Journal of Autoimmunity 59 (2015) 26–37

Contents lists available at ScienceDirect

Journal of Autoimmunity

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

Systems biologic analysis of T regulatory cells genetic pathways in murine primary biliary cirrhosis

Yin-Hu Wang [a, 1], Wei Yang [a, 1], Jing-Bo Yang [a], Yan-Jie Jia [a], Wei Tang [a], M. Eric Gershwin [b], William M. Ridgway [c, **], Zhe-Xiong Lian [a, d, *]

[a] Liver Immunology Laboratory, Institute of Immunology and The CAS Key Laboratory of Innate Immunity and Chronic Disease, School of Life Sciences and Medical Center, University of Science and Technology of China, Hefei, Anhui 230027, China
[b] Division of Rheumatology, Allergy and Clinical Immunology, University of California at Davis School of Medicine, Davis, CA 95616, USA
[c] Division of Immunology, Allergy and Rheumatology, University of Cincinnati, Cincinnati, OH 45220, USA
[d] Innovation Center for Cell Biology, Hefei National Laboratory for Physical Sciences at Microscale, Hefei, Anhui 230027, China

ARTICLE INFO

Article history:
Received 3 January 2015
Accepted 30 January 2015
Available online 17 February 2015

Keywords:
Primary biliary cirrhosis
Cholangitis
Regulatory T cells
Transcription profile and pathway analysis

ABSTRACT

$CD4^+Foxp3^+$ regulatory T cells (Tregs) play a non-redundant role in control of excessive immune responses, and defects in Tregs have been shown both in patients and murine models of primary biliary cirrhosis (PBC), a progressive autoimmune biliary disease. Herein, we took advantage of a murine model of PBC, the dominant negative transforming growth factor β receptor II (*dnTGFβRII*) mice, to assess Treg genetic defects and their functional effects in PBC. By using high-resolution microarrays with verification by PCR and protein expression, we found profound and wide-ranging differences between *dnTGFβRII* and normal, wild type Tregs. Critical transcription factors were down-regulated including *Eos*, *Ahr*, *Klf2*, *Foxp1* in *dnTGFβRII* Tregs. Functionally, *dnTGFβRII* Tregs expressed an activated, pro-inflammatory phenotype with upregulation of *Ccl5*, *Granzyme B* and *IFN-γ*. Genetic pathway analysis suggested that the primary effect of loss of TGFβ pathway signaling was to down regulate immune regulatory processes, with a secondary upregulation of inflammatory processes. These findings provide new insights into T regulatory genetic defects; aberrations of the identified genes or genetic pathways should be investigated in human PBC Tregs. This approach which takes advantage of biologic pathway analysis illustrates the ability to identify genes/pathways that are affected both independently and dependent on abnormalities in TGFβ signaling. Such approaches will become increasingly useful in human autoimmunity.

1. Introduction

Although there have been significant improvements in our understanding of the immunological events that occur in patients with primary biliary cirrhosis (PBC), there remains a paucity of data on underlying molecular mechanisms that facilitate breach of tolerance [1–6]. This problem is compounded by the fact that patients develop clinical symptomatology long after the earliest causal events that initiate disease [7]. Antibodies to mitochondrial antigens, the serologic hallmark of PBC, are found many years before diagnosis [8]. Hence, an approach to understanding of the earliest events that lead to cholangitis are critical. In this respect our laboratory has studied a murine model of PBC, *dnTGFβRII* mice [9]. These animals develop high titer autoantibodies to mitochondrial antigens of the same specificity as humans with PBC and also exhibit portal infiltrates, ductopenia, granulomas, and a number of immunological features that are shared by patients with PBC [10].

Abbreviations: dnTGFβRII, dominant negative transforming growth factor β receptor II; PBC, primary biliary cirrhosis; Tregs, regulatory T cells; mLN, mesenteric lymph node; WT, wild type; MNC, mononuclear cells; IFN-γ, interferon-γ.

* Corresponding author. Liver Immunology Laboratory, Institute of Immunology and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China. Tel./fax: +86 551 63600317.

** Corresponding author. Division of Immunology, Allergy and Rheumatology, University of Cincinnati College of Medicine, 231 Albert Sabin Drive, Cincinnati, OH 45220, USA. Tel.: +1 513 558 5551; fax: +1 513 558 3799.

E-mail addresses: wangyh8@mail.ustc.edu.cn (Y.-H. Wang), ywei0702@mail.ustc.edu.cn (W. Yang), yjingb@mail.ustc.edu.cn (J.-B. Yang), jyanjie@mail.ustc.edu.cn (Y.-J. Jia), wayne012@mail.ustc.edu.cn (W. Tang), megershwin@ucdavis.edu (M.E. Gershwin), wridg1@gmail.com (W.M. Ridgway), zxlian1@ustc.edu.cn (Z.-X. Lian).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jaut.2015.01.011
0896-8411/

In both patients with PBC and human autoimmune diseases, $CD4^+Foxp3^+$ regulatory T cells (Tregs) play a pivotal role in controlling excessive immune responses [11]. Tregs defects have been reported in both PBC patients and murine models [12,13]. Recent data suggest that *dnTGFβRII* $CD4^+Foxp3^+$ Tregs possess weaker suppressive function than wild type Tregs [14]. In fact, the immunopathology of *dnTGFβRII* mice requires defects in both Tregs and pathogenic cytotoxic $CD8^+$ T cells [15]. To better define the *dnTGFβRII* genetic Treg abnormalities, we have performed comprehensive transcriptome analysis, quantitative PCR and protein expression of various Treg populations.

2. Materials and methods

2.1. Mice

dnTGFβRII mice were derived from the vivarium at the University of California at Davis. $Foxp3^{GFP}$ mice ($Foxp3^{tm2Ayr}$) were kindly provided by Dr. A.Y. Rudensky [16]. *dnTGFβRII*; $Foxp3^{GFP}$ mice were generated by selective breeding of our *dnTGFβRII* colony with female $Foxp3^{GFP/GFP}$ mice. All mice were housed under specific pathogen-free and controlled environmental conditions in the animal facility of the School of Life Sciences of the University of Science and Technology of China. All experiments were performed following approval from the USTC Animal Care and Use Committee.

2.2. Flow cytometry, immuno-phenotype detection and intracellular staining

All mice were studied at 10–13 weeks of age. Liver, spleen or mesenteric lymph nodes mononuclear cells were isolated and prepared as described [17]. Single cell suspensions were incubated with anti-mouse CD16/32 (BioLegend, San Diego, CA). All additional flow antibodies, unless otherwise noted, were purchased from BioLegend. To identify subpopulations of $CD4^+$ T cells, cells were stained with Pacific Blue-CD3 (17A2), PE-Cy7-NK1.1 (PK136), APC-Cy7-CD4 (GK1.5) [9]. To detect Tregs related surface markers and confirm the data from gene expression profile, cells were labeled with PE-CD25 (PC61), APC-GITR (YGITR765), PE-CXCR3 (CXCR3-173), Alexa 647-CCR6 (29-2L17), PerCP/Cy5.5-ICOS (C398.4A), or PE-CD62L (MEL-14). In some cases, intracellular staining was performed using a FOXP3 Fix/Perm Buffer Set (BioLegend) to detect CTLA-4 using PE-CTLA-4 (UC10-4B9) [18]. To detect the level of intracellular cytokine and confirm the data from microarray, cells were resuspended in RPMI-1640 with 10% fetal bovine serum and stimulated with Cell Stimulation Cocktail (plus protein transport inhibitors) at 37 °C 5% CO_2 for 3 h [19]. Thence, cells were stained with surface markers by CD3, CD4, CD8β, and NK1.1 as described [17], fixed with Fixation Buffer (BioLegend), and permeabilized with Permeabilization Wash Buffer (BioLegend), and stained for intracellular PE-IFN-γ (XMG1.2), Alexa Fluro 647-Granzyme B (GB11), APC-IL-10 (JES5-16E3). For purposes of control, normal IgG isotypes were used (BioLegend). Stained cells were analyzed using a flow cytometer FACS verse (BD) and data analyzed using Flowjo software (Tree Star, Inc., Ashland, OR).

2.3. Gene-expression profiling analysis of regulatory T cells

$CD4^+$ T cells from spleens of 10-week old female *dnTGFβRII;* $Foxp3^{GFP}$ or *WT;*$Foxp3^{GFP}$ mice were first enriched by MACS using anti-CD4 microbeads (Miltenyi, Bergisch Gladbach, German) and regulatory T cells ($CD4^+Foxp3^+$) were isolated by FACS Aria (BD) to attain a purity greater than 95%. RNA was extracted with RNAiso Plus (Takara, Dalian, China) and hybridized to Affymetrix MOE 430 2.0 chips. Fluorescence was detected using an Affymetrix GeneChip Scanner 3000 and images were analyzed using Affymetrix GeneChip Operating Software (GCOS). Transcription profile chip service was provided by Shanghai Biotechnology Cooperation (Shanghai). Expression fluorescence values were log_2-transformed, and subsequent analyses conducted using SAS statistical software online (http://sas.ebioservice.com/). Differentially expressed genes were defined as equal to or more than 2 fold differences between the two groups, signal values were confirmed beyond background signals, and the genes classified using the annotation of the Gene Ontology (GO) project [20]. Heat map and blue–pink scale schemes were designed by using Multiple Experiment Viewer 4.9 software and Microsoft Excel. A scatter plot was performed using Graph Pad Prism and color labeled to facilitate identification.

2.4. Biological pathway analysis

The open source Bioinformatics software Cytoscape 3.2.0 [21–23] (http://www.cytoscape.org/) was used to visualize gene–gene interactions. We loaded a BioGRID [24] interaction network for *Mus musculus*, then imported the gene expression data we acquired before by setting Entrez Gene identifiers (IDs) as the node primary identifiers. The up-regulated genes or down-regulated genes in dnTGFβRII Tregs compared with WT Tregs (more than 2-fold change) were selected and formed two sub-networks, and their first interaction neighbor genes were shown in the interaction sub-networks. Gene Ontology (GO) annotations and P-values for different biological processes, in which the target genes were involved, were assigned and analyzed using BiNGO 3.0.2, an application for Cytoscape [25]. BiNGO was also applied to generate the GO biological process network [25]. The hypergeometric test was used as the statistical testing of BiNGO, the Bonferroni correction was used to control the family-wise error rate (FWER) [26], and the Benjamini and Hochberg correction [27] was used in multiple testing corrections to control False Discovery Rate (FDR). The P-Value significance level, the EASE score, was set as 0.01, then statistical biological process pathway analysis was performed [28].

2.5. Quantitative PCR

Regulatory T cells ($CD4^+Foxp3^+$) or non-regulatory conventional $CD4^+$ T cells ($CD4^+Foxp3^-$) from spleens of *dnTGFβRII* mice and *B6* wild type control mice were sorted using FACS Aria (BD); the purity was confirmed to be greater than 95%. Total RNA from different sorted cells was extracted with RNAiso Plus (Takara) separately, and cDNA synthesized with the PrimeScript® RT reagent Kit (Takara). Quantitative PCR was performed using SYBR® Premix Ex TaqTM II (Takara) as previously described [17]. Data were collected by an ABI

Table 1
Primers used in q-PCR.

Gene	Forward primer 5′–3′	Reverse primer 5′–3′
Foxp3	CCCATCCCCAGGAGTCTTG	ACCATGACTAGGGGCACTGTA
Ikzf2	GAGCCGTGAGGATGAGATCAG	CTCCCTCGCCTTGAAGGTC
Ikzf4	TCTGGACCACGTCATGTTCAC	ACGATGTGGGAAGAGAACTCATA
Ccl5	GCTGCTTTGCCTACCTCTCC	TCGAGTGACAAACACGACTGC
GzmB	CCACTCTCGACCCTACATGG	GGCCCCCAAAGTGACATTTATT
Cxcr3	TACCTTGAGGTTAGTGAACGTCA	CGCTCTCGTTTTCCCCATAATC
Ahr	AGCCGGTGCAGAAAACAGTAA	AGGCGGTCTAACTCTGTGTTC
Cxcr4	GAAGTGGGGTCTGGAGACTAT	TTGCCGACTATGCCAGTCAAG
Foxp1	GGTCTGAGACAAAAAGTAACGGA	CGCACTCTAGTAAGTGGTTGC
Itgae	CCTGTGCAGCATGTAAAAGAATG	CAAGGATCGGCAGTTCAGATAC
Klf2	CTCAGCGAGCCTATCTTGCC	CACGTTGTTTAGGTCCTCATCC
Vcam1	AGTTGGGGATTCGGTTGTTCT	CCCCTCATTCCTTACCACCC
Gp49a	GCAGTACAGGCAGATTCATTCT	AGTAGCATGGGTTGGCTGATT
Igfbp4	AGAAGCCCCTGCGTACATTG	TGTCCCCACGATCTTCATCTT