**Whole-genomic DNA methylation pattern revealed type I interferon contributed to the fibrosis of scleroderma by inducing Th cells polarization towards immune suppression as of Treg and release more TGF-β**

**Introduction**

Systemic sclerosis (Scleroderma, SSc) is a complex systemic autoimmune disease characterized by immune dysregulation, fibrosis, and vasculopathy. It is a rare connective tissue disease, with a prevalence rate per year of 2.8-25.3 per million in USA [[1](#_ENREF_1" \o "Mayes, 2003 #9444)]. Based on the maximum extent of skin involvement, SSc is categorised as limited cutaneous (lcSSc) if skin thickening is limited to the extremities distal to the elbows and knees with or without facial involvement, whereas SSc is categorised as diffuse cutaneous (dcSSc) if skin thickening involves areas proximal to the elbows and knees, including the trunk. Our previous research indicates that Chinese patients show 40.3 % lcSSc and 59.7 % dcSSc forms of SSc [[2](#_ENREF_2" \o "Wang, 2013 #10142)]. SSc also can be subgrouped by autoantibody subsets. The most common of the autoantibodies are directed against DNA topoisomerase I (ATA) and centromeric proteins (ACA). ATA was found in 59.9 %, ACA in 13.4 % of Chinese patients cohort [[2](#_ENREF_2" \o "Wang, 2013 #10142)].

The pathogenesis of SSc is not completely understood despite vigorous research efforts. Currently，it is widely believed that aberrant innate immunity reaction due to unknown reasons activate the different subsets of T cells, most importantly Th2, Th17, and Treg cells in SSc. These activated T-cell subsets release interleukin (IL)-4, IL-17, and transforming growth factor (TGF)-β that generate exaggerated states of fibroblast proliferation leading to the fibrosis of SSc. Moreover, it has been assumed that the unbalance between Th1 and Th2 polarize to Th2 contribute to the pathogenesis of SSc and its fibrosis. The Th1 cells release interferon (IFN)-γ that antagonize the fibrosis of SSc.

A multitude of genetic studies, ranging from candidate-gene studies to genome-wide association studies to copy number variation study [[3](#_ENREF_3" \o "McKinney, 2012 #10857)], have identified a large number of genetic susceptibility factors for SSc and its clinical phenotypes [[4-16](#_ENREF_4" \o "Aliprantis, 2007 #9391)], however, the contribution of these genetic factors to disease susceptibility is modest, only to partly explain its pathogenesis [[17](#_ENREF_17" \o "Luo, 2013 #3029)]. Similarly, circumstance factors also do so partly. Epigenetics is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence, which perfectly undertakes the interaction between the genetic factors and environmental ones. However, the contribution of the epigenetic factors to the pathological mechanism of SSc remains unknown. DNA methylation is one of the most common modifications of the epigenetic changes. Recently, using dermal fibroblast of SSc patients, Altorok et al. have found that aberrant DNA methylation status in dcSSc and lcSSc [[18](#_ENREF_18" \o "Altorok, 2015 #10855)]. Another research group shows a similar global higher hypomethylation level in peripheral blood of black South African patients by comparing systemic lupus erythematosus (SLE) with SSc [[19](#_ENREF_19" \o "Matatiele, 2015 #10854)]. These findings suggest that DNA methylation change in SSc has offered the evidences of disease susceptibility [[20-22](#_ENREF_20" \o "Broen, 2014 #3023)].

Different cell type has its special DNA methylation pattern. In early SSc, activated CD4+ and CD8+ T lymphocytes and monocytes/macrophages, and less commonly B cells, eosinophils, mast cells and NK cells, are observed in perivascular regions in the lesional skin, lungs, and other affected organs. These inflammatory cell infiltrates are detectable before the appearance of fibrosis [[23](#_ENREF_23" \o "Roumm, 1984 #3119)]. In situ hybridization studies show prominent procollagen genes expression in early-stage SSc skin in fibroblasts that are adjacent to inflammatory cells, suggesting a role for the inflammatory cells or their soluble products in inducing fibroblast activation [[24](#_ENREF_24" \o "Kahari, 1988 #3397)]. The above results indicate that antigen-driven adaptive immune responses mainly regulated by CD4+ and CD8+ T cells play a core role in SSc disease onset and contribute to its aetiology and pathogenesis [[25-29](#_ENREF_25" \o "Sakkas, 2004 #6929)].

By separating CD4+ and CD8+ T cells from peripheral blood mononuclear cells (PBMCs) of SSc patients and using whole-genome DNA methylation chip and MethylTarget sequencing, we show that SSc has its special whole-genomic DNA methylation pattern. GO analysis of hypomethylation CpG sites in CD4+ and/or CD8+ T cells of SSc showsthat there has a significant enrichment in type I interferon (IFN)-associated signaling pathway and genes, such as EIF2AK2, IFI44L, MX1, IFITM1 and the stratification analysis of SSc to do so, as well. We then focus on type I IFN-associated genes and find that there have significant increases with IFN-α and -β in the serum of SSc patients, which lead to DNA hypomethylation alterations and up-regulated expression of type I IFN-associated genes. With IFN-α and -β stimulus, T cells shift towards proinflammation as Th17 and immune suppression as of Treg, which trigger the development of SSc and its fibrogenesis. These data suggest the global hypomethylation of type I IFN-associated genes contribute to the pathogenesis of SSc and targeting type I IFN-associated genes and T cells might be the novel therapeutic approaches to treat SSc and its fibrosis.

**Materials and methods**

**Study subjects**

52 SSc patients (mean ± SD age XX years )were recruited from the outpatient dermatology clinic and impatient ward of Shanhai TCM-integrated Hospital. All patients met the criteria for SSc established by the American College of Rheumatology [[30](#_ENREF_30" \o "van den Hoogen, 2013 #9427)]. 52 age and gender matched control samples (mean ± SD age XX years) were recruited from Shanghai Changning District Tongren Hospital and Affiliated Hospital of Nantong University. None of the control subjects had a family history of autoimmune disorders. The clinical profiles of the SSc patients as well as the normal subjects are shown in Table 1. Study protocol was approved by the human ethics committee of Fudan University, and all subjects signed an informed consent form. T lymphocyte samples from each group were also paired and studied in parallel.

**Separation of CD4+ and CD8+ T cells from peripheral blood mononuclear cells (PBMCs) of study subjects**

Venous peripheral blood (about 10 ml) was collected from all study subjects and preserved with 2 mg/ml EDTA·K2. PBMCs were isolated by density-gradient centrifugation (Ficoll-PaqueTM PLUS; GE Healthcare Bio, Chicago, IL, USA). Then CD4+ and CD8+ T cells were successively separated by positive selection, using CD4 and CD8 magnetic microbeads according to the manufacturer’s protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Extraction of genomic DNA was performed using the Puregene Blood Core Kit (Qiagen, Valencia, California, USA) as described in the manufacturer’s protocol.

**Differential analysis of CD4+ and CD8+ T cell methylation status of SSc patients and matched controls**

Genome-wide DNA methylation in CD4+ and CD8+ T cells from SSc patients and controls included in this study was assessed using the Illumina Infinium HumanMethylation450 BeadChip array, which allows for the interrogation of over 485 000 methylation sites within the entire genome. This array covers 99% of RefSeq genes, with an average of 17 CpG sites per gene across the promoter region, 5’ untranslated region (5’-UTR), first exon, gene body and 3’-UTR. It also covers 96% of CpG islands. Non-CpG-methylated sites recently identified in human stem cells are also covered as well as microRNA promoter regions.

DNA methylation analysis was conducted using R software and RnBeads package in Bioconductor. In initial preprocessing step, we firstly filtered out the probes whose last 3 bases of its target sequence overlap with SNP; then we removed the cross-reactive probes whose sequence maps to multiple genomic locations. Further, we conducted the greedycut procedure and filtered out the probes whose detection p-value >0.01 in any of the samples. In addition, probes on sex chromosomes, and probes of non-CpG context as well as probes with many missing values were also filtered out. After initial preprocessing, we conducted the background subtraction using the “noob” methods implemented in the methylumi package. The methylation beta values were further normalized using the “BMIQ” normalization method.

Prior to finding of the differentially methylated sites, we conducted the Combat method using sva package to remove batch effect. After batch effect correction, we further removed the probes with little variation. Probes with IQR <=0.1 were considered as little variation and thus were filtered out. We then computed the p-values of the remaining probes with a linear regression model adding the age and gender as two covariates. Differentially methylated CpG sites were defined as sites with p-values <0.05. Furthermore, the differential methylation region (DMR) analysis was also conducted with RnBeads package and Gene Ontology analysis was also conducted with GOstats package in R using the top 500 DMRs.

**Validation of Methylation data**

CD4+ and CD8+ T cells from another 43 SSc samples as well as 41 normal controls were collected, and genomic DNA were converted with bisulfite treatment. Then net-PCR was performed to amplify the interested DNA sequence involved in differential methylation regions (DMR). Then the designed DNA fragments were sequenced by Illumina Miseq. The average sequencing depth of each CpGsite was between 58 and 6267 . Here we need to write the specific protocols used by Genesky tech.

**Differentiation expression and methylation analysis in CD4+ and CD8+ T cells**

Total RNA was extracted from cells using TRizol reagent (Invitrogen, USA) according to the manufacturer's instructions and reverse transcribed using MultiScribe reverse transcriptase kit (ABI, Foster city, CA, USA) according to the manufacturer's instructions. 4 μl of cDNA template was used as for 10 μl Quantitative real-time RT-PCR, using 5 μl of SYBR Green 1x RT-PCR mix (Qiagen) and 1μl of each primer. Reactions were performed on an ABI Q7 Sequence Detector System (ABI), with the following parameters: 94 °C, 3 min; 60 °C, 40 sec, 38 cycles. Melt curves were analyzed from 72 to 88 °C to verify homogeneity.

**T cell culture and stimulation**

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood through density-gradient centrifugation (Ficoll-Paque Plus, GE Healthcare), resuspended in RPMI medium supplemented with 10 % FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, 2 μl leukocyte Activation Cocktail, with BD GolgiPlug (BD, Franklin Lakes, NJ, USA), transferred onto a 24-well plate (2 × 106 cells/well) and cultured at 37 °C for 4~6 h. To prevent non-sepecial staining, PBMCs were preincubated with anti-human Fc blocking antibody (1 μg/ml; G28.5, BioLegend, San Diego, CA, USA) for 15 minutes. Subsequently, cells were stimulated for 6~24 h using alternatively 50 ng/ml purified human recombinant protein IFN-α4 (rIFN-α4) and/or 100 ng/ml rIFN-β (Sino biological, Beijing, China).

**Flow cytometry**

After washings with FACS (PBS+1 % FBS), cells were then stained with V450-conjugated anti-CD3 (OKT3, BD), PerCP-Cy5.5-conjugated anti-CD4 (RPA-T4, eBioscience), PE-conjugated anti-CD25 (SK1, BD), PE-Cy7-conjugated anti-CD152 (TRAP1, eBioscience), APC-conjugated anti-CD127 (FN50, eBioscience), FITC-conjugated anti-Foxp3 (TG1/CXCR3, eBioscience) antibodies, and isotype was as a control to test human Treg cells.

Also, after stimulation and washings with FACS, PBMCs were stained with V450-conjugated anti-CD3 (OKT3, BD), FITC-conjugated anti-CD4 (TG7/CCR6, eBioscience), PerCP-Cy5.5-conjugated anti-IFN γ (TG7/CCR6, eBioscience), APC-conjugated anti-IL 4, PE-conjugated anti-IL17A (1G1, eBioscience), PE-Cy7-conjugated anti-CD185 (TRAP1, eBioscience) antibodies, and also isotype was as a control to detect the secreted cytokines of human Th1, Th2, Th17, and Tfh cells.

Flow cytometry acquisition was performed on a Beckman Counter (Beckman, Brea, CA, USA) instrument to detect above-mentioned five subtype of Th cells in 20 SSc patients and 13 healthy controls. Analysis was conducted using FlowJo software (Tree Star Inc., Ashland, OR, USA).

**Isolation of Human Treg Cells and Naive T Cells**

Human PBMCs were isolated from healthy donors. Human CD4+CD25hiCD127lo Treg cells (natural Treg, nTreg) and CD4+CD127hiCD45RAhi naive T cells were sorted using a FACS ARIA II cell sorter (BD Biosciences). The purity of the isolated cells was 95–99%. In vitro expansion of Treg cells was performed in X-VIVO (Lonza) medium supplemented with 10% human AB serum, 1% GlutaMax (Gibco), 1% sodium pyruvate (Gibco), and 500 U/ml rIL-2 (R&D Systems) in the presence of anti-human CD3/CD28-conjugated Dynabeads (Invitrogen) at a bead-to-cell ratio of 4:1. The purified naive T cells were differentiated into iTreg (induced Treg) cells with 5 ng/ml TGF-β1 (R&D Systems), 100 U/ml rIL-2, and anti-human CD3/CD28-conjugated Dynabeads at a ratio of 1:1.

nTreg cells were stimulated by rIFN-α4 and rIFN-β1 for 6~12 h, then collected cell culture supernatants and cell to detect TGF-β1 concentration and mRNA levels. iTreg cells also were stimulated with rIFN-α4 and rIFN-β for 9 days, then flow cytometry to determine inducing-differentiation capability of IFN-α and IFN-β.

**The detection of cytokines concentration by [enzyme](javascript:void(0);) [linked](javascript:void(0);) [immunosorbent](javascript:void(0);) [assay](javascript:void(0);)**

IFN-α, IFN-β (PBL Assay Science, Piscataway, NJ, USA), IFN-γ and TGF-β1 (Abcam, San Francisco, USA) in human serum or cell culture supernatant by [enzyme](javascript:void(0);) [linked](javascript:void(0);) [immunosorbent](javascript:void(0);) [assay](javascript:void(0);) (ELISA) according to the instruction of the manufacturers. The concentration was calibrated from a dose response curve based on reference standards.

**Result**

**SSc has its unique whole-genomic DNA methylation pattern**

Genome-wide methylation profile of CD4+ and CD8+ T-cells in 24 SSc patients and matched normal controls were obtained with methylation 450K microarray array. The clinical information of SSc and normal samples were shown in **Table 1**.

Among purified CD4+ T cells. After quality control (See Methods), 400420 CpG sites were retained. Of which, we identified 1026 differentially methylated sites (DMS) based on our criteria. 286 of these sites were hypomethylated while 740 were hypermethylated. These probes were distributed across 621 genes, and 440 genes were hypermethylated and 175 genes were hypomethylated. In addition, 6 genes showed mixed methylation status between SSc patients and normal controls. Further, 257 of these significant differential methylated probes located at gene start or gene promoter regions, where DNA methylation changes might negatively correlate with the gene expression. These probes were distributed across 173 genes, including 117 hypermethylated and 56 hypomethylated genes.

In purified CD8+ T cells, out of a total of 400491 probes, 1830 hypermethylated and 783 hypomethylated probes were detected in SSc patients compared with normal controls. These DMS were distributed across 1567 genes, including 1086 genes with only hypermethyated sites, 412 genes with only hypomethylated sites as well as 69 genes with mixed methylation pattern of CpG sites. Among them, 795 CpG sites were located at gene start or promoter regions with 332 hypermethylated genes, 166 hypomethylated genes and 2 genes showing mixed methylation patterns. (Figure xx 交叉圆圈图).

When comparing the DMS between CD4+ T cells and CD8+ T cells, we found 330 of the DMS were shared, and 696 DMS were unique to CD4+ T cells while 2283 DMS were unique to CD8+ T cells. We found that the 330 shared DMS were showing the same directions (hyper or hypo) in CD4+ and CD8+ T cells. The shared CpG sites were distributed across 215 genes and 138 were hypermethylated, 75 were hypomethylated, and 2 genes were displaying mixed methylation patterns (Figure xx).

**A significant enrichment in type I interferon (IFN)-associated signaling pathway and genes**

To identify the biological gene ontologies influenced by the differential methylation pattern in CD4+ and CD8+ T cells between SSc patients and normals, we conducted the differential methylation enrichment analysis implemented in RnBeads package. Interestingly, we found that in both CD4+ and CD8+ subgroups, GO terms of response to type I interferon and type I interferon signaling pathway were enriched in the significantly hypomethylated CpG sites, indicating that methylation changes of type I interferon related genes were of importance in both CD4+ and CD8+ T cells and might play a role in the pathogenesis of SSc (Figure xx). While in the significantly hypermethylated CpG sites, no shared GO term was identified in CD4+ T cells and CD8+ T cells and showed strong difference between CD4+ and CD8+ T cells, indicating the differential methylation changes in the two types of T cells.

**DNA methylation next generation sequencing validate and argument the differential CpG sites**

To further confirm our findings, we then used the next generation sequencing technology to validate the 62 differentially methylated CpG sites between SSc patents and controls as well as in the subtype analysis in CD4+ and CD8+ T cells. 43 SSc patients and 41 normal controls were obtained for DMS validation in CD4+ T cells and 40 SSc patients as well as 40 normal controls were prepared for DMS verification in CD8+ T cells, including 13 patients and 12 normals from the first stage and the other samples were recruited independently. Among these CpG sites, 57 were successfully investigated and while the remainder failed due to technical limitations. However, due to the advantages of next generation sequencing technology, we also identified 154 more CpG sites besides the selected significantly methylated CpG sites. (Figure xx，线路图). In order to validate and evaluate the consistency between the microarray and next generation sequencing results, we compared the β value from the microarray data and the methylation percentage from NGS sequencing data with the same CpG sites and same samples (Figure xx). It turned out that the methylation ratios from two different platforms were almost linear and the pearson’s correlation coefficients was 0.86 and 0. 90 for CD4+ T cells and CD8+ T cells respectively, indicating the good consistency between the two platforms. Then in the differential analysis stage, we validated the 57 selected differential methylated CpGsites with the validation data. We found that 7 CpGsites were validated to be significantly differential methylated between SSc patients and normal controls in CD4+ T cells, while in the CD8+ T cell dataset, 9 CpGsites were shown to be significantly differential methylated (Table xx). In addition to the selected 57 CpG sites, we also detected another 154 CpG sites that were close to them. And among these CpG sites, we found 1 and 10 differential methylated CpG sites in CD4+ as well as CD8+ T cells. And all of the 11 CpG sites were located at promoter region of MX1 gene. Based on the validation dataset, it turned out that the CpG sites located at interferon-related genes were all validated while the methylation status of the other CpG sites were not consistent between the discovery and validation stage, and thus we then focused our further analysis on sites and genes related to the type I interferon pathway.

**Strong correlation between DNA methylation and type I interferon-induced gene expression**

To test whether DNA methylation is correlated with gene expression, we performed q-PCR experiments for quantification of the IFN-related genes’ expression. We found that in CD4+ T cells, 4 of the 5 genes showed strong higher expression in SSc patients with significant difference while the expression of EIF2AK2 is also higher in SSc patients but didn’t pass the threshold due to its small sample size (Table xx). And similarly in CD8+ T cells, we found significant higher expression of IFITM1, and marginal significant higher expression of IFI44L, MX1 as well as the PARP9 in SSc patients. It is widely known that there is strong correlation between the gene expression and methylation. And we thus combined the expression dataset with its methylation profile to test its correlation. Pearson’s correlation test was conducted and P-value < 0.05 was considered significant after FDR multiple correction. In the combined analysis of CD4+ and CD8+ T cells datasets, we found that the methylation status of three CpGsites located at IFI44L gene showed strong inverse correlation with its expression (Figure xx). And other 5 CpGsites located at PARP9, MX1 and IFI44L showed marginal significant correlation between its methylation status and gene expression. Based on these datasets, we found the expression of the type I interferon related genes were significantly higher in SSc patients both in CD4+ and CD8+ T cells, and the correlation analysis between DNA methylation and expression of IFN-related genes suggested that the DNA methylation changes might be pivotal to the regulation of IFN-related genes.

**SSc patients have significant increases of IFN-α and -β in the serum**

Based on our discovery and validation stage dataset, we can safely conclude that part of the type I interferon-related genes were hypomethylated in CD4+ and CD8+ T cells of SSc patients. In addition to the methylation and expression profile detection, we also measured the level of type I interferon (IFN- α and IFN- β) in the serum of SSc patients and normal controls through ELISA. We found that significantly higher concentration of IFN- α as well as IFN- β were shown in SSc patients than in matched controls (Figure X).

As it is widely acknowledged that high level of serum type I interferon will lead to the higher expression of its induced genes, we conducted the correlation analysis to test the association between serum IFN level and the gene expression status of type I interferon-related genes. We found that the expression of IFITM1 as well as the IFI44L gene in CD4+ T cell showed significantly positive correlation between serum type I IFN alpha level and its expression, while the expression of IFITM1, EIF2AK2, PARP9 and MX1 gene showed strongest positive correlation between type I IFN beta and its expression (Table xx). However, in the CD8+ T cell datasets, we didn’t found any significant association between gene expression and the serum type I IFN alpha/beta level of these five genes. These results indicated that the correlation between the expression of type I IFN related genes and the serum type I IFN level might be different in CD4+ and CD8+ T cells.

In addition to the correlation analysis between gene expression and serum type I Interferon level, we also conducted the correlation analysis between the gene methylation and serum type I interferon level. In CD4+ T cells, we found the CpG site located at IFITM1 gene was inversely correlated with serum IFN- α level with a marginal significant p-value, while in CD8+ T cells, two CpG sites located at PARP9 gene were inversely correlated with serum IFN- β level, and a CpGsite located at EIF2AK2 gene was also inversely correlated with serum IFN- β level (Table xx), indicating a modest inverse correlation between serum type I IFN level and methylation status of IFN-induced genes.

**High-expressed type I IFN lead to DNA hypomethylation alterations as well as elevated expression of type I IFN-associated genes**

Due to up-expression of type I IFN and a significant enrichment of type I IFN-associated genes and pathway in SSc, we speculated that maybe type I IFN-associated genes had significant over-expression. Based on these, we detected mRNA levels of type I IFN-associated genes, EIF2AK2, IFI44L, IFITM1, MX1, and PARP9 in more than 20 CD4+ T cells and CD8+ T cells from SSc patients. Compared to those in more than 20 healthy controls, mRNA levels of these genes had significant high-expression (Figure X1). Interestingly, the expression of these genes negatively correlated with their methylation status in SSc patients (Figure X1). In order to determine whether type I IFN can induce DNA hypomethylation alterations of type I IFN-associated genes, we detected the DNA methylation status of type I IFN-associated genes in Jurkat cell-line and THP-1 cell-line inducing with rIFN-α4 and rIFN-β. As expected, significant hypomethylation changes of EIF2AK2, IFI44L, IFITM1, MX1, and PARP9 were showed up (Figure X=).

**DNA hypomethylation alterations of type I IFN-associated genes, partly due to demethylase TET1 up-expression, leaded to their up-regulated expression**

THP-1 cells showed up-expression of EIF2AK2, IFI44L, IFITM1, MX1, and PARP9 genes and so did jurkat cells. We speculated whether DNA hypomethylation of type I IFN-associated genes negatively correlated with their genes expression or not. Therefore, we analyzed the correlation between DNA methylation status and mRNA expression level. As showed in figure X, the extent of demethylation in MX1 gene negative correlated with its gene expression. So did IFI44L, EIF2AK2, IFITM1 genes (figure X).

Why IFN-α and -β stimulus could induce the hypomethylation alteration of type I IFN-associated genes, we speculated that the expression of DNA methyltransferases (DNMT) or demethylases contributed to the change. Therefore, we determined the mRNA expression of human DNMT1, DNMT3A and TET1, TET2, TET3 genes in THP-1 cell line stimulated by rIFN-α4 and rIFN-β1. Compared with no stimulus, the mRNA levels of *TET1* and *TET3* genes markedly up-regulated after rIFN-α4 and rIFN-β1 stimulating for 6 h (figure Xa, Xb), while that of *DNMT3A* gene significantly down-regulated after rIFN-α4 stimulating for 12 h (figure Xa). To validated the results, we detected these DNMTs and demethylases in the CD4+ T cells and CD8+ T cells from SSc patients (n>=20). As what we think does: the mRNA level of DNMT1 gene in SSc patients was significantly high than that in healthy controls (figure Xc). Additionally, the expression level of *TET1* gene was higher than that of *DNMT1* gene in 6 SSc patients (figure Xd). Together, these results indicated that DNA hypomethylation alterations of type I IFN-associated genes inducing with IFN-α and -β, partly due to DNA demethylase TET1 up-expression.

**SSc showed high level of functional Treg cells frequencies and high level of Th17 cells, low level of Th1 cells**

A deteriorative complication of SSc is the developing fibrosis. The researches have shown that the adapative immunity involved in Th cells attributes to the fibrogenesis [[31](#_ENREF_31" \o "Wynn, 2012 #568), [32](#_ENREF_32" \o "Wynn, 2004 #569)]. So, we have utilized flow cytometry to determine CD4+CD25hiCD127lo Treg (nTreg) cells number in 20 SSc patients and 13 controls, however, there were no significance between two groups (figure X2a). Lanteri et al report that functional Treg with CD4+CD25hiCD127loCD152+ can release effective cytokines to negative regulate cell immunity[[33](#_ENREF_33" \o "Lanteri, 2009 #14036)]. Therefore, we detected functional Treg frequence in above-mentioned groups, and found that functional Treg level was significantly higher in case group than that in control (figure X2b). Radstake et al found that the frequency of CD4+CD25hiFoxP3hiCD127- Treg cells is highly increased in all SSc subgroups [[34](#_ENREF_34" \o "Radstake, 2009 #13763)]. These indicated that T cells in SSc patients maybe shift towards immune suppression as of Treg cells.

Additional, we also detected functional levels of CD4+ Th subtype cells with hallmarked cytokines staining in these case and control groups. As shown in figure X2C, Th1 cells (CD4+IFNγ+) frequencies were significantly low in case group than that in control group. However, Th2 cells (CD4+IL4+) frequencies were invariable between two groups (figure X2d) and Th17 cells (CD4+IL17A+) frequencies were also significantly higher in case group than that in control (figure X2e). These results suggested that Th1/Th2+Th17 unbalance in SSc polarized to Th2 and Th17, and the unbalance in SSc contribute to its fibrogenesis [[35](#_ENREF_35" \o "Wynn, 2011 #566)]. Interestingly, the number of Tfh cells (CD4+CD185+) in case group were significantly lower than that in control (figure X2f), which also validated that T cells in SSc patients maybe shift towards immune repression.

**With IFN-α and -β stimulus, T cells polarized towards immune suppression as of Treg cells**

The above experiments indicated that SSc patients had high Treg levels, this suggested IFN-α and -β stimulus may lead to naïve T cells differentiating towards Treg cells.Therefore, we separatedCD4+CD127hiCD45RAhi naive T cells from PBMCs of healthy controls, then induced to differentiate into iTreg cells. As shown in Fig X3a, with rIFN-α4 and -β1 stimulus for 6 h, iTreg cells level was higher than that with no stimulus. This result indicated that type I IFN could give rise to Th cells to proliferate and differatiate into more Treg through DNA hypomethylation and up-regulation of type I IFN-associated genes.Contrastly, with rIFN-α4 and –β1 stimulus, PBMCs from SSc patients had markedly lower level of IFN-γ protein (Fig X3d) and mRNA expression (Fig X3e) than those from healthy controls.

Together, type I IFN can lead to Th1/Th2 unbalance to Th2 and more Treg cells, which release more TGF-β. These two processes both accelerated SSc developing to fibrosis.

**Discussion**

[[36](#_ENREF_36" \o "Lei, 2009 #10856)]DNA methylation is an important epigenetic mechanism that can cause durable changes of gene expression that are heritable during cell division [[36-38](#_ENREF_36" \o "Watt, 1988 #11999)]. A ELISA-like study exhibits a global hypomethylation in CD4+ T cells from SLE and SSc patients [[39](#_ENREF_39" \o "Lei, 2009 #10856)], suggesting an epigenetic methylation alteration in CD4+ T cells of SSc maybe involve in its pathogenesis. Herein, we integrated whole-genome DNA methylation chip and MethylTarget methylation sequencing of target region to determine the global DNA methylation status in CD4+ T cells and CD8+ T cells from PBMCs of SSc, finally found the unique DNA methylation pattern of SSc. L;

Through comparative analysis of DNA methylation patterns in CD4+ Th cells and CD8+ Th cells of SSc, we found that the global hypomethylation alteration of type I interferon genes and their associated genes in signaling pathway shared in both Th cells. Interestingly, other studies have found that the hypomethelation of type I IFN-associated genes were occurred in SLE [[39](#_ENREF_39" \o "Lei, 2009 #10856), [40](#_ENREF_40" \o "Absher, 2013 #12008)]. As we all known, SLE is a common autoimmune disease. Although the list of type I IFN-associated genes in SLE is different from those in SSc, signaling pathway of type I IFN involved in autoimmune diseases might be in common, maybe be relative to its autoimmunity [[41-45](#_ENREF_41" \o "Renaudineau, 2011 #3036)]. What’s more, SSc patients have high expression level of type I interferon proteins such as IFN α and βin their blood (Figure X).

Hugle T. found that the relatively early age of SSc onset, like sclerosing skin disorders, in patients with multiple sclerosis (MS) has a genetic predisposition and/or an IFN-associated trigger [[46](#_ENREF_46" \o "Hugle, 2009 #11688)]. Another study also confirmed that several systemic autoimmune diseases including SSc and Sjogren syndrome were observed to activate type I IFN system, which may contribute to the vascular pathology and affect the profibrotic process [[44](#_ENREF_44" \o "Eloranta, 2010 #11684)]. Additionally, many reports indicate that DNA methylation can control the destiny and development of T cells [[47-49](#_ENREF_47" \o "Sellars, 2015 #14099)]. Nevertheless, why and how type I IFN to trigger the fibrogensis remain unknown.

Therefore, combined with our above-mentioned results and other researcher’s foregone conclusions, we assumed that type I IFN system i.e. the genes associate with type I interferon, result in their DNA hypomethylation alteration that implicated in the inherited DNA methylation variations of CD4+ and CD8+ T cells in SSc, which can modulate the immune responses to the environment, and these might be involved in Th cells developing to Treg or Th17, which consequently affect SSc fibrosis in some situations.

In order to verify this hypothesis, we ultilized the recombination of IFN α and β to induce THP-1, Jurkat cells and PBMCs and confirmedly found the hypomethylation alteration of type I IFN-associated genes due to up-regulation of type I IFN. Moreover, up-expression of TET1 gene may be the reason of hypomethylation of type I IFN-associated genes and ultimately results in their up-expresison, which then induced Th cells polarization into Treg, which trigger immune suppression and Th17 in a pathologic manner (illustrated in Fig 8). [[46](#_ENREF_46" \o "Hugle, 2009 #11688)]

Wynn TA has reported that Th17-type immunity is proinflammatory and profibrotic, however, Th1-type shows anti-fibrotic activity[[31](#_ENREF_31" \o "Wynn, 2012 #568), [35](#_ENREF_35" \o "Wynn, 2011 #566), [50](#_ENREF_50" \o "Pinto, 2011 #14038)]. One study found that Treg cells seems plausible to suppress Th17 and Th2-driven fibrosis but exacerbate TGF-β1-dependent fibrosis[[31](#_ENREF_31" \o "Wynn, 2012 #568)]. Accumulating evidence indicates that alterations in Treg frequencies and/or function may contribute to autoimmune diseases [[50](#_ENREF_50" \o "Michels-van Amelsfort, 2011 #4889)]. In our research, IFN α and βled to the hypomethelation of type I IFN-associated genes and induced Th cells differentiation to Th17 and TGF-β1-produing Treg cells, which can cause the fibrosis. In some sense, our research indicated that the therapy with type I IFN maybe result in the fibrosis as an adverse effect through producing Th17 and profibortic Treg, although Treg can allivate inflammation.

As for why SSc patients have up-regualted expression of type I interferon, we thought virus infection maybe one of the important causes. West Nile virus (WNV) infection can lead to the overexpression of type I interferon in healthy controls [[51](#_ENREF_51" \o "Pinto, 2011 #14038)]. As we known, the prevalence of WNV infection in western countries is similar with that of cytomegalovirus (CMV) in Asian countries, especially in China. Our experiment also found that high tites of CMV was present in the blood of SSc patients (data not shown). Some bacterial and viral infectious agents such as CMV have been proposed as possible triggering factors to SSc[[52](#_ENREF_52" \o "Randone, 2008 #10943), [53](#_ENREF_53" \o "Namboodiri, 2006 #10946)]. All together, these indicate that virus infection maybe the cause of the production of type I interferon.

It should be mentioned that the significantly different CpG sites of EIF2AK2、IFI44L、IFITM1、PARP9 and PRIC285 genes almost locate at less than 1500 bp from their transcription start site (TSS). This, therefore, offers the prominent convenience of DNA methylation in regulating the gene expression directly or indirectly at transcription level. Ideally, if DNA methylation of gene could control its expression[[36](#_ENREF_36" \o "Watt, 1988 #11999)], there would be negatively correlative between methylation and expression level of gene, which was luckily validated by our results about the expression levels of these genes inversely correlated with their methylation status (Figure X).

[[40](#_ENREF_40" \o "Absher, 2013 #12008)] In this study, we have separated CD4+ and CD8+ T cells, found the shared DNA hypomethylation CpG sites between them, and the up-regulation in the signaling pathway and genes related to type I interferon is common. Reports found it is distinct of DNA methylation pattern in CD4+ and CD8+ cells, respectively [[54](#_ENREF_54" \o "Bos, 2015 #14047), [55](#_ENREF_55" \o "Tserel, 2015 #14040)], and so did in SSc. Therefore, future research maybe focus on the special CpG sites in CD4+ or CD8+ cells of SSc patients.

We herein study and discuss is that DNA methylation of type I IFN system can trigger T cells into Treg or Th17 differentiation and a resultant fibrosis development. So, epigenetic treatments and diagnosis biomarkers e.g. DNA methylation already being used in oncology may soon prove beneficial also in autoimmune diseases. Autoantibodies can activate endothelial cells and fibroblasts to a profibrotic phenotype[[27](#_ENREF_27" \o "L, 2010 #5430)]. CD4+ or CD8+ T cells with different DNA methylation status can also auxiliarily lead to SSc-like fibrotic phenotype. Then, treatments directed against T cells or B cells will show promising effects to SSc in the next future.

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Here is several suggestion:

2, In the discussion section, please try to answer:

1,推测比较显著的那些基因，与SSc相关的作用机制，（pathway，Gene ontology）

2, 有没有HLA区域的位点异常甲基化，重点讲一讲

3，比较一下与上面三个文章的发现，之间有没有冲突，或者相互印证的发现

4，check一下有没有甲基化发现的区域，与SSc GWAS比较接近的区域，如果list出来。

5，也可以比较一下怎们发现的CPG区域或者基因与其他免疫性疾病（SLE,RA）发现的异常甲基化区域有相同的吗？

6，我们用了CD4和CD8，是否可以下结论CD4或CD8哪个甲基化异常更多？

5，描述一下咱们文章的limitation

6，展望一下未来的研究方向