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Melatonin mediated Foxp3-downregulation decreases cytokines production via the TLR2 and TLR4 pathways in *H. pylori* infected mice

Jianhua Luo[a,f], Jun Song[b,f], Hui Zhang[c,f], Feng Zhang[a,f], Hui Liu[e,f], Li Li[a,b,f], Zhiguang Zhang[a,b,f], Lushan Chen[a,d,f], Mi Zhang[a,f], Dacen Lin[a,f], Meifang Lin[a,f], Ruixiang Zhou[a,e,f,*]

[a] Key Laboratory of Ministry of Education for Gastrointestinal Cancer, PR China
[b] Department of Cell Biology and Genetics, PR China
[c] Fujian Center for Safety Evaluation of New Drugs, PR China
[d] Department of Pathology, Union Hospital, PR China
[e] Department of Human Anatomy, Histology and Embryology, PR China
[f] School of Basic Medical Sciences, Fujian Medical University, Fuzhou, Fujian 350108, PR China

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ABSTRACT

Melatonin has important immuno-regulatory effects in inflammatory disorders but its specific role in *Helicobacter pylori* induced gastritis remains unclear. The aim of our study was to analyze the activity of melatonin against *H. pylori* induced gastritis in vivo, and explore the underlying mechanisms. The *H. pylori* infected mice showed extensive inflammatory cell infiltration in the gastric mucosa and submucosa, along with significantly reduced spleen and thymus weight. However, 2 and 6 weeks of treatment with 25 and 50 mg/kg melatonin restored the thymus weights relative to that of the untreated mice. TLR2 was upregulated in the gastric mucosa of the infected mice, which was restored to normal levels after 2 and 6 weeks of melatonin treatment. In contrast, TLR4 levels were similar between the treated and untreated mice. Furthermore, melatonin treatment restored spleen Foxp3 and serum TGF-β1 levels that were respectively increased and decreased in the infected mice. *H. pylori* infected mice also showed a decrease in the serum levels of IL-2, IL-6, IL-10, IL-17, IFN-γ and TFN-α following 2 and 6 weeks of melatonin treatment compared to the untreated mice. Melatonin treatment also resulted in decreased CD4 + CD25 + Foxp3 + Treg cell count in the spleen. The expression of TLR2, MyD88, p-ERK, p-p38, p65, p50 and Foxp3 in the gastric tissues were lower in the untreated mice compared to mice treated with melatonin for 2 weeks. However, the expression levels evened out after 6 weeks of treatment. Taken together, melatonin alleviates *H. pylori* induced gastritis by regulating TGF-β1 and Foxp3 expression via the TLR2 and TLR4 pathways.

1. Introduction

Melatonin, a hormone secreted by the pineal gland, has multiple physiological effects such as regulation of the circadian rhythm and immuno-activation [1,2]. *Helicobacter pylori* is a gram-negative micro-aerophilic bacteria that selectively colonizes the gastric epithelium, and is considered a class I carcinogen for gastric cancer [3]. A decrease in the serum levels of melatonin synthesizing enzymes is seen in patients with symptomatic *H. pylori* infection, and melatonin can reduce the risk of carcinogenesis associated with this pathogen via its antioxidant effects [4,5]. Although melatonin can eradicate *H. pylori* [6,7], the underlying molecular mechanism is unknown. Identification of the pathways involved in the therapeutic effects of melatonin can help improve *H. pylori* clearance.

The Toll-like receptors (TLRs) 2 and 4 recognize *H. pylori* [5,8,9] and activate the MAPK and NF-κB pathways, which are the most targeted pathways in *H. pylori* induced gastritis therapy [10,11]. Other immuno-regulatory factors that can modify the course of *H. pylori* infection [12,13] include Foxp3 (regulates T cells) and TGF-β1 (immunosuppressive function to Foxp3) [14–17]. An immuno-modulatory role of melatonin is seen in inflammatory responses where it inhibits Foxp3, and is correlated with T cell differentiation [18,19]. In addition, melatonin also regulates the expression of several cytokines [20–23]. Since it is frequently present in inflamed gastric tissues [24], we hypothesized that melatonin affects the progression of *H. pylori* induced gastritis. To test this hypothesis, we established a murine model of

* Corresponding author at: Department of Human Anatomy, Histology and Embryology, School of Basic Medical Sciences, Fujian Medical University, 1 Xueyuan Road, Fuzhou, Fujian 350108, PR China.
E-mail address: rxzhou@fjmu.edu.cn (R. Zhou).

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gastritis by orally administering *H. pylori*, and treated the mice with varying concentrations of melatonin for 2 and 6 weeks.

2. Materials and methods

2.1. Bacterial strains and growth conditions

H. pylori strains were grown on Columbia agar medium (CM0331, OXOID) with sheep blood (10%) and *H. pylori* selective supplement (SR0147E, OXOID) for 48 to 72 h in a microaerophilic atmosphere (85% N_2, 10% CO_2 and 5% O_2) at 37 °C. The mouse-adapted *H. pylori* strain SS1 was kindly provided by professor Feifei She, University of Fujian Medical University.

2.2. Animal experiments

Four weeks old female C57BL/6J mice were purchased from the Shanghai SLAC Laboratory Animal Corporation. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee of Fujian medical University. Animals were housed under specific-pathogen-free conditions. *H. pylori* SS1 were harvested and suspended in Brucella broth (Oxoid Ltd., Basingstoke, England) at the density of 5×10^8 bacteria/ml. Each mouse was given an oral gavage of 100 μl bacterial suspension with a stainless steel blunt feeding needle. Melatonin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in absolute alcohol and then diluted in normal saline to the final ethanol concentration < 1%. The melatonin solution was injected between 6:00 and 6:30 PM every day for either 14 days (2 weeks group) or 42 days (6 weeks group), after which the mice were sacrificed by cervical dislocation.

2.3. Histological analysis and immunohistochemistry (IHC)

Spleen and gastric specimens were fixed in 4% paraformaldehyde for 12 h and embedded in paraffin. The paraffin blocks were cut into 4-μm sections that were dehydrated with an alcohol gradient, cleared with xylene, and stained with either hematoxylin and eosin, or by the IHC protocol with primary antibodies against TLR2 (ab2068, Abcam), TLR4 (sc-293072, SANTA CRUZ) and Foxp3 (#12653, CST). All stained sections were observed under the ZEISS light microscope (Axioplane 2, Carl Zeiss MicroImaging GmbH, Hamburg, Germany).

2.4. Enzyme linked immunosorbent assay (ELISA)

The serum level of TGF-β1 was measured using a specific ELISA kit (SMB100B, R&D) according to the manufacturer's instructions. The results were determined spectrophotometrically at 450 nm.

2.5. Cytometric bead assay (CBA)

The serum levels of IL-2, IL-6, IL-10, IL-17, IFN-γ and TFN-α were detected by flow cytometry (FACSVerse, BD) using the CBA kit (560,485, BD) according to the manufacturer's instructions.

2.6. Flow cytometry

The spleen was homogenized with core needle grinding, and the splenocytes were isolated by layering on Histopaque-1083 (Sigma-Aldrich, USA) and centrifuging at 200*g* for 30 min. The cells were stained with Mouse Regulatory T Cell Staining kit (88-8118-40, eBioscience), FITC-anti-CD4 (11-0042-81c, eBioscience) and PE-anti-CD25 antibodies (12-0251-81B, eBioscience) for 15–20 min. The cells were then re-suspended in fixation/permeabilization solution (#88-8823-88, eBioscience), and stained with APC- anti-Foxp3 antibody (17-5773-80A, eBioscience) for the intracellular antigen.

2.7. Western blotting

Spleens were lysed in tissue lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland), and the protein concentration was measured using the Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein per sample (40 μg) were separated using 12 or 15% SDS-PAGE, and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking in Tris-buffered saline (TBS) containing 0.5% bovine serum albumin (BSA), the membranes were incubated with anti-TLR2 (ab2068, Abcam), anti-MyD88 (ab2068, Abcam), anti-phosphoErk1/2 (#4370, CST), anti-Phospho-p38MAPK (#4511, CST, 1:1000 dilution), anti-NF-κB p65 (ab106502, Abcam), anti-NF-κB p50 (ab32360, Abcam, 1:1000 dilution), anti-FoxP3 (#12653,CST), anti-TGFβ1 (ab64715, Abcam), and anti-GAPDH (2118S, CST) antibodies. This was followed by incubation with the alkaline phosphatase goat anti-rabbit IgG secondary antibody (ab98505, 1:5000 dilution, Abcam). Target protein bands were detected by CDP-Star chemi-luminescence reagents (Roche Diagnostics, Mannheim, Germany), and imaged using an ImageQuant LAS 4000 mini (GE Healthcare, Chicago, IL, USA). Band intensities were quantified using ImageJ2x software (National Institutes of Health, Bethesda, MD, USA), and the band intensities relative to the internal GAPDH control were calculated.

2.8. Data analysis

The data are represented as mean ± standard deviation (SD) from at least three independent experiments. One-way ANOVA and Student's paired *t*-test were used to compare different groups, and p-values < 0.05 were considered statistically significant. All analyses were performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Gastritis development and effects of melatonin therapy

To determine the effect of melatonin on *H. pylori* induced gastritis, a mouse model of the same was developed. HE staining of the gastric tissues showed significant infiltration of lymphocytes in the mucosa and submucosa (Fig. 1A, B), indicating successful establishment of a murine chronic superficial gastritis model. Although the cumulative incidence of gastritis was increased after *H. pylori* infection, no apparent difference was observed in the gastritis phenotype. To determine the effect of melatonin therapy, the spleen and thymus weights of the mice were measured. While melatonin aggravated the significant reduction in spleen weight following *H. pylori* infection, the melatonin treated mice slowly regained their thymus in a dose dependent manner. After 6 weeks of treatment, there was no difference in the thymus weights relative to the uninfected control group (Fig. 1C).

3.2. Melatonin differentially regulated the expression of TLR2, TLR4 and MyD88 in H. pylori infected mice

The in situ TLR2 expression in the gastric tissue was significantly decreased ($p < 0.005$; Fig. 2A) in response to melatonin treatment compared to the untreated mice, and was similar to that of the uninfected controls. In contrast, melatonin therapy had minimal effect on TLR4 expression (Fig. 2B). Furthermore, the total TLR2 protein was significantly reduced in the infected mice following melatonin treatment for 2 weeks or 6 weeks compared to the untreated group. Mice receiving high dose (50 mg/kg) melatonin treatment showed the most significant downregulation of TLR2 ($p < 0.01$) relative to both the

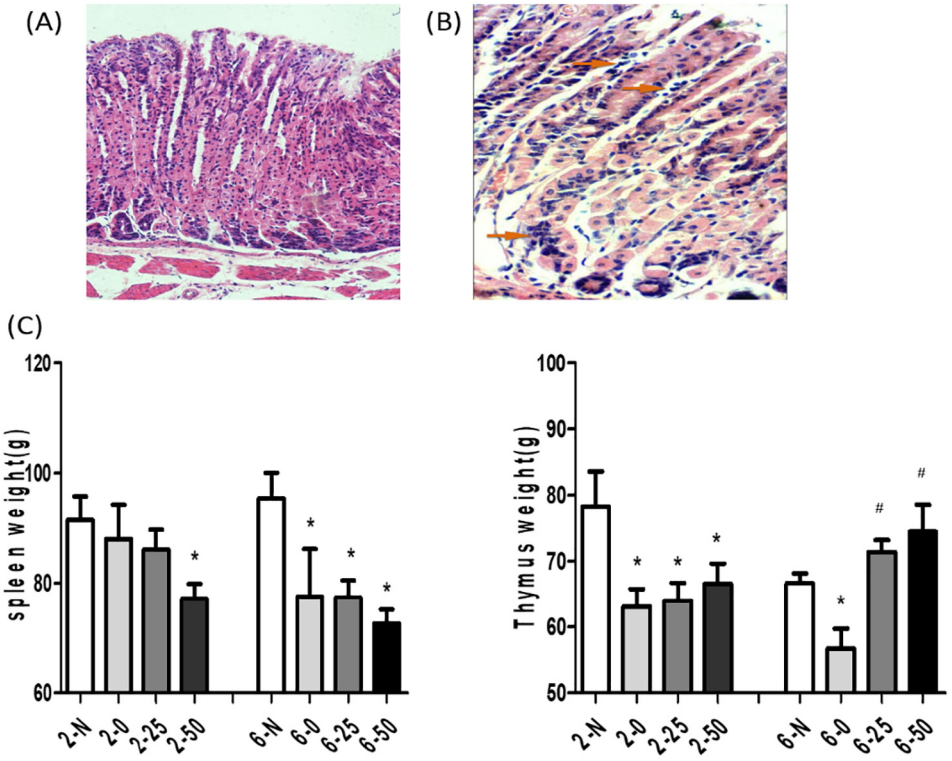

Fig. 1. The influence of melatonin on *H. pylori* induced chronic gastritis after 2 weeks and 6 weeks of treatment. HE staining of the gastric tissues of *H. pylori* infected mice at 200× (A) and 400× (B) magnification, with arrows pointing to the lymphocytes (C) Spleen and thymus weight of uninfected control (N), Brucella broth control (0), 25 mg/kg melatonin treated (25), and 50 mg/kg melatonin treated (50) groups. "*" vs N group $p < 0.05$, "#" vs 0 group $p < 0.05$.

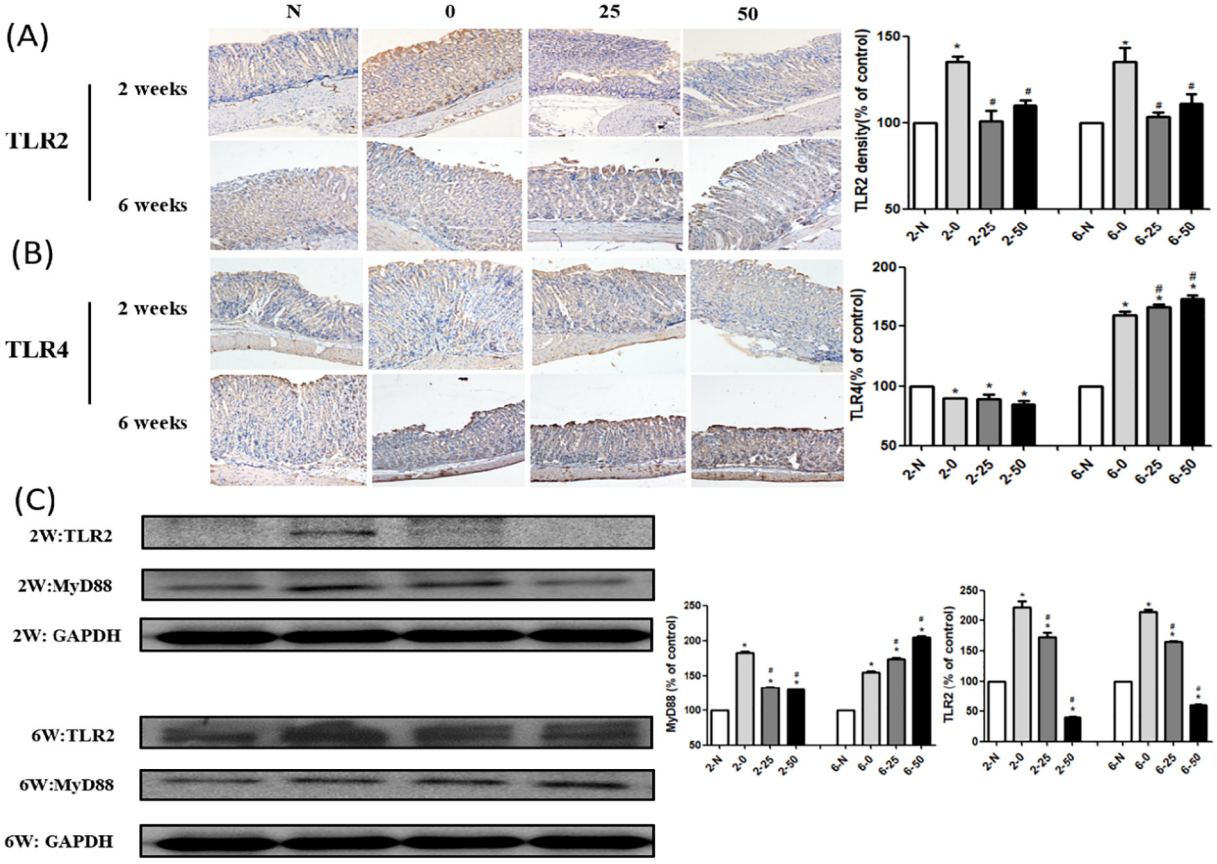

Fig. 2. The effect of melatonin on TLR2, TLR4 and MyD88 expression after 2 weeks and 6 weeks of treatment (A) IHC staining of TLR2 at 400× magnification. (B) IHC staining of TLR4 at 400× magnification. (C) Western blots showing levels of TLR2, TLR4 and MyD88 in gastric tissue. N - uninfected control, 0 - Brucella broth control, 25 - 25 mg/kg melatonin treated group, 50 - 50 mg/kg melatonin treated group. "*" vs N group $p < 0.05$, "#" vs 0 group $p < 0.05$.

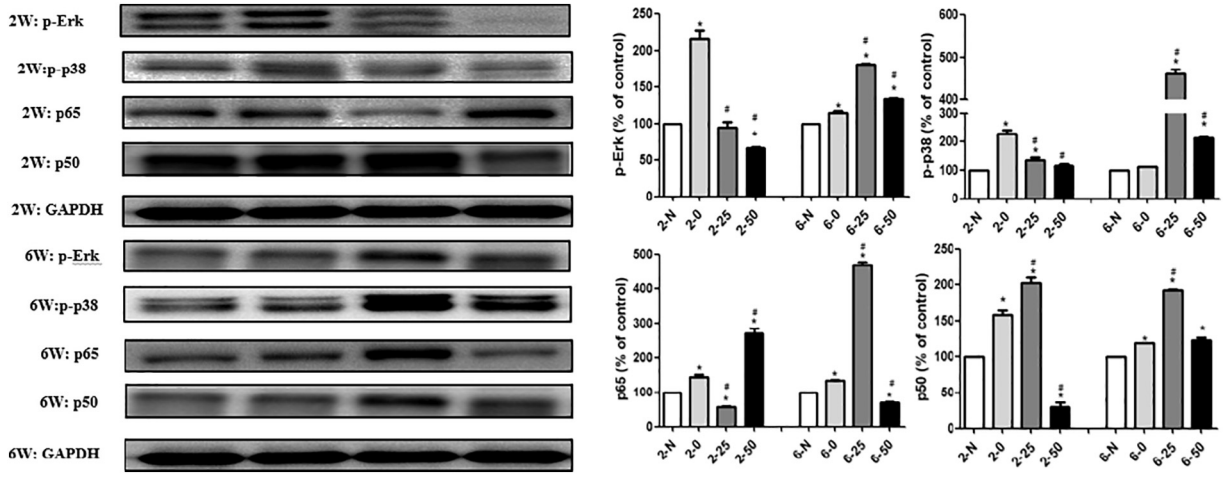

Fig. 3. The effect of melatonin on the MAPK and NF-κB pathway protein expression after 2 weeks and 6 weeks of treatment. Western blots showing p-Erk, p-p38, p65 and p50 expression in the gastric tissues of uninfected control (N), Brucella broth control (0), 25 mg/kg melatonin treated (25), and 50 mg/kg melatonin treated (50) groups. "*" vs N group $p < 0.05$, "#" vs 0 group $p < 0.05$.

culture broth and uninfected controls (Fig. 2C). MyD88, the downstream target of TLR2 and TLR4, showed similar expression tendencies as TLR2 after short-term melatonin treatment (2 weeks), but reverse trends after the long-term treatment (6 weeks) (Fig. 2C).

3.3. Melatonin affects the proteins associated with the MAPK and NF-κB pathway

To determine the role of the MAPK and NF-κB pathways in melatonin treatment, the expression levels of p-Erk, p-p38, p65 and p50, known regulators of both pathways, were analyzed in the gastric tissues. While p-Erk and p-p38 were downregulated by short-term melatonin therapy ($p < 0.05$, 2–50 mg/kg melatonin vs culture broth control; Fig. 3), long-term treatment upregulated both, especially at low-doses (6–25 mg/kg and 6–50 mg/kg vs culture broth and uninfected controls). Interestingly, the long-term treatment had a similar effect on both p65 and p50, but their expression in the short-term treatment group was inconsistent.

3.4. Melatonin regulates Foxp3 expression in spleen

Foxp3 is the lineage-defining transcription factor for Treg cells. Melatonin treatment significantly reduced the expression of Foxp3 in the splenic and peripheral blood lymphocytes compared to the untreated mice after 2 weeks (Fig. 4A, C). Furthermore, TGFβ1 expression followed similar trends in the short-term therapy. After 6-week of treatment however, no dose-time tendencies were seen in Foxp3 and TGFβ1 expression levels (Fig. 4B).

3.5. Melatonin reduces the levels of inflammatory cytokines in H. pylori infected mice

The serum levels of inflammatory cytokines involved in the Th1, Th2 and Th17 responses, such as IFN-γ, TNF-α, IL-2, IL-6, IL-10, IL-17 and TGFβ1, were also analyzed. Higher doses of melatonin (25, 50 mg/kg) for 2 and 6 weeks resulted in a significant decrease in the levels of IFN-γ ($p < 0.05$), TNF-α ($p < 0.05$), IL-2 ($p < 0.005$), IL-10 ($p < 0.005$) and IL-6 ($p < 0.05$) in *H. pylori* infected mice compared to the untreated mice (Fig. 5). No differences were seen in the levels of IL-6 in the 50 mg/kg group and of TNF-α in the 25 mg/kg group at 2 weeks.

4. Discussion

Melatonin inhibited Foxp3 expression, as well as downregulated that of TLRs and its downstream targets and cytokines, suggesting that it regulates the immune system in *H. pylori* induced gastritis via the TLR pathway. Our findings are consistent with a previous study which showed that Foxp3 expression was increased in *H. pylori* infected compared to the uninfected mice [15,17,25,26]. In addition, *H. pylori* infected mice have abundant Foxp3 expressing Treg cells and IL-10 producing B-cells in the gastrointestinal mucosa and spleen [27]. Similarly, in a previous study on patients with cleared *H. pylori* infection, the Foxp3 mRNA levels were significantly decreased in the gastric mucosa [17]. In this study, we showed for the first time that melatonin decreased Foxp3 and cytokine expression via the TLR2 or TLR4 pathway.

Our results suggest that melatonin regulates both the TLR2 and TLR4 pathways during *H. pylori* infection via two different feedback signaling loops during long-term and short-term infection. In the initial stages of the infection, TLR2 is sensitized to the circulating *H. pylori* associated pro-inflammatory factors but as the infection progresses, TLR4 becomes the dominant regulator. The infected gastric microenvironment has higher levels of gastritis-induced pro-inflammatory cytokines and Foxp3 [15,17,26,28]. Consequently, a positive feedback inflammatory signaling loop is established between Foxp3 and the cytokines, which accelerates gastritis progression. For these self-perpetuating signals to first appear, the gastric tissue must already be inflamed and responsive to *H. pylori* associated cytokine signaling.

In addition to being a key activator of innate immunity via recognition of the conserved molecular patterns on bacteria, TLRs are also master regulators of the MAPK and NF-κB pathways and contribute to a gastritic microenvironment [10,12,29]. In the *H. pylori* infected mice, there was a significant decrease in the expression of gastric p-Erk, p-p38, p65 and p50 within 2 weeks of therapy relative to both uninfected and untreated controls, which partly contributed to downregulation of NF-κB signaling. Specific NF-κB activation in the gastric epithelial cells of *H. pylori* infected mice also involved local inflammatory signaling factors, such as myeloid differentiation factor 88 (MyD88), an adaptor molecule that is common to all TLR signaling pathways with the exception of TLR3. Consistent with our findings, MyD88 is related to melatonin in the regulation of inflammatory progression [30]. In addition, short-term therapy with melatonin reduced MyD88 expression while the long-term therapy had the opposite effect.

We also observed a decrease in the serum cytokine response after melatonin therapy, characterized by downregulated INF-γ, TNF-α, IL-2,

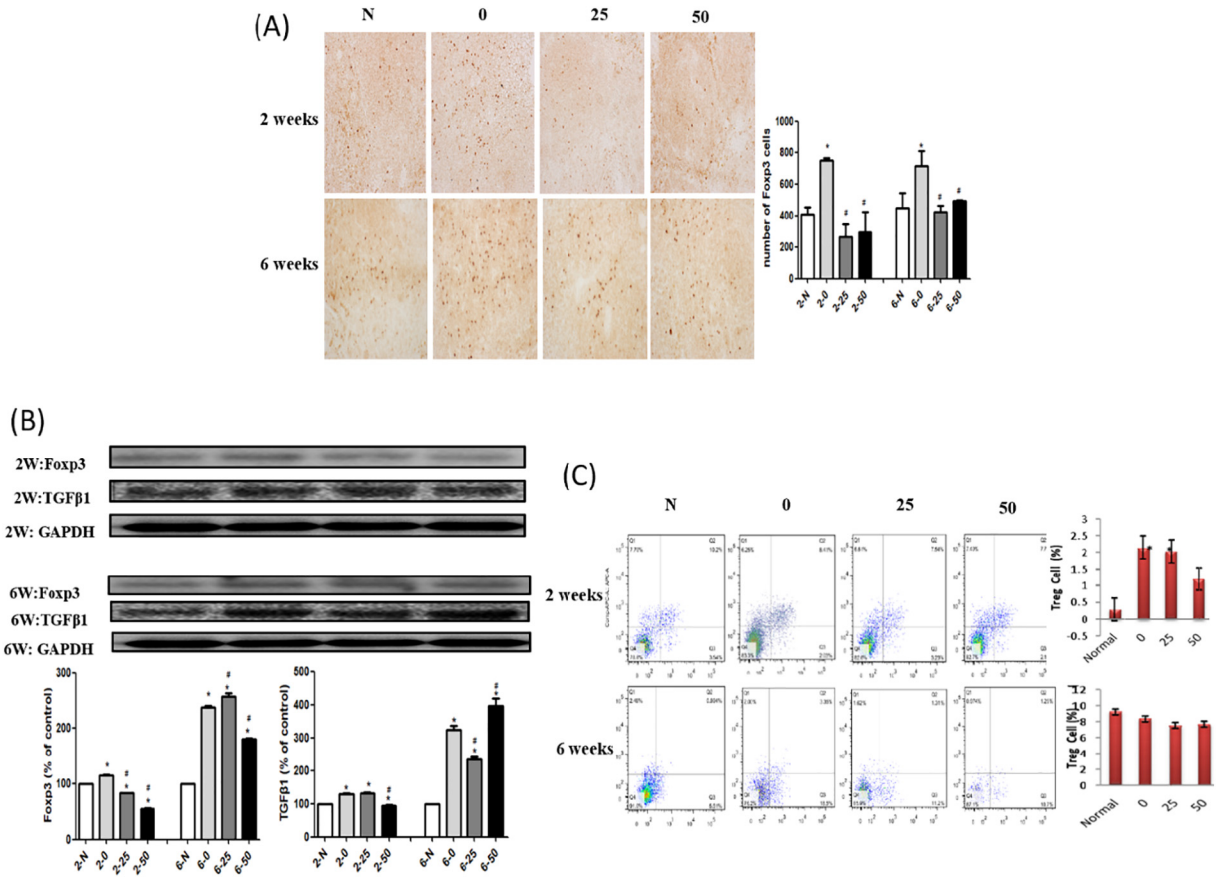

Fig. 4. The effect of melatonin on Foxp3 and TGFβ1 expression in gastric tissues after 2 weeks and 6 weeks of treatment (A) IHC staining of Foxp3 at 400× magnification. (B) Western blots showing Foxp3 and TGFβ1 levels in spleen. (C) FACS plots indicating the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen. N - uninfected control, 0 - Brucella broth control, 25 - 25 mg/kg melatonin treated group, and 50 - 50 mg/kg melatonin treated groups. "*" vs N group $p < 0.05$, "#" vs 0 group $p < 0.05$.

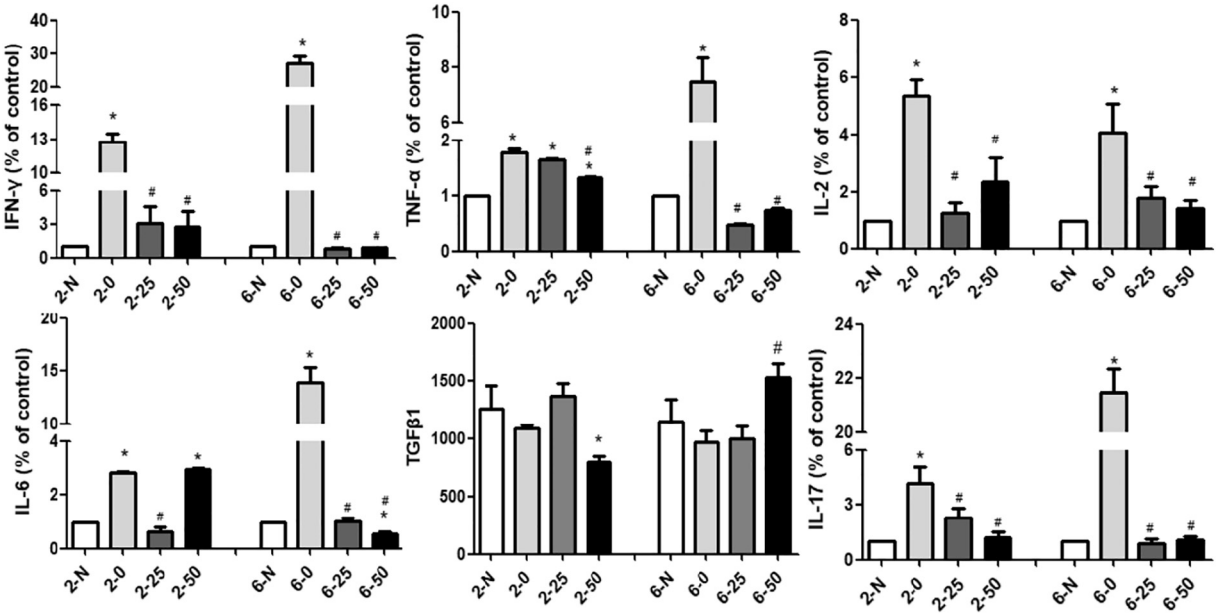

Fig. 5. The effect of melatonin on cytokine expression after 2 weeks and 6 weeks of treatment. CBA and ELISA showing the serum levels of IFN-γ, TNF-α, IL-2, IL-8, TGFβ1 and IL-17. N - uninfected control, 0 - Brucella broth control, 25 - 25 mg/kg melatonin treated, and 50 - 50 mg/kg melatonin treated groups. "*" vs N group $p < 0.05$, "#" vs 0 group $p < 0.05$.

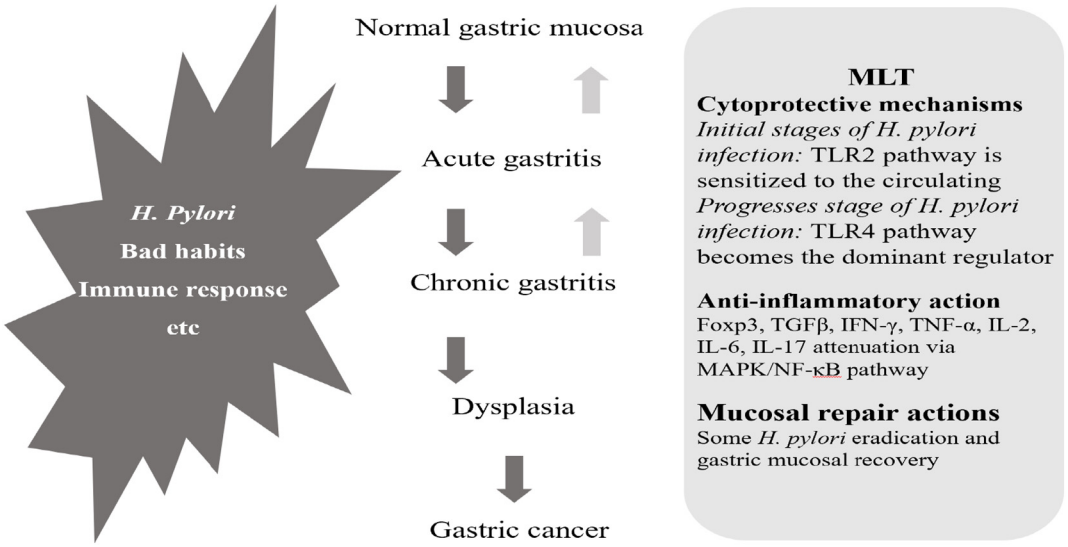

Fig. 6. Hypothetical mechanism of melatonin mediated suppression of the inflammatory responses via the indirect regulation of TLR signaling pathway.

IL-6, IL-17 and TGFβ levels compared to the untreated mice. On the basis of our findings, we hypothesize that *H. pylori* activates TLR2 and TLR4, which in turn induces the Th1, Th2 and Th17 specific cytokines. Melatonin is important for the secretion of the pro-inflammatory cytokines, while TLR2 and TLR4 are required for the optimum production of the immuno-regulatory INF-γ, TNF-α, IL-2, IL-6, IL-17 and TGFβ. These results suggest a functional specificity of the immune system through melatonin regulation of TLR signaling during *H. pylori* infection, with TLR2 and TLR4 mediating the reciprocal induction of TGFβ and downregulation of INF-γ, TNF-α, IL-2, IL-6 and IL-17.

In summary, although the molecular target of melatonin in the TLR pathway is still unknown, our study shows their cross-talk in an *H. pylori* infected mouse model. Furthermore, we were able to show that the overall gastric protective effect of melatonin is related to TLR2 suppression and TLR4 activation, which inhibited the secretion of pro-inflammatory cytokines and the inflammatory signaling cascades. Effective immuno-modulation of melatonin in *H. pylori* induced gastritis via TLR signaling warrants further investigation for a potential therapeutic use of melatonin (Fig. 6).

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