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Effects of disinfection by-products in swimming pool environments on the immunological mechanisms of respiratory diseases

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ABSTRACT

Swimming in pools is a popular and healthy recreational activity. However, potential adverse health effects from disinfection byproduct (DBP) exposure in pool water are concerning. This study evaluated how such DBP exposure affects the respiratory system. DBP exposure was simulated with an animal-specific pool environment model. Experimental animals were exposed to DBPs for a specified duration and frequency over 4 weeks. The wet and dry weights of murine lungs were measured, with no significant differences observed. There were no significant differences in interkeukin (IL)-2/4/10, and interferon-γ levels. However, IL-6 expression decreased in the experimental group. To investigate the effects of DBP exposure on immune cell response, various samples, such as bronchoalveolar lavage fluid, lymph nodes, spleen, and thymus, were collected for T-cell isolation and fluorescence-activated cell sorting. Asthma-related blood cell distribution was analyzed using a complete blood count test; no significant differences were found. Thus, DBP exposure through this model did not induce substantial lung tissue damage, major alterations in cytokine expression (besides IL-6), significant immune cell responses, or changes in asthma-associated blood cell distribution. However, considering earlier results, future studies should focus on specific types, intensity, and duration of exercise that could affect DBP exposure-related immune-inflammatory responses.

Key words: asthma, DBPs, immune response, indoor swimming, respiratory diseases, swimming pool environment

HIGHLIGHTS

- DBPs have not decoupled the effects of DBP exposure and swimming activities.
- A murine DBP exposure model was developed to test the effect of periodic exposure over a 4-week period.
- DBP exposure did not cause lung damage in this model.
- Inflammatory cytokine levels were unchanged due to DBP exposure, save for IL-6.
- There were no significant changes in asthma-related blood cell-type distribution.

INTRODUCTION

Swimming in pools is a popular recreational activity enjoyed by millions of people worldwide, and it offers several health benefits (Zwiener *et al.* 2007). However, there has been growing concern regarding the potential adverse health effects associated with exposure to disinfection byproducts (DBPs) in chemically treated pool water, primarily due to the increased indoor exposure time to commonly used disinfectants and DBPs (Zheng *et al.* 2020; Lou *et al.* 2021; Zheng *et al.* 2021). This has resulted in frequent cases of disinfectant toxicity (Chang *et al.* 2020). Moreover, the diverse nature and potential interactions between chemical substances upon exposure can cause significant public health issues (Dominici *et al.* 2010; Carlin *et al.* 2013; Rider *et al.* 2013). Currently, the most common method for pool water disinfection, which is similar to that of drinking water treatment, is 'chlorine disinfection' (Richardson *et al.* 2007). Chlorine disinfection is indispensable for effective bacteria control, ease of operation, and cost-effectiveness, making it the preferred oxidant and disinfectant in swimming pools (Park *et al.* 2010; Li *et al.* 2013; Kim *et al.* 2017; Sun *et al.* 2019). Disinfection is crucial for preventing infectious diseases; however, it leads to the formation of DBPs, which are regulated in drinking water worldwide because of their adverse health effects (Fantuzzi *et al.* 2001; Chu & Nieuwenhuijsen 2002; Glauner *et al.* 2005; Sciera *et al.* 2008; Kanan & Karanfil 2011; Fischer *et al.* 2012). Pool operators face the challenge of maintaining precise chlorine levels to ensure microbial safety and minimize DBP formation by adjusting the chlorine levels thrice a day (Yang *et al.* 2016). Despite maintaining a free available chlorine

(FAC) concentration between 1 and 5 mg/L, which is generally used to control pathogenic organisms in swimming pools, an increase in DBPs can result in a higher chlorine demand.

Compared to drinking water, which is consumed by many individuals, the risk of DBP exposure through inhalation and dermal routes is higher in swimming pools (Villanueva *et al.* 2004; Caro & Gallego 2007). This exposure is associated with respiratory symptoms such as allergies, asthma, and increased inflammation (Martin *et al.* 2003; Kaydos-Daniels *et al.* 2008; Florentin *et al.* 2011; Kim *et al.* 2014; Del Giacco *et al.* 2015). A hypothesis linking respiratory diseases to the chemical and biological agents in indoor swimming pools has been proposed since 1953; it is supported by subsequent studies (Bernard *et al.* 2007; Del Giacco *et al.* 2015; Couto *et al.* 2021). However, the challenges in designing research to distinguish between the effects of chemical and biological agents make it difficult to determine the role of indoor swimming in the occurrence of respiratory diseases (Bowen *et al.* 2007). Some studies have reported an association between increased asthma and exposure to chlorinated irritants in swimming pools (Varraso *et al.* 2002; Kohlhammer *et al.* 2006); however, these associations were based on a limited number of cases. Conflicting results from various studies make it challenging to confirm the hypothesis that asthma occurs solely because of DBP exposure, considering that asthma is a complex respiratory disease influenced by multiple environmental factors (Del Giacco *et al.* 2015). Furthermore, most studies have not thoroughly investigated the potential range of DBP concentrations in indoor swimming pool environments or statistically separated the effects of swimming activities. Therefore, independent evidence supporting immune responses associated with DBPs is lacking (Bowen *et al.* 2007), and these results require careful interpretation (Gleeson *et al.* 2011; Vlaanderen *et al.* 2017). It is important to consider that other chemical and biological agents present in the indoor swimming pool environment could affect the respiratory system and contribute to adverse health outcomes, along with the exposure duration (Westerlund *et al.* 2015). Concerns regarding asthma and swimming without any association can hinder the disinfection of indoor pool water. Addressing this issue requires close collaboration and research among experts from various fields (Westerlund *et al.* 2015).

This study is the first investigation to provide independent evidence of the inflammatory response related to DBPs, while specifically excluding swimming activities in an indoor swimming pool setting.

METHODS

Animals

Six-week-old male C57BL/6 mice (Dongnam Institute of Radiological and Medical Sciences Animal Inc., Busan, Korea) were used, following a 1-week quarantine and acclimatization period. The mice were housed in a room maintained at 23 ± 2 °C, with a relative humidity of 50 ± 5%. The lighting schedule consisted of artificial light from 08:00 to 20:00; the air within the room underwent $13 - 18$ air changes per hour. The mice were provided a standard laboratory diet and had unlimited access to water (12 mice/each group). All experimental procedures adhered to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the Dongnam Institute of Radiological and Medical Sciences (Permit Number: DI-2022-021). Animal welfare was ensured in compliance with the regulations stipulated by the National Animal Welfare Law of Korea.

Modeling of the animal swimming pool environment for DBP gas exposure

Equipment to create a simulation model of an animal swimming pool environment under DBP gas exposure was assembled (Figure 1). BALB/C mice with an average length of 6 cm (excluding the tail) were chosen based on the ratio of the average height of Korean adults (male: 172.5 cm, female: 159.6 cm; Korea Agency for Technology and Standards) to the length of the swimming pool (25 m), maintaining a ratio of 1:15. Mouse cages were constructed with a dimension of $90 \times 30 \times 30$ (width $\times$ length $\times$ height) to accommodate the mice. To eliminate external influences, an air check valve was installed to allow unidirectional airflow and a quantitative pump (JenieWell JWSE100) was implemented to prevent backflow and maintain consistent one-directional flow.

To induce the generation of DBPs, a constant-temperature stirrer (IKA C-MAG HS7) and a reaction tank measuring $30 \times 30 \times 40$ cm (width $\times$ length $\times$ height) were prepared. The residual chlorine concentration following chlorination was measured in the swimming pool using a residual chlorine analyzer (HANNA HI97771C); an initial residual chlorine concentration of 5 mg/ml or higher was ensured. After the reaction, the final residual chlorine concentration was maintained at 1 mg/ml or less. Sodium hypochlorite (MAGIC-POOL, 7510490409, Korea) was employed as the chlorine-based bleach

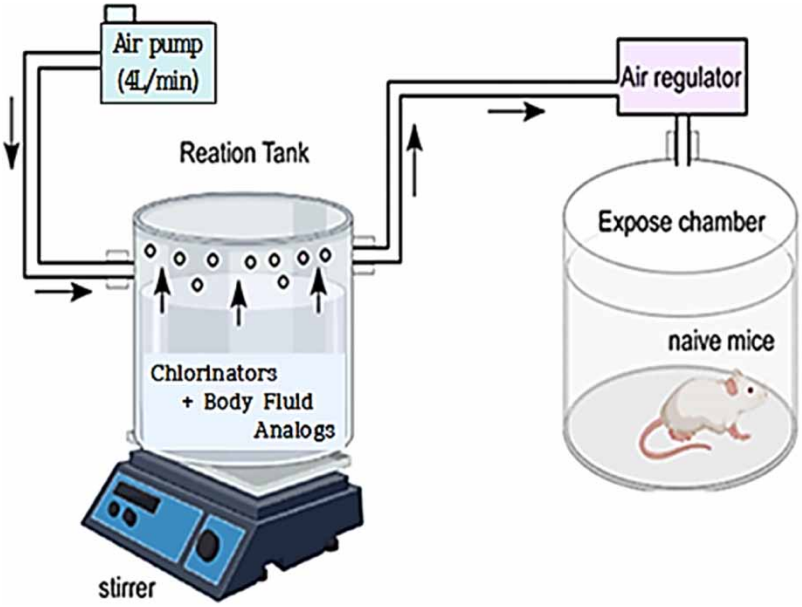

Figure 1 | Model of pool environment reproduction.

for generating DBPs. An ammonium chloride solution (12125-02-9, Korea) mimicking bodily fluids was synthesized with appropriate ratios of elements, such as creatinine, histidine, hippuric acid, uric acid, citric acid, L-arginine, and glycine (Neslihan *et al.* 2019). The synthesized solution was loaded into a syringe (NE-1000 Programmable Single Syringe Pump) for usage.

An *in vivo* model was established to investigate the effects of DBP exposure from swimming pools on respiratory diseases.

Inflammatory cell count in bronchoalveolar lavage fluid

The mice were euthanized 48 h after the final challenge through an intraperitoneal injection of Alfaxan (0.5 mg/kg; Australia), and a tracheostomy was performed. To obtain the bronchoalveolar lavage fluid (BALF), ice-cold PBS (0.5 ml) was infused into the lungs thrice and withdrawn each time through tracheal cannulation, resulting in a total volume of 1.5 ml. The total number of inflammatory cells was determined by counting the cells in at least five squares of a hemocytometer after excluding dead cells using trypan blue staining. Differential cell counts in the BALF were determined using Systemex ADVIA 2120 (Siemens Healthcare Diagnostic Inc., IL, USA) following the manufacturer's instructions. The numbers of macrophages, neutrophils, and lymphocytes were calculated by multiplying the percentages obtained from the total yield. The slides were imaged using a digital camera attached to a microscope (Nikon Eclipse 80i; Nikon Corporation, Tokyo, Japan).

Enzyme-linked immunosorbent assay

The amounts of IL-2, -4, -6, and -10 and interferon (IFN)-γ secreted in the serum of the controlled mice were quantitatively measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (BD OptEIA™ Mouse IL-2 (#555148), 4 (#555232), 6 (#555240), 10 (#555252), and IFN-γ (#555138) ELISA Kit II) according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA).

Flow cytometry analysis

The isolated cells were resuspended in 100 μl of 1% FBS solution in PBS and incubated with anti-CD3 (PE-Cy7-conjugated, BD Ms T Lym Subset Ab Cktl, #558391), anti-CD4 (PE-conjugated, BD Ms T Lym Subset Ab Cktl, #558391), anti-CD8 (FITC-conjugated, BD Ms T Lym Subset Ab Cktl, #558391), and anti-CD25 (APC-conjugated, BD MS CD25 APC, #558643) antibodies. The cell pellets were resuspended in 400 μl of 1% FBS solution in PBS and analyzed using flow cytometry (FACSAriaII cell sorter, BD Biosciences, USA).

Statistical analysis

Statistical differences between groups were analyzed using Student's two-tailed *t*-test for comparisons between two groups and one-way ANOVA for comparisons between more than two groups. All calculations were performed using GraphPad Prism software (5.0). Significance was set at $p < 0.05$.

RESULTS

Effects of DBPs on lung weight

To analyze the effects of DBP exposure on the respiratory system in a swimming pool environment, an animal-specific pool environment reproduction model was developed (Figure 1). The reaction chamber for the production of DBPs utilized a magnetic bar to stir the surrogate fluid (25 ml/h) and chlorine disinfectant (10 ml/h). The reaction was maintained at 27 – 29 °C in a temperature-controlled stirring apparatus for 72 h; the chlorine-induced gas generated was connected to an exposure chamber maintained at 27 – 29 °C. The experimental animals were exposed to the gas at a flow rate of 4 L/min for 2 h per day, 5 days a week, for 4 weeks. To determine the potential lung tissue damage caused by long-term DBP exposure, the wet and dry weights of murine lungs were measured as indicators of pulmonary impairment. The ratio of wet-to-dry tissue indicated the level of impairment in the lung tissue. No significant differences in lung ratios were observed between the two groups (Figure 2).

The effect of DBPs on the expression of cytokines associated with respiratory immune responses

ELISA was performed to determine the levels of cytokines that play key roles in the immune response to DBP exposure in a swimming pool environment. No significant changes in IL-2, IL-4, IL-10, and IFN-γ levels were observed between the experimental group (EG) and the control group (CG) (Figure 3(a), 3(b), 3(d) and 3(e)). However, the expression of IL-6 was decreased in the EG (Figure 3(c)). These data indicate that exposure to DBPs generated by this model did not influence the expression of the evaluated cytokines, except IL-6.

Alteration in the T-cell population

To observe changes in immune cells due to DBP exposure from a swimming pool environment, BALF, lymph nodes, spleen, and thymus were collected for T-cell isolation, followed by flow cytometry analysis (FACS). No significant changes were observed in the BALF and lymph nodes in the CG and the EG. However, a significant difference was observed in the number of CD4+ T cells and CD8+ T cells, which play a central role in the immune response in the spleen. In addition, significant changes were observed in the number of CD8+ T cells and Treg (CD25) T cells, but not in that of CD4+ T cells, in the

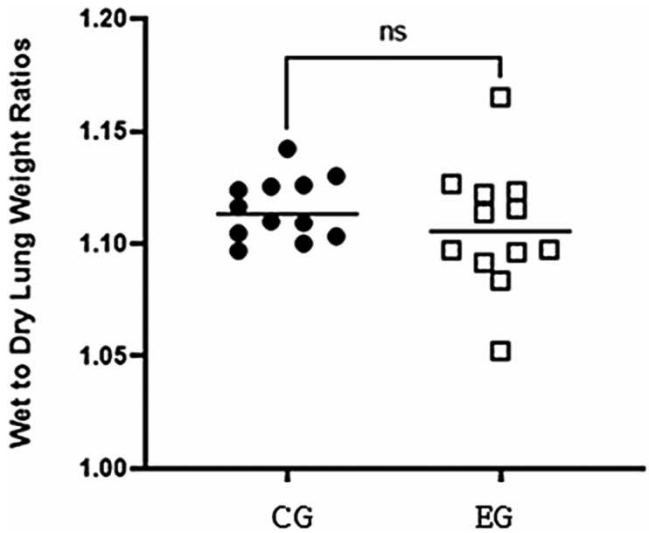

Figure 2 | Wet-to-dry lung weight ratio. Lungs were isolated from each group of mice, and the weights of wet and dry tissues were measured. The ratio was presented as wet lung tissue/dry lung tissue. Data are represented as the mean ± SEM ($n = 12$ replications/group). ns: not significant.

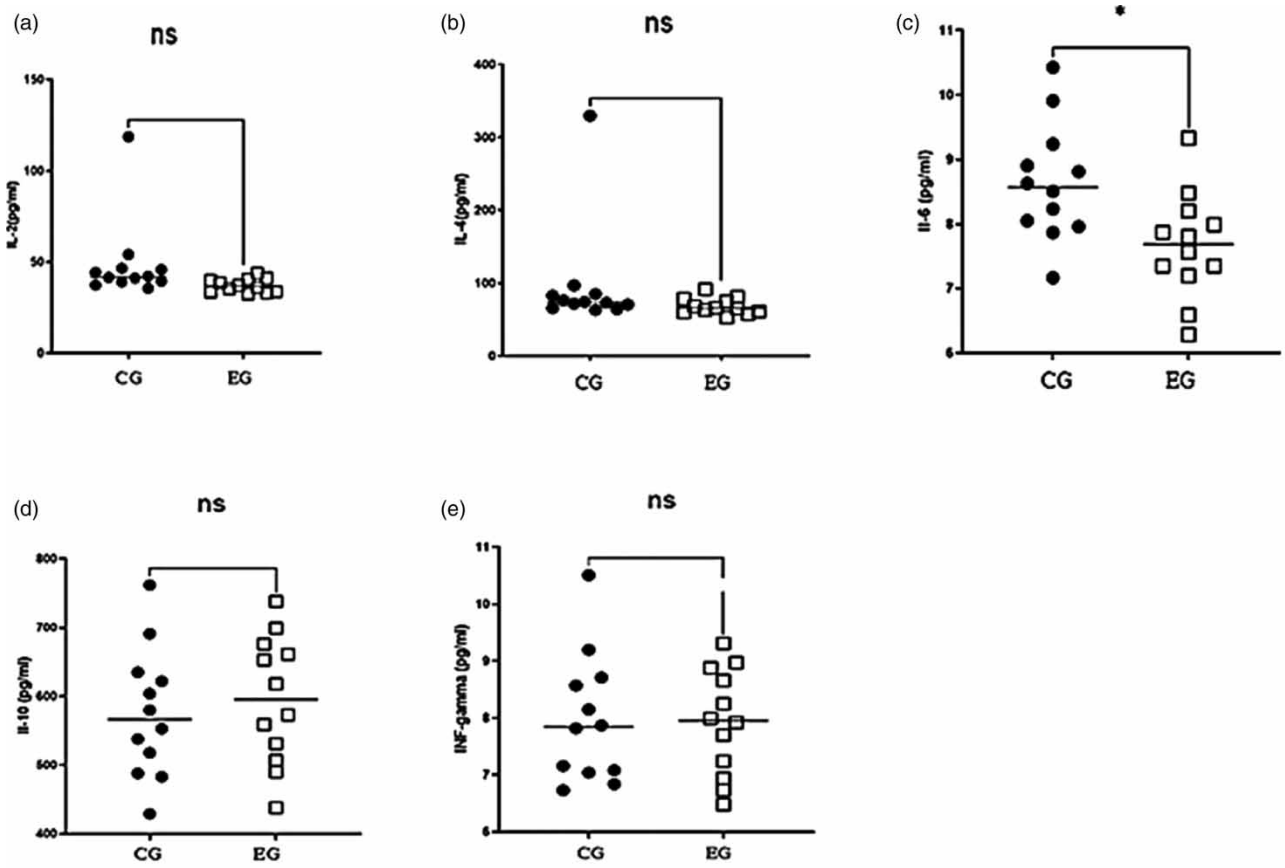

Figure 3 | Analysis of cytokines in the blood of mice. Blood serum was harvested from the control mice and analyzed using ELISA. (a) IL-2; (b) IL-4; (c) IL-6; (d) IL-10; (e) IFN-γ. Data are represented as the mean ± SEM ($n = 12$ replications/group). *$P < 0.05$ vs. CG; ns: not significant.

thymus. The observed increases or decreases in the T cells with significant changes were very small, suggesting a minimal expression indicative of an inflammatory response (asthma or allergy) (Figure 4).

Distribution of whole blood cells in the swimming pool model

To analyze changes in blood cell distribution associated with asthma in the experimental mice, we performed complete blood count (CBC) tests. No significant changes were observed in either group, suggesting the absence of an inflammatory response (Figure 5).

DISCUSSION

The use of disinfectants in swimming pools significantly impacts public health worldwide (Dehghani *et al.* 2018). The management of widespread toxicity from DBPs in swimming pool environments is becoming increasingly important, along with the increasing number of people engaging in swimming activities. A review presented various components of DBPs and showed their risks (Shakhawat *et al.* 2014). Among them, it is known that trihalomethanes (THMs), haloacetic acids (HAAs), and aldehydes are mainly present. The review suggested risks in swimming pool environments due to the complex actions of DBPs. The removal and regulation of DBPs remain challenging because of the multifactorial nature of swimming pools (Peng *et al.* 2023). In recent years, studies on the association between DBP exposure from swimming pools and asthma resulted in conflicting results without conclusive evidence (Weisel *et al.* 2009; Font-Ribera *et al.* 2014; Voisin *et al.* 2014). Individuals in occupations related to swimming pools, such as athletes, workers, and cleaners, who are frequently exposed to chlorinated environments, are concerned about the development of asthma and respiratory allergies (Medina-Ramón *et al.* 2005; Jacobs *et al.* 2007). The connection between these occupational groups and respiratory symptoms is established (Lévesque *et al.* 2006; Goodman & Hays 2008). Therefore, considering these factors, it is important to assess the relationship

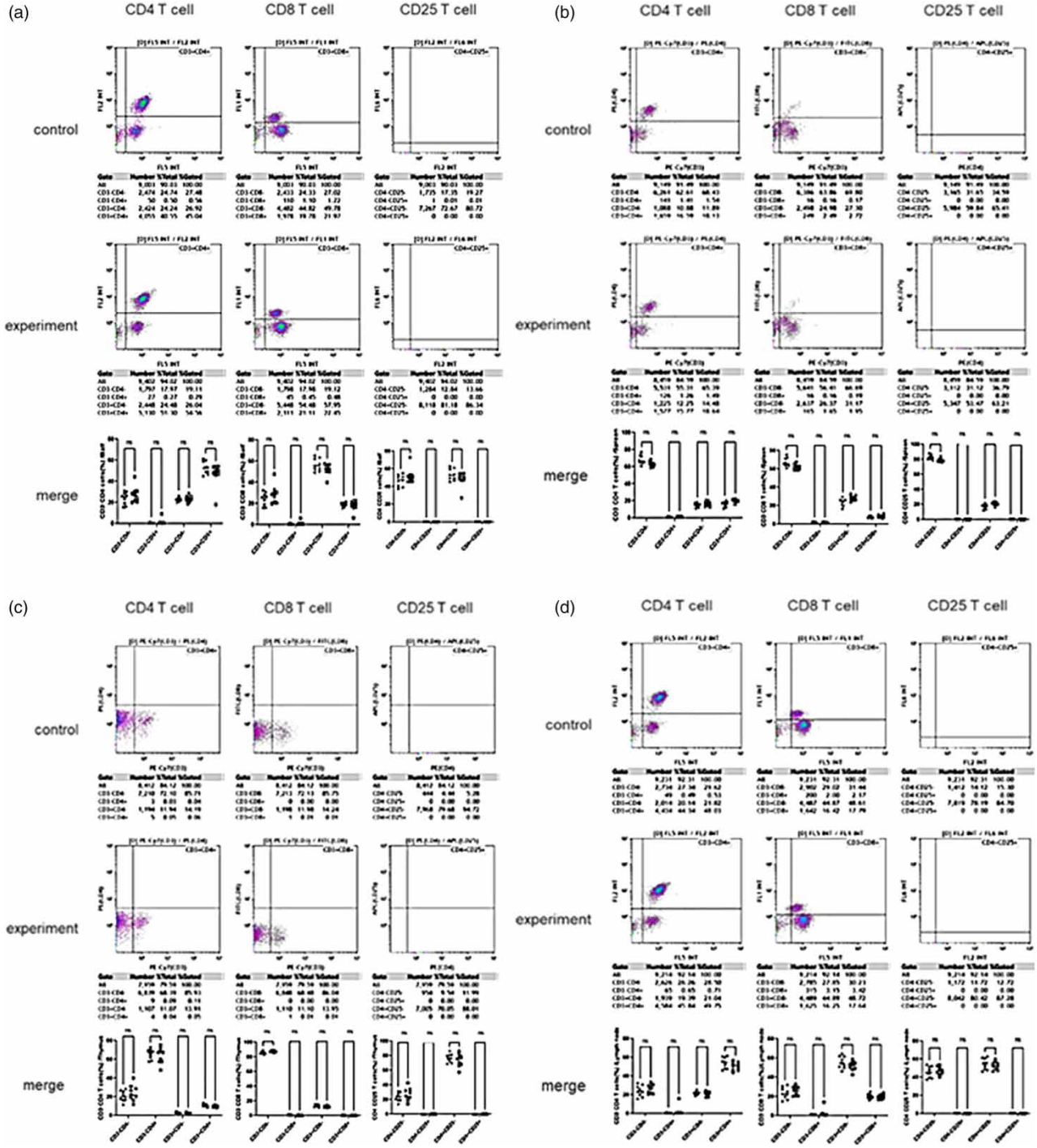

Figure 4 | FACS analysis of the isolated T cells. Cells harvested from each tissue were labeled with antibodies against CD3, CD4, CD8, and CD25 and analyzed using flow cytometry. Bottom graphs are comparison graphs of FACS data between the CG and the EG. (a) BALF, (b) spleen, (c) thymus, and (d) lymph node. The results are representative of three independent experiments. ns: not significant.

between exposure to swimming pool environments and the occurrence of asthma (Lévesque *et al.* 2006; Goodman & Hays 2008).

Cytokines, signaling molecules secreted by immune cells, play a crucial role in maintaining homeostasis between cell-mediated and humoral immune responses as well as in regulating the inflammatory process (Couto *et al.* 2021). Therefore,

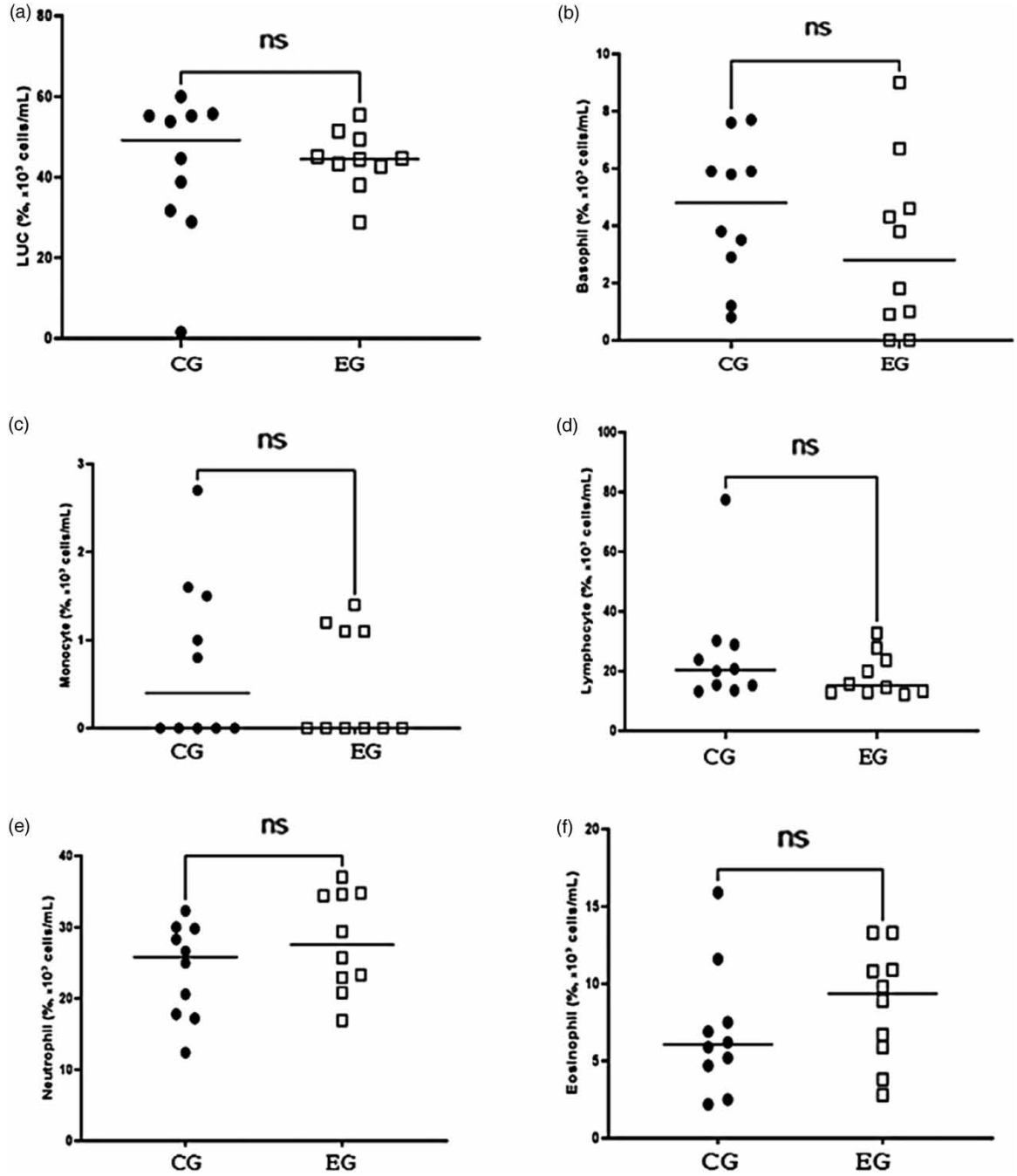

Figure 5 | Evaluation of the CBC. Blood cells harvested from control mice were analyzed using an automated blood cell analyzer. (a) Large unstained cell (LUC); (b) basophile; (c) monocyte; (d) lymphocyte; (e) neutrophile; and (f) eosinophile. ns: not significant.

to understand the changes in the immune response, we analyzed the levels of cytokines in the blood. No statistically significant differences in IL-2, IL-4, IL-10, and IFN-γ levels were observed. However, the level of IL-6a was marginally decreased in the EG; IL-6a promotes inflammatory responses. IL-4 is an immunosuppressive cytokine with a role in antitumor responses; in addition, it significantly enhances antiviral control by promoting eomesodermin (Eomes) expression in CD8+ T cells and inducing IFN-γ production (Lee *et al.* 2013; Park *et al.* 2016; Rolot *et al.* 2018). Cytokines such as IL-2, IL-4, and IFN-γ, which are beneficial for protective immune responses in disease improvement, are greatly influenced by physical activities such as swimming (Lee *et al.* 2019a). IL-6, an indicator of the inflammatory response, is affected by exercise (Lee *et al.* 2019b).

The possible mechanisms through which exercise exerts its anti-inflammatory effects include the release of IL-6 into the bloodstream from contracting muscle fibers, leading to subsequent increases in the circulating levels of IL-10 and IL-1 receptor antagonists. Exercise also promotes an increase in the number of circulating IL-10-secreting regulatory T cells. In addition, it downregulates Toll-like receptor expression in monocytes and inhibits downstream responses such as pro-inflammatory cytokine production, antigen presentation, and co-stimulatory molecule expression. Exercise reduces the number of circulating pro-inflammatory monocytes and inhibits the infiltration of monocytes and/or macrophages into adipose tissue. Regular moderate exercise is associated with a lower incidence of infection compared to that with a completely sedentary lifestyle; however, elite athletes who undergo long hours of intense training are more susceptible to infections (Gleeson *et al.* 2011).

Exercise protocols, timing, and duration are significant factors influencing the immune response (Cai *et al.* 2007). However, interpreting the effectiveness based solely on a minor decrease in the inflammatory cytokine IL-6 without a significant increase in IFN-γ, which inhibits IL-6, would not be accurate. IL-6, which promotes eosinophil infiltration, is strongly associated with various immune disorders, inflammatory diseases, and lymphatic tumors. Therefore, any significant increase or decrease observed in the T cells in the BALF, lymph nodes, spleen, and thoracic glands through FACS analysis should be considered at minimal expression levels to ascertain the presence of an inflammatory response (asthma or allergy). A study evaluating the frequency of exposure to DBPs resulting from regular attendance at swimming pools in 5,738 individuals showed no significant association with bronchial hyperresponsiveness and a lower risk for increased pulmonary function and asthma symptoms (Font-Ribera *et al.* 2011). This finding aligns with another meta-analysis that reported an unclear association between indoor swimming pool DBP exposure and asthma diagnosis (Goodman & Hays 2008; Valeriani *et al.* 2017). A study conducted on 3,223 participants to assess the relationship between respiratory symptoms and the frequency of exposure to swimming pool environments found no significant associations (Font-Ribera *et al.* 2009). However, a recent study confirmed a high prevalence of exercise-induced bronchospasm (EIB), a respiratory symptom, among athletes, especially swimmers (Boulet *et al.* 2017). This phenomenon is commonly observed in sports but is particularly pronounced in swimmers. The underlying mechanism involves increased airway resistance during exercise, leading to the release of inflammatory mediators, such as histamine, prostaglandins, and leukotrienes, from the immune cells. Increased airway ventilation enhances exposure to environmental stressors, such as air pollutants, allergens, and other stimuli, resulting in functional and structural changes in the respiratory epithelium and subsequent epithelial damage and remodeling, leading to chronic airway inflammation (Kanikowska *et al.* 2018). These changes promote the penetration of harmful substances, including allergens and viruses, creating a local microenvironment that perpetuates Th2-mediated inflammation and contributes to respiratory epithelial damage, remodeling, and induction of chronic bronchial inflammation (Holgate 2011; Kanikowska *et al.* 2018). In contrast, a higher prevalence of specific respiratory symptoms was observed in individuals occupationally exposed to swimming pool environments (excluding professional swimmers); however, chronic effects have not been reported and the causality owing to DBP exposure remains uncertain (Goodman & Hays 2008; Villanueva & Font-Ribera 2012). Considering that swimming is a recommended sport for individuals with respiratory conditions such as asthma (Uyan *et al.* 2009), it is important to elucidate the association between DBPs and respiratory diseases.

Based on the results and comprehensive analysis of previous studies, it is evident that the health benefits of swimming far outweigh the potential respiratory health risks associated with chemical contamination such as DBPs. When swimming activities are performed according to the individual's fitness level, considering the type of swimming, intensity, and duration, the risk of immune-related respiratory reactions from DBP inhalation is low. However, long-term exposure to DBPs, as highlighted in several previous studies, may increase concerns about their potential adverse health effects. Therefore, limiting exposure by maintaining proper ventilation to dilute the DBPs generated from chlorine-containing disinfectants and fostering an environment considering hygiene is essential for reaping the health benefits.

CONCLUSIONS

The swimming pool environment is reported to potentially impact respiratory health due to continuous chlorine disinfection and the presence of high levels of DBPs in both water and air, resulting from organic materials. However, the hypothesis that respiratory diseases are caused by exposure to DBPs has been a subject of diverse opinions in various studies to date. Furthermore, it has been challenging to statistically isolate the exposure effects related to physical activities such as swimming. Therefore, this study is the first to construct a simulation model of an indoor swimming pool to analyze independent evidence of the association between DBPs in swimming pool environments and immune-inflammatory responses related to respiratory

diseases, excluding from physical activities such as swimming. The impact of DBPs in swimming pool environments on the immunological mechanisms of respiratory diseases was examined, excluding physical activities such as swimming. Exposure to DBP in swimming pool environments, excluding the effects of swimming activities, did not induce respiratory diseases. The changes observed in this study were minimal when compared to those observed with exposure to chlorinated irritants in swimming pools in previous studies. Asthma, one of the most common respiratory diseases, occurs because of the complex interplay of various environmental factors and cannot be solely attributed to DBP exposure. However, considering the excessive respiratory symptoms observed in previous studies among swimmers and other individuals attending swimming pools, it is highly unlikely that these observations are coincidental or biased. Therefore, future research should investigate the specific types, intensity, and duration of exercise that may have a significant impact on immune-inflammatory responses in the presence of DBP exposure.

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AUTHOR CONTRIBUTIONS

B.-A.L. did all the work in this study. The author has read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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