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Collagen II-primed Foxp3 Transduced T Cells Ameliorate Collagen-induced Arthritis in Rats: The Effect of Antigenic Priming on T Regulatory Cell Function

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ABSTRACT

Regulatory T cells (Tregs) play a major role in the prevention of autoimmune diseases. Transfer of Foxp3 gene into conventional T cells converts their phenotype to regulatory T cells. Therefore, the question arises as to whether adoptively transferred in vitro differentiated Treg cells specific for a locally expressed antigen might have better inhibitory effects on the progression of the disease as compared with antigen-nonspecific T reg cells.

Herein, we investigated the therapeutic potential of primed and unprimed retrovirus mediated Foxp3-overexpression T cells following intravenously injected of these cells into affected rats with collagen-induced arthritis (CIA), an animal model of rheumatoid arthritis.

Our analyses demonstrate that systemic administration of collagen II primed Foxp3-transduced T cells could markedly ameliorate CIA inflammatory responses at clinical ($p<0.0014$) and pathological exchanges including cellular infiltration ($p=0.002$), bone erosion ($p=0.0013$) and synovial hyperplasia ($p=0.002$). In contrast, collagen II unprimed Foxp3-transduced T cells like as collagen II primed or unprimed GFP-transduced T cells did not reveal any beneficial effects on arthritis features as compared with untreated group ($p>0.05$).

Therefore, we believe that collagen II primed Foxp3-transduced T cells are interacting locally and systemically with immune cells which reveled with decreasing of T cells infiltration into joints along with specific CII IgG production. Considering the results described here, it appears that the using patients' T cells which previously exposed to specific antigens may have more effective therapeutic advantage in the production of induced regulatory T cells in the treatment of arthritis.

Keywords: Collagen-induced arthritis; Retroviral vectors; Rheumatoid arthritis; T regulatory cells

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease[1] which is characterized by proliferative synovitis of diarthrodial joints that leads to articular cartilage and bone destruction[2] RA is widely believed to be an autoimmune disorder where dysregulation of self-tolerance mechanisms leads to generation of auto reactive lymphocytes which target tissue-specific auto antigens derived from joint.[3] Similar to most autoimmune disorders, exact tissue-specific self-antigens and also precise pathogenic events leading to RA are not known. However, experimental findings show that inflammation, secreted-mediators by various immune system cells and some proteins including type II collagen, cartilage protein HCgp-39, proteoglycans, heat shock proteins and BiP (immunoglobulin binding protein) which expressed at high levels along with presence of auto reactive T and B cells are involved in the pathogenic process of RA.[4,5] Among the auto antigens, type II collagen from chicken or bovine were especially used to induce arthritis in appropriate strains of rodent as a most commonly studied experimental animal model of RA.[6] Nonetheless, evidence derived from both human and animal model studies have given a more central role to synovial collagen-reactive T lymphocytes in disease process[7] because they infiltrate to joint and produce proinflammatory cytokines including interferon-γ, IL-23 or IL-17 lead to increasing of immune cell recruitment into inflamed tissues and that contributes to the formation and maintenance of Th17 cells. Moreover, IL23R polymorphisms have been raised as a risk factor for ankylosing spondylitis (AS) and indicates that IL-23 is also could be involved in the pathogenesis of spondyloarthritis (SpA). [7]

Enhanced numbers of Th1 and/or Th17 cells together with decreased activity of Treg cells have been reported in joints of RA patients and also in inflamed tissues of rodents affected by CIA.[10] This pathogenic versus protective T cell imbalance seems to be a key pathophysiological event in autoimmune joint inflammation in RA. Indeed, some therapies for RA, including TNF-α antagonists have been shown to restore Th17/Treg ratios as well as enhancing Treg suppressive function.[11] In vitro generation and expansion of Treg cells with the ability to control destructive immune response against synovial antigens has been recently considered as a viable therapeutic option in RA.[12] Various methods have been employed for in vitro generation of Tregs, including polyclonal stimulation of T cells with anti-CD3/CD28 coated beads in the presence of transforming growth factor beta (TGF-beta).[13] Genetic manipulations to alter the phenotype of T cell followed by the transfer of the differentiated T cells to diseased individuals have also been suggested.[14] Thinking point in most studies that have shown modified Treg cells are inadequate to improve arthritis, is that, modified Treg cells have been created from healthy source cells. Therefore, the question arises whether the use of cells from a suffering person could be effective in improving the complications of the disease.

In the present study, the effects of Foxp3 gene transfected Treg cells from two different cell populations (unprimed and primed by collagen type II) were investigated on the improvement of the arthritis severity in rats.

MATERIALS AND METHODS

Animals

Wistar rats were purchased from the Pasteur Institute of Iran and housed in the animal facility of Department of Immunology. Arthritis was induced in female animals from one colony and all experiments were performed according to the guidelines approved by the Animal Ethics and Research Committee (N. 25220) of Tehran University of Medical Sciences, Iran. To induction of CIA and blood collection, animals were first anesthetized with intra-peritoneal (IP) injection of 0.1 mL Ketamine:Xylazine (IACUC guidelines: 92 mg/kg Ketamine+8 mg/kg Xylazine). Subsequently followed by cervical dislocation for tissue collection at the end of experiment

Production of Retroviral Vectors

The full coding sequence of mouse Foxp3 was PCR amplified from the obtained cDNA of homogenized mouse spleen and then, cloned into the bicistronic retroviral expression vector MigRI (kindly provided by Dr. Hori, S Kyoto University, Japan)[14] between BglII and EcoR1 followed by an IRES element and eGFP reporter gene. Successful cloning was confirmed by Sanger sequencing. Recombinant retroviral vectors were produced by transient co-transfection of HEK 293T cells with pMigRI (encodes Foxp3 or eGFP) and the packaging plasmid (pCL-Eco) using TurboFect

Table 1. Sequence of used primers for Foxp3 gene to amplify by PCR and CD4 to investigate the reduction of CD4+ T cells infiltration by qPCR

Symbol	Target Gene	Sequence (5'->3')	Restriction enzyme
FF	Foxp3	ACCATGCCCAACCC	BglII
FR		TCAAGGGCAGGGATTG	EcoRI
CF	CD4	TCTGGAACTGCACCGT	-
CR		CCTTCTCTGCCTTCCA	-
BF	β-actin	TGGGTATGGAATCCTGTG	-
BR		AGCAATGATCTTGATCTTCA	-

Restriction enzymes recognition sites were added to the 5' end of both Foxp3 primers. Full coding sequence of mouse Foxp3 gene were amplified by specific primers pairs for cloning. Quantitative PCR were performed to detect reduction of CD4+T cells infiltration in the joint space after cell therapy. β-actin was used as reference gene for normalization.
The Kozak sequence is underlined.

transfection reagent (Thermo Fisher Scientific). Presence of the retro viral vectors in the supernatant was confirmed by conventional PCR, and then titrated by analyzing of transduced NIH 3T3 cells by flowcytometry as descripted before.[15]

Retroviral particles were cryopreserved at -70°C.[2] Primer sequences used for PCR and qPCR are shown in Table 1.

T Cells isolation, Culture and Transduction

Single-cell suspensions of splenocytes from collagen II-immunized or unimmunized rats were prepared by nylon wool purification. T cells were adjusted to $2x10^5$ cells/mL, followed by stimulation with 100μg/mL of heat-denatured collagen II (Chondrex),[16] or 0.5 μg/mL coated anti-CD3e (BioLegend.) and 0.5 μg/mL of soluble anti-CD28 antibodies (BioLegend)[17] in the presence of 50 U/mL rIL-2 (BioLegend,) for 72 hours.[18,19] Subsequently, T cells were transduced by adding Foxp3-containing retroviral vectors at a total multiplicity of infection (MOI) of 5-10 supplemented with 2ng/mL of IL-2 and 5 μg/mL of polybrene (Sigma-Aldrich, USA) followed by centrifugation at 600g at 32°C for 1hour.[20] GFP-expressing vector was used as a control for measuring any effect of the empty vector. Overall, two kinds of viral particles were produced and used for transducing two distinct obtained T cells from collagen II primed and unprimed rats. Genetically retroviral modified T cells called as CII-primed Foxp3 transduced T cells (CIIP-FT), CII-unprimed Foxp3 transduced T cells (CIIUP-FT), CII-primed GFP transduced T cells (CIIP-GT) and CII-unprimed GFP transduced T cells (CIIUP-GT). T cells viability was determined by trypan blue dye exclusion.

Flow Cytometry of T Cells

For analysis of surface or intracellular markers, the following monoclonal antibodies from Biolegend, USA were used: APC anti- CD3 (Clone, 1F4), PE-anti-CD4 (Clone, W3/25) PE-anti-Foxp3 (Clone, 150 D). For analysis of Foxp3 gene expression, transduced cells (1×10^6 cells) were washed twice and then incubated with 1-mL Fix/Perm Buffer set (Biolegend, USA) according to the manufacturer's instructions. Cells were washed and stained with PE anti-mouse Foxp3 for 30 minutes at 4°C and samples were washed and re-suspended in PBS containing 1% fetal calf serum. Flow cytometry was performed using CyFlow Space (Sysmex Partec, Germany) and FACScalibur (BD Biosciences, USA) and analyzed using FlowJo Software (Tree Star Inc., Ashland, OR, USA)

Suppression Assays

The immunosuppressive capacity of both Foxp3-transduced T cells (CIIP-FT, CIIUP-FT) was determined by target cell suppression assays, as described previously.[21] Briefly, T cells isolated from CIA rats were labeled with 5 μM of Cell Proliferation Dye eFluor 670 (ebioscience, USA) and were plated in triplicate at $5x10^5$ cells/wells as responder cells.[22] Treg cells were added at a 1:1 ratio and cells were separately stimulated either with 100 μg/mL of CII or 0.5μg/mL of anti-CD3. After 3 days, the proliferation capacity of responder cells was evaluated by comparing the percentage of eflour+ divided cells.[23]

Collagen-Induced Arthritis (Induction, Treatment and Scoring)

The CIA was induced in female Wistar rats (aged 6 to 7 weeks old) by dorsal intradermal injections of 0.2 mL emulsified chicken CII in complete Freund's adjuvant on day 0 and receive a boost of 0.1 mL CII in incomplete Freund's adjuvant on day 7 as described previously.[2,24,25] After booster injection, each rats reaching score 4 or more were selected and randomly divided into 5 groups with 10 rats per each group. Treated group 1 (n=10) received CIIUP-GT cells, treated group 2 (n=10) received CIIP-GT cells, treated group 3 (n=10) received CIIUP-FT cells, treated group 4 (n=10) received CIIP-FT cells. All treated groups received approximately 1.5×10^6 engineered T cells intravenously which were re-suspended in 50µl of sterile PBS via the tail vein.[20] One group (n=10) served as a positive control and did not receive any cells and solution. The clinical severity was measured by a semiquantitative scoring system from 0 to 4 which is based on the observed macroscopic signs of inflammation.[2,18] Briefly, 0=normal, 1=Erythema and mild swelling in one joint, 2=Moderate redness and swelling of ankle, 3=Obvious swelling in the entire paw, and 4=maximally inflamed with deformity and/or ankylosis of the limb. Subsequently, rats were sacrificed on day 30 post T cell therapy and analyzed for joints histopathology, pro inflammatory cytokine and anti CII specific antibody levels in serum by ELISA.

CII-Specific Antibodies and Cytokine Levels in Serum

Levels of CII-specific antibodies were determined by ELISA as described before.[16,26] Barfly 1:1,000 diluted serum samples were added to wells in a 96-well plate coated with CII (10 µg/Well). After incubation at 4°C overnight, plates were washed three times with PBS- 0.05% Tween 20. Next, 1: 1,000 diluted horseradish peroxidase conjugated anti-rat IgG, IgG1 or IgG2a was added to each well and incubated for 2 hours at 4°C. After washing, 100 µL of TMB substrate was added and after adding stopping solution, absorbance was measured at 450 nm using a microplate reader. The levels of IL-6 and TNFα (Sigma Aldrich, USA) and IL-17 (Abcam, USA) were detected by using commercially available ELISA kits.

Histopathology

After euthanizing animals, one hind paw was removed from animals that had received different treatments and fixed in 4% paraformaldehyde followed by decalcificaton in 5% EDTA, as describe before.[9] Prior to sectioning, a portion of hind paw consisting of the epiphysis of tibia bone, the synovial joint and the proximal part of the ankle was cut and embedded in paraffin blocks. A series of 4 µm sections encompassing the entire length of synovial joint were prepared and a total of 6 consecutive sections were stained with H&E and assessed for inflammatory infiltrates, bone damage and synovial hyperplasia. Pathological changes were scored semi-quantitatively by three independent pathologists from 0 to 3 where 0, no changes; 1, mild; 2, moderate; 3, severe.[2] The final score for each histological appearance are the average of total 60 random fields that were reported by three pathologists.

Joints Preparation for Quantitative PCR and Flowcytometry

Ankle joints homogenate was prepared according to described protocols.[27,28] Briefly, collected ankle joints placed in 5 mL of RPMI-1640 medium plus 10% fetal calf serum supplemented with 1 mg/mL collagenase A (Roche, Switzerland) for 2 h at 37°C. Subsequently, engineered GFP+ T cells and total CD4 T cells infiltration to tissue were detected respectively with flow cytometry and with CD4 mRNA expression analyses by q-PCR as described before.[28] Primer sequences used for q-PCR are shown in Table 1.

Statistical Analysis

All data were analyzed by SPSS package software (version 20.0, Chicago, USA), and represented as mean±SD. After determination of normality of variances by Kolmogorov–Smirnov test, one-way analysis of variance (ANOVA) with Tukey post-hoc correction or Kruskal-Wallis test were used to analyze the data from multiple groups and Mann Whitney U test for gene expression and for suppression assay. *p* values less than 0.05 were considered significant

RESULT

Confirmation of Retroviral Vector Generation, Genes Expression and Biological Function

As described in Materials and Methods, we generated retroviral vectors containing mouse Foxp3 or eGFP coding sequence to transduce T cells (Figure

1A). Transfection efficiency was quantitatively determined by flow cytometry, which indicated that 80% of packaging cells express eGFP. To confirm the presence of retroviral particles in supernatant of packaging cells, we directly estimated by PCR (Figure 1B) and then viral titer was determined 2.4×10^7 viral particles/mL. Flow cytometric analysis of transduced T cells based on GFP expression indicated that, 30% of transduced T cells express eGFP. Furthermore, transduced cells were gated on eGFP to examine the Foxp3 expression (Figure 1C). Statistical analysis showed significantly (p=0.008) up-regulated gene expression in infected T cells (Figure 1D).

The Suppressive Function Is Enhanced in CII Primed T Cells

To investigate suppressive function of induced Tregs, we purified T cells from CIA rat as responder cells and after labeling with proliferation dye, stimulated separately with anti-CD3 and CII in the presence or absence of both Foxp3 transduced T cells (CIIUP-FT, CIIP-FT) (Figure 2A). The results showed that there is no significant difference between transduced Foxp3 T cells in the suppression of responding T cells proliferation (CD3 activated: p=0.12, CII activated: p=0.35). Nevertheless, responding T cells stimulated with anti CD3 showed a tendency reducing proliferation by CII unprimed T cells (Figure 2B).

Significant Reduction of Arthritis Severity in CIIP-FT Cells Receiving Rats

All rats were clinically examined and scored for disease severity from the beginning of the adaptive Foxp3 transduced T cell therapy until 30 days after booster induction. Treatment with CIIP-FT cells showed a positive effect on body weight (Data not shown) and a significant reduction in the arthritic score compared with the other groups from day 7 until 37 (p=0.0014). The reduction in the articular index of the

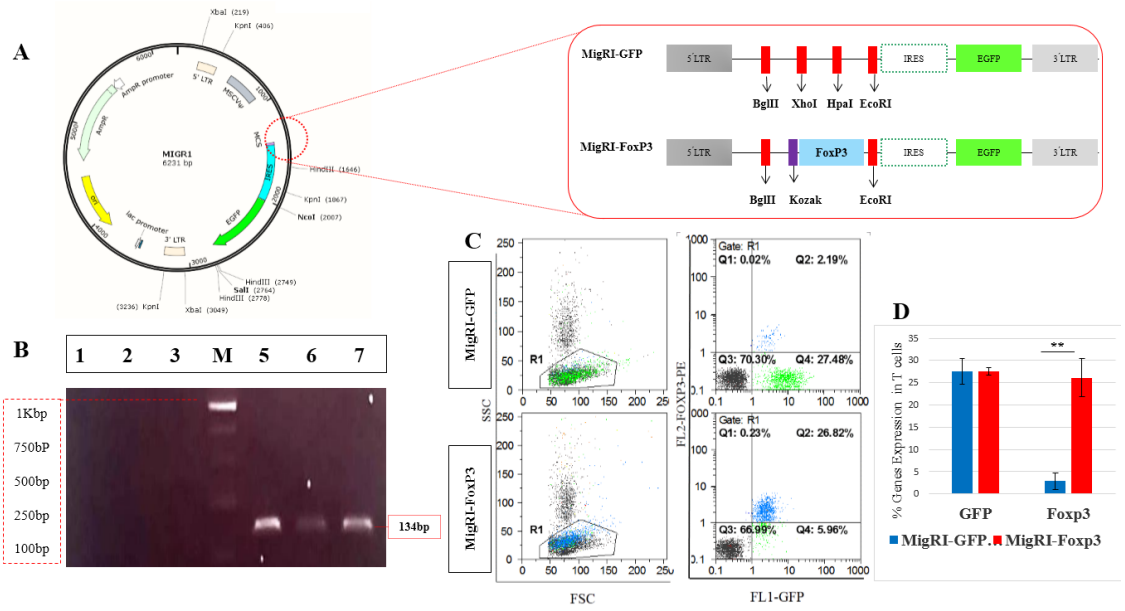

Figure 1. Construction and expression of control and recombinant retroviral vectors that encodes GFP and Foxp3 genes respectively

A); Schematic representation of control and recombinant retroviral vectors which code GFP and Foxp3-GFP respectively. The Foxp3 gene was inserted into, pMigR1, between BglII and EcoR1. (B); Retro viral vectors generation was confirmed using eGFP gene PCR amplification in supernatant directly (line 1-2) or in corresponding cDNA samples (line 6-7). The results indicate that the cell culture supernatants have only retro vectors without any plasmid DNA contamination. Lines 3 is negative and line 5 is positive controls. (C); Transduction efficiency and Foxp3 expression in T cells were analyzed by flowcytometry (D), Histograms depicting percentage of retroviral mediated Foxp3 and GFP expression on gated CD3+ Cells. The data are average of duplicate flow cytometry reading of at least five independent experiments and represent the mean values±SD.

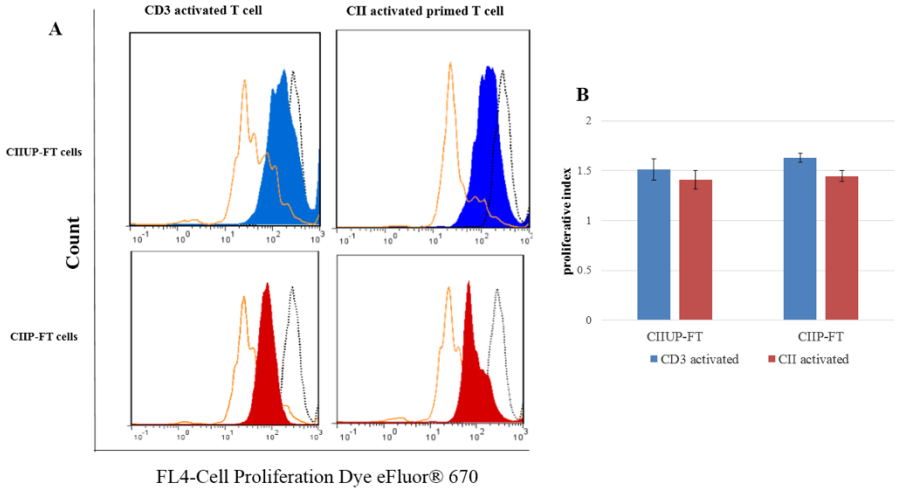

Figure 2. Suppressive activity of both CII unprimed and primed Foxp3-transduced T cells

A). Foxp3-transduced T cells (CIIUP-FT, CIIP-FT) were tested for their ability to suppress of responder T cells prolifration. responder T cells are obtained from CIA rat and stimulated with anti-CD3/CD28 antibodies and CII separately after labeling with Cell Proliferation Dye eFluor 670. Suppressive capacity of CIIUP-FT, CIIP-FT was calculated on the basis of the proliferation of responder T cells in the absence or presence of transduced T cells at a ratio of 1:1 after 3 days (B). Representative histograms shows no significant difference between both transduced T cells in suppressive activity. (CD3 activated: p=0.12, CII activated: p=0.35). The results represented as mean±SD of proliferation indices from five independent experiments per each group. CIIP-FT: CII-primed Foxp3 transduced T cells, CIIUP-FT: CII-unprimed Foxp3 transduced T cells

CIIP-FT treated group not only was seen at the end of the trial, but also was apparent throughout the treatment period (Figure 2A).These findings were confirmed by the H&E staining of histological sections which were examined to determine the treatment effects on cellular inflammation, synovial hyperplasia and bone erosion (Figure 2B). The neither articular index (p=0.9) nor histo-pathological (p= 0.7) results from CIIUP-FT cells treated rats as well as GFP transduced T cells (CIIUP-GT and CIIP-GT) were not statistically different from those obtained no treatment. In contrast, statistical analysis demonstrated significantly low levels of histological changes including cellular infiltration (p=0.002), bone erosion (p=0.0013) and synovial hyperplasia (p=0.002) in CIIP-FT-treated group. Nonetheless, the histological changes were significantly (p=0.00015) higher in CIIP-FT-treated animals compared with healthy non-CIA animals (Figure 3C).

CII-Specific IgG and Pro Inflammatory Cytokine Decreases in CIIP-FT Cell Treated Rats

In parallel with the clinical findings, we also quantified the effect of therapy on serum levels of CII-specific antibodies in terms of total IgG and subclass (IgG1, IgG2a) consistent with pro-inflammatory cytokine levels including IL-6, TNFα and IL-17, by ELISA at the termination of the experiment.

As the results shown in Table 2, no differences on IgG1 levels between all groups (p=0.2). Nonetheless, adoptive transfer of CIIP-FT cell have significantly positive effects on reducing total specific anti CII IgG, (p=0.026) anti-CII IgG2a (p=0.008) and cytokines levels including IL.6 (p=0.02), TNFa (p=0.013) and IL.17 (0.017) in contrast to other groups that these changes were not observed (Figures 4A-F).

T Cell Infiltration Decreases in CIIP-FT Cell Treated Rats

As expected, histological examination of CIA joints showed large amounts of lymphocyte infiltration in CIA joint except in treated group with CIIP-FT cell. Since previous evidence confirmed that the majority of the infiltrated lymphocyte are CD4+T cells,[29] therefore, we focused especially on the T cell infiltration in joints of different treatment groups. To further investigate, extent of CD4 gene expression was measured by qPCR

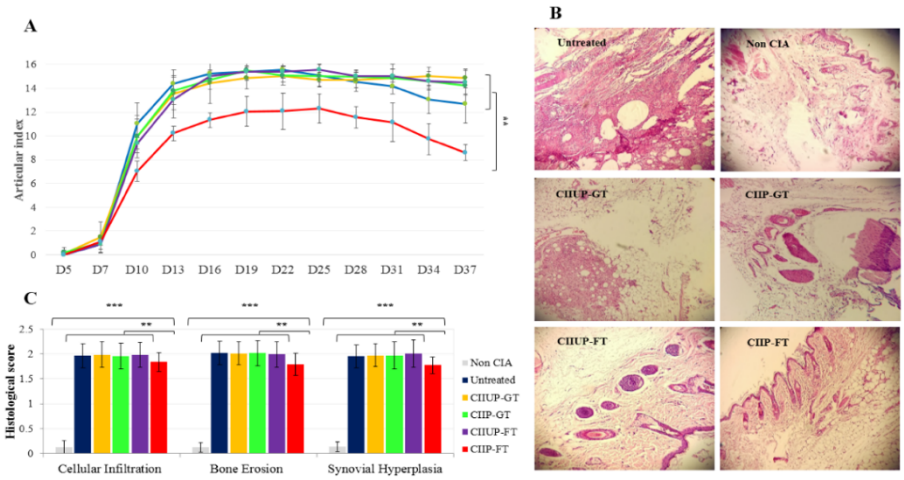

Figure 3 Significant reduction of clinical severity of CIA in rats treated with CIIP-FT therapy

The CIA was induced according to protocol and flowed by intravenously transferring of 1.5×10 6 transduced T cells on day 8 (A). The severities of arthritis were monitored daily untile day 37 and showed as articular index. The final articular index was calculated as the sum of the mean scores from the 4 limbs that were exanimated by three persons (N=10 per each group)

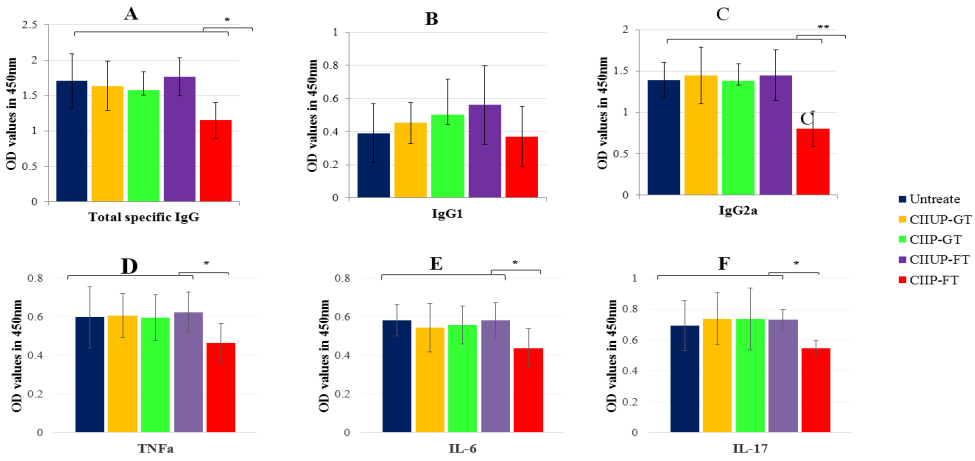

Figure 4. Levels of specific CII- IgG antibodies and pro inflammatory cytokines

Sera were collected from all samples on day 38 and the levels of specific anti- collagen II antibodies were determined by ELISA. A, B and C shows the serum levels of total IgG, IgG1 and IgG2a. D, E and F shows the levels of pro-inflammatory cytokines (TNFα, IL-6, IL-17). Results showed that transfer of CIIP-FT cells have no efficiency on IgG1 levels (p=0.2) whereas have significantly efficiency on total specific anti CII IgG, (p=0.026), anti-CII IgG2a (p=0.008) associated by cytokines levels including IL.6 (p=0.02), TNFa (p=0.013) and IL-17 (p=0.017). OD values in 450nm are expressed in the mean±SD of combined data from duplicate experiments. (p<0.05, *p<0.01). N= seven rats per groups.**

on 2 µg of total tissue-derived RNA for cDNA synthesis and calculated by livak method to confirm the striking decrease in the number of CD4+ T cells in all groups. The results of qPCR in parallel with data from histopathology relies this hypothesis that CIIP-FT cells is more benefit than CIIUP-FT cells on preventing infiltration of CD4+T cells in CIA-rats (Figure 5A). In the other hand, the ability to enter the tissue of engineered T cells were investigated using GFP expression in these cells. Flowcytomtric assay of

collagenase released single cells suspensions reveled markedly (p=0.0012) reduction in the present of the infiltrated GFP+ T cells in the joint of CIIP-FT cells treated group. However, other groups were found to have more infiltrated GFP+T cells but show a relatively worse effect instead of better effect (Figures 5B, C).

Table 2. The mean of inflammatory cytokines and CII specific IgGs in the treatment groups

	TNFa		IL-6		IL-17		Total IgG		IgG1		IgG2a	
Un treated	0.60	±0.16	0.58	±0.08	0.69	±0.16	1.57	±0.28	0.39	±0.18	1.39	±0.22
CIIUP-GT	0.61	±0.11	0.54	±0.13	0.74	±0.17	1.59	±0.18	0.45	±0.13	1.44	±0.34
CIIP-GT	0.60	±0.12	0.56	±0.10	0.74	±0.20	1.62	±0.19	0.50	±0.21	1.38	±0.21
CIIUP-FT	0.62	±0.10	0.58	±0.09	0.73	±0.07	1.59	±0.13	0.56	±0.24	1.45	±0.30
CIIP-FT	0.46	±0.10	0.44	±0.10	0.54	±0.05	1.37	±0.14	0.37	±0.18	0.89	±0.24

Reduced inflammatory cytokine and total specific collagen II IgG especially CII specific IgG2a subgroup expression were seen in CIA rats challenged by CIIP-FT cells.

Results are reported as optical density (OD) at 450nm

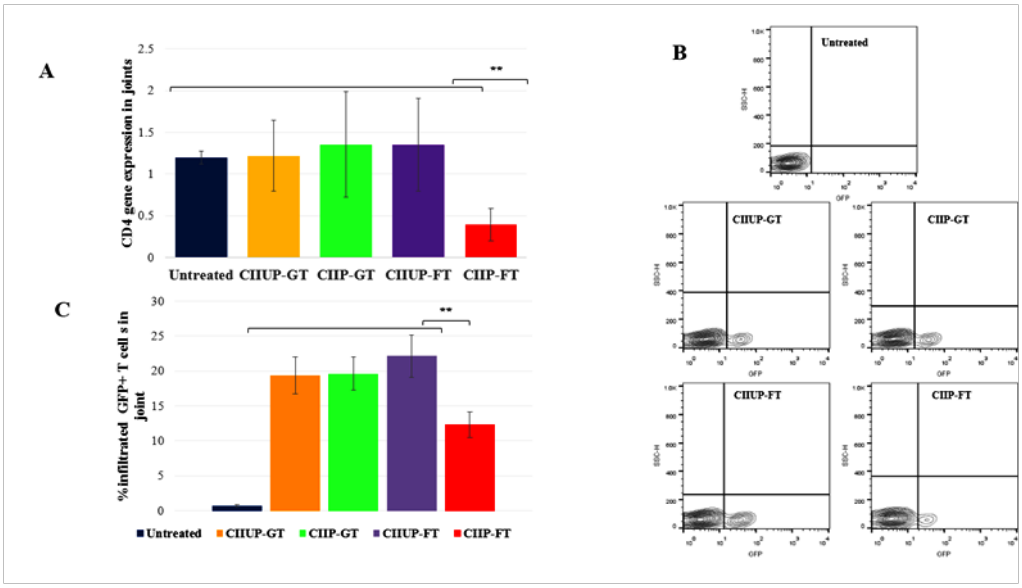

Figure 5. Cell infiltration decreases in CIIP-FT cell treated rats

Joint cells suspensions were prepared and processed for flowcytometic and qPCR analyses. to determine the degree of CD4 T cells infiltration (A) Representative qPCR results of CD4 gene mRNA showed significantly (p=0.017) decries in CIIP-FT cells treated rats. Beta-actin was used as a housekeeping gene control for comparison with CD4 mRNA expression (B). Joint cells were evaluated by flow cytometry after staining with anti CD3-APC and CD4-PE antibodies. Subsequently, cells were gated on CD3+CD4+ and analyzed for the presence of transuded GFP+ T cells in joint. (C) The differences in the percentages of transuded GFP+ T cells were markedly (p=0.0012) lower in CIIP-FT cells treated rats. Values have been expressed in the mean ± SD of duplicate experiments for flowcytometry (N=6 rats) and triplicate experiments for qPCR (N=6 rats). CIIP-FT: CII-primed Foxp3 transduced T cells, CIIUP-FT: CII-unprimed Foxp3 transduced T cells, CIIP-GT: CII-primed GFP transduced T cells and CIIUP-GT: CII-unprimed GFP transduced T cells.

DISCUSSION

Many attempts have been made to achieve a safe method to treatment of rheumatoid arthritis. One of these efforts is treatment with induced T regulatory cells which have been long considered as a therapeutic strategy in autoimmune disorders. According to some results, successful treatment with CD25 Treg cells has been reported in the animal model of colitis[30] but unlike colitis, this method is not responsible for recovery from CIA.[20] The main challenges for failing of T reg cells

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therapy can be divided into two categories. The first is the nature of the CIA, which is a systemically induced autoimmune disease and initiated mainly through CII-specific antibodies, and the second is type of efforts have been made to preserves immune system homeostasis with Foxp3 transduced T cells which obtained from normal individuals. Several studies have shown that Treg cells must have been activated via their TCRs by recognition of specific antigen to exert suppressive effects.[31] Therefore, modifying T cells obtained from RA patients seems to be an attractive therapeutic strategy with the aim of restoring immune balance and recovering tolerance to joint antigens while avoiding protective systemic immune inhibition.

In the current study we try to determine the clinical efficacy of adoptively transferred in vitro differentiated Treg cells specific or non-specific for collagen II antigen in our experimental animal models of arthritis. Collagen-induced arthritis (CIA) is a common autoimmune animal model used to study RA and can be induced in mice (DBA/1, C57BL/6)[32] or in rats (lewis, wistar)[2,33] using different protocols. Although in several studies the transfer of immune cells has been used in wistar rats [34], however, in order to develop an intensive form of inbred experimental animale model, rats were generated by repeatedly mating a male to his female descendants as described by Tave, Douglas.Female wistar rats were used in order to remove the anti-inflammatory effects of testosterone[35] and also due to the low onset of arthritis associated with severe inflammation in C57BL/6 mice that lead to the tail necrosis. Animals developed a progressive polyarthritis in four paws on day 8 after immunization with collagen II. Subsequently, animals reached score 4 or more were select to research. First retroviral vector encoding Foxp3 was constructed using an experimental protocol which used previously for the production of iTreg.[14] Isolated splenic T cells from both CII unprimed and primed rats were next activated with anti-CD3/CD28 and CII respectively and followed by transduction of cells with retroviral vectors expressing Foxp3/eGFP or eGFP alone.

The biological activity of Foxp3 transduced T cells were further confirmed by their in vitro immunosuppressive activity. We did not find any significant difference in suppressive capacities of both Tregs cells (CIIUP-FT or CIIP-FT) after general or specific stimulation with anti-CD3 or CII respectively. This finding can be explained by the fact that this effect has been observed in the ratio of 1:1, and it is better to consider the inhibitory effect of engineered cells in other incremental ratios. However, after immunization and the onset of acute inflammatory episode in the overall immunized rats, the severity of clinical sings in affected joints gradually ameliorated exclusively in the CIIP-FT cell treated group. In contrast, other treatment groups developed disease sings explosively after immunization. In compliance with the reduction of clinical symptoms severity, the pathological changes in the affected joints were observed at mild to moderate levels and also the infiltration of inflammatory cell was less intense in the CIIP-FT cell treated group compared with the other groups. This may be due to the inability of CIIUP-FT cells to control the specific inflammatory responses as well as the level of secreted inflammatory cytokines in animals.

A critical role for anti-CII specific IgG for the development of CIA has been demonstrated by previous studies[1] and it has been shown that the lgG2a subclass is a major produced antibody against CII.[36] In addition, some adoptive T cell therapy works shown a decrease in the levels of hallmarks CII-specific IgG[14] and IgG2a.[18] Nonetheless, to determine whether adaptive Treg cell therapy affected antibody responses to CII, levels of CII-specific IgG, IgG1 and IgG2a in sera were measured. Our findings are agreement with previous observations that showed levels of anti-CII-IgG1 subclass have not significantly decreased in any of the experimental groups.[18,37] While a significant difference between the specific anti-CII total IgG and IgG2a levels were detected in rats treated with CIIP FT.[16,18,23,36] Therefore, Our findings are in parallel with previous reports suggesting that IgG2a antibodies have a major role in RA pathogenesis.[38] However, we consider that this contradiction in the levels of anti-CII, depend on the strain of experimental animals as well as to the types of collagen which is used to induce arthritis.

As in previous papers has been reported the main infiltrated cells into the joint tissue are T cells using of the IHC method.[2,25] We cite this results and the effect of treatment on the rate of infiltrated cells in the tissue were directly assayed using specific RT-PCR methods for CD4 T cells. Our results showed the number of infiltrated CD4+ T cells to joints significantly reduced in CIIP-FT treated group compared to other groups. The flowcytometry analysis of joint homogenate cells showed that the engineered T cells are capable of

traveling to the inflamed joint after adoptive transfer. Despite the present in the joint, except in CIIP-FT group, they did not only cause reduction but in some cases exacerbate the symptoms of the disease. Like some researchers mentioned before for the inadequacy of therapeutic effects of polyclonal T reg cell therapy, we considered that the engineered T cells are more susceptible to the differentiation into IL-17 producing cells. Therefore, locally and systemically increasing of IL-17 production directly increases the recruitment of various immune cells to the synovial tissue and progressive joint inflammation. However, this issue needs to be reviewed carefully in order to confirm and it is currently under investigation with our colleagues.

In summary, our results significantly demonstrated that adoptive transferring of CIIP-FT cells can effectively suppress arthritis severity. Our finding data showed that although these cells presence at low amount in the joint, but they could inhibits the T cell infiltration to joints, a finding which is in corroboration with clinical, histological and qPCR results. We have also found that these cells can decrease systemic CII-specific antibody (especially IgG2a) along with pro inflammatory cytokines levels in CIA model. All of this are in contrast with CIIUP-FT which did not exert any protective effect. Altogether, these findings point to the possibility of using antigen-primed T cells in order to differentiate Tregs cells could be used as a potential way to treat arthritis.

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