

# Thimerosal induces TH2 responses via influencing cytokine secretion by human dendritic cells

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**Abstract:** Thimerosal is an organic mercury compound that is used as a preservative in vaccines and pharmaceutical products. Recent studies have shown a TH2-skewing effect of mercury, although the underlying mechanisms have not been identified. In this study, we investigated whether Thimerosal can exercise a TH2-promoting effect through modulation of functions of dendritic cells (DC). Thimerosal, in a concentration-dependent manner, inhibited the secretion of LPS-induced proinflammatory cytokines TNF- $\alpha$ , IL-6, and IL-12p70 from human monocyte-derived DC. However, the secretion of IL-10 from DC was not affected. These Thimerosal-exposed DC induced increased TH2 (IL-5 and IL-13) and decreased TH1 (IFN- $\gamma$ ) cytokine secretion from the T cells in the absence of additional Thimerosal added to the coculture. Thimerosal exposure of DC led to the depletion of intracellular glutathione (GSH), and addition of exogenous GSH to DC abolished the TH2-promoting effect of Thimerosal-treated DC, restoring secretion of TNF- $\alpha$ , IL-6, and IL-12p70 by DC and IFN- $\gamma$  secretion by T cells. These data suggest that modulation of TH2 responses by mercury and Thimerosal, in particular, is through depletion of GSH in DC. *J. Leukoc. Biol.* 81: 000–000; 2007.

**Key Words:** APC · heavy metal · immune modulation

## INTRODUCTION

Exposure to mercury is widespread in the world, and inorganic mercury, ethylmercury, and methylmercury are the predominant chemical species. The primary sources of exposure to mercury are amalgam, mercury vapors, vaccination, and seafood consumption [1–3]. Thimerosal (ethylmercurithiosalicylate) is an organic mercury compound that has been used as a preservative in vaccines, intramuscular immune globulin preparations, skin test antigens, antivenoms, ophthalmic and nasal products, and tattoo inks [1–3]. It has 49.6% mercury by weight, and following its administration, its metabolite, ethylmercury, dissociates from thiosalicylic acid and binds to blood or other tissue. The extensive use of vaccines in today's society has led to concerns about immunization safety. Today, children receive more total number of vaccinations given together during the first two years of life, leading to exposure to quantities of mercury that exceeds the safety guidelines through Thimer-

osal in vaccines. There is an increasing concern about association between the exposure to mercury (via vaccination) and the development of neurodevelopmental disorders, especially autism and learning disabilities [3–8]. This has led to Thimerosal being withdrawn from pediatric vaccines in the United States starting in 1999 (Centers for Disease Control and Prevention, 1999). Nevertheless, Thimerosal is still used in influenza, diphtheria toxoid, diphtheria toxoid and acellular pertussis, and tetanus toxoid vaccines. The majority of the studies is directed toward understanding the neurotoxic effect of Thimerosal, and few studies deal with its effect on the immune system.

The effect of mercury on the immune system has been studied mostly in rodents. These studies have revealed that subtoxic doses of mercury exposure in genetically susceptible H-2 mice strains result in the development of systemic autoimmunity characterized by lymphoproliferation with polyclonal B cell activation and hyper- $\gamma$ -globulinemia, production of autoantibodies targeting the 34-kDa nucleolar protein fibrillarin, and development of immune-complex deposits [9–15]. The different forms of mercury differ in the type and range of immune disorders, and ethylmercury (thimerosal) and inorganic mercury are similar in that they cause systemic autoimmunity, characterized by a marked increase of IgE and systemic immune-complex deposits [16, 17]. Antifibrillarin autoantibodies (AFA) and maximum levels of serum IgE are present as early as 10 days after exposure to ethylmercury in the mice [16]. Similar to the autoimmune disease induced by inorganic mercury, Thimerosal induced a distinctly increased expression of IL-4 mRNA and a large increase in TH2-dependent, Ig-secreting cells and serum Igs [18]. The increase in IL-4 has been attributed to a direct induction of IL-4 gene expression in lymphocytes by mercury [19]. Methylmercury, conversely, induces only modest titers of AFA and none of the above symptoms [16, 17, 20]. One of the possible explanations is that ethylmercury is converted much faster into inorganic mercury compared with methylmercury, leading to an earlier and more potent effect on the immune system. The immunosuppressive effects of ethyl and methyl mercury are similar and more potent than inorganic mercury in that they both cause reduction in the number and proliferative capacity of splenic T and B lymphocytes [17, 20]. Studies in humans document similar finding, where chronic exposure to low doses of ele-

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Received July 21, 2006; revised August 22, 2006; accepted October 6, 2006; doi: 10.1189/jlb.0706467.

mental mercury or mercuric salt results in excessive T cell activation, increased serum IgE, and development of antinuclear antibodies [21–26]. The sensitizing effects of Thimerosal in man are well documented. Furthermore, the incidence of allergies and autoimmunity in the population of autistic patients is also considerably higher than in control populations. A number of immunological abnormalities are found associated with autism, including T cell dysfunction, TH1/TH2 imbalance, development of autoantibodies, selective IgA deficiency, and high IgE titres [27–30]. Recent reports show that vaccinations containing Thimerosal as a preservative may lead to exacerbated eczema in children with atopic dermatitis [31, 32]. It is more important and serious that mercury exposure can accelerate or aggravate spontaneously occurring, systemic autoimmune conditions. Studies by various groups have reported such effects in a number of lupus-like mouse models [33, 34]. Exposure to mercury vapors is also known to exacerbate asthma and autoantibody production in humans. However, the mechanisms of Thimerosal-induced immune dysfunctions are unclear.

Dendritic cells (DC) are the most potent of the APCs of the immune system. They function as the sentinels of the immune system. Their responsibility is to patrol the entire body, discriminate between self and nonself, and relay information to the adaptive immune system. When responding to a threat, DC transport antigens to local lymphoid organs, where T cells specific for the antigen are activated. Because of their central role in the coordination of immunologic defenses, DC serve as the bridge between the innate and adaptive arms of the immune system [35, 36]. The nature of (pro- or anti-inflammatory) cytokines secreted by the DC in response to the threat dictates the type of the T cell responses and may result in abnormal, immunologic responses, including allergy and autoimmunity. It is well established that increased secretion of IL-10 by activated DC results in the generation of TH2 (allergic) or the T-regulatory (immunosuppressive) type of T cell response (Treg), and the secretion of IL-12 by DC generates a TH1 response through IFN- $\gamma$  induction in T cells [37–40]. There are no published reports about the effect of mercury on human DC function. Therefore, to understand the cellular basis of the immunological abnormalities associated with mercury exposure, we have examined the effect of Thimerosal on human monocyte-derived DC (MDDC) functions.

## MATERIALS AND METHODS

### Reagents

Thimerosal powder was obtained from Sigma Chemical Co. (St. Louis, MO). A 5-mM solution of Thimerosal was prepared in RPMI media, and aliquots were stored at  $-20^{\circ}\text{C}$  until used. Ultrapure LPS was purchased from List Biologicals. Thiolsalicylic acid (TSA) and glutathione (GSH) were obtained from Sigma Chemical Co.

### Isolation and culture of human MDDC

MDDC were prepared essentially as described [38]. Briefly, PBMC from buffy coat of normal, healthy donors (approved by Institutional Review Board, University of California, Irvine) were separated over Ficoll density gradient centrifugation. The blood donors were not restricted to any ethnicity or gender. The total number of donors used was 10, who were Caucasians, Hispanics, or

Asians. Cells were allowed to adhere to culture plates for 2 h. Nonadherent cells were removed. The resulting monocytes were cultured under a humidified atmosphere of 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$  in RPMI 1640 supplemented with 10% FBS, 1 mM glutamine, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 50 ng/ml human recombinant (r)GM-CSF (Peprotech, Rocky Hill, NJ), and 10 ng/ml human rIL-4 (Peprotech). Half of the medium was replaced every 2 days with fresh medium, and MDDC ( $\text{CD}14^+\text{HLA-DR}^+\text{CD}11c^+$  cells) were collected after 6 days. The purity of the MDDC obtained was  $>95\%$  (see Fig 1B). MDDC were cultured with different doses of Thimerosal (0.01–0.05  $\mu\text{M}$ ) for 6–8 h. After that, LPS (1  $\mu\text{g}/\text{ml}$ ) was added, and cells were cultured for a further 18–20 h. The next day, the cells were collected for flow cytometry, and supernatants were stored for cytokine determination.

### DC phenotype

The cells collected after Thimerosal exposure and LPS stimulation were stained for the expression of cell surface markers CD80, CD86, CD83, and HLA-DR using antibodies conjugated directly (BD Pharmingen, San Diego, CA). Ten thousand  $\text{CD}11c^+\text{HLA-DR}^+$  cells per condition were acquired using a FACSCalibur (BD Pharmingen). Analysis was done using the FlowJo software (TreeStar Inc., Ashland, OR).

### Cytokine production by DC

MDDC were cultured with various concentrations of Thimerosal and stimulated with LPS for 18–20 h as described above. Supernatants were collected and stored at  $-70^{\circ}\text{C}$  until analyzed. Cytokines TNF- $\alpha$ , IL-6, IL-12p70, and IL-10 in the supernatants were measured by specific ELISA kits (BD Pharmingen), as per the manufacturer's protocol. Briefly, Nunc Maxisorp 96-well plates were coated with specific antibody for each cytokine. After overnight incubation at  $4^{\circ}\text{C}$ , the plates were blocked with PBS containing 10% FBS, washed, and incubated with suitable dilutions of the supernatants. Bound cytokines were detected using biotinylated detection antibodies provided and HRP-conjugated streptavidin. After washing and addition of substrate, the OD in the wells was measured at 450 nm, and background values were subtracted. The average of duplicate measurements was taken. Cytokine concentrations in the samples were derived from a standard curve using purified protein of the cytokine.

### DC-T cell cultures

As mentioned above, the donors for MDDC and T cells ranged from Caucasians to Asians. At Day 6, immature DC were pulsed with Thimerosal, 0.01  $\mu\text{M}$  and 0.05  $\mu\text{M}$  for 6–8 h, followed by the addition 1  $\mu\text{g}/\text{ml}$  LPS. After 18–20 h of incubation, cells were washed. MDDC ( $1 \times 10^4$ ) were cultured with CFSE-labeled,  $1 \times 10^5$ -purified T cells (purified using the negative selection kit from Stem Cell Technology, Vancouver, BC, Canada) for 5 days. The purity of the T cells obtained ranged from 93% to 97%, as determined by CD3 staining. Proliferation was analyzed using CFSE dye dilution as an indicator of cell division. Given that each division cycle is associated with a twofold decrease in fluorescence intensity, the number of daughter cells under each CFSE fluorescence peak, corresponding to the number of divisions that the cells have undergone, was determined. Percent of proliferating cells was determined by determining the percent of responding T cells showing dilution of CFSE dye compared with total CFSE-labeled T cells. The secretion of TH1 and TH2 cytokines was assessed using ELISA.

### GSH measurement

MDDC were incubated with varying concentrations of Thimerosal + LPS or LPS or Thimerosal alone for 24 h. Then, 40  $\mu\text{M}$  Monochlorobimane (Molecular Probes, Eugene, OR) was added to the cells for 30 min at room temperature. Monobromobimane (MBBR), itself nonfluorescent, reacts with GSH to form a highly fluorescent derivative [41]. The fluorescence was measured using a Fluorimeter. The excitation and emission wavelengths were 360 nm and 460 nm, respectively.

### Cell death determination

Apoptosis of MDDC after exposure to Thimerosal was assessed by two different methods. The first method was with Annexin V and propidium iodide (PI) using an apoptosis detection kit (Calbiochem, San Diego, CA). The second method was the extraction of DNA from cells using ethanol and then staining with PI.

When apoptotic cells are fixed in precipitating fixatives such as ethanol, methanol, or acetone or permeabilized by detergent, the small-size fragments of DNA, the product of internucleosomal DNA cleavage during apoptosis, is often extracted from the cells during their staining in aqueous solutions. As a consequence of DNA loss, apoptotic cells end up with a deficit in their DNA content, and when stained with a DNA-specific fluorochrome, they can be recognized as having a fractional DNA content. The results obtained with both methods were comparable.

## Statistical analysis

Graph pad prism software was used for statistical analysis. Comparisons were done using paired *t*-test. Values of  $P < 0.05$  (two-tailed) were considered significant.

## RESULTS

### Thimerosal affects cytokine production by human MDDC

DC are central in the generation of an immune response. DC maturation results in secretion of diverse cytokines that mediate many of the functional effects of DC on other cell populations [37–40]. DC were exposed to Thimerosal (0.01–0.05  $\mu\text{M}$ ) and stimulated with LPS overnight, and the secretion of cytokines in the supernatant was determined by ELISA. TSA (nonmercury component of Thimerosal) was included as a control [42]. Thimerosal alone did not induce cytokine secretion by DC. However, Thimerosal, in a concentration-dependent manner, suppressed the secretion of proinflammatory cytokines TNF- $\alpha$  and IL-6 from LPS-stimulated DC (**Fig. 1A**). Significant inhibition of TNF- $\alpha$  and IL-6 ( $P < 0.05$ ) was observed at 0.05  $\mu\text{M}$  dose of Thimerosal. It is more important that the secretion of TH1-driving cytokine IL-12p70 was reduced significantly ( $P < 0.005$ ; Fig. 1A). In contrast, no significant inhibition of the secretion of IL-10 was observed, suggesting that Thimerosal has a differential effect on pro- and anti-inflammatory cytokines (Fig. 1A). TSA had no effect on cytokine secretion by LPS-stimulated DC, suggesting further that these effects are a result of the mercury in Thimerosal (Fig. 1A). The purity of the MDDC population used in the experiments was routinely more than 95% (Fig. 1B); therefore, the cytokine production observed is not affected significantly by the presence of other cells in the culture.

Thimerosal did not affect the maturation of DC at the concentrations used, as determined by the expression of the costimulatory molecules and maturation markers CD80, CD83, CD86, and HLA-DR. A representative flow cytometry graph is shown (Fig. 1C).

### Effect of Thimerosal on DC viability

Ethylmercury in Thimerosal induces apoptosis in T lymphocytes and neuronal cells [24–26]. To address whether the suppressive effects of Thimerosal on DC cytokine production were a result of apoptosis of DC, MDDC were cultured with LPS in the presence of varying concentrations of Thimerosal for overnight, and apoptosis was measured by Annexin V and PI staining (**Fig. 3**). Thimerosal did not induce significant ( $P > 0.05$ ) apoptosis of DC at the concentrations used in the present study. These data suggest that the inhibitory effect of

Thimerosal on cytokine production by DC is not a consequence of reduced viability of DC.

### Thimerosal suppresses the TH1 response in T cells in an allogeneic MLR

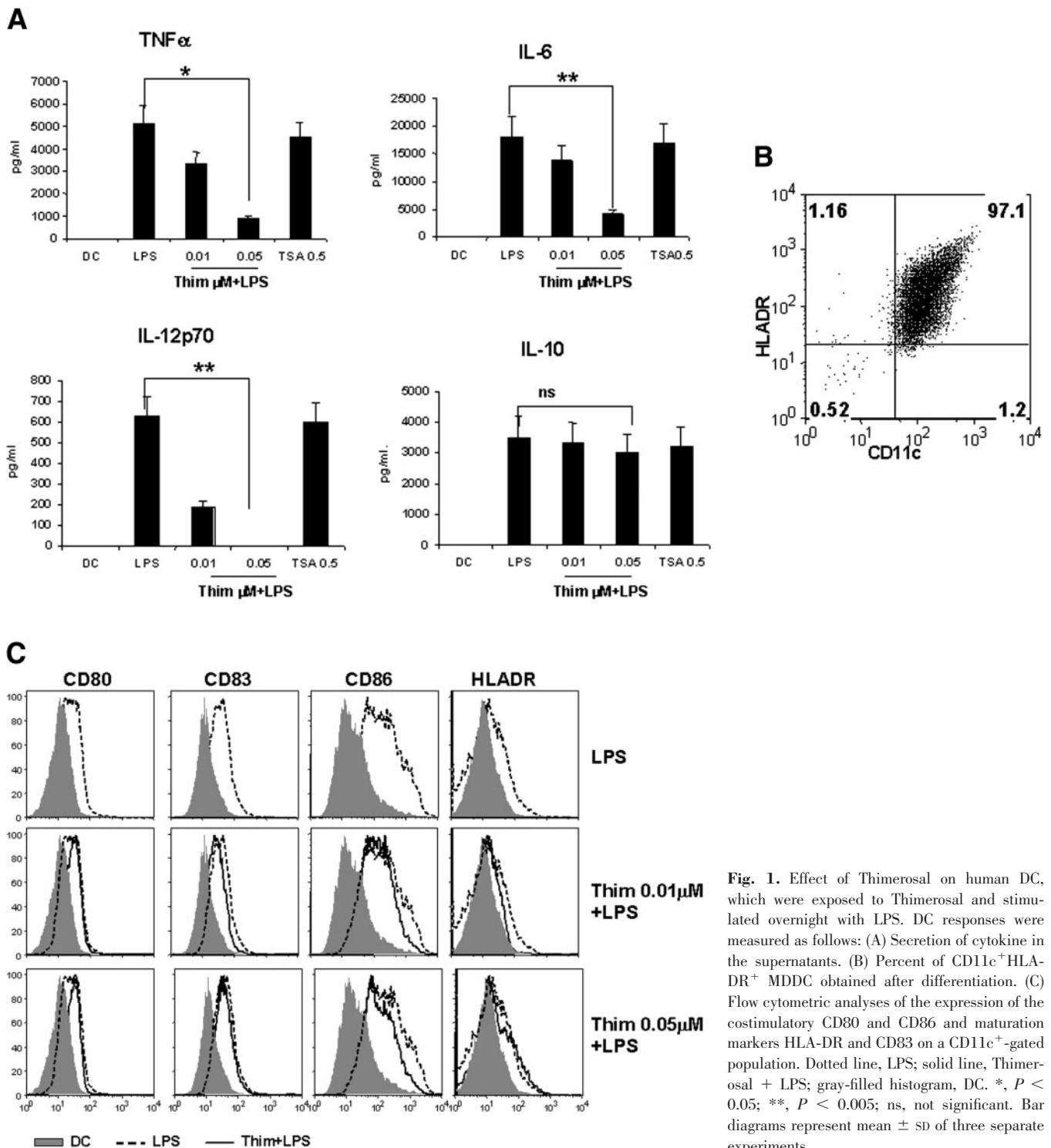
As the nature of cytokines secreted by DC governs the type of TH response and as Thimerosal altered the secretion of cytokines by DC, we investigated priming of T cells by DC, which were pre-exposed to Thimerosal. Briefly, DC were pre-exposed to Thimerosal and LPS as described in Materials and Methods and subsequently, were cultured with CFSE-labeled, allogeneic T cells. Priming was determined by measuring proliferation of T cells and production of cytokines by T cells. DC and DC stimulated with LPS induced proliferation in T cells as determined by dilution of CFSE dye using flow cytometry (Fig. 3, A and B). The proliferative responses of T cells, which were cocultured with Thimerosal-treated DC, were slightly lower than control cultures, but this difference was not statistically significant ( $P > 0.05$ ), suggesting that exposure to Thimerosal does not interfere with the ability of DC to induce T cell proliferation. The number of cell divisions was also not significantly different between Thimerosal exposed and control groups, as by Day 5, almost all responding T cells had undergone seven to eight divisions (Fig. 3A). DC, which were stimulated with LPS, but not unstimulated DC, as expected, induced a substantial increase in the secretion of IFN- $\gamma$  by T cells. In contrast, DC, which were pre-exposed to Thimerosal and subsequently stimulated with LPS, caused a significant reduction ( $P < 0.05$ ) in IFN- $\gamma$  secretion and a significant increase in IL-5 and IL-13 secretion (Fig. 3B). However, we found neither IL-10 nor IL-4 to be detectable (data not shown). Thimerosal also did not influence the number of CD4<sup>+</sup>, CD25<sup>+</sup>, and forkhead box P3<sup>+</sup> Treg cells (data not shown). These data suggest that Thimerosal influences the nature of T cell priming by DC by skewing the T cell responses towards TH2 through suppression of IFN- $\gamma$  production and enhancing IL-5 and IL-13 production.

### Thimerosal reduces intracellular GSH level in DC

Previous studies have shown that intracellular GSH levels in APCs play a central role in determining whether TH1 or TH2 cytokine response patterns predominate in immune responses [43–45]. Thimerosal is known to induce intracellular oxidative stress through depletion of intracellular GSH in lymphocytes and neuronal cells [42, 46]. Therefore, we examined the effect of Thimerosal on intracellular GSH in DC. MDDC were incubated with various concentrations of Thimerosal for 24 h, and intracellular GSH was measured by a fluorimeter, as described in Materials and Methods. Thimerosal decreased GSH level in DC as shown in **Figure 4**.

### Exogenous GSH restores DC proinflammatory cytokine secretion

Next, we investigated if the intracellular GSH depletion in DC by Thimerosal is one of the mechanisms responsible for the differential effect on cytokine secretion and thus, the TH2 bias observed in the T cells. To this end, DC were cultured with



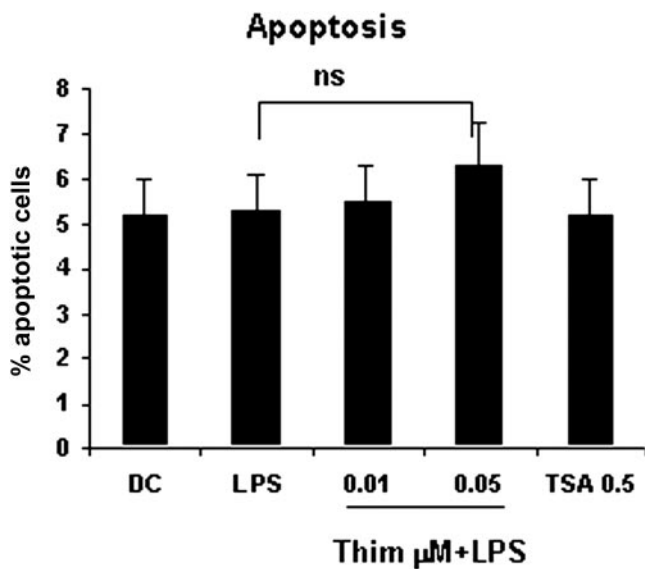
**Fig. 1.** Effect of Thimerosal on human DC, which were exposed to Thimerosal and stimulated overnight with LPS. DC responses were measured as follows: (A) Secretion of cytokine in the supernatants. (B) Percent of CD11c<sup>+</sup>HLA-DR<sup>+</sup> MDDC obtained after differentiation. (C) Flow cytometric analyses of the expression of the costimulatory CD80 and CD86 and maturation markers HLA-DR and CD83 on a CD11c<sup>+</sup>-gated population. Dotted line, LPS; solid line, Thimerosal + LPS; gray-filled histogram, DC. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; ns, not significant. Bar diagrams represent mean  $\pm$  SD of three separate experiments.

Thimerosal in the presence of varying concentrations of GSH and stimulated with LPS. DC cytokine secretion was determined by ELISA. Addition of exogenous GSH resulted in the recovery of TNF- $\alpha$ , IL-6, and IL-12 p70 production by Thimerosal-treated DC, and the maximal effect was observed at 5 mM concentration of GSH (Fig. 5). This was found to be highly significant ( $P < 0.05$  for TNF and IL-6;  $P < 0.005$  for IL-12p70). Lower concentrations of GSH were not effective, and concentrations higher than 5 mM were inhibitory to cytokine secretion. It is more important that a concomitant decrease in IL-10

levels was observed with an increase of proinflammatory cytokine secretion. These findings document DC GSH levels as a key regulator in the production of proinflammatory cytokines TNF- $\alpha$ , IL-6, and IL-12p70.

#### Exogenous GSH-treated DC restore the secretion of IFN- $\gamma$ from T cells

To investigate further if addition of exogenous GSH to DC would also restore the TH balance toward TH1, allogeneic T cells were cultured with DC treated with exogenous GSH and



**Fig. 2.** Effect of Thimerosal on DC viability. Percent of CD11c<sup>+</sup> apoptotic cells as determined by annexin V and PI staining after exposure to Thimerosal. Figures are mean  $\pm$  SD of four separate experiments.

Thimerosal for 5 days, as before. Secretion of IFN- $\gamma$  by T cells in the supernatant was determined by ELISA. We found that culture of T cells with GSH-treated DC was able to recover the suppression of IFN- $\gamma$  from T cells (**Fig. 6**). A significant decrease in the production of IL-13 was also observed (Fig. 6). Thus, intracellular GSH levels in DC influence the nature of TH responses toward TH1 or TH2 by regulating the production of proinflammatory cytokines, particularly IL-12p70, from them.

## DISCUSSION

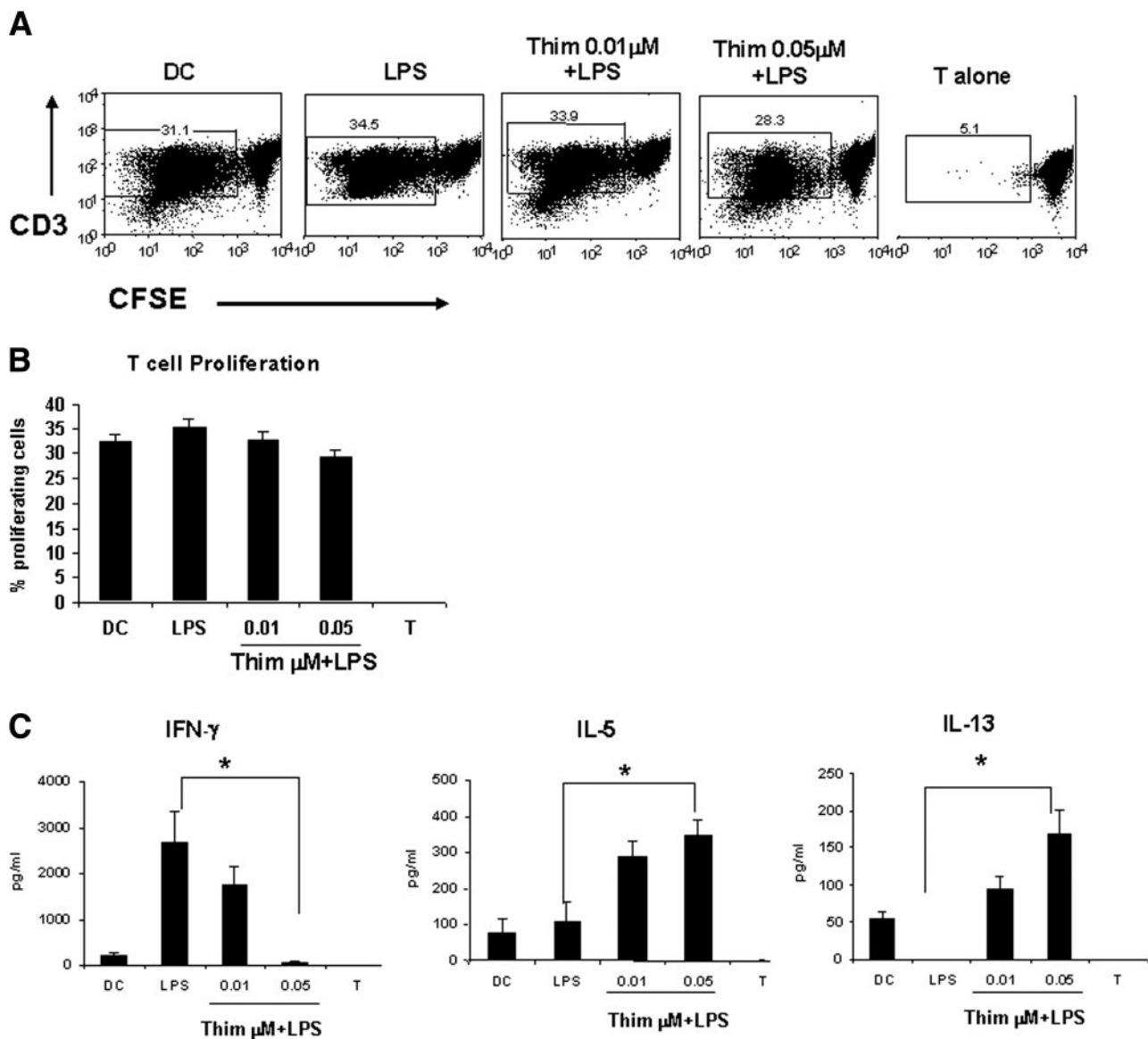
Immune modulation by heavy metals may cause serious adverse health effects in humans, although the mechanisms involved are not well understood [47, 48]. These immune-modulating properties are associated with the development of allergy and autoimmunity. Mercury is an important environmental pollutant and toxin, to which exposure has been immunosuppressive, leading to autoimmune and allergic manifestations. Mercury has been shown to induce a number of immunological and neurotoxic changes, including increased production of TH2 cytokines, increased levels of IgE, decreased activity of T cells and NK cell, suppression of IgG, production of autoantibodies to a variety of self-antigens (e.g., neural antigens), and apoptosis in microglia and astrocytes [1–12]. The underlying mechanisms by which mercury induces autoimmunity and enhances allergic inflammation are not well understood.

The present study addresses the possible mechanisms responsible for the immune dysfunction associated with mercury exposure. To this end, we have studied the effect of Thimerosal on MDDC at concentrations readily achievable in vivo. The U.S. Environmental Protection Agency (EPA) Safety of Exposure Standard is 0.1  $\mu$ g/kg body weight/day, equating to 7  $\mu$ g

for a 70-kg adult. Fully vaccinated children receive as much as 237.5  $\mu$ g mercury from vaccines in doses of up to 25  $\mu$ g each, which far exceeds the EPA safety standard. Administration of the three thimerosal-containing influenza vaccines could raise this exposure to as high as 275  $\mu$ g mercury [3, 6]. Similarly, in areas with high seafood consumption, the levels of mercury in the blood can easily reach up to 20–30  $\mu$ g/L, which far exceeds the 5.8  $\mu$ g/L safety guideline set by the EPA. In our studies, we have used concentrations of 20 ng/ml or lower, which is readily achievable during childhood vaccinations, where an infant is exposed to 20–25  $\mu$ g/kg mercury during each vaccination. In addition, the Thimerosal is administered parentally along with the vaccines; therefore, APCs at the site would be exposed to a much higher concentration of mercury than reflected at the serum level. Here, we demonstrate that Thimerosal, at mercury concentrations readily achievable in vivo, differentially influenced cytokine secretion from DC without affecting DC maturation. Except for a few reports showing that Thimerosal suppresses the release of IL-1 $\beta$  and inhibits the phagocytic capacity and oxidative burst in human monocytes, virtually nothing is known about its effect on DC [49, 50]. Our study is the first report showing the inhibition of proinflammatory cytokines TNF- $\alpha$ , IL-6, and IL-12p70 from DC by Thimerosal without significantly affecting the secretion of anti-inflammatory cytokine IL-10.

Our finding that thimerosal increased secretion of IL-5 and IL-13 and reduced IFN- $\gamma$  production by T cells is consistent with the previous reports of observed TH2 bias associated with mercury. The TH2-polarizing nature of mercury is well documented, and it has been found in the mouse that administration of Thimerosal induced increased expression of IL-4 mRNA in T cells and a large increase in TH2-dependent, Ig-secreting cells and serum Ig [14, 19]. Inorganic mercury also induces IL-4 gene expression in mouse T cells [19]. Similarly, mercury-induced oxidative stress up-regulates IL-4 gene expression in mast cells [51]. In this study, we did not detect IL-10 or IL-4 in our DC-T cell cultures. This could be a result of the strong TH1-polarizing nature of LPS, resulting in production of IL-4 and IL-10, well below the sensitivity of the ELISA kit. The underlying mechanisms responsible for TH2 polarization by Thimerosal have not been defined. Moreover, none of previous studies had focused on the role of APCs or DC, and thus, it is difficult to define their contribution in the observed TH2 bias following in vivo administration of mercury in the mouse. Our results here demonstrate DC as major perpetrators of the TH2 skewing with mercury.

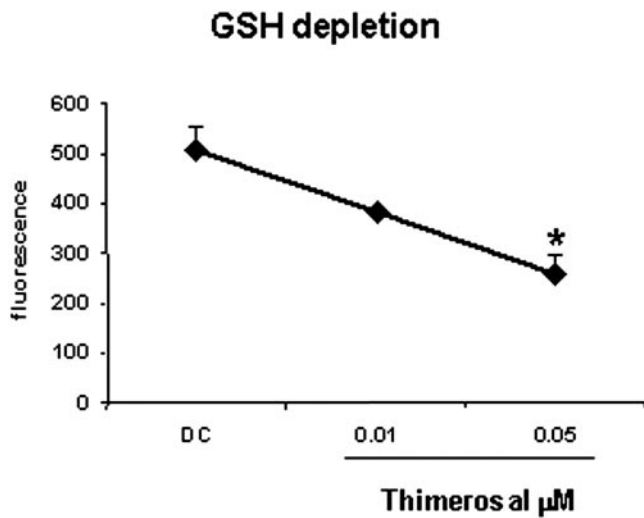
Evidence from various studies strongly suggests that restoring the TH1 cytokine secretion would suppress the TH2 responses and autoimmunity associated with mercury poisoning [52–56]. Gillespie et al. [52] show that individual Type 1 cytokines are capable of suppressing the dramatic TH2 response induced by mercury in the rat, even when they are not given until after starting mercury administration. Studies have been conducted in the mouse with the intention to attenuate or ameliorate mercury-induced autoimmunity by deviating the response toward TH1. Deviation has been accomplished by treatment with anti-IL-4 or rIFN- $\gamma$  [53, 54]. The TH2-dependent parameters, such as IgE, are reduced efficiently by such deviation, but the autoantibody response was not affected. By



**Fig. 3.** Thimerosal suppresses the TH1 response in T cells in an allogeneic MLR. Purified CD3+ T cells were cocultured with DC exposed to Thimerosal + LPS. (A) Dot blot representing the proliferation of CFSE-labeled T cells after culture with DC exposed to LPS and Thimerosal. Figure represents one of three such experiments. (B) Bar diagram showing mean  $\pm$  SD of the proliferation of T cells as determined by flow cytometry using CFSE dye. (C) TH1/TH2 cytokines in the supernatant. Figures are mean  $\pm$  SD of three separate experiments. \*,  $P < 0.05$ .

using mice with targeted mutations, Kono et al. [55] showed subsequently that IFN- $\gamma$  but not IL-4 is required for induction of mercury-induced autoimmunity. The observations of Kono et al. [55] indicate that TH1/TH2 imbalance does not play a role directly in susceptibility to mercury-induced autoimmunity and suggest that the dependence on TH1-type responses in certain autoimmune diseases is a result of the requirement for IFN- $\gamma$  for antibody production to weakly antigenic self-molecules. In contrast Bagenstose et al. [56] have shown that administration of IL-12 along with mercury resulted in a dramatic reduction of the antinucleolar antibody titers in mice. All of these studies clearly demonstrate the importance of IFN- $\gamma$  production in preventing mercury-induced allergies, although its effect on autoimmunity is controversial. Therefore, it is imperative to understand the mechanisms underlying this reduced IFN- $\gamma$  production to devise effective therapies.

As mentioned, the precise mechanisms by which mercury affects the TH responses are not known. Heavy metals, such as mercury, are known to have a high affinity for thiol groups, which leads to formation of complexes called mercaptides. Sulfhydryls are especially common in cysteine-rich proteins, and binding of mercury to thiols, even at a low concentration, may have distinct effects on cell function. GSH is a small molecule, which is present at high concentrations (mM range) inside the cells and plays key roles in basic metabolic and cell cycle-related processes. Among its many functions, this cysteine-containing tripeptide reduces protein disulfides, detoxifies free radicals and exogenous toxins, and preserves the intracellular redox balance [57, 58]. GSH depletion by Thimerosal is found in neuronal cells and lymphocytes [51, 59]. Consistent with the findings in other cells, we have observed that Thimerosal reduces intracellular GSH levels in DC. Our



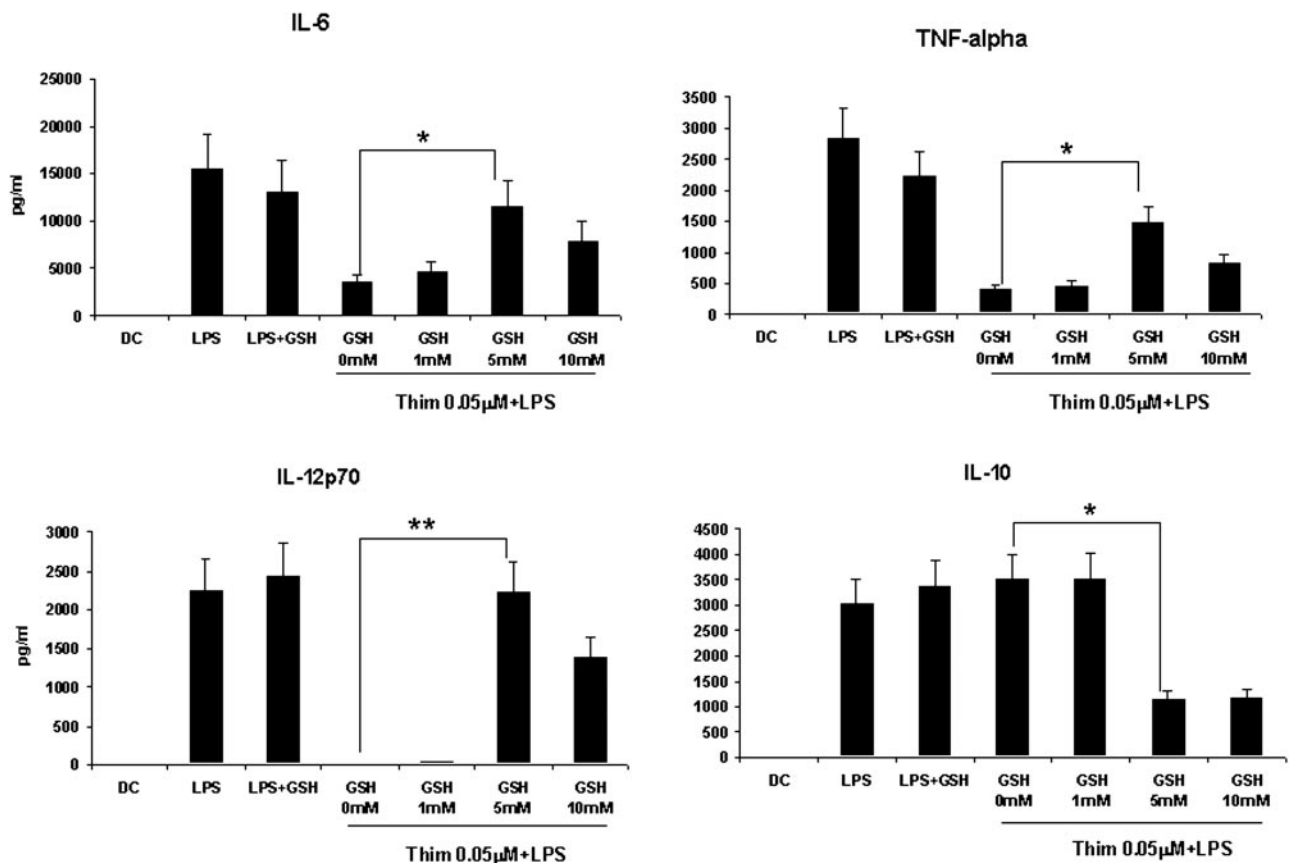
**Fig. 4.** Thimerosal reduces intracellular GSH level in DC. Measurement of intracellular GSH in DC after exposure to Thimerosal using MBBR dye. Figure represents mean  $\pm$  SD of three separate experiments. \*,  $P < 0.05$ .

studies demonstrate further that addition of exogenous GSH to DC, exposed to Thimerosal, restores the DC proinflammatory cytokine TNF- $\alpha$ , IL-6, and IL-12p70 secretion to normal levels. It is more important that this leads to recovery of IFN- $\gamma$

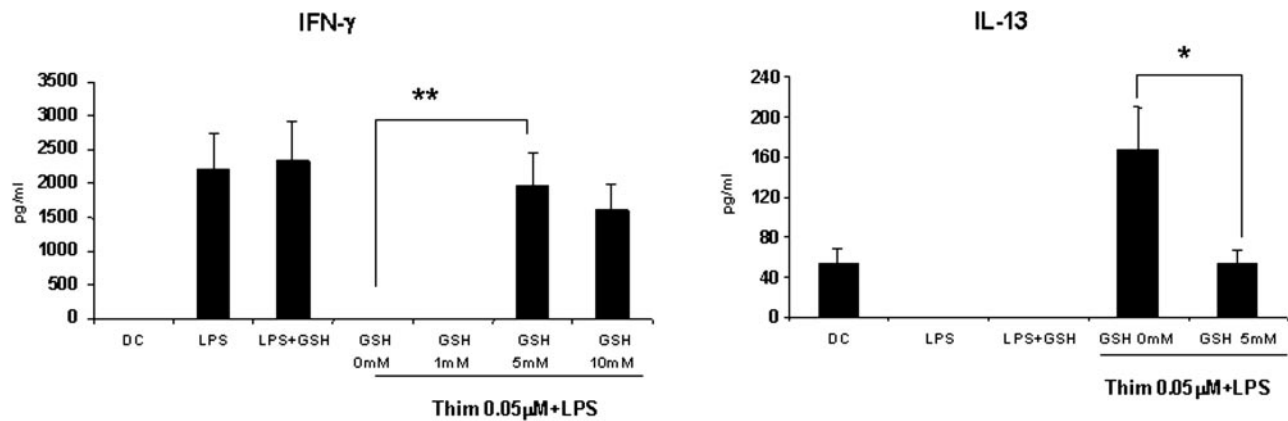
production by T cells. This suggests that intracellular GSH levels in DC define the polarization of TH responses. This is in agreement with other findings, where it has been shown that the GSH levels in murine APCs play a central role in determining the predominance of TH1 and TH2 cytokine responses in vivo through the level of IL-12 production [43–45]. Petersen et al. [43], using two immunologic models and three methods to deplete GSH, have shown that GSH depletion leads to a shift away from the typical TH1 cytokine profile and toward TH2 response patterns. Other environmental pollutants such as diesel exhaust particles have also been shown to affect GSH levels in DC, leading to a TH2-type response [60].

Depletion of GSH from DC has been shown to reduce T cell proliferation in DC-T cell cocultures [61]. However, we did not observe any significant reduction in the proliferation of T cells in our studies. One possibility is the extent of GSH depletion caused by the two agents. Short et al. [62] have demonstrated that more than a 70% decrease in GSH in murine DC reduces T cell proliferation, and a decrease of only 20% is sufficient to reduce IFN- $\gamma$  production in T cells without affecting T cell proliferation. Another possibility is a difference in the nature of depletion of GSH. Ifosfamide used in the previous study inhibits the enzyme GST, inhibiting GSH synthesis, and addition of Thimerosal leads to its oxidation.

Alternatively, mercury may affect TH responses by modulating  $\text{Ca}^{2+}$ , as it is known to increase membrane  $\text{Ca}^{2+}$  per-



**Fig. 5.** Exogenous GSH restores DC proinflammatory cytokine secretion. DC were exposed to Thimerosal in the presence of GSH and then stimulated overnight with LPS. Figure represents secretion of cytokines in the supernatants as measured by ELISA. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ . Results are mean  $\pm$  SD of three separate experiments



**Fig. 6.** Exogenous, GSH-treated DC restore the secretion of IFN- $\gamma$  from T cells. IFN- $\gamma$  and IL-13 secretion by T cells in an allogeneic MLR after coculture with DC exposed to Thimerosal in the presence of exogenous GSH. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ . Results are mean  $\pm$  SD of three separate experiments.

meability and releases  $\text{Ca}^{2+}$  from intracellular calcium stores [63]. A recently published report indicates that that transient exposure of murine bone marrow-derived DC to Thimerosal resulted in suppression of IL-6 secretion from LPS-stimulated DC through uncoupling of ATP-mediated calcium signaling [64]. This study did not examine GSH depletion in the DC. We did not find  $\text{Ca}^{2+}$  efflux as a result of Thimerosal responsible for the alterations in DC functions, as addition of  $\text{Ca}^{2+}$  channel inhibitors 2- aminoethoxydiphenyl borate and verapamil to DC inhibited thimerosal-induced  $\text{Ca}^{2+}$  mobilization but was not able to restore the DC function and TH bias (data not shown). Therefore, we tentatively favor the former hypothesis that mercury skews immune response by modulating GSH levels in DC. In summary, our findings here suggest that alterations in immune function through depletion of GSH in DC play a central role along with other factors in exacerbating autoimmune and allergic responses associated with mercury exposure in general and Thimerosal in particular.

As immune abnormalities associated with mercury have been shown to be associated with GSH depletion, the use of dietary supplements, which increase intracellular GSH, such as *N*-acetyl-L-cysteine [65], a GSH prodrug, could serve as an effective therapy to reduce previous or anticipated exposure to mercury.

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