

# Rapamycin attenuates gene expression of programmed cell death protein-ligand 1 and Foxp3 in the brain; a novel mechanism proposed for immunotherapy in the brain

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## Abstract

**Background and purpose:** Programmed cell death protein-1 (PD1) expresses on the cell surface of the activated lymphocytes and at least a subset of Foxp3+ regulatory T cells. The binding of PD1 to its ligands including PD-L1 and PD-L2 leads to deliver an inhibitory signal to the activated cells. Although PD1/PD-L signal deficiency can lead to failure in the self-tolerance and development of autoimmunity disorders, PD1 blockade with monoclonal antibodies is considered an effective strategy in cancer immunotherapy. Determining effective environmental factors such as stress conditions on the expression of PD1 and PD-L1 genes can provide an immunotherapeutic strategy to control PD1 signaling in the patients. Mammalian target of rapamycin signaling is a stress-responsive pathway in the cells that can be blocked by rapamycin. In this study, the effects of rapamycin on the expression of immunoregulatory genes were investigated in the stress condition.

**Experimental approach:** Daily administration of rapamycin (1.5 mg/kg per day) was used in the mouse model of restraint stress and the relative expression of PD1, PD-L1, and Foxp3 genes in the brain and spleen were evaluated using quantitative real-time polymerase chain reaction method.

**Findings/Results:** With our observation, daily restraint stress ceased rapamycin to decrease the expression of Foxp3 in the brain significantly. These findings would be beneficial in developing tolerance to autoimmune diseases and finding immunopathology of stress in the CNS. In another observation, daily administration of rapamycin decreased the expression of PD-L1 in the brain cells of mice. In the spleen samples, significant alteration in genes of interest expression was not detected for all groups of the study.

**Conclusion and implications:** Downregulation of the PD-L1 gene in the brain induced by rapamycin can be followed in future experiences for preventing immunosuppressive effects of PD/PD-L1 signal in the brain.

**Keywords:** Immunomodulation; PD1; Rapamycin; Stress.

## INTRODUCTION

T lymphocyte is one of the main components of cellular immunity against tumor and virally infected cells. Three main factors involved in the differentiation of naïve T lymphocytes into the effector cells, including the engagement of T cell receptor with peptide binding major histocompatibility complex, the co-stimulatory signal receiving from antigen-presenting cells, and the effect of produced cytokines. In addition to the T cell receptor, stimulatory-inhibitory receptors such as a cluster of

differentiation 28 (CD28), cytotoxic T-lymphocyte-associated protein 4, and programmed cell death protein-1 (PD1) are involved in the T cell activation (1). Inhibitory receptors such as PD1 lead to the suppression of cell growth, inactivation of the affected cells, and reduction in cell cytokine production.

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Stimulatory-inhibitory receptors can impact the progress of tumors where tumor cells express a ligand for inhibitory receptors (such as PD-L1). Also, the lack of or decrease in the cell expression of costimulatory molecules by tumor cells can suppress the function of activated T cells (2). Due to the supportive or suppressive nature of stimulatory-inhibitory receptors, the manipulation of these pathways provides a new strategy to promote T cell function for treating several diseases, including cancers, infections, and the control of T cell responses in autoimmune diseases (3). There are several monoclonal antibodies specific for PD1 or PD-L1 which are approved for the treatment of patients with advanced tumors (4).

Regulatory T cells (T-reg) which are an immunosuppressive type of CD4+ T cells are characterized by the expression of the specific transcription factor so-called Forkhead box protein P3 (Foxp3). These cells can play a role as suppressive cells in the tumor microenvironment (5,6). At least a subset of T-reg cells expresses PD1 on their cell surface. It was demonstrated that PD1 blockade by specific antibodies or PD1 signal deficiency leads to an increase in the proliferation and suppression of PD1+ T-reg cells (5).

Immune responses in an individual can be affected by endogenous and exogenous stress stimulants. There are several definitions for the concept of stress such as “a set of stressors on the brain (perception or feeling of stress) that trigger physiological responses to those factors in the body” (7). Stressors can be divided into two types, including physiological stressors such as pathogens, and toxins and neurologic stressors such as trauma, and major life events (8). Despite the common view mentioning the suppressant effects of stress on the immune system, several studies have shown that acute and chronic stresses have different effects on the immune system. Previously, it was thought that the body suppresses its immune system to save energy for other vital functions such as respond to stressors (8,9). The proposed mechanism for stress-induced immunosuppression is contributed to different mechanisms, including the decrease in the cytokine and prostaglandin and the suppression in lymphocyte proliferation. The occurrence of

these events requires a period that cannot occur in a short time of acute stress (9).

The mammalian target of rapamycin C1 (mTORC1) is a cellular pathway that senses and response to stress stimulators in the cell (10-12). Different cell signaling pathways can activate mTORC1 by triggering the phosphatidylinositol 3-kinase-protein kinase B (PI3K-Akt) pathway and eventually lead to cell growth. Rapamycin is a drug that binds to a protein called FKBP and inhibits the mTORC1 pathway. Although rapamycin inhibits interleukin-2-mediated T cell proliferation and prevents the formation of effector lymphocytes, this drug does not impair the function and survival of regulatory T lymphocytes (1). The FDA has approved the use of rapamycin in patients with advanced renal cancer carcinoma, acute renal allograft rejection, and tuberous sclerosis complex with subependymal giant cell astrocytoma (13).

Since PD1/PD-L1 pathway is a therapeutic pathway with clinical importance in patients with malignancy and autoimmune diseases, studying the factors that impact the pathway could improve the appropriate intervention in the patients. Also, PD1/PD-L1 pathway is one of the main inhibitory signals in the regulatory T cells, therefore, we designed this experiment to test whether the restraint stress and rapamycin can attenuate the expression of the genes involved in this pathway. In this study, to evaluate the effect of stress and mTOR pathway on the lymphocyte function, the expression of PD1, PD-L1, and Foxp3 genes, was assayed using the real-time polymerase chain reaction (RT-PCR) method in the mouse model of restraint stress following daily administration of rapamycin.

## MATERIALS AND METHODS

### *Animals*

Animal studies were performed according to the ethical principles and the national norms and standards for conducting Medical Research in Iran (Ethical No. IR.BMSU.REC.1397.191).

Female BALB/c mice aging 8-12 weeks were obtained from Laboratory Animal Core Facilities, Kashan University of Medical Sciences, Iran. Mice had free access to water

and food to acclimate to the surrounding condition for at least 3 days before experimentation. Animals were group-housed five per cage ( $\sim 50 \times 25 \times 25$  cm).

### Restraint stress

We used a restraint stress method based on previous studies (14,15). Mice (5 mice per group) were placed in a well ventilated 50-mL restrainer (conical tubes) and a lever adjusted for immobilization. The confined space prevented mice from moving freely but did not much press on them. After a 2-h stress cycle, mice were returned to their cages with free access to water and food. Daily exposure to stressor continued until the day mice were sacrificed and samples prepared (Fig. 1).

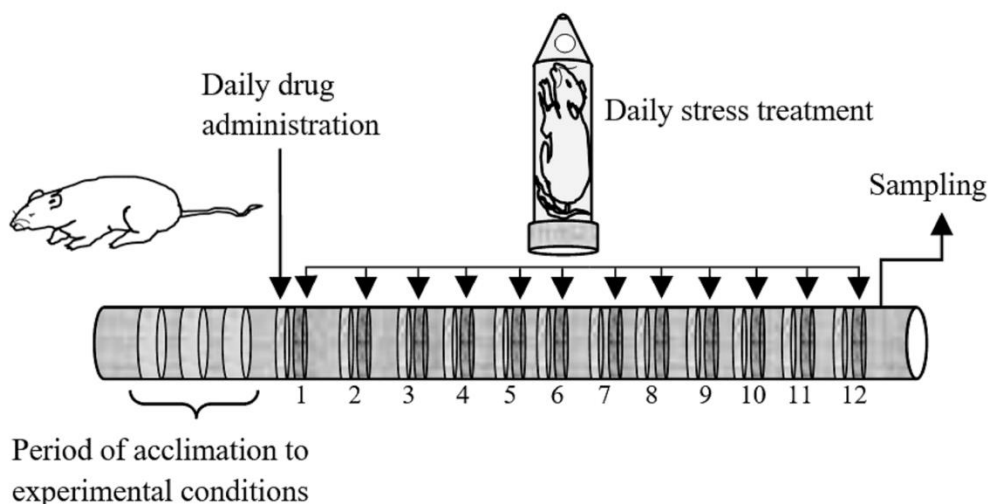
### Pharmacological studies

Mice were treated with propranolol (2 mg/kg per day) and rapamycin (1.5 mg/kg per day) using oral gavage at least for 20 min before placing them in the restrainer (conical tubes). The effective dose of propranolol and rapamycin was determined based on previous studies (16,17). The animals were divided into 6 groups (5 mice per groups) including (i) Ctrl, not exposed to any stressor and drug but stored

under the standard laboratory conditions; (ii) Str, exposed to daily stress; (iii) Rapa, received daily rapamycin; (iv) Rapa/Str, received daily rapamycin before exposing to the stressor; (v) Pro, received daily propranolol; (vi) Pro/Str, received daily propranolol before exposing to the stressor.

### RNA isolation and cDNA synthesis

Mice were sacrificed with inhalation of diethyl ether in a homemade anesthesia chamber. Brain and spleen were snap-frozen in the liquid nitrogen and then RNA was isolated from frozen spleen and brain using Denazist RNA isolation kit (Denazist, Iran) as per manufacturer's instructions. The accuracy of extracted RNA was determined using 2% agarose gel electrophoresis. Total RNA was reverse transcribed using a Parstous commercial kit (Parstus, Iran) based on the manufacturer's instruction. Briefly, 2  $\mu$ L of extracted RNA was combined with 10  $\mu$ L 2 $\times$  buffer-mix, 2  $\mu$ L enzyme-mix and 8  $\mu$ L diethylpyrocarbonate-treated water to bring the total volume to 20  $\mu$ L. The sample was mixed with quick vortex and incubated first at 25  $^{\circ}$ C for 10 min, then at 47  $^{\circ}$ C for 60 min, and finally at 85  $^{\circ}$ C for 5 min.



**Fig. 1.** Experiment timeline. Before the main experiment, mice were maintained in the cage with free access to water and food for acclimation to the experimental condition. Propranolol and rapamycin were daily administered to mice by oral gavage 0.5 h before daily restraint stress.

**Table 1.** Sequence, name, and product size of primers used in the study.

Primers	Sequence (5' to 3')	Product size (bp)	References
mGAPDH	Forward: AGGTCGGTGTGAACGGATTTG	123	18
mGAPDH	Reverse: TGTAGACCATGTAGTTGAGGTCA		
mPD1	Forward: AAATCGAGGAGAGCCCTGGA	109	19
mPD1	Reverse: CATGCCTTGAAACCGGCCTT		
mPD-L1	Forward: GGTGCGGACTACAAGCGAAT	96	20
mPD-L1	Reverse: TTCATGCTCAGAAAGTGGCTGG		
mFoxp3	Forward: CCCAGGAAAGACAGCAACCTT	89	21
mFoxp3	Reverse: TTCTACAACCAGGCCACTTG		

Foxp3, Forkhead box protein P3; GAPDH, glycerol aldehyde phosphate dehydrogenase; PD1, programmed cell death protein-1; PD-L1, ligands of PD1.

### Quantitation of gene expression by RT-PCR

Primer's sequence of PD1, PD-L1, Foxp3, and brain-derived neurotrophic factor (BDNF) was determined based on previous studies (Table 1) and synthesized by Macrogen (Macrogen, Korea). To standardize the relative assays, the expression of the glycerol aldehyde phosphate dehydrogenase (GAPDH) gene was determined as an internal control. RT-PCR mixture consisted of 2  $\mu$ L cDNA, 1.5  $\mu$ L of each reverse and forward primers, 9  $\mu$ L RT-PCR Master Mix (Biofact, China., Amplicon, Denmark) and 5  $\mu$ L ddH<sub>2</sub>O for a final volume of 19  $\mu$ L. RT-PCR was carried out using Roche thermocycler (Roche, Switzerland), the reaction was run at 94 °C for 10 min as initial denaturation followed by 45 cycles which consisted of a 30 s denaturation (94 °C), and a 40 s anneal/extension (60-62 °C; the temperature of anneal/extension was different for each set of primers).

The resulting changes in the fluorescence of amplification were relatively measured using LightCycler 96 SW 1.1 software and CT (or Cq) of the samples were obtained using Rel Quant program. The CT results of samples were entered into the Excel software in different groups. To calculate relative expression, the following equation was used:

$$\Delta C_T = C_T \text{ target gene} - C_T \text{ control gene}$$

To simplify graphs,  $\Delta C_T$  of genes was subtracted from a constant number (n) and displayed on the graphs. In other words, the value of the relative expression in the graph referred to  $n - \Delta C_T$ .

### Statistical analysis

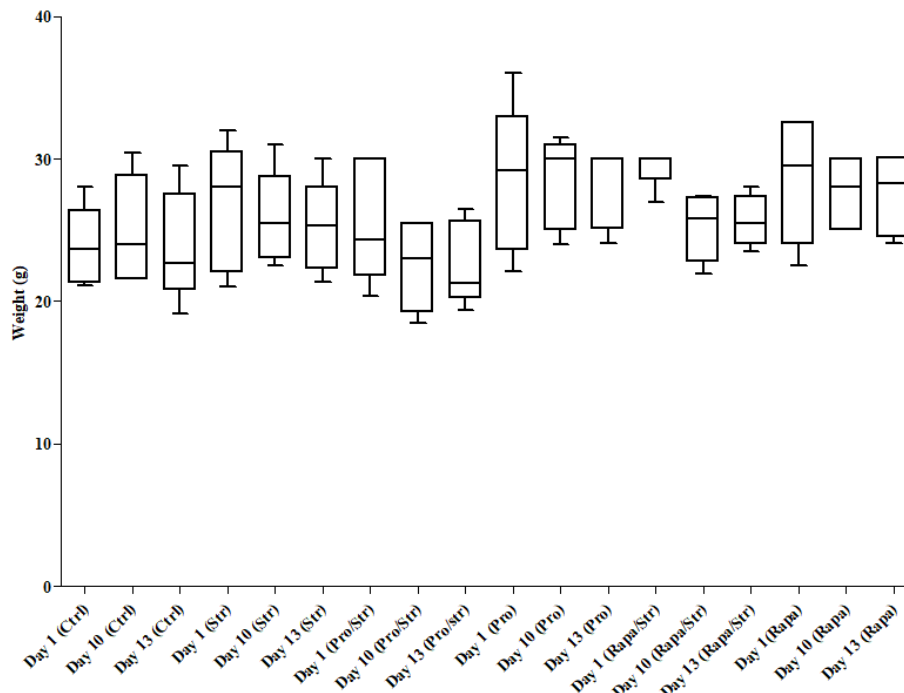
Data were analyzed using t-test and one-way analysis of variance (ANOVA) followed by

Tukey's multiple comparisons and Kruskal-Wallis (for results without using Gaussian distribution) tests using GraphPad Prism 5 and differences were considered significant at  $P < 0.05$ . *Dunn's* multiple comparison test and using median  $\pm$  IQ was used as post-test in Kruskal-Wallis test.

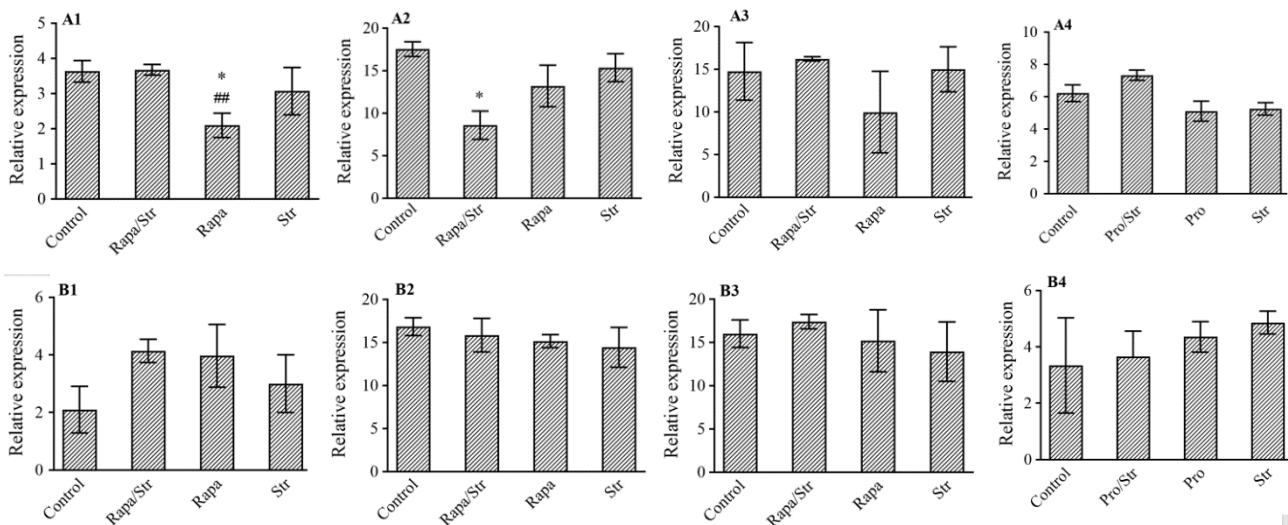
## RESULTS

Three groups of mice (Str, Pro/Str, and Rapa/Str) were exposed to daily stress for 13 days. The effect of 13-day restraint stress on the bodyweight of mice is shown in Fig. 2. Weight results in days 1, 10, and 13 were analyzed using ANOVA followed by Tukey's multiple comparison test and Kruskal-Wallis test. Although the weight loss of all groups was not statistically significant throughout the period of the experiment, predictively, for Rapa/Str and Str a weight loss was visually observed within 10 days after the beginning of the exposure to the restraint stress.

In Fig. 3, relative expression of PD1, PD-L1, Foxp3, and BDNF in the brain and spleen are illustrated. Spleen and brain of 8- to 12-week-old BALB/c mice were isolated for RNA extraction, cDNA synthesis, and quantitative RT-PCR. Mice received propranolol and rapamycin by oral gavage half an hour before daily 2-h restraint stress. Among the evaluated genes, PD-L1 and Foxp3 were significantly decreased in mice receiving rapamycin (Rapa) and mice receiving rapamycin in stress conditions (Rapa/Str), respectively (Fig. 3 A1-2). Downregulation of Foxp3 gene was not observed for the control group (Ctrl), stressed mice (Str), and rapamycin-treated mice (Rapa). In the spleen samples, significant alteration in gene expression was not detected for all groups of the study.



**Fig. 2.** Body mass of mice on days 1, 10, and 13 of the experiment (5 mice per group). The weight of all groups was not with a statistically significant dropping among days 1 and 13. Ctrl, Control; Pro, propranolol; Rapa, rapamycin; Str, restraint stress.



**Fig. 3.** Effect of restraint stress and rapamycin on the expression of PD1, PD-L1, and Foxp3 genes (5 mice per group). Relative expression of (A1) PD-L1, (A2) Foxp3, and (A3 and A4) PD1 genes in the brain. Restraint stress suppressed the expression of the Foxp3 gene but not for PD1 and PD-L1 genes in the brain of the mice treated with rapamycin. Rapamycin suppressed PD-L1 gene expression in the brain which reverted by restraint stress. The significant alteration in the gene expression of (B1) PD-L1, (B2) Foxp3, and (B3 and B4) PD1 was not observed in spleen samples of all assay groups. \* $P < 0.05$  Indicates significant differences compared to the control group and # $P < 0.01$  vs Rapa/Str. Foxp3, Forkhead box protein P3; PD1, programmed cell death protein-1; PD-L1, ligands of PD1; Pro, propranolol; Rapa, rapamycin; Str, restraint stress.

### DISCUSSION

This experiment was designed to determine the impacts of rapamycin, as an immunosuppressive drug, and stress on

regulatory T cells of the brain and spleen. Using daily restraint stress in the mouse model, the expression of PD1/PD-L1 genes, as an inhibitory signal, and Foxp3, as an important transcription factor of regulatory T cells was

assayed. Significant changes in the expression of PD-L1 and Foxp3 genes were observed in mice receiving rapamycin and mice receiving rapamycin with exposure to the restraint stress, respectively. Predictively, the weight loss was observed in the mice exposed to the stress in Str and Rapa/Str groups, but for Pro/Str, propranolol may inhibit this weight loss manifestation. The visually observed weight loss was not statistically significant for all groups of the study.

In the stress condition, the downregulation of PD-L1 expression was induced by rapamycin restored. Different cell signaling pathways that are induced by stressors might overcome the effect of rapamycin on the expression of PD-L1.

Based on the energy conservation theory, acute stress leads to an increase in the function of innate immunity and the suppression of adaptive immunity (21). Challenging this theory, firstly in the body, adaptive and innate immunity are coordinated with each other and in some cases they utilized shared components and cells, secondly, all immunosuppression mechanisms are not without energy consumption, and finally adaptive immunity are not the only component to respond to foreign antigens that innate immunity was failed to overcome on (9). The effects of chronic stress on the immune system were evaluated in several studies. It was determined that chronic stress suppresses natural killer cells activity, leukocyte proliferation, skin autograft, viral-specific T cells, and anti-mycobacterium activity of macrophages (10,22-25). According to the results of quantitative gene expression, no significant alteration in PD1 and Foxp3 gene expression (genes involving in the PD1+ regulatory cells) was detected in mice with or without stress but rapamycin decreased the expression of PD-L1 in the brain and stress condition, this drug decreased the expression of Foxp3 gene.

In the previous studies, different effects of rapamycin on the T-reg cells were reported. Prolonged treatment of rapamycin led to an increase in the thymic generation of CD4+ Foxp3+ T cells (26). The results taken from the expression of Foxp3 in this study showed that the co-administration of rapamycin and stress

might interfere with the tolerogenic function of T-reg cells in the brain. According to the role of T-reg cells in the pathogenesis of several CNS disorders such as multiple sclerosis and Alzheimer's disease (27,28), the implementation of the results would be instrumental in studies of CNS autoimmune diseases. On the other hand, rapamycin down-regulated the expression of PD-L1 in the brain, and this decrease was reverted by restraint stress. As the PD1/PD-L1 pathway is one of the most important signals in the maintenance of peripheral tolerance and inhibiting from autoimmune reactions, the obtained results support the immunosuppressant effect of stress on adaptive immunity (29). In another study, it was demonstrated that PD1 blockade in combination with rapamycin inhibitors restrains intrinsic PD1- and mTOR-signaling in hepatocellular carcinoma which leads to a decrease in the hepatoma progression (30). This finding, as a proof of principle, supported the correlation between PD1- and mTOR-signaling in the non-immune cells.

According to the gene expression profile, no significant alteration in the expression for all assayed genes was observed in the spleen as well as for PD1 in the brain. The lack of changes could be explained as a reason as brain cells might more be affected than other cells from the stress condition and brain collected samples are might better to indicate changes in the gene expression. In a similar study by Buidu and colleges, three types of stress stimulators (acute and chronic restrain and social isolation) were conducted to evaluate the effect of stress on the progression of tumor growth (31). In their results, significant stress-related changes in the primary tumor volume were not detected. It should be mentioned that the result obtained from animal models of stress might not completely be translated into the human ones, particularly for the mature and aged individuals who are much affected by life emotional and environmental factors in the different times of their life.

## **CONCLUSION**

Collectively, in this study, we demonstrated different effects of stress on the expression of

tolerogenic genes (downregulation effect for Foxp3 and upregulation effect for PD-L1), the results might be related to different regulating mechanisms involved in the expression of these two genes. Rapamycin decreased the expression of PD-L1 gene in the brain; the results can be considered as an immunotherapeutic effect of rapamycin. This effect of rapamycin can be more investigated in *in vivo* and in preclinical experimental models of challenges related to the PD1 signal such as cancer immunotherapy investigations.

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### Conflict of interest statement

The authors declared no conflict of interest in this study.

### Authors' contribution

M. Khedri conceived the study, performed experiments, analyzed the data, and wrote the paper. R.A. Taheri provided intellectual guidance and edited the paper. H. Kooshki edited the paper and provided useful comments.

## REFERENCES

1. Abbas A, Lichtman A, Pillai S. Cellular and Molecular Immunology. 7<sup>th</sup> ed. Elsevier; 2012. pp: 203-224,238,239.
2. Ccapece D, Verzella D, Fischietti M, Zazzeroni F, Alesse E. Targeting costimulatory molecules to improve antitumor immunity. *J Biomed Biotechnol.* 2012;2012:926321,1-17  
DOI: 10.1155/2012/926321.
3. Khedri M, Rafatpanah H, Abnous K, Ramezani P, Ramezani M. Cancer immunotherapy *via* nucleic acid aptamers. *Int Immunopharmacol.* 2015;29(2):926-936.  
DOI: 10.1016/j.intimp.2015.10.013.
4. Hamanishi J, Mandai M, Matsumura N, Abiko K, Baba T, Konishi I. PD-1/PD-L1 blockade in cancer treatment: perspectives and issues. *Int J Clin Oncol.* 2016;21(3):462-473.  
DOI: 10.1007/s10147-016-0959-z.
5. Kamada T, Togashi Y, Tay C, Ha D, Sasaki A, Nakamura Y, *et al.* PD-1+ regulatory T cells amplified by PD-1 blockade promote hyperprogression of cancer. *Proc Natl Acad Sci.* 2019;116(20):9999-10008.  
DOI: 10.1073/pnas.1822001116.
6. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 2003;299(5609):1057-1061.  
DOI: 10.1126/science.1079490.
7. Dhabhar FS, Mcewen BS. Acute stress enhances while chronic stress suppresses cell-mediated immunity *in vivo*: a potential role for leukocyte trafficking. *Brain Behav Immun.* 1997;11(4):286-306.  
DOI: 10.1006/brbi.1997.0508.
8. Reiche EMV, Nunes SOV, Morimoto HK. Stress, depression, the immune system, and cancer. *Lancet Oncol.* 2004;5(10):617-625.  
DOI: 10.1016/S1470-2045(04)01597-9.
9. Dhabhar FS. Effects of stress on immune function: the good, the bad, and the beautiful. *Immunol Res.* 2014;58(2-3):193-210.  
DOI: 10.1007/s12026-014-8517-0.
10. Brown DH, Zwilling BS. Activation of the hypothalamic-pituitary-adrenal axis differentially affects the anti-mycobacterial activity of macrophages from BCG-resistant and susceptible mice. *J Neuroimmunol.* 1994;53(2):181-187.  
DOI: 10.1016/0165-5728(94)90028-0.
11. Rezapour-Firouzi S, Shahabi S, Mohammadzadeh A, Tehrani AA, Kheradmand F, Mazloomi E. The potential effects of hemp seed/evening primrose oils on the mammalian target of rapamycin complex 1 and interferon-gamma genes expression in experimental autoimmune encephalomyelitis. *Res Pharm Sci.* 2018;13(6):523-532.  
DOI: 10.4103/1735-5362.245964.
12. Rezapour-Firouzi S, Kheradmand F, Shahabi Sh, Tehrani AA, Mazloomi E, Mohammadzadeh A. Regulatory effects of hemp seed/evening primrose oil supplement in comparison with rapamycin on the expression of the mammalian target of rapamycin-complex 2 and interleukin-10 genes in experimental autoimmune encephalomyelitis. *Res Pharm Sci.* 2019;14(1):36-45.  
DOI: 10.1403/1735-5362.251851.
13. Li J, Kim SG, Blenis J. Rapamycin: one drug, many effects. *Cell Metab.* 2014;19(3):373-379.  
DOI: 10.1016/j.cmet.2014.01.001.
14. Hunzeker J, Padgett DA, Sheridan PA, Dhabhar FS, Sheridan JF. Modulation of natural killer cell activity by restraint stress during an influenza A/PR8 infection in mice. *Brain Behav Immun.* 2004;18(6):526-535.  
DOI: 10.1016/j.bbi.2003.12.010.
15. Le CP, Nowell CJ, Kim-Fuchs C, Botteri E, Hiller JG, Ismail H, *et al.* Chronic stress in mice remodels lymph vasculature to promote tumour cell dissemination. *Nat Commun.* 2016;7:10634,1-14.  
DOI: 10.1038/ncomms10634.
16. Chen J, Joyal JS, Hatton CJ, Juan AM, Pei DT, Hurst CG, *et al.* Propranolol inhibition of  $\beta$ -adrenergic receptor does not suppress pathologic neovascularization in oxygen-induced retinopathy. *Invest Ophthalmol Vis Sci.* 2012;53(6):2968-2977.  
DOI: 10.1167/iovs.12-9691.

17. Leontieva OV, Paszkiewicz GM, Blagosklonny MV. Comparison of rapamycin schedules in mice on high-fat diet. *Cell Cycle*. 2014;13(21):3350-3356. DOI: 10.4161/15384101.2014.970491.
18. Zhou Y, Dai W, Lin C, Wang F, He L, Shen M, *et al*. Protective effects of necrostatin-1 against concanavalin A-induced acute hepatic injury in mice. *Mediators Inflamm*. 2013;2013:706156,1-15. DOI: 10.1155/2013/706156.
19. Banelos J, Shin S, Cao Y, Bochner BS, Morales-Nebreda L, Budinger GRS, *et al*. BCL-2 protects human and mouse Th17 cells from glucocorticoid-induced apoptosis. *Allergy*. 2016;71(5):640-650. DOI: 10.1111/all.12840.
20. He Y, Zha J, Wang Y, Liu W, Yang X, Yu P. Tissue damage-associated “danger signals” influence T-cell responses that promote the progression of preneoplasia to cancer. *Cancer Res*. 2013;73(2):629-639. DOI: 10.1158/0008-5472.CAN-12-2704.
21. Segerstrom SC, Miller GE. Psychological stress and the human immune system: a meta-analytic study of 30 years of inquiry. *Psychol Bull*. 2004;130(4):601-603. DOI: 10.1037/0033-2909.130.4.601.
22. Edwards EA, Dean LM. Effects of crowding of mice on humoral antibody formation and protection to lethal antigenic challenge. *Psychosom Med*. 1977;39(1):19-24. DOI: 10.1097/00006842-197701000-00003.
23. Bartrop R, Luckhurst E, Lazarus L, Kiloh LG, Penny R. Depressed lymphocyte function after bereavement. *Lancet*. 1977;309(8016):834-836. DOI: 10.1016/s0140-6736(77)92780-5.
24. Wistar R, Hildemann WH. Effect of stress on skin transplantation immunity in mice. *Science*. 1960;131(3394):159-160. DOI: 10.1126/science.131.3394.159.
25. Bonneau RH, Sheridan JF, Feng N, Glaser R. Stress-induced effects on cell-mediated innate and adaptive memory components of the murine immune response to herpes simplex virus infection. *Brain Behav Immun*. 1991;5(3):274-295. DOI: 10.1016/0889-1591(91)90023-4.
26. Coenen JJA, Koenen HJP, Van Rijssen E, Kasran A, Boon L, Hilbrands L, *et al*. Rapamycin, not cyclosporine, permits thymic generation and peripheral preservation of CD4+ CD25+ FoxP3+ T cells. *Bone Marrow Transplant*. 2007;39(9):537-545. DOI: 10.1038/sj.bmt.1705628.
27. Zozulya AL, Wiendl H. The role of regulatory T cells in multiple sclerosis. *Nat Clin Pract Neurol*. 2008;4(7):384-398. DOI: 10.1038/ncpneuro0832.
28. Dansokho C, Ait Ahmed D, Aid S, Toly-Ndour C, Chaigneau T, Calle V, *et al*. Regulatory T cells delay disease progression in Alzheimer-like pathology. *Brain*. 2016;139(Pt 4):1237-1251. DOI: 10.1093/brain/awv408.
29. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 2008;26:677-704. DOI: 10.1146/annurev.immunol.26.021607.090331.
30. Li H, Li X, Liu S, Guo L, Zhang B, Zhang J, *et al*. Programmed cell death-1 (PD-1) checkpoint blockade in combination with a mammalian target of rapamycin inhibitor restrains hepatocellular carcinoma growth induced by hepatoma cell-intrinsic PD-1. *Hepatology*. 2017;66(6):1920-1933. DOI: 10.1002/hep.29360.
31. Budiu RA, Vlad AM, Nazario L, Bathula C, Cooper KL, Edmed J, *et al*. Restraint and social isolation stressors differentially regulate adaptive immunity and tumor angiogenesis in a breast cancer mouse model. *Cancer Clin Oncol*. 2017;6(1):12-24. DOI: 10.5539/cco.v6n1p12.