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IMMUNE REGULATION OF INFLAMMATORY ARTHRITIS BY A NATURAL PRODUCT RESVERATROL

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Declaration

I declare that I am the author of this thesis entitled "Immune regulation inflammatory arthritis by a natural product resveratrol". This work has never previously been submitted for a higher degree.

All research was conducted at the Division of Immunology, Infection and Inflammation, University of Glasgow, under the supervision of Dr. Damo Xu.

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Abbreviations

Ab	antibody
Ag	antigen
AhR	aryl hydrocarbon receptor
ANA	antinuclear antibody
APC	antigen presenting cell
C5	complement factor 5
CCP	cyclic citrullinated peptide
CD	cluster differentiation
CFA	complete freund's adjuvant
CIA	collagen-induced arthritis
COX	cyclooxygenase
CRP	C-reactive protein
CTLA4	cytotoxic T lymphocyte activation antigen-4
DCs	dentritic cells
DLNs	draining lymph nodes
DMARDs	disease-modifying anti- rheumatic drugs
DMSO	dimethyl sulphoxide
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
FACS	fluorescent activated cell sorting
FCS	fetal calf serum
H&E	heamatoxilin and eosin
HLA	human leukocyte antigen
ICAM	inter-cellular adhesion molecule
IFA	incomplete freund's adjuvant
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LOX	lipoxygenase
LPS	lipopolysaccharide
MHC	major histocompatibility complex

MMP	matrix metalloproteinase
NBF	neutral buffered formalin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NSAIDs	non-steroidal anti-inflammatory drugs
OD	optical density
PBS	phosphate buffered saline
RA	rheumatoid arthritis
Resveratrol	3, 5, 4'-trihydroxy-stilbene
RF	rheumatoid factor
Rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SLE	systemic lupus erythematosus
Th	T helper
TNF	tumour necrosis factor
UV	ultra violet
VCAM	vascular cell adhesion molecule

Abstract

Rheumatoid arthritis (RA) is one of the most common chronic diseases worldwide. It is characterised by inflammatory cell infiltration and bone erosion. Current therapy using non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids are not very effective and can have severe side effects. New generation recombinant drugs while effective are costly. Thus, better therapeutic strategies are critically needed. Natural products with fewer side effects and which are less expensive may represent ideal drugs for clinical RA. Resveratrol is a phytoalexin mainly derived from the skin of red grapes. Many *in vitro* and *in vivo* studies have demonstrated that resveratrol has a wide-range of anti-oxidant, anti-proliferative and anti-inflammatory properties. The aim of this project was to establish whether resveratrol has an effect on the development and pathogenesis of inflammatory arthritis.

Collagen induced arthritis (CIA) is a well-studied animal model for human rheumatoid arthritis was used here to study the role of resveratrol in RA. It was first determined whether resveratrol could prevent CIA. CIA mice were administered with different doses of resveratrol before the onset of arthritis. It was found that 20mg/kg but not 15 mg/kg of resveratrol reduced CIA clinical parameters and severity of joint histology respectively. Second effect of resveratrol on established CIA was investigated and there was evidence that resveratrol, when given after the onset of CIA, could effectively suppress the progress and severity of CIA. The clinical anti-arthritic effect of resveratrol was accompanied by the reduction of collagen-specific antibodies in CIA mice. Additionally, resveratrol treatment also dramatically reduced the levels of inflammatory cytokines, in particular, IL-17, IFN-γ and TNF-α in the serum. The number of T lymphcytes was also reduced, in particular the IL-17 and IFN-γ expressing $CD4^+$T cells in DLN.

The results therefore demonstrate that resveratrol has the property to prevent and treat inflammatory arthritis by modulating the key auto-antigen-specific antibody and T cell responses. Thus, resveratrol, a natural product may have the potential to be used as a novel drug for arthritis.

1. INTRODUCTION

1.1 Rheumatoid arthritis

RA, a chronic inflammatory disease, is characterised by articular redness and swelling, together with cartilage and bone destruction, although the haematological, cardiovascular and respiratory systems are also frequently affected. This systemic autoimmune disease is induced by a combination of genetic, immune and inflammatory factors. RA is a common disease worldwide and is one of the major causes of morbidity and premature mortality.

1.2 Pathology of rheumatoid arthritis

1.2.1 Joint pathology

The joints most commonly involved in RA are those of the hands and feet, then the elbow, wrist, knee, ankle, hip and spine. Hyperplasias of synovial cells occur in the joint, which can form villi protruding into the joint cavity. Connective tissue under the synovium is infiltrated with lymphocytes, macrophages and plasma cells. Furthermore, endothelial cells highly express inter-cellular adhesion molecule (ICAM)-1, and this contributes to neovascularity [1]. Owing to this hyperplasia, inflammatory infiltration and neovascularity, the synovium forms a pannus, which gradually creeps through the whole surface of the cartilage. Finally, the joint cavity will be filled with fibrotic and calcified pannus, which leads to permanent ankylosis of the joint.

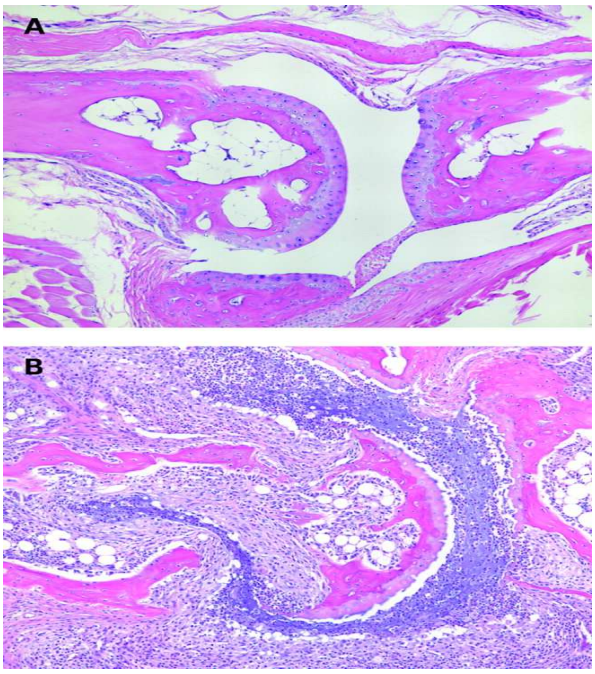


Figure 1. Histological changes observed in joint of CIA mice. It can be seen that compared with the normal joint (A), the joint suffering from arthritis (B) has significant infiltration of inflammatory cells. Bone erosion and hyperplasia are more severe. This figure is reprinted from (Utility of animal models for identification of potential therapeutics for rheumatoid arthritis) [2] with permission.

1.2.2 Non joint pathology

Because RA is a systemic disease, many organs can be affected. 25% of patients with RA have subcutaneous nodules. They can also have acute necrosis, and when serosa is involved, patients will suffer from pleurisy and pericarditis.

1.3 Symptoms of rheumatoid arthritis

Epidemiological studies indicate that RA can start at any age. However, 80% of sufferers who develop RA are initially aged between 35 and 50 and females are three times more likely than males to experience RA [3]. Although RA affects patients differently, for most people, it develops slowly and gradually. Before the typical articular symptoms appear, some sufferers can have flu-like symptom such as fever, as well as tiredness and weight loss.

1.3.1 Joints

1) Morning stiffness

   Affected joints feel stiff in the early morning for at least 1 hour after first waking up. This is called “morning stiffness” (such symptoms can also be present in the day time if there has been no movement for a long time). More then 95% of RA patients have such symptoms, and there is a positive relation between the duration of the stiffness and the severity of the disease.

2) Pain

   Joint pain is the earliest symptom. It is systemic and continuous, but the severity fluctuates. Patients with RA in the early stages can experience tenderness in their joints while in later stages sometimes brown discolouration of the skin occurs.

3) Swelling of the joint

   Swelling is caused by the excess fluid in the articular cavity and inflammation of the soft tissue surrounding the joint. Furthermore, the synovium thickens because of the inflammation. All the affected joints can swell, but the most common are the wrists, knuckles and knees.

4) Articular deformity

Joint deformity is usually present in the last stages of disease in long-term RA patients. Swan neck and boutonniere can be considered as types of deformity. Some individuals with severe RA lose joint function.

1.3.2 Non joint deformities

1) Lungs

It is common for lungs to be affected, but this occurs more often in men than in women. Sometimes lung illness can be the earliest symptom. Interstitial inflammation and fibrosis of the lung can present among RA patients, which is characterized by shortness of breath and impaired lung function. Pleural effusion is also closely associated with RA.

2) Heart

The heart can be affected both in patients with acute and with chronic RA, and pericarditis is the commonest type of heart disease in these patients. Additionally, RA can increase the risk of developing atherosclerosis, myocardial infarction and strokes.

3) Kidneys

Renal amyloidosis can occur as a consequence of vasculitis.

4) Nervous system

Peripheral neuropathy can occur due to pressure from swelling tissues, and there is a positive relationship between the severity of synovitis and neuropathy. Mononeuritis multiplex is caused by vasculitis.

5) The blood system

Felty's syndrome can be caused by RA. This is associated with an enlarged spleen, decreased neutrophils and platelets, and

anaemia. Anaemia is commonly associated with chronic inflammatory disorders and the severity of the anemia is closely related to the progress of rheumatoid arthritis.

1.4 Diagnosis of rheumatoid arthritis

The diagnosis of RA is primarily by clinical judgement and this can be supported by laboratory adjuncts.

1.4.1 Blood test

Some patients suffer from anemia with increased platelets. Increased erythrocyte sedimentation rate (ESR) and serum concentrations of C-reactive protein (CRP) reflect activity of the acute phase response and are closely related to the activity of the disease. Furthermore, the presence of rheumatoid factors (RF), the antoantibody can be indicative of RA although they can be transiently increased in a variety of inflammatory diseases. IgM is the most common isotype of RF in clinical tests, and is present in 70% of patients and is associated with the activity and severity of RA. However, RF is not an RA specific antibody. Research has indicated that 5% of a sample of the normal population had a low level of RF present [4]. Citrulline antibody (also referred to as anticitrulline antibody, anti-cyclic citrullinated peptide antibody, and anti-CCP) is most useful in investigating the aetiology of previously undiagnosed RA when traditional blood test indicates that RF is not present. Another antibody called antinuclear antibody (ANA) is frequently found in individuals with RA. 70% of patients tested are found to have these various immune-complexes present in their serum. This is more common in patients who are RF positive or in the active stage.

1.4.2 Synovial fluid

For normal people, the volume of fluid in the synovial cavity is no more than 3.5 ml. For individuals with RA, fluid increases in the

inflammatory joints with the enhanced level of white cells, and neutrophils are the predominant cell type.

1.4.3 Imaging

X-ray is useful to diagnose RA even though there may be no obvious changes at the early stage of the disease. However, as the disease advances, X-ray can show the bony erosion and subluxation. Apart from X-ray, other medical imaging techniques such as magnetic resonance imaging and ultrasound can also be used to diagnose and stage progression of RA.

1.5 Diagnostic criteria

The American College of Rheumatology has defined the following criteria for the classification of rheumatoid arthritis (1987):

1) Morning stiffness lasting more than 1 hour most mornings for at least 6 weeks.

2) Arthritic swelling present for at least 6 weeks in more than 3 out of 14 joints and soft tissues or joint groups.

3) Arthritis of hand joints, present for at least 6 weeks.

4) Symmetric arthritis, present for at least 6 weeks.

5) Subcutaneous nodules present in specific places.

6) Rheumatoid factor at a level above 95^{th} percentile.

7) Radiological changes suggestive of joint erosion

At least four of the above criteria have to be met for classification as RA [5].

1.6 Aetiology and mechanisms

1.6.1 Environmental factors

So far, no environmental factor has been defined as contributing to the development of RA, however, some microorganisms such as bacteria, viruses and mycoplasmas can affect the onset and progression of RA. RA is more likely to afflict people in western countries, suggesting that lifestyle and dietary habits should be considered as factors contributing to the incidence of rheumatic disease [6]. Moreover, recent data indicate that smoking and stress are also independent risk factors for the incidence of autoimmune disease [7, 8].

1.6.2 Genetic factors

Epidemiological studies indicate that there is a positive correlation between genetic factors and the incidence of RA. 12-30% of monozygotic twins will both develop RA, while for fraternal twins it is 4% [9]. International studies report that HLA-DR4 haploidy is closely associated with the onset of RA. Hormones also play a pivotal role, since epidemiology indicates that females are three times more likely to develop RA than males and the disease severity fluctuates during the menstrual cycle and pregnancy [10, 11].

1.6.3 Dysregulation of immunity and inflammation

Immune dysregulation is strongly associated with RA. The inflamed joint tissue is characterized by the infiltration of lymphocytes including activated $CD4^+$ T lymphocytes and antigen presenting cells (APC) and the expression of a spectrum of pro-inflammatory cytokines, such as TNF-α and IL-17 in the synovium**.** T cells, previously Th1 and now Th17 cells are thought to play a pivotal role in the initiation and persistence of inflammatory arthritis [12]. Pro-inflammatory cytokines, in particularly TNF-α are the main

pathogenic inducer that drives pathogenesis of RA [13]. It has been clinically confirmed that TNF blockage therapy can effectively control the severity and development of RA [14]. Activated T cells and macrophages can produce cytokines including tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and IL-17, which subsequently cause chronic inflammation in the synovium by different mechanisms. For instance, TNF-α can degrade the articular cartilage and bone, by stimulating osteoclast activity, leading to joint deformity. Additionally, activated B lymphcytes differentiate into plasma cells, and these release a large amount of autoantibody. These include rheumatoid factors, which may form an immune complex with self-antigens and can trigger inflammation through the activation of the complement system. The abnormality in the normal programmed cell death may be also involved in joint inflammation in RA. The excess Fas molecules, or the disproportion of Fas and Fas ligands, can interrupt the apoptosis of synovial cells. Thus, controlling the key cellular and humoral immune responses and the dominant pro-inflammatory cytokine productions may represent the possible therapeutic strategies against RA.

1.7 Treatment

Rheumatoid arthritis is normally treated with NSAIDs, and intra-articular therapies (glucocorticoids), which are not curative but merely palliative, aimed at reducing symptoms, decreasing disability and enhancing quality of life. Biological therapies, such as anti-TNF-α and anti-CD20 are effective, however, only 30 % of patients can receive these therapies and they are very expensive. This restricts their general application. Thus, economic drugs with more effciency and less side effects are still needed.

1.7.1 Non steriodal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylate (Aspirin), naproxen (Naprosyn), ibuprofen (Advil, Medipren, Motrin) and etodolac (Lodine) are medications, which can

decrease pain and inflammatory cell infiltration, but do not inhibit the progress of RA.

1.7.2 Gluco-corticosteroids

Along with NSAIDs corticosteroids are a “first line” medication. Both are fast acting but corticosteroids are more potent than NSAIDs in reducing joint inflammation. They can be used for a short period during the acute stage of the disease and the dosage depends on the severity of RA. However, corticosteroids exert many serious side effects, such as weight gain, high risk of infection, development of cataracts and thinning of the skin and bone. Also the abrupt withdrawal of corticosteroids can lead to a flare-up of the disease.

1.7.3 Disease-modifying anti-rheumatic drugs (DMARDs)

Unlike NSAIDs and corticosteroids, disease-modifying anti-rheumatic drugs (DMARDs) are “slowing acting” drugs, which are used for a long period of time. Examples of these include cytotoxic drugs methotrexate and immuno-suppressive drugs. Sometimes a number of DMARDs are used together as combination therapy.

1.7.4 Biological therapy

Compared with the traditional treatments, biological medications such as anti-TNF-α, anti-IL-1, anti-CD20 and anti-cytotoxic T lymphocyte activation antigen-4 (CTLA-4) are more directed and targeted. The best example of these is the recombinant human soluble TNF receptor (Etanercept or Enbrel) which has demonstrated extensive efficacy and limited side effects. Clinical trials indicate that newer drugs have anti-inflammatory effects and slow down the progress of bone erosion. Biological medications used in combination with traditional treatments, such as methotrexate and other DMSRDs can enhance their efficiency and reduce the negative side effects they cause.

Although the etiology of rheumatoid arthritis is still unknown, there is an increasing recognition among rheumatologists that early diagnosis and early medical intervention play a crucial role in improving outcomes. In some cases of severe joint deformity, surgery may be necessary.

1.8 Natural properties of resveratrol

The natural function of resveratrol is to protect plants against fungal infection (e.g. Botrytis cinerea) [15, 16]; environmental stress such as ultraviolet C irradiation [17] or heavy metal contamination [18]. These factors can increase the production of resveratrol [19].

1.9 Chemical and physical properties of resveratrol

Resveratrol (3, 5, 4'-trihydroxy-stilbene) is a phytoalexin first detected in the roots of white hellebore in 1940 [20]. There are two geometric isomers: cis-(Z) and trans-(T). The “trans” form is more stable than the “cis” form, which can transform into the “trans” form when exposed to ultraviolet irradiation [19]. A high concentration of resveratrol is found in the skin of red grapes [21], and it also occurs in cranberries, peanuts and pine nuts [22].

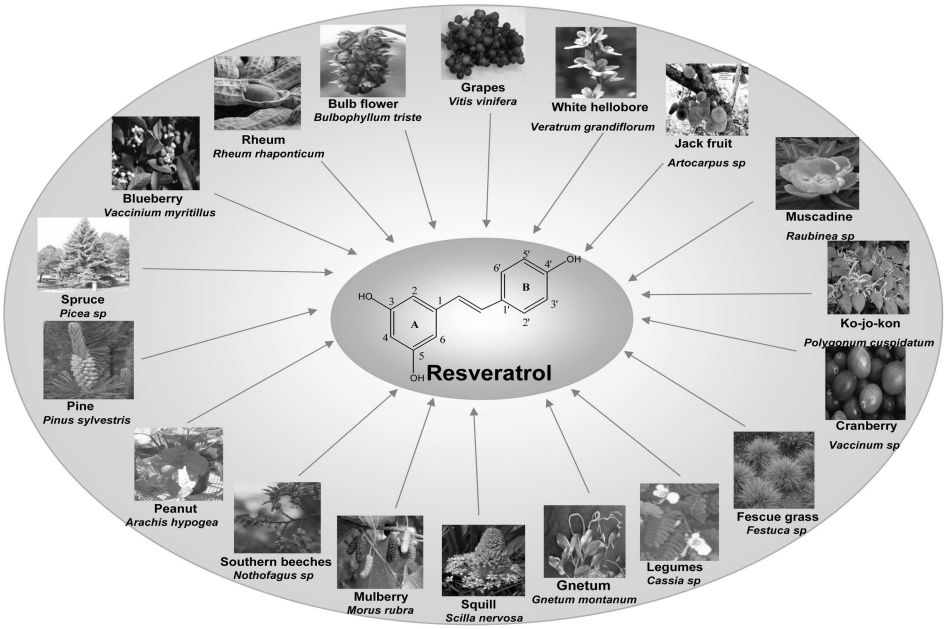


Figure2. The structure and source of resveratrol. A simple structural diagram of 3,5,4'-trihydroxy-trans-stilbene (resveratrol). This is a phytoalexin produced by a variety of plants; some indicated. This figure is reprinted from Resveratrol addiction: To die or not to die [19] with permission.

1.10 Biological effects of resveratrol

1.10.1 Anti-inflammatory effects

Some studies suggest that resveratrol could reduce C5a- induced lymphocyte recruitment, free radical formation and the production of cytokines [23]. Resveratrol also reduces signalling of IgE receptor ligation, and thus can alleviate inflammatory responses by blocking allergen mediated degranulation of mast cells [24]. In addition, resveratrol downregulates activation of the transcription of NF-κB, and suppresses LPS-induced joint inflammation and bone erosion in rabbit [25]. For example, in vitro experiments show that resveratrol can decrease TNF-α induced ICAM-1 and VCAM-1 expression in endothelial cells by regulating the activation of NF-κB [26]. The reduction of IL-4 and IFN- γ

production shows that resveratrol can block both Th1 and Th2 activation [27, 28].

1.10.2 Cardio protective effects

Since the discovery of the “French paradox” (low incidence of cardiovascular disease in southern France, perhaps due to the regular intake of red wine), resveratrol has been extensively researched and identified with effects on the cardiovascular system [23]. Three main effects have been identified: 1) resveratrol can decrease the expression of vascular cell adhesion molecule (VCAM)-1 [26]; 2) resveratrol can inhibit the proliferation of vascular smooth muscle cells [29]; 3) resveratrol can reduce platelet aggregation [19].

1.10.3 Anti-cancer effects

Animal experiments have shown that application of resveratrol can prevent melanoma developing in mice treated with chronic UVB exposure [30]. In vitro studies indicate that resveratrol can induce apoptosis of cancer cells [31]. Resveratrol can modulate a balance between cell death and proliferation and induce the apoptosis of cells which may contributtte to tumor formation. This proporty involve in the regulation of survial and apoptotic factors, including Bcl 2 as well as proteins involved in DNA synthesis, such as p53 [32].

1.10.4 Anti-aging and anti-diabetic effects

Investigation of resveratrol indicates that it can prolong the lifespan of yeast by stimulating the activity of Sir2, which can increase DNA stability and extend lifespan [33] and the fruit fly [34]. In an animal experiment, resveratrol was shown to reduce the blood sugar level in type 2 diabetes due to the ability to delay the onset of insulin resistance [35].

2. Materials and methods

2.1 Animals and reagents

Male DBA/1 mice were obtained from Harlan Laboratories UK. All mice were used at 9-10 weeks old and maintained at the Joint Animal Facilities, University of Glasgow. Mice were divided into three groups (n=10): prevention group, treatment group and control group. All animal experiments were performed in accordance with the Home Office, United Kingdom, and animal welfare guidelines.

Bovine Type II Collagen was purchased from MD Biosciences USA, which was 2mg/ml in dilute (0.1M) acetic acid. Resveratrol and other chemical were purchased from Sigma Aldrich (Poole, U.K.) unless otherwise stated. Resveratrol (>99% purity) was diluted in dimethyl sulphoxide (DMSO), and the working concentration for in vivo experiments was 20mg/kg or 15mg/kg per mouse, respectively.

2.2 Induction of experimental collagen-induced arthritis (CIA)

For Collagen-Induced Arthritis (CIA), mice were immunised by intradermal injection of 100ul Bovine Type II Collagen emulsified in CFA (Complete Freund's Adjuvant) (Difco, Detroit, MI, USA) at the base of the tail (Collagen should be emulsified in CFA at 4℃) , 2-3cm from the body on day 1. This was followed by a booster of 100ul collagen in IFA (Incomplete Freund's Adjuvant) injected at the base of the tail on day 21.

2.3 Assessment of arthritis

Mice were monitored daily for signs of arthritis. Scores were assigned based on erythema, swelling, or loss of function present in each paw on a scale of 0–3, giving a maximum score of 12 per mouse. Footpad thickness was measured with a dial calliper (Kroeplin, Munich, Germany).

2.4 Administration of resveratrol

Mice received daily i.p. injection of 100ul resveratrol diluted in DMSO. For the prevention group, mice received resveratrol each day from day 14 to day 21 (onset of arthritis), and the treatment group received resveratrol from day 21 to day 28. Control mice received the equal volume of resveratrol on day 14 or day 21 accordingly.

2.5 Histological assessment

Paws were removed and fixed in 10% neutral buffered formalin (NBF). For standard heamatoxilin and eosin (H&E) staining, the fixed footpads were decalcified in 10% solution of nitric acid in distilled water. This was replaced with fresh solution every alternate day until decalcification was completed. After decalcification, the footpads were transferred to 70% ethanol for paraffin embedding and processing. 5μm sections were stained with haematoxylin and eosin (Sigma) as described before.

2.6 Measure of cytokines and serum Abs levels

Blood samples were collected immediately after death by retro-orbital puncture and serum was separated by centrifugation (1400rpm, 10 mins) and stored at -20℃ until used. Cytokine concentrations including IL-17, TNF-α, IFN-γ, IL-6, IL-4, IL-1, IL-2 and IL-10 were determined by a commercial 20-plex mouse cytokine assay according to the manufacturer's instructions (Invitrogen, UK). Serum total antibody titres in pooled sera were detected with biotin-conjugated rat anti-mouse IgG1 or IgG2a (PharMingen, USA) followed by conjugated avidin peroxidase (Sigma) and developed with tetramethylbenzidine substrate (Kirkegaard & Perry, Gaithersburg, MD, USA). When collagen II-specific antibody was measured, the plate was coated with 2μg/ml collagen II first, after adding the serum do the same procedure as total antibody. Optical density was acquired by ELISA reader at 450 um.

2.7 Cell culture *in vitro*

On day 29 after primary immunisation, mice were sacrified and the spleen and draining lymph nodes (Axillary, Epitrochlear and Popliteal) were removed. Single-cell suspensions were prepared and were cultured at 2 $\times 10^6$ cells/ml in RPMI 1640 suppled with 100 IU/ml penicillin-streptomycin, 2mM L-glutamine and 10% FCS (all from Life Technologies, USA) at 37℃ in 5% CO_2 in a 24-well flat-bottom plate (Nunc, Roskilde, Denmark). Cells were stimulated with anti-CD3 (2μg/ml), and then were cultured in the presence or absence of 30μM of resveratrol based on our pilot result. For controls, cells were stimulated without anti-CD3 and resveratrol. 72 hours later, supernatants were collected and concentrations of IL-17, IFN-γ, GM-CSF, MIP-1α, IL-1β, TNF-α, IL-13, IL-5, IL-12, IL-4, IL-10 and IL-2 were determined by luminex.

2.8 Flow cytometry

DLN cells (1×10^6) were stained with FITC, PE or APC conjugated antibodies for CD4, IL-17 and IFN-γ with appropriate isotype-matched control (BD). All samples were pre-incubated with CD16/32 to block FcR. Then cells were analysed on FACS Calibur flow cytometry (BD). All results represented on individual mice (n=5).

2.9 Statistics

To determine the significance distribution of the clinical scores, we analysed the statistical differences between the resveratrol treated and control DBA mice models. Clinical scores, collagen-specific IgG levels were compared with a Student's t test for unpaired observation (Mann-Whitney was used as well, got the similar result). Mann-Whitney U tests were used for histological scores by SPSS software. P values less than 0.05 were considered significant.

3. Results

3.1 Resveratrol prevents the progression of inflammatory arthritis

In order to investigated whether resveratrol could prevent the development of collagen-induced arthritis (CIA), this was induced in DBA1 mice (n=10; as described in M&M) and different doses of resveratrol (REV) dissolved in DMSO or DMSO vehicle control were given by intra-peritoneal injection daily from day 14 for 6 days; all before the onset of arthritis typically at day 21.

Two doses of REV were tested (15mg/kg and 20mg/kg) in order to determine its dose effects on CIA. Meanwhile, the control mice received an equivalent volume of vehicle DMSO. Mice were monitored daily for the development of typical CIA from day 21.

3.1.1 15mg/kg resveratrol does not prevent clinical CIA

The mice that received 15mg/kg/dose of REV appeared to have no difference in disease incidence (Fig. 1A), number of diseased footpads (1B), footpad thickness (1C) and clinical score (1D) up to day 36 compared with those in DMSO controls.

Fig. 1

15mg/kg

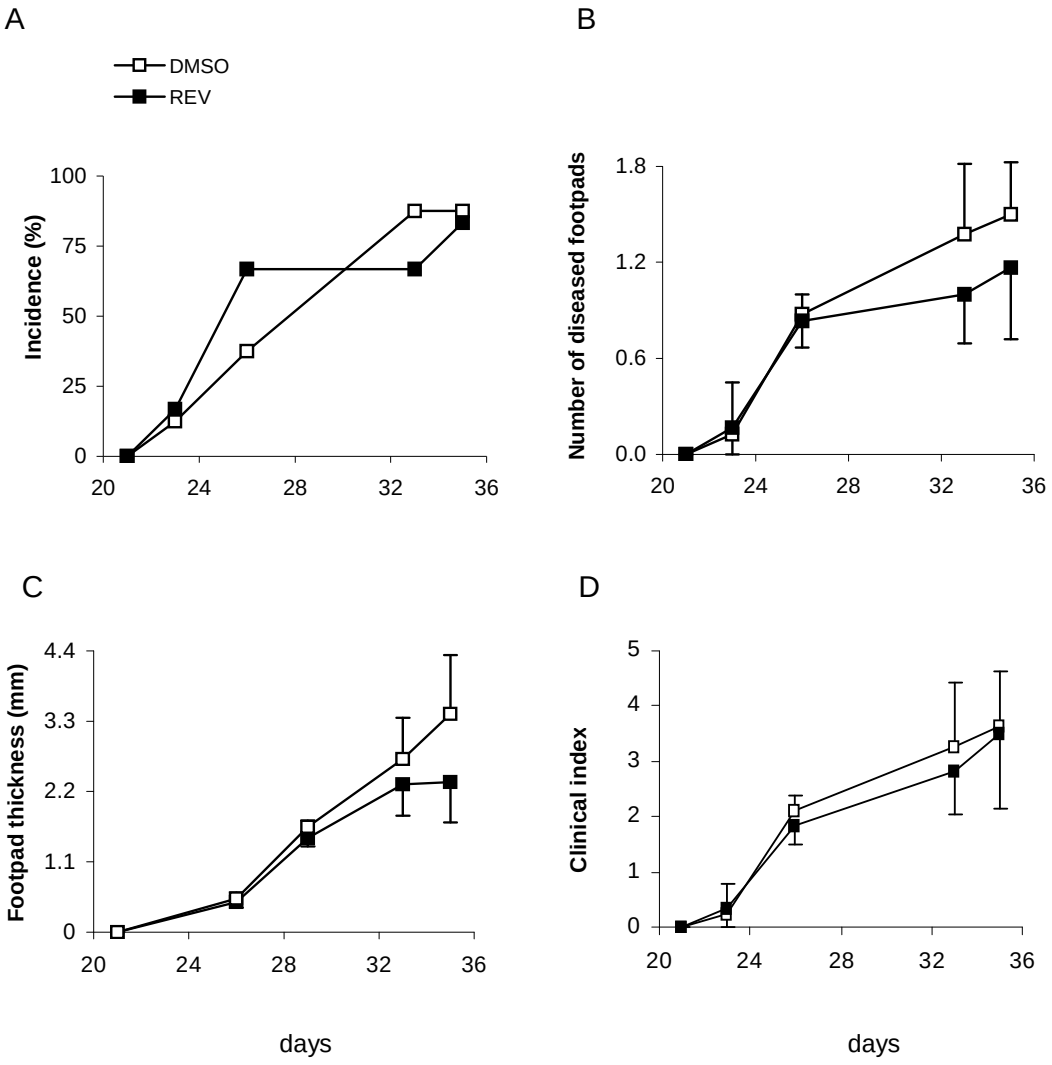


Fig.1. 15mg/kg resveratrol does not prevent clinical CIA. The CIA mice were treated with 15mg/kg of REV and the clinical parameters of arthritis in CIA mice were evaluated including incidence (A), number of diseased footpads (B), footpad thickness (C) and clinical index (D). Data were means ± SEM

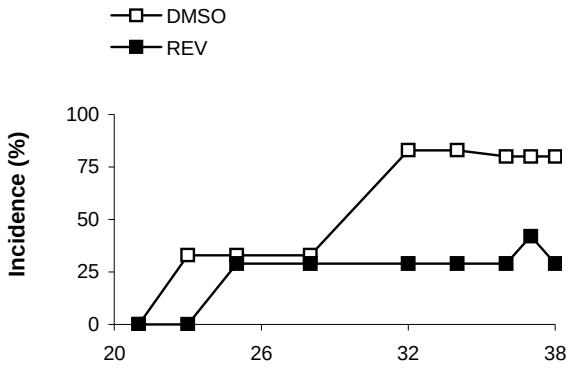
3.1.2 20mg/kg/dose of resveratrol impairs development of CIA

However, in the mice that received REV (20mg/kg), the incidence of CIA (Fig. 2A), the number of diseased footpads (2B), the footpad thickness (2C) and clinical score (2D) were attenuated compared with the controls on days 37 and 38. Thus, 20mg/kg but not 15mg/kg of REV have a preventative effect on the development and severity of CIA.

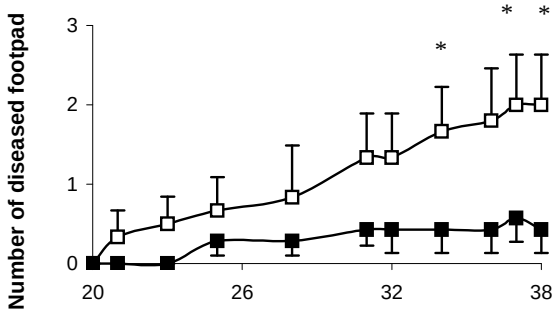
The histology of paws obtained from REV injected (20mg/kg) and DMSO control CIA mice was further examined. It was found that resveratrol treatment markedly reduced inflammatory cell infiltration and cartilage and bone erosion. This was further confirmed by significantly reduced histological score of hyperplasia, erosion and inflammatory cell infiltration in the REV-administered group compared with that in DMSO controls (2E). These observations agreed with the clinical parameters (Fig. 2A-D) and provide histological evidence that resveratrol at 20mg/kg has a preventative effect on the development and pathogenesis of CIA.

Fig. 2

A 20mg/kg



B



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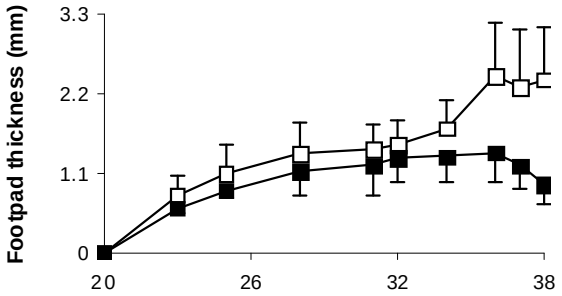

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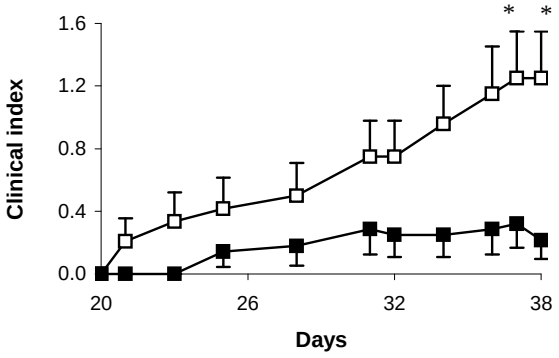


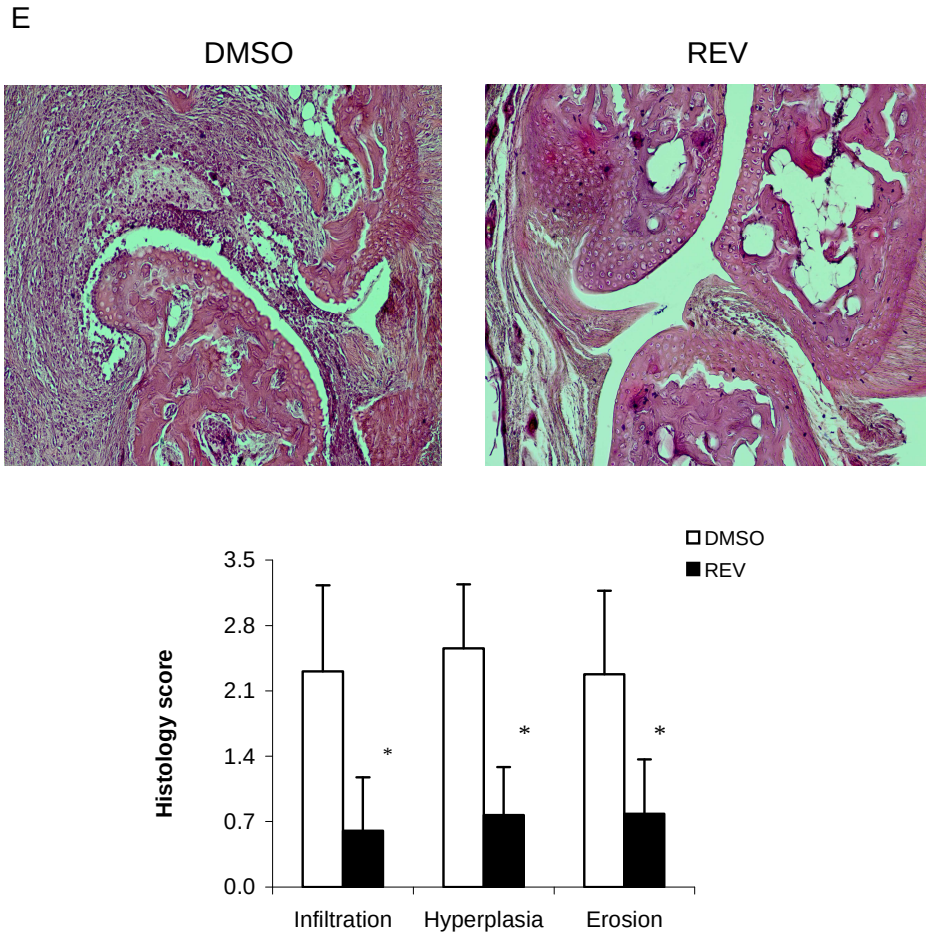


Fig.2. 20mg/kg/dose of resveratrol impairs development of CIA. 20mg/kg REV given prophylactically attenuated experimental CIA. (A-D) The clinical parameters of arthritis in CIA mice were evaluated including incidence, number of diseased footpads, footpad thickness and clinical index. Data were means ± SEM (*, $p< 0.05$; REV vs. DMSO mice by Student's t test, n=9). (E) Footpads were processed for histology (M&M). 4 um tissues were stained with H&E. Histology was analysed by scoring. Original magnification was ×40. Data are mean ± SD (*, $p< 0.001$ compared with DMSO controls by Mann-Whitney U test).

3.2 Resveratrol exerts therapeutic effects on established collagen-induced arthritis

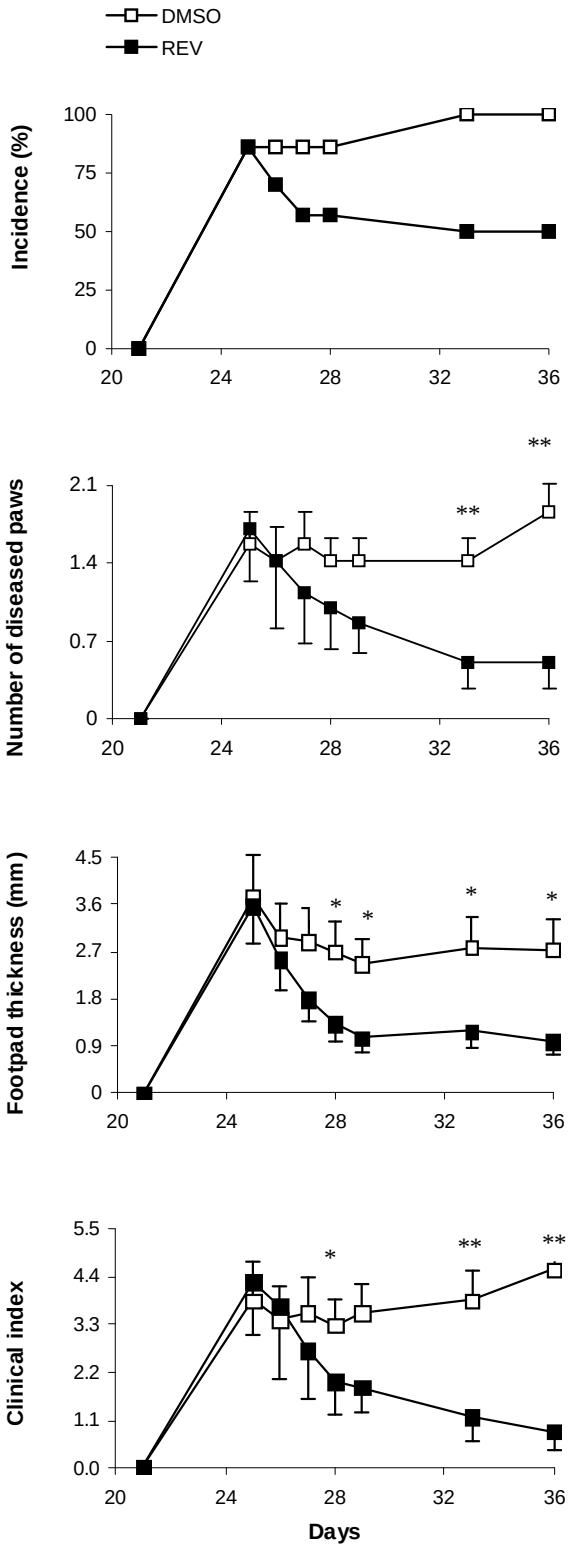
To further determine whether resveratrol (20mg/kg) can treat collagen-induced arthritis, CIA mice were treated with i.p. injection of resveratrol and DMSO control after the onset of arthritis on day 23, daily, for 6 days.

Control mice that received DMSO vehicle all developed typical severe arthritis by day 28 (Fig. 3A-D). In contrast, compared to the control mice, there was a significant time-dependent disease remission in the mice after REV treatment. This was evidenced by the impaired incidence (Fig. 3A), number of diseased footpads (3B), increased footpad thickness (3C) and overall clinical index (3D). Of potential clinical importance is that after, 6 days of treatment with REV was sufficient to sustain the reduction of clinical parameters of CIA on days 33 and 36 (Fig. 3).

These clinical observations were subsequently confirmed by the histological examination of the paws of CIA mice. Paws obtained from control mice demonstrated typical CIA tissue pathological changes, enhanced inflammatory cell infiltrations in the joint, bone erosions and hyperplasia (Fig. 3E) However, histological changes and pathology scores were significantly reduced in REV treatment mice compared to those in the controls (3E).

Fig. 3

A 20mg/kg



E

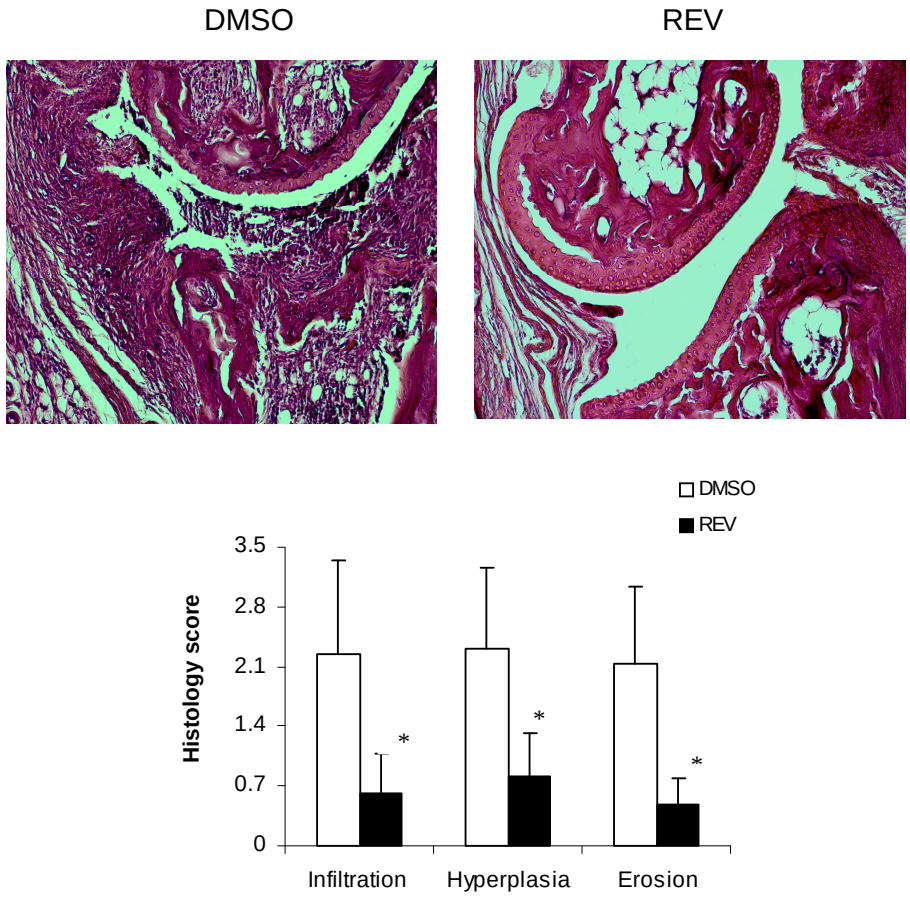


Fig.3. Resveratrol treats established CIA. (A-D) Mice were treated with resveratrol (20mg/kg) or DMSO on day 23 and monitored for disease progress, including incidence, number of diseased footpads, footpad thickness and clinical index. Data were means ± SEM (*, $p< 0.05$; **, $p< 0.01$). (E) Paws were collected for H&E staining and histology score.

3.3 Resveratrol reduces the production of collagen specific antibodies *in vivo*

Part of the inflammatory response associated with the induction of experimental arthritis is the production of antibodies against collagen antigen. In order to investigate whether the REV-mediated prevention and treatment effect on CIA involves the modulation of antibody responses, the total and collagen-specific levels of serum IgG1 and IgG2a were quantified by enzyme immunoassay (ELISA).

The collagen-specific IgG (IgG1 and IgG2a) levels were detected. Compared with the control mice, mice that received 20mg/kg of resveratrol before the development of arthritis produced less collagen-specific IgG2a and IgG1 (Fig. 4A). Similarly, 20mg/kg resveratrol also significantly decreased both collagen-specific IgG2a and IgG1 levels when administered after the development of CIA in mice (Fig. 4B). The disease severity seemed to change in parallel with the antibody levels in CIA mice.

Despite these changes in specific antibody activity, there was no difference in the serum concentrations of total serum IgG2a and IgG1 at day 36 between the resveratrol-treated and the control CIA mice (Fig. 4C).

Fig. 4

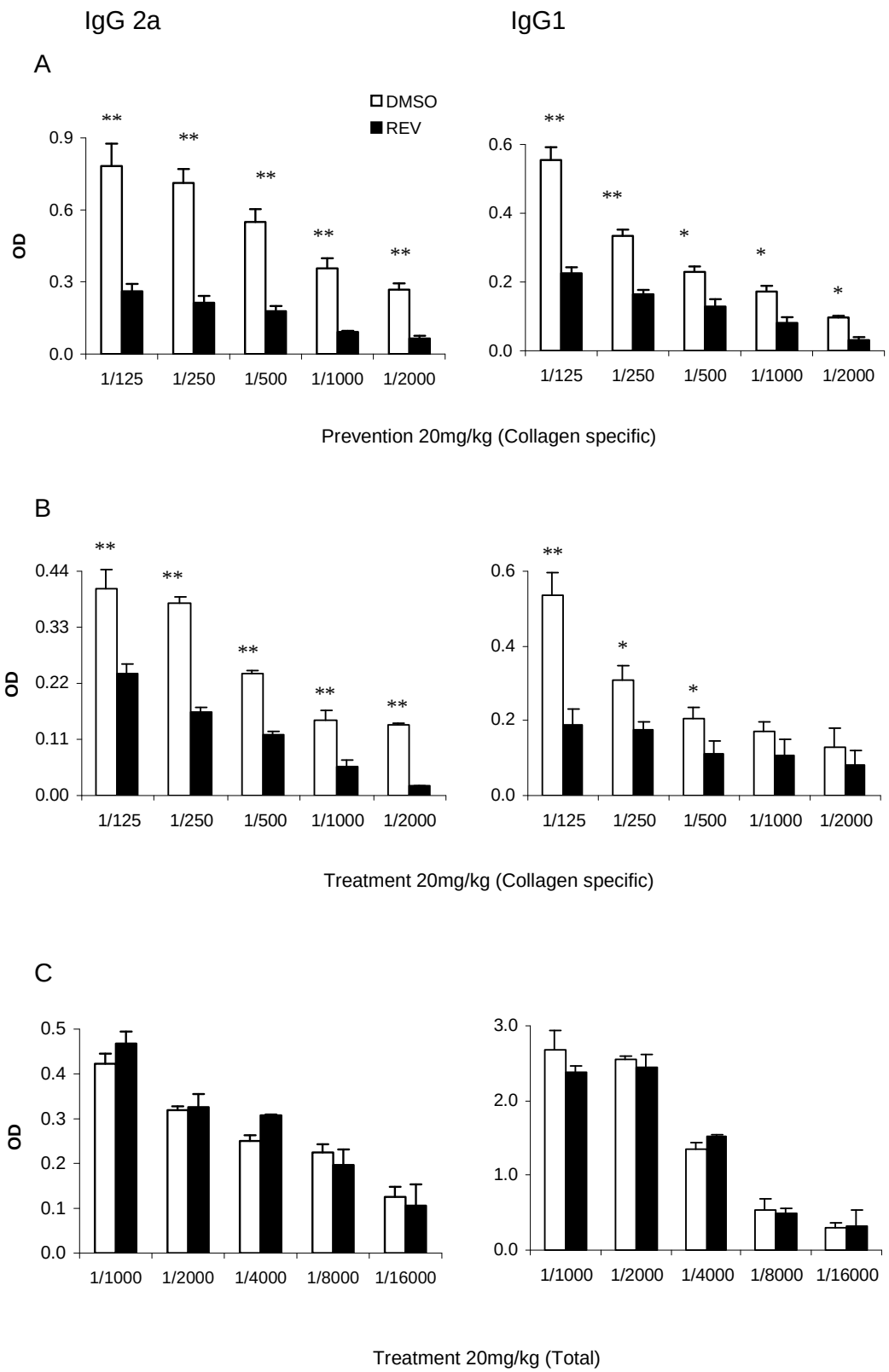

IgG 2a
IgG1
A
DMSO
REV
OD
1/125
1/250
1/500
1/1000
1/2000
Prevention 20mg/kg (Collagen specific)
B
Treatment 20mg/kg (Collagen specific)
C
1/4000
1/8000
1/16000
Treatment 20mg/kg (Total)

Fig.4. Resveratrol reduces collagen specific antibodies in CIA mice. (A-B) Anti-collagen IgG2a and IgG1 in mice serum were measured by sandwich ELISA. (C) Total IgG2a and IgG1 in the mice serum was determined by direct ELISA. Data were mean ± SD (*, $p < 0.05$; **, $p < 0.01$ compared with DMSO control by Student's t test).

3.4 Resveratrol inhibits inflammatory cytokine production in CIA mice

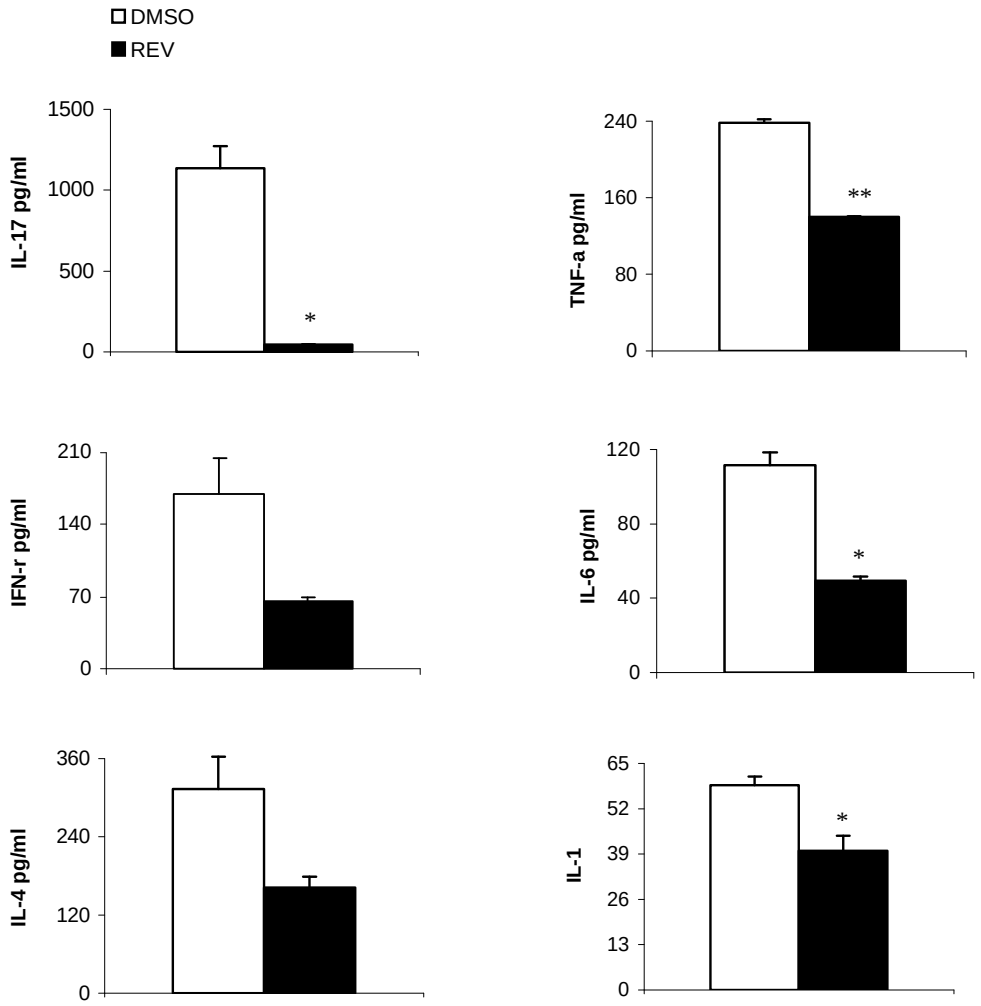
3.4.1 Preventative resveratrol selectively decreases the production of cytokines in CIA mice

Pro-inflammatory cytokines, including IL-17, TNF-α, IFN-γ and IL-6 play a critical role in the pathogenesis of RA and CIA. After 72 hours culture, we next investigated the role of REV in the regulation of inflammatory cytokine production in CIA mice (see M&M). Mice that received 20mg/kg resveratrol before the development of arthritis produced reduced levels of serum IFN-γ, TNF-α, IL-6, IL-4 and IL-1 compared to those in control mice (Fig. 5A). More importantly, a high-dose of REV-treatment almost completely abrogated IL-17 production in the serum, whereas a lower dose of REV failed to do so (data not shown).

However, REV at the concentration of 20mg/kg did not reduce the production of inhibitory cytokine IL-10 and the T-cell activation cytokine IL-2 (5B), suggesting that resveratrol selectively inhibits cytokine releases in CIA mice. These data are consistent with our observation that a higher but not a lower dose of resveratrol effectively prevents the development of CIA.

Fig. 5

A


DMSO
REV
IL-17 pg/ml
1500
1000
500
0
*
TNF-a pg/ml
240
160
80
0
**
IFN-r pg/ml
210
140
70
0
IL-6 pg/ml
120
80
40
0
*
IL-4 pg/ml
360
240
120
0
IL-1
65
52
39
26
13
0
*

B

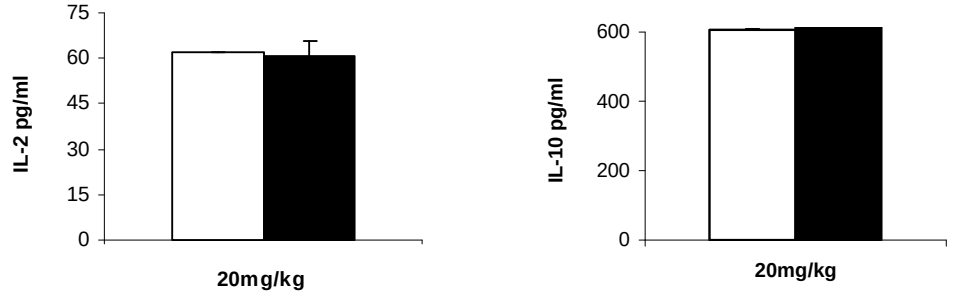

IL-2 pg/ml
75
60
45
30
15
0
20mg/kg
IL-10 pg/ml
600
400
200
0
20mg/kg

Fig. 5 Preventative resveratrol selectively decreases the production of cytokines in CIA mice. CIA mice were injected with REV on day 14 and sacrificed on day 38. Serum cytokine concentrations were analysed by luminex (A, B). Data were means ± SD (n=10. *, $p < 0.05$; **, $p < 0.01$ compared with DMSO control by Student's t test).

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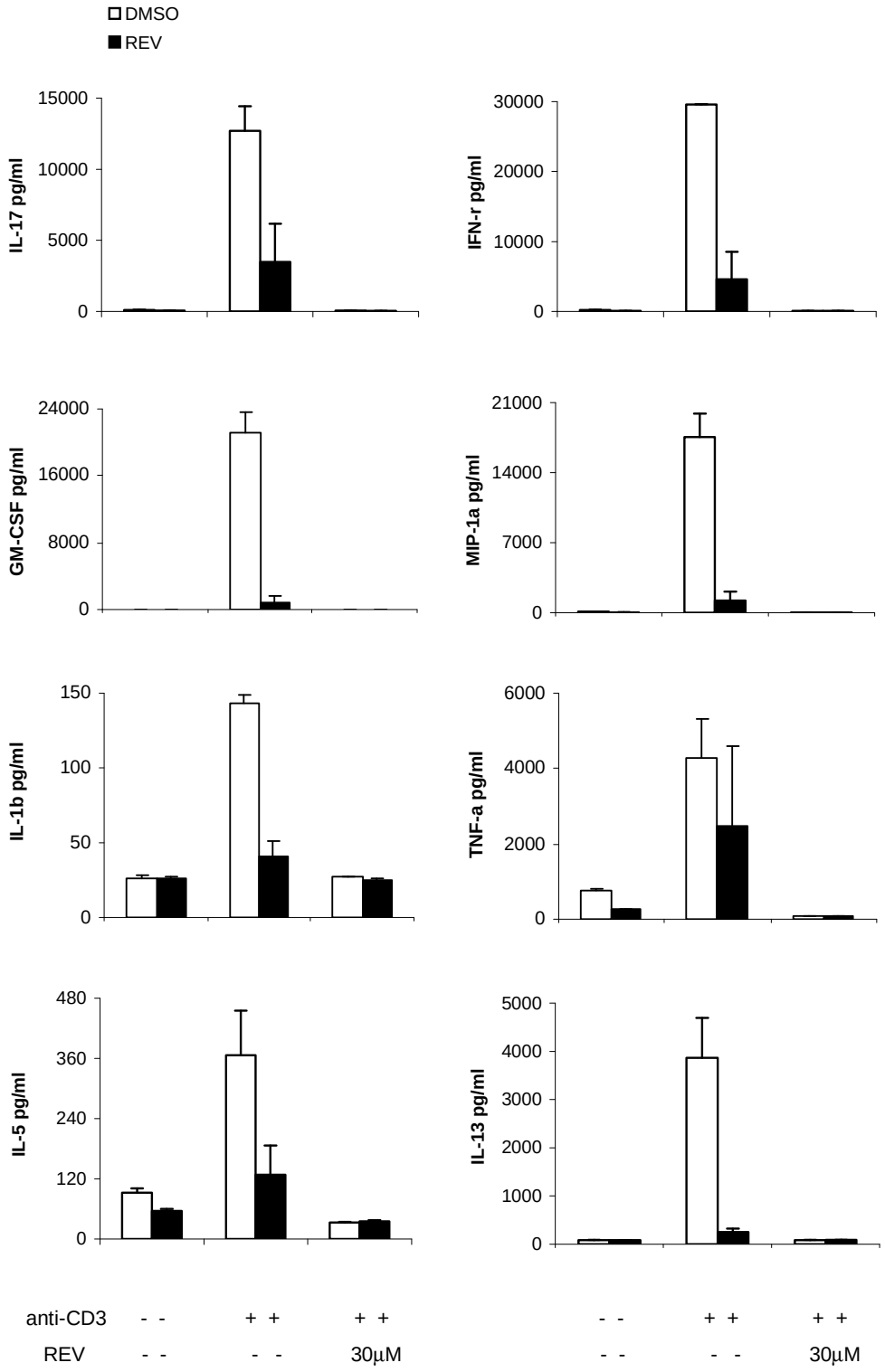
3.4.2 Resveratrol selectively decreases the production of inflammatory cytokines in DLN cells *in vitro*

Furthermore, to confirm the inhibitory effect of resveratrol on activated draining lymph node (DLN) cells, we cultured the cells with resveratrol *in vitro.* Based on our pilot experiment, 30μM of resveratrol was selected (data not shown). We found that resvertrol completely inhibited IL-17, IFN-γ, GM-CSF, MIP-1, IL-1, TNFα, IL-5 and IL-13 production in cell culture supernatant from both resveratrol-treated and untreated mice. However, it had no effect on the production of IL-12, IL-4, IL-2 and IL-10 in the same culture supernatant (Fig. 6B), suggesting again, that resveratrol selectively suppresses cytokine production.

Fig. 6

A


DMSO
REV
IL-17 pg/ml
15000
10000
5000
0
IFN-r pg/ml
30000
20000
10000
0
GM-CSF pg/ml
24000
16000
8000
0
MIP-1a pg/ml
21000
14000
7000
0
IL-1b pg/ml
150
100
50
0
TNF-a pg/ml
6000
4000
2000
0
IL-5 pg/ml
480
360
240
120
0
IL-13 pg/ml
5000
4000
3000
2000
1000
0
anti-CD3 - - + + + + - - + + + +
REV - - - - 30µM - - - - 30µM

B

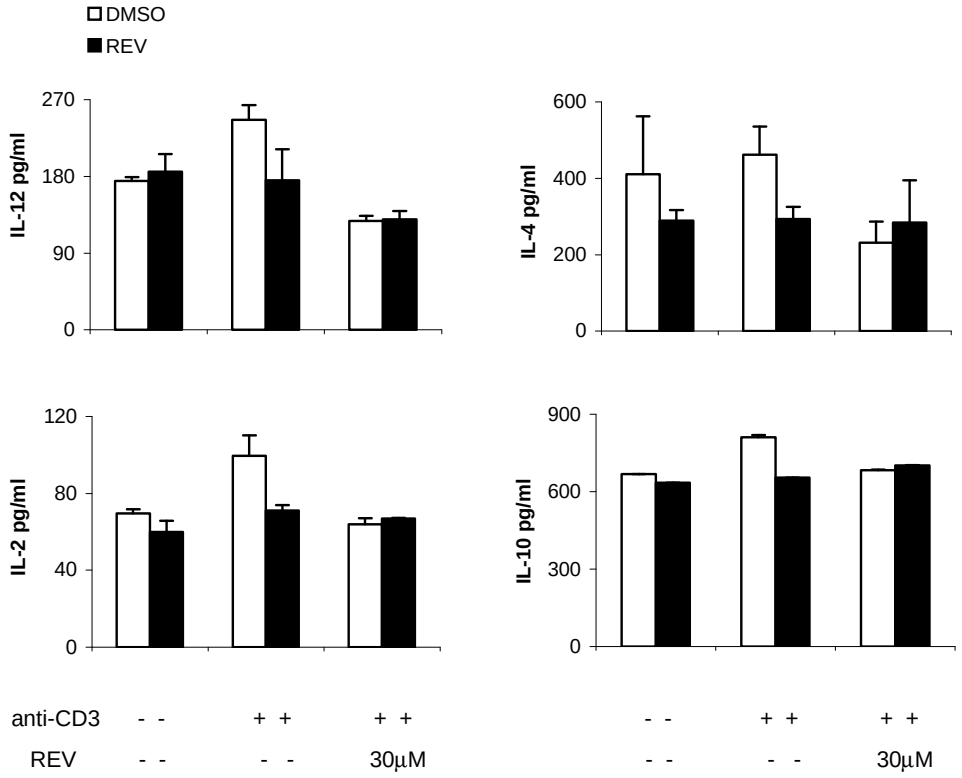


Fig.6. Resveratrol selectively decreases the production of inflammatory cytokines in DLN cells *in vitro*. (A) REV given *in vivo* and *in vitro* inhibited inflammatory cytokine production. (B) REV had no effect on the production of IL-2, IL-4 IL-10 and IL-12 in the culture.

3.4.3 Resveratrol can decrease the T cell numbers and the production of inflammatory cytokines in $CD4^+$ T cells

To investigate whether resveratrol can inhibit $CD4^+$ T-cell proliferation and production of their key cytokines, IL-17 and IFN-γ in CIA mice, we collected DLN cells from resveratrol-treated and untreated mice.

The size of the draining lymph nodes of CIA mice, which had been given resveratrol was notably smaller than the control CIA mice (data not shown). The total numbers of lymphocytes collected from REV-treated mice were also significantly reduced compared with those in control mice (Fig. 6A).

We next determined the number of $CD4^+$ T cells in these mice. Compared to the controls, the absolute numbers of $CD4^+$ cells were markedly reduced in the CIA mice treated with resveratrol (Fig. 6B).

We cultured the DLN cells with collagen antigens for 72 hours and the intracellular IL-17 and IFN-γ levels in $CD4^+$ T cells were determined by flow cytometry. Compared to the controls, the $CD4^+$ T cells from resveratrol-injected mice dramatically reduced IL-17 and IFN-γ expression in both total and single cell levels (Fig. 6C). The result suggests that resveratrol can also suppress the important arthritic Th1 cell subset function in CIA mice.

Fig. 7

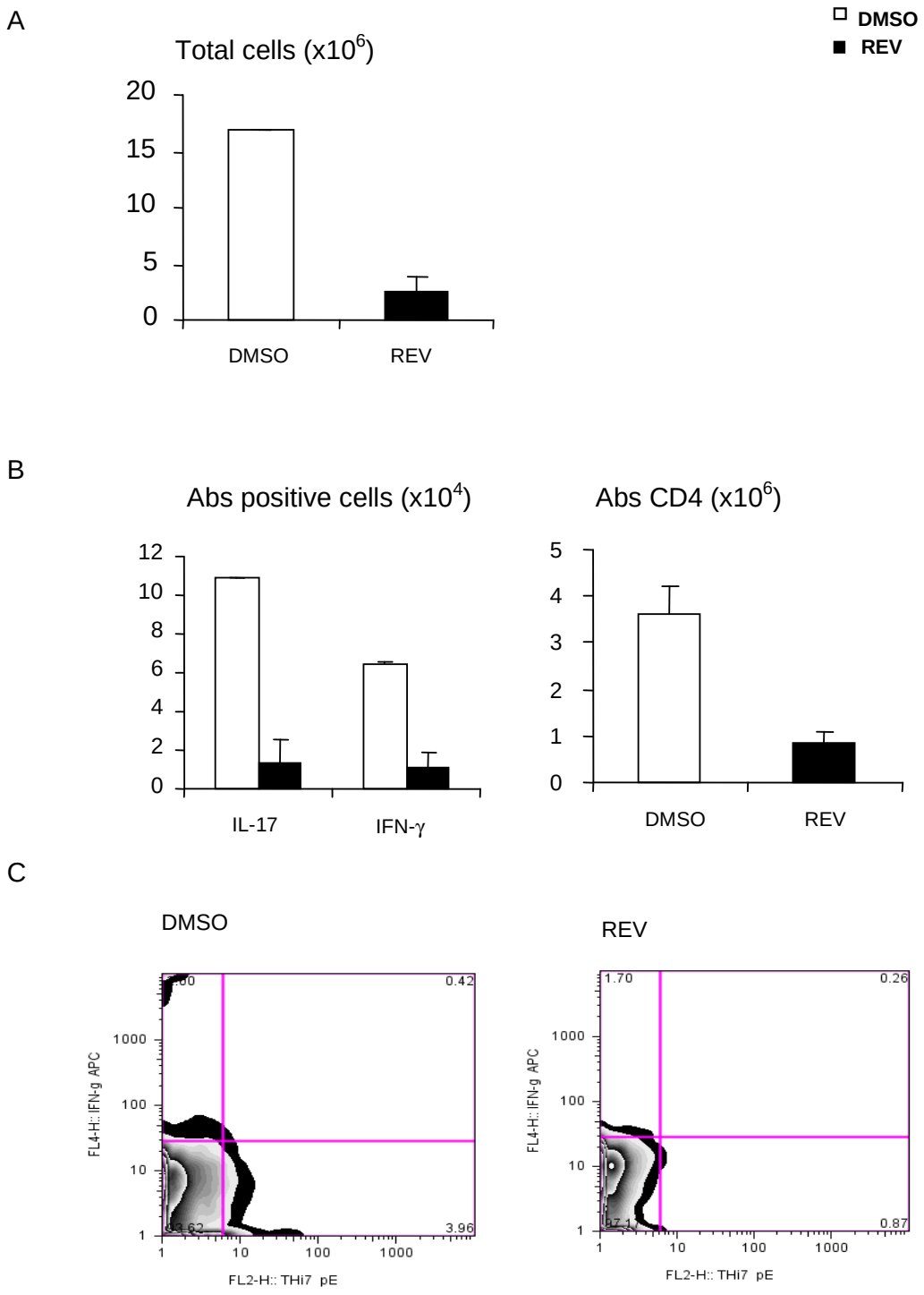

A
DMSO
REV
Total cells (x10^6)
20
15
10
5
0
DMSO
REV
B
Abs positive cells (x10^4)
12
10
8
6
4
2
0
IL-17
IFN-γ
Abs CD4 (x10^6)
5
4
3
2
1
0
DMSO
REV
C
DMSO
REV
0.42
3.96
1.70
0.26
0.87
FL4-H:: IFN-g APC
FL2-H:: THi7 pE
1000
100
10
1

Fig.7. Resveratrol can decrease the T cell numbers and the production of inflammatory cytokines in $CD4^+$ T cells. (A) The total number of lymphcytes in REV and DMSO mice. (B) The absolute number of $CD4^+$ T cells in the DLN. (C) The $IL\text{-}17^+$ and $IFN\text{-}\gamma^+$ $CD4^+$ T cells in DLN by FACS.

4. Discussion

4.1 How does REV suppress arthritic response in CIA?

Although the aetiology of RA is still unclear, B cells, T cells, macrophages and synoviocytes are closely involved in the pathogenesis of joint inflammation and bone erosion. The selective depletion of B cells with rituximab can relieve the symptoms in RA patients [36], which indicates that B cells contribute to RA [37]. This is probably because B cells can produce auto-antibodies and can also act as antigen presenting cells to promote arthritis-associated T-cell activation [37]. It has been shown that resveratrol can suppress LPS-stimulated B cell proliferation and IgG1 and IgG2a production in a dose-dependent fashion [38], and down-regulates the expression of CD80, a co-stimulatory molecular for T cell activation [38]. Our results also show *in vivo* that resveratrol inhibits the collagen-specific IgG production but not total IgG, suggesting that resveratrol may mainly target ongoing B cell response but has little influence on the general B cell homeostasis and function.

RA was previously thought to be a Th1-cell associated inflammatory disease; however, increasing evidence has demonstrated that Th17 cells play a pivotal role in RA. IL-17, a Th17-derived pro-inflammatory cytokine is highly expressed in the synovium and synovial fluid of RA patients. A significant suppression of the development of inflammatory arthritis was observed in IL-17$^{-/-}$ mice [39], and the severity of CIA can be inhibited by neutralising anti-IL17 antibodies. IL-1 is a critical arthritic cytokine in RA. It was suggested that IL-17 might contribute to the synovial inflammation and joint destruction in an IL-1-independent manner [40]. In our study, resveratrol can significantly inhibit the production of IL-17 in the serum and cell culture of CIA mice, suggesting that REV has a profound effect on IL-17 production. However, the underlying mechanism is still unknown.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that can mediate anti-inflammatory effect [41]. It is reported that AhR is required for Th17 development and resveratrol is an inhibitor of AhR [42, 43]. This may provide a possible mechanism and explanation for how resveratrol inhibits Th17 development in CIA. However, a direct link of AhR in resveratrol-inhibited Th17 function and CIA pathogenesis needs to be confirmed. Furthermore, other cells can also express IL-17, for instance monocytes and mast cells. It is currently unknown whether resveratrol suppresses the cytokine production in different cells by common or different mechanisms.

Dendritic cells (DCs) represent the most effective antigen-presenting cells in the initiation of antigen-specific T and B cell activation [44, 45]. It has been reported that resveratrol can induce a dose-dependent reduction in the proliferation of T-cell by suppressing DC maturation and function [46]. Therefore it is possible that resveratrol may produce such a wide-effect on both T and B cell responses by affecting DC functions. This hypothesis also needs to be further investigated.

IL-10 is a powerful immune inhibitory cytokine and resveratrol suppressor function may be indirect via IL-10. It has been shown that DCs treated with resveratrol completely inhibit IL-12 release and increase IL-10 secretion [47]. However, there is no difference in the production of IL-12 and IL-10 in the serum or cultured DLNs with or without resveratrol in our CIA context. This suggests that resveratrol suppress other cells but not via IL-10 induction.

Additionally, inflamed synoviocytes contribute to the pathology of arthritis, and resveratrol can directly suppress synoviocytes proliferation by inducing cell apoptosis via activating caspase-3 [48].

Nevertheless, resveratrol can suppress ongoing even established inflammatory arthritic response *in vivo*. The detailed mechanism underlying the suppression will be investigated in the future.

4.2 The effect of REV in immunity and CIA

Different dose of resveratrol may have opposite effect on immunity. The previous *in vitro* study has shown that resveratrol can modulate B cell proliferation and antibody production [49]. At a concentration of 5μmol/L resveratrol increased the percentage of $CD19^+$ B lymphocytes, however, 10μmol/L resveratrol inhibited proliferation of B cells [49]. Another study also confirmed the dose-dependent effect of resveratrol. As observed, when cultured with resveratrol (10^{-8}–10^{-5} M), the growth of human bone marrow-derived mesenchymal stem cell was increased, whereas REV (10^{-4} M) possessed a suppressive effect on cell proliferation [50]. Our results demonstrated that 20mg/kg (0.02M) dose, but not a 15mg/kg (0.015M) dose of resveratrol has preventative and therapeutic effects in CIA. This higher dose of REV can significantly reduce antibody production while a marginally lower dose REV can only slightly inhibit the production of collagen specific antibodies (data not shown). In addition to the antibody synthesis, the reduction of pro-inflammatory cytokine secretions in the 15mg/kg resveratrol treated group was less pronounced than that in the 20mg/kg group (data not shown). Histological analysis also confirms that a low dose of resveratrol has not effect on the prevention of joint inflammation and bone erosion.

Thus, the dose of resveratrol should be carefully considered when designing an experiment or in the future clinical application using this natural product.

4.3 Potential role of resveratrol in clinical application in RA

The first line drugs for RA treatment such as NSAIDs and glucocorticoids exert effects by non-specifically suppressing the inflammatory response. These drugs also have unpleasant side effects. TNF blockers are, the new generation drugs that are more effective than NSAIDs in the relief of the progress of RA, however, only 40% of subjects respond to the anti-TNF

treatment. In addition, it is very costly which may prevent its general applications. Over all, current therapies for RA cannot cure the disease but merely aim to reduce the symptoms and provide patients an acceptable quality of life. Thus, more effective, affordable drugs with minimal side effects are still needed to control this common disorder, and natural products with anti-inflammatory properties may be good candidates for this.

The pharmacology and toxicology of resveratrol have been demonstrated in rodents and in man [51, 52]. The absorption and metabolism of resveratrol have been investigated. Resveratrol is well absorbed and distributed to all the organs in the human body [53]. No detectable toxic effect was observed in rabbits and rats treated with low and high doses of high-purity resveratrol, up to 750mg/kg per day for 3 months [51]. Application to human beings has already indicated that there are no, or only minor, adverse effects in healthy volunteers even with a high dose of resveratrol [52]. Furthermore, resveratrol is commercially available as a daily healthcare product to maintain general wellbeing and health. Therefore the natural product resveratrol may represent a promising drug for RA.

4.4 Other potential natural products against RA

Apart from resveratrol, several other components extracted from plants also display anti-inflammatory properties and may have therapeutic potential against RA. Both *in vitro* and *vivo* evidence show that curcumin, the polyphenol that has been used as an ingredient in Indian food for centuries, may have potential activity against arthritis. It has been shown that curcumin inhibits synoviocyte proliferation and neutrophil activation [54]. Other investigation suggested that curcumin prevents joint inflammation before but not after the onset of arthritis [55]. Preclinical and clinical study shown that guggulsterone, a sterol extracted from gum resin can reduce pain and stiffness of the knee in osteoarthritis [56].

Withanolides, which are derived from *withania somnifera* can also inhibit experimental arthritis [57]. These agents have the common feature that they can all suppress the activation of the key transcript factor NF-κB in inflammatory response, leading to reduction of a cyclooxygenase (COX-2) expression [58-60]. Furthermore, several other compounds including 6-shogalo (a ginger rhizome extract), avemar (a component of the wheat germ) [61, 62], avocado and soybeans also exhibit different anti-arthritic properties [63]. In summary, we should like to propose that these natural products may represent potential valuable drugs for arthritic disorders.

Table 1

Molecular targets of natural products that exhibit anti-arthritic potential

Compounds	Source	Molecular targets	Reference
Boswellic acid	Boswillia serrata (Salai guggul)	NF-κB, COX-2, 5-LOX, MMP-9	[64, 65]
Berberine	Berberis bulgaris (barberry)	NF-KB, COX-2, TNF-α, IL-1β	[66]
Cekastrol	Tripterygium wilfordii	NF-KB, COX-2, MMP-9, TNF-α	[67, 68]
Curcumin	Curcuma longaa (tumeric)	NF-KB, COX-2, 5-LOX, TNF-α	
Eugenol	Syzygium aromaticum (cloves)	NF-KB, COX-2, 5-LOX, TNF-α	[69]
Guggulsterone	Commiphora mukul (guggul)	NF-KB, COX-2, MMP-9	[56, 59]
Statins	Aspergillus terreus (yeast)	NF-KB, COX-2, MMP-9	[70, 71]
Tea polyphenols	Camellia sinensis (black tea)	NF-KB, COX-2, 5-LOX, TNF-α	[72]
Ursolic acid	Ocimum sanctum (holy basil)	NF-KB, COX-2, MMP-9	[73, 74]
Withanolides	*Withania somnifera* (Ashwagendha)	NF-KB, COX-2, MMP-9, ICAM-1	[60, 75]

4.5 Future directions

In conclusion, our present study demonstrated that resveratrol could prevent and treat CIA by inhibiting collagen specific lymphocyte proliferation, autoimmune antibody production and pro-inflammatory cytokine synthesis, especially IL-17. Further studies should be focused on the molecular mechanisms underlying resveratrol's action, in particularly its role in the regulation of the key arthritic B and T cell activities *in vitro* and *in vivo*. The longer-term effect of resveratrol in treatment and

prevention of RA also needs to be assessed and compared with anti-TNF-α therapy in the next study *in vivo*. Since REV has already been applied to human beings and has shown no obvious side effects, it will be possible to test its role in RA in the near future. These studies will further enhance our understanding of pharmacological role of resveratrol and establish its potential beneficial effect on RA.

5. Reference

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