Indian Journal of Experimental Biology
Vol. 64, June 2026, pp. 465-472
DOI: 10.56042/ijeb.v64i06.25743

Investigation of the role of TLR4 agonist in M1 and M2 macrophage polarization and ıts ımmunotherapeutic effects in hepatocellular carcinoma

Eylül Akbal[1]*, Esra Erdal[2], Merve Uysal[2], Berna Yalınbalc[2], Gülsevinç Aksoy[1] & Mehmet Bertan Yılmaz[1]

[1]Çukurova University Department of Medical Biology Adana 01330 Türkiye

[2]Dokuz Eylul University Izmir International Biomedicine and Genome Institute İzmir 35340 Türkiye

Received 20 November 2025; revised 4 February 2026

Hepatocellular carcinoma (HCC) is one of the most important causes of cancer-related deaths. The availability and efficacy of treatment options for HCC patients are limited. Therefore, the number of immunotherapy studies for the treatment of advanced HCC is increasing day by day. The aim of this study was to investigate the effects of soluble factors obtained from HCC cell culture stimulated with TLR4 agonist on macrophage polarization and to reveal new immunotherapeutic targets that can be used in the treatment of HCC. Supernatants obtained from TLR4 agonist (Lipopolisakkarit, LPS) stimulated HuH7 cells and control groups were fed to THP1 cell lines and the direction of macrophage polarization was examined. The expression of NOS2 gene for M1 macrophages and ARG1 gene for M2 macrophages were analyzed by RT-PCR. The results obtained were evaluated by statistical analysis. When THP1 Control and THP1+HuH7 Supernatant groups were compared, it was found that ARG1 expression was statistically significantly increased while NOS2 expression was statistically significantly decreased in HuH7 Supernatant added THP1 cells. In THP1 cells supplemented with LPS-induced HuH7 Supernatant (THP1 HuH7+LPS Supernatant), ARG1 and NOS2 expression was significantly increased. We found that LPS stimulates TLR4, allowing the tumor environment to change and transform into M1 macrophages, the lethal form of macrophages. Increased expression of NOS2 in macrophages may indicate increased tumor lethality and decreased tumor-induced immunosuppressive effect. We think that TLR4 is a good mediator for targeted therapies.

Keywords: HuH7 cells, THP1 cells, Lipopolysaccharide, Tumor microenvironment

Introduction

HCC accounts for approximately 90% of primary liver cancers and is one of the leading causes of cancer-related deaths worldwide, ranking as the fourth most common cause[1]. The global mortality burden of HCC is projected to reach 1 million deaths annually by 2030[2]. HCC is asymptomatic in its early stages and is often diagnosed at an advanced stage. Patients diagnosed at advanced stages are not eligible for curative surgical interventions, and the availability and efficacy of treatment options for advanced HCC remain limited. Consequently, the number of studies focusing on immunotherapy for advanced HCC has been steadily increasing. However, clinical outcomes of immunotherapy associated with HCC remain limited, with the clinical objective response rate for HCC treated solely with immunological agents being only ~15-20%[3].

An increased density of tumor-associated macrophages (TAMs) in the tumor microenvironment (TME) is associated with poor prognosis in HCC, as in many other cancer types. The activation of various signaling pathways results in enhanced macrophage recruitment to the tumor site, contributing to the formation of an immunosuppressive TME and promoting tumor progression[4]. The protective phenotype in HCC has been attributed to M1 macrophages, which activate tumor-killing mechanisms. In contrast, M2 macrophages have been shown to suppress adaptive immune responses and promote tumor growth, invasion, and metastasis[5]. The differentiation between M1 and M2 macrophage phenotypes is closely related to the direction of arginine metabolism. Increased NOS2 expression in M1 macrophages contributes to the emergence of anti-tumor and pro-inflammatory effector functions through nitric oxide production. In contrast, the predominance of ARG1 activity in M2 macrophages leads to the metabolism of arginine towards polyamine and proline synthesis, thereby enhancing its immunosuppressive, angiogenic, and tissue repair-supporting functions[6].

TLR4 is responsible for the selective recognition of lipopolysaccharide (LPS), a component of the outer

Correspondence:
Phone: +905349690589
E-mail: eylulakbal@hotmail.com

membrane of Gram-negative bacteria. The expression of TLR4 is highly observed in immune cells such as monocytes, lymphocytes, and spleen cells, but it is also known to be expressed in epithelial and endothelial cells, as well as in cancer cells. Additionally, TLR4 is expressed in various liver cells, including hepatocytes, Kupffer cells, and hepatic stellate cells[7].

LPS is a well-known potent activator of the immune system, activating monocytes and neutrophils through TLR4 signaling. Previous studies have shown that LPS acts as a strong immune stimulus by activating macrophages[8]. While LPS directs macrophage polarization toward the M1 phenotype, it is known that interleukin 4 (IL-4) can induce macrophage polarization toward the M2 phenotype. M1 macrophages can elicit pro-inflammatory responses and produce pro-inflammatory-associated factors such as IL-6, IL-12, and tumor necrosis factor (TNF)[9]. Currently, some immunotherapy studies aim to utilize this characteristic of LPS to induce polarization of macrophages toward the M1 phenotype, thereby using it in cancer treatment[10].

This study aims to investigate the effects of soluble factors obtained from TLR4 agonist-stimulated HCC cell culture on macrophage polarization, with the goal of identifying new immunotherapeutic targets for the treatment of HCC.

Methods

In this study, HuH7 (HCC Cell Line, ATCC; USA) and THP1 (Acute Monocytic Leukemia Cell Line, ATCC; USA) cell lines were used. The study consists of three main steps: Application of LPS (as a TLR4 agonist) to the HuH7 cell line, Treatment of THP1 cells with the media collected from HuH7 cell cultures, Investigation of gene expression (Fig. 1).

Based on the experimental setups applied in the study, three groups were established:

- THP1 cells treated with the media from HuH7 cells (THP1 HuH7 Control),
- THP1 cells treated with the media from LPS-treated HuH7 cells (THP1 HuH7+LPS),
- THP1 cells not exposed to any stimulus except PMA (THP1 Control).

A total of 3×10^5 HuH7 cells were divided into Control and Experimental groups and cultured in RPMI medium (Gibco™, 11875093) containing 10% Fetal Bovine Serum (FBS, Gibco™, USA), 2 mM L-glutamine (Gibco™, USA), 100 U/mL penicillin

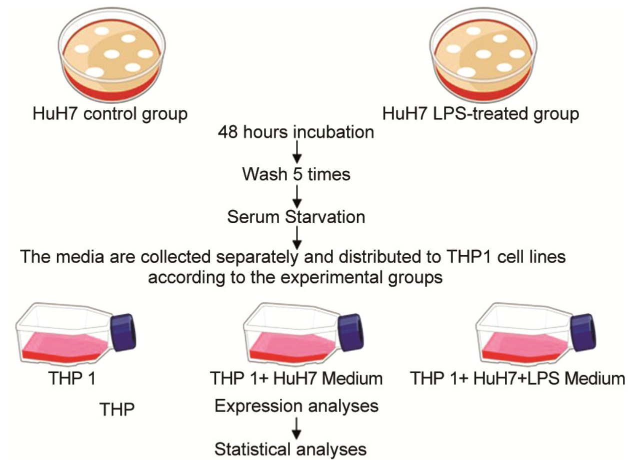

Fig. 1 — Experimental Design and Implementation Flow[11].

(Gibco™, USA), 0.1 mg/mL streptomycin (Gibco™, USA), and a 1% non-essential amino acid mixture. When the cells covered 60% of the culture surface, 100 ng/mL LPS (L2880- Sigma-Aldrich, USA) was added to the experimental group HuH7 cells. The cells were incubated for 48 hours. Before collecting the medium from HuH7 cells incubated with LPS, the culture medium was removed, and the cells were washed five times with serum-free medium. The cells were then subjected to serum starvation. The media were collected for administration to THP1 cell lines.

A total of 2×10^5 THP1 cells were divided into Control and Experimental groups and cultured in RPMI medium (Gibco™, 11875093) containing 10% Fetal Bovine Serum (FBS, Gibco™, USA), 2 mM L-glutamine (Gibco™, USA), 100 U/mL penicillin (Gibco™, USA), 0.1 mg/mL streptomycin (Gibco™, USA), and a 1% non-essential amino acid mixture. The experimental group was prepared to be combined with supernatants obtained from THP1 HuH7 cell line. The media collected from HuH7 cells were clarified by centrifugation. The clarified media were then added to the experimental THP-1 cells along with 150 ng/mL Phorbol-12-myristate-13-acetate (PMA, P 8139 Sigma-Aldrich, USA) and incubated for 48 hours[12]. At the end of the incubation, cells from both the experimental and control groups were collected for RNA isolation and expression studies. Total RNA isolation was performed using TRIzol (Thermo-Fisher, USA). RNA purity values were obtained based on the A260/A280 ratio. RNA purity ratios between 1.6 and 2.0 were considered optimal, and samples falling outside this range were re-isolated. The obtained RNA was converted into cDNA using the synthesis kit (OneScript® Plus

Table 1 — Primer Pairs Designed for Target Genes

Gene	Primer
RPL 41 (House keeping gene- Control gene)	F: AACCGCTAGCTTGCACT
	R: TCCAGAATGTCACAGGTCCA
ARG1	F: TCATCTGGGTGGATGCTCACAC
	R: GAGAATCCTGGCACATCGGGAA
NOS2	F: GCTCTACACCTCCAATGTGACC
	R: CTGCCGAGATTTGAGCCTCATG

RPL 41: Ribosomal Protein L41 gene
ARG1: Arginase 1 gene
NOS2: Nitric oxide synthase 2 gene

cDNA Synthesis Kit, ABM, Canada). For expression analysis, the SYBR Green technique was employed. Reactions were carried out using the Real-Time PCR system (Applied Biosystems 7500 Fast Real-Time System). Gene expression analyses were performed at a single predefined time point following conditioned medium exposure.

In our study, the primer sequences designed for the Real-Time PCR reactions of ARG1 and NOS2 isoforms, using the Primer BLAST and OligoAnalyzer 3.1 programs (Integrated DNA Technologies), are listed in Table 1.

For each gene, cDNA samples obtained from the experimental and control groups were tested in triplicate, and the averages of the CT values obtained from these reactions were used. The reference gene used was RPL41, a housekeeping gene. Gene expression values for ARG1 and NOS2 isoforms were determined using the $2^{-\Delta\Delta CT}$ method. ΔCT for the experimental group = CT target gene – CT reference gene, ΔCT for the control group = CT target gene – CT reference gene, ΔΔCT = Experimental group ΔCT - Control group ΔCT, and expression value = $2^{-\Delta\Delta CT}$ formula were used[13].

Statistical Analysis

The normality assumption of numerical measurements was tested using the Kolmogorov-Smirnov test. For comparison of numerical measurements between two groups, the independent samples t-test was used. For the overall comparison of numerical measurements among more than two groups, One-Way Analysis of Variance (ANOVA) was employed. After ANOVA, pairwise comparisons between groups were performed using Dunnett's test, depending on the homogeneity of variance within groups. Statistical analysis of the data was performed using GraphPad Prism 9.0 software. A *P*-value of <0.05 was considered statistically significant in all tests.

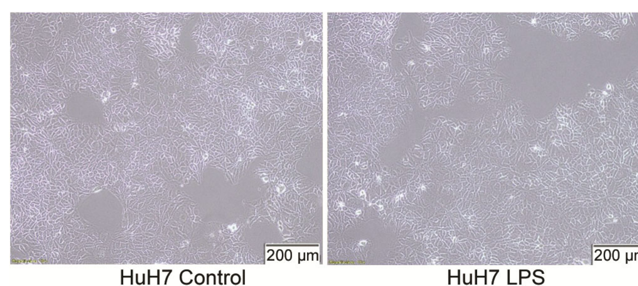

Fig. 2 — Comparison of Microscopic Images of HuH7 Cells.

Results

In this study, HuH7 and THP1 cell lines were used to investigate the functions of LPS. The HuH7 LPS cell line which was only treated with LPS and the HuH7 Control cell line which was not treated to LPS were examined microscopically. No significant differences were observed between the HuH7 Control and HuH7 LPS groups in terms of parameters such as cell count per unit area, morphological changes, and growth rate (Fig. 2).

The THP1 monocyte cell line shows M0 macrophage properties, but under appropriate conditions, it can differentiate into M1 and M2 macrophages. In the THP1 cell line with M0 macrophage properties, macrophage polarization occurs after the addition of PMA to the medium. The group that did not receive any stimulants other than PMA, which could influence polarization, was designated as "THP1 Control," while the group treated with supernatants collected from HuH7 cells was referred to as the "THP1+HuH7 Medium" group. In microscopic examinations of both groups, no visible differences were observed in terms of parameters such as cell count per unit area, morphological changes, and growth rate (Fig. 3).

A statistically significant increase in ARG1 expression was observed in THP1 cells treated with HuH7 medium compared to the THP1 Control and THP1+HuH7 Medium groups (*P*=0.045) (Fig. 4).

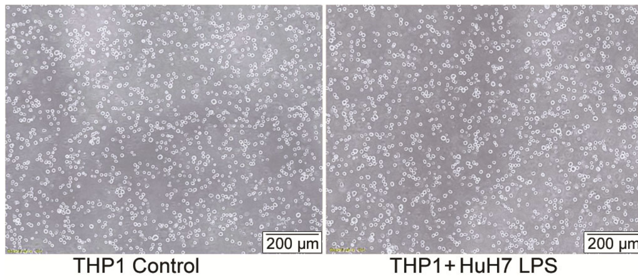

Fig. 3 — Comparison of Microscopic Images of THP1 Cells.

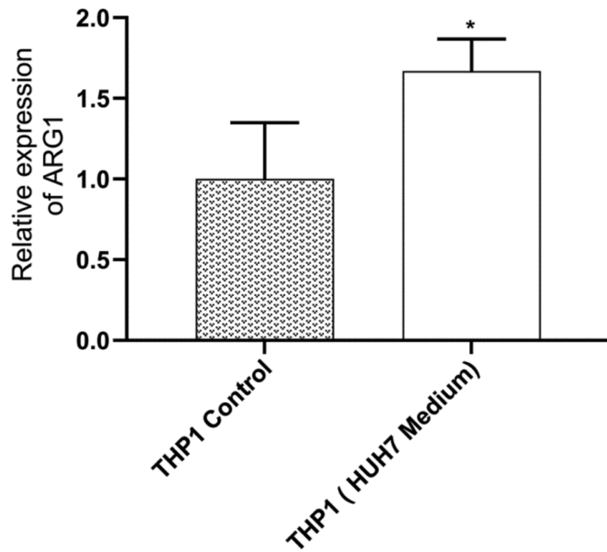

Fig. 4 — ARG1 Expression Levels in THP1 Control and THP1+HuH7 Medium Groups.

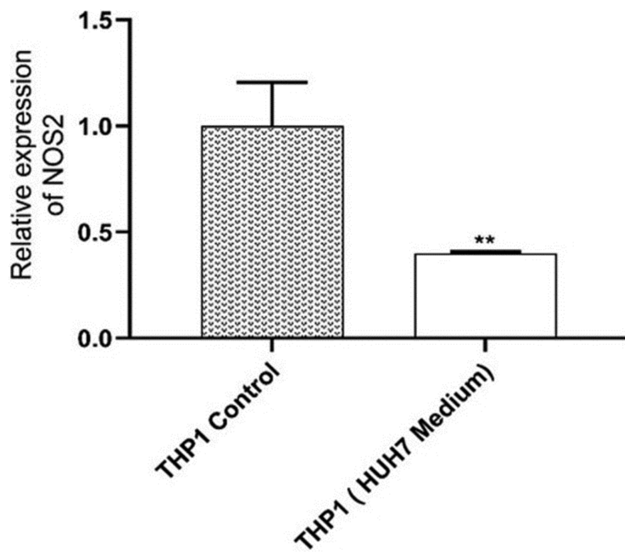

Fig. 5 — NOS2 Expression Levels in THP1 Control and THP1+HuH7 Medium Groups.

A statistically significant decrease in NOS2 expression was observed in the THP1 cells treated

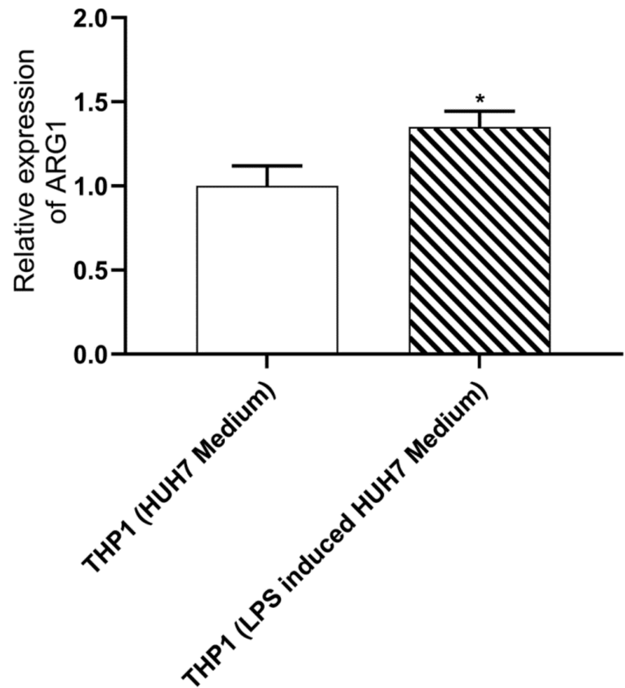

Fig. 6 — ARG1 Expression Levels in THP1 HuH7 Control and THP1 HuH7+LPS Medium Groups.

with HuH7 medium when comparing the THP1 Control and THP1+HuH7 Medium groups (P=0.007) (Fig. 5).

The expression levels of the ARG1 gene were compared between the THP1 HuH7 Control and THP1 HuH7+LPS Medium groups, and it was found that ARG1 expression was statistically significantly increased in the THP1 cells treated with LPS-induced HuH7 medium (THP1 HuH7+LPS Medium) (P=0.016) (Fig. 6).

Expression levels of the NOS2 gene were compared between the THP1 HuH7 Control and THP1 HuH7+LPS Medium groups, it was found that NOS2 expression was statistically significantly increased in THP1 cells treated with LPS-induced HuH7 medium (THP1 HuH7+LPS Medium) (P<0.000) (Fig. 7).

No statistically significant change in NOS2 expression was observed between the THP1 cells treated with LPS-induced HuH7 medium (THP1 HuH7+LPS Medium) and the THP1 Control group when comparing the THP1 Control group with the THP1 HuH7+LPS Medium group (P=0.086) (Fig. 8).

A statistically significant increase in ARG1 expression was observed in the THP1 cells treated with LPS-induced HuH7 medium (THP1 HuH7+LPS

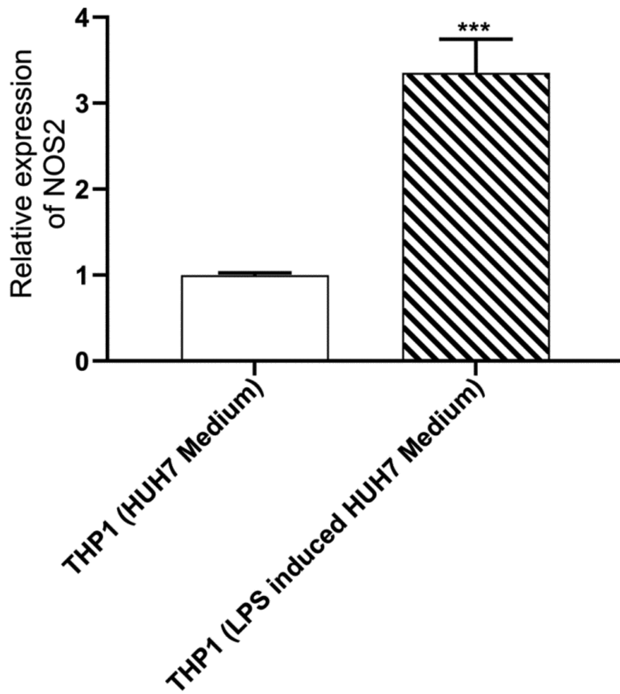

Fig. 7 — NOS2 Expression Levels in THP1 HuH7 Control and THP1 HuH7+LPS Medium Groups.

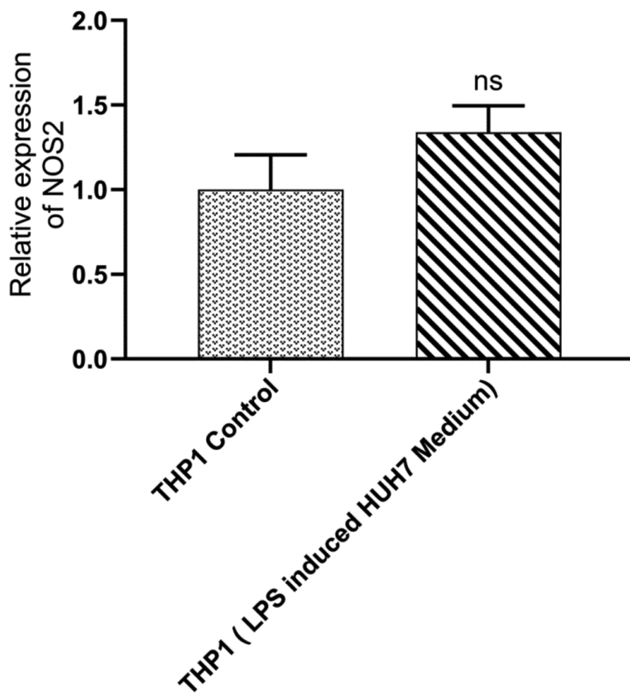

Fig. 8 — NOS2 Expression Levels in THP1 Control and THP1 HuH7+LPS Medium Groups.

Medium) compared to the THP1 Control group (P=0.003) (Fig. 9).

Comparison of ARG1 gene expression levels between the THP1 HuH7 Control, THP1 HuH7+LPS

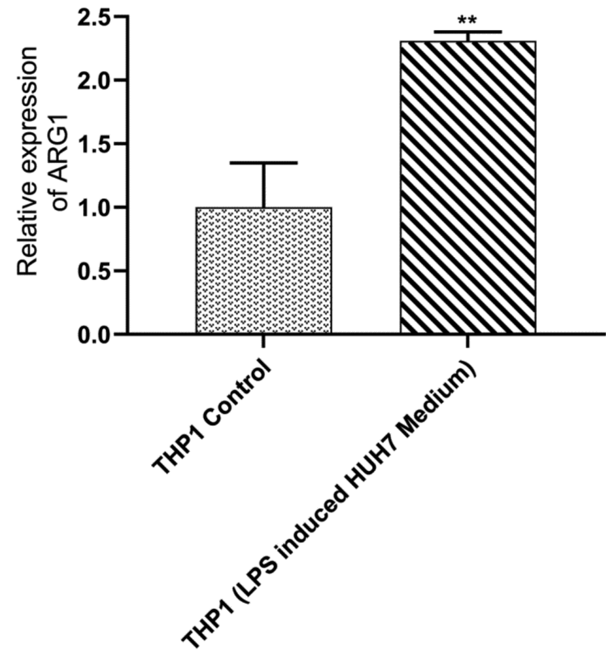

Fig. 9 — ARG1 Expression Levels in THP1 Control and THP1 HuH7+LPS Medium Groups.

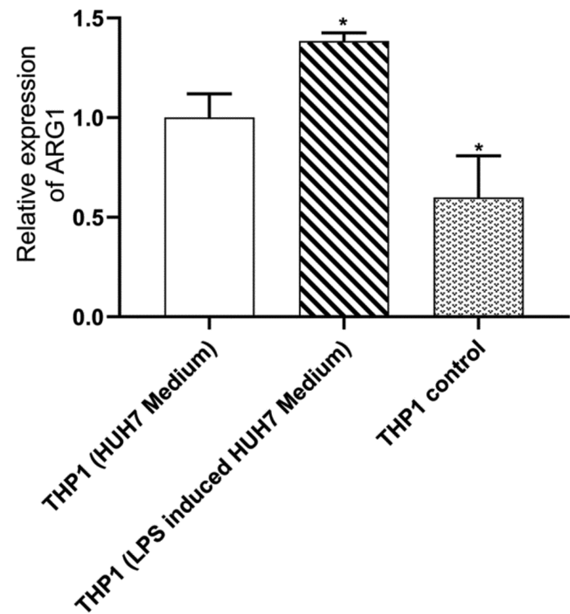

Fig. 10 — ARG1 Expression Levels in THP1 HuH7 Control, THP1 HuH7+LPS, and THP1 Control Medium Groups.

Medium, and THP1 Control groups revealed a statistically significant decrease in the THP1 Control group, both when compared to the THP1 HuH7 Control group (P=0.002) and the THP1 HuH7+LPS Medium group ($P<0.000$) (Fig. 10).

The expression levels of the NOS2 gene were compared between the THP1 HuH7 Control, THP1

Table 2 — Expression values of ARG1 and NOS2 genes by group.

Group	ARG1 Expression Levels	NOS2 Expression Levels
THP1 Control	0,72	0,81
	0,71	1,2
	0,59	2,03
THP1+HuH7 Medium	1,89	0,39
	1,51	0,39
	1,60	0,40
THP1+LPS-HuH7 Medium	1,37	3,78
	1,42	3,01
	1,34	3,25

ARG1: Arginase 1 gene
NOS2: Nitric oxide synthase 2 gene
THP1 Control: THP1 cells not exposed to any stimulus except PMA
THP1+HuH7 Medium: THP1 cells treated with the media from HuH7 cells
THP1+LPS-HuH7 Medium: THP1 cells treated with the media from LPS-treated HuH7 cells

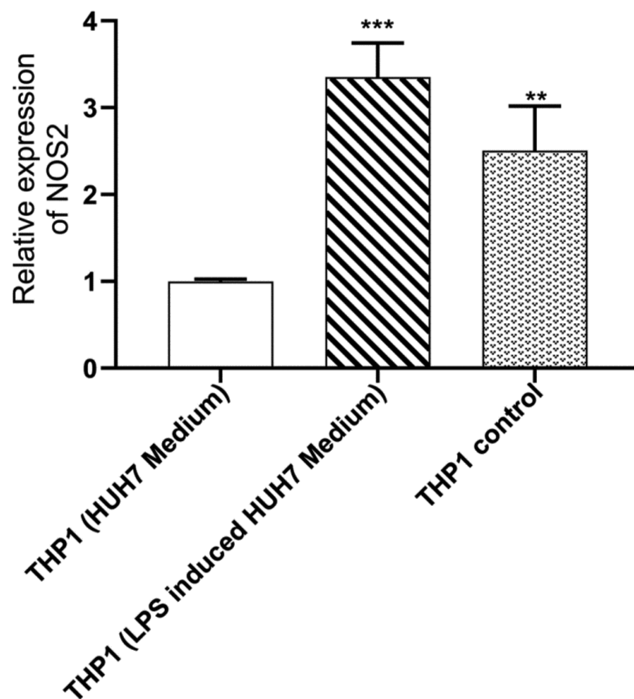

Fig. 11 — NOS2 Expression Levels in THP1 HuH7 Control, THP1 HuH7+LPS, and THP1 Control Medium Groups.

HuH7+LPS Medium, and THP1 Control groups, and it was found that NOS2 expression was statistically significantly lower in the THP1 HuH7 Control group compared to the THP1 Control group (P=0.004) and the THP1 HuH7+LPS Medium group (P<0.000) (Fig. 11).

In our study, the expression values of the ARG1 and NOS2 genes obtained from the control and experimental groups are presented in Table 2.

Discussion

In this study, we investigated how the tumor microenvironment affects the numbers of M1 and M2 macrophages by maintaining THP1 cells in two different environments and examining the gene expression profiles specific to M1 (NOS2 gene) and M2 (ARG1 gene) macrophages. We observed significant differences in the expression levels of ARG1 and NOS2 between the THP1 control group that was not treated with any stimuli other than PMA, and the experimental group treated with the conditioned medium derived from the HuH7 hepatic cell line. According to the results obtained, a statistically significant increase in the expression levels of the ARG1 gene, which is specific to M2 macrophages, was observed in THP1 cells treated with HuH7-conditioned medium compared to the THP1 control group. In our study, it was also determined that the expression levels of the NOS2 gene, which is specific to M1 macrophages, significantly decreased in THP1 cells treated with HuH7-conditioned medium, whereas a statistically significant increase was observed in the group treated with LPS-stimulated HuH7-conditioned medium.

TLR4 is expressed in different liver cell types, including hepatocytes, Kupffer cells, and hepatic stellate cells. In a study using RT-PCR to detect TLR4, it was discovered that TLR4 is functionally expressed on the surface of liver cancer cells. The TLR4 agonist LPS has been found to promote the proliferation of hepatic cells and induce apoptosis in these cells[14]. A study found that TLR4 expressed by human head and neck squamous cell carcinoma facilitates immune evasion and promotes tumor growth[15].

In a xenograft mouse model with B16 melanoma cells, the tumor response to TLR agonist stimulation was investigated. The study demonstrated that

TLR1/2 stimulation in mice led to increased secretion of TNF-α, IL-2, and IFN-γ, as well as enhanced T cell activity, particularly CD8+ T cells. This was associated with elevated granzyme B and perforin synthesis, resulting in a reduction of melanoma tumors[16]. Natural products such as *Astragalus mongholicus*, used as TLR4 agonists, have been reported to promote the maturation of dendritic cells and immune activity by targeting TLR4, leading to tumor regression in a mouse xenograft model[17]. Lamrani et al. investigated the effects of a synthetic lipid A analog, a TLR4 agonist, in a breast cancer mouse model. The study reported that the TLR4 agonist increased the production of various pro-inflammatory cytokines such as IFN-γ, TNF-α, and nitric oxide, contributing to the enhanced sensitivity of tumor cells to radiation and chemotherapeutic agents[18].

Hsu *et al*. reported that fucoidan, a TLR4 agonist, induced apoptosis through caspase 3 activation in a mouse lung cancer model[19]. Han *et al*. found that quercetin, a TLR4 agonist, reduced metastasis and invasion in colon cancer[20]. Another study reported that the LPS-TLR4 signaling pathway may cause recurrence in HCC[21]. In a study conducted by Pan *et al.* it was demonstrated that the combined use of monoclonal antibodies and TLR4 agonists contributed to a reduction in the number of M2 macrophages in the HCC microenvironment. The same researchers emphasized that combination therapies in immunotherapy would be more effective and that TLR4 agonists represent a promising target for this approach[22].

The tumor microenvironment consists of soluble factors secreted by tumor cells, receptors overexpressed on tumor cells (such as TLR4), and immune system components (such as M1/M2 macrophages). Unraveling the interactions between these factors and cells will facilitate a better understanding of tumor behavior. In our study, we designed an experimental plan aimed at uncovering this interaction and identifying immunotherapeutic targets for the treatment of HCC. Using the developed experimental plan, we observed how soluble factors secreted by HCC cells, following TLR4 receptor stimulation, influence M1/M2 macrophage polarization. To observe the natural microenvironment of HCC, no additional stimuli were added to the cell culture medium (HuH7 Control). To induce the production of soluble products that would alter the tumor microenvironment by stimulating the tumor cells, we used LPS, a TLR4 receptor agonist (HuH7+LPS group). According to our results, the expression of ARG1 and NOS2 genes was statistically increased in the HuH7+LPS group. Although both genes showed a statistically significant increase, the expression of ARG1 was approximately 2 times higher compared to the control, while the increase in NOS2 expression was approximately 3 times higher. In our study, we also examined the behavior of macrophages under their natural conditions compared to the environment with the addition of HuH7+LPS. Upon reviewing the results, we found that while there was no significant change in NOS2 expression in HuH7 cells without LPS treatment, a statistically significant increase in ARG1 expression was observed.

Based on our results, we observed that in THP1 cells co-cultured with tumor-derived medium, the synthesis of NOS2 decreased. However, this effect could be strongly reversed (with more than a 3-fold increase in expression) after LPS treatment. We found that ARG1 expression increased in the tumor environment, regardless of LPS presence, but this increase was less pronounced in the presence of LPS.

In conclusion, the data we obtained demonstrate, in line with previous studies in the literature, that the tumor microenvironment plays a significant and effective role in macrophage polarization. In our study, we observed that stimulating TLR4 receptors facilitated changes in the tumor microenvironment, and this alteration enabled the transformation of macrophages into the cytotoxic form of M1 macrophages. The increased expression of NOS2 in macrophages may indicate enhanced cytotoxicity against the tumor and a reduction in the immune-suppressive effect generated by the tumor. We believe that TLR4 is a promising target for targeted therapies. The most significant limitation of this study is the inability to analyze the expression levels of key genes in cellular pathways that affect macrophage polarization due to insufficient project funding. Another limitation of this study is that macrophage-associated gene expression was assessed at a single time point, and therefore the temporal dynamics and stability of the responses of THP1 cells to HuH7-induced conditioned medium could not be evaluated.

Conclusion

LPS-induced activation of TLR4 in tumor cells facilitated the synthesis of soluble factors that

contributed to the increased expression of NOS2 in macrophages. We observed enhanced M1 function in M0-like THP1 cells. These results demonstrate that TLR4 agonists are effective in modulating gene expression in macrophages. Therefore, we believe that TLR4 agonists, by influencing the ability of macrophages to transition between M1 and M2 forms, support the potential for these agonists to serve as immunotherapeutic targets in cancer treatment. Studies aiming to direct this polarization using natural and synthetic products could contribute to immunotherapy research. We believe that further studies identifying the soluble factors synthesized in tumor cells upon TLR4 activation are necessary to better elucidate the relationship between TLR4 and macrophage polarization.

Sources of funding

This thesis study was supported by the Research Fund of Çukurova University Rectorate with the project number TDK-2021-13762.

Ethical approval

Since this study was conducted with commercially available cell lines, ethics committee approval was not required.

Clinical trial number

Not applicable.

Conflicts of interest

All authors declare that there is no any conflict of interest.

References

1 Villanueva A. Hepatocellular carcinoma. *N Engl J Med*, 380 (2019) 1450.

2 Anstee QM, Reeves HL, Kotsiliti E, Govaere O & Heikenwalder M. From NASH to HCC: current concepts and future challenges. *Nat Rev Gastroenterol Hepatol,* 16 (2019) 411.

3 Llovet JM, Ricci S, Mazzaferro V & Bruix J. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med*, 359 (2008) 378.

4 Waidmann O, Köberle V, Bettinger D, Trojan J, Zeuzem S, Schultheiß M, Kronenberger B & Piiper A. Diagnostic and prognostic significance of cell death and macrophage activation markers in patients with hepatocellular carcinoma. *J Hepatol*, 59 (2013) 769.

5 Mehla K & Singh PK. Metabolic Regulation of Macrophage Polarization in Cancer. *Trends Cancer*, 5 (2019) 822.

6 Kieler M, Hofmann M & Schabbauer G. More than just protein building blocks: how amino acids and related metabolic pathways fuel macrophage polarization. *FEBS J.* 288 (2021) 3694.

7 Pimentel-Nunes P, Soares JB, Roncon-Albuquerque R, Jr Dinis-Ribeiro M & Leite-Moreira AF. Toll-like receptors as therapeutic targets in gastrointestinal diseases. *Expert Opin Ther Target*, 14 (2010) 347.

8 Astrakianakis G, Seixas NS, Ray R, Camp JE, Gao DL, Feng Z, Li W, Wernli KJ, Fitzgibbons ED, Thomas DB & Checkoway H. Lung Cancer Risk among Female Textile Workers Exposed to Endotoxin. *J Natl Cancer Inst, 99 (*2007) 357.

9 Sica A & Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest,* 122 (2012) 787.

10 Boushehri MS & Lamprecht A. TLR4-Based Immunotherapeutics in Cancer: A Review of the Achievements and Shortcomings. *Mol Pharm,* 15 (2018) 4777.

11 NIH Bioart Source. Accessed October 15, 2024. https://bioart.niaid.nih.gov.

12 Daigneault M, Preston JA, Marriott HM, Whyte MK & Dockrell DH. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS One,* 5 (2010) e8668.

13 Bitesizebio. What Is a Cq (Ct) Value? Accessed October 15, 2024. http://bitesizebio.com/24581/what-is-a-ct-value.

14 Li H, Li Y, Liu D & Liu J. LPS promotes epithelial-mesenchymal transition and activation of TLR4/JNK signaling. *Tumour biol,* 35 (2014) 10429.

15 Szczepanski MJ, Czystowska M, Szajnik M, Harasymczuk M, Boyiadzis M, Kruk-Zagajewska A, Szyfter W, Zeromski J & Whiteside TL. Triggering of Toll-like receptor 4 expressed on human head and neck squamous cell carcinoma promotes tumor development and protects the tumor from immune attack. *Cancer Res,* 69 (2009) 3105.

16 Geng D, Zheng L, Srivastava R, Velasco-Gonzalez C, Riker A, Markovic SN & Davila E. Amplifying TLR-MyD88 signals within tumor-specific T cells enhances antitumor activity to suboptimal levels of weakly immunogenic tumor antigens. *Cancer Res,* 70 (2010) 7442.

17 Tian Y, Li X, Li H, Lu Q, Sun G & Chen H. Astragalus mongholicus regulate the Toll-like-receptor 4 meditated signal transduction of dendritic cells to restrain stomach cancer cells. *Afr J Tradit Complement Altern Med,* 11 (2014) 92.

18 Lamrani M, Sassi N, Paul C, Yousfi N, Boucher JL, Gauthier N & Jeannin JF. TLR4/IFNγ pathways induce tumor regression via NOS II-dependent NO and ROS production in murine breast cancer models. *Oncoimmunology,* 5 (2015) e1123369.

19 Hsu HY, Lin TY, Lu MK, Leng PJ, Tsao SM & Wu YC. Fucoidan induces Toll-like receptor 4-regulated reactive oxygen species and promotes endoplasmic reticulum stress-mediated apoptosis in lung cancer. *Sci Rep.* 7 (2017) 44990.

20 Han M, Song Y & Zhang X. Quercetin suppresses the migration and invasion in human colon cancer Caco-2 cells through regulating Toll-like receptor 4/nuclear factor-kappa B pathway. *Pharmacogn Mag,* 12 (2016) 237.

21 Zhou S, Du R, Wang Z, Shen W, Gao R, Jiang S, Fang Y, Shi Y, Chang A, Liu L, Liu C, Li N & Xiang R. TLR4 increases the stemness and is highly expressed in relapsed human hepatocellular carcinoma. *Cancer Medicine,* 8 (2019) 2325.

22 Pan C, Wu Q, Wang S, Mei Z, Zhang L, Gao X, Qian J, Xu Z, Zhang K, Su R, Guo D, Zhou L & Zheng S. Combination with Toll-like receptor 4 (TLR4) agonist reverses GITR agonism mediated M2 polarization of macrophage in Hepatocellular carcinoma. *Oncoimmunology,* 6 (2022) 2073010.