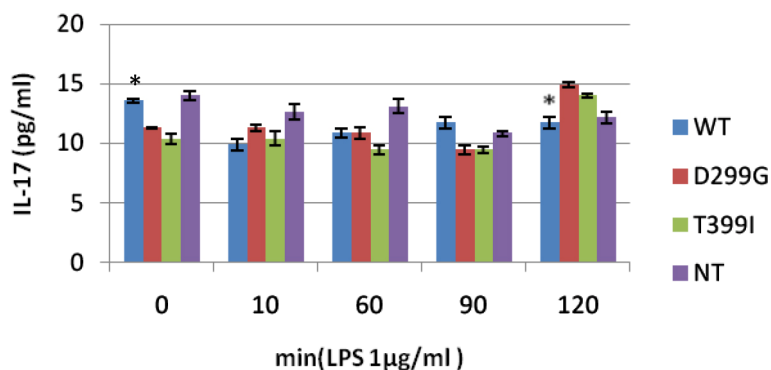


Figure 7. IL-17 secretion in transduced HCT116 cell line treated with LPS (1µg/ml) for different time. IL-17 was assayed using sandwich ELISA in cell-free supernatants. Non transfected cells (NT) were treated with transfection reagent only. Data are mean \pm SE of three separate experiments. Data are mean \pm SE of three separate experiments, * $p < 0.05$ (Compared with mutants).

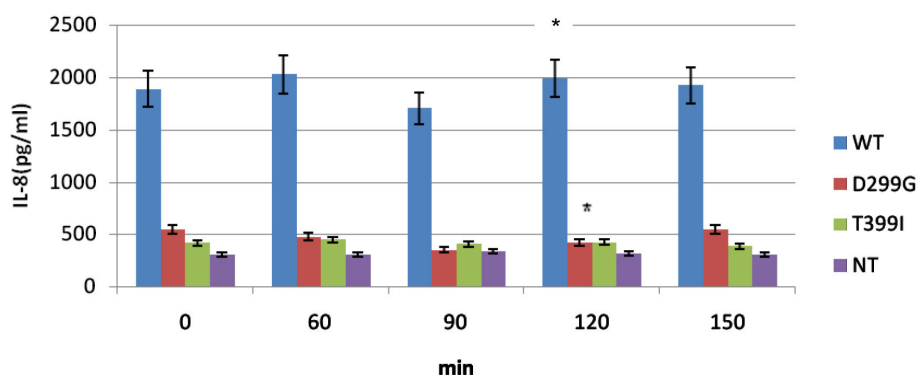


Figure 8. Transduced HCT116 cells was stimulated with LPS (1µg/ml) for different time, IL-8 were quantified using Cytometric bead array in cell-free supernatants. Non transfected cells (NT) were treated only with transfection reagent. A high level of IL-8 was detected in wild-type *TLR4* genotype. This was about four fold higher than mutants and non-transfected cells. Data are mean \pm SE of three separate experiments. *: $p < 0.05$

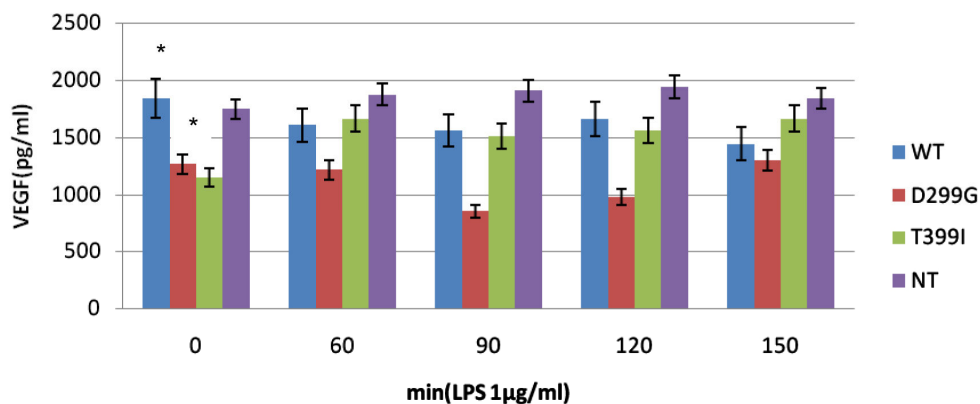


Figure 9. Transduced HCT116 cells was stimulated with LPS (1µg/ml) for different time, VEGF was quantified using Cytometric bead array in cell-free supernatants. Non-transfected cells (NT) represent cells treated with transfection reagent only. High level of basal VEGF was detected in HCT116 cells. Data are mean \pm SE of three separate experiments. *: $p < 0.05$

TGF- β also was evaluated by ELISA in culture supernatant. The amount of this cytokine was under the detection limit in three *TLR4* genotypes and non-transfected cells.

IL-6, IL-8, IL-10, VEGF and IFN γ were evaluated by Cytometric bead array (CBA). IL-6, IL-10, and IFN γ were under the detection limit and were not detected. High level of IL-8 was detected in wild-type *TLR4* genotype which was about four fold more than mutants and non-transfected cells (Figure 8).

VEGF in cell-free supernatants were assayed using CBA. As shown in Figure 9 HCT116 constitutively secreted a basal level of VEGF. The production of VEGF in D299G cells was lower than others. At the first hour of LPS exposure time, the level of VEGF was decreased in WT and D299G cells. Non transfected cells produced the highest level of VEGF which was not affected by LPS (Figure 9).

DISCUSSION

Sequence variants in the extracellular domain of *TLR4* can disrupt trafficking of this receptor to the cell membrane. Other potential effects of the mutant *TLR4* receptor include decreased ligand binding or changes in the receptor conformation that could impair signal transduction once the ligand is bound.^{13,6} Two cosegregating single nucleotide polymorphisms (SNPs) of the human *TLR4* gene, Asp299Gly and Thr399Ile, have been correlated with a hyporesponsiveness to inhaled lipopolysaccharide (LPS).¹⁴ Recognition of ligands by *TLRs* activates transcriptional factors such as NF- κ B and a complex system of molecules regulates *TLRs* and their associated signaling pathways to prevent an overactive immune response.⁵

The purpose of this study was to determine the impact of Asp299Gly and Thr399Ile *TLR4* variations on response to LPS in transfected HCT116 cells. The results of this study indicate that the activity of pNF- κ B in D299G mutant cells is higher than wild-type in response to LPS as shown by Western Blot and Dual luciferase assay. In D299G *TLR4* genotype pI κ B reached maximal activation in only 10 min after LPS stimulation and then decreased but in wild-type genotype, it reached a maximal activation by 70 min and then decreased. The level of pI κ B also was higher in D299G genotype compared to the Wild-type. This results support the idea that genetic alterations (e.g. mutations or polymorphisms) in the *TLR4* signaling

pathways can break microbial tolerance and may lead to autoimmunity *in vivo*. This is supported by genetic association studies which have reported higher frequencies of *TLR4* polymorphisms (D299G) in patients with inflammatory bowel disease.¹⁵

In a study by Savkovic *et al.* it was reported that intestinal epithelia can distinguish pathogenic from non-pathogenic bacterial strains and tolerate high concentrations of bacterial LPS without activating the inflammatory cascade. They showed that neither non-pathogenic *E. coli* (strain JM109) nor pure *E. coli* LPS (serotype 0111:B4, Sigma) activated NF- κ B transcription factors in T84 (a human colonic carcinoma cell line) cells.¹⁶ The LPS used in our study was also used in the previous published work. Variation in *TLR4* gene affects signaling molecules involved in the regulation of inflammatory response to LPS in the intestine. IL-1R-associated kinase (IRAK) plays a key role in IL-1R/Toll-like receptor (*TLR*)-mediated signaling and NF- κ B activation. In our study, the level of pIRAK protein in wild-type *TLR4* genotype was higher compared to the other cells and it was significantly decreased in response to the LPS.

Upon LPS stimulation, IRAK is quickly activated and then degraded. In the presence of LPS, IRAK protein level constantly remains at a very low level after 2 h of LPS stimulation. The rapid degradation of IRAK may serve as a negative feedback mechanism of down-regulating IL-1R/*TLR*-mediated signaling and cytokine gene transcription. Functional *TLR4* is required for LPS-mediated IRAK degradation. IRAK protein in the murine GG2EE cells harbouring a mutated *TLR4* gene does not undergo degradation upon LPS treatment.¹⁷ Our results showed that degradation of IRAK upon LPS treatment was stronger in wild-type genotype compared to the mutants.

This supports the hypothesis that in D299G mutant cells, the LPS tolerance was impaired which may lead to inflammation and commensal-associated gastrointestinal disorders such as IBD. An alternative mechanism controlling the magnitude of the response to LPS is activation of the PI3K pathway. Indeed, inhibition of PI3K/Akt pathway in THP-1 cells enhances LPS induced activation of the Mitogen-Activated Protein Kinase pathways (ERK1/2, p38 and JNK), as well as the LPS-induced NF- κ B activation.¹⁸ It has also been reported that the development of endotoxin tolerance in peritoneal macrophages from rats results in increased PI3K/AKT activity.¹⁹

The results of our study showed that the activity of AKT in wild-type was increased strongly by LPS. In D299G cells the level of pAKT was also increased after LPS treatment but not as strong as wild-type. Increased pAKT in response to LPS leads to inhibition of the inflammatory response in wild-type. This result is in agreement with our result of IRAK activity in response to the LPS. LPS-triggered *TLR4* signaling usually activates two different pathways, namely, the extracellular signal-related kinase(ERK)/c- jun-NH2-kinase(JNK)/p38 mitogen-activated protein kinase (MAPK) pathway and NF- κ B signaling pathway, resulting in gene expression and regulation of biological functions.²⁰

In our study, the level of pERK1 in wild-type was higher than D299G mutant, T399I and non-transfected cells. After LPS treatment the level of p-ERK1 was decreased until 70 minutes post stimulation and then increased at 100 minutes. It is possible that in wild-type genotype cells, *TLR4* ligation activates the MAPkinase pathway and in D299G the NF- κ B pathway is activated after LPS treatment. Further work is required to determine the effect of LPS on MAP kinase family in colorectal cancer cells with different *TLR4* genotypes. LPS can induce NF- κ B activation in human colorectal tumor cell.²¹

In lung cancer cells, *TLR4* ligation can activate p38MAPK, but not ERK1/2 and JNK1/2 in human.²² Our results also showed that LPS can induce NF- κ B activation in colorectal tumor cells and the level of this protein in D299G mutant was higher compared to the wild-type. With this result, we can explain the relationship between this polymorphism in *TLR4* and susceptibility to inflammatory intestinal disease, autoimmune disease or even cancer. The results of cytokines profile showed that HCT116 cells produced IL-17 at a low level. Secreted IL-17 in wild-type *TLR4* genotype was higher compared to the mutants and decreased in response to the LPS in a time course manner. However, in D299G and T399I *TLR4* genotypes, it was increased at two hours LPS exposure and reached to the highest level compared to the wild-type.

The production of IL-17 seems important in host protection against Gram-negative bacteria and fungal infections. IL-17 R-deficient mice are highly susceptible to infection with the extracellular pathogen *Klebsiella* and the fungus *Candida*.²³ Since the LPS which was used in this study was from a commensal strain of *E.coli*, therefore, the level of secreted IL-17

was low and in wild-type cells it was not increased in response to the LPS. However, in mutant cells the level of secreted IL-17 increased at two hours after stimulation.

TGF- β 1 also was evaluated by ELISA in culture supernatant. The amount of this cytokine was below the detection limit. This result is consistent with the result of Haller *et al.*(2000) who found that LPS did not induce TGF- α mRNA expression above control levels in response to the non-pathogenic LPS.²⁴ High level of IL-8 was detected in wild-type *TLR4* genotype which was about four fold higher than mutants and non-transfected cells. The amount of this cytokine in D299G, T399I and non-transfected cells was almost the same and was not affected by LPS. In wild-type genotype cells, even before LPS stimulation the level of IL-8 was high; it seems that the expression of *TLR4* wild-type genotype is important for IL-8 secretion. HCT116 tumor cells constitutively secreted high level of VEGF.

After LPS stimulation, VEGF was decreased in wild-type *TLR4* genotype cells but it was increased in T399I genotype. In D299G genotype cells VEGF decreased after 90 min and increased at two hours after LPS stimulation. As generally accepted VEGF is an immunosuppressive cytokine secreted by tumor cells and contributes to tumor immune escape. This results showed that *TLR4* ligation in wild-type decreased VEGF secretion but in mutants cells it was increased. This suggests that LPS stimulation in wild-type suppress tumor growth but in mutant and non-transfect cells tumor growth is promoted. In a study by He *et al.* (2007) on lung cancer cells, it was shown that *TLR4* ligation can promote cancer cells to secrete immunosuppressive cytokines (VEGF, IL8) which may contribute to their immune escape.²² As a conclusion *TLR4* mutations impact the gut homeostasis, signal transduction and cytokine profiles secreted by intestinal epithelial cells results in impairment of TLRs activation which may lead to inflammation and cancer.

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REFERENCES

1. Alpay HC, Etem EO, Kaygusuz I, Yüce H, Karlidag T, Keles E, et al. Evaluation of the polymorphism in the Toll-like receptor 4 (*TLR4*) genes of tympanosclerosis patients. *Auris Nasus Larynx* 2010; 37(1):29-32
2. Davoodi H, Seow HF. Variant Toll-Like Receptor4 (Asp299Gly and Thr399Ile Alleles) and Toll-Like Receptor2 (Arg753Gln and Arg677Trp Alleles) in Colorectal Cancer. *Iran J Allergy Asthma Immunol* 2011; 10(2):91-9.
3. Akira S, Hemmi H. Recognition of pathogen-associated molecular patterns by *TLR* family. *Immunol Lett* 2003; 85(2):85-95.
4. Bonaiuto C, McDonald PP, Rossi F, Cassatella MA. Activation of nuclear factor- κ B by β -amyloid peptides and interferon- γ in murine microglia. *J Neuroimmunol* 1997; 77(1):51-6.
5. Cario E, Podolsky D. Intestinal epithelial tolerance versus in tolerance of commensals. *Mol Immunol* 2005; 42(8):887-93.
6. Ferwerda B, McCall MB, Verheijen K, Kullberg BJ, van der Ven AJ, Van der Meer JW, et al. Functional consequences of toll-like receptor 4 polymorphisms. *Mol Med* 2008; 14(5-6):346-52.
7. Schroder J, Schumann RR. Single Nucleotide Polymorphisms of Toll-Like Receptors and Susceptibility to Infectious Disease. *Lancet infect Dis* 2005; 5(3):156-64.
8. Sun J, Turner A, Xu J, Grönberg H, Isaacs W. Genetic variability in inflammation pathways and prostate cancer risk. *Urol Oncol* 2007; 25(3):250-9.
9. Marsik C, Jilma B, Joukhadar C, Mannhalter C, Wagner O, Endler G. The Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms influence the late inflammatory response in human endotoxemia. *Clin Chem* 2005; 51(11):2178-80.
10. Arbour NC, Lorenz E, Schutte BC, Zabner JJ, Kline N, Jones M, et al. *TLR4* mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet* 2000; 25(2):187-91.
11. Franchimont D, Vermeire S, El-Housni H, Pierik M, Van Steen K, Gustot TE, et al. Deficient host-bacteria interactions in inflammatory bowel disease? The Toll-like receptor (*TLR*)-4 Asp299Gly polymorphism is associated with Crohn's disease and ulcerative colitis. *Gut* 2004; 53(7):987-92.
12. Schmidt C. Immune system's toll-like receptors have good opportunity for cancer treatment. *J Natio Cancer Instit* 2006; 98(9):574-5.
13. Lorenz E, Mira JP, Frees KL, Schwartz DA. Relevance of mutations in the *TLR4* receptor in patients with gram-negative septic shock. *Arch Intern Med* 2002; 162(9):1028-32.
14. Apetoh L, Tesniere A, Ghiringhelli F, Kroemer G, Zitvogel L. Molecular interactions between dying tumor cells and the innate immune system determine the efficacy of conventional anticancer therapies. *Cancer Res* 2008; 68(11):4026-30.
15. Papadimitraki ED, Bertsias GK, Boumpas DT. Toll like receptors and autoimmunity: a critical appraisal. *J Autoimmun* 2007; 29(4):310-8.
16. Savkovic DS, Koutsouris A, Hecht G. Activation of NF- κ B in intestinal epithelial cells by enteropathogenic *Escherichia coli*. *Am J Physiol Cell Physiol* 1997; 273(4 Pt 1):1160-7.
17. Hu J, Jacinto R, McCall C, Li L. Regulation of IL-1 receptor-associated kinases by lipopolysaccharide. *J Immunol* 2002; 168(8):3910-4.
18. Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J. Biol. Chem* 2002; 277(35):32124-32.
19. Bowling WM, Hafenrichter DG, Flye MW, Callery MP. Endotoxin tolerance alters phospholipase C-gamma 1 and phosphatidylinositol-3-kinase expression in peritoneal macrophages. *J. Surg Res* 1995; 58(6):592-8.
20. Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, et al. LPS-*TLR4* signaling to IRF-3/7 and NF- κ B involves the toll adapters TRAM and TRIF. *J Exp Med* 2003; 198(7):1043-55.
21. Wang JH, Manning BJ, Wu QD, Blankson S, Bouchier-Hayes D, Redmond HP. Endotoxin/lipopolysaccharide activates NF- κ B and enhances tumor cell adhesion and invasion through a beta 1 integrin-dependent mechanism. *Immunol* 2003; 170(2):795-804.
22. He W, Liu Q, Wang L, Chen W, Li N, Cao X. *TLR4* signaling promotes immune escape of human lung cancer cells by inducing immunosuppressive cytokines and apoptosis resistance. *Mol Immunol* 2007; 44(11):2850-9.
23. Bettelli E, Korn T, Kuchroo VK. Th17: the third member of the effector T cell trilogy. *Curr Opin Immunol* 2007; 19(6):652-7.
24. Haller D, Bode C, Hammes WP, Pfeifer AMA, SchiVrin EJ, Blum S. Non-pathogenic bacteria elicit a differential cytokine response by intestinal epithelial cell/leucocyte co-cultures. *Gut* 2000; 47(1):79-87.