Modulatory Effects of Pb$^{2+}$ on Virally Challenged Chicken Macrophage (HD-11) and B-Lymphocyte (DT40) Cell Lines In Vitro

Biyao Han,* Diego García-Mendoza, Hans van den Berg, and Nico W. van den Brink
Division of Toxicology, Wageningen University and Research, Wageningen, The Netherlands

Abstract: Elevated levels of lead have been found in waterfowl, due to human activities. Lead may cause immunomodulatory effects, but the mechanisms are largely unknown, especially after viral challenges. To characterize avian immunomodulatory hazards of lead (Pb$^{2+}$), we used chicken macrophage (HD-11) and B-lymphocyte (DT40) cell lines, in vitro models for the innate and adaptive immune systems, respectively. The cells were activated via toll-like receptor-3 by polynosinic–polycytidylic acid sodium salt (poly I:C), mimicking viral infections. Our results indicate that Pb$^{2+}$ is cytotoxic to both cell lines, macrophages being more sensitive. De novo synthesis of glutathione plays an important role in protecting macrophages from Pb$^{2+}$ intoxication, which might also be closely involved in the induction of nitric oxide after Pb$^{2+}$ exposure. Stimulatory effects on cell proliferation were noticed at noncytotoxic Pb$^{2+}$ concentrations as well. Exposure to Pb$^{2+}$ could also affect the inflammatory status by inhibiting the pro-inflammatory interferon (IFN)–γ while promoting the production of anti-inflammatory type I IFNs in both macrophages and B-cells, and increasing intracellular IgM levels in B-cells. These results suggest that the immunomodulatory effects of Pb$^{2+}$ in birds are probably closely associated with disruption of immune cell proliferation and cytokine production, potentially causing disorders of the avian immune system. Environ Toxicol Chem 2020;39:1060–1070. © 2020 The Authors. Environmental Toxicology and Chemistry published by Wiley Periodicals LLC on behalf of SETAC.

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INTRODUCTION

For decades, lead (Pb) has been identified as one of the most toxic environmental heavy metals (Gadrat et al. 1966; Brunekreef et al. 1983; Smith and Flegal 1995; Gavaghan 2002; Needleman 2004; Rahman and Singh 2019). Although overall lead emission into the environment has been reduced because of, the ban on the use of lead in paints, pipes, and gasoline, for example, elevated levels in the environment still occur. In soils and sediments of historical hunting areas in particular, the levels are higher due to the use of lead ammunition (European Chemicals Agency 2017; Williams et al. 2017). Lead pellets have been found in wetlands all over Europe, from a few up to hundreds of pellets/m$^2$ (Pain et al. 2019), potentially threatening wildlife in these habitats. One of the most vulnerable groups of animals is waterfowl. Lead pellets are often ingested by waterfowl when they are foraging; the birds mistake them for the grit used to facilitate food digestion (De Francisco et al. 2003). Ingestion of lead shot by mallard ducks has been documented in Europe, and the prevalence is closely related to the environmental densities of pellets (Mateo 2009). After ingestion by waterfowl, lead pellets dissolve in the intestinal tract, often followed by rapid uptake and distribution of Pb$^{2+}$ in various tissues including liver, kidneys, and bones (Ferreyra et al. 2014). Increased lead levels have even been detected in eggshells from wild waterfowl in contaminated areas, suggesting potential maternal exposure for avian embryos (Franson and Pain 2011; Vallverdu-Coll et al. 2015a; Pain et al. 2019). Lethal effects of lead poisoning are evident, causing mortality of millions of birds worldwide annually (De Francisco et al. 2003; European Chemicals Agency 2017). However, the sublethal effects of lead exposure on the avian immune system are still largely unknown. Lead intoxication has been reported to affect immunity and diminish resistance to infections in birds (Knowles and Donaldson 1997; Vallverdu-Coll et al. 2019), which might result in the prevalence of some zoonotic diseases including avian influenza.

Similar to mammals, the avian immune system consists of an innate system and an adaptive immune system. The innate immune system produces an initial nonspecific response to pathogens. The adaptive immune system evolves over time,
depending on the infections an organism experiences (Sharma 1997; Davison et al. 2008). Multiple types of immune cells from both the innate (e.g., macrophages, heterophils, and natural killer cells) and adaptive (e.g., B- and T-lymphocytes) immune systems collaborate to form a functional and effective immune response. Once infected by specific pathogens, the innate and the adaptive immune systems will be triggered to activate certain defense strategies within hours and days, respectively (Abbas et al. 2014). These defense strategies include, for example, production of nitric oxide by macrophages and production of antibodies (immunoglobulin [Ig]) by B-lymphocytes. For communication during immune responses, immune cells also secrete cytokines such as pro-inflammatory interferon-γ (IFN-γ), antiviral interferon-α and β (IFN-α and -β), tumor necrosis factor-α (TNF-α), interleukins (ILs; e.g., pro-inflammatory IL-18), and chemokines (e.g., chemokine [C-X-C motif] ligand 8 [CXCL8], also called IL-8; Maxwell and Robertson 1998; Genovese et al. 2013; Schat et al. 2014; Speer 2016). Different cytokines are produced by different immune cells and play specific roles in the immune response. The appropriate levels and functioning of different immune cell populations and immune mediators (e.g., cytokines and chemokines) are crucial for the communication and cooperation between different immune cells, which is the basis of an adequate immune system.

Previous studies have revealed that lead may have immunomodulating effects, potentially affecting immune organs, cellular populations, and signaling molecules in birds (Vallverdu-Coll et al. 2019). For example, mallard ducks exposed to lead showed decreased spleen mass; decreased number of heterophils and monocytes (Rocke and Samuel 1991); lower antibody levels (Trust et al. 1990; Vallverdu-Coll et al. 2015a); and lower lysozyme levels (Vallverdu-Coll et al. 2016). At the cellular level, lead can alter the normal immune function indirectly by depleting antioxidants such as glutathione (GSH) and producing oxidative stress (Vallverdu-Coll et al. 2015a), which could potentially induce damage to DNA and membrane structure (Ahamed and Siddiqui 2007). Because some immune cells such as macrophages and heterophils can undergo oxidative bursts by rapid production of reactive oxygen species (ROS) to defend against microbial pathogen infections, a chemical disturbance of redox conditions may also influence the ability to produce the immune defense response related to oxidative burst (Iles and Forman 2002). Exposure to lead has also been implicated in the modification of cytokine gene expression and production in mammals both in vivo and in vitro (Yucesoy et al. 1997; Kroeva et al. 2000; Cheng et al. 2002; Heo et al. 2007). More specifically, Pb2+ exposure was found to result in an imbalance between pro-inflammatory, cell-mediated T helper 1 (Th1) and anti-inflammatory, humoral-mediated T helper 2 (Th2) response by reducing Th1 cytokines (e.g., IFN-γ and IL-2) and enhancing Th2 cytokine (IL-4) at the same time (Lawrence and McCabe 2002; Iavicoli et al. 2004). Because the major targets of Th1 responses are cellular antigens including viruses and bacteria, whereas the major targets for Th2 responses are extracellular parasites (Degen et al. 2005), the Pb2+-induced shifting to Th2 immunity could probably weaken the host defense of viruses in birds. However, no in vitro model has been established to study the immunomodulatory effects of heavy metals in birds, which could reduce animal experiments and give more insights in the mechanisms. In addition, most studies in birds have assessed baseline immunity after lead exposure without activation of the immune system, which is crucial when one is discussing the vulnerability of birds to diseases. Among the few studies of challenges to the immune system, most of them have focused on bacterial rather than viral infections (Vallverdu-Coll et al. 2019). Therefore, the aim of the present study was to investigate the immunomodulatory effect of Pb2+ exposure in 2 chicken in vitro cell lines (macrophages and B-lymphocytes) in both nonactivated and virally activated conditions.

**MATERIALS AND METHODS**

**Experimental setup**

Two chicken immune cell lines, macrophage HD-11 cells and B-lymphocyte DT40 cells, were selected as 2 avian in vitro models for the innate and adaptive immune systems, respectively. Because both cell lines express toll-like receptor 3 (TLR3), which is able to recognize double-stranded RNA (dsRNA) related to viral infection (Peroval et al. 2013; Zou et al. 2017), polyinosinic-polycytidylic acid sodium salt (poly I:C), a synthetic analog of dsRNA (Chen et al. 2013) was used as a viral activator via TLR3.

Both general cytotoxicity and immune functional effects of Pb2+ (as lead acetate [PbAc2]) were investigated in the 2 cell lines. First, cell viability was measured by either the water-soluble tetrazolium-1 (WST-1) assay (for free-floating DT40 cells) or the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT; for attached HD-11 cells) to quantify the median effect concentration (EC50) values, used as the basis for determining the nontoxic concentration ranges in consecutive assays. Cell membrane integrity was also tested as another indicator for cytotoxicity, using the lactate dehydrogenase (LDH) leakage assay (Adan et al. 2016). Depletion of GSH and induction of ROS in the cells were tested with or without inhibition of de novo GSH synthesis, because Pb2+ has a high affinity with thiol groups (Dafre et al. 2004) and can result in oxidative stress via this mechanism (Ma et al. 2017).

Subsequently, assays were applied to assess the effects of Pb2+ on immune functions in both nonactivated cells and cells activated with 25 µg/mL poly I:C. Nitric oxide production was quantified as a primary functional parameter for macrophage (HD-11 cells). Cell proliferation was tested with prolonged WST-1/MTT assays for metabolic activity (cell activity), in a similar fashion as just described for cytotoxicity assays and by using the bromodeoxyuridine (BrdU) assay for DNA synthesis (cell divisions). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to profile the regulation of specific immune gene expression after Pb2+ exposure at the transcription level. Finally, multiple immune functional proteins were measured with enzyme-linked immunosorbent assays (ELISAs). Both released and intracellular immunoglobulin M (IgM) levels were examined for DT40 B-lymphocytes, and secretion of TNF-α by HD-11 macrophages...
was measured. The production of IFNs, including type I antiviral IFN-α and type II pro-inflammatory IFN-γ, and IL-8 was determined in the supernatant of both cell lines. All the assays were performed in 3 biological replicates.

**Cell culture**

The chicken B-lymphocyte–like cell line DT40 (ATCC® CRL2111™) derived from avian leukosis virus (Winding and Berchtold 2001) was cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% tryptose phosphate broth (Sigma-Aldrich), 10% fetal bovine serum (FBS; Sigma-Aldrich), 5% chicken serum (Sigma-Aldrich), and 0.05 mM 2-mercaptoethanol (Gibco) at 37 °C in a 5% CO2 humidified air incubator. Growth medium was renewed every 2 to 3 d.

Chicken macrophage-like cell line HD-11, transformed with MC29 virus (Beug et al. 1979), was a kind gift from J. van Baal (Department of Animal Sciences, Wageningen University, the Netherlands). The HD-11 cells were maintained in Roswell Park Memorial Institute 1640 Medium (Gibco) containing 10% FBS at 37 °C in a 5% CO2 humidified air incubator. Cells were subcultured every 2 to 3 d by detaching the cells with 5 mM ethylenediaminetetraacetic acid (EDTA; Merck) followed by a washing step to remove EDTA before seeding the cells.

**Pb2+ exposure**

Both cell lines were exposed to similar Pb2+ concentrations for comparison. In a flat-bottomed 96-well plate, 1 x 10^5 cells/well were seeded and incubated overnight. In the proliferation assay, 1 x 10^4 cells/well were used as the seeding concentration to allow for a longer exposure time. For qPCR, 1 x 10^6 cells/well were seeded in 6-well plates. A series of PbAc2 (Sigma-Aldrich) stock solutions ranging from 0.02 to 100 mM was prepared in sterile Milli-Q water and diluted 20 times to final exposure concentrations in growth medium. Exposure time was 24 h for most assays, but a 6-h exposure was performed for the ROS assay in addition to a 24-h exposure, whereas for the proliferation assay the exposure time was extended to 48 h.

To investigate the role of newly synthesized GSH, 200 µM of L-buthionine-sulfoximine (BSO; Sigma-Aldrich) was used as a GSH de novo synthesis inhibitor by blocking γ-glutamylcysteine synthetase in the GSH, ROS, and nitric oxide assays. To trigger an antiviral response, 25 µg/mL poly I:C (Sigma-Aldrich) was used in all immune functional assays. The activation of the cells started at the same time as exposure. In all immune functional assays, DT40 cells were seeded and exposed in chicken-serum–free medium to eliminate the potential effects of cytokines in chicken serum, and LDH assay cells were cultured in serum-free medium to avoid the serum-derived LDH.

**General toxicity assays**

**Cytotoxicity assays.** For HD-11 cells, 20 µL 5 mg/mL MTT solution in phosphate-buffered saline (Gibco) was added to each well after 24-h exposure to Pb2+, and the wells were incubated at 37 °C for 2 h. Then the medium was aspirated, and 100 µL dimethylsulfoxide was added to dissolve the violet formazan crystals produced by metabolically active cells. After 15 min of shaking, the plates were measured for absorbance at 562 and 620 nm using a Microplate Reader SpectraMax M2 (Molecular Devices). For DT40 cells, 20 µL WST-1 reagent was added to each well, and the wells were incubated at 37 °C for 2 h before measuring absorbance at 440 nm and a reference wavelength at 620 nm. Cell viability was expressed as percentage of negative control. Concentrations used in later assays were determined by the EC50 obtained in cell viability assays for both cell lines.

The cell membrane integrity was evaluated after Pb2+ exposure with a Pierce LDH cytotoxicity assay kit (ThermoFisher Scientific). Briefly, after incubation of the cells for 24 h in serum-free medium (to avoid serum-derived LDH) with Pb2+ exposure, cells were centrifuged, and 50 µL of supernatant was transferred into a new plate. The supernatant was incubated with 50 µL reaction mixture at room temperature for 30 min. Cells treated with lysis buffer served as the positive control for maximum LDH activity. Afterward, 50 µL of stop solution was added before measurement absorbance at 490 and 680 nm. The results were expressed as percentage of positive control.

**GSH assay.** Intracellular GSH levels were measured with the ThiolTracker™ Violet Glutathione Detection Reagent (Life Technologies/Invitrogen) in a 96-well plate setup, according to the manufacturer’s instructions. Briefly, after Pb2+ exposure for 24 h with or without 200 µM of the GSH synthesis inhibitor BSO, cells were washed with Dulbecco’s phosphate-buffered saline with calcium and magnesium (D-PBS C/M; Gibco) and labeled with 20 µM ThiolTracker™ Violet dye working solution in D-PBS C/M for 30 min at 37 °C. After the working solution was replaced with D-PBS C/M, fluorescence was measured at excitation/emission of 404/526 nm with the Microplate Reader SpectraMax M2. The GSH levels were shown as percentages relative to the Pb2+-free controls.

**ROS (2',7'-Dichlorofluorescin diacetate) assay.** 2',7'-Dichlorofluorescin diacetate (DCFDA; Sigma-Aldrich) reagent was used for measuring the intracellular level of ROS after 6- or 24-h Pb2+ exposure. After diffusion into the cell, DCFDA is deesterified by cellular esterase and oxidized to highly fluorescent 2',7'-dichlorofluorescin by ROS (Eruslanov and Kusmartsev 2010). The exposure conditions were identical to the those for the GSH assay (also with or without BSO). Because the fluorescence of DCF is only stable for approximately 6 h, the method for measurement after 24 h of exposure was modified according to the recommendation from the manufacturer. For 6-h exposure, cells were first washed with PBS and loaded with 20 µM DCFDA working solution in PBS, containing 2% FBS, for 60 min at 37 °C in the dark. After loading, the DCFDA working solution was replaced with the exposure medium containing Pb2+ and incubated for 6 h at 37 °C in the dark until measurement. For the 24-h exposure, the cells were first exposed to Pb2+ for 20 h and then the same volume of 2x DCFDA working solution was added. The plates were then incubated for another 4 h and measured. The ROS level was measured at 485 nm excitation/535 nm emission and presented as a relative ratio to the Pb2+-free controls.

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**Immune functional assays**

Nitric oxide (Griess) assay for HD-11 cells. Nitric oxide production was tested in HD-11 macrophages after Pb^{2+} exposure with the consideration of effects from de novo GSH synthesis (BSO) and activation (poly I:C). Briefly, after exposure, 100 μL of cell culture supernatant was transferred to a new plate and incubated with 50 μL 1% sulfanilamide (Sigma-Aldrich) in 5% phosphoric acid (Merck) at room temperature for 10 min in the dark. Then 50 μL of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma-Aldrich) solution was added, and the supernatant was incubated for another 10 min in the dark before absorbance was measured at 540 nm with a microplate reader. Nitric oxide production in μM was calculated according to a standard curve of NaNO₂.

**Proliferation assays.** The MTT and WST-1 assays were applied as described in the section Cytotoxicity assays. For the colorimetric BrdU assay (Roche), cells were labeled with BrdU during the last 4 h of exposure. Subsequently, floating DT40 cells were spun down by centrifuging at 300 g for 10 min. Then both DT40 and HD-11 cells were fixed and incubated with anti-BrdU-peroxidase, which binds to the BrdU-labeled newly synthesized DNA and reacts with the substrate. The reaction was terminated by adding stop solution after incubation for 30 min at room temperature. The number of proliferating cells was quantified by measuring absorbance at 450 nm and a reference wavelength at 690 nm with the microplate reader and indicated as a percentage in relative to negative controls.

**Gene expression (RT-qPCR) assay**

Expression of 7 chicken genes, including the housekeeping gene (actin-β), type I interferons (IFN-α and IFN-β), interleukins (IL-8 and IL-18), and toll-like receptors recognizing viral pathogens (TLR3 and TLR7), were tested in both cell lines by RT-qPCR with kits from Qiagen (Venlo) according to the manufacturer’s instruction. Briefly, after exposure for 24 h, cells in 6-well plates were lysed with RLT lysis buffer, and RNA was isolated using the QIAshredder and RNeasy® mini kit. The quality and quantity of the RNA obtained were assessed by Nanodrop (ND-1000; ThermoScientific). Subsequently 300 ng total RNA was reverse-translated to complementary (c)DNA with the QuantiTect® reverse transcription kit, and then RT-qPCR was applied using the Rotor-Gene® SYBR® Green PCR kit and the Rotor-Gene® 6000 cycler according to the manufacturer’s handbook. The chicken-specific primers we used were commercially available QuantiTect® primer assays, including Gg_IFNA3_1_SG, Gg_IFNB_1_SG, Gg_IL8L2_1_SG, Gg_IL18_1_SG, Gg_TLR3_1_SG, Gg_TLR7_1_SG, and Gg_ACTB_1_SG, whose efficiency was checked prior to sample measurement.

**Immune proteins**

Levels of multiple immune functional macro-biomolecules produced by the 2 cell lines, such as TNF-α by HD-11, IgM (both released and inside the cell) by DT40, together with IFN-α, IFN-γ, and IL-8 by both cell lines, were quantified with commercially available sandwich ELISA kits. The chicken IFN-γ kit was made up of a Chicken IFN-γ CytoSet™ antibody pair and a BioSource CytoSet™ Buffer Set (Invitrogen); all other ELISA kits (chicken IFN-α, TNF-α, IL-8, and IgM ELISA kits) were obtained from ELISAGenie.

Both nonactivated and activated cells were exposed to Pb^{2+} for 24 h followed by collection of supernatant. Specific immune protein levels were measured in the supernatant except for intracellular IgM in DT40 cells, which was tested in cell lysate prepared through repeating freeze–thaw cycles followed by centrifuging at 1000 g for 5 min. The ELISAs were carried out according to the manufacturer’s instructions for each kit. Levels of each immune protein (in ng/mL for IgM and pg/mL for others) were determined by comparing absorbance at 450 nm with standards from the kits. Quantities of IgM in cell lysate were normalized by total protein content in each sample, quantified with a Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific), and expressed in ng/μg protein.

**Data analysis and statistics**

GraphPad Prism 5 was used throughout the study except for heatmaps. Concentration–response curves were fitted with nonlinear regression, and the EC50 values were calculated. Significant differences between experimental groups and control groups were evaluated with one-way analysis of variance with Dunnett’s test. Gene expression was normalized against the housekeeping gene actin-β and presented as log2 fold changes in comparison with the negative control of non-activated cells by the –ΔΔCT method (Pfaffl 2001). Heatmaps and clustering analysis based on average linkage were generated by the online tool Heatmapper (2019).

**RESULTS**

**General cytotoxicity assays**

**Cytotoxicity assay.** Both cell lines showed a concentration-dependent decrease in cell viability and an increase in cell membrane damage after 24 h of Pb^{2+} exposure. The Pb^{2+} inhibited metabolic activity with an EC50 of 0.48 mM (95% confidence interval [CI] 0.34–0.68 mM) Pb^{2+} for HD-11 cells and a significantly (p < 0.05, t test) higher EC50 of 1.7 mM (95% CI 1.2–2.4 mM) Pb^{2+} for DT40 cells (Figure 1A). Similarly, Pb^{2+} reduced cell membrane integrity, especially in HD-11 cells with an EC50 of 1.7 mM (95% CI 1.4–2.2 mM) Pb^{2+}; no complete concentration–response curve could be obtained for DT40 cells up to 5 mM Pb^{2+} (Figure 1B).

**GSH levels.** In both cell types, GSH levels inside the cells significantly declined after 24 h of Pb^{2+} exposure when the de novo GSH synthesis was inhibited by BSO (Figure 2). However, no significant changes in GSH levels were observed without inhibition (Figure 2). When dosed with BSO, HD-11 cells showed a clear concentration-dependent response with an EC50 of 0.074 mM (95% CI 0.061–0.089 mM) Pb^{2+}, which is...
much lower than the EC50 for cytotoxicity of Pb$^{2+}$ as quantified by the MTT assay (shown as the vertical line in Figure 2A). In contrast, in DT40 cells, even with BSO, Pb$^{2+}$ only decreased the GSH level at the highest concentration (5 mM Pb$^{2+}$), which is higher than the EC50 of Pb$^{2+}$ cytotoxicity determined by the WST-1 assay (shown as the vertical line in Figure 2B).

**ROS.** No effects of Pb$^{2+}$ exposure on intracellular ROS levels were seen after 6 h of exposure in both cell lines and 24 h of exposure in DT40 cells (data not shown) even though GSH synthesis was blocked with BSO. When the exposure time was extended to 24 h, ROS was only significantly induced in HD-11 macrophages after treatment with the highest Pb$^{2+}$ concentration (2 mM) and BSO (Figure 3) with an EC50 of 0.41 mM (95% CI 0.22–0.76 mM) Pb$^{2+}$. No effects of Pb$^{2+}$ on ROS levels were found in DT40 cells after 24 h of exposure even with BSO (data not shown).

**Immune functional assays**

**Nitric oxide production by macrophages.** Nitric oxide produced by HD-11 macrophages after 24 h of Pb$^{2+}$ exposure was investigated in the cell culture supernatants under different conditions including poly I:C activation and GSH synthesis inhibition (Figure 4). Poly I:C triggered the production of nitric oxide to approximately 9 µM in Pb$^{2+}$-free treatments. The Pb$^{2+}$ significantly increased nitric oxide levels in all 4 conditions, especially in the 2 groups without BSO, showing induction at much lower concentrations than the EC50 of Pb$^{2+}$ cytotoxicity determined with the MTT assay. Moreover, activated
Macrophages produced more Pb²⁺-induced nitric oxide without BSO inhibiting de novo GSH synthesis.

**Cell proliferation.** Cell proliferation after 48 h of Pb²⁺ exposure was evaluated with 2 indicators: metabolic activity by the MTT (for HD-11 cells) or the WST-1 assay (for DT40 cells) and synthesis of DNA quantified by the BrdU assay. There was inhibition at cytotoxic concentrations in terms of both metabolic activity and DNA synthesis in both cell lines, although the inhibition effects were only significant in HD-11 cells (Figure 5A,B). However, Pb²⁺ exposure below cytotoxic concentrations (lower than 0.02 mM for HD-11 cells and lower than 0.2 mM for DT40 cells) showed induction of proliferation in the BrdU assay (Figure 5B,D), and this induction was only significant in nonactivated HD-11 cells (Figure 5B). Interestingly, without an effect on the EC50s, poly I:C treatment stimulated metabolic activity (Figure 5A) while inhibiting DNA synthesis in HD-11 cells (Figure 5B).

**Gene expression**

Gene expression of 6 immune functional genes was examined and visualized with a heatmap (Figure 6; detailed results are shown in the Supplemental Data, Figure S1). Clustering results suggest similar effects of Pb²⁺ exposure on gene expression in both DT40 B-cells and HD-11 macrophages. The Pb²⁺ down-regulated the expression of TLR3 and TLR7, and up-regulated the expression of IFN-α, IFN-β, IL-18, and especially IL-8. A significant difference was only found for IL-8 expression between the treatment of 0.5 mM Pb²⁺ exposure and the Pb²⁺-free control group of activated HD-11 cells.

**Functional immune proteins**

Several immune functional proteins were quantified with ELISA assays after 24 h of Pb²⁺ exposure (Figures 7 and 8).
Concentrations of IFN-γ released by DT40 cells and IL-8 released by both cell lines were lower than the detection limits of the ELISA kits (data not shown). The Pb2⁺ significantly induced IFN-α secretion from both cell lines at subcytotoxic concentrations irrespective of activation, and no significant effect of poly I:C activation was detected (Figures 7A and 8A). In HD-11 cells, IFN-γ production dropped after 24 h of Pb2⁺ treatment for both activated and nonactivated cells. Activated cells were more sensitive to Pb2⁺ exposure with an EC50 of 0.049 mM (95% CI 0.039–0.061 mM) Pb2⁺, whereas an EC50 of 0.70 mM (95% CI 0.18–2.8 mM) Pb2⁺ was obtained for nonactivated cells (Figure 7B). The HD-11 macrophages tended to release more TNF-α after activation, yet no significance was seen, and no statistical Pb2⁺-related increase was noted in either nonactivated or activated cells compared with nonexposed cells (Figure 7C). As for DT40 cells, the IgM levels in nonactivated DT40 cell lysate increased significantly after exposure to 0.5 and 1 mM Pb2⁺ and exceeded the levels in activated cells, although no EC50 value was gained due to lack of maximum effects. However, no significant changes were found in activated cells (Figure 8B). The IgM levels in DT40 cell culture supernatant were not affected by either poly I:C activation or Pb2⁺ exposure (Figure 8C).

DISCUSSION

The present study was designed to investigate cytotoxic and immune functional effects of Pb2⁺ exposure on 2 avian immune cell lines as models for the avian immune system in vitro, with the aim of mimicking a viral challenge. Compared with HD-11 macrophages, DT40 B-cells were significantly less sensitive to Pb2⁺ exposure with respect to cell viability (Figure 1A) and membrane integrity (Figure 1B). Similar patterns have been reported in primary human immune cells isolated from blood after ex vivo exposure to Pb(NO3)2 for 24 h; the monocytes from the innate system had an EC50 of approximately 3.7 mM Pb2⁺, whereas B-cells were not sensitive enough to obtain a determined EC50 value (Steffensen et al. 1994). In both cell lines, the LDH leakage caused by Pb2⁺ exposure occurred at higher concentrations than the inhibition of metabolic activity measured with the MTT or WST-1 assay. However, the LDH leakage assays were performed in serum-free medium, which
was not an optimal condition for the cells. This suggests that damage to the cell membrane is a relatively insensitive indicator of cytotoxicity. Therefore, in the present study, the results derived from the MTT or WST-1 assays were used as the basis for determining the nontoxic concentration ranges in consecutive assays.

It is possible for Pb$^{2+}$ to bind to sulfhydryl (–SH) groups, which are one of the most common functional groups of many biological molecules, including the major cellular antioxidant GSH. In this way, Pb$^{2+}$ exposure may result in elevated ROS levels (Matovic et al. 2015). Our results show that in HD-11 macrophages nontoxic concentrations of Pb$^{2+}$ depleted intracellular GSH when the de novo synthesis of GSH was blocked by BSO (Figure 2A), but the induction of ROS was very limited (Figure 3). In DT40 B-cells, no effects on either GSH (Figure 2B) or ROS levels were noticed at nontoxic concentrations of Pb$^{2+}$ even with BSO. The differences between B-cells and macrophages could be related to the nature of these 2 cell types. As macrophages are functionalized with inducible ROS and nitric oxide to deal with pathogens (so-called oxidative burst), they are equipped with a stronger protective antioxidant system for buffering oxidative stress (Brune et al. 2013). These results indicate that the cells in our experiments can compensate Pb$^{2+}$-induced GSH depletion to prevent further damage through the induction of γ-glutamylcysteine synthetase. However, some in vivo studies have indicated that Pb$^{2+}$ exposure decreased GSH levels, induced oxidative stress, and resulted in lipid peroxidation and DNA damages (Dai et al. 2012; Vallverdu-Coll et al. 2015b), which might be related to the prolonged in vivo exposure or the interaction with other elements in the organism in vivo.

Apart from cytotoxic effects, our results also indicated the modulatory effects of Pb$^{2+}$ on some functional parameters in both cell lines at subcytotoxic concentrations. For instance, Pb$^{2+}$ enhanced nitric oxide production in both nonactivated and activated HD-11 macrophages in a concentration-dependent manner (Figure 4). This could be related to the up-regulation of inducible nitric oxide synthase by Pb$^{2+}$ exposure (Liu et al. 2012; Chiang et al. 2014; Huang et al. 2019). However, when GSH synthesis was blocked by BSO, affecting the cells’ ability to protect itself from the nitric oxide–related oxidative burst, the cells produced less nitric oxide than the ones without BSO; hence it seems that the cell needs the protective antioxidants before it can mount an oxidative burst. On the other hand, the decreased synthesis of GSH may affect the uncoupling of the nitric oxide synthase enzyme, resulting in decreased nitric oxide synthase activity and reduced nitric oxide production (Kasten-Jolly and Lawrence 2014). However, contrasting effects of lead on nitric oxide production have been reported without clearly recognized mechanisms (Tian and Lawrence 1995; Sharifi et al. 2005). Induction of nitric oxide was also noted in the cadmium (Cd)$^{2+}$-exposed mouse macrophage RAW264.7 cell line at nontoxic concentrations, which was also associated with a depletion of GSH (Garcia-Mendoza et al. 2019). Both Cd$^{2+}$ and Pb$^{2+}$ are nonredox active divalent metals (Matovic et al. 2015) with a high affinity to sulfhydryl (–SH) groups and are known to share similar modes of action with respect to the redox status of cells. The impact of de novo GSH synthesis as shown in the present experiments may indicate that the impact of Pb$^{2+}$ on nitric oxide production could depend on the antioxidant status of the cells. Nitric oxide has also been identified to have more functions in the immune system besides killing of microbial pathogens, such as mediating cytokines and inducing differentiation of subpopulations of T-cells (Bogdan 2001; Murata et al. 2002). Hence, although the Pb$^{2+}$-induced nitric oxide production is rather limited compared with the induction of poly I:C, it could still have further implications on other downstream immune functions, which need to be further investigated in vivo.

As shown in Figure 5, Pb$^{2+}$ was found to alter the proliferation of both cell lines. Previous studies also highlighted similar dose-dependent proliferative effects of Pb$^{2+}$ on primary avian (Grasman and Scanlon 1995) and murine (Razani-Boroujerdi et al. 1999) lymphocytes in vitro and in vivo. The HD-11 cells tended to increase metabolic activity instead of synthesizing new DNA after viral challenge, whereas the DT40 cells

**FIGURE 8:** (A–C) Enzyme-linked immunosorbent assay results for multiple immune functional proteins produced by B-lymphocyte (DT40) cells with and without 25 µg/mL polyinosinic–polycytidylic acid sodium salt (poly I:C) activation after 24-h Pb$^{2+}$ exposure. The protein levels were tested in the cell culture supernatant, except for IgM in DT40 cell lysate (B). Results are expressed in protein concentrations (ng/mL or pg/mL) or content (ng/µg protein) as mean ± standard error of the mean (n = 3). Significant differences between treatments and Pb$^{2+}$-free controls were checked with one-way analysis of variance and are shown with different colors for different groups (*p < 0.05; **p < 0.01; ***p < 0.005). Vertical dashed lines indicate the median effect concentration of cytotoxicity after 24-h Pb$^{2+}$ exposure from the water-soluble tetrazolium-1 (WST-1) assay. PbAc$_2$ = lead acetate; IFN = interferon.
did not follow this strategy, suggesting cell type–specific responses. These results indicate that Pb²⁺-enhanced proliferation at noncytotoxic concentrations may affect the composition of immune cell populations in avian species.

More specific functional endpoints included in the present study were the gene expression of TLRs and cytokines (Figure 6). Activation of TLR3 with poly I:C (mimicking viral infection) triggers multiple pathways including the nuclear factor xB (NF-xB) and interferon receptor factors (IRFs) pathways, which may lead to the production of a series of cytokines (Abbas et al. 2014). For instance, expression of inflammatory cytokines including TNF-α and IL-8 could be induced through the NF-xB pathway, and expression of type I interferons (IFN-α and -β) could be induced through IRFs pathways (Akira et al. 2006). According to our results, the only significantly up-regulated gene after 24 h of Pb²⁺ exposure was IL-8 in activated macrophages, a pro-inflammatory chemokine recruiting heterophils (similar to neutrophils in mammals) in birds (Kogut 2002). Stimulation of IL-8 gene expression was shown to be exhibited in human peripheral blood mononuclear cells treated with PbAc₂ above 0.01 mM (Gillis et al. 2012). Other studies have suggested that this up-regulation could be closely related to the nuclear factor erythroid 2-related factor 2 pathway, which also induces ROS at the same time (Dobrakowski et al. 2016; Metryka et al. 2018).

Compared with regulation of gene expression, effects on protein levels are more closely related to potential adverse outcomes at the individual level. For the antiviral cytokine IFN-α, Pb²⁺ increased its secretion in both cell lines without significant influences of poly I:C activation (Figures 7A and 8A). Although poly I:C is known to be able to trigger type I IFN production (Matsumoto and Seya 2008), the poly I:C-induced IFN-α production could be modified by many factors such as activation time, as described earlier in the Discussion section, and the characteristics of the cell line. Given that both DT40 and HD-11 cell lines are transformed to cancer cell lines by virus (Beug et al. 1979; Winding and Berchtold 2001), they may be less sensitive than primary cells to a viral infection mimic such as poly I:C, in terms of type I IFN secretion (Dauletbaev et al. 2015). Together with the slight although insignificant up-regulation of IFN-α and -β shown in the qPCR results, our results indicate that Pb²⁺ could increase the production of antiviral type I IFNs. Secreted type I IFNs can lead the neighboring cells into an “antiviral state” as a defense response to viral infections (Kumar et al. 2011). However, prolonged production of type I IFNs could inhibit inflammation by decreasing the production of IL-1 (Guarda et al. 2011), and at the same time enhance anti-inflammatory responses by induction of Th2 cytokines like IL-10 (Ivashkov and Donlin 2014), potentially leading to disorder of the immune system. Furthermore, Pb²⁺ inhibited IFN-γ secretion in macrophages (Figure 7B), similar to previous studies (Dietert and Piepenbrink 2006; Heo et al. 2007; Valentino et al. 2007). This effect has been reported to be post-transcriptional, changing the protein levels of IFN-γ without influences on gene expression, probably through selectively interfering with the translation of messenger (m)RNAs or other biological processes (Guo et al. 1996; Heo et al. 2007; Metryka et al. 2018). Notably, the inhibitory effects of Pb²⁺ on IFN-γ production were more severe when cells were stimulated with poly I:C, suggesting that cells are less capable of IFN-γ-induced inflammatory responses after viral infections. As for DT40 B-cells, significantly increased IgM levels were found in the cell lysate of nonactivated cells after exposure to non-cytotoxic concentrations of Pb²⁺, but not in the activated cells. In addition to secreted antibody, IgM also acts as B-cell receptors (BCRs) on the cell membrane of DT40 cells recognizing antigens (Gao et al. 2002; Luo et al. 2010), which is the major component of IgM tested in the cell lysates. Hence, Pb²⁺ appeared to stimulate B-cells by increasing intracellular IgM levels and thereby potentially facilitating more surface BCRs, which might boost B-cell functions and humoral mediated Th2 response.

In summary, our results indicated that Pb²⁺ showed concentration-dependent toxic effects on both chicken B-lymphocyte and macrophage cell lines, with macrophages being more sensitive. The Pb²⁺ exposure could also affect the inflammatory status by inhibiting the pro-inflammatory IFN-γ and promoting anti-inflammatory type I IFNs, and stimulating B-cells. The immunomodulatory effects of Pb²⁺ could likely result in disorders of immune systems and inappropriate immune responses to viral pathogens, which has also been shown for bacterial and parasitic pathogens by in vivo studies (Knowles and Donaldson 1997; Vallverdu-Coll et al. 2015b). Although only 2 cell types from the immune system were studied in vitro without interaction between cell lines, our results provide a starting point for further mechanistic, long-term, and systematic studies on the immunomodulatory effects of heavy metals in avian species after viral challenges.

**Supplemental Data**—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.4702.

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**Data Availability Statement**—For access to data, please contact the corresponding author (bijiao.han@wur.nl).

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