



Iron oxide nanoparticles attenuate T helper 17 cell responses in vitro and in vivo

Yai-Ping Hsiao^a, Chien-Chang Shen^a, Chung-Hsiung Huang^{a,b}, Yu-Chin Lin^{c,*}, Tong-Rong Jan^{a,**}

^a Department and Graduate Institute of Veterinary Medicine, School of Veterinary Medicine, National Taiwan University, Taipei, Taiwan

^b National Institute of Infectious Diseases and Vaccinology, National Health Research Institutes, Miaoli, Taiwan

^c Department of Medicinal Botanicals and Health Applications, College of Biotechnology & Bioresources, Da-Yeh University, No.168, University Rd., Dacun, Changhua, Taiwan

ARTICLE INFO

Keywords:

Delayed-type hypersensitivity
Iron oxide nanoparticle
Ovalbumin
Splenocytes
Th17

ABSTRACT

Iron oxide nanoparticles (IONPs) have been shown to attenuate T helper (Th)1 and Th2 cell-mediated immunity in ovalbumin (OVA)-sensitized mice. The objective of this study is to investigate the effects of IONPs on the immune responses of Th17 cells, a subset of T cells involved in various inflammatory pathologies. For in vivo study, a murine model of delayed-type hypersensitivity (DTH) was employed. BALB/c mice received a single dose of IONPs (0.2–10 mg iron/kg) via the tail vein 1 h prior to ovalbumin (OVA) sensitization. Their footpads were subcutaneously challenged with OVA to induce DTH reactions. The expression of Th17 cell-related molecules in inflamed footpads were examined by immunohistochemistry. For in vitro study, OVA-primed splenocytes were directly exposed to IONPs (1–100 µg iron/mL), and then re-stimulated with OVA in culture. The expression of Th17 cell-related molecules were measured by reverse transcription-polymerase chain reaction and enzyme-linked immunosorbent assay. IONP administration attenuated the number of interleukin (IL)-6, IL-17, the transcription factor ROR-γ, and chemokine receptor 6 positive cells in OVA-challenged footpads, whereas the number of transforming growth factor-β, IL-23 and chemokine (C-C motif) ligand 20 positive cells was not altered. Direct exposure of OVA-primed splenocytes to IONPs suppressed the production of IL-6 and IL-17, and the mRNA expression of IL-17 and ROR-γt. These data indicate that exposure to IONPs attenuates Th17 cell responses in vivo and in vitro.

1. Introduction

Iron oxide nanoparticles (IONPs) have been exploited for potential applications in various biomedical fields, including cell labeling and transfection, contrast enhancement of magnetic resonance imaging (MRI), delivery of drugs and genes, thermal treatment, etc. [1–7]. In particular, superparamagnetic IONPs have been used in clinical as MRI contrasting agents for detection of liver tumors [7–10]. For this application IONPs are administered intravenously, resulting in a rapid uptake of the nanoparticles by the reticuloendothelial system [7,11]. Hence, the liver, spleen and other immune organs are major sites of particle deposition [12], raising concerns about the potential impacts on the immune system by medicinal preparations of IONPs.

In addition to macrophages whose functionality has been well studied to be modulated by IONPs [13–17], accumulating evidence indicates that T cells are affected by the nanoparticles. Exposure of

normal, non-sensitized mice to IONPs via intravenous or oral administrations affected the cellularity of T cells in the peripheral blood and spleen [18,19]. Systemic exposure to IONPs also increased the production of T cell-derived cytokines, including interleukin (IL)-2, IL-10, and interferon (IFN)-γ in the blood [18]. A single intravenous administration with IONPs attenuated antigen-specific immune responses, including antibody production and T cell functionality, in ovalbumin (OVA)-sensitized mice [20]. Mechanistic investigation revealed that exposure of OVA-primed splenocytes to IONPs in culture suppressed cytokine production induced by OVA re-stimulation [21], indicating that IONPs directly influenced the functionality of splenic T cells. Furthermore, both T helper (Th)1 and Th2 cell-mediated immune responses were suppressed by IONPs in OVA-sensitized murine models [22,23]. Taken together, these results indicate that T cells are a sensitive target in the immune system to IONPs.

Th17 cells, a subset of Th cells expressing high levels of IL-17, are

* Correspondence to: Y.-C. Lin, No.168, University Rd., Dacun, Changhua 51591, Taiwan.

** Correspondence to: T.-R. Jan, Department and Graduate Institute of Veterinary Medicine, School of Veterinary Medicine, National Taiwan University, No.1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan.

E-mail addresses: nubirth@mail.dyu.edu.tw (Y.-C. Lin), tonyjan@ntu.edu.tw (T.-R. Jan).

<https://doi.org/10.1016/j.intimp.2018.03.007>

Received 21 October 2017; Received in revised form 15 February 2018; Accepted 7 March 2018

Available online 14 March 2018

1567-5769/ © 2018 Published by Elsevier B.V.

known to play a crucial role in the pathophysiology of various disease conditions, including collagen-induced arthritis, contact dermatitis, dermal inflammation and inflammatory bowel disease [24–30]. IL-17 possesses strong proinflammatory properties and acts on various immune cells resulting in the production of the pro-inflammatory cytokines IL-6 and tumor necrosis factor (TNF)- α , and the chemokines CCL2, CCL3 and IL-8, thereby promoting leukocyte infiltration and tissue inflammation [31–33]. The development of Th17 cells from naïve T cells requires the cooperative effect of IL-6 and transforming growth factor (TGF)- β , which up-regulates the expression of RAR-related orphan receptor gamma (ROR- γ), a characteristic transcription factor dictating the development of Th17 cells [34–38]. In addition to IL-6 and TGF- β , IL-23 is another critical cytokine for the development and maintenance of Th17 cells, as it stabilizes and promotes the proliferation of Th17 cells [39,40].

Previous murine studies showed that systemic exposure to nano-fullerene attenuated delayed-type hypersensitivity (DTH) reactions associated with a reduced production of IL-6 and IL-17 [41]. In contrast, respiratory exposure to diesel exhaust particles aggravated pathological symptoms of DTH by enhancing the expression of several pro-inflammatory cytokines, such as IL-17 [42]. These results demonstrated that the functionality of Th17 cells associated with DTH is modulated by non-metal nanomaterials. In the present study, a murine model of DTH induced by ovalbumin (OVA) was employed to address whether Th17 immune responses were influenced by IONPs *in vivo*. Direct effects of IONPs on Th17 cells were also investigated using OVA-primed splenocytes in culture.

2. Materials and methods

2.1. Reagents and chemicals

Resovist®, a commercial medicinal preparation containing 28 mg iron/mL of IONPs, was obtained from Bayer Schering Pharma AG, (Berlin, Germany). The particle size distribution of Resovist® has been confirmed to be a monodisperse population with an average diameter of 58.7 nm [21]. The core of Resovist is comprised of Fe₃O₄ (magnetite) and γ -Fe₂O₃ (maghemite), which is coated with carboxydextran [7]. Reagents used for enzyme-linked immunosorbent assay (ELISA) were purchased from R&D Systems (Minneapolis, MN, USA). All reagents used for reverse transcription-polymerase chain reaction (RT-PCR) were obtained from Promega (Madison, WI, USA). Fetal bovine serum (FBS) and cell culture supplies were obtained from Hyclone (Logan, UT, USA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. *In vivo* experiments

2.2.1. Protocol of delayed-type hypersensitivity (DTH)

Male BALB/c mice at age of 5–6 weeks old were supplied by BioLasco (Ilan, Taiwan). Mice were randomized and housed in plastic cages containing a saw-dust bedding (5 mice per cage) and allowed to acclimatize to new housing conditions for a week prior to experimentation. The animals had free access to standard laboratory food and water. The sensitization and challenge protocol of DTH model has been described previously [22]. The mice were randomly divided into the following groups (5 mice per group), including non-sensitized (NS), ovalbumin-sensitized (OVA), vehicle-treated and OVA-sensitized (VH), and IONP-treated and OVA-sensitized (IONP) groups. The mice were either left non-sensitized (NS) or sensitized with ovalbumin (OVA) by subcutaneous injection using a sensitization solution containing 100 μ g OVA and 1 mg alum in 0.25 mL saline on day 0. The OVA-sensitized mice were intravenously administered with a single dose of IONPs (0.2–10 mg iron/kg; 0.2 mL/mouse) and/or vehicle (VH; saline) 1 h prior to OVA sensitization. To induce DTH reactions, the hind footpad of each mouse received a subcutaneous challenge of OVA (10 μ g in

20 μ L saline) on day 7. The footpads of mice were harvested on day 8. Tissue blocks of footpads were fixed in 10% neutral buffered formalin for 24 h, decalcified in a rapid decalcifier-solution (Merck, Darmstadt, Germany) for 48 h, embedded in paraffin, and then cut into 3–4 μ m sections for IHC staining. The protocol of animal experiments was approved by the Institutional Animal Care and Use Committee of the National Taiwan University (NTU-103-EL-00105).

2.2.2. Immunohistochemical (IHC) staining

Tissue sections of footpads were deparaffinized and rehydrated according to standard procedures. Antigen retrieval of the rehydrated slides was performed using Trilogy™ (Cell Marque, Hot Springs, AR, USA) following the supplier's instructions. The slides were immersed in 3% H₂O₂ in methanol to quench the activity of endogenous peroxidase and then blocked with horse serum. The slides were incubated with anti-mouse CCL-20, CCR6, IL-6, IL-17, IL-23, ROR- γ or TGF- β antibody (Abcam, MA) at 37 °C for 1–2 h. After washing, super enhancer was applied onto the slides and incubated for 1 h. The slides were treated with a poly-horseradish peroxidase (HRP) reagent and incubated for another 1 h. The slides were incubated with the HRP substrate 3-amino-9-ethylcarbazole (AEC) or 3,3'-diaminobenzidine (DAB) for 2 min and then subjected to hematoxylin counter staining (blue color) for visualization. Positive signals with dark-red (AEC) or brown (DAB) color were counted manually.

Tissue sections were also double stained for CCR6 and IL-17. Anti-mouse IL-17 antibody was applied on slides and incubated at 37 °C for 2 h, and then a secondary antibody conjugated with alkaline phosphatase (AP) was applied for 1 h. Signals of the first staining (black color) was detected using the AP substrate 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium. For the second staining, anti-CCR6 antibody was applied on the slides for 2 h, followed sequentially by super enhancer solution and poly-HRP 1 h per each step. The slides were then incubated with the AEC solution for visualizing the CCR6 staining without counter staining. Double positive signals with black and dark-red colors were enumerated manually.

Images of IHC staining displaying clear signals were used for quantitation of IHC results, three measurements per footpad and 6–8 footpads at 200-fold magnification per group were counted manually in a blinded fashion.

2.3. *In vitro* experiments

2.3.1. Preparation of ovalbumin (OVA)-primed splenocytes

OVA-primed splenocytes were prepared from BALB/c mice sensitized with OVA as described previously [21]. In brief, BALB/c mice were sensitized with OVA absorbed to alum (100 μ g OVA + 1 mg alum in 0.25 mL saline) by subcutaneous injection on days 0 and 14. The mice were sacrificed on day 15, and their spleens were harvested and made into single cell suspensions. The splenocytes were cultured in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, and 5% heat-inactivated FBS at 37 °C in 5% CO₂.

2.3.2. Measurement of cytokines by ELISA

OVA-primed splenocytes were seeded into 48-well plates (5 $\times$ 10⁶ cells/mL; 250 μ L/well). The cells were either left untreated (naïve; NA) or treated in triplicate with IONPs (1–100 μ g iron/mL) and/or VH followed by stimulation with OVA (100 μ g/mL) for 48 h. The level of IL-6 and IL-17 in the supernatants was measured by ELISA following the supplier's instructions. Three independent experiments were performed.

2.3.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Splenocytes (5 $\times$ 10⁶ cells/mL) were seeded into culture dishes (100 $\times$ 10 mm, 10 mL/dish) and received OVA stimulation and IONP treatment as described above. Three independent experiments were performed. The cells were then collected and TRI reagent (Sigma-Aldrich) was used for the extraction of total RNA. The expression level

of β -actin, IL-17 and ROR- γ t mRNA was measured by standard RT-PCR. PCR products were electrophoresed and visualized with ethidium bromide staining. Semi-quantification of the PCR products was performed using the ImageQuant 5.0 Analysis Software (Molecular Dynamics). The expression level of β -actin was used as an internal control. No DNA contamination for all extracted samples was confirmed by the absence of PCR products from a side-by-side reaction without RT. The primers used are as follows: 5'-GGTCAACCTCAAAGTCTTTAACTC-3' and 5'-TTAAAAATGCAAGTAAGTTTGCTG-3' for IL-17, 5'-ACAGCCACTGCATTCCAGTTT-3' and 5'-TCTCGGAAGGACTTGCAGACAT-3' for ROR- γ t, and 5'-AGGGAAATCGTGCCTGACATAAAA-3' and 5'-ACTCATCGTATCTCTGCTTGCTGA-3' for β -actin.

2.4. Statistical analysis

Data are expressed as the mean $\pm$ standard error (SEM) for each treatment group. Homogeneous data were evaluated by a parametric analysis of variance (ANOVA), and Dunnett's two-tailed *t*-test was employed to assess the statistical difference between IONP-treated groups and the VH group. *P* value < 0.05 was considered as statistical significance.

3. Results

3.1. IONPs attenuated Th17 immune responses in vivo

Previous results showed that IONPs at the dose range used for diagnostic imaging suppressed DTH reactions in ovalbumin (OVA)-sensitized mice [20,22]. In the present study, we firstly examined the expression of IL-17, the major Th17 cell-derived cytokine, in the footpads of DTH mice using immunohistochemical (IHC) staining. IL-17 signals were primarily detected surrounding the vasculature in the dermis layer. The number of IL-17⁺ cells in the OVA group was significantly increased, as compared to the non-sensitized (NS) control, demonstrating an enhanced Th17 immune responses in the inflamed footpads of DTH mice (Table 1). These results are in parallel with previous results showing a dampened DTH reactions in IL-17-deficient mice [47]. Notably, a single dose (0.6 and 10 mg iron/kg) of IONPs by intravenous injection prior to OVA sensitization significantly reduced the number of IL-17⁺ cells in the footpads of DTH mice, as compared to the VH control (Fig. 1A and Table 1). We further examined the expression of the orphan nuclear receptor ROR- γ , a key transcription factor dictating the development of Th17 lineage, in the inflamed footpads. In parallel with the effect on IL-17, IONPs at the same dosage (0.6 and 10 mg iron/kg) markedly reduced the number of ROR- γ ⁺ cells in the footpads (Fig. 1B and Table 1). The development of Th17 cells requires signals mediated by several cytokines, such as coordinative effects produced by

IL-6 and TGF- β [34–36]. Moreover, signals from IL-23 are crucial for maintaining Th17 effector functions and stabilizing the phenotype of Th17 cells [39,40]. We therefore examined the expression of these cytokines in the inflamed footpads. The results showed that IONPs (0.6 and 10 mg iron/kg) significantly suppressed the number of IL-6⁺ cells (Fig. 1B and Table 1), but not TGF- β ⁺ and IL-23 (Table 1). These results demonstrated that the IONP administration attenuated Th17 cell responses associated with DTH.

3.2. IONPs attenuated the infiltration of CCR6⁺ Th17 cells in inflamed footpads

Chemokine networks are involved in the regulation of leukocyte trafficking and inflammatory responses. Previous studies reported the presence of CCR6⁺ Th17 cells in the synovial T cells from individuals with idiopathic arthritis [43]. Hence, the expression of CCR6 and its ligand CCL20 in the inflamed footpads of DTH mice was examined. The results showed that the signal of CCR6 was significantly attenuated by IONPs (0.6 and 10 mg iron/kg; Fig. 1C and Table 1). The expression of CCL20 was not affected (Table 1). To further address if the reduced expression of CCR6 occurred in Th17 cells, double IHC staining for CCR6 and IL-17 was performed, and the results showed a marked reduction in the number of CCR6⁺IL-17⁺ cells in mice treated with IONPs (0.6 and 10 mg iron/kg; Fig. 1C and Table 1).

3.3. IONPs directly inhibited IL-17 cell responses in vitro

On the basis of the above in vivo results, we sought to investigate whether IONPs produced a direct effect on Th 17 cell responses in vitro. OVA-primed splenocytes were prepared from mice sensitized with OVA. The cells were directly exposed to IONP (10–100 μ g iron/mL), and then stimulated with OVA (100 μ g/mL) to activate T cells. As shown in Fig. 2A, OVA-stimulation markedly increased the levels of IL-17 in the supernatants of splenocyte cultures, as compared to the non-stimulated group (NA), indicating a successful activation of Th17 cells. In line with the in vivo effect on Th17 cell responses, IONPs (10–100 μ g iron/mL) suppressed the production of IL-17 by OVA-primed splenocytes in a concentration-dependent manner (Fig. 2A). Concordantly, IONPs demonstrated a similar inhibitory effect on the production of IL-6 by splenocytes in culture (Fig. 2B). The suppressive effect by IONPs (10–100 μ g iron/mL) on IL-17 was also confirmed at the mRNA level (Fig. 3A & B). In addition, direct exposure to IONPs (10–100 μ g iron/mL) attenuated the mRNA expression of ROR- γ t by the splenocytes (Fig. 3A & C).

Table 1

Immunohistochemical staining of IL-17⁺, IL-6⁺, TGF- β ⁺, ROR- γ ⁺, IL-23⁺, CCR6⁺, CCL-20⁺ and CCR6⁺IL17⁺ cells in the footpads of DTH mice.

	Number of positive cells			Iron oxide nanoparticles (mg Fe/kg)		
	NS	OVA	VH	0.2	0.6	10
IL-17 ⁺	4 $\pm$ 1	17 $\pm$ 2	18 $\pm$ 4	14 $\pm$ 2	9 $\pm$ 2*	7 $\pm$ 1*
IL-6 ⁺	5 $\pm$ 1	17 $\pm$ 3	18 $\pm$ 3	13 $\pm$ 2	6 $\pm$ 2*	7 $\pm$ 1*
TGF- β ⁺	3 $\pm$ 1	6 $\pm$ 1	7 $\pm$ 1	7 $\pm$ 1	5 $\pm$ 1	5 $\pm$ 1
ROR- γ ⁺	4 $\pm$ 1	12 $\pm$ 2	10 $\pm$ 2	13 $\pm$ 3	5 $\pm$ 1*	6 $\pm$ 1*
IL-23 ⁺	8 $\pm$ 1	12 $\pm$ 1	13 $\pm$ 1	11 $\pm$ 2	11 $\pm$ 1	12 $\pm$ 1
CCR6 ⁺	4 $\pm$ 1	8 $\pm$ 1	9 $\pm$ 1	7 $\pm$ 1	5 $\pm$ 1*	5 $\pm$ 1*
CCL20 ⁺	7 $\pm$ 1	13 $\pm$ 2	15 $\pm$ 2	13 $\pm$ 2	13 $\pm$ 2	13 $\pm$ 2
CCR6 ⁺ IL-17 ⁺	3 $\pm$ 1	8 $\pm$ 1	8 $\pm$ 1	6 $\pm$ 1	4 $\pm$ 1*	4 $\pm$ 1*

Staining was performed as described in the Materials and methods. The number of positive cells was counted manually. Data are expressed as the mean $\pm$ SE of 6–8 footpads per group. Three independent experiments were performed and the presented results are a representative.

* *P* < 0.05 compared to the VH group.

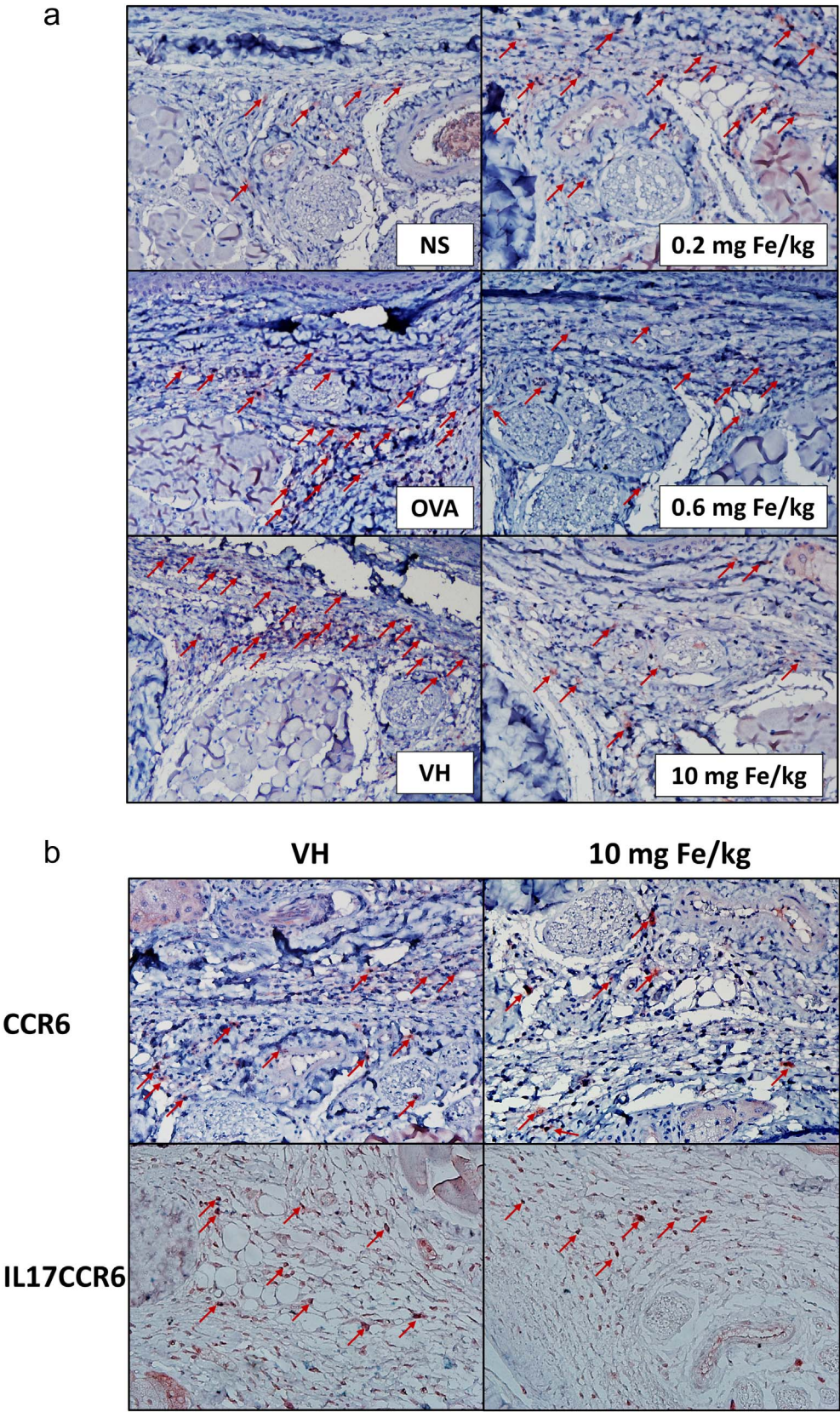


Fig. 1. The effects of IONPs on the expression of Th17 cell-related molecules in vivo. BALB/c mice were treated as described in Materials and methods. DTH reactions in footpads were induced by a subcutaneous OVA challenge. Footpad tissue sections were subjected to IHC staining for CCR6, IL-6, IL-17 and ROR- γ and double staining for IL-17 and CCR6. (A) Representative sections stained for IL-17 are shown (original magnification, $\times 400$). Arrows indicate IL-17⁺ cells with dark-red signals around the blue nuclei. (B) Representative sections stained for IL-6 and ROR- γ are shown (original magnification, $\times 400$). Arrows indicate cells with positive signals. (C) Representative sections stained for CCR6 and for both CCR6 and IL-17 are shown (original magnification, $\times 400$). Arrows indicate CCR6⁺ (dark-red) and IL-17⁺ CCR6⁺ (dark-red and black) cells in the upper and lower panels, respectively. Quantitative data are presented in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

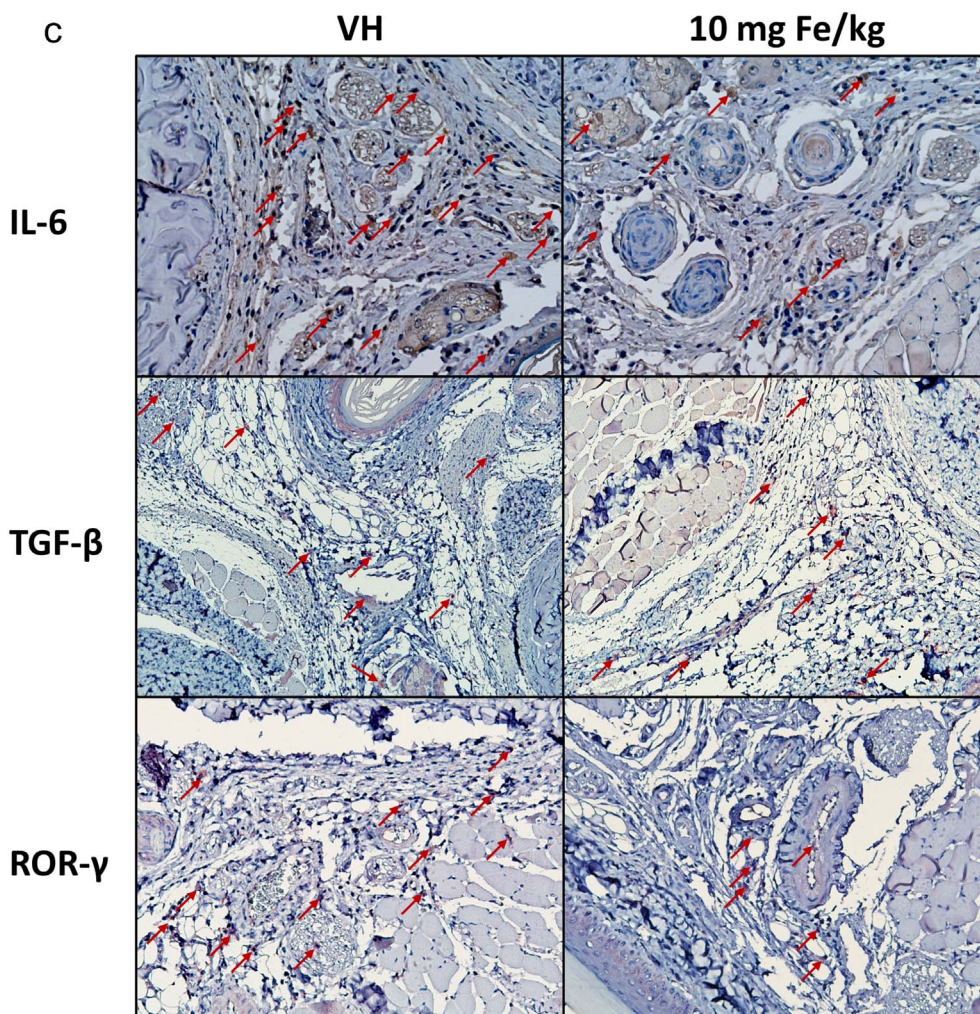


Fig. 1. (continued)

4. Discussion

Previously results showed that systemic exposure to IONPs attenuated DTH reactions by down-regulating immune responses of Th1 cells and macrophages in OVA-sensitized mice [22]. Interestingly, nano-fullerene also demonstrated a similar suppressive effect on DTH [41]. However, nano-fullerene did not affect the expression of IFN- γ , a Th1 signature cytokine. Instead, nano-fullerene attenuated the activation of Th17 cells associated with DTH, as evidenced by a reduced IL-17 expression in mice treated with nano-fullerene. To date, it remains mostly unknown if IONPs influence Th17 immune responses. Results of the present study showed that a single intravenous administration with IONPs (0.6 and 10 mg iron/kg) markedly reduced the number of infiltrated IL-17⁺ cells in the inflamed footpads. Although previous results on IFN- γ are inconsistent between nano-fullerene and IONPs, Th17 cells are affected by both nanomaterials. As Th17 cells play a critical role in the immunobiology of DTH [47] and many other inflammatory conditions [44–46], systemic exposure to nanomaterials may potentially impact on the pathophysiology of inflammatory diseases involving Th17 cells.

In addition to the *in vivo* results, we further investigated whether IONPs induced direct effects on Th17 cells. Results from cell culture experiments revealed that exposure of OVA-primed splenocytes to IONPs resulted in a reduced expression of the Th17 signature cytokine and transcription factor, namely IL-17 and ROR- γ t, respectively. These results demonstrated a direct suppressive effects by IONPs on Th17 cell responses.

The retinoid-related orphan receptor ROR- γ t is a key transcription factor dictating the development of Th17 lineage and [37,38]. The present study showed that administration of IONPs reduced the number of ROR- γ ⁺ cells in the footpads of DTH mice and direct exposure of OVA-primed splenocytes to IONPs *in vitro* inhibited the expression of ROR- γ t mRNA, suggesting a potential impact by IONPs on the development of Th17 cells. The development of the Th17 lineage also requires the cooperative effect mediated by IL-6 and TGF- β [34–36]. Notably, the production of IL-6 by OVA-primed splenocytes was attenuated by IONP exposure. In addition, the number of IL-6⁺ cells, but not TGF- β ⁺, was reduced by IONPs in the footpads of DTH mice, suggesting that IL-6, instead of TGF- β , played a more predominant role in the underlying immunological mechanisms for IONP-mediated suppression of the Th17 immune responses associated with DTH.

The CCR6/CCL20 chemokine axis is involved in the immunobiology of various inflammatory disorders [43,48–51]. CCR6 is expressed by several populations of T cells, such as Th17 cells [43,49]. In the present study, we investigated whether the CCR6/CCL20 pathway was modulated by IONPs. The signal of both CCR6 and CCL20 was elevated in the footpads of DTH mice, demonstrating that the CCR6/CCL20 axis may contribute to the DTH reactions. The signal of CCR6 in the footpads of DTH mice was attenuated in IONP-treated mice, whereas CCL20 was unaltered. We further addressed whether the expression of CCR6 by Th17 cells was affected by IONPs, results from IHC double staining demonstrated that IONPs reduced the number of IL-17⁺ cells capable of expressing CCR6. These results are in line with the observed suppressive effects by IONPs on the infiltration of IL-17⁺ cells. It is suggested

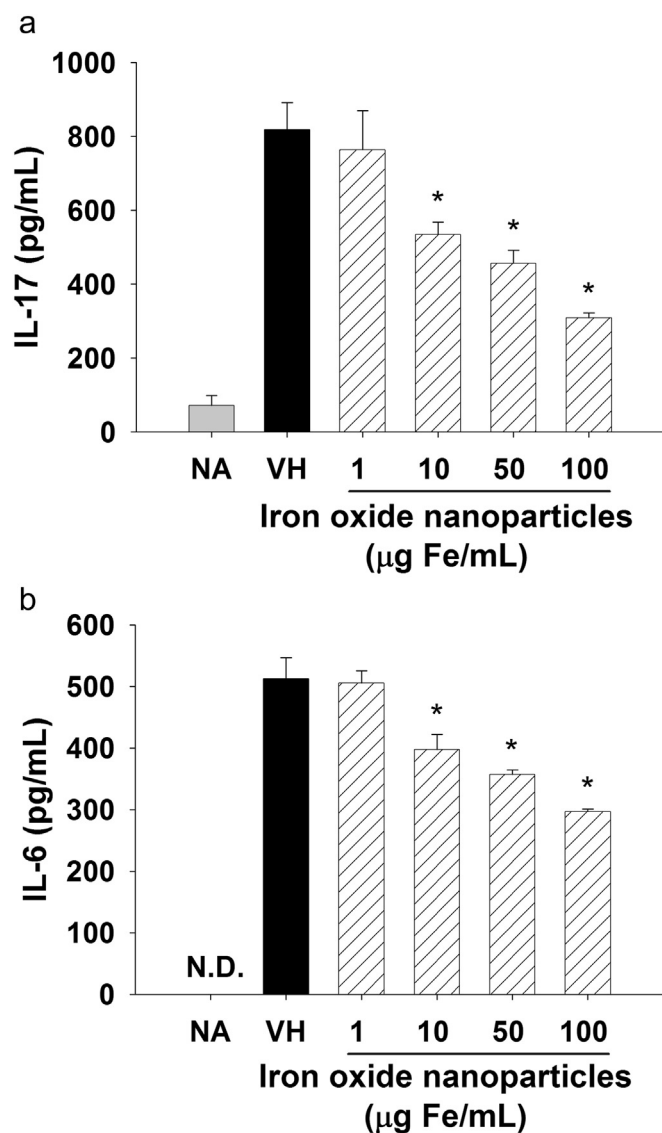


Fig. 2. IONPs inhibited antigen-induced IL-6 and IL-17 production by splenocytes in vitro. Ovalbumin (OVA)-primed splenocytes (5×10^6 cells/mL) were prepared as described in the Materials and methods. The splenocytes were either left untreated (naïve; NA) or treated with IONPs (1–100 $\mu\text{g iron/mL}$) and/or vehicle (VH; saline) followed by restimulation with OVA (100 $\mu\text{g/mL}$) for 48 h. The levels of (A) IL-17 and (B) IL-6 in the supernatants were measured by ELISA. Three independent experiments were performed, and similar results were observed. Results are a representative of three independent experiments. * $P < 0.05$ compared to the VH group.

that IONP-mediated effects on IL-17⁺ cell infiltration may be attributed to the reduced recruitment of CCR6⁺ Th17 cells to the inflamed site.

Pulmonary exposure to IONPs activated macrophages and induced lung inflammation in normal, non-antigen-sensitized animals, but attenuated allergen-induced Th2 cell-mediated allergic responses in OVA-sensitized mice [14,16,23]. Moreover, systemic exposure to IONPs attenuated the immune responses of macrophages and Th1 cells associated with DTH in OVA-sensitized mice [22]. Hence, in addition to proinflammatory effects, IONPs possessed immunomodulatory property on T cell immunity, in which both Th1 and Th2 cell responses were attenuated. Using the same DTH model and OVA-primed splenocytes, we further showed that IONPs suppressed Th17 cell responses associated with DTH. The suppressive effect of IONPs on the expression of key Th17-related molecules was further demonstrated in OVA-primed splenocytes in vitro. IL-17 is known to stimulate the expression of proinflammatory cytokines, including IL-1, IL-6 and TNF- α , under many inflammatory conditions [31–33]. We therefore speculate that the

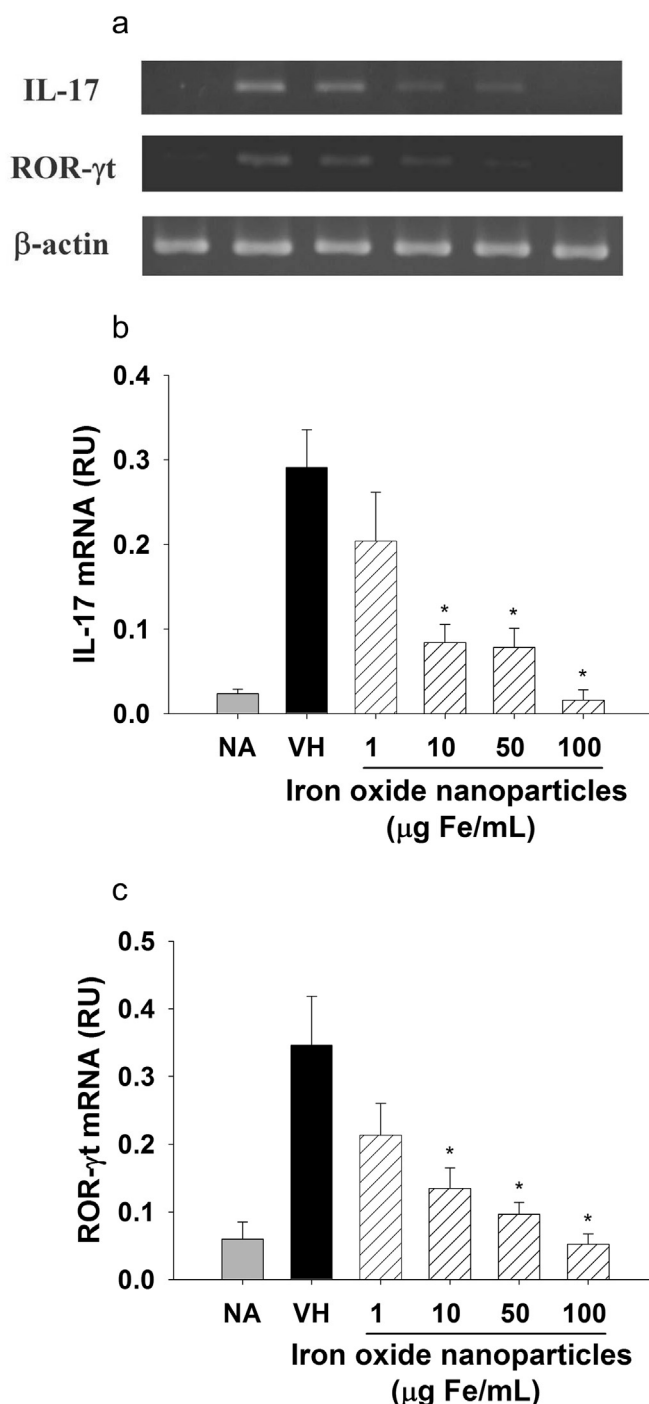


Fig. 3. IONPs suppressed the mRNA expression of IL-17 and ROR- γ t by OVA-primed splenocytes in vitro. Splenocytes (5×10^6 cells/mL) were treated as described in Fig. 2. The mRNA expression of IL-17 and ROR- γ t was measured by RT-PCR. (A) Representative photos of RT-PCR products are shown. The levels of (B) IL-17 and (C) ROR- γ t expression are expressed as mean $\pm$ SEM of three independent experiments. * $P < 0.05$ compared to the VH group.

diminished Th17 cell responses may be an upstream event contributing to a reduced expression of proinflammatory cytokines by macrophages in inflamed footpads and the suppressive effect of IONPs on DTH [22]. Results from the present and previous studies suggest that T cells, including Th1 and Th17, are both sensitive targets to systemic IONP exposure. Together, these lines of evidence highlight a need for a more comprehensive investigation on the potential impact of medicinal nanomaterials on T cell-mediated immunity.

In conclusion, the present study demonstrated that a single systemic

exposure of OVA-sensitized mice to IONPs suppressed Th17 immune responses associated with DTH, as evidenced by a decreased infiltration of CCR6⁺, IL-6⁺, IL17⁺ and ROR- γ ⁺ cells in inflamed footpads. In addition, IONPs caused a direct suppressive effect on the expression of IL-6, IL-17 and ROR- γ t by OVA-primed splenocytes in culture. Collectively, these results indicate that IONPs attenuate Th17 cell responses in vitro and in vivo.

Acknowledgments

This work was supported by grant 104-2320-B-002-024-MY3 from the Ministry of Science and Technology, Executive Yuan, Taiwan.

Conflict of interest

The authors declare no conflicts of interest relevant to this study.

References

- [1] A. Ito, M. Shinkai, H. Honda, T. Kobayashi, Medical application of functionalized magnetic nanoparticles, *J. Biosci. Bioeng.* 100 (1) (2005) 1–11.
- [2] D.L. Huber, Synthesis, properties, and applications of iron nanoparticles, *Small* 1 (5) (2005) 482–501.
- [3] A.K. Gupta, M. Gupta, Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications, *Biomaterials* 26 (18) (2005) 3995–4021.
- [4] D. Hautot, Q.A. Pankhurst, C.M. Morris, A. Curtis, J. Burn, J. Dobson, Preliminary observation of elevated levels of nanocrystalline iron oxide in the basal ganglia of neuroferritinopathy patients, *Biochim. Biophys. Acta* 1772 (1) (2007) 21–25.
- [5] G. Liu, P. Men, P.L. Harris, R.K. Rolston, G. Perry, M.A. Smith, Nanoparticle iron chelators: a new therapeutic approach in Alzheimer disease and other neurologic disorders associated with trace metal imbalance, *Neurosci. Lett.* 406 (3) (2006) 189–193.
- [6] J.W. Bulte, T. Douglas, B. Witwer, S.C. Zhang, E. Strable, B.K. Lewis, H. Zywicke, B. Miller, P. van Gelderen, B.M. Moskowitz, I.D. Duncan, J.A. Frank, Magnetodendrimers allow endosomal magnetic labeling and in vivo tracking of stem cells, *Nat. Biotechnol.* 19 (12) (2001) 1141–1147.
- [7] B. Hamm, T. Staks, M. Taupitz, R. Maibauer, A. Speidel, A. Huppertz, T. Frenzel, R. Lawaczek, K.J. Wolf, L. Lange, Contrast-enhanced MR imaging of liver and spleen: first experience in humans with a new superparamagnetic iron oxide, *J. Magn. Reson. Imaging* 4 (5) (1994) 659–668.
- [8] T.J. Vogl, R. Hammerstingl, W. Pegios, B. Hamm, A. Eibl-Eibesfeldt, B. Woessmer, J. Lissner, R. Felix, The value of the liver-specific superparamagnetic contrast medium AMI-25 for the detection and differential diagnosis of primary liver tumors versus metastases, *RöFo* 160 (4) (1994) 319–328.
- [9] L. Josephson, J. Lewis, P. Jacobs, P.F. Hahn, D.D. Stark, The effects of iron oxides on proton relaxivity, *Magn. Reson. Imaging* 6 (6) (1988) 647–653.
- [10] S.J. McLachlan, M.R. Morris, M.A. Lucas, R.A. Fisco, M.N. Eakins, D.R. Fowler, R.B. Scheetz, A.Y. Olukotun, Phase I clinical evaluation of a new iron oxide MR contrast agent, *J. Magn. Reson. Imaging* 4 (3) (1994) 301–307.
- [11] P. Reimer, M. Muller, C. Marx, D. Wiedermann, R. Muller, E.J. Rummeny, W. Ebert, K. Shamsi, P.E. Peters, T1 effects of a bolus-injectable superparamagnetic iron oxide, SH U 555 A: dependence on field strength and plasma concentration—preliminary clinical experience with dynamic T1-weighted MR imaging, *Radiology* 209 (3) (1998) 831–836.
- [12] J. Wang, Y. Chen, B. Chen, J. Ding, G. Xia, C. Gao, J. Cheng, N. Jin, Y. Zhou, X. Li, M. Tang, X.M. Wang, Pharmacokinetic parameters and tissue distribution of magnetic Fe(3)O(4) nanoparticles in mice, *Int. J. Nanomedicine* 5 (2010) 861–866.
- [13] C.-H. Yeh, J.-K. Hsiao, J.-L. Wang, F. Sheu, Immunological impact of magnetic nanoparticles (Ferucarbotran) on murine peritoneal macrophages, *J. Nanopart. Res.* 12 (1) (2010) 151–160.
- [14] E.J. Park, H. Kim, Y. Kim, J. Yi, K. Choi, K. Park, Inflammatory responses may be induced by a single intratracheal instillation of iron nanoparticles in mice, *Toxicology* 275 (1–3) (2010) 65–71.
- [15] O. Lunov, T. Syrovets, B. Buchele, X. Jiang, C. Rucker, K. Tron, G.U. Nienhaus, P. Walther, V. Mailander, K. Landfester, T. Simmet, The effect of carboxydextran-coated superparamagnetic iron oxide nanoparticles on c-Jun N-terminal kinase-mediated apoptosis in human macrophages, *Biomaterials* 31 (19) (2010) 5063–5071.
- [16] M.T. Zhu, W.Y. Feng, B. Wang, T.C. Wang, Y.Q. Gu, M. Wang, Y. Wang, H. Ouyang, Y.L. Zhao, Z.F. Chai, Comparative study of pulmonary responses to nano- and submicron-sized ferric oxide in rats, *Toxicology* 247 (2–3) (2008) 102–111.
- [17] J.K. Hsiao, H.H. Chu, Y.H. Wang, C.W. Lai, P.T. Chou, S.T. Hsieh, J.L. Wang, H.M. Liu, Macrophage physiological function after superparamagnetic iron oxide labeling, *NMR Biomed.* 21 (8) (2008) 820–829.
- [18] B.A. Chen, N. Jin, J. Wang, J. Ding, C. Gao, J. Cheng, G. Xia, F. Gao, Y. Zhou, Y. Chen, G. Zhou, X. Li, Y. Zhang, M. Tang, X. Wang, The effect of magnetic nanoparticles of Fe(3)O(4) on immune function in normal ICR mice, *Int. J. Nanomedicine* 5 (2010) 593–599.
- [19] J. Wang, B. Chen, N. Jin, G. Xia, Y. Chen, Y. Zhou, X. Cai, J. Ding, X. Li, X. Wang, The changes of T lymphocytes and cytokines in ICR mice fed with Fe₃O₄ magnetic nanoparticles, *Int. J. Nanomedicine* 6 (2011) 605–610.
- [20] C.C. Shen, C.C. Wang, M.H. Liao, T.R. Jan, A single exposure to iron oxide nanoparticles attenuates antigen-specific antibody production and T-cell reactivity in ovalbumin-sensitized BALB/c mice, *Int. J. Nanomedicine* 6 (2011) 1229–1235.
- [21] C.C. Shen, H.J. Liang, C.C. Wang, M.H. Liao, T.R. Jan, A role of cellular glutathione in the differential effects of iron oxide nanoparticles on antigen-specific T cell cytokine expression, *Int. J. Nanomedicine* 6 (2011) 2791–2798.
- [22] C.C. Shen, H.J. Liang, C.C. Wang, M.H. Liao, T.R. Jan, Iron oxide nanoparticles suppressed T helper 1 cell-mediated immunity in a murine model of delayed-type hypersensitivity, *Int. J. Nanomedicine* 7 (2012) 2729–2737.
- [23] M. Ban, I. Langonne, N. Huguet, Y. Guichard, M. Goutet, Iron oxide particles modulate the ovalbumin-induced Th2 immune response in mice, *Toxicol. Lett.* 216 (1) (2013) 31–39.
- [24] C.L. Langrish, Y. Chen, W.M. Blumenschein, J. Mattson, B. Basham, J.D. Sedgwick, T. McClanahan, R.A. Kastelein, D.J. Cua, IL-23 drives a pathogenic T cell population that induces autoimmune inflammation, *J. Exp. Med.* 201 (2) (2005) 233–240.
- [25] H. Park, Z. Li, X.O. Yang, S.H. Chang, R. Nurieva, Y.H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, C. Dong, A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17, *Nat. Immunol.* 6 (11) (2005) 1133–1141.
- [26] J.S. Stumhofer, A. Laurence, E.H. Wilson, E. Huang, C.M. Tato, L.M. Johnson, A.V. Villarino, Q. Huang, A. Yoshimura, D. Sehly, C.J. Saris, J.J. O'Shea, L. Hennighausen, M. Ernst, C.A. Hunter, Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system, *Nat. Immunol.* 7 (9) (2006) 937–945.
- [27] K. Hirota, M. Hashimoto, H. Yoshitomi, S. Tanaka, T. Nomura, T. Yamaguchi, Y. Iwakura, N. Sakaguchi, S. Sakaguchi, T cell self-reactivity forms a cytokine milieu for spontaneous development of IL-17⁺ Th cells that cause autoimmune arthritis, *J. Exp. Med.* 204 (1) (2007) 41–47.
- [28] J.R. Chan, W. Blumenschein, E. Murphy, C. Diveu, M. Wiekowski, S. Abbondanzo, L. Lucian, R. Geissler, S. Brodie, A.B. Kimball, D.M. Gorman, K. Smith, R. de Waal Malefyt, R.A. Kastelein, T.K. McClanahan, E.P. Bowman, IL-23 stimulates epidermal hyperplasia via TNF and IL-20R2-dependent mechanisms with implications for psoriasis pathogenesis, *J. Exp. Med.* 203 (12) (2006) 2577–2587.
- [29] L. Geboes, L. Dumoutier, H. Kelchtermans, E. Schurgers, T. Mitera, J.C. Renaud, P. Matthey, Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice, *Arthritis Rheum.* 60 (2) (2009) 390–395.
- [30] D. Yen, J. Cheung, H. Scheerens, F. Poulet, T. McClanahan, B. McKenzie, M.A. Kleinschek, A. Owyang, J. Mattson, W. Blumenschein, E. Murphy, M. Sathe, D.J. Cua, R.A. Kastelein, D. Rennick, IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6, *J. Clin. Invest.* 116 (5) (2006) 1310–1316.
- [31] J.K. Kolls, A. Linden, Interleukin-17 family members and inflammation, *Immunity* 21 (4) (2004) 467–476.
- [32] W. Ouyang, J.K. Kolls, Y. Zheng, The biological functions of T helper 17 cell effector cytokines in inflammation, *Immunity* 28 (4) (2008) 454–467.
- [33] Y. Iwakura, S. Nakae, S. Saijo, H. Ishigame, The roles of IL-17A in inflammatory immune responses and host defense against pathogens, *Immunol. Rev.* 226 (2008) 57–79.
- [34] B. Stockinger, M. Veldhoen, Differentiation and function of Th17 T cells, *Curr. Opin. Immunol.* 19 (3) (2007) 281–286.
- [35] L.A. Fouser, J.F. Wright, K. Dunussi-Joannopoulos, M. Collins, Th17 cytokines and their emerging roles in inflammation and autoimmunity, *Immunol. Rev.* 226 (2008) 87–102.
- [36] M. Veldhoen, R.J. Hocking, C.J. Atkins, R.M. Locksley, B. Stockinger, TGF β in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells, *Immunity* 24 (2) (2006) 179–189.
- [37] X.O. Yang, B.P. Pappu, R. Nurieva, A. Akimzhanov, H.S. Kang, Y. Chung, L. Ma, B. Shah, A.D. Panopoulos, K.S. Schluns, S.S. Watowich, Q. Tian, A.M. Jetten, C. Dong, T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR α and ROR γ , *Immunity* 28 (1) (2008) 29–39.
- [38] I.I. Ivanov, B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelletier, J.J. Lafaille, D.J. Cua, D.R. Littman, The orphan nuclear receptor ROR γ directs the differentiation program of proinflammatory IL-17⁺ T helper cells, *Cell* 126 (6) (2006) 1121–1133.
- [39] C.T. Weaver, L.E. Harrington, P.R. Mangan, M. Gavrieli, K.M. Murphy, Th17: an effector CD4 T cell lineage with regulatory T cell ties, *Immunity* 24 (6) (2006) 677–688.
- [40] S. Aggarwal, N. Ghilardi, M.H. Xie, F.J. de Sauvage, A.L. Gurney, Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17, *J. Biol. Chem.* 278 (3) (2003) 1910–1914.
- [41] K. Yamashita, M. Sakai, N. Takemoto, M. Tsukimoto, K. Uchida, H. Yajima, S. Oshio, K. Takeda, S. Kojima, Attenuation of delayed-type hypersensitivity by fullerene treatment, *Toxicology* 261 (1–2) (2009) 19–24.
- [42] M. Sakai, K. Yamashita, N. Takemoto, Y. Ohshima, M. Tsukimoto, Y. Shinkai, K. Takeda, S. Oshio, S. Kojima, Diesel exhaust (DE) aggravates pathology of delayed-type hypersensitivity (DTH) induced by methyl-bovine serum albumin (mBSA) in mice, *J. Toxicol. Sci.* 34 (5) (2009) 483–492.
- [43] E.V. Acosta-Rodriguez, L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, F. Sallusto, G. Napolitani, Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells, *Nat. Immunol.* 8 (6) (2007) 639–646.
- [44] M. Feuerer, K. Eulenburg, C. Loddenkemper, A. Hamann, J. Huehn, Self-limitation of Th1-mediated inflammation by IFN- γ , *J. Immunol.* 176 (5) (2006) 2857–2863.
- [45] I.M. Irmiler, M. Gajda, R. Brauer, Exacerbation of antigen-induced arthritis in IFN- γ -deficient mice as the result of unrestricted IL-17 response, *J. Immunol.* 179

- (9) (2007) 6228–6636.
- [46] B. Afzali, G. Lombardi, R.I. Lechler, G.M. Lord, The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease, *Clin. Exp. Immunol.* 148 (1) (2007) 32–46.
- [47] S. Nakae, Y. Komiyama, A. Nambu, K. Sudo, M. Iwase, I. Homma, K. Sekikawa, M. Asano, Y. Iwakura, Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses, *Immunity* 17 (3) (2002) 375–387.
- [48] B. Homey, M.C. Dieu-Nosjean, A. Wiesenborn, C. Massacrier, J.J. Pin, E. Oldham, D. Catron, M.E. Buchanan, A. Müller, R. deWaal Malefyt, G. Deng, R. Orozco, T. Ruzicka, P. Lehmann, S. Lebecque, C. Caux, A. Zlotnik, Up-regulation of macrophage inflammatory protein-3 alpha/CCL20 and CC chemokine receptor 6 in psoriasis, *J. Immunol.* 164 (12) (2000) 6621–6632.
- [49] J.M. Larsen, C.M. Bonefeld, S.S. Poulsen, C. Geisler, L. Skov, IL-23 and T(H)17-mediated inflammation in human allergic contact dermatitis, *J. Allergy Clin. Immunol.* 123 (2) (2009) 486–492.
- [50] K. Hirota, H. Yoshitomi, M. Hashimoto, S. Maeda, S. Teradaira, N. Sugimoto, T. Yamaguchi, T. Nomura, H. Ito, T. Nakamura, N. Sakaguchi, S. Sakaguchi, Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model, *J. Exp. Med.* 204 (12) (2007) 2803–2812.
- [51] T.J. Paradis, S.H. Cole, R.T. Nelson, R.P. Gladue, Essential role of CCR6 in directing activated T cells to the skin during contact hypersensitivity, *J. Invest. Dermatol.* 128 (3) (2008) 628–633.