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M1/M2 Liver Macrophage Polarization After Antigenic Stimulation with Lipopolysaccharide (LPS) in Rats*

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Objective: In our study, we aimed to detect M1/M2 macrophage polarization in rat liver tissue by immunohistochemical staining after antigenic stimulation with lipopolysaccharide (LPS).

Materials and Methods: Twenty-four Spraque-Dawley male 10-week-old rats were used in the study. Rats were randomly divided into 4 groups (n=6): Control-24 group, Control-48 group, LPS-24 group and LPS-48 group. Rats in the control groups were administered 1 mL of saline intraperitoneally (i.p) and the animals were decapitated after 24 or 48 hours. The rats in the LPS groups were administered with 5 mg/kg LPS (in 1 mL saline) i.p. After 24 or 48 hours, the rats were decapitated and the study was terminated.

Results: Inflammatory cell infiltration and hyperemia were detected in liver tissues of rats in LPS-24 and LPS-48 groups as a result of examination of sections stained with hematoxylin-eosin (H&E). Sinusoidal dilatation was also noted in the LPS-24 group. CD68 immunoreactivity was found to be significantly increased in LPS-treated rat liver sections when compared to control groups ($P<0.001$). CD68 immunoreactivity was significantly increased in the LPS-48 group compared to the LPS-24 group ($P<0.001$). While no change was observed in the LPS-24 group in terms of CD163 and CD204 immunoreactivities compared to the control groups, CD163 and CD204 immunoreactivities were increased in the LPS-48 group compared to LPS-24, but this increase was not statistically significant ($P>0.05$).

Conclusion: It was determined that the polarization of liver macrophages increased in the direction of M1 macrophages after antigenic stimulation with LPS in rats.

Anahtar Kelimeler: *LPS, antigen, liver, rat, macrophage*

Sıçanlarda Lipopolisakkarit ile Antijenik Uyarım Sonrası M1/M2 Karaciğer Makrofaj Polarizasyonu

Amaç: Çalışmamızda, Lipopolisakkarit (LPS) ile yapılacak antijenik uyarım sonrası sıçan karaciğer dokusunda M1/M2 makrofaj polarizasyonunun immunohistokimyasal boyamalarla saptanması amaçlanmıştır.

Gereç ve Yöntem: Çalışmada 24 adet Spraque-Dawley cinsi 10 haftalık erkek sıçan kullanıldı. Sıçanlar rastgele 4 gruba ayrıldı (n=6): Kontrol-24 grubu, Kontrol-48 grubu, LPS-24 grubu ve LPS-48 grubu. Kontrol gruplarındaki sıçanlara 1 mL saline intraperitoneal (i.p) olarak uygulandı ve hayvanlar 24 veya 48 saat sonra dekapite edildi. LPS gruplarındaki sıçanlara ise 5 mg/kg LPS (1 mL saline içerisinde) i.p olarak uygulandı ve 24 veya 48 saat sonra sıçanlar dekapite edilerek çalışma sonlandırıldı.

Bulgular: Hematoksilen-eozin (H&E) ile boyanmış kesitlerin incelenmesi sonucu LPS-24 ve LPS-48 gruplarındaki sıçanların karaciğer dokularında inflamatuar hücre infiltrasyonu ve hiperemi tespit edildi. LPS-24 grubunda ayrıca sinüzoidal dilatasyon dikkat çekmekteydi. LPS uygulanan sıçan karaciğer kesitlerinde CD68 immünreaktivitesi kontrol gruplarıyla kıyaslandığında istatistiksel olarak anlamlı bir şekilde artmış ($P<0.001$) bulundu. LPS-48 grubunda ise LPS-24 grubuna kıyasla CD68 immünreaktivitesi anlamlı şekilde artmıştı ($P<0.001$). LPS- 24 grubunda CD163 ve CD204 immünreaktiviteleri açısından kontrol grupları ile karşılaştırıldığında herhangi bir değişiklik izlenmezken, LPS-48 grubunda CD163 ve CD204 immünreaktiviteleri LPS-24'e kıyasla artmış olarak izlendi, fakat bu artış istatistiksel açıdan önemli değildi ($P>0.05$).

Sonuç: Sıçanlarda LPS ile yapılan antijenik uyarım sonrası karaciğer makrofaj polarizasyonunun M1 makrofajları yönünde arttığı belirlenmiştir.

Key Words: *LPS, antijen, karaciğer, sıçan, makrofaj*

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Introduction

Macrophages, the cells responsible for phagocytosis, are distributed in various tissues and take part in very important mechanisms such as immune response and inflammation (1, 2). Hepatic macrophages play an important role in maintaining homeostasis (2-4). Recently, studies have been conducted on how macrophages change in liver development and two types of liver macrophage development have been

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reported. In the prenatal period, CD163(+) and CD204(+) positive cells, known as Kupffer cells, are very few. The majority of hepatic macrophages observed in this period are CD68(+) positive macrophages with severe phagocytic activity, and it is reported that this type of macrophages originate from the vitellus sac. In the postnatal period, while the number of CD68(+) positive macrophages decreases, the number of CD163(+) positive Kupffer cells increases. This change in the number and type of macrophages in liver development is remarkable considering the different functions of macrophages (4). Liver macrophages are variable cells that can adapt to stimuli (3). In current studies, a new macrophage polarization concept called M1/M2 macrophage polarization is accepted (4-6). M1 macrophages mainly stimulated by IFN-ɣ, having high phagocytic capacity and cytotoxicity; They are CD68(+) positive macrophages. CD163(+) positive and CD204(+) positive M2 type macrophages are stimulated by IL-4 and play a role in reparative fibrosis (4). There is not enough information about how M1/M2 polarization is formed in liver macrophages during systemic inflammation. In experimental acute inflammation models, systemic inflammation induced by LPS has been found to be very successful (7). Therefore, in our study, we aimed to reveal M1/M2 macrophage polarization in rat liver tissue by immunohistochemical methods in systemic inflammation induced by LPS.

Materials and Methods

Research and Publication Ethics: The research budget of this study, which was approved by the Firat University Animal Experiments Ethics Committee (31.01.2018, 2018/2), was met by the Firat University Scientific Research Projects Unit (FUBAP) (Project no: TF.18.62).

Experimental Procedure: All experimental stages of the study were performed in the Fırat University Experimental Research Center (FUDAM) unit and in the Histology and Embryology Laboratory of Fırat University Faculty of Medicine. Twenty-four 10-week-old Sprague-Dawley male rats weighing 200-220 g were randomly divided into 4 groups with 6 animals in each group. Group I: Control-24 hour group; 1 mL of saline was administered intraperitoneally (i.p) and was decapitated after 24 hours. Group II: Control-48 hour group; 1 mL of saline was administered i.p. and decapitated 48 hours later. Group III: LPS-24 hour group; 5 mg/kg LPS (*Escherichia coli* 026:B6, Sigma, USA) (in 1ml saline) was administered i.p and decapitated after 24 hours. Group IV: LPS-48 hour group (n:6); 5 mg/kg LPS (in 1 mL saline) was administered i.p and decapitated 48 hours later. For decapitation processes i.p. xylazine (10 mg/kg) + ketamine (75 mg/kg) anesthesia was administered. After decapitation, liver tissues of the rats were quickly removed. Liver tissues of all groups were fixed in 10% formaldehyde solution for histological evaluations.

Histological and Immunohistochemical Evaluations: Rat liver tissues fixed in 10% formaldehyde were dehydrated, cleared with xylol and embedded in paraffin. Afterwards, tissue sections of 5 mm thickness from the prepared paraffin blocks were taken to the milled and polylysine slides, and the sections taken to the grinding slides were stained with hematoxylin and eosin (H&E) stains. Liver sections stained with H&E were evaluated under the light microscope. On the other hand, for the detection of M1/M2 macrophage polarization in liver tissues, immunohistochemical staining procedure (8) was performed on liver tissues taken on polylysine slides. Deparaffinized tissues were passed through graded alcohol series and boiled in citrate buffer solution at pH:6 in a microwave oven (750 W) for 7+5 minutes for antigen retrieval. After boiling, the tissues, were washed with PBS (Phosphate Buffered Saline, P4417, Sigma-Aldrich, USA) and then incubated with hydrogen peroxide block solution to prevent endogenous peroxidase activity (Hydrogen Peroxide Block, TA-125-HP, Lab Vision Corporation, USA). Primary antibodies CD68, CD204 and CD163 (YID1080, YID 3530, YID 0991, YLBiont, Shanghai, China, respectively) were used in these stainings. After the primary antibody incubation, the tissues were washed with PBS and incubated with secondary antibody (Biotinylated Goat Anti-Polyvalent (anti-mouse / rabbit IgG), TP–125-BN, Lab Vision Corporation, USA) in a humid environment at room temperature. Tissues were washed with PBS and incubated with Streptavidin Peroxidase (TS–125-HR, Lab Vision Corporation, USA) and then taken into PBS. 3-amino-9-ethylcarbazole (AEC) Substrate + AEC Chromogen (AEC Substrate, TA-015-HAS; AEC Chromogen, TA-002-HAC, Lab Vision Corporation, USA) solution is dripped onto the tissues and the image signal is obtained under the light microscope. Tissues that were counterstained with hematoxylin were passed through PBS and distilled water and closed with the appropriate closure solution (Large Volume Vision Mount, TA-125-UG, Lab Vision Corporation, USA).

All preparations were evaluated and photographed under the Leica DM500 light microscope (Leica DFC295). In the evaluation of immunohistochemical staining, all immunopositive cells in 10 randomly selected areas were counted at x400 magnification in each preparation.

Statistical Analysis: IBM SPSS 22.0 package program was used in the analysis of the data obtained in the study. Shapiro-Wilk normality analysis was performed to determine whether the values of the groups showed normal distribution, and as a result of the test, it was determined that the values in all parameters showed normal distribution. One-way Analysis of Variance (ANOVA) and post hoc Tukey tests were applied to the data. One-way ANOVA was used to compare group means, and Tukey test was used to

determine differences between groups. P<0.05 values were considered statistically significant. Data were presented as mean±standard deviation.

Results

Histopathological Changes: As a result of the light microscopic evaluation of the liver sections of the Control-24 and Control-48 groups, it was observed that the tissue samples of the two groups were similar to each other and the hepatocyte cords were in a normal structure. Vena centralis, hepatocytes, sinusoidal widths and Kupffer cells were in normal histological appearance (Figure 1A,1B). When the liver sections of the LPS-24 group were examined; sinusoidal dilatation, hyperemia and inflammatory cell infiltration were observed (Figure 1C,1D). In the LPS-48 group, increased hyperemia and inflammatory cell infiltration were detected in some areas compared to the LPS-24 group (Figure 1E, 1F).

Immunohistochemical Evaluations: In rat liver tissue sections, CD68, CD204 and CD163 immunoreactivities were detected in sinusoidal cells. Histocore values (Histoscore = severity x prevalence) of all immunohistochemical stainings are presented in Table 1.

CD68 immunoreactivity; It was similar in the Control-24 (Figure 2A) and Control-48 (Figure 2B) groups (P=0.972). CD68 immunoreactivity was statistically significantly increased in the LPS-24 (Fig. 2C) (P<0.001) and LPS-48 (Figure 2D) (P<0.001) groups compared to the control groups. In the LPS-48 group, increased CD68 immunoreactivity was detected compared to the LPS-24 group (P<0.001).

As a result of examining the immunohistochemical staining for CD204 immunoreactivity under light microscopy; similar staining patterns were observed in the Control-24 (Figure 3A) and Control-48 (Figure 3B) groups (P=0.994). Compared to the control groups, there was no change in CD204 immunoreactivity in the LPS-24 (Figure 3C) group (P>0.05), while a statistically insignificant increase in CD204 immunoreactivity was detected in the LPS-48 (Figure 3D) group (P=0.394). There was no significant difference in CD204 immunoreactivity between the LPS-24 and LPS-48 groups (P=0.375).

CD163 immunoreactivity in liver tissue; It was similar in the Control-24 (Figure 4A) and Control-48 (Figure 4B) groups (P=0.998). Compared to the control groups, there was no change in CD163 immunoreactivity in the LPS-24 (Figure 4C) group (P=0.993), while an increase in CD163 immunoreactivity was observed in the LPS-48 (Figure 4D) group, but this increase was not statistically significant (P=0.056). CD163 immunoreactivity was increased in the LPS-48 group compared to the LPS-24 group, but this increase was not statistically significant (P=0.206).

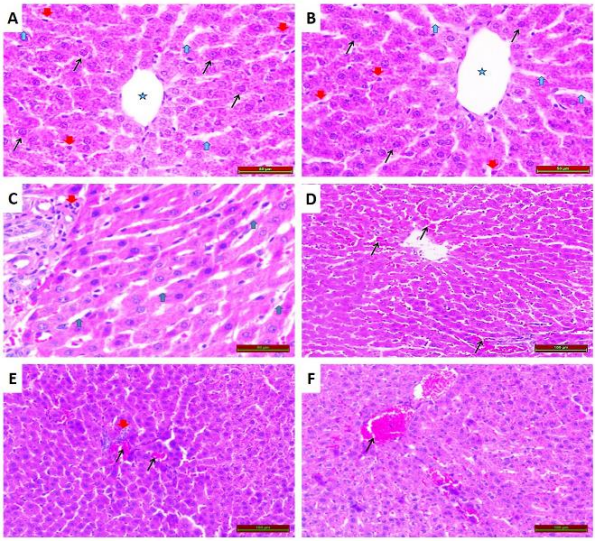

Figure 1. Histopathological changes (H&E) in liver tissues. A. In the Control-24 group and B. In the Control-48 group, vena centralis (blue star), hepatocytes (black thin arrow), sinusoids (blue thick arrow) and Kupffer cells (red thick arrow) are observed in normal structure. C. Sinusoidal dilatation (blue thick arrow) and inflammatory cell infiltration (red thick arrow) are observed in the LPS-24 group. D. Hyperemia (black thin arrow) is observed in the LPS-24 group. E. Hyperemia (black thin arrow) and inflammatory cell infiltration (red thick arrow) are observed in the LPS-48 group. F. Hyperemia (black thin arrow) is observed in the LPS-48 group.

Table 1. CD68, CD204 and CD163 immunoreactivity histoscore of all groups

	CD68	CD204	CD163
Control-24	13.54±3.57[a]	9.54±3.56	10.08±1.83
Control-48	14.12±4.15[a]	9.20±1.28	10.25±4.24
LPS-24	36.62±4.39[b]	9.50±1.10	10.62±2.66
LPS-48	44.08±6.05[c]	11.58±8.02	12.41±3.33

Values are given as mean ± standard deviation. Different lettering (a, b, c) in the columns means statistically significant difference.

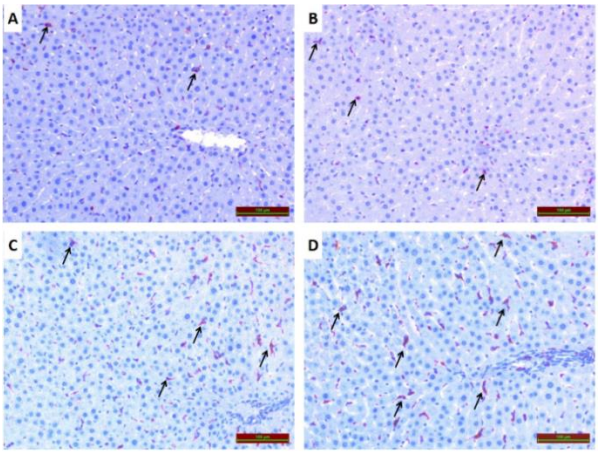

Figure 2. CD68 immunoreactivity in liver tissue. A. Control-24. B. Control-48. Sections from C. LPS-24 and D. LPS-48 groups. Red/brown cytoplasmic staining shows immunopositive cells. The preparations were counterstained with Harris hematoxylin. Immunopositive cells are marked with arrows.

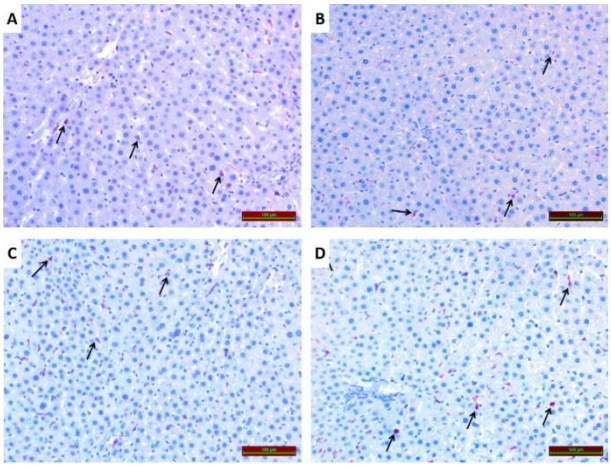

Figure 3. CD204 immunoreactivity in liver tissue. A. Control-24. B. Control-48. Sections of C. LPS-24 and D. LPS-48 groups. Red/brown cytoplasmic staining indicates immunopositive cells. The preparations were counterstained with Harris hematoxylin. Immunopositive cells are marked with arrows.

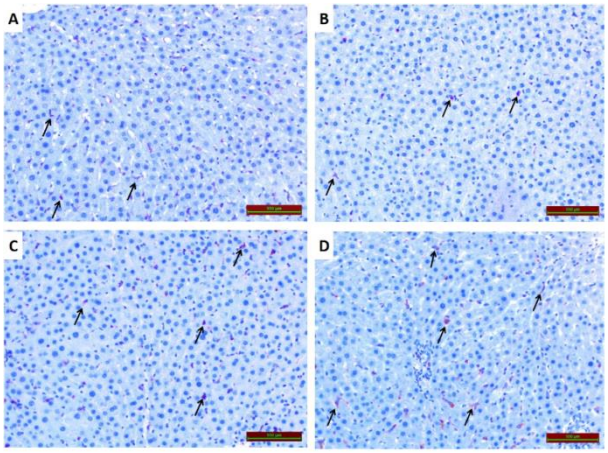

Figure 4. CD163 immunoreactivity in liver tissue. A. Control-24. B. Control-48. Sections of C. LPS-24 and D. LPS-48 groups. Red/brown cytoplasmic staining indicates immunopositive cells. The preparations were counterstained with Harris hematoxylin. Immunopositive cells are marked with arrows.

Discussion

Macrophages, which originate from the hepatic microenvironment and are the main cellular components of the liver, are cells with high adaptation capacity and are rapidly affected by the deterioration of hepatic homeostasis. They play an important role in acute and chronic liver diseases, injury and repair processes. Macrophages, whose main functions are the production of phagocytosis and inflammatory mediators, and whose basic activities are controlled by surface receptors, are involved both in physiological processes and in various pathological conditions such as infection, inflammation, cancer and atherosclerosis (9-11). There are two distinct macrophage polarization phenotypes, which are classically activated M1 macrophages and alternatively activated M2 macrophages (12-15). M1 macrophages; They are CD68 positive, stimulated mainly by IFN-ɣ, and have high phagocytosis ability and cytotoxicity. M2 type macrophages are CD163 positive and CD204 positive, and they are mainly stimulated by IL-4 and serve in reparative fibrosis (16).

LPS, one of the components of gram-negative bacteria, is a dangerous endotoxin that strongly induces cytokine release in the inflammatory response in the host (17). The elimination of LPS in the liver is mediated mainly by Kupffer cells (18). LPS stimulates Kupffer cells via TLR4 signaling to release proinflammatory cytokines such as TNF-α and IL-1 (19-21). Activation of TLRs has been reported to induce M1 polarized macrophage response inducing proinflammatory activation (22). TLR4 activation is thought to cause cellular hyperactivation and accumulation of neutrophils in narrow capillaries of the liver (23). In an experimental study in rats, it was reported that LPS administration caused inflammatory cell infiltration (24). In our study, hyperemia and inflammatory cell infiltration were observed in rat liver tissue after LPS stimulation. We think that these histopathological changes in the liver tissue may be related to the above-mentioned mechanism. In our study, it was determined that CD68 positive M1 macrophages in the rat liver after LPS stimulation were statistically significantly higher when compared to the control groups. Moreover, it was determined that CD68 immunoreactivity was significantly increased in the LPS-48 group compared to the LPS-24 group. This significant and sustained increase is probably due to activation of CD68 positive M1 macrophage cells with high phagocytosis ability and cytotoxicity via TLR 4 signaling. Thus, elimination of LPS, a dangerous endotoxin, and tissue defense will be facilitated.

In a study with mouse bone marrow cell cultures from the femur, it was shown that macrophages that were not induced by immunohistochemical staining had M1 and M2 profiles. As a result of the application of LPS to in vitro mouse cultures, it was determined that the macrophages were polarized to M1 macrophages. Uninduced macrophages show a low M1 and M2 profile, while macrophages show a high M1/M2 ratio when induced by LPS. The findings of this study are highly similar to our study, in which the number of M1 macrophages increased after induction with LPS (25). In another study, it was determined that macrophage immunophenotypes showed different distribution and kinetics in cholestatic rat liver lesions induced by alpha naphthyl isothiocyanate. It was observed that CD163 positive macrophages increased in the early period, while CD204 and CD68 positive macrophages increased continuously throughout the experiment (26). In a study conducted in cattle liver with Fasciola hepatica infection, it was found that CD68 and CD163 immunopositive macrophages increased and CD204 immunopositive macrophages decreased in fibrotic livers compared to control livers (27). Macrophages, whose main activities are controlled by surface receptors, including phagocytosis and production of inflammatory mediators, also play an important role in various pathological conditions such as inflammation, infection, atherosclerosis and cancer (28, 29).

Heterogeneity has been reported to be one of the most important features of macrophages. In different diseases, macrophages can be polarized to different phenotypes. For example, in most tumors, macrophages are thought to be polarized to the M2 phenotype (30).

It has been reported that M1-like macrophages are more dominant in the early stages of inflammatory responses, while M2-like macrophages are more frequently associated with the chronic inflammation process (31-33).

It is thought that M1 macrophages are responsible for resistance against Listeria monocytogenes and Salmonella infections, generally together with intracellular pathogens (34, 35). When the effect of new treatments for acute inflammation was investigated, it was determined that systemic inflammation induced by LPS was the best model (36). Therefore, in our study, we aimed to determine the M1/M2 macrophage polarization in the liver in systemic inflammation induced by LPS, and to reveal it by immunohistochemical methods.

In conclusion, this study revealed the immunohistochemical distribution of M1 and M2 macrophages in vivo in rat liver at 24 and 48 hours after LPS stimulation. CD68 immunoreactivity with an increase in M1 polarization in vivo in rat liver at 24 and 48 hours after LPS stimulation; It was determined that it was statistically significantly higher when compared to the control groups. On the other hand, CD204 and CD163 immunoreactivity, which determines M2 polarization; When compared to the control groups, no change was observed in the LPS-24 group, while an increase was determined in the LPS-48 group, but this increase was not statistically significant. It was determined that after antigenic stimulation with lipopolysaccharide, rat hepatic macrophage phenotypes increased in the direction of M1 polarization, which has high phagocytosis ability and cytotoxicity. We believe that the information obtained as a result of this study may also be useful for the examination of experimentally created rat liver lesions.

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