International Immunopharmacology 59 (2018) 53–60

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Dysfunction of regulatory T cells in patients with ankylosing spondylitis is associated with a loss of Tim-3

Mingfei Wang[a,1], Chengyi Liu[a,1], Allen Bond[b], Jun Yang[c], Xu Zhou[a], Jian Wang[d,*], Bin Ji[a,**]

[a] Department of Orthopedics, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China
[b] DICAT Biomedical Computation Centre, British Columbia, Canada
[c] Department of Spine Surgery, Changzheng Hospital, Second Military Medical University, Shanghai, China
[d] Department of Orthopedics, Pudong New Area People's Hospital, Shanghai, China

ARTICLE INFO

Keywords:
Tim-3
Treg
Arthritis
Ankylosing spondylitis

ABSTRACT

An expansion of regulatory T cells (Tregs) in ankylosing spondylitis (AS) was observed. However, AS patients continue to exhibit aberrant inflammation. In this study, we collected PBMCs from 26 AS patients and 26 healthy controls, and investigated the functional capacity of Treg cells from these subjects. In AS patients, the frequency of $CD4^+CD25^+Foxp3^+CD127^-$ Treg cells was slightly increased compared to healthy controls, but the level of Foxp3 MFI in AS patient $CD4^+CD25^+Foxp3^+CD127^-$ Treg cells was significantly lower than that in healthy control $CD4^+CD25^+Foxp3^+CD127^-$ Treg cells. Tim-3^+ Treg cells were previously shown to present stronger suppressive capacity than Tim-3^- Treg cells. Here, we discovered that the Tim-3^+ cell frequency in $CD4^+CD25^+Foxp3^+CD127^-$ Treg cells was significantly lower in AS patients. In both healthy volunteers and AS patients, Tim-3^+ Treg cells demonstrated higher transcription of Foxp3, IL-10 and TGF-β, higher secretion of IL-10 and TGF-β, and stronger inhibition of conventional T cell inflammation, than Tim-3^- Treg cells. In some but not all functional aspects, the Tim-3^+ Treg cells from healthy controls were more potent than the Tim-3^+ Treg cells from AS patients. Collectively, these results demonstrated two Treg-related impairments in AS patients. First, the frequency of the more potent Tim-3^+ Treg cells was lower in AS patients, and second, some of Tim-3^+ Treg-mediated functions were less potent in AS patients. Interestingly, the ratio of Tim-3^-/Tim-3^+ Treg cells in AS patients was directly correlated with the Bath ankylosing spondylitis disease activity index (BASDAI) score, the C-reactive protein (CRP) level, and the erythrocyte sedimentation rate (ESR). Given the fact that Tim-3^+ Treg cells presented potent suppressive functions, Tim-3^+ Treg cells and Tim-3^+ Treg-mediated mechanisms might be potential candidates for immunotherapies in AS patients.

1. Introduction

Ankylosing spondylitis (AS) is a rheumatic disease caused by long-term inflammation in the spine and the sacroiliac joints. The chronic inflammation at the affected areas can induce abnormal bone growth and bony fusion, resulting in pain, stiffness, and distortion in the spinal cord [1]. Both genetic association and environmental factors are thought to contribute to the atypical inflammation in AS. The HLA-B27 gene is strongly associated with AS, as 80% of AS patients belong to the HLA-B27 haplotype [2,3]. At the same time, only 1% to 5% of all HLA-B27 carriers develop AS [4]. Bacterial infections with *Chlamydia*, *Salmonella*, *Yersinia*, *Shigella* and *Campylobacter*, as well as subclinical intestinal inflammation and inflammatory bowel disease, have been associated with increased AS risk [5,6]. In addition, T cell response to the autoantigen aggrecan is found in AS patients [7]. Together, these lines of evidence suggest that AS can develop from the breaking of self-tolerance and atypical inflammation in genetically predisposed individuals.

Regulatory T (Treg) cells are critical in maintaining self-tolerance and suppressing excessive inflammation. The canonical Treg cells express the forkhead/winged helix transcription factor P3 (Foxp3), which is a transcriptional activator of multiple Treg-associated genes [8]. Treg cells secrete interleukin (IL)-10 and/or transforming growth factor (TGF)-β, which are pleiotropic cytokines = suppressing multiple inflammatory responses, including the maturation and proliferation, of conventional T (Tconv) cells and B cells [9,10]. The canonical Treg cells

* Correspondence to: J. Wang, Department of Orthopedics, Pudong New Area People's Hospital, 490 Chuanhuan South Road, Shanghai, China.
** Correspondence to: B. Ji, Department of Orthopedics, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, 164 Lanxi Road, Shanghai, China.
E-mail addresses: jianwang1962@126.com (J. Wang), drbinji@sina.com (B. Ji).
[1] These authors contributed equally to the work.

https://doi.org/10.1016/j.intimp.2018.03.032
Received 18 January 2018; Received in revised form 29 March 2018; Accepted 29 March 2018

also express CD25 (IL-2 receptor alpha chain), and may impair T cell proliferation by depleting local IL-2 concentration [11]. In addition, Treg cells express cytotoxic T lymphocyte-associated protein 4 (CTLA-4), which binds and downregulates CD80/CD86 [12]. Impairment in Treg-mediated suppression has been demonstrated in multiple autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, and type 1 diabetes [13–15]. In AS, the peripheral blood Treg cells was shown to present lower Foxp3 expression, little STAT5 phosphorylation upon IL-2 stimulation, and more extensive methylation at CNS2, of which the demethylation is required for the maintenance of Foxp3 expression [16].

T cell immunoglobulin and mucin-domain containing molecule 3 (Tim-3) is a transmembrane protein expressed on Treg cells and Tconv cells. Despite the fact that Tim-3 is best recognized as a marker of exhausted T cells, there is surprisingly a lack of evidence supporting the notion that Tim-3 could directly mediate T cell inhibition [17,18]. Interestingly, Tim-3$^+$ Treg cells appear to represent a more potent subset of Treg cells, with higher expression of IL-10, granzymes, and perforin, and higher Foxp3 expression than Tim-3$^-$ Treg cells [19–21]. When incubated with Tconv cells, Tim-3$^+$ Treg cells displayed superior suppression capacity than Tim-3$^-$ Treg cells [19–21]. In tumor-infiltrating Treg cells, the Tim-3$^+$ population was significantly enriched [20,22]. The reversal of T cell exhaustion by anti-Tim-3 treatment in tumor immunotherapies, therefore, is possibly caused by the suppression of Tim-3$^+$ Treg-mediated regulation [20].

Although it was identified that Treg cells in AS patients displayed functional impairments [16,23,24], the underlying mechanism of Treg dysfunction is yet unclear. In this study, we compared the Treg cells from healthy volunteers and AS patients, and discovered that the AS patients displayed a downregulation in the frequency and function of the Tim-3$^+$ Treg subset.

2. Methods

2.1. Patients and controls

This study was approved by the Institutional Review Board of Putuo Hospital. All AS patients and healthy volunteers provided written informed consent. Newly diagnosed untreated AS patients fulfilling the 1984 modified New York criteria for AS [25], and age- and sex-matched healthy volunteers, were consecutively enrolled at Putuo hospital. All participants provided peripheral blood samples. Bath ankylosing spondylitis disease activity index (BASDAI), C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR) levels of the AS patients and healthy volunteers, if applicable, were evaluated on the same day of the sampling. The characteristics of the participants are listed in the Table 1.

2.2. Cell culture conditions

In vitro cell incubation was performed in RPMI 1640 supplemented with 5 mM Hepes, 2 nM L-glutamine, 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.5 mM sodium pyruvate, and 0.05 mM non-essential amino acids (all from Thermo Fisher Scientific) in 96-well round bottom plates. In selected cell cultures, 50 ng/mL PMA and 1 μg/mL ionomycin (Sigma Aldrich), or Human CD3/CD28 T cell activator (Stemcell) were added for stimulation, as specified in each experiment.

Table 1
Characteristics of study participants.

	Healthy	AS	P
N	26	26	
Age (y)	31.5 ± 10.2	33.5 ± 8.4	> 0.05
Sex (M/F)	18/6	18/6	
BASDAI (cm)	N/A	5.9 ± 1.5	
CRP (mg/L)	3.2 ± 1.4	26.3 ± 12.7	< 0.001
ESR (mm/h)	10.4 ± 3.5	39.8 ± 14.0	< 0.001

Data were represented as mean ± SD where applicable. Differences in age, CRP, and ESR levels between healthy volunteers and AS patients were calculated using Student's *t*-test.

2.3. Flow cytometry

Phenotyping of the Treg cells was performed in a LSR II system (BD), and cell subset isolation was performed in a FACSAria cell sorter (BD). PBMCs were first isolated from blood by Ficoll-Paque (Amersham Biosciences) centrifugation method, washed twice in PBS supplemented with 2% FBS, and labeled with fluorophore-conjugated anti-human CD3, CD4, CD25, CD127, and Tim-3 antibodies (BioLegend) and a Violet dead cell dye (Invitrogen), for 30 min in dark. The cells were then labeled with anti-human Foxp3 antibodies using the Foxp3 Fixation/Permeabilization Buffer set (eBioscience) according to the manufacturer's protocol. For sorting, cells were not fixed or permeabilized. T cell–depleted accessory cells were first obtained by negative selection of PBMCs using anti-CD2–coated beads (111.13; Dynal), and then irradiated at 3300 rad.

2.4. Treg suppression assay

CD4$^+$CD25$^-$ Tconv cells, Tim-3$^+$CD4$^+$ CD25$^+$CD127$^-$ Treg cells, and Tim-3$^-$CD4$^+$CD25$^+$CD127$^-$ Treg cells were sorted and re-suspended at 5×10^5 cells/mL. 50 μL/well Tconv cells were then added to each well of the 96-well round bottom plate, with sequentially diluted Treg cells. All wells were topped up with culture medium to a final volume of 200 μL/well. Cells were stimulated with anti-CD3/CD28 beads and incubated for 72 h. The plates were then centrifuged at 400 *g* for 5 min. 100 μL supernatant was removed for cytokine ELISA, using the Human IFN-γ and TNF-α Quantikine ELISA kits (R&D Systems). The cells were resuspended and pulsed with 0.1 μCi/well ^{3}[H]-thymidine (Sigma Aldrich) for 5 h, after which the radioactivity was counted.

2.5. Examination of Treg-associated gene transcription and cytokine secretion

For Foxp3 mRNA, the sorted Tim-3$^+$CD4$^+$CD25$^+$CD127$^-$ and Tim-3$^-$CD4$^+$CD25$^+$CD127$^-$ Treg cells from PBMCs were lysed directly ex vivo. For IL-10 and TGF-β mRNA, the sorted Tim-3$^+$CD4$^+$CD25$^+$CD127$^-$ and Tim-3$^-$CD4$^+$CD25$^+$CD127$^-$ Treg cells were stimulated with PMA and ionomycin for 5 h and then lysed. Total RNA was collected using the RNeasy mini kit (Qiagen) and was reverse-transcribed using the High-Capacity cDNA RT Kit (Applied Biosystems). The resulting cDNA was processed with the Taqman PreAmp Master Mix (Applied Biosystems), followed by the Taqman gene expression assay. Relative mRNA levels were calculated using the $2^{-\Delta Ct}$ method with the actin gene for calibration.

2.6. Statistical analyses

Mean ± SD was presented where applicable. Student's *t*-test was used for one-way comparisons between two groups. ANOVA was used for two-way comparisons between two or more groups. Bonferroni's post-test was then applied. Pearson correlation was used for examining the correlation between two parameters. *P* value smaller than 0.05 was required for statistical significance.

3. Results

3.1. Tim-3 expression was downregulated in CD4$^+$CD25$^+$Foxp3$^+$CD127$^-$ Treg cells from AS patients

First, we examined the Tim-3 expression in Treg cells from AS