Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

CR6-interacting factor 1 controls autoimmune arthritis by regulation of signal transducer and activator of transcription 3 pathway and T helper type 17 cells

Jin-Sil Park,[1] Si-Young Choi,[1] Sun-Hee Hwang,[1] Sung-Min Kim,[1] JeongWon Choi,[1] Kyung-Ah Jung,[1] Ji Ye Kwon,[1] Young-Yun Kong,[2] Mi-La Cho[1,3,4,a] and Sung-Hwan Park[1,5,a]

[1]*The Rheumatism Research Center, Catholic Research Institute of Medical Science, The Catholic University of Korea, Seoul,* [2]*School of Biological Sciences, Seoul National University, Seoul, South Korea,* [3]*Department of Medical Life Science, College of Medicine, The Catholic University of Korea, Seoul,* [4]*Department of Biomedicine & Health Sciences, College of Medicine, The Catholic University of Korea, Seoul, Korea and* [5]*Divison of Rheumatology, Department of Internal Medicine, The Catholic University of Korea, Seoul, South Korea*

doi:10.1111/imm.13042
Received 28 May 2018; revised 31 October 2018; accepted 11 December 2018.
[a]These authors contributed equally to this work.
The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/Iyv9f4
Correspondence: Sung-Hwan Park, Division of Rheumatology, Department of Internal Medicine, School of Medicine, The Catholic University of Korea, Seoul St. Mary's Hospital, Seoul, South Korea.
Email: rapark@catholic.ac.kr
and
Mi-La Cho, Department of Medical Lifescience, College of Medicine, The Catholic University of Korea, Seoul, South Korea.
Email: iammila@catholic.ac.kr
Senior author: Mi-La Cho and Sung-Hwan Park

Summary

CR6-interacting factor 1 (CRIF1) is a nuclear protein that interacts with other nuclear factors and androgen receptors, and is implicated in the regulation of cell cycle progression and cell growth. In this study, we examined whether CRIF1 exerts an immunoregulatory effect by modulating the differentiation and function of pathogenic T cells. To this end, the role of CRIF1 in rheumatoid arthritis, a systemic autoimmune disease characterized by hyperplasia of synovial tissue and progressive destruction of articular cartilage structure by pathogenic immune cells [such as T helper type 17 (Th17) cells], was investigated. p3XFLAG-CMV-10-CRIF1 was administered to mice with collagen-induced arthritis 8 days after collagen type II immunization and the disease severity and histologic evaluation, and osteoclastogenesis were assessed. CRIF1 over-expression in mice with collagen-induced arthritis attenuated the clinical and histological signs of inflammatory arthritis. Furthermore, over-expression of CRIF1 in mice with arthritis significantly reduced the number of signal transducer and activator of transcription 3-mediated Th17 cells in the spleen as well as osteoclast differentiation from bone marrow cells. To investigate the impact of loss of CRIF1 in T cells, we generated a conditional CRIF1 gene ablation model using CD4-cre transgenic mice and examined the frequency of Th17 cells and regulatory T cells. Deficiency of CRIF1 in $CD4^+$ cells promoted the production of interleukin-17 and reduced the frequency of regulatory T cells. These results suggest a role for CRIF1 in modulating the activities of Th17 cells and osteoclasts in rheumatoid arthritis.

Keywords: CR6-interacting factor 1; rheumatoid arthritis; suppressor of cytokine signaling 3; signal transducer and activator of transcription 3; T helper type 17 cells.

Abbreviations: APC, allophycocyanin; CIA, collagen-induced arthritis; CII, collagen type II; CRIF1, CR6-interacting factor 1; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; IL, interleukin; LIF, leukemia inhibitory factor; PE, phycoerythrin; PerCP, peridinin chlorophyll protein complex; RANKL, receptor activator of nuclear factor κB ligand; RA, rheumatoid arthritis; SOCS3, suppressor of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; Th17, T helper type 17; TRAP, tartrate-resistant acid phosphatase; Treg, regulatory T; α-MEM, α-minimum essential medium

© 2018 John Wiley & Sons Ltd,

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by inflamed and hyperplastic synovial tissue, autoantibody production, and progressive destruction of articular cartilage and bone structure.[1,2] The pathogenesis of RA is mediated by multiple cytokines, proteolytics and prostanoids. In inflamed RA joints, pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α induce the differentiation and activation of osteoclasts, specialized bone-resorbing cells from bone marrow, leading to destruction of both cartilage and the bone matrix.[3]

Development of RA is associated with inflammatory cell infiltration, and T cells are implicated in the inflamed and hyperplastic synovia in patients with RA.[4] Among the various subtypes of effector T cells, T helper type 17 (Th17) cells are distinguished from Th1 and Th2 cells by their production of IL-17A, IL-17F, and IL-21.[5,6] The Th17 cells are linked to various autoimmune disorders, such as RA, inflammatory bowel disease, multiple sclerosis, systemic lupus erythematosus, and allergic responses.[7–11] Interleukin-6 plays a role in the development of Th17 cells by activating signal transducer and activator of transcription 3 (STAT3). In RA, IL-17 promotes the activity of pathogenic cells by inducing the production of pro-inflammatory cytokines including IL-1, IL-6 and tumor necrosis factor-α[12–14] and activated STAT3 is present in inflamed synovium in animal models of RA.[15,16]

CR6-interacting factor 1 (CRIF1) is a nuclear protein that interacts with CR6/GADD45, nuclear factors including Nur77, the androgen receptor, and NRF2, and participates in the regulation of cell-cycle progression and cell growth.[17–20] CRIF1 functions as a transcriptional co-activator of STAT3 by modulating its DNA-binding ability.[21] In addition, CRIF1 is essential for the integration of mitochondrial oxidative phosphorylation polypeptides into the mitochondrial membrane.[22] However, it is unknown whether CRIF1 has therapeutic potential in autoimmune diseases, especially in RA.

In this study, we investigated the effects of CRIF1 on the development of autoimmune arthritis using an animal model. Over-expression of CRIF1 exerted therapeutic effects by regulating Th17 and regulatory T (Treg) cells as well as osteoclast differentiation in mice with collagen-induced arthritis (CIA). Deletion of CRIF1 in CD4 cells promoted Th17 cell differentiation by activating phosphorylated STAT3.

Materials and methods

Animals

Four- to six-week-old male DBA/1J mice were purchased from Orient Bio Inc. (Seongnam, Korea). CD4-Cre mice and CRIF1 flox mice were obtained from Professor Rho Hyun Seong and Professor Young-Yun Kong (Seoul National University, Korea). CD4 cell-specific CRIF1 conditional knockout mice were generated by crossing CRIF1 flox mice with CD4-Cre mice. All experiments were performed using 6- to 16-week-old mice. Animals were maintained under specific pathogen-free conditions at the Institute of Medical Science of the Catholic University of Korea and were fed standard mouse chow and water. All experimental procedures were examined and approved by the Animal Research Ethics Committee of the Catholic University of Korea; the procedure conformed to all National Institutes of Health of the USA guidelines (Permit number: 2016-0025-03, 2016-0247-02).

Plasmid constructs

Full-length mouse CRIF1 cDNA was amplified by polymerase chain reaction from pcDNA3.1$^+$ DYK mouse CRIF1 vector (GenScript, Piscataway, NJ) using gene-specific primers. The amplified mouse CRIF1 cDNA fragment was cloned into the N-terminal FLAG tagged p3XFLAG-CMV-10 vector between *Hin*dIII and *Bgl*II. The FLAG-tagged p3XFLAG-CMV-10 vector was obtained from Professor Mi-Ock Lee (Seoul National University, Korea).

Arthritis induction and treatment with vector

The CIA was generated as described previously.[23] On day 7 after the first immunization, mice were injected intravenously with 100 μg of p3XFLAG-CMV-10-CRIF1 or control vector in 2 ml of saline within 5 seconds. After 8 days, the same mice were injected intramuscularly with 100 μg of the p3XFLAG-CMV-10-CRIF1 or control vector in the left leg with electrical stimulation (electroporation). The intramuscular injection was performed using a 31-gauge needle insulin syringe for hydrodynamics-based procedures. Two days later, the mice were injected intramuscularly with 100 μg of p3XFLAG-CMV-10-CRIF1 or control vector in the right leg with electroporation.

Clinical assessment of arthritis

The arthritis index, which indicates the onset and severity of joint inflammation, of the mice was scored twice weekly for up to 8 weeks after the primary immunization. The severity of arthritis was assessed on a scale of 0–4 as described previously.[24] The mean arthritis index was compared between the control and experimental groups.

Histopathological analysis

Joint tissues were fixed in 10% neutral-buffered formalin, decalcified in a histological decalcifying agent (Calci-Clear Rapid; National Diagnostics, Atlanta, GA), embedded in paraffin, and sectioned. The sections (5 μm thick) were

© 2018 John Wiley & Sons Ltd,

stained with hematoxylin & eosin and safranin O. The extent of inflammation, bone damage, and cartilage damage was scored as described previously.[25]

Measurement of immunoglobulin

Blood was removed from the orbital sinus of the mice and the sera were stored at − 20°C until use. The amounts of total IgG and collagen type II (CII) -specific IgG in sera were measured using a mouse IgG enzyme-linked immunosorbent assay (ELISA) quantification kit (BethylLab Co., Montgomery, TX). The absorbance at 405 nm was measured on an ELISA microplate reader (Molecular Devices, Sunnyvale, CA).

In vitro osteoclastogenesis and tartrate-resistant acid phosphatase staining

Bone marrow cells were isolated from the tibias and femurs of mice by flushing the bone marrow cavity with α-minimum essential medium (α-MEM) (Welgene, Gyeongsangbuk-do, Korea). The cells were incubated in α-MEM containing 10% fetal bovine serum (Gibco, Carlsbad, CA) for 12 hr. Next, the cells were seeded in 48-well plates at 2×10^5 cells/well and cultured with α-MEM in the presence of 10 ng/ml recombinant human macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN) for 3 days to form macrophage-like osteoclast precursor cells. After 3 days, these osteoclast precursor cells were cultured in the presence of recombinant human macrophage colony-stimulating factor (10 ng/ml) and soluble recombinant human receptor activator of nuclear factor κB ligand (RANKL; 10 or 30 ng/ml; PeproTech, London, UK) for 4 days to generate osteoclasts. Tartrate-resistant acid phosphatase (TRAP) staining was performed using a commercial TRAP kit (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. Multinucleated cells with three or more nuclei were scored as osteoclasts.

Confocal microscopy

Spleen tissues were snap-frozen in liquid nitrogen and stored at −70°C. Spleen tissue sections (5 μm thick) were fixed in acetone. To stain IL-17$^+$, STAT3$^+$, suppressor of cytokine signaling 3 (SOCS3), and phosphorylated (p)-STAT3 (S727 or Y705) in CD4$^+$ cells the following antibodies were used: Alexa Fluor® 488-labeled anti-CD4 (BioLegend, San Diego, CA), anti-STAT3 (Cell Signaling, Danvers, MA) with an Alexa Fluor 555-conjugated secondary antibody (Invitrogen, Carlsbad, CA), anti-SOCS3 (Abcam, Cambridge, UK) with a phycoerythrin (PE) -conjugated secondary antibody (Southern Biotech, Birmingham, AL), and PE-labeled anti-p-STAT3 (pTyr705 or pSer727; BD Biosciences, San Jose, CA). To stain Treg cells, fluorescein isothiocyanate (FITC) -labeled anti-FoxP3, PE-labeled anti-CD4 (both from eBioscience, San Diego, CA), and allophycocyanin (APC) -labeled anti-CD25 (BioLegend) antibodies were used. After incubation with the appropriate antibodies at 4° overnight, sections were analyzed using an LSM 510 Meta confocal microscopy system (Carl Zeiss, Oberkochen, Germany). Positive cells were counted visually at high magnification by four investigators.

Isolation of splenocytes and splenic CD4$^+$ T cells and stimulation

Splenocytes were prepared from mouse spleens by sieving through a 40-μm cell strainer (Falcon, Durham, NC). Red blood cells were lyzed using hypotonic ACK buffer (0·15 mM NH_4Cl, 1 mM KCO_3, and 0·1 mM ethylenediaminetetraacetic acid, pH 7·4). To isolate splenic CD4$^+$ cells, splenocytes were incubated with CD4-coated magnetic beads and isolated on magnetic-activated cell sorting separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were cultured in the presence of plate-bound anti-CD3 (0·5 μg/ml) and soluble anti-CD28 (1 μg/ml) for 3 days to stimulate the T-cell receptor.

Real-time polymerase chain reaction

A LightCycler 2·0 instrument (Roche Diagnostics, Penzberg, Germany; software version 4.0) was used for polymerase chain reaction amplifications. All reactions were performed with SensiFAST SYBR® Hi-ROX (Bioline USA Inc., Taunton, MA) according to the manufacturer's instructions. The following primers were used: IL-17A, 5′-CCTCA AAGCTCAGCGTGTCC-3′ (sense) and 5′-GAGCTCACTT TTGCGCCAAG-3′ (antisense); STAT3, 5′-CCGTCTGGAA AACTGGATAACTTC-3′ (sense) and 5′-CCTTGTAGGA CACTTTCTGCTGC-3′ (antisense); IL-6, 5′-AACGATGAT GCACTTGCAGAAA-3′ (sense) and 5′-TCTGAAGGACTC TGGCTTTGTC-3′ (antisense); SOCS3, 5′-CCTTTGACAA GCGGACTCTC-3′ (sense) and 5′-GCCAGCATAAAAACC CTTCA-3′ (antisense); and β-actin, 5′-GTACGACCAGAG GCATACAGG-3′ (sense) and 5′-GATGACGATATCGCTG CGCTG-3′ (antisense). The mRNA levels were normalized to that of β-actin mRNA.

Enzyme-linked immunosorbent assay

The IL-17 and IL-10 levels in culture supernatants were determined by sandwich ELISA (R&D Systems). Alkaline phosphatase (Sigma-Aldrich) was used for color development. Absorbance at 405 nm was measured on an ELISA microplate reader (Molecular Devices).

Intracellular staining and flow cytometry

To determine the frequency of Th17 cells, peridinin chlorophyll protein complex (PerCP) -conjugated anti-CD4

antibody (eBioscience) -stained cells were intracellularly immunostained with an FITC-conjugated anti-IL-17 antibody (eBioscience). For Treg cells, PerCP-conjugated anti-CD4 and APC-conjugated anti-CD25 (BioLegend) antibody-stained cells were immunostained with a PE-conjugated anti-Foxp3 antibody (eBioscience). Before intracellular staining for IL-17, the cells were stimulated with 25 ng/ml phorbol-12-myristate-13-acetate and 250 ng/ml ionomycin (all from Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences) for 4 hr. Intracellular staining was conducted using an intracellular staining kit (eBioscience) according to the manufacturer's protocol. Events were collected using a FACSCalibur (BD Biosciences, San Diego, CA), and the data were analyzed with FLOW JO software, version 7·6 (Treestar, Ashland, OR).

Statistical analysis

All statistical analyses were performed using GRAPHPAD PRISM (version 4 for Windows; GraphPad Software, San Diego, CA). Normally distributed continuous data were analyzed by parametric Student's *t*-test. Differences in mean values among the groups were subjected to an analysis of variance test. Data are presented as means ± standard deviation. A value of $P < 0{\cdot}05$ (two-tailed) was considered to indicate statistical significance.

Results

CRIF1 controls the severity of autoimmune arthritis

To determine whether over-expression of CRIF1 modulates the severity of arthritis *in vivo*, p3XFLAG-CMV-10-CRIF1 was administered to mice with CIA at 8 days after CII immunization. Injection of p3XFLAG-CMV-10-CRIF1 vector into CIA resulted in a significantly decreased arthritis score ($P < 0{\cdot}001$) and incidence ($P < 0{\cdot}05$) compared with the control mice (Fig. 1a,b). Histological sections of the joints stained with haematoxylin & eosin and safranin O showed that joint inflammation, bone damage, and

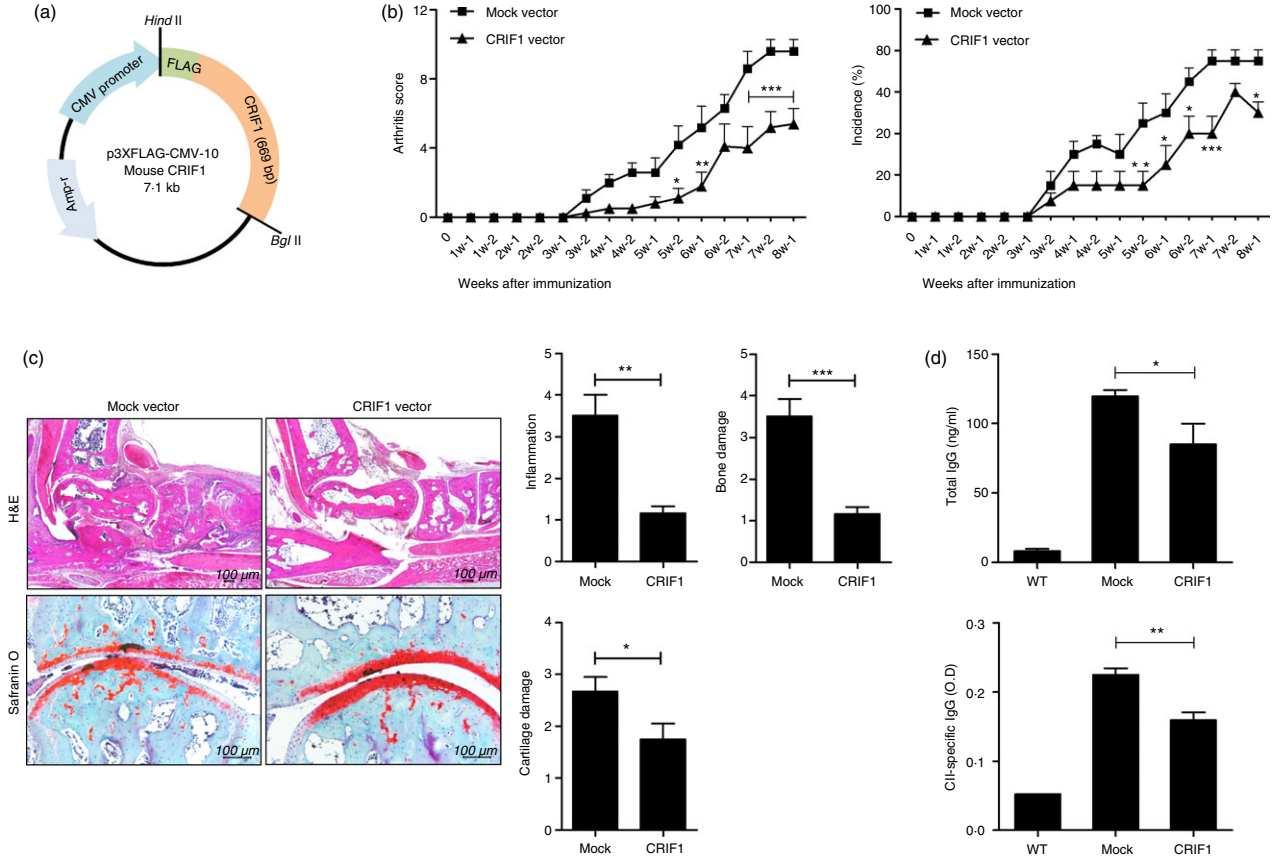

Figure 1. Over-expression of CR6-interacting factor 1 (CRIF1) ameliorates the severity of autoimmune arthritis. (a) Map of the expression vector p3XFLAG-CMV-10-CRIF1. (b) Seven days after the first immunization, p3XFLAG-CMV-10-CRIF1, or the control vector, was administered to the mice by hydrodynamic intravenous injection. After 8 days, the mice were injected intramuscularly with p3XFLAG-CMV-10-CRIF1 or the control vector ($n = 5$/group). Arthritis development was assessed using the arthritis score (left) and incidence (right). (c) Sections of articular tissue were prepared from mice treated as described in (b) 60 days after the first immunization and stained with haematoxylin & eosin and safranin O. Representative histological features are shown. The graphs depict the degree of inflammation, bone damage, and cartilage damage. (d) Serum concentrations of IgG and collagen type II-specific IgG were measured by ELISA. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$ versus p3XFLAG-CMV-10-CRIF1 vector group. Data are means ± SD.

© 2018 John Wiley & Sons Ltd,

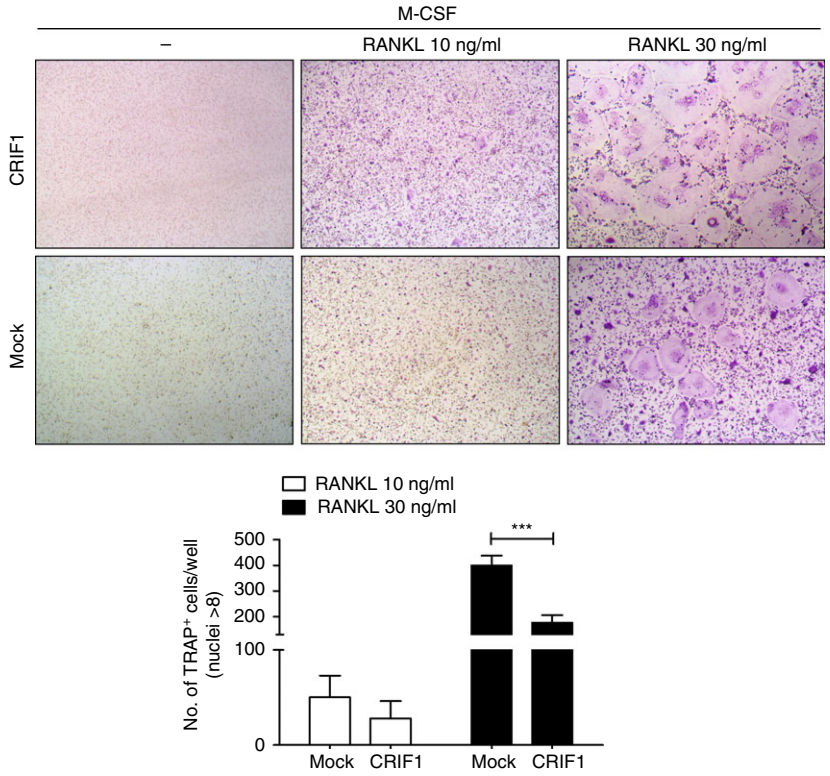

Figure 2. CR6-interacting factor 1 (CRIF1) inhibits osteoclastogenesis in mice. Bone marrow cells were isolated from mice treated with p3XFLAG-CMV-10-CRIF1 or the control vector 60 days after the first immunization and cultured with macrophage colony-stimulating factor (M-CSF) for 3 days to induce osteoclast precursor cells. The cells were cultured with M-CSF and RANKL (10 or 30 ng/ml) for 4 days and stained for TRAP activity (original magnification, ×100). Representative photographs from each group are shown. The number of $TRAP^+$ cells with at least eight nuclei (osteoclasts) was counted under a light microscope. ***$P < 0{\cdot}001$ versus p3XFLAG-CMV-10-CRIF1 vector group. Data are means ± SD.

cartilage damage were significantly ameliorated ($P < 0{\cdot}01$, $P < 0{\cdot}001$, and $P < 0{\cdot}05$, respectively) compared with control mice (Fig. 1c). The serum levels of IgG ($P < 0{\cdot}05$) and CII-specific IgG ($P < 0{\cdot}01$) in mice injected with p3XFLAG-CMV-10-CRIF1 vector were significantly lower than those in control mice (Fig. 1d). Devastating inflammation-driven cartilage and bone destruction in RA is mainly caused by abnormal activation of osteoclasts. Over-expression of CRIF1 in mice with CIA significantly ($P < 0{\cdot}001$) reduced osteoclast differentiation, as determined by enumerating $TRAP^+$ cells (Fig. 2). These results suggest that CRIF1 modulates the development of inflammatory arthritis *in vivo*.

CRIF1 controls the development of arthritis by suppressing Th17 cells *in vivo*

To determine whether over-expression of CRIF1 affects the population of Th17 cells, we examined the number of $CD4^+$ $IL\text{-}17^+$ cells in the spleens of mice treated with p3XFLAG-CMV-10-CRIF1 or the control vector by confocal microscopy. The number of Th17 cells was lower in spleens from p3XFLAG-CMV-10-CRIF1-treated CIA mice compared with the control mice ($P < 0{\cdot}05$) (Fig. 3a). To investigate whether CRIF1 plays a role in the regulation of STAT3, a key transcription factor in Th17 differentiation, the frequencies of total and p-STAT3 (S727) -positive T cells in the spleens of p3XFLAG-CMV-10-CRIF1-treated CIA mice were analyzed by confocal microscopy. The numbers of $CD4^+$ $STAT3^+$ and $CD4^+$ p-STAT3 $(S727)^+$ cells were decreased in p3XFLAG-CMV-10-CRIF1-treated CIA mice compared with the control mice ($P < 0{\cdot}05$) (Fig. 3a,b). By contrast, the expression of SOCS3, a negative regulator of Th17 cells,[7] in T cells was increased in p3XFLAG-CMV-10-CRIF1-treated CIA mice compared with the control mice ($P < 0{\cdot}05$) (Fig. 3c). The mRNA levels of IL-17 ($P < 0{\cdot}05$), STAT3 ($P < 0{\cdot}05$), and IL-6 ($P < 0{\cdot}01$) were decreased, whereas those of SOCS3 were non-significantly increased in splenocytes from p3XFLAG-CMV-10-CRIF1-treated CIA mice compared with those in splenocytes from control mice (Fig. 3d). These results suggest that over-expression of CRIF1 exerts an anti-inflammatory effect by regulating STAT3 and SOCS3, which suppresses Th17 cells.

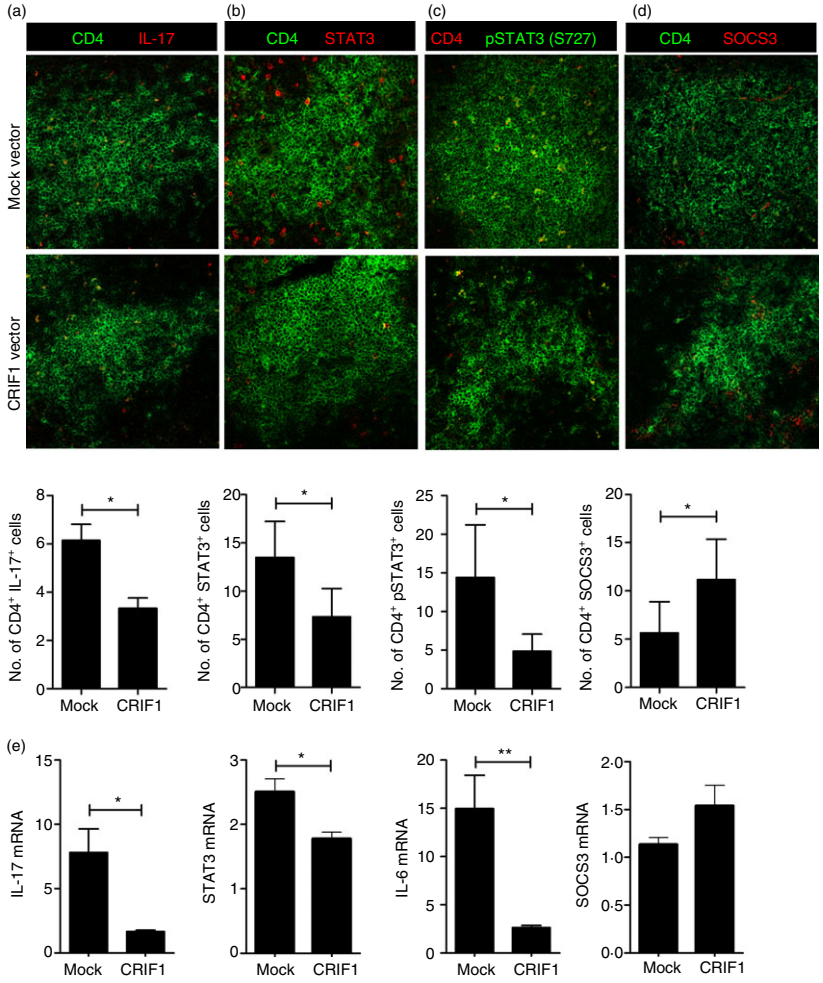

Figure 3. CR6-interacting factor 1 (CRIF1) reduces the frequency of T helper type 17 (Th17) cells in mice with collagen-induced arthritis (CIA). After the first immunization, p3XFLAG-CMV-10-CRIF1, or the control vector, was administered by hydrodynamic intravenous injection and electroporation. (a–d) Sixty days after the first immunization, spleen tissues were isolated and stained for CD4 and signal transducer and activator of transcription 3 (STAT3) (a), CD4 and phosphorylated STAT3 (p-STAT3) (S727) (b), CD4 and suppressor of cytokine signaling 3 (SOCS3) (c), and CD4 and interleukin-17 (IL-17) (d). Each of the cell subsets was analyzed in four independent quadrants by laser confocal microscopy. The distributions of the cell populations in each group are shown. (e) Splenocytes were isolated from mice treated with p3XFLAG-CMV-10-CRIF1 or the control vector 60 days after the first immunization. RNA was extracted from the splenocytes and IL-17, STAT3, IL-6, and SOCS3 mRNA levels were determined by real-time polymerase chain reaction. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ versus p3XFLAG-CMV-10-CRIF1 vector group. Data are means ± SD.

CRIF1 increases the Treg cell population

To determine whether over-expression of CRIF1 regulates the population of Treg cells, which ameliorate the inflammatory response,[11,26] the numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ cells in spleens from p3XFLAG-CMV-10-CRIF1 were analyzed by confocal microscopy. The number of Treg cells was higher in spleens from p3XFLAG-CMV-10-CRIF1-treated CIA mice compared with the control mice (Fig. 4).

CRIF1 deficiency promotes Th17 cell differentiation by activating STAT3

To investigate the impact of loss of CRIF1 in T cells, we generated a conditional CRIF1 gene ablation model using CD4-cre transgenic mice. $CRIF1^{\Delta CD4}$ mice exhibited a higher frequency of $CD4^+$ $IL\text{-}17^+$ Th17 cells ($P < 0{\cdot}01$) whereas the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells was lower compared with wild-type mice (Fig. 5a). Following T-cell receptor stimulation with anti-CD3 and anti-CD28, splenic $CD4^+$ T cells isolated from $CRIF1^{\Delta CD4}$ mice were more prone to differentiation toward Th17 cells compared with cells from wild-type mice ($P < 0{\cdot}05$). By contrast, Treg cells were less prone to differentiation toward Th17 cells compared with those from wild-type mice ($P < 0{\cdot}05$) (Fig. 5b). The IL-17 level was increased ($P < 0{\cdot}05$) whereas that of IL-10 ($P < 0{\cdot}001$) was decreased in the culture supernatants of splenic $CD4^+$ T cells from $CRIF1^{\Delta CD4}$ mice compared with those from wild-type mice (Fig. 5c). To verify the expression of p-STAT3 under

© 2018 John Wiley & Sons Ltd,

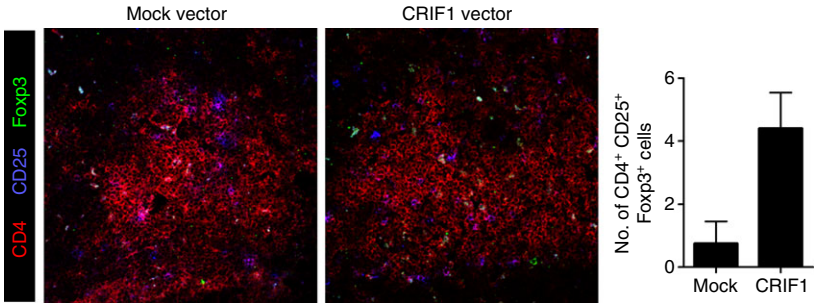

Figure 4. CR6-interacting factor 1 (CRIF1) increases the frequency of regulatory T (Treg) cells in mice with collagen-induced arthritis (CIA). After the first immunization, p3XFLAG-CMV-10-CRIF1, or the control vector, was administered to the mice by hydrodynamic intravenous injection and electroporation. Sixty days after the first immunization, spleen tissues were isolated from mice and stained for CD4, CD25, and Foxp3. Treg cells were enumerated in four independent quadrants by confocal laser microscopy. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$ versus p3XFLAG-CMV-10-CRIF1 vector group. Data are means ± SD.

CRIF1-deficient conditions, the numbers of p-STAT3 $(Y705)^+$ T cells in spleens from $CRIF1^{\Delta CD4}$ mice were analyzed by confocal microscopy. The spleens from $CRIF1^{\Delta CD4}$ mice harbored a larger number of $CD4^+$ p-STAT3 $(Y705)^+$ cells than did the spleens from wild-type mice (Fig. 5d). These results suggest that CRIF1 participates in the development of Th17 and Treg cells by regulating STAT3.

Discussion

In the present study, we investigated the effect of CRIF1 on inflammation in a murine model of autoimmune arthritis. *In vivo*, over-expression of CRIF1 significantly ameliorated the clinical and histologic signs of arthritis by reciprocally regulating Th17 and Treg cells and suppressing osteoclast

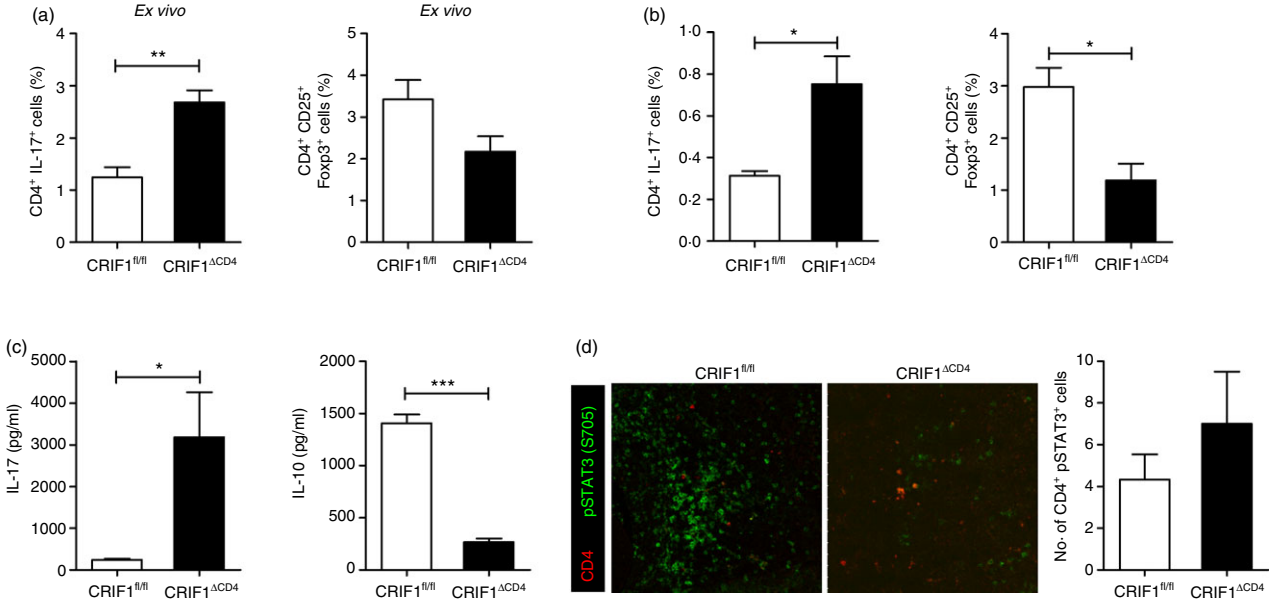

Figure 5. CR6-interacting factor 1 (CRIF1) deficiency increases the number of T helper type 17 (Th17) cells by activating signal transducer and activator of transcription 3 (STAT3). (a) Splenocytes were isolated from the spleens of $CRIF1^{\Delta CD4}$ and wild-type mice and subjected to intracellular staining for $CD4^+$ IL-17^+ cells and $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells by flow cytometry. Values are percentages of positive cells. (b) $CD4^+$ cells were isolated from the spleens of $CRIF1^{\Delta CD4}$ and wild-type mice and stimulated with anti-CD3 (0·5 μg/ml) and anti-CD28 (1 μg/ml) antibodies for 3 days. Next, the cells were re-stimulated with RMA and ionomycin in the presence of GolgiStop for 4 hr to detect intracellular interleukin-17 (IL-17). Cells were stained for IL-17, CD4, CD25, and Foxp3 and analyzed by flow cytometry. Values are percentages of positive cells. (c) $CD4^+$ cells were isolated from the spleens of $CRIF1^{\Delta CD4}$ and wild-type mice and stimulated with anti-CD3 (0·5 μg/ml) and anti-CD28 (1 μg/ml) antibodies for 3 days. IL-17 and IL-10 levels were measured by ELISA. (d) Spleen tissues isolated from $CRIF1^{\Delta CD4}$ and wild-type mice were stained for CD4 and p-STAT3 (Y705). $CD4^+$ pSTAT3 $(Y705)^+$ cells were enumerated in four independent quadrants by confocal laser microscopy. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$ versus $CRIF1^{\Delta CD4}$ mice. Data are means ± SD.

differentiation. The frequency of Th17 cells was markedly increased by conditional knockout of CRIF1 in CD4 cells, whereas the number of Treg cells was decreased. These *in vitro* and *in vivo* results indicate that CRIF1 controls the development of Th17 cells by regulating STAT3 phosphorylation.

CRIF1 is a specific transcriptional co-activator of STAT3. CRIF1 knockout results in embryonic lethality due to defective cellular proliferation and increased cell death. In addition, in cultured blastocysts from CRIF1-knockout mice, the expression of the STAT3 target genes Myc, c-FOS, JunB, and SOCS3 was down-regulated.[21] In this study, we focused on the association of CRIF1 with SOCS3 in T-cell development. Interleukin-6 induces production of Th17 cells by activating STAT3. Interestingly, IL-6 promotes the activation of STAT3 as well as the expression of SOCS3, a key physiological negative regulator of STAT3-activating cytokines.[27,28] Loss of SOCS3 negatively regulates phosphorylation of STAT3 and its binding to the IL-17A and IL-17F promoter region in response to IL-23, which results in greatly increased Th17 cell polarization and cytokine expression.[7] Because CRIF1 deficiency controls the expression of SOCS3, CRIF1 may modulate the activity of Th17 cells. Furthermore, CRIF1 enhances the transcriptional activity of STAT3 by inducing production of leukemia inhibitory factor (LIF) and oncostatin M.[21] LIF, which is produced by neural progenitor cells, exerts an immunoregulatory effect by inhibiting the inflammatory response.[29] Cao *et al.* demonstrated that LIF inhibits the differentiation of Th17 cells by up-regulating SOCS3 and extracellular signal-regulated kinase, which is correlated with impaired STAT3 phosphorylation in mouse and human.[30] In addition, oncostatin M, a member of the IL-6 family, is induced by stimulation with IL-21 immune complex, which suppresses CIA through IL-2–STAT5 signaling in splenocytes and regulates Th17 and Treg cells reciprocally by activating SOCS3 and STAT5 through a negative feedback loop of SOCS3 for inhibition of STAT3.[31] These results suggest that CRIF1 functions as a negative regulator of STAT3 by increasing SOCS3 activity. Therefore, fortification of CRIF1 could ameliorate arthritis by modulating the activity of STAT3 and SOCS3 in T cells to suppress Th17 cells. Further studies should identify the mechanism by which CRIF1 modulates Th17 differentiation.

Mitochondria are complex organelles that play integrated roles in energy metabolism by regulating important anabolic and catabolic pathways. Recently, the role of CRIF1 in mitochondria has gained attention. CRIF1 is a mitochondrial protein that participates in the synthesis and insertion of oxidative phosphorylation polypeptides by interacting with the mitoribosomal large subunit.[22] Until recently, the activity of effector and regulatory T cells was thought to be mediated by transcription factors and epigenetic mechanisms. However, accumulating evidence suggests that changes in basic cellular metabolism also influence T-cell proliferation and cell-fate decisions. Activated T cells must adjust their metabolic programs to satisfy the demand for biosynthetic precursors and provide energy to participate in the immune response.[32–34] For example, activated $CD4^+$ T cells are highly anabolic and exhibit increased glycolysis and glucose uptake to generate ATP and synthesize amino acids, lipids, complex carbohydrates, and ribonucleotides. In contrast, Treg cells produce energy by lipid oxidation and can function and proliferate in the absence of glucose.[35–37] We did not assess the relationship between mitochondrial dysfunction and T-cell development. However, our findings that splenic $CD4^+$ T cells from $CRIF1^{\Delta CD4}$ mice are more likely to differentiate into Th17 cells and that the number of Treg cells and IL-10 production are significantly reduced suggest that CRIF1 plays a role in T-cell development. Further studies are needed to understand fully the role of CRIF1 in T cells.

In conclusion, our data are the first to demonstrate that CRIF1 alleviates arthritis by suppressing Th17 cells and osteoclastogenesis *in vivo*. Loss of CRIF1 in CD4 cells promotes Th17 cell differentiation by activating phosphorylated STAT3 and reduces the frequency of Treg cells and the production of IL-10 *in vitro*. These findings suggest that CRIF1 has therapeutic potential with regard to RA by inhibiting Th17 cells and preventing bone destruction.

Acknowledgments

This study was supported by a grant from the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI14C1894).

Authors' contributions

JSP participated in the study design, experiments, data interpretation, and writing the manuscript. JSP, SYC, SHH, SMK, JWC, KAJ, and JYK carried out the experiments, acquired the data, and performed the statistical analysis. YYK, MLC, and SHP participated in the study design and data interpretation. MLC and SHP conceived and designed the study, interpreted the data, and made critical revisions of the manuscript for important intellectual content. All authors read and approved the final manuscript.

Disclosures

The authors declare that they have no competing interests.

References

1 Feldmann M, Brennan FM, Maini RN. Rheumatoid arthritis. *Cell* 1996; **85**:307–10.
2 Firestein GS. Invasive fibroblast-like synoviocytes in rheumatoid arthritis Passive responders or transformed aggressors?. *Arthritis Rheum* 1996; **39**:1781–90.

© 2018 John Wiley & Sons Ltd,

3 Strand V, Kavanaugh AF. The role of interleukin-1 in bone resorption in rheumatoid arthritis. *Rheumatology (Oxford)* 2004; **43**(Suppl 3):iii10–6.

4 Mellado M, Martinez-Munoz L, Cascio G, Lucas P, Pablos JL, Rodriguez-Frade JM. T cell migration in rheumatoid arthritis. *Front Immunol* 2015; **6**:384.

5 Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006; **441**:235–8.

6 Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, *et al.* Interleukin (IL) -22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 2006; **203**:2271–9.

7 Chen Z, Laurence A, Kanno Y, Pacher-Zavisin M, Zhu BM, Tato C, *et al.* Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc Natl Acad Sci U S A* 2006; **103**:8137–42.

8 Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, *et al.* Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 2007; **26**:371–81.

9 Wei L, Laurence A, Elias KM, O'Shea JJ. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J Biol Chem* 2007; **282**:34605–10.

10 Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. *Annu Rev Immunol* 2009; **27**:485–517.

11 Miossec P. IL-17 and Th17 cells in human inflammatory diseases. *Microbes Infect* 2009; **11**:625–30.

12 Fossiez F, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, Maat C, *et al.* T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med* 1996; **183**:2593–603.

13 Sutton C, Brereton C, Keogh B, Mills KH, Lavelle EC. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J Exp Med* 2006; **203**:1685–91.

14 McGeachy MJ, Chen Y, Tato CM, Laurence A, Joyce-Shaikh B, Blumenschein WM, *et al.* The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells *in vivo*. *Nat Immunol* 2009; **10**:314–24.

15 Nowell MA, Richards PJ, Fielding CA, Ognjanovic S, Topley N, Williams AS, *et al.* Regulation of pre-B cell colony-enhancing factor by STAT-3-dependent interleukin-6 trans-signaling: implications in the pathogenesis of rheumatoid arthritis. *Arthritis Rheum* 2006; **54**:2084–95.

16 de Hooge AS, van de Loo FA, Koenders MI, Bennink MB, Arntz OJ, Kolbe T, *et al.* Local activation of STAT-1 and STAT-3 in the inflamed synovium during zymosan-induced arthritis: exacerbation of joint inflammation in STAT-1 gene-knockout mice. *Arthritis Rheum* 2004; **50**:2014–23.

17 Chung HK, Yi YW, Jung NC, Kim D, Suh JM, Kim H, *et al.* CR6-interacting factor 1 interacts with Gadd45 family proteins and modulates the cell cycle. *J Biol Chem* 2003; **278**:28079–88.

18 Park KC, Song KH, Chung HK, Kim H, Kim DW, Song JH, *et al.* CR6-interacting factor 1 interacts with orphan nuclear receptor Nur77 and inhibits its transactivation. *Mol Endocrinol* 2005; **19**:12–24.

19 Suh JH, Shong M, Choi HS, Lee K. CR6-interacting factor 1 represses the transactivation of androgen receptor by direct interaction. *Mol Endocrinol* 2008; **22**:33–46.

20 Kang HJ, Hong YB, Kim HJ, Bae I. CR6-interacting factor 1 (CRIF1) regulates NF-E2-related factor 2 (NRF2) protein stability by proteasome-mediated degradation. *J Biol Chem* 2010; **285**:21258–68.

21 Kwon MC, Koo BK, Moon JS, Kim YY, Park KC, Kim NS, *et al.* Crif1 is a novel transcriptional coactivator of STAT3. *EMBO J* 2008; **27**:642–53.

22 Kim SJ, Kwon MC, Ryu MJ, Chung HK, Tadi S, Kim YK, *et al.* CRIF1 is essential for the synthesis and insertion of oxidative phosphorylation polypeptides in the mammalian mitochondrial membrane. *Cell Metab* 2012; **16**:274–83.

23 Park JS, Jeong JH, Byun JK, Lim MA, Kim EK, Kim SM, *et al.* Regulator of calcineurin 3 ameliorates autoimmune arthritis by suppressing Th17 cell differentiation. *Am J Pathol* 2017; **187**:2034–45.

24 Barnett ML, Kremer JM, St Clair EW, Clegg DO, Furst D, Weisman M, *et al.* Treatment of rheumatoid arthritis with oral type II collagen. Results of a multicenter, double-blind, placebo-controlled trial. *Arthritis Rheum* 1998; **41**:290–7.

25 Rosloniec EF, Cremer M, Kang A, Myers LK. Collagen-induced arthritis. *Curr Protoc Immunol* 2001; **20**:15.5.1–15.5.24. Chapter 15, Unit 15 5.

26 Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* 2010; **28**:445–89.

27 Lang R, Pauleau AL, Parganas E, Takahashi Y, Mages J, Ihle JN, *et al.* SOCS3 regulates the plasticity of gp130 signaling. *Nat Immunol* 2003; **4**:546–50.

28 Croker BA, Krebs DL, Zhang JG, Wormald S, Willson TA, Stanley EG, *et al.* SOCS3 negatively regulates IL-6 signaling *in vivo*. *Nat Immunol* 2003; **4**:540–5.

29 Pluchino S, Zanotti L, Brambilla E, Rovere-Querini P, Capobianco A, Alfaro-Cervello C, *et al.* Immune regulatory neural stem/precursor cells protect from central nervous system autoimmunity by restraining dendritic cell function. *PLoS ONE* 2009; **4**:e5959.

30 Cao W, Yang Y, Wang Z, Liu A, Fang L, Wu F, *et al.* Leukemia inhibitory factor inhibits T helper 17 cell differentiation and confers treatment effects of neural progenitor cell therapy in autoimmune disease. *Immunity* 2011; **35**:273–84.

31 Lee SY, Cho ML, Oh HJ, Ryu JG, Park MJ, Jhun JY, *et al.* Interleukin-2/anti-interleukin-2 monoclonal antibody immune complex suppresses collagen-induced arthritis in mice by fortifying interleukin-2/STAT5 signalling pathways. *Immunology* 2012; **137**:305–16.

32 Fox CJ, Hammerman PS, Thompson CB. Fuel feeds function: energy metabolism and the T-cell response. *Nat Rev Immunol* 2005; **5**:844–52.

33 Jones RG, Thompson CB. Revving the engine: signal transduction fuels T cell activation. *Immunity* 2007; **27**:173–8.

34 Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 2009; **324**:1029–33.

35 Chang CH, Curtis JD, Maggi LB Jr, Faubert B, Villarino AV, O'Sullivan D, *et al.* Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* 2013; **153**:1239–51.

36 Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, Mason EF, *et al.* Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory $CD4^+$ T cell subsets. *J Immunol* 2011; **186**:3299–303.

37 Shi LZ, Wang R, Huang G, Vogel P, Neale G, Green DR, *et al.* HIF1α-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Exp Med* 2011; **208**:1367–76.