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RESEARCH PAPER

Sublingual immunotherapy by Nanogold in mice model of asthma

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ABSTRACT

Objective(s): It has been shown that Nanogold particles have anti-inflammatory effects in different Rheumatologic, neurologic and gastrointestinal disease. They inhibit the synthesis of pro-inflammatory cytokines and also infiltration of inflammatory cells. Sublingual immunotherapy is a well-known effective, safe and clinically effective method way of immune response regulation which results in long-lasting symptoms reduction. This research was designed to find the immunological effects of sublingual immunotherapy using Nanogold in mice model of asthma.

Materials and Methods: Twenty BALB/c mice were divided into four groups including one group of non-sensitized mice and three groups of asthmatic mice which were treated sublingually with PBS, Nanogold and Beclomethasone. IL-4 and IFN-γ levels were measured in serum and spleen cells supernatant using ELISA. BAL fluid inflammatory cells differential counting and lungs histological analysis were also done.

Results: The results revealed that there was significant increase in level of IFN-γ and decrease in level of IL-4 in serum and spleen cells supernatant of Nanogold treated group ($p<0.05$). These findings indicates the shift of Th2/Th1 balance towards Th1 cells which is protective against asthma. In addition, histological and BAL fluid analysis demonstrated the reduction of cells and eosinophilic infiltration.

Conclusion: Based on our results, sublingual immunotherapy by Nanogold has significant anti-inflammatory roll in asthmatic mice. Thus, Nanogold is a potentially valuable agent for controlling the underlying inflammation in asthma. However, further investigations is recommended to find more details about its effects.

Keywords: *Asthma, Gold, Nanomedicine, Sublingual immunotherapy*

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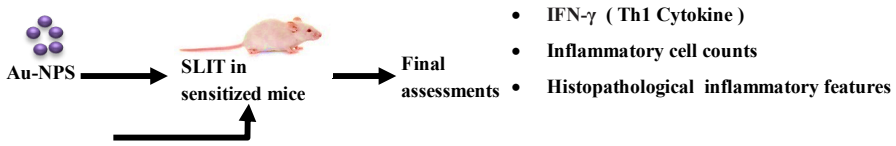
Graphical Abstract



INTRODUCTION

Asthma is a well-known chronic disease affecting about 300 million people around the world. Recent studies predicted that the prevalence will be even more in the next decade. Although billions of dollars are spent each year to manage the asthma, it's still associated with morbidity, mortality and also has adverse effects on the quality of life and productivity at work and schools [1, 2]. In asthma, the interactions of the innate and adaptive immune systems (macrophages, eosinophils, mast cells, Th2 cells and Th17 cells) act together with epithelial cells

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to develop inflammation in conducting airways. These cells can augment the inflammatory response by producing inflammatory mediators, such as Th2- associated cytokines (interleukin [IL]-4, IL-5, IL-9 and IL-13), Th17-associated cytokines, and some other pro-inflammatory cytokines [3, 4]. This process is also associated with bronchial hyper-reactivity, mucus overproduction, airway wall remodeling and airway narrowing [4, 5] which finally lead to signs and symptoms of asthma such as wheeze, shortness of breath and chest tightness. Nanotechnology, as an emerging field of progress, is attracting much attention because of its extensive application in different fields such as medicines.

Thanks to their novel size-dependent properties and surface properties for tissue penetration, therapeutic Nanoparticles (NPs) had an indisputable impact in medical sciences with great consideration in scientific research [6, 7].

Many researches have been done to study the effects of NPs on pulmonary responses. Even though it has been shown that NPs such as CeO2 NPs, NiO NPs, ZnO NPs, and CuO NPs may induce the inflammation [8], other studies revealed that healthy individuals respond differently to NP exposure in comparison with individuals with a pulmonary disorder such as asthma. For example, a recent study showed that exposure to Iron Oxide NPs increases the numbers of neutrophils, eosinophils, and lymphocytes in the airways of non-sensitized mice while exposing sensitized mice to these NPs causes cellular reduction in the alveolar space [9].

Gold (Au) has been used for several years for therapeutic purposes [6]. Nanogold have been studied and widely used in medical fields such as prevention, diagnosis and drug delivery systems [7, 10]. Recent studies revealed that Nanogold have anti-inflammatory effects in different disease. Although Nanogold has interactions with different types of immune cells, the precise mechanism requires further research.

However, the cellular uptake of Nanogold in chronic inflammation can occur via Macrophage phagocytosis. It has been shown that Nanogold can significantly inhibit synthesis of pro-inflammatory cytokines such as tumor necrosis factor-a (TNF-α), IL-1β, cyclooxygenase-2 (COX-2) as well as inhibition of infiltration of inflammatory cells in joint in Rheumatoid arthritis [10].Another study showed that Nanogold can inhibit pro-inflammatory cytokines expression such as TNFα and IL-6 within the fat tissue which are produced by the adipose tissue macrophages [11].

The anti-inflammatory effects of Nanogold in gastrointestinal inflammatory disease [12], Osteoarthritis [13], cognitive disease and neuroinflammation have been documented [14]. Although avoiding the allergens and other triggers is a common way for preventing the signs and symptoms of asthma, strict avoidance of exposure to most triggers is impossible in daily life and can't guarantee a successful treatment [15]. Anti-inflammatory therapies such as Glucocorticoids are the major therapy for asthma, but they have many side effects. In addition, they are not always effective for patients.

Furthermore, in spite the reality that asthma therapies such as inhaled corticosteroids and long-acting β-agonists can effectively reduce the bronchoconstriction and symptoms, little improvement has been observed in the past decade regarding new drugs discovery [16, 17]. Therefore, the scientific research community turned its attention towards new anti-inflammatory therapies [17].

Sublingual immunotherapy (SLIT) is recognized as one of the best etiology-based treatment of allergic diseases. It is a safe and clinically effective method which results in long-lasting symptoms reduction [18, 19]. Other advantages of SLIT are convenient administration and also prevention of new allergic sensitizations and disease progression[20, 21]. It has been shown that SLIT may shift the allergy promoting Th2 cells toward Th1 cells which are protective against asthma [22]. However, to our best knowledge no publications can be found in the literature that discusses the immunological effects of Nanogold in the treatment of asthma.

Thus, the present study was designed to investigate the effect of SLIT using Nanogold in asthmatic BALB/c mice.

MATERIALS AND METHODS

Materials

Commercial available suspension of Nanogold particles (stabilized suspension in citrate buffer, core size: 20nm) was purchased from Sigma-Aldrich, USA (product number: 741965). These particles were prepared by adding hydrogen tetrachloroaurate (HAuCl4) to PBS (10 mM, pH 7.2) solution.

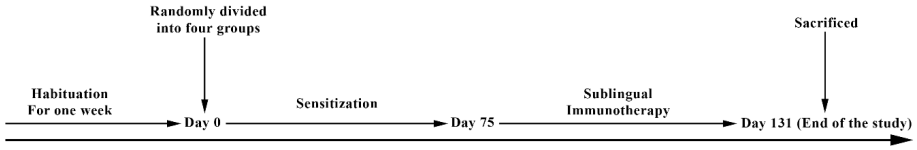

Fig 1. Schematic representation of the experimental model. Time course study of asthma induction and treatment with Beclomethasone and AuNPs in mice model of asthma

Also, UV–vis spectroscopy showed that particle concentration was ~ 11 nM (*20* µg/ml) with maximum absorption peak at 520 nm. Beclomethasone (APO-Beclomethasone Nasal Spray Toronto, Canada) was prepared by being dissolved in sterile phosphate-buffered saline (PBS). BCA protein assay kit was provided by Parstous, IRAN. IL-4 and IFN- γ were measured by ELISA kit according to manufacturer's instructions (Zell Bio Company, Germany). Other chemical solvents and reagents were commercial products of analytical or reagent grade and were used without further purification.

Animals

The guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) were followed in this research. The experimental protocol was approved Animal Ethics Committee of Islamic Azad University of Mashhad (IR.IAU.MSHD.REC.1396.148). Twenty Balb/c male mice (6-8 weeks) were purchased from the Razi Institute (Mashhad, Iran).The animals were maintained at 24±2 on 12h light-dark automatically rhythm. After one week of habituation, we divided the mice randomly into four groups (*n* = 5 per group). First group was non-sensitized non-treated control group that passed all steps, but was nebulized with PBS (as the placebo). Other groups were sensitized and treated with PBS, Beclomethasone, and Nanogold.

Induction of asthma by* ovalbumin *(OVA) sensitization and challenge

Methods of animal sensitization were similar to our previous study[16] and the guidance of European Respiratory Society task force. Briefly, mice in all groups except the control group were injected intraperitoneally with aluminum hydroxide and ovalbumin. Then, inhalation of OVA (and PBS for control group) for 15 courses were given to the animals over 75 days. Samples including bronchoalveolar lavage fluid (BALF) and lung tissues were collected 48 h after the final OVA/PBS challenge.

Treatment with Nanogold and beclomethasone

An overview of treatment is shown in Fig 1. Mice in Nanogold group were treated 5 days per week for 8 consecutive weeks using sublingual administration of Nanogold particles(20 µg/kg) dissolved in sterile phosphate buffer (10 mM, pH 7.2).

Total volume of each administration was 50 µl per mouse. The Beclomethasone (150 mg/kg body weight) was nebulized into the animal box every day for 8 weeks.

Cytokine assay

Levels of IL-4 (Th2 hallmark cytokine) and IFN-γ (Th1 hallmark cytokine) in the serum and also spleen cells supernatants were measured using specific enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Blood was sampled from mouse periorbital venous sinus and its serum was separated and was rapidly frozen at -80°C until assay. Spleen cells of each mice were harvested in RPMI-1640 medium (Gibco) with 10% fetal bovine serum containing penicillin and streptomycin using standard procedures. Cell supernatants were collected after 72 hours for evaluation of above mentioned cytokines.

Bronchoalveolar lavage and cytological evaluation

Mice trachea were lavaged using PBS (4 ml) to collect the BAL fluid for differential counts of inflammatory cells. The procedure of BAL collection was described in our previous study [23]. The BAL fluid were examined for inflammatory cells differential counts and cytokines assay. For differential cell count, a smear was prepared and stained with May Grunwald-Giemsa. Based on standard morphological criteria at least 300 cells were studied. Total cell and *differential cell counts of* inflammatory cells (macrophages, lymphocytes, eosinophils, and neutrophils) were measured and compared among different groups.

Table 1. Cytokine assay Comparing mean level of cytokines of serum and splenocyte culture supernatants and the IFN-γ/IL-4 ratio in different treatment groups (A: Non-asthmatic group - B: PBS treated group - C: Nanogold treated group - D: Beclomethasone treated group). Values are presented as mean + S.D

	Serum			Spleen cell culture		
	IL-4 (pg/ml)	IFN-γ (pg/ml)	IFN-γ/IL-4 ratio	IL-4 (pg/ml)	IFN-γ (pg/ml)	IFN- γ /IL-4 ratio
A	5.28 + 1.28 [b]	9.15 + 3.22	1.7	16.95 + 2.77 [b]	86.72+ 28.21	5.1
B	14.10 + 2.08 [a]	12.38 + 3.18	0.8	51.87 + 11.85 [a]	119.67+31.72	2.3
C	7.67 + 1.69 [b]	17.33 + 4.20 [a]	2.2	21.63+ 2.54 [c]	168.52+32.46 [a, b]	7.7
D	7.92 + 2.55 [b]	16.00 + 2.68 [a]	2.0	9.80 + 2.51 [b]	159.23+15.47 [a]	16.2

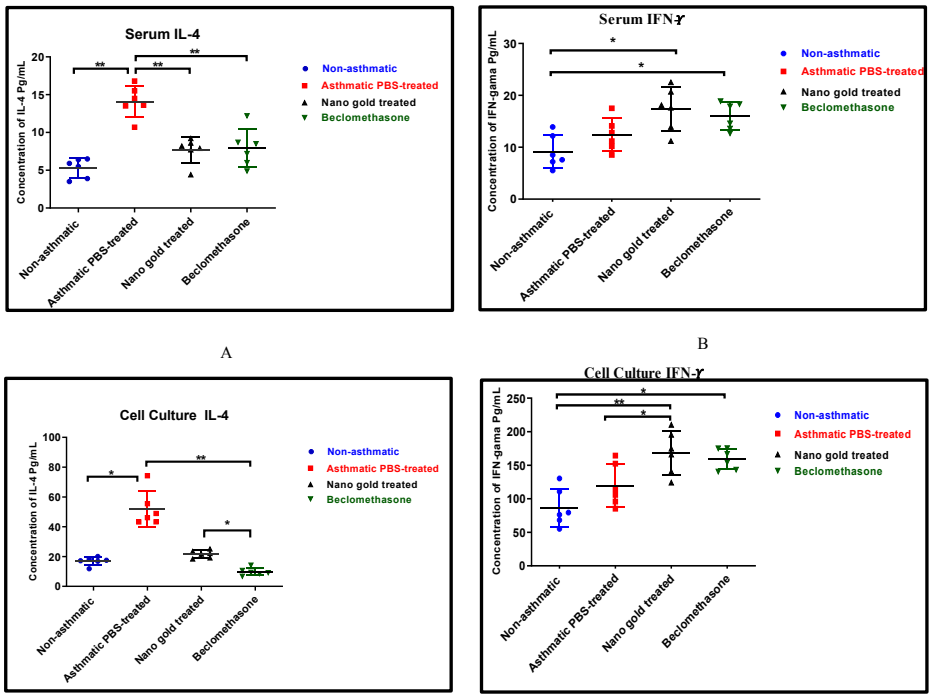

Fig 2. Comparing the effect of treatment on the levels of cytokines in different groups (*P<.05 and **P< .01)

Histological examination

Histological examination of lung tissues were performed based on the manufacturer's protocol. Both lungs were perfused with 5 ml of 10% buffered formalin (pH 7.4) through the trachea. Lung tissues were excised and samples were taken from the upper portion of left lung. Then, tissues were embedded in paraffin, and cut at 3 mm thickness. Tissue sections were stained with hematoxylin and eosin (H&E) and were analyzed by an animal pathologist. Perivascular cell infiltration, formation of mucus plaque, smooth muscle hyperplasia and collagen deposition were measured semi-quantitatively for further analysis.

Statistical Analysis

GraphPad Prism 5 (version 5.01, GraphPad Software, Inc.) was used for statistical analysis. The data have been expressed as mean ± standard deviation. We compared the means using one-way analysis of variance (ANOVA) and two- way ANOVA followed by Tukey-Kramer test and the p-value ≤0.05 was considered statistically significant.

RESULTS

The results of different cytokines, cellular and histological assessments are explained and analyzed in this sections. Charts, tables and Figs are used to show and compare the outcomes in different groups.

Cytokine levels in the serum and splenocyte culture

IL-4 as a key inflammatory cytokine in shifting of T-cells to Th2 subgroups, and also IFN-γ which is an inflammatory marker of Th1 were measured in order to find any deviation in Th1/Th2 balance. Th1 dominant milieu is in favor of asthma.

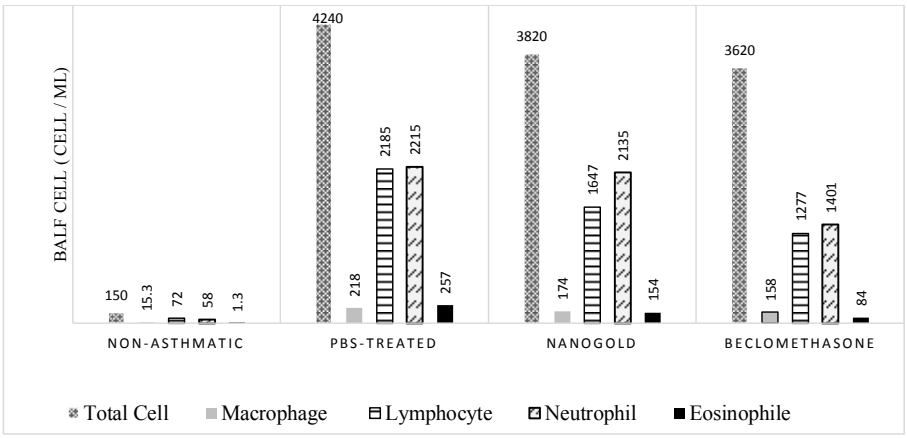

Fig 3. Effect of Nanogold and beclomethasone on the bronchoalveolar lavage fluid inflammatory cells counts per milliliter

Table 1 contains mean concentrations of IL-4 and IFN-γ in the serum and splenocyte culture supernatant. As it is shown, treatment with Nanogold led to IL-4 downregulation and IFN-γ upregulation compared with the normal group and there is a significant difference among mean level of different groups. As it is shown in Fig 2, there is a significant decrease in the serum level of IL-4 in the Nanogold treated group comparing with the PBS treated group ($p<0.05$). However, no statistically significant difference is seen in the level of this cytokine in splenocyte culture supernatants in this regard. Further data analysis revealed that Nanogold treated group had significantly higher serum levels of IFN-γ ($p<0.05$). In addition, splenocyte culture supernatant levels of IFN-γ were also significantly higher in Nanogold treated group comparing to both PBS and Beclomethasone treated groups ($p<0.05$).

IFN-γ/IL-4 ratio in the serum and BAL fluid is shown in table 1. This ratio changed in serum from 0.5 in PBS treated/Non-asthmatic to 1.3 in Nanogold/Non-asthmatic. Furthermore, this ration increased in the splenocyte culture supernatant from 0.44 in PBS treated/Non-asthmatic to 1.52 in Nanogold/Non-asthmatic.

BALF Cellular infiltration

Inflammatory cells of BALF were counted to inspect the effect of Nanogold on cellular infiltration as a part of asthma pathophysiology. As shown in Fig 3, total counts of inflammatory cells in the BALF were higher in PBS treated group in comparison to the Non-asthmatic group. Treatment with beclomethasone decreased the total counts of inflammatory cells. However, there was no significant difference in total inflammatory cell counts of Nanogold-treated group versus the PBS-treated group.

Effect of Nano gold on lung histological changes

The histological features of each group are shown in Fig 4. Structure of lung tissues of non-asthmatic group were well defined with no significant inflammatory cells infiltration, damage or edema (A). However, a remarkable perivascular

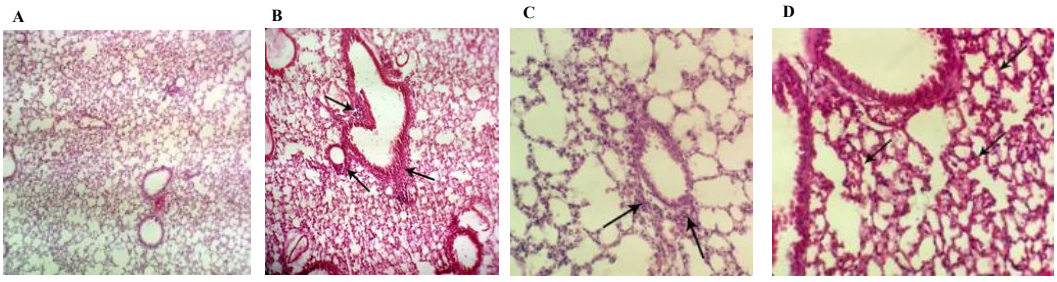

Fig 4. Formalin-fixed lung sections were stained with hematoxylin and eosin (H&E). Magnification is ×40 in A, ×100 in B, C, and D. (A) Non-asthmatic group. (B) PBS treated group: arrows show remarkable infiltration of inflammatory cells. (C) Nanogold treated group: arrows show mild peribronchial inflammation. (D) Beclomethasone treated group: Histology of the lung was normal however alveolar capillaries were seemed congested (arrows).

inflammation, edema, bronchial smooth muscle hypertrophy and squamous metaplasia of airways epithelium was seen in the lung tissues of asthmatic mice in PBS-treated group (B). As it is shown, the Nanogold treated group had weak perivascular inflammatory cell infiltration (C) and significantly less inflammation in comparison to the PBS treated group. On the other hand, the histological studies did not show significant histological difference between the normal group and Nanogold treated group (D).

DISCUSSION

We designed this clinical trial to assess the effect of sublingual immunotherapy (SLIT) using Nanogold particles on inflammatory cells and mediators in asthmatic mice. Attractive properties of Nanogold such as different biological effects [24], low cytotoxicity [25] made it a key element in the therapy of various inflammatory disease. In addition, these particles showed anti-inflammatory effects in treatment of some diseases. The present study revealed that SLIT with Nanogold diminished the inflammatory cytokine production and inhibited the infiltration of inflammatory cells in asthmatic mice. Significant decrease of Th2 related cytokines such as IL4 and also increase of IFN-γ which is an inflammatory marker of Th1 was seen. This shows that the underlying inflammatory response shifted from Th2 toward Th1 cytokines which is an effective strategy in asthma treatment. The overall findings suggest that SLIT with Nanogold could have anti-inflammatory effects in asthma.

One of most remarkable results to emerge from our results is that the levels of IL-4 in serum and splenocyte culture medium supernatant was significantly suppressed in mice that received Nanogold. IL-4 as a key inflammatory cytokine is involved in asthma via its essential role in many cellular mechanisms by IgE isotype-switching in B cells and eosinophil chemotaxis [26]. It is also a critical factor in the regulation of T-cell deviation towards Th2 subgroup.

In addition, it causes airway remodeling through regulating numerous genes involved in collagen and fibronectin synthesis which are fundamental elements of airway remodeling. So, the inhibition of IL-4 can play a crucial role in shifting the Th1/Th2 balance toward Th1 subgroup and also preventing airway remodeling in patients with severe asthma [26, 27]. Moreover, there was a reduction in eosinophils count of BAL in Nanogold treated group. Our results shared a number of similarities with the study that was designed by Barreto et al. [28] to evaluate the potential effect of nasal-instilled Nanogold to prevent allergen-induced asthma in mice. In this research, Nanogold clearly inhibited allergen-induced accumulation of inflammatory cells as well as the Th2 pro-inflammatory cytokines such as IL-4 in the lung.

Further data analysis revealed that the serum and splenocyte culture medium supernatant levels of IFN-γ were significantly elevated in Nanogold treated group. IFN-γ/IL4 ratio which is regarded as an index for treatment response, was also increased in Nanogold and Beclomethasone treated groups.

There was no significant difference between the effect of the Nanogold and Beclomethasone in this regard. These findings suggest a suppressing effect of the Nanogold on the immune deviation from Th2 toward Th1 response. The results are consistent with previous studies which showed that nanoparticles such as peanut extract nanoparticles [29] and lipopolysaccharide-loaded PLGA nanoparticles [16] can suppress the Th2-associated immune responses and upregulate the IFN-γ.

The anti-inflammatory response of SLIT by Nanogold was accompanied by a trend towards a decrease in total number of inflammatory cells of BAL. However, controversial results have been reported in literature. Gosens et al. reported that a single dose (250 nm) of gold nanoparticles delivered in the rat's lung by intratracheal instillation could induce a significant increase in the percentage of neutrophils after 24 hours [30]. The reason for this disagreement among results is unclear. However, A possible explanation is that the Nanogold can show different immunogenicity in organisms depending on the technical issues such as dose, duration of treatment and surface modification of particles [28, 31].

Histological examination of lung tissues revealed a mild inflammation in Nanogold treated group. This finding is consistence with the research done by Hussain et al. which revealed that gold nanoparticles causes a mild neutrophilic inflammation, edema and limited epithelial damage[32]. However, the inflammation in Nanogold treated group was less than PBS-treated group and was similar to the Beclomethasone

treated group which is an evidence for anti-inflammatory role of Nanogold. These findings are in line with results of Barreto et al. [28] research, in which treatment with Nanogold inhibited eosinophil-rich inflammatory leukocyte infiltration in the peribronchiolar space of the lung tissues.

Sublingual immunotherapy as a generally acceptable and fundamental method for asthma immunotherapy, has been widely studied. On the other hand, it has been shown that Nanogold can have anti-inflammatory and immunomodulatory effects in different tissues when applied subcutaneous and intraperitoneally [31]. However, the knowledge about the effects of systemic application of Nanogold on pulmonary system is mostly limited to the toxicological aspects of it and no data is available in current literature regarding its immunomodulation properties in asthma. In our research, Nanogold showed anti-inflammatory activity when administered sublingually.

It is plausible that a number of limitations might have influenced our results. Unfortunately it wasn't possible to measure all important cytokines that take part in asthma pathophysiological process to achieve more precise results. Further studies on animal and human subjects is recommended to learn more about the anti-inflammatory effects of Nanogold in asthma. We also suggest more extensive works for the future that involve different treatment doses and also longer duration of treatment to learn more about details of remaining issues.

For asthmatic patients, providing a better quality of life depends on relieving the symptoms of their disease.

Inflammation, as the key to asthma pathogenesis, has been the main target for biopharmaceutical researches. So, the necessity of developing new therapies which can target more specific inflammatory key cells and mediators is apparent.

The evidences of this study revealed that Nanogold can alter the asthma inflammation by redirecting the Th1/Th2 toward the Th1 dominant pattern. Taken together, these findings suggest that Nanogold has potentially safe immunotherapeutic effects that can assist in controlling asthma inflammation.

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