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14. ABSTRACT Our major research achievements/findings were as follows: (i) Farnesylthiosalicylic acid (FTS) therapy, a first-in-class oral selective RAS inhibitor, provides a significant immunomodulatory effect in the rat adjuvant-induced arthritis (AIA) model by all clinical and laboratory outcome parameters. (ii) Therapy with FTS as an add-on to the methotrexate (MTX) is more effective compared to monotherapy. (iii) The FTS derivative, F-FTS, showed higher therapeutic efficacy compared to FTS in AIA. (iv) The functional genomics studies showed that FTS therapy primarily inhibits the TH17 immune response to pathogenic antigens. (v) FTS semi-prophylactic therapy in the mouse collagen-induced arthritis (CIA) model was a highly effective therapy that was non-inferior to combined FTS+MTX therapy. (vi) The therapeutic effect of FTS treatment in the CIA model was also coupled with inhibition of the in vivo IL-6, IL-17 and IL-22 (Th17 type) response to collagen. (vii) The in vitro studies revealed that FTS is predominantly a potent inhibitor of generation and expansion of TH17 type cells. In conclusion: our original findings strongly imply that oral selective RAS inhibitors are potent inhibitors of the TH17-driven autoimmune response in animal models of RA, signifying a strong translational horizon for these compounds.		
15. SUBJECT TERMS Ras GTPases; Rheumatoid Arthritis (RA); Farnesylthiosalicylic acid (FTS); T helper cells, disease-modifying anti-rheumatic drugs (DMARDs); targeted synthetic DMARDs		

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1. INTRODUCTION:

Ras GTPases are molecular switches that regulate key cellular processes and in T cells they are necessary for proper TCR and CD28-dependent activation following antigen recognition. Reduced Ras signaling has been associated with T cell anergy and defective IL-2 production (1, 2). Importantly, synovial T cells from patients with RA display augmented activation of the Ras/Raf/ ERK pathway (3). Thus, Ras GTPases appear to be a promising molecular target for inhibiting T cell activation in RA. Based on an innovative concept Kloog (the partnering PI) and colleagues discovered (4) a potent non-toxic inhibitor of Ras, Farnesylthiosalicylic acid (FTS). In collaboration with Concordia Pharmaceuticals Inc., FTS was developed into an oral drug, Salirasib®. The drug was already tested in the clinic for the treatment of cancers with oncogenic mutations in KRAS and NRAS. No dose-limiting toxicities or major adverse events were reported during Salirasib® treatment, in phase I clinical trials of patients with advanced solid cancer. Thus, Salirasib® is the only available successful Ras GTPase inhibitor that reached clinical trials, which moreover received an orphan drug designation by the FDA for the treatment of pancreatic cancer (5, 6). In this project, our objective was to complete the following tasks:

- (i) Test in the rat AIA model of RA (rheumatoid arthritis) the prophylactic and therapeutic efficacy of FTS on relevant clinical outcomes and immunological parameters.
- (ii) Validate in CIA mouse model the prophylactic/therapeutic effects of FTS and the FTS derivative F-FTS.
- (iii) Test the effect of FTS as an add-on therapy to MTX in the AIA and CIA models.
- (iv) To explore in vitro the effects of FTS and its derivative F-FTS on a wide range of mouse T cell functions.

2. KEYWORDS:

Ras GTPases; Rheumatoid Arthritis (RA); Farnesylthiosalicylic acid (FTS); Adjuvant-induced Arthritis (AIA); T cells; T-helper cells, T regulatory cells (Treg), disease-modifying antirheumatic drugs (DMARDs); targeted synthetic DMARDs (tsDMARDs).

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major Task 1: Studying the immunomodulatory effects of the small molecule Ras-inhibitor Farnesylthiosalicylic acid (FTS/Salirasib) and its derivatives in the adjuvant-induced arthritis (AIA) rat model. **100% completion.**

Major Task 2: To validate in the collagen induced arthritis (CIA) DBA/1 mouse model the prophylactic/therapeutic effects of FTS and FTS derivatives. **100% completion.**

Major Task 3: To test the effect of FTS as an add-on therapy to MTX in the AIA rat model. **100% completion.**

Major Task 4: To study in vitro the effects of FTS and its derivative (F-FTS) on a wide range of mouse T cell functions and signaling networks following TCR stimulation. **100% completion** - to be achieved in full during the 4th year (approved extension period at no cost).

Milestone #1: Write and publish a co-authored manuscript on the therapeutic value of Ras inhibitors in the AIA and CIA models. This milestone was accomplished on time during this reporting period (months 21-33 per SOW).

Journal publications: We published a comprehensive manuscript in *Frontiers in Immunology* that is a leading journal in its field and the official Journal of the International Union of Immunological Societies (IUIS). This multidisciplinary open-access journal publishes only rigorously peer-reviewed research across basic, translational and clinical immunology and has an excellent impact factor of 6.43.

1. Zayoud M, et al. *Front Immunol* 2017. 8:799. doi:10.3389/fimmu.2017.00799 (See attached manuscript reprint in appendix) (1).

Conference papers: We also presented our accomplishments in two prominent international meetings in the fields of Rheumatology (EULAR 2017) and Immunology (IMMUNOLOGY 2017TM), as detailed in item 6 of the report.

1. Zayoud M, et al. Farnesylthiosalicylic acid reduces disease severity in the collagen type-II induced arthritis mouse model by inhibiting Ras Signaling in pathogenic T cells. *The Journal of Immunology* 2017. 198(1 Supplement):224.227-224.227 (2).

2. Zayoud M, et al. RAS SIGNALING INHIBITION ATTENUATES ARTHRITIS IN ANIMAL MODELS OF RHEUMATOID ARTHRITIS BY DOWN MODULATING THE PATHOGENIC TH17 CELL RESPONSE. *Annals of Rheumatic Diseases (EULAR conference Supplement)* 2017. doi: 10.1136/annrheumdis-2017-eular.2843 (3).

Milestone #2: Co-authored comprehensive manuscript describing the mechanisms (cellular and molecular) that control/mediate the therapeutic (Immunomodulatory) effects of small molecule Ras inhibitors in the CIA mouse model of RA, including mechanistic data derived from the in vitro work in Task 4. During months 42-48, we indeed wrote a comprehensive manuscript describing the cellular and molecular mechanisms that regulate the immunomodulatory effects of FTS based on our in vivo data in the CIA model and the in vitro data from the Task 4 (see appendix). We plan to publish it in a leading journal in the fields of Rheumatology & Immunology within the next 6 months.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

OVERALL PROJECT SUMMARY (First, Second and Third Year):

In *Major Task 1* of the revised SOW, we determined in the Lewis rat AIA model of the prophylactic and therapeutic effects of FTS.

Subtask 1A: Analyze the prophylactic/therapeutic effects of FTS on clinical AIA progression and joint pathology (**months 1-15, completed**).

As detailed in the interim Technical reports of the first and second year, we have already defined the clinical effects of prophylactic dosing of FTS and its derivative F-FTS on the progression of AIA in Lewis rats. In this set of experiments, we compared the therapeutic effects of the oral Ras-inhibitors to treatment with a conventional DMARD, methotrexate (MTX). Adjuvant induced arthritis in Lewis rats is an experimental model of polyarthritis that has been extensively employed for preclinical testing of numerous anti-arthritic agents. The arthritis is induced by injection of an arthrogenic preparation of complete Freund's adjuvant (CFA), prepared by suspending powdered heat-killed Mycobacterium tuberculosis in mineral oil at 10 mg/ml. The hallmark of this model is consistent onset and progression of robust and easy to measure polyarticular inflammation associated with marked tissue/synovial inflammation and subsequent articular bone resorption (4). In our hands, in agreement with previous works, clinically evident arthritis of the ankle joint usually developed ~9-11 days' post CFA injection that progressed, in untreated control animals, into severe poly-arthritis within a few days.

We employed two dosing models:

- Prophylactic – start on day +1 and continue until the end of experiment.
- Therapeutic – start on disease onset (day +9) until study termination.

Clinical Assessment: To assess disease progression, both clinical scoring (0-4 scale for each paw, 0-16 scale for total arthritis severity) and caliper measurements of ankle joint width were used, as previously described.

Histopathological Assessment: At study termination, the tibiotarsal joint was transected at the level of the medial and lateral malleolus for Histopathological Assessment. Ankle joints were then collected into 10% paraformaldehyde, for at least 24 hours, and then placed in a decalcifier solution. 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 & eosin. Arthritic ankles were then given scores on a scale of 0-5 for inflammation and bone resorption by an experienced pathologist blinded to the animal treatment protocol, as previously described (4).

i. As detailed in previous interim reports, we determined the clinical efficacy of prophylactic dosing with FTS suppresses the clinical signs of AIA. In the experimental arm, we started daily treatment with oral FTS (100 mg/kg) from day +1 post CFA immunization. Control rats received oral vehicle solution of 0.5% carboxy methyl cellulose (CMC). In the MTX arm, the rats were given weekly *i.p* injection of MTX (0.5 mg/kg). AIA progression and severity was scored using the clinical index of 0 to 16. In parallel, we also assessed disease progression by caliper measurements of ankle joint width prior to the onset of arthritis, and then every other day until the study termination on day +21 post CFA injection. We found and reported in the previous interim report and our recent publications (1, 3) that the arthritis scores and ankle inflammatory swelling were significantly reduced by FTS treatment group compared to CMC vehicle treated rats. Importantly, we also found that the clinical effect of FTS, assessed by the above outcomes measure, was non inferior to MTX therapy (Figure 1A and B). **Responsible PI: Yoel Kloog, Tel Aviv University.**

ii. We also previously found that the histopathological assessment of Ankles of arthritic CMC vehicle treated rats showed extensive infiltration of joints tissue with mononuclear cells (inflammation scores ranging from 4 to 5, n=8), and significant bone destruction (bone resorption scores ranging from 4 to 5). In contrast, the histopathological assessment of joint sections from FTS-treated rats showed significant reduction in joint inflammation (inflammation scores ranging from 2 to 3, n=8), and significantly less destruction of trabecular and cortical bone in the distal tibia (bone resorption scores ranging from 2 to 3). **Responsible PI: Yoel Kloog, Tel Aviv University**

iii. We also performed immunohistochemistry assessment of deparaffinized and rehydrated arthritic rat ankle joint sections for the infiltrations of T lymphocyte into the synovial tissue, as detected using anti-CD3 antibodies. Our findings (reported on 2nd year report), were that the arthritic joints of CMC vehicle treated rats displayed extensive infiltration of the synovial tissue with CD3+ T cells that was significantly reduced in the joints of FTS treated mice (data not shown). This implies that FTS and its derivative target the T cell response and subsequent homing into target synovial tissues.

Responsible PI: Itamar Goldstein, Tel Aviv University.

iv. The FTS derivative, 5-Fluoro-FTS (F-FTS), has been previously shown by studies from Prof. Kloog's lab to be a highly potent immunomodulatory drug (5). Thus, during the second year of the project, we investigated more broadly the relative therapeutic efficacy of F-FTS.

In a set of experiments comparing "head-to-head" the clinical effects of F-FTS vs. FTS, we found that prophylactic dosing with F-FTS (60mg/kg) from day +1 post CFA injection had a significant therapeutic effect on the clinical outcome of AIA without obvious *in vivo* toxicity. The efficacy of F-FTS was indeed superior compared to FTS treatment ($P<0.05$ by one-way ANOVA and post-hoc Bonferroni's Multiple Comparison Test (Figure 2) (see relevant data in 2nd year report and recent publication (1)).

Responsible PI: Yoel Kloog, Tel Aviv University.

v. As we reported in the 2nd year, we next tested the efficacy of FTS when the treatment was given in the "therapeutic scheme" from day +9 (onset of arthritis in the hind paws). We found that

therapeutic dosing of FTS did not effectively reduce disease severity as measured by both clinical disease scoring and ankle swelling per caliper measurements. As expected, dexamethasone therapeutic dosing (positive control) was effective in decreasing the severity of AIA compared to CMC vehicle treatment ($p < 0.01$). Additionally, in a single exploratory study we also tested FTS as an add-on therapy to MTX (0.5mg/kg) in the therapeutic dosing model compared to this combined treatment in the prophylactic protocol. We found that while prophylactic FTS+MTX dosing almost completely prevented arthritis development, the therapeutic FTS+MTX dosing schedule had no significant effects on disease outcome measures. During the current reporting period we also performed a single exploratory study to test the efficacy of F-FTS in the therapeutic-dosing-scheme, also with negative results (data not shown). Thus, our data imply that this latter dosing scheme is imprecise for predicting the "true" therapeutic potential of certain agents in RA.

Responsible PI: Yoel Kloog, Tel Aviv University.

Subtask 1B: To analyze the effects of FTS treatment on the cytokine profile and relevant isolated T cell subsets including, TH17, TH1 and Foxp3+ regulatory T cells and related molecular markers (months 4-15).

vi. Next, we analyzed the effects of the various treatment protocols on the immune response to CFA, particularly on the CD4+ T cell response. At study termination both peripheral blood samples and spleens were collected and analyzed by flow cytometry for treatment effects on major lymphocytic populations. As detailed in previous reports, we observed a trend towards an increased CD4+ to CD8+ T cell ratio in the spleens of FTS treated rats compared to control rats. This effect was likely due to a statistically significant increase in the percentage of CD4+ Foxp3+ regulatory T cells (Treg) in the spleens of FTS treated rats (see relevant figures in previous reports). As both TH1, TH17 and double positive TH1/Th17 cells have been postulated to be instrumental in the pathogenesis of T cell dependent autoimmune responses, both in animal models and humans, we analyzed the effects of FTS and other treatments on the induction of these T cell subsets in arthritic rats. Our data show that FTS therapy was associated with significantly lower numbers of TH17 and TH1/TH17 cells in the spleens of treated rats compared to CMC vehicle treated mice. This effect was even more significant when we administered FTS, as an add-on therapy to MTX, with strong positive correlation with the clinical outcome data (see also recent publications (1-3)).(Figure 3)

Responsible PI: Itamar Goldstein, Tel Aviv University.

vii. In accordance with the intracellular cytokine detection data, we also found that on day +14 both FTS and MTX alone typically reduced serum IL-17 levels by ~50%, whereas the combined treatment of FTS+MTX reduced IL-17 levels even more significantly by >90%, as compared to CMC control treated arthritic rats. We also found that CFA injection indeed induced a substantial increase in CRP levels in CMC vehicle treated arthritic rats. In contrast, the combined FTS+MTX completely abolished this increase at the two-time points (day +10 and +14 of the study). While both FTS and MTX reduced CRP levels at day +10, they were less effective, as a single agent, in suppressing the induction of CRP on day +14. Moreover, our CRP data show that it directly correlated with the extent of target tissue inflammation (ankle diameter) that was strongly inhibited by the combined treatment (See relevant data and figures in the 2nd year report and publications).

Responsible PI: Yoel Kloog, Tel Aviv University.

viii. As detailed in previous reports, we studied the *in vitro* cytokine response to re-stimulation with the pathogenic mycobacterial antigen Bfsp65. Thus, on day +12, after the onset of clinical arthritis, spleens and draining superficial inguinal and para-aortic lymph nodes (LNs) were harvested and a single-cell suspension of a mixture of spleen and LN cells was obtained. Subsequently, the isolated cells were cultured at 37°C for 5 days in a six-well plate (5×10^6 cells/well) with or without Recombinant mycobacterial Bfsp65 protein (5µg/ml), as previously described (6). We detected a strong induction of IL-17 secretion following *in vitro* antigenic re-stimulation with Bfsp65, principally in the T cell cultures of untreated mice. Importantly, *in vivo* prophylactic FTS and F-FTS treatment resulted in significant reduction Bfsp65-specific IL-17 secretion compared to control CMC treatment. Moreover, F-FTS treatment was more effective in the suppression of the pathogenic antigen-specific IL-17 response. **Responsible PI: Yoel Kloog, Tel Aviv University.**

xiii. In accordance with the revised current SOW, to gain a more comprehensive insight into the "molecular mechanism" that mediate the therapeutic action of small molecule Ras-inhibitors in AIA, we analyzed the changes in gene expression (mRNAs) mediated by FTS and F-FTS treatment in pure CD4+ T cells by GeneChip arrays. Thus, at the end of the *in vitro* antigenic re-challenge, as detailed above, we harvested the cells and isolated the CD4+ T cell population using Rat CD4 MicroBeads and the MACS cell separation platform (Miltenyi Biotec, Germany), according to the manufacturer's instructions. The purified CD4+ T cells were lysed with the TRIzol® Reagent (Invitrogen, Inc.), and total high quality RNA was purified using the Direct-zol™ RNA Kit (Zymo Research Corporation) for subsequent downstream analysis. Gene expression was determined using the GeneChip® Rat Gene 2.0 ST Array System (Affymetrix, Inc.), according to the manufacturer's instructions. Gene level RMA sketch algorithm was used for crude data generation (Affymetrix Expression Console and Partek Genomics Suite 6.2). Genes were analyzed using unsupervised hierarchical cluster analysis (Spotfire DecisionSite for Functional Genomics; Somerville, MA, USA) to get a first assessment of the data, and filtered according to fold change calculations. The primary microarrays data from this research have been deposited in the NCBI Gene Expression Omnibus data repository under accession number GSE100280.

The results of the advanced bioinformatics analysis of the various CD4+ T cell samples revealed that the antigenic re-stimulation with Bfsp65, as expected, induced a robust upregulation of multiple ($n = 50$) genes in CD4+ T cells (see previous report and Figure 5 in our recently published manuscript). Importantly, the analysis of the signal intensity of this list of Bfsp65-induced differentially expressed genes in CD4+ T cells of FTS vs. CMC treated rats (≥ 2 fold-change), revealed a widespread FTS-dependent reduction in the intensity of the Bfsp65-induced transcription of a large percentage of these 50 genes ($P < 0.001$, by chi-square test). Notably, this list included a large number of acknowledged immune response genes, such as genes encoding pro-inflammatory cytokines (e.g., Il22, Il17a/f, Ifng, Csf2/GM-CSF, Lta, and Il1a).

Next, to determine, in unbiased manner, the biological processes and molecular functions that mediate the therapeutic effect of FTS, we performed additional in-depth bioinformatics analysis. Thus, we computed the overlaps between our gene list and relevant annotated gene sets within the HALLMARK and gene ontology (GO) collections of the Molecular Signatures Database of the Broad Institute (Massachusetts Institute of Technology) using the GSEA software web site v6.1. <http://software.broadinstitute.org/gsea/msigdb/index.jsp>

By this analysis, we determined that the list of genes upregulated significantly (≥ 2 -fold change, FDR $q < 0.05$) following Bhs65 re-stimulation of CD4⁺ T cells isolated from CMC treated control rats exhibited significant overlap with a large number of curated relevant immune response and cell proliferation gene sets. The top overlapping annotated gene sets included: (i) cytokine activity (GO); immune system processes (GO); G2M_checkpoint (HALLMARK); inflammatory response (HALLMARK); response to tumor necrosis factor (GO); response to IFN-gamma (GO); positive regulation of cell proliferation (GO); IL6_JAK_STAT3_signaling (HALLMARK); K-RAS signaling up (HALLMARK); and others (see Figure 5B in our recently published manuscript). To validate our GeneChip® data, we analyzed by qPCR the relative quantity of mRNA transcripts of five highly relevant inflammatory response genes differentially induced by Bhs65 re-stimulation in CMC vs. FTS treated CD4⁺ T cells (ccl20, il22, il17A, il17F, and Csf2). Our results, confirmed that the induced transcription of all these genes, during the recall response of TH17 cells to Bhs65, was significantly inhibited by FTS therapy ($P < 0.05$, by t-test). Moreover, we also validated in relevant culture supernatants, by ELISA kits, a strong induction of IL-17A and IL-22 protein expression following in vitro Bhs65 re-challenge that was significantly inhibited by in vivo FTS therapy.(Figure 4)

In conclusion, bioinformatics of the transcriptomics data, indeed, allowed us to gain further insight into the biology behind the effect of FTS. Namely, down modulation of the *in vivo* induction of a pathogenic TH17 response (IL-22- and IL-17-driven) to CFA/Bhs65 (See also relevant data and figures in the 2nd year report and our recent paper (1)).

Responsible PIs: Itamar Goldstein & Yoel Kloog, Tel Aviv University.

In **Major Task 2** of the revised SOW we proposed to validate in collagen induced arthritis mouse model the prophylactic/therapeutic effects of FTS and its derivative, F-FTS.

Subtask 2A: Analyze the prophylactic/therapeutic effects of FTS and F-FTS on the clinical scoring, histopathology of ankle joints, and serum IL-17A/IL-6 levels at arthritis onset (**months 16-42**).

CIA is the most widely studied animal model of RA, as it shares several pathological features with RA, and Collagen type-II is (CII) is a major antigen in human cartilage, the target tissue of RA. This model has been used widely to identify potential pathogenic mechanisms of autoimmunity relevant to RA, including the role of specific T cell subsets in disease pathogenesis and progression, as well as to design and test new drugs and therapeutic modalities. For example, the CIA model has been instrumental in the testing and development of the new biologically based therapeutics, such as those that target the pathogenic cytokines TNF, IL-6 and IL-17 produced by macrophages and T cells that are the dominant immune cell mediators of RA pathogenesis. CIA can be established in the genetically susceptible, DBA/1 (H-2q) mouse strain, by immunization with CII emulsified in CFA (on study day 0 and 21). The ensuing pathogenesis shares several clinical and immunological features with RA, including synovial hyperplasia, mononuclear cell infiltration, cartilage degradation, and like RA, the disease is dependent on MHC class II genes and T cells. In our hands, in agreement with previous studies, clinically evident arthritis of the ankle joint usually developed ~10 days after the second booster immunization with CII (namely ~30-32 days after the primary immunization), which progressed, in control animals, into severe polyarthritis within a few days.

We employed two dosing schemes:

- Semi Prophylactic – start dosing 3 days before booster immunization until study end.
- Therapeutic – start dosing at arthritis onset (~ day +30) until study end.

Clinical Assessment: Disease progression was assessed by a clinical scoring index (0-16 scale) starting at arthritis onset and subsequently every other day until study termination.

i. In the previous reporting period, we performed initial studies to determine whether prophylactic dosing with FTS attenuated the clinical signs of CIA, and also assessed its "effect size" compared to a recognized DMARD, MTX. The preliminary observation suggested that FTS was a highly potent therapy that was at least as effective as MTX therapy. In this reporting period we also compared the clinical efficacy of the FTS derivative, 5-Fluoro-FTS (F-FTS), against both FTS and MTX in the CIA model. Thus, 8-week-old DBA/1 male mice were given an initial injection of type 2 collagen on day 0, and arthritis was induced with a second injection on day 21. Mice in the three experimental arms were treated starting day +18, as follows: **(i)** FTS at 100mg/kg daily; **(ii)** F-FTS at 60mg/kg daily; or **(iii)** weekly i.p injection of MTX (1 mg/kg). Mice treated with CMC vehicle solution served as controls. CIA severity was scored using a clinical index of 0 to 16 (0-4 scale for each paw). Our results from these large experiments were that F-FTS therapy was non-inferior to FTS and MTX therapies. Although, FTS displayed a trend towards being more potent than F-FTS in its clinical efficacy, the effect did not reach the statistical threshold of $P < 0.05$ (Figure 5)

Given these data and our ethical duty to balance the needs of the study with that of the animals' welfare, in compliance with the principle of reduction (per USAMRMC ACURO guidelines), we decided that we will concentrate on FTS as the lead compound to be investigated in the CIA model. Thus, in current reporting period, we focused on expanding and repeating the experiments assessing the clinical efficacy of FTS vs. no active drug (0.5% CMC vehicle). We found that FTS monotherapy was very efficacious, considerably reducing arthritis scores compared to CMC treated mice (Figure 5A). Importantly, the area under curve (AUC) of the clinical scores of mice in the active drug treatment arm was significantly reduced, by ~80%, compared to the control arm (Figure 5B; $P < 0.001$ for FTS vs. CMC by Student's t-test).

Responsible PIs: Itamar Goldstein & Yoel Kloog, Tel Aviv University.

ii. For Histopathological Assessment at study termination, the tibio-tarsal joint was transected at the level of the medial and lateral malleolus. Next, we collected the ankle joints into 4% paraformaldehyde for at least 24 hours and then placed them in a decalcifier solution. When decalcification was completed, we transected the ankle joints in the longitudinal plane and the joints were paraffin embedded, sectioned and stained with hematoxylin & eosin (Figure 3A). Next, an experienced pathologist, blinded to the animal treatment protocol, scored the various joint sections on a scale of 0-5 for inflammation, pannus formation and bone resorption (Figure 3B), as previously described (4). Our pathology scoring results show that while immunization against CII in control animals induced extensive tissue inflammation, pannus formation and bone resorption (all with an average scores > 4), the therapy with FTS significantly reduced joint pathology scores for all these three parameters (all with an average score < 1.5).

Responsible PI: Itamar Goldstein, Tel Aviv University.

iii. (a) The titers of anti-CII antibody are a very useful parameter/marker to probe for effective immunization and the induction of T cell-dependent CII-specific autoreactive pathogenic B cells (7). Thus, by a readymade "gold standard" anti-CII IgG ELISA kit (Chondrex, Inc., USA) we determined in the sera of mice from the various treatment groups the relative levels of these pathogenic autoantibodies. Our results show that FTS therapy significantly inhibited the induction of a prominent anti-CII antibody response post immunization with CII and adjuvants ($p < 0.001$, for both time points, by Student's t-test).

(b) Previous studies have suggested that auto-antibodies against self citrullinated proteins are centrally involved in the pathogenesis of rheumatoid arthritis, and it was reported that polyclonal antibody responses to anti-citrullinated proteins, found in joints, develop in the early stages of CIA creating neo-antigens that further boost the "epitope spreading" during the autoimmune response(8). Therefore it was very informative to analyze the effect of FTS therapy on the induction of such anti-citrullinated peptide antibodies (ACPA). By clinical-grade commercial ELISA kits we tested sera, collected at day +48 post immunization, from actively treated and control mice. Our finding clearly show that FTS therapy resulted in a significant reduction in the levels of ACPA ($P < 0.001$ for FTS vs. CMC, by Student's t-test). This observed strong positive correlation between these two validated immunological disease-relevant parameters/markers and the clinical scoring data further confirms our working hypothesis that FTS is a potent anti-arthritis drug.

Responsible PI: Itamar Goldstein, Tel Aviv University.

iv. The cytokines IL-6 and IL-17 have been shown to mediate and mark the systemic inflammation and arthritis in rodent models (9, 10). Importantly, our published results in the AIA model indicate a substantial inhibition of the IL-6 driven TH17 response to be a major mechanism of action for FTS and F-FTS therapy (1). In this reporting period, we also probed the effect of FTS on the induction of these autoimmunity-relevant cytokines in the CIA model of RA. Thus, sera from FTS and vehicle CMC treated mice were collected at day +33, at the onset of arthritis, and analyzed for IL-17A and IL-6 levels by ready-made ELISA kits. (eBioscience Inc.). We find that at the early stages of arthritis development FTS significantly reduces the upregulation of IL-6 observed in the control arm (~60% inhibition, $p < 0.001$ by Student's t-test). More importantly, FTS therapy completely suppresses IL-17 induction to below the detection limit of the ELISA kit compared to $>60\text{pg/ml}$ levels in the control mice (Figure 4C). This observation strongly indicates that the suppression of the IL-6-dependent TH17 response is an important general mechanism of action for our novel small molecule Ras-inhibitors, as we show in the two animal models of rat AIA and mouse CIA.

Responsible PI: Yoel Kloog, Tel Aviv University.

Subtask 2B: To analyze the effects of the Ras inhibitors on relevant immune cells and T cell subsets including FOXP3+ Treg and TH17 cells, the cytokines response by multiplex analysis, and relevant molecular markers such p-AKT and p-ERK, and (mo. 16-45).

v. In this reporting period, we expanded the analysis of the effects of FTS treatment on the "major" lymphocytic cell populations. Thus, at study end we harvested spleens and prepared single cell suspensions for downstream polychromatic flow cytometry analysis. The cells were next immunostained for the markers CD3, B220, CD4 and CD8. We found that neither CFA/CII

immunization by itself nor the treatment protocol (FTS vs CMC) result in a significant effect on the percentage of total CD3⁺ T cells, total B cells, and the ratios of CD4⁺ to CD8⁺ T cells in the spleen as compared to naïve mice. **Responsible PI: Itamar Goldstein, Tel Aviv University.**

vi. During previous reporting periods, in initial experiment, we used the Luminex xMAP bead-based multiplex immunoassay technology to gain a comprehensive insight into the effect of FTS treatment on the CII-specific TH cell-dependent cytokine response. Thus, we first prepared a single cell suspension from spleen and lymph node cells of mice immunized with CII and treated with oral FTS or CMC vehicle. The various cell samples were immediately stimulated with the relevant T cell pathogenic antigen, heat denatured bovine CII (from Chondrex Inc.), and the cells were cultured for 72 hours. At the end of culture, the supernatants were collected and we determined the induction of a large number of relevant cytokines, using the ProcartaPlex Mouse Th1/Th2/Th9/Th17/Th22/Treg Cytokine Panel (17 analytes), per manufacturer's recommendations (eBioscience Inc.). We found that the re-stimulation of CII-specific effector T cells from immunized untreated arthritic mice (CMC control treatment arm) induced a strong upregulation of many autoimmunity related cytokines (IL-6, TNF, GM-CSF, IFN- γ , IL-9, IL-17A, and IL-22). Importantly, this induction was significantly inhibited in T cells from mice treated with FTS. Figure 6

Responsible PI: Itamar Goldstein, Tel Aviv University.

vii. 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 (11). Th17 cells also secrete IL-22, a cytokine generally considered as pro-inflammatory in the context of RA and its levels are elevated in the blood and synovia of RA patients (12). Mice deficient in IL-22 are less susceptible to CIA and IL-22 can induce proliferation of synovial fibroblasts and promote the generation of osteoclasts, and more recently it was reported that IL-22 plays a pathogenic role during the initiation phase in CIA (13-15). As our multi-Plex data indicated a strong induction of IL-17 and IL-22 during CIA development, we focused in the current reporting period, 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), single-cell suspensions of splenocytes and inguinal lymph nodes (LN) were prepared by mechanical disaggregation followed by lysis of red blood cells. For intracellular cytokine staining, the isolated lymphocytes were activated for 5 hrs, ex vivo, with Cell Stimulation Cocktail reagent in the presence of a commercial Protein Transport Inhibitor Cocktail, according to the manufacturer's protocol (all reagents from eBioscience). After harvesting, T cells were surface stained with fluoro-chrome conjugated anti-mouse CD3 and CD4 mAbs, washed, fixed, and permeabilized using BD Cytfix/Cytoperm™ kit, per manufacturer's instructions (BD Biosciences). Intracellular staining was performed with fluoro-chrome conjugated mAbs against IL-17A and IL-22 (from eBioscience). The results of the intracellular staining showed a significant reduction in the percentage of IL-17A/IL-22 double positive TH17 cells and IL-22⁺ single positive TH cells in FTS treated mice compared to relevant control mice. 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. These original findings imply that targeting the induction of IL-22 expression in TH17 cells is a relevant mechanism of action for FTS therapy.

Responsible PIs: Itamar Goldstein & Ronit Pinkas-Kramarski, Tel Aviv University.

viii. In addition, we also analyzed specifically the antigen-specific recall response to CII, directly linked to the pathogenesis of CIA. Thus, 8-12-week-old DBA/1 male mice were immunized with CII in CFA, and treated from Day +1 with oral FTS or control CMC vehicle for 10 days. At the end of study, spleens were collected, processed into a single cell suspension, and splenocytes were labeled with the CFSE dye. The cells were then stimulated *in vitro* with heat denatured bovine CII (Chondrex Inc.) or control medium and culture for additional 96 hrs. Our results conclusively show that the proliferation of CD4+ T cells, as determined by the CFSE-dye dilution flow cytometric assay, was significantly reduced in cultures from FTS vs. CMC treated. Likewise, the ELISA of IL-17 supernatants that were collected from these cultures highlighted the strong suppression of the *in vivo* induction of the TH17 response to CII. We found that FTS therapy was associated with a significantly reduced secretion of IL-6, IL-22 and IL-17A, as compared to relevant control cultures. Taken together these new data allow us to conclude that FTS is a potent suppressor of the TH17 response to CII immunization – central to the pathogenesis of CIA. Figure 6

Responsible PIs: Itamar Goldstein & Ronit Pinkas-Kramarski, Tel Aviv University.

ix. As the balance between the inductions of antigen-specific pathogenic TH17 versus peripheral CD4+Foxp3+ regulatory T cells (Treg) can influence the outcome of the T cell response to self-antigens (16), we also determined, during the previous reporting period, the percentage of Treg cells in the spleens of the various mice groups. Our results show that FTS therapy was associated with significantly increase in Foxp3+ Treg cells percentage in the spleens of treated rats compared to CMC vehicle treated mice (see relevant data in 2nd year technical report). Importantly, the ratio of Treg to Th17 cells was significantly increased ($P < 0.01$) in FTS vs. CMC treated mice (see related text and figures in previous report). These results strongly support our working hypothesis that FTS is a potent immunomodulatory drug, potentially applicable to the treatment of human RA.

Responsible PI: Itamar Goldstein, Tel Aviv University.

x. In the second year report, we already demonstrated, as projected, that FTS therapy is coupled with effective blockade of the RAS signaling cascade in lymphocytes. We used p-ERK and p-AKT levels in splenocytes of CII immunized mice at necropsy, as molecular marker of active Ras-GTPase signaling. Our analysis of p-ERK and p-AKT levels in CD4+ T cells by the Phospho-specific Flow Cytometry method proved that FTS therapy significantly reduced, *in vivo*, in freshly isolated splenic CD4+ T cells both p-ERK and p-AKT levels.

Responsible PI: Yoel Kloog & Ronit Pinkas-Kramarski, Tel Aviv University.

In **Major Task 3**, we proposed to test the effect of FTS as an add-on therapy to MTX therapy in the AIA rat model.

Subtask 3A: Analyze the prophylactic/therapeutic effects of FTS and F-FTS on the clinical scoring, histopathology of ankle joints, and serum IL-17A/IL-6 levels at arthritis onset (**months 6-24, completed**).

i. As detailed in the previous reporting period and in our published manuscript and throughout the text of major task 1, we also discovered that treatment with FTS as an add-on to MTX provided a very strong protective effect, such as that the combined treatment almost completely inhibited the development of clinically-evident arthritis (clinical score) and ankle joint swelling (caliper

measurements of ankle joint diameter). While monotherapy with each compound alone reduced these clinical outcome measure by approximately 50%, the combined MTX+FTS therapy reduced these disease severity parameters by >80% (see Figure 1A in the attached manuscript. The difference in efficacy of the combined FTS+MTX therapy vs. monotherapy was of statistical significance in repeated studies ($P < 0.05$, by one way ANOVA with post hoc Bonferroni's multiple comparison test).(1)).

Responsible PI: Ronit Pinkas-Kramarski, Tel Aviv University.

ii. To further evaluate the effects of FTS as an add-on treatment to MTX on arthritis development, we examined ankle joint sections stained with hematoxylin and eosin from the various treatment arms as detailed above. The ankles sections were given scores of 0–5 for bone resorption and inflammation, as previously described (4). As reported in our manuscript, the histological joint tissue sections from 0.5% CMC vehicle treated rats showed extensive infiltration with mononuclear cells (inflammation scores ranging from 4 to 5, $n = 5$), and significant bone destruction (bone resorption scores ranging from 4 to 5). In comparison, the sections from FTS treated rats showed reduced joint tissue infiltration by immune cells (inflammation scores ranging from 2 to 3, $n = 5$), and less destruction of trabecular and cortical bone in the distal tibia (bone resorption scores ranging from 2 to 3). More importantly, the tissue sections from rats treated with FTS as an add-on to MTX showed only minimal joint tissue infiltration with immune cells (average inflammation score of ~ 1.5) and only rare areas of trabecular or cortical bone resorption not readily apparent on low magnification (average bone resorption scores of < 1.5). This superior efficacy of the combined treatment in preventing tissue inflammation and bone damage was statistically significant as well ($P < 0.05$, by Student's t-test).

Responsible PI: Itamar Goldstein & Ronit Pinkas-Kramarski, Tel Aviv University.

Subtask 3B: Analyze the effects of the combined MTX and FTS treatment on the serum cytokine profile and relevant T cell subsets including TH17 cells and FOXP3+ Treg (**months 6-24, completed**). The results of this aim have been partly reported in the 2nd year as well as in our recent manuscript (see details in Milestone #1 and the paper's reprint in the appendix material).

iii. IL-17⁺ and IL-17⁺IFN- γ ⁺ Th17-type cells have been shown to be instrumental for the pathogenesis of autoimmune responses (17). Thus, we determined in the second year the effects of FTS, MTX, and the combined treatment protocols on the induction of IL-17 and IFN- γ producing TH cells in the lymphatic system (spleen) during AIA development (see Figure 2A in manuscript (17)). We find that CFA immunization in control CMC-vehicle treated rats induces a significant induction of IL-17⁺ (~ 20 -fold increase; $P < 0.001$) and of IL-17⁺IFN- γ ⁺ Th17 cells (4.6 ± 0.34 vs. 0.04 ± 0.01 , $P < 0.01$, by Student's t-test) compared to naive unimmunized rats. FTS monotherapy significantly reduces the induction of these pathogenic Th17 cells in the spleens of FTS treated rats compared to control CMC-vehicle treated arthritic rats ($P < 0.001$). More importantly, FTS as an add-on to MTX therapy induces a more significant reduction in the percentage of the highly pathogenic IL-17⁺ IFN- γ ⁺ double positive TH17 cells, as compared to either drug alone $P < 0.01$ (see Figures 4 in published paper). Thus, the TH17 cell response data showed a positive and significant correlation with the clinical outcomes data of the various treatment protocols. Next, we analyzed the effects of FTS and the different treatments on the induction of IFN- γ ⁺ (only) TH1 cells in the spleens. We find that, in contrast to the TH17 data, CFA immunization did not produce a significant increase in splenic TH1 cells percentage

compared to naive unimmunized rats. Additionally, FTS monotherapy or as an add-on to MTX did not significantly reduce TH1 cells numbers in the spleens of the various rat groups.

Responsible PI: Itamar Goldstein, Tel Aviv University.

iv. Next, to further investigate the immunomodulatory effect of the combined treatment with MTX vs. FTS monotherapy on the TH17 response to BhSP65/CFA, we determined the levels of the key TH17 cell cytokines, IL-17A and IL-22, in serum samples collected at day 14 of the experiment. We find that monotherapy with FTS or MTX significantly reduces serum IL-17 levels compared to control CMC-treated arthritic rats ($P < 0.001$, by Student's t-test). Furthermore, FTS+MTX combined therapy was extra potent in suppressing IL-17 levels compared to single agent ($P < 0.01$). We also find a strong upregulation of serum IL-22 at day +14 in control CMC treated rats, which is significantly inhibited by either FTS or MTX therapy ($P < 0.001$). Interestingly, FTS as an add-on to MTX inhibits IL-22 production very effectively compared to MTX alone (~75% suppression by the combined FTS and MTX vs. MTX monotherapy, $P < 0.01$).

Responsible PI: Itamar Goldstein, Tel Aviv University.

In **Major Task 4** we analyzed in vitro the effects of FTS and F-FTS on various T cell-signaling events and T cell differentiation programs following TCR stimulation (**months 25-42**).

i. In this reporting period we studied the effects of FTS on the proliferation of T cells. Thus, we purified CD4+ T cells by negative selection from spleens of naïve 8-12-week-old DBA/1 male mice, using the MACS® magnetic cell separation technology (all reagents were from Miltenyi Biotec, Germany). Next, the pure CD4+ T cells were labeled with CFSE and activated by plate-bound anti-CD3 and soluble anti- CD28 (All from eBioscience Inc.) in a fully supplemented complete RPMI medium for 3 days. The cultures were treated with the indicated concentrations FTS dissolved in DMSO or vehicle alone. At the end of culture the CD4+ T cells were harvested and analyzed by FACS for CFSE-dilution (Figure 7A). These data allowed us to determine the effect of the different doses of FTS on T cell proliferation. We found a significant inhibition of proliferation starting at a low concentration of 12.5µM and peaking at ~50µM of FTS (Figure 7B). Of note the inhibition was not significantly more effective at concentrations in the range of 100-150µM (data not shown).

Responsible PI: Ronit Pinkas-Kramarski, Tel Aviv University.

ii. Our results and major achievements in Tasks 1-3 highlight that a major *in vivo* mechanism of action of the small molecule oral Ras-inhibitors, FTS and F-FTS, is potent inhibition of the TH17 response to pathogenic antigens. Treatment with Ras-inhibitors was associated with a significant inhibition of IL-17A and IL-22 levels in the serum of actively treated animals as compared to relevant control arthritic rats. Moreover, our ELISA and transcriptomics data show that the TH17-type recall response to Bhsp65 or CII were in immunized rats and mice, respectively, were significantly diminished in T cells derived from FTS treated animals. Importantly, the superior clinical efficacy F-FTS treatment positively correlated with its superior capacity to down modulate the pathogenic antigen-specific TH17 response (i.e. reduced IL-17 secretion).

Thus, to further study and validate these in vivo data we stimulated pure CD4+ T cells with plate-bound anti-CD3 and soluble anti- CD28 and cultured them for 72 hrs. At the end of culturing, the supernatants were collected and analyzed for secreted IL-17A and IL-22 by readymade ELISA kits. Our results show that following polyclonal stimulation FTS suppressed the strong induction

of the TH17-class cytokines, IL-7 and IL-22, secretion by CD4+ T cells detected in control cultures (Figure 8). In agreement with our in vitro proliferation data, we find a significant inhibition of IL-7 and IL-22 secretion starting at a low concentration of 12.5 μ M. This inhibition was rather effective (>50%) at this FTS dose, with a small incremental increase at 25-50 μ M of FTS (Figure 8). Once more, the inhibition of TH17 cytokines production was not significantly augmented by higher concentrations of FTS in the 100-150 μ M range (data not shown).

Responsible PI: Itamar Goldstein, Tel Aviv University.

iii. The stimulation of T cell receptor (TCR) as well as of the co-stimulatory receptor CD28 induces activation of RAS and its downstream effectors. To investigate the ability of FTS to inhibit Ras signaling following T cell activation in vitro. CD4+ T were activated for 30 min by plate-bound anti-CD3 and soluble anti-CD28 with or w/o FTS treatment. Using the RayBio® Mouse MAPK Pathway Phosphorylation Array we detected the relative phosphorylation levels of 17 relevant MAPK pathway proteins. We found that the FTS treatment inhibit significantly the phosphorylation of multiple critical kinases such as Erk, Akt, p38 and mTOR (figure 9A). To validate our RayBio® Mouse MAPK Pathway Phosphorylation Array data, we then 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 cells activation protocol as above; we found that at +30 min (the timepoint of pick pERK upregulation) FTS treatment decreased significantly ($P < 0,001$) the phosphorylation level of ERK1/2, Akt and p38, as compared to control medium (Figure 9B). Thus, FTS inhibits phosphorylation of multiple downstream effectors of RasGTPases that are induced following TCR/CD28 co-stimulation.

Responsible PI: Ronit Pinkas-Kramarski, Tel Aviv University.

iv. Our results show that FTS indeed suppressed the secretion of all three TH17 related cytokines. Thus, to investigate whether FTS modulates *de novo* Th17 cell differentiation, we cultured primary naïve CD4+ T cells under Th17-polarizing conditions in the presence of different concentrations of FTS or DMSO (vehicle control). On day +5 the CD4+ T cells were analyzed by flow cytometry for intracellular cytokine production. Our data showed that FTS in a dose dependent mode, starting at 1.56 μ M, significantly suppressed the production of IL-17A (Figure 10). Moreover, FTS treatment even at a low dose inhibited the production of IL-17A, IL-22 and GM-CSF (Figure 10 A, B and C) .

Responsible PI: Itamar Goldstein, Tel Aviv University.

v. To investigate whether FTS can enhance the expansion of "naturally occurring" regulatory T cells (Treg) or affect their suppressive IL-10 dependent functions, we stimulated CD4+ T cells obtained from spleens and draining lymph nodes (DLNs) of naïve mice with plate-bound anti-CD3 and soluble anti-CD28, and then treated the various cultures with FTS or vehicle for 72 hrs. At culture end, to determine Treg numbers the cells were analyzed by FACS for Foxp3 and CD25 co-expression. We found that FTS treatment following T cell polyclonal stimulation significantly affected the ex vivo expansion of Foxp3+ Treg cells as compared to counterpart vehicle-treated cells (figure 11A and B). Moreover, the ELISA analysis of supernatants collected at culture end for IL-10 levels showed that FTS treatment significantly enhanced IL-10 secretion by CD4+ T cells compared to control cultures (figure 11C). Thus, in vitro FTS treatment increased the expansion of Treg cells and reciprocally of total IL-10 production.

Other achievements:

i. Milestone #1: Published Manuscript on the therapeutic value of Ras inhibitors in the AIA and CIA animal models.

As detailed in this report and previous reports, we have generated multiple clinical, immunological and molecular data clearly showing that the small molecule Ras-inhibitors, FTS and F-FTS, are potent disease modifying drugs in two major animal models of RA and that using FTS therapy as an add-on to MTX therapy significantly increased efficacy compared to monotherapy. In accordance with the SOW, during this reporting period we published a comprehensive manuscript that summarizes our novel findings (*1*).

ii. Conference papers and presentations: As detailed in item 6 in the current report our data and research achievements were presents in three important international scientific conferences. **IMMUNOLOGY 2017™** meeting (May 12-16, Washington, DC); **EULAR 2017** (June 14-17, Madrid, Spain), and **12th World Immune Regulation Meeting 2018** (March 14-17, Davos, Switzerland).

iii. Milestone #2: We wrote a comprehensive manuscript describing the mechanisms (cellular and molecular) that control/mediate the therapeutic (Immunomodulatory) effects of small molecule Ras inhibitors in the CIA mouse model of RA, including mechanistic data derived from the in vitro work in Task 4 (see attached appendix). We plan to publish it in a leading journal in the fields of Rheumatology & Immunology within the next 6 months.

Responsible PIs: Itamar Goldstein & Yoel Kloog / Ronit Pinkas-Kramarski, Tel Aviv University.

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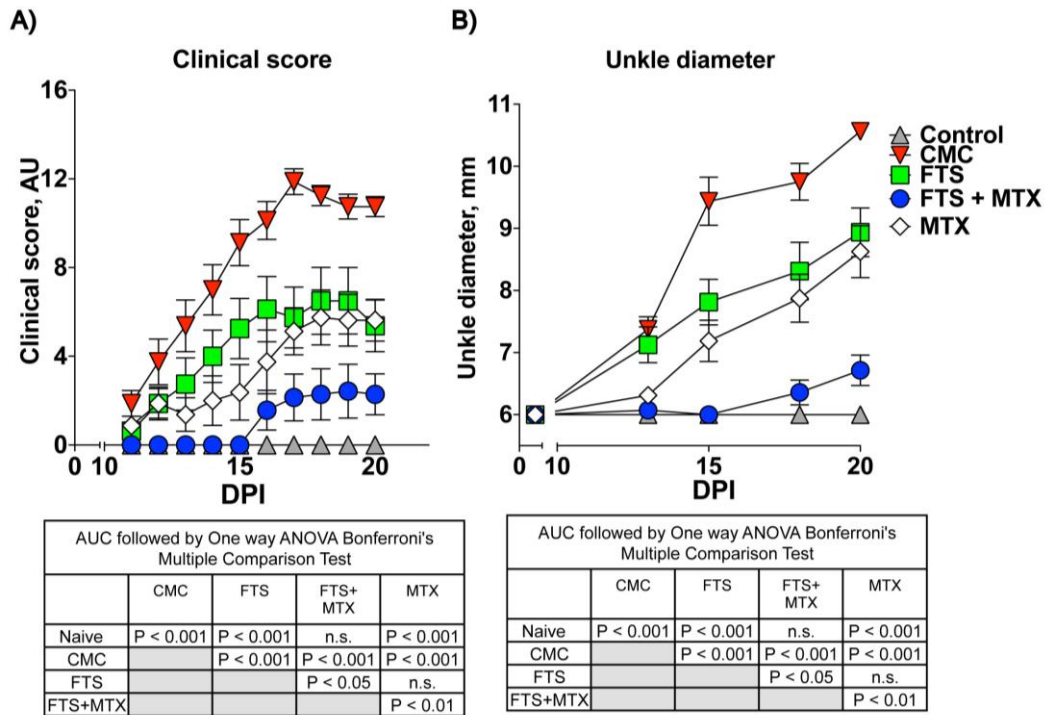


Figure 1: FTS treatment reduces the severity of adjuvant-induced arthritis. Rats were immunized with CFA and then graded regularly for signs of arthritis by a clinical disease severity score (A), and by caliper measurements of ankle joint diameter (B). Rats in the experimental arms were treated starting day +1 with either oral FTS (100 mg/kg), 0.5% CMC vehicle solution, weekly i.p injection of MTX (0.5 mg/kg), or FTS combined with MTX. Statistical analysis of the AUC of the effect of the various treatments on the clinical score (A) and ankle diameter (B), were done by one way ANOVA with post-hoc Bonferroni's multiple comparison test. The results of the analysis are summarized in the table inset. One representative of three independent experiments is depicted. (*P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001).

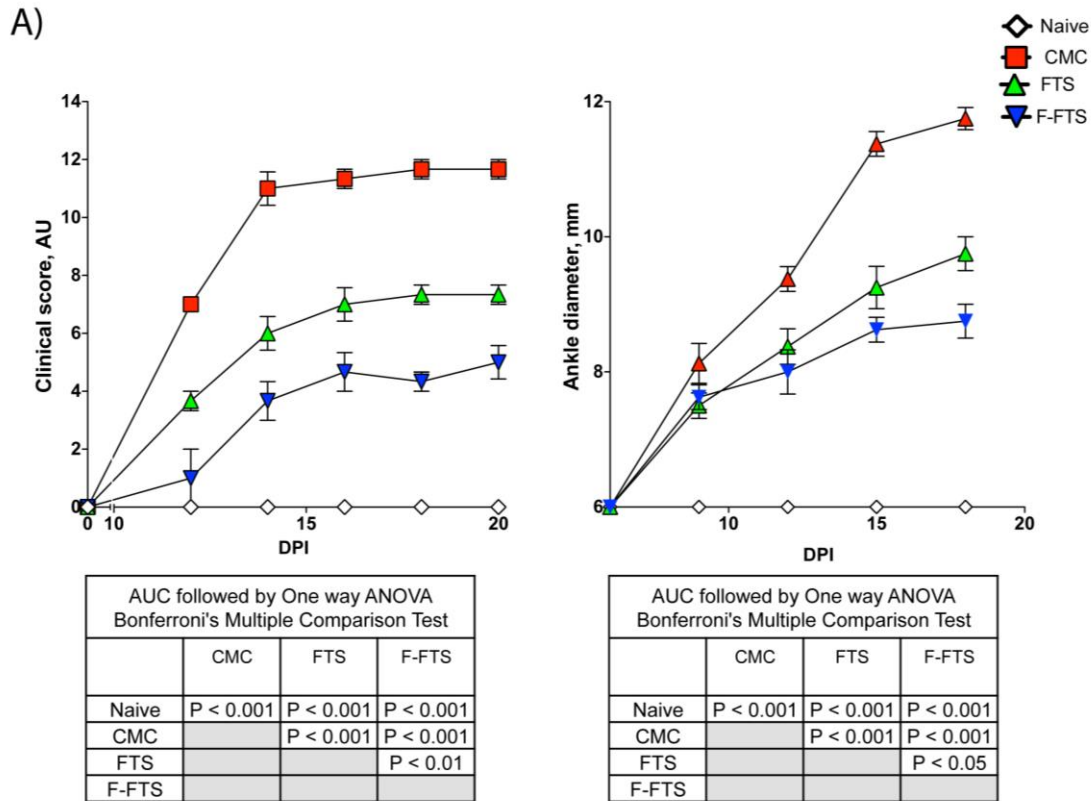


Figure 2: F-FTS is significantly more effective than FTS in attenuating multiple outcome measures of AIA. (A) Rats immunized with CFA were treated starting day +1 with either oral FTS (100 mg/kg), F-FTS (60mg/kg) or 0.5% CMC vehicle solution. Arthritis severity was graded by clinical scores (left panel) and ankle diameter (right panel). Statistical analysis of the AUC of the effect of the treatments on the clinical outcomes were done by one way ANOVA with post-hoc Bonferroni's multiple comparison test, and the results are summarized in the table insets. One representative experiments out of two performed. Bar graphs depict mean \pm SD. Statistical significance was assessed by the *Student's t* test (*P \leq 0.05, **P \leq 0.01, and ***P \leq 0.001).

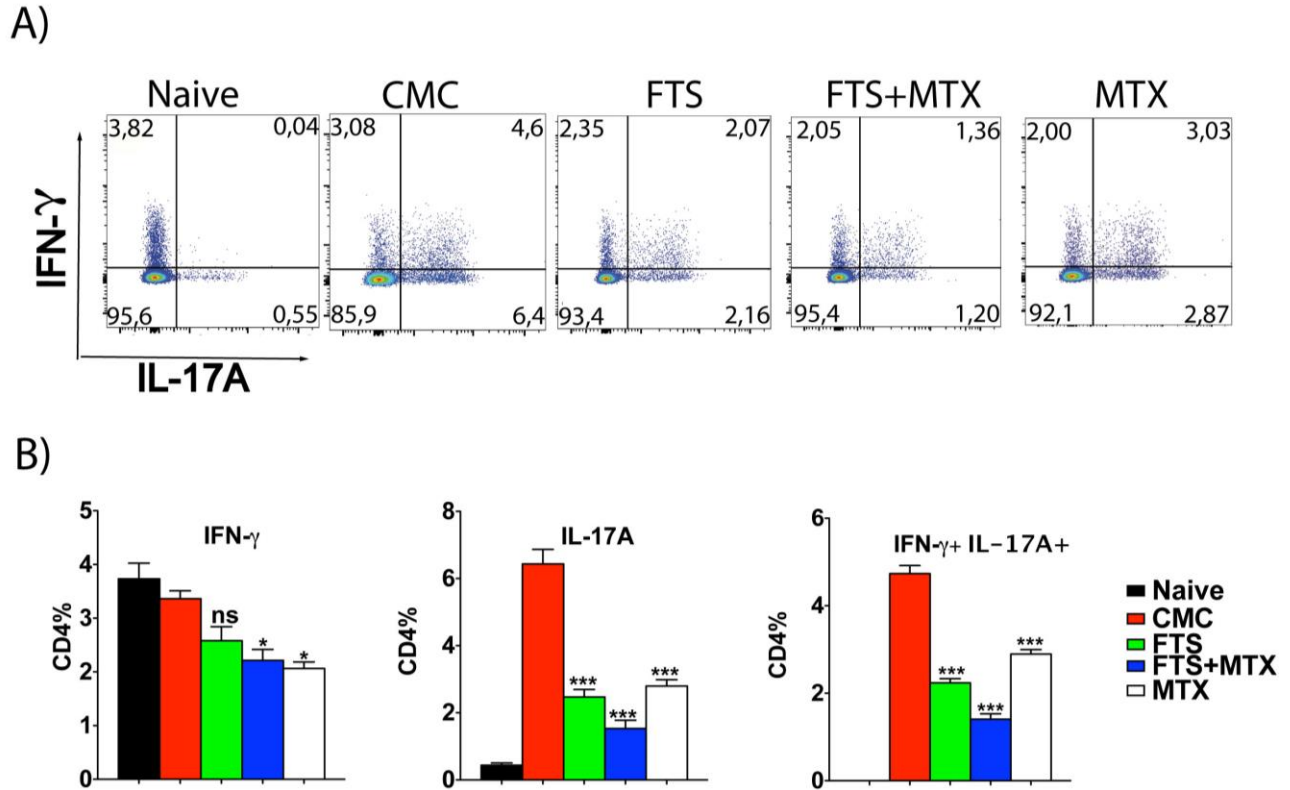
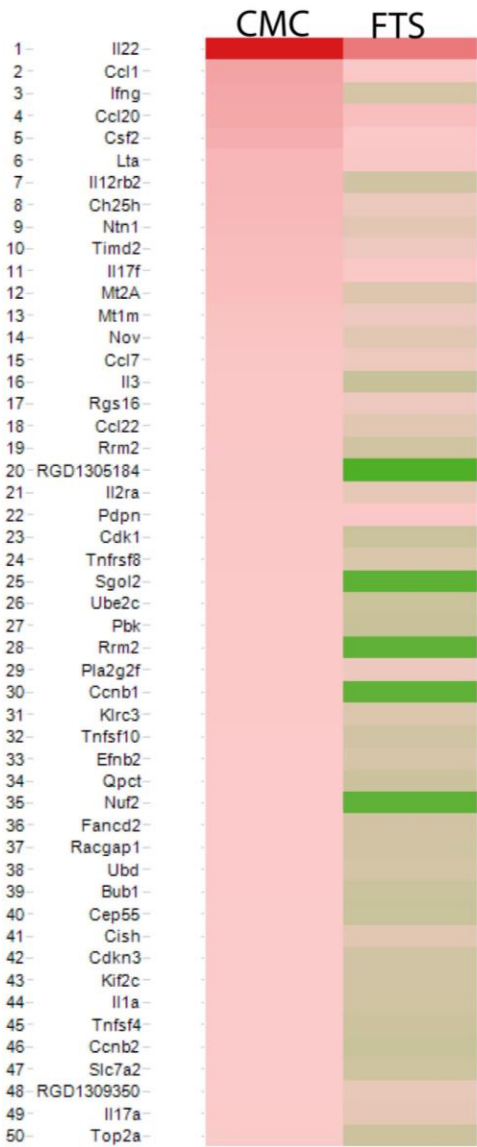
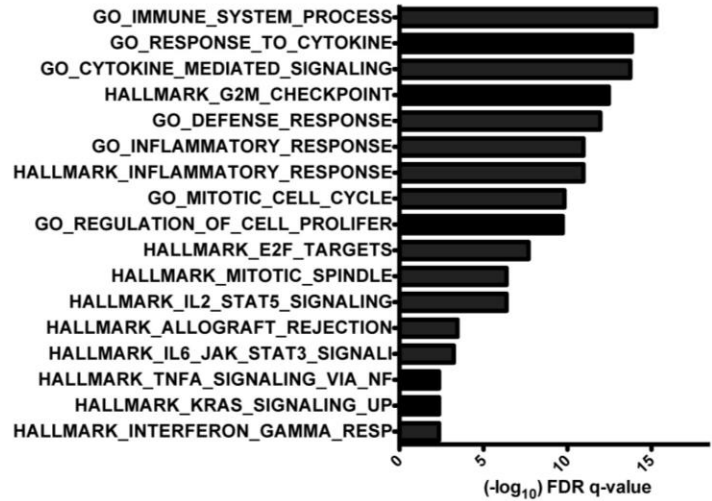


Figure 3. Immunomodulatory effect of FTS treatment on the induction of Th1, Th17 and Treg subsets post CFA immunization. (A) Representative flow cytometry plots of intracellular staining for Th1 and Th17 cytokines producing CD4+ T cells. (B) Bars represent the percentage (mean \pm SD) of the indicated Th-subsets in spleens of the various rat groups harvested at day 20 post CFA immunization (n=5 per group). Statistical significance was assessed by one way ANOVA with post-hoc Bonferroni's multiple comparison test (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$).

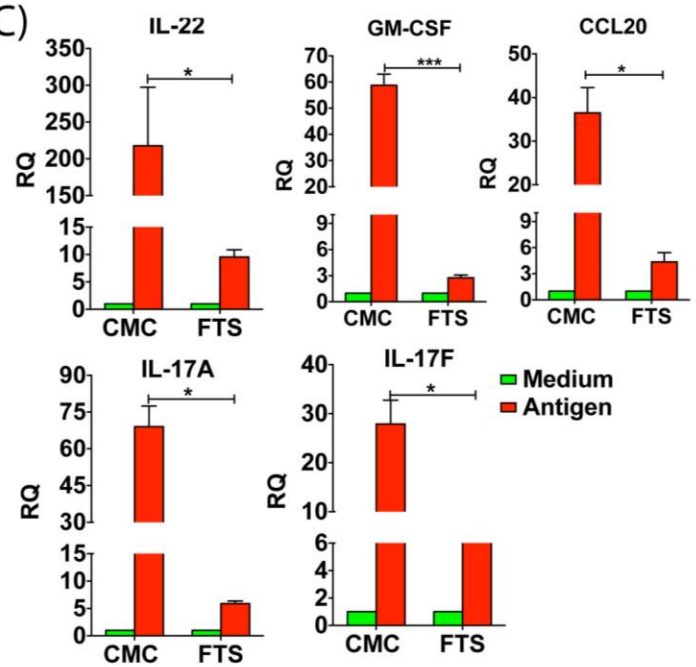
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B)



C)



D)

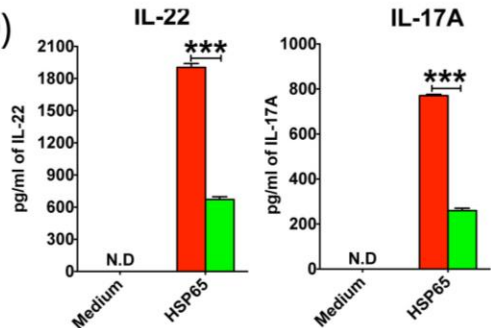


Figure 4: FTS therapy down modulates the Bhsp65-induced transcription of multiple genes tightly linked to inflammatory and immune processes. Following CFA-immunization, rats were treated with CMC or FTS starting from day +1 post AIA induction. Single cell suspensions from draining LNs and Spleen of treated rats (harvested on day +14 post disease induction), were stimulated with Bhsp65 (5 μ g/ml) or control PBS and cultured for additional 72 hrs. At the end of cultures, CD4+ T cells were isolated and high quality total RNA was extracted from the purified cells. **(A)** Heat map analysis depicting the expression matrix of the 50 genes significantly induced by Bhsp65 stimulation (>2FC, FDR $q < 0.05$) in the CMC control group as compared to their expression in FTS treated group (n=2 per group). **(B)** Next, we computed using the GSEA software, the overlaps between our gene list and relevant validated gene sets. Bars represent the FDR q-value (cutoff of < 0.05) for the statistical significance of the overlap between the annotated Gene set and our list. **(C)** Purified total RNA was analyzed by qPCR for the relative mRNA expression (normalized to Actb) of five inflammatory response genes (Ccl20, Il22, Il17A, Il17F, and Csf2) induced by Bhsp65 re-stimulation of CD4+ T cells treated in vivo with CMC vs. FTS. **(D)** Supernatants from the same cultures were tested by ELISA kits for levels of secreted 17A and IL-22 by readymade ELISA kits. Bar graphs depict mean \pm SD, and statistical significance was analyzed by *Student's t* test (*P ≤ 0.05 , **P ≤ 0.01 , and ***P ≤ 0.001). Data shown are representative of a typical experiment out of n=3.

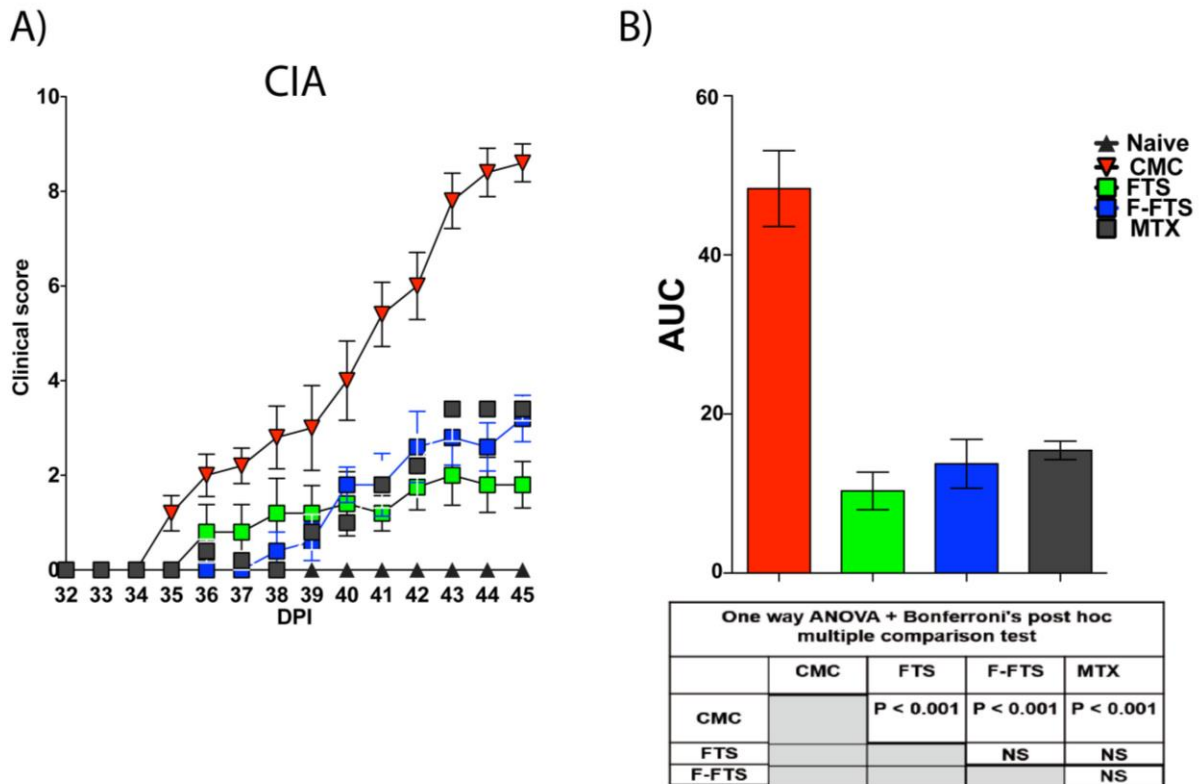


Figure 5: Prophylactic treatment with FTS, , F-FTS and MTX reduces the severity of collagen-induced arthritis (CIA). 8-12 week-old DBA/1 male mice were given an initial injection of CFA/collagen type-2 (CII) on day 0, and arthritis was induced with a second booster injection of IFA/CII on day +21. Mice in the experimental arms (n=8 per group) were treated starting day +18 with daily FTS (100mg/kg), daily F-FTS (60mg/kg), weekly i.p injection of MTX (1 mg/kg), or 0.5% CMC vehicle solution (control treatment). **(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 AUC \pm S.D. for the clinical CIA disease scores from days post immunization (DPI) +30 to +45, and a representative study of three independent experiments done is depicted (**n=8 per treatment group**). Inset shows the results of the statistical analysis of the differences in area under the curve (AUC) values for the different treatment arms. Statistical analysis was performed by One way ANOVA with Bonferroni's post hoc multiple comparison test

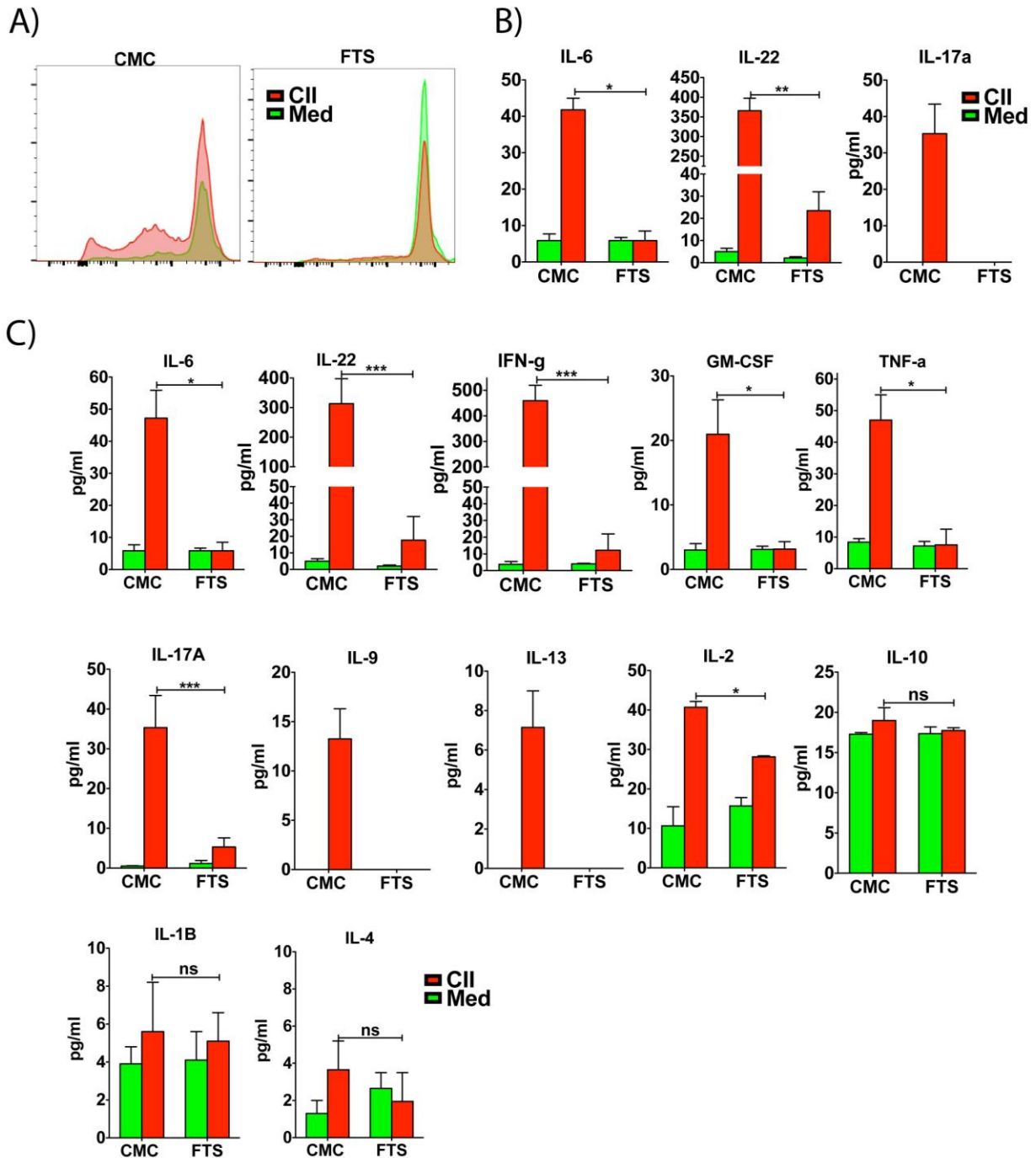


Figure 6. FTS therapy mediates significant inhibition of the T cell recall response to CII. 8- 12-week-old DBA/1 male mice were immunized with CFA/type II collagen on day 0 and treated from Day +1 of the study with oral FTS or CMC vehicle for 10 days. Single-cell suspensions of splenocytes were prepared by mechanical disaggregation. Splenocytes were labeled with CFSE and stimulated *in vitro* with heat denatured bovine CII or control medium, and then cultured for additional 96 hours. **(A)** Cells were harvested and stained with anti-mouse CD3 and CD4 mAbs. And cells were analyzed for CII-specific

proliferation of CD3+CD4+ T cells by flow cytometry (green for medium and red for CII). **(B)** In parallel, supernatants were collected and the levels of IL-6, IL-22 and IL-17A were determined by commercial ELISA kits. **(C)** Splenocytes isolated from mice treated with FTS or CMC (up day 18) were isolated at day 32 DPI and cultured heat denatured bovine CII or control medium for additional 96 hours, supernatants were collected and we determined the levels of a large number of important cytokines by the ProcartaPlex Mouse Th1/Th2/Th9/Th17/Th22/Treg Cytokine Panel. Bars represent mean \pm S.D. of triplicates from a representative experiment, and red bars represent CII stimulated cultures while green bars represent medium control cultures. Data were analyzed for statistical significance t-test for FTS treatment compared to CMC control treatment Values of $P < 0.001$, $P < 0.01$, and $P < 0.05$ were marked by three, two and one asterisks, respectively. (N.D: not detectable)

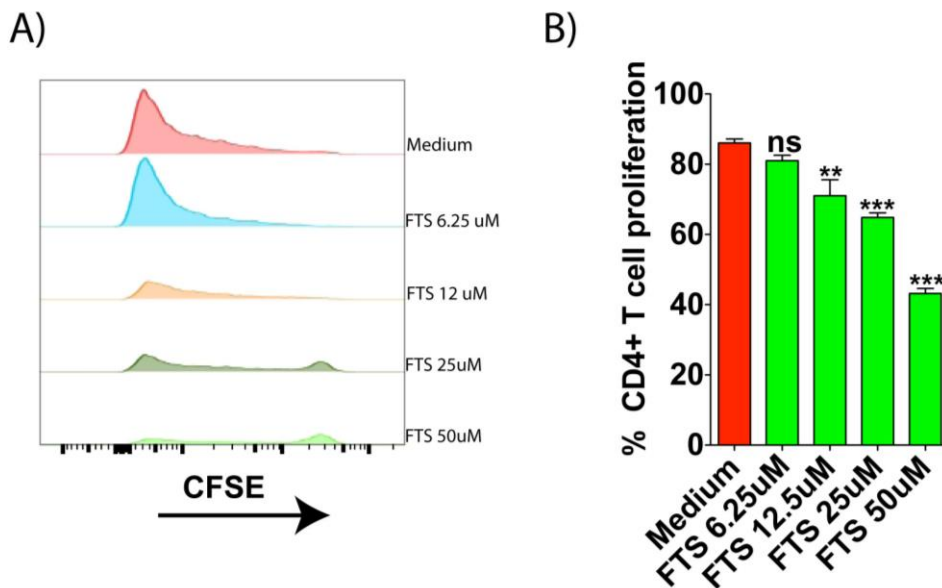


Figure 7. FTS inhibits T cell proliferation following in vitro polyclonal stimulation. CD4+ T cells were purified by negative selection from spleens of naïve 8-12-week-old DBA/1 male mice using magnetic cell separation technology (MACS, Miltenyi Biotec, Germany). CD4+ T cells were labeled with CFSE and activated by plate-bound anti-CD3 and soluble anti- CD28 in fully supplemented RPMI for 3 days. During T cell activation cells were treated with the indicated concentrations FTS dissolved in DMSO. (A and B) Representative and Statistical analysis proliferated CD4+ T-cells with different dose of FTS. One representative of two independent experiments is shown. Data were analyzed for statistical significance t-test for FTS treatment compared to CMC control treatment Values of $P < 0.001$, $P < 0.01$, and $P < 0.05$ were marked by three, two and one asterisks, respectively

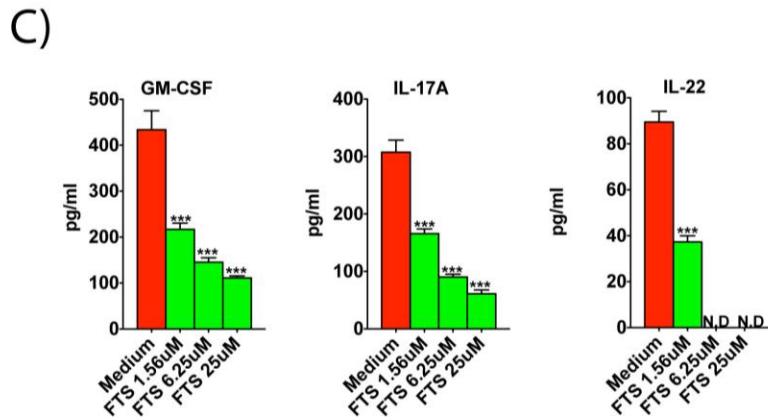
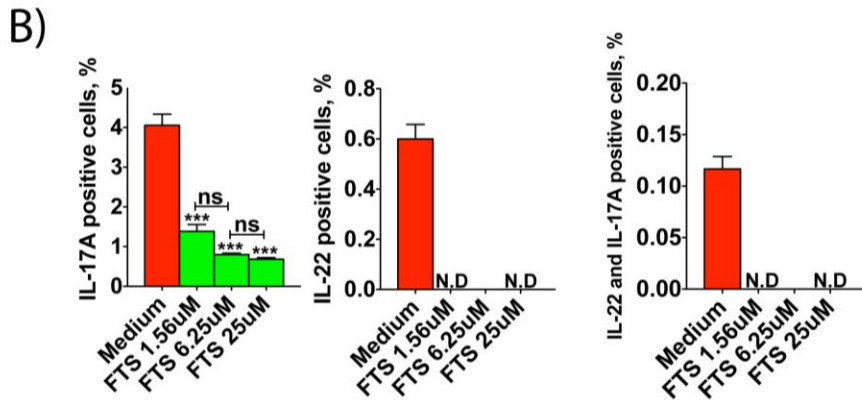
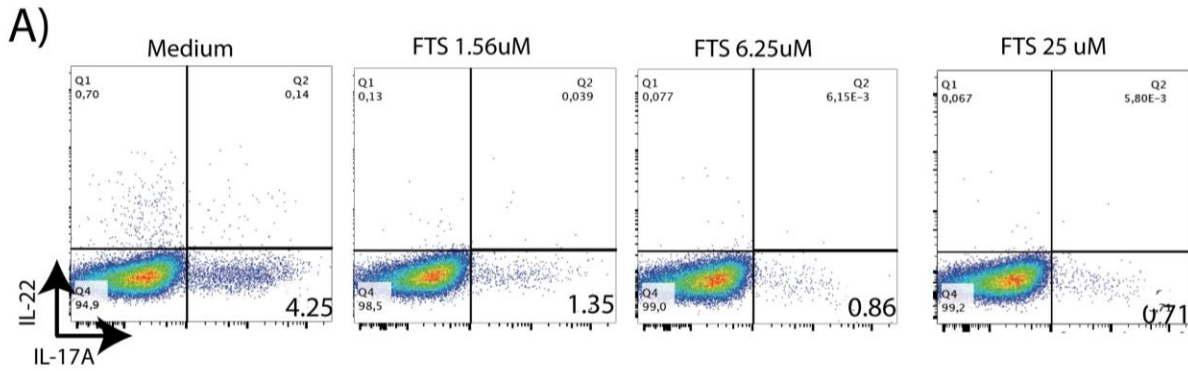


Figure 8. FTS reduce the production of Th17 related cytokines following in vitro Th17 expansion with IL-23 . CD4⁺ T cells were purified by negative selection from spleens of naïve 8-12-week-old DBA/1 male mice using magnetic cell separation technology (MACS, Miltenyi Biotec, Germany). CD4⁺ T cells were plate-bound anti-CD3 and soluble anti- CD28 in fully supplemented RPMI with IL-23 for 5 days. During T cell activation cells were treated with the indicated concentrations FTS dissolved in DMSO. **A)** Representative flow cytometry plots of intracellular staining of CD4⁺ T cells for IL-17A and IL- 22 cytokines production. **(B)** Bars represent the mean percentage (\pm S.D) of the indicated TH17- subsets. **(C)** At the end of culture, the supernatants were collected and tested for secreted GM-CSF, IL-17A and IL-22 by ELISA. One representative study out of 3 independent experiments is depicted. Statistical significance was calculated by the Student's t test (***) $P \leq 0.0001$, by Student's t-test).

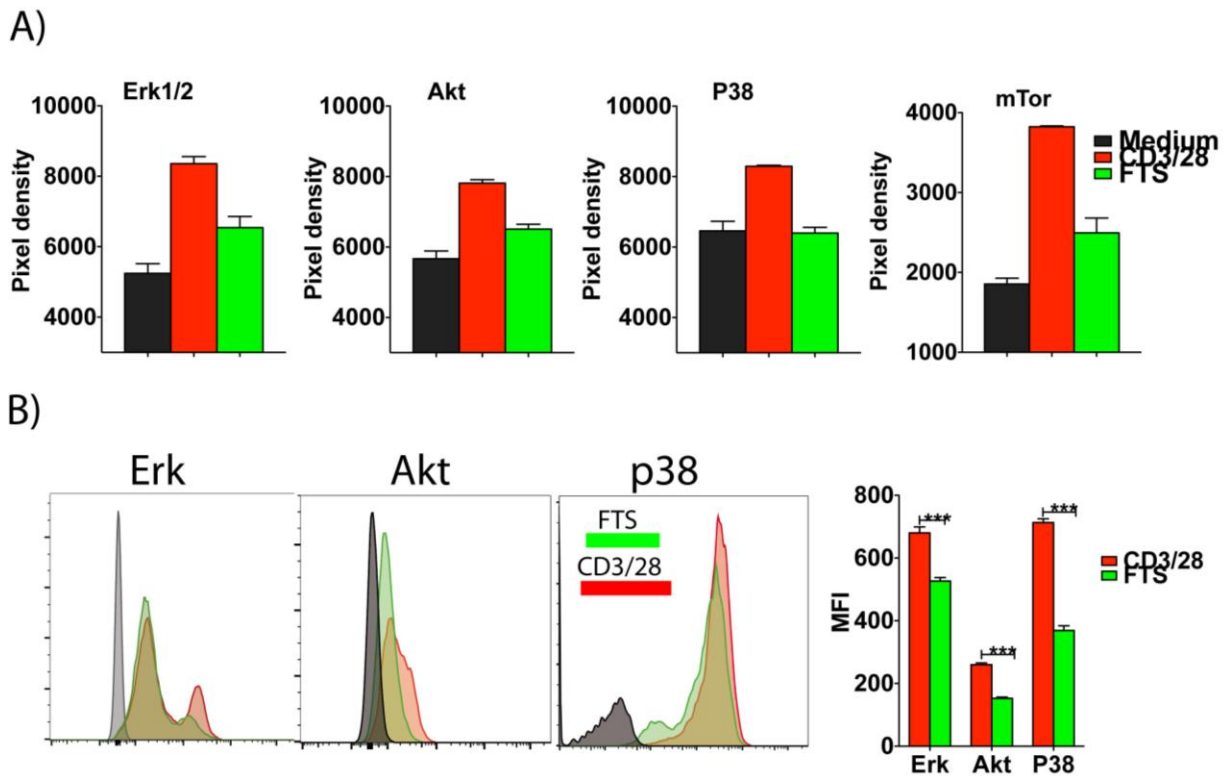


Figure 9: Treatment of T-cells with FTS *in-vitro* modulate, the downstream signaling of Ras pathway. A) Isolated mouse CD4+ T cells were stimulated with anti CD3/28 antibody with(green) or without (red) FTS 10uM for 30 min . (A) protein extracts of cell lysates were analyzed by RayBio® Mouse MAPK Pathway Phosphorylation Array. (B) Activation of Erk ,AKT and P38 were determined with phospho-specific antibodies by intracellular flow cytometry, red : activated cells in medium, green : activated cells with 10uM FTS and gray: unstained cells. Data were analyzed for statistical significance t-test for FTS treatment compared to CMC control treatment Values of $P < 0.001$, $P < 0.01$, and $P < 0.05$ were marked by three, two and one asterisks, respectively.

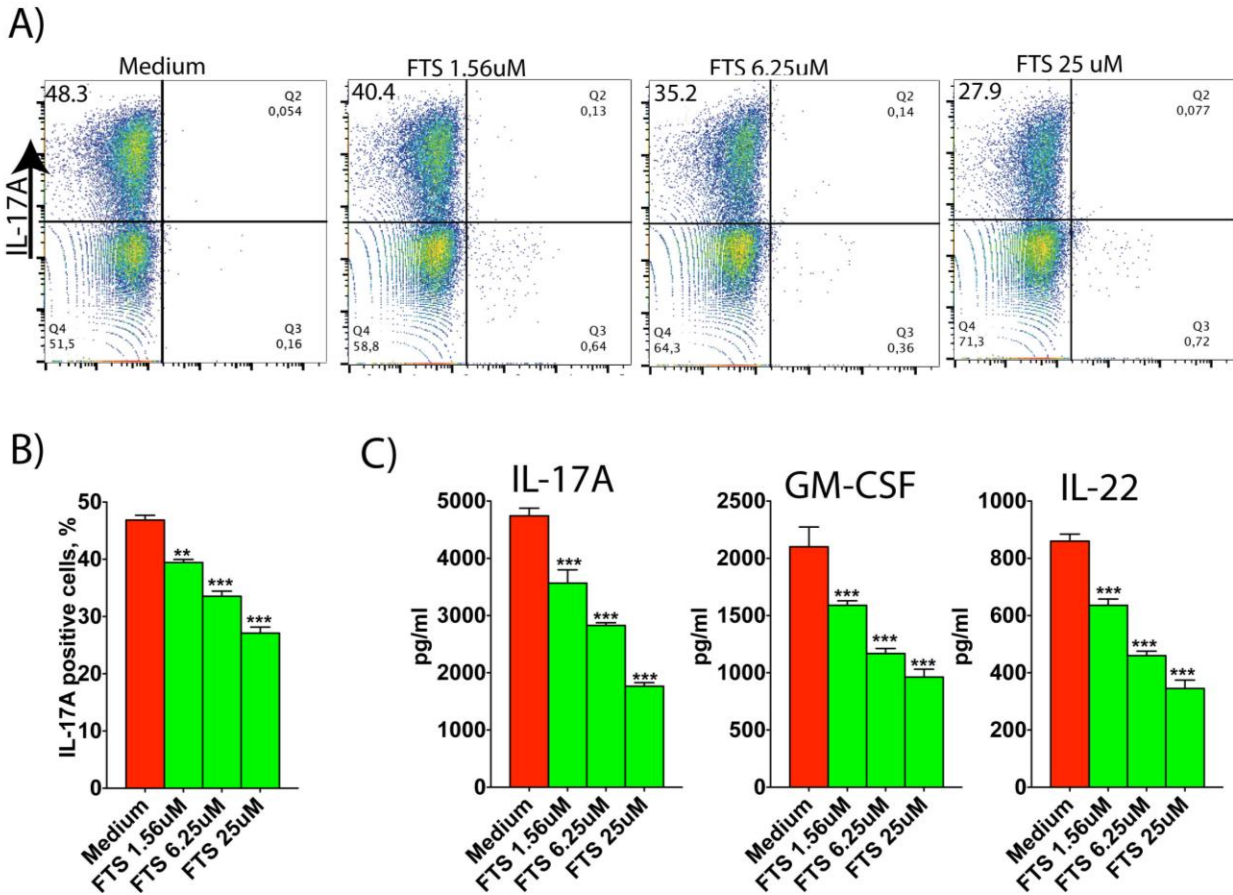


Figure 10: Treatment of naïve CD4⁺ T-cells with FTS *in-vitro* modulates *de novo* differentiation to TH17 cells. Naïve CD4⁺ T cells were activated for 5 days with anti-CD3/anti-CD28 antibodies in the presence of Th17 polarization cytokines or Tregs polarization cytokines. FTS was added to the cultures at the indicated concentrations. **(A)** Representative flow cytometry plots of staining of IL-17A producing cells. **(B)** Bars represent the mean percentage (\pm S.D) of the indicated Th17 cells. **(C)** 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 3 independent experiments is depicted. Statistical significance was calculated by the Student's t test ($P < 0.001$, $P < 0.01$, and $P < 0.05$ were marked by three, two and one asterisks, respectively)

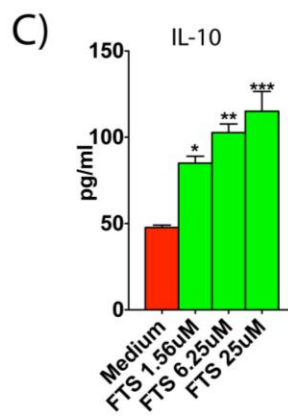
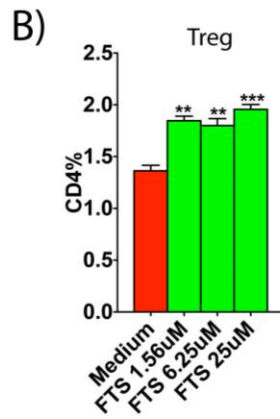
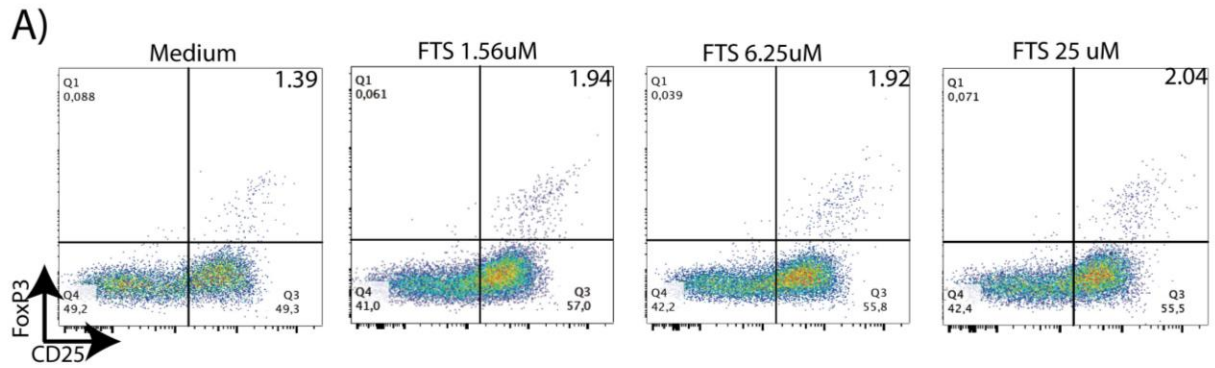


Figure 11. FTS enhance the expansion of regulatory T cells in vitro following polyclonal activation. Isolated CD4⁺ T cells were from spleens of naïve 8-12-week-old DBA/1 male mice were activated with plate-bound anti-CD3 and soluble anti- CD28 in fully supplemented RPMI with 3 days. During T cell activation cells were treated with the indicated concentrations FTS dissolved in DMSO. **A)** Representative flow cytometry plots of staining of Tregs. **(B)** Bars represent the mean percentage (\pm S.D) of the indicated Tregs. **(C)** At the end of culture, the supernatants were collected and tested for secreted IL-10 by ELISA. One representative study out of 3 independent experiments is depicted. Statistical significance was calculated by the Student's t test ($P < 0.001$, $P < 0.01$, and $P < 0.05$ were marked by three, two and one asterisks, respectively)

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Training activity for Ph.D. graduate student Morad Zayoud M.D. included one-on-one work with Dr. Goldstein (mentor) and participation in conferences, workshops and seminars, as detailed:

1. New Horizons in Immune Dynamics 2017 (July 18, Weizmann Institute, Israel).
2. IMMUNOLOGY™ 2017 meeting (May 12-16, Washington, DC) of the "The American Association of Immunologists".
3. The annual meeting of the Israeli society of Rheumatology 2017 (April 27-28, Israel).
4. 12th World Immune Regulation Meeting 2018 (March 14 – 17, Davos, Switzerland).

Focused on "Development and Maintenance of Immune Activation and Tolerance with a special focus on Autoimmunity".

All other members of the project's team participate in lab/project team internal seminars, supervised by the PIs, and designed to advance their professional, research and academic skills.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

“Nothing to Report.”

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state “Nothing to Report.” objectives.

“Nothing to Report.”

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state “Nothing to Report.” Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The results and achievements of our research project have the potential to make significant contributions to both basic immunology and the clinical care of rheumatoid arthritis (RA). *Short-term impact:* The results of this project improve our insight on the role of Ras signaling (and its blockade) on the T cell response in preclinical animal models of RA. Our in vivo results show that Ras signaling is important for the induction of T helper (TH) 17-type effector responses, while its blockade inhibits this autoimmune response and enhances peripheral immune tolerance by increasing the ratio of FoxP3+ regulatory T cells (Treg). *Long-term impact:* The results of this project strongly imply that FTS (Salirasib) and/or its more potent analogue, F-FTS, have the potential to become important novel targeted synthetic anti-rheumatic drug. Obviously, the development of FTS into an effective (and cheaper) oral drug for RA may ultimately impact the clinical care of RA patients. The prevalence of RA in adult Americans is estimated at ~1.5 million. Regarding the prevalence of RA in veterans, available epidemiological data indicates that ~1% of patients seen each year in outpatient settings have a diagnosis of RA. FTS (Salirasib) is the only available successful Ras inhibitor that reached Phase II clinical trials, and consequently received an orphan drug designation by the FDA and EMA for the treatment of pancreatic cancer. Our results signify that its introduction into the field of rheumatology should be a significant opportunity for patients and scientists alike.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.” Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

“Nothing to Report.”

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.” Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*

- *adoption of new practices.*

“Nothing to Report.”

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

“Nothing to Report.”

- 5. CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

“Nothing to Report.”

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

As stated in the 2nd year Annual Technical Report and SOW our research aims for the third year (CY17) of the award were as follows:

- i. To validate in the collagen induced arthritis (CIA) mouse model the prophylactic and/or therapeutic effects of FTS and the FTS derivative, F-FTS (Task 2).
- ii. To analyze the in vitro effects of FTS and its derivative F-FTS on various T cell-signaling events following TCR stimulation.

Unfortunately, there was an unexpected delay with ACURO approval of our recent animal use protocol due to a lengthy review time. The animal protocol PR130028.05 entitled, "Studying the Immunomodulatory Effects of Ras-Inhibitors in the Collagen Induced Arthritis Mouse Model," IACUC protocol number 04-17-005 was therefore approved by the USAMRMC Animal Care and Use Review Office (ACURO), only at the end of APRIL 2017. Thus, our animal experiments, as described above (aim i) could be continued only after the 1st of May 2017. Thus. We estimated a 9-12 months delay in finalizing the animal studies related to Major Tasks 2 and 4 and completing Milestone #2, and requested a no-cost extension of 12 months of the award performance period. The request for extension and the revised SOW were approved on Aug. 2017.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

“Nothing to Report.”

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

“Nothing to Report.”

Significant changes in use or care of vertebrate animals

“Nothing to Report.”

Significant changes in use of biohazards and/or select agents

“Nothing to Report.”

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Journal publications (peer-reviewed research papers):

M. Zayoud, V. Marcu-Malina, E. Vax, J. Jacob-Hirsch, G. Elad-Sfadia, I. Barshack, Y. Kloog, I. Goldstein, Ras Signaling Inhibitors Attenuate Disease in Adjuvant-Induced Arthritis via Targeting Pathogenic Antigen-Specific Th17-Type Cells. *Frontiers in immunology* 8, 799 (2017) published on line July 2017. (04/18/17 received; 06/23/17 accepted)

Acknowledgement of federal support: yes.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Conference papers:

1. Morad Zayoud, Einva Vax, Galit Elad Sfadia, Yoel Kloog and Itamar Goldstein. Farnesylthiosalicylic acid reduces disease severity in the collagen type-II induced arthritis mouse model by inhibiting Ras Signaling in pathogenic T cells. *The Journal of Immunology* 198(1 Supplement):224.227-224.227. Published online May 1, 2017.

This abstract, summarizing our major accomplishments in this research award, was selected for publication and Poster presentation in the prestigious international **IMMUNOLOGY™ 2017** meeting (May 12-16, Washington, DC) of the "The American Association of Immunologists". This conference is the largest annual gathering of immunologists worldwide.

2. M. Zayoud, E. Vax, G. Elad Sfadia, V. Marcu-Malina, Y. Kloog, I. Goldstein. RAS SIGNALING INHIBITION ATTENUATES ARTHRITIS IN ANIMAL MODELS OF RHEUMATOID ARTHRITIS BY DOWN MODULATING THE PATHOGENIC TH17 CELL RESPONSE. *Annals of Rheumatic Diseases (Supplement 1)* 2017. DOI: 10.1136/annrheumdis-2017-eular.2843

This abstract was accepted for publication and poster display/presentation in The European League against Rheumatism Congress, **EULAR 2017** (14-17 June, Madrid, Spain). This presentation summarized our findings and accomplishments in the AIA and CIA models of RA with a translational emphasis. The abstract was also selected for presentation, in a GUIDED POSTER TOUR: "New drivers in RA and SPA pathophysiology" attended by scientists from academic institutions and industry, as well as physician/rheumatologists.

Website(s) or other Internet site(s)

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

"Nothing to Report."

• **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

"Nothing to Report."

- **Inventions, patent applications, and/or licenses**
Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

“Nothing to Report.”

- **Other Products**
Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
 - *data or databases;*
 - *models;*
 - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
 - *and other.*

“Nothing to Report.”

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Name: **Itamar Goldstein**
Project Role: Initiating Primary Investigator
Researcher Identifier (e.g. ORCID ID): 0000-0001-5393-186X
Nearest person month worked: “no change”
Contribution to Project:
Dr. Goldstein has supervised all aspects of the project, including mentorship of PhD graduate students and co-authoring of all manuscripts and other publications.

Name: **Morad Zayoud**
Project Role: Ph.D. Graduate Student
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: “no change”
Contribution to Project:
Dr. Zayoud has performed the experiments in the AIA and CIA models and analyzed relevant data, and co-authored the Manuscript and other publications in partial fulfillment of the requirements for his Ph.D. degree from Tel Aviv University.

Name: **Einav Vax**
Project Role: Research Associate
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: “no change”
Contribution to Project:
Einav has managed all technical aspects of the project, and participated in the majority of the experiments in the AIA and CIA models.

Name: **Ronit Pinkas-Kramarski**
Project Role: Partnering Primary Investigator
Researcher Identifier (e.g. ORCID ID): 0000-0002-1000-369X
Nearest person month worked: “no change”
Contribution to Project:
Prof. Pinkas-Kramarski co-supervised all aspects of the project and directly supervised Galit Elad Sfadia and Itzhak Ben Moshe.

Name: **Galit Elad Sfadia**
Project Role: Research Associate
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: “no change”
Contribution to Project:
Galit has participated in the experiments of the AIA and CIA models and contributed to the analysis of relevant data.

Name: **Itzhak Ben Moshe**
Project Role: Technical Assistant
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: “no change”
Contribution to Project:
Itzhak has participated in all the in experiments in animal models and helped collect the data.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

“Nothing to Report.”

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

“Nothing to Report.”

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

- 9. APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Title

Farnesylthiosalicylic acid modulates MAPK-dependent induction of proinflammatory programs in T cells and suppresses collagen induced arthritis

Morad Zayoud^{1,2,3}, Einav Vax^{1,3}, Galit Elad-Sfadia⁴, Iris Barshak^{3,5}, Ronit Pinkas-Kramarski⁴ and Itamar Goldstein^{1,2,3}

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³Sackler School of Medicine, Tel-Aviv University, Tel-Aviv 6997801, Israel

⁴Department of Neurobiology, The George S. Wise Faculty of Life Sciences & Sagol School of Neuroscience, Tel-Aviv University, Tel-Aviv

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Keywords

Ras GTPases; Rheumatoid Arthritis (RA); Farnesylthiosalicylic acid (FTS); Collagen induced Arthritis (CIA); T-helper cells (TH)

Correspondence to:

Itamar Goldstein, email: Itamar.Goldstein@sheba.health.gov.il

Abstract

The Ras family of GTPases plays an important role in signaling nodes downstream to T cell receptor and CD28 activation, potentially lowering the threshold for TCR activation by autoantigens. Somatic mutation in NRAS or KRAS may cause a rare autoimmune disorder coupled with abnormal expansion of lymphocytes. T cells from Rheumatoid Arthritis (RA) patients show excessive activation of Ras/MEK/ERK pathway. The small molecule Farnesylthiosalicylic acid (FTS) interferes with the interaction between RasGTPases and their prenyl-binding chaperones to inhibit proper plasma membrane localization. To investigate the immunomodulatory effect of FTS in the DBA/1 mouse collagen type-II induced arthritis (CIA) model. Arthritis was induced in 8-10 week old male DBA/1 mice by immunization with collagen type-II (CII) and complete Freund's adjuvant (CFA). Animals were treated semi-prophylactically with once daily oral FTS (100 mg/kg); weekly i.p injection of MTX (0.5 mg/kg), or daily oral vehicle solution (control). Arthritis severity was scored daily from disease onset until study termination and multiple immunological biomarkers of inflammation were analyzed. Our data from the mouse CIA model show that the therapeutic efficacy of FTS was similar to MTX, and both drugs significantly reduced arthritis severity and joint pathology compared to CMC controls. Importantly, FTS significantly inhibited the upregulation of serum IL-6 and IL-17A compared to control arthritic mice. Moreover FTS therapy induced a significantly lower induction of pathogenic Th17 cells in the spleens of FTS treated mice compared to CMC vehicle treated arthritic mice. The in depth analysis of the effect of FTS on the T cell cytokine response to CII, revealed strong suppression of CII antigen specific response in vitro as well as of the production of IL-22, IL-17, IL-9, GM-CSF and TNF. In conclusion: our original findings strongly imply that oral selective RAS inhibitors are potent inhibitors of the TH17-driven autoimmune response in animal models of RA, signifying a strong translational horizon for these compounds.

Introduction

Rheumatoid Arthritis (RA) is a prototypical systemic inflammatory autoimmune disorder that principally leads to erosive synovitis with destruction of cartilage and joints.[1]. The pathogenesis of RA has not full been fully elucidated. Immune mechanism that include autoreactive Th17- and Th1-type effector T cells, autoreactive B cells that produce Anti-citrullinated protein antibodies (ACPA), and dysregulation of various mitogen-activated protein kinase pathways have been postulated to contribute to the development of RA in human as well as in pre-clinical experimental animals models of arthritis [2-8]

Ras-GTPases are evolutionary conserved molecules vital for regulation of key cellular processes, such as proliferation, differentiation, and motility. In T cell signaling via the T cell antigen receptor (TCR) and the major co-stimulatory receptor CD28 is tightly associated with the activation of the Ras-dependent signaling cascade. Moreover, there is evidence that the threshold for T cell activation following antigen recognition is partly set by the level of Ras-dependent ERK1/2 phosphorylation [9]. Dysregulation of the Ras/Raf/MEK/ERK1/2 cascade has been furthermore associated with T cell anergy, and accordingly increased expression of activate oncogenig Ras was shown to reverse anergy and to restore IL-2 production [10-12]. Moreover, T cells from patients with RA have been found to express significantly higher levels of K-Ras and its downstream effector B-Raf that mediate the increased levels of phospho-ERK1/2 observed in patients' T cells. [13, 14], Importantly, K-Ras is critical for T-cell activation and engineering its overexpression in CD4+ T cells of healthy donors can increase TCR-sensitivity to low-affinity antigens, including many clinically relevant autoantigens (e.g., citrullinated vimentin peptides) [13, 14].

Importantly, patients presenting with a rare form of autoimmune lymphoproliferative disorder have somatic, gain-of-function mutations in KRAS or NRAS, restricted mainly to their immune cells. These RAS-associated Autoimmune Leukoproliferative disorder (RALD) patients present with peripheral lymphadenopathy, significant splenomegaly, and autoimmunity. The production of a variety of autoantibodies is usually detected, including Anti-nuclear, rheumatoid factor, and anti-cardiolipin [15]. Moreover, T cells of RALD patients, are resistant to to IL-2 withdrawal, as persistent

RAS/ERK signaling decreases the intracellular levels of negative inhibitors of cell cycle progression, permitting proliferation even at limiting IL-2 levels [15] .

Activating mutations in the proto-oncogenes *KRAS* and *NRAS* are common driver mutations in human cancers [16]. The posttranslational modifications of the addition of the farnesyl isoprenoid molecule to the conserved Cysteine 186 is vital for the proper function of Ras [17]. More recent studies demonstrated that specific protein chaperones with a prenyl-binding hydrophobic pocket are vital for effective Ras-GTP signaling from the plasma membrane and for proper subcellular compartmentalization of Ras proteins . In agreement with this concept, Kloog and colleagues discovered a potent non-toxic inhibitor of active (GTP-bound) Ras proteins, the small molecule Farnesylthiosalicylic acid (FTS) that competes for Ras chaperon interactions, by effectively dislodging Ras proteins from the PM and decreasing downstream signaling [12, 18]

Our current understanding of the molecular mechanisms of Ras signaling in T and B cells strongly suggests that pharmacological modulation of the Ras pathway might be an effective therapeutic approach for autoimmune disorders. Over the past decade the effects of FTS and related analogues have been extensively studied in multiple pre-clinical animal models of autoimmunity [19-22]. Importantly, we have very recently demonstrated that FTS displayed significant immunomodulatory effects in the rat adjuvant induced arthritis model (AIA) coupled with inhibition of the Th17-type effector response to a dominant arthritogenic antigen [23].

Collagen induced arthritis (CIA) is a widely studied animal model of RA, sharing several clinical and immunological features with RA, such as synovial hyperplasia, mononuclear cell infiltration, cartilage degradation and bony erosions, and like RA, the disease is dependent on MHC class II genes and T cells [24]. Central to the pathogenesis of this model is the development of pathogenic autoantibodies. This model has been widely employed to identify potential pathogenic mechanisms of autoimmunity relevant to RA and test potential therapies.

Here we tested the therapeutic and immunomodulatory effects of the small molecule FTS, a first-in-class oral selective inhibitor of RAS signaling in the CIA model.

Multiple outcome and molecular parameters were evaluated, such as: clinical scoring of arthritis severity, histopathological scoring, proinflammatory serum cytokine levels and the ability to develop a robust CII-specific Th17-Type recall response.

Results

Treatment with FTS reduces the severity of arthritis in CIA

CIA was generated in 8-week-old DBA/1 male mice as previously described [25]. Clinically evident arthritis of the ankle joints usually developed around 10 days after the second CII booster immunization [25], and progressed into severe polyarthritis within a few days. We employed a "semi-prophylactic" dosing scheme and started dosing three days before booster immunization (day +18) until study end (~ day +45). The observed disease incidence in the control group was usually > 90%. In our initial experiments, we also assessed the "effect size" of FTS therapy compared to the classic DMARD, MTX. The various mice groups were treated starting day post induction (DPI) 18, as follows: (i) oral FTS at 100mg/kg daily; (ii) oral F-FTS (iii) weekly i.p injection of MTX at 1 mg/kg, and (iiii) oral 0.5% carboxy methyl cellulose (CMC) vehicle solution. CIA severity was scored using a clinical index of 0 to 16 (0-4 scale for each paw), starting at arthritis onset and subsequently every other day until study end. We found that FTS monotherapy was very efficacious, significantly reducing the arthritis scores as compared to CMC control therapy (Figure 1A). Importantly, FTS and MTX showed a comparable effect size, both significantly reducing arthritis severity and joint swelling (Figure 1A and B). The area under curve (AUC) mean of the clinical scores of mice in the two active drug treatment groups were significantly reduced (by ~80%) compared to the control treatment arm (Figure 1B; $P < 0.001$ for both FTS and MTX vs. CMC by Student's t-test).

At study termination, day post induction (DPI) 45, mice were scarified and Histopathological analysis of the paws was performed by H&E and Safranin O staining (Figure 2A).

Our pathology scoring results show that immunization against CII in control mice induced extensive joint tissue inflammation, pannus formation, bone resorption and cartilage damage (all with an average scores of ~ 4 out 5), whereas the therapy with FTS significantly reduced joint pathology scores for all these four parameters (figure 2B).

Next, we tested whether FTS can reduce disease severity after arthritis onset. Thus, dosing in a therapeutic scheme was started when >60% of mice developed symptoms of arthritis, usually by DPI 31. As shown in Figure 3, therapeutic dosing with FTS produced a significant reduction in disease progression, such as that the mean AUC for the severity scores of mice treated with FTS was significantly reduced compared

to the control treatment arm (Figure 1B; $P < 0.001$ for both FTS and MTX vs. CMC by Student's t-test).

FTS inhibits the production of pro-inflammatory cytokines in CIA

The cytokines IL-6, IL-17 and IL-22 have been shown to mediate the systemic inflammation and arthritis in rodent models and in RA patients [26-28]. Importantly, our published results in the AIA model indicate a substantial inhibition of the IL-6-driven TH17 response to be a major mechanism of action for FTS therapy in this model [23]. Thus, we probed the effect of FTS on the induction of these arthritis-relevant cytokines in the CIA model of RA. Sera from FTS and CMC treated mice were collected at the onset of arthritis (~ DPI 32) and analyzed for IL-17A and IL-6 levels by ready-made ELISA kits. We found that at the early stages of arthritis development FTS therapy significantly reduced the upregulation of IL-6 compared to control mice (~60% inhibition, $p < 0.001$ by Student's t-test) (Figure 4A). Importantly, while significant induction of IL-17A (mean > 60 pg/ml) was observed in control mice, FTS therapy completely suppressed IL-17 induction to below the limit of detection (Figure 4B). Furthermore FTS inhibited the upregulation of the pro-inflammatory IL-22 in the sera of treated mice (Figure 4C).

Analyzing the effect of FTS therapy on major splenic lymphocytic populations and relevant Th cell subsets

Next, we analyzed the effects of FTS treatment on the "major" lymphocytic cell populations. Thus, at study end we harvested spleens and prepared single cell suspensions for downstream polychromatic FACS analysis. We found that neither CII immunization by itself nor 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 to naïve mice and CMC treated mice (Figure 5A and B).

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[29]. Th17 cells also secrete IL-22, a cytokine generally considered as pro-inflammatory in the context of RA and its levels are elevated in the blood and synovia of RA patients[30]. 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), single-cell suspensions of splenocytes and inguinal lymph nodes (LN). By multicolor

Staining for relevant intracellular cytokines and cell surface antigens, 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 to relevant control mice (Figure 6A and B). 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 pathogenic TH1/TH17-type recall response to CII

To study the effect of FTS treatment on the CII-specific Th cell response of 8-12-week-old DBA/1 male mice immunized with CII, we treated the mice from DPI 3 with oral FTS or CMC-vehicle. 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 culture for additional 96 hrs. Our results show that the proliferation of antigen-specific CD4+ T cells, as determined by the CFSE-dilution assay, was significantly reduced in cultures from FTS vs. CMC treated mice (Figure 8A). Equally, we found that FTS therapy was associated with a significant inhibition of IL-6, IL-22 and IL-17A secretion, as compared to relevant control cultures (Figure 8B).

To further address the full impact of FTS therapy on the cytokine response to CII we analyzed the recall response of splenocytes and LN cells at disease onset (DPI 32). Thus, the various cell samples were immediately stimulated with heat denatured bovine CII, and cultured for additional 96 hours. At the end of culture, the supernatants were collected and the levels of a large number of relevant cytokines (17 analytes) was determined by the ProcartaPlex Mouse Th1/Th2/Th9/Th17/Th22/Treg Cytokine Panel. We found that the re-stimulation of CII-specific effector T cells from immunized control arthritic mice (CMC group) induced a strong upregulation of multiple pro-inflammatory cytokines (IL-6, TNF, GM-CSF, IFN- γ , IL-9, IL-17A, and IL-22) (figure 8C). Importantly, this induction was significantly inhibited in the corresponding cell cultures from FTS treated mice.

Discussion

In this manuscripts we demonstrated that FTS therapy, a first-in-class oral selective RAS inhibitor, provides a significant immunomodulatory effect in the CIA model by multiple clinical and laboratory outcome parameters. The therapeutic effect of FTS treatment in the CIA model was coupled with attenuation of the IL-6, IL-17 and IL-22 cytokine response (Th17-type) to C-II immunization.

Adjuvant induced arthritis was the first described experimental model of autoimmune arthritis. While it shares several pathological features with RA such as symmetric joint involvement, immune cell infiltrates, cartilage degradation, synovial hyperplasia, 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 auto-antibodies producing B cells are less relevant to its pathogenesis [31, 32]. To further bridge the gap between the AIA model and RA and to increase the capacity of our pre-clinical investigations to predict the actual translational potential of oral Ras inhibitors, we decided to investigate the therapeutic efficacy of FTS in the collagen-induced arthritis (CIA) mouse model. CIA is the most widely studied animal model of RA, as it shares several pathological similarities with RA such as symmetric joint involvement, prominent synovitis destruction of cartilage and bone erosions, immune cell infiltrates, pannus formation, genetically regulated by MHC and non- MHC genes and the pathogenic role of both T cells and auto-antibodies producing B cells. This model has been widely studied to identify pathogenic mechanisms relevant to RA and to evaluate new therapeutic modalities[31, 32]

We demonstrated that FTS therapy, a first-in-class oral selective RAS inhibitor, provides a significant immunomodulatory effect in the CIA model by multiple clinical and laboratory outcome parameters. The therapeutic effect of FTS treatment in the CIA model was coupled with attenuation of the IL-6, IL-17 and IL-22 cytokine response (Th17-type) induced by C-II immunization. Our data from initial experiments showed that F-FTS therapy was non-inferior to FTS and MTX monotherapies. Although, FTS displayed a trend towards being more potent than F-FTS in its clinical efficacy, the effect did not reach the statistical threshold of $P < 0.05$. Given these data and our ethical duty to balance the needs of the study with that of the

animals' welfare, in compliance with the ethical principle of reduction, we decided to focus on FTS as the lead compound to be investigated in the CIA model.

Proinflammatory cytokines such as IL-6, IL-17A and IL-22 have been postulated to play a main role in the pathophysiology of RA[28, 33, 34]. Indeed, we found that FTS is a strong inhibitor of the upregulation of IL-6 and IL-17A in the sera of CIA mice that is usually induced by C-II in the CIA model, consistent with our previous findings in the AIA model. This observation strongly indicates that the suppression of the IL-6-dependent TH17 response is an important general mechanism of action for our novel small molecule Ras-inhibitors. Moreover, previously it was reported that IL-22 is elevated in the serum of patients with established RA, and that the serum level of IL-22 correlate with disease activity measures such as Clinical Disease Activity Index (CDAI) and Disease Activity Score in 28 joints (DAS28)[30]. Our novel observation that FTS inhibited the upregulation of IL-22 in the sera of the CIA mice and the IL-22 recall response to C-II re-challenge in vitro further verifies that FTS is a strong candidate immunomodulatory drug for the treatment of RA.

IL-17 is produced by a distinct subpopulation of CD4⁺ helper T cells that has been designated TH17[35]. Abundant evidence indicates that T cells are required for initiation and/or chronicity, both in RA and in pre-clinical animal models of RA. IL-17 neutralization with antibodies or the genetic ablation of IL-17 or IL-17R is associated with the attenuation of arthritis in several experimental murine models of arthritis[36]. TH17 cells also secrete IL-22, a cytokine generally considered pro-inflammatory because of its co-expression with IL-17 by TH17 cells. In the context of RA it has been shown that elevated numbers of TH17 (IL-17a and IL-22 producing) as well as TH22 (IL-22 producing) in peripheral blood of RA patients positively correlate with a more aggressive disease course[37]. In agreement with these human data, we also observed upregulation of CD4⁺ TH17 (IL-17⁺ and IL-17⁺/IL22⁺) and Th22 (IL-22⁺) cells in the spleens of untreated mice post C-II immunization, and more importantly FTS treatment significantly downregulated the frequency of TH17 and TH22 cells in relevant lymphoid tissues. Thus, our present cytokine data from the CIA mouse model, and the comparable previous data from the AIA rat model, are consistent with a pathogenic role for the TH17 related cytokines IL-17A/F and IL-22 in the pathogenesis of murine arthritis. Specifically, these data strongly imply that the antigen-specific T cell response in these two murine models of arthritis is associated

with an antigen specific TH17 response, and especially that the therapeutic effect of the oral Ras-inhibitors is very likely due to their ability to attenuate the polarization of the antigen-specific T cell response into a predominantly Th17/Th22 pathogenic response. Additionally, by our cytokine array data FTS treatment can also down modulate the induction of other arthritis associated cytokines such as IFN- γ , TNF- α and GM-CSF.

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Disclosure of conflicts of interest.

The authors have no conflict of interest.

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Figure 1: Prophylactic treatment with FTS, , F-FTS and MTX reduces the severity of collagen-induced arthritis (CIA). 8-12 week-old DBA/1 male mice were given an initial injection of CFA/collagen type-2 (CII) on day 0, and arthritis was induced with a second booster injection of IFA/CII on day +21. Mice in the experimental arms (n=8 per group) were treated starting day +18 with daily FTS (100mg/kg), daily F-FTS (60mg/kg), weekly i.p injection of MTX (1 mg/kg), or 0.5% CMC vehicle solution (control treatment). **(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 AUC \pm S.D. for the clinical CIA disease scores from days post immunization (DPI) +30 to +45, and a representative study of three independent experiments done is depicted (**n=8 per treatment group**). Inset shows the results of the statistical analysis of the differences in area under the curve (AUC) values for the different treatment arms. Statistical analysis was performed by One way ANOVA with Bonferroni's post hoc multiple comparison test

Figure 2: Histopathological assessment of ankles of arthritic mice at study termination. CIA was induced in 8-12-week-old DBA/1 male mice as detailed above. Mice in the experimental arms (n=8 per group) were treated semi-prophylactically, starting from day +18, with either oral FTS (100 mg/kg) or control CMC vehicle solution (0.5%). **(A)** Shown are representative images out of 3 experiments performed. Scale bars = 20 μ m. **(B)** Bar graphs depict the mean (\pm S.D) 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 vs. CMC therapy was calculated by Student's t-test (*P \leq 0.05 **P \leq 0.01).

Figure 3: therapeutic treatment with FTS reduces the severity of collagen-induced arthritis (CIA). 8-12 week-old DBA/1 male mice were given an initial

injection of CII) on day 0, and arthritis was induced with a second booster injection of IFA/CII on day +21. Mice in the experimental arms (n=6 per group) were treated starting day +31 with daily FTS (100mg/kg) or 0.5% CMC vehicle solution (control treatment). **(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 AUC \pm S.D. for the clinical CIA disease scores from days post immunization (DPI) +30 to +45, and a representative study of two independent experiments done is depicted (**n=6 per treatment group**). Inset shows the results of the statistical analysis of the differences in area under the curve (AUC) values for the different treatment arms. Statistical analysis was performed by the *Student's t* test (**P<0.001)

Figure 4: FTS therapy reduces the levels of proinflammatory cytokines in CIA. Sera from mice treated with FTS or CMC were collected at day 32 post immunization, and analyzed by commercial ELISA kits for the level of: (A) IL-6, (B) IL-17 and (C) IL-22. Bars represent mean \pm S.E.M of triplicates. Statistical analysis was performed by the Student's t test (**P<0.001)(N.D: not detectable)

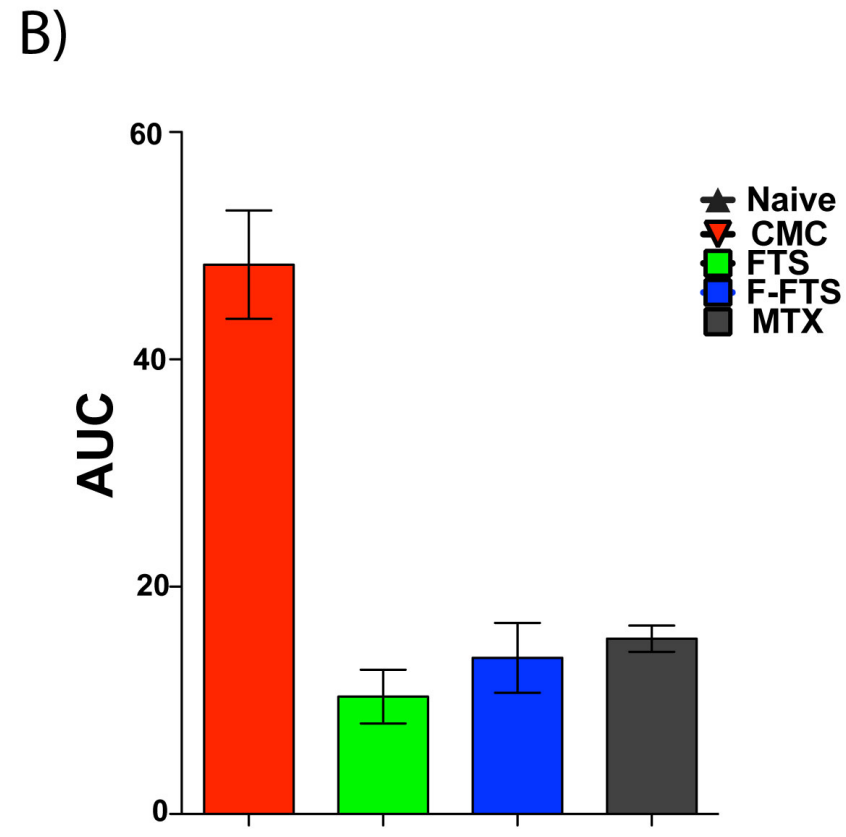
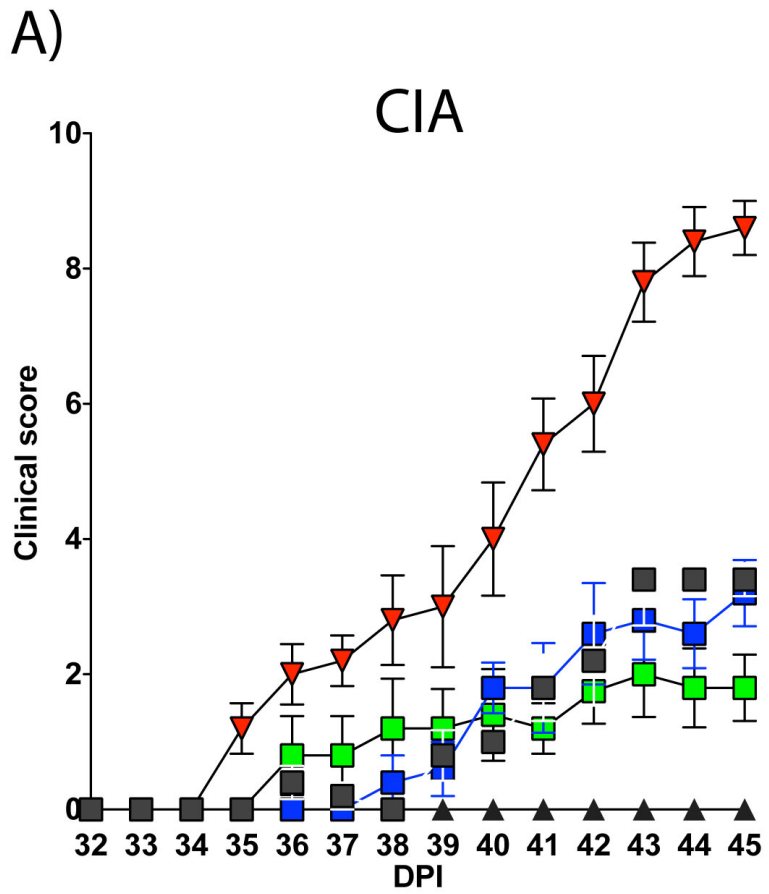
Figure 5. Effects of FTS therapy on major splenic lymphocytic populations. At study end (day +45), single cell suspension of spleens and LNs from the various groups of treated mice were analyzed by flow cytometry for CD3+ CD4+, and CD8+ T cell frequencies. **(A and B)** Representative FACS bivariate pseudo color dot plots of T cells (CD3+) and B cells (CD45R+) events, and bar graphs with pooled data from all mice studied (n = 6 per group) - respectively. Bars represent the mean percentage of the indicated cell population \pm S.D. **(C and D)** Representative FACS bivariate pseudo color dot plots of CD8+ cytotoxic T cells and CD4+ T cells, and bar graphs with pooled data from all mice studied - respectively. Shown is a representative study out of 3 performed.

Figure 6. Analysis of the immunomodulatory effect of FTS therapy on IL-17A/IL-22 producing TH17 cells in CIA. At study end (day +45), single cell suspension of spleens and LNs from the various groups of treated mice were prepared, activated with Cell Stimulation Cocktail reagent, and then analyzed by flow cytometry for intracellular cytokine production. **(A)** Representative flow cytometry plots of intracellular staining of CD4+ T cells for IL-17A and IL- 22 cytokines

production. **(B)** Bars represent the mean percentage (\pm S.D) of the indicated TH17-subsets in spleens of the various mice groups (n=6 per group). ***P \leq 0.001, by the Student's t test.

Figure 7. FTS therapy mediates significant inhibition of the T cell recall response to CII. 8- 12-week-old DBA/1 male mice were immunized with CFA/type II collagen on day 0 and treated from Day +1 of the study with oral FTS or CMC vehicle for 10 days. Single-cell suspensions of splenocytes were prepared by mechanical disaggregation. Splenocytes were labeled with CFSE and stimulated *in vitro* with heat denatured bovine CII or control medium, and then cultured for additional 96 hours. **(A)** Cells were harvested and stained with anti-mouse CD3 and CD4 mAbs. And cells were analyzed for CII-specific proliferation of CD3+CD4+ T cells by flow cytometry (green for medium and red for CII). **(B)** In parallel, supernatants were collected and the levels of IL-6, IL-22 and IL-17A were determined by commercial ELISA kits. **(C)** Splenocytes isolated from mice treated with FTS or CMC (up day 18) were isolated at day 32 DPI and cultured heat denatured bovine CII or control medium for additional 96 hours, supernatants were collected and we determined the levels of a large number of important cytokines by the ProcartaPlex Mouse Th1/Th2/Th9/Th17/Th22/Treg Cytokine Panel. Bars represent mean \pm S.D. of triplicates from a representative experiment, and red bars represent CII stimulated cultures while green bars represent medium control cultures. Data were analyzed for statistical significance t-test for FTS treatment compared to CMC control treatment Values of P<0.001, P<0.01, and P<0.05 were marked by three, two and one asterisks, respectively. (N.D: not detectable)

Figure 1



One way ANOVA + Bonferroni's post hoc multiple comparison test

	CMC	FTS	F-FTS	MTX
CMC		P < 0.001	P < 0.001	P < 0.001
FTS			NS	NS
F-FTS				NS

Figure 2

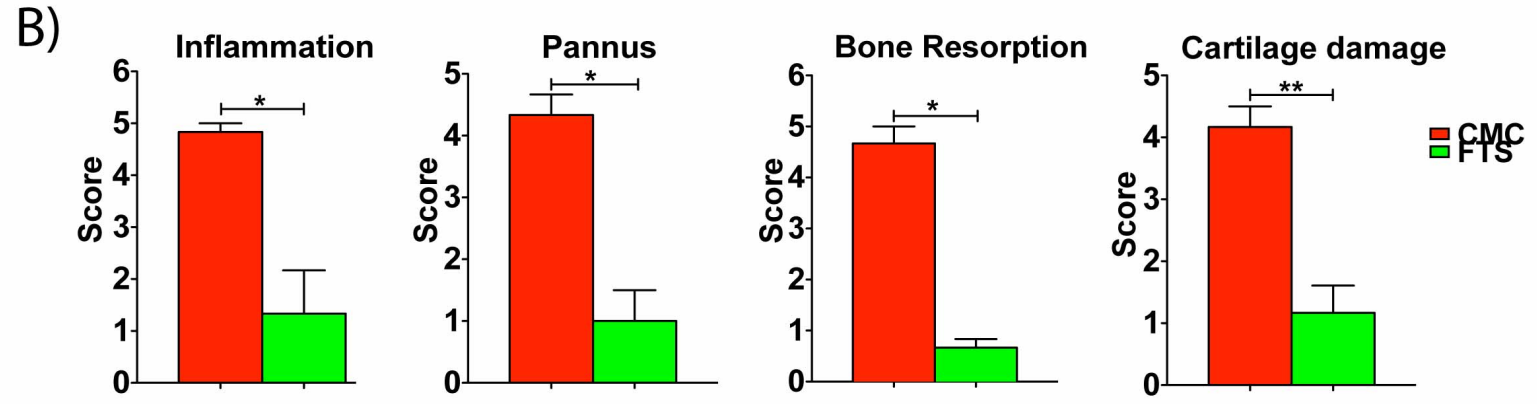
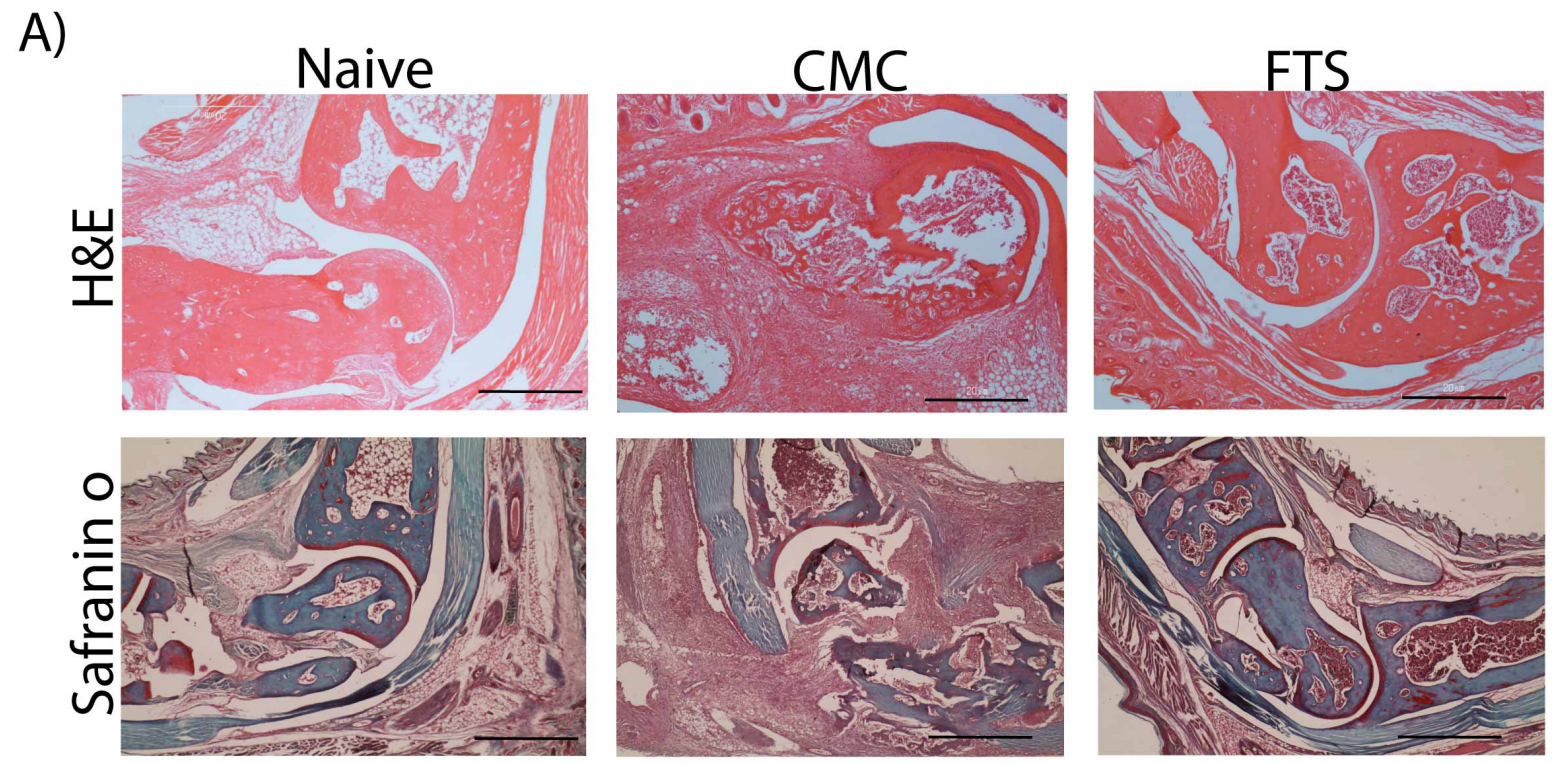
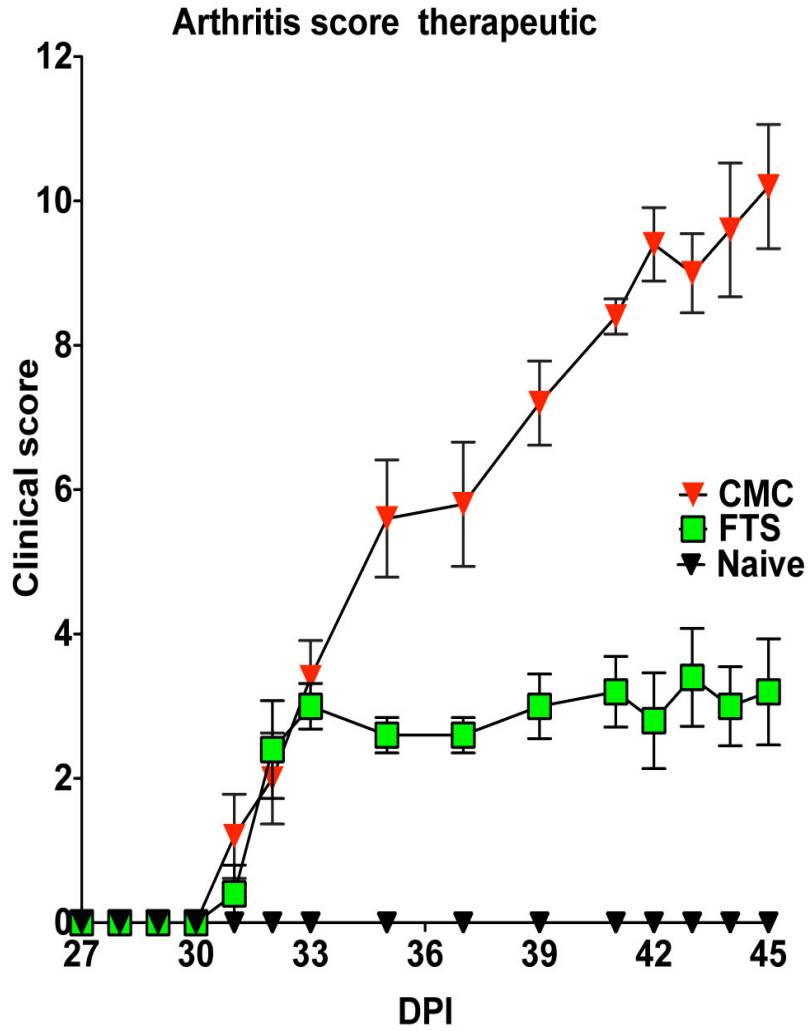


Figure 3

A)



B)

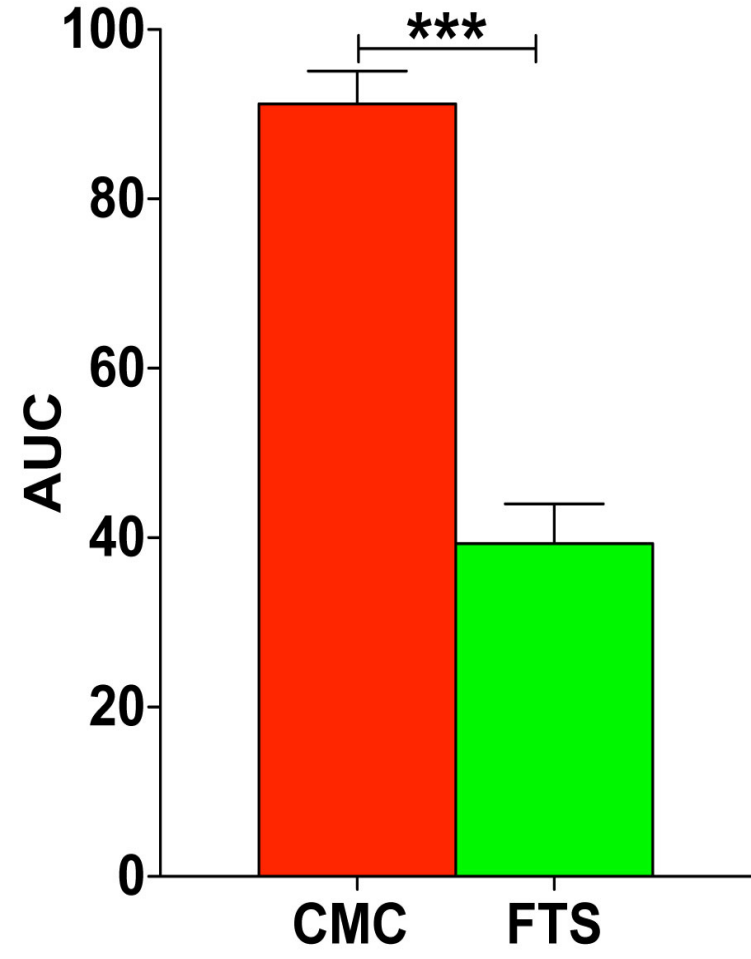


Figure 4

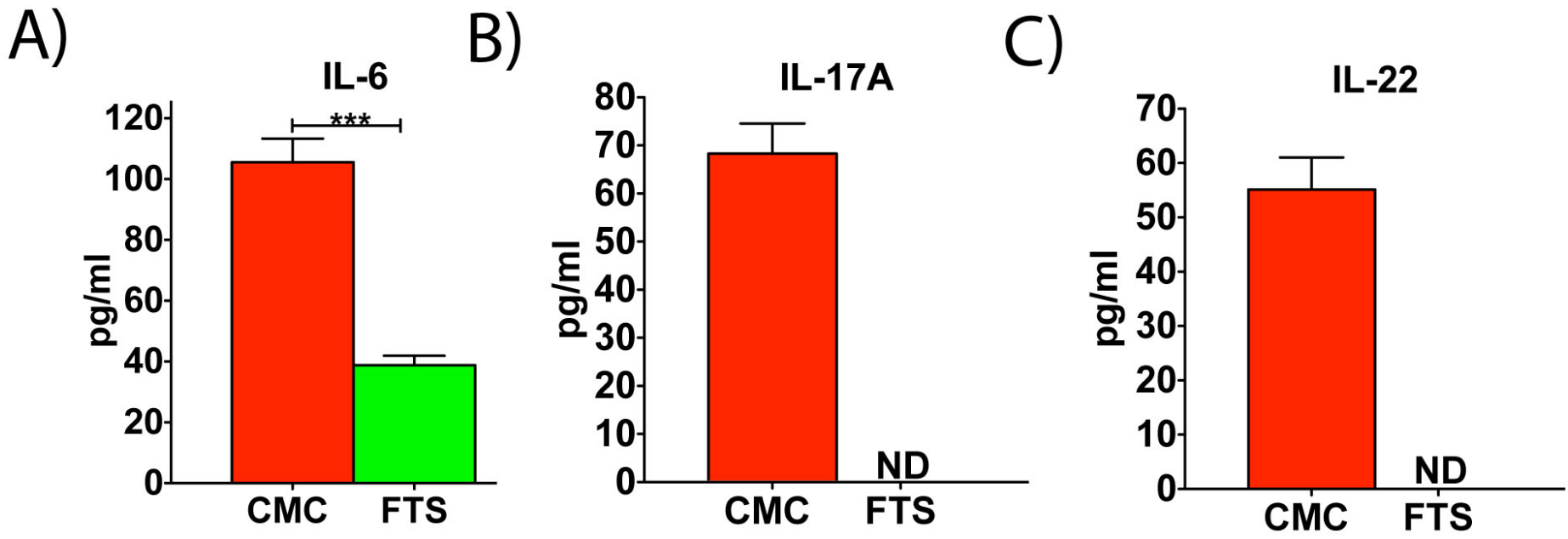


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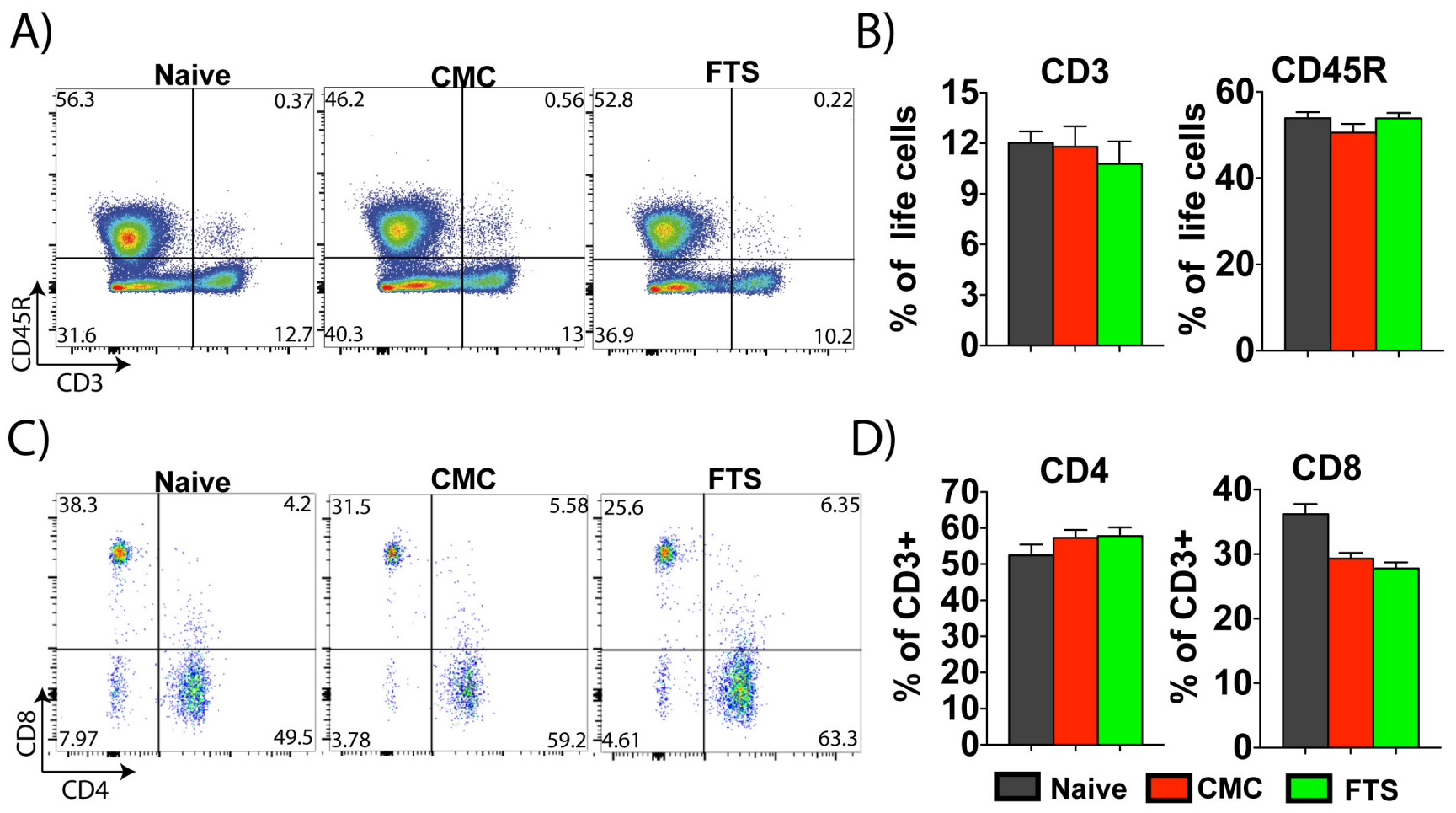


Figure 6

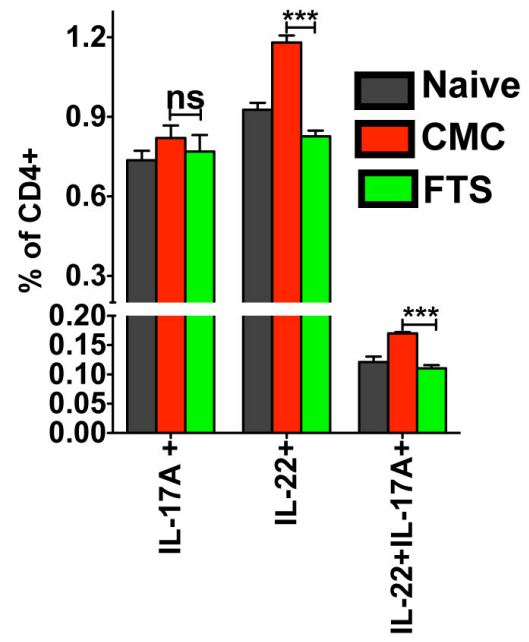
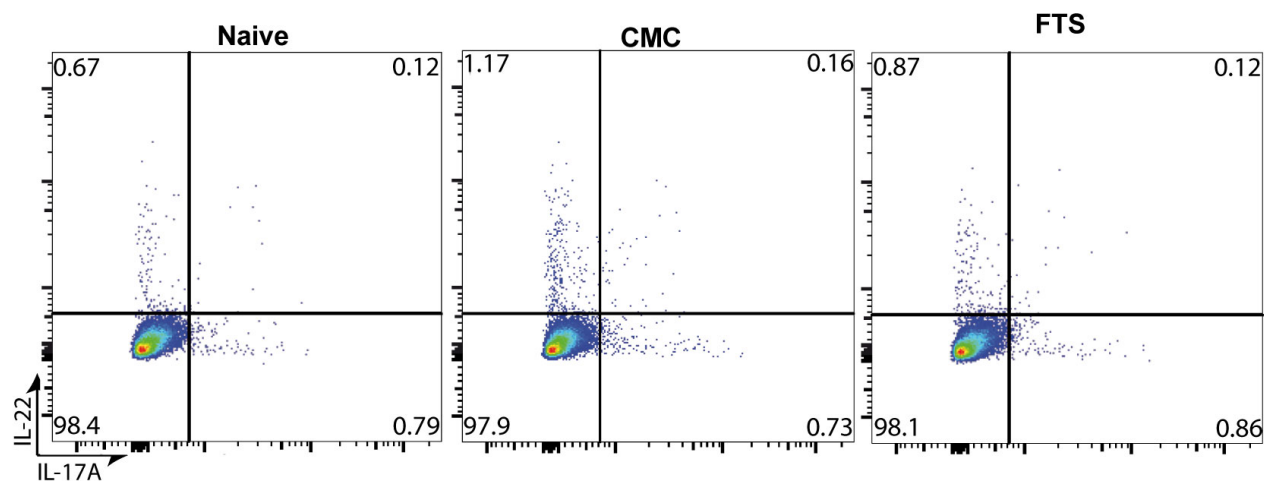


Figure 7

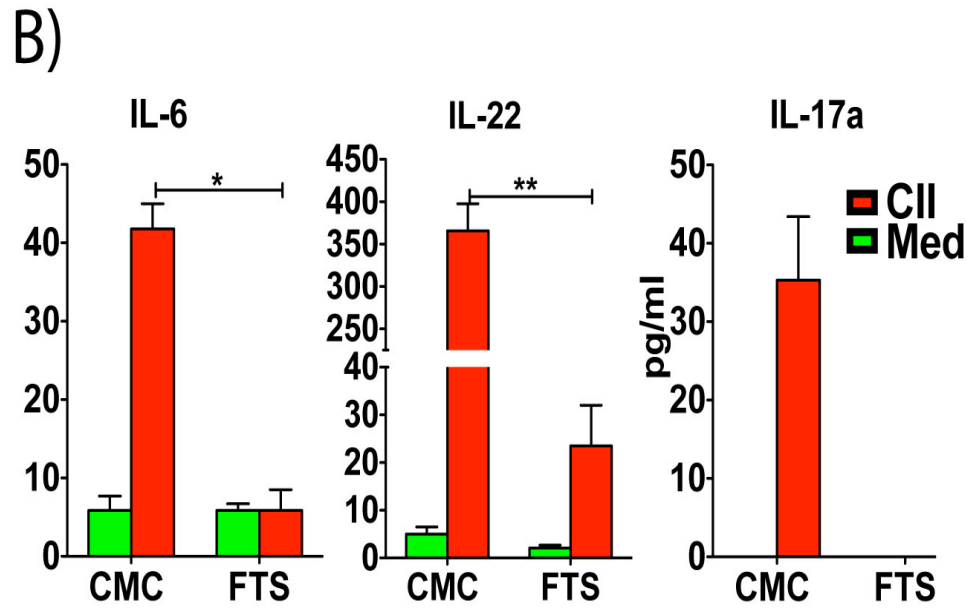
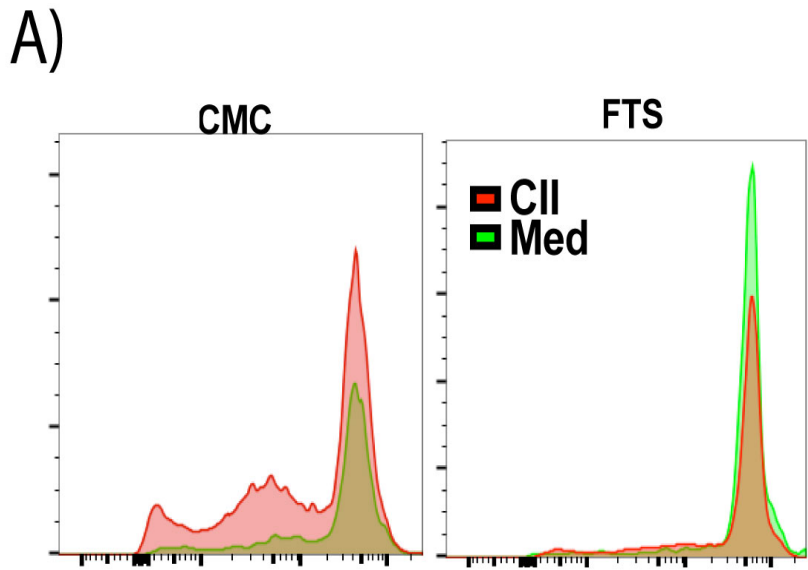
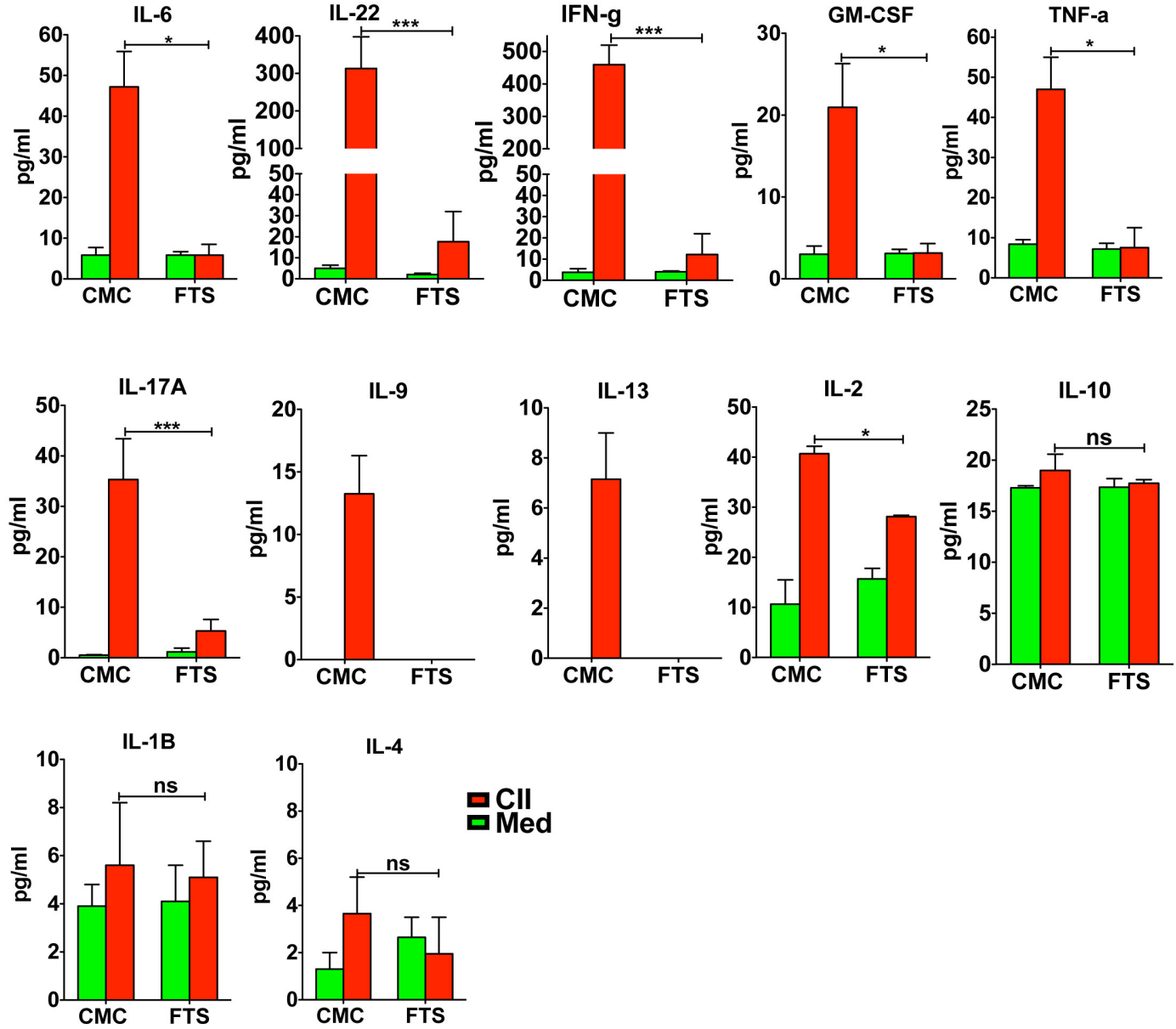


Figure 7

C)





Ras Signaling Inhibitors Attenuate Disease in Adjuvant-Induced Arthritis *via* Targeting Pathogenic Antigen-Specific Th17-Type Cells

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The Ras family of GTPases plays an important role in signaling nodes downstream to T cell receptor and CD28 activation, potentially lowering the threshold for T-cell receptor activation by autoantigens. Somatic mutation in *NRAS* or *KRAS* may cause a rare autoimmune disorder coupled with abnormal expansion of lymphocytes. T cells from rheumatoid arthritis (RA) patients show excessive activation of Ras/MEK/ERK pathway. The small molecule farnesylthiosalicylic acid (FTS) interferes with the interaction between Ras GTPases and their prenyl-binding chaperones to inhibit proper plasma membrane localization. In the present study, we tested the therapeutic and immunomodulatory effects of FTS and its derivative 5-fluoro-FTS (F-FTS) in the rat adjuvant-induced arthritis model (AIA). We show that AIA severity was significantly reduced by oral FTS and F-FTS treatment compared to vehicle control treatment. FTS was as effective as the mainstay anti-rheumatic drug methotrexate, and combining the two drugs significantly increased efficacy compared to each drug alone. We also discovered that FTS therapy inhibited both the CFA-driven *in vivo* induction of Th17 and IL-17/IFN- γ producing “double positive” as well as the upregulation of serum levels of the Th17-associated cytokines IL-17A and IL-22. By gene microarray analysis of effector CD4⁺ T cells from CFA-immunized rats, re-stimulated *in vitro* with the mycobacterium tuberculosis heat-shock protein 65 (Bhsp65), we determined that FTS abrogated the Bhsp65-induced transcription of a large list of genes (e.g., *Il17a/f*, *Il22*, *lfn*, *Csf2*, *Lta*, and *Il1a*). The functional enrichment bioinformatics analysis showed significant overlap with predefined gene sets related to inflammation, immune system processes and autoimmunity. In conclusion, FTS and F-FTS display broad immunomodulatory effects in AIA with inhibition of the Th17-type response to a dominant arthritogenic antigen. Hence, targeting Ras signal-transduction cascade is a potential novel therapeutic approach for RA.

Keywords: Ras GTPases, rheumatoid arthritis, farnesylthiosalicylic acid, adjuvant induced arthritis, T-helper cells

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic inflammatory autoimmune disorder that principally affects synovial joints (1). Th17 cells have been postulated to play a key role in the pathogenesis of several autoimmune diseases (2) and in animal models of human autoimmune diseases including autoimmune colitis (3), experimental autoimmune encephalomyelitis (4), collagen-induced arthritis (5), and rat adjuvant-induced arthritis (6).

Ras-GTPases are molecular switches that regulate key cellular processes, such as proliferation, differentiation, apoptosis, and motility. In T cells, Ras-family GTPases (e.g., K/N-Ras) are crucial for proper T-cell receptor (TCR)-dependent activation following antigen recognition. Defective activation of Ras/Raf/MEK/ERK1/2 cascade has been associated with T cell energy, and accordingly increased expression of active Ras was shown to reverse energy and to restore IL-2 production (7–9). Moreover, T cells from patients with RA have been found to express significantly higher levels of K-Ras and its downstream effector B-Raf that mediate the increased levels of phospho-ERK1/2 observed in RA patients' T cells (10, 11). Importantly, over expression of K-Ras in CD4⁺ T cells from healthy donors enabled the induction of autoreactive T cells that reacted citrullinated vimentin-derived peptides, postulated to be a pathogenic autoantigen in RA. Thus, hyperactivity of the Ras signal transduction cascade has been postulated to increase TCR-sensitivity to low-affinity antigens, including many clinically relevant autoantigens (10, 11). Interestingly, previous reports suggest that inner plasma membrane (PM)-anchored Ras proteins can transfer from antigen-presenting B cells to T cells both through the immunological synapse and through actin supported long-range PM extensions termed tunneling nanotubes. These findings further support the importance of Ras GTPases in immunity, by showing that active Ras-GTPase signals can spread between immune cells (12, 13) and even from cancer cells expressing oncogenic Ras to T cells (14).

Activating mutations in the proto-oncogenes *KRAS* and *NRAS* are frequent in human cancers (15, 16). This has led to ongoing efforts to develop drugs that target Ras signaling (16–20). To be active, Ras GTPases have to associate with membranes, and hence they require several posttranslational modifications in their carboxy-terminal domain, such as the addition of the hydrophobic farnesyl isoprenoid molecule to Cysteine 186 that is conserved in all Ras family members (16, 21–23). Based on an innovative concept, Kloog and colleagues (24, 25) discovered a potent non-toxic inhibitor of active (GTP-bound) Ras proteins, the small molecule farnesylthiosalicylic acid (FTS/Salirasib).

In recent years, it has been discovered that following posttranscriptional processing Ras proteins interact with prenyl-binding chaperones (26–29). These chaperones with prenyl-binding hydrophobic pockets are vital for proper PM localization and effective downstream Ras signaling (30). In agreement with this concept, it was found that FTS, by competing for Ras-chaperon interactions, effectively dislodges the oncogenic Ras proteins from the PM and inhibits Ras mediated oncogenesis (31–33).

The central role of Ras signaling in T cells strongly suggests that targeting Ras might be an effective therapeutic approach for

this disease. Over the past decade the effects of FTS and related analogs have been extensively studied in multiple pre-clinical animal models of autoimmune. For example, FTS can attenuate disease manifestations in experimental autoimmune encephalomyelitis (34, 35), Type 1 diabetes in NOD mice (36), experimental colitis (37), and other autoimmune diseases such as systemic lupus erythematosus (38). Preliminary studies by Aizman et al. (39) in the adjuvant-induced arthritis (AIA) model in rats suggest that prophylactic treatment with FTS may attenuate the clinical score of the disease; however, the biology behind the effect of FTS was not comprehensively elucidated. AIA is an experimental animal model of polyarthritis, which can be induced in inbred Lewis rats by immunization with Complete Freund's adjuvant containing *Mycobacterium tuberculosis* (Mtb). Importantly, mycobacterial heat-shock protein 65 (Hsp65) reactive T cells have been implicated in the pathogenesis of AIA. The AIA model and human RA have many overlapping characteristics, such as genetic susceptibility, T cell dependence, and pathogenic contribution of synovial CD4⁺ cells. Therefore, this model has been extensively employed for preclinical testing of numerous anti-arthritic agents, including biologics used for latest therapy in RA (40, 41). As previous studies imply that the main mechanism of action of FTS is down modulation of the T cell response (36), and the major role of T cells in AIA pathogenesis (42), we chose this pre-clinical model to assess the therapeutic potential of FTS in human RA.

Here, we provide a comprehensive insight into the molecular mechanisms that mediate the therapeutic action of small molecule Ras-inhibitors in AIA. Moreover, we determined that prophylactic treatment with FTS as an add-on to methotrexate (MTX) inhibits almost completely the development of AIA by all clinical and immunological/molecular outcome measures.

MATERIALS AND METHODS

Animals

Lewis rats obtained from Harlan Biotech (Rehovot, Israel). All rats were subjected to regular health status controls. Male rat, 8 weeks of age were used for experiments. All animal experiments were conducted in accordance with relevant laws of the state of Israel and guidelines of the Tel Aviv University and approved by the Institutional Animal Care and Use Committee (Approval # L-14-018).

Arthritis Induction and Drug Administration

To induce AIA, the rats have been injected intradermal (i.d.) at the base of the tail with 100 μ l CFA produced by suspending heat-killed *M. tuberculosis* (Difco) in mineral oil at 10 mg/ml. For drug administration, on day +1 after AIA induction, the rats were randomly divided into four groups. Rats in the first group were treated daily (starting on day 1 after AIA induction) with oral (intragastric) FTS (100 mg/kg). Rats in the second group (control) received oral vehicle [0.5% carboxy methyl cellulose (CMC)] daily. Rats in the third group were treated with i.p MTX (0.5 mg/kg) on days 3, 10, and 17 after AIA induction.

Rats in the fourth group (FTS + MTX) were treated daily (starting on day 1 after AIA induction) with oral (intragastric) FTS (100 mg/kg) as well with i.p MTX (0.5 mg/kg) on days 3, 10, and 17 after AIA induction. 20 days after the experiment has been terminated. As negative control, we used naïve healthy rats without any treatment. For determining the clinical scores, each paw is scored on a scale of 0–4 for the degree of swelling, erythema, and deformity of the joints with a maximum total score of 16 (43).

Lymphocyte Isolation, Intracellular Cytokine Staining, and Flow Cytometric Analysis

Single-cell suspensions of splenocytes and inguinal lymph nodes (LN) 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 as indicated with the following anti-rat CD monoclonal antibodies (mAbs): CD3-PE, CD4-FITC, CD8-PE-Cy7, and CD25-PE (all from eBioscience). For intracellular cytokine staining, freshly isolated lymphocytes were activated for 5 h *ex vivo* in RPMI medium with 1× Cell Stimulation Cocktail in the presence of a commercial Protein Transport Inhibitor Cocktail according to the manufacturer's protocol (eBioscience). Thereafter, cells were surface-stained with anti-CD4-FITC, washed, fixed, and permeabilized using BD Cytotfix/Cytoperm™ kit, per manufacturer's instructions (BD Biosciences). Intracellular staining was performed with anti-rat IL-17A-PE and IFN- γ -APC (both mAbs from eBioscience) and the BD Perm/Wash™ Buffer. For Treg cells staining, the cells were surface stained for CD4 and CD25, washed, fixed, and permeabilized using the Foxp3 Staining Buffer Set, and then immunostained with anti-mouse/rat Foxp3-APC mAbs (all reagents from eBioscience). Samples were acquired using a FACSAria flow cytometer (BD Bioscience) and further analyzed using the FlowJo v10 software (TreeStar, Inc.).

Measurement of Cytokines and CRP in Serum and Cell Culture Supernatant by ELISA

The serum levels of the inflammatory mediator CRP were determined by the rat specific CRP Elisa Kit (R&D Systems, Inc.). The levels of cytokines IL-17A and IL-22 from the collected rats sera and in cell culture supernatant were determined by rat specific IL-17A (homodimer) ELISA Ready-SET-Go kit (eBioscience) and by the rat IL-22 ELISA Kit (R&D Systems, Inc.), respectively.

Processing and Evaluation of Joint Histology

At study termination, the tibiotarsal joint was transected at the level of the medial and lateral malleolus for Histopathological Assessment. Ankle joints were then collected into 4% paraformaldehyde, for at least 24 h, and then placed in a decalcifier solution with 10% hydrochloric acid. 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. Tissue section slides of arthritic ankles were examined by an experienced pathologist, blinded to the animal treatment protocol, and scored for inflammation and bone resorption on a scale of 0–5, as previously described (44).

Antigen-Specific T Cell Activation and Cell Cultures

Freshly isolated splenocytes were plated in 24 well plates at 2×10^6 and re-challenged *in vitro* with Bhsp65 (5 μ g/ml) or control vehicle, and cultured for additional 72 h in complete RPMI medium supplemented with 10% FCS. At culture termination, we first collected the supernatants for relevant analyses and then harvested the cells and isolated the CD4⁺ T cell population using Rat CD4 MicroBeads and the MACS cell separation platform (Miltenyi Biotec, Germany), according to the manufacturer's instructions.

GeneChip Microarray Analysis

The purified CD4⁺ T cell was lysed with the TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was purified using the Direct-zol™ RNA Kit (Zymo Research Corporation, Irvine, CA, USA) for subsequent downstream analysis. Gene expression was determined using the GeneChip® Rat Gene 2.0 ST Array System (Affymetrix, Inc.), according to the manufacturer's instructions. The 2100 Bioanalyzer (Agilent Technologies) was used to determine RNA quality, and biotinylated target DNA was prepared from each suitable RNA sample according to the manufacturer's instructions. Gene level RMA sketch algorithm was used for crude data generation (Affymetrix Expression Console and Partek Genomics Suite 6.2). Genes were analyzed using unsupervised hierarchical cluster analysis (Spotfire DecisionSite for Functional Genomics; Somerville, MA, USA) to get a first assessment of the data, and filtered according to fold change calculations. For further functional bioinformatics analysis to discover molecular processes and biological pathways enriched in the experimental dataset, we used the Gene Set Enrichment Analysis (GSEA) software available online (<http://software.broadinstitute.org/gsea/msigdb/annotate.jsp>). The primary microarrays data from this research have been deposited in the NCBI Gene Expression Omnibus data repository under accession number GSE100280.

Reverse Transcription and Real-time qPCR

Total RNA was extracted from CD4⁺ cells using TRIzol® Reagent (Invitrogen, Thermo Fisher Scientific, Inc.), and total RNA was purified using Direct-zol™ RNA Kits (Zymo Research Corporation), followed by treatment with 1 U of RNase-free DNase (Roche). Reverse transcription reactions were performed on 1 μ g total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The quantitative PCR (qPCR) analyses for the relative mRNA expression of Ccl20, Il22, Il17A, Il17E, and Csf2 (normalized to Actb) were performed with premade QuantiTect bioinformatically validated

primer sets (from Qiagen) and the SYBR Green Real-Time PCR Kit (Applied Biosystems) on an ABI Prism 7900 SDS Instrument (Applied Biosystems), as recommended by the manufacturers, in reactions containing 50–100 ng cDNA. All reactions were done in triplicates and relative mRNA quantities (RQ) were determined using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Statistically significant differences between group means were determined either by the one-way ANOVA test with Bonferroni's *post hoc* multiple comparison test or the Student's *t*-test, as appropriate, using the Prism win V.5.02 software (GraphPad Software, Inc.).

RESULTS

FTS Treatment Attenuates Disease Severity in AIA

To determine whether treatment with FTS suppresses the clinical signs of AIA, we started daily treatment of rats from day +1 after immunization in the experimental arm with oral FTS (100 mg/kg). Control rats received oral solution of 0.5% CMC (vehicle). As a "positive control," we treated a group of Lewis rats with weekly *i.p.* injection of the anti-rheumatic drug MTX (0.5 mg/kg). AIA progression and severity was scored on a clinical index of 0–16 (0–4 scale for each paw). In parallel, we also assessed arthritis severity and progression by successive caliper measurements of ankle joint diameter. We found that in the FTS treatment arm the arthritis was significantly attenuated ($P < 0.001$) as compared to CMC vehicle treated rats by both clinical scoring and ankle joint diameter measurements (Figures 1A,B). The clinical effects of FTS, with respect to arthritis severity and joint swelling, were of similar magnitude to the effects of MTX. Moreover, we also discovered that treatment with FTS as an add-on to MTX provided a very strong protective effect such as that the combined treatment almost completely inhibited the development of clinically evident arthritis (Figure 1A) and ankle joint swelling (Figure 1B). To further evaluate the effects of FTS on arthritis development, we examined ankle joint sections stained with hematoxylin and eosin from the various treatment arms (Figure 1C). Ankles of rats treated with adjuvant were given scores of 0–5 for bone resorption and inflammation, as previously described (44). The histological joint tissue sections from 0.5% CMC vehicle treated rats showed extensive infiltration with mononuclear cells (inflammation scores ranging from 4 to 5, $n = 5$), and significant bone destruction (bone resorption scores ranging from 4 to 5). In comparison, the sections from FTS treated rats showed less joint tissue infiltration by mononuclear cells (inflammation scores ranging from 2 to 3, $n = 5$), and less destruction of trabecular and cortical bone in the distal tibia (bone resorption scores ranging from 2 to 3). Moreover, the tissue sections from rats treated with FTS as an add-on to MTX showed only mild joint tissue infiltration with mononuclear cells (inflammation scores ranging from 2 to 3) and only rare areas of trabecular or cortical bone resorption not readily apparent on low magnification (average bone resorption scores of ≤ 2).

FTS Inhibits the Upregulation of Pathogenic Serum Cytokines and Relevant Biomarkers

To investigate the immunomodulatory effect of FTS on the response to immunization with mTb containing CFA, we analyzed the levels of the key TH17 cell cytokines, IL-17A and IL-22, in serum samples collected at day 14 of the experiment. We found that treatment with FTS or MTX alone significantly reduced ($P < 0.001$) serum IL-17 levels compared to control CMC-treated arthritic rats (Figure 2A). Furthermore, combined treatment with FTS and MTX was extra potent in suppressing IL-17 upregulation, as compared to single agent therapy ($P < 0.01$). As predicted CFA immunization also induced a strong upregulation of serum IL-22 as compared to naïve healthy unimmunized littermate rats. The IL-22 response observed in CMC treated rats was significantly inhibited by either FTS or MTX therapy ($P < 0.001$) (Figure 2B). Interestingly, treatment with FTS alone or as an add-on to MTX had a more significant capacity to inhibit IL-22 production, as compared to MTX as a single agent ($P < 0.01$ for FTS and FTS + MTX vs. MTX).

In parallel, we analyzed the levels of serum CRP, a typical acute phase reactant protein in humans and in the rat (45). As expected, CFA injection induced a substantial increase in CRP levels in control CMC treated rats compared to naïve healthy littermate rats. Next, we found that FTS, MTX, and FTS as an add-on to MTX treatments equally and significantly reduced the arthritis-associated upregulation of serum CRP levels at day 14 of the experiments (Figure 2C).

Characterizing the Immunomodulatory Effects of FTS on the *In Vivo* T Cell Response

The use of this adjuvant model also offers an opportunity to study the pathological changes in a variety of tissues other than the joints. For example, the splenomegaly that develops as part of the systemic inflammation induced by CFA (40, 44). Thus, at study end, we could study the effects of the immunization and the various treatment protocols on the cellular immune response to CFA, principally analyzing by polychromatic flow cytometry the single cell suspension of spleens and peripheral blood samples for changes in CD4⁺ and CD8⁺ T cell percentages. Our results show that CFA immunization itself induced a significant decline ($P < 0.05$) in the percentage of circulating CD3⁺ T cells but not in splenic T cells. However, the various treatment regimens had only a marginal effect on the percentage of circulating and splenic CD3⁺ T cells. Moreover, we found that the various treatments had no significant effects on CD3⁺, CD4⁺, and CD8⁺ T cells percentage both in the spleen and the blood at study end. Nevertheless, we observed a trend ($P = 0.08$) for an increased CD4⁺ to CD8⁺ T cell ratio in the FTS therapy arm (Figures 3A–C).

As both IL-17⁺ and IL-17⁺IFN- γ ⁺ Th17-type cells have been shown to be instrumental for the pathogenesis of autoimmune responses (46), we analyzed the effects of FTS and other treatment protocols on the induction of IL-17 and IFN- γ expressing T cells in arthritic rats spleens by intracellular cytokine staining.

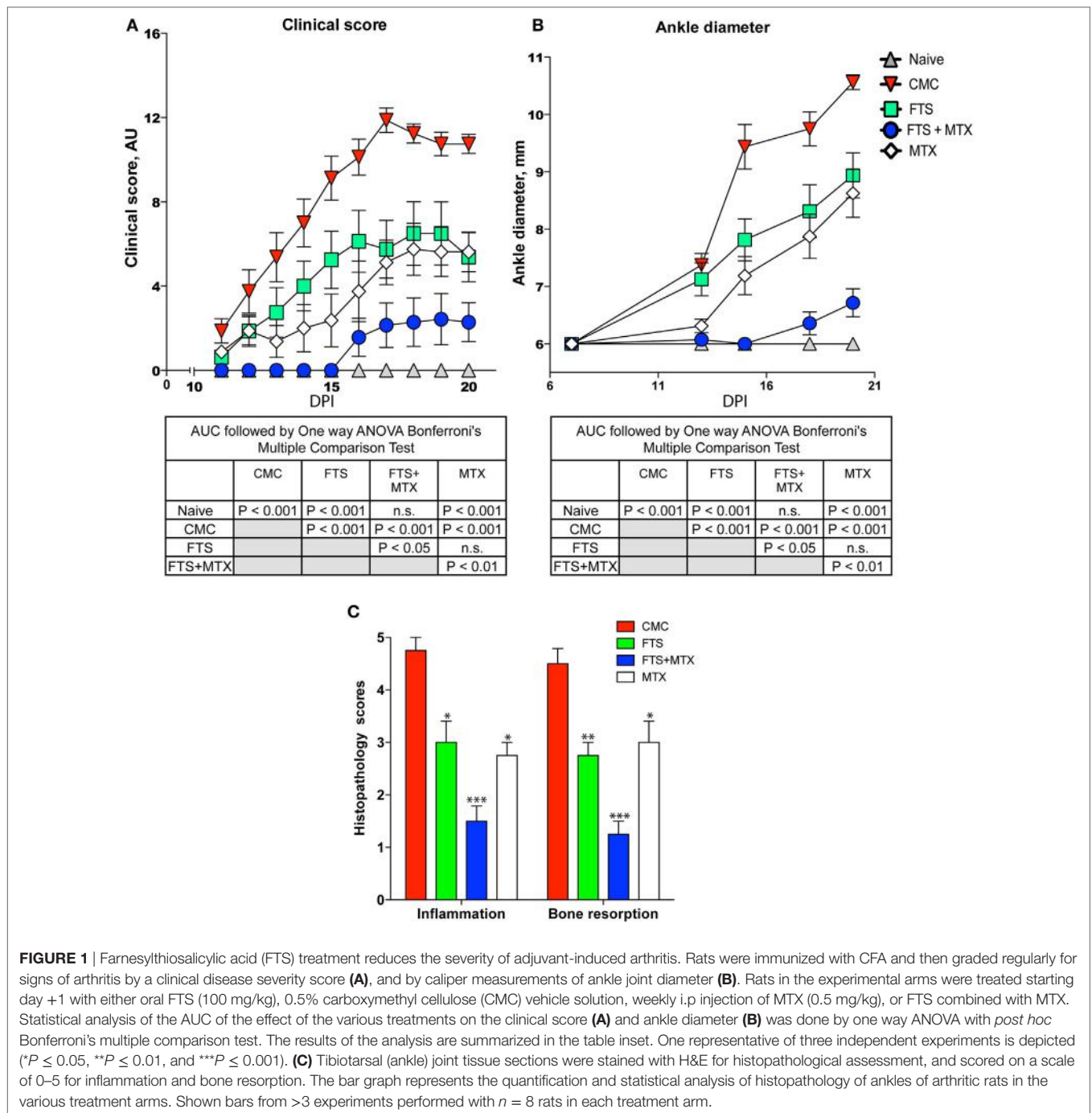


FIGURE 1 | Farnesylthiosalicylic acid (FTS) treatment reduces the severity of adjuvant-induced arthritis. Rats were immunized with CFA and then graded regularly for signs of arthritis by a clinical disease severity score (A), and by caliper measurements of ankle joint diameter (B). Rats in the experimental arms were treated starting day +1 with either oral FTS (100 mg/kg), 0.5% carboxymethyl cellulose (CMC) vehicle solution, weekly i.p injection of MTX (0.5 mg/kg), or FTS combined with MTX. Statistical analysis of the AUC of the effect of the various treatments on the clinical score (A) and ankle diameter (B) was done by one way ANOVA with *post hoc* Bonferroni's multiple comparison test. The results of the analysis are summarized in the table inset. One representative of three independent experiments is depicted ($*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$). (C) Tibiotarsal (ankle) joint tissue sections were stained with H&E for histopathological assessment, and scored on a scale of 0–5 for inflammation and bone resorption. The bar graph represents the quantification and statistical analysis of histopathology of ankles of arthritic rats in the various treatment arms. Shown bars from >3 experiments performed with $n = 8$ rats in each treatment arm.

Our data show that CFA immunization induced a significant induction of IL-17⁺ (~20-fold increase; $P < 0.001$) and of IL-17⁺IFN- γ ⁺ Th17 cells (4.6 ± 0.34 vs. 0.04 ± 0.01 , $P < 0.01$) compared to naive unimmunized rats. Moreover, FTS therapy alone as well as the other treatments all induced a significantly lower induction of pathogenic Th17 cells in the spleens of FTS treated rats compared to CMC vehicle treated arthritic rats $P < 0.001$. Interestingly, FTS as an add-on to MTX therapy induced a more significant reduction in the percentage of the highly pathogenic IL-17⁺IFN- γ ⁺ T cells, as compared to either

drug alone $P < 0.01$ (Figures 4A,B). Thus, the Th17 cell response data showed a positive and significant correlation with the clinical outcomes data of the various treatment protocols.

Next, we analyzed the effects of FTS and the different treatments on the induction of IFN- γ ⁺ Th1 cells in the spleens. We found that, in contrast to the Th17 data, CFA immunization did not produce a significant increase in splenic Th1 cells percentage compared to naive unimmunized rats. Additionally, FTS therapy did not significantly reduce the induction of Th1 cells in the spleens of treated rats compared to CMC control arthritic rats.

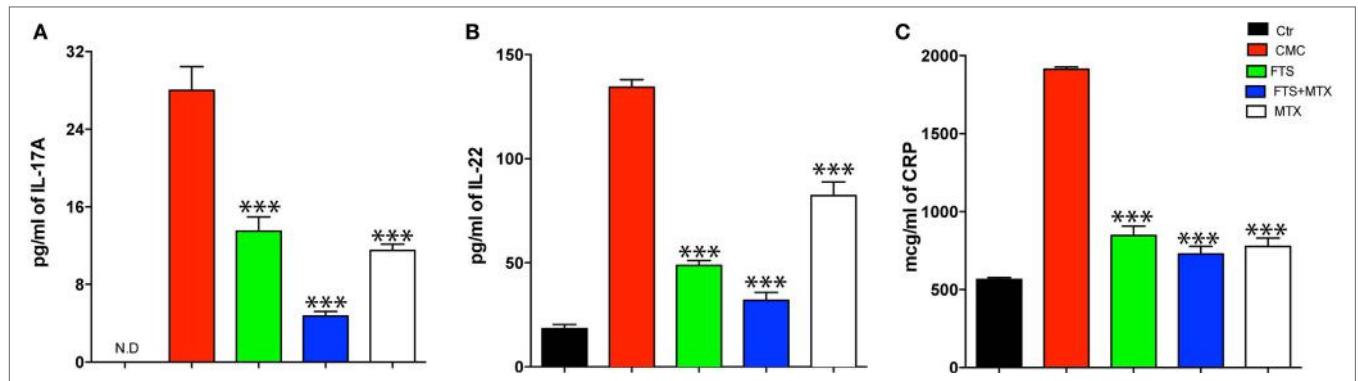


FIGURE 2 | Farnesylthiosalicylic acid (FTS) treatment reduces serum Th17-cytokines and CRP levels during arthritis development. Sera from rats treated with carboxymethyl cellulose (CMC), FTS, FTS + MTX, or MTX was collected at Day 14 post adjuvant injection (day +3 from arthritis onset) and analyzed for **(A)** IL-17A, **(B)** IL-22, and **(C)** CRP levels by ELISA kits. Bars represent mean \pm SD of triplicates from a representative experiment out of three performed ($n = 4$ rats analyzed per group). ND, not detectable. Statistical significance among groups was analyzed by the Student's *t*-test ($*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$).

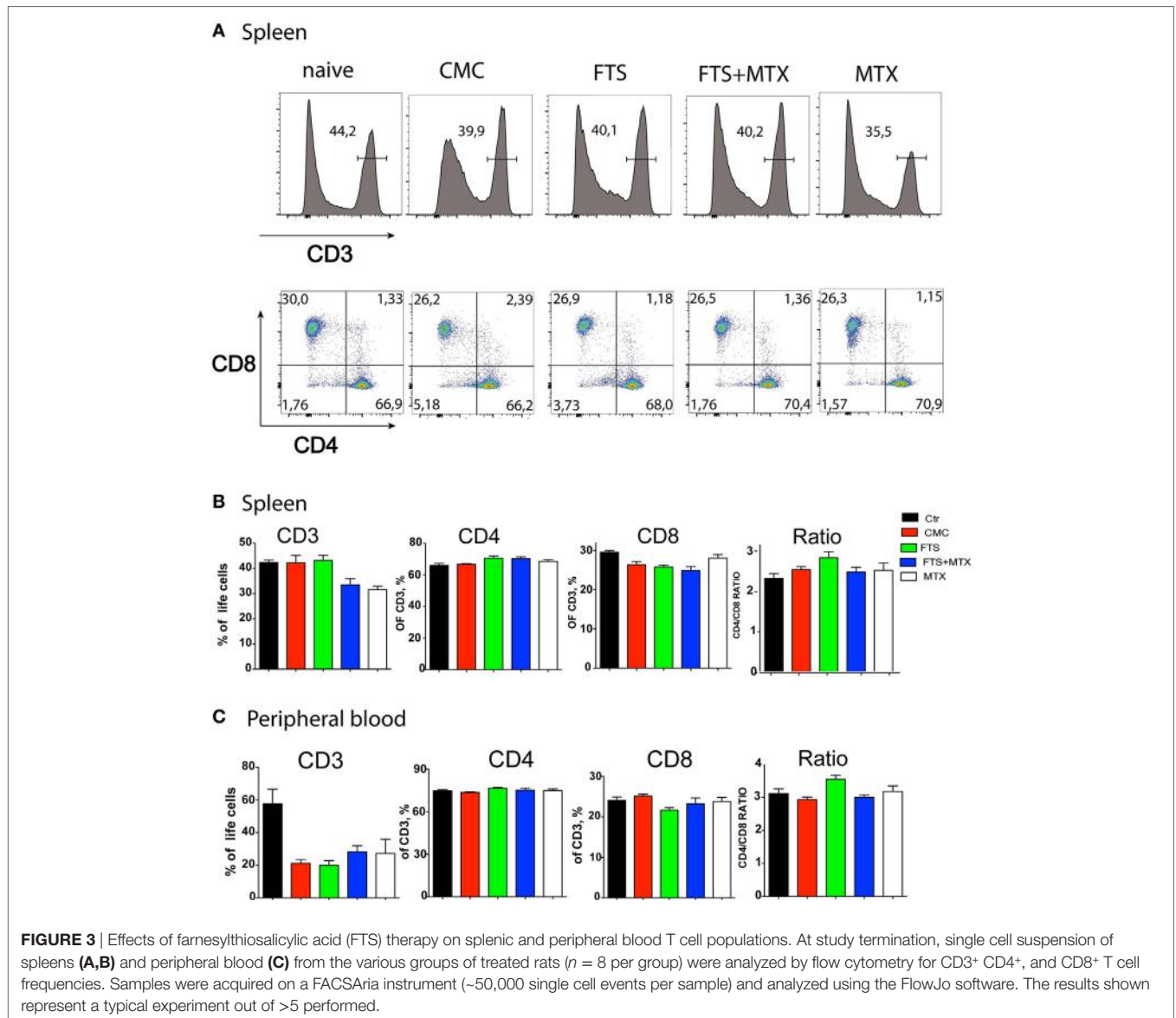


FIGURE 3 | Effects of farnesylthiosalicylic acid (FTS) therapy on splenic and peripheral blood T cell populations. At study termination, single cell suspension of spleens **(A,B)** and peripheral blood **(C)** from the various groups of treated rats ($n = 8$ per group) were analyzed by flow cytometry for CD3⁺ CD4⁺, and CD8⁺ T cell frequencies. Samples were acquired on a FACSAria instrument (~50,000 single cell events per sample) and analyzed using the FlowJo software. The results shown represent a typical experiment out of >5 performed.

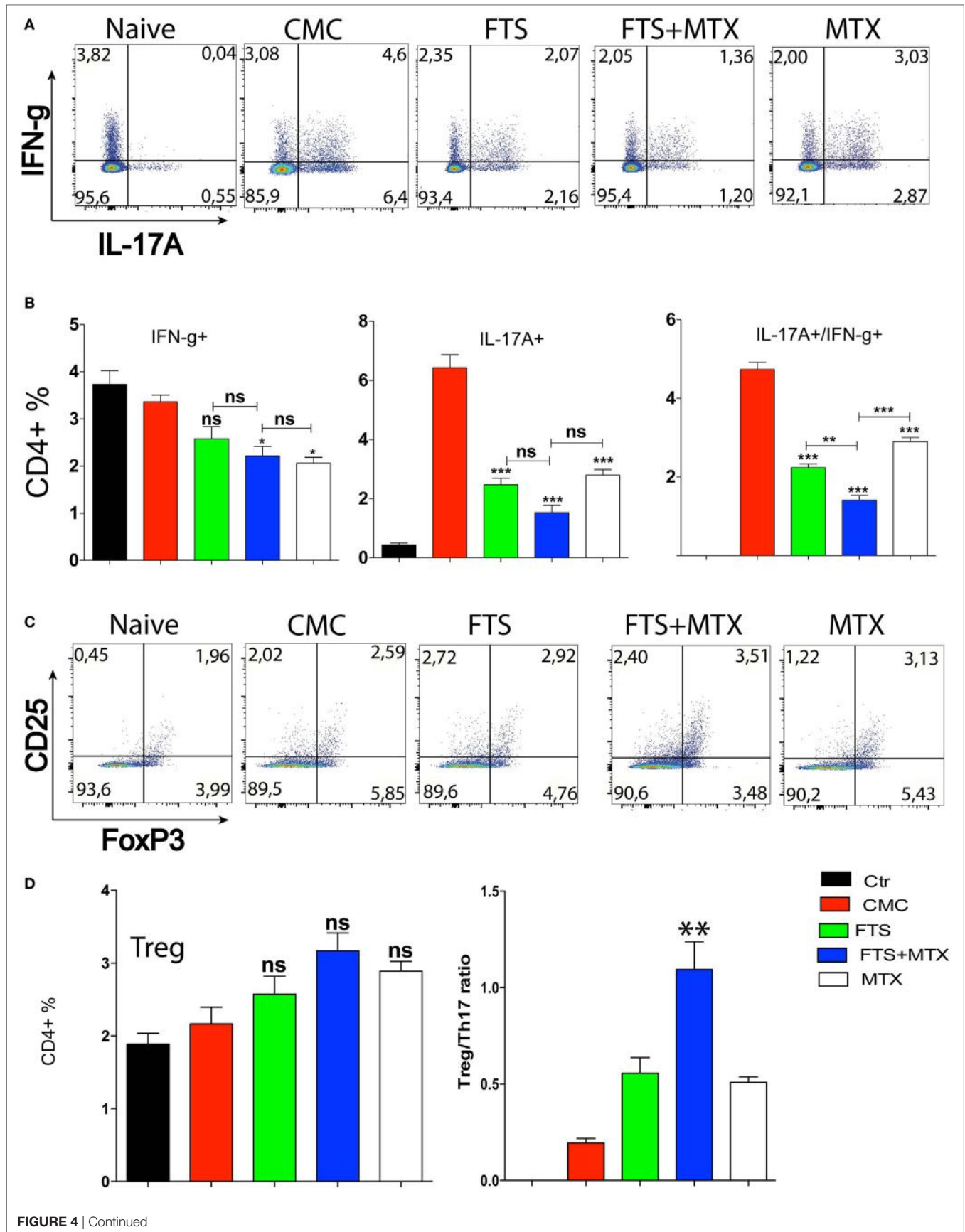


FIGURE 4 | Continued

FIGURE 4 | Continued

Immunomodulatory effect of farnesylthiosalicylic acid (FTS) treatment on the induction of Th1, Th17, and Treg subsets post CFA immunization. **(A)** Representative flow cytometry plots of intracellular staining for Th1 and Th17 cytokines producing CD4⁺ T cells. **(B)** Bars represent the percentage (mean \pm SD) of the indicated Th-subsets in spleens of the various rat groups harvested at day 20 post CFA immunization ($n = 5$ per group). **(C)** Representative flow cytometry plots for Treg cells identification (CD3⁺CD4⁺FoxP3⁺ events). **(D)** Left hand Bars graph represent the percentage (mean \pm SD) of Treg cells in the various rat groups ($n = 5$ per group). Right hand plot represents the Treg to Th17 ratio in these rats. Statistical significance was assessed by one way ANOVA with *post hoc* Bonferroni's multiple comparison test (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$).

The combined FTS and MTX therapy only induced a marginally significant reduction in the percentage of Th1 cells as compared to FTS alone therapy (Figures 4A,B). Our results suggest that the improved clinical outcome produced by FTS therapy is less dependent on the inhibition of Th1 polarization.

As the balance between the inductions of antigen-specific pathogenic Th17 versus peripheral CD4⁺Foxp3⁺ regulatory T cells (Treg) can influence the outcome of the T cell response (47), we also determined the percentage of Treg cells in the spleens of the various rats. We found a statistically insignificant trend of increased Treg cells percentage in the spleens of FTS and FTS + MTX treated rats vs. CMC treated control arthritic rats (Figures 4C,D). Importantly, the ratio of Treg to Th17 cells was significantly increased ($P < 0.01$) when FTS and MTX therapy were combined, but not by the single drug therapy with either FTS or MTX ($P = 0.07$).

FTS Therapy Modulates the Bhs65-Induced Inflammatory Genes Expression Program of CD4⁺ T Cells from Treated Rats

The mycobacterial heat-shock protein 65 (Bhs65), present in CFA, has been implicated in the immune-pathogenesis of AIA (41, 48). Hence, we used *in vitro* antigenic re-stimulation with Bhs65 as an additional approach to study the effects of FTS therapy on the antigen-specific T cell response at the molecular level. Briefly, following immunization with CFA the animals were treated with FTS or CMC vehicle. At day +12 (onset of obvious clinical arthritis), the rats were euthanized, spleen, and draining superficial inguinal and para-aortic LN were harvested, and a single-cell suspension of a mixture of spleen and LN cells was prepared. Subsequently, the cells were cultured for four days with or without recombinant Bhs65 protein (5 μ g/ml), as previously described (49) and total RNA was purified at termination of the experiment.

To gain a more comprehensive insight into the gene networks that are associated with the therapeutic action of Ras-inhibitor in AIA, we analyzed by GeneChip[®] microarrays the changes in global gene expression (primarily mRNAs) associated with FTS treatment. Thus, at day +12, we purified CD4⁺ T cells from the spleen and relevant draining LNs of FTS treated and control rats, re-stimulated them with Bhs65 *in vitro* and then cultured them for additional 4 days. At the end of experiment, total RNA was purified and used for the downstream transcriptome analysis.

The bioinformatics analysis of the various CD4⁺ T cell samples showed that the antigenic re-stimulation with Bhs65, as expected, induced a robust upregulation of multiple ($n = 50$) genes in CD4⁺ T cells (Figure 5A). As seen in the plotted heat

map, depicting the signal intensity of the list of Bhs65-induced and differentially expressed genes (transcripts with \geq twofold change; 2FC) in CD4⁺ T cells of CMC as well as FTS treated rats. The plot shows a widespread FTS-dependent reduction in the intensity of the Bhs65-induced transcription of a large percentage of these 50 genes ($P < 0.001$, by chi-square test). Notably, this list includes a large number of recognized immune response genes, such as genes encoding pro-inflammatory cytokines (e.g., Il22, Il17a/f, Ifng, Csf2/GM-CSF, Lta, and Il1a).

Next, to determine, in unbiased manner, the biological processes and molecular functions that mediate the therapeutic effect of FTS, we performed additional in-depth bioinformatics analysis. Thus, we computed the overlaps between our gene list and relevant annotated gene sets within the HALLMARK and gene ontology (GO) collections of the Molecular Signatures Database of the Broad Institute (Massachusetts Institute of Technology) using the GSEA software web site v6.1 (50).

By this analysis, we determined that the list of genes upregulated significantly ($>$ twofold change, FDR $q < 0.05$) following Bhs65 re-stimulation of CD4⁺ T cells isolated from CMC treated control rats exhibited significant overlap with a large number of curated relevant immune response and cell proliferation gene sets. The top overlapping annotated gene sets included: (i) cytokine activity (GO); immune system processes (GO); G2M_checkpoint (HALLMARK); inflammatory response (HALLMARK); response to tumor necrosis factor (GO); response to IFN-gamma (GO); positive regulation of cell proliferation (GO); IL6_JAK_STAT3_signaling (HALLMARK); K-RAS signaling up (HALLMARK); and others (see also Figure 5B).

To validate our GeneChip[®] data, we analyzed by qPCR the relative quantity of mRNA transcripts of five highly relevant inflammatory response genes differentially induced by Bhs65 re-stimulation in CMC vs. FTS treated CD4⁺ T cells (ccl20, il22, il17a, il17f, and Csf2). As shown in Figure 5C, the qPCR data confirmed that the induced transcription of all these genes, during the recall response of TH cells to Bhs65, was significantly inhibited by FTS therapy ($P < 0.05$, by *t*-test). In agreement with our gene transcription data, we also detected by ELISA a strong induction of IL-17A and IL-22 protein expression following *in vitro* Bhs65 re-stimulation of control CD4⁺ T cells (Figure 5D), whereas this antigen-dependent induction of IL-17A and IL-22 secretion was strongly inhibited in equivalent cultures of FTS treated rats ($P < 0.001$, by *t*-test).

The FTS Derivative 5-Fluoro-FTS (F-FTS) Shows a Higher Therapeutic Effect than FTS
Fluoro-FTS, although presumed to have a rather similar mechanism of action as the parent compound, has been previously

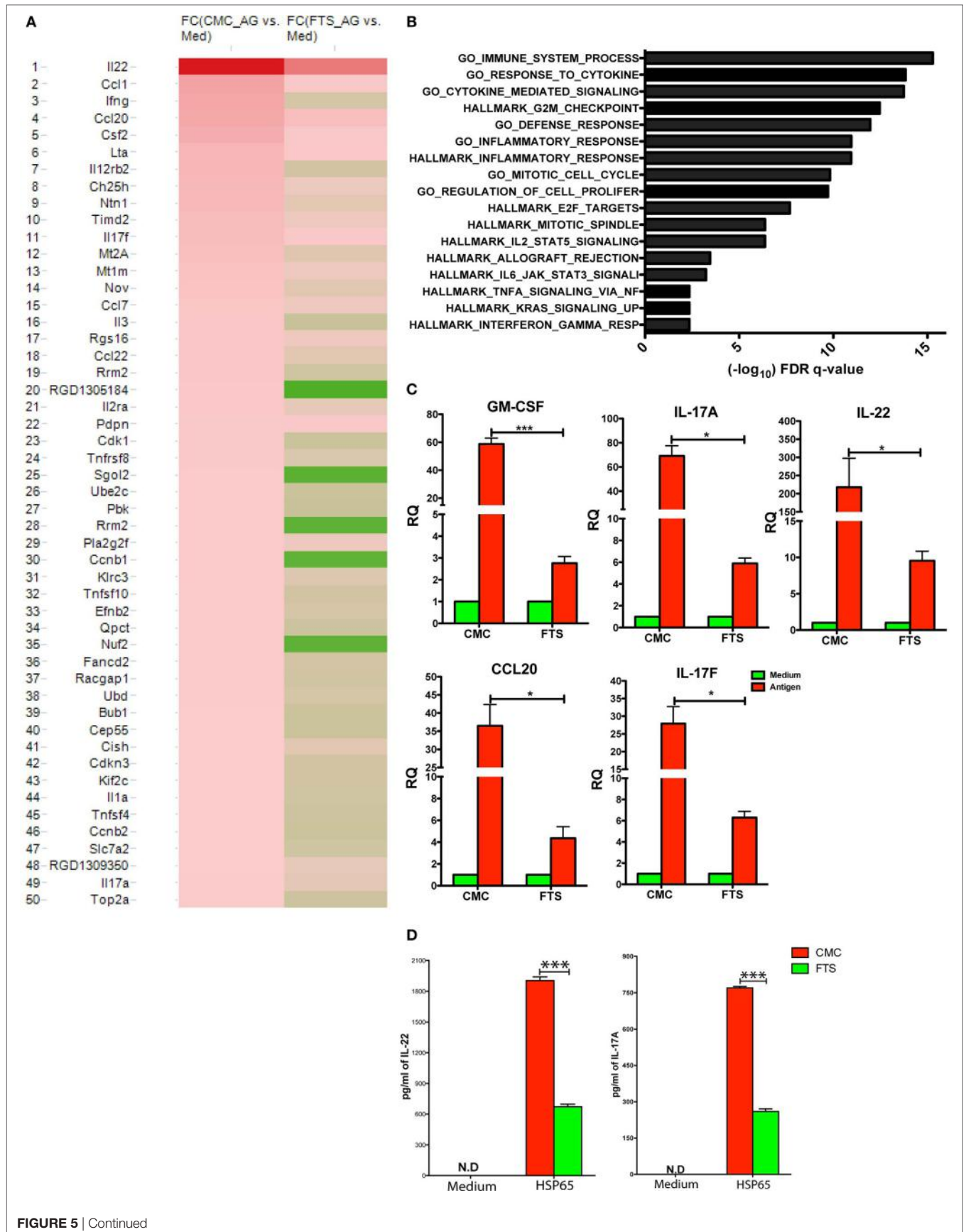


FIGURE 5 | Continued

Farnesylthiosalicylic acid (FTS) therapy down modulates the Bhsp65-induced transcription of multiple genes tightly linked to inflammatory and immune processes. Following CFA-immunization, rats were treated with carboxymethyl cellulose (CMC) or FTS starting from day +1 post AIA induction. Single cell suspensions from draining LNs and spleen of treated rats (harvested on day +14 post disease induction) were stimulated with Bhsp65 (5 µg/ml) or control PBS and cultured for additional 72 h. At the end of cultures, CD4⁺ T cells were isolated and high quality total RNA was extracted from the purified cells. **(A)** Heat map analysis depicting the expression matrix of the 50 genes significantly induced by Bhsp65 stimulation (>2FC, FDR $q < 0.05$) in the CMC control group as compared to their expression in FTS treated group ($n = 2$ per group). In this two-color heat map, the brightest red and green colors represent the top up- or down-regulated mRNAs, respectively. **(B)** Next, we computed using the GSEA software web site v6.1 software, the overlaps between our gene list and relevant validated gene sets. Bars represent the FDR q -value (cutoff of <0.05) for the statistical significance of the overlap between the annotated Gene set and our list. **(C)** Purified total RNA was analyzed by quantitative PCR for the relative mRNA expression (normalized to Actb) of five inflammatory response genes (Ccl20, Il22, Il17A, Il17F, and Csf2) induced by Bhsp65 re-stimulation of CD4⁺ T cells treated *in vivo* with CMC vs. FTS. **(D)** Supernatants from the same cultures were tested by ELISA kits for levels of secreted 17A and IL-22 by readymade ELISA kits. Bar graphs depict mean \pm SD, and statistical significance was analyzed by Student's t -test ($*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$). Data shown are representative of a typical experiment out of $n = 3$.

shown to be a more potent inhibitor of oncogenic Ras signaling as compared to FTS (51). Moreover, F-FTS treatment was previously shown to strongly attenuate the development of type-1 diabetes in NOD mice (36). However, the *in vivo* therapeutic efficacy of F-FTS and FTS has not been previously compared. Thus, we designed a set of experiments to investigate the relative therapeutic efficacy of F-FTS compared to FTS. The data from these studies demonstrate that both F-FTS and FTS therapy following CFA immunization produced a significant attenuation of disease development and severity, as assessed by the clinical score and ankle joint diameter measurements, as compared to CMC treated control rats. Importantly, the therapeutic efficacy of F-FTS was significantly superior compared to FTS (**Figure 6A**). Next, we show that F-FTS therapy produced a robust reduction of serum IL-17 and IL-22 levels post CFA immunization, as compared to control immunized rats (**Figure 6B**). Moreover, the inhibitory effect on IL-22 induction was more pronounced with F-FTS vs. FTS therapy ($P < 0.001$, by t -test).

Next, we analyzed the effect of F-FTS therapy on the induction of antigen-specific Th17 cells. Thus, as already detailed above we employed *in vitro* antigenic re-stimulation with Bhsp65 to test this question. Our results demonstrated that *in vivo* therapy with either F-FTS or FTS was coupled with significant inhibition of IL-17 production during the recall response of Bhsp65-specific T cells compared to the robust IL-17 recall response in control CMC treated rats cultures ($P < 0.001$ for both drugs vs. CMC, by one-way ANOVA). Moreover, as shown in **Figure 6C**, F-FTS was a more potent inhibitor compared to FTS ($P < 0.05$), which positively correlated with its superior therapeutic efficacy.

DISCUSSION

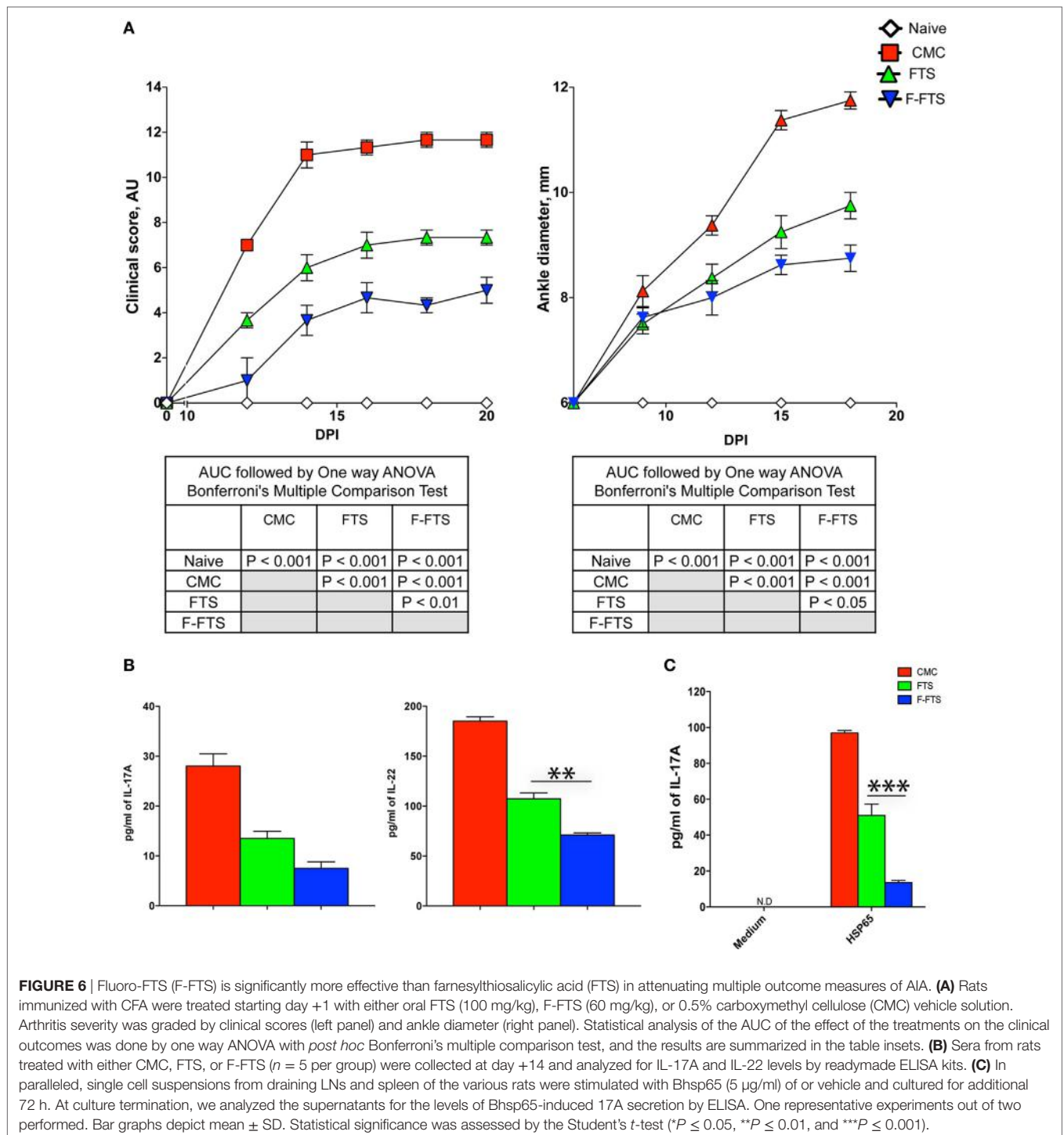
In this study, we further demonstrate the therapeutic efficacy of the orally active small-molecule inhibitors of PM-localization of Ras GTPases, FTS, and its more potent derivative F-FTS, in the rat AIA model of RA. Therapy with either FTS or F-FTS significantly reduced ankle swelling and clinical arthritis scores. Moreover, the therapeutic efficacy of Ras inhibitors was comparable to that of the mainstay disease modifying anti-rheumatic drug (DMARD), MTX. Importantly, FTS treatment as an add-on to MTX therapy resulted in a robust attenuation of arthritis development. The histopathological assessment of the ankle joints confirmed that FTS treatment reduced ankle joint inflammation and bone resorption. The clinical efficacy of the Ras inhibitors strongly correlated with reduced serum levels of IL-17 and IL-22 together

with a reduced recall Th17-response to Bhsp65-antigen stimulation in splenocytes from FTS or F-FTS treated rats. Moreover, the bioinformatics of the gene microarrays further demonstrated the wide-ranging efficacy of FTS to down-modulate the TCR activation induced transcription of a large set of canonical pro-inflammatory genes in effector CD4⁺ T cells following *in vitro* Bhsp65 re-challenging.

Initial findings in a previous study showed that FTS treatment reduced the clinical arthritis score in the AIA model, and that this treatment was associated with reduced levels of various pro-inflammatory cytokines (IFN- γ , TNF- α , IL-6, and IL-17) in the serum at study end. Our current work was designed to obtain new and pertinent data on the effects of oral Ras inhibitors on the pathogenic T cell response to CFA, and more specifically to the arthritogenic mycobacterial antigen, Bhsp65. We also provide new insight on the molecular effects of *in vivo* Ras-signaling blockade on the transcriptomes of Bhsp65-specific CD4⁺ T cells. In the present study, we also tested in parallel the therapeutic effects of F-FTS (FTS derivative) and of FTS as an add-on to the drug MTX.

The Ras family of GTPases play an important role in signaling nodes downstream to TCR and CD28 activation (52). Perturbation of K-Ras-signaling lowers the threshold for TCR activation and thus may support abnormal responses to autoantigens (10). For example, somatic mutation in NRAS or KRAS genes in hematopoietic cells can cause a rare autoimmune disorder, characterized by lymphadenopathy and splenomegaly due to abnormal expansion of lymphocytes (53, 54). Therefore, our working hypothesis was that our Ras inhibitors might chiefly target the T cell response to CFA injection. As extensive research in murine arthritis models strongly indicates a central role for the Th17-type cells (55, 56), we focused on the effects of FTS on the induction of the Th17 response.

Our data indeed show that CFA injection was linked to a robust induction of Th17 cells, and that importantly FTS therapy reduced the frequency of Th17 cells in relevant lymphoid tissues (draining LNs and spleen). The two chief CD4⁺ T cells subsets suppressed by FTS were IL17⁺ (classical Th17 cells) and IL-17⁺IFN- γ ⁺ “double positive” cells. The inhibitory effect on the induction of these IL-17 producing T cell subsets was comparable to the effect of MTX therapy, while the combined FTS and MTX was significantly more potent in this regard. In contrast, FTS therapy had no significant effect on the induction IFN- γ ⁺CD4⁺ T cells (classical Th1 cells). Accordingly, we also detected reduced serum IL-17 levels at arthritis onset in FTS treated rats. Moreover,



FTS and F-FTS therapy strongly attenuated Bhs65-induced IL-17 production by CD4⁺ T cells (both per ELISA and per GeneChip[®] array data).

Although the prototype Th17 cell cytokines, IL-17A/F, have been strongly linked to autoimmunity in multiple animal models, there has been recent evidence that a fraction of Th17 cells also co-express IFN- γ at the site of inflammation with reported functional consequences (57). Our data are the first work that

shows that CFA immunization induces significant expansion of both classical Th17 cells and Th cells with a Th17/Th1 “double phenotype.” Moreover, we clearly determine by intracellular cytokine staining that FTS treatment strongly targets the expansion of this unique and likely pathogenic T cell subset in relevant lymphoid tissues.

By functional enrichment analysis of the GeneChip[®] array data, utilizing the GSEA platform (50), we identified a list

of validated immune/inflammatory response genes that are “silenced” by FTS therapy specifically in pathogenic CD4⁺ Th cells responding to Bhp65. For example, besides the classical Th17 cytokine genes (*Il7* and *Il22*), we also identified that FTS targeted other pro-inflammatory cytokine genes (e.g., *Ifng*, *Cfs2*, *Lta*, *Il1a*) as well as chemokines genes that are chemotactic for effector T cells, monocytes, and dendritic cells (*Ccl1*, *Ccl20*, *Ccl7*, and *Ccl22*). Thus, these data allowed us to discover additional interesting genes, molecular processes, and biological pathways that expand our insight into the mechanism of action of FTS.

The Th17-allied cytokine IL-22 has been shown to play a pathogenic role in murine models of arthritis, particularly in promoting the early inflammatory responses to CFA and enhancing cartilage and bone damage (58–60). Moreover, levels of IL-22 and Th22 cells are increased in the synovial tissue and in the blood of RA patients, and correlate with disease activity (61, 62). Importantly, IL-22 mainly secreted locally by T cells, can induce synovial fibroblasts' proliferation and RANKL (Receptor Activator of NF- κ B Ligand) expression, and consequently promote their differentiation into osteoclasts (63). Our data clearly show that *in vivo* treatment with Ras inhibitors strongly attenuated the CFA-induced upregulation of serum IL-22 as well as the transcription and secretion of IL-22 by purified effector CD4⁺ T cells re-challenged *in vitro* with the mycobacterial antigen, Bhp65.

Our novel finding that the combined MTX and FTS treatment was more potent, both by clinical and laboratory outcome measures, as compared to monotherapy with either drug alone in AIA, is highly relevant to RA treatment. MTX is commonly referred to as the cornerstone of modern RA therapy as a large percentage of patients are effectively treated with MTX alone or in combination with other drugs. Moreover, the majority of anti-RA biologics are more effective as a combination therapy with a conventional DMARD, predominantly MTX, as compared to biologics monotherapy (64). Thus, our findings strongly fit into this clinical concept: i.e., combination therapy with MTX is superior to monotherapy with the targeted-synthetic immunomodulatory compound, FTS.

Additionally, we performed for the first time a “head-to-head” comparison of the therapeutic efficacy of F-FTS vs. FTS. Our findings demonstrate that F-FTS has a superior therapeutic efficacy compared to its parent compound, FTS, at a lower dose (60 mg/kg compared to 100 mg/kg, respectively). Moreover, our ELISA data

show, in agreement with the clinical outcome data, that F-FTS was a more potent inhibitor of the *in vivo* Th17 response to CFA injection. Thus, the results from the latter set of studies add to the translational impact of the paper.

In conclusion, FTS and F-FTS, two targeted synthetic Ras-GTPases inhibitors, exhibited a potent immunomodulatory effect in the classical animal model of arthritis, AIA, which was further enhanced by combination therapy with MTX. The therapeutic effect was coupled with *in vivo* inhibition of CFA-induced generation of Th17-polarized, IL-17 and IL-22 secreting, lymphocytes. Thus, Ras-signaling-blockade is a promising novel therapeutic approach for RA, justifying further preclinical evaluation of these compounds in RA patients.

ETHICS STATEMENT

All animal experiments were conducted in accordance with relevant laws of the state of Israel and guidelines of the Tel-Aviv University and approved by the Institutional Animal Care and Use Committee (Approval # L-14-018), and by the USAMRMC Animal Care and Use Review Office (protocol PR130028.03).

AUTHOR CONTRIBUTIONS

MZ, VM-M, YK, and IG designed the research and analyzed data; MZ, VM-M, EV, GE-S, and JJ-H performed the research; YK and IB contributed new reagents/analytic tools; and MZ and IG wrote the paper. IG approved the final version of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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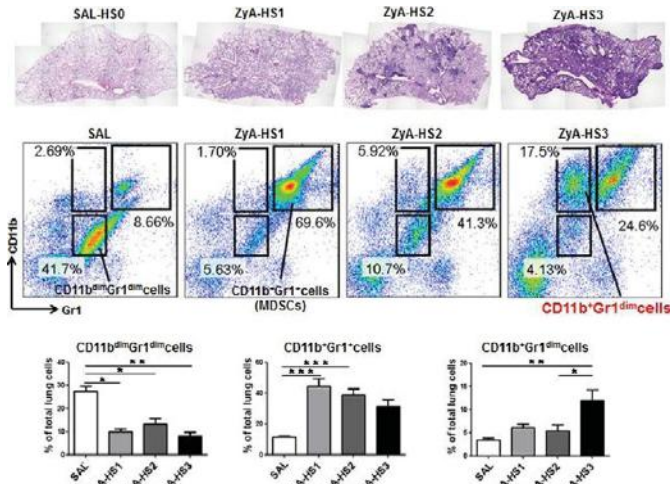


Figure 1

Disclosure of Interest: None declared
DOI: 10.1136/annrheumdis-2017-eular.1429

FRI0081 A MACAQUE MODEL OF RHEUMATOID ARTHRITIS BY IMMUNIZATION WITH CITRULLINATED PEPTIDES: LESSONS FOR THE HUMAN DISEASE

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Background: Recent evolution in the understanding of rheumatoid arthritis (RA) mechanisms is the role of antibodies directed against citrullinated (cit) proteins (ACPAs). The shared epitope (SE) on the MHC class II is the main genetic risk factor of RA and favors presence of ACPAs. Mouse models dependent on cit peptides immunization require transgenic expression of the SE and are controversial. Non-human primates are ideal to study the interaction between ACPA and RA since 8% carry, similarly to humans, the SE called the H6 haplotype. **Objectives:** The goal of this study was to develop a new animal model of RA based on immunization of genetically predisposed macaques against cit peptides to generate an ACPA-mediated model of arthritis. **Methods:** Six macaques were intra dermally (ID) immunized with 4 peptides: vimentin (59–71) and (66–78), α fibrinogen (79–91) and aggrecan (89–103). H6 animals were immunized with either cit (n=2) or arginine (arg) (n=2) containing peptides. Two non H6 animals were immunized with cit peptides. These peptides are known to induce a T cell response in RA patients carrying the SE. T-cell response was assessed with Interferon γ ELISPOT and B-cell response by ELISA. An intra articular (IA) boost was done 30 weeks after initial immunization with either incomplete Freund's adjuvant (IFA) alone, IFA and cit peptides and IFA plus non relevant peptides. **Results:** In the macaques, the T-cell response was specific to cit or arg peptides (depending on the peptides used for immunization). Surprisingly, the presence of the H6 epitope did not influence the response. Conversely the antibodies generated in response to the peptides were cross-reactive between the cit and arg peptides. Since no clinical response was observed, an IA boost was performed with the same 4 cit peptides and IFA adjuvant. This led to a prolonged neutrophil-rich mono-arthritis preferentially in H6 animals (Figure). Conversely, animals



boosted with IFA alone only or with IFA plus myelin oligodendrocyte glycoprotein (MOG) peptides and previously immunized with MOG peptides presented with a transient mono-arthritis. Histological analysis revealed a local mononuclear infiltrate in one of the two animals that had prolonged knee monoarthritis. There was no clinical polyarthritis but 2 animals displayed synovial proliferation in 1 MCP and 1 MTP, respectively.

Conclusions: Immunization of macaques with cit peptides, then IA boost with the same cit peptides plus IFA, induced a prolonged monoarthritis. Shared epitope bearing did not restrict the T-cell response but seemed to favor the prolonged swelling after the IA boost. Neutrophil infiltration of the joint occurred similarly to what is seen in RA. Further use of neutrophil chemo-attractant might lead to a poly-articular disease. This macaque model of RA appears unique to study the events occurring during the pre-clinical phase of RA.

Disclosure of Interest: None declared
DOI: 10.1136/annrheumdis-2017-eular.2843

FRI0082 RAS SIGNALING INHIBITORS ATTENUATE ARTHRITIS IN ANIMAL MODELS OF RHEUMATOID ARTHRITIS BY DOWN MODULATING THE PATHOGENIC TH17 CELL RESPONSE

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Background: Ras-GTPases are vital for normal T cell activation, and downstream effectors of Ras include the MEK/ERK, PI3-kinase/AKT, mTOR/p70S6-kinase, and NF- κ B pathways. Somatic mutations in NRAS cause an autoimmune lymphoproliferative disorder and T cells from Rheumatoid Arthritis (RA) patients exhibit perturbation of the Ras/MEK/ERK pathway. The small molecule Farnesylthiosalicylic acid (FTS) inhibits the interaction between Ras-GTPases and prenyl-binding chaperones vital for proper plasma membrane localization and downstream signaling [1]. Previous pre-clinical studies suggest that FTS has an immunomodulatory effect in various animal models of autoimmunity [2].

Objectives: To test in the Lewis rat adjuvant induced arthritis (AIA) and in the DBA/1 mouse collagen type-II induced arthritis (CIA) models the therapeutic immunomodulatory effect of FTS alone or combined with methotrexate (MTX).

Methods: Arthritis was induced in 8–12 week old male Lewis rats by complete Freund's adjuvant (CFA) injection and in male DBA/1 mice by collagen type-II (CII) immunization. Animals were treated prophylactically with once daily oral FTS (100 mg/kg); weekly i.p injection of MTX (0.5 mg/kg), oral FTS combined with MTX, or daily oral vehicle solution (0.5% carboxy methyl cellulose; CMC). Arthritis severity was scored daily from disease onset until study termination. In addition, we measured multiple disease- and drug-related immunological/molecular biomarkers.

Results: AIA severity was significantly reduced by FTS treatment compared to CMC controls (Figure 1A, $P < 0.001$). Combining FTS and low dose MTX significantly increased its therapeutic efficacy compared to each drug alone (Figure 1A, $P < 0.05$). FTS or FTS+MTX treatment also suppressed the upsurge in serum IL-17 and CRP compared to ailing controls. Global gene expression analysis of relevant splenic CD4+ T cells revealed that FTS is a potent inhibitor of pro-inflammatory and TH17 related gene networks. Next, our data from the mouse CIA model show that the therapeutic efficacy of FTS was non-inferior to MTX and it significantly reduced arthritis severity compared to controls (Figure 2, $P < 0.001$). Importantly, FTS significantly inhibited the production of pathogenic anti-CII autoantibodies and upregulation of serum IL-6 and IL-17A compared to control arthritic mice. The in depth, multiplex, analysis of the effect of FTS on the T cell cytokine response to CII, revealed strong suppression of IL-22, IL-17, IL-9, GM-CSF and TNF production. Noteworthy, FTS therapy positively correlated with reduced Ras-GTP, p-ERK and p-AKT levels in splenic lymphocytes (drug related biomarkers).

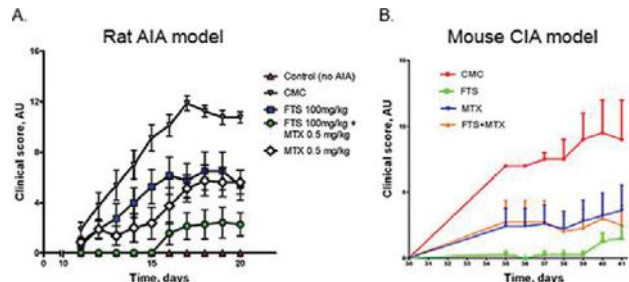


Figure 1. FTS attenuates arthritis severity in the (A) rat AIA and (B) mouse CIA models

Conclusions: FTS, a first-in-class oral selective Ras-GTPases inhibitor, exhibits a potent immunomodulatory effect in two classical murine model of arthritis, coupled with the inhibition of the TH17 response to relevant arthritogenic-antigens. Thus, Ras-signaling-blockade is a promising novel therapeutic approach for RA.

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Disclosure of Interest: None declared

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FRI0083 **REDUCED INCREASE OF ACPA IGG-FC GALACTOSYLATION DURING PREGNANCY IN COMPARISON TO TOTAL IGG: AN EXPLANATION WHY AUTOANTIBODY POSITIVE RA-PATIENTS IMPROVE LESS DURING PREGNANCY?**

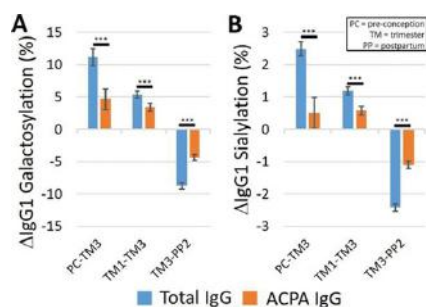
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Background: Rheumatoid arthritis (RA) disease activity (DAS28-CRP) improves less during pregnancy in autoantibody positive patients.¹ The most specific autoantibodies for RA are anti-citrullinated protein antibodies (ACPAs), which mainly occur as the immunoglobulin (Ig) G isotype. An association with DAS28-CRP and the pregnancy-associated improvement is well established for the Fc glycosylation of total IgG, in particular for galactosylation (Gal) and sialylation (SA).² The Fc glycosylation of ACPAs – mainly present as IgG – has been reported to be different from the total IgG Fc glycosylation.³

Objectives: We sought to determine whether the change in ACPA IgG glycosylation during pregnancy is different from that of total IgG, and whether this relates to the improvement of RA during pregnancy.

Methods: ACPA positive patient sera (n=152) were obtained within the framework of the PARA cohort, a prospective study designed to investigate pregnancy-associated improvement of RA. ACPA IgG was isolated using microscale affinity chromatography. Trypsin digested ACPA IgG was measured using nano-liquid chromatography mass spectrometry, and compared to total IgG.

Results: Pregnancy-associated changes in the levels of glycosylation were observed for all ACPA IgG subclasses. Pregnancy-associated glycosylation changes were less pronounced during pregnancy and after delivery in ACPA IgG (Gal +5%; SA +0.5%) compared to total IgG (Gal +11%; SA +2.5%; Figure 1), but – for total IgG – not different between ACPA+ and ACPA- patients. No association of the change in DAS28-CRP with the change in ACPA IgG or total IgG galactosylation was observed for ACPA+ patients, whereas a strong association of total IgG galactosylation was observed for ACPA- patients.



Conclusions: During pregnancy the increase in galactosylation of ACPA IgG was less pronounced than that of total IgG, whereas the increase in the galactosylation of total IgG was not different between ACPA+ and ACPA- patients. Since it is known that changes in IgG galactosylation are associated with improvement of RA during pregnancy and since ACPA is thought to be of pathogenic significance in RA, our data might provide an explanation why ACPA+ RA patients are less likely to improve during pregnancy.

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Disclosure of Interest: None declared

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FRI0084 **THE TONSIL MICROBIOME IS INVOLVED IN RHEUMATOID ARTHRITIS**

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Background: Rheumatoid arthritis (RA) is a prevalent systemic autoimmune disease characterized by the production of autoantibodies¹. The tonsil has been demonstrated to be a site of citrullination, and tonsillectomy has been reported to be a potential treatment of RA, suggesting the possibility that the tonsil could be a site of autoimmunity generation in RA^{2,3}. The dysbiosis of gut microbiome and the associated host immune response has been implicated in the initiation and progression of RA^{4–6}. However, there is no in-depth studies on the role of tonsil microbiota in RA. Thus, studies of the characteristics of tonsil microbiome in RA patients, the underlying mechanisms, as well as specific markers for the diagnosis and therapeutic evaluation for RA, are critical for the early diagnosis and prevention of RA.

Objectives: Therefore, we aimed to define the association of RA with tonsil microbiome as well as a microbial and metabolite profile that could predict disease status.

Methods: 16S rRNA gene sequencing was utilized on 220 tonsil swab samples (121 RA patients and 99 healthy controls) as well as 78 fecal samples (68 RA and 10 controls). Analysis of microbial taxa and metabolic pathway were performed to characterise and compare the tonsil microbiome of RA patients and healthy subjects

Results: Results showed that the tonsil harbored a unique microbiome relative to that present in the fecal samples. Patients with RA exhibited different tonsil microbiome from controls. A taxon-level analysis suggested that the relative abundance of 26 microbial clades were significantly altered, with 7 taxa increased and 19 taxa decreased in RA samples. Noticeably, we observed an expansion of rare microbial lineages as well as an alteration in microbial cladogenesis within RA patients. RA tonsil microbiota was associated with smoke, anti-peripheral factor, rheumatoid factors, disease duration and activity. Furthermore, we identified that 86 genes associated with bacterial metabolic pathway were enriched in RA tonsil microbiome.

Conclusions: Our results demonstrated that the RA tonsil microbiome differs from that of healthy controls, which correlates with systemic autoimmune changes and may potentially drives initiation of RA.

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Disclosure of Interest: None declared

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FRIDAY, 16 JUNE 2017

Rheumatoid arthritis - prognosis, predictors and outcome

FRI0085 **NUMBER OF PEPTIDE-SPECIFIC ANTI-CITRULLINATED PEPTIDE ANTIBODIES IN SYNOVIAL FLUID AND IN SYNOVIAL FLUID IMMUNE COMPLEXES ASSOCIATE WITH DEGREE OF TRIAMCINOLONE HEXACETONIDE FOR KNEE SYNOVITIS IN RHEUMATOID ARTHRITIS**

A. Sohrabian¹, L. Mathsson Alm^{1,2}, M. Hansson³, J. Lysholm⁴, M. Cornillet⁵, A. Knight⁶, K. Skriner⁷, G. Serre⁵, A. Larsson⁸, T. Weitoft⁹, J. Rönnelid¹.

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Background: We have described a planar microarray for the determination



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Farnesylthiosalicylic acid reduces disease severity in the collagen type-II induced arthritis mouse model by inhibiting Ras Signaling in pathogenic T cells

Morad Zayoud, Einva Vax, Galit Elad Sfadia, Yoel Kloog and Itamar Goldstein

J Immunol May 1, 2017, 198 (1 Supplement) 224.7;

Article

Info & Metrics

Abstract

Background Ras proteins are vital for normal T cell activation, and downstream effectors of Ras include the MEK/ERK, PI3-kinase/AKT, and NF- κ B pathways. T cells from Rheumatoid Arthritis patients exhibit abnormal activation of the Ras/MEK/ERK pathway. The small molecule Farnesylthiosalicylic acid (FTS) blocks the interaction between Ras proteins and their prenyl binding chaperones, attenuating plasma membrane localization and signaling.

Objectives To investigate the immunomodulatory effect of FTS alone or combined with methotrexate (MTX) in the DBA/1 mouse collagen type-II induced arthritis (CIA) model.

Methods Arthritis was induced in 8–10 week old male DBA/1 mice by immunization with collagen type-II (CII) and complete Freund's adjuvant (CFA). Animals were treated semi-prophylactically with once daily oral FTS (100 mg/kg); weekly i.p injection of MTX (0.5 mg/kg), FTS combined with MTX, or daily oral vehicle solution (control). Arthritis severity was scored daily from disease onset until study termination and multiple immunological markers of inflammation were analyzed.

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Results Our data from the mouse CIA model show that the therapeutic efficacy of FTS was similar to MTX, and both drugs significantly reduced arthritis severity compared to CMC controls. Importantly, FTS significantly inhibited the production of pathogenic anti-CII autoantibodies and upregulation of serum IL-6 and IL-17A compared to control arthritic mice. The in depth, multiplex, analysis of the effect of FTS on the T cell cytokine response to CII, revealed strong suppression of IL-22, IL-17, IL-9, GM-CSF and TNF production. Importantly, FTS therapy positively correlated with reduced p-ERK1/2 and p-AKT levels in splenic lymphocytes.

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