Dimethyl Phthalate Induces Blood Immunotoxicity Through Oxidative Damage And Caspase-Dependent Apoptosis

Zhenxing Chi
Harbin Institute of Industry: Harbin Institute of Technology
Hongwei Lin
Harbin Institute of Industry: Harbin Institute of Technology
Xuanlin Meng
Shanghai Jiao Tong University
Jieqiong Zhou
Harbin Institute of Technology
Li Xiang
Hong Kong Baptist University
Guodong Cao
Hong Kong Baptist University
Pengfei Wu
Hong Kong Baptist University
Xingchen Zhao
333zxc333@163.com
Chinese Academy of Sciences https://orcid.org/0000-0001-6441-9394
Zongwei Cai
Hong Kong Baptist University

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Abstract

Dimethyl phthalate (DMP), a low molecular weight phthalate ester, is present in ectoparasiticides, plastics, and insect repellants, has been linked to neurotoxic, reproductive, and endocrine disruptive responses. However, its blood immunotoxic effects and mechanism remain poorly understood. In this study, rats were exposed to graded concentrations of DMP through intragastric administration to assess the blood immunotoxic effects using a combination assay of biomarker, cytometry, and transcriptomics. DMP treatment altered the redox status of rats, causing that oxidative damage. Significantly decreased blood cell counts and disordered antibody and cytokine secretion were observed, suggesting the suppressed immune defense and destructed inflammatory regulation. Flow cytometry showed for lymphocytes, especially $CD3^{+}CD4^{+}$ T cells, apoptosis/necrosis occurred positively related to DMP exposure level. Transcriptomics revealed responses that were in line with oxidative damage effects. Overexpression of the Bcl-2 family genes and activation of the Fas/FasL pathway trigger downstream caspase cascade, causing reactive oxygen species signaling mediated apoptosis/necrosis. This is the first report on immunotoxic effects of low molecular weight phthalate esters.

Introduction

The widespread distribution of organic pollutants in eco-environments has posed an ever-growing threat to human and ecological health in the past decades. Among these pollutants, phthalate esters (PAEs), which are broadly used in agriculture, commerce, and industry as agrochemicals and plasticizers, have drawn growing concerns. They can gradually migrate from the products (especially aged products) and easily find their way to the environment during the manufacturing, application, and disposal of relevant products, with concentrations of up to over one thousand micrograms per liter or kilogram in the environment (Dong et al., 2020; Fatoki and Ogunfowokan, 1993; Kaewlaoyoong et al., 2018; Olujimi et al., 2012; Wu et al., 2019).

Their direct or indirect release in the environment produces higher possibilities of human exposure. PAEs enter the human body through breathing, ingestion, and skin contact. After entering the human body, PAEs are absorbed into the blood and then transported to all parts of the body, which may lead to diseases and threaten human health. Toxicological studies have shown that PAEs have adverse effects, such as reproductive toxicity(van' t Erve et al., 2019), endocrine dysplasia(Guo and Kannan, 2013), neurotoxicity(Ran et al., 2019), and cardiovascular diseases(Mariana et al., 2016). However, the toxic effects of low molecular weight PAEs on blood immune function are rarely studied. This work evaluated whether low molecular weight PAEs like DMP induce immune-mediated hematotoxicity and the related mechanism. ROS-related blood cell and pro-inflammatory cytokine responses were recorded to provide novel insights into the mechanisms of DMP-induced immunotoxicity in rats. Also, this study elucidated the hematotoxicity and immunological impairment through linking differentially expressed genes (DEGs) to toxic endpoints, which could be helpful in an occupational exposure and ecological risk assessments of DMP.

Experimental

Rat exposure

The rats were randomly divided into two groups with or without antioxidant treatment. The details of chemicals and exposure are described in Supplementary Material Text S1.

Oxidative stress and immune function

The reactive oxygen species (ROS) biomarkers, blood cells and immune factors were measured on the Thermo Scientific Varioskan LUX Multimode Microplate Reader, Hanfang HF-3800 animal hematology analyzer, and Agilent BioTek Gen5 microplate reader. Details are described in Text S1.

Cell apoptosis/necrosis

The lymphocytes and $CD3^{+}CD4^{+}$ T cells were detected using a BD FACSCalibur flow cytometer (Becton Dickinson Biosciences, Franklin Lakes, US) and observed under an Olympus CX63 fluorescence microscope (Tokyo, Japan). Details are described in Text S1.

Transcriptome

Experimental details of the transcriptomic analyses were described in Text S1. The total number of DEGs, both upregulated and downregulated, for each treatment is provided in Table S1.

Statistical analysis

The data were expressed as the average of three runs ± standard deviations (SDs). Significance analysis are described in Text S1.

Results And Discussion

Effects of DMP on blood antioxidant capacity

After DMP treatment, the ROS content and activity of plasma SOD changed conversely along with increasing DMP dose (Fig. S1), demonstrating DMP inhibited the activity of SOD and are toxic to the antioxidant defense system. It should be noted that the ROS production is not significant at doses of 50 and 250 mg/Kg, probably ascribed to the fact that enzymatic antioxidants or lipids have consumed excess ROS. The unpaired electron of radicals may damage the lipids of cellular membranes, which is initiated by a process known as LP. To testify to the assumption, we measured the content of MDA, which is the product of LP. The MDA level developed conversely with SOD, showing that DMP induced ROS targeted at the fatty acids in membrane phospholipids and destructed the cellular membranes. These findings align with our (Chi et al., 2021; Li et al., 2019) and Zhang's *in vitro* results, the latter of whom firstly discovered oxidative damage of bisphenol S(Zhang et al., 2016b). Compared with heavy metals(Matović et al., 2015) and other industrial additives like sodium fluoride(Umarani et al., 2015), DMP

is much less toxic to the antioxidative system. In contrast, dibutyl phthalate(Wang et al., 2020), also a phthalate ester, has apparent less toxicity than DMP.

Haemocytes, antibody, and pro-inflammatory cytokines

Having shown that DMP invokes oxidative stress, we questioned whether it triggers immunohematological responses. As illustrated in Fig. 1, the numbers of RBC, WBC, and LYM without antioxidants are lower than that in the control group, demonstrating a downward tendency with increasing DMP concentration. Therefore, DMP is bio-active enough to inhibit the proliferation of hemocytes and initiate an inflammatory response. In quest of the DMP immunohematological toxicity mechanism, we used two important *in vivo* non-enzymatic antioxidants (vitamin C and E) as antagonists. Apparently, these antioxidants are effective stimulators of hemocyte proliferation. ROS likely participates in a conglomerate of steps that lead to innate immune activation.

The pro-inflammatory cytokine and immunoglobulin levels are usually tested to predict the immunomodulatory effects of exogenous substances and the possibility of inflammation-mediated toxicity. Alterations in antibody and cytokine expression were observed, suggesting inflammation effects of DMP (Fig. 1). Compared to the untreated, IgG, IL-4, -6, and IFN-γ are negatively associated with the exposure dose; however, IL-2 experiences hormesis and stays unchanged at the highest dose. In addition, antibody IgG production is elevated at light concentration and then declined compared to the untreated. All the phenomena suggest suppressed immune defense and destructed inflammatory regulation. What is surprising is that the antioxidants generate a significant change in IL-2 and -6 production with and suppression. The administration of antioxidants may polarize the balance between the Th1 and Th2 cytokines towards one specific pathway(Elsabahy and Wooley, 2013; Liu et al., 2009). Together, it is concluded that DMP is reactive enough to initiate an inflammatory response, and antioxidants may worsen immune balance.

Immune function

We determined induction of apoptosis/necrosis in lymphocytes using flow cytometry analysis. The treatment of rats with DMP resulted in lymphocyte apoptosis/necrosis. As shown in Fig. 2A, treatment with DMP generated a population shift from normal cells to apoptotic/necrotic ones. There is a significant increase in Annexin V and PI staining, with the percentages going up from 1.79-7.07-9.62 to 18.93% as the exposure dose rises.

We further investigated the T helper cell subset CD4 to determine if the single subpopulation was biased toward the production of ROS. It seems that DMP is a potent inhibitor of $CD3^+CD4^+$ T cell proliferation or differentiation. Zone Q2 in Fig. 2B shows the fluorescence level of $CD3^+CD4^+$ T cell. Analysis of the $CD3^+CD4^+$ population demonstrated a remarkable diminution in treated rats and an approximate 2.5-fold decrease. It is thus suspected that the apoptotic/necrotic lymphocytes are mainly $CD3^+CD4^+$ cells. Therefore, the decrease of $CD3^+CD4^+$ T cell content makes a cytokine decline, which indirectly leads to a deterioration in immunoglobulin secretion and immune function.

Feature, GO functional, and KEGG pathway analysis of transcriptomic responses

Exposure to DMP caused changes in global transcript expression (Fig. S2). Of all the transcripts, 570 overlapped across all treatment groups, either upregulated or downregulated. Interestingly, the medium and low dose treatments have the lowest and highest dysregulated transcripts, respectively. This phenomenon is consistent with the recent study revealing hormesis of dysregulated genes in 17α-Ethinylestradiol-treated rainbow trouts(Schultz et al., 2021) and is also consistent with the above toxicity features.

We further analyzed the 570 DEGs using GO and KEGG pathway enrichment analysis. The results of GO analysis show that exposure to DMP dysregulated a high number of genes in biological process, cell component, and molecular function. Among all the enriched genes, organonitrogen compound metabolic process, cytoplasm, intracellular part, cytoplasmic part, intracellular, and protein binding take percentages above 35% (Fig. 3A).

It's known that intracellular components, especially mitochondria and cytoplasm, are the primary sources of ROS production(Zhang et al., 2016a). One of the most important cellular responses to ROS is protein (organonitrogen compound) oxidation, which changes in thiol/disulfide pairs affect protein function. The ROS-dependent signaling processes, such as cytokine production, are also likely to be affected. Therefore, it's pretty evident that DMP would pose a significant risk on the blood immunology of rats.

KEGG pathway enrichment (Fig. 3B) as well as mechanism prediction (Fig. S3) analysis manifests that signal pathway alterations are closely involved in the metabolism following DMP exposure. Considering the hints from KEGG and GO analysis, it is likely that intracellular and cytoplasmic ROS is the cornerstone that contributes to metabolic dysfunction and inflammatory signaling. Cytosolic ROS are high possibly formed through NOX (nicotinamide adenine dinucleotide phosphate oxidase) activities, which influence metabolic processes like pentose phosphate pathway activity, glycolysis, and downstream oxidative phosphorylation, and autophagy(Forrester et al., 2018; Panieri and Santoro, 2016). Moreover, these disorders are also likely to be associated with lipid metabolism, a biological process where estrogen and xenoestrogen (like PAEs) exert regulatory control(Mankidy et al., 2013; Palmisano et al., 2017). The metabolomic disorders, intricately intertwined with the inflammation effects, result in the apoptosis/necrotic of immunocytes.

Expression level of genes related to ROS and immunocyte apoptosis

To further explore the potential relationship between ROS and the immunotoxic as well as the potential apoptosis pathways, the expression of Bcl-2 family genes (Bcl-2, Bax, Bid, and Bak-1) was analyzed. As shown in Fig. 4A, the expression levels of all the markers increase in folds with increasing dose, demonstrating an overexpression in blood cells. It should be noted that although all the pro-apoptotic

genes overexpressed after exposure, the expression of the anti-apoptotic gene Bcl-2 also increased. Therefore, DMP may cause apoptosis through the endoplasmic reticulum pathway and meantime, the anti-apoptotic gene also over-expressed as antagonism.

Apoptosis can be initiated through overexpression of death receptors (Fig. 4B) which are categorized as the TNF family members. Fas is a known death receptor and participates in the process of apoptosis with the involvement of FasL (Fas ligand) and FADD (Fas-associated death domain protein), which is also highly related to ROS and cytokines (Lenzi et al., 2018; Matés et al., 2012). Besides, previous observations have shown that FasL plays a regulatory role on the during the environmental toxicant-induced cell apoptosis(Wang and Su, 2018), which is in agreement with our findings.

Among the pro-apoptotic caspases, caspase-2, -8, and -9 are the activators of apoptosis, and caspase-3, -6, and -7 are the executors(Li and Yuan, 2008). Activation of caspases (Fig. 4C) may lead to disorder of cytoskeleton, resulting in chromatin concentration, cell structure disintegration, and apoptosis. When these enzymes are activated, DNA enzymes are subsequently activated to degrade DNA and induce apoptosis.

Conclusion

In summary, we have demonstrated DMP-induced ROS-related apoptosis/necrosis in blood immune cells in a concentration-dependent manner. DMP inhibited the activity of antioxidants, increased the content of ROS and led to oxidative stress in a dose-dependent way. The blood immune function was also damaged, as evidenced by the decreased number of blood cells. Results of the immunoactive substance analysis showed disorder in immune function after DMP entering the body. Furthermore, DMP could induce lymphocyte apoptosis/necrosis and induce overexpression of the Bcl-2 family genes as well as Fas, FasL, and FADD genes. Activation of the upstream promoted the expression of caspase-2, 8, 9, and caspase-3, 6, 7 genes (Fig. 4D). Thus, the apoptosis occurred through the endoplasmic reticulum and death receptor Fas/FasL pathways.

Declarations

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Conflicts of interest/Competing interests Not applicable.

Ethics approval This was approved by the Experimental Animal Welfare Ethics Committee, Harbin Institute of Technology.

Consent to participate This was approved by all the authors.

Consent for publication This was approved by all the authors.

Availability of data and material Data can be obtained upon request.

Code availability Not applicable.

Authors' contributions Zhenxing Chi: Investigation, Methodology, Supervision, Funding acquisition, Project administration, Writing - original draft, Resources. Hongwei Lin: Investigation, Data curation. Xuanlin Meng: Data curation, Methodology. Jieqiong Zhou: Resources. Li Xiang: Data curation. Guodong Cao: Data curation. Pengfei Wu: Data curation. Xingchen Zhao: Methodology, Conceptualization, Validation, Writing - review & editing. Zongwei Cai: Resources.

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Figures

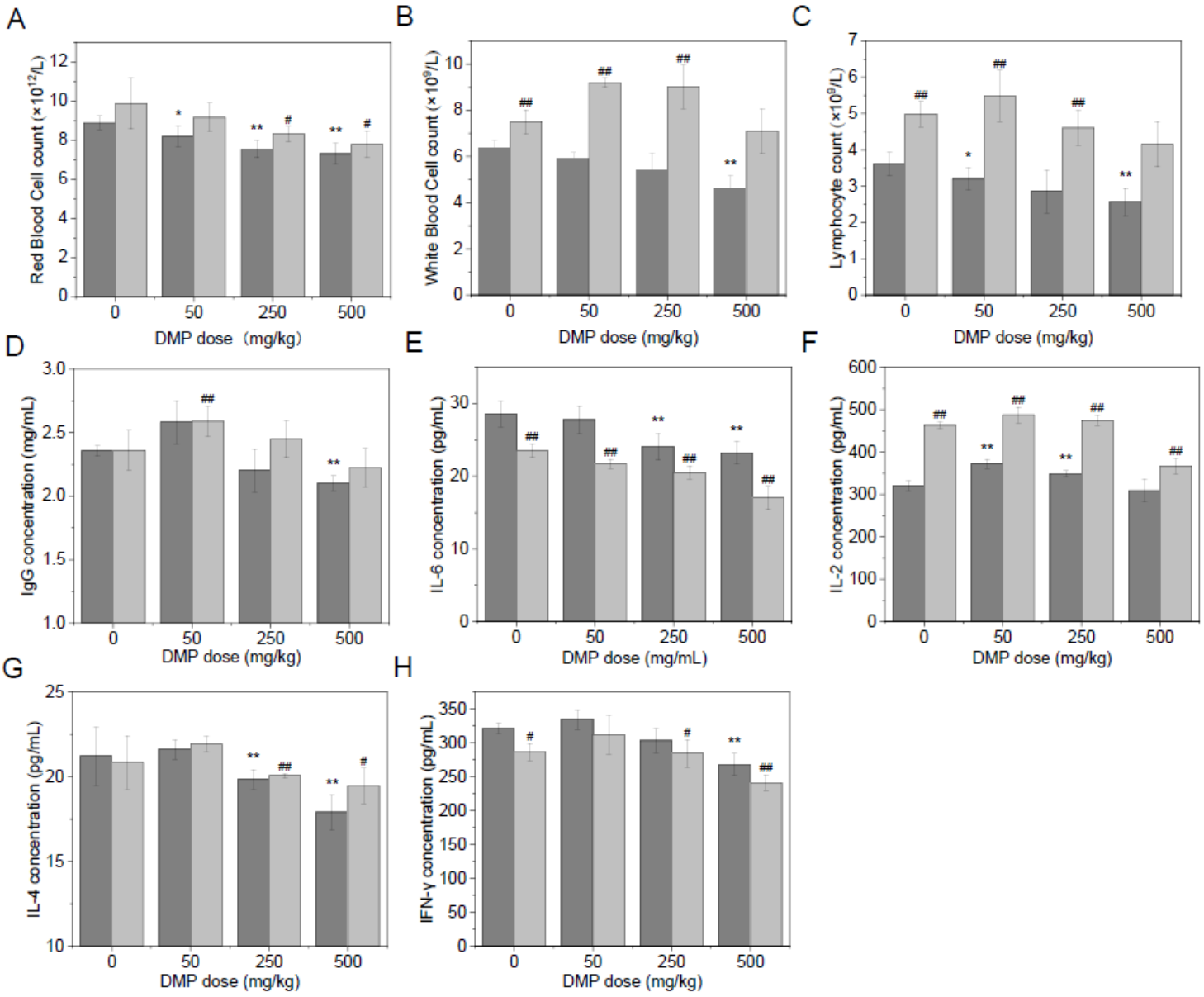

Figure 1

(A-C) Blood cell counts and (D-H) immune factors in in peripheral blood of rats. Lighter and darker colors represent samples with and without antioxidant treatment, respectively. Data were presented as mean ± SD, n = 3.

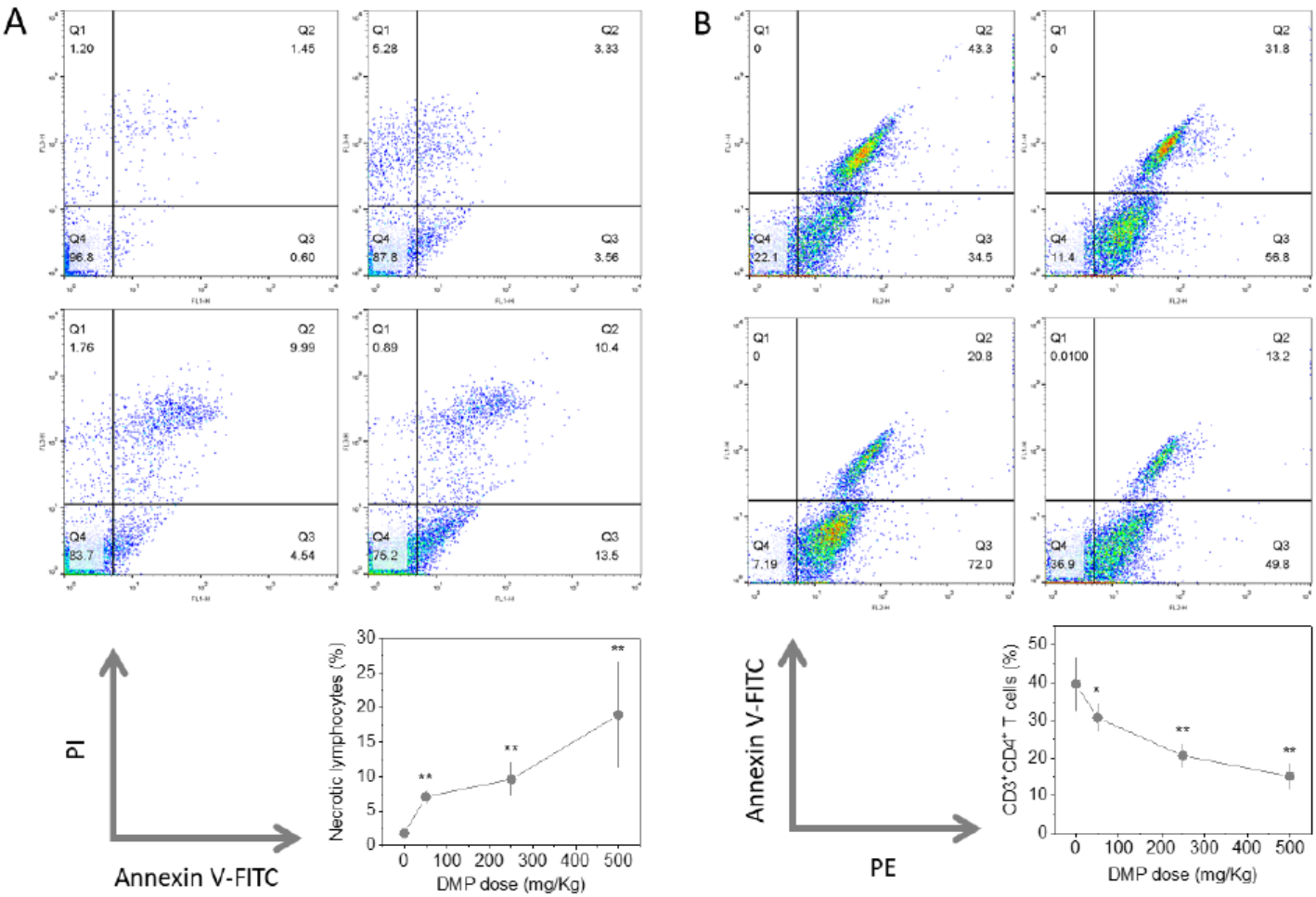

Figure 2

Apoptosis/necrosis of (A) peripheral blood and (B) $CD3^{+}CD4^{+}$ lymphocytes. C, L, M, and H stand for the control, 50, 250, and 500 mg/Kg groups, respectively.

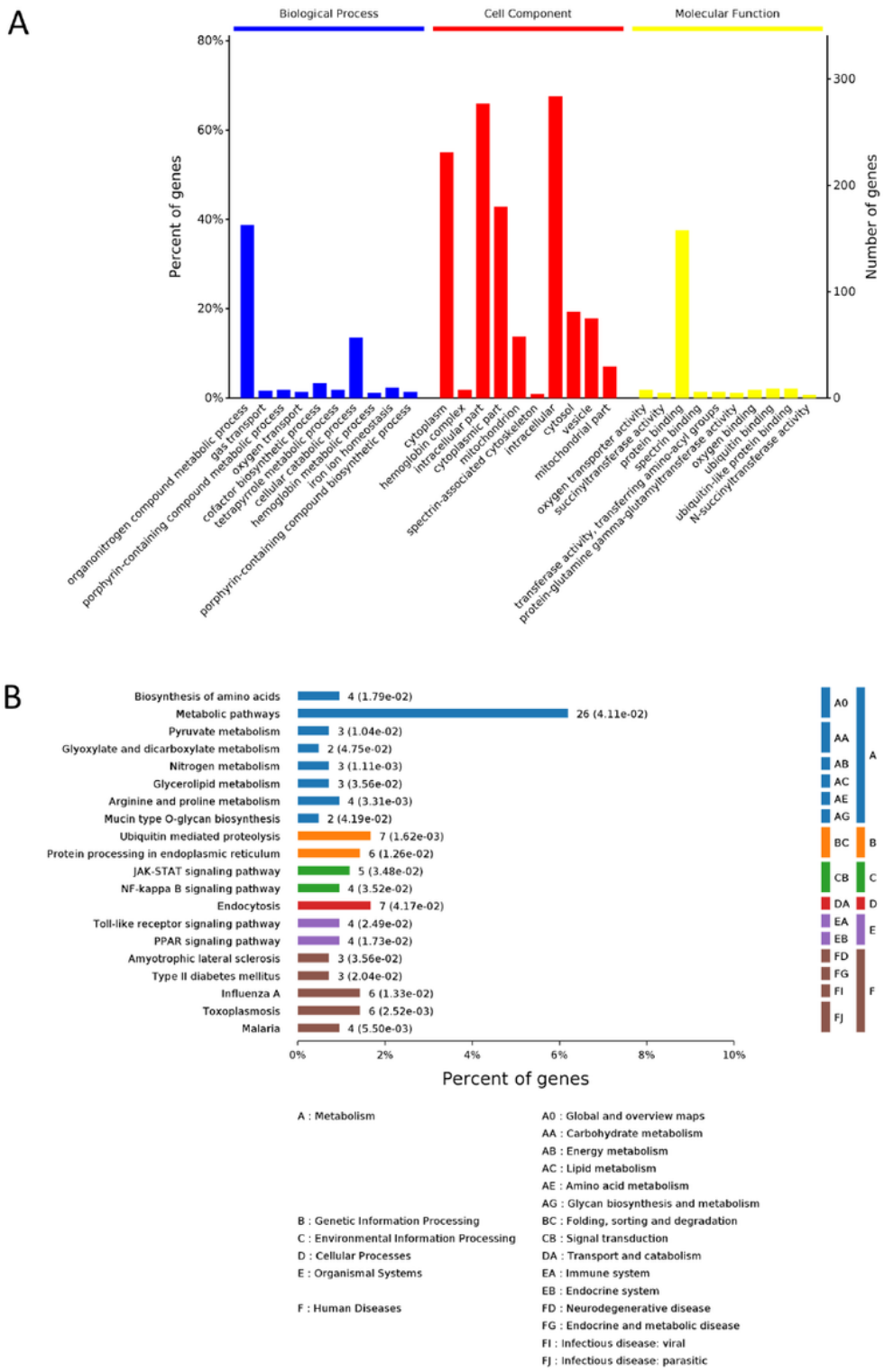

Figure 3

(A) The enriched GO terms and (B) KEGG pathway enrichment of DEGs. Data were shown as the percentage of DEGs shared by the three groups.

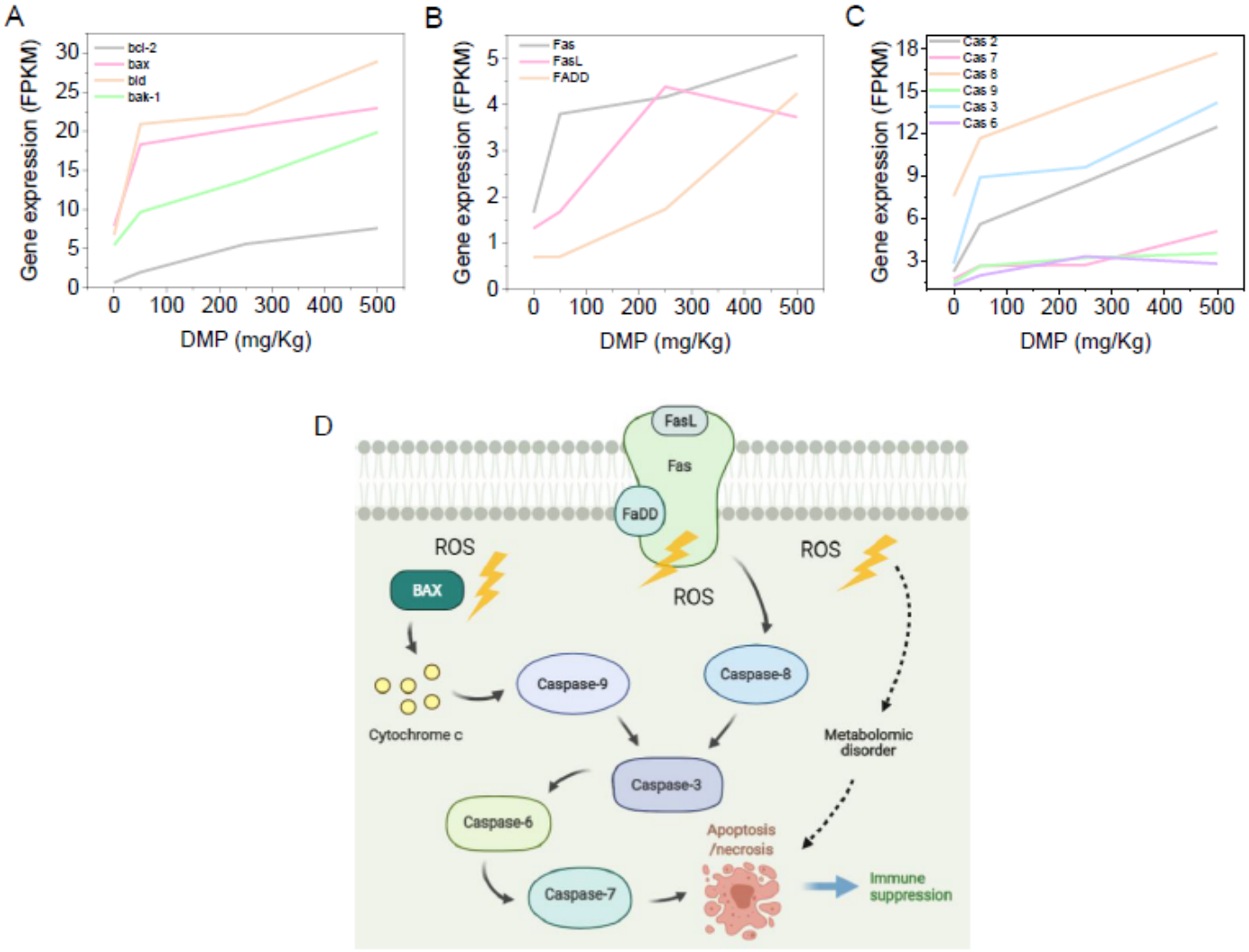

Figure 4

mRNA expression level of (A) bcl-2 family, (B) Fas-related, and (C) caspase genes. Results are expressed as FPKM. (D) Proposed model highlighting the mechanistic basis of the blood immunotoxicity induced by DMP.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata.doc