Inhibition of Ras GTPases prevents Collagen-Induced Arthritis by Reducing the Generation of Pathogenic CD4⁺ T Cells and the Hyposialylation of Autoantibodies

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Objective. RasGTPases are master regulators of multiple intracellular signaling cascades. Perturbation of this pathway has been implicated in the pathogenesis of rheumatoid arthritis (RA). In this study we aimed to define the therapeutic potential of a novel RasGTPases inhibitor, farnesylthiosalicylate (FTS), in the preclinical mouse model of collagen-induced arthritis (CIA) and better delineate its immunomodulatory effects both ex vivo and in the mouse.

Methods. We analyzed in vitro the immunomodulatory effects of FTS on various CD4⁺ T-cell functions such as activation, proliferation, T-helper polarization, and production of proinflammatory cytokines. Using the CIA model, we further determined the efficacy of FTS to inhibit clinical, histopathologic, and diverse immunological outcomes of arthritis.

Results. FTS treatment of CD4⁺ T cells in vitro effectively targeted distinct kinases (extracellular signal-regulated kinase 1/2, p38, protein kinase B/AKT, and mammalian target of rapamycin), the production of interleukin (IL)-17A, IL-22, and granulocyte-macrophage colony-stimulating factor, and Th17 polarization. FTS therapy in the mouse CIA model significantly reduced clinical disease severity and joint inflammation/damage by histology. Importantly, FTS suppressed the in vivo induction of splenic IL-17⁺IL-22⁺ Th17 cells and the secretion of proinflammatory cytokines. The production of pathogenic autoantibodies and their abnormal hyposialylation was significantly attenuated by FTS therapy. Importantly, in vivo generation of collagen type-II specific effector CD4⁺ T cells was likewise repressed by FTS therapy.

Conclusion. The RasGTPases inhibitor FTS attenuates the production of proinflammatory cytokines by in vitro–activated T cells and is a potent immunomodulatory compound in the CIA model, primarily targeting the generation of autoreactive Th17 cells and the production of autoantibodies and their subsequent pathogenic hyposialylation.

INTRODUCTION

Rheumatoid arthritis (RA) is a prototypical systemic inflammatory autoimmune disorder that mainly leads to erosive synovitis with destruction of cartilage and joints (1). Although the pathogenesis of RA has not been fully elucidated, immune mechanisms that include autoreactive Th17- and Th1-type effector T cells, autoreactive B cells producing anticitrullinated protein antibodies (ACPA), and dysregulation of various protein kinase pathways have been postulated to contribute to the development of RA and experimental arthritis in relevant animal models (2-8).

GTPases of the Ras family are evolutionary conserved proteins that regulate vital cellular processes (9,10). Downstream signaling from the T-cell antigen receptor (TCR) and the co-stimulatory receptor CD28 is coupled with activation of the Ras signaling cascade, and Ras-dependent phosphorylation of selected members of the mitogen-activated protein kinase (MAPK) pathway, primarily extracellular signal-regulated kinase (Erk)1/2 has been suggested to regulate the threshold for TCR activation (11). Disruption of the Ras/Erk1/2 cascade has been linked to T-cell anergy (12,13). Moreover, T cells from patients with RA have been found to have significantly higher levels of phosphorylated Erk1/2 (11,14). Importantly,

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overexpression of mutated K-Ras in CD4+ T cells increased TCR sensitivity to low-affinity antigens, including to the clinically relevant autoantigen, citrullinated vimentin peptides (11,14).

Posttranslational addition of the farnesyl isoprenoid molecule to the conserved Cysteine 186 of Ras is vital for proper function (15). More recent studies demonstrated that specific protein chaperones with a prenyl-binding hydrophobic pocket facilitate appropriate subcellular compartmentalization of Ras GTPases and their signaling from the plasma membrane (16). Farnesylthiosalicylate (FTS) is a nontoxic competitor for the interactions between Ras and its chaperons, effectively dislodging RasGTPases from the plasma membrane to reduce downstream signaling (16,17). Over the past decade, FTS and related analogues were shown to have a significant therapeutic effect in several animal models of autoimmunity (18-20). More recently, using the rat adjuvant-induced arthritis (AIA) model, we demonstrated that prophylactic, but not therapeutic, dosing with FTS can attenuate the severity of disease (21).

Collagen-induced arthritis (CIA) is a widely studied preclinical animal model of RA that is widely employed to identify and test novel therapies for RA. It shares several clinical and immunological features with RA, such as synovial hyperplasia, mononuclear cell infiltration, cartilage degradation, and bony erosions. Similar to RA, major histocompatibility complex class II genes and CD4+ T cells are vital to the pathogenesis of CIA. Unlike AIA, the development of arthritis in CIA depends on the production of pathogenic autoantibodies (22-24).

In this study, we determined the distinct effect of FTS in vitro treatment on a variety of CD4+ T-cell functions and Th17 polarization program. In parallel, we defined the therapeutic effectiveness of oral FTS and its in vivo mechanism of action.

**MATERIALS AND METHODS**

**Animals.** Male DBA/1 mice, 8 to 12 weeks old, were obtained from a commercial breeder, ie, Harlan Laboratories Ltd. (now Envigo). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Tel Aviv University (Approval L-14-023).

**Arthritis induction and drug administration.** Mice were first injected, on day 0, with immunization-grade Bovine Type II Collagen (CII) emulsion in Complete Freund’s adjuvant (CFA) with Mycobacterium tuberculosis H37 RA at 2 mg/ml, and arthritis was induced with a booster injection of incomplete Freund’s adjuvant/CII on day +21 (all reagents from Chondrex, Inc) as previously described (23). Mice in the experimental arms were treated starting day +18 with daily oral FTS (100 mg/kg), weekly intraperitoneal injection of methotrexate (MTX) (1 mg/kg), or 0.5% carboxymethylcellulose (CMC) vehicle solution (control treatment). CIA severity was graded by a validated clinical score (0-16 scale), starting from day +30 slightly prior to clinical arthritis onset.

**Lymphocyte isolation, intracellular cytokine staining, and flow cytometric analysis.** Single-cell suspensions of splenocytes and inguinal lymph nodes (LNs) were prepared by mechanical disaggregation followed by lysis of red blood cells with a commercial ammonium chloride buffer (Gibco, Thermo Fisher Scientific, Inc). T cells were stained with a combination of fluochrome-conjugated monoclonal antibodies (mAb), all obtained from eBioscience (Thermo Fisher Scientific). For intracellular cytokine staining, lymphocytes were activated for 5 hours in RPMI medium with Cell Stimulation Cocktail (eBioscience, Thermo Fisher Scientific) per the manufacturer’s protocol. Then, cells were stained, washed, fixed, and permeabilized using a BD Cytofix/Cytoperm kit (BD Biosciences). For Tregs, cells were stained, washed, fixed, and permeabilized with anti-Foxp3 mAbs (eBioscience). For phospho-protein analysis, cells (1 × 10^6/sample) were surface stained and fixed with 4% paraformaldehyde for 45 minutes at room temperature and permeabilized using ice-cold methanol for 30 minutes, washed, and then immunostained with fluochrome-conjugated mAb for 45 minutes at room temperature. Stained cells were resuspended in 200 µl of FACS buffer and acquired using a FACS Aria flow cytometer (BD Biosciences) and further analyzed using the FlowJo version 10 software (TreeStar, Inc).

**In vitro T-cell proliferation assay.** Freshly isolated mouse CD4+ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE), activated by plate-bound anti-CD3 and soluble anti-CD28, and cultured in complete RPMI medium for 3 days, and treated with different concentration of FTS or vehicle. At the end of the experiment, the CD4+ T cells were harvested and analyzed for CFSE dilution by FACS.

**In vitro T-cell differentiation.** Naive CD4+ T cells were stimulated as detailed above, and cultured for 5 days in complete RPMI medium supplemented with Th17-polarizing cytokines (IL-23 at 50 ng/ml, IL-6 at 50 ng/ml, and IL-1β at 20 ng/ml; all from PeproTech, Inc) or vehicle. The various cultures were treated with increasing concentration of FTS or vehicle.

**Processing and evaluation of joint histology.** At study termination, the tibialartial joint was transected at the level of the medial and lateral malleolus for histopathological assessment. Ankle joints were then collected for histopathological assessment. When decalcification was completed, the ankle joint was transected in the longitudinal plane, and joints were processed for paraffin embedding, sectioned, and stained with hematoxylin and eosin (H&E) or Safranin O. Tissue section slides were examined by an experienced pathologist, blinded to the animal treatment protocol, and scored for inflammation, bone resorption, and cartilage damage on a scale of 0 to 5, as previously described (25,26).

**Flow cytometry.** Single-cell suspensions of splenocytes were stained for CD4 and CD25 expression, washed, fixed, permeabilized, and immunostained with anti-Foxp3 mAbs. For Tregs, cells were stained, washed, fixed, and permeabilized with anti-Foxp3 mAbs (eBioscience). For phospho-protein analysis, cells (1 × 10^6/sample) were surface stained and fixed with 4% paraformaldehyde for 45 minutes at room temperature and permeabilized using ice-cold methanol for 30 minutes, washed, and then immunostained with fluochrome-conjugated mAb for 45 minutes at room temperature. Stained cells were resuspended in 200 µl of FACS buffer and acquired using a FACS Aria flow cytometer (BD Biosciences) and further analyzed using the FlowJo version 10 software (TreeStar, Inc).

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In vitro CII-specific T-cell recall responses. Single-cell suspension of splenocytes (1 × 10^6/ml) were cultured in complete RPMI medium pulsed with T-cell proliferation-grade Bovine Type-II Collagen per the manufacturer’s protocol (Chondrex, Inc) or in control medium. The various cultures were treated with different concentration of FTS or vehicle as indicated and cultured for 96 hours at 37°.

Mouse cytokine multiplex panel and ELISA kits. Th1/Th2/Th9/Th17/Th22/Treg-related cytokines were detected in cell culture supernatant using ProcartaPlex Technology (Invitrogen) per the manufacturer’s instructions. IL-6, IL-17, and IL-22 in mouse serum or culture supernatants were determined by ELISA Ready-SET-Go kits (eBioscience, Thermo Fisher Scientific), and granulocyte-macrophage colony-stimulating factor (GM-CSF) by Quantikine ELISA kit (R&D Systems, Inc).

Detection of specific antibodies in serum. Total anti-CII levels were analyzed by mouse anti-Bovine type II Collagen immunoglobulin G (IgG) by commercial ELISA kits (Chondrex). To determine ACPA levels, the sera samples were serially diluted and incubated in 96-well microplates that were precoated with synthetic citrullinated peptides (QUANTA Lite CCP3 IgG; Inova Diagnostics, Inc) for 60 minutes at room temperature. Bound immunoglobulin was detected using horse radish peroxidase (HRP)-conjugated anti-mouse IgG (Biolegend) and the colorimetric HRP substrate, ABTS, and analyzed by an Infinite 200 PRO microplate reader at 450 nm (Tecan Group Ltd).

ACPAs and anti-CII glycosylation (sialylation) level. The sialylation level of ACPAs and anti-CII was determined with the biotinylated Sambucus nigra lectin (b-SNA; binds preferentially to sialic acid) ELISA kit, per manufacturer instructions (Vector Laboratories). Briefly, ELISA microtiter plate was precoated with synthetic citrullinated peptide (Inova Diagnostics, Inc.) or with type CII (Chondrex), blocked with Carbo-Free blocking solution, and incubated for 60 minutes with the indicated serum samples. Next, b-SNA in phosphate buffer saline was applied to the wells and incubated for 30 minutes at room temperature. After extensive washing, VECTASTAIN (streptavidin-HRP/ABTS system) was used to determine sialylation level.

Statistical analysis. Statistically significant differences were determined either by the one-way analysis of variance test with Bonferroni’s post hoc multiple comparison test or the Student’s t test, as appropriate, using the Prism Windows version 5.02 software (GraphPad Software, Inc).

RESULTS

FTS inhibits phosphorylation of distinct downstream effectors of Ras following T-cell activation. The stimulation of T-cell receptors as well as of the co-stimulatory receptor CD28 induces activation of Ras and its downstream effectors. Thus, we analyzed the ability of FTS to inhibit relevant signaling pathways downstream of Ras following T-cell activation. CD4+ T cells were activated for 30 minutes by plate-bound anti-CD3 and soluble anti-CD28 and treated with FTS or vehicle (dimethyl sulfoxide). Using the RayBio Mouse MAPK Pathway Phosphorylation Array, we determined the relative phosphorylation level of 17 different kinases, including well-established Ras effectors (Figure 1A). We found that FTS indeed inhibits the phosphorylation of the core downstream effectors of RasGTPases, whereas the inhibition of other less relevant kinases included in the array was marginal (JNK, CREB, etc). To validate our kinase phosphorylation array data, we also used the phospho-specific flow cytometry (phospho-flow) method to analyze the phosphorylation level of the three key kinases: Erk, Akt, and p38. Using the same T-cell activation protocol, as described above, we indeed confirmed that FTS treatment significantly decreased (P < .001) the phosphorylation level of Erk1/2, Akt, and p38 immediately after T-cell activation as compared with vehicle treatment (Figure 1B).

Next, to determine the effect of therapeutically relevant doses of FTS on T-cell proliferation, we labeled CD4+ T cells with the dye CFSE and activated them in vitro by plate-bound anti-CD3 and soluble anti-CD28 and then cultured them in a complete RPMI medium for 72 hours. The cultures were treated with the indicated concentrations of FTS or vehicle. At the end of culture, the CD4+ T cells were harvested and analyzed by FACS for CFSE dilution (Figure 1C). We found that significant inhibition of T-cell proliferation was evident only starting at drug concentration greater than 12.5 μM and peaking at ~50 μM. Moreover, the analysis of 7-aminoactinomycin D staining for quantitation and exclusion of dead cells revealed that FTS significantly reduced CD4+ T-cell viability only at concentrations greater than 25 μM (data not shown).

FTS modulates T-cell proliferation, cytokine production, as well as the de novo differentiation of pathogenic Th17 cells. Autoreactive CD4+ T cells that secrete IL-17A, IL-22, and GM-CSF (Th17 cells) are important for the induction of autoimmunity (27-29). Thus, we next asked whether FTS could reduce the production of proinflammatory cytokines by preformed Th17 cells. CD4+ T cells obtained from spleens of naive mice were stimulated in vitro by plate-bound anti-CD3 and soluble anti-CD28 and then cultured in an IL-23–containing medium. The various cultures were treated with graded concentrations of FTS or vehicle for 5 days. At the end of culturing, the cells were analyzed by flow cytometry for intracellular production of IL-17A and IL-22. We observed a significant reduction in the percentage of IL-17A and IL-22 single-positive Th17 cells in FTS versus vehicle-treated cultures (Figure 2A and B). In parallel, we determined the secretion of IL-17A, IL-22, and GM-CSF into the supernatants by commercial ELISA kits. We found that FTS significantly reduced the production of all three inflammatory cytokines, most prominently of IL-22 (Figure 2C). Importantly, all these inhibitory effects were significant even at the lowest FTS concentration tested, 1.56 μM.
Next, we investigate whether FTS could restrict IL-6–regulated Th17 cell induction. Thus, we cultured primary naive CD4+ T cells in a medium containing Th17-polarizing cytokines (IL-6/IL-23/IL-1β) and treated the different cultures with graded concentrations of FTS or only vehicle. Five days later the cells were harvested and CD4+ T cells were reactivated and analyzed by flow cytometry for intracellular production of IL-17A and IL-22. Our data show that FTS significantly inhibited the de novo generation of IL-17A–producing T cells in a dose-dependent mode, starting from 1.56 µM (Figure 2D and E). Correspondingly, FTS treatment even at the lowest dose tested here inhibited the production of all three Th17-related cytokines (IL-17A, IL-22, and GM-CSF; Figure 2F).

**Figure 1.** Farnesylthiosalicylate (FTS) inhibits phosphorylation of mitogen-activated protein kinases (MAPKs) and T-cell proliferation following T-cell antigen receptor (TCR) stimulation. Isolated mouse CD4+ T cells were stimulated with anti-CD3/28 antibodies and treated with FTS 10 µM (green) or vehicle (red) for 30 minutes. A, Bar graphs depict the relative phosphorylation level (arbitrary units of intensity per densitometry) of the indicated kinases included in the RayBio Mouse MAPK Pathway Phosphorylation Array, as analyzed per the manufacturer’s protocol. B, Flow cytometry data expressed as overlay histograms depicting phosphorylated (p)-extracellular signal-regulated kinase (Erk)1/2, p-AKT, and p-p38 levels, determined by phosho-flow-cytometry with phospho-specific antibodies, as detailed in the Methods section. T cells treated with FTS or control media are shown in green and red, respectively, whereas unstained counterpart samples are in grey. C, CD4+ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and activated by plate-bound anti-CD3 and soluble anti-CD28 monoclonal antibodies (mAbs), treated with the indicated concentration of FTS, and cultured for 72 hours. Shown is a representative experiment (CFSE-dilution flow cytometry histograms) out of two independent experiments, and the bar graphs depict mean ± SD of triplicates. Samples were analyzed for statistical significance by Student’s t test (***P < .001, **P < .01, and *P < .05).

**FTS demonstrates a significant therapeutic effect in CIA.** CIA was induced in 8-week-old DBA/1 male mice as previously described (23). We employed a “semiprophylactic,” once-daily dosing scheme starting just before booster immunization at 18 days postinduction (days postinduction [DPI] +18) and continuing up until study end (DPI +45). The observed disease incidence in the CMC vehicle–treated control group was usually more than 90%. In our initial experiments, we also assessed the “effect size” of FTS therapy compared with the classic immunomodulatory drug, MTX. The severity of CIA in four limbs was assessed using a validated clinical score of 0 to 16 (23), starting from arthritis onset and subsequently every other day until study end. We found that FTS monotherapy induced a substantial therapeutic effect, significantly reducing arthritis scores as compared with CMC control therapy (Figure 3A). Importantly, FTS and MTX showed a comparable therapeutic effect size (Figure 3A and B). The mean area under curve of the clinical scores in the two active drug treatment arms were significantly reduced (by ~80%) compared with the control vehicle treatment arm (Figure 3B; P < .001 for both FTS and MTX vs. CMC by Student’s t test).
At study termination (DPI +45), the mice were sacrificed and histopathological analysis of the paws was performed by both H&E and Safranin O staining (Figure 3C). The pathology results show that immunization against CII in control mice induced extensive joint tissue inflammation, pannus formation, bone resorption, and cartilage damage (all with average scores of ~4 out 5), whereas FTS therapy significantly reduced the joint pathology scores for all four parameters (Figure 3D).

FTS inhibits the production of proinflammatory cytokines. IL-6 is an acute-phase cytokine-induced following CFA/CII injection and is pivotal for the development of arthritis in the CIA model, and importantly it also regulates the differentiation of pathogenic autoantigen-specific Th17 cells that secrete the key proinflammatory cytokines IL-7 and IL-22 (30,31). Thus, we probed the effect of FTS on the induction of these arthritis-pertinent cytokines in the CIA model of RA. Sera from FTS- and CMC-treated mice were collected at the onset of arthritis (DPI +32 days) and analyzed for IL-6, IL-17, and IL-22 levels by ready-made ELISA kits. We found that at the initial stages of arthritis development, semiprophylactic FTS therapy significantly reduced IL-6 blood levels (~60% inhibition, \( P < .001 \) by Student’s t test) compared with control CMC-treated mice (Figure 4A). Importantly, although high levels of IL-17A and IL-22 (>50 pg/ml) were observed in the sera of control mice, FTS therapy completely inhibited the induction of

**Figure 2.** Farnesylthiosalicylate (FTS) reduces differentiation of Th17 cells and production of Th17-associated cytokines. CD4+ T cells were purified from spleens and lymph nodes of naive 8- to 12-week-old DBA/1 male mice, activated by anti-CD3/anti-CD28 monoclonal antibodies (mAbs) and cultured in complete medium supplemented with interleukin (IL)-23 for 5 days. The cultures were treated with the indicated concentrations of FTS. A. Representative bivariate pseudo color dot plots of intracellular cytokine staining for IL-17A and IL-22 production in CD4+ T cells (flow cytometry data). B. Bars represent the mean percentage (±SD) of IL-17A and IL-22 single-positive Th17 cells. C. At the end of culture, the supernatants were collected and tested for secreted granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-17A and IL-22 by ELISA. Next, only naive CD4+ T cells were activated for 5 days with anti-CD3/anti-CD28 mAbs in the presence of Th17 polarization cytokines or Treg cell polarization cytokines, and treated with FTS at the indicated concentrations. D. Representative flow cytometry plots of staining of IL-17A producing cells. E. Bars represent the mean percentage (±SD) of the indicated Th17 cells. F. At the end of culture, the supernatants were collected and tested for secreted IL-17A, IL-22, and GM-CSF by ELISA. One representative study out of three independent experiments is depicted. Statistical significance was calculated by the Student’s t test (**P < .01, ***P < .001).
Figure 3. Treatment with farnesylthiosalicylate (FTS) reduces the severity of collagen-induced arthritis (CIA) and immune injury of joints. A cohort of 8- to 12-week-old DBA/1 male mice were given an initial injection of Complete Freund’s adjuvant (CFA)/Bovine Type II Collagen (CII) on day 0, and arthritis was induced with a second booster injection of incomplete Freund’s adjuvant/CII on day +21. Mice in the experimental arms (n = 8 per group) were treated starting day +18 with daily FTS (100 mg/kg), weekly intraperitoneal injection of methotrexate (MTX; 1 mg/kg), or 0.5% carboxymethylcellulose (CMC) control vehicle solution. 

A, CIA severity was graded by a validated clinical score (0-16 scale), starting from day +30 just prior to clinical arthritis onset. B, Bar graphs depict mean value of area under the curve ± SD for the clinical CIA disease scores from days postinduction (DPI) +32 to +45 in a representative study out of three independent experiments done (n = 8 per treatment group). C, At the end of the experiment, the mice were euthanized and histopathological analysis of the paws was performed by both hematoxylin and eosin (H&E) and Safranin O staining. Shown are representative images out of three experiments performed (scale bars = 20 μm). D, Bar graphs depict the mean (±SD) for the histological scores (1-5 scale), with separate scores for inflammation, pannus formation, bone resorption, and cartilage damage. The statistical significance of differences in scores for FTS versus CMC therapy was calculated by Student’s t test (*P ≤ .05, **P ≤ .01).
both IL-17 and IL-22 to below the limit of detection of the ELISA kits (Figure 4B and C).

FTS reduces the production and alters the sialylation of pathogenic autoantibodies. Anti-CII antibodies are arthritogenic and are essential for the pathogenesis of CIA. The titer of anti-CII antibodies is a very useful immunological marker to probe for effective induction of T-cell–dependent autoreactive B cells (23). Thus, we determined in the sera of mice from the various treatment groups the relative levels of anti-CII (IgG type) by means of a ready-made ELISA kit. We found that FTS therapy significantly inhibited the induction of a robust anti-CII antibodies response postimmunization compared with control CMC-treated mice (Figure 4D).

Autoantibodies against self-citrullinated proteins are highly involved in the pathogenesis of RA. Similarly, a polyclonal antibody response to anticitrullinated proteins develop in the early stages of CIA, creating neoantigens that further boost the destructive autoimmune response (32). Therefore, it was very informative to analyze the effect of FTS therapy on the induction of ACPA. We decided to use a clinical-grade commercial CCP3.1 ELISA kit, which was designed by combinatorial peptide engineering to contain multiple

Figure 4. Farnesylthiosalicylate (FTS) decreases production of proinflammatory cytokines and the levels of autoantibodies and their hyposialylation. Sera from mice treated with FTS or carboxymethylcellulose (CMC) were collected at day postinduction (DPI) +32, and analyzed by commercial ELISA kits for the level of IL-6 (A), IL-17 (B), IL-22 (C), circulating anti-CII antibodies (D), and anticitrullinated peptides antibodies (ACPA) (E). Next, we performed analysis of sialylation (SA) levels of anti-CII and ACPA autoantibodies using a biotinylated Sambucus nigra lectin–based assay. The data are presented as optical density (OD) at 450 nm. Bars represent means ± SD of triplicates. Statistical analysis was performed by the Student’s t test (**P < .01, ***P < .001).
citrullinated epitopes to increase specific immunoreactivity and validity for the detection of ACPA in patients’ sera. Our findings show that FTS therapy resulted in a significant reduction in serum ACPA levels in sera collected at study end from FTS-treated mice versus control mice. Of note, we observed a clear positive correlation between the signal intensity and the sera dilution factor and a clear separation between the results of CII-immunized mice and unimmunized mice (Figure 4D).

Recent studies show that CII-specific autoantibodies from immunized mice have a lower sialic acid content within their Fc region than that of polyclonal IgG. Terminal sialylation has been linked to the regulation of IgG conformation and postulated to interfere with their pathogenicity (33,34). Importantly, ACPA isolated from patients with severe RA disease display lower levels of Fc-region sialylation compared with autoantibodies isolated from patients with milder RA disease or in clinical remission (35). To investigate the effect of FTS treatment on the sialylation of the pathogenic autoantibodies in the CIA model, we determined the level of sialic acid content of ACPA and anti-CII antibodies using a b-SNA–based commercial assay. Our data show that although FTS therapy resulted in reduced production of anti-CII and ACPAs, the sialylation level for these two autoantibodies was...

Figure 5. Effects of farnesylthiosalicylate (FTS) therapy on major splenic lymphocytic populations. At study end (day +45), single-cell suspension of spleens and lymph nodes from the various groups of treated mice were analyzed by flow cytometry for CD3+, CD4+, and CD8+ T-cell frequencies. A and C, Representative bivariate pseudo color dot plots of total T cells (CD3+), B cells (CD45R+/B220+), CD8+ cytotoxic T cells, and CD4+ T cells, respectively. B and D, Bar graphs show pooled data from all mice studied (n = 6 per group). Bars represent the mean percentage of the indicated T-cell population ± SD. E, Representative FACS plots of intracellular staining of CD4+ T cells for interleukin (IL)-17A and IL-22 cytokines production. F, Bars represent the mean percentage (±SD) of the indicated Th17 subsets in spleens of the various mice groups (n = 6 per group). ***P ≤ .001 by the Student’s t test. Abbreviations: CMC = carboxymethylcellulose, ns = not significant.
in fact significantly increased in FTS-treated mice compared with controls (Figure 4E).

Analyzing the effect of FTS therapy on relevant Th cell subsets. Next, we analyzed the effects of FTS treatment on the main lymphocytic cell populations. Thus, at study end we harvested the spleens and prepared single-cell suspensions for downstream polychromatic flow cytometry analysis. We found that neither CII immunization by itself nor the FTS therapy had a significant effect on the percentage of CD3+ T cells, B220+ B cells, and on the ratios of CD4+ to CD8+ T cells in the spleen, as compared with naive nonimmunized mice and immunized CM- treated mice (Figure 5A-D).

Ample evidence indicates that Th17 cells are required for initiation and progression of autoimmune arthritis both in the mouse CIA model and in patients with RA (8,36,37). Th17 cells also secrete IL-22, a cytokine generally considered as proinflammatory in the context of RA, and its levels are elevated in the blood and synovia of patients with RA (24,28,38,39). Thus, we focused on the effect of FTS therapy on the important effector population of IL-17A and IL-22 producing Th17 cells. At study end (day +45), we prepared single-cell suspensions of splenocytes and inguinal LNs and reactivated them as described above. Subsequently, we

Figure 6. Farnesylthiosalicylate (FTS) therapy inhibits the induction of Bovine type-II Collagen (CII)-specific cytokine recall responses at early (days post induction [DPI] +13 and late (DPI +32) time points. Eight- to twelve-week-old DBA/1 male mice were immunized with CII as detailed above and treated with FTS or carboxymethylcellulose (CMC). Splenocytes were isolated at two time points: DPI +13 (early) and DPI +32 (arthritis onset). Single-cell suspensions (1 x 10^6/ml) of splenocytes were prepared by mechanical disaggregation and then cultured with heat-denatured bovine CII or control medium for an additional 96 hours as detailed in the Methods section. A, Splenocytes isolated at DPI +13 were labeled with carboxyfluorescein succinimidyl ester (CFSE) and stimulated in vitro with heat-denatured bovine CII or control medium, and then cultured for an additional 96 hours. Subsequently, the cells were harvested, immunostained for CD4 and CD3 expression, and analyzed by FACS for CFSE dilution in CD4+ T-cells. B, At culture end, supernatants were collected and analyzed for interleukin (IL)-6, IL-22, and IL-17A levels by commercial ELISA kits. C, Supernatants from DPI +32 splenocytes rechallenged in vitro with heat-denatured bovine CII or control medium and cultured for 96 hours (as detailed above) were collected and analyzed for the levels of multiple cytokines by the ProcartaPlex Mouse Th1/Th2/Th9/Th17/Th22/Treg Cytokine Panel (Invitrogen, Carlsbad, CA, USA). Bars represent mean ± SD of triplicates from a representative experiment (red bars represent CII, and green bars represent control medium). Data were analyzed for statistical significance of FTS compared with CMC/ control treatment by Student’s t test. ***P < .001, **P < .01, and *P < .05. Abbreviation: GM-CSF = granulocyte-macrophage colony-stimulating factor, IFN-g = interferon-γ, ns = not significant, Med = control Medium, TNF-a = tumor necrosis factor-a.
analyzed the cells by multicolor flow cytometry for intracellular cytokines staining. We found a significant reduction in the percentage of IL-17A/IL-22 double-positive Th17 cells and IL-22+ single-positive T effector cells in FTS-treated mice compared with relevant control mice (Figure 5E and F). Interestingly, our results did not demonstrate a significant change in the numbers of IL-17 single-positive Th17 cells in the samples of FTS-treated mice.

**FTS therapy inhibits the early (DPI +13) and late (DPI +32) CII-specific recall response during CIA development.**

To study the effect of FTS treatment on the induction of CII-specific Th17/Th22 cells at an early time point, we immunized 8- to 12-week-old DBA/1 male mice with CII and treated them from DPI +3 with daily oral FTS or CMC. On DPI +13, the spleens and inguinal LNs were collected, processed into a single-cell suspension, and labeled with CFSE. Next, the cells were stimulated in vitro with heat-denatured bovine CII or control medium and were cultured for an additional 96 hours. The CFSE-dilution assay shows that FTS significantly reduced the in vivo induction (ie, precursor frequency) of CII-specific CD3+CD4+ effector T cells as compared with control splenocytes (Figure 6A). Correspondingly, we found that FTS therapy was associated with a significant inhibition of the cytokine recall response of splenocytes to CII, significantly suppressing the secretion of IL-6, IL-22, and IL-17A (Th17-related cytokines) at this early time point (DPI +13 to +16) as compared with relevant control cultures (Figure 6B).

Furthermore, we studied the effect of FTS on the CII-specific cytokine recall response of splenocytes at another key time point, DPI +32, upon clinical disease onset. Thus, the various cell samples were immediately stimulated with heat-denatured bovine CII and cultured for an additional 96 hours. At the end of culture, the supernatants were collected, and the levels of a large number of relevant cytokines (17-analytes) were determined using the ProcartaPlex Mouse Th1/Th2/Th9/Th17/Th22/Treg Cytokine Panel. We found that although a CII rechallenge of splenic T cells of immunized “arthritic” vehicle-treated mice induced a strong upregulation of multiple proinflammatory cytokines (IL-6, tumor necrosis factor [TNF]-α, GM-CSF, interferon [IFN]-γ, IL-9, IL-17A, and IL-22), this induction was significantly suppressed (>75% inhibition) in counterpart cultures from FTS-treated mice (Figure 6C). Interestingly, the induction of IL-10, considered a major immune regulatory cytokine, was not inhibited by FTS.

**DISCUSSION**

In this manuscript, we show that FTS has the ability to modulate the activation of the MAP kinases, Erk1/2, and p38, in CD4+ T cells following in vitro CD3/CD28 co-stimulation. It can also inhibit the in vitro differentiation of Th17 cells from naïve CD4+ T cells. Moreover, we demonstrate that FTS therapy, a first-in-class oral Ras inhibitor, provides a significant immunomodulatory effect in the CIA model as assessed by clinical outcome parameters and multiple immunological markers. Importantly, the therapeutic effect of FTS treatment in the CIA model was coupled with the attenuation of the induction of the “acute-phase” cytokine IL-6, and the ensuing generation of artheriogenic CII-specific Th17 cells postimmunization with bovine CII that likely regulate the production of harmful autoantibodies.

The original data presented in the results section show for the first time that following in vitro CD3/CD28 co-stimulation FTS treatment indeed reduces the phosphorylation of two important MAPks (Erk1/2 and p38) and of the principal kinases in the AKT/m-TOR pathway. Several transcription factors, such as ROR-γ/ ROR-α, STAT3, and IRF4, are postulated to regulate in the differentiation of “pathogenic” autoreactive Th17 cells (29,40,41). Nevertheless, several studies demonstrated that kinases downstream of RasGTPases, including Erk1/2, p38, and Akt also play an essential role in the induction of Th17-associated cytokine (IL-6, IL-17A/F, and TNF-α) in CD4+ T cells. Moreover, studies in relevant animal models show an association between excessive activation of these kinases and the development of autoimmunity (42-45). In this regard, we also determined that treatment with FTS reduces the production of such cytokines (IL-17A, IL-22, and GM-CSF) in effector CD4+ T cells following TCR stimulation, and decreases the in vitro IL-6-dependent polarization of naïve CD4+ T cells into Th17 cells.

Although Erk1/2 activation, downstream of Ras, has been postulated to promote cell proliferation, our novel data show that FTS significantly inhibits T-cell proliferation only at high concentrations greater than 10 μM that are unfeasible in situ. Conversely, the production of the cytokines IL-17A and IL-22 was strongly inhibited starting at much lower concentrations of ~1.5 μM. Regarding this, studies suggest that p38 (a MAPK member) signaling in CD4+ T cells plays a pivotal role in regulating IL-17 production at the translational level by controlling the eukaryotic translation initiation factor (46). Hence, it is possible that the strong inhibition of cytokine secretion from Th17 cells by FTS is dependent on its capacity to reduce p38 MAPK phosphorylation. These findings validate our working hypothesis that FTS effectively inhibits key signaling pathways downstream of Ras that are critical for cognate T-cell activation by foreign and self-antigens.

In a previous publication, we investigated the therapeutic effect of FTS in the rat AIA model. Although AIA shares several pathological and immunological characteristics with RA, including symmetrical joint involvement, immune cell infiltrates, synovial hyperplasia, bone erosions, genetic linkage, and T-cell dependency, the model also diverges from RA as the damage to cartilage is less severe, bone destruction is more prominent, and autoantibodies are less relevant to its pathogenesis (47,48). To improve the applicability of our preclinical studies to predicting the translational potential of FTS, we studied the therapeutic effect of FTS in the mouse CIA model. This model is the leading preclinical model of RA as it shares many pathological features with RA, and articular cartilage that is a major target tissue in RA is mostly made of CII.
It can be established in the genetically susceptible DBA/1 mouse strain by immunization with CII (47,48). Here we determined that FTS had a robust therapeutic effect in CIA, significantly reducing both the clinical arthritis scores and all relevant pathological domains including synovial hyperplasia, immune cell infiltration, and cartilage/bone degradation.

Abundant evidence indicates that T cells are required for initiation and/or chronicity both in RA and in CIA. IL-17 is produced by a distinct subpopulation of effector CD4+ T cells that has been designated Th17 (45). IL-17 neutralization with antibodies or the genetic ablation of IL-17 or IL-17R is associated with the attenuation of CIA (46). Th17 cells also secrete IL-22, a cytokine generally considered proinflammatory. In the context of RA, it has been shown that elevated numbers of Th17 (IL-17A– and IL-22–producing) and Th22 cells (IL-22–producing) in peripheral blood of patients with RA positively correlates with a more aggressive disease course (47). In agreement with these human data, we also observed induction of Th17 (IL-17+ and IL-17+/IL22+) and Th22 (IL-22+) cells in the spleens of untreated mice post-CII immunization; more importantly, FTS treatment significantly reduced their frequency. Thus, our present cytokine data from the CIA mouse model are consistent with our working hypothesis that the therapeutic effect of the oral Ras-inhibitors is very likely linked to their ability to attenuate the differentiation and expansion of artheriogenic, antigen-specific Th17/Th22 cells.

As already mentioned, the cytokines IL-6, IL-17A, and IL-22 have been postulated to play a key role in the pathophysiology of RA (5,39,49). Interestingly, we found that FTS is a strong inhibitor of the systemic induction of IL-6 and IL-17A in IL-22–producing) and Th22 cells in the spleens of untreated mice post-CII immunization; more importantly, FTS treatment significantly reduced their frequency. Thus, our present cytokine data from the CIA mouse model are consistent with our working hypothesis that the therapeutic effect of the oral Ras-inhibitors is very likely linked to their ability to attenuate the differentiation and expansion of artheriogenic, antigen-specific Th17/Th22 cells.

Previous studies have suggested that autoantibodies against self-citrullinated proteins are centrally involved in the pathogenesis of RA (32). Similarly, polyclonal antibody responses to both CII and citrullinated joint proteins develop in CIA, creating neoantigens that further boost the “epitope spreading” during the experimental autoimmune process. Importantly, we found that FTS therapy resulted in a significant downmodulation of the generation of anti-CII and ACPA. In this regard, effector functions of IgG antibodies partly depend on their Fc glycosylation pattern. For instance, the accumulation of nongalactosylated IgG antibodies in the sera of patients with RA positively correlates with severity of the disease, whereas antibodies with sialylated Fc probably contribute to the immunomodulatory effects of high-dose pooled healthy human serum IgG therapy. It has been postulated that the enzyme β-galactoside α2,6-sialyltransferase 1 (St6ga1) that catalyzes the posttranslational transfer of sialic acid to the N-glycans of IgG is regulated by the Th17 cytokines, IL-21 and IL-22, such that they repress the expression of St6ga1 in CII-specific plasmablasts before the onset of arthritis (33). More recently, it was shown that preadministration of sialylated anti-CII–specific IgG attenuated disease manifestation in CIA (34). Our data also show that FTS treatment prevents the reduction in sialylation levels of ACPA and anti-CII autoantibodies in the CIA model, further proving that FTS therapy is associated with multiple anti-inflammatory outcomes.

Our data from in vitro experiments demonstrate that FTS is a potent inhibitor of generation and expansion of the pathogenic Th17-type cells. Moreover, the data from the CIA model show that the therapeutic efficacy of FTS was similar to MTX, and both drugs significantly reduced arthritis severity and joint pathology compared with CMC controls. Importantly, FTS significantly inhibited the upregulation of serum proinflammatory cytokines as well as reduced the titer of pathogenic autoantibodies compared with control arthritic mice. Additionally, FTS therapy induced a significantly lower induction of pathogenic Th17 cells in the spleens of treated mice compared with control mice. The in-depth analysis of the effect of FTS in vivo therapy on the recall T-cell cytokine response to CII revealed strong suppression of the induction of CII-specific Th cells that can secrete proinflammatory cytokines such as IL-22, IL-17, IL-9, GM-CSF, and TNF-α.

In summary, our current in vitro work and preclinical studies in mice revealed that the Ras GTPase inhibitor, FTS, has a few interconnected modes of action. It strongly inhibits the generation and expansion of the pathogenic CII-specific Th17 cells, the induction of artheriogenic autoantibodies specific for CII and citrullinated proteins, and, moreover, it downmodulates the harmful hyposialylation of these autoantibodies. These extensive in-depth preclinical data imply a strong translational potential for FTS as a new treatment for RA and other related autoimmune disorders.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

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