Basic nutritional investigation

Consumption of hydrolyzable tannins-rich pomegranate extract suppresses inflammation and joint damage in rheumatoid arthritis

Meenakshi Shukla, M.Sc.,a Kalpana Gupta, Ph.D.,a Zafar Rasheed, Ph.D.a,† Khursheed A. Khan, Ph.D.,b and Tariq M. Haqqi, Ph.D.a,*,†

a Division of Rheumatic Diseases, Department of Medicine, Case Western Reserve University/University Hospitals of Cleveland, Cleveland, Ohio, USA
b Department of Kulliyat, Faculty of Unani Medicine, Aligarh Muslim University, Aligarh, India

Manuscript received September 25, 2007; accepted March 12, 2008.

Abstract

Objective: Although consumption of dietary supplements containing pomegranate extract (POMx) by patients with arthritis is on the rise, the efficacy of such preparations in suppressing joint inflammation and damage is not known. The present study was designed to evaluate a standardized preparation of POMx using collagen-induced arthritis (CIA) in mice, a widely used animal model of rheumatoid arthritis.

Methods: CIA-susceptible DBA/1 mice were fed POMx by gavage before and after immunization with chicken type II collagen. Severity of clinical arthritis was scored using a visual scoring system. Arthritic joints were analyzed by histopathology and graded. Lysates were generated from mouse joints and levels of anti–type II collagen immunoglobulin G and inflammatory cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor-α were quantified by enzyme-linked immunosorbent assay. The effect of POMx on lipopolysaccharide-induced nitric oxide production was determined by Griess reaction and mitogen-activated protein kinase activation was studied by western immunoblotting in mouse macrophages.

Results: Consumption of POMx potently delayed the onset and reduced the incidence of CIA in mice. Severity of arthritis was also significantly lower in POMx-fed animals. Histopathology of the arthritic joints from POMx-fed mice demonstrated reduced joint infiltration by inflammatory cells, and the destruction of bone and cartilage were alleviated. Levels of IL-6 were significantly decreased in the joints of POMx-fed mice with CIA. In mouse macrophages, POMx abrogated multiple signal transduction pathways and downstream mediators implicated in the pathogenesis of rheumatoid arthritis.

Conclusion: Our studies suggest that inhibition of a spectrum of signal transduction pathways and the downstream pathogenic cellular response by POMx or compounds derived from it may be a useful approach for the prevention of the onset and severity of inflammatory arthritis. © 2008 Elsevier Inc. All rights reserved.

Keywords: Pomegranate; Collagen-induced arthritis; Rheumatoid arthritis; Interleukin-6; Tumor necrosis factor-α

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that results from a complex interplay of genetic and environmental factors. Clinically RA manifests itself as a systemic, chronic inflammatory disease characterized by synovial inflammation and erosion of bone and cartilage, which lead to the destruction of affected joints. The normal synovial lining consists of one to two cell layers but increases to at least 10 cell layers in RA joints. Patients with RA have type II collagen (CII)-reactive T cells and antibodies, which sug-
gests that CII is a candidate autoantigen in disease pathogenesis [1]. RA affects 0.5–1% of the world population, with more women being affected than men [2]. The preclinical stages of RA pathogenesis are poorly understood, but it is now clear that inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6 are overexpressed in RA joints and play an important role in its pathogenesis [3–5]. In addition, the current view of the cytokine network in RA joints supports the notion that TNF-α activates a cytokine cascade characterized by the simultaneous production of proinflammatory cytokines such as IL-1β and IL-6, whereas anti-inflammatory cytokines such as IL-10 and soluble TNF receptor are suppressed [2,6]. In RA, joint-infiltrating macrophages produce TNF-α and other inflammatory cytokines that potentiate inflammation [2,4]. TNF-α has been shown to play a pivotal role in synovitis and joint destruction in animal models of arthritis and in RA [6,7]. This is supported by published studies showing that the long-term use of biological agents targeting TNF-α give rise to sustained improvements in symptoms and signs of RA and, furthermore, that TNF-α blockade protects joints from structural damage [8]. Although TNF-α has been hypothesized to be the “master cytokine” driving joint inflammation, about 50% of patients with RA do not respond to anti-TNF therapy [9]. In addition, it has been shown that mice lacking TNF-α gene can still develop severe joint inflammation and destruction, demonstrating experimentally that TNF-α is not rate limiting for chronic inflammatory joint disease [10]. The development of anti–TNF-α antibodies or soluble receptor molecules to treat RA emerged in part as a consequence of the growing appreciation of the severity of this condition and in part to the significant progress made in understanding the important role TNF-α and inflammatory cytokine-signaling network play in the pathogenesis of this disease.

Although recently developed anti–TNF-α agents are well tolerated and have a good overall safety profile, pitfalls in the use of biological agents include the costs of these treatments, tuberculosis, and in some cases even malignancies [11,12]. Despite optimal use of currently available antirheumatic agents, most patients with RA live with chronic pain and severe functional decline because these therapies focus primarily on preventing joint inflammation and soft tissue swelling, but are not effective in preventing cartilage breakdown and the joint destruction associated with RA.

The pomegranate has been used for centuries as a therapeutic agent for the treatment of inflammatory diseases and disorders of the digestive tract by practitioners of the Ayurvedic and Unani systems of medicine [13]. More recently standardized extracts of pomegranate have been shown to have anti-inflammatory and cartilage-sparing effects in vitro and cancer-preventing and cardiovascular disease—preventing effects in vivo [14–19]. The major effect of the pomegranate extract (POMx) was shown to be the reduction of oxidative stress, inhibition of the p38–mitogen-activated protein kinase (p38-MAPK) pathway, and inhibition of the activation of transcription factor nuclear factor-κB (NF-κB). Activation of p38-MAPK and NF-κB is intimately associated with an increased gene expression of TNF-α, IL-1β, monocyte chemoattractant protein-1 (MCP1), inducible nitric oxide synthase (NOS), and cyclo-oxygenase-2 agents that are critical mediators of joint inflammation and the pathogenesis of RA [20,21].

Previous studies from our group have shown that a standardized pomegranate extract (POMx) rich in hydrolysable tannins and anthocyanins is highly effective in exerting human cartilage-sparing effects in vitro [19]. In this study we have compared the efficacy of a commercial standardized preparation of POMx in a mouse model of RA. The collagen-induced arthritis (CIA) in mice is a well-characterized animal model and has been used for more than two decades as an experimental model with which to study RA. Joint histopathology in this animal model shows features that are remarkably similar to those seen in RA joints. The significance of the model also lies in the fact that CIA is the major constituent protein of cartilage in the diarthrodial joints, the primary site affected in RA [22,23]. Because of the many compelling similarities between CIA and RA, CIA is an excellent model to develop and test preventive and therapeutic approaches for the prevention and/or treatment of arthritis in humans [24].

Materials and methods

Mice

In the present study, we used 8-wk-old male DBA/1 Lac J mice (Jackson Laboratory, Bar Harbor, ME, USA) with an average body weight of 20.0 g. The mice were maintained under specific pathogen-free conditions with free access to tap water and a commercial diet. All animal experiments were undertaken with the approval of the Institutional Animal Care and Use Committee and were in accordance with approved guidelines for the care and use of laboratory animals.

Chemical composition of POMx

POMx is produced from pomegranates (Punica granatum L., Wonderful variety) grown in California by Paramount Farms. POMx is produced in a two-step process: 1) extraction of fruit residue after pressing for juice and 2) solid-phase extraction to produce a powder with a high concentration of polyphenols. Extraction is performed during fruit harvest using pressed pomegranate fruit and arils. The powder extract used in this study contains on average 86.0% ellagitannins, 2.5% ash, 3.2% sugars, 1.9% organic acids as citric acid equivalents, 0.8% nitrogen, and 1.2% moisture. The approximate percent distribution of pomegranate polyphenols is as follows: 1) ellagitannins as punicalagins and punicalins, 19%; 2) free ellagic acid, 4%; and
3) oligomers composed of 2–10 repeating units of gallic acid, ellagic acid, and glucose in different combinations, 77%.

**CII, antibodies, and enzyme-linked immunosorbent assays**

Chicken CII was purchased from Chondrex (Seattle, WA, USA). Antibodies were purchased from Cell Signaling Technology (Denvers, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Kits for performing cytokine enzyme-linked immunosorbent assays (ELISAs) were purchased from eBiosciences (San Diego, CA, USA) and R&D Systems (St. Paul, MN, USA). An anti-CII antibody ELISA kit was purchased from Chondrex. All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

**Immunization of mice and induction of CIA**

After 1 wk of acclimatization, animals were randomly divided into three groups of eight animals each: group 1 received water (control), group 2 received 13.6 mg/kg of POMx, and group 3 received 34 mg/kg of POMx. Mice were given the stated dose of POMx dissolved in water by gavage for 10 d before the induction of arthritis, with animals in the control group receiving equal amounts of water the same way. This regimen was continued after immunization with CII for the induction of arthritis. CIA was induced in all three groups of mice by immunization with chicken CII (Chondrex) as previously described [1]. In brief, mice received intradermal immunization with 100 μg of chicken CII emulsified in complete Freund’s adjuvant and were boosted 3 wk later with chicken CII emulsified in incomplete Freund’s adjuvant. Mice were scored for the severity of arthritis using a previously described visual scoring system [25]. Briefly, grade 0 indicated no swelling or erythema; grade 1, mild swelling and erythema or digital inflammation; grade 2, moderate swelling and erythema confined distal to the midpaw; grade 3, more pronounced swelling and erythema with extension to the ankle; and grade 4, severe swelling, erythema, and joint rigidity of the ankle, foot and digits. Thus a mouse could have a maximum arthritis severity score of 16.

**Histopathology of the joints**

Affected limbs were harvested and dissected free of soft tissue as much as possible. These were then decalcified before embedding in paraffin. Sections were stained with hematoxylin and eosin and scored by an investigator blinded to the treatment for synovitis, pannus formation, and bone and cartilage destruction. The sections were scored as previously described [26]. Briefly, grade 0 indicate normal; grade 1, mild inflammation, mild hyperplasia of the synovial lining layer, and mild cartilage destruction without bone erosion; and grades 2–4, increasing degrees of cellular infiltration, synovial lining hyperplasia, pannus formation, and bone and cartilage destruction.

**Mouse macrophages and stimulation**

Mouse macrophages (RAW 264.1, ATCC, Rockville, MD, USA) were cultured in complete Minimum Essential Medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS). Confluent (80%) macrophage cultures were serum starved and then pretreated with POMx (20 μg/mL) for 2 h and stimulated with lipopolysaccharide (LPS; 50 ng/mL) for different periods of time. Cell lysates were prepared and used for western immunoblotting and culture supernatants were used for the measurement of cytokines by ELISA. POMx concentrations used in these studies were derived from pilot studies employing RAW 264.1 cells.

**Determination of NO production**

Culture supernatants from LPS-stimulated macrophages were used for the determination of nitrite levels by the Griess reaction as an indicator of NO production using a commercially available kit (Molecular Probes/Invitrogen).

**Protein determination**

Total protein content was determined using the Bio-Rad (Hercules, CA, USA) detergent-compatible protein assay kit according to the instructions. Bovine serum albumin (Sigma-Aldrich) was used as a standard.

**Enzyme-linked immunosorbent assay**

Harvested limbs were dissected free of soft tissue as much as possible and then snap-frozen in liquid nitrogen. Cell-free lysate was then prepared as previously described [1]. Total lysate protein or culture supernatant was used to measure levels of anti-CII antibodies (Chondrex), IL-6 (eBiosciences), IL-1β, and TNF-α (R&D Systems) using sandwich ELISAs according to the instructions of the manufacturer. Limits of detection (sensitivity) for the kits were 4 pg/mL for IL-6, 7.8 pg/mL for IL-1, and 5.1 pg/mL for TNF-α. ELISA results were quantitated by absorbance at 450 nm using a Synergy HT microplate reader (BioTek Instruments, Winooski, VT, USA).

**Western immunoblotting**

Cell lysates were prepared from harvested joints and stimulated macrophages (lysate buffer: 50 mM Tris·HCl, pH 7.4; 150 mM NaCl; 1% Triton X-100; 0.1% sodium dodecylsulfate; 0.5% sodium deoxycholate; 1 mM ethylene-diaminetetra-acetic acid; 1 mM ethylene glycol-bis-(2-aminoethylether)-N,N,N′,N′-tetraacetic acid [Roche, Nutley, NJ, USA]; Complete Protease [Roche]; and phosphatase inhib-
protector cocktail as previously described [1,27]). Total lysate protein (25 μg/lane) was resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Bio-Rad), blocked with skim milk, and probed with primary and secondary antibodies (Cell Signaling Technologies, Santa Cruz Biotechnology). Immunoreactive proteins were visualized by enhanced chemiluminescence (GE Healthcare, Milwaukee, WI, USA) as previously described [28]. Images were captured and analyzed using Alpha-Innotech Imaging System and software (San Leandro, CA, USA).

Luciferase reporter assay

Mouse macrophages were seeded at 3 × 10⁵ cells/mL in six-well plates in Minimum Essential Medium (with FBS and without antibiotic) the day before transfection. On the next day, cells were transiently transfected using the NF-κB luciferase reporter gene construct (Panomics, Fremont, CA, USA) and Lipofectamine 2000 according to the protocol provided by the manufacturer (Invitrogen). Fresh growth media with 10% FBS was added 5 h after the incubation of cells and incubation continued for 18 h in a tissue culture incubator (total incubation time 23 h). Cells were then starved for 6 h in Minimum Essential Medium without FBS and stimulated by LPS (100 ng/mL) for 18 h. To determine the effect of POMx, cells were pretreated with POMx (40 μg/mL) for 2 h before stimulation with LPS. Cells were then harvested, washed with phosphate buffered saline, and lysed by adding 200 μL of lysis buffer and the cell lysates were prepared according to instructions provided with the kit (Luciferase Assay kit, Promega, Madison, WI, USA). Luciferase activity in the cell lysates was measured using a tube luminometer (Promega) and kit-supplied substrate and the results were expressed as relative light units. Macrophages stimulated with LPS in the presence of polymyxin
B (10 μg/mL) were used as positive controls for the inhibition of LPS-induced activation of NF-κB.

**Statistical analysis**

Arthritis scores and histologic scores were compared by the Mann-Whitney U test using the GraphPad statistical software package (San Diego, CA, USA). Cytokine and NO levels were compared using unpaired two-tailed Student’s t test (GraphPad Software). \( P < 0.05 \) was considered statistically significant.

**Results**

**Consumption of POMx reduces incidence and severity of CIA in mice**

In these experiments we determined the ability of POMx to prevent the development of inflammatory arthritis in DBA/1 mice. Arthritis was induced by immunizing DBA/1 mice with heterologous CII emulsified in complete Freund’s adjuvant followed by a booster dose 3 wk later with CII emulsified in incomplete Freund’s adjuvant. Mice were dosed orally with POMx 10 d before immunization for the induction of arthritis and then continued to receive POMx orally until termination of the experiment. Mice orally fed 13.6 or 34 mg/kg of POMx displayed significant reductions in disease incidence (Fig. 1A) and delay in the onset of disease (Fig. 1B). Clinical severity of arthritis was also reduced in mice fed POMx (Fig. 1C) based on reduced paw swelling, erythema, and joint rigidity as determined by the mean visual arthritis score (\( P < 0.05 \)). There was no apparent toxicity or weight loss in mice receiving POMx orally (Fig. 1D). Control mice (water fed) showed a loss in body weight at 3 wk but the difference in body weight did not reach statistical significance when compared with the body weights of POMx-fed mice at any of the time periods used in this study (\( P > 0.05 \)).

Histopathologic analysis was performed on affected paws (fore or hind paws) harvested from control mice (no treatment) and mice with CIA receiving water or POMx. Representative images of joint sections stained with hematoxylin and eosin from these groups are shown in Figure 2A. These data show that in treatment groups oral consumption of POMx resulted in statistically significant reductions in synovitis, pannus, and bone erosion scores when compared with water-fed mice immunized the same way to develop arthritis (Fig. 2B).

**POMx inhibits macrophage production of inflammatory mediators**

It has previously been shown that activated macrophages infiltrate the joint and produce several inflammatory mediators in the joint microenvironment (reviewed by Burmester et al. [29]). One of these mediators is NO, which has been shown to be produced by activated macrophages, and high levels of NO have been detected in arthritic joints [29]. Based on this knowledge we characterized the effect of POMx on the activation of mouse macrophages. Mouse macrophages were cultured, pretreated with POMx, and then stimulated with LPS and the production of the second messenger nitric oxide in the culture supernatant was determined. POMx at the concentration used dramatically inhibited the production of NO (\( P < 0.001 \)) compared with cultures stimulated with LPS alone (Fig. 3). Because high-level expression of NO affects the cellular response to injury and its high levels can be pathogenic, these results suggest that POMx may inhibit joint damage by suppressing the production of NO.

**POMx effect on production of anti-CII antibodies in mice**

We also determined the presence and level of anti-CII antibodies by CII-specific ELISA in the arthritic joints of POMx-fed mice and non-POMx-fed mice with arthritis. There was no statistically significant difference in the levels of anti-CII-specific antibodies in any of the groups (\( P > 0.05 \), results not shown). This suggests that the reduced incidence and severity of arthritis in the POMx-fed mice was due to modulation mainly of the cellular immune response.

**POMx effect on inflammatory cytokines in arthritic joints**

Because the cytokine milieu is a critical mediator of the pathogenesis of arthritis, the production of IL-1β, IL-6, and TNF-α was examined by ELISA in the arthritic joint lysate prepared from joints isolated from POMx-fed and non-POMx-fed mice with CIA. Previous studies have suggested that these cytokines play a pivotal role in RA [30–33]. Our results (Fig. 4) showed that in arthritic joints isolated from non-POMx-fed mice levels of the inflammatory cytokine IL-6 were higher in the joints of non-POMx-fed mice compared with levels present in the arthritic joints of POMx-fed mice. Although levels of IL-1β and TNF-α were low in the arthritic joints of POMx-fed mice, the difference was not statistically significant when compared with levels detected in the joints of non-POMx-fed mice. None of these cytokines were detected in the joint lysates of untreated control mice (results not shown). Low-dose POMx feeding was more effective in inhibiting the production of IL-1β, whereas the higher dose tested was more effective in suppressing the production of TNF-α and IL-6 (Fig. 4). The reason behind this observation is not clear but suggests a window beyond which the consumption of POMx may not be useful. Taken together, these results suggest that the reduced incidence and severity of CIA may be related to the lower level of IL-6 in POMx-fed mice. An absence of significant differences in the levels of TNF-α in the arthritic joints of untreated and POMx-treated mice suggests that
Fig. 2. (A) PomX inhibits synovitis, pannus formation, and joint degradation in collagen-induced arthritis. Representative joint tissue sections stained with hematoxylin and eosin were obtained from (A) control DBA/1 mice (no treatment), (B) water-fed DBA/1 mice immunized to develop arthritis, (C) DBA/1 mice orally fed with PomX (13.4 mg/kg) daily and immunized to develop arthritis, and (D) DBA/1 mice orally fed with PomX (34 mg/kg) daily and immunized to develop arthritis. (B) Histopathologic scores of synovitis, pannus formation, and bone and cartilage erosions in collagen-induced arthritis-susceptible DBA/1 mice. Values are means ± SEs and differ without a common letter (P < 0.05). PomX, pomegranate extract.
POMx constituents exert their joint-sparing effects independently of the suppression of TNF-α, possibly by modulating the production of other inflammatory mediators in arthritis.

**POMx inhibits MAPK signal transduction pathways in mouse macrophages**

High levels of proinflammatory cytokines are present in RA joints and can induce the production of joint-degrading mediators by macrophages and other cell types, thereby exacerbating arthritis [29]. Activation of MAPK signal transduction pathways plays an important role in the production of such molecules in RA [34,35]. To determine whether POMx affects inflammatory stimuli-mediated MAPK signal transduction in mouse macrophages, we used immunoblotting to characterize lysates generated from mouse macrophages. Mouse macrophages were pretreated with POMx (20 μg/mL) and stimulated with 50 ng/mL of LPS for different periods of time, and lysates were prepared for analysis. Results of studies employing immunoblotting with highly specific antibodies showed that, when mouse macrophages were stimulated with LPS alone, phosphorylation of Jun N-terminal kinase (JNK) was rapid and intense, reached a peak 15–30 min after stimulation, and then declined (Fig. 5A). In contrast, in mouse macrophages stimulated in the presence of POMx, LPS-induced phosphorylation of JNK was delayed, with weakly phosphorylated JNK being detected at 30 min after stimulation and then barely detectable at 120 min after stimulation (Fig. 5A). Also, the phospho-JNK/total JNK analysis showed that phosphorylation of JNK molecules was higher in cells stimulated with LPS alone (Fig. 5B) compared with cells stimulated with LPS in the presence of POMx (Fig. 5C). Taken together, these data indicate that POMx inhibited the LPS-induced activation of JNK pathways, whereas levels of total JNK were not affected (Fig. 5A and results not shown). Phosphorylation of the downstream signaling molecule activating transcription factor-2 (ATF-2), a target of the stress-activated protein kinase (SAPK)/JNK signaling pathways [21], was also inhibited in mouse macrophages pretreated with POMx (not shown). These data provide support to the hypothesis that POMx exerts its anti-inflammatory effect by modulating activation of the JNK pathway in macrophages.

**POMx inhibits activation of NF-κB in mouse macrophages**

The activation of NF-κB is essential for the induction and expression of many proinflammatory genes, such as inducible NOS (NOS2) and IL-6, which may be relevant to the pathogenesis of arthritis. Activation of NF-κB typically requires the phosphorylation of the inhibitory protein IκB by the IκB kinase complex, which results in the degradation of IκB. This releases NF-κB and allows it to translocate to the nucleus, where it binds to the promoters of target genes. Because our results showed that treatment with POMx inhibited the LPS-induced production of NO by mouse macrophages, we determined whether POMx also inhibits the activation of NF-κB in mouse macrophages. We used the NF-κB–Luc reporter vector to transfect the mouse macrophages and treated the transfected macrophages with POMx, and then these cells were stimulated with LPS. Activation of NF-κB was determined by measuring the activity of the reporter enzyme in the macrophages. These results are shown in Figure 6 and clearly demonstrate that POMx was effective in suppressing the LPS-induced activation of NF-κB in mouse macrophages in vitro.

**Discussion**

Rheumatoid arthritis is a chronic inflammatory disease of the connective tissue and is characterized by synovial hyperplasia with local invasion of the bone and cartilage. The disease is largely driven by the recruitment of activated T and B cells and macrophages to the afflicted joints. Proinflammatory cytokines such as IL-6 and TNF-α produced by these activated cells contribute to the irreversible joint damage seen in RA. In view of the historical use and currently reported anti-inflammatory effects of POMx in several model systems [14–19], we explored the potential of consumption of POMx for preventing joint damage in RA. In this study we demonstrate that consumption of POMx robustly prevents the development of CIA in mice (an animal model of RA) by selectively inhibiting a spectrum of signal transduction pathways and cytokines critical to the development and maintenance of inflammation in RA. Our results demonstrate that consumption of POMx reduces the incidence and severity of CIA in mice (Fig. 1). We also demonstrate that POMx abrogates the phosphorylation of...
JNK and production of proinflammatory cytokines and inhibits cytokine-induced NO production in mouse macrophages. The c-Jun N-terminal protein kinase MAPKs (JNK) is a subgroup of the evolutionarily conserved family of serine/threonine protein kinases. JNK is activated by treatment of cells with inflammatory cytokines (such as TNF-α) and by exposure of cells to endotoxin. Previous studies have demonstrated that inhibition of JNK inhibits joint damage in inflammatory arthritis [36]. Thus our in vitro and in vivo data collectively indicate that POMx potently inhibits a range of cellular responses, including the activation of JNK, that play critical roles in driving synovitis, pannus formation, and joint destruction in RA. In all experiments there were no signs of toxicity in any of the groups as evidenced by weight gain (Fig. 1D), alanine aminotransferase, or serum creatinine levels (data not shown). No mortality due to POMx in any of the groups was observed.

Dietary supplements are taken orally and, hence, our use of gavage feeding was chosen to maximize the delivery so that the bioactivity of the administered dose could be determined independently of other factors. Examination of the data reveals that this regimen was effective in protecting mice from the development of CIA because statistically significant arthritis inhibition and joint-sparing effects were demonstrated in POMx-fed mice compared with water-fed mice (Figs. 1 and 2). These results provide the first in vivo evidence of antiarthritic efficacy of a chemically complex mixture of pomegranate fruit that is commercially available. Because in these studies pretreatment was required to inhibit the incidence and severity of joint inflammation, these data support the use of POMx for arthritis prevention and not for arthritis treatment in the face of active inflammation. Future studies will address the disease-modifying aspect of POMx consumption.

Macrophages are present in RA joints and play an important role in the pathogenesis of RA (reviewed by Szekanecz and Koch [37]). These activated cells produce TNF-α, NO, and other inflammatory mediators that exci-
Using biochemical techniques and immunoblotting, we demonstrate that POMx inhibits LPS-induced activation of the JNK and NF-κB signal transduction pathways and downstream activation of transcription factors and the production of NO in mouse macrophages. NO is synthesized from L-arginine by the enzyme NOS. After...
induction of the inducible isoform of NOS by endotoxin or inflammatory cytokines, NO is overproduced, resulting in cytotoxic and bacteriotoxic effects. The role of NO in inflammation is also evident from its inhibitory effects on T-cell activity, chemotaxis of monocytes and migration of neutrophils, and production of prostaglandin E2 and thromboxane in macrophages (reviewed by Stichtenoth and Frolich [38]). Studies with animal models of inflammatory arthritis and in patients with RA have demonstrated an important role for high levels of NO in the pathogenesis of the disease [38]. Our novel data presented in this report suggest that POMx-mediated inhibition of macrophages could contribute to its efficacy in inhibiting CIA as shown in this study and possibly in human RA.

Transcription factors such as NF-κB are ubiquitously expressed and can induce and repress gene expression by binding to κB elements present in the promoters of target genes including the genes that control apoptosis, adhesion, inflammation, and tissue remodeling (reviewed by Perkins [39]). Activation of NF-κB involves the phosphorylation of its inhibitory protein IκB by the IκB kinase complex, which results in its degradation and release of NF-κB. It has been shown that the incidence and severity of CIA were treated with a small molecule inhibitor of NF-κB [41]. Attenuation of murine CIA by administration of a selective small molecule inhibitor of NF-κB correlated with the reduction in levels of inflammatory cytokines such as IL-6 in the paw tissues of arthritic mice [42]. These data provide support to the present results by showing that POMx inhibited the endotoxin-induced activation of NF-κB in mouse macrophages. The significantly low levels of IL-6 detected in the arthritic joints of POMx-fed mice compared with control mice (P < 0.005) may be related to the in vivo inhibitory effect of POMx on NF-κB activation. However, this remains to be investigated.

In addition, low levels of inflammatory cytokines detected in the arthritic joints of the POMx-fed mice support the hypothesis that the observed reduction in joint inflammation scores was likely due to the inhibition of inflammatory cytokine production in the joints. In contrast to the ineffectiveness of high-dose POMx on the production of IL-1β in arthritic joints, low-dose feeding of POMx was effective in suppressing the production of IL-1β (Fig. 4A), whereas a high dose was needed to suppress the production of TNF-α (Fig. 4C). These results suggest that the constituents of POMx are needed in different concentrations to suppress the damaging effects of IL-1β and TNF-α in arthritis and other diseases associated with abnormal production of these cytokines. Identification of these components of POMx will open new avenues for the development of effective preventive therapies against arthritis. Moreover, our demonstration of the arthritis-preventive and joint-protective effects of POMx consumption provides support to the use of POMx or pomegranate-containing dietary supplements for the prevention of inflammatory arthritis.

Conclusions

The results of these translational studies and studies reported previously [19] together provide strong and compelling evidence to support further clinical testing of POMx for the prevention of RA and osteoarthritis. However, we must mention that, to our knowledge, no clinical trials in patients with RA have yet been done to document the safety and efficacy of POMx or other extracts of pomegranate available for over-the-counter use.

References

[33] Bhan D, Nair MG, Heber D. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J Nutr Biochem 2005;16:360–7.