ORIGINAL RESEARCH

A role of cellular glutathione in the differential effects of iron oxide nanoparticles on antigen-specific T cell cytokine expression

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Background: Accumulating evidence indicates that iron oxide nanoparticles modulate immune responses, and induce oxidative stress in macrophages. It was recently reported that iron oxide nanoparticles attenuated antigen-specific immunity in vivo, though the underlying mechanism remains elusive. The present study investigates the direct effect of iron oxide nanoparticles on antigen-specific cytokine expression by T cells, and potential underlying mechanisms.

Methods: Ovalbumin-primed splenocytes were exposed to iron oxide nanoparticles, followed by restimulation with ovalbumin. Cell viability, cytokine production, and cellular levels of glutathione and reactive oxygen species were measured.

Results: The splenocyte viability and the production of interleukin-2 and interleukin-4 were unaffected, whereas interferon-γ production was markedly attenuated by iron oxide nanoparticles (10–100 μg iron/mL) in a concentration-dependent manner. Iron oxide nanoparticles also transiently diminished the intracellular level of glutathione, with a peak response at 6 hours posttreatment. The effects of iron oxide nanoparticles on interferon-γ and glutathione were attenuated by the presence of N-acetyl-L-cysteine, a precursor of glutathione. However, iron oxide nanoparticles did not influence the generation of reactive oxygen species.

Conclusion: Iron oxide nanoparticles induced a differential effect on antigen-specific cytokine expression by T cells, in which the T helper 1 cytokine IFN-γ was sensitive, whereas the T helper 2 cytokine interleukin-4 was refractory. In addition, the suppressive effect of iron oxide nanoparticles on interferon-γ was closely associated with the diminishment of glutathione.

Keywords: iron oxide nanoparticle, T cell, antigen-specific, glutathione, cytokine

Introduction

Nanomaterials have been increasingly applied in numerous fields, including nanomedicine. Among various biomedical nanoparticles, superparamagnetic iron oxide nanoparticles have been used in clinical settings as a contrasting agent to enhance magnetic resonance imaging.[1,2] In addition, iron oxide particles have shown promising potential for cell labeling, cancer therapy, and drug delivery.[2–4] It has been well-documented that iron oxide nanoparticles administered systemically are rapidly engulfed by the reticuloendothelial system, with the liver and spleen being the main distribution sites for the particles.[5–7] Because phagocytes are one of the major cell groups exposed to iron oxide nanoparticles, the potential effect of nanoparticles on the functionality of immune cells and host immune competency is a concern, and is under intensive investigation.

Accumulating evidence indicates that exposure to iron oxide nanoparticles causes apoptosis and alters the functionality of macrophages.[8–12] Primary macrophages exposed

to iron oxide nanoparticles in culture showed a marked increase of apoptosis, accompanied by elevated generation of reactive oxygen species (ROS).[10] Similarly, exposure of the murine macrophage cell line J774 to iron oxide nanoparticles resulted in an increased production of intracellular ROS, with subsequent cell injury and apoptosis.[13] Murine studies further showed that a single intratracheal instillation of iron oxide nanoparticles had proinflammatory and prooxidative effects, as evidenced by a marked infiltration of inflammatory cells into the lungs and a diminished level of intracellular glutathione in bronchoalveolar lavage cells.[12] Collectively, these results have demonstrated the immunomodulatory and cytotoxic effects of iron oxide nanoparticles on macrophages, possibly through oxidative stress-related mechanisms.

In addition to macrophages, other immune cells are also sensitive to iron oxide nanoparticles. For example, the function of dendritic cells to process antigens and stimulate T cells were suppressed by iron oxide nanoparticles.[14] Several animal studies have reported that T cells are another target in the immune system sensitive to iron oxide nanoparticles. Both oral and intravenous administrations of iron oxide nanoparticles to normal nonsensitized mice altered T cell cellularity.[15,16] The serum levels of interleukin (IL)-2, IL-10, and interferon (IFN)-γ were elevated in mice intravenously treated with iron oxide nanoparticles.[16] Furthermore, it was recently reported that a single systemic administration of iron oxide nanoparticles attenuated the serum production of antigen-specific immunoglobulin (IgG)$_1$ and IgG$_{2a}$ and the expression of IL-4 and IFN-γ by splenocytes, in ovalbumin (OVA)-sensitized mice.[17] These results demonstrate that exposure to iron oxide nanoparticles influences T cell functionality in both nonsensitized and antigen-sensitized mice. However, the underlying mechanisms remain elusive, and whether iron oxide nanoparticles produce a direct effect on T cells remains unclear.

The present study investigated the direct effect of iron oxide nanoparticles on antigen-specific T cell reactivity by using OVA-primed splenocytes in culture. Furthermore, in light of the available evidence showing the cytotoxic and prooxidative effects of iron oxide nanoparticles on macrophages, the potential role of oxidative stress and cellular glutathione in iron oxide nanoparticle-mediated effects on T cells was also addressed.

Materials and methods
Reagents and chemicals
All reagents were obtained from Sigma-Aldrich Corporation (St Louis, MO), unless otherwise stated. Reagents and antibodies for enzyme-linked immunosorbent assay were purchased from BD Biosciences – Pharmingen (Becton, Dickinson and Company, San Diego, CA). Fetal bovine serum and cell culture supplies were purchased from Thermo Fisher Scientific (Logan, UT). A commercial preparation of carboxydextran-coated iron oxide nanoparticles containing 28 mg iron (Fe)/mL, namely ferucarbotran (Resovist®; Schering AG, Berlin-Wedding, Germany), was used in the present study. According to its package insert, the hydrodynamic diameters of the coated particles range from 45 nm to 60 nm. Results from confirmatory experiments revealed that Resovist exhibited a monodisperse population of particles with an average diameter to be 58.7 nm, using a particle size analyzer (Zetasizer Nano S, Malvern Instruments Ltd, Malvern, Worcestershire, United Kingdom). The crystalline core of ferucarbotran is composed of magnetite and maghemite.[5]

Animals
Male BALB/c mice (5–6 weeks old) were purchased from BioLasco Taiwan Co, Ltd (Taipei, Taiwan). On arrival, the mice were randomly transferred to plastic cages containing aspen bedding, with five mice per cage, and quarantined for at least 1 week. The mice were housed in an environment in which temperature (22°C ± 2°C), humidity (50% ± 20%), and light (12-hour light/dark cycle) were controlled. Food and water were supplied ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of the National Taiwan University.

OVA-primed splenocytes
BALB/c mice were sensitized twice with OVA by intraperitoneal injection on days 0 and 14. On these days, each mouse was injected with 250 μL sensitization solution, containing 20 μg OVA and 2 mg alum (as adjuvant) in saline. On day 15, the mice were euthanized and their spleens were harvested aseptically and made into single cell suspensions as described previously.[17] The obtained OVA-primed splenocytes were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 5% heat-inactivated fetal bovine serum. In all cases, splenocytes were cultured at 37°C in 5% carbon dioxide.

Measurement of splenocyte viability
The viability of splenocytes was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay described previously.[18] In brief, splenocytes (5×10^6 cells/mL) were seeded into 96-well plates (100 μL/well)

and treated with iron oxide nanoparticles (1–100 µg Fe/mL) and/or vehicle (VH; Roswell Park Memorial Institute medium). Each iron oxide treatment was administered in quadruplicate. The iron oxide concentration range used corresponded with 0.11–11 times the estimated plasma concentration (9 µg/mL) at a dose of 447 µg Fe/kg.[19] For the next 44 hours, the cells were either left unstimulated or were stimulated with OVA (100 µg/mL). An MTT stock solution (5 mg/mL in phosphate buffered saline) was then added to each well (10 µL/well) and incubated for 4 hours. The formed formazan was dissolved with 0.1 N acid-isopropanol (100 µL/well), and optical density was measured at 570 nm, and at 630 nm as a background reference, using a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA).

Cytokine measurement

Splenocytes (5×10^6 cells/mL) were cultured in 48-well plates (250 µL/well) and treated with iron oxide nanoparticles (1–100 µg Fe/mL) and/or VH, followed by stimulation with OVA (100 µg/mL) for 48 hours. Each iron oxide treatment was administered in triplicate. The supernatants were harvested and quantified for IL-2, IL-4, and IFN-γ by enzyme-linked immunosorbent assay. The level of cytokines in the VH group was designated as 100%, and the percentage of inhibition induced by treatment of iron oxide nanoparticles was calculated according to the following formula:

% of inhibition

$$= ([\text{Cytokine level}_{VH} - \text{Cytokine level}_{\text{Iron oxide nanoparticles}}] / \text{Cytokine level}_{VH}) \times 100\%$$

Flow cytometric analysis of intracellular glutathione

Splenocytes (5×10^6 cells/mL) were seeded into 48-well plates (250 µL/well) and treated with iron oxide nanoparticles (1–100 µg Fe/mL) and/or VH, followed by stimulation with OVA (100 µg/mL) for 6 hours. Each iron oxide treatment was administered in triplicate. The cells were incubated with monochlorobimane (20 µM) for 20 minutes. The single cell fluorescence for each sample was measured at emission of 525 nm and excitation of 355 nm using a flow cytometer (BD LSRFortessa cell analyzer; BD Biosciences – Immunocytometry Systems, Becton, Dickinson and Company, San Jose, CA). The data were analyzed using the software Flowjo 5.7 (Tree Star, Inc, Ashland, OR).

ROS measurement

Splenocytes (5×10^6 cells/mL) were preincubated with 20 µM dichlorofluorescin diacetate for 30 minutes at 37°C. The splenocytes preloaded with dichlorofluorescin diacetate were cultured in a 96-well opaque plate (100 µL/well) and treated with iron oxide nanoparticles (1–100 µg Fe/mL) and/or VH, followed by stimulation with OVA (100 µg/mL) for 6 hours. Each iron oxide treatment was administered in triplicate. Each iron oxide treatment was administered in triplicate. To measure the levels of intracellular ROS, the cells were lysed with dimethyl sulfoxide (10 µL/well) and measured at excitation of 488 nm and emission of 525 nm using a microplate reader.

Statistical analysis

The mean ± standard error was determined for each treatment group in the individual experiments. Normality and homoscedasticity of data were tested by the Shapiro–Wilk test. Homogeneous data were then evaluated by a parametric analysis of variance, and Dunnett's two-tailed *t*-test was used to compare the results for the treatment groups with those of the control group. For experiments with *N*-acetyl-*L*-cysteine pretreatment (NAC), the data were evaluated by two-way analysis of variance and Duncan's multiple range test, using the Statistical Analysis System (v 9.1; SAS Institute Inc, Cary, NC). Statistical significance was defined as a *P* value of less than 0.05.

Results
No effect of iron oxide nanoparticles on splenocyte viability

Using an MTT assay, it was examined whether iron oxide nanoparticles caused cytotoxicity. OVA-primed splenocytes were exposed to iron oxide nanoparticles (1–100 µg Fe/mL) and/or VH, followed by stimulation with OVA (100 µg/mL). For nonstimulated controls, the splenocytes were exposed to iron oxide nanoparticles but did not receive OVA stimulation. The results showed that regardless of whether cells were or were not stimulated by OVA, the iron oxide nanoparticles did not influence the viability of splenocytes (Figure 1).

Differential effects of iron oxide nanoparticles on the expression of antigen-specific cytokines

The effects of iron oxide nanoparticles on the expression of three cytokines predominantly expressed by T cells, namely IL-2, IFN-γ, and IL-4, were examined. The production of cytokines by unstimulated splenocytes was very low, whereas OVA stimulation strongly induced the expression of the three cytokines (Figure 2A–C, naïve versus VH). The presence of iron oxide nanoparticles (10–100 µg Fe/mL) did not influence the production of IL-2 and IL-4, whereas IFN-γ was
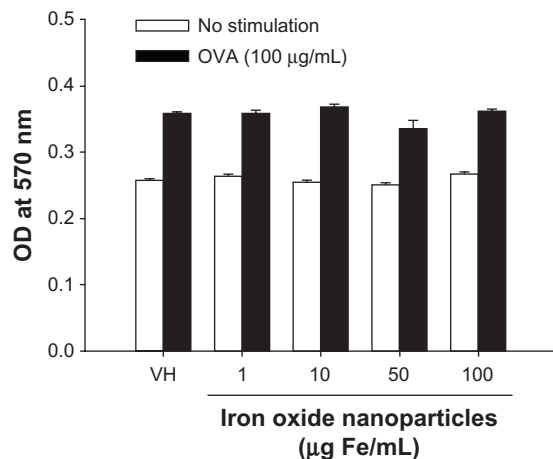
Figure 1 No effect of iron oxide nanoparticles on viability of splenocytes. Splenocytes (5×10^6 cells/mL) were treated with iron oxide nanoparticles (1–100 μg iron [Fe]/mL) and/or vehicle (VH; Roswell Park Memorial Institute medium) and then either left unstimulated or stimulated with ovalbumin (OVA; 100 μg/mL) for 44 hours. The viability of splenocytes was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay.
Notes: Data are expressed as the mean ± standard error of quadruplicate cultures. Results are representative of three independent experiments.
Abbreviation: OD, optical density.

markedly suppressed in a concentration-dependent manner (Figure 2A–C).

Previous reports have indicated that iron oxide nanoparticles cause oxidative stress in macrophages.[10,13] Therefore the potential role of oxidative stress as a possible mechanism for the effect of iron oxide nanoparticles on IFN-γ was investigated. For this purpose we used NAC, a thiol antioxidant as well as a precursor of glutathione. The presence of NAC (1 mM) markedly attenuated iron oxide nanoparticle-mediated inhibition of IFN-γ production (Figure 2D). In the absence of NAC, the magnitude of inhibition induced by 10, 50, and 100 μg Fe/mL of iron oxide nanoparticles on IFN-γ was 45.1%, 86.1%, and 95.4%, respectively (Figure 2C). In the presence of NAC, these percentages were attenuated to 18.3%, 39.8%, and 87.8%, respectively (Figure 2D).

Attenuation of iron oxide nanoparticle-mediated inhibition of IFN-γ by thiol, but not nonthiol, antioxidants

In addition to NAC, several thiol and nonthiol antioxidants were employed to further address the involvement of oxidative stress. Both NAC and exogenous glutathione (1–4 mM of each) were found to significantly attenuate iron oxide nanoparticle (50 μg Fe/mL)-mediated suppression of IFN-γ in a concentration-dependent manner (Table 1). In contrast, the nonthiol antioxidants pyruvate (1–4 mM), dimethylthiourea (4 mM), and tiron (100 μM) did not reverse the effects of iron oxide nanoparticles (Table 1).

Diminishment of intracellular glutathione by iron oxide nanoparticles

Based on the results showing the effectiveness of thiol antioxidants, the influence of iron oxide nanoparticles on intracellular levels of glutathione in splenocytes was examined. Exposure of splenocytes to iron oxide nanoparticles (50 μg Fe/mL) markedly decreased the monochlorobimane fluorescence with a peak response at 6 hours postexposure, indicating a diminished level of intracellular glutathione (Figure 3A). The effect of iron oxide nanoparticles (1–100 μg Fe/mL) on glutathione diminishment was concentration-dependent, and was significantly reversed by the presence of NAC (1 mM) (Figure 3B). In addition to glutathione, the level of intracellular ROS was also measured in splenocytes exposed to iron oxide nanoparticles. At the time point (6 hours) showing peak glutathione diminishment, no significant changes in ROS levels were detected in splenocytes exposed to iron oxide nanoparticles up to 100 μg Fe/mL (Figure 4).

Discussion

Although iron oxide nanoparticles have been shown to affect the functionality and apoptosis of macrophages,[8–12] evidence pertaining to their effects on other immune cells is limited. It was previously reported that systemic exposure of OVA-sensitized mice to iron oxide nanoparticles attenuated the production of antigen-specific antibodies and T cell cytokines.[17] In the present study, it was further investigated whether iron oxide nanoparticles induced a direct effect on T cells in culture. The data demonstrated that direct exposure of OVA-primed splenocytes to iron oxide nanoparticles in culture resulted in a marked suppression of IFN-γ expression and a decrease in intracellular glutathione levels. These results provide evidence that iron oxide nanoparticles produce a direct effect on antigen-specific T cell responses.

In contrast to the suppressive effect on IFN-γ, iron oxide nanoparticles did not influence OVA-induced production of IL-2 and IL-4. Previous research has established that IFN-γ and IL-4 are signature cytokines expressed by T helper (Th)1 and Th2 cells, respectively.[20] The current findings suggested a differential sensitivity between Th1 and Th2 cells, with Th1 cells being a more sensitive target to the nanoparticles. This notion is in line with a recent report showing that antigen-specific IgG_{2a} and IFN-γ were slightly more sensitive than IgG_1 and IL-4 regarding suppression by iron oxide nanoparticles in vivo.[17] On the basis of these results, it was speculated that exposure to iron oxide nanoparticles may
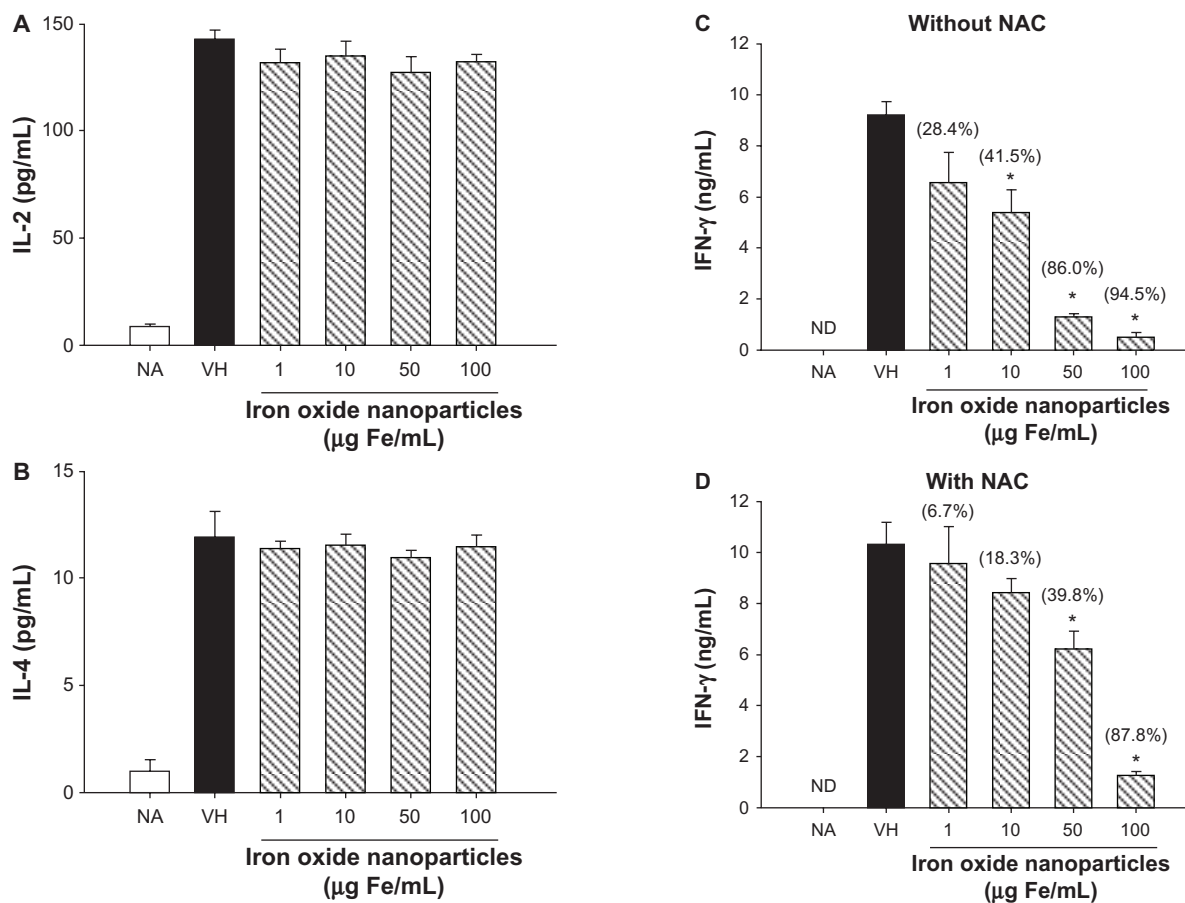
Figure 2 Differential effects of iron oxide nanoparticles on the production of antigen-specific interleukin (IL)-2, IL-4, and interferon (IFN)-γ by splenocytes. (**A–C**) Splenocytes (5×10^6 cells/mL) were either left untreated (naïve; NA) or treated with iron oxide nanoparticles (1–100 μg iron [Fe]/mL) and/or vehicle (VH) followed by stimulation with ovalbumin (100 μg/mL) for 48 hours. (**D**) Splenocytes were pretreated with *N*-acetyl-*L*-cysteine (NAC; 1 mM) prior to the treatment of iron oxide nanoparticles and ovalbumin stimulation described above. The levels of (**A**) IL-2, (**B**) IL-4, and (**C** and **D**) IFN-γ in the supernatants were measured by enzyme-linked immunosorbent assay.
Notes: Data are expressed as the mean ± standard error of triplicate cultures. The level of cytokines in the VH group was designated as 100%, and the percentage of inhibition induced by iron oxide nanoparticles (10–100 μg Fe/mL) was calculated against this standard (as indicated in parentheses). *$P < 0.05$, comparison with VH group. Results are representative of three independent experiments.
Abbreviation: ND, no data.

switch the Th1/Th2 immunobalance toward Th2-dominant immunity in response to antigen stimulation. The potential effect of iron oxide nanoparticles on immune responses mediated by Th1 and Th2 cells warrants further investigations using appropriate models.

These data on antigen-specific T cell responses contradict the findings of a previous study, which reported that a single intravenous administration of iron oxide nanoparticles to normal nonsensitized mice elevated the serum level of IFN-γ.[15] Although the mechanism responsible for these contradictory results remains to be elucidated, apparently a crucial factor dictating the effect of iron oxide nanoparticles on IFN-γ production may be the absence or presence of antigen sensitization. In the authors' studies, IFN-γ production is antigen-specific. In contrast, the elevated IFN-γ in nonsensitized mice has been interpreted as a possible proinflammatory status induced by iron oxide nanoparticles.[15]

Iron oxide nanoparticles reportedly cause cytotoxicity in macrophages.[13] In the present study, the cell viability of splenocytes was monitored using an MTT assay, which showed comparable viability between nanoparticle-treated and VH-treated groups. Hence, the effect of iron oxide nanoparticles on cytokine expression evidently should not be attributed to a general cytotoxic mechanism.

The authors' mechanistic studies revealed that both the IFN-γ suppression and the glutathione diminishment induced by iron oxide nanoparticles were significantly reversed by NAC, a glutathione precursor. Glutathione plays a critical role in maintaining the cellular homeostasis of redox balance. Both NAC and glutathione can function as ROS scavengers.[21] Notably, iron oxide nanoparticles have been reported to cause oxidative stress in several cells, including those of immune origin.[10,13] It was therefore also evaluated whether ROS were induced in the present study's experimental conditions.

Table 1 Effects of thiol and nonthiol antioxidants on iron oxide nanoparticle-mediated suppression of interferon-γ production by splenocytes

Antioxidants	Iron oxide nanoparticle-mediated inhibition of interferon-γ production (%)[a]
Control	85.8 ± 2.6
N-acetyl-L-cysteine (mM)	
1	39.3 ± 8.9*
2	35.6 ± 6.4*
4	27.9 ± 4.8*
Glutathione (mM)	
1	41.5 ± 6.2*
2	36.1 ± 8.5*
4	29.7 ± 4.1*
Pyruvate (mM)	
1	82.3 ± 6.9
4	84.3 ± 3.1
Dimethylthiourea (mM)	
4	89.7 ± 1.0
Tiron (mM)	
0.1	86.6 ± 1.2

Notes: [a]Splenocytes (5×10^6 cells/mL) were pretreated with iron oxide nanoparticles (50 μg iron/mL) in the absence (control) or presence of N-acetyl-L-cysteine, glutathione, pyruvate, dimethylthiourea or tiron, followed by stimulation with ovalbumin (100 μg/mL) for 48 hours. The levels of interferon-γ in the supernatants were measured by enzyme-linked immunosorbent assay. The percentage of inhibition induced by iron oxide nanoparticles was calculated as described in Materials and methods. Data are expressed as the mean ± standard error of six samples pooled from two experiments; *$P < 0.001$ compared to the control.

Figure 3 Diminishment of intracellular glutathione in the presence of iron oxide nanoparticles in splenocytes. (**A**) Splenocytes (5×10^6 cells/mL) were treated with iron oxide nanoparticles (50 μg iron [Fe]/mL) and/or vehicle (VH) followed by stimulation with ovalbumin (100 μg/mL) for 1–12 hours. (**B**) Splenocytes were treated with iron oxide nanoparticles (1–100 μg Fe/mL) and/or VH, followed by stimulation with ovalbumin (100 μg/mL) for 6 hours in the absence or presence of N-acetyl-L-cysteine (NAC; 1 mM). The levels of intracellular glutathione were measured as monochlorobimane (MCB) fluorescence by flow cytometry.
Notes: Data are expressed as the mean ± standard error of triplicate cultures. *$P < 0.05$, comparison with matched control group without nanoparticle treatment. #$P < 0.05$, comparison with matched nonNAC group. Results are representative of three independent experiments.

However, all three nonthiol antioxidants which were used, namely pyruvate, dimethylthiourea, and tiron, failed to counteract the effects of iron oxide nanoparticles. Moreover, iron oxide nanoparticles did not alter the intracellular level of ROS in splenocytes restimulated with OVA, as measured by dichlorofluorescin fluorescence. Overall, these results provided evidence to differentiate the respective contributions of glutathione and ROS in iron oxide nanoparticle-mediated effects, with glutathione rather than ROS playing a central role.

Similar findings were reported from a previous study on human cardiac endothelium, in which ROS was not found to play a positive mediating role. In that study, iron oxide nanoparticles were found not to induce cytotoxicity and ROS production in human cardiac endothelial cells.[22] Another recent report showed that titanium dioxide nanoparticles induced ROS production in both *Escherichia coli* MG1655 and *Cupriavidus metallidurans* CH34, but caused cytotoxicity only in *E. coli* MG1655.[23] Overall, these results suggest that the induction of ROS production and the contribution of ROS in metal oxide nanoparticle-mediated cytotoxic effects vary across different cells.

By contrast, previous research has documented that glutathione plays a pivotal role in various T cell functions,
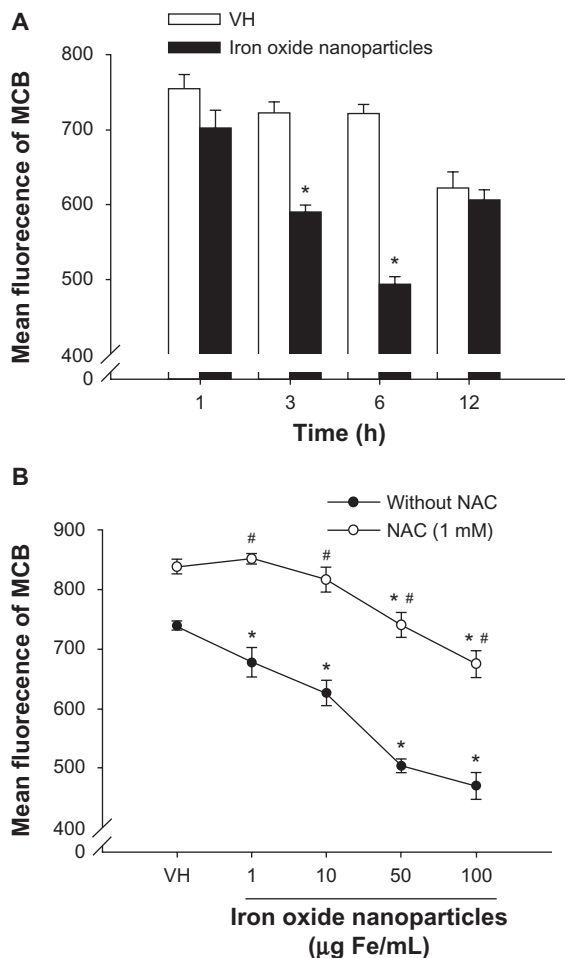
such as the regulation of cytokine expression.[24,25] Glutathione is considered the hallmark redox buffer in living cellular systems. Depletion of cellular glutathione in mice fed with a liquid control diet containing 30% ethanol-derived calories was found to downregulate the production of antigen-specific IFN-γ when also upregulating IL-4 production, but did not affect the level of IL-2.[24] In addition, depletion of intracellular glutathione by buthionine sulfoximine differentially influenced the functionality of Th1/Th2 cell clones. Specifically, IL-2-induced DNA synthesis was attenuated in the IFN-γ producing Th cell clone 29, whereas DNA synthesis in the IL-4 producing Th cell clone D10. G4.1.HD was not affected.[26] The current findings were
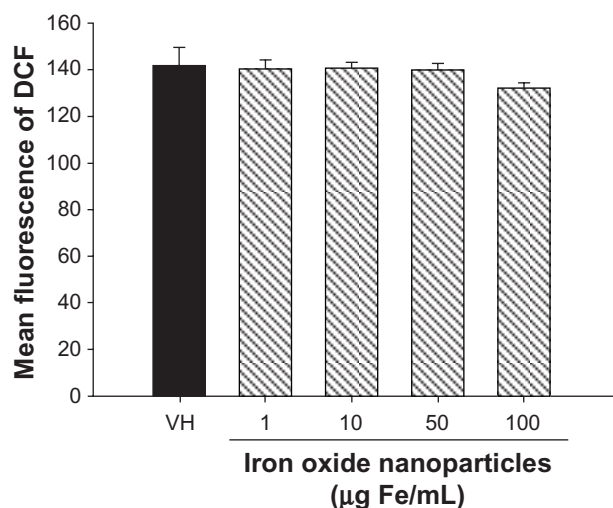
Figure 4 No effect of iron oxide nanoparticles on the intracellular levels of reactive oxygen species. Splenocytes (5×10^6 cells/mL) preloaded with dichlorofluorescin (DCF) diacetate (20 µM) were treated with iron oxide nanoparticles (1–100 µg iron [Fe]/mL) and/or vehicle (VH), followed by stimulation with ovalbumin (100 µg/mL) for 6 hours. The levels of intracellular reactive oxygen species were measured as DCF fluorescence using a microplate reader.
Notes: Data are expressed as the mean ± standard error of triplicate cultures. Results are representative of three independent experiments.

congruent with these reports and showed that iron oxide nanoparticles diminished the intracellular glutathione and suppressed the production of IFN-γ, whereas IL-2 and IL-4 were unaffected.

Conclusion

The present study demonstrated that direct exposure of antigen-primed splenocytes to iron oxide nanoparticles resulted in a marked suppression of the expression of antigen-specific IFN-γ, accompanied by a decrease in intracellular glutathione. Both the IFN-γ suppression and glutathione diminishment were reversed by the thiol antioxidant NAC, but not by nonthiol antioxidants. These findings constitute the first reported evidence of the critical role played by glutathione in the immunosuppressive effect of iron oxide nanoparticles on T cells. Accumulating evidence suggests that iron oxide nanoparticles may drive macrophages toward proinflammatory responses and affect T cell reactivity.[8–10,15–17] Thus, it would be prudent to further investigate the immunopharmacology and immunotoxicology of these nanoparticles, and to consider their possible biomedical application in vivo.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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