

AD_____

AWARD NUMBER: W81XWH-14-1-0610

TITLE: Studying the Immunomodulatory Effects of Small Molecule Ras-Inhibitors in Animal Models of Rheumatoid Arthritis

PRINCIPAL INVESTIGATOR: Yoel Kloog

RECIPIENT: Tel Aviv University
TEL AVIV 69978
Israel

REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE October 2015		2. REPORT TYPE Annual		3. DATES COVERED 30Sept2014-29Sept2015	
4. TITLE AND SUBTITLE Studying the Immunomodulatory Effects of Small Molecule Ras-Inhibitors in Animal Models of Rheumatoid Arthritis				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-14-1-0610	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Yoel Kloog Email: kloog@post.tau.ac.il				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) . Tel Aviv University TEL AVIV 69978 Israel				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT attached					
15. SUBJECT TERMS-					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)
			UU		

[SF298]

Note: An abstract is required to be provided in Block 14

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 TCR-dependent activation following antigen recognition. Defective Ras GTPases signaling has been associated with T cell anergy, and accordingly increased expression of active Ras was shown to reverse anergy and to restore IL-2 production. Importantly, T cells from patients with Rheumatoid Arthritis (RA) display augmented activation of the Ras/Raf/MEK/ERK1/2 signaling pathway, and accordingly overexpression of active K-RAS in normal CD4+ T cells has been shown to promote T cells reactivity to relevant autoantigen in RA. Thus, Ras GTPases appear to be a promising molecular target for inhibiting T cell activation in RA. Based on an innovative concept Kluog (the partnering PI) and colleagues discovered a potent non-toxic inhibitor of Ras, Farnesylthiosalicylic acid (FTS). This small molecule does not belong to the class of farnesyltransferase inhibitors (FTIs) that failed in clinical trials. It interferes with the interactions between Ras and distinct prenyl-binding chaperone proteins that are vital for the proper plasma membrane (PM) localization and signaling dynamics of Ras-GTPases, and indeed FTS dislodges the classical H/N/K-Ras GTPases from the PM and inhibits their effective downstream signaling. In multiple preclinical animal studies it has been shown that FTS effectively inhibited *in vivo* tumor growth of oncogenic K/N-Ras-dependent cancers. Thus, in collaboration with Concordia Pharmaceuticals Inc., FTS was developed into an oral drug, Salirasib®. The drug has been 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 pancreatic cancer, hematological malignancies (NCT00867230; M.D. Anderson Cancer Center, TX), and in phase II clinical study in non-small-cell lung cancer patients (NCT00531401; Memorial Sloan Kettering Cancer Center, NY). Thus, Salirasib® is the only available successful Ras GTPases inhibitor that reached Phase II clinical trials, and moreover received an orphan drug designation by the FDA for the treatment of pancreatic cancer.

Importantly, we have extensively studied the effects of FTS and related derivatives (e.g., FTS-Amide and FTS-methoxymethylester), in multiple pre-clinical animal models of autoimmune inflammation, such as: experimental autoimmune encephalomyelitis; Type-1 diabetes; colitis and others. More recently, our preliminary studies in the adjuvant-induced arthritis (AIA) rat model – a classical animal model for RA – imply that FTS attenuates disease manifestation, as assessed by: clinical scores; MRI imaging; histopathology; and serum levels of pro-inflammatory cytokines. Thus, our working hypothesis is that Salirasib® has a good potential to be a “silver bullet” drug for RA and other T cell-dependent autoimmune disorders.

Our objectives are to further test this hypothesis in the AIA model as well as in another established animal model of RA, the collagen type-II induced arthritis (CIA) in DBA/1 (H-2_q) mice. In parallel we will study *in vitro* the effects of FTS and its different newer derivatives on a wide range of T cell functions and signaling networks implicated in RA. For the therapeutic treatment protocol, FTS will be administered orally by gavage, once daily, starting immediately after disease initiation. Multiple modalities will be used to assess joint inflammation/damage and the immune response, as follows: arthritis clinical scores; MRI scans, micro-CT; histopathology examination by a blinded pathologist; serum cytokine profiles; T cell subset analysis (e.g.,

Foxp3+ Treg, Th1, Th17, etc.) by polychromatic flow cytometry. Additionally, the activation of different Ras downstream effectors and relevant cellular programs will be analyzed by: Western blotting, Affymetrix GeneChip® whole-transcript arrays, and quantitative real-time PCR analysis. The proposed project is highly relevant to the FY13 PRMRP topic area of Rheumatoid Arthritis (RA). The short-term impact of our research will be an improved understanding of the role of Ras GTPases in shaping, tuning, and regulating the T cell response, and the effects of Ras inhibitors on the pathogenesis of inflammatory arthritis in two important pre-clinical models of RA. We envision that the long-term impact of our proposed research plan will be the introduction of a new class of synthetic drugs, orally available small molecule Ras-inhibitors such as Salirasib®, to advance the clinical management of RA patients with conceivably fewer side effects and reduced healthcare system costs as compared to current biologic drugs.

Table of Contents

	<u>Page</u>
1. Introduction	5
2. Keywords	5
3. Overall Project Summary	5-12
4. Key Research Accomplishments	12-13
5. Conclusion	13-14
6. Publications, Abstracts, and Presentations	14
7. Inventions, Patents and Licenses	14
8. Reportable Outcomes	14-15
9. Other Achievements	15
10. References	15-16
11. Appendices	16-29

- 1. INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Ras GTPases are molecular switches that regulate key cellular processes and in T cells they are necessary for proper TCR-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, 4). 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 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 has been 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, and moreover received an orphan drug designation by the FDA for the treatment of pancreatic cancer (5, 6). In the first year our aim was to complete Major Task 1: "Test in the rat AIA model of RA (rheumatoid arthritis) the prophylactic and therapeutic efficacy of FTS (a targeted synthetic DMARD) on relevant clinical outcomes and immunological parameters".

- 2. KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

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

- 3. OVERALL (First Year) PROJECT SUMMARY:**

In *Major Task 1* of the SOW we proposed to test in the Lewis rat Adjuvant induced arthritis (AIA) model of Rheumatoid Arthritis (RA) the prophylactic and therapeutic effects of FTS.

Subtask A: Analyze the prophylactic / therapeutic effects of FTS on clinical AIA progression and joint pathology.

In the first set of experiments we studied extensively the clinical effects of prophylactic dosing of FTS and its derivative F-FTS on the progression of AIA in Lewis rats as compared to vehicle control treatment and treatment with methotrexate (MTX) that is an effective immunosuppressive drug widely used in RA patients.

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, including drugs that are currently being tested in clinical trials or are currently used as novel therapeutics in RA (7). 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 hallmarks of this model

are reliable onset and progression of robust and easy to measure polyarticular inflammation associated with marked tissue/synovial inflammation and subsequent articular bone resorption.

In our hands, in agreement with previous works, clinically evident arthritis in the hind paws (ankle joint) usually developed 9-10 days post CFA injection and progressed, in control animals, into a severe polyclonal arthritis within a few days.

We employed two dosing models:

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

Clinical Assessment:

To assess disease progression, both clinical scoring (0-16 scale) caliper measurements of ankle joint width were done once prior to the onset of arthritis, and subsequently every other day until the study was terminated.

Histopathological Assessment:

At 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 (8).

- i. To determine whether prophylactic dosing with FTS suppresses the clinical signs of AIA, we started daily treatment of rats from day +1 in the experimental arm with oral FTS (100 mg/kg). Control rats received oral solution of 0.5% carboxy methyl cellulose (CMC vehicle). As a "positive control" we treated a group of Lewis rats with weekly *i.p* injection of MTX (0.5 mg/kg). AIA progression and severity was scored on a clinical index of 0 to 16 (0-4 scale for each paw). In parallel, we also assessed disease progression by a more "objective" method, using caliper measurements of ankle joint width prior to the onset of arthritis, and then every other day until the study was terminated on day 21 post CFA injection. As shown in Figure 1, the arthritis was significantly less severe, as evident by both clinical scoring and caliper measurements of ankle joints, in the FTS treatment group compared to CMC vehicle treated rats. Importantly, the clinical effects of FTS, with respect to arthritis severity and joint swelling, were of similar magnitude to the effects of MTX. Of note the latter drug is a FDA approved disease-modifying antirheumatic drugs (DMARDs) that is considered a cornerstone in the treatment of rheumatoid arthritis and other rheumatic diseases (9).
- ii. To further evaluate the effects of FTS on the joints of rats with AIA, we examined tissue sections stained with hematoxylin & eosin (Figure 2). Ankles of rats treated

with adjuvant were given scores of 0-5 for bone resorption and inflammation. The tissue sections from 0.5% 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 comparison, the sections from FTS-treated rats showed less joint tissue infiltration by mononuclear cells (inflammation scores ranging from 2 to 3, n=8), and less destruction of trabecular and cortical bone in the distal tibia (bone resorption scores ranging from 2 to 3). These set of experiments using at least 8 rats per group and repeated >3 times lead us to conclude that prophylactic FTS dosing was an effective therapy that reduced both the clinical severity (Figure 1A) and ankle joint swelling as measured by caliper (Figure 1B).

Responsible PI: Itamar Goldstein, Tel Aviv University.

- iii. In accordance with our Institutional Animal Care and Use Committee (IACUC) guidelines, the PIs are required to ensure that the 3Rs principles are implemented. Thus, to comply with the principle of reduction, the partnering PIs have agreed to combine some experiments (subtasks) related to task 3 at this stage. Because, we already had a MTX and FTS treatment groups in Task 1 related experiments, we could easily test the efficacy of FTS as an add-on therapy to MTX during this report period and ahead of schedule. The results of these experiments were outstanding, as we found that the combined treatment of FTS + MTX almost completely inhibited the development of clinically evident arthritis as well as ankle joint swelling by caliper measurements (Figure 1A&B). Moreover the tissue sections from FTS+ MTX treated rats 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). In comparison, as detailed above, the ankle joint tissue sections from CMC vehicle treated rats, generally showed extensive infiltration with mononuclear cells and bone destruction.

Responsible PI: Itamar Goldstein, Tel Aviv University.

- iv. In an exploratory experiment we compared "head-to-head" (n=6 rats per group) the efficacy of FTS versus its newer derivative 5-Fluoro-FTS (F-FTS). The latter compound has been previously shown by Kloog's lab to be a more potent oral inhibitor of RAS signaling compared to the parent compound (10). We found that prophylactic dosing with F-FTS (60mg/kg) from day +1 post CFA injection had a protective effect on the clinical severity of AIA and was not associated with obvious *in vivo* toxicity. The efficacy of F-FTS was minimally superior compared to FTS treatment per the statistical analysis using one way ANOVA and post-hoc Bonferroni's Multiple Comparison Test (Figure 3). To further explore the therapeutic potential of F-FTS in RA models, including its effects on the various disease outcomes we have requested Ricerca Biosciences, LLC to prepare 50 grams of 5-Fluoro-FTS (Concord, OH 44077, USA). We plan (pending the shipment of the 5-Fluoro-FTS lot to TAU with the certificate of analysis and

supporting analytical data) to complete this set of experiments during the first 6 months of the second year of the award.

Responsible PI: Yoel Kloog, Tel Aviv University.

- v. In the next set of experiments we tested the efficacy of FTS when the treatment was given in the "therapeutic model", meaning that therapy was initiated at day +9 (onset of arthritis in the hind paws). As shown in figure 4 A&B, 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, therapeutic dosing with dexamethasone (positive control) was effective in decreasing the severity of AIA compared to CMC vehicle (negative control). 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 dosing of FTS+MTX showed a much lower efficacy in the two clinical assessment parameters (Figure 4C).

To further explore this issue we plan to test the therapeutic efficacy of the newer FTS derivative, F-FTS, once it will be synthesized in a large quantity (see above) either as a single agent or as an add-on to MTX. Obviously, given our current data we cannot anticipate the results. If we find that the efficacy of F-FTS started only at the disease onset is minimal even as an add-on to MTX, it may suggest that in the AIA model inhibiting the RAS-ERK pathway in lymphocytes long after T cell antigenic stimulation has been induced (by a very robust stimulus) cannot reverse target organ inflammation and damage. The observation that FTS+MTX treatment in the therapeutic dosing model displayed only minimal efficacy (Figure 4C), implies that the AIA therapeutic dosing model may be imprecise for predicting the activity of certain agents in RA, such as MTX that is the corner-stone drug in present RA therapy. **Responsible PI: Yoel Kloog, Tel Aviv University.**

Subtask B: 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 (tissue levels of p-ERK and Ras-GTP).

- vi. Activation of the RAS-ERK pathway, marked by elevated levels of p-ERK, has been postulated to be pivotal in regulating T-cell sensitivity to antigenic stimulation (refs). Increased pERK activity sustains several positive feedback loops important for T cells activation, survival and proliferation (refs). Inhibition of RAS-ERK pathway is shown to induce T cell anergy coupled with defective IL-2 production (ref). Importantly, T cells isolated from synovia of RA patients have been found to express higher levels of K-Ras, B-Raf and their downstream effector phospho-ERK1/2 [refs]. Therefore, to verify that FTS therapy was indeed associated with effective blockade of the RAS-ERK pathway in lymphocytes, we analyzed p-ERK levels in splenocytes (mostly lymphocytes) at necropsy, as a molecular marker for effective targeting of Ras signaling. Our results show that

p-ERK levels were indeed significantly reduced in splenic lymphocytes *in vivo* (Figure 5). This reduction positively correlated with the good clinical efficacy of FTS as well as of FTS combined with MTX. Of note, the therapeutic dosing of FTS and MTX from day +9 was also associated with effective targeting the RAS-ERK pathway *in vivo*, although it was clinically less effective compared to parallel prophylactic dosing. This result implies that in the AIA therapeutic dosing model blocking the RAS-ERK pathway long after T cell antigenic stimulation has been induced is associated with a limited protective effect with respect to target tissue inflammation.

Responsible PI: Yoel Kloog, Tel Aviv University.

- vii. Next, we analyzed the effects of the various treatment protocols on the immune response to CFA, particularly on the CD4⁺ 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, particularly useful is the splenomegaly that occurs as a marker of the systemic inflammation induced by CFA (7, 8). Thus, at study termination both peripheral blood samples were collected and spleens were harvested. A single cell suspension was subsequently obtained by means of mechanical dissociation. Peripheral blood and spleen cells were then immunostained with relevant cell markers and analyzed by polychromatic flow cytometry for changes in leukocyte populations (T cells, granulocytes, CD4⁺T cells and CD8⁺ T cells). Our results show that both in peripheral blood and in spleen, adjuvant injection resulted in a statistically significant increase in granulocyte percentage (reflecting the induction of extramedullary hematopoiesis in the spleen by CFA) that was not significantly affected by the various treatments. In addition, there was a trend towards increase in the ratio of CD4 to CD8 T cells in the spleens of FTS treated rats which was mainly due to an increase in the percentage of CD4⁺ T cells in the spleen (Figure 6). Importantly, we also found that this trend was associated with statistically significant increase in the percentage of CD4⁺ Foxp3⁺ regulatory T cells (Treg) in the spleens of FTS treated vs. vehicle treated arthritic rats (Figure 7).

Responsible PI: Itamar Goldstein, Tel Aviv University.

- viii. As both TH1 and more recently 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. For intracellular cytokine detection, the T cells isolated from spleens were activated with 20ng/ml PMA and 0.8 μ M ionomycin in the presence of 2 μ g/ml monensin. Subsequently, the cells were fixed, permeabilized and immune-stained with anti-IFN- γ (TH1 cells) and IL-17 (TH17 cells) flouochrome-tagged monoclonal antibodies (mAbs). Our data show that FTS therapy was associated with significantly lower numbers of CD3⁺ CD4⁺ TH1 and TH17 cells in the spleens compared to CMC vehicle treated mice. This anti-inflammatory effect of FTS therapy was more significant when the drug was administered as an add-on therapy to MTX, such as the increase in the percentage of TH1 and TH17 cells

following CFA injection was minimal, as compared to naive non-arthritic control rats (Figure 8A&B). These immunological data positively correlated with the observed clinical outcomes of the various treatment protocols, highlighting the finding that the combined FTS+MTX treatment was associated with a stronger suppression of ankle joint swelling as compared to each drug alone (see also Figures 1 and 2). **Responsible PI: Itamar Goldstein, Tel Aviv University.**

- ix.** To further address the effects of FTS on the normal inflammatory response to CFA we analyzed the levels of the cytokines IL-17 and IL-6 in serum samples collected at various time points. In accordance with the intracellular cytokine detection data, we 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 suppressed IL-17 levels >90%, as compared to CMC/vehicle treated arthritic rats. In general the levels of IL-17 in day +20 sera were low approaching the limit of detection of rat IL-17 by the ELISA test, likely reflecting the termination of the acute immune response to CFA, such as it was difficult to draw unequivocal conclusions on the effects of the different treatments at this late time point (Figure 9A). Regarding serum IL-6 levels, in repeated experiments using readymade validated ELISA kits (Rat IL-6 Platinum ELISA from eBioscience, Inc., USA), we could only detect low levels of this cytokine approaching the limit of detection of rat IL-6 (20-30 pg/mL) in all day +10 (arthritis onset) and day +16 (disease peak) sera tested regardless of the animal treatment protocol (data not shown).

Responsible PI: Yoel Kloog, Tel Aviv University.

- x.** Because it is known that IL-6 stimulates the acute phase production of C-reactive protein (CRP) in the liver, we decided to measure by ELISA serum levels of CRP as a 'surrogate' marker for IL-6 levels and systemic immune activation to CFA. We found as predicted that CFA injection induced a substantial increase in CRP levels in CMC vehicle treated arthritic rats compared to naïve healthy littermate rats. In contrast, the combined FTS+MTX completely abolished this increase at the two time points analyzed (days +10 and +14). Both FTS and MTX reduced CRP levels at day +10, but were less effective, as a single agent, in suppressing the observed CFA-induced increase in this classical acute phase reactant on day +14 (Figure 9B). These findings also showed strong positive correlation with the ankle swelling data, directly reflecting target tissue inflammation that was indeed strongly inhibited by the combined treatment and only partially prevented by FTS or MTX alone.

Responsible PI: Yoel Kloog, Tel Aviv University.

- xi.** Our most recent set of experiments were planned to gain additional mechanistic insights into the effects of FTS on AIA outcome by comparing the gene expression (transcriptome) profiles of the draining lymph node cells (superficial inguinal and para-aortic) of the various experimental groups (n=3 per treatment group). The rats were immunized intradermally with 100µl of CFA at the base of the tail and treated starting day +1 (prophylactic dosing) with FTS, FTS+MTX,

MTX or CMC vehicle. The rats were sacrificed on day +15 at the peak of the arthritis in the hind paw, and their draining lymph nodes were harvested downstream analysis. Total RNA was extracted from LN cells using Trizol (Invitrogen Inc., USA) and purified using the Direct-zol™ RNA Kits (Zymo Research Inc., USA). These RNA samples are presently being processed at our Genomics Core Facility, as the input for the amplification and generation of biotin-labeled fragment cRNA for expression analysis using the Rat GeneChip® Gene 2.0 ST Array System, according to the manufacture's guidelines. This array system has been designed to provide the ability to evaluate whole-transcriptome gene expression both at the gene and exon levels, allowing the study of transcript variants and alternative splicing events. We predict that in the next 3-months we will finalize the gene array analysis with high coverage across the entire transcript (>17,000 Entrez genes), enabling us to define by various genomics and bioinformatics approaches the molecular pathways and gene networks that mediate the in vivo biological effects of FTS therapy on the lymphocytic response to CFA in the AIA model.

Responsible PI: Itamar Goldstein, Tel Aviv University.

- xii.** To further address the effect of FTS treatment on serum levels of an array of rat inflammation-related cytokines we used the Quantibody array platform (Quantibody Rat Inflammation Array 1, RayBiotech, Inc., USA), a multiplexed sandwich ELISA-based quantitative array platform that enables accurate determination of the concentration of the following cytokines in serum: IFN- γ , TNF- α , IL-4, IL-6, IL-10, IL-1- α , IL-1- β , IL-2, IL-13, and MCP-1. Based on previous studies we analyzed the serum samples from arthritic rats at the onset of disease (day +10) for the levels of these cytokines. However, by this Quantibody Rat Inflammation Array 1, we could only detect low levels for the majority of the analytes, regardless of the AIA treatment protocol (we analyzed selected samples from two different experiments). This precluded us from drawing concrete conclusions on the effects of FTS on these cytokines at this stage. The problem we have encountered in our attempts to reliably detect treatment dependent changes in serum cytokines other than IL-17 by two different methodologies, classical ELISA and multiplexed sandwich ELISA, may cause some delay finalizing all the aims of major Task 1/ Subtask B. To resolve this issue we plan to use a newer Luminex xMAP bead-based immunoassay technology that theoretically should offer higher sensitivity and specificity compared to traditional ELISA based approaches. Unlike traditional ELISA, the Luminex xMAP capture antibodies are covalently attached to the bead surface, allowing for a greater surface area coupled with a free 3-D solution/liquid environment to react with the analytes. Moreover, it allows assay flexibility in a multiplex array format, and hence less limitations in regard to sampling volume, cost, and labor. Thus in the next 3-months we will further attempt to address the effects of FTS treatment on a 12-Plex array of rat serum cytokines at multiple time points (days +5, +10 and +14) using The Bio-Plex Pro Rat Cytokine Th1/Th2 12-Plex array Immunoassay (from Bio-Rad Laboratories, Inc., USA).

Responsible PI: Itamar Goldstein, Tel Aviv University.

- xiii.** As stated in the Specific Aim 2: "Targeting Ras signaling with FTS and FTS derivatives in the collagen induced arthritis (CIA) mouse model", we have initiated on time the necessary regulatory review and approval processes for animal studies described in Task 2 to be initiated during the second year. Accordingly, the animal protocol titled "Studying the Immunomodulatory Effects of Small Molecule Ras-Inhibitors in the Adjuvant/Collagen Induced Arthritis Model", IACUC protocol L-14-023 / USAMRMC proposal PR130028.04, has already been approved by TAU-IACUC (11/05/2014). Presently, this protocol has completed the preliminary review and has been forwarded for final review by the USAMRMC Animal Care and Use Review Office, for compliance with Federal and Department of Defense regulations and guidelines concerning the use of animals. **Responsible PIs: Itamar Goldstein & Yoel Kloog, Tel Aviv University.**

Regarding changes to originally proposed methods, we decided not to perform new imaging studies of the ankle joint by MRI and/or micro-CT, outlined in the SOW, as additional clinical outcome assessment tools. This decision stems from the fact that in the period between the original award application due date and the award start date, the Kloog Lab (partnering PI) performed additional analyses of the MRI and micro-CT imaging data obtained during the early AIA experiments described in the background section of the original award application. These data were also recently published by the Kloog Lab (11), a few months before this award start date. Subsequently, we think that the current published imaging data is sufficient to support our working hypothesis that prophylactic dosing with FTS reduces joint inflammation (MRI data) and bone erosions (micro-CT data). The imaging data showed a strong positive correlation with the clinical outcome assessment of disease progression/severity, particularly with ankle joint swelling and erythema. Moreover, we realized that the histopathological outcome assessment of the ankle joints, is a more sensitive and accurate outcome measure to assess the effects of therapy on disease severity. Importantly, we also recognized that the imaging data does not provide any additional mechanistic insight into the *in vivo* therapeutic efficacy of FTS on the development of a robust polyclonal arthritis following the injection of CFA to Lewis rats. Lastly, as the proposed imaging studies are associated with substantial distress to the animals, and given our duty to balance the needs of the study with that of the welfare of the animals in compliance with refinement strategies required by TAU-IACUC and the USAMRMC Animal Care and Use Review Office, we have decided that the information to be gained from additional imaging studies may be ethically unacceptable. As detailed above, we furthermore do not think that this adjustment represents a major change from the original approved SOW, and the funds allocated for imaging will be used for more informative outcome measures such as: serum markers of inflammation (IL-17 ELISA kits, cytokines arrays, CRP), immunological studies of relevant T cell subsets, and analysis of relevant molecular pathways by WB, and gene networks by GeneChip® Rat arrays and qRT-PCR of various gene transcripts.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Treatment with FTS in the prophylactic protocol significantly reduces (~ 50% inhibition) the clinical outcome of AIA as assessed by clinical scoring and ankle swelling by caliper measurements (moderate efficacy)
- Treatment with FTS in the prophylactic protocol significantly reduces joint inflammation and bone resorption in the ankle joint per the histopathological assessment.
- Treatment with FTS as an add-on to low dose MTX in the prophylactic dosing model almost completely suppressed (~90% inhibition) the development of AIA, as assessed by clinical scoring and ankle swelling by caliper measurements (high efficacy).
- Treatment with FTS as an add-on to low dose MTX in the prophylactic dosing model significantly reduces joint inflammation and bone resorption in the ankle joint per the histopathological assessment.
- Treatment with FTS alone or as an add-on to low dose MTX in the therapeutic dosing model showed minimal efficacy with respect to the clinical outcome of AIA (clinical scoring and ankle diameter) and joint histopathology.
- FTS prophylactic treatment was associated with statistically significant increase in the percentage of CD4+ Foxp3+ Treg cells in the spleens of FTS treated compared to CMC/vehicle treated arthritic rats.
- FTS prophylactic therapy was associated with lower percentage of TH1 and TH17 cells in arthritic rats' spleens and this immunosuppressive effect was more pronounced when FTS was prophylactically co-administered with MTX.
- FTS prophylactic therapy was associated with reduced serum IL-17 levels at the peak of the disease ~50% inhibition of the CFA-induced increase compared to normal control levels, whereas the combined treatment of FTS+MTX inhibited IL-17 increase from normal levels by approximately 90%.
- FTS prophylactic therapy reduced the observed increase in the IL6-dependent acute phase reactant, CRP, at the onset of AIA (day +10), and the combined FTS+MTX treatment completely abolished this CFA-induced increase as compared to control naïve rats.

5. CONCLUSION:

During the first year of the award we had two major conclusive findings with significant medical implications: (i) the prophylactic treatment with the small molecule FTS, a first-in-class oral selective inhibitor of RAS signaling, provides a protective effect on the severity of AIA by all outcome measures (Clinical assessment, Histopathological assessment, serum markers of inflammation, etc.), and (ii) the remarkable discovery that prophylactic dosing of FTS as an add-on to MTX provides a very strong protective effect (~90% effect) against the development of AIA by all clinical and laboratory outcomes measured.

We consider these findings important with respect to the future medical treatment of RA patients in the Veterans Health Administration's primary care and specialty clinics for the following reasons:

- Given the excellent track record of the AIA model for predicting activity of anti-RA drugs in patients, these major findings strongly supports our view that FTS and/or its newer derivative F-FTS particularly in combination with MTX have a good potential to show clinical efficacy in RA patients.
- The finding that drug combination of FTS and MTX can suppresses autoimmune arthritis following CFA injection, is highly relevant to modern RA therapy, as methotrexate is presently the cornerstone of RA therapy being used alone or in various drug combination in a large percentage of patients. Importantly, most biologics or new synthetic targeted DMARDs are more effective in combination with MTX and are not routinely prescribed as monotherapy.
- Most clinical trials of new RA drugs are designed to prove clinical efficacy of the new drug as an add-on to MTX. Thus, our finding imply that FTS can indeed show superior efficacy in clinical trials as an add-on to MTX therapy compared to MTX + placebo therapy.
- The oral formulation of FTS, the drug Salirasib (Concordia Pharmaceuticals, Inc., USA), received an orphan drug designation by the FDA for the treatment of pancreatic cancer, as it showed no dose-limiting toxicities or major adverse effects in phase I clinical trials that recruited patients with advanced cancers (5, 6). This fact together with our present findings may help speed the clinical development of the drug as a first-in-class oral DMARD.

Our future aims are to accomplish the goals and tasks, as described in the original project narrative and the approved SOW for the second and third year of the award. Briefly, our plans are as follows: (i) To validate in the collagen induced arthritis (CIA) mouse model the prophylactic and/or therapeutic effects of FTS and FTS derivatives, (ii) to gain further mechanistic insight on a immunological and molecular level into the effects of FTS and its analogues on a wide range of mouse T cell functions and biologically relevant molecular pathways, and (iii) to publish a comprehensive manuscript describing the mechanisms (immunological and molecular) that mediate the therapeutic (Immunomodulatory) effects of small molecule Ras inhibitors in the AIA and CIA models of RA, including mechanistic data derived from the *in vitro* work detailed in Task 4 (To analyze the effects of FTS on signaling events downstream of T cell antigen receptor stimulation).

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the first year:

“Nothing to report.”

b. List presentations made during the first year

“Nothing to report.”

7. INVENTIONS, PATENTS AND LICENSES:

“Nothing to report.”

8. REPORTABLE OUTCOMES:

We provide herewith a list of reportable outcomes representing a scientific advance that makes a meaningful contribution toward the understanding and treatment of Rheumatoid Arthritis.

- Prophylactic treatment with the small molecule oral inhibitor of RAS signaling, FTS, has a protective effect on the severity of adjuvant-induced arthritis by all outcome measures: disease score, ankle swelling, Histopathological assessment, serum IL-17 and CRP levels, percentage of splenic Treg, TH1 and TH17 cells and target tissue levels of RAS-GTP and p-ERK. This reportable outcome supports our understanding of T cell dependent autoimmune processes (including RA) by showing a vital role for the RAS-ERK cascade in the development of experimental arthritis. It also supports our view that FTS and/or its derivatives should be further developed and tested as a novel class of targeted synthetic DMARDs.
- Prophylactic treatment with FTS as an add-on to MTX showed a very strong protective effect against the development of adjuvant-induced arthritis by all the outcome measures (as detailed above), which was moreover significantly superior to FTS or MTX therapy alone. Importantly, this new combination therapy practically suppressed all clinical sign of disease and the increase of serum markers of inflammation (CRP and IL-17 levels) induced by CFA in the arthritic control rat groups. This is particularly relevant to RA treatment, as methotrexate is presently referred to as the "cornerstone of therapy" and is used alone or in various combination therapies in most RA patients treated at Veterans Health Administration's primary care and specialty clinics. For example, most of the new generation RA drugs (biologics and targeted synthetic DMARDs) are routinely prescribed as an add-on therapy to MTX as they show reduced efficacy as monotherapy. Thus, this reportable outcome strongly implies that FTS like other new RA drugs can in combination with MTX significantly improve the efficacy of oral DMARD therapy in RA patient.

9. OTHER ACHIEVEMENTS: “Nothing to report.”

10. REFERENCES:

1. P. E. Fields, T. F. Gajewski, F. W. Fitch, Blocked Ras activation in anergic CD4+ T cells. *Science* **271**, 1276-1278 (1996).
2. Y. Zha *et al.*, T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase- α . *Nat Immunol* **7**, 1166-1173 (2006).
3. K. Singh *et al.*, K-RAS GTPase- and B-RAF kinase-mediated T-cell tolerance defects in rheumatoid arthritis. *Proc Natl Acad Sci U S A* **109**, E1629-1637 (2012).
4. K. Singh *et al.*, ERK-dependent T cell receptor threshold calibration in rheumatoid arthritis. *J Immunol* **183**, 8258-8267 (2009).
5. E. Bustinza-Linares, R. Kurzrock, A. M. Tsimberidou, Salirasib in the treatment of pancreatic cancer. *Future Oncol* **6**, 885-891 (2010).
6. D. Laheru *et al.*, Integrated preclinical and clinical development of S-trans, trans-Farnesylthiosalicylic Acid (FTS, Salirasib) in pancreatic cancer. *Invest New Drugs* **30**, 2391-2399 (2012).
7. A. Bendele, Animal models of rheumatoid arthritis. *J Musculoskelet Neuronal Interact* **1**, 377-385 (2001).

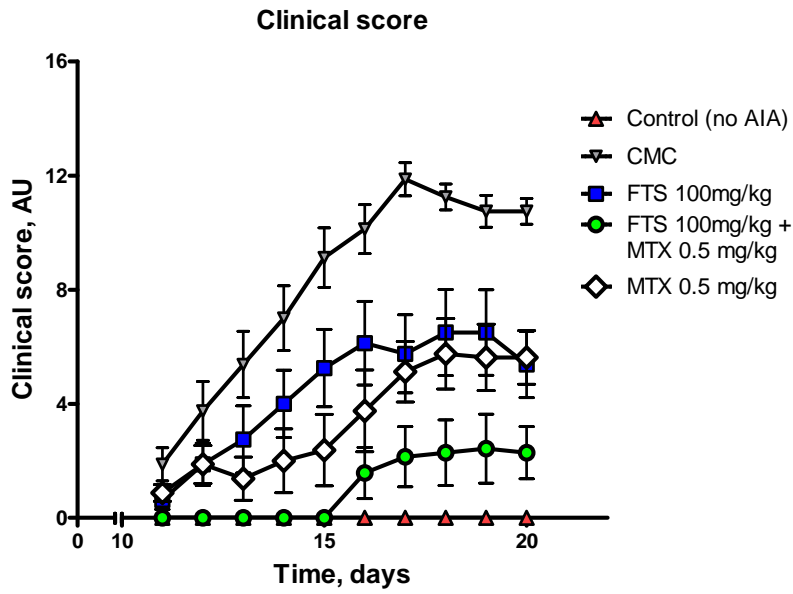
8. A. Bendele *et al.*, Animal models of arthritis: relevance to human disease. *Toxicol Pathol* **27**, 134-142 (1999).
9. E. S. Chan, B. N. Cronstein, Methotrexate--how does it really work? *Nat Rev Rheumatol* **6**, 175-178 (2010).
10. A. Mor, Y. Kloog, G. Keren, J. George, Ras inhibition increases the frequency and function of regulatory T cells and attenuates type-1 diabetes in non-obese diabetic mice. *Eur J Pharmacol* **616**, 301-305 (2009).
11. E. Aizman *et al.*, Therapeutic effect of farnesylthiosalicylic acid on adjuvant-induced arthritis through suppressed release of inflammatory cytokines. *Clin Exp Immunol* **175**, 458-467 (2014).

11. APPENDICES:

Attached Figures (9 figures with legends)

Figure 1

A



One way ANOVA + Bonferroni's Multiple Comparison Test				
	CMC	FTS	FTS+MTX	MTX
No AIA	P < 0.001	P < 0.001	n.s.	P < 0.001
CMC		P < 0.001	P < 0.001	P < 0.001
FTS			P < 0.05	n.s.
FTS+MTX				P < 0.01

B

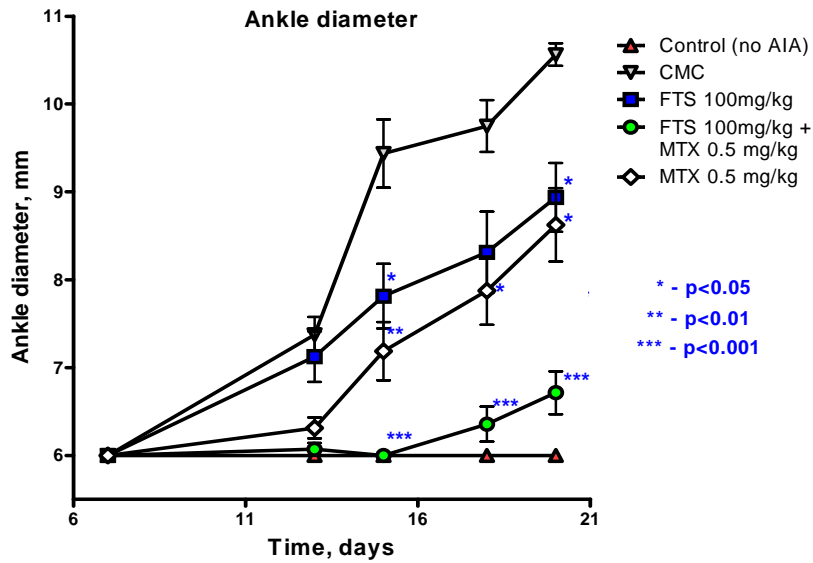


Figure 1

C



Figure 1: Prophylactic treatment with farnesylthiosalicylic acid (FTS) has a protective effect on the severity of adjuvant-induced arthritis (AIA). Rats were immunized with CFA and then graded daily for signs of arthritis by a clinical disease severity score **(A)** and every other day by caliper measurements of ankle joint width **(B)**. Rats in the experimental arms (n=8 per group) were treated starting day +1 with oral FTS (100 mg/kg), 0.5% carboxy methyl cellulose (CMC) vehicle solution, weekly i.p injection of MTX (0.5 mg/kg), or FTS combined with MTX. Statistical analysis of the effect of the various treatments on the clinical scoring **(A)** was done using One way ANOVA with Bonferroni's Multiple Comparison Test, and the results are summarized in the table inset. This analysis confirmed that treatment with FTS or MTX alone was protective compared to CMC, and moreover that the combined treatment (FTS+MTX) was superior compared to each compound alone. Statistical analysis of the effects of the various therapies on ankle joint diameter **(B)** was done by Student t-test at each time point. This analysis further confirmed that both FTS and MTX significantly reduce ankle swelling and that the combined treatment was significantly more potent. **(C)** Representative photographs of hind paws of FTS treated and CMC vehicle treated arthritic rats at study termination (day +20). Hind paws of a naive non-immunized rat are also shown for comparison. Black lines mark the ankle joint region and simulate ankle diameter.

Figure 2

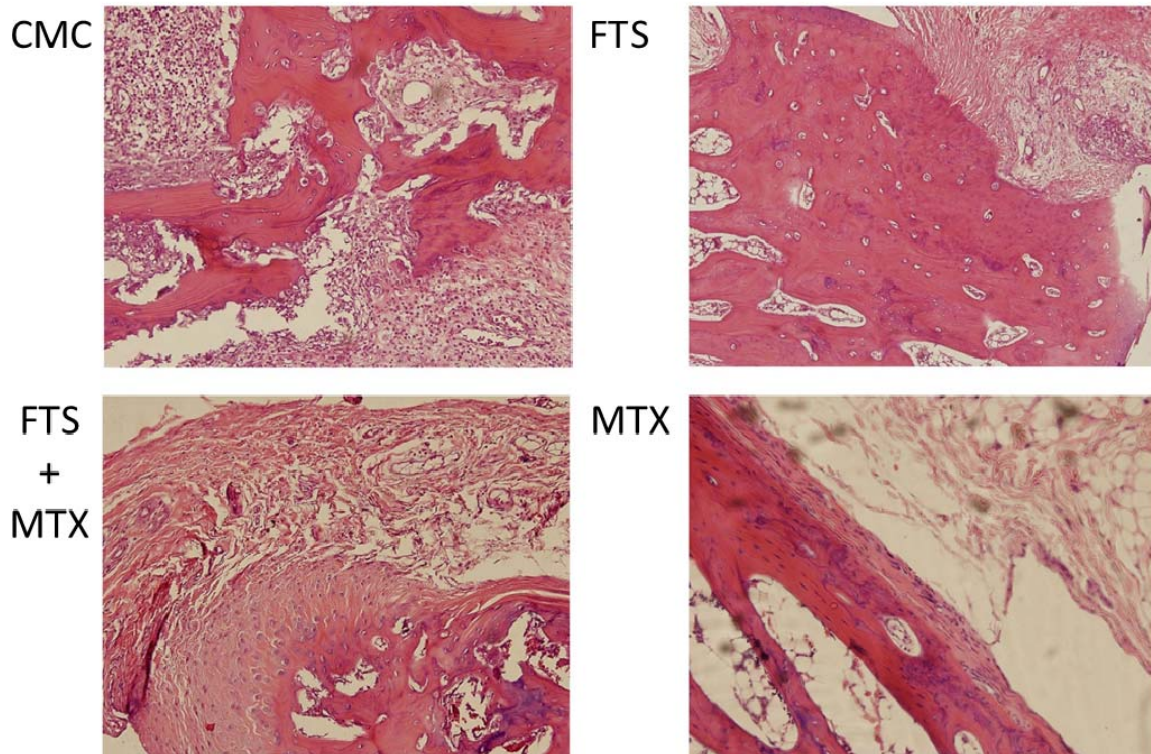


Figure 2. Representative histopathology of ankles of arthritic mice. The tibiotarsal (ankle) joint was transected at the level of the medial and lateral malleolus for Histopathological Assessment. Ankle joints were collected into 10% paraformaldehyde, for at least 24 hours and then placed in a decalcifier solution. Next, the joints were transected in the longitudinal plane and processed for paraffin embedding, sectioned and stained with H&E. Arthritic ankles were given scores on a scale of 0-5 for inflammation and bone resorption. The left upper image is a typical tissue section of CMC vehicle treated arthritic rat, showing extensive infiltration of joint tissue with mononuclear cells (inflammation score of 5) and severe bone destruction with full thickness defects in cortical bone and distortion of remaining cortical surfaces (bone resorption scores of 5). In comparison, the images of typical tissue sections from FTS (upper right), FTS+MTX (lower left), and MTX (lower right) treated rats show less joint tissue infiltration by mononuclear cells (inflammation scores ranging from 2 to 3), and less destruction of cortical bone surfaces (bone resorption scores ranging from 1 to 3). Shown are representative images out >3 experiments performed with n=8 rats in each treatment arm (magnification $\times 200$).

Figure 3

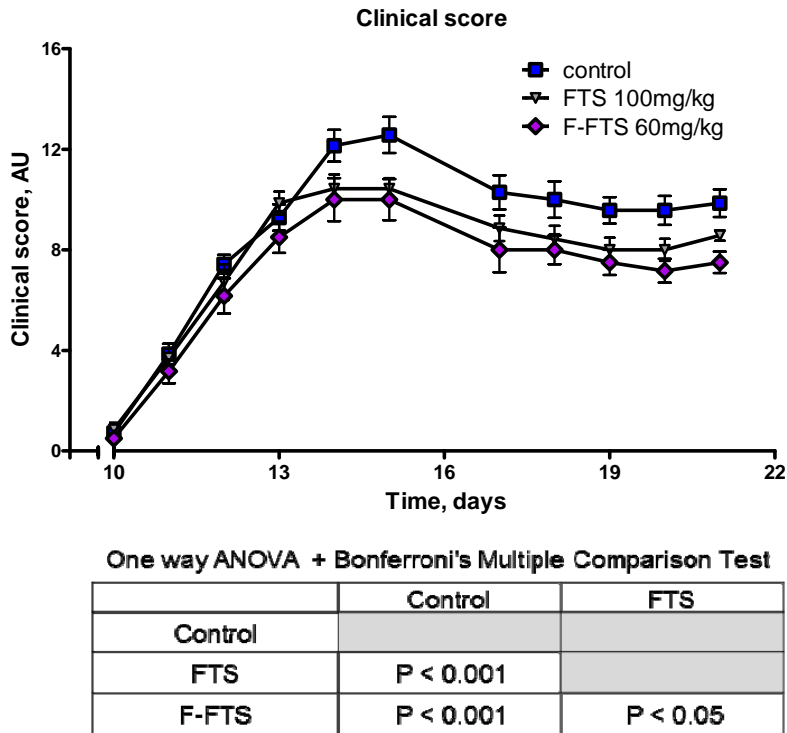


Figure 3. Comparison between clinical effect of FTS and Fluoro-FTS (F-FTS) on disease severity in AIA model. Rats (n=6) were treated daily starting day +1 with FTS (100mg/kg), 5-Fluoro-FTS (60mg/kg) or 0.5% CMC vehicle (control group) starting from day +1 post CFA injection. Thereafter, rats were graded daily for signs of arthritis by a clinical disease severity score starting from day +10 (usual time point of arthritis onset). Statistical analysis of the results was performed using *One way ANOVA and Bonferroni's Multiple Comparison Test* between the groups (n=6 per group) and the comparison is summarized in the table inset.

Figure 4

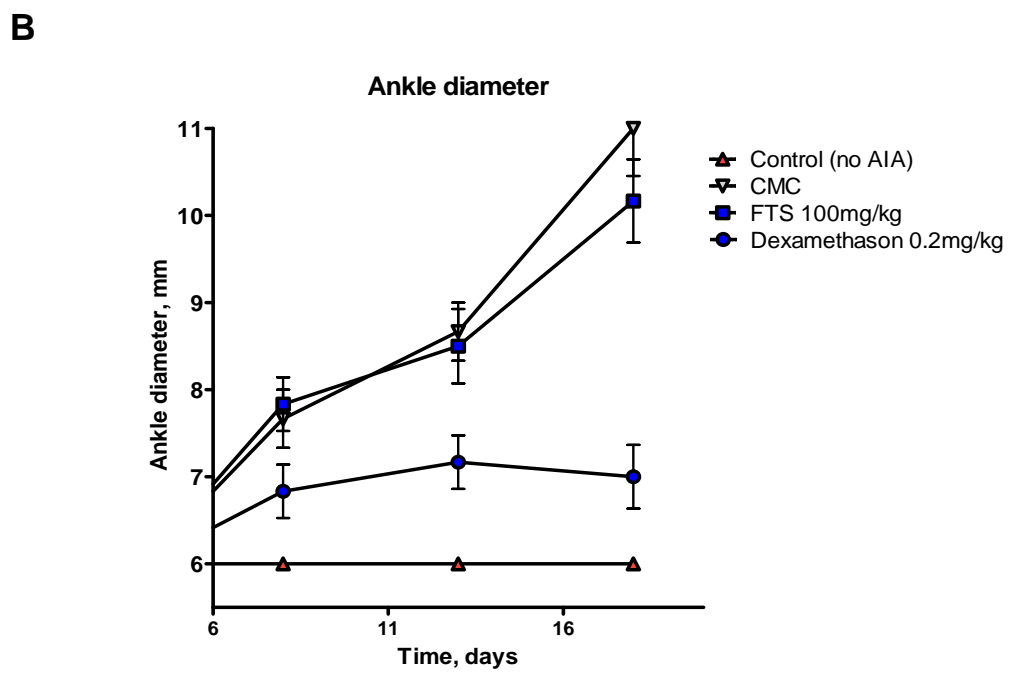
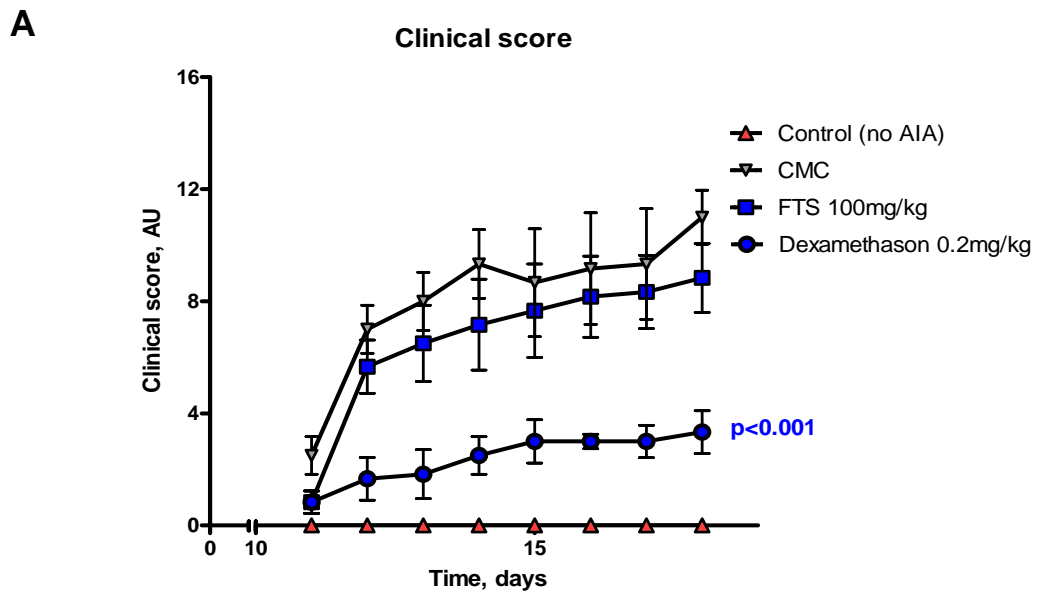


Figure 4

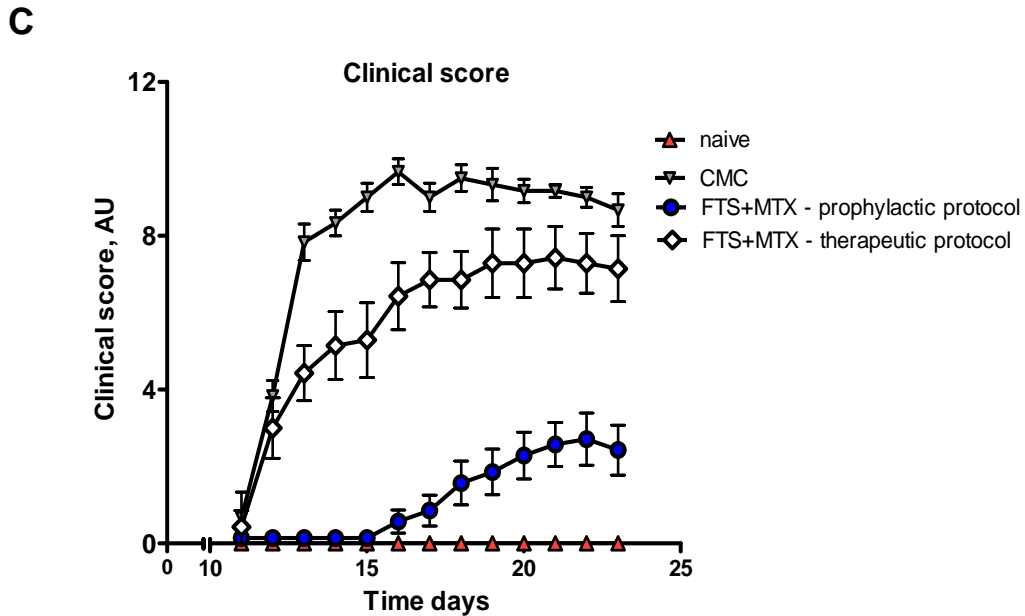


Figure 4. Clinical effects of FTS on the severity of AIA in the therapeutic dosing model. Rats were immunized with CFA and then graded regularly for signs of arthritis by a clinical disease severity score **(A)** and by caliper measurements of ankle joint width **(B)**. Rats in the experimental arms (n=8 per group) were treated starting day +9 with oral FTS (100 mg/kg), 0.5% CMC vehicle solution and 0.2mg/kg Dexamethasone daily. Statistical analysis of the effect of the various treatments on the clinical scoring **(A)** was done using One way ANOVA with Bonferroni's Multiple Comparison Test, the AIA severity was significantly lower ($p<0.001$) only in the Dexamethasone vs. CMC treatment groups. Ankle diameter was also significantly lower ($p<0.01$) only for Dexamethasone treatment (by Student's t-test). **(C)** Results of a single exploratory study comparing the protective effects of FTS as an add-on therapy to MTX in the prophylactic dosing vs. therapeutic dosing protocols, demonstrating superior efficacy for the prophylactic protocol.

Figure 5

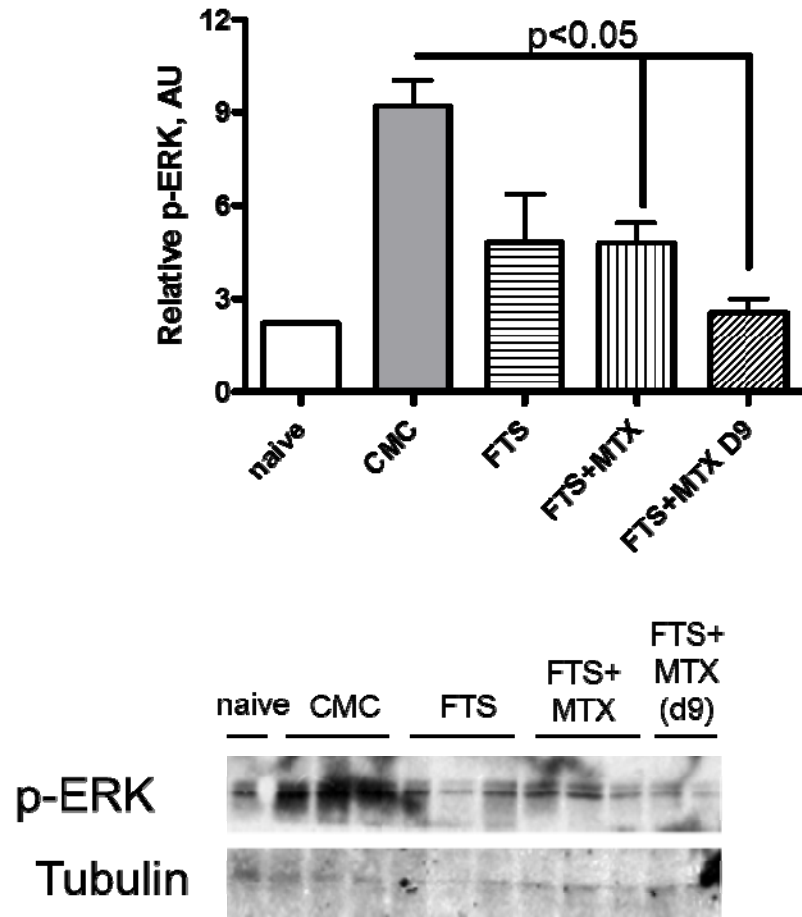


Figure 5. FTS and FTS add-on to MTX treatments both reduce p-ERK expression in rat splenocytes (lymphocytes) in adjuvant arthritic rats. Following CFA injection rats were treated with CMC (vehicle), FTS (100mg/kg) or FTS (100mg/kg) plus MTX (0.3mg/kg; weekly) starting from day +1 post AIA induction as well as FTS plus MTX starting from day +9 (therapeutic protocol) post AIA induction. Lysates of single cell suspension of spleens (harvested on day +21 post disease induction) were analyzed by western blotting for relative p-ERK levels. The upper panel show the results of the densitometric analysis of a typical experiments done in triplicates. The levels of p-ERK were normalized to tubulin levels and the bars represent mean \pm SEM of triplicates (arbitrary units; A.U). *P<0.05 by Student's t-test for all treatment groups compared to CMC vehicle treated mice.

Figure 6

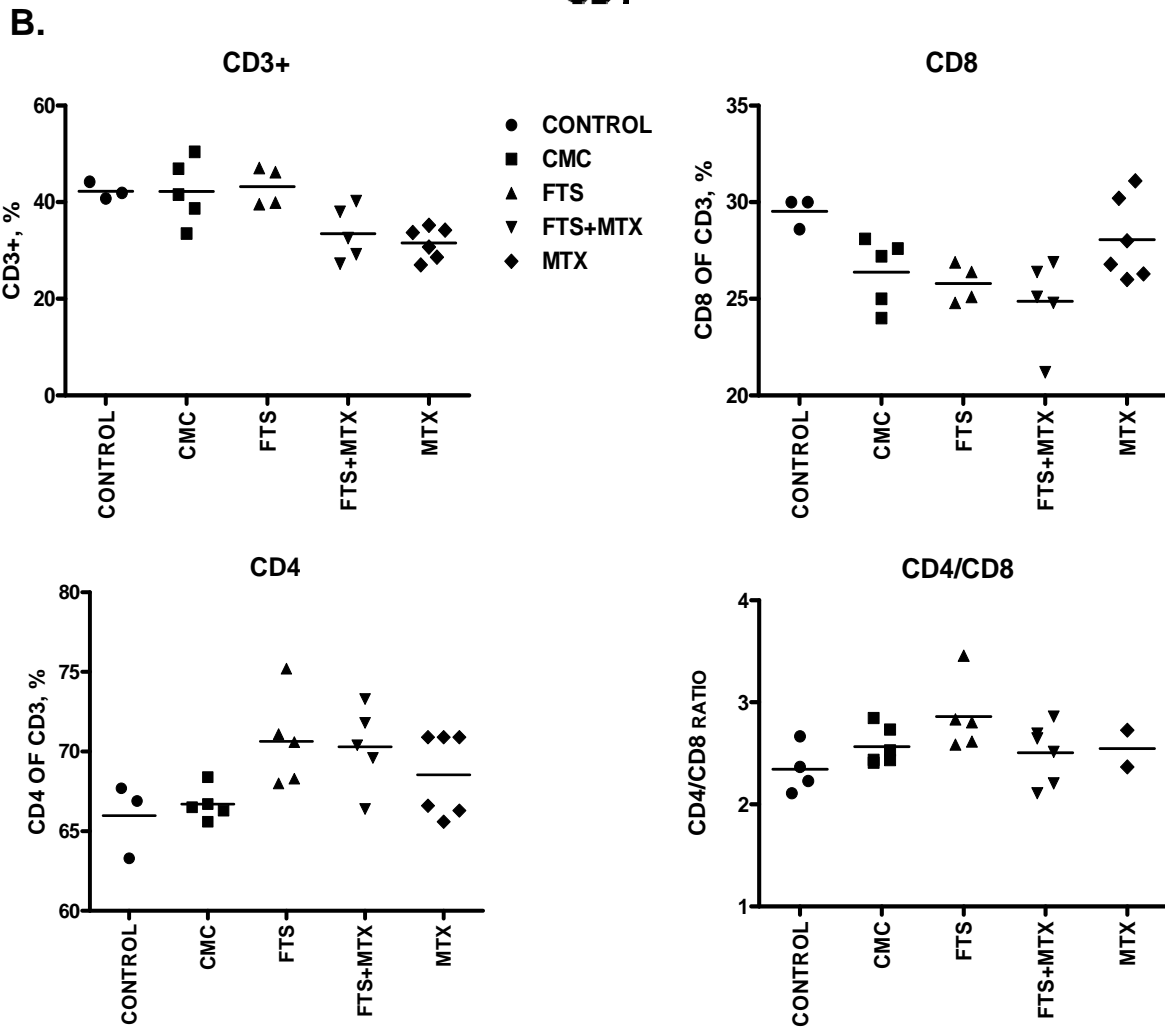
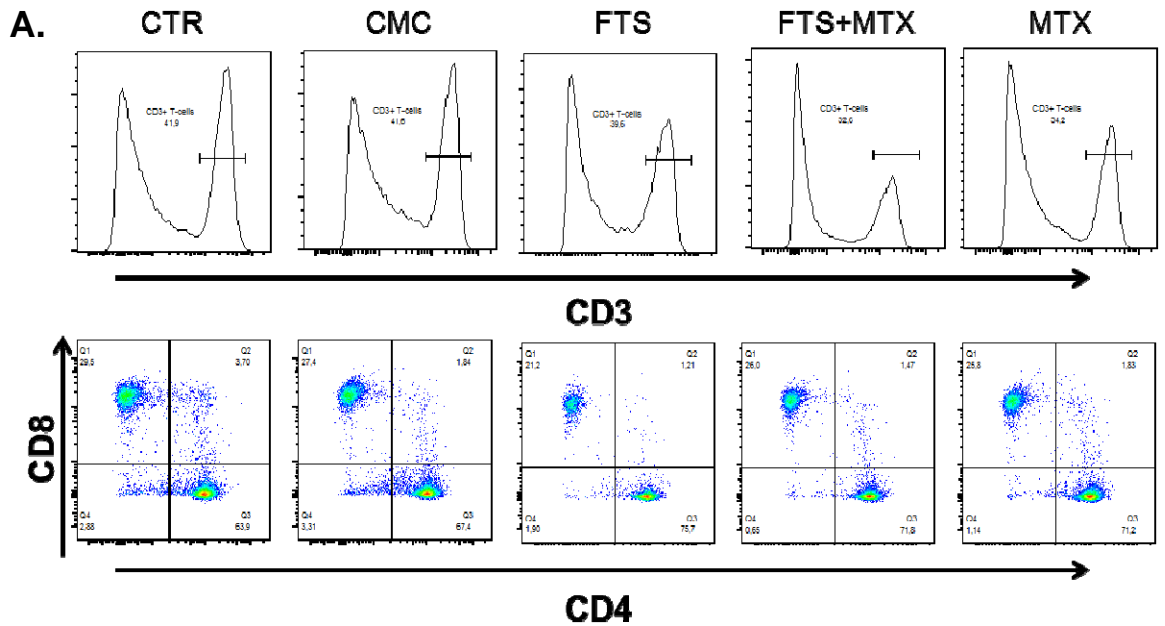


Figure 6

C.

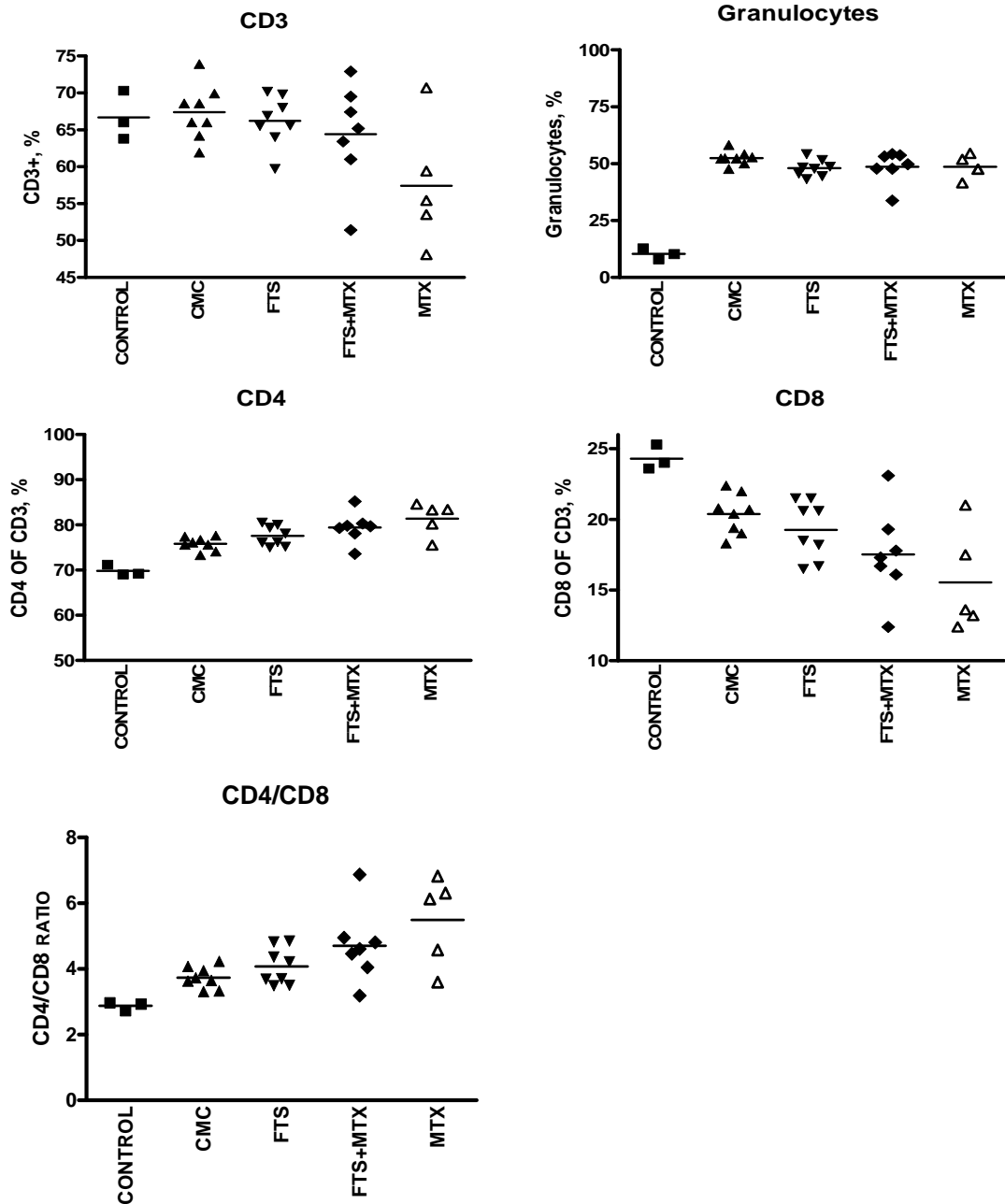


Figure 6. FTS effects on various leukocyte populations in peripheral blood and Spleen during AIA progression. Analysis of changes in leukocyte populations (Granulocytes, CD3+ T cells, CD4+ and CD8+ T cells) both in spleen (A-B) and peripheral blood (C) by flow cytometry, at study termination (day +20). Rats (n=8 per group) were treated with CMC, FTS, FTS+MTX or MTX. Three non-immunized rats were used as naïve control reference. The data were acquired on a FACSARIA instrument (~10,000 single cell events) and analyzed using FlowJo software. The results shown represent a typical experiment out of >3 performed.

Figure 7

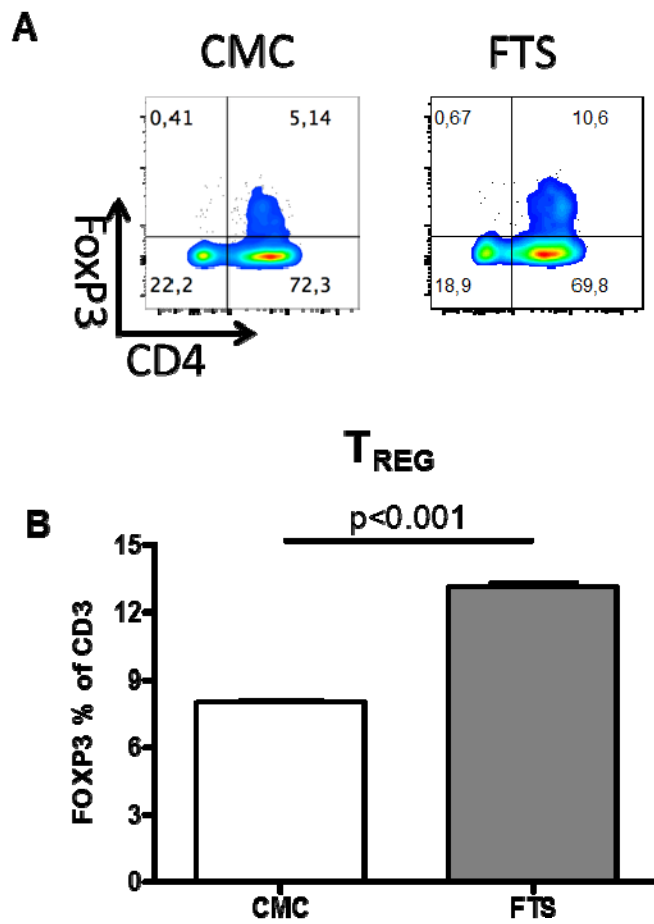


Figure 7. Prophylactic FTS dosing promotes regulatory T cell expansion (Treg) compared to CMC vehicle treated arthritic mice. (A) Representative flow cytometry plots for the effect of FTS treatment on Treg cells (CD3+CD4+FoxP3+) percentage in splenocytes at study termination (day +20). **(B)** Bars represent the mean \pm SD of Treg percentage (n=8 per group) of rats treated with CMC or FTS. Statistical analysis was done by Student's t-test.

Figure 8

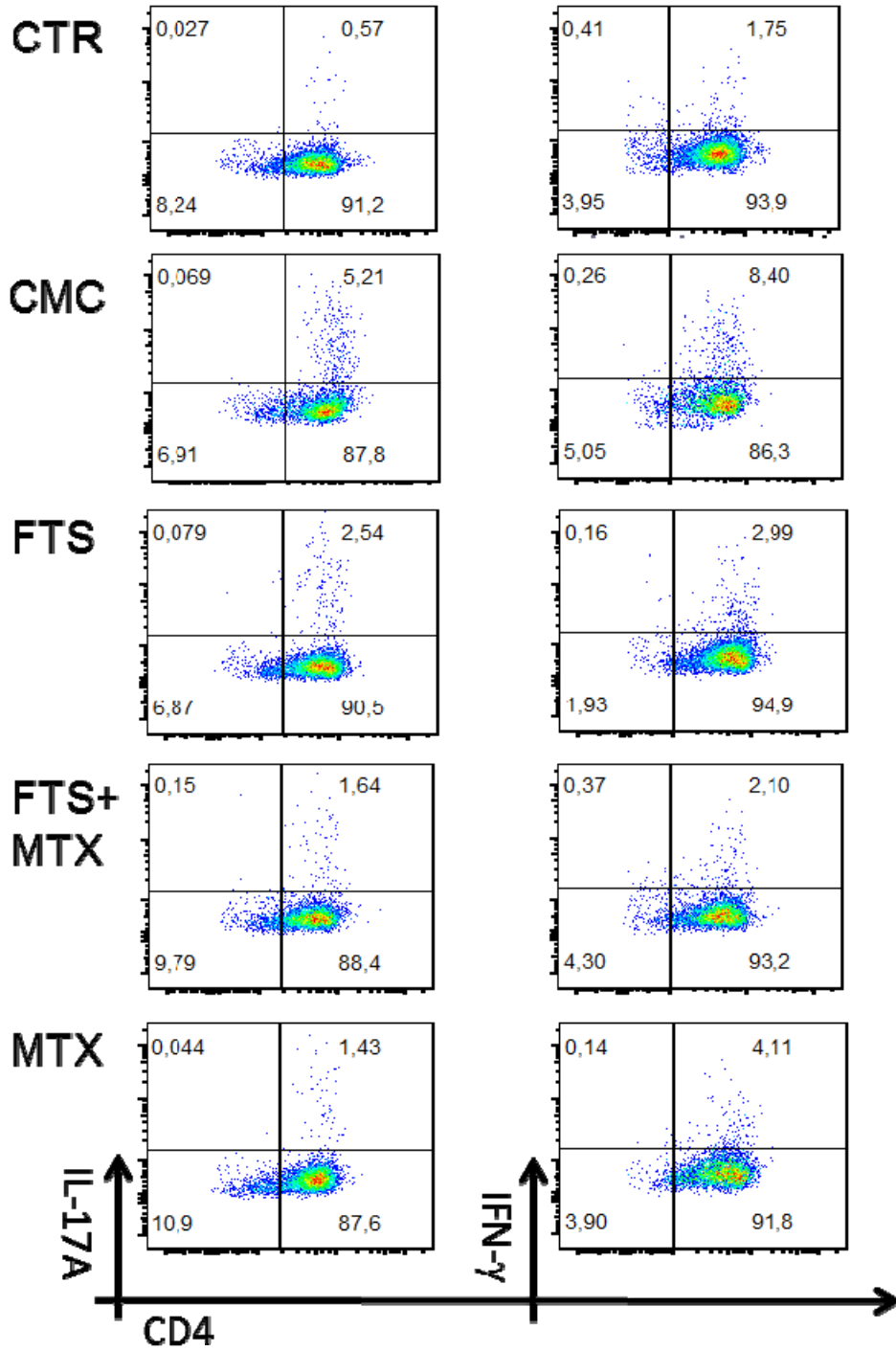


Figure 8

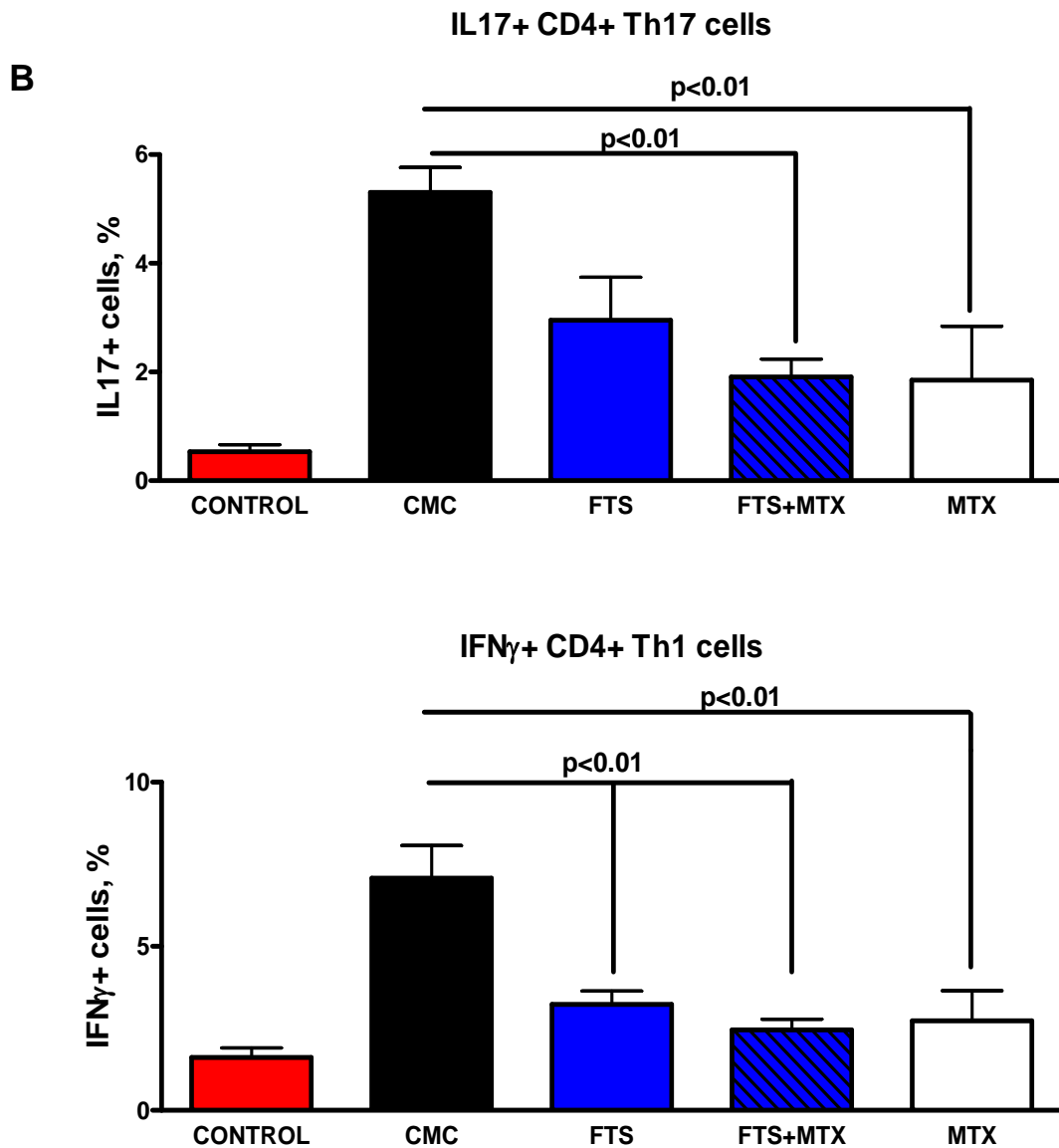


Figure 8: Prophylactic FTS treatment decreases the percentage of TH1 and TH17 cells in spleens of rats immunized with CFA. (A) Representative flow cytometry plots of intracellular staining to identify IL-17A and IFN- γ producing CD4+T cells in single cell suspensions of spleens harvested at day 20 post CFA injection from rats treated with CMC vehicle, FTS, FTS+MTX or MTX compared to naïve control (CTR) rats. **(B)** Bars represent the mean \pm S.D (n=8 per group) of TH17 (upper graph) and TH1 cells percentage (lower graph) detected in the various groups. A representative experiment out of >3 performed. Statistical analysis was done by Student's t-test.

Figure 9

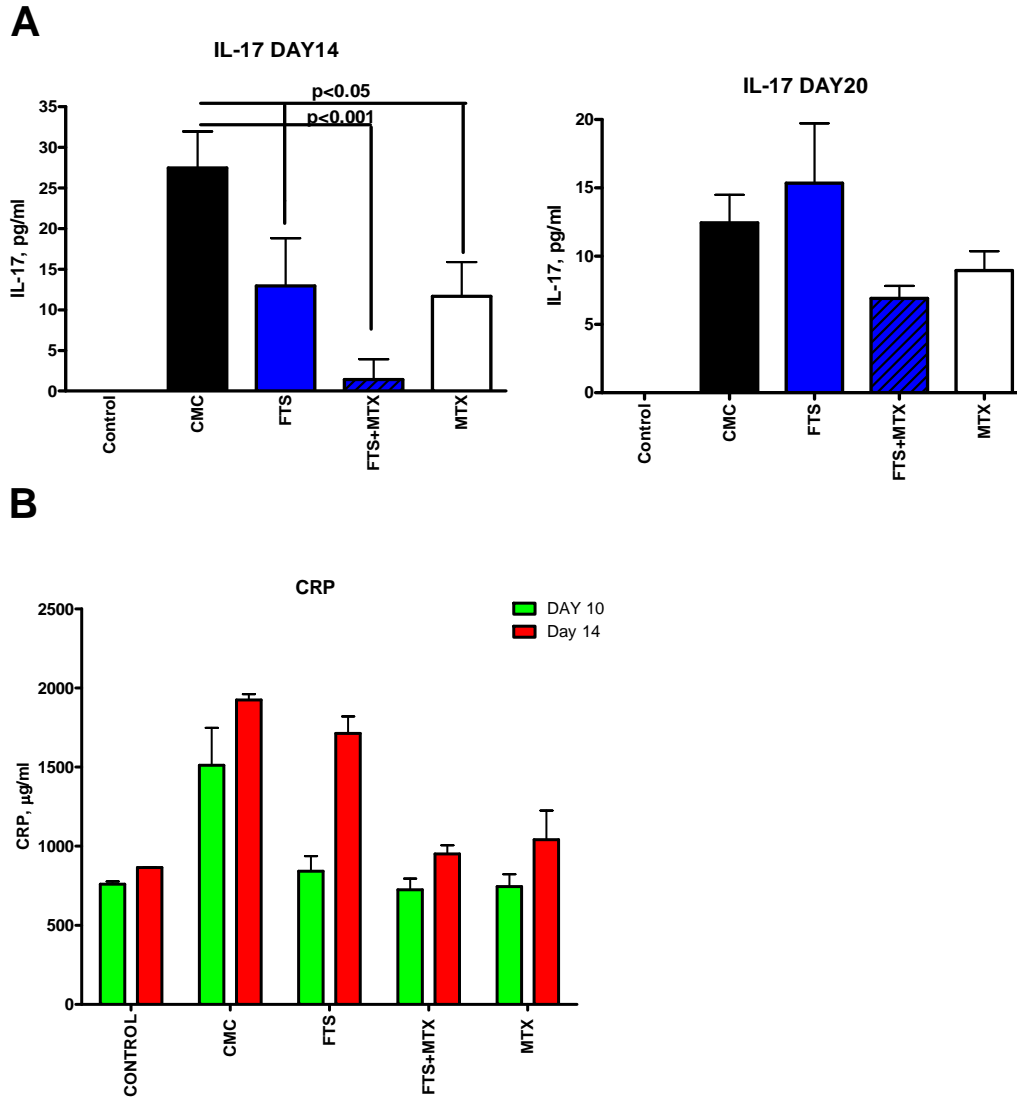


Figure 9. FTS prophylactic dosing reduces the levels of IL-17A and of the acute phase reactant CRP in rats with AIA. Sera from rats treated with CMC, FTS, FTS+MTX or MTX was collected at various time points post adjuvant injection and analyzed for IL-17A and CRP levels by ELISA. Bars represent mean \pm S.D of triplicates from a representative experiment out of 3 performed (n=4 rats analyzed per group).