Final report of project entitled "Effect of the food additive tBHQ on the immune system via xenosensors (OTKA-PD 108297)

Introduction:

In our body, the most important detoxifying organ is the liver, which plays major role in the removal of foreign substances (xenobiotics). In addition to metabolic pathways, it is becoming more and more evident, that entering xenobiotics parallel with the liver are also sensed by the immune system. If a xenobiotic enters to the cell, it activates xenosensing receptors (xenosensors). Since xenosensors are present also in immune cells, one can assume that these receptors upon activation with xenobiotics modulate immune response as well. This is supported by a fact, that immunotoxicity of many xenobiotics can be explained by their binding to xenosensors in immune cells e.g. TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) binding to AHR (arylhydrocarbon receptor). Studies with AHR knock-out mice showed, that TCDD itself is not responsible for the immunotoxic effect, but its activated xenosensor (AHR) is. TCDD-activated AHR in T cells displaces the balance of the autoreactive Th17 subpopulation responsible for autoimmune symptoms **[6*]**.

In our experiments we tested a phenolic compound called *tert*-butyl-hidroquinone (tBHQ, E319) for its immune effects. This molecule is used to preserve foodstuffs in the EU and the United States due to its antioxidant effect. As a preservative, it appears as a variety of oil-based foods, margarines, pastas, chips additives, as well as fragrances and cosmetics. According to the EU rules, maximum allowed tBHQ in the food is 200mg/kg (for certain special oils, for frozen fish it can be up to 1g/kg) and maximum daily intake is 0.7mg/kg. Since daily intake depends on eating habits, it is difficult to ascertain whether there is dose overdose in the population. tBHQ can activate various xenosensor mediated signalling pathways, including the aforementioned AHR and NRF2 (Nuclear factor (erythroid-derived 2)-like 2) pathways. Since these xenosensors beside metabolic and antioxidant processes, are also involved in the regulation of the immune system, it is also believed that this food component has immunmodulatory effect. In *in vivo* studies, found in the literature uses 1-2% tBHQ as a reference dose, so we used this concentration range in our experiments also. It is well-known that tBHQ activates both xenosensors (NRF2 and AHR). During our experiments we studied the effect of tBHQ on T cell differentiation and we also investigated the involvement of the mentioned nuclear receptors in T cell dependent immune responses.

Objectives:

During our research, the immunmodulatory effect of two xenosensors, namely NRF2 and AHR were investigated using tBHQ xenobiotic.

I. In our preliminary experiments, we examined whether the two xenobiotics (TCDD and tBHQ) affect the AHR / NRF2 pathway in heterogenic splenocytes and spleen-derived CD4+ cells and affect the differentiation of T cells.

II. In vivo studies:

1. We investigated the effect of per os administered tBHQ on the gene expression of splenic

* Numbers in square brackets identify publications/presentations of this project given in the final publications list and also in the reference list at the end of this report.

CD4+ cells using microarray experiments and RT-PCR.
2. In addition to the gene expression changes, the effect of oral tBHQ treatment was examined on the ratio of different T-cell populations and on the activation process of T cells.
III. In vitro experiments:
1. The effect of tBHQ on the Th17 and Treg cell differentiation was studied *in vitro*. Using specific AHR and NRF2 antagonists, we investigated the role of AHR and NRF2 xenosensors on tBHQ mediated cell differentiation.
2. Part of our in vitro studies focused on the effect of tBHQ on the extracellular vesicle.
3. Effect of tBHQ on autophagy was also studied.

Methods:

Animals: Male Balb/c mice (wild type, age between 12-13 weeks, 20-30g) were housed in normal light-cycle room, maintained on AIN 76-A semi purified diet (MP Biomedicals, Solon, OH, USA) or AIN-76-A diet mixed with tBHQ (1.5% w/w) for 20 days and allowed water and food *ad libitum*. After the treatment time, mice were sacrificed using CO_2 suffocation.

Transcriptomic analysis: Spleen of tBHQ treated and control animals were taken out and CD4+ cells were isolated with magnetic separation using autoMACS separator (Miltenyi Biotec, Germany). RNA derived from CD4+ cells was subjected to microarray analysis using Agilent platform. Microarray analysis was performed as it is published in **[1, 5, 8]**.
Microarray validation: Microarray data were validated with 47 genes using Taqman assays (Taqman Array Fast Plate, Applied Biosystems, USA).

Ex vivo cell characterisation experiments: Splenic CD4+ cells were isolated from *per os* treated and control mice with magnetic separation using AutoMACS separator (Miltenyi Biotec, Germany). After isolation, cells derived from tBHQ treated animals and cells derived from untreated mice were treated with brefeldin A and were split in two. One half of each group was subjected to 3h PMA/IONO activation (25ng/ml phorbol myristate acetate, 1μg/ml ionomycin respectively), other half was further processed without activation. Number of various T cell subsets was investigated with FACS analysis (FACS Calibur, Beckton Dickinson, USA).

Cell culture experiments: Isolated $CD4^+$ cells were differentiated with specific differentiation kits on anti-CD3 coated activation plates either into Treg (FlowCellect™ Mouse Treg Differentiation Tool Kit, FCIM025166, Merck, Millipore Germany) or Th17 cells (FlowCellect™ Mouse Th17 Differentiation Tool Kit FCIM025163, Merck, Millipore, Germany) in the presence/absence of 10 μM tBHQ, specific AHR inhibitor 6, 2, 4'-trimetoxi-flavone, (TMF, 1 μM) or specific NRF2 inhibitor trigonelline (TRIG, 1μM). The cells were analyzed by flow cytometry using FlowCellect™ Mouse Viable Treg characterization kit (FCIM025168, Merck Millipore, Germany) for Treg and FlowCellect™ Mouse Th1/Th17 Intracellular Cytokine kit (FCIM025168, Merck Millipore, Germany) for Th17 cells.

Western blot analysis of tBHQ treated CD4+ T cells for autophagy marker LC3-II: $CD4^+$ T cells were treated with 0.2, 2, 20 μM tBHQ for 24h. Cells were harvested in ProteoJET Mammalian Cell Lysis Reagent (Fermentas, USA) supplemented with protease and phosphatase inhibitors. Immunoblot analysis was carried out using primary (LC3A

Rabbit mAb, Cell Signaling, USA), secondary (Anti-rabbit IgG, HRP-linked Antibody, Cell Signalling, USA) antibodies and enhanced chemoluminescence system (Pierce ECL Plus, USA). The blots were scanned and the immune complexes were quantified with ChemiDoc Imaging System (Bio-Rad, USA).

Analysis of extracellular vesicles: A lymphoid cell line (CCRF-CEM, human T cell lymphoblast-like cell 4 line) was used to investigate the effect of tBHQ on the extracellular vesicle secretion. Cells were treated with 0.2, 2, or 20 μM tBHQ for 24 hours and secreted extracellular vesicles were analyzed from the supernatant media. Microvesicular and exosomal fraction was separated as it was published **[4]**. The size distribution and concentration of the isolated microvesicles/exosomes were determinated by qNANO instrument (IZON, New Zeeland).

Results:

I. The results of preliminary experiments designed to select xenobiotics. Intraperitoneal treatment both with 50μg/kg of TCDD or 30mg/kg tBHQ for 3 consecutive days caused significant changes in CD4+ fractions. Since immune effects of tBHQ are not well known and it is an important food additive, we used this molecule in further experiments.

II. Results of transcriptomic studies after per os tBHQ treatment. Using Agilent cDNA microarray platform we found significant gene expression changes in case of 269 genes (max fold change=30; minimum fold change=0.1; median 1.7, $_{nom}p<0.05$). The microarray data were validated with Taqman plates for 47 genes (correlation coefficient $r^2= 0.089$). Network analysis with GSEA (gene set enrichment analysis) for KEGG pathways and ORA (over representation analysis) for other mouse data sets showed a significant change for terms connected with immune system/immune regulation (Figure 1.) **[2, 3, 7, 9, 10, 11]**.

III. Results of *ex vivo* cellular studies after per os tBHQ treatment. The number of Th1, Th2, Th17 and Treg cells derived from *per os* treated and control animals was analysed by flow cytometry using specific cell surface and intracellular markers. Splenic CD4+ cells of both the tBHQ treated and control animals were divided into two and were activated for short term (3h) with PMA/IONO or left not activated. Thus, four test groups were generated; tBHQ-untreated and not activated (C); tBHQ not treated, but activated (C_PMA/IONO Treated with tBHQ but not activated (tBHQ); and tBHQ-treated and activated (tBHQ, PMA/IONO) groups.

Our results show that the number of CD4+ cells (Th1, Th2, Th17 and Treg) originated from control animals did not change significantly after short term PMA/IONO activation. In case of CD4+ cells isolated from tBHQ treated animals, both the number of Th1, Th2 or Th17 cells were significantly elevated after activation with PMA/IONO compared to treated, not activated samples (Figure 2A/B/C). In case of Tregs a significant decrease was measured after PMA/IONO activation in the cell count compared to treated, not activated group (Figure 2D). Comparing the CD4+ cells from untreated control and tBHQ treated animals, only changes in the Th2 and Treg numbers were observed. Based on these, it can be concluded that treatment with tBHQ increases significantly the activation sensitivity of CD4+ cells and reduce response time for activation stimulus compared to cells from untreated animals (Figure 2) **[11, 12]**.

IV. Studying the involvement of AHR and NRF2 receptors in tBHQ effects using *in*

***vitro* cell culture.**

AHR and NRF2 have synergistic effect on Th17 differentiation: In the absence of inhibitors, tBHQ-treated (tBHQ) samples showed a significant increase in Th17 cell counts compared to the control. In comparison to the increased Th17 cell count (tBHQ), a significantly lower Th17 cell count was measured in both the TMF (tBHQ + TMF), trigonelline (tBHQ + TRIG) or samples treated with both inhibitors (tBHQ + TRIG + TMF). tBHQ therefore increases the number of Th17 cells, but this effect is inhibited by blocking either the AHR or the NRF2 pathway. Thus, in tBHQ mediated Th17 differentiation, both xenosensors (NRF2, AHR) play role and have synergistic effect on cells (Figure 3B).

AHR and NRF2 have antagonistic effect on Treg differentiation: The number of Treg cells decreased significantly with tBHQ (tBHQ) compared to untreated control (C). However, if AHR was inhibited by TMF (tBHQ + TMF) this effect was absent. The opposite effects were observed with the NRF2 inhibitor trigonelline (tBHQ + TRIG). The presence of this inhibitor caused even greater decrease in cell count than tBHQ (tBHQ) inhibitor alone. Thus, it can be concluded that NRF2 also plays role in mediating the tBHQ effect, but its effect is opposite to AHR, since it stimulates Treg differentiation (Figure 3A) **[2, 3, 7, 9, 10, 11].**

V. The effect of tBHQ on autophagocytosis. The effect of tBHQ on phagocytosis was investigated by Western blot technique using LC3-II autophagosome marker. CD4+ cells were treated in culture with 0.2μM, 2μM or 20 μM tBHQ and after 24h immunoblot analysis was carried out for LC3-II protein. LC3-II expression showed a concentration-dependent increase compared to β-actin.

VI. Effect of tBHQ on extracellular vesicle secretion. To test whether tBHQ modulates extracellular vesicle secretion by immune cells, CCRF-CEM cells (human T *cell* lymphoblast-like *cell* 4 line) were treated with this substance for 24h and vesicles were prepared as described **[5]**. Our results measured with resistive pulse sensing (IZON, New Zealand) show a concentration dependent change of vesicle (exosome) secretion between 0.2 μM, and 20 μM, with a maximum at 2 μM.

Summarizing our results, we demonstrated that:

1. Long-term (20 days), *per os* administered tBHQ induces the gene expression of 269 genes and many of them have role in immune or metabolic processes.
2. Long-term, *per os* administered tBHQ increases the activation sensibility of CD4+ T cells;

   2a. number of effector T cells (Th1, Th2,Th17) increases rapidly and immediately (in 3 hours) after the beginning of activation, in the contrary to T cell originated from control animals, where no significant increase was measured upon activation in this time frame.

   2b. number of Treg cells decreases rapidly under the same conditions compared to the control cells, where no change was observed in this time frame.
3. Based on our in vitro studies:

   3a. both NRF2 and AHR play role in the transmission of tBHQ effects in CD4+ T cells

   3b. Also tBHQ modulates extracellular vesicle secretion in concentration dependent

manner

3c. tBHQ increases expression of LC3 II autophagocytosis marker in CD4+ cells

Figure 1. Network analysis of significantly enriched gene sets after per os tBHQ treatment in BALB/c mice. Blue and red circles represent down-and upregulation of the associated KEGG pathways, respectively (p<0.05, false discovery rate <0.25).

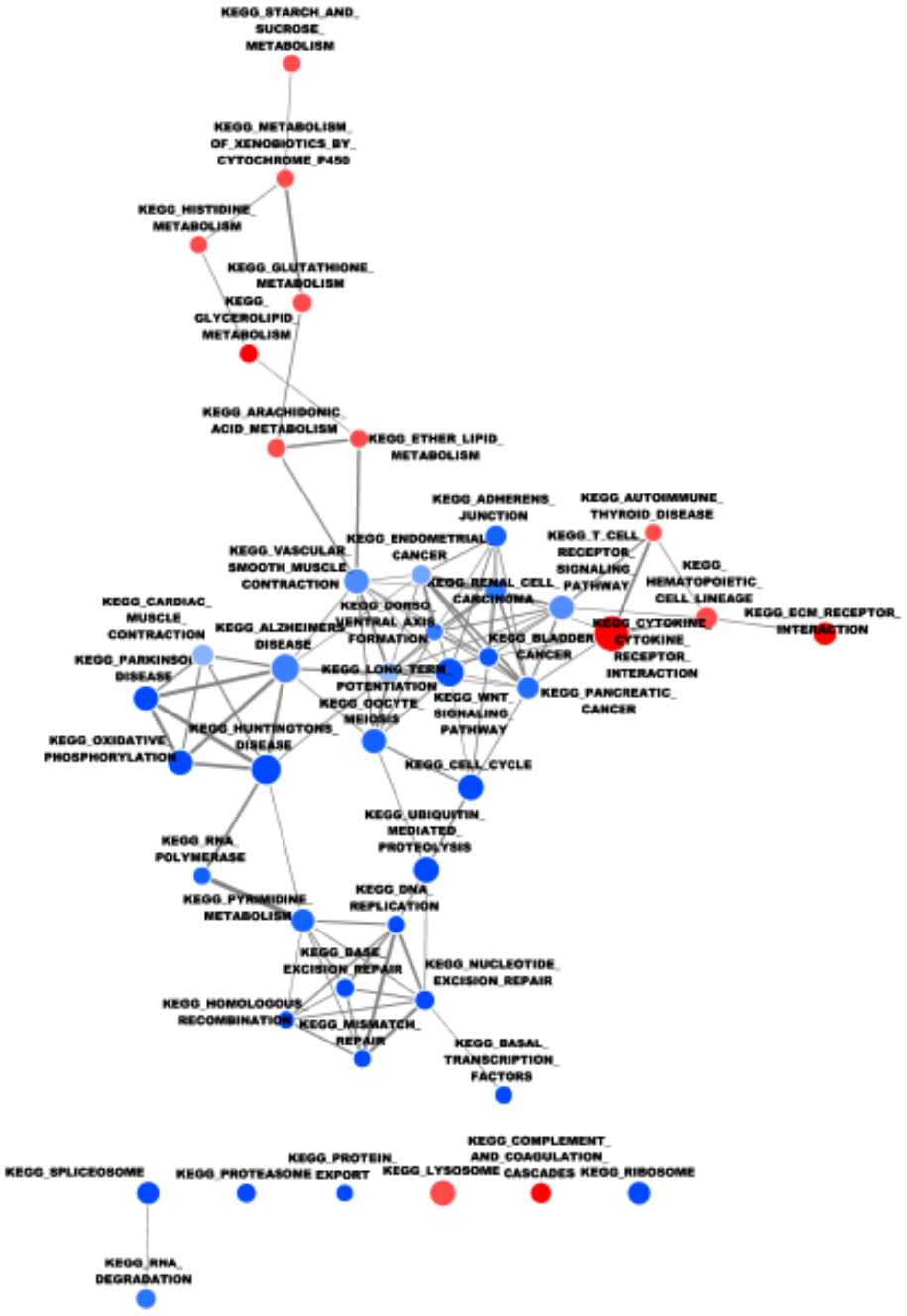


Figure 2. Activation of CD4$^+$ T cells after *per os* treatment with tBHQ. Splenic CD4+ cells were isolated from tBHQ treated or control BALB/c mice. Control and tBHQ treated cells were activated for 3 hours either with PMA/Ionomycin, or were further processed without activation and number of Th1 (A), Th2 (B), Th17 (C) or Treg (D) cells were determined by FACS analysis

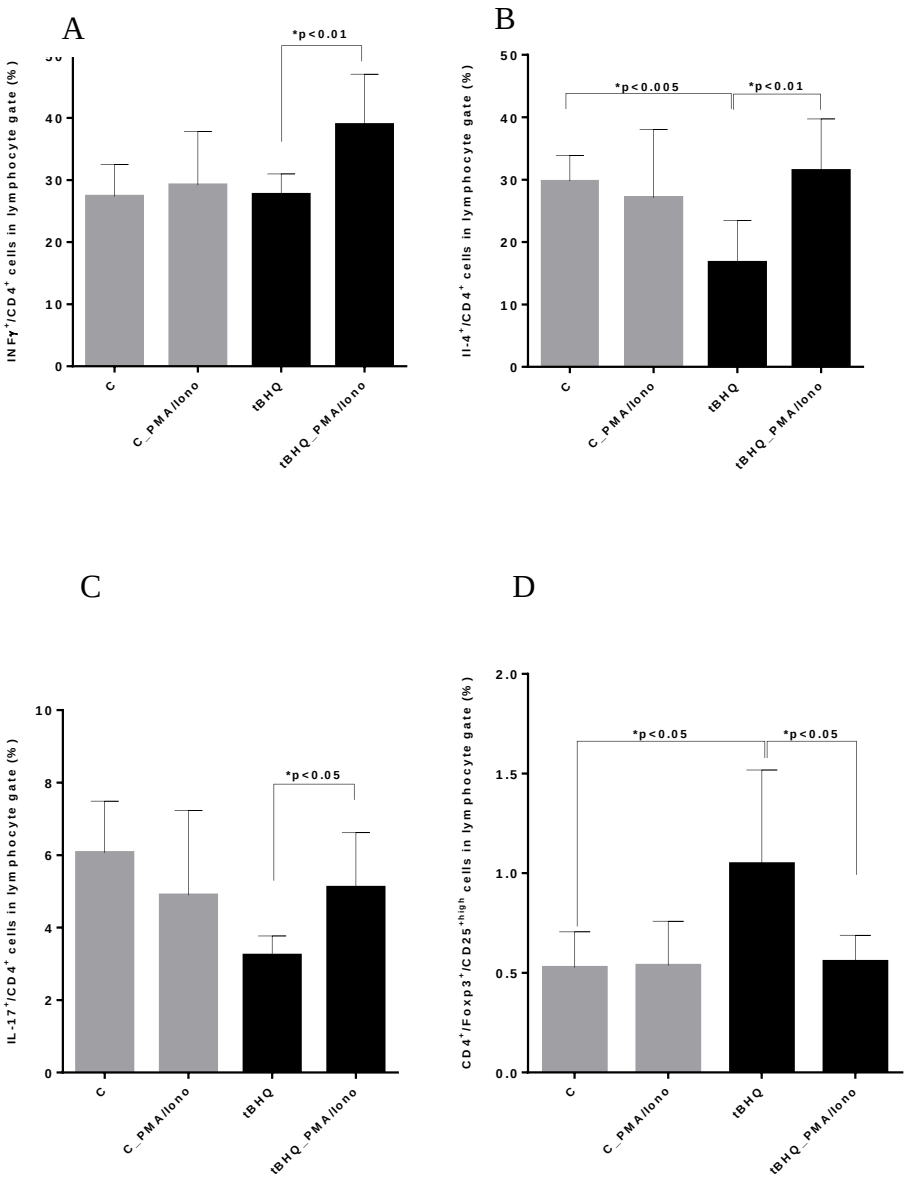
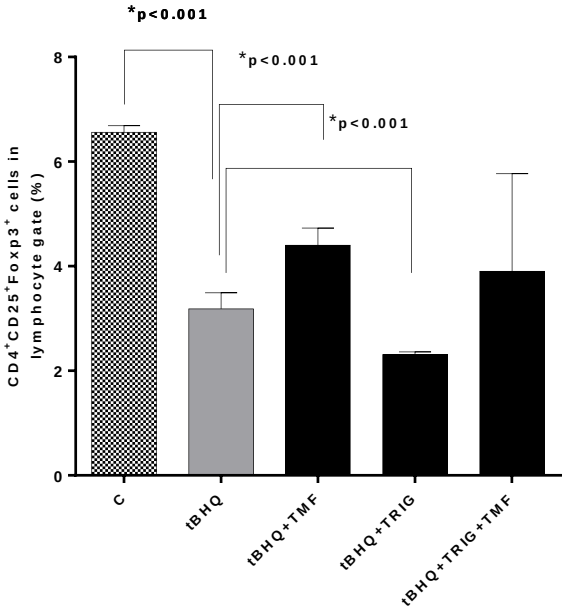


Figure 3. Involvement of AHR and NRF2 receptors in Th17 and Treg activation after tBHQ treatment. Splenic $CD4^+$ cells were differentiated either into Treg (A) or Th17 (B) cells in the presence/absence of 10 μM tBHQ, 1 μM TMF or 1 μM TRIG. Th17 or Treg cell number was determinated with FACS analysis ($p<0.05$).

A



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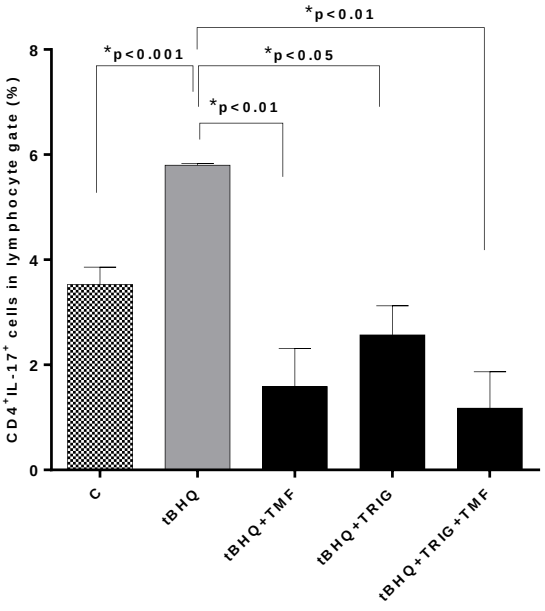

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2. Békés Márta: ***Xenoszenzorok hatása az immunsejtek differenciációjára***, Orvosképzés, (90):1, p200, 2015
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7. Békés Márta Petschner Péter, Fekete Nóra, Pállinger Éva, Pálóczi Krisztina, Buzás Edit, Tamási Viola: ***Effect of xenosenzors on the differentiation of immune cells***, Magyar Immunológiai Társaság 45. Vándorgyűlésére, 2016
8. Petschner P, Juhasz G, Tamasi V, Adori C, Tothfalusi L, Hökfelt T, Bagdy G: ***Chronic venlafaxine treatment fails to alter the levels of galanin system transcripts in normal rats***, Neuropeptides., 2016
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10. Békés Márta: ***AHR és NRF2 xenoszenzorok T-sejt differenciációra gyakorolt hatása***, Orvosképzés, (92):1, p177, 2017
11. Békés Márta: ***AHR and NRF2 xenoszenzorok T-sejt differenciációra gyakorolt hatása***, Orvosképzés, (92):2, p383, 2017

Student activities, prizes of the project

Márta Békés:

2017 TDK (I. prize)

OTDK (II. prize)

Rector's competition: "tBHQ szerepe a T-sejtek differenciációjában" (ongoing ranking)

Diploma work: „tBHQ szerepe a T-sejtek differenciációjában" (grade 5 (excellent)

2016 TDK (I. prize)

OTDK (presentation)

Rector's competition: „Xenoszenzorok szerepe az immunsejtek differenciációjánan" (Rector's praise)