Splenocytes with fucosylation deficiency promote T cell proliferation and differentiation through thrombospondin-1 downregulation

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Abstract

Fucosylation plays a critical role in cell-to-cell interactions and disease progression. However, the effects of fucosylation on splenocytes and their interactions with T cells remain unclear. In this study, we aimed to explore the transcriptome profiles of splenocytes deficient in fucosyltransferase (FUT) 1, an enzyme that mediates fucosylation, and investigate their impact on the proliferation and differentiation of T cells. We analyzed and compared the transcriptomes of splenocytes isolated from *Fut1* knockout (KO) mice and those from wild-type (WT) mice using RNA-seq. Additionally, we examined the effects of *Fut1* KO splenocytes on CD4 T cell proliferation and differentiation, in comparison to WT splenocytes, and elucidated the mechanisms involved. The comparative analysis of transcriptomes between *Fut1* KO and WT splenocytes revealed that thrombospondin (THBS)-1, among the genes related to immune response and inflammation, was the most highly downregulated gene in *Fut1* KO splenocytes. The reduced expression of THBS1 was further confirmed using qRT-PCR and flow cytometry. In coculture experiments, *Fut1* KO splenocytes promoted the proliferation of CD4 T cells and drove their differentiation towards Th1 and Th17 cells, compared to WT splenocytes. Moreover, the levels of IL-2, IFN-γ and IL-17 were increased, while IL-10 was decreased, in T cells cocultured with *Fut1* KO splenocytes compared to those with WT splenocytes. These effects of *Fut1* KO splenocytes on T cells were reversed when THBS1 was replenished. Taken together, our results demonstrate that splenocytes with *Fut1* deficiency promote CD4 T cell proliferation and Th1/Th17 differentiation at least in part through THBS1 downregulation.

Introduction

Fucosylation is a biological process that involves the transfer of a fucose residue to oligosaccharide chains carried by cell-surface glycoproteins or glycolipids, and is regulated by fucosyltransferases (FUTs) (1). Depending on the site of the oligosaccharide chain to which the fucose is added, fucosylation is categorized into two types, core fucosylation and terminal fucosylation, which are mediated by different FUTs. These fucosylated carbohydrate moieties play a crucial role in regulating various biological processes within cells, including cell survival, adhesion, cellular signaling and function, and cell-to-cell or cell-to-environment interactions. In effect, recent research has revealed that altered fucosylation in cells and tissues is associated with a number of diseases, ranging from inflammatory conditions such as collagen-induced arthritis (2), rheumatoid arthritis (3, 4), allergic airway inflammation (5), bacterial intestinal inflammation (6) and dry eye disease (7) to multiple types of cancers (8).

In our previous study (7), we observed increased inflammatory responses on the ocular surface and activation of CD4 T cells in *Fut1*knockout (KO) mice with a deficiency in terminal fucosylation mediated by FUT1, resulting in a dry eye disease phenotype. While the role of FUT1-mediated fucosylation has been previously investigated in vascular endothelial cells (3, 9) and synovial fibroblasts at the cellular level (4), its effects on immune cells and their interactions with T cells remain unclear. Therefore, in this current study, we analyzed the transcriptome profiles of splenocytes in *Fut1* KO mice compared to wild-type (WT) mice, and investigated the impact of these profiles on T cell proliferation and differentiation.

Materials and methods

Cell culture and reagents

Under the approval of the Institutional Animal Care and Use Committee of Seoul National University Biomedical Research Institute, spleens and corneas were collected from 8-week-old *Fut1* KO mice (C57BL/6 background, B6.129-Fut1tm1Sdo/J; Jackson laboratory, Bar Harbor, ME) and C57BL/6 mice (Jackson laboratory), respectively. The confirmation of*Fut1* gene knockdown in *Fut1* KO mice was performed following the genotyping protocols provided by Jackson laboratory (10).

Splenocytes were prepared by mashing the spleen, and *Fut1* gene knockdown was further confirmed in the cells using real-time RT-PCR (**Fig. S1**). CD4 cells were isolated from splenocytes using mouse CD4 microbeads (CD4 (L3T4) MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany). CD4 cells were then cocultured with splenocytes at a ratio of 1 : 1 on plates coated with 5 μg/mL mouse anti-CD3 and anti-CD28 mAbs (BD pharmingen, San Jose, CA) in RPMI1640 media (Welgene, Daegu, Korea) containing 10% fetal bovine serum (Gibco/Thermo Fisher Scientific, Waltham, MA) for 5 d.

Recombinant mouse thrombospondin-1 (THBS1) protein (5 μg/mL) (R&D Systems, Minneapolis MN) was added to some cocultures.

RNA sequencing

Total RNA was isolated from *Fut1* KO and C57BL/6 WT splenocytes, and RNA quality was assessed using Agilent 2100 bioanalyzer with the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, The Netherlands). A total of 500 ng of RNA from each group was used for RNA sequencing.

For RNA sequencing, library construction was performed using the QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen, Vienna, Austria) according to the manufacturer's instructions. The completed library was purified to remove PCR components. High-throughput sequencing was carried out as single-end 75 sequencing using the NextSeq 500 platform (Illumina, San Diego, CA).

For data analysis, the QuantSeq 3' mRNA-Seq reads were aligned using Bowtie2. Bowtie2 indices were either generated from genome assembly sequence or the representative transcript sequences for aligning to the genome and transcriptome. The resulting alignment file was used for transcript assembly, abundance estimation and detection of differential gene expression. Differentially expressed genes (DEGs) were determined based on counts from unique and multiple alignments using coverage in Bedtools (11). The Read Count data were processed using the quantile normalization method with EdgeR within R using Bioconductor (12). Gene classification was performed based on searches conducted by DAVID (http://david.abcc.ncifcrf.gov/) and Medline databases (http://www.ncbi.nlm.nih.gov/).

qRT-PCR

Splenocytes or corneal tissues were lysed using RNA isolation reagent (RNA Bee, Tel-Test, Friendswood, TX) and homogenized with an ultrasound sonicator (Ultrasonic Processor, Cole Parmer Instruments, Vernon Hills, IL). Total RNA was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany), and first-strand cDNA was synthesized by reverse transcription (High Capacity RNA-to-cDNA™ Kit, Applied Biosystems, Carlsbad, CA). Real-time amplification was performed using TaqMan® Universal PCR Master Mix (Applied Biosystems) on an automated instrument (ABI 7500 Real Time PCR System, Applied Biosystems) for *Fut1* or *Thbs1* . The data were normalized to*Gapdh* and expressed as fold changes relative to controls. All PCR probe sets were purchased from Applied Biosystems (TaqMan® Gene Expression Assay kits, Applied Biosystems).

ELISA

The cell-free supernatants from the cell cultures were collected after centrifugation, and their concentrations of IL-2, IFN-γ, IL-17 and IL-10 were measured using the DuoSet® ELISA kits (R&D Systems, Minneapolis MN).

Carboxyfluorescein succinimidyl ester (CFSE) assay

For cell proliferation assay, CD4 cells were incubated with 5 μM CFSE (CellTrace CFSE Cell Proliferation Kit, Invitrogen/Thermo Fisher Scientific, Waltham, MA) at 37°C for 10 min. After PBS washing, the CFSE-labeled $CD4^+$ cells were seeded onto anti-CD3- and anti-CD28-coated plates, and cocultured with *Fut1* KO or C57BL/6 WT splenocytes as described above. The CFSE fluorescence was measured using a flow cytometer (Cytek Aurora Flow Cytometer, Cytek biosciences, Fremont, CA).

Flow cytometry

The cells were stained with fluorescence-conjugated Abs against THBS1 (Cat # MA5-13398, Invitrogen/Thermo Fisher Scientific, Waltham, MA), CD4, IFN-γ, IL-17 or Foxp3 (eBioscience, San Diego, CA). For intracellular staining of cytokines, the cells were treated with an intracellular fixation buffer (eBioscience IC Fixation Buffer, Invitrogen/Thermo Fisher Scientific) before staining. For Foxp3 staining, eBioscience Foxp3/Transcription Factor Staining Buffer Set (Cat # 00-5523-00, Invitrogen/Thermo Fisher Scientific) was used. The stained cells were assayed using a flow cytometer (Cytek Aurora Flow Cytometer, Cytek biosciences) and data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Statistical analysis

Statistical analysis was performed using Prism software (GraphPad, San Diego, CA). The normal distribution of data in each group was assessed using the Shapiro-Wilk test and the Kolmogorov-Smirnov test. For comparisons between more than two groups, one-way ANOVA with Tukey's test or Kruskal-Wallis test with Dunn's multiple-comparisons test was employed to determine significant differences in mean values. Student's*t* -test was used for comparisons between two groups. The data were presented as mean ± SD. Statistical significance was defined as $p< 0.05$.

Results

Comparative transcriptome analysis of Fut1 KO vs. WT splenocytes

To investigate the impact of FUT1-mediated terminal fucosylation on the immune response in splenocytes, we isolated splenocytes from *Fut1*KO and WT mice, and analyzed both transcriptomes using QuantSeq 3' mRNA sequencing (**Fig. 1A**) (13). The comparative analysis of the transcriptomes identified a total of 124 DEGs with a fold change of > 2 and a p value < 0.05. Among these DEGs, 69 transcripts were significantly upregulated, whereas 55 transcripts were downregulated in *Fut1* KO splenocytes when compared to WT splenocytes (**Fig. 1A-D**).

Further categorization of the DEGs based on gene ontology revealed that 7 genes related to immune or inflammatory response were upregulated, while 4 genes were downregulated in *Fut1* KO splenocytes compared to WT splenocytes (**Fig. 1C, E**). The up-regulated genes in the immune response or inflammation categories were *Gp2, Hist1h2bk, Sdc1, Cfb, Fas* and *Mx1* , and the down-regulated genes included*Thbs1, Orm3, Ccl24* and *Tnfrsf11a* (**Fig. 1E).**Remarkably, *Thbs1* , which encodes thrombospondin-1, was the most highly down-regulated gene (4.8-fold) in *Fut1* KO splenocytes compared to WT splenocytes.

Reduced THBS1 expression in Fut1 KO splenocytes compared to WT splenocytes

To validate the RNA-seq data, we performed real-time RT-PCR assays on*Fut1* KO and WT splenocytes. The results confirmed a 5.9-fold or greater increase in THBS1 transcript levels in *Fut1* KO splenocytes compared to WT splenocytes (**Fig. 1F**). In contrast, THBS1 transcript levels in the corneal tissue did not show any significant difference between *Fut1* KO and WT mice (**Fig. 1F**).

Moreover, we assessed the proportion of THBS1-expressing cells in splenocytes using flow cytometry. The percentage of $THBS1^+$ cells among total splenocytes was significantly higher in *Fut1* KO mice than in WT mice (**Fig. 1G**).

Taken together, the results indicate that FUT1 deficiency in splenocytes leads to a reduction in the expression of THBS1 at both mRNA and protein levels.

Impact of Fut1 KO splenocytes and THBS1 on T cell proliferation and differentiation

Next, to investigate the effects of *Fut1* KO splenocytes and their reduced levels of THBS1 on the proliferation and differentiation of CD4 T cells, we cocultured $CD4^+$ cells isolated from WT mice with either *Fut1* KO splenocytes or WT splenocytes (**Fig. 2A**). The CFSE dilution assay revealed that the proliferation of $CD4^+$ cells cocultured with*Fut1* KO splenocytes was significantly higher than those cocultured with WT splenocytes (**Fig. 2B**). Remarkably, the addition of THBS1 to the coculture reversed the effects of *Fut1*KO splenocytes on CD4+ cell proliferation (**Fig. 2A**).

Similar findings were observed with CD4 T cell differentiation. The coculture of $CD4^+$ cells with *Fut1* KO splenocytes significantly induced the differentiation of $CD4^+$ cells into IFN-γ^+CD4^+ Th1 cells and IL-17^+CD4^+ Th17 cells, compared to the coculture with WT splenocytes (**Fig. 2C**). The replenishment of THBS1 in $CD4^+$ cell-*Fut1* KO splenocyte cocultures effectively suppressed the differentiation of $CD4^+$ cells into Th1 and Th17 subsets (**Fig. 2C**). However, neither *Fut1* KO splenocytes nor THBS1 affected the differentiation of $CD4^+$ cells into $Foxp3^+$ regulatory T cells (Tregs) (**Fig. 2D**).

Furthermore, we examined the levels of inflammatory cytokines in the coculture supernatants. The secretion of IL-2, IFN-γ and IL-17 were significantly increased, while IL-10 was reduced, in $CD4^+$ cells cocultured with *Fut1* KO splenocytes compared to those cocultured with WT splenocytes (**Fig. 2E**). THBS1 was also effective in reducing the levels of IL-2, IFN-γ and IL-17, as well as enhancing IL-10, in the coculture of $CD4^+$ cells and *Fut1* KO splenocytes (**Fig. 2E**).

These results suggest that *Fut1* KO splenocytes promote CD4 T cell proliferation and the differentiation towards Th1 and Th17 cells through reduced levels of THBS1 at least in part.

Discussion

Our data demonstrated that *Fut1* KO splenocytes exhibited distinct transcriptome profiles from WT splenocytes, with *Thbs1* showing the most significant down-regulation in *Fut1* KO splenocytes. Since THBS1 was effective in inhibiting CD4 T cell proliferation and Th1/Th17 differentiation, *Fut1* KO splenocytes more significantly enhanced the proliferation of CD4 T cells and the differentiation into Th1/Th17 subsets compared to WT splenocytes.

Previous studies have investigated the effects of fucosylation on immune cells. For instance, T cells treated with the pan-fucosylation inhibitor 2-deoxy-2-fluorofucose (2FF) were shown to be more easily activated by peptide-major histocompatibility complex (MHC) (14), while treatment with the terminal FUT1/2 inhibitor, 2-deoxy-D-galactose (2-D-gal), promoted Th17 cell differentiation (1). Also, 2FF was reported to upregulate MHC class II, CD83, CD86 and CD40 on dendritic cells (DCs) (14). Moreover, the overexpression of type I Lewis antigens, which are terminal fucosylated carbohydrate epitopes, in colon cancer cells induced an immunosuppressive phenotype in DCs (15). In contrast, it was reported that 2-D-gal suppressed the expression of MHC class II and CD86 on macrophages and disrupted the interaction between antigen-presenting cells (APCs) and T cells (2). In addition to these previous findings, our study showed that deficiency in FUT1-mediated terminal fucosylation in splenocytes potentiated the stimulating effects of splenocytes as APCs on CD4 T cell proliferation and Th1/Th17 differentiation due to the reduced expression of THBS1. Further studies are needed to identify the specific cell types within splenocytes that exhibit altered THBS1 production in response to FUT1 deficiency, and to investigate the underlying molecular pathways through which FUT1-mediated fucosylation regulates THBS1 expression in these cells.

THBS1 is a matricellular glycoprotein that plays crucial roles in angiogenesis, cancer and inflammation. It is secreted by diverse cell types, including endothelial cells, fibroblasts, adipocytes, smooth muscle cells and APCs, reflecting the involvement of THBS1 in regulating multiple cellular processes associated with tissue repair and inflammation resolution (16,17). While THBS-1 has long been investigated in the context of vascular health and disease, recent research has shed light on its emerging role in the immune response. Especially, THBS1 derived from APCs was reported to suppress the capacity of APCs to allosensitize CD4 T cells, promoting the survival of corneal allografts (18). It was also observed that THBS1 expression by

APCs was necessary for TGF-β_2-expressing APCs to induce Foxp3$^+$ Tregs (19). Furthermore, exogenous THBS1 was found to inhibit the differentiation of CD4 T cells into Th17 cells and stimulate their differentiation towards Tregs (20), confirming the role of THBS1 as a negative regulator of pro-inflammatory T cell activation. Consistent with these findings, in our study, THBS1 significantly abrogated the effects of *Fut1* KO splenocytes on the induction of CD4 T cell proliferation and Th1/Th17 differentiation, although it did not affect Treg differentiation.

In conclusion, we herein demonstrate that *Fut1* deficiency in splenocytes leads to reduced THBS1 secretion, thereby promoting CD4 T cell proliferation and driving Th1/Th17 differentiation. These findings contribute to our understanding of the interplay between splenocyte fucosylation, THBS1 expression and T cell responses. Future strategies aimed at modulating fucosylation in APCs to upregulate THBS1 expression may hold promise as potential therapeutic approaches for the management of T-cell-mediated immune disorders.

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Figure captions

Figure 1. *Fut1* KO splenocytes exhibit decreased levels of THBS1 in comparison to WT splenocytes.

A. Experimental protocol. Splenocytes were isolated from WT or*Fut1* KO mice and subsequently subjected to assays. Scatter plot from RNA sequencing. The plot displays the normalized data in a logarithmic scale for each gene, comparing WT splenocytes on the x-axis to *Fut1* KO splenocytes on the y-axis.

B. Volcano plot comparison of gene expression between*Fut1* KO and WT splenocytes. The x-axis indicates the fold change in a logarithmic scale, while the y-axis indicates the statistical significance of the difference in expression (p value from at test). Red dots represent 69 transcripts that were upregulated ($>$ 2-fold, p-value < 0.05), while green dots represent 55 transcripts that were downregulated in *Fut1* KO splenocytes compared to WT splenocytes.

C. Bar chart comparison of gene expression based on gene ontology categories. The y axis represents the percentage of DEGs out of the total genes in each category. The number displayed above each bar represents the count of genes upregulated (red bar) or downregulated (green bar) in the specific category.

D, E. Heat maps of RNA sequencing data of *Fut1* KO splenocytes compared to WT splenocytes. The heat map in D represents the complete list of DEGs, while the heat map in E presents the DEGs within the gene ontology category of immune or inflammatory response. *Thbs1* exhibited the most pronounced downregulation in *Fut1*KO splenocytes when compared to WT splenocytes.

F. qRT-PCR for Thbs1 in splenocytes or corneal tissues collected from WT and *Fut1* KO mice. The mRNA levels are presented as fold changes relative to the levels in the WT control group.

G. Representative and quantitative flow cytometry results for $THBS1^+$ cells in WT or *Fut1* KO splenocytes.

***p < 0.001, ****p < 0.0001, ns: not significant, as analyzed by one-way ANOVA with Tukey's test (F) or Student'st -test (G)

Figure 2. *Fut1* KO splenocytes enhance T cell proliferation and differentiation, while replenishing THBS1 reverses these effects.

A. Experimental design. $CD4^+$ cells isolated from WT mice were cocultured with either splenocytes isolated from WT mice or splenocytes from *Fut1* KO mice for 5 d, after which the cells were subjected to further assays. In some cocultures, 5 μg/mL recombinant THBS-1 was added.

B. CFSE dilution assay for $CD4^+$ cell proliferation

C, D. Representative and quantitative flow cytometry results for IFN-γ^+CD4^+ and IL-17^+CD4^+ cells (C) and for $Foxp3^+CD4^+$ cells (D)

E. ELISA for secreted levels of IL-2, IFN-γ, IL-17 and IL-10 in the cocultures of $CD4^+$ cells and splenocytes.

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: not significant, as analyzed by one-way ANOVA with Tukey's test or Kruskal-Wallis test with Dunn's multiple-comparisons test (D)