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Regulation of T cell lineage commitment by SMAR1 during inflammatory & autoimmune diseases

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***Background & objectives*: CD4$^+$ T cells are involved in abnormal inflammatory responses causing adverse effects to the body. Th17 cells play a major role in immune disorders and the exact mechanism by which CD4$^+$ T cells regulate its effector Th1 and Th17 phenotype at chromatin level is not clearly understood. This study was aimed to understand the role of matrix associated region (MAR) binding protein SMAR1 (scaffold/matrix attachment region binding protein 1) in T cell differentiation during inflammatory and autoimmune condition using SMAR1 transgenic mice as model.**

***Methods*: Wild type (C57BL/6J) and SMAR1 transgenic mice were used for isolation of T cells and further identification of different T cell lineages, along with histological analysis. Further, we studied autoimmune and inflammatory diseases using chemically induced and T cell transfer model of colitis and rheumatoid arthritis to better understand the role of SMAR1 in immune responses.**

***Results*: SMAR1 transgenic mice were resistant to dextran sodium sulphate (DSS) induced colitis with decreased expression of Th1 and Th17 specific cytokines. Overexpression of SMAR1 repressed Th17 response by negatively regulating RORγt and IL-17 expression. Downregulation of SMAR1 upregulated signal transducer and activator of transcription 3 (pSTAT3) and IL-17 expression that caused generation of more proinflammatory Th1 and Th17 cells leading to inflammation and disease.**

***Interpretation & conclusions*: Our results show an important role of SMAR1 in regulating CD4$^+$ T cell differentiation during inflammatory disorders via regulation of both Th1 and Th17 signaling pathways. This study reveals a critical role of SMAR1 in maintaining the proinflammatory immune responses by repressing Th1 and Th17 cell function and it gives the novel insight into immune regulatory mechanisms.**

Key words Colitis - IL-17 - rheumatoid arthritis - RORγt - SMAR1 - T cell differentiation

Naive CD4$^+$ T cells give rise to T helper cell subsets with functions that are determined to their respective roles in host defense. Depending on the nature of antigen and cytokine secretion of antigen presenting cells, the naïve T cells are differentiated into its various subtypes. For effective and specific immune response, T helper cell mediated immune response is very important, as it affects the overall

immune response to antigens[1]. Following activation, naïve $CD4^+$ T cells differentiate into different lineages of helper T (Th) cells that are characterized by its regulation and distinct biological functions[2]. Till the last decade, T cell mediated immune functions were considered to be binary, involving interferon-γ (IFN-γ) secreting Th1 cells and interleukin-4 (IL-4) secreting Th2 cells, which are primarily effective against viral and intracellular antigens and extracellular parasitic infections, respectively[3]. The focus on IL-17 increased when, the precise cell source of IL-17 was identified in mouse[4]. These cells were named Th17 cells and were associated with a key role in inflammation and matrix destruction[5]. It was found that a combination of immunoregulatory cytokine transforming growth factor beta (TGF-β) and the pro-inflammatory and pleotropic cytokine IL-6 is required to induce IL-17 in naïve T cells[6]. Initial documentation of chromatin modifications of IL-17 locus suggests hyper-acetylation of Histone 3 at Lysine 4 near the promoter of both IL-17A and IL-17F gene in Th17 polarized naïve T cells. Changes in the chromatin loops correlate with the external signals and this contributes for the overall activation/suppression of the locus[7]. Nuclear matrix and associated proteins facilitate this chromatin loop and spatio-temporal arrangement of chromatin by tethering the chromatin to the nuclear matrix and allowing the transcriptional co-activators/repressors to function[8]. Our group is working on one of the nuclear matrix attachment region (MAR) binding protein (MARBP) called SMAR1 (scaffold/matrix attachment region binding protein 1) that interacts with regulatory regions (promoters/enhancers) of the gene and potentially controls the transcriptional activity[9]. MAR, also known as scaffold/matrix attachment regions (S/MARs), are sequences in the DNA of eukaryotic chromosomes, where the nuclear matrix attaches. S/MARs mediate structural organization of the chromatin within the nucleus[10]. These elements constitute anchor points of the DNA for the chromatin scaffold and serve to organize the chromatin into structural domains[11]. Studies on individual genes led to the conclusion that the dynamic and complex organization of the chromatin mediated by S/MAR elements plays an important role in the regulation of gene expression. Role of SMAR1 in immune responses was studied in detail as SMAR1 was identified as an immunomodulator[9]. To better understand the role of SMAR1 in T cell response, transgenic mice were developed, where SMAR1 was overexpressed under a CMV promoter, showed significant fluctuations in the immune responses[12,13].

The present study was undertaken to show the role of SMAR1 as a critical regulator of T cell homeostasis during immune responses and hence, as a good candidate for immunotherapy in autoimmune and inflammatory disorders.

Material & Methods

Mice: This experiment was conducted in Chromatin and Disease Biology Laboratory and experimental animal facility at National Centre for Cell Science (NCCS), Pune, India, from March 2012 to January 2014. SMAR1 transgenic mice (SMAR1 Tg.) were generated at National Institute of Immunology, New Delhi[14]. Wild type C57BL/6J and all other mice were inbred in the experimental animal house facility of the institute. Animal experiments were done with mice 6-10 wk of age and protocols were approved by the Institutional Animal Ethical Committee, NCCS, Pune.

Flow cytometry: Anti-CD4, anti-CD8, anti-CD62L, anti-CD44, anti-CD25, anti-IFNγ, anti-IL-17, and anti-IL-4 were purchased from BD Bioscience, USA. For intracellular cytokine staining (ICS), *in vitro* cultured cells were re-stimulated with phorbol myristate acetate (PMA) (Sigma, USA, 50 ng/ml) and ionomycin (Sigma, 1 mM) for four h. Golgistop (BD Bioscience) was added during the last three h of re-stimulation and $CD4^+$ T cells were stained for intracellular-specific cytokines and sorted by standard procedures[15].

Cytokine assay: Experimental colitis was induced using DSS in six wk old WT and SMAR1 Tg. mice; after 10 days, mice were sacrificed and colon was isolated. WT and SMAR1 Tg. mice without DSS feed were kept as control. Cells were harvested and intraepithelial lymphocytes were isolated. Supernatants were collected and IL-17, IL-6, IFN-γ and IL-12 analyses were done using mouse ELISA kits (R & D Systems, USA). IL-12, IL-6 and IFNγ were measured with OptEIA kits (BD Pharmingen, USA), and IL-17 was measured with DuoSet ELISA kits (R&D Systems, USA). Cytokine mRNA levels were measured by real-time quantitative PCR[6,7].

Isolation of naïve T cells by FACS and differentiation towards Th17 cells: Spleen and lymph nodes of 6-7 wk old C57BL/6J and SMAR1 Tg. mice were processed for single cell suspension and sorted into naïve $CD4^+CD62L^{hi}CD25^-$ T cells with a BD FACS Aria[16]. For Th17 differentiation, IL-6 (20 ng/ml), TGF-β (5 ng/ml), anti-IFNγ (clone-XMG1.2) and anti IL-4 (10 ng/ml) (R & D system, USA) treatment was given. After 24 h, Th17 cells were treated with 10 ng/ml of IL-

23 and observed under microscope for differentiation and blasting at the resting stage after five days of culture[16].

Real-time PCR analysis: Total RNA was extracted from TGF-β and IL-6 treated naïve $CD4^+$ T cells of WT and SMAR1 Tg. mice using RNeasy Mini kit (Invitrogen, USA). Real-time PCR reactions were set up with SYBR Green (Bio-Rad Laboratories, USA) using primers - forward and reverse SMAR1, RORγt (RAR-related orphan receptor gamma t), IL-17; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as control[17].

Colitis induction and histological analysis: Animals were given normal sterile water (control) or three per cent (w/v) dextran sulphate sodium (DSS) (Mw 500.00/η/=0.50, MP Biomedical, USA) continuously for 10-11 days to induce colitis. The animals were weighed daily and monitored for signs of distress associated with rectal bleeding. Histological evaluation of colitis severity was done[18] and sections were stained with hematoxylin and eosin (H&E). Analysis of colitis was performed using clinical and macroscopic parameters like length and weight of the colon. A histopathological score was blindly assessed for the level of colitis. The scoring system was as follows: (1) 1-25%, (2) 26-50%, (3) 51-75%, and (4) 76-100% based on the percentage of tissue affected by inflammation or crypt damage[18].

Cell sorting for purification of $CD4^+CD45RB^{hi}$ cells and injection in severe combined immune deficiency (SCID) mice: A single-parameter histogram (gated on singlet lymphoid cells based on FSC vs. SSC) was used to sort $CD4^+$ singlet cells. The $CD45RB^{hi}$ population, identified as the 40 per cent of cells exhibiting the highest CD45RB staining was sorted. The injection of purified $CD4^+CD45RB^{hi}$ population was done by re-suspending sorted cells in the residual volume (500 µl) of 1X PBS. Weight of the recipient mice was recorded and 0.5×10^6 cells were injected intraperitoneally (ip)[19].

Studies on human samples from patients with autoimmune disorders: Synovial fluid samples were collected from 50 rheumatoid arthritis patients and healthy individuals at Sancheti Hospital, Pune. Peripheral blood mononuclear cells (PBMC) from the synovial fluid of rheumatoid arthritis patients and healthy individuals were isolated using Ficoll layering followed by $CD4^+$ T cell isolation by negative selection with human $CD4^+$ T cell enrichment kit (BD Biosciences, USA) utilizing the magnetic separation. $CD4^+$ T cells were harvested in TRIzol (TRI) reagent (Sigma-Aldrich, USA) for RNA isolation, reverse transcribed to cDNA and checked for the expression of SMAR1 and cytokine IL-17 transcript by quantitative real time PCR[6,11].

Immunoblot analysis: Protein (50 µg) from colonic LP T cell lysates was resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After transferring the protein to PVDF membrane it was probed using pSTAT3 (Santa Cruz, Dallas, TX), SMAR1 (Bethyl, Montgomery, TX), and β-actin (Santa Cruz, Dallas, TX) antibodies using a standard protolcol[10,11].

Statistical analysis: For all experiments, unless stated otherwise, the unpaired student's t test was applied with GraphPad Prism software (La Jolla, CA) to all data points. Correlation was tested with Pearson's correlation test.

Results

SMAR1 overexpression rescues severity of colitis: To understand the physiological relevance of SMAR1 mediated IL-17 regulation, it was assumed that overexpressed SMAR1 would inhibit the inflammatory responses in acute colitis model. For this, colitis was induced in SMAR1 Tg. and WT mice with 3 per cent w/v of DSS in sterile distilled water and the control group received only sterile distilled water. Three animals were housed per cage and DSS/water consumption was monitored daily. The animals were sacrificed by carbon dioxide narcosis on day 10. Gross examination of colon did not show any typical symptoms of colitis including opaque and drastic shortening of the colon length in SMAR1 Tg. mice (Fig. 1A). Further investigation of these mice showed that they have significantly more weight at 10^{th} day of DSS treatment compared with wild type (Fig. 1B). H and E staining showed normal structure of the colon, with normal crypts containing abundant goblet cells and without any major infiltrates (Fig. 1C) and not showed severe signs of colitis including shortened colon length at necropsy (Fig. 1D).

SMAR1 Tg. mice shows decreased IL-17, IL-6 and IFN-γ in DSS induced colitis: There was a significant decrease of IL-6, IL-17 and IFN-γ (Fig. 2A, B and C, respectively) production on day 10 after DSS treatment in SMAR1 Tg. mice, whereas no difference was observed in IL-12 production (Fig. 2D) in intraepithelial lymphocytes cultures between DSS treated WT and SMAR1 Tg. mice.

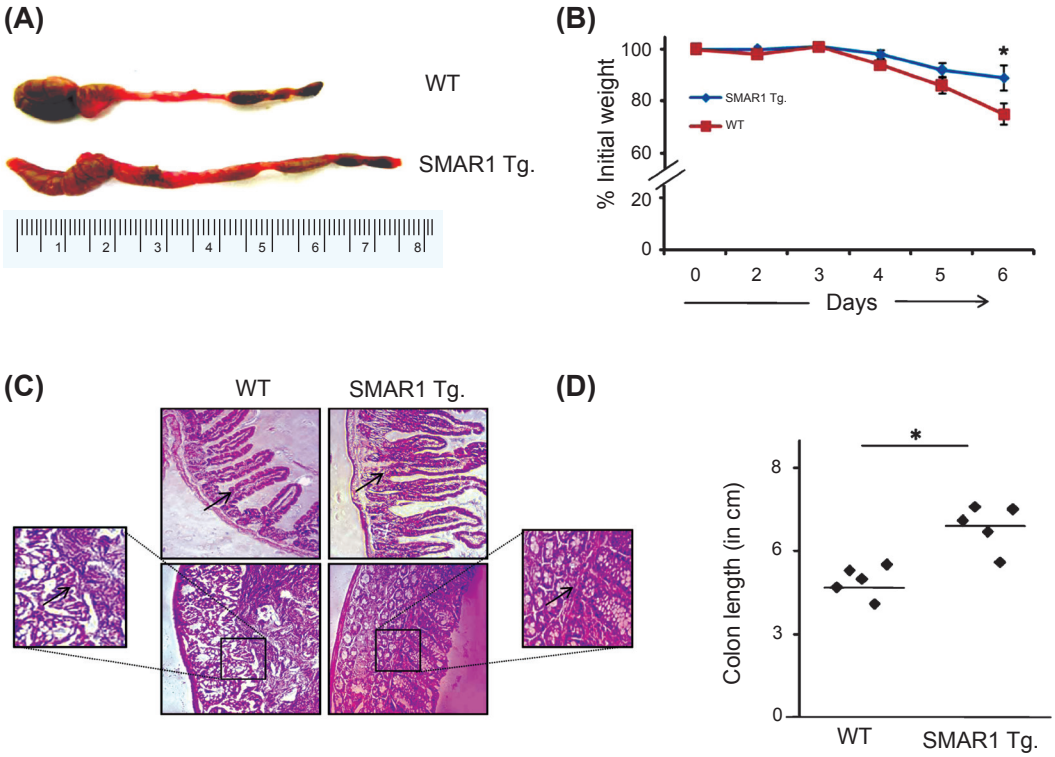

Fig. 1. Comparison of the colon architecture in SMAR1 transgenic (Tg.) and control mice of DSS induced colitis. **(A)** Colon size of control and SMAR1 Tg. mice after 10 days of DSS induced colitis are shown at the same scale. **(B)** Body weight loss of WT and SMAR1 Tg. mice treated with DSS and weighted every alternate day. Values are mean ± SEM (n=6). **(C)** Histological analysis using H & E staining of colon from untreated and DSS treated wild type (WT) and SMAR1 Tg. mice. Displayed images show colon cross-sections with similar region shown by arrows. Inset shows 10×magnification 10 days after colitis induction. **(D)** Colon length of DSS treated mice was measured 10 days after DSS treatment. Data represent the mean ± SEM of n=5 mice/genotype. *P* value were calculated with unpaired student's t test; **P*<0.005.

Reduced SMAR1 level during colonic inflammation mediated by Th1 and Th17 cells: SMAR1 Tg. mice have been shown to develop resistance to a T cell dependent intestinal autoimmune inflammation. We, therefore, reasoned that SMAR1 might be controlling the generation and/or function of proinflammatory Th1 and Th17 cells. First, a profile of total $CD4^+$ T cells from spleen and mesenteric lymph nodes (MLNs) of WT donor and SCID recipient was obtained (Fig. 3A). The isolation of $CD4^+CD45RB^{hi}$ T cells from WT donor was performed using BD-FACS ARIA sorters, the purity of $CD4^+CD45RB^{hi}$ T cells population ranged between 95 to 98 per cent. Transfer of WT $CD4^+CD45RB^{hi}$ T cells initiated the wasting disease in SCID mice, and the colon presented with infiltrating lymphocytes and extensive mucosal and transmural injury (Fig. 3B). Staining (H&E) showed atypical structure of the colon with crypts damage containing oedema, loss of goblet cells and major infiltrates (Fig. 3C). As expected $CD4^+CD45RB^{hi}$ T cells led to severe inflammation associated with significant weight loss (Fig 3D). Regarding cytokine producing cells in the colonic lamina propria (LP) of SCID mice reconstituted with WT $CD4^+CD45RB^{hi}$ T cells; there was a significant increase of IFN-γ^+ and IL-17^+ T cells after 7^{th} wk post transfer (Fig. 3E). Although, the gut pathology allowed the evaluation of effector cytokine production by Th1 and Th17 cells in this scenario, this phenotype was associated with significant reduction in the frequency of $CD4^+SMAR1^+$ T cells in the colon LP compartment of SCID mice reconstituted with WT $CD4^+CD45RB^{hi}$ T cells at 5^{th} and 7^{th} wk post transfer (Fig. 3F). Immunoblot analysis of protein lysate from colonic LP T cells on 1^{st}, 3^{rd}, 5^{th} and 7^{th} wk post transfer revealed enhanced expression of pSTAT3 and reduced expression of SMAR1 (Fig. 3G). It is now well recognized that STAT3 mediated inflammation in the intestine causes increased production of inflammatory cytokines contributes to colitis pathology[20]. These

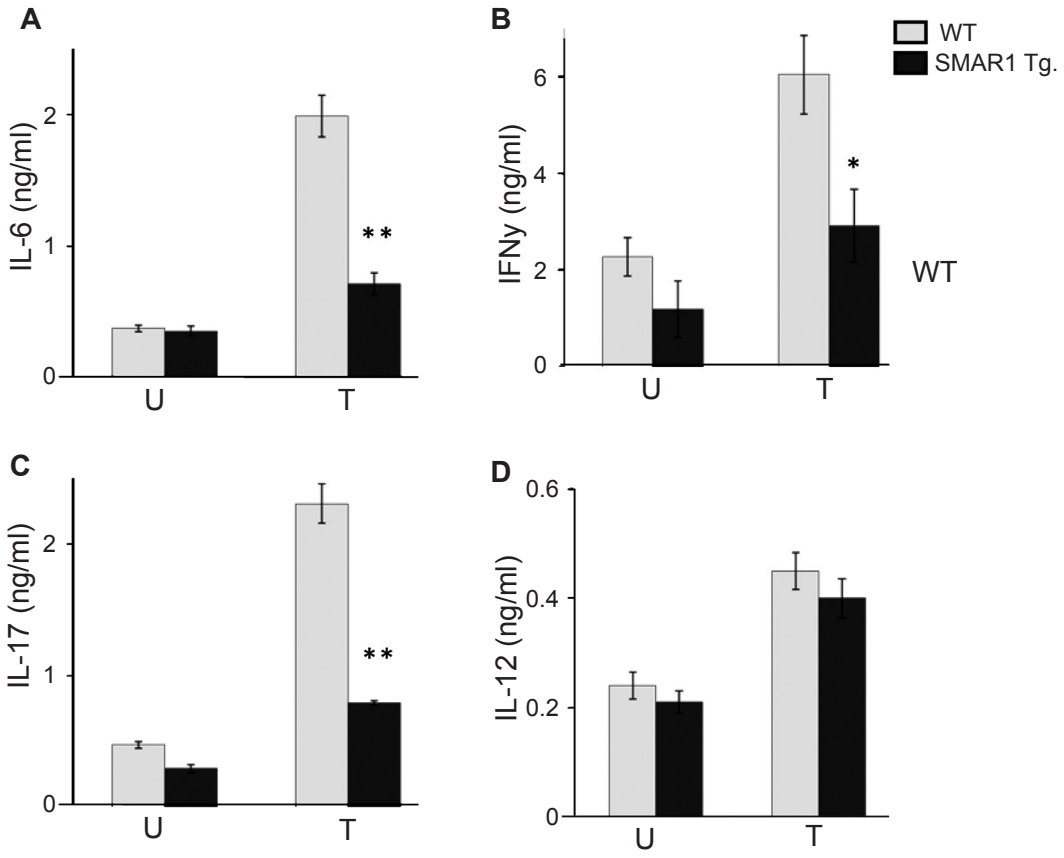

Fig. 2. Reduced effector cytokine production in SMAR1 Tg. mice during DSS colitis. Quantification of *ex vivo* production of IL-6 **(A)**, IFN γ **(B)**, IL-17 **(C)** and IL-12 **(D)** by purified intraepithelial lymphocytes from untreated (U) and DSS treated (T) colons. Data represent the mean ±SEM (n=6). Unpaired student t test was used for statistical analysis. $P^{*}<0.05$, $^{**}<0.001$ compared with WT.

results indicated that the expression of SMAR1 in $CD4^+$ T cells resulted in a deficiency in Th1 and Th17 cells; and defined the immunological mechanisms responsible for the induction as well as regulation of intestinal inflammation.

SMAR1 overexpression can inhibit Th17 differentiation: Naïve T cells, isolated from SMAR1 Tg. and control WT mice were kept under Th17 condition for five days and induction of Th17 lineage specific transcription factor RORγt and cytokine IL-17 was measured. SMAR1 Tg. mice had perturbed Th17 polarization as evident by the reduced induction of RORγt. RORγt induction was 3.5 fold higher in WT mice compared to 1.5 fold induction in SMAR1 Tg. mice (Fig. 4A). Flow cytometric analysis for Th17 cells from WT and SMAR1 Tg. mice was done for protein level expression of RORγt and effector cytokine IL-17. Notably, SMAR1 Tg. Th17 cells significantly downregulated the expression of RORγt in comparison with WT Th17 cells (Fig. 4B). Additionally, SMAR1 Tg. Th17 cells expressed lower effector cytokine IL-17 compared with WT Th17 cells (Fig. 4B). The data suggest a perturbed polarization of Th17 cells in SMAR1 Tg. mice compared with the WT mice in the RORγt and IL-17 axis.

SMAR1 is downregulated in systemic inflammatory disorders: Taken together, our results suggested a role of SMAR1 in regulating the $CD4^+$ T cell lineage commitment. To analyze the physiological significance of the above experimental observations under *in vivo* human disease conditions, SMAR1 level was analyzed in the $CD4^+$ T cells isolated from the synovial fluid of the patients suffering from autoimmune and inflammatory disorders like RA. A total of 50 samples from the patients suffering RA were analyzed. Fig. 5 represents the cumulative data for the expression of SMAR1 and IL-17 from patients samples compared with healthy individuals. Transcript level of SMAR1 is downregulated by about four folds in patient samples compared to healthy subjects, with an inverse association of IL-17, which is upregulated by about 10 folds in RA patients' samples.

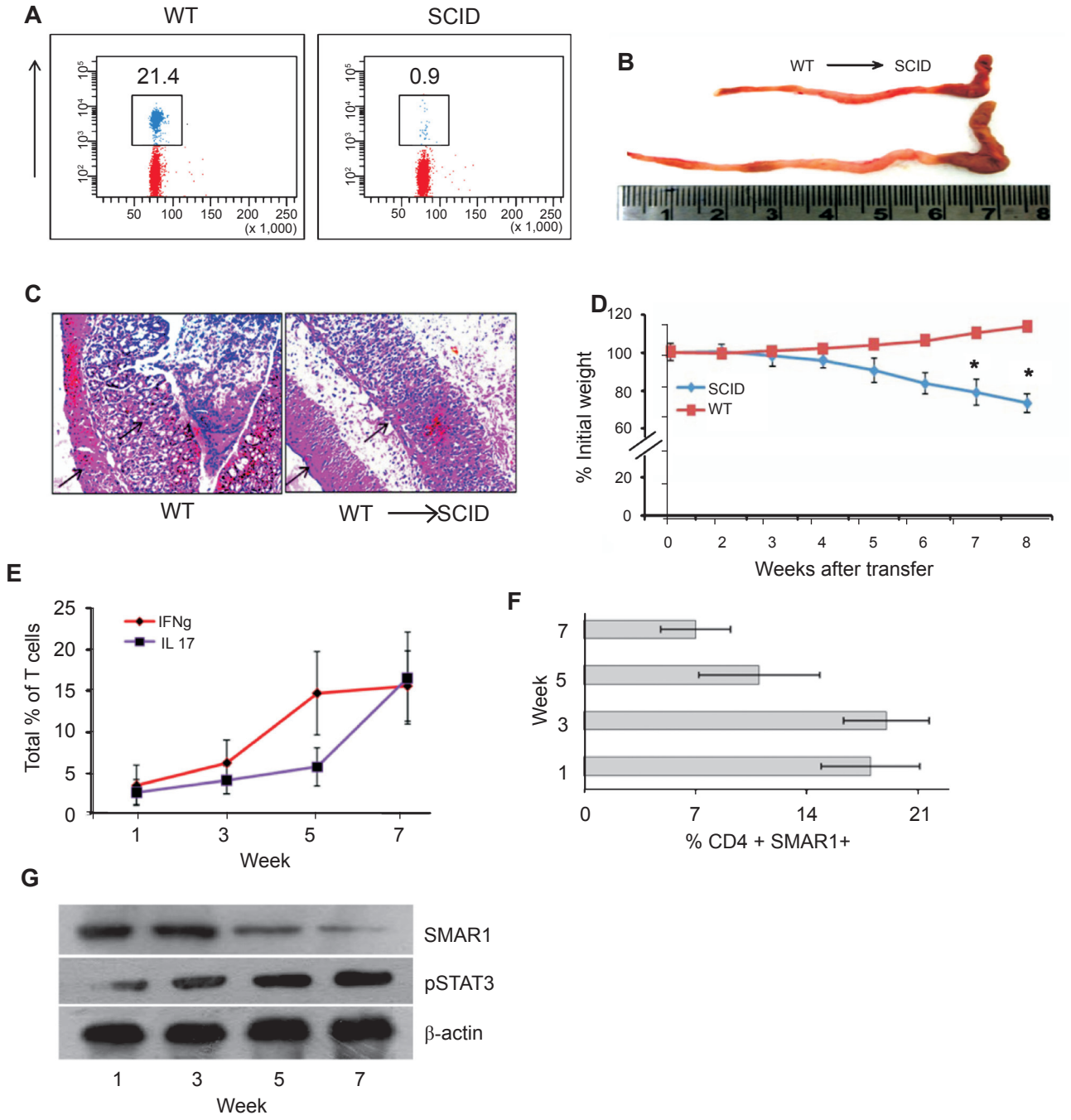

Fig. 3. Reduced SMAR1 level during colonic inflammation mediated by Th1 and Th17 cells. **(A)** Frequencies of $CD4^+$ T cells from the spleen and mesenteric lymph nodes (MLNs) of WT and SCID mice evaluated by flow cytometry. **(B)** Representative colon gross anatomy of WT and WT $CD4^+CD45RB^{hi}$ T cells transferred to SCID mice (WT → SCID). **(C)** H & E staining of colon sections from the mice in **B** of a similar region (arrow) with 10× magnification. **(D)** Sorted $CD4^+CD45RB^{hi}$ T cells from WT mice were transferred in to 6-wk old SCID mice, and the weight of the mice assessed weekly. $^*P<0.002$; determined by unpaired student t test. Values are mean ± SEM (n=6). (E) Frequencies of $IFN\gamma^+$ and $IL\text{-}17^+$ cells among total T cells isolated from colon lamina propria (LP) sections from the mice in **B.** Values are mean ± SEM (n=6). **(F)** Frequencies of $CD4^+SMAR1^+$ cells among total colon LP cells after 1st, 3rd, 5th and 7th week isolated from colon sections of WT $CD4^+CD45RB^{hi}$ T cells transferred to SCID mice. Values are mean ± SEM (n=6). **(G)** Total protein lysate, prepared from the colonic LP T cells from the treated mice as described in **(F)**, analyzed for the pSTAT3 and SMAR1 expression by Western blotting showing decreased SMAR1 and increased pSTAT expression. β-actin was used as a control. Data representative of three independent experiments each with four mice per group.

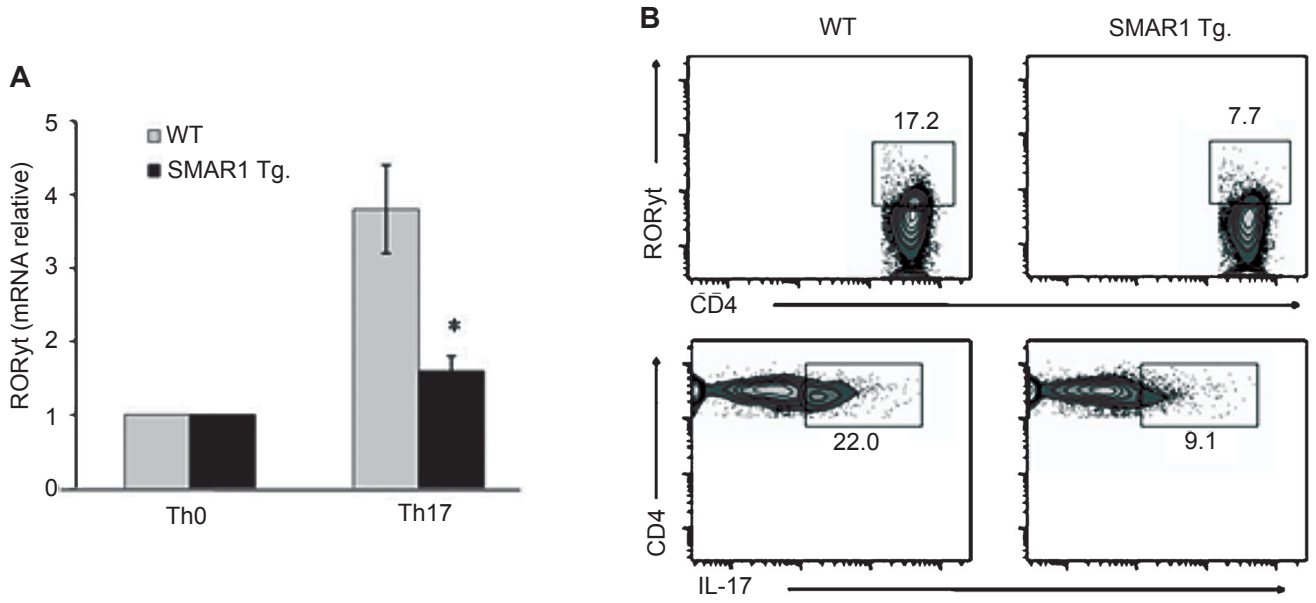

Fig. 4. SMAR1 overexpression inhibits Th17 polarization. **(A)** Naïve T cells were polarized towards Th17 and checked quantitatively for lineage specific transcriptional factor RORγt after 5 days of polarization. Naïve T cells were sorted out from SMAR1 Tg. mice and WT mice and difference of RORγt induction was shown. Values are normalized with naïve T cells. Figure is a representation of three independent experiments. $^{*}P<0.002$ (Calculate using unpaired student t test) **(B)** SMAR1 Tg. Th17 cells express low level of RORγt and IL-17. Cells were harvested and then stained for congenic markers, CD4, RORγt and IL-17. Numbers in quadrant refer to the per cent of each subset. Data representative of four experiments.

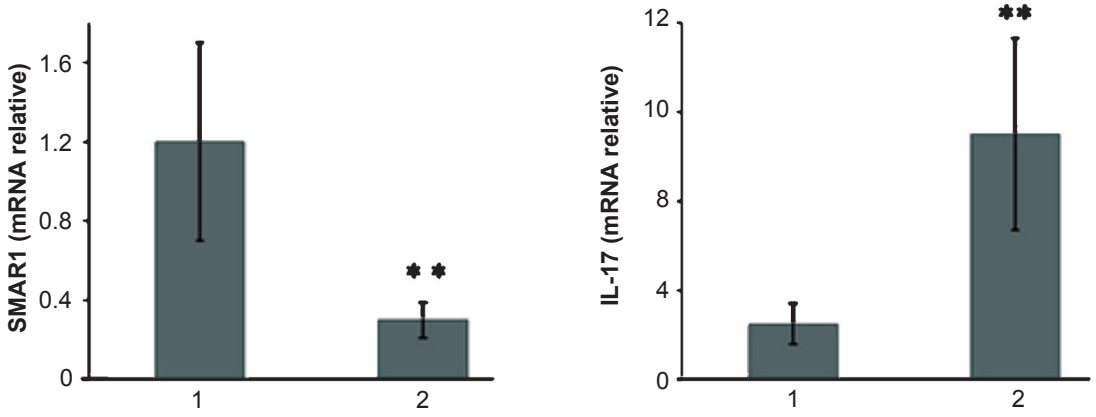

Fig. 5. SMAR1 downregulated in human patients suffering from systemic inflammatory disorders. $CD4^{+}$ T cells were isolated from the synovial fluid of the control normal individuals (1) and rheumatoid arthritis patients (2) and the transcript levels of SMAR1 and IL-17 are quantitatively analyzed using real time PCR. Fifty samples each were analyzed and the result is the cumulative data of the same. Statistical significance was verified with unpaired students t test. $^{**}P<0.001$ compared with healthy (1).

Discussion

Regulation of T cells and fate determining intrinsic factors are of utmost importance due to direct consequences of immune responses in inflammatory and autoimmune disorders. T cell development and its differentiation to various subsets is regulated by nuclear matrix proteins, which modulates chromatin architecture and gene rearrangement. SMAR1, a nuclear matrix protein, was initially shown to attach with the DNA at the MAR regions in T-cell receptor-β (TCR-β) gene[21]. Overexpression of SMAR1 in the thymocytes exhibited reduced rearrangement of

TCR-β gene with elevated number of early double negative thymocytes[22]. They also exhibited reduction in the proportion of thymocytes expressing either CD4 or CD8 co-receptors. Differentiation of naive T cells into effector T cells is a complex mechanism of the immune defense of the body, particularly at the chromatin level. When naive T cells are confronting a pathogenic epitope presented on the surface of major histocompatibility complex (MHC) molecule, it undergoes changes at the chromatin level[3,23]. This modulation at the chromatin level ultimately leads to the expression of a particular set of cytokines which marks the T cells for its functionality and effectiveness[24]. The signals from the MHC-peptide-TCR complex cause activation, proliferation and differentiation of T cells to a particular T cell lineage[25]. IL-17 is a pro-inflammatory cytokine particularly secreted by Th17 cells and is important in eliciting the immune defense against many pathological infections. TGF-β and IL-6 are needed for the polarization of Th17 both *in vivo* and *in vitro*[26-29]. Distinct nature of activation of IL-17 loci and polarization by external cytokine makes a unique model to understand the chromatin modulation by MAR binding proteins in T cell lineage commitment.

We proposed SMAR1 Tg. mice as a good model to study autoimmune and inflammatory diseases, associated with Th17 mediated immune response. Mice generated with overexpressed SMAR1 have perturbed immune response, which confirms the immuno-modulatory function of the protein. SMAR1 Tg. mice display defective T cell maturation and both $CD4^+$ and $CD8^+$ single positive T cells are reduced, and percentage of double positive ($CD4^+CD8^+$) cells remains unchanged. Thus, overexpression of SMAR1 perturbed both $CD4^+$ and $CD8^+$ T cell differentiation[12]. Th17 cells are attributed to immune disorders and targeting the Th17 cells using IL-17 neutralizing antibodies can be a good clinical therapy. We used chemically induced colitis model in mice to understand the role of SMAR1 in T cell development and differentiation during inflammatory responses. The mechanism of protective role of SMAR1 in chemically induced colitis is yet to be understood. As per our observation, SMAR1 downregulation in $CD4^+$ T cells is necessary for Th17 polarization and this downregulation culminates in the activation of Th17 lineage specific transcription factors and cytokines like pSTAT3, RORγt and IL-17 leading to inflammation. SMAR1 Tg. mice had perturbed Th17 polarization as evident by the reduced induction of RORγt and IL-17. The data suggest that SMAR1 has regulatory role in Th17 polarization and it can inhibit the secretion of IL-17 in Th17 polarized cells.

Most therapies in autoimmune and inflammatory disorders are aimed at general suppression of the inflammatory response, thus preventing permanent tissue damage. In addition, a specific approach targeting naturally occurring Th17 population may stimulate immune regulatory event. We chose RA as a model of autoimmune disorder, where Th17 cells played a key role in its regulation[4,15]. In RA patients, the percentage of Th17 cells was increased by 25 per cent in synovial fluid from an affected organ compared to peripheral blood[30]. Therefore, the regulation and functions of Th17 cells can be an effective immunotherapy for RA. In addition to T cell modulating cytokines, MAR binding proteins such as SMAR1 could be an interesting target to reduce IL-17 producing Th17 cells in RA and other inflammatory disorders. The aberrant expressions of MAR binding proteins make them a reliable marker for diagnosis of diseased conditions. Nuclear matrix proteins are also a potential candidate for the use as tumour prognostic factors and targets of anticancer drugs through apoptosis[21]. Therefore, we focused on the role of SMAR1 in the development of immune response, unraveling the novel mechanism of gene regulation. Chromatin changes due to nuclear matrix proteins have consequences in the regulation of gene expression. Thus, study of a cell intrinsic factor like nuclear matrix proteins and targeting it in the therapy of inflammatory and autoimmune disorders is of high importance.

Conflicts of Interest: None.

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