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Mercury increases IL-1 β and IL-18 secretion and intensifies coronary arteritis in an animal model of Kawasaki disease

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Kawasaki disease (KD) is a multisystem vasculitis that predominantly targets the coronary arteries in young children. Epidemiological data suggest both environmental and genetic factors contribute to the susceptibility and severity of the disease. Mercury (Hg) is a known environmental pollutant and a Ca²⁺ signaling modulator. Ca²⁺ signaling regulates the activation of NLRP3 inflammasome. Using the *Lactobacillus casei* cell wall extract (LCWE) induced coronary arteritis mouse model of KD; we studied the effect of mercury on inflammasome activation and its impact on the immunopathogenesis of KD. Mercury enhances the expression of inflammasome activation resulting in caspase-1 mediated secretion of IL-1 β and IL-18 cytokines. *In vivo*, the administration of mercury together with disease inducing LCWE exacerbates disease resulting in increased incidence and severity of coronary arteritis compared to LCWE alone. Mercury can act as a novel danger signal modulating Ca²⁺ signaling to increase IL-1 β and IL-18 secretion and intensifies coronary arteritis in an animal model of KD.

KEYWORDS

Kawasaki disease (KD), inflammasome, mercury, inflammation, coronary arteritis, NLRP3 inflammasome, calcium signaling, ITPKC/IP3-3KC/IP3KC

Introduction

Kawasaki disease (KD) is a multisystem vasculitis that predominantly targets the coronary arteries in infants and young children (1, 2). It is characterized by prolonged fever and multisystem inflammation with polymorphous skin rash, oral mucosal changes (red, cracked lips, and strawberry tongue), non-purulent conjunctival injection, extremity changes (redness and swelling), and cervical lymphadenopathy. Epidemiological data suggest an environmental trigger that causes disease in genetically predisposed

individuals (3, 4). During the acute phase of KD, large amounts of pro-inflammatory cytokines, including inflammasome mediated cytokines IL-1 β and IL-18, are produced (5). Genetic studies in KD have associated inositol triphosphate 3-kinase C (ITPKC), an enzyme in the Ca²⁺ signaling pathway, with susceptibility and severity of disease (6). We have shown that ITPKC regulates the secretion of IL-1 β and IL-18 in KD (5), suggesting that KD is an inflammasome mediated disease.

Various environmental agents have been associated with outbreaks of KD (7). Environmental triggers include both infectious and non-infectious origins. One such non-infectious environmental pollutant associated with the pathogenesis of KD is mercury (8). Mercury (Hg) is a known Ca²⁺ signaling modulator (9–11). Ca²⁺ signaling regulates the activation of NLRP3 inflammasome. Mercury is a natural component of our environment, making about 0.5 parts per million of the earth's crust. It is found in three main forms: elemental mercury or quicksilver (Hg⁰), inorganic mercury (Hg⁺ and Hg²⁺), and organic (such as methyl, ethyl, and phenyl) mercury. Inorganic mercury is derived from the oxidization of elemental mercury, while the biomethylation of inorganic mercury compounds produces the organic derivatives by microorganisms in aquatic sediments and soils. The human body can absorb all three forms of mercury. However, the human population is primarily exposed to Hg⁰ and organic mercury, which are converted to inorganic mercury *in vivo* (12).

LCWE-induced coronary arteritis in mice is an animal model for KD. It is similar to children with KD in its time course, susceptibility in the young, vessel pathology, and response to IVIG therapy. Using inorganic mercuric chloride (HgCl₂) as a novel danger signal, we studied its role in NLRP3 expression and secretion of IL-1 β and IL-18 *in vitro* and *in vivo*. HgCl₂ treatment enhances the expression of NLRP3 and secretion of IL-1 β and IL-18 cytokines in LCWE-primed bone marrow derived macrophages (BMDM). The HgCl₂ mediated secretion of IL-1 β and IL-18 is caspase-1 mediated.

Furthermore, we show that extracellular and intracellular Ca²⁺ mobilization is critical for HgCl₂ mediated secretion of IL-1 β and IL-18. LCWE-injected mice exposed to HgCl₂ produce more IL-1 β and IL-18 compared to those given HgCl₂ or LCWE alone. Additionally, HgCl₂ intensifies coronary arteritis *in vivo* with increased incidence and severity of the disease. These results, together with our previous work, show that Ca²⁺ signaling is critical in KD immunopathogenesis and that genetic (ITPKC) and environmental (Hg) factors can directly contribute to the regulation of intracellular calcium mobilization affecting susceptibility to and severity of KD.

Results

Mercury is non-toxic to immune cells at low concentrations

HgCl₂ is a known toxic agent that can cause cell death at high concentrations (10, 11). To determine the cell toxicity of HgCl₂ on

immune cells, we treated mouse splenocytes with various concentrations (10 μ M to 1M) of HgCl₂. Incorporation of propidium iodine as a marker of cell death was then determined by flow cytometry. Low concentrations of HgCl₂ up to 100 μ M do not cause cell death (Figures 1A, B). Non-toxic doses under 20 μ M were used in this study for *in vitro* experiments.

Mercury increases IL-1 β and IL-18 secretion in LCWE-primed cells

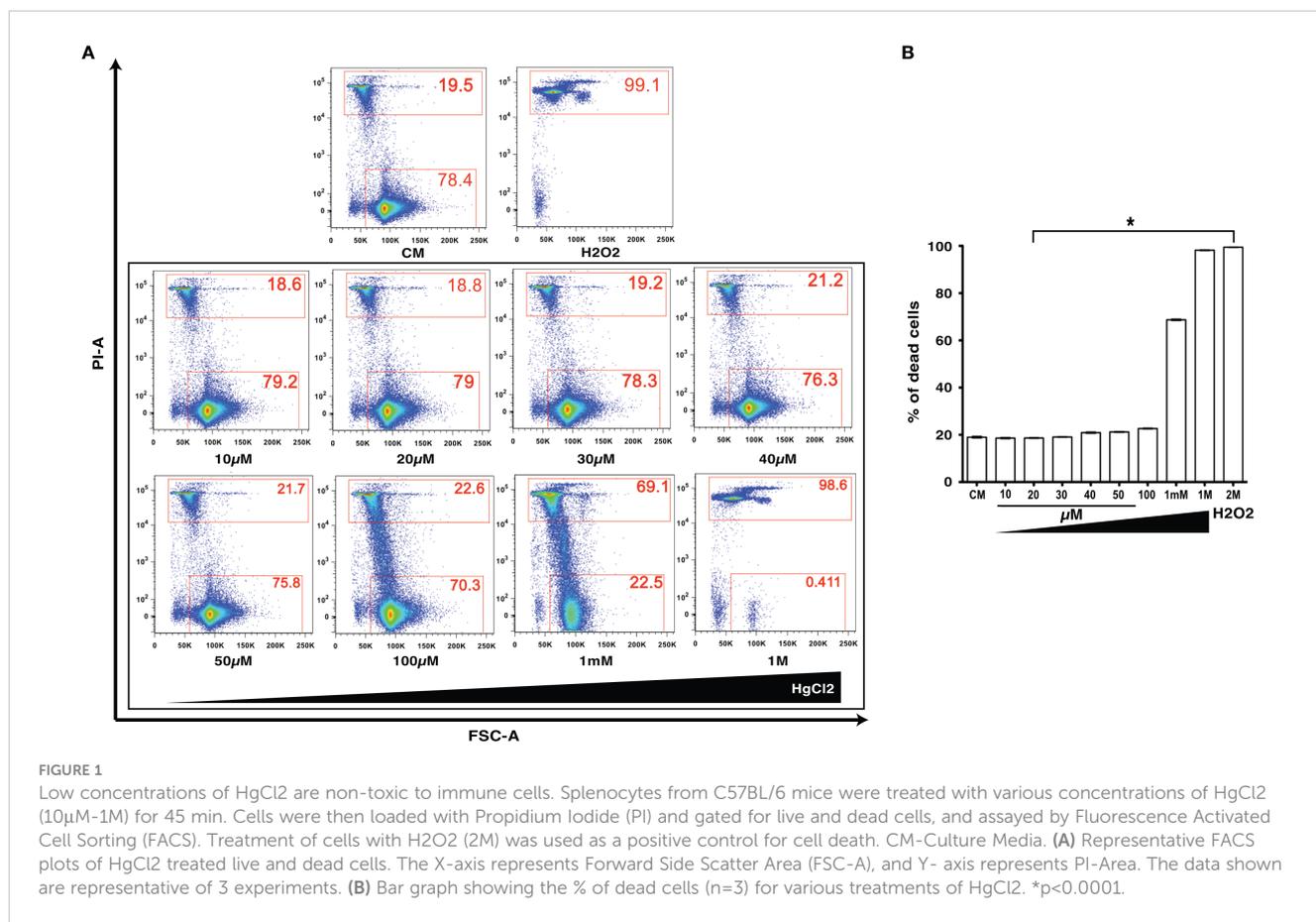
To determine if mercury could regulate the secretion of IL-1 β and IL-18, we treated LCWE-primed BMDMs with HgCl₂. HgCl₂ increases the secretion of IL-1 β cytokine in LCWE-primed BMDMs (Figure 2A) compared to LCWE or HgCl₂ treatment alone. Similar to previous observations (13), stimulation with LCWE alone does not lead to the secretion of IL-18. However, when LCWE-primed BMDMs were treated with HgCl₂, there was the secretion of IL-18 cytokine. IL-18 secretion increases in a dose-dependent fashion with increasing concentrations of HgCl₂ (Figure 2B). Furthermore, treatment with LCWE but not HgCl₂ increases the protein expression of NLRP3 (Figure 2C). When LCWE-primed cells were treated with HgCl₂, there is increased protein expression of NLRP3 from BMDMs (Figure 2C). When HgCl₂ was chelated using Dimercaptosuccinic Acid (DMSA) (Figure 2D) or Dimercaptopropionic Acid (DMPS) (data not shown), secretion of inflammasome mediated cytokines was significantly reduced. These results suggest that HgCl₂ amplifies activation in LCWE-primed immune cells leading to the secretion of inflammasome mediated cytokines IL-1 β and IL-18.

Mercury is a novel danger signal leading to the production of IL-1 β and IL-18

Previous studies show that LCWE contains a TLR2 ligand (14). To determine if HgCl₂ treatment could activate inflammasomes with a known TLR2 ligand, we used Pam3Cys to prime BMDMs. Similar to LCWE, treatment of HgCl₂ with Pam3Cys primed cells leads to the secretion of both IL-1 β and IL-18 cytokines (Figures 3A, B). Taken together, the results suggest that HgCl₂ at low concentrations could act as a novel danger signal in providing optimal activation for the secretion of IL-1 β and IL-18.

Mercury treatment increases intracellular Ca²⁺ mobilization

HgCl₂ is a known agent of Ca²⁺ mobilization (15). Studies have shown that Ca²⁺ mobilization is critical for NLRP3 inflammasome activation (5, 16–19). Therefore, to assess whether HgCl₂ mediated inflammasome activation is Ca²⁺ dependent, we used extracellular and intracellular Ca²⁺ inhibitors and measured the secretion of IL-1 β and IL-18. When live BMDMs from C57BL/6 were assessed for intracellular Ca²⁺ levels using confocal spinning disc microscopy, HgCl₂ treated cells acquired more intracellular Ca²⁺ when



compared to untreated cells (Figure 4A). We next examined whether Ca²⁺ mobilization plays a role in HgCl₂ mediated activation of the inflammasome.

Mercury mediated production of IL-1β and IL-18 is dependent on extracellular Ca²⁺ mobilization

Extracellular Ca²⁺ channels were blocked using Ca²⁺ channel inhibitor 2-APB and PLC pathway inhibitor U73122. Both IL-1β and IL-18 production in cells treated with 2-APB and U73122 was significantly reduced (Figures 4B, C), suggesting that extracellular Ca²⁺ mobilization is vital for HgCl₂ mediated IL-1β and IL-18 secretion in LCWE-primed BMDM.

Mercury mediated production of IL-1β and IL-18 is dependent on intracellular Ca²⁺ mobilization

Intracellular Ca²⁺ channels and receptors were blocked using Ca²⁺ channel inhibitor Tg and IP3R-specific inhibitor XeC, respectively. Tg and XeC treatment inhibited the secretion of IL-1β and IL-18 cytokines (Figure 4C). The secretion of both IL-1β and IL-18 in cells treated with

both extracellular and intracellular Ca²⁺ inhibitors was drastically downregulated (Figures 4B, C). These results emphasize the critical role of both extracellular and intracellular Ca²⁺ mobilization in HgCl₂ mediated activation of inflammasomes, possibly NLRP3 inflammasome.

Mercury induced secretion of IL-1β is caspase-1 dependent and NLRP3 mediated

Inflammasome mediated secretion of IL-1β and IL-18 cytokines are caspase-1 mediated (20). Therefore, to determine if HgCl₂ induced secretion of IL-1β is caspase-1 dependent, Z-YVAD-FMK, a known caspase-1 specific inhibitor, was used. LCWE-primed and HgCl₂-activated BMDMs treated with the inhibitor produced significantly less IL-1β cytokine than untreated cells (Figure 5). As HgCl₂ mediated activation is Ca²⁺ dependent, we treated cells with XeC, a specific inhibitor for IP3R and Z-YVAD-FMK. There was further inhibition of secreted IL-1β cytokine (Figure 5) compared to Z-YVAD-FMK or XeC treatment alone. These results show that HgCl₂ induced secretion of IL-1β is caspase-1 mediated and is IP3R- dependent. Furthermore, when NLRP3 inflammasome activation is inhibited with a specific NLRP3 inhibitor MCC950 in LCWE with or without HgCl₂-treated BMDMs, there is a complete abrogation of IL-1β and IL-18 cytokines secretion (Figures 6A, B).

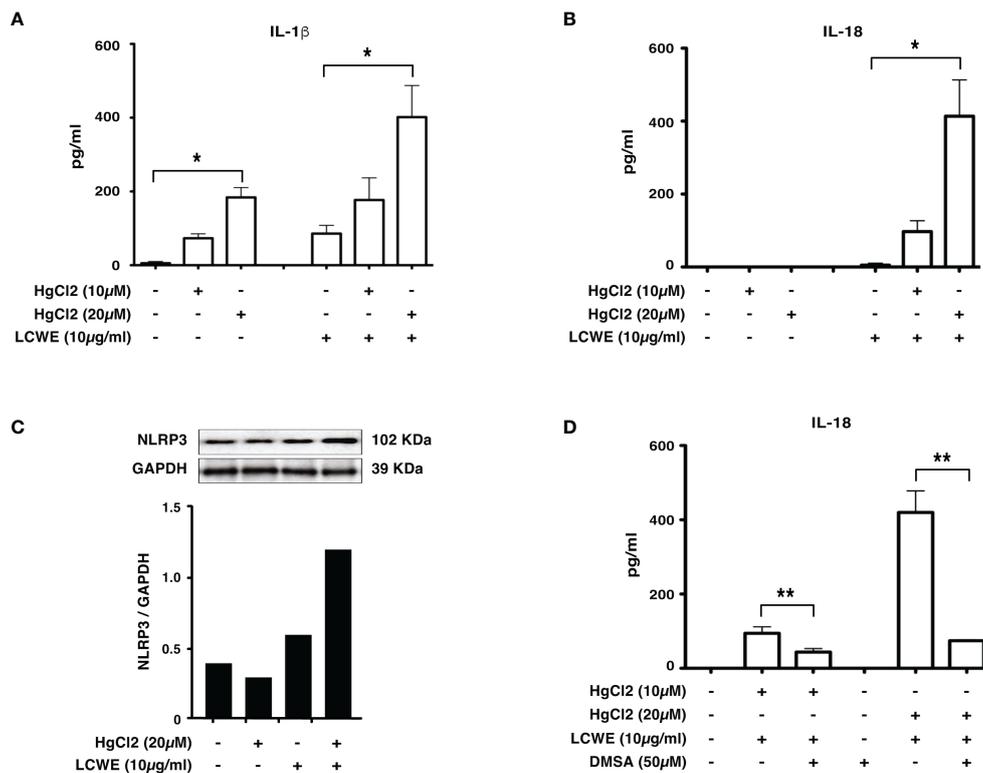


FIGURE 2

HgCl₂ increases NLRP3 expression and increases IL-1 β , IL-18 secretion in LCWE-primed immune cells. BMDMs from C57BL/6 mice were treated with LCWE (10mg/ml) for 18 hrs. 30-45 min prior to 18 hrs, cells were treated with/without HgCl₂ (10 μ M or 20 μ M). Secretion of IL-1 β and IL-18 in supernatants was measured by ELISA, and protein expression of NLRP3 in cells was determined by Western blot analysis. (A) Levels of IL-1 β from supernatants of BMDMs, measured by ELISA (n=3). *p<0.0001. (B) Levels of IL-18 from supernatants of BMDMs, measured by ELISA (n=3). *p<0.0001. (C) Representative western blot of NLRP3 protein expression from BMDMs. Densitometry was measured, and relative amount of protein normalized to GAPDH are shown in the bar graphs. (D) Levels of IL-18 from supernatants of BMDMs, that were treated with/without Hg chelating agent DMSA (50 μ M) (n=3). **p<0.005.

Mercury exacerbates coronary artery disease in an animal model of KD

To determine the effect of HgCl₂ on the LCWE induced coronary arteritis disease model of KD, we administered HgCl₂ to LCWE-injected mice. A single dose of HgCl₂ was administered subcutaneously concurrent with LCWE. We assayed circulating IL-1 β and IL-18 levels serially and determined the incidence and severity of coronary arteritis as per histologic grading protocol. In mice given LCWE and HgCl₂, there was increased production of both IL-1 β and IL-18 in the peripheral blood starting at 48 hrs post injection (Figures 7A, B). Treatment with mercury also resulted in more severe coronary arteritis. In comparison to mice injected with LCWE alone, those injected with LCWE and HgCl₂ exhibited more severe and concentrated cellular infiltrates in the cardiac tissues. On the other hand, in animals injected with just HgCl₂, only minimal cellular infiltrates were observed (Figure 8). Furthermore, both disease incidence (n=10/10) and severity (Mean \pm SD = 1.95 \pm 0.15, p<0.0001) were significantly increased in mice injected with LCWE and HgCl₂ when compared to LCWE (n=7/10) (Mean \pm SD = 0.65 \pm 0.47), or HgCl₂ (n=0/10) injected mice alone (n=10 in each group) (Table 1).

Discussion

It is well established that genetic factors are associated with KD (7). Genes associated with Ca²⁺ signaling and its pathway have been associated with the severity and susceptibility of KD (6). Alterations in ITPKC increase intracellular Ca²⁺ levels due to increased IP3s (6). Intracellular Ca²⁺ levels are critical for the activation of NLRP3 inflammasome (16). We have shown that ITPKC regulates NLRP3 expression in KD, suggesting that genetic factors that alter Ca²⁺ mobilization or signaling can be involved in the severity of KD (5). Similar to genetic factors, environmental factors can change Ca²⁺ mobilization. Mercury (Hg) is one such environmental factor (12). Both inorganic (HgCl₂) and organic (MeHg) forms of mercury can increase intracellular Ca²⁺ mobilization in murine and Jurkat T-cells at low concentrations (10). Furthermore, mercury is one of the many environmental agents associated with KD (9). The clinical symptoms of KD, which form the basis of clinical diagnosis, resemble the clinical features of mercury hypersensitivity in children called Acrodynia or pink disease (8, 9, 21–24).

Using mercury as a novel danger signal, we studied the effect of mercury on inflammasome activation in the LCWE induced coronary arteritis murine model of KD. Treating BMDMs with

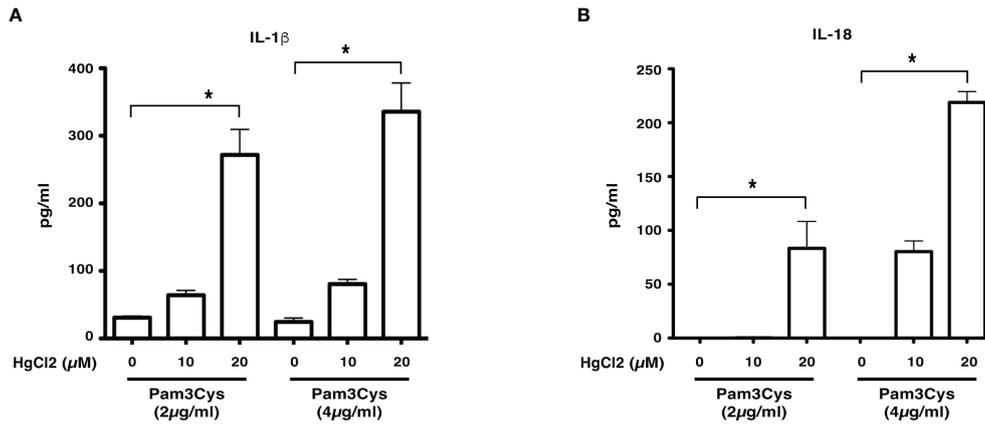


FIGURE 3 HgCl₂ is a novel danger signal mediating the secretion of IL-1β and IL-18. BMDMs from C57BL/6 mice were treated with TLR-2 ligand Pam3Cys (2µg/ml or 4µg/ml) for 18 hrs. 30-45 min prior, cells were treated with/without HgCl₂ (20µM). Secretion of IL-1β and IL-18 in supernatants was measured by ELISA. **(A)** Levels of IL-1β from supernatants of BMDMs, were measured by ELISA (n=3). **(B)** Levels of IL-18 from supernatants of BMDMs, measured by ELISA (n=3). *p<0.005.

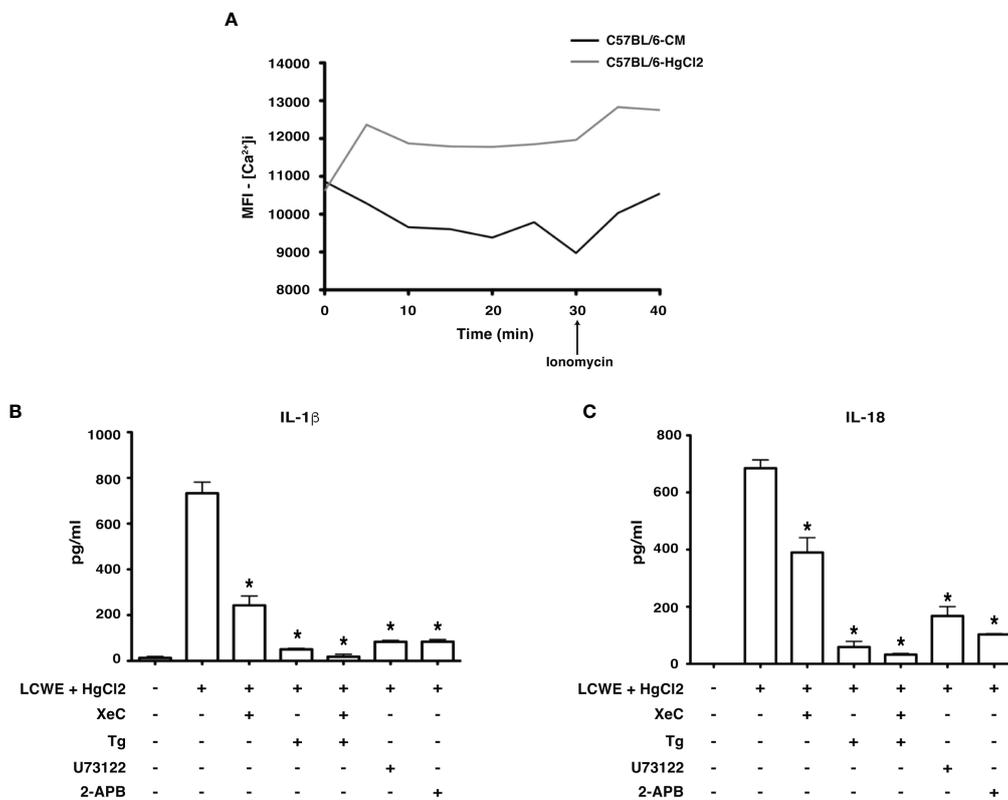
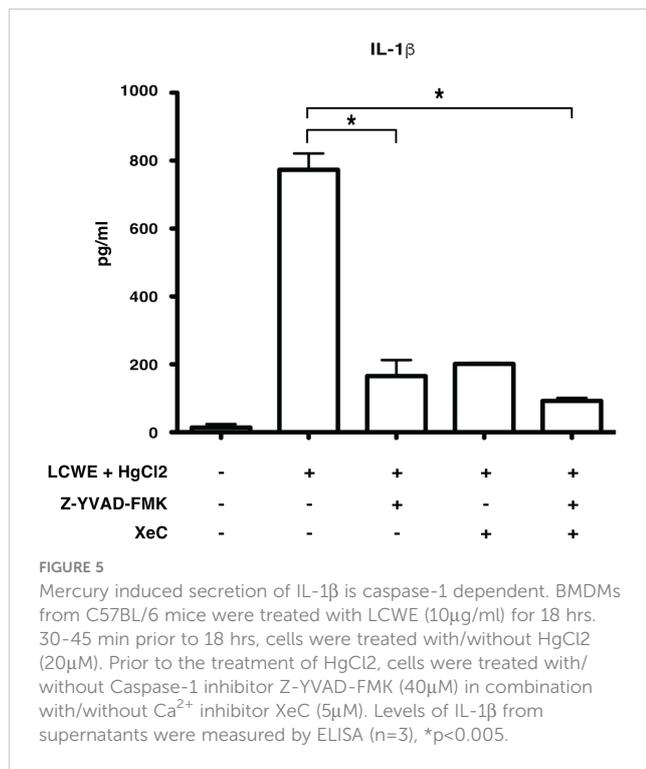


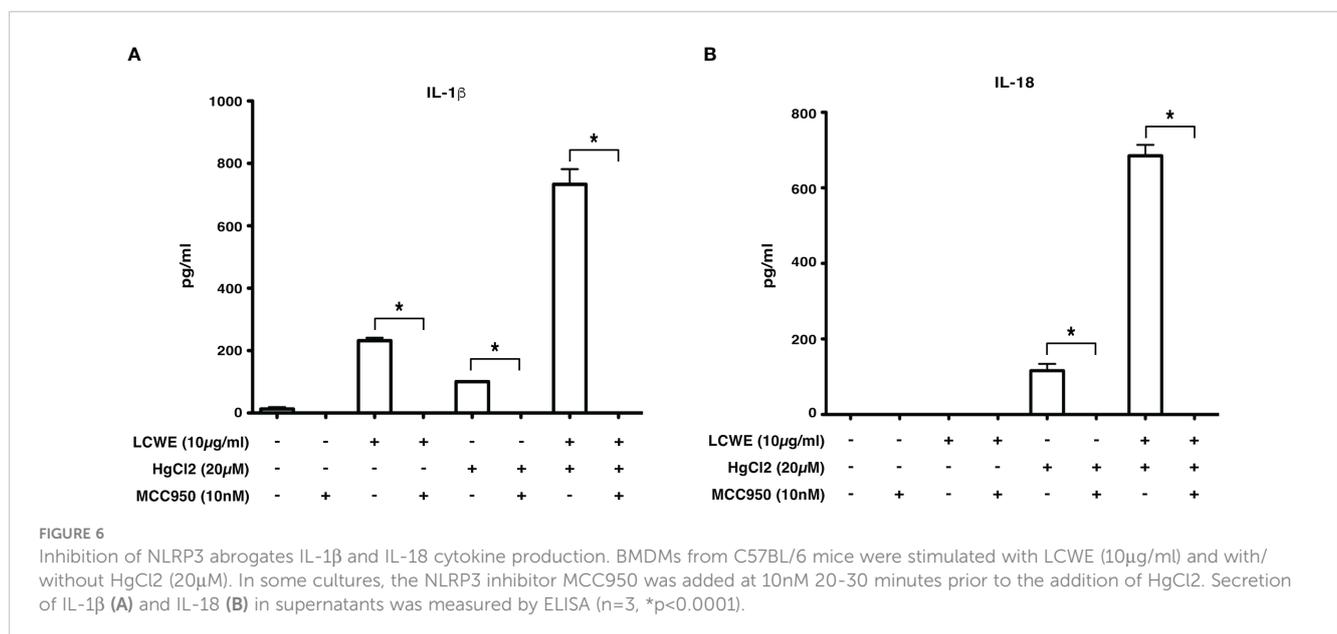
FIGURE 4 HgCl₂ regulates Ca²⁺ mobilization leading to secretion of IL-1β and IL-18. **(A)** [Ca²⁺]_i levels in treated with/without HgCl₂. BMDMs from C57BL/6 were loaded with calcium indicator dye Fluo-4AM and treated with/without HgCl₂ (20µM). Live [Ca²⁺]_i levels were continuously measured using confocal spinning disc microscopy for 40 minutes. Ionomycin (1µM) was added at 30min. The average Mean Fluorescence Intensity (MFI) (Y-axis) from 5 independent fields was measured and plotted against time on the X-axis. The data shown is a representative graph of 2 experiments. BMDMs from C57BL/6 mice were treated with LCWE (10µg/ml) and treated with/without HgCl₂ (20µM). Prior to the treatment of HgCl₂, cells were treated with/without Ca²⁺ inhibitors XeC (5µM), Tg (100nM), U73122 (10µM), and 2-APB (100µM). **(B)** Levels of IL-1β from supernatants of BMDMs, measured by ELISA (n=3). *p<0.05. **(C)** Levels of IL-18 from supernatants, measured by ELISA (n=3). *p<0.05. Statistical analysis between cultures of LCWE + HgCl₂ - stimulated BMDM with/without Ca²⁺ inhibitors is done using the Unpaired t-test.



mercury alone does not activate the inflammasome. However, when primed with LCWE, mercury exacerbates the expression of NLRP3 and subsequent secretion of IL-1β and IL-18 cytokines (Figure 2). Similar to previous observations (13), LCWE treatment alone leads to activation of NLRP3 and leads to the secretion of IL-1β but not IL-18. Mercury serves as a second signal for inflammasome mediated production of pro-inflammatory cytokines IL-1β and IL-18 (Figures 2, 3). Indeed, when mercury along with LCWE were injected *in vivo*, there is increased secretion of IL-1β and IL-18 (Figures 7A, B). Furthermore, we observed an increase in disease severity and coronary arteritis in mice injected with LCWE and

HgCl2 compared to mice injected with a single agent (Figure 8 and Table 1). These results suggest that mercury intensifies KD symptoms in the LCWE mouse model. Previous studies show that extracellular and intracellular Ca²⁺ mobilization is critical for activating the NLRP3 inflammasome (16). Importantly, intracellular Ca²⁺ sensing IP3R receptor is crucial in the activation of the NLRP3 inflammasome (17). Therefore, to further dissect the mechanism of inflammasome activation by HgCl2, we used both intracellular and extracellular Ca²⁺ inhibitors. Tg, XeC are for intracellular channels, where XeC is specific for IP3R receptor and U73122, 2-APB for extracellular Ca²⁺ inhibitors. The activation of IL-1β and IL-18 cytokines after stimulation with LCWE and HgCl2 was dependent on both extracellular and intracellular Ca²⁺, suggesting that HgCl2 mediated activation could be mediated by the NLRP3 inflammasome. Looking back at the past forty years, outbreaks or peak incidences of KD in Japan have been observed to correlate with wind patterns. Peak periods in KD cases coincide with the shift to northwesterly winds originating in central Asia as it crosses Japan and traverses through the North Pacific, suggesting that potential triggering factors for KD may be carried in the wind (25). Industrial pollution of Hg distributed in the atmosphere is measured as geo-anthropogenic emissions, with striking similarities between the geo-anthropogenic emissions of mercury and the prevalence of KD worldwide. As an environmental pollutant, mercury is widely dispersed and can travel long distances in the atmosphere. Interestingly, northwestern winds originating in central Asia would travel through China, the largest producer of mercury in the world (26). With its extensive effects on immune cells and as a known modulator of Ca²⁺ mobilization, mercury may be the environmental trigger contributing to immune activation in KD (Figure 9).

The confluence of environmental triggers, both infectious and non-infectious, has taken on added significance during the COVID-19 pandemic, with the emergence of a multisystem inflammatory syndrome in children (MIS-C), a severe



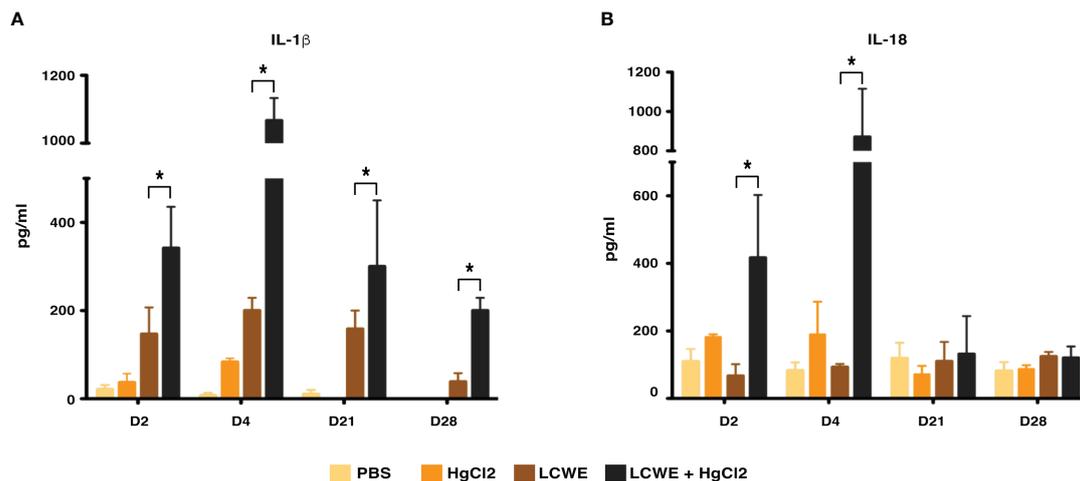


FIGURE 7

HgCl₂ exacerbates secretion of IL-1 β and IL-18 in LCWE treated mice *in vivo*. C57BL/6 mice were injected with PBS (1ml, i.p), LCWE (1mg i.p), HgCl₂ (1.25 mg/Kg body weight, s.c) or LCWE + HgCl₂. Serum was collected on days 2, 4, 21, and 28. Levels of IL-1 β (A) and IL-18 (B) from serum were measured by ELISA (n=5 in each group, *p<0.005).

phenotype of KD, seen in children as a post-COVID hyperinflammatory syndrome. The key role of inflammasome activation has important implications for therapy, with a growing recognition of the importance of IL-1 blockade in the management of affected children.

Materials and methods

Mice

Four weeks old C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed under specific pathogen-free conditions at the University Health Network (Toronto, Canada). All animal studies were performed according to guidelines and procedures approved by Animal Care Committee at both the Hospital for Sick Children Research Institute and the University Health Network (Toronto, Canada).

Live cell analysis by flow cytometry

Splenocytes (1×10^6 cells) were treated with various concentrations of HgCl₂ (10 μ M-1M) for 45 minutes. Cells were loaded with propidium iodide (PI) (ThermoFisher) to determine live/dead cells. The cells were acquired using BD™ LSR II Analyzer (BD Biosciences, Mississauga, ON, Canada). Data were analyzed using FlowJo v9 (Tree star, OR, USA) software.

Bone marrow derived macrophages

BMDMs were obtained by differentiating bone marrow progenitors from the tibia and femur of 8-12 weeks old wild type C57BL/6 mice in RPMI-1640 (VWR, Mississauga, ON, Canada) supplemented with 10% FBS, 1mM Sodium pyruvate, 0.1mM non-essential amino acid, 50 μ M 2-ME, 2mM L-glutamine and 10mM HEPES, 100 U/ml penicillin and 100 μ g/ml streptomycin

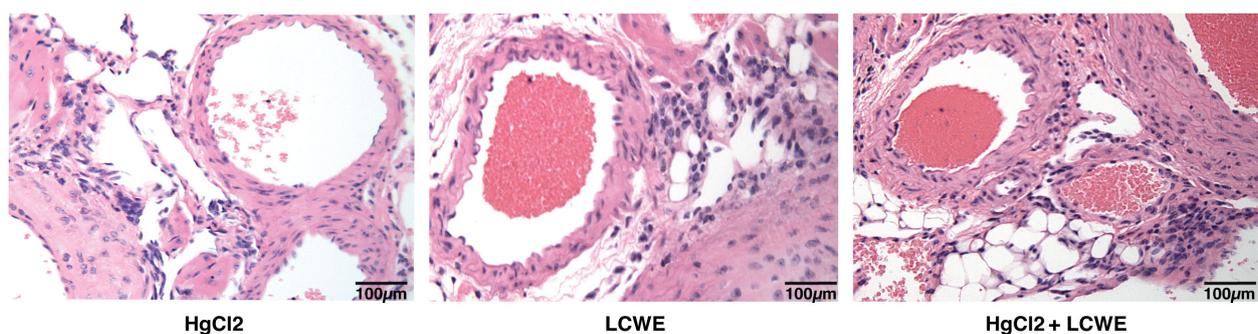


FIGURE 8

Mercury exacerbates coronary artery disease in LCWE-injected murine model of KD. C57BL/6 mice were injected either with HgCl₂ alone (s.c., 1.25mg/Kg body weight), LCWE alone (i.p., 1 mg/mouse) or with both LCWE and HgCl₂. Heart sections were prepared 28 days later and stained with haematoxylin and eosin to evaluate the presence of coronary artery inflammation. LCWE-injected and treated with HgCl₂ mice exhibited more intense and concentrated cellular infiltrates in comparison to mice injected only with either HgCl₂ or LCWE. (200x magnification).

TABLE 1 Mercury increases the incidence and severity of coronary arteritis in mice.

Coronary Arteritis ^a					
PBS	HgCl ₂	LCWE		LCWE+HgCl ₂	
Incidence	Incidence	Incidence	Severity (Mean ± SD)	Incidence	Severity (Mean ± SD)
0/10	0/10	7/10	0.65 ± 0.47	10/10	1.95 ± 0.15 ^b

Incidence of coronary arteritis 28 days after disease induction is presented as a total number of mice with histologic evidence of inflammation over the total number of mice injected. Disease severities were significantly increased in mice injected with LCWE and HgCl₂ when compared to LCWE alone.

^aCellular infiltrates are evaluated based on a semi-quantitative scoring system from 0–4: 0 = no infiltrate; 1 = minimal cellular infiltrate; 2 = moderate cellular infiltrate; 3 = extensive transmural cellular infiltrate; 4 = extensive cellular infiltrate extending well beyond the coronary artery wall. Inflammation was determined based on the presence of monocytes, lymphocytes, and neutrophils, as morphologically identified.

^bp<0.0001.

(ThermoFisher, Mississauga, ON, Canada) (complete medium), and 20ng/ml of M-CSF (Sigma Aldrich, MO, USA) for 7 days. The culture medium was replaced with fresh complete medium and M-CSF every 3 days. BMDMs were re-plated in 24 well plates (VWR International) in complete medium at 2.5x10⁵ cells/ml 1 day before experimental assays.

Activation of IL-1 β and IL-18 secretion

BMDMs from C57BL/6 were cultured at 2.5x10⁵ cells/ml in 24 well plates in complete RPMI alone, LCWE (10mg/ml), or Pam3Cys (2 or 4 μ g/ml) for 18 hours. HgCl₂ (10 μ M–20 μ M) (Sigma Aldrich)

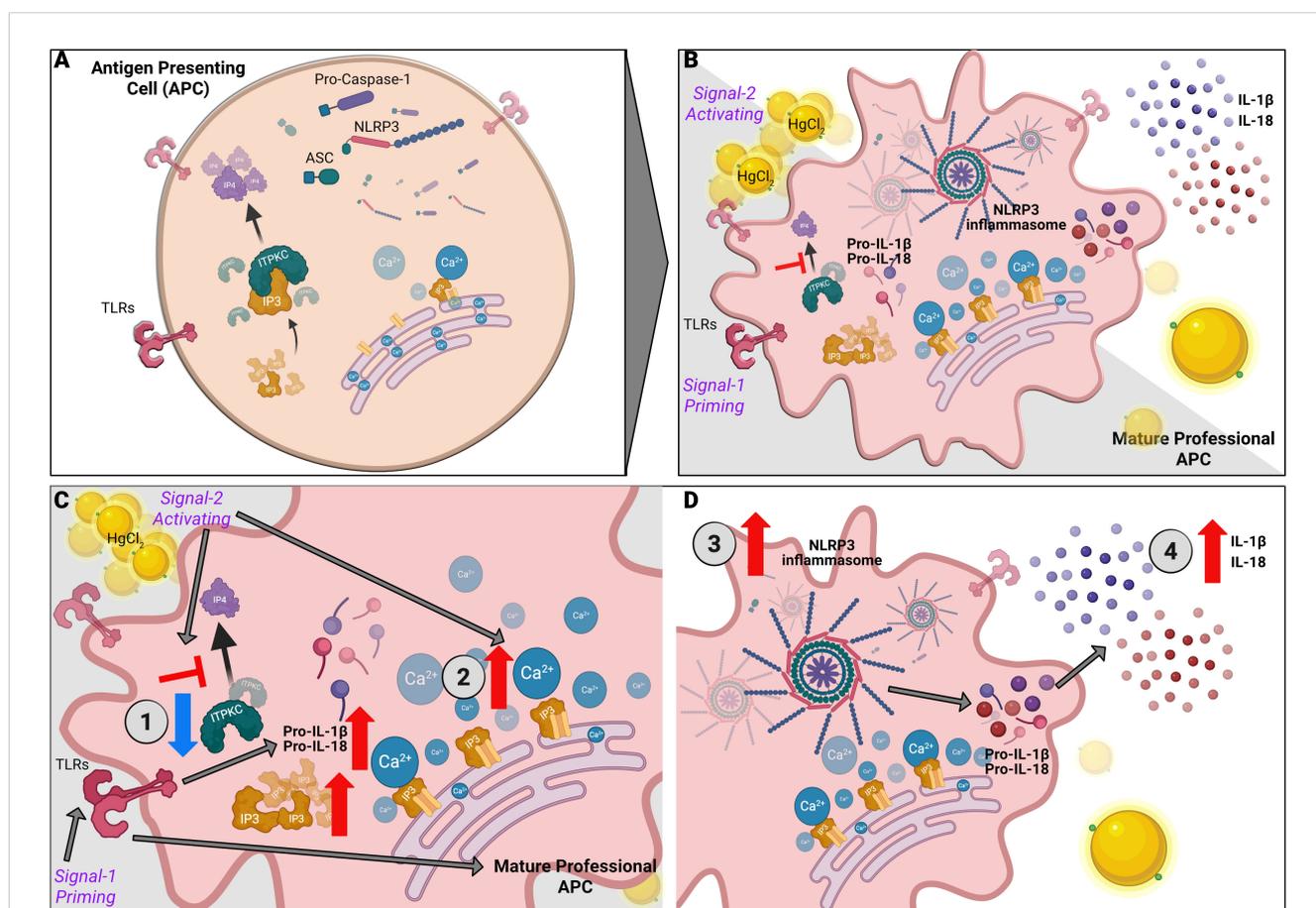


FIGURE 9

Mercury mediated regulation of Ca²⁺ mediated NLRP3 inflammasome in KD. NLRP3 inflammasome activation is a two-step process with priming and an activation signal. (A) Unstimulated APCs. (B) Signal one triggers a pattern recognition receptor on APCs such as Toll-like receptors (TLRs) to increase pro-IL-1 β and pro-IL-18 via the NF- κ B pathway. Signal two mediates the assembly and activation of the inflammasome, a large molecular platform composed of an NLR protein (such as NLRP3), the adaptor ASC and pro-inflammatory caspases (such as caspase 1). Activation of caspase 1 cleaves pro-IL-1 β and pro-IL-18 into their respective active cytokines. The following steps illustrate the contribution of HgCl₂ and ITPKC to the disease model: (C) 1. ITPKC regulates the phosphorylation of IP₃ to IP₄. Decreased ITPKC (knockout mice or humans with CC genotype) results in increased IP₃, which binds to IP₃R. 2. Release of intracellular Ca²⁺ from the endoplasmic reticulum (ER). (D) 3. Intracellular Ca²⁺ increases NLRP3 expression and inflammasome activation leading to 4. Increased production of IL-1 β and IL-18 from their pro-forms. Mercury acts as signal 2, leading to increased intracellular Ca²⁺ and NLRP3 expression and activation, leading to step 4.

was added during the last 30–45 min of incubation for activation of inflammasomes. Supernatants and cell lysates were collected and assayed for IL-1 β and IL-18 by ELISA (eBiosciences, San Diego, CA) and for NLRP3 protein by immunoblotting, respectively. To chelate HgCl₂, cells were treated with 50 μ M meso-2,3-Dimercaptosuccinic acid (DMSA) (Sigma Aldrich).

Intracellular and extracellular Ca²⁺ channels were inhibited with 5 μ M Xestospongine C (XeC), 100 nM Thapsigargin (Tg), 10 μ M U73122, 100 μ M 2-Aminoethoxydiphenylborane (2-APB). All Ca²⁺ inhibitors were purchased from Tocris Biosciences, Bristol, UK. For inhibition of caspase-1, irreversible inhibitors Z-YVAD-FMK (40 μ M) (Abcam, Toronto, ON) and MCC950 (10 nM) (Cayman Chemical, Ann Arbor, MI) were used to inhibit NLRP3 inflammasome. Where appropriate, inhibitor agents were added 15–20 minutes prior to the addition of HgCl₂.

In vivo studies

Four-week-old C57BL/6 mice were injected with either PBS alone or LCWE (1 mg/mouse) intraperitoneally as per protocol (27). A single injection of 0.1 ml HgCl₂ (1.25 mg/kg body weight) was administered subcutaneously (28) to animals treated with or without LCWE. Both LCWE and HgCl₂ were administered concurrently. Animals were sacrificed on days 2, 4, 21, and 28. Blood was collected, and serum was extracted, aliquoted, and stored at -80°C until use. Heart sections were obtained on day 28, stained with Hematoxylin and Eosin (H&E), and assessed histologically by a blinded reviewer for coronary arteritis. A semi-quantitative score from 0–4 was assigned as follows: 0 = no infiltrate; 1 = minimal cellular infiltrate; 2 = moderate cellular infiltrate; 3 = extensive transmural cellular infiltrate; 4 = extensive cellular infiltrate extending well beyond the coronary artery wall. Inflammation was determined based on the presence of monocytes, lymphocytes, and neutrophils, as morphologically identified. LCWE was prepared, and concentration was determined as previously described (27, 29).

Immunoblots

Proteins from lysates of BMDMs were extracted in Radio-Immunoprecipitation (RIPA) Buffer (Sigma Aldrich) containing HaltTM protease and phosphatase inhibitor cocktail (Fisher Scientific, Napean, ON, Canada). Immunoblots were prepared with Bolt[®] Bis-Tris Plus Gel (ThermoFisher), and western blot analysis was carried out according to standard protocols, with antibodies specific for rabbit polyclonal raised against human NLRP3 (1:6500, sc-66846, Santa Cruz Biotechnology Inc., Santa Cruz, CA). GAPDH was used as a loading control (AM4300, ThermoFisher). Relative protein levels were normalized to GAPDH as determined by densitometry using Image J software (1.8v, NIH).

Quantification of secreted cytokines

All ELISAs were carried out as per the manufacturer's protocol. The following ELISAs were used in this study: Mouse IL-1 β and

IL-18 in cell culture supernatants or serum were measured using mouse-IL-1 β Ready-SET-Go![®] and mouse-IL-18 Platinum ELISA kits (ThermoFisher).

Calcium analysis by confocal microscopy

BMDMs were plated on 4-chambered glass dishes (Fisher Scientific) at 0.1 $\times 10^6$ cells per chamber. Cells were loaded with Fluo-4/AM (ThermoFisher in Ca²⁺ free DMEM media (ThermoFisher) supplemented with 10% FBS, 1 mM Sodium pyruvate, 0.1 mM non-essential amino acid, 50 μ M 2-ME, 2 mM L-glutamine and 10 mM HEPES (complete-DMEM) and maintained in 37°C. Live images of untreated cells were taken at t=0. Cells were then treated with 20 μ M HgCl₂ in Ca²⁺ free DMEM at t=0. Cells were imaged for 40 min with acquisition at 15-sec intervals. Ionomycin (1 μ M) (Sigma Aldrich) was added to the medium at the end of 30 min, and cells were imaged for the remaining time. Images were acquired using Olympus 1X81[®] motorized inverted fluorescence microscope with a Hamamatsu C9100-13 black-thinned EM-CCD camera and Yokogawa CSU X1- spinning disc confocal imaging system using the 488-nm laser and emission in the range of 500–600 (Carl Zeiss, Toronto, ON, Canada). Images were analyzed using Volocity software (Perkin Elmer, Woodbridge, ON, Canada). Absolute intensity for cells in 5 independent fields at different time points was obtained, and the average is displayed as the mean fluorescence intensity (MFI) of all cells in the fields.

Statistical analyses

Paired *t*-test, unpaired *t*-test, or two-way ANOVA were used as appropriate for analysis of both *in vivo* and *in vitro* experiments using GraphPad Prism v5.0 and v9.0.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by The Hospital for Sick Children Research Institute Animal Care Committee.

Author contributions

MA and RY designed the study and drafted the manuscript. MA, TD, and ST performed experiments. MA and TD collected and analyzed data. MA, TD, ST, and RY reviewed, revised, and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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