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Differential immunomodulatory effects of six pesticides of different chemical classes on human monocyte-derived macrophage functions

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1

¹ Abbreviations: Catalase (CAT), Dimethyl sulfoxide (DMSO), Enzyme-linked immunosorbent assay (ELISA), Hank's balanced salt solution (HBSS), Heme oxygenase 1 (HMOX1), Human monocyte-derived macrophages (hMDMs), Interleukin- (IL-), Lactate dehydrogenase (LDH), Lipopolysaccharide (LPS), nicotinamide adenine dinucleotide phosphate (NADPH), NADPH oxidase 2 (NOX2), NADPH dehydrogenase quinone 1 (NQO1), Nuclear factor erythroid 2-related factor 2 (NRF2), Peripheral blood mononuclear cells (PBMC), Protein kinase C (PKC), Reactive oxygen species (ROS), Serum-free medium (SFM), Superoxide dismutase (SOD), Toll-like receptor (TLR), Tumor Necrosis Factor (TNF), 12-O-Tetradecanoylphorbol 13-acetate (TPA), Non-opsonized zymosan (ZNO)

1 **1. Introduction**

2 Pesticides are widely used in agriculture for the prevention of fungal diseases or the elimination of plant pests
3 such as insects and mites. Pesticides are also used to eliminate unwanted plants (grass, weeds, and brush) in
4 ornamental landscaping, parks, and gardens, and to control vector-borne diseases (e.g., malaria, dengue). This
5 intensive use of pesticides causes environmental contamination and increases the risk of exposure to the general
6 population through pesticide residues in water (10% exposure risks) and food (90% exposure risk) (Jakubowski
7 and Trzcinka-Ochocka, 2005; Kapka-Skrzypczak et al., 2011; Baldi et al., 2014; Bureau et al., 2020). Many studies
8 have shown a positive correlation between the presence of pesticide residues in food, their transfer into the body,
9 and certain pathologies (MacIntosh et al., 2001; Curl et al., 2003, Lu et al., 2006). *In utero* exposure to pesticides
10 has also been confirmed (Krieger, 2010, Tagiyeva et al., 2010). Agricultural workers can be exposed to pesticides,
11 as shown in studies on field crops (Lebailly et al., 2009; Aprea et al., 2016), vineyards (Baldi et al., 2006; Fustinoni
12 et al., 2014), and fruit crops such as apples (Hines et al., 2011; Moon et al., 2013; Kim et al., 2015; Lee et al.,
13 2018; Bureau et al., 2021). Significant exposures to pesticides can lead skin irritation, suggesting dysregulation of
14 skin immune cells (Campbell et al., 2003; Basilicata et al., 2013; Campbell et al., 2017). Pesticides can induce
15 immune cell dysfunction, resulting in immunosuppression leading to infectious diseases (Peden-Adams et al.,
16 2007), or immunostimulation leading to inflammatory and allergic reactions and autoimmune diseases (Yasunaga
17 et al., 2015; Dhouib et al., 2016; Ogasawara et al., 2017). Most *in vitro* immunotoxicology studies have focused
18 on the proliferation and activity of lymphocyte subsets and natural killer cells. For example, several studies have
19 shown that organophosphates and carbamates promote allergies, autoimmune diseases, asthma and respiratory
20 infections inhibiting the cytotoxic activities of cells such as Natural Killer and cytotoxic T lymphocytes (Thrasher
21 et al., 1993; Wilson et al., 2004; Li, 2007; Fukuyama et al., 2009; Corsini et al., 2013; Jorsaraei et al., 2014).
22 Pesticide can also affect the functional state of macrophages (Corsini et al., 2006; Kurpios-Piec et al., 2015; Weis
23 et al., 2019).

24 Macrophages are a group of heterogeneous cells that are partly derived from blood monocytes. In addition to
25 their role in triggering inflammation, macrophages play a key role in resolving inflammation, contribute to
26 repairing and remodeling tissue, and participate in restoring tissue homeostasis (Aggarwal et al., 2014; Hussell
27 and Bell, 2014). Alterations in any of these functions can lead to serious or even fatal consequences (vulnerability
28 to infections, chronic inflammation, defective wound healing, metabolic and autoimmune diseases, and some
29 cancers) (Belchamber and Donnelly, 2017; Fricker and Gibson, 2017; He et al., 2017; Xie et al., 2019). Increased

30 pesticide use thus raises environmental and public health concerns. Emerging evidence suggests that exposure to
31 environmental contaminants plays a role in the development of cancers and neurological, skin, and metabolic
32 diseases (Thrasher et al., 2002; Colosio et al., 2003; Alavanja et al. 2004; Basilicata et al., 2013; Campbell, Baker
33 et al., 2017; Sharma et al., 2018; Hamann et al. 2019), which are related to immune system dysregulation, and
34 potentially to macrophage dysfunction. While the polarization of macrophages by various exogenous or
35 endogenous stimuli, bacterial products, cytokines, chemokines, and necrotic or apoptotic cells is well documented
36 (Gordon and Pluddemann, 2017), few studies have addressed the functional changes of human macrophages by
37 environmental xenobiotics, particularly pesticides. For on murine macrophage cultures, it has been shown that
38 propanil inhibited the activation of the effector functions of these cells (Salazar et al., 2008). Exposure to
39 dithiocarbamates significantly reduced the LPS-induced release of Tumor necrosis factor (TNF)- α in the THP-1
40 human monocytic cell line (Corsini et al., 2006). In the same cell line, chlorpyrifos, an organophosphate
41 insecticide, was shown to induce TNF- α mRNA expression and secretion at high doses (30 and 100 $\mu\text{mol.L}^{-1}$)
42 (Proskocil et al. 2019). In contrast, other *in vitro* studies in murine macrophages showed that a concentration of
43 chlorpyrifos between 0.01 and 10 $\mu\text{mol.L}^{-1}$ would reduce macrophage lysosomal enzyme activity and LPS-induced
44 production of interleukin (IL-) 1β and TNF- α inflammatory cytokines (Helali et al. 2016). It has been suggested
45 that organophosphate and carbamate insecticides inhibit enzymes with serine esterase activity and thus modulate
46 immune response (Corsini et al., 2013; Mokarizadeh et al., 2015). As a result signal transduction pathways may
47 be modulated and the activation of effector functions of immune cells such as the phagocytic function of
48 macrophages may be inhibited (Pipy et al., 1982; Pipy et al., 1983; Dhouib et al., 2016). Dithiocarbamate pesticides
49 such as mancozeb, maneb, zineb, ziram and thiram modulate macrophage functions in macrophage lineages or
50 murine macrophages. Depending on the nature of the pesticide, experimental design and dose, studies show that
51 macrophages are activated and an inflammatory response is induced (Weis et al., 2019) or, conversely, the
52 activation of macrophages is decreased, along with their ability to produce inflammatory mediators (Corsini et al.,
53 2006; Kurpios-Piec et al., 2015).

54 These controversial studies on macrophages have led us to evaluate the impact of several pesticides belonging
55 to different chemical classes, whose effects on the polarization and effector functions of human macrophages
56 remains little studied. In this study, we used human monocyte-derived macrophages (hMDMs) from several
57 screened donors, in contrast to most studies that have used murine or human macrophage cell lines. Indeed, some
58 studies report that cell lines, such as RAW 264.4, are not sufficient for initial screening and assessment of immune
59 reactivity of test products. These cell lines unlike human primary cell-derived macrophages present an incomplete

60 picture of the immunomodulatory properties of the test substances and, may in fact be misleading with respect to
61 the overall bioactive potential of the substances (Merly and Smith, 2017). *In vitro*, we studied the
62 immunomodulatory effects of two insecticides: chlorpyrifos and thiacloprid, and four fungicides: thiophanate,
63 boscalid, dithianon, and captan (Table 1) which have in common their wide use in fruit arboriculture, in particular
64 in the treatment of apple orchards in the South of France. Up to 40 pesticide applications can be made between
65 March and the end of August each year in apple orchards. Half of them are fungicides against *Venturia inaequalis*,
66 which causes apple scab. In 2019, the quantities of each pesticide sold in France were listed: chlorpyrifos (174
67 tons), thiacloprid (0.7 tons), thiophanate (65 tons), boscalid (99 tons), dithianon (72 tons), captan (106 tons). A
68 recent study of apple orchards in 4 regions of France including the South of France, conducted during the 2016
69 and 2017 treatment seasons showed that the contamination of workers and seasonal workers per day was
70 approximately 5.50 mg captan and 3.33 mg dithianon for operators, 24.39 mg captan and 1.84 mg dithianon for
71 re-entry workers, and 5.82 mg captan and 0.74 mg dithianon for harvesters (Bureau et al., 2021). One criticism of
72 this study is that only captan and dithianon were used as exposure markers, but pesticides are known to be present
73 in apples from southern France. These data as well as data on residues found by our partners on apple fruits and
74 leaves (data not presented) led us to test concentrations of pesticides ranging from 0.01 to 10 $\mu\text{mol.L}^{-1}$. Our study
75 seeks to understand how certain pesticides used on apple trees in the South of France, present in apples and
76 therefore potentially ingested by consumers, can impact the immune system by deregulating the functions of
77 macrophage, key cells of the immune system. The immunomodulatory effects of pesticides such as captan,
78 boscalid, dithianon, and thiacloprid are poorly known. We studied the cytotoxicity of the different pesticides at
79 different doses and their effects on cytokine expression in hMDMs stimulated or not with LPS to evaluate the
80 impact of the tested products on the capacity of macrophages to produce cytokines in normal or inflammatory
81 conditions. Finally, we measured the oxidative stress of these cells by studying their ROS production. The study
82 of mRNA expression levels of oxidative stress genes in hMDMs after exposure to these pesticides allowed us to
83 assess the impact of these xenobiotics on the activation of antioxidant mechanisms by pesticides. The aim of this
84 study is to better evaluate the immunotoxic risk induced by the different chemical classes of pesticides that can be
85 found in fruit arboriculture.

86 **Table 1**

87 List of pesticides studied

Name	Chemical family	Class
Chlorpyrifos	Organophosphate	Insecticide (NS)

Thiacloprid	Neonicotinoid	Insecticide (S)
Thiophanate	Benzimidazole	Fungicide (S)
Boscalid	Carboxamide	Fungicide (S)
Dithianon	Quinone	Fungicide (S)
Captan	Phtalimide	Fungicide (NS)

88 NS: non-systemic action; S: systemic action

89 2. Materials and methods

90 2.1. Reagents

91 The two insecticides (chlorpyrifos, thiacloprid) and the four fungicides (methyl-thiophanate, boscalid,
92 dithianon, captan) used in fruit trees were purchased from Sigma-Aldrich (France). These pesticides were
93 dissolved in dimethyl sulfoxide (DMSO) at a final concentration below 0.01% (v/v). DMSO, as well as
94 lipopolysaccharide (LPS), 12-O-Tetradecanoylphorbol 13-acetate (TPA) and zymosan A of *Saccharomyces*
95 *cerevisiae* were also purchased from Sigma-Aldrich (France). Purity of the compounds ranged from 95.2% to
96 99.6% according to manufacturer specification.

97 2.2. Isolation of human monocyte-derived macrophages (hMDMs)

98 Primary human monocytes were obtained from healthy blood donors (EFS - Etablissement français du sang,
99 Toulouse, France) and purified by a density gradient centrifugation method on Lymphoprep (Abcys) as described
100 by Martin-Blondel et al. (2009). Written informed consent was obtained from donors under EFS contract
101 n°21PLER2017-0002. In accordance with Articles L1243-4 and R1243-61 of the Public Health Code, the contract
102 was approved by the French Ministry of Science and Technology. The blood was screened for possible infections:
103 HIV (AIDS), HBV/HCV (Hepatitis B and C), HTLV (Leukemia/Lymphoma) and *Treponema Pallidum* (Syphilis).
104 Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient. Monocytes were isolated from the
105 PBMC by adherence to plastic plates for 2 h in a macrophage serum-free medium (M ϕ -SFM; Life technologies)
106 at 37°C in a humidified atmosphere containing 5% CO₂. After 2 h, the plates were rinsed twice using Hank's
107 Balanced Salt Solution (HBSS, without calcium or magnesium) to remove nonadherent cells. The resulting
108 adherent cells in the M ϕ -SFM medium were >85% monocytes. After 24 h of culture in M ϕ -SFM medium the
109 human monocytes were differentiated into human monocyte-derived macrophages (hMDMs), characterized by
110 decreased ROS production and myeloperoxidase activity. All protocols were performed on screened blood from
111 at least on 3 donors.

2.3. Human monocyte-derived macrophages treatments

The concentrations of pesticides assessed in our study were: 0, 0.01, 0.1, 1 and 10 $\mu\text{mol.L}^{-1}$. These doses were calculated according to: data on the amounts of pesticide residues found in apples, the CANEPA study (Bureau et al., 2021), and the maximum residue limits (MRLs) and acceptable daily intakes (ADIs) published by the European Food Safety Agency (EFSA) for the Joint Food and Agriculture Organization of the United Nations/World Health Organization Meeting on Pesticide Residues (Lukowicz et al., 2018; European Food Safety et al., 2020; Smith et al., 2020). Pesticides solutions ($100 \mu\text{mol.L}^{-1}$) were prepared in DMSO and stored at -20°C . Each aliquot was thawed and used once only. The required dilutions were prepared immediately prior to use in the culture media so that the DMSO level did not exceed a final concentration of 0.01%. This concentration of DMSO did not affect the different assays (viability, ROS, and cytokine production).

hMDMs cells were plated in 96-well plates at 1.5×10^5 monocytes /well or in 48-well plates at 3×10^5 monocytes/well, depending on the protocol. For evaluation of the expression levels of mRNA by qRT-PCR, the hMDMs were treated for 4 h at 37°C in 5% CO_2 with increasing concentrations of pesticides (0, 0.01, 0.1, 1 and $10 \mu\text{mol.L}^{-1}$) or DMSO as vehicle control (0.01% v/v final concentration). For all other protocols, the hMDMs were treated for 24 h at 37°C in 5% CO_2 with increasing concentrations of the different pesticides (0, 0.01, 0.1, 1 and $10 \mu\text{mol.L}^{-1}$) or DMSO as vehicle control (0.01% v/v final concentration).

2.4. Evaluation of cell viability by alamarBlue and LDH assay

Cell viability was determined by measuring lactate dehydrogenase (LDH) levels and by the alamarBlue assay, which has been shown to be more sensitive than MTT (Hamid et al., 2004) . Monocytes were plated in 96-well plates at a concentration of 1.5×10^5 cells/well and treated for 24 h at 37°C with 5% CO_2 , with either the presence or absence of increasing concentrations of pesticides. After 24 h of culture, 50 μL of medium was removed for the LDH assay, then 15 μL of alamarBlue reagent (ThermoFisher Scientific, Inc. cat. DAL1025) was added to 150 μL of cultured M ϕ -SFM, and the cells were incubated for another 2 h at 37°C . Absorbance at 570 nm was measured using a microplate reader (EnVision Perkin Elmer). Percentage of cell viability was calculated using the formula $[(A_t/A_0) \times 100]$, where A_0 = absorbance of cells treated with 0.01% DMSO medium, A_t = absorbance of cells treated with various concentrations of the pesticides. Total cellular LDH was obtained by lysing cells with lysis buffer solution for 45 min before measuring LDH activity. LDH in 50 μL culture medium or cell lysate samples was measured using LDH cytotoxicity assay kit (Pierce) according to the manufacturer's instructions. The percentage of cell lysis of the treated cells was calculated using the following equation: % Cell lysis =

141 [(Compound-treated LDH Activity – Spontaneous LDH activity) / (Maximum LDH Activity – Spontaneous LDH
142 activity)] x 100]. Each experiment was repeated at least four times in triplicate.

143 2.5. Assessment of oxidative hyperactivity

144 To evaluate the impact of pesticides on the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-
145 dependent oxidative hyperactivity (NOX2) of hMDMs, monocytes plated in 96-well plates (1.5×10^5
146 monocytes/well) were cultured for 24 h with either the presence or absence of increasing concentrations of
147 pesticides as described in section 2.3. After 24 h, the culture medium was removed and adherent cells were washed
148 with HBSS and the respiratory burst of macrophages (ROS production) was assessed by chemiluminescence in
149 the presence of 5-amino-2,3-dihydro-1,4-phthalazinedione ($66 \mu\text{mol.L}^{-1}$, luminol; Sigma-Aldrich) using a
150 thermostatically (37°C) controlled luminometer (EnVision of Perkin Elmer). The generation of
151 chemiluminescence was monitored continuously for 90 min in basal conditions after challenging of the
152 macrophages, either with 12-O-tetradecanoylphorbol-13 acetate (TPA, 100 nmol.L^{-1}) or with unopsonized
153 zymosan (ZNO, 50 ng/well), a polysaccharide prepared from the cell wall of *Saccharomyces cerevisiae*. Statistical
154 analysis was performed using the area under the chemiluminescence curve expressed in counts per seconds.

155 2.6. Pro and anti-inflammatory cytokine measurements

156 Monocytes were plated in 48-well plate at 3×10^5 monocytes/well and treated for 24 h at 37°C with 5% CO_2 ,
157 with either the presence or absence of increasing concentrations of six pesticides as described in section 2.3. After
158 24 h of culture, the medium was removed, and adherent cells were washed with HBSS and then cultured for 4 h
159 with either the presence or absence of LPS (10 ng.mL^{-1}) to stimulate pro-inflammatory cytokines production. The
160 release of cytokine was evaluated using enzyme-linked immunosorbent assay (ELISA) for IL-1 β , TNF- α , IL-10
161 (Invitrogen™) as recommended by the manufacturer's protocol.

162 2.7. Analysis of antioxidant gene expression in macrophages

163 Monocytes plated in 48-well plates with 3×10^5 monocytes/well were treated for 4 h at 37°C in 5% CO_2 , with
164 either the presence or absence of increasing concentrations of pesticides. After 4 h, the culture medium was
165 removed, and the adherent cells were washed with HBSS. The cells were then lysed in a solution of RLT buffer
166 with 1% β -Mercapto-ethanol, and the total RNA was obtained with EZ-10 Spin Column (Miniprep Super Kit, Bio
167 Basic) in accordance with the manufacturer's protocol. Reverse transcription to obtain cDNA was performed using
168 LightCycler SYBR Green I Master (Roche Diagnostics) according to the manufacturer's recommendations
169 (Thermo Electron, Saint Herblain, France). Quantitative real-time PCR was performed for 50 cycles (10 seconds

170 at 95°C, 10 seconds at 60°C, and 10 seconds at 72°C) on a LightCycler 480 system. Primers (at a final
 171 concentration of 2 μmol.L⁻¹) were designed with the software Primer 3 and are listed in table 2. *GAPDH* gene was
 172 used as the internal reference gene. Threshold cycle values of the target genes were normalized to those of *GAPDH*
 173 and the relative change in gene expression was calculated with the 2^{-ΔΔCt} method.

174 **Table 2**

175 Human primer sequences used in qPCR analysis

Gene	Antisense	Sense
<i>HMOX1</i>	TCC-GAT-GGG-TCC-TTA-CAC-TC	CAA-GGA-AGC-CAG-CCA-AGA-GA
<i>NQO1</i>	TGG-AAG-CCA-CAG-AAA-TGC-AGA	AGG-ACC-CTT-CCG-GAG-TAA-GAA
<i>CAT</i>	AGC-TTA-GCG-TTC-ATC-CGT-GT	TCC-AAT-CAT-CCG-TCA-AAA-CA
<i>SOD2</i>	CAG-CCT-CTT-ACT-GGG-CAA-AG	CCA-GGT-CCG-TCA-ACA-GTT-TT

176 *2.8. Statistical analysis*

177 Results are shown as means ± standard errors of the means (SEM). Statistical differences between two groups
 178 (treated and vehicle) were evaluated using one-way analysis of variance followed by Bonferroni's means multiple
 179 comparison method. Differences were statistically accounted for when p<0.05. In the figures, the following
 180 symbols are used: * p<0.05; ** p<0.01.

181 **3. Results**

182 *3.1. Effect of the pesticides on cytotoxicity in hMDMs*

183 The cytotoxicity of pesticides on hMDMs was assessed by measuring the metabolic activity of these cells by
 184 the alamarBlue (resazurin) assay, and by measuring the integrity of their membranes by the level of LDH released
 185 into the culture medium, as shown in Fig. 1. Cell viability was measured after exposure to different concentrations
 186 of pesticides ranging from 0.01 to 10 μmol.L⁻¹ or to DMSO (control) for 24 h. ¹ Figure 1 shows an absence of cell
 187 lysis assessed by analysis of LDH level in the culture medium for thiacloprid, thiophanate, and boscalid (between
 188 0.01 and 10 μmol.L⁻¹), compared to the control group (DMSO alone) after 24 h of exposure of hMDMs to these
 189 pesticides. Conversely, we showed a reduction in hMDMs viability after exposure to 10 μmol.L⁻¹ of chlorpyrifos
 190 or captan (10%) compared with the control group, and 22% after exposure to 10 μmol.L⁻¹ of dithianon. Cell
 191 permeability assessed with alamarBlue shows that 20% of hMDMs show an increase in dye permeability after
 192 exposure of hMDMs to chlopyrifos, boscalid and dithianon at any dose studied between 0.01 and 10 μmol.L⁻¹.
 193 Thiacloprid and captan have no effect at 0.01 μmol.L⁻¹. Thiophanate, which has no effect at 0.01 and 0.1 μmol.L⁻¹
 194 ¹, increased dye permeability after exposure of hMDMs at 1 and 10 μmol.L⁻¹ (Fig. 1).

195 3.2. Effect of the pesticides on basal ROS production by hMDMs

196 In response to an inflammatory signal, macrophages produce reactive oxygen species (ROS) via the activation
197 of a NADPH oxidase, NOX2. Macrophages controlled production of ROS (superoxide anion: O_2^- , hydroxyl
198 radical: HO, hydrogen peroxide: H_2O_2) plays an essential role in the defense against pathogens. Conversely, an
199 overproduction of ROS or a deficiency of antioxidant systems results in an inefficient immune response.
200 Macrophages treated with $0.01 \mu\text{mol.L}^{-1}$ chlorpyrifos increased ROS production (Fig. 2). Macrophages treated
201 with $1 \mu\text{mol.L}^{-1}$ thiophanate increased ROS production. dithianon and captan significantly ROS production
202 especially in macrophages treated with $10 \mu\text{mol.L}^{-1}$. Thiacloprid and boscalid did not alter hMDMs ROS
203 production.

204 3.3. Effect of the pesticides on TPA- or ZNO-induced ROS production

205 Figure 3 shows the effect of pesticides on ROS production induced by 2-O-tetradecanoylphorbol-13-acetate
206 (TPA) and by unopsonized zymosan particles (ZNO) from the cell wall of *Saccharomyces cerevisiae*. TPA directly
207 activates NOX2 through protein kinase C (PKC) mobilization. The ROS production triggered by ZNO is coupled
208 to the phagocytosis of the yeast walls by macrophages. Chlorpyrifos and thiophanate increased TPA-induced ROS
209 production at $0.01 \mu\text{mol.L}^{-1}$. Dithianon inhibited TPA-induced ROS production at $10 \mu\text{mol.L}^{-1}$. Captan decreased
210 TPA-induced ROS at $1 \mu\text{mol.L}^{-1}$, and completely inhibited this ROS production at $10 \mu\text{mol.L}^{-1}$. Boscalid and
211 thiacloprid had no effect on TPA-induced ROS production. Interestingly, only chlorpyrifos, dithianon and captan
212 had an immunosuppressive effect on ZNO-induced ROS production. Chlorpyrifos decreased ZNO-induced ROS
213 production at 1 and $10 \mu\text{mol.L}^{-1}$. Captan decreased ZNO-induced ROS production at $10 \mu\text{mol.L}^{-1}$. Dithianon
214 completely inhibited oxidative hyperactivity induced by ZNO at $10 \mu\text{mol.L}^{-1}$. In contrast, thiacloprid increased
215 ROS production at 1 and $10 \mu\text{mol.L}^{-1}$ (Fig. 3).

216 3.4. Effect of the pesticides on LPS-induced pro-inflammatory cytokines production

217 In response to aggression or infection, macrophages can produce pro- and anti-inflammatory cytokines to
218 initiate an inflammatory response, eliminate the pathogen and repair tissue. The direct effect of the six pesticides
219 was therefore evaluated on the production of two pro-inflammatory cytokines (IL- 1β , TNF- α) and of an anti-
220 inflammatory cytokine (IL-10) which plays a role in inflammation resolution. After 24 h of treatment of hMDMs,
221 the studied pesticides were unable to trigger the production of IL- 1β , TNF- α pro-inflammatory cytokines nor the
222 production of the IL-10 anti-inflammatory cytokine compared to control cells whatever doses.

223 In order to assess whether the pesticides studied could interfere with the ability of hMDMs to produce cytokines
224 in response to an inflammatory stimulus, macrophages pre-treated with the pesticides at 0.01, 0.1, 1 and 10 $\mu\text{mol.L}^{-1}$
225 $^{-1}$ for 24 h were stimulated by lipopolysaccharides (LPS) from *Escherichia coli* (10 ng.mL^{-1}) to induce the
226 production of pro-inflammatory cytokines such as IL-1 β , TNF- α . Fig. 4 shows that none of the pesticides studied
227 increased the ability of macrophages to produce IL-1 β , TNF- α pro-inflammatory cytokines. However, we observed
228 that captan at 10 $\mu\text{mol.L}^{-1}$ decreased the capacity of hMDMs to produce IL-1 β and TNF- α by more than 90%.
229 Dithianon inhibited the production of IL-1 β and TNF- α in a dose-dependent manner at 1 and 10 $\mu\text{mol.L}^{-1}$.
230 Treatment of hMDMs with chlorpyrifos also decreased the production of IL-1 β at 0.01 and 0.1 $\mu\text{mol.L}^{-1}$ and TNF-
231 α at 0.1 and 1 $\mu\text{mol.L}^{-1}$, whereas thiophanate, thiacloprid and boscalid had no effect on LPS-induced production
232 of these cytokines. Fig. 4 shows that hMDMs in culture had a lower capacity to produce IL-10 than IL-1 β and
233 TNF- α in response to LPS. However, it also showed that boscalid and thiacloprid, which did not inhibit the
234 production of pro-inflammatory cytokines, increased IL-10 production at 0.01 $\mu\text{mol.L}^{-1}$ and 0.1 $\mu\text{mol.L}^{-1}$,
235 respectively. The figure 4 also shows that thiophanate had no effect on IL-10 production whereas dithianon and
236 captan inhibited this production at 1 and 10 $\mu\text{mol.L}^{-1}$.

237 3.5. Effect of the pesticides on gene expression of molecules involved in the control of oxidative stress

238 In phagocytic cells such as macrophages, reactive oxygen species (ROS) are formed upon activation of NOX2
239 NADPH oxidase during the phagocytic process. In parallel, ROS are produced by the mitochondrial enzyme
240 complex of the respiratory chain and *via* other cytosolic sources or within different organelles. To protect the cells
241 from oxidative stress and maintain cell homeostasis, antioxidant enzymes such as heme oxygenase (HMOX1),
242 NADPH quinone 1 (NQO1), catalase and superoxide dismutase are generated. Since chlorpyrifos, dithianon and
243 captan induced or suppressed the dose-dependent production of ROS by hMDMs, we evaluated whether those
244 three pesticides modulated the expression of antioxidant genes. To achieve this, cells were exposed for 4 h to
245 pesticides at different doses and the expression levels of heme oxygenase (*HMOX1*), NADPH dehydrogenase
246 quinone 1 (*NQO1*), catalase (*CAT*) and superoxide dismutase (*SOD2*) mRNAs were evaluated. Fig. 5 shows that
247 chlorpyrifos did not induce any antioxidant genes at any concentration between 0.01 and 10 $\mu\text{mol.L}^{-1}$. Exposure
248 of hMDMs at 1 $\mu\text{mol.L}^{-1}$ dithianon and captan increased the mRNA level of *HMOX1* and *NQO1* by more than
249 nine times. While captan had no effect on the expression of catalase and *SOD2* mRNAs, dithianon, which weakly
250 induced *CAT* and *SOD2* at a dose between 0.1 and 10 $\mu\text{mol.L}^{-1}$.

251 4. Discussion

252 Macrophages can respond to various inflammatory stimuli, such as a bacterial and fungal infection, viruses,
253 and parasites by producing ROS and cytokines. ROS production through activation of phagocytic NADPH oxidase
254 (NOX2) is crucial in the defense against infectious diseases and is involved in inflammation and multiple signaling
255 pathways (Segal et al., 2012). Our results show that boscalid and thiacloprid at 0.01, 0.1, 1 and 10 $\mu\text{mol.L}^{-1}$ had
256 no effect on the basal production of ROS by macrophages. Thiophanate (1 $\mu\text{mol.L}^{-1}$) and chlorpyrifos (0.01
257 $\mu\text{mol.L}^{-1}$) significantly increased the basal production of ROS. This increase in ROS production by macrophages
258 was not accompanied by an increased production of TNF- α and IL-1 β inflammatory cytokines. These results
259 suggest that these pesticides would activate, at the indicated doses, the NADPH oxidase signaling pathway in
260 phagocytes without affecting the signaling components involved in TNF- α and IL-1 β production. Conversely,
261 dithianon which had no effect at 0.01, 0.1 and 1 $\mu\text{mol.L}^{-1}$, suppressed the basal production of ROS at a dose of 10
262 $\mu\text{mol.L}^{-1}$. This suppression could be explained by the toxicity caused by dithianon on cell membrane integrity by
263 increasing LDH levels from macrophages by 20%. However, chlorpyrifos did not suppress the basal production
264 of ROS at a dose of 10 $\mu\text{mol.L}^{-1}$ despite the 10% cell lysis assessed by LDH. Chlorpyrifos, captan and dithianon
265 did not inhibit the basal secretion of TNF- α , IL-1 β and IL-10 at a dose of 10 $\mu\text{mol.L}^{-1}$. At this dose, dithianon and
266 captan completely suppressed TPA-induced oxidative bursts while only dithianon also completely inhibited
267 zymosan-induced ROS production. TPA is a diacylglycerol mimetic that triggers ROS production by acting
268 downstream of receptors coupled to NADPH oxidase activation, *via* a PKC-dependent signaling pathway
269 (Fontayne et al., 2002; Makni-Maalej et al., 2013). Zymosan, a cell-wall preparation from *Saccharomyces*
270 *cerevisiae*, is mainly composed of β -glucans, α -mannans, chitin, proteins, and lipids (Dostert and Tschopp, 2007).
271 Dectin-1 is the most important receptor for β -glucans (Brown et al., 2002). In human monocytes, dectin-1 has been
272 identified as the major pattern recognition receptor (PRR) for zymosan-induced activation of NADPH oxidase and
273 ROS production, and PKC δ has been identified as an important signaling component downstream of dectin-1
274 (Elsori et al., 2011). However, zymosan activates respiratory burst in macrophages through other receptors such
275 as TLR2, and a collaboration has been established between dectin-1 and TLR2 (Gantner et al., 2003). NADPH
276 oxidase activation by fungal or fungal extracts such as zymosan is controlled by the phosphorylation of its p47phox
277 cytosolic component on serines located between Ser303 and Ser379 (El-Benna et al., 2009). It has been shown on
278 human polynuclear cells that PKC δ and other PKC isoforms would be involved in zymosan induced dectin-1
279 dependent NADPH oxidase activation by phosphorylating p47phox on Ser315 and Ser328. It has also been shown
280 that p38MAPKinase and MEK1/2 would be involved in NADPH oxidase activation by zymosan (Elsori et al.,

281 2011) by phosphorylating the Ser345 residue of p47phox subunit independent of PKC isoforms (Makni-Maalej et
282 al., 2013). Thus, dithianon which has inhibited zymosan- and TPA-induced ROS production, could act by
283 inhibiting PKC-dependent and PKC-independent pathways such as p38MAPKinase and MEK1/2 involved
284 respectively in dectin-1 and TLR2-mediated zymosan signaling and NADPH oxidase activation process in
285 macrophages. Captan, which at 1 $\mu\text{mol.L}^{-1}$ inhibits TPA-induced ROS production without inhibiting zymosan-
286 induced ROS generation, and at 10 $\mu\text{mol.L}^{-1}$ inhibits TPA-induced ROS production and only weakly inhibits
287 zymosan-induced ROS generation, would have no effect on PKC-independent pathways involved in zymosan
288 signaling and NADPH oxidase activation (Fig. 6). Chlorpyrifos, at 10 $\mu\text{mol.L}^{-1}$, also slightly decreased the ROS
289 production induced by zymosan and TPA. It could act on dectin-1 and TLR2-mediated signaling pathways of
290 macrophages. Chlopyrifos at 0.01 $\mu\text{mol.L}^{-1}$ increased basal ROS production and ROS production towards TPA.
291 Similar results were found for the pesticide ziram (Parny et al., 2021). At a dose of 0.01 $\mu\text{mol.L}^{-1}$, ziram induced
292 a slight increase in ROS production as well as an increase in phagocytosis towards unopsonized zymosan. These
293 results were correlated with a slight increase in the expression of the dectin-1 receptor, involved in zymosan
294 phagocytosis. This suggests that chlorpyrifos could, similarly, activate the expression of dectin-1 on the cell
295 surface, and thus induce ROS production at 0.01 $\mu\text{mol.L}^{-1}$. The effect of chlopyrifos on dectin-1 is not known,
296 further studies are therefore needed to assess the effect of chlorpyrifos on the polarization of human monocyte-
297 derived macrophages. When macrophages engulf foreign agents such as microbes, and other particles and are
298 exposed to inflammatory stimuli, they secrete various cytokines depending on their polarization state.
299 Macrophages can adopt different polarization states determined by the multiple extrinsic and intrinsic factors of
300 their microenvironment. Classically activated macrophages (M1 polarization) have a pro-inflammatory and
301 tumoricidal phenotype. These macrophages release pro-inflammatory cytokines such as TNF- α and IL-1 β (Arango
302 Duque and Descoteaux, 2014). The many subtypes of alternately activated macrophages (M2 polarization) can be
303 induced depending on the intrinsic and extrinsic factors to which they are subjected. Thus, depending on the nature
304 and type of stimulus, these macrophages express different levels of anti-inflammatory cytokines such as IL-10 and
305 TGF- β that can promote diametrically opposed functions, trigger resolution of inflammation, and tissue repair, or
306 suppress T cell activation. In macrophage activation, the level of production of IL-10 seems to be linked to their
307 polarization status (Murray, 2017). Thus, M1 macrophages produce low levels of IL-10 in response to LPS,
308 whereas M2 macrophages produce high levels of IL-10 and lesser pro-inflammatory cytokines (TNF- α , IL-1 β) in
309 response to LPS. Our results show that under our experimental conditions, macrophages derived from human
310 monocytes produced more TNF- α and IL-1 β and less IL-10 in response to LPS treatment. Based on the type of

311 macrophage response to LPS after exposure to pesticides compared to the control group, the pesticides used in this
312 study can be classified into three groups. The first group, thiophanate, boscalid and thiacloprid, did not affect the
313 ability of macrophages to produce TNF- α , IL-1 β and IL-10 in response to LPS and thus did not modify the initial
314 polarization of macrophages. The second group, chlorpyrifos, had a biphasic effect on the production of
315 inflammatory cytokines TNF- α and IL-1 β : significant dose-dependent inhibition at 0.01 and 0.1 $\mu\text{mol.L}^{-1}$, and no
316 effect at 1 and 10 $\mu\text{mol.L}^{-1}$, and no effect on the production of IL-10. The third group, captan and dithianon strongly
317 inhibited the production of TNF- α and IL-1 β at 10 $\mu\text{mol.L}^{-1}$ and also inhibited the secretion of IL-10 by 50%.
318 Dithianon also inhibited the production of TNF- α and IL-1 β at 1 $\mu\text{mol.L}^{-1}$ without affecting the membrane integrity
319 of the cells. Finally, considering the TNF- α / IL-1 β ratio versus IL-10, we have shown that these two pesticides
320 decrease the inflammatory response of macrophages to LPS without increasing the production of IL-10. Other
321 studies have shown that exposure of murine macrophage or human macrophage-like cells to chlorpyrifos or
322 methyl-thiophanate can modulate the production of inflammatory cytokines TNF- α and / or IL-1 β induced by
323 LPS. Regarding chlorpyrifos, Helali et al. have shown an inhibition of LPS-induced TNF- α and IL-1 β productions
324 by mouse peritoneal macrophages in a dose-dependent manner between 0.01 and 10 $\mu\text{mol.L}^{-1}$ (Helali et al., 2016).
325 Unlike these results, we report for human monocytes after LPS activation an inhibition of TNF- α release (only at
326 0.01 and 0.1 $\mu\text{mol.L}^{-1}$) and of IL-1 β (at 0.01, 0.1 and 1 $\mu\text{mol.L}^{-1}$) while higher concentrations (1 and 10 $\mu\text{mol.L}^{-1}$)
327 for TNF- α and the concentration of 10 $\mu\text{mol.L}^{-1}$ for IL-1 β had no inhibitory effect. Recently, it was shown that
328 high concentration methyl-thiophanate (290 $\mu\text{mol.L}^{-1}$ /100 $\mu\text{g.mL}^{-1}$) increased the production of IL-1 β , TNF- α pro-
329 inflammatory cytokines, and inhibited the production of the IL-10 anti-inflammatory cytokine by the murine
330 macrophage cell line, RAW 264.7 (Weis et al., 2019). Under our experimental conditions, methyl-thiophanate in
331 the dose range studied, 0.01 to 10 $\mu\text{mol.L}^{-1}$, had no effect on the production of these cytokines by human
332 macrophages. Several studies have also established that exposure to pesticides could induce oxidative damage in
333 humans (Sharma et al., 2013; Jablonska-Trypuc et al., 2017). This is the case for chlorpyrifos which induce
334 oxidative stress, *in vitro*, in neuronal cells (Xu et al., 2017; Zhao et al., 2019) and zebrafish (Jin et al., 2015). When
335 cells are exposed to oxidative stress, a defense mechanism induces the expression and regulation of a number of
336 antioxidant enzymes to protect them from damage caused by free radicals. Many of these antioxidant enzymes,
337 e.g. as Heme oxygenase-1 (HMOX1), NADPH quinone oxidoreductase (NQO1) (Bellezza et al., 2018),
338 superoxide dismutase 2 (SOD2) (Dong et al., 2008; Sun et al., 2015) and catalase (Ishii et al., 2002), are under the
339 control of the transcription factor Nuclear factor erythroid 2-related factor 2 (Nrf2). The nuclear receptor PPAR γ
340 is also involved in the induction of superoxide dismutase and catalase, and peroxisome proliferator response

341 elements and Nrf2-antioxidant response elements coexist in the promoter region of these genes (Zhao et al., 2015).
342 Biochemical alterations, particularly in antioxidant enzymes have been reported in some pesticide-exposed
343 populations (Sharma et al., 2013). Studies have also shown that certain carbamates and organophosphates can alter
344 antioxidant index levels. It was shown that cartap (thiocarbamate) and malathion (organophosphate) were able to
345 increase the expression levels of SOD and CAT in Wistar rats (Gupta et al., 2020) while carbofuran (carbamate)
346 decreased the expression of these enzymes in the brains of rats (Jaiswal et al., 2016). In our experimental
347 conditions, we have shown that chlorpyrifos between 0.01 and 10 $\mu\text{mol.L}^{-1}$ had no effect on the gene expression
348 of antioxidant enzymes studied, *HMOX1*, *NQO1*, *SOD2* and *CAT*. An *in vitro* study has shown that at higher doses
349 (50 and 100 $\mu\text{mol.L}^{-1}$) chlorpyrifos induced ROS production and triggered antioxidant defense mechanisms in
350 JEG-3 cells through an upregulation of catalase activity, an increase of *HMOX1* mRNA levels and NRF2 at both
351 mRNA and protein levels (Chiapella et al., 2013). Another recent study showed that chlorpyrifos at 50 $\mu\text{mol.L}^{-1}$
352 could activate *in vitro* cell pyroptosis in human neuroblastoma SH-SY5Y cells and oxidative stress through down-
353 regulation of the SIRT1 / PGC-1 α / Nrf2 pathway (Zhao et al., 2019). In this study we also showed that captan
354 (0.1 and 1 $\mu\text{mol.L}^{-1}$) and dithianon (between 0.01 and 1 $\mu\text{mol.L}^{-1}$) increased mRNA levels of antioxidant enzymes,
355 *NQO1* and *HMOX1*, which are known to be under the control of Nrf2-ARE signaling pathways. These results
356 suggest that low doses of these pesticides would trigger oxidative stress in macrophages and that adaptive
357 activation of the Nrf2 / ARE pathway would be insufficient (notably at 10 $\mu\text{mol.L}^{-1}$) to attenuate the oxidative
358 toxicity and the inhibition of macrophage functions like oxidative burst resulting in the release of reactive oxygen
359 and the production of inflammatory and anti-inflammatory cytokines in response to inflammatory or infectious
360 stimuli. In another study, we showed similar effects with a dithiocarbamate, ziram, which also inhibited at 10
361 $\mu\text{mol.L}^{-1}$ the pro-inflammatory functions of hMDMs *via* zinc and its oxidative properties by polarizing the
362 macrophages towards an M2-type anti-inflammatory phenotype despite bacterial (LPS) or fungal (zymosan)
363 aggression (Parny et al., 2021).

364 **5. Conclusion**

365 Our findings demonstrate that among the tested pesticides, only boscalid between 0.01 and 10 $\mu\text{mol.L}^{-1}$ had no
366 effect on human monocyte-derived macrophages effector functions. Thiacloprid promoted the ability of
367 macrophages to produce ROS in response to zymosan and induced the production of IL-10 in response to LPS. On
368 the other hand, chlorpyrifos and thiophanate increased the production of ROS at low doses (0.01-0.1) while at high
369 doses, chlorpyrifos, captan and dithianon have shown immunosuppressive effects through the reduction of ROS
370 and pro-inflammatory cytokines such as IL-1 β and TNF- α production. Thus, these data suggest that exposure to

371 these pesticides including chlorpyrifos, dithianon and captan may compromise immune responses to infection and,
372 in particular the clearance of microbial pathogens by macrophages. Given that the doses of captan and dithianon
373 can range from 18 to 81 $\mu\text{mol.L}^{-1}$ and 2.5 to 11.2 $\mu\text{mol.L}^{-1}$ respectively in workers (Bureau et al., 2021) and that
374 some MLRs, such as dithianon can reach doses of 10 $\mu\text{mol.L}^{-1}$ (European Food Safety et al., 2020), these three
375 pesticides could contribute to immune system dysregulation. The information obtained from this study could be
376 used to guide policy and regulations relating to the doses of these pesticides to minimize occupational health risks.

377 **CRedit authorship contribution statement**

378 **Melissa Parny:** Conceptualization, Methodology, Writing - original draft. **Agnès Coste:** Resources, Writing
379 - review & editing. **Agnès Aubouy:** Writing - review & editing. **Mouna Rahabi:** Writing - review & editing.
380 **Melissa Prat:** Writing - review & editing. **Bernard Pipy:** Conceptualization, Methodology, Writing - original
381 draft, Supervision. Investigation, **Michel Treilhou:** Conceptualization, Writing - review & editing, Supervision,
382 Project administration, Funding acquisition.

383 **Declaration of competing interest**

384 The authors declare that they have no known competing financial interests or personal relationships that could
385 have appeared to influence the work reported in this paper.

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603 **FIGURE LEGENDS**

604 **Fig.1.** Effect of the pesticides on the cytotoxicity of human monocyte-derived macrophages (hMDMs)

605 Monocytes were treated with each pesticide (chlorpyrifos, thiacloprid, thiophanate, boscalid, dithianon, captan) at
606 different concentrations (0.01, 0.1, 1 and 10 $\mu\text{mol.L}^{-1}$) for 24 h. Cells treated with DMSO alone (0.01%) were used
607 as controls. The toxicity of each pesticide to hMDMs was measured by alamarBlue and LDH assay. The data were
608 shown as the mean \pm SEM of three healthy triplicate donors. * $p < 0.05$, ** $p < 0.01$ compared to cells treated with
609 DMSO alone (Bonferroni multiple comparison test).

610 **Fig.2.** Effect of pesticide exposure on the production of ROS by hMDMs

611 The cells were treated with each pesticide (chlorpyrifos, thiacloprid, thiophanate, boscalid, dithianon, captan) at
612 different concentrations (0.01, 0.1, 1 and 10 $\mu\text{mol.L}^{-1}$) for 24 h. Cells treated with DMSO alone (0.01%) were used
613 as controls. The production of reactive oxygen species was determined by chemiluminescence as described in
614 "Materials and Methods". The data were shown as the mean \pm SEM of three healthy donors made of triplicate.
615 * $p < 0.05$, ** $p < 0.01$ compared to cells treated with DMSO alone (Bonferroni multiple comparison test).

616 **Fig.3.** Impact of the pesticides on TPA- or ZNO- induced ROS production

617 Monocytes were treated with each pesticide (chlorpyrifos, thiacloprid, thiophanate, boscalid, dithianon, captan) at
618 different concentrations (0.01, 0.1, 1 and 10 $\mu\text{mol.L}^{-1}$) for 24 h. Cells treated with DMSO alone (0.01%) were used
619 as controls. The production of reactive oxygen species was determined on cells stimulated with TPA or ZNO by
620 chemiluminescence as above. The data was shown as the mean \pm SEM of three healthy donors made in triplicate.
621 * $p < 0.05$, ** $p < 0.01$ compared to cells treated with DMSO alone (Bonferroni multiple comparison test).

622 **Fig.4.** Impact of the pesticides on IL-1 β , TNF- α and IL-10 cytokine secretion by LPS- stimulated hMDMs

623 Monocytes were treated with each pesticide (chlorpyrifos, thiacloprid, thiophanate, boscalid, dithianon, captan) at
624 different concentrations (0.01, 0.1, 1 and 10 $\mu\text{mol.L}^{-1}$) for 24 h. To simplify the figure, only the 10 $\mu\text{mol.L}^{-1}$ dose
625 has been represented. Cells treated with DMSO alone (0.01%) were used as controls. The level of IL-1 β , TNF- α
626 and IL-10 was measured in the culture medium by ELISA. To induce inflammation, after 24 h, LPS were added
627 to induce the production of pro-inflammatory cytokines such as IL-1 β and TNF- α . The data were shown as the
628 mean \pm SEM of three healthy donors made in triplicate. * $p < 0.05$, ** $p < 0.01$ compared to cells treated with DMSO
629 alone (Bonferroni multiple comparison test).

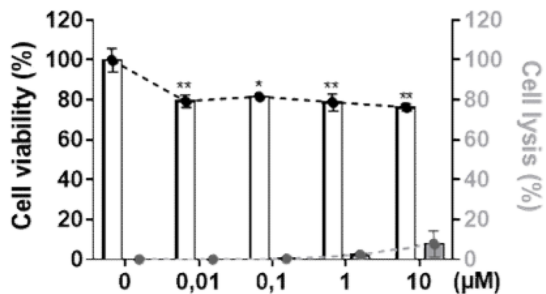
630 **Fig.5.** Effect of chlorpyrifos, dithianon and captan on the expression of genes involved in the control of oxidative
631 stress

632 Monocytes were treated with an insecticide (chlorpyrifos) or with the two fungicides (dithianon, captan) at
633 different concentrations (0.01, 0.1, 1 and 10 $\mu\text{mol.L}^{-1}$) or with DMSO alone (0.01%) as control for 4 h. Gene
634 expression of the antioxidant enzymes (heme oxygenase (*HMOX1*), NADPH quinone dehydrogenase 1 (*NQO1*),
635 catalase (*CAT*) and superoxide dismutase 2 (*SOD2*)) was evaluated in the hMDMs by qRT-PCR as described in
636 the section "Materials and Methods". The results were represented in fold induction relative to control (DMSO).
637 The data were shown as the mean \pm SEM of three healthy donors made in triplicate. * $p < 0.05$, ** $p < 0.01$ compared
638 to cells treated with DMSO alone (Bonferroni multiple comparison test).

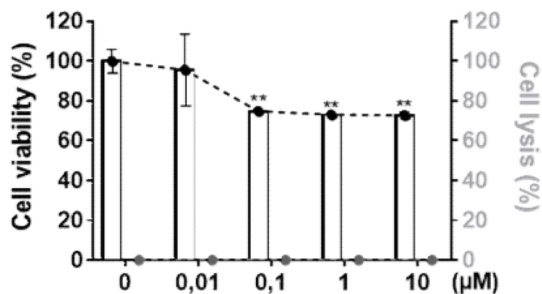
639 **Fig 6.** Effect of chlorpyrifos, dithianon and captan on protein kinase C (PKC) dependent and independent signaling
640 pathways

641 Chlorpyrifos and dithianon inhibited both PKC-dependent and independent pathways while captan inhibited only
642 the PKC-dependent pathway.

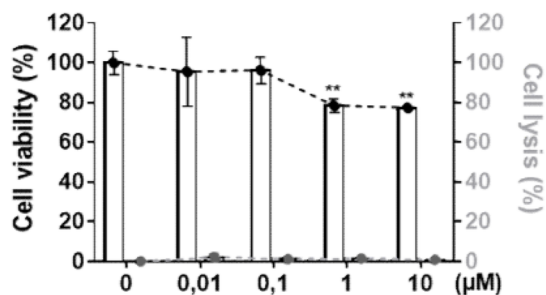
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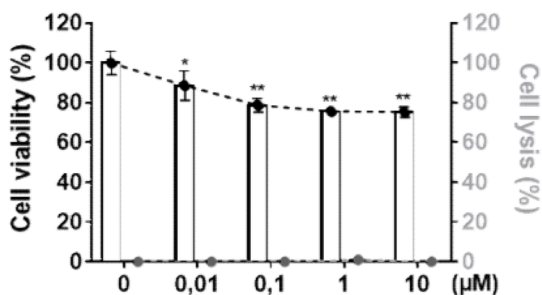
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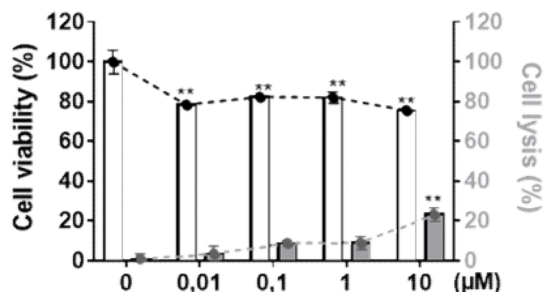
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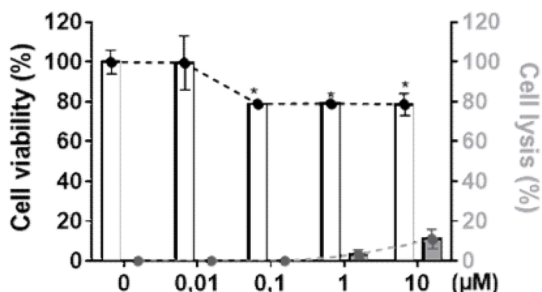
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dithianon



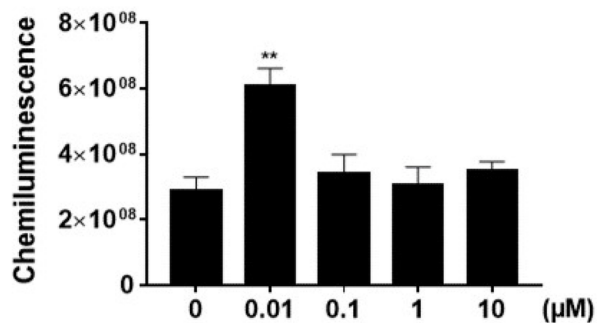
captan



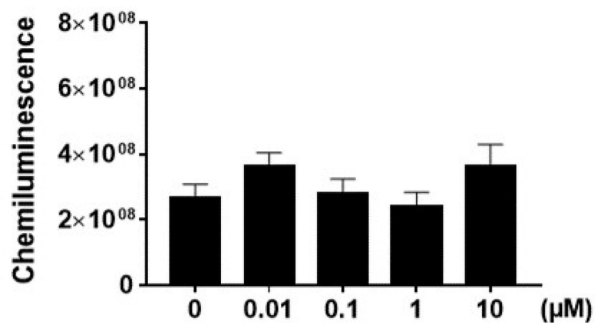
□ alamarBlue

■ LDH

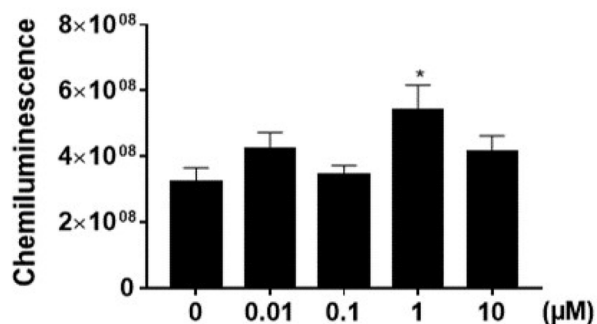
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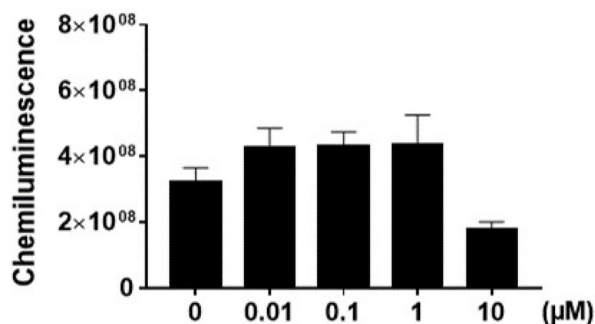
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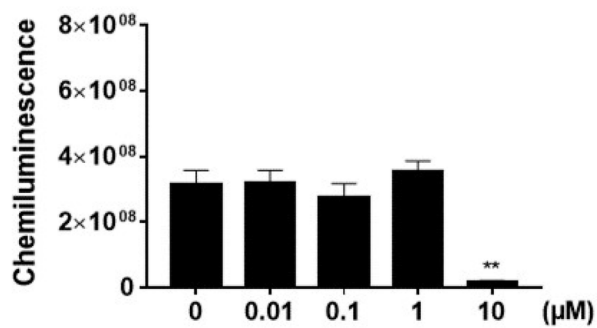
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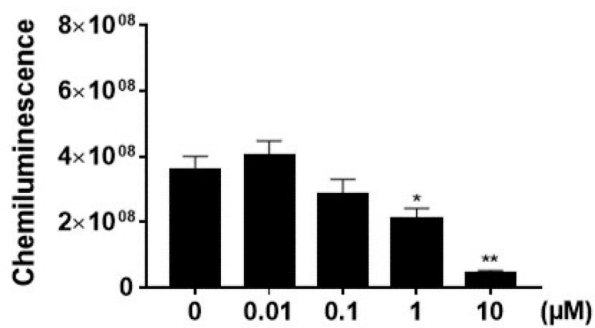
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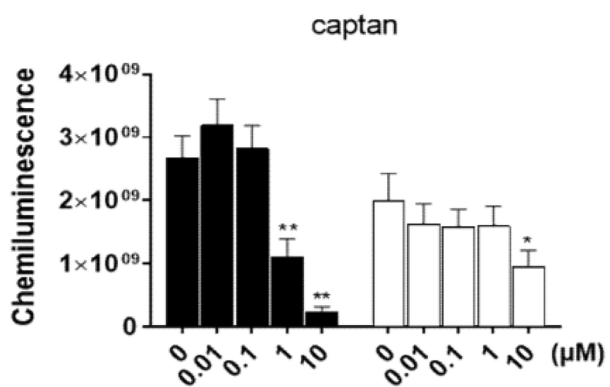
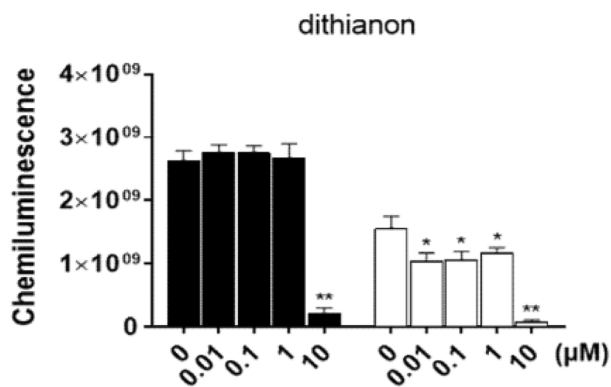
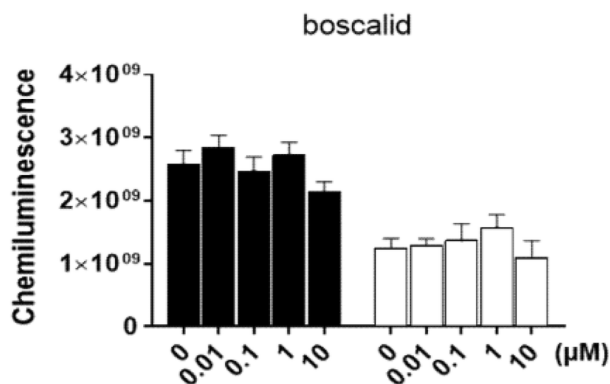
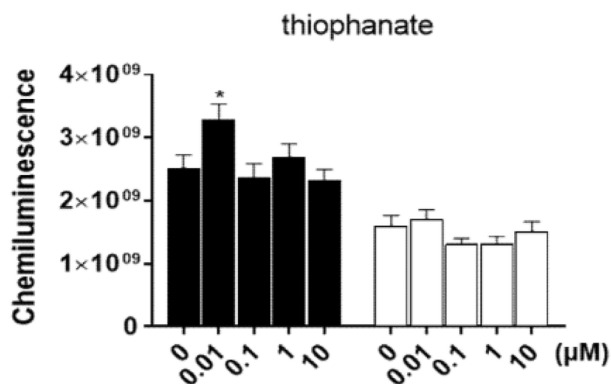
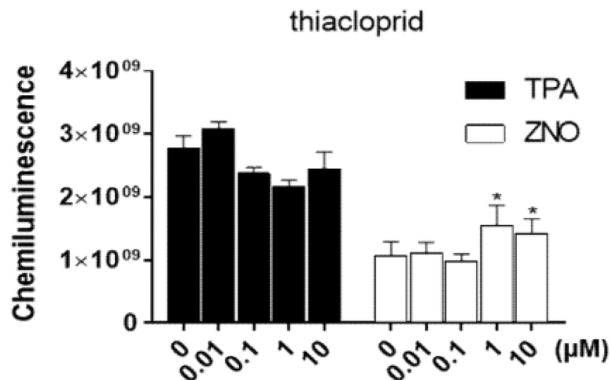
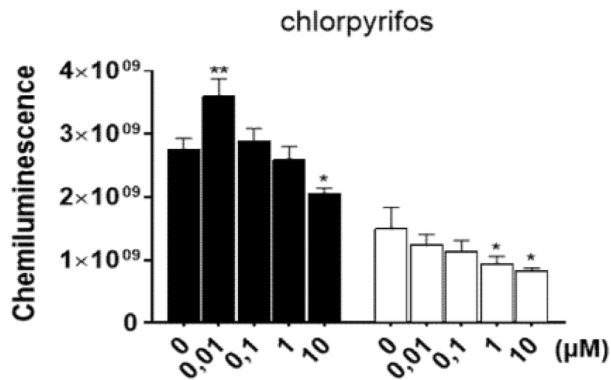


dithianon



captan

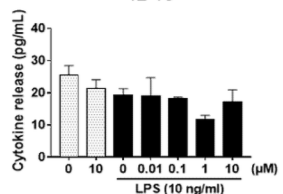
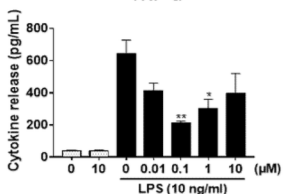
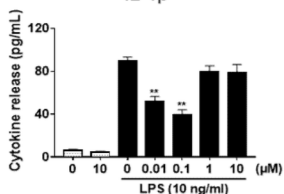




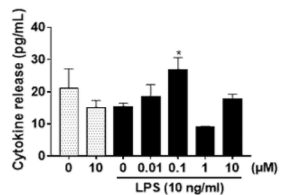
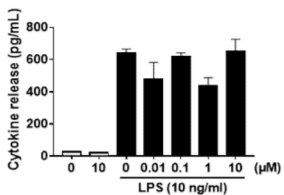
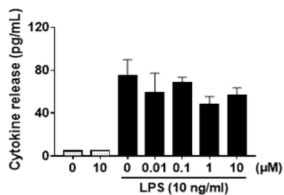
IL-1 β TNF- α

IL-10

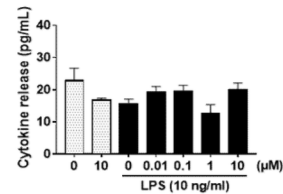
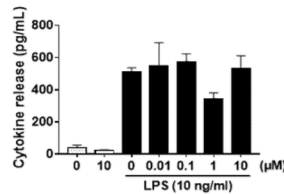
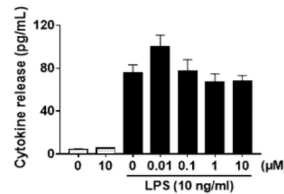
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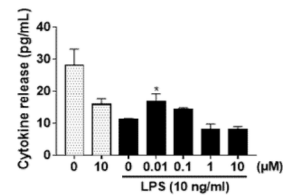
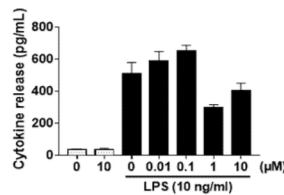
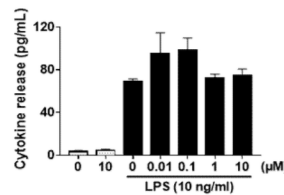
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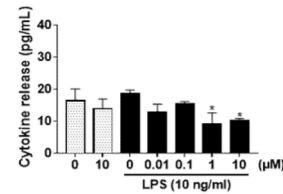
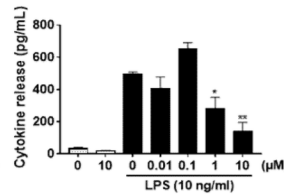
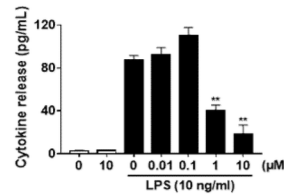
thiophanate



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