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ORIGINAL ARTICLE

Preclinical efficacy of sodium narcistatin to reduce inflammation and joint destruction in rats with adjuvant-induced arthritis

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Abstract Current therapies for the treatment of rheumatoid arthritis (RA) do not work for all patients, can lose efficacy over time, and can have significant side effects. The discovery of new, effective therapies for RA remains an unmet medical need. The Amaryllidaceae isocarbostyril narciclasine was previously shown to prophylactically reduce paw swelling in rats with adjuvant-induced arthritis (AA). In this study, the efficacy of sodium narcistatin (SNS), a water-soluble cyclic phosphate pro-drug of narciclasine, was assessed in AA rats for anti-inflammatory and bone-sparing properties after disease onset. AA rats were given daily intraperitoneal injections of SNS (1.75, 3.5, or 5 mg/kg/day, in 500 µl sterile endotoxin-free saline) or saline from disease onset through severe disease stages. Footpad widths and radiographic scoring were used as indicators of inflammation and joint destruction, respectively. Ex vivo cytokine production by peripheral blood mononuclear cells (PMBC), splenocytes, and draining lymph node (DLN) cells were determined using ELISAs. SNS treatment dose-dependently reduced joint inflammation (~70%) and bone loss (~50%) compared with AA controls. SNS treatment also reduced spleen weight (without affecting body weight), pro-inflammatory cytokine production by PMBC, splenocytes, and DLN cells, and site-dependently altered T-helper (Th)1-/Th2-type and anti-inflammatory cytokine profiles. SNS dramatically reduces inflammation and has bone-sparing properties, possibly by reducing immune cell pro-inflammatory cytokine production. Our findings support the development of SNS as a therapeutic for RA.

Keywords Adjuvant-induced arthritis · Sodium narcistatin · Rheumatoid arthritis · Cytokines · Bone destruction · Inflammation

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Introduction

During the last 20 years, fundamental changes have occurred in the treatment of rheumatoid arthritis (RA). The effective use of methotrexate and the arrival of biologics, in particular anti-tumor necrosis factor (TNF)-α compounds, have made the goal of remission feasible for many RA patients [1–3]. Despite the monumental success of the biologics as a whole, 14–38% of RA patients have no response to first-line anti-TNF-α therapies and 45% of patients placed on these anti-TNF-α treatments are forced to discontinue their use within 2 years due to adverse events or loss

of efficacy [1–3]. Therefore, there is still an urgent need for the development of new efficacious drugs that prevent joint pathology without compromising host defense.

In RA, myelomonocytic cells, primarily macrophages and neutrophils, play key roles in the production and perpetuation of synovial inflammation by producing inflammatory cytokines, proteolytic enzymes and calprotectin [4, 5]. Systemic activation of macrophages precedes and correlates with arthritis induction and progression and the development of joint erosions [6, 7]. Furthermore, treatments targeting activated macrophages or their products have been the most effective therapeutics in ameliorating the disease [8, 9].

In synovial lining hyperplasia, the numbers of both macrophage-like and fibroblast-like synovial cells (FLS) increase [10, 11] due to proliferation, reduced apoptosis, and recruitment from other fibroblast pools [12]. The thickened synovial lining may transform into pannus, a tissue mass that invades into cartilage and subchondral bone [13]. In response to pro-inflammatory cytokines, resident FLS interact with diverse cell types to promote inflammation and joint destruction [14]. Pannus tissue has many tumor-like features, including attachment to cartilage, invasive growth, and FLS phenotypic changes similar to those of transformed cells [15]. Further, FLS from RA patients can grow in vitro in an anchorage-independent manner, a property that correlates closely with tumorigenicity in vivo [16]. FLS are responsible for the progressive destruction of articular cartilage and bone. FLS also help to recruit inflammatory cells and sustain the inflammatory response. The synovium undergoes a striking transformation characterized by inflammatory cell infiltration and synovial lining hyperplasia. Neutrophils predominate in the synovial fluid, while T cells, macrophages, B cells, plasma cells, and dendritic cells accumulate in the synovial sublining [17].

Narciclasine and similar Amaryllidaceae isocarbostyrils were developed as promising anti-cancer therapies that specifically target tumor cells [18]. In vitro, narciclasine's anti-inflammatory and anti-proliferative properties selectively induce apoptosis in cancer cells, but not normal fibroblasts, perhaps by activation of death receptors (DR) [19, 20]. If narciclasine could exert similar effects on FLS and the immune system, then this class of drug would have exciting anti-rheumatic therapeutic potential. Two papers provide support for anti-proliferative and anti-inflammatory effects of narciclasine in RA. Mimiki and coworkers reported that narciclasine reduces inflammation in the AA model of RA when administered prior to disease onset [5]. In addition, Yui and coworkers demonstrated narciclasine inhibits LPS—induced macrophage production of TNF-α [21], currently the most successful therapeutic target for aggressive RA.

While the narciclasine plant extract [22–24] and the organic synthesis procedures [18, 25] are available, narciclasine is sparingly soluble in most organic and aqueous solvents, limiting its development for medical applications. We have succeeded in synthesizing a cyclic phosphate prodrug of narciclasine, designated sodium narcistatin (SNS), which retains narciclasine's anti-inflammatory properties and is water soluble [19] (Fig. 1). This study examines the disease-modifying effects of SNS on established AA disease pathology. We report that SNS reduced joint inflammation, bone loss, and joint deformities in the Lewis rat AA model. Further, SNS shifted cytokine production toward anti-inflammatory and T-helper (Th) 2 profiles in secondary immune organs. Collectively, these findings suggest that SNS or its other cation derivatives [19] have exciting potential as effective disease-modifying therapeutics for the treatment of RA, at least in part, via immune-mediated mechanisms.

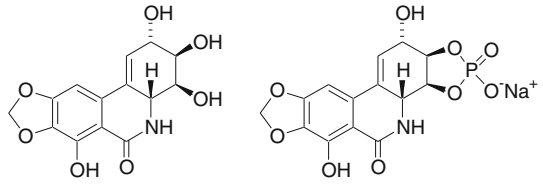

Fig. 1 The chemical structures of narciclasine (**1**) and sodium narcistatin (**2**)

Materials and methods

Chemicals and adjuvant

Sodium narcistatin was synthesized as previously described [19]. All tissue culture media, serum, and antibiotic were purchased from Invitrogen (San Diego, CA). Complete Freund's adjuvant (CFA) was prepared by emulsifying *Mycobacterium butyricum* (0.03 g heat-killed lyophilized; Difco, Detroit, MI) in 10 ml sterile mineral oil (Sigma Chemical Company, Saint Louis, MO). The suspensions were treated with an F-50 sonic dismembraner (Fisher Scientific, Pittsburg, PA) for 5 min to ensure a uniform bacterial cell wall suspension. All animals within each experimental group in this study developed arthritis with similar timing of disease onset and severity.

Animals

Adult male Lewis rats [200–250 g; Charles River Laboratories (Raleigh, NC)] were dual housed and allowed to acclimate to the Banner Research Institute's (BRI) vivarium for 7 days prior to initiating experiments. Animals were maintained on a 12-h off/on light schedule with food and water available ad libitum. CFA (100 µl) was injected intradermally

into the base of the tail to induce AA. To ensure easy assess to food and water, rodent chow (Purina Lab Diet 5001) was placed on the bottom of the cage and long-stemmed sipper tubes were used to deliver water. Animals were observed daily for adequate weight gain and general health. All rats were observed to eat and drink and continued normal grooming behavior throughout the experiments. Despite the preclinical weight loss seen in the CFA-challenged animals, their weights stabilized and the arthritic animals were able to maintain their weight and remain active throughout the course of the experiments. Protocols for animal use and care in this study were approved by the Banner Sun Health Research Institute's Animal Use and Care Committee prior to beginning the experiments and complied with NIH guidelines for the humane use and care of research animals.

Experimental design

Rats were randomly assigned into 1 of 6 experimental groups with 4 animals per group as follows: (1–4) saline vehicle, low (L)-, medium (M)-, and high (H)-dose SNS (arthritic; 0, 1.75, 3.5, and 5.0 mg/kg/day, i.p., respectively, given in a total volume of 500 µl), (5) non-treated CFA (arthritic; control for stress of injections), and (6) non-treated animals (non-arthritic, control for baseline immune values). The experiment was replicated a total of 3 times with an $N = 4$ per experiment. The data from each group in the replicate experiments were compared using a one-way ANOVA, and there were no statistical differences between the replicated experiments ($P > 0.05$); therefore, the data from the repeat experiments were combined giving an $N = 12$ per treatment group. No differences were found between the untreated and vehicle-treated CFA animals, so their data were collapsed and treated as one control group. CFA injections were given on experimental day 1 to all arthritic groups. Daily treatment with saline or SNS was started on day 10 and continued through day 28 post-CFA immunization. Untreated animals were handled, but received no injections.

Assessment of arthritis

Joint inflammation in arthritic rats was assessed by routine methods, as previously described [26]. Briefly, dorsoplantar widths of the hind feet were measured beginning 1 week prior to CFA administration, continued every other day until disease onset, then daily from disease onset through the day of killing using a Mitutoyo Corporation dial thickness gauge. Right and left footpad measurements from each rat were averaged, then mean footpad widths from animals within each group were averaged and expressed as a mean ± standard error of the mean (SEM). Radiographs were taken the day of killing using the following settings: 400 nN, 50 kvp, and 0.4 s exposure, at 40 cm and X-OMAT processor. X-rays were evaluated using a grading scale modified from Ackerman and coworkers [27]. In short, 2 independent observers subjectively rated each of the radiographs on the scale: 0 (normal), 1 (slight), 2 (mild), 3 (moderate), and 4 (severe) abnormalities in the tissue. Radiographs were scored for the following: (1) swelling as indicated by the width of soft tissue shadows and alterations in the normal configuration of the soft tissue planes, (2) osteoporosis as measured by bone density (recognized by increases in radiolucency relative to uninvolved adjacent bone), (3) cartilage loss shown by narrowing of the joint spaces, (4) destruction of bone (erosions), and (5) heterotopic ossification defined as formation of new bone (fine ossified lines paralleling normal bone, but not contiguous with calcified area of the bone). The radiographic scores for each category were summed for both hind limbs, giving a maximum score of 40, the scores were averaged within the treatment groups, and then expressed as a mean ± SEM.

Immune cell cultures

Inflammatory, Th1 and Th2 cytokine production by peripheral blood mononuclear cells (PBMCs), spleen and lymph nodes that drain the hind limbs (DLNs) were assessed ex vivo using non-arthritic, and saline- and H-SNS-treated arthritic animals.

To obtain PBMCs, blood was collected into a 7-ml lithium heparin-coated Vacutainer® tube using cardiac puncture. Tubes were inverted 7 times and the cells spun down at 3,000 rpm for 15 min at 10°C. The buffy coat was removed using a 2-ml pipette and placed into a sterile 15-ml tube containing 10 ml of NH_4Cl hypotonic buffer for 3 min to lyse the red blood cells. The PBMCs were centrifuged and resuspended in 10 ml Hank's balanced salt solution (HBSS), washed 3 times with 10 ml HBSS, and after the final spin, resuspended into complete media (RPMI 1640 media supplemented with 5% fetal calf serum and 1% antibiotic/antimycotic).

The spleen and DLNs (inguinal and poplitial lymph nodes that drain the hind limbs) were aseptically dissected and placed into HBSS. Spleens were placed in a stomacher bag containing 10 ml HBSS and homogenized for 30 s at normal speed (Seward, London, UK). Spleen cells were triturated with a 10-ml pipette 3 times, washed with an additional 10 ml HBSS, and passed through a 70-µm nylon filter (BD Biosciences, Bedford, MA) to remove extraneous connective tissue. The collected cells were centrifuged for 7 min at 1,500 rpm and resuspended in 5 ml NH_4Cl hypotonic buffer for 3 min to lyse red blood cells. The splenocytes were washed 2 times with 10 ml HBSS, centrifuged, and resuspended into complete media.

DLNs were pooled from both sides of the body, placed into 5 ml HBSS, teased apart using forceps, and triturated with a pipette. The cell suspensions were passed through nylon mesh to remove cell debris, centrifuged, and washed with 5 ml HBSS. Cells were pelleted by centrifugation at 1,500 rpm and then resuspended in complete media.

PBMCs, splenocytes, and DLNs were plated in a total volume of 2 ml at a cell concentration of 2×10^6 cells/ml in 24-well plates (Falcon, Oxnard, CA). Plates were incubated at 7% CO_2, 37°C for 24 h. Supernatants were collected into storage vials, and the vials, frozen and stored at −70°C until cytokine analyses.

Measurement of ex vivo cytokine production

Duo-set sandwich ELISA kits for the detection of IL-2, IL-4, IL-6, IL-10, IFNγ, and TNFα were obtained from R&D Systems (Minneapolis, MN) and performed according to the manufacturer's specifications. In brief, high-binding flat-bottom microtiter 96-well plates (Nunc Industries, Naperville, IL) were precoated with capture antibody in coating buffer (0.1 M phosphate-buffered saline, PBS, pH 7.4), sealed with plate film (Denville Scientific, South Plainfield, NJ), and incubated overnight at room temperature (RT). Plates were washed with 0.1 M PBS-0.5% Tween 20 (PBS-Tw20) and blocked (PBS-1% bovine serum albumin) for 1 h. Standards/samples were plated in duplicate, and the plates were incubated for 2 h at RT. Plates were washed 3 times with PBS-Tw20, biotinylated secondary detection antibody was added, and the plates were incubated for 2 h. Plates were washed 3 times, and streptavidin enzyme conjugate was added to the wells and then incubated for 30 min. Plates were washed 5 times, developed for 1 h using tetramethylbenzidine (TMB) reagent (BD Biosciences, Bedford, MA). The chromagen reaction was stopped with 1 N sulfuric acid. Cytokine concentrations in each sample were compared with a standard curve determined for each plate using an absorbance reader (Ceres 900 HDI, Bio Tek Instruments Incorporated, Winooski, VT) set at 450 nm. The duplicate samples were averaged. For each lymphoid compartment, cytokine concentrations within each treatment group were expressed as a mean ± SEM in pg/ml.

Statistical analysis

Dorsoplantar footpad widths were subjected to a two-way repeated measures analysis of variance (ANOVA) ($P < 0.05$), and significant ANOVAs were subjected to Bonferroni post hoc testing. The radiographic analysis was completed using Krüskal-Wallis statistical analysis followed by Dunn post hoc testing. Group differences for cytokine measures were determined using a one-way ANOVA coupled to Bonferroni post hoc testing for significant ANOVAs ($P < 0.05$).

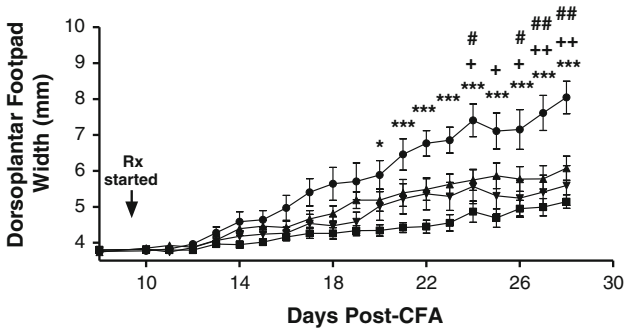

Fig. 2 Dorsoplantar footpad widths expressed in mm ± SEM ($N = 12$/group) 28 days post-immunization from arthritic rats treated daily with saline (*filled circle*), H-SNS (*filled square*), M-SNS (*filled inverted triangle*), or L-SNS (*filled triangle*) from disease onset through day 28. Treatment with H-, M-, or L-SNS significantly decreased (*, +, or #, respectively) the soft tissue swelling of the dorsoplantar footpads between day 23 and 28 post-immunization compared with vehicle-treated rats. Footpads were analyzed using a repeated measure two-way ANOVA followed by multiple comparison Bonferroni post hoc testing. SNS, sodium narcistatin; (*, +, or # $P < 0.05$; ++ or ## $P < 0.01$, *** $P < 0.001$). *Rx* treatment

Results

SNS reduces hind limb inflammation

SNS treatment reduced signs of inflammation (i.e., redness and swelling) in the hind limbs of arthritic rats compared with saline-treated arthritic controls on day 28 post-CFA immunization (Fig. 2). An increase in mean dorsoplantar footpad widths was apparent by day 10 in all CFA-treated rats and continued to increase through the effector phase of the disease in control rats (filled circles). Dorsoplantar footpad measurements demonstrated that treatment with H-, M-, or L-SNS (filled squares, inverted triangles, or triangles, respectively) significantly decreased soft tissue swelling compared with saline-treated arthritic rats (filled circles; Fig. 2) [H-SNS vs. Saline: day 20: $P < 0.05$ (*); day 21–28: $P < 0.01$ (++); M-SNS vs. Saline: day 24, 26: $P < 0.05$ (+); day 27–28: $P < 0.01$ (++); L-SNS vs. Saline: day 24: $P < 0.05$ (#); day 27–28: $P < 0.01$ (##)]. The anti-inflammatory effects of SNS on mean dorsoplantar foot pad widths were dose-dependent, with the highest dose having the greatest efficacy.

SNS reduces hind limb bone loss

Representative radiographs from non-arthritic (non-AA) and arthritic rats treated with saline or H-, M-, or L-SNS (Fig. 3a) revealed significant bone loss, soft tissue swelling, periosteal bone formation coupled to a narrowing of the joint spaces

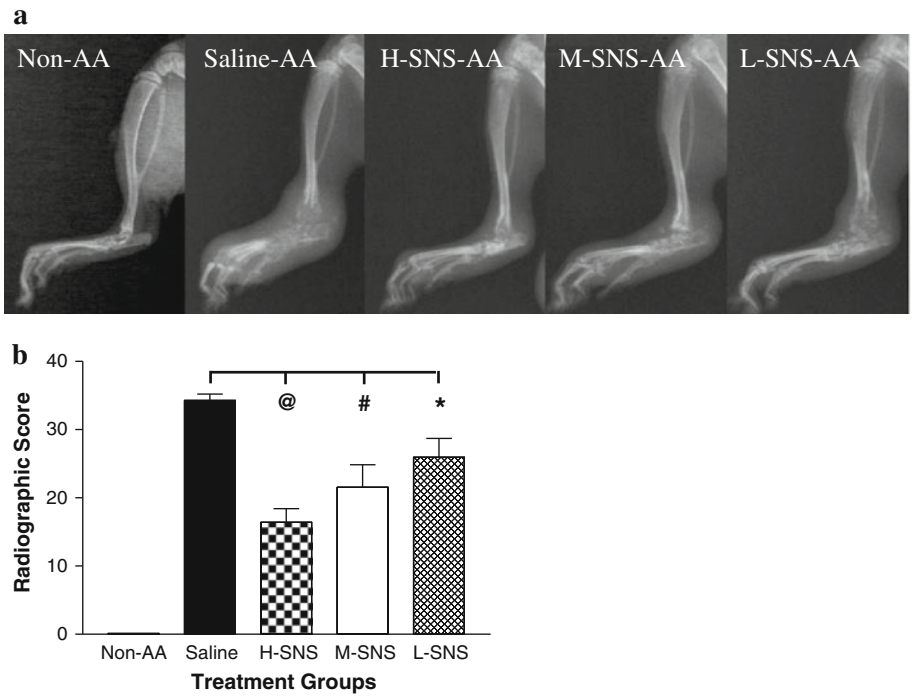

Fig. 3 Hind limb radiographs. **a** At 28 days post-immunization, representative radiographs of hind limbs from non-arthritic (non-AA) and arthritic rats treated daily with saline or high (H)-, medium (M)-, or low (L)-dose sodium narcistatin (SNS) from day 10 to 28. The ankle joint from non-AA controls was normal. There was significant bone loss, soft tissue swelling, periosteal bone formation, joint space narrowing between the metatarsals, and reduced bone radiolucency in saline-treated arthritic (saline-AA) compared with non-arthritic (non-AA) rats. Joint space narrowing and soft tissue swelling was apparent in all arthritic animals, but was greatest and lowest with saline (saline-AA) and high-dose SNS (H-SNS-AA) treatment, respectively. H-, M-, or L-SNS treatment dose-dependently decreased soft tissue swelling, as indicated by reduced hind limb shadow widths. Bone destruction was significantly reduced (mainly less osteoporosis and fewer erosions, as well as increased bone luminescence) and cartilage preservation in H-, M-, and L-SNS-treated (H-, M-, and L-SNS-AA) compared with saline-treated (saline-AA) rats. **b** Saline-treated rats had the highest radiographic scores, indicative of severe inflammation and joint destruction. With H-, M-, or L-SNS treatment, radiographic scores were dose-dependently decreased ($^{@}P < 0.001$, $^{\#}P < 0.01$, $^{*}P < 0.05$, respectively) compared with saline-treated controls. ($N = 12$)

between the metatarsals, and decreased bone radiolucency in saline-treated AA animals. In contrast, radiographs from SNS-treated AA rats demonstrated a dose-dependent, drug-induced sparing of bone, reduced soft tissue swelling, lower periosteal bone formation, less narrowing of the joint spaces, and increased bone density compared with saline-treated arthritic rats. Radiographic analysis of the ankle joints 28 days post-CFA challenge confirmed the destructive joint changes in all AA groups and the ameliorating effects of SNS on joint pathology (Fig. 3b). SNS treatment lowered radiographic scores compared with the saline-treated AA rats (H-SNS vs. Saline: $P < 0.001$; M-SNS vs. Saline: $P < 0.01$; L-SNS vs. Saline: 16.34, $P < 0.05$). Effects of SNS on joint destruction were dose-dependent, with the highest dose having the greatest disease ameliorating effects.

SNS reduces spleen weight without effecting body weight

The mean body weight from untreated non-arthritic rats steadily increased during the experiment (data not shown). By day 28, body weights of the arthritic rats were reduced (23%) compared with non-arthritic control rats (Fig. 4a). This decreased body weight measure is disease-specific, as regardless of the dose, there were no differences between the body weights of the arthritic rats given SNS compared with those given saline (Fig. 4a). On day 28, there was a disease-specific increase in spleen weight (Fig. 4b) and spleen weight/body weight ratio (Fig. 4c) in the saline-treated AA animals compared with the non-arthritic controls (Fig. 4b, Saline vs. Non-AA: $P < 0.001$; Fig. 4c; Saline vs. Non-AA: $P < 0.001$). Treatment of arthritic rats with H- or M-SNS attenuated the increase in spleen weight and spleen weight/body weight ratio (Fig. 4b and c, respectively) that occurred with arthritis development (Fig. 4b, H-SNS vs. Saline, $P < 0.001$; M-SNS vs. Saline, $P < 0.001$; Fig. 4c, H-SNS vs. Saline, $P < 0.001$; M-SNS vs. Saline, $P < 0.05$).

Immune cell cytokine production is altered with AA and by treatment with H-SNS

To determine the effects of H-SNS on cytokine profiles, ex vivo cytokine production in PBMCs, splenocytes, and DLN

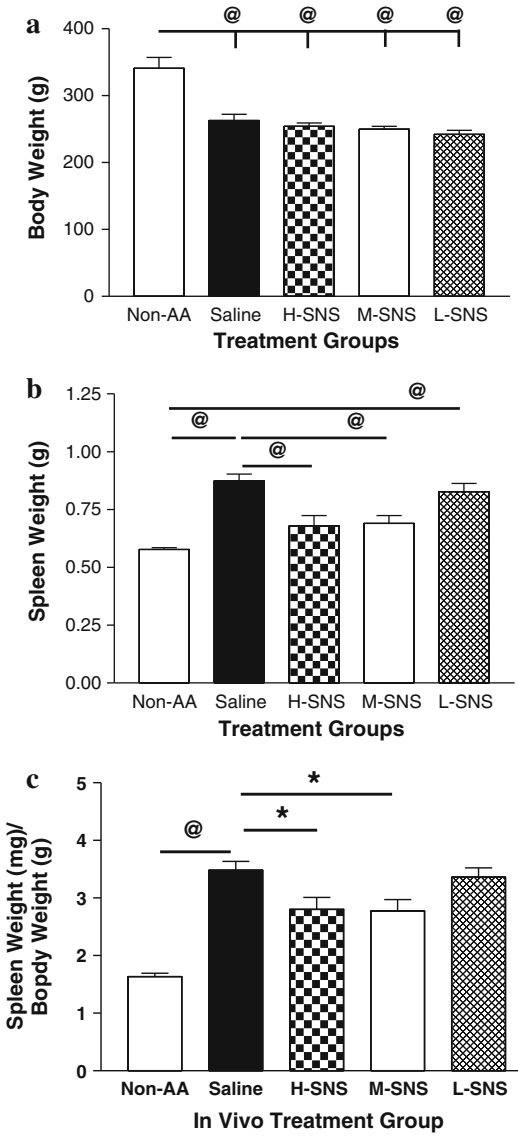

Fig. 4 Mean body weights (**a**) and spleen weights expressed as mean ± SEM in grams (g) (**b**) or normalized for body weight (mg/g ± SEM) (**c**) 28 days post-immunization from non-arthritic (non-AA) and arthritic rats receiving daily treatments of saline or high (H)-, medium (M)-, or low (L)-dose sodium narcistatin (SNS) initiated at disease onset (day 10) and continued until day 28 ($N = 12$/group). There was a disease-induced decrease ($^{@}P < 0.001$) in body weights of saline-treated arthritic compared with non-arthritic rats (**a**). Chronic SNS treatment did not significantly alter body weight compared with saline-treated controls (**a**). In contrast, there was a disease-specific increase ($^{@}P < 0.001$) in spleen weights in saline-treated compared with non-AA rats that was not affected by treatment with L-SNS (**b**). However, treatment with H- or M-SNS attenuated ($^{@}P < 0.001$) the disease-specific rise in spleen weight (**b**). Normalized spleen weights (**c**) revealed a similar response pattern in all treatment groups, with increased ($^{@}P < 0.001$) normalized spleen weight with saline and L-SNS treatment over non-AA rats, and H- and L-SNS treatment reducing ($^{*}P < 0.05$) the effect of AA on normalized spleen weights

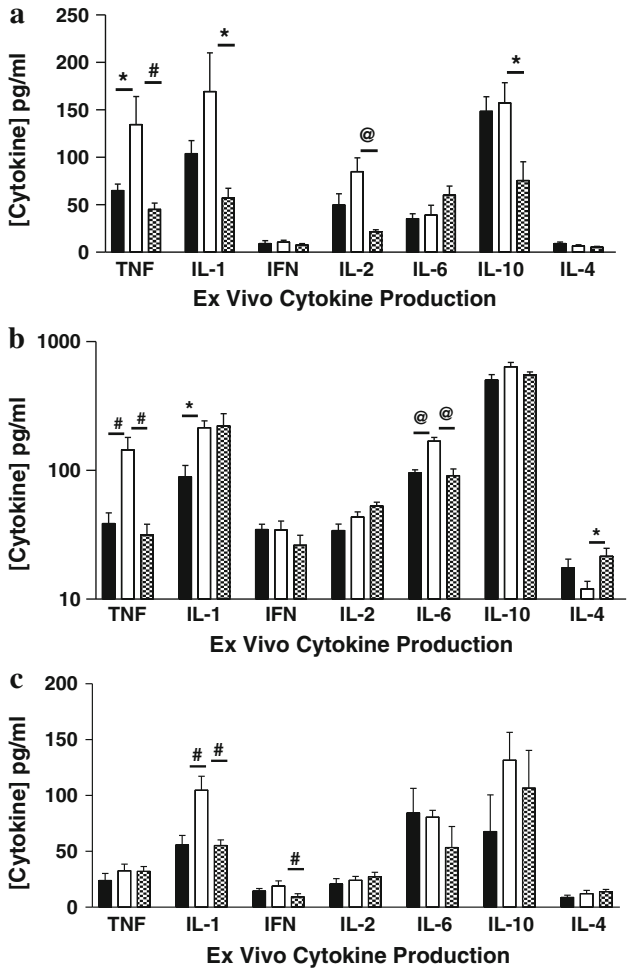

Fig. 5 Ex vivo cytokine production by peripheral blood mononuclear cells (PBMCs) (**a**), splenocytes (**b**), and draining lymph node (DLN) cells (**c**) from non-arthritic (non-AA) (*black*), and saline-treated (*white*) and high (H)-dose sodium narcistatin (SNS) (checkered)-treated arthritic rats 28 days post-CFA is expressed as means ± SEM ($N = 12$/group). With saline treatment, TNFα levels from PBMCs rose ($^{*}P < 0.05$) over non-AA controls, an effect reversed ($^{\#}P < 0.01$) by H-SNS treatment (**a**). H-SNS treatment lowered ($^{*}P < 0.05$, $^{@}P < 0.001$ and $^{*}P < 0.05$, respectively) IL-1β, IL-2, and IL-10 concentrations from PBMCs over saline treatment (**a**). No disease or treatment effects on IFNγ, IL-6, or IL-4 production by PBMCs were observed (**a**). Splenocyte TNFα, IL-1β, and IL-6 secretion was greater ($^{\#}P < 0.01$, $^{*}P < 0.05$, and $^{@}P < 0.001$, respectively) with saline treatment than non-arthritic controls, effects reversed by H-SNS treatment for TNFα ($^{\#}P < 0.01$) and IL-6 ($^{@}P < 0.001$) (**b**). H-SNS treatment increased splenocyte IL-4 levels ($^{*}P < 0.05$) over saline-treated concentrations (**b**). No disease or treatment effects were observed for IFNγ, IL-2, or IL-10 (**b**). IL-1β production by cultured DLN cells was augmented ($^{\#}P < 0.01$) in saline-treated arthritic compared with non-arthritic controls, an effect blocked ($^{\#}P < 0.01$) by H-SNS treatment (**c**). H-SNS treatment also reduced DLN cell IFNγ production; no disease or treatment effects on TNFα, IL-2, IL-6, IL-10, or IL-4 were seen (**c**)

cells was evaluated at day 28 post-immunization with CFA without further stimulation in culture. There was a 200 and 75% increase ($P < 0.05$) in TNFα and IL-1 production by cultured PBMCs from saline-treated AA rats (Fig. 5a) compared with non-AA rats, respectively. H-SNS treatment blocked the disease-specific rise in TNFα and IL-1 (H-SNS

vs. Saline, $P < 0.01$ and $P < 0.05$, respectively). There was a non-significant trend toward increased IL-2 production in cultured PBMCs from saline-treated arthritic rats compared with non-arthritic controls. H-SNS treatment reduced IL-2 production by PBMCs compared with saline-treated arthritic rats ($P < 0.001$). Cultured IL-10 levels in PBMCs were similar in non-arthritic and saline-treated arthritic rats, but treatment with H-SNS significantly reduced ($P < 0.05$) IL-10 production by PBMCs (Fig. 5a). Finally, low levels of IFNγ, IL-6, and IL-4 were released from unstimulated PBMCs but were similar in all treatment groups (Fig. 5a). No effects of AA or drug treatment were observed in the IL-10/TNF ratio from PBMCs (Fig. 6a).

Cultured splenocytes from saline-treated AA rats produced significantly greater ($P < 0.01$, $P < 0.05$, and $P < 0.001$, respectively) levels of TNFα, IL-1, and IL-6 than non-AA rats (Fig. 5b). Treatment with H-SNS blocked ($P < 0.01$ and $P < 0.001$, respectively) the rise in TNFα and IL-6, but had no effect on IL-1, compared with the saline-treated AA rats. In H-SNS-treated rats, splenocytes produced higher ($P < 0.05$) IL-4 concentrations than non-arthritic and saline-treated arthritic animals (Fig. 5b). There were no significant treatment or disease effects on IFNγ, IL-2, or IL-10; however, IL-10 production was high in all treatment groups. Interestingly, the ratio of anti- to pro-inflammatory macrophage cytokine production (IL-10/TNFα) (Fig. 6b), an indicator of anti-inflammatory drug effects, demonstrated a significant shift toward an anti-inflammatory milieu in the H-SNS arthritic rats compared with their saline-treated arthritic counterparts (H-SNS/saline, $P < 0.05$).

Development of AA increased the release of IL-1 from DLN cells compared with non-arthritic controls ($P < 0.01$), an effect that was reversed by treatment with H-SNS (Fig. 5c; $P < 0.01$). There were no differences between the levels of TNFα, IL-2, IL-6, IL-10, IL-4, or IFNγ released by DLN cells from arthritic and non-arthritic rats (Fig. 5c). However, H-SNS treatment in arthritic rats significantly reduced the IFNγ production compared to saline-treated arthritic rats (Fig. 5c; $P < 0.05$). The IL-10/TNF ratios from DLN cells were similar in all treatment groups (Fig. 6c).

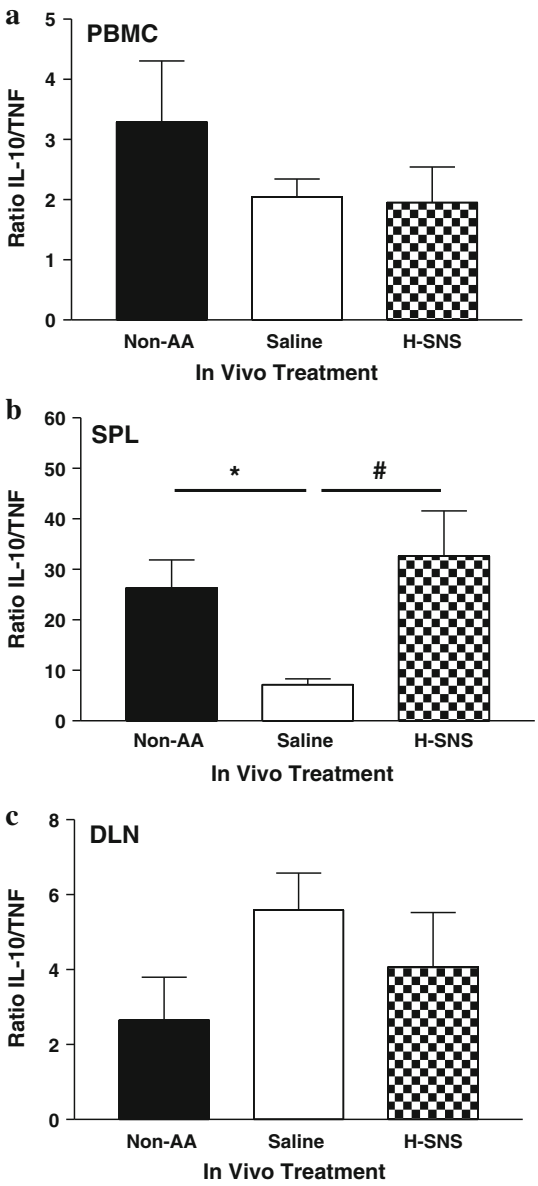

Fig. 6 IL-10/TNFα ratios based on ex vivo cytokine production from 24-h cultures of peripheral blood mononuclear cells (PBMC) (**a**), splenocytes (SPL) (**b**), and draining lymph node (DLN) cells (**c**) from non-arthritic (non-AA), and saline or high-dose sodium narcistatin (H-SNS). IL-10/TNFα ratios in PBMC (**a**) or DLN (**c**) cultures did not differ between treatment groups, although there was a trend toward an increased ratio by DLN cells from saline- and H-SNS-treated arthritic rats (**c**). In contrast, splenic IL-10/TNFα ratios (**b**) were lower ($^{*}P < 0.05$) in the saline-treated group compared with the non-AA rats, an effect reversed ($^{\#}P < 0.01$) by H-SNS treatment. *IL* interleukin; *TNF* tumor necrosis factor

Discussion

The ability of SNS to attenuate the development of severe disease pathology in AA was examined. Daily SNS treatment, from disease onset through severe disease, reduced joint inflammation and dramatically decreased bone and cartilage damage. The inhibition of joint soft tissue swelling and inflammation is consistent with a previous report [5] where experiments were carried out using the parent compound, narciclasine, in a different rat AA model. This report demonstrated that prophylactic narciclasine treatment reduced inflammation during the acute inflammatory disease phase; however, the effectiveness of narciclasine was lost by day 21 post-CFA challenge. Our finding that treatment with SNS effectively reduced inflammation and joint destruction when administered after disease onset

through severe disease (day 28) in AA rats is novel. The discrepancy in efficacy between these studies may reflect differences in animal models, drug kinetics, bioavailability, and timing/length of drug delivery. Our findings suggests that SNS, and presumably other cyclic phosphate derivatives of narciclasine, has potent anti-inflammatory and bone-sparing properties, indicating their potential as RA therapeutics.

SNS treatment was well tolerated by the arthritic rats. No overt signs of toxicity were apparent based on body weight, grooming behaviors, and activity levels; further, no pathology was apparent with gross evaluation of internal organs (data not shown) compared to untreated AA rats. We previously reported a disease-specific increase in whole spleen weight with arthritis development compared with non-arthritic rats [26]. This is consistent with reports that spleen-derived immune cells play a significant role in the disease pathology [28–30]. In this study, treatment with SNS blocked this disease-specific increase in spleen size, suggesting the ability of SNS to alter immune functions related to the processing of the arthritogenic antigens and development of effector immune cell functions. This result is also consistent with the established disease-specific anti-proliferative properties of SNS described in cancer cells [19]. These data suggest that the effects of SNS are mediated in part by its effects on lymphoid organs. Future studies will be needed to determine the effects of SNS on clonal expansion, negative selection, homing, and immunocyte activation in the primary and secondary lymphoid organs of arthritic animals.

Reduced footpad inflammation and altered cytokine profiles suggest that SNS mediates its anti-inflammatory effects, in part, by inhibiting macrophage pro-inflammatory cytokine production. Disease-induced increases in PMBC and splenocyte TNFα were reduced following in vivo SNS treatment. Our findings are consist with a study demonstrating narciclasine inhibits LPS- or bacteria-induced TNFα production by macrophages [21]. Given that anti-TNFα therapies can effectively reduce pathology in collagen-induced arthritis (CIA) [31], transgenic mice that over-express TNFα [32], and RA patients (reviewed in [9]), the inhibition of macrophage-derived TNF-α production may partially explain the decrease in disease severity observed in this study. In addition, in vivo SNS treatment reduced IL-1β production in DLN cells and the PBMCs. IL-1β plays a pivotal role in bone destruction, as blocking IL-1β with its receptor antagonist, IL-1ra, reduces bone destruction in CIA [33, 34] and RA patients [8]. Thus, SNS targets two key cytokines that promote inflammation and joint destruction in RA.

Our findings also indicate that SNS alters the balance between Th1 and Th2 immune responses. SNS treatment reduced PBMC production of IL-2, increased splenocyte production of IL-4, and decreased DLN cell production of IFN-γ. Although these cytokine changes were less robust than those for TNF-α and IL-1β, these changes would be expected to promote Th2 cell development and dampen Th1 cell development, driving a shift toward humoral- over cellular-mediated immunity.

The mechanisms responsible for mediating SNS anti-arthritic effects have not been elucidated; however, others report that narciclasine inhibits the cytotoxic activities of calprotectin [5] and reduces TNFα production [21]. More recently, Dumont and coworkers [20] reported that narciclasine causes cancer cell-specific apoptosis by inducing the initiator caspases of death receptor pathways for Fas and death receptor 4 (DR4). Thus, in cancer cells, narciclasine appears to activate DR pathways via receptors for FasL and TNF-related apoptosis-inducing ligand (TRAIL) selectively expressed in the cancer cells. However, RA synovial fibroblasts express functionally active DRs for FasL and TRAIL [35], suggesting another potential mechanism for SNS joint-sparing effects through selective activation of DRs in the arthritic synovium.

In RA, FLS develop anti-apoptotic protective mechanisms, including changes in DR and mitochondrial apoptosis pathways that alter expression of downstream modulators of DRs and transcriptional regulators (reviewed in [14]). Fibroblast-like synoviocytes from RA patients express a variety of DRs of the TNF-receptor family, Fas/CD95 [36], TRAIL-R1 (DR4) and R2 (DR5) [35, 37, 38], and TNFR1 [39]; however, they remain relatively resistant to receptor-induced apoptosis.

Antigen-specific lymphocytes involved in promoting FLS proliferation and invasion of joint tissue also express DRs that are essential for negative selection of autoreactive T cells in the thymus [40]. Negative selection is essential for self-tolerance, and its breakdown may lead to development of autoimmune diseases, including RA. Negative selection is defective in mice deficient in TRAIL [41] and confers sensitivity to CIA due to failure to delete specific relevant T cells and to properly silence activated T cells [40]. Blockade of TRAIL with systemic treatment of DR5 exacerbated arthritis. Further, injection of TRAIL-expressing adenovirus into arthritis joints reduces arthritis [42, 43], and treatment with anti-TRAIL receptor antibody is effective in treating bone-erosive disease [38]. These findings support a role for TRAIL in development of arthritis.

In TRAIL-deficient mice, immature CD4+CD8+ cells expressing high levels of heat-stable antigen are resistant to anti-CD3 antibody-mediated cell death [40]. TRAIL−/− mice also failed to reduce ovalbumin-specific cells following exposure to ovalbumin. Blockade of TRAIL promotes accumulation of splenic T cells in S-G2/M of the cell cycle [42], which confers resistance to DR-mediated apoptosis [44]. These findings support an important role for DRs in

negative selection. If SNS is confirmed to act as a death receptor ligand for TRAIL or Fas receptors in RA, it could affect disease outcome by altering T cell as well as synoviocyte functions. Future studies will explore this potential mechanism for the anti-arthritic effects of SNS.

In conclusion, we report for the first time that SNS treatment of rats with AA, from disease onset through development of severe disease, dramatically reduced inflammation and joint destruction in AA. Chronic SNS treatment was well tolerated at the doses and over the time course of treatment in the arthritic rats used in this study, prevented the disease-associated increase in spleen weight, and shifted cytokine production in secondary immune organs toward anti-inflammatory and Th2 profiles. Given that the best current drug therapies are not effective in a large subset of RA patients, have significant side effects, are expensive, and are not orally bioavailable, the protective effects of SNS in AA rats and its potential for oral administration suggest that this drug warrants further investigation as a potential novel therapeutic for RA.

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