Organophosphorus Pesticide Malathion and Immunotoxicity: A More Sensitive Toxicological Target and Current Recommendations

Minhan Lou

Zhejiang Academy of Medical Sciences

Ling Yong

China National Center for Food Safety Risk Assessment

Weifeng Mao

China National Center for Food Safety Risk Assessment

Xiao Xiao

China National Center for Food Safety Risk Assessment

Chunlai Liang

China National Center for Food Safety Risk Assessment

Wei Wang

China National Center for Food Safety Risk Assessment

Hui Yang

China National Center for Food Safety Risk Assessment

Xudong Jia

China National Center for Food Safety Risk Assessment

Zhaoping Liu

China National Center for Food Safety Risk Assessment

Yin Wang

Zhejiang Academy of Medical Sciences

Ning Li

China National Center for Food Safety Risk Assessment

Yan Song (✉ songyan@cfssa.net.cn)

China National Center for Food Safety Risk Assessment <https://orcid.org/0000-0002-5847-0254>

Research

Keywords: Malathion (MLT), Immunotoxicity, Immunosuppression, Humoral immunity, IgG, LOALE, ADI

Posted Date: December 8th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-122196/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Female mice were randomly divided into five groups. Both the negative control and the positive control groups of mice were daily gavaged with corn oil, and those in the positive control group were intraperitoneally injected with 200 mg/kg bw of cyclophosphamide (CY) 24 h before the end of the study. In the dosage groups, MLT was daily gavaged at doses of 16, 65 and 258 mg/kg bw for 30 days, respectively. After exposure, the body weight, the absolute and relative weight of organs, the hematology and clinical chemistry parameters, the splenocyte number, the bone marrow cell classification, histopathology, as well as the cellular immunity, humoral immunity, and non-specific immunity functions of mice were studied. Comparisons between multiple groups were carried out by using one-way ANOVA followed by Bonferroni post hoc comparisons tests when equal variances were assumed and Dunnett's T3 post hoc tests when equal variances assumption was not met.

Results

Compared with the negative control group, the low dose of MLT significantly reduced the levels of IL-10, neutrophil, polychromatic erythroblast and monocyte series ($P<0.05$), induced atrophy in white pulp of spleen (1/10), which showed an immunosuppression in mice, and increased the absolute and relative weight of thymus ($P<0.05$). Inhibition of the PFC response, liver injury and thymus atrophy were observed in the high-dose group. What's more, the IgG level was abnormally elevated with the increase of dose and presented a dose-response relation ($P<0.05$).

Conclusions

The immunotoxicity of low-dose of MLT is clear from the evidence and the LOAEL of MLT is preliminarily considered to be 16 mg/kg bw. It is much lower than that of the NOAEL (29 mg/kg bw)

based on a 2-year chronic toxicity test and carcinogenicity test in rats, which was used to derive the ADI by Joint WHO/FAO Meeting on Pesticide Residues (0.3 mg/kg bw). It indicates that the immune system is a more sensitive target of MLT toxicity.

Keywords

Malathion (MLT), Immunotoxicity, Immunosuppression, Humoral immunity, IgG, LOALE, ADI.

Background

Malathion has been widely used in agriculture and household pest control for years, and sometimes it is used to treat head lice on human as well (1, 2). Since the long-term use of MLT, it is distributed in the environment, although the UV light (3) and some microorganisms in the soils (4) continuously degrade it. In some cases, workers without protective gear exposed to MLT through inhalation or contact the air, which was contained with spraying MLT. Except for occupational exposure, evidences suggested that the use of MLT for the household pest control purpose and the ingestion of water and food containing MLT increase the human health risks (5). Therefore, the possible adverse health outcomes caused by MLT were of concern. Based on the previous studies, MLT was linked with various diseases including allergic contact urticaria (6), depression (7), hypothyroidism (8), prostate cancer (9, 10), and the International Agency for Research on Cancer (IARC) classified it as “probably carcinogenic to humans” (Group 2A) (11). Experimental evidences showed that this commonly-used pesticide had the potential of neurotoxicity (12), developmental toxicity (13), genotoxicity (14) and immunotoxicity (15), and these conclusions were particularly important for pesticide safety management.

Acting as a defensive barrier to the body, the immune system is highly sensitive to xenobiotics, which is reflected in some cases that the doses of xenobiotics causing adverse reactions to the immune system (organs) are often lower than their general toxic doses. Therefore, it is particularly important to focus on the study of immunotoxicity of pesticides and develop a comprehensive, scientific and specific immunotoxicity testing guidelines for the safety assessment and management. Compared with other countries, the United States has put forward higher requirements on pesticide registration management. In addition to the acute toxicity, sub-chronic toxicity, chronic toxicity, reproductive and developmental toxicity, genetic toxicity, and metabolic studies of pesticides, the immunotoxicity of pesticides are also required in registration. The United States Environment Protection Agency (US EPA) has made several revisions to its previous guidelines for evaluating the immunotoxicity of pesticides (16-18). However, other countries have not put forward a clear guideline for immunotoxicity assessment, which maybe the direction of their further efforts.

In the present study, the immunotoxicity of MLT was systematically and fully evaluated with reference to the current guidelines of pesticides immunotoxicity evaluation, for the purpose of providing a scientific basis for the management of MLT and other new pesticides.

Materials and methods

Materials

MLT (purity 95%) was purchased from Hebei Hengshui Beifang Pesticide Chemical Co., Ltd. (Hebei, China). Cyclophosphamide (CY) was purchased from Jiangsu Hengrui Pharmaceutical Co., Ltd (Jiangsu, China). Sheep red blood cell (SRBC) was obtained from Beijing Laboratory Biology technology Co.,

Ltd. (Beijing, China). FITC hamster anti-mouse CD3e, APC rat anti-mouse CD4, APC rat anti-mouse CD19, PE rat anti-mouse CD8a, PE rat anti-mouse CD49b, lysing buffer and Mouse CBA flex sets(IL-2, IL-4, IL-5, IL-10, IFN- γ , TNF) were obtained from Becton, Dickinson and Company (Franklin Lakes, NJ, USA.). Mouse IgG, IgA and IgM ELISA kits were purchased from GenWay Biotech, Inc. (San Diego, CA, USA.).

Animals

In this study, 6-8 weeks old female BALB/c mice (SPF grade, with body weight of 18-22 g) were used. Animals were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China) and maintained in a temperature-controlled (20-25°C), relative humidity-controlled (40-70%), artificially illuminated light/dark cycle-controlled (10/14 h) and air exchange-controlled (10-15 times h⁻¹) environment. All animals were observed within 3 days of arrival. Nutritional propagation diets and water were available ad libitum during the 30-day feeding studies. At the end of treatments, mice were humanely sacrificed..

Experimental design

After 3 days of adaptive feeding, mice were randomly divided into different groups with 10 mice/ each based on their body weight. In this study, the negative control group, three dosage groups and the positive control group were set. The highest dosage of MLT in the study was mainly based on the result of the oral acute toxicity in mice, 1/4 LD₅₀. The dosages of the low, the middle and the high dose groups were 16 mg/kg bw, 65 mg/kg bw, 258 mg/kg bw of MLT, respectively. Both the negative control group and the positive control group of mice were daily gavage with corn oil, and the mice in the positive control

group were intraperitoneal injected with 200 mg/kg bw of cyclophosphamide (CY) 24 h before the end of the study. Housed the animals by group with corresponding doses gavage during the study.

Preparation of splenocyte suspensions

Mice were sacrificed and the spleens were removed under sterile conditions on day 30, which had been immunized on day 25 by intraperitoneal injection with 0.2 ml of 2% (v/v) SRBC suspensions in sterilizing saline, and transferred to petri dishes containing 2 ml of HBSS. Finely ground the spleens and transferred the cell suspensions into tubes. Centrifuged for 10 min at 1 000 rpm and washed twice in HBSS at 4°C. Splenocytes were diluted to 5×10^6 cells ml^{-1} in the culture medium (Roswell Park Memorial Institute, RPMI, 1640 medium with phenol red supplemented) (Gibco, Grand Island, NY, USA) for backup.

Body weight and feeding quantity

All animals were weighted at weekly intervals and the dose of gavage was adjusted according to the body weight in time. At the end of the experiment, the weights of the mice were recorded and the mice were humanely sacrificed.

Hematology and clinical chemistry parameters

For the purpose of hematological analysis, the whole blood was collected from the retro-orbital plexus and transferred into tubes which with ethylenediaminetetraacetic acid dipotassium (EDTA-K^2) anticoagulant inside. A COULTER Ac. T diff2 Hematology Analyzer (Beckman Coulter Corporation) was used to measure the following parameters: white blood cell count (WBC), red blood cell count (RBC),

116 hematocrit (HCT), hemoglobin (HGB), mean corpuscular hemoglobin (MCH), mean corpuscular volume
117 (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), red blood cell
118 distribution width (RDW), prothrombin consumption test (PCT), mean platelet volume (MPV) and
119 platelet distribution width (PDW); the count and percentage of monocyte (MON), lymphocyte (LYM),
120 neutrophil (NEUT), basophil (BAS) and eosinophil (EOS).

121 Blood for clinical chemistry analysis was collected as stated, without the presence of anticoagulant. Spun
122 the blood sample to obtain serum (4×10^3 rpm min⁻¹, 10 minute) in the Low Speed Centrifuge Model
123 800D (Surgifriend Medicals, England) and tested the clinical parameters with an automatic clinical
124 analyzer (Hitachi 7080, Hitachi High-Technologies Corporation): aspartate aminotransferase (AST),
125 alanine aminotransferase (ALT), albumin (ALB), total protein (TP), albumin to globulin (A/G), glucose
126 (GLU), alkaline phosphatase (ALP), creatinine (CRE), urea nitrogen (BUN), cholesterol (CHO),
127 triglyceride (TG), calcium (CA), sodium (NA), potassium (K).

128 Peripheral blood lymphocytes phenotyping

129 Collected whole blood in the same way and transferred into tubes with anticoagulant (EDTA-K²) inside.
130 Two kinds of three-color combinations of antibodies (antibodies of CD3e, CD19 and CD49b or
131 antibodies of CD3e, CD4 and CD8) were used to stain 50 µl of blood cell suspension, respectively, for
132 20 min at room temperature in dark. Added into 2 ml of lysing buffer, spun and kept for 20 min at room
133 temperature in dark. Centrifuged twice (1×10^3 rpm min⁻¹, 5 min), spun the samples in 0.5 ml of PBS and
134 analyzed on FACSCalibur flow cytometer using CellQuest software (Becton, Dickinson, and Company,
135 USA). Appropriate isotype controls were used for compensation controls.

136 Bone marrow cell classification

137 Bone marrow cells were taken from the left femurs of mice and made the smears. The percentages of
138 promyelocytes, myelocytes, metamyelocytes, stab granulocytes, segmented granulocytes, basophilic
139 normoblast, polychromatic normoblast, orthochromatic normoblast, lymphocytic series, monocytic
140 series and others were determined.

141 Splenocyte account

142 Removed the spleens under aseptic conditions and placed it in culture dishes containing Hank's solution.
143 Ground the spleens and transferred the cell suspensions into tubes. Centrifuged the cell suspensions for
144 10 min at 1 000 rpm and washed twice in HBSS at 4°C. Then the splenocytes were suspended in 1 ml of
145 complete culture medium to count the number of splenocytes.

146 Pathology

147 Animals were humanly sacrificed and dissected at the end of experiment. Liver, kidney, spleen, thymus,
148 lymph glands (axillary lymph nodes, cervical lymph nodes, mesenteric lymph nodes), Peyer's patches
149 and bone marrow were removed. Weighted the organs and calculated the relative weights, and counted
150 the number of Peyer's patches on small intestine. Fixed the organs in 10% neutral buffered formalin,
151 processed for paraffin embedding, sectioned at 5µm, made into slices with HE staining observed under
152 the optical microscope, and all the results were transformed to data through an image analysis system. In
153 addition, the accurate measurements of the splenic periarterial lymphatic sheaths, splenic germinal center,
154 lymph follicles in the Peyer's patches and germinal center in the Peyer's patches were also required.

155 Humoral immunity functions

156 Plaque-forming cell (PFC) assay

157 Mice had been immunized on day 25 with 0.2 ml of 2% (v/v) SRBC suspensions in sterile saline by
158 intraperitoneal injection. Five days later, the splenocyte suspensions were prepared as stated. Transferred
159 25 μ l of splenocyte suspensions in RPMI 1640 medium (5×10^6 cells ml^{-1}), which were supplemented
160 with 10% fetal bovine serum and 1% penicillin-streptomycin solution, to a glass tube containing with 50
161 μ l of 10% (v/v) SRBC in SA buffer solution and 0.5 ml of agar solution (0.5 g ml^{-1} in HBSS, pH 7.2-
162 7.4), and poured onto slides. The slides were inverted on a special frame after the mixtures were solidified
163 and incubated (37°C, 1.5h). Added guinea pig sera to the slot between the glass slides and the bottom of
164 the frame. Incubated for another 1.5 hour (37°C), then plaque production was counted and the results
165 were expressed as the number of PFC/ 10^6 splenocytes.

166 Serum immunoglobulin quantification

167 The total IgA, IgG and IgM levels in serum were determined by ELISA kits. The test steps were
168 completely in accordance with the instructions. Mice were immunized as stated. 100 μ l of blank, standard
169 or diluted serum samples were respectively added into the 96-well plates in duplicate, incubated (room
170 temperature, 1 h), and washed 4 times with wash solution. Added enzyme-antibody conjugate (100 μ l
171 well^{-1}), incubated (room temperature, 30 min), and washed 4 times with wash solution. Added TMB
172 substrate solution (100 μ l well^{-1}), incubated (room temperature, 10 min) and added stop solution (100 μ l
173 well^{-1}) to prevent further reactions. Finally, determined the absorbance (450 nm) of the contents in each
174 well within 15min, using an ELISA Reader (BioTek., USA.).

175 Hemolysis test

176 Collected whole blood and got the serum as stated. 1 ml of SA buffer solution, 0.5 ml of 10% (v/v) SRBC,
177 1 ml diluted guinea pig complement (1:8 diluted with SA buffer solution) and 2 µl mouse sera were
178 mixed. Set the control tube without mouse serum. Kept the tubes in a water bath (at 37°C, 15 to 30 min)
179 and put them in an ice bath immediately to stop the reaction. Centrifuged (2 000 r min⁻¹, 10 min) collected
180 1 ml of supernatant and added into 3 ml Drabkin solution (1.0 g of NaHCO₃, 0.05 g of KCN and 0.2 g
181 of K₃Fe (CN)₆ solved in 1000 ml still water). Meanwhile, 0.25 ml of 10% (v/v) SRBC and 3.75 ml
182 Drabkin solution were thoroughly mixed and placed for 10 min as the positive control. Determined the
183 absorbance (540 nm) and got the HC₅₀ value by the formula: $HC_{50} = (OD1/OD2) \times 500$ (OD1 = the
184 OD value of the sample well subtract that of the control well, OD2 = the OD value of the positive control
185 well subtract that of the control well).

186 Serum cytokine measurement

187 Collected whole blood without anticoagulant, obtained the serum as stated and assayed for the levels of
188 cytokines by Mouse CBA Flex sets, including IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF. The assay was
189 fully enforced the descriptions in the manufacture's instruction. Transferred 50 µl of mixed capture beads
190 into the tubes, added 50 µl of standard, blank or serum samples to the appropriate tubes, added PE
191 detection reagent into each tube and incubated in dark (room temperature, 2 h). After incubation, washed
192 samples and spun in 300 µl of wash buffer. Analyzed on FACSCalibur flow cytometer by CellQuest
193 software.

194 Cellular immunity functions

Mitogen-induced splenic lymphocyte proliferation

ConA and LPS were dissolved in distilled water ($100\ \mu\text{g ml}^{-1}$), respectively. The splenocyte suspensions ($3\times 10^6\ \text{cells ml}^{-1}$) were prepared as stated and 1 ml of the suspensions were added into the 24-well plate to culture with 75 μl of the mitogen solutions (at $37\ ^\circ\text{C}$, with 5% CO_2 , 68 h). In the control well, added 75 μl of distilled water to replace the mitogen. Four hours before the terminal of incubation, discarded 0.7 ml of the supernatant from each well and added 0.7 ml of RPMI 1640 immediately, added 50 $\mu\text{l well}^{-1}$ of fresh prepared MTT solution ($5\ \text{mg ml}^{-1}$, dissolved in PBS, pH 7.2) and kept on incubation. 4 hours later, 1 ml well^{-1} of acid-isopropanol (4 ml of 1 mol L^{-1} HCl added to 96 ml of isopropanol) was added and mixed to make sure the purple crystallize were dissolved. Transferred the solution to 96-well plates in triplicate and determined the absorbance (570 nm) of the contents in each well. The calculation results were that the OD value of the sample well minus the OD value of the control well.

Delayed-type hypersensitivity (DTH)

Mice were immunized as stated. On day 30, determined the thickness of left rear footpads of mice with Vernier caliper (Changchun, Jilin Province, China). Hypodermically injected the left rear footpads with 20 μl of 20% (v/v) SRBC and measured the thickness 24 h later. The extent of DTH was expressed by the difference of footpad thickness before and after hypodermic injection.

Cytotoxic T-lymphocyte (CTL) assay

Splenocyte suspensions were prepared ($6\times 10^7\ \text{cells ml}^{-1}$) as the effector cells. Added 0.5 ml of splenocyte suspensions and an equal amount of target cells, P815 cell suspensions ($1.2\times 10^6\ \text{cells ml}^{-1}$), into the

24-well round-bottom plate and incubated for 5 days at 37 °C with 5% CO₂. Collected the cultures, washed in Hank's solution and resuspended the cells in RPMI 1640 medium (2×10^7 cells ml⁻¹). Added 100 µl of effector cells (2×10^7 cells ml⁻¹) to triplicate wells in round-bottom plates and added 100 µl of target cells (2×10^5 cells ml⁻¹) in each well for a total of 200 µl well⁻¹. Spontaneous LDH release and total LDH release of target cells were determined by adding culture solution, 2.5% Triton solution and effector cell suspension, respectively, as well as the effector cell control. All plates were incubated for 6 h (37 °C with 5% CO₂) and spun (1 000 r min⁻¹, 5 min). Transferred 100 µl well⁻¹ of supernatant to another 96-well round-bottom plate, added 50 µl of fresh prepared LDH substrate solution in each well, and incubated at room temperature for 30 min in dark. 50 µl per well of 1 mol L⁻¹ HCl solution were added to stop the reaction. The absorbance (492 nm) was determined, and the results were calculated by the equation: % CTL cell activity = $[(E - S - C)/(M - S)] \times 100$ (where E = experimental release of effector/target co-culture; S = spontaneous target cell LDH release; C = release of effector cell control; M = total target cell LDH release).

Non-specific immunity

NK cell activity assay

100 µl of splenocyte suspensions (2×10^7 cells ml⁻¹) was prepared as stated and plated in 96-well round-bottom plate as effector cells. 100 µl of YAC-1 cell suspensions (4×10^5 cells ml⁻¹) was prepared and added as target cells. Added 100 µl of culture solution and 2.5% Triton solution, respectively, to determine the spontaneous LDH release and the total LDH release of target cells. All tests were performed in triplicate. The plates were incubated for 4 h at 37°C with 50% CO₂ and spun for 5min at 1

234 500 r min⁻¹. 100 µl well⁻¹ of supernatant was removed to another 96-well flat-plate, incubated with
 235 100µl of freshly prepared LDH substrate solution (0.05 mol L⁻¹ lithium lactate, 6.6×10⁻⁴ mol L⁻¹ 2p-
 236 iodophenyl-3p-nitrophenyl tetrazolium chloride, 2.8×10⁻⁴ mol L⁻¹ phenazine metosulphate, and 1.3×10⁻
 237 ³ mol L⁻¹ nicotineamide nucleotide NAD in 0.2 mol L⁻¹ Tris-HCl buffer, pH 8.2) at room temperature in
 238 dark for 10 min. Stopped the reaction by adding 30 µl well⁻¹ of HCL (1 mol L⁻¹). Determined the
 239 absorbance (490 nm) and calculated the results with the equation: % NK cell activity =
 240 $[(E - S)/(M - S)] \times 100$ (where E=experimental release of effector/target co-culture;
 241 S=spontaneous target cell LDH release; and M=total target cell LDH release).

242 The Carbon-clearance test

243 The activity of macrophage was performed as carbon-clearance test. On day 30, mice were injected with
 244 diluted ink (10 mg ml⁻¹) via caudal vein. At 2 min or 10 min after the injection, 10 µl of the blood was
 245 collected as stated and added into 2 ml of 1 mg ml⁻¹ Na₂CO₃ solution. Then the absorbance (600 nm) of
 246 the mixed samples were measured and the index of phagocytosis was calculated by the formula:
 247 *Index of phagocytosis* = $[body\ weight / (liver\ weight + spleen\ weight)] \times$
 248 $[(\lg OD1 - \lg OD2) / (t2 - t1)]^{1/3}$ (OD1: the absorbance (600 nm) of 2 min after the injection of ink,
 249 OD2: the absorbance (600 nm) of 10 min after the injection of ink).

250 Statistical analysis

251 All data were analysed by using SPSS software (version 17.0). The values were presented as means ±
 252 SD. Comparisons between multiple groups were carried out by using one-way ANOVA followed by
 253 Bonferroni post hoc comparisons tests when equal variances assumed and Dunnett's T3 post hoc tests

when equal variances assumption was not met. $P < 0.05$ were considered to statistically significant difference.

Results

Effects on body and organ weights

As seen in Table 1, there was no significant difference in periodic body weights among each group in all weeks ($P > 0.05$).

The relative weight of kidney, the absolute and relative weights of liver in the high-dose group were significantly greater than those in the negative control group and other dosage groups ($P < 0.05$); the relative and absolute weights of thymus in the low-dose group and the middle-dose group were significantly higher than those in the negative control group ($P < 0.05$) (Table 2).

Table 1 The body weight of MLT exposed mice at different time points

Group	Week 0	Week 1	Week 2	Week 3	Week 4
Negative Control	19.4±0.9	19.1±1.1	19.9±1.1	19.1±1.1	19.4±0.9
Low Dose Group	19.6±1.9	19.4±1.6	20.3±1.7	19.7±1.7	20.2±1.7
Middle Dose Group	19.5±0.9	19.6±1.0	20.2±0.9	20.0±1.2	19.7±1.3
High Dose Group	19.2±1.3	19.4±1.1	19.3±0.9	19.4±1.1	20.0±1.1
Positive Control	19.4±1.3	19.6±1.1	19.8±0.9	20.1±1.2	20.0±1.0

Note: Data are given as mean ± SD (n=10).

Table 2 Effects on the number of Peyer's lymph gland, terminal body weight and organ weight of mice

	Negative control	Low-dose	Middle-dose	High-dose	Positive control

	group	group	group	group	group
Terminal body weight (g)	18.6±1.0	18.6±1.4 ^e	19.2±1.4 ^e	18.1±1.1	17.6±0.9 ^{b, c}
Number of Peyer's Patches (n)	8.4±1.0	7.3±0.9	8.8±2.1	8.8±2.2	7.9±2.0
Liver					
Absolute weight (g)	0.736±0.061 ^d	0.727±0.069 ^d	0.764±0.056 ^d	0.861±0.110 ^{a, b, c, e}	0.708±0.427 ^d
Relative weight (%)	3.978±0.390 ^d	3.957±0.187 ^d	3.994±0.376 ^d	4.760±0.659 ^{a, b, c, e}	4.036±0.215 ^{c, d}
Kidney					
Absolute weight (g)	0.208±0.168	0.203±0.023	0.213±0.017	0.221±0.024	0.216±0.015
Relative weight (%)	1.201±0.064 ^{d, e}	1.090±0.085 ^{d, e}	1.109±0.070 ^{d, e}	1.222±0.129 ^{a, b, c}	1.228±0.074 ^{a, b, c}
Spleen					
Absolute weight (g)	0.059±0.006 ^e	0.064±0.010 ^e	0.064±0.008 ^e	0.056±0.007	0.034±0.006 ^{a, b, c}
Relative weight (%)	0.319±0.029 ^e	0.343±0.043 ^e	0.336±0.048 ^e	0.307±0.025 ^e	0.191±0.031 ^{a, b, c, d}
Thymus					
Absolute weight (g)	0.026±0.005 ^{b, c}	0.034±0.010 ^{a, e}	0.035±0.009 ^{a, e}	0.030±0.010	0.021±0.007 ^{b, c}
Relative weight (%)	0.141±0.027 ^{b, c}	0.182±0.047 ^a	0.181±0.045 ^a	0.167±0.049	0.118±0.041
Lymph gland					
Absolute weight (g)	0.006±0.002	0.006±0.003	0.007±0.003 ^d	0.005±0.002	0.027±0.012
Relative weight (%)	0.031±0.010	0.035±0.014	0.038±0.016	0.027±0.012	0.030±0.016

Note: Data are given as mean ± SD (n = 10). ^a: statistically significant different from the negative control group at $P < 0.05$; ^b: statistically significant different from the low dose group at $P < 0.05$; ^c: statistically significant different from the middle dose group at $P < 0.05$; ^d: statistically significant different from the high dose group at $P < 0.05$; ^e: statistically significant different from the positive control group at $P < 0.05$.

Effects on hematology and clinical chemistry parameters

The high-dose of MLT caused a significant decrease in the WBC count, compared with the result of the negative control group ($P < 0.05$), and showed a liner dose-response relationship (Fig. 1).

274 Although the PLT count of the high-dose group was significantly lower than that in the low-dose group
 275 ($P<0.05$), there was no significant difference when compared with the negative control group ($P>0.05$). The
 276 NEUT level of the low-dose and middle-dose groups were significantly lower than that of the negative control
 277 group ($P<0.05$). Moreover, the count and percentage of BAS in the medium-dose group were higher than
 278 that of the negative control group ($P<0.05$) (Table 3).

279 **Table 3 Effects on hematology parameters in mice after exposure**

Group	Negative control group	Low dose group	Middle dose group	High dose group	Positive control group
RBC ($\times 10^{12} \text{ L}^{-1}$)	10.67 $\pm$ 1.14	10.8 $\pm$ 1.14	10.59 $\pm$ 0.82	10.27 $\pm$ 1.48	10.70 $\pm$ 2.11
HGB (g L^{-1})	174.2 $\pm$ 16.6	172.6 $\pm$ 20.2	174.9 $\pm$ 12.7	171.1 $\pm$ 20.9	176.3 $\pm$ 37.3
HCT (%)	49.3 $\pm$ 5.2	49.2 $\pm$ 5.1	50.6 $\pm$ 3.6	48.1 $\pm$ 6.6	50.7 $\pm$ 9.9
MCV (fL)	46.2 $\pm$ 1.0	487.5 $\pm$ 1.1	47.8 $\pm$ 0.7	46.9 $\pm$ 1.1	47.5 $\pm$ 0.9
MCH (pg)	16.4 $\pm$ 0.4	16.6 $\pm$ 0.4	16.6 $\pm$ 0.7	16.7 $\pm$ 0.6	16.4 $\pm$ 0.4
MCHC (g L^{-1})	353.5 $\pm$ 6.8	350.2 $\pm$ 11.5	346.1 $\pm$ 13.7	356.1 $\pm$ 8.7	346.3 $\pm$ 10.8
RDW (%)	13.38 $\pm$ 0.31	13.15 $\pm$ 0.25	13.32 $\pm$ 0.36	13.69 $\pm$ 0.56	13.63 $\pm$ 0.39
PLT ($\times 10^9 \text{ L}^{-1}$)	717.2 $\pm$ 92.8	806.7 $\pm$ 76.7 ^d	758.4 $\pm$ 70.1	645.3 $\pm$ 158.1 ^b	687.4 $\pm$ 105.8
PCT (%)	0.20 $\pm$ 0.03	0.22 $\pm$ 0.05	0.21 $\pm$ 0.04	0.18 $\pm$ 0.06	0.20 $\pm$ 0.05
MPV (fL)	2.77 $\pm$ 0.35	2.75 $\pm$ 0.43	2.73 $\pm$ 0.31	2.76 $\pm$ 0.36	2.88 $\pm$ 0.36
PDW (%)	17.64 $\pm$ 0.75	17.45 $\pm$ 0.93	17.57 $\pm$ 0.89	17.82 $\pm$ 0.83	17.69 $\pm$ 0.85
WBC ($\times 10^9 \text{ L}^{-1}$)	8.11 $\pm$ 2.47 ^{d, e}	7.49 $\pm$ 1.39 ^e	7.08 $\pm$ 2.08 ^e	6.18 $\pm$ 2.29 ^{a, e}	3.81 $\pm$ 1.98 ^{a, b, c, d}
LYM ($\times 10^9 \text{ L}^{-1}$)	4.89 $\pm$ 1.29 ^e	4.34 $\pm$ 2.09 ^e	5.35 $\pm$ 1.59 ^e	4.65 $\pm$ 1.40 ^e	2.41 $\pm$ 1.81 ^{a, b, c, d}
(%)	62.46 $\pm$ 15.64	67.20 $\pm$ 9.23	70.24 $\pm$ 10.08 ^e	66.55 $\pm$ 12.43	58.02 $\pm$ 18.18 ^c
MON ($\times 10^9 \text{ L}^{-1}$)	0.37 $\pm$ 0.24 ^e	0.39 $\pm$ 0.31 ^e	0.42 $\pm$ 0.24 ^e	0.38 $\pm$ 0.19 ^e	0.08 $\pm$ 0.12 ^{a, b, c, d}
(%)	5.39 $\pm$ 4.46	5.35 $\pm$ 3.57	5.40 $\pm$ 3.07	5.87 $\pm$ 3.78	2.98 $\pm$ 4.51

NEUT ($\times 10^9 \text{ L}^{-1}$)	2.82 $\pm$ 2.21 ^{b, c, e}	1.41 $\pm$ 0.35 ^a	1.66 $\pm$ 0.66 ^a	2.05 $\pm$ 1.36	1.32 $\pm$ 0.59 ^a
(%)	31.36 $\pm$ 18.93 ^d	26.60 $\pm$ 12.56	23.44 $\pm$ 10.92 ^c	27.18 $\pm$ 15.12	38.28 $\pm$ 19.15 ^c
EOS ($\times 10^9 \text{ L}^{-1}$)	0.01 $\pm$ 0.03	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
(%)	0.40 $\pm$ 0.58	0.26 $\pm$ 0.11	0.23 $\pm$ 0.10	0.15 $\pm$ 0.13	0.42 $\pm$ 0.16
BAS ($\times 10^9 \text{ L}^{-1}$)	0.02 $\pm$ 0.04 ^c	0.04 $\pm$ 0.05 ^d	0.06 $\pm$ 0.07 ^{a, d}	0.00 $\pm$ 0.00 ^{b, c}	0.00 $\pm$ 0.00
(%)	0.39 $\pm$ 0.34 ^c	0.59 $\pm$ 0.34 ^{d, e}	0.69 $\pm$ 0.58 ^{a, d, e}	0.25 $\pm$ 0.14 ^{b, c}	0.30 $\pm$ 0.40 ^{b, c}

Note: Data are given as mean $\pm$ SD (n = 10). ^a: statistically significant different from the negative control group at $P < 0.05$; ^b: statistically significant different from the low dose group at $P < 0.05$; ^c: statistically significant different from the middle dose group at $P < 0.05$; ^d: statistically significant different from the high dose group at $P < 0.05$; ^e: statistically significant different from the positive control group at $P < 0.05$.

There were no significant differences in ALT levels between the treated groups and the negative control group ($P > 0.05$), however, ALT levels in the high-dose group were significantly higher than those in the low-dose and the middle-dose groups ($P < 0.05$). Compared with the negative control group, the serum TP level significantly descended in the high-dose group ($P < 0.05$), and the serum TP levels of the middle dose group and the high dose group were significantly lower than that of the low dose group ($P < 0.05$). The ALB levels in the high-dose group were significantly lower than those in the negative control group and other dose groups ($P < 0.05$), and there was a significant dose-response relationship among the three dosage groups ($P < 0.05$). Although the GLU level in the middle-dose group was significantly higher than that in the negative control group and the high dose group ($P < 0.05$), there were no dose-response relationships among the groups. The blood glucose indexes were within the normal range of laboratory history, so it had no biological significance. The sera CHO level in the high-dose group was higher than that in the negative control group and the low-dose group ($P < 0.05$) (Table 4).

Table 4 Effects on clinical chemistry parameters of mice after exposure

Group	Negative control group	Low dose group	Middle dose group	High dose group	Positive control group
ALT (U L ⁻¹)	28.4±4.2	22.3±2.0 ^{d, e}	22.0±1.0 ^{d, e}	31.9±11.8 ^{b, c}	33.7±10.7 ^{b, c}
AST (U L ⁻¹)	151.7±35.4	137.9±24.1 ^e	128.8±20.0 ^e	150.0±32.6	165.4±31.2 ^{b, c}
TP (g L ⁻¹)	63.45±3.81 ^d	65.21±3.59 ^{c, d}	59.86±5.04 ^{b, e}	56.95±3.26 ^{a, b, c, e}	65.62±3.49 ^{c, d}
ALB (g L ⁻¹)	39.38±1.74 ^d	40.22±0.80 ^{c, d, e}	38.45±1.12 ^{b, d}	36.58±2.35 ^{a, b, c, e}	38.33±1.67 ^{b, d}
ALP (U L ⁻¹)	114.5±6.6	122.4±14.4	110.7±20.3	111.8±18.0	114.6±10.8
GLU (mmol L ⁻¹)	1.97±0.90 ^{c, e}	2.54±1.23	3.52±0.84 ^{a, d}	1.85±1.57 ^{c, e}	2.60±1.03 ^{a, d}
BUN (mmol L ⁻¹)	7.67±0.75	7.32±0.92	8.00±0.86	7.33±1.56	8.78±1.13
CRE (μmol L ⁻¹)	49.66±3.00	49.37±4.55	48.49±5.12	49.29±9.61	56.47±15.69
CHO (mmol L ⁻¹)	2.08±0.20 ^d	2.14±0.26 ^d	2.21±0.31	2.46±0.30 ^{a, b}	2.20±0.24
TG (mmol L ⁻¹)	0.97±0.21	0.98±0.17	0.96±0.13	1.02±0.25	1.14±0.20
Na (mmol L ⁻¹)	130.5±8.2	130.4±8.8	133.1±6.2	132.8±7.6	135.4±16.5
K (mmol L ⁻¹)	6.5±0.8	6.6±0.8	6.4±0.6	6.6±0.3	6.5±0.3
CA (mmol L ⁻¹)	1.4±0.1	1.4±0.1	1.4±0.1	1.4±0.0	1.4±0.1

Note: Data are given as mean ± SD (n = 10). ^a: statistically significant different from the negative control group at *P*<0.05; ^b: statistically significant different from the low dose group at *P*<0.05; ^c: statistically significant different from the middle dose group at *P*<0.05; ^d: statistically significant different from the high dose group at *P*<0.05; ^e: statistically significant different from the positive control group at *P*<0.05.

Effects on phenotypic analysis of peripheral blood lymphocytes

Compared with the negative control group, the percentage of B lymphocytes (CD³⁺CD¹⁹⁺) was increased with the dosage of MLT and showed statistical difference in the high-dose group (*P*<0.05) (Fig.2); the percentage of Ts lymphocytes (CD³⁺CD⁸⁺) in the middle-dose group was higher than that of the negative control group (*P*<0.05), but there was no dose-response relationship (Table 5) .

Table 5 Effects on phenotypic analysis of peripheral blood lymphocytes in mice

Group	CD3 ⁺ CD19 ⁺ (%)	CD3 ⁺ CD19 ⁻ (%)	CD3 ⁺ CD49 ⁺ (%)	CD3 ⁺ CD4 ⁺ (%)	CD3 ⁺ CD8 ⁺ (%)	CD4 ⁺ /CD8 ⁺
Negative control group	29.2±4.7 ^{d, e}	63.5±5.5 ^e	5.5±1.9	52.4±5.8 ^e	11.1±1.0 ^c	4.8±0.8 ^e
Low-dose group	30.4±10.6 ^e	62.8±11.2 ^e	6.4±2.1	52.0±11.1 ^e	10.9±1.3	4.8±1.2 ^e
Middle-dose group	36.1±9.2 ^e	56.8±10.0 ^e	7.4±3.2	47.3±10.3 ^e	9.5±1.3 ^a	5.1±1.4 ^e
High-dose group	37.0±7.3 ^{a, e}	56.0±6.8 ^e	6.1±1.0	46.1±6.2 ^e	10.0±1.8	4.7±1.0 ^e
Positive control group	7.5±8.4 ^{a, b, c, d}	84.6±7.9 ^{a, b, c, d}	5.4±2.9	74.2±8.8 ^{a, b, c, d}	10.4±1.9	7.5±2.1 ^{a, b, c, d}

Note: Data are given as mean ± SD (n = 10). ^a: statistically significant different from the negative control group at $P<0.05$; ^b: statistically significant different from the low dose group at $P<0.05$; ^c: statistically significant different from the middle dose group at $P<0.05$; ^d: statistically significant different from the high dose group at $P<0.05$; ^e: statistically significant different from the positive control group at $P<0.05$.

Effects on the number of splenocytes and marrow cells

There was no significant difference in the number of splenocytes ($P>0.05$) (Table 6).

Table 6 Effects on the splenocytes count and parameters of humoral immunity, cellular immunity and non-specific immunity of mice under MLT exposure

Group	Negative control group	Low dose group	Middle dose group	High dose group	Positive control group
Number of splenocyte (10 ⁸ /g spleen)	7.09±2.01 ^e	9.76±2.81 ^e	8.24±1.81 ^e	8.10±2.06 ^e	1.89±1.38 ^{a, b, c, d}
PFC (/10 ⁶ splenocytes)	30.0±16.5 ^{d, e}	24.8±23.4	22.4±14.4	14.0±15.9 ^a	15.2±8.4 ^a
HC ₅₀	276.2±74.8	260.2±56.3	256.2±71.3	242.7±39.6	227.8±48.8
IgA (μg ml ⁻¹)	8.64±6.52	5.68±6.48 ^d	4.96±3.84 ^d	11.84±5.04 ^{b, c, e}	0.00±0.00 ^d
IgG (μg ml ⁻¹)	1440.0±657.0 ^d	1340.5±1251.5 ^d	1840.5±688.0 ^d	4503.0±1915.0 ^{a, b, c, e}	1246.4±533.0 ^d
IgM (μg ml ⁻¹)	92.4±61.6	97.6±39.2	114.4±38.6	147.6±70.0	124.3±51.8
IL-2(pg ml ⁻¹)	2.90±0.89	2.74±0.36	2.57±1.06	3.10±0.58	2.63±0.98
IL-4 (pg ml ⁻¹)	2.16±0.31	2.07±0.42	2.25±0.43	2.43±0.31	2.52±0.26

IL-5 (pg ml ⁻¹)	15.91±6.10 ^c	13.66±5.93	10.75±3.58 ^a	13.41±6.90	14.69±4.09
IL-10 (pg ml ⁻¹)	11.85±5.43 ^{b, c, e}	6.28±5.11 ^{a, d}	7.94±3.87 ^{a, d}	12.50±9.25 ^{b, c, e}	7.88±3.27 ^{a, d}
IFN (pg ml ⁻¹)	6.49±3.87	5.38±2.03	6.06±3.73	7.38±5.05	4.27±1.28
TNF (pg ml ⁻¹)	7.50±1.26	8.40±4.12	8.41±3.54	7.91±2.32	8.16±1.59
ConA-induced splenocyte proliferation	0.61±0.22 ^e	0.66±0.10 ^e	0.79±0.22 ^e	0.81±0.20 ^e	0.21±0.16 ^{a, b, c, d}
LPS-induced splenocyte proliferation	0.40±0.18 ^e	0.32±0.11 ^e	0.41±0.16 ^e	0.36±0.17 ^e	0.02±0.10 ^{a, b, c, d}
NK cell activity	14.06±16.18	8.61±9.86	4.15±8.44	12.63±5.87	13.08±36.40
Macrophage phagocytosis	6.41±1.60	5.73±1.02	6.40±1.11	5.67±1.76	5.62±4.43
CTL cell activity	3.48±3.54	6.46±9.80	7.72±17.40	2.76±7.12	4.74±5.53
Increase in foot pad thickness (mm)	0.058±0.043 ^e	0.069±0.034 ^e	0.043±0.037	0.074±0.054 ^e	0.019±0.033 ^{a, b, d}

Note: Data are given as mean ± SD (n = 10). ^a: statistically significant different from the negative control group at $P<0.05$; ^b: statistically significant different from the low dose group at $P<0.05$; ^c: statistically significant different from the middle dose group at $P<0.05$; ^d: statistically significant different from the high dose group at $P<0.05$; ^e: statistically significant different from the positive control group at $P<0.05$.

The number of polychromatic normoblast and monocyte series in the medium-dose group were statistically lower than that in the negative control group ($P<0.05$) (Table 7).

Table 7 Effects on classification of bone marrow cells in MLT-exposed BALA/c mice

Group	Negative control	Low dose group	Middle dose group	High dose group	Positive control
Myeloid series					
Promyelocyte	0.9±0.9	0.7±0.7	1.5±1.5 ^e	1.0±1.9	0.1±0.3 ^c
Myelocyte	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Metamyelocyte	0.2±0.6	0.1±0.3	0.1±0.3	0.2±0.4	0.0±0.0
Stab granulocyte	12.0±5.2 ^e	11.0±3.6	12.7±4.5 ^e	9.7±5.0	6.7±5.7 ^{a, c}

Segmented granulocyte	21.2±6.7 ^e	21.0±7.8 ^e	24.6±5.9 ^e	27.6±12.1 ^e	48.2±8.3 ^{a, b, c, d}
Erythrocytes series					
Basophilic erythroblast	2.0±2.5	1.2±1.5	1.0±0.9	1.0±0.8	1.1±1.4
Polychromatic erythroblast	6.0±4.2 ^{b, c, d, e}	2.3±1.6 ^a	2.4±1.5 ^a	1.4±1.5 ^a	1.2±1.5 ^a
Orthochromatic erythroblast	6.7±4.0	7.9±4.5	6.9±3.6	7.0±2.4	7.2±3.7
Lymphocytic series	50.0±10.9 ^e	55.1±6.6 ^e	50.6±7.6 ^e	52.6±13.1 ^e	35.5±8.0 ^{a, b, c, d}
Monocytic series	1.0±0.8 ^{b, c, d, e}	0.1±0.3 ^a	0.2±0.4 ^a	0.5±0.7 ^{a, e}	0.0±0.0
Other myeloid cell series	0.0±0.0	0.1±0.3	0.0±0.0	0.0±0.0	0.0±0.0

Note: Data are given as mean ± SD (n=10). ^a: statistically significant different from the negative control group at $P<0.05$; ^b: statistically significant different from the low dose group at $P<0.05$; ^c: statistically significant different from the middle dose group at $P<0.05$; ^d: statistically significant different from the high dose group at $P<0.05$; ^e: statistically significant different from the positive control group at $P<0.05$.

Histopathological examination

Histological examinations were performed and pathological changes were observed, such as atrophy of splenic white pulp in the low-dose group (1/10); hepatocellular necrosis in a small lesion with a little of inflammatory cells in filtrates in the medium-dose group (1/10); edema of hepatocytes (8/10) and mild atrophy in thymus in the high-dose group (1/10). In the positive control group, more pathology changes were found, including: moderate splenic atrophy (3/10), reduction or disappearance of germinal center in lymphonodulus, mild splenic atrophy (7/10); mild thymic atrophy (7/10) and moderate thymic atrophy (3/10); lymphoid node atrophied, follicle structure vanished or unclear, lymphocyte reduction, fibrous tissue hyperplasia (10/10); Peyer's patches atrophy, follicle structure unclear, reduction or disappearance of germinal center (10/10); decrease in the number of bone marrow stem cells and adipose hyperplasia (10/10) (Fig.3). The splenic germinal center in the medium-dose group were significantly greater than

that in the negative control group ($P<0.05$), however, there was no dose-response relationship (Table 8).

Tissues and organs not mentioned above had no visible pathological changes.

Table 8 Effects on spleen and Peyer's patches in BALA/c mice after 30 days of feeding

Group	Negative control	Low dose group	Middle dose group	High dose group	Positive control
Spleen					
Periarterial lymphatic sheath (μm)	470.46 $\pm$ 58.00 ^e	462.73 $\pm$ 62.56 ^e	486.12 $\pm$ 73.08 ^e	474.15 $\pm$ 69.01 ^e	292.85 $\pm$ 61.46 ^{a, b, c, d}
Germinal center (μm)	87.82 $\pm$ 79.19 ^c	95.78 $\pm$ 40.47	129.17 $\pm$ 41.78 ^a	114.86 $\pm$ 20.41	120.23 $\pm$ 35.14
Peyer's patch					
Lymphoid follicle (μm)	473.28 $\pm$ 80.13	493.18 $\pm$ 69.98	504.61 $\pm$ 119.99	473.21 $\pm$ 86.22	383.32 $\pm$ 45.53
Germinal center (μm)	286.59 $\pm$ 85.80 ^e	314.34 $\pm$ 142.89 ^e	281.13 $\pm$ 157.24 ^e	306.06 $\pm$ 77.61 ^e	221.08 $\pm$ 57.34 ^{a, b, c, d}

Note: Data are given as mean $\pm$ SD (n = 10). ^a: statistically significant different from the negative control group at $P<0.05$; ^b: statistically significant different from the low dose group at $P<0.05$; ^c: statistically significant different from the middle dose group at $P<0.05$; ^d: statistically significant different from the high dose group at $P<0.05$; ^e: statistically significant different from the positive control group at $P<0.05$.

Humoral immunity

PFC and HC₅₀

The number of plaque-forming cells was decreased with the rising dose of MLT, and was significantly lower in the high-dose group than that in the negative control group ($P<0.05$) (Fig. 4). No significant change of HC₅₀ was observed (Table 6).

Serum immunoglobulin and cytokines

IgG level in the high-dose group was significantly higher than that in the negative control, the low-dose and the middle dose groups, while there was no dose-response relationship. But the sera IgA level of the high dose group was higher than that in the low-dose and the middle-dose groups ($P<0.05$). And there was no significant difference in IgM level among the groups ($P>0.05$) (Table 6, Fig. 5).

As shown in Table 6, IL-5 level of the middle-dose group was significantly lower than that of the negative control group ($P<0.05$) ; IL-10 levels in the low-dose and the medium-dose groups were significantly lower than that in the high-dose group, and IL-10 level in the low-dose group was significantly lower than that in the negative control group ($P<0.05$).

Cellular immunity

The mitogen-induced splenic lymphocyte proliferation, CTL cell activity, and DTH reaction were used to detect cellular immunity. Compared with the negative control group, CY inhibited the mitogen-induced splenic lymphocyte proliferation and the reaction of DTH. None of these parameters in the high-dose group were found to be significantly different (Table 6).

Non-specific immunity

No significant changes were observed in either the carbon clearance test or the NK cell activity test ($P>0.05$) (table 6).

Discussion

A comprehensive and systematic immunotoxicological evaluation of pesticides will help managers further understand the possible health risks caused by the pesticides and formulate reasonable and

standardized management regulations. The purpose of this study was to explore the effects of MLT on the immune system, including the extent of these adverse reaction, and provide the basis data for the standardized management of pesticides.

As a powerful immunosuppressant, CY was commonly used to establish an immune suppressive animal model in the immunotoxicology study of food additives or chemicals (19). According to the previous study, it had been shown that a single dose of 200 mg/kg bw of CY via intraperitoneal injection 24h before the terminal of study was recommended, since it was able to effectively reduce the immune functions of animals (20). In our study, mice in the positive control group were treated with this method for the purpose of establishing an immunosuppressed model. Compared with the negative control group, CY significantly decreased the spleen weight, WBC value, LYM value, MON value, NRUT value, % B cells, PFC/10⁶ splenocytes, mitogen-induced splenic lymphocyte proliferation, and the reaction of DTH as well, and significantly increased the GLU value, % T cells, % Th cells and Th/Ts ratio. In addition, histopathology changes in spleen, thymus, lymph nodes and Peyer's lymph nodes were also observed. These results were consistent with the previous studies on the immunosuppressive model, which were induced by CY (21, 22). It could be concluded that the immunosuppressed animal model had been successfully established as the positive control in this study.

The WBC count with differential measured the amount of leukocytes and the percentages of each type, including the percentage of NEUT, basophils, eosinophils, monocytes and lymphocytes; and provided information about the immune system (23). With the increase of MLT concentration, the leukocyte level decreased and showed an obvious dose-response relationship in this study. Both the count and the

percentage of BAS in the high-dose group were significantly lower than those in the other two MLT-treated groups. What's more, as a major effector in both inherent and adaptive immunity of mammals, the NEUT plays a particularly important role in complex immune regulation (24). In general, the concentration of serum NEUT is in a steady-state in the body, and the NEUT level in the blood will decrease when NEUT enter the tissues where inflammation occurs, or bone marrow cells are functionally impaired (25). The reduction of NEUT levels of the low-dose and the middle-dose groups suggested that a relative lower dose of MLT had a negative impact on the neutrophil homeostasis, in turn, the risk of infection might also be dramatically increased.

Peripheral blood lymphocyte phenotype analysis is the most sensitive method to test the immunotoxicity of chemicals with 91% accuracy (26). The present results showed that high-dose of MLT increased the percentage of B lymphocytes (CD3⁺CD19⁺), while the LYM count and the proportion of T lymphocytes (CD3⁺CD19⁻) in the same group had no significant change. It might reflect that the MLT (256 mg/kg bw) had a potential to disrupt the lymphocyte homeostasis in BALA/c mice and the B lymphocyte was much more sensitive to MLT, when compared with the T lymphocyte.

ALT level is commonly used as liver injury biomarker for estimating hepatic injury in experimental animals and clinical diagnosis of patients (27). According to the clinical chemistry parameters in the present study, the ALT level in the high-dose group was significantly increased while the levels of ALB and TP were reduced. In the meantime, hepatocyte necrosis in a small lesion with a little of inflammatory cell infiltration in the middle-dose group, as well as edema of hepatocytes in the high-dose group were observed. Similar results had been reported in 2 cases of drug-induced hepatic injury (DILI)

(28) and some hepatotoxicity studies of MLT, including histopathological and hematological changes (29-31). In addition to that, the absolute and relative weights of liver in the high-dose group were increased, which might be induced by the metabolism of MLT. All the evidence listed above indicated that MLT (258 mg/kg bw) can induce liver injury in mice.

Except for the hepatic damage that had been discussed before, atrophy in white pulp of spleen (1/10) in the low-dose group and mild atrophy of thymus in the high-dose group (1/10) were also cases for concern. Similar results were reported in the study of Ojha *et al.*, that both acute and chronic exposure (LD₅₀ and 0.5 LD₅₀, respectively) of MLT caused splenic pathological changes (32). What's more, their study clearly demonstrated that the DNA damage index of the spleen in the MLT-treated group was statistically significant when compared with the control group. The results of another two studies supported the adverse effect induced by MLT to the thymocytes in vitro. Investigators exposed murine thymocytes to MLT and found a concentration-dependent increase of apoptosis after 12 h incubation (33). Further research, carried out by Olgun *et al.*, later revealed that oxidative stress induced by MLT played a key role in apoptotic of cell death in thymocytes (33). In the high-dose group, the mild atrophy of thymus didn't result in the significant decrease of the absolute and relative weight of it, which might be associated with increased thymus connective tissue.

Either PFC assay or sera immunoglobulin enzyme-linked immunosorbent assay (ELISA) were recommended by US EPA to assess the potential immunotoxicity of chemicals (34). In particular, as one of the most sensitive methods, PFC assay has been proved to have a tight association with immunotoxicity (78% concordance) (35). Humoral immunity tests showed that, with the increase of

MLT concentration, the number of plaques per 10^6 splenocytes was decreased in a dose-dependent manner, and showed the maximum inhibition in the high-dose group. Considering the mild splenic atrophy (1/10) in the high-dose group, the observed linear dose-response relationship in Fig.5 indicated that splenic toxicity of MLT was strengthened with the increasing of concentration. Similar trend was found in a 30-day study (36), which demonstrated the higher dose of MLT will suppress the humoral immunity response. However, the serum IgG level was abnormally elevated in the high-dose group, which was inconsistent with the conclusion of PFC assay. Similar trend of serum IgG level was only found in one study of workers in Poland, as early as 1985, who were occupationally exposed to organophosphorus pesticides, which showed an elevation of serum IgG level as compared to the nonexposed participants(37). The possible reasons for the dramatic difference in the distribution of IgG in serum and spleen might be as follows: a) the impaired function of spleen resulted in a decrease in the level of IgG synthesized in the lymph nodes of the spleen, but the function of lymph nodes in other tissues was normal, b) the repeated high dose of MLT as an antigen stimulated the plasma cells (effector B cells) to secrete more IgG, c) intestinal metabolizing toxicants of MLT might cause IgG elevation, d) immune dysfunction with unknown mechanism. The mechanisms behind the results is unknown and need to be further explored.

Furthermore, IL-10 levels in the low-dose and medium-dose groups were significantly decreased and IL-5 level in the medium-dose group was much lower than that in the negative control group. The IL-10 was mainly produced by T lymphocytes, B lymphocytes, macrophages, granulocytes, dendritic cells (DCs), etc. (38), and was a recognized inflammatory and immunosuppressive factor involved in

inflammatory response and immune reaction. Therefore, the reduction of IL-10 levels in the low-dose and the middle-dose groups were considered to be related to the decrease of neutrophils, which were the majority of the granulocytes and had the function of secreting IL-10.

In addition, exposure to MLT had no effect on the phagocytic function of splenic macrophages, the mitogen-induced splenic lymphocyte proliferation, the cytotoxic T-lymphocyte response, the serum half hemolysis value, the increase of foot pad thickness and the NK cell activity, which indicate that the cellular immune functions and non-specific immune functions in BALA/c mice might not sensitive to MLT.

MLT mainly had adverse effects on immunopathological and humoral immune parameters, and could induce the disruption of immune homeostasis in BALB/c mice at a relative low dose. It was preliminarily concluded that the LOAEL of MLT on immune system was 16 mg/kg bw. In accordance with the "Guidelines for the Acceptable Daily Intake (ADI) of Pesticides" published by the Ministry of Agriculture (MOA) of the People's Republic of China (Notice No. 1825), the safety factor was set at 1000 due to the uncertainty of derivation from LOAEL to NOAEL, and the differences of interspecific and intraspecific (39). Derived from the LOAEL, the ADI of malathion was 0.016 mg/kg bw. It is much lower than the current regulation of 0.3 mg/kg bw, which was assigned by the Joint WHO/FAO Meeting on Pesticide Residues in 1997 (40) and reaffirmed in 2016 (41). The conclusion was based on the NOAEL of 29 mg/kg bw in a chronic study of toxicity and carcinogenicity in rats, using a 100-fold safety factor. It suggests that the immunotoxicity is more sensitive to malathion than other toxicity observation

end points. Therefore, it may be considered necessary to include immunotoxicity assessment information in the pesticide registration management.

Conclusions

In view of the need of pesticides safety management, the result of this study can be a considerable reference in laying down a residue criterion of malathion, deriving a more reliable ADI and developing an effective and feasible guidelines for pesticide immunotoxicity test.

Abbreviations

MLT, Malathion; **CY**, Cyclophosphamide; **PFC**, Plaque-forming cell; **DTH**, Delayed-type hypersensitivity; **LOAEL**, Lowest observed adverse effect level; **ADI**, Acceptable daily intake; **UV**, Ultraviolet; **IARC**, International Agency for Research on Cancer; **US EPA**, United States Environment Protection Agency; **NK cell**, Natural killer cell; **SRBC**, Sheep red blood cells; **ELISA**, Enzyme-linked immunosorbent assays; **ConA**, Concanavalin A; **LPS**, Lipopolysaccharide; **HBSS**, Hanks' balanced salt solution; **PBS**, Phosphate buffered saline; **SPF**, Specific pathogen free; **RPMI**, Roswell park memorial institute; **EDTA**, Ethylenediaminetetraacetic; **WBC**, White blood cell count; **RBC**, Red blood cell; **HGB**, Hemoglobin; **HCT**, Hematocrit; **MCV**, Mean corpuscular volume; **MCH**, Mean corpuscular hemoglobin; **MCHC**, Mean corpuscular hemoglobin concentration; **RDW**, Red blood cell distribution; **PLT**, Platelet count; **PCT**, Prothrombin consumption test; **MPV**, Mean platelet volume; **PDW**, Platelet distribution width; **LYM**, Lymphocyte; **MON**, Monocyte; **NEUT**, Neutrophil; **EOS**, Eosinophil; **BAS**, Basophilic granulocyte; **ALT**, Alanine aminotransferase; **AST**, Aspartate aminotransferase; **TP**, Total protein; **ALB**, Albumin; **ALP**, Alkaline phosphatase; **GLU**, Glucose; **BUN**, Brea nitrogen; **CRE**,

Creatinine; **CHO**, Cholesterol; **TG**, Triglyceride; **NA**, Sodium; **K**, Potassium; **CA**, Calcium; **FITC**, Fluorescein isothiocyanate; **IgG**, Immunoglobulin G; **IgA**, Immunoglobulin A; **IgM**, Immunoglobulin M; **TMB**, Tetramethylbenzidine; **IL-2**, Interleukin-2; **IL-4**, Interleukin-4; **IL-5**, Interleukin-5; **IL-10**, Interleukin-10; **IFN- γ** , Interferon- γ ; **TNF**, Tumor necrosis factor; **MTT**, Thiazoyl blue tetrazolium bromide solution; **CTL**, Cytotoxic T-lymphocyte; **LDH**, Lactate dehydrogenase; **MOA**, Ministry of Agriculture; **NOAEL**, No observed adverse effect level; **DCs**, dendritic cells; **WHO**, World Health Organization; **FAO**, Food and Agriculture Organization of the United Nations.

Reference

1. Koch E, Clark JM, Cohen B, Meinking TL, Ryan WG, Stevenson A, et al. Management of Head Louse Infestations in the United States-A Literature Review. *Pediatric dermatology*. 2016;33(5):466-72.
2. Wananukul S, Chatproedprai S, Tempark T, Wananukul W. Clinical response and safety of malathion shampoo for treatment of head lice in a primary school. *Journal of the Medical Association of Thailand = Chotmailhet thangphaet*. 2011;94(4):465-9.
3. Li W, Zhao Y, Yan X, Duan J, Saint CP, Beecham S. Transformation pathway and toxicity assessment of malathion in aqueous solution during UV photolysis and photocatalysis. *Chemosphere*. 2019;234:204-14.
4. Ishag AE, Abdelbagi AO, Hammad AM, Elsheikh EA, Elsaid OE, Hur JH, et al. Biodegradation of Chlorpyrifos, Malathion, and Dimethoate by Three Strains of Bacteria Isolated from Pesticide-Polluted Soils in Sudan. *Journal of agricultural and food chemistry*. 2016;64(45):8491-8.

- 508 5. Kim KH, Kabir E, Jahan SA. Exposure to pesticides and the associated human health effects. The
509 Science of the total environment. 2017;575:525-35.
- 510 6. Sharma VK, Kaur S. Contact sensitization by pesticides in farmers. Contact dermatitis.
511 1990;23(2):77-80.
- 512 7. Beard JD, Umbach DM, Hoppin JA, Richards M, Alavanja MC, Blair A, et al. Pesticide exposure
513 and depression among male private pesticide applicators in the agricultural health study. Environmental
514 health perspectives. 2014;122(9):984-91.
- 515 8. Shrestha S, Parks CG, Goldner WS, Kamel F, Umbach DM, Ward MH, et al. Pesticide Use and
516 Incident Hypothyroidism in Pesticide Applicators in the Agricultural Health Study. Environmental health
517 perspectives. 2018;126(9):97008.
- 518 9. Koutros S, Beane Freeman LE, Lubin JH, Heltshe SL, Andreotti G, Barry KH, et al. Risk of total
519 and aggressive prostate cancer and pesticide use in the Agricultural Health Study. American journal of
520 epidemiology. 2013;177(1):59-74.
- 521 10. Band PR, Abanto Z, Bert J, Lang B, Fang R, Gallagher RP, et al. Prostate cancer risk and exposure
522 to pesticides in British Columbia farmers. The Prostate. 2011;71(2):168-83.
- 523 11. Guyton KZ, Loomis D, Grosse Y, El Ghissassi F, Benbrahim-Tallaa L, Guha N, et al.
524 Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate. The Lancet
525 Oncology. 2015;16(5):490-1.
- 526 12. Salama M, Lotfy A, Fathy K, Makar M, El-Emam M, El-Gamal A, et al. Developmental neurotoxic

527 effects of Malathion on 3D neurosphere system. *Applied & translational genomics*. 2015;7:13-8.

528 13. Ji Q, Lee J, Lin YH, Jing G, Tsai LJ, Chen A, et al. Atrazine and malathion shorten the maturation
529 process of *Xenopus laevis* oocytes and have an adverse effect on early embryo development. *Toxicology*
530 *in vitro* : an international journal published in association with BIBRA. 2016;32:63-9.

531 14. Flessel P, Quintana PJ, Hooper K. Genetic toxicity of malathion: a review. *Environmental and*
532 *molecular mutagenesis*. 1993;22(1):7-17.

533 15. Voccia I, Blakley B, Brousseau P, Fournier M. Immunotoxicity of pesticides: a review. *Toxicology*
534 *and industrial health*. 1999;15(1-2):119-32.

535 16. EPA. Health Effects Test Guidelines OPPTS 870.7800 Immunotoxicity. In: EPA US, editor.
536 Washington1998. p. 1-11.

537 17. EPA. Biochemicals test guidelines. OPPTS 880.3800 Immune response. In: U.S.EPA, editor.
538 Washington1996. p. 1-6.

539 18. EPA. Biochemicals test guidelines. OPPTS 880.3550 Immunotoxicity. In: U.S.EPA, editor. 1996. p.
540 1-12.

541 19. Hinton DM. US FDA "Redbook II" Immunotoxicity Testing Guidelines and Research in
542 Immunotoxicity Evaluations of Food Chemicals and New Food Proteins. *Toxicologic Pathology*.
543 2000;28:467-78.

544 20. Yan S, Xudong J, Wenming C, Qiannan Z, Yongning L, Lin Y, et al. Comparison research of
545 immunosuppression models induced by different ways and doses of cyclophosphamide in mice. *Chinese*

- 546 Journal of Food Hygiene. 2013;25(3):218-25.
- 547 21. Shand F. The Immunopharmacology of Cyclophosphamide. Immunopharmac. 1979;1:165-71.
- 548 22. Marxfeld HA, Kuttler K, Dammann M, Groters S, van Ravenzwaay B. Variance of body and organ
549 weights in 28-day studies in mice. Regulatory toxicology and pharmacology : RTP. 2019;108:104472.
- 550 23. George-Gay B, Parker K. Understanding the complete blood count with differential. Journal of
551 perianesthesia nursing : official journal of the American Society of PeriAnesthesia Nurses.
552 2003;18(2):96-114; quiz 5-7.
- 553 24. Scapini P, Cassatella MA. Social networking of human neutrophils within the immune system.
554 Blood. 2014;124(5):710-9.
- 555 25. Mayadas TN, Cullere X, Lowell CA. The Multifaceted Functions of Neutrophils. Annual Review
556 of Pathology: Mechanisms of Disease. 2014;9(1):181-218.
- 557 26. Risk Assessment in Immunotoxicology: I. Sensitivity and Predictability of Immune Tests, 18 (1992).
- 558 27. McGill MR. The past and present of serum aminotransferases and the future of liver injury
559 biomarkers. EXCLI journal. 2016;15:817-28.
- 560 28. Radovanovic M, Dushenkovska T, Cvorovic I, Radovanovic N, Ramasamy V, Milosavljevic K, et
561 al. Idiosyncratic Drug-Induced Liver Injury Due to Ciprofloxacin: A Report of Two Cases and Review
562 of the Literature. The American journal of case reports. 2018;19:1152-61.
- 563 29. Severcan C, Ekremoglu M, Sen B, Pasaoglu OT, Akyurek N, Severcan SM, et al. Acute effects of

564 different doses of malathion on the rat liver. *Clinical and experimental hepatology*. 2019;5(3):237-43.

565 30. Flehi-Slim I, Chargui I, Boughattas S, El Mabrouk A, Belaid-Nouira Y, Neffati F, et al. Malathion-
566 induced hepatotoxicity in male Wistar rats: biochemical and histopathological studies. *Environmental*
567 *science and pollution research international*. 2015;22(22):17828-38.

568 31. Hana RS, Saed N. Alteration in Oxidants, Antioxidants and Cytokines Levels in Blood of Malathion
569 Exposed Human and Animal Groups and the Effect of Flaxseed Oil in Alleviating Malathion Toxic
570 Effects. *European Journal of Biotechnenology and Bioscience*. 2013;1(1):8-19.

571 32. Ojha A, Yaduvanshi SK, Pant SC, Lomash V, Srivastava N. Evaluation of DNA damage and
572 cytotoxicity induced by three commonly used organophosphate pesticides individually and in mixture,
573 in rat tissues. *Environmental Toxicology*. 2013;28(10):543-52.

574 33. Olgun S, Gogal RM, Jr., Adeshina F, Choudhury H, Misra HP. Pesticide mixtures potentiate the
575 toxicity in murine thymocytes. *Toxicology*. 2004;196(3):181-95.

576 34. Johnson CW, Williams WC, Copeland CB, DeVito MJ, Smialowicz RJ. Sensitivity of the SRBC
577 PFC assay versus ELISA for detection of immunosuppression by TCDD and TCDD-like congeners.
578 *Toxicology*. 2000;156(1):1-11.

579 35. Michael I.Luster, Christopher Portier, D. Gayla Pait, Gary J. Rosenthal, Dori R.Germolec,
580 Emanuela Corsini, et al. Risk Assessment in Immunotoxicology II. Relationships between Immune and
581 Host Resistance Tests. *Fundamantal And Applied Toxicology*. 1993;21:71-82.

582 36. Victor J. Johnson, Alan M. Ossenberrg, Kwang Lee, Blakley BR. Increased T-lymphocyte dependent

583 antibody production in female SJL J mice following exposure to commercial grade malathion.

584 Toxicology. 2002;170:119-29.

585 37. Kossmann S, Konieczny B, Panek E. Immuno-electrophoretic Data and Serum Immunoglobulin G,

586 A and M Levels in Workers Engaged in the Production of Organophosphate Pesticides. Medycyna pracy.

587 1985;36(1):27-30.

588 38. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. Nature reviews

589 Immunology. 2010;10(3):170-81.

590 39. Planting Industry Management Department MoA. Guidelines for the acceptable daily intake (ADI)

591 of pesticides. In: (MOA) TmoaotPsRoC, editor. Beijing2012.

592 40. Pesticide residues in food-1997. Report. Report of the Joint Meeting of the FAO Panel of Experts

593 on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. Report No.:

594 FAO Plant Production and Protection Paper, 145, 1998.

595 41. Pesticide residues in food-2016. Report of the Joint Meeting of the FAO Panel of Experts on

596 Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. Report No.:

597 FAO Plant Production and Protection Paper, 227, 2017.

598 **Declarations**

599 Ethics approval and consent to participate

600 The study was approved by Animal Experimental Welfare & Ethical Inspection Committee,

601 China National Center for Food Safety Risk Assessment. Animal experiments and feeding processes

were operated according to the laboratory animal administration rules of the Ministry of Science and Technology of the People's Republic of China.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Key Research and Development Program of China (2018YFC1603003) and Special Fund for China Agro-scientific Research in the Public Interest (200903054-06).

Authors' Contributions

YS, NL conceived and designed the experiments. YS, LY, WFM, XX, CHLL, WW, HY, XDJ and ZHPL performed the experiments. MHL, YS, YW and WW analyzed the data. YS, CHLL and WW contributed reagents/materials/analysis tools. MHL and YS wrote the paper. All authors read and approved the final manuscript.

Authors' Contact information

Minhan Lou Email: louminhan@zjams.com.cn

622 Ling Yong Email: yongling@cfsa.net.cn

623 Weifeng Mao Email: maoweifeng@cfsa.net.cn

624 Xiao Xiao Email: xiaoxiao@cfsa.net.cn

625 Chunlai Liang Email: liangchunlai@cfsa.net.cn

626 Wei Wang Email: wangwei@cfsa.net.cn

627 Hui Yang Email: yanghuig@cfsa.net.cn

628 Xudong Jia Email: jiaxudong@cfsa.net.cn

629 Zhaoping Liu Email: liuzhaoping@cfsa.net.cn

630 Yin Wang Email: wy3333@163.com

631 Ning Li Email: lining@cfsa.net.cn

632 Yan Song Email: songyan@cfsa.net.cn

633 **Acknowledgements**

634 Not applicable.

635 **Figure titels and legends**

636 **Fig. 1 Effect on the number of WBC of BALA/c mice.** Note: *statistically significant different from the
637 negative control group and the positive control group at $P<0.05$. The number of WBC was decreased with
638 the increasing of MLT concentration, and the WBC level in the high-dose group was significantly lower
639 than that in the negative control group.

Fig. 2 Effects on T lymphocyte (CD3⁺CD19⁻) and B lymphocyte (CD3⁻CD19⁺) after exposed to MLT for 30 days.

Fig. 3 Histopathology of various tissues of control groups and MLT treated groups in BALA/c mice.

Photomicrographs of histological examination of liver, kidney, spleen, thymus, bone marrow, mesenteric lymph node and Peyer's lymph node (a, b, c, d, e, f and g) (100×) of BALA/c mice in the negative control group, the low-dose group, the medium-dose group, the high-dose group and the positive control group.

Fig. 4 Effects on the numbers of plaque-forming cells (PFC) after exposure to MLT. Note: * statistically significant different from the negative control group at $P<0.05$. The PFC number per 10^6 splenocytes of the high-dose group was significantly lower than that in the negative control group.

Fig. 5 The serum IgG level of each group. Note: *statistically significant different from the negative control group, the low-dose group, medium-dose group and the positive control group at $P<0.05$. The serum IgG level was abnormal elevated in the high-dose group.

Figures

Figure 1

Effect on the number of WBC of BALA/c mice. Note: *statistically significant different from the negative control group and the positive control group at $P < 0.05$. The number of WBC was decreased with the increasing of MLT concentration, and the WBC level in the high-dose group was significantly lower than that in the negative control group.

Figure 1

Effect on the number of WBC of BALA/c mice. Note: *statistically significant different from the negative control group and the positive control group at $P < 0.05$. The number of WBC was decreased with the increasing of MLT concentration, and the WBC level in the high-dose group was significantly lower than that in the negative control group.

Figure 2

Effects on T lymphocyte (CD3+CD19-) and B lymphocyte (CD3-CD19+) after exposed to MLT for 30 days.

Figure 2

Effects on T lymphocyte (CD3+CD19-) and B lymphocyte (CD3-CD19+) after exposed to MLT for 30 days.

Figure 3

Histopathology of various tissues of control groups and MLT treated groups in BALA/c mice. Photomicrographs of histological examination of liver, kidney, spleen, thymus, bone marrow, mesenteric lymph node and Peyer's lymph node (a, b, c, d, e, f and g) (100×) of BALA/c mice in the negative control group, the low-dose group, the medium-dose group, the high-dose group and the positive control group.

Figure 3

Histopathology of various tissues of control groups and MLT treated groups in BALA/c mice. Photomicrographs of histological examination of liver, kidney, spleen, thymus, bone marrow, mesenteric lymph node and Peyer's lymph node (a, b, c, d, e, f and g) (100×) of BALA/c mice in the negative control group, the low-dose group, the medium-dose group, the high-dose group and the positive control group.

Figure 4

Effects on the numbers of plaque-forming cells (PFC) after exposure to MLT. Note: * statistically significant different from the negative control group at $P < 0.05$. The PFC number per 10⁶ splenocytes of the high-dose group was significantly lower than that in the negative control group.

Figure 4

Effects on the numbers of plaque-forming cells (PFC) after exposure to MLT. Note: * statistically significant different from the negative control group at $P < 0.05$. The PFC number per 10⁶ splenocytes of the high-dose group was significantly lower than that in the negative control group.

Figure 5

The serum IgG level of each group. Note: *statistically significant different from the negative control group, the low-dose group, medium-dose group and the positive control group at $P < 0.05$. The serum IgG level was abnormal elevated in the high-dose group.

Figure 5

The serum IgG level of each group. Note: *statistically significant different from the negative control group, the low-dose group, medium-dose group and the positive control group at $P < 0.05$. The serum IgG level was abnormal elevated in the high-dose group.