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Review of the doctoral dissertation by M.Sc. Leila Khani Khouzani entitled "Functional outcomes of flame retardant action in immune cells" conducted under the supervision of Łukasz Pułaski, PhD, DSc, Prof. IBM PAS

I. Scope of the Dissertation

The subject of the doctoral dissertation submitted for review concerns the consequences of the action of flame retardants (FRs) on immune system cells. Although it is currently well established that flame retardants significantly affect both animal and human organisms, their impact on cells of the immune system remains insufficiently characterized. Therefore, this dissertation – structured as a collection of publications, aims to address this existing research gap.

In two published articles (one review, IF^{2023} = 8.2, MEiN points = 200; and one original research paper, IF^{2025} = 8.0, MEiN points = 200) and two manuscripts (both original research articles, IF^{2025} = 9.7, MEiN points = 140; and IF^{2025} = 6.9, MEiN points = 140), the doctoral candidate investigates the immunotoxic effects of tetrabromobisphenol A (TBBPA), bisphenol A (BPA), octabromodiphenyl ether (DecaBDE), tris(2-butoxyethyl) phosphate (TBEP), and tricresyl phosphate (TCP). The dissertation presents a coherent thematic scope and aligns well with the proposed title.

The first article (Leila Khani, Leonardo Martin, Łukasz Pułaski, Cellular and physiological mechanisms of halogenated and organophosphorus flame retardant toxicity, Science of the Total Environment 2023, 897:165272. DOI: 10.1016/j.scitotenv.2023.165272)

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is a comprehensive review of the toxicity of both halogenated and organophosphorus FRs in animal organisms and human cells. The author highlights that FRs – once considered relatively non-toxic, are now known to exert adverse effects on numerous biological processes. The doctoral candidate appropriately emphasizes the cellular-level mechanisms of FR action, many of which arise from their pronounced lipophilicity. The review describes how FRs disrupt membrane structure and key elements of intracellular signaling pathways. It then outlines how these disturbances manifest at the organismal level, contributing to hormonal dysfunction, metabolic disturbances, and negative effects on reproductive and immune system function. In summary, the publication provides an extensive synthesis of current knowledge on FR toxicity, presenting shared mechanisms across structurally diverse compounds. The article stands out due to its comprehensive nature – reviews that integrate both major FR classes and describe their effects across multiple biological levels are uncommon. The candidate correctly notes that, despite their chemical diversity, different FRs often target similar biological processes. This holistic presentation of current evidence, combined with identification of key knowledge gaps, constitutes a valuable and original contribution to the field and provides an excellent introduction to the candidate's subsequent experimental work.

The second article (Leila Khani, Maciej Studzian, Leonardo Martins, Michał Gorzkiewicz, Łukasz Pułaski, Tetrabromobisphenol A, but not bisphenol A, disrupts plasma membrane homeostasis in myeloid cell models – A novel threat from an established persistent organic pollutant, Science of the Total Environment 2025, 961:178284. DOI: 10.1016/j.scitotenv.2024.178284) is an original research paper comparing the effects of TBBPA and BPA on physicochemical membrane properties in immune-system–derived cell lines such as HL-60, THP-1, and Mono Mac 6. The key finding is that exposure to TBBPA unlike BPA, disturbs plasma membrane homeostasis. TBBPA markedly increases both rotational and lateral lipid mobility in the plasma membrane, enhancing membrane fluidity while reducing overall lipid order and polarity. Interestingly, an opposite trend was observed in the internal membranes of the same cells. In contrast to TBBPA, BPA at comparable concentrations exhibited no significant effects on membrane properties. The author concludes that the plasma membrane of macrophages and other immune cells may represent

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a previously underappreciated target of TBBPA toxicity. This finding is important because it reveals a novel mechanism of environmental toxicity for TBBPA, one based on disruption of membrane biophysics in immune cells. Although the literature contains reports suggesting possible membrane-related effects of TBBPA, no previous study has directly compared TBBPA with BPA while providing a deeper mechanistic analysis.

The third work submitted for review, a manuscript (Leila Khani, Maciej Studzian, Leonardo Martin, Lukasz Pulaski, The brominated flame retardant DecaBDE inhibits low-density lipoprotein macropinocytosis in human M2 macrophages, Archives of Toxicology – manuscript under review), investigates the effects of widely used brominated flame retardants (BFRs) including DecaBDE, TBBPA, and hexabromocyclododecane (HBCD) on lipid metabolism in primary human macrophages. The author demonstrates that at 5 μM, both DecaBDE and HBCD strongly inhibit LDL uptake by M2 macrophages. The candidate suggests that this effect is not due to direct inhibition of LDL endocytic channels but rather results from altered expression of genes encoding elements of the macropinocytosis pathway and interference with kinases PI3Kγ, Akt3, WNK1, and SGK1. Experiments revealed that DecaBDE inhibits uptake of large particles characteristic of macropinocytosis while having no effect on receptor-mediated endocytosis or bacterial phagocytosis. Compared with other BFRs, HBCD showed a weaker effect and TBBPA showed none. In summary, the author demonstrates that DecaBDE can interfere with lipid homeostasis by suppressing an alternative pathway of cholesterol uptake in macrophages. This finding is relevant to atherosclerosis and lifestyle diseases associated with lipid accumulation. Literature reports indicate that DecaBDE may promote foam-cell formation through increased TLR4 expression and LDL uptake; however, the candidate's research complements this by showing that DecaBDE simultaneously inhibits the macropinocytic pathway of LDL internalization. Thus, the study presents an original mechanism of immunotoxic action of BDE-209, expanding existing knowledge. Particularly innovative is the examination of DecaBDE's impact on macropinocytosis in immune cells – a phenomenon not previously described.

The fourth manuscript (Leila Khani, Maciej Studzian, Leonardo Martin, Lukasz Pulaski, TBEP and TCP impair metabolic and immune functions in human macrophages: a novel redox-related activity with potential immunotoxic consequences, Environment

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International – manuscript under review) investigates the effects of two organophosphorus flame retardants, TBEP and TCP, on the metabolic and immune functions of human macrophages. Contrary to the commonly held view that FRs exert pro-oxidative effects, the author shows that TBEP and TCP decrease the production of reactive oxygen species (ROS) in primary human macrophages. This effect occurs rapidly and is not attributable to direct ROS scavenging or to increased expression of antioxidant enzymes. The candidate's experiments demonstrate that TBEP and TCP do not inhibit NADPH oxidase activity but instead induce mitochondrial membrane hyperpolarization without altering superoxide levels within the mitochondrial matrix. These disturbances resulted in pronounced impairment of key macrophage functions. TCP halved cytoplasmic ATP levels and nearly abolished extracellular hydrogen peroxide release. While bacterial phagocytosis remained unaffected, TCP drastically reduced the macrophages' ability to kill internalized *Staphylococcus aureus*. These effects occurred at micromolar concentrations relevant to real-world exposure. The findings highlight a novel immunotoxic mechanism of TBEP and TCP involving disruption of redox homeostasis and impairment of macrophage antimicrobial functions. The literature lacks previous reports describing such effects of TBEP or TCP on immune cells. The observation that these compounds exert antioxidative effects in macrophages; therefore, represents an original contribution. The author correctly notes that this mechanism has not been previously described for the FRs examined.

II. Assessment of the Doctoral Candidate's Theoretical Knowledge

The dissertation of MSc Leila Khani Khouzani presents an extensive literature review on flame retardants (publication no. 1). The author thoroughly describes the characteristics of FRs, their chemical classification, intended purpose, and the scale of their production and environmental contamination. The doctoral candidate outlines the main categories of FRs (brominated, organophosphorus, etc.) and provides examples (e.g., DBDE, HBCD, TBBPA, TCP), demonstrating both the relevance and reliability of her theoretical background.

In the section devoted to the immune system, the author explains its functions and emphasizes the importance of maintaining balance among them for human health (publication

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no. 1). The dissertation correctly notes that the impact of FRs on the immune system remains poorly understood, which justifies the research questions posed. The broad inclusion of the most recent scientific sources and the clear placement of the topic within the context of immunotoxicity confirm the candidate's solid and comprehensive theoretical knowledge.

III. Assessment of the Candidate's Ability to Conduct Independent Scientific Research

The doctoral candidate presented a clearly formulated research problem and objective – namely, to investigate the effects of various FRs on immune system cells. In my opinion, the selection of experimental models was well justified. The candidate conducted experiments both on immune-cell lines (HL-60, THP-1, Mono Mac 6) and on macrophages isolated from human donors. Although the concentrations used were relatively high, they remain sufficiently close to levels detectable in the environment.

The application of appropriate controls (DMSO solvent control) and reference compounds (e.g., comparison of TBBPA with BPA) reflects methodological rigor. The author employed advanced analytical techniques, and it is evident that she possesses extensive proficiency in modern research methodologies.

In publication 2, MSc Leila Khani Khouzani used a broad array of fluorescence-based methods. In manuscript 1 submitted for review, she performed endocytosis measurements, whereas in manuscript 2 she conducted assays of ROS production, mitochondrial membrane potential, ATP levels, and macrophage bactericidal activity.

I consider the data analysis to have been performed using appropriate statistical approaches, with clear attention to consistency with the proposed hypotheses. For example, inhibition of LDL uptake by DecaBDE was demonstrated using multiple independent assays, and the candidate employed a wide range of macropinocytosis inhibitors.

The experimental design, comprehensive data interpretation, and well-supported conclusions confirm the doctoral candidate's capability for independent scientific research.

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IV. Assessment of the Originality of the Scientific Contribution

The dissertation contains numerous original findings, several of which were highlighted in Section I of this review. Notably innovative is the application of advanced biophysical methods in immunotoxicology to study how FRs affect cellular structures. The candidate's research demonstrated that TBBPA specifically increases lipid mobility in the plasma membrane of monocytes and decreases membrane polarization – effects not observed for the structurally similar BPA.

In manuscript 1 (the third work submitted for evaluation), the phenomenon of DecaBDE- and HBCD-induced inhibition of LDL macropinocytosis in M2 macrophages was described for the first time. The resulting findings have meaningful implications from a biomedical perspective.

Another significant innovation of the dissertation is the demonstration that certain organophosphorus FRs (TBEP and TCP) act in an atypical manner, particularly by reducing ROS production in macrophages through a mechanism associated with mitochondrial hyperpolarization. In particular, TCP markedly decreases intracellular ATP levels and hydrogen peroxide production while simultaneously impairing the macrophages' ability to kill internalized bacteria. These results open new research perspectives regarding immunotoxic factors associated with FR exposure.

Based on my expertise as a reviewer and on the publications and manuscripts submitted for evaluation, I conclude that the candidate's findings are original and significantly advance the understanding of FR mechanisms of action.

V. Comments, Questions, and Suggestions

A general remark regarding the entire set of works submitted by MSc Leila Khani Khouzani is that all presented publications and manuscripts are very well prepared visually, and the included graphical schemes significantly facilitate understanding of the discussed findings.

A general methodological suggestion for future research on FRs is that the doctoral candidate should consider using culture media without phenol red and serum depleted of steroid hormones (CH-FBS, charcoal-stripped fetal bovine serum). Phenol red is a known

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estrogenic compound. Moreover, standard serum is rich in various steroid hormones, and since FRs are well-established endocrine-disrupting chemicals (EDCs), such background levels of hormones may influence experimental outcomes. This is particularly relevant in studies involving molecular pathways that may be influenced by estrogens or androgens, such as PPARs, AhR, WNT, and others.

For the mechanisms investigated in the present dissertation, this issue is not critical, as they are largely independent of steroid hormones. Additionally, immune cells express very low levels of estrogen and androgen receptors. Nevertheless, I emphasize this point in case the candidate plans to continue research on deeper molecular pathways or on other cell types.

Below I provide detailed comments and suggestions for each publication and manuscript.

Publication 1

For publication 1, which is a review of studies on FRs, I have no major remarks. I consider the selection of literature appropriate. Within the broader context of the dissertation, the inclusion of a review focused on the immune system is clearly justified; however, I am curious why the second major system discussed is the reproductive system. Could the candidate provide a rationale for this choice?

Publication 2

• In publication no. 2, the candidate conducted experiments exclusively on leukemic cell lines (HL-60, THP-1, MM6). Were similar experiments on non-transformed, primary cells considered, or are such studies planned for the future?

• The candidate investigated the effects of TBBPA using fluorescent probes. In my 2016 study (Szychowski KA et al., *Environ Sci Pollut Res Int.* 2016;23(12):12246–52), I demonstrated that the fluorescent probe H_2DCFDA spontaneously reacts with TBBPA, producing fluorescence even in the absence of cells. Did the candidate verify possible interactions between TBBPA and the fluorescent probes used in her experiments?

• The concentration of 25 μM used in the study is relatively high, although justified in light of Figures 2, 3, and 4. Nevertheless, could the candidate comment on the concentration ranges detected in the human organism? What is the biologically relevant range?

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• In future studies, the candidate may consider investigating the effects of TBBPA on cholesterol synthesis and metabolism, as this may influence the organization of lipid microdomains and help explain the mechanism underlying increased membrane fluidity.

Manuscript 1

• I believe that the manuscript could reference earlier studies reporting enhanced lipoprotein uptake induced by DecaBDE in a TLR4-dependent manner. Could the candidate comment on the findings by Zhi et al.? (Hui Zhi et al., *Food Chem Toxicol.* 2018;121:367–373.)

• The concentration of 5 μM used in the experiments does not appear excessive; however, was its potential cytotoxicity evaluated? Were optimization studies performed, or was the concentration based on previous experiments?

• Only mRNA expression analysis was performed. Drawing strong mechanistic conclusions based solely on transcript levels is controversial, as mRNA does not necessarily reflect protein abundance. Does the candidate plan to perform protein-level validation in future studies?

Manuscript 2

• As in publication 2, did the candidate check whether the tested compounds interact with the fluorescent probes used in the assays?

• To better elucidate the mechanism of TBEP and TCP action, deeper investigation of molecular pathways would be required. Could the candidate propose proteins or signaling pathways that should be examined in future studies?

VI. Final Recommendation

Based on the conducted assessment, I conclude that the dissertation meets all scientific and formal requirements. The work is well documented, and the research presented holds substantial cognitive and scientific value. In accordance with Article 187 of the Act of 20 July 2018 on Higher Education and Science, I request that the Scientific Council admit MSc Leila Khani Khouzani to the subsequent stages of the procedure for awarding the doctoral degree in the field of medical and health sciences, in the discipline of medical sciences.

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VII. Recommendation for Distinction

In my opinion, the dissertation is characterized by high scientific merit and an original contribution to research on the immunotoxicity of flame retardants, which justifies granting it a distinction. In light of the above, I request that the Scientific Council award a distinction to the doctoral dissertation of MSc Leila Khani Khouzani.

Rzeszów, 4 December 2025

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