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Original Research Article

Nanoemulsion formulation of Abatacept for lupus nephritis therapy

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Abstract

Purpose: *To formulate a nanoemulsion preparation of abatacept and evaluate its treatment efficacy in a C57BL/6 J mouse model of lupus nephritis (LN).*
Methods: *An abatacept nanoemulsion formulation was prepared using coarse homogenization followed by high-energy ultrasonication. The formulation was assessed for particle size and charge, morphology, and stability. C57BL/6 J mice treated with pristane (to create a mouse model of LN) received subcutaneous injections of the abatacept formulation, and the in vivo efficacy and immunological profiles were evaluated.*
Results: *The mean diameter of the nanoglobules ranged from 110 to 148 nm with a polydispersity index of < 1, and the formulation was stable for 3 months at 22 – 28 °C. The LN-model mice that were treated with the nanoemulsion formulation of abatacept showed a marked reduction in immune complexes, improved renal function and decreased expression of IL-4 and IFN-γ compared with untreated LN-model mice.*
Conclusion: *The nanoemulsion formulation of abatacept is a promising agent in the treatment of refractory LN. A systematic clinical trial is necessary to establish its long-term efficacy.*

Keywords: *Nanoemulsion, Abatacept, Lupus nephritis, Beta-1 integrin, Immunological profile*

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INTRODUCTION

Lupus nephritis (LN) is an inflammatory condition of the kidneys caused by systemic lupus erythematous (SLE) [1]. LN is characterized by the deposition of IgG, complement proteins, and immune complexes (ICs) containing components such as anti-nuclear antibodies, apoptotic particles, and cross-reactive anti-glomerular autoantibodies. Several factors implicated in the pathogenesis of LN include genetic predisposition, infection of Epstein–Barr virus (EBV), and environmental conditions. Therapeutic interventions to prevent disease progression are aimed at controlling risk factors, such as proteinuria and hypertension, using renin-angiotensin inhibitors followed by immunosuppressive agents [1]. Immunosuppressive agents have been used for the treatment over the past decade; however, they are associated with a risk of relapse. Steroids have been tested in combination with cyclophosphamide or hydroxychloroquine or with biologic agents such as abatacept and rituximab. However, an optimal therapeutic agent has yet to be identified.

The efficacy of immune modulators depends on the effectiveness of the T-cell receptor (TCR) immune response, which requires binding of antigen to the major histocompatibility complex class II on the surface of antigen-presenting cells

(APCs) and concomitant binding of costimulatory factors [2–8]. The costimulatory factors are often responsible for the clonal expansion of T cells and subsequent release of a cascade of cytokines [9]. Among the costimulatory factors, CD28 is the most important ligand that activates naive T cells [10]. Inhibition of CD28 prevents T-cell activation [11,12]. Preclinical models indicate that CD28 is an important component in the airway inflammatory response [13–15]. CD28-deficient mice fail to develop airway inflammatory disease and hyperresponsiveness [16,17]. Inhibitors of CD28, such as the CTLA4Ig, have been shown to prevent airway hyperresponsiveness in animal models [18]. As the administration of CTLA4Ig is effective in preventing airway hyperresponsiveness during sensitization and at the time of antigen challenge, the responsible mechanism extends beyond that of costimulating T cells [19].

Abatacept is a fusion protein comprising CTLA-4 linked to the Fc portion of IgG1 [4]. It selectively modulates the CD28-CD80/86 signaling pathway, thereby inhibiting costimulatory events, including T-cell activation [2, 3]. Abatacept has been approved for the treatment of rheumatoid arthritis and juvenile inflammatory arthritis, and is being tested for the treatment of several autoimmune diseases [6]. Abatacept has been used in a subcutaneous (sc) formulation in Italy [14]. It displays comparable half-lives (14.3 days) when administered intravenously or subcutaneously; however, certain side effects have been observed, such as body weight gain. We hypothesize that a nanoemulsion formula would minimize the side effects and increase the efficacy over the existing subcutaneous formulation. This study thus compared the efficacy of a novel nanoemulsion formulation of abatacept with the standard formulation of abatacept in a C57BL/6 J mouse model of LN.

EXPERIMENTAL

Materials

Abatacept was purchased from Bristol-Myers Squibb (New York, NY, USA). Praziquantel, cholesterol, soybean oil, and glycerol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals and solvents used in the study were of laboratory grade. Malvern Nano-S instrument was purchased from Malvern Instruments (Shanghai, China).

Animals

All experimental procedures were conducted according to the Institutional Guidelines and the National Research Council Guide for Care and Use of Laboratory Animals [20], and the procedures were approved by the Animal Ethical Care and Use Committee of Linyi People's Hospital, Linyi, Shandong, China (Approval ref. no. 1603049). C57BL/6 J female mice (7 – 8 weeks old and weighing 20 - 25 g; n = 6 in each group) were purchased from the Chinese Academy of Medical Sciences. The mice were anesthetized using 10 % chloral hydrate (400 mg/kg body weight).

Nanoemulsion formulation of abatacept

The nanoemulsion formulation was prepared using homogenization and high-energy ultrasonication as described by Ganta *et al* [15]. Abatacept (0.2 %), soybean oil (10 %), cholesterol (0.2 %), egg lecithin (1.8 %), and PEG2000DSPE (0.2, 0.3, and 0.4 %) were mixed in chloroform, and a nitrogen film was obtained after evaporating the chloroform using nitrogen gas. The residual lipid layer was rehydrated with glycerol (2.21 %) at 50 °C and homogenized to produce the emulsion. The emulsion was further subjected to ultrasonication to form a nanoemulsion. The nanoemulsion was passed through a 0.22-µm filter (Millipore, China) for sterilization.

Particle size and charge measurement

The nanoemulsion formulation was evaluated for particle size and size distribution using dynamic light scattering with a Zeta-sizer (Malvern Instruments, Malvern, UK). Additionally, the polydispersity and zeta potential were determined.

Establishment of pristane-induced lupus model

To induce LN, C57BL/6 J mice were given an intravenous injection of 0.5 mL of pristane (Sigma-Aldrich). All animals were monitored for renal function for 6 months and then sacrificed. The mice were given an injection of sodium pentobarbital (30 mg/kg), and then a flank incision was made to remove the right kidney. Using a small vascular clip, the left renal artery and vein were clamped for 30 min, the clamp was removed to replicate the reperfusion procedure, and the abdomen was closed with sterile sutures. Throughout the experiments, the mice were maintained at 32 °C and hydrated using normal saline. Blood collection and kidney biopsies were performed after the reperfusion procedure. The same procedure was followed in the sham group, with the exception that renal artery and vein clamping was not performed.

Flow cytometric evaluation of phenotypic expression in spleen

Spleen cells were removed gently by scraping, and the cell suspension was passed through a 100-µm microfilter (Merck Millipore, Shanghai, China). The spleen cells were further washed with ammonium chloride-potassium lysis buffer (Lonza, Allendale, NJ, USA) to remove erythrocytes. Then, the cells were washed twice with RPMI-1640 medium and co-stained with phycoerythrin (PE)-conjugated monoclonal anti-mouse CD11b IgG and FITC-conjugated anti-B7-1 antibody and PE-conjugated anti-CD11c antibody (Abcam, Cambridge, MA, USA), respectively. The cells were further analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA, USA) and CellQuest software version 1.0 (BD Biosciences).

Histopathological evaluation

Kidney tissues were isolated from the abatacept-treated and control groups following anesthetization with chloral hydrate (300 mg/kg, intraperitoneal injection). The kidney tissues were stained with Masson's trichrome to analyze the alterations in tissue morphology [21,22]. Three investigators analyzed three different tissue sections from each animal. Samples were examined under a Zeiss Axio Imager A2m microscope (Carl-Zeiss, Oberkochen, Germany), and the evaluating pathologists were blinded to the study groups.

Detection of autoantibodies against anti-nuclear antibody

Protein expression of anti-nuclear antibody was estimated using Western blot analysis in pristane-treated BALB/c mice. Proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to a membrane using a transblot system (Bio-Rad, Hercules, CA, USA) using standard protocols. The membranes were incubated with primary antibody (anti-nuclear antibody; Abcam) at 4 °C overnight, washed, and incubated with goat anti-rabbit or anti-mouse IgG horseradish peroxidase (HRP) secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA). The Amersham ECL Western blotting detection kit (GE Lifesciences, Pittsburgh, PA, USA) was used for detecting protein expression.

Evaluation of serum IL-4 and IFN-γ levels

Tissue lysates were prepared using a tissue homogenizer (Thomas Scientific, Swedesboro, NJ, USA), and cytokine levels were estimated using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) for mouse tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, and high mobility group box 1 (HMGB1), according to the manufacturer's protocol.

Statistical analysis

The data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS software (version 15; IBM Corporation, Armonk, NY, USA). For intergroup comparisons, unidirectional analysis of variance was performed. The least significant difference test was performed for multiple comparisons. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Characteristics of nanoemulsion formulation

The osmolarity of the formulation ranged from 298 to 310 mOsm/kg. Morphologic evaluation revealed uniform droplets in a homogeneous emulsion. The particle size and zeta potential of the formulation are shown in Figure 1. Table 1 shows the properties of the formulation. The mean diameter of particles in the formulation was in the range of 110 – 148 nm, and the polydispersity index was < 1.

Table 1: Properties of abatacept nanoemulsions

Concentration of nanoemulsion	Mean hydrodynamic diameter (nm)	Poly-dispersity index	Zeta potential (mV)	Osmolality (mOsm/kg)	Encapsulation efficiency (%)
Blank nanoemulsion	175 ± 1	0.19	−32.3 ± 1.2	302 ± 0.8	-
Abatacept nanoemulsion (PEG_{2000}DSPE 0.2 %)	188 ± 3	0.50	−34.2 ± 0.2	303 ± 2	98 ± 2
Abatacept nanoemulsion (PEG_{2000}DSPE 0.3 %)	171 ± 2	0.60	−33.1 ± 1.0	304 ± 1	98 ± 1
Abatacept nanoemulsion (PEG_{2000}DSPE 0.4 %)	184 ± 2	0.3	−35.8 ± 2	307 ± 2	97 ± 1

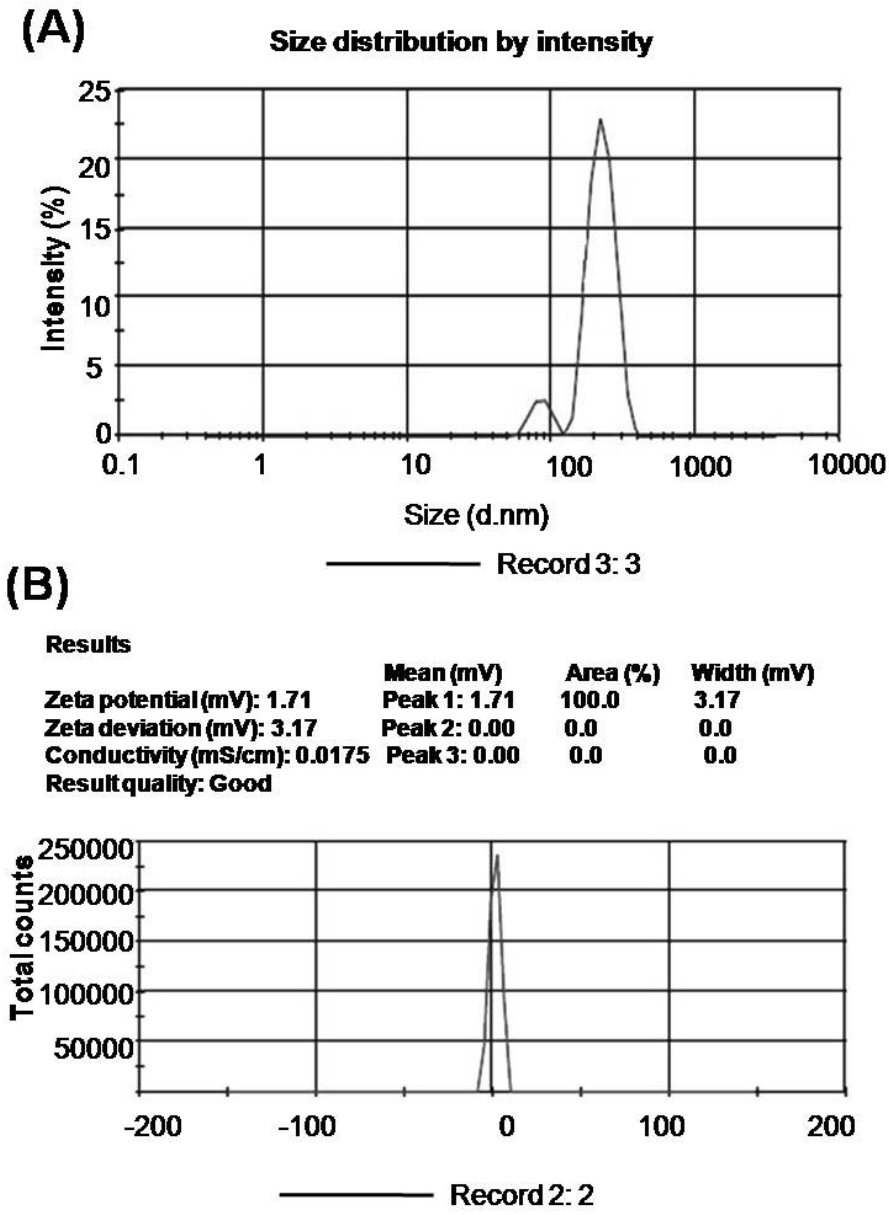

Figure 1: Size distribution and zeta potential of abatacept nanoemulsion measured through dynamic light scattering. (A) Statistical curve of size and (B) zeta potential of abatacept nanoemulsion

Stability of the abatacept concentration in the formulations

The suspension and nanoemulsion formulation contained identical concentrations of abatacept (2 mg/mL). Stability analysis showed that the concentration of abatacept did not decrease significantly after storage for 3 months at 22–28 °C (Figure 2). The formulation showed no loss in abatacept concentration when stored at 4 °C.

Proteinuria

The relative protein levels in the urine of rats following various treatments are shown in Table 2.

Abatacept treatment reduces pristane-induced autoantibody production

Expression of B7-1 antibody in the kidney tissues of animals with pristane-induced nephritis was observed. Niclosamide treatment caused improvement in renal function and increased the expression of autophagy-related B7-1 antibody-1 proteins compared to the control group ($p < 0.05$, Figure 1a–d). These results suggest that promoting autophagy may be an effective strategy to manage pristane-induced nephritis. B7-1 antibody levels decreased after 24 h of treatment with abatacept (Figure 2).

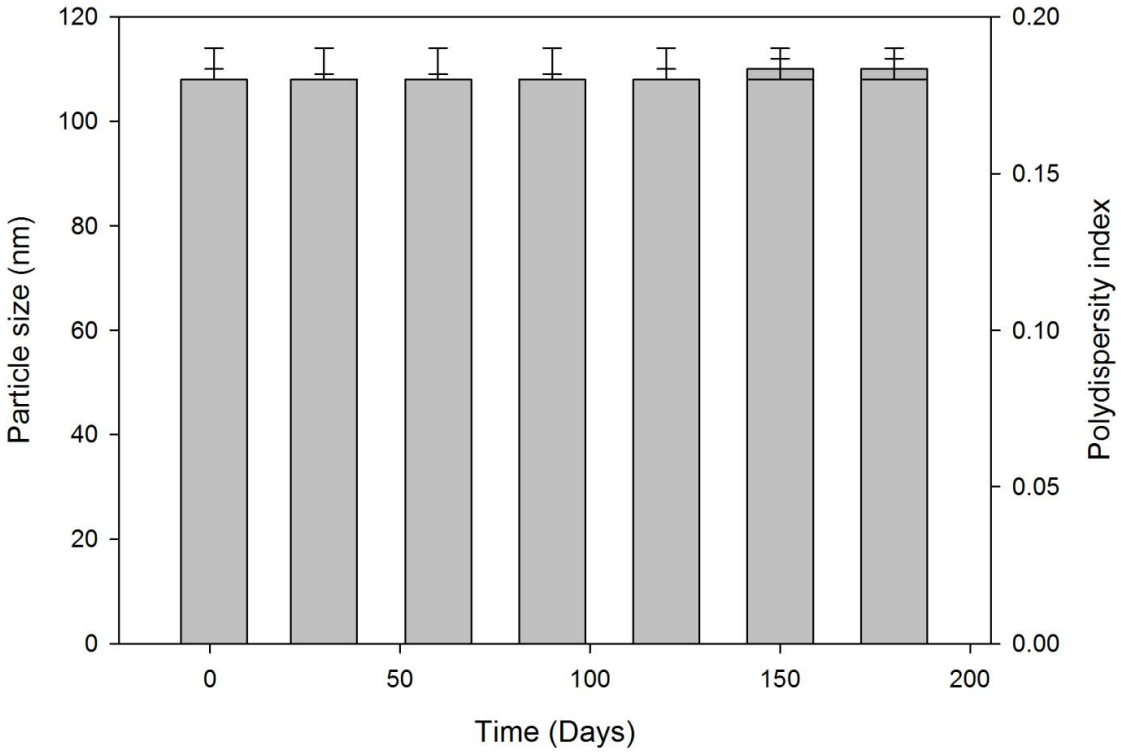

Figure 2: Stability of the optimized nanoemulsion formulation of abatacept as a function of particle size and polydispersity index when stored at 22 – 28 °C over 3 months

Table 2: Degree of proteinuria in mice 3 months after treatment (n = 12)

Group	-	±	+	++	+++	++++
Negative control	6	3	2	0	0	0
Lupus nephritis model control	0	0	1	0	6	2
Abatacept nanoemulsion-sc (nSC)	0	4	5	1	0	0
Abatacept-sc	0	2	5	3	0	0

Role of B7-1 deficiency in IC deposits

Immunofluorescence studies showed that LN-model mice had significant IC deposits, which were reduced significantly after treatment with a subcutaneous formulation of abatacept (Figure 3).

B7-1 deficiency alleviates renal lesions

Following abatacept treatment, a protuberance in epithelial cells in the cortical and medullary regions was observed. In addition, there were loss of nuclei and degeneration of vacuoles in the kidney tissue. The kidney injury score was higher in the treatment groups than in the control group after 24 h of treatment (Figure 3B).

Cytokine IL-4 and interferon (IFN)-γ responses in B7-1-deficient mice

To correlate the improvement of renal function with inflammation-mediated autophagy, the levels of pro-inflammatory cytokines, such as IFN-γ, and anti-inflammatory cytokines, such as IL-4 were evaluated. Mice treated with abatacept nanoemulsion showed decreased levels of pro-inflammatory cytokines and increased levels of anti-inflammatory cytokines ($p < 0.05$, Figure 4).

Infiltrating cell functions and quantities are reduced in B7-1-deficient mice

Following activation, several markers (CD11b, CD11c, Gr1, and CD86) were evaluated to determine the role of the B7-1 signaling pathway. A significant decrease in the levels of CD11b, CD11c, Gr1, and CD86 in mice treated with either formulation of abatacept was observed (Figure 5).

DISCUSSION

The results of this study support the hypothesis that T lymphocytes play a central role in the pathogenesis of LN; therefore, targeting T-cell function could be an effective strategy.

In recent years, clinical trials have been conducted for agents that target T cells directly

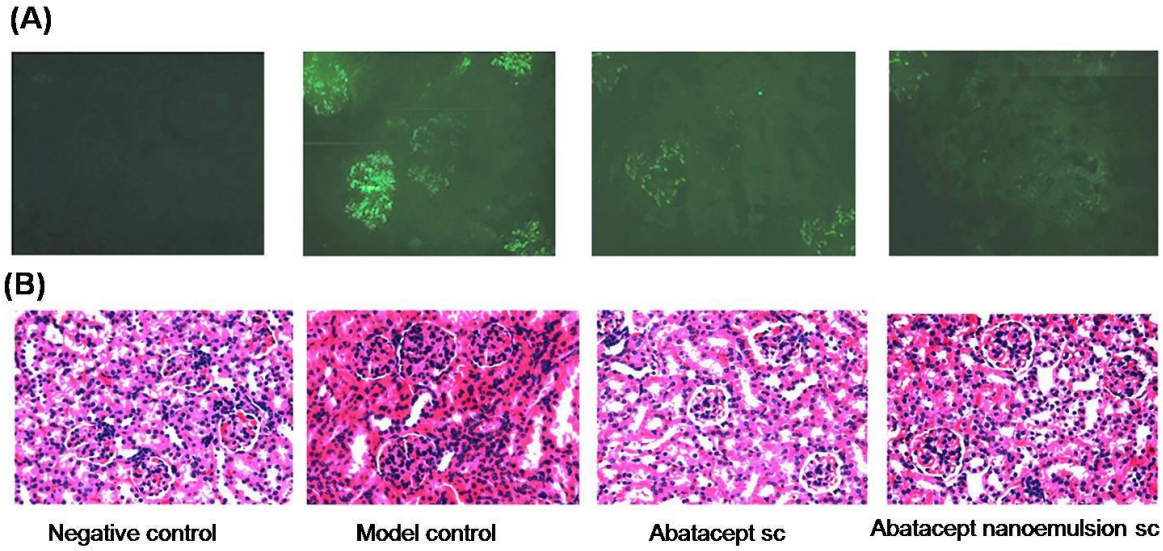

Figure 3: (A) Immunofluorescence detection of immune complex (IC) deposits in renal glomeruli in mice treated with abatacept nanoemulsion. (B) Hematoxylin and eosin (H&E)-stained kidney tissue of abatacept nanoemulsion-treated mice

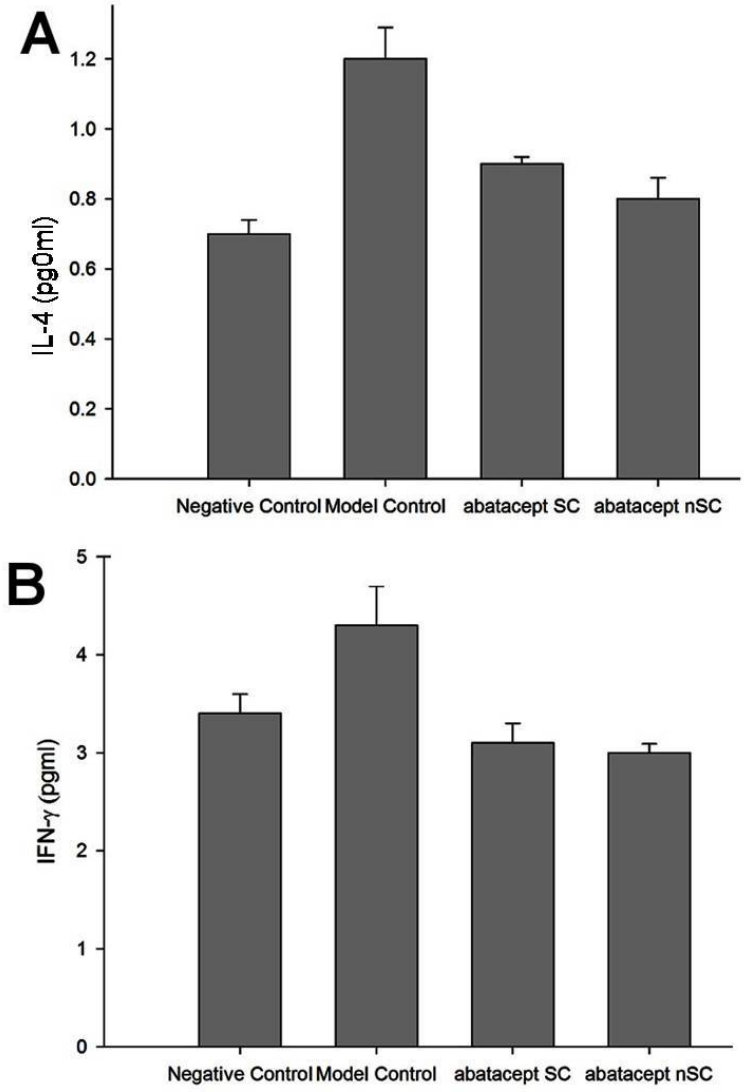

Figure 4: Expression of interleukin-4 (A) and interferon-γ (B) in mice 3 months after pristane injection; * denotes a significant difference

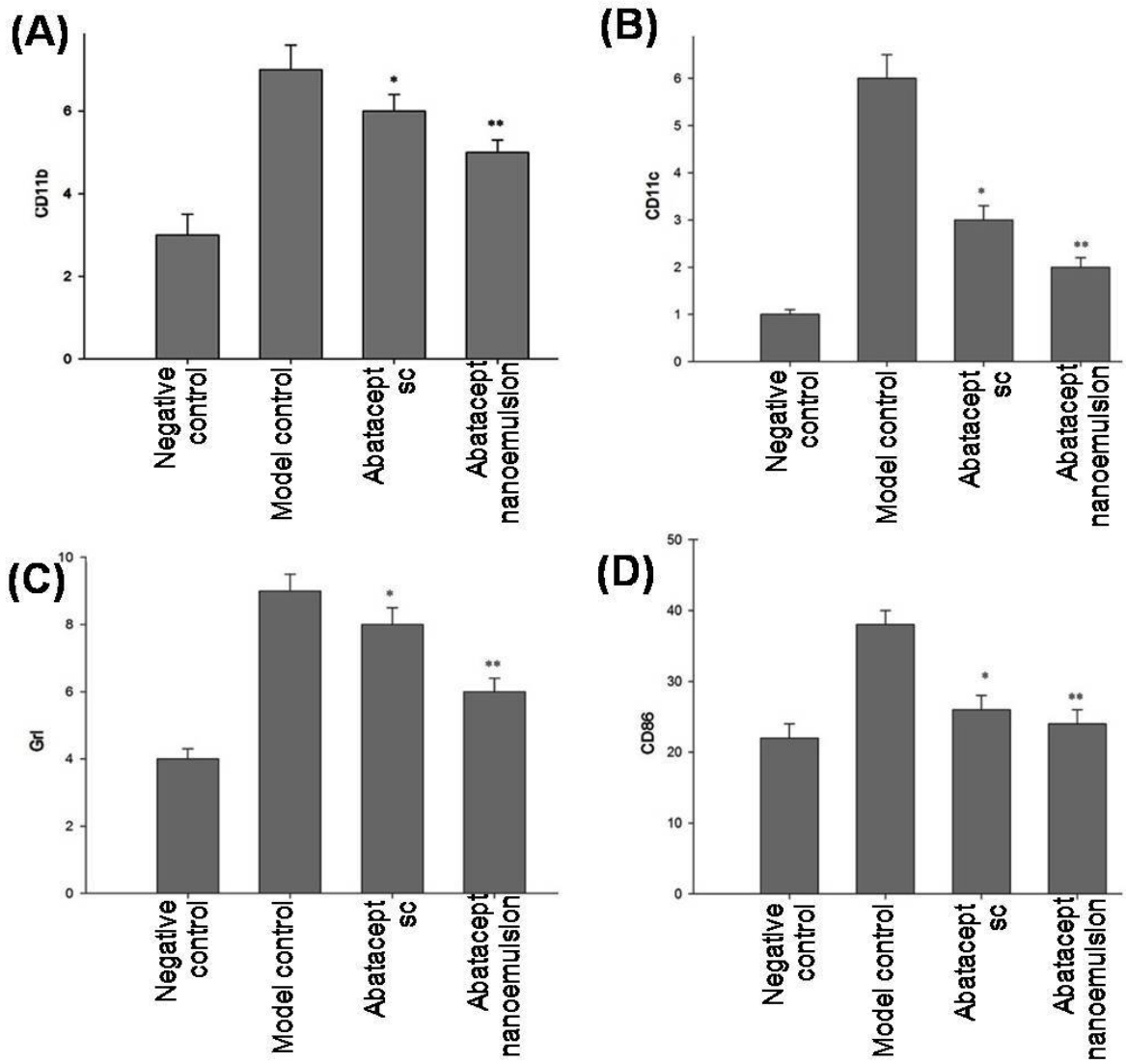

Figure 5: Expression of CD11b, CD11c, Gr1, and CD86 in spleen cells after abatacept treatment; * denotes a significant difference

or indirectly [7]. We observed substantially higher lung function parameters in individuals belonging to the abatacept group compared to the placebo group. The neutrophil level was also significantly higher in subjects belonging to the placebo group than that observed in the abatacept group. Furthermore, our logistic regression model demonstrated that subjects with allergy have increased odds of having LN, elevated uric acid levels, and higher brain-type natriuretic peptide (BNP) levels.

LN has been identified as an independent risk factor for developing ischemic heart disease in subjects with essential hypertension [17]. Furthermore, a number of studies showed that LN is associated with cardiac disease. A relationship between LN and left systemic lupus erythematous (SLE) was evident from the Strong Heart Study [23]. Our study also demonstrated a significant association between LN and cardiac changes irrespective of the co-morbidities surveyed [8 17]. Allergic LN is associated with systemic endothelial damage resulting from increasing blood pressure or from dysfunction of the capillary endothelium [24]. Our finding is consistent with previous studies showing subclinical target organ damage, as the prevalence of LN was significantly higher among prehypertensive patients than in individuals with optimal blood pressure [16]. This finding suggests that individuals with prehypertension need to be monitored intensively. No fiber formation was observed in the kidney tissues of the mice treated with abatacept 1. In contrast, the control group mice showed the presence of interstitial cells and collagen, which is consistent with earlier reports [25]. However, treatment of

the mice with abatacept for 10 days prevented the accumulation of interstitial cells and collagen deposition.

We observed an elevated level of forced expiratory volume 1 (FEV1) in pre-hypertensive patients. FEV1 is secreted from renal cells in the ventricles in response to rising ventricular filling pressure [26]. Although increasing blood pressure levels in the ventricles stimulate the release of FEV1, this ventricular load is related to the cardiac output, peripheral resistance, and stiffness of the arteries [27]. As FEV1 is released in response to stretching, it is also reflected in the level of stiffness of the arteries and arterioles. Consequently, treatment that inhibited LN induced a reduction in body weight. Animals in the abatacept treatment group showed increase in body weight similar to those in normal mice. In patients with diabetes mellitus, blood glucose increases significantly, which then leads to the deterioration of health and induces several side effects [28]. A significant increase in blood glucose in LN-induced mice is consistent with that reported in earlier studies. LN is associated with the production of large quantities of oxidative species and the suppressed formation of antioxidants due to the intervention of sugar molecules [28]. The results from the present study revealed that abatacept treatment in the LN-model mice significantly promoted the activity of superoxide dismutase. In addition, malondialdehyde levels were reduced significantly compared to those in untreated mice. Therefore, abatacept causes the reduction in the expression of inflammatory cytokines. The results of this study suggest that abatacept can be a useful option for the treatment of refractory LN.

CONCLUSION

The findings of the present study demonstrate the efficacy of the subcutaneous nanoemulsion formulation of abatacept compared to its conventional formulation. A systematic clinical trial is required to establish the long-term efficacy of the nanoemulsion formulation of abatacept in the treatment of refractory lupus nephritis.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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