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SPECIAL ARTICLE

Induction of the 'ASIA' syndrome in NZB/NZWF1 mice after injection of complete Freund's adjuvant (CFA)

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Adjuvants, commonly used in vaccines, may be responsible for inducing autoimmunity and autoimmune diseases, both in humans and mice. The so-called 'ASIA' (Autoimmune/inflammatory Syndrome Induced by Adjuvants) syndrome has been recently described, which is caused by the exposure to a component reproducing the effect of adjuvants. The aim of our study was to evaluate the effect of injection of complete Freund's adjuvant (CFA) in NZB/NZWF1 mice, a lupus-prone murine model. We injected 10 NZB/NZWF1 mice with CFA/PBS and 10 with PBS, three times, 3 weeks apart, and followed-up until natural death. CFA-injected mice developed both anti-double-stranded DNA and proteinuria earlier and at higher levels than the control group. Proteinuria-free survival rate and survival rate were significantly lower in CFA-treated mice than in the control mice ($p = 0.002$ and $p = 0.001$, respectively). Histological analyses showed a more severe glomerulonephritis in CFA-injected mice compared with the control mice. In addition, lymphoid hyperplasia in spleen and lungs, myocarditis, and vasculitis were observed in the former, but not in the latter group. In conclusion, the injection of CFA in NZB/NZWF1 mice accelerated autoimmune manifestations resembling 'ASIA' syndrome in humans. *Lupus* (2012) **21,** 203–209.

Key words: adjuvants; ASIA syndrome; NZB/NZWF1 mice; systemic lupus erythematosus; vaccines

Introduction

Some adjuvants may induce autoimmunity and autoimmune diseases both in humans and mice.[1–4] Adjuvants are substances commonly used in vaccines that enhance specific immune response against antigens, but little is known about their mechanisms of action. Adjuvants may act in several ways: 1) they promote the translocation of antigens to the lymph nodes where antigens can be recognized by T cells; 2) they induce a progressive release of the antigen, hindering its clearance and leading to a longer exposure of the antigen to antigen-presenting cells; 3) they increase the local reactions in the site of injection by mimicking danger signals;[2,5–10] 4) they induce the release of inflammatory cytokines; 5) they interact with toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs) including NALP3 inflammasome.[2,3,6,11,12]

The immunological consequence of these actions is stimulation of the innate and adaptive immune response, with increased activation of dendritic cells, macrophages, T and B cells, their proliferation and migration to the site of injection.[2]

Notably, adjuvants based on microbial components of bacterial walls such as liposomes or lipopolysaccharides, or endocytosed nucleic acids such as single-stranded DNA (ssDNA), double-stranded RNA (dsRNA), or unmethylated CpG dinucleotide-containing DNA may mimic a natural infection, whereas aluminium- or oil-based adjuvants may aggravate tissue damage at the injection site. Complete Freund's adjuvant (CFA) is one of the most commonly used adjuvants because it is able to induce an intense and persistent immune response due to its composition, which includes heat-killed

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and dried *M. tuberculosis*, paraffin oil, and mannide monooleate. Mycobacterium tuberculosis-derived antigens, particularly cell wall components and fragmented DNA,[13] attract macrophages and other cells to the injection site, enhancing the immune response.[2] Thus, the administration of microbial products together with this adjuvant greatly increases antibody response.

Recently, it has been highlighted that siliconosis, macrophagic myofasciitis (MMF), Gulf war syndrome (GWS), and post-vaccination events are related to each other since all these conditions probably occur when genetically predisposed individuals are exposed to a component that reproduces the effect of adjuvants. Notably, it has been recently proposed to group them under a common syndrome called 'Autoimmune (Autoinflammatory) Syndromes Induced by Adjuvants' (ASIA).[1]

Systemic lupus erythematosus (SLE) is a systemic, multifactorial, autoimmune disease involving many organ systems. Patients with SLE develop autoantibodies, particularly antinuclear and anti-double-stranded DNA (dsDNA), which can be found in tissues as immune complexes.[14–17]

NZB/NZWF1 mice are a murine model of human SLE; these mice develop a lupus-like glomerulonephritis (GLN) within 5–7 months of age.[18] As in humans, anti-dsDNA antibodies are found at high levels in the circulation and deposited as immune complexes in glomeruli of NZB/NZWF1.[18]

Genetic predisposition, defective apoptosis and abnormalities in TLR signalling, B and T-cell tolerance, complement activation, cytokine regulation, and endothelial cell functions are all factors involved in lupus pathogenesis both in humans and in NZB/NZWF1 mice.[14,18,19]

The aim of our study was to evaluate the effect of CFA on NZB/NZWF1 mice.

Materials and methods

Eight-week-old NZB/NZWF1 mice (Harlan Laboratories) were subdivided into two groups of 10 mice. Group 1 was treated with 100 μl of CFA (Sigma, St Louis, USA) in 100 μl of PBS, and group 2 with 200 μl of PBS, by subcutaneous injections on posterior feet, three times, 3 weeks apart, starting from the 10th to the 16th week of age. All mice were bred until natural death occurred. The study was approved by the Institutional Animal Care and Use Committee.

Disease progression was monitored by weekly urine sample collection, in order to evaluate proteinuria levels, using multi reactive strips (Bayer). Blood samples were collected from the caudal vein before every injection (at 10th, 13th, and 16th weeks of age), on weeks 19th, 21st, and at death. Blood samples were centrifuged at 3000 rpm for 10 min, to obtain serum to evaluate circulating anti-dsDNA antibody levels, by a standardized home-made enzyme-linked immunosorbent assay (ELISA) test.

Briefly, the coating was performed in three phases: addition of poly-L-lysine to block the DNA and incubation at 37°C, addition of calf thymus DNA and incubation at room temperature (RT), and addition of poly-L-glutamate to neutralize the negative free charges of DNA and incubation at RT. After washing circles, 3% BSA/TBS was added as blocker and incubated at 4°C overnight. Duplicated serum samples were added at a dilution of 1:100 in 1% BSA/TBS and incubated at RT; after washings anti-mouse IgG diluted 1:10,000 in 1% BSA/TBS was added and incubated at 37°C. After washing cycles p-nitrophenil-phosphate was added and the plates were read at 405 nm. All products for the ELISA were purchased from Sigma, St Louis, USA. The values were expressed as mean optical density (OD) of the double of every serum sample.

Kidneys, hearts, lungs, and spleens were harvested at death, and fixed in formalin for histological analyses by haematoxylin/eosin staining, to evaluate histological abnormalities.

Statistical analyses

Statistical analyses were performed using SPSS 16 software. Differences between groups were evaluated by student *t*-test, for normally distributed variables, and continuous variables by Pearson's correlation. Survival rates were evaluated by Kaplan-Meier method using Mantel–Cox test for comparison.

Results

Both groups developed anti-dsDNA autoantibodies. However, in group 1 anti-dsDNA antibodies were detectable earlier than in the mice of group 2. Anti-dsDNA antibody levels, expressed as mean OD $\pm$ SD, were higher in

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CFA-treated mice compared with the control mice: week 13 0.09 ± 0.03 vs. 0.12 ± 0.09, $p =$ n.s.; week 16 0.39 ± 0.19 vs. 0.16 ± 0.08, $p = 0.002$; week 19 0.53 ± 0.17 vs. 0.28 ± 0.23, $p = 0.019$; week 21 0.69 ± 0.15 vs. 0.39 ± 0.15, $p = 0.003$ (Figure 1a).

Similarly, group 1 mice developed proteinuria earlier and at higher levels than group 2. In fact, four out of 10 CFA-treated mice developed traces of proteinuria (15 mg/dl) at the 13th week of age, and another two at the 16th week. At the 17th week of age, 60% of CFA-treated mice had proteinuria $\geq$300 mg/dl. In group 2, traces of proteinuria occurred in the first mouse at the 18th week of age.

Proteinuria levels, expressed as mean mg/dl±SD, were higher in CFA-treated mice compared with the control mice: week 13 6 ± 7.75 vs. 0, $p = 0.005$; week 16 43.50 ± 90.95 vs. 0, $p = 0.004$; week 21 720 ± 383.41 vs. 50.63 ± 41.36, $p = 0.003$ (Figure 1b).

Notably, all mice in group 1, and five in group 2, died after developing the maximum detectable levels of proteinuria ($\geq$300 mg/dl).

Survival rates

Proteinuria-free survival rate (proteinuria $<$300 mg/dl) was significantly lower in group 1 than in group 2 ($p = 0.002$) (Figure 1c). At the 20th week of age, 90% of the mice in group 1 had proteinuria levels $\geq$300 mg/dl compared with only 30% in group 2 ($p = 0.002$). Proteinuria $\geq$300 mg/dl occurred at the 23rd week of age in the last mouse of the CFA-treated group and at the 39th week of age in the last mouse of PBS-injected group. Mean proteinuria-free survival rate (weeks $\pm$ SD) was significantly lower in CFA-treated mice than in those injected with PBS (18.3 ± 2.4 vs. 24.4 ± 6.5, $p = 0.004$).

Survival rate was significantly lower in group 1 than in the control group (Figure 1d). The first death in group 1 was recorded in a 15-week-old mouse, whereas the first death in the control group occurred in a 20-week-old mouse. The last death in CFA-treated group occurred in a 25-week-old mouse; by contrast, the last death in the control group occurred in a 39-week-old mouse. At the 21st

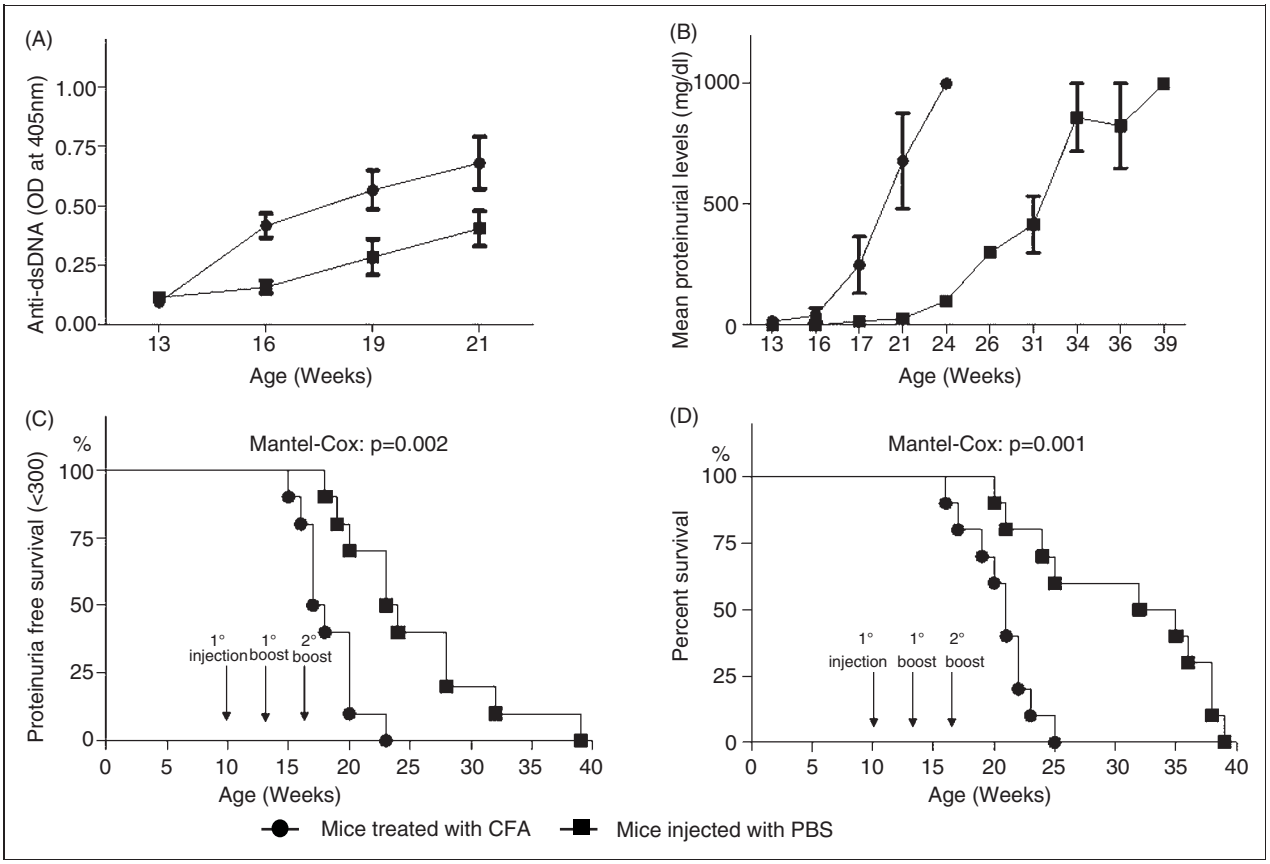

Figure 1 Comparison between mice treated with CFA and those injected with PBS. a) mean anti-dsDNA autoantibodies levels (OD at 405 nm); b) mean proteinuria levels (mg/dl); c) proteinuria free survival rate ($<$300 mg/dl); d) and survival rate. CFA, complete Freund's adjuvant; PBS, phosphate buffer salt; dsDNA, double stranded DNA; OD, optical density.

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week of age the survival rate was 40% in group 1 vs. 75% in group 2. When the last mouse of the CFA-treated group died, only 40% of control mice were dead ($p = 0.001$) (Figure 1d). Mean survival (weeks $\pm$ SD) was significantly lower in CFA-treated mice than in those injected with PBS (group 1 vs. group 2: 20.6 ± 2.7 vs. 30.5 ± 8, $p = 0.004$).

Histological analyses

Histological examinations showed that CFA-treated mice had more significant organ damage not only in the kidneys, but also in the lungs, heart, and spleen, in comparison with mice injected with PBS (Figure 2).

In fact, CFA-treated mice had more severe renal abnormalities than control mice (Figure 2a and 2b, respectively). In the former group the glomeruli showed segmental widening of the mesangial stalk, with an increase in mesangial cells and matrix, and proliferation of endocapillary cells, leading to the occlusion of capillary lumen and, in turn, a reduction in the glomerular surface area (Figure 2a); by contrast, control mice had only mild glomerular abnormalities (Figure 2b).

Lung involvement was more severe in group 1 than in group 2 (Figure 2c and 2d, respectively). In the lung tissue of four CFA-treated mice a more prominent lymphoid cell proliferation and hyperplasia around and near the bronchus, and infiltrating cells in peripheral zone were observed (Figure 2c). No abnormalities were detected in the lungs of the control mice (Figure 2d).

Hyperplasia with follicular cell proliferation was more prominent in the spleens of group 1 mice compared with those of PBS injected mice (Figure 2e and 2f, respectively). In three CFA-treated mice, myocarditis, characterized by focal necrotizing area with infiltrating cells, was detected (Figure 2g). In contrast, the hearts of control mice were normal (Figure 2h).

Notably, three mice of group 1 developed necrotizing lesions in the tail (Figure 3a); histological examination showed phlogistic necrotizing inflammatory areas, subcorneal pustule-like lesions in the epithelium, and vasculitis of small and medium veins (Figure 3b).

Discussion

In our study, CFA injection in NZB/NZWF1 mice caused an acceleration of the autoimmune process, with a worsening of GLN and a shortening of survival time. Indeed, all CFA-treated mice died within 24 weeks of age compared with only 40% in the control group.

ASIA is a new syndrome, recently described by Shoenfeld et al.,[1] who also proposed a set of four major classification criteria: 1) exposure to an external stimulus before the occurrence of clinical manifestations; 2) appearance of typical manifestations; 3) disease improvement after the removal of the inciting agent; and 4) development of typical histological lesions at biopsy of the involved organs.

Our mice met three of these criteria; criterion 1: injection of CFA worsened clinical manifestations; criterion 2: typical manifestations such as autoantibodies, GLN, lymphoid tissue hyperplasia, myocarditis, and vasculitis appeared after the exposure; and criterion 4: histological analyses revealed typical immune inflammatory lesions in the tissues (Figure 2). By contrast, our mice did not satisfy the criterion number 3. In our experiment we used lupus-prone mice, where CFA injection resulted in the aggravation of lupus. As expected in this model, when lupus was established, it progressed unrelentlessly.

Patients affected with chronic rheumatic or autoimmune diseases, such as SLE, have a twofold higher risk of infection than healthy individuals, due to abnormalities in immune system regulation and secondary to immunosuppressive therapy.[14,16,20–24] Many studies have shown that vaccination may trigger or worsen autoimmune and rheumatic diseases, because of the release of proinflammatory cytokines after the injection.[20,25–29] Notably, not only the antigens of the vaccines, but also adjuvants and preservatives may enhance antigenic stimulation.[2,20,29–32]

Many in vivo studies using animal models have shown that adjuvants can induce autoimmunity.[2,3,32] Subcutaneous injection of mineral oil induced sclerosing lipogranulomas, a chronic local inflammatory reaction in BALB/c mice.[3,33] Pristane (2,6,10,14-tetramethylpentadecane) and mineral oil mixtures induced plasmacytomas in susceptible strains of mice.[34] Mice and rats treated with pristane, incomplete Freund's adjuvant, or squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) developed chronic arthritis;[35] moreover, a single injection of one of these adjuvants induced lupus-related autoantibodies in nRNP/Sm and to Su in BALB/c mice.[36,37]

In humans, vaccines have been reported to potentially induce autoantibodies, inflammatory reactions, and autoimmune diseases such as arthritis, neuronal damage, fatigue, encephalitis, myocarditis, and vasculitis.[3,32,38,39] These adverse events

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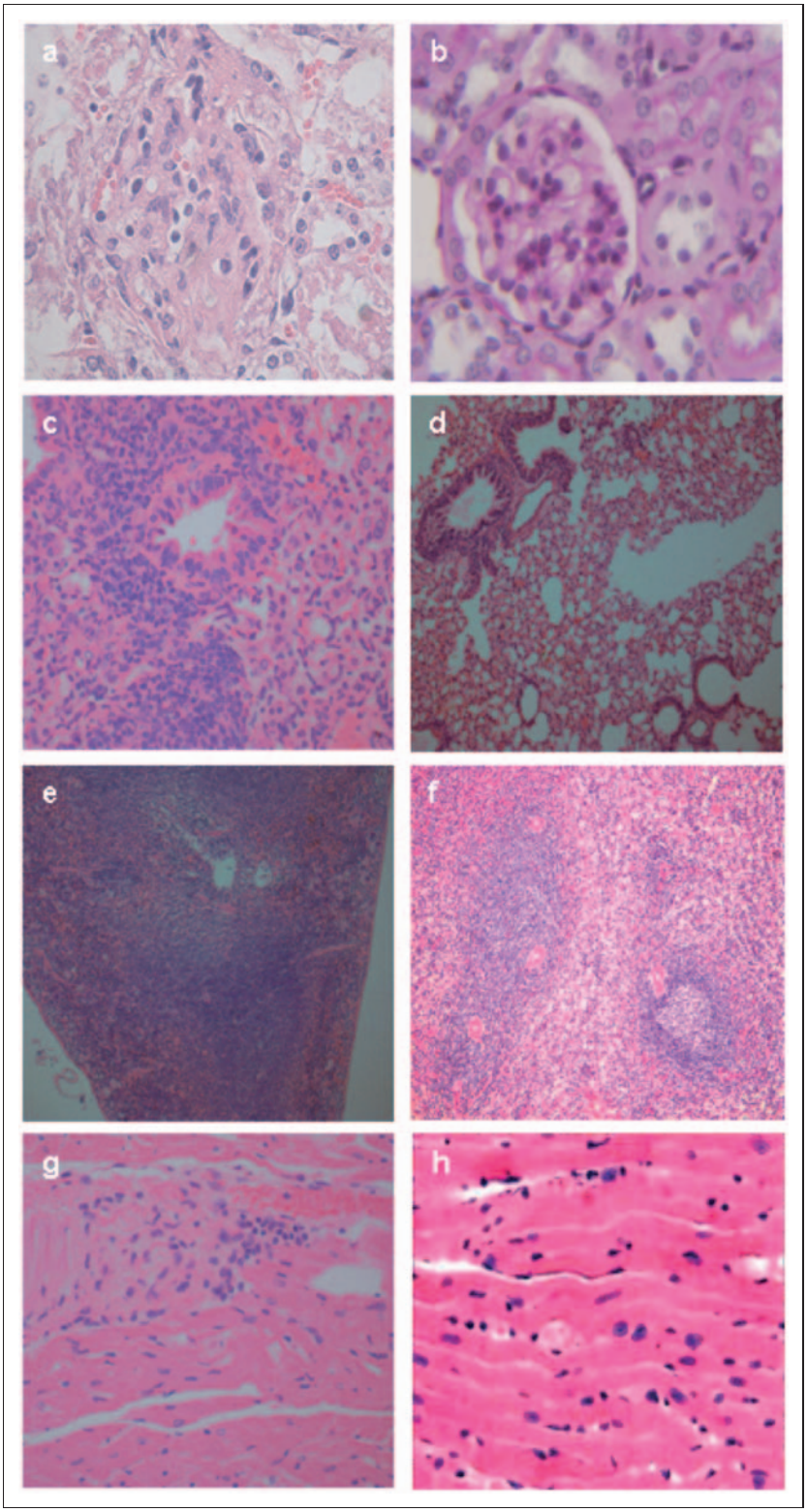

Figure 2 Histological analyses of **a)** kidney from a CFA-treated mouse (renal lesions characterized by increase in mesangial cells number and expansion in mesangial matrix); **b)** kidney from a control mouse (renal damage milder than in CFA-treated mice); **c)** lung from a CFA-treated mouse (clusters of infiltrating mononuclear cells around bronchia and vascular structures); **d)** lung from a control mouse (normal tissue); **e)** spleen from a CFA-treated mouse (hyperplasia with follicular cell proliferation); **f)** spleen from a control mouse (hyperplasia milder than CFA-treated mice); **g)** right ventricle from a CFA-treated mouse (myocarditis, characterized by highly infiltrating cells in the necrotizing zone and presence of some giant nucleated cells); **h)** heart from control mouse (normal tissue). CFA, Complete Freund's Adjuvant.

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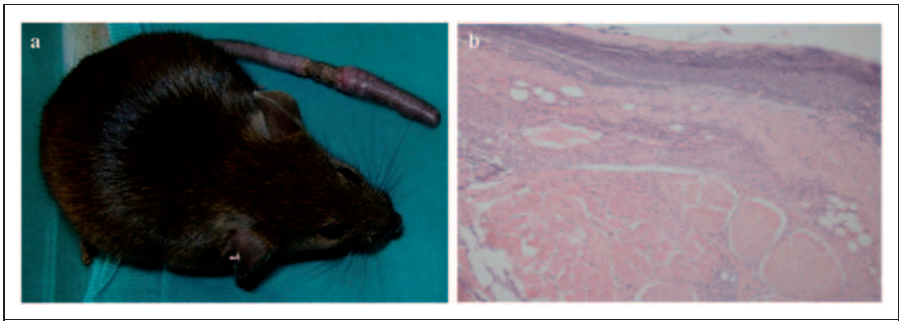

Figure 3 CFA-treated mouse tail: **a)** bulges of necrotic tissue in the tail; **b)** superficial and epithelial necrotic zones and vein with neutrophilic infiltration of the wall. CFA, Complete Freund's Adjuvant.

can occur weeks or even months or years after vaccination.[32,39] Mineral oils may induce sclerosing lipogranulomas also in humans.[33] Silicone has been implicated in the induction of scleroderma, SLE, and rheumatoid arthritis.[40] Finally, alum, aluminium hydroxide and squalene are all reported as cofactors in causing chronic fatigue syndrome, polymyalgia, MMF, and GWS.[41,42] Adjuvants may also induce the production of antibodies against themselves, as reported in US military personnel with GWS, in whom high levels of anti-squalene circulating antibodies were found.[42–45]

CFA is the most active adjuvant due to its composition. which includes components of *M. tuberculosis*. Vaccination with CFA might evoke an excessive immunological reaction, mainly in patients affected with autoimmune diseases, because of the concomitant presence of two different antigens: the vaccine antigen and the *M. tuberculosis* components of CFA.[46,47]

For this reason, CFA should be avoided as an adjuvant during immunization of autoimmune-prone mice in experimental studies; in addition, we suggest avoiding CFA even in non-autoimmune-prone mice due to its autoimmune potential.[2]

The benefits that can derive from studying the relationship among vaccines, adjuvants, and autoimmunity may include some indications for the preparation and use of vaccines, in addition to the comprehension of disease mechanisms involved in autoimmunity,[48,49] improving SLE prognosis.[50–52]

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Conflict of interest

The authors declare that they have no conflicts of interest.

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