Current Topics in Toxicology
Vol. 14, 2018

Original Communication

Protein kinase C activation *via* serotonin receptor induces IL-8 in antigen-presenting cells stimulated with diazinon

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ABSTRACT

Many allergic diseases and neutrophilic inflammations are multifactorial. The susceptibility to these diseases is caused by the interaction between genetic and environmental factors. However, a significant increase in the prevalence of these diseases has been observed in recent decades, possibly due to environmental factors. In the present study, we examined diazinon (DZN) as an agricultural organophosphate pesticide to identify their immunomodulatory effects as a possible environmental factor. We measured the levels of IFN-γ, IL-4/5, IL-17, IL-5 and IL-8 as indicators for Th1, Th2 and Th17 responses and eosinophilic and neutrophilic inflammation, respectively. We found that DZN increases the production of IL-8 from a human acute monocytic leukemia cell line (THP-1). Furthermore, the IL-8 production was increased in human macrophages stimulated with DZN. A 5-hydroxytryptamine (5-HT) receptor antagonist or a protein kinase C (PKC) inhibitor was able to suppress the IL-8 production from THP-1 cells stimulated by DZN. These observations suggest that DZN binds to 5-HTR in human monocytic cells, thereby producing IL-8 *via* PKC activation. DZN may exacerbate human neutrophilic inflammation.

KEYWORDS: organophosphorus, diazinon, protein kinase C, antigen-presenting cell, IL-8, serotonin.

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INTRODUCTION

It is important to evaluate the immunomodulatory effects of environmental chemicals. Chemical substances, aside from protein antigens, modify immune responses by targeting antigen-presenting cells (APCs) [1]. APCs then interact with protein antigens and process them. Dendritic cells (DCs), macrophages, B cells and monocytes are all defined as APCs.

Immature DCs are located in the peripheral tissues, where they scan for pathogen entry. Under inflammatory conditions, DCs undergo a maturation process induced by cytokines and co-stimulatory molecules such as CD80, CD86 and bacterial or viral products. DCs at different maturation stages show distinct chemokine expression patterns. At first, DCs produce CXCL1, CXCL2 and interleukin-8 (IL-8) [2]. IL-8 attracts effector cells such as neutrophils, cytotoxic T cells and natural killer (NK) cells. DCs then migrate to the draining lymph nodes to present the processed antigens to T cells, using major histocompatibility complex (MHC) class II molecules, T cell receptors and co-stimulatory molecules. Following stimuli, naïve $CD4^+$ T cells undergo rapid clonal expansion, followed by differentiation into functionally distinct T-helper (Th) cell subsets such as T-helper (Th)1, Th2 and Th17 cells.

Immune modulators/adjuvants change the nature of DCs to regulate T cell priming. These include many substances such as lipopolysaccharide (LPS), forskolin and β-glucan [3]. Many environmental chemicals, such as agricultural chemicals, food

additives and dyes, have been reported to cause aberrant immune responses [4-7]. Allergies and neutrophilic inflammation are induced by immune responses. Allergic diseases include food allergy, atopic dermatitis, allergic rhinitis and eosinophilic airway inflammation. The etiology of some diseases, such as neutrophilic airway inflammation, psoriasis, periodontal disease, endometriosis and acne vulgaris, is likely to be neutrophilic inflammation. Of note, the prevalence of these diseases has increased in recent decades. Organophosphates (OPs) are chemical agents widely used as insecticides. The primary target of OP insecticides is the inhibition of acetyl-cholinesterase (AChE) activity. Exposure to OPs leads to the build-up of acetylcholine (Ach) at the terminus of a presynaptic fiber, which induces acute symptoms such as increasing saliva, small pupils, bradycardia and sweating. There are many cohort studies evidencing a relationship between OP exposure and the incidence of many diseases, including cancer, reproductive disorders, Parkinson's disease, Alzheimer's disease and respiratory diseases [6, 8-10]. Previous studies have shown that exposure to OPs increased the frequency of these chronic diseases due to their effects as AChE inhibitors [11]. However, recent studies have shown that the inhibition of AChE does not necessarily contribute to the incidence of these chronic diseases [12, 13]. Studies on the relationship between OPs and G protein-coupled receptors (GPCRs) are now in progress [9, 14].

GPCRs are G_s-, G_q- or $G_{i/o}$-coupled. When GPCRs detect extracellular ligands, internal signal transduction pathways are induced. G_s activates intracellular adenylate cyclase, which increases the concentration of cyclic adenosine 3', 5'-monophosphate (cAMP). This leads to the activation of protein kinase A (PKA), which activates multiple downstream effectors. $G_{i/o}$ inhibits adenylate cyclase, and G_q stimulates phospholipase C (PLC) to activate protein kinase C (PKC).

The 5-hydroxytryptamine (5-HT) receptors (5-HTR) belong to the GPCR family. There have been some reports regarding the relationship between 5-HTR and chronic diseases triggered by exposure to OPs, the details of which remain to be elucidated [9, 15].

Diazinon (DZN; O,O-Diethyl O-[4-methyl-6-(propan-2-yl) pyrimidin-2-yl] phosphorothioate) (Figure 1) is an OP commonly used as an agricultural pesticide. *In vivo* studies suggest that high levels of DZN exposure change the number of monocytes, $CD4^+$ and $CD8^+$ lymphocytes in the peripheral blood or in the spleen. These changes may induce chronic diseases [16-18].

In the present study, we performed a screening assay to evaluate the immunomodulatory effects induced by environmental chemicals.

MATERIALS AND METHODS

Reagents

Diazinon was obtained from WAKO (Osaka, Japan).

Preparation of human peripheral blood mononuclear cells

Human peripheral blood was collected from healthy volunteer blood donors under the approval of the Research Ethics Committee of Saitama Medical University (#787-II). Human peripheral blood mononuclear cells (PBMCs) were separated from adult blood buffy coat specimens by Ficoll-Paque PLUS (GE Healthcare, Buckinghamshire, UK) centrifugation.

Mixed lymphocyte reaction

For the human two-way mixed lymphocyte reaction (MLR), 0.75×10^6 PBMCs were incubated with 0.75×10^6 HLA-DR nonshared allogeneic PBMCs in the presence or absence of DZN to be tested in 200 µL of RPMI 1640 medium containing 10% human serum, 50 IU/mL penicillin, 50 µg/mL streptomycin and 1% L-glutamine in a round-bottomed 96-well plate for 7 days in a humidified atmosphere under 5% CO_2 at 37 °C.

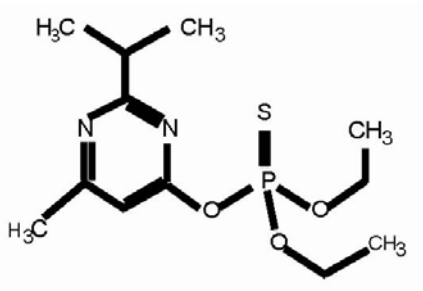

Figure 1. Structural formula of DZN.

Culture of a human acute monocytic leukemia cell line (THP-1 cells)

THP-1 was maintained at a density of 10^6 cells/ml in RPMI 1640 medium containing 10% fetal calf serum, 50 IU/mL penicillin, 50 µg/mL streptomycin and 1% L-glutamine in 6-well plates in a humidified atmosphere under 5% CO_2 at 37 °C. After washing with phosphate-buffered saline (PBS), they were cultured in AIM-V serum-free medium (Thermo Fisher Scientific, Waltham, MA, USA) and incubated with DZN to be tested. After pre-incubation with 5-HT receptor antagonists or a PKC inhibitor, the THP-1 cells were washed to remove the residual agent before adding DZN.

In brief, following 60 min to 24 h of pre-incubation with inhibitors or antagonists, the cells were rapidly washed with RPMI 1640 and then cultured with inhibitors or antagonists in AIM-V medium. After stimulation with phorbol-12-myristate-13-acetate (PMA), THP-1 cells can differentiate into a macrophage-like phenotype. The THP-1 cells were cultured in 6-well plates for 18 h in the presence of 50 ng/mL PMA, and the adherent cells were washed and then cultured with AIM-V medium.

Viability and IL-8 production of THP-1 cells stimulated with DZN

To determine the optimum concentration and duration of exposure to DZN, we examined the effect of DZN on cell viability. THP-1 cells (2.0×10^4 cells) were incubated with various concentrations of DZN (100-1000 µg/mL) for 2-32 h in AIM-V medium. The cells were then stained with trypan blue, and the numbers of living and total cells were counted. The cell viability was calculated as the percentage of living cells. The culture supernatants were assayed for IL-8 using a commercial enzyme-linked immunosorbent assay kit (ELISA; R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

IL-8 production of PMA-treated THP-1-derived macrophage-like cells stimulated with DZN

PMA-treated THP-1-derived macrophage-like cells (1.0×10^5 cells) were incubated in the presence or absence of 1 mg/mL DZN in AIM-V medium in a flat-bottomed 96-well plate for 8 h. The levels of IL-8 were measured using a commercial ELISA kit.

IL-8 production of human macrophages stimulated with DZN

PBMCs (1.0×10^6 cells) were seeded in a flat-bottomed 96-well plate and then incubated in 100 µL of RPMI 1640 medium containing 10% human serum for 1 h. Following incubation, the PBMCs were washed twice to remove non-adherent cells. Adherent cells were defined as human macrophages. These cells were stimulated with various concentrations of DZN in the presence or absence of LPS (100 pg/mL) in AIM-V medium for 8 h. The supernatant was then collected for the IL-8 ELISA.

IL-8 production of THP-1 cells or human macrophages stimulated with 5-HT

THP-1 cells (1.0×10^5 cells) or human macrophages as described above were cultured in a flat-bottomed 96-well plate in AIM-V medium and then exposed to a series of concentrations of 5-HT for 8 h. Following incubation, the supernatant was collected for the IL-8 ELISA.

Effects of 5-HT receptor antagonists on the IL-8 production of THP-1 cells stimulated with DZN

THP-1 cells (1.5×10^6 cells) were cultured in a flat-bottomed 12-well plate in 10% FCS-containing medium and then exposed to a series of concentrations of the 5-HTR1/2/5/6/7 antagonist Methiothepin (Sigma-Aldrich), the 5-HTR4 antagonist GR113808 (abcam, Cambridge, MA, USA) or the 5-HTR7 antagonist SB269970 (abcam) for 24 h. Following incubation, the cells were washed and then cultured with varying concentrations of 5-HTR antagonist for 8 h. The THP-1 cells (1.0×10^5 cells) were then seeded in a flat-bottomed 96-well plate in AIM-V medium and stimulated with 1 mg/mL DZN for 8 h. The supernatant was then collected for the IL-8 ELISA.

Effects of a PKC inhibitor on IL-8 production induced by DZN

THP-1 cells were exposed to a series of concentrations of the PLC inhibitor Gö 6983 (abcam) for 1 h. Following incubation, the cells were washed and then cultured with varying concentrations of Gö 6983. These THP-1 cells (1.0×10^5 cells) were seeded in a flat-bottomed 96-well plate in AIM-V medium and then stimulated

with 1 mg/mL DZN for 8 h. The supernatant was then collected for the IL-8 ELISA.

Cytokine ELISA

The culture supernatants were assayed for interferon gamma (IFN-γ), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-17 (IL-17) and IL-8 using ELISA kits (R&D Systems).

Statistical analyses

Statistical analyses between two groups were performed with Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Differentiation of T cells

Previous studies have shown that environmental chemicals contribute to the differentiation of effector T cells [19]. The DO11.10 mouse model is often used to evaluate the non-self-antigen-dependent differentiation of T cells because of the high clonal frequency in TCR-transgenic mice. In human models, it is impossible to design such an assay because of the low clonal frequency of T cells responsive to specific non-self-antigens. Therefore, an MLR assay is often selected in human models [20]. The clonal frequency of allo-HLA-DR-specific T cells is relatively high (i.e. $>10^{-4}$) in the HLA-DR-non-shared combination model. Because the majority of activated T cells in MLR are naïve $CD4^+$ T cells, immunomodulatory effects can be evaluated by an MLR assay.

An MLR assay was performed in the presence of various concentrations of DZN. The culture supernatant fluids obtained after 7 days' culture were subjected to an ELISA to detect typical cytokines such as IFN-γ, IL-4/5 and IL-17, for Th1, Th2 and Th17 responses, respectively. No significant differences in the levels of the cytokines were observed with DZN (Figure 2).

The viability and IL-8 production of THP-1 cells stimulated with DZN

Next, we performed a screening assay to evaluate the levels of inflammatory cytokines produced by

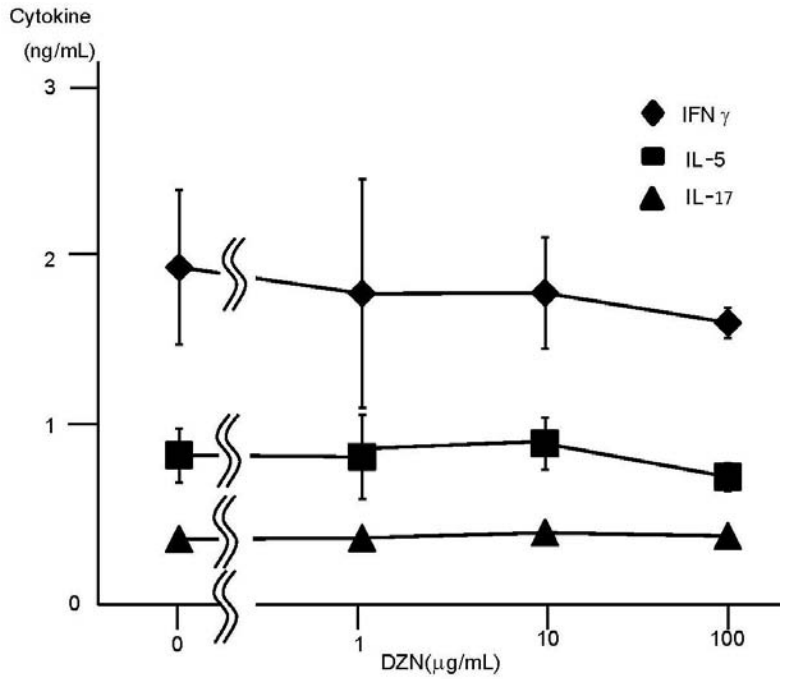

Figure 2. The differentiation of $CD4^+$ T cells stimulated by DZN was evaluated using an MLR assay. For the human two-way MLR, 0.75×10^6 cells of PBMCs were incubated with 0.75×10^6 HLA-DR non-shared allogeneic PBMCs in the presence or absence of various chemicals for 7days. The culture supernatant fluids obtained after seven days' culture were subjected to an ELISA to detect the cytokines IFN-γ (♦), IL-5 (■), and IL-17 (▲).

THP-1 cells by incubating the cells with DZN. DZN was able to induce the production of IL-8 by THP-1 cells. However, DZN was unable to induce the production of IL-6 or IL-23 (data not shown). We then evaluated the levels of IL-8 in the culture of THP-1 cells stimulated with various concentrations of DZN for 2-32 h. Stimulation with 1 mg/mL DZN led to a significant increase in the levels of IL-8 (Figure 3).

To determine the optimum concentration and duration of exposure to DZN, we assessed the effect of DZN on the cell viability (Figure 3B). THP-1 cells were incubated for various periods of time (2-32 h) with various concentrations of DZN (100 μg/mL-1 mg/mL) and then subjected to the cell viability assay. While the cell viability after incubation with 1 mg/mL DZN for <8 h was more than 95%, the viability was significantly decreased after 16 h. Based on these results, THP-1 cells were subsequently cultured with 1 mg/mL DZN for 8 h to evaluate the production of IL-8.

OPs can cause the accumulation of Ach due to their role as cholinesterase inhibitors. We therefore evaluated whether or not THP-1 cells stimulated

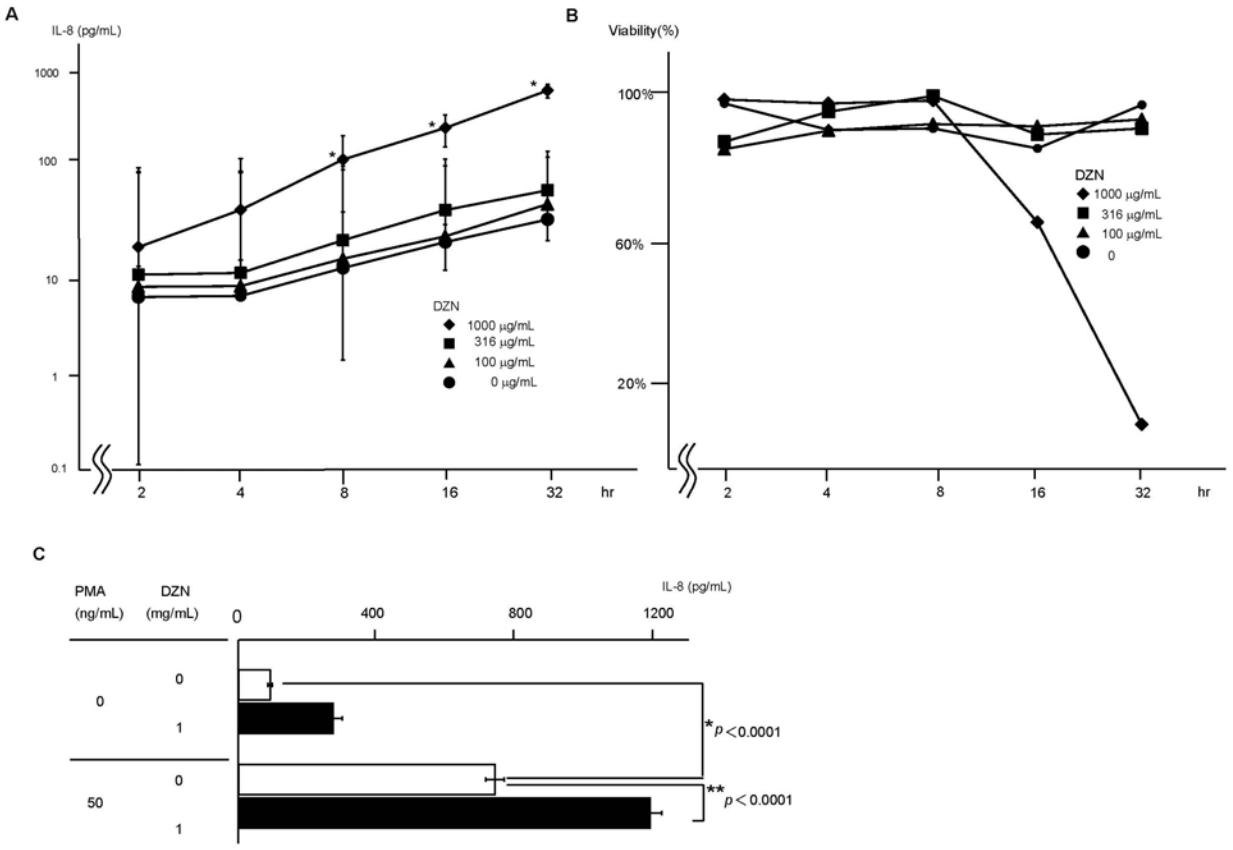

Figure 3. The viability and IL-8 production of THP-1 cells stimulated with DZN. (**A**) THP-1 cells were incubated with DZN (100-1000 μg/mL) for 2-32 h. The culture supernatants were subjected to an ELISA to determine the levels of IL-8. Experiments were performed independently at least three times. Data are shown as the mean ± SD. *$p < 0.01$ compared with samples without DZN at 8 to 32 h. (**B**) The viability of THP-1 cells stimulated with DZN was computed as the percentage of living cells. The cell viability (%) was calculated with the following formula: 100 × living cells / total cells. The data are representative of one out of three independent experiments. (**C**) PMA-treated THP-1-derived macrophage-like cells were incubated in the presence or absence of DZN for 8 h. The levels of IL-8 were measured using an ELISA. The experiments were performed independently at least three times. Data are shown as the mean ± SD. *$p < 0.0001$ compared with samples without PMA. **$p < 0.0001$ compared with PMA-stimulated samples without DZN.

with Ach were able to produce IL-8. Our findings showed that Ach stimulation did not increase the levels of IL-8 (data not shown).

IL-8 production in PMA-treated THP-1 cells stimulated with DZN

THP-1 cells reportedly show a macrophage-like phenotype after treatment with PMA. We evaluated the IL-8 production in PMA-treated THP-1 cells stimulated with DZN. Our findings showed that the level of IL-8 in PMA-treated THP-1 cells was higher than that in mock-treated THP-1 cells ($p < 0.0001$; Figure 3C). Furthermore, the addition of DZN increased the level of IL-8 in PMA-treated THP-1 cells over that in THP-1 cells ($p < 0.0001$; Figure 3C).

IL-8 production in human macrophages stimulated with DZN

Next, we examined whether or not DZN increases the level of IL-8 not only in PMA-treated THP-1 cells but also in human macrophages as physiological APCs. The production of IL-8 was significantly higher in the culture of human macrophages stimulated with 1 mg/mL DZN than in controls ($p < 0.01$; Figure 4A). Furthermore, low concentrations of DZN (0.01 μg/mL) together with LPS (100 pg/mL) dose-dependently increased the IL-8 level in human macrophages ($p < 0.05$; Figure 4B).

IL-8 production in 5-HT-stimulated and PMA-treated THP-1 cells or human macrophages

5-HT ligand binding to 5-HTR is known to induce its own internalization [21, 22]. Recent studies by Slotkin *et al.* have shown that exposure to OPs altered the expression of 5-HTR or 5-HT transporter genes [15, 23]. We therefore hypothesized that the increased IL-8 production in APCs stimulated with DZN is mediated by the 5-HT pathway. To test our hypothesis, we evaluated the production of IL-8 in a culture of APCs stimulated with various concentrations of 5-HT. The production of IL-8 was significantly increased in the culture of both PMA-treated THP-1 cells and human macrophages ($p < 0.05$; Figure 5A, $p < 0.001$; Figure 5B, respectively).

Effects of 5-HT receptor antagonist on IL-8 production in THP-1 cells stimulated with DZN

THP-1 cells were preincubated for 24 h with various concentrations of Methiothepin and GR113808 to block the activity of endogenous 5-HT1/2/4/5/6/7 receptors. THP-1 cells were thereafter stimulated

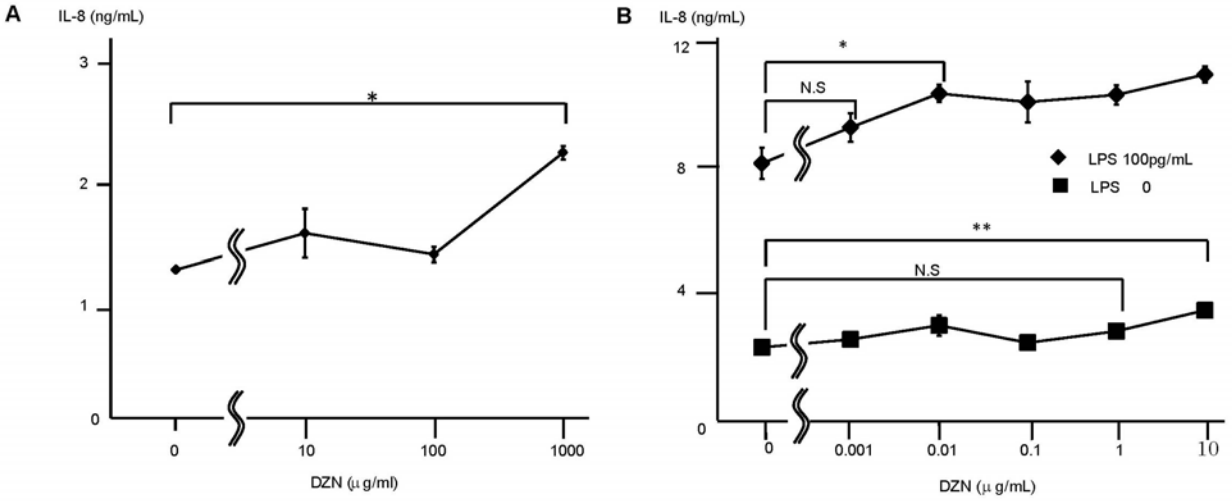

Figure 4. IL-8 production in human macrophages stimulated with DZN. (**A**) Human macrophages were stimulated with DZN (10-1000 μg/mL) for 8 h. Data are shown as the mean ± SD. *$p < 0.01$ compared with samples without DZN. (**B**) Human macrophages were stimulated with DZN (0.001-10 μg/mL) in the presence or absence of LPS (100 pg/mL) for 8 h. The culture supernatants were subjected to an ELISA to determine the levels of IL-8. All experiments were performed independently at least three times. Data are shown as the mean ± SD. *$p < 0.05$ compared with LPS-stimulated samples without DZN. **$p < 0.05$ compared with samples without DZN. N.S., not significant.

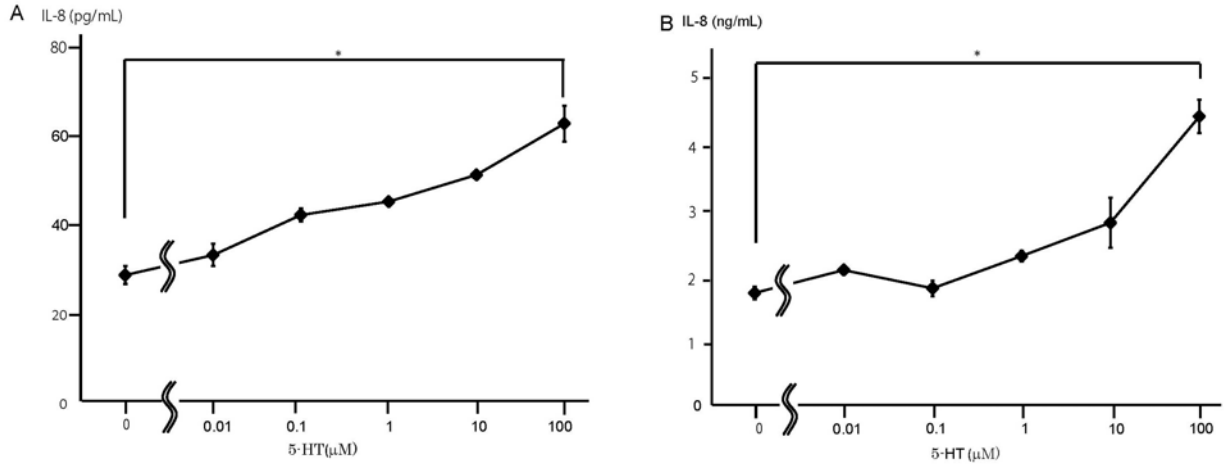

Figure 5. IL-8 production in antigen-presenting cells stimulated with 5-HT. (**A**) THP-1 cells were stimulated with 5-HT (0.01-100 μM) for 8 h. The culture supernatants were subjected to an ELISA to determine the levels of IL-8. All experiments were performed independently at least three times. Data are shown as the mean ± SD. *$p < 0.05$ compared with samples without 5-HT. (**B**) Human macrophages were stimulated with 5-HT (0.01-100 μM) for 8 h. The culture supernatants were subjected to an ELISA to determine the levels of IL-8. All experiments were performed independently at least three times. Data are shown as the mean ± SD. *$p < 0.001$ compared with samples without 5-HT.

with DZN. As shown in Figure 6, both antagonists significantly attenuated the DZN-induced IL-8 production ($p < 0.0001$).

Effects of PKC inhibitor on IL-8 production induced by DZN

5-HT-induced signal transduction is mediated by different classes of GPCR as well as ionotrophic receptors. While 5-HTR3 is an ionotrophic receptor, 5-HTR1/2/4/5/6/7 are GPCRs. Our findings suggest that the production of IL-8 might be mediated through the cAMP or PLC pathway. We first determined the intracellular cAMP levels in DZN-stimulated THP-1 cells in accordance with our previously published report [3]. Consequently, DZN stimulation was unable to increase the cAMP levels, whereas a positive control did (data not shown). 5-HTR1/2 agonist is known to increase the PKC activity. We therefore examined whether or not the PKC inhibitor agent Gö 6983 was able to suppress the IL-8 levels in DZN-stimulated THP-1 cells. Our findings showed that IL-8 production was indeed suppressed by Gö 6983 ($p < 0.0001$; Figure 7A), and the viability of a culture incubated with 10 μM Gö 6983 exceeded 95% (Figure 7B).

DISCUSSION

APCs play an important role in both innate and acquired immune responses against pathogens. In the present study, we evaluated the activity of immune modulators/adjuvants using MLR. Human naïve $CD4^+$ T cells recognize self-antigens presented by allo-HLA-DR and undergo subsequent clonal expansion. The differentiation of naïve $CD4^+$ T cells depends on the balance of monokines secreted by DCs and membrane proteins expressed on DCs. For example, LPS alters the differentiation of naïve DCs *via* toll-like receptor 4 (TLR4) due to MyD88 signaling. In this process, interleukin-12 (IL-12) secreted by DCs or a specific Notch ligand isoform expression on DCs induces Th1 differentiation [24]. The MLR analysis in the present study revealed no immune modulator/adjuvant activities of DZN.

We also evaluated the innate immune responses against DZN using THP-1 cells. THP-1 cells resemble primary monocytes and macrophages in their morphological and functional properties. THP-1 is a human leukemia monocytic cell line that has been widely used to evaluate the cytokines, chemokines and signaling pathways of monocytes or macrophages [25]. Peripheral blood monocytes can produce IL-8, which stimulates neutrophil migration and promotes the adhesion of monocytes to endothelium cells [26, 27]. IL-8 is produced by vascular epithelium cells, intestinal endothelium cells and T cells [28]. The production of IL-8 is regulated by signal transduction pathways such as NF-κB, MAPK, JAK-STAT and ROS [29].

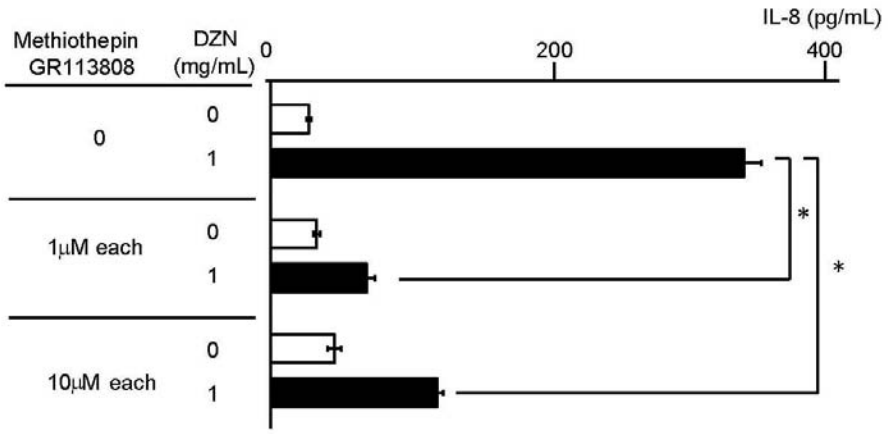

Figure 6. Effects of 5-HT receptor antagonist on IL-8 production in THP-1 cells stimulated with DZN. THP-1 cells were cultured in Methiothepin and GR113808 for 24 h and then stimulated with DZN for 8 h. The culture supernatants were subjected to an ELISA to determine the levels of IL-8. All experiments were performed independently at least three times. Data are shown as the mean ± SD. **p* < 0.0001 compared with DZN-stimulated samples without 5-HT antagonist drugs.

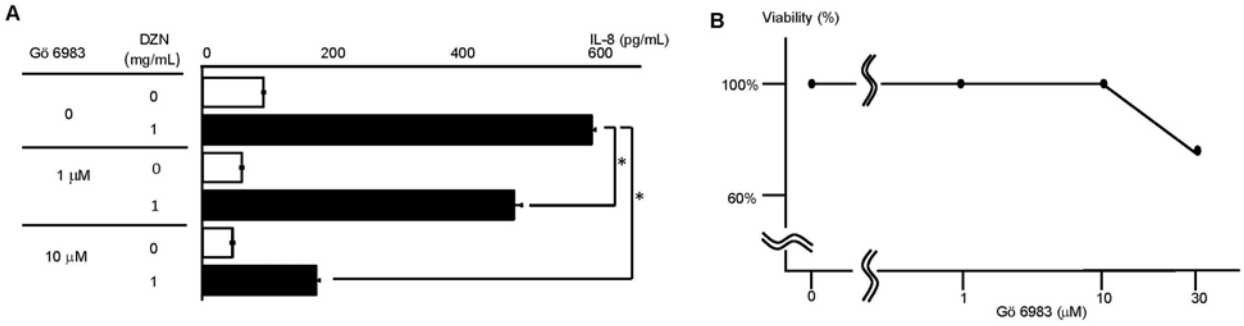

Figure 7. Effects of PKC inhibitor on IL-8 production induced by DZN. (**A**) THP-1 cells were cultured in Gö 6983 for 1 h and then stimulated with DZN for 8 h. The culture supernatants were subjected to an ELISA to determine the levels of IL-8. All experiments were performed independently at least three times. Data are shown as the mean ± SD. **p* < 0.0001 compared with DZN-stimulated samples without Gö 6983. (**B**) The viability of DZN-stimulated THP-1 cells with Gö 6983 (1-30 μM) was computed as the percentage of living cells. The cell viability (%) was calculated with the following formula: 100 × living cells / total cells. The data are representative of one out of three independent experiments.

Our findings showed that the IL-8 production increased in cultures of THP-1 cells or human macrophages stimulated with DZN. The production of IL-8 and cell viability varied with the incubation period, allowing us to determine the optimum condition for the experiments. For example, the production of IL-8 markedly increased in the culture stimulated with DZN between 0.1 to 1 mg/mL for 8 to 32 h (Figure 3A). Based on the findings for the cell viability (Figure 3B), we decided to measure the IL-8 production under THP-1 culture conditions of 1 mg/mL DZN for 8 h.

We found that PMA-treated THP-1 cells stimulated with DZN produced increased levels of IL-8, as was the case in human macrophages (Figure 3C). Furthermore, low concentrations of DZN were able to increase the level of IL-8 in LPS-stimulated human macrophages (Figure 4B). These results suggest that the effect of DZN was augmented under inflammatory conditions.

Some studies have reported that exposure to OPs contributes to the incidence of neurological disorders [30]. Immune cells are known to express neurotransmitters and their receptors [3, 31, 32] suggesting that OPs might influence the immune system. Ogasawara *et al*. reported that DZN was able to activate APCs, such as murine bone marrow-derived macrophages (BMDM), murine bronchoalveolar lavage fluid (BALF) cells and the murine monocyte/macrophage cell line RAW264.7, thereby exacerbating the inflammatory responses [33]. However, how DZN affects immune cells remains unclear.

5-HT is a major monoamine neurotransmitter in the central nervous system and is associated with many functions, including the immune function, gastrointestinal function, platelet aggregation, mood and sleep [34]. Slotkin *et al*. reported that DZN administration to animals modulated the 5-HT receptor and 5-HT transporter gene expression [15, 23]. This result suggests that OPs may be ligands of 5-HT receptors. Therefore, as a working hypothesis, we postulated that 5-HT receptor may be related to the IL-8 production of DZN-stimulated THP-1 cells.

Indeed, 5-HT increased the IL-8 secretion in THP-1 cells and human macrophages (Figure 5A, B). The 5-HT receptors consist of 7 general receptor classes including a total of 14 known serotonin receptors. They transmit their signals *via* multiple intracellular messengers. 5-HTR3 is a ligand-gated ion channel, while all of the other 5-HT receptors are GPCRs that induce an intracellular secondary messenger cascade to enhance excitatory or inhibitory responses.

The production of IL-8 was suppressed when THP-1 cells preincubated with methiothepin and GR113808 were stimulated with DZN. In contrast, when THP-1 cells preincubated with SB269970 were stimulated with DZN, the IL-8 expression remained stable (data not shown). It was previously reported that the frequency of 5-HTR subtype expression was dependent on the cell differentiation and maturation stages [35, 36]. Studies have shown that immature DCs express 5-HTR1 or 5-HTR2, and agonists of 5-HTR1 or 5-HTR2 were able to regulate the chemotaxis of immature DCs [35, 36]. Furthermore, a 5-HTR7 agonist reportedly increased the production of IL-8 in mature DCs. In contrast to these previous findings, however, we found that 5-HTR7 antagonist was unable to suppress the IL-8 production in THP-1 cells, possibly due to the low expression of 5-HTR7 in THP-1 cells. Taken together, these present and previous findings suggest that THP-1 cells might express 5-HTRs other than 5-HTR7.

5-HTR4/6/7 are associated with G_s protein-mediated stimulation of adenylyl cyclase and induce an increased intracellular cAMP level. 5-HTR1 can mediate PLC or PKC [37]. 5-HTR2 is G_q-coupled and activates PLC or PKC [38]. There were no statistically significant differences in the intracellular cAMP levels in THP-1 cells stimulated with different concentrations of DZN. Therefore, as a working hypothesis, we postulated that DZN might be involved in the activation of PLC or PKC. We found that a PKC inhibitor decreased the IL-8 production in THP-1 cells. PKC is a downstream signaling factor of PLC and is known to contribute to monocyte behavior, including their differentiation, apoptosis and immune responses [39]. While it is well known that PMA induces PKC activation and plays a significant role in altering the cell morphology, our study showed that PMA was able to induce IL-8 production as a functional change. DZN stimulation augmented the IL-8 production in PMA-treated THP-1-derived macrophage-like cells. PKC is known to play a role in the differentiation of monocytes into macrophages, but our results indicate that DZN was able to activate PKC in PMA-treated THP-1 cells and human macrophages, thereby inducing the production of IL-8 (Figure 3C). Taken together, our findings clearly indicate that DZN activated PKC *via* 5-HTR1/2 and induced IL-8 production.

The incidence of neutrophilic airway inflammation, such as bronchial asthma or chronic obstructive pulmonary disease (COPD), has been reported to be associated with exposure to OPs in farmers [40, 41]. Gottipati *et al*. reported that the production of IL-8 was increased in THP-1 cells stimulated with agricultural dust extract [42]. Because agricultural dust can include agricultural chemicals, such as OPs, the increased IL-8 production might be due to OP exposure.

In general, acute respiratory failure in cases of OP poisoning is considered to be due to the excessive build-up of Ach in the nervous systems. Acute cholinergic toxicity can induce respiratory complications, such as alveolar edema,

bronchoconstriction and bronchorrhea, through muscarinic effects [43]. Bronchorrhea due to alveolar fluid occurs frequently after OP poisoning. While β-receptor stimulation is known to induce the ejection of fluid from the alveoli, animal studies have shown that the effect of salbutamol in OP-poisoned guinea pigs was transitory [44]. This result suggests that OPs induce the internalization of GPCR. However, respiratory failure occurs more than 24 h after the exposure to OPs without cholinergic signs, suggesting that Ach may not be involved in late-onset respiratory failure [43].

Pepe *et al*. reported that IL-8 is the most important factor that affects the severity of human airway neutrophilic inflammation [45]. Furthermore, Ghanei *et al*. reported that workers using OPs showed emphysematous lesions on lung computed tomography [46]. COPD includes emphysema and chronic bronchitis, and IL-8 is considered to play a key role in the inflammatory mechanisms underlying COPD [47]. These findings suggest that OPs may induce subacute and delayed pulmonary sequelae *via* neutrophilic inflammation.

Our results support the notion that molecular and cellular mechanisms of neutrophilic inflammation are induced by exposure to OPs. Furthermore, in the presence of LPS, DZN can exert its activity even at a low level, suggesting that inflammatory conditions exacerbate DZN-induced toxicity.

5-HT and its receptors are present not only in lymph nodes but also in immune cells [48]. Many reports have described the contribution of 5-HT to immunity. Several have shown that antipsychotics belonging to the 5-HTR antagonist family, such as chlorpromazine and risperidone, have anti-inflammatory effects [49]. Therefore, some 5-HT-5HTR-related agents may be clinically useful for treating the aberrant immune responses induced by exposure to OPs.

Recent reports have revealed the existence of hetero-dimerization between 5-HTR and other GPCRs such as dopamine receptors. Furthermore, these receptors function interactively with each other in coordinated or antagonistic activities [50]. These findings suggest that DZN may be involved with other GPCRs. Further details regarding the mechanisms underlying the activity of these agents remain to be elucidated.

CONCLUSION

DZN was able to increase the IL-8 production by binding to 5-HTR in APCs. This finding suggests that DZN might induce neutrophilic inflammation independent of cholinergic toxicity, providing a foundation for the development of more effective treatments for OP toxicity.

ACKNOWLEDGEMENT

This work was partially funded by a MEXT KAKENHI Grant (number 15K15375) to S.M. and a MEXT KAKENHI Grant (number 16K08412) to M.K.

AUTHORS' CONTRIBUTIONS

N.K. performed the experiments. M.K. and S.M. conceived and designed the experiments. N.K. and S.M. wrote the manuscript. All authors discussed the results and commented on the manuscript.

CONFLICT OF INTEREST STATEMENT

S.M. is an employee of iMmno, Inc. The other authors declare no conflicts of interest regarding the publication of this manuscript.

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