Cadmium and wild boar: Environmental exposure and immunological impact on macrophages

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A R T I C L E   I N F O

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A B S T R A C T

Cadmium (Cd²⁺) is regarded as one of the most toxic heavy metals, which can enter the food chain through environmental contamination and be bioaccumulated. Its exposure in Ligurian wild boars was monitored between 2016–2020 and revealed high level of this heavy metal in different provinces. In one of these polluted area, 21 wild boars were additionally sampled and the relationship between hepatic and renal Cd²⁺ concentration suggested that majority of these animals presented chronic intoxication. Cd²⁺ exposure of wild boar might lead to an immunosuppression status, thus in vitro experiments on wild boar monocyte-derived macrophages (moMΦ) were carried out. Effects of Cd²⁺ scalar doses were evaluated through viability and adsorption assays, ELISA, qPCR. Moderate doses of this environmental pollutant (20 μM) were absorbed by moMΦ, with subsequent reduction of their viability. This heavy metal did not trigger release of either IFN-β, anti-inflammatory or pro-inflammatory cytokines by moMΦ, instead 24 h treatment with 20 μM of Cd²⁺ resulted in down-regulated expression of TNF-α, IL-12p40, several TLRs, CD14, MD2, BD2, MyD88, p65, and NOS2. The results of our monitoring activity suggested that wild boar can be useful to monitor environmental exposure of this heavy metal and can help in understanding the type of contamination. In addition, in vitro experiments on wild boar moMΦ revealed that Cd²⁺ exposure negatively affected the immune function of these cells, likely leading to increased susceptibility to infection.

Abbreviations: Cd²⁺, cadmium; moMΦ, monocyte-derived macrophages; IFN, Interferon; TLR, Toll-like receptor; IL, Interleukin; TNF, tumor necrosis factor; LDH, lactate dehydrogenase; Arg-1, arginase 1; NOS2, nitric oxide synthase 2; MyD88, myeloid differentiation factor 88; MD2, myeloid differentiation protein 2; BD, beta defensin; LPS, lipopolysaccharide; PAMPs, pathogen associated molecular patterns; iNOS, inducible nitric oxide synthase.

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1. Introduction

Cadmium (Cd\(^{2+}\)) is one of the most toxic environmental and industrial heavy metals; many human activities lead to Cd\(^{2+}\) production, such as the combustion of fossil fuels, run-off from agricultural land, leachate from landfill sites, electroplating to protect steel from corrosion, and the manufacture of Nickel–Cd batteries, pigments, stabilizers and alloys. This heavy metal has been reviewed by the International Register of Potentially Toxic Chemicals of the United Nations Environment Program and included on the list of chemical substances considered to be potentially dangerous at the global level, indeed it has been reported to be carcinogenic and mutagenic [1–3]. Several studies highlighted a link between cadmium exposure and cancer in humans, and the main affected organs are liver, prostate, breast, lungs, kidney, skin and pancreas [3–5]. This toxic heavy metal can modulate the activity of cellular enzymes, initiate oxidative stress, suppress mitochondrial functions, disrupt calcium, homeostasis, negatively modulate the immune response and act as an endocrine disruptor, in particular of the thyroid and nervous system [6–8]. A link between cadmium exposure and cancer was highlighted by several in vivo studies using rodents as animal model [4].

Cadmium is not eliminated from ecosystems and, because of its long half-life (15–30 years), it can enter the food chain through environmental contamination of soil, enhancing the bioaccumulation along all the trophic levels of the ecological pyramid. In cows and ewes, effects on reproduction of the species should well reflect the effects of this environmental pollutant on human innate immune system.

2. Materials and methods

2.1. Wild boar sampling

During the hunting season from 2016 to 2020, 1271 wild boars (Sus scrofa) were killed by hunters or found dead in the different areas of Liguria Region (Fig. 1). After death, livers were dissected by hunters and immediately refrigerated. Samples were transported to the laboratory by hunters; occasionally kidney samples were also collected. At the laboratory, samples were immediately stored at –20 °C until analyzed.

In a selected area (Chiavari municipality, within Genova province), additional sampling was carried out: 21 wild boars from a polluted area in that municipality were examined.

Animals were sampled after being hunted or found dead. No wild boar was sacrificed for this study in order to determine Cd\(^{2+}\) contents in its selected organs; thus, approval of the ethics committee was not required to monitor Cd\(^{2+}\) environmental contamination in the Liguria Region. Wild boars were preserved in suitable conditions and transported to the laboratories of IZS of Piemonte, Liguria and Valle d’Aosta. Necroscopies were carried out, during which samples of kidney and liver were immediately frozen and stored at –20 °C.

2.2. Chemical analysis of wild boar organs

An aliquot (approximately 1 g) of tissue samples was homogenized and then was transferred to a Teflon® microwave vessel and mixed with 5 mL of 65 % nitric acid (Sigma-Aldrich S.r.l., Milano, cat. V001338) and 1.5 mL of hydrogen peroxide (Merck Millipore, Germany, cat. 1,086,001,000). The samples then were digested using a laboratory microwave oven. The extract was filtered and diluted to 25 mL with ultrapure water. Determination of Cd\(^{2+}\) contents was carried out using Analytical Yena 650 Plus Atomic Absorption Spectrometer with graphite furnace, at 228.8 nm with a current of 4 mA. Quantification was obtained by standard addition method. In brief, calibration was carried out by scalar addition of standards (certified standard solution at 10 mg/L by Ultra Scientific) to the matrix solution. The data were plotted as absorbance versus the amount of the standard added. The least squares line intersects the x-axis at the negative of the concentration of the sample. The quantification limit (LOQ) was 0.020 mg/kg. For testing the purity of the reagents and possible contamination, “blanks” was analyzed for each run, using the same procedure.

2.3. Generation of wild boar monocyte-derived macrophages and cadmium treatment

Five healthy wild boars, 9–12 months of age, were used as blood donors for in vitro experiments. Animals were kept at the University of Sassari, Faculty of Veterinary Medicine (Sassari, Italy). EDTA blood was collected by puncture of the cranial vena cava; blood sampling was approved by the local ethics committee, in accordance with the Guide of Use of Laboratory Animals issued by the Italian Ministry of Health, as we previously described [26].

Wild boar peripheral blood mononuclear cells (PBMCs) were prepared by diluting 20 mL of EDTA blood in 10 mL of phosphate buffered saline (PBS), layering it over 20 mL of Histopaque-1077 (Sigma-Aldrich, Saint Louis, MO, USA), and centrifuging it at 1400 x g for 30 min at room temperature (RT), in a rotating bucket centrifuge, without braking. PBMCs were aspirated from the plasma-Histopaque interface and
Live cells per well. Cells were exposed to scalar doses of Cd μM. Cultures were obtained from PBMCs, using methods previously described with slight modifications [26,28]. In brief, PBMCs were cultured for 7 days in RPMI-1640 (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 100 U/mL streptomycin, and 100 μg/mL penicillin (complete RPMI, cRPMI), and with 50 ng/mL of recombinant human M-CSF (hM-CSF) (Thermo Fisher Scientific, Waltham, MA, USA), using Petri dishes. Wild boar MoMΦ were then harvested, washed, re-suspended in cRPMI and seeded in 12-well plates (Greiner CELLSTAR, Sigma-Aldrich) (8 × 10^5 live cells per well) or 8-well chamber slide (Thermo Fisher Scientific, Waltham, MA, USA) (1 × 10^5 live cells per well). Cells were incubated at 37°C 5% CO₂ for further 24 h before treatment. Different Cd²⁺ concentrations (Carlo Erba reagents srl, Milano, cat 505,548) were tested: 2 μM or 20 μM, as previously used on swine epithelial cells [20]. In selected experiment, two additional Cd²⁺ concentrations were investigated: 0.02 or 0.2 μM.

2.4. Cadmium uptake

MoMΦ ability to adsorb Cd²⁺ was investigated by atomic absorption spectroscopy. Cells were left untreated (control) or treated with scalar doses of cadmium (0.02, 0.2, 2, 20 μM). 24 h later, the intracellular concentration of Cd²⁺ was checked using a graphite furnace atomic absorption spectroscopy (model ZEEnit 650 P, Analytik-Jena, Germany) with inverse Zeeman-effect background correction system, as we previously described [20]. In brief, culture supernatants were removed, and cells were lysed in 400 μL/well of tissue lysis buffer ATL (Qiagen, Milan, Italy); then the cell lysate was digested with 600 μL of a solution of nitric acid 69% and hydrogen peroxide 30% 5:1 ratio, filtered through a 0.20 μm paper filter, finally diluted in 5 mL with ultrapure water. Intracellular Cd²⁺ concentration was expressed as μg Cd²⁺/10⁶ cells.

2.5. Cd²⁺ impact on moMΦ morphology

Wild boar moMΦ were cultured in 8-well chamber slides at 1 × 10^5 live cells per well. Cells were exposed to scalar doses of Cd²⁺ (0, 0.02, 0.2, 2, 20 μM) and 24 h later moMΦ were fixed with 4% paraformaldehyde, washed in PBS, and labeled with DAPI (Roche Diagnostics GmbH, Mannheim, Germany) to visualize nuclei [28]. Microscopy was performed using a Leica SP5 Confocal Microscope (Leica Microsystems, Wetzlar, Germany) equipped with a HCX PL APO lambda blue 63 × 1.40 OIL UV objective. Nuclei and DIC image were acquired using simultaneous UV-diode (405 nm) for DAPI signal and argon laser (488 nm) as light source. Images were acquired on a format of 1024 × 1024 pixels and were processed with LAS AF Lite software (Leica Microsystems) as previously described [28].

2.6. Viability assay

Impact of cadmium on wild boar moMΦ viability was evaluated using a non-radioactive cytotoxicity assay, as previously described [29]. Cells were seeded in 12 well plate, left untreated (control) or cultured 24 h in the presence of scalar doses of cadmium (0.02, 0.2, 2, 20 μM). LDH (lactate dehydrogenase) levels in culture supernatants were determined using a Cytotox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) following manufacturer’s instructions; a lysis solution provided by the manufacturer used as positive control. Absorbance was read at 492 nm, using an Epoch microplate reader (BioTek, Winoosky, USA).

2.7. Detection of cytokine levels in culture supernatants

Wild boar moMΦ were cultured in 12 well plates at 8–10 × 10⁵ live cells per well. Cells were exposed to scalar doses of Cd²⁺ (0, 2, 20 μM) for 24 h, then cytokine levels in culture supernatants were determined as previously described [28,30]. The simultaneous measurement of IL-1α, IL-1 β, IL-6, IL-10, IL-12, and TNF-α in culture supernatants were performed using Porcine Cytokine/Chemokine Magnetic Bead Panel Quantikine assay (Merck Millipore, Darmstadt, Germany) and a Bioplex MAGPIX Multiplex Reader (Bio-Rad, Hercules, USA), according to the manufacturer’s instructions. The measurement of IFN-β was instead performed using a sandwich enzyme immunoassay (porcine IFN-β ELISA kit, Mybiosource, San Diego, CA, USA), according to manufacturer’s directions. Absorbance was read with an Epoch microplate reader (BioTek).

2.8. Gene expression

Gene expression in moMΦ after Cd²⁺ exposure was also monitored.
Changes in mRNA expression profiles of IL-1β, IL-6, IL-10, IL-12p40, TNF-α, IFN-β, IFN-α1, TLR3, TLR4, TLR5, TLR7, TLR8, TRL9, MyD88, p65, CD14, MD2, BD1, BD2, Arg-1, NOS2 in wild boar moMΦ stimulated with two different Cd^{2+} concentrations were evaluated as previously described [20,28,31]. Wild boar moMΦ were cultured in 12 well plates at 8–10 × 10^5 live cells per well. Cells were left untreated (control) or treated with two different doses of cadmium (2 or 20 μM). 0, 3, 6, 24 h post-treatment, culture supernatants were removed, and cells were lysed with buffer RTL (Qiagen). Then, total RNA was extracted using the RNeasy Mini Kit (Qiagen) and eluted in 100 μL of ultrapure RNase-free water (Sigma). 250 ng of purified RNA was used as template for cDNA synthesis, as previously described [32]. Gene expression was evaluated by RT-qPCR, using primer sets reported in Table S1 [28,31,47–52]. Real-time PCR amplification was performed in a CFX96™ Real-Time System after the reverse transcription step, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene [20]. In each sample, the relative expression of the test genes was calculated using the widely adopted 2^{-ΔΔCq} method, with Cq acronym of quantification cycle [20].

2.9. Data analysis and statistics

In vitro experiments were performed in technical duplicate (multiplex ELISA, sandwich ELISA, qPCR) or triplicate (cytotoxic test). In vitro experiments repeated at least three times with different blood donors (at least three biological replicates). Adsorption test was repeated using five diverse blood donors. Data were presented as median with interquartile range or means with standard deviations (SD) quoted to indicate the uncertainty around the estimate of the group mean. Graphical and statistical analysis was performed using GraphPad Prism 7.02 (GraphPad Software Inc, La Jolla, USA) and Minitab (Minitab Inc., Coventry, UK). All data were checked for normality using the Anderson Darling test and analysed by the parametric one-way ANOVA followed by Dunnett’s
multiple comparison test or the non-parametric Mann-Whitney test or Kruskal-Wallis followed by Dunn’s multiple comparison test.

3. Results

Samples with a cadmium concentration higher than 0.50 mg/kg were considered contaminated (as reported in EC Reg. 1881/2006 for liver of cattle, sheep, pigs, poultry and horses) and the total prevalence attested to 25 %. As shown by Fig. 2, over the years, the number of samples tested increased, revealing a greater prevalence of positivity in eastern Liguria. Imperia was the least polluted province by Cd % (125 out of 436) respectively, were the provinces most exposed to Cd contamination, with maximum concentrations of 13.56 mg/kg for Savona in 2016, higher than those detected was 1.28 mg/kg in 2017. La Spezia was instead the area with the highest concentration was 6.48 mg/kg in 2020; Genoa and Savona, despite having a positivity rate of 20 % (103 out of 511) and 29 % (125 out of 436) respectively, were the provinces most exposed to Cd contamination, with maximum concentrations of 13.56 mg/kg for Savona in 2016, higher than those observed in 2015.

In one of the polluted areas (within Chiavari municipality) several hunters notified that wild boar, belonging to different age classes, presented poor body condition with anaemia, cachexia and weakness (personal communication), thus further sampling was carried out from that area. Most of the tested subjects (11 out of 21) presented Cd$^{2+}$ levels which exceeded the limits of current legislation (Reg. EC 1881/2006 Cd$^{2+}$: 0.50 mg/kg for liver and 1 mg/kg for kidney), as reported in Table S1. The relationship between hepatic and renal concentration was also monitored because it is considered a valid index of intoxication degree (Chronic < 1 / Acute > 1) [33] and our data suggested that most animals presented chronic intoxication of this heavy metal (Fig. 3, Table S2). Indeed, the toxicity index in animals tested from adjacent contaminated areas (Chiavari municipality) showed a value of 0.21, while the average toxicity index of the control group (animals from the Imperia area), was around 0.11.

Chronic exposure of this environmental pollutant might negatively affect wild boar immune system. Thus, in the second part of this study, we investigated Cd$^{2+}$ impact on wild boar moMF, which are phagocytic cells at the frontline of defence against foreign invaders [21].

First, ability of these cells to uptake this heavy metal and the subsequent impact on moMF viability were evaluated, using doses we previously tested on IPEC-J2 cells [20], which were defined as ‘low’ (2 μM) or ‘moderate’ (20 μM) by Luevano and Demodaran [19]. Nevertheless, moMF are key players of innate immunity and highly responsive to environmental cues [34], thus initially other two lower concentrations were included in our in vitro experiments: 0.2 μM and 0.02 μM. Our results revealed a significant (P < 0.05) increase of Cd$^{2+}$ intracellular levels after 24 h (P < 0.05) of exposure at 20 μM of Cd$^{2+}$ concentrations (Fig. 4a, Figure S1). Then Cd$^{2+}$ impact on wild boar moMF viability was investigated using a non-radiolabelled immunoassay. Cells were exposed to Cd$^{2+}$ (0, 0.02, 0.2, 2 or 20 μM) and 24 h later LDH levels in culture supernatants were quantified using a cytotoxicity non-radioactive assay. Cell viability decreased when Cd$^{2+}$ was added at 2 or 20 μM (Fig. 4b). Morphology was next investigated using confocal microscopy: MoMF presented with a spherical shape with short hairy protrusions on their surface, as observed in our previous study [28], irrespective of Cd$^{2+}$ treatment (Fig. 4c).

Then, Cd$^{2+}$ immunological impact on wild boar moMF was assessed. We opted to test only low (2 μM) and moderate Cd$^{2+}$ (20 μM) doses, considering that at lower concentration Cd$^{2+}$ was neither absorbed or affected moMF’s viability. Pro-inflammatory or anti-inflammatory cytokines levels in culture supernatants of moMF were determined using multiplex ELISA. A small increase in IL-1α, IL-1 β and TNF-α were observed in moMF treated with low but not moderate doses of Cd$^{2+}$ (Fig. 5a, Figure S2). No changes in IL-6, IL-10, IL-12 levels were appreciated 24 h post stimulation with either low (2 μM) or moderate (20 μM) doses of this heavy metals.

Cd$^{2+}$ modulation of key cytokines gene expression was also quantified using qPCR. This heavy metal did not alter expression of pro-inflammatory IL-1 β or IL-6 at any tested time points, whereas a small increase of IL-1 β was observed 3 h post-exposure (Fig. 5, Figure S3). On the contrary, 20 μM of Cd$^{2+}$ induced small down-regulation of IL-12p40 and TNF-α 24 h post-treatment (Fig. 5, Figure S3). A small increase in IL-10 gene expression was observed 3 h post-treatment with high doses of this environmental pollutant, although no modulations were appreciated at later time points (Fig. 5, Figure S3).

Type I IFNs play a crucial role in the fight against viral infections [35], and in this study we assessed whether this heavy metal affect its induction/synthesis. Release of IFN-β was investigated using a sandwich enzyme immunoassay, whereas induction of both IFN-α1 and IFN- β

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**Fig. 3.** Cd$^{2+}$ levels in wild boar’s liver and kidney from a polluted area of Liguria. Cd$^{2+}$ concentration (mg/kg) in liver (a), kidney (b) of wild boar collected from a polluted area (within Chiavari municipality) and a control area (within Imperia province) of Liguria. (c) Cd intoxication index, determined as the ratio between cadmium concentration in liver and kidney. Values are presented as median with interquartile range. Data were compared using a Mann-Whitney test; ** p < 0.01, * p < 0.05.
gene expression was monitored over time through qPCR. No statistically significant differences were observed between untreated and \(\text{Cd}^{2+}\)-treated moM\(\Phi\), with the exception of a small down-regulation of IFN-\(\alpha\) expression 6 h post-treatment with moderate doses of this heavy metal (Fig. 5, Figure S3).

\(\text{Cd}^{2+}\) modulation of Toll like receptors (TLRs) expression was next investigated. These receptors are expressed by different immune cells and can recognize a broad range of pathogen associated molecular patterns (PAMPs). TLR3, TLR7, TLR8, TLR9 are intracellular TLRs and recognize nucleic acids derived from bacteria and viruses, whereas TLR4 and TLR5 are located on the cell-surface and recognize bacterial lipopolysaccharide (LPS) or flagellin, respectively [36, 37]. Treatment with 20 \(\mu\)M of this heavy metal induced a statistically significant down-regulation of TLR3 (at 24 h), TLR4 (at 24 h), TLR7 (at 6, and 24 h), TLR8 (at 24 h), TLR9 (at 24 h) (Fig. 6, Figure S4). Low doses of this heavy metal did not result in modulation of any of the tested TLRs (Fig. 6, Figure S5).

We subsequently investigated \(\text{Cd}^{2+}\) ability to modulate gene expression of other key innate immunity molecules, such as the adaptor protein myeloid differentiation factor 88 (MyD88), p65 (a subunit of transcription factor NF-\(\kappa\)B) [38], and molecules with antimicrobial activities: CD14 and myeloid differentiation protein 2 (MD2) (both involved in LPS recognition by TLR4 [39]), and the host antimicrobial peptides beta defensin 1 (BD1) and 2 (BD2) [40]. Treatment with 20 \(\mu\)M of \(\text{Cd}^{2+}\) reduced expression of MyD88 (24 h), p65 (24 h), MD2 (24 h), CD14 (24 h), and BD2 (24 h) (Fig. 6, Figure S5). Reduced expressions of BD2 and CD14 were also triggered by low doses of this heavy metal (24 h) (Fig. 6, Figure S5). Enhanced expression of BD1 was induced by low doses of \(\text{Cd}^{2+}\) after 6 h of treatment, although no differences between treated and untreated moM\(\Phi\) were detected at later time points (Fig. 6, Figure S5).

Finally, we investigated the impact of this heavy metal on two enzymes involved in macrophage arginine metabolism: induction of both arginase 1 (Arg-1) and nitric oxide synthase 2 (NOS2) were monitored over time [56]. NOS2 encodes for the enzyme inducible nitric oxide synthase (iNOS), which generates nitric oxide (NO) from arginine,
Fig. 5. Induction and release of a key cytokines by moMΦ in response to Cd$^{2+}$. Wild boar moMΦ were left untreated (0) or treated with different Cd$^{2+}$ concentration. a) At 24 h post-stimulation, levels of IL-1α, IL-1β, IL-6, IL-10, IL-12, TNF-α in culture supernatants were quantified using a multiplex ELISA, whereas levels of IFN-β were determined using a sandwich enzyme immunoassay. Mean data and SD from three independent experiments using different wild boar are presented. Values for Cd$^{2+}$-stimulated samples were compared to untreated control (0) using or a one-way ANOVA followed by Dunnett’s multiple comparison test or a Kruskal–Wallis multiple comparison test. (b) At 3, 6, and 24 h post-stimulation, gene expression levels of IL-1β, IL-6, IL-10, IL-12p40, TNF-α, IFN-β, and IFN-α1 genes were determined using RT-qPCR. At each time point, data were normalized on the values of untreated control and expressed as $2^{-\Delta\Delta Cq}$, with $\Delta Cq = Cq (target \ gene) - Cq (reference \ gene)$, and $\Delta\Delta Cq = \Delta Cq (Cd^{2+}$-treated samples) — $\Delta Cq$ (untreated sample, moMΦ). Heatmap displays mean data from five independent experiments using different blood donor wild boar. The colors in the cells represent the relative magnitude of gene expression. The yellow color represents the average magnitude of gene expression. The green color represents the smallest value, and the brightest orange represents the highest value.
whereas Arg-1 is an enzyme that hydrolyses arginine to ornithine and urea. Arg-1 expression was not affected by treatment with either low (2 μM) or moderate doses (20 μM) of this heavy metal, whereas NOS2 expression was down-regulated at both 6 h and 24 h post-treatment with moderate Cd^{2+} doses (Fig. 6, Figure S5).

4. Discussion

Cd^{2+} is a non-infectious stressor, a toxic pollutant that can be bio-accumulated, thus it is recommended to monitor its level in the environment [5]. Wildlife species accumulate environmental pollutants, thus are frequently used as bioindicators for habitat contamination [10, 11]. Liguria region is a highly urbanized area, where human–wildlife interface increased over the last years, facilitating the spread of infectious diseases from wildlife to humans [41]. The data collected during the monitoring activity (2016–2020) showed a prevalence of contaminated samples (Cd^{2+} concentration higher than 0.50 mg/kg) in the eastern part of Liguria. Additional samplings were carried out in one polluted area to better understand the type of contamination. The relationship between hepatic and renal Cd^{2+} concentration of the 21 additionally sampled wild boar was determined: the [Cd^{2+}] liver/ [Cd^{2+}] kidney ratio was lower than 1 in 20 out of 21 tested subjects. These results support the thesis of a cadmium chronic exposure, since the liver is the first site of cadmium absorption [33]. The results of our monitoring activity suggest that wild boar can be useful to monitor Cd^{2+} environmental exposure in highly urbanized area, such as Liguria, and can help in understanding Cd^{2+} type of contamination (chronic or acute). These monitor activity can be of crucial importance to guarantee food products safety and prevent cadmium poisoning. Previous studies in humans and rodents showed that this heavy metal triggered macrophage immune dysfunctions [16,42], whereas other studies reported that cadmium polarize lung macrophages toward a pro-inflammatory phenotype, with subsequent exacerbation of lung injury [43].

Thus, in the second part of this study, we investigated Cd^{2+} effects on wild boar moMΦ, with the aim to further elucidate Cd^{2+} modulation of the immune system. Macrophages are key elements of the innate immune system, at first line of defence to foreign invaders [21]. Humans and pigs share many physiological and immunological characteristics [22]. In particular, pig macrophages resemble human macrophages more than rodent’s [23–25], thus discoveries in this wild species should well reflect the effects of this environmental pollutant on human innate immune system.

Wild boar moMΦ were able to adsorb Cd^{2+}, with subsequent decrease in percentages of live cells. Similar findings were reported in our previous study on IPEC-J2 cells, where adsorption of this heavy metal led rapidly to cell death [20]. Treatment of porcine intestinal cells with 20 μM of Cd^{2+} resulted in complete detachment of cell monolayer [20], whereas we observed that 24 h post treatment with moderate doses more than 50 % of wild boar moMΦ were still alive. This difference is probably linked to higher ability of IPEC-J2 to absorb that metal compared to moMΦ.

In the above-mentioned study, we observed that absorption of this heavy metal by porcine intestinal cells was related to a significant modulation of key pro-inflammatory genes [20], thus we investigated Cd^{2+} modulation of pro-inflammatory and anti-inflammatory cytokines in moMΦ. Proinflammatory properties of subtoxic doses of this heavy metal have been reported not only in our previous study on porcine intestinal cells [20], but also in several murine and human cell lines or primary cells (reviewed in [44]). In particular, Cd^{2+} doses similar to those used in our study (10 μM) lead to increased secretion of IL-1β by murine macrophages (RAW 264.7 macrophages) [45]. We observed that 3 h post-exposure to this heavy metal induced a slight increased expression of two pro-inflammatory cytokines (IL-1β and IL-6), although without statistical significance. Nevertheless, no statistically significant increase in any pro-inflammatory cytokines was observed in culture supernatants of Cd^{2+}-treated moMΦ and 24 h post-exposure to moderate Cd^{2+} doses lead to down-regulation of TNF-α and IL-12p40 expression. In addition, moderate dose of this heavy metal induced down-regulated expression of IFN-α. We might speculate that wild boar moMΦ initially respond to this foreign invader through induction of pro-inflammatory cytokines, but then cadmium affect macrophage’s viability and negatively modulate the ability of this phagocytic cells to combat invading microbes.

Cadmium-mediated macrophage immune dysfunction is also supported by our observation on TLRs expression: 24 h exposure to moderate doses of this heavy metal resulted in down-regulated expression of TLR3, TLR4, TLR7, TLR8, TLR9, p65, MYD88, MD2, CD14, BD1, BD2, Arg1, NOS2 genes were determined using qPCR. At each time point, data were normalized on the values of untreated control and expressed as 2^−ΔΔCq (ΔCq = Cq (target gene) −Cq (reference gene), and ΔΔCq = ΔCq (Cd^{2+}-treated samples) −ΔCq (untreated sample, moMΦ)). Heatmaps display mean data from five independent experiments using different blood donor wild boar. The colors in the cells represent the relative magnitude of gene expression. The yellow color represents the average magnitude of gene expression. The green color represents the smallest value, and the brightest orange represents the highest value.

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was investigated: NOS2 encodes for the enzyme iNOS, which generates nitric oxide (NO) from arginine, whereas Arg-1 encode for an enzyme which catalyzes the hydrolysis of arginine to ornithine, with subsequent increase of polyamine synthesis, promoting tissue repair and remodeling [54]. iNOS and Arg-1 are regarded as hallmark of classical (M1) or alternative (M2) polarization, respectively [54]. M1 macrophages are mainly associated with pro-inflammatory and antimicrobial activities, on the other hand M2 macrophages are mostly involved in immunosuppression and wound healing functions [46]. A previous study showed that exposure to high doses of cadmium (50 μM CdCl₂) for 3 h triggered up-regulation of TNF-α, NOS2, and down-regulation of IL-10, and Arg-1 in THP-1 macrophages, suggesting that this heavy metal polarize macrophages toward a pro-inflammatory classically activated phenotype [43]. On the contrary, we observed that exposure to this environmental pollutant resulted in null modulation of Arg-1 and instead decrease NOS2 expression (6 h and 24 h post-treatment). Differences are maybe linked to the type of macrophages used in the two different studies: THP-1 [43] and wild boar M0M0 (our study). Our data suggest that wild boar M0M0 exposed to Cd²⁺ did not polarize toward a pro-inflammatory M1 phenotype, on the contrary moderate dose of this heavy metal decreased macrophage’s antimicrobial defenses, supporting the other data generated in this study.

In conclusion, this wild species can help understanding how environmental factors, such heavy metal, shape immunity and can be a valid model to study the effects of this environmental pollutant on human innate immune system. Overall our data revealed that the exposure of macrophages to moderate doses (20 μM) of Cd²⁺ affected their viability and lead to down-regulated expression of pro-inflammatory cytokines, several TLRs, p65, NOS2 and other molecules with microbicidal activities, indicating that exposure to this heavy metal negatively affect macrophage’s immune functions, which potentially increased susceptibility to infection.

**Author statement**

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**Declaration of Competing Interest**

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

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**Appendix A. Supplementary data**

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2022.01.009.

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